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JANUARY 1974

VOLUME 63 NUMBER 1

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ROLLING OUT THE '74 MODEL

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Edward G. Feldmann

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REVIEW ARTICLE

Stability of Solids and Solid Dosage Forms

J. T. CARSTENSEN

Keyphrases □ Solids and solid dosage forms—stability, review, decomposition schemes, equations and rate constants, effect of moisture □ Stability of solids and solid dosage forms—review, decomposition schemes, equations and rate constants, effect of moisture □ Decomposition of solid dosage forms—review, degradation schemes, equations and rate constants, effect of moisture □ Moisture effect—decomposition of solid dosage forms, review

The topic of stability of pharmaceutical solids has not been covered extensively in the pharmaceutical literature. Only four review articles [Garrett (1, 2), Lachman (3), and Carstensen (4)] appeared in the last two decades [except for the partial coverage in the yearly literature review articles in the *Journal of Pharmaceutical Sciences* (5–12)]. The stability of pure compounds has been covered to an extent in chemical journals, and a brief outline of stability of one- and two-component systems will be given first.

STABILITY OF SINGLE CRYSTALLINE SOLIDS

The decomposition of pure solids adhering to Scheme I gives curves that are (*a*) initially steep and then approach 100% decomposition or an asymptote, or (*b*) characterized by an induction period, giving a sigmoid appearance.

There are many theories accounting for this, *e.g.*, Young's (13), but the shape denoted in *a* can usually be accounted for by geometrical contractive consideration (topochemical reactions), whereas the shape denoted in *b* may be accounted for by either nucleation theories or liquid layer theories.

CONTRACTING GEOMETRIES

The relation of decomposition to geometrical aspects was first proposed by Langmuir (14), who postulated that reactions occurred at boundaries and surfaces. Jacobs and Tompkins (15) reviewed the treatment of geometric contractions; the three most predominant cases will be outlined here.

In the contracting cylinder (Fig. 1), it is assumed that the radius of intact chemical substance decreases linearly with time, *i.e.*:

$$r = r_0 - k_1 t \qquad \text{(Eq. 1)}$$

where r_0 is the initial radius. At time t, there will be a decomposition product, B, outside of r and an unchanged chemical substance, A, inside of r. The fraction decomposed at time t is then given by:

$$x = \frac{hn\rho\pi[r_0^2 - r^2]}{hn\rho\pi r_0^2} \qquad \text{(Eq. 2)}$$

where n is the number of particles and h is their height; n and h cancel out, so that the amount retained is obtained from Eqs. 1 and 2:

$$1 - x = 1 + (k_1/r_0)^2 t^2 - (2k_1/r_0)t \qquad \text{(Eq. 3)}$$

or:

$$\sqrt{(1 - x)} = 1 - (k_1/r_0)t \qquad \text{(Eq. 4)}$$

If Fig. 2 is considered to represent a sphere, then in a similar fashion:

$$x = \frac{(4/3)\pi r_0^3 - (4/3)\pi(r_0 - k_3 t)^3}{(4/3)\pi r_0^3} = 1 - \frac{1 - k_3 t^3}{r_0} \qquad \text{(Eq. 5)}$$

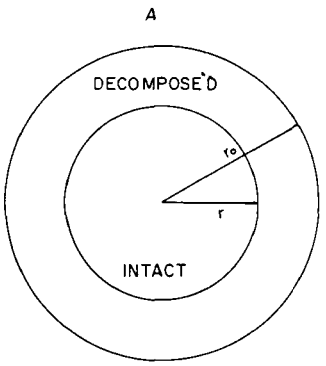

Figure 1—*Model of cylinder or sphere during decomposition.*

or:

$$(1 - x)^{1/3} = 1 - (k_3/r_0)t \qquad \text{(Eq. 6)}$$

A similar form is achieved if the starting point is a cube, and this is frequently referred to as the contracting cube equation.

Roginski and Schul'tz (16) showed that Eq. 6 held for potassium permanganate. Another geometric equation, Eq. 7, was derived by Jander (17). The equations may be summarized as:

$$(1 - x)^{1/2} = 1 - \gamma t \qquad \text{(Eq. 3}a\text{)}$$

$$(1 - x)^{1/3} = 1 - \gamma t \qquad \text{(Eq. 6}a\text{)}$$

$$(1 - x)^{1/3} = 1 - (\gamma t)^{1/2} \qquad \text{(Eq. 7)}$$

where γ has unit of time^{-1} (hr^{-1}).

A set of curves of the types described in Eqs. 3*a*, 6*a*, and 7 is shown in Fig. 2*a* and Table I. They are compared with a curve of the type:

$$1 - x = e^{-kt} \qquad \text{(Eq. 8)}$$

which is a straightforward first-order case. All curves in Fig. 2*a* are made to fit the point [3,0.28]. For small values of γt, Eqs. 3*a* and 6*a* become:

$$1 - x \sim 1 - \frac{2k_1 t}{r_0} \qquad \text{(Eq. 3}b\text{)}$$

$$1 - x \sim 1 - \frac{3k_3 t}{r_0} \qquad \text{(Eq. 6}b\text{)}$$

compared to the expansion of Eq. 8 which, for small kt values, becomes:

$$1 - x = 1 \doteq kt \qquad \text{(Eq. 8}a\text{)}$$

so that $k \sim 3k_3/r_0$ or $k \sim 2k_1/r_0$. The former comparison is shown in Table I and Fig. 2*b*. *The similarity may account for many decompositions in solid dosage forms approaching first-order kinetics.*

There are a few pharmaceutical examples of the utility of Eq. 7 such as the work of Horikoshi and Himuro (18). They studied the dehydration reactions of a series of glucuronic acid derivatives (Table II). Their data for dehydration of glucuronamide hydrate are shown in Figs. 3–5. It is seen that proper selection of treatment (*i.e., via* Eqs. 3*a*, 6*a*, or 7) leads to

$$A_{\text{solid}} \longrightarrow B_{\text{solid}} + C_{\text{gas}}$$

Scheme I

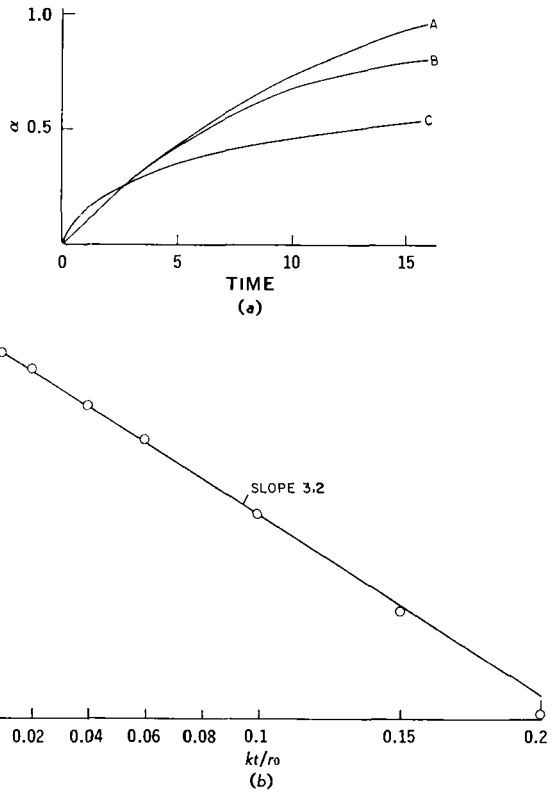

Figure 2—(a) *Decomposition curves from Table I.* A *is contracting cube or rectangle,* B *is product layer diffusion controlled, and* C *is first-order decomposition. Decomposition product is plotted* versus *time.* (b) *Data following Eq. 6 plotted* via *Eq. 8 (Table I).*

rate constants that can be treated by an Arrhenius equation.

SIGMOID DECOMPOSITION CURVES

The reaction in Scheme I can give rise to sigmoid curves when the mole fraction decomposed (x) is plotted *versus* time. There is an initial induction period (A) as shown in Fig. 6, characterized by a lag time at levels of x at $0 < x < 0.1$. This is followed by an acceleratory period (B) at levels of x at $0.1 < x < 0.5$. This is, finally, followed by the decay period (D), which is less reproducible than the two former periods. The inflection point is usually at $x = P/P_\infty = 0.5$.

The most general model explaining this type of de-

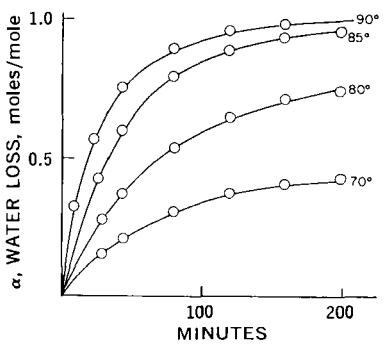

Figure 3—*Decomposition of glucuronamide hydrate. Adapted, with permission, from Ref. 18.*

Table I—Decompositions According to Eqs. 3*a*, 6*a*, 7, and 8

Time, t	γ = 0.05 hr^{-1}, Eq. 3*a*	γ = 0.035 hr^{-1}, Eq. 6*a*	γ = 0.00359 hr^{-1}, Eq. 7	γ = 0.11 hr^{-1}, Eq. 8	k_3t/r_0, Eq. 6	$1 - x$, Eq. 6	$\ln(1 - x)$
1	0.1	0.1	0.17	0.10	0.01	0.970	−0.030
2	0.19	0.20	0.23	0.20	0.02	0.941	−0.061
3	0.28	0.28	0.30	0.28	0.04	0.885	−0.122
5	0.44	0.44	0.35	0.42	0.06	0.830	−0.186
10	0.75	0.73	0.47	0.67	0.10	0.729	−0.316
15	0.94	0.89	0.55	0.81	0.15	0.614	−0.488
20			0.61		0.20	0.512	−0.669
25			0.66				

Table II—Activation Energies of Dehydration of Glucuronic Acid Derivatives (18)

Reaction	Activation Energy	Symbols
$GN \cdot H_2O \rightarrow GN + H_2O$	38.6 kcal/mole	GN = glucuronamide hydrate
$GK \cdot 2H_2O \rightarrow GK \cdot H_2O + H_2O$	4.3 kcal/mole	GK = potassium glucuronate
$GK \cdot H_2O \rightarrow GK + H_2O$	18.5 kcal/mole	
$GI \cdot 2H_2O \rightarrow GI + 2H_2O$	12.2 kcal/mole	GI = glucuronate isoniazone

composition curve for reactions of the type in Scheme I has been the Prout–Tompkins (19) model. The assumption is made that the decomposition is governed by formation and growth of active nuclei. The existence of nuclei and their growth have been substantiated by several investigators (19–23); they occur on the surface as well as inside (24) the crystals during decomposition. Prout and Tompkins (19) assumed that the reaction probability is proportional to the number of nuclei; they also assumed that the nuclei propagated with a certain probability α and terminated with a probability β. If the initial number of potential nucleus formation sites is N_0 and N is the number at time t, then:

$$dN/dt = k[N_0 - N] \qquad \text{(Eq. 9)}$$

At time t, there remain $N_0 - N$ sites to be used up. If for the moment one disregards termination, then this can be integrated with the result:

$$N = N_0[1 - \exp(-kt)] \sim N_0kt \qquad \text{(Eq. 10)}$$

where the approximation is valid for small kt values. Differentiating Eq. 10 gives:

$$dN/dt = kN_0 \qquad \text{(Eq. 11)}$$

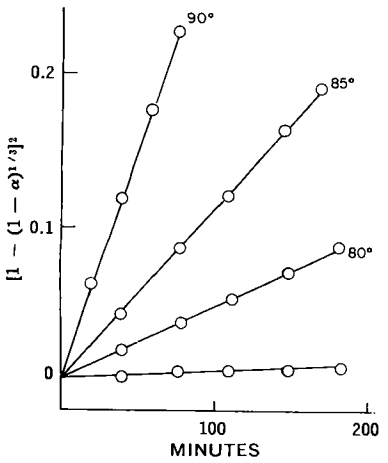

Figure 4—*Data in Fig. 3 plotted according to Eq. 7. Adapted, with permission, from Ref. 18.*

Since, however, each nucleus has a probability α of branching, this is more properly written:

$$dN/dt = kN_0 + \alpha N \qquad \text{(Eq. 12)}$$

Eventually, branches will merge (with a probability of β), so Eq. 12 should include this as well. If, in addition, the initial potential nucleation sites are used up rapidly, Eq. 12 becomes:

$$dN/dt = [\alpha - \beta]N \qquad \text{(Eq. 13)}$$

in which α and β are functions of t (and x); hence Eq. 13 cannot be directly integrated. Since $\alpha = \beta$ at $x = 0.5$ and since $\alpha = 1$ and $\beta = 0$ at $t = 0$ ($x = 0$), the functionality $\beta = 2x\alpha$ is a reasonable trial function in the model. Hence:

$$dN/dt = \alpha[1 - 2x]N \qquad \text{(Eq. 14)}$$

The decomposition rate is now assumed proportional to the number of nuclei, so that:

$$dx/dt = k_1N \qquad \text{(Eq. 15)}$$

Inserting this into Eq. 14 gives:

$$\frac{dN}{dt} = \frac{dN}{dx}\frac{dx}{dt} = \frac{\alpha[1 - 2x]}{k_1}\frac{dx}{dt} \qquad \text{(Eq. 16)}$$

so:

$$N = (\alpha/k_1)[x - x^2] \qquad \text{(Eq. 17)}$$

assuming that $N_0 \ll N$ and that $N = 0$ implies that x is approximately equal to zero. Inserting Eq. 17 into Eq. 15 then gives:

$$\ln[x/(1 - x)] = \gamma t + C \qquad \text{(Eq. 18)}$$

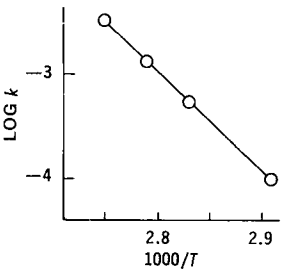

Figure 5—*Arrhenius plotting of the data in Fig. 4. For legend, see Table II. Adapted, with permission, from Ref. 18.*

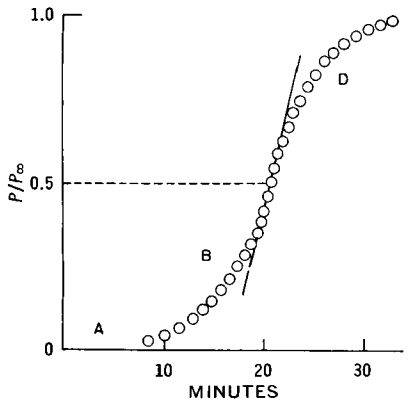

Figure 6—*Decomposition of silver permanganate. Adapted, with permission, from Ref. 26.*

where C is the lag time term and the logarithmic relation should be apparent beyond that point. Data on silver permanganate (26) are shown graphically in Figs. 7 and 8. The linearity according to Eq. 18 is apparent, and the rate constant adheres to an Arrhenius relation.

There are pharmaceutical examples of this type of degradation. In the work by Kornblum and Sciarrone (27), aminosalicylic acid was shown to decompose by a pattern similar to that described in Eq. 18. The decomposition of aspirin anhydride reported by Garrett *et al.* (28) is another example where Prout–Tompkins (19) kinetics may apply at the higher temperatures studied (60–70°).

To establish curves of the type discussed, it is necessary to assay at frequent intervals in the early stages; the problem of thermal equilibration is difficult to gauge when solids are stored at temperatures above 25° (29).

SYSTEMS WITH LIQUID DECOMPOSITION PRODUCT LAYERS

If the general reaction is as shown in Scheme II rather than Scheme I, the decomposition pattern changes. Carstensen and Musa (30) reported on the decomposition of a series of substituted benzoic acids, all decomposing into a liquid and a gas. For example, aminobenzoic acid decomposes into aniline and carbon dioxide. The aminobenzoic acid dissolves in the formed aniline to an extent of S moles/mole of aniline, so that at a particular time t, when x moles are decomposed, there are Sx moles of aminobenzoic acid in solution and $[1 - x - Sx]$ moles in the solid state. By denoting k_s and k_a as the first-order rate constants of the decomposition in the solid and liquid states, respectively, the following equation governs the decomposition:

$$dx/dt = k_s[1 - x - Sx] + k_aSx = k_s + \Gamma x \quad \text{(Eq. 19)}$$

where:

$$\Gamma = -[k_s + k_sS - k_aS] \quad \text{(Eq. 20)}$$

Integrated, this gives:

$$\ln[1 + (\Gamma x/k_s)] = \Gamma t \quad \text{(Eq. 21)}$$

so that Γ/k_s can be found as an adjustable parameter. Since Γ is known from the slope, k_s can be found through division. S can be determined from the point on the curve in Fig. 9 where liquefaction takes place (this can be done fairly accurately by visual means). Beyond this point, the decomposition is simply a first-order decomposition in solution, and k_a can be found from this as well as from Eq. 20 (with knowledge of S and k_s). The data obeyed Eq. 21 and there was agreement between values of k_a found *via* Eq. 20 and *via* the part of the decomposition curve beyond the liquefaction point. The curves for this type of decomposition are, therefore, sigmoid as well, in that the part up to the liquefaction point is upward convex by the equation:

$$x = \frac{k_s}{\Gamma}\{e^{\Gamma t} - 1\} \quad \text{(Eq. 22)}$$

and the part beyond the liquefaction point [where $x = x' = 1/(1 + S)$ at $t = t'$] is downward convex by

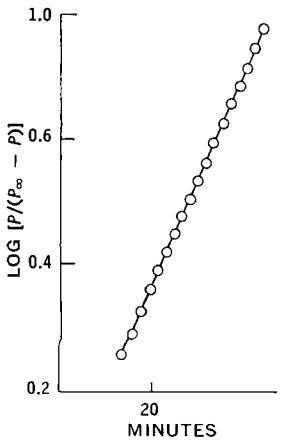

Figure 7—*Data from Fig. 6 plotted according to Eq. 18. Adapted, with permission, from Ref. 26.*

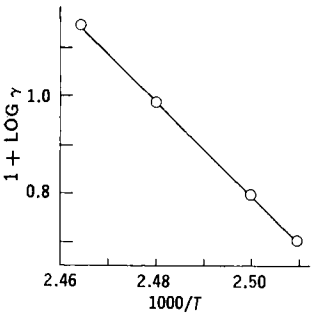

Figure 8—*Data from Fig. 7 plotted by Arrhenius plotting. Adapted, with permission, from Ref. 26.*

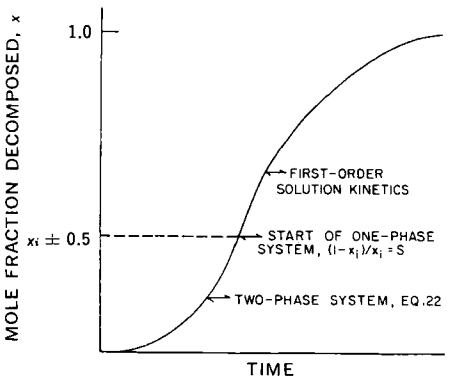

Figure 9—*Solid-state decomposition of aminobenzoic acid. Adapted, with permission, from Ref. 30.*

$$A_{solid} \longrightarrow B_{gas} + C_{liquid}$$

Scheme II

the equation:

$$(1 - x)/(1 - x') = e^{-k_s(t-t')} \qquad \text{(Eq. 23)}$$

The data by Carstensen and Musa (30) are indicative (although not proof) of the existence of an isokinetic point for solid reactions (31), as shown in Fig. 10.

The decomposition of a series of vitamin A derivatives was studied (32) and found to be zero order. The decomposition rate constants, k_A, were found to be related to the melting point, T_m, of the compounds by the equation (Fig. 11):

$$\log k_A = \theta\frac{1}{T_m} + \sigma \qquad \text{(Eq. 24)}$$

The results are rationalized *via* the assumption of a liquid layer theory; similar theories have been proposed (33–43). Gluzman and coworkers (34–43) showed evidence of pseudoliquid layers at temperatures far below the melting temperature of the compounds studied. Carstensen and Musa (30) also found that a series of *p*-substituted benzoic acids adhered to the Guillory–Higuchi (32) equation (Eq. 24) (Fig. 11).

DECOMPOSITION OF ONE-COMPONENT PHARMACEUTICAL SYSTEMS

There are two main purposes for conducting studies of solid decomposition in pharmaceutics: (*a*) to elucidate the mechanism of a reaction, and (*b*) to predict stability. In general, pharmaceutical systems do not lend themselves to studies through many half-lives, so fractional order assignment is not possible in most cases. Distinction between first and zero order is, however, both possible and important; in extrapolatory techniques, wrong conclusions can be drawn by wrong order assignments. Statistical methods for these purposes have been described (1, 44).

Zero-order patterns are explained by being either the initial part of curves such as the ones shown in Fig. 2 or the induction period of curves such as the ones shown in Fig. 6. There are, of course, times where first and zero order cannot be distinguished in

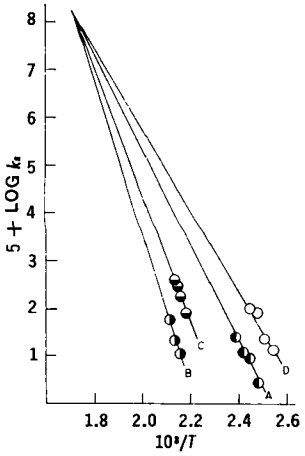

Figure 10—*Plot showing possible isokinetic point in solid-state decomposition of* p-*substituted benzoic acids. Key: A, amino; B, dimethylamino; C, hydroxy; and D, methylamino.*

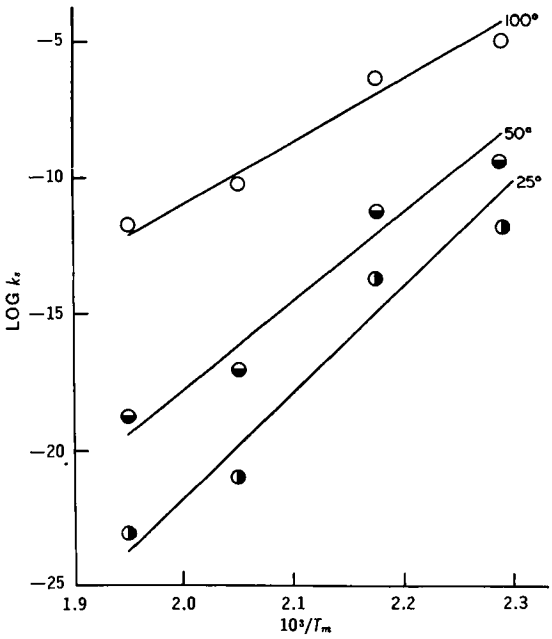

Figure 11—*Decomposition rate constants in solid state* (k_s) *as a function of melting point* (T_m). *Reprinted, with permission, from Ref. 30.*

any way: in general, when $x < 0.15$, $\ln(1 - x) \sim -x$ (45). An example is the work by Brownley and Lachman (46) where the formation of hydroxymethylfuraldehyde from lactose was studied. Another case is the bacitracin study by Gross *et al.* (47, 48) (Fig. 12). Neither plotting of decomposition-time curves nor Arrhenius plotting will reveal the actual order. Adherence to Arrhenius plotting was reported by Garrett (49) in the case of fumagillin decomposition; the case of vitamin A derivatives reported by Guillory and Higuchi (32) was already mentioned. Nakanishi *et al.* (50) found aluminum aspirin to decompose by zero-order kinetics and found good Arrhenius correlation.

First-order cases can be explained by inspection of

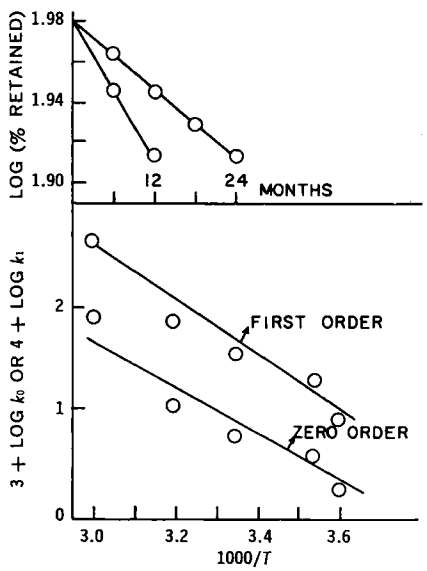

Figure 12—*Bacitracin decomposition. Adapted, with permission, from Refs. 47 and 48.*

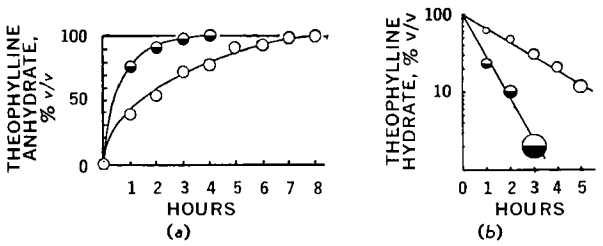

Figure 13—*Dehydration kinetics of theophylline hydrate.* (a) *Anhydrate formation at 40°* (◒) *and theophylline hydrate at 40°* (○). (b) *Logarithmic plotting of the data. Reprinted, with permission, from Ref. 53.*

Fig. 2. If the reaction goes to completion, any of the curves corresponding to Eq. 3*a*, 6*a*, or 7 may be approximated by Eq. 8; in particular, at higher temperatures where thermal lag may be operative, an apparent first-order scheme is perfectly sound. The same applies to decompositions following Prout-Tompkins kinetics (Eq. 18) as long as lag times are short. Carstensen and Pothisiri (51) found the decomposition of aminosalicylic acid *in vacuo* [as opposed to atmospheric decomposition (27)] to be first order and amenable to Arrhenius plotting.

The approach to equilibrium reported by Takahashi and Yamamoto (52) in the case of isomerization of vitamin D_2 to isocalciferol and isotachysterol is the consequence of the chemistry of reaction, as is the high temperature equilibrium approached by fumagillin reported by Garrett (49). In other cases, there are *apparent* approaches to equilibrium that may be of either chemical or physical origin, as will be discussed later. The bacitracin work by Gross *et al.* (47, 48) can be viewed in this light. Again, any of the curves in Fig. 2 may be approximated by an equation such as:

$$x = x_\infty[1 - e^{-kt}] \qquad \text{(Eq. 25)}$$

where x_∞ is an apparent equilibrium level; if analytical methods have a coefficient of variation of more than 5%, distinction between this and the particular topochemical scheme may be difficult to establish.

DEHYDRATION KINETICS

Although loss of water of crystallization is not a clearcut "chemical decomposition," it is of pharmaceutical importance. The case of dehydration of some glucuronic acid derivatives was reported by Horikoshi and Himuro (18) and their results are tabulated in Table II. Shefter and Kmack (53) reported on the dehydration kinetics of theophylline hydrate, and their results are shown in Fig. 13.

Shefter and Kiral[1] showed that the thermal decomposition of sodium bicarbonate is an $A \rightarrow B \rightarrow C$ reaction (Scheme III), in that twice the appearance rate of sodium carbonate (Na_2CO_3), followed by X-ray techniques, does not equal the disappearance rate of sodium bicarbonate ($NaHCO_3$), except at the beginning and at the end; the intermediate $[NaHCO_3]^+$ is not detectable by X-ray techniques.

[1] E. Shefter and R. Kiral, to be published.

$$2NaHCO_3 \longrightarrow 2[NaHCO_3]^+ \longrightarrow Na_2CO_3 + H_2O + CO_2$$

Scheme III

$$A_{solid} + B_{solid} \longrightarrow 2C_{solid}$$

Scheme IV

In the case of dehydration of hydrates, Garner (54) suggested that water in coordination complexes is more strongly held than "structural" water (*i.e.*, interstitial water) and that the surface may dehydrate first and that the formed interface serves as a barrier through which subsequent water must diffuse. Kohlschütter and Nitschmann (55) showed by X-ray techniques that cupric sulfate pentahydrate, when dehydrated *in vacuo*, forms a dehydrated product with no apparent crystalline structure, whereas dehydration at (low) water vapor pressure produces a crystalline end-product. It is assumed that *in vacuo* the evaporation of water molecules leaves a surface of ionic network, which is stable in only a few cases (zeolites); it will mostly rearrange to a phase of ill-defined crystalline structure.

Zawadski (56) and Garner (54) showed interfaces of the same type to be important in carbon dioxide loss from carbonates as well.

TWO-COMPONENT SYSTEMS

When two compounds are involved in a decomposition reaction, Scheme IV, a "reaction layer" of thickness ζ forms between A and B. A molecules must diffuse through this layer to reach and react with B molecules if it is assumed that the B molecules are the less mobile. This diffusion is governed by Fick's first law, *i.e.*, the rate of A diffusion is:

$$\frac{dA}{dt} = \frac{\pi([A] - 0)}{\zeta} \qquad \text{(Eq. 26)}$$

where π is the permeability and the reaction rate is assumed to be much greater than the diffusion rate. A loss of an A molecule means an increase of the thickness of the layer by:

$$dA = ad\zeta \qquad \text{(Eq. 27)}$$

so:

$$\frac{d\zeta}{dt} = \frac{q}{\zeta} \qquad \text{(Eq. 28)}$$

which is integrated to:

$$\zeta^2 = 2qt \qquad \text{(Eq. 29)}$$

This is comparable to Eq. 7. In actuality, a is not independent of ζ. The complete expression was derived by Cohn (57) and Rastogi and coworkers (58–61) and is:

$$\zeta^2 = Qte^{-\alpha\zeta} \qquad \text{(Eq. 30)}$$

They studied the reaction of trinitrophenol (picric acid) and α-naphthol; their data, plotted *via* Eq. 30, are shown in Fig. 14. It is seen from the figure that the temperature relationship is akin to an Arrhenius relationship. The factor Q, of course, is related to

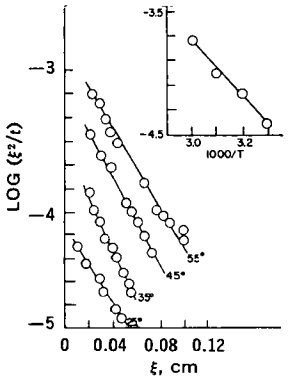

Figure 14—*Solid-state reaction between trinitrophenol and α-naphthol, according to Eq. 30. Adapted, with permission, from Ref. 58.*

diffusion, so that activation energy may be different from that usually encountered in chemical reactions, as indeed it is (60 kcal/mole).

Interactions of solids with oxygen were reported by Garrett (49) and Tingstad and Garrett (62), who established (Scheme V) that fumagillin (A) oxidized *via* a steady-state intermediate (AO_2) which reacted with A to form AO:

$$A + O_2 \underset{k_-}{\overset{k_+}{\rightleftharpoons}} AO_2 \xrightarrow[k_2]{+A} 2AO$$

Scheme V

If $k_2[A] \gg k_- \gg k_+[A]$, then:

$$\frac{d[AO]}{dt} = -2\frac{d[A]}{dt} = \frac{k_2[O_2]}{k_-}[A]^2 \qquad \text{(Eq. 31)}$$

so that the reaction should be second order and follow:

$$\frac{1}{[A]} = \frac{1}{[A]_0} - kt \qquad \text{(Eq. 32)}$$

The data are plotted in this way in Fig. 15, and Fig. 16 shows that the rate constants follow an Arrhenius relation.

DECOMPOSITIONS INVOLVING MOISTURE

The work by Kornblum (63) and by Kornblum and Sciarrone (27) included a study of the effect of atmospheric moisture. Figure 17 shows that added

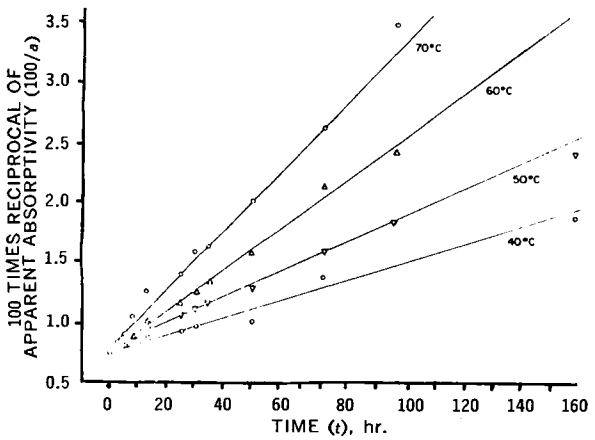

Figure 15—*Second-order decomposition of fumagillin in presence of oxygen. See Eq. 32. Reprinted, with permission, from Ref. 49.*

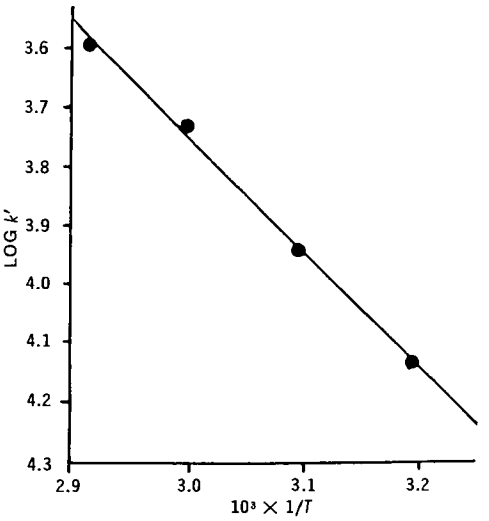

Figure 16—*Arrhenius plot of the data in Fig. 15. Reprinted, with permission, from Ref. 49.*

moisture decreases the lag time and increases the zero-order rate constant. Leeson and Mattocks (64) showed that in the presence of abundant moisture, the decomposition of aspirin can be accounted for by considering the moisture adsorbed, being saturated by aspirin, and the sole source of decomposition. They took into account that the "solution" gets more acid as the reaction progresses. Denoting by k_H the hydrogen-catalyzed hydrolysis rate constant and by K the ionization constant of acetic acid, and assuming that the volume, V, of moisture adsorbed follows a Freundlich isotherm:

$$V = k'p^n \qquad \text{(Eq. 33)}$$

they arrived at the following expression for decomposition at time t:

$$\log[a_0(\sqrt{d} - \sqrt{c_0})/\{a(\sqrt{a} + \sqrt{c})\}] = ([\sqrt{d_0}kp^{3n/2}]/2.3)t \qquad \text{(Eq. 34)}$$

where a denotes aspirin content, c is salicylic acid content, $d = a + c = a_0 + c_0$, p is water vapor pressure, and $k = k_H k'\sqrt{Kk'}$. Figure 18 shows that Eq. 34 was followed well. The slopes of the lines α are given by:

$$\log \alpha = (3n/2) \log p + \log[\sqrt{d_0}k/2.3] \qquad \text{(Eq. 35)}$$

and Fig. 19 shows that this relation held well at all temperatures. From the intercept it is possible to

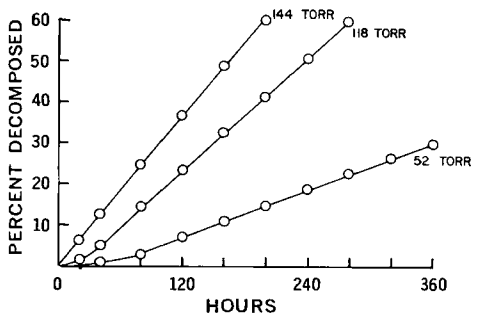

Figure 17—*Decomposition* versus *time curves for aminosalicylic acid in atmospheres containing moisture: 144, 118.4, and 52.3 torr. Adapted, with permission, from Ref. 27.*

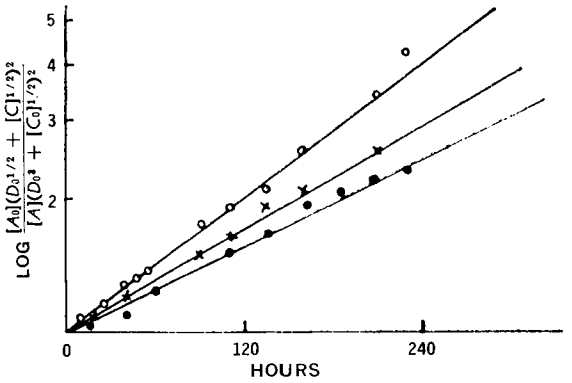

Figure 18—*Decomposition of aspirin at different vapor pressures. Key: ○, 233 torr; ×, 200 torr; and ●, 181 torr. See Eq. 34. Reprinted, with permission, from Ref. 64.*

calculate $k_H k'^{3/2}$ (knowing the value of K). This value should depend on temperature *via* the following equation:

$$\log k_H + 1.5 \log k' = \frac{-E}{2.3R}\frac{1}{T} + q \qquad \text{(Eq. 36)}$$

where E is the sum of activation and adsorption energies. The latter is negligible if the water is not chemisorbed. The value of E found (15 kcal) is indeed close to the 15.6 kcal found by Edwards (65) for aspirin decomposition in solution. In this manner, the Leeson-Mattocks (64) study provides complete accounting of the decomposition *via* solution kinetics in a saturated, sorbed moisture layer.

Kornblum (63) and Kornblum and Sciarrone (27) found that the lag time disappeared for the aminosalicylic acid system and that the rate constant increased with increasing moisture; they, however, could not quantitatively account for the decomposition as occurring in a saturated, sorbed moisture layer. They suggested that the layer was "stagnant" and might not be completely saturated; *i.e.*, the decomposition might be diffusion controlled. Carstensen (66) showed that, in such a case, the saturation term (S) should be replaced by:

$$C = S[1 - e^{-\alpha l}]/\alpha \qquad \text{(Eq. 37)}$$

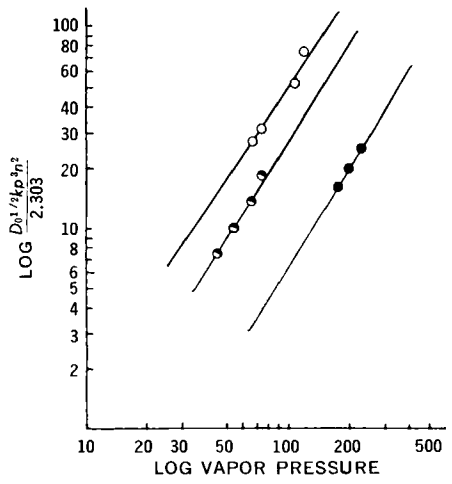

Figure 19—*Aspirin data in Fig. 18 plotted according to Eq. 35 at different vapor pressures. Key: ○, 233 torr; ◒, 200 torr; and ●, 181 torr. Reprinted, with permission, from Ref. 64.*

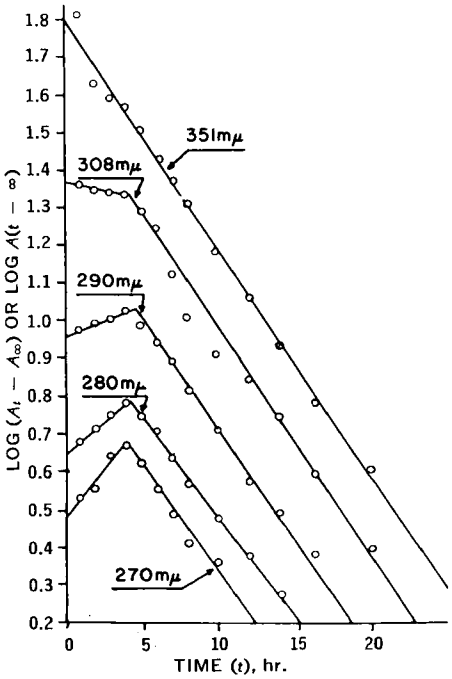

Figure 20—*Pseudo-first-order photolysis of fumagillin. Reprinted, with permission, from Ref. 67.*

where $\alpha = k/D$, l is the layer thickness, k is the rate constant, and D is the diffusion coefficient.

PHOTOLYSIS

There are a few examples of photolysis of pharmaceutical compounds, notably the work by Eble and Garrett (67). From their work shown in Fig. 20, it can be seen that the decomposition is first order; although no general statement can be made to this effect, it is frequently true. DeMerre and Wilson (68) found the photodecomposition of cyanocobalamin to be first order, and Carstensen (69) found that vitamin A in gelatin-coated beadlets decomposed in a first-order type of reaction. Savard *et al.* (70) reported on photodecomposition of steroids.

DECOMPOSITION OF SOLID DOSAGE FORMS

From what has been stated regarding pure compounds, it would appear that both zero- and first-order patterns of decomposition might be expected in solid dosage forms. The theories relating to decomposition in sorbed moisture layers should be particularly attractive for solid dosage forms since moisture is always present to some extent. This would lead one to expect that zero-order patterns would predominate. The development pharmacist and chemist need to establish the order with which a dosage form decomposes and whether or not Arrhenius plotting is possible. A large number of one- and two-component systems have already been quoted where this was possible.

Haynes *et al.* (71) reported on first-order decomposition of chlortetracycline capsules. Carstensen (72) reported on the first-order decomposition of vitamin A in tablets, and Tardif (73) reported on first-

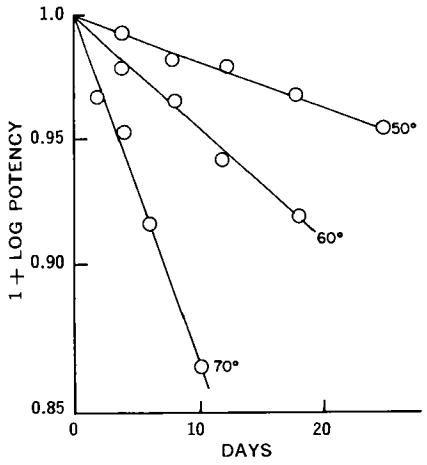

Figure 21—*Pseudo-first-order plots of thermal decomposition of ascorbic acid in a solid dosage form. Adapted, with permission, from Ref. 73.*

order decomposition of vitamins B_1, C, and A in tablets. In the latter two studies, there was adherence to an Arrhenius equation. The data by Tardif are shown in Figs. 21 and 22.

Enezian (74) showed that aspirin in tablets made in a microcrystalline cellulose base decomposed by a first-order pattern and that the rate constants adhered to an Arrhenius equation. The data by Leyden *et al.* (75) regarding reserpine also imply first-order decomposition. In fact, there is a general tendency to expect first-order decomposition; nomograms and stability designs assuming first-order decomposition have been constructed (76, 77), although these are not necessarily restricted to solid dosage forms.

In some cases, it is (as mentioned) difficult to distinguish between first and zero order. The decomposition of lactose, aside from the quoted work of Brownley and Lachman (46), was also reported (78–80) with much the same conclusions. Distinction between orders is also difficult in the decomposition of thiamine, riboflavin, and niacinamide (81–83), thiamine mononitrate (84), and ascorbic acid (85), all decompositions relating to solid dosage forms. On the other hand, the data relating to decomposition in tablets of vitamin A, cyanocobalamin, ascorbic acid, and pantothenic acid (81–83) are first order. In the case of cyanocobalamin, Macek and Feller (86) showed that the decomposition at higher temperatures is catalyzed by thiamine decomposition products and, hence, is complicated.

EQUILIBRIA IN SOLID DOSAGE FORM STABILITY

The earlier quoted bacitracin work (47) also covered stability of troches containing bacitracin. From the data (Table III and Fig. 23), it appears that equilibrium at 65% retained is implied. Equilibria are much more common in solid dosage forms than in solutions or pure solids, and there are several reasons why this is the case. It may only be an apparent equilibrium (and, indeed, dictated by a more correct topochemical relation) but frequently it offers a sound procedure for extrapolation in predictive work.

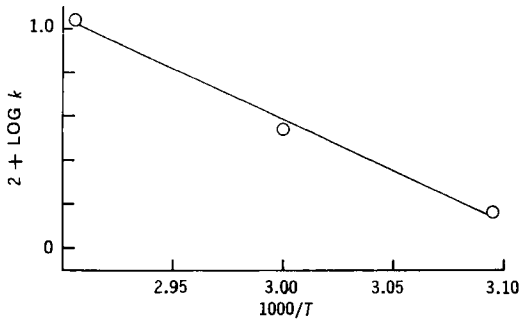

Figure 22—*Arrhenius plots of the slopes from Fig. 21. Adapted, with permission, from Ref. 73.*

Actual reasons for equilibria in solid dosage forms may be chemical, as shown earlier for vitamin D_2 (52). Takahashi and Yamamoto (52) found that various excipients (dicalcium phosphate and talc) accelerated the approach to equilibrium and tied this in with surface acid on the excipients; experiments in hexane solution showed this to be the case in solution as well. Other compounds (ascorbic acid, folic acid, thiamine hydrochloride, and pyridoxine hydrochloride), having a pKa in the dry state of 2–4, also catalyzed the isomerization; but calcium pantothenate and niacinamide, having a "dry pKa" of 4.8–6.8, did not catalyze the isomerization. Carstensen *et al.* (87) showed that vitamin A in gelatin beadlets and vitamin E succinate (deesterification) approach equilibria in solid dosage forms and that the data can be plotted *via* a Van't Hoff (rather than an Arrhenius) plot (Fig. 24). In this case, two kinetic parameters (rate constant and equilibrium level) are sought. Simple procedures for attaining them have been published (87).

Some equilibria are predicated on competitive adsorption on a carrier [thiamine on microcrystalline cellulose (88)] (Fig. 25).

Kornblum and Zoglio (89) demonstrated that impurities present on the surface of solid excipients could give rise to equilibrium kinetics in solid dosage forms and described a suspension technique whereby the effect of particular batches of calcium stearate on the stability of aspirin could be predicted. It is assumed that there are surface impurities in an excipient and that they go into solution in the sorbed moisture layer where they can react with the active ingredient, *A*; the reaction continues until the impurity (*I*) is exhausted (Scheme VI):

$$A + I \longrightarrow C$$

Scheme VI

so:

$$-dC/dt = dI/dt = -kIA = -k'I \qquad \text{(Eq. 38)}$$

where the amount of active ingredient dissolved in the moisture layer, *A*, is the saturation concentration and hence constant. Equation 38 can be integrated to:

$$I = I_0 e^{-k't} \qquad \text{(Eq. 39)}$$

The amount of active compound lost is equivalent to

Table III—Potency of Bacitracin Troches

Number of Months Stored	Potency Retained, A		A − 65, 25°	log(A − 65), 25°
	40°	25°		
0	100	100	35	1.544
3	69	93	23	1.447
6	67	80	15	1.177
12	66	78	13	1.114
18	64	75	10	1.000
24	63	69	4	0.610

the amount of impurity lost (*i.e.*, $V[I_0 - I]$, where V is the volume of the sorbed layer). If the active component only degrades *via* Scheme VI, then the maximum loss is VI_0, so that $A_\infty = A_0 - VI_0$, and mass balance then dictates:

$$A - A_\infty = VI_0 e^{-k't} \qquad \text{(Eq. 40)}$$

The equilibrium level is *independent of temperature* whereas, of course, k' is temperature dependent. If this type, as is often the case, is superimposed on a regular decomposition, then time–potency curves will intersect the potency axis at lower than initial (and theoretical) potency; this is then referred to as "manufacturing loss," although it may truly be a "raw material loss."

If a limited amount of moisture is present, then Eq. 40 may still apply where now I_0 is the limited amount of moisture present. This can be superimposed on a first- or zero-order reaction to give a less than 100% intercept at time zero. The bend in the curve in this case would be a measure of the amount of water present (and would be temperature independent). To this end, it is important to be able to distinguish between free and bound moisture; Armstrong and Griffiths (90) published a method for such a differentiation.

DECOMPOSITIONS INVOLVING MOISTURE

Aspirin tableted in a microcrystalline cellulose base degrades by a first-order reaction as shown by Enezian (74). Other published data (91–93) imply that the trend reverts to a zero-order pattern with higher moisture contents. As pointed out earlier, the nature of a raw material can greatly influence the stability of a dosage form. Table IV, taken from the

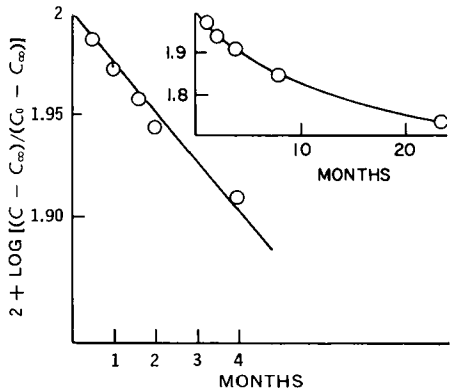

Figure 23—*Equilibrium treatment of data by Gross* et al. *(47).*

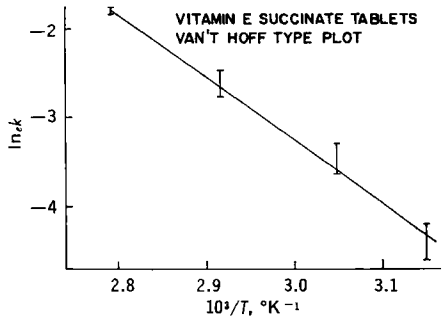

Figure 24—*Van't Hoff plot of vitamin E succinate decomposition in a solid dosage form. Adapted, with permission, from Ref. 87.*

work of Gold and Campbell (93), shows the effect of different talcs on the stability of aspirin tablets. Carstensen *et al.* (94) showed that vitamin A beadlets compressed in lactose degrade with rate constants that are proportional to the third power of the water content. *Stability programs should always include samples that have artificially been stressed by moisture addition. One purpose of a stability program should be to define the stability of the dosage form as a function of moisture content.*

SOLID-SOLID INTERACTIONS IN DOSAGE FORMS

There are only a few implied cases of solid-solid interactions as such in pharmaceutical dosage forms, although such interactions apparently would occur in many instances. The work by Troup and Mitchner (95) is classical and is illustrated in Fig. 27. It was concluded that in solid dosage forms containing aspirin and phenylephrine, there is a correlation between loss rates of both compounds. Chromatography proved the existence of acetylated phenylephrine in the dosage form, so that the acetic acid liberated in the aspirin decomposition resulted in acetylation of the phenylephrine; this is borne out by Fig. 27. Jacobs *et al.* (96) showed that a similar phenomenon takes place in aspirin-codeine combinations in solid

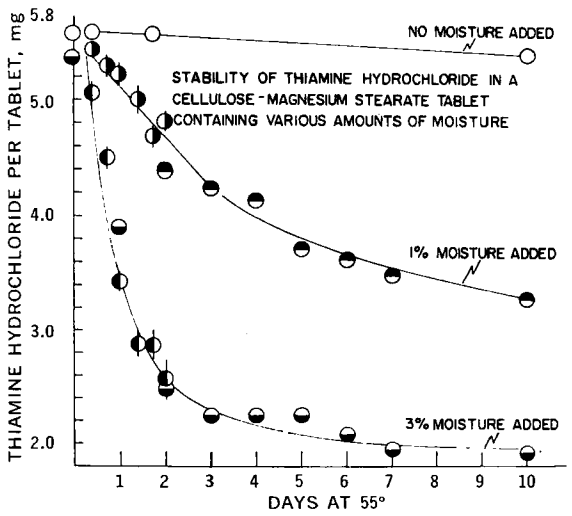

Figure 25—*Equilibrium attained in adsorption process in solid dosage form. Adapted, with permission, from Ref. 88.*

Table IV—Free Salicylic Acid (Percent) in Aspirin Tablets with Various Sources of Talc (93)

Weeks	Talc A	Talc B	Talc C	Talc D
0	0.1	0.1	0.1	0.1
1	0.13	0.30	0.93	0.28
2	0.23	0.69	2.61	0.57
4	0.41	1.07	5.85	1.12
8	0.32	2.05	13.00	1.46
12	0.80	3.35	25.80	2.96

dosage forms. Eppich *et al.* (97) showed that aspirin in alkaline environment degrades by a topochemical relation that adheres to Eq. 3*a* above 46° and to Eq. 7 below 46°.

The detection of solid–solid interactions in drug dosage form development programs is of great importance. By detecting so-called *incompatibilities* early in a development program, it is possible to formulate dosage forms with ingredients that are least likely to interact; this type of screening program is usually referred to as a "compatibility program" or a "preformulation screening program." A detailed, simple program was described by Carstensen *et al.* (98), and Table V shows the "general performance" of several excipients in 3 years of program performance.

The test simply consists of mixing drug and excipients in the stated amounts in a sealed vial and storing for 14 days at 55°. Half of the vials have moisture (5%) added to them, so that there is a dry and a "wet" rating. The appearance is gauged visually but, in later years, TLC has been employed as well to detect chemical interactions. Although the method provides a good preformulation screen, it is crude in quantitative aspects.

Lach and coworkers (99–107) developed methods for screening by using diffuse reflectance spectroscopy. In most of these investigations, *solutions* of drug were exposed to the adsorbent and equilibrated (often for 24 hr.) at 30°. These solutions were then vacuum dried (often at 40°). These "incompatibilities" are hence solvent mediated; they are an excellent quantitative refinement of the "wet" ratings referred to above but do not throw light on the dry ratings.

Compatibility tests give information about the chemical and physical stability that might result from storage of a dosage form containing the tested combination; one cannot necessarily draw conclusions regarding therapeutic availability from such tests. For instance, Su (108) showed that certain adsorbates on montmorillonite are of an ion-dipole nature and, hence, are not chemisorbed. They nevertheless cause shifts in diffuse reflectance spectroscopy spectra. The alleged interaction between silica gel and ascorbic acid (99–101) was shown (109) to be an interaction with moisture in that the ascorbic acid loss is directly proportional to the unbound moisture in the system. Bioassays furthermore showed that the bioavailability of ascorbic acid is unaffected by the presence of silica gel.

Blaug and Huang (110) demonstrated the interaction between dextroamphetamine sulfate and spray-dried lactose in a quantitative fashion by use of a (ethanol-mediated) diffuse reflectance spectroscopy technique.

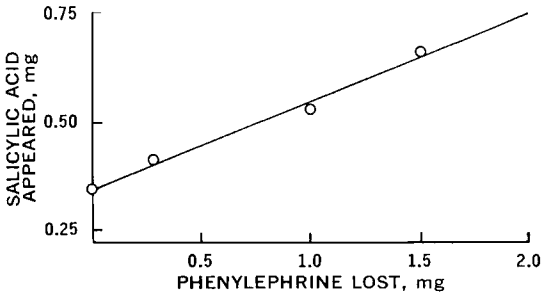

Figure 27—*Correlation between aspirin and phenylephrine stability (4 weeks, 70°) in solid dosage forms. Adapted, with permission, from Ref. 95.*

LIGHT SENSITIVITY OF SOLID DOSAGE FORMS

It was shown (111) that photolysis of surface molecules should give rise to first-order decay; free radicals were shown (112) to be of importance in this type of reaction. Other reports (113–120) established that the decay pattern consists of three consecutive first-order decays (Fig. 28) and stressed that the fad-

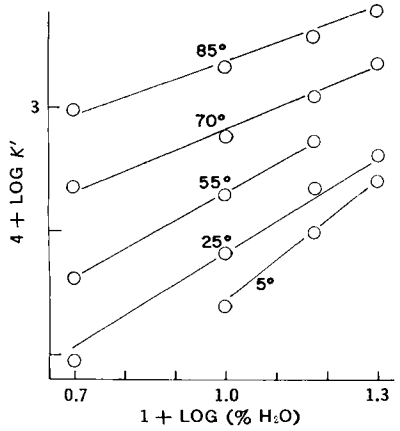

Figure 26—*Effect of moisture on vitamin A beadlet stability. Adapted, with permission, from Ref. 94.*

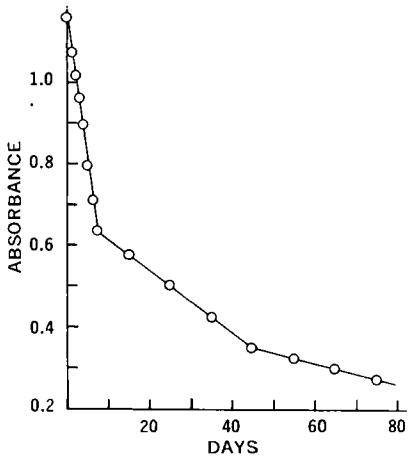

Figure 28—*Fading of the surface of tablets colored with FD&C Blue No. 1. Adapted, with permission, from Ref. 114.*

Table V—Categories for Two-Component Systems[a] (98)

		Identical	17–27 Months at 25° Worse	10 Days at 55° Worse	Total Score 25°	Total Score 55°
Drug *per se*	Dry	15	4	1	38	31
	5% water	9	8	3	49	38
Drug + magnesium stearate	Dry	16	3	1	34	30
	5% water	15	4	1	43	35
Drug + calcium stearate	Dry	13	4	3	37	32
	5% water	12	5	3	38	35
Drug + stearic acid	Dry	15	5	0	42	31
	5% water	7	11	2	60	38
Drug + talc	Dry	14	5	1	38	30
	5% water	10	8	2	45	34
Drug + acid-washed talc	Dry	12	8	0	44	31
	5% water	10	9	1	49	35
Drug + lactose	Dry	12	5	3	38	32
	5% water	9	7	4	65	56
Drug + calcium phosphate, dibasic, anhydrous	Dry	12	6	2	46	36
	5% water	9	8	3	66	53
Drug + starch	Dry	12	5	3	39	34
	5% water	10	5	5	40	37
Drug + mannitol	Dry	10	7	3	39	31
	5% water	8	7	5	47	45
Drug + terra alba	Dry	14	6	0	41	28
	5% water	11	6	3	50	45
Drug + sugar 4x	Dry	12	6	2	41	34
	5% water	9	7	4	63	61

[a] Values in fourth and fifth columns represent scoring from 1 to 6 for degree of color change (1 = unchanged, 6 = substantially changed) and from 1 to 6 for degree of fineness change.

ing is not necessarily three different mechanisms with different rates but that the probable causes are alteration in tablet surface and fading of subsequent layers. The photolysis is a *surface phenomenon* and, in most cases, the interior of the tablet will be unaffected.

It has been established (113–118) that when the fading has penetrated to a depth of 0.03 cm, the tablet surface appears white and that the faded layer does not become thicker upon further light exposure. This has also been demonstrated for plastics (121–123). The ratio of absorbed to scattered light, θ, relates to the fraction, R, absorbed to reflected light by the relation (124):

$$\theta = [1 - R^2]/2R \qquad \text{(Eq. 41)}$$

By use of this relation, Everhard and Goodhart (125) and Goodhart *et al.* (126, 127) showed that a suitable presentation of data consists of using "color difference" as the ordinate and "footcandle hours" as the abscissa.

APPEARANCE STABILITY

Appearance can be quantitated by reflectance measurements (98); tristimulus tests (which give percent reflectance X, Y, and Z at three wavelength bands) can be performed on tablet surfaces, and the parameter x, y, or z, where $x = 100X/(X + Y + Z)$, can be used for plotting. Plots such as the one shown in Fig. 29 result. After evaluation of the asymptote, plotting of log $[x/x_\infty]$/log $[x_0/x]$ usually gives a straight line (Fig. 30). The slopes of these lines are a type reaction constant k, and these can be plotted in Arrhenius plots; constants at 25° can be calculated from accelerated data (Fig. 31).

SUMMARY

It has been shown that solids *per se* decompose ei-

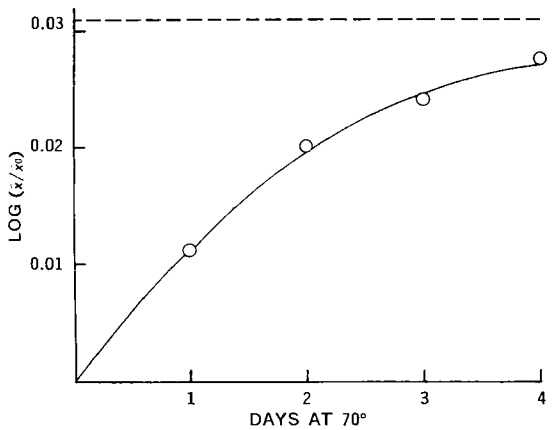

Figure 29—*Reflectance meter readings as a function of age of tablet. Adapted, with permission, from Ref. 98.*

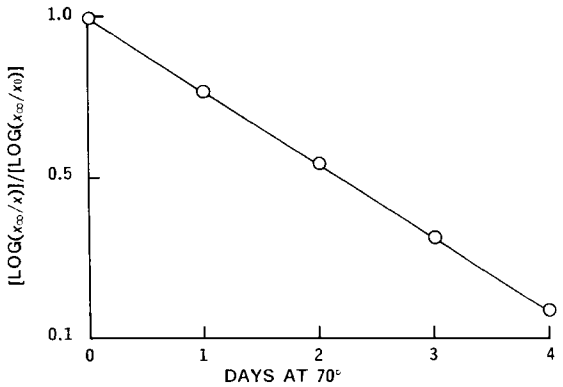

Figure 30—*Data in Fig. 29 plotted after adjustment for infinity value. Adapted, with permission, from Ref. 98.*

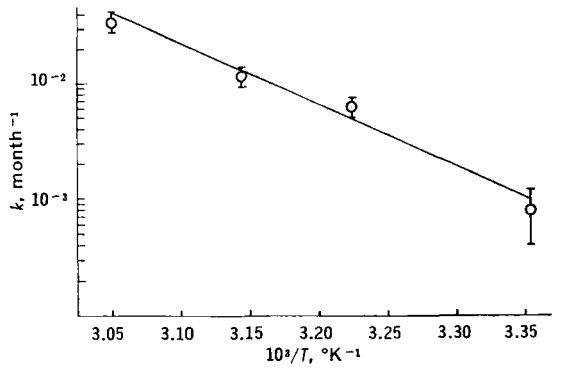

Figure 31—*Data from plots of the type shown in Fig. 30, plotted in formal Arrhenius fashion. Adapted, with permission, from Ref. 98.*

ther topochemically, in which case the decomposition appears *first order*, or by Prout-Tompkins kinetics, in which case *S-shaped curves* result. The logarithm of rate constants is, in a homologous series, often proportional to the reciprocal of the absolute melting temperature. When moisture is present, decomposition is often accounted for by solution kinetics of a saturated, sorbed solution, in which case, barring complications, the decomposition is *zero order*. If the decomposition is diffusion controlled, then it still appears zero order, but the solubility term is replaced by a smaller, constant concentration. In either event, the rate constants are amenable to *Arrhenius plotting*. In the case of interacting substances, the equation $[1 - (1 - x)^{1/3}]^2 = kt$ often holds.

These considerations often apply to solid dosage forms, with the one added feature that *equilibria* frequently occur. If these are of strictly chemical nature, then a *Van't Hoff plot* can be used for prediction of shelflife. Equilibria due to limited moisture content and limited impurity content have equilibrium levels that are temperature independent. Equilibria may also be due to competitive adsorption. The outlined principles allow meaningful prediction by accelerated testing methods when reaction patterns have been elucidated.

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Structure–Activity Relationship Studies on Inhibition of Histamine Uptake by Rabbit Blood Platelets

JOUKO TUOMISTO*, E. J. WALASZEK*, and E. E. SMISSMAN‡×

Abstract □ The correlation of histamine uptake inhibition with small changes in inhibitor structure was studied to determine the chemical requirements for the inhibitor. ^{14}C-Histamine was added to platelet-rich rabbit plasma *in vitro* with or without inhibitor, and the total accumulation of radioactivity into the platelets was counted. Several amino acids, amines, and their derivatives possessing an imidazole ring, an indole nucleus, or an aliphatic chain were studied. The results indicate that the most important function for the inhibitory effect is a protonated amino group. If the positive charge is decreased or if a negatively charged group is in the vicinity of the amino function, the activity is decreased. The results are the same with indole, imidazole, and aliphatic derivatives. The secondary binding, which evidently takes place through the heterocyclic ring or aliphatic chain, appears to be quantitatively less important, although it contributes to the relative potency of different amines. It is suggested that the primary binding is due to ionic bond formation between the positively charged nitrogen and some negative group in the carrier and the secondary binding to some weak forces such as a π-bond or hydrophobic forces. A few amines, such as butylamine and β-phenethylamine, increase rather than inhibit histamine uptake in long incubations, although they too are inhibitory in short incubations. This effect appears to be due in part to histamine catabolism inhibition, which prevents the fall in histamine concentration in the plasma, which then overcomes the uptake inhibiting effect of the substances.

Keyphrases □ Histamine uptake by rabbit blood platelets, inhibition—correlation between inhibitor activity and structure □ Inhibition of histamine uptake by rabbit blood platelets—structure–activity relationships □ Blood platelet uptake of histamine, inhibition—structure–activity relationships □ Amines, as inhibitors of histamine uptake by rabbit blood platelets—correlation between activity and structure

Histamine is taken up by blood platelets by what appears to be an active process (1). It is probable that a common mechanism operates for several amines such as 5-hydroxytryptamine, tryptamine, and phenethylamines, although the affinity of 5-hydroxytryptamine appears to be by far the highest (1, 2). Other amines inhibit the uptake of histamine (1). Because their structures differ from each other, it was decided to compare the chemistry and activity of some compounds related to the inhibitory amines to determine which parts of the molecule are essential to the uptake inhibition. This finding could then be correlated with the affinity of the compound to the carrier operating at the cellular membrane (3–6). Both imidazole and indole derivatives as well as some aliphatic compounds were studied.

EXPERIMENTAL

Male albino rabbits, weighing 2.0–2.5 kg, were bled, and platelet-rich plasma was prepared as previously described (7). Radioactive histamine was added to ice-cold plasma to give the final concentration of 10^{-6} *M*. Two-milliliter portions of plasma were immediately added to incubation tubes containing 0.2-ml saline solutions of the substances to be tested. All concentrations refer to the final concentration in 2.2-ml samples. After incubation for 80 min at 37° under an atmosphere of oxygen containing 5% carbon dioxide, the platelets were sedimented by centrifugation for either 30 min at 2000×*g* or 5 min at 20,000×*g* and were washed once with 2 ml of saline to remove the extracellular radioactivity. Washed platelets were solubilized[1] and the radioactivity was determined in a scintillation counter[2]. Results are given as percent inhibition calculated compared to a control sample incubated with the solvent at the same time and under the same conditions. All experiments were performed in duplicate, and the difference between duplicates was usually 0–2%.

Chemicals—The following were used: *n*-propylamine[3], *n*-butylamine[3], isobutylamine[3], *n*-pentylamine[3], isopentylamine[3], *n*-hexylamine[3], phenylacetic acid[3], *dl*-α-aminobutyric acid[4], *dl*-β-aminobutyric acid[4], γ-aminobutyric acid[4], α-aminoisobutyric acid[4], β-aminoisobutyric acid[4], *l*-histidine hydrochloride[5], melatonin[5], *N*-methyltryptamine hydrogen oxalate[5], tryptamine hydrochloride[6], 5-hydroxytryptamine creatinine sulfate[6], β-phenethylamine hydrochloride[7], 2-mercaptoethylamine[7], bufotenine[7],

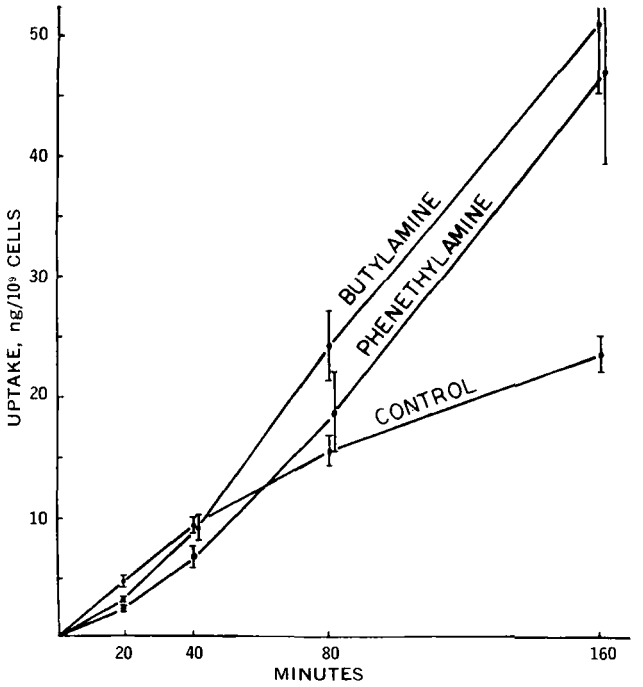
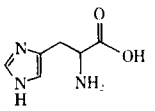
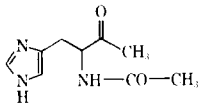
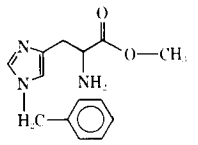
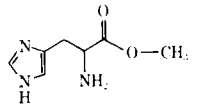
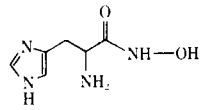
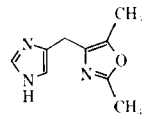
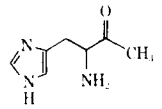
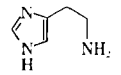


Figure 1—*Time course of the inhibition and increase of histamine uptake by 10^{-3} M n-butylamine and 10^{-3} M β-phenethylamine. Mean ± SE of six experiments.*

[1] Soluene-100.
[2] Packard Tri-Carb 3320.
[3] Matheson, Coleman and Bell.
[4] Nutritional Biochemicals Corp.
[5] California Corporation for Biochemical Research.
[6] Sigma Chemical Co.
[7] Calbiochem.

Table I—Inhibition of ^{14}C-Histamine Uptake (in Percent) by Some Histamine Analogs[a]

Inhibitor	10^{-5} *M*	10^{-4} *M*	10^{-3} *M*
I. Histidine 	—	—	−0.5 ± 2.2 (6)
II. 4-Imidazolyl-3-(*N*-acetyl)-amino-2-butanone 	−2.8 ± 1.7 (5)	7.3 ± 2.1 (5)	8.5 ± 2.8 (6)
III. 3-(1′-*N*-Benzyl-4′-imidazolyl)-2-aminopropionic acid methyl ester 	—	8.3 (3)	31.4 ± 3.2 (6)
IV. Histidine methyl ester 	—	20.9 ± 2.3 (5)	37.3 ± 3.6 (8)
V. Histidine hydroxamic acid 	—	25.7 ± 3.3 (7)	35.4 ± 3.5 (7)
VI. 4-(4-Imidazolylmethyl)-2,5-dimethyloxazole 	−0.5 ± 2.5 (5)	12.7 ± 2.9 (7)	44.8 ± 4.1 (8)
VII. 4-Imidazolyl-3-amino-2-butanone 	6.4 ± 2.6 (6)	21.8 ± 2.7 (8)	58.9 ± 2.2 (8)
VIII. Histamine (nonradioactive) 	—	27.4 (6) —	58.8 (2) 49.1 ± 3.7 (7)[b]

[a] Platelets were incubated in plasma for 80 min at 37° under oxygen with 5% carbon dioxide. Values are the means ± *SE*. Number of experiments is given in parentheses. [b] The latter is an earlier result using 160 min incubation.

methylamine hydrochloride[8], ethylamine hydrochloride[8], ethanolamine[9], histamine-(ring-2-^{14}C) hydrochloride[10], and scintillation chemicals[11]. The other substances were synthesized according to Smissman and Weis (8).

RESULTS AND DISCUSSION

Inhibition by Histamine Analogs (Table I)—Nonradioactive histamine, 10^{-3} *M*, inhibited ^{14}C-histamine uptake about 50%, and two of the other imidazoles (VI and VII) were comparable to histamine. Histidine was totally ineffective, and the effect of Compound II was negligible. Other compounds were intermediate in their ability to block histamine uptake. Thus, compounds that have an amino group capable of being protonated and that do not have a negative ion directly adjacent to the protonated nitrogen were effective. If the ability of the side-chain amino function to protonate was abolished by amide formation, no inhibition of histamine uptake was observed (compare Compounds II and VII). If the ability of an adjacent carboxyl to form an anion was removed through ester formation, the compound became effective again (compare Compounds I and IV).

Inhibition by Tryptamine Analogs (Table II)—Both tryptamine and 5-hydroxytryptamine were very potent histamine uptake inhibitors. As in the case of histidine, the amino acid 5-hydroxytryptophan, IX, was completely devoid of effect. The *N*-acetylated derivative, melatonin, was also ineffective, as was Compound XI, in which the amino group is also acylated. Although *N*-methyltryptamine and bufotenine were active compounds, *N*-monomethylation and *N,N*-dimethylation of 5-hydroxytryptamine decreased the activity compared to that of the parent compound.

Inhibition by Aliphatic Compounds (Table III)—An aromatic or heterocyclic ring was not an absolute necessity for uptake inhibition, since simple aliphatic amines had some effect. In the aliphatic series, the potency increased with an increase in chain length, with the exception of *n*-butylamine. Methylamine was ineffective and *n*-hexylamine was the most effective of the aliphatic amines studied.

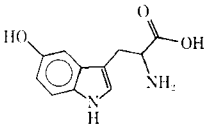
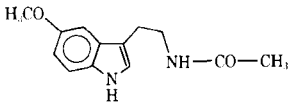
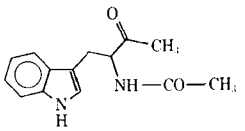
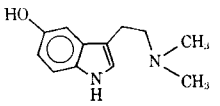
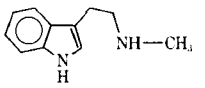
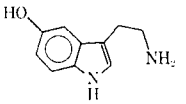
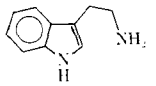
When a hydroxyl or thiol group is introduced two carbons from the amino function in the aliphatic amines, there is an increase in potency over that of the parent amine.

The aliphatic amino acids were ineffective in their ability to inhibit histamine uptake (Table III).

Effect of Butylamines and β-Phenethylamine—*n*-Butylamine and 3-methylbutylamine (isopentylamine) in high concentrations significantly increased histamine uptake. Since β-phenethylamine has a similar effect and also inhibits histamine metabolism (1, 9), the time course of their effects was studied (Fig. 1). Both *n*-butylamine and β-phenethylamine inhibited histamine uptake during short incubation periods ($p < 0.001$). After 1 hr, the rate of histamine uptake in control samples decreased, but it continued lin-

[8] Eastman Organic Chemicals.
[9] K & K Laboratories.
[10] Amersham/Searle Corp.
[11] Packard Instrument Co.

Table II—Inhibition of ^{14}C-Histamine Uptake (in Percent) by Some Tryptamine Analogs[a]

Inhibitor		10^{-5} *M*	10^{-4} *M*	10^{-3} *M*
IX. 5-Hydroxy-tryptophan		—	—	0.1 ± 0.5 (5)
X. Melatonin		—	−1 ± 0.8 (5)	−6.0 ± 3.8 (5)
XI. 4-Indolyl-3-(*N*-acetyl)-amino-2-butanone		—	−1.5 ± 1.1 (4)	1.6 ± 2.3 (3)
XII. Bufotenine		14.7 ± 2.1 (4)	35.0 ± 2.1 (4)	—
XIII. *N*-Methyl-tryptamine		18.8 ± 1.8 (4)	46.4 ± 1.8 (4)	—
XIV. 5-Hydroxy-tryptamine		15.8 ± 3.7 (6)	58.0 ± 1.1 (6)	86.0 ± 0.6 (3)
XV. Tryptamine		23.8 ± 1.0 (6)	62.2 ± 1.1 (6)	70.6 ± 3.7 (2)

[a] Platelets were incubated in plasma for 80 min at 37° under oxygen with 5% carbon dioxide. Values are the means ± *SE*. Number of experiments is given in parentheses.

early or increased in samples containing *n*-butylamine or β-phenethylamine.

To determine if the uptake increasing activity of *n*-butylamine and β-phenethylamine could be due to acidic metabolites, butyric acid, phenylacetic acid, and other aliphatic and aromatic carboxylic acids were tested. They were found to be inactive in concentrations that could result from oxidation of the amine to the corresponding carboxylic acid in plasma ($<10^{-3}$ *M*). However, at a concentration of 10^{-2} *M*, all tested acids showed some activity with the exception of acetic acid, which may be rapidly removed by metabolic processes. At a concentration of 3×10^{-2} *M*, phenylacetic and butyric acids more than doubled histamine uptake during 80 min of incubation.

It can be concluded from the results that the most important group for histamine uptake inhibition is a protonated amino function. The amount of substitution on the amino nitrogen does not appear to be critical since amines other than primary amines are also active.

Since 5-hydroxytryptamine and tryptamine are by far the most effective of the compounds studied, it can be assumed that the indole ring is a favorable function for a fit to the carrier-receptor. This is in agreement with the fact that 5-hydroxytryptamine is taken up far more effectively by platelets than either histamine (1) or catecholamines (10–12). Since tryptamine and 5-hydroxytryptamine show only a minor difference in uptake inhibition, the 5-hydroxyl group evidently is not critical. Both in this laboratory and in earlier work (1), tryptamine was found to be more effective in low concentrations as an uptake inhibitor whereas 5-hydroxytryptamine became relatively more potent with increasing concentrations. A possible explanation is that in low concentrations 5-hydroxytryptamine is rapidly and effectively taken up itself and stored in the amine granules (13). Therefore, during the prolonged incubation, there is no longer an effective concentration of 5-hydroxytryptamine in the incubation medium unless the capacity of the storage mechanism is exceeded. Tryptamine, however, is stored much less effectively (14). Thus the molecules taken up by the platelets could leak out again, giving inhibition even with lower concentrations.

On the basis of these results, the following type of attachment is suggested for the amine carrier operating on the outer membrane of the platelet. The amine molecule is attached primarily by ionic binding through its positively charged nitrogen to some negatively charged group in the carrier. This type of binding is suggested to be the most powerful because various amines having no other active groups cause some inhibition, whereas molecules having the preferred binding function except for the charged nitrogen are ineffective. Secondary binding seems to occur through the aromatic ring. The indole ring appears preferable to others, but the imidazole ring and possibly even an aliphatic carbon chain seem to replace it to some extent. Amphetamine was reported earlier to inhibit histamine uptake very effectively (1); thus the phenyl ring can also replace the indole ring. Because of these relatively flexible requirements and because no substance lacking an amino nitrogen was an inhibitor, it is suggested that this secondary binding is relatively weak and might be due to π-binding, charge transfer binding, or hydrophobic binding.

Because of the small difference observed between 5-hydroxytryptamine and tryptamine, the 5-hydroxyl group evidently has no special role in binding, although it evidently is important for binding in storage granules (14). The role of the heterocyclic ring is not excluded and binding through the indole ring could explain the increased activity of the tryptamine compounds. The reasons for the time course effect noted with butylamine and β-phenethylamine are under study. It is known that histamine is partly metabolized during the incubation (1, 9) and that phenethylamine inhibits this metabolism, which increases its availability in plas-

Table III—Inhibition of ^{14}C-Histamine Uptake (in Percent) by Some Aliphatic Compounds[a]

Inhibitor		10^{-4} *M*	10^{-3} *M*
XVI. Methylamine	$CH_3—NH_2$	−0.6 ± 1.1 (4)	−21.3 ± 4.4 (4)[b]
XVII. Ethylamine	$CH_3—CH_2—NH_2$	0.6 ± 1.0 (5)	17.0 ± 3.9 (5)
XVIII. *n*-Propylamine	$CH_3—(CH_2)_2—NH_2$	18.6 ± 3.5 (4)	30.8 ± 5.8 (4)
XIX. Isobutylamine	$CH_3—CH_2—CH(CH_3)—NH_2$	20.9 ± 1.0 (3)	42.8 ± 1.5 (3)
XX. *n*-Butylamine	$CH_3—(CH_2)_3—NH_2$	15.6 ± 6.1 (4)	−53.8 ± 13.7(4)[b]
XXI Isopentylamine	$(CH_3)_2—CH(CH_2)_2—NH_2$	25.7 ± 2.6 (3)	−31.8 ± 3.1 (3)[b]
XXII. *n*-Pentylamine	$CH_3—(CH_2)_4—NH_2$	26.1 ± 1.6 (4)	14.0 ± 5.1 (4)
XXIII. *n*-Hexylamine	$CH_3—(CH_2)_5—NH_2$	37.4 ± 1.6 (8)	39.7 ± 7.5 (8)
XXIV. Ethanolamine	$HO—CH_2—CH_2—NH_2$	6.6 ± 1.5 (4)	29.6 ± 4.6 (3)
XXV. 2-Mercapto-ethylamine	$HS—CH_2—CH_2—NH_2$	32.9 ± 8.1 (4)	76.5 ± 6.3 (3)
XXVI. α-Aminobutyric acid	$CH_3—CH_2—CH(COOH)—NH_2$	—	1.6 ± 1.0 (5)
XXVII. β-Aminobutyric acid	$CH_3—CH(CH_2—COOH)—NH_2$	–	3.4 ± 2.0 (3)
XXVIII. γ-Aminobutyric acid	$HOOC—(CH_2)_3—NH_2$	—	−0.5 ± 1.0 (3)[b]

[a] Platelets were incubated in plasma for 80 min at 37° under oxygen with 5% carbon dioxide. Values are the means ± *SE*. Number of experiments is given in parentheses. [b] Negative number shows an increase in uptake.

Table IV—Effect of Some Carboxylic Acids on Histamine Uptake by Platelets[a]

Acid	10^{-3} *M*	10^{-2} *M*	3×10^{-2} *M*
Control	100	100	100
Aliphatic			
Acetic acid	99.6 ± 1.2	103.8 ± 0.7	105.5 ± 2.2
Propionic acid	101.9 ± 1.4	116.0 ± 4.9	39.6 ± 4.6
Butyric acid	113.9 ± 2.1	174.0 ± 8.6	223.5 ± 11.0
Aromatic			
Phenylacetic acid	102.2 ± 0.3	174.2 ± 4.7	212.3 ± 8.8
Indoleacetic acid	101.6 ± 2.9	129.9 ± 3.3	142.7 ± 16.7

[a] Incubation conditions were as in Table I. Acids were dissolved in 0.9% saline and neutralized with solid sodium bicarbonate to pH 7. Uptake is given as percent of control incubated with saline only. Values are the means ± *SE* of three to four duplicate experiments.

ma during the incubation. High concentrations of monocarboxylic acids increase 5-hydroxytryptamine uptake (15), and a similar increase in histamine uptake was found in this study (Table IV). Although the concentrations required were high, these findings may be relevant to the increase of histamine uptake by the corresponding amines. This interpretation is supported by the preliminary finding in these laboratories that monoamine oxidase inhibitions reversed the effect of butylamine and phenethylamine on histamine uptake.

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xTo whom inquiries should be directed.

Synthesis and Antimicrobial Properties of 17β-Amino-4-aza-5α-androstane and Derivatives

W. E. SOLOMONS[x] and N. J. DOORENBOS

Abstract □ To develop further structure–activity relationships relative to antimicrobial activity in 4-azasteroids, 17β-amino-4-aza-5α-androstane and derivatives were synthesized. While using the Leuckart–Wallach reductive amination to place a β-amine function in the 17-position, the 5-double bond of the 4-aza-5-en-3-one system was reduced stereospecifically and a dimethylaminoethyl group was eliminated from the 4-nitrogen in the same system. These observations should give insight into the mechanism of the Leuckart–Wallach reaction. Antimicrobial studies showed that the 17β-amino grouping almost completely abolishes activity.

Keyphrases □ 17β-Amino-4-aza-5α-androstane and derivatives—synthesis, screened for antimicrobial activity □ Azasteroids—synthesis of 17β-amino-4-aza-5α-androstane and derivatives, screened for antimicrobial activity □ Antimicrobial activity—4-azasteroids synthesized and screened □ Structure–activity relationships—4-azasteroids and antimicrobial activity

Previous studies showed that certain 4-azasteroids possess high antimicrobial activity (1). A study of structure–activity relationships among these steroids strongly indicated that polar functional groups in the vicinity of the 17-position greatly decreased antimicrobial potency (2–4). Since these findings, potent compounds with ester and ether groups in the 17- and 20-positions were synthesized (1, 5, 6). However, no basic amine or quaternary nitrogen functional groups have been substituted in this region of the molecule. To establish structure–activity relationships in this region of the molecule, the synthesis of 17β-amino-4-aza-5α-androstane and its derivatives was undertaken.

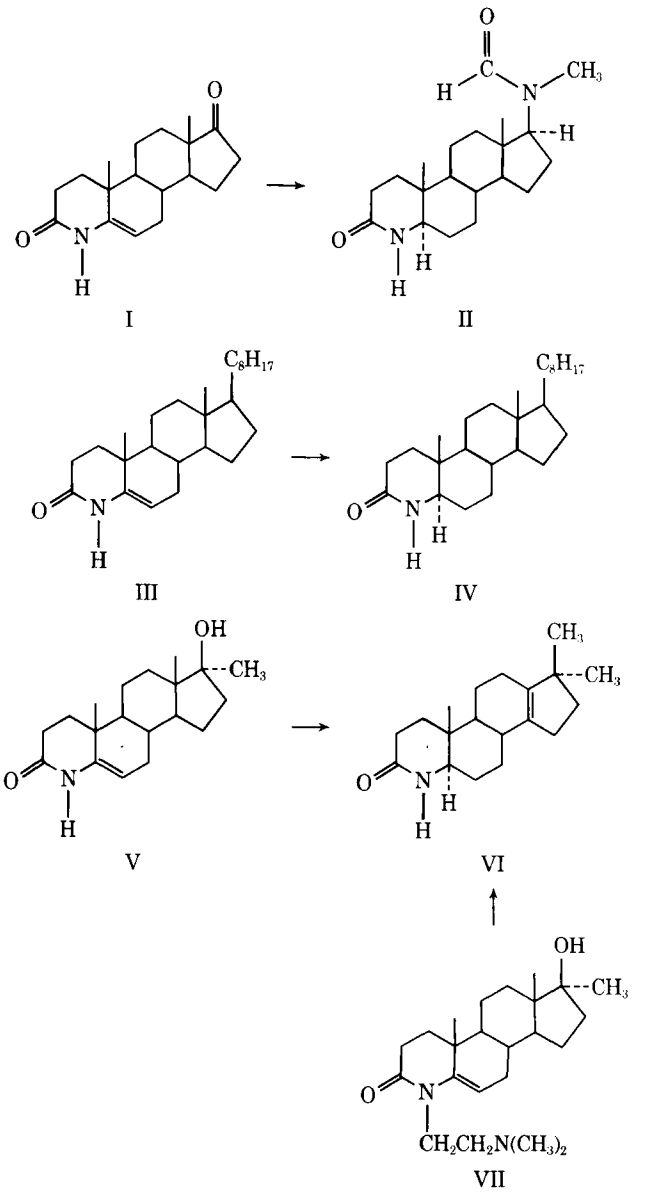
SYNTHESIS

While synthesizing the title compounds, a novel reaction was observed. This reaction proved to be of practical value in obtaining the target compounds. Scheme I outlines this reaction and the studies made of it.

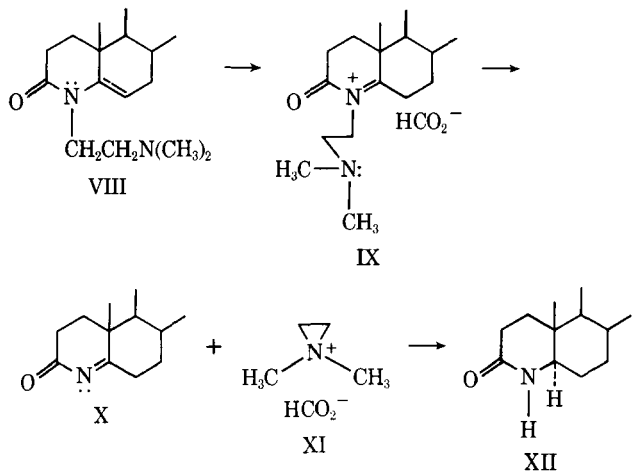
Compound I was produced by ozonolysis of androst-4-en-3,17-dione to 3,5-*seco*-4-norandrostan-5,17-dion-3-oic acid, followed by ring closure in concentrated ammonium hydroxide. The Leuckart–Wallach reductive amination of I was attempted to determine the stability of the 4-aza-5-en-3-one system under the rigorous conditions of this reaction. The formation of 17β-amine groups from 17-keto steroids by this reaction has been thoroughly studied (7–9). The product obtained from I was shown by IR, UV, NMR, and elemental analysis to have Structure II. It has been rigorously demonstrated (7–9) that this reaction gives an amide group with 17β-stereochemistry from 17-keto steroids.

The question of the configuration of the 5-position was answered by reacting Compound III under the same conditions as I. Melting point, mixed melting point, TLC, and optical rotation data proved IV to be the same as the known 4-aza-5α-cholestan-3-one (10), usually produced by catalytic hydrogenation of III. This method proved to be superior to catalytic hydrogenation (with stable molecules such as III) because of shorter reaction times and a cleaner product. Low-pressure hydrogenation of gram quantities of III usually required long reaction times (as much as 2 weeks in some instances) and large quantities of catalyst and gave a product contaminated with starting material.

On the other hand, attempts to convert more complex molecules such as V and VII to the corresponding saturated lactams by formic acid reduction gave rearrangement products. The Wagner–Meerwein rearrangements in the D-ring were not surprising (11). The elimination of the 4-dimethylaminoethyl group of VII was unexpected, but it can be rationalized if the reduction of the 5-double bond occurs according to the mechanisms for the Leuckart–Wallach reductive amination proposed either by Leonard and Sauers (12) or more recently by Lukasiewicz (13). Both mechanisms require the formation of a positive charge on the nitrogen, so the mechanism depicted in Scheme II is a possibility. The compounds obtained from V and VII were identical in physical properties and possessed superimposable NMR spectra. The successful completion of the reactions (Scheme I) indicated the route of



Scheme I

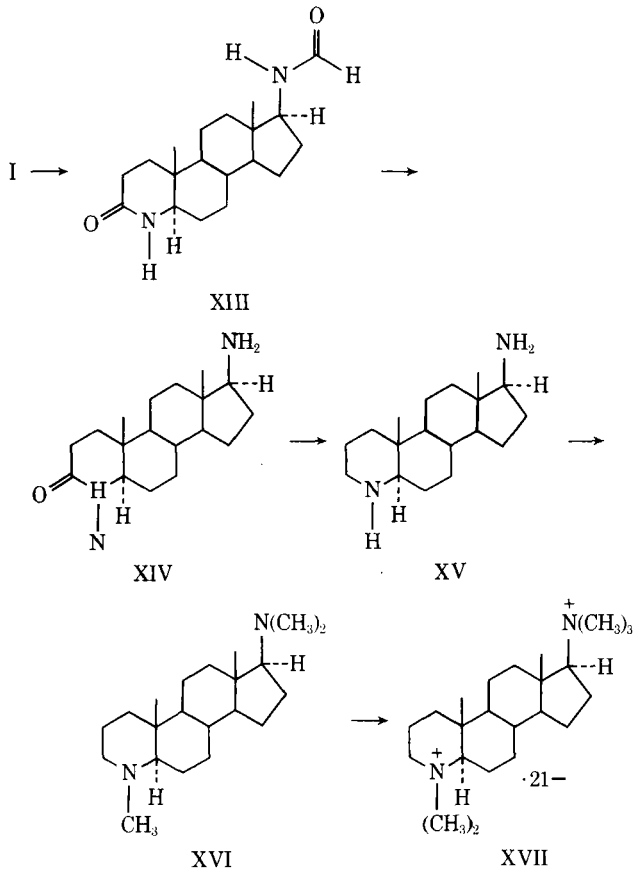


Scheme II

synthesis (Scheme III) to be taken for the title compounds. The Leuckart–Wallach reductive amination of I with formic acid and formamide gave XIII, which was converted to XIV by a simple acid-catalyzed hydrolysis. Lithium aluminum hydride reduction of XIV gave XV, one of the target compounds, 17β-amino-4-aza-5α-androstane. Two derivatives were prepared from XV, 17β-dimethylamino-4-methyl-4-aza-5α-androstane (XVI) and the corresponding bisquaternary salt XVII.

Another compound, 4,20-diaza-5α-cholestane, was similarly synthesized (Scheme IV) and may be considered as either an androstane or a cholestane.

The Leuckart–Wallach reaction has been used under various conditions and with slightly varying reagents (14). The reaction from I to XVIII is a variation utilizing the substrate, I, isohexylamine, and formic acid. The IR spectrum of XVIII showed replacement of the 17-ketone with the amide, but the UV spectrum



Scheme III

showed incomplete reduction of the 5-double bond. The reduction was completed by heating XVIII with formamide and formic acid, producing XIX. Compound XIX was converted to XX by lithium aluminum hydride reduction in tetrahydrofuran.

BIOLOGICAL

A twofold serial dilution assay was used to determine the approximate antimicrobial potency of Compounds XV, XVI, XVII, and XX. For comparison, ND-502 (4-methyl-4-aza-5α-cholestane), a 4-azasteroid of known activity, was tested by the same procedure.

The cell cultures were obtained from the Department of Biology, University of Mississippi. The strains of *Staphylococcus aureus* and *Escherichia coli* are identified by the numbers K-257 and ATCC-4157, respectively. *Saccharomyces cerevisiae, Aspergillus niger,* and *Bacillus cereus* cultures have been part of University stock cultures for many years and are typed periodically by students using microscopic and biochemical methods.

The first tube of each series contained 100 μg of sample; therefore, succeeding tubes contained 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 μg. The level of activity of the compounds was determined by choosing the last tube in which there was no noticeable growth of the organism. No statistical analyses were employed. To ensure that the cells were viable under test conditions, tubes containing no steroid were used as blanks.

The biological data are presented in Table I. The growth medium for the bacteria was nutrient broth; for the fungi, it was mycophil broth.

EXPERIMENTAL[1]

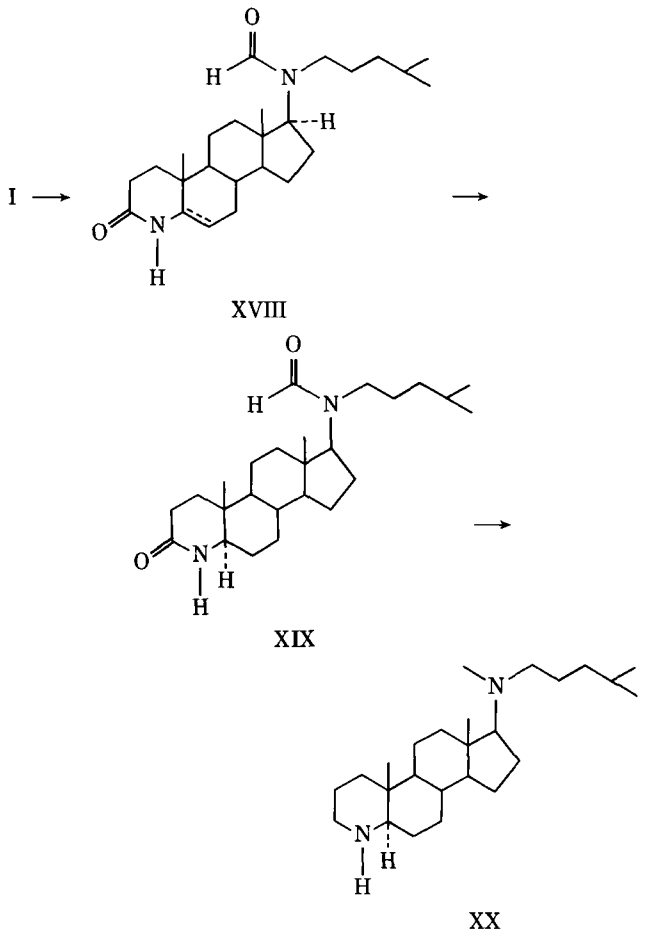
4-Aza-5-androstene-3,17-dione (I)—A solution of 50.0 g (0.174 mole) of 4-androstene-3,17-dione in 75 ml of ethyl acetate and 300 ml of acetic acid was ozonized with 2.9 equivalents of ozone at ice bath temperature. After addition of 20 ml of 30% hydrogen peroxide and 20 ml of water and stirring for 12 hr, the solution was mixed with 500 ml of ether and washed with 500 ml of water. After extracting the water phase with 3 × 125 ml of ether, combining the ether fractions, washing with 3 × 300 ml of water, and extracting with 300 ml of 5 *M* sodium hydroxide, the basic solution was made acidic to blue litmus by slowly adding water–phosphoric acid (50:50 v/v) with cooling. After extracting the oily precipitate with 3 × 200 ml of *n*-butanol, washing the extract with 3 × 200 ml of water, and evaporating the solvent, 26.2 g (49%) of a viscous oil was obtained. The IR spectrum (liquid film) showed major bands at 3700–3050 (broad OH, carboxyl group), 1740 (17-ketone), and 1720 (5-ketone and carbonyl of the carboxyl group) cm^{-1}.

A solution of 33.0 g (0.107 mole) of 3,5-*seco*-4-norandrostane-5,17-dione-3-oic acid thus obtained in 350 ml of concentrated ammonium hydroxide was heated under nitrogen for 22 hr at 170° in a steel pressure vessel. After sitting at room temperature for 12–20 hr, the crystalline precipitate was collected by filtration and washed with concentrated ammonium hydroxide, giving 20.5 g (66%) of I. Recrystallization from *sec*-butanol gave I, mp 346–348°; $[\alpha]_D^{24}$ +56.6° (chloroform); UV_{max} (95% ethanol): 235 nm (ϵ 13,500); IR: 3190, 3050 (NH), 2910 (CH), 1750 (17-ketone), 1680 (C=C), and 1670 (lactam carbonyl) cm^{-1}; NMR ($CDCl_3$): δ 8.68 (broad, NH), 5.00 (broad vinyl), 1.18 (s, 3, C-19 methyl), and 0.96 (s, 3, C-18 methyl).

Anal.—Calc. for $C_{18}H_{25}NO_2$: C, 75.22; H, 8.77; N, 4.87. Found: C, 75.37; H, 8.97; N, 4.93.

17β-(*N*-Methylformamido)-4-aza-5α-androstan-3-one (II)—A mixture of 2.0 g (6.95 mmoles) I, 2 ml (0.039 mole) of 90% formic

[1] All melting points were determined on a Thomas-Hoover capillary melting-point apparatus and the elemental analyses were obtained from Midwest Microlab, Inc., Indianapolis, Ind., or from Alfred Bernhardt Mikroanalytisches Laboratorium, West Germany. The wavelength of absorption in the UV spectra was determined on a Perkin-Elmer model 202, and the absorbance was determined on a Beckman model DU spectrophotometer. The IR spectra were recorded on a Perkin-Elmer model 257 spectrophotometer, and all spectra were determined in potassium bromide unless indicated otherwise. For the NMR spectra, sample solutions in most instances were 40 mg of sample/0.5 ml of chloroform-*d*. Tetramethylsilane was the internal reference. Ozone was generated by a Welsbach model T-816 ozone generator. Specific rotations were determined on a Perkin-Elmer model 141 automatic polarimeter as 2% solutions in chloroform.



Scheme IV

acid, and 20.0 g (0.34 mole) of *N*-methylformamide in a glass pressure bottle was heated under nitrogen at 170–180° for 16 hr. After cooling, the reaction mixture was dissolved in 200 ml of chloroform, washed with 3 × 200 ml of water, dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo* to give 2.14 g (87%) of a slightly yellow powder. For an analytical sample, a small quantity was recrystallized from ethanol, giving needles, mp 287–288.5°; $[\alpha]_D^{25}$ +12.0° (chloroform); UV_{max} (95% ethanol): transparent at 235 nm; IR: 3465, 3405, 3265, 3170, 3055 (NH and H_2O), and 1648 (amide and lactam carbonyl) cm^{-1} and absence of 17-ketone absorption; NMR ($CDCl_3$): δ 8.20 (s, 1, formyl), 6.41 (broad, 1, NH exchanged with D_2O), 3.0–4.0 (m, 2–3, *N*-methines), 2.93 (s, 3, *N*-methyl), 0.93 (s, 3, C-19 methyl), and 0.76 (s, 3, C-18 methyl).

Anal.—Calc. for $C_{20}H_{32}N_2O_2$: C, 72.25; H, 9.70; N, 8.43. Found: C, 72.07; H, 9.77; N, 8.18.

4-Aza-5α-cholestan-3-one (IV)—A mixture of 2.0 g (5.0 mmoles) 4-aza-5-cholesten-3-one (III), 20.7 g (0.351 mole) *N*-methylformamide, and 2.0 ml (0.039 mole) 90% formic acid in a glass pressure bottle was heated under nitrogen for 16 hr at 170–185°. The cooled reaction mixture was dissolved in 200 ml of chloroform, washed with 3 × 100 ml of 10% potassium carbonate and 3 × 100 ml of distilled water, dried over magnesium sulfate, filtered, and evaporated *in vacuo* to give 1.9 g (95%) of a pale-yellow solid. The UV spectrum taken in 95% ethanol showed the absence of enamine lactam absorption at 235 nm. Recrystallization from acetone (distilled from potassium permanganate and from sodium hydroxide pellets) gave fine, colorless needles, mp 245–249°; $[\alpha]_D^{20}$ +44.4° (chloroform). Reported values (10) are: mp 240–251°, after one recrystallization; mp 250–252°, after several recrystallizations; and $[\alpha]_D^{25}$ +45°. The IR spectrum gave major bands at 3170, 3050 (NH), and 1672 (lactam carbonyl) cm^{-1}. Preparation of the same compound by low-pressure hydrogenation with platinum oxide gave a compound having a melting point of 246–253° and a mixed melting point with the above material of 246–249°. TLC of these compounds, separately and mixed spotting of 40–80 μg on a silica gel thin-layer sheet with development in benzene–methanol–ethyl acetate (84:5:11), was performed. Iodine vapors were used for detection. The material obtained by hydrogenation and the mixture gave two spots: one corresponding to a small amount of starting material and one to the product. The product of reduction with *N*-methylformamide and formic acid corresponded to the major product from hydrogenation and gave only one spot.

17-Dimethyl-4-aza-18-nor-5α-androstan-13-en-3-one (VI)—*From 17β-Hydroxy-17α-methyl-4-aza-5-androsten-3-one (V) (15)*—After heating a mixture of 2.00 g (6.6 mmoles) of V, 20.0 g (0.444 mole) of formamide, and 3 ml (0.059 mole) of 90% formic acid in a glass pressure bottle under nitrogen for 16 hr at 175–185°, the cooled reaction mixture was dissolved in 200 ml of chloroform and washed with 2 × 150 ml of water, 2 × 100 ml of 20% sodium carbonate, and 3 × 150 ml of water. It was then dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo*, yielding 1.82 g of a hard, tan solid. The UV spectrum taken in 95% ethanol showed the absence of enamine lactam absorption at 235 nm, and the IR spectrum indicated that dehydration of the tertiary alcohol had occurred. Two crystallizations from acetonitrile gave colorless prisms, mp 237–240°; IR: 3262, 3150, 3040 (NH), 1684 (lactam carbonyl), and 1631 (13,14-double bond) cm^{-1}; NMR ($CDCl_3$): δ 7.13 (broad, 1, NH exchanged with D_2O), 0.96 (s, 6,17-dimethyl), 0.89 (s, 3, C-19 methyl), and no vinyl proton absorption.

Anal.—Calc. for $C_{19}H_{29}NO$: C, 79.39; H, 10.17; N, 4.87. Found: C, 79.46; H, 10.20; N, 4.79.

From 4-Dimethylaminoethyl-17β-hydroxy-17α-methyl-4-aza-5-androsten-3-one (VII) (16)—After heating a mixture of 2.20 g (5.4 mmoles) of VII, 45.2 g (1.00 mole) of formamide, and 8 ml (0.156 mole) of 90% formic acid at 170–180° for 72 hr, the cooled reaction mixture was rinsed into a separator with methanol and shaken with 200 ml of cold 10% sodium carbonate. This mixture was extracted with 3 × 80 ml of chloroform, and the chloroform extracts were combined, washed with 3 × 150 ml of water, dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo*, yielding 2.19 g of an oil which solidified. After drying 24 hr in the presence of phosphorus pentoxide at 54° *in vacuo*, the substance weighed 1.51 g. The IR spectrum indicated absence of the hydroxyl function, and the UV spectrum indicated reduction of the 5-double bond. Recrystallization twice from acetonitrile gave 424 mg of prisms, mp 239–242°. The IR spectrum of this compound was the same as that of the substance obtained above, and the NMR spectra of the two substances were superimposable.

Anal.—Calc. for $C_{19}H_{29}NO$: C, 79.39; H, 10.17; N, 4.87. Found: C, 79.43; H, 10.35; N, 4.65.

The NMR spectrum of the starting material clearly showed the presence of the 17β-hydroxyl group, the vinyl proton of the 5-double bond, the dimethylaminoethyl side chain, and the 17α-methyl and angular methyl groups.

17β-Formamido-4-aza-5α-androstan-3-one (XIII)—A mixture of 5.0 g (17.4 mmoles) of I, 3 ml (0.059 mole) of 90% formic acid, and 20 g (0.444 mole) of formamide in a glass pressure bottle was heated under nitrogen at 170–180° for 16 hr. After cooling, the reaction mixture was taken up in 200 ml of chloroform and washed with 2 × 130 ml of water, 3 × 130 ml of 10% potassium carbonate, and 3 × 130 ml of water. It was then dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo* to give 5.33 g (96%) of a white solid. A portion of this material (1.8 g) was heated in acetonitrile and filtered; the filtrate was evaporated slowly (5 days) and filtered to give 127 mg (2.3%) of white powder, mp 295–304° dec.; UV_{max} (95% ethanol): no absorption at 235 nm; IR: 3250, 3218, 3020 (NH), 1673, and 1658 (lactam and amide carbonyls) cm^{-1}.

Anal.—Calc. for $C_{19}H_{30}N_2O_2$: C, 71.66; H, 9.50; N, 8.80. Found: C, 71.45; H, 9.42; N, 8.88.

17β-Amino-4-aza-5α-androstan-3-one (XIV)—After refluxing 1.12 g (3.5 mmoles) XIII, 16 ml methanol, and 8 ml (0.096 mole) of concentrated hydrochloric acid for 3 hr, adding 150 ml of water, and extracting with 3 × 100 ml of chloroform, 50 ml of 32% potassium carbonate was added to the acidic water phase. The basic solution was extracted with 3 × 95 ml of chloroform, and the chloroform extracts were combined, washed with 3 × 150 ml of water, dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo* to give 0.75 g (73%) of a hard, pale-yellow solid, mp 225–240°. After subliming 0.31 g of this substance a dis-

tance of 2.6 cm three times at 0.005 mm and a maximum temperature of 215°, 210 mg (49% calculated as if the entire sample were similarly processed) of a white solid was recovered, mp 204–240°; $[\alpha]_D^{25}$ +47.3° (chloroform); IR: 3417, 3267, 3187, 3047 (NH), and 1653 (lactam carbonyl) cm^{-1}; NMR ($CDCl_3$): δ 7.08 (broad, NH of lactam), 1.25 (s, NH_2), 0.92 (s, C-19 methyl), and 0.67 (s, C-18 methyl).

Anal.—Calc. for $C_{18}H_{30}N_2O$: C, 74.44; H, 10.41; N, 9.64. Found: C, 74.14; H, 10.35; N, 9.59.

TLC[2] of this substance by spotting 40 μg and developing with chloroform–methanol–concentrated ammonium hydroxide (85:14:1) gave a single well-defined spot that was detected by iodine vapors.

17β-Amino-4-aza-5α-androstane (XV)—When 4.0 g (0.105 mole) of lithium aluminum hydride was added to a solution of 1.60 g (5.5 mmoles) XIV in 150 ml of dry tetrahydrofuran in a 300-ml three-necked flask fitted with a nitrogen inlet and outlet, condenser, and mechanical stirrer, the mixture was refluxed for 36 hr. After decomposing the excess lithium aluminum hydride by dropwise addition of 40–50 ml of saturated sodium sulfate, the mixture was filtered and the filtrate was evaporated *in vacuo*, giving 1.30 g (86%, quite crude) of a yellow semisolid. Purification was accomplished by subliming 0.92 g through a distance of 2.6 cm to a cold finger at an initial temperature of 95° and 0.005 mm. The first material collected, an oil, was removed before collecting a white solid (0.56 g) while raising the temperature to 156°. This substance recrystallized from acetonitrile, giving 0.31 g (28% calculated as if the whole sample were similarly processed) of pearly white leaflets, mp 156.5–157.5°; $[\alpha]_D^{23}$ +38.2° (chloroform); IR: 3335, 3235 (NH), and 1580 (NH_2 bending vibrations) cm^{-1} and absence of carbonyl absorption; NMR ($CDCl_3$): δ 1.23 (s, NH_2, exchanged with D_2O), 0.99 (s, C-19 methyl), and 0.68 (s, C-18 methyl).

Anal.—Calc. for $C_{18}H_{32}N_2$: C, 78.20; H, 11.67; N, 10.13. Found: C, 78.11; H, 11.50; N, 10.11.

17β-Dimethylamino-4-methyl-4-aza-5α-androstane (XVI)—A solution of 0.50 g (1.8 mmoles) XV, 5.0 ml (0.098 mole) of 90% formic acid, and 5.0 ml (0.063 mole) of formalin was refluxed for 6 hr and warmed at 40–45° for an additional 12 hr. After cooling, concentrated ammonium hydroxide was added dropwise until the solution turned red litmus blue. The precipitate was collected and dried *in vacuo* at 50° for several hours to give 0.52 g of product.

Purification Method A—The solid was recrystallized from acetonitrile, yielding 0.40 g (69%) of a fluffy, white powder, mp 149–151°; IR: 2960, 2930, 2850, 2830, 2805, 2755, and 2695 cm^{-1} (Bohlmann bands, CH stretching at 3 and 5, hydrogens adjacent and *trans*, diaxial to the nonbonded pair of electrons on nitrogen), and no NH stretching; NMR ($CDCl_3$): δ 2.28 (s, 17β-dimethylamino), 2.20 (s, 4-*N*-methyl), 0.98 (s, C-19 methyl), and 0.81 (s, C-18 methyl).

Anal.—Calc. for $C_{21}H_{38}N_2$: C, 79.18; H, 12.02; N, 8.79. Found: C, 79.11; H, 11.93; N, 8.78.

Purification Method B—A procedure similar to Method A was used for 0.42 g (1.9 mmoles) XV. After workup and drying *in vacuo*, 0.57 g of crude material was obtained, which was sublimed through a distance of 2.6 cm at 0.005 mm and a maximum wax bath temperature of 150°, giving a white solid, 0.51 g (85%), identical to that obtained with Method A.

Anal.—Calc. for $C_{21}H_{38}N_2$: C, 79.18; H, 12.02; N, 8.79. Found: C, 79.00; H, 11.95; N, 8.57.

17β-Dimethylamino-4-methyl-4-aza-5α-androstane Bismethiodide (XVII)—After refluxing 0.5 g (1.6 mmoles) of XVI in 70 ml of acetonitrile and 7.0 g (0.049 mole) of methyl iodide for 48 hr, the solution was reduced to one-half its volume and stored at −15° for 2 days, yielding prisms. These prisms were rapidly collected and dried for several days at 54° *in vacuo* in the presence of phosphorus pentoxide. Recrystallization from acetonitrile gave 317 mg (34%) of colorless prisms, mp 279.5° (after drying powdered material at 100°, 0.025 mm, for 48 hr) with liberation of a gas. The IR spectrum gave major bands at 3640–3100 (broad doublet), 2930, 2839 (CH), and 1615 cm^{-1}.

Anal.—Calc. for $C_{23}H_{44}I_2N_2$: C, 45.85; H, 7.36; I, 42.13; N, 4.64. Found: C, 45.63; H, 7.20; I, 42.02; N, 4.40.

[2] Silica gel layer from Eastman Kodak Co.

Table I—Inhibitory Concentration (Micrograms per Milliliter)

Microorganisms	Steroids[a] XV	XVI	XVII	XX	ND-502
S. cerevisiae	50	50	–	–	12.5
A. niger	–	–	–	–	50.0
Staph. aureus	–	–	–	–	12.5
B. cereus	–	–	–	–	25.0
E. coli	–	–	–	–	–

[a] No inhibition at 100 μg/ml.

17β-(*N*-Isohexylformamido)-4-aza-5α-androstan-3-one (XIX)—In a glass pressure bottle, 2.0 g (6.95 mmoles) of I, 6.0 g (0.059 mole) of isohexylamine, and 2.74 ml (0.053 mole) of 90% formic acid were heated under nitrogen at 175–180° for 16 hr. After cooling, the reaction mixture was taken up in 200 ml of chloroform and washed with 3 × 100 ml of water, 2 × 100 ml of 20% potassium carbonate, and 3 × 100 ml of water. It was then dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo*. *N*-Formylisohexylamine formed during the reaction and was removed from the product by distillation at 0.025 mm and a maximum temperature of 200° (wax bath), giving 2.86 g of a hard glass which lacked 17-ketone absorption at 1740 cm^{-1} in the IR spectrum. However, the UV spectrum showed only partial reduction of the 5-double bond. This solid was added to 20.0 g (0.444 mole) of formamide and 3 ml (0.059 mole) of 90% formic acid, heated under nitrogen at 170–180° for 16 hr, cooled, and worked up as before, including the high vacuum distillation, giving 2.40 g of a substance which showed no UV absorption at 235 nm. Recrystallization twice from ethyl acetate yielded 0.53 g (19%) of fine, shiny leaflets. These leaflets were boiled in 25 ml of acetone and sufficient chloroform to effect solution. After storing at −15° for 24 hr, warming to room temperature, and filtering, 147 mg (5.2%) of white powder was collected, mp 241–243°; IR: 3171, 3060 (NH), and 1670 (very intense lactam and amide carbonyls) cm^{-1}; NMR ($CDCl_3$): δ 8.56 (s, 1, formyl), 0.95 (s, 3, C-19 methyl), 0.90 (d, 6, left half superimposed on the C-19 methyl singlet), and 0.72 (s, 3, C-18 methyl).

Anal.—Calc. for $C_{25}H_{42}N_2O_2$: C, 74.58; H, 10.51; N, 6.96. Found: C, 74.38; H, 10.40; N, 6.70.

4,20-Diaza-5α-cholestane (XX)—To a slurry of 100 ml of tetrahydrofuran and 2.0 g (0.053 mole) of lithium aluminum hydride in a 500-ml three-necked flask fitted with condenser, drying tube, dropping funnel, and mechanical stirrer was added dropwise 0.92 g (2.3 mmoles) XIX in 50 ml tetrahydrofuran. After rinsing the dropping funnel with 50 ml of tetrahydrofuran, the mixture was refluxed for 36 hr and cooled in an ice bath; the complex was decomposed by adding dropwise 1.7 ml of water followed by 2.0 ml of 20% sodium hydroxide and then 6.0 ml of water. After stirring an additional 3 hr, the solid salts were removed by decanting tetrahydrofuran three times, filtering, and concentrating the filtrate *in vacuo* to give 1.04 g of an oil which solidified. Drying under vacuum in the presence of phosphorus pentoxide and recrystallization three times from acetonitrile gave 478 mg (56%), mp 101–102°; $[\alpha]_D^{27}$ +24.8° (chloroform); IR: 3270 (NH) cm^{-1} and absence of carbonyl absorption; NMR ($CDCl_3$): δ 2.21 (s, 3, *N*-methyl), 0.88 (s, 3, C-19 methyl), 0.83 (d, 6, isopropyl group in side chain), and 0.75 (s, 3, C-18 methyl) and a proton under the broad methylene absorption exchangeable with D_2O.

Anal.—Calc. for $C_{25}H_{46}N_2$: C, 80.15; H, 12.38; N, 7.48. Found: C, 80.38; H, 12.31; N, 7.58.

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[x]To whom inquiries should be directed. Present address: Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee Medical Units; Memphis, TN 38163

Comparative Study of Seven Media for Sterility Testing

MOHAMED A.-F. ABDOU

Abstract □ A comparative study of the efficacy of sterility testing of pharmaceuticals according to two systems is described. The culture media used in the first system were: dithionite–thioglycollate (HS-T) broth, recommended by the Nordic Pharmacopoeia Board, and a peptone liver digest medium or a peptone liver digest agar. The media used in the second system were: fluid thioglycollate medium and soybean–casein digest medium, both prescribed by the USP XVIII, and Sabouraud liquid medium or Sabouraud dextrose agar. The supplemental use of one of the two latter media has been recommended, because the USP XVIII media were considered inadequate. A total of 180 cultures of fastidious bacteria, yeasts, and molds was investigated. The first system was found superior to the second.

Keyphrases □ Sterility testing—comparison between seven media divided into two systems, 180 cultures of bacteria, yeasts, and molds □ Culture media for sterility testing—comparison between seven media divided into two systems, 180 cultures of bacteria, yeasts, and molds □ Medium for sterility testing—two systems compared using 180 cultures of bacteria, yeasts, and molds

For sterility testing of pharmaceutical products, the FDA, USP XVI, and USP XVII prescribed fluid thioglycollate medium and Sabouraud liquid medium. A collaborative study performed by 12 laboratories in the United States and Canada showed that soybean–casein digest medium is superior to Sabouraud liquid medium (1). Accordingly, USP XVIII and the First Supplement to NF XIII replaced Sabouraud liquid medium with soybean–casein digest medium. In Germany, the Subcommittee for Microbiology, a Division of the Pharmaceutical Committee of the Confederation of the Pharmaceutical Industry, recommended the use of three media: fluid thioglycollate medium, soybean–casein digest medium, and Sabouraud liquid medium for sterility testing. The Nordic Pharmacopoeia Board recommended dithionite–thioglycollate (HS-T) broth[1].

In 1972, the author developed peptone liver digest medium and peptone liver digest agar to replace Sabouraud liquid medium and Sabouraud dextrose agar. Dithionite–thioglycollate (HS-T) broth and both peptone liver digest medium and peptone liver digest agar contain neutralizing constituents and supplementary mineral salts. To find out which media are most reliable for sterility testing of pharmaceuticals, the efficacy of these seven media was investigated.

MATERIALS AND METHODS

Microorganisms[2]—To simulate low levels of contamination which might possibly occur in a contaminated pharmaceutical preparation being tested for sterility, the inoculum was diluted so that every vessel (test tube or petri dish) of medium contained about 50–200 colony-producing units as determined by a plate count. When petri dishes were used, the inoculum was suspended in buffer solution and 0.1 ml was spread out on the surface of the solidified medium which had been poured the day before.

The following microorganisms were employed:

I. 18 strains belonging to 15 species of the genus *Bacillus*.
II. 22 strains belonging to 12 species of the genus *Clostridium*.
III. 48 strains belonging to 40 species of the following 22 genera: *Bacteroides, Comamonas, Corynebacterium, Desulfovibrio,*

[1] This medium was developed recently by Dr. Clausen, University of Oslo, Oslo, Norway.

[2] The 180 cultures used included the following: 78 from the American Type Culture Collection, 31 from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; 14 from the National Collection of Industrial Bacteria, Aberdeen, Scotland; 10 from the National Collection of Type Cultures, London, England; four from the Institute of Food Microbiology, Chiba University, Chiba, Japan; one from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.; one from the Commonwealth Mycological Institute, Kew, England; and 41 cultures from other sources.

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[x]To whom inquiries should be directed. Present address: Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee Medical Units; Memphis, TN 38163

Comparative Study of Seven Media for Sterility Testing

MOHAMED A.-F. ABDOU

Abstract □ A comparative study of the efficacy of sterility testing of pharmaceuticals according to two systems is described. The culture media used in the first system were: dithionite–thioglycollate (HS-T) broth, recommended by the Nordic Pharmacopoeia Board, and a peptone liver digest medium or a peptone liver digest agar. The media used in the second system were: fluid thioglycollate medium and soybean–casein digest medium, both prescribed by the USP XVIII, and Sabouraud liquid medium or Sabouraud dextrose agar. The supplemental use of one of the two latter media has been recommended, because the USP XVIII media were considered inadequate. A total of 180 cultures of fastidious bacteria, yeasts, and molds was investigated. The first system was found superior to the second.

Keyphrases □ Sterility testing—comparison between seven media divided into two systems, 180 cultures of bacteria, yeasts, and molds □ Culture media for sterility testing—comparison between seven media divided into two systems, 180 cultures of bacteria, yeasts, and molds □ Medium for sterility testing—two systems compared using 180 cultures of bacteria, yeasts, and molds

For sterility testing of pharmaceutical products, the FDA, USP XVI, and USP XVII prescribed fluid thioglycollate medium and Sabouraud liquid medium. A collaborative study performed by 12 laboratories in the United States and Canada showed that soybean–casein digest medium is superior to Sabouraud liquid medium (1). Accordingly, USP XVIII and the First Supplement to NF XIII replaced Sabouraud liquid medium with soybean–casein digest medium. In Germany, the Subcommittee for Microbiology, a Division of the Pharmaceutical Committee of the Confederation of the Pharmaceutical Industry, recommended the use of three media: fluid thioglycollate medium, soybean–casein digest medium, and Sabouraud liquid medium for sterility testing. The Nordic Pharmacopoeia Board recommended dithionite–thioglycollate (HS-T) broth[1].

In 1972, the author developed peptone liver digest medium and peptone liver digest agar to replace Sabouraud liquid medium and Sabouraud dextrose agar. Dithionite–thioglycollate (HS-T) broth and both peptone liver digest medium and peptone liver digest agar contain neutralizing constituents and supplementary mineral salts. To find out which media are most reliable for sterility testing of pharmaceuticals, the efficacy of these seven media was investigated.

MATERIALS AND METHODS

Microorganisms[2]—To simulate low levels of contamination which might possibly occur in a contaminated pharmaceutical preparation being tested for sterility, the inoculum was diluted so that every vessel (test tube or petri dish) of medium contained about 50–200 colony-producing units as determined by a plate count. When petri dishes were used, the inoculum was suspended in buffer solution and 0.1 ml was spread out on the surface of the solidified medium which had been poured the day before.

The following microorganisms were employed:

I. 18 strains belonging to 15 species of the genus *Bacillus*.
II. 22 strains belonging to 12 species of the genus *Clostridium*.
III. 48 strains belonging to 40 species of the following 22 genera: *Bacteroides, Comamonas, Corynebacterium, Desulfovibrio,*

[1] This medium was developed recently by Dr. Clausen, University of Oslo, Oslo, Norway.

[2] The 180 cultures used included the following: 78 from the American Type Culture Collection, 31 from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; 14 from the National Collection of Industrial Bacteria, Aberdeen, Scotland; 10 from the National Collection of Type Cultures, London, England; four from the Institute of Food Microbiology, Chiba University, Chiba, Japan; one from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.; one from the Commonwealth Mycological Institute, Kew, England; and 41 cultures from other sources.

Table I—Growth Ability of Vegetative Cells and Spores of 18 *Bacillus* Strains[a] in Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, and Dithionite–Thioglycollate (HS-T) Broth at 32°

Growth Intensity	Number of Strains in Soybean–Casein Digest Medium						Fluid Thioglycollate Medium						Dithionite–Thioglycollate (HS-T) Broth					
	Days of Incubation						Days of Incubation						Days of Incubation					
	2		7		10		2		7		10		2		7		10	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
−	5	3	2	2	2	2	5	4	2	1	2	1	3	3	1	1	1	1
±	4	2	1	0	0	0	7	4	1	1	1	1	1	0	0	0	0	0
+	4	5	2	2	2	1	6	7	7	6	3	2	0	0	0	0	0	0
++	5	8	11	8	8	4	0	3	8	8	11	9	4	6	2	2	1	1
+++	0	0	2	6	6	10	0	0	0	2	1	5	10	9	13	11	14	12
++++	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2	4	2	4
Evaluation[b]	16	22	30.5	36	36	43	9.5	15	23.5	28.5	28.5	35.5	38.5	39	51	53	52	54

[a] Ten National Collection of Industrial Bacteria strains, seven American Type Culture Collection strains, and one National Collection of Type Cultures strain, representing 15 saprophytic species. A, only vegetative cells were tested; and B, only spores were tested. [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

Table II—Growth Ability of Vegetative Cells and Spores of 22 *Clostridium* Strains[a] in Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, and Dithionite–Thioglycollate (HS-T) Broth at 32°

Growth Intensity	Number of Strains in Soybean–Casein Digest Medium						Fluid Thioglycollate Medium						Dithionite–Thioglycollate (HS-T) Broth					
	Days of Incubation						Days of Incubation						Days of Incubation					
	2		7		10		2		7		10		2		7		10	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
−	22	22	22	22	22	22	21	21	21	21	21	21	1	1	0	0	0	0
±	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
+	0	0	0	0	0	0	1	1	0	0	0	0	2	4	0	0	0	0
++	0	0	0	0	0	0	0	0	1	1	1	1	14	4	2	2	0	1
+++	0	0	0	0	0	0	0	0	0	0	0	0	5	7	17	10	13	8
++++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	10	9	13
Evaluation[b]	0	0	0	0	0	0	1	1	2	2	2	2	45	36	67	74	75	78

[a] Nine National Collection of Type Cultures strains, seven American Type Culture Collection strains, one National Collection of Industrial Bacteria strain, and five other strains, representing 12 saprophytic and pathogenic species. A, only vegetative cells were tested, and B, only spores were tested. [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

Diplococcus, Haemophilus, Halobacterium, Lactobacillus, Leuconostoc, Micrococcus, Mima, Moraxella, Mycobacterium, Nocardia, Pasteurella, Pediococcus, Pseudomonas, Sarcina, Staphylococcus, Streptococcus, Streptomyces, and *Thiobacillus.*

IV. 38 strains of yeasts including: (*a*) 21 strains belonging to 19 species of the genera *Candida, Pichia, Rhodotorula, Saccharomyces, Schizosaccharomyces, Torulopsis,* and *Trichosporon*; and (*b*) 17 strains not identified which were recovered from pharmaceutical products and production plants.

V. 54 strains of molds belonging to 48 species of the following 32 genera: *Allescheria, Alternaria, Aspergillus, Aureobasidium, Beauveria, Byssochlamys, Cercosporella, Chrysosporium, Cladosporium, Claviceps, Coccidioides, Colletotrichum, Cunninghamella, Curvularia, Epidermophyton, Fusarium, Gaeumannomyces, Geotrichum, Gliocladium, Hemispora, Keratinomyces, Marasmius, Monosporium, Mucor, Neurospora, Penicillium, Phoma, Rhinocladiella, Rhizopus, Trichoderma, Trichophyton,* and *Verticillium.*

Media—The following fluid and semisolid media were used.

Soybean-Casein Digest Medium—pH after sterilization 7.3 ± 0.2.

Fluid Thioglycollate Medium—pH after sterilization 7.1 ± 0.2.

Dithionite-Thioglycollate (HS-T) Broth[3]—pH after sterilization 7.1 ± 0.2.

Sabouraud Liquid Medium—Contained 2% dextrose, pH after sterilization 5.7 ± 0.1.

Peptone Liver Digest Medium—This medium contained the following: mycological peptone[4], 15.0 g; liver digest[5], 5.0 g; dextrose, 19.8 g; yeast extract[6], 6.0 g; malt extract[7], 4.0 g; sodium chloride, 2.92 g; sodium glycerophosphate, 5.2 g; potassium phosphate, monobasic, 1.0 g; polysorbate 80, 3.0 g; lecithin, 0.3 g; magnesium sulfate (hexahydrate), 0.25 g; cobaltous sulfate (hexahydrate), 0.001 g; manganese chloride ($MnCl_2 \cdot 4H_2O$), 0.001 g; agar[8] No. 1, 0.75 g; and distilled water, 1000 ml. The pH after sterilization was 5.8 ± 0.1.

Eight-milliliter amounts of each medium were transferred to 15 × 160-mm test tubes.

The following solid media were used.

Sabouraud Dextrose Agar—Contained 4% dextrose, pH after sterilization 5.7 ± 0.1.

Peptone Liver Digest Agar—This medium was prepared by adding 19.8 g of dextrose and 20.0 g of agar to 1 liter of peptone liver digest medium. The pH after sterilization was 5.8 ± 0.1.

Twenty-milliliter quantities of each of the two solid media were transferred to petri dishes (90 mm diameter). All media were prepared 1 or 2 days before they were required.

Incubation—Vessels inoculated with bacteria were incubated at 32 ± 2° for 14 days. Those inoculated with yeasts or molds were incubated at 26 ± 2° for 2 weeks. Each vessel was examined visually for growth on Days 2, 7, 10, and 14.

[3] Available from Oxoid Limited, London SE 1, England.

[4] Oxoid, code L 40.
[5] Merck, No. 5402.
[6] Merck, No. 3753.
[7] Merck, No. 5391.
[8] Oxoid, code L 11.

Table III—Growth Ability of 48 Strains of Nonspore-Forming Bacteria[a] in Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, and Dithionite–Thioglycollate (HS-T) Broth at 32°

Growth Intensity	Number of Strains in Soybean–Casein Digest Medium, Days of Incubation			Number of Strains in Fluid Thioglycollate Medium, Days of Incubation			Number of Strains in Dithionite–Thioglycollate (HS-T) Broth, Days of Incubation		
	2	7	10	2	7	10	2	7	10
−	23	13	11	21	10	10	13	5	5
±	12	5	3	11	3	1	3	3	1
+	5	12	12	7	12	13	12	4	4
++	6	12	12	8	18	10	12	16	7
+++	2	6	10	1	5	14	8	16	25
++++	0	0	0	0	0	0	0	4	6
Evaluation[b]	29	56.5	67.5	31.5	64.5	75.5	61.5	101.5	117.5

[a] Thirty-eight American Type Culture Collection strains, four Institute of Food Microbiology strains, three National Collection of Industrial Bacteria strains, and three other strains, representing 40 saprophytic and pathogenic species (22 genera). [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

Table IV—Growth Ability of 38 Yeast Strains[a] in/on Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, Dithionite–Thioglycollate (HS-T) Broth, Sabouraud Liquid Medium, Peptone Liver Digest Medium, Sabouraud Dextrose Agar, and Peptone Liver Digest Agar at 26°

Growth Intensity	Number of Strains in/on Soybean–Casein Digest Medium, Days of Incubation			Fluid Thioglycollate Medium, Days of Incubation			Dithionite–Thioglycollate (HS-T) Broth, Days of Incubation			Sabouraud Liquid Medium, Days of Incubation			Peptone Liver Digest Medium, Days of Incubation			Sabouraud Dextrose Agar, Days of Incubation			Peptone Liver Digest Agar, Days of Incubation		
	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10
−	25	11	5	24	11	6	15	3	3	15	0	0	6	0	0	4	0	0	1	0	0
±	4	9	5	6	6	5	8	7	0	3	5	0	9	1	0	3	0	0	5	0	0
+	9	7	9	4	4	6	4	2	5	12	9	2	3	1	1	8	1	0	6	1	0
++	0	7	10	4	10	5	11	11	3	8	13	10	15	10	2	18	9	3	16	4	1
+++	0	4	9	0	7	16	0	15	27	0	11	26	5	20	14	5	28	34	10	28	12
++++	0	0	0	0	0	0	0	0	0	0	0	0	0	6	21	0	0	1	0	5	25
Evaluation[b]	11	37.5	58.5	15	48	66.5	30	72.5	92	29.5	70.5	100	52.5	105.5	131	60.5	103	112	70.5	116	138

[a] Seven American Type Culture Collection and 13 Centraalbureau voor Schimmelcultures strains representing 19 saprophytic and pathogenic species (seven genera), and 18 nonclassified strains isolated from pharmaceutical products and production plants. [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

RESULTS AND CONCLUSIONS

As shown in Table I, dithionite-thioglycollate (HS-T) broth is undoubtedly the best of the three media for the recovery (in respect to rate and yield) of the 18 *Bacillus* strains tested. Just one strain, namely *B. pasteurii* NCIB 8841, did not grow either in dithionite-thioglycollate (HS-T) broth or in soybean-casein digest medium or fluid thioglycollate medium. At least a 7-day incubation was required to obtain the best recovery of the species of the genus *Bacillus*. It did not make any difference whether the inoculum was composed of vegetative cells or spores. Fluid thioglycollate medium contains mercaptoacetate (thioglycollate) which was reported (2) to be definitely inhibitory to the growth of some *Bacillus* species; this inhibition was confirmed. However, the degree of inhibition or noninhibition appears to be influenced by other constituents of the medium; *e.g.*, in dithionite-thioglycollate (HS-T) broth, mercaptoacetate seems to be nontoxic. In view of the facts that not only *B. pasteurii* NCIB 8841 but also *B. coagulans* NCIB 9365 did not grow in soybean-casein digest medium and that some fastidious species (with respect to nutritive requirements) showed only poor and slow growth in both soybean-casein digest medium and fluid thioglycollate medium, it would seem that the two latter media are definitely inferior to dithionite-thioglycollate (HS-T) broth for the recovery of the *Bacillus* species.

Table II makes it clear that dithionite-thioglycollate (HS-T) broth is far better than the two other media with regard to the recovery of the 22 *Clostridium* strains tested. From the quantitative point of view, optimal recovery of all 12 species of the genus *Clostridium* tested was attained after 7-10 days of incubation. As expected, soybean-casein digest medium was found to be not at all suitable as a recovery medium for strictly anaerobic microorganisms, especially *Clostridia*.

Fluid thioglycollate medium inhibited the growth of almost all of the *Clostridium* species tested. These findings are somewhat analogous to previous results (3), where sodium thioglycollate was inhibitory to the germination of spores of several *Clostridium* species on solid agar medium. However, thioglycollate proved to be noninhibitory on the growth of *Clostridia* in dithionite-thioglycollate (HS-T) broth, as demonstrated in Table II.

Table III shows that dithionite-thioglycollate (HS-T) broth is by far the best of the three tested media with respect to the recovery of nonspore-forming bacteria, especially fastidious species. Five strains will not grow in dithionite-thioglycollate (HS-T) broth, but these are chemolithotrophic and halophilic bacteria which will grow neither in soybean-casein digest medium nor in fluid thioglycollate medium. Furthermore, it was not possible with fluid thioglycollate medium to recover five other strains which also belong to five species of bacteria. The recovery with soybean-casein digest medium was poorer than with fluid thioglycollate medium. Hence, it follows that dithionite-thioglycollate (HS-T) broth will give a better recovery than soybean-casein digest medium and fluid thioglycollate medium together.

A 10-day incubation is required to obtain optimal recovery of nonspore-forming bacteria. Prolonging the incubation to 14 days did not increase the recovery for dithionite-thioglycollate (HS-T)

Table V—Growth Ability of 54 Mold Strains[a] in/on Soybean–Casein Digest Medium, Fluid Thioglycollate Medium, Dithionite–Thioglycollate (HS-T) Broth, Sabouraud Liquid Medium, Peptone Liver Digest Medium, Sabouraud Dextrose Agar, and Peptone Liver Digest Agar at 26°

Growth Intensity	Number of Strains in/on																				
	Soybean–Casein Digest Medium			Fluid Thioglycollate Medium			Dithionite–Thioglycollate (HS-T) Broth			Sabouraud Liquid Medium			Peptone Liver Digest Medium			Sabouraud Dextrose Agar			Peptone Liver Digest Agar		
	Days of Incubation			Days of Incubation			Days of Incubation			Days of Incubation			Days of Incubation			Days of Incubation			Days of Incubation		
	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10	2	7	10
−	11	3	3	14	10	5	12	7	7	8	2	1	12	2	1	17	1	1	17	3	1
±	28	2	1	20	4	9	15	2	1	11	0	1	8	1	0	10	1	0	8	1	2
+	11	20	7	16	11	8	14	5	3	25	10	5	22	4	2	19	4	2	20	3	1
++	4	24	19	4	23	7	13	21	4	9	22	12	10	8	5	4	15	5	4	10	2
+++	0	5	24	0	6	25	0	19	39	1	20	35	2	27	16	4	32	43	4	24	19
++++	0	0	0	0	0	0	0	0	0	0	0	0	0	12	30	0	1	3	1	13	29
Evaluation[b]	33	84	117.5	34	77	101.5	47.5	105	128.5	51.5	114	134.5	52	149.5	180	44	134.5	153	48	147.5	179

[a] Nineteen American Type Culture Collection strains, 18 Centraalbureau voor Schimmelcultures strains, one Northern Utilization Research and Development strain, one Commonwealth Mycological Institute strain, and 15 other strains, representing 48 saprophytic, pathogenic, and phytopathogenic species (32 genera). [b] Evaluation according to the following point system: − = no point, ± = 0.5 point, + = 1 point, ++ = 2 points, +++ = 3 points, and ++++ = 4 points.

broth, soybean-casein digest medium, or fluid thioglycollate medium. As shown in Table IV, the best recovery of yeasts was attained with peptone liver digest agar and peptone liver digest medium. Although all strains of yeasts tested were able to grow in Sabouraud liquid medium and on Sabouraud dextrose agar, these two media have a drawback insofar as they contain no constituents that will neutralize preservatives and enhance the growth of impaired cells. Furthermore, both growth rate and abundance in peptone liver digest medium and on peptone liver digest agar are greater than in Sabouraud liquid medium and on Sabouraud dextrose agar, respectively. In none of the other three media was it possible to recover all of the tested yeast strains. Growth was not observed in dithionite-thioglycollate (HS-T) broth (three strains, two osmophilic and one nonidentified), in soybean-casein digest medium (five strains, three osmophilic and two nonidentified), and in fluid thioglycollate medium (six strains, two osmophilic, three nonidentified, and one pathogenic). Therefore, the number of strains of yeasts recovered with peptone liver digest medium or peptone liver digest agar was higher than that recovered with both soybean-casein digest medium and fluid thioglycollate medium. A 10-day incubation was necessary to obtain the best recovery of yeasts when using peptone liver digest medium or peptone liver digest agar.

Of the seven media evaluated in Table V, peptone liver digest medium and peptone liver digest agar proved to be superior to the other five. Just one rare species of mold, namely *Marasmius foetidus* CBS 20847, did not grow either in peptone liver digest medium or on peptone liver digest agar. Although in Sabouraud liquid medium and on Sabouraud dextrose agar, 53 out of 54 strains of molds were able to grow, both of these media have the disadvantage mentioned previously. Like yeasts, molds also were able to grow in peptone liver digest medium and on peptone liver digest agar more quickly and more abundantly than in Sabouraud liquid medium or on Sabouraud dextrose agar. In soybean-casein digest medium, three strains belonging to three species of molds did not grow: eight other strains belonging to eight species showed slow and poor or very poor growth. In dithionite-thioglycollate (HS-T) broth, seven strains belonging to six species did not grow; four other strains belonging to four species showed slow and faint or very faint growth. Generally, the growth of molds in fluid thioglycollate medium was not as abundant as that in peptone liver digest medium. Furthermore, in fluid thioglycollate medium, five strains belonging to four species did not grow; 17 other strains belonging to 13 species showed slow and poor growth (Table V). Accordingly, neither soybean-casein digest medium nor fluid thioglycollate medium, nor both together, can replace either peptone liver digest medium or peptone liver digest agar. The heaviest growth of molds was obtained after a 10-day incubation when using the two latter media.

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Structure Relationship for Binding of Sulfonamides and Penicillins to Bovine Serum Albumin by Fluorescence Probe Technique

PAR-LIN HSU, JOSEPH K. H. MA, H. W. JUN, and LOUIS A. LUZZI[x]

Abstract □ The binding constants and the number of binding sites for the binding of 11 sulfonamide and seven penicillin derivatives to bovine serum albumin were determined using a fluorescence probe method. The results demonstrate that these drugs bind to hydrophobic sites on the serum albumin. The binding affinities of sulfonamide and penicillin are greatly affected by their side-chain substitutions. The binding is enhanced by the hydrophobic substitutions but decreased by the hydrophilic substitutions on the parent molecules.

Keyphrases □ Sulfonamides—relationship between structure and binding to bovine serum albumin, fluorescence probe technique, binding constants □ Penicillins—relationship between structure and binding to bovine serum albumin, fluorescence probe technique, binding constants □ Binding, sulfonamides and penicillins to bovine serum albumin—effect of structure, fluorescence probe technique, binding constants □ Fluorescence probe technique—used to study relationship between sulfonamide and penicillin structure and their binding to bovine serum albumin

The fact that plasma protein-bound sulfonamides and penicillins are devoid of antimicrobial activity is well established (1–3). It has also been found that these drugs bind predominantly to the albumin fraction of plasma (4, 5). Although various binding mechanisms have been proposed (6, 7), the techniques commonly used for binding studies have generally yielded somewhat limited information on the nature involved in the formation of the complex. The use of fluorescence probes has provided insight into the mechanisms and sites of binding of small molecules and has been shown (8) to be a convenient tool for competition studies of small molecules for protein binding sites.

A fluorescence probe (9, 10) is defined as a compound that undergoes changes in one or more of its fluorescence properties when bound to certain proteins. The observation (8) that certain probes bind to a number of highly hydrophobic protein sites led to the proposition that these compounds might be useful for investigation of the hydrophobic nature of protein (9–11). An additional observation (12) suggested that the fluorescence intensity of the probes in protein dispersion is decreased by the introduction of certain drug molecules. This is taken as an indication that competition between drug and probe does occur and that the drug falls into the same type of binding category as characterized by the probe (12).

1-Anilinonaphthalene-8-sulfonic acid has been used as a fluorescence probe to detect binding at the hydrophobic sites of proteins. Competition with a drug for these sites reduces the fluorescence yield of the probe–protein complex. In the present investigation, 11 sulfonamide and seven penicillin analogs were studied by the probe technique; the emphasis was on their structure–activity relationships.

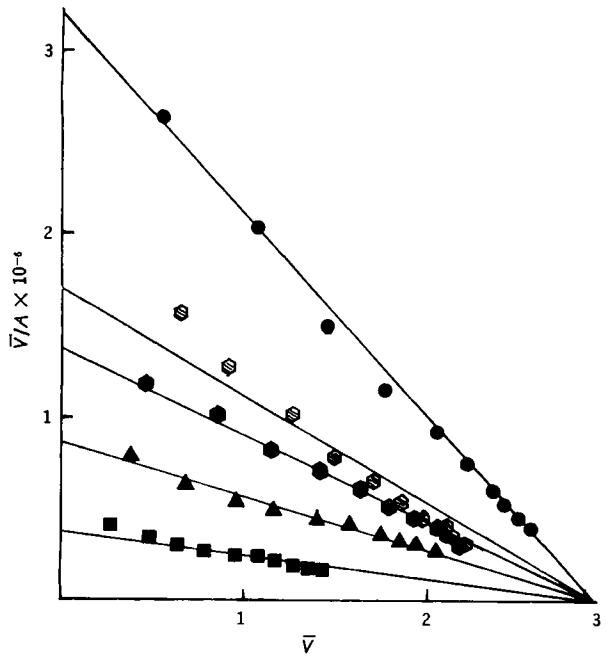


Figure 1—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.45. Key: ●, in the absence of drug; ○, in the presence of 2 × 10^{-4} M sulfaphenazole; ○, in the presence of 1 × 10^{-4} M sulfamethoxypyridazine; ▲, in the presence of 5 × 10^{-4} M sulfamethizole; and ■, in the presence of 5 × 10^{-4} M sulfadimethoxine.*

EXPERIMENTAL

Materials—All sulfonamides[1] were recrystallized from ethanol–water mixtures. All penicillins[2] were used without further purification. 1-Anilinonaphthalene-8-sulfonic acid[3] (recrystallized twice from water), spectrograde methanol[4], bovine serum albumin[5] (crystalline), and double-distilled water were also used. All other chemicals[6] were reagent grade and were used without further purification.

[1] Sulfisoxazole, Roche, Lot 634069; sulfadimethoxine, Roche, Lot 256050; sulfamethoxazole, Roche, Lot 797031; sulfamethazine, American Pharmaceutical Co., Lot 2915; sulfamerazine, American Pharmaceutical Co., Lot 2915; sulfapyridine, American Pharmaceutical Co., Lot 2915; sulfadiazine, American Pharmaceutical Co., Lot 1784; sulfamethoxypyridazine, Lederle, Lot 1906-JU88; sulfaphenazole, Purdue-Frederick Co., Lot RM9902; sulfamethizole, Ayerst Laboratories, Lot p2935A; and sulfisomidine. Ciba-Geigy, Lot H7531.

[2] Sodium dicloxacillin, Bristol, Lot 71F1026; sodium cloxacillin, Bristol, Lot 71F1009; sodium oxacillin, Bristol, Lot 71F381; sodium methicillin, Bristol, Lot 71F378; potassium penicillin G, Upjohn, Lot 3035K; ampicillin, Wyeth Laboratories, Lot W-703632; and potassium phenoxymethyl penicillin, Abbott, Lot 07-159-CD.

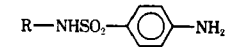
[3] Aldrich Co., Milwaukee, WI 53233

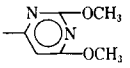
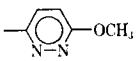
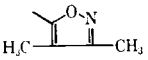
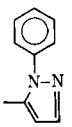
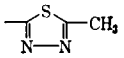
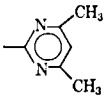
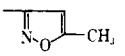
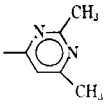
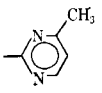
[4] Fisher Scientific Co., Fair Lawn, N.J.

[5] Nutritional Biochemical Co., Cleveland, OH 44128. Concentration was determined by measuring the absorbance of solutions at 280 nm, using $E_{1cm}^{1\%}$ value of 6.6.

[6] J. T. Baker Co.

Table I—Binding Data for Sulfonamides to Blood Fractions



Compound	R	Blood Fraction	Binding Data[a]		Reference
1-Anilinonaphthalene-8-sulfonic acid		Bovine albumin[b]	$n = 2.9$	$k = 1.1 \times 10^6/M$	This study
Sulfadimethoxine		Human serum	$n_1 = 2.12$	$k_B = 7.1$	19
		Bovine albumin	$n = 2$	$\log k = 5.4$	20
		Human plasma	$n = 2.0$	$k = 1 \times 10^4/M$	21
		Bovine albumin	$n = 2.5$	$k = 2.30 \times 10^4/M$	22
		Bovine albumin[b]	$n = 2.9$	$k = 1.5 \times 10^4/M$	This study
Sulfamethoxypyridazine		Human albumin	83%		2
		Human plasma	80%		23
		Human albumin	$n = 70$	$k = 9.78 \times 10^3/M$	24
		Human serum	$n_1 = 1.95$	$k_B = 37.1$	19
		Bovine albumin	$n = 2$	$\log k = 4.8$	20
		Bovine albumin[b]	$n = 2.9$	$k = 1.4 \times 10^4/M$	This study
Sulfisoxazole		Human albumin	84%		2
		Bovine albumin	$n = 2.0$	$\log k = 5.0$	20
		Bovine albumin	$n = 2.5$	$k = 1.47 \times 10^4/M$	22
		Bovine albumin[b]	$n = 2.9$	$k = 9.6 \times 10^3/M$	This study
Sulfaphenazole		Human plasma	90%		23
		Bovine albumin	$n = 2.0$	$\log k = 3.3$	20
		Bovine albumin	$n = 2.5$	$k = 1.07 \times 10^4/M$	22
		Bovine albumin[b]	$n = 2.9$	$k = 5.6 \times 10^3/M$	This study
Sulfamethizole		Bovine albumin	$n = 2.0$	$\log k = 4.3$	20
		Bovine albumin[b]	$n = 2.9$	$k = 5.2 \times 10^3/M$	This study
Sulfamethazine		Human albumin	58%		16
		Human albumin	$m = 0.66$	$\log k = 7.9$	25
		Bovine albumin[b]	$n = 2.9$	$k = 2.9 \times 10^3/M$	This study
Sulfamethoxazole		Human serum	$n_1 = 3.26$	$k_B = 449$	19
		Bovine albumin[b]	$n = 2.9$	$k = 1.6 \times 10^3/M$	This study
Sulfisomidine		Bovine albumin	$n = 2$	$\log k = 3.1$	20
		Human plasma	$n = 1.1$	$k = 5 \times 10^3/M$	21
		Bovine albumin	$n = 2.5$	$k = 3.60 \times 10^3/M$	22
		Bovine albumin[b]	$n = 2.9$	$k = 1.4 \times 10^3/M$	This study
Sulfamerazine		Human albumin	57%		16
		Human albumin	$m = 0.72$	$\log k = 2.8$	25
		Bovine albumin	$n = 2$	$\log k = 3.7$	20
		Bovine albumin[b]	$n = 2.9$	$k = 6.6 \times 10^2/M$	This study
Sulfapyridine		Human plasma	45%		1
		Bovine albumin	$n \times k = 4.2 \times 10^3/M$		26
		Bovine albumin[b]	$n = 2.9$	$k = 4.5 \times 10^2/M$	This study
Sulfadiazine		Human plasma[c]	55%		1
		Human albumin[d]	32%		16
		Human albumin	33%		2
		Human serum	$n_1 = 1.86$	$k_B = 775.6$	19
		Bovine albumin	$n = 2$	$\log k = 2.0$	20
		Human albumin	$n_1 = 1$	$k_1 = 2.4 \times 10^3/M$	27
			$n_2 = 2$	$k_2 = 3.5 \times 10^2/M$	
		Bovine albumin[b]	$n = 2.9$	$k = 4.2 \times 10^2/M$	This study

[a] n, n_1, and n_2 = number of binding sites; k, k_1, k_2, and k_B = binding constants; % = percentage of drug bound to blood fractions; and m = slope for the Freundlich-type equation (17), obtained by plotting logarithmic concentrations of bound drug *versus* free drug. [b] The pH and temperature of the solutions were 7.45 and 27°, respectively. [c] Drug and plasma concentrations were 10 and 7%, respectively. [d] Drug and plasma albumin concentrations were 10 and 3%, respectively.

Methods—The binding of the probe, 1-anilinonaphthalene-8-sulfonic acid, to bovine serum albumin was determined by measuring the increase in fluorescence following the titration of the protein solution with the probe as described by Brand *et al.* (8) and modified as described below. The fluorescence measurements were made at 475 nm with a spectrophotofluorometer[7], using an excitation wavelength of 375 nm. The temperature of all measurements was maintained at 27 ± 1°.

Solutions of bovine serum albumin were prepared in 0.05 *M* phosphate buffer at pH 7.4. The probe, 1-anilinonaphthalene-8-sulfonic acid, was dissolved in methanol at a concentration of 1×10^{-3} *M*. Successive aliquots of 2 μl of probe solution were added directly to the cell, which contained 2 ml of bovine serum albumin; addition was made by means of a microsyringe[8]. Titrations with probe were carried out at both low (1.38×10^{-6} *M*) and high (1.38×10^{-5} *M*) bovine serum albumin concentrations.

Drug binding was determined by titrating 2 ml of the low protein solution with successive additions of 2 μl of 1×10^{-3} *M* 1-anilinonaphthalene-8-sulfonic acid in the presence of drug compound.

Because of their low solubilities in water and alcohol, all of the sulfonamides examined, except sulfisoxazole and sulfaphenazole, were dissolved in 0.1 *N* NaOH to make a concentration of 0.1 *M* with ensuing conversion to their sodium salts. Ten microliters of each sulfonamide solution was injected separately into 2 ml of 1.38×10^{-6} *M* bovine serum albumin prior to titration as pre-

[7] Aminco-Bowman, American Instrument Co., Silver Spring, Md.

[8] Hamilton, Hamilton Co., Whittier, Calif.

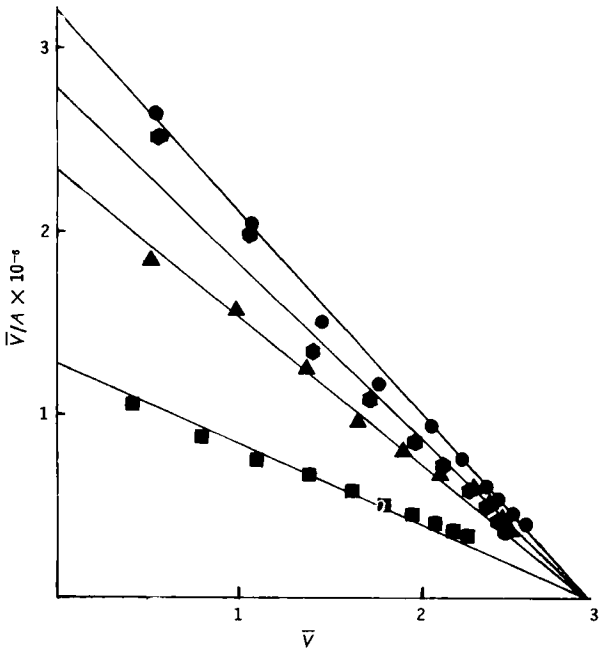


Figure 2—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.45. Key: ●, in the absence of drug; ○, in the presence of 5 × 10⁻⁴ M sulfadiazine; ▲, in the presence of 5 × 10⁻⁴ M sulfamerazine; and ■, in the presence of 5 × 10⁻⁴ M sulfamethazine.*

viously described (13). The final concentration of each sulfonamide solution was 5×10^{-4} *M*, except for sulfaphenazole and sulfamethoxypyridazine which were 2×10^{-4} and 1×10^{-4} *M*, respectively. The final pH ranged from 7.40 to 7.45. Sulfisoxazole and sulfaphenazole were dissolved in methanol; the final pH and the ionic strength of the protein solutions, to which sulfisoxazole and sulfaphenazole were added, were controlled by the addition of 0.1 *N* NaOH to the buffer solution to eliminate those variables.

In the case of ampicillin, dimethyl sulfoxide was used to effect dissolution of the drug and the remaining penicillins were dissolved in double-distilled water. All solutions were 0.1 *M* with respect to the individual penicillin.

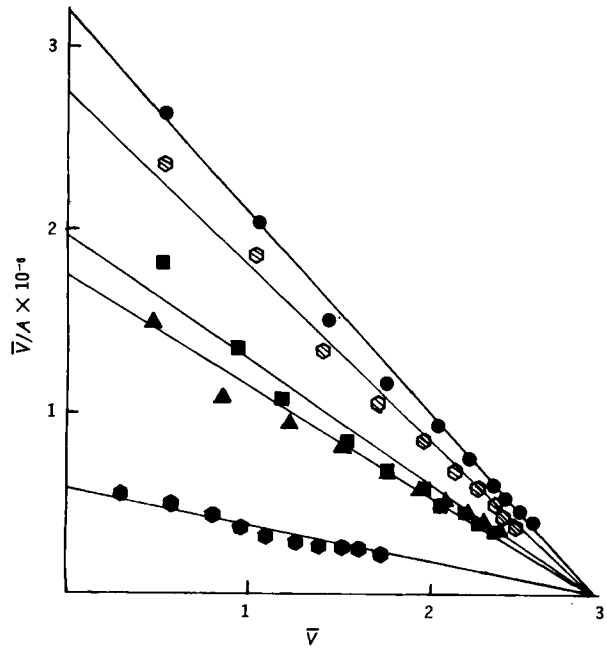


Figure 3—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.45. Key: ●, in the absence of drug; ○, in the presence of 5 × 10⁻⁴ M sulfapyridine; ■, in the presence of 5 × 10⁻⁴ M sulfisomidine; ▲, in the presence of 5 × 10⁻⁴ M sulfamethoxazole; and ○, in the presence of 5 × 10⁻⁴ M sulfisoxazole.*

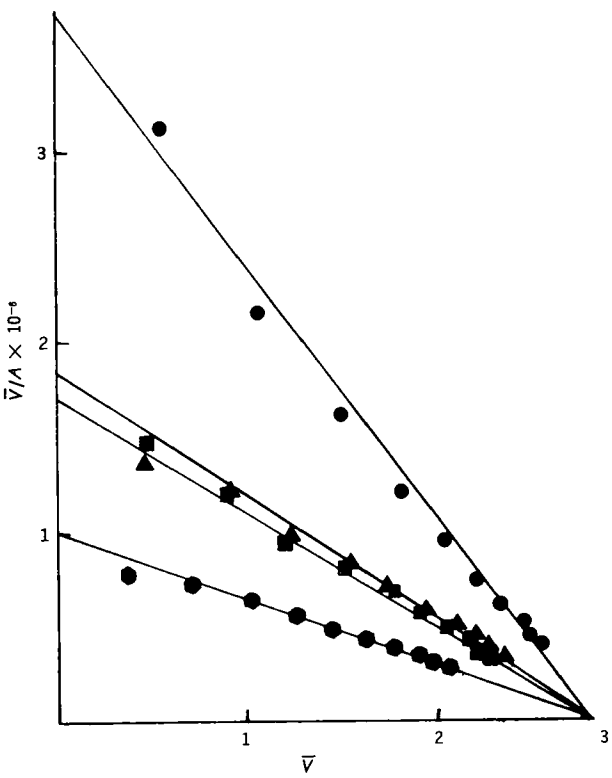


Figure 4—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.40. Key: ●, in the absence of drug; ▲, in the presence of 5 × 10⁻⁴ M potassium phenoxymethyl penicillin; ■, in the presence of 5 × 10⁻⁴ M potassium penicillin G; and ○, in the presence of 5 × 10⁻⁴ M ampicillin.*

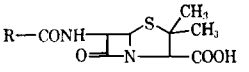
RESULTS AND DISCUSSION

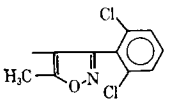
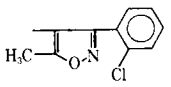
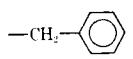
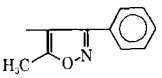
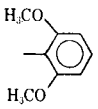
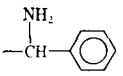
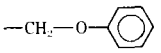
Binding of Sulfonamides to Bovine Serum Albumin—Scatchard plots of the results with sulfonamides (Figs. 1–3) were constructed by using the Scatchard equation (14) as previously described (13). The common intercept indicates that binding occurs at the same sites on the albumin where the probe is bound. The binding affinities are characterized by the binding constants calculated from the equation of Klotz *et al.* (15). These binding constants are compared with the results from published data (Table I). Although the results varied, the rank order of all data seems to be in agreement.

Sulfadimethoxine, sulfamethoxypyridazine, sulfisoxazole, sulfaphenazole, and sulfamethizole can be classified as highly bound, with affinities higher than $5 \times 10^3/M$. Sulfamethazine, sulfamethoxazole, and sulfisomidine can be classified as moderately bound, with affinities between 5×10^3 and $1 \times 10^3/M$; sulfamerazine, sulfapyridine, and sulfadiazine are weakly bound, with affinities below $1 \times 10^3/M$. These differences in affinity may be due to differences in structure (Table I). With sulfamethazine, sulfamerazine, and sulfadiazine, it is seen that the binding affinities increase from 4.2×10^2 to 6.6×10^2 to $2.9 \times 10^3/M$ with the increasing number of methyl groups on the pyrimidine ring. This indicates that methyl groups on the pyrimidine ring increase the phenomenon of protein binding. A similar conclusion was also given by Van-Dyke *et al.* (16).

Sulfisomidine, having two methyl groups on its pyrimidine ring, has a higher binding constant than sulfapyridine, which has none. Sulfisoxazole has two additional methyl groups on its oxazole ring when compared with sulfamethoxazole and, therefore, exhibits a higher binding constant even though the *p*-aminobenzenesulfonamide moiety is substituted at a different position on the oxazole ring. Sulfadimethoxine, having two methoxyl groups

Table II—Binding Data for Penicillins to Blood Fractions



Compound	R	Blood Fraction	Binding Data[a]	Reference
Dicloxacillin		Human serum	97.9%	28
		Bovine albumin[b]	$n = 2.8$ $k = 7.5 \times 10^3/M$	This study
Cloxacillin		Human serum	94%	29
		Human serum	95.2%	28
		Bovine albumin[b]	$n = 2.8$ $k = 7.2 \times 10^3M$	This study
Penicillin G		Human albumin	58%	7
		Human albumin, fraction V	60%	7
		Bovine albumin	52%	7
		Human serum	60.7%	30
		Human serum	66.7%	31
		Human serum	59%	29
		Bovine serum	37.7%	32
		Human serum	48%	33
		Human serum	48.7%	34
		Bovine albumin[b]	$n = 2.8$ $k = 5.2 \times 10^3/M$	This study
Oxacillin		Human serum	94.2%	28
		Human serum	93.1%	29
		Human serum	87%	35
		Bovine serum	58.4%	32
		Bovine albumin[b]	$n = 2.8$ $k = 4.5 \times 10^3/M$	This study
Methicillin		Human serum	49%	30
		Human serum	37.3%	28
		Human serum	49.3%	29
		Human serum	22%	35
		Bovine albumin[b]	$n = 2.8$ $k = 2.9 \times 10^3/M$	This study
Ampicillin		Human serum	18%	30
		Human serum	22.5%	28
		Human serum	18%	29
		Bovine serum	24.2%	32
		Bovine albumin[b]	$n = 2.8$ $k = 2.1 \times 10^3/M$	This study
Phenoxymethyl penicillin		Human serum	79.5%	30
		Bovine albumin, fraction V	$n_1 = 1$ $n_2 = 3$ $n_3 = 82$ $k_1 = 1887/M$ $k_2 = 14/M$ $k_3 = 15.2/M$	36
		Human serum	83.4%	31
		Human serum	78.5%	28
		Human serum	79.7%	29
		Bovine serum	53.8%	32
		Human serum	75.5%	33
		Human serum	74.4%	34
		Bovine albumin[b]	$n = 2.8$ $k = 1.7 \times 10^3/M$	This study

[a] n, n_1, n_2, and n_3 = number of binding sites; k, k_1, k_2, and k_3 = binding constants; and % = percentage of drug bound to blood fractions. [b] The pH and temperature of the solutions were 7.40 and 27°, respectively.

on its pyrimidinyl ring, has a higher binding constant than sulfamethoxypyridazine, which has one on its pyridazinyl ring.

Scholtan (17), studying the structure effects of sulfonamides on their binding to serum, also found that binding is strengthened by the presence of alkyl, alkoxyl, and halogen groups in the sulfonamide structure. Furthermore, he reported that binding was strengthened by the substitution of an oxygen atom for a sulfur atom but was reduced by amino substitution. These effects suggest that the binding of sulfonamide to serum proteins is of a hydrophobic nature.

Jardetzky and Wade-Jardetzky (18) used an NMR relaxation technique to investigate the binding of sulfonamides and found that sulfaphenazole may bind to albumin either by its *p*-aminobenzenesulfonamide moiety or by its phenyl ring. The fact that sulfaphenazole is highly bound to albumin is probably due to the phenyl substitution on the pyrazole ring as a result of strengthening hydrophobic bonding.

Binding of Penicillins to Bovine Serum Albumin—The Scatchard plots of the data for each penicillin are shown in Figs. 4 and 5. The plots, both in the absence and presence of penicillin, intercept at the same point on the X axis, indicating the competition between the probe and penicillins at the same or adjacent hydrophobic sites. The binding affinities, calculated from the Klotz equation (12, 15), are shown in Table II.

According to Kunin (31, 34), penicillins with binding constants less than $4.0 \times 10^3/M$ do not bind to a therapeutically significant degree. In the present studies, sodium dicloxacillin, sodium cloxacillin, potassium penicillin G, and sodium oxacillin are strongly bound to albumin, with binding constants greater than $4.0 \times 10^3/M$. Sodium methicillin, ampicillin, and potassium phenoxymethyl penicillin are less strongly bound, with binding constants of less than $4.0 \times 10^3/M$.

A comparison of published data with the data reported here shows that the relative binding affinities of these drugs (Table II) are generally in good agreement.

The binding affinities of penicillins vary with different side-chain substituents. These side-chain groups all possess a phenyl ring, which has been shown by NMR studies (37) to be the primary binding site.

From the binding affinities determined by the probe technique for sodium dicloxacillin, sodium cloxacillin, and sodium oxacillin, it is seen that the binding is enhanced by halogen substitution on the phenyl ring. Since the oxacillins are shown generally to have greater binding affinities than the group of penicillins at large, it is clear that the methoxazole ring has an enhancing effect on the binding of these drugs to proteins. The methoxazole ring is also present in both sulfisoxazole and sulfamethoxazole, and they also bind more strongly than the class as a whole.

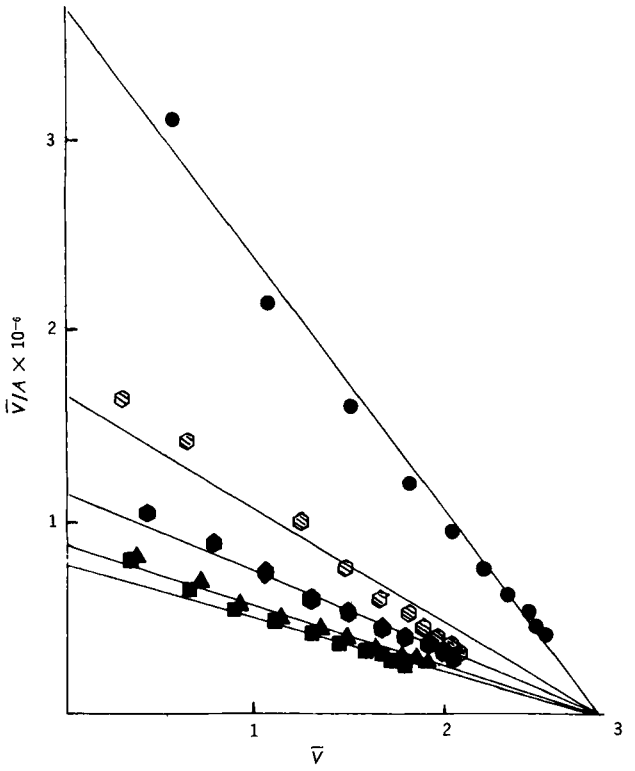


Figure 5—*Scatchard plots of 1-anilinonaphthalene-8-sulfonic acid binding to bovine serum albumin at 27° and pH 7.40. Key: ●, in the absence of drug; ○, in the presence of 5 × 10⁻⁴ M sodium oxacillin; ▲, in the presence of 5 × 10⁻⁴ M sodium cloxacillin; ■, in the presence of 5 × 10⁻⁴ M sodium dicloxacillin; and ○, in the presence of 5 × 10⁻⁴ M sodium methicillin.*

In contrast to the effect of halogen substitution, an amino substitution may reduce the binding affinity. As shown in Table II, the binding constant of potassium penicillin G was found to be $5.2 \times 10^3/M$ at pH 7.40 while that for ampicillin was found to be $2.0 \times 10^3/M$.

Phillips *et al.* (38) used the "hydrated electron" to detect the ionic binding of the carboxylic group of penicillin. They found that ionic binding was greatly reduced with similar compounds without the phenyl ring. This led them to suggest that hydrophobic binding may facilitate ionic binding. It is suggested at this time that the reverse may also be true and, as suggested by Brand and Gohlke (28), 1-anilinonaphthalene-8-sulfonate may act as an anion to augment the binding constants to the value found in the present study (Table I).

SUMMARY AND CONCLUSIONS

In summary, it seems that the R groups may play an important role in the binding of sulfonamides to hydrophobic protein sites. The methyl groups on the pyrimidine rings of sulfamerazine and sulfamethazine, the methyl groups on the pyrimidine ring of sulfisomidine, and the methyl group on the isoxazole ring of sulfisoxazole significantly increase the binding to albumin. The benzene substitution of sulfaphenazole and methoxy substitutions of sulfadimethoxine and sulfamethoxypyridazine may also enhance the binding to albumin.

The binding of penicillins is enhanced by halogen substitution on the side chain while being greatly reduced by amino substitution. Since the binding constants of penicillins with identical parent molecules differ with different side chains, it may also be concluded that the side chains of penicillin are involved in the binding to albumin.

These relationships between the structure and binding affinities and the agreement between the present study and previously published data show that the fluorescence probe technique can rapidly yield valuable binding information.

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* To whom inquiries should be directed.

New Fluorescence Probes for Drug–Protein Binding Studies

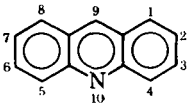
JOSEPH K. H. MA, PAR-LIN HSU, and LOUIS A. LUZZI[x]

Abstract □ 9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine and 9-anilino-6-chloro-2-methoxyacridine were synthesized from 6,9-dichloro-2-methoxyacridine. The fluorescence characteristics of these compounds along with *N*-phenylsulfanilate in various solvents and in their bovine serum albumin mixtures were studied using a fluorescent technique. The results show that the fluorescence of these acridine derivatives is essentially a combination of the fluorescence of acridine and the substitution effects. In polar solvent, the fluorescence is mainly characterized by the acridine residue; in nonpolar solvents and in bovine serum albumin mixtures, the substitution effects are the dominant factors. It was found that 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine and *N*-phenylsulfanilate possess the main features of fluorescence probes. In binding to bovine serum albumin, the quantum yields of 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine and *N*-phenylsulfanilate increased 20-fold and 26-fold, respectively. However, neither 9-anilino-6-chloro-2-methoxyacridine nor diphenylamine showed significant interaction with bovine serum albumin. These findings indicate that, as an anion, the probes may interact with positively charged residues on the protein molecules. The potential for using 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine as a fluorescence probe was tested with several drug compounds.

Keyphrases □ Acridine derivatives—synthesized, evaluated as potential fluorescence probes for drug-protein binding studies □ 9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine—synthesized, evaluated as potential fluorescence probe for drug-protein binding studies □ Fluorescence probes, potential—synthesis of acridine derivatives, evaluation in drug-protein binding studies □ Drug-protein binding—synthesis, evaluation of acridine derivatives as fluorescence probes

Fluorescence probes have been defined as: "small molecules which undergo changes in one or more of their fluorescence properties as a result of noncovalent interaction with protein or other macromolecules" (1). In general, the fluorescence of a probe varies in intensity and in spectral characteristics with the polarity of the solvent. The emission maximum is shifted toward the blue, and the fluorescence increases as the polarity of solvent decreases (2, 3). In aqueous solution, the same effects occur with the addition of protein. These effects have been shown to indicate that the binding of the probe to the protein is hydrophobic in nature. Because of the large molecule of the protein molecule and the rather complicated binding process, it is the authors' belief that more than one probe is required for the complete study of certain binding systems. The number of probes available at this time, however, is limited, and the finding of new probes became necessary. Therefore, several acridine compounds and *N*-phenylsulfanilate were studied in this laboratory.

The fluorescence of acridine (I) and its derivatives is varied by different substitutions on the acridine residue. It was reported (4, 5) that acridine shows pronounced shifts in fluorescence maximum with increasing dielectric constant of the solvent. However, the introduction of an anilino group or of certain substituted anilino groups at the 9-position (I) results in a compound that is nonfluorescent in aqueous solution but strongly fluorescent when absorbed in organic solution or on the solid phase (6). Similar compounds in which the anilino group is replaced by a benzylamino or a phenyl group are brightly fluorescent in aqueous solution, in contrast with the anilino-substituted compounds (6).



I

Acridine fluoresces very weakly in cyclohexane and benzene but shows intense fluorescence in ethanol, butanol, and water (4, 5). These solvent effects were interpreted (7) to indicate that an electron of the unshared electron pair on the nitrogen atom in acridine undergoes a transition to an antibonding π molecular orbital. This excited state, in turn, undergoes a radiationless transition to the "triplet state"; consequently, such a compound does not exhibit fluorescence. When the unshared electrons on the nitrogen atom become involved in hydrogen bonding with proton-donating solvents, transitions to the triplet state become less likely or nonexistent and, therefore, enhanced fluorescence is observed in a proton-donating solvent. In other studies (8, 9) the effect of acetic acid and trichloroacetic acid on the absorption and fluorescence of acridine in benzene was also examined and the changes in fluorescence quality was ascribed to hydrogen bond formation.

The substituted acridine compounds mentioned exhibit different features in their fluorescence characteristics. This is probably because the substitutions increase the hydrophobicity of these compounds, and the changes of structures may influence the dipole moment and/or the charge-transfer character of these compounds and, therefore, their fluorescence. The study of fluorescence changes in these compounds brought about by solvent and protein binding effects may provide useful information in understanding the interaction between proteins and small molecules.

EXPERIMENTAL

Materials—The following were used: acridine[1], 3,6-diaminoacridine[1], 6,9-dichloro-2-methoxyacridine[1], *N*-phenylsulfanilic acid sodium salt[2], dansic acid[3], and bovine serum albumin[4]. 9-Anilino-6-chloro-2-methoxyacridine and 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine were prepared in this laboratory from 6,9-dichloro-2-methoxyacridine by a method previously described (10). Concentrations of the protein were determined by measuring the absorbance at 280 nm and by using $a = 0.66$ ($E_{1\,cm}^{1\%} = 6.6$) (11); the molecular weight of 69,000 was used to determine molar concentrations. Methanol and other solvents were spectroscopic

[1] Aldrich Chemical Co.
[2] Pfaltz & Bauer, Inc.
[3] Pierce Chemical Co.
[4] Nutritional Biochemicals Corp.

Table I—Solvent Effects on Fluorescence of 6,9-Dichloro-2-methoxyacridine (II), 9-Anilino-6-chloro-2-methoxyacridine (III), and 9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine (IV)[a]

Solvent	Solvent Dielectric Constant[b], debyes	II			III			IV		
		λ_{ex}, nm	λ_{em}, nm	Relative[c] Intensity	λ_{ex}, nm	λ_{em}, nm	Relative Intensity	λ_{ex}, nm	λ_{em}, nm	Relative Intensity
pH 7.4 phosphate buffer	—	400	465	12.7	395	465	1.2	390	465	1.0
Methanol	32.6	400	452	197.3	395	452	1.4	390	450	1.7
Dimethyl-acetamide		400	450	150.3	395	450	1.7	415	515	2.3
Acetone	20.7	400	445	125.0	395	445	1.1	415	492	7.7
Ethyl acetate	6.0	400	440	96.0	415	520	0.9	415	482	46.7
Benzene	2.3	400	440	117.3	415	490	8.7	415	472	75.6

[a] A concentration of 2×10^{-6} *M* was used in all samples. [b] "Handbook of Chemistry and Physics," 51st ed., R. C. Weast, Ed., The Chemical Rubber Co., Cleveland, Ohio, 1970–1971, pp. E61–64. [c] The relative intensities of all solutions are compared to the fluorescence of IV in pH 7.4 buffer, of which the value is taken to be 1.

grade, and all other chemicals were reagent grade or of special purity.

Synthesis—*9-Anilino-6-chloro-2-methoxyacridine Hydrochloride*—A mixture of 6.0 g of 6,9-dichloro-2-methoxyacridine and 30 g of phenol was heated in a flask to 70° over an oil bath. After all of the starting material was dissolved in phenol, 2 g of aniline was added to the mixture. The reaction was completed by refluxing the mixture at 140° for 12 hr with constant stirring using a magnetic stirrer. The product was cooled and washed thoroughly with anhydrous ether to remove phenol. A yellow product was recrystallized from 95% ethanol and dried *in vacuo*, mp 300–301° dec., *m/e* 335.

Anal.—Calc. for $C_{20}H_{16}Cl_2N_2O$: C, 64.70; H, 4.34; Cl, 19.15; N, 7.55. Found: C, 64.55; H, 4.30; Cl, 19.15; N, 7.45.

9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine Hydrochloride—This compound was prepared from 6,9-dichloro-2-methoxyacridine and *p*-aminobenzoic acid using the same procedures as already described, mp 335–337° dec., *m/e* 378.

Anal.—Calc. for $C_{21}H_{16}Cl_2N_2O_3$: C, 60.74; H, 3.88; Cl, 17.10; N, 6.75. Found: C, 60.64; H, 3.98; Cl, 17.19; N, 6.61.

9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine Sodium Salt—This compound was prepared by adding an excess amount of sodium hydroxide into the above compound (pH 9). The bright-yellow crystal was recrystallized from aqueous solution and dried *in vacuo*.

Anal.—Calc. for $C_{21}H_{14}ClN_2NaO_3$: C, 62.92; H, 3.50; N, 6.99; Na, 5.74. Found: C, 62.75; H, 3.65; N, 6.82; Na, 5.69.

The purity of these compounds was tested by TLC, and no impurity was found.

Apparatus and Methods—Fluorescence measurements were made with a spectrophotofluorometer[5] equipped with a 150-w xenon lamp and a lp21 photomultiplier tube. The relative fluorescence intensities were recorded directly from fluorometer readings. Spectra were recorded with an *X–Y* recorder.[6] The entrance slit for the excitation light and the exit slit for fluorescence emission were 3 and 4 mm, respectively.

Stock solutions of all probes were prepared using methanol as the solvent. Fluorescence titrations were performed manually with microsyringes[7]. The temperature of all fluorescence measurements was controlled to 25 ± 0.1°. Quantum yield determinations were made using Eq. 1 (12):

$$\Phi_u = \Phi_s\left(\frac{a_u A_s d_s \lambda_s}{a_s A_u d_u \lambda_u}\right) \qquad \text{(Eq. 1)}$$

where:

a = emission peak area corrected for solvent blank
A = absorbance at excitation wavelength
d = dilution factor in dilution of sample used for absorbance measurements to concentration used in fluorescence measurements
λ = excitation wavelength
Φ = quantum yield
s, u = subscripts denoting standard (s) and unknown (u)

Although the quantum yield can be obtained by absolute methods (13), it is more convenient to use comparative methods in which compounds of known quantum yield are used as reference standards. Dansic acid (5-dimethylamino-1-naphthalenesulfonic acid) was used as the reference standard, taking its quantum yield to be 0.36 at an excitation wavelength of 320 nm (14).

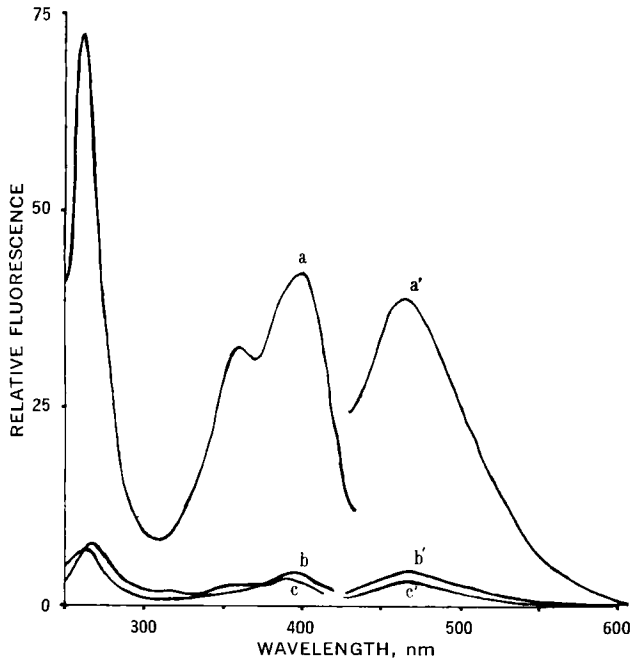


Figure 1—*Excitation and emission spectra of 2 × 10⁻⁶ M probes in pH 7.4 phosphate buffer. Key: a and a′, excitation (λ_{em} = 465 nm) and emission (λ_{ex} = 400 nm) curves of 6,9-dichloro-2-methoxyacridine; b and b′, excitation (λ_{em} = 465 nm) and emission (λ_{ex} = 395 nm) curves of 9-anilino-6-chloro-2-methoxyacridine; and c and c′, excitation (λ_{em} = 465 nm) and emission (λ_{ex} = 390 nm) curves of 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine.*

RESULTS AND DISCUSSION

The excitation and emission spectra of 6,9-dichloro-2-methoxyacridine (II), 9-anilino-6-chloro-2-methoxyacridine (III), and 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine (IV) measured in pH 7.4 phosphate buffer are shown in Fig. 1. 6,9-Dichloro-2-methoxyacridine was highly fluorescent in the buffer solution, but the intensity was quenched considerably when the 9-chloro group was replaced by an anilino or a carboxyanilino group. The emis-

[5] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[6] Omnigraphic, Houston Instrument, Bellaire, Tex.
[7] Hamilton.

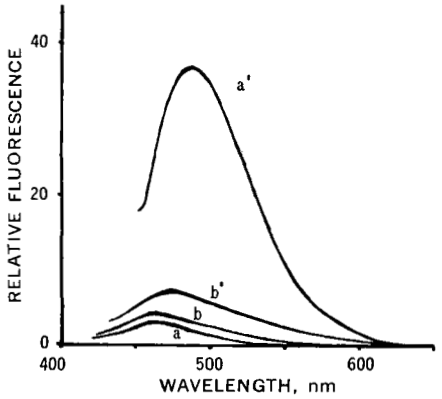


Figure 2—*Emission spectra of 2 × 10⁻⁶ M probes in the presence and absence of 6.9 × 10⁻⁶ M bovine serum albumin. Key: a and a', emission curves of 9-(4'-carboxyanilino)-6-chloro-2-methoxyacridine in the absence and presence of bovine serum albumin; and b and b', emission curves of 9-anilino-6-chloro-2-methoxyacridine in the absence and presence of bovine serum albumin.*

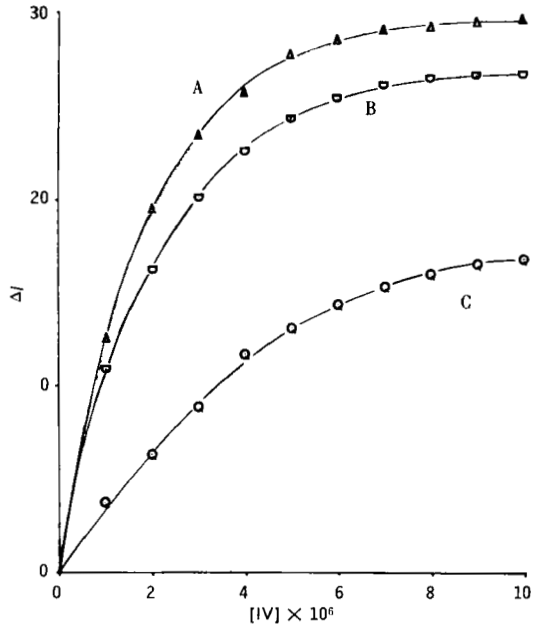


Figure 3—*Plots of* ΔI versus *9-(4'-carboxyanilino)-6-chloro-2-methoxyacridine (IV) concentration for solutions containing 1.38 × 10⁻⁶ M bovine serum albumin in the absence and presence of drug compound, where* ΔI *is the corrected fluorescence intensity of the solution from the residue fluorescence of bovine serum albumin and the fluorescence of IV in the absence of bovine serum albumin at the same wavelengths* (λ_{ex} = *420 nm,* λ_{em} = *485 nm). Key: A, in the absence of drug; B, in the presence of 5 × 10⁻⁵ M salicylic acid; and C, in the presence of 5 × 10⁻⁵ M sodium warfarin.*

sion wavelength, however, remained the same at 465 nm. The excitation maxima for II, III, and IV were 400, 395, and 390 nm, respectively. Acridine fluoresced much stronger than II and had excitation and emission maxima at 355 and 442 nm in pH 7.4 buffer, respectively.

The fluorescence characteristics of II in various solvents are shown in Table I. The emission maximum shifted toward the blue as the polarity of solvent decreased. The intensity changes, however, did not follow the probe pattern. As shown in Table I, the relative intensities in methanol, dimethylacetamide, and acetone are higher than those in either more polar or more nonpolar solvents. Since the fluorescence of acridine has been known to decrease as the polarity of solvent decreases (4, 5), it is concluded that the fluorescence of II is essentially a combination of the fluorescence of acridine and the substitution effects. In contrast to acridine, the presence of the chloro and methoxy groups of II apparently causes an increase of intensity with a decrease in the polarity of the solvent as a result of an increase in its hydrophobicity. This is further substantiated by the results obtained for III and IV (Table I). The fluorescence of III is quite similar to II with the exception of having a much lower intensity in the same solvent. Compound IV is practically nonfluorescent in buffer solution but brightly fluoresces in nonpolar solvents. Furthermore, in buffer and methanol the spectral characteristics of II, III, and IV are very similar, but in less polar solvents the emission maxima of III and IV are shifted to longer wavelengths. Further increases in the polarity of the solvent result in a blue shift accompanied by an increase in emission intensity. This effect is particularly clear in the results for IV.

The interactions of acridine and some derivatives with bovine serum albumin also were studied. The emission spectra of III and IV in the presence of 6.9 × 10⁻⁶ *M* bovine serum albumin in pH 7.4 buffer are shown in Fig. 2. The emission maxima for III and IV are 475 and 485 nm, respectively. The quantum yield data of II, III, and IV measured in pH 7.4 phosphate buffer and the data of their bovine serum albumin mixtures are listed in Table II. Compound IV produced the highest quantum yield increases in binding to bovine serum albumin (20-fold). The protein mixture of II had a twofold increase in quantum yield, whereas III increased about three times in the presence of protein. The manifold increase in fluorescence accompanied by the blue shifts of the emission maximum in various solvents and in the protein mixture indicates that IV, 9-(4'-carboxyanilino)-6-chloro-2-methoxyacridine, should be useful as a probe for drug–protein binding studies.

The fluorescence of the bovine serum albumin mixtures of acridine and 3,6-diaminoacridine was also examined. These compounds do not seem to interact with bovine serum albumin. In polar solvents, the fluorescence of II, III, and IV is mainly charac-

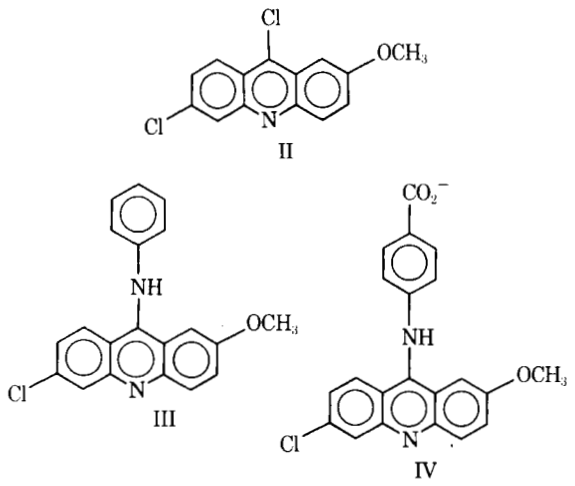


Table II—Quantum Yield of 6,9-Dichloro-2-methoxyacridine (II), 9-Anilino-6-chloro-2-methoxyacridine (III), 9-(4'-Carboxyanilino)-6-chloro-2-methoxyacridine (IV), and *N*-Phenylsulfanilate (V) and of Their Bovine Serum Albumin Mixtures in pH 7.4 Phosphate Buffer

Compound	Concentration, *M*	Bovine Serum Albumin Concentration, *M*	Excitation, nm	Emission, nm	Quantum Yield
II	2 × 10⁻⁶	—	400	465	0.044
II	2 × 10⁻⁶	6.9 × 10⁻⁶	400	460	0.092
III	2 × 10⁻⁶	—	395	465	0.021
III	2 × 10⁻⁶	6.9 × 10⁻⁶	400	475	0.060
III	2 × 10⁻⁶	69.0 × 10⁻⁶	400	475	0.053
IV	2 × 10⁻⁶	—	390	465	0.006
IV	2 × 10⁻⁶	6.9 × 10⁻⁶	430	485	0.120
IV	2 × 10⁻⁶	69.0 × 10⁻⁶	430	485	0.119
V	4 × 10⁻⁴	—	332	457	0.006
V	4 × 10⁻⁴	6.9 × 10⁻⁶	338	405	0.155

Table III—Fluorescence of *N*-Phenylsulfanilate in pH 7.4 Phosphate Buffer

Concentration, *M*	Excitation, nm	Emission, nm	Relative Intensity
1×10^{-5}	300	465	11.0
2×10^{-5}	304	467	17.0
5×10^{-5}	311	460	25.0
15×10^{-5}	324	465	29.1
25×10^{-5}	328	460	30.0
35×10^{-5}	332	458	30.0
45×10^{-5}	332	457	30.2
55×10^{-5}	332	456	30.6

terized by the acridine residue; in nonpolar solvents and in protein mixtures, the substitution effects are the dominant factors.

The fluorescence of a probe is affected by the solvent or protein environment through dipole–dipole interaction, hydrogen bonding, and charge transfer, *etc.* Probes with greater dipole moment in the excited state than the ground state show solvent-dependent emission spectra reflecting the mobility and polar character of the solvent (15–18). This is true since solvent molecules may relax about the excited state prior to emission and this, in turn, tends to lower the energy of the excited state, resulting in a red shift at the fluorescence emission (15–18). Other probes, such as acridine, may be sensitive to the proton-donating or proton-accepting character of the solvent.

The fluorescence of acridine is characterized by the hydrogen-bonding effect of the solvent (4, 5). This effect, however, is reduced by the chloro, methoxy, anilino, and carboxyanilino substitutions as in the cases of II, III, and IV. These substituents, which are more sensitive to the polarity of the solvent, tend to change the dipole moment of II, III, and IV in the excited state and, therefore, alter their fluorescence. As shown in Table I, III still shows the hydrogen-bonding effect by the solvent, whereas IV clearly shows a solvent-relaxation effect. This difference may be due to the difference of the intramolecular charge-transfer character of these compounds. The fluorescence changes of IV by the solvent and protein environment are very similar to those of *N*-arylaminonaphthalinesulfonates, the probes that were reported (19–21) to possess the intramolecular charge-transfer character.

By carefully examining the interactions of III and IV with bovine serum albumin, it is believed that the binding occurs on the anilino group rather than on the acridine residue. The carboxylic group in IV must be directly involved in the binding. Hydrophobic forces do exist in the binding, but the difference in binding affinity of III and IV is somewhat greater than can be accounted for solely on the basis of hydrophobic interaction. This fact is further strengthened by the binding studies of diphenylamine and *N*-phenylsulfanilate to bovine serum albumin. The results will be discussed later.

It was reported (1, 22) that the strong interactions between 5-dimethylaminonaphthalene-1-sulfonamide and carbonic anhydrase were due to the loss of amino proton by 5-dimethylaminonapthalene-1-sulfonamide; *i.e.*, the binding species is the anion rather than the neutral molecule. In fact, most probes used in drug–protein binding studies have an anionic group while, on the other hand, proteins such as bovine serum albumin are known to bind strongly with small organic molecules with anionic groups (23). These acid groups may interact with positively charged residues in the protein to form complexes, and the binding is reinforced by the hydrophobic interactions between the protein and the small molecule. The most strongly hydrophobic sites on the protein are probably primarily involved in the binding. The fluorescence change of the probe when bound to protein could be due to changes in the charge of protein molecules (24).

Use of 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine (IV) as a probe was tested with several anionic drugs, two of which are reported here. As an example, the competitive displacements of the probe by salicylic acid and sodium warfarin in binding to bovine serum albumin are shown in Fig. 3. Other drugs such as phenylbutazone, dicumarol, and the sulfonylureas can also displace the probe from its binding sites on bovine serum albumin.

Fluorescence studies of diphenylamine and *N*-phenylsulfanilate were also carried out in this laboratory. Table III shows the concentration effects of *N*-phenylsulfanilate on the fluorescence measured in pH 7.4 phosphate buffer. At low concentrations, the excitation maximum of *N*-phenylsulfanilate is close to that of bovine serum albumin (excitation maximum 290 nm). At 3.5×10^{-4} *M*, the excitation maximum ceases to increase with increasing excitation. Therefore, the concentration chosen for quantum yield and spectral measurements was 4×10^{-4} *M*, where the excitation maxima of *N*-phenylsulfanilate in the absence and presence of bovine serum albumin are 332 and 338 nm, respectively.

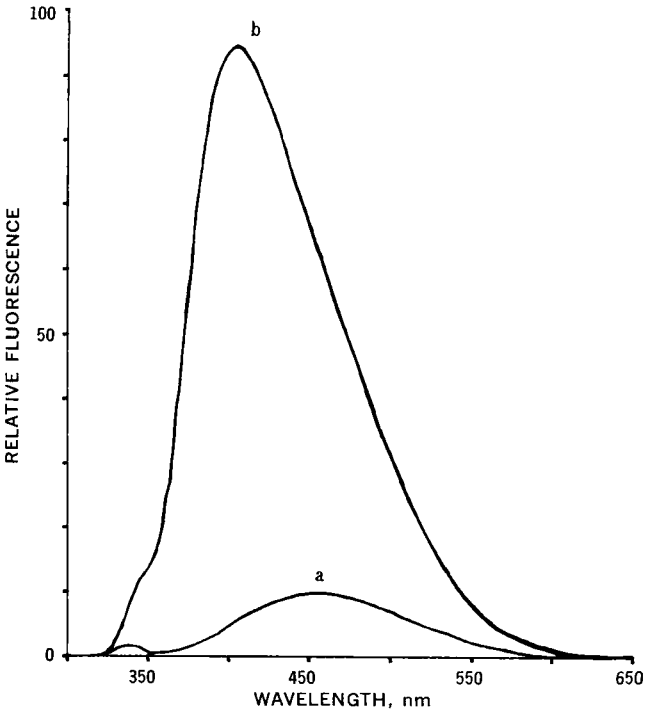


Figure 4—*Emission spectra of* 4×10^{-4} M *N-phenylsulfanilate. Key: a, in the absence of bovine serum albumin* (λ_{ex} = 332 nm, λ_{em} = 457 nm); *and b, in the presence of* 6.9×10^{-6} M *bovine serum albumin* (λ_{ex} = 338 nm, λ_{em} = 405 nm).

Diphenylamine is highly fluorescent in nonpolar as well as polar solvents. The addition of bovine serum albumin did not affect the fluorescence of diphenylamine. In the case of *N*-phenylsulfanilate, however, the fluorescence was changed dramatically upon addition of bovine serum albumin. The emission spectra of *N*-phenylsulfanilate in the absence and presence of 6.9×10^{-6} *M* bovine serum albumin are shown in Fig. 4. In binding to the protein, the quantum yield of *N*-phenylsulfanilate increased from 0.006 to 0.155 while its fluorescence emission maximum shifted from 457 to 405 nm. The fact that *N*-phenylsulfanilate and 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine strongly interact with bovine serum albumin, whereas diphenylamine and 9-anilino-6-chloro-2-methoxyacridine do not, suggests that the acid groups of these compounds are indeed involved in the binding.

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Abstract □ A GLC assay technique, previously applied to the evaluation of the influence of nitrofurazone on pathological vaginal odors, was refined and tested in a study of vaginal secretion odors in normal women before and after administration of a suppository. The principal methodological refinements included considering changes in the concentrations of seven frequently encountered malodorants and testing results for their statistical validity. The technique was sufficiently sensitive to indicate statistically significant ($p < 0.05$) changes in a group of 10 subjects.

Keyphrases □ Vaginal odors—method and GLC assay technique for evaluating odor changes, suppository induced □ Odors, vaginal—method and GLC assay technique for evaluating odor changes, suppository induced □ GLC—detection of changes in vaginal odors

Established techniques exist to determine the type and the number of microorganisms that may produce odors in the vaginal secretions. However, formation of malodorous compounds *in vivo* may depend also on the secretions of changing composition. Reduction in bacterial or fungal counts *in vitro* or *in vivo* cannot be *a priori* equated with the activity in odor suppression; lack of such correlations in axillary odors was reported (1, 2).

Earlier work showed that a combination of GLC and sensory techniques permits an evaluation of the reduction of infection-induced vaginal odors by nitrofurazone (3, 4), as well as a comparison of the number of malodorous compounds in normal secretions (5). Even such a simple indicator as the number of malodorous compounds was sufficient to assess gross changes.

It was felt that further and more quantitative refinements in the odor evaluation technique were desirable. The degree of the malodorosity of each component should be considered. Changes should be objectively quantified by observing the concentration changes (GLC peak areas) of the same odorant. Finally, spurious and arbitrary interpretations should be avoided by application of some simple statistical tests so that an estimate of the significance of the changes can be obtained.

The usefulness of these refinements was investigated in a study of odor changes following an intravaginal administration of a suppository.

EXPERIMENTAL

Sampling—Samples of vaginal secretions were obtained using heat-sterilized and deodorized Teflon inserts (Fig. 1). The insert carried 480 circular smooth-edged pits, each 0.15 cm (0.06 in.) wide and 0.07 cm (0.03 in.) deep. A Teflon slip-ring collar allowed adjustment of the depth of insertion. The pits and the surface of the insert served as mechanical traps for mucus. After 20 min, the insert was removed, placed in a clean glass container (Fig. 1), and stored in a freezer until analyzed.

Analysis—Inserts, still in their glass containers, were brought to room temperature. A total volume of 6000 ml of high purity ("zero" grade) helium was passed through the vessel at a rate of 100 ml/min. The collection arrangement is shown in Fig. 1. The vapor-saturated helium then entered a vapor collector, containing 5 g of highly purified, high surface area (>300 m^2/g), nonpolar styrene–divinyl copolymer[1], where organic vapors were absorbed but water vapor was retained to a negligible extent. Vapors then were removed from this collector and transferred in one single injection into a gas chromatograph, using procedures and devices described previously (6).

The chromatographic partition column was a 6.09-m (20-ft), 0.31-cm (0.125-in.), stainless steel tubing packed with 2.5% Carbowax 20M on 60–80-mesh Chromosorb G support, acid washed and dimethylsiloxane treated. The carrier gas was high purity helium introduced at 60 ml/min; the temperature was elevated linearly from 60 to 180° at 2°/min. The effluent was split, with one half flowing to a hydrogen flame-ionization detector and the other half flowing to a sniffing port supplied with a controlled flow of air humidified to reduce nose irritation (7).

An analyst, specialized in the sensory evaluation of GLC effluents, characterized the odors of the separated components as

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Table I—Example of a Computer-Transcribed Odorogram of Vaginal Odors[a]

Peak	Retention Time, min	Peak Area	Percent Peak Area	Odor Note	Hedonic Code	Peak	Retention Time, min	Peak Area	Percent Peak Area	Odor Note	Hedonic Code
1	1.57	−0.00	0.00	—	—	36	23.21	−0.00	0.00	—	—
2	1.72	−0.00	0.00	—	—	37	24.06	8.96	0.13	Aldehydic	P
3	1.85	−0.00	0.00	—	—	38	24.51	−0.00	0.00	—	—
4	2.43	−0.00	0.00	—	—	39	26.10	−0.00	0.00	—	—
5	2.54	−0.00	0.00	—	—	40	26.75	18.56	0.28	Unpleasant	XX
6	2.72	−0.00	0.00	—	—	41	27.04	58.24	0.87	Acidic	XX
7	2.94	1.20	0.02	Pleasant	P	42	27.40	92.16	1.38	Acidic	XX
8	3.19	−0.00	0.00	—	—	43	28.00	−0.00	0.00	—	—
9	3.90	−0.00	0.00	—	—	44	29.25	−0.00	0.00	—	—
10	4.40	14.52	0.22	Yeasty	N	45	30.30	32.64	0.49	Acidic	XX
11	4.75	−0.00	0.00	—	—	46	31.75	788.48	11.83	Sweet	P
12	5.42	3.36	0.09	Fatty	X	47	33.87	3.64	0.06	Sweet	XX
13	5.73	−0.00	0.00	—	—	48	34.46	−0.00	0.00	—	—
14	5.97	−0.00	0.00	—	—	49	36.24	−0.00	0.00	—	—
15	6.46	−0.00	0.00	—	—	50	37.69	896.00	13.45	Fruity	P
16	7.33	−0.00	0.00	—	—	51	39.19	134.40	2.02	Spicy	P
17	8.45	−0.00	0.00	—	—	52	40.40	16.00	0.24	Cheesy	XXX
18	8.78	−0.00	0.00	—	—	53	40.84	14.72	0.22	Cheesy	XXX
19	9.07	−0.00	0.00	—	—	54	41.55	3.84	0.06	Unpleasant	XX
						55	42.31	152.04	2.28	Acidic	XX
20	10.37	26.24	0.39	Unpleasant	XX	56	42.94	102.40	1.54	Fruity	P
21	10.91	−0.00	0.00	—	—	57	43.96	900.00	13.51	Pungent	X
22	11.67	−0.00	0.00	—	—	58	44.69	880.64	13.22	Pungent	X
23	12.37	−0.00	0.00	—	—	59	45.78	409.60	6.15	Spicy	P
24	13.04	−0.00	0.00	—	—	60	46.22	−0.00	0.00	—	—
25	14.12	−0.00	0.00	—	—	61	47.30	−0.00	0.00	—	—
26	14.93	25.60	0.38	Odor	N	62	48.85	950.00	14.26	Bitter	XXX
27	16.43	32.00	0.48	Unpleasant	XX	63	50.10	51.20	0.77	Pleasant	P
28	17.06	−0.00	0.00	—	—	64	50.73	900.20	13.51	Fruity	P
29	17.88	−0.00	0.00	—	—	65	52.01	−0.00	0.00	—	—
30	18.52	2.32	0.03	Fruity	P	66	52.85	17.02	0.27	Unpleasant	XX
31	19.00	55.04	0.83	Bitter	XXX	67	54.15	28.16	0.42	Unpleasant	XX
32	19.48	−0.00	0.00	—	—	68	55.31	−0.00	0.00	—	—
33	20.37	−0.00	0.00	—	—	69	55.81	−0.00	0.00	—	—
34	20.76	−0.00	0.00	—	—	70	59.67	−0.00	0.00	—	—
35	22.06	43.52	0.65	Fatty	X	71	60.06	−0.00	0.00	—	—

[a] Sample number: Subject 10, 1 pm, day before treatment. Total sample size: 666,300 in.^{2}, odorous peaks only. Peak areas of nonodorous peaks are not indicated. The areas are in square inches.

they emerged from the column through the sniffing port, using the method described earlier (3–5, 8). This method was further refined by using, in addition to the odor descriptors, a hedonic category scale: X, XX, and XXX designated unpleasant odors in the order of increasing intensity of unpleasantness; P, PP, and PPP designated pleasant odors in the order of increasing pleasantness; and N was used for odors that were neither primarily unpleasant nor pleasant[2].

In the described arrangement, odorants preconcentrated from a 6000-ml sample of the vapors are evaluated in one single analysis. Therefore, each compound present in the sample is delivered for the sensory assay, even after some subsequent dilution in the GLC column and the sniffing port, at a concentration 50–100 times higher than in the original sample vapor space. Components exhibiting odor do so at an enhanced intensity which facilitates their odor characterization. On the other hand, if a component is not odorous when it emerges from the port, its concentration in the original sample was much below its odor threshold and, therefore, it is not odor relevant.

Schedule—Ten healthy subjects were used[3]. Usual precautions (5) were taken to reduce the possibility of interferences by extraneous factors (cosmetics, spiced foods, *etc.*).

Three samples were taken from each subject at regular intervals on 2 consecutive days. The samples "before" treatment were taken at 9 am, 1 pm, and 5 pm of the "pretreatment" day; the suppository was administered at 7 am on the subsequent or "treatment" day, and samples were again taken at 9 am, 1 pm, and 5 pm. Thus, they represented secretions 2, 6, and 10 hr after treatment. Additionally, 60 samples representing usual (nontreated) vaginal conditions were taken from the same 10 subjects on different days under the same precautions as on the pretreatment day to collect more information on the normal chromatograms.

The vaginal suppository[4] contained 0.0004 g phenylmercuric acetate, 0.0004 g methylbenzethonium chloride, and 0.002 g methylparaben in a water-dispersible base.

Data Reduction—Areas of odorous peaks were measured with an electronic integrator[5], and the accumulated data were transferred to punched cards and printed out in tabular form. A sample analysis is shown in Table I. From the calibration of the instrument with *n*-hexane, peak area multiplied by 5 yields the approximate concentrations of the respective odorants in nanograms per liter of gas space in equilibrium with the sample[6].

RESULTS AND DISCUSSION

The effect of an odor change may be reflected by the GLC–sensory assays in several ways. The number of the odorous or malodorous chemical species may increase or decrease, and their relative malodorosity may change. This may become evident in the odor assay of the effluents at the exit from the GLC partition column or by a more exact procedure of comparing the GLC areas of

[2] Several years of experience with this technique has demonstrated that clearly unpleasant odorants (amines, lower fatty acids, sulfides, mercaptans, *etc.*) are uniformly judged unpleasant by different analysts when presented, without identification, for sensory ratings in a form of GLC effluents. Ratings are more individualistic on odors that are pleasant or neutral. It was shown previously (9) that, even with a minimum of experience, individuals reasonably reliably distinguish three to four levels of odor intensity.

[3] Clinical data on the subjects are available from the authors upon request.

[4] Norforms, Norwich Pharmacal Co.

[5] Hewlett-Packard.

[6] One nanogram of an organic substance gives a GLC peak with an area of 0.2 in.^{2}.

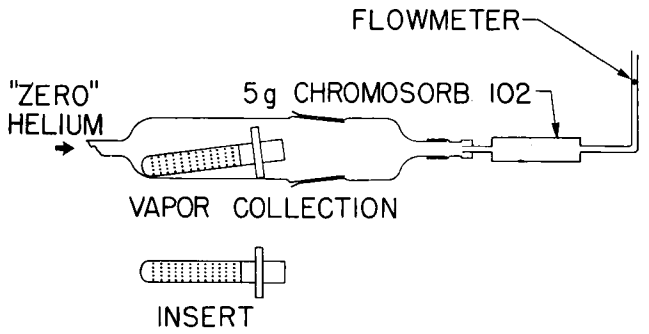

Figure 1—*Vaginal insert (bottom) and the arrangement for transfer of vapors from insert to an organic vapor collector.*

the corresponding peaks in different samples. With the latter procedure, the GLC positions of odorants most commonly occurring in the samples are ascertained first.

Baseline Data—The gas chromatograms of 90 samples taken from 10 subjects on days not influenced by any treatment, including the 30 samples taken on the day before the experimental treatment, served to characterize the GLC profiles of usual secretions, with particular emphasis on malodorous compounds.

An overview on the number and GLC positions of the usually occurring malodorants was obtained from inspection of Fig. 2. This histogram indicates the frequency of occurrence of malodorous chemical species for each minute of retention time. As an example, such a compound was noted at the 8th min in 31 out of 90 analyzed samples. A Kovats index (10) scale (indicated at the top) provides retention values that are largely independent of the flow rates, temperatures, and the amount of stationary phase in the column, as long as the same phase is used.

There were seven distinct retention time points where malodorants frequently appeared (arrows) and seven additional points (stars) with less pronounced frequency-of-occurrence maxima. The seven most frequently occurring compounds had estimated Kovats indexes of 960, 1100, 1300, 1470, 1720, 1750, and 1840.

The following possible indicators derivable from the GLC–sensory assays were compared for their ability to reflect changes in samples from the pretreatment to the treatment day: *(a)* total

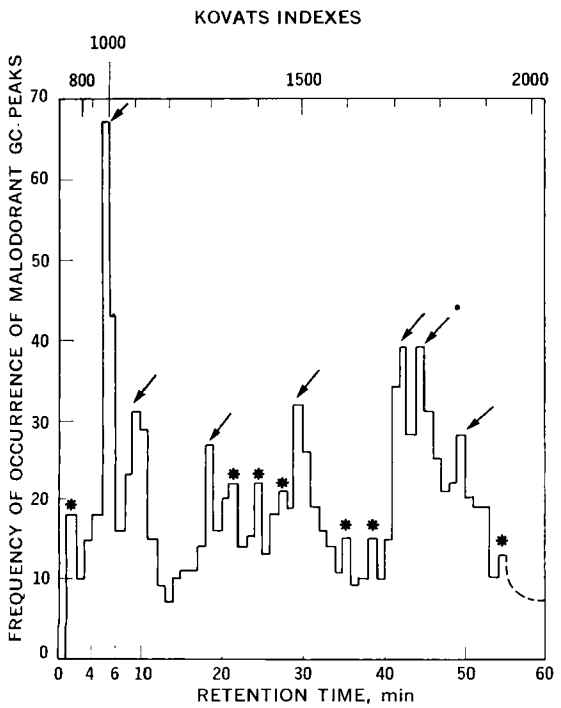

Figure 2—*Histogram of malodorant peak occurrences in gas chromatograms of vaginal vapors (10 subjects, 90 chromatograms, all samples on days not affected by treatment).*

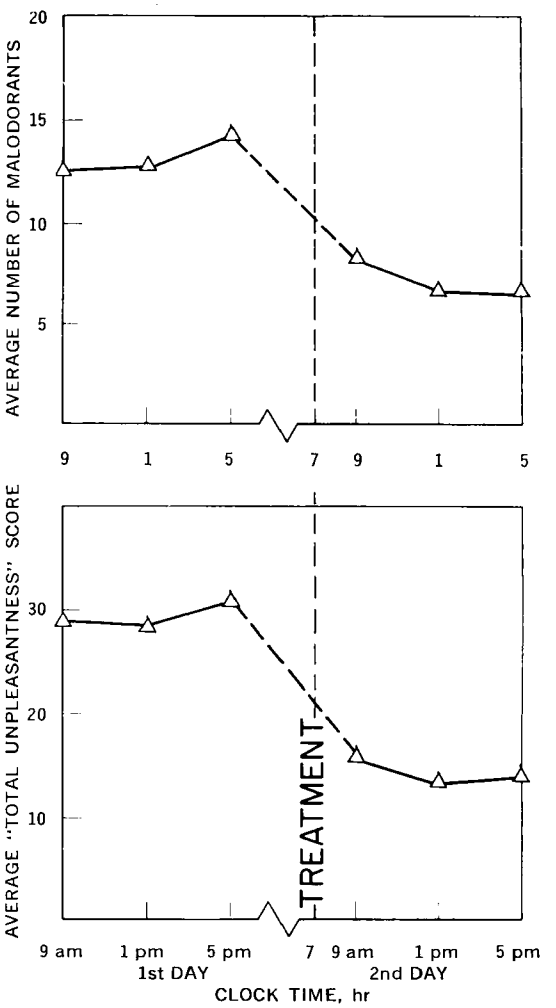

Figure 3—*Change in the number of malodorants and average total malodorosity score during the experiment.*

number of peaks, *(b)* total number of odorous peaks, *(c)* total number of malodorous peaks, *(d)* sum of GLC areas of all odorous peaks, and *(e)* GLC areas of the seven most frequently occurring malodorants.

Total Number of Peaks—This value represents the minimum number of chemical species present in the sample. The number of peaks ranged from 22 to 87, with an average number of 55. This value was not influenced by the treatment to a statistically significant degree (Wilcoxon sum of ranks test).

Total Number of Odorous Peaks—The number of peaks accompanied by a detectable odor ranged from 2 to 38. It also was not significantly influenced by the treatment (Wilcoxon sum of ranks test).

Total Number of Malodorous Peaks—Figure 3 indicates the average number of malodorous peaks per sample for different time points in the experiment. Since each malodorous peak in each gas chromatogram was scored for the degree of malodorosity on an arbitrary three-point scale (X, XX, and XXX), a derived indicator was possible: a cumulative malodorosity score which is

Table II—Sum of Ranks on GLC–Sensory Data[a]

Hours after Treatment	Wilcoxon Sum of Ranks Test, p	
	Number of Malodorants	Malodorosity Score
2	Insignificant	Insignificant
6	<0.05	<0.002
10	<0.05	<0.002

[a] Ten pairs of observations.

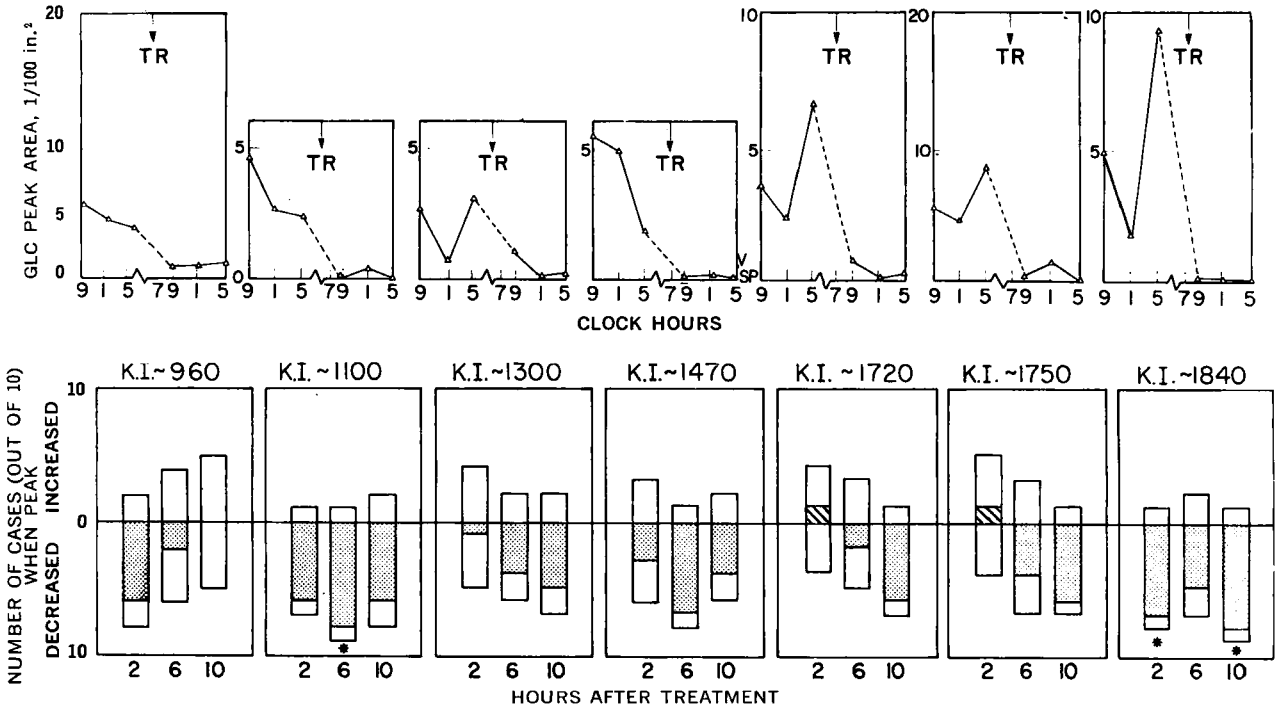

Figure 4—*Effect of suppository on concentrations of seven malodorants most frequently occurring in vapors of vaginal odors of 10 subjects. Kovats indexes of peaks to which each set of diagrams belongs are indicated by K.I. numbers between the two rows of diagrams. The treatment point is indicated by arrows marked TR. Above: median values of peak areas. Below: histogram of frequencies of the direction of peak size changes. Key:* □, *excess of decreases over increases; and* □, *excess of increases over decreases.*

the sum of scores per sample. As an example, it is 38 for the gas chromatogram of Table I. The rationale was that a few high-score peaks may have as much odor significance as a larger number of low-score peaks. The cumulative malodorosity is also plotted in Fig. 3.

The statistical significance of observed changes was estimated using the Wilcoxon sum of ranks test. The 10 samples taken at 9 am on the day before treatment were coranked with 10 samples taken at 9 am of the treatment day, *etc.* (Table II). This procedure was used to minimize the possible effect of daily cyclic activities. The cumulative malodorosity score indicated higher significance levels than the number of malodorous peaks.

Concentration of Odorous Compounds—The average concentration of all odorous compounds, regardless of their odor type, was 2.1×10^{-6} g/liter of sample vapor for samples taken on days not influenced by any treatment. It was not influenced by the treatment to a statistically significant degree.

Concentrations of Seven Malodorants—The upper part of Fig. 4 reflects, separately for each of the seven most frequently occurring malodorants, the changes in median concentration during the experiment. The median value was used to combine the data from the subjects because the peak areas varied from 0 (no peak observed or the peak was nonodorous) to as high as several hundred units. In such a situation, an arithmetic average is inappropriate and a logarithmic average is impossible because of zero values. A median value of zero results when six or more samples from 10 subjects do not exhibit the particular peak.

Table III—Statistical Significance Test on Observed Changes of Concentrations of Seven Malodorants[a]

Hours after Suppository	Lowest Rank Sum (after Suppository)	z Value	Probability that Effect Occurred by Chance Only
2	1204	3.80	<0.002
6	1231	3.41	<0.002
10	1151	4.55	<0.002

[a] Wilcoxon stratified rank test of seven strata, 10 pairs per stratum.

The correlations between odors and concentrations do not necessarily follow a normal statistical distribution. Therefore, nonparametric tests are preferred for evaluation of the statistical significance of the observed changes. The individual data were paired, each pair consisting of one pretreatment sample and the sample taken from the same subject at the same hour of the day of the treatment. This procedure should minimize the effect of daily cyclic activities. The Wilcoxon stratified rank test (11) was used, with seven malodorants serving as seven strata, each containing 10 pairs. This procedure tends to equalize the relative influence of the malodorants, removing the difference in the average concentration levels of the seven malodorants as a factor in the comparison. While some odorants may be more critical than others, the seven malodorants were weighted equally.

Table III presents the statistical results. The change in concentration of the more frequently occurring malodorants jointly reached statistically significant levels.

The lower graphs in Fig. 4 represent a simpler test. The relative direction of change in the peak areas of each malodorant was the only factor considered; decrease upon treatment was plotted downward, and increase was plotted upward. The shaded areas indicate the relative balance of the two directions. The Sign test was used to evaluate the statistical significance of the changes. Data for all seven malodorants were pooled separately for the 9 am samples of the pretreatment and treatment day, *etc.* Table IV

Table IV—Sign Test on Direction of Change in GLC Peak Areas of Seven Malodorants

Hours after Treatment	Total Number of Cases in Which a Change in Peak Areas Occurred	Number of Cases in Which the Peak Area Decreased	Number of Cases in Which the Peak Area Increased	Statistical Significance Level
2	62	42	20	<0.05
6	64	47	17	<0.01
10	63	47	16	<0.01

Table V— Individual Differences in Changes of Concentration of the Seven Most Frequently Occurring Malodorants

GLC Kovats Index of Malodorant	Sampling Time	Number of Cases (out of 10) where Malodorant Content Increased after Treatment									
			Subject Identification								
		Number	1	2	3	4	5	6	7	8	9	10
≈960	9 am	2		x			x					
	1 pm	4			x	x	x	x				
	5 pm	4		x		x	x	x				
≈1100	9 am	1										x
	1 pm	1						x				
	5 pm	2				x						x
≈1300	9 am	3					x			x		x
	1 pm	2							x	x		
	5 pm	2	x							x		
≈1470	9 am	3			x			x				x
	1 pm	1						x				
	5 pm	2			x							x
≈1720	9 am	4					x	x		x	x	
	1 pm	3							x	x	x	
	5 pm	1									x	
≈1750	9 am	5			x		x	x			x	x
	1 pm	3					x		x	x		
	5 pm	1									x	
≈1840	9 am	1						x				
	1 pm	2								x	x	
	5 pm	1										x
		Total[a]	1	2	4	4	7	8	3	7	6	7

[a] Out of 21 comparison pairs per subject.

indicates that this method of data analysis also shows statistically significant changes.

Table V brings out the degree of variability of response in different subjects. Since the response to treatment varied considerably from subject to subject, this variability should be considered in any similar testing study and should have a bearing on the total number of subjects used to reach statistical significance.

CONCLUSIONS

A refined form of GLC–sensory assaying of vaginal odors indicated that approximately 14 malodorants appeared frequently in vapors of the vaginal secretions of 10 normal healthy women. Seven malodorants appeared with considerable frequency, and changes in their concentrations were used to follow the effect of a vaginal treatment.

This refined method may provide a more quantitative basis for evaluating vaginal treatments in terms of malodorant concentration changes.

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*Present address: Chicago Medical School, Chicago, IL 60612

xTo whom inquiries should be directed.

Reaction of Haloperidol with 5-(Hydroxymethyl)-2-furfuraldehyde, an Impurity in Anhydrous Lactose

CASIMIR A. JANICKI[x] and HAROLD R. ALMOND, Jr.

Abstract □ In the investigation of haloperidol direct compression tablets, using anhydrous lactose as one excipient, a 5–8% loss was encountered on initial assays. Analysis of the lactose by UV, GLC, and mass spectrometry confirmed the presence of 5-(hydroxymethyl)-2-furfuraldehyde. Haloperidol was reacted with 5-(hydroxymethyl)-2-furfuraldehyde and furfural, and conversion to addition compounds resulted. Although both addition compounds were characterized, they could not be isolated from the direct compression tablets.

Keyphrases □ Haloperidol—reaction with 5-(hydroxymethyl)-2-furfuraldehyde impurity in anhydrous lactose □ 5-(Hydroxymethyl)-2-furfuraldehyde as impurity in anhydrous lactose—reactions with haloperidol □ Lactose, anhydrous, containing 5-(hydroxymethyl)-2-furfuraldehyde as contaminant—reaction with haloperidol

Direct compression as a method of tablet manufacture continues to receive attention in the industry because it offers distinct economic advantages over conventional methods. Interest is also high because the tablets produced usually have significantly faster dissolution rates since there are no collodial binders to envelop the granules. The use of lactose (beadlets) and dextrose[1] as excipients in this method of manufacture was recently proposed (1). Spray-dried lactose, anhydrous lactose, and microcrystalline cellulose have also been used as excipients in the direct compression technique.

During an investigation of haloperidol[2] direct compression tablets, using anhydrous lactose as one excipient, a 5–8% drug loss was encountered in initial assays. This problem was unexpected since it had been demonstrated (2) that anhydrous lactose could be used successfully in the manufacture of tablets of the related butyrophenone trifluperidol hydrochloride {4′-fluoro-4-[4-hydroxy-4-(α,α,α-trifluoro-*m*-tolyl)piperidino]butyrophenone} and no evidence of a reaction between trifluperidol hydrochloride and anhydrous lactose was observed.

The anhydrous lactose was then tested for the presence of 5-(hydroxymethyl)-2-furfuraldehyde (I), since other workers (3, 4) had reported problems caused by the aldehyde in spray-dried lactose forming Schiff bases with drugs containing a primary amine. In a related study, Brownley and Lachman (5) demonstrated a relationship between the presence of I and the discoloration of spray-dried lactose. UV absorption spectra of the various lots of anhydrous lactose used in this study indicated the presence of I even though the lactose used was white in color.

Haloperidol, being a tertiary amine, could not form a Schiff base with I. However, when haloperidol was reacted with I, a Claissen–Schmidt condensation compound resulted. A similar compound resulted when haloperidol was similarly reacted with 2-furfural. The condensation compounds were characterized.

EXPERIMENTAL[3]

The haloperidol tablets and capsules were assayed for intact drug by the official method (6). The assay is stability indicating for haloperidol. Haloperidol tablets were manufactured using formulas similar to those given previously (2). They contained about 85% anhydrous lactose, 15% starch, and 0.7% calcium stearate.

Nonspecific Assay for Haloperidol—Weigh accurately a portion of tablet powder, equivalent to about 1 mg of haloperidol, and transfer it to a 120-ml (4-oz) bottle. Add exactly 50 ml of chloroform and 30 ml of methyl orange solution (a saturated solution in pH 5 buffer, 0.2 *M* sodium phosphate adjusted to pH with 1 *M* sodium hydroxide). Shake on a mechanical shaker for 15 min, centrifuge, aspirate, and discard the aqueous layer. Transfer exactly 5 ml of the chloroform layer to exactly 10 ml of acidified methanol (5% v/v hydrochloric acid in methanol), and mix. Concomitantly determine the absorbance of the solution and of a solution of haloperidol standard, taken through the assay, in 1-cm cells at the maximum at about 522 nm.

Assay and Identification of I—Anhydrous lactose was assayed for I by a published UV method (7). Further identification and confirmation of the UV data were obtained by the GLC method as follows.

Ten percent aqueous solutions of lactose were used. Aqueous solutions of I were used as standards. The analyses were performed using a dual-column chromatograph[4] equipped with dual flame-ionization detectors. Injector port and detector temperatures were 280 and 300°, respectively. Helium flow was 60 ml/min. The glass column was 1.82 m (6 ft) × 2 mm i.d., containing 3% cyclohexanedimethanol succinate on 100–120-mesh silanized, acid-washed, flux-calcined diatomite[5]. The column temperature was programmed from 90 to 250° at 20°/min, holding at 250° until all components were eluted.

The column has to be conditioned as follows prior to use. Maintain the column at 250° overnight with a helium flow of about 5 ml/min. Connect the column outlet to the detector and inject 10-μl samples of water with the column at 250° and normal helium flow until a reproducible peak is obtained.

Reaction of Haloperidol with 5-(Hydroxymethyl)-2-furfuraldehyde—Haloperidol (2 g, 0.0053 mole), I (3 g, 0.0229 mole), and methanol (3 g) were added to a 120-ml (4-oz) screw-capped bot-

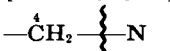
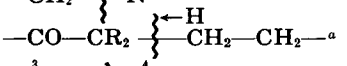
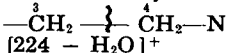
[1] PAF 2011.
[2] Marketed as Haldol.

[3] 5-(Hydroxymethyl)-2-furfuraldehyde was obtained from Aldrich Chemical Co., Milwaukee, Wis., and the anhydrous lactose was obtained from Sheffield Chemical Co. Melting points were determined in a Thomas–Hoover melting-point apparatus and are uncorrected. IR spectra were determined as potassium bromide dispersions in a Perkin-Elmer 521 grating IR spectrophotometer. NMR spectra were determined with a Varian A-60 spectrometer, using tetramethylsilane as the internal standard. All spectra were obtained in $CDCl_3$ in about 10% concentration. Mass spectra were obtained with a Hitachi Perkin-Elmer RMU-6 mass spectrometer. The samples were introduced by the direct inlet technique. UV spectra were obtained on a Beckman DK-2A or Cary 14 recording spectrophotometer. Microanalyses were performed by Cilag-Chemie, Schaffhausen, Switzerland.

[4] Perkin-Elmer 900.

[5] Hi-EFF-8BP on Gas Chrom Q, Applied Science Laboratories, State College, Pa.

Table I—Partial Mass Spectra of Haloperidol and Its Furfural Adducts

Fragment	*m/e* (% of base peak) Adduct II	Adduct III	Haloperidol
M^+	453 (0.3)	483 (0.9)	375 (2.3)
$[M - H_2O]^+$	435 (0.9)	465 (0.5)	357 (1.3)
$[M—FC_6H_4CO]^+$	330 (0.4)	360 (0.3)	252 (0.3)
	243 (5)	273 (2)	165 (15)
 [a]	237 (1.5)	237 (22)	237 (71)
	224 (100)	224 (100)	224 (100)
$[224 - H_2O]^+$	206 (46)	206 (27)	206 (19)
$[224 - Cl]^+$	189 (5)	189 (9)	189 (3)
$Cl—C_6H_4—CO^+$	139 (13)	139 (11)	139 (9)
$F—C_6H_4—CO^+$	123 (37)	123 (32)	123 (34)
$F—C_6H_4^+$	95 (20)	95 (13)	95 (16)
$C_5H_{10}N^+$	84 (10)	84 (8)	84 (12)
$C_3H_6N^+$	56 (24)	56 (16)	56 (26)
$C_2H_4N^+$	42 (64)	42 (60)	42 (87)

[a] McLafferty rearrangement (8).

tle. Water (5 ml) was added, and the mixture was stored in an 80° oven for about 60 hr. The cooled solution was transferred to a separator, made alkaline with sodium hydroxide solution (10%), and extracted with chloroform. The chloroform solution was evaporated to about 5 ml, diluted with 200 ml of ether, and then filtered. Ethereal hydrogen chloride was added, and a tan solid was obtained. Isolation of the free base resulted in an oil which would not recrystallize. Regeneration of the salt from the free base resulted in 1.9 g (68% of theory) of a white solid, mp 219–222°.

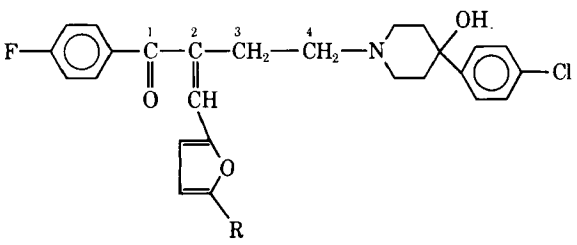
Anal.—Calc. for $C_{27}H_{27}ClFNO_4 \cdot HCl$: C, 62.31; H, 5.42; N, 2.69. Found: C, 61.49; H, 5.45; N, 2.59.

Reaction of Haloperidol with Furfural—Haloperidol (2 g, 0.0053 mole), furfural (5 g, 0.038 mole), and methanol (3 g) were mixed in a 120-ml (4 oz) screw-capped bottle. Water (5 ml) was added, and the mixture was stored in an 80° oven for about 60 hr. The cooled solution was transferred to a separator, made acidic with hydrochloric acid (10%), and extracted with chloroform. The remainder of the procedure was the same as described previously. Regeneration of the salt from the free base resulted in 2.1 g (81% of theory), mp 231–233°.

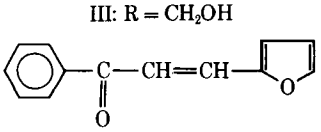
Anal.—Calc. for $C_{26}H_{25}ClFNO_3 \cdot HCl$: C, 63.68; H, 5.34; N, 2.86. Found: C, 63.30; H, 5.37; N, 2.85.

STRUCTURE DETERMINATION

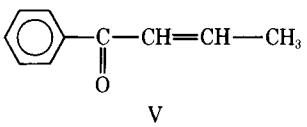
The mass spectra of the adducts of haloperidol with furfural (II) and hydroxymethylfurfural (III) are presented in Table I along with that of haloperidol. In addition to confirming the molecular weights and exhibiting many fragmentations seen in haloperidol, the base peak at *m/e* 224 and the strong *m/e* 123 peak are of particular interest. These fragments localize the point of attachment of the furfural moiety to either C-2 or C-3 of the butyrophenone.



II: R = H
III: R = CH_2OH



IV



V

The NMR spectra are in general agreement with these structures, showing one vinyl proton at δ 5.7 as well as the expected ratio of aliphatic to aromatic protons.

The IR spectra show a shift of the carbonyl stretch absorption to lower frequencies relative to haloperidol. This implies an extension of the conjugation of the aryl ketone.

The UV spectra of the adducts corroborate this extended conjugation. Comparisons with spectra of 3-(2-furyl)acrylophenone (IV), crotonophenone (V), and haloperidol show (Table II) the close resemblance of the adduct spectra with that of the furylacrylophenone (IV). Therefore, the furfural moiety must be attached to C-2 of the butyrophenone, confirming the assigned structures of II and III.

RESULTS AND CONCLUSIONS

Determination of I in Lactose—The UV spectra of 1% aqueous solutions of the various lots of anhydrous lactose used in the manufacture of the tablets were similar to the hydroxymethylfurfural spectra previously reported (7). GLC *analysis of the* aqueous solutions of the lactose resulted in a peak whose retention time, about 6.5 min, was identical to that of I. Furthermore, when the mass spectrum of that peak was compared to a spectrum of I, they were identical, proving that the lactose did indeed contain 5-(hydroxymethyl)-5-furfuraldehyde.

Tablet Assays—The initial batch of direct compression haloperidol tablets assayed satisfactorily. This batch was manufactured with a lot of anhydrous lactose containing less than 2 mg I/kg. These tablets possessed excellent physical characteristics, such as small tablet weight variation, uniform drug distribution, fast disintegration times, and rapid dissolution rates. In addition, high temperature and high humidity had no adverse effects on either the physical or chemical properties of the tablets. Subsequent batches of tablets were manufactured with lots of anhydrous lactose with higher amounts of I (10 times or more).

A comparison of the assay results for the initial batch of direct

Table II—Comparison of UV Absorption Spectral Data

Compound	λ_{max} (ϵ)	
Furfural adduct, II	258 (11,200), 332 (17,200)	
Hydroxymethylfurfural adduct, III	258 (8000), 336 (24,400)	
3-(2-Furyl)acrylophenone, IV	260 (8500), 344 (26,800)	(9)
Crotonophenone, V	256 (17,400)	(10)
Haloperidol	247 (11,900)	

Table III—Effect of Aging on Haloperidol Direct Compression Tablets

Batch	Label Amount, mg Haloperidol/ Tablet	Hydroxymethyl Furfural Content of Lactose, mg/kg	Percent Label Amount			
			Nonspecific Assay	Initial Assay	3 Months at 40°	3 Months at 60°
A	0.5	<2	100	102	101	101
B	0.5	29	98	92	93	93
C	1	29	97	93	92	91
D	2	29	97	93	94	94

compression tablets, which assayed satisfactorily, with three other batches of tablets, which assayed low, is given in Table III. Also included are results obtained by the nonspecific assay for haloperidol. The nonspecific colorimetric assay demonstrates that the tertiary nitrogen of haloperidol is still present in the dosage form. Without this assay, a question of whether the correct amount of drug had been added could have been raised. The stability of the dosage forms is also shown in Table III. The data show that haloperidol is lost initially and that the degradation does not proceed further with aging at elevated temperatures.

Haloperidol capsules were manufactured with a lot of anhydrous lactose containing 240 mg I/kg lactose. The nonspecific assay demonstrated that the drug was present as shown in Table IV, while the stability-indicating assay showed that the haloperidol content was low. The low assays were verified by repeated assays and remained essentially constant even after aging for 1 year. Capsules were then manufactured with the identical manufacturing directions but using hydrous lactose. They assayed satisfactorily (Table IV). The only other ingredient in the capsule besides the haloperidol and lactose was a small amount of the lubricant calcium stearate.

Extensive TLC work did not demonstrate the presence of the haloperidol–I condensation products in the tablets or capsules. But the fact that haloperidol could react easily with either I or furfural indicates that hydroxymethylfurfural in anhydrous lactose can be responsible for the low assay for direct compression haloperidol tablets. The nonspecific colorimetric assay demonstrates that the basic nitrogen of haloperidol remains in the dosage form, while the stability-indicating assay for haloperidol clearly indicates that there is a definite loss of haloperidol in the dosage forms.

Reactions—It was previously demonstrated (2) that anhydrous lactose could be used successfully in the manufacture of trifluperidol hydrochloride tablets. No evidence of a reaction between trifluperidol hydrochloride and anhydrous lactose was observed. Haloperidol is chemically related to trifluperidol hydrochloride, both drugs belonging to the class of compounds known as the butyrophenones.

When it became apparent that haloperidol could easily react with I and furfural, it was decided to see if the same reaction could occur with trifluperidol hydrochloride. When trifluperidol hydrochloride was reacted with either I or furfural, no condensation products were obtained. Instead, trifluperidol was recovered intact from the reaction mixtures. The postulated Claissen–Schmidt condensation between haloperidol and I or furfural is a base-catalyzed reaction. Therefore, the free base of trifluperidol and furfural were reacted. A condensation compound of the same type obtained for haloperidol resulted.

Furthermore, compounds that resemble haloperidol structurally but do not contain the carbonyl group adjacent to the *p*-fluorophenyl group do not react with either I or furfural. For example, pimozide, 1-[1-[4,4-bis(*p*-fluorophenyl)butyl]-4-piperidyl]-2-benzimidazolinone, does not react with either furfural or hydroxymethylfurfural.

Table IV—Assay of Haloperidol Capsules

Batch	Label Amount, mg Haloperidol/ Capsule	Hydroxymethylfurfural Content of Lactose, mg/kg	Percent Label Amount	
			Nonspecific Assay	Stability Assay
E	1	240	—	92
F	2	240	96	90
G	1	0	—	100
H	2	0	102	102

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[x] To whom inquiries should be directed.

Influence of Vehicle Dielectric Properties on Acetaminophen Bioavailability from Polyethylene Glycol Suppositories

SHRIKANT N. PAGAY, ROLLAND I. POUST[x], and JOHN L. COLAIZZI

Abstract □ The rectal absorption of acetaminophen from an aqueous solution and from several polyethylene glycol base dosage forms was studied in six human subjects using urinary excretion measurements. A relationship was observed between acetaminophen bioavailability and the dielectric properties of the vehicles utilized. The *in vitro* dissolution rates of acetaminophen in these dosage forms were also studied and were somewhat related to the time of occurrence of the maximum urinary excretion rate.

Keyphrases □ Acetaminophen absorption from polyethylene glycol suppositories—effect of vehicle dielectric properties □ Absorption, acetaminophen from polyethylene glycol suppositories—effect of vehicle dielectric properties □ Polyethylene glycol dielectric properties—effect on acetaminophen absorption from suppositories □ Suppositories, acetaminophen—effect of polyethylene glycol vehicle dielectric properties on drug absorption □ Bioavailability, acetaminophen from polyethylene glycol suppositories—effect of vehicle dielectric properties □ Dielectric properties of polyethylene glycols—effect on acetaminophen absorption from suppositories

The importance of drug release from suppository bases has been recognized by several workers (1–3) who attempted to identify the physicochemical properties of drugs and bases that most greatly influence rectal absorption. Lowenthal and Borzelleca (4) measured plasma salicylate levels following rectal administration of salicylic acid and sodium salicylate in dogs and found that the free acid and its salt were equally well absorbed from cocoa butter suppositories. However, with water-miscible bases, salicylic acid gave higher plasma levels. The effect of formulation variables on the rectal absorption of salicylates was studied (5) and compared to the rectal and oral routes of administration. Based upon urinary salicylate excretion data, both aspirin and sodium salicylate were found to be equally bioavailable from the oral and rectal routes. Aspirin was released more rapidly from water-miscible bases, such as polyethylene glycol and polyoxyethylene (4) sorbitan monostearate with glyceryl monolaurate, than from oily bases. It was found that cocoa butter gave a more rapid release of sodium salicylate, and it was concluded that water-soluble sodium salicylate was more readily released from a suppository base than was the slightly soluble aspirin.

The effect of water-soluble bases on the rectal absorption of several sulfonamides also was reported (6). These bases caused a reduction in the absorption rate of sulfonamides which was shown to depend on the partitioning behavior of the drug between lipid and water-soluble materials. These differences were related to the dielectric constants of the bases. The absorption rate constants for sulfonamides, when released from several water-miscible bases, were correlated with the reciprocal of the dielectric constants of these bases. Thus, from these studies, it appears that water-soluble bases tend to decrease the polarity of the aqueous phase which decreases the partitioning into the lipoidal membrane.

Shangraw and Walkling (7) studied the effect of vehicle dielectric properties on the rectal absorption of acetaminophen in humans. Using urinary excretion measurements, they reported rectal absorption from a microcrystalline cellulose–carboxymethylcellulose gel, a propylene glycol suspension, and a cocoa butter suppository. The cumulative amount of acetaminophen excreted over 6 hr was about the same when the drug was given in the gel and in the cocoa butter suppository and significantly greater than with the propylene glycol suspension. These authors concluded that propylene glycol, having dielectric properties favoring high solubility, retains more drug in the vehicle and causes reduced rectal absorption as compared to either cocoa butter or aqueous gel bases, which have dielectric properties yielding extremely low solubility in the vehicle and subsequently show a higher drug absorption.

It appears that the release of drugs and subsequently their absorption from rectal dosage forms are somewhat related to their solubility in the base, which in turn is related to the respective dielectric properties of the drug and the base. The purposes of this study were: (*a*) to determine the bioavailability of acetaminophen from rectal dosage forms using several polyethylene glycol bases having varying dielectric properties and an aqueous solution; and (*b*) to determine the effects of the dielectric properties of the bases on bioavailability more quantitatively.

EXPERIMENTAL

Dosage Form Preparation—The rectal suppositories were prepared by the fusion method. Each suppository weighed an average of 2.3 g and contained 0.130 g of acetaminophen[1]. The suppositories were assayed for acetaminophen content, which was within ±3% of the specified dose. The bases were polyethylene glycols[1] whose average molecular weights were 400, 1000, 4000, and 6000. The polyethylene glycol 400 formulation was prepared as a 2.3-g liquid. A 5-ml aqueous solution containing 0.130 g of acetaminophen was also utilized.

Bioavailability Studies—Six healthy male subjects, 24–34 years old and 60–85 kg, were employed. Participants were required to observe a normal diet, follow normal sleep habits, drink plenty of water, and abstain from any alcoholic beverages and other drugs during the 24 hr preceding rectal administration of acetaminophen. Upon arising, the subjects ingested 180 ml of water and evacuated the bowel. The bladder was voided, and the urine was collected to serve as a blank specimen. For each study, the subjects inserted one suppository into the rectum. The polyethylene glycol 400 formulation and the aqueous solution of acetaminophen were administered through a rectal tube[2] attached to a calibrated 5-ml plastic hypodermic syringe[3].

[1] Ruger Chemical Co., Hillside, NJ 07205
[2] Davol Inc., Providence, RI 02901
[3] Becton, Dickinson, and Co., Rutherford, NJ 07070

Table I—Mean Rate of Acetaminophen Excretion (Milligrams per Hour ± Standard Error) from Various Formulations in Six Human Subjects

Collection Interval, hr	Formulations				
	Polyethylene Gycol 400	Polyethylene Glycol 1000	Polyethylene Glycol 4000	Polyethylene Glycol 6000	Aqueous Solution
0–1	1.6 ± 0.51	1.4 ± 0.43	1.3 ± 0.29	1.6 ± 0.41	7.7 ± 1.25
1–2	3.7 ± 0.21	3.7 ± 0.29	3.9 ± 0.96	5.5 ± 0.90	12.9 ± 1.05
2–3	6.0 ± 0.29	7.9 ± 0.81	8.4 ± 1.12	8.6 ± 0.69	13.0 ± 0.58
3–4	8.3 ± 1.34	9.8 ± 1.13	8.6 ± 0.67	10.2 ± 0.58	11.5 ± 1.12
4–5	7.6 ± 1.15	7.9 ± 1.31	9.4 ± 0.77	10.8 ± 1.03	9.5 ± 0.27
5–6	6.3 ± 0.78	6.6 ± 0.52	9.2 ± 0.65	8.6 ± 0.89	7.6 ± 0.54
6–7	5.6 ± 1.05	6.6 ± 1.19	6.7 ± 0.54	7.8 ± 0.82	5.7 ± 0.95
7–8	4.4 ± 0.42	4.1 ± 0.56	5.9 ± 0.66	6.8 ± 0.42	4.1 ± 0.71
8–9	4.0 ± 0.52	4.1 ± 0.49	5.1 ± 0.49	5.1 ± 0.26	3.2 ± 0.52
9–10	3.2 ± 0.43	2.7 ± 0.22	4.3 ± 0.33	3.9 ± 0.20	2.2 ± 0.33

After drug administration, the subjects collected complete voids every hour for 10 hr and then pooled all voids up to 24 hr. During the first 10 hr, the subjects were instructed to ingest 180 ml of water after each collection. A normal lunch and supper were eaten immediately following the 4- and 10-hr collections, respectively.

Initially, only the four polyethylene glycol formulations were tested in four subjects. Each subject followed a different treatment sequence to balance any possible sequential effects. The remaining subjects participated at a later time and followed separate sequences. All six subjects were administered the aqueous solution as their last formulation.

Analytical—Acetaminophen content in the suppositories was determined by the method described in NF XIII (8). This method was modified slightly by using aqueous instead of hydroalcoholic solutions. The samples for the dissolution rate measurements were also analyzed by the same procedure.

Acetaminophen and its metabolites were assayed using a literature procedure (9, 10). The analytical procedure involves acid hydrolysis of acetaminophen and its metabolites present in urine to *p*-aminophenol, which reacts with phenol in the presence of ammonia and sodium hypochlorite to form an indophenol dye, which has a spectrophotometric absorbance maximum at 625 nm[4].

Dissolution Studies—The suppositories were tested for the rate at which the drug in the dosage form dissolved, using a slightly modified "beaker method" (11) in which a stainless steel holder was used to maintain the suppository in a vertical position at the bottom of a 100-ml beaker. Eighty milliliters of distilled water was used as the dissolution medium and was maintained at 37°. A four-blade, stainless steel stirrer was used to stir the fluid at 25 rpm. One-milliliter samples were withdrawn at appropriate intervals and assayed to obtain a dissolution profile.

Dielectric Constants—The dielectric constants of all vehicles were determined on a chemical oscillometer[5]. The dielectric cell was calibrated by measuring instrument readings for several organic solvents whose dielectric constants are accurately reported in the literature. The dielectric constants for the polyethylene glycol vehicles were evaluated from a calibration plot of instrument readings *versus* the reported dielectric constants of the standards employed. For those polyethylene glycol vehicles that solidify at room temperature, the material was melted and the liquid was poured in the dielectric cell to the top. After the polyethylene glycol base solidified and equilibrated to room temperature, the dielectric constant was measured.

RESULTS

The mean rates of excretion of acetaminophen for six subjects for all formulations are summarized in Table I. The standard error is listed with each mean value.

The peak or maximum rates of excretion of acetaminophen occurred at 3 hr when the drug was given rectally in solution. The peak excretion rates with polyethylene glycols 400 and 1000 occurred at 4 hr, while the peak rate occurred at 5 hr with the polyethylene glycol 4000 and 6000 formulations. The initial rates of excretion (first 3 hr) were much higher from the aqueous solution than from any of the polyethylene glycol formulations. An analysis of variance performed on the rates for each of the first 3 hr indicated that there were indeed significant differences due to changes in the formulations (F at 1st hr = 16.66; df = 4, 25; $p < 0.01$. F at 2nd hr = 29.60; df = 4, 25; $p < 0.01$. F at 3rd hr = 10.90; df = 4, 25; $p < 0.01$). Moreover, the magnitude of the peak rate of excretion for the aqueous solution was larger than those of most polyethylene glycol formulations. Although polyethylene glycol 6000 showed a longer time to achieve peak excretion than the other polyethylene glycol bases, the rate at this time, 10.797 mg/hr, was greater than the others, even than those exhibiting earlier peak times.

Figure 1 shows the mean cumulative amount of acetaminophen excreted as a function of time following rectal administration of various formulations. There was a fairly wide variation in the total amount of drug excreted after 24 hr following drug administration in the various formulations. The greatest amount of drug was recovered when it was administered rectally in an aqueous solution, followed by the various polyethylene glycol formulations.

Table II shows the results of an analysis of variance of the cumulative excretion of acetaminophen from 1 to 24 hr following rectal administration of acetaminophen in various formulations to

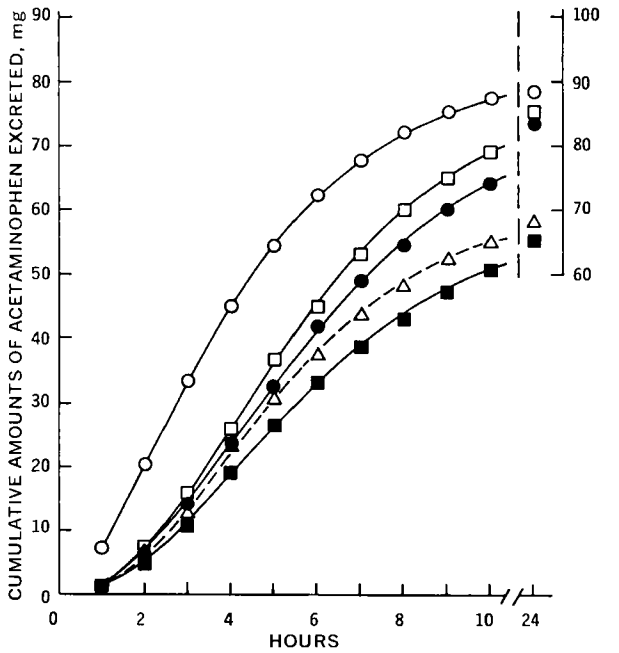

Figure 1—*Effects of various vehicles on the mean cumulative amounts of acetaminophen excreted for six subjects during the 24 hr following rectal administration. Key:* ○, *aqueous solution;* □, *polyethylene glycol 6000;* ●, *polyethylene glycol 4000;* △, *polyethylene glycol 1000; and* ■, *polyethylene glycol 400.*

[4] Beckman model DB-G, Beckman Instruments, Fullerton, CA 92634
[5] Model V, Sargent-Welch Scientific Co., Skokie, IL 60076

Table II—Results of Statistical Analysis of the Cumulative Excretion of Acetaminophen at Hourly Intervals to 10 hr and Total Excretion Values for Studies Involving All Formulations and Those Involving Only Polyethylene Glycol Bases

Hours	All Formulations		Polyethylene Glycol Formulations	
	F^a	p	F^b	p
1	16.66	<0.01	0.12	>0.05
2	27.31	<0.01	0.94	>0.05
3	27.25	<0.01	1.52	>0.05
4	20.83	<0.01	2.10	>0.05
5	17.10	<0.01	3.14	<0.05
6	17.35	<0.01	4.83	<0.05
7	14.42	<0.01	4.47	<0.05
8	12.77	<0.01	5.20	<0.01
9	10.90	<0.01	5.15	<0.01
10	10.05	<0.01	5.51	<0.01
24	8.48	<0.01	8.05	<0.01

[a] Degrees of freedom (4, 25). [b] Degrees of freedom (3, 20).

the same group of subjects. The results revealed significant differences at the 99% level in acetaminophen excretion at all intervals. These differences were due to formulation differences and not to variation among subjects. An analysis of variance was also performed to test differences among only the polyethylene glycol bases (Table II). The results revealed that statistically significant differences in cumulative amounts of acetaminophen excretion occurred at all time periods after 4 hr. These differences were the result of differences among the various formulations and not of variations among the subjects.

Figure 2 shows the rate of dissolution of acetaminophen in terms of the percent of drug undissolved as a function of time from rectal suppositories containing polyethylene glycol 1000, 4000, and 6000. Each dissolution profile represents an average of three determinations. Based on the percent of acetaminophen undissolved at the 5-, 10-, and 15-min sampling times, it appears that acetaminophen dissolved most rapidly when formulated with the polyethylene glycol 1000 base, followed by polyethylene glycol 4000 and polyethylene glycol 6000 bases, respectively. A t-test was

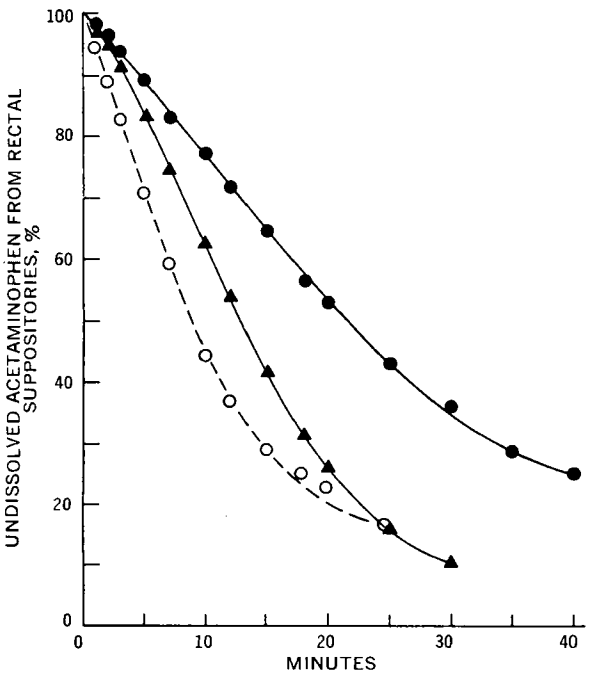

Figure 2—*Dissolution of acetaminophen from three different suppository formulations in 80 ml distilled water at 37° and 25 rpm. Key: ●, polyethylene glycol 6000; ▲, polyethylene glycol 4000; and ○, polyethylene glycol 1000.*

Table III—Results of Statistical Analysis of Dissolution Rates of Each Possible Combination of Pairs of Polyethylene Glycol 1000, 4000, and 6000–Acetaminophen Rectal Suppositories at 5-, 10-, and 15-min Sampling Times

Combination of Polyethylene Glycols	t Value	p
4000 and 1000 (5 min)	11.803	<0.01
6000 and 4000 (5 min)	8.463	<0.05
6000 and 1000 (5 min)	25.935	<0.01
4000 and 1000 (10 min)	6.895	<0.05
6000 and 4000 (10 min)	7.873	<0.05
6000 and 1000 (10 min)	21.280	<0.01
4000 and 1000 (15 min)	5.610	<0.05
6000 and 4000 (15 min)	8.847	<0.05
6000 and 1000 (15 min)	24.086	<0.01

performed on each possible combination of pairs of formulations at three sampling times to determine if there were significant differences in the dissolution profiles of these formulations. The results (Table III) reveal that the differences in the percent undissolved among any two formulations are statistically significant at each interval. These differences were also apparent when the time for half the drug to dissolve (t_{50}) was determined graphically for each formulation. These values were as follows: polyethylene glycol 1000, 8 min; polyethylene glycol 4000, 13 min; and polyethylene glycol 6000, 22 min.

Table IV lists the dielectric constants of the vehicles employed and the total amounts of acetaminophen excreted in 24 hr from each studied formulation. It would appear that, as the dielectric values increase, there is a decline in bioavailability up to some point around a dielectric value of 13 or more, beyond which an increase in bioavailability occurs as observed when water was employed as the vehicle. This effect is more clearly illustrated in Fig. 3, which shows a plot of acetaminophen bioavailability as a function of dielectric constant along with a plot of acetaminophen solubility in water–dioxane mixtures as a function of dielectric constants as reported previously (12). Although it may be rather presumptuous to connect the closed circles with any type of curve, the solid line has been shown to illustrate more clearly the trend and not to assume any type of mathematical relationship. As mentioned previously, it appears that there might be an inflection point in the bioavailability–dielectric relationship in a dielectric range of 10–20 similar to the one that is apparent from the solubility–dielectric curve.

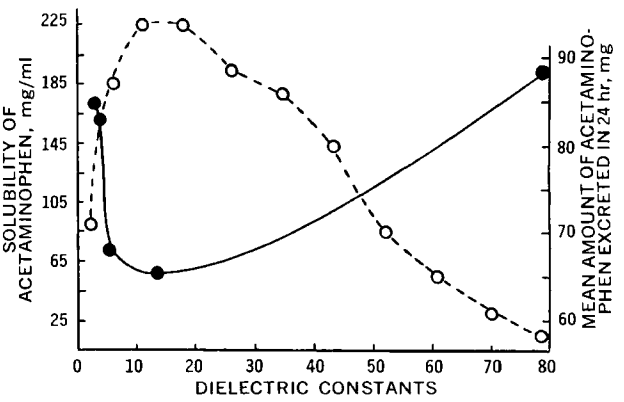

Figure 3—*Comparison of acetaminophen bioavailability and solubility (solubility–dielectric profile from Ref. 12) as a function of vehicle dielectric constant. Key: ○, solubility data; and ●, bioavailability data.*

Table IV—Mean Total Amounts (Milligrams ± Standard Error) of Acetaminophen Excreted in 24 hr for Each Formulation and Dielectric Constants of the Vehicle for Each Formulation

Formulation	Total Amount Excreted, mg	Dielectric Constant
Polyethylene glycol 6000	84.7 ± 1.66	3.0
Polyethylene glycol 4000	83.2 ± 3.03	3.6
Polyethylene glycol 1000	68.0 ± 4.62	5.3
Polyethylene glycol 400	65.7 ± 5.41	13.6
Water	88.2 ± 4.28	78.5

DISCUSSION

The statistical analyses of the results of this study reveal that alteration in the composition of the vehicles employed leads to differences in the absorption pattern of rectally administered formulations of acetaminophen. This conclusion is based on the urinary excretion rates and cumulative urinary excretion of acetaminophen over 24 hr. The rate of urinary excretion is a function of the blood level of the drug and of its metabolites at any given time and, thus, serves as an indication of absorption.

The peak excretion time may be used as an approximate indicator of the relative absorption rates of a drug for comparing different formulations administered by the same route and in equal doses (13). The peak excretion time of 3 hr obtained with the aqueous solution is in accord with significantly higher excretion rates observed during the first 3 hr as compared with the polyethylene glycol bases which showed 4- and 5-hr peak excretion times.

The dissolution rate studies show that one factor controlling the initial rate of absorption is the speed with which the polyethylene glycol bases release the drug into the rectal fluid. Figure 2 shows that the rate of dissolution of polyethylene glycol 1000 suppositories, as reflected by a t_{50} of about 8 min, is faster than polyethylene glycol 4000 (t_{50} = 13 min) and polyethylene glycol 6000 (t_{50} = 22 min). Polyethylene glycol 1000 showed a peak excretion rate at 4 hr, while polyethylene glycol 4000 and 6000 showed peak excretion rates at 5 hr. Although no dissolution was involved with the polyethylene glycol 400 formulation, relatively slower attainment of peak excretion rates as compared to water might be due to slower releasing characteristics of the polyethylene glycol vehicles. Thus, the respective peak excretion times appear to show some correlation with the rates of dissolution, indicating that dissolution may play at least a partial role in the release of acetaminophen from polyethylene glycol suppository bases.

The results of the present study, as illustrated by the cumulative excretion data, appear to indicate that the extent of rectal absorption of acetaminophen is related to its solubility-dielectric profile. This is not an unexpected finding, especially when considered in the light of the previously discussed literature reports (6, 7). Kakemi *et al.* (6) employed solvents with dielectric constants ranging from 10.8 to 53.5. If the assumed bioavailability-dielectric relationship of the present study, as shown in Fig. 3, is correct, then the findings of the two studies would be in agreement in the dielectric range reported previously (6). This would be expected because of the reported similarity in dielectric-related solubility of acetaminophen to the sulfonamides.

Thus, the present findings are in agreement with earlier reports which relate rectal drug absorption to the dielectric properties of the vehicle. However, this study presents a more definitive relationship between rectal absorption and vehicle dielectric properties. All vehicles employed were aqueous or water miscible, thus eliminating the use of a lipoidal substance in the lower dielectric range. Also, the dielectric constants of all vehicles employed were accurately determined, permitting the construction of a plot (Fig. 3) relating bioavailability to the dielectric properties of the vehicle. This relationship below a dielectric constant of 13.6 is rather well defined. It is apparent from Fig. 3 that the decline in the dielectric-related solubility of acetaminophen with decreasing dielectric constants coincides with an increase in the total amount of drug excreted, as might be expected from the previously discussed concepts. Above a dielectric constant of 13.6, the relationship is not as well defined. However, based on the work of Shangraw and Walkling (7) and the results of the present study in the lower dielectric range, a gradual increase in total acetaminophen excretion coinciding with the gradual decline in dielectric-related solubility might be expected in the higher dielectric range.

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[x]To whom inquiries should be directed.

Electrokinetic Studies of Bacteria IV: Effect of Acridines on *Streptococcus faecalis*

HANS SCHOTT

Abstract □ The effect of the following acridines on the electrophoretic mobility of *Streptococcus faecalis* was studied at constant values of pH and ionic strength: acridine hydrochloride, 9-hydrazinoacridine acetate, 9-aminoacridine hydrochloride, acriflavine hydrochloride, and proflavine dihydrochloride. Increasing times of contact of the bacteria with acridines rendered the electrophoretic mobility less negative or more positive due to the interaction with nucleic acids released from the bacteria through the action of the acridines. Stirring of the bacterial suspensions minimized this effect. Hence, electrophoretic mobilities were measured on samples withdrawn from stirred bacterial suspensions at successive time intervals after the addition of the acridines, and the mobilities were extrapolated to zero time. The mobility–concentration curves had similar shapes for all acridines. With increasing concentration of the acridinium salts, the negative mobilities or ζ-potentials of the bacteria decreased slowly at first but at a much faster rate as they approached zero. When the neutralization of the carboxylate groups in the bacterial cell wall by chemisorption of acridinium ions was complete, the ζ-potential was reduced to zero because the resultant acridinium carboxylates were only slightly dissociated. Further concentration increases inverted the electric charge of the bacteria to positive by physical adsorption of the entire acridinium salts. The positive mobilities increased with the acridine concentrations, rapidly at first but at slower rates for still higher concentrations. The acridine concentrations required to reduce the mobility or ζ-potential of the bacteria to zero were lower in more acid media. For four of the five acridines, there was an inverse relationship between these concentrations and the base strength of the acridines. The acridines of highest pKa were most strongly chemisorbed by the carboxylate groups in the cell wall, saturating them at the lowest concentrations. Relatively high concentrations of acridines caused the bacteria to agglomerate. This agglutination was not due to a reduction in the absolute value of the ζ-potential; it was probably caused by nucleic acids leaked from the bacteria or by their complex coacervates with the acridines, possibly through bridging. Aminoacridine hydrochloride and proflavine dihydrochloride did not inhibit the growth of *S. faecalis* even at concentrations 10 times greater than those at which they reduced the ζ-potential to zero. This suggests that the primary site of attack of these acridines is not the cell wall.

Keyphrases □ Electrokinetics—effect of acridines on electrophoretic mobility of *Streptococcus faecalis* □ Electrophoretic mobility, *Streptococcus faecalis*—effect of acridines, ζ-potential □ *Streptococcus faecalis*—effect of acridines on electrophoretic mobility □ Acridines—effect on electrophoretic mobility of *Streptococcus faecalis* □ Zeta-potential—calculated for *Streptococcus faecalis*, effect of acridines

Previous work had shown that *Streptococcus faecalis* is negatively charged at pH values above 2.3 owing to carboxylate groups in the cell wall (1–3). The interaction with a surface-active quaternary ammonium halide antiseptic consisted of chemisorption which progressively neutralized the charge of the bacteria with increasing concentration, followed by physical adsorption which reversed their charge to positive (2).

Acridine derivatives were selected to examine the effect of the base strength of a series of compounds containing basic nitrogen atoms, or the extent of ionization of their salts, on their adsorption by the bacterial cell wall. Acridines have been investigated extensively by Albert and coworkers (4–6). They range from weak to strong bases and are often available as hydrochlorides. While the site of the antibacterial action of the acridines is not the cell wall (6), it is interesting to ascertain if there is any relationship between their adsorption at the cell wall as studied by electrophoresis and their bacteriostatic effectiveness. Because of the porosity of the cell wall, acridines also come into contact with the cytoplasmic membrane. Cetyltrimethylammonium bromide modified the permeability of the membrane of *S. faecalis*, promoting the leakage of nucleic acids (2). This may be the mechanism of its antibacterial action.

EXPERIMENTAL

Materials—The following acridines were used as received: acridine hydrochloride[1], 9-aminoacridine hydrochloride[1], acriflavine hydrochloride (3,6-diamino-10-methylacridinium chloride hydrochloride)[1], proflavine dihydrochloride (3,6-diaminoacridine dihydrochloride)[1], and 9-hydrazinoacridine[2] (5). (The ring numbering is that of the International Union of Chemistry.) Cetrimonium (cetyltrimethylammonium) bromide was purified as described previously (2), and the salts used were ACS reagent grade. Water was redistilled in an all-quartz still and boiled out just prior to use.

Procedures—The bacteria, *S. faecalis* (ATCC 9790), the low-phosphate liquid growth medium, and the electrokinetic techniques were described previously (1). Electrophoretic mobilities were measured with the glass cell (3).

The bacteria were harvested after 20–25 hr incubation at 37°, which brought them into the stationary phase of their growth cycle and produced an uncorrected absorbance of 0.70 ± 0.02 at 640 nm. The suspensions were centrifuged for 15 min at 3000 rpm. The bacteria were freed of adhering nutrients by resuspending the sediment twice in 0.020 *M* potassium acetate and centrifuging for 15 min at 3000 rpm. They were suspended in potassium acetate or potassium chloride solutions, and acridine solutions were added to bring the suspensions to the desired concentration and ionic strength. The suspensions containing acridines were usually kept in the dark prior to measuring electrophoretic mobility because of the light sensitivity of many acridines. However, exposure to light produced no significant effect on mobility in those instances where comparison measurements were made with suspensions exposed to light and kept in the dark.

The inhibitory effect of two of the acridines was assessed by the pour plate method as described previously (2, 3). The presence of nucleic acids in solution was estimated using a visible–UV recording spectrophotometer[3] equipped with 1-cm quartz cells. The single change in procedure, namely, stirring of bacterial suspensions after drug addition, will be discussed.

The *Streptococcus pyogenes* culture[4] was incubated in the same liquid growth medium as *S. faecalis* in a shaking bath at 37° for 26 hr, at which time the uncorrected absorbance at 640 nm was 0.38–0.43. Purification methods were the same as for *S. fae-*

[1] K & K Laboratories, Plainview, N.Y.

[2] Supplied by Professor Adrien Albert, John Curtin School of Medical Research, Australian National University, Canberra City, Australia. The author thanks him for suggesting the use of 9-hydrazinoacridine and for supplying a sample.

[3] Perkin-Elmer double-beam spectrophotometer, Coleman 124.

[4] From the culture collection of the Department of Microbiology of Temple University Schools of Pharmacy and Dentistry.

Table I—Influence of Stirring on the Effect of 9-Aminoacridine Hydrochloride and of Proflavine Dihydrochloride on the Electrophoretic Mobility of *S. faecalis* at Constant Ionic Strength

Concentration of 9-Aminoacridine Hydrochloride[a], *M*	pH	Stirring Level	Electrophoretic Mobility[b] (Specific Conductance[c]) after Contact Times of: 5 min	15 min	35 min	45 min	60 min
0.0060	7.1	Occasional slow	−0.78 ± 0.03 (2750)	−0.58 ± 0.03 (2750)	0 (2750)	+0.56 ± 0.01 (2750)	+0.40 ± 0.01 (2710)
0.0060	7.1	Continuous slow	−0.78 ± 0.04 (2750)	−0.63 ± 0.02 (2750)	0 (2750)	+0.53 ± 0.02 (2750)	+0.49 ± 0.03 (2680)
0.0060	7.1	Continuous fast	−0.86 ± 0.04 (2750)	−0.46 ± 0.02 (2620)	−0.40 ± 0.02 (2600)	−0.43 ± 0.03 (2560)	−0.53 ± 0.03 (2540)
			10 min	30 min	40 min	60 min	
0.0082	6.9	None	−0.69 ± 0.03 (2710)	+0.69 ± 0.02 (2710)	+0.64 ± 0.03 (2710)	+0.55 ± 0.02 (2680)	
0.0082	7.1	Occasional slow	−0.65 ± 0.03 (2710)	+0.59 ± 0.03 (2680)	+0.56 ± 0.04 (2650)	+0.56 ± 0.03 (2620)	
0.0082	7.1	Continuous slow	−0.58 ± 0.02 (2710)	+0.23 ± 0.01 (2680)	+0.48 ± 0.03 (2650)	+0.40 ± 0.02 (2620)	
0.0082	7.0	Continuous fast	−0.61 ± 0.03 (2710)	−0.53 ± 0.01 (2600)	−0.45 ± 0.02 (2580)	−0.42 ± 0.02 (2580)	
Concentration of Proflavine Dihydrochloride[d], *M*			5 min	15 min	25 min	40 min	
0.0015	5.2	None	−1.20 ± 0.05 (690)	−1.08 ± 0.04 (690)	−0.82 ± 0.03 (690)	−0.80 ± 0.05 (690)	
0.0015	5.2	Continuous fast	−1.02 ± 0.02 (690)	−0.91 ± 0.03 (690)	−0.88 ± 0.03 (690)	−0.84 ± 0.04 (690)	

[a] Ionic strength adjusted to 0.03 *M* with potassium acetate. [b] Units of (micrometers per second)/(volts per centimeter); average of 10 or more measurements ± standard deviation of the average. [c] Units of μmhos per centimeter. [d] Ionic strength adjusted to 0.0065 *M* with potassium acetate.

Table II—Effect of Ionic Strength, Time of Contact, Stirring, and pH on Electrophoretic Mobility of *S. faecalis* in Potassium Acetate Buffers

Medium, Potassium Acetate[a], *M*	pH	Specific Conductance, μmhos/cm	Mobility ± *s*[b], (μm/sec)/(v/cm)	Contact Time[c], min
0.010	6.8	1060	−2.30 ± 0.07	30
0.010	7.0	1060	−2.56 ± 0.13	5
		1060	−2.61 ± 0.10	15
		1000	−2.57 ± 0.07	25
		1000	−2.40 ± 0.06	40
0.010	7.0	1060	−2.56 ± 0.13	5
		1060	−2.61 ± 0.10	15
		1000	−2.57 ± 0.07	25
		1000	−2.40 ± 0.06	40
0.030	7.0	2870	−1.86 ± 0.08	10
		2870	−1.73 ± 0.08	20
		2930	−1.82 ± 0.10	30
		2930	−1.70 ± 0.11	45
		2930	−1.70 ± 0.08	60
0.030	7.0	3080	−1.83 ± 0.11	5
		3080	−1.75 ± 0.06	15
		3080	−1.72 ± 0.04	5[d]
		3080	−1.78 ± 0.06	15[d]
0.030	7.0	3050	−1.82 ± 0.06	5
		3050	−1.72 ± 0.04	15
		3050	−1.74 ± 0.08	25
		3050	−1.73 ± 0.07	40
0.030	7.1	3050	−1.73 ± 0.06	5
		3020	−1.73 ± 0.05	15
		3020	−1.75 ± 0.06	25
		3050	−1.69 ± 0.04	40
0.030	7.0	2930	−1.78 ± 0.05	5[e]
		2930	−1.72 ± 0.10	20[e]
		2930	−1.75 ± 0.04	30[e]
0.030 + 0.044 *M* acetic acid	4.7	3000	−1.70 ± 0.10	5
		3000	−1.72 ± 0.08	30

[a] Each entry refers to a different batch of *S. faecalis*. [b] Average of 10 measurements ± the standard deviation of the average. [c] No stirring unless otherwise indicated. [d] With constant fast stirring. [e] With constant slow stirring.

Table III—Electrophoretic Mobility of *S. faecalis* as a Function of Proflavine Dihydrochloride Concentration at Constant Ionic Strength

Concentration of Proflavine Dihydrochloride, *M*	pH	Specific Conductance, μmhos/cm	Mobility[a], (μm/sec)/(v/cm)
Ionic Strength 0.01 *M*[b]			
0	6.8	1060	−2.40[c]
0.0005	6.0	1040	−1.94[c]
0.0010	5.7	1160	−1.31
0.0015	5.5	1080	−0.76
0.0025	5.0	1060	+0.45
0.0050	4.8	1080	+0.87
Ionic Strength 0.03 *M*[b]			
0	7.0	3000	−1.78[c]
0.000025	6.9	2930	−1.71[c]
0.000050	6.8	3010	−1.77[c]
0.00010	6.8	2880	−1.55
0.00020	6.7	2810	−1.12[c]
0.00025	6.7	2940	−1.27[c]
0.00040	6.4	2990	−1.35[c]
0.00050	6.4	2930	−1.07
0.00100	6.2	2870	−0.78
0.0012	6.0	2940	−0.42
0.0014	6.0	2890	−0.48
0.0015	6.0	2930	+0.37[d]
0.0016	6.0	2930	+0.63[d]
0.0018	5.8	2990	+0.43
0.0020	5.8	2950	+0.56
0.0030	5.6	2710	+0.54
0.0040	5.4	2990	+0.80[c]
0.0045	5.4	3020	+0.92
0.0050	5.2	3000	+1.55

[a] Extrapolated to zero time from four sets of 10 measurements performed after 5, 15, 25, and 40 min, respectively. [b] Ionic strength adjusted with potassium acetate. [c] No significant trend with time. [d] Sign of electric charge changed with time.

calis. The bacteria were suspended in the acridine solutions and stirred for the specified time, after which the suspensions were diluted with distilled water. Aliquots were treated by the pour plate method as described (3).

RESULTS AND DISCUSSION

Electrophoresis—*Effect of Stirring*—Preliminary experiments designed to determine optimum contact times of bacterial suspensions with acridines prior to measuring the electrophoretic mobility gave poorly reproducible mobility values; they were found to undergo systematic changes with time in the presence of acridines. This led to the evaluation of the effect of stirring, as summarized in Table I. Fast stirring refers to the use of a magnetic stirrer at the highest speed which did not produce a vortex,

Table IV—Electrophoretic Mobility of *S. faecalis* as a Function of Acriflavine Hydrochloride Concentration at Constant Ionic Strength[a]

Concentration of Acriflavine Hydrochloride, *M*	pH	Specific Conductance, μmhos/cm	Mobility[b], (μm/sec)/(v/cm)
0.0010	4.7	3530	−1.69
0.0020	4.7	3460	−1.02
0.0025	4.7	3430	−0.53
0.0030	4.7	3240	+0.36
0.0030	5.6	3150	−0.48
0.0070	4.7	3060	+0.70
0.0090	4.8	3000	+0.80
0.0150	4.5	2950	+1.11

[a] Ionic strength adjusted to 0.03 *M* and pH adjusted to values listed with potassium acetate and potassium chloride. [b] Extrapolated to zero time from four sets of 10 measurements performed after 5, 15, 30, and 45 min, respectively.

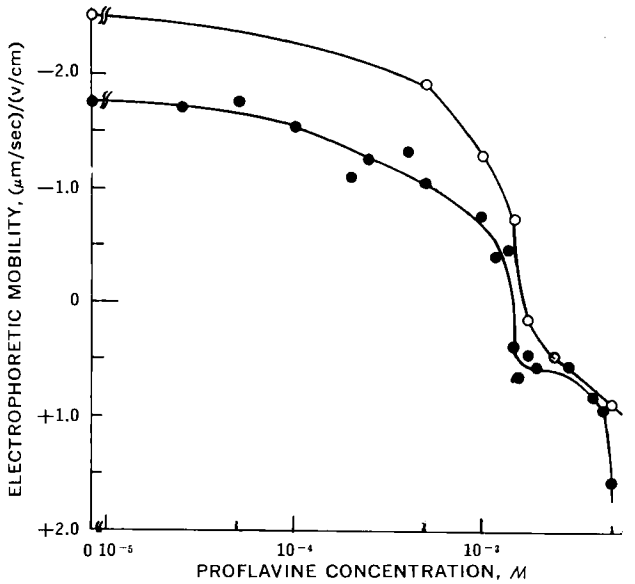

Figure 1—*Electrophoretic mobility of* S. faecalis *as a function of proflavine dihydrochloride concentration. Key:* ○, μ = *0.01* M; *and* ●, μ = *0.03* M.

approximately 10 rps of the magnetic bar. Slow stirring refers to approximately 2 rps.

Negative mobility indicates a negatively charged cell wall, which caused the bacterium to migrate to the positive pole. With or without stirring, mobilities and, hence, ζ-potentials usually became increasingly less negative or more positive with increasing times of contact of the suspended bacteria with acridines. All five acridines showed this effect, which was due to leakage of nucleic acids from the bacteria caused by the acridines, similar to what had been observed with cetyltrimethylammonium bromide (2). The increase in absorbance at 260 nm with time of contact with acridines paralleled the increase in mobility of the bacteria, indicating that nucleic acids released by the acridines were the cause of the change in mobility. In the absence of acridines, no extracellular nucleic acids could be detected spectroscopically, nor did the mobilities change with time in stirred and unstirred suspensions (Table II).

Continuous fast stirring of bacterial suspensions to which acridines had been added produced the smallest increase in mobility with time. Evidently, stirring dispersed the nucleic acids released by a given bacterium, removing them from its vicinity fast enough to prevent substantial adsorption of these nucleic acids or of their complex coacervates with acridines at the cell wall. According to previous work (2), adsorption at the cell wall of nucleic acids released by the action of cetyltrimethylammonium bromide on *S. faecalis* in unstirred suspensions, probably in the form of complex coacervates with the cationic surfactant, changed the charge density of the cell wall to more positive values. In unstirred suspensions with added acridines, nucleic acids just released and/or their coacervates with acridines were slow to diffuse away from the bacteria, increasing the opportunity for their adsorption by the cell wall, with resultant changes in mobility.

To correct this effect, mobilities were measured on samples withdrawn at regular intervals after the addition of the acridines, while the bulk of the bacterial suspension was continuously stirred. Reported mobilities are the values extrapolated to zero time. Even in rapidly stirred suspensions, the charge of the bacterial cell wall occasionally underwent a change of sign with increasing time of contact, usually from negative to positive. This was observed especially for 9-aminoacridine hydrochloride, proflavine dihydrochloride, and acridine hydrochloride; it occurred near dye concentrations producing zero mobility.

Effect of Ionic Strength—The addition of indifferent electrolytes, *i.e.*, electrolytes whose cations and anions are not specifically chemisorbed by ionic groups in the surface of the bacteria, generally reduces the absolute values of the ζ-potential or electrophoretic mobility (1). Increasing ionic strength likewise reduces them. As is shown in Table II, a threefold increase in the ionic

Table V—Electrophoretic Mobility of *S. faecalis* as a Function of 9-Aminoacridine Hydrochloride Concentration at Constant Ionic Strength[a]

Concentration of 9-Aminoacridine Hydrochloride, *M*	pH	Specific Conductance, μmhos/cm	Mobility[b], (μm/sec)/(v/cm)
0	7.1	3020	−1.73[c]
0.0005	7.1	2910	−1.35[c]
0.0010	7.1	2890	−0.98[c]
0.0020	7.0	2870	−1.06[c]
0.0050	7.0	2850	−0.57
0.0070	7.1	2810	−0.32
0.0080	7.1	2750	−0.56
0.0085	7.0	2750	−0.36[d], −0.34[d]
0.0090	7.0	2680	+0.29[c]
0.0100	7.0	2680	+0.46
0.0115	7.0	2680	+0.62
0.0150	6.9	2500	+0.67[c]
0.0200	6.9	2460	+0.71[c]

[a] Ionic strength adjusted to 0.03 *M* with potassium acetate. [b] Extrapolated to zero time from four sets of 10 measurements performed after 5, 20, 35–40, and 55–60 min, respectively. [c] No significant trend with time. [d] Sign of electric charge changed with time.

strength of the buffer solution at constant pH reduced the absolute value of the mobility by 30%.

Table II also shows the excellent reproducibility of the electrophoretic mobility measurements and, in the absence of acridines, their independence of time of contact with the bacteria and of stirring. Five series of measurements, made over 10 months in 0.030 *M* potassium acetate blanks, gave a grand average mobility of −1.751 (μm/sec)/(v/cm), with a standard deviation of less than 1%. Three series of measurements in 0.010 *M* potassium acetate blanks gave a grand average of −2.509, with a standard deviation of 1.5%.

Changing the pH from 7.0 to 4.7 at constant ionic strength produced no significant change in mobility in the absence of acridines. The zero point of charge of *S. faecalis* is at a pH of about 2.3 (1).

Effect of Acridines at Constant Ionic Strength—The data for proflavine dihydrochloride (Table III) show that the presence of acridines did not alter the lowering of the absolute value of the electrophoretic mobility by an increase in ionic strength as long as the bacteria remained negatively charged. After charge inversion, however, an increase in ionic strength unexpectedly caused increases in mobility.

A threefold increase in ionic strength (μ) shifted the concentration–mobility curve toward more positive mobilities and lower concentrations (Fig. 1). This reduced the proflavine dihydrochloride concentration producing zero mobility by 24%. Thus, a drug concentration of 0.0015 *M* changed the mobility to −0.76 (μm/sec)/(v/cm) at $\mu = 0.01$ *M* but to +0.37 at $\mu = 0.03$ *M*.

Clumping or aggregation of bacteria was sometimes observed at high concentrations of acridines, *e.g.*, above 0.001 *M* for proflavine dihydrochloride at $\mu = 0.03$ *M*, in 0.01 *M* hydrazinoacridine acetate, and in 0.03 *M* acridine hydrochloride. It became more severe as the time of contact of the dissolved acridines with the suspended bacteria increased. Time of contact affected agglomeration more than mobility.

This aggregation is not a flocculation caused by reduction in the absolute value of the ζ-potential due to adsorption of acridines at the cell wall. Clumping occurred only at relatively high acridine concentrations, regardless of the magnitude of the mobility. In the case of proflavine dihydrochloride, for instance, aggregation was observed from small negative through zero to high positive mobility values. At $\mu = 0.03$ *M*, the compound produced severe clumping at a concentration of 5×10^{-3} *M*, where the ζ-potential was +21 mv according to Eq. 5 of Ref. 1, but none at 7×10^{-5} *M*, where the interpolated ζ-potential was −21 mv. Aggregation seems to be caused by nucleic acids released from the bacteria through the action of the acridines or by nucleic acid–acridine complex coacervates, possibly *via* a bridging mechanism (7, 8).

Acriflavine hydrochloride is dibasic. Thus, in a 0.015 *M* solution, the maximum amount of potassium acetate consistent with an ionic strength of 0.03 *M* gave a pH of only 4.7. To maintain constant conditions in all suspensions, with acriflavine hydrochloride concentration as the only variable, the pH was adjusted to 4.7 with potassium acetate; potassium chloride was added to maintain the ionic strength at 0.03 *M* where necessary. The results are listed in Table IV. One measurement, at an acriflavine hydrochloride concentration of 0.0030 *M*, was made at pH 5.6 in addition to the usual 4.7 value. The charge of the bacteria in the

Table VI—Electrophoretic Mobility of *S. faecalis* as a Function of Acridine Hydrochloride Concentration at Constant Ionic Strength[a]

Concentration of Acridine Hydrochloride, *M*	pH	Specific Conductance, μmhos/cm	Mobility[b], (μm/sec)/(v/cm)
0	6.7	4220	−1.70
0.0050	4.1	4000	−1.59
0.0100	3.5	3500	−1.22
0.0300	3.1	2600	−0.40[c]

[a] Ionic strength adjusted to 0.03 *M* with potassium chloride. [b] Extrapolated to zero time from four sets of 10 measurements performed after 5, 15, 30, and 45 min, respectively. [c] Sign of electric charge changed with time.

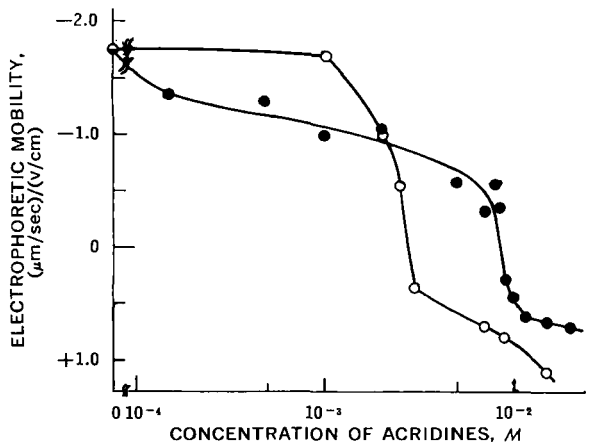

Figure 2—*Electrophoretic mobility of* S. faecalis *as a function of the concentrations of acriflavine and aminoacridine. Key:* ○, *acriflavine hydrochloride; and* ●, *9-aminoacridine hydrochloride.*

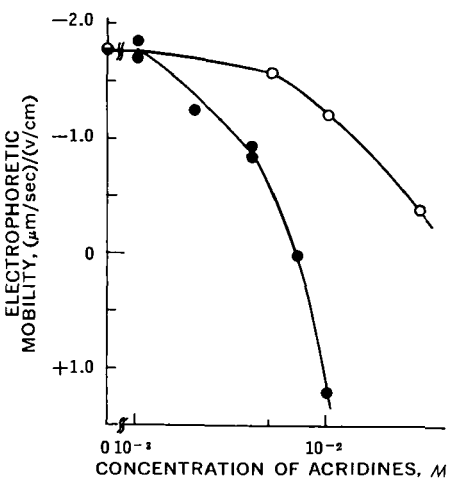

Figure 3—*Electrophoretic mobility of* S. faecalis *as a function of the concentrations of acridine and hydrazinoacridine. Key:* ○, *acridine hydrochloride; and* ●, *9-hydrazinoacridine acetate.*

Table VII—Electrophoretic Mobility of *S. faecalis* as a Function of 9-Hydrazinoacridine Concentration at Constant Ionic Strength[a]

Concentration of 9-Hydrazinoacridine, *M*	pH	Specific Conductance, μmhos/cm	Mobility ± *s*[b], (μm/sec)/(v/cm)
0	4.7	3000	−1.72 ± 0.10[c], −1.68 ± 0.07[d]
0.001	4.5	3060	−1.71 ± 0.08[c], −1.84 ± 0.09[d]
0.002	4.6	2810	−1.25 ± 0.07[c], −1.23 ± 0.04[d]
0.004	4.7	2930	−0.96 ± 0.06[c], −0.84 ± 0.05[d]
0.007	4.4	3000	0[c], 0[d]
0.010	4.4	3120	+1.14 ± 0.06[c], +1.38 ± 0.08[d]

[a] Ionic strength adjusted to 0.03 *M* with potassium acetate; pH adjusted with acetic acid. [b] Each number represents the average of 10 measurements ± the standard deviation of the average. [c] Measured after 7–10-min contact time. [d] Measured after 20–23-min contact time.

medium of higher pH was still negative while it had already been inverted to positive in the more acid medium. The acriflavine concentration required to reduce the ζ-potential of the bacteria to zero is apparently higher in the less acid medium, similar to what had been observed for cetyltrimethylammonium bromide (2).

The results obtained with 9-aminoacridine hydrochloride and acridine hydrochloride are summarized in Tables V and VI, respectively. Acridine is the weakest base of those studied. A solution containing 0.0295 *M* potassium acetate and 0.0005 *M* acridine hydrochloride produced a precipitate, presumably of the free base. Therefore, no attempts were made to buffer acridine hydrochloride solutions with potassium acetate. The hydrochloride was dissolved in water, and potassium chloride was added to maintain the ionic strength at 0.03 *M*. The resultant solutions, although acidic, had pH values above the isoelectric point of *S. faecalis*, which is at 2.3 (1).

Since the sulfate and hydrochloride of 9-hydrazinoacridine are poorly soluble in water (5), the compound was used as the acetate. The ionic strength was adjusted to 0.03 *M* with potassium acetate while enough acetic acid was added to bring the pH value to 4.4–4.7 and to dissolve the base. The data of Table VII indicate that the effect of contact time on mobility was less pronounced for hydrazinoacridine acetate than for aminoacridine hydrochloride and proflavine dihydrochloride.

Acridine Concentrations Producing Zero Mobility—The uptake of acridines by the cell wall probably occurs in two stages analogous to those described for cetyltrimethylammonium bromide (2). The first stage is chemisorption by ion exchange between the acridinium hydrochlorides and the carboxylic acid or potassium carboxylate groups in the cell wall (1). It reduces the electrophoretic mobility of the bacteria to near zero, since the resultant acridinium carboxylates are only slightly dissociated. The second stage, physical adsorption of the acridinium hydrochlorides by the cell wall, inverts the sign of the ζ-potential to positive. The transition between these two processes corresponds to the steepest portions of the acridine concentration *versus* electrophoretic mobility plots in Figs. 1–3.

The bacterial suspensions used in the electrophoresis measurements contained very small amounts of bacteria, not nearly enough to lower significantly the concentration of the dissolved acridines by adsorption. Therefore, the initial tabulated concentrations of acridines are substantially the same as their equilibrium concentrations.

The concentrations of the different acridine derivatives required to reduce the electrophoretic mobility of the bacteria and, hence, the ζ-potential to zero were obtained by interpolation in Figs. 1–3. These concentrations are inversely related to the affinity of the compounds for the cell wall: lower concentrations correspond to stronger adsorption. The concentrations producing zero mobility are listed in Table VIII together with the pKa values of the acridine derivatives. By applying the equation (4):

$$\text{percentage ionized} = \frac{100}{1 + \text{antilog (pH} - \text{pKa)}} \quad \text{(Eq. 1)}$$

the acridine derivatives are seen to be fully ionized at the pH values prevailing in the bacterial suspensions. For dibasic acridine derivatives, this refers only to the more basic of the two cationic groups.

Table VIII—Acridine Derivative Concentrations Required to Reduce Electrophoretic Mobility (*U*) of *S. faecalis* to Zero[a]

Compound	pKa in Water at 20°[b]	Concentration for *U* = 0	
		M	at pH
Acridine hydrochloride	4.8	0.046	About 3.1
9-Hydrazinoacridine acetate	7.15	0.0070	4.4
9-Aminoacridine hydrochloride	9.99	0.0086	7.0
Acriflavine hydrochloride	12	0.0028	4.7
Proflavine dihydrochloride	9.65	0.00145	6.0

[a] At an ionic strength of 0.03 *M*. [b] From Refs. 4 and 5.

For acridine, hydrazinoacridine, and acriflavine, the concentration required for producing zero mobility increases with decreasing base strength (*cf.*, Table VIII). Aminoacridine does not follow this inverse relationship, probably because the effect of its concentration on electrophoretic mobility was studied in a neutral medium whereas the other compounds were studied in acid media. As shown previously, the concentration for producing zero mobility is lowered by a decrease in pH. In the case of cetyltrimethylammonium bromide, this concentration was lowered from 7×10^{-4} to 3×10^{-4} *M* when the pH was decreased from 7.0 to 3.4 at constant ionic strength (2). If a drop in pH from 7.0 to 4.4–4.7 caused a proportional decrease in the aminoacridine concentration for producing zero mobility, the inverse relationship between this concentration and the pKa would most likely be extended to include aminoacridine. This inverse relationship is expected because the more basic compounds, of higher pKa, are chemisorbed more avidly by the carboxylate groups in the cell wall and, therefore, tie them up or neutralize them at lower concentrations. At the point of complete saturation or neutralization of the carboxylate groups, the ζ-potential of the cell wall is reduced to zero.

Of the five acridine compounds listed in Table VIII, proflavine is the only one whose pKa and concentration for producing zero mobility do not follow the inverse rank order. A decrease in pH from 6.0 to 4.4–4.7 would reduce its concentration for producing zero mobility even more, thereby further accentuating the discrepancy with the other four acridine derivatives.

Antibacterial Action of Acridines—As can be seen from Table IX, *S. faecalis* was remarkably resistant to 9-aminoacridine hydrochloride and to proflavine dihydrochloride, even though these compounds were reported (4) to possess marked antibacterial activity. Therefore, the susceptibility to cetyltrimethylammonium bromide of the lot of *S. faecalis* used in the present experiments was measured and compared with that of another lot of the same strain used 2 years earlier (2). The data of Table IX and those of Table III of Ref. 2 show that the minimum inhibitory concentration of cetyltrimethylammonium bromide was low and of comparable magnitude for the two lots. Thus, the lack of susceptibility to antibacterial agents of the present lot of *S. faecalis* was not general, and it is unlikely that it was due to mutation.

As a further check on the reliability of the susceptibility testing, a bacterium reported to be quite susceptible to acridines (4), *S. pyogenes*, was treated with different concentrations of aminoacridine hydrochloride, proflavine dihydrochloride, and cetyltrimethylammonium bromide, using the same pour plate technique. Growth was completely inhibited by low concentrations of all

Table IX—Effect of Antiseptics on Growth of Bacteria by Pour Plate Method[a]

Organism	Compound	Concentration[b], *M*	pH	Viability[c], %
S. faecalis	9-Aminoacridine hydrochloride	0.0001	6.8	106 (106), 92 (93)
		0.005[d]	7.1	93 ((89))
		0.01	6.6	84 (105) ((86)), 98 (84) ((98))
		0.01[d]	7.0	98 ((102))
		0.02[d]	7.0	78 ((65))
		0.05	6.2	106 (106)
		0.06[d]	6.8	65 ((89))
		0.08[d]	6.8	79 ((66))
		0.10	6.2	84 (105)
		0.10[d]	6.7	70 ((81))
	Proflavine dihydrochloride	0.0001	3.9	88 (96), 104 (82)
		0.001	2.8	67 (72), 74 (71)
		0.005	1.9	93
		0.005[d]	5.2	100 (95)
		0.01	1.7	75 (77)
		0.01[d]	4.6	((97))
		0.02[d]	4.0	49, ((79))
		0.04[d]	1.5	((73))
	Cetyltrimethylammonium bromide[e]	1×10^{-5}	7.0	0 (0)
		1×10^{-4}	7.0	0 (0)
S. pyogenes	9-Aminoacridine hydrochloride	1×10^{-6}	7.0	112
		1×10^{-4}	7.0	0 (0) ((0))
		1×10^{-3}	6.9	0 (0)
	Proflavine dihydrochloride	1×10^{-6}	5.1	0 (0)
		1×10^{-4}	4.2	0 (0) ((0))
	Cetyltrimethylammonium bromide[e]	1×10^{-5}	6.9	0 (0)
		1×10^{-4}	6.9	0 (0)

[a] Contact time of bacteria with compounds = 30 min. [b] Solvent for compound and medium for blank was redistilled water unless otherwise indicated. [c] Average colony count of three or more plates expressed as percentage of average count of three or more plates incubated with blank samples, *i.e.*, bacterial suspensions treated under identical conditions except for the absence of antiseptic compounds. Figures without parentheses, between single parentheses, and between double parentheses refer to suspensions stirred continuously and slowly, continuously and fast, and unstirred, respectively. Commas separate duplicate sets of results. [d] Solvent for compound and medium for blank was 0.03 *M* potassium acetate. [e] Solvent for compound and medium for blank was 0.01 *M* potassium acetate.

three antibacterials (*cf.*, Table IX). These two observations corroborate the finding that *S. faecalis* is quite resistant to aminoacridine hydrochloride and proflavine dihydrochloride.

These two acridines, which were strongly chemisorbed at the cell wall of *S. faecalis*, did not inhibit its growth even at concentrations exceeding by more than an order of magnitude the concentrations at which they reduced the ζ-potential of the bacterium to zero, *i.e.*, the concentrations at which their chemisorption to the cell wall was completed. This implies that the primary site of attack of the acridines on the bacteria is not the cell wall, a conclusion corroborated by the extensive literature on the mode of action of acridines (6, 9). They combine with nucleic acids and inhibit the enzymes involved in nucleic acid synthesis, possibly in the cytoplasmic cell membrane.

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Antipseudomonal Effect of Polymyxin and Phenylethanol

R. M. E. RICHARDS* and R. J. McBRIDE[x]

Abstract □ The effect of subinhibitory concentrations of phenylethanol and polymyxin, alone and in combination, on the growth rate of phenylethanol-sensitive and -resistant or polymyxin-sensitive and -resistant *Pseudomonas aeruginosa* was determined. The combination had an additive effect on sensitive *P. aeruginosa*, while the behavior of the resistant strains indicated that cross-resistance occurs between polymyxin and phenylethanol, which was confirmed by sterilization time measurements. It is suggested that there is no cross-resistance between polymyxin and either benzalkonium chloride or chlorhexidine acetate.

Keyphrases □ *Pseudomonas aeruginosa*, phenylethanol sensitive and resistant or polymyxin sensitive and resistant—effect of subinhibitory concentrations of phenylethanol and polymyxin, evaluation of components and combination □ Polymyxin—effect on *Pseudomonas aeruginosa*, alone and in combination with phenylethanol □ Phenylethanol—effect on *Pseudomonas aeruginosa*, alone and in combination with polymyxin □ Preservatives—evaluation of phenylethanol and polymyxin, alone and in combination, against *Pseudomonas aeruginosa*

Polymyxin B sulfate at a concentration of 1000 units/ml was reported to kill *Pseudomonas aeruginosa* inocula within 30 min (1). Kohn *et al.* (2) reported that polymyxin was unsatisfactory as an ophthalmic preservative since it required 18 hr to sterilize for *P. aeruginosa*; these authors recommended that such preservatives should sterilize a large inoculum of *P. aeruginosa* in less than 1 hr. The difference between the results may be due to differences in resistance between the test organisms. Some ophthalmic formulations contaminated with 10^6 *P. aeruginosa*/ml did not pass this test; but if phenylethanol was included in addition to the preservative, the preparations sterilized such inocula within 1 hr (3, 4). Combinations of phenylethanol with benzalkonium chloride, chlorhexidine acetate, or phenylmercuric nitrate were found to be more effective than the single preservatives against *P. aeruginosa* resistant to either phenylethanol or the other member of the combination (5). Phenylethanol–benzalkonium chloride and phenylethanol–chlorhexidine acetate combinations evaluated at subinhibitory concentrations had an effect against *P. aeruginosa* greater than additive, *i.e.*, greater than the sum of the effects of the single substances used alone (6).

The objectives of this study were to determine if: (*a*) phenylethanol enhances the effect of polymyxin against *P. aeruginosa*, (*b*) the combination has an effect greater than additive against *P. aeruginosa*, and (*c*) the combination is more effective against *P. aeruginosa* resistant to either component in the combination.

EXPERIMENTAL

Materials—Reagent grade phenylethanol[1] and polymyxin B sulfate[2] BP were used. *P. aeruginosa*[3] (NCTC 6750) was the test organism, Oxoid[4] nutrient broth No. 2 was the growth medium, and 37° was the incubation temperature. Maintenance of stock cultures was as previously described (7).

Preparation of Resistant Cells—Phenylethanol-resistant *P. aeruginosa* and log phase cultures of phenylethanol-resistant cells were prepared as described previously (5).

Polymyxin-resistant *P. aeruginosa* was prepared as follows: 0.1 ml of an overnight culture of *P. aeruginosa* was used to inoculate 100 ml of nutrient broth containing 1 unit/ml of polymyxin and incubated for 2 days. An inoculum of 0.1 ml of the resulting culture was used to inoculate 100 ml of broth containing 5 units polymyxin/ml. A similar process was used to inoculate media containing 10, 20, 50, 100, and 200 units/ml in succession. Selected cultures were stored in a refrigerator at 4° until required.

Log phase cultures of polymyxin-resistant *P. aeruginosa* were prepared by adding 1 ml of refrigerated *P. aeruginosa* resistant to 20 units polymyxin/ml to 100 ml of medium containing 10 units polymyxin/ml, and the product was incubated overnight in a water bath shaken at 105 throws/min. Then 1.0 ml of the resulting culture was added to 100 ml of prewarmed medium containing 3 units polymyxin/ml/ml. When the extinction at 420 nm in a 1-cm cell was approximately 0.35, 1.0 ml of the culture was transferred to each of four flasks containing 100 ml of medium with 3 units polymyxin/ml. Sufficient polymyxin and phenylethanol to give a final concentration of 3 units/ml and 0.2%, respectively, alone and in combination, were added to three flasks when the extinction at 420 nm was approximately 0.12 in each flask; water was added as a control to the fourth flask.

Growth Rate Studies—The effect of phenylethanol concentration on the growth of *P. aeruginosa* was described previously (8). A similar procedure was adopted with polymyxin to determine the growth rates of *P. aeruginosa* in nutrient broth containing 1, 2, 3, 4, 5, 6, 7, or 8 units polymyxin/ml. The growth rate for each concentration of polymyxin is expressed as a percentage of the growth rate of the control culture containing no polymyxin.

The effect of phenylethanol on polymyxin action was observed

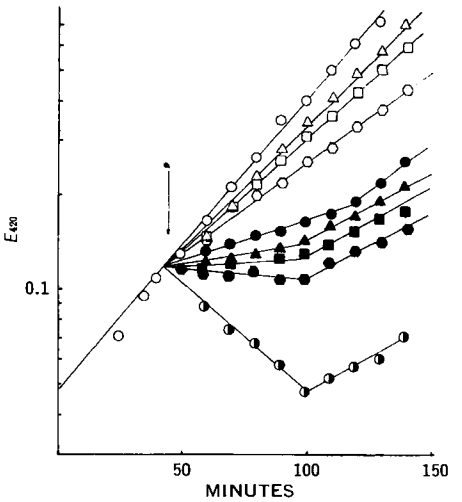

Figure 1—*Effect of various concentrations of polymyxin B sulfate on* P. aeruginosa. *Polymyxin was added at the time indicated by the arrow to give the following concentrations. Key: ○, water added; △, 1 unit polymyxin/ml; □, 2 units polymyxin/ml; ◇, 3 units polymyxin/ml; ●, 4 units polymyxin/ml; ▲, 5 units polymyxin/ml; ■, 6 units polymyxin/ml; ◆, 7 units polymyxin/ml; and ◑, 8 units polymyxin/ml.*

[1] British Drug Houses, Poole, Dorset, England.

[2] Burroughs Wellcome Ltd., Dartford, England.

[3] National Collection of Type Cultures, Colindale, London, England.

[4] Oxo Ltd., London, England.

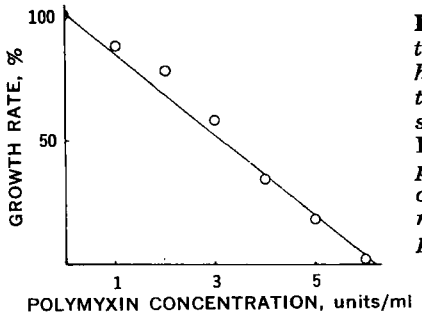

Figure 2 — *Correlation between subinhibitory concentrations of polymyxin B sulfate and growth of* P. aeruginosa *expressed as a percentage of the growth rate in the absence of polymyxin.*

by comparing the growth of *P. aeruginosa* in nutrient broth containing 0.15% phenylethanol after the addition of 3 or 5 units polymyxin/ml with similar cultures in nutrient broth only.

In a test for additivity between polymyxin and phenylethanol, four mixtures, calculated to be equipotent, were added to separate cultures of *P. aeruginosa* and the subsequent growth was followed. The effect of each mixture on *P. aeruginosa* was the difference between the growth rate of the organisms in the control culture and that in the medium containing the mixture. This effect was expressed as a percentage of the control rate and is referred to as the percentage effect.

Sterilization Times—The times required for aqueous solutions of polymyxin and phenylethanol, both alone and in combination, to sterilize large inocula of phenylethanol-sensitive and -resistant or polymyxin-sensitive and -resistant *P. aeruginosa* were determined as described previously (9, 10). The sterilization times for aqueous solutions of benzalkonium chloride and chlorhexidine acetate were also determined.

DISCUSSION

The effect of polymyxin on the growth rate differs from that reported for phenylethanol (8). In subinhibitory concentrations of phenylethanol, *P. aeruginosa* grew at a uniform rate. Figure 1 shows that in the presence of 4 units polymyxin/ml or more, there is an initial slow growth rate or lysis followed by a faster growth rate. Garrett and Brown (7) obtained nonlinear rates with *E. coli* and chloramphenicol and attributed this result to consumption of the antibiotic, development of resistant mutants, or the presence of antibiotic-resistant strains in the original inoculum. The same authors also suggested that the initial growth rate of the culture should be used for evaluation purposes. Figure 2 shows the linear correlation between growth of *P. aeruginosa* and concentration of polymyxin, indicating that the minimum inhibitory concentration of polymyxin against log phase cells is 6 units/ml. This linearity of effect is similar to that of phenylethanol (8) on *P. aeruginosa* and of chloramphenicol, tetracycline (11), or spectinomycin (12) on *E. coli*. Garrett *et al.* (11), in explaining this rectilinear regression as opposed to the more usual curvilinear regression, suggested that only a small fraction of the receptor sites in the biophase need be blocked for total inhibition of bacterial growth.

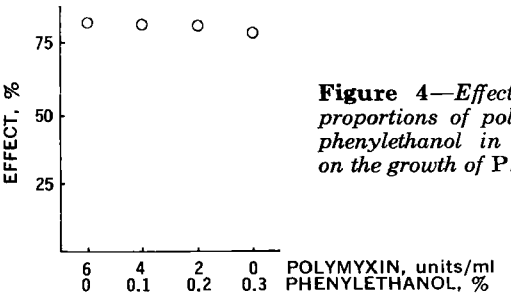

Figure 4—*Effect of relative proportions of polymyxin and phenylethanol in combination on the growth of* P. aeruginosa.

Figure 3 shows the effect of polymyxin on the growth of *P. aeruginosa* in nutrient broth containing 0.15% phenylethanol. A polymyxin concentration of 3 units/ml produces a single response in nutrient broth, but in the phenylethanol medium it produces a bilinear effect similar to that of 6 units polymyxin/ml (Fig. 1). Similarly, a concentration of 5 units polymyxin/ml shows lysis initially in the phenylethanol medium such as occurs with 8 units polymyxin/ml in nutrient broth only. Increasing the concentration of phenylethanol in the medium did not increase the effect of polymyxin but did increase the time during which the initial slow growth rate or lysis occurred.

Figure 3 also shows that 0.15% phenylethanol and 3 units polymyxin/ml have very similar effects on the growth in nutrient broth, suggesting that these concentrations are equipotent. When 3 units polymyxin/ml was added to nutrient broth containing 0.15% phenylethanol, the initial difference in growth rate from the control culture expressed as a percentage of the control growth rate was double that in nutrient broth only. With the minimum inhibitory concentrations of 0.3% phenylethanol and 6 units polymyxin/ml, both drugs have similar concentration effects on growth rate correlations. This implies that 0.1% phenylethanol is equivalent in effect to 2 units polymyxin/ml in its effect on the growth rate of log phase *P. aeruginosa* and that combinations of the substances would have an additive effect. The results in Fig. 4 show that the effects of four mixtures, which should show similar effects if additive, are analogous. This supports the hypothesis that the drugs are additive in their effect on *P. aeruginosa* and have the same mechanism of action.

The polymyxin-resistant and phenylethanol-resistant strains of *P. aeruginosa* in log phase cultures are not so readily affected as this type culture by polymyxin and phenylethanol, alone and in combination (Fig. 5). This result indicates cross-resistance between polymyxin and phenylethanol and contrasts with the effect of benzalkonium chloride and chlorhexidine acetate on phenylethanol-resistant *P. aeruginosa* (5). Such results suggest that there

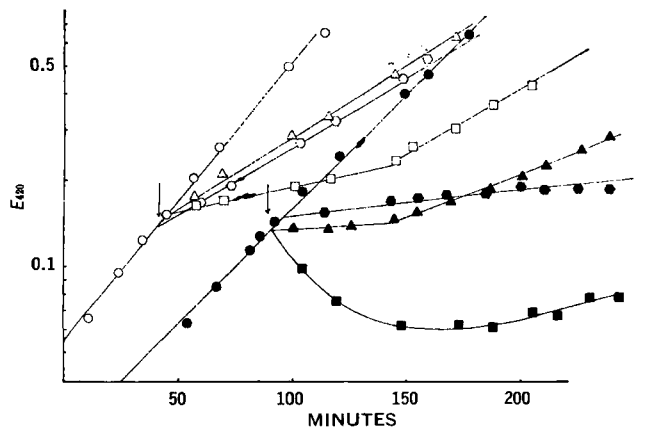

Figure 3—*Effect of 3* (△) *and 5* (□) *units polymyxin B sulfate/ml on* P. aeruginosa *growing in nutrient broth (open symbols) and in nutrient broth containing 0.15% phenylethanol (closed symbols). Sufficient phenylethanol to give 0.15%* (○) *was also added to each medium. Additions were made at the times indicated by the arrows.*

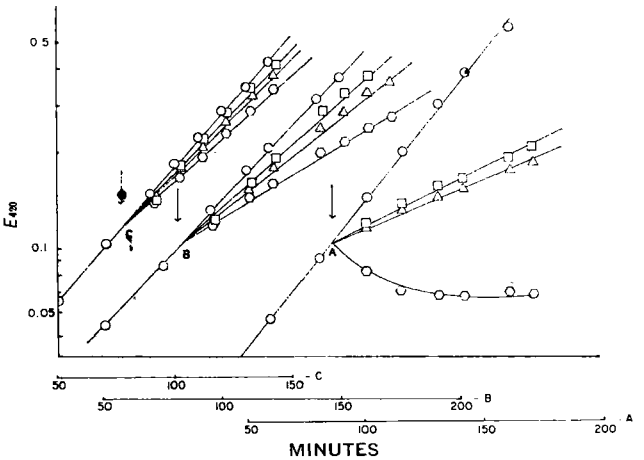

Figure 5—*Effect of 3 units polymyxin/ml* (□), *0.2% phenylethanol* (△), *and a combination of both* (○) *on the growth of* P. aeruginosa (*A*), P. aeruginosa *resistant to phenylethanol* (*B*), *and* P. aeruginosa *resistant to polymyxin* (*C*). *Drugs were added at the times indicated by the arrows.*

Table I—Sterilization Times for *P. aeruginosa*, Sensitive and Resistant to Either Phenylethanol or Polymyxin, in Solutions of Polymyxin and Phenylethanol, Alone and in Combination, Benzalkonium Chloride, and Chlorhexidine Acetate

Antibacterial(s) Concentration(s)	*P. aeruginosa* NCTC 6750		*P. aeruginosa* Resistant to 0.6% Phenylethanol		*P. aeruginosa* Resistant to 200 units Polymyxin/ml	
	Cells × 10^5/ml	Sterilization Time, min	Cells × 10^5/ml	Sterilization Time, min	Cells × 10^5/ml	Sterilization Time, min
Benzalkonium chloride, 0.005%	6.7	30	4.2	<5	6.9	25
Chlorhexidine acetate, 0.005%	6.7	40	4.2	<5	6.9	15
Phenylethanol, 0.4%		24 hr		>24 hr		>24 hr
Polymyxin, 25 units/ml	13.0	15	8.5	>300	5.3	>24 hr
Phenylethanol, 0.4%, and polymyxin, 25 units/ml		15		240		>24 hr
Polymyxin, 100 units/ml				240		>24 hr
Phenylethanol, 0.4%, and polymyxin, 100 units/ml	—	—	12.0	45	5.8	>24 hr
Polymyxin, 300 units/ml						>24 hr
Phenylethanol, 0.4%, and polymyxin, 300 units/ml	—	—	—	—	6.9	>24 hr

is no cross-resistance between polymyxin and benzalkonium chloride or chlorhexidine acetate.

Lacey (13) divided combinations of drugs which are additive or synergistic (greater than additive) into six classes according to the presumed site of action of the drug, the presumed route by which the drug arrives at the site, and the presumed biochemical sequence blocked. The characteristics used to decide into which class a combination of two drugs falls are the effect of the combination on a homogeneous population, the pattern of cross-resistance, and a comparison of the drug antagonists. Polymyxin and phenylethanol combinations are additive in effect and show cross-resistance, so they would be in one of two possible classes, as defined by Lacey, that have the same mode of action. The factor that decides whether the combination is Class 1 (same route) or Class 2 (different routes) is the route by which the drugs arrive at the site. The factor that decides the class is whether the effect of one drug can be stopped by an antagonist having no effect on the other; no potent antagonists have been reported for either drug. The phenylethanol–chlorhexidine and phenylethanol–benzalkonium chloride combinations do not fit readily into Lacey's classification. The greater-than-additive effect of the combinations indicates that the drugs have different sites of action. The lack of cross-resistance or one-way resistance suggests that the drugs have different routes, *i.e.*, Class 6 (13). Although cross-resistance is not found in Class 6, an increased sensitivity to one drug may accompany resistance to the other. This is known as "collateral sensitivity" (14) and was exhibited by phenylethanol with benzalkonium chloride and chlorhexidine acetate. Barber (15) stated that combinations used for their synergistic effect in antibacterial chemotherapy belong to Class 6.

Sterilization times were determined to confirm that the results obtained with subinhibitory concentrations also pertain when inhibitory concentrations are used. Table I gives the results of attempts to determine suitable polymyxin–phenylethanol concentrations which would sterilize large inocula of sensitive and resistant *P. aeruginosa* within 1 hr. If a culture was sterilized in less than 1 hr by a combination, that strain was not tested with the higher concentrations of polymyxin. The results corroborated the findings of the growth studies in that: (*a*) the type culture was more affected than the resistant strains, and (*b*) the combination of phenylethanol and polymyxin was more effective than either preservative alone against the type culture and phenylethanol-resistant culture. The polymyxin-resistant strain was the most resistant tested and was not sterilized by any combination. An attempt was made to determine the minimum inhibitory concentration of the polymyxin-resistant strain, but growth occurred in a medium containing 3000 units polymyxin/ml, which was the highest concentration tested. The effect of benzalkonium chloride and chlorhexidine acetate on the three strains shows that there is no cross-resistance between polymyxin and these bactericides against *P. aeruginosa*. The lack of cross-resistance between polymyxin and benzalkonium chloride is in agreement with previous results (16).

It was suggested (5) that, among the desirable properties of an antibacterial combination, the spectrum of activity should include pathogenic Gram-positive and Gram-negative organisms and should also be effective against organisms resistant to one component of the combination. The present results show that the additive combination of polymyxin and phenylethanol would not be a suitable combination because of cross-resistance. Furthermore, both substances have weak activity against Gram-positive organisms. Polymyxin does not show cross-resistance with benzalkonium chloride or chlorhexidine acetate, but collateral sensitivity was not shown because the sterilization times for benzalkonium-resistant and chlorhexidine-resistant *P. aeruginosa* were not determined against polymyxin.

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* Present address: Pharmacy Department, University of Rhodesia, P.O. Box MP 167, Salisbury, Rhodesia.

ˣ To whom inquiries should be directed.

Modified Fluorescence Assay for Oxidized and Reduced Glutathione in Tissue

WILLIAM M. HADLEY, WILLIAM F. BOUSQUET[x], and TOM S. MIYA

Abstract □ A previously reported enzymatic-fluorometric assay for reduced glutathione and oxidized glutathione was modified through the use of heat precipitation of sample protein and hydrogen peroxide oxidation of reduced glutathione to oxidized glutathione. These modifications were made to eliminate two critical pH adjustments present in the original assay and the possibility of interference with the assay of oxidized glutathione by the perchloric acid protein precipitant and the cupric-ion oxidant used in the original assay. The reduced glutathione and oxidized glutathione content of Harding–Passey melanoma was determined using the modified assay. The reduced glutathione content was similar to literature values. The precision of the assay was not adversely affected by these modifications.

Keyphrases □ Glutathione, oxidized and reduced—modified fluorometric determination in tissue □ Spectrophotofluorometry—determination of oxidized and reduced glutathione in tissue

Reduced glutathione, a tripeptide (λ-glu-cys-gly), is found widely distributed in tissue (1). It also exists in an oxidized form in which two reduced molecules are joined through a disulfide bridge. To be maximally useful, any assay for these species should measure both in the same sample.

An enzyme, glutathione reductase (EC 1.6.4.2), reduces oxidized glutathione in the presence of reduced nicotine adenine dinucleotide phosphate. One mole of reduced nicotine adenine dinucleotide phosphate is required for conversion of each mole of oxidized glutathione to 2 moles of reduced glutathione (2, 3). This relationship was used by Halprin and Ohkawara (4) to develop an enzymatic assay for reduced glutathione and oxidized glutathione. The amount of oxidized glutathione present in the sample was determined by following the decrease in reduced nicotine adenine dinucleotide phosphate fluorescence. The reduced glutathione content of another aliquot of the sample was determined after its oxidation to oxidized glutathione. This assay as described requires two critical, time-consuming adjustments of the sample pH. The first pH adjustment with potassium hydroxide removes the perchloric acid as potassium perchlorate, and the second optimizes the rate of oxidation of reduced glutathione to oxidized glutathione by cupric ion. When the original assay was modified in this laboratory so that larger volumes of sample could be used, it was found that standards and various volumes of tissue sample did not consistently yield linear fluorescence changes. It was suspected that cupric ion or perchloric acid might be interfering with the oxidized glutathione assay, since cupric ion is known to inhibit glutathione reductase (5) and perchloric acid is a protein precipitant.

The objective of this work was to modify the reduced glutathione–oxidized glutathione assay developed by Halprin and Ohkawara (4) to eliminate the perchloric acid used for protein precipitation and the cupric ion used for reduced glutathione oxidation. By eliminating these two substances, the possibility of their interfering with the assay of oxidized glutathione would be removed and the two critical pH adjustments would be eliminated. Heat precipitation of tissue protein and hydrogen peroxide oxidation of reduced glutathione were investigated as means of accomplishing these objectives. The assay in its modified form was used to determine the oxidized glutathione and reduced glutathione content of Harding–Passey melanoma tissue.

EXPERIMENTAL

Materials—Reduced and oxidized glutathione[1], glutathione reductase[1] (yeast) in 2.8 *M* ammonium sulfate, reduced nicotine adenine dinucleotide phosphate tetrasodium[1], tetrasodium edetate[1], and tromethamine[1] were used. All other chemicals were analytical reagent grade. Stock solutions of reduced nicotine adenine dinucleotide phosphate were prepared in deionized, double-distilled water containing 1% sodium bicarbonate.

Method—Melanoma tissue was rapidly excised from mice and frozen on dry ice. The tissue was prepared for assay by homogenization in five volumes of phosphate buffer (0.05 *M*, pH 6.4) at the temperature of melting ice. The homogenate was placed in a water bath at 100° for 4 min to precipitate protein and was then centrifuged in an ultracentrifuge[2] using a No. 30 rotor (3000×*g*, 5750 rpm for 10 min, 0°). A portion of the supernate was divided into four 1.0-ml samples. The first sample was assayed directly for oxidized glutathione and the second sample was assayed for oxidized glutathione after a known quantity of oxidized glutathione was added as an internal standard. To the third sample were added a known quantity of reduced glutathione and 30% hydrogen peroxide sufficient to make the solution 0.12% (v/v) in hydrogen peroxide. Only hydrogen peroxide sufficient to make 0.12% (v/v) was added to the fourth sample. After 3 hr at room temperature, the third and fourth samples were placed in a water bath (100°, 5 min) to drive off residual hydrogen peroxide; they were then assayed for oxidized glutathione.

Samples were assayed for oxidized glutathione as in the original assay by following the decrease in fluorescence[3] of reduced nicotine adenine dinucleotide phosphate (340-nm excitation, 450-nm emission, uncorrected). The assay mixture contained 40 nanomoles of reduced nicotine adenine dinucleotide phosphate, 0.05 *M* (pH 7.4) tromethamine buffer (containing tetrasodium edetate, 0.005 *M*, and magnesium chloride, 0.03 *M*), and standards or tissue extracts containing 2–25 nanomoles of oxidized glutathione in a total volume of 3.45 ml. The reaction components were placed in a spectrophotofluorometer cell and the fluorescence was allowed to stabilize. This value was recorded as the initial fluorescence. Glutathione reductase was then added (0.025 ml, 0.3 unit, in 2.8 *M* ammonium sulfate), and the solution was thoroughly mixed. A second fluorescence value was recorded 10 min later. All samples were assayed in triplicate. Blanks contained pH 6.4 phosphate buffer instead of the sample solution and served to correct for

[1] Calbiochem, Los Angeles, Calif.
[2] Beckman Spinco model L or L2-50, Spinco, Beckman Instruments, Palo Alto, Calif.
[3] Aminco-Bowman spectrophotofluorometer or SPF-125.

Table I—Influence of Heat on the Oxidized Glutathione Content of Solutions Containing Reduced or Oxidized Glutathione[a]

Glutathione	Heat Exposure	Oxidized Glutathione Content, nanomoles ± SE[b]	t Value[c]
Oxidized (10 nanomoles)	−	10.00 ± 0.07	
Oxidized (10 nanomoles)	+	9.87 ± 0.15	0.62 (NS)
Reduced (20 nanomoles)	−	0.06 ± 0.06	
Reduced (20 nanomoles)	+	0.05 ± 0.05	0.12 (NS)

[a] Triplicate samples, dissolved in pH 6.4 phosphate buffer, were placed in a water bath (100°) for 4 min (+) before assay for oxidized glutathione or were assayed directly for oxidized glutathione (−). [b] Standard error. [c] (NS) = not significantly different ($p > 0.05$) from unheated samples.

nonenzymatic oxidation of reduced nicotine adenine dinucleotide phosphate. Oxidized glutathione used as an internal standard allowed the conversion of fluorescence units to oxidized glutathione content. Reduced glutathione was used as an internal standard to check on the completeness of oxidation of sample reduced glutathione by hydrogen peroxide.

RESULTS AND DISCUSSION

Heat Stability of Reduced Glutathione and Oxidized Glutathione—The proposed use of heat as a protein precipitant necessitated verification of the heat stability of oxidized glutathione and reduced glutathione. Solutions containing known amounts of either oxidized glutathione or reduced glutathione in pH 6.4 phosphate buffer were assayed for oxidized glutathione content after exposure in a water bath at 100° for 4 min. Reduced glutathione was previously reported to be stable under these conditions (6). Data from this experiment (Table I) indicate that there is no significant destruction of oxidized glutathione or conversion of reduced glutathione to oxidized glutathione by heat exposure of these species at 100° for 4 min.

Evaluation of Reduced Glutathione Oxidation to Oxidized Glutathione by Hydrogen Peroxide—The oxidation of reduced glutathione to oxidized glutathione by hydrogen peroxide was reported previously to be complete (4). Hydrogen peroxide (30%) (to make 0.12% v/v) was added to a solution containing known amounts of reduced glutathione and oxidized glutathione in pH 6.4 phosphate buffer (both reduced glutathione and oxidized glutathione were placed in the same solution to more closely approximate the condition of the tissue homogenate). After 3 hr at room temperature, the solution was heated (100° water bath for 5 min) before assay for oxidized glutathione to drive off excess hydrogen peroxide. If the hydrogen peroxide is not driven off, reduced glutathione values are abnormally high. Data from this experiment (Fig. 1) indicate that the conversion of reduced glutathione to oxidized glutathione is linear over the range of concentrations used (4-12 nanomoles).

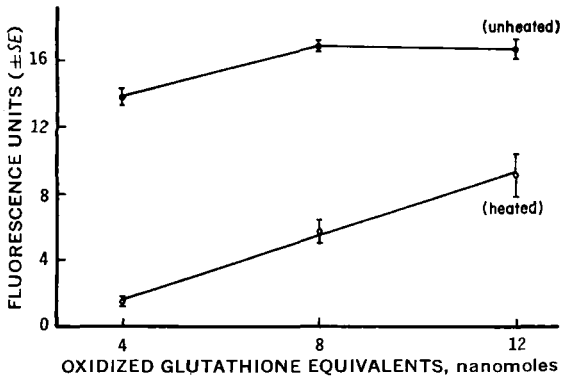

Figure 1—*Evaluation of reduced glutathione oxidation to oxidized glutathione by hydrogen peroxide. An oxidized glutathione–reduced glutathione mixture (1:2) in phosphate buffer was treated with hydrogen peroxide (0.12%, 3 hr). One aliquot was heated (100°, 5 min) to drive off excess hydrogen peroxide and then both aliquots were assayed (triplicate) for oxidized glutathione. Reduced glutathione (nanomoles) is divided by two to obtain oxidized glutathione equivalents (nanomoles). The fluorescence change due to the oxidized glutathione originally added was subtracted from the values shown on the figure.*

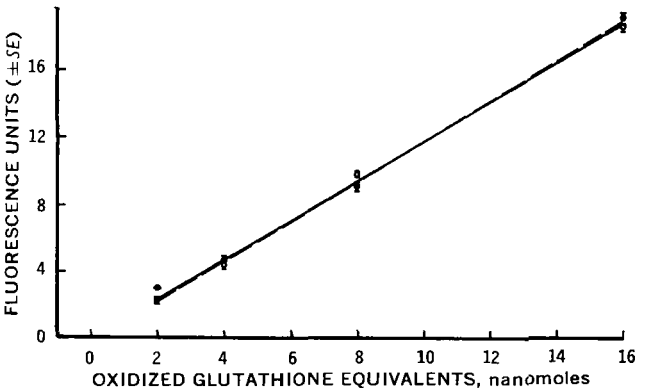

Figure 2—*Linearity and completeness of oxidation of reduced glutathione to oxidized glutathione by hydrogen peroxide. Solutions containing either reduced glutathione (●) (4, 8, 16, and 32 nanomoles) or oxidized glutathione (○) (2, 4, 8, and 16 nanomoles) were assayed as described under* Method.

Evaluation of Assay Linearity—The decrease in reduced nicotine adenine dinucleotide phosphate fluorescence produced by reduced glutathione (4, 8, 16, and 32 nanomoles) after oxidation by hydrogen peroxide and by oxidized glutathione (2, 4, 8, and 16 nanomoles) was determined. The two curves obtained (Fig. 2) were not significantly different [$F = 0.06$, $df_{1,16}$, by two-way analysis of variance (7)] and were linear. This experiment indicates that the oxidation of reduced glutathione to oxidized glutathione is complete under the conditions employed.

The decrease in reduced nicotine adenine dinucleotide phosphate fluorescence produced by various volumes of tissue homogenate supernate (0.025, 0.05, 0.075, 0.100, and 0.200 ml) from a Har-

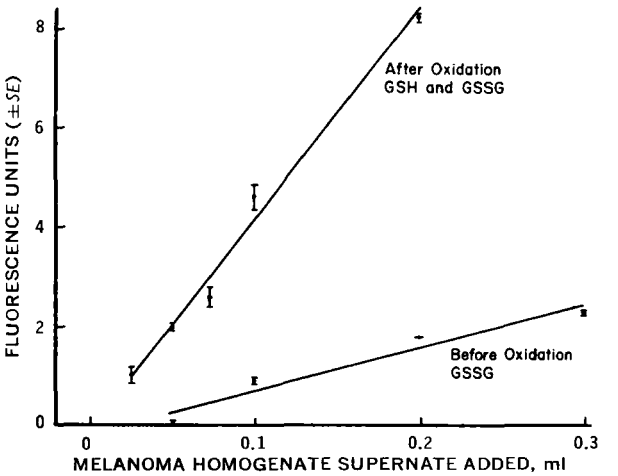

Figure 3—*Linearity of the assay for tissue oxidized glutathione (GSSG) before and after oxidation of tissue reduced glutathione (GSH) to oxidized glutathione by hydrogen peroxide. Melanoma homogenate supernate, prepared as described under* Method, *was divided into two aliquots. One aliquot was assayed directly for oxidized glutathione. The other aliquot was treated with hydrogen peroxide.*

Table II—Reduced and Oxidized Glutathione Content of Harding–Passey Melanoma Tissue[a]

Tissue Oxidized Glutathione, nanomole/mg ± *SE*[b]	Tissue Reduced Glutathione, nanomoles/mg ± *SE*	Ratio Reduced Glutathione to Oxidized Glutathione
0.141 ± 0.029	1.473 ± 0.054	12.5

[a] Melanomas were excised from four mice and triplicate samples were assayed for oxidized glutathione and reduced glutathione following tissue preparation as described under *Method*. [b] Standard error.

ding–Passey melanoma homogenate, prepared as described under *Method*, was determined both before and after addition of hydrogen peroxide. As shown in Fig. 3, the fluorescence change produced is linear with respect to the volume of supernate employed both before and after the oxidation of reduced glutathione to oxidized glutathione.

Determination of Oxidized Glutathione and Reduced Glutathione Values for Harding–Passey Melanoma—The Halprin and Ohkawara assay (4) as modified through the use of heat precipitation of sample protein and hydrogen peroxide oxidation of reduced glutathione was performed on a Harding–Passey melanoma homogenate supernate prepared as described under *Method*. The value for reduced glutathione (Table II) is similar to the value of 1.4 nanomoles/mg (sulfosalicylic acid-soluble sulfhydryl) reported (6) for this tissue. More than 90% of the glutathione in the melanoma is in the reduced form, which is similar to the percentage reported for other tissues (4, 9). This is indicated by the experimentally determined ratio, reduced glutathione–oxidized glutathione of 12.5, obtained in the present study.

The enzymatic–fluorometric assay for reduced glutathione and oxidized glutathione previously described (4) was modified to eliminate the use of a strong acid protein precipitant and of cupric ion for the oxidation of reduced glutathione. These procedures were replaced by the use of heat as a protein precipitant and of hydrogen peroxide as an oxidant. These modifications obviate the necessity of a critical pH adjustment and remove the possibility of copper interference with glutathione reductase activity.

It was determined that heat treatment of reduced glutathione- and oxidized glutathione-containing solutions does not interfere with the precision of oxidized glutathione determination and does not convert reduced glutathione to oxidized glutathione. It appears that heat precipitation of protein is an acceptable substitute for perchloric acid precipitation of protein and is free of the mentioned disadvantages.

Substitution of hydrogen peroxide for cupric ion in the oxidation of reduced glutathione to oxidized glutathione eliminates the need for making a critical pH adjustment and the possibility of cupric-ion interference with the reduced glutathione assay. Cupric ion (10^{-5} *M*) was reported to inhibit glutathione reductase by approximately 50% (5). Hydrogen peroxide appears to be an acceptable substitute for cupric ion in that the oxidation of reduced glutathione standards is complete and the oxidation of reduced glutathione to oxidized glutathione in tissue samples is linear.

The reduced glutathione content determined with the modified assay for Harding–Passey melanoma was nearly identical to the 1.4-nanomoles/mg (sulfosalicylic acid-soluble sulfhydryl) value reported (8) for this tissue. No literature value for oxidized glutathione in the Harding–Passey melanoma is available; but human epidermis, which has a similar amount of reduced glutathione to Harding–Passey melanoma, was reported (4) to have 0.078–0.111 nanomole/mg of oxidized glutathione, values similar to those determined experimentally for the melanoma with the modified assay.

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[x]To whom inquiries should be directed.

Behavior of Erythrocytes in Various Solvent Systems VII: Water–Monohydric Alcohols

SHIAO-HUAN KU and DONALD E. CADWALLADER ˣ

Abstract □ The hemolytic behavior of human erythrocytes in water–monohydric alcohol solutions was investigated. Complete hemolysis of erythrocytes occurred in all methanol, ethanol, *n*-propanol, and isopropanol solutions. Sodium chloride was effective in preventing complete hemolysis in solutions containing up to 18% methanol, 11% ethanol, 4% *n*-propanol, and 8% isopropanol. The addition of sodium chloride to solutions containing more than these concentrations of alcohol did not prevent hemolysis, discoloration, and/or precipitation of human red blood cells. The addition of 2% sodium chloride did not appreciably influence the hemolytic action of methanol, ethanol, *n*-propanol, or isopropanol, while 5% sodium chloride lowered the alcohol concentrations needed to cause 100% hemolysis. The data were used to calculate Van't Hoff *i* values for sodium chloride in the various alcohol solutions. Studies in aqueous ternary systems containing two alcohols (in 1:1 proportion) indicated an additive effect for the hemolytic action of alcohols.

Keyphrases □ Erythrocytes, human—hemolysis in water–monohydric alcohol solutions □ Hemolysis of human erythrocytes—water–monohydric alcohol solutions □ Alcohol (monohydric)–water solutions—effect on hemolysis of human erythrocytes

It is well known that, to prepare a safe and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous solvent. For this reason, investigations have been made to study the hemolytic effects of aqueous solutions of glycerin, propylene glycol, and liquid polyethylene glycols on rabbit and human erythrocytes and the hemolytic effects of aqueous solutions of dimethyl sulfoxide, liquid amides, and tetramethylurea on human erythrocytes (1–6).

This report is concerned with the investigation of water–monohydric alcohol systems. The alcohols studied were methanol, ethanol, *n*-propanol, and isopropanol. Next to water, the monohydric alcohols are probably the most widely used pharmaceutical solvents. One extensive article (7) on nonaqueous solvents for use in parenteral products surveyed the physical properties, toxicities, and parenteral application of ethanol. It was reported that this solvent had occasional use in parenteral products, particularly the digitalis glycosides. USP XVIII (8) states that a digoxin preparation containing 9–11% alcohol may be used for intramuscular or intravenous administration.

The purpose of this investigation was to observe the behavior of human erythrocytes in various water–monohydric alcohol solutions utilizing the hemolytic method. By comparison of standard hemolysis curves obtained for human blood in aqueous saline solutions and those obtained from experiments using sodium chloride–water–monohydric alcohol solution, it was possible to calculate the hemolytic isotonic coefficient for sodium chloride in various water–alcohol solutions.

EXPERIMENTAL

Materials—Ethanol, absolute[1], and reagent grades of methanol[2], *n*-propanol[3], isopropanol[2], and sodium chloride[2] were used.

Preparation of Solutions—All alcohol solutions were volume-in-volume percentage preparations. Sodium chloride solutions were prepared on a weight-in-volume basis. Necessary dilutions were made from stock solutions, and distilled water was used to prepare all solutions.

Collection of Blood—The blood samples used for all experiments were obtained from the forearm veins of several 20–25-year-old Caucasian and Oriental subjects. Fresh blood samples were used in all experiments. Approximately 10 ml of blood was obtained from the donors and placed in a 50-ml round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min, and then the blood was decanted into a 50-ml conical flask and aerated by swirling the flask gently for about 5 min.

Quantitative Determination of Percent Hemolysis—In each experiment, the hemolytic method was used to determine the degree of hemolysis of erythrocytes in the various alcohol solutions. This quantitative method is based on the fact that a hypotonic solution liberates oxyhemoglobin in direct proportion to the number of cells hemolyzed. Into each of two test tubes was transferred 5 ml of standard sodium chloride solution (0.32, 0.34, . . ., 0.46, 0.48%) or 5 ml of the mixed solvent system being tested. After the test tubes were brought to a constant temperature by placing in a water bath (37 ± 0.5°), 0.05 ml of blood was pipeted into each tube. Each tube was shaken on a vibratory mixer for approximately 5 sec to ensure thorough mixing and then was allowed to stand 45 min at 37°. The tubes were centrifuged at approximately 2500 rpm, and the light absorbance of the supernatant liquid was measured using a photoelectric colorimeter[4] equipped with a No. 54 filter. To find the percent hemolysis, these absorbance readings were divided by the absorbance readings for 0.05 ml of blood in 5 ml of distilled water (standard for 100% hemolysis) and multiplied by 100. A blank, made by placing 0.05 ml of blood in 5 ml of 0.9% NaCl solution, was used to cancel any light absorbance inherent to the blood sample. Both the standard and the blank were subjected to the same conditions of standing for 45 min at 37° followed by centrifuging.

Calculations of *i* Values—Through use of the hemolytic method, concentrations of sodium chloride and the alcohol solutions giving the same degree of hemolysis could be determined. Once these concentrations were ascertained, it was possible to calculate isotonic coefficients (*i* values) through use of the following equation:

$$\begin{pmatrix}i \text{ value for NaCl} \\ \text{in water}\end{pmatrix}\begin{pmatrix}\text{grams of NaCl in} \\ \text{100 ml of water}\end{pmatrix} = \begin{pmatrix}i \text{ value for NaCl} \\ \text{in alcohol solution}\end{pmatrix}\begin{pmatrix}\text{grams of NaCl in} \\ \text{100 ml of alcohol solution}\end{pmatrix} \quad \text{(Eq. 1)}$$

The value of *i* for sodium chloride was taken as 1.86, which is the accepted *i* value for 0.9% NaCl in water (9).

Curves showing the degree of hemolysis in sodium chloride–water solutions and sodium chloride–water–alcohol solutions were plotted on rectangular coordinate graph paper. From these curves, it was possible to determine the concentrations of sodium chloride in g/100 ml of water and the alcohol solvent causing 25,

[1] U.S. Industrial Chemical Co.
[2] J. T. Baker Chemical Co.
[3] Mallinckrodt Chemical Works.
[4] Klett-Summerson.

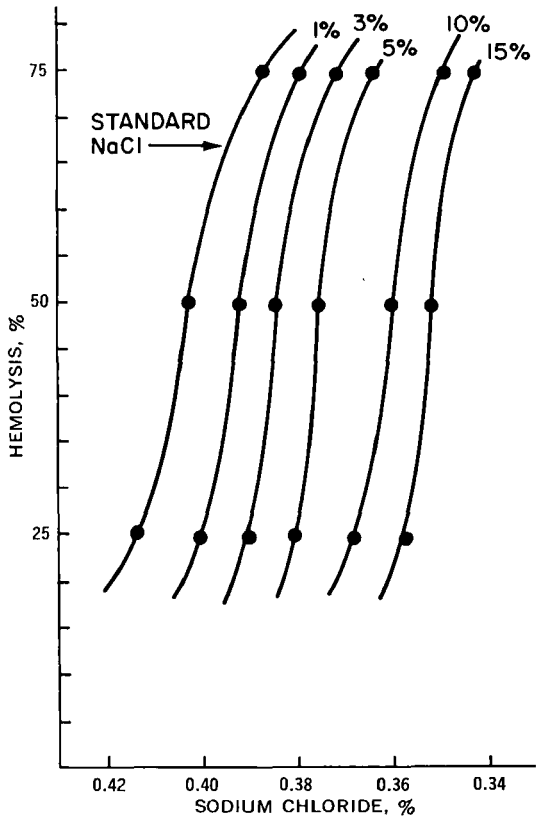

Figure 1—*Hemolysis of human erythrocytes after 45 min at 37° in various methanol solutions.*

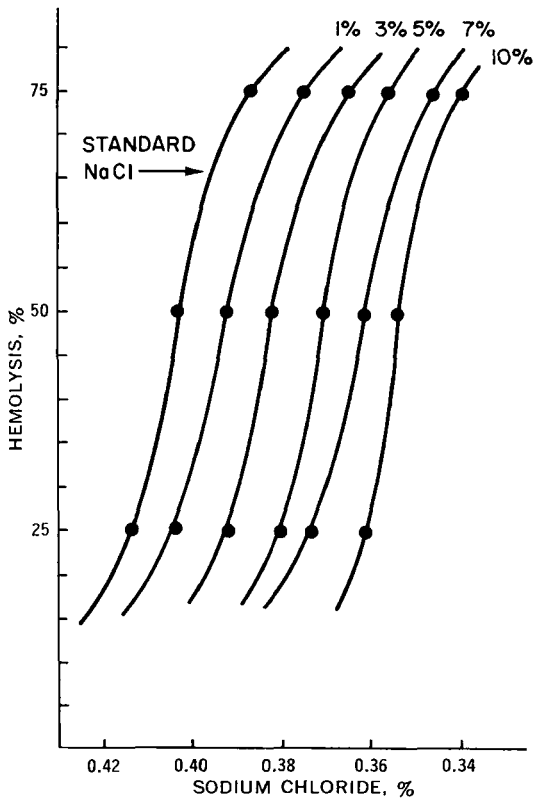

Figure 2—*Hemolysis of human erythrocytes after 45 min at 37° in various ethanol solutions.*

50, and 75% hemolysis. These values were inserted into Eq. 1, thereby giving the values of i for sodium chloride in a particular water-alcohol solution at concentrations producing 25, 50, and 75% hemolysis. The various i values for sodium chloride in aqueous methanol, ethanol, *n*-propanol, and isopropanol solutions are shown in Table I.

Preparation of Hemolysis Curves—Approximately 17 experiments employing human blood were carried out. A standard hemolysis curve (left-hand side of Figs. 1–4) was constructed from the average readings of these experiments. Hemolysis curves of the various alcohol solutions (Figs. 1–4) were constructed using the i values previously calculated from Eq. 1 and shown in Table I. Through a rearrangement of Eq. 1, the grams of sodium chloride/100 ml in an alcohol solution causing 25% hemolysis were calculated using Eq. 2:

$$\begin{pmatrix}\text{grams of NaCl in}\\ \text{100 ml of}\\ \text{alcohol solution}\\ \text{causing 25%}\\ \text{hemolysis}\end{pmatrix} = \frac{\begin{pmatrix}\text{1.86-}i\text{ value for}\\ \text{NaCl in water}\end{pmatrix}\begin{pmatrix}\text{grams of NaCl in 100 ml of water}\\ \text{causing 25\% hemolysis}\end{pmatrix}}{i\text{ value for NaCl in alcohol solution}} \quad \text{(Eq. 2)}$$

Similar calculations were carried out at 50 and 75% hemolysis. By plotting these three points, the hemolysis curves for the various alcohol solutions were constructed (Figs. 1–4).

RESULTS

Water–Monohydric Alcohol Solutions—Quantitative hemolytic determinations were carried out with methanol, ethanol, *n*-propanol, and isopropanol employing human blood. Complete hemolysis and/or denaturation of human erythrocytes occurred in all aqueous alcohol test solutions ranging in concentration from 0.0 to 100% after 45 min at 37°. Normal red solutions were evident up to concentrations of 35% methanol, 25% ethanol, 12% *n*-propanol, and 17% isopropanol; however, red-brown discolorations were observed in solutions containing 36–40% methanol, 26–28% ethanol, 13–14% *n*-propanol, and 18–20% isopropanol. Very heavy pinkish-red to pinkish-brown turbidity and/or precipitates occurred when blood was placed in solutions containing 50, 60, and 70% methanol; 30, 40, and 50% ethanol; 15–30% *n*-propanol; and 25–40% isopropanol. In solutions containing 80% or more metha-

Table I—Values of i for Sodium Chloride in Various Water–Alcohol Solutions, Calculated from Concentrations Causing 25, 50, and 75% Hemolysis of Human Erythrocytes at 37° [a]

Percent (v/v) Alcohol	Percent Hemolysis			Average i
	25	50	75	
Methanol				
1	1.92	1.91	1.90	1.91
3	1.97	1.95	1.94	1.95
5	2.02	2.00	1.98	2.00
10	2.09	2.08	2.06	2.08
15	2.15	2.13	2.10	2.13
Ethanol				
1	1.90	1.91	1.92	1.91
3	1.96	1.96	1.97	1.96
5	2.02	2.02	2.02	2.02
7	2.06	2.07	2.08	2.07
10	2.13	2.12	2.12	2.12
n-Propanol				
1	1.91	1.92	1.89	1.91
3	1.97	1.99	1.95	1.97
Isopropanol				
1	1.92	1.91	1.91	1.91
3	1.95	1.97	1.95	1.96
5	2.01	2.01	1.99	2.00
7	2.02	2.01	2.00	2.01

[a] Each i value represents an average of at least two blood samples.

Table II—Critical Concentrations (Percent v/v) of Aqueous Alcohol Solutions at which Hemolysis, Complete Hemolysis, Discoloration, and/or Precipitation of Blood Components Occurred and the Effect of the Addition of Sodium Chloride to These Solutions[a]

Alcohol	Maximum Concentration Allowing No Hemolysis[b], %	Complete Hemolysis[c], %			Discoloration[d] and/or Precipitation[e], %			
	0.9% NaCl	0.9% NaCl	2% NaCl	5% NaCl	No NaCl	0.9% NaCl	2% NaCl	5% NaCl
Methanol	18	22	22.5	20	40	35	35	30
Ethanol	11	14	14	12	29	25	25	18
n-Propanol	4	6	6	5	13.5	12	10	8
Isopropanol	8	10	10	8	19	15	15	12

[a] Each value represents an average of at least two blood samples. [b] These alcohol concentrations represent the critical concentrations above which hemolysis occurred (greater than 5%) and below which there was complete protection of human red cells. [c] Essentially 100% hemolysis as indicated by hemolysis readings of 95–105% of 100% hemolysis standard. [d] Discoloration occurred which resulted in brownish-red color. [e] Turbidity occurred which resulted in brick-red precipitate after centrifuging.

nol, 60% or more ethanol, 35% or more *n*-propanol, and 45% or more isopropanol, hemagglutination occurred immediately, which resulted in a very deep red-brown clumped precipitate and a very pale greenish-yellow supernate.

Aqueous Monohydric Alcohol Solutions Containing 0.9% NaCl—Upon addition of 0.9% NaCl, hemolysis of human erythrocytes was essentially prevented (less than 5%) in solutions containing 0.0–18% methanol, 0.0–11% ethanol, 0.0–4% *n*-propanol, and 0.0–8% isopropanol. Hemolysis occurred in solutions containing 19–22% methanol, 12–14% ethanol, 5–6% *n*-propanol, and 9–10% isopropanol (Fig. 5). Red-brown solutions resulted when blood was added to saline solutions containing 35% methanol, 25% ethanol, 12% *n*-propanol, and 15% isopropanol. More pronounced discoloration and precipitation occurred in solutions containing higher concentrations of alcohols.

Values of *i* for Sodium Chloride—Through the addition of hypotonic quantities of sodium chloride (0.32, 0.34, ..., 0.46, 0.48%) to various water-alcohol solutions, it was possible to modify the fragility of human erythrocytes. When blood was added to the saline solutions containing 0.0–15% methanol, 0.0–10% ethanol, 0.0–3% *n*-propanol, and 0.0–7% isopropanol, typical sigmoid hemolysis curves resulted (*viz.*, Figs. 1–4). These curves were constructed in the manner described in the *Experimental* section. The *i* values for sodium chloride in various water-alcohol solutions were calculated using Eq. 1 and are presented in Table I.

Aqueous Monohydric Alcohol Solutions Containing 2 or 5% NaCl—The addition of 2% NaCl did not appreciably influence the hemolytic action of methanol, ethanol, *n*-propanol, or isopropanol, while 5% NaCl lowered the alcohol concentrations needed to cause 100% hemolysis (Table II).

Alcohol-Alcohol (1:1) Combinations—Quantitative hemolytic determinations were also carried out in ternary systems containing two alcohols (in 1:1 proportion) in water containing 0.9% NaCl (Table III).

DISCUSSION

The concentrations of methanol, ethanol, *n*-propanol, and isopropanol in water that are isoosmotic to 0.15 *M* NaCl and human blood, according to osmotic calculations (10)[5], are approximately 0.93, 1.33, 1.74, and 1.74%, respectively. Regardless of the alcohol content in aqueous solution, all monohydric alcohol solutions studied failed to prevent hemolysis of human erythrocytes. These experimental data point out that when water-alcohol systems are used as vehicles for intravenous solutions, the finished product should not be assumed isotonic or hypertonic with respect to blood, even when there is a high concentration of alcohol present. All alcohol solutions investigated freely penetrated human red blood cells and caused complete hemolysis and/or denaturation of human erythrocytes in solutions void of sodium chloride after 45 min at 37°.

$$[5]\text{osmotic factor} = \frac{(\text{number of particles from one molecule of solute})\ (\text{grams of solute in 100 ml of solution})}{(\text{grams} - \text{molecular weight of solute})}$$

Zanowiak and Husa (11) reported that complete hemolysis of human blood cells occurred in 7% *n*-propanol and in 10% methanol, ethanol, or isopropanol solutions even in the presence of 0.2% (0.03 *M*) NaCl; addition of 0.6% (0.1 *M*) NaCl offered complete protection to human erythrocytes from hemolysis in 10% methanol and allowed approximately 70% hemolysis in 10% ethanol and 100% hemolysis in 7% *n*-propanol. In their investigations, only the highest practical concentrations of certain monohydric alcohols were employed.

In this report, the critical concentration for an individual solvent is defined as that concentration above which hemolysis (more than 5%) is not prevented by the addition of 0.9% NaCl and below which there is complete protection of human red cells. The present investigations showed that the inclusion of 0.9% (0.15 *M*) NaCl was effective in preventing hemolysis of human erythrocytes in aqueous alcohol solutions containing up to 18% methanol, 11% ethanol, 4% *n*-propanol, and 8% isopropanol. These concen-

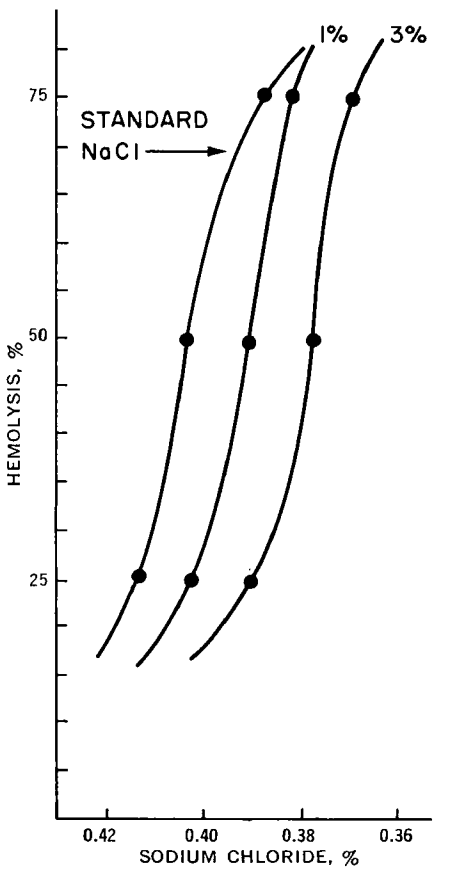

Figure 3—*Hemolysis of human erythrocytes after 45 min at 37° in various* n-*propanol solutions.*

Table III—Critical Concentrations (Percent v/v) of Aqueous Alcohol–Alcohol (1:1) Solutions Containing 0.9% Sodium Chloride Affecting Human Erythrocytes[a]

Solvent System	Critical Concentrations[b], %		Concentration Range Causing 5–100% Hemolysis, %
	Theoretical[c]	Experimental	
Methanol–ethanol (1:1)	14.5	15	16–20
Methanol–*n*-propanol (1:1)	11	7	8–12
Methanol–isopropanol (1:1)	13	12	13–16
Ethanol–*n*-propanol (1:1)	7.5	7	8–10
Ethanol–isopropanol (1:1)	9.5	10	11–13
n-Propanol–isopropanol (1:1)	6	6	7–10

[a] Each value is an average of at least two blood samples. [b] These total alcohol concentrations represent the critical concentrations above which hemolysis occurred (greater than 5%) and below which there was complete protection of human red cells. [c] These concentrations obtained by taking one-half the summation of the critical concentrations (see Table II) of the two alcohols in a particular ternary system. For example, the critical concentrations of ethanol and methanol are 11 and 18%, respectively. The theoretical critical concentration would be 29%/2 or 14.5%.

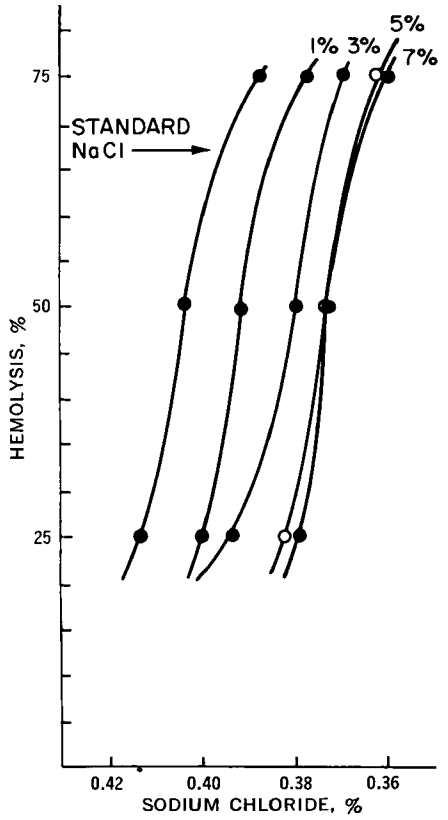

Figure 4—*Hemolysis of human erythrocytes after 45 min at 37° in various isopropanol solutions.*

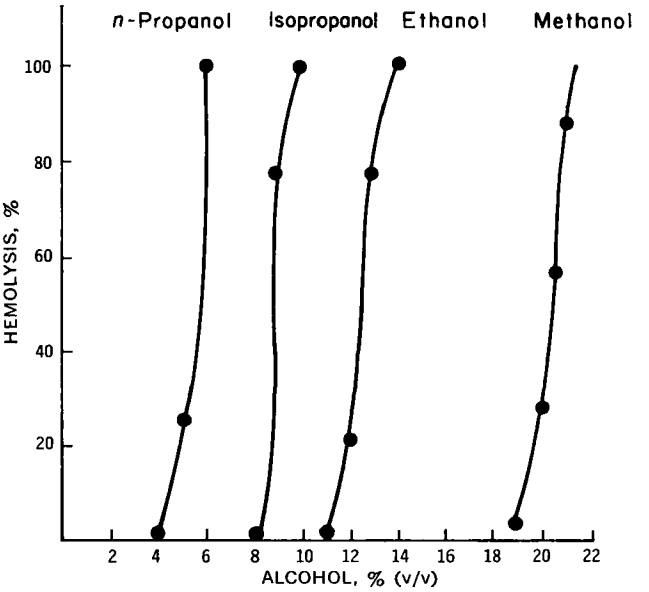

Figure 5—*Hemolysis of human erythrocytes after 45 min at 37° in various water–alcohol solutions containing 0.9% NaCl.*

trations represent the critical concentrations of the alcohols. Hemolysis in these or lower concentrations of alcohol is an osmotic phenomenon since the presence of an isotonic amount of sodium chloride in these solutions prevented hemolysis. Above these critical concentrations, a drastic increase in hemolysis occurred. As alcohol concentrations increased (Table II), further destruction of blood cells occurred resulting in brown-red discoloration and/or precipitation of blood components. The transition from nonhemolytic concentration to destructive concentrations was very abrupt, as evidenced by the hemolysis curves in Fig. 5.

The Van't Hoff factor or isotonic coefficient can be expressed as the ratio of any colligative property of a real solution to that of an ideal solution of a nonelectrolyte (12). The isotonic coefficients (*i* values) for sodium chloride in aqueous solutions of methanol, ethanol, *n*-propanol, and isopropanol were found to be in the range of 1.91–2.13 (Table I) for human blood. An *i* value greater than 1.86 would be an indication that the alcohol contributed slightly to the tonicity of the extracellular aqueous solutions. The slightly higher *i* values obtained in this study indicated that alcohols themselves offered very little protection to red blood cells against osmotic hemolysis. From these experimental *i* values, it appears that the order in which the alcohols, in the presence of sodium chloride, protect human erythrocytes against hemolysis is: methanol > ethanol > isopropanol > *n*-propanol. This is in agreement with Hober and Orakov (13) who found that the permeability of erythrocytes decreased in the order: methanol < ethanol < propanol < butanol. It would appear from these data that the ability of alcohols to contribute to the tonicity of aqueous solutions is dependent on the molecular weight and the isomeric structure present. All monohydric alcohols investigated apparently were able to penetrate the erythrocyte membrane and therefore have little or no effective concentration in the extracellular solution. Hemolytic activity increases with an increase in molecular weight of the monohydric alcohols. The isomeric alcohol was noted to possess weaker hemolytic activity than the corresponding normal alcohol.

The addition of 2% NaCl did not appreciably influence the hemolytic action of methanol, ethanol, *n*-propanol, and isopropanol at their critical concentrations, while 5% NaCl even lowered the alcoholic concentrations needed to cause 100% hemolysis (Table II). This indicated that at the critical concentration the particular alcohol was having a direct effect on the cell membrane which was not inhibited by increased tonicity; in fact, the higher salt concentration appeared to have an additional weakening effect on the membrane.

When two alcohols were combined to make an aqueous ternary solvent system, the critical concentration at which hemolysis decreased was very close to the concentration predicted by taking

one-half the summation of the critical concentrations of the two individual alcohols (Table III). The effect of the alcohols on red cells appeared to be strictly an additive one and did not depend on one of the alcohols being at or near its critical concentration.

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[x] To whom inquiries should be directed.

Influence of Cetrimonium Bromide on Base-Catalyzed Hydrolysis of *p*-Substituted Ethyl Benzoates

I. K. WINTERBORN[*], B. J. MEAKIN, and D. J. G. DAVIES[x]

Abstract □ The effect of cetrimonium bromide at a concentration above its CMC on the base-catalyzed hydrolysis of eight *p*-substituted ethyl benzoates was investigated. The *p*-substituents were chosen on the basis of their Hammett substituent constants and included nitro, cyano, acetyl, fluoro, hydrogen, methoxy, amino, and dimethylamino. Of these, the nitro and cyano esters showed an increase in rate in the presence of cetrimonium bromide whereas all the others showed a decrease in rate. Micellar rate constants were calculated from both kinetic and gel filtration data. Treatment of the results according to the Hammett relationship for the effect of *p*-substituents on the rate of aromatic side-chain reactions led to the hypothesis that the observed rate modifications are dependent not only on the *p*-substituent and the surface pH of the micelle but also on the dielectric constant at the surface of the micelle.

Keyphrases □ Cetrimonium bromide—effect on base-catalyzed hydrolysis of eight *p*-substituted ethyl benzoates, Hammett considerations □ Benzoates, ethyl, *p*-substituted—effect of cetrimonium bromide on base-catalyzed hydrolysis, Hammett considerations □ Hydrolysis, base catalyzed, *p*-substituted ethyl benzoates—effect of cetrimonium bromide, Hammett considerations

In recent years, there have been a number of investigations into the modifying effect of surfactants on the rates of organic reactions, both with regard to their influence on drug stability and as models for enzymatic and other biological reactions, and several reviews have been written on the subject (1–3). Much published work, however, is not easily interpreted and the mechanisms by which surfactants exert their influences are not fully understood.

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$$k_{obs} = k_m F_m + k_w F_w \qquad \text{(Eq. 1)}$$

where k_{obs}, k_m, and k_w are the observed, micellar, and aqueous reaction rate constants, respectively, and F_m and F_w are the fractions of the ester associated with the micelles and the aqueous phase, respectively.

The rate of the micellar reaction depends upon the site of association of the drug with the micelle. Penetration of the substrate into the hydrocarbon interior would result in protection from attack and would reduce the rate considerably. NMR (4) and UV (5) spectroscopic measurements, however, indicate that simple aromatic esters are solubilized at, or close enough to, the micellar surface for electrostatic interactions between the surface and attacking species to have an effect; therefore, the ionic nature of the surfactant is of importance. Simple electrostatic theory (6) would predict that the base-catalyzed hydrolysis of an uncharged ester will be enhanced in the presence of cationic micelles and retarded in the presence of anionic micelles; conversely, the acid-catalyzed hydrolysis will be enhanced by anionic and retarded by cationic micelles.

Contrary to this theory, it was previously reported that the presence of cetrimonium bromide (I) at concentrations above its critical micelle concentration (CMC) increases the rate of base-catalyzed hydroly-

one-half the summation of the critical concentrations of the two individual alcohols (Table III). The effect of the alcohols on red cells appeared to be strictly an additive one and did not depend on one of the alcohols being at or near its critical concentration.

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[x] To whom inquiries should be directed.

Influence of Cetrimonium Bromide on Base-Catalyzed Hydrolysis of *p*-Substituted Ethyl Benzoates

I. K. WINTERBORN[*], B. J. MEAKIN, and D. J. G. DAVIES[x]

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Contrary to this theory, it was previously reported that the presence of cetrimonium bromide (I) at concentrations above its critical micelle concentration (CMC) increases the rate of base-catalyzed hydroly-

sis of ethyl *p*-nitrobenzoate and *p*-nitrophenyl acetate but decreases the rate for *p*-aminophenyl acetate and ethyl *p*-aminobenzoate (7). This difference in the nature of the effect of I was postulated to be dependent upon the net charge on the *p*-substituent, the nitro group with a net δ^- charge causing an increase in rate and the amino group with a net δ^+ charge causing a decrease in rate. The nature of the rate modification, therefore, appeared to be dependent upon the combined inductive and mesomeric effect of the *p*-substituent on the benzene ring, electron-withdrawing groups having a negative charge and electron-releasing groups having a positive charge, influencing the orientation of the ester with respect to the micellar surface.

This report concerns the investigations into the effect of 4.8×10^{-3} *M* I, which is above the CMC under the experimental conditions used, on the base-catalyzed hydrolysis of eight *p*-substituted ethyl benzoates under identical conditions. These esters have different combined inductive and mesomeric effects as defined by their Hammett substituent constants (8).

EXPERIMENTAL

Esters—The eight esters used are listed in Table I, together with the values of their Hammett substituent constant (8). The esters that were commercially available were reagent grade; the others were synthesized using standard methods. In each case, they were further purified by two recrystallization or redistillation processes and stored under anhydrous conditions. Full details of the preparatory and purification techniques are available (9).

Surfactant—Cetrimonium bromide (cetyl trimethyl ammonium bromide) (I) was prepared and purified as previously described (7).

Buffer Solutions—Delory and King's carbonate–bicarbonate buffer at pH 9.20 was used for all kinetic investigations (10).

Water—Freshly distilled water from an all-glass still was used.

pH Measurements[1]—The pH meter and electrode systems were standardized prior to each pH determination using sodium tetraborate buffer (11).

Kinetic Investigations—All experiments were carried out using a 4×10^{-5} *M* solution of the appropriate ester at pH 9.2 and 25°, alone and in the presence of 4.8×10^{-3} *M* surfactant.

Two hundred milliliter samples of carbonate–bicarbonate buffer alone and of buffer containing 5.0×10^{-3} *M* I at pH 9.20 were prepared and equilibrated to 25°. The pH was measured and, if necessary, adjusted to pH 9.20 by the addition of up to 0.5 ml of the appropriate standard solution. To 96 ml of the solution in a 100-ml volumetric flask was added 4 ml of a 10^{-3} *M* aqueous solution of the ester. The flask was then stoppered and placed in a constant-temperature bath at 25 ± 0.1°. A sample was immediately removed for assay and at appropriate time intervals afterward.

Assay Procedures—All esters were assayed by direct spectrophotometric[2] measurement of the samples removed from the reaction flask by a method similar to that of Higuchi *et al.* (12). The addition of surfactant on occasion caused a change in the λ_{max} and extinction values, resulting in slight modifications of the basic method. In these cases, the validity of the procedure was confirmed for the assay in the presence and absence of surfactant (9).

Treatment of Results—In all cases, the hydrolysis followed first-order kinetics. The observed first-order rate constants (k_{obs}), each with its associated standard error, were obtained from the log percentage concentration–time data by means of a computerized least-squares regression analysis. Duplicate experiments were performed in all cases, and no significant differences in rates were observed (9).

[1] Performed with a Pye Dynacap pH meter fitted with a Pye Ingold 405 combined electrode and 622 thermal resistor.

[2] Unicam SP500 spectrophotometer was used for all measurements.

Table I—Effect of 4.8×10^{-3} *M* I on the Rates of Hydrolysis of *p*-Substituted Ethyl Benzoates with Different Hammett Substituent Constant Values at a Concentration of 4×10^{-5} *M* in Carbonate–Bicarbonate Buffer at pH 9.2 and 25°

p-Substituent	Hammett Substituent Constant, σ	Rate Constant in Absence of Surfactant, min^{-1}	Rate Constant in Presence of Surfactant, min^{-1}
Dimethylamino	−0.83	2.74×10^{-6}	4.98×10^{-7}
Amino	−0.66	3.41×10^{-6}	1.17×10^{-6}
Methoxy	−0.27	1.34×10^{-5}	5.96×10^{-6}
Hydrogen	0.00	4.68×10^{-5}	3.03×10^{-5}
Fluoro	0.06	7.04×10^{-5}	3.93×10^{-5}
Acetyl	0.50	2.19×10^{-4}	1.70×10^{-4}
Cyano	0.66	4.08×10^{-4}	5.11×10^{-4}
Nitro	0.78	6.02×10^{-4}	8.12×10^{-4}

Partition Coefficients (K_p)—Partition coefficients for the esters between surfactant micelles and the aqueous phase were obtained by the gel filtration technique of Herries *et al.* (9, 13). Determinations at 25 and 50° were carried out in sodium chloride solutions of the same ionic strength as the buffers used in the kinetic experiments. This technique was used because the more conventional methods of determination, such as equilibrium dialysis and solubility, cannot be used due to the lack of suitable membranes for relatively low molecular weight ionic surfactants and the instability of some esters even at more neutral pH values. This instability requires determinations to be completed within hours rather than days, and it is also the reason for carrying out

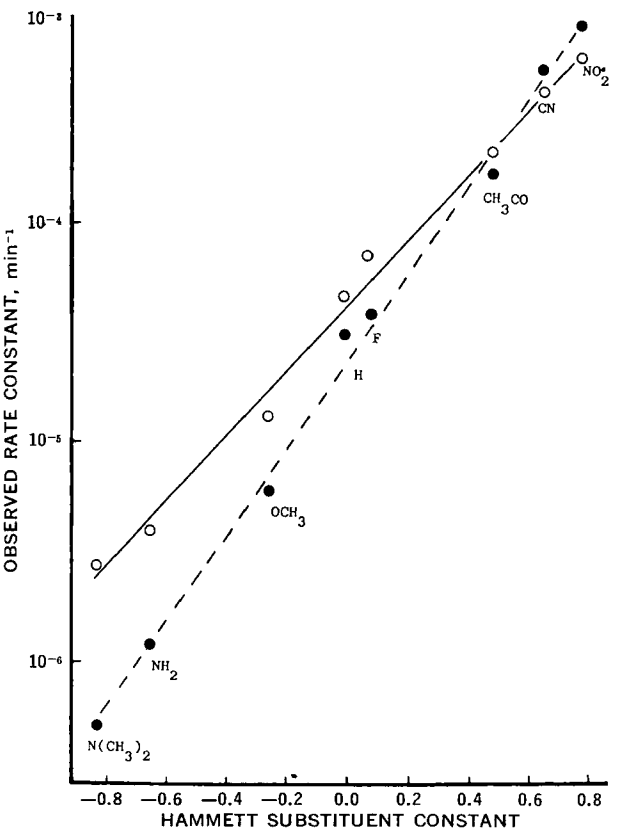

Figure 1—*Plots of the observed rate constant against Hammett substituent constant for the hydrolysis of* p*-substituted ethyl benzoates at a concentration of* 4×10^{-5} M *in carbonate–bicarbonate buffer at pH 9.2 and 25° in the absence* (○—) *and in the presence* (●– –) *of* 4.8×10^{-3} M *of I.*

Table II—Values of the Partition Coefficient, K_p, and Micellar Rate Constant, k_m, for the Different Esters Calculated from Gel Filtration Data and Corrected Values K_p' and k_m' Calculated from Kinetic Data

p-Substituent	Hammett Constant, σ	Partition Coefficient by Gel Filtration, K_p	Corrected Partition Coefficient, K_p'	Micellar Rate Constant, min^{-1}, from Gel Filtration, k_m	Corrected Micellar Rate Constant, min^{-1}, k_m'
Dimethylamino	−0.83	2268	5670	-1.07×10^{-7}	2.61×10^{-7}
Amino	−0.66	686	1715	-8.30×10^{-7}	3.81×10^{-7}
Methoxy	−0.27	1234	3085	2.29×10^{-6}	4.51×10^{-7}
Hydrogen	0.00	601	1503	1.34×10^{-5}	2.36×10^{-5}
Fluoro	0.06	691	1728	1.19×10^{-5}	2.85×10^{-5}
Acetyl	0.50	523	1308	1.13×10^{-4}	1.48×10^{-4}
Cyano	0.66	384	960	6.22×10^{-4}	5.76×10^{-4}
Nitro	0.78	666	1665	1.00×10^{-3}	8.88×10^{-4}

gel filtration in neutral salt solution. For an unionized ester, the partition coefficient should be independent of pH.

RESULTS AND DISCUSSION

The results for the hydrolysis of the esters in the absence and presence of I (Table I) are the mean of the duplicate experiments. It can be seen that the presence of I causes an increase in rate for the *p*-nitro and *p*-cyano substituents but a decrease for all other substituents studied.

The Hammett relationship for the effect of *p*-substituents on the rates of aromatic side-chain reactions, such as the hydrolysis of ethyl benzoate, may be represented by Eq. 2:

$$\log \frac{k}{k_o} = \sigma\rho \qquad \text{(Eq. 2)}$$

where k and k_o are the rate constants for the reaction of the substituted and unsubstituted compounds, respectively; σ is the Hammett substituent constant and is dependent upon the substituent but independent of the reaction conditions; and ρ is the reaction constant, which is dependent upon the reaction, the conditions under which it takes place, and the nature of the side chain undergoing reaction (14).

If this relationship is obeyed, a plot of the rate constant on a log scale *versus* the Hammett substituent constant σ should be linear with a slope equal to the reaction constant ρ. Plots of this form for the eight *p*-substituted ethyl benzoates both in the presence and absence of I are shown in Fig. 1, the calculated values of ρ being 1.98 and 1.49, respectively.

Consideration of the kinetics of micellar reactions as represented by Eq. 1 indicates that four variables (k_m, k_w, F_m, and F_w) can influence the observed rate constant. Therefore, the good linear relationships shown in Fig. 1 are perhaps somewhat surprising.

From the plot of log k_{obs} against σ in the absence of I, it is clear that k_w is directly related to the substituent constant. Values of F_m and F_w are dependent upon the partition coefficients for the

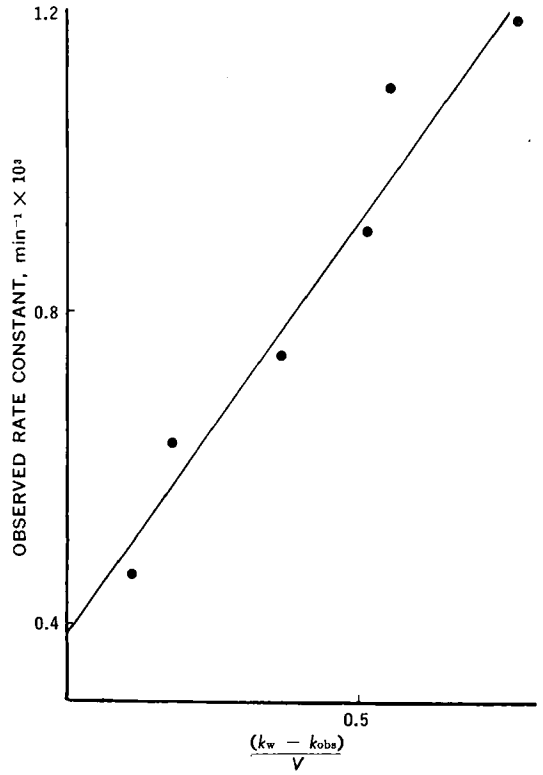

Figure 2—*Plot of* k_{obs} *against* $(k_w - k_{obs})/V$ *to determine the partition coefficient between micelles of I and the aqueous phase for ethyl* p*-aminobenzoate in glycine buffer at pH 10.39 and 50°.*

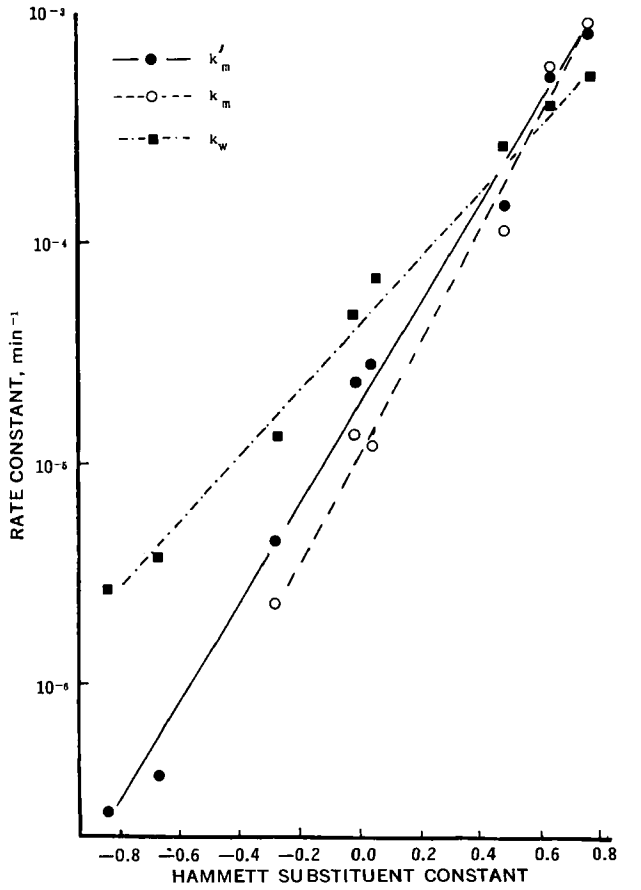

Figure 3—*Plots of aqueous and micellar rate constants against Hammett substituent constant for the hydrolysis of* p*-substituted ethyl benzoates in carbonate–bicarbonate buffer at pH 9.2 and 25°.*

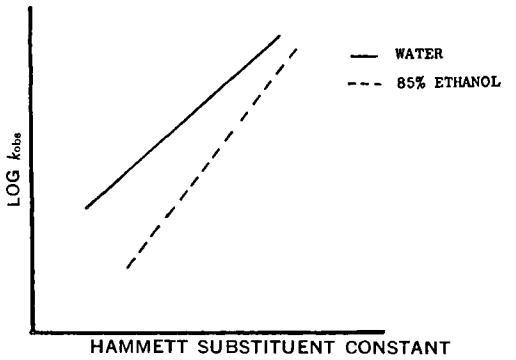

Figure 4—*Diagrammatic representation of data plotted according to the Hammett relationship to show the effect of dielectric constant on the base-catalyzed hydrolysis of* p-*substituted ethyl benzoates.*

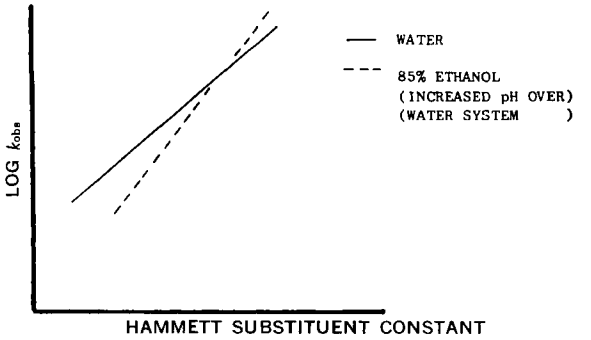

Figure 5—*Diagrammatic representation of data plotted according to the Hammett relationship to show the effect of decreased dielectric constant and increased pH on the base-catalyzed hydrolysis of* p-*substituted ethyl benzoates.*

esters between I micelles and the aqueous phase. Values of the partition coefficients, K_P's, as measured by the gel filtration technique are shown together with substituent constants in Table II. Although K_P varies with the substituent, there is no direct correlation with σ, which is indicative that the micellar rate constant, k_m, is varying for each individual ester.

The micellar rate constants may be calculated from a knowledge of K_P, k_w, k_{obs}, and V, the volume fraction of the micellar phase, by application of Eq. 3[3]:

$$k_{obs} = k_w \frac{(1-V)}{(1-V)+K_pV} + k_m \left(1 - \frac{(1-V)}{(1-V)+K_pV}\right) \quad \text{(Eq. 3)}$$

Micellar rate constants calculated from Eq. 3 are shown in Table II. The calculated rate constants for the amino and dimethylamino substituents are *negative* and cannot be accounted for by errors in the experimental rate constants. The most likely explanation of these negative rates is that the value of K_P obtained from gel filtration does not represent the true partitioning in the hydrolyzing system.

An alternative estimate of K_P can be obtained from the kinetic data by application of Eq. 4:

$$k_{obs} = \frac{(1-V)}{K_p}\left(\frac{k_w - k_{obs}}{V}\right) + k_m \quad \text{(Eq. 4)}$$

This equation may be obtained by rearrangement of Eq. 3, assuming that k_m is constant for any given ester and set of hydrolysis conditions and is independent of surfactant concentration. Since this estimate of K_P is determined from its influence on the observed rate of reaction, its value should be more appropriate to this discussion.

At low surfactant concentration, V is small and $(1 - V)$ approaches unity. A plot of k_{obs} against $(k_w - k_{obs})/V$ should be linear with a slope of $1/K_P$ and intercept k_m. The only data available for a plot of this form are those presented previously for the effect of surfactant concentration on the hydrolysis of ethyl *p*-aminobenzoate in glycine buffer of pH 10.39 at 50° (7). Figure 2 shows data plotted according to Eq. 4. The linear relationship is reasonable with a slope of 1.047×10^{-3} and an associated standard error of 1.26×10^{-4}, giving a K_P value of 956 compared with a value of 381 obtained by gel filtration at 50°. Since the K_P value obtained from the kinetic data differs markedly from that obtained by gel filtration, it appears that the latter does not reflect the binding that occurs during hydrolysis. In the absence of relevant kinetic data to calculate the partition coefficients at 25°, it was assumed that the value at 25° determined by gel filtration has the same "error" as that determined at 50° (*i.e.*, a factor of 2.5 too low), which gives a "corrected" value, K_P', for the *p*-amino substituent of 1715, giving a corrected micellar rate, k_m', of 3.81×10^{-7} min^{-1}. If it is further assumed that all of the partition coefficients determined by gel filtration have this same error and the micellar rate constants are recalculated, the values given in Table II are obtained. Figure 3 shows a plot of these corrected k_m' values together with k_w on a log scale against the substituent constant; also shown is a plot for the k_m values calculated using the K_P values from gel filtration, ignoring the negative values. The Hammett reaction constants, ρ's, for k_m and k_m' obtained from the slopes of these lines were 2.50 and 2.25, respectively.

It is apparent that the nature and magnitude of the modifying effect of I on the base-catalyzed hydrolysis of *p*-substituted ethyl benzoates are dependent upon the σ value of the *p*-substituent and, therefore, on the electron distribution in the ester as a result of the combined inductive and mesomeric effects of the substituent. Previously it was suggested that the electron distribution exerts this influence by governing the site and/or orientation of the ester with respect to the micellar surface (7). The present results favor an alternative mechanism to account for the observed rate modifications based on the values of the Hammett reaction constants.

The value of the Hammett reaction constant, ρ, is known to vary with the dielectric constant of the medium (15). For the hydroxyl-ion-catalyzed hydrolysis of *p*-substituted ethyl benzoates in various aqueous organic solvent mixtures, ρ increases with a decrease in the dielectric constant (14-16), ρ values in the region of 2.0–2.5 being reported for solvents with dielectric constants lying between 30 and 55. The increased values of the reaction constant of 1.98, 2.25, and 2.50 for k_{obs} in the presence of surfactant, k_m', and k_m, respectively, over that of 1.49 for the aqueous reaction in the absence of surfactant micelles could therefore be associated with a decrease in dielectric constant at the micellar surface.

Although no values are presently available for this surfactant (I) system, Mukerjee and Ray (17) estimated the dielectric constant at the surface of dodecylpyridinium micelles to be approximately 36 by examination of charge transfer spectra. Values of this order are not inconsistent with those postulated in the Bockris-Devanathan-Müller model of the electrical double layer, where values of 6–12 and approximately 30 were calculated for the inner and outer Helmholz layers, respectively (18, 19), the latter being the supposed site for charge transfer reactions (20). This leads to the postulation that in these systems the ester molecules are not penetrating the micelle but are adsorbed close to the micellar surface in the region of the outer Helmholz plane.

From theoretical considerations, the rate of base-catalyzed hydrolysis of *p*-substituted ethyl benzoates should decrease when the dielectric constant of the medium is decreased (21). This was confirmed by the work of Tommila and coworkers (22-24). Thus, data for the base-catalyzed hydrolysis of *p*-substituted ethyl benzoates in water and a medium of lower dielectric constant, plotted according to the Hammett relationship, will be as shown diagrammatically in Fig. 4. The rates of hydrolysis of all of the esters are decreased in the lower dielectric constant system, the magnitude of the effect increasing with the decreasing Hammett σ value. A decrease in dielectric constant cannot, therefore, account for the increase in rate observed for the *p*-nitro and *p*-cyano substituents. If the pH of the lower dielectric constant system is increased, *i.e.*, if there is a greater hydroxyl-ion concentration in the region immediately adjacent to the micellar surface than that of the aqueous system, the pattern of results shown in Fig. 5

[3] For derivation of this equation, see the *Appendix*.

could be obtained. This is similar to the experimental findings shown in Fig. 3.

From a knowledge of the micellar ζ-potential, the increased pH at the shear plane of the micelle over that in the bulk solution may be calculated by application of the Boltzmann distribution theory (25). The ζ-potential values for I are not available in the literature but, in general, values of ζ for ionic surfactants are found to be between 50 and 120 mv; *e.g.*, sodium lauryl sulfate = −101 mv in the absence of added salt and = −68 mv in the presence of 0.1 *M* sodium chloride (26) and the values for decyl-, dodecyl-, and tetradecyltrimethylammonium chlorides in the absence of added salt are 57, 75, and 118 mv, respectively (27). If a value of 50 mv for the ζ-potential of I as micelles is assumed, then the increased pH at the shear plane over that in the bulk phase will be 0.84 of a pH unit. Assuming ideal kinetic behavior, *i.e.*, the slope of a log k_{obs} *versus* pH plot is unity, the pH increase required to produce the corrected micellar rate constant of 8.88×10^{-4} min^{-1} for the *p*-nitro compound compared to the aqueous rate constant of 6.0×10^{-4} min^{-1} is 0.3 of a pH unit, leaving 0.54 of a pH unit to compensate for the reduced rate due to the lower dielectric constant at the micellar surface.

Thus, a mechanism to account for the observed effect of I on the base-catalyzed hydrolysis of *p*-substituted ethyl benzoates based on a low dielectric constant at the micellar surface combined with an increased hydroxyl-ion concentration in accordance with electrostatic theory is feasible. Although this hypothesis is founded on estimated micellar rate constants, due to the lack of accurate partition coefficients, we consider that the results available are sufficient to warrant the interpretation presented.

Further work to validate these hypotheses is now proceeding in these laboratories.

APPENDIX: DERIVATION OF EQ. 3

From Eq. 1:

$$k_{obs} = k_w F_w + k_m F_m \qquad \text{(Eq. A1)}$$

$$\therefore k_{obs} = k_w F_w + k_m(1 - F_w) \qquad \text{(Eq. A2)}$$

The partition coefficient, K_p, for the esters between cetrimonium micelles and the aqueous phase may be represented by Eq. A3:

$$K_p = \frac{C_m}{C_w} = \frac{N_m}{N_w} \cdot \frac{1-V}{V} \qquad \text{(Eq. A3)}$$

where C_m and C_w are the concentrations of the ester in the micellar and aqueous phases, respectively; N_m and N_w are the moles of substrate in the micellar and aqueous phases, respectively, for unit volume of system; and V is the volume fraction of the micellar phase.

By definition, Eq. A4 may be written:

$$F_w = \frac{1}{1 + \frac{N_m}{N_w}} \qquad \text{(Eq. A4)}$$

By combining Eqs. A3 and A4, one obtains:

$$F_w = \frac{1}{1 + \frac{K_p V}{1-V}} = \frac{(1-V)}{(1-V) + K_p V} \qquad \text{(Eq. A5)}$$

Substituting for F_w in Eq. A2 gives:

$$k_{obs} = k_w \frac{(1-V)}{(1-V) + K_p V} + k_m \left(1 - \frac{(1-V)}{(1-V) + K_p V}\right) \qquad \text{(Eq. A6)}$$

V is calculated from the concentration of micelles in g ml^{-1} and the partial specific volume of the surfactant in micellar form. The latter is obtained from density measurements (9).

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ˣ To whom inquiries should be directed.

Synthesis and Physical Properties of Substituted 4-Dimethylaminomethyl-1-phenyl-1-nonen-3-ones Possessing Antitumor Properties

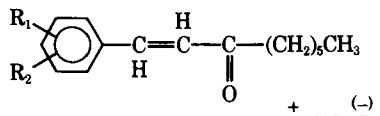
J. R. DIMMOCK[x] and W. G. TAYLOR

Abstract □ Substituted 4-dimethylaminomethyl-1-phenyl-1-nonen-3-ones were synthesized using the Mannich reaction. The spectral properties and elimination reactions of some prepared compounds were examined. Evidence is presented that the product obtained from the Mannich reaction using 1-(*p*-dimethylaminophenyl)-1-nonen-3-one underwent aromatic rather than alkyl side-chain substitution.

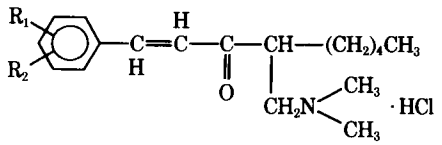
Keyphrases □ 4-Dimethylaminomethyl-1-phenyl-1-nonen-3-ones, substituted—synthesized as possible antitumor agents, physical properties, elimination reactions □ Mannich bases from styryl ketones—synthesis and physical properties of substituted 4-dimethylaminomethyl-1-phenyl-1-nonen-3-ones possessing antitumor properties □ Ketones, unsaturated—synthesis and physical properties of substituted 4-dimethylaminomethyl-1-phenyl-1-nonen-3-ones possessing antitumor properties

In studies of the synthesis of novel antineoplastic agents, it was of interest to prepare a series of nuclear-substituted styryl ketones (I) and the related Mannich bases (II), both groups being α,β-unsaturated ketones. Conjugated olefinic ketones may be regarded as alkylating agents due to their ability to undergo addition reactions with such biologically important nucleophiles as amines and thiols. Chemical studies demonstrated that Michael-type addition reactions of nucleophiles occur with α,β-unsaturated ketones (1, 2). In addition, enzyme-catalyzed alkylation of the thiol group of cysteine with α,β-unsaturated carbonyl compounds has been observed (3, 4). Furthermore, the α,β-olefinic keto group has been implicated as the principal functional group in different compounds possessing antibacterial (5) and antitumor (6–8) activity.

This investigation involved the preparation of the water-soluble Mannich bases from the precursor unsaturated ketones. Mannich bases exhibit various biological responses (9) and have shown activity against certain tumors, including the Ehrlich ascites carcinoma (10), and in the sarcoma 180 screen (11).



I: $R_1 = R_2 = H$, Cl, $N(CH_3)_2$, $\overset{+}{N}(CH_3)_3\overset{(-)}{I}$



II

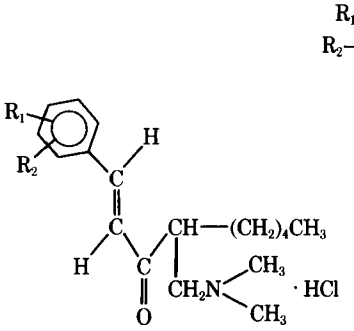
IIa: $R_1 = R_2 = H$
IIb: R_1 = 2-Cl, $R_2 = H$
IIc: R_1 = 4-Cl, $R_2 = H$
IId: R_1 = 2-Cl, R_2 = 6-Cl
IIe: R_1 = 2-Cl, R_2 = 4-Cl
IIf: R_1 = 3-Cl, R_2 = 4-Cl

RESULTS AND DISCUSSION

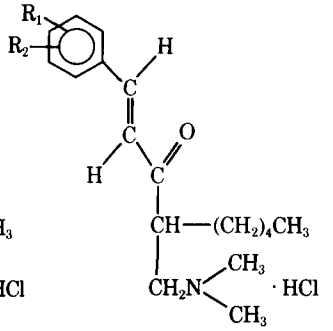
The synthesis and mass spectrometry of the unsaturated ketones (I) were described previously (12). This paper deals with the synthesis, chemical properties, and spectroscopy of the Mannich bases (II). The series of Compounds II was prepared by the Mannich reaction between the unsaturated ketone (I), paraformaldehyde, and dimethylamine hydrochloride in yields ranging from 29 to 43% (Table I). Products other than the desired Mannich base are possible (13, 14) but were not observed in this investigation. It is well known that the choice of solvent (15, 16) and the time of heating under reflux (17, 18) affect the yield of a Mannich base. Table II summarizes the various reaction conditions employed; in general, a 9-hr reflux in ethanol gave the best results.

The Mannich bases, isolated as crystalline hydrochlorides, were stable at room temperature and the structures were confirmed by elemental analysis and spectroscopy. Table III gives some spectroscopic details of these compounds. High-resolution IR spectroscopy has shown that (*E*)-benzylideneacetone (19) and related (*E*)-1-(substituted phenyl)-1-alken-3-ones (20, 21) are capable of existing as s-*cis* (IX) or s-*trans* (X) rotational isomers. The higher frequency band at approximately 1695 cm^{-1} was assigned to the s-*cis* rotational isomer, and the s-*trans*-isomer normally absorbed at 1670 cm^{-1}. In the cases of IIa and IIc, strong carbonyl stretching frequencies were observed at 1655 and 1660 cm^{-1}, respectively, and were identified as due to the s-*trans*-conformation. In addition, both compounds showed weaker absorptions at higher frequencies, denoting the presence of the s-*cis*-isomer. When an *ortho*-chlorine atom was present (IIb, IId, and IIe), only the s-*cis* rotational isomer was seen, and a chlorine atom in the *meta*-position (IIf), shown by UV studies to exert a buttressing effect, gave a spectrum in which only the s-*cis*-isomer was observed.

A strong absorption in the IR spectrum of all of the Mannich bases appeared in the 965–980-cm^{-1} range due to the carbon-hydrogen out-of-plane deformation bands associated with an olefinic bond possessing the *E*-configuration. The NMR spectra indicated doublets at δ 7.70–8.10 and 6.82–6.93 due to the protons at positions 1 and 2, respectively, with olefinic coupling of 16 Hz confirming the *E*-configuration for these compounds. The *N*-methyl protons of the hydrochloride salts resonated as a broad

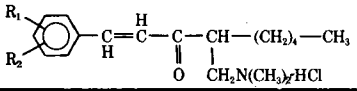


IX: s-*trans*



X: s-*cis*

Table I—Physical Properties of (*E*)-4-Dimethylaminomethyl-1-(substituted phenyl)-1-nonen-3-one Hydrochlorides



Compound	R_1	R_2	Yield, %	Melting Point	Recrystallization Solvent[a]	R_f[b]	Molecular Formula	Analysis, %	
								Calc.	Found
II*a*	H	H	43	138.5–139°	A	0.51	$C_{18}H_{28}ClNO$	C 69.77 H 9.11 N 4.52	69.85 9.38 4.70
II*b*	2-Cl	H	42	95–95.5°	B	0.53	$C_{18}H_{27}Cl_2NO$	C 62.78 H 7.91 N 4.07	62.51 8.05 3.99
II*c*	4-Cl	H	40	139.5–140°	C	0.51	$C_{18}H_{27}Cl_2NO$	C 62.78 H 7.91 N 4.07	62.68 7.95 4.14
II*d*	2-Cl	6-Cl	33	108–108.5°	D	0.52	$C_{18}H_{26}Cl_3NO$	C 57.07 H 6.92 N 3.70	57.25 7.11 3.82
II*e*	2-Cl	4-Cl	35	119–119.5°	C	0.51	$C_{18}H_{26}Cl_3NO$	C 57.07 H 6.92 N 3.70	57.28 6.97 3.67
II*f*	3-Cl	4-Cl	29	111.5°	C	0.50	$C_{18}H_{26}Cl_3NO$	C 57.07 H 6.92 N 3.70	56.84 6.89 3.64

[a] A = ethanol, B = ethyl butyrate, C = acetone, and D = ethyl acetate. [b] On silica gel plates using a mixture of *n*-butanol–acetic acid–water (12:3:5 v/v).

Table II—Reaction Conditions Employed in the Preparation of Mannich Bases II*a*–II*f* from the Corresponding Styryl Ketone, Paraformaldehyde, and Dimethylamine Hydrochloride

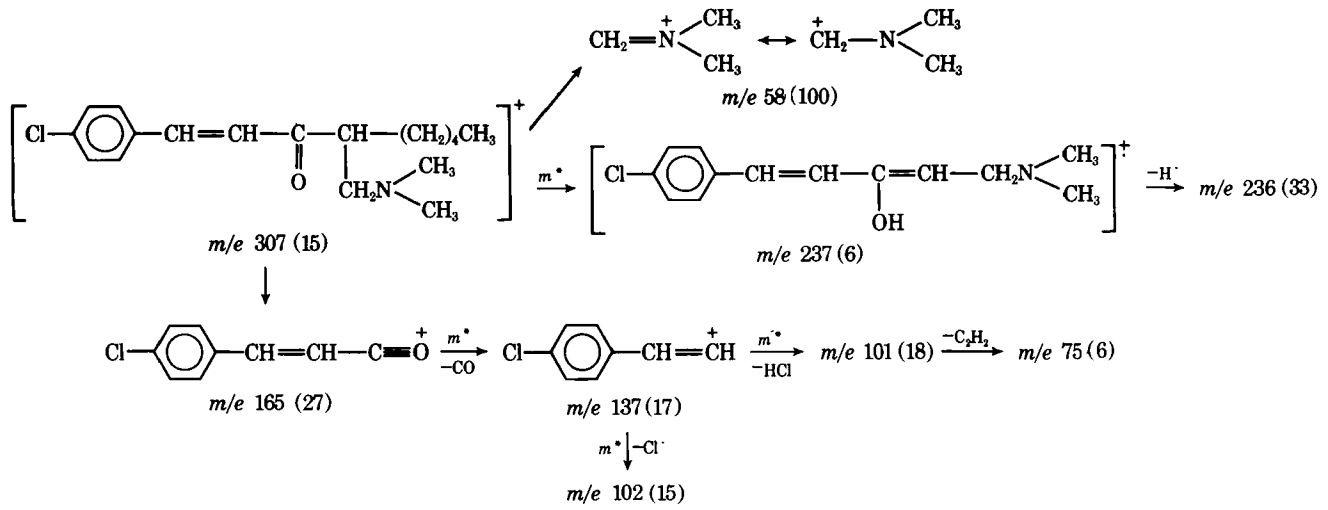
Compound	Styryl Ketone, mole	Paraformaldehyde, mole	Dimethylamine Hydrochloride, mole	Solvent[a] (Volume)	Time of Heating under Reflux, hr	Yield[b], %
II*a*	0.025	0.025	0.025	A (12.5 ml)	9	43
	0.025	0.025	0.025	A (12.5 ml)	21	38
	0.225	0.225	0.225	A (125 ml)	9	45
II*b*	0.025	0.025	0.025	A (12.5 ml)	9	42
II*c*	0.025	0.025	0.025	A (12.5 ml)	9	40
	0.025	0.025	0.025	A (12.5 ml)	21	36
	0.025	0.0375	0.0275	A (4 ml)	2.5	2.5
	0.025	0.025	0.025	I (6.5 ml)	3	13
II*d*	0.025	0.025	0.025	A (12.5 ml)	9	33
II*e*	0.025	0.025	0.025	A (12.5 ml)	9	35
	0.150	0.150	0.150	A (75 ml)	9	34
II*f*	0.025	0.025	0.025	A (12.5 ml)	9	29
	0.025	0.025	0.025	A (4 ml)	3.5	0
	0.025	0.0375[c]	0.025	A (12.5 ml)	6	10
	0.150	0.150	0.150	A (75 ml)	9	45
	0.025	0.025	0.025	I (6.5 ml)	6.5	0

[a] A = ethanol, and I = isoamyl alcohol. [b] Yields based on material obtained, melting within 2° of the analytical sample. [c] Source of formaldehyde was a 37% (w/v) aqueous solution.

Table III—Spectroscopic Properties of Compounds II*a*–II*f*

Compound	IR Spectra[a], cm^{-1}			NMR Spectra[b], δ		Mass Spectra (Relative Intensity)			UV Spectra[c], λ_{max} nm (ε)		
	C=O	C=C	H C=C H	C_1H	C_2H	$M^+ - HCl$	Aryl C_3–C_4 Cleavage Ion	Base Peak			
II*a*	1690 w 1655 s	1630 s	970 s	7.75	6.82	273 (6)	131 (13)	58	299.5 (21,720)	227.5 (8610)	
II*b*	1690 s	1615 s	975 s	8.10	6.90	307 (11)	165 (8)	58	290 (17,770)	230 (9320)	213 (11,650)
II*c*	1690 m 1660 s	1630 s	965 s	7.76	6.83	307 (15)	165 (27)	58	302.5 (24,290)	232 (8860)	228 (9210)
II*d*	1690 s	1615 s	980 s	7.77	6.92	341 (11)	199 (11)	58	273.5 (10,950)	222.5 (13,660)	
II*e*	1690 s	1615 s	975 s	8.05	6.93	341 (10)	199 (14)	58	297.5 (20,420)	235 (10,470)	218.5 (9630)
II*f*	1690 s	1625 s	975 s	7.70	6.84	341 (8)	199 (8)	58	297.5 (24,330)	237 (14,200)	232.5 (14,070)

[a] Determined as potassium bromide disks. [b] Determined in deuteriochloroform (0.5 *M*), $J_{1,2}$ = 16 Hz. [c] Solvent utilized was 95% ethanol.



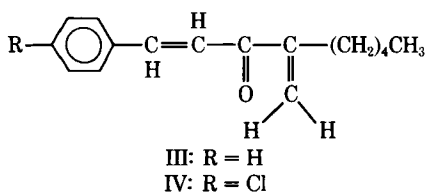
Scheme I—Mass spectral fragmentation pathway of 1-(p-chlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one (IIc)

absorption near δ 2.8. Both IIa and IId appeared as two doublets with a coupling constant of 4 Hz. The remaining Mannich-base salts showed *N*-methyl signals indicating chemical shift nonequivalence, but splitting in each of the two lines was not observed. The *N*-methyl signals of IIa as the free base appeared as a singlet at δ 2.2. Addition of deuterium oxide to the NMR probe showed the disappearance of the low field proton on the charged nitrogen atom and the appearance of the *N*-methyl signals as a singlet. This evidence suggests that coupling of the *N*-methyl protons to the proton of the quadrivalent nitrogen atom occurs, and values of 5–6 Hz for coupling between the proton of a charged nitrogen atom and the protons on the adjacent carbon atom have been observed (22).

The UV spectral data (Table III) indicate that the Mannich bases have absorption maxima in the 273.5–302.5-nm region due to $\pi \rightarrow \pi^*$ transitions of the styryl ketone chromophore (21, 23). Compounds containing an *ortho*-chlorine atom (IIb, IId, and IIe) showed a reduction in the molar absorptivity at the absorption maxima, which is associated with steric hindrance of the aromatic ring with the unsaturated ketone group (24–26). When a *meta*-chlorine atom is present (IIf), the molar absorptivity indicates the existence of a buttressing effect.

The base peak in the mass spectra of all of the Mannich bases was an ion at *m/e* 58, considered to be a reasonance-stabilized species (Scheme I). This ion may be formed by a homolytic bond cleavage process beta to the ion radical site on the nitrogen atom, since this β-cleavage process is known to give prominent peaks in the mass spectra of *N*-alkyl amines (27). The fragmentation patterns[1] for the *para*-chloro Mannich base (IIc) are given in Scheme I. The figures in parentheses after the *m/e* values indicate the relative intensity of the peak compared to the base peak at *m/e* 58 designated 100.

Scheme I shows an ion at *m/e* 237, considered to arise by a McLafferty rearrangement of the γ-hydrogen atom to the carbonyl oxygen. This McLafferty-rearrangement product may lose a hydrogen atom to give the peak at *m/e* 236. An α-cleavage product at *m/e* 165 decomposed with the loss of 28 atomic mass units, corresponding to a loss of carbon monoxide, to give an ion at *m/e* 137 designated as a vinylic cation. This ion at *m/e* 137 lost chlorine and hydrogen chloride ions to give peaks at *m/e* 102 and 101, respectively. It was considered that the ion at *m/e* 101 could lose acetylene to give the ion at *m/e* 75.



III: R = H
IV: R = Cl

In the mass spectra of 1-(*o*-chlorophenyl)-1-nonen-3-ones, a prominent ion corresponding to the loss of chlorine from the molecular ion was observed (12). These peaks were not noted with IIb, IId, and IIe. In addition, the McLafferty-rearrangement products of these compounds did not lose a chlorine atom. It is possible that the loss of *ortho*-chlorine atoms in these Mannich bases may be suppressed due to the favorable fragmentation pathway leading to the ion at *m/e* 58.

TLC showed a mixture of two compounds when an aqueous solution of IIa, as the hydrochloride salt, was neutralized with aqueous sodium hydroxide solution. Resolution of the mixture by column chromatography indicated the presence of IIa as the free base and of the dienone III. The identity of III was elucidated mainly by NMR spectroscopy.

Storage of IIa as the free base at room temperature or by heating the free base at 120° facilitated the formation of III. TLC also indicated that IV was formed when a pure sample of IIc was neutralized with weak bases such as sodium carbonate, ammonium hydroxide, or triethylamine. This elimination mechanism of Mannich bases may partly explain the biological activity of certain members of this class of compounds. The dialkylamino portion could assist transportation of the drug molecule to a site of action and liberate the corresponding unsaturated ketone (28). Furthermore, exchange reactions of Mannich bases with nucleophilic reagents by an elimination–addition mechanism to give sulfides (29) may have possible significance *in vivo*.

It was decided, therefore, to synthesize the eliminated product IV and to compare its antitumor properties to that of IIc. Quaternization of IIc (free base) with methyl iodide gave the corresponding quaternary ammonium compound, which underwent the Hofmann elimination reaction to give IV. A yield of 35% was obtained when benzene–water was used as the solvent mixture, whereas a 75% yield of IV resulted when aqueous alkali alone was employed. The dienone IV was identified mainly by NMR spectroscopy, and the major fragmentation pathways[2] in the mass spectrum are shown in Scheme II.

In contradistinction to the mass spectral fragmentation pathway of the Mannich bases IIa–IIf, an M − 1 peak in the spectrum of IV was observed. In the mass spectra of the unsaturated ketones I, it was shown that, with the exception of the 2,6-dichloro compound, an *ortho*-substituent was lost followed by ring closure to give the benzopyrylium ion (12). The structure of the M − 1 peak at *m/e* 261 is considered to be a substituted benzopyrylium ion. The McLafferty-rearrangement product at *m/e* 192, like the corresponding ion from IIc, had low relative intensity in comparison to the McLafferty-rearrangement ion from 1-(*p*-chlorophenyl)-1-nonen-3-one (V), which had a relative intensity of 42%. Substi-

[1] A complete listing of the mass spectral peaks for Compounds IIa–IIf is available from the authors.

[2] A complete listing of the mass spectral peaks for IV is available from the authors.

tution in the alkyl chain at the α-carbon atom to the carbonyl group, therefore, suppresses the McLafferty-rearrangement process. The α-cleavage ion at m/e 165 in IV, like V, represented the base peak. Decomposition of the base peak into several fragments, confirmed by the appropriate metastable peaks, is shown in Scheme II. In addition, ions at m/e 233 (12), 219 (79), and 205 (16) probably result from M − C_2H_5, M − C_3H_7, and M − C_4H_9 cleavage processes with charge retention on the aromatic fragment.

In an attempt to convert the unsaturated ketone VI to the corresponding Mannich base VII, a yellow solid with a sharp melting point was isolated in low yield. Elemental analysis was in agreement with the proposed structure of VII as the free base, and the mass spectrum gave a prominent peak (60% intensity) at m/e 316 corresponding to the molecular weight of VII. However, NMR spectroscopy showed that in both VI and the product there was a triplet (J = 6.5 Hz) integrating for two protons at δ 2.50 and 2.80, respectively, assigned to the methylene protons at the C-4 position. This would indicate that aminomethylation had not occurred at the predicted position of the alkyl side chain. Sharp *N*-methyl singlets, each integrating for six protons, appeared at δ 2.23 and 3.00. In the δ 6.4–8.0 region, five protons were present; but if the isolated product had Structure VII, then six protons would be expected to appear in this region due to the four aromatic and two olefinic hydrogen atoms. The dimethylaminomethyl group was substituted into the aromatic ring, and Structure VIII was assigned to the product (Scheme III). It was felt that the electron-withdrawing unsaturated keto group would favor substitution in the *meta*-position of the aromatic ring similar to the observation that a Mannich reaction using *p*-hydroxyacetophenone gave substitution of the dialkylaminomethyl group in the *meta*-position of the aromatic ring in contradistinction to substitution of this group in the side chain (30). In addition, the *p*-dimethylamino group would enhance substitution at this position in the aromatic ring.

EXPERIMENTAL[3]

Aminomethylations of (*E*)-1-(Substituted phenyl)-1-nonen-3-ones—The compounds (Table I) were prepared by the following general method. The appropriate α,β-unsaturated ketone (12) (0.025 mole), paraformaldehyde (0.025 mole), and dimethylamine hydrochloride (0.025 mole) in absolute ethanol (12.5 ml) were heated to reflux temperature. Concentrated hydrochloric acid was added until pH 3 was reached, and the mixture was then heated under reflux for 9 hr. The solvent was then removed *in vacuo*, and the residue was dissolved in water (200 ml) and extracted with ether. The ether extracts were washed with water, and the combined aqueous extracts were cooled, basified with aqueous sodium hydroxide solution, and extracted with benzene. Removal of the solvent gave the free Mannich base as a yellow oil. The free base was dissolved in anhydrous ether, the pH was adjusted to 4 with ethanolic hydrochloric acid, and the resultant mixture was cooled to −10°. The precipitate was collected, dried, and recrystallized to yield the appropriate (*E*)-4-dimethylaminomethyl-1-(substituted phenyl)-1-nonen-3-one hydrochloride as colorless prisms (Table I). The effect of yield obtained by varying the experimental conditions is indicated in Table II.

Chromatography of (*E*)-4-Dimethylaminomethyl-1-phenyl-1-nonen-3-one—Neutralization of an aqueous solution of (*E*)-4-dimethylaminomethyl-1-phenyl-1-nonen-3-one hydrochloride, using the general synthetic procedure described earlier, gave an oil which was examined by TLC on silica gel using benzene–acetic acid–95% ethanol (10:1:1 v/v). Two products were present, R_f 0.86 and 0.11. The less mobile major component was identified as (*E*)-4-dimethylaminomethyl-1-phenyl-1-nonen-3-one. IR (film): 2780 s (N—CH_3), 1690 s (C=O), 1660 s (C=O), 1610 s (C=C), 1580 s (aromatic C=C), 1495 m (aromatic C=C), and 980 s [(*E*) HC=CH] cm^{-1}; NMR ($CDCl_3$): δ 7.50 (m, 6, C_1H, C_6H_5), 6.80 (d, 1, $J_{2,1}$ = 16 Hz, C_2H), 3.27–2.00 [m, 9, C_4H, $CH_2N(CH_3)_2$], 1.77–1.02 [m, 8, $(CH_2)_4$], and 0.83 (m, 3, C_9H_3) ppm.

After 3 months at room temperature, 0.25 g of the mixture was chromatographed on silica gel using a 2 × 20-cm column. Elution with petroleum ether (50 ml) and benzene (100 ml) gave a yellow syrup (0.09 g), which was shown by GLC to be 95% of one component and had a R_f value of 0.86 using the TLC conditions described previously. Nitrogen was absent (Lassaigne's test), and spectroscopy indicated the syrup to be essentially (*E*)-4-methylene-1-phenyl-1-nonen-3-one (III). IR (film): N—CH_3 absent, 1670 s (C=O), 1615 s (C=C), 1580 s (aromatic C=C), 1500 m (aromatic C=C), and 980 s [(*E*) CH=CH] cm^{-1}; NMR (carbon tetrachloride): δ 7.33 (m, 7, C_1H, C_2H and C_6H_5), 5.90 (s, 1, C_4CH), 5.64 (s, 1, C_4CH), 2.30 (t, 2, J = 6 Hz, C_5H_2), 1.70–1.07 [m, 6, $(CH_2)_3$], and 0.90 (m, 3, C_9H_3) ppm. A further elution of the column with chloroform (50 ml), acetone–chloroform mixtures (100 ml), and acetone (400 ml) gave an oil (0.1 g), shown by TLC and NMR to be a mixture of IIa and III. The mixture was heated at 120° for 2 hr, and the NMR spectrum of the product showed strong singlets at 5.90 and 5.64 ppm and very small absorptions for C_1H, C_2H, and $N(CH_3)_2$ of the Mannich base IIa.

GLC of the Mannich base and the hydrochloride salt indicated three peaks. TLC of the hydrochloride salt on silica gel, using *n*-butanol–acetic acid–water (12:3:5 v/v) as the developing system, gave one spot (Table I). With the benzene–acetic acid–95% ethanol mixture (10:1:1 v/v), the hydrochloride salt remained at the point of application and no spots appeared for a compound with an R_f value of 0.86.

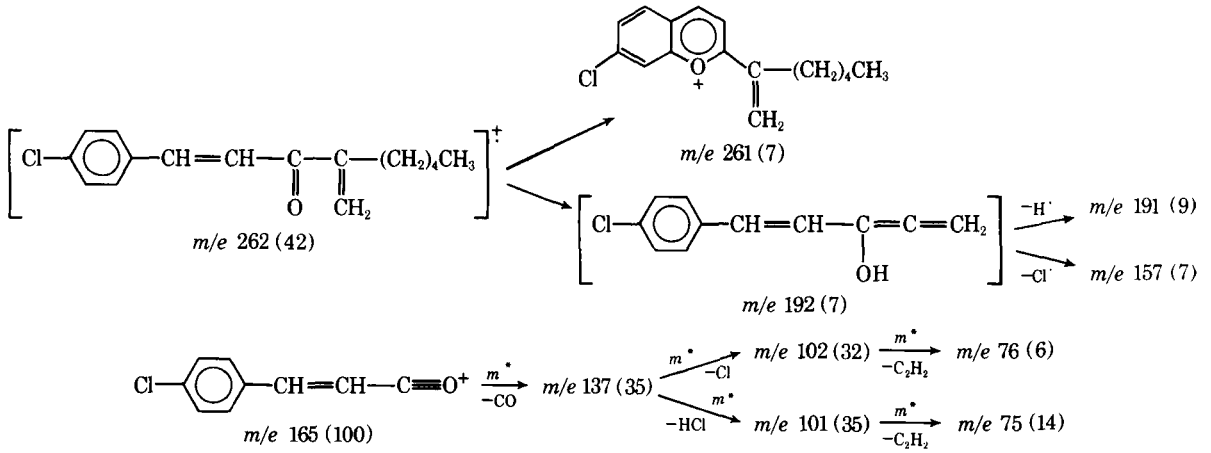
(*E*)-1-(*p*-Chlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one Methiodide—(*E*)-1-(*p*-Chlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one (8.37 g, 0.027 mole), obtained from the hydrochloride by basifying with aqueous sodium hydroxide solution at 0° and extracting with benzene, was dissolved in methyl iodide (7.72 g, 0.054 mole) and ethanol (25 ml) and heated under reflux for 20 hr. The crystals deposited at −10° were recrystallized from ethanol to give the required methiodide (8.43 g, 60%), mp 170° dec., as colorless prisms; IR (KBr): 1690 s (C=O), 1660 s (C=O), 1615 s (C=C), and 970 s (HC=CH) cm^{-1}; NMR ($CDCl_3$, 0.5 *M*): δ 7.87 (m, 3, C_1H and aromatic H), 7.35 (m, 3, C_2H and aromatic H), 4.37–3.93 (m, 2, CH_2N^+), 3.83–3.17 [broad s, 10, C_4H and $^+N(CH_3)_3$], 2.00–1.05 [m, 8, $(CH_2)_4$], and 0.83 (m, 3, C_9H_3) ppm.

Anal.—Calc. for $C_{19}H_{29}ClINO$: C, 50.73; H, 6.50; I, 28.22; N, 3.11. Found: C, 50.89; H, 6.68; I, 28.10; N, 3.17.

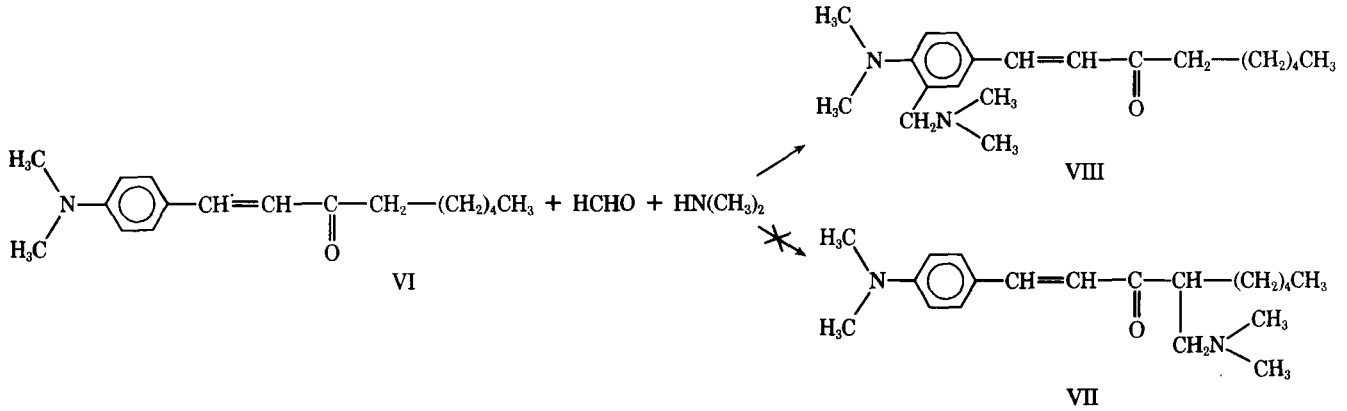
(*E*)-1-(*p*-Chlorophenyl)-4-methylene-1-nonen-3-one (IV)—A mixture of (*E*)-1-(*p*-chlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one methiodide (1.31 g, 0.003 mole), sodium hydroxide (0.30 g, 0.0075 mole), benzene (7 ml), and water (25 ml) was heated under reflux for 1.5 hr. On cooling, the pH of the aqueous phase was adjusted to 6 and the benzene extract was separated. The aqueous solution was extracted with benzene, and the combined organic extracts were removed to give a solid. This solid was recrystallized from ethanol to give the dienone IV (0.271 g, 35%) as off-white prisms, mp 64–65°; UV_{max} (95% ethanol): 228 (ϵ 12,560) and 308 (ϵ 20,970) nm; IR (KBr): 1660 s (C=O), 1600 s (C=C), and 985 s (HC=CH) cm^{-1}; NMR (carbon tetrachloride, 0.5 *M*): δ 7.30 (m, 6, C_1H, C_2H, and aromatic H), 5.90 (s, 1, C_4CH), 5.67 (s, 1, C_4CH), 2.33 (t, 2, J = 6 Hz, C_5H_2), 1.73–1.10 [m, 6, $(CH_2)_3$], and 0.87 (m, 3, C_9H_3) ppm; mass spectrum: 262 (M^+,

[3] Melting points were determined on a Gallenkamp MF-370 apparatus and are uncorrected. The NMR spectra were determined using a Varian T-60 spectrometer with tetramethylsilane as the internal standard. IR absorption spectra were recorded on a Unicam SP-200G spectrophotometer previously calibrated with polystyrene. Band intensities are denoted as s (strong), m (medium), and w (weak). Mass spectra were determined at 70 ev on an AE1 MS-12 single-focusing mass spectrometer operated by Mr. D. Bain, Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. The instrument used a heated inlet system operating near the melting point of the compound, and samples were introduced by the direct probe technique. UV spectra were recorded by Mr. N. Johnson, College of Pharmacy, University of Saskatchewan, Saskatoon, using a Cary model 14 spectrophotometer with 95% ethanol and 1-cm quartz cells. Elemental analyses were performed by Dr. F. B. Strauss, Microanalytical Laboratories, Oxford, England, and by Mr. R. M. Smith, College of Pharmacy, University of Saskatchewan, Saskatoon, using a Coleman model 33 carbon–hydrogen analyzer.

TLC plates, 0.5 mm in thickness, were prepared using silica gel G (E. Merck and Co.) and subsequently heated at 120° for 2 hr prior to use. The chromatograms were developed (20–120 min) and the compounds were detected with a spray composed of a 1% (w/v) aqueous solution of potassium permanganate containing 1% sulfuric acid. Column chromatography was performed using neutral silica gel powder (SilicAR CC-7, 200–325 mesh, Mallinckrodt Chemical Works). GLC was undertaken on a Pye 104 model 64 instrument equipped with flame-ionization detector, using nitrogen as the carrier gas (flow rate of 50 cm^3/min). A 1.52 m (5 ft) × 0.63 cm (0.25 in.) o.d. glass column, containing 4% SE-30 absorbed onto acid-washed and silanized Chromosorb W, 100–200 mesh, was used. The isothermal operating temperature was 220° while the detector temperature was held at 25–30° above the oven temperature during the operation. Organic extracts were washed with water and dried over anhydrous magnesium sulfate.



Scheme II—Mass spectral fragmentation pathway of 1-(p-chlorophenyl)-4-methylene-1-nonen-3-one (IV)



Scheme III—Mannich reaction using 1-(p-dimethylaminophenyl)-1-nonen-3-one

relative intensity 42%).

Anal.—Calc. for $C_{16}H_{19}ClO$: C, 73.13; H, 7.29. Found: C, 73.12; H, 7.10.

In a separate synthesis, a mixture of the methiodide (4.498 g, 0.010 mole), sodium hydroxide (2.80 g, 0.070 mole), and water (70 ml) was heated for 0.5 hr at 70°. Hydrochloric acid was added until the pH was 6, and extraction with benzene afforded a tan solid (2.26 g) which, on recrystallization, gave the pure dienone (1.965 g, 75%).

Mannich Reaction between (*E*)-1-(*p*-Dimethylaminophenyl)-1-nonen-3-one, Paraformaldehyde, and Dimethylamine Hydrochloride—A mixture of (*E*)-1-(*p*-dimethylaminophenyl)-1-nonen-3-one (6.483 g, 0.025 mole), paraformaldehyde (0.751 g, 0.025 mole), dimethylamine hydrochloride (2.039 g, 0.025 mole), and alcohol (25 ml) was heated to reflux temperature, the pH was adjusted to 4, and the mixture was heated under reflux for 9 hr. On cooling, the mixture was stirred at room temperature overnight; removal of the solvent gave a yellow residue which was extracted with benzene. The benzene extract contained unreacted ketone (0.82 g) from melting-point and mixed melting-point evidence. The aqueous extract was cooled in an ice bath, basified with aqueous sodium hydroxide solution, and extracted with benzene to give a yellow solid. TLC on silica gel [*n*-butanol–acetic acid–water (12:3:5 v/v)] suggested two components, R_f 0.50 and 0.84. Recrystallization of the solid from alcohol gave the material with the R_f value of 0.50 (0.14 g, 1.8%). A further recrystallization gave fluffy, yellow needles (0.024 g) identified as 1-(*p*-dimethylamino-*m*-dimethylaminomethylphenyl)-1-nonen-3-one, mp 71.5–72°; IR (KBr): 1650 m (C=O) and 1595 s (C=C) cm^{-1}; NMR ($CDCl_3$): 7.87–7.37 (m, 3), 6.87–6.47 (m, 2), 3.33 (s, 2), 3.00 [s, 6, $N(CH_3)_2$], 2.80 (t, 2, J = 6 Hz), 2.23 [s, 6, $N(CH_3)_2$], and 0.90 (m, 3, C_9H_3) ppm; mass spectrum: 316 (M^+, 60), 134 (100), and 58 (75).

Anal.—Calc. for $C_{20}H_{32}N_2O$: C, 75.59; H, 10.18; N, 8.84. Found: C, 75.52; H, 9.93; N, 8.59.

Attempts to form a crystalline hydrochloride were unsuccessful. Experiments designed to prepare the desired Mannich base (VII) using isoamyl alcohol, acetic acid, or dimethyl sulfoxide as the solvent gave black tars which were not examined.

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Hydrolysis of Steroid Oximes: Mechanism and Products

RICHARD E. HUETTEMANN and ARVIN P. SHROFF[x]

Abstract □ The conversion of 17α-acetoxy-6α-methyl-4-pregnen-3,20-dione 3-oxime to the corresponding diketone in acidic media was found to be a first-order reaction at 37°. The effects of incorporating ester groupings at the oxime function or at the C-17 position and modifications in ring B were also investigated. Only the length of the ester chain at the oxime function had a profound effect on the rate constant. From these observations, it is proposed that two competing mechanisms of hydrolysis are involved for the oxime esters.

Keyphrases □ Steroid oximes—hydrolysis mechanisms and products, effects of ester substituents □ 17α-Acetoxy-6α-methyl-4-pregnen-3,20-dione 3-oxime—conversion to diketone, effects of ester substituent at oxime function and C-17 position, modifications in ring B, mechanisms of hydrolysis □ Oximes, steroid—hydrolysis mechanisms and products, effects of ester substituent and ring B modifications □ Hydrolysis—steroid oximes, mechanisms and products

Buhler *et al.* (1) reported that the acid hydrolysis of 11β-hydroxy-11α-methyl-5β-pregnane-3,20-dione dioxime proceeds rapidly to the corresponding 3,20-diketone by the stepwise mechanism shown in Scheme I. The rate of hydrolysis for the 3-oxime could not be measured, but they reported the rate to be 5.5×10^{-4} sec^{-1} for the 20-oxime. The half-life for the overall hydrolysis (Scheme I) was about 21 min in gastric juice.

Several compounds with the 3-oximino function were synthesized in this laboratory (Table I) and reported (2) to be potent progestational agents. The observation of Buhler *et al.* (1) suggested that these oximes might be hydrolyzed rapidly in gastric fluid and that the resulting 3-keto compounds would be responsible for the observed biological activity. Therefore, a study was initiated to measure the kinetic rate of 3-oximino hydrolysis at pH 1.5 and 37° and to determine if other modifications at the oxime function or around the steroid nucleus affected the rate of hydrolysis.

EXPERIMENTAL[1]

Apparatus—A constant-temperature water bath and a spectrophotometer[2] were used.

Buffers—A pH 1.5 buffer, prepared according to USP XVIII (3), was used.

Procedure for Hydrolysis—A stock solution of each compound (Table I) at a concentration of 300 μg/ml was prepared in purified dioxane. A 30-ml aliquot of this solution was further diluted with 20 ml of buffer solution, and then a 5-ml aliquot was placed in each of three 10-ml glass ampuls. The ampuls were sealed and placed in a constant-temperature water bath maintained at 37°. At specified time intervals, the ampuls were removed and each was extracted separately with methylene chloride. The organic layers were transferred to another vial and evaporated, and the residues were saved for their respective assays. The first ampul residue was assayed by the oxime colorimetric method, and the second ampul residue was assayed by the carbonyl colorimetric method. The residue from the third ampul was used for TLC.

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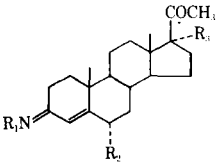
Table I—Oximino Steroids Studied at pH 1.5 and 37°



Compound	R_1	R_2	R_3
1	OH	CH_3	$OCOCH_3$
2	CH_3COO	CH_3	$OCOCH_3$
3	C_2H_5COO	CH_3	$OCOCH_3$
4	$CH_3(CH_2)_4COO$	CH_3	$OCOCH_3$
5	OH	CH_3	$OCOCH_3$ (6,7 unsaturated)
6	OH	H	$OCOCH_3$ (6,7 unsaturated)
7	OH	CH_3	$OCOC_2H_5$

fate and 200 mg of sodium carbonate into a 1000-ml volumetric flask, and dilute to volume with distilled water.

N-1-Naphthylethylenediamine Dihydrochloride Solution—Place 100 mg of *N*-1-naphthylethylenediamine dihydrochloride into a 100-ml volumetric flask, and dilute to volume with distilled water. This solution should be prepared fresh daily.

Sodium Acetate Solution—Transfer 50 g of sodium acetate into a 100-ml volumetric flask, and add approximately 80 ml of distilled water. Heat to effect solution, and then dilute to volume with distilled water.

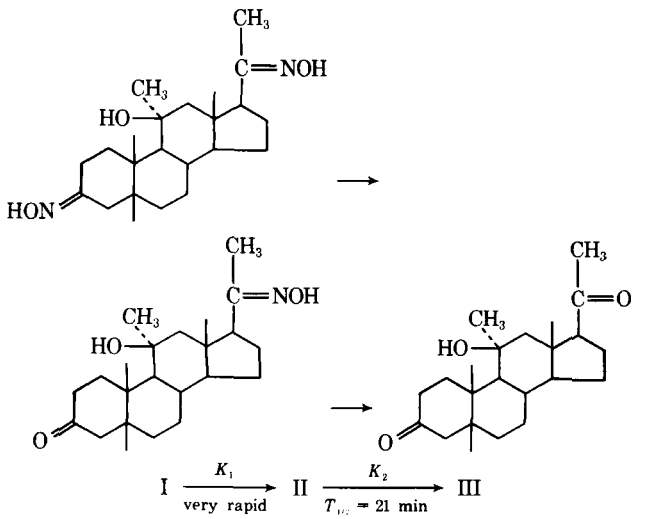
Assay—Evaporate 3.0 ml of the methylene chloride stock solution of a steroid oxime being studied (standard). To both this residue and the residue from the hydrolysis experiment, add 1.0 ml of ethanol and 2.0 ml of 2% aqueous sulfuric acid. Heat both vials at 85° for 30 min and allow them to come to room temperature. To each vial, add 2.5 ml of sulfanilamide solution, 2.5 ml of iodine solution, 5.0 ml of sodium acetate solution, 2.5 ml of sodium thiosulfate solution, and 5.0 ml of *N*-1-naphthylethylenediamine dihydrochloride solution. (*Note:* Stopper and shake the vial after the addition of each solution.) Quantitatively transfer the contents of each vial into 100-ml volumetric flasks, and dilute to volume with distilled water. Determine the absorbance of the standard and the sample at 505 nm, using water as the reference, and calculate the amount of oxime in the sample.

Carbonyl Colorimetric Assay Method—*Isoniazid Solution*—Transfer 500 mg of isoniazid into a 1-liter volumetric flask. Add 800 ml of methanol and 0.63 ml of concentrated hydrochloric acid. Swirl to dissolve, and dilute to volume with methanol.

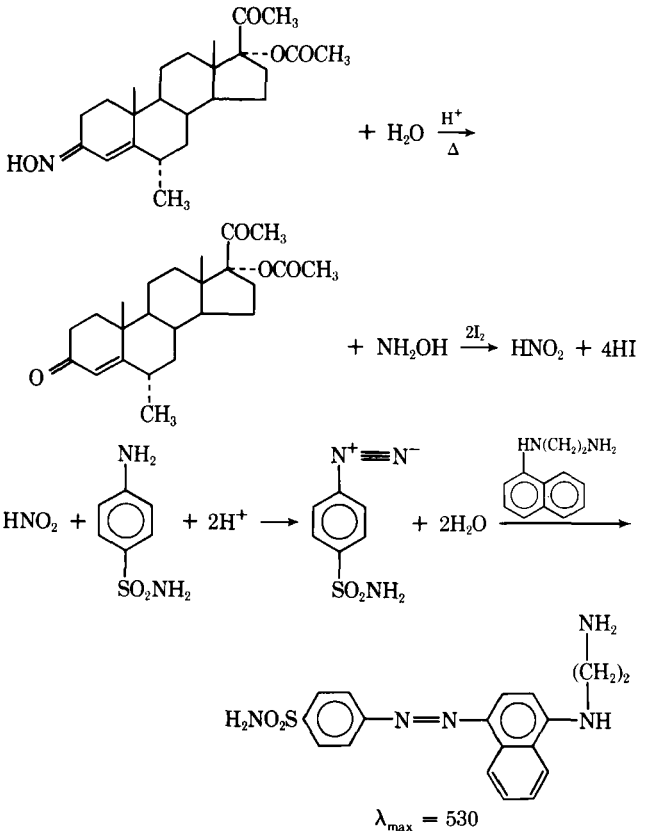
3-Ketosteroid Standard Solution—Weigh 100 mg of the appropriate 3-ketosteroid (compounds listed in Table I) into a 100-ml volumetric flask, and dilute to volume with methanol.

Assay—Transfer 1.0 ml of the appropriate 3-ketosteroid standard solution into a vial, and evaporate to dryness. To this residue and to the residue obtained from the hydrolysis experiment, add 25 ml of isoniazid solution. Heat both vials at 60° for 5 min, and then allow them to come to room temperature. Determine the absorbance of the standard and the sample at 370 nm *versus* reagent blank, and calculate the amount of ketosteroid in the sample.

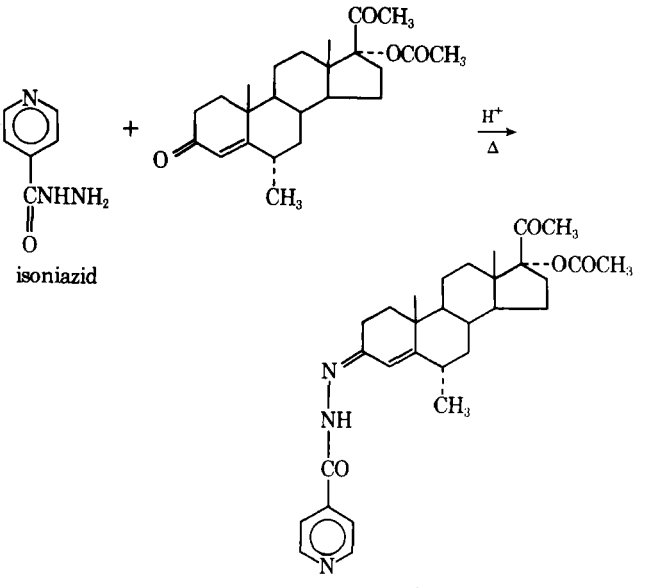
TLC Procedure—The steroid residue from the hydrolysis experiment was dissolved in 1.0 ml of methylene chloride, and a 5.0–10.0-μl sample was spotted on a 20 × 20-cm TLC plate[3], 250



Scheme I—Stepwise hydrolysis of 11β-hydroxy-11α-methyl-5β-pregnane-3,20-dione dioxime



Scheme II—Mechanism for the oxime chromophore formation



Scheme III—Mechanism for the carbonyl chromophore formation

[3] Analtech, Wilmington, Del.

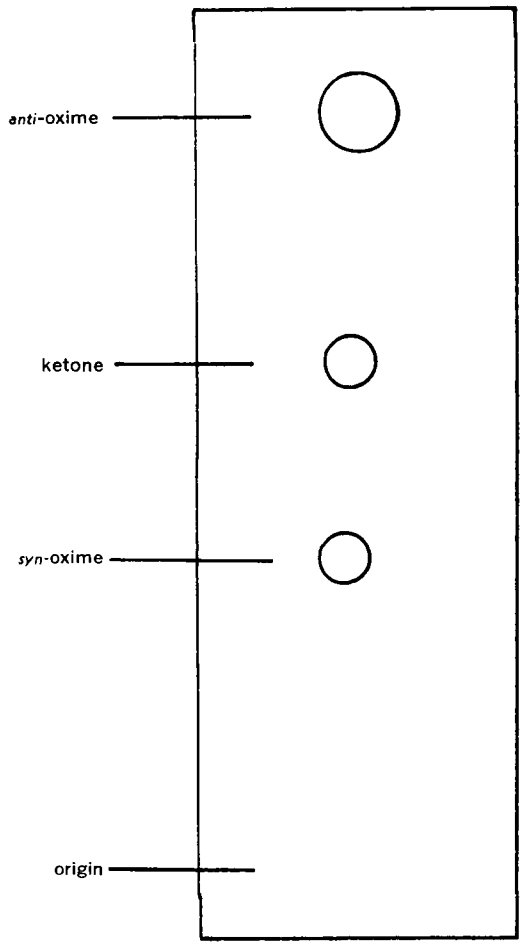
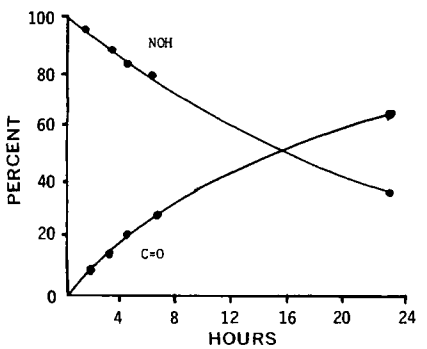


Figure 1—*Thin-layer chromatogram depicting the separation of oxime from its corresponding ketone.*

μm thick. Chromatograms were developed in 7 × 22 × 22-cm glass tanks containing 200 ml of hexane–ethyl acetate (1:1). After development the plates were air dried, sprayed with 5% methanolic sulfuric acid, and heated in an oven at 100° for 15 min. The plates were then examined under UV light (366 nm) for qualitative identification of various steroids. Reference steroids were used wherever necessary.

DISCUSSION

The sensitivity of the oxime colorimetric method (10 μg/ml), the carbonyl colorimetric method (10 μg/ml), and the TLC procedure was satisfactory for detection of minor changes during hydrolysis. The mechanism of chromophore formation and separation on TLC is shown in Schemes II and III and Fig. 1. Both chromophores were stable for at least 3 hr.



Hours	NOH, %	C=O, %
0	100	0
1.5	96	8
3	88	13
4	84	21
6	80	28
23	37	64

Figure 2—*Plot of time* versus *oxime and ketone concentrations for hydrolysis of Compound 1.*

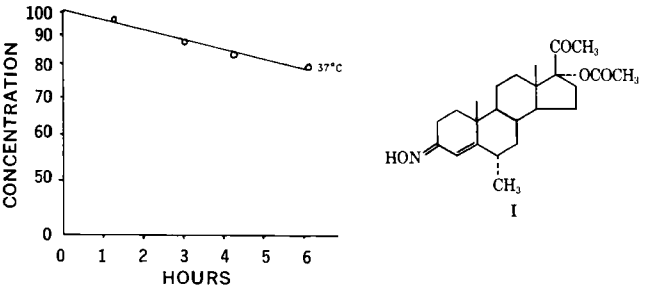


Figure 3—*Typical first-order plot obtained for Compound 1 at 37°.*

Initially, the kinetics of hydrolysis at pH 1.5 and 37° was investigated for 17α-acetoxy-6α-methylpregn-4-en-3,20-dione 3-oxime (Compound 1 of Table I). The rate was monitored quantitatively by TLC. The data are graphically depicted in Fig. 2. Spectroscopic identification of the hydrolyzed product, isolated by TLC, revealed it to be the 3-ketone, 17α-acetoxy-6α-methylpregn-4-en-3,20-dione. The first-order kinetics obeyed by this reaction are shown by Fig. 3.

Similar hydrolysis was carried out with Compounds 2–7 (Table I), which have modifications on the 3-oximino function at C-17 and in ring B of the steroid nucleus. It was found that changes at C-17 or in ring B did not influence the rate of hydrolysis of the 3-oxime (K in hr^{-1}: Compound 1, 0.038; Compound 5, 0.039; Compound 6, 0.032; and Compound 7, 0.035). However, ester substitutions at the 3-oximino function had a profound effect on the kinetics (K in hr^{-1}: Compound 1, 0.038; Compound 2, 0.068; and Compound 3, 0.163) (Fig. 4). The first-order plot reveals that ester groups initially increase the rate of hydrolysis of the 3-oxime.

For example, methyl and ethyl esters of Compound 1 showed a marked increase in the initial rate of hydrolysis over Compound 1 but after 6 hr the slope (Fig. 4) was parallel to that for Compound 1. On the other hand, the pentyl ester, Compound 4, had such a fast first-order rate of hydrolysis that its overall rate appeared to be identical with that of Compound 1. In fact, under experimental conditions, no pentyl ester could be detected after the first 30 min.

These results suggest that in the case of oxime esters there were two mechanisms by which the hydrolysis was proceeding. To study this in-depth, Compound 2 was chosen and the TLC technique was used because of its sensitivity and ability to separate

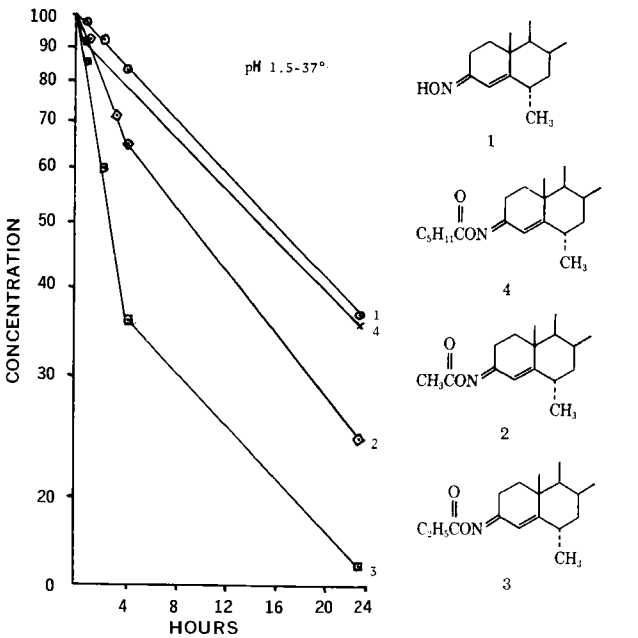


Figure 4—*Comparison of first-order plots for Compounds 1–4 showing the loss of total oxime with time (pH 1.5, 37°).*

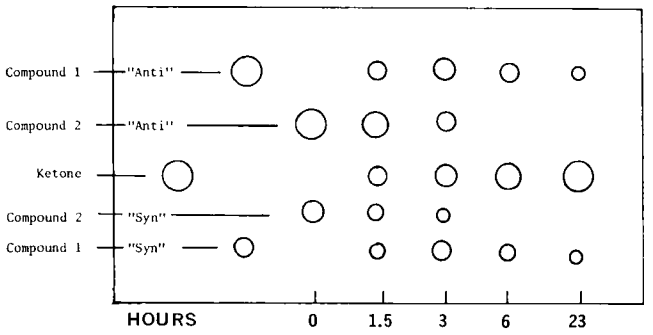


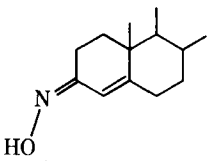
Figure 5—*Thin-layer chromatogram depicting the products obtained on acid hydrolysis of Compound 2.*

all possible hydrolysis products. Figure 5 illustrates a typical thin-layer chromatogram obtained for Compound 2 at various time intervals. It reveals that Compound 2 hydrolyzed rapidly to Compound 1 and its corresponding 3-ketone. After 6 hr, all ester was consumed and one could detect only Compound 1 and its 3-keto analog. The two spots observed on TLC for Compound 1 are attributed to *syn*- and *anti*-isomers (I and II, respectively).

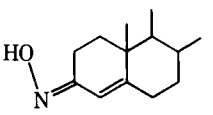
These TLC observations suggest that the mechanism of hydrolysis must be as depicted in Scheme IV. The time study also indicated that rapid hydrolysis of oxime ester to the corresponding ketone (K_1 of Scheme IV) took place in the early stages of the reaction but hydrolysis to Compound 1 (K_2 of Scheme IV) took place at a relatively slower rate. The overall conversion to the ketone, therefore, must be a combination of $K_1 + K_3$ (Scheme IV).

From the few oximes studied, it can be stated that the longer the alkyl chain of the ester group the faster is the rate of hydrolysis. The inductive effect is believed to play a significant role. The longer the ester group the greater is the +I effect on the nitrogen atom, making it more susceptible to attack by a proton and resulting in faster overall hydrolysis.

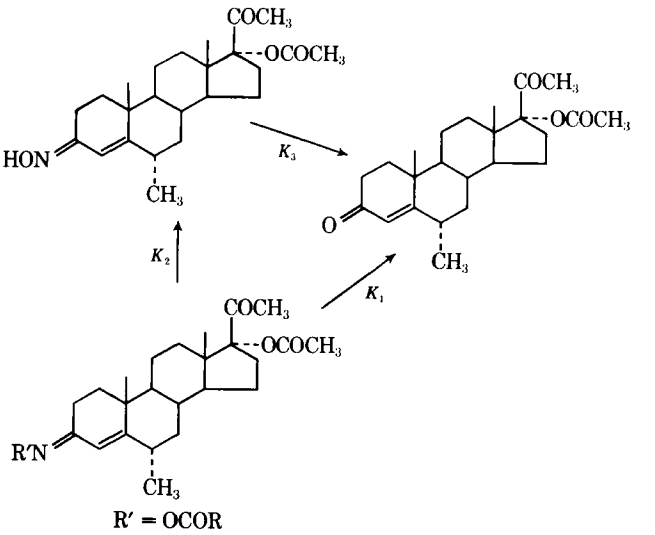
In conclusion, these experiments suggest that the oxime and its derivatives undergo hydrolysis at different but measurable rates. In view of the contrary findings of Buhler *et al.* (1) on other compounds, experience dictates that each compound should be individually evaluated; general prediction based on limited data may be erroneous. The rate of hydrolysis for the various compounds



I: *syn*-isomer



II: *anti*-isomer



Scheme IV—Proposed mechanism for the hydrolysis of 3-oximino esters

studied suggests that their *in vivo* biological performance is not necessarily dependent upon their conversion to ketones in gastric fluid.

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[x] To whom inquiries should be directed.

Reaction of *N*,3,3-Trimethyl-1-phenyl-1-indanpropylamine Hydrochloride with 7-Chloro-4-nitrobenzofurazan and Its Application to Spectrophotofluorometric Assay

RAMAZAN TURDIU, MELVIN H. PENNER[x], and LESTER CHAFETZ

Abstract □ The reaction of *N*,3,3-trimethyl-1-phenyl-1-indanpropylamine hydrochloride, a secondary amine, with 7-chloro-4-nitrobenzofurazan yielded a well-defined, crystalline, fluorescent derivative which was isolated and characterized. A sensitive spectrophotofluorometric method was developed based on this reaction and was applied to the assay of capsules and compatibility samples.

Keyphrases □ *N*,3,3-Trimethyl-1- phenyl-1-indanpropylamine hydrochloride—reaction with 7-chloro-4-nitrobenzofurazan, isolation and characterization of derivative, application to spectrophotofluorometry □ 7-Chloro-4-nitrobenzofurazan—used as reagent for spectrophotofluorometric analysis of *N*,3,3-trimethyl-1-phenyl-1-indanpropylamine hydrochloride, isolation and characterization of derivative □ Spectrophotofluorometry—reaction of *N*,3,3-trimethyl-1-phenyl-1-indanpropylamine hydrochloride with 7-chloro-4-nitrobenzofurazan

N,3,3-Trimethyl-1-phenyl-1-indanpropylamine hydrochloride (I) is currently being investigated for its antisecretory and antidepressant properties. A reliable and sensitive method was necessary for the quantitation of this drug formulated with a variety of excipients and at concentrations encountered during compatibility studies, stability testing, and clinical evaluations.

It was immediately apparent that the measurement of its UV absorption lacked sufficient sensitivity. The spectrum showed only benzenoid absorption and λ_{max} = 272 nm (ϵ = 1300). A useful "handle" for the analysis of this compound proved to be the secondary amine function. No fluorescence could be detected when this amine was allowed to react with 1,2-naphthoquinone-4-sulfonic acid according to the procedure of Pesez and Bartos (1).

The reaction of 7-chloro-4-nitrobenzofurazan (II) with amino acids and primary and secondary amines to yield highly fluorescent derivatives which were stable in aqueous solution was described (2) (Scheme I). Unlike fluorogenic reagents, such as dansyl chloride, commonly employed with amines, 7-chloro-4-nitrobenzofurazan does not fluoresce and therefore the blanks are low. In addition, the resulting derivatives are excited by visible light at 470 nm and fluoresce at 510 nm.

Since the first paper (2), several reports appeared describing the utility of this reagent; however, most have been primarily of a qualitative nature and precise details regarding reaction conditions are lacking. Two-dimensional TLC on silica gel plates was used to separate the benzofurazan derivatives of a number of phenethylamine drugs (3) in which the derivatives of primary amines were yellow, having an absorption maximum in methanol of 447–464 nm, while the reaction products with secondary amines were reddish with a λ_{max} in methanol of 470–480 nm. The same technique was applied to the detection of methamphetamine and its metabolite, 1-phenyl-2-aminopropane, in urine (4). The structures of the aminobenzoxadiazoles of 11 phenethylamine drugs were characterized by mass spectrometry (5). This fluorogenic reagent has also been utilized for the detection of sulfonamides (6), alkaloids and other nitrogen-containing drugs (7), and thiols (8).

Recently, *N*-methyl- and *N*,*N*-dimethylcarbamate insecticides were determined quantitatively (9) with 7-chloro-4-nitrobenzofurazan. After hydrolysis of the carbamates in 0.1 *M* sodium bicarbonate, the liberated alkyl amines were reacted with the fluorogenic reagent to yield aminobenzofurazans separable by TLC. Quantitation was achieved *via* fluorometric scanning of the developed chromatoplates.

Study of the reaction of 7-chloro-4-nitrobenzofurazan with I led to a sensitive and reproducible fluorometric assay. Reaction conditions have been optimized so that essentially complete derivatization occurs.

EXPERIMENTAL[1]

Isolation and Characterization of Benzofurazan Derivative of I—The reaction was first performed on a milligram scale to isolate and characterize the product obtained. Compound I, 16.5 mg, was dissolved in 0.6 ml methanol–water (1:2) and to this was added 1.5 ml of a 1.0% solution of the reagent in methanol. Upon the addition of 0.1 ml 1 *N* sodium hydroxide to the clear yellow solution, a red flocky precipitate began to emerge, and the reaction mixture was allowed to stand overnight at room temperature. The precipitate was washed with a small amount of methanol and recrystallized from chloroform–methanol (1:2).

Pure material was isolated by preparative TLC, employing cyclohexane–ethyl acetate (1:1) as the solvent system. The intensely fluorescent main zone appearing at R_f 0.47 was eluted with methanol, the solution volume was concentrated, and the product was allowed to crystallize. After drying in an Abderhalden apparatus at 100° for 6 hr, a bright-red crystalline solid was obtained, mp 175–176°; λ_{max} CH_3OH: 480 (log ϵ 4.41) and 340 (3.91) nm. A molecular weight of 456 was obtained by mass spectrometry; the fragmentation pattern showing other significant peaks at *m/e* 426, 207, 191, 129, 114, 91, and 77 was consistent with the proposed structure. The purity of the derivative was verified by TLC in an

[1] A Farrand Mark 1 spectrophotofluorometer equipped with a 150-w xenon arc lamp, two grating monochromators, a 1P28 photomultiplier, and a solid-state photometer was used to record all fluorescence measurements. Melting points were taken in a capillary tube on a Thomas-Hoover melting-point apparatus and are uncorrected. Spectral data were obtained with a Cary model 14 recording spectrophotometer. TLC used chromatoplates obtained from Analtech, Inc., coated with 0.25-mm layers of silica gel GF. The plates were activated before use by heating at 105° for 30 min. The reagent, 7-chloro-4-nitrobenzofurazan, was synthesized according to a reaction scheme described previously (2).

Table I—Spectral Data for the Aminobenzofurazan in Various Solvents

Solvent	Absorption Maximum, nm	ϵ	Excitation Maximum, nm	Fluorescence Maximum, nm	Fluorescence Intensity[a]
Cyclohexane	453 325	21,400 8,000	465	490	55
Chloroform	472 338	25,200 8,000	470	510	85
Methanol	480 340	25,700 8,200	470	525	5

[a] Fluorescence intensities measured with an instrument range setting of 3- and 10-nm slits.

alternate solvent system consisting of chloroform–methanol–concentrated ammonia (95:5:3), where a single fluorescent zone at R_f 0.78 was detected.

Anal.—Calc. for $C_{27}H_{28}N_4O_3$: C, 71.03; H, 6.18; N, 12.27. Found: C, 70.68, 70.70; H, 6.20, 6.13; N, 12.06, 12.27.

Assay of I in Solid Samples—*7-Chloro-4-nitrobenzofurazan Solution*—Prepare a fresh 0.25% solution of the reagent in methanol daily.

Sodium Acetate Buffer—Dissolve 2.5 g of reagent grade sodium acetate in 50 ml alcohol USP.

Standard Preparation—Dissolve about 8 mg of I standard, accurately weighed, in 50.0 ml methanol. Make a 10:50 dilution with methanol.

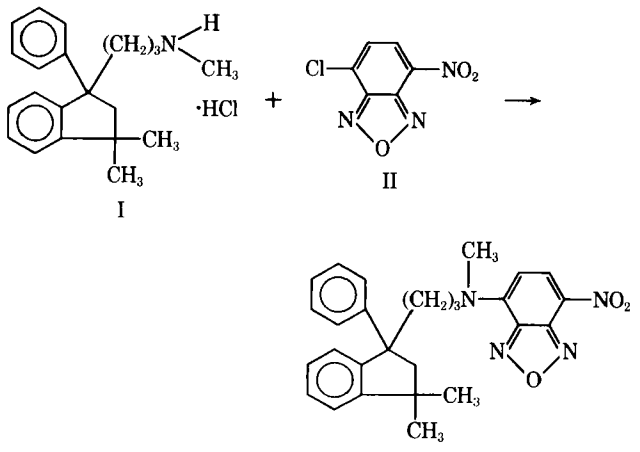
Sample Preparation—Carefully transfer the equivalent of about 8 mg of active, accurately weighed, into a 50-ml glass-stoppered centrifuge tube. Extract the solids with 2 × 25 ml methanol, shaking each time for about 10 min. Centrifuge and decant the supernatant liquid into a 50-ml volumetric flask, filtering through a small pledget of glass wool. Dilute to volume with methanol. Make a 10:50 dilution with methanol.

For those samples containing ascorbic acid or oxone, which interfere with the derivative formation, presumably by destroying the reagent, use the following extraction procedure. Transfer the equivalent of approximately 8 mg of I, accurately weighed, into a 125-ml separator containing 25 ml water and add 2.5 ml of 1 *N* sodium hydroxide. Extract for 2 min with 3 × 25 ml of chloroform. Wash the combined chloroform extracts with 10 ml water, filter through sodium sulfate into a 100-ml volumetric flask, and dilute to volume with chloroform. Withdraw 2.0-ml aliquots of each chloroform extract, transfer to a 15-ml glass-stoppered centrifuge tube, add 0.5 ml 0.1 *N* methanolic hydrochloric acid, and evaporate to dryness with the aid of a gentle stream of nitrogen. Redissolve the residue with 5.0 ml methanol.

Procedure—Pipet 1 ml of each sample and standard solution and 1 ml of methanol (to serve as a reagent blank) into separate, appropriately labeled, 10-ml volumetric flasks. To each in turn, add 0.5 ml sodium acetate buffer and 0.2 ml fluorogenic reagent. Mix, stopper well, and place in a 70° water bath for 45 min. Remove the flasks from the bath and pipet 1 ml acetic acid into each. Dilute to volume with methanol. Further dilute 3.0 ml of each sample, standard, and blank solution to 50.0 ml with chloroform. Determine the fluorescence of each sample (F_U), standard (F_S), and blank (F_B) preparation. Use a suitable sensitivity setting with an excitation wavelength of 470 nm, a fluorescence wavelength of 510 nm, and 10-nm slits. For the assay of the dosage form:

$$\text{milligrams active ingredient per capsule} = \frac{F_U - F_B}{F_S - F_B} \times \frac{A}{B} \times \frac{C}{4} \qquad \text{(Eq. 1)}$$

where F_U, F_S, and F_B are as defined previously; A is the standard weight in milligrams; B is the sample weight in milligrams; and C is the average net capsule weight in milligrams.



Scheme I

RESULTS AND DISCUSSION

Effect of Solvent on Spectral Characteristics of Benzofurazan Derivative—The wavelength of maximum absorption of the derivative is dependent on the polarity of the solvent. As the polarity of the solvent increases, the peak shifts to longer wavelength due to ionization of the nitro group, and the absorption increases. Since the absorption spectrum of the derivative showed a marked solvent effect, the fluorescence spectrum was checked for shifts in emission wavelength and intensity differences due to different solvents (Table I).

Linearity of Fluorescence with Concentration—The sensitivity of the reaction is illustrated by the data shown in Fig. 1, which relates fluorescence intensity to the concentration of I. A linear relationship was obtained over the concentration range of 0–50 μg of amine in the reaction mixture. After dilution, this corresponds to 0–0.3 μg of amine/ml of the final solution read. Each point on the curve represents the average fluorometric response of duplicate reaction mixtures. Readings were taken with a sensitivity range setting of the photometer of 10.

Optimum Reaction Conditions—Although the reaction employed for the isolation and characterization of the derivative involved the use of sodium hydroxide, the resulting solution pH was not suitable for quantitative purposes. At this pH, high blanks were observed, apparently due to decomposition of the reagent. It was also ascertained that the amine derivative was much less stable at this pH than when sodium acetate was utilized for pH control. The solution must be sufficiently alkaline to convert the amine hydrochloride to the free base since the salt gives little or no reaction, as indicated by the very low fluorescence response.

The optimum amounts of sodium acetate and fluorogenic reagent were arrived at experimentally by varying each of these two

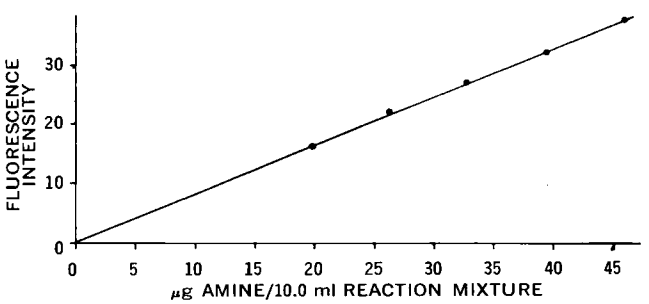


Figure 1—*Fluorescence intensity produced by the reaction of scalar amounts of amine with 7-chloro-4-nitrobenzofurazan in sodium acetate-buffered solution at 70° for 45 min.*

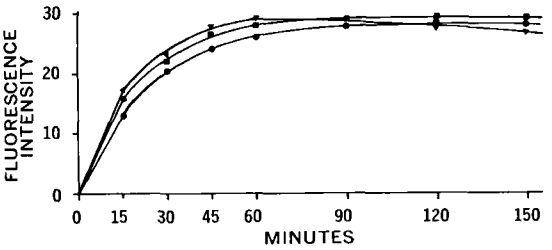


Figure 2—*Optimization of sodium acetate content. Fluorescence intensity was monitored up to 2.5 hr as a function of the amount of sodium acetate (5%): —▼—, 0.2 ml; —■—, 0.5 ml; and —●—, 1 ml. Concentration of amine = 30 μg in all cases.*

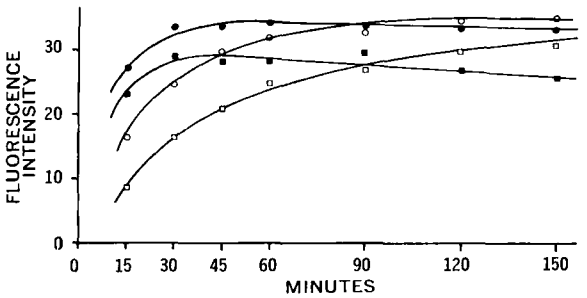


Figure 3—*Variation of fluorescence intensity as a function of the amount of reagent: —□—, 0.1 mg; —○—, 0.2 mg; —●—, 0.5 mg; and —■—, 1.0 mg. Amount of amine = 30 μg in all cases.*

variables until the maximum fluorescence was obtained. The readings obtained over a 2.5-hr reaction period, employing 0.2, 0.5, and 1.0 ml of a 5% sodium acetate solution, are plotted in Fig. 2. The use of 0.5 ml of buffer resulted in an apparent pH of 7.4 and was chosen as the optimum amount since it resulted in the highest and most reproducible response. Four simultaneous reaction mixtures, each containing 30 μg of amine and 0.5 ml sodium acetate solution but varying amounts of reagent, were reacted at 70° up to 2.5 hr. The fluorescence measured was plotted against time to produce the family of curves presented in Fig. 3. The reaction mixture containing 0.5 mg reagent produced the maximum response after approximately 30 min and remained relatively constant. Although virtually complete derivatization has occurred in the final procedure after 45 min at 70°, the addition of 1 ml of acetic acid was found to be a convenient means of halting the reaction. This simple expedient resulted in more reproducible readings.

Assay of I in Compatibility Samples—Weighed amounts of active ingredient were admixed with tartaric acid, sodium carbonate, oxone, and ascorbic acid. These additives were chosen as representative acid, base, oxidizing, and reducing agents to warn of potential solid-solid incompatibilities in the dosage form. The solids were triturated in a glass mortar in a 1:1 weight ratio and assayed. The results of these assays and recovery experiments performed without grinding are presented in Table II. Quantitative recovery of the amine was only obtained when the triturated samples were transferred with the aid of solvent since adsorption of the active ingredient on the glass surface apparently occurred.

Table II—Assay of Compatibility Samples

Additive	Percent Recovery of Active Ingredient
Tartaric acid	95.6, 93.5, 88.2, 88.4[a]
	100.1, 99.9, 99.9, 100.6[b]
	100.5, 99.0[c]
Sodium carbonate	93.8, 95.2[a]
	99.8, 101.7[c]
Lactose	89.7, 88.7, 87.4, 89.7[a]
	99.5, 98.6, 99.3, 99.1[b]
	98.7, 99.1[c]
Ascorbic acid	90.3, 91.4[a]
	98.2, 98.4, 99.6[b]
	101.2, 104.2[c]
Oxone	86.3, 88.2[a]
	98.3, 102.1[b]
	98.3, 99.8[c]

[a] Solids were triturated in a glass mortar and a weighed portion was assayed. [b] Solids were triturated in a glass mortar, and the entire sample was quantitatively transferred with solvent. [c] Recovery of known amounts of active ingredient mixed with additives without grinding, and the entire sample was quantitatively transferred with solvent.

Table III—Assay Values of Capsule Formulations

Declared, mg/capsule	100.0	25.0	5.0	1.0
Found, mg/capsule	101.9	24.1	5.0	0.99
	100.7	25.1	5.3	1.0
	99.3	24.5	5.1	1.0
	98.6	24.7	5.1	1.0
	100.4	24.5	5.0	
	99.0			
	98.2			
	100.0			
	99.6			
Average, mg/capsule	99.7	24.6	5.1	1.0
Relative standard deviation, %	1.2	1.5	2.4	0.5

The addition of a small amount of methanolic hydrochloric acid to the chloroform extracts of the ascorbic acid and oxone compatibility samples was employed to convert the free amine base extracted to the hydrochloride salt. This was necessary since the free base is somewhat volatile, and low recoveries even of extracted standards were obtained after evaporation of the chloroform to dryness.

Quantitation of Active Ingredient in Capsule Formulations—Four capsule formulations were prepared for dose finding studies at labeled concentrations of 100, 25, 5, and 1 mg active ingredient/capsule. The capsules were assayed *via* direct methanolic extraction (Table III). Pooling the results of all 23 capsule assays resulted in an average recovery of 99.9% with a relative standard deviation of 1.9%.

SUMMARY AND CONCLUSION

The reaction of I with 7-chloro-4-nitrobenzofurazan was carefully investigated and resulted in the isolation and characterization of the expected derivative. Upon optimizing the reaction conditions, whereby essentially complete derivatization takes place and a highly fluorescent species is formed, a sensitive reproducible analytical method was developed for the quantification of the active ingredient in a variety of solid mixtures.

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Relationship of Composition of Nonaqueous Binary Solvent Systems and Dielectric Constant

A. K. AMIRJAHED* and MARTIN I. BLAKE[x]

Abstract □ Five nonaqueous binary solvent systems are described. Each system is composed of a polar and a nonpolar component, both of which are inert and do not exhibit leveling effects. The dielectric constant of each system is related to the composition of the polar component expressed as mole fraction, percent weight by weight and volume by volume. Consequently, it is possible to prepare a solvent mixture with a specific dielectric constant within the range of values of dielectric constant provided by the system. The formulas relating dielectric constant to the particular expression of the composition of the polar component are reported.

Keyphrases □ Nonaqueous binary solvent systems—relationship between composition of polar component and dielectric constant, equation □ Dielectric constant of nonaqueous binary solvent system—relationship to composition of polar component, equation □ Solvent systems, nonaqueous binary—relationship between composition of polar component and dielectric constant, equation

The literature on nonaqueous titrimetry has expanded considerably over the last 3 decades. Solvents and their merits, solvent–solute interactions, titrants and their advantages, electrode systems, and different combinations of electrodes have been studied extensively, and these and similar topics were reviewed (1). The scope of nonaqueous solvent chemistry, the role of the solvent in chemical reactions, and related topics were discussed (2), and a thorough treatment of the electronic theory of acids and bases, acidic and basic radicals, neutralization, displacement, and acid and base catalysis was presented (3). Kolthoff and Bruckenstein (4) considered acid–base strength and equilibria in nonaqueous solutions.

Excellent reviews of the literature on nonaqueous titrimetry (5–8) and on its use in pharmacy (9) were presented. Theory, titrants and their standardization, solvents, and applications in general and in pharmaceutical analysis were covered (9). In all, wherever nonaqueous solvents are discussed the importance of the dielectric constant of the solvent is also emphasized and qualitative inferences are usually given. However, a rigid quantitative relationship is seldom indicated. In fact, in potentiometric titrations in nonaqueous media as well as any other scientific study where such media are employed and where the dielectric constant of the medium is of importance, a binary solvent system that is well defined in terms of the dielectric constant and the corresponding composition should be useful.

THEORETICAL

When assuming an ideal mixture of two solvents, one nonpolar (referred to as Solvent 1) and the other polar (referred to as Solvent 2) where there is no change in the volume of the two solvents upon mixing and the effect of electrical forces of surrounding molecules on each other is negligible, the following terms can be defined:

M_1 = gram molecular weight of Solvent 1
M_2 = gram molecular weight of Solvent 2
n_1 = moles of Solvent 1
n_2 = moles of Solvent 2
X_1 = mole fraction of Solvent 1 in the mixture of the two solvents
X_2 = mole fraction of Solvent 2 in the mixture of the two solvents
$\bar{V}_1$ = molar volume of pure Solvent 1 = M_1/ρ_1, in which ρ designates density
$\bar{V}_2$ = molar volume of pure Solvent 2 = M_2/ρ_2, in which ρ designates density
$\bar{P}_1$ = molar polarization of pure Solvent 1
$\bar{P}_2$ = molar polarization of pure Solvent 2
P_m = total molar polarization of solvent mixture
$P_m = X_1\bar{P}_1 + X_2\bar{P}_2$
M_m = weight of 1 mole of mixture
$M_m = (n_1M_1 + n_2M_2)/(n_1 + n_2) = [(n_1M_1)/(n_1 + n_2)] + [(n_2M_2)/(n_1 + n_2)]$
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$V_m = X_1\bar{V}_1 + X_2\bar{V}_2$
$\rho_m = (X_1M_1 + X_2M_2)/(X_1\bar{V}_1 + X_2\bar{V}_2)$, with ρ_m = (weight of 1 mole of mixture)/(volume of 1 mole of mixture)

Debye (10) reasoned that $P_m = X_1\bar{P}_1 + X_2\bar{P}_2$ and $\bar{P}_1$ is constant. $\bar{P}_2$ is considered constant here based on the assumptions of ideality of the mixture of the two solvents; however, it is not constant in real mixtures.

For nonpolar liquids and dilute solutions of polar solutes in nonpolar solvents, Eq. 1 is available (11):

$$P_m = [(D - 1)/(D + 2)](M/\rho) \qquad \text{(Eq. 1)}$$

where P_m is the molar polarization. This quantity has the dimensions of volume and is really a molar polarizability. D is the dielectric constant, M is the gram molecular weight of the dielectric, and ρ is the density of the dielectric. The total molar polarization quantity, P_m, includes contributions from both the induced dipoles (induction effect) and the permanent dipoles (orientation

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Table I—Best Values for Coefficients a, b, c, and d of $D_m = (aX_1 + bX_2)/(cX_1 + dX_2)$ Calculated by the Computer

Binary Solvent System	a	b	c	d
Benzene–acetonitrile	4.5840×10^{-3}	3.7530×10^{-2}	2.1153×10^{-3}	1.0000×10^{-3}
Benzene–nitromethane	4.5561×10^{-3}	3.5975×10^{-2}	2.2796×10^{-3}	1.0000×10^{-3}
Benzene–nitrobenzene	1.7589×10^{-2}	1.9965×10^{-1}	7.9076×10^{-3}	5.7292×10^{-3}
Benzene–acetone	1.6931×10^{-1}	9.9052×10^{-1}	7.7332×10^{-2}	4.7205×10^{-2}
Benzene–methyl isobutyl ketone	1.8045×10^{-1}	9.9064×10^{-1}	7.9600×10^{-2}	7.7053×10^{-2}

effect). If P_m, M, and ρ for a certain solvent or solvent mixture are known, the dielectric constant, D, can be calculated. Thus, D also has contributions from the induced dipoles and the permanent dipoles and as such can be used as a physical index representing the total intermolecular interactions in a solvent or solvent mixture.

From the formula $P_m = [(D - 1)/(D + 2)](M/\rho)$ and the fact that $V_m = M_m/\rho_m$, the following can be derived:

$$P_m = [(D_m - 1)/(D_m + 2)]V_m \quad \text{(Eq. 2)}$$

where D_m is the dielectric constant of the mixture, and:

$$P_m D_m + 2P_m = V_m D_m - V_m \quad \text{(Eq. 2a)}$$

$$D_m(P_m - V_m) = -2P_m - V_m \quad \text{(Eq. 2b)}$$

$$D_m = (-2P_m - V_m)/(P_m - V_m) = (2P_m + V_m)/(V_m - P_m) \quad \text{(Eq. 2c)}$$

$$V_m + 2P_m = X_1\overline{V}_1 + X_2\overline{V}_2 + 2X_1\overline{P}_1 + 2X_2\overline{P}_2 \quad \text{(Eq. 2d)}$$

$$V_m + 2P_m = X_1(\overline{V}_1 + 2\overline{P}_1) + X_2(\overline{V}_2 + 2\overline{P}_2) \quad \text{(Eq. 2e)}$$

$$V_m - P_m = X_1\overline{V}_1 + X_2\overline{V}_2 - X_1\overline{P}_1 - X_2\overline{P}_2 \quad \text{(Eq. 2f)}$$

$$V_m - P_m = X_1(\overline{V}_1 - \overline{P}_1) + X_2(\overline{V}_2 - \overline{P}_2) \quad \text{(Eq. 2g)}$$

$$D_m = [X_1(\overline{V}_1 + 2\overline{P}_1) + X_2(\overline{V}_2 + 2\overline{P}_2)]/[X_1(\overline{V}_1 - \overline{P}_1) + X_2(\overline{V}_2 - \overline{P}_2)] \quad \text{(Eq. 2h)}$$

Let:

$$\overline{V}_1 + 2\overline{P}_1 = a \quad \text{(Eq. 3a)}$$

$$\overline{V}_2 + 2\overline{P}_2 = b \quad \text{(Eq. 3b)}$$

$$\overline{V}_1 - \overline{P}_1 = c \quad \text{(Eq. 3c)}$$

$$\overline{V}_2 - \overline{P} = d \quad \text{(Eq. 3d)}$$

Then for an ideal binary solvent system:

$$D_m = (aX_1 + bX_2)/(cX_1 + dX_2) \quad \text{(Eq. 4)}$$

The D_m values measured for a series of binary solvent systems of a nonpolar and a polar solvent can be used to find the best values of a, b, c, and d with the aid of a computer. Once these values are found, the entire composition of the binary solvent system and the corresponding dielectric constant can be represented by this formula.

EXPERIMENTAL

Selection of Solvent Systems—After studying and evaluating the dielectric constant, the acidity and/or basicity, the autoionization, the leveling effect, the chemical reactivity, and the toxicity of a large number of solvents, the following were selected as the polar components of the five binary solvent systems to be investigated: acetonitrile, nitromethane, nitrobenzene, acetone, and methyl isobutyl ketone. Benzene was selected as the nonpolar component in the following solvent systems: benzene–acetonitrile, benzene–nitromethane, benzene–nitrobenzene, benzene–acetone, and benzene–methyl isobutyl ketone. All reagents were of the highest purity grades available, and they were used without further purification and/or drying.

Preparation of Solvent Mixtures—Mixtures of the polar solvent in benzene were prepared in 100-ml volumetric flasks. The flask was weighed and the calculated volume of polar solvent was pipeted into the volumetric flask. The weight of the flask and the solvent was determined, and benzene was added to the mark. The final weight was then determined. This procedure permitted calculation of the weight by weight and volume by volume percentages of the polar component. The mole fractions of the nonpolar component and the polar component were also calculated. All measurements were performed at a constant room temperature of 25 ± 1°.

Determination of Dielectric Constant of Solvent Mixtures—The Heterodyne-Beat method was employed for determining the dielectric constant, D, of each mixture. This method permitted the determination of values on solutions having low conductance. The procedure and apparatus used in these experiments were previously described (12).

After 1 hr of warm-up, the cell beaker and the plates of the variable capacitor were rinsed with pure benzene and pure acetone and dried in a stream of air. The cell beaker was then introduced into the variable capacitor, the top of the variable capacitor was replaced, and the whole unit, with the plate-columns of the variable capacitor separated (position a), was carefully introduced into the constant-temperature (25°) water bath. Fifteen minutes was allowed for temperature equilibration, and then the instrument was adjusted to indicate a constant frequency of the variable oscillator (by maintaining a circle on the oscilloscope). The precision air capacitor was read at this point. The variable capacitor plate-columns were then brought together (position b). The capacitance of the precision air capacitor was changed by rotating the dial, and the reading was taken when a stable circle appeared on the oscilloscope again. The difference between the two readings of the two positions ($b - a$) was designated as ΔC. At the beginning of the experiment, the ΔC was determined with just the dry, empty, cell beaker in place. This ΔC was designated as ΔC_{air}. The desired solvent mixture was introduced next and the measured ΔC was designated as ΔC_{liq}. The dielectric constant of that particular binary solvent sample was then calculated from $D = (\Delta C_{liq})/(\Delta C_{air})$. Each ΔC determination was repeated at least six times. The amount of solution in the beaker was always more than enough to cover the plates of the capacitor completely. The capacitor was handled with care to avoid any damage to the plates and potential change in ΔC.

All measurements were made at 25°, and the values reported were the averages of at least six trials. The data from two replicates were pooled. Each replicate represented an entire experimental determination of D for all of the benzene–polar component mixtures of the individual binary solvent system from low D to the highest D measurable by the instrument. The pooled data were fed to a computer[1] to determine the best-fitting polynomial that would express D as a function of the percent weight by weight or volume by volume of the polar component of the binary solvent system, and the best values were determined for the coefficients a, b, c, and d in Eq. 4.

RESULTS AND DISCUSSION

The solvents used in nonaqueous titrimetry vary widely in their properties: the dielectric constant, the acidity and/or basicity, the autoionization, the leveling effect, the chemical reactivity, and the toxicity. Certain solvents are used for the titration of acids or bases but generally not both. Some inert solvents have been used for titration of both acids and bases. The solvents selected as the polar component of the binary solvent systems are inert and do not present any leveling effects. They also provide a varied range of dielectric constants and chemical structures. Ac-

[1] IBM 360/44 computer running under HASP and OS.

Table II—D_m Expressed as a Polynomial Function of Percent Weight by Weight (in Grams) of the Polar Component of the Binary Solvent System in 100.00 g of Total Mixture

Benzene–acetonitrile:

$$D_m = 2.28880 + 0.29719588W + 0.84850605 \times 10^{-3}W^2 - 0.94192253 \times 10^{-6}W^3 - 0.20513955 \times 10^{-7}W^4$$

Benzene–nitromethane:

$$D_m = 2.24461 + 0.20290456W - 0.18107903 \times 10^{-2}W^2 + 0.70412945 \times 10^{-4}W^3 - 0.38935198 \times 10^{-6}W^4$$

Benzene–nitrobenzene:

$$D_m = 2.29464 + 0.14154240W + 0.11201036 \times 10^{-2}W^2 - 0.26201666 \times 10^{-5}W^3 + 0.97894765 \times 10^{-7}W^4$$

Benzene–acetone:

$$D_m = 2.27947 + 0.14625993W + 0.24731895 \times 10^{-3}W^2 + 0.52095944 \times 10^{-5}W^3 - 0.37250925 \times 10^{-7}W^4$$

Benzene–methyl isobutyl ketone

$$D_m = 2.27923 + 0.77094202 \times 10^{-1}W + 0.32896261 \times 10^{-3}W^2 - 0.16212037 \times 10^{-5}W^3 + 0.12050527 \times 10^{-7}W^4$$

Table III—D_m Expressed as a Polynomial Function of Percent Volume by Volume (in Milliliters) of the Polar Component of the Binary Solvent System in 100.00 ml of Total Mixture

Benzene–acetonitrile:

$$D_m = 2.28849 + 0.26297646V + 0.91613647 \times 10^{-3}V^2 + 0.30119845 \times 10^{-5}V^3 - 0.32592511 \times 10^{-7}V^4$$

Benzene–nitromethane:

$$D_m = 2.23619 + 0.25477603V - 0.23317571 \times 10^{-2}V^2 + 0.86627173 \times 10^{-4}V^3 - 0.55120893 \times 10^{-6}V^4$$

Benzene–nitrobenzene:

$$D_m = 2.29588 + 0.19525744V + 0.10431213 \times 10^{-2}V^4 - 0.98546458 \times 10^{-6}V^3 + 0.35515077 \times 10^{-7}V^4$$

Benzene–acetone:

$$D_m = 2.27430 + 0.13201515V + 0.29015152 \times 10^{-3}V^2 + 0.55827506 \times 10^{-5}V^3 - 0.30885781 \times 10^{-7}V^4$$

Benzene–methyl isobutyl ketone:

$$D_m = 2.27783 + 0.69132964 \times 10^{-1}V + 0.40893065 \times 10^{-3}V^2 - 0.22678710 \times 10^{-5}V^3 + 0.18502331 \times 10^{-7}V^4$$

cording to Kucharsky and Safarik (1), a wide range of potential measurements is also possible in most of these solvents. The lack of leveling effect and the inertness of the solvents were judged to be the two most important factors. Benzene is also an inert nonleveling solvent of very low dielectric constant and, therefore, a good choice for the nonpolar component of the binary solvent system. Thus, a mixture of benzene and any one of the polar components would in effect act more as a nonreactive medium in which the acid molecules may be dispersed and suspended as monomer, dimer, trimer, and/or higher polymolecular aggregates. The degree of the intramolecular aggregation of the acid molecules depends on the polarity (dielectric constant) of the particular solvent mixture and the possibility of the acid and/or the solvent molecules forming intra- and/or intermolecular hydrogen bonding. One can safely assume that the effect and extent of polarity and intermolecular attractions among the solvent molecules themselves can be summarized and included in the dielectric constant. Thus, the dielectric constant of the solvent system is an important physical constant for characterizing the particular solvent mixture.

Selection of binary solvent systems of a polar component and a nonpolar component provides the investigator with a range of available dielectric constants (limited by those of the pure components) out of which it is possible to select a mixture with definite composition and resultant dielectric constant. When media of varying dielectric constants are needed, this approach is more versatile and effective than using pure solvents of different dielectric constants, since in the latter case the solvent molecular structure differs with every new dielectric constant used. In the former case the molecular species of the solvent system are the same and only their ratio varies with the different dielectric constant values employed. Furthermore, it is virtually impossible to find solvents that cover a range of dielectric constants with a regular incremental increase of dielectric constant as those of, for example, the benzene-acetonitrile solvent system (D = 5, 10, 15, 20, 25, 30, and 35).

Binary solvent systems have been used to advantage in nonaqueous titrimetry. In such cases, the acidity or basicity of the polar component and the resulting leveling effect may be undesirable factors. Even in cases where binary mixtures of inert solvents are used, the composition of the mixture most suitable for titrations is selected empirically. However, a systematic study of a binary solvent system could result in the determination of the exact composition of the binary solvent system (of two inert nonleveling solvents) most suitable for the titrations.

Mixtures of the five binary solvent systems (benzene–acetonitrile, benzene–nitromethane, benzene–nitrobenzene, benzene–acetone, and benzene–methyl isobutyl ketone) were prepared and the D_m was measured. The computer running under HASP and OS used the pooled data (the two replicates together for each binary solvent system) and determined the best values for the coefficients a, b, c, and d (Table I). The same computer facilities determined the polynomial functions that would fit the data best when the dielectric constant was expressed as a function of the percent weight by weight (Table II) and volume by volume (Table III) of the polar component. These tables give the final form of the polynomial for each of the five binary solvent systems. From these polynomials, one can provide the calculated values of D_m for percent values (weight by weight or volume by volume increasing in increments of 5, for example) of the polar component of the binary solvent system. An enlarged graph of these values accurately plotted enables the investigator to read the composition of the binary solvent system (in percent weight by weight or volume by volume) corresponding to the D_m value desired and make up the solvent mixture of a definite dielectric constant by the gravimetric or volumetric methods. The polynomial functions are reported using W to represent the percent weight by weight of grams of polar component in 100.00 g of total mixture and V to represent the percent volume by volume of milliliters of polar component in 100.00 ml of total mixture.

The calculations were first performed by the computer on each replicate separately. The comparison of the results indicated that the differences between the replicates were minor and of no significance. The data for the two replicates of each binary solvent system were then pooled to produce the final results reported here.

SUMMARY

This investigation was initiated, planned, and performed to study some binary solvent systems that are inert and, therefore, do not present the leveling effect and the related problems. Five binary solvent systems were selected. Each was composed of benzene and a polar component with dielectric constant values ranging from 2.29 for pure benzene to 12.80 (pure methyl isobutyl ketone), 20.97 (pure acetone), 34.82 (pure nitrobenzene), 35.90 (pure nitromethane), and 37.5 (pure acetonitrile). Several mixtures of each binary solvent system were prepared and their dielectric constants were measured. The data from measurements of the dielectric constant, D_m, of the different mixtures of each binary solvent system were pooled (two replicates), and the best fitting curve for the data was obtained using a computer. Two curves were obtained for each system: one representing D_m as a function of the percent volume by volume and the other representing D_m as a function of the percent weight by weight of the polar component. From these two curves, it is possible to calculate how much of the polar component and benzene should be combined to produce a mixture having the desired dielectric constant.

Furthermore, a formula was derived to express the dielectric constant of the mixture as a function of the mole fractions of benzene (X_1) and the polar component (X_2). The same computer facility was used to determine the best value of each coefficient of the formula. Thus, five sets of coefficients were obtained. The five

binary solvent systems are completely defined in this manner. The calculated D_m values of the particular binary solvent system can be plotted (on a large graph) against the percent volume by volume and weight by weight of the polar component of the system. It is possible, then, to select and prepare any mixture of the binary solvent system having a definite dielectric constant.

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* Present address: College of Pharmacy, University of Toledo, Toledo, OH 43606

ˣ To whom inquiries should be directed.

Pharmacokinetics of Pentazocine in the Rhesus Monkey

K. A. PITTMANˣ **and G. A. PORTMANN**

Abstract □ The time course of pentazocine concentration was followed in the plasma and cerebrums of individual monkeys by obtaining serial samples of both tissues after intravenous administration of tritiated drug. Plasma concentrations of pentazocine were also followed in a monkey after portal vein infusion. A three-compartment open-system model adequately fits the observed plasma data. The use of the constants derived from that model to predict the time course of pentazocine concentration in the brain results in a good fit with the observed cerebral concentrations. As expected, portal vein infusion caused a reduction in the area below the plasma concentration–time curves and in the percentage of pentazocine excreted in the urine. The relative proportions of known metabolites and their conjugates in urine were determined.

Keyphrases □ Pentazocine—pharmacokinetics, correlation between plasma and brain concentrations, rhesus monkey □ Pharmacokinetics—pentazocine, correlation between plasma and brain concentrations, rhesus monkey □ Plasma concentration, pentazocine—correlated with brain concentration, rhesus monkey □ Brain concentration, pentazocine—correlated with plasma concentration, rhesus monkey

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The rhesus monkey was used because the metabolism of pentazocine has been studied more thoroughly in this species than in others and is known to be similar to that in man. Moreover, its brain is sufficiently large to permit the removal of multiple samples of cerebral tissue.

EXPERIMENTAL

Chemicals—Pentazocine [1,2,3,4,5,6 - hexahydro - *cis* - 6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocin-8-ol] and pentazocine labeled with tritium in the 4-position were pre-

binary solvent systems are completely defined in this manner. The calculated D_m values of the particular binary solvent system can be plotted (on a large graph) against the percent volume by volume and weight by weight of the polar component of the system. It is possible, then, to select and prepare any mixture of the binary solvent system having a definite dielectric constant.

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* Present address: College of Pharmacy, University of Toledo, Toledo, OH 43606

ˣ To whom inquiries should be directed.

Pharmacokinetics of Pentazocine in the Rhesus Monkey

K. A. PITTMANˣ **and G. A. PORTMANN**

Abstract □ The time course of pentazocine concentration was followed in the plasma and cerebrums of individual monkeys by obtaining serial samples of both tissues after intravenous administration of tritiated drug. Plasma concentrations of pentazocine were also followed in a monkey after portal vein infusion. A three-compartment open-system model adequately fits the observed plasma data. The use of the constants derived from that model to predict the time course of pentazocine concentration in the brain results in a good fit with the observed cerebral concentrations. As expected, portal vein infusion caused a reduction in the area below the plasma concentration–time curves and in the percentage of pentazocine excreted in the urine. The relative proportions of known metabolites and their conjugates in urine were determined.

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EXPERIMENTAL

Chemicals—Pentazocine [1,2,3,4,5,6 - hexahydro - *cis* - 6,11-dimethyl-3-(3-methyl-2-butenyl)-2,6-methano-3-benzazocin-8-ol] and pentazocine labeled with tritium in the 4-position were pre-

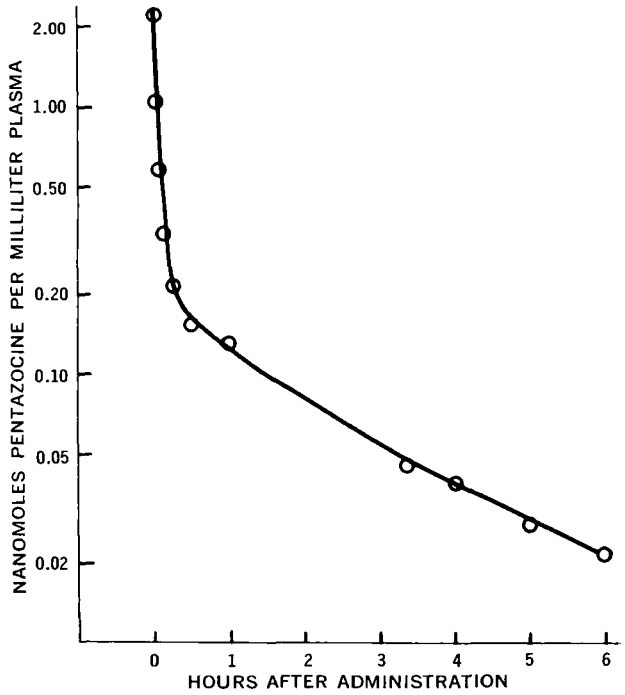


Figure 1—*Plasma levels of pentazocine* versus *time in a 3.75-kg monkey (No. 5635) after 2.04 mg iv (7148 nmoles). The equation fitting the curve is* $2.7e^{-21t} + 0.12e^{-0.82t} + 0.10e^{-0.26t}$. *Key: O, experimental values; and ——, predicted curve.*

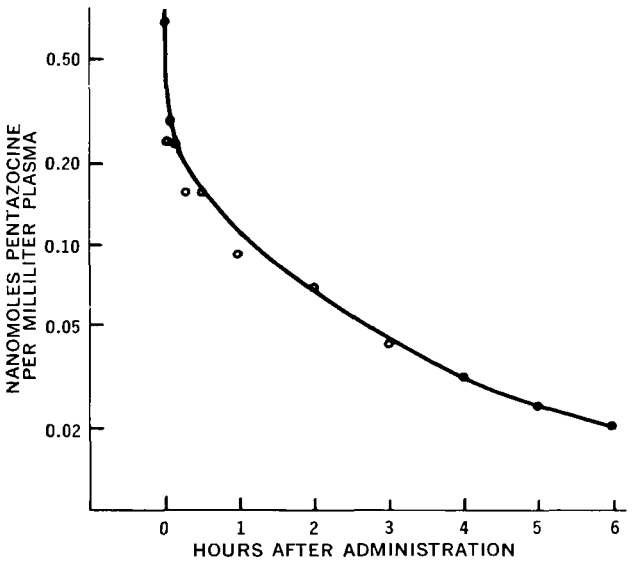


Figure 2—*Plasma levels of pentazocine* versus *time in a 4.78-kg monkey (No. 5269) after 2.41 mg iv (8444 nmoles). The equation fitting the curve is* $0.54e^{-25t} + 0.17e^{-1.1t} + 0.074e^{-0.22t}$. *Key: O, experimental values; and — , predicted curve.*

pared[1]. All other chemicals were analytical reagent grade or better.

Doses—Doses were prepared as 0.1% (w/v) solutions of tritiated pentazocine in lactic acid. Their specific activity was different in each experiment, being approximately 190 Ci/mole for the systemic intravenous doses, administered at about 0.5 mg/kg, and approximately 316 Ci/mole for the portal infusion dose, administered at 1.4 mg/kg.

Animals—Young adult female rhesus monkeys (*Macaca mulatta*), weighing 3.8–4.8 kg, were used. The animals were maintained on the premises for at least 3 months before use and were normal and healthy in all respects.

Design and Execution—One monkey (No. 4851) was restrained in a primate chair, its bladder was catheterized, and an in-dwelling venous catheter was placed in a saphenous vein. The dose was injected rapidly through the saphenous catheter. For the first 6 hr after administration of the dose, sterile Ringer's lactate solution was infused at about 1 ml/min *via* the saphenous catheter. Blood samples were drawn from surface veins on either arm at intervals for 6 days. Urine and feces were collected at intervals for 7 days; the urinary catheter was removed after the first 24 hr.

This monkey was returned to its cage for recovery, and 2.5 months later it was used for the portal vein infusion experiment. By this time, there was no trace of radioactivity in the plasma or excreta. For the portal infusion experiment, the monkey was anesthetized with 3% sodium pentobarbital, its peritoneal cavity was opened, and a cannula was placed in a small vein feeding the portal vein. The end of the cannula was slipped past the junction of the small vein with the portal vein, and the body of the cannula was tied in place in the small vein. The dose (5.94 mg in 3.7 ml) was infused[2] at rates of 10 ml/hr for 15 min and of 3.4 ml/hr for 21 min. Lactated Ringer's solution was used to rinse in the dose, and this infusion was continued for the course of the experiment. Blood was drawn from a saphenous vein cannula, and urine was collected by bladder catheter as before.

In the experiments in which serial brain samples were to be taken, Monkeys 5635 and 5269 first were anesthetized lightly with sodium pentobarbital. Then a urinary catheter was inserted, the animals were tied to a restraining board, a femoral vein was exposed, and in-dwelling venous catheters were placed in the femoral vein and in the saphenous vein of the opposite leg. Ringer's lactate was infused as before through a three-way stopcock. A syringe containing 5% sodium thiopental was attached to the second inlet of the stopcock, and, when required, the minimum amount of this solution necessary to maintain the animal under light anesthesia was injected into the saphenous vein. After insertion of the venous catheters, the scalp was reflected, the right and left lateral aspects of the parietal bone were opened with a 1.27-cm (0.5-in.) trephine, and incisions were made through the dura. Except for the time during which a sample was being obtained, the trephined holes were plugged with gelatin sponge[3] to reduce leakage of cerebral-spinal fluid, and the scalp was pulled over the skull to reduce drying and capillary hemorrhage.

Brain samples were obtained by aspiration with a syringe attached to an 11-gauge stainless steel cannula with a 90° point and an inside bevel. The cannula was inserted through the incision in the dura, deep enough into the cerebrum to obtain an adequate sample but not so deep as to injure the opposite leptomeninges. The average sample weight was 52 mg, with the lowest being 26 and the highest 86. After each puncture, fresh gelatin sponge was fitted into the hole in the skull, the scalp was laid in place, the cannula was washed with water and 70% alcohol and dried, and a fresh syringe was fitted to the cannula. To reduce the effect of trauma caused by one biopsy upon the following biopsies, only four samples were taken from each cerebral hemisphere and these were taken in a radial pattern as follows: anterio-medial, anterio-lateral, posterio-lateral, and posterio-medial. Blood samples were taken from the femoral vein cannula.

At the end of the experiment, the animals were killed by the injection of excess sodium thiopental and the brain was exposed, removed intact, and examined for hemorrhage or edema and for the location of the biopsy sample tracts. The tracts left from biopsy were filled with normal clotting, and some edema had occurred around the incision with only minimal swelling of the cerebrum above the normal limit of the dura.

Analytical Methods—Total radioactivity in blood and in cerebral samples was determined by combustion analysis. Measured volumes of blood were dried on filter paper and wrapped in filter paper. Weighed samples of cerebrum were air dried on weighing paper and wrapped in filter paper. These packages were com-

[1] Dr. N. F. Albertson and Dr. G. D. Diana, Sterling-Winthrop Research Institute.

[2] Holter infusion pump, model RD-174, Extracorporeal Medical Specialties, Inc.

[3] Gelfoam, The Upjohn Co.

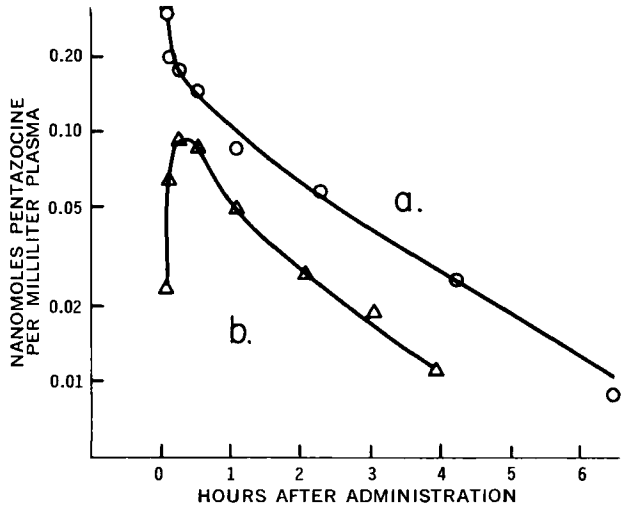


Figure 3—(*a*) *Plasma levels of pentazocine* versus *time in a 3.97-kg monkey (No. 4851) after 2.0 mg iv (7010 nmoles). The equation fitting the curve is* $1.1e^{-23t} + 0.086e^{-1.6t} + 0.13e^{-0.39t}$. *Key:* O, *experimental values; and* ——, *predicted curve.* (*b*) *Plasma levels of pentazocine* versus *time in a 4.23-kg monkey (No. 4851) after a portal infusion of 5.94 mg (20,800 nmoles). Key:* △, *experimental values.*

pressed[4] into hard pellets and combusted[5] for the recovery of tritium as tritiated water. The count rate in the least active sample was about 100 times the background count rate. Total radioactivity in plasma and urine was determined by the direct counting of appropriate samples.

Pentazocine in plasma was determined by adding 0.25 mg pentazocine and 40 mg of an equal mixture of sodium bicarbonate and sodium carbonate to 1 ml of plasma, extracting five times with 5 ml of benzene, reducing the combined benzene extracts to dryness, taking the residue up in a small volume of methanol, and spotting the solution on 5 × 20-cm commercial silica gel plates[6]. The plates were developed in benzene–methanol–isopropylamine (95:5:3 by volume). After development, the band corresponding to pentazocine was scraped, pelleted, and combusted in a manner similar to the brain samples.

Pentazocine and metabolites in urine were determined by adding unlabeled standards to urine samples, desalting the samples by a column[7] method (7), separating pentazocine and metabolites on thin-layer plates in the solvent system already described, and determining the tritium content of the appropriate bands from each plate. The same analysis was also performed on urine samples after enzyme treatment to hydrolyze conjugates (7).

All counts of radioactivity were corrected to disintegrations by a channels-ratio quench correction procedure (8).

Table I—Pharmacokinetic Analysis of the Time Course of Pentazocine Concentrations in the Plasma of Three Rhesus Monkeys Given the Drug Intravenously[a]

Derived Value[b]	Monkey Number (mg/kg)		
	5635 (0.54)	5269 (0.50)	4851 (0.50)
V_C (liters)	2.45	10.8	5.33
W_{FC} (kg)	1.40	1.55	—[d]
K (hr^{-1})	4.43	1.53	3.04
K_{CF} (hr^{-1})	13.3	14.9	14.1
K_{FC} (hr^{-1})	2.18	8.33	4.51
K_{CS} (hr^{-1})	1.88	1.05	2.25
K_{SC} (hr^{-1})	0.479	0.475	1.02
Area: dose (hr kg/ml)[c]	34.6×10^{-5}	29.0×10^{-5}	24.7×10^{-5}

[a] Pentazocine was administered intravenously in aqueous solution, and blood samples taken at various times thereafter for the determination of plasma pentazocine. See text for experimental details. [b] V_C = volume of central compartment, W_{FC} = weight of fast compartment, K = sum of all excretion and metabolic rate constants, K_{CF} = rate constant for movement of pentazocine from central to fast compartment, K_{FC} = rate constant for movement of pentazocine from fast to central compartment, K_{CS} = rate constant for movement of pentazocine from central to slow compartment, and K_{SC} = rate constant for movement of pentazocine from slow to central compartment. [c] The area was determined by integration of the equations describing the plasma concentration–time curves for each monkey. [d] Brain pentazocine levels were not determined.

RESULTS AND DISCUSSION

Concentrations of total radioactivity in blood samples were compared to such concentrations in the corresponding cerebral

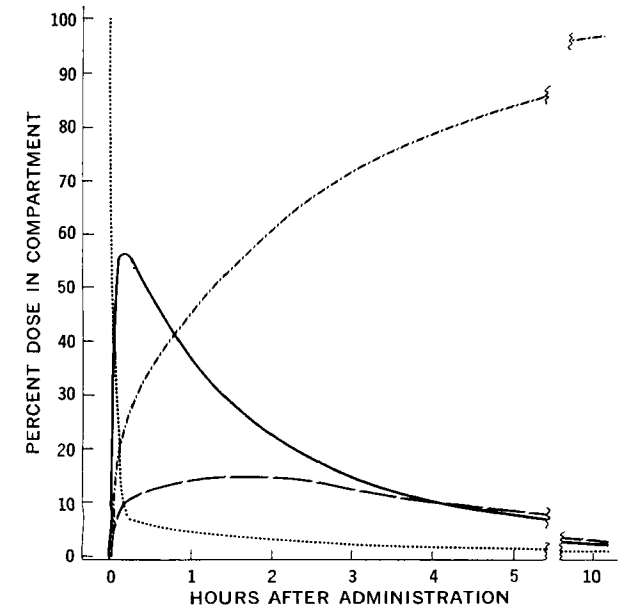


Figure 4—*Calculated percent of a pentazocine dose in various compartments* versus *time in Monkey 5635 after 0.54 mg/kg iv. Key: - - -, central compartment;* ——, *fast compartment; — —, slow compartment; and —·—, metabolized and excreted pentazocine.*

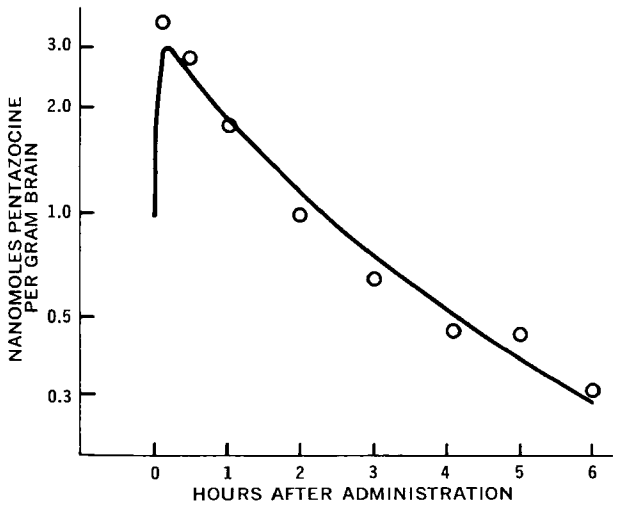
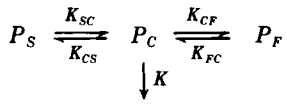


Figure 5—*Pentazocine brain levels* versus *time for Monkey 5635 after 2.04 mg iv (7148 nmoles). The experimental values* (O) *and calculated curve for the fast compartment are shown.*

[4] Stokes tablet machine, model A, F. J. Stokes Machine Co.
[5] Packard Tri-Carb sample oxidizer, model 300, Packard Instrument Co.
[6] Silica gel F-254, E. Merck, Darmstadt, Germany.
[7] XAD-2.



Scheme I

samples. From early to late sampling times, blood values ranged from one-fourth to three-fourths of the brain values. Therefore, the small amount of blood expected to be present in the cerebral samples would not affect the determination of total radioactivity therein significantly. Based on these data and on the fact that only pentazocine and none of its metabolites enter the brain of the rhesus monkey (2) as well as other species (1, 3, 4), the concentration of total radioactivity in the cerebral samples was used as an adequate estimate of pentazocine concentration.

Graphical analysis of semilogarithmic plots of the time course of pentazocine concentration in the plasma of intravenously dosed monkeys (Figs. 1–3) revealed the presence of three first-order components. Therefore, a three-compartment model (Scheme I) was postulated to account for pentazocine kinetics in the rhesus monkey. First-order rate constants (K_{CS}, K_{SC}, K_{CF}, and K_{FC}) control pentazocine (P) distribution in the central (C), fast (F), and slow (S) compartments. The overall elimination rate constant (K) is the sum of the first-order excretion and metabolism rate constants. By using standard analytical procedures (9–11), the plasma data were used to calculate the volume of the central compartment, the rate constants, and the area under the curves (Table I). Elimination half-lives ($0.693/K$) for pentazocine from the monkeys were 0.16 (Monkey 5635), 0.45 (Monkey 5269), and 0.23 (Monkey 4851) hr, while the corresponding half-lives for the slowest plasma exponential were 2.7, 3.2, and 1.8 hr.

One might expect from this information that a large proportion of the dose is distributed to the tissues. The theoretical distribution of pentazocine in Monkey 5635 for 10 hr after an intravenous dose was calculated from the data in Table I and is shown in Fig. 4. The rapid distribution of pentazocine from the central to the fast compartment results in the accumulation of almost 60% of the dose in the fast compartment within 10 min after administration. At this time, only about 10% of the dose remains in the central compartment, the rest being metabolized, excreted, or in the slow compartment. From earlier work (2), it was suspected that the tissues served as an important reservoir for pentazocine after parenteral administration. Both the tissues of the fast and slow compartments serve as important reservoirs, as revealed by the calculations summarized in Fig. 4.

Since the high concentrations of pentazocine found in cerebral samples of two parenterally dosed monkeys and the changes in those concentrations with time (Figs. 5 and 6) both indicate that the brain is likely to be a part of the fast compartment of this model, the plasma data obtained in Monkeys 5635 and 5269 were

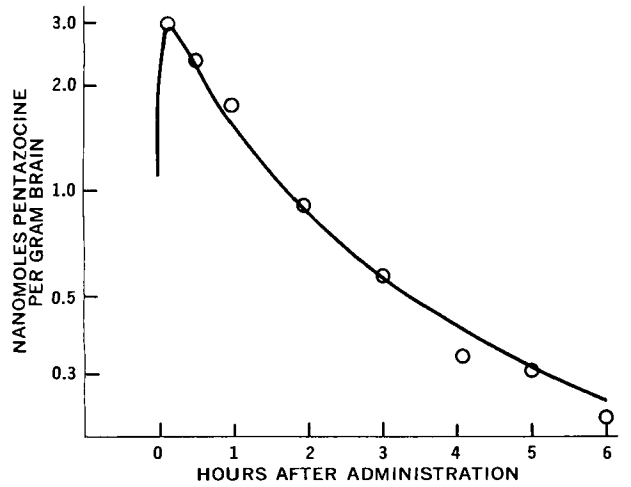
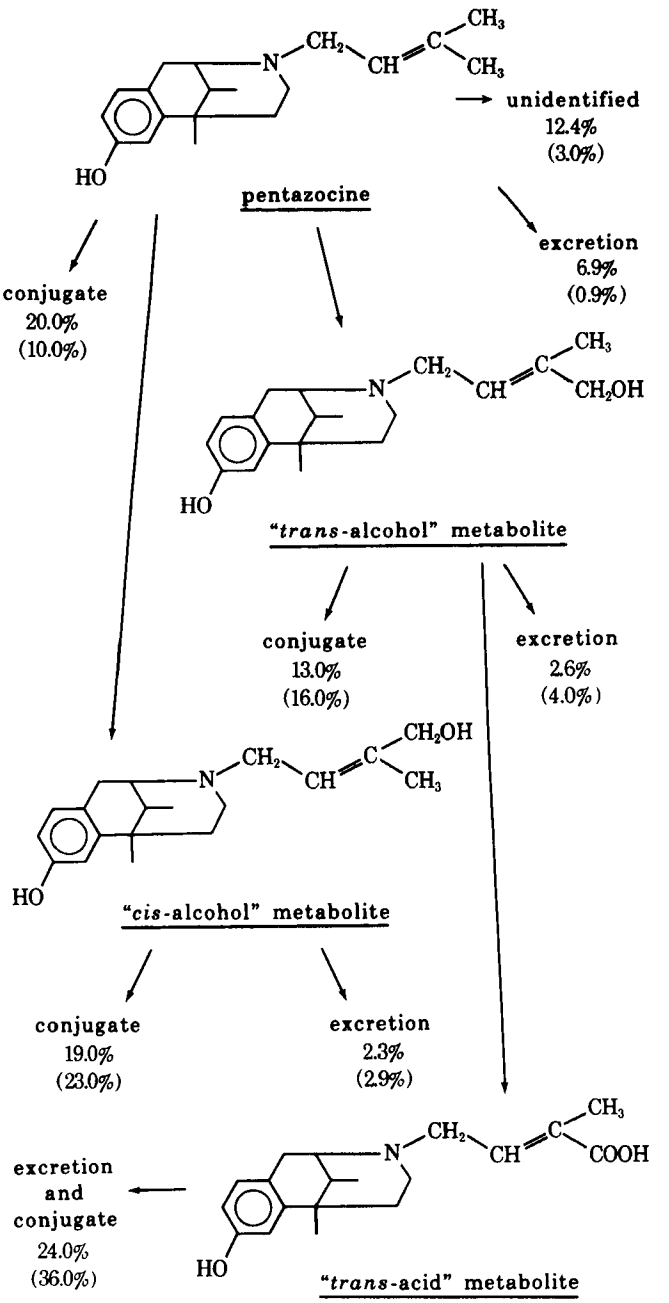


Figure 6—*Pentazocine brain levels* versus *time for Monkey 5269 after 2.41 mg iv (8444 nmoles). The experimental values* (O) *and calculated curve for the fast compartment are shown.*



Scheme II—Pentazocine biotransformation in Monkey 4851 after 0.5 mg/kg iv. Values are expressed as percent of the total amount excreted for 6.9 days. Values in parentheses were obtained for 265 min after a portal infusion of 1.17 mg/kg in the same monkey.

used to predict the time course of pentazocine concentration in the brain. This prediction, which is based on the model, was then compared with the data obtained by repeated sampling of cerebral tissue. The quantity of pentazocine in the fast compartment was calculated according to the model, and a weight term (W_{FC}) was used as a scaling factor for conversion to concentration. Due to differential partitioning and binding of drugs in various tissues within a single pharmacokinetic compartment, any such term is fictitious (12). Therefore, the weight of distribution of the fast compartment (Table I) which was used is as biologically meaningless as the volume of distribution of the central compartment. The results indicate a very close fit between predicted and observed values (Figs. 5 and 6). The fact that such good fits were obtained indicates that the use of a three-compartment model is appropriate for the data and, more importantly, that it is possi-

ble to predict brain concentrations of pentazocine accurately from the plasma data. The information available from studies with other species (1, 3, 4) suggests that the distribution of pentazocine between brain and plasma is similar in all species. Provided that uptake saturation or irreversible effects do not occur, the time course of brain concentrations of pentazocine could be predicted for any species from plasma data and the pharmacokinetic model. No saturation effects were noted in this study with a dose comparable to that used in man.

It is possible that a three-compartment model also is necessary to describe pentazocine pharmacokinetics in man. Data obtained in a clinical study of pentazocine in man[8] indicate that the time course of plasma concentrations of pentazocine after an intravenous dose is similar to that in monkeys. Half-lives for the slowest plasma exponential of pentazocine in man were reported to be about 2 hr in one study (5) and to range between 1.7 and 6 hr in another study (13, 14) after intravenous injection. It seems likely that brain concentrations (and therefore analgesia) of pentazocine in man could be predicted from plasma concentrations and based on a pharmacokinetic analysis.

The so-called "first-pass effect" (15) is quite evident when plasma pentazocine concentrations after injection of the drug into the systemic circulation are compared to concentrations after portal vein infusion (Fig. 3). The relative area under the plasma curve after portal vein infusion is only 14% (3.54×10^{-5} hr kg/ml) of that after intravenous injection (Table I). This effect also is reflected in the relative proportions of pentazocine and its metabolites excreted in the urine (Scheme II). After portal vein infusion, the amount of pentazocine excreted, relative to the dose, is only 13% of that excreted after intravenous injection. The excretion of conjugated pentazocine is also reduced, but the proportions of other metabolites are increased. This dramatic effect of hepatic metabolism upon plasma levels was noted in earlier work (2). Our data[8] and that of others (5, 13, 14) show that the same effect occurs in man.

The metabolism of pentazocine in the monkey as observed in this study agrees closely with that reported earlier (16). The relative proportions of each metabolite plus conjugate excreted are quite similar.

CONCLUSIONS

In the rhesus monkey, brain concentrations of pentazocine are in rapid equilibrium with plasma concentrations of pentazocine. Brain concentrations of pentazocine at any point in time can be predicted on the basis of a three-compartment pharmacokinetic model and known plasma concentrations. The rhesus monkey provides an excellent model for the pharmacokinetic study of drugs with central nervous system activity, because multiple samples of cerebral tissue can be obtained from one animal during the experiment.

[8] K. A. Pittman and G. A. Portmann, unpublished data.

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[x] To whom inquiries should be directed. Present address: Albany Medical College, Albany, NY 12208

Analysis of Acetaminophen and Barbiturate Combinations by Differentiating Nonaqueous Titration

MARTIN I. BLAKE[x], JAMES HUNT*, and HAROLD J. RHODES

Abstract □ The difference in pKa values for acetaminophen and the barbituric acids permits differentiation of mixtures containing these drugs. Ion-exchange chromatography is used to separate acetaminophen and barbiturate from other active and inert constituents. Titrations are performed nonaqueously in dimethylformamide using tetrabutylammonium hydroxide as the titrant and a titrimeter equipped with a calomel and platinum electrode system.

Keyphrases □ Acetaminophen-barbiturate in combination—analysis by differentiating nonaqueous titration □ Barbiturate-acetaminophen in combination—analysis by differentiating nonaqueous titration □ Titrimetry, differentiating nonaqueous—analysis of acetaminophen-barbiturate in combination

Acetaminophen is a frequently used analgesic-antipyretic, alone as well as in combinations with other similar drugs in liquid and solid dosage forms. A number of studies have dealt with procedures for the analysis of the acetaminophen content in these preparations. These were referred to in an earlier paper (1), which described a nonaqueous titration procedure for dosage forms containing acetaminophen. Tablets were assayed by dissolving an aliquot of the powdered tablet mass in dimethylformamide and titrating potentiometrically with 0.1 *N* sodium methoxide. Additives and excipients in the formulation apparently did not interfere with the analysis. A drops dosage form was assayed similarly after evaporation of the solvent. An elixir dosage form was analyzed by extracting the acetaminophen from an aliquot by passage through a strong anion-exchange resin column. The weakly acidic acetaminophen was removed by the resin, while all nonionic components were washed from the column. Elution of the acetaminophen was effected with a solution of acetic acid in alcohol. After evaporation of the solvent, the residue was analyzed in the same manner as the tablets.

A previous report (2) described a differentiating titration procedure for acetaminophen and aspirin combinations. An aliquot of the powdered tablet mass was titrated potentiometrically in dimethylformamide with triethyl-*n*-butylammonium hydroxide. Preliminary separation of the components was not necessary.

More recently, a nonaqueous titration procedure for mixtures containing acetaminophen and salicylamide was described (3). Dimethylformamide was the titration solvent, and tetrabutylammonium hydroxide was the titrant. Titrations were performed potentiometrically, and the method was applied to synthetic mixtures and an assortment of commercially available complex solid dosage forms. Preliminary extraction of the active components was unnecessary.

The present report describes procedures for analyzing acetaminophen and barbiturate combinations. The difference in pKa values between acetaminophen, 9.92 (4), and barbiturates (phenobarbital), 7.42 (5), is sufficiently large to permit a satisfactory differentiating titration by the method described for the other combinations. The method is applied to several complex dosage forms.

EXPERIMENTAL

Apparatus—Titrations were performed potentiometrically with a titrimeter[1] equipped with a calomel and platinum electrode system. A 50-ml buret (1 cm i.d.) served as a chromatographic column. It contained a glass wool plug at the base to support the resin column.

Reagents and Chemicals—A strong anion-exchange resin[2] (200–400 mesh), acetaminophen, phenobarbital, and dimethylformamide were obtained from commercial sources and were the best quality available. All dosage forms were obtained commercially. All other chemicals and solvents employed in this study were reagent grade, and they were used without further purification.

Tetrabutylammonium hydroxide, 0.1 *N*, in benzene–methanol was prepared according to Cundiff and Markunas (6). The solution was standardized by dissolving 1 mEq of benzoic acid in 30 ml of dimethylformamide and titrating potentiometrically. The solution was restandardized at least weekly during the study.

Differentiating Titration of Synthetic Mixtures—Synthetic mixtures of acetaminophen and phenobarbital were prepared by weighing about equal amounts (50–200 mg) of each component into a 150-ml beaker and dissolving the mixture in 25 ml of dimethylformamide with the aid of magnetic stirring. The solution was titrated potentiometrically with 0.1 *N* tetrabutylammonium hydroxide.

Analysis of Acetaminophen–Barbiturate Dosage Forms—*Preparation of Column*—A strong anion-exchange resin column was prepared as described earlier (7).

Chromatography of Synthetic Mixtures—Synthetic mixtures containing approximately equal quantities (60–100 mg) of acetaminophen and phenobarbital were dissolved in 25 ml of 50% ethanol with the aid of magnetic stirring. The solution was transferred quantitatively to the resin column. The flow rate was adjusted to 1 ml/min. When the level of the solution for analysis reached the surface of the resin, three 20-ml portions of distilled water were added to the column. The washings were discarded and the resin column was then eluted with three 20-ml portions of

[1] Fisher, model 35, or Sargent-Welch recording titrator.
[2] Dowex 1-X8 (or equivalent).

Table I—Analysis of Acetaminophen and Phenobarbital, Alone and in Synthetic Mixtures

Run	Acetaminophen Recovery, %	Phenobarbital Recovery, %
1	101.1 ± 1.32[a]	—
2	—	101.1 ± 0.5
3	101.0 ± 0.7	102.4 ± 1.0
4[b]	97.1 ± 0.5	—
5[b]	—	101.7 ± 1.1
6[b]	97.8 ± 1.6	98.0 ± 0.5

[a] Standard deviation based on at least five determinations. [b] Runs 4, 5, and 6 were subjected to the ion-exchange chromatographic step prior to analysis.

50% acetic acid in ethanol. The eluate was collected in a 150-ml beaker and evaporated to dryness with the aid of a jet of clean air directed over the surface of the liquid. The residue was dissolved in 50 ml of dimethylformamide and titrated potentiometrically with 0.1 *N* tetrabutylammonium hydroxide.

Chromatography of Dosage Forms—Two tablet dosage forms and one capsule dosage form were analyzed by the described procedure. Twenty tablets were powdered or the contents of 20 capsules were removed as completely as possible, and the powder mass was weighed.

An aliquot of the tablet or powder mass containing about 150 mg of acetaminophen was transferred to a beaker containing 25 ml of 50% ethanol. The mixture was stirred magnetically for 10 min and was then transferred quantitatively to the resin column, using several 10-ml aliquots of 50% ethanol to aid in the transfer. The column was washed with three 20-ml portions of distilled water and was then eluted with 50% acetic acid in ethanol as described previously. The residue obtained from the evaporation of the eluate was titrated as described.

Since the ratio of acetaminophen to phenobarbital may range from 10:1 to as high as 30:1, it was necessary to analyze for the individual components in two aliquots: one containing the equivalent of about 150 mg of acetaminophen and the other of at least 80 mg of barbiturate.

RESULTS AND DISCUSSION

Earlier reports from this laboratory were concerned with the nonaqueous differentiating titration of mixtures of weak acids as synthetic combinations and as components in complex dosage forms. These included salicylic acid and benzoic acid (8), aspirin and barbiturates (9, 10), acetaminophen and aspirin (2), and acetaminophen and salicylamide (3). For each combination, the difference in pKa values was sufficiently large to permit satisfactory differentiating titrations in a nonaqueous solvent.

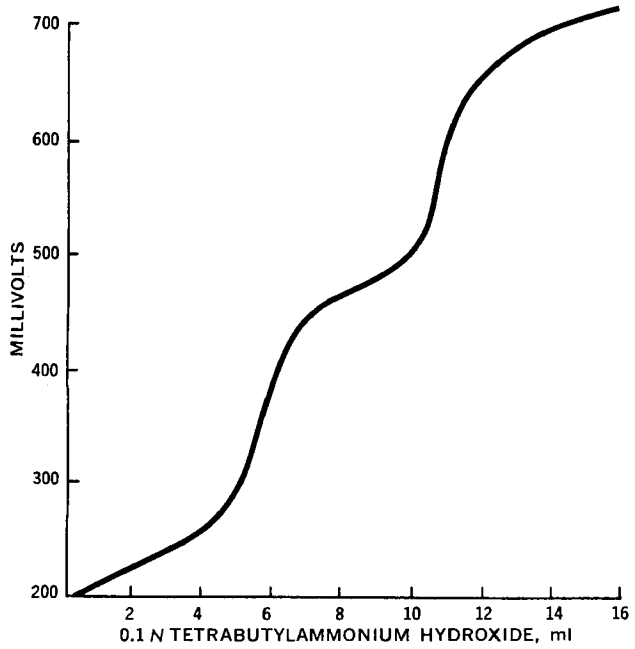

Figure 1—*A typical titration curve for a mixture containing acetaminophen and phenobarbital.*

Table II—Analysis of Dosage Forms Containing Acetaminophen and a Barbiturate by Differentiating Nonaqueous Titration

Dosage Form	Labeled Composition, mg/unit	Recovery, %
Tablet A	Phenobarbital, 16 Acetaminophen, 162 Phenacetin, 162 Atropine sulfate, 0.00065 Scopolamine hydrobromide, 0.0011 Hyoscyamine hydrobromide, 0.0325	102.5 ± 1.0 101.0 ± 0.2
Tablet B	Allobarbital, 15 Acetaminophen, 300	102.7 ± 0.9 103.2 ± 1.1
Capsule A	Sodium butabarbital, 10 Acetaminophen, 300 Mephenesin, 200	101.6 ± 0.8 103.6 ± 0.8

Acetaminophen and barbiturates are typical weak acids frequently used in analgesic-antipyretic preparations that very often contain other therapeutic agents. The difference in pKa values permits the differentiation by nonaqueous titration. Data are reported in Table I for the analysis of the individual components as well as for synthetic mixtures. A typical titration curve for the synthetic mixture is shown in Fig. 1. The first end-point corresponds to phenobarbital, and the second end-point is due to acetaminophen.

Since the presence of other components, active or inert, in complex dosage forms may interfere with the differentiating titration, an ion-exchange chromatographic procedure was developed for the separation of acetaminophen and the barbiturate from the other active and inert constituents. Solutions of acetaminophen and phenobarbital, individually and as mixtures, in 50% alcohol were subjected to ion-exchange chromatography. The strong anion-exchange resin removes all anions from solution, while all cations and soluble nonionic components appear in the eluate. The eluent, 50% acetic acid in alcohol, displaces the acetaminophen and phenobarbital from the column since the acetic acid is a stronger acid than either component. Other anions such as chloride and sulfate, which represent acids stronger than acetic acid, will not be displaced. The eluate, after removal of the solvent, is titrated nonaqueously as described in the *Experimental* section. Recovery data are shown in Table I. This procedure was then applied to one capsule and two tablet dosage forms.

For Tablet A (Table II), the bromide ion, sulfate ion, and any excipient anions are retained by the resin along with the acetaminophen and phenobarbital. The phenacetin, atropine, scopolamine, hyoscyamine, and any excipient cations and nonionic components appear in the eluate. Elution with acetic acid removes the acetaminophen and phenobarbital. In the case of Tablet B, allobarbital and acetaminophen are readily separated from the dosage form in the manner described. For Capsule A, the butabarbital anion and acetaminophen are retained by the column; the sodium cation appears in the eluate. Mephenesin, which is nonionic, also appears in the eluate. Because of the large ratio of acetaminophen to barbiturate in the dosage forms, it was necessary to use separate aliquots of the powder mass for analysis of each component. A sample size was taken that would require 3–10 ml of titrant for each analysis. For the barbiturate determination, titrations were carried out only to the first end-point; for acetaminophen, the difference between the first and second end-points was used for calculating recovery data.

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* Present address: Pharmacy Service, Hines Veterans Administration Hospital, Hines, IL 60141

x To whom inquiries should be directed.

Analysis of Acetaminophen, Phenylephrine Hydrochloride, Diphenhydramine Hydrochloride, and Ascorbic Acid in a Capsule Preparation

FABRIZIO De FABRIZIO

Abstract □ A column chromatographic procedure was developed for separating acetaminophen, phenylephrine hydrochloride, and diphenhydramine hydrochloride in combination with ascorbic acid. While acetaminophen, which passes through an alginic acid column with the washings, is determined spectrophotometrically at 257 nm, phenylephrine hydrochloride and diphenhydramine hydrochloride are both eluted from the column with 0.01 *N* hydrochloric acid and determined simultaneously at two different wavelengths. Ascorbic acid is analyzed by a visual titration method, employing 2,6-dichloroindophenol as the titrant.

Keyphrases □ Acetaminophen—method for separation and analysis in combination with phenylephrine hydrochloride, diphenhydramine hydrochloride, and ascorbic acid □ Phenylephrine hydrochloride—method for separation and analysis in combination with acetaminophen, diphenhydramine hydrochloride, and ascorbic acid □ Diphenhydramine hydrochloride—method for separation and analysis in combination with acetaminophen, phenylephrine hydrochloride, and ascorbic acid □ Ascorbic acid—method for separation and analysis in combination with acetaminophen, phenylephrine hydrochloride, and diphenhydramine hydrochloride

Acetaminophen, phenylephrine hydrochloride, diphenhydramine hydrochloride, and ascorbic acid are used alone and in combination with other drugs in various pharmaceutical preparations. Several methods are available (1–7) for the analysis of these therapeutically active ingredients as pure substances and in combination. Smith (8) recently described a method for the separation and determination of sympathomimetic amines, antihistamines, and phenothiazines in various mixtures using polystyrene cation and quaternary ammonium anion resins packed into two assembled columns. Although this method might have served as the basis for the present study, a previously reported analytical procedure (9), which employed an alginic acid column with UV spectrophotometry and which was adequate for the separation and determination of acetaminophen, phenylephrine hydrochloride, codeine phosphate, and pyrilamine maleate in tablets or powder, was chosen. Therefore, the present report concerns the feasibility of utilizing alginic acid (10) for cation exchange in the analysis of pharmaceutical products. This investigation found that acetaminophen and ascorbic acid were not retained by the alginic acid but passed through the prepared column; phenylephrine hydrochloride and diphenhydramine hydrochloride were both eluted with 0.01 *N* hydrochloric acid and were simultaneously determined at 236 and 273 nm. Finally, ascorbic acid was analyzed by a visual titration method in an aliquot of the original solution using 2,6-dichloroindophenol.

Table I—Recovery of Phenylephrine Hydrochloride and Diphenhydramine Hydrochloride Mixed Standard in 50% Ethanol in 0.5% (v/v) Acetic Acid

	Phenylephrine Hydrochloride		Diphenhydramine Hydrochloride	
Mixture	Amount Taken, mg	Recovery, %	Amount Taken, mg	Recovery, %
1	7.5	98.3	15.0	98.3
2	7.5	101.8	15.0	99.5
3	15.0	99.4	30.0	101.4
4	15.0	99.1	30.0	98.2
5	7.5	98.7	15.0	98.3
6	15.0	100.5	30.0	101.2
7	7.5	101.3	15.0	100.7
8	7.5	101.4	15.0	98.6
9	15.0	99.6	30.0	98.3
10	7.5	99.4	15.0	98.8
Average, %	—	99.9	—	99.8
SD, %		±1.23		±1.29

EXPERIMENTAL

Apparatus—A recording spectrophotometer[1] with matched UV

[1] Beckman DB.

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Acetaminophen, phenylephrine hydrochloride, diphenhydramine hydrochloride, and ascorbic acid are used alone and in combination with other drugs in various pharmaceutical preparations. Several methods are available (1–7) for the analysis of these therapeutically active ingredients as pure substances and in combination. Smith (8) recently described a method for the separation and determination of sympathomimetic amines, antihistamines, and phenothiazines in various mixtures using polystyrene cation and quaternary ammonium anion resins packed into two assembled columns. Although this method might have served as the basis for the present study, a previously reported analytical procedure (9), which employed an alginic acid column with UV spectrophotometry and which was adequate for the separation and determination of acetaminophen, phenylephrine hydrochloride, codeine phosphate, and pyrilamine maleate in tablets or powder, was chosen. Therefore, the present report concerns the feasibility of utilizing alginic acid (10) for cation exchange in the analysis of pharmaceutical products. This investigation found that acetaminophen and ascorbic acid were not retained by the alginic acid but passed through the prepared column; phenylephrine hydrochloride and diphenhydramine hydrochloride were both eluted with 0.01 *N* hydrochloric acid and were simultaneously determined at 236 and 273 nm. Finally, ascorbic acid was analyzed by a visual titration method in an aliquot of the original solution using 2,6-dichloroindophenol.

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2	7.5	101.8	15.0	99.5
3	15.0	99.4	30.0	101.4
4	15.0	99.1	30.0	98.2
5	7.5	98.7	15.0	98.3
6	15.0	100.5	30.0	101.2
7	7.5	101.3	15.0	100.7
8	7.5	101.4	15.0	98.6
9	15.0	99.6	30.0	98.3
10	7.5	99.4	15.0	98.8
Average, %	—	99.9	—	99.8
SD, %		±1.23		±1.29

EXPERIMENTAL

Apparatus—A recording spectrophotometer[1] with matched UV

[1] Beckman DB.

Table II—Recovery of Acetaminophen, Phenylephrine Hydrochloride, Diphenhydramine Hydrochloride, and Ascorbic Acid from Synthetic Mixtures in the Presence of the Excipient

Mixture	Acetaminophen		Phenylephrine Hydrochloride		Diphenhydramine Hydrochloride		Ascorbic Acid	
	Added, mg	Recovery, %	Added, mg	Recovery, %	Added, mg	Recovery, %	Added, mg	Recovery, %
A	590.6	98.6	8.8	99.2	15.7	98.3	74.7	98.9
B	600.0	99.0	7.9	101.3	15.4	99.5	75.3	98.3
C	584.3	98.9	8.0	98.0	15.8	101.4	75.3	99.2
D	599.1	99.8	7.3	100.5	14.7	98.2	75.4	97.9
E	600.0	100.3	7.9	99.7	15.2	98.3	74.9	100.9
F	579.9	98.7	7.5	101.3	14.9	101.2	75.1	99.0
G	589.3	99.8	7.4	98.7	15.8	100.7	75.8	98.3
H	578.4	98.2	8.7	101.4	15.3	98.7	75.4	98.6
I	595.5	98.9	8.1	99.0	15.6	98.3	74.3	99.1
J	577.9	101.0	8.4	100.0	14.8	98.8	75.1	98.8
Average, %		99.3		99.9		99.3		98.8
SD, %		±0.87		±1.29		±1.29		±0.80

1- and 4-cm cells was used. The glass column, 30 × 1.8 cm with a 5-cm stem, was fitted with a buret key.

Reagents—The following were used: alginic acid[2] cation-exchange resin, 40–100 mesh; 0.01 *N* hydrochloric acid in water; 2 *N* hydrochloric acid in water; 0.1 *N* sodium hydroxide in water; 50% ethanol in 0.5% (v/v) acetic acid in water; and 0.5% 2,6-dichloroindophenol in water.

Standard Solutions—The following were used: acetaminophen, 0.006 mg/ml in 0.01 *N* sodium hydroxide; phenylephrine hydrochloride, 0.015 mg/ml in 0.01 *N* hydrochloric acid; diphenhydramine hydrochloride, 0.03 mg/ml in 0.01 *N* hydrochloric acid; and ascorbic acid, 1.5 mg/ml in 50% ethanol in 0.05% (v/v) acetic acid.

Column Preparation—About 4 g alginic acid was slurried in water and allowed to soak 4 hr. The slurry was poured into a glass column and allowed to settle. The column was washed with 2 *N* hydrochloric acid until the absorbance of the eluate (path length 4 cm) was less than 0.005 at 235 and 273 nm, and it was then washed with distilled water until the eluate was neutral to litmus solution. Finally, 15 ml of 50% ethanol was passed through the column.

Sample Treatment—The average weight of 20 capsules was determined, and an accurately weighed portion of powder, equivalent to about 1.5 capsules[3], was transferred to a 50-ml volumetric flask. Then 50% ethanol–acetic acid solution was added to the mark. The mixture was stirred for 3 min and then centrifuged for 2 min.

Determination—*Ascorbic Acid*—Dilute 1 ml of standard ascorbic acid solution (containing 1.5 mg ascorbic acid) in a small erlenmeyer flask with 10 ml of 50% ethanol–acetic acid solution. Titrate with the dye solution to a pink color, which persists for 15 sec. Since this volume of dye represents 1.5 mg ascorbic acid, the ascorbic acid equivalent of 1 ml of dye solution is equal to 1.5 divided by the volume in milliliters of the dye solution in this titration.

Dilute a 1-ml aliquot of the centrifuged sample solution in a small erlenmeyer flask with 10 ml of solvent and titrate immediately with the standard dye solution to a faint pink end-point, which persists for 15 sec.

Acetaminophen—Pipet a 10-ml aliquot of the centrifuged sample solution onto the prepared alginic acid column. Place a 500-ml volumetric flask beneath the column and start collecting the eluate at a rate of 1 ml/min. Then add 200 ml water divided into four portions, allowing each portion to sink into the resin. Remove the flask and dilute to volume with water. Dilute further with 0.01 *N* sodium hydroxide to give a concentration of acetaminophen equal to about 0.006 mg/ml. Scan the sample and standard solutions between 360 and 220 nm in 1-cm cells against 0.01 *N* sodium hydroxide as the reference. Determine the absorptivity at the maximum (about 257 nm) for acetaminophen standard and calculate the concentration of acetaminophen in the sample solution.

Phenylephrine Hydrochloride and Diphenhydramine Hydrochloride—Elute the column with 0.01 *N* hydrochloric acid at a rate of 2 ml/min. Discard the first 10 ml of eluate, collecting the rest in a 100-ml volumetric flask to volume. This solution contains phenylephrine and diphenhydramine. Determine the absorbances of the sample solution at 236 and 273 nm in 4-cm cells against 0.01 *N* hydrochloric acid as reference. Determine the absorptivities at the same wavelengths for both phenylephrine hydrochloride and diphenhydramine hydrochloride standard and calculate their concentrations in the sample solution.

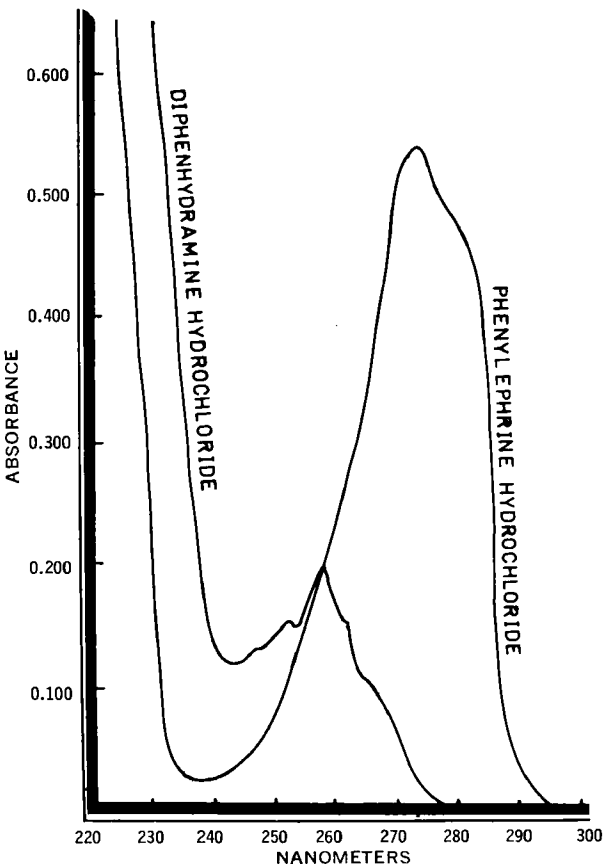

Figure 1—*Spectra of 0.015 mg/ml of phenylephrine hydrochloride and of 0.03 mg/ml of diphenhydramine hydrochloride in 0.01* N *HCl.*

[2] British Drug Houses.

[3] The analysis was applied to capsules containing 400 mg acetaminophen, 5 mg phenylephrine hydrochloride, 10 mg diphenhydramine hydrochloride, and 50 mg ascorbic acid; these capsules were marketed as "Flustop."

RESULTS AND DISCUSSION

The presented method reaffirms that ion-exchange chromatography is an excellent general procedure for quantitatively separating alkaloid bases and organic acids. Initially, the choice of the most suitable solvent for readily dissolving all of the active ingredients as well as providing a solution with pH 3–4 (the optimum pH range for alginic acid absorption) was important. An equivalent mixture of 0.5% (v/v) acetic acid in water and ethanol was the most appropriate. Although this study did not present particular difficulties because a previous method served as its basis, attention was focused on the possibility of finding two different wavelengths where the interferences of phenylephrine hydrochloride and diphenhydramine hydrochloride, contained in the same eluate, were as low as possible. Several standard solutions, containing these two active ingredients in the same weight ratio as in the commercial formulation, were subjected to a spectrophotometric test at 285 nm (peak for diphenhydramine hydrochloride), 273 nm (peak for phenylephrine hydrochloride), 252, 243, and 236 nm. It was found that the most favorable results were obtained by using wavelengths at 273 and 236 nm, which correspond to the maximum and the minimum absorbances for phenylephrine hydrochloride, respectively (Fig. 1). The results of 10 determinations of mixtures containing phenylephrine hydrochloride are shown in Table I.

In the determination of acetaminophen, the effects of ascorbic acid, starch[4], and the alcoholic-acetic acid solvent which passed through the alginic acid column together with acetaminophen were investigated. Standard mixtures of these three ingredients in an alcoholic-acetic solution were subjected to the described procedure. It was found that they did not affect the results at 257 nm because they were in such high dilution. The accuracy of the proposed method was based upon results of 10 synthetic mixtures containing all ingredients and prepared in a manner similar to commercial formulations (Table II).

In the quantitative evaluation of ascorbic acid, the use of 2,6-dichloroindophenol yielded reasonable results. This titrant did not interfere with the other components present in this formulation. The possibility of utilizing other methods for the determination was not investigated.

This method provides a simple and rapid means by which the concentration of each component of this preparation is quantitatively determined.

[4] Starch was used as the excipient.

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Quantitative Determination of Resorcinol and Phenol in Resorcinol–Phenol–Boric Acid Solution

V. DAS GUPTA[x] and LINDLEY A. CATES

Abstract □ The quantitative determination of resorcinol and phenol in resorcinol–phenol–boric acid solution is reported. They are assayed by combining a bromination technique with NMR spectroscopy. There is no interference from other ingredients of the solution (acetone, alcohol, boric acid, and water).

Keyphrases □ Resorcinol and phenol in resorcinol–phenol–boric acid solution—analysis, bromination technique and NMR spectroscopy □ Phenol and resorcinol in resorcinol–phenol–boric acid solution—analysis, bromination technique and NMR spectroscopy □ NMR spectroscopy—analysis, phenol and resorcinol in resorcinol–phenol–boric acid solution

A colorless resorcinol–phenol–boric acid solution is used externally as an antifungal preparation. No method for analyzing resorcinol and phenol quantitatively in each other's presence has been reported. Most available methods (1, 2) such as the bromination technique and UV spectroscopy are useful for the combined determination of both ingredients. Two GLC procedures (3, 4) for the quantitative determination of phenolic compounds have also been reported. A method was suggested for the quantitative determination of resorcinol in Castellani's paint by condensing the former with 4-dimethylaminobenzaldehyde and measuring the intense violet color in acetone (5). Recently, the quantitative determination of resorcinol monoacetate in creams and lotions by GLC was reported (6).

This paper reports the quantitative determinations of resorcinol and phenol in resorcinol–phenol–boric acid solution. The method is based on the bromination technique for combined results. The individual quantities of resorcinol and phenol are then computed from the bromination value and the molar ratio obtained from the NMR spectroscopic analysis.

EXPERIMENTAL

Chemicals and Reagents—All chemicals and reagents were USP, NF, or ACS grade.

Preparation of Resorcinol–Phenol–Boric Acid—The solution

RESULTS AND DISCUSSION

The presented method reaffirms that ion-exchange chromatography is an excellent general procedure for quantitatively separating alkaloid bases and organic acids. Initially, the choice of the most suitable solvent for readily dissolving all of the active ingredients as well as providing a solution with pH 3–4 (the optimum pH range for alginic acid absorption) was important. An equivalent mixture of 0.5% (v/v) acetic acid in water and ethanol was the most appropriate. Although this study did not present particular difficulties because a previous method served as its basis, attention was focused on the possibility of finding two different wavelengths where the interferences of phenylephrine hydrochloride and diphenhydramine hydrochloride, contained in the same eluate, were as low as possible. Several standard solutions, containing these two active ingredients in the same weight ratio as in the commercial formulation, were subjected to a spectrophotometric test at 285 nm (peak for diphenhydramine hydrochloride), 273 nm (peak for phenylephrine hydrochloride), 252, 243, and 236 nm. It was found that the most favorable results were obtained by using wavelengths at 273 and 236 nm, which correspond to the maximum and the minimum absorbances for phenylephrine hydrochloride, respectively (Fig. 1). The results of 10 determinations of mixtures containing phenylephrine hydrochloride are shown in Table I.

In the determination of acetaminophen, the effects of ascorbic acid, starch[4], and the alcoholic-acetic acid solvent which passed through the alginic acid column together with acetaminophen were investigated. Standard mixtures of these three ingredients in an alcoholic-acetic solution were subjected to the described procedure. It was found that they did not affect the results at 257 nm because they were in such high dilution. The accuracy of the proposed method was based upon results of 10 synthetic mixtures containing all ingredients and prepared in a manner similar to commercial formulations (Table II).

In the quantitative evaluation of ascorbic acid, the use of 2,6-dichloroindophenol yielded reasonable results. This titrant did not interfere with the other components present in this formulation. The possibility of utilizing other methods for the determination was not investigated.

This method provides a simple and rapid means by which the concentration of each component of this preparation is quantitatively determined.

[4] Starch was used as the excipient.

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This paper reports the quantitative determinations of resorcinol and phenol in resorcinol–phenol–boric acid solution. The method is based on the bromination technique for combined results. The individual quantities of resorcinol and phenol are then computed from the bromination value and the molar ratio obtained from the NMR spectroscopic analysis.

EXPERIMENTAL

Chemicals and Reagents—All chemicals and reagents were USP, NF, or ACS grade.

Preparation of Resorcinol–Phenol–Boric Acid—The solution

Table I—Assay of Resorcinol–Phenol–Boric Acid Solution

Experiment	Total Volume of 0.1 *N* Bromine Solution Used	Molar Ratio of Resorcinol–Phenol Found[a]	Average Volume of Solution Used against	
			Resorcinol[b]	Phenol[b]
1	20.62	1.920:1		
2	20.53	1.919:1		
3	20.65	1.912:1		
Average	20.60 (99.09%)[c]	1.917:1 (Calc. 1.899:1)	13.54	7.06
Average deviation (%)	0.23	0.17	(98.41%)	(98.47%)

[a] Average of at least four integrations as obtained by NMR spectroscopy. [b] The equivalent weights of both (resorcinol and phenol) equal one-sixth of their molecular weights. [c] The solution contains 10% resorcinol and 4.5% phenol.

was prepared according to directions in NF X (7) for Castellani's paint, except that no dye was added since many practitioners prefer this solution without the dye.

Determination of Total Volume of 0.1 *N* Bromine Solution Required for Analysis of Combined Phenol and Resorcinol—A 5.0-ml portion of resorcinol–phenol–boric acid was diluted to 100.0 ml with water. Then a 5.0-ml portion of the diluted solution was transferred to a glass-stoppered bottle. The remainder of the procedure was the same as previously reported (1), starting with: "Add 30 ml. of 0.1 *N* bromine . . .." Results are presented in Table I.

Determination of Molar Ratio of Phenol–Resorcinol Present in Resorcinol–Phenol–Boric Acid—Samples (0.8 ml) of solution were pipeted into an analytical NMR tube and examined[1] from δ 6 to 8, with peak field position being referred to tetramethylsilane

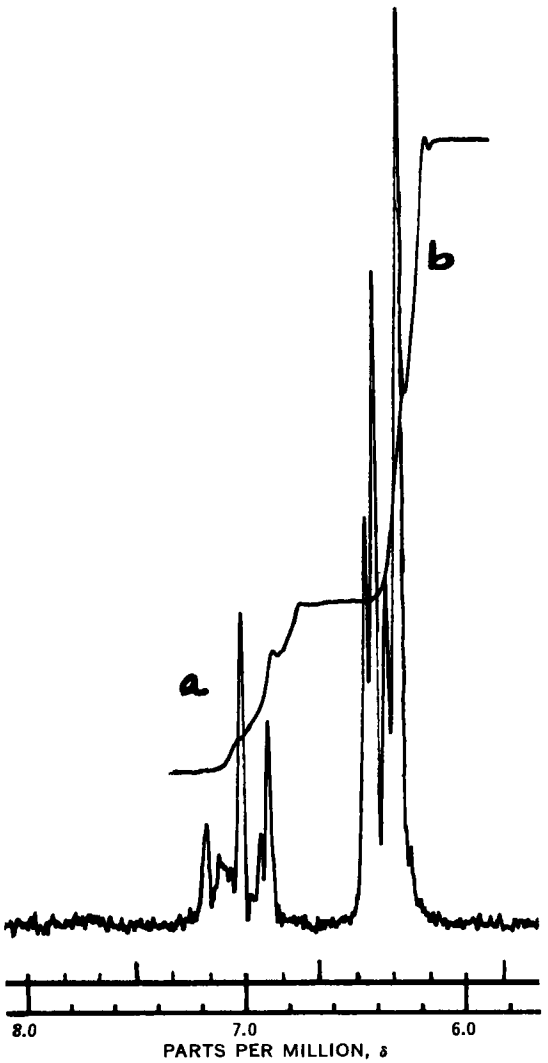
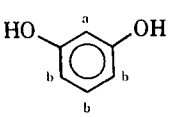


Figure 1—*NMR spectrum of resorcinol in deuterium oxide.*



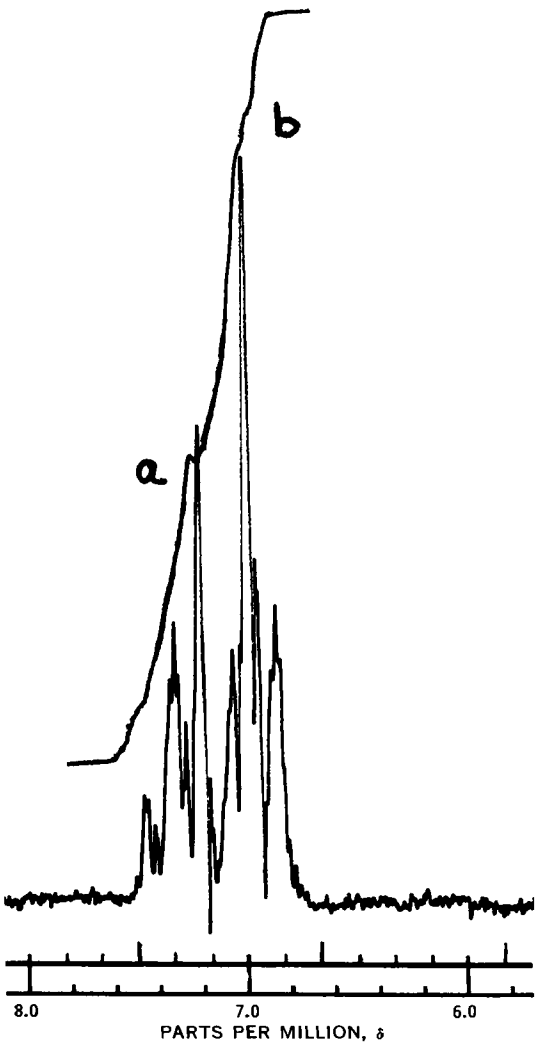
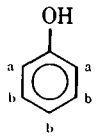


Figure 2—*NMR spectrum of phenol in deuterium oxide.*



[1] Varian T-60 NMR spectrometer.

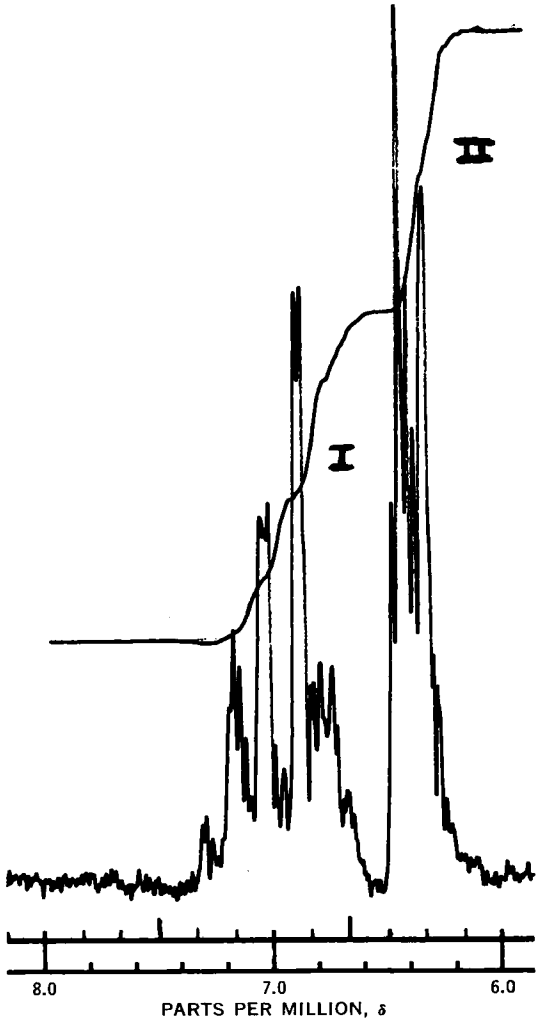


Figure 3—*NMR spectrum of resorcinol–phenol–boric acid solution. Key: I, integral for the C-2 proton of resorcinol and the five protons of phenol; and II, integral for the C-4, C-5, and C-6 protons of resorcinol.*

at 0 ppm. The peaks of interest were integrated at least four times, and the average was used to calculate the molar ratios of resorcinol to phenol as follows:

$$\text{resorcinol} = \frac{A_r}{3} \qquad \text{(Eq. 1)}$$

$$\text{phenol} = \frac{[A_{pr} - (A_r/3)]}{5} \qquad \text{(Eq. 2)}$$

where A_r = integral value of the signal representing three of the four aromatic protons in resorcinol, and A_{pr} = integral value of the signal representing the aromatic protons in phenol and one resorcinol aromatic proton. Results are presented in Table I.

DISCUSSION AND CONCLUSION

The results (Table I) indicate that resorcinol and phenol can be assayed when combined together. The combined result on phenol and resorcinol and the result on phenol are slightly low, probably due to slight impurities in the phenol.

Table II—Pure Mixtures of Resorcinol and Phenol Using NMR Spectra Taken in Deuterium Oxide[a]

Sample	Molar Ratio of Resorcinol to Phenol	
	Known	Found
1	1:1	12.33:12.42
2	1:1	11.33:11.27
3	2:1	12.2:6.1
4	1:2	6.3:12.5

[a] It is not necessary to use deuterium oxide because water gives similar results.

The ability to determine relative amounts of resorcinol and phenol in a mixture is based on the chemical shifts produced by certain protons contained in these similar substances. As seen in Fig. 1, resorcinol gives two NMR signals which are assigned to the more highly deshielded C-2 proton and the three remaining protons whose multiplet occurs more upfield. However, all five protons in phenol give a signal[2] at a downfield position (Fig. 2). Thus, in a mixture of resorcinol and phenol, there appears to be sufficient difference between the absorptions produced by the five aromatic protons of phenol plus the C-2 proton of resorcinol (about δ 6.6–7.6) and the C-4, C-5, and C-6 protons of resorcinol (about δ 6.0–6.6) to permit determination of their relative peak areas and, therefore, calculation of their molar ratios. To test this concept, several known resorcinol–phenol mixtures of varying concentration in deuterium oxide were examined. The results (Table II) indicate that the procedure was applicable. It was later found that the other ingredients in the product (acetone, ethanol, boric acid, and water) do not interfere with the ratio determination and, although there are slight differences in spectra due to solvent effect, the pertinent data can be obtained as well from the solution *per se* (Fig. 3) as from samples prepared (*i.e.*, using deuterium oxide as a solvent) for this product.

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[x] To whom inquiries should be directed.

[2] Although phenol actually gives two signals, integrating for two (C-2 and C-6) and three (C-3, C-4, and C-5) protons, this is not important to this analysis.

Determination of Purity of Levodopa and Levodopa Products by GLC

J. R. WATSON

Abstract □ A simple GLC procedure for the detection and quantitation of possible impurities in levodopa raw material and commercial dosage forms is presented. The sample is reacted with *N,O*-bis(trimethylsilyl)acetamide in acetonitrile at ambient temperature for 30 min, and the silyl derivatives are separated on a methyl silicone column under isothermal conditions. Peak areas are measured using an electronic digital integrator of wide input signal range capacity. The results obtained by applying the procedure to the analysis of several multicomponent simulated mixtures were in good agreement with theoretical values. Only 3-methoxy-4-hydroxyphenylalanine was detected as a contaminant in each of the five commercial products examined.

Keyphrases □ Levodopa bulk, capsules, and tablets—GLC analysis, 3-methoxy-4-hydroxyphenylalanine detected in commercial products □ 3-Methoxy-4-hydroxyphenylalanine—detected as impurity in commercial levodopa capsules and tablets, GLC □ GLC—analysis, levodopa bulk, capsules, and tablets

Since the institution of levodopa (L-3,4-dihydroxyphenylalanine) as a potent agent for the long-term therapy of Parkinson's disease, several methods have been proposed for its quantitative determination in the presence of its metabolic products in biological media. Urinary and plasma levodopa levels were measured by derivatization of the purified extract of the drug to the *N*-trifluoroacetyl-*n*-butyl ester followed by GLC using electron-capture detection (1). The major metabolite in plasma, 3-methoxy-4-hydroxyphenylalanine, was determined with a modified amino acid analyzer technique (2), while Curzon *et al.* (3) and Sharpless *et al.* (4) quantitated both this compound and the parent levodopa in plasma and in cerebrospinal fluid (5) by fluorometry. A colorimetric assay method for levodopa based on its reaction with isonicotinic acid hydrazide (isoniazid) in alkaline medium was also reported (6). However, none of these methods was really designed for the routine purity screening of the drug and of its pharmaceutical products.

In view of the several routes of synthesis currently being used for the manufacture of levodopa, a number of possible by-products, with unknown biological activity, could be associated with the drug. This, coupled with the shortcomings of other methods, points to a clear requirement for an analytical procedure that would embody the virtues of sensitivity, rapidity, specificity, and amenability to a multiple-analysis setup for the quantitative estimation of levodopa contaminants.

Since several authors reported facile quantitative silylation and successful GLC of amino acids (7–9) and of catecholamines (10, 11), it seemed reasonable to expect levodopa and its intermediates, which, in general, combine the structural features of both amino acids and catechols, to undergo similar transformations to yield silylated products which could be eluted easily.

This paper reports a rapid GLC method suitable for the routine quality control detection and quantitation of tyrosine, 3,4-dimethoxyphenylalanine, 3-methoxy-4-hydroxyphenylalanine, 3-hydroxy-4-methoxyphenylalanine, and 3,4,6-trihydroxyphenylalanine (6-hydroxydopa) in the levodopa raw material and in commercial products. The procedure involves silylation of the drug or drug mixture with *N,O*-bis(trimethylsilyl)acetamide in acetonitrile at room temperature for 30 min prior to isothermal chromatography on a methyl silicone column and measurement of the component peak areas by electronic digital integration.

EXPERIMENTAL

Materials—The following were used: 3,4-dimethoxyphenylalanine[1], 3-hydroxy-4-methoxyphenylalanine[1], L-tyrosine[2], levodopa[3], L-3-methoxy-4-hydroxyphenylalanine (monohydrate)[4], and DL-6-hydroxydopa (monohydrate)[5].

Preparation of Samples—*Response Factor Calibration Mixtures*—For each intermediate, varying quantities ranging from 1 to 4 mg were accurately weighed into four separate 10-ml volumetric flasks, each containing an accurately known amount of levodopa (50–70 mg).

Simulated Mixtures—Appropriate amounts of each desired component (0–4 mg) were accurately weighed into a 10-ml volumetric flask along with a precisely known quantity of levodopa (50–70 mg).

Commercial Products—The formulations were sampled as follows: 10 capsules were emptied and their contents were weighed and thoroughly mixed. In the case of the tablets, 10 were selected at random, weighed, and finely powdered. An amount of powder equivalent to about 60–70 mg of levodopa was accurately weighed into a 10-ml volumetric flask.

Derivatization Procedure—Each sample of drug or drug mixture was treated with *N,O*-bis(trimethylsilyl)acetamide reagent[6] (2.5 ml), diluted to volume with "silylation grade" acetonitrile[6], and allowed to stand for 30 min in the dark at room temperature with occasional shaking. At the end of this period, the oily globules dissolved to give a clear homogeneous solution or, in the case of the commercial products, a clear supernate.

Gas Chromatography—Five microliters of solution was injected into a gas chromatograph[7] (flame-ionization detector), fitted with a 5% OV-101[6] on Chromosorb W/HP[8] (100–120 mesh) U-shaped glass column [1.82 m (6 ft) × 6 mm o.d.] preconditioned at 265° for 18 hr. The support was coated using a Hi-Eff[9] fluidizer maintained at 150° for 2 hr with a suitable nitrogen flow to ensure gentle yet thorough drying of the packing material. Temperatures were: column, 170° (isothermal); injection port, 255°; and detector, 260°. Gas flows were: nitrogen, 60 ml/min; hydrogen, 40 ml/min; and air, 380 ml/min.

The detector signal was fed to a continuous balance dual pen,

[1] Courtesy of Norwich Pharmacal Co.
[2] Wyeth Inc.
[3] Monsanto Chemical Co. This material was found by GLC to contain about 0.27% of 3-methoxy-4-hydroxyphenylalanine, but due allowance was made for this in the calculation of the results.
[4] Monsanto Chemical Co.
[5] Chemical Dynamics Corp.
[6] Pierce Chemical Co.
[7] Bendix series 2500.
[8] Chromatographic Specialties Ltd.
[9] Applied Science Laboratories.

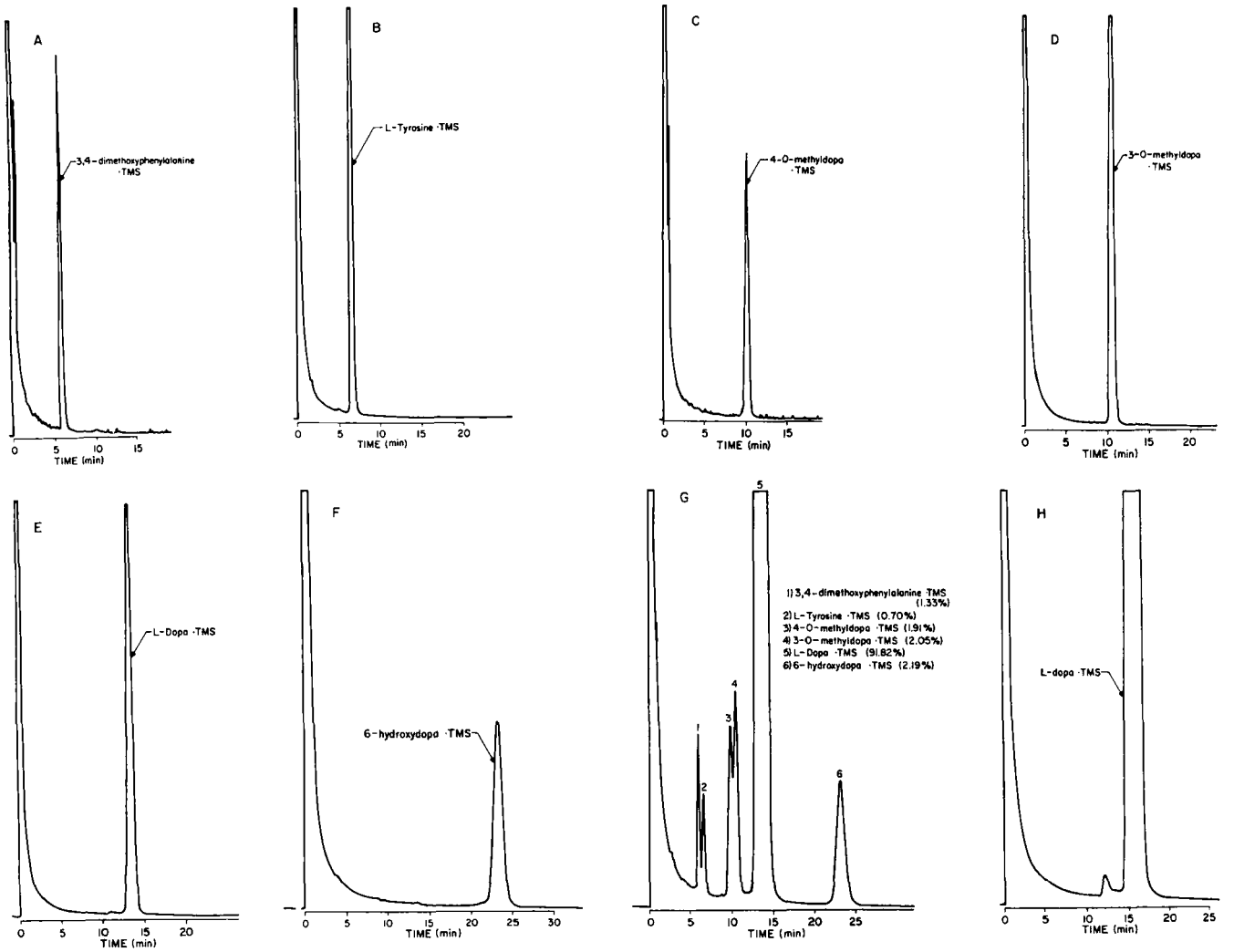

Figure 1—*Gas chromatograms of the trimethylsilyl (TMS) derivatives of levodopa and its possible contaminants. Key: A, pure 3,4-dimethoxyphenylalanine; B, pure* L-*tyrosine; C, pure 3-hydroxy-4-methoxyphenylalanine (4-O-methyldopa); D, pure 3-methoxy-4-hydroxyphenylalanine (3-O-methyldopa); E, pure levodopa* (L-*dopa); F, pure 6-hydroxydopa; G, simulated mixture of components A–F; and H, commercial levodopa product (500-mg capsule) contaminated with 3-O-methyldopa (about 0.25%).*

1-mv recorder[10] with variable chart speed set at 0.508 cm (0.20 in.)/min and connected to an automatic printout electronic digital integrator[11] with a selected input signal range of 0–100 mv. The attenuation was kept constant at 20×10^{-11} afs.

Calculations—The response factor (relative to levodopa) for an impurity I is given by Eq. 1:

$$f_I = \frac{\text{area ratio } (I_s/\text{levodopa}_s)}{\text{weight ratio } (I/\text{levodopa})} = \frac{Y_{I_S}}{X_I} \qquad \text{(Eq. 1)}$$

where the subscript s denotes the silyl derivative. For the computation of the impurity levels in simulated mixtures and commercial dosage forms, the weight ratio X_I is determined by Eq. 2:

$$X_I = \frac{Y_{I_S}}{f_I} \qquad \text{(Eq. 2)}$$

and is expressed as a weight unit fraction of the actual levodopa content. For a mixture consisting of a number of impurity components, $I, J \ldots Z$, the total recovered relative weight units, taking levodopa as unity, is expressed in Eq. 3:

$$1 + X_I + X_J + \ldots + X_Z = T \qquad \text{(Eq. 3)}$$

The level of impurity I can be calculated as a percentage of the total weight of drug found:

$$I = \frac{100X_I}{T} \qquad \text{(Eq. 4)}$$

Similarly:

$$\%J = \frac{100X_J}{T} \qquad \text{(Eq. 5a)}$$

$$\%Z = \frac{100X_Z}{T} \qquad \text{(Eq. 5b)}$$

and:

$$\%\,\text{levodopa} = \frac{100}{T} \qquad \text{(Eq. 5c)}$$

RESULTS AND DISCUSSION

In this study, the most efficacious procedure for the silylation of levodopa and its by-products involved the use of *N,O*-bis(trimethylsilyl)acetamide in acetonitrile. In this system, complete derivatization of levodopa was achieved in about 15 min at room temperature, although it was preferable to allow about 30 min since L-tyrosine and 3,4-dimethoxyphenylalanine, which might be present as contaminants, appeared to require a somewhat longer reaction period. Other solvents such as pyridine or dimethylformamide could be used but, since more time was needed (about 2 hr), they were considered to be less feasible as media for a routine

[10] Minneapolis-Honeywell, Elektronik 19 strip-chart recorder.
[11] Kent, model Chromalog 2.

Table I—GLC Data on Levodopa and Possible Contaminants

Compound	Molecular Weight (Fully Silylated Derivative)	Retention Time of Derivative, min	Relative[a] Retention Time of Derivative	Relative[a] Response Factor of Derivative
3,4-Dimethoxyphenylalanine	369.58	6.15	0.447	0.604 (±5.3%)
L-Tyrosine	397.70	6.80	0.495	0.886 (±1.8%)
3-Hydroxy-4-methoxyphenylalanine	427.73	10.25	0.745	0.754 (±3.4%)
3-Methoxy-4-hydroxyphenylalanine	427.73	10.90	0.793	0.726 (±3.0%)[b]
Levodopa	485.88	13.75	1.000	1.000
6-Hydroxydopa	574.05	23.40	1.702	0.919 (±2.4%)[b]

[a] Relative to levodopa. [b] Calculated for the anhydrous form.

Table II—Detector Linearity Data for Impurity Concentrations[a] in the Range of About 0.1–1%

Compound	Concentration, %	Area Counts	Concentration, %	Area Counts	Concentration, %	Area Counts	Concentration, %	Area Counts	Concentration, %	Area Counts
3,4-Dimethoxyphenylalanine	1.45	13,781	0.73	6911	0.29	2703	0.15	1287	0.07	593
L-Tyrosine	1.07	11,139	0.54	5612	0.21	2208	0.11	1058	0.06	496
3-Hydroxy-4-methoxyphenylalanine	1.62	14,364	0.81	7093	0.32	2796	0.16	1427	0.08	657
3-Methoxy-4-hydroxyphenylalanine	1.96	16,084	0.98	8211	0.39	3370	0.20	1581	0.10	743
6-Hydroxydopa	1.63	16,499	0.82	8147	0.33	3255	0.16	1501	0.08	732

[a] Calculated as a percentage of total weight of drug found.

Table III—Analysis of Simulated Mixtures

Compound	1		2		3		4		5	
	% Calc.	% Found	% Calc.	% Found	% Calc.	% Found	% Calc.	% Found	% Calc.	% Found
3,4-Dimethoxyphenylalanine	1.55	1.45	2.42	2.27	3.18	2.93	—	—	1.33	1.12
L-Tyrosine	0.66	0.57	2.36	2.33	—	—	1.23	1.07	0.70	0.51
3-Hydroxy-4-methoxyphenylalanine	—	—	2.38	2.13	—	—	2.37	2.14	1.91	1.62
3-Methoxy-4-hydroxyphenylalanine	3.83	4.09	—	—	2.38	2.12	—	—	2.05	1.96
Levodopa	93.96	93.89	92.84	93.27	89.33	89.82	94.56	95.16	91.82	92.81
6-Hydroxydopa	—	—	—	—	5.11	5.13	1.84	1.63	2.19	1.98

screening program. Other silylation reagents such as hexamethyldisilazane or trimethylsilylimidazole in toluene were also generally satisfactory, but these systems required a number of hours at reflux temperature before complete derivatization was effected. Although the structures of the derivatives[12] obtained with *N,O*-bis(trimethylsilyl)acetamide were not elucidated, the preponderance of evidence in the literature (7–11) suggests that, with this reagent and its fluorinated analog, silylation at all vulnerable sites to give the silylamine–silyl ester normally occurs with amino acids. However, it is known that *N*-silylated derivatives, like other silylamines, hydrolyze very readily and must be carefully protected from moisture. Therefore, it is conceivable that, while the fully *N,O*-silylated compound might be formed *in situ* under the present conditions, it suffers hydrolysis in the syringe or at the injection port to afford the fully *O*-silylated product (with free amino group). In any case, with the proposed method, one sharp GLC peak was obtained for the silyl derivative of each intermediate and of levodopa itself (Figs. 1A–1F). An unambiguous separation of the components of a synthetic mixture of six compounds is shown in Fig. 1G.

As well as conferring increased volatility and thermal stability, the replacement of the active hydrogen atoms by trimethylsilyl groups has the additional effect of eliminating, or at least substantially reducing, the polarity of the molecule. Intermolecular adsorptive interactions between relatively nonpolar solutes and nonpolar stationary phases, such as the methyl silicone used in this study, are rather weak so that one can usually safely assume a strictly molecular weight-dependent elution order. That this is the case with silylated hydroxyphenylalanines on OV-101 is clearly illustrated in Table I, which lists the retention time and relative (to levodopa) retention time of each compound in ascending order of molecular weight calculated as the fully *N,O*-silylated derivative. For the chromatography of these compounds, it was necessary to maintain the injection port temperature above 250°. At lower temperatures, incomplete elution of the less volatile derivatives occurred, resulting in reduced responses and reappearance of these peaks as ghosts or breakdown products which could have invalidated subsequent analyses.

Each response factor (relative to levodopa) in Table I represents the average of four determinations at varying weight ratios of compound to levodopa. The quantities weighed out were selected to represent an impurity range of about 1–10%, even though levels above 1% would not normally be encountered in the analysis of the raw material and of commercial dosage forms. To establish the detector linearity below 1%, appropriate dilutions were made with acetonitrile to give impurity concentrations down to about 0.1%. The area counts of the peaks were found to be proportional to those obtained with the higher levels (Table II). It can be reasonably assumed that the limit of detectability for each component is at least 0.1%. The precision of each measured value is expressed in terms of its coefficient of variation, which is given

[12] Characterization of these compounds using mass spectrometry and other selected techniques is presently in progress.

Table IV—Determination of the Impurity Levels in Commercial Dosage Forms

Sample Number and Dosage Form	Manufacturer	Dosage Level, mg/unit	Percent Impurity[a]
I, capsules	A	250	0.99
	A	250	0.97
II, capsules	A	500	0.35
	A	500	0.35
III, capsules	B	500	0.25
	B	500	0.25
IV, tablets	B	500	0.23
	B	500	0.26
V, capsules	C	250	0.20
	C	250	0.21

[a] Only one impurity, 3-methoxy-4-hydroxyphenylalanine, was detected and is expressed as a percentage of total drug found.

beside its respective response factor value. Theoretically, it is evident that the greater the increase in molecular weight from the underivatized compound to the trimethylsilyl product, relative to the analogous change effected with levodopa, the greater is the detector response. For example, 3,4-dimethoxyphenylalanine with only two available sites for trimethylsilyl attack (carboxylic and amino functions) would give less derivatized product per unit weight and, therefore, a lower response factor than 3-methoxy-4-hydroxyphenylalanine, which has an additional phenolic site, or than levodopa, which can incorporate four trimethylsilyl groups. In this study, the value obtained for 6-hydroxydopa was less than that expected on the basis of five susceptible sites for trimethylsilyl attack. The reason for this anomaly was not investigated but is probably related to some interplay between instrumental parameters and elution characteristics of the compound.

The results of the GLC analyses of five simulated mixtures are given in Table III. Each quoted experimental value represents the average of duplicate injections and is expressed as a percentage of total amount of drug recovered. The theoretical percentages were calculated on a similar basis. Even with mixtures having closely spaced component peaks, such as 3,4-dimethoxyphenylalanine and tyrosine or 3-methoxy-4-hydroxyphenylalanine and 3-hydroxy-4-methoxyphenylalanine, good accuracy was achieved, although in some cases slight underestimations can be noted. While each intermediate examined is indeed a possible contaminant in levodopa samples, more than one impurity was never encountered in any given sample of raw material or finished dosage form. Thus, the inclusion of simulated Mixture 5 (Fig. 1G) was not intended to illustrate a practical situation but simply to demonstrate the feasibility of the procedure as a reliable qualitative and quantitative monitor of purity.

The results of the analyses of five commercial products in both capsule and tablet forms from three manufacturers at two dosage levels are presented in Table IV. A typical chromatogram of such a sample, after silylation of an aliquot of the powder with *N,O*-bis(trimethylsilyl)acetamide reagent in acetonitrile, is shown in Fig. 1H. As readily observed, 3-methoxy-4-hydroxyphenylalanine, but no other impurity, was detected in each product. Of these, Product I had the highest level (about 1%). The three- to fivefold difference existing between the 3-methoxy-4-hydroxyphenylalanine levels in Product I and the four remaining preparations again raises the seemingly ubiquitous question of the importance of sound pharmaceutical workmanship. While the presence of the latter contaminant will not result in a significant loss of therapeutic activity, it may represent a potential hazard (3), especially since levodopa is administered in high doses and chronically.

CONCLUSIONS

There appears to be no entirely adequate method for the detection and quantitation of the impurities in levodopa raw material and commercial dosage forms. To our knowledge, previously proposed GLC methods utilized conversion of the drug to its trifluoroacetylbutyl derivative but, in our experience, this process is not only time consuming but gives rise to a number of reaction by-products which tend to vitiate the area measurements of the relevant peaks. Therefore, there seemed to be a need for a more specific and accurate method suitable for routine screening of the drug, simpler in its manipulative aspects, and, perhaps, adaptable to automation. The present GLC procedure should represent a step forward in this direction.

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Calculation of Plasma Level *versus* Time Profiles for Variable Dosing Regimens

PAUL J. NIEBERGALL[x], EDWIN T. SUGITA, and ROGER L. SCHNAARE

Abstract □ Equations are presented and flow charts are given for obtaining plasma level *versus* time profiles utilizing a high-speed digital computer for drug dosing regimens in which both dose and dosing interval can vary. Examples are given for drugs that can be described by both the one- and two-compartment open models.

Keyphrases □ Dosage regimens—equations for obtaining plasma level-time profiles in which dose and interval vary □ Drug regimens—equations for obtaining plasma level-time profiles in which dose and interval vary □ Pharmacokinetic parameters—one- and two-compartment open model, plasma level-time profiles in which dose and interval vary, equations

The utilization of pharmacokinetic parameters to obtain the optimal dosage regimen for a drug is an important application of biopharmaceutics. Since most drugs are generally given in multiple doses over a period of time, efforts have been devoted toward the development of equations useful for the prediction of: (*a*) plasma levels of drug *versus* time (1–4), (*b*) maximum and minimum plasma levels once equilibrium has been established (5, 6), and (*c*) the average equilibrium plasma level for a drug given in multiple doses at constant dosing intervals. The equations developed by Swintosky *et al.* (6) and by Wiegand *et al.* (3) allow the initial dose to be different from the maintenance doses. However, no equation developed to date allows the drug to be administered at varying time intervals.

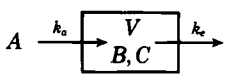
Giving a drug at uniform time intervals equal to its biological half-life ($T_{1/2}$) and using an initial dose twice the maintenance dose (assuming the absorption rate to be much greater than the elimination rate) would give the most nearly optimal dosing regimen with little or no accumulation (8). However, most drugs are not given in this manner. In hospitals, for one reason or another, drugs are frequently given twice (bid), three times (tid), or four times (qid) daily. The most common definitions given for these terms in hospital formularies are: bid = 10–6 (10 am and 6 pm) or 10–10, tid = 10–2–6 or 9–1–5, and qid = 10–2–6–10 or 9–1–5–9. Thus, in the typical hospital, drugs given either tid or qid are given every 4 hr during the day, with 12–16 hr between the last dose of one day and the first dose of the next day. In many instances this is true regardless of the $T_{1/2}$ of the drug. For example, the authors found that in a number of hospital formularies both tetracycline ($T_{1/2}$ of 6–10 hr) (9) and potassium penicillin G ($T_{1/2}$ of approximately 0.5 hr) (10) are directed to be given qid at 10–2–6–10. Family physicians frequently direct drugs to be taken near meal times and possibly also at bed time; drug package inserts frequently contain directions for the drug to be taken three or four times daily, with no definition being given for these terms. Because of the frequency with which drugs are taken at varying dosing intervals, it is of interest to obtain plasma level *versus* time profiles for drugs given under such conditions.

Multiple-dosing regimens can be simulated on analog computers (11–14), and the authors programmed an analog computer[1] to simulate both varying dose and time interval dosing regimens. However, the method is extremely cumbersome. Kirschner *et al.* (15) recently published a program for simulating variable dosing regimens on an analog/hybrid computer which, although it has the advantage of permitting the almost instantaneous viewing of the plasma level *versus* time profile, is expensive and not readily available. Due to the general availability of high-speed digital computers, the authors wrote FORTRAN IV programs for both the one- and two-compartment open models which simulate plasma level *versus* time profiles for dosing regimens in which both dose and dosing interval may be varied at will. Readily available plotting subroutines can be used to give automatic plotting of plasma levels *versus* time. In addition, the programs can be modified readily for drugs whose pharmacokinetic parameters vary with time.

THEORETICAL

The plasma level *versus* time profile for most drugs can be described adequately by viewing the body as consisting of either one or two compartments, with the drug being absorbed, distributed, and eliminated from the body through a series of first-order processes.

One-Compartment Open Model—In Model I, A is the amount of drug at the absorption site, k_a is the absorption rate constant, V is the volume of distribution, B is the amount of drug in the plasma, C is the concentration of drug in the plasma, and k_e is the elimination rate constant. The differential equations for this model are well known and have been solved repeatedly in the literature under the conditions that at time $t = 0$, $A = A_0$ and $B = 0$, which would correspond to the situation when a single dose of



Model I

[1] TR-20, Electronic Associates, Inc. West Long Branch, N.J.

Table I—Definition of Terms Used in Scheme I

Term	Definition
NODOSE[a]	Number of doses administered
OUT[a]	Time increment during the dosing interval for which plasma levels are to be calculated
KA[a]	First-order absorption rate constant
KE[a]	First-order elimination rate constant
AO(I)[a]	FD/V for dose number I
TAU(I)[a]	Dosing interval for dose number I
AEND	Drug at absorption site at the end of a dosing interval
CO	Plasma level at the start of a dosing interval
TIME	Counter for incrementing time
TEND	Time at the end of a dosing interval
I	Counter for number of doses given
NO(I)	Number of times that plasma levels will be calculated for dose number I
TPRIME	Time after the start of a dosing interval
TMAX(I)	Time at which the plasma level will reach a maximum for dose number I
AMAX(I)	Actual total time from the start of the dosing regimen until the plasma level reaches a maximum during dose number I
CMAX(I)	Maximum plasma level during dose number I
J	Counter for number of times the plasma levels will be calculated during a dosing interval
T(I,J)	Time for plasma level number J in dosing interval I
C(I,J)	Plasma level number J in dosing interval I
A(I,J)	Drug at absorption site for reporting time number J in dosing interval I

[a] Input variables.

drug is given to a subject. In a multiple-dosing study, this would be true for only the first dose; at the start of any dose n after the first, at time $t = 0$, $A = A_0$ and $B = B_0$. By using these conditions, a general set of equations was developed which describe both A and B as a function of time during any dosing interval n, for which $n \geq 1$ (Eqs. 1 and 2):

$$A_n = (A_0)_n e^{-k_a t} \qquad \text{(Eq. 1)}$$

$$B_n = \frac{k_a(A_0)_n}{(k_a - k_e)}(e^{-k_e t} - e^{-k_a t}) + (B_0)_n e^{-k_e t} \qquad \text{(Eq. 2)}$$

in which A_0 represents the amount of drug at the absorption site at the start of the dosing interval, t represents time after the start of the dosing interval, and B_0 is the amount of drug in the plasma at the start of the dosing interval. During the first dosing interval, A_0 is equal to the dose given times the fraction of the dose absorbed, and $B_0 = 0$. Under these conditions, Eqs. 1 and 2 reduce to the familiar equations for a single-dose study.

Equation 2 is the equivalent of Eq. 8 of Wiegand *et al.* (3), which was developed for the situation when two doses of drug were given. To solve their Eq. 8 for the situation in which $n > 2$, Wiegand *et al.* had to assume that the dosing intervals remained constant. In the present study, Eq. 2 was developed further through the use of a high-speed digital computer to have the advantage of permitting the dosing intervals τ to vary. This method utilizes the facts that: (*a*) after the first dose has been given, the B_0 term for dosing interval n is equal to the amount of drug in the plasma at the end of dosing interval $n - 1$; (*b*) after the first dose, the term A_0, which represents the amount of drug at the absorption site at the start of dosing interval n, is equal to D, the dose given, times F, the fraction of the dose absorbed, plus the amount of drug remaining at the absorption site from the previous dose or, as given in Eq. 3:

$$(A_0)_n = (FD)_n + (A_0 e^{-k_a \tau})_{n-1} \qquad \text{(Eq. 3)}$$

Thus, Eqs. 1 and 2 are used to calculate both A and B as a function of time during dosing interval $n - 1$. The values of A and B at the end of this dosing interval, $t = \tau$, are saved in the computer memory banks. During dosing interval n, the saved value of B calculated at $t = \tau$ of interval $n - 1$ is recalled from storage and serves as the B_0 term of Eq. 2, in which B as a function of time is calculated for interval n. The value of A calculated at $t = \tau$ of dosing interval $n - 1$ is recalled from storage and added to the FD term for dosing interval n. The sum represents the A_0 term of Eq. 1 for calculating A as a function of time during dose interval n. This process is repeated as many times as necessary and does not depend upon the dosing intervals being constant.

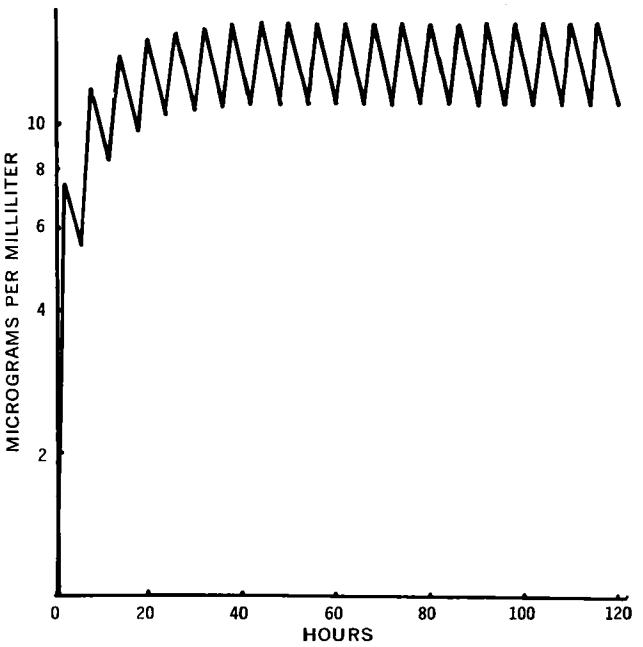


Figure 1—*Plasma level* versus *time profile for a drug with* $k_a = 1.0\ hr^{-1}$, $k_e = 0.116\ hr^{-1}$, *and* $A_0' = 10\ \mu g/ml$. *Drug was administered every 6 hr around the clock.*

The mass of drug in the plasma at any time t after the start of a dosing interval can be calculated using Eqs. 1-3 and converted into plasma levels by dividing by V, the volume of distribution. This necessitates knowing the values of F and V, which are not always available. The collection of terms, FD/V, is readily available from a plot of plasma level *versus* time for a single-dose study and, if the model is correct, is directly proportional to the dose given. Due to the relative ease with which the term FD/V can be obtained, the authors chose to solve directly for plasma concentration. Dividing both sides of Eq. 2 by V gives:

$$C_n = \frac{k_a(A_0')_n}{(k_a - k_e)}(e^{-k_e t} - e^{-k_a t}) + (C_0)_n e^{-k_e t} \qquad \text{(Eq. 4)}$$

in which C_0 is the plasma concentration at the start of the nth dosing interval, and A_0' is equal to A_0/V. The term A_0 in Eqs. 1 and 2 must represent the same quantity in this model. Thus, Eqs. 1 and 3 become:

$$(A')_n = (A_0')_n e^{-k_a t} \qquad \text{(Eq. 5)}$$

and:

$$(A_0')_n = (FD/V)_n + (A_0' e^{-k_a \tau})_{n-1} \qquad \text{(Eq. 6)}$$

The time needed to attain a maximum plasma level for any single dosing interval n can be obtained by differentiating Eq. 4 with respect to time and setting the derivative equal to zero to give:

$$(T_{max})_n = \frac{2.303}{(k_a - k_e)} \log\left(\frac{k_a^2(A_0')_n}{k_e[k_a(A_0')_n + (k_a - k_e)(C_0)_n]}\right) \qquad \text{(Eq. 7)}$$

Substituting Eq. 7 into Eq. 4 gives the maximum plasma concentration attained for dosing interval n:

$$(C_{max})_n = \frac{(A_0')_n k_a}{k_e}\left(\frac{k_a^2(A_0')_n}{k_e[k_a(A_0')_n + (k_a - k_e)(C_0)_n]}\right)^{-\frac{k_a}{(k_a - k_e)}} \qquad \text{(Eq. 8)}$$

Table II—Portion of the Output from the Authors' FORTRAN IV Program Based upon Scheme I

DRUG GIVEN EVERY 6 HOURS AROUND THE CLOCK

DOSE NO. 1 TMAX = 2.44 HRS. ACTUAL = 2.44 HRS.

TIME (HRS.)	PLASMA CONC.	DOSING INTERVAL	CMAX	CMIN
1.00	5.912E 00	6 HRS.	7.538E 00	5.612E 00
2.00	7.439E 00			
3.00	7.424E 00			
4.00	6.906E 00			
5.00	6.257E 00			
6.00	5.612E 00			

DOSE NO. 2 TMAX = 1.98 HRS. ACTUAL = 7.98 HRS.

TIME (HRS.)	PLASMA CONC.	DOSING INTERVAL	CMAX	CMIN
7.00	1.092E 01	6 HRS.	1.191E 01	8.424E 00
8.00	1.191E 01			
9.00	1.141E 01			
10.00	1.045E 01			
11.00	9.415E 00			
12.00	8.424E 00			

DOSE NO. 3 TMAX = 1.81 HRS. ACTUAL = 13.81 HRS.

TIME (HRS.)	PLASMA CONC.	DOSING INTERVAL	CMAX	CMIN
13.00	1.343E 01	6 HRS.	1.417E 01	9.826E 00
14.00	1.414E 01			
15.00	1.339E 01			
16.00	1.222E 01			
17.00	1.099E 01			
18.00	9.826E 00			

During the first dosing interval, $C_0 = 0$, and Eqs. 6 and 7 reduce to the usual equations for T_{max} and C_{max} for a single-dose study.

The flow diagram from which this FORTRAN IV computer program[2] was written is shown in Scheme I, and the computer variables are defined in Table I. The output from the program is a matter of personal preference. A sample of the output used by the authors is shown in Table II.

To illustrate the desirability of being able to vary dosing intervals to correspond to those dosing intervals in actual use, the plasma level profile was generated for a drug with: $k_a = 1.0\ hr^{-1}$, $k_e = 0.116\ hr^{-1}$ ($T_{1/2} = 6$ hr), and $A_0' = 10\ \mu g/ml$ [obtained by dividing by k_a the intercept on the Y-axis of a semilogarithmic plot of concentration *versus* time for a single-dose study and multiplying by $(k_a - k_e)$]. This drug would theoretically be given at its half-life of 6 hr. The plasma level *versus* time profile for this drug, given at 6-hr intervals around the clock, was generated using the computer program based upon Scheme I. Data for the 20th dosing interval obtained using this program and Eq. 13 of Wiegand *et al.* (3) are given in Table III to demonstrate the validity of the method. The complete plasma level *versus* time profile is given in Fig. 1. Since no loading dose was used, the drug accumulates and finally equilibrates with a maximum plasma level of 16.5 $\mu g/ml$ and a minimum plasma level of 11.2 $\mu g/ml$.

This drug given every 6 hr around the clock would be given four times daily. If, however, four times daily is defined as qid as in all formularies examined by the authors, the drug would be given at 4-hr intervals during the day, with the last dose at 9 or 10 pm. Use of the common 10-2-6-10 regimen would result in the plasma level *versus* time profile shown in Fig. 2. The broken lines in Fig. 2 indicate the equilibrium maximum and minimum plasma level attained on the every 6-hr dosing schedule. For 6 hr of every day the plasma level on the 10-2-6-10 dosing schedule is above and for 8 hr it is below the respective daily maximum and minimum plasma levels attained on the every 6-hr dosing schedule. The marked fluctuations demonstrated in Fig. 2 would be undesirable for drugs with narrow ranges between minimal effective and toxic plasma levels (*i.e.*, theophylline and procainamide) but would probably not be important if the range between effective and toxic plasma levels were wide enough. Therefore, the clinical importance of a marked daily fluctuation in plasma levels would have to be assessed for a given drug.

Two-Compartment Open Model—Although Wagner and Metzler (4) showed that for many drugs the one-compartment open model can be used for predicting plasma levels for doses given at constant intervals, the two-compartment open model (Model II)

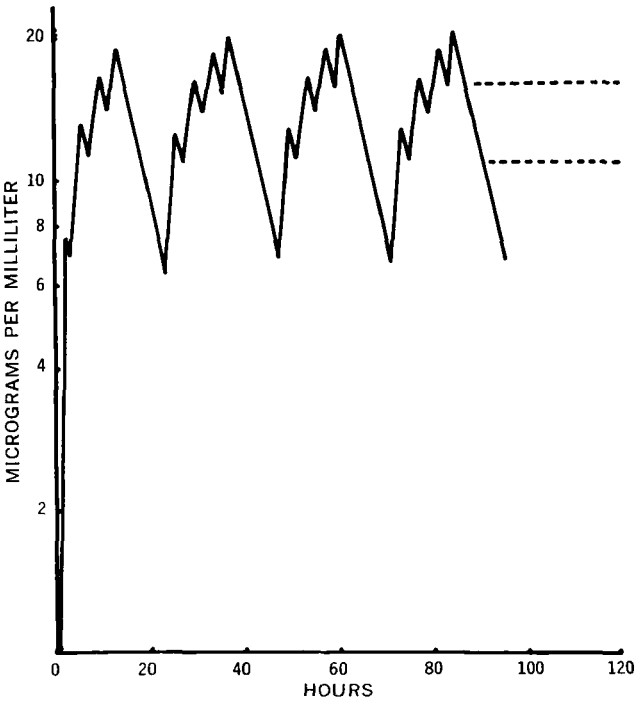
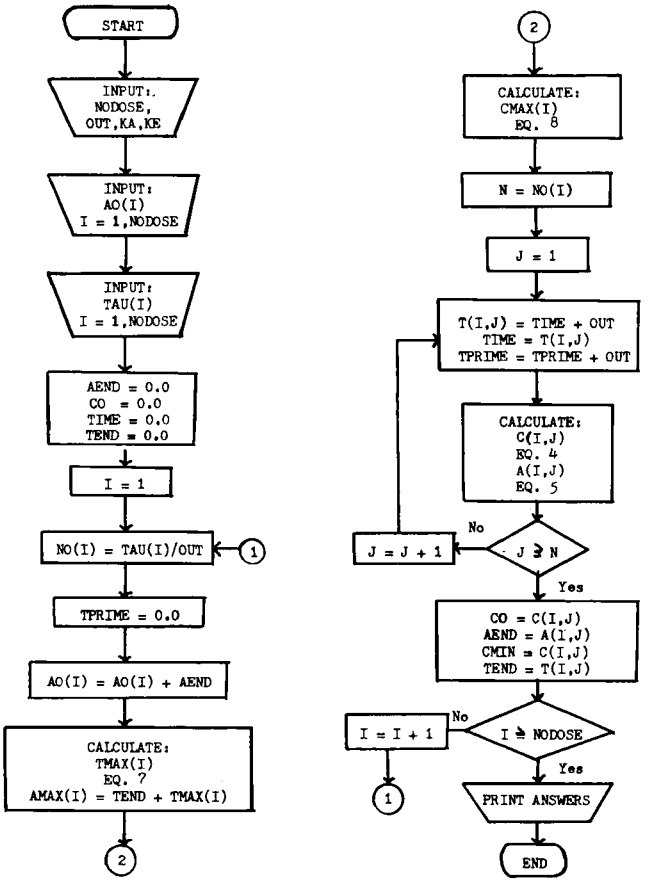


Figure 2—*Plasma level* versus *time profile for a drug with* $k_a = 1.0\ hr^{-1}$, $k_e = 0.116\ hr^{-1}$, *and* $A_0' = 10\ \mu g/ml$. *Drug was administered at 10–2–6–10. Broken lines indicate equilibrium maximum and minimum plasma levels obtained for the same drug given every 6 hr around the clock.*

[2] Available from the authors upon request.



Scheme I—Flow diagram for the one-compartment open model

might have to be used in some instances. In Model II, A represents drug at the absorption site, k_a is the first-order absorption rate constant, k_{bt} and k_{tb} are the first-order disposition rate constants, k_e is the elimination rate constant, M_1 and M_2 represent the amount of drug in each compartment, V_1 and V_2 are the volumes of distribution for each compartment, and C_1 represents the plasma level of the drug. Integrating the differential equations

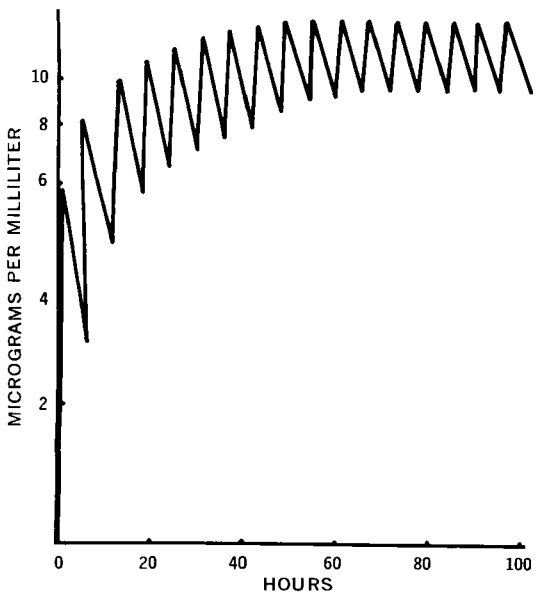
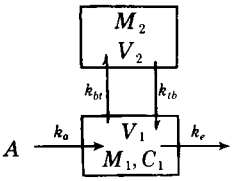


Figure 3—*Plasma level* versus *time profile for a drug with* $k_a = 2.0\ hr^{-1}$, $k_{bt} = 0.226\ hr^{-1}$, $k_{tb} = 0.255\ hr^{-1}$, $k_e = 0.123\ hr^{-1}$, $A_0 = 500$ *mg, and* $V_1 = 60$ *liters. Drug was administered every 6 hr around the clock.*



Model II

describing this model under the conditions that at the start of any given dosing interval n, time $t = 0$, $M_1 = M_1^0$, and $M_2 = M_2^0$ gives:

$$A_n = (A_0)_n e^{-k_a t} \quad \text{(Eq. 9)}$$

$$(M_1)_n = \left(\frac{P_2\alpha - P_1\alpha^2 - P_3}{(\alpha - \beta)(k_a - \alpha)}\right)e^{-\alpha t} + \left(\frac{P_2\beta - P_1\beta^2 - P_3}{(\alpha - \beta)(\beta - k_a)}\right)e^{-\beta t} - \left(\frac{P_1 k_a^2 - P_2 k_a + P_3}{(\beta - k_a)(k_a - \alpha)}\right)e^{-k_a t} \quad \text{(Eq. 10)}$$

$$(M_2)_n = \left(\frac{Q_2\alpha - Q_1\alpha^2 - Q_3}{(\alpha - \beta)(k_a - \alpha)}\right)e^{-\alpha t} + \left(\frac{Q_2\beta - Q_1\beta^2 - Q_3}{(\alpha - \beta)(\beta - k_a)}\right)e^{-\beta t} - \left(\frac{Q_1 k_a^2 - Q_2 k_a + Q_3}{(\beta - k_a)(k_a - \alpha)}\right)e^{-k_a t} \quad \text{(Eq. 11)}$$

in which:

$$P_1 = (M_1^0)_n \quad \text{(Eq. 12)}$$

$$P_2 = (M_1^0)_n(k_a + k_{tb}) + k_{tb}(M_2^0)_n + k_a(A_0)_n \quad \text{(Eq. 13)}$$

$$P_3 = k_a k_{tb}[(A_0)_n + (M_1^0)_n + (M_2^0)_n] \quad \text{(Eq. 14)}$$

$$Q_1 = (M_2^0)_n \quad \text{(Eq. 15)}$$

$$Q_2 = (M_2^0)_n(k_{bt} + k_e + k_a) + k_{bt}(M_1^0)_n \quad \text{(Eq. 16)}$$

$$Q_3 = k_a k_{bt}[(A_0)_n + (M_1^0)_n + (M_2^0)_n] + k_a k_e (M_2^0)_n \quad \text{(Eq. 17)}$$

$$\alpha = \tfrac{1}{2}\left[(k_{bt} + k_e + k_{tb}) + \sqrt{(k_{bt} + k_e + k_{tb})^2 - 4k_{tb}k_e}\right] \quad \text{(Eq. 18)}$$

$$\beta = \tfrac{1}{2}\left[(k_{bt} + k_e + k_{tb}) - \sqrt{(k_{bt} + k_e + k_{tb})^2 - 4k_{tb}k_e}\right] \quad \text{(Eq. 19)}$$

At the start of the first dosing interval, $M_1^0 = M_2^0 = 0$ and $A_0 = FD$, where F is the fraction of the dose D that is absorbed. After the first dose has been given, the terms M_1^0 and M_2^0 at the start of dosing interval n are equal to the amounts of drug left in their respective compartments at the end of dosing interval $n - 1$, and $(A_0)_n$ is given by Eq. 3. Plasma concentrations can be obtained by dividing the amount of drug in Compartment 1 by its volume of distribution or:

$$(C_1)_n = (M_1)_n / V_1 \quad \text{(Eq. 20)}$$

The flow diagram from which the FORTRAN IV computer pro-

Table III—Validity of Scheme I; Data for the 20th Dosing Interval for a Drug with $k_a = 1.0\ hr^{-1}$, $k_e = 0.116\ hr^{-1}$, and $A_o' = 10\ \mu g/ml$

Time after Start of Dose, hr	Plasma Levels, μg/ml	
	Present Work	Literature[a]
0	11.22	11.22
1	15.92	15.92
2	16.35	16.35
3	15.37	15.37
4	13.98	13.98
5	12.55	12.55
6	11.22	11.22

[a] Equation 13 of Wiegand *et al.* (3).

Table IV—Definition of Terms Used in Scheme II

Term	Definition
KA[a]	First-order absorption rate constant
KE[a]	First-order elimination rate constant
KBT[a]	First-order rate constant for transfer from Compartment 1 into Compartment 2
KTB[a]	First-order rate constant for transfer from Compartment 2 into Compartment 1
V1[a]	Volume of distribution for Compartment 1
NODOSE[a]	Number of doses to be administered
OUT[a]	Time increment during the dosing interval for which plasma levels are to be calculated
AO(I)[a]	Dose for interval number I
TAU(I)[a]	Dosing interval for dose number I
M10	Amount of drug in Compartment 1 at the start of a dosing interval
M20	Amount of drug in Compartment 2 at the start of a dosing interval
AEND	Drug at the absorption site at the end of a dosing interval
TIME	Counter for incrementing time
I	Counter for number of doses given
NO(I)	Number of times that plasma levels will be calculated for dose number I
TPRIME	Time after the start of a dosing interval
J	Counter for the number of times the plasma levels will be calculated during a dosing interval
T(I,J)	Time for plasma level number J in dosing interval I
A(I,J)	Drug at absorption site for reporting time number J in dosing interval I
M1(I,J)	Amount of drug in Compartment 1 for reporting time number J in dosing interval number I
M2(I,J)	Amount of drug in Compartment 2 for reporting time number J in dosing interval I
C1(I,J)	Plasma level number J in dosing interval I

[a] Input variables. AO(I) and V1 should be in the appropriate units in order to yield the plasma concentration in the units desired, *i.e.*, AO(I) in milligrams and V1 in liters would give plasma levels in micrograms per milliliter.

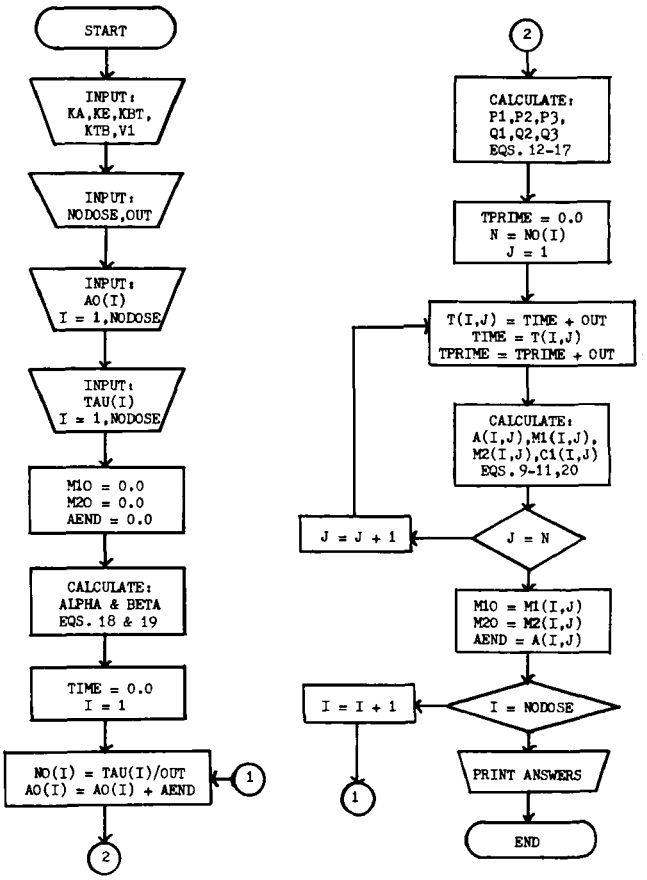
gram was written for this model is shown in Scheme II, and the computer variables are defined in Table IV.

As an example, data were generated for a drug given every 6 hr around the clock with: k_a = 2.0 hr^{-1}, k_{bt} = 0.226 hr^{-1}, k_{tb} = 0.255 hr^{-1}, k_e = 0.123 hr^{-1}, FD = 500 mg (assuming complete absorption), and V_1 = 60 liters. The computer program for the 20th dosing interval is given in Table V. The values obtained are exactly the same as those obtained with Eq. 5 of Wagner and Metzler (4). The complete plasma level *versus* time profile is shown in Fig. 3. Since no loading dose was used, the drug accumulates and finally equilibrates with a maximum plasma level of 13.9 μg/ml and a minimum plasma level of 9.7 μg/ml. The plasma level *versus* time profile was also generated for a 10-2-6-10 dosing regimen (Fig. 4). As with the one-compartment open model, the 10-2-6-10 dosing schedule gave a much more irregular pattern than the schedule in which the drug was given every 6 hr around the clock. Once again, the clinical significance of this would have to be assessed for individual drugs.

SUMMARY

The presented method for generating plasma level *versus* time profiles is general and can be used for studying time-release systems with either zero- or first-order release, constant intravenous infusions, urinary excretion patterns, or any other type of dosing regimen; it is not restricted to the one- or two-compartment open models. Although the authors used a digital computer to generate the plasma level *versus* time profiles, a programmable desk calculator with a sufficient number of program steps and storage registers can be used also. The authors, for example, have programmed a 258-step, 20-storage register calculator[3] for the one-compartment open model.

[3] Model 1775, Monroe Co., Orange, N.J.



Scheme II—Flow diagram for the two-compartment open model

The method can be summarized as follows:

1. Integrate the differential equations describing the model under the condition that at time t = 0, drug is present in each compartment and at the absorption site.

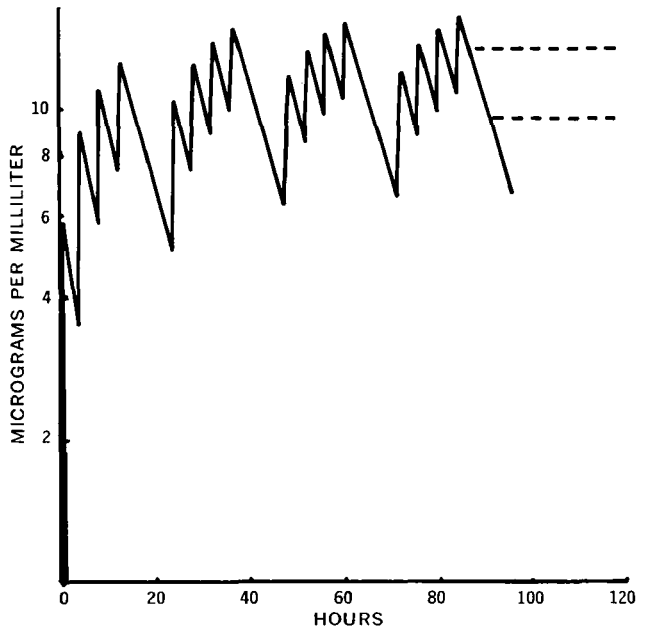


Figure 4—*Plasma level* versus *time profile for a drug with* k_a = *2.0* hr^{-1}, k_{bt} = *0.226* hr^{-1}, k_{tb} = *0.255* hr^{-1}, k_e = *0.123* hr^{-1}, A_0 = *500 mg, and* V_1 = *60 liters. Drug was administered at 10–2–6–10. Broken lines indicate the equilibrium maximum and minimum plasma levels obtained with the same drug given every 6 hr around the clock.*

2. During the first dosing interval, initialize the amount of drug in all compartments to zero. The amount of drug at the absorption site is proportional to the dose given and should be expressed in terms appropriate for the model. For example, in the one-compartment open model, the authors chose to use the collection of terms FD/V to represent the dose given. If this collection of terms is not directly proportional to the dose, it should be determined from a single-dose study at the dose given.

3. Using the integrated expressions, generate the amount of drug in each compartment and at the absorption site as a function of time.

4. At the start of the next dosing interval, initialize the amount of drug in each compartment equal to the amount of drug in that compartment at time $t = \tau$ of the previous dosing interval. The amount of drug at the absorption site is set equal to the sum of the amount of drug remaining at the absorption site at time $t = \tau$ of the previous dosing interval plus the amount given in terms appropriate for the model.

5. Repeat Steps 3 and 4 as often as desired.

In the authors' present digital computer programs, all rate constants are entered once and considered to remain constant throughout the dosing period. The amount of drug given and the length of the dosing interval must be entered for each dosing interval. If any rate constant is known to vary from dose to dose, that rate constant can be entered for each dosing interval in the same manner that the dose given and the dosing interval are entered. This is accomplished through the use of a FORTRAN DIMENSION statement and the use of subscripted variables for the terms in question[4].

NOMENCLATURE

A = amount of drug at the absorption site
A' = A/V, in which V is the volume of distribution
A_0 = amount of drug at the absorption site at the start of a dosing interval
A_0' = A_0/V, in which V is the volume of distribution
B = amount of drug in the plasma for the one-compartment open model
B_0 = amount of drug in the plasma at the start of a dosing interval for the one-compartment open model
C = plasma concentration of drug for the one-compartment open model
C_0 = plasma concentration of drug at the start of a dosing interval for the one-compartment open model
C_1 = plasma concentration for the two-compartment open model
C_{max} = maximum plasma level attained during a dosing interval for the one-compartment open model
D = dose given at the start of a dosing interval
F = fraction of a dose that is absorbed
k_a = first-order absorption rate constant
k_e = first-order elimination rate constant
k_{bt} = first-order rate constant for transfer of drug from Compartment 1 into Compartment 2
k_{tb} = first-order rate constant for transfer of drug from Compartment 2 into Compartment 1
M_1 = amount of drug in Compartment 1 (plasma)
M_1^0 = amount of drug in Compartment 1 at the start of a dosing interval
M_2 = amount of drug in Compartment 2 (tissue)
M_2^0 = amount of drug in Compartment 2 at the start of a dosing interval
n = number of the dosing interval
t = time after the start of a dosing interval
T_{max} = time needed to reach the maximum plasma level for a given dosing interval for the one-compartment open model
V = volume of distribution for the one-compartment open model
V_1 = volume of Compartment 1
V_2 = volume of Compartment 2
τ = length of a dosing interval

[4] Anyone having difficulties in doing this should feel free to contact the authors.

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[x] To whom inquiries should be directed.

Instrument for Producing Standardized Skin Abrasions

THOMAS J. HALEY[x] and JOHN HUNZIKER

Abstract □ An instrument is described which will produce a standardized skin abrasion in the Draize test for skin irritants. The same degree of abrasion without bleeding can be produced on each application.

Keyphrases □ *Skin abrasions—instrument for producing standardized abrasions* □ Abrasions, skin—instrument for producing standardized abrasions

In 1944, Draize *et al.* (1) introduced a method for the study of skin irritation but did not specify an instrument for producing a standard abrasion. Draize (2) indicated that such abrasions should be minor and not produce bleeding. Abrasions have been produced with hypodermic needles, scapels, and other sharp objects, but no standard instrument has been used. This factor was probably involved in the poor correlation between intra- and interlaboratory results of a skin irritation study recently reported (3). To overcome these difficulties, an instrument was designed which produces a standard abrasion for skin irritant studies.

EXPERIMENTAL

The skin abrader and its specifications are illustrated in Fig. 1. The essential features are as follows: the pins must be 0.15 cm (0.06 in.) long and in a straight line; the handle must be flattened for a better hand fit; the angle of the handle must be 72°, and the angle of the handle to the pin base plate must be 10°. The pins can be removed when dull and replaced easily because they are attached with low temperature silver solder. The angle of the handle to the base plate makes certain that the latter lies flat to the skin, thus assuring the same degree of penetration through the stratum corneum as the instrument is pulled across the skin area. Moreover, the length of the pins prevents bleeding while removing the upper skin area when the instrument is drawn firmly but gently over the area to be abraded. The abrasion is produced by three strokes in one direction, followed by three more at a 90° angle to the first ones.

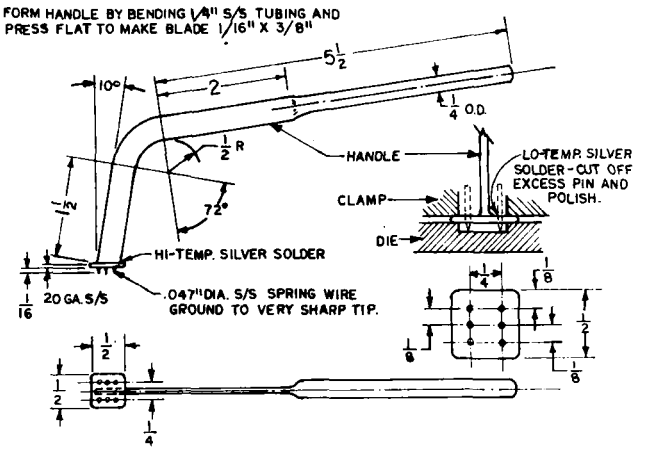

Figure 1—*Specifications for the skin abrader.*

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[x] To whom inquiries should be directed.

Stability of Hydrocortisone in Polyethylene Glycol Ointment Base

ALBERT E. ALLEN and V. DAS GUPTA ˣ

Abstract □ The degradation of 1% hydrocortisone in polyethylene glycol ointment USP was investigated. The stability tests were conducted on two different batches of ointments at 23, 45, and 60° for about 4 months. Decomposition occurred at the C-17 side chain as assayed by the blue tetrazolium reaction, as well as in ring A as determined by UV spectroscopy. The decomposition of the C-17 side chain was much faster (average half-life of 186 days at 60°) as compared with ring A (average half-life of 505 days at 60°). Decomposition of the C-17 chain appeared to be first order but was zero order for ring A. The energy of activation was determined to be 9.7 kcal/mole. Based on these studies, the shelflife for a 1% hydrocortisone ointment in polyethylene glycol base is suggested to be about 6 months. The shelflife may be extended to about 9 months with the addition of a 5% excess of the active ingredient at the time of manufacture.

Keyphrases □ Hydrocortisone ointment—stability, shelflife, polyethylene glycol base □ Polyethylene glycol base—stability, shelflife of hydrocortisone ointment □ Ointment, hydrocortisone—stability, shelflife, polyethylene glycol base

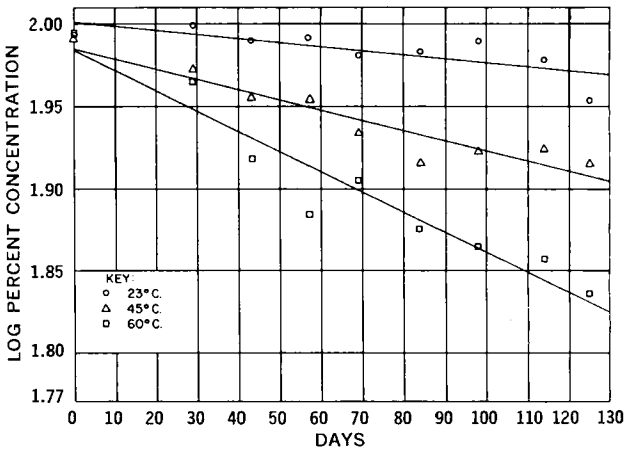


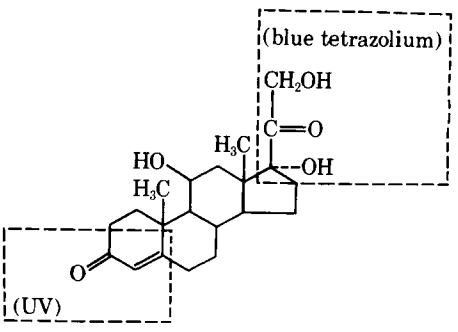
Figure 1—*First-order kinetic plot of hydrocortisone (Sample 1) at various temperatures, showing decomposition of the C-17 side chain.*

A study in this laboratory evaluated the diffusion of hydrocortisone from a series of commercially available bases[1], water, and polyethylene glycol. The maximum quantity of hydrocortisone diffused from the polyethylene glycol ointment base (1). It appears that no company is marketing hydrocortisone ointment in a polyethylene glycol base, and this may be due to the instability of hydrocortisone in such a base. Degradation of the C-17 dihydroxyacetone side chain of certain corticosteroids has been studied under various conditions for many years. A base-catalyzed anaerobic degradation of cortisone was reported (2). Deoxycorticosterone acetate and 21-acetoxypregn-5-en-3β-ol-20-one were converted by ethanolic potassium hydroxide in the presence of air to the corresponding ethianic acids (3); similar results using 6β,21-diacetoxypregn-4-ene-3,20-dione also were found (4).

These conversions were confirmed (5) by investigations into the kinetics of the base-catalyzed degradation of prednisolone, which suggested that it was a complex reaction resulting from at least three parallel pseudo-first-order reactions. The degradation of hydrocortisone hemisuccinate at 70° was studied over a narrow pH range and found to be a first-order, consecutive reaction (6).

The stability of cortisone and hydrocortisone was investigated (7) using UV spectrophotometry and phenylhydrazine methods to detect alterations in ring A and blue tetrazolium to detect deterioration of the C-17 side chain; both cortisone and hydrocortisone were stable and underwent no change on heating at 100° for 1 hr or at 130° for 2 hr. The stability of cortisone and hydrocortisone at various pH values was also studied; heating these steroids at pH 9 caused only slight decomposition (7).

Various factors influencing the stability of corticosteroids in aqueous suspensions and solutions were investigated (8), and the effects of some solid buffering agents on the stability of prednisolone were determined. Corticosteroids similar to prednisolone should not be exposed to materials capable of producing an elevated pH during formulation according to this report (8). Some factors influencing the rate of disappearance of prednisolone from aqueous solution were investigated, and decomposition was found to be markedly accelerated by contaminants in the buffer reagents, with the evidence strongly indicating that the contaminants were trace metals (9). A mix-



Scheme I

[1] Acid Mantel, Dome Laboratories, West Haven, Conn.; Aquaphor, Duke Laboratories, S. Norwalk, Conn.; cold cream, The Upjohn Co., Kalamazoo, Mich.; HEB, Barnes-Hind Pharmaceuticals Inc., Sunnyvale, Calif.; hydrophilic petrolatum, Emerson Laboratories, Dallas, Tex.; petrolatum, McKesson Laboratories, Bridgeport, Conn.; and vanishing cream, Neobase, Burroughs Wellcome and Co., Triangle Park, N.C.

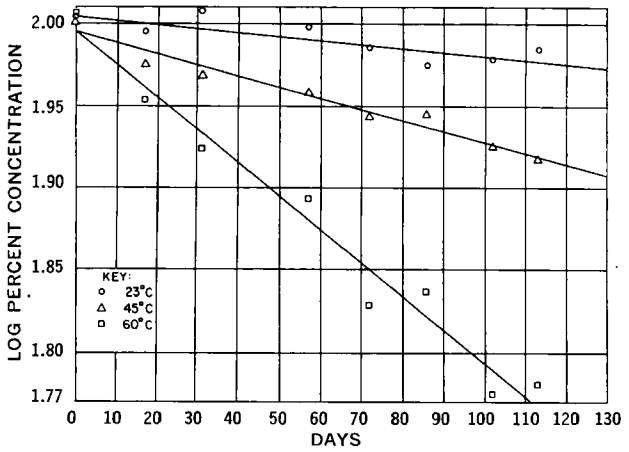


Figure 2—*First-order kinetic plot of hydrocortisone (Sample 2) at various temperatures, showing decomposition of the C-17 side chain.*

ture with a pH of <3.5 or >8.0 promoted the hydrolysis of hydrocortisone sodium succinate (10).

The stability of prednisolone in an organic medium containing 50% camphor, 25% *m*-cresyl acetate, and 25% *p*-chlorophenol was studied (11). This medium containing 1% prednisolone has been used as a pulp-capping agent to reduce thermal sensitivity in dental restorations. Due to the slightly acidic nature of the vehicle, a shelflife of 2–5 years was predicted for this product (11). Prednisolone in anhydrous form was observed to be stable in liquid paraffin but not in water (12).

The purpose of this study was to investigate the stability of hydrocortisone in a polyethylene glycol base.

EXPERIMENTAL[2]

Chemicals and Reagents—All chemicals and reagents were USP, NF, or ACS grade except for the 10% tetramethylammonium hydroxide solution[3]. All were used without further purification.

Ointment Preparation—Two separate batches of ointments were prepared by mechanically incorporating 1% hydrocortisone into polyethylene glycol ointment USP (1) using the trituration method.

Assay Procedures—After the initial assay, each ointment was divided into three parts and stored at 23 ± 2, 45 ± 2, and 60 ± 2°.

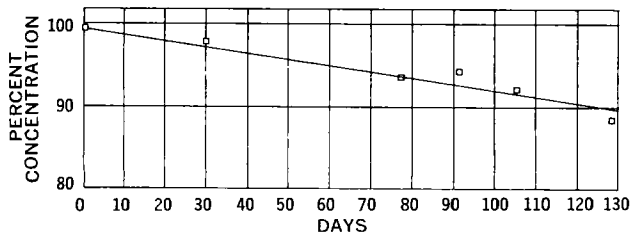


Figure 3—*Zero-order kinetic plot of hydrocortisone (Sample 1) at 60°, showing decomposition of ring A.*

[2] Ointment samples were incubated in ovens manufactured by National *Appliance Co., model 320,* serial FSR-6. A Beckman DB-GT grating spectrophotometer was used to measure all absorbance values at 247 nm. A Bausch & Lomb Spectronic 20 was used to measure all absorbance values at 525 nm. A Beckman IR-8 spectrophotometer was used for all IR determinations.

[3] Eastman Kodak.

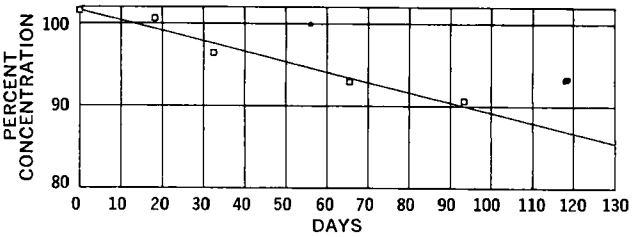


Figure 4—*Zero-order kinetic plot of hydrocortisone (Sample 2) at 60°, showing decomposition of ring A.*

At specified intervals, samples were taken and assayed. The ointment bases (without hydrocortisone) were assayed at the same time. To follow the decomposition of hydrocortisone, two methods of assay were used.

Blue Tetrazolium Assay Method for Detection of Degradation of C-17 Side Chain (Scheme I)—All blue tetrazolium assays were performed using the USP XVII (13) method for total steroids.

UV Spectrophotometric Method for Alterations in Ring A (Scheme I)—For the standard solution, weigh exactly 100.0 mg hydrocortisone USP and dissolve in sufficient ethanol to make 100.0 ml. Dilute 2.0 ml of this solution to 100.0 ml with distilled water.

For the assay preparation, dissolve exactly 200.0 mg of each ointment in enough distilled water to make 100.0 ml of the solution.

For the assay procedure, concomitantly determine the absorbances of the standard and assay solutions at 247 nm (the wavelength of maximum absorbance) in a suitable spectrophotometer, using distilled water as the blank.

Calculations—For both methods of assay, results were calculated by comparing absorbance readings of the samples with the standard reading. The assay results were treated theoretically and are presented in Figs. 1–5.

THEORETICAL

The order of reactions was determined graphically (Figs. 1–4). Before plotting, the regression analysis was conducted on the data to determine the best possible order of reaction.

The UV data (decomposition of ring A) at 23 and 45° were not treated graphically since the samples were satisfactory (loss of potency was less than 10%).

The energy of activation (E_a) on data from the blue tetrazolium assay was determined using the Arrhenius equation (Eq. 1):

$$\log \frac{K_2}{K_1} = \frac{E_a}{2.303R} \frac{(T_2 - T_1)}{T_2 T_1} \qquad \text{(Eq. 1)}$$

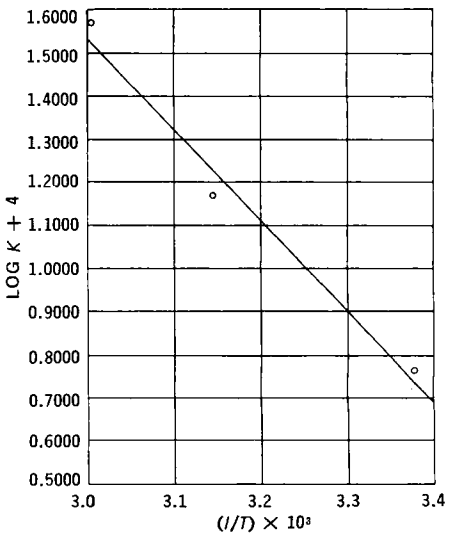
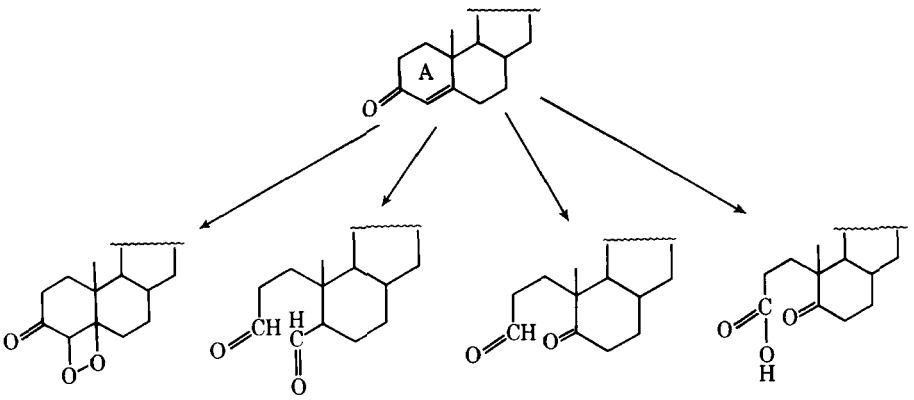


Figure 5—*Arrhenius plot of hydrocortisone using average* K *values from Samples 1 and 2 (see Figs. 1 and 2).*



Scheme II

The average K values (decomposition of C-17 side chain) for Samples 1 and 2 were used for the Arrhenius plot (Fig. 5). For the best line, the method of least squares was used. The energy of activation was determined to be 9.7 kcal/mole.

The average K value at room temperature (Figs. 1 and 2) was used to estimate the stability of the ointment under "shelf conditions" using Eq. 2:

$$t = \frac{2.303}{K} \log \frac{c_i}{c_t} \qquad \text{(Eq. 2)}$$

where t is time in days, c_i is the initial concentration, and c_t is the concentration at time t.

When substituting the data from the blue tetrazolium assay (decomposition of C-17 side chain) at 23° (average K = 5.78×10^{-4}/day) and using 100 and 90% for c_i and c_t, respectively, the expiration date is approximately 182 days. By increasing c_i to 105% (*i.e.*, by adding 5% excess), the shelflife is approximately 267 days or about 9 months.

DISCUSSION

Alterations in the hydrocortisone molecule apparently occurred at both ring A and the C-17 side chain (Figs. 1–4). The degradation of the dihydroxyacetone moiety was greater and of greater concern in stability studies. The degradation of the C-17 side chain has been extensively investigated and was well documented previously.

The degradation of the C-17 side chain appears to be first order. Although slow (the average half-life was 505 days at 60° *versus* 186 days using the blue tetrazolium assay), alterations in ring A were noticed (Figs. 3 and 4). The order of reaction for the degradation of ring A appears to be zero (see *Theoretical* and Figs. 3 and 4). To determine the scheme of degradation of ring A, the samples were inspected by IR spectroscopy. Except for the area between 1500 and 2000 cm^{-1}, the field was overshadowed by the base vehicle. A consistent trend was a progressive increase in absorption at 1715 cm^{-1} and diminution at 1635 cm^{-1} as samples were viewed from 23 to 45 to 60°. One possibility for the degradation of ring A is shown in Scheme II.

The activation energy of hydrocortisone was determined to be about 9.7 kcal/mole (see *Theoretical*).

Finally, the shelflife of the ointment at room temperature appears to be about 6 months (see *Theoretical*). Nevertheless, by adding an excess of 5% at the time of manufacture, hydrocortisone ointment, if prepared in polyethylene glycol ointment USP, should be reasonably stable for about 9 months. This length of time hardly justifies manufacture on a commercial basis considering the combined lag times involved in warehousing and shipping from the manufacturer through a wholesaler to a dispensing pharmacy and, ultimately, to the patient.

Although it has a limited shelflife, conditions at some hospitals and pharmacies may justify the manufacture of this product for their own use.

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[x] To whom inquiries should be directed.

Design and Evaluation of a Rotating Filter–Stationary Basket *In Vitro* Dissolution Test Apparatus II: Continuous Fluid Flow System

ASHOK C. SHAH[x] and JOHN F. OCHS

Abstract □ The rotating filter-stationary basket apparatus reported previously for the determination of dissolution rates in fixed fluid volumes was modified for use as a continuous fluid flow dissolution test system. The modified apparatus, consisting of a smaller size fluid container, provides a convenient means of maintaining sink conditions during the dissolution process by a continuous exchange of a portion of the dissolution medium with fresh solvent. Equations are developed to calculate the total amount of drug dissolved as a function of time from continuous-flow dissolution rate profiles. Dissolution rate results for several tablet samples of an antidiabetic drug evaluated under continuous fluid flow conditions are comparable with the results obtained under fixed fluid volume conditions, and they also correlate with *in vivo* blood sugar-lowering activity of the tablets.

Keyphrases □ Dissolution test apparatus—design and evaluation of a continuous fluid flow system, rotating filter-stationary basket □ Continuous fluid flow system—design and evaluation of rotating filter-stationary basket dissolution test apparatus □ Drug dissolution profiles—using continuous-flow system, design and evaluation of rotating filter-stationary basket apparatus, equations developed

In the design of a reliable *in vitro* dissolution test model that can positively characterize the role of the dissolution process in drug bioavailability, it is essential that the model is capable of simulating test conditions that are likely to prevail during the *in vivo* dissolution process. One such condition is the maintenance of nearly perfect sink conditions during *in vitro* dissolution rate determinations; *i.e.*, the drug concentration in the dissolution medium does not exceed 10–20% of solubility. This condition is required because in a dissolution rate-limited drug absorption process, there is no appreciable build-up of drug concentration in GI fluids—they act as an infinite sink. It has been suggested, therefore, that unless similar sink conditions are embodied in the *in vitro* dissolution test model, *in vitro* results will bear little relation to the *in vivo* observations (1, 2).

The types of systems employed for maintaining sink conditions during the dissolution process are: (*a*) fixed fluid volume, (*b*) multiple phase, and (*c*) continuous fluid flow. In the fixed fluid volume system, dissolution studies are performed in a finite volume of aqueous dissolution medium, a volume sufficient to maintain solution concentration below 10–20% of drug solubility. Simplicity in the design and operation of this system has probably favored its extensive use in a number of dissolution test methods (3–7), including those recognized by the official *compendia* (8, 9). However, the fixed fluid volume system is unsuitable for relatively insoluble and/or high dosage drugs because of the practical limits up to which large fluid volumes can be conveniently used in a dissolution test.

In the multiple-phase system, the drug upon dissolution into an aqueous medium is either partitioned into a water-immiscible organic phase or adsorbed by a solid adsorbent. Since both approaches provide sink conditions by constant removal of dissolved drug from the dissolution medium, they may be employed in the dissolution rate determinations of all drugs regardless of their solubility and strength per unit dose. Although the feasibility of these multiple-phase systems has been demonstrated (2, 10, 11), additional parameters involved in these systems, such as selection of proper organic phase or adsorbent, rate of partitioning and adsorption, and miscibility of organic and aqueous phases, will obviously complicate the dissolution test methodology. Niebergall *et al.* (12) stressed the importance of selecting the proper type of organic phase in a multiple-phase dissolution test method.

The continuous fluid flow system provides a relatively simple and convenient test system applicable for the determination of dissolution rates of all drugs under sink conditions, regardless of their solubility and dosage strength. As in the multiple-phase system, the sink conditions in this system are maintained by constant removal of the dissolved drug from the dissolution medium, but none of the above-mentioned partitioning and adsorption parameters are involved. The constant removal of the dissolved drug in this system is achieved by continuous elimination of the filtered dissolution medium from the dissolution chamber and simultaneous addition of fresh solvent into the chamber. Several continuous-flow dissolution test apparatus were reported in the literature (13–18). In all of these apparatus, continuous filtration of effluent dissolution medium is performed by wire mesh screen, sintered glass, membrane, or other similar static filter elements. As demonstrated in previous work (19), continuous filtration through such a static filter may not only create serious operational problems but may also introduce analytical errors in the dissolution rate results due to the occurrence of filter clogging, diminishing fluid flow rates, and escape of solid particles into the filtrate.

A rotating filter–stationary basket dissolution test apparatus, described previously (19), incorporates a dynamic, nonclogging, microporous filter medium. This apparatus, with a relatively large-volume (1.5 liters) fluid container, was designed primarily as a fixed fluid volume dissolution test apparatus. While it can be employed as a continuous-flow system, replacement of a fraction of the large volume of dissolution medium requires excessively fast fluid flow

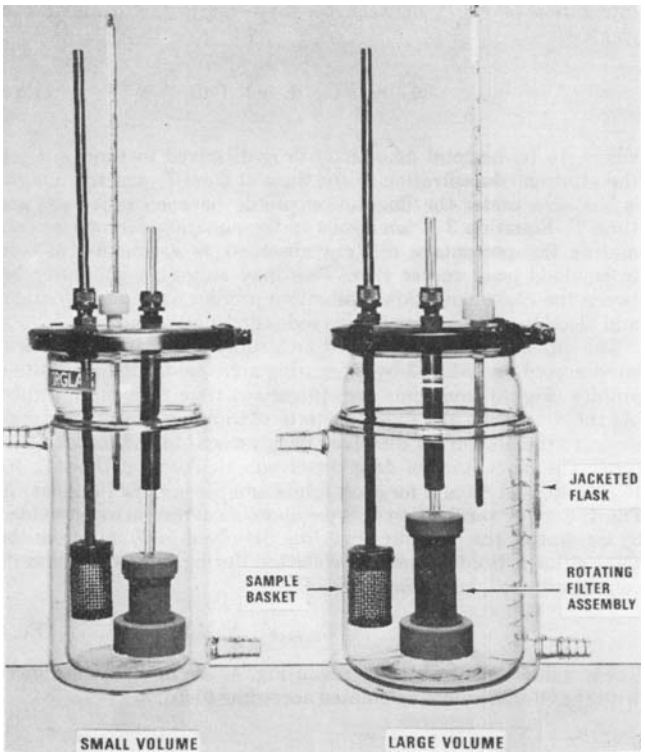

Figure 1—*Photograph of the small- and large-volume rotating filter–stationary basket apparatus.*

rates and consumption of appreciable quantities of solvent media. Fluid replacement at a rate of 20%/min for 1 liter of bulk dissolution medium, for instance, requires a flow rate of 200 ml/min; if the test is continued for 30 min, a total of 7 liters solvent media is consumed. These conditions are obviously not very convenient for routine dissolution experiments. This report describes the design of a small-volume rotating filter–stationary basket apparatus and its application as a continuous fluid flow dissolution test system.

EXPERIMENTAL

Test Samples—Samples from five different tablet lots of a highly insoluble antidiabetic drug were employed. The formulation of all five lots was identical, but each tablet lot was manufactured using a different particle-size bulk drug. The disintegration time of these tablet samples was less than 45 sec. These tablet samples were also employed in a previous study (19).

Apparatus—The basic design of the small-volume continuous-flow apparatus is similar to the large-volume rotating filter–stationary basket apparatus reported previously (19). Each consists of a rotating filter assembly, a stationary sample basket, and a closed jacketed flask (Fig. 1). A detailed description of these components was given previously (19). Modifications incorporated in the fabrication of the continuous-flow apparatus are a smaller diameter flask and a shorter size rotating filter assembly which suspends closer toward one side of the flask. The filter assembly was shortened by reducing the size of two of its parts: the filter head and the cylindrical filter element. Suspension of the assembly closer toward one side of the flask was necessary to accommodate both the filter assembly and the sample basket inside the small diameter flask. The small-volume apparatus can hold up to 700 ml of dissolution medium, and at least 300 ml is required to keep the filter assembly and the sample basket completely immersed in the liquid.

The broken lines in the schematic diagram of the continuous-flow apparatus (Fig. 2) represent the fluid flow system, with arrows pointing the direction of liquid flow. According to this arrangement, the dissolution medium is filtered continuously through the rotating filter assembly and withdrawn from the upper end of the pilot tube. Then it is circulated through a spectrophotometer flow cell by means of flexible tubing passing through a multichannel peristaltic pump[1] and finally discarded or collected in a separate container. Fresh solvent is simultaneously pumped into the flask at the same rate as the liquid elimination rate, so a portion of the dissolution medium in the flask is constantly being replaced with the fresh solvent without changing the volume of the fluid in the flask.

Procedure—Dissolution characteristics of antidiabetic tablet samples were evaluated by continuous fluid flow and fixed fluid volume test systems using the small- and large-volume rotating filter–stationary basket apparatus, respectively.

In continuous-flow experiments, 300 ml of simulated intestinal fluid USP (without pancreatin) was equilibrated at 37° and 400 rpm stirring speed in the small-volume apparatus. Approximately 3 liters of the fluid was maintained at 37° in a separate thermostated reservoir. The peristaltic pump was set to regulate continuous addition of fresh solvent from the reservoir into the dissolution flask at the rate of 38 ml/min and liquid removal from the flask at the same rate through the filter assembly. During the dissolution experiment, minor adjustments in fluid flow rates were required to maintain a constant liquid level corresponding to 300 ml bulk fluid volume in the flask. The dissolution experiment was started by lowering the sample basket containing two 2.5-mg tablets through the opening in the Plexiglas cover. A Plexiglas disk attached to the basket-holding rod fits inside the cover opening (Fig. 2) to hold the sample basket stationary at a precise level inside the flask. Dissolution rate profiles were obtained by continuous automated recording of the absorbance upon circulation of the dissolution medium through the spectrophotometer flow cell. To calculate the amount of drug dissolved as a function of time, areas under the dissolution profile from zero to various time intervals were measured using a planimeter. The total amount of drug dissolved in 60 min was also determined from the total volume of the effluent fluid collected during this period and the overall concentration of the total effluent.

The procedure employed in the fixed fluid volume dissolution test experiments was similar to the previous studies (19). One

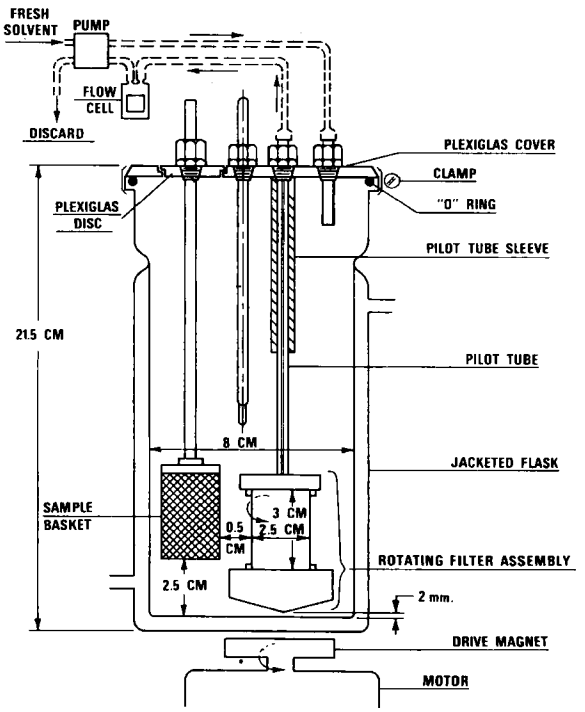

Figure 2—*Schematic diagram of the small-volume rotating filter–stationary basket apparatus.*

[1] Model 1210, Harvard Apparatus Co., Millis, MA 02054

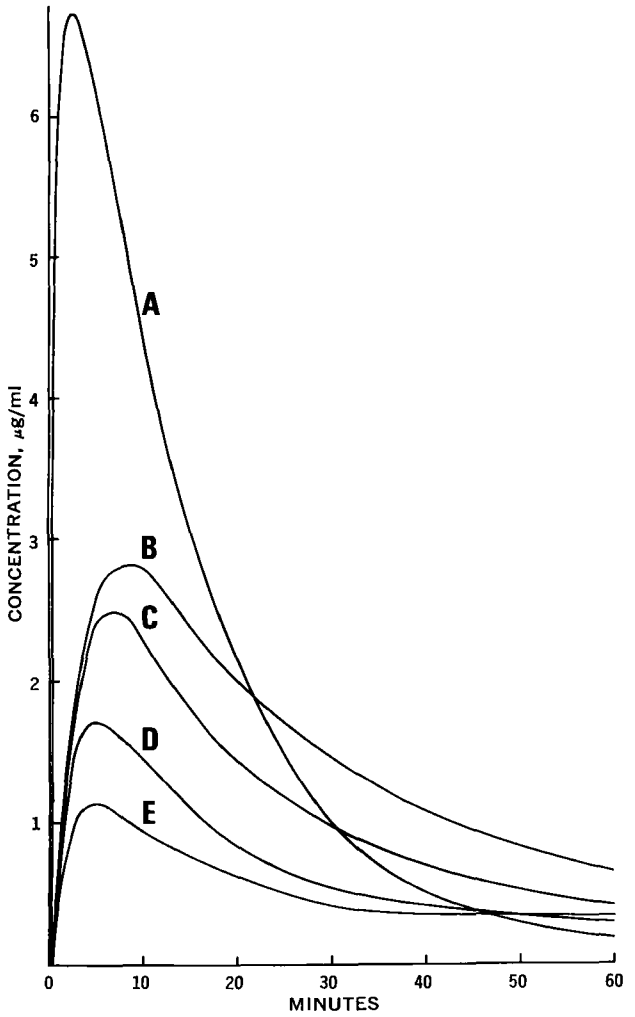

Figure 3—*Spectrophotometric recordings of the continuous-flow dissolution profiles for five antidiabetic tablet samples.*

liter of simulated intestinal fluid USP (without pancreatin) was equilibrated at 37° and 400 rpm stirring in the large-volume apparatus. The sample basket containing one 2.5-mg tablet was introduced into the apparatus. The dissolution rates were monitored by automated spectrophotometric analysis of the dissolution fluid samples.

RESULTS AND DISCUSSION

Dissolution profiles for the five tablet samples determined by the continuous fluid flow system are shown in Fig. 3. The initial rise followed by a decline in solution concentrations indicates occurrence of drug dissolution initially at a faster rate and later at a slower rate than the rate of elimination of the dissolved drug. For these continuous-flow dissolution profiles, the rate of dissolution as a function of time may be expressed as:

$$\frac{dA}{dt} = V\left(\frac{dC}{dt} + KC\right) \qquad \text{(Eq. 1)}$$

where dA/dt is the rate of dissolution, V is the fluid volume in the dissolution flask, K is the first-order rate constant for elimination of the dissolved drug from the flask, dC/dt is the slope of the dissolution profile at time t, and C is the concentration of drug in the dissolution medium at time t. Since the elimination rate constant equals the ratio of fluid flow rate ν, to the fluid volume V, substitution of ν/V for K in Eq. 1 yields:

$$\frac{dA}{dt} = V\frac{dC}{dt} + \nu C \qquad \text{(Eq. 2)}$$

Integration of Eq. 2 between the time limits $t = 0$ and $t = T$ yields:

$$A_T = VC_T + \nu\int_0^T C\,dt \qquad \text{(Eq. 3)}$$

where A_T is the total amount of drug dissolved in time T, C_T is the solution concentration in the flask at time T, and the integral is the area under the dissolution profile between time zero and time T. Equation 3 is analogous to the equation derived for estimating the percentage of drug absorbed as a function of time from blood level curves (20). This may suggest a similarity between the continuous-flow dissolution profiles as an *in vitro* index and blood level data as an *in vivo* index of drug availability.

The amount of drug dissolved as a function of time was calculated according to Eq. 3 by measuring areas under the dissolution profiles (Fig. 3) from time zero to various time periods, multiplying these areas by the fluid flow rate (38 ml/min), and then adding it to the amount of dissolved drug present in the flask at that time. The percentage of drug dissolved calculated at times 5, 10, 15, 20, 40, and 60 min for each tablet sample is shown (circles) in Fig. 4. Further verification of these dissolution results was provided by estimating the percentage of drug dissolved in 60 min from the total effluent fluid volume V_e collected during this period and its concentration C_e according to Eq. 4:

$$A_{60\,\text{min}} = C_{60\,\text{min}}V + C_eV_e \qquad \text{(Eq. 4)}$$

These values, shown by squares in Fig. 4, are in close agreement with the 60-min values calculated according to Eq. 3.

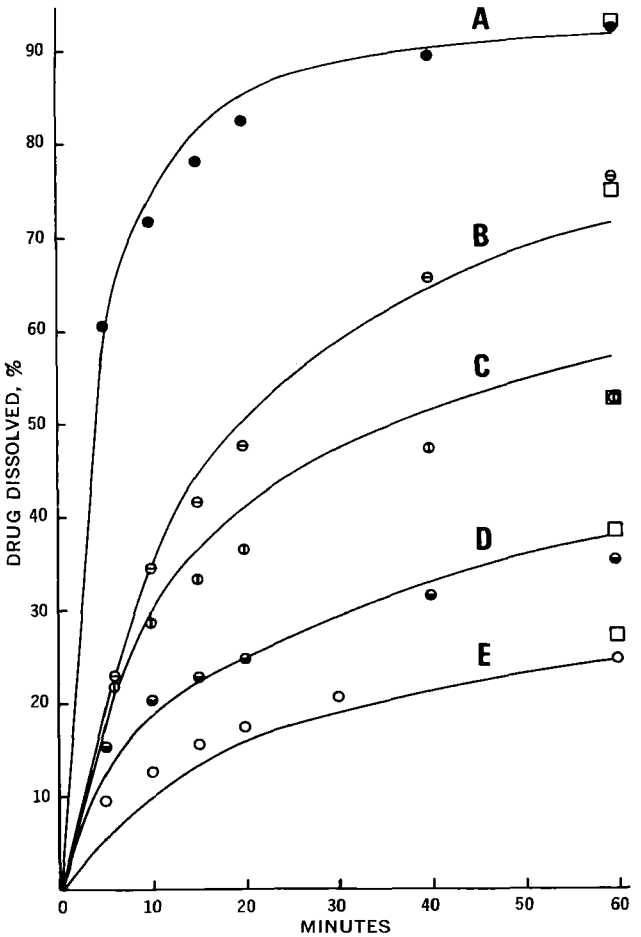

Figure 4—*Comparison of dissolution rate results for tablet samples obtained by continuous-flow and fixed fluid volume systems. Lines represent the results obtained by fixed fluid volume experiments and points are the values calculated from continuous-flow experiments.*

Table I—Correlation between Continuous-Flow Dissolution Rates of Antidiabetic Tablets and Their Blood Sugar-Lowering Response in Dogs

Sample	Percent Dissolved in 60 min	Total Blood Sugar-Lowering Response in 24 hr, mg %
A	92.3	293
B	76.2	256
C	52.5	250
D	35.3	225
E	24.7	167

Dissolution profiles for the tablet samples determined by the fixed fluid volume process are represented by lines in Fig. 4. These results also seem to be in reasonable agreement with the continuous-flow dissolution test results. However, although the stirring speed of 400 rpm was employed in both the continuous-flow and fixed fluid volume experiments, because of the different size apparatus and liquid volumes employed in these two experiments the actual intensity of liquid agitation may not be the same. Nevertheless, for the tablet samples examined, it seems that the rate of dissolution is not significantly influenced by minor differences in the intensity of liquid agitation.

The dissolution rate results for the antidiabetic tablet samples obtained by the continuous fluid flow test system are listed in Table I along with the previously reported results for the blood sugar-lowering activity of these tablets (19). Comparison of these two sets of data indicates a rank-order correlation between the *in vitro* dissolution rates of the tablets and their *in vivo* activity ($\gamma = 0.9191$).

In the continuous-flow dissolution experiments, the test sample consisted of two 2.5-mg tablets; in the fixed fluid volume experiments, one 2.5-mg tablet was employed. Selection of the sample size was based on the consideration of maintaining sink conditions in the fixed fluid volume system while simulating conditions that may arise in testing relatively insoluble high dosage drugs by the continuous-flow system. Since the solubility of this drug is 20 μg/ml, dissolution of 2.5 mg drug in 1 liter fixed fluid volume will yield solution concentrations up to 12.5% of saturation. Similarly, it is evident from the continuous-flow dissolution profiles (Fig. 3) that except for the fast dissolving Sample A, solution concentration never exceeded more than 14% of saturation. For the fast dissolving Sample A, the peak solution concentration reached was about 34% of saturation. In this case, the sink conditions were maintained by repeating the test at a faster fluid flow rate of 90 ml/min, which lowered the peak solution concentration to about 19% of saturation.

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[x] To whom inquiries should be directed.

Heat Production by Ultrasonic Equipment

DONALD M. SKAUEN

Abstract □ Temperature profiles were developed for several ultrasonic instruments over varying time intervals with two volumes of water. A wide range of temperatures at different power levels was noted.

Keyphrases □ Ultrasonic instruments—temperature profiles of heat production during insonation □ Insonation—temperature profiles of heat production for six ultrasonic instruments □ Heat production—temperature profiles for six ultrasonic instruments during insonation

It is well known to many users of ultrasonic equipment that heat is developed in an irradiated liquid due to mechanical energy emission. Literature indicating the extent of heat development by standard equipment in liquid systems is not readily available. In theory, if the energy output of the ultrasonic probe is known, calculations can be made to determine heat loss to the environment. In practice, this is difficult for the ordinary user because of the unreliability of the data available for most equipment.

This study was conducted to determine temperature profiles of various equipment found in biological laboratories. It is hoped that the results will afford the scientist adequate data for selection of optimal instrument parameters to avoid thermal problems.

EXPERIMENTAL

Equipment—Six instruments[1] (Instruments A–F) were studied.

Method—Distilled water (25 and 100 ml) was placed into 50- and 120-ml polyethylene containers. The ultrasonic probes were immersed to a depth of 1.27 cm into the liquid, the power levels were set, and the water was irradiated for intervals of 30, 60, 120, 180, 240, and 300 sec. The temperature after insonation was recorded at each time interval. All experiments were run in duplicate.

In the case of Instrument F, the probe was immersed to within 0.6 cm of the bottom of the plastic container prior to activation rather than at a depth of 1.27 cm from the top of the liquid since it operates on a different principle from the other instruments.

RESULTS AND DISCUSSION

The temperature profiles for the instruments studied are shown in Tables I–VII. Wherever possible, three power levels (low, medium, and high) were selected.

The probe immersion depth of 1.27 cm was selected on the basis of a previous report (1) and was verified using Instrument E at a power setting of 10 (Fig. 1). Maximum cavitational intensity values were obtained when the probe was immersed to a depth of no more than 1.27 cm.

Instrument F was studied because it is widely used as a cell disrupter and because the trade literature implies that it is an ultrasonic instrument. When used by most individuals, the probe is immersed to the greatest extent possible to expose the sample to maximum shear forces. For this reason, the probe was immersed to within 0.6 cm of the bottom in this study.

Instrument C—Conditions of operation were 20 kc/sec, 1.27-cm probe, and 75 w.

At the highest power of 8, the maximum temperature recorded during 300 sec was 74.8° in a volume of 25 ml while 59.3° was obtained in 100 ml (Table I). The temperatures at the two lower powers, 3 and 5, were considerably less, with a maximum of 37.8° attained in a 25-ml volume at a power of 5.

Instrument D—Conditions of operation were 20 kc/sec, 1.27-cm probe, and 125 w.

At high and medium power and at 25-ml volume, the curves are almost superimposed and are at about the same levels as for Instrument C. The high and low power values for 100-ml volumes are also about the same as those recorded for Instrument C with the intermediate power level much higher (Table II).

Instrument E—Conditions of operation were 20 kc/sec, 1.27-cm probe, and 185 w.

At the high power of 10 and a volume of 25 ml, the temperature reached a maximum of 95° after 240 sec and appeared to level off thereafter. By contrast with Instrument C where the intermediate and low level powers produced small temperature changes, Instrument E showed significant temperature changes at both lower powers. A similar result occurred in the 100-ml volume, the maximum temperature reaching 60° at high power (Table III).

Instrument A—Conditions of operation were 20 kc/sec, 0.95-cm probe, and 300 w.

The maximum temperatures reached with this 0.95-cm probe in 25 and 100 ml of water were 66 and only 38.3°, respectively,

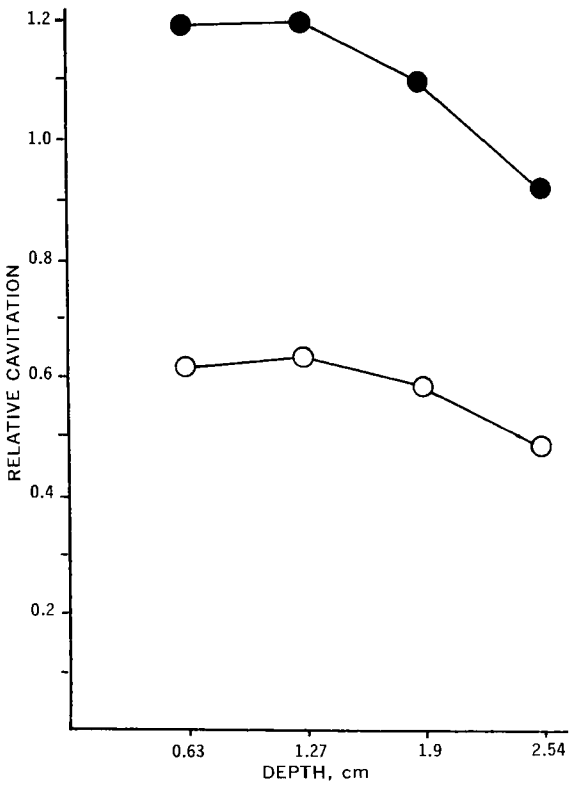

Figure 1—*Effect of depth of immersion upon cavitational intensity (Instrument E). Key: ○, 30 sec; and ●, 60 sec.*

[1] Instrument A, Biosonik III, Bronwill Scientific Co., Rochester, N.Y.; Instrument B, Biosonik IV, Bronwill Scientific Co., Rochester, N.Y.; Instrument C, Sonifier S-75, Branson Sonic Power Co., Danbury, Conn.; Instrument D, Sonifier S-125, Branson Sonic Power Co., Danbury, Conn.; Instrument E, Sonifier W-185, Branson Sonic Power Co., Danbury, Conn.; and Instrument F, Polytron, Bronwill Scientific Co., Rochester, N.Y.

Table I—Increase in Temperature during Insonation of Water Using Instrument C with a 1.27-cm Diameter Probe

Seconds	25 ml Power Level			100 ml Power Level		
	3	5	8	3	5	8
0	25°	25°	25°	25°	25°	25°
30	25.8°	26°	34°	25°	25°	28.5°
60	26.8°	27°	41°	25.5°	25.5°	32°
120	28.5°	29°	53.5°	26°	26°	40.3°
180	29.8°	31°	64.5°	26°	27°	47°
240	31.3°	33.8°	71.3°	26°	28°	53.3°
300	34.8°	37.8°	74.8°	26.5°	29°	59.3°
Average difference[a]	±1.3°	±0.8°	±1.6°	0°	0°	±0.3°

[a] The average temperature difference between duplicate runs.

Table II—Increase in Temperature during Insonation of Water Using Instrument D with a 1.27-cm Diameter Probe

Seconds	25 ml Power Level			100 ml Power Level		
	3	5	8	3	5	8
0	25°	25°	25°	25°	25°	25°
30	26.5°	34.2°	36°	26°	28°	30°
60	28.3°	43°	43°	27°	32°	34°
120	29.5°	54°	54°	29.5°	38°	42°
180	31.3°	63°	62.3°	32°	44°	49°
240	33.8°	69.5°	69°	34°	50°	56°
300	35°	73.5°	72.5°	37°	56°	62°
Average difference[a]	±1.6°	±3.6°	±1.6°	0°	±0.7°	±0.1°

[a] The average temperature difference between duplicate runs.

Table III—Increase in Temperature during Insonation of Water Using Instrument E with a 1.27-cm. Probe

Seconds	25 ml Power Level			100 ml Power Level		
	3	7	10	3	7	10
0	25°	25°	25°	25°	25°	25°
30	30°	35°	42°	26.5°	27.5°	29.3°
60	34°	45°	55.6°	27.5°	30.3°	33.5°
120	42°	61°	78°	30°	35°	41.3°
180	48.5°	74°	90°	32°	39.3°	48°
240	54.5°	82.5°	95°	34°	44°	54.5°
300	60°	88°	95°	36°	48°	60°
Average difference[a]	±0.7°	±0.4°	±0.4°	±0°	±0.2°	±1.6°

[a] The average temperature difference between duplicate runs.

Table IV—Increase in Temperature during Insonation of Water Using Instrument A with a 0.95-cm Probe

Seconds	25 ml Power Level			100 ml Power Level		
	20	40	60	20	40	60
0	25°	25°	25°	25°	25°	25°
30	28.5°	27.5°	30.3°	26°	25.8°	26.5°
60	31.3°	30.8°	35.3°	26.8°	27°	28.3°
120	36.0°	36.5°	44.5°	28.5°	28.8°	30.5°
180	41.2°	41.5°	52.8°	30.3°	30.5°	33.3°
240	46.5°	46.3°	60°	31.5°	32.5°	35.5°
300	49.3°	50.8°	66°	33.5°	34°	38.3°
Average difference[a]	±1.2°	±1.1°	±1.2°	±0.5°	±0.1°	±0.8°

[a] The average temperature difference between duplicate runs.

Table V—Increase in Temperature during Insonation of Water Using Instrument B with a 0.95-cm Probe

Seconds	25 ml Power Level—Hi			100 ml Power Level—Hi		
	20	40	60	20	40	60
0	25°	25°	25°	25°	25°	25°
30	27°	29°	28°	27°	27°	26°
60	29°	33°	32°	28°	28°	27°
120	33°	40°	37°	29°	29°	28°
180	38°	45°	44°	31°	31°	31°
240	42°	50°	49°	32°	33°	32°
300	47°	53°	53°	34°	35°	34°
Average difference[a]	±1.1°	±1.2°	±1.1°	0°	±0.2°	±0.5°

[a] The average temperature difference between duplicate runs.

Table VI—Increase in Temperature during Treatment of Water Using Instrument F

Seconds	25 ml Power Level			100 ml Power Level		
	3	7	10	3	7	10
0	25°	25°	25°	25°	25°	25°
30	27°	29°	30°	26°	27°	28°
60	28°	31°	31°	27°	28°	28.5°
120	30°	34°	34°	28.5°	30°	31°
180	32°	35°	35°	29.5°	31°	32.5°
240	33°	36°	36°	30°	32°	34°
300	33.5°	36°	37°	31°	33°	35°
Average difference[a]	±1.5°	±0.3°	±0.4°	0°	±0.3°	±0.9°

[a] The average temperature difference between duplicate runs.

Table VII—Increase in Temperature during Insonation of Water Using Instrument A with a 0.4-cm Diameter Probe

Seconds	10 ml Power Setting		25 ml Power Setting	
	20	30	20	30
0	25°	25°	25°	25°
30	35.5°	25.5°	26°	25.5°
60	26.5°	26.5°	26.5°	26°
120	28°	28°	27°	27°
180	29.5°	29.5°	27.5°	27.5°
240	31°	31°	28°	28°
300	32.5°	32°	29°	29°
Average difference[a]	±0.2°	±0.8°	0°	±0.8°

[a] The average temperature difference between duplicate runs.

after 5 min irradiation and full power. At lower power settings, the temperature increases were linear but less than at full power (Table IV).

Instrument B—Conditions of operation were 20 kc/sec, 0.95-cm probe, and 300 w.

Maximum temperatures were obtained at power settings of 40 and 60 using the 0.95-cm probe in 25 ml of water. Temperature increases with 100-ml volumes were minimal at all three power levels, with the highest temperature reaching 35° (Table V). These experiments were carried out at the instrument panel setting of Hi.

Instrument F—Temperature profiles for 25- and 100-ml volumes of water treated by Instrument F are shown in Table VI. Maxima are not as high as those attained by some other instruments. The maximum of 37° obtained in the 25-ml volume was only slightly higher than that obtained with the 100-ml volume.

Microprobes—The only available instrument with a microprobe was Instrument A with a 0.4-cm probe. Since microprobes are widely used, temperature profiles for power levels of 20 and 30 in 10 and 25 ml of water were prepared. As shown in Table VII, the temperature increases are minor with the highest at 32.5°.

Cavitation is responsible for much of the heat produced during the ultrasonic irradiation of a liquid with a step-horn transducer. If the equipment is well designed, little if any heat is contributed by the horn itself. Since many users of step-horn probes are interested in the disruptive effects of cavitation which is accompanied by heat, any additional heat contributed by the probe should be avoided.

The results indicate that where modest increases in temperature can be tolerated and where agitation rather than power is important, the irradiations of up to 5 min can be conducted at the low power settings of Instruments C, D, A (100 ml), E at lowest power (100 ml), B at all power levels (100 ml), A with the microprobe, and F at all power levels.

If power is significant and the system is thermolabile, then cooling will be necessary at all high power settings of all of the instruments studied except Instruments F, A with the microprobe, and B (100 ml).

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Influence of Dispersion Method on Dissolution Rate of Digoxin–Lactose and Hydrocortisone–Lactose Triturations I

CHAREONSRI AMPOLSUK, JAMES V. MAURO, ALAN A. NYHUIS, NAVNIT SHAH, and CHARLES I. JAROWSKI[x]

Abstract □ Spreading of digoxin or hydrocortisone over a lactose surface by frictional pressure produced 1:20 triturations with significantly enhanced dissolution rates in simulated gastric fluid. Triturations prepared by simple blending or solvent deposition exhibited slower dissolution rates. These observations may help explain the variations in dissolution rate observed in manufactured tablet and capsule dosage forms of these two drugs.

Keyphrases □ Digoxin–lactose triturations—effect of simple blending, solvent deposition, and spreading by frictional pressure on dissolution rates □ Hydrocortisone–lactose triturations—effect of simple blending, solvent deposition, and spreading by frictional pressure on dissolution rates □ Triturations, digoxin–lactose and hydrocortisone–lactose—effect of simple blending, solvent deposition, and spreading by frictional pressure on dissolution rates □ Dissolution rates, digoxin–lactose and hydrocortisone–lactose triturations—effect of simple blending, solvent deposition, and spreading by frictional pressure

Recent publications pointed out the wide variation in digoxin absorption from tablets produced by various manufacturers (1–3). Furthermore, significant variations in blood levels were noted between batches made by the same manufacturer (1). Studies of the blood levels of digoxin (3) and urine recovery (4) showed that the absorption efficiency from tablets was only 75% that of the drug in solution. The hydrophobicity and low aqueous solubility of digoxin account to a significant degree for these variations.

Similar problems are encountered with tablets of hydrocortisone. The biological availability of hydrocortisone from tablet dosage forms is an ongoing problem for the Food and Drug Administration (5, 6).

In the manufacture of tablet and capsule dosage forms of potent drugs such as digoxin and hydrocortisone, triturations are commonly employed. Such triturations are usually prepared by simple blending or solvent deposition. A method for ensuring rapid and reproducible dissolution rates of these steroidal drugs from their triturations is desirable. The use of such triturations in the manufacture of tablets and capsules should result in more rapid and reproducible rates of dissolution. These, in turn, definitely should help to correct the blood level variations currently encountered.

This report describes the dissolution of digoxin and hydrocortisone from lactose triturations prepared by the following methods: (*a*) simple blending, (*b*) solvent deposition, and (*c*) frictional pressure. The third method was included because steroidal compounds might be spreadable on the diluent surface by frictional force.

EXPERIMENTAL

Materials—The following were obtained from commercial sources: digoxin USP[1]; lactose USP[2]; sodium chloride USP[3]; hydrochloric acid, reagent grade[4]; alcohol USP[5]; and hydrocortisone USP[6].

Equipment—The following pieces of equipment were used: a constant-temperature water bath[7], a double-beam spectrophotometer[8], and a disintegration–dissolution apparatus (USP).

Table I—Dissolution Rates of Digoxin–Lactose (Three Batches) and Hydrocortisone–Lactose (Two Batches) in Simulated Gastric Fluid at 37°

	Percentage Dissolved							
	Simple Blend		Simple Blend of Ground Drug		Solvent-Deposited Drug		Frictionally Deposited Drug	
Minutes	Digoxin	Hydrocortisone	Digoxin	Hydrocortisone	Digoxin	Hydrocortisone	Digoxin	Hydrocortisone
15	30.3 ± 0.4	57.0 ± 0.8	47.7 ± 0.3	54.0 ± 1.1	61.0 ± 0.4	73.2 ± 0.5	74.3 ± 0.3	94.8 ± 0.3
30	41.4 ± 6.6	65.0 ± 3.1	52.4 ± 1.2	63.6 ± 1.4	66.2 ± 7.6	77.2 ± 6.6	83.0 ± 4.4	96.3 ± 2.2
45	41.8 ± 1.0	81.0 ± 2.3	53.6 ± 1.7	72.4 ± 2.4	67.9 ± 2.1	81.1 ± 2.1	86.0 ± 3.1	96.8 ± 1.7
60	53.3 ± 5.5	84.9 ± 4.6	61.1 ± 5.8	79.2 ± 4.1	75.0 ± 3.9	84.8 ± 2.6	91.7 ± 3.5	98.6 ± 2.2
75	—	86.2 ± 3.1	—	83.4 ± 2.6	—	84.6 ± 0.8	—	98.8 ± 1.7
90	60.1 ± 4.0	87.4 ± 2.8	67.3 ± 3.8	86.0 ± 3.5	80.8 ± 4.4	85.9 ± 5.1	96.9 ± 4.5	99.2 ± 2.4
120	65.4 ± 5.2	—	72.3 ± 3.9	—	84.7 ± 6.8	—	99.4 ± 5.2	—

Preparation of Drug–Lactose Triturations—The digoxin and hydrocortisone powders were passed through a 325-mesh screen. Microscopic examination revealed that the major portion of the powdered digoxin was in the 5–40-μm range. The major portion of the hydrocortisone powder was in the 15–30-μm range. In both powders, aggregation of fine particles was noted.

The drug–lactose triturations were prepared in a weight ratio of 1:20. Manual bottle tumbling for 15 min was conducted in preparing the *simple blend*. Mechanical shaking of the lactose dilu-

[1] Burroughs Wellcome & Co., Research Triangle Park, N.C.
[2] Matheson Co., East Rutherford, N.J.
[3] Merck & Co., Rahway, N.J.
[4] J.T. Baker Chemical Co., Phillipsburg, N.J.
[5] Commercial Solvents Corp., New York, N.Y.
[6] Pfizer & Co., New York, N.Y.
[7] Precision Scientific Co., Chicago, Ill.
[8] Coleman Hitachi, Coleman Instruments Co., Maywood, Ill.

ent through a series of sieves ranging from 40 to 400 mesh established its average diameter to be 132 μm (7).

The *solvent-deposited* samples were prepared by dissolving the drug in a sufficient quantity of either 80% ethanol (digoxin) or absolute ethanol (hydrocortisone) and uniformly wetting the lactose with the solution. After drying at 32°, the sample was passed through a 60-mesh screen with the aid of a spatula.

By the conventional geometric build-up procedure, the powdered drugs were energetically hand triturated with powdered lactose. It was reasoned that the application of such frictional force would uniformly spread the drug on the lactose surface. The average diameter of the drug–lactose blends prepared with a mortar and pestle was approximately 66 μm. It was difficult to differentiate the drug from the diluent under microscopic examination. Interestingly, the absence of fine particle aggregates was noted.

A fourth drug–lactose trituration was prepared by pestle grinding the finely powdered drugs alone. After passage through a 325-mesh screen, the powders were examined microscopically. Insignificant size reduction was achieved by pestle grinding these fine powders. As before, fine particle aggregates were observed. The sieved powders were geometrically diluted with powdered lactose without additional frictional pressure. The intent in this instance was to expose the drugs to a similar pestle grinding action but not to spread them onto the lactose surface with frictional force.

Dissolution Studies—A modification of the procedure of Monkhouse and Lach (8) was used to determine the dissolution rates of the various powder blends. Two hundred milliliters of simulated gastric fluid without pepsin (USP XVIII) was added to a 400-ml beaker and permitted to equilibrate at 37 ± 0.5° in a constant-temperature bath. The dissolution medium was stirred at 60 rpm using the basket recommended for the USP dissolution test. The basket was vertically centered to a depth of 2.54 cm (1 in.) from the bottom of the beaker. At zero time, the drug–trituration (digoxin–lactose, 84 mg, or hydrocortisone–lactose, 210 mg) was spread over the surface of the dissolution medium and the apparatus was set in motion. Five-milliliter samples of solution were periodically withdrawn through a sintered-glass filter of medium porosity immersed at all times in the dissolution medium. The withdrawn aliquot was then passed through a filter[9] (0.45 μm), and the drug concentrations were measured spectrophotometrically using simulated gastric fluid as the blank. Five milliliters of simulated gastric fluid was added to the dissolution medium to replace the volume of sample withdrawn for assay.

Spectrophotometric Absorption and Calibration Curves for Digoxin and Hydrocortisone in Simulated Gastric Fluid—Twenty milligrams of digoxin was dissolved in 10 ml of hot 80% ethanol. Sufficient simulated gastric fluid was added to make 1 liter. This solution was used to determine absorption spectral and calibration curves. The concentrations of digoxin in the dissolution study obeyed Beer's law at 224 nm. Incorporation of a 20-fold excess of lactose did not alter the absorbance values for digoxin.

Fifty milligrams of hydrocortisone was dissolved in 20 ml of ethanol, and sufficient simulated gastric fluid was added to make 1 liter. This solution was used to determine the absorption spectral curve and to establish 248 nm as the wavelength for the calibration curve. Again the concentrations of hydrocortisone in the dissolution study obeyed Beer's law, and the incorporation of a 20-fold excess of lactose did not alter the absorbance values.

RESULTS AND DISCUSSION

The dissolution rates of the various triturations are shown in Table I. The superiority of the frictionally prepared digoxin–lactose trituration is clearly evident. Thus, 91.7 ± 3.5% of the digoxin had dissolved within 60 min. The remaining digoxin blends did not attain 90% dissolution up to 120 min. Similarly, the frictionally prepared hydrocortisone–lactose trituration showed the fastest dissolution rate. At the end of 15 min, 94.8 ± 0.3% of the hydrocortisone had dissolved. The remaining hydrocortisone–lactose blends did not attain 90% dissolution up to 90 min.

The failure of the solvent-deposited triturations to match or exceed the dissolution rate of the frictionally applied drug–lactose blends was surprising. Deposition of the drug in a molecular state of subdivision on a 20-fold excess of powdered lactose would be expected to be a highly efficient method of increasing the total surface of the drug. Monkhouse and Lach (8) achieved enhanced dissolution of aspirin solvent deposited on 10% starch plus 10% lactose. Adverse adsorption effects can be ruled out as an explanation since lactose is soluble in simulated gastric fluid. A likely explanation for the observed results is inefficient solvent evaporation. Solvent migration during the drying step could account for a smaller total drug surface as compared to that obtained by the frictional method. Sodium warfarin was shown (9) to migrate during the drying step after wet granulation with lactose.

The frictional method of preparing triturations of steroidal, sparingly soluble drugs is worthy of further study. The reported observations can help to explain the batch-to-batch variations in dissolution rate encountered in the manufacture of capsules and tablets. Frictional forces are applied to varying degrees in the manufacture of such dosage forms. Procedures such as milling, blending, slugging, granulating, and tableting do impart frictional force on the drug and the excipients. The intensity and length of time for each operation will vary. In light of the reported results, it is not surprising that such procedural variations could account for the dissolution rate variations encountered.

Developmental experimental studies are underway in this laboratory to establish efficient mechanical methods for the manufacture of triturations, capsules, and tablets possessing reproducible physical properties.

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[x] To whom inquiries should be directed.

[9] Millipore.

Effects of Solids-Loading on Moisture Permeability Coefficients of Free Films

JAMES W. PARKER*, GARNET E. PECK[x], and GILBERT S. BANKER

Abstract □ The effects on the water vapor transmission properties of free polymer films by the incorporation of dispersed solid filler materials (solids-loading) were studied. Two mineral talcs and titanium dioxide were separately incorporated, in a range of concentrations, in a standard plasticized cellulosic film comprised of hydroxypropyl methylcellulose and ethylcellulose (3:1). The nature of the additive dispersed solid filler material and its general surface chemistry, particle size, particle-size distribution, and concentration were found to influence the water permeation properties of the free films. Film thickness did not provide a standard variable of analysis for films containing various concentrations of dispersed solid or the same concentration of different solids. The mass of polymer in any standard cross section of film did provide a meaningful method of analyzing solids-loading effects. Over the solids-loading range studied, the rate of water vapor transmission decreased as solids-loading increased for both talc systems. The titanium dioxide films had a minimum water vapor transmission rate at an intermediate solids-loading value. A linear increase in the permeability coefficient was observed as the amount of polymer in the film cross section increased.

Keyphrases □ Solids-loading, two mineral talcs and titanium dioxide—effect on moisture permeability coefficients of cellulose free films □ Films, hydroxypropyl methylcellulose-ethylcellulose—effect of incorporating dispersed solid fillers (talcs and titanium dioxide) on moisture permeability coefficient □ Permeation (moisture) of cellulose films—effect of incorporating dispersed solid fillers (talcs and titanium dioxide) □ Conspergents—effects on moisture permeability of cellulose films

To expedite the coating of particles or tablets, water-insoluble or slowly soluble materials are often added in suspension in the polymer film-forming solution to increase solids-loading, to accelerate correspondingly the build-up of the film coat structure, and to reduce coating time and costs. Such filler coating materials may, however, greatly change the properties of the resultant films and their subsequent applications and uses.

The development and pharmaceutical applications of synthetic polymers as film structures have been recognized only in the last 15–20 years, resulting in film coating of pharmaceutical dosage forms for product protection or controlled drug release. Virtually nothing has been published that quantitatively indicates the effects of conspergents (dispersed nonpolymeric nonfilm-forming additives) upon the properties of pharmaceutical polymer films. Only very recently have pharmaceutical scientists begun to examine the structural, mechanical, and physiochemical properties of films related to film application, performance, and use (1, 2).

Table I—Additives Selected for Evaluation

Additive	Chemical Composition	Median Particle Size, μm[a]
Italian talc[b]	Hydrous magnesium silicate	20.5
Talc USP	Hydrous magnesium silicate	12.5
—	Titanium dioxide	2.7

[a] Coulter counter determination. [b] Lot 3MEA 064, Mead Johnson Co., Evansville, Ind.

EXPERIMENTAL

Materials—A hydroxypropyl methylcellulose[1] and a low molecular weight ethylcellulose[2] were the two polymers used. A 3% (w/v) concentration of hydroxypropyl methylcellulose-ethylcellulose (3:1) was used to cast free films from a solvent system of methylene chloride-methanol-isopropanol (4:3:3). A surfactant, polysorbate 20[3], 14.3%, and a plasticizer, propylene glycol, 34.1%, on a w/w basis calculated from the nonvolatile percent, were also included in each formulation. The water-insoluble additive agents (conspergents) selected for dispersion in the previously described polymer system are shown in Table I.

Preparation of Conspergent Suspension in Polymer Organosol—The conspergent was blended with all of the solvent including the propylene glycol and surfactant in a single-speed blender[4] for 5 min. This produced complete dispersion of the insoluble additive at low viscosity. The suspension was then transferred to a magnetic stirrer[5], and the polymer was added slowly with stirring. Then the suspension was covered and allowed to solvate for 24 hr with continued stirring at a speed setting of 4. The suspension was again placed in the blender for 3 min, after which the films were immediately cast. Although slight aeration resulted from redispersion in the blender in this step, the cast films were observed to be devoid of air bubbles.

Preparation of Free Films—Several methods were evaluated for casting free films. A glass substrate (polished side of a pill tile) was selected as yielding the smoothest, most uniform films. The glass substrate was coated by hand using disposable wipers with a 2% (v/v) silicone release agent[6] in xylene, which was then baked on at 250° for 2 hr. Plexiglas strips, 12.7 × 0.93 × 1.27 cm (5 × 0.37 × 0.5 in.), were used to partition the substrate into cells so that different volumes could be cast, yielding several film thicknesses for each formulation. The film casting was done in an airtight glove box, which served as a casting chamber to house the films during drying and to facilitate control of temperature and relative humidity. The temperature inside the chamber was controlled by circulating water at the required temperature through a radiator inside the chamber. The relative humidity inside the chamber was in the range of 40–50% as checked by the wet-dry bulb technique (3), and the temperature was controlled at 30 ± 2°. The films were allowed to dry for 24 hr in the chamber; they were then removed and stored in an oven in a desiccator with anhydrous calcium sulfate desiccant[7] at approximately 0% relative humidity and 30 ± 1° for 1 week prior to evaluation and testing.

Density Determination of Free Films—An indirect method of determining the film density was used employing film thickness and the relation $D = m/V$, where D = density of free film, m = weight of film sample in grams, and V = volume of film sample (cm^3) or (standard area) × (measured average thickness). The thickness of each film disk was measured in the center and

[1] Methocel 50 cps, 60 HG, Dow Chemical Co., Midland, Mich.
[2] Ethocel 50 cps standard ethoxy, Dow Chemical Co., Midland, Mich.
[3] Polyoxyethylene sorbitan monolaurate (Tween 20), Atlas Chemical Industries, Wilmington, Del.
[4] Model F.C.1 14, Waring Corp., New York, N.Y.
[5] La Pine Scientific Co., Chicago, Ill.
[6] 200 Fluid, Dow Chemical Co., Midland, Mich.
[7] Drierite, W. A. Hammond Drierite Co., Xenia, Ohio.

Table II—Italian Talc Water Vapor Transmission Data for Films

Film Density, g/cm³	Additive[a], %	Weight of Exposed Film, mg	Polymer, %	Weight of Polymer Exposed, mg	Film Thickness, mils	Film Thickness, cm × 10⁻³	Rate of Moisture Transport, mg/hr[b]	R_{wvt}, × 10⁻⁵[c]	Permeability Coefficient, × 10⁻⁷
1.176	0	13.75	59.45	8.17	2.79	7.08	6.37	13.3	9.43
		15.83		9.41	3.21	8.15	6.00	12.6	10.2
		18.06		10.74	3.66	9.30	5.66	11.8	11.0
		20.43		12.15	4.14	10.52	5.27	11.0	11.6
		22.56		13.41	4.58	11.62	5.22	10.9	12.7
1.190	10	14.52	56.12	8.15	2.91	7.39	5.79	12.1	8.95
		16.43		9.22	3.29	8.36	5.65	11.8	9.88
		16.43		10.84	3.87	8.36	5.26	11.0	10.8
		10.31		11.96	4.27	10.85	5.09	10.7	11.6
		21.32		12.66	4.52	11.48	4.90	10.3	11.8
1.204	20	22.50	53.13	11.95	4.46	11.32	4.73	9.89	11.2
		25.31		13.45	5.01	12.73	4.72	9.87	12.6
		29.58		15.72	5.86	14.88	4.40	9.21	13.7
		40.22		21.37	7.97	20.23	4.12	8.62	17.5
1.232	40	21.17	48.03	10.17	4.10	10.41	4.96	10.4	10.8
		23.27		11.18	4.51	11.44	4.65	9.73	11.1
		34.88		16.75	6.75	17.15	4.13	8.65	14.8
		36.73		17.64	7.11	18.06	4.07	8.52	15.4
1.260	60	21.13	43.82	9.26	4.00	10.16	4.61	9.65	9.80
		24.56		10.76	4.65	11.81	4.38	9.17	10.8
		26.89		11.78	5.09	12.93	4.32	9.04	11.7
		29.24		12.81	5.54	14.06	4.19	8.76	12.3
		37.44		16.41	7.09	19.00	3.74	7.83	14.1
		41.06		17.99	7.77	19.74	3.71	7.76	15.3
		44.05		19.30	8.34	21.18	3.60	7.53	16.0
1.288	80	26.51	40.29	10.68	4.91	12.47	4.32	9.05	11.3
		28.60		11.52	5.30	13.45	4.27	8.92	12.1
		41.37		16.67	7.66	19.46	4.17	8.73	17.0
		44.92		18.10	8.32	21.13	4.07	8.51	18.0
		50.90		20.51	9.43	23.94	4.04	8.44	20.2
1.316	100	22.96	37.28	8.56	4.16	10.57	4.17	8.72	9.21
		25.65		9.56	4.65	11.81	4.19	8.77	10.4
		27.70		10.33	5.02	12.75	4.18	8.74	11.2
		38.90		14.50	7.05	17.91	3.59	7.51	13.5
		46.88		17.48	8.50	21.58	3.34	6.98	15.1

[a] Percent (w/w) of polymer. [b] Rate of moisture transport = slope of the line in milligrams per hour. [c] Water vapor transmission rate = grams per (hour × square centimeters area × millimeters mercury). Table values are multiplied by 10^{-5} to produce experimental values.

around the periphery with a micrometer[8], reading to 1×10^{-5} in., and the weighings were carried out on an analytical balance. All thickness and weight measurements were made after residual solvent had been removed from the samples by storing the films as previously described for 1 week.

Water Vapor Transmission Study—Water vapor transmission rates were determined for all film formulations studied. The design of the transmission cell was very similar to the permeation cell used by Patel *et al.* (4) and Gore (5). It consisted of a 7-dram, screw-capped, cylindrical glass bottle with a circular hole, 1.5 cm in diameter, in the screw cap. A circular piece of film was placed between two rubber gaskets, which were then fixed inside the screw cap. Each rubber gasket also had a circular opening of 1.45 cm diameter. The area of the exposed film was 1.65 cm². The bottle contained approximately 5 ml of supersaturated sodium tartrate solution, which gave an internal relative humidity of 91% at 30°, equivalent to a vapor pressure of 28.96 mm Hg (6). The bottles were filled with the solution so that the distance from the film to the liquid surface was 4.75 cm.

The bottles were tightly sealed with the screw caps and placed in a desiccator over anhydrous calcium sulfate. The desiccator was placed in an oven at 30° and the bottles were allowed to equilibrate for 12 hr, at which time each cell was weighed on an analytical balance. These weights were labeled time-zero. Subsequent weighings were made in the same manner at 12-hr intervals for 72 hr. The amount of moisture transmitted through the film at any time is given by the loss of weight of the transmission cell at that time. Ten samples of four or more different film thicknesses were used for transmission measurements for each formulation.

An extensive review of the mathematical derivation and theory for calculating rates of moisture permeation was covered by several workers (4, 7–9). In this work, the water vapor transmission properties of the formulations studied were evaluated as functions of time, film thickness, weight of polymer exposed, and conspergent concentrations. The temperature, area of the film exposed, and vapor pressure gradient across the film were held constant.

The amount of moisture transmission due to the diffusion across the film membrane was expressed by Fick's law (10). Since one primary objective was to observe the effect of film thickness upon the water vapor transmission, this rate was calculated using the equation (7) modified by excluding the film thickness. Thus:

$$R_{wvt} = W/(A\,\Delta p) \qquad \text{(Eq. 1)}$$

where:

R_{wvt} = rate of water vapor transmission
W = amount of moisture transmitted per unit time in grams per hour
A = area of film exposed in square centimeters
Δp = vapor pressure gradient across film (28.96 mm Hg)

The water vapor transmission rate, R_{wvt}, is defined as the amount of moisture transmitted per unit time through a film of unit area when the film is subjected to a vapor pressure gradient of unity. R_{wvt} was expressed in the following dimensions: grams of moisture per (hours × square centimeters of film × millimeters mercury) at 30°. The value of W was calculated as the slope of the line resulting from a plot of moisture transmitted *versus* time. The variables A and Δp were maintained constant.

The permeability constant for each formulation was also deter-

[8] L. S. Starrett Co., Athol, Mass. [No. 25-209 gauge, No. 653 base, and 0.63-cm (0.25-in.) flat tip].

Table III—Water Vapor Transmission Data for Talc USP Films

Film Density, g/cm³	Additive[a], %	Weight of Exposed Film, mg	Polymer, %	Weight of Polymer Exposed, mg	Film Thickness, mils	Film Thickness, cm × 10⁻³	Rate of Moisture Transport, mg/hr[b]	R_{wvt}, × 10⁻⁵[c]	Permeability Coefficient, × 10⁻⁷
1.165	0	13.62	59.45	8.10	2.79	7.08	6.37	13.3	9.44
		15.68		9.32	3.21	8.15	6.00	12.6	10.2
		17.89		18.64	3.66	9.30	5.66	11.8	11.0
		20.24		12.03	4.14	10.52	5.27	11.0	11.6
		22.35		13.29	4.58	11.62	5.22	10.9	12.7
1.184	10	11.22	56.12	6.30	2.26	5.74	7.11	14.9	8.54
		12.06		6.77	2.43	6.17	6.43	13.5	8.30
		24.26		13.61	4.89	12.41	4.82	10.1	12.5
		33.68		18.90	6.78	17.23	4.65	9.74	16.8
		44.32		24.87	8.93	22.67	4.26	8.93	20.2
1.203	20	17.50	53.13	9.30	3.47	8.81	5.20	10.9	9.59
		19.37		10.29	3.84	9.75	5.06	10.6	10.3
		24.91		13.23	4.94	12.54	4.75	9.94	12.5
		26.60		14.13	5.27	13.39	4.26	8.92	11.9
		27.85		14.80	5.52	14.02	4.31	9.03	12.7
1.241	40	17.99	48.03	8.64	3.46	8.70	5.01	10.5	9.20
		19.67		9.45	3.78	9.60	4.91	10.3	9.86
		25.33		12.17	4.87	12.36	4.38	9.17	11.3
		35.53		17.07	6.83	17.34	3.92	9.21	14.2
		36.90		17.72	7.09	18.01	3.80	7.95	14.3
1.280	60	22.15	43.82	9.71	4.13	10.48	3.81	7.99	8.37
		30.50		13.37	5.68	14.43	3.43	7.18	10.4
		36.67		16.07	6.83	17.35	3.30	6.91	12.0
		38.38		16.82	7.15	18.16	3.23	6.77	12.3
1.318	80	24.35	40.29	9.81	4.41	11.19	3.67	7.69	8.60
		32.62		13.14	5.90	14.99	3.44	7.21	10.8
		35.21		14.19	6.37	16.18	3.28	6.88	11.1
		45.96		18.52	8.32	21.12	3.03	6.34	13.4
		48.97		19.73	8.86	22.50	2.92	6.11	13.8
1.356	100	25.28	37.38	9.42	4.45	11.29	3.47	7.26	8.19
		35.71		13.31	6.28	15.95	3.12	6.54	10.4
		40.73		15.18	7.16	18.19	3.11	6.52	11.9
		43.10		16.07	7.58	19.25	3.08	6.45	12.4
		46.24		17.24	8.13	20.65	2.95	6.17	12.7
		48.05		17.91	8.45	21.46	2.85	5.97	12.8
		51.21		19.09	9.01	22.87	2.79	5.85	13.4

[a] Percent (w/w) of polymer. [b] Rate of moisture transport = slope of the line in milligrams per hour. [c] Water vapor transmission rate = grams per (hour × square centimeters area × millimeters mercury). Table values are multiplied by 10^{-5} to produce experimental values.

mined and may be given by (7):

$$P_{erm} = R_{wvt}t \qquad \text{(Eq. 2)}$$

where P is the permeability constant, and R_{wvt} and t are as previously defined. The units for the permeability constant are (grams × centimeters) per (square centimeters × hours × millimeters mercury). The permeability constant should be independent of thickness in films that exhibit Fickian diffusion.

Determination of Percent Polymer Free Films—Knowledge of the weight content of each component in each film formulation permitted calculation of the percent polymer by weight contained in each formulation:

hydroxypropyl methylcellulose	4.500
ethylcellulose	1.500
propylene glycol	3.108 (3.0 ml)
surfactant	0.984 (0.9 ml)
	10.092

$$\text{percent polymer} = \frac{6.000}{10.092 + \text{additive}} \times 100 \qquad \text{(Eq. 3)}$$

Determination of Weight of Polymer Exposed for Water Vapor Transmission Studies—As previously described, the densities of the films were determined. By using the density–volume relationship and knowing the density, area, and thickness of each film formulation, it was possible to calculate the weight of each film disk subjected to water vapor determination. By knowing the weight of this film disk and the percent by weight of polymer it contained, it was thus possible to calculate the weight of polymer exposed to water vapor transmission in the film disk volume.

RESULTS AND DISCUSSION

Evaluation and Selection of Film Formulations and Film-Casting Techniques—The selection of a film-forming technique for uniform films is of paramount importance in research where film thickness must be accurately determined and controlled. The importance of this aspect of the research is compounded when the film is not homogeneous in nature but is actually a suspension of insoluble additives in the film-forming agent, such as the conspergents in Table I suspended in the cellulose combination studied.

The water-insoluble additive must be uniformly dispersed throughout the dried film to prevent concentrated areas of unequal strength or weakness and to provide a film specimen that will yield reproducible results over a given area. The method of film casting considered most acceptable for this work was to disperse the conspergent (Table I) in the solvent system. This produced complete dispersion of the conspergent unhampered by high viscosity which resulted after the suspending cellulosic suspending agents were added.

Several methods were evaluated for the production of free films, and the results indicated that a glass substrate previously coated with a releasing agent produced the most suitable films for this research. The release agent enhanced the smoothness of the glass substrate in addition to facilitating removal of the dried films from the substrate with minimal stress imparted to the films. The release agent was applied in such a diluted concentration, 2%, that negligible quantities of the agent were deposited on

Table IV—Water Vapor Transmission Data for Titanium Dioxide Films

Film Density, g/cm³	Additive[a], %	Weight of Exposed Film, mg	Polymer, %	Weight of Polymer Exposed, mg	Film Thickness, mils	Film Thickness, cm × 10⁻³	Rate of Moisture Transport, mg/hr[b]	R_{wvt}, × 10⁻⁵[c]	Permeability Coefficient, × 10⁻⁷
1.166	0	13.63	59.45	8.10	2.79	7.08	6.37	13.3	9.44
		15.69		9.33	3.20	8.15	6.00	12.6	10.2
		17.91		10.65	3.66	9.30	5.66	11.8	11.0
		20.26		12.04	4.14	10.52	5.27	11.0	11.6
		22.38		13.30	4.58	11.62	5.22	10.9	12.7
1.211	10	18.69	56.12	10.49	3.68	9.35	5.58	11.7	10.9
		21.55		12.09	4.25	10.78	5.16	10.8	11.6
		25.65		14.39	5.05	12.83	5.13	10.7	13.8
		33.84		18.99	6.67	16.93	4.78	10.0	17.0
1.255	20	18.05	53.13	9.59	3.43	8.71	4.83	10.1	8.81
		21.74		11.55	4.13	10.49	4.63	9.69	10.2
		23.38		12.42	4.44	11.28	4.58	9.60	10.8
		33.26		17.67	6.32	16.05	4.06	8.49	13.6
		36.53		19.41	6.94	17.63	3.74	7.84	13.8
1.433	60	23.00	43.82	10.08	3.83	9.72	5.47	11.5	11.1
		26.80		11.74	4.46	11.33	5.29	10.9	12.4
		47.29		20.72	7.87	19.99	4.62	9.67	19.3
		49.45		21.67	8.23	20.90	4.60	9.63	20.1
1.521	80	31.38	40.29	12.64	4.92	12.49	5.63	11.8	14.7
		49.51		19.95	7.76	19.71	5.23	11.0	21.6
		65.81		22.08	8.59	21.82	5.01	10.5	22.9
		64.64		26.04	10.13	25.73	4.99	10.4	26.9
		67.12		27.04	10.52	26.72	4.82	10.1	26.9
1.610	100	27.39	37.28	10.21	3.95	10.03	5.87	12.3	12.3
		41.13		15.33	6.09	15.47	5.33	11.2	17.3
		42.54		15.86	6.30	16.00	5.11	10.7	17.1
		50.04		15.65	7.41	18.82	5.12	10.7	20.2
		67.77		25.26	10.04	25.49	4.47	9.36	23.8

[a] Percent (w/w) of polymer. [b] Rate of moisture transport = slope of the line in milligrams per hour. [c] Water vapor transmission rate = grams per (hour × square centimeters area × millimeters mercury). Table values are multiplied by 10^{-5} to produce experimental values.

the film surface. Furthermore, since the release agent was baked onto the surface of the substrate at 250° for 2 hr, the probability of its transference was reduced.

In contrast with the technique of peeling the dried film from an aluminum substrate or uncoated glass substrate (7, 11), the dried films were lifted from the silicone-coated glass substrate virtually free of adhesion. This low order of adhesion and ease of film removal from the substrate were of great importance relative to maintaining the integrity of the film and preventing prestressing or distortion.

Determination of Free Film Densities—The densities of the films were determined for all film formulations (Tables II–IV). Figure 1 illustrates the linear relationship observed between film density and percent additive for the 0, 20, 60, and 100% additive formulations, and Table V shows the statistical constants determined for the relationship between film density and percent additive (w/w) with respect to polymer weight. The film densities for the 40 and 80% additive formulations were calculated from the established relationship between film density and percent additive for each additive material as described in the *Experimental* section. An average of five or more thickness determinations of each film specimen was made for each experimental density determined ±0.00001 in.

As seen in Fig. 2, the order of particle size decreases from Italian talc to talc USP to titanium dioxide. The median particle size for these additives was given in Table I. Figure 1 shows a linear increase in film density with decreasing additive particle size for all three insoluble film additives studied. This effect would be ex-

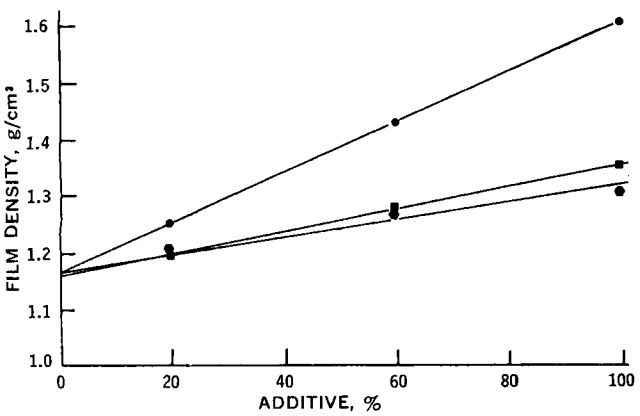

Figure 1—*Density of free films as a function of percent additive (w/w) with respect to polymer. Key: ●, titanium dioxide; ■, talc USP; and ◆, Italian talc.*

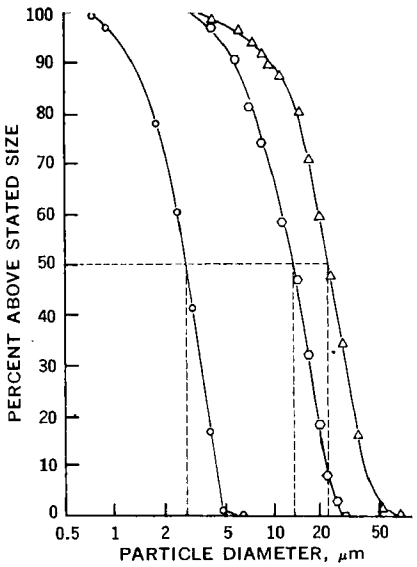

Figure 2—*Coulter counter particle-size analysis. Key: ○, titanium dioxide; △, Italian talc; and ○, talc USP.*

Table V—Statistical Constants for the Relationship between Film Density and Percent Additive (w/w) with Respect to Polymer

Additive	Correlation Coefficient, r	Slope of the Line, $m \times 10^{-3}$	Y Intercept
Italian talc	0.96	1.395	1.176
Talc USP	0.98	1.912	1.165
Titanium dioxide	0.99	4.440	1.166

pected based on the closer packing of the particles as the particle size decreases. However, it is also noted that the film density difference between the two talcs was not great. Although the median particle-size difference was substantial, the talc particle distributions overlapped each other. On the other hand, the film density of the titanium dioxide films was substantially greater than either of the talc films at any solids-loading value. The change in density with a change in solids-loading was greatest with the titanium dioxide films.

The amount of the moisture transmitted through all of the films was found to be directly proportional with time over 72 hr and was also related to the film thickness and composition. Figure 3 shows the relationship between time and milligrams of water vapor transmitted for free films containing 10% Italian talc (w/w) with respect to polymer. A similar relationship was observed for all film formulations that contained conspergents found in Table I.

It was determined (Fig. 4) that the rate of water vapor transmission across the film membranes for the solids-loaded films, in milligrams per hour, was directly related to film thickness in the thickness range studied. Statistical analysis showed the correlation coefficients for standard films (no conspergent) and conspergent films to be 0.98 or better, with two formulations showing a correlation coefficient of 0.92 and 0.93.

As seen in Tables II–IV, the rate of water vapor transmission, in grams per (hours × square centimeters × millimeters mercury), hereafter referred to as R_{wvt}, was determined for each formulation studied. Figures 5–7 show the relationship between this R_{wvt} and the weight (milligrams) of polymer exposed in the film disk (see *Experimental*). Since the films studied were two-component systems of homogeneous polymer–plasticizer dispersed with varying concentrations of heterogeneous conspergent additive, film thickness did not necessarily reflect the amount of polymer in the cross section of a standard area film disk.

As seen in Fig. 4, the rate of moisture transport decreased with increasing film thickness. Figure 5 illustrates the decrease in the R_{wvt}, which was consistently observed as the weight of polymer exposed in the Italian talc films increased (film thickness was increased and/or conspergent concentration was decreased). Figure 5 also demonstrates that the change in rate of moisture transport

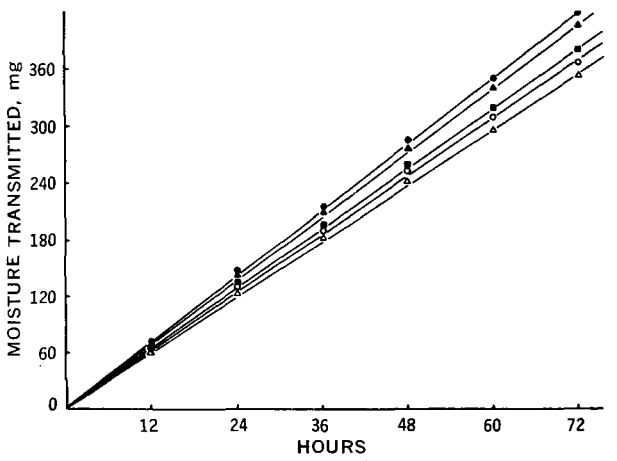

Figure 3—*Water vapor transmission as a function of time for 10% Italian talc free films. Key [film thickness (mils)]: ●, 2.91; ▲, 3.29; ■, 3.87; ○, 4.27; and △, 4.52.*

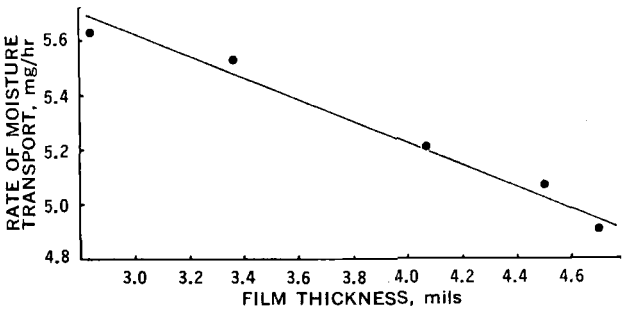

Figure 4—*Relationship between rate of moisture transport and film thickness for 10% Italian talc free films.*

can indeed be meaningfully expressed as a function of the amount of polymer in the film.

A consistent decrease in the R_{wvt} as a function of increasing Italian talc additive concentration was observed, at any mass of polymer exposed (Fig. 5), with the exception of the 80% formulation. The 0 and 10% additive formulations showed a much more marked decrease in R_{wvt} with an increase in weight of polymer exposed in comparison to the 20, 40, 60, and 100% additive formulations, which approached a plateau in rate of change of decreasing R_{wvt} with an increase in weight of polymer exposed. The 20, 40, 60, and 100% additive curves were displaced to lower values (Fig. 5), an indication of a decreasing transmission level with an increase in additive concentration at any weight of polymer in the film cross section.

Thus, the films containing 0 and 10% Italian talc additive with increasing film thickness or increasing polymer weight in the cross section produced substantial decreases in rate of water vapor transmission, whereas increasing film thickness or mass of polymer in the cross section of the films containing 20% and more additive did not produce an equally substantial decrease in R_{wvt}. Increasing dispersed additive concentration in the film structure rather consistently reduced the water vapor transmission rate, due probably to the increased hydrophobicity of the film. However, possibly due to the reduced integrity of the films containing 20% or more additive, increasing film thickness did not substantially increase protection to water vapor transmission.

In a similar manner, Fig. 6 shows a consistent decrease in R_{wvt} as a function of increasing talc USP additive concentration and as a function of increasing weight of polymer in the film disk exposed to moisture transport. A plateau in rate of change of decreasing R_{wvt} was reached at the 60, 80, and 100% additive formulations (Fig. 6).

Figure 7 illustrates the rate of change of R_{wvt} with increasing amounts of polymer exposed for titanium dioxide films. A different phenomenon is observed here. As with the two mineral talcs, the R_{wvt} decreased with increasing weight of polymer exposed, but the relationship between decrease in rate of R_{wvt} and percent additive was different. The R_{wvt} decreased as it did in the talc formulations until it reached the 20% additive formulation, at which concentration the decrease of R_{wvt} appeared to have reached its maximum. The 60% additive formulation showed a rate of decrease of R_{wvt} similar to that of the 10% additive formu-

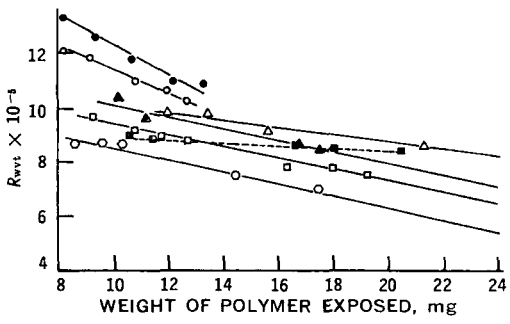

Figure 5—*Rate of water vapor transmission as a function of weight of polymer exposed for Italian talc free films. Key (percent additive): ●, 0; ○, 10; △, 20; ▲, 40; □, 60; ■, 80; and ○, 100.*

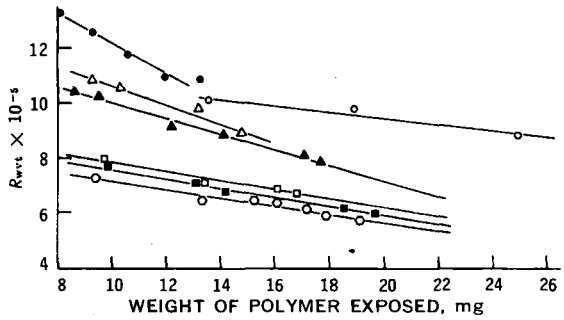

Figure 6—*Rate of water vapor transmission as a function of weight of polymer exposed for talc USP free films. Key (percent additive): ●, 0; ○, 10; △, 20; ▲, 40; □, 60; ■, 80; and ○, 100.*

lation, and the 80 and 100% additive formulations showed an even greater R_{wvt} than the lower titanium dioxide concentrations in the thickness range studied.

The difference in change of R_{wvt} and percent additive between pigment films containing titanium dioxide and additive films such as those containing mineral talcs in film formulations may be due to the striking difference in the conspergent surface characteristics, the surface of the talc particles being more complex and having a variation of chemical groups in its molecules (12). The elemental contents of talcs vary greatly among themselves according to their sources and, therefore, give rise to a more varied and complex surface than that shown by titanium dioxide. The surface differences, in addition to particle-size effects, could affect the cohesive forces within the film, film structure, and the influence imparted by an additive to the permeability properties of films.

Obviously, numerous factors affect the water permeability through conspergent loaded films, such as particle packing, particle distribution, film microstructure, and overall film hydrophilicity/hydrophobicity. Interaction between these variables could also be expected. Accordingly, it is not possible to predict the effect of solids-loading on the permeability properties of films comprised of different conspergent materials.

Figures 8–10 show the relationship between the permeability coefficient and weight of polymer exposed for the three conspergent systems. Figure 8 shows a linear increase in the permeability coefficient as the amount of polymer in the film cross section exposed to moisture transport increases and suggests that the permeability coefficient is independent of percent additive in the formulations for Italian talc and predominantly dependent on the weight of polymer exposed, with the coarsest additive's 80% formulation again being the only exception.

Figure 9 also shows a linear increase with a positive slope for this relationship, but the permeability coefficient also appears to be a function of the additive concentration for talc USP film formulations. No systematic relationship between solids-loading and permeability was noted for the titanium dioxide films (Fig. 10). An apparent permeability minimum was reached at 20% loading, after which permeability increased. This finding could be explained by the Chatfield (13) theory, which states that pigmentation decreases the moisture permeability of films until the critical pigment volume is reached. In Fig. 10, this critical pigment volume appears to be approximately 20%, above which concentration permeability increases. The presence of pigment particles in the film lengthens the path through which the moisture passes and is equivalent to a thicker unpigmented film. Above the critical pigment volume concentration, there is insufficient film-producing medium in the film structure to bind the pigment particles together so there are pores in the film through which the moisture passes.

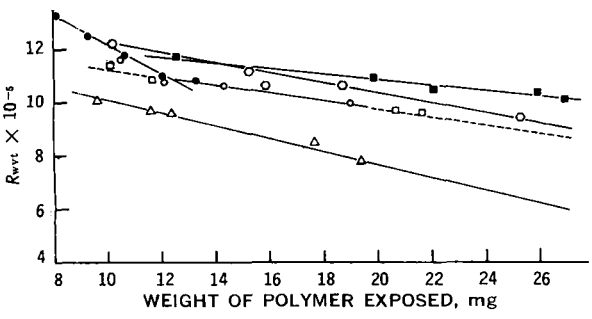

Figure 7—*Rate of water vapor transmission as a function of weight of polymer exposed for titanium dioxide free films. Key (percent additive): ●, 0; ○, 10; △, 20; □, 60; ■, 80, and ○, 100.*

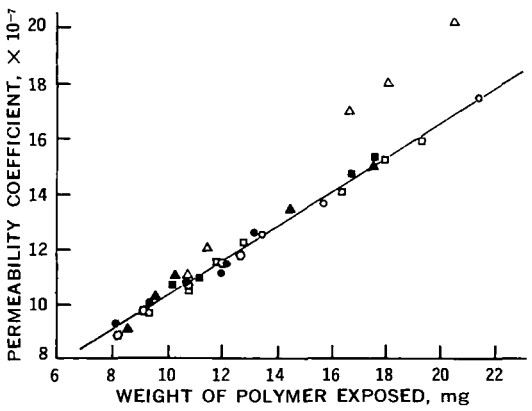

Figure 8—*Permeability coefficient as a function of weight of polymer exposed for Italian talc free films. Key (percent additive): ●, 0; ○, 10; ○, 20; ■, 40; □, 60; △, 80; and ▲, 100.*

In this study, the relationship between permeability and film thickness for the three conspergents studied clearly indicated a linear correlation existed between these properties. No statistically significant differences were found between the mineral talcs, but the titanium dioxide containing films showed a trend away from Fickian behavior at higher solids-loading. This again supports Chatfield's (13) theory in that pigment particles in the film above the critical pigment concentration give rise to pores in the film due to insufficient binding.

SUMMARY AND CONCLUSIONS

The nature of the dispersed film additive, its particle size, its surface characteristics, and the concentration of insoluble additives must be considered in evaluating the water vapor transmission properties of free polymeric films containing dispersed solids.

When analyzing the moisture permeation properties of free films containing dispersed solids, it was noted that film thickness was not a standard variable, since films of the same thickness containing the same volumes of different dispersed solids or different percentages of the same dispersed solid vary in the percentage or weight of polymer in any standard cross section of free

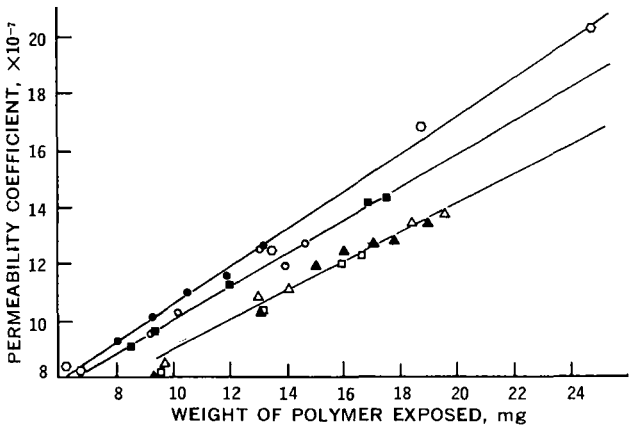

Figure 9—*Permeability coefficient as a function of weight of polymer exposed for talc USP free films. Key (percent additive): ●, 0; ○, 10; ○, 20; ■, 40; □, 60; △, 80; and ▲, 100.*

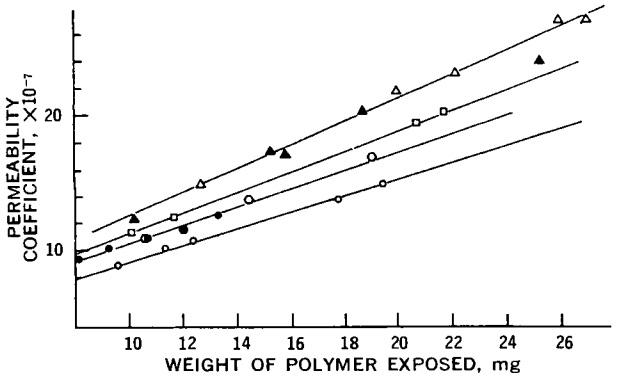

Figure 10—*Permeability coefficient as a function of titanium dioxide content (w/w with respect to polymer) in free films. Key (percent additive): ●, 0; ○, 10; ○, 20; □, 60; △, 80; and ▲, 100.*

film. A meaningful way of analyzing moisture permeation of free films containing dispersed solids has been suggested in which the permeability coefficient, which incorporates film thickness, is related to the mass of polymer in the film cross section analyzed. This type of analysis permits the comparison of permeability and water vapor transmission behavior of films containing different dispersed solids. In one talc-containing film system, no significant change in permeability was observed with a change in weight of polymer contained in the cross-sectional area of the films at different solids-loading stages; in another talc system, there was an effect on the permeability coefficient with a change in this variable. Titanium dioxide film systems appeared to exert a different effect on permeability properties, as predicted by Chatfield's (13) theory.

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* Present address: The Upjohn Company, Kalamazoo, Mich.

ˣ To whom inquiries should be directed.

Quantitative Assessment of Surface Mottling of Colored Tablets

N. A. ARMSTRONG[x] and G. A. MARCH*

Abstract □ A photographic technique was developed to measure the degree of mottling of calcium phosphate tablets colored with FD&C Blue No. 1 dye. Optimization of the photographic process is described, and mottling values calculated by this method are shown to correlate well with the visual appearance of the tablets. Although the technique was developed primarily with a view to investigating factors that affect mottling, it could form the basis of a quality control procedure for use with colored tablets where mottling may be a cause for concern.

Keyphrases □ Surface mottling of colored tablets—measurement, photographic technique and equipment □ Mottling, surface of colored tablets—measurement, photographic technique and equipment □ Tablets with colored surfaces—measurement of mottling, photographic technique and equipment

Colorants are widely used in the manufacture of compressed tablets for elegance and ease of identification. In the case of tablets prepared by a wet granulation process, however, it is difficult to attain a reproducible, uniform surface coloration. The wet mass generally is uniformly colored before granulation, but during drying the colorants migrate with the granulating fluid and accumulate at the drying surface. Compression of these nonuniformly colored granules results in mottled tablets.

To quantify some factors which influence tablet mottling, it was necessary to develop a reproducible technique for assessing the uniformity of color dispersion exhibited by a tablet. Currently used methods (1, 2) for the measurement of overall tablet color are based on the Kubelka–Munk equation, which can only be applied to light reflected from a uniformly colored surface; therefore, these methods are unsuitable for the evaluation of mottled tablets. For this evaluation, one requires an apparatus capable of measuring variations in light reflectance along a predetermined path across a tablet surface. Two apparatus appeared to be suitable. One of these[1] utilizes the reflectance of an intense beam of light from the tablet surface. Although convenient, it had to be abandoned since the light beam caused fading of the dye. The second apparatus[2] measures the absorbance of a photographic negative image of the tablet.

This report describes a method using the second apparatus which provides reproducible measurement of tablet mottling and includes the development of a standardized photographic process to eliminate artifacts caused during the production of a photographic negative.

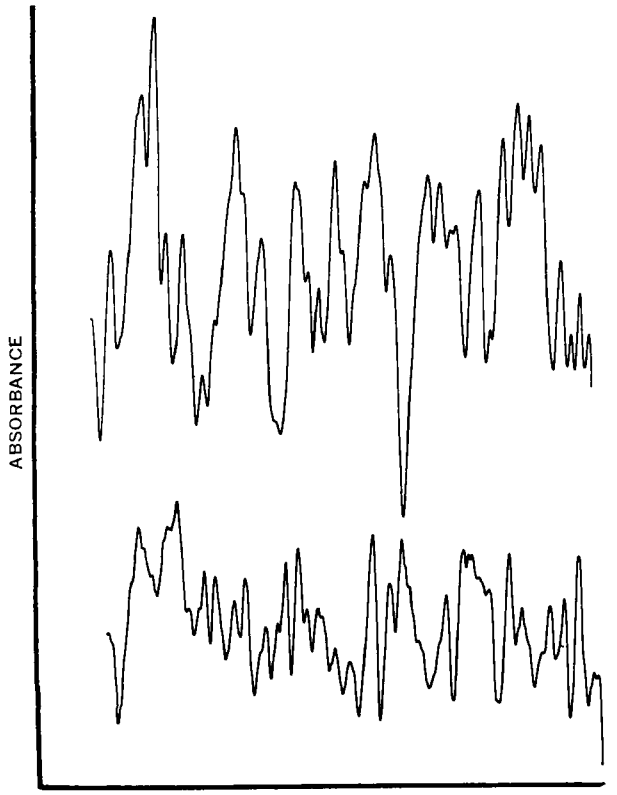

Figure 1—*Microdensitometer traces of mottling across the surface of (bottom) a slightly mottled (8.5 units) tablet and (top) a more severely mottled (24.5 units) tablet of calcium phosphate colored with FD&C Blue No. 1 dye.*

EXPERIMENTAL

Tablet Preparation—Dibasic calcium phosphate NF was colored with 0.0333% (w/w) FD&C Blue No. 1 dye (92% dye content)[3] dissolved in water (100 ml). The colored powder was granulated with polyvinylpyrrolidone[4] (15% w/w in water) and sieved through a 2.8-mm mesh screen using an oscillating granulator[5]. A portion of the wet granulate was dried for 20 min in a fluid-bed drier[6] (air inlet temperature 60°). The remaining material was spread to a depth of 1 cm and dried in a tray drier[7] for 8 hr at 60°.

The cake of tray-dried granules was broken down by an oscillating granulator fitted with a 1.70-mm mesh screen, but the fluid-bed dried granules were not comminuted further. Sieve fractions of each granulation were collected by shaking for 20 min on a vibratory sieve shaker fitted with British standard sieves[8]. Each fraction was lubricated with magnesium stearate BP (1%) colored with the same dye (0.0333%) and compressed into tablets by a single-stroke tablet machine[9] fitted with 12.5-mm diameter flat-faced punches. Each tablet weighed 660 mg and had a thickness of 4.00 mm.

[1]Vitatron densitometer, Fisons Scientific Apparatus Ltd., Loughborough, United Kingdom.

[2]Joyce Loebl microdensitometer Mark III CS, Joyce Loebl and Co. Ltd., Team Valley, Gateshead, United Kingdom.

[3]D. F. Anstead Ltd., Billericay, United Kingdom.

[4]Plasdone-25, Badische Anilin and Soda-Fabrik A. G., Ludwigshafen am Rhein, West Germany.

[5]Type 143A, Apex Construction Co., London, United Kingdom.

[6]Type SSE65, Apex Construction Co., London, United Kingdom.

[7]Model VA, L. A. Mitchell Ltd., Manchester, United Kingdom.

[8]Pascall Engineering Co. Ltd., Crawley, United Kingdom.

[9]Model E2, Manesty Machines Ltd., Liverpool, United Kingdom.

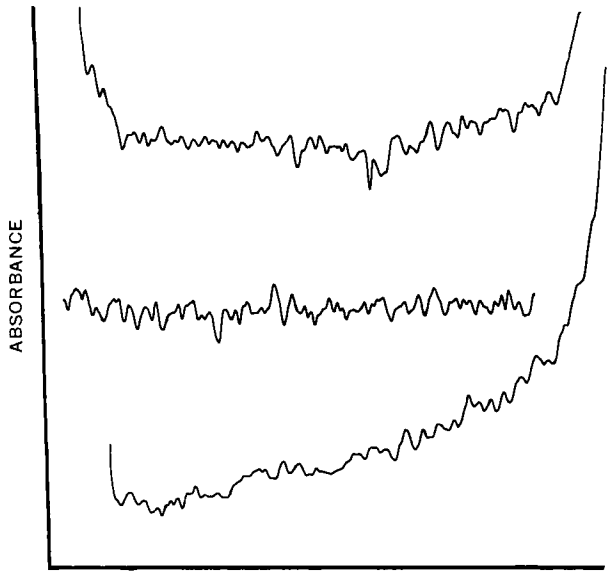

Figure 2—*Effect of developer type and agitation during development on the uniformity of density exhibited by negatives of a uniformly illuminated gray card. Key: top, a fast developer with agitation; middle, a slow developer with agitation; and bottom, a slow developer without agitation.*

Preparation of Photographic Negatives by a Standardized Method—A standard camera unit[10], consisting of a camera with a 127-mm lens, camera stand, baseboard, and lamp supports, was illuminated by four floodlights (150 w, 240–250 v), one mounted at each corner of the baseboard. A tablet set in a circular orifice cut in a gray neutral test card[11] was mounted at the center of the baseboard. Photographs were taken using 35-mm fine grain roll-film[12], with optimum exposure (4.0 sec). Contrast was enhanced by the use of a red gelatin filter[13]. After exposure, the film was placed in a daylight development tank[14] containing developer[15] (760 ml). The tank was inverted 15.0 sec after the film had been placed in the developer solution and then inverted (0.5 sec) at 5.0-sec intervals throughout the development process (10.00 min). The temperature of the developer solution was maintained at 20.0° by partially submerging the tank in a water bath. Agitation of the developer solution was ceased 10.0 sec before the end of the development period and the film was fixed[16], washed, and dried.

Method of Assessing Mottling—The microdensitometer[2] is designed to produce a graphic record of the changes in absorbance along a predetermined path across a transparent specimen such as a photographic negative. Absorbance (A) is defined as $A = \log P_0/P_t$, where P_0 and P_t are, respectively, the incident and radiant flux of a measured area of specimen.

A photographic negative image (14.30 mm diameter) developed by the standard method was scanned by the microdensitometer along paths (1.166 mm wide) across four diameters orientated at 45° to each other. The dimensions of the rectangular scanning beam were 1.166 × 0.136 mm. Typical traces obtained from a severely mottled tablet and a more uniformly colored specimen are shown in Fig. 1. An arbitrary baseline was drawn on each graph, and the ordinate of the trace was measured at equidistant intervals along this abscissa, giving some 45 values per graph. (Each interval was equivalent to about 0.25 mm on the actual tablet surface.) A numerical assessment of mottling was made by calculating the standard deviation of these values around a mean, the standard deviation being independent of the position of the baseline. Finally, the average was calculated from the four standard deviation values obtained from each tablet.

[10]Polaroid MP-3.
[11]Kodak Ltd., Kingsway, London, United Kingdom.
[12]Ilford Pan F, Ilford Ltd., Ilford, United Kingdom.
[13]No. 25 Wratten, Kodak Ltd., London, United Kingdom.
[14]Johnsons of Hendon Ltd., London, United Kingdom.
[15]Ilford ID11, Ilford Ltd., Ilford, United Kingdom.
[16]Ilford Hypam fixing solution, Ilford Ltd., Ilford, United Kingdom.

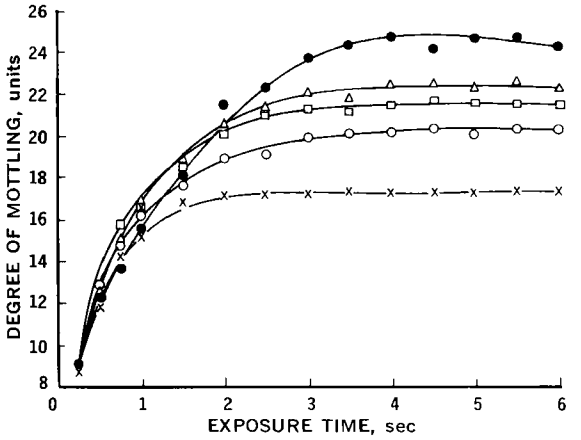

Figure 3—*Effect of development time on the numerical values obtained for the mottling on the surface of a tablet. Key (development time): ×, 8 min; ○, 9 min; □, 10 min; △, 11 min; and ●, 12 min.*

Effect of Developer Type and Agitation on Development Uniformity—Three sets of photographic negatives of a uniformly illuminated neutral test card were prepared. One set was developed by the standard method using a slow acting developer[15]. Another set was prepared by a similar process, but the tank was not inverted during development. The third set was developed for 2.00 min in a fast developer[17] by the agitation process described in the standardized method.

Effect of Exposure and Development Time on the Mottling Value—Negatives of a mottled tablet were prepared at a range of exposure levels by the standard method. Other negatives of this subject were prepared by the same process but were developed for different periods.

Negatives of other mottled tablets exhibiting different levels of color saturation were prepared at a range of exposure levels by the standard method.

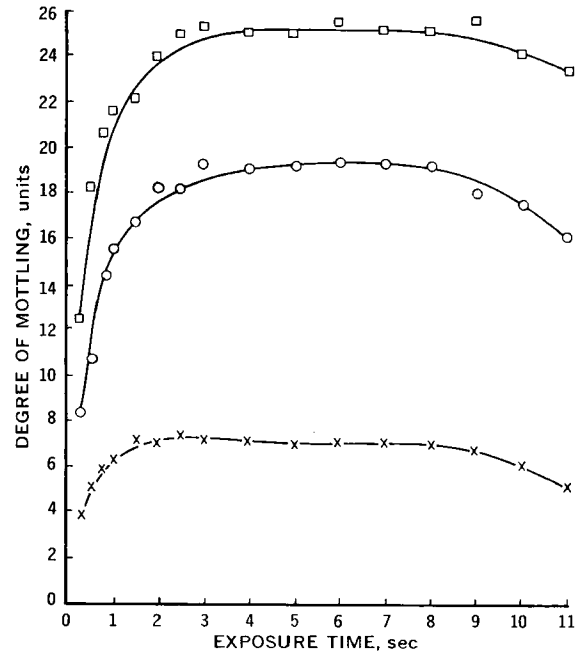

Figure 4—*Effect of exposure time on the numerical values obtained for the mottling on the surface of tablets exhibiting different levels of color saturation and mottling. Key: □, dark-blue, severely mottled tablet; ○, blue, mottled tablet; and ×, pale-blue, relatively uniformly colored tablet.*

[17]Ilford Contrast FF.

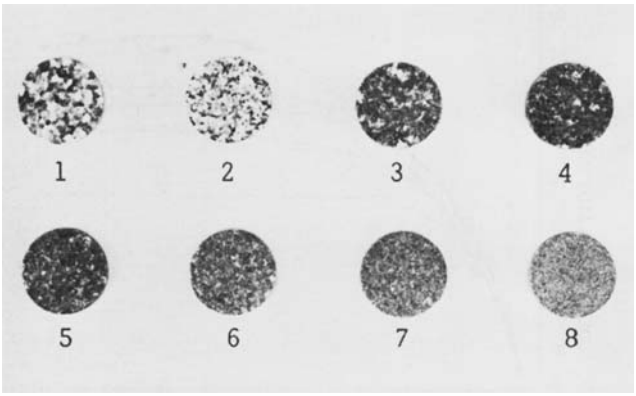

Figure 5—*Effect of drying method and granule size on the mottling shown by calcium phosphate tablets colored with FD&C Blue No. 1 dye (numbers refer to Table I).*

RESULTS AND DISCUSSION

The irregular traces (Fig. 1) representing the changes in absorbance across tablet images were evaluated by the method utilized by Bricout (3) to quantify similar traces representing the random distribution of silver grains in a photographic negative. The traces in that study were obtained with a high power microdensitometer. Mottling, being a random distribution of color over a tablet surface, was not constant and the standard deviation value for each path across a tablet was different. However, reproducible results could be obtained by evaluating four diametrical paths per tablet.

Since this method depends on changes in absorbance across a photographic negative, it is essential to minimize variations in density due to insufficient agitation of the developer (Fig. 2). This problem is well recognized in photography, and many elaborate methods have been devised to ensure reproducible vigorous agitation during the development process (4). The inversion technique used in this investigation is based on that described by the British Standards Institution (5) and gave adequate turbulence using a slow acting developer[15] (Fig. 2). The negatives of a uniformly illuminated test card had a mottling value of 1.9; this figure, representing background mottling due to silver grains and development artifacts, was subtracted from all subsequent mottling determinations. The background value was constant for each of the 36 negatives of a roll-film and reproducible from batch to batch. The inversion technique could not, however, be used to produce sufficient agitation (Fig. 2) for a faster developer[17].

It has been shown that there is a linear relationship between the logarithm of the exposure time and the density exhibited by a uniformly exposed negative (6), unless a film is underexposed or overexposed. Since density is also affected by the development time, both development and exposure time affect the contrast of a negative and, consequently, the numerical value for mottling assigned to the negative image of a tablet. Prolongation of development (Fig. 3) increased density and, hence, negative contrast and mottling values, but development times greater than 11 min caused excessive granularity of the negatives through agglomeration of silver particles. These negatives could not detect fine detail, and values for mottling were not constant. There was less variation between the values for mottling obtained with negatives developed for 8 min, but the mean value was less reproducible. This was due to slight variations in the time required to transfer the film to and from the developer solution. A development time of 10.00 min was found to be satisfactory and was selected as standard.

Reproducible results could only be obtained when the film had been adequately exposed, and the time required to attain an adequate level of exposure varied according to the depth of color of the tablet. Light-colored tablets, which reflect more light, required a shorter exposure time before reaching the region of linearity than those of greater color saturation (Fig. 4). Tablets of dibasic calcium phosphate colored with FD&C Blue No. 1 were prepared from fluid-bed dried granules of closely defined size. Visual examination showed that the tablets prepared from coarse material were a darker color and more mottled than those prepared from smaller granules. The minimum exposure level suitable for very severely mottled dark-blue tablets was 4.0 sec (Fig. 4). This level was insufficient to produce a decrease in values for mottling of paler, less mottled tablets due to overexposure (longer than 8.0 sec).

The high degree of mottling exhibited by tablets prepared from coarse granules is thought to be due to their relatively low surface area–volume ratio. On drying, dye migration to the periphery of the granule produced a layer with a high concentration of colorant. On fracture, there was a marked contrast between the color of the periphery of a large granule and its color-deficient interior; therefore, the tablets were severely mottled. Figure 5 is a photograph of this range of tablets, and Table I shows the corresponding values for mottling.

The correlation between experimentally determined mottling values and the visual appearance of those tablets will not necessarily apply to tablets incorporating a dye of a different color. This is because the human eye is not equally sensitive to light from all regions of the spectrum (7) but is most sensitive to yellow (wavelength 570 nm) and least sensitive to blue (400 nm) and red (700 nm) light. Blue and red objects, therefore, appear to reflect less light than those colored yellow. Sumner (8) showed that differences in light reflectance have a much greater influence on the legibility of print than have differences in color. Consequently, yellow and white mottled tablets appear to the eye to be more uniformly colored than those prepared with blue or red colorants. A photographic film, however, is equally sensitive to light of all visible wavelengths and a photographic negative would show all nonuniformly colored tablets to be mottled, irrespective of their actual color.

Although the foregoing technique was primarily developed to facilitate investigation of the factors affecting mottling, it could form the basis of a quality control procedure for colored tablets where mottling is a problem. For a specific product, it is possible that a range of tablets could be produced showing quantified degrees of mottling and these could be used as reference standards.

Table I—Effect of Drying Method and Granule Size on Mottling Shown by Calcium Phosphate Tablets Colored with FD&C Blue No. 1 Dye[a]

Tablet Number	Method of Drying	Granule Size Range, μm	Mottling Value
1	Tray dried	710–1000	38.8
2	Tray dried	355–600	23.1
3	Fluid bed dried	1400–1680	21.1
4	Fluid bed dried	1000–1400	19.8
5	Fluid bed dried	710–1000	17.3
6	Fluid bed dried	600–710	14.0
7	Fluid bed dried	355–600	7.0
8	Fluid bed dried	250–355	5.5

[a] Photographs of these tablets are shown in Fig. 5.

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*Present address: Merck Sharp & Dohme Research Laboratories, Hoddesdon, United Kingdom.

xTo whom inquiries should be directed.

Influence of Sunscreening Agents on Color Stability of Tablets Coated with Certified Dyes I: FD&C Red No. 3

B. R. HAJRATWALA

Abstract □ The influence of a protective coating of certain sunscreening agents upon the photostability of FD&C Red No. 3 used to color coat tablets was studied. The sunscreening agents used were glyceryl *p*-aminobenzoate, 2-ethoxyethyl *p*-methoxycinnamate, benzocaine, *m*-homomenthyl salicylate, *n*-octyl salicylate, and amyl salicylate. Alcohol film-, modified sugar-, and film-coating methods were developed and used to apply the sunscreening agents. Tablet samples were exposed to intensified artificial light of a measured intensity. At certain intervals, the change in reflectance of the surface of the colored tablets was measured using a reflectance instrument. Visual observations were also made. Kinetic studies were performed, and approximate shelflives of the various colored tablets were calculated using the Kubelka-Munk equation. The greatest protection against fading was observed with 2-ethoxyethyl *p*-methoxycinnamate as the protective agent.

Keyphrases □ Sunscreening agents—effect on color stability (shelflife) of tablets coated with FD&C Red No. 3 □ Tablets—three methods for coating with sunscreening agents, effect of agents on color stability (shelflife) of FD&C Red No. 3 □ Dyes, color stability of coated tablets—effect of sunscreening agents on FD&C Red No. 3

The stability of certified dyes used in pharmaceuticals has been of continued interest to the pharmaceutical formulator. Within the last decade, reflectance measurements have been used to determine the stability of various dyes. The effect of light on creams, powders, granules, and coated tablets was studied (1). Lachman and coworkers (2–7) carried out extensive quantitative studies on color-coated tablets. The fading of dyes was observed to follow an apparent first-order reaction, but their work did not take into account the sensitivity of the human eye.

Several other studies were made to quantitate the dye-fading phenomenon. These studies utilized various equations such as the Kubelka-Munk equation (8, 9) based on kinetic principles or the Adams-Nickerson or McAdam equations (10) based on color differences and chromaticity coordinates. Recently, a fadeometer was employed for the rapid determination of color stability (11). Some attempts were made to stabilize dye fading in tablets, using derivatives of benzophenones (6, 12, 13), resorcinol (12), and sulfonic and acrylic acids (13). Sunscreening agents, *e.g.*, salicylates, benzoates, and cinnamates, are mainly used for protection from sunburn, to control the degree of tanning, and for protection of colors in textiles and plastics. These agents could provide protection against color fading if applied as a coating on tablets coated with certified dyes.

The purposes of this study were to: (*a*) develop a procedure for the application of these sunscreening agents to tablets coated with certified dyes; (*b*) determine the thickness of the coating of the sunscreening agents on the tablets; (*c*) determine the amount of sunscreening agent applied on tablets in the form of a coating; (*d*) evaluate the influence of sunscreening agents of the salicylate, benzoate, and cinnamate types on tablets coated with the FD&C Red No. 3; and (*e*) predict the color shelflife of tablets.

EXPERIMENTAL

Materials—Six sunscreening agents were selected: glyceryl *p*-aminobenzoate[1], benzocaine, 2-ethoxyethyl *p*-methoxycinnamate[2], *m*-homomenthyl salicylate, *n*-octyl salicylate, and amyl salicylate[3]. The dye used was FD&C Red No. 3. All compounds were used as received without further purification.

Tablet Preparation—Tablets were prepared according to the following formula:

calcium phosphate, dibasic	99.4%
magnesium stearate	0.5%
talc	0.1%
acacia solution, 25%	*q.s.*

The granules were prepared according to commonly employed tableting techniques, using 25% acacia solution as the granulating agent. Tablets weighing 365 mg, with a thickness of 3.45 mm and a hardness[4] of approximately 13 kg/6.45 cm^2 (1 in.^{2}), were compressed using a 1.06-cm (0.42-in.) deep concave punch[5].

Coating Procedure—Ten subcoats and 25 smoothing coats were applied to core tablets, in lots of 6 kg, using medium

[1] Escalol 106, Van Dyk & Co.
[2] Giv-Tan-F, Sindar Corp.
[3] Amyl Salicylate Extra, Fristsche Brothers, Inc.
[4] Pfizer hardness tester, Chas. Pfizer Co.
[5] Stokes model B-2 rotary machine.

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strength gelatin syrup and white subcoating dusting powder. After tablets were completely dry, the color coating was applied. Plain coating syrup (170 g sucrose and 100 ml deionized water) and color syrup (0.300 g dye in 100 ml plain coating syrup) in the following ratios were applied. Five coats of 25 ml each in 15:1 parts, followed by 10 coats of 25 ml each in 10:1 parts, followed by 10 coats of 27 ml each in 5:1 parts, and finally 12 coats of 27 ml each in 2:1 parts of plain syrup and color syrup, respectively.

Application of Sunscreening Agents—Since the sunscreening agents used were insoluble in water, the following three coating methods were developed: alcohol film, modified sugar, and film. The coating was performed on the thoroughly dried color-coated tablets in batches of 500 g. One coat of 2 ml and four coats of 1.5 ml coating solution were applied in each case. After drying thoroughly, tablets were polished, where mentioned, with carnauba wax pieces in a canvas-lined pan. In each case, a set of control tablets was coated using the same solutions without the sunscreening agent.

Alcohol Film Coating—The formula of 5% sunscreening agent and ethanol (95%) *q.s.* was used for all sunscreening agents except benzocaine, which was applied in 0.5% concentration. In earlier experiments, it was found that 5% benzocaine caused mottled tablets.

Tablets coated with glyceryl *p*-aminobenzoate had a "wet" feeling even after drying for 24 hr at ambient temperature. Tablets coated with 2-ethoxyethyl *p*-methoxycinnamate and *m*-homomenthyl salicylate were shiny even before they were polished. A portion of the tablets coated with *m*-homomenthyl salicylate was left unpolished to compare the difference between polished and unpolished tablets.

Modified Sugar Coating—The coating solution consisted of one part of alcohol film-coating solution and 10 parts of diluted color syrups [color syrup–plain syrup (1:2)]. The resulting coating solutions were turbid with the exception of those containing glyceryl *p*-aminobenzoate and benzocaine, which were clear solutions.

Film Coating—The coating solution consisted of equal parts of Solutions A and B. Solution A consisted of 10% cellulose acetate phthalate, 4% polyethylene glycol 4000, and acetone *q.s.* Solution B consisted of 10% sunscreening agent and ethanol (95%) *q.s.*

The finished coat was uniform, hard, smooth, and glossy. However, tablets coated with salicylates had a mottled appearance after 24 hr of drying at ambient temperature. The mottled appearance was due to cracking of the film coating. A reported formula (12) for film coating was tried but did not appear satisfactory for these sunscreening agents.

Equipment—The following were used: a light cabinet used by Vaidya (12), similar to that described by Lachman *et al.* (14); a light meter[6] (range 0–5000 foot-candles); a spectrophotometer[7] with reflectance attachment (12, 14) with magnesium carbonate blocks; and a micrometer caliper[8].

Exposure to Light—Adequate samples of each set of color-coated tablets were placed in flat, white, paper trays in the light cabinet. The lower surfaces of samples were marked so as to distinguish the surface exposed to incident light. The intensity of light was maintained at a constant level of 1000 foot-candles while the temperature remained at 26 ± 2°. Regular checks were made of light intensity and temperature. Heat build-up was prevented by a constantly operating fan.

Measurement of Color Change—Samples were withdrawn from the light cabinet at designated time intervals, and the reflectance of the tablets was measured using the transmittance scale. Measurements were made between 450 and 575 nm for each sample at appropriate wavelength intervals and at the maximum for the dye (535 nm). The color of tablet samples was also evaluated by visual observation at various time intervals. This procedure is in keeping with a previous report (15) which described the importance of visual observation as a method for following color change.

Determination of Sunscreening Agents per Tablet—One hundred tablets were extracted with 150 ml ether. The ethereal layer was washed with 3 × 100 ml of deionized water to remove traces of color and water-soluble compounds. The clear ethereal layer was separated and evaporated to dryness. The residue was dissolved in alcohol, dilutions were made, and the agent was determined quantitatively at 295 nm for glyceryl *p*-aminobenzoate, at 292.5 nm for benzocaine, at 311 nm for 2-ethoxyethyl *p*-methoxycinnamate, at 305 nm for *m*-homomenthyl salicylate, and at 306 nm for *n*-octyl salicylate and amyl salicylate.

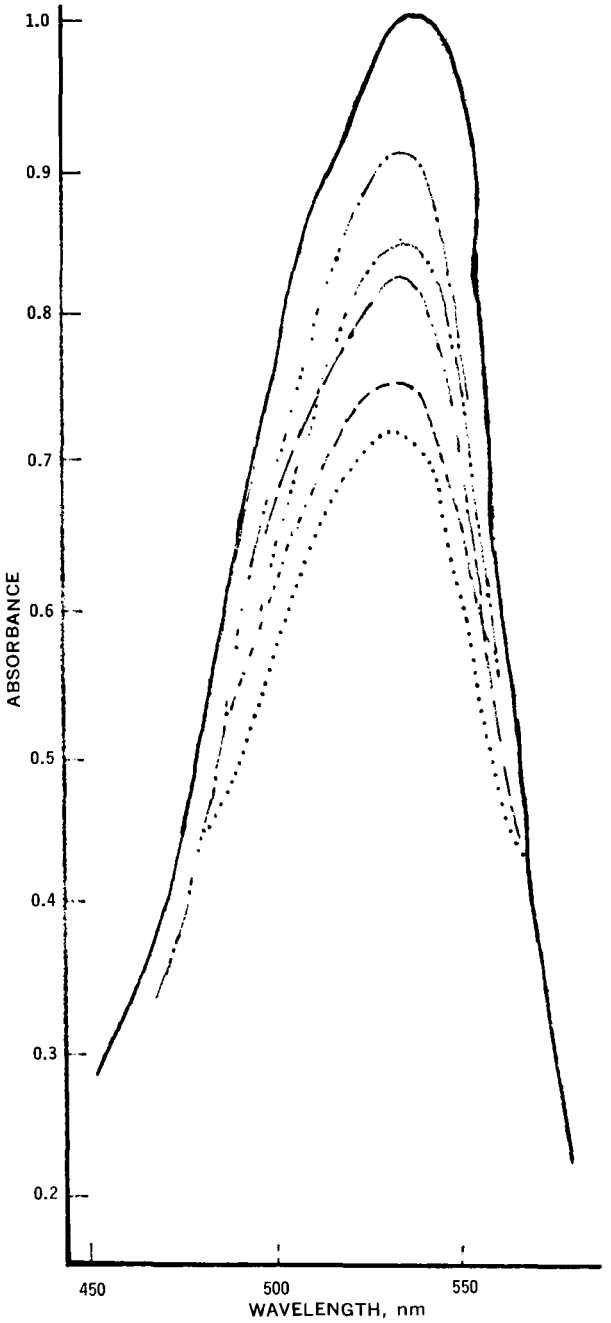

Figure 1—*Visible absorption spectra of control tablets color coated with FD&C Red No. 3 by alcohol film coating, after intervals of storage under 1000 foot-candles of illumination. Key: ——, initial; —·—, after 5 days; —··—, after 10 days; — — —, after 20 days; - - -, after 30 days; and ..., after 40 days.*

RESULTS AND DISCUSSION

The range[9] of thickness of five coatings of sunscreening agent applied by the alcohol film-coating method was 15–20 μm; by the modified sugar-coating method it was 300–400 μm, and by the film-coating method it was 30–40 μm. The greater thickness and

[6] General Electric, type 213.
[7] Beckman DU.
[8] Starrett model 436.

[9] Range of 25 readings on individual tablets.

Table I—Rate Constants for Fading of Color-Coated Tablets with FD&C Red No. 3 and Approximate Shelflife to Objectionable Fading at 50 Foot-Candles

Sunscreening Agent[a]	Alcohol Film Coating		Modified Sugar Coating		Film Coating	
	$k \times 10^5$, (foot-candle day)$^{-1}$	Shelflife, days $\times 10^{-2}$	$k \times 10^5$, (foot-candle day)$^{-1}$	Shelflife, days $\times 10^{-2}$	$k \times 10^5$, (foot-candle day)$^{-1}$	Shelflife, days $\times 10^{-2}$
Control	5.1	2.4	6.1	3.8	3.0	2.4
Glyceryl *p*-aminobenzoate	3.5	0.6	6.6	2.9	2.2	0
Benzocaine	1.9	6.7	4.2	2.9	5.8	2.0
2-Ethoxyethyl *p*-methoxycinnamate	3.1	3.4	2.7	6.0	2.5	8.0
m-Homomenthyl salicylate (n.p.)	3.0	6.0	—[b]	—[b]	—[b]	—[b]
m-Homomenthyl salicylate	2.1	6.1	2.0	6.6	2.7	0
n-Octyl salicylate	1.4	3.6	1.9	6.8	2.7	0
Amyl salicylate	6.3	2.0	3.8	5.0	3.2	0

[a] n.p. = not polished; all others were polished. [b] Not studied.

range with the modified sugar-coating method are due to the presence of sugar.

Amount of Sunscreening Agent Applied on Tablets as a Coating—The theoretical yield for tablets coated by the alcohol film-coating method was 400 μg/tablet, except for tablets coated with benzocaine where it was 40 μg/tablet. The theoretical yield for tablets coated by the modified sugar-coating method was 40 μg/tablet; for tablets coated by the film-coating method, it was 400 μg/tablet.

The average (of three assays) amount of sunscreening agent found was approximately two-thirds of the theoretical amount. The remaining unaccounted for sunscreening agent was probably "lost" in the coating procedure by adhering to the inside of the coating pan, hand, *etc.* The only exceptions were:

1. Tablets coated with glyceryl *p*-aminobenzoate. *Alcohol film method*—The amount present was only 40–50 μg/tablet, and it appears that a definite chemical reaction occurred which resulted in a change of hue. *Modified sugar-coating method*—No hue change was observed apparently because the amount present was very little (8–10 μg/tablet). *Film-coating method*—the hue change was marked, and the amount found ranged from 300 to 310 μg/tablet. In no case was any attempt made to isolate degradation product(s) of the dye or the sunscreening agent.

2. Tablets coated with salicylates using film-coating method. It was not possible to recover more than 10–20 μg/tablet.

Influence of Sunscreening Agents—The reflectance curves of tablets were recorded, and a typical curve is shown in Fig. 1. To evaluate the effect of sunscreening agents on FD&C Red No. 3, the Kubelka-Munk equation (Eq. 1) was used:

$$\ln \theta_t = -ktI + \ln \theta_t' \qquad \text{(Eq. 1)}$$

where $\theta_t = (1 - R)^2/2R$ in which R is the reflectance at the absorbance maximum at time t, I is the intensity in foot-candles, and k is an apparent first-order rate constant; θ_t' is θ_t at $t = 0$. The theoretical concepts of the Kubelka-Munk equation and its wide use were described elsewhere (8, 9).

Figure 2 shows typical plots of θ_t *versus* product of time and intensity. The fading of FD&C Red No. 3 fits a first-order rate equation in the presence and absence of sunscreening agents. The first-order rate constants were calculated (Table I), and the rate constant for this dye is different than those reported previously (3, 8). The difference is due to the differences in the overall composition of the coating plus the background material. Previous investigators included the dye and UV absorber within the granulation, while in this study they were applied to the tablet as coatings.

Visual observations were made on all tablets (Table II). A scale value of 1, 2, or 3 (fading less than, similar to, or greater than the control, respectively) was arbitrarily assigned. The time at which objectionable fading or appearance was seen, in the opinion of the author, was also noted. To determine the shelflife of coated tablets, the light intensity in a well-illuminated room was taken as 50 foot-candles. By substituting $I = 50$ in Eq. 1, the approximate shelflife in days was calculated for the minimum acceptable value of θ_t (Table I).

When compared with values for control tablets (Table I), it is apparent that the rate constant decreases to almost half when

Table II—Visual Observations of Tablets Coated with FD&C Red No. 3 Exposed to an Intensity of 1000 Foot-Candles of Light over 40 Days

Sunscreening Agent	Coating Type[a]		
	Alcohol Film	Modified Sugar	Film
Control	Noticeable fading becoming objectionable within 20–30 days		
Glyceryl *p*-aminobenzoate	1A	3	1A
Benzocaine	1	2–3	3
2-Ethoxyethyl *p*-methoxycinnamate	2	1	1
m-Homomenthyl salicylate (not polished)	1	Not studied	Not studied
m-Homomenthyl salicylate	1	1	2A
n-Octyl salicylate	2	1	2A
Amyl salicylate	2	2	2A

[a] 1 = fading less than control, 1A = fading less than control with change of hue, 2 = fading similar to control, 2A = fading similar to control but with mottled appearance due to cracked film, and 3 = fading greater than control. All polished except as noted.

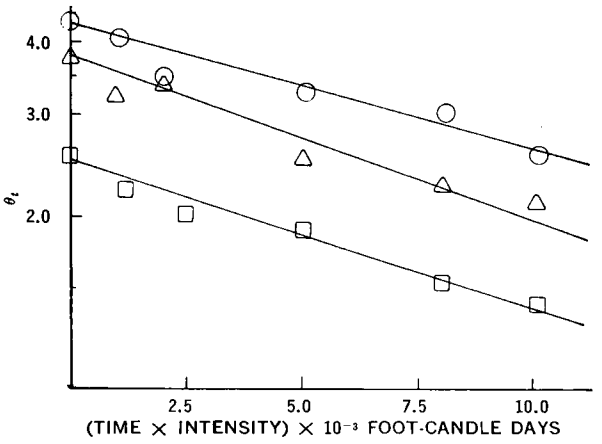

Figure 2—*Plots of* θ_t versus *the product of time and intensity for tablets color coated with FD&C Red No. 3. Key:* ○, *control, alcohol film coating;* △, *glyceryl* p*-aminobenzoate, modified sugar coating; and* □, *benzocaine, film coating.*

Table III—Relative Protective Action of Sunscreening Agents for FD&C Red No. 3

Sunscreening Agent	Type of Coating	Days × 10^{-2}
2-Ethoxyethyl *p*-methoxycinnamate	Film	8.0
n-Octyl salicylate	Modified sugar	6.8
Benzocaine	Alcohol film	6.7
m-Homomenthyl salicylate	Modified sugar	6.6
None (control)	Average of three methods	2.9

film coating is applied. However, a decrease in the rate constant does not necessarily indicate an increase in shelflife.

Glyceryl p-*Aminobenzoate*—When tablets were coated by either alcohol film coating or film coating, a distinct change of hue was noticed. The hue change was not observed with the modified sugar method, perhaps because of the small amount of glyceryl *p*-aminobenzoate found in the tablets coated with the modified sugar coating or perhaps because the combination with sugar had some influence on the rate of hue change. The rate constant is not much different from the control except for tablets coated with the alcohol film coating where it is considerably smaller. The hue change is the reason for the decrease in shelflife to as much as a fourth. The shelflife is zero for tablets coated by the film-coating method since the color change is immediately noticed following the application of the sunscreening agent.

Benzocaine—For FD&C Red No. 3 protected with benzocaine, the rate constant varied considerably from one method to another. It is lowest for the alcohol film-coating method which reduced color change to about half of that found for the control tablets.

2-Ethoxyethyl p-*Methoxycinnamate*—This compound appears to have a distinct protective action on FD&C Red No. 3 when applied as a tablet coating by either the modified sugar-coating or the film-coating method. The longest shelflife of 800 days was calculated for tablets coated by the film-coating method. A brilliant shine was also noticed.

m-*Homomenthyl Salicylate*—Tablets coated with FD&C Red No. 3 appear to be most effectively protected with *m*-homomenthyl salicylate when it is applied by the alcohol film-coating or the modified sugar-coating method.

Polished tablets had a smaller rate constant than unpolished tablets. This may be due to the fact that polished tablets can "reflect" light whereas unpolished tablets "absorb" light. Thus, polished tablets should have a longer shelflife. However, based on visual observation, there did not seem to be any significant difference.

Tablets coated by the film-coating method for all salicylates appeared mottled after 24 hr of drying, due to cracking of the film.

n-*Octyl Salicylate*—FD&C Red No. 3 is best protected by *n*-octyl salicylate when applied by the modified sugar-coating method. Although the rate constant is not much different from that for the alcohol film method, a longer shelflife (almost double) for tablets was found where the coating had been applied by the modified sugar-coating method. The rate constant obtained for the alcohol film-coating method was lower than that obtained for any other sunscreening agent by any other method.

Amyl Salicylate—When applied by the alcohol film method, it yielded the highest rate constant among all sunscreening agents. The shelflife is even lower than that of the control. When coated by the modified sugar-coating method, the shelflife is about a third better than the control.

SUMMARY AND CONCLUSION

The photostabilizing effect of six sunscreening agents (glyceryl *p*-aminobenzoate, benzocaine, 2-ethoxyethyl *p*-methoxycinnamate, *m*-homomethyl salicylate, *n*-octyl salicylate, and amyl salicylate) on FD&C Red No. 3 was evaluated by exposing tablets to intense measured light. Three processes were developed for application of these agents on color-coated tablets: alcohol film-coating, modified sugar-coating, and film-coating methods. The film-coating method was not satisfactory for glyceryl *p*-aminobenzoate and all of the salicylates used.

The Kubelka-Munk equation was used to study the kinetics of fading. FD&C Red No. 3 followed a first-order rate of fading. The approximate shelflives for color-coated tablets with and without sunscreening agents were calculated.

The relative protective action of sunscreening agents for FD&C Red No. 3 in terms of shelflife is listed in Table III.

It is quite difficult to correlate rate constants obtained from plots of θ_t *versus* time–intensity units with shelflives obtained from data based on visual observation. It was not uncommon to observe that the rate constant was essentially the same for two sets of tablets whose shelflife varied from zero days to several hundred days. Visual observation together with scientifically sound constants or units is an invaluable aid to the pharmaceutical formulator.

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Present address: Department of Pharmacy, University of Otago, Dunedin, New Zealand.

Aspirin Stability in Substituted and Nonsubstituted Polyhydric Alcohols in Absence and Presence of Added Water

H. W. JUN[x], C. W. WHITWORTH, and L. A. LUZZI

Abstract □ The degradation of aspirin in methoxypolyethylene glycol 350, 550, and 5000 and unsubstituted polyethylene glycol 400 was studied at four temperatures. The rate of degradation was slower in methoxypolyethylene glycols than in unsubstituted polyethylene glycol. The data were then compared to those obtained in the presence of varying amounts of water added to the bases. The effect of water on the rate of degradation of aspirin was greater in methoxypolyethylene glycol than in polyethylene glycol. Slower degradation of aspirin in methoxypolyethylene glycol was due to the fact that the transesterification pathway is partly blocked in this base. A conclusion is made that the stability of aspirin in polyhydric alcohols may be achieved by blocking the free hydroxyl group in the molecules.

Keyphrases □ Aspirin stability—in substituted and nonsubstituted polyhydric alcohols, effect of added water □ Stability, aspirin—in substituted and nonsubstituted polyhydric alcohols, effect of added water □ Degradation, aspirin—in substituted and nonsubstituted polyhydric alcohols, effect of added water □ Methoxypolyethylene glycols—aspirin stability, effect of added water, degradation rates of aspirin

The degradation of aspirin in various pharmaceutical dosage forms has been generally considered to be due to a hydrolytic reaction in the presence of water. Edwards (1) and Garrett (2) extensively studied the hydrolysis of aspirin in aqueous solutions.

Recently, it was reported (3) that when aspirin was incorporated into polyethylene glycol suppository bases, it decomposed to the extent that the product could not meet stability standards. The mechanism responsible for degradation of aspirin in polyethylene glycol base was reported to be due in part to a transesterification reaction between acetyl groups of aspirin and hydroxyl groups in polyethylene glycols (4). Therefore, in the apparent absence of water, it was expected that degradation of aspirin would be less when incorporated in substituted polyethylene glycol than polyethylene glycol *per se*. This is because in substituted materials such as methoxypolyethylene glycol, the transesterification pathways are partly blocked. The purpose of this work was to study the degradation of aspirin in polyethylene glycol and in the substituted derivative, methoxypolyethylene glycol. The effects of added water on the degradation of aspirin in both bases were also studied.

EXPERIMENTAL

Materials—Aspirin USP[1] and polyethylene glycol 400[2] were used as received. Monosubstituted methoxypolyethylene glycol 350, 550, and 5000 were supplied by the manufacturer[3]. Chloroform[4] was spectrograde.

Sample Preparation—The samples were prepared by incorporating 12% of aspirin by weight in methoxypolyethylene glycol 350, 550, and 5000 and in polyethylene glycol 400. When effects of water were studied, a predetermined amount of distilled water was added to the bases prior to incorporation of aspirin. Samples were kept in airtight containers and stored at 4, 24, 36, and 45°.

Table I—Degradation of Aspirin in Polyethylene Glycol 400 as a Function of Added Water at Various Temperatures

	Aspirin Remaining, %					
Days	0% Water	0.5% Water	1% Water	3% Water	5% Water	10% Water
			At 4°			
0	99.5	99.5	99.5	99.5	99.5	99.5
6	99.3	99.0	99.4	99.4	99.3	99.5
18	99.0	99.1	99.0	99.2	98.3	99.2
27	98.8	98.9	98.6	98.8	98.9	99.0
41	98.9	98.9	99.0	99.0	99.0	98.9
56	98.1	98.2	99.8	97.9	98.2	98.5
			At 24°			
0	99.6	99.6	99.6	99.6	99.6	99.6
9	97.8	97.8	98.1	97.4	97.6	98.0
17	96.6	96.6	96.7	96.8	96.9	97.1
24	95.6	95.6	95.4	95.9	96.1	95.6
31	95.1	95.3	95.4	95.2	95.3	95.0
43	93.8	93.7	93.9	94.2	94.0	03.5
57	92.4	92.5	92.7	92.5	92.8	91.6
72	91.2	91.3	91.4	91.6	91.8	90.5
90	90.1	89.9	90.2	90.3	90.2	88.5
			At 36°			
0	99.5	99.5	99.5	99.5	99.5	99.5
3	97.5	97.6	97.7	97.7	97.7	97.9
10	93.8	93.9	93.6	94.1	93.8	93.4
16	91.7	91.9	92.1	91.8	92.1	90.9
22	89.7	89.9	90.0	89.5	90.6	88.4
31	86.8	86.9	86.9	86.8	86.9	84.4
			At 45°			
0	99.5	99.5	99.5	99.6	99.6	99.6
1	97.0	97.6	97.1	—	—	—
3	—	—	—	95.4	95.9	94.7
4	93.0	93.7	92.5	—	—	—
7	—	—	—	91.3	92.3	90.0
8	89.7	90.1	88.1	—	—	—
11	—	—	—	87.0	88.2	85.5
12	86.8	86.4	84.6	—	—	—
17	—	—	—	80.3	83.5	79.4
18	81.6	81.7	80.7	—	—	—
25	—	—	—	73.5	76.4	72.1
27	76.4	75.3	74.1	—	—	—
33	—	—	—	67.9	70.4	65.6
39	69.4	69.6	66.4	—	—	—
41	—	—	—	63.2	64.8	58.8
49	—	—	—	57.4	57.8	50.7

[1] Merck & Co., Rahway, N.J.
[2] Matheson, Coleman and Bell, Norwood, Ohio.
[3] Union Carbide, New York, N.Y.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.

Table II—Degradation of Aspirin in Methoxypolyethylene Glycol 350 and in the Presence of Added Water at Various Temperatures

Days	Aspirin Remaining, %					
	0% Water	0.5% Water	1% Water	3% Water	5% Water	10% Water
			At 4°			
0	99.6	99.6	99.6	99.6	99.6	99.6
6	99.7	99.7	99.6	99.4	99.5	99.5
12	99.5	99.2	99.5	99.2	99.2	99.4
21	99.5	99.4	99.4	99.3	99.3	98.4
34	99.4	99.3	99.3	99.3	99.1	98.9
55	99.2	99.3	99.2	99.0	99.0	98.8
			At 24°			
0	99.6	99.6	99.6	99.6	99.6	99.6
3	99.3	99.3	99.0	99.0	99.0	98.8
8	99.1	99.0	98.8	97.8	98.2	97.4
13	98.8	98.7	98.5	98.1	99.8	97.6
20	98.0	98.1	98.1	97.8	97.1	96.4
29	97.5	97.6	97.5	97.0	96.2	95.6
36	97.3	97.2	97.2	95.4	95.7	94.6
44	96.8	96.9	97.1	95.8	95.5	94.6
53	96.7	96.5	96.3	95.2	94.5	93.2
65	95.6	96.0	95.6	04.5	94.0	92.0
			At 36°			
0	99.6	99.6	99.6	99.6	99.6	99.6
3	98.7	98.7	98.8	98.7	98.3	98.4
10	96.9	97.0	06.7	96.2	95.3	94.6
16	95.3	95.7	95.4	94.9	94.1	92.0
22	94.8	94.5	94.5	93.3	92.1	90.1
31	93.0	92.6	92.3	90.8	89.4	85.7
			At 45°			
0	99.5	99.5	99.5	99.5	99.5	99.5
3	98.0	97.3	97.4	96.8	96.4	96.0
8	95.2	95.3	95.0	93.7	93.3	92.0
14	92.5	92.4	92.1	90.2	89.3	87.2
21	89.7	89.4	89.1	86.8	85.2	81.2
28	87.4	86.6	86.4	83.5	81.6	76.2
36	84.0	83.6	83.2	80.0	77.8	70.7
44	81.8	80.5	80.0	76:2	74.2	61.1
55	78.4	77.2	77.1	72.2	68.8	58.9

At various intervals, the samples were assayed.

Analytical Method—UV spectrophotometric analysis, as reported by Tinker and McBay (5), was employed to measure aspirin and salicylic acid. Samples (approximately 2.4 g) were taken at specific intervals and dissolved in 100 ml chloroform containing 1% acetic acid. A further appropriate dilution was made, and the absorbance of the dilution was read[5] at 278 nm for aspirin and 308 nm for salicylic acid. At these wavelengths, standard curves were prepared for aspirin and salicylic acid. The quantification of aspirin and salicylic acid was based on the standard method of simultaneous spectrophotometric determinations.

RESULTS AND DISCUSSION

Aspirin undergoes degradation in polyethylene glycol at least in part *via* a transesterification reaction (4). In the presence of water, however, it was expected that the mechanism of degradation would probably be more complex. Tables I and II summarize the data obtained in the present study. Under all experimental conditions, the degradation of aspirin showed a temperature dependency.

In Fig. 1, the solid lines indicate the amount of aspirin remaining in polyethylene glycol 400 at four temperatures. The reaction appears to follow the first-order process. When samples were stored at 4°, the degradation was relatively slow. When varying amounts of water were added to the base, differences were observed in the rate of degradation. The dotted lines indicate the degradation of aspirin in the same base with 10% water added. The effect of water on the rate of degradation of aspirin in this base was generally small. At lower temperatures (4 and 24°), no significant effects were observed while the differences were some-

[5] Beckman DU spectrophotometer.

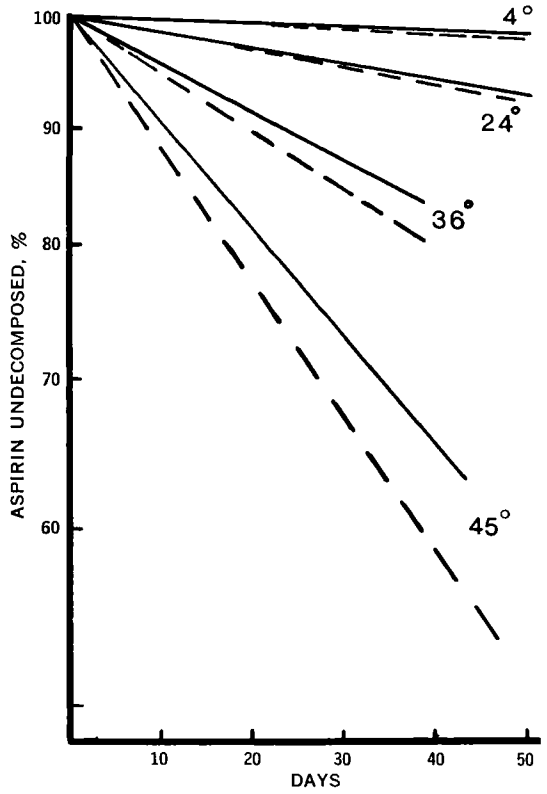

Figure 1—*Degradation of aspirin in polyethylene glycol 400 at four temperatures (solid lines). Dotted lines are for degradation of aspirin in the base in the presence of 10% water.*

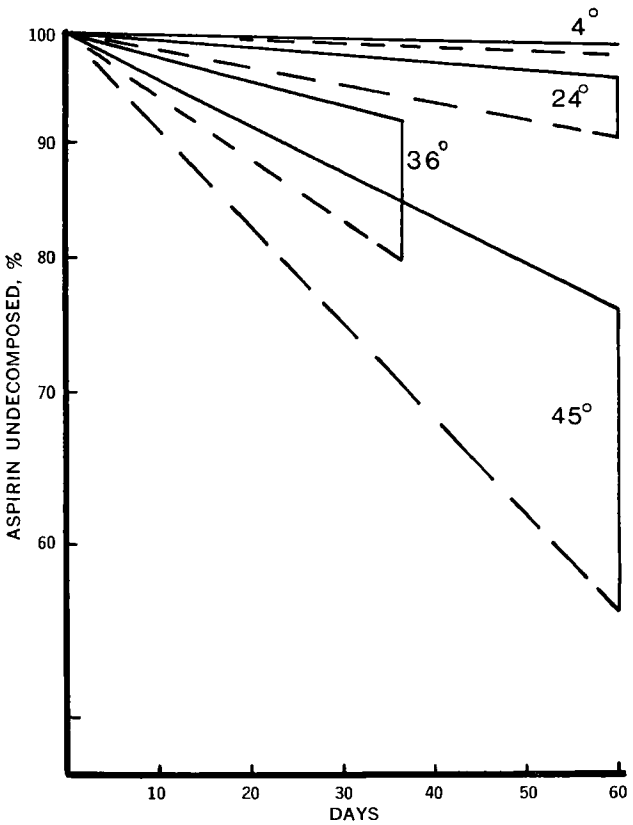

Figure 2—*Degradation of aspirin in methoxypolyethylene glycol 350 at four temperatures (solid lines). Dotted lines are for degradation of aspirin in the base in the presence of 10% water.*

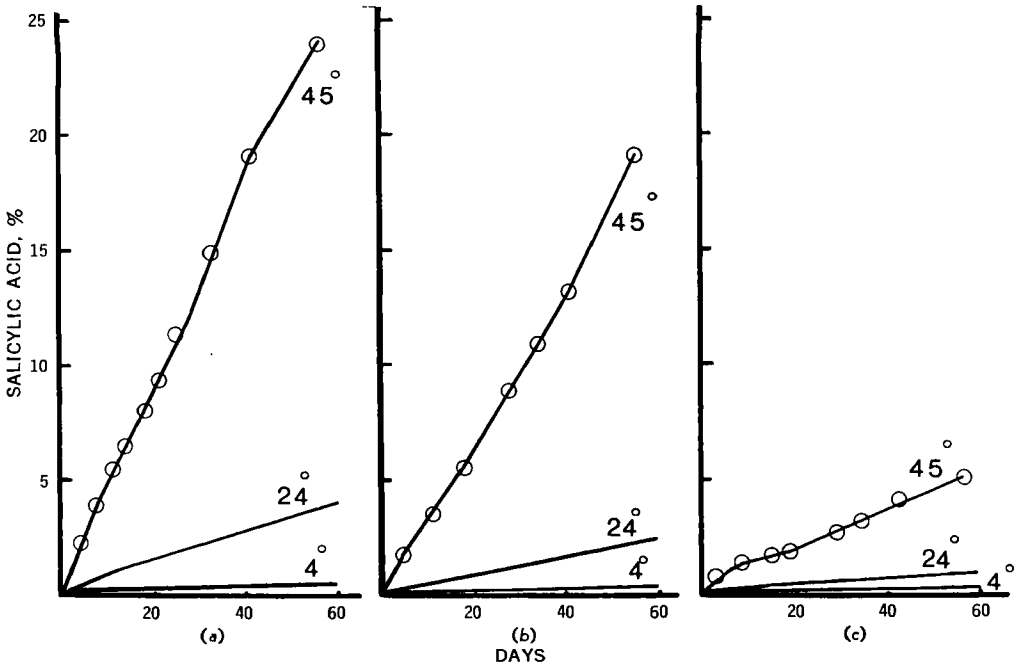

Figure 3—*Degradation of aspirin in methoxypolyethylene glycol 350 (3a), 550 (3b), and 5000 (3c) at three temperatures.*

what greater at higher temperatures. When smaller amounts of water (0.5–5%) were added to the base, the rates of degradation fell between the solid and dotted lines. A small effect of added water on the degradation of aspirin in polyethylene glycol suggests that aspirin molecules may be entrapped in polyethylene glycol in the presence of water, thus reducing a molecular contact between water and aspirin.

The greater effect of water on the degradation of aspirin in methoxypolyethylene glycol as compared to polyethylene glycol is probably due primarily to hydrolysis effects.

Figure 2 shows degradation of aspirin in methoxypolyethylene glycol 350 (solid lines) and in a mixture of methoxypolyethylene 350 and water (dotted lines) at four temperatures. In methoxypolyethylene glycol, aspirin appeared to degrade more slowly than in unsubstituted polyethylene glycol (compare Figs. 1 and 2). After 31 days at 36°, aspirin decomposed about 13% in polyethylene glycol but only about 7% in methoxypolyethylene glycol. This is attributed to the fact that transesterification pathways were partly blocked in methoxypolyethylene glycol. When 10% water was added to the bases, however, the difference in the amount of aspirin decomposed in methoxypolyethylene glycol was considerably more pronounced than that in polyethylene glycol (compare Figs. 1 and 2). After 31 days at 36°, aspirin degraded 15% in polyethylene glycol and 14% in methoxypolyethylene glycol. The greater effect of water on degradation of aspirin in methoxypolyethylene glycol may be due to the fact that in methoxypolyethylene glycol a transesterification reaction was partly blocked and when water was added the unreacted aspirin became available for hydrolysis.

Figure 3 shows degradation of aspirin in methoxypolyethylene glycol 350, 550, and 5000 at three temperatures, as indicated by the rate of appearance of salicylic acid. In methoxypolyethylene glycol 350, aspirin degraded 25% in 50 days at 45° as compared to 19 and 5% in methoxypolyethylene glycol 550 and 5000, respectively. At lower temperatures, the differences have the same relationship. In higher molecular weight methoxypolyethylene glycols, fewer hydroxyl radicals per unit weight are available for the transesterification reaction. The finding supports the hypothesis that aspirin degrades in polyhydric alcohols at least in part *via* a transesterification reaction.

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[x] To whom inquiries should be directed.

CMC of Polysorbates

LUCY S. C. WAN[x] and PHILIP F. S. LEE

Abstract □ The CMC of a series of polysorbates was determined using a surface tension method. The CMC values obtained did not vary to any appreciable extent from those determined by other techniques, indicating that the procedure adopted was reliable, simple, and fast. The application of the usual correction factors was not necessary in the calculation of the CMC values. Deviations for corrected and uncorrected CMC values were considered insignificant.

Keyphrases □ Polysorbates—CMC determination using surface tension method, values compared to other techniques □ CMC, polysorbates—determined using surface tension method, values compared to other techniques □ Surfactants—CMC determination of polysorbates using surface tension method, values compared to other techniques

The fundamental micellar properties of the polysorbate group of nonionic surfactants have been widely investigated. Becher (1) tabulated the critical micelle concentration (CMC) values of some polysorbates in aqueous solutions, as determined by iodine solubilization and light-scattering methods. A differential refractive index method has also been used (2), while Bloor *et al.* (3) and Mittal (4) employed a surface tension method to determine the CMC of polysorbates 40 and 20, respectively. Since the polysorbates are essentially heterodisperse in character (5), any "definite" values assigned to their determined micellar properties must be looked upon with caution, because such values differ among research workers and especially among different batches of the same material. In a study involving polysorbates, in solubilization and emulsification processes, the CMC values were determined by a rapid surface tension method without the need for elaborate experimental details and calculations.

EXPERIMENTAL

Materials—Polysorbates 20[1], 40[1], 60[1], 80[1], 65[1], and 85[1] were used as supplied. Distilled water from an all-glass still was used throughout this study.

Aging Effects on Surface Tension—Nonionic surfactants exhibit marked surface activity at much lower concentrations than ionic surface-active agents with identical hydrophobic groups and, therefore, have lower CMC values (6). The effect has been attributed to a gradual diffusion of solute molecules from the bulk to the surface and orientation of the surface molecules before equilibrium is attained. From a preliminary experiment, an equilibrium of 15 min was found to be sufficient so this period was utilized for subsequent experiments.

Measurement of Surface Tension—An aqueous stock solution of 0.10% (w/v) concentration was prepared for each polysorbate. From each stock solution, appropriate dilutions were made to produce solutions of concentrations ranging from 0.0001 to 0.050% (w/v). Polysorbates 60, 65, and 85 formed hazy solutions at these dilutions. All solutions were allowed to stand overnight in their containers before measurements of their surface tension (γ) values were made. Surface tension data, as a function of concentration (C), were determined employing the ring-detachment method[2]. An accuracy check on the tensiometer was made by measuring the γ of pure water; γ_{water} = 72.0, 72.1, 72.0, and 72.0 dynes/cm, giving an average γ of 72 dynes/cm at 25 ± 0.5° which is in agreement with the literature value (3, 7).

The following standardized procedures were adopted:

1. All measurements were made at 25 ± 0.5°. All reported values were averaged.
2. All glass apparatus used, as well as the platinum ring, were cleaned with freshly prepared chromic acid and then thoroughly rinsed with distilled water.
3. Each solution contained in the dish was set aside for 10 min prior to the actual measurement of γ. This procedure was to compensate for any adsorption by the glass apparatus. The dish was then thoroughly drained, a fresh volume of the same solution was added, the ring was submerged just below the liquid surface, and the whole was allowed to equilibrate undisturbed for 15 min before measurement of γ. This procedure was to make allowance for aging effects mentioned earlier.

RESULTS AND DISCUSSION

The objective of this investigation was to establish a relatively fast and accurate method for the CMC determination of heterodispersed nonionic surfactants. Figure 1 shows the plot of γ against

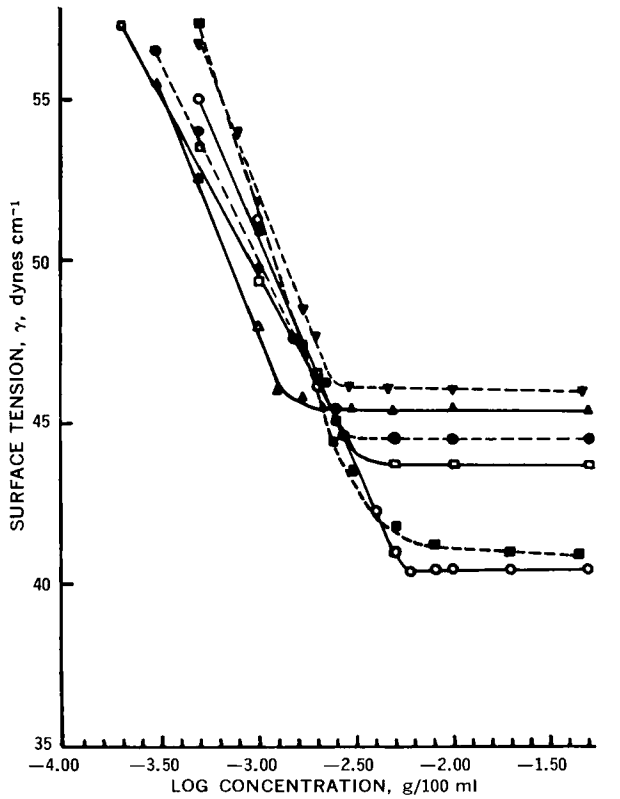

Figure 1—*Change of surface tension with concentration of various polysorbate solutions. Key:* ○, *polysorbate 20;* □, *polysorbate 40;* ●, *polysorbate 60;* ▲, *polysorbate 80;* ■, *polysorbate 65;* ▼, *polysorbate 85;* – – –, *turbid solution; and* —, *clear solution.*

[1] Tween 20, 40, 60, 80, 65, and 85, respectively; Honeywill-Atlas Ltd., London, England.

[2] Du Nouy tensiometer, Cambridge Instrument Co.

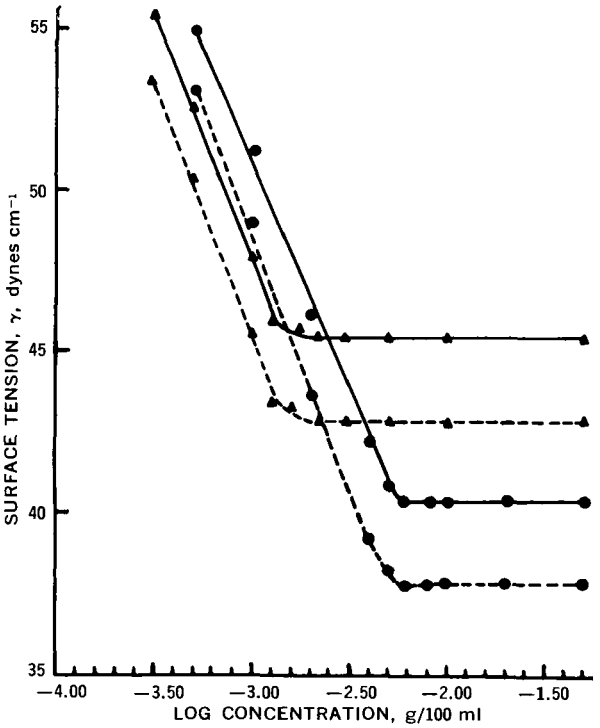

Figure 2—*Comparison between plots of surface tension* versus *log concentration for polysorbate 20 and 80 solutions, with and without correction factor. Key: ●, polysorbate 20; ▲, polysorbate 80; - - - -, with correction factor; and —, without correction factor.*

log C where γ values were obtained without using the correction factor as suggested previously (8).

All plots show that γ varies linearly with log C up to a certain concentration characteristic of each compound, when γ remains essentially constant with further increases in concentration. The CMC of each compound is determined graphically from the intersection of the extrapolated linear portions of each plot. The results can be seen in column A of Table I. For comparison, the CMC values determined by light-scattering and iodine solubilization techniques stated by Becher (1) are shown in column B. The CMC of polysorbate 65 is given as a concentration range because there is no sharp break in its γ *versus* log C plot.

Figure 2 compares the plot obtained by employing the correction factor of Harkins and Jordan (8) in the calculation of γ with those calculated without it. The results indicate that although there is an expected shift toward a lower γ value for each concentration plotted, the break in the γ *versus* log C curve and, therefore, the CMC does not vary. This is also true for the other polysorbates studied.

An interesting observation can be related to the data obtained. Since all of the polysorbates used were soluble in water beyond their CMC's, a CMC–HLB (hydrophilic-lipophilic balance) relationship, as stated by Schott (9), can be empirically established. Since the CMC is a function of composition within a homologous series of surfactants, the more hydrophilic a surfactant is, the larger its HLB value and, therefore, the higher its CMC due to its lower tendency to form micelles.

Table I—CMC of Aqueous Solutions of Various Polysorbates at 25 ± 0.5°

Surfactant	CMC, g/100 ml	
	A	B[a]
Polysorbate 20	0.0060	0.0060
Polysorbate 40	0.0031	0.0029
Polysorbate 60	0.0028	0.0027
Polysorbate 80	0.0014	0.0013
Polysorbate 65	0.0040–0.0060	—
Polysorbate 85	0.0023	—

[a] Values taken from Ref. 1.

These results are also related to the fact that increasing the length of the hydrophobic chain for the same number of ethylene oxide units produces a shift of the γ curve and of the CMC to the lower concentration. The data obtained are consistent with this general behavior of nonionic surfactants. However, a reversal in the order seems to occur with polysorbates 65 and 85. This reversal may be attributed to the much slower attainment of equilibrium surface tension with long-chain members of the same homologous series (10).

From the findings and the data available so far, it appears that the CMC value of each polysorbate does not deviate widely despite the variety of physical methods employed for its determination. However, due to the heterodisperse nature of these compounds and the fact that they are often used as the unpurified, commercially available grade, no definite CMC value ought to be assigned with any degree of certainty to any member of the series. Even among chemically pure compounds, errors and limitations inherent in the method chosen will contribute to variations of the same determination. Nevertheless, a prior knowledge of the CMC of any polysorbate compound will be of immense help in elucidating some of their micellar characteristics.

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[x] To whom inquiries should be directed.

Kinetics of Heparin Removal from Circulation of the Minipig

P. A. HARRIS[x] and K. L. HARRIS

Abstract □ Techniques were developed for kinetic studies in unanesthetized, unrestrained minipigs weighing less than 45.4 kg (100 lb). By using ^{35}S-heparin and ^{3}H-heparin, the disappearance from the plasma of different amounts of heparin injected intravenously into the minipig was determined. Serial plasma samples were assayed for radioactivity, and the resulting plasma radioactivity-time curves were analyzed for calculation of half-lives of disappearance ($t_{1/2}$) and volume of distribution (V_d). The plasma radioactivity followed a monoexponential decay. Over the 1–50-mg heparin range, an increase in $t_{1/2}$ from 3.4 to 39 min was observed. Essentially no differences in the V_d were observed among the various doses. This volume was approximately 1.5 times the estimated plasma volume. The observed kinetics are consistent with previous suggestions that the reticuloendothelial system may be involved in the disposition of heparin.

Keyphrases □ Heparin—kinetics of removal from circulation of minipig □ Minipig—kinetics of heparin removal from circulation, suitability as test animal □ Anticoagulants—kinetics of heparin removal from circulation of minipig □ Pharmacokinetics—heparin removal from circulation of minipig

Heparin, a strongly acidic mucopolysaccharide, is well known and is exploited for its pharmacological properties as an anticoagulant and a lipemia-clearing factor. Although these effects of administered heparin are well characterized, the physiological role of endogenous heparin remains unclear. Little also is known regarding the relationship between heparin blood concentrations and its pharmacological effect. The kinetics of heparin removal from the blood were reported in a retrospective study and literature analysis (1). It appears clear that there are species differences in both quantitative and qualitative parameters of heparin removal. In man, the $t_{1/2}$ of removal varies with the dose. With a dose of 100 units/kg iv, the $t_{1/2}$ is approximately 1 hr; with 400 units/kg iv, it is approximately 2.5 hr. The plasma concentration-time curves decline exponentially in any case; therefore, a saturation mechanism does not seem likely.

Another report (2) showed that, although the $t_{1/2}$ of heparin removal is inversely related to dose, the $t_{1/2}$ of heparin's anticoagulant effect is not affected by dose. The former was measured by bioassay with a whole blood partial thromboplastin time assay, while the latter was estimated by heparin's effect on the whole blood partial thromboplastin of patients. *Recently*, a study (3) showed that heparin is removed biexponentially in man. The method utilized for heparin analysis was a metachromatic assay combined with aminoacridine precipitation of heparin from the plasma. This study did not examine the dose dependency of heparin kinetics. Nearly all dose dependency studies to date have used coagulation assays such as blood clotting time or partial thromboplastin time for heparin, which only indirectly estimates heparin concentration (1).

The present study was undertaken as part of an effort to define the suitability of the minipig as an experimental animal in investigations of the physiological role of heparin.

EXPERIMENTAL

Animal Procedures—Techniques were developed for kinetic studies in unanesthetized, unrestrained minipigs[1] weighing less than 45.4 kg (100 lb). The animal was placed on its back in a V-shaped trough and held in position while a 13-gauge needle was introduced into the jugular vein by percutaneous puncture. A Teflon catheter was passed through the needle for a distance of 20 cm toward the heart. The needle was removed and a stopcock was attached to the catheter. The catheter extending from the skin was placed dorsally around the neck to the back and protected by securing it to the neck with adhesive tape. After the catheter and stopcock were secured, the animal was returned to its feet and released. It was then possible to approach the animal at any time to administer the dose or collect blood samples without unduly disturbing it. The dose of heparin was injected into the catheter, and the catheter was flushed well with 0.15 *M* saline. Twelve to 15 blood samples (3 ml) were collected serially up

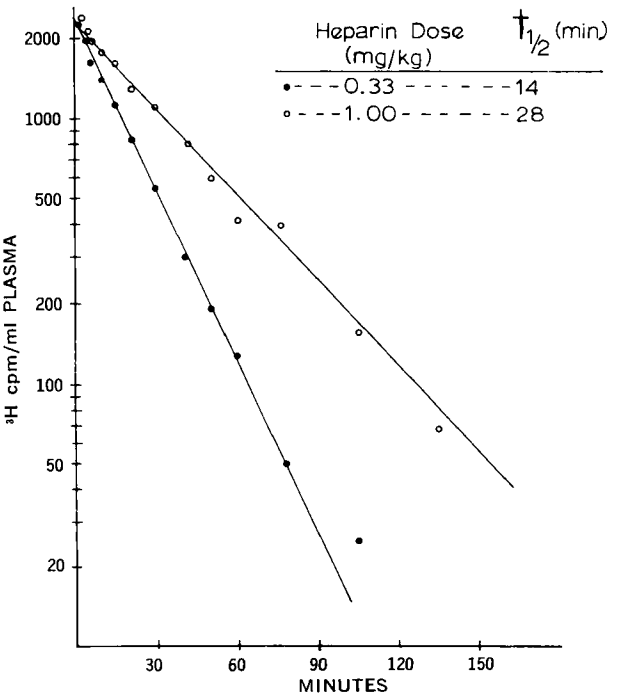

Figure 1—*Radioactivity in the plasma with time resulting from the intravenous injection of 2.76 × 10⁶ cpm of ^{3}H-heparin at two dose levels of heparin.*

[1] The minipigs used were of the genetic line produced by the Hormel Institute of the University of Minnesota.

to 180 min. At the end of the experiments, the catheter was removed. No evidence of infection developed at the site of catheterization. In subsequent experiments, catheters were positioned in a similar manner. Five animals were studied, three of them with both ^{3}H-heparin and ^{35}S-heparin.

Dose Preparation—^{35}S-Heparin[2] was purified by a procedure involving precipitation with 9-aminoacridine. A literature method (4) was modified slightly to redissolve the 9-aminoacridine–heparin complex with 4 *M* NaCl instead of a strongly alkaline solution. The high salt concentration was removed by dialysis. Recovery was approximately 90%. ^{3}H-Heparin[3] was purified in a similar manner with 75% yield. The authenticity of the purified labeled heparins was verified by agarose gel electrophoresis[4] against a reference standard of nonradioactive heparin[2]. Solutions of heparin containing 1 μg/μl in barbital buffer (0.1 *M*, pH 8.6) were prepared and subjected to electrophoresis according to the method of Jaques *et al.* (5). The electrophoretograms were stained with toluidine blue and scanned for radioactivity with a silicon avalanche detector[5]. Doses were prepared in 0.15 *M* saline. Appropriate amounts of unlabeled heparin were added to the ^{3}H-heparin and ^{35}S-heparin for total dosage adjustment.

Sample Analysis—Blood was collected into tubes containing edetate (EDTA) as an anticoagulant. The plasma was separated by centrifugation. One milliliter plasma was added to 12 ml scintillation fluid[6] for analysis by liquid scintillation spectrometry[7]. Counts were corrected for quenching.

RESULTS AND DISCUSSION

The purified ^{3}H-heparin and ^{35}S-heparin on electrophoresis yielded a single band each when stained with toluidine blue. The radioactive heparin mobility and the hue of their stains corresponded to unlabeled heparin. All radioactivity was associated with the band corresponding with the stains.

Curves of plasma radioactivity *versus* time are shown in Fig. 1. The plasma radioactivity followed a monoexponential decay. Data utilizing the ^{3}H-heparin are shown, but similar curves were obtained with ^{35}S-heparin.

The resulting plasma radioactivity–time curves were analyzed for calculating $t_{1/2}$ and V_d (Table I). It is apparent that $t_{1/2}$ increases with increasing dose, but at higher doses there appears to be much less change in $t_{1/2}$ as indicated by the similarity in the $t_{1/2}$ of the 0.95–2.80-mg/kg doses. There was no significant change in the V_d after different doses. The V_d was approximately 1.5 times the plasma volume. Results obtained with ^{3}H-heparin are similar to those obtained with ^{35}S-heparin.

The results of the electrophoresis of the labeled heparins suggest a high degree of purity and authenticity of the heparin used in these experiments. The magnitude of the V_d suggests also that the measured radioactivity was associated with intact heparin because the volumes are similar to those reported by others using unlabeled heparin (2, 3, 6).

The lack of differences in the kinetics of removal of ^{3}H-heparin and ^{35}S-heparin suggests that heparin is removed from the circulation intact rather than being broken down by sulfatases or mucopolysaccharidases in plasma. The kinetics in swine are similar to those reported in man in that the $t_{1/2}$ increases with increasing doses while the V_d remains unchanged. The removal rate in swine appears to be more rapid than those reported in man (2). However, the dose range used in this study was much smaller than those studied in man. The upper range in this study corresponds with the lower range studied in man. The $t_{1/2}$ of these higher doses in swine approach those encountered in man.

Table I—Disposition Kinetics of ^{3}H-Heparin and ^{35}S-Heparin in the Minipig

Animal	Isotope	Dose, mg/kg	$t_{1/2}$, min	V_d, ml/kg
1	^{35}S	0.06	3.4	65
	^{35}S	2.80	38	65
2	^{35}S	0.30	9.2	68
	^{35}S	1.47	32	71
3	^{35}S	0.20	8.5	69
	^{35}S	0.95	28	77
4	^{35}S	0.32	13	61
	^{35}S	1.27	25	59
5	^{3}H	0.33	14	53
	^{3}H	1.00	28	60
1	^{3}H	0.25	11.6	65
	^{3}H	1.25	31	67
2	^{3}H	0.25	12.6	72
	^{3}H	1.25	22	66
3	^{3}H	0.05	4.3	58
	^{3}H	2.50	39	65

Estes *et al.* (1) believed that their compiled data were consistent with the hypothesis that heparin may leave the plasma by uptake into the reticuloendothelial system. Piper (7) and Monkhouse (8) also suggested involvement of the reticuloendothelial system in the elimination of heparin. Another study (9) implicated mast cells in the uptake of heparin. It has been speculated that the combination of the large "stiff" heparin molecule with plasma protein molecules may be capable of uptake as a foreign particle (1). If this is true, then models for the uptake of particles (10) may be expected to apply to heparin. Our kinetic data are consistent with previous studies in that they indicate that heparin may be removed from the plasma by the reticuloendothelial system in the minipig.

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[x] To whom inquiries should be directed.

[2] Cal-Biochem, Los Angeles, Calif.
[3] Abbott, Chicago, Ill.
[4] Turner, Palo Alto, Calif.
[5] Nucleye, General Electric Co., King of Prussia, Pa.
[6] Aquasol, New England Nuclear, Boston, Mass.
[7] Mark II spectrometer, Nuclear-Chicago, Chicago, Ill.

Mass Spectra of Steroid Sapogenins

A. M. DAWIDAR and M. B. E. FAYEZ[x]

Abstract □ A study of the mass spectra of 15 natural and synthetic steroid sapogenins confirmed the general modes of fragmentation in the side chain and revealed additional features due to breakdown of ring D and to special structural and stereochemical characteristics.

Keyphrases □ Sapogenins, steroid—mass spectra and fragmentation patterns □ Mass spectrometry—fragmentation patterns of steroid sapogenins □ Steroid sapogenins—mass spectra and fragmentation patterns

This note reports examples of steroid sapogenins which behave under electron impact in accordance with the generalizations put forward by Budzikiewicz *et al.* (1). In addition, other breakdown pathways were identified, and their detection in compounds of unsettled nature may be diagnostically useful.

DISCUSSION

The commonly recognized (1) steroid sapogenin fragmentation products are the spiroketal ring F-containing ion species *a* and *b* (appearing at *m/e* 139 and 115, respectively, for the ring F-unsubstituted compounds, the first being the strongest ion in the spectra) and *c–h*, comprising an intact steroid nucleus. All of these species, conveniently explicable (1) as resulting from transition states involving cleavage of any of the C—C and C—O bonds attached to C-22, are well represented in the mass spectra of the compounds examined (I–XIII, Table I). However, the C-23 brominated derivatives XIV and XV show ion species *a* and *b*, with the appropriate mass shifts, only with very low intensities due to loss of the halogen content (partly as HBr) from the molecular ions as primary reactions. For the same reason, no evidence is available for ion species *f* and *h* in the spectrum of XIV and for *f*, *g*, and *h* in that of XV. The absence of species *h* in the spectrum of 23b-bromotigogenin acetate (XIV)—where the bromine atom is axially bound—is likely due to the unfavorable configuration of the C-23 hydrogen atom which would participate in the breakdown mechanism (1); a different situation may exist in 23ξ-bromodeoxytigogenin which shows ion *h* in a published (2) spectrum.

Another mode of cleavage is observed to have representation in nearly all spectra and may have general utility. This cleavage occurs by fission of the C-13/C-17 and C-14/C-15 bonds, with elimination of the entire spiroketal side chain together with ring D and an additional hydrogen atom, and is analogous to the principal type of fragmentation known (3) in sterols. The primary product of this breakdown is ion species *i*, which shows intact in some spectra but only as an artifact [resulting from loss of the C-3 substituent with or without subsequent expulsion of 54 mass units (butadiene) in a retro-Diels–Alder reaction, leading to ion *j*] in others (Table I).

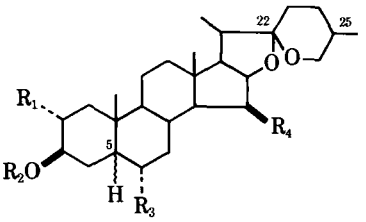
The spectra are complicated by presence of satellite ions resulting from the various species by losses of the nuclear substituents (as water or acetic acid). The loss of the C-3 substituent is frequently followed by collapse of ring A in a retro-Diels–Alder type of reaction, which is inhibited, obviously, where a double bond or additional hydroxy group(s) is present in or close to the ring. The latter type of breakdown of ring A is observed to take place in most ion species comprising the steroid nucleus arising from smilagenin acetate (III) and sarsasapogenin (IV) as opposed to tigogenin acetate (I) and neotigogenin acetate (II), a fact that may be associated with the specific stereochemistry at C-5.

The presence of additional functions remote from ring A appears to induce other special types of reactions (Scheme I). Thus, in addition to the expectable loss of CO from some ion species of hecogenin (X) and 9-dehydrohecogenin acetate (XI), the spectrum of the latter compound contains an appreciable ion at *m/e* 229, which may be the outcome of a γ-hydrogen transfer to the C-12 carbonyl group with loss of ring D to give ion *k*. Another peak (*m/e* 332) in the same spectrum may be due to an additional McLafferty hydrogen transfer with loss of the spiroketal side chain, giving structure *l* for the resulting ion. The spectrum of

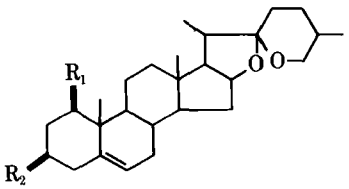
Table I—Mass Spectral Data

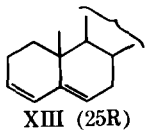
Compounds	Ion Species[a]									
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*
Tigogenin acetate (I)	139[1]	115	315[2]	344[2]	329[2]	386	389	399	—[2]	161
Neotigogenin acetate (II)	139[1]	115	315[2]	344	329[2]	386[2]	389	399	—[2]	161
Smilagenin acetate (III)	139[1]	115	315[2]	344[2,4]	329[2]	386[2,4]	389[2]	399[2,4]	—	161
Sarsasapogenin (IV)	139[1]	115	273[3,4]	302[3,4]	287[3,4]	344[3,4]	347[3,4]	357[3,4]	233[3]	161
Yamogenin acetate (V)	139	115	313	—[1,2]	—[2]	—[2]	—[2]	—[2]	—[2]	—
Gitogenin diacetate (VI)	139[1]	115	373[2]	402[2]	387[2]	444	447	457	—[2]	—
Chlorogenin diacetate (VII)	139[1]	115	373[2]	402[2]	387[2]	444	447	457[2]	—[2]	—
Ruscogenin diacetate (VIII)	139[1]	115	—[2]	—[2]	—[2]	—[2]	445	—[2]	—[2]	—
Digitogenin 2,3-diacetate (IX)	139[1]	115	389[2]	418[2]	403[2,3]	460[2]	463[2,3]	473	—	—
Hecogenin (X)	139[1]	115	287[3]	316[3,5]	301[3,5]	358[3]	361[3]	371[3]	247[3]	175
9-Dehydrohecogenin acetate (XI)	139[1]	115	327[2,4,5]	356[2]	341[2,4,5]	398[2]	401	411[5]	287[2,5]	173
3β-Chloro-25D-spirost-5-ene (XII)	139[1]	115	289[6]	318[6]	303[6]	360[6]	363[6]	373[6]	249	—
25D-Spirosta-3,5-diene (XIII)	139[1]	115	253	282[1]	267	324	327	337	213	—
23b-Bromotigogenin acetate (XIV)	218	—	315[2]	344	329[2,4]	—	389[2,4]	—	—	161
23,23-Dibromosarsasapogenin acetate (XV)	—	273	315[2]	344[2]	329	—	—	—	—	161

[a] Additional information pertaining to observed fragment ions in the various spectra. [1] Base peak (100% intensity ion). Satellite ions resulting from indicated ion species by losses of: [2] acetic acid, [3]water, [4]butadiene (54 mass units) in addition to loss of acetic acid or water, [5]carbon monoxide, and [6]hydrogen chloride.



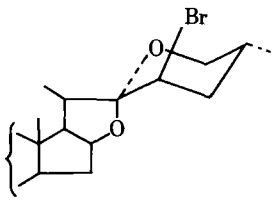
I: $R_1 = R_3 = R_4 = H$, $R_2 = COCH_3$ (5α, 25R)
II: $R_1 = R_3 = R_4 = H$, $R_2 = COCH_3$ (5α, 25S)
III: $R_1 = R_3 = R_4 = H$, $R_2 = COCH_3$ (5β, 25R)
IV: $R_1 = R_2 = R_3 = R_4 = H$ (5β, 25S)
VI: $R_1 = OCOCH_3$, $R_2 = COCH_3$, $R_3 = R_4 = H$ (5α, 25R)
VII: $R_1 = R_4 = H$, $R_2 = COCH_3$, $R_3 = OCOCH_3$ (5α, 25R)
IX: $R_1 = OCOCH_3$, $R_2 = COCH_3$, $R_3 = H$, $R_4 = OH$ (5α, 25R)



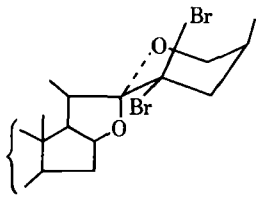


XIII (25R)

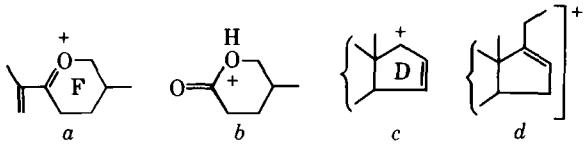
V: $R_1 = H$, $R_2 = OCOCH_3$ (25S)
VIII: $R_1 = R_2 = OCOCH_3$ (25R)
XII: R_1 = H, R_2 = Cl (25R)



XIV (3β-$OCOCH_3$; 5α-H)



XV (3β-$OCOCH_3$; 5β-H)



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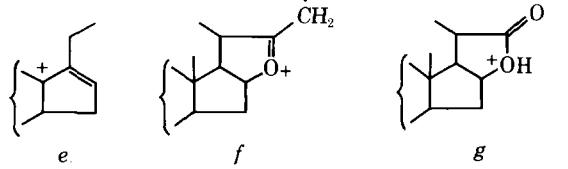

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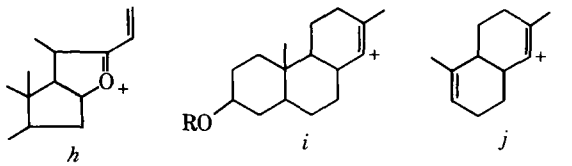
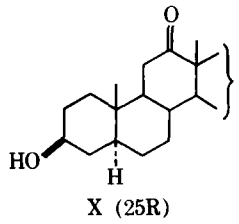
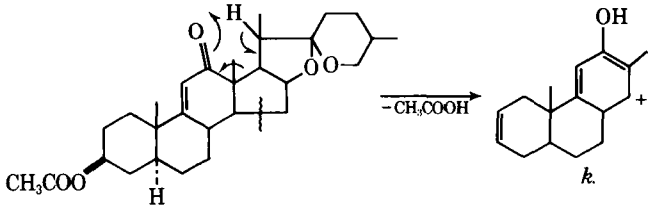


h i j

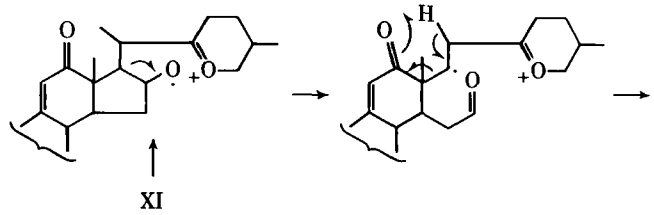
digitogenin 2,3-diacetate (IX) contains two prominent ions at m/e 126 and 168 which, presumably, comprise the spiroketal side chain and result from unusual modes of fragmentation. In the absence of more direct evidence from labeling experiments, it may be assumed that the first ion results by cleavage across ring E, leading to structure *m*, and the second by cleavage across ring D, giving *n*. Although the C-15 hydroxyl group is not directly involved according to the proposed mechanisms, the presence of this function seems to be the sole factor leading to these ion species.



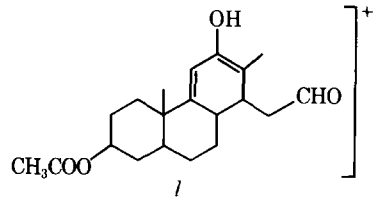
X (25R)



XI



XI



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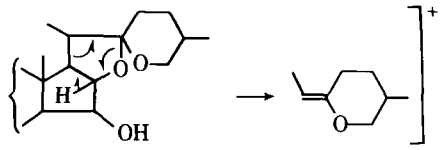
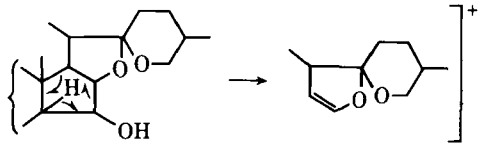


IX *m*



IX *n*

Scheme I

EXPERIMENTAL[1]

All compounds used in this study are known and were obtained by isolation from natural sources, by synthesis using published methods, or as gifts. Melting points are uncorrected. Optical rotations were measured in chloroform solution. Purity of the samples used in the mass spectrometric study was ascertained by TLC using the conditions given.

TLC Conditions—The following four solvent systems were used: A, adsorbent of silver nitrate-impregnated alumina, solvent of cyclohexane–ethyl acetate (10:1); B, adsorbent of alumina G, solvent of *n*-hexane–ethanol (10:1); C, adsorbent of silica gel G, solvent of *n*-hexane–ethyl acetate (20:1); and D, adsorbent of silica gel G, solvent of cyclohexane–ethyl acetate–isopropanol (20:1:2). The spray reagents used were chlorosulfonic acid–acetic acid (1:3) and *p*-anisaldehyde–acetic acid–sulfuric acid (1:100:2).

TLC Data—*Compound I*—mp 204–206° [lit. (4) mp 204°], $[\alpha]_D$

[1] The mass spectra were measured on an MS-9 spectrometer, using an electron energy of 70 ev at a temperature of 140–150°.

−71.6° [lit. (4) $[\alpha]_D$ −73°], R_f 0.62 (A), isolated from *Trigonella foenum-graecum* L. seeds (5).

Compound II—mp 173–175° [lit. (6) mp 172–174°], $[\alpha]_D$ −77.8° [lit. (6) $[\alpha]_D$ −73.4°], R_f 0.60 (A), isolated from *Agave filifera* var. *compacta* L. leaves (7).

Compound III—mp 149–151° [lit. (4) mp 150°], $[\alpha]_D$ −62.6° [lit. (4) $[\alpha]_D$ −60°], R_f 0.55 (A), isolated from *Agave ghiesbrechtii* Koch. leaves (7).

Compound IV—mp 201–204° [lit. (4) mp 199–200°], $[\alpha]_D$ −76° [lit. (4) $[\alpha]_D$ −78°], R_f 0.80 (B), isolated from *Yucca filamentosa* L. leaves (8).

Compound V—mp 184–186° [lit. (9) mp 182°], $[\alpha]_D$ −118° [lit. (9) $[\alpha]_D$ −113°], R_f 0.40 (A).

Compound VI—mp 233–237° [lit. (10) mp 237–242°], $[\alpha]_D$ −91.7° [lit. (10) $[\alpha]_D$ −98°], R_f 0.57 (A), isolated from *Trigonella foenum-graecum* L. seeds (5).

Compound VII—mp 155–157° [lit. (11) mp 155°], $[\alpha]_D$ −38° [lit. (11) $[\alpha]_D$ −38°], R_f 0.40 (A), isolated from *Furcraea gigantea* Vent. leaves (12).

Compound VIII—mp 190–193° [lit. (13) mp 192–194°], $[\alpha]_D$ −80.6° [lit. (13) $[\alpha]_D$ −85°], R_f 0.25 (A), isolated from *Ruscus alexandrinus* cladophylls (14).

Compound IX—mp 240–242° [lit. (15) 241.5–242°], $[\alpha]_D$ −101° [lit. (15) $[\alpha]_D$ −104°], R_f 0.64 (B).

Compound X—mp 257–259° [lit. (4) mp 254–258°], $[\alpha]_D$ +12° [lit. (4) $[\alpha]_D$ +10°], R_f 0.59 (B), isolated from *Yucca aloifolia* L. leaves (8).

Compound XI—mp 226–228° [lit. (4) mp 227°], $[\alpha]_D$ −9.5° [lit. (4) $[\alpha]_D$ −7°], R_f 0.08 (A).

Compound XII—mp 208–211° [lit. (16) mp 213°], $[\alpha]_D$ −109° [lit. (16) $[\alpha]_D$ −101°], R_f 0.61 (C), obtained as an artifact from *Balanites aegyptiaca* L. leaves (17).

Compound XIII—mp 162–163° [lit. (18) mp 164°], $[\alpha]_D$ −179° [lit. (18) $[\alpha]_D$ −175°], R_f 0.72 (C), obtained as an artifact from *Trigonella foenum-graecum* L. seeds (5).

Compound XIV—mp 225–228° [lit. (19) mp 220–230°], $[\alpha]_D$ −78° [lit. (19) $[\alpha]_D$ −81°], R_f 0.78 (D), obtained by synthesis (19).

Compound XV—mp 238–240° [lit. (20) mp 242–244°[, $[\alpha]_D$ −68.3° [lit. (20) $[\alpha]_D$ −73.1°], R_f 0.78 (D), obtained by synthesis (20).

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[x] To whom inquiries should be directed. Present address: College of Pharmacy, Howard University, Washington, DC 20001

Effect of Solvents on NMR Spectra of Penicillins

JOHN M. PADFIELD[x] and I. W. KELLAWAY

Abstract □ High-resolution NMR spectra of penicillin G and ampicillin in deuterium oxide, water, deuterated dimethyl sulfoxide, and deuterated dimethyl sulfoxide–deuterium oxide were examined. The α-amino group of ampicillin could not be observed in these solvents but was observed using the pivaloyl derivative of ampicillin in deuterochloroform. No evidence for intramolecular hydrogen bonding between the protons of the α-amino group and the oxygen of the β-lactam carbonyl group was obtained. The amino group does not restrict the rotation of the side chain, which has been shown to be important in interaction with phospholipids.

Keyphrases □ Penicillins—effect of solvents on NMR spectra □ Solvents—effect on NMR spectra of penicillins □ NMR spectroscopy—effect of solvent on spectra of penicillins

Assignments of the protons in penicillins were reported by several workers (1-4). In a recent study on the interaction of penicillins with phospholipids (5), an attempt was made to observe the signal due to the α-amino group of ampicillin to assess the possibility of intramolecular hydrogen bonding. This was not possible in deuterium oxide (D_2O), and the spectra were observed in deuterated dimethyl sulfoxide [$(CD_3)_2SO$] and a dimethyl sulfoxide–deuterium oxide [$(CD_3)_2SO$–D_2O] mixture.

EXPERIMENTAL

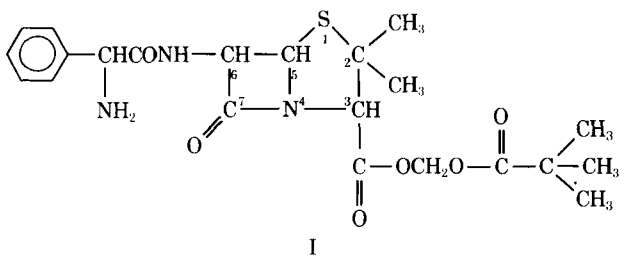
Materials—Potassium penicillin G[1], sodium ampicillin[2], and pivaloyl ampicillin[2] (I) were laboratory reference grade.

Deuterium oxide (99.95%), deuterated dimethyl sulfoxide, and deuterochloroform ($CDCl_3$) were used as solvents; tetramethylsilane was used as the external reference.

NMR Measurements—The spectra were obtained at 31° using a spectrometer[3] operating in a field-sweep mode.

RESULTS AND DISCUSSION

In their original report, Green *et al.* (2) observed anomalous behavior for the β-lactam protons at 60 MHz; further studies at 100 MHz (3) indicated that their original interpretation was incorrect. Penicillin G appeared to give a double doublet at τ 4.35 for the C_6-proton and a single doublet at τ 4.45 for the C_5-proton. Coupling of the amide and C_6-protons is probably responsible for the double doublet. Accidental coincidence of chemical shifts was postulated as being responsible for the two-proton singlet at τ 4.49 for ampicillin. Kan *et al.* (6) did not report the presence of a double doublet for the C_6-proton of penicillin G at 220 MHz.



I

The chemical shifts (Table I) indicate that, whereas a doublet was observed for each proton of the β-lactam ring in penicillin G, only one singlet was observed for both protons in ampicillin. The presence of an electron-attracting group such as $-NH_2$ may transfer its effect to the β-lactam ring to produce the observed differences. Collapse of this doublet was also observed for penicillin G solutions in dimethyl sulfoxide and its mixture was deuterium oxide (Table I). In both antibiotics, the proton most affected by changes in solvent was the C_3-proton; upfield shifts of 41 and 33 Hz were observed for penicillin G and ampicillin, respectively, when deuterium oxide was replaced by dimethyl sulfoxide.

An amide signal for ampicillin could only be detected in deuterated dimethyl sulfoxide as a broad signal at τ 1.32. The same signal for penicillin G could be detected in water (τ 1.45, broad), deuterated dimethyl sulfoxide (τ 1.28, doublet, J_{BX} = 8 Hz), and deuterated dimethyl sulfoxide–deuterium oxide (τ 1.27, doublet, J_{BX} = 7 Hz). This lends further evidence to differences in coupling of the amide group to the C_6-proton on substitution of the amino group into the side chain. In deuterium oxide, the amide group was unobservable for both antibiotics. Hydrogen bonding with the solvent cannot be eliminated in view of the presence of this signal for both penicillin G and ampicillin in dimethyl sulfoxide, when the amide signal would shift to be coincident with that due to deuterium oxide. However, exchange of the amide proton with deuterium oxide is a more likely explanation since the signal in deuterated dimethyl sulfoxide disappears upon addition of a drop of deuterium oxide. The exchange appears less rapid in penicillin G since the amide proton can be observed initially in the mixed solvent, which may be due to its coupling with the C_6-proton.

Different magnetic environments for the protons result from solvent interaction, giving rise to line shifts. The differences in chemical shifts observed between the systems in water and deuterium oxide may be due to differences in the dielectric constant; differences in the dipole moment are negligible. It was reported (7) previously that solvent effects on the NMR spectrum of acetonitrile correlated with dielectric constants.

The signal due to the amino protons of ampicillin was unobservable even in different solvents; the spectrum of the nonzwitteri-

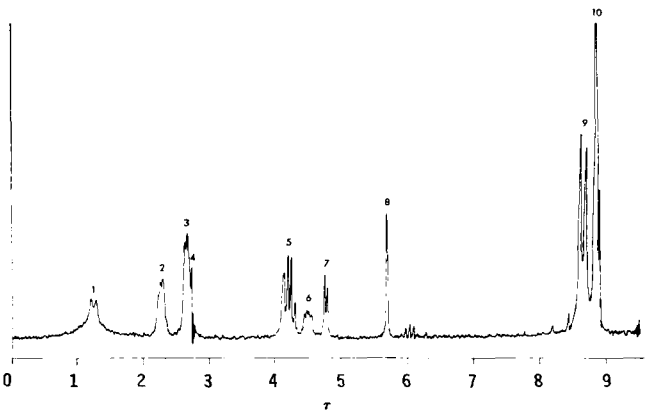
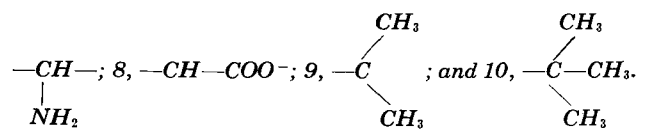


Figure 1—*100-MHz NMR spectrum of pivaloyl ampicillin in deuterochloroform at 31°. Key: 1, —NH—; 2, —NH_2; 3, C_6H_5—; 4, $CDCl_3$; 5, —O—CH_2—O—; 6, —CH—CH—; 7,*  *; and 10,* .

[1] Gift of Glaxo Research Ltd, Greenford, England.
[2] Gift of Beecham Research Laboratories, Betchworth, England.
[3] Varian Associates HA-100.

Table I—Effect of Solvents on 100-MHz Signals[a] of Antibiotic Protons (τ Values) (J Values in Hz in Parentheses)

Antibiotic and Solvent	C_6H_5—	—CH—X[b]	C-6	C-5	C-3	C-2—$(CH_3)_2$	
Penicillin G							
Deuterium oxide	2.60	6.32	4.44 d (4.0)	4.46 d (4.0)	5.69	8.39	8.44
Water	2.45	6.16	—[c]	—[c]	—[c]	8.20	8.35
Deuterated dimethyl sulfoxide	2.73	6.45	4.60 s	4.66 s	6.10	8.44	8.54
Deuterated dimethyl sulfoxide–deuterium oxide	2.72	6.46	4.63 s	4.63 s	6.05	8.44	8.54
Ampicillin							
Deuterium oxide	2.48	5.26	4.45 s	4.45 s	5.72	8.41	8.48
Water	2.44	—[c]	—[c]	—[c]	—[c]	8.24	8.30
Deuterated dimethyl sulfoxide	2.64	5.48	4.57 s	4.57 s	6.05	8.45	8.56
Deuterated dimethyl sulfoxide–deuterium oxide	2.49	5.30	4.46 s	4.46 s	—[c]	8.29	8.40

[a] s = singlet, and d = doublet. [b] X = H in penicillin G, and X = —NH_2 in ampicillin. [c] Not observable under water peak.

onic pivaloyl ampicillin (I) in deuterochloroform was therefore examined (Fig. 1). Decoupling experiments were performed in which signals 2 and 7 were separately irradiated and the changes in each, if any, were observed. The occurrence of intramolecular hydrogen bonding between the α-amino group and the β-lactam carbonyl oxygen would be expected to result in complex signals for the amino protons and the α-CH-proton due to changes in their coupling. Despite the presence of a doublet for the α-CH-proton, irradiation provided no evidence of the complex signals required to postulate intramolecular bonding. James *et al.* (8) found no evidence of intramolecular hydrogen bonding in the crystal of ampicillin trihydrate. A recent study (9) suggested that the nonuniform concentration dependence observed for the β-lactam protons may indicate intermolecular hydrogen bonding, which would aid the postulation of self-association of penicillin G.

It would appear that the amino group does not restrict the rotation of the side chain, which has been shown to be important in interaction with phospholipids (5, 10, 11).

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[x]To whom inquiries should be directed. Present address: School of Pharmacy, University of Bath, Claverton Down, Bath, BA2 7AY, United Kingdom.

Physical Properties of Four Polymorphic Forms of Sulfanilamide I: Densities, Refractive Indexes, and X-Ray Diffraction Measurements

HWAING OU LIN*§, NORMAN C. BAENZIGER‡, and J. KEITH GUILLORY*x

Abstract □ Densities, refractive indexes, and X-ray crystallographic data are tabulated for four crystalline forms of sulfanilamide. These data suggest that three of the forms have very similar structures.

Keyphrases □ Sulfanilamide—densities, refractive indexes, and X-ray diffraction data for four polymorphic forms □ Polymorphs—sulfanilamide, physical properties □ X-ray diffraction—four polymorphic forms of sulfanilamide

In a previous article (1), IR spectra, heats of transition, and differential thermal analysis (DTA) thermograms were reported for four polymorphic forms of sulfanilamide. The properties of these crystalline species differed somewhat from those previously reported in the literature. Consequently, it was deemed essential to obtain additional information for characterizing the polymorphs.

EXPERIMENTAL

Crystal Structures—The Weissenberg camera and Precession camera were employed for taking single-crystal diffraction pictures. One end of a fine fiber of glass was fixed on a goniometer head using Canada balsam. A selected crystal was mounted on the other end of the glass fiber. The goniometer head holding the crystal was then transferred to the Weissenberg or Precession camera. Adjustments were made on the goniometer so that the chosen axis of the crystal was coincident with the axis of rotation of the goniometer. The Weissenberg zero-layer-line and first-layer-line pictures and Precession pictures were taken after the crystal was well aligned. Cell dimensions and monoclinic angles were determined by careful measurements on these pictures for each crystal sample. In addition, cell dimensions and errors for the α- and γ-forms were calculated by a least-squares computer program of back-reflection Weissenberg single-crystal diagrams. The errors of cell dimensions for the β- and δ-forms were then determined by comparing the values of cell dimensions and errors for the α- and γ-forms obtained by direct measurements and computer calculations. The number of molecules per unit cell for each crystal form was calculated according to Eq. 1:

$$Z = \frac{\text{unit cell volume} \times \text{density} \times \text{Avogadro's number}}{\text{molecular weight}} \quad \text{(Eq. 1)}$$

The calculation and interpretation of X-ray pictures were performed according to procedures described by Buerger (2) and McLachlan (3).

Refractive Indexes—Refractive indexes were determined with a Zeiss polarizing microscope. The microscope used for examination of the crystals was equipped with polarizing prisms below and above a rotating, graduated, circular stage, and with accessories including a Bertrand lens and first-order red and quartz wedge compensators. Crystals were immersed in a series of liquids of known refractive indexes and were examined between crossed polars under the microscope. One of the component vibrations of light of the crystal was selected, and the Becke test was applied for the refractive index determinations. A crystal has the same refractive index as the medium in which shadow boundaries are at a minimum. This operation and test were repeated after rotating the stage 90°, and a second refractive index of the substance was obtained. The precision of each determination of refractive index was within ±0.004.

RESULTS AND DISCUSSION

Data obtained from the X-ray crystallographic study of sulfanilamide polymorphs are reproduced in Table I. These data indicate that the δ-, β-, and γ-forms in this study correspond to the α-, β-, and γ-forms reported by Watanabe (4). The α-form in this investigation is a form that was not reported previously (1).

The unit cell volumes of these polymorphs are calculated to be 768.7, 758.8, 769.2, and 1544.7 Å^3 for the α-, β-, γ-, and δ-forms, respectively. The similarity in cell dimensions and monoclinic angles in the α- and β-forms indicates that the structures of these two forms are similar. This conclusion is further supported by the observation that they have very nearly the same heat of transition (1). The slightly greater unit cell volume of the α-form suggests that the packing of this form is less compact and its structure is more easily broken down. Therefore, the α-form undergoes a phase transition to the γ-form at a lower temperature than does the β-form.

Data obtained from optical crystallographic studies of sulfanilamide and sulfanilamide-d_4 polymorphs are listed in Table II. The data for the refractive indexes of the δ-, β-, and γ-forms are in agreement with data on the α-, β-, and γ-forms reported previously (5, 6). A careful comparison of refractive indexes between the α- and β-forms in this study reveals that the α-form has lower refractive indexes on the average than the β-form. These results imply that light passes through a crystal of the α-form at a slightly higher speed. In other words, the α-form is less compact in terms of molecular packing. The slightly lower refractive indexes of sulfanilamide-d_4 polymorphs compared to the corresponding undeuterated forms can be interpreted to mean that substitution of deuterium for hydrogen in sulfanilamide increases the hydrogen bond distances. Therefore, the molecular packings are less compact in sulfanilamide-d_4 crystals.

The crystal densities of sulfanilamide polymorphs were determined to be 1.505, 1.523, 1.510, and 1.499 g/cm^3 for the α-, β-, γ-, and δ-forms, respectively. The lower value of the density for the α-form, as well as observations obtained from solubility and dissolution studies (7), indicates that there is a correlation between these physical measurements and the results of the X-ray diffraction studies. Both groups of data provide evidence that the α-form is less compact and less stable as compared with the β-form. The small differences in crystal structures between the α- and β-forms apparently have a substantial influence on their physical properties.

Significant changes in physical characteristics resulting from minor differences in crystal structure were reported previously. Katayama (8) studied the structures of two polymorphic forms of chloroacetamide. In spite of very small variations in the cell's dimensions and in the monoclinic angles of the α-and β-chloroacetamide polymorphs, their solubility behaviors are quite different.

The α-, β-, and γ-forms of sulfanilamide are monoclinic and contain four molecules in each unit cell. Transformations from the α- and β-forms to the γ-form at transition temperatures probably involve only bond bending and bond dilation within a cluster. The δ-form, on the other hand, is orthorhombic and contains eight molecules in each unit cell. The transformation from the δ-form to the γ-form may, therefore, be expected to involve a molecular reconstructive process. Such processes, as a rule, involve more work, and more energy has to be absorbed during the phase

Table I—X-Ray Crystallographic Data for Sulfanilamide Polymorphs

Polymorphic Forms	Crystal Systems	Cell Dimensions, Å	Unit Cell Numbers	Monoclinic Angles
α-Form	Monoclinic	$a = 9.042 \pm 0.003$ $b = 9.034 \pm 0.002$ $c = 10.06 \pm 0.02$	4	110°42′
β-Form	Monoclinic	$a = 8.95 \pm 0.02$ $b = 9.06 \pm 0.02$ $c = 9.96 \pm 0.02$	4	110°
γ-Form	Monoclinic	$a = 7.783 \pm 0.006$ $b = 12.944 \pm 0.003$ $c = 7.95 \pm 0.02$	4	106°1′
δ-Form	Orthorhombic	$a = 14.81 \pm 0.02$ $b = 5.65 \pm 0.02$ $c = 18.46 \pm 0.02$	8	—

Table II—Optical Crystallographic Data for Sulfanilamide and Sulfanilamide-d_4 Polymorphs

	Polymorphic Forms	Refractive Indexes α	β	γ
Sulfanilamide polymorphs	α-Form	1.580	1.644	—
	β-Form	1.560	1.680	—
	γ-Form	—	1.676	>1.800
	δ-Form	1.548	1.624	
Sulfanilamide-d_4 polymorphs	α-Form	1.576	1.640	—
	β-Form	1.556	1.672	—
	γ-Form	—	1.664	>1.800
	δ-Form	1.540	1.620	—

transformation. Consequently, the transformation energy is higher for the δ-form as compared with the α- and β-forms.

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§ Present address: Johns Hopkins University, Baltimore, Md.

ˣ To whom inquiries should be directed.

Effect of Multiple Doses of Cadmium on Glucose Metabolism in the Rat

DIONYSSIS S. ITHAKISSIOS*, WAYNE V. KESSLERˣ, JAMES N. ARVESEN‡, and GORDON S. BORN

Abstract □ The effect of multiple doses of cadmium on carbohydrate metabolism in rats was studied. The treated groups received two dose levels, 0.25 and 0.50 mg cadmium/kg ip every 2nd day for 20 doses. Plasma glucose, immunoresponsive insulin levels, and body weights were determined before treatment and after 10 and 20 doses of cadmium. After the last dosage, D-glucose-^{14}C (uniformly labeled) was administered to a randomly chosen subgroup and a carbon dioxide radiorespirometry experiment was performed. Statistically significant differences in blood glucose and insulin levels were not detected at any time, indicating no effect of cadmium on the pancreatic secretory activity. There was no effect on body weights. However, cadmium increased the evolution of respiratory $^{14}CO_2$. It is postulated that cadmium affects the rate of glucose metabolism *in vivo* by affecting mechanisms such as glycolysis and the tricarboxylic acid cycle.

Keyphrases □ Cadmium—effect of multiple doses on glucose metabolism, rat □ Glucose metabolism—effect of multiple doses of cadmium, rat □ Glycolysis—possible effect of multiple doses of cadmium on glucose metabolism, rat

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Table I—Effect of Cadmium on Plasma Glucose, Insulin, Insulinogenic Index, and Weight of Rats

Parameter	Replicate	Saline Control			Cadmium Treated			
		Before Treatment	After 10 Doses	After 20 Doses	Dose, mg Cadmium/kg	Before Treatment	After 10 Doses	After 20 Doses
Plasma glucose, mg/100 ml	1	149[a] ±18	154 ±29	182 ±29	0.25	174 ±35	150 ±19	209 ±43
	2	126 ±14	159 ±90	123 ±18		128 ±70	160 ±80	124 ±11
					0.50	128 ±10	160 ±12	129 ±14
Insulin, μunits/ml	1	35.1 ±17.5	36.9 ±17.4	32.4 ±11.5	0.25	33.1 ±19.6	24.2 ±7.6	44.2 ±13.6
	2	37.9 ±9.0	42.6 ±11.0	43.7 ±9.0		52.0 ±9.0	50.5 ±11.0	55.3 ±15.0
					0.50	55.5 ±16.0	47.1 ±16.0	57.6 ±11.0
Insulinogenic index[b]	1	0.24 ±0.13	0.24 ±0.10	0.18 ±0.07	0.25	0.22 ±0.15	0.16 ±0.05	0.21 ±0.09
	2	0.31 ±0.11	0.27 ±0.06	0.36 ±0.09		0.41 ±0.08	0.32 ±0.07	0.45 ±0.12
					0.50	0.43 ±0.11	0.29 ±0.10	0.45 ±0.10
Weight, g	1	215 ±10	257 ±10	271 ±9	0.25	218 ±4	250 ±8	266 ±6
	2	105 ±4	176 ±7	203 ±12		105 ±3	175 ±9	198 ±14
					0.50	104 ±4	165 ±10	195 ±18

[a] Mean of eight animals ± standard deviation. [b] Insulinogenic index = $\frac{\text{insulin, } \mu\text{units/ml}}{\text{blood glucose, mg/100 ml}}$.

Table II—Cadmium Concentrations in Tissues after 20 Intraperitoneal Administrations of 0.25 mg Cadmium/kg Every 2nd Day

Tissue	Saline Control, ppm	Cadmium Treated, ppm
Blood	0.29[a] ± 0.02	2.30 ± 0.24
Kidneys	0.40 ± 0.09	86.82 ± 13.37
Spleen	0.50 ± 0.13	16.24 ± 1.02
Liver	0.57 ± 0.01	120.38 ± 1.30
Pancreas	0.94 ± 0.06	33.46 ± 2.99

[a] Mean of four animals ± standard deviation. Wet basis.

form complexes with different ligands on a metalloprotein. It is not clear to what extent the properties of these complexes can be used to predict the identity of the metal ligands of metalloproteins (1). Cadmium binds more strongly than zinc to metallothionein, and an excess of cadmium can displace zinc (2–4). The parts of a protein with high cysteinyl content are where cadmium and zinc may interact, and this interaction may affect the secondary and tertiary structure of the protein.

In previous studies, investigators (1, 5–8) found that cadmium introduces changes in the three-dimensional structure of enzymes and influences their rate or mechanism of action by activation, inactivation, uncoupling reactions, or other actions. In addition, it was found that cadmium selectively damages pancreatic β-cells (9, 10) and produces changes in blood glucose (5).

Since cadmium may affect the secretory activity of pancreatic β-cells and the rate of reaction of enzymes involved in carbohydrate and cellular energetic metabolism, the following *in vivo* investigation concerned the effect of multiple doses of cadmium on glucose and insulin blood levels and the evolution of respiratory $^{14}CO_2$.

EXPERIMENTAL

Sixteen female rats[1], weighing approximately 200 g, were assigned randomly to two groups, control and treatment, of eight animals each. Animals were housed individually in metal cages. The animals were maintained for 7 days under laboratory conditions prior to experimentation. They were allowed free access to food[2] and tap water.

A dose of 0.25 mg cadmium/kg ip was administered to the treatment group every 2nd day for 20 doses. The cadmium was given as cadmium acetate in water. Two months later, this study was replicated with a different shipment of animals, weighing approximately 100 g. In this replicate, an additional group of eight rats was given twice the previous dose (0.5 mg/kg). Control animals received saline solution instead of cadmium. Plasma glucose and immunoresponsive insulin levels as well as body weights were measured for each animal immediately prior to treatment with cadmium and 24 hr after the 10th and 20th doses.

Twenty-four hours after completion of the last blood sampling, all animals from the first replicate were assigned randomly to two groups, each subdivided into control and treatment animals, to allow for a carbon dioxide respiration experiment and for determination of cadmium concentrations in the pancreas, liver, kidneys, spleen, and blood.

Insulin and Glucose Determination—The immunoresponsive insulin of the plasma was determined quantitatively using a commercially available radioimmunoassay kit[3], which closely resembles the method described by Hales and Randle (11). The method uses an antibody specific to insulin, which is reacted with plasma immunoresponsive insulin and a known amount of ^{125}I-insulin to form an insoluble insulin-antibody complex. This complex is filtered and measured for radioactivity by liquid scintillation spectrometry. The level of radioactivity from ^{125}I-insulin recovered in

[1] Sprague-Dawley descendants, Laboratory Supply Co., Indianapolis, Ind.
[2] Wayne Lab-Blox, Allied Mills, Chicago, Ill.
[3] Amersham/Searle Co., Chicago, Ill.

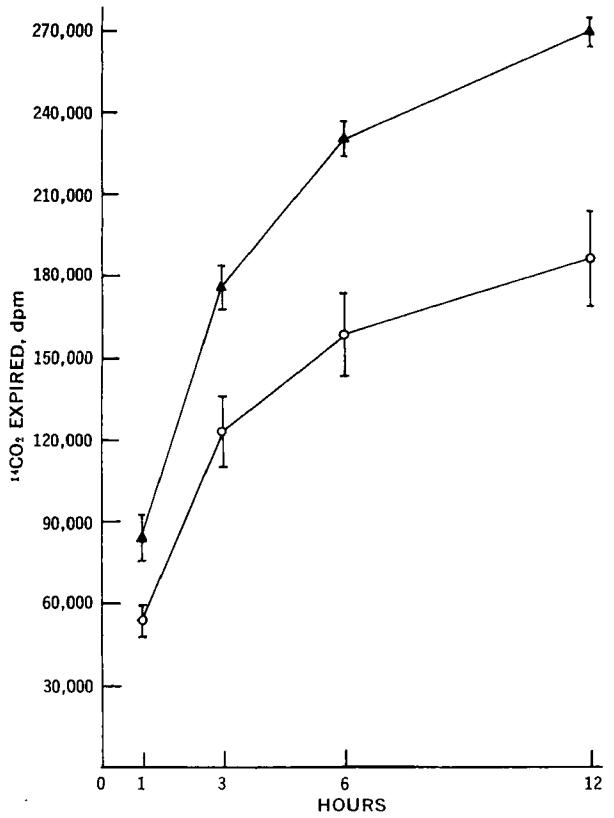

Figure 1—*Amount of $^{14}CO_2$ expired by rats receiving* D-*glucose-U-^{14}C. Key:* ○, *control; and* ▲, *0.25 mg cadmium/kg every 2nd day for 20 doses.*

the complex is related inversely to the concentration of immunoresponsive insulin in the plasma sample.

Plasma glucose was measured with a glucose analyzer containing a digital readout[4]. The principle of this method involves the conversion of glucose to its oxidation products (gluconic acid and hydrogen peroxide) by a glucose oxidase enzyme. The rate of oxygen consumed during the reaction is proportional to the glucose concentration in the sample. The oxygen is detected with an oxygen-sensitive electrode.

Radiorespirometric Study—Eight 15.24-cm, controlled-ventilation, glass metabolism chambers[5] were used for $^{14}CO_2$ collection. Carbon dioxide and water were removed from the incoming air stream (500 ml/min) with a drying tube containing anhydrous calcium sulfate followed by asbestos coated with sodium hydroxide. The chamber exhaust air was dehydrated with anhydrous calcium sulfate, and $^{14}CO_2$ was trapped in 100 ml of a solution of 2-ethoxyethanol and 2-aminoethanol (2:1). Primary and secondary carbon dioxide traps were used, although the efficiency of the primary traps approached 100%.

Prior to the carbon dioxide respiration experiment, the animals were fasted for 24 hr. On the day of testing, each animal was given 3.0 ml po of a 50% (w/v) glucose–water solution containing 4.82 μCi/3.0 ml of D-glucose-U-^{14}C (U = uniformly labeled). Upon completion of dosing, the animals were immediately placed in the metabolism chambers where neither water nor food was provided. Trapping solutions for $^{14}CO_2$ were changed at 1, 3, 6, and 12 hr.

A liquid scintillation counter[6] utilizing internal standardization was used for the determination of ^{14}C activity. The counting efficiencies for the scintillator used[7] ranged from 72 to 75%, the counting error was less than 1%, and the instrument error was minimized by counting each sample twice.

The radiochemical purity of the D-glucose-U-^{14}C was greater than 99%. The purity was determined by paper chromatography, autoradiography (12), and liquid scintillation counting of the spots placed in 15 ml of scintillator[8].

Tissue Digestion—All samples were dissolved by heating with concentrated nitric acid. They were filtered into volumetric flasks and analyzed for cadmium with an atomic absorption spectrophotometer[9].

Table III—Analysis of Variance of Data for Radiorespiration Study

Source	*df*	*MS*	*F*
Cadmium treatment (A)	1	44153.936	218.390[a]
Time (B)	3	71142.130	351.876[b]
AB	3	403.067	1.996[b]
Residual	24	202.180	
Total	31		

[a] At the 0.05 significance level, a significant $F = 4.26$. [b] At the 0.05 significance level, a significant $F = 3.01$.

RESULTS AND DISCUSSION

The effects of multiple doses of cadmium on plasma glucose and insulin levels and body weight are shown in Table I. The insulinogenic index is the ratio of serum immunoresponsive insulin to blood glucose concentration. This index indicates the pancreatic secretory activity. A reduced insulinogenic index implies a decreased pancreatic secretory activity (13). Control animals, which had received repeated doses of saline, were examined at the same time intervals.

The results shown in Table I were analyzed by analysis of covariance. Each replicate and each dose level were analyzed separately. The value before treatment was taken as a covariate for the values after 10 and 20 doses. The significance of the covariate and the difference in treatment effects *versus* control were determined for each case. When the F value of the covariate was lower than 1.0, the analysis was performed on the original cell means; with F values higher than 1.0, the analysis was performed on the adjusted cell means. Since the F values of treatment for $p = 0.05$ with 3 and 27 *df* were significant in all cases, a Newman-Keuls multiple comparison on the adjusted or unadjusted means was performed. The analysis showed that there were no significant differences in glucose, insulin, insulinogenic index, or weight at the $p = 0.05$ level after 10 or 20 doses of cadmium. This was the case for both replicates and for both dose levels.

Analysis of covarince was also run on the combined results from both replicates. Again no significant differences were found.

The cadmium concentrations in the blood, kidneys, spleen, liver, and pancreas were determined, and the results are shown in Table II. The cadmium concentrations in the various tissues of the treated animals were higher than those in controls. The levels varied from 10 times higher for blood to as much as 200 times higher for liver. The relative uptake of the various tissues also was different between the control and treatment groups.

Results from the carbon dioxide radiorespiration study showed that cadmium increased the evolution of respiratory $^{14}CO_2$. The data (Fig. 1) were analyzed by a two-factor analysis of variance (treatment and time effects) to assess the difference between the control and treatment groups. The analysis was done with the square roots of the cumulative amounts (dpm) of $^{14}CO_2$ collected, which provided a response with a variance approximately independent of its mean. The F value for the treatment by time interaction was not significant even at the 10% level (Table III); the main effect difference between control and treatment groups was highly significant over all time periods.

Since cadmium is known to accumulate in the pancreatic islet tissue and repeated administration of cadmium may selectively produce destruction of β-cells (9, 10, 14), a decrease in the insulin secretory activity of the pancreas was expected. The data did not prove this, in spite of the high accumulation of cadmium in the pancreas of treated animals (Table II). Further studies on the effects of multiple cadmium administration on insulin secretion

[4] Beckman, model 2001.
[5] Delmar Scientific Laboratories, Maywood, Ill.
[6] Tri-Carb, Packard Instrument Co., Downers Grove, Ill.
[7] Toluene–2-ethoxyethanol (1:1) and 0.4% 2,5-diphenyloxazole.
[8] Toluene–*p*-dioxane–2-ethoxyethanol (1:3:3), 1% 2,5-diphenyloxazole, and 8% naphthalene.
[9] Perkin-Elmer model 306.

from the perfused pancreas as well as from isolated islets are required to clarify this point.

The significant increase in the evolution of respiratory $^{14}CO_2$ by the animals treated with cadmium indicates that multiple dosages of cadmium alter carbohydrate metabolism. The affected mechanisms may involve glycolysis, the tricarboxylic acid cycle, respiration, or a combination of these. It was reported (15–17) that cadmium ions uncouple oxidative phosphorylation. This uncoupling may be due to the effect of cadmium on the transport of ions across the mitochondrial membrane (16) or to the blocking of some free active site (15). Uncoupling oxidative phosphorylation increases the adenosine diphosphate concentration relative to that of adenosine triphosphate. This causes an increase in the rate of glycolysis and the tricarboxylic acid cycle since it activates certain enzymes such as phosphorylase *a*, adenosine triphosphate:D-fructose 6-phosphate 1-phosphotransferase, adenosine triphosphate:pyruvate phosphokinase, and isocitrate dehydrogenase.

With the increase in the rates of glycolysis and the tricarboxylic acid cycle after cadmium treatment, there may be a concomitant increase in labeled glucose utilization. With the information available, it is not possible to explain further the effect of cadmium on the evolution of carbon dioxide *in vivo* and further work is indicated.

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* Fellow, Purdue Research Foundation.

‡ Department of Statistics, School of Science, Purdue University, West Lafayette, IN 47907

ˣ To whom inquiries should be directed.

Synthesis and Hypotensive Properties of 4-Amino-6,7-dimethoxyisoquinoline

GEORGE C. WRIGHTˣ and ROBERT P. HALLIDAY

Abstract □ The compound 4-amino-6,7-dimethoxyisoquinoline hydrochloride, a structural isomer of amiquinsin hydrochloride (4-amino-6,7-dimethoxyquinoline hydrochloride), was synthesized. It was shown to be hypotensive in anesthetized normotensive dogs and produced an antihypertensive effect in unanesthetized renal hypertensive dogs.

Keyphrases □ 4-Amino-6,7-dimethoxyisoquinoline—synthesis and hypotensive properties, structural isomer of amiquinsin hydrochloride □ Amiquinsin hydrochloride—synthesis and hypotensive properties of the structural isomer 4-amino-6,7-dimethoxyisoquinoline □ Antihypertensive agents, potential—synthesis and screening of 4-amino-6,7-dimethoxyisoquinoline, a structural isomer of amiquinsin hydrochloride

Since the hypotensive properties of amiquinsin hydrochloride [1] (4-amino-6,7-dimethoxyquinoline hydrochloride hydrate) (I) were well known (1), there was a desire to prepare the isomeric 4-amino-6,7-dimethoxyisoquinoline hydrochloride (II) for hypotensive screening. This paper discusses the synthesis of II and several related intermediates, as well as the hypotensive properties of II.

The key intermediate, 2,3-dihydro-6,7-dimethoxy-4(1*H*)-isoquinolone (IV), in the synthesis of II was prepared *via* a two-step process (Scheme I). Reductive condensation of veratraldehyde and glycine gave *N*-veratrylglycine (III) which, upon subsequent cyclization in polyphosphoric acid, gave IV [2]. Although the intermediate Schiff-base product of veratraldehyde and glycine was not isolated, it was necessary to heat briefly the reaction solution prior to reduction to avoid formation of *N,N*-diveratrylglycine (VII). The reductive alkylation at room temperature

[1] Norwich Pharmacal Co.

[2] The present synthesis of IV is more direct than the four-step synthesis used in the similar preparation of 2,3-dihydro-6,7-dimethoxy-1-methyl-4(1*H*)-isoquinolone (3).

from the perfused pancreas as well as from isolated islets are required to clarify this point.

The significant increase in the evolution of respiratory $^{14}CO_2$ by the animals treated with cadmium indicates that multiple dosages of cadmium alter carbohydrate metabolism. The affected mechanisms may involve glycolysis, the tricarboxylic acid cycle, respiration, or a combination of these. It was reported (15–17) that cadmium ions uncouple oxidative phosphorylation. This uncoupling may be due to the effect of cadmium on the transport of ions across the mitochondrial membrane (16) or to the blocking of some free active site (15). Uncoupling oxidative phosphorylation increases the adenosine diphosphate concentration relative to that of adenosine triphosphate. This causes an increase in the rate of glycolysis and the tricarboxylic acid cycle since it activates certain enzymes such as phosphorylase *a*, adenosine triphosphate:D-fructose 6-phosphate 1-phosphotransferase, adenosine triphosphate:pyruvate phosphokinase, and isocitrate dehydrogenase.

With the increase in the rates of glycolysis and the tricarboxylic acid cycle after cadmium treatment, there may be a concomitant increase in labeled glucose utilization. With the information available, it is not possible to explain further the effect of cadmium on the evolution of carbon dioxide *in vivo* and further work is indicated.

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* Fellow, Purdue Research Foundation.

‡ Department of Statistics, School of Science, Purdue University, West Lafayette, IN 47907

ˣ To whom inquiries should be directed.

Synthesis and Hypotensive Properties of 4-Amino-6,7-dimethoxyisoquinoline

GEORGE C. WRIGHTˣ and ROBERT P. HALLIDAY

Abstract □ The compound 4-amino-6,7-dimethoxyisoquinoline hydrochloride, a structural isomer of amiquinsin hydrochloride (4-amino-6,7-dimethoxyquinoline hydrochloride), was synthesized. It was shown to be hypotensive in anesthetized normotensive dogs and produced an antihypertensive effect in unanesthetized renal hypertensive dogs.

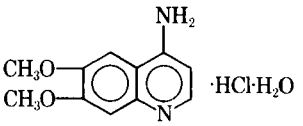
Keyphrases □ 4-Amino-6,7-dimethoxyisoquinoline—synthesis and hypotensive properties, structural isomer of amiquinsin hydrochloride □ Amiquinsin hydrochloride—synthesis and hypotensive properties of the structural isomer 4-amino-6,7-dimethoxyisoquinoline □ Antihypertensive agents, potential—synthesis and screening of 4-amino-6,7-dimethoxyisoquinoline, a structural isomer of amiquinsin hydrochloride

Since the hypotensive properties of amiquinsin hydrochloride [1] (4-amino-6,7-dimethoxyquinoline hydrochloride hydrate) (I) were well known (1), there was a desire to prepare the isomeric 4-amino-6,7-dimethoxyisoquinoline hydrochloride (II) for hypotensive screening. This paper discusses the synthesis of II and several related intermediates, as well as the hypotensive properties of II.

The key intermediate, 2,3-dihydro-6,7-dimethoxy-4(1*H*)-isoquinolone (IV), in the synthesis of II was prepared *via* a two-step process (Scheme I). Reductive condensation of veratraldehyde and glycine gave *N*-veratrylglycine (III) which, upon subsequent cyclization in polyphosphoric acid, gave IV [2]. Although the intermediate Schiff-base product of veratraldehyde and glycine was not isolated, it was necessary to heat briefly the reaction solution prior to reduction to avoid formation of *N,N*-diveratrylglycine (VII). The reductive alkylation at room temperature

[1] Norwich Pharmacal Co.

[2] The present synthesis of IV is more direct than the four-step synthesis used in the similar preparation of 2,3-dihydro-6,7-dimethoxy-1-methyl-4(1*H*)-isoquinolone (3).



I

gave VII in 32% yield. Direct synthesis of VII from III and 3,4-dimethoxybenzyl chloride (VIII) established the *N,N*-diveratrylglycine structure.

Intermediate IV was previously prepared by an alternative four-step synthesis (2), without an improvement in yield over that of the presently described method.

The intermediate 6,7-dimethoxy-4-isoquinolinol (V) (2) was obtained through aromatization of IV with palladium-on-charcoal in refluxing toluene. Initially unsuccessful attempts were made to convert V to 4-amino-6,7-dimethoxyisoquinoline hydrochloride (II) through usual methods. Alternatively, the hydrazone (VI) of IV was prepared and subjected to aromatization (palladium-on-charcoal in refluxing alcohol), with the cleavage of the hydrazine moiety of VI to give the desired II.

Compound II was hypotensive in anesthetized normotensive dogs and produced an antihypertensive effect when administered to unanesthetized renal hypertensive dogs. In comparison with the activity of I, II was equipotent in regard to pressure lowering in the anesthetized dog; however, the duration of hypotensive effect was shorter. In the renal hypertensive dog, II was less active than I in regard to both potency and duration.

EXPERIMENTAL [3]

***N*-Veratrylglycine (III)**—To a solution of glycine (10.8 g, 0.14 mole) in water (110 ml) was added a solution of veratraldehyde (24.9 g, 0.15 mole) in ethanol (60 ml). The resultant solution was heated to boiling and cooled in an ice bath to room temperature. With the addition of 5% palladium-on-charcoal (2.0 g), the mixture was hydrogenated in a Parr apparatus at room temperature for 1 hr; a quantitative drop in pressure was observed. The reaction solution was filtered of catalyst, and the filtrate was evaporated under reduced pressure. The solid residue was triturated with hot 95% ethanol (70 ml) and cooled in an ice bath. The white crystalline product, III, was collected and washed with ethanol and ether, mp 200–202°, yielding 21.4 g (68%).

***N*-Veratrylglycine Hydrochloride (III-HCl)**—A mixture of III (70 g, 0.31 mole) and ethanol (700 ml) was treated with hydrogen chloride (2-propanol) solution (250 ml). The mixture was heated on the steam bath, and water (50 ml) was added to obtain solution. The solution was cooled, and the product was collected and washed with 95% ethanol and ether, mp 207–210°, yielding 55 g (68%).

Anal.—Calc. for $C_{11}H_{15}NO_4 \cdot HCl$: C, 50.48; H, 6.16; N, 5.35. Found: C, 50.27; H, 6.26; N, 5.30.

***N,N*-Diveratrylglycine (VII)**—*Method A*—To a solution of glycine (7.5 g, 0.10 mole) in water (120 ml) was added a solution of veratraldehyde (16.6 g, 0.10 mole) in ethanol (45 ml) and platinic oxide (0.3 g). The mixture was hydrogenated in a Parr apparatus at room temperature for 3.5 hr. The reaction mixture was cooled, and the product was collected and washed with water. Recrystallization from ethanol gave 6.0 g (32%) of a white crystalline solid, mp 196–199°.

The hydrochloride of VII was prepared by the same procedure as described for III-HCl, mp 187–189°.

Anal.—Calc. for $C_{20}H_{25}NO_6 \cdot HCl$: C, 58.32; H, 6.36; Cl, 8.61; N, 3.40. Found: C, 58.45; H, 6.47; Cl, 8.68; N, 3.48.

Method B—A mixture of III (6.6 g, 0.029 mole), VIII (5.8 g, 0.031 mole), anhydrous sodium carbonate (3.0 g, 0.028 mole), and dimethylformamide (150 ml) was heated at 85–90° overnight with mechanical stirring. The reaction mixture was filtered hot, and the filtrate was evaporated to dryness under reduced pressure. The amorphous residue, washed twice with ether, was treated with 2-propanol and diluted with ether. A cream-colored solid was isolated, mp 185–188°, yielding 2.6 g (23%). The IR absorption spectrum [4] was identical to that of authentic VII. Recrystallization from methanol gave a white crystalline solid, mp 197–200°; the mixed melting point with authentic VII was 199–202°.

2,3-Dihydro-6,7-dimethoxy-4(1*H*)-isoquinolone Hydrochloride (IV-HCl)—To polyphosphoric acid (730 g) was added, portionwise, III (60 g, 0.27 mole) with mechanical stirring. The mixture was heated (85–90°) on the steam bath for 2 hr and allowed to stand overnight. The reaction syrup was gradually triturated with ice (3 liters) and water (1 liter) and then treated with benzene (1.5 liters) and solid sodium carbonate (880 g) to pH 6–7. The organic layer was separated, and the aqueous layer was further extracted with four 1-liter portions of benzene. After drying over magnesium sulfate and charcoal, the filtered extracts were treated with hydrogen chloride while being cooled in an ice bath. Recrystallization of the product from 97% ethanol gave white crystals, yielding 11.9 g (18%), mp 235–237° dec. [lit. (2) mp 237–238°, from methanol].

Anal.—Calc. for $C_{11}H_{13}NO_3 \cdot HCl$: C, 54.21; H, 5.79; N, 5.75. Found: C, 54.09; H, 5.81; N, 5.64.

6,7-Dimethoxy-4-isoquinolinol Hydrochloride (V-HCl)—A mixture of IV (30 g, 0.15 mole), toluene (1500 ml), and 5% palladium-on-charcoal (15 g) was heated on the steam bath for 22 hr. The insoluble product was extracted from the catalyst with hot dimethylformamide (340 ml). The extract was evaporated to dryness under reduced pressure, and the residue was washed with ether. The product in 95% ethanol (1200 ml) was heated with charcoal for 7 min and filtered. The cooled filtrate was treated with hydrogen chloride; then one volume of ether was added, and a cream-colored solid was deposited. The isolated solid melted at 239–244° and yielded 17.7 g (49%). Recrystallization from 95% ethanol gave the hydrochloride, mp 242–244° dec. [lit. (2) mp 264–265°, from methanol].

Anal.—Calc. for $C_{11}H_{11}NO_3 \cdot HCl$: C, 54.67; H, 5.01; N, 5.80. Found: C, 54.69; H, 5.01; N, 5.64.

2,3-Dihydro-6,7-dimethoxy-4(1*H*)-isoquinolone Hydrazone (VI)—To a mixture of IV (64.0 g, 0.31 mole) and ethanol (525 ml) was added hydrazine hydrate (29.0 ml, 0.58 mole). The reaction solution was refluxed for 1.5 hr, and the solution was cooled in an ice bath for 2 hr. The resultant brown, crystalline solid was collected and washed with ethanol and ether, yielding 48.1 g (70%), mp 156–159°. Recrystallization from ethanol gave pale-yellow crystals, mp 155–158°.

Anal.—Calc. for $C_{11}H_{15}N_3O_2$: C, 59.71; H, 6.83; N, 18.99. Found: C, 59.51; H, 6.81; N, 18.48.

4-Amino-6,7-dimethoxyisoquinoline Hydrochloride (II-HCl)—A mixture of VI (38 g, 0.17 mole), ethanol (600 ml), and 5% palladium-on-charcoal (19 g) was refluxed for 16 hr. The hot solution was filtered, cooled, and treated with dry hydrogen chloride. The resultant tan, crystalline solid was collected and washed with cold 2-propanol. The product was recrystallized from a mixture of ethanol and methanol (with charcoal), yielding 13 g (32%), mp 249–251° dec. A second crop of crystals was isolated, yielding 12 g (29%), mp 251–254° dec.

Anal.—Calc. for $C_{11}H_{12}N_2O_2 \cdot HCl$: C, 54.89; H, 5.44; Cl, 14.73; N, 11.64. Found: C, 54.63; H, 5.36; Cl, 14.59; N, 11.40.

The NMR spectrum [5] in D_2O showed δ 3.76, singlet (two CH_3O); 6.41 and 6.71, singlets (two aromatic); and 7.25 and 7.88, singlets (two heteromatic).

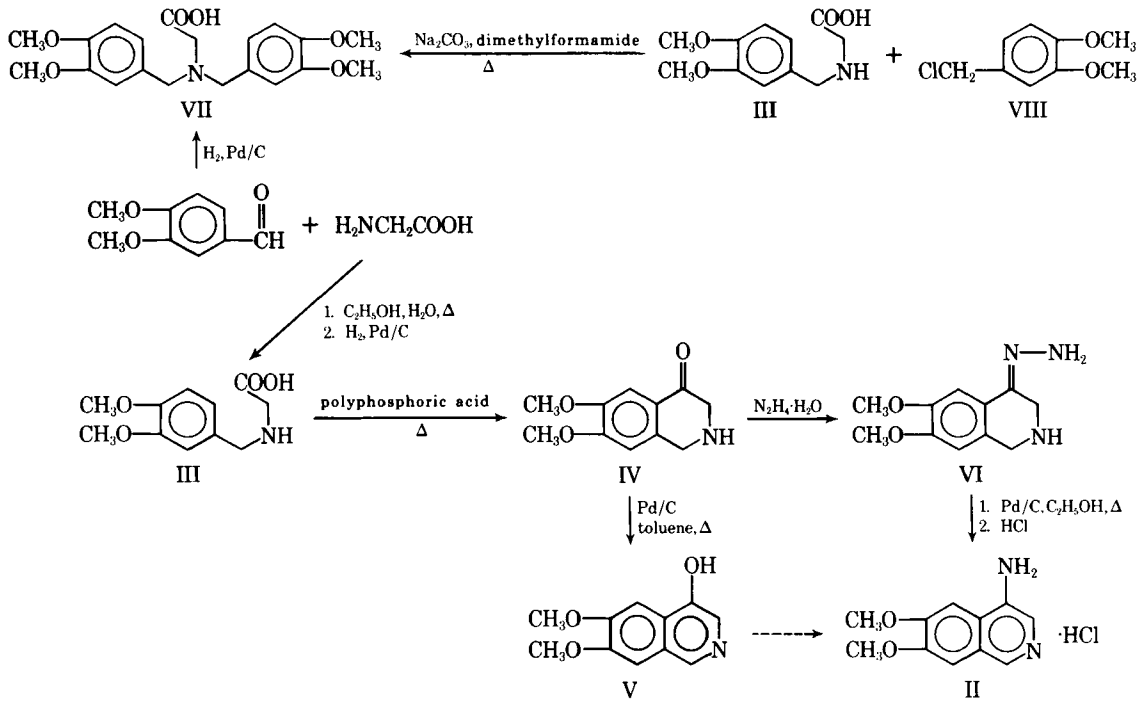
RESULTS AND DISCUSSION

Compounds I and II were evaluated for hypotensive activity in

[3] Melting points were determined on a Fisher-Johns hot-stage apparatus and are uncorrected.

[4] The IR spectrum (Nujol) was determined on a Perkin-Elmer model 137B spectrophotometer.

[5] Determined on a Varian A-60A spectrophotometer with tetramethylsilane as the internal standard.



Scheme I

barbiturate-anesthetized mongrel dogs. Blood pressure was recorded from a cannulated femoral artery. The compounds were administered intravenously in water. At a dose of 5 mg/kg, II showed a maximum blood pressure fall of 47% for 90 min; at 10 mg/kg, it showed a maximum fall of 85% for >210 min. Compound I, at 1 mg/kg, showed a maximum blood pressure fall of 17% for >270 min; at 10 mg/kg, it showed a maximum fall of 51% for >270 min.

The compounds were further evaluated for antihypertensive activity in renal hypertensive dogs (4). Systolic blood pressure was measured by the indirect tail-cuff method (5).

When I and II were administered to unanesthetized, renal hypertensive dogs at a dose of 20 mg/kg perorally, a reduction in systemic arterial pressure was observed. Compound I produced an average decrease in systolic blood pressure of 36% which persisted longer than 6 hr, and II produced an average decrease in systolic pressure of 14% with an average duration of 3 hr.

These findings indicate that II may be equipotent to I with regard to blood pressure lowering in the anesthetized dog when given intravenously but is of shorter duration. When compared in renal hypertensive dogs, II is less active than I with regard to both potency and duration.

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[x] To whom inquiries should be directed.

Alteration of Maternal Behavior in Mice following Administration of Cyproterone Acetate

ELVERA R. ABBATIELLO

Abstract □ Administration of a long-acting progestational, antiandrogenic compound, cyproterone acetate, to mice at randomly selected stages of gestation altered the maternal behavior at the time of parturition. This reversal of behavior resulted in cannibalization of all offspring of treated mothers immediately or shortly after birth. Gross examination of the newborn pups revealed no anatomical malformations, thus eliminating this factor in the mutilation of the offspring by the mothers. Caesarian section of other groups of treated mothers confirmed that the fetuses were normal in size, weight, and appearance. The possibility exists that cyproterone acetate, by altering the hormonal balance of the mother, may cause atypical maternal behavior.

Keyphrases □ Cyproterone acetate—effects on maternal behavior in mice, cannibalism □ Antiandrogenic agents, cyproterone acetate—effect on maternal behavior in mice, cannibalism □ Behavior, maternal—effects of cyproterone acetate in mice □ Cannibalism, drug induced—following administration of cyproterone acetate to pregnant mice

Administration of a long-acting progestational, antiandrogenic compound such as cyproterone acetate to pregnant mice revealed that the male offspring of these treated mothers follow a female pattern of development (1). A number of experiments involving cyproterone acetate treatment of pregnant rabbits demonstrated dose-dependent destruction of the Wolffian ducts and inhibition of the prostate glands and external genitalia in males which were indistinguishable from those in normal females (2).

The mechanism of action of antiandrogenic agents has been explained as a competition for the receptors of androgens at the target organs (3–5). In this manner, the antiandrogen prevents the testicular hormone from exerting its normal effects on the organism without interfering with the secretion of the hormone.

All experiments and documented observations were restricted to the developmental response of the offspring as a result of drug exposure during gestation. The present investigation was undertaken, therefore, to determine the effects of cyproterone acetate on the behavioral response of the mother to her offspring.

EXPERIMENTAL

The test animals were Swiss-Webster mice[1], approximately 65 days of age and weighing between 25 and 30 g. Upon arrival, the female mice were housed individually and the males were housed in groups, allowing them to adjust to their surroundings.

All animals were handled in a similar manner in terms of environmental control, housing, feeding[2], cleaning, and other necessary manipulations. At the end of 1 week, the females were randomly assigned to one of the following groups:

Group 1—Control (50 female mice, no treatment).

Group 2—Placebo (50 female mice divided into five subgroups according to day of treatment).

Group 3—Experimental (50 female mice divided into five subgroups according to day of treatment).

At 75 days of age, the animals were mated by introducing the males into the cages of the females in a 1:2 ratio. Copulation was determined by observing the animals for mating behavior and was confirmed by the presence of a vaginal plug or spermatozoa in the vaginal washings. This time was designated Day 1 of pregnancy.

The days randomly chosen for injections were Days 7, 10, 12, 15, and 17 of gestation. All injections were administered subcutaneously into the right hindleg; each animal within a subgroup received only one injection, and it received that on the day assigned to its group.

Female mice in the experimental subgroups were treated on the designated day with a dose of 0.5 mg cyproterone acetate[3] suspended in 0.25 ml benzyl benzoate[4] plus 50% peanut oil[5]. Female mice in the placebo subgroups were treated on the designated day with 0.25 ml of the vehicle.

Following treatment, the course of pregnancy was permitted to continue undisturbed until the animals came to term. This precaution was necessary since handling of pregnant mice has been shown to affect the behavior of the offspring (6, 7). The mothers delivered their offspring unassisted. As soon as delivery was complete, the offspring were examined for manifestations of gross anatomical malformations and the mother was observed for characteristic signs of maternal behavior (cleaning the pups, carrying them to the nesting area, hovering over them, and nursing them).

RESULTS

The results obtained in two separate experiments using cyproterone acetate are shown in Table I. The second experiment was a replication of the first in terms of methodology and was run to confirm the findings of the initial experiment.

In Experiment 1, all mothers in each of the five treatment groups mutilated and killed their offspring immediately or within 24 hr following delivery. When the mothers were observed during delivery, they appeared to be experiencing no unusual difficulty. However, in all instances when cannibalism did not occur immediately, the mother would push the newborn pup to a corner of the cage and ignore it, making no attempt to clean it. Neither did any of the treated mothers carry the surviving offspring to a nesting corner nor attempt to nurse them. Within 24 hr, all surviving offspring in the first experiment and most offspring in the second experiment were cannibalized by the mothers.

In those cases where the animals were not immediately cannibalized, they were able to be examined for gross anatomical malformations. None was observed in any offspring, although they were subsequently destroyed by the mothers.

The results obtained in the experimental animals were in contrast to those obtained in both the placebo and control groups where the percentage of cannibalism was less than 10.

By using smaller groups of pregnant mice and injecting them randomly with a single dose of cyproterone acetate (Days 7, 10, 12, 15, and 17 of gestation), an attempt was made to confirm that the fetuses, just prior to delivery, were alive and fully developed. Caesarian section was performed on Day 19 of gestation. The results are summarized in Table II.

[1] Camm Research Institute, Wayne, N.J.
[2] Standard laboratory animal diet, Rockland Mouse/Rat Diet (Complete), Tekland, Inc., Monmouth, Ill.
[3] Berlin Laboratories, Division of Schering, Bloomfield, N.J.
[4] Eastman Organic Chemicals, Rochester, N.Y.
[5] Magnus, Mabee and Reynard, Inc., New York, N.Y.

Table I—Effect of a Single Dose of Cyproterone Acetate on Maternal Behavior

Group	Litter Size, Mean ± *SD*	Percent Offspring Killed at Birth, Mean ± *SD*	Length of Gestation, days
Control, 50 mothers	8.2 ± 1.5	8.0 ± 1.4	20–21
Placebo (Days 7, 10, 12, 15, and 17), 50 mothers	8.3 ± 1.8	6.4 ± 1.5	20–21
Cyproterone acetate, 0.5 mg/animal			
Experiment 1 (Days 7, 10, 12, 15, and 17), 50 mothers	7.7 ± 2.1	100.0 ± 0	21–22
Experiment 2 (treatment as in Experiment 1), 50 mothers	8.1 ± 1.8	94.4 ± 1.2	21–22

Table II—Comparison of Fetal Viability, Fetal Mortality, and Litter Size in Treated *versus* Untreated Mice[a]

Group	Number of Pregnant Mice	Percent Fetal Mortality, Mean ± *SD*	Litter Size, Mean ± *SD*
Control	15	3.2 ± 1.1	12.2 ± 3.2
Placebo (Days 7, 10, 12, 15, and 17), total	15	2.4 ± 1.0	12.5 ± 2.1
Cyproterone acetate (Days 7, 10, 12, 15, and 17), total	15	2.9 ± 1.8	12.3 ± 3.6

[a] Caesarian section performed on Day 19 of gestation.

No gross malformations were noted in any of the fetuses, whether alive or dead, except that in most cases the dead fetus was smaller in size. In each major group, there was also at least one incompletely formed fetus, possibly due to death during the earlier stages of development. In all instances, the findings were consistent and not unusual.

DISCUSSION

The fact that cyproterone acetate reversed the maternal behavior of female mice to such an extent that all offspring were destroyed by the mothers at birth is indeed significant. Cyproterone acetate is a progestational, antiandrogenic compound which has been investigated in rats at a much higher dosage than that employed in this study. The action of cyproterone acetate is one of competitive inhibition with testosterone in peripheral tissues, and it has little effect on testosterone uptake by neural tissues (8).

If one were to postulate that increased amounts of testosterone are secreted during pregnancy, then cyproterone acetate could raise the blood titer of testosterone by inhibiting its uptake in peripheral tissues. Since cyproterone acetate has a prolonged duration of action and since neural uptake of testosterone is not inhibited, the possibility presents itself that increased amounts of testosterone entering the brain tissue might account for the alteration in maternal behavior.

Estrogen and prolactin interaction have also been implicated in the maternal behavior of mice toward their newborn pups (9). Administration of progesterone several days before delivery was believed to be responsible for altering the balance of these hormones, thereby resulting in destruction of 50% of the litters by the mothers. Since cyproterone acetate is a progestational compound, it is conceivable that the cannibalistic behavior observed in the mothers following treatment with cyproterone acetate was a result of some drug-induced hormonal imbalance.

There are, of course, other possible hypotheses to account for this type of behavior; however, further investigations are necessary to clarify the underlying mechanism of this particular aspect of abnormal behavior.

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Phytochemical Screening of *Cannabis sativa* L. I: Constituents of an Indian Variant

M. LEONARD MOLE, Jr., and CARLTON E. TURNER[x]

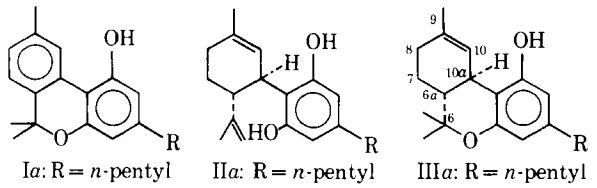
Abstract □ Δ^9-*trans*-Tetrahydrocannabivarin, a mixture of sterols (campesterol, stigmasterol, and β-sitosterol), and the amino acid L-proline were isolated from an Indian variant of *Cannabis sativa* L. Characterizations were accomplished by the usual spectral methods, except for the sterols which were subjected to GLC–mass spectral analysis.

Keyphrases □ Tetrahydrocannabivarin—isolation and characterization from *Cannabis sativa* L. (Indian variant) □ Sterols (campesterol, stigmasterol, and β-sitosterol)—isolation and characterization from *Cannabis sativa* L. (Indian variant) □ L-Proline—isolation and characterization from *Cannabis sativa* L. (Indian variant)

Until the 1940's, the chemistry of *Cannabis sativa* L. remained relatively unexplored and only in the 1960's, with newly accessible modern separation techniques and instrumentation, was major structural work on the cannabinoids accomplished (1). Two groups of workers independently reported confirmation of the structure of the first cannabinoid, cannabinol (I*a*) (2, 3).

However, the structure of cannabidiol (II*a*), the second cannabinoid to be fully characterized, remained obscure until 1963 when the double bond in the terpene moiety was shown (4) to be between carbon atoms 9 and 10 and not between 7 and 8 as previously thought (5, 6).

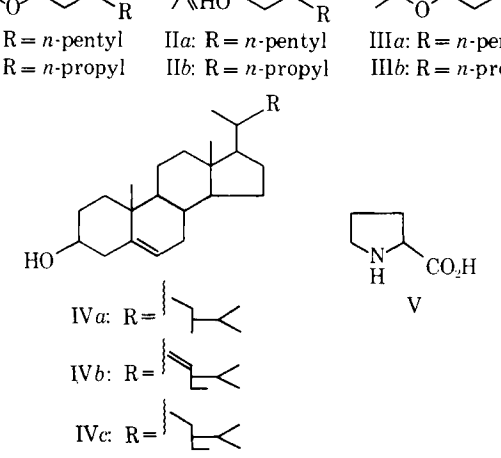
Since that time, about 20 cannabinoids have been isolated and the syntheses of most have been accomplished. Included in this group is the constituent considered to be principally responsible for the psychotomimetic activity of marijuana, (−)-Δ^9-*trans*-tetrahydrocannabinol (III*a*). Also among those isolated are the propyl homologs (*i.e.*, a propyl side chain instead of pentyl) including tetrahydrocannabivarin (III*b*) (7, 8), cannabivarin (I*b*) (8), and cannabidivarin (II*b*) (9).



I*a*: R = *n*-pentyl
I*b*: R = *n*-propyl

II*a*: R = *n*-pentyl
II*b*: R = *n*-propyl

III*a*: R = *n*-pentyl
III*b*: R = *n*-propyl



V

The pharmacological significance of tetrahydrocannabivarin, although only one-fifth as active as III*a* in a catalepsy test on mice (7), has yet to be fully evaluated when taken by humans in the crude state, *e.g.*, smoking marijuana. Gill (7) also reported its effects to be more rapid in onset and decay than III*a* when tested on isolated organs.

In an earlier article from these laboratories (10), significant amounts of tetrahydrocannabivarin and cannabivarin were reported to be present in an Indian variant[1] of *C. sativa* L. It was felt that the isolation of one or both of these constituents would be worthwhile for the following reasons: (*a*) earlier workers had used tinctures or extracts of hashish of generally unknown origin (7–9), (*b*) a supply of III*b* for pharmacological testing could be obtained, and (*c*) confirmation of earlier work might be achieved. The results of these efforts are reported, as well as the isolation of sterols and the amino acid L-proline.

EXPERIMENTAL[2]

Indian plant material, small stems, leaves, and flowering tops which passed through a 14-mesh sieve, was used. Material coded CIX-71, 220 g, and material coded CIF-71, 910 g, were combined and extracted with 95% ethanol for 3 days. The extract was concentrated at aspirator pressure and finally *in vacuo* overnight. The final weight of the dark residue was 106 g. This thick mass was triturated three times with 200-ml portions of hexane. The hexane-soluble portion was concentrated, leaving 55 g of dark gum.

Isolation of Tetrahydrocannabivarin (III*b*)—The cannabinoid-rich gum (hexane soluble), 40 g, was dissolved in 250 ml of hexane, and 175 g of silica gel was added to this solution with swirling. The hexane was evaporated *in vacuo* and the residue was pulverized in a mortar. This somewhat free-flowing material was placed in three equal portions on top of three previously packed columns containing approximately 450 g of silica gel each. The final dimensions were 44 mm × 55 cm. One-liter fractions were taken from each column; combinations were made using GLC analysis. Elution was as follows: fractions 1–15, hexane; 16–

[1] This variant was grown at the University of Mississippi from seed stock produced in the garden. Seeds were obtained originally by Professor Norman J. Doorenbos from Dr. C. K. Atal of the Regional Research Laboratory, Fammutawi, India. Voucher specimens are located in the *C. sativa* L. Herbarium of the School of Pharmacy, University of Mississippi.

[2] GLC analyses were performed using the Beckman GC-45 and GC-72-5 equipped with flame ionization. Glass columns were packed with 2% OV-17 on Chrom "WHP." The inlet temperature was 230°, the column temperature was 210°, and the detector temperature was 250°. Nitrogen was used as the carrier gas at a flow rate of 15 ml/min. IR spectra were taken either on a Beckman IR 33 or a Perkin-Elmer 257 spectrophotometer. UV spectra were taken on a Beckman Acta III. A Dupont 21-492 mass spectrometer was used to obtain mass spectra, and NMR spectra were taken on a Jeol C-60HL. Optical rotations were taken on a Perkin-Elmer 141 polarimeter. Melting points are uncorrected. Column chromatography was performed using Grace grade 923 silica gel, 100–120 mesh, as adsorbent (Davison Chemical, Baltimore, Md.).

18, hexane–benzene (9:1); 19–22, hexane–benzene (4:1); 23–33, hexane–benzene (3:2); 34–44, benzene; 45–49, benzene–chloroform (9:1); 50–53, benzene–chloroform (4:1); 54–56, benzene–chloroform (3:2); 57–59, chloroform; and 60–63, chloroform–methanol (9:1). Solvents were recycled except where solvent system changes were made that would have led to an unwanted system [such as in changing from hexane–benzene (3:2) to benzene].

TLC and GLC analyses of fractions 33 and 34 (2.20 g) indicated that they consisted of one major component (III*b*) and two minor components (I*b* and II*b*). Further purification was accomplished on a column of silica gel impregnated with silver nitrate (20% of total weight). A column, 25 mm × 30 cm, was prepared with 90 g of the adsorbent. Elution collecting 200-ml fractions was as follows: fractions 1–7, benzene; 8–9, ether; and 10, ether–methanol (9:1). Fraction 1, 1.38 g containing 78% III*b* by GLC analysis, was subjected to another chromatography on 59 g of 20% silver nitrate-impregnated silica gel. The column measured 25 mm × 18 cm and elution, taking 150-ml cuts, was as follows: fractions 1–3, hexane; 4–6, hexane–benzene (99:1); 7–9, hexane–benzene (95:5); 10–12, hexane–benzene (9:1); 13–18, hexane–benzene (3:1); 19–21, hexane–benzene (1:1); 22–26, benzene; and 27–28, ether.

Fraction 22, after evaporation of solvent, yielded 250 mg of a colorless oil which darkened rapidly. It was shown by GLC analysis to contain 94% III*b* (relative retention time of 0.28 with 4-androstene-3,17-dione as internal standard). NMR ($CDCl_3$): δ 0.92 (t, J = 7 Hz, aliphatic CH_3), 1.12 and 1.47 (gem-CH_3's), 1.75 (olefinic CH_3), 2.48 (t, J = 8 Hz, C-1′ H's), 3.30 brd, J = 10 Hz, C-10H), 5.56 (s, OH), 6.30 (d, J = 2 Hz, aromatic H), 6.46 (d, J = 2 Hz, aromatic H), and 6.53 (m, C-10H); mass spectroscopy (70 ev, inlet temperature 185°): m/e M^+ 286 (100%), 271 (74), 258 (6), 243 (53), 218 (7.5), 203 (48), and 165 (18), and m^* 257, 206.8, and 189.1; IR (sodium chloride plates): 3410, 2945, 1635, 1590, 1520, 1435, 1190, 1040, 910, and 730 cm^{-1}; $[\alpha]_D^{24}$ −143° (c 1.08 in carbon tetrachloride): [lit. (7) $[\alpha]_D^{20}$ −128° (c 1.0 in carbon tetrachloride)]; λ_{max} (methanol): 279 nm (log ϵ 3.51).

Isolation of a Mixture of Sterols [Campesterol (IV*a*), Stigmasterol (IV*b*), and Sitosterol (IV*c*)]—Fractions 49 and 50 of the first chromatography described were combined (420 mg) and recrystallized three times from ethanol, yielding white crystals (50 mg), mp 134–135°. The NMR spectrum was typical of sterols. TLC showed only one spot, the R_f value of which corresponded to that of cholesterol[3]. NMR ($CDCl_3$): δ 0.70–1.02 (CH_3's) and 5.43 (olefinic H's); IR: 3430, 3320, 3020, 2930, 1640, and 1463 cm^{-1}; GLC–mass spectral data[4]: peak 1 (campesterol), M^+ 400 (31), 382 (91), 367 (32), 281 (11), 207 (34), and 43 (100); peak 2 (stigmasterol), M^+ 412 (20), 394 (52), 351 (17), 255 (61), 133 (42), and 55 (100); peak 3 (β-sitosterol), M^+ 414 (28), 406 (49), 381 (19), 255 (25), and 43 (100).

Isolation of L-Proline (V)—The hexane-insoluble portion, 51 g, of the plant extract was dissolved in chloroform and filtered. The filtrate was extracted with 5% HCl, and the acid extract was made basic with concentrated ammonium hydroxide. This basic solution was concentrated to a paste under vacuum, which was dissolved in 100 ml of methanol and filtered. To the filtrate was added 100 ml of chloroform, and the precipitate was filtered. Concentration of the filtrate left 16.7 g of brown crystalline material, which was dissolved in 20 ml of methanol and chromatographed on 500 g of silica gel (44 mm × 55 cm). Elution was as follows: fractions 1–4, approximately 400 ml of methanol each, and 5–6, approximately 700 ml of ethanol–ammonium hydroxide (9:1) each.

Fraction 5, 125 mg, slowly crystallized; however, an attempt to recrystallize this fraction from methanol–chloroform resulted in an oil separating out. The supernate was decanted and allowed to evaporate slowly, leaving a mixture of oil and white crystals. The crystals were quite soluble in chloroform whereas the oil was not; therefore, separation was achieved. Evaporating the chloroform left 70 mg of an oil which was now insoluble in chloroform. This oil was partitioned between chloroform and water. Evaporation of water left 67 mg of crystalline solid. Three recrystallizations from ethanol–acetone afforded 18 mg of slightly colored needles, mp 220° (instant change), 233–234° dec. (melt) (apparatus preheated to 220°), no depression on admixture with authentic L-proline (mp 233–235°); IR: 3415, 2975, 1616, 1560, and 1375 cm^{-2}; NMR (D_2O): δ 4.73 (t, J = 7.5 Hz, 1H, N—$CHCO_2$), 3.93 (t, J = 6 Hz, 2H, N—CH_2), and 2.62 (m, 4H, CH_2CH_2); $[\alpha]_D^{24.5}$ −71.1° (c 0.95 in water) [lit. (11) $[\alpha]_D$ −85.0 (c 1.0)]; mass spectroscopy (70 ev, inlet temperature 252°): m/e M^+ 115 (3), 87 (2), 70 (100), 43 (19), and 41 (14)[5].

RESULTS AND DISCUSSION

(−)-Δ^9-*trans*-Tetrahydrocannabivarin (III*b*) was isolated from an Indian variant of *C. sativa* L. and purified by extensive column chromatography. To the knowledge of the authors, this represents the first isolation of this cannabinoid from plant material of confirmed origin. Physical data agree well with those published (7, 8). The mass spectrum shows fragmentations that are expected by analogy with those reported for the pentyl homolog (12–14). These fragmentations were discussed in detail previously (13, 14). In addition, the NMR spectrum agreed well with published data, the outstanding feature being the highly deshielded vinyl proton (C-10), δ 6.54. This is quite characteristic of the 9,10-double bond, as discussed previously (15) in reference to III*a*.

There were, however, some minor discrepancies in the IR spectrum when compared with that previously observed (7). It was reported that the IR spectra of III*a* and III*b* were essentially the same, except for the presence of a band at 2950 cm^{-1} in III*a* and some difference in the relative intensities of the two portions of the doublet at 1038 and 1048 cm^{-1}. By contrast, in the present study the spectra of the two compared well except for a more intense band for III*a* at 2950 cm^{-1} and the presence of two bands for III*b* of medium intensity at 910 and 730 cm^{-1}. Perhaps these discrepancies can be attributed to the different phases used when obtaining the spectra.

Additional column chromatography after III*b* was isolated afforded fractions rich in sterols. Constant melting material was obtained by recrystallization; however, GLC analysis indicated a mixture. Further analysis by GLC–mass spectrometry revealed three components whose mass spectra matched well with those corresponding to campesterol (IV*a*), stigmasterol (IV*b*), and β-sitosterol (IV*c*). The latter two are probably the most commonly occurring sterols in higher plants. To the authors' knowledge, this is the first report of sterols having been isolated from *C. sativa* L. of known origin grown under controlled conditions. Fenselau and Hermann (16) reported isolation of a mixture of these three sterols, which were characterized by GLC–mass spectroscopy, from red oil extract of Yugoslavian *Cannabis*.

Column chromatography of the polar portion of the extract yielded L-proline (V), which was purified in the somewhat unorthodox manner discussed previously. Assignment of structure was based on the mass spectrum which showed the base peak, m/e 70 (M − 45), characteristic of L-proline (17, 18). Likewise, the IR and NMR spectra matched those of an authentic sample. Even though this commonly occurring amino acid would be expected to be found in *C. sativa* L., this is the first report of its having been isolated[6].

SUMMARY

Chemical screening of an Indian variant of *C. sativa* L. resulted in the isolation of Δ^9-*trans*-tetrahydrocannabivarin, three sterols (campesterol, stigmasterol, and β-sitosterol), and the amino acid L-proline. To the authors' knowledge, this is the first report of these compounds having been isolated from marijuana plant material of known origin.

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[3] Silica gel with two solvent systems: ethyl acetate and chloroform–benzene (3:2).

[4] The authors thank Dr. Paul Schiff, Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh, for the GLC–mass spectroscopic analysis and his helpful comments.

[5] The authors thank Dr. Roger Foltz and Dr. Al Fentiman, Battelle Memorial Institute, Columbus, Ohio, for a mass spectrum and suggestions.

[6] Lousberg and Salemink (19) reported the composition of hydrolyzed amino acids in *C. sativa* L. determined with an amino acid analyzer.

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[x]To whom inquiries should be directed.

Sulfide, Disulfide, and Sulfone Derivatives of 2-Phenylcinchoninic Acid as Antimalarial Congeners

CHARLES I. SMITH[x] and PILAN LIN HSU

Abstract □ The diethyl ester of bis[6-(2-phenylcinchoninic acid)] sulfone, the corresponding sulfide and disulfide derivatives, and the disulfide acid were prepared. The results of chemotherapeutic testing for antimalarial activity are reported.

Keyphrases □ Bis[6-(2-phenylcinchoninic acid)] sulfone diethyl ester and sulfide and disulfide analogs—synthesized and screened as potential antimalarial agents □ Antimalarial agents, potential —synthesis and screening of bis[6-(2-phenylcinchoninic acid)] sulfone diethyl ester and sulfide and disulfide analogs □ Sulfones, aryl—synthesis and screening of bis[6-(2-phenylcinchoninic acid)] sulfone diethyl ester as a potential antimalarial agent

Elslager *et al.* (1) reported the relationship between chemical structure and antimalarial activity in a series of compounds related to 4,4′-sulfonyldianiline (I), also known as DDS and dapsone. Other investigators reported the synthesis and testing of a variety of sulfones with varying degrees of activity as antimalarial agents (2, 3).

As part of an effort directed toward the synthesis of potential antimalarial agents, it was decided to prepare bis[6-(2-phenylcinchoninic acid)] sulfone diethyl ester (II) as an analog of dapsone. Several structurally related compounds, bis[6-(2-phenylcinchoninic acid)] sulfide diethyl ester (III), bis[6-(2-phenylcinchoninic acid)] disulfide (IV), and bis[6-(2-phenylcinchoninic acid)] disulfide diethyl ester (V), were also prepared for possible structure–activity comparisons.

The synthetic approach to III and IV started with the condensation of the appropriately substituted amines, 4,4′-thiodianiline and *p*-aminophenyl disulfide, respectively, with benzaldehyde and pyruvic acid *via* the Doebner reaction (4). The intermediate acids were converted to the esters, III and V, by a standard procedure (5) for the preparation of aromatic esters. The sulfone was prepared by dichromate oxidation (6) of the corresponding sulfide.

Chemotherapeutic screening of these substances as potential antimalarial agents disclosed no significant antimalarial activity and no detectable host toxicity under the test conditions.

EXPERIMENTAL[1]

4,4′-Thiodianiline[2] and *p*-aminophenyl disulfide, prepared according to the method of Price and Stacy (7), were used.

Bis[6-(2-phenylcinchoninic Acid)] Sulfide Diethyl Ester (III) —To a stirred solution of benzaldehyde (21.2 g, 0.200 mole) in warm ethanol (80 ml), 4,4′-thiodianiline (21.6 g, 0.100 mole) was added rapidly and the resulting mixture was heated at reflux for 10 min. A solution of redistilled pyruvic acid (17.6 g, 0.200 mole) in ethanol (18 ml) was added dropwise over 30 min. The resulting mixture was refluxed and stirred continuously for an additional 6 hr, cooled to room temperature, and stirred overnight. After

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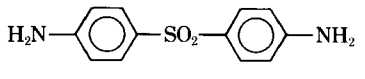

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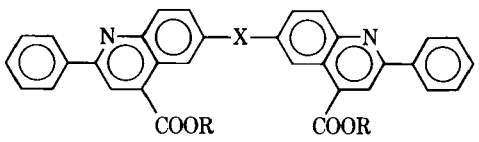

II: X = SO_2, R = C_2H_5

III: X = S, R = C_2H_5

IV: X = S—S, R = H

V: X = S—S, R = C_2H_5

evaporation of the ethanol, the residue was stirred thoroughly with a hot solution of sodium carbonate (10.6 g in 200 ml of water) and the hot slurry was filtered. The residue was treated twice more in a similar fashion with half of the previous quantity of hot sodium carbonate solution and subsequent filtrations. The combined filtrates were acidified with a slight excess of acetic acid, and the resulting precipitate was filtered and dried in air. The precipitated crude acid (30 g), ethanol (1200 ml), and concentrated sulfuric acid (32 ml) were refluxed for 10 hr. The ethanol was evaporated and the residue was diluted with water and filtered. The resulting crude ester was dissolved in ether, and the ethereal solution was washed twice with a saturated solution of sodium carbonate. The ethereal solution was evaporated, and the residue was dissolved in a minimum amount of ethyl acetate. This solution was placed on a silica gel[3] column and developed with cyclohexane–ethyl acetate (2:1) as eluant. Fractions judged similar by TLC were combined and evaporated to dryness. The residue containing the major component was recrystallized from ethanol to obtain 2.5 g (4.3%) of yellow crystals, mp 147–149.5°.

Anal.—Calc. for $C_{36}H_{28}N_2O_4S$: C, 73.95; H, 4.83; N, 4.79; S, 5.48. Found: C, 74.04; H, 4.81; N, 4.88; S, 5.78.

Bis[6-(2-phenylcinchoninic Acid)] Disulfide (IV)—This acid was prepared in a similar fashion from benzaldehyde (16.1 g, 0.152 mole), *p*-aminophenyl disulfide (19 g, 0.076 mole), and redistilled pyruvic acid (13.7 g, 0.156 mole). The crude acid, obtained by acidification of the carbonate extracts was recrystallized from dioxane to yield 3 g (7%) of a yellow solid, mp 287–289°.

Anal.—Calc. for $C_{32}H_{20}N_2O_4S_2$: C, 68.56; H, 3.60; N, 5.00; S, 11.44. Found: C, 68.57; H, 3.57; N, 4.88; S, 11.32.

Bis[6-(2-phenylcinchoninic Acid)] Disulfide Diethyl Ester (V)—A mixture of IV (1.8 g, 0.0032 mole), ethanol (75 ml), and concentrated sulfuric acid (2 ml) was heated under reflux for 10 hr and then concentrated to about 10 ml under reduced pressure. The ester was precipitated by the addition of concentrated ammonium hydroxide (10 ml) and water (10 ml), filtered, dried, and recrystallized from ethanol to yield 0.47 g (24%) of V as yellow crystals, mp 138–140°.

Anal.—Calc. for $C_{36}H_{28}N_2O_4S_2$: C, 70.11; H, 4.58; N, 4.54; S, 10.40. Found: C, 70.15; H, 4.58; N, 4.48; S, 10.15.

Bis[6-(2-phenylcinchoninic Acid)] Sulfone Diethyl Ester (II)—A cooled (15°) mixture of potassium dichromate (0.35 g), water (5 ml), and concentrated sulfuric acid (32 ml) was added slowly to a solution of III (0.58 g, 0.0010 mole), kept below 20° by external cooling, in acetic acid (5 ml, initially warmed to dissolve the ester). After the addition was completed, the reaction mixture was allowed to stand at room temperature for 90 min and then was poured into ice water (55 ml) with stirring, filtered, and washed with water until free of acid. The precipitate was recrystallized from ethanol to obtain 0.24 g (39%) of II, mp 205–207°.

Anal.—Calc. for $C_{36}H_{28}N_2O_6S$: C, 70.12; H, 4.58; N, 4.54; S, 5.20. Found: C, 70.07; H, 4.61; N, 4.72; S, 5.14.

Antimalarial Testing—Compounds II–V were screened for antimalarial activity and toxicity against *Plasmodium berghei* in mice and against *Plasmodium gallinaceum*, Strain B, in chicks according to standard procedures[4]. Results indicated that these compounds lacked noteworthy antimalarial activity and also toxicity to the hosts in doses up to 640 mg/kg in mice and in the range of 100–160 mg/kg in chicks.

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[x] To whom inquiries should be directed.

[3] SilicAR, CC-7, 200-325 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.

[4] Antimalarial testing, through the courtesy of Dr. Thomas R. Sweeney, Walter Reed Army Institute of Research, Washington, D.C., was performed at the University of Miami by previously published methods (8).

New Compounds: Synthesis of *p*-Dialkylaminoalkoxybenzoxylidides as Possible Local Anesthetics

CARLOS RODRIGUEZ-SALAZAR

Abstract □ *p*-Dialkylaminoalkoxybenzoxylidides were prepared by two methods. In the first, the potassium salt of *p*-hydroxy-2′,6′-benzoxylidide was allowed to react with dimethylaminoethyl chloride. In the second method, the sodium salt of methyl *p*-hydroxybenzoate was allowed to react with an excess of 1,2-dibromoethane; after hydrolysis, the resulting *p*-(2-bromoethoxy)benzoic acid was transformed into the acid chloride and then condensed with 2,6-dimethylaniline. Finally, the xylidide obtained was condensed with diethylamine.

Keyphrases □ *p*-(2-Dimethylaminoethoxy)-2′,6′-benzoxylidide—synthesized as possible local anesthetic □ *p*-(2-Diethylaminoethoxy)-2′,6′-benzoxylidide—synthesized as possible local anesthetic □ Anesthetics, local, potential—synthesis of *p*-dialkylaminoalkoxybenzoxylidides

Some *p*-dialkylaminoalkoxybenzoic esters possess local anesthetic activity (1–3). In the present investigation, Compounds I and II, in which the ester group has been replaced by a xylidide group, were prepared.

EXPERIMENTAL[1]

Method A—p-*Acetoxy-2′,6′-benzoxylidide*—Ninety grams (0.5 mole) of *p*-acetoxybenzoic acid was treated with 120 g (1 mole) of thionyl chloride. The acid chloride formed was condensed with 60.5 g (0.5 mole) of 2,6-dimethylaniline according to the method of Lofgren and Lundquist (4). The acetoxybenzoxylidide formed was filtered, washed with water, and recrystallized (ethanol–water), yielding 92%, mp 168–169°.

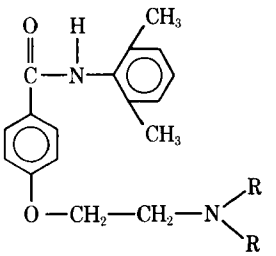
Anal.—Calc. for $C_{17}H_{17}NO_3$: C, 72.08; H, 6.00; N, 4.94. Found: C, 72.01; H, 6.15; N, 4.83.

p-*Hydroxy-2′,6′-benzoxylidide*—A mixture of 113 g (0.4 mole) of *p*-acetoxybenzoxylidide in ethanol (500 ml) and 16 g (0.4 mole) of sodium hydroxide in water (100 ml) was refluxed for 5 hr and then neutralized. The excess solvent was removed, and the solid residue was washed with water and recrystallized (ethanol–water), yielding 95%, mp 225–228°.

Anal.—Calc. for $C_{15}H_{15}NO_2$: C, 74.68; H, 6.22; N, 5.80. Found: C, 74.55; H, 6.27; N, 5.75.

p-*(2-Dimethylaminoethoxy)-2′,6′-benzoxylidide* — Twenty-four grams (0.1 mole) of *p*-hydroxybenzoxylidide was treated with 5.6 g (0.1 mole) of potassium hydroxide in methanol (200 ml), followed by 11.8 g (0.11 mole) of β-dimethylaminoethyl chloride. The mixture was refluxed for 6 hr and the excess solvent was evaporated. The resulting solid was washed with water and dissolved in dilute hydrochloric acid. The solution was treated with carbon and made alkaline with sodium hydroxide. The solid formed was washed with water and recrystallized (ethanol–water), yielding 63%, mp 147–148°.

Anal.—Calc. for $C_{19}H_{24}N_2O_2$: C, 73.07; H, 7.69; N, 8.97.



I: R = CH_3
II: R = C_2H_5

Found: C, 73.16; H, 7.57; N, 8.96.

Method B—p-*(2-Bromoethoxy)benzoic Acid*—This compound was prepared from methyl *p*-hydroxybenzoate and ethylene bromide according to the method of Caldwell and Jackson (5), mp 176–180°.

p-*(2-Bromoethoxy)-2′,6′-benzoxylidide*—Seventy grams (0.285 mole) of *p*-(2-bromoethoxy)benzoic acid in 200 ml of benzene was treated with 66 g (0.54 mole) of thionyl chloride, and the mixture was heated under reflux for 4 hr. Removal of the excess thionyl chloride and solvent under reduced pressure and treatment of the residual acid chloride with 2,6-dimethylaniline gave 79 g of solid, mp 204–206°.

Anal.—Calc. for $C_{17}H_{18}BrNO_2$: C, 58.62; H, 5.17; Br, 22.97; N, 4.02. Found: C, 58.50; H, 5.19; Br, 22.79; N, 3.95.

p-*(2-Diethylaminoethoxy)-2′,6′-benzoxylidide* — Thirty-five grams (0.47 mole) of dimethylamine and 5 g of potassium iodide were added to a solution of 130 g (0.34 mole) of *p*-(2-bromoethoxy)-2′,6′-benzoxylidide in 250 ml of dimethylformamide. The mixture was refluxed for 6 hr; part of the solvent was removed under vacuum and the remaining solvent was added to cold water. After filtering, the solid was dissolved in 10% hydrochloric acid, and the solution was treated with charcoal and then precipitated with sodium hydroxide. The product was crystallized (ethanol–water) to give 100 g (87%) of white crystals, mp 143–145°.

Anal.—Calc. for $C_{21}H_{28}N_2O_2$: C, 74.18; H, 8.24; N, 8.24. Found: C, 74.14; H, 8.13; N, 8.27.

[1] Melting points were determined with a Fisher-Johns block melting-point apparatus and are uncorrected. Elemental analyses were performed by Microanalytical Laboratory, Oxford, England. IR spectra were taken on a Perkin-Elmer model 137 spectrophotometer and were as expected.

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Naltrexone Zinc Tannate: A Prolonged-Action Narcotic Antagonist Complex

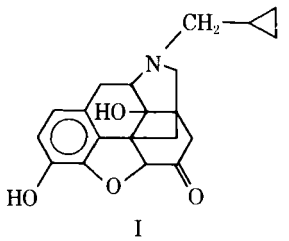
Keyphrases □ Naltrexone zinc tannate—prepared as prolonged-action narcotic antagonist □ Narcotic antagonists—preparation and evaluation of naltrexone zinc tannate □ Drug dependence—preparation and evaluation of naltrexone zinc tannate as prolonged-action narcotic antagonist □ Zinc tannate complex with naltrexone—prepared as prolonged-action narcotic antagonist

Sir:

No fully satisfactory therapeutic regimen is yet at hand for the treatment of narcotic dependence, a major sociomedical problem. Psychotherapy alone may be useful for some drug users but demands strong motivation on the part of the subject and, in any event, would hardly be practicable on a scale adequate to treat the numerous addicts [an estimated 150,000 in New York City alone (1)] currently at large in the United States. Methadone is useful but introduces its own complications since it is basically a substitute for heroin and is itself an addicting drug subject to abuse (1, 2).

A potentially more promising therapeutic approach involves the use of narcotic antagonist drugs. A pure narcotic antagonist has no effects of its own in the dosages used but blocks the effects, including the euphoric effect, of heroin and other narcotics. A drug-dependent subject, first relieved of his or her physical addiction by a brief period of methadone detoxification and then treated with a narcotic antagonist, would not experience a euphoric reaction to heroin. The psychological dependence would not be reinforced and, ideally, time would be available for other treatment modes and social changes to reduce and eventually eliminate dependence on drugs. The difficulty with existing narcotic antagonist drugs is that they are too short acting since, unlike methadone, they provide no incentive to the subject to return for frequent maintenance therapy (1, 3, 4). A need has thus been indicated for injectable narcotic antagonist preparations with a prolonged duration of action (1, 3).

Our approach to this problem has involved development of sparingly soluble salts and salt complexes of existing narcotic antagonists inasmuch as precedents in other therapeutic areas show that intramuscular injection of such form can provide slow release of drug and a useful prolongation of action (5–7). We prepared numerous complexes of the antagonists naloxone and cyclazocine (8), evaluated their duration of action in mice, and found zinc tannate complexes to provide the most significant prolongation of effect (8). Naloxone zinc tannate showed a prolongation of action much greater than that shown by naloxone pamoate (8, 9)[1,2].



I

Since naltrexone (I), a close analog of naloxone, has been reported to be essentially as pure an antagonist as is naloxone and to be two to three times as potent with about twice the duration (9), we then prepared complexes of this drug. We found naltrexone zinc tannate to provide a highly promising increase in duration of action in mice. Duration was further enhanced when the complex was incorporated in an aluminum monostearate gel.

Our methods were essentially as described earlier (8). Naltrexone tannate was prepared by stirring an aqueous solution of naltrexone hydrochloride[3] with an aqueous solution of an equivalent amount of gallotannic acid [assuming 5 equivalents/mole and an approximate molecular weight of 1700 (10)] just neutralized with 0.2 *N* NaOH. The resultant precipitate of naltrexone tannate was washed with water and dried *in vacuo* over phosphorus pentoxide.

Naltrexone zinc tannate was prepared by stirring an aqueous suspension of the tannate with 1 equivalent of 1 *M* solution of zinc sulfate and washing and drying as before.

To analyze for naltrexone, an aqueous suspension of a weighed amount of complex was treated with a pH 10.4 phosphate buffer, sodium chloride was added, and the pH was adjusted to 9.5–10 with 20% NaOH. The aqueous mixture was extracted with chloroform containing 1% 2-propanol, and the organic extract was shaken with 0.1 *N* HCl. The acid solution was diluted to standard volume, the absorbance at 281 nm was determined with a UV spectrophotometer[4], and naltrexone content was read off a standard curve.

Zinc content was determined by shaking a suspension of a weighed amount of complex in 1 *N* HCl, centrifuging, serially diluting an aliquot of the supernatant solution with distilled water and 0.1 *N* HCl, and measuring the responses of an atomic absorption flame-emission spectrophotometer[5] in comparison with the dose–response of standard zinc solutions (Fisher standard reference solution of zinc oxide in dilute nitric acid, serially diluted with 0.1 *N* HCl).

[1] Naloxone pamoate showed a significant increase in duration of action only when incorporated in an aluminum monostearate gel. We thank Dr. H. Blumberg and Dr. A. Rubin for information on the composition of this gel.

[2] W. R. Martin, NIMH Addiction Research Center, and L. S. Harris and W. L. Dewey, Medical College of Virginia, personal communications.

[3] Endo Laboratories.

[4] Beckman model DU-2.

[5] Jarrell-Ash model 82-270.

Table I—Analytical Data: Amount of Tannic Acid in Complexes Assumed by Difference

Naltrexone Complex	Melting Point	Drug, %		Zinc, %	Mole Ratios		Dissociation, % at 37°[a]
		Calc.	Found		Drug/ Acid	Zinc/ Acid	
Hydrochloride	>250°	90.3	—	—	—	—	100
Tannate	197–199°	50.1	49.4	—	4.9	—	38.2
Zinc tannate	>250°	—	31.3	4.19	2.4	1.7	11.5

[a] Percent dissociation is percent of drug dissociating from complex suspended in isotonic phosphate buffer, pH 7.3.

Table II—Duration of Antagonism for Dose Containing 4.0 or 40.0 mg/kg Naltrexone Base Administered Intramuscularly at Indicated Time before Tests

Dose Containing 4.0 mg/kg Naltrexone Base

Naltrexone Complex	Vehicle	Percent Antagonism[a,b] at: Hours			Days						
		4	8	16	1	2	3	4	5	6	7
Hydrochloride	Peanut oil	100	76	28	0	—	—	—	—	—	—
Tannate	Peanut oil	100	76	37	19	5	—	—	—	—	—
Zinc tannate	Peanut oil	99	94	86	67	31	43	29	7	—	—
Zinc tannate	Aluminum monostearate gel	—	—	—	100	91	72	53	23	29	0

Dose Containing 40.0 mg/kg Naltrexone Base

Naltrexone Complex	Vehicle	Percent Antagonism[a,b] at (Days):											
		1	2	3	5	7	9	12	14	15	17	19	21
Hydrochloride	Peanut oil	39	18	—	—	—	—	—	—	—	—	—	—
Tannate	Peanut oil	84	59	25	—	—	—	—	—	—	—	—	—
Zinc tannate	Peanut oil	100	—	96	39	18	9	—	—	—	—	—	—
Zinc tannate	Aluminum monostearate gel	100	—	94	83	78	86	86	74	73	68	71	39

[a] Each value represents the mean of 12 mice. Each mouse was used only once. [b] Percent antagonism to a standard dose of 20 mg/kg morphine sulfate administered intraperitoneally 30 min before test.

To determine *in vitro* dissociation, a weighed amount of complex calculated to contain 15 mg of naltrexone was suspended in 10 ml isotonic phosphate buffer, pH 7.3 (125 ml of 0.2 *M* KH_2PO_4, 85 ml of 0.2 *N* NaOH, and 2 g NaCl, diluted with distilled water and pH adjusted to 7.3 with additional 0.2 *N* NaOH; final volume 500 ml). The suspension was stirred (magnetic stirrer) for 1 hr in a 37.0 ± 0.1° bath and filtered, and an aliquot of the filtrate was bought to a pH of about 10 with 20% NaOH and treated with an additional 2 g NaCl. The mixture was extracted with chloroform containing 1% 2-propanol, and the naltrexone content was determined spectrophotometrically as before. Analytical and dissociation data are given in Table I.

Antagonism of analgesia was determined by the tail-flick procedure of Harris and Pierson (12) with the mouse substituted for the rat as the test animal (11). Dose–response curves and ED_{80} (antagonism) values were obtained based on measurement of reaction time 40 min after intramuscular administration of naltrexone preparation and 30 min after intraperitoneal administration of 20 mg/kg of morphine sulfate, which was the ED_{80} (analgesia) dose under normal conditions. Surprisingly, naltrexone hydrochloride, tannate, and zinc tannate, administered by intramuscular injection in peanut oil suspension, all showed the same ED_{80} (antagonism) value, *i.e.*, the dose producing 80% antagonism at 40 min contained 0.02 mg/kg naltrexone base.

Duration of antagonist activity was evaluated by dosing mice intramuscularly with naltrexone preparations, challenging groups of these mice at the intervals indicated in Table II with a 20-mg/kg ip dose of morphine sulfate, and testing their reaction time in the tail-flick test 30 min thereafter. Naltrexone preparations were injected in peanut oil suspension and also incorporated in an aluminum monostearate gel. The gel was prepared by slowly heating, with stirring, a mixture of 2 g aluminum monostearate[6] in 100 ml of peanut oil to 125° and then allowing the gel to cool to room temperature[1]. Duration of activity was determined at dose levels calculated to contain 4.0 and 40.0 mg/kg of naltrexone base (Table II). At least 12 mice were used for each determination (at each dose level and time interval), and no mice were used for more than one determination. Thus, mice were not repeatedly exposed to the test procedure; each mouse was exposed only to two control tail flicks and one test tail flick.

It is clear that duration of activity *in vivo* followed percent dissociation *in vitro* (Table I) quite well in this limited series, although there were exceptions in more extensive series of naloxone and cyclazocine complexes (8). Naltrexone tannate showed some in-

[6] Mallinckrodt.

crease in duration relative to the hydrochloride salt, more clearly at 40.0 than at 4.0 mg/kg. The zinc tannate complex in peanut oil showed at least a threefold prolongation of the activity of the hydrochloride, and there was a further roughly threefold prolongation of activity when the zinc tannate was incorporated in the aluminum monostearate gel. Indeed, naltrexone zinc tannate in the gel medium still evidenced 70% antagonism 19 days after injection of the 40.0-mg/kg dose. The mice showed no behavioral or other adverse drug effects even at the 40-mg/kg dose.

This work is continuing. We earlier speculated as to the nature of the zinc tannate complexes (8) and indicated that the metal ion appeared to bond covalently to the same sites as were involved in ionic bonding with drug since the zinc displaced an equivalent amount of drug from the complex and the equivalents ratios remained substantially constant. We recently found[7] that by varying the method of preparation, we can increase the amount of zinc in the complex without further reducing the amount of drug. The resultant complexes show reduced percent dissociation *in vitro* and increased duration of activity *in vivo*. We also prepared analogous complexes containing aluminum in place of zinc which show somewhat enhanced duration of activity.

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Allan P. Gray[x]
*Donald S. Robinson**

Department of Pharmacology
University of Vermont
Burlington, Vt.

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[x]To whom inquiries should be directed. Present address: Chemistry Research Division, ITT Research Institute, Chicago, IL 60616

[7] Unpublished data.

Conversion of Apocodeine to Apomorphine and Norapomorphine in Rats

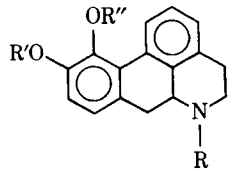
Keyphrases □ Apocodeine—metabolic conversion to apomorphine and norapomorphine, rats □ Apomorphine and norapomorphine—urinary metabolites of apocodeine, rats □ Norapomorphine and apomorphine—urinary metabolites of apocodeine, rats

Sir:

There is renewed and increased interest in the biological activities of aporphine compounds. This has resulted in part from the finding that apomorphine (I) has some usefulness in the treatment of Parkinson's disease (1–8). Apocodeine (II) is an important homolog of I. The relatively poor dopaminergic activity of II (and III) in pigeons and dogs (9, 10) led Cannon *et al.* (10) to theorize that both phenolic groups of I are required to exert central emetic effects. Recently, however, Lal *et al.* (11) reported that II induced stereotyped behavior and caused reversal of reserpine-induced catalepsy in rats similar to that of I. While its activity is described as intermittent, II is clearly one-fourth as active as I (11). Lal *et al.* (11) suggested that these latter observations might be explained by *in vivo* conversion of II to I. Our recent experiments show that I and norapomorphine (IV) are metabolites of II in rats.

Male Sprague–Dawley[1] rats (175–300 g) were injected with II-HCl (30 mg/kg ip); urine, separate from feces, was collected for 48 hr following injections and frozen. The first and second 24-hr samples (4–12 ml) were made up to 12 ml with distilled water; 3 ml of 3 *N* hydrochloric acid was added to each, and the mixtures were heated at 100° for 1 hr. The hydrolysates were adjusted to pH 7.0 with 1 *N* sodium hydroxide and extracted with three 10-ml portions of ethyl acetate. The separators were rinsed with 10 ml of ethyl acetate and the combined extracts were reduced to dryness *in vacuo;* the residues were dissolved in 1–2 ml of acetone for TLC analysis.

Sample and blank urine extracts were spotted on silica gel GF_{254} plates[2] (250 μm) and then developed in the following solvent systems: 1, chloroform–methanol (85:15); 2, acetone; and 3, acetone–methanol (1:1). Detection was *via* quenching of 254 nm-induced fluorescence and visualization of plates allowed to stand in the air for 12–24 hr. Authentic I[3]



I: R = CH_3; R′, R″ = H
II: R, R′ = CH_3; R″ = H
III: R, R′, R″ = CH_3
IV: R, R′, R″ = H

[1] Sprague–Dawley Co., Madison, Wis.
[2] Analtech, Newark, Del.
[3] S. B. Penick, New York, N.Y.

crease in duration relative to the hydrochloride salt, more clearly at 40.0 than at 4.0 mg/kg. The zinc tannate complex in peanut oil showed at least a threefold prolongation of the activity of the hydrochloride, and there was a further roughly threefold prolongation of activity when the zinc tannate was incorporated in the aluminum monostearate gel. Indeed, naltrexone zinc tannate in the gel medium still evidenced 70% antagonism 19 days after injection of the 40.0-mg/kg dose. The mice showed no behavioral or other adverse drug effects even at the 40-mg/kg dose.

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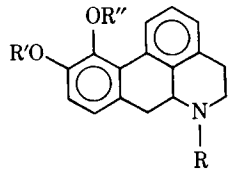
Keyphrases □ Apocodeine—metabolic conversion to apomorphine and norapomorphine, rats □ Apomorphine and norapomorphine—urinary metabolites of apocodeine, rats □ Norapomorphine and apomorphine—urinary metabolites of apocodeine, rats

Sir:

There is renewed and increased interest in the biological activities of aporphine compounds. This has resulted in part from the finding that apomorphine (I) has some usefulness in the treatment of Parkinson's disease (1–8). Apocodeine (II) is an important homolog of I. The relatively poor dopaminergic activity of II (and III) in pigeons and dogs (9, 10) led Cannon *et al.* (10) to theorize that both phenolic groups of I are required to exert central emetic effects. Recently, however, Lal *et al.* (11) reported that II induced stereotyped behavior and caused reversal of reserpine-induced catalepsy in rats similar to that of I. While its activity is described as intermittent, II is clearly one-fourth as active as I (11). Lal *et al.* (11) suggested that these latter observations might be explained by *in vivo* conversion of II to I. Our recent experiments show that I and norapomorphine (IV) are metabolites of II in rats.

Male Sprague–Dawley[1] rats (175–300 g) were injected with II-HCl (30 mg/kg ip); urine, separate from feces, was collected for 48 hr following injections and frozen. The first and second 24-hr samples (4–12 ml) were made up to 12 ml with distilled water; 3 ml of 3 *N* hydrochloric acid was added to each, and the mixtures were heated at 100° for 1 hr. The hydrolysates were adjusted to pH 7.0 with 1 *N* sodium hydroxide and extracted with three 10-ml portions of ethyl acetate. The separators were rinsed with 10 ml of ethyl acetate and the combined extracts were reduced to dryness *in vacuo;* the residues were dissolved in 1–2 ml of acetone for TLC analysis.

Sample and blank urine extracts were spotted on silica gel GF_{254} plates[2] (250 μm) and then developed in the following solvent systems: 1, chloroform–methanol (85:15); 2, acetone; and 3, acetone–methanol (1:1). Detection was *via* quenching of 254 nm-induced fluorescence and visualization of plates allowed to stand in the air for 12–24 hr. Authentic I[3]



I: R = CH_3; R′, R″ = H
II: R, R′ = CH_3; R″ = H
III: R, R′, R″ = CH_3
IV: R, R′, R″ = H

[1] Sprague–Dawley Co., Madison, Wis.
[2] Analtech, Newark, Del.
[3] S. B. Penick, New York, N.Y.

Table I—TLC Mobilities of Authentic Apomorphine and Norapomorphine and Urinary Apocodeine Metabolites

TLC System	$R_f \times 10^2$		
	Authentic		
	Apomorphine	Norapomorphine	Apocodeine Metabolites[a]
Chloroform–methanol (85:15)	33	8	32, 8
Acetone	40	8	40, 8
Acetone–methanol (1:1)	68	43	68, 43

[a] Identified in 12 separate experiments with 12 animals.

Table II—Conversion of Apocodeine to Apomorphine and Norapomorphine in Rats

Metabolite	Percent Conversion[a]	
	Range[b]	Mean
Apomorphine	8.1–11.0	9.6
Norapomorphine	15.8–18.4	17.2
Totals	23.9–29.4	26.8

[a] From separate analyses of 24-hr urines of three rats. [b] Data were corrected for losses due to extraction (13); losses due to hydrolysis were: I, 18% (five experiments); and II, 9% (two experiments).

and IV [prepared according to the literature procedure (9)] yielded blue-green-colored spots, presumably due to formation of *ortho*-quinone-type oxidation products (12). Quantitation of I and IV was by TLC fluorescence quenching (13).

TLC examination of extracts of hydrolyzed urine[4] obtained from animals injected with II-HCl revealed the presence of I and IV (Table I). In addition, these metabolites seemed to be entirely excreted (as their conjugates) during the first 24 hr following injection. Quantitative evaluation of these results gave the data indicated in Table II. Compound II shows about one-fourth the central nervous system activity of I in rats (11), and one-quarter of the dose of II appears to be converted to I and IV. Although the ability of IV to induce stereotyped behavior and reverse reserpine-induced catalepsy in rats does not seem to have been evaluated, the structural similarities of I and IV suggest that IV might have significant activity. This suggestion is somewhat contradicted by the apparent low order of activity of IV causing compulsive gnawing in mice (9).

The observations reported here support the suggestion (11) that the pharmacological activities of II in the rat may be due to its conversion to I and/or IV. The *in vivo* formation of I and IV from II also has interesting and important implications with respect to the design of apomorphine prodrugs and the mechanism of action of I and a number of its analogs.

[4] During preliminary experiments, no free I and IV were detected, as indicated from TLC analysis of ethyl acetate extracts of unhydrolyzed urine.

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Robert V. Smith[x]
Melvin R. Cook

Division of Medicinal Chemistry and Natural Products
College of Pharmacy
University of Iowa
Iowa City, IA 52242

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[x] To whom inquiries should be directed.

1974

January 25–27
American Medical Association National Leadership Conference, Marriott Motor Hotel, Chicago, Ill. (Contact American Medical Association Public Affairs Division, 535 N. Dearborn St., Chicago, IL 60610)

February 5–7
Interdisciplinary National Conference on Biomedical Research Problems in a Changing World, Washington, D.C. (Contact R. Friedenberg, Suite 807, Medi-Science, Inc., 7979 Old Georgetown Road, Bethesda, MD 20014)

February 19
Stephen Wilson Pharmacy Seminar, Wayne State University, Engineering Society, Detroit, Mich.

February 24–March 1
American Association for the Advancement of Science, Annual Meeting, San Francisco, Calif.

February 25–26
Uniform Methods of Clinical Data Collection, Processing, and Analysis Workshop, Drug Information Association, Crystal City Marriott, Arlington, Va.

March 4–8
Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Pittsburgh Section, American Chemical Society, Pittsburgh, Pa.

March 10–14
Society of Toxicology, Washington, D.C. (Contact R. A. Scala, Medical Research Division, Esso Research and Engineering Co., Linden, NJ 07036)

March 23–April 7
American Medical Association International Health Conference, Sydney, Australia (Contact Australian Scientific Conference, American Medical Association, 535 N. Dearborn St., Chicago, IL 60610)

March 24–25
American Society for Clinical Pharmacology and Therapeutics, Maui Surf Hotel, Maui, Hawaii (Contact Elaine Galasso, 1718 Gallagher Rd., Norristown, PA 19401)

March 28–29
American Society for Clinical Pharmacology and Therapeutics, San Francisco Hilton, San Francisco, Calif. (Contact Elaine Galasso, 1718 Gallagher Rd., Norristown, PA 19401)

March 31–April 5
American Chemical Society, National Meeting, Los Angeles, Calif. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

April 3–5
Pharmaceutical Manufacturers Association, Boca Raton Hotel, Boca Raton, Fla.

April 7–12
Federation of American Societies for Experimental Biology, Convention Hall, Atlantic City, N.J.

April 15–18
Pharmacology of Thermoregulation, International Symposium, Paris, France (Contact P. Lomax, Department of Pharmacology, University of California Medical School, Los Angeles, CA 90024)

April 22–26
European Conference on Biochemical and Instrument Analysis, Munich, Germany (Contact Secretary General, Dr. Rosmarie Vogel, D-8000 Muenchen 2, Nussbaumstrasse 20, West Germany)

May 3–4
American College of Clinical Pharmacology, Annual Meeting, Chalfonte-Haddon Hall, Atlantic City, N.J. (Contact Secretary, American College of Clinical Pharmacology, 2 E. 103rd St., New York, NY 10029)

May 12–15
Proprietary Association, Annual Meeting, The Greenbrier, White Sulphur Springs, W. Va.

May 18–22
Association of Faculties of Pharmacy of Canada, Annual Meeting, Ottawa, Canada (Contact F. W. Teare, Faculty of Pharmacy, University of Toronto, Toronto, Ontario, M5S 1A1, Canada)

June 12–14
Annual Summer Symposium on On-Line Analytical Chemistry, Division of Analytical Chemistry, American Chemical Society, Ft. Collins, Colo. (Contact R. A. Hagstrom, Olin Mathieson Chemical Corp., 275 Winchester Ave., New Haven, CT 06504)

June 16–20
National Medicinal Chemistry Symposium, Division of Medicinal Chemistry, American Chemical Society, University of New Hampshire, Durham, N.H. (Contact Dr. Robert E. Lyle, Department of Chemistry, Parsons Hall, University of New Hampshire, Durham, NH 03824)

June 17–20
European Congress on Thermography, The European Thermographic Association and The American Thermographic Society, Amsterdam, The Netherlands (Contact Secretariat, 1st European Congress on Thermography, Organisatic Bureau Amsterdam, N.V., P.O. Box 7205, Amsterdam, The Netherlands)

June 23–27
American Medical Association, Annual Convention, Palmer House, Chicago, Ill.

June 24–26
International Symposium on Mass Spectrometry in Biochemistry and Medicine, Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy (Contact Alberto Frigerio, Istituto di Ricerche Farmacologiche, 20157 Milan-Via Eritrea, 62, Italy)

June 25–28
Canadian Federation of Biological Sciences, Annual Meeting, Hamilton, Ontario, Canada (Contact D. T. Armstrong, Department of Physiology, University of Western Ontario, London 72, Ontario, Canada)

June 26–28
Sources of Drug Information for the Health Professions, Drug Information Association, Annual Meeting, Palmer House, Chicago, Ill.

August 3–9
American Pharmaceutical Association, Annual Meeting, Chicago, Ill.

August 8–13
American Podiatry Association, Annual Meeting, Marriott Motor Hotel, Atlanta, Ga.

September 2–7
General Assembly of the International Pharmaceutical Federation, Rome, Italy

September 8–13
American Chemical Society, National Meeting, Atlantic City, N.J. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

September 8–13
International Conference on Alcohol, Drugs, and Traffic Safety, Toronto, Canada (Contact Dr. W. S. Schmidt, 33 Russell St., Toronto, M5S 2S1, Canada)

September 9–13
International Symposium on Medicinal Chemistry, Medicinal Chemistry Section, Royal Netherlands Chemical Society, Noordwijkerhout, The Netherlands (Contact Secretariat, Merck Sharp and Dohme B. V., External Relations, Waarder-weg 39, P.O. Box 581, Haarlem, The Netherlands)

September 24–27
National Congress of Pharmaceutical Sciences, Hungarian Pharmaceutical Association, Hungarian Academy of Sciences, Budapest, Hungary [Contact Office for Conference Organization (Motesz), H-1361 Budapest, POB 32, Hungary]

October 8–12
American Society of Hospital Pharmacists, Midyear Meeting, Hollywood, Fla.

October 20–24
American Public Health Association, New Orleans, La.

November 10–14
APhA Academy of Pharmaceutical Sciences, National Meeting, Fairmont Hotel, New Orleans, La.

November 10–14
American Dental Association, Annual Meeting, Washington, D.C.

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INDUSTRY'S LAST OPPORTUNITY

Statement by the American Pharmaceutical Association Board of Trustees, January 8, 1974

Several years ago, the American Pharmaceutical Association adopted a policy position which advocated appropriate amendment of state laws in order to restore the pharmacist to his rightful role as the health care practitioner primarily responsible for drug product selection. This action was based upon a number of factors including: *(a)* general recognition that today legal constraints in this area are not serving the purposes for which they were originally enacted, and indeed the original basis has long ceased to exist; *(b)* drug industry marketing, distribution, and pricing policies have been largely skewed to take advantage of and to exploit these state laws and regulations; and *(c)* a general recognition that the pharmacist, more than the physician or any other member of the health care team, is in the best position to judge drug product quality on the basis of his training, experience, and direct contact with the very products involved.

Society was ripe and ready for just such a proposal at the time when APhA introduced it. State and local government agencies, legislative bodies, and consumer groups were only a few of those outside the immediate health care complex that responded with interest and enthusiasm to the position espoused by APhA.

But, alas, most of the drug industry was not prepared to accept this change in what had come to be a very comfortable and generally lucrative system of drug distribution. Virtually all industry advertising and promotion practices had been built up about a marketing system which depended upon the prescriber as the dominant—if not the sole—determiner of both the drug substance to be prescribed, as well as the specific drug product (*i.e.*, manufacturer source) to be dispensed by the pharmacist.

As a consequence, a majority within the pharmaceutical industry made the decision to resist this movement; and vigorously resist it they have. In every forum and field—whether it was professional meetings, journals, conferences, legislative hearings, the public press, smoke-filled rooms, or the lobbies of congress—opposition from the drug industry has been persistent, consistent, and insistent.

And basically, just what is it that they have opposed so vehemently over these past several years? Simply stated, they have fought a transfer from the physician back to the pharmacist of the primary responsibility of comparing, choosing, and selecting a quality drug product at reasonable cost for the patient. What is involved is not an abrogation of responsibility, but a transfer of responsibility from one health professional to another—that is, to the health professional having the best qualifications and training to perform this particular function.

While busily fighting their battles, however, the drug industry is in danger of losing the war.

The public, consumers, and congress appear to have grown even more impatient and frustrated than has pharmacy with the obstructionist tactics that have impeded progress to a more effective, efficient, and economical system of drug distribution.

The Nixon Administration, as disclosed in HEW Secretary Weinberger's recent testimony before the Kennedy Senate Committee, has plans to restrict drug reimbursements under government-administered programs to the lowest cost medications generally available. In essence, the major political forces in both the executive and legislative branches of the federal government now are prepared to abolish all forms of drug product selection whether it be by physician, pharmacist, or government formulary (the latter constituting another frequently made proposal which the drug industry has repeatedly opposed).

Hence, by not accepting drug product selection by the pharmacist as a step forward in orderly and logical progress, the drug industry has spawned a proposal which is less than ideal to pharmacy, to medicine, and, in our opinion, even to the public. At this writing, the precise HEW proposal has not yet appeared in the *Federal Register*. Therefore, it is impossible to comment upon specific points, and, in fact, APhA itself may well find that certain provisions in the HEW proposal conflict with Association policy position, necessitating the filing of objections regarding any such aspects. However, while pharmacy may be unhappy with certain approaches in the HEW plan on professional grounds, it appears to us that from industry's economic viewpoint, this latest turn of events means that, despite the skirmishes they may have won, the industry's entire marketing system now teeters on the brink of total disintegration.

"On the brink" we say, because there still appears to be an opportunity for industry to reverse its position, to support constructive approaches to drug product selection, and to adopt positions which foster both quality and economy in the drug supply. But time is rapidly running out. The industry will need to respond as vigorously and quickly in this positive approach, as it has during the past few years in mobilizing its battle plan of opposition.

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REVIEW ARTICLE

Camptothecin

MAURICE SHAMMA[x] and V. St. GEORGIEV

Keyphrases □ Camptothecin—structure elucidation, synthesis, pharmacology □ Alkaloids, pentacyclic—structure elucidation, synthesis, and pharmacology of camptothecin □ Antitumor activity, pentacyclic alkaloid camptothecin—structure, synthesis, pharmacology

Camptothecin is an alkaloid with a novel ring system. As will be discussed later, it has been reported to possess antileukemic and antitumor activities in animals and humans. In 1966, Wall *et al.* (1) described the isolation and structural determination of this new alkaloid, which was assigned the unusual structure I. The alkaloid was obtained from the stem wood of the tree *Camptotheca acuminata* (Nyssaceae), which grows almost exclusively in mainland China (2, 3). This review will focus on the isolation and structural determination of camptothecin and some related compounds and the biogenesis and synthesis of camptothecin.

ISOLATION AND STRUCTURAL ELUCIDATION

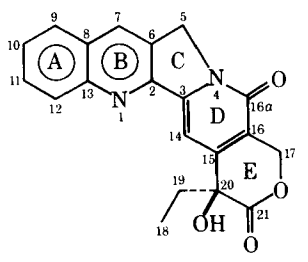
Camptothecin—Camptothecin, mp 264–267° dec., $[\alpha]_D^{25}$ +31.3° [chloroform–methanol (8:2)], showed in its mass spectrum a molecular ion at *m/e* 348.1117 corresponding to the formula $C_{20}H_{16}N_2O_4$ (calc. 348.1111). The UV spectrum exhibited λ_{max} 220, 254, 290, and 370 nm (ϵ 37,320, 29,230, 4980, and 19,900). The IR spectrum had bands corresponding to the presence of hydroxyl (3440 cm^{-1}), δ-lactone (1760–1745 cm^{-1}), lactam (1660 cm^{-1}), and aromatic (1610 and 1588 cm^{-1}) functions. In the NMR spectrum (dimethyl sulfoxide-d_6), signals were present at δ 0.91 (3H, $-CH_2-CH_3$), 1.90 [2H, >C(—OH)—CH_2—CH_3], 5.45 (2H, Ar—CH_2O—), and 5.28 (2H, Ar—CH_2N<).

A mono-*O*-acetyl derivative could be obtained following acetylation, while exhaustive hydrogenation with Adams catalyst in acetic acid gave rise to the dodecahydro compound II (1). In the presence of sodium hydroxide, the sodium salt III was readily formed. Treatment of camptothecin (I) with thionyl chloride yielded chlorocamptothecin (IV), which could be hydrogenolized with palladium-on-carbon to 20-deoxycamptothecin (V). Finally, reduction of camptothecin with sodium borohydride led to the lactol VI (4).

The structure and absolute configuration of the alkaloid were conclusively established through X-ray analysis of the iodoacetate salt (1, 5). The molecular formulas and melting points for camptothecin and a number of its derivatives and analogs are given in Table I.

Very recently, the Indian plant *Mappia foetida* Miers. was found to be an alternative source of camptothecin (6).

10-Hydroxycamptothecin and 10-Methoxycamptothecin—Further examination of the alkaloidal ex-



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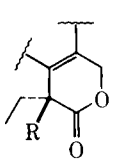
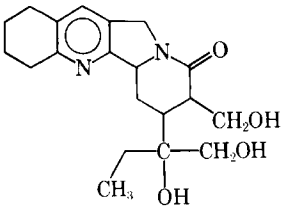
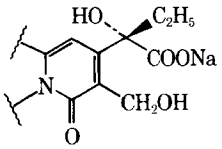


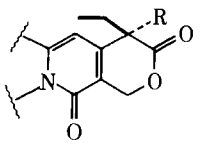
Ia: R = $OCOCH_3$
Ib: R = $OCOCH_2Cl$
Ic: R = $OCOCH_2I$
Id: R = Cl



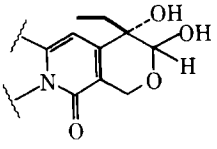
II



III



IV: R = Cl
V: R = H



VI

Table I—Molecular Formulas and Melting Points of Camptothecin and Derivatives

Alkaloid	Formula	Melting Point	Reference
Camptothecin (I)	$C_{20}H_{16}N_2O_4$	264–267°	1
Camptothecin acetate (I*a*)	$C_{22}H_{18}N_2O_5$	271–274°	1
Camptothecin chloroacetate (I*b*)	$C_{22}H_{17}ClN_2O_5$	245–248°	1
Camptothecin iodoacetate (I*c*)	$C_{22}H_{17}IN_2O_5$	238–240°	1
Chlorocamptothecin (I*d*)	$C_{20}H_{15}ClN_2O_3$	248–250°	1
10-Hydroxycamptothecin (VII)	$C_{21}H_{18}N_2O_5$	268–270°	7
9-Methoxycamptothecin (XIII)	$C_{21}H_{18}N_2O_5$	258–260°	6
10-Methoxycamptothecin (VIII)	$C_{21}H_{18}N_2O_5$	254–255°	7

tract of *C. acuminata* resulted in the isolation of two additional alkaloids of the same group: *(a)* 10-hydroxycamptothecin (VII), $C_{20}H_{16}N_2O_5$, mp 268–270°; and *(b)* 10-methoxycamptothecin (VIII), $C_{21}H_{18}N_2O_5$, mp 254–255° (7).

The exact positions of the hydroxyl group in VII and of the methoxyl group in VIII were established from the NMR spectra of VIII and the synthetic analogs IX–XII possessing a methoxyl function at each possible site in ring A (7, 8).

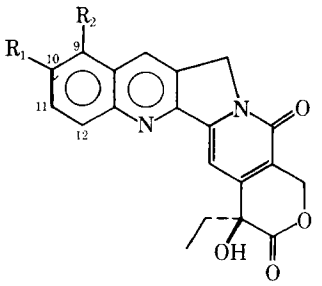
9-Methoxycamptothecin—This alkaloid has been isolated from *M. foetida* Miers. as a minor component of an alkaloidal mixture consisting chiefly of camptothecin (I). Structure XIII was suggested for the new alkaloid (6).

BIOGENESIS

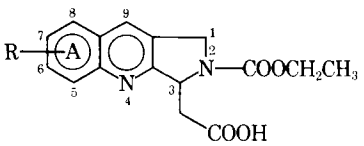
The biogenesis of camptothecin (I) involves the formation of the 1,3-dihydro-2*H*-pyrrolo[3,4*b*]quinoline system (XIV) which was unknown in natural products chemistry prior to the characterization of the alkaloid. The numbering system is based on the Le Men–Taylor model (9).

Careful inspection of the camptothecin molecule reveals structural similarities with such indole alkaloids as rubescine (XV) (10), hunterburnine methiodide (XVI) (5), and dihydrocorynantheol (XVII) (5) as well as with several *Adina* alkaloids (11). Although less obvious, camptothecin (I) and ajmalicine (XVIII) are also related in that both contain the familiar 10-carbon unit XIX characteristic of the Corynanthe family of indole alkaloids (9, 12).

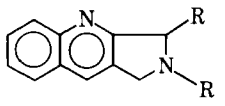
This relationship suggested that camptothecin, like the indole alkaloids, is of tryptamine–terpene origin. Using this premise, Wenkert *et al.* (13) then treated the biogenesis of camptothecin as simply an extension of that for the indole alkaloids (14–22). Two rearrangements of the indole alkaloid isositsirikine (XX) were envisioned to lead to camptothecin (13).



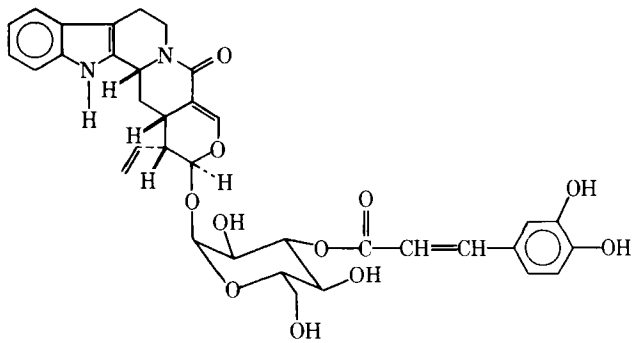
VII: R_1 = OH, R_2 = H
VIII: R_1 = OCH_3, R_2 = H
XIII: R_1 = H, R_2 = OCH_3



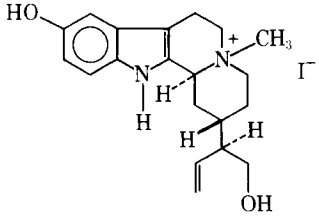
IX: R = 5-OCH_3
X: R = 6-OCH_3
XI: R = 7-OCH_3
XII: R = 8-OCH_3



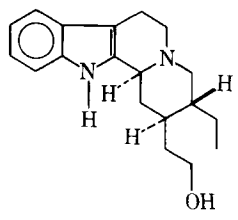
XIV



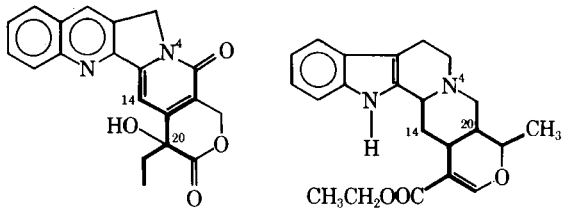
XV



XVI

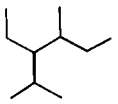


XVII

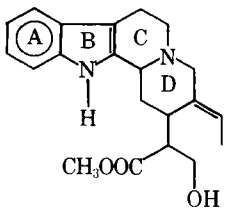


I

XVIII

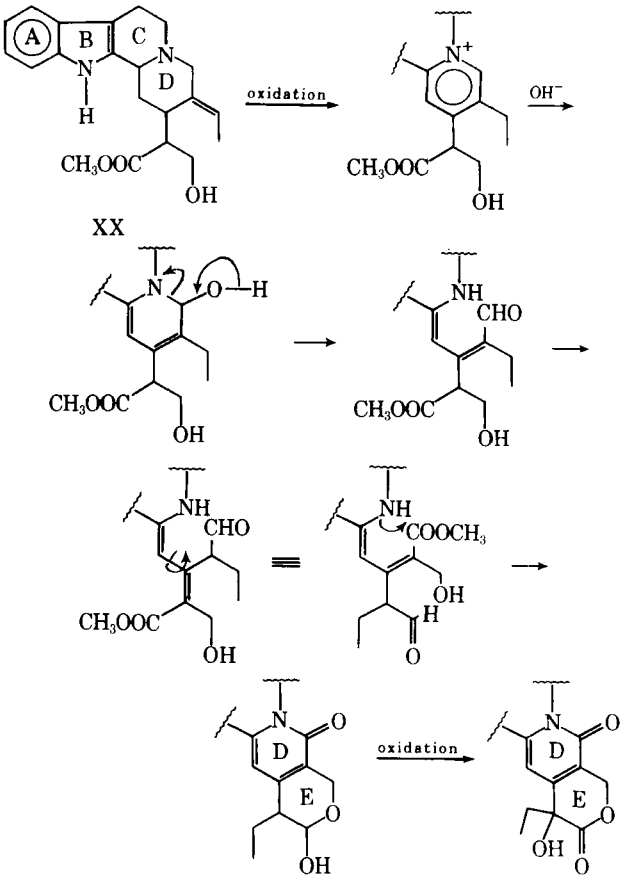


XIX



XX

One sequence involves the conversion of the tetrahydrocarboline system (rings A, B, and C) into the pyrroloquinoline moiety through a series of known reactions (Scheme I) (23–25). The second sequence includes cleavage of ring D of isositsirikine followed by recyclization to give the carbon skeleton appropriate to rings D and E of camptothecin (Scheme II).

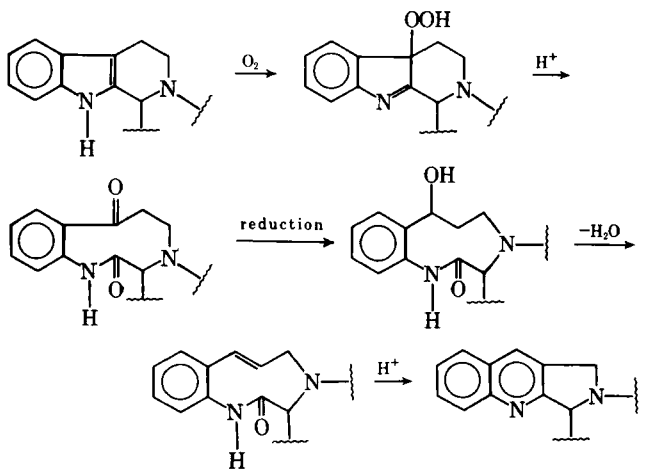


Scheme II

SYNTHESIS OF CAMPTOTHECIN

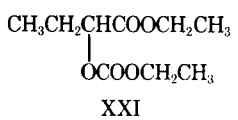
Early reports ascribing promising antitumor activity to camptothecin, coupled with the inaccessibility of *C. acuminata*, generated considerable interest in the total synthesis of the alkaloid. Additionally, a structure involving an α-hydroxy-δ-lactone together with a pyridone system fused to a five-membered ring presents an interesting challenge in organic synthesis. Synthetic efforts culminated recently in several independent preparations of (±)-camptothecin.

The Stork–Schultz Synthesis—Stork and Schultz (26) reported the first total synthesis of (±)-camptothecin in 1971. The starting material was the tricyclic quinoline carboxylic acid XXV, incorporating potential rings A, B, and C of camptothecin, which was obtained by Friedländer condensation of anthranilaldehyde with the known pyrrolidinone XXIV. Following hydrolysis and esterification, the resulting amino ester XXVI was condensed with carbethoxyacetyl chloride to give the diester amide XXVII. Dieckmann cyclization furnished the β-keto ester XXVIII, and hydrolysis and decarboxylation afforded the β-keto lactam XXIX. The β-hydroxy lactam XXX, obtained through reduction of XXIX with sodium borohydride, was then dehydrated to the unsaturated lactam XXXI.

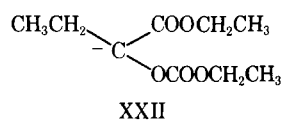


Scheme I

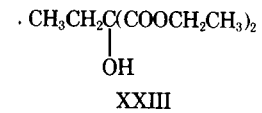
The stage was now set for the critical annelation of ring E of the camptothecin molecule. To begin with, the use of 1 equivalent of lithium diisopropylamide at dry ice–acetone temperature on the carbonate ester XXI generated the desired anion XXII. Low temperature was necessitated by the fact that the anion tended to rearrange to the tartronic ester XXIII at room temperature. Michael addition of the anion XXII to the unsaturated lactam XXXI generated the γ-lactone XXXII in 85% yield. The carbox-



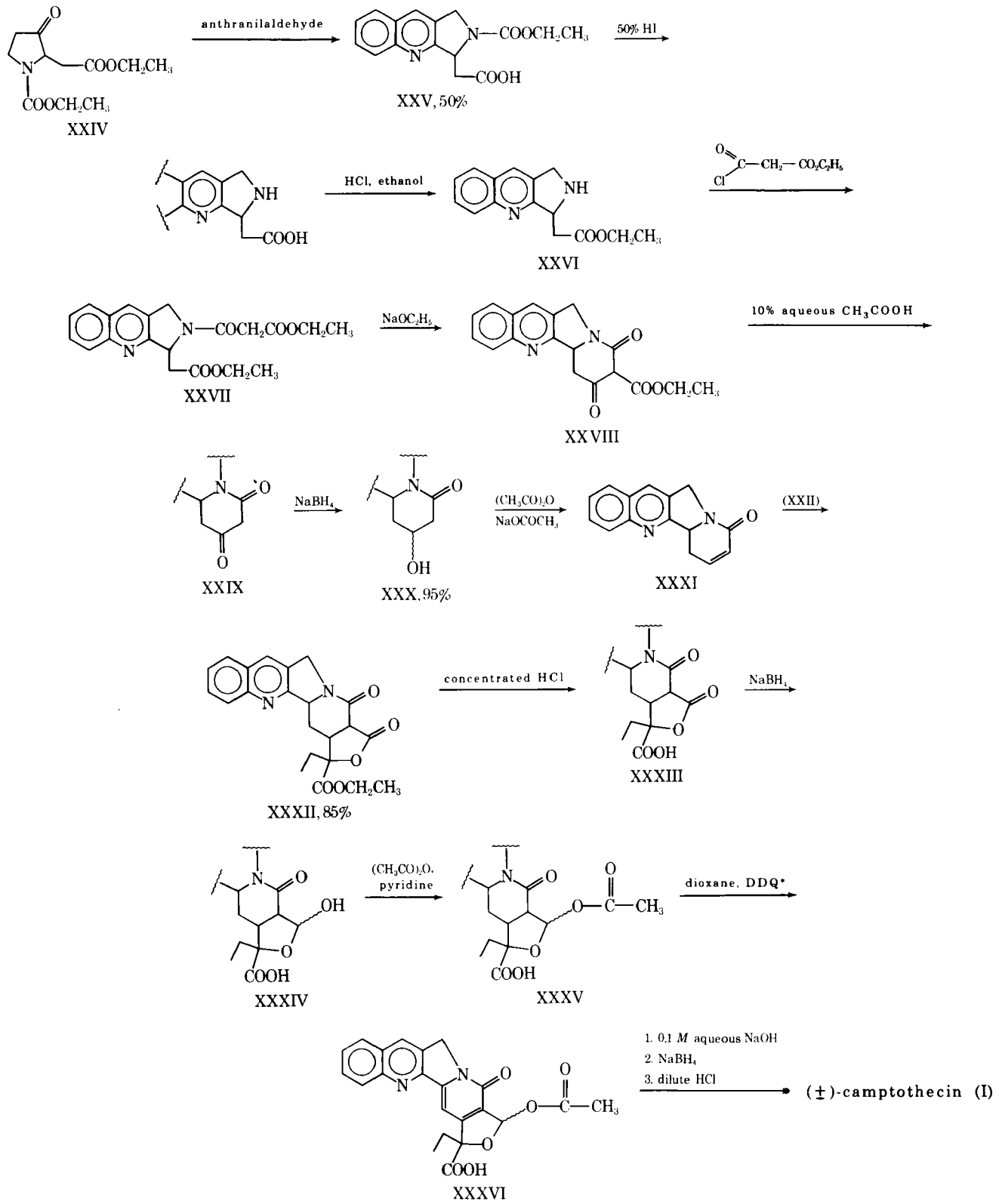
XXI



XXII



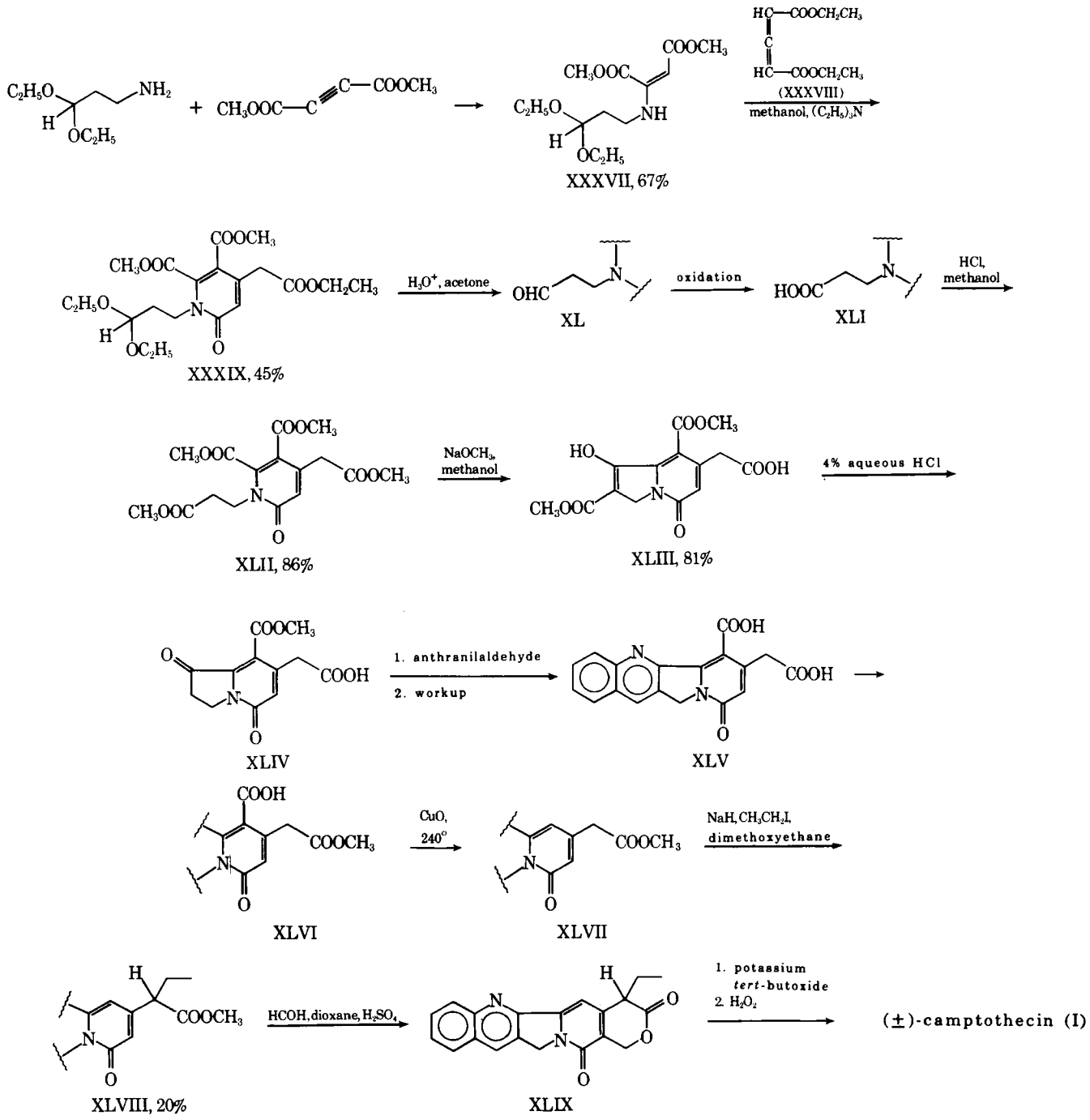
XXIII



*Scheme III—*DDQ = 2,3-dichloro-5,6-dicyanobenzoquinone*

ylic acid XXXIII derived from hydrolysis of the ethyl ester function in XXXII was then reduced to the hemiacetal XXXIV. *O*-Acetylation (XXXV) and dehydrogenation subsequently supplied the pyridone XXXVI. The pyridone XXXVI was converted to (±)-camptothecin through hydrolysis with dilute sodium hydroxide, sodium borohydride reduction, and acidification with dilute hydrochloric acid (Scheme III).

The Volkmann-Danishefsky-Eggler-Solomon Synthesis—The first step in this synthesis (27) was the preparation of the enamino ester XXXVII through the reaction of β-aminopropionaldehyde di-

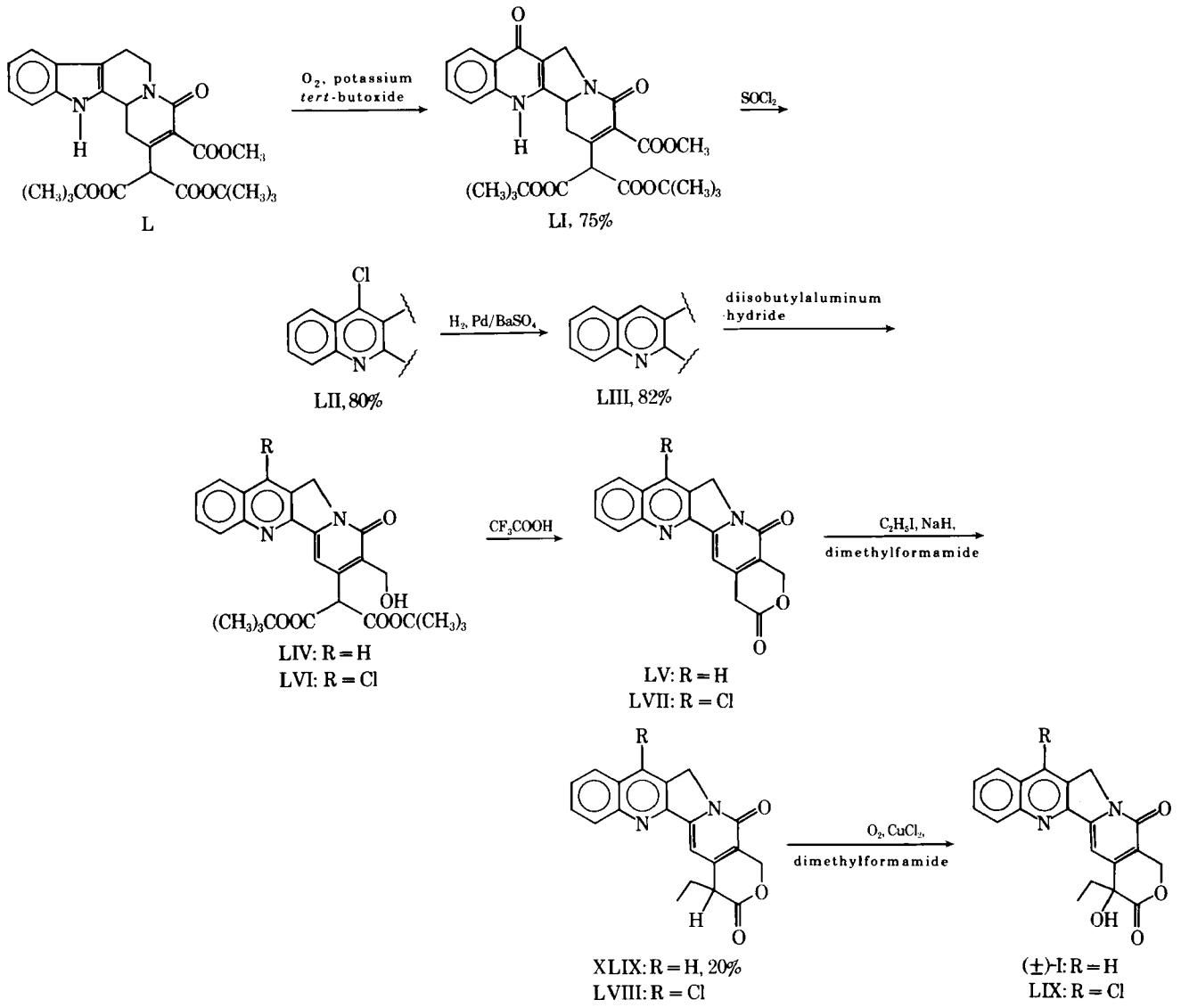


Scheme IV

ethyl acetal with dicarbomethoxyacetylene. Condensation of XXXVII with dicarbethoxyallene (XXXVIII) in methanol containing triethylamine afforded the pyridone triester XXXIX in what amounts to a new pyridone synthesis. Acid hydrolysis achieved the quantitative transformation of this material to the aldehyde XL.

The next two steps, oxidation and esterification–transesterification, were directed toward the formation of the tetraester XLII, which possesses the appropriate substituents for the construction of ring C of camptothecin. Dieckmann ring closure of XLII led reproducibly, in 81% yield, to the enolic acid ester XLIII which, following hydrolysis and selective decarboxylation, gave rise to the keto acid XLIV. Friedländer condensation with anthranilaldehyde was accompanied by hydrolysis of the ester function. The resulting tetracyclic diacid XLV was selectively esterified to the monocarboxylic acid ester XLVI, and subsequent decarboxylation provided the tetracyclic ester XLVII in 29% yield from the enolic acid ester XLIII.

C-Ethylation of XLVII gave the ethylated monoester XLVIII, which was converted to (±)-20-deoxycamptothecin (XLIX) after treatment with paraformaldehyde in dioxane containing a trace of sulfuric



Scheme V

acid. Hydrogen peroxide oxidation of the anion of XLIX then afforded (±)-camptothecin (I) (Scheme IV).

The Winterfeldt Synthesis—It was noted earlier in this review that the biogenetic pathway to camptothecin most probably involves the oxidation of an indole double bond followed by recyclization and aromatization to a quinoline system (9, 13). A biogenetically patterned synthesis of (±)-camptothecin based on such a series of transformations was carried out by Winterfeldt and coworkers (28–30).

The previously known (31) indolic intermediate L readily underwent the indole-quinolone conversion upon oxidation (30) so that Compound LI was obtained in 75% yield. The required quinoline analog LIII was then obtained through the classical sequence involving treatment with thionyl chloride to give LII and hydrogenolysis using palladium–barium sulfate.

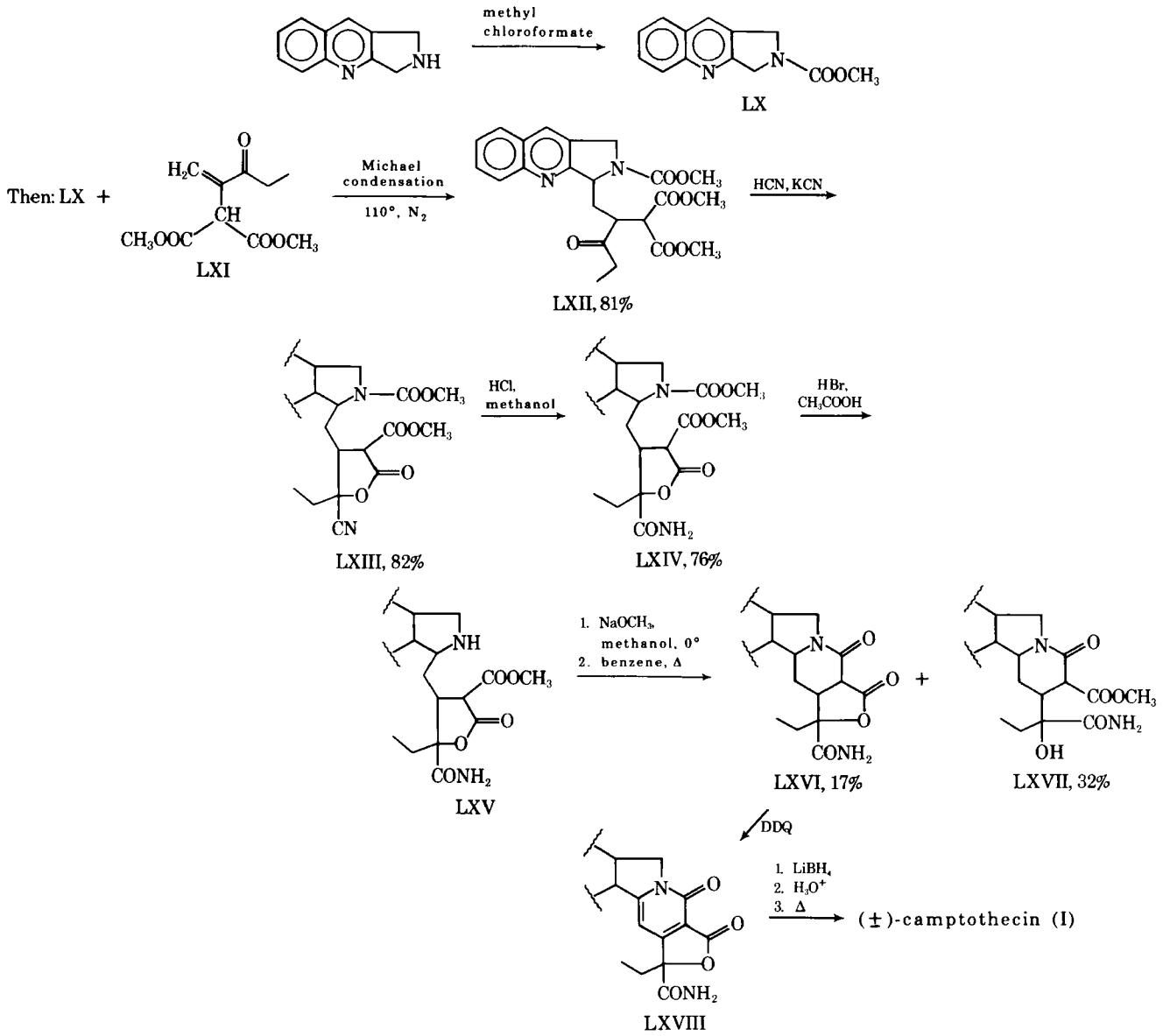
Selective reduction of LIII using diisobutylaluminum hydride led to the alcohol LIV which, in turn, led to the δ-lactone LV. C-Alkylation of LV followed by oxidation of the resulting (±)-20-deoxycamptothecin (XLIX) with oxygen and cupric chloride supplied (±)-camptothecin (Scheme V).

In a parallel sequence, 7-chlorocamptothecin (LIX) was prepared through similar reactions (LVI→LVII→LVIII→LIX).

The Wani–Wall–Levine Synthesis—1,3-Dihydro-2-methoxycarbonyl- 2*H*-pyrrolo[3,4*b*]quinoline (LX), which served as the starting material for this synthesis (32), undergoes a crucial Michael condensation with the keto diester LXI to produce the urethan LXII in 81% yield. This urethan possesses nearly all of the functionalities necessary for the eventual formation of rings D and E of camptothecin.

Treatment of LXII with potassium cyanide in liquid hydrogen cyanide generated a cyanohydrin, which immediately lactonized to a mixture of diastereoisomeric cyano lactones LXIII. The diastereoisomeric mixture of amides LXIV was obtained upon treatment of LXIII with methanolic hydrogen chloride.

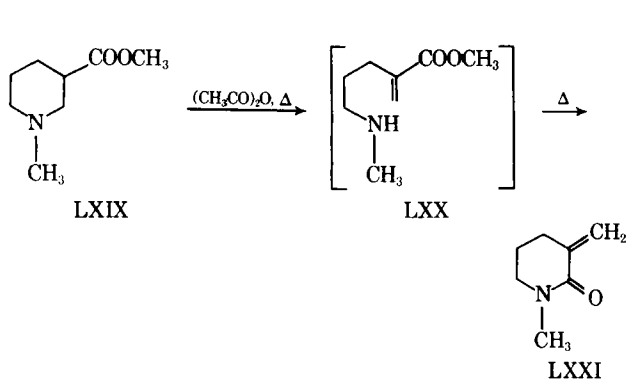
To construct ring D of camptothecin, the *N*-carbo-



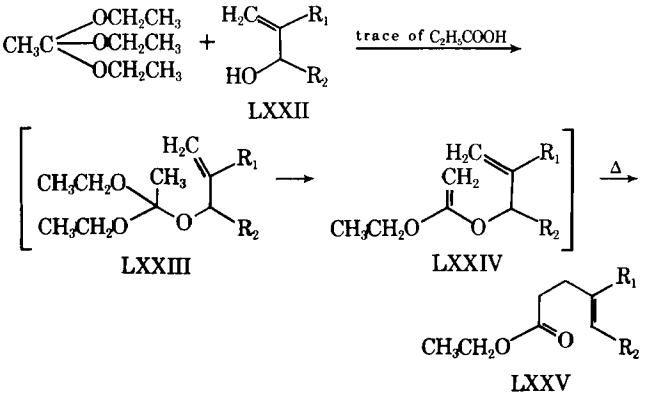
Scheme VI

methoxy group of LXIV was selectively removed using hydrogen bromide in glacial acetic acid, and the resulting amine LXV was refluxed with base. A separable mixture of lactams LXVI and LXVII was thus obtained. The remainder of the synthesis involved the oxidation of LXVI into LXVIII and reduction of the lactone carbonyl in the latter compound with lithium borohydride. Acidification of the reaction mixture followed by heating on a steam bath gave rise to (±)-camptothecin (I) (Scheme VI).

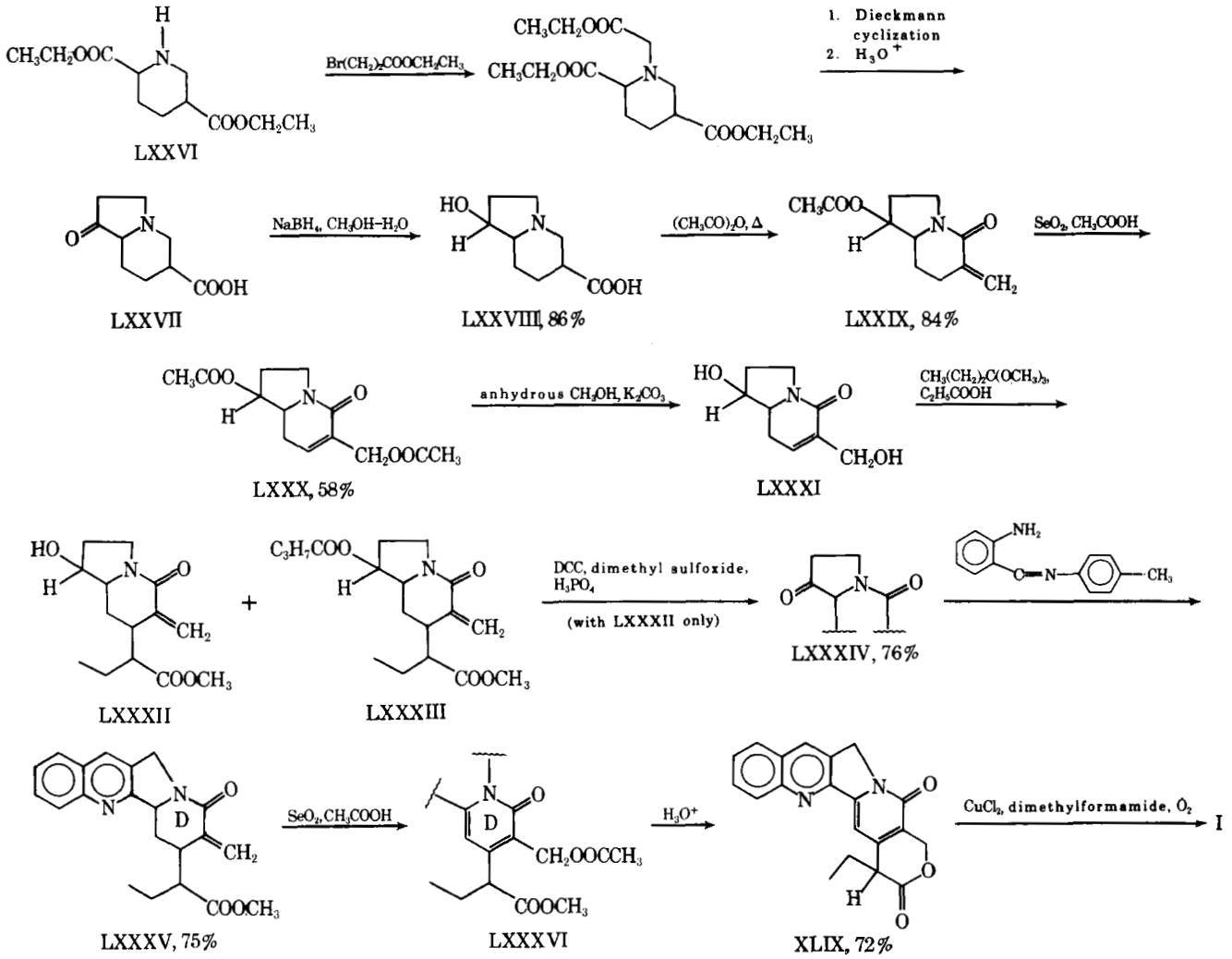
The Rapoport–Tang Synthesis—Two known reactions were of primary importance in this synthesis (33). First, the α-methylene-lactam rearrange-



Scheme VII



Scheme VIII



Scheme IX

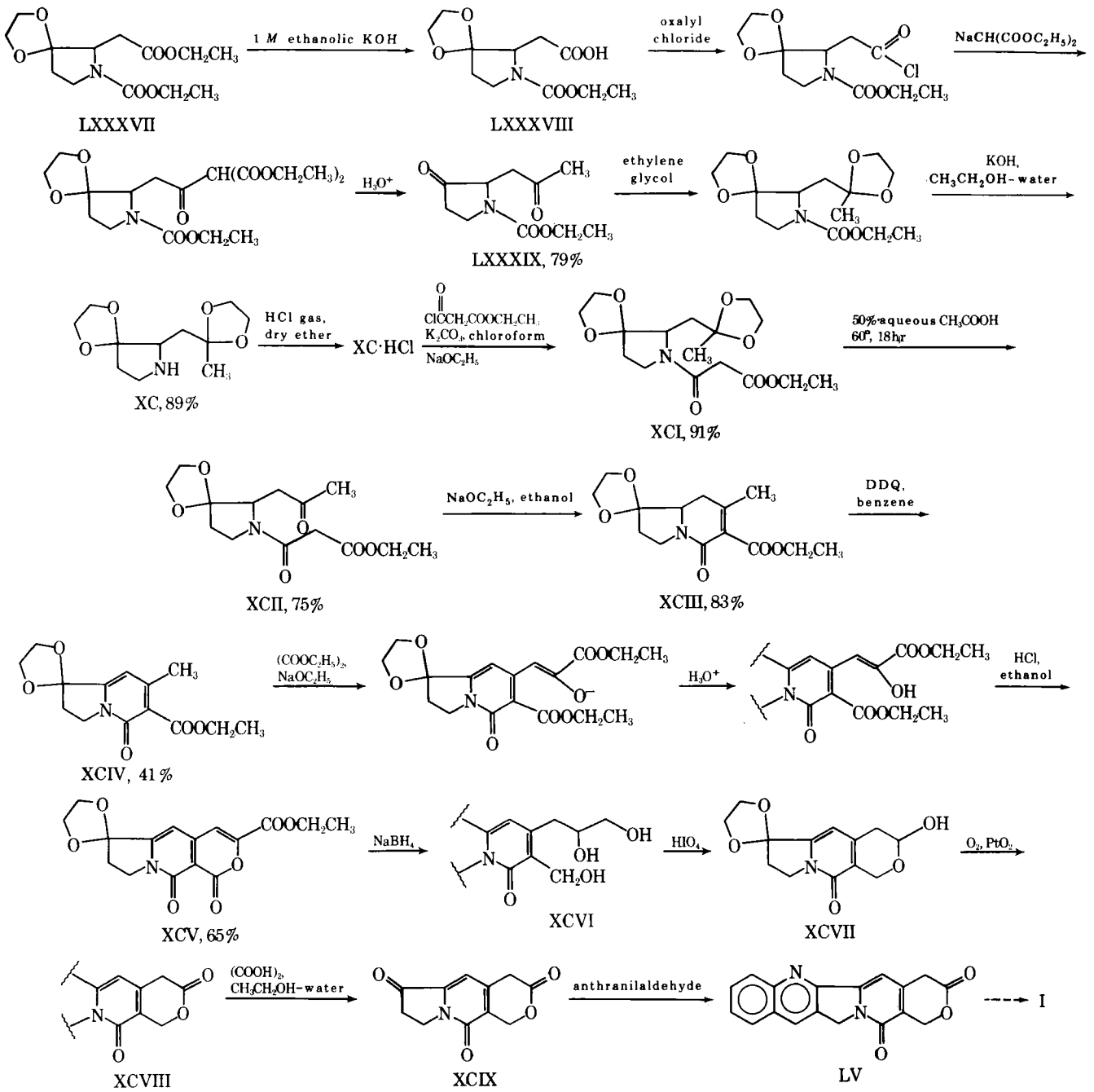
ment (34), when applied to a simple β-amino ester such as 1-methylnipecotinate (LXIX), led to the unsaturated lactam LXXI through the probable intermediacy of the α,β-unsaturated ester LXX (Scheme VII).

Second, the new Johnson *et al.* (35) modification of the Claisen thermal rearrangement had been proven to be an efficient method for the preparation of substituted olefins. The method simply involves heating an allylic alcohol, *e.g.*, LXXII, with an excess of an orthoester such as ethyl orthoacetate in the presence of a trace of a weak acid, usually propionic acid. Evidently, the mixed orthoester LXXIII is formed, which loses ethanol to form the ketene acetal LXXIV, which then rearranges to the olefinic ester LXXV (Scheme VIII).

Immediately after initial trials involving the above two transformations using model compounds (described later), a total synthesis of (±)-camptothecin was initiated (33). The first steps of this synthesis dealt with the formation of rings C and D of the alkaloid. The ketonic β-amino acid LXXVII obtained from the diester LXXVI was reduced with sodium borohydride in methanol–water to the alcoholic β-amino acid LXXVIII. When refluxed with acetic anhydride, LXXVIII underwent the α-methylene lactam rearrangement to afford the piperidone acetate LXXIX as a diastereoisomeric mixture. The diacetate LXXX, obtained *via* selenium dioxide in acetic acid oxidation of LXXIX, was converted to the diol LXXXI in an anhydrous methanol–potassium carbonate medium. Application of the Johnson *et al.* (35) modification of the Claisen rearrangement, using trimethyl orthobutyrate, then gave rise to two products, the free alcohol LXXXII and the butyrate ester LXXXIII, which could readily be converted to the desired alcohol LXXXII.

Following oxidation of LXXXII with dicyclohexylcarbodiimide (DCC) in dimethyl sulfoxide, the AB ring system of camptothecin was constructed *via* Friedländer condensation of the ketone LXXXIV and *N*-(2-aminobenzylidene)-*p*-toluidine.

The remaining transformations in the synthesis, involving aromatization of the ring D as well as formation of the ring E, were accomplished through selenium dioxide oxidation of the α-methylene lactam LXXXV, hydrolysis, lactonization to XLIX, and finally introduction of the C-20 tertiary alcohol function using oxygen–cupric chloride in dimethylformamide (Scheme IX).



LXXXVII LXXXVIII LXXXIX, 79% XC, 89% XC·HCl XCI, 91% XCII, 75% XCIII, 83% XCIV, 41% XCV, 65% XCVI XCVII XCVIII XCIX LV

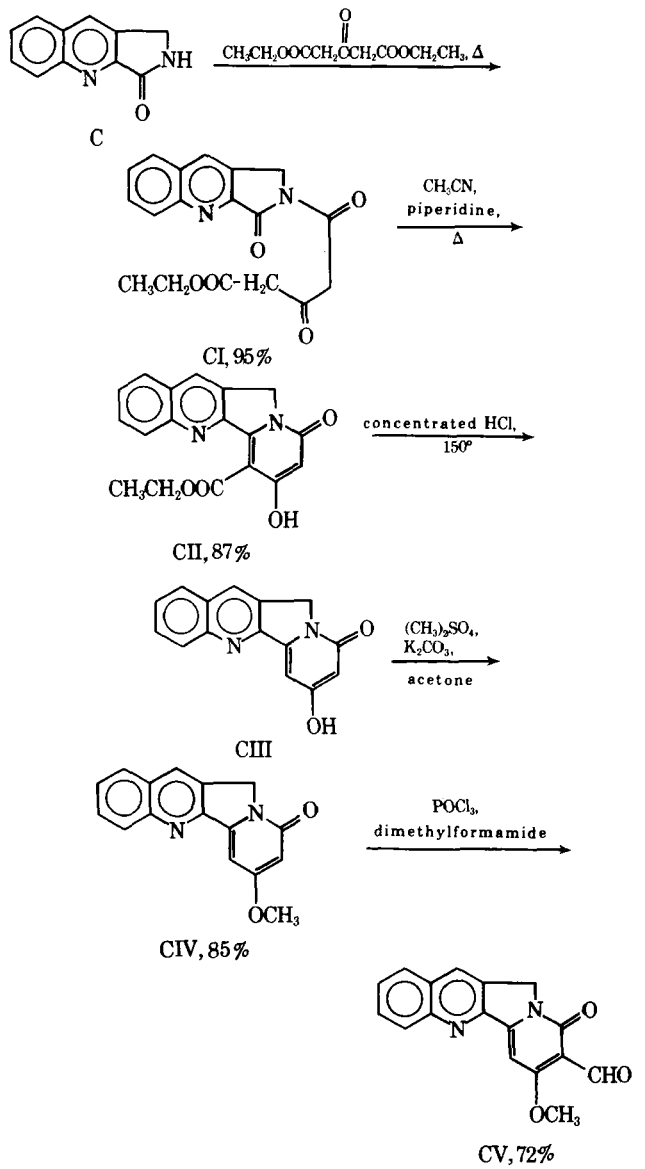
Scheme X

The Shamma–Smithers–Georgiev Synthesis—Since rings A and B in camptothecin primarily impart to the alkaloid its near total insolubility in the usual organic solvents, the construction of these two rings was reserved for the terminal stages of the synthesis (36).

Prolonged treatment of the urethan LXXXVII with 1 *M* ethanolic potassium hydroxide gave the carboxylic acid LXXXVIII in 91% yield. The acid chloride of LXXXVIII was converted to the dione LXXXIX by reaction with the sodium salt of diethyl malonate and acid hydrolysis of the resulting diester. The amine XC, obtained in 89% yield after alkaline hydrolysis of the diethylene ketal of LXXXIX, was acylated to the amide XCI in 91% yield, using carbethoxyacetyl chloride in basic solution.

The critical selective hydrolysis of XCI to the monoketone XCII was achieved in 75% yield using 50% aqueous acetic acid. Treatment of XCII with sodium ethoxide furnished the dihydropyridone XCIII in what was, in effect, a new synthesis of dihydropyridones. Subsequent oxidation of XCIII with excess 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) produced the pyridone XCIV containing rings C and D of camptothecin.

Attention was then turned to the construction of the δ-lactone ring E of camptothecin. Base-catalyzed condensation of the pyridone XCIV with diethyl oxa-

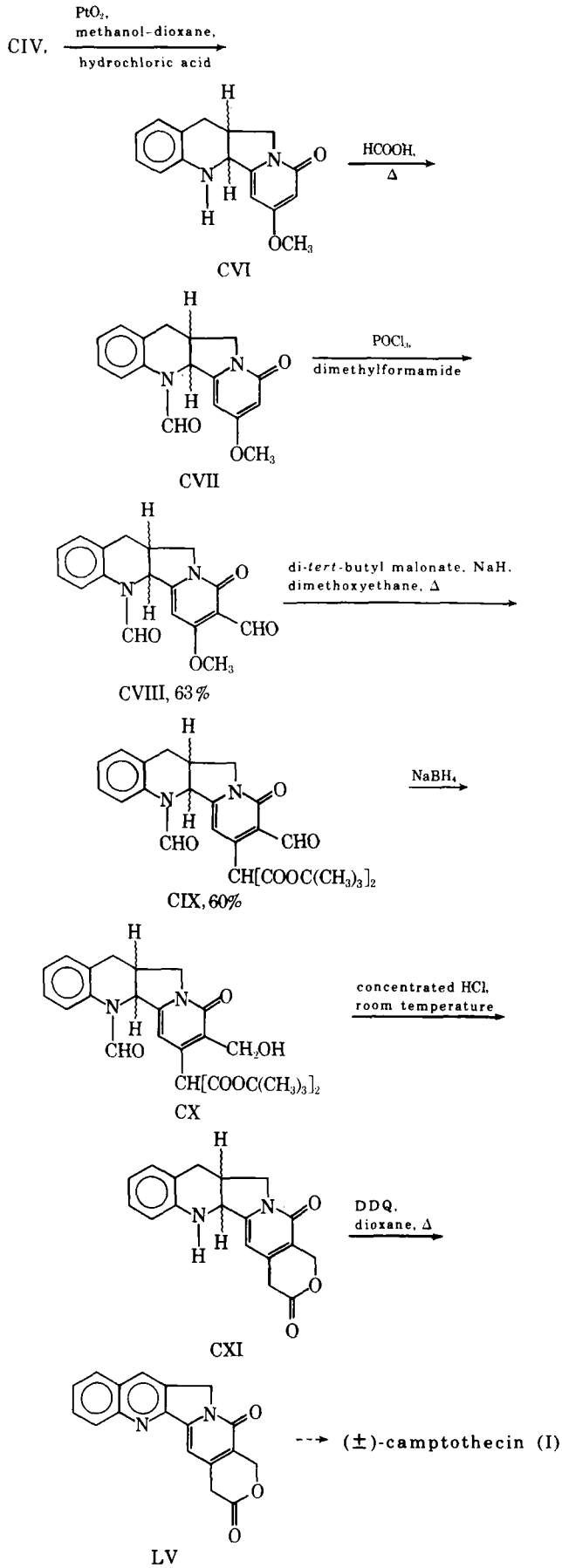


Scheme XI

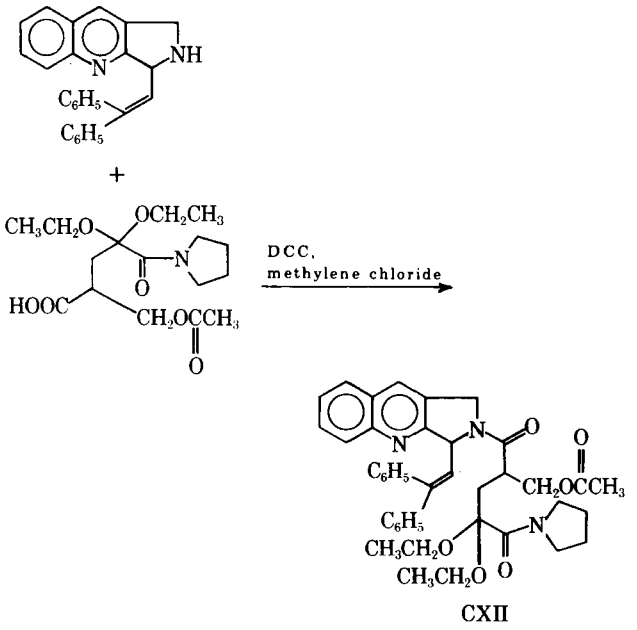
late and acidification of the resulting product afforded the vinyl lactone XCV in 65% yield. The conversion of the latter compound to the δ-lactone XCVIII was carried out utilizing sodium borohydride succeeded by periodic acid. The catalytic oxidation of the resulting hemiacetal XCVII led to the desired δ-lactone XCVIII. The sodium borohydride reduction of the vinyl lactone XCV proceeded through the intermediacy of the triol XCVI, thus indicating a marked susceptibility of the pyrone carbonyl to nucleophilic attack.

Hydrolysis of the ketal function of XCVIII and condensation of the resulting keto lactone XCIX with anthranilaldehyde afforded the known 20-deoxy-2-deethylcamptothecin (LV) (28, 29), which had been previously converted to (±)-camptothecin (I) (28, 29) (Scheme X).

The Sugasawa-Toyoda-Sasakura Synthesis—3-Oxodihydro-2*H*-pyrrolo[3,4*b*]quinoline (C) served as a starting material for this synthesis (37). Heating of C in diethyl acetonedicarboxylate at 160–165° gave rise to the imido ester CI. Intramolecular cycli-



Scheme XII



Scheme XIII

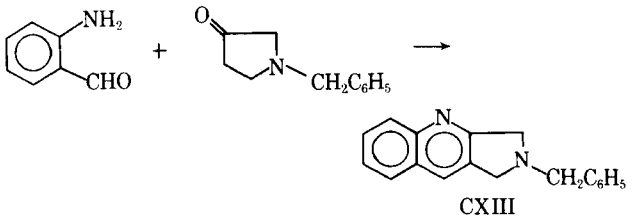
zation of CI to the hydroxypyridone ester CII proceeded smoothly. Hydrolysis and decarboxylation of CII, followed by methylation of the hydroxyl group of the resulting Compound CIII, furnished the 4-methoxy-2-pyridone CIV which possesses the ABCD ring system of camptothecin.

To construct ring E of the alkaloid, the generation of an active substituent next to the methoxyl group was desirable. Thus, treatment of CIV with phosphorus oxychloride in dimethylformamide afforded the methoxyaldehyde CV (Scheme XI).

Initial attempts to build ring E according to an annelation method previously developed (37) (discussed later) were not successful because of the lability of CV under basic conditions. The 4-methoxy-2-pyridone CIV was, therefore, reduced with Adams catalyst in methanol–dioxane containing hydrochloric acid. *N*-Formylation of the resulting Compound CVI, followed by a Vilsmeir C-formylation, gave rise to the corresponding aldehyde CVIII. When the latter compound was treated with di-*tert*-butyl malonate and sodium hydride in refluxing dioxane, the expected malonate ester CIX was obtained. Crude CIX was hydrogenated with sodium borohydride to give CX, which was treated directly with concentrated hydrochloric acid at room temperature to furnish CXI through lactone formation and *N*-deformylation. Dehydrogenation of CXI with 2,3-dichloro-5,6-dicyanobenzoquinone afforded 20-deoxy-2-deethylcamptothecin (LV), which was subsequently ethylated (ethyl iodide–sodium hydride) and oxidized (O_2, copper acetate, triethylamine), thus giving (±)-camptothecin (I) (Scheme XII)

SYNTHESIS OF CAMPTOTHECIN ANALOGS

A variety of simpler analogs of camptothecin have been prepared, and these preparations are discussed in the present section. The work described here is essentially a summary of transformations carried out as models preceding the total synthesis of the alkaloid.



Scheme XIV

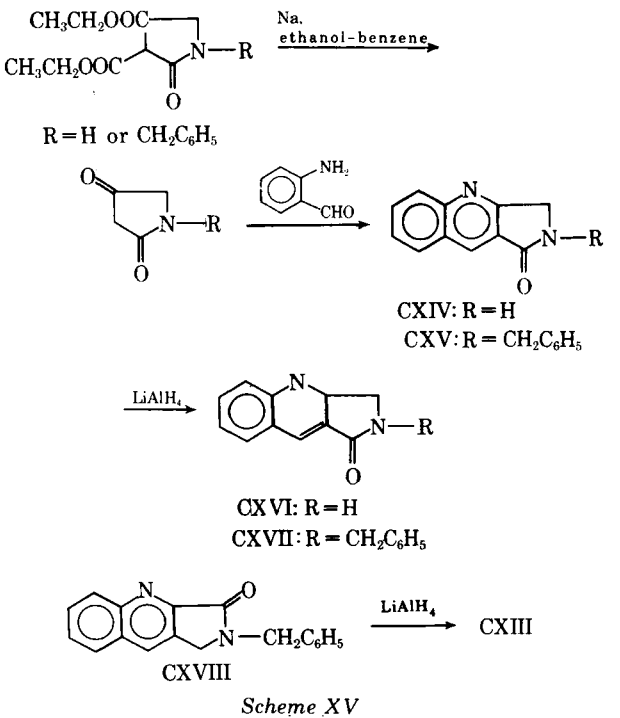
Analogs Containing the ABC Ring System of Camptothecin—In an early attempt at a total synthesis of camptothecin, the preparation of the polyfunctional analog CXII, which incorporates the ABC ring system of the alkaloid as well as some structural features of rings D and E, was described (38) (Scheme XIII).

In a separate study, analog CXIII was prepared *via* Friedländer condensation of anthranilaldehyde and 1-benzyl-3-oxopyrrolidine (39) (Scheme XIV). Lithium aluminum hydride reduction of the tricyclic analogs CXIV and CXV gave lactams CXVI and CXVII, while analog CXVIII was converted mainly to the amine CXIII (39) (Scheme XV).

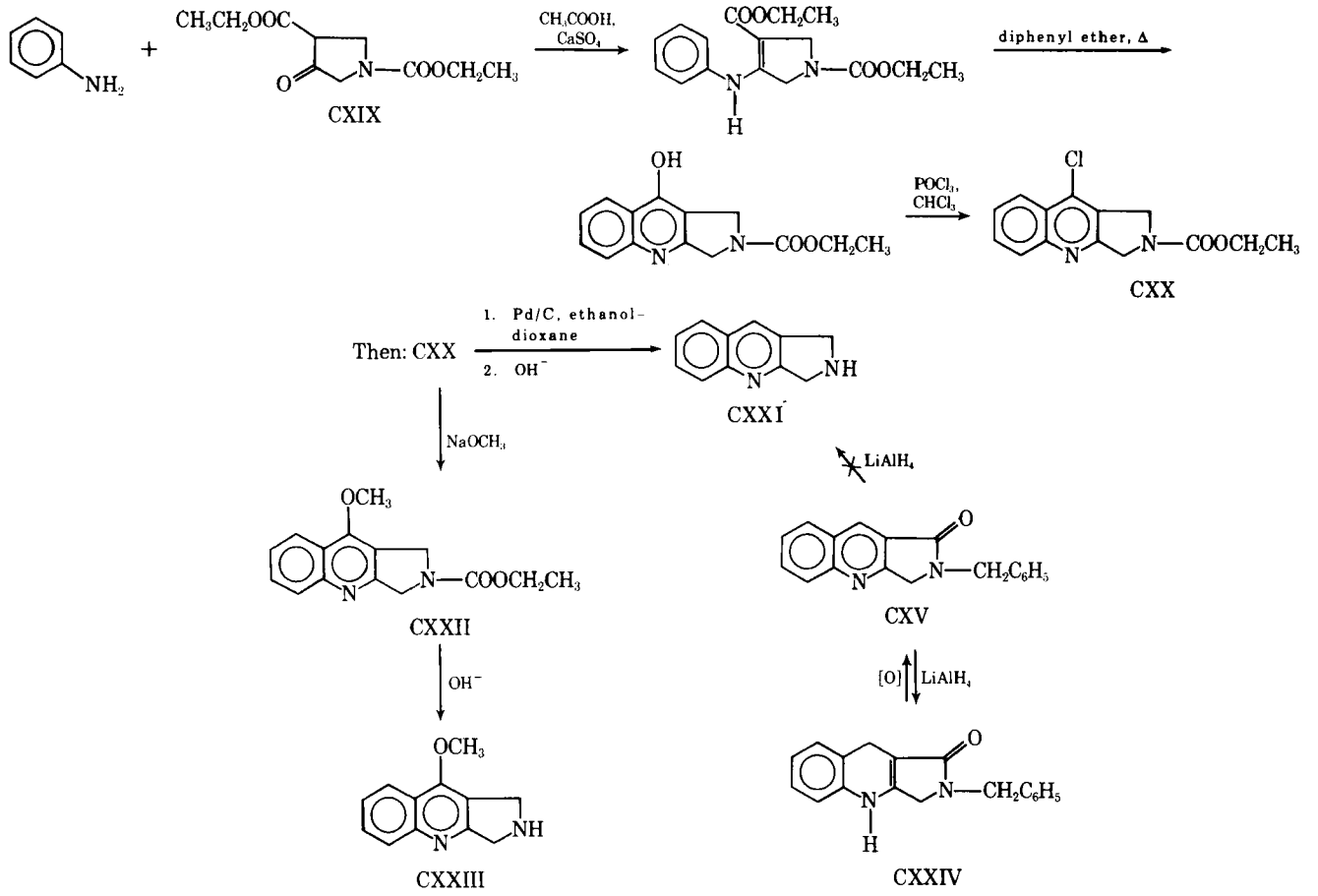
The tricyclic bases CXXI and CXXIII have been prepared using aniline and 1,4-diethoxycarbonyl-3-oxopyrrolidine (CXIX) as starting materials (40). Lithium aluminum hydride reduction of Compound CXV afforded 2-benzyl-1-oxo-2,3,4,9-tetrahydro-1*H*-pyrrolo[3,4*b*]quinoline (CXXIV) instead of the desired CXXI (40) (Scheme XVI). These results are in close analogy with an earlier observation already discussed (39).

Several *N*-substituted analogs of CXXI have also been reported (41, 42).

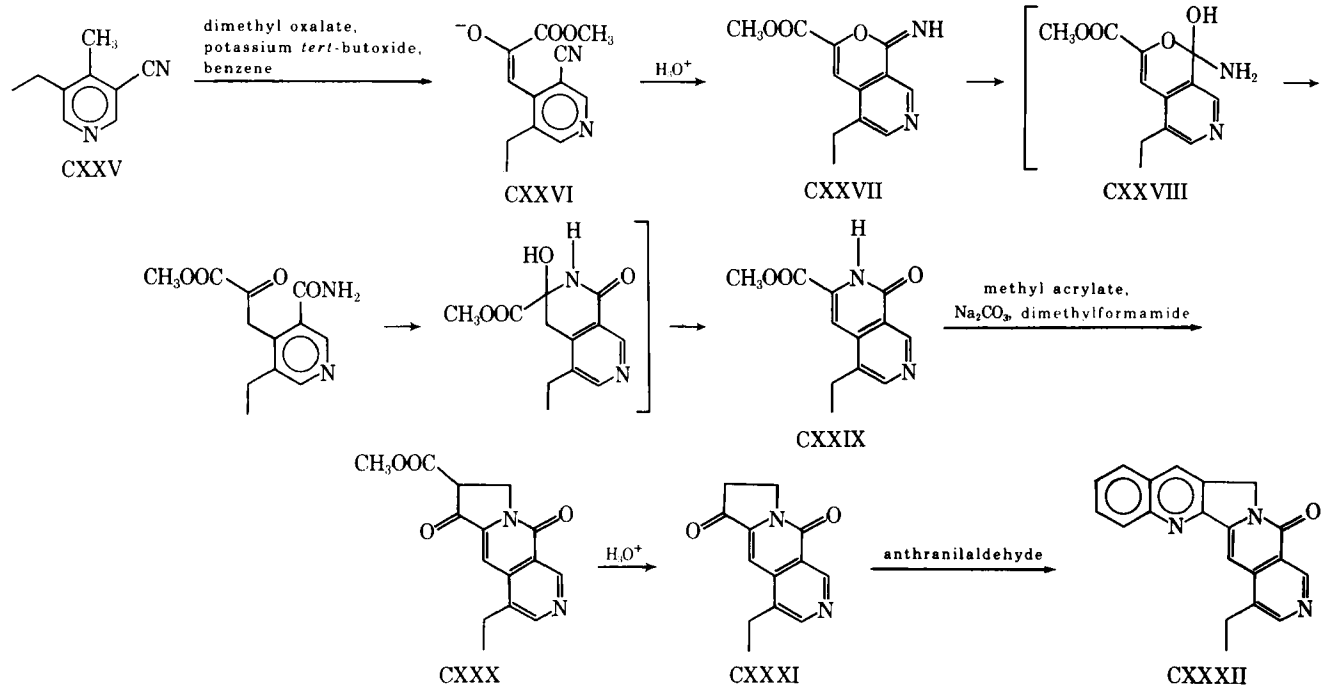
Analogs Containing the ABCD Ring System of Camptothecin—The pentacyclic base CXXXII has been prepared (13) as a potential precursor of the alkaloid. 4-Methyl-5-ethylnicotinonitrile (CXXV) was condensed with dimethyl oxalate, and the resulting



Scheme XV



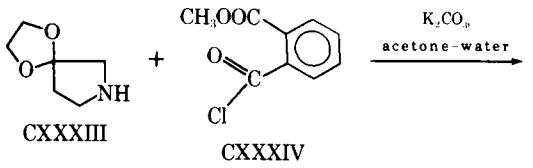
Scheme XVI



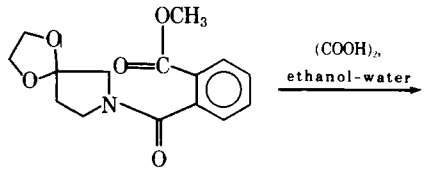
Scheme XVII

enolate salt CXXVI was cyclized in aqueous acid to the azaisocarbostyril CXXIX through the intermediacy of CXXVII and CXXVIII. Condensation of CXXIX with methyl acrylate produced the diester CXXX, which was converted to the ketone CXXXI through hydrolysis and decarboxylation. Finally, Friedländer condensation using anthranilaldehyde led to the desired pentacyclic base CXXXII (Scheme XVII).

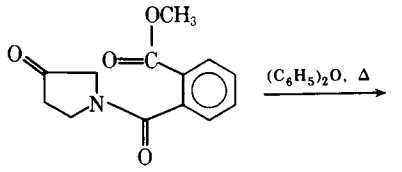
Another approach (43) to a pentacyclic system



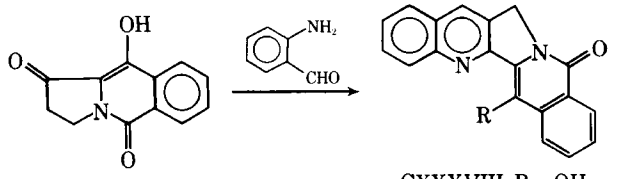
CXXXIII CXXXIV



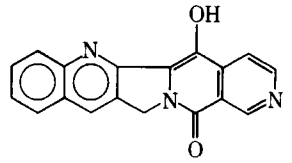
CXXXV



CXXXVI

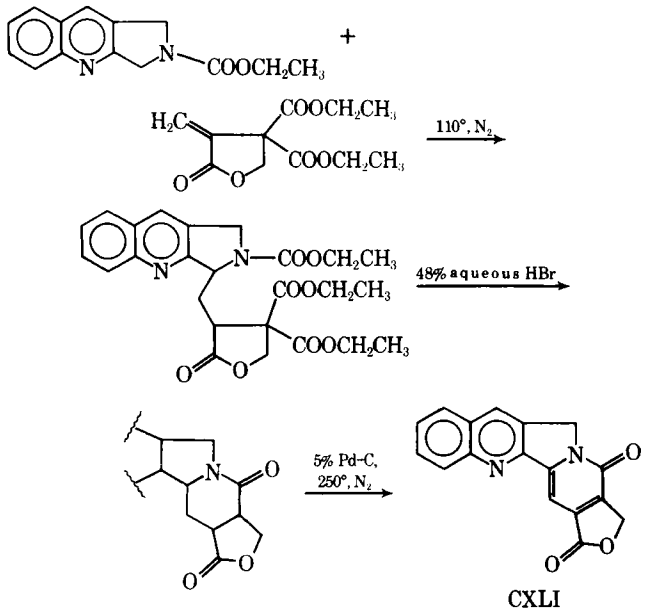


CXXXVII CXXXVIII: R = OH
CXXXIX: R = H



CXL

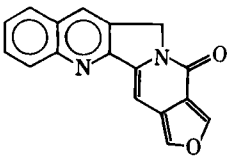
Scheme XVIII



CXLI

Scheme XIX

bearing resemblance to camptothecin involved condensation of the 3-pyrrolidinone ethylene ketal CXXXIII with the acid chloride of phthalic acid monomethyl ester CXXXIV to afford the ester amide



CXLII

CXXXV. Deketalization to CXXXVI followed by cyclization engendered the tricyclic ketone CXXXVII which, upon Friedländer condensation, led to the pentacyclic analog CXXXVIII (Scheme XVIII).

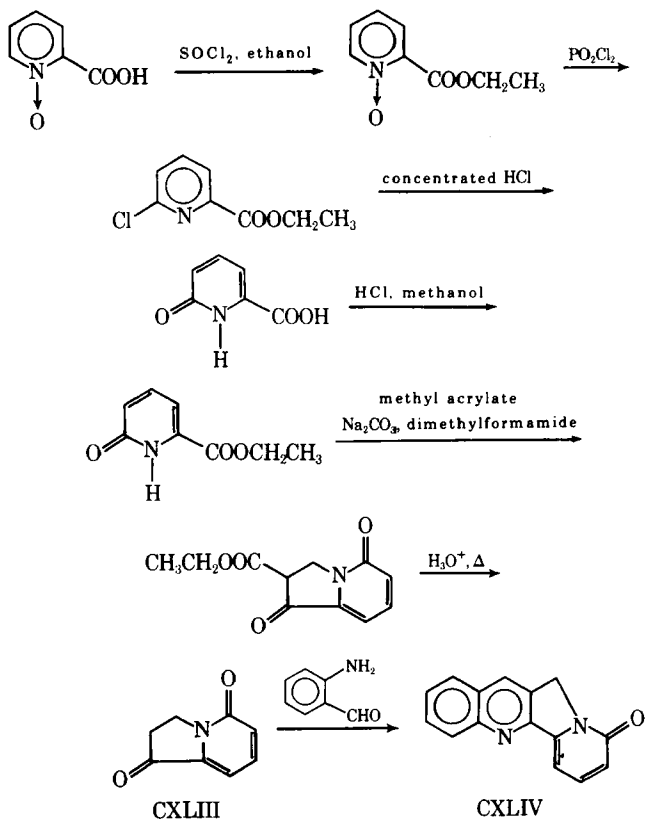
Using parallel routes, the pentacyclic species CXXXIX and CXL have also been prepared (43) (Scheme XVIII). Analog CXLI, which possesses a γ-lactone rather than the required δ-lactone, has also been prepared (44) (Scheme XIX).

Yet another pentacyclic analog of camptothecin is the furanoid derivative CXLII whose synthesis was reported in brief form (45).

Friedländer condensation of anthranilaldehyde with 3-oxopyrrolidino[1,2*f*]-2-pyridone (CXLIII) resulted in formation of the ABCD ring system CXLIV of camptothecin (46) (Scheme XX).

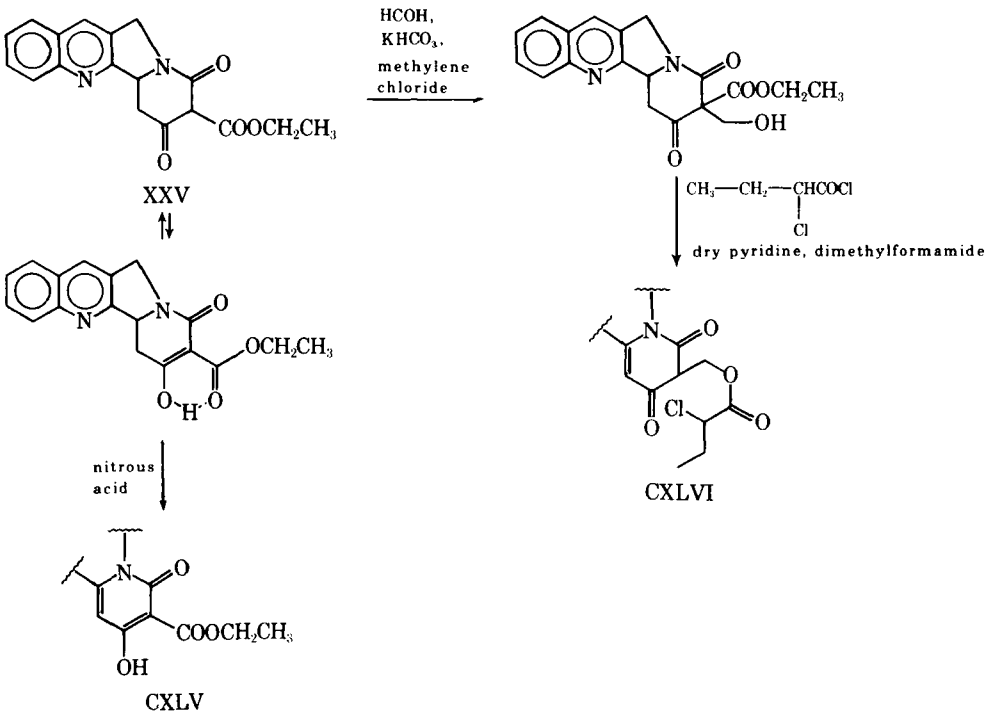
Two camptothecin analogs, CXLV and CXLVI, have been prepared (47) using the key intermediate XXV (Scheme XXI).

Condensation of base CXXII (already discussed) with β-formylpropionic acid diethyl acetal, followed by treatment with dilute hydrochloric acid and then intramolecular cyclization, gave rise to the lactam CXLVII. Following dehydrogenation, the tetracyclic analog CXLVIII was obtained (40) (Scheme XXII).



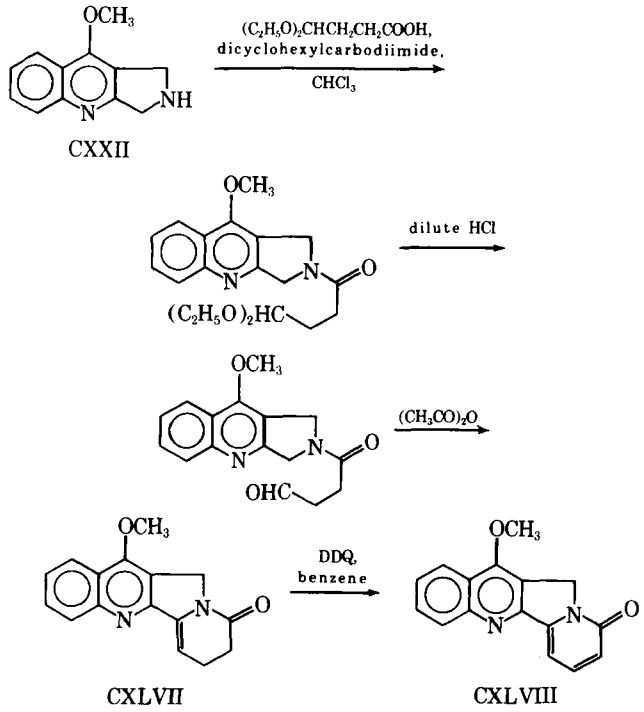
CXLIII CXLIV

Scheme XX



Scheme XXI

An unusual and interesting entry into rings ABCD of camptothecin, provided by Borch *et al.* (48), involved in the key step the condensation of the imminium salt CLI with the anion CLII. *N,N*-Dimethyl-3-cyanoquinaldamide (CL) was first prepared by Friedländer condensation between *o*-aminobenzaldehyde and ethyl 3-cyanopyruvate, followed by treatment of the resulting ester CXLIX with dimethylamine in ethanol. The next step involved the preparation of the enamine CLIII using the imminium salt CLI and the triester ion CLII. Catalytic hydrogenation of CLIII furnished the corresponding C-3 aminomethyl derivative which, without isolation, underwent an intramolecular enamine exchange-cyclization reaction to give the camptothecin analog CLIV (Scheme XXIII).



Scheme XXII

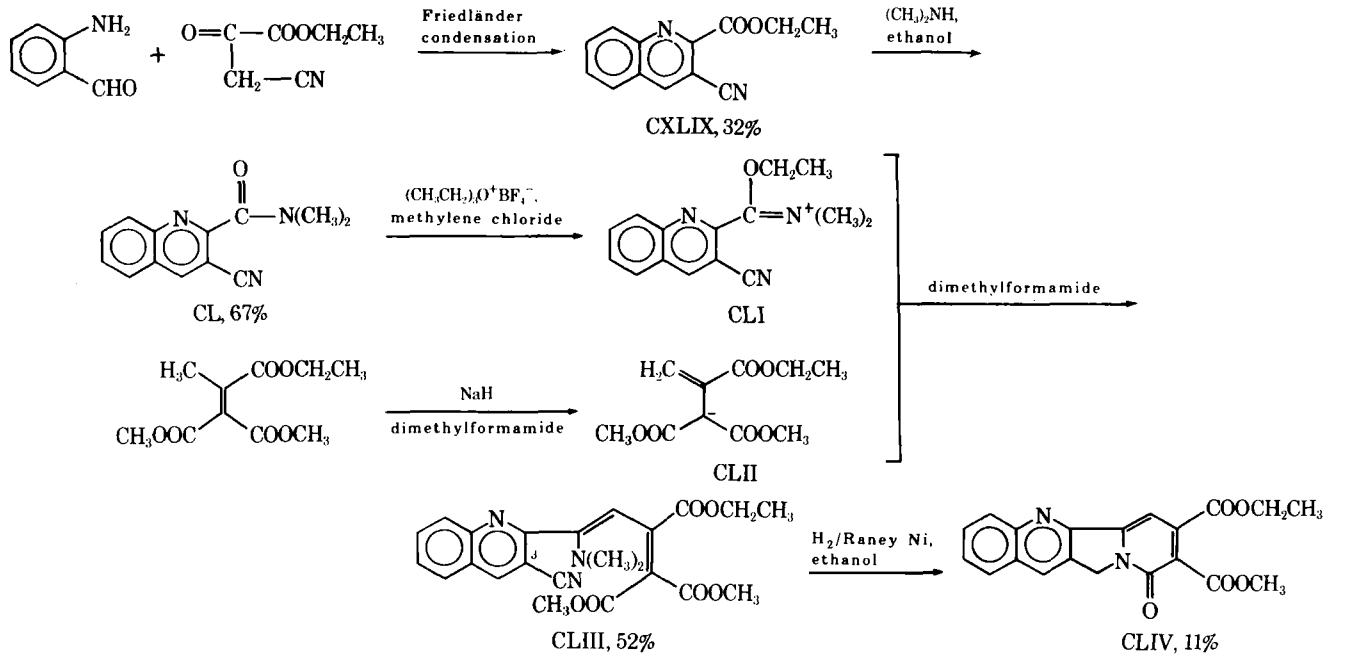
Finally, the tetracyclic pyridone CLVI has been synthesized (49) *via* Friedländer condensation of anthranilaldehyde with the pyridone CLV (Scheme XXIV).

Compounds Possessing Rings D and/or E of Camptothecin—Since the presence of an α-hydroxy-δ-lactone in camptothecin was thought to be responsible for the pharmacological activity of the alkaloid (50), several efforts at the synthesis of this system have been recorded in the literature.

Bromination of the keto diester CLVII (51) under anionic conditions in the presence of sodium hydride followed by dehydrobromination gave a mixture of olefins CLVIII and CLIX. Michael condensation of the olefinic mixture with nitroethane furnished the intermediate CLX. Ethylene ketal formation followed by catalytic reduction of the nitro group then generated lactam CLXI, which was converted to the bicyclic pyridone δ-lactone CLXII through a series of steps paralleling those described under *The Wani-Wall-Levine Synthesis* (Scheme XXV).

The synthesis of the δ-lactone CLXIII has been achieved through the condensation of triethyl 3-ethyl-1(2)-pentene-1,1,3-tricarboxylate with paraformaldehyde (52), and the preparation of the δ-lactone CLXIV has also been reported (53) (Scheme XXVI).

In a study (54) considered as a direct forerunner to a synthesis of camptothecin, the glycol CLXVII was obtained from nipecotic acid (CLXV) *via* methylene



Scheme XXIII

lactam rearrangement to CLXVI, epoxidation, and hydrolysis. The ultimate compound, CLXVIII, which incorporates rings D and E of the alkaloid, was then prepared through a sequence of reactions (Scheme XXVII) bearing close similarity to that applied in the synthesis of camptothecin and described under *The Rapoport–Tang Synthesis.*

Two additional analogs, CLXIX and CLXX, were prepared by a similar route (54). Another variation of this same α-methylene lactam approach, which led to the tricyclic analogs CLXXI and CLXXII, is described in Scheme XXVIII (54).

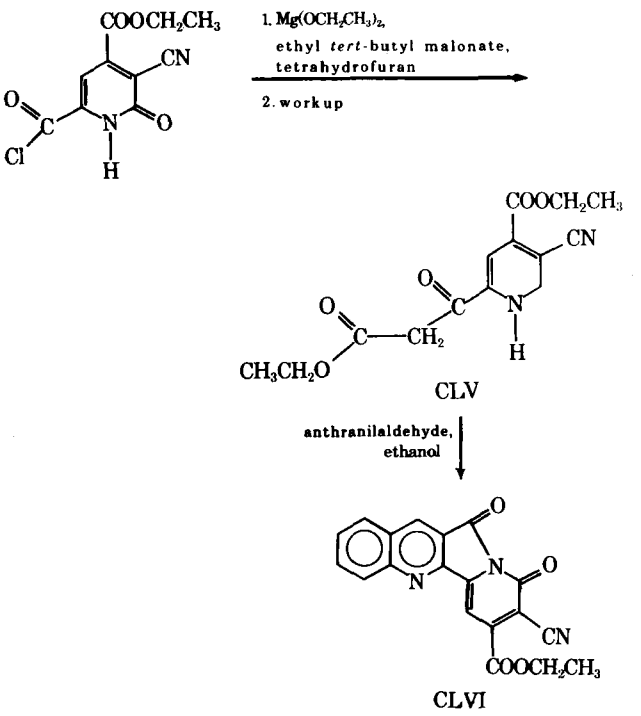
By using 4-methoxy-1-methyl-2-(1*H*)-pyridone (CLXXIII) as the starting material, a method for E ring annelation was developed by Sugasawa *et al.* (37). Vilsmeir formylation (phosphorus oxychloride in dimethylformamide) of CLXXIII supplied the pyridone aldehyde CLXXIV in 73% yield. The terminal compound CLXXV, having the DE ring system of camptothecin, was prepared through a sequence of reactions (Scheme XXIX) similar to that described under *The Sugasawa–Toyoda–Sasakura Synthesis.*

Some efforts aimed at the construction of ring E analogs of camptothecin have also been described (55).

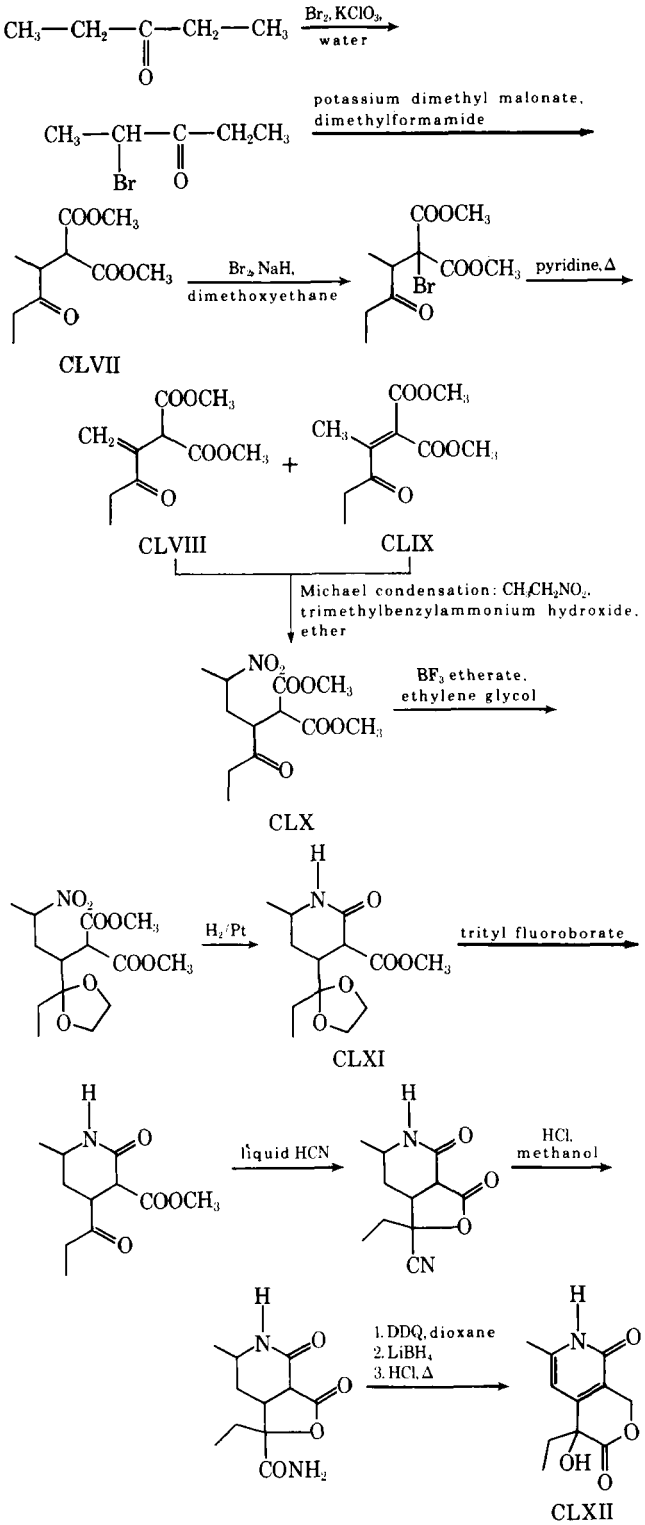
Indole–Quinolone Conversions—Following the biogenetic-type conversion of indole derivatives into quinolones, which culminated in a synthesis of camptothecin (see *The Winterfeldt Synthesis*), it was found that treatment of the tetracyclic indole CLXXVI with potassium *tert*-butoxide in dimethylformamide in the presence of oxygen resulted in formation of the quinolone CLXXVII, which was then transformed to the chloroquinoline CLXXVIII (31) (Scheme XXX). A possible mechanism for the transformation of CLXXVI to CLXXVII is given in Scheme XXXI (30).

In a recent development, the camptothecin analog CLXXX was obtained through the indole–quinolone conversion from the indole compound CLXXIX (56).

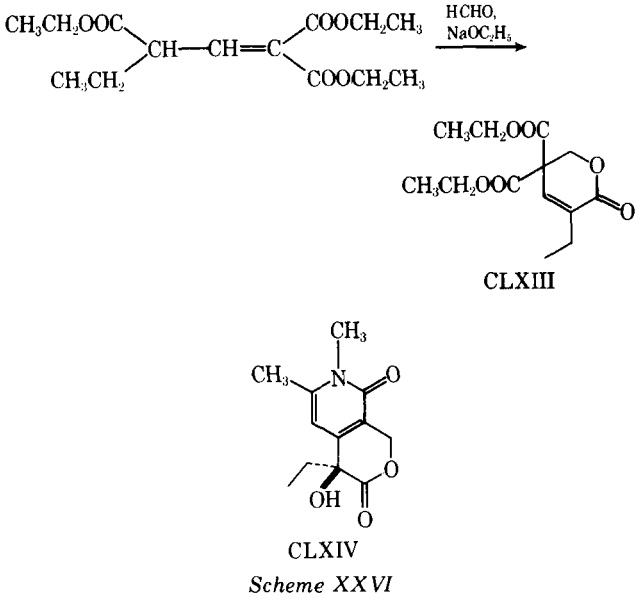
One more indole compound, which was recently synthesized as a potential intermediate to camptothecin, is the hemiketal CLXXXI (57). In a separate study, several model compounds (CLXXXII–CLXXXV) were synthesized through the indole–quinolone conversion of the corresponding indole derivatives (58).



Scheme XXIV



Scheme XXV



Scheme XXVI

PHARMACOLOGY

In the late 1950's, crude extracts of the tree *C. acuminata* (Nyssaceae) were observed to possess antitumor activity during extensive screening (59). At a later time, definite activity was demonstrated for the main alkaloid camptothecin. Tested against leukemia L-1210 in mice, camptothecin gave life prolongation as high as 100% on a daily dose of 0.25–1.0 mg/kg (60). Against rat Walker 256 carcinosarcoma, concentrations as low as 1.25 mg/kg gave significant inhibition of growth (1, 61). Moderate cytotoxic activity, ED_{50} = 0.07 mg/ml, against KB cell culture was also noted.

The effects of camptothecin on DNA have been investigated (62, 63). Some additional biological tests of camptothecin are also available (64, 65).

Following the completion of extensive preliminary testing, camptothecin was administered experimentally to 18 cancer patients at the Baltimore Cancer Research Center (59). The patients were suffering from various advanced solid tumors and were given intravenous injections of camptothecin as the sodium salt. These injections were administered approximately every 2 or 3 weeks in dosages proportional to the body weight of the patient. The most favorable responses were noted in those patients suffering from advanced GI tumors. Improvement was noted in 11 cases. In five of these, at least a 50% mass decrease in the tumor was observed. Side effects noted in these patients were generally tolerable and consisted mainly of alopecia and GI disorders.

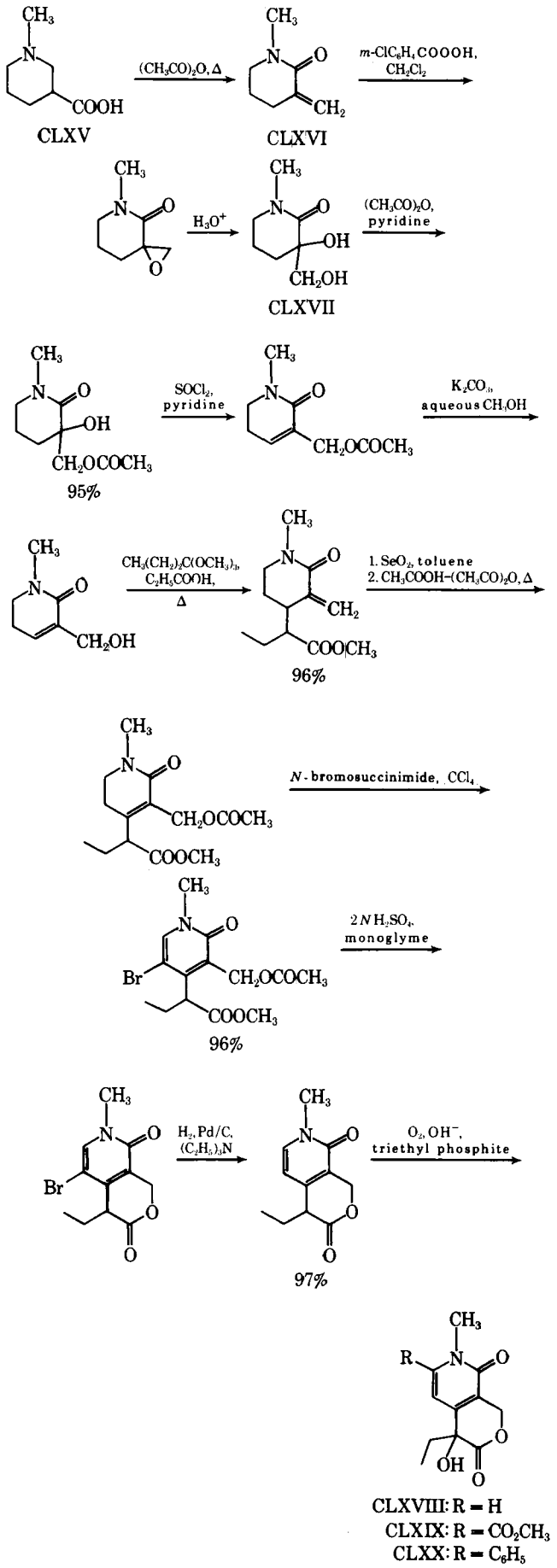
These encouraging results were not corroborated, however, in more recent tests at the Mayo Clinic in Minnesota (66). Camptothecin was used in the treatment of 61 patients with advanced GI adenocarcinoma. Only two of these (3%) showed partial objective responses after 2 months.

ADDENDUM

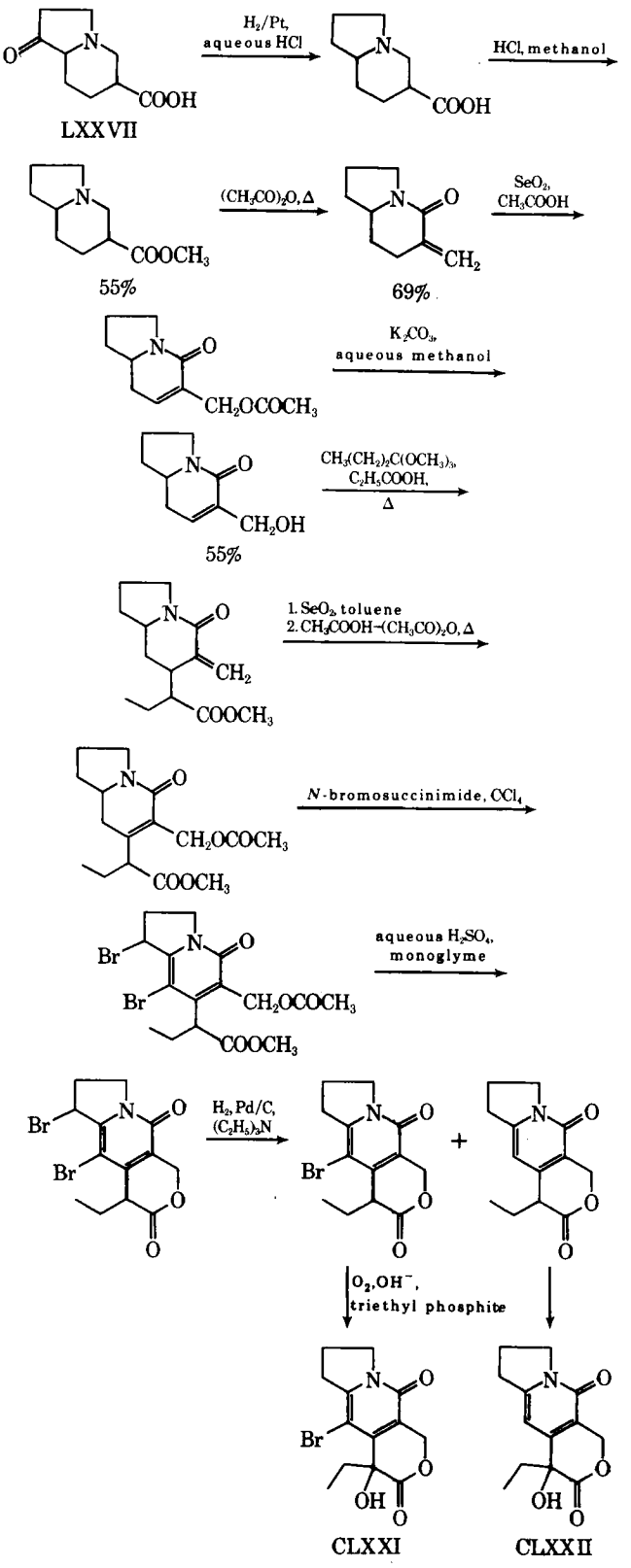
Two additional syntheses of camptothecin have appeared and are discussed below.

The Kende Synthesis—The first goal of this synthesis was the preparation of the furoic acid CLXXXVII starting from furfuraldehyde dimethyl acetal (CLXXXVI) (Scheme XXXII) (67).

The next two steps were aimed toward the construction of the pentacyclic amide CLXXXVIII, which underwent a facile and unusual alkaline hy-

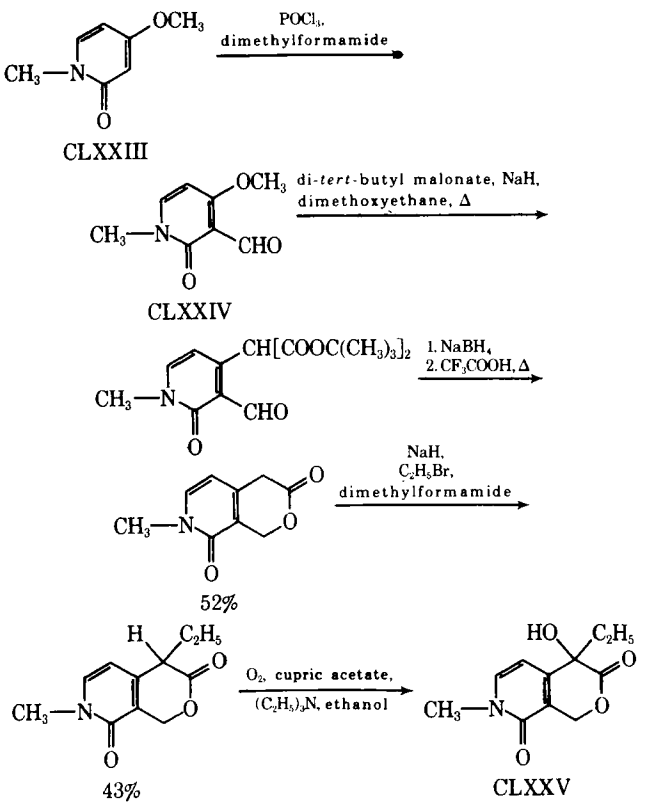


Scheme XXVII

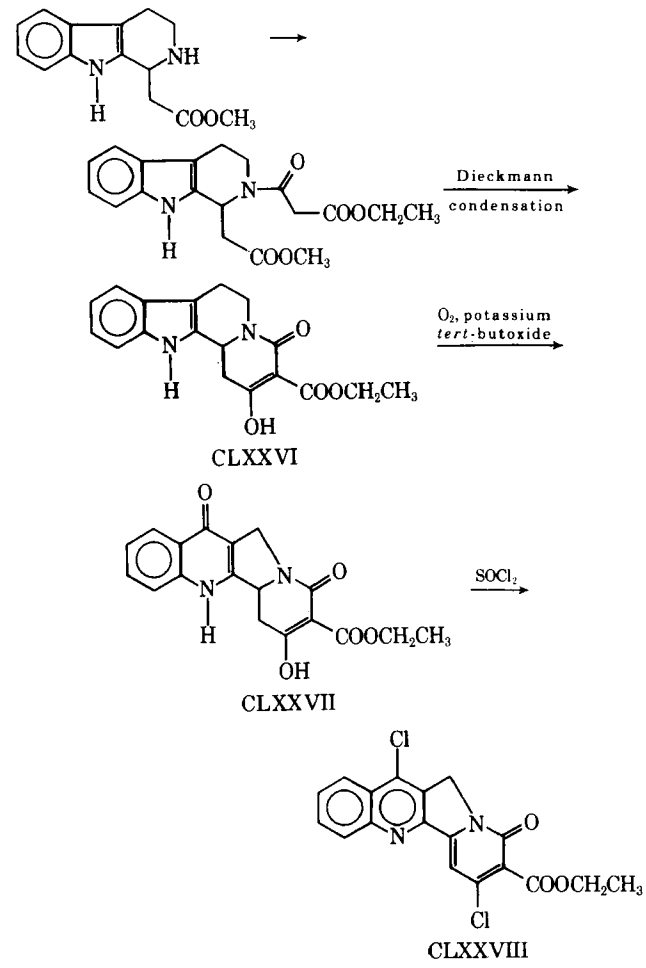


Scheme XXVIII

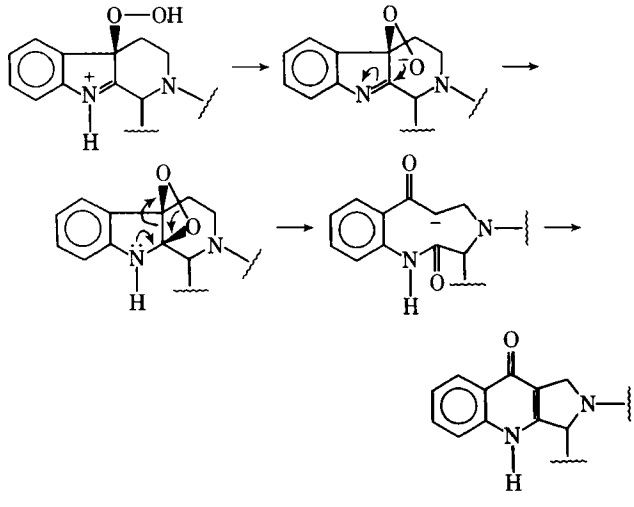
drolysis with loss of one carbon atom to yield the hydroxyamide CLXXXIX, probably by the mechanism indicated. The final steps involved construction of the known tetracyclic ester XLVIII (27), which had been previously converted to (±)-camptothecin (Scheme XXXIII).



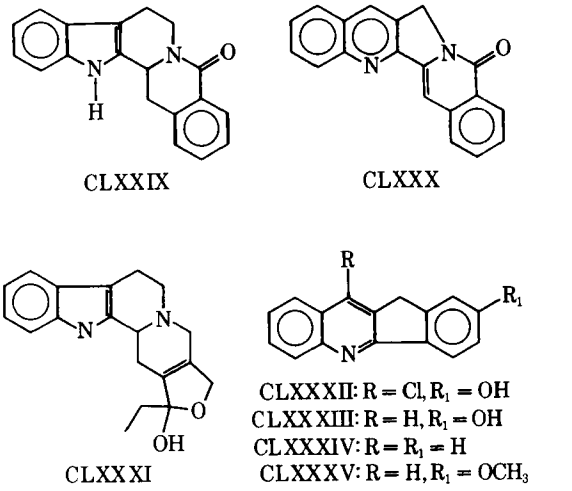
Scheme XXIX

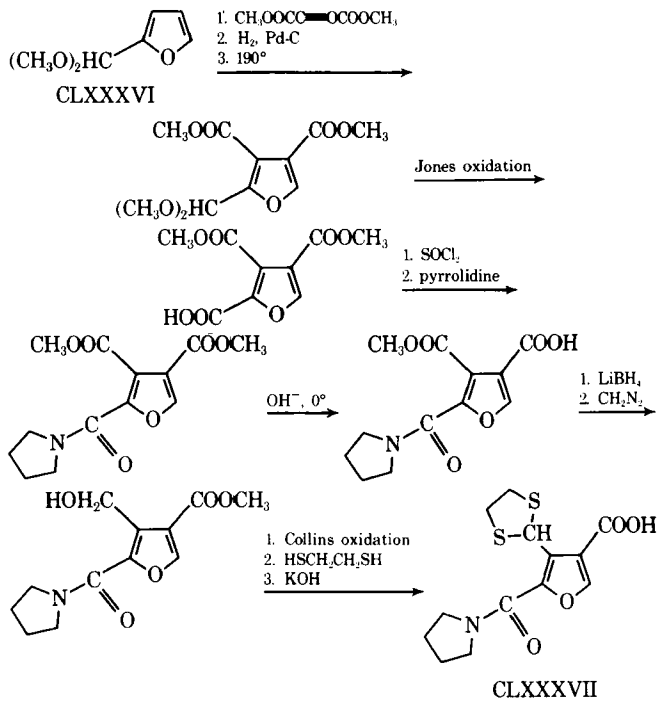


Scheme XXX

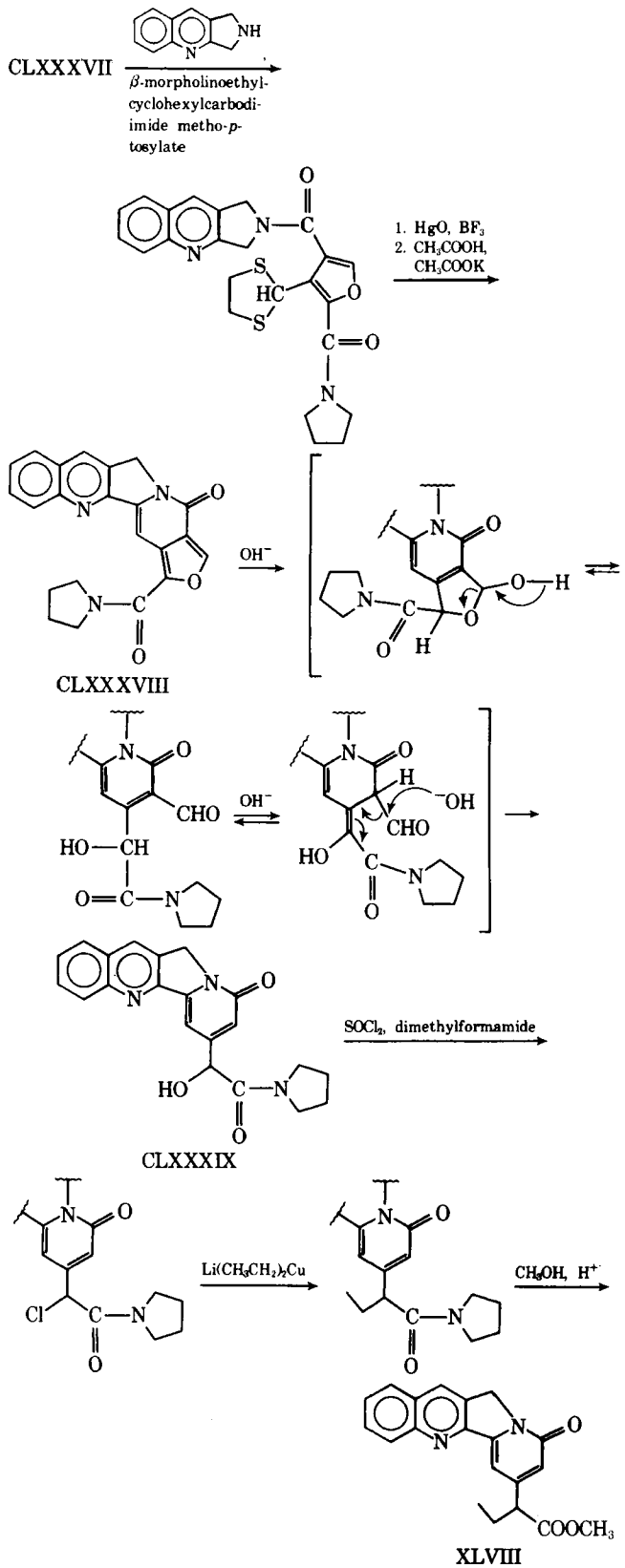


Scheme XXXI

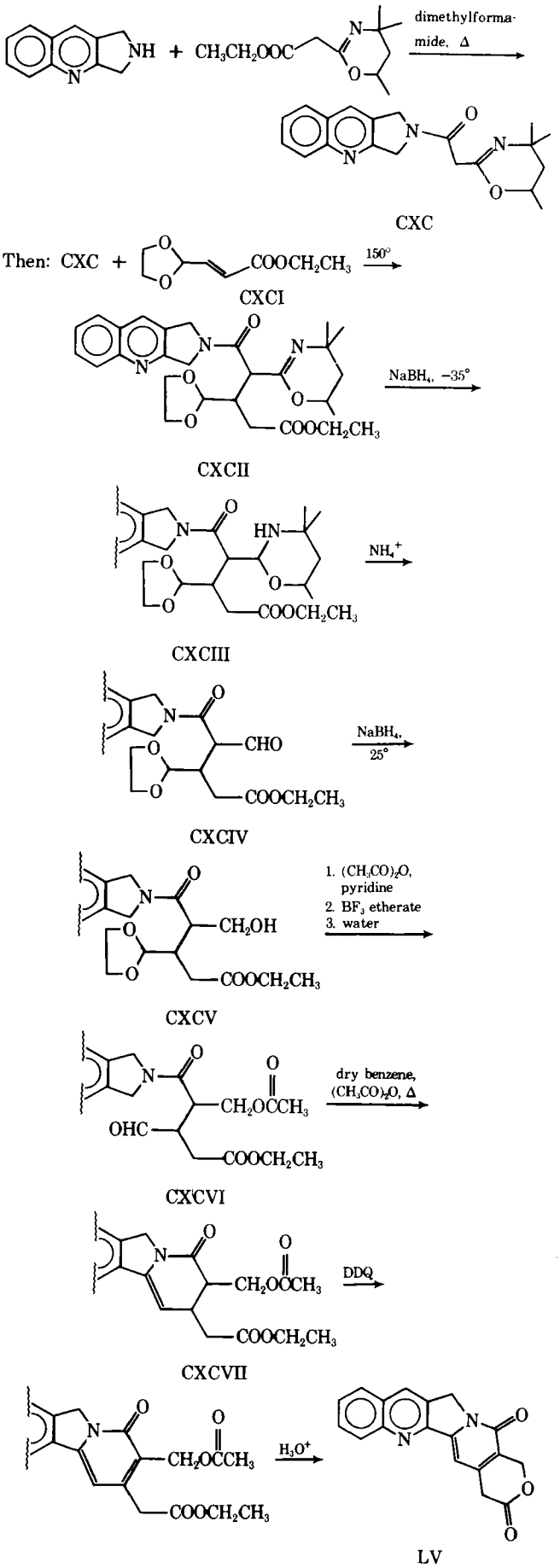




Scheme XXXII

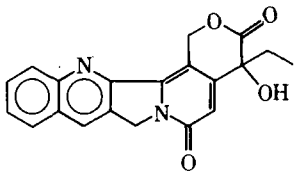


Scheme XXXIII



Scheme XXXIV

The Meyers Synthesis—The synthesis achieved by Meyers and coworkers required initially the preparation of the oxazine amide CXC, which underwent Michael addition with the unsaturated ester CXCI, thus furnishing the oxazine ester CXCII. Sodium bo-



CXCVIII

rohydride reduction to the tetrahydro derivative CXCIII, followed by cleavage to the aldehyde CXCIV, produced, following borohydride reduction, the hydroxy ester CXCV. Cyclodehydration of the aldehyde CXCVI led to the dihydropyridone CXCVII which, after oxidation and hydrolysis, was converted to deoxydeethylcamptothecin (LV). The latter compound had been previously converted to racemic camptothecin (27) (Scheme XXXIV).

Miscellaneous—The synthesis of isocamptothecin (CXCVIII) (69), which is not a natural product, as well as of other camptothecin analogs (70) has been reported. A review of the chemistry, pharmacology, and syntheses of camptothecin has appeared recently (71).

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*To whom inquiries should be directed.

RESEARCH ARTICLES

Comparison of Criteria for Content Uniformity

BRUCE FLANN

Abstract □ The criterion of lot quality for pharmaceutical unit doses based on the percentage of defectives has been generally used in the evaluation of tests determining content uniformity. This criterion is rejected in favor of joint criteria based on the deviation of the lot mean content from label claim and on the standard deviation of the unit content within the lot. By utilizing methods of simulation by computer, several tests by attributes, including those tests currently in the USP and the NF, are compared with selected tests by variables for reliability, flexibility, and simplicity. (Robustness with respect to type of distribution is rejected as a criterion because it is dependent on the definition of lot quality.) Several tests by variables are quite superior to tests by attributes with respect to reliability and flexibility. Tests for mean content and for weight variation are also examined.

Keyphrases □ Content uniformity—comparison of criteria, percentage of defectives compared to deviation of lot mean content from label claim and standard deviation of within lot unit content, computer simulations □ Statistical evaluation, content uniformity criteria—percentage of defectives method compared to joint use of deviation of lot mean content from label claim and standard deviation of within lot unit content, computer simulations □ Tablets and capsules, content uniformity—comparison of criteria for determination, computer simulations, statistical evaluations

The Pharmaceutical Manufacturers Association of the United States, the United States Pharmacopeial Convention, and the National Formulary Board have pioneered in the field of quality control of the finished pharmaceutical with official recognition of the need for a criterion of content uniformity among single-dosage units. Since 1965, the USP (1) and NF (2) have described, for some tableted pharmaceuticals, a test of this uniformity. Subsequently, they have included a special variation for application to capsules.

A review by Olson and Lee (3) mentioned that the statistics of acceptance sampling was developed during World War II for the armament industry, and shortly after the technique was applied by the pharmaceutical industry to the control of tablet weight (4). In 1960, F. Wiley of the Food and Drug Administration presented data (5) showing that the test controlling tablet weight variation was not controlling content variation. He suggested the replacement of this test with a test for content uniformity. Both old and new tests required counting the number of units beyond an acceptance range (for weight or assay, respectively) and the number beyond double-the-range where the midpoint of the acceptance range was a function either of average tablet weight or average assay.

The tests subsequently accepted by the NF and the USP are of the sequential type requiring 10 or 30 assays. Decisions are based on the number of assays beyond the range 85–115% of label claim. Also, in some cases a sample is automatically rejected if any assays are beyond the range 75–125% of label claim.

Those tests, which are based on the number of measurements (made on single units from a sample) outside an acceptance range, are called tests by attributes. Examples are described in military specifications (6). Other tests, which are based on the magnitude of one or more variables describing the sample, are called tests by variables. A well-known variation of this class of test is based on the magnitude of "the absolute deviation of the mean from the target value plus a multiple of the standard deviation," as described in another set of military specifications (7) and by Lieberman and Resnikoff (8).

Generally, it is accepted that the tests by variables are the more reliable tests of dispersion; *i.e.*, conclusions are less subject to the vagaries of random sampling, at least where the variable is normally distributed. However, there is ample evidence that the content of tablets is not always normally distributed (4, 9–11). Papers comparing the two types of tests can be divided into two groups: those that suggest that most pharmaceutical lots have an approximately

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normal distribution so that the more reliable test by variables should be preferred (4, 12, 13) and those that suggest that sufficient pharmaceutical lots have a nonnormal (and unknown) distribution so that a distribution-independent test by attributes should be preferred (14, 15).

Dunnett and Crisafio (4) compared the performance of several possible tests for weight variation by generating 200 groups of pseudoweights from numbers in a table of random normal deviates. To obtain the weights, they combined the gaussian random numbers with a preselected standard deviation for the lot (by definition, lot mean weight equals 100%). They assessed lot quality by computing the percentage of defective tablets in the lot, *a defective tablet being defined as one that differs in weight from the lot mean by more than 5% of the lot mean.* Then they plotted, for each test, the percentage of samples passing the test against the percentage of defective tablets in the lot. By repeating this process for a series of standard deviations, the *OC* (operating characteristic) curve was estimated.

Subsequently, other authors (14, 16), utilizing computer simulation, obtained estimated *OC* curves for several tests for content uniformity. The method was analogous to that of Dunnett and Crisafio (4).

In the above, lot quality has been equated with percentage defective; in the following article, lot quality is equated jointly with both the deviation of the lot mean content from label claim and the standard deviation of the content of individual units about the lot mean content. Hence, the *OC* curves currently in the literature are two dimensional, giving the probability of a sample from a lot passing the test as a function of the percent defective, *i.e.*, the percentage of the units in a lot with an assay beyond an acceptance range. In contrast, the following curves are three dimensional, giving the probability of a sample from a lot passing as a function of the mean assay of the lot and of the standard deviation of the assay of the units of the lot.

In this article, *OC* curves for a selection of reasonable tests, initially restricted to those requiring 20 assays, are estimated by computer simulation, with the restriction that the simulated assays have a normal distribution about the lot mean. Both the lot mean and the standard deviation are varied systematically. Subsequently, the curves for the more promising tests are obtained for four nonnormal distributions already suggested in the literature (9, 14). Then the effect of varying the number of assays per sample is studied. And, finally, sequential variations of these tests are compared.

The general use of the normal distribution in this work does not imply any assumption of normality in the content distribution in pharmaceutical lots. There is ample evidence (4, 11) that an appreciable proportion of the many distributions of drug content are significantly nonnormal. Even an extreme binodal distribution is possible, although presumably very rare. This same evidence shows that the normal distribution is a reasonable distribution to be used initially in the simulation of pharmaceutical assays.

For practical reasons, a criterion for content uniformity can only be applied in conjunction with a criterion for mean content. These criteria can be applied as a single merged criterion, as two criteria combined in a single expression, or as separate criteria.

EXPERIMENTAL

The simulations were carried out in FORTRAN IV G on a large computer[1]. Storage of the program required 50K bytes, and additional storage of approximately 2–6K bytes was required for the results of each test. Up to 11 min of computing time was required for a run. The results, stored on magnetic tape, were then used in conjunction with other programs to generate instruction tapes for an incremental plotter[2] which, in turn, produced the appended graphs.

Estimation of the operating characteristic curves was made as follows:

1. Twenty-five values of δ, generally increasing from 0.0 in steps of 0.5, were selected to represent the percentage deviation of the lot mean from label claim. All values of δ were taken as positive, since the *OC* curves for lots with means below label claim will be mirror images of those with means above label claim.
2. Twenty-four or more values of σ, generally increasing from 0.5 in steps of 0.5, were selected to represent the standard deviation of the lot about its mean, expressed as a percentage of label claim.
3. Values of δ and σ were taken systematically to form 600 or more pairs of lot parameters.
4. Thirty numbers were randomly generated from a population of numbers with mean 0.0, standard deviation of 1.0, and a selected probability distribution, either normal or nonnormal, as discussed below.
5. By using the first or next pair of lot parameters, 30 assays, expressed as deviations from label claim, were derived by multiplying each of the random numbers by σ and adding to δ.
6. Then using the numbers of assays specified by the test, each sample was evaluated by each test. The result was stored according to the values of the parameters and test number. If the test was of the sequential type, the number of assays required was also stored.
7. Then the program returned to the next pair of parameters under Step 5 until all pairs of parameters had been used. Then the program returned to the generation of the next set of 30 assays under Step 4 and so on until a thousand sets of assays had been generated.
8. The results were expressed as percentages of samples passing according to the values of the parameters and of the test numbers. For sequential tests, the average numbers of assays required were similarly calculated.
9. The *OC* curves were drawn.
10. The *ASN* (average sample number) curves for sequential tests were also drawn in cross section or in a three-dimensional representation.

The following scheme was used to generate pseudorandom numbers of mean 0.0, standard deviation 1.0, and the selected probability distribution.

A library function, RANDU, is available. Upon input of a suitable random integer, it generates both a new integer for the next cycle and a number between 0.0 and 1.0, which has uniform probability of being equal to any value in this range. To generate a normally distributed random number, another library function, GAUSS, calls RANDU 12 times and adds the 12 numbers to obtain a new number, which belongs to a population with mean 6.0 and a virtually normal distribution. Subtracting 6.0 from this number and multiplying by a normalizing factor yield the desired gaussian random number. (GAUSS cannot generate numbers more than 5 standard deviations from the mean, and presumably the frequency will be low before this limit is reached.)

The subroutine GAUSS cannot be used directly when nonnormal distributions are desired. Instead, the desired distribution is

[1] IBM 360/85 computer (System-Dimensions Ltd., Ottawa, Canada).
[2] Calcomp 663 plotter.

Table I—Percentage[a] of Single-Dose Units more than 15.05% from Label Claim

Standard Deviation about Mean Assay, %	Deviation of Population Mean Assay from Label Claim, %												
	0.0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0
1.0														1.9
2.0											0.5	2.0	6.2	15.2
3.0								0.3	0.9	2.1	4.5	8.5	15.3	
4.0					0.2	0.5	1.1	2.1	3.9	6.3	10.2	15.4		
5.0	0.2	0.2	0.4	0.7	1.3	2.1	3.5	5.2	7.6	11.1	15.5			
6.0	1.1	1.2	1.6	2.2	3.2	4.6	6.4	8.7	11.8	15.5	20.1			
7.0	3.1	3.2	3.8	4.6	5.9	7.5	9.6	12.4	15.6	19.4				
8.0	6.1	6.2	6.8	7.7	8.9	10.9	13.1	15.9	19.1					
9.0	9.4	9.7	10.1	11.0	12.6	14.4	16.6	19.3						
10.0	13.2	13.2	13.9	14.9	16.2	17.9	20.1							
11.0	16.9	17.2	17.7	18.6	19.9									
12.0														

[a] Only percentages between 0.15 and 20.55% are included in Table I. The sample size is 30,000.

expressed as the sum of n components, all with normal distributions, where the ith component is present in proportion P_i and has a mean of $\bar{\epsilon}_i$ and a standard deviation of σ_i'.

Since the sum of the proportions must equal 1.0 and, by choice, the mean and standard deviation of the random numbers are to be 0.0 and 1.0, respectively, the values of P_i, $\bar{\epsilon}_i$, and σ_i' must be found so that:

$$\sum^{n} P_i = 1.0 \qquad \text{(Eq. 1)}$$

$$\sum^{n} P_i \bar{\epsilon}_i = 0.0 \qquad \text{(Eq. 2)}$$

$$\sum^{n} P_i(\bar{\epsilon}_i^2 + \sigma_i'^2) = (1.0)^2 \qquad \text{(Eq. 3)}^3$$

Now a scale of unit length can be subdivided into n regions such that the boundaries of the ith subregion are located at $\Sigma^{n-i-1} P_i$ and $\Sigma^{n-i} P_i$. The program calls the next random number from RANDU and determines the subregion in which it falls. If this is the ith subregion, the next gaussian number to be generated is assigned to the ith component, *etc.*

As a crosscheck, the program[4] obtains the mean, standard deviation, coefficient of skewness, and coefficient of kurtosis of the population of numbers generated.

To compare different tests, certain arbitrary numerical limits are required. The value 15.05, found in the description of the tests in several tables, reflects the 85.0–115.0% acceptance range for single-dosage units in the current pharmacopeial tests. Similarly, the value 25.05, required only for a few tests, reflects the rejection range for single-dosage units. The value 7.55 was chosen as the limiting value for the standard deviation and for the deviation of the mean from label claim, both expressed as a percentage of label claim. By use of these arbitrary limits, the tests were described so that the specifications implied by the different tests were as nearly equivalent as possible, thus simplifying the intercomparison.

Also, to compare tests, the four following criteria were developed: reliability, robustness, flexibility, and simplicity.

Ideally a test for content uniformity will always pass or always fail a particular lot of tablets according to its degree of uniformity. In practice, as can be seen from the figures, there is a region of limited uniformity in which a lot sometimes passes and sometimes fails, depending on the vagaries of random sampling. The smaller this region, the more reliable is the test.

An *ad hoc* estimator of the reliability of the various tests is given by Eq. 4, in which "area" refers to the area between that contour indicated by the subscript and the axes in the *OC* curves found in Figs. 2–5:

$$\text{reliability} = \left(1.0 - \frac{\text{area}_{5\%} - \text{area}_{95\%}}{2 \times \text{area}_{50\%}}\right) \times 100\% \qquad \text{(Eq. 4)}$$

This definition arbitrarily equates the case, where the area between the 5 and 95% contours is twice that between the 50% contour and the axes, with 0% reliability.

Robustness of a statistical test refers to its ability to come to the same conclusion, on the average, for populations of a given quality, regardless of the violation of assumptions. Here an arbitrary measure of the robustness of selected tests with respect to type of distribution was obtained by estimating the deviation of the *OC* curve (still in three dimensions) for a test as applied to a set of four populations, each with a particular nonnormal distribution from the corresponding curve for normal distributions. This was carried out by computer simulation as already described except that the output was in the form of tables, the 12 columns of which correspond to the 12 integral values of the deviation of the mean from label claim from 0.0 to 11.0 inclusive and the 14 rows of which correspond to the standard deviation of units about population mean from 1.0 to 14.0 inclusive. The values in the body of the tables give the percentage of times for which the samples from respective populations passed the particular test. After tables were generated for four nonnormal and the normal distribution, four tables of differences were obtained by subtracting the normal table from the four nonnormal tables. Then the absolute values of all the numbers in each table of differences were added. This sum is proportional to the volume between the curves, and the larger it is the less robust is the test. This sum was arbitrarily divided by 4000 and subtracted from 1.0. As shown by Eq. 5, this difference multiplied by 100% was taken as a measure of robustness:

$$\text{robustness} = \left[1.0 - \frac{\sum^{n=4} \begin{pmatrix}\text{volume}\\ \text{between}\\ \text{curves}\end{pmatrix}}{4000}\right] \times 100\% \qquad \text{(Eq. 5)}$$

As with the coefficient of reliability, a value of 100% would indicate a completely robust test while unsatisfactory tests would have a low or even negative value for this coefficient.

[3] Define f_i as the frequency of the ith component in an unlimited number, N, of the random numbers. Then:

$$P_i = f_i/N$$

By the definition of variance:

$$(\sigma_i')^2 = [\Sigma(\epsilon_i - \bar{\epsilon}_i)^2]/f_i$$
$$= [\Sigma(\epsilon_i)^2]/f_i] - (\bar{\epsilon}_i)^2$$

Therefore:

$$\sum^{n} P_i[(\bar{\epsilon}_i^2) + (\sigma_i')^2] = \sum^{n} P_i[\Sigma(\epsilon_i)^2]/f_i$$
$$= \left\{\sum^{n}[\Sigma(\epsilon_i)^2]\right\}/N$$

Since, by choice, the mean of all the numbers is 0.0, the latter quantity is the variance of these numbers.

[4] The details of the program and the flowsheet are available on request.

Table II—Comparison of the Distribution of Simulated Assays with That Predicted for a Normal Population

Standard Deviation about Population Mean[a], %	Percentage of Assays beyond the Range 84.95–115.05%		Standard Deviation of Experimental Results, Calculated[b]
	Experimental	Calculated	
5.0	0.20	0.26	0.03
6.0	1.10	1.21	0.06
7.0	3.14	3.16	0.10
8.0	6.06	6.04	0.14
10.0	13.15	13.23	0.20
12.0	21.03	20.98	0.24

[a] In this case, the population mean is the label claim (100%). [b] Calculated through Eq. 6.

Flexibility, the adaptability of the basic test criteria to different test formats without, in effect, altering the definition of acceptable lot quality, was not quantified.

And simplicity, the ease with which the basic test criteria or the format are understood, remembered, and applied, was not quantified either.

TESTS EXAMINED AND RESULTS

The normality of the pseudorandom numbers can be tested when the computer program includes the requirement that the number of defective units in each group of 30 assays for each combination of lot mean and standard deviation be stored by addition, and then the sum for 1000 groups of 30 can be converted to a percentage. Representative values of those obtained are given in Table I. Certain of these values, restricted to those for populations with mean equal to the label claim, are compared with calculated values in Table II. The calculated values were obtained by interpolation in a table of the normal distribution (17), where z equals the ratio of 15.05% to the standard deviation of the lot about the population mean. The standard deviation to be expected in the proportion of experimental, *i.e.*, computer-generated, assays beyond the acceptance range can be calculated with the aid of Eq. 6:

$$\sigma'^2 = P(1 - P)/N \qquad \text{(Eq. 6)}$$

as derived in Ref. 18, for binominal distributions. The experimental and calculated values are in good agreement. There is an indication of a low frequency in the tails of the distribution, but this will have a trivial effect on the *OC* curves.

For all computer simulations of 1000 groups of 30 numbers from a given distribution, the same set of random numbers was used for practical reasons. However, for different distributions, different sets of numbers were generated.

Test of Mean Content—Very frequently, pharmacopeial monographs specify that one weigh, powder, and mix 20 dose units and assay an aliquot. The lot is accepted if the assay is not beyond certain limits. Although the variance is larger, this assay has the same expected value as the mean of the assays of 20 individual dose units. By computer simulation, the probability of acceptance by this latter test for lots of various means and standard deviations is shown graphically in Fig. 1. In this and other cases, the normal distribution of content within lots is used unless the nonnormal is specified.

Tests for Content Uniformity—The numbering system for distinguishing between tests uses a three-component number with components joined by hyphens. The first component, the type number, lies between 100 and 199 if the test is a test by variables; otherwise, it lies between 0 and 99. The second component indicates the number of possible conclusions. And the third number indicates the number of assays required by the test. For sequential tests, this number can take the form 10/20, 10/30, or 10/20/30. Variations of sequential tests are distinguished by a letter following the number of assays. Generally, tests are described and discussed in the order in which the results appear in the tables. Comparison of types of tests are generally made with variations

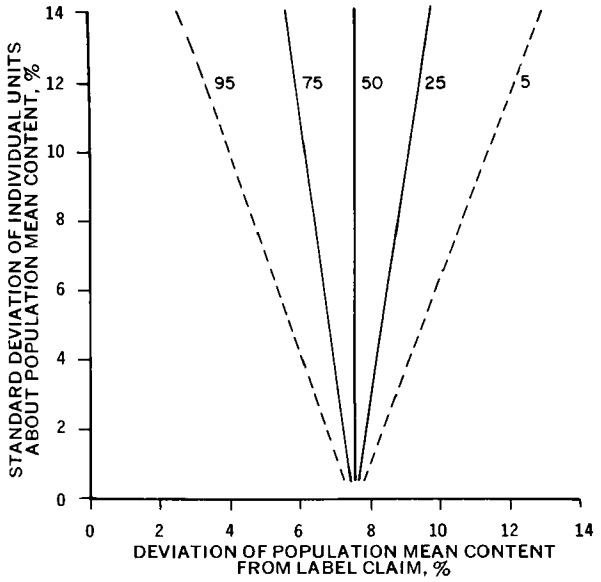

Figure 1—*The* OC *curve of an idealized form of the pharmacopeial test for mean content. The sample passes if the mean of 20 individual assays is not greater than 7.55% from label claim. The five contour lines, labeled 95, 75, 50, 25, and 5, respectively, indicate the probability of samples passing the test, expressed as a percentage.*

requiring a fixed number of assays, generally 20 assays. All tests are described in terms of percentages of label claim.

Comparison of Nonsequential Tests for Reliability and Robustness—

Test 10-2-20: If the mean assay for 20 dose units is beyond the range 92.45–107.55% of label claim or if two or more individual assays are beyond the range 84.95–115.05%, the lot fails.

Test 80-2-20: If seven or more individual assays from 20 dose units are beyond the range 92.45–107.55% of label claim, the lot fails.

Test 90-2-20: If the mean assay for 20 dose units is beyond the range 92.45–107.55% of label claim, or if three or more individual assays are beyond the range 87.74–112.26%, or if any individual assays are beyond the range 81.88–118.12%, the lot fails.

Test 100-2-20: If the mean assay for 20 dose units is beyond the range 92.45–107.55% of label claim or if the standard deviation of these assays is greater than 7.55%, the lot fails.

Test 120-2-20: If the mean assay for 20 dose units is beyond the range 92.45–107.55% or if the sum of the (positive) deviation of the mean assay from label claim (100%) plus 2.0 times the standard deviation of the same assays is greater than 15.05%, the lot fails.

Test 121-2-20: This test is the same as 120-2-20, except the factor 1.8 is used instead of 2.0.

Test 124-2-20: This test is the same as 121-2-20, except 2.4 times the mean deviation is used instead of 1.8 times the standard deviation.

Test 130-2-20: If the sum of the (positive) deviation of the mean assay of 20 dose units from label claim (100%) plus 0.90 times the standard deviation of these assays is greater than 7.55%, the lot fails.

Test 134-2-20: This test is the same as 130-2-20 except 1.2 times the mean deviation is used instead of 0.90 times the standard deviation.

Test 140-2-20: If the sum of the square of the deviation of the mean assay of 20 dose units from label claim (100%) plus the variance, *i.e.*, the square of the standard deviation of these assays, is greater than $(7.55\%)^2$, the lot fails.

Test 150-2-20: If the mean of the (absolute) deviations of the assays of 20 dose units from label claim (100%) is greater than 7.55%, the lot fails.

Test 160-2-20: If the mean square of the deviations of the assays of 20 dose units from label claim (100%) is greater than $(7.55\%)^2$, the lot fails.

Table III—Comparison of Basically Different Tests for Content Uniformity (All Tests Require 20 Assays)

Test	Class of Test	Abbreviated Description of Test[a], Sample Passes If:	Estimate of Reliability[b], %	Estimate of Robustness[c], %	*OC* Curve, Figure
10-2-20	By attributes	$\lvert\bar{X}\rvert \leq 7.55$, $(No._{20} > 15.05\%) \leq 1$	49	46	2A
80-2-20	By attributes	$(No._{20} > 7.55\%) \leq 6$	41	−3	2B
90-2-20	By attributes	$\lvert\bar{X}\rvert \leq 7.55$, $(No._{20} > 12.26\%) \leq 2$ and $(No._{20} > 18.11\%) = 0$	55		2C
100-2-20	By variables	$\lvert\bar{X}\rvert \leq 7.55$, $S \leq 7.55$	53	73	3A
120-2-20	By variables	$\lvert\bar{X}\rvert \leq 7.55$, $(\lvert\bar{X}\rvert + 2S) \leq 15.05$	60		See 3B[d]
121-2-20	By variables	$\lvert\bar{X}\rvert \leq 7.55$, $(\lvert\bar{X}\rvert + 1.8S) \leq 15.05$	59	73	3B
124-2-20	By variables	$\lvert\bar{X}\rvert \leq 7.55$, $(\lvert\bar{X}\rvert + 2.4M) \leq 15.05$	58		See 3B[e]
130-2-20	By variables	$\lvert\bar{X}\rvert + 0.9S \leq 7.55$	52		3C
134-2-20	By variables	$\lvert\bar{X}\rvert + 1.2M \leq 7.55$	52		See 3C[e]
140-2-20	By variables	$(\bar{X})^2 + S^2 \leq (7.55)^2$	58		See 3E[e]
150-2-20	By variables	$\Sigma\lvert\bar{X}\rvert/N \leq 7.55$	51	40	3D
160-2-20	By variables	$(\Sigma X^2)/N \leq (7.55)^2$	59	73	3E
170-2-20	By variables	$(\Sigma\lvert X^3\rvert)/N \leq (7.55)^3$	60		3F

[a] The symbols used in this column have the following meanings: X = deviation of individual assays from label claim expressed as a percent; $\bar{X}$ = mean value of X; S = standard deviation of X about $\bar{X}$; M = mean deviation of X about $\bar{X}$; N = number of values of X involved, *i.e.*, 10, 20, or 30, in the generalized format of certain tests by variables. Similarly, superscript or subscript 10, 20, or 30 indicates the total number of assays in question. [b] Reliability is defined by Eq. 4 in the text. [c] Robustness is defined by Eq. 5 in the text. The estimates of robustness have been brought forward from Table V. [d] Similar to Fig. 3B; however, the contour lines are closer to the origin. [e] Virtually the same as the quoted figure.

Table IV—Characterization of Normal and Nonnormal[a] Populations Utilized in Evaluating the Robustness of Certain Tests for Content Uniformity

	Description of Components				Description of Population Generated[b]			
					Moments about Zero		Coefficient of	
Type of Distribution	Number of Components	Proportion of Population	Standard Deviation (Relative)[c]	Mean (Relative)[c]	First	Second	Skewness	Kurtosis
Normal	1	1.000	1.000	0.000	0.003	1.008	−0.004	2.886
Platykurtic	2	0.500 0.500	0.830 0.830	1.000 −1.000	0.003	1.000	−0.005	2.280
Leptokurtic	2	0.500 0.500	0.500 2.000	0.000 0.000	−0.007	0.992	−0.059	5.149
Leptokurtic skewed	2	0.910 0.090	1.000 1.000	−0.317 3.205	0.011	1.023	1.037	4.486
Platykurtic	10	0.100 (all)	1.000 (all)	$[-2.250 + (n - 1) \times 0.500]$, where $n = 1, 2, \ldots, 10$	−0.008	0.999	−0.009	2.456

[a] The nonnormal populations correspond to those suggested by Haynes *et al.* (9, 14). [b] Based on a sample of 30,000 numbers. [c] The relative values must be divided by a normalizing factor before use so that Eq. 3 of the text is not violated.

Table V—Estimation of Robustness of Selected Tests for Content Uniformity (All Tests Require 20 Assays)

			Scores[b] for Nonnormal Distributions[c]					Estimate of Relative Robustness,[d] %
Test	Class of Test	Abbreviated Description of Test[a], Sample Passes If:	Platykurtic	Leptokurtic	Leptokurtic Skewed	Platykurtic (10 Components)	Total Score	
10-2-20	By attributes	$\lvert\bar{X}\rvert \leq 7.55$, $(No._{20} > 15.05\%) \leq 1$	279	645	1015	202	2141	46
80-2-20	By attributes	$(No._{20} > 7.55\%) \leq 6$	407	1853	1686	173	4119	−3
100-2-20	By variables	$\lvert\bar{X}\rvert \leq 7.55$, $S \leq 7.55$	131	423	428	104	1086	73
121-2-20	By variables	$\lvert\bar{X}\rvert \leq 7.55$, $(\lvert\bar{X}\rvert + 1.8S) \leq 15.05$	124	390	453	92	1059	73
150-2-20	By variables	$(\Sigma\lvert\bar{X}\rvert)/N \leq 7.55$	269	949	1004	161	2383	40
160-2-20	By variables	$(\Sigma X^2)/N \leq (7.55)^2$	108	423	434	120	1085	73

[a] Table III, Footnote *a*, defines the symbols required by this column. [b] The "score" is proportional to the scalar volume between *OC* curves of the nonnormal distribution and the normal distribution. [c] The distributions are described in Table IV. [d] Robustness is defined by Eq. 5 in the text.

Test 170-2-20: If the mean cube of the (absolute) deviations of the assays of 20 dose units from label claim (100%) is greater than $(7.55\%)^3$, the lot fails.

The numerical results obtained for these tests are summarized in Table III. Figures 2 and 3 compare the application of the tests to samples from normal populations. The *ad hoc* estimates of reliability are derived from these figures. By using the four nonnormal populations described in Table IV, the estimates of robust-

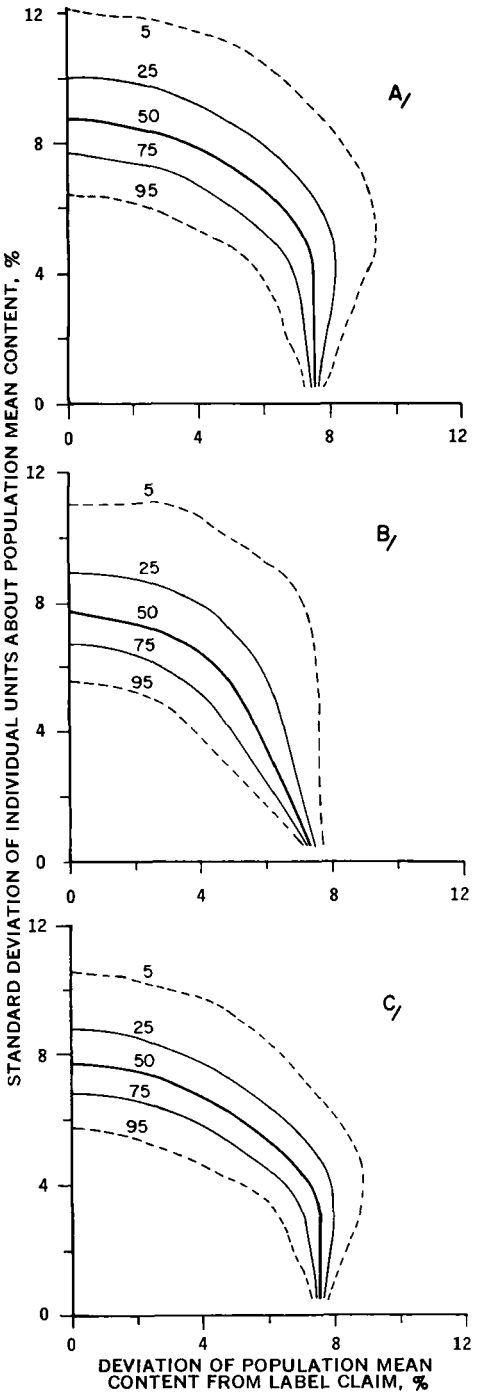

Figure 2—*The* OC *curves for three tests by attributes, each requiring 20 assays, for content uniformity. The tests are: (A) No. 10-2-20,* $|\bar{X}| \leq 7.55$, $(No._{20} > 15.05\%) \leq 1$; *(B) No. 80-2-20,* $(No._{20} > 7.55\%) \leq 6$; *and (C) No. 90-2-20,* $|\bar{X}| \leq 7.55$, $(No._{20} > 12.26\%) \leq 2$, $(No._{20} > 18.11\%) = 0$. *The symbols are defined in Footnote* a, *Table III.*

ness are derived within Table V which, in turn, is derived from tables giving in detail the differences between the *OC* curves obtained with normal and nonnormal distributions. Since the significance of the estimates of robustness with respect to pharmaceutical quality is questioned, only a few values were derived for comparison with earlier work by Haynes *et al.* (14).

The results of Table III clearly show that the reliabilities of the tests by attributes are lower than most of the tests by variables. Nevertheless, sequential variations of these tests were examined to facilitate comparison with the work by Haynes *et al.* (14). On the other hand, Test 170-2-20 is not considered further, despite the high efficiency, because it seems unlikely that the adverse effects of unit doses varying from label claim will vary as the third power of this variation. Tests 120-2-20 and 124-2-20 also are not considered further because of their resemblance to Test 121-2-20. Test 90-2-20, suggested by Haynes *et al.* (14), is not considered further because it is not fundamentally different from Test 10-2-20 when lot quality is measured in terms of mean content and standard deviation. Also, those tests with relatively low reliabilities (<56%) are not considered further.

This latter decision is somewhat arbitrary in view of the differences in shape and position of the 50% contour line for the various tests. Test 100-2-20 probably has a moderately low reliability simply because it encloses a relatively high proportion of points representing high standard deviations. However, the premise of the test, that the acceptable limits for the deviation of the mean and for the standard deviation are mutually independent, seems unlikely. (In contrast, Tests 130-2-20 and 134-2-20 seem to be unduly harsh with respect to lots with both a moderately high deviation of the mean and a moderately high standard deviation, say 5%.)

Comparison of Basically Different Types of Sequential Tests for Reliability and Robustness—For practical reasons, only those tests requiring 10 assays with an optional 20 additional assays are considered. In the following discussion, *acceptance range* means the range 92.45–107.55% of label claim.

Test 11-2-10/30: If the mean assay for 10 dose units is beyond the acceptance range, or if two or more individual assays are beyond the range 84.95–115.05%, or if any assay is beyond 74.95–125.05%, the lot fails. If the mean assay is not beyond the acceptance range and no individuals are beyond the range 84.95–115.05%, the lot passes. Otherwise, a total of 30 assays is required. If the mean assay of the 30 is beyond the acceptance range or two or more individuals are beyond the range 84.95–115.05%, the lot fails. Otherwise it passes.

Test 80-2-10/30: If more than five of the first 10 individual assays are beyond the acceptance range, *i.e.*, 92.45–107.55%, the lot fails. If none is beyond the acceptance range, the lot passes. Otherwise, a total of 30 assays is utilized. If more than nine of the 30 assays are beyond the acceptance range, the lot fails. Otherwise it passes.

Test 121-2-10/30: If the mean assay for 10 dose units is beyond 1.1 times the acceptance range or if the absolute value of the mean assay plus 1.8 times the standard deviation of the individuals is greater than 1.3 times 15.05%, the lots fails. If the mean assay is less than 0.9 times the acceptance range and if the absolute value of the mean assay plus 1.8 times the standard deviation of the individuals is less than 0.6 times 15.05%, the lot passes. Otherwise, a total of 30 assays is utilized. If the mean assay of the 30 is beyond the acceptance range or if the absolute value of the mean assay plus 1.8 times the standard deviation of the individuals is greater than 15.05%, the lot fails. Otherwise it passes.

Test 160-2-10/30A: If the mean of the squares of the deviations of the first 10 individual assays from label claim (100%) is greater than 2.0 times $(7.55\%)^2$, the lot fails. If this mean is greater than 1.2 times $(7.55\%)^2$ and the standard deviation of the individual assays is less than 2.0%, the lot fails. If this mean is less than 0.33 times $(7.55\%)^2$, the lot passes. If this mean is less than 0.8 times $(7.55\%)^2$ and the standard deviation of the individual assays is less than 2.0%, the lot passes. Otherwise, a total of 30 assays is utilized. If the corresponding mean of the squares of the deviations of the 30 assays is greater than $(7.55\%)^2$, the lot fails. Otherwise it passes.

The above four tests and the corresponding numerical results are summarized in Table VI and Fig. 4. The *ad hoc* estimates of reliability are derived from Figs. 4A–4D. The quantity, the maximum average sample size, is derived from Figs. 4E–4H and refers only to the special case where the lot standard deviation equals the deviation of the lot mean from label claim. Tables giving the average sample size, for the sequential tests, as a function of lot mean and standard deviation are available on request.

Reliability of Typical Tests as a Function of Number of Assays Required—Table VII compares the reliabilities of five types of tests for 10, 20, and 30 assays. Figure 5 indicates the location of the 5, 50, and 95% contours for each test. The table includes abbreviated descriptions of the tests, all of which have been described before in principle.

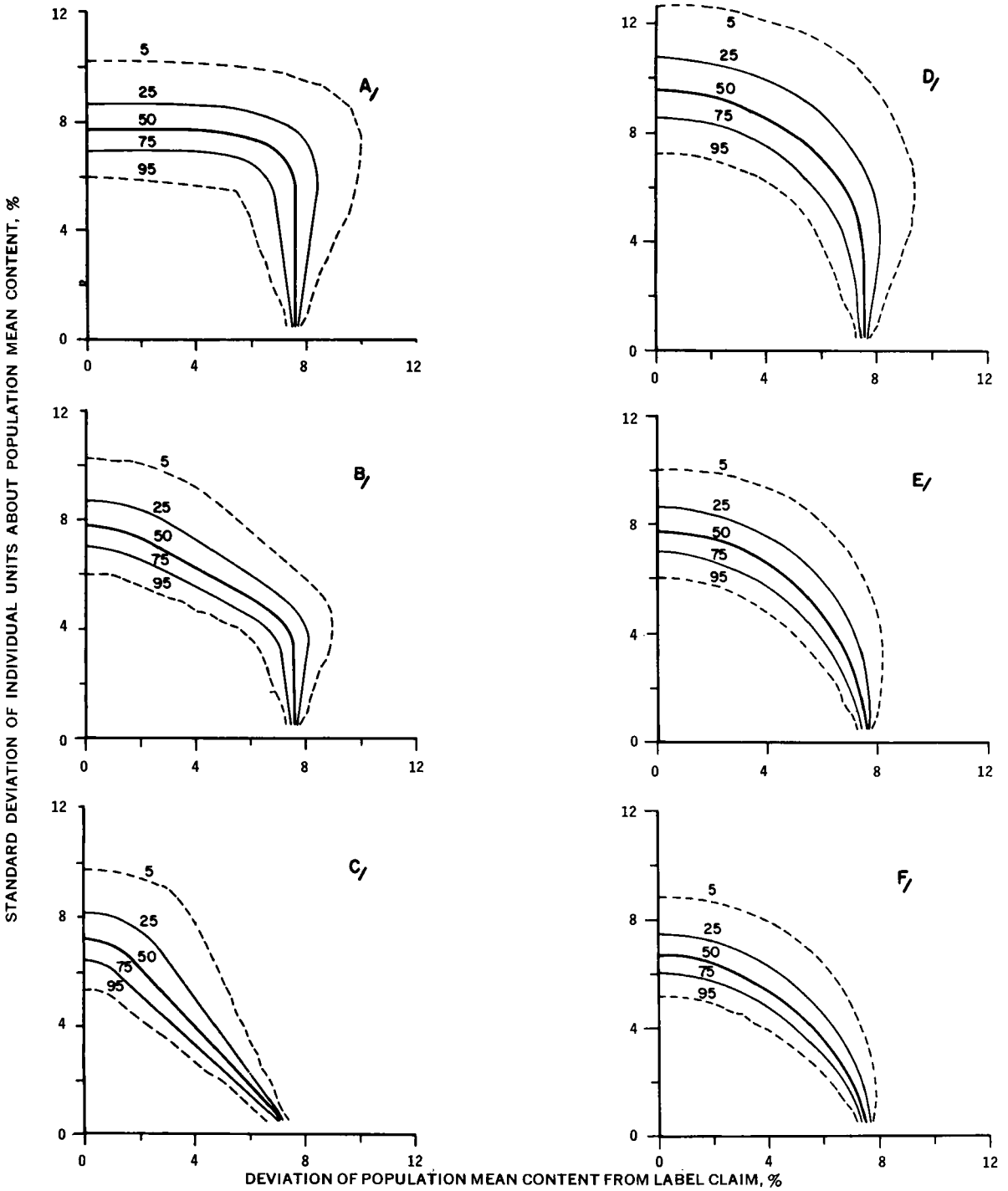

Figure 3—*The* OC *curves for six tests by variables, each requiring 20 assays, for content uniformity. The tests are: (A) No. 100-2-20,* $|\bar{X}| \leq 7.55$, $S \leq 7.55$; *(B) No. 121-2-20,* $|\bar{X}| \leq 7.55$, $(|\bar{X}| + 1.8S) \leq 15.05$; *(C) No. 130-2-20,* $|\bar{X}| + 0.9S \leq 7.55$; *(D) No. 150-2-20,* $\Sigma|\bar{X}|/N \leq 7.55$; *(E) No. 160-2-20,* $(\Sigma X^2)/N \leq (7.55)^2$; *and (F) No. 170-2-20,* $(\Sigma|X^3|)/N \leq (7.55)^3$.

Flexibility of Different Types of Tests for Content Uniformity—Besides the simple format of a fixed number of assays with a simple pass-fail conclusion, sequential formats and formats with three or more graded conclusions may be used advantageously on occasion. An acceptable type of test must not only permit these other formats without introducing too much complexity but must do so without appreciably changing the definition of an acceptable lot, *i.e.*, without changing appreciably the location of the 50% contour. As discussed later in connection with Tables VI and VII, it is already apparent that tests by attributes do not have this flexibility. Only those tests by variables that still appear promising are considered further.

The sequential tests studied were limited, for practical reasons, to those requiring a minimum of 10 assays and a maximum of either 20 or 30, with the number of assays required increasing in steps of 10 or a single step of 20. Graded-conclusion tests were limited to those requiring 10 or 20 assays and having four possible conclusions.

Representative sequential tests are listed in Table VI. These tests can always be designed to have virtually the same reliability

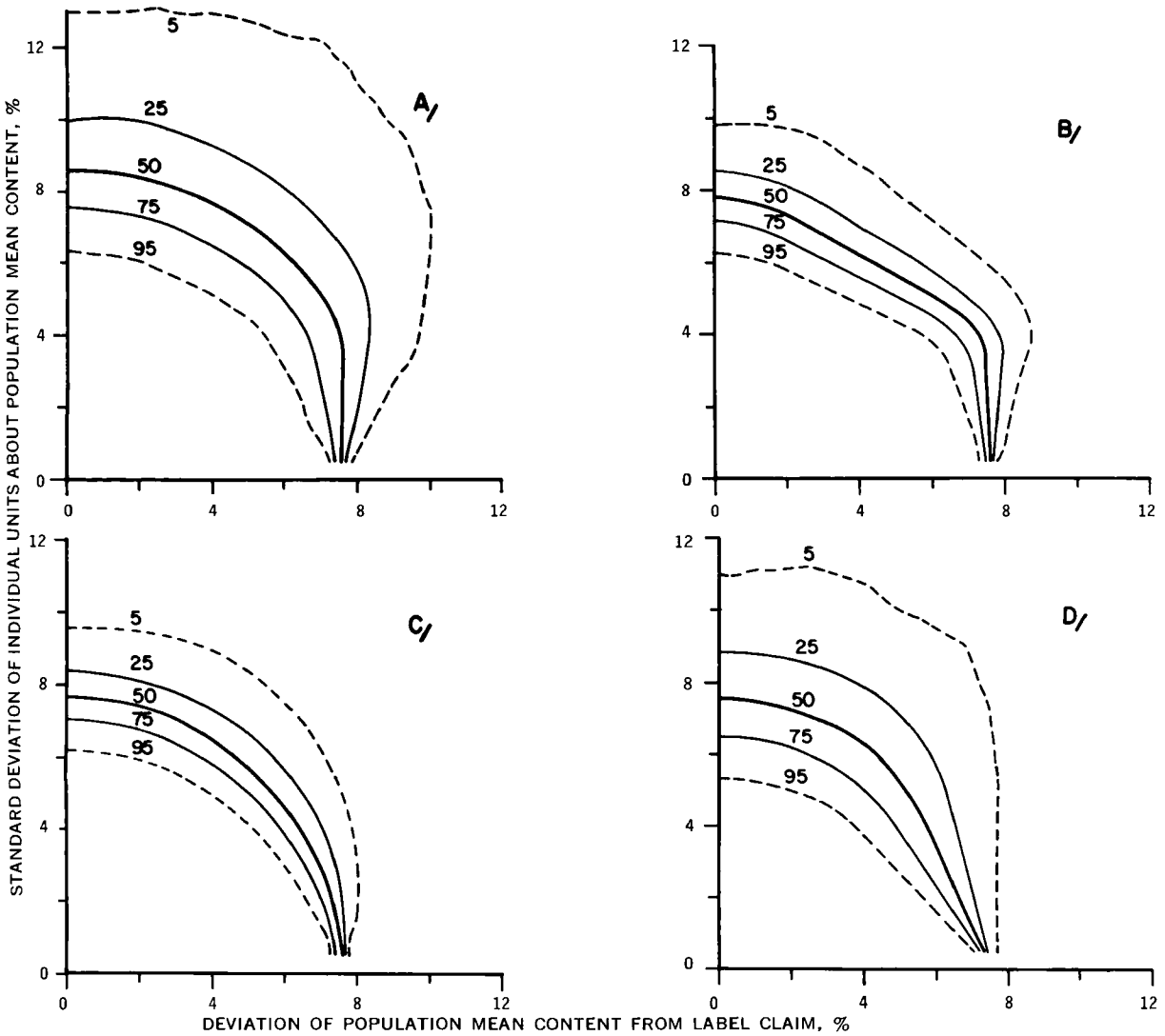

Figure 4A–D—*The* OC *curves for the four sequential tests for content uniformity as completely described in Table VI. The tests and basic criteria are: (A) No. 11-2-10/30,* $|\bar{X}| \leq 7.55$, $(No._n > 15.05\%) \leq R$; *(B) No. 121-2-10/30,* $|\bar{X}| \leq 7.55$, $(|\bar{X}| + 1.8S) \leq 15.05$; *(C) No. 160-2-10/30A,* $(\Sigma X^2)/N \leq (7.55)^2$; *and (D) No. 80-2-10/30,* $(No._n > 7.55) \leq (3n/10)$.

as the corresponding test that has the number of assays equal to the maximum possible number in the sequential version, but there remains the question of whether this design is wasteful by requiring redundant assays. In designing and evaluating sequential tests, it appeared likely that the width of the zone in which additional assays should be required (column 4 of Table VI) should be a function of the magnitude of the standard deviation of the units in the lot. This latter quantity is, in practice, unknown but can be estimated with the standard deviation of the sample. In Table VIII, three types of sequential designs are evaluated, their limitations are observed, and then a modification is suggested.

Three graded-conclusion tests, corresponding to the sequential tests listed as the second variation in Table VIII, are listed in Table IX, and their curves that correspond to *OC* curves are given in Fig. 6.

Comparison of Pharmacopeial Tests for Content Uniformity—The following pharmacopeial tests for content uniformity were altered by the inclusion of the restriction on the magnitude of the mean assay. In contrast with the above sequential tests, the magnitude of the mean assay cannot directly influence the number of assays required.

Test 12-2-10/30: This is the test for tablets in USP XVII (1) and NF XII and XIII (2). This test is identical to Test 11-2-10/30 except that there is no special restriction with respect to assays beyond the range 74.95–125.05%.

Test 13-2-10/30: This is the test for tablets in USP XVIII (1). If the mean assay for 10 tablets is beyond the acceptance range, if any individual assays are beyond the range 74.95–125.05%, or if three or more assays are beyond the range 84.95–115.05%, the lot fails. If the mean assay is not beyond the acceptance range and no more than one is beyond the range 84.95–115.05%, the lot passes. Otherwise, a total of 30 assays is required. If the mean assay of the 30 is beyond the acceptance range or three or more are beyond the range 84.95–115.05%, the lot fails. Otherwise it passes. (Test 13-2-10/30 allows one more defective unit than either Test 11- or 12-.)

Test 14-2-10/30: This is the test for capsules in USP XVIII (1) and NF XIII (2). If the mean assay of 10 capsules is beyond the acceptance range, if any individual assays are beyond the range 74.95–125.05%, or if four or more are beyond 84.95–115.05%, the lot fails. If the mean assay is not beyond the acceptance range and not more than one individual assay is beyond the range 84.95–115.05% (but not beyond 74.95–125.05%), the lot passes. Otherwise, a total of 30 assays is required. If the mean assay of the 30 is beyond the acceptance range, if any assays are beyond the range 74.95–125.05%, or if four or more are beyond the range 84.95–115.05%, the lot fails. Otherwise it passes.

The *OC* curves and the *ASN* curves for these three pharmacopeial tests are shown, in a three-dimensional representation, in Figs. 7 and 8 with, for contrast, the curves of the sequential tests by variables listed in Table VIII. When using Eq. 4, the values for the respective reliabilities of the pharmacopeial tests are 28, 15, and 19% while those of the tests by variables are 64 or 65%.

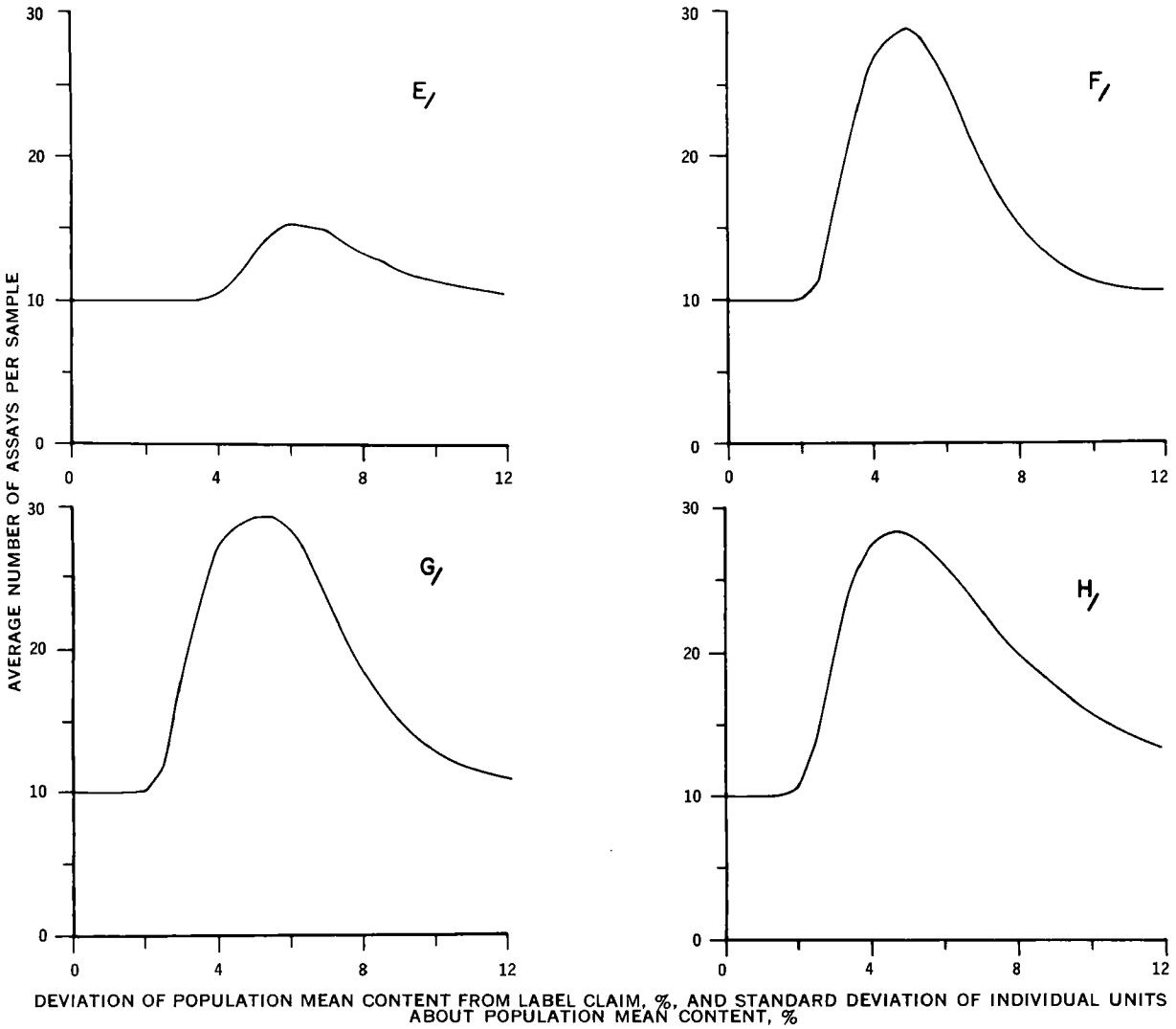

Figure 4E–H—*The* ASN *curves corresponding to the diagonals (starting at the origin) of the graphs in Figs. 4A–4D, taken in the same order. Note that the mean content and the standard deviations will be equal.*

The inclusion, in Test 11-2-10/30, of the restriction that no assays be beyond the range 74.95–125.05% produces a maximum increase in the probability of failing the test of 0.5% when the probability is between 25 and 75% and the lot mean equals label claim. The effect is, therefore, of marginal significance. However, other tests (not listed) show that this restriction increases the probability of failing by approximately 5% for two of the current pharmacopeial tests, 13-2-10/30 and 14-2-10/30. This double restriction, in which the principal criterion corresponds to the definition of defectiveness, is different in principle from Test 90-2-20, suggested by Haynes *et al.* (14), in which two restrictions were evenly balanced about the definition of defectiveness.

DISCUSSION

Error—The location of the contour lines in the *OC* curves is subject to an uncertainty originating in the pseudorandom nature of the simulated assays used in the evaluation of the performance of the test in question. This uncertainty is essentially experimental error. The standard deviation of this error, σ'', for an individual point can be evaluated with Eq. 7, a modified form of Eq. 6. For this equation, N samples, randomly chosen from a population with a given mean and standard deviation, each has a probability, P, of passing the test. The letter, G, refers to the gradient (at the corresponding point) of the appropriate three-dimensional surface. The reciprocal of the gradient, G, converts the standard deviation in percent passing to a unit of length both in the plane perpendicular to the *percent passing* axis and in the direction perpendicular to the contour line:

$$\sigma''^2 = [P(1 - P)/N]/G^2 \qquad \text{(Eq. 7)}$$

As already mentioned, in this work N equals 1000. For the 5, 25, 50, 75, and 95% contours, σ'' equals (0.7%), (1.4%), (1.6%), (1.4%), and (0.7%)/G, respectively. By inspection of the figures, one can see that this quotient is roughly constant for corresponding points on the different contours of a figure and that its magnitude could be used as a measure of reliability.

This value for σ'' applies only to a single point based on its own series of random numbers. Nevertheless, one might surmise, since all the derived points on the *OC* curves are based on the same series of random numbers, that experimental error will affect neighboring portions of the *OC* curve in a corresponding manner, especially for tests by variables.

The location of contours within the figures has been made by linear interpolation within the tables. Presumably the linear aspect of this interpolation has been the source of some of the irregularities in the curves in certain figures.

Criteria of Lot Quality—The definition of lot quality, ideally, requires a pharmacological basis, but for practical reasons pharmaceutical chemists have, for years, temporized. As early as 1951, Smith (19) indicated that the generally accepted criterion of quality with respect to tablet weight was based on the percentage of defectives, *i.e.*, the percentage beyond certain limits in weight. Dunnett and Crisafio (4) used this criterion in the comparison of

Table VI—Estimation of Robustness and Reliability of Selected Tests for Content Uniformity (All Tests Are of the Sequential Type Requiring 10 or 30 Assays)

Test	Class of Test	Basic Test Criteria[a]	Sequential Criteria[a], 30 Assays Required If:	Estimated Robustness[b], %	Estimated Reliability[c], %	Maximum Average Sample Size[d]
11-2-10/30	By attributes	$\lvert\bar{X}\rvert \leq 7.55$ and number of deviations greater than 15.05%	$\lvert\bar{X}_{10}\rvert \leq 7.55$, $(No._{10} > 15.05\%) = 1$, and $(No._{10} > 25.05\%) = 0$	45	28	15
80-2-10/30	By attributes	Number of deviations greater than 7.55%	$(No._{10} > 7.55\%) =$ 1, 2, 3, 4, or 5	−3	37	28
121-2-10/30	By variables	$\lvert\bar{X}\rvert \leq 7.55$, and $(\lvert\bar{X}\rvert + 1.8S) \leq 15.05$	$(\lvert\bar{X}_{10}\rvert \leq 1.1 \times 7.55)$ and $(\lvert\bar{X}_{10}\rvert + 1.8S \leq 1.3 \times 15.05)$ while either $(\lvert\bar{X}_{10}\rvert \leq 0.9 \times 7.55)$ or $(\lvert\bar{X}_{10}\rvert + 1.8S \geq 0.6 \times 15.05)$	69	64	29
160-2-10/30A	By variables	$(\Sigma X^2)/N \leq (7.55)^2$	Case I: $-S_{10} < 2.0$; then $0.8 \times (7.55)^2 \leq (\Sigma^{10} X^2)/10 \leq 1.2 \times (7.55)^2$ Case II: $-S_{10} \geq 2.0$; then $0.33 \times (7.55)^2 \leq (\Sigma^{10} X^2)/10 \leq 2.0 \times (7.55)^2$	78	65	29

[a] Table III, Footnote *a*, defines the symbols required by these columns. [b] Robustness is defined by Eq. 5 in the text. It is determined in an analogous manner to that of Table V. [c] Reliability, defined by Eq. 4 in the text, is derived from Fig. 4. [d] Maximum average sample size is derived from the original data of those tables represented in Figs. 4E–H.

Table VII—Reliability *versus* Number of Assays, and Intercept of 50% Contour on the Standard Deviation Axis *versus* Number of Assays

Test	Class of Test	Abbreviated Description of Test[a]	Estimated Reliability[b], %			Intercept of 50% Contour with Standard Deviation Axis, %		
			10 Assays	20 Assays	30 Assays	10 Assays	20 Assays	30 Assays
10-2-10	By attributes	$\lvert\bar{X}_{10}\rvert \leq 7.55$, $(No._{10} > 15.05\%) = 0$	19	—	—	8.2	—	—
11-2-20[c]	By attributes	$\lvert\bar{X}_{20}\rvert \leq 7.55$, $(No._{20} > 15.05\%) = 0$	—	48	—	—	7.2	—
10-2-30	By attributes	$\lvert\bar{X}_{30}\rvert \leq 7.55$, $(No._{30} > 15.05\%) \leq 1$	—	—	58	—	—	7.9
121-2-...	By variables	$\lvert\bar{X}\rvert \leq 7.55$, $(\lvert\bar{X}\rvert + 1.8S) \leq 15.05$	31	59	65	7.6	7.8	7.9
140-2-...	By variables	$(\bar{X})^2 + S^2 \leq (7.55)^2$	32	58	65	7.4	7.5	7.5
160-2-...	By variables	$(\Sigma X^2)/N \leq (7.55)^2$	30	59	65	7.8	7.7	7.7
80-2-10	By attributes	$(No._{10} > 7.55\%) \leq 3$	1	—	—	8.1	—	—
80-2-20	By attributes	$(No._{20} > 7.55\%) \leq 6$	—	41	—	—	7.8	—
80-2-30	By attributes	$(No._{30} > 7.55\%) \leq 9$	—	—	37	—	—	7.6

[a] Table III, Footnote *a*, defines the symbols required by this column. [b] Reliability, defined by Eq. 4 in the text, is derived from Fig. 5 except for the 140 series. [c] Test 10-2-20, as listed in Table III, is similar except a single assay beyond 15.05% is permitted. The reliability is 49%, and the intercept of the 50% contour on the standard deviation axis is 8.8%.

the reliabilities of several tests for weight variation. Subsequently, authors employed the analogous criterion, *i.e.*, the percentage of assays beyond certain limits, in the evaluation of tests for content uniformity.

Regardless of whether units with assays beyond these limits are called defectives or outsiders, this classification implies that all units within the range are pharmacologically equally satisfactory and those outside the range are equally unsatisfactory. However, data, as recently reviewed by Ritschel (20), indicate that variations in drug content, modified by the accumulative effect of a succession of units administered at intervals, are reflected by variations in blood levels which, in turn, are reflected by variations in pharmacological activity. While these relationships will not necessarily be linearly proportional, they will be generally continuous functions, increasing and decreasing in unison.

This reasoning eliminates the use of an *acceptance range* as an *intrinsic* measure of unit quality and *percentage beyond* as an *intrinsic* measure of lot quality. Rather, it suggests the use of a simple function of the deviation of unit content from label claim as a measure of unit quality and the pooled magnitude as a measure of lot quality.

Of the various possible functions, the second moment of the deviations about label claim has a certain elegance and reasonableness, but it is an unfamiliar and unproven function. Mean drug content and standard deviation are familiar quantities which can be independently related to different aspects of the manufacturing process. Also, without any restriction on the nature of the distribution, they are intrinsic descriptive parameters for each pharmaceutical lot (although they do not completely characterize it). In addition, the mean blood level will be generally proportional to the mean unit content; the standard deviation of the blood level, especially where there is an accumulative effect, will be generally proportional to the standard deviation of the unit content. Therefore, mean drug content and standard deviation are used here jointly as indexes of lot quality (although they do not necessarily completely characterize lot quality in the pharmacological sense).

If mean content and standard deviation did completely characterize lot quality in the pharmacological sense, then the charac-

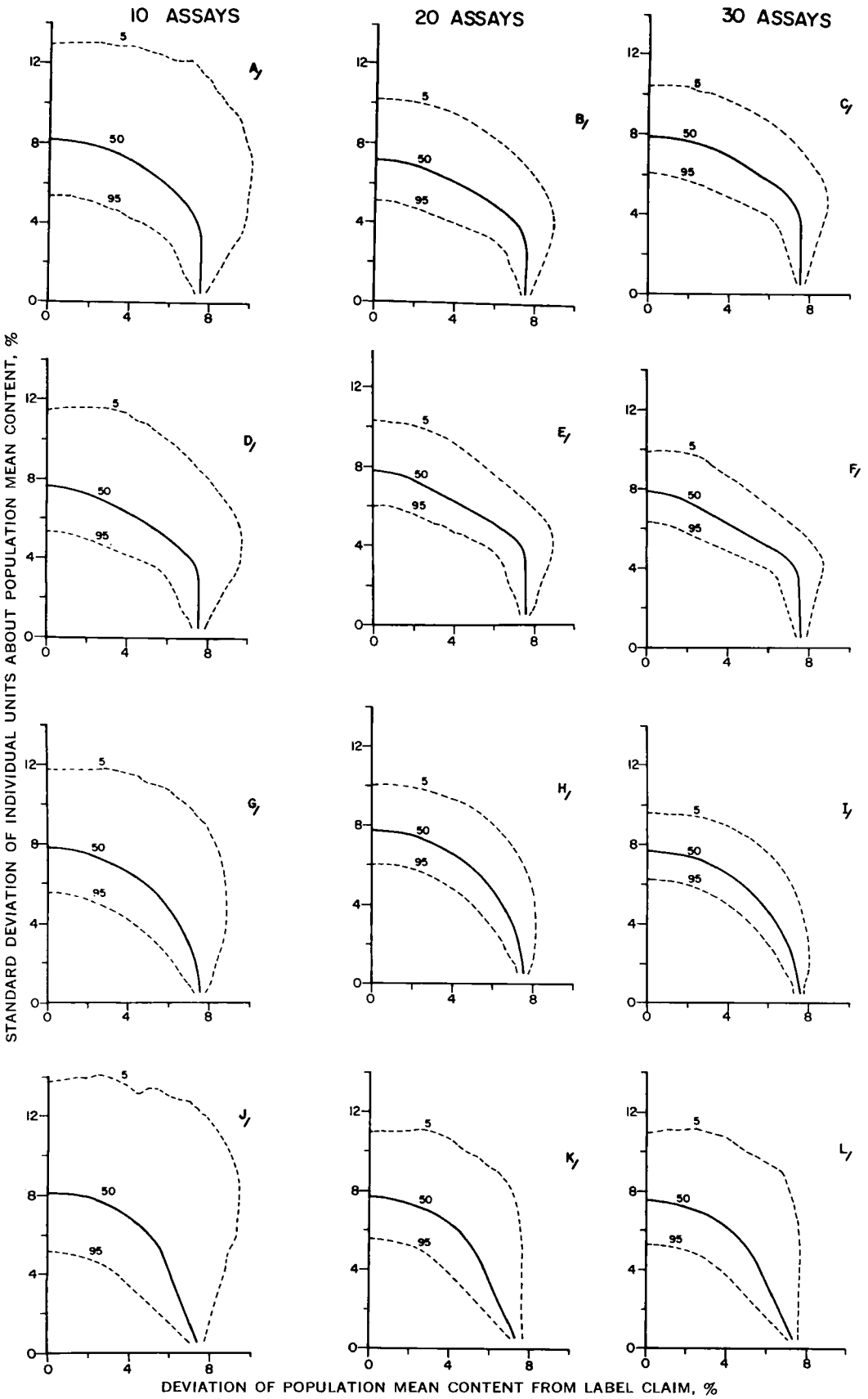

Figure 5—*The* OC *curves for four types of tests for content uniformity, showing the variation with 10, 20, and 30 assays. The tests and criteria are: (A) No. 10-2-10,* $|\bar{X}_{10}| \leq 7.55$, $(No._{10} > 15.05\%) = 0$; *(B) No. 11-2-20,* $|\bar{X}_{20}| \leq 7.55$, $(No._{20} > 15.05\%) = 0$; *(C) No. 10-2-30,* $|\bar{X}_{30}| \leq 7.55$, $(No._{30} > 15.05\%) \leq 1$; *(D, E, F) No. 121-2-. . .,* $|\bar{X}| \leq 7.55$, $(|\bar{X}| + 1.8S) \leq 15.05$; *(G, H, I) No. 160-2-. . .,* $(\Sigma X^2)/N \leq (7.55)^2$; *(J) No. 80-2-10,* $(No._{10} > 7.55\%) \leq 3$; *(K) No. 80-2-20,* $(No._{20} > 7.55\%) \leq 6$; *and (L) No. 80-2-30,* $(No._{30} > 7.55\%) \leq 9$.

Table VIII—Flexibility of Sequential Format[a]

Primary Test Criteria	First Variation		Second Variation	
	Test	30 Assays Required If:	Test	30 Assays Required If:
$\lvert\bar{X}\rvert \lesssim 7.55$ and $(\lvert\bar{X}\rvert + 1.8S) \lesssim 15.05$	121-2-10/30A	$(\lvert\bar{X}_{10}\rvert \lesssim 7.55 + 0.63S)$ and $(\lvert\bar{X}_{10}\rvert + 1.8S \lesssim 15.05 + 0.45S)$ while either $(\lvert\bar{X}_{10}\rvert \gtrsim 7.55 - 0.63S)$ or $(\lvert\bar{X}_{10}\rvert + 1.8S \gtrsim 15.05 - 1.65S)$	121-2-10/30B	$(\lvert\bar{X}_{10}\rvert \lesssim 1.1 \times 7.55)$ and $(\lvert\bar{X}_{10}\rvert + 1.8S \lesssim 1.4 \times 15.05)$ while either $(\lvert\bar{X}_{10}\rvert \gtrsim 0.9 \times 7.55)$ or $(\lvert\bar{X}_{10}\rvert + 1.8S \gtrsim 0.6 \times 15.05)$
$(\Sigma X^2)/N \lesssim (7.55)^2$	160-2-10/30	$0.33(7.55)^2 \lesssim (\Sigma^{10}X^2)/10$ and $(\Sigma^{10}X^2)/10 \lesssim 2.0(7.55)^2$	160-2-10/30C	$(1.0 - 0.15S) \times 0.9 \times (7.55)^2 \lesssim (\Sigma^{10}X^2)/10$ and $(\Sigma^{10}X^2)/10 \lesssim (1.0 + 0.15S) \times 1.1 \times (7.55)^2$
$(\bar{X})^2 + K \cdot S^2 \lesssim (7.55)^2$, where $K = 1.0$ generally	140-2-10/30	$0.33(7.55)^2 \lesssim (\bar{X}_{10})^2 + S^2$ and $(\bar{X}_{10})^2 + S^2 \lesssim 2.0(7.55)^2$	140-2-10/30B	$0.9 \times (7.55)^2 \lesssim (\bar{X}_{10})^2 + 3.0 \times S^2$ and $(\bar{X}_{10})^2 + 0.5 \times S^2 \lesssim 1.1 \times (7.55)^2$

[a] For the six variations listed in the table, the reliabilities are 64 or 65%. Of the first three variations, Test 121-2-10/30A is considered too complicated for routine use while the other two call for redundant assays when S is small. The second three variations are considered satisfactory.

Table IX—Several Multiconclusion Tests for Content Uniformity (All Tests Require 10 Assays)

Test	Sample Passes If[a]	Basic Test Criteria	Sample Fails If[b]	Results and Comments
121-4-10	$\lvert\bar{X}_{10}\rvert < 0.9 \times 7.55$ and $\lvert\bar{X}_{10}\rvert + 1.8S < 0.6 \times 15.05$	$\lvert\bar{X}\rvert \lesssim 7.55$ and $\lvert\bar{X}\rvert + 1.8S \lesssim 15.05$	$\lvert\bar{X}_{10}\rvert > 1.1 \times 7.55$ or $\lvert X_{10}\rvert + 1.8S > 1.4 \times 15.05$	See Fig. 6A; the acceptance and the rejection regions overlap each other equally at the 5% contours
160-4-10B	$(\Sigma^{10}X^2)/N < (1.0 - 0.15S) \times 0.9 \times (7.55)^2$	$(\Sigma X^2)/N \lesssim (7.55)^2$	$(\Sigma^{10}X^2)/10 > (1.0 + 0.15S) \times 1.1 \times (7.55)^2$	See Fig. 6B; similar to above except at the 2% contour; the rejection region is poorly defined
140-4-10	$(\bar{X}_{10})^2 + 3.0S^2 < 0.9 \times (7.55)^2$	$(\bar{X})^2 + S^2 \lesssim (7.55)^2$	$(\bar{X}_{10})^2 + 0.5S^2 > 1.1 \times (7.55)^2$	See Fig. 6C; the acceptance and the rejection regions overlap each other equally at the 15% contours

[a] Samples that do not pass but are within the basic test criteria are classed as *probably acceptable.* [b] Samples that are beyond the basic test criteria but do not fail are classed as *probably unacceptable.*

terization would be distribution free; however, experimental difficulties preclude the determination of the completeness of characterization. (The question whether lot quality as characterized by certain parameters is distribution free should not be confused with the question whether an acceptance test is distribution free, *i.e.*, robust.)

Recently, Oie *et al.* (21) tested, with aspirin (acetylsalicylic acid) tablets, for a correlation between a controlled interdose variation and plasma levels, but the results were not conclusive.

Criteria for Selecting Tests for Quality Control—The primary criterion must be reliability. Due to the random variation in sampling, tests on successive samples from the same lot do not always provide the same conclusion. The risks this involves for both the manufacturer and the regulatory agency are apparent, and the risks must be minimized, at least, to the point where the improvement in the definition of quality becomes trivial in terms of the additional work involved. The estimates of reliability show that some tests are much more reliable than others. Where several tests have the required degree of reliability, the most economical test is to be preferred.

The second criterion must be flexibility. The individual test cannot be flexible in itself; rather the principle on which the test is based must be adaptable to other formats without substantially changing the definition of acceptability, *i.e.*, without substantially changing the location of the 50% contour. Furthermore, since lot quality is taken as a function both of dispersion and of deviation of the mean, the principle of a test should permit the variation of the relative influence of the dispersion and the deviation of the mean. The following two minor aspects of flexibility should also be considered: *(a)* the test, or at least the basic principle of the test, should be applicable to other measurements besides content uniformity, in particular, to weight variation; and *(b)* the test should be directly applicable to the situation where the analytical methods with the necessary precision for single-tablet assays do not have the necessary accuracy for the determination of the mean content. While the criterion of flexibility is difficult to quantify, its use should permit the ranking of tests with comparable reliabilities.

The third criterion is simplicity with respect both to appreciation by persons with quite limited training in statistics and to calculation manually or by desk calculators and computers. This criterion underlies the initial selection of the tests to be examined.

In more specific terms than those preceding Eq. 5, robustness of a test refers to independence, for a given lot quality, from the assumption of normality. Unfortunately, in the development (14, 15) of the current pharmacopeial tests (1, 2), considerable weight was attached to the relative robustness where lot quality was expressed as percentage defective. Until either the relative robustness has been shown to be independent of the definition of lot

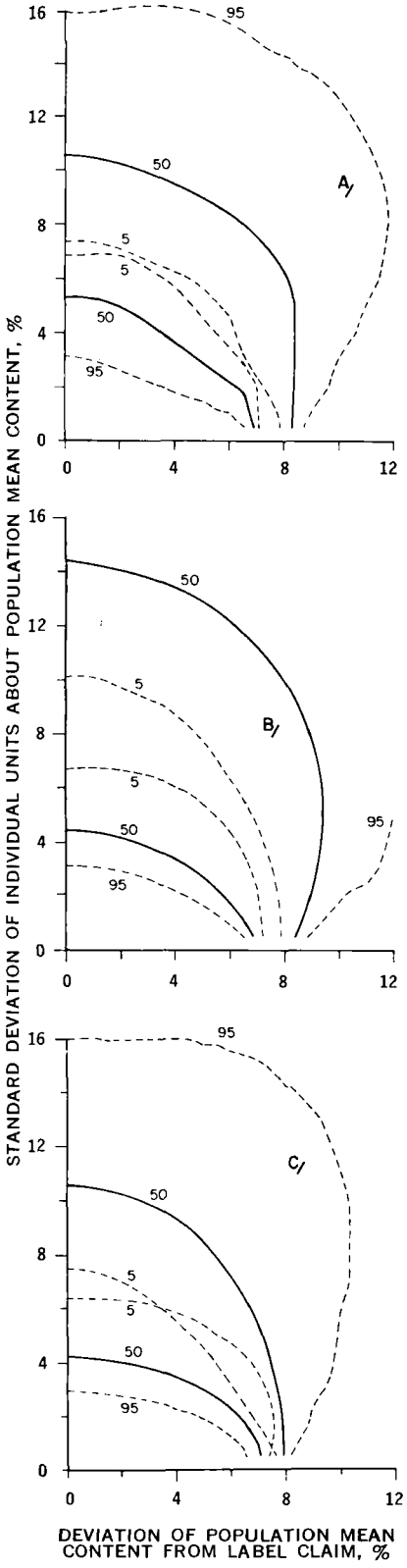

Figure 6—*The analog of* OC *curves for three multigrade tests by variables, each requiring 10 assays, for content uniformity, as completely described in Table IX. The tests and basic criteria are: (A) No. 121-4-10,* $|\bar{X}| \leq 7.55$, $(|\bar{X}| + 1.8S) \leq 15.05$; *(B) No. 160-4-10,* $(\Sigma X^2)/N \leq (7.55)^2$; *and (C) No. 140-4-10,* $(\bar{X})^2 + S^2 \leq (7.55)^2$. *The three contours that intersect the axes closer to the origin indicate the probability of passing the test. The other three contours indicate the probability of failing the test.*

quality or the definition of lot quality is established in pharmacological terms, robustness should not be used as a criterion for selecting tests.

Test of Mean Content—The *OC* curve for the test of mean content (based on the mean of 20 assays) is given in Fig. 1 for comparison with those of the various tests for content uniformity. The figure includes lots with high (14%) standard deviations, not because they are common but because they occasionally do occur, usually where the active ingredient is in a macroscopically nonhomogeneous phase.

It is, perhaps, of greater importance that the figure illustrates the limitations of the usual approach to determining mean content as described in Refs. 1 and 2. The *OC* curve actually depicts the optimum performance of the test. If the mixture from the 20 units is not homogeneous with respect to the size of the weighed portion or if the analytical method (for two or three assays) has a relatively high coefficient of variation, the uncertainty is increased.

While tests for mean content have not been systematically considered, one is suggested here for comparison with the tests for content uniformity. If the absolute deviation from label claim of the mean assay for 10 dose units is greater than 7.55% plus twice the "standard deviation of the ten assays divided by the square root of 10," the lot fails. If the absolute deviation is less than 7.55% minus twice the "standard deviation divided by the square root of 10," the lot passes. Otherwise, 30 additional dose units are assayed (singly or in aliquots of powdered and mixed units). If the absolute deviation is greater than 7.55%, the lot fails. Otherwise it passes.

This test can only be used without modification if the analytical method with the necessary precision for determining the dispersion is sufficiently accurate for the determination of the mean.

Tests for Content Uniformity—As shown by Table III and Figs. 2 and 3, the conclusions reached, based on 20 assays, by the various classes of tests were not radically different. In most of the graphs, the 50% contour forms a rough quarter-circle about the origin. To some degree, this was intentional because it simplifies the intercomparison.

In two cases, for reasons inherent in the test definition, the 50% contours do not form a rough quarter-circle. As shown by Fig. 3A, the 50% contour for Test 100-2-20 is composed basically of a straight line reflecting the condition limiting the mean assay and a second straight line reflecting the condition limiting the standard deviation. In contrast, as shown by Fig. 3C, the 50% contour for Test 130-2-20 (and, by analogy, Test 134-2-20) is a single diagonal line reflecting the single condition limiting the sum of two linear components. For subjective reasons mentioned earlier, these tests are not considered further.

The data of Table III show that the three tests by attributes (Fig. 2) are generally not as reliable as the tests by variables (Fig. 3). This is undoubtedly a consequence of the loss of information in conversion of assay values to single binary digits, *i.e.*, 0 or 1, in the cases of Test 10-2-20 (a modified form of the current pharmacopeial tests) and Test 80-2-20. Test 90-2-20 (Fig. 2C), as suggested by Haynes *et al.* (14), loses much less information with respect to the distribution of assay values and has a correspondingly higher reliability.

Haynes *et al.* (14) showed that tests by attributes (based on the number of defectives) were more robust than a test by variables based on standard deviation, while the data of Table III indicate the converse. This, apparently, reflects the change in criteria of lot quality.

Table VI and Figs. 4A–4D show that sequential tests by variables are decidedly more reliable than the two sequential tests by attributes, one of which, Test 11-2-10/30, is very similar to the current pharmacopeial tests.

By comparison of the maximum average sample size as listed in Table VI or as shown by Figs. 4E–4H, it is apparent that the sequential mechanism of Test 11-2-10/30 fails in its purpose, *i.e.*, to require the maximum number of assays in borderline cases. Apparently, this defect can also be traced back to the loss of information in conversion of assay values to single binary digits.

The values for robustness for sequential tests are virtually the same as for the corresponding values for the tests requiring 20 assays.

Table VII and Fig. 5 show that the reliabilities of the sequential tests just discussed closely approach those for the corresponding

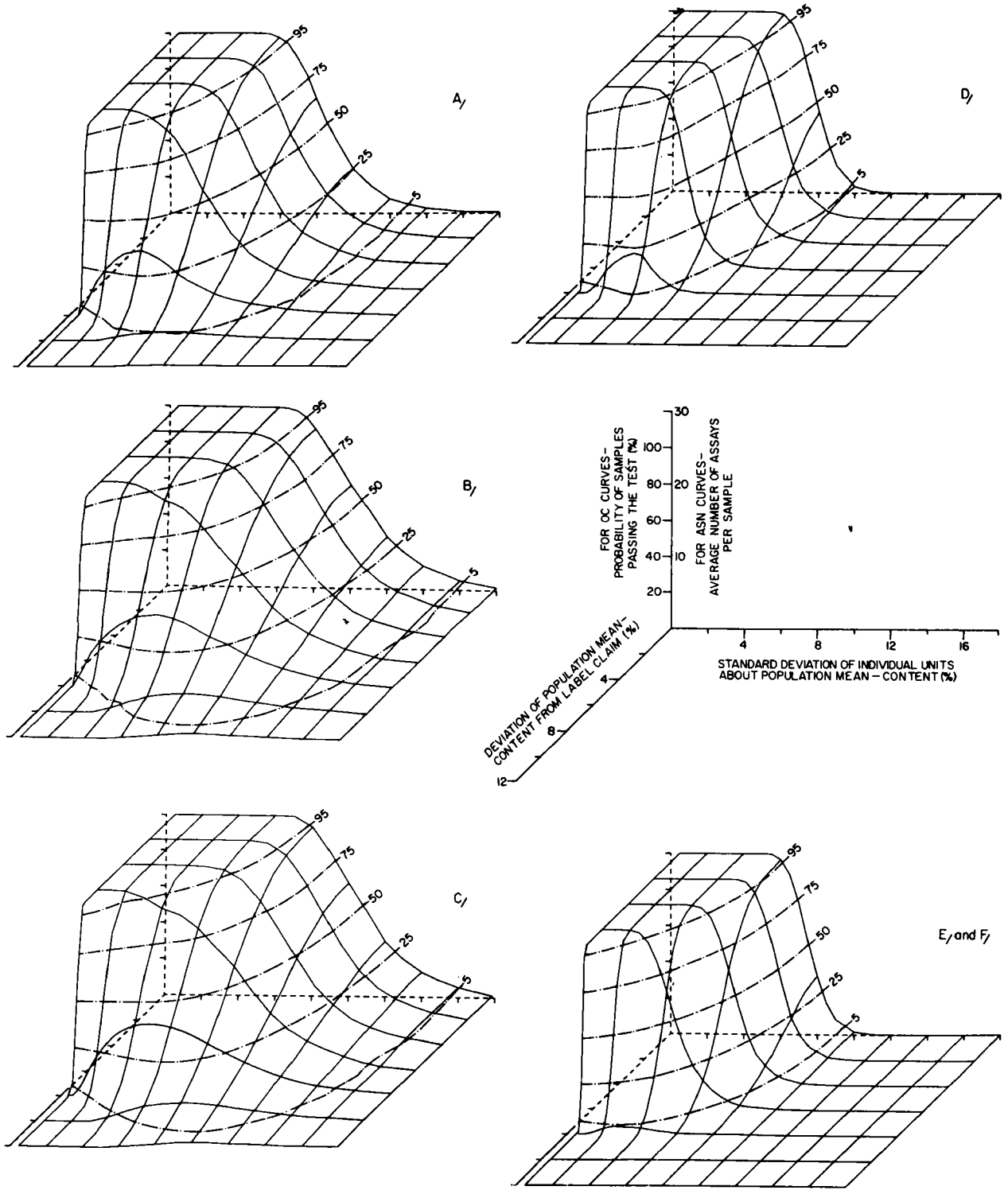

Figure 7—*The* OC *curves for the three current pharmacopeial tests and the three preferred tests by variables for content uniformity. The tests and their source or their basic criteria are: (A) No. 12-2-10/30, NF XIII; (B) No. 13-2-10/30, USP XVIII;* (C) *No. 14-2-10/30, NF and USP method for capsules; (D) No. 121-2-10/30B,* $|\bar{X}| \leq 7.55$, $(|\bar{X}| + 1.8S) \leq 15.05$; *(E) No. 160-2-10/30C,* $(\Sigma X^2)/N \leq (7.55)^2$; *and (F) No. 140-2-10/30B,* $(\bar{X})^2 + S^2 \leq (7.55)^2$. *Since E is nearly identical to F, it has not been included as such.*

tests requiring 30 assays, except for Test 10-2-10/30. This is further evidence that the "sequential mechanism" of this test is unsatisfactory.

Figure 5 also shows the relatively large improvement in reliability obtained in going from 10 to 20 assays compared with going from 20 to 30 assays.

Table VIII contains the essence of attempts to improve the sequential format of the more promising classes of tests. If a class has the desired flexibility, it is possible to design the sequential format so that, for lots with parameters in the vicinity of those of the 50% contours, the number of assays called for is equal or close to the maximum while for other lots the required number of assays is rapidly decreased to the minimum as the lot parameters move away from this contour. The *ASN* curves of Figs. 8D–8F show that the preferred tests by variables do have this flexibility, in contrast with the current pharmacopeial tests (Figs. 8A–8C).

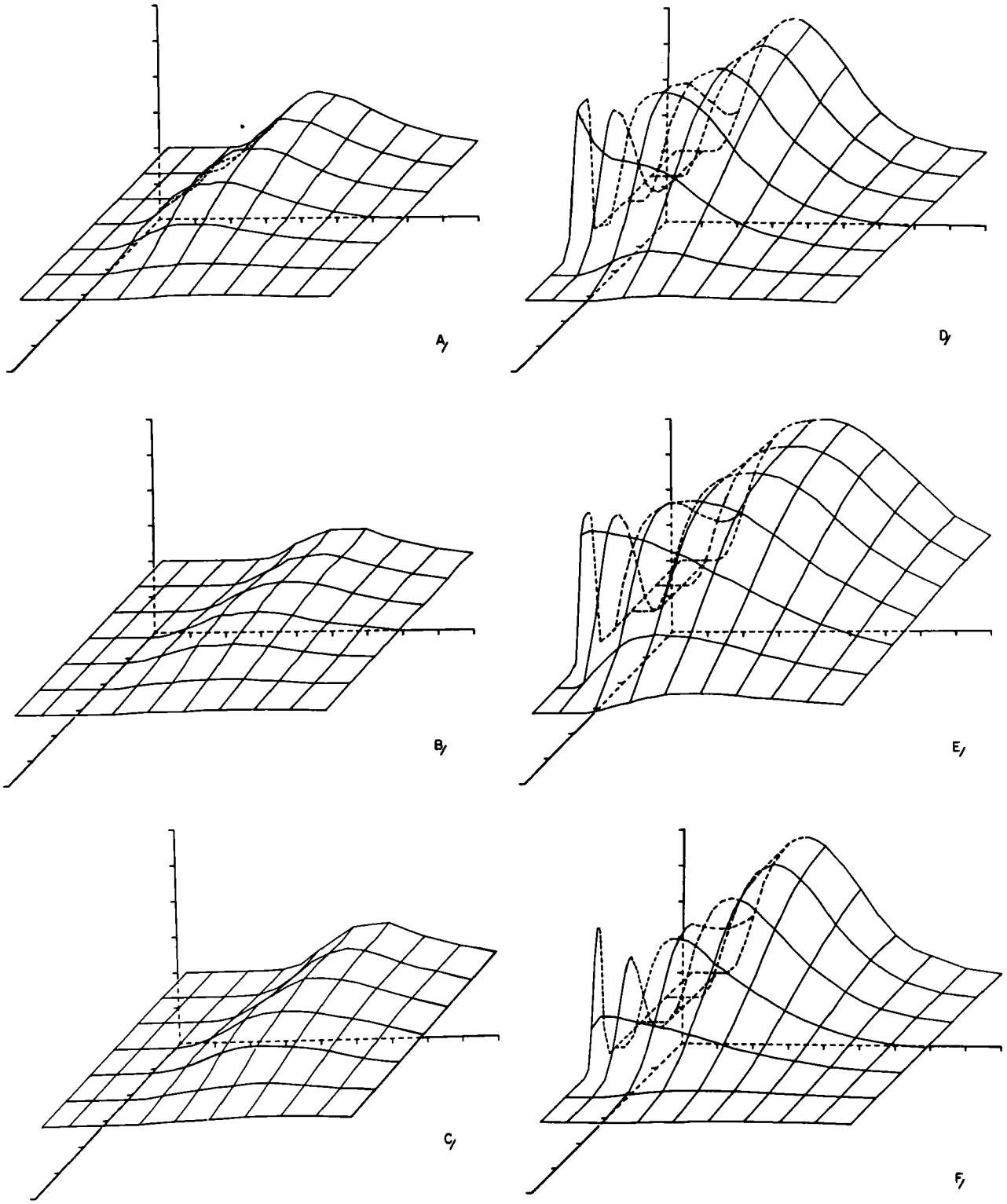

Figure 8—*The* ASN *curves corresponding to the* OC *curves of Fig. 7.*

Presumably this sequential format could be improved further, but these improvements must not be made at the expense of simplicity.

Perhaps a finer test of the flexibility of the various classes of tests is the adaptability to the graded-conclusion format. Arbitrarily, the format is considered satisfactory if the 5% contour of the acceptance region is within and parallel to the 50% contour of the corresponding sequential test while the 5% contour of the rejection region is beyond and parallel to the same 50% contour. Table IX and Fig. 6 show that Test 121-4-10 is successful in this respect. Presumably with further adjustment of parameters, Tests 140-4-10 and 160-4-10 would be successful also.

Since those tests by attributes that classify lots according to the number of assays beyond a single limit have relative difficulty in classifying lots into even two groups, they have not been considered for graded-conclusion formats which would divide lots into three or more groups. More complex tests by attributes, similar to the one suggested by Haynes *et al.* (14), might be convertible to the graded-conclusion format; but due to inherent inflexibility, it is doubtful that they could fulfill the above criterion.

The primary comparison to be made in this work is between the current pharmacopeial tests for content uniformity and the best of the known potential alternatives. The criteria for the comparison are reliability, flexibility, simplicity, and reasonableness.

The reliabilities of the current pharmacopeial tests are in the range 15–30%, while those of the preferred tests by variables are approximately 65%.

The criterion of flexibility requires the following: *(a)* the definition of acceptability, *i.e.*, the location of the reference contour be independent of sample size; *(b)* the sequential format and graded-conclusion formats be successful; and *(c)* the relative importance of mean assay and dispersion be variable. Table VII and Figs. 4A–4D and 5 show that the location of the 50% contour for tests similar in principle to the current pharmacopeial tests varies considerably. In contrast, the preferred tests by variables form a homologous series with virtually the same 50% contour. With respect to the sequential format, for lots of intermediate quality, a sequential test should consistently call for the full complement of assays to estimate reliably the lot quality. The *ASN* curves of Fig. 8 show that the tests by variables successfully meet this condition while, in contrast, the tests by attributes call for the full complement, at best, only 30% of the time. Figure 6 shows that the preferred tests can be used in a graded-conclusion format; but the corresponding format for the tests by attributes, where the four-way classification would depend on whether there was zero, one, two, or three defectives in 10 assays, was not considered sufficiently promising for investigation. The relative importance of the mean assay and the relative importance of content uniformity in the pharmacopeial tests and in Test 121-2-10/30 are independently variable since they are controlled by completely separate criteria. Also, in one preferred test by variables, Test 140-2-10/30, the relative importance is varied by changing the proportionality constant k. However, in the test based on the mean-square deviation, Test 160-2-10/30, the relative importance of mean assay and content uniformity cannot be varied. (In some cases, single-tablet assay procedures can yield relative assays only, and the mean assay is determined by a separate procedure. In this circumstance, it becomes arithmetically simpler to use either Test 121-2-10/30 or 140-2-10/30, which are based on the mean and the standard deviation, rather than the other tests which are based on individual assay values.)

The emphasis of the criterion of simplicity has perhaps changed in the last decade, at least where the calculations are handled by a large or small computer. The current pharmacopeial tests for content uniformity are manually simpler than the tests by variables, but it is doubtful whether simplification ever justified the increase in likelihood of an erroneous conclusion. The above tests are intended to be sufficiently simple for routine use.

In summary, the tests based on $|\bar{X}| + 1.8S$ and on $(\bar{X})^2 + S^2$ are decidedly more reliable and more flexible than the current pharmacopeial tests but suffer a minor loss of simplicity with respect to manual calculation.

New Tests for Weight Variation—As already mentioned above, Wiley (5), in 1960, suggested replacement of the weight variation test with a test for content uniformity. This replacement has been carried out in the USP and NF, where the unit dosage is not more than 50 mg and where there is a suitable analytical method. The weight variation test (22, 23) generally applies to the others. This is consistent with the view that the weight variation test is a crude but simple measure of content variation.

Comer *et al.* (24) suggested, as an alternative approach, that one assume that unit weight and the proportion of drug in a unit vary *independently* from unit to unit, then estimate the variance in weight with 100 units and the variance in proportion with nine units, and then combine these values to obtain a relatively accurate estimate of the variance of the drug content. This latter value can then be employed in one of several tests for content and content uniformity. However, the validity of the assumption that unit weight and proportion of drug vary independently is not yet established. Where the density of the drug is different from that of the other components or where there is an interaction between the drug and other components on mixing, unit weight may be a dependent function of proportion.

A third approach is to use the weight variation test in parallel to, but independently of, the test for content uniformity as a test for *good manufacturing practices.*

In the references cited and in general, the test of weight variation is a particular variation of: "Weigh n groups of 10 units. The sample passes if not more than n units are more than x% by weight from the sample mean and none are more than $2x$%." This is a test by attributes, the same in principle as the pharmacopeial test for content uniformity except for one aspect. The calculation of the deviations is based on the sample mean instead of the theoretical or *target* weight, *i.e.*, the weight corresponding to 100% assay. The target weight is readily calculated since the mean sample weight and mean assay are known (subject to experimental error). Perhaps it is time to bring the older test for weight variation onto the same footing as the test for content uniformity.

The search for an improved test for weight variation would seem to parallel that for content uniformity, except that presumably more weights would be required. For reasons discussed under *Tests for Content Uniformity*, tests based on $|\bar{X}| + 1.8S$ and $(\bar{X})^2 + S^2$ (where $\bar{X}$ is the deviation of the mean weight from the target weight and S is the standard deviation of the weights of the unit dose) are indicated.

Extensions—This work is meant only as a comparative study of selected basic types of tests. The optimum number of assays, the most efficient sequential arrangement, and the maximum practical number of grades were not investigated. Indeed, the computer program requires modification before certain variations, such as a 10/20/40 format for the sequential tests, can be studied.

Neither the question of the optimum values of the acceptance and rejection ranges nor the question of the desirability of more tolerant standards for pharmaceuticals with inherently high standard deviation of the unit content has been considered because these are pharmacological questions.

In this discussion, the distinction between the variance of the assay values and the variance of the content of the units assayed has been ignored. Similarly, the distinction between the mean of the assay values and the mean content of the units assayed has been ignored. Generally, the accuracy and precision of the analytical method are such that the distinction is secondary. Also, USP XVIII (25) states, with respect to tolerances: "These limits allow for assay error, for unavoidable variations in manufacturing and compounding, and for deterioration to an extent considered insignificant under practical conditions." In any event, the curves would seem to be valid for either case provided that one is consistent, *i.e.*, provided both lot and sample are described in terms of either absolute or assay values. In actual practice, the true mean and variance are not known so that the assay values must be used. Recently, the effect of analytical error on the conclusion of the USP XVII test for content uniformity was determined (26, 27).

There remains the question as to whether more reliable tests, for a given number of assays, can be designed. In converting the assay values into the numbers utilized in the tests, there is a loss of information. This becomes apparent if one tries to use these numbers to regenerate the original assay values. Presumably, the most reliable tests will be those utilizing, to the greatest degree, all available information. Perhaps such tests, besides involving the mean and standard deviation, will utilize the coefficients of skewness and kurtosis. However, due to nonrandom variations in the actual mean and variance from one portion of a lot to another, the limitations in obtaining a representative sample may mask small improvements in the test for content uniformity.

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Use of 2-(4′-Hydroxybenzeneazo)benzoic Acid to Study the Binding of L-Thyroxine to Serum Albumins

RALPH I. NAZARETH, THEODORE D. SOKOLOSKI[x], DONALD T. WITIAK, and ALLEN T. HOPPER*

Abstract □ A study was made to investigate the use of the dye 2-(4′-hydroxybenzeneazo)benzoic acid as a potential agent that would reflect the binding of L-thyroxine to serum albumins. The dye is a strong visible absorbing material which interacts with serum albumins to give characteristic spectrophotometric peaks and, as such, provides the basis for an extremely convenient assay to measure amounts, free and bound, after their separation in the presence of serum albumin and potential competitive inhibitors. The results obtained showed that the dye and L-thyroxine compete for the same binding site on bovine and rat serum albumins; thus the dye can be used to gauge the displacement of L-thyroxine from serum albumin by potential competitive inhibitors. Additionally, a method was introduced to evaluate statistically differences, between data sets, in y-intercepts obtained by linear regression. The method used should have general application.

Keyphrases □ L-Thyroxine binding to serum albumins—measured using 2-(4′-hydroxybenzeneazo)benzoic acid, spectrophotometry □ Serum albumin binding of L-thyroxine—measured using 2-(4′-hydroxybenzeneazo)benzoic acid, spectrophotometry □ 2-(4′-Hydroxybenzeneazo)benzoic acid—used to measure L-thyroxine binding to serum albumins

2-(4′-Hydroxybenzeneazo)benzoic acid (I) is a dye that interacts with serum albumins to give characteristic spectrophotometric peaks (1). The intensity of these peaks can be related to the extent of binding of the dye to serum albumin and, therefore, could represent an extremely convenient means of studying displacement reactions. The binding of I to serum albumins and its displacement from serum albumin by chlorophenoxyacetic acids were studied previously (2, 3). Moriguchi *et al.* (4) also studied the binding of I to bovine serum albumin and described a procedure that utilized I in determining the binding of acidic drugs. Drugs studied with bovine serum albumin were substituted benzoic acids (5) and sulfonamides (6). This study was made to investigate the use of I as a potential agent that would reflect the binding of L-thyroxine to serum albumins. The objective was to determine if I and L-thyroxine are bound to the same site on serum albumin so that the dye could be used to gauge the displacement of L-thyroxine from serum albumin. The results using bovine and rat serum albumins are presented here.

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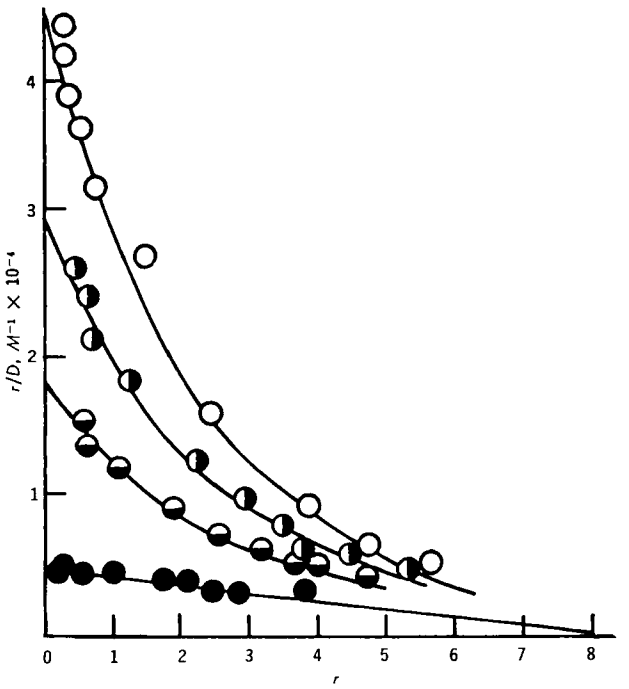

Figure 1—*Scatchard plot for the binding of I to bovine serum albumin (1.0 × 10⁻⁴ M) in the presence of various total amounts of* L-*thyroxine. Key:* ○, *I alone;* ◑, *3.33 × 10⁻⁵ M* L-*thyroxine;* ◒, *6.66 × 10⁻⁵ M* L-*thyroxine; and* ●, *16.66 × 10⁻⁵ M* L-*thyroxine.*

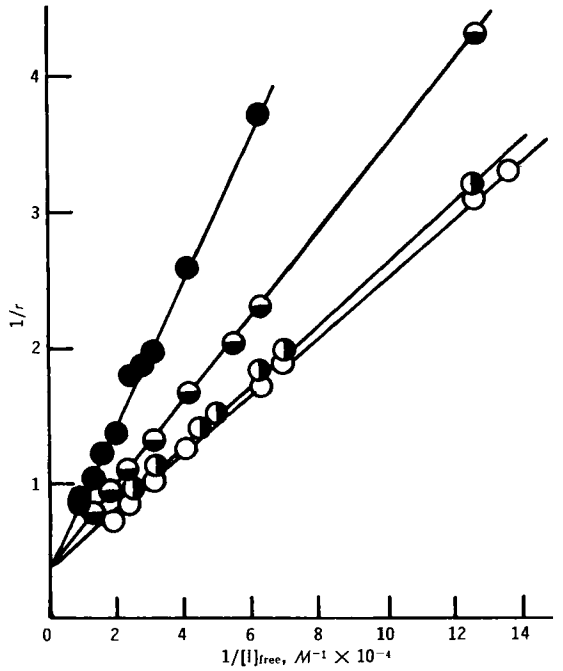

Figure 2—*Relationship between 1/*r (*moles bovine serum albumin per mole I*) *and the reciprocal of free I concentration at low* r *values in the presence of* L-*thyroxine. Key:* ○, *no thyroxine;* ◑, *1.66 × 10⁻⁵ M* L-*thyroxine;* ◒, *3.33 × 10⁻⁵ M* L-*thyroxine; and* ●, *6.66 × 10⁻⁵ M* L-*thyroxine.*

EXPERIMENTAL

Materials—Bovine serum albumin[1] (Fraction V, fatty acid poor), 2-(4′-hydroxybenzeneazo)benzoic acid[1], rat serum albumin[2] (Fraction V), L-thyroxine[3], and dimethyl sulfoxide[3] were used as purchased without further purification. All final solutions were made with 0.1 *M* sodium phosphate buffer, pH 7.4, using analytical grade chemicals. The dimethyl sulfoxide stock solution of L-thyroxine (1.0×10^{-3} *M*) was diluted with appropriate amounts of phosphate buffer to give the desired final concentrations.

Competitive Binding between I and L-Thyroxine to Bovine Serum Albumin—The binding of I to this albumin in the presence and absence of L-thyroxine was determined by varying the concentration of I from 7.0×10^{-5} to 1.6×10^{-3} *M* at constant bovine serum albumin and L-thyroxine concentrations. The albumin concentration was held at 1.0×10^{-4} *M* (using molecular weight 67,000) in all studies, and four fixed concentrations of L-thyroxine were used (1.66×10^{-5}, 3.33×10^{-5}, 6.66×10^{-5}, and 16.66×10^{-5} *M*). Ultrafiltration at ambient temperatures was used to separate the free amount of I. Twelve milliliters of solution was placed in the ultrafiltration cell[4]. The ultrafiltrate (1.2 ml) was collected and analyzed spectrophotometrically after suitable dilution. In the absence of L-thyroxine, five separate determinations were made where the concentration of free I was determined from the absorbance of the ultrafiltrate at 350 nm. The ϵ for I is 17,682 at this wavelength in phosphate buffer. In the presence of L-thyroxine, the ultrafiltrate was analyzed spectrophotometrically at 400 nm after adjusting the pH of the solution to 10.2 with 2 *N* sodium hydroxide. At pH 10.2, L-thyroxine does not contribute to the 400-nm absorbance of I. The ϵ for I at 400 nm is 21,630.

Competitive Binding between I and L-Thyroxine to Rat Serum Albumin—The binding of I to this albumin in the presence and absence of L-thyroxine was determined by varying the concentration of I from 5.0×10^{-5} to 1.6×10^{-3} *M* at constant rat serum albumin and L-thyroxine concentrations. The albumin concentration was held at 1.0×10^{-4} *M* in all studies, and three fixed concentrations of L-thyroxine were used (3.33×10^{-5}, 6.66×10^{-5}, and 16.66×10^{-5} *M*). Ultrafiltration was used to determine the amount of free I by spectrophotometric analysis at 350 nm in the absence of L-thyroxine and at 400 nm in the presence of L-thyroxine, as already described for the competitive binding to bovine serum albumin.

Treatment of Binding Data—The concentration of bound I in the ultrafiltration studies was determined by taking the difference between the total amount added and the amount found free after ultrafiltration. Corrections were made for the small amount of ligand bound to the ultrafiltration membrane. Scatchard plots were constructed and computer analyzed for binding parameters assuming that two independent classes of binding sites exist on the serum albumin. The computer-generated constants gave *n* (maximum number of sites) and *K* (association constant) values for each class.

Statistical Analysis of Linear Regression Intercepts (7)—To determine whether or not the intercept values for two sets of regression data are the same, use was made of the Student *t* test. In this test, the difference between the average intercepts for two linear regressions is divided by the pooled standard deviation for the data sets:

$$t = \frac{I_1 - I_2}{s_{\text{pooled}}} \qquad \text{(Eq. 1)}$$

In Eq. 1, I_1 is the mean intercept for data set 1, I_2 is the mean intercept for data set 2, and s is the pooled standard deviation for the two data sets. In a regression analysis of y on x, the variance in the intercept, s_I^2, for a data set is given by:

$$s_I^2 = MSE \cdot G/N \qquad \text{(Eq. 2)}$$

where N is the number of observations, G (that takes into account the effect on error variance of the distance of the intercept from the mean $\bar{X}$) is given by:

$$G = 1 + \frac{Nx^{-2}}{\sum_{i=1}^{N}(x_i - \bar{x})^2} \qquad \text{(Eq. 3)}$$

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Pentex Inc., Kankakee, Ill.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Amicon model 12 cell with a PM-10 membrane.

Table I—Statistical Analysis of Linear Regression Intercepts at $p = 0.01$

System	Number of Observations, N	Mean Square Error[a]	Calculated F Ratio for Mean Standard Error[b]	Theoretical F Ratio for Mean Standard Error[c]	Variance in Intercept[d], s_I^2	Calculated t[e]	Theoretical t[c]
In bovine serum albumin							
I alone	11	3.06×10^{-3}	—	—	8.73×10^{-4}	—	—
I + thyroxine (1.66×10^{-5} *M*)	9	2.09×10^{-4}	14.62	5.61	8.87×10^{-5}	0.69	2.92
I + thyroxine (3.33×10^{-5} *M*)	11	2.70×10^{-3}	1.13	5.35	7.17×10^{-4}	1.18	2.88
I + thyroxine (6.66×10^{-5} *M*)	14	8.41×10^{-3}	2.75	5.11	1.34×10^{-3}	0.78	2.83
In rat serum albumin							
I alone	6	1.04×10^{-4}	—	—	1.55×10^{-4}	—	—
I + thyroxine (3.33×10^{-5} *M*)	5	6.81×10^{-5}	1.53	28.70	9.02×10^{-5}	2.27	3.50
I + thyroxine (6.66×10^{-5} *M*)	10	2.77×10^{-3}	26.62	7.01	1.34×10^{-3}	1.97	3.05
I + thyroxine (16.66×10^{-5} *M*)	8	1.35×10^{-4}	1.29	9.15	1.71×10^{-4}	1.80	3.17

[a] Calculated by Eq. 4. [b] Mean standard error for I/mean standard error for system. [c] From Ref. 9, degrees of freedom $= N - 2$. [d] Calculated by Eq. 2. [e] From Eq. 1.

and MSE is the mean square error given as:

$$MSE = \sum_{i=1}^{N}(y_i - y_{calc})^2/(N - 2) \qquad \text{(Eq. 4)}$$

The numerator of Eq. 4 is the sum of squares of the errors *(SSE)*, where y_{calc} is the regression calculated value for y. The pooled standard deviation used in Eq. 1 is defined as:

$$s_{pooled} = \sqrt{s_{I,1}^2 + s_{I,2}^2} \qquad \text{(Eq. 5)}$$

where subscripts refer to individual data sets. Substituting Eq. 2 into Eq. 5 gives:

$$s_{pooled} = \sqrt{MSE_1 \cdot G_1/N_1 + MSE_2 \cdot G_2/N_2} \qquad \text{(Eq. 6)}$$

If MSE_1 is essentially equal to MSE_2, Eq. 6 simplifies to:

$$s_{pooled} = \sqrt{\frac{SSE_1 + SSE_2}{N_1 + N_2 - 4}\left(\frac{G_1}{N_1} + \frac{G_2}{N_2}\right)} \qquad \text{(Eq. 7)}$$

Therefore, to use Eq. 7 (and Eq. 1), it had to be established that the errors (mean square error values) in the two data sets being compared were essentially the same. To determine whether or not the errors were statistically different, the F ratio for the mean square error in the two data sets was calculated and compared to the theoretical F values at $p = 0.01$. If the calculated F ratios were less than the theoretical values, it was concluded that the errors were not statistically different and that the use of Eq. 7 (and Eq. 1) was valid.

RESULTS AND DISCUSSION

Competitive Binding between I and L-Thyroxine on Bovine Serum Albumin—The binding of the azo dye, I, to bovine serum albumin in the presence and absence of fixed amounts of L-thyroxine gave the results plotted in Fig. 1. In the presence of L-thyroxine, the amount of bound dye decreases and more dye is displaced as the concentration of the hormone increases. At a concentration of 16.66×10^{-5} *M* L-thyroxine, it appears that I is dis-

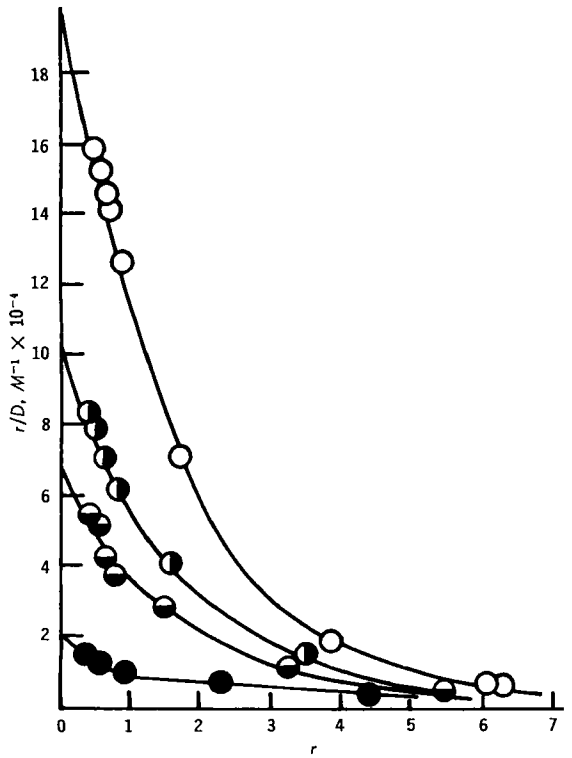

Figure 3—*Scatchard plot for the binding of I to rat serum albumin (1.0×10^{-4} M) in the presence of various total amounts of L-thyroxine. Key:* ○, *I alone;* ◑, *3.33×10^{-5} M L-thyroxine;* ◒, *6.66×10^{-5} M L-thyroxine; and* ●, *16.66×10^{-5} M L-thyroxine.*

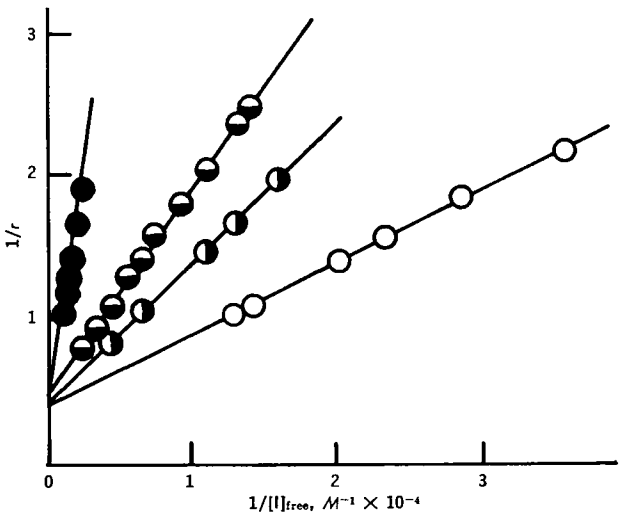

Figure 4—*Relationship between 1/r (moles rat serum albumin per mole I) and the reciprocal of free I concentration at low r values in the presence of L-thyroxine. Key:* ○, *no thyroxine;* ◑, *3.33×10^{-5} M L-thyroxine;* ◒, *6.66×10^{-5} M L-thyroxine; and* ●, *16.66×10^{-5} M L-thyroxine.*

placed from its primary site since an apparent linear Scatchard plot exists at this L-thyroxine level, giving $n = 8$ and $K = 640$ by regression analysis which approaches the values of n_2 (7) and K_2 (1310 M^{-1}) for I as determined by a computer fit of the binding of the dye to this albumin. The n_1 and K_1 values for I are 1.7 and 23,000 M^{-1}, respectively. By using values for r (moles bound I per mole albumin) of approximately 1 and less, where the first class of binding sites is primarily affected, the data of Fig. 1 were replotted as $1/r$ *versus* $1/[I]_{free}$ to determine if L-thyroxine and I are competing for the same binding site on bovine serum albumin. If the same extrapolated $1/r$ intercept is obtained for each concentration of L-thyroxine as found for I alone, the conclusion can be made that the same site is involved (8).

Figure 2 shows the effect of three different total concentrations of L-thyroxine on the binding of I to bovine serum albumin. Linear regression analysis of the data of Fig. 2 produces intercepts of 0.38 for I alone and of 0.36, 0.35, and 0.34 for L-thyroxine at 1.66×10^{-5}, 3.33×10^{-5}, and 6.66×10^{-5} M, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table I). The validity of the use of the test is described in the *Experimental* section. In the case of 1.66×10^{-5} M L-thyroxine, the t value suggests no difference in intercepts, but statistical methods cannot be used here because the F distribution limit for the variances of the intercepts falls outside the theoretical range. It is assumed that this intercept is not really different since the other concentrations of L-thyroxine give statistically valid interpretations. The conclusion is, therefore, made that L-thyroxine competes with I at the same binding site on bovine serum albumin.

Competitive Binding between I and L-Thyroxine on Rat Serum Albumin—The binding of I to rat albumin in the presence and absence of fixed amounts of L-thyroxine gave the results plotted in Fig. 3. In the absence of inhibitor, computer-generated values were: $n_1 = 2.2$, $n_2 = 8.1$, $K_1 = 83{,}610$ M^{-1}, and $K_2 = 1295$ M^{-1}. In the presence of L-thyroxine, the amount of bound dye decreases and more dye is displaced as the concentration of the hormone increases. At a concentration of 16.66×10^{-5} M L-thyroxine, it appears that I is almost completely displaced from its primary site since an almost linear Scatchard plot exists at this L-thyroxine level. By using values for r (moles bound I per mole rat albumin) of approximately 1 and less, where the first class of sites for I would most likely be involved, the data of Fig. 3 were replotted as $1/r$ *versus* $1/[I]_{free}$ to determine if L-thyroxine and I are competing for the same binding site in rat serum albumin. Figure 4 shows the effect of three different total concentrations of L-thyroxine on the binding of I to the albumin. Linear regression analysis of the data of Fig. 4 produces intercepts of 0.37 for I alone and of 0.41, 0.49, and 0.40 for L-thyroxine at 3.33×10^{-5}, 6.66×10^{-5}, and 16.66×10^{-5} M, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table I). In the case of 6.66×10^{-5} M L-thyroxine, the t value suggests no difference in intercepts, but statistical methods cannot be used here because the F distribution limit for the variances of the intercepts falls outside the theoretical range. It is assumed that this intercept is not really different since the other concentrations of L-thyroxine give statistically valid interpretations. The conclusion is, therefore, made that L-thyroxine competes with I at the same binding site on rat serum albumin.

The results of the competitive binding studies between I and L-thyroxine to bovine and rat serum albumins show that I and L-thyroxine compete at the same binding site on both albumins. Therefore, it should be possible to use I as a model to mirror the binding of L-thyroxine to serum albumin. Use of this property of I on the binding of hypolipemic agents to serum albumins is described in a subsequent report (10).

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*Present address: Battelle Memorial Institute, Columbus, OH 43210

x To whom inquiries should be directed.

Biological Significance of Serum Albumin Binding Parameters Determined *In Vitro* for Clofibrate-Related Hypolipemic Drugs: Use of 2-(4′-Hydroxybenzeneazo)benzoic Acid to Mirror L-Thyroxine Binding to and Displacement from Serum Albumins

RALPH I. NAZARETH, THEODORE D. SOKOLOSKI[x], DONALD T. WITIAK, and ALLEN T. HOPPER*

Abstract □ Clofibrate [ethyl 2-(*p*-chlorophenoxy)-2-methylpropionate] has been shown to be an effective lipid-lowering drug in man and to exert its action by multiple modes. To explore further the possibility that albumin binding parameters determined *in vitro* are predictive of hypolipemic activity determined *in vivo*, the binding of several cyclic and acyclic analogs of the corresponding carboxylic acid of clofibrate to bovine, rat, and human serum albumins was studied. This report compares the differential hypolipemic effects of the esters of these analogs with data derived from studies involving their ability to undergo hydrolysis by serum esterases *in vitro*, as well as with the pKa's, log *P* values, and albumin binding parameters of their respective free acids. The latter study involved the use of 2-(4′-hydroxybenzeneazo)benzoic acid as an optical probe which mirrors thyroxine binding *in vitro* as well as the use of the free acids alone and their ability to displace radiolabeled thyroxine. These investigations indicate that albumin binding parameters probably are important for drug transport *in vivo* but that hypolipemic activity is related to mechanisms other than thyroxine displacement.

Keyphrases □ Clofibrate-related hypolipemic drugs—*in vitro* serum albumin binding parameters determined using 2-(4′-hydroxybenzeneazo)benzoic acid, thyroxine displacement, relationship to hypolipemic activity □ Hypolipemic drugs related to clofibrate—*in vitro* serum albumin binding parameters, determined using 2-(4′-hydroxybenzeneazo)benzoic acid, thyroxine displacement, relationship to hypolipemic activity □ Serum albumin binding—clofibrate-related hypolipemic drugs, determined using 2-(4′-hydroxybenzeneazo)benzoic acid, thyroxine displacement □ 2-(4′-Hydroxybenzeneazo)benzoic acid—used to determine serum albumin binding of clofibrate-related hypolipemic drugs, thyroxine displacement

Clofibrate [ethyl 2-(*p*-chlorophenoxy)-2-methylpropionate, I] has been shown to be an effective lipid-lowering drug in man (1, 2). This drug rapidly undergoes hydrolysis *in vivo*, and the corresponding acid, II, is presumed to be the active drug (3). Studies *in vivo* with I and *in vitro* with II suggest that clofibrate may be exerting its effect by multiple modes of action (4). Aryloxyisobutyrates were reported (5) to cause a reduction of L-thyroxine binding to albumin in the range of $r < 1$ (r = moles drug bound per mole albumin). While clofibrate was reported to both alter (6) and not alter (7, 8) the distribution of labeled thyroxine *in vivo*, causing a reduction in plasma concentration with a concomitant increase in liver concentration, it was suggested that any alteration in distribution of thyroxine *in vivo* caused by clofibrate may have physiological significance and, in fact, correlate with *in vitro* binding studies. For example, the suggestion was made (6) that the displacement effects observed for hypolipemic drugs in the range r = 0–1 are likely of relevance to therapy whereas effects at $r > 1$ may be critical in the evaluation of toxicological studies. Studies by Thorp (9) involving interspecies differences in the binding of these drugs also led him to conclude that albumin binding studies *in vitro* could be used to predict the effects of a given compound on serum cholesterol levels *in vivo*.

To explore further the possibility that albumin binding parameters determined *in vitro* are predictive of hypolipemic activity determined *in vivo*, the binding of several cyclic and acyclic analogs of II to bovine, rat, and human serum albumins was studied. A comparative analysis was recently described (10) of the hypocholesterolemic and hypotriglyceridemic activity of the corresponding ethyl esters of these analogs in a hyperlipemic rat model in which hyperlipemia was induced by intraperitoneal injection of tyloxapol[1]. Under these conditions, clofibrate (I), ethyl 1,4-benzodioxane-2-carboxylate (V), ethyl 5-chloro-2,3-dihydro-2-benzofurancarboxylate (VII), and ethyl 6-chlorochroman-2-carboxylate (XI) significantly reduced serum cholesterol levels. The deschloro analogs IX and XIII were inactive. In these same hyperlipemic rats, esters I, V, and XI also significantly reduced plasma triglyceride levels. Deschloro analogs IX and XIII were inactive, and the apparent hypotriglyceridemic effect of chloro analog VII was not statistically significant. Acyclic analog III, administered as the DL-compound, also showed no significant hypocholesterolemic or hypotriglyceridemic activity in these hyperlipemic rats.

In this report, the differential hypolipemic effects of esters I, III, V, VII, IX, XI, and XIII are compared with data derived from studies involving their ability to undergo hydrolysis by serum esterases *in vitro*, as well as with the pKa's, log *P* (*P* = octanol–water partition coefficient) values, and albumin binding parameters of the respective free acids. Albumin binding studies also were carried out with optically pure L(*S*)-IV and D(*R*)-IV, alicyclic homolog XV, and 4-ketochroman (XVII) to determine whether stereochemistry and these minor structural modifications had a major influence on albumin binding parameters.

Since it was shown previously (11) that the optical probe 2-(4′-hydroxybenzeneazo)benzoic acid (XVIII) could be used as a model to mirror the binding of thyroxine to bovine and rat serum albumins, acid

[1] Triton WR-1339.

Table I—Molar Absorptivity and Wavelength Maximum Used to Study the Hydrolysis of Esters by Rat Plasma *In Vitro* at 37°

Ester	ϵ (Wavelength, nm)
I	11,600 (226)
III	13,047 (226)
VII	7,196 (231)
XI	8,965 (229)
XVI	19,290 (226)

XVIII also was utilized in these studies to gauge the displacement of thyroxine from serum albumins by the various analogs. Use of XVIII in place of thyroxine to estimate relative differences in competitive binding by the various analogs is simple and has the additional advantage of decreased expense over the usual assays for the hormone utilizing radiolabeled compound (12) or enzymatic reactions (13). To substantiate further the validity of results obtained in competitive binding studies of the various analogs using XVIII, the displacement of ^{125}I-thyroxine from rat serum albumin by II, XII, and XIV (12, 14) was investigated.

EXPERIMENTAL

Materials—Bovine serum albumin (Fraction V, fatty acid poor), human serum albumin (Fraction V), and 2-(4′-hydroxybenzeneazo)benzoic acid (XVIII) were used as purchased[2]. Rat serum albumin[3] (Fraction V), L-thyroxine[4], dimethyl sulfoxide[4], isooctane[5] (2,2,4-trimethylpentane, spectral grade), and ^{125}I-thyroxine[6] (specific activity 200 μCi/ml) were used without further purification. 1-Octanol[7] was purified as described under *Octanol–Water Partition Coefficient Determination.* All final solutions were made with 0.1 *M* sodium phosphate buffer, pH 7.4, using analytical grade chemicals. All carboxylic acids were synthesized as described by Witiak *et al.* (15–18). Dimethyl sulfoxide stock solutions of the carboxylic acids (1.0×10^{-2} *M*) were diluted with appropriate amounts of phosphate buffer to give the desired final concentrations.

Hydrolysis of Esters by Rat Plasma *In Vitro*—The hydrolysis of esters I, III, VII, XI, and XVI by rat plasma *in vitro* at 37° was determined according to the method of Thorp[8]. Blood collected from the abdominal aorta of anesthetized male Sprague–Dawley rats (180–200 g) was placed in tubes (Vacutainer) containing a small amount of ethylenediaminetetraacetic acid. The blood was immediately centrifuged for 15 min, and the supernatant plasma was separated and pooled. The esters were dissolved in spectral grade methanol to give 0.05 *M* solutions. The incubation system used was as follows: 0.1–0.3 ml of the ester solution was added to 8 ml of rat plasma, and the mixture was incubated at 37° on a metabolic shaker. Samples (1.0 ml) were removed at various times (0, 5, 10, and 60 min) for determination of the amounts of the parent esters present.

For the assay procedure, 1 ml of each sample was placed in a 10-ml glass-stoppered centrifuge tube. One-half milliliter of 3 *N* hydrochloric acid and 5 ml of an isooctane–absolute ethanol mixture (95:5 v/v) were added to each tube. The tubes were stoppered, shaken by hand, and allowed to stand. After separation of the layers, the UV absorption of the extracts was determined against a blank consisting of the solvent mixture. The wavelengths used for the esters as well as their ϵ values are given in Table I. This UV absorption of the extract is due to the presence of both ester and free acid. An aliquot of the isooctane–ethanol extract was extracted with an equal volume of 2% sodium bicarbonate to remove the free acid selectively. The amount of free acid was determined from the reduction in the UV absorption of the organic phase by comparison with suitable standards, blanks, and recoveries (at zero time) from rat plasma.

pKa and ϵ of Carboxylic Acids—The pKa's of the carboxylic acids were determined potentiometrically by titrating 0.02% solutions prepared by diluting 0.4% stock solutions in dimethyl sulfoxide with double-distilled water. Sodium hydroxide (0.01 *N*) was the titrant, and a pH meter[9] was used to measure the pH.

The molar absorptivity, ϵ, for the carboxylic acids was determined by measuring[10] the absorbance of solutions made by diluting 1.0×10^{-2} *M* dimethyl sulfoxide solutions with phosphate buffer. Reference solutions consisted of equivalent dilutions of dimethyl sulfoxide with buffer. The wavelength maxima at which ϵ was determined are listed in Table II. All studies were made at ambient temperature.

Binding of Carboxylic Acids to Serum Albumin—The binding of the carboxylic acids to bovine, human, and rat serum albumins was determined by the ultrafiltration technique (11) at ambient temperatures, using phosphate buffer solutions having a constant concentration of serum albumin (1.0×10^{-4} *M*, molecular weight 67,000) and a varying analog concentration (from 2.0×10^{-4} to 3.0×10^{-3} *M*). The ultrafiltrate was analyzed spectrophotometrically after suitable dilution. The ϵ value and wavelength of measurement are given in Table II.

Competitive Binding between XVIII and II, L(*S*)-IV, D(*R*)-IV, XIV, and XV on Bovine Serum Albumin—The binding of XVIII to bovine serum albumin in the presence of four analogs [L(*S*)-IV, D(*R*)-IV, XIV, and XV] was determined by varying the concentration of XVIII from 3.0×10^{-5} to 1.6×10^{-3} *M* at fixed albumin (1.0×10^{-4} *M*) and analog (1.0×10^{-4} *M*) concentrations. Three fixed concentration levels of II were used: 1.0×10^{-4}, 2.0×10^{-4}, and 5.0×10^{-4} *M*. Ultrafiltration was used to study binding where the amount of XVIII free was determined by spectrophotometric analysis at 350 nm. None of the analogs absorbs at this wavelength in phosphate buffer.

I: R = CH_2CH_3
II: R = H

III: R = (DL)-CH_2CH_3
IV: R = H

V: R = CH_2CH_3
VI: R = H

VII: R = CH_2CH_3, X = Cl
VIII: R = H, X = Cl
IX: R = CH_2CH_3, X = H
X: R = X = H

XI: R = CH_2CH_3, X = Cl
XII: R = H, X = Cl
XIII: R = CH_2CH_3, X = H
XIV: R = X = H

XV

XVI: R = CH_2CH_3
XVII: R = H

XVIII

[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Pentex, Inc., Kankakee, Ill.
[4] Eastman Kodak Co., Rochester, N.Y.
[5] Matheson, Coleman and Bell, Norwood, Ohio.
[6] Amersham-Searle Corp., Arlington Heights, Ill.
[7] Aldrich Chemical Co., Milwaukee, Wis.
[8] J. M. Thorp, Biological Research Department, Imperial Chemical Industries Ltd., Alderly Park, Macclesfield, Chesire, England, personal communication.

[9] Sargent, model DR.
[10] With a Cary model 16 spectrophotometer.

Table II—Molar Absorptivity and pKa for Various Analogs Used in Serum Albumin Binding Studies

Analog	ϵ (Wavelength, nm)	pKa
II	1018 (279)	4.46
L(*S*)-IV	1372 (279)	4.35
D(*R*)-IV	1400 (279)	4.35
VI	2300 (277)	4.23
VIII	2228 (279)	4.36
XII	1873 (283)	4.26
XIV	1613 (279)	4.14
XV	1048 (279)	3.90
XVII	3029 (337)	3.90

Table III—Octanol–Water Partition Coefficients of Analogs

Analog	ϵ (Wavelength, nm)	Log *P*
II	1038 (280)	2.57
D(*R*)-IV	1565 (281)	2.31
VI	2300 (277)	1.40
VIII	3010 (290)	2.11
XII	2120 (285)	2.40
XIV	1825 (282)	1.90
XV	1100 (281)	2.90
XVII	3240 (330)	1.77

Moriguchi Method—For comparative purposes, the binding of the carboxylic acids to bovine serum albumin also was studied by an indirect method (19, 20). The binding constants of the analogs were evaluated spectrophotometrically in terms of their ability to displace competitively 2-(4′-hydroxybenzeneazo)benzoic acid (XVIII). Bovine albumin and XVIII stock solutions were made in 0.05 *M* sodium phosphate buffer, pH 7.4. Stock analog solutions (1.0×10^{-4} *M*) were made in the phosphate buffer with 10% dimethyl sulfoxide. Solutions containing analogs (0, 1.0×10^{-4}, 1.5×10^{-4}, 2.0×10^{-4}, and 2.5×10^{-4} *M*), XVIII (1.0×10^{-4} *M*), and bovine serum albumin (5.0×10^{-5} *M*) were prepared, and the absorbances were measured at 482 nm 1 hr after preparation of the solutions. The results were analyzed according to Eq. 1:

$$\Delta E = E' - (E + E'') \qquad \text{(Eq. 1)}$$

where:

E' = absorbance of the solutions containing 1.0×10^{-4} *M* XVIII and 5.0×10^{-5} *M* bovine serum albumin with 0, 1.0×10^{-4}, 1.5×10^{-4}, 2.0×10^{-4}, and 2.6×10^{-4} *M* analogs at 482 nm

E = absorbance of the solution of 1.0×10^{-4} *M* XVIII at 482 nm

E'' = absorbance of the solution of 5.0×10^{-5} *M* bovine serum albumin at 482 nm

Log K_b, where K_b is the binding constant of the analog (presumably to the primary binding site), is determined from the ΔE value obtained for the various analogs through use of theoretical curves relating log K_b to ΔE as described by Moriguchi (20).

Competitive Binding between XVIII and II, XII, and XIV on Rat Serum Albumin—The binding of XVIII to rat serum albumin in the presence of two analogs (XII and XIV) was determined by varying the concentration of XVIII from 5.0×10^{-5} to 1.6×10^{-3} *M* at fixed albumin (1.0×10^{-4} *M*) and analog (2.0×10^{-4} *M*) concentrations. The effect of two fixed concentration levels (2.0×10^{-4} and 5.0×10^{-4} *M*) of II was studied under the same conditions. Ultrafiltration was used to study binding, and the amount of XVIII free was determined by spectrophotometric analysis at 350 nm.

Binding of Thyroxine to Rat Serum Albumin—Equilibrium dialysis with ^{125}I-thyroxine was used to study the binding of thyroxine to rat serum albumin (12, 14). Twenty-centimeter (8-in.) lengths of 2.1-cm (0.83-in.) dialysis tubing were soaked in 0.1 *M* nitric acid for 24 hr and then in 0.01 *M* nitric acid for 3 days to remove metal impurities. The tubing was thoroughly rinsed with distilled water and was finally stored in deionized water at 4°. Before use, the tubing was rinsed with glass-distilled water. The stock solution of thyroxine (1.0×10^{-3} *M*) was prepared in 0.04 *N* sodium hydroxide. The range of thyroxine concentrations used was from 2.6×10^{-6} to 2.6×10^{-5} *M*. These solutions were prepared by appropriate dilutions of the stock thyroxine solution using 0.1 *M* sodium phosphate buffer, pH 7.4, containing 3.0×10^{-4} *M* ethylenediaminetetraacetic acid. ^{125}I-Thyroxine (2.0 μCi) was added to the nonradioactive thyroxine solutions, and 5 ml of this solution was placed in 50-ml lusteroid centrifuge tubes (28 × 102 mm). Five milliliters of the rat serum albumin solution (0.05 g rat serum albumin/100 ml) was placed in the dialysis bag. The tubes were covered with aluminum foil and placed on a metabolic shaker at 37°. After 16 hr, the radioactivity in 2-ml samples from each compartment was counted[11]. The fraction bound was obtained from the relationship: (counts per minute inside − counts per minute outside)/total counts per minute.

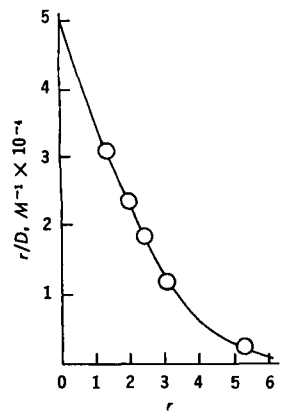

Figure 1—*Scatchard plot for the binding of XII to human serum albumin (1.0×10^{-4} M) at pH 7.4. Key: O, experimental values; and ——, computer fit assuming two independent classes of binding sites exist on serum albumin.*

Competitive Binding between Thyroxine and II, XII, and XIV on Rat Serum Albumin—The binding of thyroxine to rat serum albumin in the presence of two analogs (II and XIV) was determined by varying the concentration of thyroxine from 2.6×10^{-6} to 2.6×10^{-5} *M* at a fixed albumin concentration (7.5×10^{-6} *M*), employing an analog concentration of 1.0×10^{-4} *M*. Under similar experimental conditions, two fixed concentration levels of XII were used (1.0×10^{-4} and 2.0×10^{-4} *M*). Stock solutions of the analogs were made in 0.1 *M* sodium phosphate buffer, pH 7.4, containing 3.0×10^{-4} *M* ethylenediaminetetraacetic acid. Equilibrium dialysis, followed by analysis of ^{125}I-thyroxine as already described, was used to determine the fraction of thyroxine bound.

Octanol–Water Partition Coefficient Determination—Partition coefficients of the carboxylic acid analogs were determined according to the method of Hansch[12]. 1-Octanol was purified by washing with 10% sodium hydroxide, 10% sulfuric acid, and, finally, 10% sodium bicarbonate solution. After drying over magnesium sulfate, the octanol was distilled under reduced pressure and then saturated with distilled water. Analogs were dissolved in octanol, and 10 ml of the resulting solution was placed in a 250-ml glass-stoppered centrifuge bottle. Two hundred milliliters of the appropriate buffer solution (pH two units below the pKa of the analog) was added; the bottle was stoppered and the phases were mixed by inverting and shaking by hand for 2 min. After centrifugation for 1 hr, samples of the octanol phase were removed by pipet and analyzed spectrophotometrically. The wavelengths used for these measurements and the ϵ values for the various analogs are listed in Table III.

RESULTS

Hydrolysis of Esters by Rat Plasma *In Vitro*—The hydrolysis of esters I, III, VII, XI, and XVI by rat plasma *in vitro* was carried out at 37°. All of these esters were rapidly hydrolyzed, in accord with the results obtained by Thorp (3) for clofibrate. The mean percent and standard deviation of the ester hydrolyzed in 5

[11] Baird-atomic well counter, model 810C, with a Baird-atomic spectrometer, model 530.

[12] C. Hansch, Department of Chemistry, Pomona College, Claremont, CA 91711, personal communication.

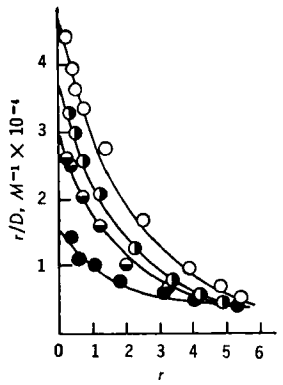

Figure 2—*Scatchard plot for the binding of XVIII to bovine serum albumin (1.0×10^{-4} M) in the presence of various total amounts of II. Key: ○, XVIII alone; ◑, 1.0×10^{-4} M II; ◒, 2.0×10^{-4} M II; and ●, 5.0×10^{-4} M II.*

min at 37° were as follows: I, 98.6 ± 0.2%; III, 98.1 ± 0.1%; VII, 97.8 ± 0.5%; XI, 96.3 ± 0.5%; and XVI, 99.0 ± 0.3%.

Binding of Carboxylic Acid Analogs to Bovine, Rat, and Human Serum Albumins—At the pH of the binding studies (7.4), all of the analogs exist primarily in the anionic form since their pKa's range from 3.90 to 4.46 (Table II). It is apparent that the species bound must be the charged form for each analog. A Scatchard plot for the binding of each analog to the various serum albumins gives the same general nonlinear relation, excluding analog X which binds to only one site and, therefore, gives a linear Scatchard plot. A typical or representative result is shown in Fig. 1 for the binding of XII to human serum albumin. The solid line in Fig. 1 is the computer fit for the data using the binding parameters generated assuming two independent classes of binding sites exist on serum albumin. It is apparent that this assumption adequately describes the binding of this analog to human serum albumin. Similar computer fits were obtained for each of the other analogs with all serum albumins used. The computer-generated values for n_1, K_1, n_2, and K_2 best describing the binding of the analogs to bovine, rat, and human serum albumins are given in Table IV. The parameters are related by the well-known equation:

$$r = \frac{n_1 K_1 D_f}{1 + K_1 D_f} + \frac{n_2 K_2 D_f}{1 + K_2 D_f} \qquad \text{(Eq. 2)}$$

in which r is the number of moles of analog bound per mole of albumin; n_1 and n_2 are the maximum number of sites in the first and second class of mutually independent sites, respectively; K_1 and K_2 are the binding constants in each class, and D_f is the concentration of free analog in equilibrium with bound analog.

For all analogs and a particular albumin, the binding constant for the first class of sites is larger than that for the second binding class. With bovine serum albumin, analogs L(S)-IV, VI, and XIV have the larger K_1 values, ranging from four to five times the K_1 value for II. With rat serum albumin, XIV has a primary binding constant approximately twice that of the other analogs. With human serum albumin, XIV has a K_1 less than the one observed for II, but again differences in binding constants for the various

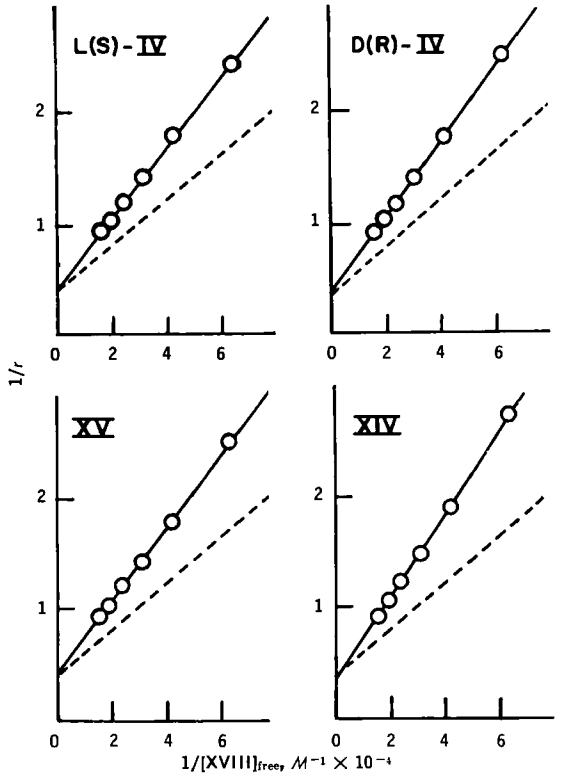

Figure 4—*Relationship between 1/r (moles bovine serum albumin per mole XVIII) and $1/[XVIII]_{free}$ at low r values in the presence of 1.0×10^{-4} M L(S)-IV, D(R)-IV, XIV, and XV. The broken line is XVIII alone, having an intercept of 0.38.*

analogs are comparatively small. It is apparent that there are minor species differences in the albumin binding parameters for a given analog. From these data, determined *in vitro* for human serum albumin, one can calculate the percent of II (derived from the *in vivo* hydrolysis of clofibrate) that would be expected to be bound to human serum albumin *in vivo* by using a computer program developed by Garten and Wosilait (21). Assuming a total therapeutic serum concentration of 170 mg/liter for clofibrate (22) and a total albumin concentration of 6.0×10^{-4} *M*, 93% of the free acid II is predicted to be bound to serum proteins *in vivo*. This value is in agreement with results obtained by Thorp (3) who found that II was 96% bound to 4% bovine serum albumin (6.0×10^{-4} *M*) *in vitro*. Whereas the therapeutic serum concentrations for all experimental drugs in humans (or animals) are not known, the relatively small differences in log *P* values observed for these analogs leads to the prediction that similar serum concentrations would be attained if equivalent oral doses were administered. When using the same therapeutic serum concentra-

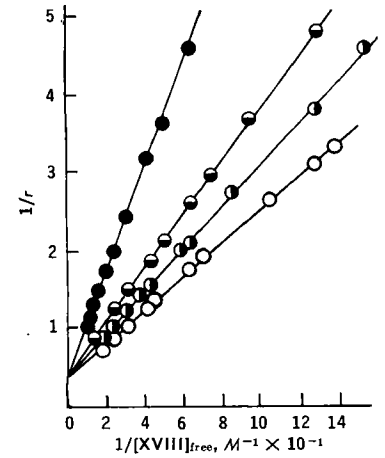

Figure 3—*Relationship between 1/r (moles bovine serum albumin per mole XVIII) and $1/[XVIII]_{free}$ at low r values in the presence of II. Key: ○, no II; ◑, 1.0×10^{-4} M II; ◒, 2.0×10^{-4} M II; and ●, 5.0×10^{-4} M II.*

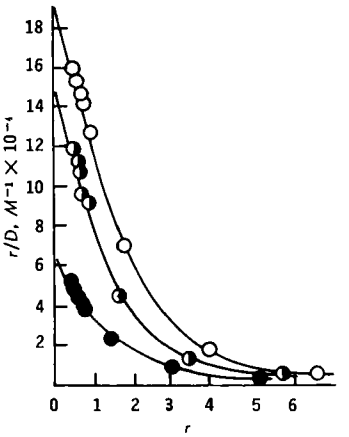

Figure 5—*Scatchard plot for the binding of XVIII to rat serum albumin in the presence of various total amounts of II. Key: ○, XVIII alone; ◑, 2.0×10^{-4} M II; and ●, 5.0×10^{-4} M II.*

Table IV—Binding Parameters of Analogs to Bovine, Rat, and Human Serum Albumins (1.0×10^{-4} *M*) at pH 7.4 (0.1 *M* Phosphate Buffer)

Albumin	Parameter	Analog II	L(*S*)-IV	D(*R*)-IV	VI	VIII	X	XII	XIV	XV	XVII
Bovine	n_1	4.0	1.5	2.1	2.0	3.3	—	3.1	2.1	3.7	2.6
	n_2	9.7	7.8	15.1	4.3	6.1	—	11.4	10.4	7.6	9.0
	K_1, $M^{-1} \times 10^{-4}$	1.28	5.97	2.64	4.56	1.16	—	3.78	6.46	1.55	2.64
	K_2, $M^{-1} \times 10^{-2}$	3.3	3.8	1.4	6.3	2.5	—	2.3	2.3	2.9	1.7
Rat	n_1	2.6	2.3	2.2	2.2	3.6	2.8	4.1	1.6	3.3	3.3
	n_2	18.0	3.5	6.9	10.2	13.8	—	19.2	5.8	8.7	8.0
	K_1, $M^{-1} \times 10^{-4}$	2.94	1.81	2.43	2.27	1.83	0.34	1.88	5.06	2.93	2.41
	K_2, $M^{-1} \times 10^{-2}$	4.4	5.0	6.5	2.1	1.7	—	1.1	5.9	4.6	1.7
Human	n_1	1.8	1.3	1.7	1.5	2.3	—	3.1	2.4	2.8	2.7
	n_2	9.0	6.2	8.0	10.0	10.2	—	3.4	12.9	3.6	2.7
	K_1, $M^{-1} \times 10^{-4}$	2.47	2.40	1.56	3.18	2.59	—	1.52	1.09	1.35	2.19
	K_2, $M^{-1} \times 10^{-2}$	4.7	4.8	4.1	1.5	3.9	—	8.8	0.96	6.2	5.3

Table V—Statistical Analysis of Linear Regression Intercepts at $p = 0.01$

System	Number of Observations	Variance of Intercept[a], s_I^2	Mean Square Error	*F* Ratio for Mean Square Error	Theoretical *F* Ratio[b]	Theoretical *t* Value[b]	Calculated *t* Value[a]
Bovine serum albumin:							
XVIII alone	11	8.73×10^{-4}	3.06×10^{-3}	—	—	—	—
XVIII + II (1.0×10^{-4} *M*)	10	8.20×10^{-4}	2.51×10^{-3}	1.22	5.47	2.90	0.74
XVIII + II (2.0×10^{-4} *M*)	14	7.80×10^{-4}	3.94×10^{-3}	1.29	4.39	2.83	1.22
XVIII + II (5.0×10^{-4} *M*)	13	3.86×10^{-4}	2.30×10^{-3}	1.33	4.63	2.84	0.19
XVIII + L(*S*)-IV (1.0×10^{-4} *M*)	9	8.44×10^{-4}	2.60×10^{-3}	1.77	6.72	2.92	1.39
XVIII + D(*R*)-IV (1.0×10^{-4} *M*)	9	1.94×10^{-3}	6.01×10^{-3}	1.96	6.72	2.92	0.23
XVIII + XIV (1.0×10^{-4} *M*)	9	3.32×10^{-4}	1.02×10^{-3}	2.99	5.61	2.92	2.06
XVIII + XV (1.0×10^{-4} *M*)	9	6.47×10^{-4}	2.00×10^{-3}	1.53	6.72	2.92	1.10
Rat serum albumin:							
XVIII alone	6	1.55×10^{-4}	1.04×10^{-4}	—	—	—	—
XVIII + II (2.0×10^{-4} *M*)	6	1.33×10^{-4}	1.27×10^{-4}	1.22	16.00	3.36	4.80
XVIII + II (5.0×10^{-4} *M*)	6	3.77×10^{-4}	2.40×10^{-4}	2.30	16.00	3.36	3.35
XVIII + XII (2.0×10^{-4} *M*)	6	6.75×10^{-4}	6.91×10^{-4}	6.62	16.00	3.36	2.72
XVIII + XIV (2.0×10^{-4} *M*)	8	4.49×10^{-4}	8.80×10^{-4}	2.04	15.20	3.17	1.20
Thyroxine and rat serum albumin:							
Thyroxine alone	6	1.19×10^{-3}	1.23×10^{-3}	—	—	—	—
Thyroxine + II (1.0×10^{-4} *M*)	4	9.68×10^{-4}	8.08×10^{-4}	1.52	18.00	3.71	1.43
Thyroxine + XII (2.0×10^{-4} *M*)	5	1.16×10^{-3}	9.76×10^{-4}	1.26	28.70	3.50	1.13
Thyroxine + XII (1.0×10^{-4} *M*)	5	1.52×10^{-3}	1.07×10^{-3}	1.15	28.70	3.50	0.39
Thyroxine + XIV (1.0×10^{-4} *M*)	7	8.04×10^{-4}	7.56×10^{-4}	1.63	11.40	3.25	2.25

[a] Calculated by a reported method (11). [b] From "Scientific Tables," K. Diem and C. Lentner, Eds., J. R. Geigy S. A., Basle, Switzerland, 1970.

tion employed for clofibrate, computer analysis of data obtained *in vitro* using human serum albumin predicted that all analogs would be 85–95% bound to serum proteins in humans.

Competitive Binding between XVIII and II, L(*S*)-IV, D(*R*)-IV, XIV, and XV on Bovine Serum Albumin—The binding of XVIII to bovine serum albumin was studied in the presence of a constant total concentration of II where three levels of II were used (1.0×10^{-4}, 2.0×10^{-4}, and 5.0×10^{-4} *M*). The results represented as Scatchard plots are given in Fig. 2. As the concentration of II increases, displacement of XVIII from its binding sites also increases. By using values of *r* (moles XVIII bound per mole bovine serum albumin) of approximately 1 and less, where the first class of sites is primarily affected, the data of Fig. 2 were replotted as $1/r$ *versus* $1/[\text{XVIII}]_{\text{free}}$ to determine if II and XVIII are competing for the same binding site on the albumin. If the same extrapolated $1/r$ intercept is obtained for each concentration of II as found for XVIII alone, the conclusion can be reached that the same site is involved (23). Figure 3 shows the effect of the three different total concentrations of II on the binding of XVIII to bovine serum albumin. The linear relationships obtained at the three levels of II extrapolated to essentially the same intercept on the $1/r$ axis as found for XVIII alone (*i.e.*, 0.38). Linear regression analysis of the data yielded $1/r$ intercepts of 0.35, 0.43, and 0.39 for II at 1.0×10^{-4}, 2.0×10^{-4}, and 5.0×10^{-4} *M*, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student *t* test (Table V). The validity of the use of the test was described previously (11).

Four other analogs [L(*S*)-IV, D(*R*)-IV, XIV, and XV] were studied to determine if they also competed with XVIII at the same binding site on bovine serum albumin. Reciprocal plots using data at low *r* values gave the results presented in Fig. 4 where a constant total level of analog was used (1.0×10^{-4} *M*). The intercepts obtained for L(*S*)-IV, D(*R*)-IV, XIV, and XV by regression analysis were 0.44, 0.39, 0.30, and 0.42, respectively. The values for these intercepts also are not statistically different at $p = 0.01$ as shown by the Student *t* test (Table V).

Moriguchi Method—The binding of the analogs by bovine serum albumin using Moriguchi's method gave the results presented in Table VI. For purposes of comparison, the K_1 values obtained for binding of the analogs to bovine serum albumin using ultrafiltration are also presented in Table VI. In terms of decreasing K_1 values, the results obtained using Moriguchi's method rank the analogs as follows: XII, XVII, XIV, VIII, XV, II, D(*R*)-IV, and L(*S*)-IV. The results obtained using ultrafiltration, on the other hand, rank the analogs as follows: XIV, L(*S*)-IV, XII, XVII, D(*R*)-IV, XV, II, and VIII. There is no apparent similarity in the ordering of the analogs using the two methods.

Competitive Binding between XVIII and II, XII, and XIV to Rat Serum Albumin—The binding of XVIII to rat serum albumin was studied in the presence of a constant total concentration of II where two levels of II were utilized (2.0×10^{-4} and 5.0×10^{-4} *M*). The results, represented as Scatchard plots, are given in Fig. 5. To determine if the same binding site is involved in the displacement, reciprocal plots were constructed for those binding data taken at $r = 1$ or less, where the strong primary site for XVIII would likely be involved. Plots of $1/r$ *versus* $1/[\text{XVIII}]_{\text{free}}$

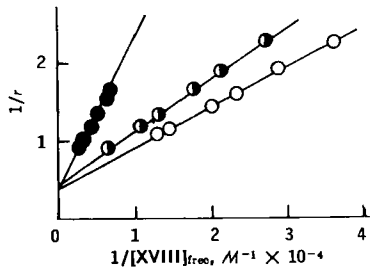

Figure 6—*Relationship between 1/r (moles rat serum albumin per mole XVIII) and 1/[XVIII]free at low r values in the presence of II. Key: ○, no II; ◑, 2.0 × 10⁻⁴ M II; and ●, 5.0 × 10⁻⁴ M II.*

at the different concentrations of II are given in Fig. 6. Linear regression analysis of the data yielded $1/r$ intercepts of 0.37 for XVIII alone and 0.45 for II at levels of 2.0×10^{-4} and 5.0×10^{-4} M. The value for the intercept is statistically different at $p = 0.01$ for II at a level of 2.0×10^{-4} M, but it is not statistically different for II at a level of 5.0×10^{-4} M as shown by the Student t test (Table V).

Two additional analogs, XII (an active hypolipemic agent) and XIV (an inactive analog), were studied to determine if they also competed with XVIII at the same binding site on rat serum albumin. Reciprocal plots using data taken at low r values gave the results presented in Fig. 7, where a constant total level (2.0×10^{-4} M) of analog was used. The $1/r$ intercepts obtained for XII and XIV by regression analysis were 0.46 and 0.36, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table V).

Binding of Thyroxine to Rat Serum Albumin—The binding of thyroxine to rat serum albumin gave results plotted in Fig. 8. The solid line in Fig. 8 is the computer fit for the data using the binding parameters generated assuming that two independent classes of binding sites exist. It is apparent that this assumption adequately describes the binding of thyroxine to rat serum albumin. The computer-generated values are: $n_1 = 0.49$, $K_1 = 1.51 \times 10^6$ M^{-1}, $n_2 = 3.5$, and $K_2 = 8.2 \times 10^4$ M^{-1}.

Competitive Binding between Thyroxine and II, XII, and XIV on Rat Serum Albumin—The binding of thyroxine to rat serum albumin was studied in the presence of a constant total concentration of XII where two levels of XII were employed (1.0×10^{-4} and 2.0×10^{-4} M). The results, represented as Scatchard plots, are given in Fig. 9. As the concentration of XII increases, displacement of thyroxine also increases. To determine if the same binding site is involved in the displacement, reciprocal plots were constructed for those binding data taken at low r values, where the strong primary site for thyroxine would most likely be involved. Plots of $1/r$ *versus* $1/[\text{thyroxine}]_{free}$ at the different concentrations of XII are given in Fig. 10. The linear relationships obtained at the two levels of XII extrapolated to essentially the same intercept on the $1/r$ axis as that found for thyroxine alone (0.57). Linear regression analysis of the data yielded $1/r$ intercepts of 0.59 and 0.62 for XII at levels of 1.0×10^{-4} and 2.0×10^{-4} M, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table V).

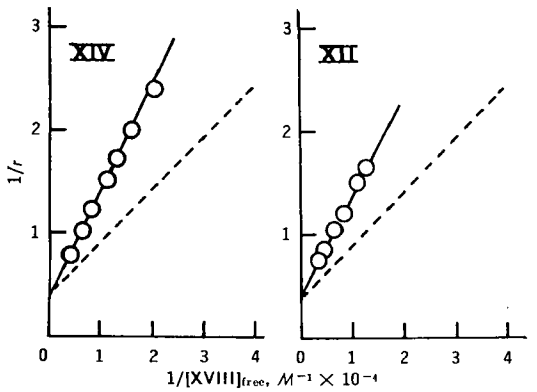

Figure 7—*Relationship between 1/r (moles rat serum albumin per mole XVIII) and 1/[XVIII]free at low r values in the presence of 2.0 × 10⁻⁴ M XII and XIV. The broken line is XVIII alone, having an intercept of 0.37.*

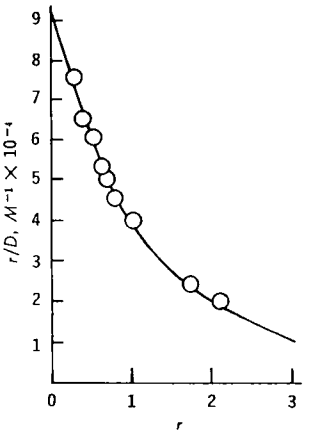

Figure 8—*Scatchard plot for the binding of L-thyroxine to rat serum albumin (7.5×10^{-6} M) at pH 7.4. Key: ○, experimental values; and ——, computer fit assuming two independent classes of binding sites exist on serum albumin.*

Two other analogs (II and XIV) were studied to determine if they also competed with thyroxine at the same binding site on rat serum albumin. Reciprocal plots using data at low r values gave the results presented in Fig. 11, where a constant total level (1.0×10^{-4} M) of analog was used. The intercepts obtained for II and XIV by regression analysis were 0.50 and 0.67, respectively. The values for the intercepts are not statistically different at $p = 0.01$ as shown by the Student t test (Table V).

Octanol-Water Partition Coefficients of Analogs—The octanol–water partition coefficients of the analogs were determined at a pH two units below the pKa of each analog. The log P values for the analogs are presented in Table III. The experimental log P values are in fairly good agreement with the calculated log P values, using the Fujita *et al.* (24) log P value for phenoxyacetic acid (1.21) as a reference.

DISCUSSION

Clofibrate is rapidly hydrolyzed by tissue and serum esterases *in vivo* and *in vitro* to the active hypolipemic acid II (3). Like I, esters III, VII, XI, and XVI also rapidly undergo hydrolysis by rat plasma *in vitro*. Therefore, the differences in hypolipemic activity observed previously (10) cannot be explained on the basis of differential rates of hydrolysis to the active free acids.

To explore further the possibility that the differential hypolipemic effects of these analogs are related to the physicochemical properties of the free acids, the pKa's for a number of these analogs were determined. As expected, the pKa's of the analogs do not differ greatly and fall in the 3.90–4.46 range (Table II). Hence, there is no simple relationship between biological activity and pKa among these structurally modified analogs of clofibrate. The log P values for the carboxylic acid analogs [II, D(*R*)-IV, VI, VIII, XII, XIV, XV, and XVII], experimentally determined at a pH two units below their respective pKa's, are shown in Table III. These values are in agreement with those values calculated from the experimentally determined log P value for phenoxyacetic acid (24) and may be used to predict distribution of drugs *via* a random-walk process to sites of action (25). While the two most

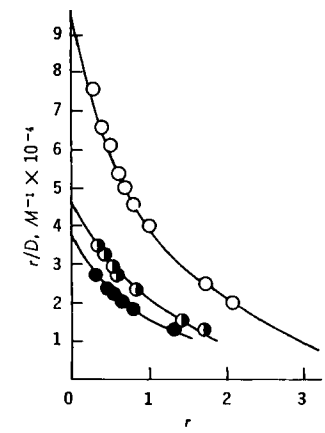

Figure 9—*Scatchard plot for the binding of L-thyroxine to rat serum albumin (7.5×10^{-6} M) in the presence of various total concentrations of XII. Key: ○, L-thyroxine alone; ◑, 1.0×10^{-4} M XII; and ●, 2.0×10^{-4} M XII.*

Table VI—Comparison of K_1 Obtained by the Moriguchi Method with K_1 Obtained by Ultrafiltration for Binding of Analogs to Bovine Serum Albumin

Analog	K_1, $M^{-1} \times 10^{-4}$ (Moriguchi Method)	K_1, $M^{-1} \times 10^{-4}$ (Ultrafiltration Method)
XII	7.94	3.78
XVII	6.31	2.64
XIV	6.31	6.46
VIII	2.51	1.16
XV	2.00	1.55
II	1.59	1.28
D(*R*)-IV	1.00	2.64
L(*S*)-IV	0.63	5.97

active hypotriglyceridemic and hypocholestrolemic drugs (I and XI) also have the largest log P values for their corresponding carboxylic acids, the relative activity of the other analogs shows no apparent relationship to partition coefficients. Because of their similar pKa's and the lack of correlation of log P with hypolipemic activity, it seems that differences in activity observed *in vivo* are not due to major differences in absorption and distribution but rather to structural differences that either enhance or decrease the affinity of the molecule for certain enzymes at sites of action or loss.

Several mechanisms of action have been proposed for the hypolipemic effect of clofibrate. Of immediate interest is the thyroxine displacement mechanism proposed by Platt and Thorp (26). In this hypothesis, the free acid hydrolysis product of clofibrate is proposed to exert its effect by displacing thyroxine from its binding proteins in the plasma and the liver. The result is presumed to be increased lipid metabolism with concomitant lowering of serum lipid levels because of the hyperthyroid effect in the liver.

To explore the hypothesis that these compounds exert their action indirectly by displacing thyroxine from plasma proteins, the binding parameters for these analogs as well as the nature of the sites to which they are bound were investigated, and these results were compared with binding studies involving thyroxine and dye XVIII which, as previously shown, mirrors the binding of thyroxine (11). Since prevous studies in these laboratories detected species differences in the binding of dye XVIII and acids II, L(*S*)-IV, and D(*R*)-IV (27), a thorough investigation of the binding of the compounds to bovine, rat, and human serum albumins was in order before any further conclusions concerning the relationship between albumin binding parameters and biological activity could be made. Bovine serum albumin has been used by several workers for studying the binding of thyroxine (13, 28, 29) and its displacement by drug entities (27, 30). Studies utilizing rat serum albumin are important since the biological evaluation of these compounds in rats has been performed. Studies utilizing human serum albumin are important since such studies may ultimately reflect or be predictive of hypolipemic activity in humans (9). The results show that thyroxine binds to rat serum albumin at site n_1 with $K_1 = 1.51 \times 10^6\ M^{-1}$ and that this value is similar to the binding of thyroxine at site n_1 on bovine serum albumin ($K_1 = 1.6 \times 10^6\ M^{-1}$) (29). Similarly, the results obtained for the binding of the analogs to bovine, rat, and human serum albumins (Table IV) also show only minor species differences. For all studies involving albumin derived from these three species, both active and inactive analogs bind similarly at site n_1. Since the primary site n_1 is the major site for thyroxine binding, the possibility that these compounds may be able to displace the hormone *in vitro* was investigated.

To test differences in the ability of the analogs to displace thyroxine, the optical probe 2-(4′-hydroxybenzeneazo)benzoic acid (XVIII) was used as a convenient means to mirror thyroxine binding to serum albumin in order to determine whether earlier reported work (27) had biological relevance. In a previous paper (11), it was shown that thyroxine competed with XVIII at the same binding site on bovine and rat serum albumins. Competitive binding studies between XVIII and five analogs [II, L(*S*)-IV, D(*R*)-IV, XIV, and XV] to bovine serum albumin were carried out to determine whether the five analogs compete with XVIII at the same binding site on the albumin. The results (Figs. 3 and 4) show that all analogs compete with XVIII at the same binding site on

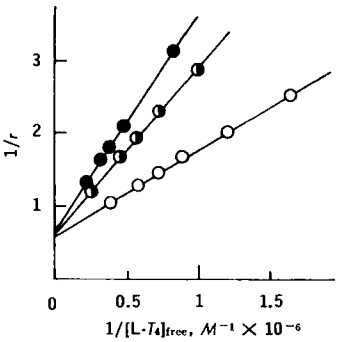

Figure 10—*Relationship between 1/r (moles rat serum albumin per mole* L-*thyroxine) and the reciprocal of free* L-*thyroxine concentration* (L-T_4) *at low r values in the presence of XII. Key:* ○, *no XII;* ◑, *1.0* × 10^{-4} M *XII; and* ●, *2.0* × 10^{-4} M *XII.*

bovine albumin. It may be concluded, therefore, that these acids could theoretically displace thyroxine from its binding site on the albumin. However, the primary binding constant for thyroxine is much greater than the primary binding constant for any analog studied. Similar results were obtained for rat serum albumin using analogs II, XII, and XIV; however, binding studies in greater detail were carried out with bovine serum albumin owing to the high cost of rat serum albumin.

The slope of a plot of $1/r$ *versus* $1/[\text{XVIII}]_{\text{free}}$ at low r values is $1/nK$, where n would approximate the number of primary sites for XVIII on bovine serum albumin and K is the apparent primary binding constant for XVIII. The reciprocal plot for XVIII alone, having an intercept of 0.38, shows n to be 2.6. The computer fit of the Scatchard curves shows n_1 to be 1.7 ± 0.2 (average ± average deviation of five determinations). Reciprocal plots assuming one class of sites normally generate a high value for n as compared to a multiple-class model, so a high n value in the reciprocal treatment was expected (31). The slope of the reciprocal plot for XVIII alone predicts K_1 to be 18,800 M^{-1}, whereas the computer fit for the Scatchard treatment of the two class assumption gives $K_1 = 23{,}000 \pm 3000$ (average ± average deviation of five determinations). A lower value for K in the reciprocal treatment assuming only one class of sites is to be expected (31).

In the presence of an inhibitor, the binding constant, K, obtained for the binding of XVIII should be related to the binding constant in the absence of the inhibitor, K', by Eq. 3 (31):

$$K = \frac{K'}{1 + K_c(C_f)} \qquad \text{(Eq. 3)}$$

Thus, K for XVIII in the presence of the inhibitor depends on the binding constant, K_c, and the free concentration of the inhibitor, (C_f). Therefore, the greater the value of $K_c(C_f)$, the greater is the slope of the reciprocal plot. The slopes of the reciprocal plots for XVIII in the presence of 1.0×10^{-4} *M* II, L(*S*)-IV, D(*R*)-IV, XIV, and XV are 2.7×10^{-4}, 3.2×10^{-4}, 3.4×10^{-4}, 3.7×10^{-4}, and 3.4×10^{-4} *M*, respectively. The effectiveness of these compounds

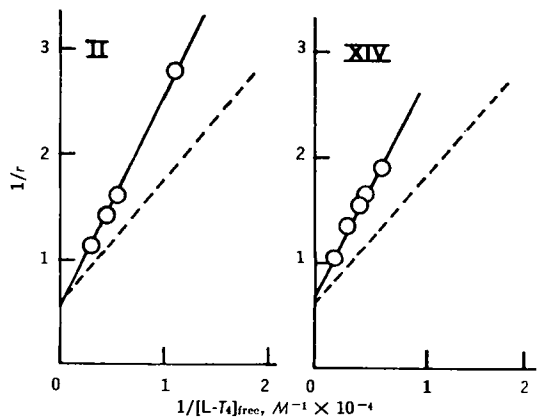

Figure 11—*Relationship between 1/r (moles rat serum albumin per mole* L-*thyroxine) and the reciprocal of free* L-*thyroxine concentration* (L-T_4) *at low r values in the presence of 1.0* × 10^{-4} M *II and XIV. The broken line is* L-*thyroxine alone, having an intercept of 0.57.*

to displace thyroxine from bovine serum albumin stand in the order XIV, XV, D(*R*)-IV, L(*S*)-IV, and II based on the assumption that the concentration of the inhibitor free (C_f) is the same in each case and that the small differences in the observed slopes are significant.

Moriguchi and coworkers (19, 20) also used XVIII in their binding studies with bovine serum albumin. These studies were carried out at low concentrations of XVIII, and the maximum r value that was obtained was about 1.5. They indicated that their data were best fitted by the equation:

$$\log \{r/(n - r)\} = m \log A + m \log K \qquad \text{(Eq. 4)}$$

where r represents the moles of bound drug A per mole of total albumin, A is the concentration of unbound A, K is the binding constant, m is an empirical parameter, and n is the number of binding sites on a single molecule of albumin. This equation is based on the assumption that binding to serum albumin is affected by electrostatic interactions altering binding affinity. Such an assumption differs from the one used in this study, which assumes that all binding sites are independent but that more than one class of sites exist. The validity of Eq. 4, where n had to assume a value of 2.0, was based solely on best fit of data. This assumption is not valid since the results of previous investigations (11) showed that r values much greater than 2.0 can be obtained. The results obtained for the binding of the analogs to bovine serum albumin using Moriguchi's method are listed in Table VI; comparison of these values with those derived from ultrafiltration showed differences in rank ordering of the analogs. From these data, it was concluded that Moriguchi's method is not applicable.

Recognizing that binding to human serum albumin is pertinent and that binding might depend on species differences, the binding of the analogs to human serum albumin was also studied. In humans, three carrier proteins for thyroxine were identified: *(a)* thyroxine binding prealbumin, which has an anodal mobility greater than that of serum albumin; *(b)* thyroxine binding globulin, with an electrophoretic mobility between α_1- and α_2-globulin; and *(c)* serum albumin (32). When tracer quantities of ^{131}I-thyroxine are added to serum and electrophoresis is performed in a glycine–acetate buffer at pH 8.6 (33), approximately 30% of the radioactivity is associated with the prealbumin, 60% with thyroxine binding globulin, and the remaining 10% with serum albumin (34). The prealbumin and binding globulin preparations are not available commercially, so the present studies were done with human serum albumin. The results obtained (Table IV) show no marked differences in K_1 among the analogs; *i.e.*, active and inactive analogs bind similarly. The percent of each analog that would be expected to be bound to serum albumin was calculated using the parameters listed in Table IV through the use of a published computer program (21), as described in the *Results* section. There is no dramatic difference in the amount of each analog that would be expected to be bound to human serum albumin, although active analogs II, VIII, and XII showed a slightly larger percent bound than analog XIV which was inactive in the hyperlipemic rat model (10). Furthermore, the differential hypocholesterolemic and hypotriglyceridemic activity observed for VIII could not be predicted on the basis of these albumin binding studies.

In the rat, thyroxine is bound to both an α-globulin and albumin (35), and it seemed that attempts to correlate hypolipemic activity *in vivo* using rats with rat serum binding parameters would have greater validity. The results obtained for the binding of analogs to rat serum albumin (Table IV) show that both active and inactive analogs have similar binding parameters. There are no marked differences in K_1 among the analogs except X and XIV. Analog X differs from the other analogs in that it binds to only one site and has a low K_1 value. Why this analog is the only one that binds in this fashion is not understood. Analog XIV has a primary binding constant that is approximately twice that of the other analogs. Newman *et al.* (10) showed that both X and XIV are biologically inactive in hyperlipemic rats *in vivo*. Therefore, it would seem that Thorp's displacement mechanism cannot be invoked to differentiate the varying activity among the analogs, since most analogs essentially bind to the same extent to rat serum albumin and differences in binding by X and XIV do not account for their inactivity *in vivo*. Furthermore, the differential hypocholesterolemic effects of I, D(*R*)-III, and L(*S*)-III in normocholesterolemic Swiss Webster rats (15) cannot be accounted for on the basis of differential binding of the corresponding free acids to rat serum albumin *in vitro*. In fact, D(*R*)-IV binds to a slightly greater extent than L(*S*)-IV, but ester L(*S*)-III is active *in vivo*.

To substantiate this conclusion, the binding of thyroxine to rat serum albumin was determined by equilibrium dialysis at 37° using ^{125}I-thyroxine. These studies were necessary to confirm that data obtained using the optical probe XVIII indeed can be used to determine thyroxine binding to, and displacement from, serum albumin. Competitive binding studies between thyroxine and three analogs (II, XII, and XIV) were carried out to determine if the three analogs do indeed displace thyroxine at the same binding site on rat serum albumin. The results (Figs. 10 and 11) show that the three analogs compete with thyroxine at the same binding site on rat serum albumin. From earlier studies (11) utilizing the optical probe XVIII and rat serum albumin, it was shown that thyroxine displaces XVIII from the same binding site on rat serum albumin; from the present studies, it was shown that the analogs used also displace XVIII from the same binding site on rat serum albumin. Therefore, it was concluded that the analogs could displace thyroxine from its binding site on this albumin. The results of competitive binding studies between thyroxine and the analogs to rat albumin show that the analogs do displace thyroxine from the same (primary) binding site on the albumin. This finding confirms the results obtained using the probe XVIII and substantiates the validity of the use of the probe XVIII to mimic thyroxine binding to rat serum albumin. Analogs II and XII are biologically active while analog XIV is inactive in hyperlipemic rats (10); since all three analogs displace thyroxine from its binding site on rat serum albumin under the experimental conditions *in vitro*, and the ester XIII of analog XIV is inactive but readily undergoes hydrolysis by serum esterases *in vitro*, it seems that thyroxine displacement determined *in vitro* is not related to activity *in vivo*. Rather, albumin binding *in vivo* likely is important for drug transport, and the mode of action of these compounds involves their selective influence on lipid regulating enzymes. Work designed to probe the differential enzymatic mechanisms of action of clofibrate and related analogs is continuing in these laboratories.

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BENITO CASU*x and PAOLO VENTURA‡

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Current chemical methods for penicillin assay, such as those based on iodometric (1, 2) and acidimetric (3) titrations, are indirect and nonspecific. UV spectrophotometry is of limited applicability in penicillin analysis. The aminopenicillanic moiety of penicillins displays only end-absorption in the UV region. Only the degradation products of penicillins and a few compounds related to penicillins (including cephalosporins) display characteristic UV absorption bands (4). Because of the lack of specificity of the iodometric and acidimetric methods, it is also a common practice to characterize penicillins from their IR spectra. Such spectra, usually obtained on solid samples, are excellent "fingerprints" for individual penicillins, the elucidation of the structure of penicillins having actually been one of the oldest applications of IR spectroscopy (5).

It should be attractive to use the IR spectra for both the qualitative and quantitative analysis of penicillins. However, the IR spectra run on solid samples are not easily amenable to quantitative measurements. In fact, the absorbance of the IR bands is strongly affected by scattering and crystallinity effects (6). The logical approach to an IR quantitative analysis is to work on samples in solution. An IR assay in chloroform solution of a number of semisynthetic penicillins in the acid form was reported (7). Since most important penicillins are in the salt form, this method requires acidification and solvent extraction. Moreover, the chloroform method does not apply to ampicillins (7).

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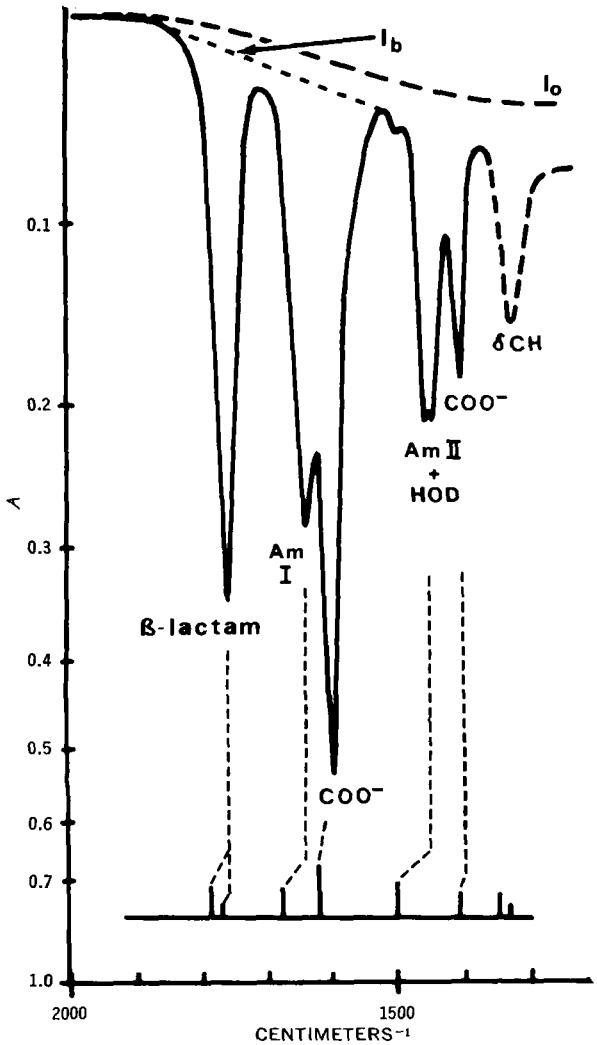


Figure 1—*IR absorption in the carbonyl region of potassium penicillin G in deuterium oxide solution (c 0.040 g/ml; l = 0.055 mm). The stick diagram in the bottom of the figure refers to band positions in the solid (KBr disk).*

ation as an IR solvent for most penicillins. However, water strongly absorbs the IR radiation except in the region between 1300 and 1000 cm^{-1} (8). On the other hand, deuterium oxide is more transparent than water in a number of analytically important regions of the IR spectrum.

The present work was undertaken to evaluate the feasibility of a direct IR analysis of penicillins in deuterium oxide solution. Such an approach looked promising because this solvent is sufficiently transparent in the carbonyl (1900–1400 cm^{-1}) region, where typical penicillin bands, including the key band of the β-lactam group, occur. The β-lactam band in a deuterium oxide–phosphate buffer was already shown (9) to be suitable for kinetic studies of the enzymic cleavage of a few penicillins. Moreover, unpublished work from these laboratories has shown that the bands of the unionized carboxyls, carboxylate ions, and amides lend themselves to the quantitative analysis of such groups in uronic acids and *N*-acetylated amino sugars.

An IR spectrophotometric method that has proved

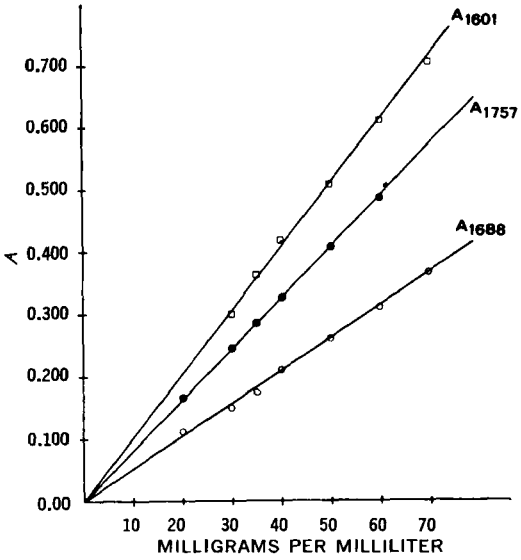


Figure 2—*Absorbance–concentration relationship for the β-lactam (1757 cm^{-1}), cyclic amide (1688 cm^{-1}), and carboxylate-ion (1601 cm^{-1}) bands of hetacillin in deuterium oxide (l = 0.055 mm, I_0 measured from a baseline).*

adequate for all water-soluble penicillins was then worked out. The water-insoluble penicillins were handled in 0.5 *N* DCl or, more conveniently, in dimethyl sulfoxide. The present method in solution appears to provide a rapid, specific, and accurate alternative to the currently used methods for penicillin assay.

EXPERIMENTAL[1]

Assay in Deuterium Oxide Solution—Forty milligrams of each penicillin sample was weighed in a ground-stoppered 5-ml flask, from a precision pipet or a self-filling microburet. Most sodium and potassium salts of penicillins easily dissolve in deuterium oxide. Part of the solution was transferred *via* an ordinary Lauer lock syringe in an IR cell of about 0.050-mm thickness, fitted with calcium fluoride windows. [The actual thickness of the sample cell used for the present work was 0.0552 mm, as measured by the interference-fringes (10) method.] A similar cell was filled with deuterium oxide. The IR spectrum of the solution was run from 2000 to 1300 cm^{-1}, using the cell filled with the solvent alone as the reference.

The absorbance of the analytical (β-lactam) band in the 1760-cm^{-1} region was measured either from a reference 100% (I_0) line, obtained by recording the spectrum with deuterium oxide in both the sample and the reference cell, or by the baseline method (10). The chosen baseline is shown in Fig. 1.

The penicillin content was calculated from the measured absorbance (*A*) of the β-lactam band by referring to a calibration curve such as that in Fig. 2, obtained using solutions of a reference sample of the same penicillin at different concentrations in deuterium oxide (2–6% w/v). Since the absorbance of the analytical band varies linearly with concentration, reference can be made to the absorbance value (A_{st}) measured for a single (4% w/v) solution of the standard sample. The percent concentration of penicillin in the sample (*c* %) can thus be obtained using the

[1] Penicillins were from different commercial sources. Their iodine consumption was 100 ± 2% of theoretical, except for sodium ampicillin which gave 95 ± 1.5%. Deuterium oxide (99.75%) and dimethyl sulfoxide were from C. Erba, Milan, Italy. Since the water content of the latter solvent did not exceed 0.5% (IR spectrophotometric analysis in the 3500-cm^{-1} region), dimethyl sulfoxide was used without any further purification. Deuterium chloride (~38%, 99.5% D) was from Ciba, Basel, Switzerland. An IR spectrophotometer (small grating), Perkin-Elmer model 337, was used.

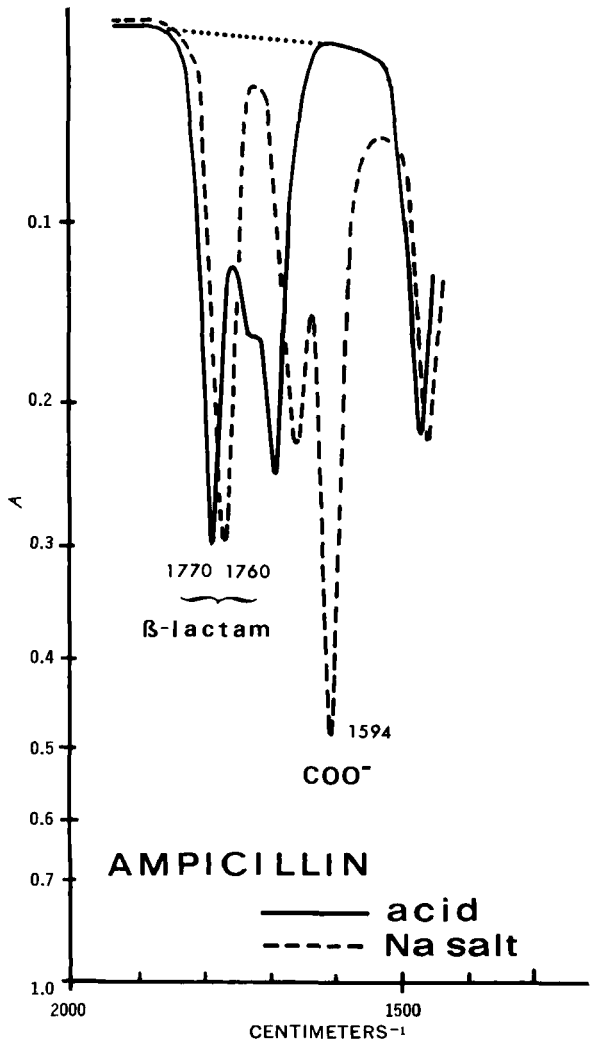


Figure 3—*IR spectrum of ampicillin (anhydrous), freshly dissolved in 0.5 N DCl, compared with the spectrum of the sodium salt in deuterium oxide (experimental conditions as in Fig. 1).*

simple relationship:

$$c\,\% = \frac{A}{A_{st}} \times 100 \qquad \text{(Eq. 1)}$$

Assay in Deuterium Chloride Solution—The penicillin samples were dissolved in deuterium chloride (approximately 0.5 *N*) prepared by diluting a concentrated (~38% w/v) deuterium chloride solution with deuterium oxide; the analyses were conducted as described for deuterium oxide solutions. Since deuterium chloride shows some background absorption in the analytical region, unbalanced solvent can shift the I_0 line. The 100% transmittance was then measured exclusively from a baseline as shown in Fig. 3 for ampicillin.

Assay in Dimethyl Sulfoxide Solution—The penicillin samples were dissolved directly in anhydrous dimethyl sulfoxide, and the analyses were conducted as described for deuterium oxide solutions. The absorbance of the β-lactam band was measured either from the usual I_0 line (solvent alone) or from a baseline as shown in Fig. 4.

RESULTS

Spectra of Penicillin Salts in Deuterium Oxide—The carbonyl region of the IR spectrum of potassium penicillin G is shown in Fig. 1. The reported band assignment is based on established spectra-structure correlations (11). The comparison of the spectrum of penicillin G in deuterium oxide with the corresponding spectrum in a potassium bromide disk (Fig. 1) shows that most bands shift toward lower frequencies on going from the solid to deuterium oxide solution. The largest shift (about 60 cm^{-1}), shown by the amide II band, is associated with the substitution of the amide hydrogen with deuterium. The hydrogen-deuterium (H-D) exchange of amides usually occurs within a few minutes, leading to the species —COND— (12). Since such an exchange also produces HOD, which absorbs at about 1460 cm^{-1}, and since the absorbance of the composite band shown by penicillins near the same frequency does not increase with time after the first run, it was assumed that the H-D exchange of the amide hydrogens of penicillins was complete before recording the spectrum (*i.e.*, before less than 10 min after dissolution of the sample in deuterium oxide).

The partial IR spectra in deuterium oxide of a number of water-soluble penicillins are shown in Fig. 5. The spectra share with that of penicillin G a β-lactam band at about 1760 cm^{-1}, but the spectra differ to some extent from each other and can be used as fingerprints for the individual penicillins. The β-lactam band is well resolved from the other carbonyl bands at lower frequencies. Only penicillin derivatives having ester groups (such as pivampicillin, Fig. 6) show a band partially superimposed on that of β-lactam. As can be seen in Fig. 1 for penicillin G, the characteristic splitting observed for the β-lactam band in solid-phase spectra of most penicillin salts (9) is no longer shown in deuterium oxide solution.

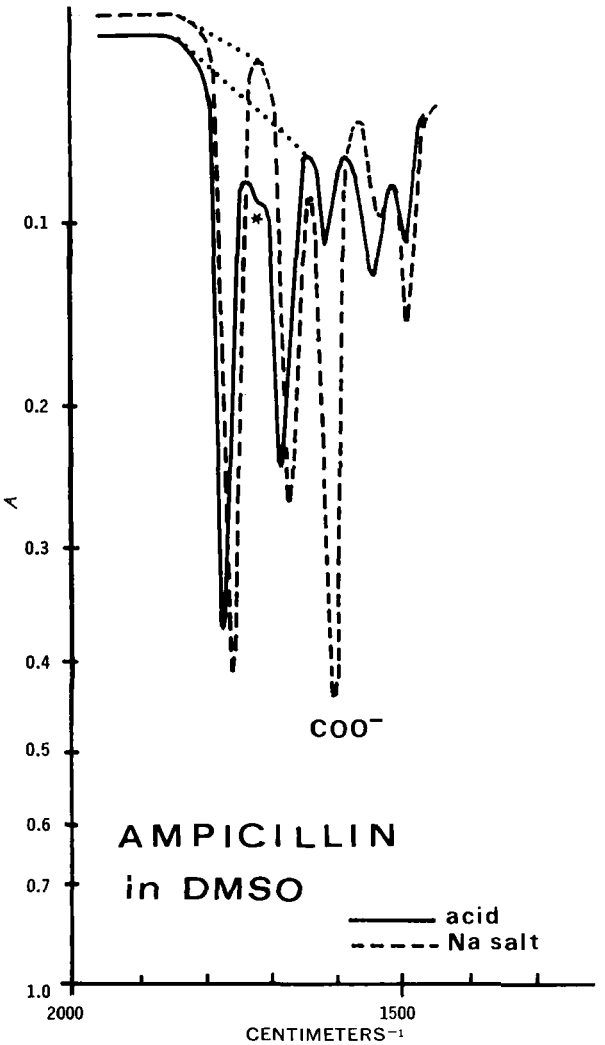


Figure 4—*IR spectra in dimethyl sulfoxide (DMSO) of ampicillin (acid, anhydrous) and its sodium salt (experimental conditions as in Fig. 1). The band of the unionized carboxyl groups is labeled with an asterisk.*

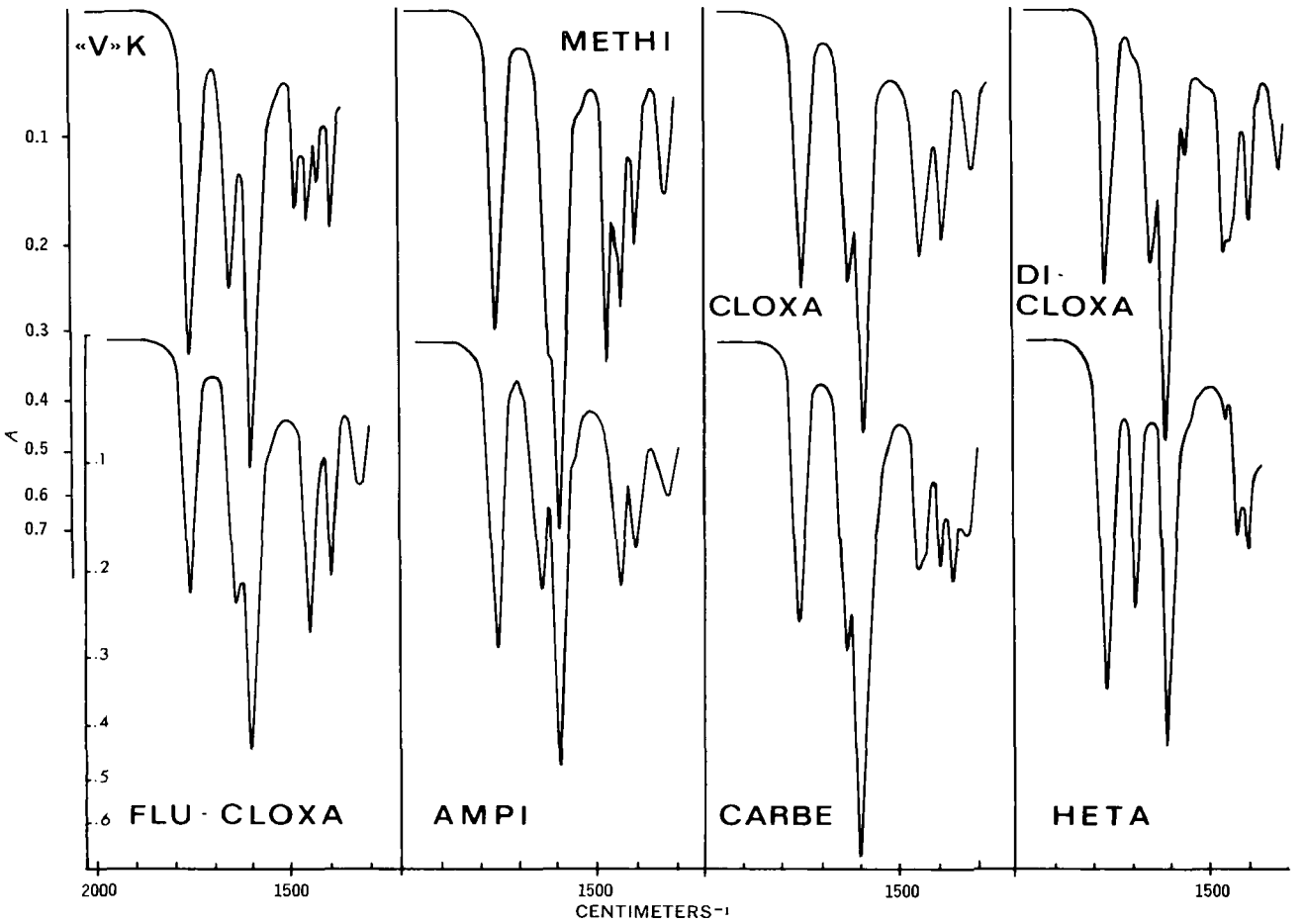


Figure 5—*IR absorption in the carbonyl region of alkaline metal salts of some common penicillins in deuterium oxide solution (concentration and cell thickness as in Fig. 1).*

The frequency and the absorptivity data for the β-lactam, amide, and carboxylate bands for a number of common penicillins in deuterium oxide are given in Table I. Data for 6-aminopenicillanic acid are included for comparison purposes. Except for the atypical 6-aminopenicillanic acid, the β-lactam band is displayed at fairly constant frequency (1760 ± 2 cm^{-1}). The amide group bands are more sensitive to the molecular environment. The amide I frequencies are in the 1670–1625-cm^{-1} range, the highest value being shown by pivampicillin and the lowest one by methycillin. The amide (γ-lactam) group of hetacillin absorbs at fairly high frequency (1688 cm^{-1}). The assignment of the amide II band frequency is uncertain because of overlapping HOD and CH_3 bands. The absorption frequency of the carboxylate ion is quite constant (asymmetrical stretching frequency, ν_{as}, = 1601–1595 cm^{-1}; symmetrical stretching frequency, ν_s, = 1405–1397 cm^{-1}).

To a certain extent also, the absorptivity of the carbonyl bands is dependent on the molecular environment of the groups. The absorptivity value for a well-resolved β-lactam band deviates from its average value (545 $mole^{-1} \cdot cm^{-1}$) up to about ±10%, the largest values found with phenoxymethyl penicillin and the lowest one found with ampicillin[2]. The absorptivity values for hetacillin and pivampicillin are consistently higher than the average, mainly because of interference by adjacent bands (due to the γ-lactam and the ester groups, respectively).

As shown in Fig. 2 for hetacillin, the main carbonyl bands of penicillins in deuterium oxide follow Beer's law, and each of them can be used for the direct quantitative analysis of individual penicillins. The 1760-cm^{-1} band, being common to all penicillins and associated with intact β-lactam groups, was chosen as the analytical band. Most functional groups of common penicillins give bands at lower frequencies and were not expected to interfere with the β-lactam band.

The reproducibility of the absorptivity data, as evaluated in a number of tests for the investigated penicillins, was well within 2%. The present IR method was also tested for accuracy against established iodometric methods, using conversion factors experimentally determined on USP or BP standards. As shown by the data in Table II referring to a sample of cloxacillin, the accuracy and precision of the IR method are of the same order of magnitude as those of the iodometric method. The absorptivity values calculated for cloxacillin samples of different purity (as evaluated iodometrically) were essentially the same, showing that impurities do not interfere with the IR analysis.

The absorbance of the β-lactam band does not vary within the time (~10 min) required for the measurements. No appreciable decrease in intensity was observed within 2 hr, unless the solution was acidified. On standing more than 2 hr, most penicillins undergo decomposition even in neutral solution, with a corresponding decrease of intensity of the β-lactam band. After 2 days, the absorbance at 1760 cm^{-1} decreased 20–60%, depending on the individual penicillin.

Spectra in Deuterium Chloride—In an attempt to extend the IR analysis to water-insoluble penicillins and related compounds in aqueous solution, the acid forms of ampicillins and cephalexin were run in acidic (deuterium chloride) solution. A 0.5 *N* acid concentration was chosen as a reasonable compromise between dissolving power of the acid and convenience of handling of solvent and solutions. Since deuterium chloride has some background absorption in the analytical region, a stronger concentration of acid (which would have decreased the instrument response below acceptable limits for quantitative work) was avoided. A reduced

[2] The absorptivity value obtained for penicillin G (566) is significantly lower than the one (614) derived by Zugaza and Hidalgo (9) in connection with their kinetic study. However, their spectrum seems superimposed on a pattern of interference fringes, a drawback that usually prevents the measurement of *absolute* absorbance values.

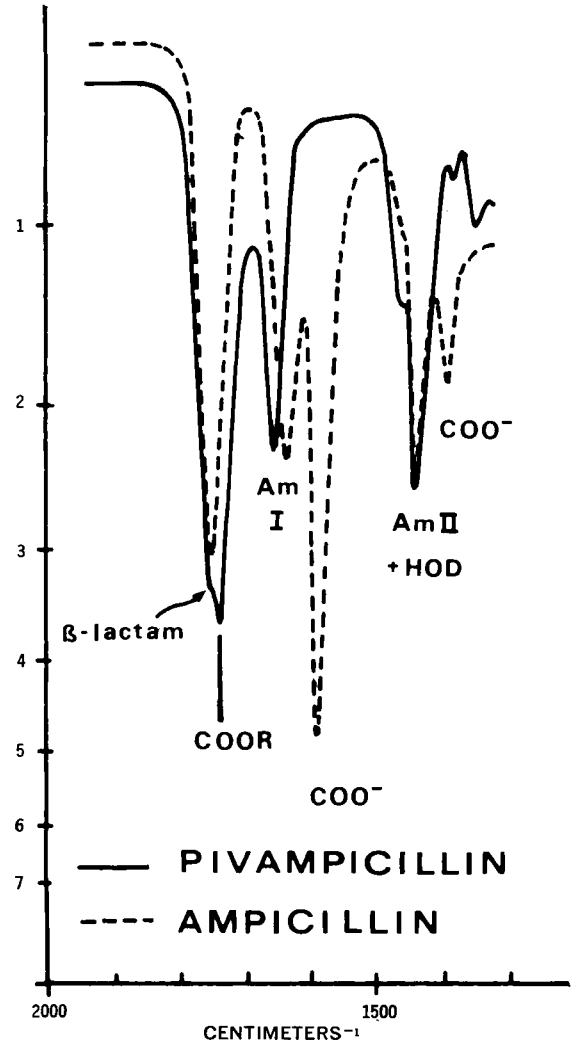


Figure 6—*Comparison of the spectrum in deuterium oxide of pivampicillin hydrochloride with that of sodium ampicillin (same experimental conditions as in Fig. 1).*

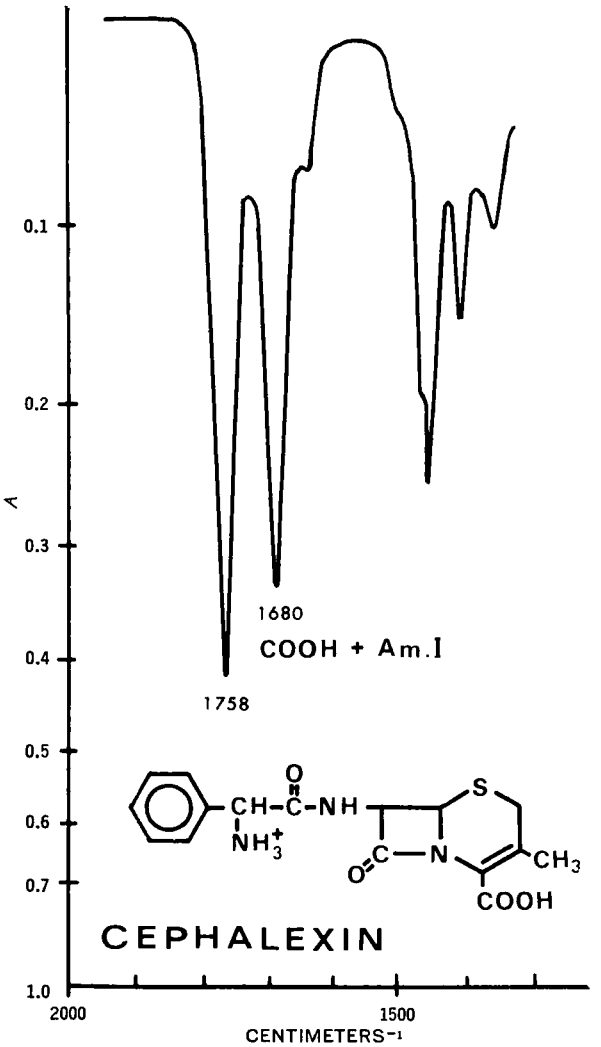


Figure 7—*IR spectrum of cephalexin in 0.5 N DCl (experimental conditions as in Fig. 1).*

acidity usually also reduces the rate of decomposition that most penicillins undergo in acidic solution (4).

Figure 3 shows the spectrum of a sample of ampicillin (anhydrous) freshly dissolved in 0.5 *N* DCl. By comparison with the superimposed spectrum of the corresponding sodium salt in neutral solution, it can be seen that the β-lactam and the amide bands shift toward higher frequencies on acidification. The most important difference between the spectra in acid and in neutral solution is the complete disappearance, in deuterium chloride solution, of the bands of the ionized carboxyl; these bands are replaced by the less intense band of the unionized carboxyl in the 1710-cm^{-1} region. The spectrum of ampicillin trihydrate in 0.5 *N* DCl differs from that of anhydrous ampicillin in the same solvent only by an increased HOD absorption in the 1460-cm^{-1} region.

Due to rapid decomposition in acidic solution, the spectra of ampicillins do not lend themselves to a direct quantitative analysis, which is possible for the more stable cephalexin (Fig. 7).

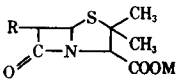
Figure 8 shows the spectral changes undergone by ampicillin on standing up to 40 hr in 0.5 *N* DCl solution. The β-lactam band decreases in intensity until completely disappearing, with a corresponding increase in intensity of the unionized carboxyl bands in the 1710-cm^{-1} region. The increase of the carboxyl band is compatible with the proposed formation of carboxylated products on acid degradation of ampicillin (13, 14).

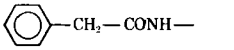
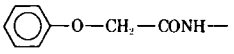
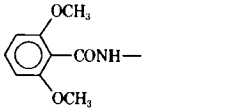
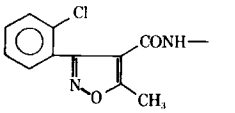
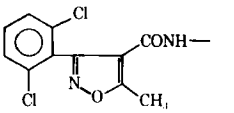
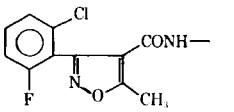
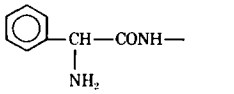
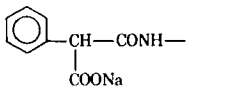
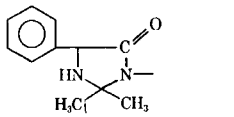
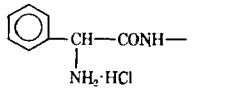
As shown in Fig. 9, extrapolation to zero time of a set of absorbance data obtained within less than 30 min allows one to obtain a "zero-time" absorbance value which can be used in a conventional way to calculate the ampicillin content in the original sample. Although in the present work such an approach was not thoroughly evaluated for its analytical potential, the accuracy of the quantitative data appears of the same order of magnitude as that obtained in deuterium oxide solution for water-soluble penicillins.

Spectra in Dimethyl Sulfoxide—A direct assay of most water-insoluble penicillins, as well as of a number of water-soluble penicillins, turned out to be possible using dimethyl sulfoxide as a solvent. Such a solvent is relatively transparent in the carbonyl region. Since water dissolved in dimethyl sulfoxide absorbs at 1655 cm^{-1} (8), care must be taken to avoid excessive exposure to atmospheric water vapor. However, since the water band is rather apart from the analytically important region for penicillins, the solvent can be conveniently handled, even in the open, without introducing interferences that could not be eliminated using the baseline technique.

Among the investigated penicillins, the acid form of ampicillin (anhydrous and trihydrated) and the cloxacillin salts almost instantaneously dissolve in dimethyl sulfoxide in the analytical concentration range (1–8% w/v). Water-soluble sodium and potassium salts of a few penicillins require some time to dissolve in dimethyl sulfoxide at room temperature (sodium ampicillin, 15 min; carbenicillin, 30 min; and potassium penicillin G, 12 hr). Potassium phenoxymethyl penicillin, potassium hetacillin, and cephalexin are practically insoluble in dimethyl sulfoxide. Figure 10 shows the spectrum in dimethyl sulfoxide of sodium cloxacillin, compared with that in deuterium oxide. The spectrum in dimethyl sulfoxide, being that of the nondeuterated species, is more reminiscent of the solid-phase spectrum than that in deuterium oxide.

Table I—Frequency ($\bar{\nu}$, cm^{-1}) and Absorptivity (a, $mole^{-1} \cdot cm^{-1}$) of the β-Lactam, Amide, and Carboxyl Bands of Some Penicillins (Sodium or Potassium Salts) in Deuterium Oxide[a]



Compound	R	M	Molecular Weight, Anhydrous	β-Lactam ν	β-Lactam a	Amide I, ν	Amide II a	Amide II ν	Carboxylate Ion $\nu_{as}COO^-$ ν	Carboxylate Ion $\nu_{as}COO^-$ a	Carboxylate Ion ν_sCOO^-, ν
6-Aminopenicillanic acid	$-NH_2$	K	254.3	1747	—[b]	—	—	—	1599	—[b]	1405
Penicillin G		K	372.5	1761	566	1639	450	1445	1601	868	1402
Phenoxymethyl penicillin		K	388.5	1760	585	1656	412	1435	1599	953	1400
Methicillin		Na	402.4	1762	566	1625 sh	617	1439	1600	1368	1401
Cloxacillin		Na	457.9	1761	551	1638	475	—[c]	1601	987	1402
Dicloxacillin[d]		Na	593.3	1760	568	1641	477	—[c]	1600	1082	1397
Flucloxacillin		Na	476.9	1760	536	1638	490	—[c]	1600	1002	1400
Ampicillin		Na	371.4	1760	527	1640	367	1445	1595	843	1403
Carbenicillin		Na	422.4	1758	537	1638	541	—[c]	1597	1459	1403
Hetacillin		Na	427.6	1757	648	1688 (γ-lactam)	440	—	1601	813	1401
Pivampicillin		—[e]	500.0	1760	714	1676	423	—[c]	—	—	—

[a] The absorptivity data were measured from the I_0 line (deuterium oxide *versus* deuterium oxide). The values are expressed on an anhydrous basis and were normalized to 100% when purity (as determined by iodometric assay) was less than 100%. Quantitative data are not given for the amide II and ν_sCOO^- bands because of interferences by HOD and CH_3 bands. [b] Data not given because of uncertain purity of the compound. [c] Uncertain assignment. [d] Contains some unionized carboxyl. [e] M = $-CH_2OC(=O)C(CH_3)_3$.

A comparison of the spectrum of ampicillin in the acid form in dimethyl sulfoxide and that of the corresponding sodium salt is shown in Fig. 4. The spectrum of the acid shows bands attributable to both an unionized carboxyl and a carboxylate ion. In the solid state, ampicillin is thought to be in a zwitterionic form (15), and its solid-phase IR spectrum (KBr disk) actually shows bands in the 1620–1560-cm^{-1} region, suggesting an ionic bond (intra- or intermolecular) between the carboxyl and the amino groups (11). The spectrum of ampicillin in dimethyl sulfoxide suggests that these ionic bonds are partially broken by the solvent, most likely by formation of a hydrogen bond between the carboxyl and dimethyl sulfoxide. On addition of a few drops of deuterium oxide to the dimethyl sulfoxide solution of ampicillin, the band of the unionized carboxyl disappeared while that of the carboxylate ion increased in intensity.

Since the carboxyl–dimethyl sulfoxide association is expected to be broken by water, the observed changes on addition of deuterium oxide support this contention.

The frequency and absorptivity of the β-lactam, amide, and carboxyl bands of some penicillins in dimethyl sulfoxide are reported in Table III. The β-lactam group and the carboxylate ion absorb at somewhat higher frequencies (~10 cm^{-1}) than in deuterium oxide. The amide I band of the sodium salt of ampicillin displays an even higher high/frequency shift (+46 cm^{-1}) relative to deuterium oxide.

An interesting feature of the spectra in dimethyl sulfoxide is that the bands are narrower than in deuterium oxide (and deuterium chloride). The absorptivity values are correspondingly higher, providing a better sensitivity for quantitative analysis.

The β-lactam band of the investigated penicillins in dimethyl sulfoxide follows Beer's law and lends itself to quantitative applications. Reproducibility of the absorbance values was within 1.5%. Precision (evaluated for a cloxacillin sample by comparison with a reference sample titrated iodometrically) was better than ±2%. Such a precision may be lowered with increasing water content of the solvent. In fact, the β-lactam band shifts toward lower frequency and broadens on addition of water to the solution. Such an effect, illustrated by the shift of the β-lactam band of hydrat-

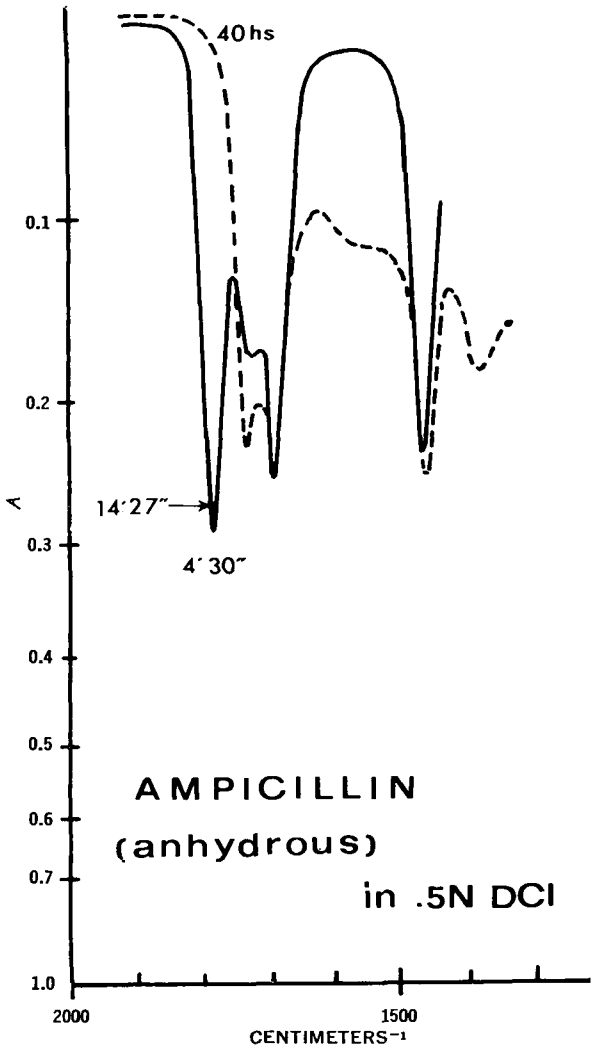


Figure 8—*IR spectra of ampicillin (anhydrous) in 0.5 N DCl at various times after dissolution. For clarity, only the absorbance of the β-lactam band is indicated for the spectrum after 14 min 27 sec (experimental conditions as in Fig. 1).*

ed ampicillin relative to anhydrous ampicillin (Table III), implies that the absorptivity of the analytical band is different for different water contents in the solution. From a practical point of view, satisfactory analyses can be performed whenever the water content is essentially the same in the sample and standard solutions.

DISCUSSION AND CONCLUSIONS

The present work has shown that the assay of most common penicillins can be performed in a very simple way by IR spectro-

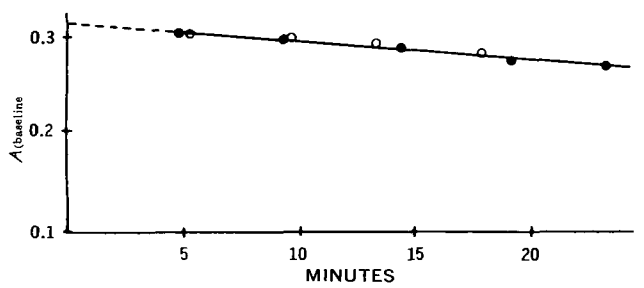


Figure 9—*Absorbance at 1770 cm^{-1}* versus *time data for ampicillin (anhydrous) in 0.5 N DCl (experimental conditions as in Fig. 1). Data refer to sets of data taken on two samples of the same ampicillin.*

Table II—Comparison of Quantitative Data Obtained for a Sample of Cloxacillin Using the IR and the Iodometric Methods[a]

Test	IR	Δ%	Iodometry	Δ%
1	98.20	−0.03	97.61	−0.68
2	97.41	−0.82	97.81	−0.48
3	98.59	+0.39	98.99	+0.70
4	97.81	−0.39	98.10	−0.19
5	97.13	−1.10	98.50	+0.21
6	98.20	−0.03	99.18	+0.89
7	98.99	+0.76	97.61	−0.68
8	99.18	+0.95	98.10	−0.19
9	98.59	+0.36	98.70	+0.41
Average	98.23		98.29	
Standard deviation		0.69		0.59
Standard error		±0.23		±0.22

[a] Percentages, relative to an international standard (BP) of cloxacillin.

photometry in deuterium oxide or dimethyl sulfoxide solution. All that is required is weighing the sample, dissolving it in the appropriate solvent, running the spectrum in the carbonyl region, and converting the absorbance of the β-lactam band to concentration values. This conversion can be done by reference to a calibration curve or to a single value from a standard sample. A complete analysis can usually be carried out in less than 15 min.

Accuracy and precision of the IR method in solution compare

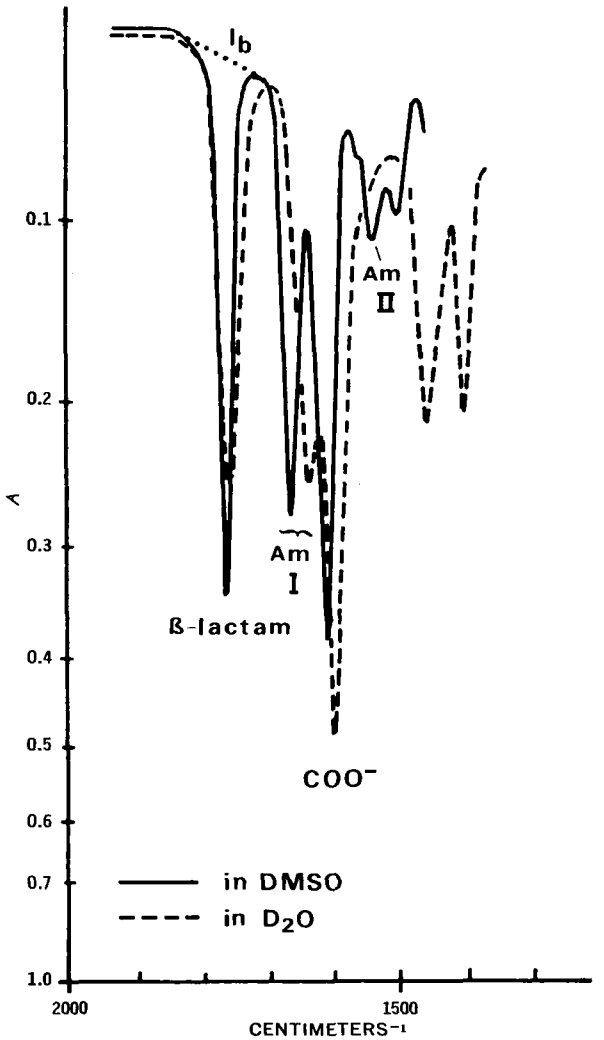
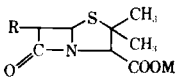
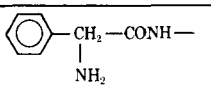
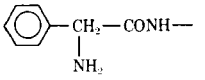
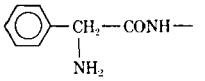
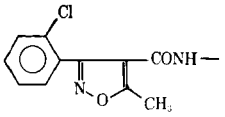


Figure 10—*IR spectra of sodium cloxacillin in dimethyl sulfoxide (DMSO) and in deuterium oxide (experimental conditions as in Fig. 1).*

Table III—Frequency ($\bar{\nu}$, cm^{-1}) and Absorptivity (a, $mole^{-1} \cdot cm^{-1}$) of β-Lactam, Amide, and Carboxyl Bands of Some Penicillins in Dimethyl Sulfoxide Solution



Compound	R	M	Molecular Weight	β-Lactam		Amide I		Carboxylate (ν_{as})	
				$\bar{\nu}$	a	$\bar{\nu}$	a	$\bar{\nu}$	a
Ampicillin[a] trihydrate		H	349.4	1771	696	1685	—[b]	1618	294
Ampicillin		H	349.4	1775	708	1690	413	1618	190
Ampicillin		Na	371.4	1774	755	1686	489	1621	793
Cloxacillin[a]		Na	457.8	1764	820	1664	661	1611	928

[a] Data calculated on the anhydrous basis. [b] Data not obtained because of interference by water.

well with those of current iodometric procedures. Due to the different absorption patterns of the amide and carboxylate groups of different penicillins in the carbonyl region of the spectrum, the specificity of the present method is definitely superior to that of the iodometric one. A further advantage of the IR method is that usually its precision is not lowered by common penicillin impurities. The precision of the iodometric method decreases with decreasing purity of the penicillin sample (16). A further advantage of the IR assay is that it lends itself to a direct investigation of the stability of penicillins in solution. Such an approach is of special interest in the case of aqueous solutions, since, apart from small isotope effects, penicillins in deuterium oxide and deuterated acids are expected to behave as in water and protonated acids. The kinetic measurements can be performed by either repetitive scanning of the spectrum or working at a fixed wavelength, by the "time-drive" technique.

Since the solvent and the cells are relatively transparent in the analytical region, the analysis can be done using an IR instrument of any class.

Most inorganic salts and organic compounds that are usually associated with penicillins in pharmaceutical preparations do not interfere with the assay on the β-lactam band, unless they possess carbonyl groups (such as ester groups) absorbing near the 1760–1670-cm^{-1} region. Glucose and inorganic phosphates are among the noninterfering partners of penicillins. A large excess of water in the sample can increase the background absorption on the low frequency side of the analytical region, both in deuterium oxide (HOD band at about 1460 cm^{-1}) and in dimethyl sulfoxide (water band at 1655 cm^{-1}). However, the baseline technique of measuring the absorbance of the β-lactam band usually allows for these potential interferences.

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(16) P. Finholt, G. Jurgensen, and H. Kristiansen, *J. Pharm. Sci.*, **54**, 387(1965).

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ˣ To whom inquiries should be directed.

GLC Determination of Ibuprofen [(±)-2-(*p*-Isobutylphenyl)propionic Acid] in Plasma

DAVID G. KAISER[x] and GARRETT J. VANGIESSEN

Abstract □ To evaluate the pharmacokinetics and drug availability from various dosage formulations, a method for the determination of ibuprofen [(±)-2-(*p*-isobutylphenyl)propionic acid] in human plasma was required. A simple, rapid, sensitive, and specific procedure, based on a benzene extraction of the acidified specimen and subsequent GLC analysis of the methyl esters of the extract residue, was developed. The method is sensitive to 0.5 μg ibuprofen/ml plasma. Statistical analyses indicate an average recovery of 94.8 ± 6.6% *(SD)*, which is adequate to differentiate assay error from normal biological variation. Mass spectrometric analysis, in conjunction with GLC, confirmed the specificity of the method for intact drug. The procedure was successfully applied to drug absorption studies in humans.

Keyphrases □ Ibuprofen—GLC analysis in human plasma □ (±)-2-(*p*-Isobutylphenyl)propionic acid (ibuprofen)—GLC analysis in human plasma □ GLC—analysis, ibuprofen in human plasma

Based on a variety of tests in animals, ibuprofen[1] [(±)-2-(*p*-isobutylphenyl)propionic acid, I] was observed (1, 2) to be one of the more potent orally active anti-inflammatory, antipyretic, and analgesic agents of a large number of substituted 2-phenylalkanoic acids. The extensive toxicological and biochemical studies (3, 4) in animals and man (5) were comprehensively reviewed (6).

To study the absorption, metabolism, and excretion of these agents in animals and man, Adams and Cliffe (7) developed a method based on paper chromatographic separation and reaction of the drug with bromcresol purple. The lower level of assay detection sensitivity was estimated to be 5–10 μg drug/ml serum, and assay precision was ±15%. Unfortunately, the assay required 48 hr for completion. Since I was selected for extensive biological evaluation (8), a simple, rapid, sensitive, and specific GLC method of analysis for this compound in human plasma was developed. Subsequent to these investigations, Nash *et al.* (9) described a GLC method for the measurement of a structurally related compound, fenoprofen [*dl*-2-(3-phenoxyphenyl)propionic acid], in human plasma. After extraction from the acidified plasma, the compound was converted to the silyl ester and measured *via* GLC utilizing a flame-ionization detector. The lower level of assay detection sensitivity for measurement of fenoprofen in human plasma was 0.25 μg/ml, and assay precision was approximately ±10%.

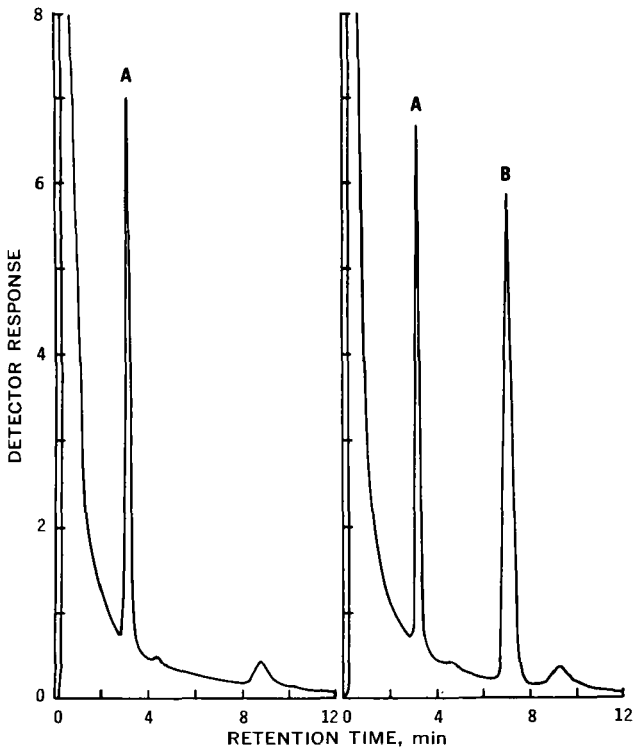
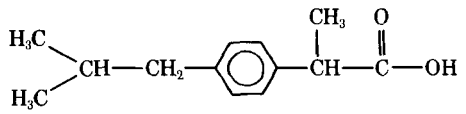


Figure 1—*Gas–liquid chromatograms of human plasma extracts. Left: normal plasma specimen. Right: plasma specimen from subject at 1 hr after single-dose oral administration of 200 mg ibuprofen. Key: A, naphthalene internal standard; and B, ibuprofen methyl ester.*

EXPERIMENTAL

Reagents and Materials—The I used in this study was synthesized[2], and naphthalene, triethylamine, and hydrocarbon-stabilized chloroform were used as supplied[3]. The stock solution of 1,1′-carbonyldiimidazole[4] (II) (65 mg/ml) and naphthalene (100 μg/ml) in hydrocarbon-stabilized chloroform was prepared fresh daily. Stock solutions of I in hydrocarbon-stabilized chloroform (100 μg/ml), triethylamine (III) in methanol (10% v/v), aqueous hydrochloric acid (1 *N*), and aqueous sodium hydroxide (1 *N*, saturated with hydrocarbon-stabilized chloroform) were stored in glass containers. All other solvents were analytical reagent grade. Diethylene glycol succinate[5] on 80–100-mesh Diatoport-S (6% w/w) was used as supplied.

Instrumentation—A two-speed reciprocating shaker[6] was used for shaking the samples in the horizontal position. A mixer[7] was used to aid in preparing the methyl esters. Chromatographic measurements were made with a gas chromatograph[8] equipped



I

[1] Motrin, The Upjohn Co., Kalamazoo, Mich.

[2] Within the Research Division of Boots Pure Drug Co. Ltd., Nottingham, England.

[3] Matheson, Coleman and Bell, Milwaukee, Wis.

[4] Aldrich Chemical Co., Milwaukee, Wis.

[5] LAC-728, Hewlett-Packard Co., Avondale, Pa.

[6] Eberbach and Sons, Ann Arbor, Mich.

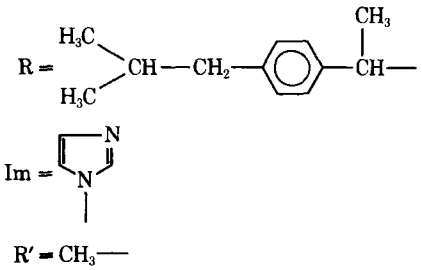
[7] Vortex model K-500, Scientific Industries, Inc., Queen's Village, N.Y.

[8] F&M model 400, Hewlett-Packard Co., Avondale, Pa.

$$R—COOH + Im—CO—Im \longrightarrow Im—CO—R + CO_2 + ImH$$

$$R—CO—Im + R'O^-H^+ \longrightarrow R—CO—OR' + Im^-H^+$$

where:



Scheme I—Reaction scheme for esterification of carboxylic acids

with a hydrogen flame-ionization detector and a −0.2-1.0-mv recorder[9]. All cylinders of gases used for chromatography (*i.e.*, helium, hydrogen, and oxygen) were fitted with filters containing molecular sieve 4A.

Chromatographic Conditions—All chromatography was conducted on U-shaped or coiled stainless steel columns [1.82 m (6 ft) × 0.63 cm (0.25-in.) o.d.] of 6% (w/w) diethylene glycol succinate on 80-100-mesh Diatoport-S. All newly prepared columns were preconditioned at 200° for: *(a)* 1 hr without carrier gas flow, and *(b)* 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and detector block were maintained isothermally at 150, 190, and 210°, respectively. Helium, hydrogen, and oxygen flow rates were 60, 40, and 400 ml/min, respectively. Under these conditions, naphthalene and the I methyl ester have retention times of 3.4 and 6.8 min, respectively (Fig. 1).

Assay Procedure—*Preparation of Standards*—Pipet aliquots of the I chloroform stock solution equivalent to 2, 5, 10, 20, 40, and 60 μg into glass-stoppered centrifuge tubes. Evaporate to dryness with a gentle stream of nitrogen gas. Add 1 ml of control plasma to each centrifuge tube and mix well with the mixer. Prepare an appropriate blank. Extract all standards in the same manner as described for the plasma specimens.

Preparation of Samples—Place 1 ml of plasma in a glass-stoppered centrifuge tube. Add 0.25 ml 1 *N* aqueous hydrochloric acid and 5 ml benzene and shake in the horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm. Transfer a 4-ml aliquot of the benzene layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with a gentle stream of nitrogen gas. Wash down the walls of the centrifuge tube with 0.5 ml chloroform and evaporate to dryness with nitrogen. Add 0.1 ml of II reagent to each benzene extract residue. Rotate each tube to permit the reagent to contact the lower 2.54 cm (1 in.) of the centrifuge tube wall, allowing the reagent to react for at least 1 min. Add 0.1 ml 10% (v/v) III in methanol and mix thoroughly using the mixer. Allow the reagent to react for 5 min. Add 1 ml 1 *N* aqueous sodium hydroxide and shake vigorously by hand. Centrifuge for 10 min at 2000 rpm. Inject a 1-μl aliquot of the chloroform layer for analysis into the chromatograph.

Calculations—The peak heights for naphthalene and the I methyl ester are measured. Peak height ratios are obtained by dividing the peak height of the I methyl ester by the peak height of naphthalene. Calibration curves from known concentrations of I in plasma are prepared by plotting peak height ratios *versus* free acid concentration, expressed as micrograms per milliliter of plasma. Values for unknown concentrations of I in plasma specimens, obtained in the same manner, are then read directly from the graph or calculated from the slope of the standard curve.

Drug Administration to Man—Informed written consent was obtained from each of 20 normal human male volunteers prior to participation in this study. All subjects were between the ages of 22 and 50 years; they ranged in body weight from 63.6 to 81.8 kg and in height from 1.66 to 1.88 m. All subjects were fasted for 16 hr prior to drug administration. Each received a 200-mg dose of I, as a solution in polysorbate 80, contained in soft elastic gelatin capsules. Food was withheld for an additional 4 hr. Blood specimens (10 ml) were withdrawn in heparinized syringes at predetermined time intervals from 0 to 24 hr after drug administration. The plasma was harvested and stored at −18°.

[9] Electronik 15, Honeywell, Inc., Philadelphia, Pa.

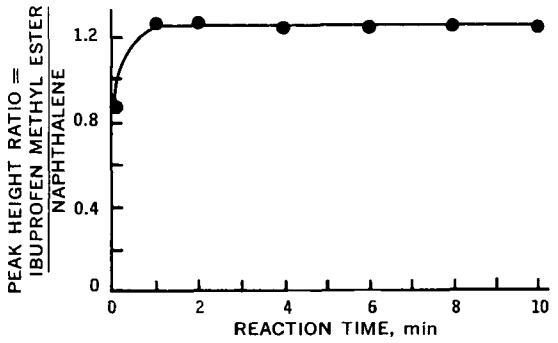


Figure 2—*Effect of reaction time (1,1'-carbonyldiimidazole in chloroform) on formation of ibuprofen methyl ester (reaction time for triethylamine in methanol = 10 min).*

RESULTS AND DISCUSSION

Esterification of Ibuprofen—Studies by Ko and Royer[10] indicated that II was a useful reagent for the facile esterification of free fatty acids found in serum extract residues. The general reaction scheme for the esterification (10) of carboxylic acids is presented in Scheme I. A series of samples, containing known amounts of I, was prepared to determine the optimal reaction times for: *(a)* imidazolide formation and *(b)* methyl ester formation from the imidazolide. The results indicated that imidazolide formation was completed within 1 min (Fig. 2) and that methyl ester formation was completed almost instantaneously (Fig. 3). Reaction times of 1 and 5 min, respectively, were selected for convenience. Synthesis of standard material indicated that the methyl ester of I was a liquid at room temperature. Attempts to isolate crystalline material were unsuccessful. GLC (using a solid sample injector) indicated that the material submitted for elemental analysis was greater than 98% pure.

Anal.—Calc. for $C_{14}H_{20}O_2$: C, 76.33; H, 9.15. Found: C, 76.16; H, 9.08.

IR and mass spectrometric analysis, before and after GLC, supported the proposed structure and confirmed that the I methyl ester chromatographed as the intact molecule.

Selection of Internal Standard—Pilot studies, using 2-(*p*-isobutylphenyl)acetic acid as an internal standard, showed that the methyl ester had a retention time of about 8.8 min as compared to 6.8 min for I methyl ester. As shown in Fig. 1, benzene extracts of plasma specimens from normal human subjects contained an interfering material of approximately the same retention time as the methyl ester 2-(*p*-isobutylphenyl)acetic acid. Naphthalene was subsequently selected to replace 2-(*p*-isobutyl

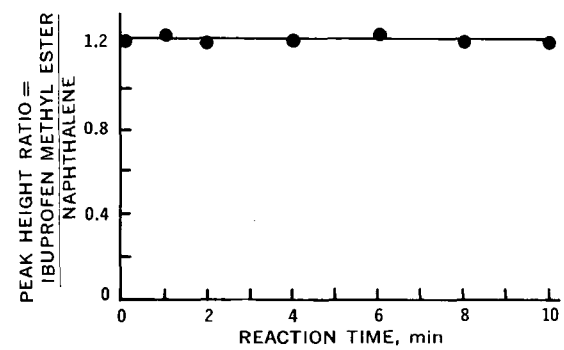


Figure 3—*Effect of reaction time (triethylamine in methanol) on formation of ibuprofen methyl ester (reaction time of 1,1'-carbonyldiimidazole in chloroform = 10 min).*

[10] H. Ko and M. E. Royer, The Upjohn Co., Kalamazoo, Mich., personal communication.

Table I—Recovery of Ibuprofen from Human Plasma

Added, μg/ml	Found, μg/ml	Recovery, %
2.0	1.82	91.0
5.0	4.42	88.4
10.0	9.45	94.5
20.0	17.60	88.0
40.0	42.76	106.9
60.0	57.96	96.6
80.0	78.64	98.3
	Mean ± *SD* =	94.8 ± 6.6

phenyl)acetic acid as the internal standard. Although naphthalene eluted from the chromatograph in a region where there was no interference, it only served as a measure of sample injection reproducibility.

Solution Stability of 1,1′-Carbonyldiimidazole—A chloroform solution of II was allowed to stand at room temperature for a total of 5 days. Forty-eight hours after the preparation of the solution, it began to turn yellow. Esterification reactions conducted at predetermined time intervals indicated that the reagent, as observed under these experimental conditions, was stable for at least 24–48 hr. Ancillary studies using NMR techniques suggested that the diimidazole was slowly degrading to imidazole in the chloroform solution.

Assay Sensitivity and Specificity—At a sensitivity of 1.28×10^{-9} amp/mv, 1.2 μg of I as its methyl ester produced a full-scale response. However, under the described assay conditions, the lower limit of detection sensitivity for I in extracts of human plasma is 0.5 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under these assay conditions, a linear relationship between detector response and concentration is obtained for I over the 0–80-μg/ml range. Quantification from a standard curve was adequate. Analysis of plasma specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay is identical to known I methyl ester.

Recovery Experiments—Known amounts of I in chloroform were evaporated to dryness in centrifuge tubes, and water or plasma was added. The samples were thoroughly mixed and extracted with benzene. All extract residues were esterified and analyzed chromatographically. The results (Table I) indicated that recovery of I from plasma was essentially quantitative (94.8 ± 6.6%) as compared to simple aqueous samples.

Plasma Levels of Ibuprofen in Man—Results from the measurement of plasma I concentrations in 20 normal human subjects, after single-dose oral drug administration, demonstrated the utility of the analytical methodology (Fig. 4). A peak mean (±*SD*) level of I (21.8 ± 4.2 μg/ml) was observed at 1 hr after drug administration, indicating rapid drug absorption from the polysorbate 80 solution. Beyond 12 hr, plasma drug concentrations were below the level of assay detection sensitivity (*i.e.*, less than 0.5 μg/ml), indicating rapid drug disappearance from peripheral circulation. The plasma drug disappearance half-life, as estimated graphically from the average plasma drug concentrations, was 1.93 hr. The combined results from these investigations showed that the GLC method could be used for: *(a)* evaluating pharmacokinetics, *(b)* evaluating drug availability from various dosage formulations, and *(c)* selecting an optimum dosage regimen for I administration to man.

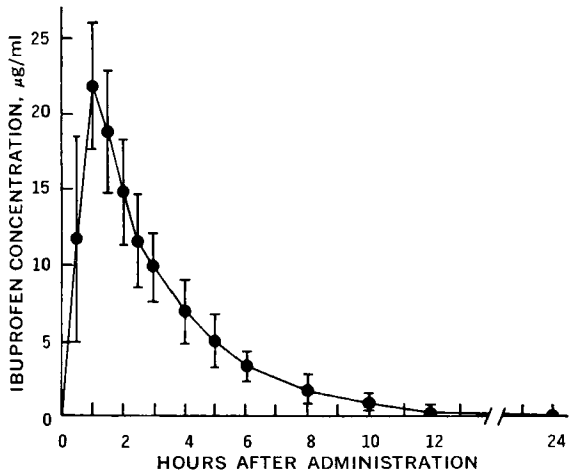


Figure 4—*Average* (±SD) *plasma concentrations of ibuprofen* versus *time in humans* (n = *20*) *after single-dose oral administration of 200 mg drug as a solution in polysorbate 80.*

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[x] To whom inquiries should be directed.

In Vitro Hydrolysis of Oxazepam Succinate Half-Ester by a Stereospecific Soluble Esterase from Different Animal Species

MARIO SALMONA, CARLO SARONIO, ROBERTO BIANCHI, FRANCA MARCUCCI, and EMILIO MUSSINI[x]

Abstract □ The presence of a stereospecific soluble esterase that hydrolyzes the three forms of oxazepam succinate half-ester [(+), (−), and (±)] in some tissues of rats, mice, and guinea pigs was demonstrated. The enzymatic activity was determined by a titrimetric method. The K_m values obtained were 2.5×10^{-3} *M* for the (+)-isomer, 3.3×10^{-3} *M* for the racemate, and 14.3×10^{-3} *M* for the (−)-isomer in tissues showing enzymatic activity. Studies employing several inhibitors or activators indicate that the enzyme probably belongs to the C-esterase group.

Keyphrases □ Oxazepam succinate half-ester—*in vitro* hydrolysis by stereospecific soluble esterase from rats, mice, and guinea pigs □ Stereospecific esterase in liver, kidney, and brain of rats, mice, and guinea pigs—*in vitro* hydrolysis of oxazepam succinate half-ester □ Enzymatic activity, stereospecific soluble esterase in rat, mouse, and guinea pig—*in vitro* hydrolysis of oxazepam succinate half-ester □ Esterase, stereospecific, in rats, mice, and guinea pigs—*in vitro* hydrolysis of oxazepam succinate half-ester

A recent derivative of oxazepam, the succinate half-ester which differs from the parent compound due to its high solubility in water, shows interesting pharmacodynamic and kinetic properties (1–4). The racemic compound has been resolved into the two optically active isomers (5). These enantiomers show quantitatively different pharmacological effects. Mussini *et al.* (6) recently demonstrated that the (+)-isomer shows a more powerful antipentylenetetrazol effect than the (−)-isomer while the racemate exhibits intermediate activity. More recently, de Angelis *et al.* (7), studying the effects of oxazepam succinate half-ester in the central nervous system (CNS), also showed that the (+)-isomer is more active than the (−)-form while the activity of the racemate lies between the two. The tests used to evaluate their activity in mice were potentiation of narcosis, spontaneous motor activity, antipentylenetetrazol and antistrychnine activities, analgesic effect, and muscle relaxant activity.

Since the pharmacological activity of oxazepam succinate half-ester may depend on the availability of oxazepam (6), an attempt was made to localize the distribution of the esterase that metabolizes this compound in three animal species and to confirm its stereospecificity *in vitro*. Furthermore, various inhibitors were used to investigate the type of esterase involved in the hydrolysis of the ester linkage between oxazepam and succinate.

EXPERIMENTAL

Animals—Male Sprague–Dawley rats (200–250 g), male albino Swiss mice (20–25 g), and male albino guinea pigs (300–350 g), fed *ad libitum*, were used in all experiments.

Substrates—α-Naphthyl acetate was synthesized from acetic anhydride and α-naphthol[1] by shaking an ice-cold aqueous alkaline solution of α-naphthol with acetic anhydride. The acetyl derivative, crystallized from water had a melting point of 48.5°.

The following substrates were also used: oxazepam succinate half-esters[2], neostigmine methylsulfate[1], physostigmine[1] (eserine), diisopropylfluorophosphate[1] (I), edetate[1] (ethylene diaminetetraacetate) (II), *p*-chloromercuribenzoate[3] (III), mercuric chloride[4], sodium fluoride[5], and lanthanum nitrate hexahydrate[5] [$La(NO_3)_3 \cdot 6H_2O$].

Buffers—For the enzymatic determinations, either naphthyl acetate or the oxazepam succinate half-ester was dissolved in 0.025 *M* phosphate buffer, pH 7.6. For the experiments concerning the pH-activity profile, the following buffers were used: 0.025 *M* sodium acetate for pH 5.0–6.5, 0.025 *M* sodium phosphate for pH 6.5–8, 0.025 *M* sodium pyrophosphate for pH 8–9.5, and 0.025 *M* sodium borate for pH values above 9.5. The pH of all solutions was adjusted to the desired value before the experiments.

Preparation of Microsomes—After killing the animals, the liver, kidneys, heart, lungs, and brain were immediately removed and homogenized in ice-cold 1.15% KCl solution (1:4 w/v) with a Teflon glass homogenizer.

The homogenate was centrifuged at 9000×*g* for 20 min, and the supernatant fraction was further centrifuged[6] at 105,000×*g* for 1 hr. The supernatant fractions containing the "soluble" enzymes were collected separately, and the microsomes were suspended in 1.15% KCl solution.

Blood Collection—Animals were killed by decapitation, and blood samples were collected in heparinized test tubes. After centrifugation at 9000×*g* for 10 min, the erythrocytes and plasma were collected separately and tested for enzymatic activities.

Protein Analysis—Proteins were determined according to the method of Lowry *et al.* (8).

Enzymatic Activities—The esterase activity of the aqueous extracts was determined by a titrimetric method[7] (9). The volume of the reaction mixture was always 15 ml. A volume of 0.100–0.300 ml or a suitable dilution of enzymatic preparation was added to the reaction vessel. The final concentration of α-naphthyl acetate or of oxazepam succinate half-ester in the vessel was 10^{-3} *M*. The reaction mixture also contained 0.45% NaCl.

Sodium hydroxide at a concentration of 0.005 *M* was used for the titrations, which were carried out at 37° for 3 min, maintaining the pH at 7.6. The initial rate of hydrolysis was determined from the titration curves as micromoles of sodium hydroxide utilized per milliliter of extract per minute.

Studies with Inhibitors (or Activators)—To investigate the effect of a number of inhibitors (or activators) of esterases on the enzyme metabolizing the oxazepam succinate half-ester, the normal reaction mixture was added together with 1 ml or a suitable dilution of inhibitor (or activator) to obtain a final concentration range of 10^{-8}–10^{-3} *M*. The titration was then carried out as usual.

The I_{50} values, *i.e.*, the concentration of inhibitors producing 50% of inhibition, were obtained graphically by plotting the per-

[1] Merck, Darmstadt, Germany.
[2] RV 1206 (±), RV 1208 (+), and RV 1210 (−), supplied by U. Ravizza, Muggiò, Milan, Italy.
[3] Shuchardt, München, Germany.
[4] Industria Chimica Italo-Svizzera, Milan, Italy.
[5] Carlo Erba, Milan, Italy.
[6] Beckman model L ultracentrifuge, rotor 40′.
[7] pH-Stat TTT Ic apparatus, Radiometer, Copenhagen, Denmark.

Table I—Esterase Activity in Rats (Units per Milligram Protein)[a]

Tissue	α-Naphthyl Acetate	Oxazepam Succinate Half-Ester (±)	(+)	(−)
Plasma	0.146 ± 0.010	—	—	—
Brain				
Soluble fraction	0.285 ± 0.020	—	—	—
Microsomes	0.773 ± 0.030	—	—	—
Liver				
Soluble fraction	0.032 ± 0.040	0.021 ± 0.003	0.053 ± 0.003	0.017 ± 0.003
Microsomes	1.043 ± 0.072	—	—	—
Kidney				
Soluble fraction	0.962 ± 0.270	—	—	—
Microsomes	5.340 ± 0.180	—	—	—

[a] Results are expressed as mean ± standard error of six determinations. A pool of three rats was used for each experiment.

Table II—Esterase Activity in Mice (Units per Milligram Protein)[a]

Tissue	α-Naphthyl Acetate	Oxazepam Succinate Half-Ester (±)	(+)	(−)
Plasma	0.121 ± 0.012	—	—	—
Brain				
Soluble fraction	0.485 ± 0.070	—	—	—
Microsomes	3.680 ± 0.470	—	—	—
Liver				
Soluble fraction	0.427 ± 0.023	0.015 ± 0.001	0.040 ± 0.002	0.004 ± 0.001
Microsomes	0.868 ± 0.074	—	—	—
Kidney				
Soluble fraction	1.925 ± 0.020	1.111 ± 0.030	1.501 ± 0.025	0.925 ± 0.014
Microsomes	1.820 ± 0.045	—	—	—

[a] Results are expressed as mean ± standard error of six determinations. A pool of 10 mice was used for each experiment.

Table III—Esterase Activity in Guinea Pigs (Units per Milligram Protein)[a]

Tissue	α-Naphthyl Acetate	Oxazepam Succinate Half-Ester (±)	(+)	(−)
Plasma	0.407 ± 0.031	—	—	—
Brain				
Soluble fraction	0.056 ± 0.001	0.023 ± 0.001	0.006 ± 0.001	0.019 ± 0.001
Microsomes	0.202 ± 0.054	—	—	—
Liver				
Soluble fraction	0.490 ± 0.010	0.143 ± 0.010	0.380 ± 0.050	0.113 ± 0.020
Microsomes	6.640 ± 0.298	—	—	—
Kidney				
Soluble fraction	0.605 ± 0.001	0.025 ± 0.003	0.031 ± 0.001	0.018 ± 0.001
Microsomes	1.550 ± 0.328	—	—	—

[a] Results are expressed as mean ± standard error of six determinations. A pool of three guinea pigs was used for each experiment.

centage of inhibition as a junction of inhibitor concentration on semilogarithmic paper.

RESULTS

Tissue Distribution of Enzyme—To find the exact distribution of the enzyme metabolizing the oxazepam succinate half-ester, the total amount of esterase activity present in the extracts was initially determined by means of α-naphthyl acetate, since it can be metabolized, although at a different rate, by all esterases (10–12). Then the extracts were analyzed for oxazepam succinate half-ester hydrolyzing activity. Tables I–III show the tissue distribution of the enzyme in rats, mice, and guinea pigs, respectively.

Esterase activity was not detected with respect to the oxazepam succinate half-ester in the heart, lungs, and erythrocytes of these three animal species. In contrast to oxazepam succinate, enzyme activity against α-naphthyl acetate was found in both supernatant fractions and in the microsomes of the tissues studied.

The distribution of oxazepam succinate esterases was not the same in the three animal species; when the enzyme was present, it was only in the soluble fractions of the cells, *i.e.*, in the supernatant fraction of the 105,000×*g* preparation. Although the enzyme always showed a stereospecific activity on oxazepam succinate half-ester, only in the soluble fraction of liver does this difference seem to be important.

K_m Values for (+)- and (−)-Isomers and for the Racemate in Tested Tissues—The K_m values obtained were 2.5×10^{-3} *M* for the (+)-isomer, 3.3×10^{-3} *M* for the racemate, and 14.3×10^{-3} *M* for the (−)-isomer. The K_m values for the three forms of oxazepam succinate half-ester did not differ significantly among the three species studied (Fig. 1).

Studies with Inhibitors (or Activators) of Esterases—The enzymatic preparations were first tested with neostigmine, physostigmine, and I. These compounds did not show any inhibitory activity toward the enzyme. However, I was not hydrolyzed by the enzymatic preparations. Cupric sulfate and mercuric chloride inhibited the enzymatic preparations very strongly (Fig. 2); the pI_{50} values for the two salts were 5 and 6, respectively. Compound III activated the enzyme when used at low concentrations in the 10^{-6}–10^{-4} *M* range, but at 10^{-3} *M* this activation dimin-

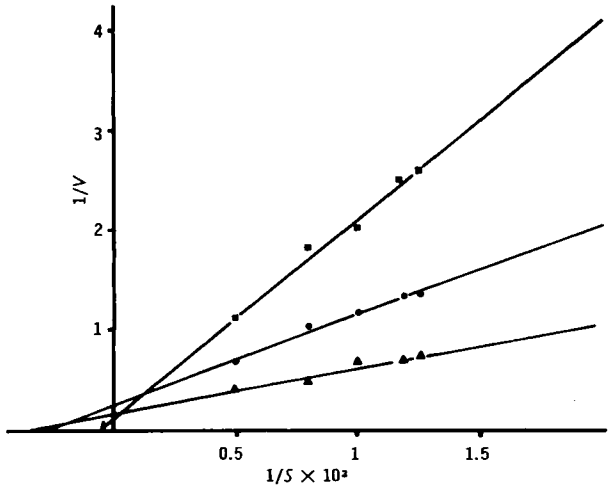

Figure 1—*Double reciprocal plot of the three forms of oxazepam succinate half-ester. In all tissues with oxazepam succinate esterase activity, the* K_m *values do not differ significantly in the three animal species studied. The* K_m *values are* 3.3×10^{-3} M *for the racemate* (●), 2.5×10^{-3} M *for the* (+)*-isomer* (▲), *and* 14.3×10^{-3} M *for the* (−)*-isomer* (■). V *is the initial velocity of the reaction in micromoles per minute per milligram protein.* S = [*oxazepam succinate half-ester* (M)].

ished (Fig. 3). The maximum activation occurred at a concentration of 10^{-5} *M*. Compound II and sodium fluoride (Fig. 3) also activated the enzymatic preparations at low concentrations, although this effect was not comparable with that of III.

pH-Activity Profile for Oxazepam Succinate Half-Ester Esterases—The pH-activity profile for oxazepam succinate half-ester of enzymatic preparations is bell-shaped and has a "plateau" between pH 7.5 and 8 (Fig. 4).

DISCUSSION

The enzyme that is able to metabolize the succinate half-ester of oxazepam is located only in the supernatant fraction of the 105,000×*g* preparations, which means that the enzyme is not particulate but belongs to the soluble enzymes of the cell. The different distribution of enzymes in the three species studied may be due to a different phylogenetical development or to a peculiar difference of enzymatic content in these three species. In man, for instance, it is well known that many genetic differences in esterasic response can be found (13–17) for different substrates.

The behavior of the enzymatic preparations against the inhibitors or activators studied seems very similar to that found in previous studies (18–22). In fact, the enzymatic preparations are not inhibited by organophosphates while they are activated by III at low concentrations; at higher concentrations, a degradation of enzymatic protein probably occurs. It must be emphasized that while Bergmann and Rimon (21) reported a maximum of 800% activation, only 400% activation was found in the present preparations. On the other hand, Tashian (20) reported an activation at high concentrations, *i.e.*, 10^{-3} *M*. Furthermore, sodium fluoride and II at low concentrations activate the enzymatic preparations. The II-dependent activity is quite unusual, since Björklund *et al.* (23) reported a II-dependent A-esterase in human blood.

Although there is no evidence in the literature of enzymatic activation by sodium fluoride, since this compound normally inhibits pseudocholinesterases (24, 25), the present findings may be interesting with regard to the further characterization of the enzymes, probably belonging to the C-esterase group. The lanthanum salt does not inhibit the enzymatic preparations even at a concentration of 10^{-3} *M*. Mercuric chloride and cupric sulfate strongly inhibit the enzymatic activity at very low concentrations; in the experimental conditions, the pI_{50} values were 5 and 6, respectively. The type of inhibition involved was not identified since this will be done after purification of the enzyme to eliminate any possible interaction.

The results obtained suggest that the hydrolysis of oxazepam succinate half-ester may show specific aspects *in vivo* in the three animal species tested.

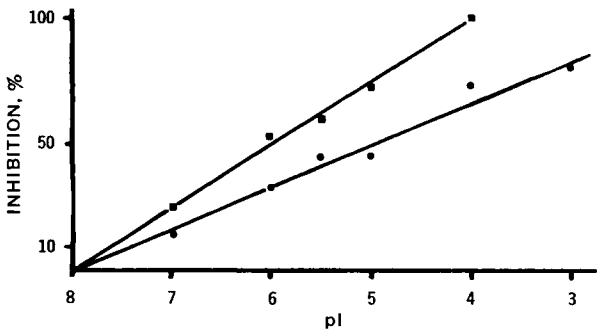

Figure 2—*The pI–activity curve of the enzymatic preparations. Key:* ●, *mercuric chloride; and* ■, *cupric sulfate. pI = −log of concentration of inhibitor.*

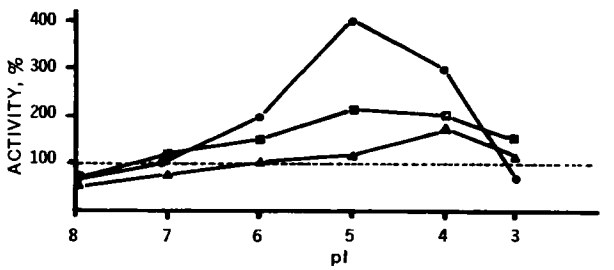

Figure 3—*Effect of* p-*chloromercuribenzoate* (●), *edetate* (▲), *and sodium fluoride* (■) *on the enzymatic preparations. The horizontal line indicates the standard level of activity.*

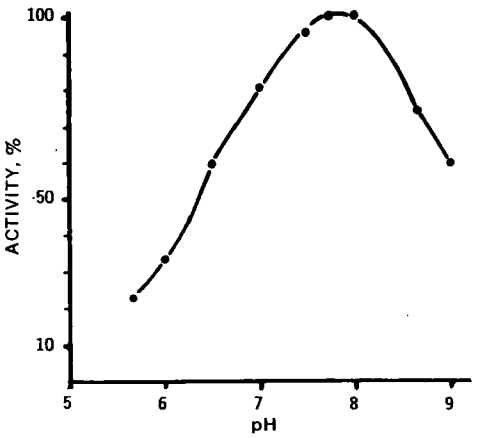

Figure 4—*The pH–activity profile of enzymatic preparations, showing a "plateau" between pH 7.5 and 8.*

Table IV—Behavior of Enzymatic Preparations Hydrolyzing Oxazepam Succinate Half-Ester against Inhibitors (or Activators)

Enzymatic Preparations	Activity
Neostigmine	No inhibition
Physostigmine	No inhibition
Diisopropylfluorophosphate	No inhibition, no esterase activity for this compound
p-Chloromercuribenzoate	Activation at 10^{-5} *M*
Edetate	Activation at 10^{-4} *M*
Sodium fluoride	Activation at 10^{-5} *M*
Mercuric chloride	Inhibition, $pI_{50} = 5$
Cupric sulfate	Inhibition, $pI_{50} = 6$
Lanthanum nitrate hexahydrate	No inhibition

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[x] To whom inquiries should be directed.

Disposition of Hydralazine in Man and a Specific Method for Its Determination in Biological Fluids

SYLVIA B. ZAK*x, M. FRED BARTLETT*, WILLIAM E. WAGNER‡, THOMAS G. GILLERAN*, and GEORGE LUKAS*

Abstract □ A sensitive analytical procedure was developed for the measurement of the concentrations of hydralazine in human plasma and urine. The method is based on the formation of the hydrazone derivative of hydralazine with *p*-methoxybenzaldehyde. Specificity of the procedure for unchanged drug was demonstrated by inverse isotope dilution analysis following administration of hydralazine-^{14}C to humans. Plasma levels and urinary excretion of hydralazine were measured in healthy subjects after single oral therapeutic doses.

Keyphrases □ Hydralazine-^{14}C—plasma levels and urinary excretion in man, spectrophotometric and inverse isotope dilution analysis in biological fluids □ Plasma levels—hydralazine-^{14}C after oral administration, man □ Urinary excretion—hydralazine-^{14}C after oral administration, man □ Spectrophotometry—analysis, hydralazine in plasma and urine □ Inverse isotope dilution—analysis, hydralazine in plasma and urine

Hydralazine[1] (1-hydrazinophthalazine), a potent, peripherally acting vasodilator with a prompt and general blood pressure-lowering effect, has been used for the treatment of essential hypertension for many years. Although many pharmacological and clinical papers have appeared, relatively little is known concerning its biological disposition.

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The method published by Perry *et al.* (3) utilized

[1] Apresoline, Ciba-Geigy Corp.

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[x] To whom inquiries should be directed.

Disposition of Hydralazine in Man and a Specific Method for Its Determination in Biological Fluids

SYLVIA B. ZAK*x, M. FRED BARTLETT*, WILLIAM E. WAGNER‡, THOMAS G. GILLERAN*, and GEORGE LUKAS*

Abstract □ A sensitive analytical procedure was developed for the measurement of the concentrations of hydralazine in human plasma and urine. The method is based on the formation of the hydrazone derivative of hydralazine with *p*-methoxybenzaldehyde. Specificity of the procedure for unchanged drug was demonstrated by inverse isotope dilution analysis following administration of hydralazine-^{14}C to humans. Plasma levels and urinary excretion of hydralazine were measured in healthy subjects after single oral therapeutic doses.

Keyphrases □ Hydralazine-^{14}C—plasma levels and urinary excretion in man, spectrophotometric and inverse isotope dilution analysis in biological fluids □ Plasma levels—hydralazine-^{14}C after oral administration, man □ Urinary excretion—hydralazine-^{14}C after oral administration, man □ Spectrophotometry—analysis, hydralazine in plasma and urine □ Inverse isotope dilution—analysis, hydralazine in plasma and urine

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The method published by Perry *et al.* (3) utilized

[1] Apresoline, Ciba-Geigy Corp.

the reaction of hydralazine with ninhydrin. Schulert (4) found that the ninhydrin method gave incomplete recovery and variable results, so he developed an alternative method based on the condensation of hydralazine with *p*-hydroxybenzaldehyde. Unfortunately, other investigators (5) encountered difficulties in their attempts to repeat Schulert's procedure and had to introduce modifications to obtain reproducible results.

Both methods were tested in this laboratory, but neither the original nor the modified method of Schulert afforded the desired reproducibility with the necessary accuracy, particularly at low plasma concentrations. Furthermore, the earlier investigators did not provide evidence that the reported methods were specific for unchanged hydralazine in the presence of its metabolites. Therefore, it became necessary to develop new methodology that would satisfy these criteria and be sufficiently simple to be of value in large-scale plasma level studies.

The development of a new, specific assay method for hydralazine plasma levels and the results obtained in healthy human subjects by its use comprise the subject of this report.

METHODS

Protocol—Healthy adult male volunteers participated in the studies after a complete medical history had been recorded for each individual. No medication was permitted for at least 3 days prior to the study. Control blood and urine specimens were obtained after an overnight fast and, subsequently, hydralazine was administered.

A pool of six volunteers participated in the studies involving nonradioactive hydralazine. A 100-mg oral dose, consisting of four 25-mg commercial tablets, was given to three subjects. A 75-mg oral dose (three 25-mg commercial tablets) was administered to three subjects on a different occasion. In addition, three individuals were given a 20-mg intramuscular dose of the commercial parenteral preparation. Certain individuals participated in more than one study; at least 1 week elapsed between successive experiments. The subjects were permitted to have a fat-free breakfast without coffee at 2 hr postadministration and resumed their normal eating habits at 5 hr.

In the study involving hydralazine-^{14}C, a 100-mg oral dose of the appropriately diluted radioactive material in capsules was administered to a different group of four subjects. These subjects had a simple fat-free meal without coffee at 4 hr postadministration and resumed their normal eating habits at 8 hr.

Determination of Hydralazine Concentrations in Plasma by the Spectrophotometric ("Cold") Method—Blood specimens (15 ml) were withdrawn with heparinized syringes prior to and at specified times after administration of hydralazine. They were transferred immediately to refrigerated, heparinized centrifuge tubes. The specimens were centrifuged in a refrigerated centrifuge, and the plasma was removed and mixed with 0.2 ml of 6 *N* HCl. This part of the procedure had to be completed within 40 min. The acidified specimens were frozen and stored in a freezer until they were analyzed. Under these conditions, hydralazine levels remained unchanged for 4 days.

Aliquots (6 ml) of plasma were transferred to a glass-stoppered centrifuge tube and diluted with 6 ml of 0.4 *N* HCl. To each sample, 0.2 ml of a 10% solution of ascorbic acid in water and 0.5 ml of a 1% solution of *p*-methoxybenzaldehyde in ethanol were added. The mixture was incubated for 30 min at 70°. The tubes were cooled to room temperature, and the pH was adjusted to 9.0 with 5 *N* NaOH. The resulting hydrazone derivative was extracted by shaking each tube on a mechanical shaker for 20 min with 30 ml of benzene–methanol (93:7). The organic phase was separated by centrifugation, and a 25-ml aliquot was transferred to another centrifuge tube. The solvent was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 3 ml of heptane and mixed with 1.5 ml of 0.2 *N* HCl. The hydrazone derivative was reextracted into the aqueous phase by shaking for 20 min. The mixture was centrifuged, the organic phase was removed by aspiration, and the aqueous phase was transferred with a Pasteur pipet into a microcell. The absorbance was read in a spectrophotometer[2] at 355 nm against 0.2 *N* HCl.

The value thus obtained was converted into concentration using a standard curve of solutions of authentic hydrazone derivative in 0.2 *N* HCl. To generate the standard curve, the concentrations in these solutions had to be determined without delay because breakdown of the hydrazone occurred after 20 min in 0.2 *N* HCl. However, in the presence of plasma extracts and excess reagents, the compound was stable for at least 60 min.

Plasma samples containing known amounts of hydralazine also were carried through the entire procedure along with the samples obtained in the study.

In the experiment using ^{14}C-labeled hydralazine, 50-ml blood specimens were withdrawn. Plasma was obtained as described previously, and 6-ml aliquots were analyzed for hydralazine. The remaining amounts of plasma were used for measurement of radioactivity and for inverse isotope dilution analysis.

Determination of Hydralazine Concentrations in Urine—The spectrophotometric method described in detail for plasma was used with two modifications.

1. To 2 ml of urine were added 1 ml of 0.4 *N* HCl, 0.2 ml of a 10% aqueous solution of ascorbic acid, and 0.5 ml of a 1% ethanolic solution of *p*-methoxybenzaldehyde. Incubation at 70° and subsequent extraction at alkaline pH were carried out without modification.
2. Following the evaporation of the 25-ml aliquot of benzene–methanol extract to dryness, the residue was dissolved in 6 ml of heptane and reextracted with 3 ml of 0.2 *N* HCl. Subsequent steps were identical in the plasma and urine methods.

Preparation of *p*-Methoxybenzaldehyde Hydrazone of Hydralazine—To a solution of hydralazine (720 mg) in 7.5 ml of ethanol, 5 ml of water was added. The mixture was heated to 50°, and *p*-methoxybenzaldehyde (0.3 ml in 2.5 ml of ethanol) was added slowly with constant stirring. The resulting thick, yellow mass was stirred with an additional 5 ml of water. A few minutes later, 8 ml of 5% sodium bicarbonate was added and the mixture was cooled to room temperature. The precipitate was filtered, washed with ice-cold water and ethanol, and dried under reduced pressure. The crude product was recrystallized from 400 volumes of 60% aqueous methanol, giving the analytical sample, mp 130–132°; UV (0.1 *N* HCl containing 1% methanol): 299 and 358 nm (ϵ 23,300 and 31,200, respectively); UV (chloroform): 295 and 373 nm (ϵ 22,450 and 25,350, respectively); mass spectroscopy (mol. wt. 278): *m/e* 278, 277, 171 (base peak), 145, and 134; NMR (dimethyl sulfoxide-d_6): 3.8 (3H, s, OCH_3), 7.0 (2H, d, J = 9 Hz, aromatic protons *ortho* to OCH_3), 7.8 (2H, d, J = 9 Hz, aromatic protons *meta* to OCH_3), 8.42 (1H, s, heterocyclic ring proton), 7.5–8.5 (5H, multiplet, four aromatic protons of the bicyclic ring system and CH of the side chain), and 11.8 (1H, NH) ppm.

Anal.—Calc. for $C_{16}H_{14}N_4O$: C, 69.0; H, 5.1; N, 20.2. Found: C, 68.63; H, 5.27; N, 20.24.

Preparation of Capsules Containing Hydralazine-^{14}C—Hydralazine-1-^{14}C (specific activity 12.73 μCi/mg) was obtained commercially[3]. The purity of the material was tested by TLC and measurement of radioactivity and was found satisfactory.

A mixture of 656.1 mg of unlabeled hydralazine and 43.9 mg of hydralazine-^{14}C was dissolved in 35 ml of water. The solution was freeze dried in a lyophilizer (Virtis), and the resulting powder was stored under nitrogen. Exactly 100-mg aliquots corresponding to 80 μCi of ^{14}C were transferred into commercial gelatin capsules for use in the plasma level studies.

Determination of Total Radioactivity—The plasma or urine concentration of total ^{14}C isotope, corresponding to the sum of unchanged hydralazine and its metabolites, was measured by transferring appropriate aliquots to scintillation vials, adding 20 ml of Bray's solution (6), and counting in a liquid scintillation spectrometer[4]. Results were corrected for quenching by the external standard technique.

Feces were homogenized with water. The mixture was freeze

[2] Zeiss.
[3] Mallinckrodt Nuclear, St. Louis, Mo.
[4] Packard Tri-Carb model 3380.

Table I—TLC Behavior of Hydralazine and Its *p*-Methoxybenzaldehyde Hydrazone Derivative

Solvent System	R_f Value	
	Hydralazine	Hydrazone
Cyclohexane–acetone (5:4)		0.83
Benzene–ethanol–acetic acid (220:10:1)		0.76
Toluene–ethyl acetate (10:9)		0.73
3 *N* HCl–methanol–ascorbic acid (44:6:1)	0.53[a]	

[a] Hydralazine was spotted on methanol-washed, silica gel-coated glass plates under a current of nitrogen. The plates were developed in hydrochloric acid–methanol–ascorbic acid flushed with nitrogen before use. Considerable decomposition of hydralazine occurred on the plates when other solvent systems were used.

dried in a lyophilizer (Virtis), and aliquots of the dry residue were combusted in a sample oxidizer[5]. The resulting ^{14}C-labeled carbon dioxide was collected in vials containing 1.5% of 2,5-diphenyloxazole and 0.1% of *p*-bis(*o*-methylstyryl)benzene in toluene and measured in the liquid scintillation spectrometer.

Plasma samples obtained from subjects who had been given hydralazine-^{14}C were analyzed for unchanged hydralazine by the spectrophotometric method described previously. At the completion of these analyses, each sample was also assayed for its radioactivity content; appropriate aliquots were withdrawn and counted in the usual manner using Bray's solution.

Determination of Hydralazine Concentrations in Plasma by Inverse Isotope Dilution Analysis—The derivatization and extraction steps essentially followed the procedure outlined earlier in the spectrophotometric method for the assay of hydralazine. The few modifications were found advantageous because of the larger volume of plasma used.

Unlabeled hydralazine carrier (220 μg) was added to measured aliquots (13–14 ml) of plasma containing hydralazine-^{14}C and its metabolites. Ascorbic acid (50 mg), 3 ml of 1 *N* HCl, 0.02 ml of a 10% ethanolic *p*-methoxybenzaldehyde solution, and finally 5 ml of ethanol were added to each plasma sample. The hydrazone derivative was formed by incubation of the mixture for 15 min at 65°. The tubes were cooled to room temperature, and the resulting hydrazone was extracted at pH 9 into benzene–methanol (93:7). The extract was concentrated to a small volume under a stream of nitrogen. The residue was applied in streaks to methanol-washed silica gel thin-layer plates and chromatographed.

The solvent systems suitable for the chromatography of hydralazine[6] and its *p*-methoxybenzaldehyde hydrazone, as well as the corresponding R_f values, are summarized in Table I. The zone corresponding to the R_f of the hydrazone was scraped off and eluted with ethyl acetate. The amount of the material present in the eluate was determined by measurement of its absorbance at 355 nm. The specific activity of the eluate was then calculated from measurement of its radioactivity content. The chromatography, elution, and measurements were repeated until a steady-state specific activity was obtained. Usually three or four cycles were carried out. Finally, the plasma concentration of hydralazine was estimated from the steady-state specific activity values using Eq. 1:

$$C_p\ (\mu g/ml) = \frac{C_c \times SA_{obs}}{SA_{adm}} \qquad \text{(Eq. 1)}$$

where C_p is the plasma concentration of hydralazine before dilution with carrier, C_c is the concentration of the carrier in the plasma to which it was added, SA_{obs} is the observed steady-state specific activity of the purified sample, and SA_{adm} is the specific activity of the administered drug.

RESULTS

Development of Improved Assay Method—The condensation of the hydrazino side chain of hydralazine with several aromatic aldehydes (cinnamaldehyde, salicylaldehyde, 3,4,5-trimethoxybenzaldehyde, and 1-naphthaldehyde) was explored to find an improved assay method for the drug. Unfortunately, none of these derivatives had a markedly higher absorptivity in either acidic or alkaline solution than the *p*-hydroxybenzaldehyde hydrazone. The derivative that had relatively the highest absorptivity with the lowest plasma blank values was the reaction product with *p*-methoxybenzaldehyde. In addition, its extractability, compared to that of the corresponding *p*-hydroxy derivative, was advantageous for permitting further refinements in the methodology. Thus, the *p*-methoxybenzaldehyde hydrazone became the derivative of choice.

The specific improvements over Schulert's method (4) included evaporation of the solvent used for the initial extraction of the hydrazone derivative from plasma, dissolution of the residue in the nonpolar heptane, and reextraction of the hydrazone into aqueous hydrochloric acid solution. A considerable amount of interfering material was eliminated through these purification steps without causing an appreciable loss of the desired product. Changes in the pH of the reaction and extractions led to a greater stability of the resulting color and increased the overall reproducibility. Therefore, when this new procedure was used for analyses of plasma samples containing 0.1 μg/ml of hydralazine, absorbance values greater than two times plasma blank readings were obtained. The absorbance values were proportional to the concentration in the 0.1–5.0-μg/ml range.

Plasma containing known amounts of hydralazine gave the following absorbance values (average ± *SE*): 0.15 μg/ml, 0.055 ± 0.0003; 0.25 μg/ml, 0.089 ± 0.0012; 0.5 μg/ml, 0.177 ± 0.0026; and 1.0 μg/ml, 0.364 ± 0.0037. The values represent 38 determinations

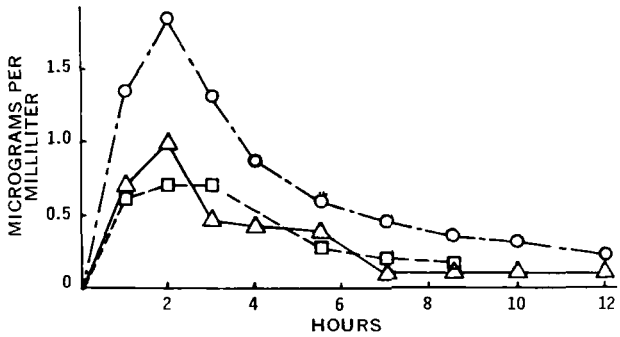

Figure 1—*Concentration of hydralazine-^{14}C and its metabolites in the plasma of Subject 1 after an oral dose of 100 mg (80 μCi). Key: ○, total radioactivity; △, unchanged hydralazine by cold method; and □, unchanged hydralazine by inverse isotope dilution analysis.*

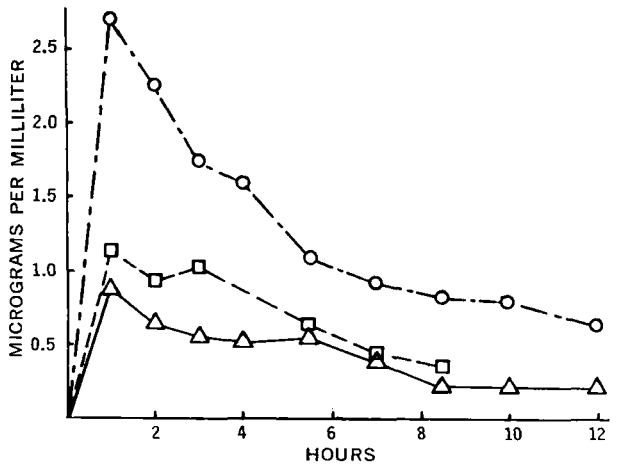

Figure 2—*Concentration of hydralazine-^{14}C and its metabolites in the plasma of Subject 2 after an oral dose of 100 mg (80 μCi). Key: ○, total radioactivity; △, unchanged hydralazine by cold method; and □, unchanged hydralazine by inverse isotope dilution analysis.*

[5] Packard Tri-Carb.

[6] J. E. Francis, Ciba-Geigy Corp., Ardsley, N.Y., personal communication.

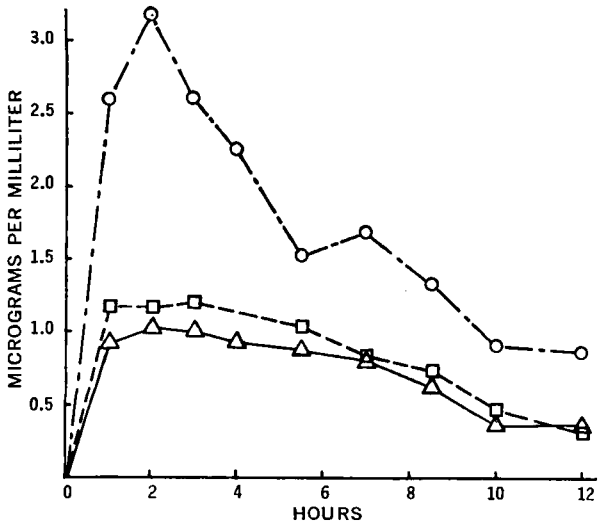

Figure 3—*Concentration of hydralazine-^{14}C and its metabolites in the plasma of Subject 3 after an oral dose of 100 mg (80 μCi). Key: ○, total radioactivity; △, unchanged hydralazine by cold method; and □, unchanged hydralazine by inverse isotope dilution analysis.*

at each concentration after subtraction of the individual blank readings. The plasma blanks had an average absorbance value of 0.038 ± 0.0025. Recoveries, calculated from the 38 measurements, were (average ± *SE*): 96.0 ± 1.6%, 0.15 μg/ml; and 98.0 ± 1.0%, 1.0 μg/ml. The individual recovery values closely approximated a normal distribution at both concentrations (expected value of Pearson's coefficient of skewness: 0.0; found, 0.32 and 0.45, respectively; expected value of the moment coefficient of kurtosis: 3.0; found, 2.56 and 2.94, respectively). These data demonstrate the good reproducibility of the assay method.

Schulert recommended that blood specimens containing hydralazine be processed at a weakly acidic pH (6.5) in view of the instability of the free base form of the drug. In the present experiments, some degree of hemolysis could not be prevented under these conditions and the resulting pink color of the plasma interfered with the analysis, particularly at low drug concentrations. Therefore, it was necessary to study the stability of hydralazine in whole blood. At room temperature, hydralazine (0.25 μg/ml) was stable in plasma at pH 7 for 20 min but appreciable losses were encountered after storage for 30 min. However, in refrigerated, heparinized human whole blood the drug was stable for at least 40 min. Based on this finding, it was concluded that it was not necessary to acidify the blood before separation of the plasma provided that the specimens were cooled to 0° immediately after withdrawal and centrifuged in a refrigerated centrifuge and the plasma samples were acidified within 40 min from withdrawal.

In acidified human urine, hydralazine was stable for 60 min at room temperature and for at least 2 weeks at −20°.

Plasma Levels of Hydralazine—In preliminary studies, hydralazine was administered to groups of healthy individuals. Plasma levels of hydralazine were measured after oral administration of 100 mg to three subjects. Absorption of the drug was

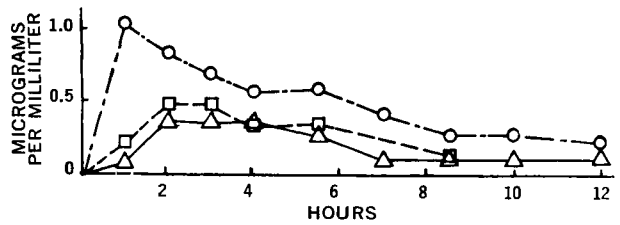

Figure 4—*Concentration of hydralazine-^{14}C and its metabolites in the plasma of Subject 4 after an oral dose of 100 mg (80 μCi). Key: ○, total radioactivity; △, unchanged hydralazine by cold method; and □, unchanged hydralazine by inverse isotope dilution analysis.*

Table II—Excretion of Hydralazine-^{14}C and Metabolites after a 100-mg Oral Dose

Time Interval, hr	Cumulative Excretion of Radioactivity, % of Dose			
	Subject 1	Subject 2	Subject 3	Subject 4
Urine:				
0–4	49.1	40.4	33.1	5.5
0–8	50.8	60.5	54.6	30.8
0–12	54.7	68.0	62.6	43.4
0–24	NA[a]	76.5	73.9	49.6
0–48	55.8[a]	78.5	79.7	50.9
0–72	56.0[a]	80.9	80.9	51.5
Feces:				
0–48	NA	NA	2.9	11.9

[a] NA = not available. The 12–24-hr urine fraction of Subject 1 was not available for analysis. Consequently, subsequent data on that individual underestimate the true magnitude of cumulative excretion.

rapid in all cases, maximum concentration being attained at 1 hr postadministration. Peak levels were 0.76, 0.95, and 1.03 μg/ml. Subsequently, a rapid decline occurred; at 24 hr, hydralazine concentrations were below the limit of detection in all subjects.

Similarly, maximum plasma concentrations of unchanged hydralazine after the administration of 75 mg were attained rapidly, ranging between 0.7 and 0.8 μg/ml. The limited amount of data of the experiments with the 100- and 75-mg doses represents a fairly good correlation between dose and the resulting peak levels. Individual half-lives could not be determined accurately in these experiments because of the fluctuating pattern of plasma levels. The appearance of secondary peaks may be related to biliary recirculation or changes in the volume of distribution of hydralazine. The available data are insufficient to permit a better understanding of these phenomena.

When hydralazine (20 mg) was administered intramuscularly to a group of three subjects, maximum concentrations of 0.16–0.61 μg/ml of the drug were attained within 1 hr. The fluctuating pattern of the plasma levels after the parenteral dose indicated that the intersubject differences and the occasional appearance of secondary peaks observed in the oral studies were probably not associated with a variability of GI absorption.

Confirmation of Specificity of Assay Method in Human Studies—An experiment was designed to assess the degree of

Table III—Urinary Excretion of Hydralazine

Dose and Route	Subject	Time Interval, hr	Cumulative Excretion, % of Dose
100 mg po	B.B.	0–6	9.0
		0–10	10.2
		0–24	10.5
	M.C.	0–6	6.6
		0–10	6.9
		0–24	7.3
	R.L.	0–6	3.7
		0–10	3.7
		0–24	3.7
75 mg po	B.B.	0–6	9.9
		0–12	10.7
		0–24	12.9
	S.T.	0–6	8.4
		0–12	8.4
		0–24	8.4
	K.S.	0–6	3.0
		0–12	3.1
		0–24	3.7
20 mg im	B.B.	0–4	8.2
		0–8	9.6
		0–12	9.6
	B.C.	0–4	5.7
		0–8	7.7
		0–12	7.7
	K.S.	0–4	10.6
		0–8	12.3
		0–12	13.8

specificity of the new method (hereafter called the "cold" method). Four individuals were each given a 100-mg oral dose of hydralazine-^{14}C (80 μCi). Figures 1–4 show the time course of plasma levels of total radioactivity and unchanged hydralazine in these subjects. In all four cases, the plasma levels of total radioactivity, representing the sum of unchanged drug and its metabolites, reached maxima within 2 hr postadministration. These peak values ranged between 1.6 and 3.2 μg/ml, expressed as the amount of radioactivity that would correspond to unchanged hydralazine. The levels declined slowly and were still measurable at 12 hr postadministration. In each instance, higher levels were found for total radioactivity than for unchanged drug, indicating that hydralazine underwent extensive metabolism.

Some levels of unchanged hydralazine were measured by the cold method. Peak plasma levels were attained in all four individuals in 1–2 hr postadministration, indicating rapid GI absorption. In three of the four subjects, these peak levels ranged between 0.9 and 1.3 μg/ml. These values are comparable to the ones found after an oral dose of 100 mg of nonradioactive hydralazine. However, in the fourth subject, peak levels reached only 0.38 μg/ml. The levels declined rapidly, and in Subjects 1 and 4 the levels were not detectable after 5.5 hr postadministration. In Subjects 2 and 3, the decline was less rapid and a half-life of 6–8 hr could be estimated, although the measured values did not fall exactly on a monoexponential curve.

After each sample had been carried through the various purification steps of the cold method and their absorbance values had been determined, the radioactivity content of the solutions was also measured. The values obtained by the assay of radioactivity were essentially superimposable on those obtained by the cold method. The close agreement shows that the material carried through the cold method and assayed as apparent hydralazine did not contain appreciable amounts of nonradioactive endogenous compounds absorbing at the wavelength employed.

Analysis of each sample by the cold method and by determination of its radioactivity content could not exclude the possibility of interference by possible metabolites of hydralazine that might have retained the ^{14}C-label and contributed to the UV readings. Therefore, as a definitive proof of the specificity of the cold method, the assay of most plasma samples was repeated by inverse isotope dilution analysis. The latter method is highly specific because it incorporates multiple TLC, thereby separating the hydrazone derivative of hydralazine from compounds that might affect the results obtained during the cold method. The results of these determinations are also shown in Figs. 1–4. The excellent agreement between the data obtained by the cold method and by inverse isotope dilution analysis demonstrates the specificity of the cold procedure.

Excretion of Hydralazine and Its Metabolites—Urinary excretion of radioactivity was measured in the four subjects administered 100 mg orally of hydralazine-^{14}C (Table II). In 72 hr, 51–81% of the dose was excreted, with most excretion taking place during the first 24 hr. Feces collected over 48 hr in two of the four subjects contained 2.9 and 11.9% of the dose, respectively. The findings of the urinary and fecal excretion pattern were confirmed recently[7].

At variance with the recent observations of Lesser *et al.* (7), unchanged hydralazine comprised only a small fraction of the urinary radioactivity, ranging from 3 to 14% of the dose. Similar results were obtained in the studies using nonradioactive hydralazine, the urinary excretion of unchanged drug ranging from 3.7 to 13.8% of the dose (Table III).

DISCUSSION

The biological disposition of the antihypertensive drug hydralazine in man has not been the subject of detailed examination. Previous studies had been confined to hypertensive patients and mostly involved chronic administration of high doses. The primary objective of the present work was to obtain information on the biological disposition of hydralazine in healthy subjects after administration of a single therapeutic dose.

The analytical methodology developed in this laboratory has sufficient sensitivity for the determination of plasma levels of hydralazine after single oral therapeutic doses. Furthermore, it was demonstrated with the aid of inverse isotope dilution analysis that the method is specific for the assay of unchanged hydralazine in the presence of its metabolites.

The rate of decline of hydralazine plasma levels showed a marked intersubject variation. Fluctuating plasma levels and the occasional appearance of secondary peaks made the exact determination of the half-lives difficult. Nevertheless, in certain individuals the half-life was estimated to be in the 2–4-hr range whereas in others it approximated 6–8 hr. Pharmacogenetic factors may be partly responsible for the observed differences. Acetylation, a pathway subject to genetic control (8), has been known to constitute one *in vivo* metabolic transformation of hydralazine (1). Statistically significant differences in the average 2-hr plasma levels between genetically fast and slow acetylator individuals have been reported (5). Unfortunately, the latter study was limited to the 2-hr plasma levels, and the data do not lend themselves to conclusions regarding the time course of hydralazine in plasma. To the extent that acetylation represents a major metabolic pathway of hydralazine in man, genetic differences could conceivably affect the half-life of the drug. However, according to recent findings in these laboratories[8] and by Reidenberg *et al.* (9), the half-life of hydralazine does not necessarily correlate with the acetylator phenotype.

The data obtained in the present investigation describe the biological disposition of hydralazine in 10 healthy subjects. The results indicate rapid absorption and elimination; the latter process occurs primarily by metabolic transformation[8] of the drug and excretion of the metabolites mainly in the urine.

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[x] To whom inquiries should be directed.

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Comparison between Theoretical and Experimental Electric Dipole Moments of Selected *N,N*-Dimethylaniline Derivatives

JOHN M. CLAYTON*§, GEORGE E. BASS*, WILLIAM P. PURCELL*x, and CLIFTON C. THOMPSON*‡

Abstract □ Electric dipole moments of nine *N,N*-dimethylaniline derivatives were determined experimentally in benzene solution according to literature methods. The theoretical dipole moments of these compounds were calculated from molecular orbital approximations according to the Del Re σ-electron treatment, the Hückel π-electron method, the Pariser–Parr–Pople treatment, the complete neglect of differential overlap method, and selected combinations of these methods. Parameters not previously reported were determined for the nitro group by reproduction of experimental dipole moments for use in the Del Re calculations. Comparisons between the experimental and theoretical moments indicate that the combination of the Del Re σ-electron and Hückel π-electron methods gave the best reproduction of the experimental dipole moment values while being considerably less expensive in terms of computer size and time, as well as requiring less time in preparing the data than more sophisticated methods examined.

Keyphrases □ *N,N*-Dimethylaniline derivatives—comparison between theoretical and experimental electric dipole moments □ Electric dipole moments, *N,N*-dimethylaniline derivatives—comparison between theoretical and experimental values □ Dipole moments, electric, *N,N*-dimethylaniline derivatives—comparison between theoretical and experimental values □ Electronic parameters—comparison between theoretical and experimental electric dipole moments for *N,N*-dimethylaniline derivatives □ Quantum mechanics—comparison between theoretical and experimental electric dipole moments for *N,N*-dimethylaniline derivatives

In view of the current interest in correlations between selected physicochemical properties and biological activities of diverse series of compounds (1–5), several investigators have also become interested in the potential applications of quantum mechanical indexes in such studies (6–12). Due to the variety of available methods and levels of sophistication of quantum mechanical calculations, interest was generated in determining the method of choice for the calculation of selected electronic parameters for a series of compounds for subsequent use in structure–activity studies. The basis for selection of a method of choice for this study consisted of the following three criteria: *(a)* the accuracy of the method; *(b)* the labor involved in the calculation, including the availability of the parameters required for each method and the relative ease of parameter determination as well as the complexity of setting up the data required for each method; and *(c)* the cost per calculation for each compound relative to the accuracy, which is primarily dependent, of course, upon the computer size and the computer time required for each calculation.

As Pullman suggested (13), the comparison of charges *per se* is: "a very complicated problem if only because their theoretical definitions are not equivalent in the different procedures." Also, the charges are not directly measurable quantities. Since the interest of this study is primarily in parameters based on the distribution of electronic charges, the basis selected for relative comparison of the methods was the closely related measurement more readily accessible to a direct experimental check, the molecular electric dipole moments. Pullman (13) carried out a somewhat similar study for the nucleic acid bases. There is, however, a great difference in the nature of the compounds studied, particularly since the compounds of interest in this study contain both electron-withdrawing and electron-releasing substituent groups.

The theoretical methods selected for comparison were: *(a)* the Del Re σ-charge calculations, *(b)* the Hückel π-electron molecular orbital approximations, *(c)* a summation of the Del Re σ- and Hückel π-electron calculations, *(d)* the Pariser–Parr–Pople self-consistent-field π-electron calculations, *(e)* a combination of the Del Re σ- and Pariser–Parr–Pople π-electron methods, and *(f)* the complete neglect of differential overlap all-electron treatment (CNDO/II) method.

The compounds selected for this comparative study were the *N,N*-dimethylaniline series shown in Table I. These compounds present simple, conjugated π-electron systems which allow for a relatively easy and complete π-electron theoretical calculation. Since this series also consists of electron-releasing and electron-withdrawing substituent groups, the

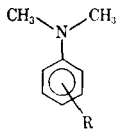

Table I—Experimental Electric Dipole Moments of Selected *N,N*-Dimethylanilines[a]

Compound	R	μ_S[b]	μ_G[c]	Experimental Error
I	H	1.59	1.59	±0.02
II	*m*-CH_3	1.51	1.52	±0.003
III	*p*-CH_3	1.12	1.13	±0.02
IV	*m*-OCH_3	2.18	2.18	±0.02
V	*p*-OCH_3	1.77	1.78	±0.04
VI	*m*-NO_2	5.27	5.30	±0.02
VII	*p*-NO_2	7.09	7.13	±0.03
VIII	*m*-Cl	3.19	3.19	±0.02
IX	*p*-Cl	3.32	3.32	±0.03

[a] In benzene solution at 25 ± 0.01°. [b] Dipole moment calculated by the method of Smith (14). [c] Dipole moment calculated by the method of Guggenheim (15).

study encompasses a fairly wide range of electronic properties.

For comparison with theoretical values, experimental electric dipole moment values for the subject compounds were determined (Table I). The range in electronic properties over the series is exemplified by the range in dipole moment values of 1.12–7.10 Debyes.

EXPERIMENTAL

Measurement of Electric Dipole Moments—The dielectric constants of the series of *N,N*-dimethylaniline compounds in Table I were measured in benzene solution by the heterodyne beat method (16, 17). The compounds were very carefully purified and dried *in vacuo* at 25° over phosphorus pentoxide for 48 hr. Criteria for purity included constant melting points on three successive recrystallizations, single peak on the gas chromatograph, and constant refractive indexes on successive distillations. Seven solutions of approximately 50 ml each and ranging from approximately 0.007 to 0.10% (w/w) in spectroscopic grade benzene[1] were prepared from each compound. Weighings were made to 10^{-5} g on a semimicro balance[2]. Flasks containing the solutions were kept in a desiccator over anhydrous calcium sulfate[3] until used. All solutions were measured within 2 hr after preparation.

Dielectric constants of the solutions were measured at 25.00 ± 0.01° on a dipolemeter[4] with the cell thermostated with a circulator[5]. The 20-ml jacketed, gold-plated (DFL 1) cell was calibrated with benzene, carbon tetrachloride, and cyclohexane at 20° using dielectric constant values (18) of 2.2832, 2.2368, and 2.0230, respectively. Refractive indexes were measured at 25.00 ± 0.01° using the D line of sodium on a precision laboratory refractometer[6] thermostated with a circulator[5]. All temperatures were measured with thermometers calibrated against a National Bureau of Standards certified thermometer.

From the indexes of refraction, dielectric constants, and weight fractions of the seven solutions and the solvent, the electric dipole moment (μ) of each compound was calculated according to the methods of Smith (14) (μ_S) and Guggenheim (15) (μ_G).

Theoretical Electric Dipole Moments—Net atomic charges were determined from the electron charge densities of the atoms calculated by the various methods according to Eq. 1:

$$Q_r = N - q_r \qquad \text{(Eq. 1)}$$

where Q_r is the net charge on atom r, N is the number of electrons contained in the valence orbital of the atom prior to its bonding with other atoms, and q_r is the charge density at atom r. The summations of the products of the value of Q at each atom and the approximate component of its molecular geometry (d_x, d_y, and d_z) multiplied by a conversion constant (4.77) to obtain Debye units yield the three-dimensional components (μ_x, μ_y, and μ_z) of the dipole moment (Eqs. 2–4):

$$\mu_x = 4.77\Sigma(Q_r d_x) \qquad \text{(Eq. 2)}$$

$$\mu_y = 4.77\Sigma(Q_r d_y) \qquad \text{(Eq. 3)}$$

$$\mu_z = 4.77\Sigma(Q_r d_z) \qquad \text{(Eq. 4)}$$

The resultant theoretical moment for each compound was then calculated according to Eq. 5:

$$\mu = \sqrt{\mu_x^2 + \mu_y^2 + \mu_z^2} \qquad \text{(Eq. 5)}$$

RESULTS AND DISCUSSION

Initial calculations were made on the compounds in the series using the simplest of methods, the Del Re (19) σ-electron treatment. By this method, only the σ-electrons are treated. The parameters used in the calculations were those of Del Re (19) and Berthod and Pullman (20) with the exception of those for the nitro group.

Table II—Del Re Parameterization of the Nitro Group

Compound	Experimental Dipole Moment, Debyes[a]	Calculated Dipole Moment, Debyes[b]	$\Delta\mu$[c]
Nitrobenzene	4.25	4.16	−0.09
m-Nitroaniline	4.90	4.99	+0.09
p-Nitroaniline	5.60	5.60	0.00

[a] Taken from McClellan (21). [b] Calculated from Hückel molecular orbital π-electron charge densities using reported parameters (20, 23) and from Del Re σ-electron charge densities using reported parameters (19, 20). [c] $\Delta\mu$ = experimental dipole moment − calculated dipole moment.

Since the bond parameters (γ_p and ϵ_{pq}) necessary for treatment of the nitro group (C—N and N—O bonds) were not available in the literature, parameterization of this group was necessary. Since these parameters are based on their ability to reproduce experimental electric dipole moment values, closely related molecules (namely, nitrobenzene, *m*-nitroaniline, and *p*-nitroaniline) whose dipole moments were reported previously (Table II) (21), were selected for use in the parameterization of this group. These molecules were selected because of their simplicity and similarity to the molecules to be studied with the derived parameters.

Since γ_p is an empirical measure of the ability of atom p to polarize atom q and should not change with the change in bond type, the value of γ_p (0.10) was selected to be the same as that for other C—N and C—O bonds. Therefore, the values of ϵ_{pq} were varied to obtain a consistent theoretical reproduction of the experimental dipole moments of all three molecules. Parameters used in these calculations were those reported by Berthod and Pullman (20) and Berthod *et al.* (23) for the Hückel π-electron molecular orbital calculations and by Del Re (19) and Berthod and Pullman (20) for the Del Re calculations. Theoretical dipole moments were calculated as already described[7].

These calculations resulted in the evaluation of ϵ_{C-N} and ϵ_{N-O} to be 0.50 and 1.00, respectively. As shown in Table II, all calculated dipole moments are in excellent agreement with the experimental values.

The σ-electron dipole moments (μ_σ) for the series of dimethylanilines were then calculated from the atomic net charges obtained through these calculations (Table III). As should be anticipated, the overall agreement between the observed (μ_{obs}) and calculated moments is generally poor. The deviation between these values ($\Delta\%\mu_\sigma$) varies from a low value of 21% for the *p*-methoxy compound to a high value of 98% for the *p*-methyl derivative. The average deviation for the series is 64%.

The second level of calculation was the Hückel π-electron molecular orbital treatment (HMO) (22). According to this method, only the π-electrons are treated. The parameters used in these calculations[8] were those determined by Berthod and Pullman (20) and Berthod *et al.* (23). These values were selected in order to use parameters consistent with those used in the Del Re study.

Again, the π-electron electric dipole moments ($\mu_{\pi(HMO)}$) were calculated from the atomic net charges (Table III). The overall deviations between the calculated and observed dipole moments are somewhat lower from Hückel molecular orbital π-electron calculations than from the σ-electron calculations. This indicates a greater contribution of the π-electrons to the overall dipole moment than that of the σ-electrons. The deviations ($\Delta\%\mu_{\pi(HMO)}$) range from a low of 1% for the *p*-methyl derivative to a high of 91% for the *p*-methoxy derivative. The average deviation for this method is 46%.

A third treatment of the data was the calculation of the dipole moments from a summation of the Del Re σ- and the Hückel π-

[1] Matheson, Coleman and Bell.
[2] Sartorius Selectra.
[3] Drierite.
[4] Wissenschaftlich-Technische Werkstatten DM01.
[5] Haake NBe.
[6] Bausch & Lomb Inc.

[7] All calculations were made on the IBM 1620[11] computer using programs written by Dr. G. E. Bass and Dr. K. Sundaram and the All-India Institute of Medical Sciences.
[8] Calculations were performed on the IBM 1620[11] computer using a program written by Dr. K. Sundaram and modified by Dr. G. E. Bass.

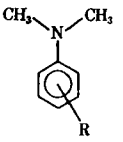

Table III—Theoretical Electric Dipole Moments of *N,N*-Dimethylanilines Calculated from the Del Re σ-Electron and Hückel Molecular Orbital π-Electron Charges and Comparison with Observed Values

R	μ_{obs}[a]	μ_σ	$\Delta\%\mu_\sigma$[b]	$\mu_{\pi(HMO)}$	$\Delta\%\mu_{\pi(HMO)}$[b]	$\mu_{\sigma+\pi(HMO)}$	$\Delta\%\mu_{\sigma+\pi(HMO)}$[b]
H	1.59	0.06	96	1.13	29	1.19	25
m-CH_3	1.51	0.08	95	1.13	25	1.15	24
p-CH_3	1.12	0.02	98	1.13	1	1.11	1
m-OCH_3	2.18	1.43	34	1.16	47	2.12	3
p-OCH_3	1.77	1.40	21	0.16	91	1.31	26
m-NO_2	5.28	1.41	73	3.44	35	4.82	9
p-NO_2	7.10	1.45	80	3.96	44	5.40	24
m-Cl	3.19	1.90	40	0.99	69	2.35	26
p-Cl	3.32	1.93	42	0.79	76	2.72	18

[a] Observed dipole moments were measured in benzene solution at 25 ± 0.01°. [b] $\Delta\%\mu = (\mu_{obs} - \mu_{calc})/\mu_{obs} \times 100$.

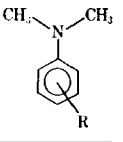

Table IV—Theoretical Electric Dipole Moments of *N,N*-Dimethylanilines Calculated from Del Re σ-Electron and Pariser–Parr–Pople π-Electron Charges and Comparison with Observed Values

R	μ_{obs}[a]	μ_σ	$\mu_{\pi(PPP)}$	$\Delta\%\mu_{(PPP)}$[b]	$\mu_{\sigma+\pi(PPP)}$	$\Delta\%\mu_{\sigma+\pi(PPP)}$[c]
H	1.59	0.06	0.79	50	0.85	46
m-CH_3	1.51	0.08	0.79	48	0.81	46
p-CH_3	1.12	0.02	0.79	29	0.77	31
m-OCH_3	2.18	1.43	0.68	69	2.01	8
p-OCH_3	1.77	1.40	0.33	81	1.62	8
m-Cl	3.19	1.90	0.79	75	2.41	24
p-Cl	3.32	1.93	0.79	76	2.72	18

[a] Observed moments were measured in benzene solution at 25 ± 0.01°. [b] $\Delta\%\mu = (\mu_{obs} - \mu_{calc})/\mu_{obs} \times 100$.

electron net charges ($\mu_{\sigma+\pi(HMO)}$). The results of this treatment are also shown in Table III. By this method, the calculated dipole moments are in very good agreement with the observed values; the range of the deviations ($\Delta\%\mu_{\sigma+\pi(HMO)}$) goes from 1% for the *p*-methyl derivative to 26% for the *m*-chloro compound. The overall average deviation for the series is only 17%.

The next level of treatment used was the Pariser–Parr–Pople self-consistent-field (PPP-SCF) π-electron method (24–27). Only the π-electrons are treated in this method. The parameters used in the calculations[9] were developed by Nagata *et al.* (28). The geometry of each molecule was derived from the bond distances and bond angles reported by Pople and Gordon (29). Two-center Coulomb integrals were calculated by the Mataga–Nishimito approximation (30).

Similar to the other treatments, dipole moments ($\mu_{\pi(PPP)}$) were calculated from the atomic net charges (Table IV). Missing in this treatment are the nitro derivatives of the series which were deleted due to a convergence problem in the calculations. By this method, the deviations between the observed and calculated values ($\Delta\%\mu_{\pi(PPP)}$) range from 29% for the *p*-methyl compound to 81% for the *p*-methoxy congener. The average deviation for the series was 61%, indicating poor agreement for the moments calculated by this method and considerably poorer agreement than that obtained for the Hückel molecular orbital π-electron moments.

Similar to the Hückel treatment, the Del Re σ-electron charges were summed with the Pariser–Parr–Pople π-electron charges, and the dipole moments ($\mu_{\sigma+\pi(PPP)}$) were recalculated from these values. Very good agreement (Table IV) between the observed and calculated values was obtained. The deviations ($\Delta\%\mu_{\sigma+\pi(PPP)}$) range from a low of 8% for the *m*-methoxy and *p*-methoxy compounds to a high of 46% for the unsubstituted and *m*-methyl derivatives. The overall average deviation for the series by this method is 26%, indicating relatively good agreement between the calculated and observed moments.

The highest level of sophistication used in this study was the complete neglect of differential overlap treatment (CNDO/II) of Pople and Segal (32, 33). This treatment is essentially an extension of the Pariser–Parr–Pople self-consistent-field formalism to include all valence electrons (π and σ). The approximations and assumptions used in this method are for the most part those used in the solutions by the Pariser–Parr–Pople method, with the exception that the molecules need not be planar for the complete neglect of differential overlap treatment. Although they are used in a slightly different manner for inclusion in the integrals of the Roothaan equations, the parameters necessary for solution by this method are those used in the Pariser–Parr–Pople formalism. The parameters used in these calculations[10] were those reported by Nagata *et al.* (28). The three-dimensional geometries of the molecules were calculated using the bond distances and bond angles reported by Pople and Gordon (29).

The chloro derivatives were not included in this treatment since the available facilities are not capable of handling second-row elements. From these calculations, the electric dipole moments were calculated in two ways. The first method is similar to the other calculations in that the atomic net charges were used to determine the theoretical moments ($\mu_{CNDO/II(ac)}$) (Table V). By this method, the overall agreement between the calculated and observed values appears to be very good with the largest difference ($\Delta\%\mu_{(ac)}$) being 55% for the *m*-methyl derivative and the smallest difference being 4% for the *m*-nitro congener. The average percentage deviation for the series is 37.

The second method is denoted as the "complete dipole." By this method, the dipole moment calculated from the atomic charges is corrected for the atomic polarization resulting from the 2*s* and 2*p* orbital mixing (33). This method is considered to be one of the most accurate of the crude theoretical dipole moment calculations. By this method the calculated moments ($\mu_{CNDO/II(cd)}$) are in excellent agreement with the observed values (Table V). The largest deviation ($\Delta\%\mu_{(cd)}$) between the two is

[9] Calculations were carried out on the IBM 360/40 computer using a program written by Dr. T. Janiszewski (31) and modified by Dr. G. E. Bass.

[10] Calculations were carried out on the Sigma 9 computer using a program written by Dr. G. A. Segal (34).

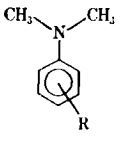

Table V—Theoretical Electric Dipole Moments of *N,N*-Dimethylanilines Calculated from the Complete Neglect of Differential Overlap Treatment and Comparison with Observed Values

R	μ_{obs}[a]	$\mu_{CNDO/II(ac)}$[b]	$\Delta\%\mu_{(ac)}$[c]	$\mu_{CNDO/II(cd)}$[d]	$\Delta\%\mu_{(cd)}$[c]
H	1.59	0.80	50	0.88	45
m-CH_3	1.51	0.68	55	0.72	52
p-CH_3	1.12	0.68	39	0.76	32
m-OCH_3	2.18	1.25	43	2.39	10
p-OCH_3	1.77	0.89	50	1.93	9
m-NO_2	5.28	5.04	4	5.90	12
p-NO_2	7.10	5.92	17	6.81	4

[a] Observed dipole moments were measured in benzene solution at 25 ± 0.01°. [b] (ac) indicates that the dipole moment was calculated from the atomic charges. [c] $\Delta\%\mu = (\mu_{obs} - \mu_{calc})/\mu_{obs} \times 100$. [d] (cd) indicates that the dipole moment is the calculated "complete dipole."

52% for the *m*-methyl compound, and the smallest deviation is 4% for the *p*-nitro derivative. The small overall average deviation (23%) by these calculations indicates that this method gave the second best agreement of those used in this comparative study.

A summary of the results of this comparative study is presented in Table VI. Listed beside the method used in the dipole moment calculations is the average percentage deviation between the theoretical and observed electric dipole moments. On this basis of comparison, the highest level of sophistication of the calculations (complete neglect of differential overlap) gave only the second best agreement between the values (second lowest deviation), while a combination of the much less sophisticated treatments (Del Re plus Hückel molecular orbital) led to a slightly better agreement (average deviation of 17 *versus* 23%). As a result of this study, it is concluded that the less sophisticated methods gave the best "accuracy" (correlation with experimental electric dipole moments) in the treatment of this series of compounds while being considerably less expensive in terms of computer size and time as well as in the time required to prepare the data.

Table VI—Average Deviations between Theoretical and Observed Electric Dipole Moments of Selected *N,N*-Dimethylanilines

Method of Calculation	Average Deviation[a], %
Del Re σ-electron net charges	64
Hückel molecular orbital π-electron net charges	46
Pariser–Parr–Pople π-electron net charges	61
Summation of Del Re σ- and Hückel molecular orbital π-electron net charges	17
Summation of Del Re σ- and Pariser–Parr–Pople π-electron net charges	26
Complete neglect of differential overlap atomic net charges	37
Complete neglect of differential overlap complete dipole	23

[a] Average of $(\mu_{obs} - \mu_{calc})/\mu_{obs} \times 100$ for the series.

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§ Present address: Food and Drug Administration, National Center for Toxicological Research, Jefferson, AR 72079

[x] To whom inquiries should be directed.

Kinetic Approach to Drug–Protein Binding

J. S. ROBERTSON[x] and B. W. MADSEN

Abstract □ Since estimates in the literature for the fundamental binding parameters of dicumarol to bovine serum albumin show wide disparity, these phenomena were reinvestigated, using a nonequilibrium partitioning technique. A three-phase system was used consisting of an aqueous drug–protein phase and a separate aqueous receiving phase, both in mutual contact with a third overlying immiscible organic phase. The phases were contained in a partitioned, temperature-controlled cylindrical glass cell which could be rotated along its horizontal axis to effect gentle yet thorough mixing and drug transfer. Free (unbound) drug diffused from the protein phase sequentially into the other phases where it was analyzed. This technique allowed estimates of free and bound drug to be made over a very wide range of drug–protein ratios, using a single drug–protein solution, in a short time. Data analysis using an iterative, nonlinear, least-squares computer program indicated that there were two classes of binding sites with the following characteristics; $n_1 = 2.3 \pm 0.15$, $K_1 = (1.8 \pm 0.23) \times 10^5$ M^{-1}; $n_2 = 14.0 \pm 0.71$, $K_2 = (3.0 \pm 0.36) \times 10^3$ M^{-1}. A computer-solved theoretical treatment of drug transfer within the cell showed good correlation with the experimental results. The rotating cell technique possesses a flexibility in adjustment of operational parameters which should suit it to the study of the binding characteristics of various compounds.

Keyphrases □ Dicumarol—reinvestigation of binding to bovine serum albumin, nonequilibrium partitioning technique, three-phase system □ Bovine serum albumin binding of dicumarol—reinvestigated using nonequilibrium partitioning technique, three-phase system □ Drug–protein binding—dicumarol and bovine serum albumin, kinetics determined using nonequilibrium partitioning technique □ Binding of dicumarol to bovine serum albumin—kinetics determined using nonequilibrium partitioning technique, three-phase system

Methods used to study drug–protein interactions can be grouped under four headings: subtractive, direct, electrostatic, and other (1). All methods have their advantages and disadvantages, and the selection of a particular approach depends mainly on the system under study and the aims of the experiment.

The major drawback of direct methods, where both free and bound drug concentrations are determined in the presence of protein, is that the protein can create assay problems. Therefore, most reports are based on the subtractive approach, in which free drug is separated from the protein prior to assay; by subtraction of free drug from the total quantity of drug added to the system, bound drug is obtained. Such methods include equilibrium dialysis, equilibrium partitioning, ultrafiltration, ultracentrifugation, and gel filtration. Of these, equilibrium dialysis is the most frequently used.

Equilibrium methods can involve considerable labor and cost of chemicals since they require the preparation of many drug–protein samples to cover an adequate range of binding ratios—each sample provides but one data point. There is also a time delay inherent in this approach, which may allow protein denaturation or microbial growth. Recently, there has been a growing interest in kinetic methods to circumvent some of these limitations.

Stein (2) introduced a dynamic dialysis method based on the use of an automated analyzer which reduced the experimental time from hours to minutes. He reported that the binding data of methyl orange to bovine serum albumin obtained by this technique were similar to those obtained (3) using equilibrium dialysis. While this work pioneered the kinetic approach, only one binding system was investigated over a very limited range. Although the technique eliminated prolonged waiting time, individual drug–protein solutions covering all the binding ratios still had to be prepared.

A flow dialysis technique was reported (4–6) which both reduced experimental time and enabled a number of data points to be obtained from a single drug–protein solution. The binding of a number of drugs was studied, and membrane effects were a disturbing factor.

Meyer and Guttman (7) reported preliminary work on a nonequilibrium dialytic method. They later published details of their studies on the various factors influencing the method, and they investigated the binding behavior of a number of compounds including phenol red, methyl orange, warfarin, caffeine, 8-nitrotheophylline, 8-chlorotheophylline, and salicylic acid (8, 9). In their technique, the drug and protein were placed together inside a membrane sac supported in drug-free buffer; unbound drug diffused into the buffer solution at a rate proportional to its concentration in the sac. The concentration of drug in the external buffer phase was kept at a low level by sampling at selected time intervals and replacement with fresh buffer. This method allowed rapid, easy generation of a large series of free and bound

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drug concentrations, and it showed good correlation with literature reports using more standard techniques.

Farrell *et al.* (10) introduced a dynamic dialysis technique, with a mode of operation analogous to that of a hemodialysis unit. They claimed that their approach possessed the advantages (among others) of rapid evaluation of binding characteristics and ease of temperature adjustment to measure enthalpy and entropy effects. The method represents a considerable refinement over the initial approach of Stein (2), and their work included many more binding systems.

Blatt *et al.* (11) described a continuous diafiltration technique which was a blending of the better features inherent in the more orthodox methods of dialysis and ultrafiltration. Drug transfer across a semipermeable membrane was hastened by application of pressure, and the drug–protein solution volume was maintained constant with a reservoir assembly. This method was extended by sequentially assaying the eluate until equilibrium was reached (12).

The work described in this report is a further application of a nonequilibrium method—*viz.*, kinetic partitioning, to investigate a drug whose binding properties have proven troublesome to characterize by the more standard techniques.

EXPERIMENTAL

Chemicals—Dicumarol[1] (I) recrystallized from cyclohexanone gave an uncorrected melting point of 286–289° (13). TLC, using silica gel H[2] with the solvent system acetone–ethyl acetate–petroleum ether (b.p. 60–80°)–water (100:100:33:3.5 v/v), showed a single spot of R_f 0.62 when viewed under UV light (of wavelength 254 nm) or when sprayed with phosphomolybdic acid reagent (14).

Bovine serum albumin[3] (II) was used as received. The molecular weight of II was assumed to be 69,000. 2-Octanol was redistilled before use (178–180° fraction collected). All other chemicals were of laboratory reagent grade.

UV Spectrophotometry—UV determinations were carried out using a spectrophotometer[4]. The organic phase absorbance was measured at 324 nm in a 4-cm cell, and the alkaline phase absorbance was measured at 315 nm in a 1-cm cell. Frequently, the spectrum of an alkaline phase sample was scanned from 200 to 450 nm as a precaution against false absorption.

From an increasing absorptivity and change in the spectrum, it appeared that some form of association of I was taking place in the organic phase; it became significant at concentrations greater than 20 μg/ml, and care was taken that this concentration was not exceeded during a kinetic run and assay. When background absorption of either phase was troublesome, a corrected absorbance ($A_{corr} = A_\lambda - A_{360}$) was used, where A_λ was the assay wavelength for the particular phase (15, 16).

Solutions of I and II were assayed spectrophotometrically for total I. Solutions were prepared with known amounts of I ranging from 0.595 to 8.33 × 10^{-5} *M* and a constant amount of II (2.38 × 10^{-5} *M*). A standard curve was then prepared by plotting the absorbances at 325 nm. While the presence of II affected the absorption of I, a Beer's plot was approximately linear in the 0–2 molar ratio (I/II) range. By diluting the sample from the rotating cell to a concentration of 2.38 × 10^{-5} *M* for II, the concentration of I was readily found by linear interpolation.

The interaction between I and II was further investigated by difference spectroscopy (17). Changes in UV absorption at various molar ratios of I (0.595–8.33 × 10^{-5} *M*) and II (2.38 × 10^{-5} *M*) were examined at 315 and 340 nm.

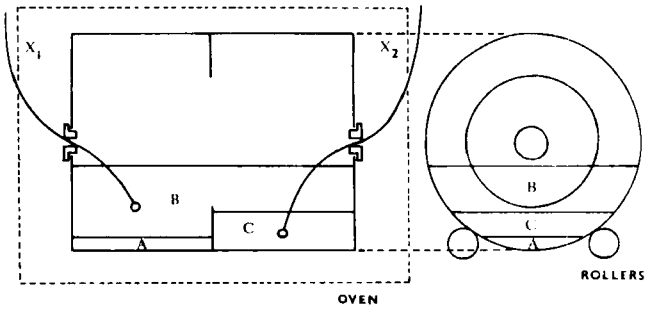


Figure 1—*Diagram of the rotating cell. Key:* A, *aqueous drug–protein phase;* B, *organic solvent phase;* C, *aqueous alkaline phase; and* X_1, X_2, *weighted nylon sampling tubes inserted into the cell* via *small holes in rubber stoppers. A motor-driven roller rotated the cell at 40 rpm; the thermostated oven temperature was maintained by an electric heating element and fan at each end.*

Computing—An analog computer[5] coupled to a recorder[6] was used to estimate rate constants. All extensive numerical calculations were performed on an automatic computer[7], using USASI FORTRAN IV[8] as the programming language.

Rotating-Cell Methods—A diagram of the rotating cell is shown in Fig. 1. The apparatus was previously described (18) except that an all-glass cell was used in the present investigation and the whole assembly was enclosed in an oven maintained at 37 ± 0.6°. All experiments using the cell were conducted at this temperature. The cell was designed with a central partition keeping separate the two aqueous phases, with the only connection between the two being *via* the overlying organic phase. The drug was initially placed in *A*, and the time course of transfer was followed by assaying phases *B* and *C*; the drug concentration in *A* was obtained by subtraction.

Phase *A* contained 0.05 *M* tromethamine adjusted to pH 7.4 (at 37°) and sufficient sodium chloride for an ionic strength of 0.15 to simulate physiological conditions. Phase *B* consisted of a mixture of cyclohexane–2-octanol (1:1 v/v). This ratio was based on the necessity for *B* to be immiscible, to be not too volatile, to be cheap, to present minimal problems in assay, and to ensure sufficient polarity for rapid transfer of I. Phase *C* was always 0.1 *N* NaOH, giving an alkaline to organic distribution ratio of 365:1. During an experiment, sample removal prevented any large accumulation of I in alkali, so that back-transfer from phase *C* to *B* could be considered negligible. The volumes of phases *A*, *B*, and *C* were 25, 360, and 125 ml, respectively. The choice of volumes for phases *A* and *B* was dictated by the wish to obtain a high *B* to *A* phase volume ratio. This ratio determined the net rate of transfer of I from phase *A* to *B* and thus the experimental time. The one all-glass rotating cell was used in all work to reduce variability from this source.

Preparation of Phases—Two liters of 0.1 *N* NaOH was shaken with 100 ml of organic solvent, sealed, and allowed to equilibrate in the oven (at 37°) overnight prior to a run.

Similarly with the buffer, 50 ml was shaken in a separating flask with 10 ml of organic solvent and allowed to equilibrate overnight. Presaturation of contiguous phases had an important bearing on the interphase rate constants. In addition, presaturation of the alkali phase was important from an assay viewpoint. 2-Octanol absorbs at λ = 280 nm and so interfered with the assay for I (at 315 nm). When alkali was presaturated with organic phase, the 2-octanol absorbance amounted to approximately 0.04 at 315 nm in 1-cm cells. By presaturating phases and using a blank, a correction was unnecessary.

Steinhardt and Reynolds (1) previously commented that the presence or absence of protein at the membrane interface in equi-

[1] Sigma Chemical Co., St. Louis, Mo.
[2] E. Merck, A-G, Darmstadt, Germany.
[3] Fraction V, lot 110C-0300, Sigma Chemical Co.
[4] Unicam SP800, Unicam Instruments Ltd., Cambridge, England.
[5] Solartron Electronic Group Ltd., Surrey, England.
[6] Model 2D2 X-Y, F. L. Mosley Co., Pasadena, Calif.
[7] 32K IBM 7040.
[8] A listing of the programs is available on request.

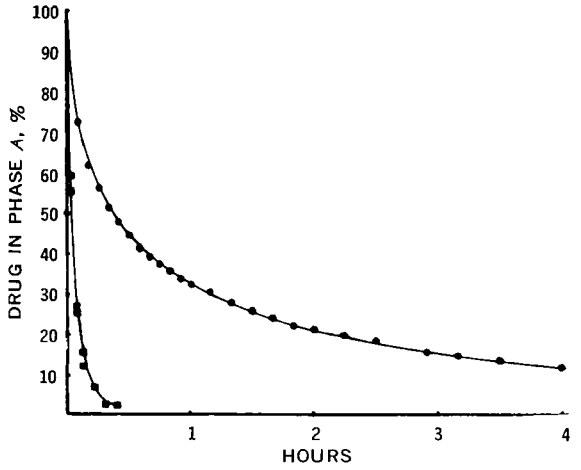


Figure 2—*Loss of dicumarol from the aqueous buffer phase with* (●) *and without* (■) *bovine serum albumin.*

librium dialysis could affect the transfer rate of free drug. This type of phenomenon was observed for the transfer of I between phases in the rotating cell. Preequilibration of phase *B* with 25 ml of protein solution depressed the two forward rate constants slightly, and this effect seemed to be independent of II concentration from 0.1 to 4% (w/v). Therefore, in cases where no protein was added to the aqueous phase, *e.g.*, for rate constant determination, the organic layer was shaken with the corresponding protein solution.

This organic layer was periodically checked to determine if extraction of any UV-absorbing substances (which might interfere with the assay) had occurred from the protein solutions. When the aqueous phase was at pH 7.4, no such substances were detected.

Inadequate cleaning of the cell reduced the rate of loss of drug from phase *A*, possibly due to an adsorption phenomenon. When the cell and all glassware were cleaned with chromic acid, this was not a problem. Prior to an experiment, adequate stock solutions of each phase were placed in the oven and time was allowed for temperature equilibration.

Preparation of the Cell—A volume of 125 ml of preequilibrated alkali was added to the right side of the cell; then 340 ml of organic solvent was gently added from the left side. The cell was plugged to prevent evaporation of any solvent, carefully placed on the rollers in the oven to avoid spillage, and rotated for 1 hr. The rotation of the glass cell was periodically checked and found to be constant at 40 rpm. Twenty-five milliliters of phase *C* was removed and replaced with fresh solvent. This sample served as a blank. The cell was then rotated for another hour to ensure complete temperature equilibration of the cell and contents.

Compound I, in separate amounts of 2.1, 6.07, 11.24, and 22.0 mg, was weighed directly into a standard 25-ml flask to prevent loss. The dissolution of crystalline I at physiological pH was very time consuming and it was better first to add a few drops of 1 *N* NaOH for this step. To avoid any slight pH change in the buffer, the appropriate quantity of 0.2 *M* HCl was added later. If protein was to be present, an amount sufficient to produce 1% (w/v) was inserted and the contents were made up to volume with buffer. The pH of the final solution was checked frequently at this stage and was 7.8 at room temperature (20°). The buffer capacity was adequate to maintain constancy with up to 1% (w/v) protein. This solution was then given a minimum of 1 hr to heat to 37°.

The two nylon sampling tubes connected to the exterior of the oven were then inserted in the cell *via* the narrow holes in the stoppers. Subsequently, all additions and withdrawals were made *via* these tubes to minimize heat loss. Absorption of drug and protein by the nylon tubes was tested for and found to be unimportant under the prevailing conditions of agitation.

Commencement of Transfer—When ready, the rotation was stopped, drug solution was pipeted in, and the standard flask and pipet were washed with a final 20 ml of organic solvent required to make up the 360-ml organic phase. Then the rotor was switched on and timing was begun. This injection phase was dead time and was done as quickly as possible, commonly taking about 1 min.

If phase *A* contained I alone, typical sampling times were 2, 5, 9, 14, 20, and 25 min; if protein was present, usually 30–40 samples were taken at frequent intervals for up to 6 hr. The sequence followed was removal first of organic phase and then of alkali and then replacement in the reverse order, usually taking about 2 min. The sample volumes were 10 ml for phase *B* and 25 ml for phase *C*. The interval between replacement of fresh solvent and the withdrawal of the next sample, if greater than 1–2 min, was adequate to ensure homogeneity of the phases.

At the end of a run, the total content of the cell (510 ml) was measured to guard against cumulative error in sampling and replacement and the possibility that a sample had not been replaced. The pH of the drug–protein phase was checked to ascertain whether accidental spillage of alkali into the buffer phase could have occurred. This solution was then assayed spectrophotometrically for total I.

The possibility of denaturation of the protein having occurred due to intimate contact with the organic phase was examined by testing for changes in the UV spectrum and in viscosity (1), using a viscometer[9] (19). No changes were observed.

In a further experiment, a solution containing I (10.7 mg) and II was incubated at 37° for 11 hr prior to a transfer experiment.

The pKa_2 of I was estimated by a solubility method (20). A partitioning experiment was designed between the buffer and organic phases at 37° in the pH range 6.54–8.62. Both phases were assayed spectrophotometrically, and the concentration data were then rearranged according to an equation given by Martin (21):

$$\frac{K_a + [H_3O^+]}{C_w} = \frac{K + 1}{C}[H_3O^+] + \frac{K_a}{C} \qquad \text{(Eq. 1)}$$

where K_a = the dissociation constant, C_w = concentration of I in the aqueous phase, C = sum of the concentrations of I in each phase, and K = true distribution coefficient.

RESULTS AND DISCUSSION

The loss of I from phase *A*, both with and without II, is shown in Fig. 2. A model of the transfer process in the absence of protein is shown in Scheme I. The kinetics of such a scheme can be described by the following differential equations:

$$dA/dt = -k_5A + k_6B \qquad \text{(Eq. 2)}$$

$$dB/dt = k_5A - k_6B - k_7B \qquad \text{(Eq. 3)}$$

$$dC/dt = k_7B \qquad \text{(Eq. 4)}$$

Evaluation of the rate constants by analog computer gave the means and standard deviations as: $k_5 = 0.324 \pm 0.009$ min^{-1}, $k_6 = 0.027 \pm 0.003$ min^{-1}, and $k_7 = 0.054 \pm 0.001$ min^{-1}.

$$\boxed{A} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \boxed{B} \xrightarrow{k_7} \boxed{C}$$

Scheme I

Calculations with Protein Present—From the initial weighing and the terminal assay for I in the buffer phase of the rotating cell, the original (A_0) and final (A_{final}) values for total drug in phase *A* were known. Between these two values, there was a whole series of values (A_i) which could be calculated as follows.

At the first sampling interval, the amount of drug in phase *A* was simply the original minus the quantities in phases *B* and *C*:

$$A_1 = A_0 - (B_1 + C_1) \qquad \text{(Eq. 5)}$$

Estimation of A_2 was similar, except that allowance had to be made for drug lost from the system by sampling:

$$A_2 = A_0 - \left(\frac{B_1}{36} + \frac{C_1}{5}\right) - (B_2 + C_2) \qquad \text{(Eq. 6)}$$

In general, if R_i equaled the total drug remaining in the cell at

[9] Ostwald.

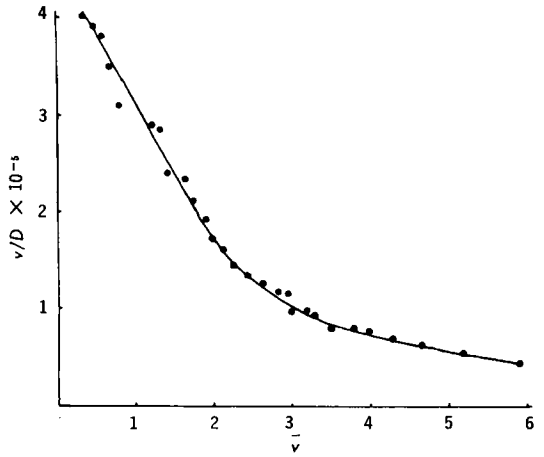


Figure 3—*Scatchard plot of I–II interaction obtained from a single experiment. The small scatter of the points is indicative of the precision of the technique.*

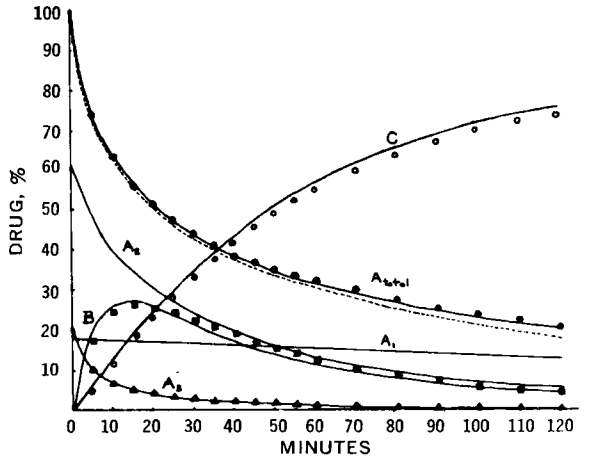


Figure 4—*Comparison of observed and predicted drug levels changing with time for each compartment shown in Scheme II. The solid curves were obtained by solution of the differential Eqs. 17–20, while the points are experimenal. The dotted line is the predicted* A_{total} *obtained by altering* k_7 *(Scheme II) by +15%.*

any time, then since $R_0 = A_0$:

$$R_{i+1} = R_i - \left(\frac{B_i}{36} + \frac{C_i}{5}\right) \qquad \text{(Eq. 7)}$$

and:

$$A_i = R_i - (B_i + C_i) \qquad \text{(Eq. 8)}$$

The series of values (A_i) so calculated represented the total amount of drug (free plus bound) in A at each of the sampling times (t_i) based on the original weight A_0.

From Eq. 7, it was evident that the error in R_i was cumulative. If the error in R_2 was $\pm S_2$, comprised of errors in timing, assay, and model assumptions, the error in R_3 was augmented by that in R_2 and so on. Hence, the probable error in A_i also increased in the series, and the calculated amount at any time A_i could have varied by $\pm\Sigma|S_i|$, with the largest error occurring in A_{final}. Furthermore, since A_i decreased with time, the absolute error increased and the relative error increased even more. To minimize this effect, the values for A_i were calculated on the basis of the $A_{final,\ assay}$, which, being obtained directly, was subject only to assay error. The largest correction required was when A_0 = 22 mg and samples were taken at 37 time intervals. In this case, $A_{final,\ predicted}$ = 0.78 mg and $A_{final,assay}$ = 0.64 mg, which gave a relative error of 22%. When $A_{final,\ assay}$ was used to determine A_0, then $A_{0,\ predicted}$ = 21.86 mg, which meant a maximum probable error of 0.6% for the A_i series.

In the presence of protein, the total amount of drug in phase A (A_{total}) was made up of both bound (A_{bound}) and free (A_{free}) species.

Rearranging Eq. 2 gave Eq. 9:

$$A_{free} = \frac{k_6 B - dA_{total}/dt}{k_5} \qquad \text{(Eq. 9)}$$

The value of the expression dA_{total}/dt at each sampling time was obtained from Eq. 10 for closely spaced intervals (Fig. 2):

$$\left(\frac{dA_{total}}{dt}\right)_{i+1} \cong \frac{(A_{i+2} - A_i)}{(t_{i+2} - t_i)} \qquad \text{(Eq. 10)}$$

Substitution in Eq. 9 then gave a series of A_{free} values for the various sampling times, and the bound drug was found by subtraction:

$$A_{i(bound)} = A_{i(total)} - A_{i(free)} \qquad \text{(Eq. 11)}$$

$A_{i(free)}$ and $A_{i(bound)}$ were converted to the molar quantities D_f, $\bar{v}$, and $\bar{v}/D_f$ to allow construction of a Scatchard plot, where D_f = molar free drug concentration and $\bar{v}$ = ratio of moles of drug bound per total moles of protein. From such a Scatchard plot (Fig. 3), estimates for the binding constants were obtained to initiate the nonlinear regression computer program.

The expression used to calculate $\bar{v}$ was:

$$\bar{v} = \sum_i^N \frac{n_i K_i D_f}{1 + K_i D_f} \qquad \text{(Eq. 12)}$$

where N = number of classes of binding sites, n_i = number of binding sites in the ith class, and K_i = association constant for the ith class.

A least-squares approach, similar to that of Fletcher and Spector (22), was adopted to obtain best fit, regressing $\bar{v}$ onto D_f and minimizing the sum of squares of the deviations R, where:

$$R = \sum_i^n \omega_i(\bar{v}_{calc} - \bar{v}_{obs})^2 \qquad \text{(Eq. 13)}$$

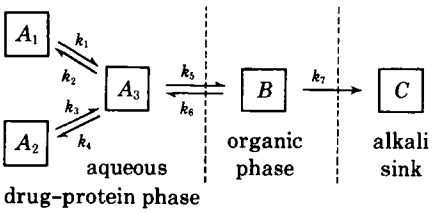
and n = number of data points, ω_i = weighting term for the ith data point, $\bar{v}_{calc}$ = drug bound per total moles of protein calculated by Eq. 12 from observed values of D_f, and $\bar{v}_{obs}$ = observed drug bound per total moles of protein. After preliminary trials, ω_i was assigned a value of unity and the function was minimized[10].

The results of these computations on pooled data (130 data points) from four experiments indicated that there were two classes of binding sites for I, with n_1 = 2.3 ± 0.15[11], K_1 = (1.8 ± 0.23) × 10^5 M^{-1}, n_2 = 14.0 ± 0.71, and K_2 = (3.0 ± 0.36) × 10^3 M^{-1}. Due to the relatively small difference between the values of n_1K_1 and n_2K_2, a minimum of 20–30 samples for each experiment was found necessary to allow estimation of the parameters with an acceptable standard deviation. For comparison, a previous report (24) of these parameters, obtained using equilibrium dialysis, gave n_1 = 3.8 ± 0.6 and K_1 = (7 ± 1) × 10^5 M^{-1}, the values being estimated by eye; it was assumed that contribution from the weaker class of sites would not interfere with estimates of the first class.

Theoretical Treatment—From the foregoing, Scheme II was taken to represent the transfer of I within the rotating cell when II was present. To generate theoretical data on the distribution of drug with time, initial levels for each compartment shown in Scheme II had to be calculated. The amount of drug originally placed in the buffer phase (11.24 mg = $A_1 + A_2 + A_3$) was known, and Eq. 12 relates A_1 to A_3 for i = 1 and A_2 to A_3 for i = 2. These three equations were solved iteratively giving values of 2.441 × 10^{-4}, 8.214 × 10^{-4}, and 2.745 × 10^{-4} M for A_1, A_2, and A_3, respectively. Phases B and C were zero.

[10] Using FUNMIN, a library subprogram, Basser Computing Centre, Sydney University, which uses the Simplex method of Nelder and Mead (23).

[11] Best estimate ± standard deviation.



Scheme II—Representation of drug transfer in the cell. Compartment A_1 *represents drug bound on the first class of sites on II, and* A_2 *represents drug bound on the weaker second class. Both of these compartments and the organic phase* (B) *equilibrate with compartment* A_3*, which is the free drug in the buffer phase.*

Differential equations describing drug transfer in Scheme II were derived by first considering the equilibration of Compartments A_1 and A_3. From Eq. 12 for one class of sites:

$$\frac{A_1}{P_t} = \frac{n_1 K_1 A_3}{1 + K_1 A_3} \qquad \text{(Eq. 14)}$$

where P_t = molar protein concentration.

Rearranging Eq. 14:

$$A_1 = K_1 A_3 (n_1 P_t - A_1) \qquad \text{(Eq. 15)}$$

therefore:

$$\frac{A_1}{A_3(n_1 P_t - A_1)} = K_1 = \frac{k_2}{k_1} \qquad \text{(Eq. 16)}$$

so the forward-transfer rate was given by $k_1 A_1$ and the back-transfer rate was given by $k_2 A_3 (n_1 P_t - A_1)$. At equilibrium, these two rates would be equal, as implied by Eq. 16. For nonequilibrium cases, the net rate of change was found from the difference in these rates:

$$\frac{dA_1}{dt} = k_2 A_3 (n_1 P_t - A_1) - k_1 A_1 \qquad \text{(Eq. 17)}$$

The other equations were derived similarly and are:

$$\frac{dA_2}{dt} = k_4 A_3 (n_2 P_t - A_2) - k_3 A_2 \qquad \text{(Eq. 18)}$$

$$\frac{dA_3}{dt} = k_1 A_1 + k_3 A_2 + k_6 B - k_5 A_3 - k_2 A_3 (n_1 P_t - A_1) - k_4 A_3 (n_2 P_t - A_2) \qquad \text{(Eq. 19)}$$

$$\frac{dB}{dt} = k_5 A_3 - k_6 B - k_7 B \qquad \text{(Eq. 20)}$$

A problem arose in assigning values to k_1 and k_3. Individual rate constants for drug-protein interactions are not known with any accuracy. Froese *et al.* (25) reported a dissociation rate constant of about 1 sec^{-1} for an analogous system. A digital computer solution for the system shown in Scheme II, with k_1 and k_2 having such large absolute values as 1 sec^{-1} and 1×10^6 sec^{-1}, respectively, would have been very slow, costly, and associated with a large degree of error. Provided the assumed value of k_1 did not make it the rate-limiting step, its absolute value rapidly became of diminishing importance in regard to total transfer. Accordingly a value of 0.01 sec^{-1} was assumed for k_1 and k_3 as a computing compromise. When this value was doubled, the maximum deviation between predicted levels for the different compartments at the various experimental times shown was less than 2%.

The values of k_2 and k_4 were calculated from the derived association constants, while the values of k_5, k_6, and k_7 were known. The system of Eqs. 17–20 was solved by computer[12]. The results are shown in Fig. 4. The dotted line in this figure was also computer generated from the equations but with an "error" of +15% in the value of k_7, the rate-limiting step. The resulting deviation from the experimental data was an indication that the proposed model was realistic, being sensitive to an error of this magnitude.

[12] Using DHPCG, IBM System/360 Scientific Subroutine Package. This *subroutine required* rate constants and initial compartmental values for input and computed the levels in each compartment at appropriate time intervals.

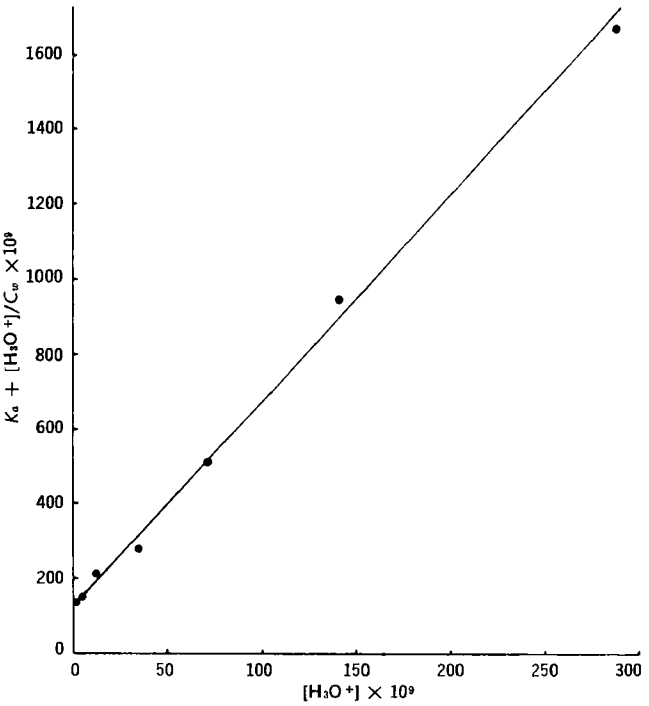


Figure 5—*Partitioning of I between the organic and buffer phases at 37° in the pH range 6.54–8.62.*

Chignell and Starkweather (26) reported that prolonged incubation together increased the affinity of human serum albumin for I, particularly in the strongly bound sites. This phenomenon was investigated for the I-II system, but no obvious difference in binding behavior was observed. In this report (26) the data for the control (*i.e.*, no incubation) were taken from a literature source.

The solubility method for determination of pKa was selected because it was felt that data obtained in solvent mixtures such as 95% ethanol (27) and dimethylformamide-water (28) should not be extrapolated to purely aqueous systems (20, 29). The apparent pKa_2 of I at 37° in the described buffer was estimated to be 6.9. The very low solubility of the neutral molecule [0.5 mg/liter (28)] made estimation of pKa_1 vague, but it would seem to be at least 1.5 units below this figure. Therefore, at pH 7.4 most of I existed as the diionized salt with somewhat less in the monoionized form and a very low concentration of nonionized species. To account for the observed high transfer rate of I from buffer to organic phase, it seemed necessary to assume that the species in the organic phase was the monoionized form. This hypothesis was supported by the two-phase partitioning experiment (Fig. 5) with K_a in Eq. 1 given a value of 6.9. On the basis of this graph, it would not be necessary to postulate any species other than the diionized and monoionized forms for transfer between phases *A* and *B*.

A marked change in spectral properties at a molar ratio of 1.5 (I/II) was noted in the 340-nm region but not at 315 nm (Fig. 6) as reported previously (17). Whatever the reasons for this change, it ought not to be assumed to reflect a change in binding strength (1).

The rotating cell technique would seem a suitable method for the study of drug-protein binding. In common with other dynamic dialysis methods, it has the advantages over equilibrium techniques of a saving in time and materials since only one drug-protein solution need be prepared to produce data for the whole range of binding ratios. There is no need to make estimates or assumptions regarding drug-membrane binding, because the "membrane" is assayed. Also, the properties of the organic phase can be more accurately controlled than can those of dialysis tubing. Compared with other kinetic techniques, the apparatus is relatively simple, with the advantage of readily adjustable pa-

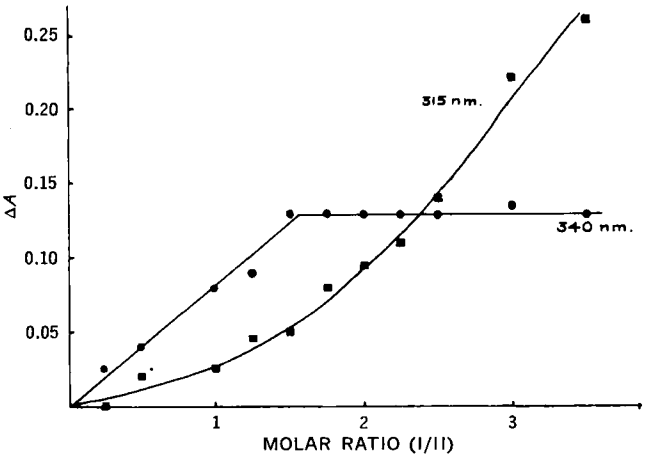


Figure 6—*Differential absorption of the I–II interaction, with the sharp discontinuity at a molar ratio of 1.5.*

rameters. The polarity of the organic phase can be altered to give the desired drug transfer rate, as can the phase volume ratios, protein concentration, and sink conditions. The latter parameter can be altered to minimize back transfer of drug instead of assuming that keeping the external phase drug concentration below 10% of the internal free drug concentration achieves this objective (8). The organic phase has a further advantage in allowing substances to pass on the basis of polarity rather than of dialytic permeation. The latter process, unlike the former, permits passage of water-soluble UV-absorbing substances present in proteins, which can interfere with a UV assay process.

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[x] To whom inquiries should be directed.

Kinetic and Thermodynamic Aspects of *In Vitro* Interphase Transfer of Sulfonamides I: Influence of Methyl Group Substitution on Transfer of Unionized Sulfonamides

G. E. SCHUMACHER[x] and JANARDAN B. NAGWEKAR

Abstract □ The influence of methyl group substitution on the kinetic and thermodynamic aspects of the interphase transfer of three unionized sulfonamides was studied in a two-phase *in vitro* system composed of an aqueous pH 4.3 buffer and *n*-octanol. By applying the theory of absolute reaction rates to the interphase transfer process, the forward parameters (k_f, ΔH_f^*, and ΔS_f^*) increased but the back parameters (k_b, ΔH_b^*, and ΔS_b^*) decreased as the lipophilic nature of the sulfonamides increased. In terms of the net thermodynamic parameters for the process, both ΔH and ΔS increased as the lipophilicity increased. The entropic energy contribution ($T\Delta S$) dominated the ΔH contribution, however. Thus, the observed increase in the ratio of k_f/k_b with increased lipophilicity appeared to be due to an increase in ΔS for transfer from aqueous to alcohol phases resulting from substitution of the methyl group. Based upon these kinetic and thermodynamic parameters, possible mechanisms for the interphase transfer of unionized sulfonamides were proposed.

Keyphrases □ Sulfonamides (sulfadiazine, sulfamerazine, and sulfamethazine)—effect of pyrimidine ring methyl on interphase transfer, water-*n*-octanol system □ Transfer of unionized sulfonamides (sulfadiazine, sulfamerazine, and sulfamethazine)—effect of pyrimidine ring methyl on interphase transfer □ Thermodynamic parameters—interphase transfer of unionized sulfonamides (sulfadiazine, sulfamerazine, and sulfamethazine), water-*n*-octanol system

In recent years, several investigators developed the application of *in vitro* models in the study of the transfer of solutes across immiscible phases (1–8). These studies have been valuable in constructing models and developing kinetic equations to evaluate the influence of variables such as partition coefficient, dissociation constant, pH, phase polarity, and phase constituents on the diffusion rates of solutes. The thermodynamics and mechanisms of interphase transfer, however, do not appear to have been examined in pharmaceutical systems.

The purpose of this investigation was to determine in *in vitro* systems the kinetic and thermodynamic parameters for the interphase transfer (aqueous ↔ octanol) of sulfadiazine and its homologs, sulfamerazine and sulfamethazine, and to use these parameters to gain insight into possible mechanisms for the interphase transfer of these compounds at the molecular level. These three sulfonamides are considered to be suitable compounds for this investigation, because they differ from each other only in the hydrophobic nature of the pyrimidine aromatic moiety and because they are ampholytes which exist in cationic, unionized, and anionic forms over the physiological pH range encountered during GI absorption. Thus, in support of the objectives of this study, the manner in which factors such as the partition coefficient, interface composition, solute structure and charged form, and thermodynamic parameters contribute to the interphase transfer characteristics of sulfonamides can be studied in two-phase systems composed of various aqueous buffer and alcohol phases. The differences in interphase transfer rates demonstrated by the homologs and their charged forms may be attributed to differences in activation entropies and enthalpies resulting from variations in intermolecular forces between solute and solvent.

This portion of the study examines the effect on the kinetic and thermodynamic parameters of the interphase transfer of sulfonamides resulting from methyl group substitution on the pyrimidine moiety of the unionized sulfonamide nucleus. These results may reveal the possible role of the hydrophobic interaction between a drug molecule and solvent while crossing the interface and they may provide an additional dimension to the understanding of the mechanisms responsible for the transfer of drugs across biological membranes.

EXPERIMENTAL[1]

Sulfadiazine, sulfamerazine, and sulfamethazine were specially synthesized commercially, purified, and analyzed by differential scanning calorimetry[2]. The following reagents were also used in the highest quality obtainable: *n*-octanol, acetic acid, potassium chloride, and hydrochloric acid.

Diffusion Experiments—All aqueous buffer solutions and organic solvents were mutually saturated prior to beginning the diffusion experiments. To approximate the ionic strength of the GI tract (9), all aqueous buffer solutions were adjusted to 0.15 with potassium chloride. To study the diffusion of the unionized form of the sulfonamides, 4×10^{-4} *M* solutions of each sulfonamide were prepared in an aqueous acetate buffer solution[3] adjusted to pH 4.3.

A suitable glass cell capable of containing two immiscible solvents and a stirring apparatus were used for conducting the diffusion experiments. The glass cell consisted of a 120-ml (4-oz) square, wide-mouth prescription-type bottle which presented a cross-sectional interfacial area of 25 cm². The cap of the bottle was perforated to allow insertion of a double-bladed, glass stirring shaft. The loss of organic solvent that occurred due to the perfo-

[1] Diffusion experiments were conducted with the aid of a Phipps and Bird six-unit stirrer (Phipps and Bird, Inc., Richmond, Va.) containing a speed controller, a water bath equipped with a thermostatic control, glass diffusion cells, and specially prepared glass stirrers. The quantitative analysis of sulfonamides in solution was performed with a Beckman model DU UV spectrophotometer (Beckman Instruments, Fullerton, Calif.). All pH determinations were made on a Corning model 12 research pH meter (Corning Scientific Instruments, Corning, N.Y.).

[2] Matheson, Coleman and Bell, Norwood, Ohio.

[3] The acetate buffer solution contained 0.02 *M* acetic acid, 0.124 *M* potassium chloride, 0.006 *M* sodium hydroxide, and distilled water.

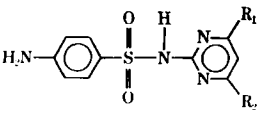

Table I—Structures and Physical Data for the Sulfonamides

Sulfonamide	Adjusted Literature[a] pK_1	Adjusted Literature[a] pK_2	R_1	R_2	pI Range[b]	Apparent Partition Coefficient[c]
Sulfadiazine	1.86	6.34	H	H	3.86–4.34	0.91
Sulfamerazine	2.12	6.92	CH_3	H	4.12–4.92	1.45
Sulfamethazine	2.22	7.24	CH_3	CH_3	4.22–5.24	2.09

[a] Adjusted values obtained by applying the Debye–Hückel equation for ionic strength of 0.15 to the literature values. [b] pH range in which the sulfonamide is more than 99% in the unionized form. [c] Expressed in terms of the ratio of solute concentrations in octanol–aqueous pH 4.3 acetate buffer phases, as obtained from interphase transfer experiments, at 36°.

ration was negligible, if any. One of the 2.0-cm blades was positioned at the midheight of each immiscible phase. The stirring blades were rotated at a constant speed of 48 rpm by using the six-unit stirrer which allowed six simultaneous experiments to be run at any one temperature. The studies at 24° were performed in a temperature-controlled room, while those at 30 or 36° were performed in a water bath maintained at the desired temperature ±0.5°.

For experiments involving the net transfer of solute from an aqueous to an organic phase, 50 ml of a sulfonamide-containing aqueous pH 4.3 buffer solution was pipeted into the glass cell; at pH 4.3 the sulfonamides are essentially in an unionized form. The stirring blades were clamped into place prior to pipeting 50 ml of *n*-octanol gently over the sulfonamide solution. A blank composed of the aqueous buffer solution and organic solvent was prepared in the same manner. Four 1-ml samples of organic phase were removed from the diffusion cells containing the sulfonamide at 15–30-min intervals, depending upon the rate of transfer, by using 1-ml tuberculin syringes. Samples of organic phase were also obtained from the diffusion cell containing the blank at the corresponding time. To maintain an equal volume of both phases in the system, a 1-ml sample of aqueous buffer phase was removed at the time the organic phase was sampled using a different tuberculin syringe. To measure the partition coefficient, diffusion was allowed to proceed to equilibrium, which required 5–8 hr depending upon the rate of transfer. The final sample was then taken from the organic phase and the blank. All samples of the organic phase were diluted to 10 ml with ethanol, their absorbance was measured at 270 nm using a UV spectrophotometer, and the concentration was determined from a previously prepared Beer's law plot for the sulfonamide. The procedure for studying the net diffusion of solute from the organic phase to the aqueous phase was the same as already described, except that the sulfonamide was initially contained in the organic phase.

Determination of Partition Coefficients of Sulfonamides—Apparent partition coefficients for the unionized form of the sulfonamides were determined at 24, 30, and 36° by measuring the concentration of sulfonamide in the organic phase at equilibrium. The partition coefficient was expressed as the ratio of equilibrium concentrations in the organic–aqueous phases.

Determination of Dissociation Constants and pH for Studying Unionized Form of Sulfonamides—The dissociation constants of the sulfonamides were determined by a titrimetric procedure at 30°. They showed a close approximation to the values obtained from the literature by using the Debye–Hückel equation to compensate for the ionic strength of 0.15 of the solutions used in the study (10–12). By ignoring activity coefficients, since they were unavailable, adjusted literature values, pK_1 and pK_2, were taken to represent the pH values at half-protonation and half-dissociation of the sulfonamides, respectively. The pH range in which these amphoteric compounds were present as essentially unionized species was obtained by adding 2 units to pK_1 and subtracting 2 units from pK_2.

RESULTS

Dissociation Constants and Partition Coefficients of Sulfonamides—The structures of the three sulfonamides, their adjusted dissociation constants, and the pH range in which the compounds are more than 99% present in their unionized form are summarized in Table I. Based on the pI range of the compounds, pH 4.3 was chosen as a suitable value for the aqueous buffer phase for studying the unionized form of the three sulfonamides.

The apparent partition coefficients of the sulfonamides in the pH 4.3 acetate buffer–*n*-octanol system are also included in Table I. The partition coefficient increased as the number of methyl groups substituted on the pyrimidine moiety of the sulfonamides increased. Since the partition coefficient has long been recognized as a significant factor in membrane penetration and interphase transfer (1, 13–17), this property was expected to play a role in the interpretation of the experimental interphase transfer data.

Kinetic and Thermodynamic Data—Sulfadiazine, sulfamerazine, and sulfamethazine were used to examine the influence of methyl group substitution in these molecules (Table I) on their interphase transfer characteristics. The interphase transfer of sulfonamide in the experimental two-phase system was described by a reversible kinetic scheme (Scheme I).

$$S_w \underset{k_b}{\overset{k_f}{\rightleftharpoons}} S_a$$

Scheme I

In this scheme, S_w and S_a represent the amounts of sulfonamide in the aqueous buffer phase and alcohol phase, respectively, and k_f and k_b are the apparent first-order rate constants for forward and back transfer, respectively.

For graphical presentation, the integrated rate equation (18) describing the interphase transfer denoted in Scheme I, when the sulfonamide is initially present in the aqueous phase, is given as Eq. 1:

$$\log [(Sa)\text{eq} - (Sa)] = \log (Sa)\text{eq} - \frac{(k_f + k_b)t}{2.3} \qquad \text{(Eq. 1)}$$

In this equation, (Sa)eq is the concentration of sulfonamide in the

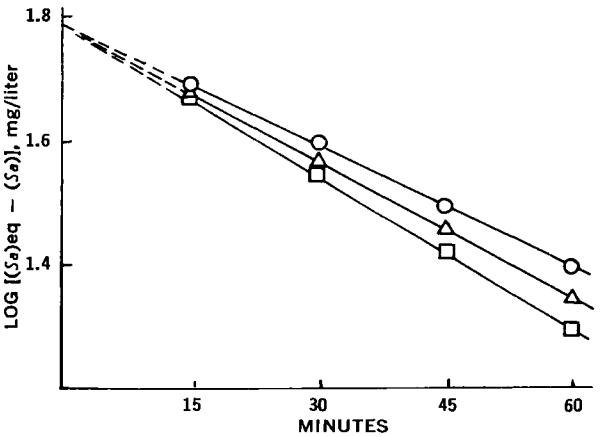

Figure 1—*Transfer of sulfamethazine from an aqueous pH 4.3 buffer to* n-*octanol. Data are the average of four experiments at each temperature. Key:* ○, *25°;* △, *30°; and* □, *36°.*

Table II—Kinetic and Thermodynamic Parameters of Activation for the Interphase Transfer of Unionized Sulfonamides in a Two-Phase System at 36°

Sulfonamide	k, hr^{-1}		ΔH^*, cal/mole		ΔS^*, cal/mole-deg		ΔF^*, cal/mole		Ea, cal/mole	
	k_f	k_b	ΔH_f^*	ΔH_b^*	ΔS_f^*	ΔS_b^*	ΔF_f^*	ΔF_b^*	Ea_f	Ea_b
Sulfadiazine	0.473	0.518	4099	4145	−46.8	−46.5	18,574	18,515	4713	4759
Sulfamerazine	0.672	0.456	4374	3147	−45.2	−50.0	18,355	18,590	4988	3761
Sulfamethazine	0.896	0.428	5060	2379	−42.4	−52.6	18,174	18,639	5674	2993

octanol phase at equilibrium, and *(Sa)* is the concentration in that phase at the time of sampling, t. A typical plot of experimental data using Eq. 1 is shown in Fig. 1. The slope of the line, as obtained by the method of least squares, is $(k_f + k_b)/2.3$.

The method for obtaining the k_f and k_b values, as well as the energy of activation for the forward *(Ea_f)* and back *(Ea_b)* reactions, was described (18). Accordingly, k_f and k_b are calculated from the following equations:

$$k_f = \frac{(Sa)\text{eq}}{(Sw)_0}(k_f + k_b) \qquad \text{(Eq. 2)}$$

$$k_b = (k_f + k_b) - k_f \qquad \text{(Eq. 3)}$$

where $(Sw)_0$ is the initial concentration of sulfonamide in the aqueous buffer phase.

The thermodynamic parameters of activation for the interphase transfer process noted in Scheme I were obtained by applying the theory of absolute reaction rates to the diffusion process, as depicted in Scheme II (19), where S_w' and X represent a sulfonamide molecule and solvent molecule(s) of octanol, respectively. This scheme provides for the inclusion of an intermediate state composed of a transient association of reacting molecules, S_w' and X, which is termed an "activated complex" $[S_w'\text{-}X]$, prior to the diffusion of the solute to a new equilibrium position. Assuming that transfer across the interface is rate limiting (2, 4, 8), the formation of the activated complex is considered to be the rate-limiting step for the diffusion process across the interface.

$$S_w' + X \rightleftharpoons [S_w'\text{-}X] \longrightarrow S_a$$

Scheme II

A summary of the kinetic and thermodynamic parameters for the interphase transfer of the three sulfonamides at 36° is shown in Table II.

The ΔF^* for the forward or back transfer was obtained from Eq. 4 (18):

$$k = \frac{RT}{Nh}e^{-\Delta F^*/RT} \qquad \text{(Eq. 4)}$$

where R, T, N, and h have the usual meanings. The ΔH^* for the forward or back transfer was obtained from Eq. 5 (18):

$$\Delta H^* = Ea - RT \qquad \text{(Eq. 5)}$$

where Ea is the energy of activation; Ea is obtained from the slope of the Arrhenius plot of log k *versus* $1/T$. These plots for the interphase transfers are shown in Figs. 2 and 3. Since these plots yielded reasonable least-squares lines, the ΔH^* is considered to be constant over the temperature range of 24–36°. As noted in Table III, the forward parameters (k_f, ΔH_f^*, and ΔS_f^*) increased but the back parameters (k_b, ΔH_b^*, and ΔS_b^*) decreased as the lipophilic nature of the three sulfonamides increased.

Davies (1) developed the concept of partition coefficient energy in interpreting interphase transfer data. He considered the difference in free energy of activation for forward and back transfer ($\Delta F_f^* - \Delta F_b^*$) to represent the net free energy of transfer (ΔF) of solute across an interface. He related the partition coefficient, rate constants, and the net free energy of interphase transfer as follows:

$$K_w^0 = \frac{k_f}{k_b} = \frac{e^{-\Delta F_f^*/RT}}{e^{-\Delta F_b^*/RT}} = e^{\Delta F/RT} \qquad \text{(Eq. 6)}$$

$$K_w^0 = \frac{k_f}{k_b} = e^{(\Delta H/RT - \Delta S/R)} \qquad \text{(Eq. 7)}$$

The relationships between the rate constants, net thermodynamic parameters, and partition coefficients (K_w^0) for the interphase transfer of the three sulfonamides are recorded in Table III.

Thermodynamic Contributions of Methyl Group Substitution to Interphase Transfer of Sulfonamides—To assess the role of the methyl group in altering the net thermodynamic parameters for interphase transfer, the thermodynamic contributions resulting from the systematic addition of methyl groups to sulfadiazine are recorded in Table IV. Since sulfamerazine differs from sulfadiazine and sulfamethazine differs from sulfamerazine only

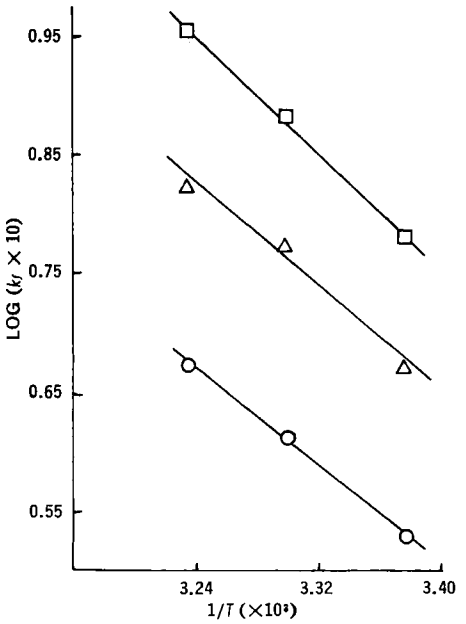

Figure 2—*Arrhenius plots of the transfer of sulfonamides from an aqueous pH 4.3 buffer to* n-*octanol. Key:* ○, *sulfadiazine;* △, *sulfamerazine; and* □, *sulfamethazine.*

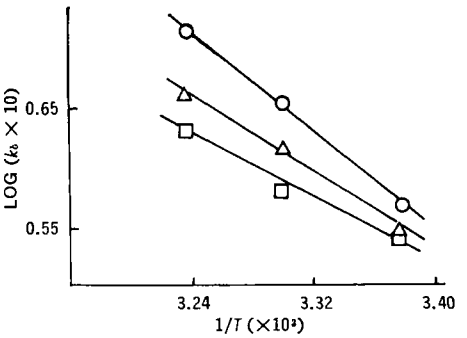

Figure 3—*Arrhenius plots of the transfer of sulfonamides from* n-*octanol to an aqueous pH 4.3 buffer. Key:* ○, *sulfadiazine;* △, *sulfamerazine; and* □, *sulfamethazine.*

Table III—Kinetic Parameters, Net Thermodynamic Parameters, and Apparent Partition Coefficients for the Interphase Transfer of Three Unionized Sulfonamides in a Two-Phase System at 36°

Sulfonamide	k_f/k_b[a] or K_w[0]	ΔH[b], cal/mole	ΔS[b], cal/mole-deg	$T\,\Delta S$[b], cal/mole	ΔF[b], cal/mole
Sulfadiazine	0.91	−46	−0.3	−93	59
Sulfamerazine	1.45	1227	4.8	1483	−245
Sulfamethazine	2.09	2681	10.2	3152	−465

[a] As defined by Eqs. 6 and 7. [b] Net values representing the difference between activation parameters for forward transfer minus back transfer. Values are calculated from the data in Table II.

Table IV—Contribution of Methyl Groups to Net Thermodynamic Parameters for the Interphase Transfer of Unionized Sulfonamides at 36°

ΔH, cal/mole		ΔS, cal/mole-deg		$T\,\Delta S$, cal/mole		ΔF, cal/mole	
SMR[a] minus SD	SMT[b] minus SMR	SMR[a] minus SD	SMT[b] minus SMR	SMR[a] minus SD	SMT[b] minus SMR	SMR[a] minus SD	SMT[b] minus SMR
1273	1454	5.1	5.4	1576	1669	−294	−230

[a] The difference in net values between sulfamerazine (SMR) and sulfadiazine (SD). [b] The difference in net values between sulfamethazine (SMT) and sulfamerazine (SMR).

in the addition of a single methyl group, the difference in any given thermodynamic value between these two sets is considered to result from the contribution of the methyl group.

DISCUSSION

As observed in Table II, the large free energy of activation (ΔF_f^* or ΔF_b^*) for interphase transfer of the sulfonamides, approximately 18 kcal/mole, suggests that the energy barrier for transfer across the interface is great. Values of this magnitude were also observed (1, 4) in studying the diffusion of inorganic ions across an interface composed of water and various organic phases. This value is considerably higher than the ΔF^* values for ordinary bulk diffusion, which generally fall within the range of 2–3 kcal/mole (19).

The negative entropy of activation (ΔS_f^* or ΔS_b^*) for interphase transfer (Table II) was also observed by others (1, 4). Since most substances diffuse in homogeneous bulk solutions with ΔS^* values within a range of −5 to +5 eu (19), the large negative ΔS^* values observed for crossing the interface appear to influence interphase transfer rates and ΔF^* values significantly.

Based upon the kinetic and thermodynamic parameters presented in this and the following paper (20), it is possible to consider a model of the activated complex formed for interphase transfer. Neither the nature of the activated complex nor the number of solvate molecules interacting with the solute during formation of the activated complex for diffusion has been clarified in the literature, however. Conway (21) suggested that it is difficult to ascribe a unique set of initial and final states to the formation and cleavage of the activated complex because the process probably includes not only the rearrangement of the solvent structure around the solute but also the movement of the solute into the rearranged region. Others suggested that a number of hydrogen bonds are broken during formation of the activated complex (19, 22).

For these reasons, the possibility of the interaction of more than one solvate molecule with the sulfonamide molecule is considered in Schemes III and IV. To interpret the data in Table II, as well as the experimental evidence on the role of the escaping tendency of solvate molecules into the interface, presented more extensively in the next paper (20), these schemes are postulated as possible mechanisms for the interphase transfer of unionized sulfonamides. S_{sw} and S_{sa} represent the solvated sulfonamide molecule in the aqueous and alcohol phases, respectively; S_{psw} and S_{psa} represent the partially desolvated sulfonamide molecule in the aqueous and alcohol phases, respectively, at the interface; ROH and HOH represent alcohol and water molecules in the interface, respectively; and [S_{psw}-ROH] and [S_{psa}-HOH] represent the activated complex for the forward and back transfer, respectively, at the interface.

As depicted in Scheme III and as discussed more completely in the following paper (20), an interaction between a partially desolvated sulfonamide molecule and alcohol molecules during the formation of the activated complex for forward transfer occurs as a result of two types of intermolecular forces. Alcohol molecules are assumed to associate with the sulfonamide molecule primarily through hydrogen bonding with some of the several possible sites, including the sulfamyl oxygen(s) and the nitrogen and hydrogen(s) of the *para*-amino group, and secondarily through hydrophobic interaction between the hydrocarbon chain of the alcohol and the nonpolar groups of the sulfonamide. However, since the three sulfonamides do not differ in substitution on the phenyl group (Table I), the contribution to ΔH_f^* and ΔS_f^* due to hydrogen bonding by alcohol molecules at the *para*-amino group and the hydrophobic interaction at the phenyl moiety is not expected to differ significantly for the three homologs. But the sulfonamides do differ in the number of methyl groups substituted on the pyrimidine ring. Sulfamerazine, with the addition of one methyl group, should be more solvated by water molecules than sulfadiazine, which contains no methyl group, due to the greater iceberg formation (23) induced by the methyl group. It has been recognized for some time that the structural order of water increases near nonpolar solutes in aqueous phases (23–26). Therefore, it is conceivable that the association of sulfonamide and alcohol molecules through hydrogen bonding at the sulfamyl oxygens and hydrophobic interaction with the pyrimidine moiety may result in releasing more water molecules upon desolvation in the case of sulfamerazine as compared to sulfadiazine. The addition of two methyl groups for sulfamethazine should result in even greater solvation, with the subsequent release of water molecules, than is suggested for sulfamerazine. For back transfer, as discussed previously for Scheme IV, it is assumed that water molecules associate with the partially desolvated sulfonamide molecule through hydrogen bonding with some of the several possible sites, including the sulfamyl oxygen(s), pyrimidine nitrogen(s), and the nitrogen and hydrogen(s) of the *para*-amino group. In addition, icebergs may form around the nonpolar groups during transfer of solute from alcohol to aqueous phases. This formation is expected to be greatest for sulfamethazine, due to the presence of two methyl groups on the pyrimidine moiety, and least for sulfadiazine, with no methyl group substitution.

These schemes may explain the variations in ΔH^* and ΔS^*

$$S_{sw} \rightleftharpoons S_{psw}$$
$$S_{psw} + \mathrm{ROH} \rightleftharpoons [S_{psw}\text{-ROH}] \longrightarrow S_{sa}$$

Scheme III—Transfer from water to alcohol

$$S_{sa} \rightleftharpoons S_{psa}$$
$$S_{psa} + \mathrm{HOH} \rightleftharpoons [S_{psa}\text{-HOH}] \longrightarrow S_{sw}$$

Scheme IV—Transfer from alcohol to water

values observed in Table II. The increase in ΔH_f^* and ΔS_f^* values with increased lipophilicity of the solute may be attributed to the increasingly positive enthalpy and entropy contributions that can be expected to arise from the release of water molecules upon hydrophobic interaction between the nonpolar group of the octanol molecules and the pyrimidine moiety of the sulfonamides as the lipophilic nature of the homologs increased. For back transfer, ΔH_b^* and ΔS_b^* values decreased as the lipophilicity of the three sulfonamides increased. This may be attributed to the increasingly negative enthalpy and entropy contributions expected to result from increasing iceberg formation around the methyl groups of the sulfonamides.

These considerations may also suggest an explanation for the observation that the ΔH_f^* and ΔS_f^* values in Table II for sulfamerazine and sulfamethazine are greater than the corresponding ΔH_b^* and ΔS_b^* values. It is possible that the number of water molecules released in forming the activated complex for forward transfer is greater than the number of alcohol molecules displaced in forming the activated complex for back transfer. Furthermore, it is possible that the association of water molecules with the solute during the back-transfer process is greater than the association of alcohol molecules with the solute during the forward-transfer process. For sulfadiazine, however, which lacks a methyl group, the thermodynamic parameters for forward and back transfer are similar.

In terms of the kinetic data and the thermodynamic parameters for interphase transfer (Tables III and IV), increasing the lipophilicity of the sulfonamide by methyl group substitution resulted in an increase in the forward rate constant and a decrease in the backward rate constant due to an increase in the apparent partition coefficient of the unionized sulfonamides. Although increases in lipophilicity of the solute were accompanied by increases in both the net enthalpy and entropy, the entropy contribution and, consequently, the entropic energy $(T\ \Delta S)$ dominated the enthalpy contribution. Thus, as predicted by Eqs. 6 and 7, the ratio of forward to backward rate constants (k_f/k_b) increased with increased lipophilicity due to increases in the entropy for transfer from aqueous to alcohol phases resulting from substitution of the methyl group. This resulted in decreases in the net free energy of interphase transfer as the lipophilicity and apparent partition coefficient of the unionized sulfonamides increased. This pattern relating increases in lipophilicity and partition coefficient to decreases in the free energy of transfer from polar to nonpolar phases was observed by other investigators (13, 15, 17).

These observations are consistent with the proposal for the formation of the activated complex discussed in Schemes III and IV. The increased lipophilicity of the sulfonamides was associated with an increased liberation of water molecules upon hydrophobic interaction during formation of the activated complex for forward transfer and an increased association, or restriction, of water molecules upon formation of the activated complex for back transfer. This should result in a net positive enthalpy and entropy for interphase transfer, as observed in Tables III and IV. The data corroborate the common observation that there is a relationship between interphase transfer rates and the partition coefficient. Since the partition coefficient reflects the net gain in enthalpy and entropy during transfer, variations in interphase transfer rates are related to variations in the degree of interaction of solute and solvate molecules during formation of the activated complex for forward and back transfer.

It is recognized that the octanol phase used in the simple model system employed in this study does not simulate the complexity of biological membranes composed of proteins, phospholipids, cholesterol, water, and other constituents. However, as inferred by Diamond and Wright (17), it appears that the intermolecular forces governing the permeation of solutes through cell membranes are the same forces governing the partitioning of solutes between immiscible phases. Thus, these results and conclusions are expected to provide some insight into the mechanisms for the passive transfer of solutes across aqueous-membrane interfaces.

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[x]To whom inquiries should be directed.

Kinetic and Thermodynamic Aspects of *In Vitro* Interphase Transfer of Sulfonamides II: Influence of Interface Composition on Transfer of Unionized Sulfonamides

G. E. SCHUMACHER[x] and JANARDAN B. NAGWEKAR

Abstract □ The influence of interface composition on the kinetic and thermodynamic aspects of the interphase transfer of unionized sulfamethazine was studied in a two-phase *in vitro* system composed of an aqueous pH 4.3 buffer and various alcohol phases. By applying the theory of absolute reaction rates to the interphase transfer process, variations in the ΔH^* and ΔS^* values for the various two-phase systems were considered to be the results of differences in the escaping tendency of the alcohols into the interface and the influence of this parameter on the degree of hydrogen bonding and hydrophobic interaction between solute and solvate molecules in the various systems. The influence of the solubility parameter of the alcohol phase on the interphase transfer process was also examined. Based upon the observed kinetic and thermodynamic parameters, possible mechanisms for the interphase transfer of unionized sulfamethazine were proposed.

Keyphrases □ Sulfonamides (sulfamethazine)—effect of interface composition on interphase transfer, water-alcohol systems □ Transfer of unionized sulfonamides (sulfamethazine)—effect of interface composition on interphase transfer □ Thermodynamic parameters—interphase transfer of unionized sulfonamides (sulfamethazine), water-alcohol systems

In a previous paper (1), a scheme was proposed for the interphase transfer (water ↔ *n*-octanol) of three sulfonamides (sulfadiazine, sulfamerazine, and sulfamethazine) from the data obtained in terms of the thermodynamic parameters of activation. This scheme incorporated the concept of the formation, at the interface, of an "activated complex" composed of a partially desolvated solute molecule and solvate molecules. Basic to the postulation of the mechanism for formation of the activated complex is the concept of the escaping tendency of solvate molecules into the interface. Therefore, in support of this concept, the influence of interface composition on the kinetic and thermodynamic parameters for the interphase transfer of unionized sulfamethazine was examined. In addition, the influence of the concept of the solubility parameter on interphase transfer was studied.

EXPERIMENTAL

The apparatus used, the method of performing the diffusion experiments, and the procedure for determining the partition coefficients in the various two-phase systems were discussed previously (1).

Materials—Sulfamethazine[1] was specially synthesized commercially, purified, and analyzed by differential scanning calorimetry. The following reagents were also used in the highest quality obtainable: *n*-octanol, *n*-octane, *n*-heptyl alcohol, *n*-heptane, *n*-amyl alcohol, *n*-pentane, acetic acid, potassium chloride, and hydrochloric acid.

Determination of Interfacial Tension—The interfacial tension of the various aqueous-organic systems at 27° was measured with a tensiometer[2], according to the procedure recommended by the manufacturer.

Constituents of Aqueous and Alcohol Phases—The constituents of the aqueous and alcohol phases used are summarized in Tables I and II. A pH 4.3 acetate buffer[3], adjusted to an ionic strength of 0.15 to approximate the value of the fluids of the GI tract (2), was used for the aqueous phase because, as explained in the previous study (1), sulfamethazine was more than 99% in the unionized form at this pH.

RESULTS

Partition Coefficients of Sulfamethazine—The apparent partition coefficients of the unionized form of sulfamethazine in the various pH 4.3 acetate buffer-organic systems are summarized in Table III. Since the partition coefficient has long been recognized as a significant factor in membrane penetration and interphase transfer (3–8), and since it was an important parameter for interpreting previous results (1), this property was expected to play a role in the interpretation of the experimental interphase transfer data.

Interfacial Tension of Various Solvents—The interfacial tensions of the various solvents are summarized in Table IV. Since interfacial tension has been implicated as a possible factor in the transfer of drugs across an aqueous-membrane interface (10–12), this parameter of the various two-phase systems was determined as a potential aid in the interpretation of the interphase transfer data.

Kinetic and Thermodynamic Data—The kinetic scheme for the reversible interphase transfer of sulfamethazine in the various two-phase systems, as well as the methods for obtaining the kinetic and thermodynamic data, were discussed in the previous paper (1).

A summary of the kinetic and thermodynamic parameters at 36° for the interphase transfer of sulfamethazine in the various two-phase systems is given in Table V. Arrhenius plots for the interphase transfers are shown in Figs. 1 and 2.

By using the concept of the partition coefficient energy (1, 3), the difference in thermodynamic parameters of activation for forward minus back transfer (*e.g.*, ΔF_f^* minus ΔF_b^*), termed net thermodynamic parameters (*e.g.*, ΔF), was calculated (Table VI). The relationships between the partition coefficient, rate constants, and net thermodynamic parameters of interphase transfer are given in Eq. 1:

$$K_w^0 = \frac{k_f}{k_b} = e^{\Delta F/RT} = e^{(\Delta H/RT - \Delta S/R)} \qquad \text{(Eq. 1)}$$

DISCUSSION

As shown in Table V, the large positive free energy of activation (ΔF_f^* or ΔF_b^*) and the large negative entropy of activation (ΔS_f^* or ΔS_b^*) suggest that the energy barrier for interphase

[1] Matheson, Coleman and Bell, Norwood, Ohio.

[2] DuNouy model 20, Fisher Scientific Co., Pittsburgh, Pa.

[3] The acetate buffer solution contained 0.02 *M* acetic acid, 0.124 *M* potassium chloride, 0.006 *M* sodium hydroxide, and distilled water.

Table I—Constituents of the Aqueous Phases

Aqueous Phase	Percent (v/v)[a]	Mole Fraction
I. Aqueous buffer	97.80	0.996
Pentyl alcohol	1.65[b]	0.003
Pentane	0.55[b]	0.001
II. Aqueous buffer	99.88	0.99986
Heptyl alcohol	0.11[c]	0.00013
Heptane	0.01[c]	0.00001
III. Aqueous buffer	99.80	0.99975
Heptyl alcohol	0.20[d]	0.00025
IV. Aqueous buffer	99.950	0.999947
Octanol	0.045[e]	0.000047
Octane	0.005[e]	0.000006
V. Aqueous buffer	99.95	0.999947
Octanol	0.05[d]	0.000053

[a] Concentrations of the organic solvents are expressed on the basis of volumetric additions by pipet or buret. [b] The composition of a mixture composed of 3 parts pentyl alcohol and 1 part pentane, which was added to the aqueous buffer to saturate the buffer at 25° with respect to the mixture. [c] The composition of a mixture composed of 11 parts heptyl alcohol and 1 part heptane, which was added to the aqueous buffer to saturate the buffer at 25° with respect to the mixture. [d] The composition of alcohol required to saturate the aqueous buffer at 25° with respect to the alcohol. [e] The composition of a mixture composed of 9 parts octanol and 1 part octane, which was added to the aqueous buffer to saturate the buffer at 25° with respect to the mixture.

transfer is great and that a significant constraint is placed upon the solute molecule during interphase transfer. Others (3, 13) observed values of this magnitude in studying the diffusion of inorganic ions across various aqueous–organic interfaces.

In the previous paper (1), a proposal was advanced for the mechanism of interphase transfer of sulfonamides based on the thermodynamic parameters of activation of three sulfonamide homologs and the concept of escaping tendency of solvate molecules. The proposed schemes incorporated the observations of Davies (3), who suggested that an exchange of aqueous and organic solvate molecules occurs during formation of the activated complex, and of Blank (14), who suggested that some form of desolvation of the solute molecule is an important factor during interphase transfer. The schemes for the forward and back transfer of solute across the interface included the formation of an activated complex composed of a partially desolvated solute molecule and solvate molecules. Furthermore, the evidence for a twofold interaction between solute and solvate molecules, including hydrogen bonding and hydrophobic interaction, was discussed. In these schemes (Schemes I and II), as presented previously, S_{sw} and S_{sa} represent the solvated sulfonamide molecule in the aqueous and alcohol phases, respectively: S_{psw} and S_{psa} represent the partially desolvated sulfonamide molecule in the aqueous and alcohol phases, respectively, at the interface; ROH and HOH represent alcohol and water molecules in the interface, respectively; and

Table II—Constituents of the Alcohol Phases

Alcohol Phase	Percent (v/v)	Mole Fraction	Solubility Parameter of Phase at 25°[a]
I. Pentyl alcohol	72.2	0.62	10.51
Pentane	24.0	0.19	
Aqueous buffer	3.8[b]	0.19	
II. Heptyl alcohol	88.7	0.77	10.57
Heptane	9.1	0.08	
Aqueous buffer	2.2[b]	0.15	
III. Heptyl alcohol	96.2	0.77	11.07
Aqueous buffer	3.8[b]	0.23	
IV. Octanol	88.9	0.76	10.34
Octane	8.9	0.08	
Aqueous buffer	2.2[b]	0.16	
V. Octanol	98.0	0.85	10.63
Aqueous buffer	2.0[b]	0.15	

[a] Calculated using the equation of Burrell (20). [b] The concentration required to saturate the alcohol phase at 25° with respect to the aqueous buffer.

Table III—Apparent Partition Coefficients of Unionized Sulfamethazine in the Various Two-Phase Systems at 36°

Alcohol Phase	Apparent Partition Coefficient[a]
Octanol–octane	1.63
Octanol	2.20
Heptyl alcohol–heptane	2.59
Heptyl alcohol	3.00
Pentyl alcohol–pentane	5.12

[a] Expressed in terms of the ratio of solute concentration in alcohol–aqueous pH 4.3 acetate buffer phase, as obtained from interphase transfer experiments.

Table IV—Interfacial Tension of the Solvents at 27°

Alcohol Phase	Interfacial Tension[a], dynes/cm
Octanol–octane	8.7
Octanol	8.2
Heptyl alcohol–heptane	7.8
Heptyl alcohol	7.4
Pentyl alcohol–pentane	4.3

[a] Measured against an aqueous pH 4.3 acetate buffer, both phases being mutually saturated. Values are corrected for Du Nouy error.

[S_{psw}-ROH] and [S_{psa}-HOH] represent the activated complex for the forward and back transfer, respectively, at the interface.

As previously noted, basic to the postulation of the mechanism for formation of the activated complex is the concept of the escaping tendency of solvate molecules. In the case of forward transfer, as described previously, the sulfonamide molecule associates with alcohol molecules. The greater the escaping tendency of the alcohol molecules into the interface, the greater will be the opportunity for association with sulfonamide molecules due to an increase in the number of alcohol molecules per unit volume of interface. Table IV shows that the interfacial tension between the aqueous and alcohol phases decreases as the alcohol chain length decreases. Furthermore, Laiken and Nemethy (15) noted that the free energy for transfer of alcohol molecules from a hydrocarbon to an aqueous phase became more favorable as the chain length decreased. These observations suggest that the escaping tendency of alcohols increases as their hydrocarbon chain length decreases, thus resulting in the increased possibility of interaction with solute. The same general principles may apply for back transfer. The escaping tendency of water molecules into the interface increases as the alcohol chain length decreases.

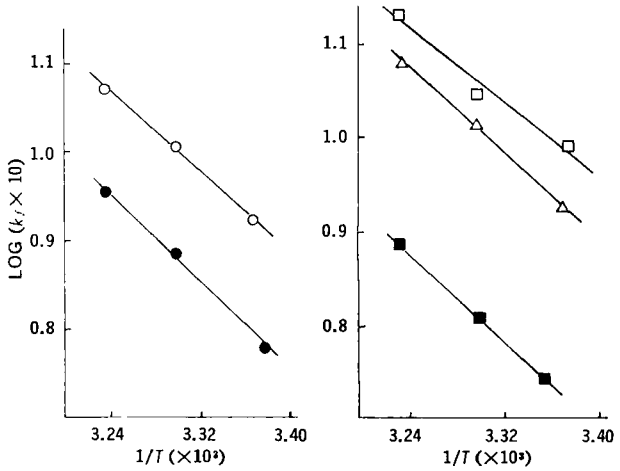

Figure 1—*Arrhenius plots of the transfer of sulfamethazine from an aqueous pH 4.3 buffer to various alcohol phases. Key:* ●, *octanol;* ○, *heptyl alcohol;* ■, *octanol–octane;* △, *heptyl alcohol–heptane; and* □, *pentyl alcohol–pentane.*

Table V—Kinetic and Thermodynamic Parameters of Activation for the Interphase Transfer of Unionized Sulfamethazine in Various Two-Phase Systems at 36°

Alcohol Phase	k, hr^{-1}		ΔH^*, cal/mole		ΔS^*, cal/mole-deg		ΔF^*, cal/mole		Ea, cal/mole	
	k_f	k_b	ΔH_f^*	ΔH_b^*	ΔS_f^*	ΔS_b^*	ΔF_f^*	ΔF_b^*	Ea_f	Ea_b
Octanol–octane	0.769	0.472	4992	3742	−43.0	−48.0	18,273	18,573	5606	4356
Heptyl alcohol–heptane	1.189	0.458	4355	2683	−44.2	−51.5	18,006	18,591	4969	3297
Pentyl alcohol–pentane	1.325	—[a]	3962	—[a]	−45.2	—[a]	17,939	—[a]	4576	—[a]
Octanol	0.896	0.408	5060	1940	−42.4	−54.1	18,174	18,662	5674	2554
Heptyl alcohol	1.187	0.396	4726	1746	−43.0	−54.8	18,007	18,680	5340	2360

[a] Not determined.

Table VI—Kinetic Parameters, Net Thermodynamic Parameters, and Apparent Partition Coefficients for the Interphase Transfer of Unionized Sulfamethazine in a Two-Phase System at 36°

Alcohol Phase	k_f/k_b[a] or K_w[a]	ΔH^b, cal/mole	ΔS^b, cal/mole-deg	$T\,\Delta S^b$, cal/mole	ΔF^b, cal/mole
Octanol–octane	1.63	1250	5.0	1545	−300
Octanol	2.20	3120	11.7	3615	−488
Heptyl alcohol–heptane	2.59	1572	7.3	2256	−585
Heptyl alcohol	3.00	2980	11.8	3646	−673

[a] As defined by Eqs. 6 and 7 of Ref. 1. [b] Net values representing the difference between activation parameters for forward transfer minus back transfer. Values are calculated from the data in Table V.

The data in Table V show that, although the magnitude of the ΔH^* and ΔS^* values for the interphase transfer of sulfamethazine varied with the composition of the alcohol phase, the values for ΔH^* and ΔS^* were always positive and negative, respectively, for both forward and back transfer. The positive ΔH_f^* suggests that the formation of the activated complex is dominated by an endothermic process. As depicted in Scheme I and as already explained, the activated complex formed for the forward transfer of partially desolvated sulfamethazine involved not only hydrogen bonding with alcohol molecules, which is an exothermic process, but also hydrophobic interaction, which is an endothermic process resulting from the release of water molecules (16, 17). Since the resultant ΔH_f^* is positive, this suggests that the positive enthalpy contribution from the hydrophobic interaction dominates the negative enthalpy contribution from hydrogen bonding. The negative ΔS_f^* values suggest that the constraint placed upon the sulfamethazine molecules during the formation of the activated complex for forward transfer, which contributes to a negative entropy, is greater than the positive entropy contribution resulting from the disorder caused by the release of water molecules.

As depicted in Scheme II, the activated complex formed for the back transfer of partially desolvated sulfamethazine involved hydrogen bonding with water molecules (which may result in displacing some alcohol molecules) and the possible formation of icebergs around the nonpolar groups, such as the methyl groups on the pyrimidine moiety of the sulfonamide. The displacement of alcohol molecules is expected to be an endothermic process, while the association of water molecules and iceberg formation is exothermic. The positive ΔH_b^* values suggest that the endothermic process is dominant. Since the alcohol molecules solvate sulfamethazine not only through hydrogen bonding, as do water molecules, but also through considerable induced dipole–induced dipole interaction, this may result in the greater energy required for the displacement of alcohol molecules as compared to the energy gained from the association of water molecules. Because of the smaller size of the water molecules, it is possible that several water molecules solvate the sulfamethazine molecule as compared to the fewer number of alcohol molecules displaced. The negative ΔS_b^* values suggest that the negative entropy of solvation dominated the positive entropy of disorder resulting from the displacement of alcohol molecules.

These considerations may also suggest an explanation for the observation that the ΔH_f^* and ΔS_f^* values recorded in Table V are greater than the corresponding ΔH_b^* and ΔS_b^* values. It is possible that the number of water molecules released in forming the activated complex for forward transfer is greater than the number of alcohol molecules displaced in forming the activated complex for back transfer. Furthermore, it is possible that the association of water molecules during the back-transfer process is greater than the association of alcohol molecules during the forward-transfer process.

Based on these considerations, the variations in the magnitude of the ΔH^* and ΔS^* values (Table V) for the interphase transfer of sulfamethazine in various aqueous–alcohol systems are the result of differences in the escaping tendency of the alcohols into the interface and the influence of this parameter on the degree of hydrogen bonding and hydrophobic interaction between solute and solvate molecules in the various systems. Thus, despite small variations in the mole fraction of alcohol in the various alcohol phases (Table II), the data in Table V suggest that the escaping tendency of alcohol is a primary factor in differentiating the thermodynamic parameters when comparing the interphase transfer of sulfamethazine in systems containing octanol–octane, heptyl alcohol–heptane, and pentyl alcohol–pentane or when comparing systems containing octanol and heptyl alcohol. Since the escaping tendency of alcohol and water molecules into the interface increases as the alcohol chain length decreases, the possibility of interaction between solute and solvate molecules in forming the activated complexes for interphase transfer improves as the chain length decreases. Therefore, it is postulated that the ΔH_f^* values decrease as the alcohol chain length decreases due to the increasingly negative enthalpy contributions arising from the increasing frequency of hydrogen bond interactions between solute and alcohol molecules for a given unit of interfacial region. Contrary to this observation is the possibility that the positive ΔH_f^* contribution might be expected to increase with the increased frequency of solute and solvate interaction due to the liberation of water molecules upon hydrophobic interaction. However, it is postulated that most of the additional alcohol molecules do not interact with the nonpolar methyl groups of the sulfamethazine molecule but rather with the aromatic moieties. This is not expected to result in the release of a significant number of

$$S_{sw} \rightleftharpoons S_{psw}$$
$$S_{psw} + \mathrm{ROH} \rightleftharpoons [S_{psw}\text{-ROH}] \longrightarrow S_{sa}$$

Scheme I—Transfer from water to alcohol

$$S_{sa} \rightleftharpoons S_{psa}$$
$$S_{psa} + \mathrm{HOH} \rightleftharpoons [S_{psa}\text{-HOH}] \longrightarrow S_{sw}$$

Scheme II—Transfer from alcohol to water

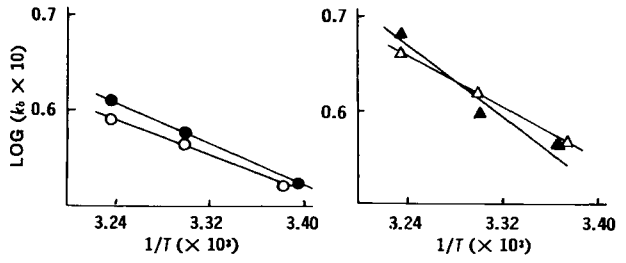

Figure 2—*Arrhenius plots of the transfer of sulfamethazine from various alcohol phases to an aqueous pH 4.3 buffer. Key: ●, octanol; ○, heptyl alcohol; ▲, octanol–octane; and △, heptyl alcohol–heptane.*

water molecules from the partially desolvated sulfonamide molecules because the aromatic moieties do not promote iceberg formation to the same extent as do alkyl groups (17, 18). Thus, this endothermic contribution is considered to be small in comparison to the exothermic interaction resulting from the hydrogen bonding of alcohol molecules. This increase in the number of interactions with decreases in alcohol chain length also results in a decrease in the ΔS_f^* values due to the increasingly negative entropy contribution arising from the constraint placed upon the interacting solute and solvate molecules. This trend is reinforced by a decrease in the opposing ΔS_f^* contribution (resulting from the release of water molecules) as the alcohol chain length decreases.

For back transfer, the escaping tendency of water molecules into the interface increases, and the possibility of interaction between solute and water molecules thus increases, as the alcohol chain length decreases. Therefore, the ΔH_b^* and ΔS_b^* values decrease as a result of the increasingly negative enthalpy and entropy contributions arising from the increasing frequency of solute–water association per unit of interface volume.

By using the data in Table V, it is possible to examine further the role of escaping tendency in the thermodynamics of the transfer process. The interphase transfer characteristics of sulfamethazine may be compared in two-phase systems in which the alcohol phase is composed of either an alcohol and its parent hydrocarbon or the alcohol alone. For this purpose, the data for the octanol–octane and the heptyl alcohol–heptane systems were compared to data obtained for the corresponding octanol and heptyl alcohol systems. In the case of forward transfer, the ΔH_f^* and ΔS_f^* values were greater for systems containing a given alcohol alone than for mixtures of the alcohol and its parent hydrocarbon. For back transfer, the pattern was reversed. The ΔH_b^* and ΔS_b^* values were greater for mixtures of a given alcohol and its parent hydrocarbon than for systems containing the alcohol phase alone.

These observations may be explained in terms of differences in the interface composition of the two types of systems. At the interface, the structural packing of heterogeneous components, especially when composed of molecules with dissimilar intermolecular bonding forces, is considered to be less rigid and coherent than a structure composed of homogeneous molecules (9). Therefore, a film of octanol molecules at the interface, which demonstrates intermolecular hydrogen bonding, is expected to be more coherent and rigid than a film composed of octanol and octane, since the latter demonstrates weaker dipole-induced dipole intermolecular forces. Therefore, based on these considerations, the escaping tendency of alcohol molecules into the interface can be expected to be greater from systems containing mixtures of a given alcohol and its parent hydrocarbon than from systems containing the alcohol alone.

In the case of forward transfer, the possibility of interaction between a partially desolvated sulfonamide molecule and alcohol molecules in forming the activated complex, as in Scheme I, is greater from systems containing the mixtures. This should result in lower ΔH_f^* and ΔS_f^* values for transfer into mixtures than into alcohol phases alone because of the increasingly negative enthalpy and entropy contributions arising from the increasing frequency of association of solute and alcohol molecules for a given unit of interface volume as discussed previously. For back transfer, the escaping tendency of water molecules into the interface is expected to be greater for systems containing a given alcohol alone than for a mixture of the alcohol and its parent hydrocarbon, as suggested by the interfacial tension data (Table IV). Furthermore, since the concentration of alcohol molecules in the interface is also greater for mixtures, the sulfamethazine molecule is expected to be less desolvated of alcohol molecules in mixtures. This should result in the displacement of more alcohol molecules upon formation of the activated complex for back transfer. Thus, the ΔH_b^* and ΔS_b^* values for the back transfer from single-component alcohol phases are expected to be less than for the corresponding phases composed of mixtures, as a result of the more negative enthalpy and entropy contributions arising from the increasing frequency of solute–water interaction in single-component systems as well as the more positive enthalpy and entropy contributions from the displacement of alcohol molecules from the solute in mixtures.

Since the solubility parameter (δ) is an index of the affinity of a solute for a solvent, Khalil and Martin (19) studied the interphase transfer of salicylic acid between water and various alkyl hydrocarbons, alkyl alcohols, and aromatic alcohols. They observed that the forward rate constants for salicylic acid increased as the difference in δ between the solute and solvent decreased. This pattern was not observed in the present study. Three alcohol phases, octanol, heptyl alcohol–heptane, and pentyl alcohol–pentane, were prepared in such a manner that the δ values for the three phases were similar at 25°: 10.63, 10.57, and 10.51, respectively (Table II). Although the range for δ values at 25° is only 1.1%, assuming that the change in δ values is negligible during the interphase transfer experiments, the range for the forward rate constants at 36° for transfer of sulfamethazine into each of the three alcohol phases was 48%, using the k_f value for octanol as a base (Table V). A comparison of the data for transfer into octanol and heptyl alcohol–heptane phases, with values of 10.63 and 10.57, respectively, shows that a 0.5% difference in δ values at 25° yields a 33% variation in k_f values at 36°, again using octanol as a base. These observations suggest that the role of the solubility parameter in interphase transfer is less important than properties of the system, such as the escaping tendency and the thermodynamic parameters associated with the interaction of solute and solvate.

An examination of Table VI corroborates the common observation that there is a relationship between interphase transfer rates and the partition coefficient. Although the net enthalpy and entropy for interphase transfer are positive, the partition coefficients and the ratio of forward to back rate constants (k_f/k_b) are greater than unity because the net entropic energy ($T/\Delta S$) exceeds the net enthalpy, resulting in a preferential transfer of unionized sulfamethazine from aqueous to alcohol phases. Thus, variations in the partition coefficient and the interphase transfer rates are related to variations in the degree of interaction of solute and solvate molecules during formation of the activated complex for forward and back transfer.

As noted previously (1), although the alcohol phases used in the simple model system employed do not simulate the complexity of biological membranes, the intermolecular forces influencing the partitioning of solutes between immiscible phases are expected to be the same forces influencing the permeation of solutes through cell membranes (8). Thus, these results are expected to provide some insight into the mechanisms for the passive transfer of solutes across aqueous–membrane interfaces.

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Measurement of Drug Displacement by Continuous Ultrafiltration

DAVID S. CAMPION[x] and ROBERT OLSEN

Abstract □ Adaptation of the continuous ultrafiltration technique to allow precise measurement of the displacement of one ligand by another from binding sites on human serum albumin is described. The displacement of sodium urate and methyl orange by sodium salicylate is demonstrated with analysis of the number of binding sites and association constants for these reactions.

Keyphrases □ Drug displacement from human serum albumin—measurement of sodium urate and methyl orange displacement by sodium salicylate using continuous ultrafiltration □ Human serum albumin binding—displacement of sodium urate and methyl orange by sodium salicylate, measured using continuous ultrafiltration □ Binding of sodium urate and methyl orange to human serum albumin—displaced by sodium salicylate, binding measured by continuous ultrafiltration □ Ultrafiltration, continuous—measurement of sodium urate and methyl orange displacement from human serum albumin by sodium salicylate

Displacement of one substance by another from shared binding sites on the albumin molecule is increasingly being recognized as an important factor modifying expected physiological and pharmacological activity. The introduction of a continuous ultrafiltration technique (1) greatly facilitated the study of ligand–protein interactions, and the present study extends this simple ultrafiltration technique to allow a precise study of drug displacement over a wide range of ligand concentration. The displacement from human serum albumin (albumin) of the ligands, sodium urate and methyl orange, sodium *p*-[(*p'*-dimethylaminophenyl)azo]benzenesulfonic acid, by sodium salicylate was used as a model, but the technique is useful over a wide range of interactions. The only requirement is that an appropriate membrane be available to retain the macromolecule in the ultrafiltration chamber.

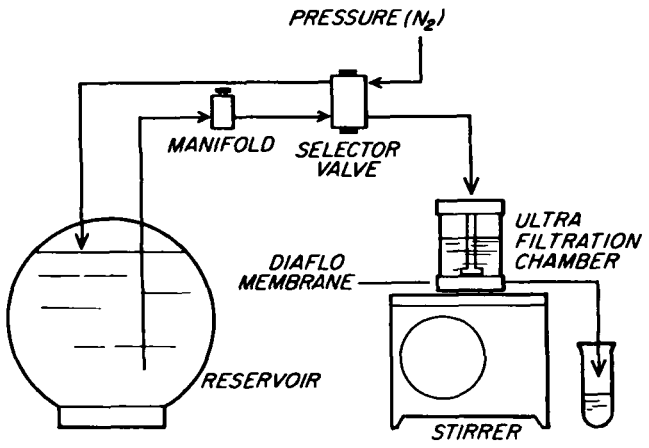

Figure 1—*Experimental setup, continuous filtration.*

EXPERIMENTAL

Crystallized human serum albumin[1] (electrophoretically pure, <0.05 m*M* of free fatty acids per m*M* of albumin) dried to constant weight was used in all studies. Methyl orange[2] was recrystallized from ethanol, dried to constant weight, and dissolved in phosphate buffer at pH 7.4. Sodium urate was made from uric acid triturated with 0.1 *N* NaOH and buffered with phosphate buffer to pH 7.4. The ionic strength of the final solutions was 0.16. Ultrafiltration was performed (Fig. 1) using an ultrafiltration chamber[3] connected through a concentration dialysis selector valve[4] to a nitrogen gas cylinder, which provided pressure for ultrafiltration, and to a 2.4-liter reservoir. A manifold in the line to the reservoir was used as a flow switch. During the experiment, since this was a closed system, the ultrafiltrate leaving the chamber was replaced by an identical volume of solution from the reservoir, with the chamber volume remaining constant. An ultrafiltration membrane[5] with a 10,000 molecular weight cut-off was used. All experiments were done at 22.5°. Methyl orange was

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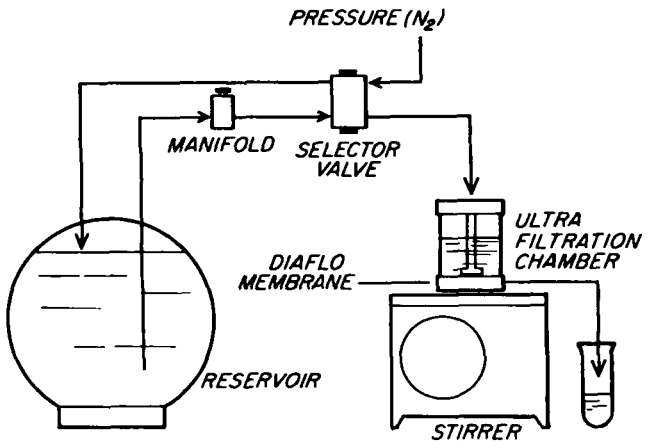

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Table I—Procedures to Establish Binding of Ligand *A* to Protein *P*, and Extent of Displacement by Competing Ligand *B*

Procedure	Ultrafiltration Chamber	Reservoir	Plot of (Total *A*) *versus* (Free *A*)	Interpretation
1	Buffer only	$(A)^a$	$dF/dT^b = 1$	No binding of *A* to membrane, no reflection of *A* at membrane
2	(B)	(A), $(B)^a$	$dF/dT = 1$	No effect of *B* on the ultrafiltration of *A*
3	(P)	(A)	$dF/dT < 1$	Binding of *A* by *P*
			$dF/dT = 1$	No binding of *A* by *P*
4	(P)	(B)	Ultrafiltration until (B) in ultrafiltrate equals (B) in reservoir	Establishes the equilibrium: $(P) + (B) \rightleftharpoons (PB)$
At the end of Step 4, the chamber now containing (P) and (PB) in equilibrium with (B) is connected to a new reservoir:				
5	(P), (PB), (B)	(A), (B)	$dF/dT > dF/dT$ of Step 3	Displacement of *A* by *B*
			$dF/dT = dF/dT$ of Step 3	No displacement of *A* by *B*

[a] (A) and (B) represent constant concentrations of the primary ligand *A* and competing ligand *B*, respectively. [b] $dF/dT = d(\text{free } A)/d(\text{total } A)$.

measured spectrophotometrically in the acid phase at 510 nm. Urate was measured using the method of Liddle *et al.* (2). Sodium salicylate[6] dissolved in pH 7.4 phosphate buffer, ionic strength of 0.16, was made as a standard solution of 0.25 and 0.5 m*M*.

Binding of Primary Ligand—The following ultrafiltration procedure was used to study the binding of the primary ligand, ligand *A* (in this case, sodium urate or methyl orange), to albumin. A known concentration of ligand *A* in buffer was placed in the reservoir (Fig. 1). The same reservoir solution *(R)* was then used in two separate continuous ultrafiltration experiments. In the first (Procedure 1, Table I), only buffer was in the ultrafiltration chamber; in the second (Procedure 3, Table I), an albumin solution *(P)* was in the chamber. Procedure 1 examines whether there is binding of sodium urate or methyl orange to the membrane or reflection of these ligands by the membrane. Correction for such effects may then be made in the analysis of Procedure 3 where binding of the ligand is examined.

Having filled the tubing and selector valves with the reservoir solution, the flow was stopped by the manifold switch and the ultrafiltration chamber connected by selector switch to the gas cylinder, allowing equal pressurization of the whole system. The selector valve was then changed to connect the reservoir to the chamber, the manifold switch was simultaneously opened, and ultrafiltration commenced. Ultrafiltration was carried out at 0.703 kg/cm² (10 lb/sq in.) pressure; the ultrafiltrate, which formed at the rate of 10–15 ml/hr, was collected in 2–4-ml volumes in previously dried and weighed tubes, the volume collected (v_i) being determined by reweighing (to four decimal places) and converting to volume, allowing for temperature effects on volume.

In essence, a series of separate ultrafiltration experiments at constant *(P)* but increasing concentration of ligand *A* were performed in sequence. However, in each collection period, the concentration of total, free, and bound *A* could be precisely calculated or measured. Since *(R)*, *(P)*, and the collection volumes (v_i) were accurately determined, the only limit on accuracy in this technique is ligand analysis.

Calculation of Primary Ligand-Albumin Interaction—Let v_i = volume in liters of the *i*th ultrafiltrate collected, V = volume of solvent in chamber in liters, *(R)* = ligand *A* concentration in the reservoir (moles per liter), and $(M)_i$ = ligand *A* concentration in the *i*th collection (moles per liter). Since this is a closed system, the volume of solution entering the chamber in the *i*th period exactly equals the volume leaving (v_i). Therefore:

$$\text{moles into chamber} = v_i(R) \quad \text{(Eq. 1)}$$

$$\text{moles out of chamber} = v_i(M)_i \quad \text{(Eq. 2)}$$

$$\text{moles retained} = v_i(R) - v_i(M)_i \quad \text{(Eq. 3)}$$

At the end of the *i*th period, the:

$$\text{increase in total concentration in chamber during } i\text{th period} = \frac{1}{V}[v_i(R) - v_i(M)_i] \quad \text{(Eq. 4)}$$

At the end of the *n*th period:

$$\text{total moles/liter in chamber} = \frac{1}{V}\sum_{i=1}^{n}[v_i(R) - v_i(M)_i] \quad \text{(Eq. 5)}$$

During the *n*th period, the:

$$\text{ligand } A \text{ concentration in ultrafiltrate collected} = \text{moles/liter } \textit{free} \text{ in chamber} \quad \text{(Eq. 6a)}$$

$$= (M)_n \quad \text{(Eq. 6b)}$$

$$= \frac{1}{V}\sum_{i=1}^{n}[v_i(R) - v_i(M)_i] - (M)_n \quad \text{(Eq. 7)}$$

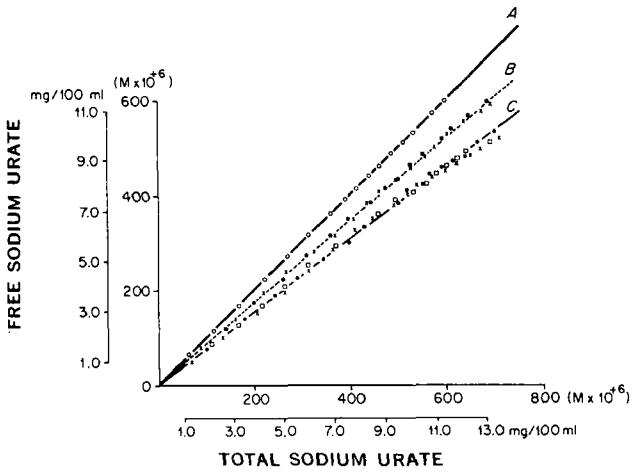

Figure 2—*Binding of sodium urate to albumin and its displacement by sodium salicylate. Key:* A, *buffer only in chamber (Procedure 1, Table I);* B, *5 g/100 ml albumin and 0.5 mM salicylate (Procedures 4 and 5); and* C, *5 g/100 ml albumin in chamber (Procedure 3). The conditions were: temperature, 22.5°; ionic strength, 0.16; and pH, 7.4.*

[6] Eastman Organic Chemicals.

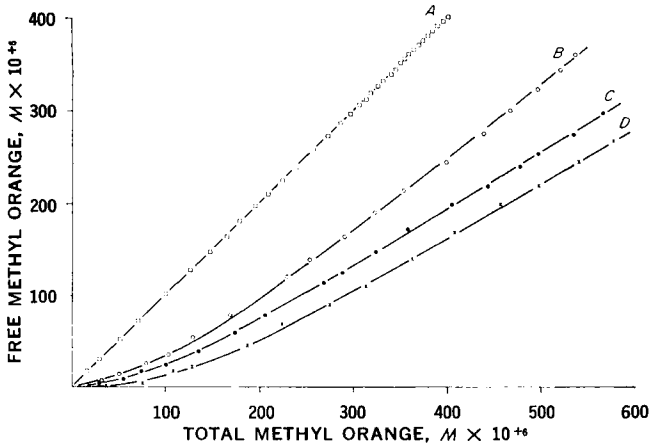

Figure 3—*Binding of methyl orange to albumin. Key:* A, *buffer only in chamber (Procedure 1, Table I);* B, *0.2 g/100 ml albumin and 2.5 mM salicylate (Procedures 4 and 5);* C, *0.2 g/100 ml albumin and 0.5 mM salicylate (Procedures 4 and 5); and* D, *0.2 g/100 ml albumin in chamber (Procedure 3). The conditions were: temperature, 22.5°; ionic strength, 0.16; and pH, 7.4.*

A plot may then be constructed of (total *A*) *versus* (free *A*).

Displacement of Primary Ligand *A* by Displacing Ligand *B*—It is necessary first to establish an equilibrium between ligand *B* and the protein *(P)* solution (Procedure 4, Table I). This is accomplished by connecting a reservoir containing only ligand *B* to the ultrafiltration chamber containing the protein solution *(P)*. Ultrafiltration is then carried out until the reaction *(B)* + *(P)* ⇄ *(PB)* reaches equilibrium. It is essential that at least three times the chamber volume be passed from the reservoir through the chamber. The protein and the *PB* complex are then in equilibrium with ligand *B* at the concentration of *B* in the reservoir. A simple test of this is that the *(B)* in the ultrafiltrate and the *(B)* in the reservoir are equal.

The reservoir and tubing are then filled with the ligand *A* solution but now also containing ligand *B* at the same concentration as was just used to equilibrate the protein. The ultrafiltration process is then carried out as described in the section *Binding of Primary Ligand,* and calculations of (total *A*), (free *A*), and (bound *A*) now reflect binding under a specified *free* concentration of ligand *B*. The total ligand *B* concentration to which this free level corresponds may be determined by measurement of (*B*) in the chamber, and (bound *B*) is obtained by subtraction of *(B)* in the reservoir or ultrafiltrate from (total *B*).

Since a competing ligand could conceivably have an effect on the ultrafiltration of the primary ligand, it is necessary in practice also to perform Procedure 2 (Table I) where the ultrafiltration of *A* in the presence of *B* is examined. A plot of (total *A*) *versus* (free *A*) from Procedure 2 should be identical to a similar plot of Procedure 1.

RESULTS

In the first series of experiments performed, ligand *A* was sodium urate and ligand *B* was sodium salicylate. Figure 2 clearly demonstrates the line of identity of free sodium urate *versus* total sodium urate (Procedure 1, Table I), indicating that there was no binding of sodium urate to the membrane and no sieving or reflection of sodium urate by the membrane. This line was identical in the experiment (Procedure 2) in which sodium urate plus salicylate was ultrafiltered. Sodium salicylate, therefore, had no effect on the process of ultrafiltering the sodium urate. With 5.0 g/100 ml albumin in the chamber (Procedure 3), the free sodium urate concentration *(F)* no longer equaled the total urate *(T)*, ($dF/dT < 1$), indicating binding of urate. After equilibrating 5.0 g/100 ml albumin with 0.5 m*M* sodium salicylate (Procedure 4) and exposing the albumin to 0.5 m*M* salicylate during the ultrafiltration with sodium urate in the reservoir (Procedure 5), less urate was bound to the albumin at all levels of total sodium urate than when salicylate was not present. This indicated displacement of sodium urate by salicylate.

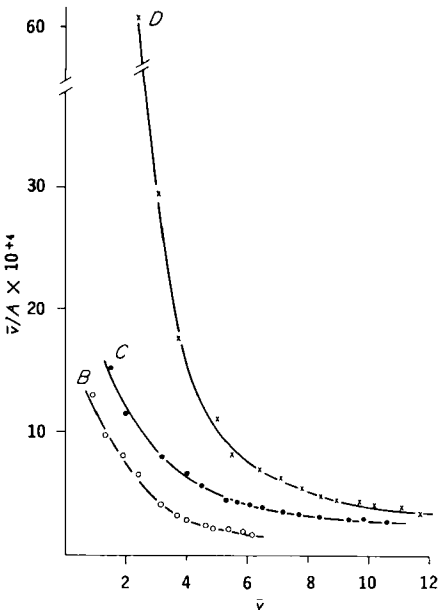

Figure 4—*Scatchard plot of methyl orange–albumin interaction, where* $\bar{v}$ = *moles bound per mole albumin, and* A = *free methyl orange. Points correspond to experimental points of Fig. 3. Key:* B, *without salicylate;* C, *0.5 mM salicylate; and* D, *2.5 mM salicylate. Line was computed from* n *and* k *values of Table II and formula in text (Eq. 8).*

Comparable experiments with methyl orange were performed, and Fig. 3 illustrates the line of identity of (total) *versus* (free) methyl orange, the binding of methyl orange to 0.2 g/100 ml albumin, and the displacing effect of 0.5 and 2.5 m*M* sodium salicylate.

DISCUSSION

The continuous ultrafiltration technique (1) allows, in a single ultrafiltration experiment, a precise investigation of ligand–macromolecule interaction over a wide range of ligand concentration. From one or two experiments, a Scatchard plot may then be constructed, analysis of which gives the number of binding sites *(n)* and their respective association constants *(k)*. From the latter, the free energy of reaction is derived so that precise information as to the molecular interaction is obtained. The value of the Scatchard plot in analyzing the factors responsible for decreased binding was recently discussed (3).

Part of a Scatchard plot derived from a single methyl orange–albumin experiment, together with those plots determined in the presence of 0.5 and 2.5 m*M* salicylate, is given in Fig. 4. These

Table II—Number of Binding Sites (*n*) and Association Constants (*k*) of Methyl Orange–Albumin and Sodium Urate–Albumin Interaction at 22.5°, Ionic Strength 0.16, and pH 7.4

Interaction	n_1	k_1	n_2	k_2
Methyl orange–albumin	3.5	37×10^4	13.5	3.7×10^3
Methyl orange–albumin + 0.5 m*M* sodium salicylate	2.5	12.4×10^4	14	2.8×10^3
Methyl orange–albumin + 2.5 m*M* sodium salicylate	2.0	6.8×10^4	10	2.5×10^3
Sodium urate–albumin	1.0	4.5×10^2	—	—
Sodium urate–albumin + 0.5 m*M* sodium salicylate	1.0	2.1×10^2	—	—

curves were interpreted in terms of two sets of binding sites, and the derived n and k values are listed in Table II. The points on Fig. 4 are experimental; the lines are drawn from the equation:

$$\frac{\bar{v}}{A} = \frac{n_1 k_1}{1 + k_1 A} + \frac{n_2 k_2}{1 + k_2 A} \qquad \text{(Eq. 8)}$$

using the derived n and k values. It is clear that the addition of salicylate at a free concentration of 0.5 mM affects the first set of binding sites; at the higher concentrations of free salicylate (2.5 mM), methyl orange is displaced from both the first and second sets of binding sites. The urate albumin interaction was analyzed previously (4), and the effect of 0.5 mM sodium salicylate on the derived n and k values is shown in Table II.

After determining n and k values, it is possible to predict fractional binding at any concentration of protein and ligand. The fraction bound (β) is given by:

$$\beta = \frac{1}{1 + \frac{1}{(P)nk} + \frac{(A)}{n(P)}} \qquad \text{(Eq. 9)}$$

(P) and (A) being protein and free ligand concentrations, respectively.

Preequilibration of the protein with the competing ligand (ligand B) (Procedure 4, Table I) is necessary, because useful data on displacement may only be obtained when the binding of the primary ligand is studied in the presence of a constant concentration of the competing ligand.

A full discussion of the analyses that allow the association constant of the displacing ligand (ligand B), in this case of salicylate–albumin interaction, to be determined was made by Edsall and Wyman (5).

This method is ideally suited to the *in vitro* determination of drug–protein interaction and drug displacement. It is fast, and analysis of a few experiments yields as much information on the molecular interactions involved as many conventional ultrafiltration, equilibrium dialysis, or gel filtration experiments. Adequate analysis of the data allows a precise prediction of free drug concentration to be made in the presence of varying concentrations of displacing compounds. The results, if indicating displacement, may also alert one clinically to potentiation of action and/or to toxicity when the therapeutic regimen requires the patient to take more than one drug.

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*To whom inquiries should be directed.

2-Nitrophenylhydrazine: A Selective Reagent for Colorimetric Determination of Carboxylic Acid Anhydrides and Chlorides

JAMES W. MUNSON

Abstract □ Carboxylic acid anhydrides and chlorides react with 2-nitrophenylhydrazine to form the corresponding hydrazides. These hydrazides in aqueous basic solution give a blue color which can be utilized for colorimetric analysis. Both acid anhydrides and acid chlorides undergo this reaction in water, while only the acid chlorides react readily in acetonitrile. The use of acetonitrile as the solvent allows the analysis of acid chlorides in the presence of acid anhydrides. A variety of other carboxylic acid derivatives and carbonyl compounds gave no interference. Suggested analytical procedures for acid anhydrides and acid chlorides are given along with analytical data.

Keyphrases □ 2-Nitrophenylhydrazine—colorimetric reagent for determination of carboxylic acid anhydrides and chlorides □ Carboxylic acid anhydrides and chlorides—colorimetric determination using 2-nitrophenylhydrazine □ Colorimetry—determination, carboxylic acid anhydrides and chlorides using 2-nitrophenylhydrazine

The analysis of carboxylic acid anhydrides and carboxylic acid chlorides has generally been accomplished by one of three methods: direct titration, condensation with a nucleophile and subsequent back-titration of excess nucleophile, or condensation with a nucleophile and subsequent spectrophotometric determination of the product.

Carboxylic acid anhydrides have been titrated directly with sodium methoxide in a nonaqueous medium (1). Carboxylic acid chlorides are readily hydrolyzed to the parent carboxylic acid with the addition of water. The parent acid can be then titrated directly with a standard base. However, any mineral acid present, such as hydrochloric acid, will also be titrated and lead to higher values (2). Acid anhydrides are also hydrolyzed by water, but the reaction is slower than that of acid chlorides.

Anhydrides have been determined by condensation with excess aniline to form the anilide. The unreacted aniline is determined *via* titration with a standard acid (3). An interference encountered in this procedure is the formation of anilides from carboxylic acids which may be present. Other nucleophiles such

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This method is ideally suited to the *in vitro* determination of drug–protein interaction and drug displacement. It is fast, and analysis of a few experiments yields as much information on the molecular interactions involved as many conventional ultrafiltration, equilibrium dialysis, or gel filtration experiments. Adequate analysis of the data allows a precise prediction of free drug concentration to be made in the presence of varying concentrations of displacing compounds. The results, if indicating displacement, may also alert one clinically to potentiation of action and/or to toxicity when the therapeutic regimen requires the patient to take more than one drug.

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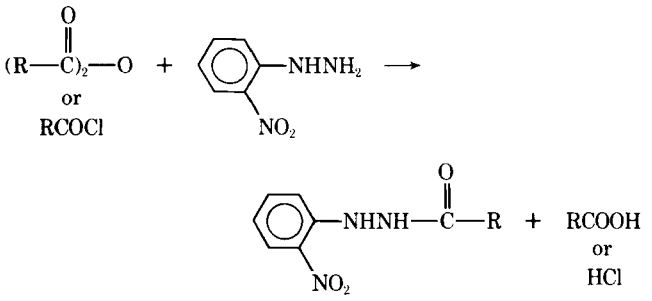


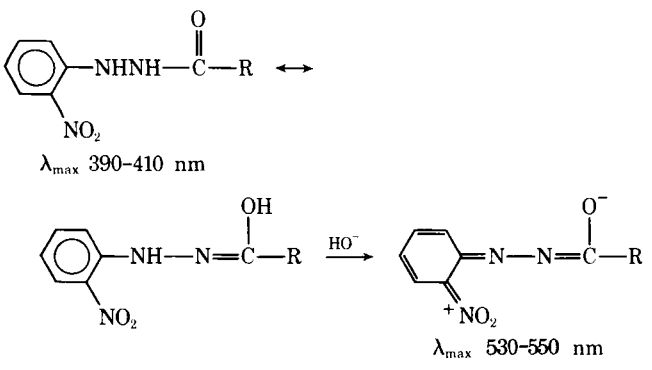
Scheme I

as *p*-nitroaniline (4) and 2,4-dichloroaniline (5) have been used to minimize this interference. Morpholine has also been used successfully as a nucleophile, with the excess morpholine being titrated with a standard methanolic hydrochloric acid solution (6). Moreover, anhydrides have been determined by reaction with excess *m*-chloroaniline and determination of the unreacted amine by titration with sodium nitrite (7).

Acid anhydrides and acid chlorides have also been determined by the conventional ferric hydroxamate procedure (8). A neutral hydroxylamine reagent is preferred because esters and amides do not react readily under these conditions. However, higher yields were obtained when an alkaline hydroxylamine reagent was used. Acid anhydrides, acid chlorides, and phosgene have been determined by reaction with a known excess of piperidine (9). The excess piperidine was determined by reaction with 3,5-dinitrobenzoyl chloride and subsequent colorimetric determination of the 3,5-dinitrobenzamide.

A fluorescence analysis for acid chlorides and acid anhydrides involves condensation of the substrate with 2-carboxyisonitrosoacetanilide in an alkaline aqueous acetone medium (10). The resulting products fluoresce when exposed to UV light. This method is suitable for measuring small quantities (1×10^{-12} mole) of substrate. However, the fluorescence produced is not stable.

Legradi (11) recently reported that acid anhydrides and acid chlorides react with 2-nitrophenylhydrazine to form a blue color in basic media. It was also reported that esters, imides, amides, and carboxylic acids do not react under the same conditions. The reactions involved are shown in Scheme I. The resulting hydrazide ionizes in basic solution to give a blue color. A resonance form of the chromophore is given in Scheme II (11). In acidic solutions, these hydrazides exhibit an absorption maximum between 390 and 410 nm; in basic solutions, the maxima are shifted to 530–550 nm.



Scheme II

This paper describes the development of an analytical method for acid anhydrides and acid chlorides utilizing this reaction. A method for the determination of acid chlorides in the presence of acid anhydrides is also described.

EXPERIMENTAL[1]

Materials—Unless otherwise stated, chemicals were analytical reagent grade and were used directly. All water was redistilled from alkaline permanganate. Standard pH buffer solutions were prepared according to Bates (12). Acetonitrile[2] was redistilled from phosphorus pentoxide; the fraction boiling at 81.5° was collected. Acetone[3] was refluxed with potassium permanganate, dried with anhydrous sodium sulfate, and redistilled, bp 56° [lit. (13) bp 56–60°].

2-Nitrophenylhydrazine hydrochloride[2] was recrystallized from methanol, mp 195–199° dec.

Anal.—Calc. for $C_6H_7N_3O_2 \cdot HCl$: C, 38.00; H, 4.26; N, 22.16. Found: C, 38.26; H, 4.37; N, 21.97.

2-Nitrophenylhydrazine[2], mp 90° [lit. (11) mp 90°] was used without further purification. Acetamide[4] was recrystallized from benzene, mp 81° [lit. (14) mp 81°]. Benzoic anhydride[5] was recrystallized from benzene–methanol, mp 41–42° [lit. (15) mp 42°]. Phthalic anhydride[2] was recrystallized from an ether–methanol solution, mp 128° [lit. (16) mp 132°]. Succinimide[4] was recrystallized from methanol, mp 124° [lit. (17) mp 123–125°]. *p*-Nitrophenyl acetate was synthesized by the method of Chattaway (18), mp 81–82° [lit. (18) mp 83°]. Succinic anhydride[5] was recrystallized from an ether–methanol solution, mp 116–117° [lit. (19) mp 119°]. Maleic anhydride[6], mp 52–53° [lit. (20) mp 52°], was used without further purification.

The following liquids were redistilled at atmospheric pressure: acetyl chloride[4], bp 51–52° [lit. (21) bp 50–55°]; acetic anhydride[4], bp 137–138° [lit. (22) bp 139°]; acetophenone[5], bp 198–200° [lit. (23) bp 201°]; benzaldehyde[3], bp 176–177° [lit. (24) bp 175–180°]; benzoyl chloride[7], bp 194° [lit. (21) bp 194–196°]; butyrolactone[5], bp 203–204° [lit (25) bp 206°]; ethyl acetate[4], bp 76° [lit. (26) bp 77°]; phthaloyl dichloride[2], bp 271° [lit. (27) bp 281°]; pivaloyl chloride[5], bp 104° [lit. (28) bp 103–104°]; propionic anhydride[2], bp 162° [lit. (29) bp 168°]; and propionyl chloride[2], bp 78–79° [lit. (21) bp 75–80°]. Thionyl chloride[6], *p*-toluoyl chloride[2], *p*-toluenesulfonyl chloride[2], and sodium perchlorate[8] were used directly.

Synthesis of Acetyl- and Phthalyl-2′-nitrophenylhydrazides—Acetyl-2′-nitrophenylhydrazide and phthalyl-2′-nitrophenylhydrazide were synthesized by the following method. The appropriate anhydride (0.01 mole) was dissolved in 40 ml of benzene, and 0.015 mole of 2-nitrophenylhydrazine was suspended in 25 ml of benzene. The anhydride solution was added dropwise to the stirred suspension of 2-nitrophenylhydrazine. The resultant mixture was allowed to stand at room temperature for 30 min. The precipitated hydrazide was collected on a filter, washed with benzene and 0.1 *N* hydrochloric acid, and recrystallized from a methanol–water mixture.

1. Acetyl-2′-nitrophenylhydrazide, mp 140–141° [lit. (30) mp 140°].

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[1] All melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are corrected. Boiling points are uncorrected. Spectral measurements were made with either a Cary model 15 or a Beckman model DU spectrophotometer. The Cary model 15 was fitted with a thermostated cell compartment that maintained the temperature constant to ±0.1° with a circulating water bath (Precision Scientific Co.). Thermometers for use at 25.0° were calibrated against a thermometer carrying an ASTM certificate. The pH measurements were made with a Radiometer pH meter model 52 equipped with a Radiometer combination electrode (GK2321C). All elemental analyses were performed by Baron Consulting Co., Orange, Conn.

[2] Aldrich Chemical Co.
[3] J. T. Baker Chemical Co.
[4] Allied Chemical Co.
[5] Eastman Organic Chemicals.
[6] Fisher Chemical Co.
[7] Matheson, Coleman and Bell.
[8] G. F. Smith Chemical Co.

Table I—Effect of Solvent Composition on Reaction of Phthalic Anhydride and 2-Nitrophenylhydrazine

Solvent[a]	t_∞[b], sec
Water	300
Methanol	1500
Ethanol	1500
2-Propanol	2500
tert-Butanol	3250
Acetonitrile	—[c]

[a] Solvent composition is: methanol 5 ml, acetonitrile 2 ml, solvent listed—add a sufficient quantity to make 25 ml. [b] Time required to reach maximum absorbance. Conditions were: 2-nitrophenylhydrazine, 0.005 *M*; and phthalic anhydride, 0.0001 *M*. [c] No reaction within 2 hr.

Anal.—Calc. for $C_8H_9N_3O_3$: C, 49.22; H, 4.66; N, 21.53. Found: C, 49.44; H, 4.93; N, 21.80.

2. Phthalyl-2′-nitrophenylhydrazide, mp 291° [lit. (31) mp 293–294°].

Anal.—Calc. for $C_{14}H_{11}N_3O_5$: C, 55.81; H, 3.69; N, 13.95. Found: C, 56.05; H, 3.76; N, 13.92.

Kinetics of 2-Nitrophenylhydrazine Decomposition—The decomposition of 2-nitrophenylhydrazine in aqueous solutions was followed by measuring the decrease in absorbance at 435 nm as a function of time. Pseudo-first-order rate constants were calculated from plots of log $(A_\infty - A_t)$ *versus* time, where A_∞ = the absorbance at infinity, and A_t = the absorbance at the time interval taken. Small contributions of the buffer to the decomposition of the substrate were eliminated by measuring the rate at different buffer concentrations and extrapolating to the rate constant at zero buffer concentration. All solutions were adjusted to an ionic strength of 0.15 *M* with sodium perchlorate.

Effect of Solvent on Extent of Reaction—The effect of solvent on the extent of reaction was measured by reacting phthalic anhydride with 2-nitrophenylhydrazine in various solvent mixtures. One-milliliter aliquots of the reaction mixture were removed as a function of time and made basic with sodium hydroxide. The final concentration of hydroxide was approximately 0.1 *M*. The absorbances of the final solutions were measured at 550 nm. Solvent compositions are listed in Table I.

Determination of Reaction Time and Reaction Yields for Anhydrides—Reaction mixtures were prepared by mixing appropriate volumes of stock solutions of 2-nitrophenylhydrazine hydrochloride, phosphate buffer, anhydride, and water. Stock solutions of anhydrides were freshly prepared in acetonitrile. One-milliliter aliquots were removed and diluted to 10 ml with an aqueous sodium hydroxide solution, the final concentration of hydroxide being 0.1 *M*. The visible spectra of these solutions were measured to determine the wavelength of maximum absorbance for each anhydride. All reactions were carried out using the following conditions: 2-nitrophenylhydrazine, 0.0023 *M*; phosphate buffer, 0.005 *M*; anhydride, approximately 0.0001 *M*; pH 6.10 (±0.05); and 25°. Reaction yields were determined by reacting different concentrations of the anhydride for a sufficient time, removing an aliquot, making it basic, and measuring the absorbance at an appropriate wavelength. The absorbances were plotted against the apparent concentration of hydrazide in the spectral solution (complete conversion of anhydride to hydrazide was assumed). At least four different substrate concentrations were used for each anhydride.

Determination of Reaction Time and Reaction Yields for Acid Chlorides—Reaction mixtures were prepared by mixing stock solutions of 2-nitrophenylhydrazine (in acetonitrile), acid chloride (freshly prepared in acetonitrile), and acetonitrile. One-milliliter aliquots were removed as a function of time and diluted to 10 ml with an aqueous sodium hydroxide solution, giving a final hydroxide concentration of 0.1 *M*. The visible absorption spectra of these solutions were measured to determine the wavelength of maximum absorption. Reaction yields were obtained by plotting absorbance against apparent concentration of hydrazide in the spectral solution (complete conversion of the acid chloride to hydrazide was assumed). At least four different substrate concentrations were used for each acid chloride. All reactions were carried out under the following conditions: 2-nitrophenylhydrazine, 0.004 *M*; acid chloride, approximately 0.0001 *M*; and 25°.

Table II—Reaction of Acid Anhydrides with 2-Nitrophenylhydrazine in Aqueous Solution

Substrate	t_∞[a], min	λ_{max}, nm	Apparent Molar Absorptivity[b], liters mole^{-1} cm^{-1}
Acetic anhydride	<2	530	5710
Phthalic anhydride	<2	550	7330
Maleic anhydride	4	550	3500
Propionic anhydride	10	530	4800
Succinic anhydride	10	530	1330
Benzoic anhydride	60	540	1190

[a] Time required for maximum color yield. [b] In 0.1 *N* sodium hydroxide. Conditions were: 2-nitrophenylhydrazine, 0.0023 *M*; phosphate buffer, 0.005 *M*; pH 6.10 (±0.05); and 25°.

Determination of Interferences—The possibility of interference by other carboxylic acid derivatives and carbonyl compounds was investigated by reacting various substrates in the aqueous and acetonitrile systems. Aliquots were removed at 30 and 60 min and made basic. The appearance of a blue color (or absorbance between 520 and 570 nm) was used to indicate interference. Concentrations of substrates used were 10–100 times greater than those used in determining reaction yields for the anhydrides and acid chlorides.

Investigation of Decomposition Products of 2-Nitrophenylhydrazine—One hundred milligrams of 2-nitrophenylhydrazine was dissolved in 50 ml distilled water. A saturated sodium hydroxide solution was added dropwise until the solution was decolorized. The solution was adjusted to pH 7 and extracted with three 25-ml portions of ether. The ether fractions were combined, dried with anhydrous sodium sulfate, and evaporated to dryness. The aqueous fraction was made basic (pH 10–11) and the extraction procedure was repeated. The aqueous fraction was then made acidic (pH 1–2) and extracted with ether as before. Only the acidic fraction yielded a significant residue. This residue was recrystallized from hot water, yielding white needles melting at 155° and having the following elemental composition: C, 53.55%; H, 3.69%; and N, 30.08%.

Suggested Analytical Procedures—*Anhydrides*—Prepare the reaction mixture by mixing 5.0 ml of a stock solution of 2-nitrophenylhydrazine hydrochloride (0.110 g in 50.0 ml methanol), 5.0 ml of phosphate buffer solution (1.69 g of dried reagent grade potassium dihydrogen phosphate, KH_2PO_4, and 1.76 g of dried reagent grade disodium hydrogen phosphate, Na_2HPO_4, dissolved in 1000 ml distilled water), and sufficient distilled water to make 25.0 ml. Pipet 5.0 ml of the reaction mixture into a 10.0-ml volumetric flask. Add 1.0 ml of a suitably diluted solution of the anhydride, mix thoroughly, and allow to stand at 25° for the appropriate time (Table II). Add 1.0 ml of 1.0 *N* sodium hydroxide and dilute to 10.0 ml with distilled water. Measure absorbance at the appropriate wavelength.

Table III—Reaction of Acid Chlorides with 2-Nitrophenylhydrazine in Acetonitrile

Substrate	t_∞[a], min	λ_{max}, nm	Apparent Molar Absorptivity[b], liters mole^{-1} cm^{-1}
Acetyl chloride	<2	530	7908
Propionyl chloride	<2	530	6560
Pivaloyl chloride	3	530	5990
Phthaloyl chloride	4	550	7420
Benzoyl chloride	6	540	9090
p-Toluoyl chloride	9	540	8910

[a] Time required for maximum reaction. [b] In 0.1 *N* sodium hydroxide (aqueous). Conditions were: 2-nitrophenylhydrazine, 0.004 *M*; and 25°.

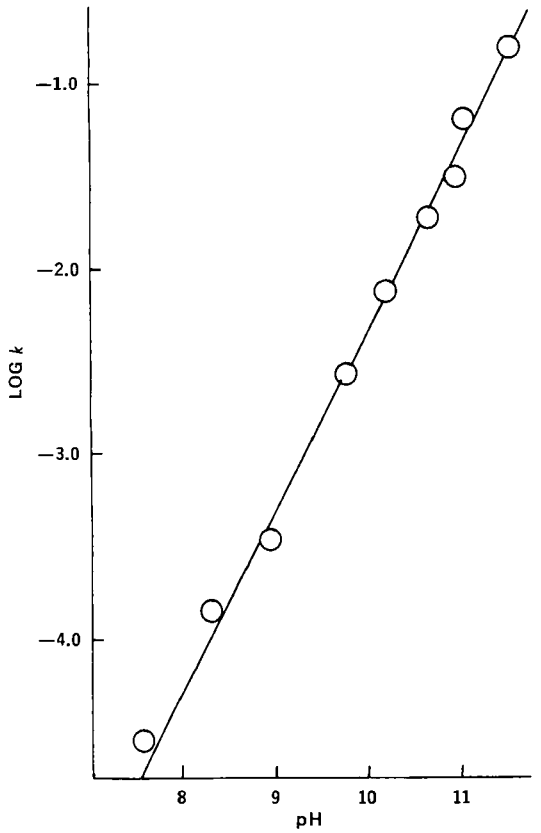

Figure 1—*The pH–rate profile for the decomposition of 2-nitrophenylhydrazine in aqueous solutions at 25.0° and ionic strength of 0.15* M.

Acid Chlorides—Pipet 2.0 ml of a 0.04 *M* solution of 2-nitrophenylhydrazine in acetonitrile into a 10.0-ml volumetric flask. Pipet 2.0 ml of a suitably diluted solution of the acid chloride in acetonitrile into the flask. Mix and allow to react for the appropriate time at 25° (Table III). Add 1.0 ml of 1.0 *N* sodium hydroxide and dilute to volume with distilled water. Measure the absorbance at the appropriate wavelength.

RESULTS AND DISCUSSION

Effect of Solvent on Extent of Reaction—Table I gives the effect of different solvents on the reaction of phthalic anhydride and 2-nitrophenylhydrazine (I). Since the solvolysis of the substrate was a possible competing reaction in the analysis, this study was performed to find a solvent that would give a minimum amount of solvolysis while not severely lowering the reaction rate. The reaction rate decreased with a decrease in the dielectric constant of the solvent within the hydroxylic solvent series. However, yields of the 2-nitrophenylhydrazide were not significantly different. Therefore, water was used as the solvent for the rest of the anhydride studies. Acetonitrile (an aprotic solvent) gave no reaction within 2 hr; this finding was utilized in later experiments on the analysis of the acid chlorides. Further work on the solvent requirement for the reaction is currently underway.

Table IV—Reaction of Anhydrides with 2-Nitrophenylhydrazine in Acetonitrile

Substrate	Half-Life[a], hr
Acetic anhydride	10
Propionic anhydride	10
Maleic anhydride	44
Phthalic anhydride	90
Succinic anhydride	>150
Benzoic anhydride	>150

[a] Taken from plots of absorbance *versus* time. Conditions were: 2-nitrophenylhydrazine, 0.004 *M*; and 25°.

Table V—Compounds Not Reacting within 1 hr at 25° in Either the Aqueous or Acetonitrile System

Acetone[a]	*p*-Nitrophenyl acetate
Acetophenone[a]	Butyrolactone
Benzaldehyde[a]	Acetamide
Succinimide	Thionyl chloride
Ethyl acetate	*p*-Toluenesulfonyl chloride

[a] Reacted to form precipitates but did not produce a blue color in aqueous sodium hydroxide. For other possible interferences, consult Ref. 37.

Kinetics of 2-Nitrophenylhydrazine Decomposition—In early investigations, it was found that I in solution underwent a decoloration from orange to colorless as the solution is made basic. Addition of an acid failed to reverse this color change, suggesting that a decomposition is involved rather than an acid–base equilibrium. A partial pH–rate profile for the decomposition of I in aqueous solutions is given in Fig. 1. The slope of the line indicates that the reaction is first order with respect to hydroxide. The only product isolated from the basic degradation mixture is consistent with the compound 1-hydroxy-1,2,3-benzotriazole, mp 155° [lit. (32) mp 159–160°].

Anal.—Calc. for $C_6H_5N_3O$: C, 53.33; H, 3.74; N, 31.09. Found: C, 53.55; H, 3.69; N, 30.88.

The presence of this compound is consistent with the findings of Nietzki and Braunschweig (33). A similar product, 6-nitro-1-hydroxy-1,2,3-benzotriazole, was isolated as a basic decomposition product of 2,4-dinitrophenylhydrazine (34). However, in the case of 2,4-dinitrophenylhydrazine, dinitrobenzene and *m,m'*-dinitroazoxybenzene were also isolated. For a review of the condensation reactions of I and related compounds, the reader is referred to Ref. 35.

The rapid decomposition of I at basic pH presents a potential problem because it would be difficult to maintain a sufficient concentration of nucleophile under these conditions. The pKa for I was found to be 3.46 at 25° by potentiometric titration. This value agrees well with a literature value of 3.50 (36). Because of this low pKa, I will be essentially in the free base form at pH values as low as 6 or 7. From Fig. 1, it can be seen that this compound is reasonably stable at these pH values. Therefore, all further investigations of I reactions in water were carried out between pH 6 and 7. An additional benefit of working at this pH is that the hydroxide-catalyzed hydrolysis of the anhydrides is minimized.

Reaction Time and Yields for Anhydrides—The results of the study of the reaction of anhydrides and I are given in Table II. Multiple determination of the apparent molar absorptivity of the acetic anhydride reaction gave excellent agreement (5710 ± 100 liters $mole^{-1}$ cm^{-1}). The molar absorptivities of acetyl-2′-nitrophenylhydrazide and phthalyl-2′-nitrophenylhydrazide in 0.1 *N* sodium hydroxide were found to be 6850 and 8570 liters $mole^{-1}$ cm^{-1}, respectively. These values indicate that yields are less than quantitative, probably due to a competing hydrolysis reaction, and that standards should be run with each unknown. An attempt to analyze cinnamic anhydride under these conditions was not successful due to the insolubility of the substrate. The color of the basic solutions was stable for at least 3 hr.

Table VI—Calibration Data for Determination of Acetic Anhydride by Reaction with 2-Nitrophenylhydrazine in Aqueous Solution

10^4C, M[a]	A_{530}[b]	$\bar{A}_{530}$
0.336	0.175, 0.175, 0.175	0.173
0.672	0.370, 0.378, 0.378	0.375
0.840	0.480, 0.480, 0.480	0.480
1.008	0.570, 0.581, 0.583	0.578
1.681	0.946, 0.953, 0.958	0.952
2.689	1.501, 1.501, 1.575	1.526

[a] Concentration in the spectral solution. This represents a 10-fold dilution of the original anhydride solution. [b] One-centimeter cells.

Table VII—Calibration Data for Determination of Acetyl Chloride by Reaction with 2-Nitrophenylhydrazine in Acetonitrile

10^4C, M[a]	A_{530}[b]	$\bar{A}_{530}$
0.298	0.246, 0.240, 0.270	0.252
0.349	0.255, 0.257, 0.260	0.257
0.596	0.485, 0.500, 0.495	0.493
0.948	0.760, 0.758, 0.756	0.758
1.190	0.928, 0.925, 0.925	0.926
1.49	1.203, 1.208, 1.195	1.202
1.75	1.367, 1.390	1.379

[a] Concentration in spectral solution. This represents a fivefold dilution of the original acetyl chloride solution. [b] One-centimeter cells.

Acid chlorides also react under these conditions, but the yields are very low, probably due to rapid hydrolysis of the substrate. No further work was carried out on the reaction of acid chlorides in water.

Reaction of Acid Chlorides with 2-Nitrophenylhydrazine in Acetonitrile—Table III presents the results of the reaction of various acid chlorides and I in acetonitrile. The apparent molar absorptivity of the product of the acetyl chloride reaction is slightly higher than that of acetyl-2′-nitrophenylhydrazide. While the yields are very reproducible, this slightly higher value (7908 liters $mole^{-1}$ cm^{-1}) is unexplained at this time. The reaction products of the acetonitrile reaction are currently under investigation. As a product is formed in this reaction, an equivalent amount of 2-nitrophenylhydrazine hydrochloride is formed which precipitates from solution. This effect is more noticeable at higher substrate concentrations but does not interfere in the analysis.

Reaction of Acid Anhydrides with 2-Nitrophenylhydrazine in Acetonitrile—Based on the early observation that phthalic anhydride did not react with I in acetonitrile, a study was carried out to measure the extent of the reaction of anhydrides in this system over a longer period. The large difference in the reaction times between acid chlorides and anhydrides in acetonitrile (Table IV) suggests that acid chlorides could be measured in the presence of anhydrides in an acetonitrile system with as little as 1% error. This analysis is feasible if the anhydride and acid chloride are of comparable concentrations. In the event that the anhydride is in a large excess of the acid chloride, the interference from the anhydride may become too great for accurate analysis.

Reaction of Other Compounds with 2-Nitrophenylhydrazine—A variety of carboxylic acid derivatives, carbonyl compounds, and related substrates were examined for reaction in both the aqueous and acetonitrile systems. Absorbance at 520–570 nm or the production of a blue color was used to indicate reaction. Table V lists those compounds that gave no reaction within 1 hr at 25°. The concentrations of substrates used were 10–100 times those used in the anhydride and acid chloride studies. Acetone, acetophenone, and benzaldehyde reacted with I in the aqueous system, as evidenced by the formation of a precipitate. However, no blue color was formed on the addition of sodium hydroxide. This reaction, probably hydrazone formation, represents a possible interference since it consumes nucleophile. Legradi (37) reported that acetyl and benzoyl acetone, substituted benzaldehydes, and formaldehyde reacted with I to give colors ranging from orange to violet.

Results of Suggested Analytical Procedures—*Anhydrides*—A variety of solutions of acetic anhydride (freshly prepared in acetonitrile) were subjected to the suggested procedure (Table VI). Over the concentration range used, the calibration curve is linear; the linear regression line for all data is $A_{530} = 5715.5C - 0.008$, with a correlation coefficient of 0.999 and a standard error of 0.016.

Acid Chlorides—The results of several determinations of acetyl chloride are presented in Table VII. The calibration curve is linear over this concentration range; the linear regression line for all data is $A_{530} = 7908C + 0.005$, with a correlation coefficient of 0.999 and a standard error of 0.018.

The reaction of I with acid chlorides and anhydrides is potentially useful for the analysis of these substrates. The low nucleophilicity and consequent high selectivity of the nucleophile make it an excellent choice for analysis of these substrates in the presence of other carboxylic acid derivatives. Since the nucleophile decomposes to colorless products under the final analysis conditions, large concentrations of it can be used without causing an interference in the final measurement.

Throughout this study, very low concentrations of nucleophile were used. Obviously, faster reactions can be achieved at higher nucleophile concentrations, but the possibility of interferences should be investigated under these conditions. However, solubility of I becomes a limiting factor in aqueous reaction mixtures.

Other functional groups that can be converted easily to acid chlorides and anhydrides may be analyzed using this reaction. Legradi (38) utilized this reaction as a spot test for acids after first converting the acid to acid chloride by the action of thionyl chloride. Higher molecular weight acids such as fatty acids were analyzed by this method in this laboratory. However, yields are not entirely reproducible and further work is in progress.

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Present address: College of Pharmacy, University of Kentucky, Lexington, KY 40506

Anticancer Activity of Zoanthids and the Associated Toxin, Palytoxin, against Ehrlich Ascites Tumor and P-388 Lymphocytic Leukemia in Mice

R. J. QUINN*, MIDORI KASHIWAGI‡, RICHARD E. MOORE*x, and TED R. NORTON‡

Abstract □ Crude alcoholic extracts of zoanthids, indigenous to Hawaii, have been shown to have general antitumor activity against Ehrlich ascites tumor in mice. The toxic component, palytoxin, was effective in the control and cure of Ehrlich ascites at extremely low dosages using several different methods of administration and displayed marginal activity against P-388 lymphocytic leukemia.

Keyphrases □ Zoanthids—antitumor activity of crude extracts, palytoxin as potent antitumor component □ Anticancer agents, potential—pharmacological testing of zoanthids and palytoxin against Ehrlich ascites tumor and P-388 leukemia □ Palytoxin—pharmacological studies as antitumor agent

Observations of antitumor activity in extracts of marine invertebrates (1–3), particularly the phylum Coelenterata (4, 5), have now been extended to extracts of a number of species belonging to the family Zoanthidae, order Zoantharia. The water-soluble materials, from ethanolic extracts of eight Hawaiian species representing three genera of zoanthids, have been found to inhibit growth of Ehrlich ascites carcinoma in Swiss Webster mice.

The specific Ehrlich ascites activity increased with increasing toxicity of the extracts, suggesting that the toxic component itself may be responsible for the antitumor activity. Palytoxin, the toxic principle of "limu-make-o-Hana," a zoanthid from Maui, Hawaii (6), which has been identified as *Palythoa toxica* Walsh and Bowers (7), was found to be a potent antitumor agent that completely controlled Ehrlich ascites carcinoma in mice at doses as low as 84 ng/kg and exhibited an inhibitory effect on the tumor in doses as low as 5.25 ng/kg. Several different modes of administration and injection schedules were used to evaluate its activity against Ehrlich ascites tumor. Palytoxin was tested also against P-388 lymphocytic leukemia in mice but showed only marginal activity.

RESULTS AND DISCUSSION

The activities of the crude extracts against Ehrlich ascites tumor are given in Table I. In this laboratory, during the past 5 years, more than 2500 mice were used as infected controls and none survived for as long as 30 days. Consequently, the presence of any survivors in the treated group at 30 days indicates significant activity. The absence of observable abdominal distension from ascitic fluid was the basis for describing survivors as nonascitic (8).

Since the specific Ehrlich ascites activity generally increased with increasing toxicity of the extracts, the antitumor activity may be primarily due to palytoxin. In this study, the most toxic and Ehrlich ascites-active sample was obtained from an unidentified zoanthid from Haleiwa, Oahu; nontoxic extracts, at least at the levels tested, with low Ehrlich ascites activity were obtained from *Zoanthus pacificus*, *Palythoa psammophilia*, and an unidentified zoanthid from a tidepool adjacent to Makapuu Beach, Oahu. The palytoxins from *Palythoa tuberculosa*, *Palythoa vestitus*, and the zoanthid from Haleiwa were found to have UV spectra identical with that of palytoxin from *P. toxica*. Whether the palytoxins from these various sources are all structurally identical or subtly different remains to be demonstrated. Recently, it was found that the toxicity of *P. tuberculosa* is seasonally dependent and, more importantly, is linked with the reproductive cycle of the zoanthid because the toxin appears to be associated with the bisexual and female polyps and is located in the eggs of the female polyps (9). This finding might explain the low toxicity of the specimen of *P. toxica* that was collected in September at the Halona Blowhole, Oahu, since all previous specimens of this organism had shown toxicities comparable to that of *P. toxica* from Muolea, Maui.

Only a few Hawaiian zoanthids have been adequately described in the literature (7), and all have been classified in the three genera *Palythoa*, *Zoanthus*, and *Isaurus*. In this work, six known and two new species of zoanthids[1] were collected and evaluated for anticancer activity. For a preliminary chemotaxonomic identification of each organism, the sterol complement of the zoanthid was isolated, analyzed by GLC and mass spectrometry, and compared with those from known zoanthids. The two unidentified zoanthids from Haleiwa and Makapuu, as well as *P. toxica*, contained essentially a single sterol (24-methylenecholesterol), the

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Table I—Activity of Crude Extracts against Ehrlich Ascites Tumor

Species	Month of Collection	Dose, mg/kg	Number of Mice	Deaths due to Toxicity	Survivors at 30 Days	
					% Alive	% Non-ascitic
P. psammophilia Walsh and Bowers	March	80	5	1	25	25
		40	5	—	60	40
		22.7	5	—	0	0
		11.4	5	—	40	20
		5.7	5	—	0	0
P. tuberculosa Esper	January	22.3	5	5	—	—
		4.54	5	—	100	60
		4.48	5	—	100	100
		2.27	5	—	20	0
		1.14	5	—	0	0
P. vestitus Verrill	December	22.9	5	1	100	100
		11.6	5	—	100	100
		4.64	5	—	40	0
P. toxica Walsh and Bowers	September	3.3	5	—	100	80
		1.67	5	—	0	0
		0.83	5	—	0	0
Z. pacificus Walsh and Bowers	March	160.4	5	—	60	40
		79.3	5	—	20	0
		59.4	5	—	20	20
		29.7	5	—	0	0
		17.4	5	—	0	0
Zoanthid from Haleiwa	July	1.78	5	5 (15 min)	—	—
		0.36	5	4 (chronic, Days 6–9)	100	100
		0.33	5	—	100	100
		0.165	5	—	100	100
		0.064	6	—	100	0
Zoanthid from Makapuu	January	45.4	5	—	20	0
		40	5	—	0	0
		21.7	5	—	40	40
		20	5	—	20	20
		11.4	5	1	50	25
		10	5	—	0	0
I. elongatus Verill	October	13	5	2	25	25
		6.7	5	1	0	0

same as reported previously for a Tahitian *Palythoa* species (10). In contrast, *P. tuberculosa* (10), *P. psammophilia*, and *P. vestitus* elaborate an identical complex mixture of sterols containing no detectable amounts of 24-methylenecholesterol while *Z. pacificus* [formerly *Z. confertus* (10)] and *Isaurus elongatus* contain sterol mixtures different both from each other and from the *Palythoa* species[2]. The genus *Palythoa* might well be comprised of two different genera.

The behavior of palytoxin in the standard screening procedure is shown in Table II. Palytoxin completely inhibited the growth of Ehrlich ascites tumor in 100% of mice treated with doses as low as 84 ng/kg and gave some nonascitic survivors at 30 days with doses down to the 5.25-ng/kg level. The mice from two experiments were kept for 3 months to check the long-term effects. At 3.37×10^{-1} μg/kg, given twice, eight out of the 10 mice (two deaths due to toxicity) survived 30 days and were nonascitic and all of these mice were alive and nonascitic at 90 days. At 8.44×10^{-2} μg/kg, given 20 times, nine out of the 10 mice (one death due to toxicity) survived 30 days and were nonascitic and eight out of these nine mice survived the 90-day period in good health. These results would indicate that all tumor cells were killed in 16 of the 17 mice (94%) examined and that the disease was cured.

Palytoxin gave some life extension using the P-388 lymphocytic leukemia screen; however, the best figure obtained was only a 32% life extension and does not appear sufficient to warrant further work with this system. Some crude extracts were examined by the National Cancer Institute's screening service against P-388 and human epidermoid carcinoma of the nasopharynx (KB cell culture). The zoanthid from Haleiwa was active against KB with an ED_{50} of 3.0×10^{-2} μg/ml, while both *P. psammophilia* and *Z. pacificus* were inactive, having ED_{50}'s greater than 100 μg/ml. In the P-388 screen, however, *P. psammophilia* was active, having now reached a confirmed active status, while both *Z. pacificus* and the zoanthid from Haleiwa were inactive, suggesting that the antileukemia agent in *P. psammophilia* is not palytoxin.

The correlation between the antitumor properties of palytoxin and its occurrence in the eggs of female *P. tuberculosa*[3] is a fascinating one. The presence of highly toxic substances in the reproductive organs of animals is not unusual. Tetrodotoxin is associated with the ovaries of the puffer fish and has its highest concentration just before spawning. It has been isolated (under the name tarichatoxin) from the eggs of western American newts of the genus *Taricha* (11). Palytoxin might be expected, therefore, to have some function in cell growth and cell differentiation and its antitumor activity could be a consequence of this biological function.

The experimental data from a study of different modes of drug administration in the control of Ehrlich ascites tumor with palytoxin are given in Table III. Palytoxin appears to be systemic in action; not only is it effective in the screening procedure where Ehrlich ascites cells and drug are administered intraperitoneally, but it is effective also when Ehrlich ascites cells are injected subcutaneously, resulting in the formation of a solid tumor, and the drug is administered intraperitoneally. Some effectiveness is displayed when the tumor is peritoneal and the drug is administered subcutaneously. In the latter case, however, the skin around the injection site became calloused and it appeared that drug distribution was poor. Delay of treatment until 4 days after tumor inoculation resulted in some control; however, the survival rate was not 100% as observed when palytoxin was administered 20 hr after inoculation. The results indicate, however, that palytoxin can control, to some degree, well-established tumor proliferation. In one experiment, two doses, administered 20 and 27 hr after in-

[2] R. E. Moore, K. Gupta, and P. J. Scheuer, unpublished work.

[3] Dr. Y. Hashimoto, Laboratory of Marine Biochemistry, Faculty of Agriculture, University of Tokyo, Tokyo, Japan, personal communication.

Table II—Activity of Palytoxin against Ehrlich Ascites and P-388 Leukemia

Dose, μg/kg	Number of Doses	Ehrlich Ascites Carcinoma				P-388 Lymphocytic Leukemia		
		Number of Mice	Deaths due to Toxicity	Survivors at 30 Days		Number of Mice	Deaths due to Toxicity	T/C, %
				% Alive	% Nonascitic			
2.7	1	10	10[a]	—	—	—	—	—
1.35	1	10	10[a]	—	—	—	—	—
6.75×10^{-1}	1	10	0[a]	—	—	—	—	—
4×10^{-1}	20	—	—	—	—	5	1	125
3.37×10^{-1}	2	10	2[b]	100	100[c]	—	—	—
2×10^{-1}	20	—	—	—	—	5	0	123
1×10^{-1}	20	—	—	—	—	5	0	132
8.44×10^{-2}	20	10	1[b]	100	100[d]	5	0	128
2.11×10^{-2}	20	10	0	90	80	5	0	122
5.25×13^{-3}	20	10	0	20	20	5	0	118
2.65×10^{-3}	20	10	0	0	0	5	0	110

[a] Toxicity tests, no Ehrlich ascites cells. [b] Observed toxicity after Ehrlich ascites cell inoculation. [c] One hundred percent alive and nonascitic at 90 days. [d] Eighty-nine percent alive and nonascitic at 90 days.

Table III—Effectiveness of Palytoxin Using Different Methods of Administration

Dose, μg/kg	Number of Doses	Number of Mice	Deaths due to Toxicity	Survivors at 30 Days	
				% Alive	% Nonascitic
Different routes of administration: Ehrlich ascites cells intraperitoneally, drug intraperitoneally					
1.08×10^{-1}	20	5	—	100	100
8.64×10^{-3}	20	5	—	100	80
4.32×10^{-3}	20	5	—	20	20
1.73×10^{-3}	20	5	—	0	0
Ehrlich ascites cells intraperitoneally, drug subcutaneously					
1.3×10^{-1}	20	10	—	40	30
Ehrlich ascites cells subcutaneously, drug intraperitoneally					
1.73×10^{-1}	8 (1/day)	10	1	Average tumor weight on 9th day 84 mg (range: 39–119 mg)	
Control		10	—	Average tumor weight on 9th day 446 mg (range: 262–824 mg)	
Delayed treatment: drug treatment commenced 4 days after Ehrlich ascites cell inoculation					
3.46×10^{-1}	20	8	—	75	75
1.73×10^{-1}	20	8	1	28.6	14.3
Minimum number of doses required:					
5.4×10^{-1}	1	8	—	37.5	25
5.4×10^{-1}	4(2/day)	8	—	62.5	62.5
3.37×10^{-1}	2(2/day)	10	2	100	100

oculation, resulted in complete tumor control; however, the survival rate was not as good in other experiments. Palytoxin displays a very strong kill of tumor cells as it approaches the toxic level.

In Table IV the Ehrlich ascites activities of palytoxins from three different sources are compared. This table illustrates one difficulty associated with cancer chemotherapy. The results in Tables I–III were obtained when using mice from a different source than those recorded in Table IV. The mean survival times for control animals in the three runs prior to the change were 18, 22, and 19 days as opposed to 12, 9, and 11 days after the change. The latter mice appear more susceptible to Ehrlich ascites tumor, and the established tumor appears more resistant to the action of drugs.

From the data, however, there do not appear to be any significant differences among the three samples of palytoxin. Although palytoxin was not as effective in this study as in the prior studies, it still gave essentially cures, albeit not 100% of the treated group.

Further work is continuing on the isolation of the P-388 leukemia active material from *P. psammophilia*.

EXPERIMENTAL

Extraction Procedure—Extraction was generally carried out by allowing the animals to soak in 95% ethanol at room temperature. The solution was decanted, and the process was repeated until no further material was extracted. The combined extract was evaporated under reduced pressure to remove ethanol, and the aqueous suspension was extracted with chloroform. The aqueous suspension was filtered or centrifuged as necessary to remove insoluble material and either evaporated under reduced pressure or freeze dried.

P. psammophilia was extracted by a different procedure. The collected samples were stored in a small volume of 95% ethanol. The animals were removed from the storage liquid, and the polyps were freed of coral substrate. The wet zoanthids (323 g) were homogenized in a blender, in two batches, with 28.5% ethanol (1.6 liters) for 10 min at highest speed; they were then combined with the storage liquid (490 ml) and allowed to stand at room temperature for 4 days. The supernate was decanted and the residue centrifuged (2000 rpm for 5 min). The combined supernate (1.74 liters) was evaporated under reduced pressure (bath temperature

Table IV—Comparison of Various Palytoxin Preparations against Ehrlich Ascites Tumor

Source of Palytoxin[a]	Dose, μg/kg	Number of Mice	Deaths due to Toxicity	Survivors at 30 Days	
				% Alive	% Non-ascitic
P. toxica from Muolea, Maui	1.28	5	5	—	—
	6.4×10^{-1}	5	5	—	—
	3.2×10^{-1}	5	0	100	100
	3.2×10^{-1}	5	0	80	60
	1.6×10^{-1}	5	0	80	80
	8×10^{-2}	5	0	0	0
	2×10^{-2}	5	0	0	0
	5×13^{-3}	5	0	0	0
P. mammilosa from Jamaica	1.28	5	5	—	—
	6.4×10^{-1}	5	5	—	—
	3.2×10^{-1}	5	1	100	100
	3.2×10^{-1}	5	1	75	50
	1.6×10^{-1}	5	0	80	80
	8×10^{-2}	5	0	0	0
	2×10^{-2}	5	0	0	0
	5×13^{-3}	5	0	0	0
P. spp. from Tahiti	1.28	5	5	—	—
	6.4×10^{-1}	5	5	—	—
	3.2×10^{-1}	5	4	100	100
	3.2×10^{-1}	5	0	80	80
	1.6×10^{-1}	5	0	0	0
	8×10^{-2}	5	0	40	40
	2×10^{-2}	5	0	60	60
	5×10^{-3}	5	0	0	0

[a] The authors thank Dr. Tatsuo Higa and Professor Paul J. Scheuer for samples of palytoxin from *P. mammilosa* and the Tahitian *P.* spp.

45°) to 330 ml of aqueous suspension and centrifuged (20,000×*g*, 10 min), and the aqueous solution was freeze dried to give a solid (14.24 g) which was used for the tests. Soaking the residue with an additional 1 liter of 28.5% alcohol yielded 2.7 g solid material.

Isolation of Palytoxin from Toxic Zoanthids—The palytoxins were isolated from the extracts of *P. tuberculosa*, *P. vestitus*, and the unidentified zoanthid from Haleiwa as previously described (6). All three toxins showed UV spectra indistinguishable from that of palytoxin from *P. toxica*.

Bioassay Method Using Ehrlich Ascites Tumor[4]—*Method 1 (Standard Screening Procedure)*—This method consisted of intraperitoneal injection of Ehrlich ascites cells and intraperitoneal injection of drug. The method used was previously described (4).

Method 2—This method consisted of intraperitoneal injection of Ehrlich ascites cells and subcutaneous injection of drug. The method was the same as Method 1 except that drug was administered subcutaneously on the back of the mouse.

Method 3—This method consisted of subcutaneous injection of Ehrlich ascites cells and intraperitoneal injection of drug. Ehrlich ascites cells (10^6) in 0.2 ml volume (Hank's solution) were inoculated subcutaneously at slightly above the midabdominal region of the mouse. The mice developed a solid tumor at the site of inoculation in about 3–5 days. Drug injection was initiated 18–20 hr after Ehrlich ascites cell inoculation, and daily injections of 0.1 ml volume of drug were given intraperitoneally for up to 10 days. At the end of 9–14 days, before the first sign of ulceration of any solid tumor, the animals were sacrificed and their tumor weights were taken.

Bioassay Method Using Lymphocytic Leukemia (Standard Screening Procedure)—P-388[5] was transplanted in male or female DBA/2 mice[6] every 7 days.

Ascitic fluid was drawn from the intraperitoneal cavity of the DBA/2 mice 7 days after the previous transfer. The ascitic fluid was diluted with Hank's saline solution to 10^6 cells/0.1 ml. Then cells (10^6) were implanted intraperitoneally into female BDF_1 mice (20–23 g)[6]. Drug treatment was commenced 18–20 hr after inoculation and was administered twice daily for 10 days. Mean survival time was used as the activity parameter, and the results were expressed as the percentage of the mean survival time of the controls.

[4] Data in Tables I–III obtained using Swiss Webster mice from University of Hawaii Animal Colony and that in Table IV using Swiss Webster mice from Simonsen Labs., Gilroy, Calif.

[5] The authors thank Dr. Jonathan L. Hartwell, Natural Products Section, Cancer Chemotherapy, National Cancer Institute, Bethesda, Md., for arranging to supply the P-388 system.

[6] From Simonsen Labs., Gilroy, Calif.

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xTo whom inquiries should be directed.

Synthesis and Antimicrobial Activity of *N*-Substituted *N'*-Cyano-*O*-(triorganostannyl)isoureas

EUGENE J. KUPCHIK*x, MICHAEL A. PISANO‡, DILIPKUMAR K. PARIKH*, and MARY ANN D'AMICO‡

Abstract □ Twenty-five *N*-substituted *N'*-cyano-*O*-(triorganostannyl)isoureas were synthesized by the reaction of (triphenylstannyl)cyanamide or (trimethylstannyl)cyanamide with various organic isocyanates. The IR spectrum of each compound was obtained over the 4000–30 cm^{-1} range, and some bands were assigned. Six compounds were tested for, and found to exhibit, antifungal activity. One of these compounds was also investigated for antibacterial activity and was observed to be inhibitory toward Gram-positive species.

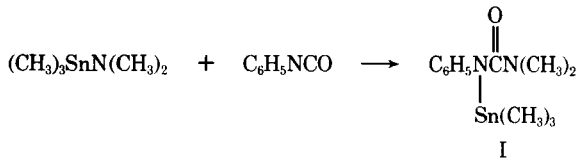
Keyphrases □ *N'*-Cyano-*O*-(triorganostannyl)isoureas, *N*-substituted—synthesis, IR spectra, antimicrobial activity □ Antifungal activity—synthesis and evaluation of six *N*-substituted *N'*-cyano-*O*-(triorganostannyl)isoureas □ Antibacterial activity—synthesis and evaluation of *N*-phenyl-*N'*-cyano-*O*-(triphenylstannyl)isourea

The reaction of dimethylaminotrimethylstannane with phenyl isocyanate was reported (1) to afford a 1:1 addition compound, which was assigned, on the basis of its IR spectrum, Structure I (Scheme I). The IR spectrum of I contained a band at 1660 cm^{-1}, which was assigned to a carbonyl stretching mode of a fully substituted urea. Recently, (triphenylstannyl)-cyanamide was reported (2) to form a 1:1 addition compound with phenyl isocyanate, but no suggestion was made as to its structure. As in I, the tin in this compound could be bonded to nitrogen (Structure II); another possibility, however, is a structure in which the tin is bonded to oxygen (Structure III).

It was considered of interest to synthesize a number of (triorganostannyl)cyanamide–organic isocyanate addition compounds and to study their IR spectra to determine whether the tin is bonded to nitrogen or to oxygen. Since some organotin compounds were previously found to exhibit antifungal and antibacterial activity (3), it was also of interest to determine the antifungal and antibacterial activity of some of these compounds.

RESULTS AND DISCUSSION

Synthesis—Thirteen (trimethylstannyl)cyanamide–organic isocyanate addition compounds were prepared by allowing (trimethylstannyl)cyanamide to react separately with 13 different organic isocyanates (1:1 mole ratio) in acetonitrile at room temperature (Table I). In addition, 12 (triphenylstannyl)cyanamide–organic isocyanate addition compounds were prepared in a similar manner by allowing (triphenylstannyl)cyanamide to react separately with 12 different organic isocyanates (Table II). The IR spectra of these compounds are summarized in Tables III and IV. The assignments are based largely on the work of others (4–10). None of the compounds exhibited a band near 1660 cm^{-1} due to the carbonyl group of a urea (11); this band appears at 1667 cm^{-1} in 1-cyano-3-phenylurea (12). The absence of carbonyl absorption and the presence of a band that can be assigned to the C=N group (13), especially in the cases of Compounds 1–6 (Table III) which do not contain interfering phenyl absorptions, suggest that the compounds are *N*-substituted *N'*-cyano-*O*-(triorganostannyl)isoureas rather than 1-cyano-3-phenyl-3-(triorganostannyl)ureas. The trimethylstannyl compounds (Table III) contained both the ν_{as} (SnC) band and the ν_s (SnC) band, indicating that the trimethyltin group is nonplanar in these compounds (17).



Scheme I

Unlike the tin–oxygen bond in many organotin alkoxides (14), the tin–oxygen bond in these compounds was found to be stable in water. For example, 88% of *N*-phenyl-*N'*-cyano-*O*-(triphenylstannyl)isourea (III) was recovered unchanged after being stirred in water–tetrahydrofuran (2:50)(pH 5.5) for 24 hr at 26°. The hydrolytic stability of III may be due to intramolecular or intermolecular coordination between tin and the nitrogen of the CN group. Compound III was unstable in 6 *N* hydrochloric acid at 26°, with one of the products formed being triphenyltin chloride.

Biological Results—Six of the *N*-substituted *N'*-cyano-*O*-(triorganostannyl)isourea compounds were investigated for the inhibition of growth of 10 fungus species. One compound was also investigated for antibacterial activity. Table V lists the antifungal activity of the compounds tested. From the data presented, it can be seen that the *N*-substituted *N'*-cyano-*O*-(triphenylstannyl)isoureas (Compounds 18 and 26) were more active than the trimethylstannyl compounds (Compounds 1, 7, 9, and 10). Complete inhibition of *Aspergillus niger* and *Trichophyton mentagrophytes* occurred in the presence of 100 μg/ml of Compound 18, *N*-cyclohexyl-*N'*-cyano-*O*-(triphenylstannyl)isourea. At a similar concentration, Compound 26, *N*-phenyl-*N'*-cyano-*O*-(triphenylstannyl)isourea (III), totally inhibited both *Saccharomyces cerevisiae* and *T. mentagrophytes*. The latter compound was also investigated for antibacterial activity (Table VI). The data show that Compound 26 was active against Gram-positive but not Gram-negative species. The Gram-positive bacteria displayed several degrees of sensitivity, with *Micrococcus agilis* being completely inhibited at 1 μg/ml, whereas for *Bacillus subtilis* 10 μg/ml was required for a similar effect. *Staphylococcus aureus* was the least sensitive of the Gram-positive species tested, 100 μg/ml of Compound 26 being required for complete inhibition of growth.

EXPERIMENTAL[1]

***N*-Ethyl-*N'*-cyano-*O*-(trimethylstannyl)isourea (Compound 1)**—A mixture of (trimethylstannyl)cyanamide (15) (0.819 g, 0.004 mole), ethyl isocyanate (0.33 ml, 0.0042 mole), and acetonitrile (25 ml) was stirred at 26° for 24 hr and filtered to give 0.82 g (72%) of Compound 1, mp 158–162°. Two recrystallizations from tetrahydrofuran–*n*-pentane gave the analytical sample, mp 160–161°.

[1] Melting points were determined with a Mel-Temp capillary melting-point apparatus and are uncorrected. The IR data were obtained using KBr pellets with a Beckman IR 8 spectrophotometer. The far IR data were obtained with a Perkin-Elmer model FIS-3 IR spectrophotometer (polyethylene pellets) and with a Perkin-Elmer model 21 double-beam IR spectrophotometer fitted with a cesium bromide prism and purged with nitrogen (KBr pellets). Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

Table I—*N*-Substituted *N'*-Cyano-*O*-(trimethylstannyl)isoureas[a]

$$RN{=}C(OSn(CH_3)_3)NHCN$$

Compound	R	Yield[b], %	Melting Point[c]	Formula	Analysis, %	Calc.	Found
1	C_2H_5	72	160–161°	$C_7H_{15}N_3OSn$	C	30.47	30.15
					H	5.48	5.60
					N	15.23	15.02
					Sn	43.02	43.14
2	n-C_3H_7	85	148–150°	$C_8H_{17}N_3OSn$	C	33.14	33.69
					H	5.91	5.96
					N	14.49	14.04
					Sn	40.93	40.62
3	n-C_4H_9	73	148–150°	$C_9H_{19}N_3OSn$	C	35.56	35.24
					H	6.30	6.34
					N	13.82	13.82
					Sn	39.05	38.87
4	$(CH_3)_3C$	86	154–156°	$C_9H_{19}N_3OSn$	C	35.56	35.70
					H	6.30	6.43
					N	13.82	14.08
					Sn	39.05	38.77
5	cyclo-C_6H_{11}	89	187–188°	$C_{11}H_{21}N_3OSn$	C	40.04	39.94
					H	6.41	6.63
					N	12.73	12.42
					Sn	35.97	35.91
6	$CH_2{=}CHCH_2$	88	140–141°	$C_8H_{15}N_3OSn$	C	33.37	33.71
					H	5.25	5.41
					N	14.59	14.78
					Sn	41.23	40.89
7	C_6H_5	70	168°	$C_{11}H_{15}N_3OSn$	C	40.78	40.83
					H	4.67	4.80
					N	12.97	12.69
					Sn	36.63	36.77
8	m-$CH_3C_6H_4$	75	151–152°[d]	$C_{12}H_{17}N_3OSn$	C	42.65	42.88
					H	5.07	5.28
					N	12.43	12.63
					Sn	35.12	35.31
9	p-$CH_3C_6H_4$	88	173–176°	$C_{12}H_{17}N_3OSn$	C	42.65	42.73
					H	5.07	5.24
					N	12.43	12.20
					Sn	35.12	35.56
10	p-$C_2H_5OC_6H_4$	73	170–171°	$C_{13}H_{19}N_3O_2Sn$	C	42.43	42.22
					H	5.21	5.28
					N	11.42	11.66
					Sn	32.25	32.14
11	p-FC_6H_4	89	174–175°	$C_{11}H_{11}FN_3OSn$	C	38.98	38.78
					H	3.27	4.08
					N	12.45	12.40
					Sn	35.02	35.03
12	p-ClC_6H_4	82	176–178°	$C_{11}H_{14}ClN_3OSn$	C	36.86	36.68
					H	3.94	3.94
					N	11.72	11.77
					Sn	33.11	32.90
13	m-$O_2NC_6H_4$	35	165–166°	$C_{11}H_{14}N_4O_3Sn$	C	35.81	35.71
					H	3.82	3.77
					N	15.18	14.95
					Sn	32.17	32.17

[a] Unless otherwise indicated, the recrystallization solvent was tetrahydrofuran–*n*-pentane. [b] Based on material that melts within 5° of the analytical sample. [c] Analytical sample. [d] Recrystallized from acetonitrile.

Compounds 2–13 were prepared in a similar manner.

***N*-Ethyl-*N'*-cyano-*O*-(triphenylstannyl)isourea (Compound 14)**—A mixture of (triphenylstannyl)cyanamide (16) (1.56 g, 0.004 mole), ethyl isocyanate (0.33 ml, 0.0042 mole), and acetonitrile (25 ml) was stirred at 26° for 24 hr and filtered to give 2.63 g (72%) of Compound 14, mp 173–174°. Recrystallization from tetrahydrofuran–*n*-pentane gave the analytical sample, mp 174°.

Compounds 15–25 were prepared in a similar manner.

Biological Methods—The organotin compounds were individually dissolved in tetrahydrofuran, and 1% solutions of each were sterilized by filtration (Seitz) (0.1-μm porosity). Dilutions of the sterile solutions were prepared and added to liquefied potato dextrose agar[2] to yield final concentrations of 100, 10, and 1 μg/ml. The agar, containing the appropriate organotin compound, was poured into sterile petri dishes and allowed to solidify overnight. The agar surface was inoculated centrally with a 2-mm² portion of the growth from a 10-day-old culture of each fungus[3] with the exception of *S. cerevisiae* in which case 2-day-old cultures were employed. Inoculated plates were incubated for 14 days, and observations of growth inhibition were made by measuring the diameters of the colonies present on the agar surface.

One organotin compound, *N*-phenyl-*N'*-cyano-*O*-(triphenylstannyl)isourea (III), was also investigated for antibacterial activity. It was incorporated into sterile tryptic soy agar[2] at levels of 100, 10, and 1 μg/ml. The agar was also seeded with a 1:100 dilu-

$$C_6H_5NC(=O)NHCN \quad \text{with } Sn(C_6H_5)_3 \text{ on N}$$

II

$$C_6H_5N{=}C(OSn(C_6H_5)_3)NHCN$$

III

[2] Difco.

[3] All fungi were obtained from the American Type Culture Collection (ATCC), Rockville, Md.

Table II—*N*-Substituted *N'*-Cyano-*O*-(triphenylstannyl)isoureas[a]

$$RN{=}C(OSn(C_6H_5)_3)NHCN$$

Compound	R	Yield[b], %	Melting Point[c]	Formula	Analysis, %	
					Calc.	Found
14	C_2H_5	72	174°	$C_{22}H_{21}N_3OSn$	C 57.18	57.09
					H 4.58	4.76
					N 9.09	9.35
					Sn 25.68	25.76
15	*n*-C_3H_7	61	175–177°	$C_{23}H_{23}N_3OSn$	C 58.02	58.26
					H 4.87	5.11
					N 8.82	8.65
					Sn 24.93	24.71
16	*n*-C_4H_9	73	157–158°	$C_{24}H_{25}N_3OSn$	C 58.81	58.94
					H 5.14	5.00
					N 8.57	8.47
					Sn 24.21	24.09
17	$(CH_3)_3C$	84	160–161°	$C_{24}H_{25}N_3OSn$	C 58.81	59.29
					H 5.14	4.99
					N 8.57	8.36
					Sn 24.21	23.96
18	cyclo-C_6H_{11}	89	181–183°	$C_{26}H_{27}N_3OSn$	C 60.50	60.50
					H 5.27	5.45
					N 8.14	7.83
					Sn 22.99	23.09
19	$CH_2{=}CHCH_2$	86	167–170°[d]	$C_{23}H_{21}N_3OSn$	C 58.26	58.18
					H 4.46	4.44
					N 8.86	9.09
					Sn 25.03	25.19
20	*m*-$CH_3C_6H_4$	91	162–163°[e]	$C_{27}H_{23}N_3OSn$	C 61.87	61.76
					H 4.42	4.54
					N 8.02	7.84
					Sn 22.64	22.83
21	*p*-$CH_3C_6H_4$	80	158–159°[e]	$C_{27}H_{23}N_3OSn$	C 61.87	61.67
					H 4.42	4.53
					N 8.02	7.91
					Sn 22.64	22.91
22	*p*-$C_2H_5OC_6H_4$	81	165–168°[d]	$C_{28}H_{25}N_3O_2Sn$	C 60.68	60.85
					H 4.55	4.55
					N 7.58	7.28
					Sn 21.42	21.28
23	*p*-FC_6H_4	89	156–158°[d]	$C_{26}H_{20}FN_3OSn$	C 59.13	59.11
					H 3.81	3.79
					N 7.95	7.87
					Sn 22.47	22.59
24	*p*-ClC_6H_4	80	130–132°[d]	$C_{26}H_{20}ClN_3OSn$	C 57.40	57.38
					H 3.70	3.77
					N 7.71	7.93
					Sn 21.79	21.92
25	*m*-$O_2NC_6H_4$	52	160–162°[e]	$C_{26}H_{20}N_4O_3Sn$	C 56.25	56.48
					H 3.63	3.86
					N 10.09	10.13
					Sn 21.38	21.59

[a] Unless otherwise indicated, the recrystallization solvent was tetrahydrofuran–*n*-pentane. [b] Based on material that melts within 5° of the analytical sample. [c] Analytical sample. [d] Washed with ether. [e] Recrystallized from acetonitrile.

Table III—IR Spectra of *N*-Substituted *N'*-Cyano-*O*-(trimethylstannyl)isoureas[a]

Compound	NH	C≡N	C=N	$Sn(CH_3)_3$	
				ν_{as}	ν_s
1	3268 s, 3106 m	2165 s	1550 s	550 s	500 s
2	3268 m, 3106 m	2179 s	1555 s	553 s	516 m
3[b]	3279 m, 3049 m	2183 s	1560 s	546 s	521 m
4	3322 m, 3086 w	2174 s	1541 s	553 s	513 s
5[c]	3268 s, 3067 w	2174 s	1541 s	553 s	493 m
6[d]	3268 s	2179 s	1538 s	544 s	518 w
7[e]	3257 m	2198 s	1524 s[f]	556 s	503 s
8[g]	3268 m	2179 s	1531 s[f]	544 s, br	—[h]
9	3257 w	2188 s	1515 s[f]	553 s	503 s
10	3257 m	2198 s, 2179 s	1527[f]	553 s	516 s
11[k]	3257 m	2198 s, 2169 s, sh	1538[f]	559 s	510 s
12[l]	3247 w	2188 s, 2169 s, sh	1515[f]	541 s	495 s
13[m]	3257 w	2188 s	1504[f]	550 s, br	—[h]

[a] Values are expressed in centimeters^{-1}; s = strong, m = medium, w = weak, br = broad, and sh = shoulder. The data were obtained using KBr pellets. [b] A band at 479 m was present. [c] Bands were present at 614 s, 571 s, 529 w, 474 w, and 453 w. [d] Bands were present at 3106 m, 599 w, 559 m, and 454 s. [e] Bands were present at 613 s, 568 s, and 535 m. [f] This assignment is uncertain due to the presence of aromatic C=C bands in this region. [g] A band was present at 614 s. [h] The band may be overlapped by the broad ν_{as} band. [k] A band was present at 524 s. [l] A band was present at 463 s. [m] Bands were present at 614 s and 568 s.

Table IV—IR Spectra of *N*-Substituted *N'*-Cyano-*O*-(triphenylstannyl)isoureas[a]

Compound	NH	C≡N	C=N[b]	C_6H_5 Ring Vibration	$Sn(C_6H_5)_3$ ν_{as}	$Sn(C_6H_5)_3$ ν_s
14	3436 m	2188 s	1560 s	451 s	276 s	225 m
15	3401 m	2165 s	1560 s	455 s	277 s	229 m
16	3401 m	2174 s	1558 s	455 s	277 s	227 m
17	3401 m	2183 s, 2160 s	1546 s	455 s	277 s	230 s
18	3436 m, 3413 m	2174 s	1538 s	444 s	279 s	224 s
19	3413 m	2179 s	1538 s	448 s	275 s	227 s
20	3367 m	2179 s	1558 s	453 s	272 s	225 m
21	3390 m	2198 s	1555 s	455 s	276 s	230 m
22	3367 s	2193 s	1550 s	453 s	279 s	223 m
23	3367 m	2188 s	1553 s	453 s	276 s	228 m
24	3367 w	2193 s	1555 s	444 s	278 s	230 m
25	3401 m	2208 s, 2160 s	1515 s	461 s	270 s	217 s

[a] Values are expressed in centimeters^{-1}; s = strong, m = medium, and w = weak. The $Sn(C_6H_5)_3$ values were obtained using polyethylene pellets; the other data were obtained using KBr pellets. [b] This assignment is uncertain due to the presence of aromatic C=C bands in this region.

Table V—Antifungal Activity of *N*-Substituted *N'*-Cyano-*O*-(triorganostannyl)isourea Compounds

Compound[a]	*Aspergillus niger* (12845)[b] 1[c]	10	100	*Chaetomium globosum* (6205) 1	10	100	*Cladosporium carpophilum* (12117) 1	10	100	*Fusarium moniliforme* (10052) 1	10	100	*Myrothecium verrucaria* (9095) 1	10	100
1	−	−	+	−	−	+	−	−	2+	−	−	+	−	+	+
7	−	−	+	−	−	+	−	−	2+	−	−	+	−	+	+
9	−	−	+	−	−	+	−	−	2+	−	−	+	−	+	+
10	−	−	+	−	−	+	−	+	2+	−	−	+	−	−	+
18	−	+	2+	−	+	+	+	+	+	−	+	+	−	+	+
26	−	+	+	−	+	+	−	+	+	−	+	+	+	+	+

Compound[a]	*Penicillium notatum* (9179) 1	10	100	*Rhizopus stolonifer* (10404) 1	10	100	*Saccharomyces cerevisiae* (9896) 1	10	100	*Trichoderma viride* (8678) 1	10	100	*Trichophyton mentagrophytes* (9129) 1	10	100
1	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+
7	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+
9	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+
10	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+
18	−	+	+	−	+	+	−	−	+	−	−	+	+	+	2+
26	−	+	+	−	+	+	−	−	2+	−	−	+	+	+	2+

[a] Except for Compound 26, the numbers correspond to the compounds in Tables I and II; Compound 26 is *N*-phenyl-*N'*-cyano-*O*-(triphenylstannyl)isourea (III). [b] Numbers in parenthesis indicate ATCC designation of each fungus. [c] Indicates concentration of compounds employed in micrograms per milliliter; − indicates no inhibition of growth, + indicates partial inhibition of growth, and 2+ indicates complete inhibition of growth.

tion of an 18-hr-old culture of the desired bacterial species[4]. The plates were incubated for 24 hr at 37°, except for *Micrococcus agilis* which was maintained for 72 hr at 25°. Observations of bacterial growth were made following the appropriate incubation period.

A solvent control was included in all experiments, as were growth controls for all fungal and bacterial species. The latter were employed for comparative purposes to determine the extent of inhibition exhibited by the organotin compounds.

Table VI—Antibacterial Activity of *N*-Phenyl-*N'*-cyano-*O*-(triphenylstannyl)isourea (III)

Organism	Concentration, μg/ml 1	10	100
Bacillus subtilis	+[a]	2+	2+
Escherichia coli	−	−	−
Micrococcus agilis	2+	2+	2+
Staphylococcus aureus	−	+	2+

[a] − indicates no inhibition of growth, + indicates partial inhibition of growth, and 2+ indicates complete inhibition of growth.

[4] Obtained from the culture collection of the Department of Biology, St. John's University.

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PHARMACEUTICAL ANALYSIS

Determination of Calcium Pantothenate in Chewable Multivitamin Tablets

L. L. SHANKLEˣ, A. E. TROUP, and R. N. DUVALL

Abstract □ A hybrid procedure was developed for the determination of calcium pantothenate in a chewable multivitamin tablet. Applicable operations and techniques were adopted from different published sources. The resulting procedure incorporates solvent extraction, chromatography, hydrolysis, and color development. This method proved to be more accurate and precise than any published method investigated.

Keyphrases □ Calcium pantothenate in chewable multivitamin tablets—analysis, procedure using various analytical techniques □ Multivitamin tablets, chewable—analysis of calcium pantothenate, procedure using various analytical techniques □ Pantothenate, calcium, in chewable multivitamin tablets—analysis, procedure using various analytical techniques

In this laboratory, the determination of calcium pantothenate by wet methods has led to many difficulties. It has been necessary to modify and revise existing procedures continually to obtain reliable results on various dosage forms, as well as on similar dosage forms containing different pharmaceutic aids.

Most often the ninhydrin method (1) or an adaptation proved to be suitable. This method, however, could not be made applicable to certain chewable multivitamin tablet formulations containing relatively high levels of natural sweeteners. The most serious objection to this method was the pronounced variability. A statistical analysis showed the coefficient of variation to be over 6%. Analysis of variance studies indicated significant differences in the variability between replicates, days, and operators. Therefore, this method was considered unapplicable.

The iodine method (2, 3) was the next to be investigated. The lack of precision was also a problem with this procedure (3). Some developmental work was done to improve the precision. Subsequently, a statistical evaluation was undertaken which yielded a high coefficient of variation of 8.4%.

Finally, the naphthoquinone method (4) was tried. In this case, the chromatographic separation achieved by Panalaks and Campbell could not be duplicated. The pantothenate was partially eluted with the boric acid wash and tailed badly during the elution step. Consequently, poor results were obtained.

A search of the literature did not reveal any alternative procedures that were considered compatible with techniques and equipment available in this laboratory. However, during this in-depth study of available methods, it became evident that certain techniques involving sample cleanup and detection employed by various authors were very effective. The possibility that a hybrid procedure possessing combined advantages could be developed seemed promising. Such a procedure did, in fact, evolve from the combination of carefully selected existing steps and techniques.

This procedure, when applied to the product mentioned, supplied results significantly more accurate and precise than those provided by any published method tried. No special apparatus is required. Puri-

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This procedure, when applied to the product mentioned, supplied results significantly more accurate and precise than those provided by any published method tried. No special apparatus is required. Puri-

Table I—Hydrolysis of Calcium Pantothenate *versus* pH at 100°

pH	Percent Remaining after 1 hr
11.0	47
12.0	23
13.0	0.57
13.5	None detected

fication of reagents and solvents is not necessary, and analysis time is comparable to most other existing wet methods.

The purpose of this paper is to describe this method. It is hoped that it will provide an additional alternative to anyone encountering similar assay problems.

EXPERIMENTAL

Reagents—The following were used: ion-exchange resin[1], 100–200 mesh; and activated magnesium silicate[2], 60–100 mesh.

Boric Acid—Dissolve 100 g boric acid in distilled water and dilute to 2 liters.

Naphthoquinone Solution—Dissolve 250 mg sodium 1,2-naphthoquinone-4-sulfonate in distilled water and dilute to 50 ml. This reagent should be prepared fresh daily.

Formaldehyde Solution—Mix three parts 6 *N* hydrochloric acid, four parts acetic acid, and four parts 0.6 *M* formaldehyde.

Thiosulfate Solution—Dissolve 2.5 g sodium thiosulfate pentahydrate in water and dilute to 100 ml with water.

Standard Solution—Dissolve 120 mg calcium pantothenate in water and dilute to 100 ml with water.

Procedure—Slurry pack 5 cm of ion-exchange resin over 5 cm activated magnesium silicate in a chromatographic tube, 1.5 cm i.d., fitted with a stopcock. Place pledgets of glass wool at the bottom and top of the column and between the two chromatographic materials. Just prior to using the columns, wash with 10 ml 0.1 *N* HCl and then with 100 ml of water.

Next, prepare an aqueous extract of the sample containing about 1.2 mg calcium pantothenate/ml. Transfer 10.0 ml of sample and standard to 40-ml centrifuge tubes (Oak Ridge) containing 13 g sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$). Shake the tubes on a mechanical shaker for 15 min. The shaker used was of the oscillating type with a 4.5-cm travel and 30 cpm. To each tube, add exactly 20 ml of benzyl alcohol and shake another 15 min. After centrifugation, transfer 15.0 ml of the clear upper benzyl alcohol layer to another centrifuge tube. Add 10.0 ml toluene and 12.0 ml water to each tube and again shake 15 min. Centrifuge to obtain a clear lower layer.

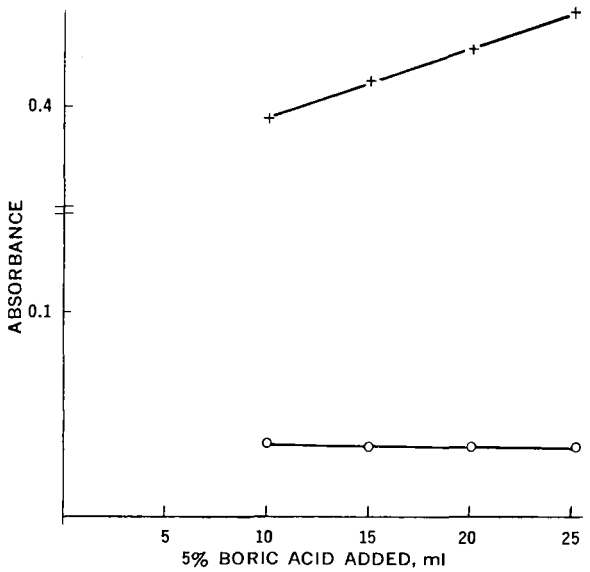

Figure 1—*Effect of boric acid concentration on assay sensitivity. Key: +, standard; and O, reagent blank.*

[1] 50W-X4, BioRad Labs, Richmond, Calif.

[2] Florisil, Fisher Scientific Co., Fair Lawn, N.J.

Table II—Effect of Cooling Conditions on Precision

Cooling Conditions		
After Hydrolysis	After Color Development	Coefficient of Variation, %
Ice bath	Ice bath	0.64
Bench top, room temperature	Ice bath	1.57
Ice bath	Bench top, room temperature	1.15

Pipet 10-ml aliquots of the lower aqueous layer onto the previously prepared chromatographic columns and allow to percolate through at full flow. Collect all column effluents in 100-ml volumetric flasks. Wash the calcium pantothenate through the column with six 5-ml portions of water. To each receiver plus a reagent blank (40 ml water in 100-ml volumetric flask), add exactly 25 ml boric acid solution and 8 ml of 3.5 *N* NaOH. The pH of the solutions at this point should be about 13.5. Heat the flasks at 100° for 1 hr and then cool to near room temperature in an ice bath.

Adjust each solution to pH 8.0 by adding a predetermined volume of 3 *N* HCl. After adding 4.0 ml of the naphthoquinone reagent, heat the flasks at 100° for 10 min and cool in an ice bath as before. To each flask, add exactly 4 ml each of the formaldehyde and thiosulfate reagents and let stand 15 min. Bring to volume with water, mix, and determine the absorbance on a suitable spectrophotometer[3] *versus* a reagent blank at the maximum wavelength, at about 465 nm, in a 1-cm cell.

RESULTS AND DISCUSSION

Color Reaction—The detection method was selected first and the remainder of the procedure was designed to accommodate it. The naphthoquinone color reaction as carried out by Panalaks and Campbell (4) was chosen. Experience had shown this technique to be highly precise and accurate, and it is relatively convenient. The various parameters, *e.g.*, heating conditions, pH, and concentration of reagents, associated with the color development were investigated and confirmed as optimum. The color is stable for at least 1.5 hr.

Chromatography—β-Alanine, a degradation product of pantothenate, is the substance actually involved in the color reaction. Any of this material present due to degradation must be removed to render the method specific for intact pantothenate. The technique chosen employs the dual column proposed by Schmall and Wollish (1). Considerable experience with this column had revealed it to be consistently effective. The free β-alanine is removed by the ion-exchange portion of the column. Although its full potential was not determined, the resin has a demonstrated capability of removing an amount of β-alanine equivalent to 6 mg calcium pantothenate. Therefore, the stability of the products could be followed to a 50% degradation level. In addition, some interfering tablet ingredients, *e.g.*, riboflavin, are removed by the activated magnesium silicate portion.

Several authors (2, 3, 5) recommended slurrying to remove fines from chromatographic materials to increase precision. This practice was, therefore, incorporated into the presented procedure.

Solvent Extraction—Interfering materials that were not removed by the column necessitated the addition of the benzyl alcohol extraction step. A 65% reduction in response was noted when this step was omitted. This technique, proposed by Panalaks and Campbell (4), effectively eliminates interferences and was adopted without modification. Since the problem was solved in this manner, no attempt was made to determine the source of

[3] Beckman DU.

Table III—Statistical Evaluation of Procedure

Synthetic Sample	Calcium Pantothenate, mg		Recovery, %	Mean Recovery, %	*SD*	Coefficient of Variation, %
	Added	Found				
I	10.18	10.10	99.2			
		10.10	99.2			
		10.14	99.6			
		9.92	97.4			
		—	—	98.8	0.9848	0.97
II	7.49	7.59	101.3			
		7.59	101.3			
		7.65	102.1			
		7.69	102.6			
		7.57	101.1	101.7	0.6419	0.65
III	4.98	5.06	101.6			
		5.08	102.0			
		5.04	101.2			
		4.94	99.1			
		5.04	101.2	101.0	1.128	1.07

the interference. It is possible, therefore, that some formulations may not require this treatment.

Hydrolysis—The rate of hydrolysis of pantothenate increases uniformly with pH up to about 13, above which it tends to level off (Table I). A pH of 13.5 was chosen for the hydrolysis medium to reduce analysis time. The blank value in this case was unaffected by variations in pH.

The half-life of calcium pantothenate at 100° and pH 13.5 is approximately 5 min. The hydrolysis is at least 99.9% complete after 50 min.

Heating and Cooling Conditions—To provide the 100° heating conditions necessary for both hydrolysis and color development, several flasks were placed on a screen over an opening of a steam bath and a bell-shaped cover fashioned of aluminum foil placed over them. This technique provides very uniform and homogeneous heating conditions and eliminates bumping problems. Ice bath cooling conditions were employed not only for expediency but also to improve precision (Table II).

Boric Acid—Originally the boric acid was added only to facilitate adjustment and preservation of pH. It became evident, however, that the intensity of the measured color was directly proportional to the concentration of this reagent. The relationship of response *versus* the amount of boric acid present is shown in Fig. 1; the absorbance of the reagent blank was independent of boric acid concentration. Therefore, to achieve maximum sensitivity, the boric acid concentration was adjusted as high as practical. Consequently, to achieve precise results, analytical precision is required when adding this reagent.

Accuracy and Precision—To evaluate the method for accuracy and precision, synthetic samples were assayed. These samples were prepared by adding accurately known amounts of calcium pantothenate to a placebo, containing all tablet ingredients with the exception of calcium pantothenate. Three levels of pantothenate were selected. The first was the level present in the product of interest and the remaining two were equal to 75 and 50%, respectively, of the first. Thus, the stability-indicating ability of the assay was included in the evaluation. As mentioned, the ion-exchange column was effective in removing free β-alanine over this range of degradation (Table III). It is evident from these data that the assay adheres to a linear model. That is, similar recoveries were obtained at all three levels. The assay is stability indicating, as well as adequately accurate and precise.

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[x]To whom inquiries should be directed.

Detection and Determination of Chrysotile in Talc USP

HARRY A. ROSE

Abstract □ A procedure for the determination of chrysotile in talc was developed. It depends on the adsorption of a sulfonphthalein dye by chrysotile but not by talc. The final measurement is a spectrophotometric determination of the dye left in solution after the adsorption step.

Keyphrases □ Chrysotile—determination in talc USP □ Talc USP —determination of chrysotile as impurity

The wide use of talc as a dusting powder and as a diluent in capsule contents has caused concern about possible impurities, especially asbestos, in the talc. Cralley *et al.* (1) found asbestos in some cosmetic talcs, and asbestos in talc was implicated as a cause of stomach cancer in Japan (2).

Talc, a hydrated magnesium silicate, may ideally be represented as $Mg_3(OH)_2Si_4O_{10}$. It may be contaminated with other minerals such as quartz, dolomite, chlorite, and the various forms of asbestos. Of the asbestos minerals, chrysotile is currently of most physiological concern. Chrysotile, also a hydrated magnesium silicate, may ideally be represented as $Mg_6(OH)_8Si_4O_{10}$. The structure of both minerals is apparently made up of sheets of silicon and oxygen atoms interspersed with sheets of magnesium and oxygen atoms, the difference being in the added magnesium and oxygen in the chrysotile structure.

USP XVIII (3) is rather vague about the chemistry of talc and apparently would admit a talc containing some chlorite. The concern is for rather gross impurities such as excesses of pH or water-soluble iron. Thus, a rather substantial contamination of the talc with chrysotile could occur, and no cause for rejection would appear unless the product became gritty.

Several tests can be proposed to detect the chrysotile in talc, but each suffers from some difficulty, all relating to the large ratio of talc to chrysotile which should be present. Obviously, a simple determination of magnesium, hoping to find the excess due to the chrysotile, would fail because of the natural variability of the talc itself. X-ray diffraction, which was used (4) very elegantly for measuring air pollution by chrysotile, becomes uncertain at low percentages when much talc is present. Optical microscopic examination suffers because the particles approach the limit of resolution of the instrument.

The test described here detects chrysotile contamination in talc but not the amphibole types of asbestos such as tremolite. The test depends on the adsorption of bromcresol purple by chrysotile and the lack of adsorption of that dye by talc. Although other sulfonphthalein dyes of this series have similar behavior, bromcresol purple was selected because of its convenient range for change of color to the acid form.

EXPERIMENTAL

Reagents—Bromcresol purple[1] was used as received. A stock solution of this dye was made by dissolving 0.15 g in 250 ml of 95% ethanol and diluting to 500 ml with water. The working solution was made by taking 20 ml of the stock solution plus 40 ml of 95% ethanol and diluting to 100 ml with pH 4.2 acetate buffer. This working solution, when 10 ml is diluted to 25 ml with pH 4.2 acetate buffer, should have an absorbance of 0.325 at 422 nm.

Chrysotile[2] was used as received except for reduction in particle size to that of the talc being tested.

Procedure—Weigh three 0.2000 ± 0.0010-g samples of talc USP. To Sample 2, add about 3 mg of chrysotile, and to Sample 3, add about 6 mg of chrysotile, both accurately weighed. To each sample, add 10 ml of 50% ethanol–water, and also provide a 10-ml sample of 50% ethanol–water to serve as a blank. Give all samples and the blank an ultrasonic treatment[3] for 2 min at 70 w of power. After the samples cool to room temperature, add 10 ml of working dye solution to each and to the blank. Mix and allow to stand for 30 min with occasional shaking. Then centrifuge the samples and blank, and filter through a 45-μm filter[4]. Dilute a 10-ml aliquot of each filtrate to 25 ml with pH 4.2 acetate buffer. Then determine the optical absorbance at 422 nm in a suitable spectrophotometer, and plot the absorbance against milligrams of chrysotile added (Fig. 1). By extrapolation to a baseline, the level of which is indicated by the blank, the chrysotile content of the talc may be determined.

RESULTS AND DISCUSSION

The assay design used here follows that used for an X-ray diffraction determination of chrysotile in air pollution studies (4). The present design differs in that instead of a positive slope for additions of chrysotile (increased peak height), a negative slope is obtained (increased adsorption and, hence, lower amount of dye remaining). The advantage of using this design is that, in effect, an internal standard is being used which can compensate for variations in the dye used, and it makes a fixed calibration curve unnecessary.

The double-separation step involving centrifugation and filtration was needed because the finest talc and chrysotile particles could not be removed in a reasonable time by centrifugation alone. On the other hand, attempted direct filtration rapidly resulted in a plugged filter before enough filtrate could be collected.

Figure 1 shows a typical determination on a talc spiked with

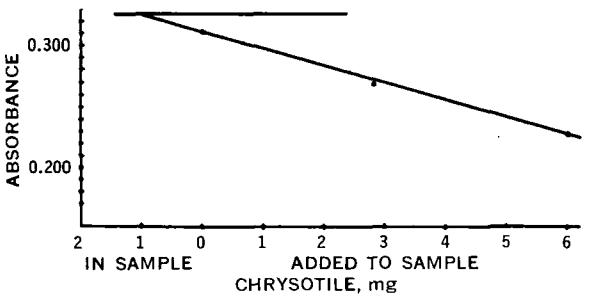

Figure 1—*Extrapolated graph of additions of chrysotile plotted against absorbance.*

[1] Matheson, Coleman and Bell.
[2] Johns Manville Research Center.
[3] Sonifier cell disrupter, model W185D, Heat Systems Ultrasonics, Inc., Plainville, N.Y. The microtip adapter was used.
[4] Millipore.

0.5% chrysotile. The result from the assay in Fig. 1 indicates 1.1 mg chrysotile in a 200-mg sample, or 0.55%. All tests for precision of the assay were done on spiked samples. A limited number of determinations on a sample containing 0.5% chrysotile indicated a standard deviation of ±0.06%.

To eliminate the possibility that a peculiarity of the chrysotile sample used was responsible for the dye adsorption, another sample was obtained[5]. This sample gave substantially the same adsorption behavior as the first chrysotile sample.

SUMMARY

A determination of chrysotile impurity in talc USP was developed. The assay is useful at least at the level of 0.5% chrysotile. An internal standard method is used which eliminates the need for a standard calibration curve. The assay depends on the adsorptive property of the chrysotile for a sulfonphthalein dye.

[5] Geology Department, Indiana University.

REFERENCES

(1) L. J. Cralley, M. M. Key, D. H. Groth, W. S. Lainhart, and R. M. Ligo, *Amer. Ind. Hyg. Ass. J.*, **29**, 350(1968).

(2) R. R. Merliss, *Science*, **173**, 1141(1971).

(3) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 708.

(4) A. L. Rickards, *Anal. Chem.*, **44**, 1872(1972).

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Selective Determination of Phenylpropanolamine Hydrochloride in Pharmaceutical Dosage Forms by Reaction with Ninhydrin

D. BURKE, V. S. VENTURELLA ˣ, and B. Z. SENKOWSKI

Abstract □ A colorimetric method for the determination of phenylpropanolamine hydrochloride, based on the reaction with ninhydrin, was applied to various forms of pharmaceutical products. The method is applicable to pharmaceutical products that do not contain other primary or secondary amines. The determination is dependent upon the combined hydroxyl and primary amino moieties. It is postulated that phenylpropanolamine hydrochloride reacts with ninhydrin under the experimental conditions by a mechanism similar to that for amino acids. A comparison of the molar absorptivities of phenylpropanolamine hydrochloride with those of amino acids, such as glycine and phenylalanine, clearly demonstrates the similarity of the reaction mechanism to the classic ninhydrin mechanism.

Keyphrases □ Phenylpropanolamine hydrochloride formulations—colorimetric analysis, ninhydrin reagent □ Colorimetry—analysis, phenylpropanolamine hydrochloride in mixed formulations □ Ninhydrin reagent—used to determine phenylpropanolamine hydrochloride in mixed formulations

In the past few years, phenylpropanolamine hydrochloride has been used widely in many different pharmaceutical preparations as an adrenergic agent. This popularity can be attributed to the fact that it produces vascular and bronchial effects with little, if any, central effects. It is used in asthma and as a nasal decongestant, both locally and orally (1). Phenylpropanolamine hydrochloride can be found in many formulations including tablets, capsules, syrups, elixirs, suspensions, nasal jellies, and nasal solutions.

Recently, because of the increased use of phenylpropanolamine hydrochloride in complex mixtures, it has become necessary to develop a specific and rapid method for its determination. The method described in this report is dependent upon a colorimetric response to concentrations and, therefore, does not require prior separation from other active ingredients present in many allergenic and cold preparations. By contrast, many reported methods require long and tedious separations prior to quantitative determination.

A review of the literature (2–14) showed that a great variety of methods had been used to determine the phenylpropanolamine hydrochloride content in pharmaceutical formulations quantitatively. The methods used were diatomaceous earth[1] separation followed by spectrophotometry, GLC, extraction and titration, anion-exchange chromatography followed by titration with hydrochloric acid, and periodate oxidation to benzaldehyde.

EXPERIMENTAL[2]

Reagents—*Citrate Buffer, pH 5.0*—Dissolve 21.008 g of citric acid USP in 200 ml of water. Add 200 ml of 1.0 *N* sodium hydroxide solution and dilute to 500 ml with distilled water. Adjust the pH with sodium hydroxide or hydrochloric acid, if necessary.

Potassium Cyanide—Accurately weigh 0.1628 g of potassium cyanide, transfer to a 250-ml volumetric flask, and dilute to volume with water.

[1] Celite.

[2] The sample preparation given for layered tablets, capsules, and combination tablets is typical of that required for most dosage forms.

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[2] The sample preparation given for layered tablets, capsules, and combination tablets is typical of that required for most dosage forms.

Table I—Constituents of Color Development Vessels

Added Solution	Volume Added, ml		
	Blank	Sample	Standard
Working sample solution	—	1.0	—
Working standard solution	—	—	1.0
Citrate buffer	0.5	0.5	0.5
Ninhydrin color reagent	1.2	1.2	1.2
Water	1.0	—	—

Table II—Analysis of a Layered Tablet Formulation[a] with Aspirin

Sample	Assay, mg/Tablet	Percent Claim
1	6.26	100.3
2	6.40	102.3
3	6.27	100.3
4	6.36	101.8
5	6.45	103.1

Mean 6.35 mg/tablet
$\sigma \pm 0.08$ mg/tablet
(ts:95%) $\pm$ 0.22 mg/tablet

[a] Phenylpropanolamine hydrochloride, 6.25 mg/tablet, present as an adsorbate.

Ethanol—Use 60% (v/v) ethanol.

Potassium Cyanide-2-Methoxyethanol Solution—Dilute 1 ml of the potassium cyanide reagent to 25 ml with 2-methoxyethanol[3].

2-Methoxyethanol-Ninhydrin Solution (5.0%)—Accurately weigh 1.25 g of ninhydrin (triketohydrindene hydrate), transfer to a 25-ml volumetric flask, and dilute to volume with 2-methoxyethanol.

Potassium Cyanide-Ninhydrin-2-Methoxyethanol Solution—Mix 5 ml of the 2-methoxyethanol-ninhydrin reagent with 25 ml of the potassium cyanide-2-methoxyethanol reagent.

Standard Reference Solution—Weigh accurately approximately 188 mg of phenylpropanolamine hydrochloride and transfer to a 1-liter flask. Dissolve in, and dilute to volume with, distilled water. Pipet 25 ml of this solution into a 50-ml volumetric flask and add 20 ml of citrate buffer. Adjust to pH 5.0 with sodium hydroxide or hydrochloric acid and dilute to volume with pH 5.0 buffer.

Sample Solution for Layered Phenylpropanolamine Hydrochloride (Adsorbate) and Aspirin Tablets—Weigh accurately the equivalent of approximately 37.5 mg of phenylpropanolamine hydrochloride from a ground, representative tablet mass. Transfer the powder to a blender[4] and add 100 ml of 0.2 N sodium hydroxide. Blend for 2.5 min at a speed suitable to prevent splashing. Let stand for 5 min and repeat the procedure.

Transfer the blender contents quantitatively to a 200-ml volumetric flask with the aid of 0.2 N sodium hydroxide. Dilute to volume with the same reagent. Centrifuge approximately 80 ml of the mixture for 5 min at 6000 rpm. Filter the centrifugate through fluted filter paper. Transfer exactly 25 ml of the filtrate to a 50-ml beaker, and titrate with 0.4 N hydrochloric acid to pH 5.0 $\pm$ 0.2, measured with a suitable pH meter. To a second 25 ml of solution contained in a 50-ml volumetric flask, add exactly the same volume of titrant and dilute to volume with pH 5.0 buffer. Use this prepared solution for the final colorimetric reaction.

Sample Solution for Phenylpropanolamine Hydrochloride Capsules and for Tablets Containing Phenylpropanolamine Hydrochloride, Atropine Sulfate, Chlorpheniramine Maleate, and Acetaminophen—Determine the average weight of 20 tablets or capsules and reduce them to a fine powder. Weigh accurately a portion of ground sample equivalent to about 37.5 mg of phenylpropanolamine hydrochloride, and transfer to a 200-ml volumetric flask. Add 100 ml of 0.10 N hydrochloric acid to the flask and shake mechanically for 30 min. Dilute to volume with 0.10 N hydrochloric acid and filter. Pipet 25 ml of the filtrate into a 50-ml volumetric flask. Add 20 ml of pH 5.0 buffer. Adjust the solutions to pH 5.0 $\pm$ 0.2 before diluting to volume.

[3] Methyl Cellosolve.
[4] Waring.

Table III—Data Obtained for a Combination Tablet[a]

Sample	Assay, mg/Tablet	Percent Claim
1	24.87	99.7
2	24.82	99.5
3	25.30	101.2
4	25.90	103.6
5	25.90	103.6

Mean 25.36 mg/tablet
$\sigma \pm 0.53$ mg/tablet
(ts:95%) $\pm$ 1.47 mg/tablet

[a] Composition: phenylpropanolamine hydrochloride, 25 mg; atropine sulfate, 0.08 mg; chlorpheniramine maleate, 1.33 mg; and acetaminophen, 300 mg.

Table IV—Assay of Phenylpropanolamine Hydrochloride in a Sulfonamide Preparation and a Timed-Release Formula

Sample	Active Formula	Phenylpropanolamine Hydrochloride Found	Percent Claim
1	Trisulfapyrimidines USP (500 mg), acetaminophen (120 mg), and phenylpropanolamine hydrochloride (12.5 mg)	12.9 mg/5 ml[a]	103.2
2[b]	Chlorpheniramine maleate (8 mg), phenylpropanolamine hydrochloride (50 mg), and isopropamide iodide (2.5 mg)	50.55 mg/capsule[c]	101.1

[a] Results of duplicate assays. [b] Timed-release formula. [c] Results of triplicate assays.

Colorimetry—Into separate test tubes of 1.8 cm i.d. (about 30 ml capacity), add, by pipet, sample, standard, buffer, and color reagent as given in Table I.

Transfer the tubes to a 1-liter beaker containing 750 ml of vigorously boiling water. Add several glass beads to each tube and allow the color to develop for 30 min. Remove the tubes from the bath and cool immediately under cold running water for 5 min. Add 10 ml of 60% (v/v) ethanol. Transfer the contents to individual 25-ml volumetric flasks and dilute to volume with the same solvent. Filter the solutions and determine the absorbance of the clear filtrates at 570 nm *versus* the blank in the reference cell.

The content of phenylpropanolamine hydrochloride per capsule or tablet can be found using Eq. 1:

$$\text{phenylpropanolamine hydrochloride (milligrams)} = \frac{A_u}{A_s} \times \frac{W_s \times W_0}{W \times 5} \quad \text{(Eq. 1)}$$

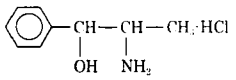
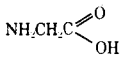
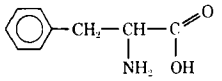
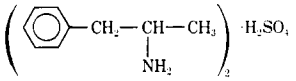
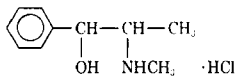
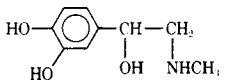
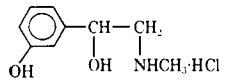
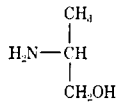
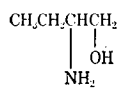
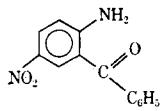
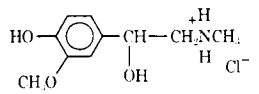
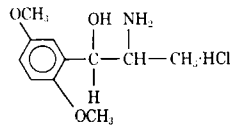
where:

A_u = absorbance of sample solution
A_s = absorbance of standard reference solution
W_s = weight of standard phenylpropanolamine hydrochloride (milligrams)
W = weight of sample (milligrams)
W_0 = average capsule or tablet weight (milligrams)
5 = dilution factor

RESULTS

Analyses of tablet formulations containing phenylpropanolamine hydrochloride adsorbate and aspirin (layered) clearly demon-

Table V—Molar Absorptivity Values for Phenylpropanolamine Hydrochloride, Related Amino Acids, and Amines in 60% Ethanol

Compound	Structure	Concentration, moles/liter × 10^5	A_{570}	ε
Phenylpropanolamine hydrochloride		2.00 2.61	0.387 0.516	19,510[a]
Glycine		2.49	0.492	19,759
Phenylalanine		2.44 2.16	0.425 0.400	17,986[a]
Amphetamine sulfate		1.94	Negligible	—
Ephedrine hydrochloride		2.16 6.53	0.106 0.311	4,628[a]
Epinephrine		8.56	0.179	2,009
Phenylephrine hydrochloride		7.80 9.04	0.200 0.220	2,552[a]
2-Aminopropanol		7.00	0.327	4,671
3-Aminopropanol		6.74	0.592	8,776
1-Amino-2,3-propanediol		2.16 1.93	0.302 0.262	13,778[a]
2-Aminobutanol		14.04 7.00	0.822 0.396	5,751[a]
2-Amino-5-nitrobenzophenone		2.06	Negligible	—
4-Hydroxy-3-methoxy-α-(methylaminomethyl)-benzyl alcohol hydrochloride		3.10 2.70 9.48	0.082 0.067 0.261	2,627[a]
Methoxamine hydrochloride		2.01 1.98	0.406 0.403	20,151[a]

[a] Average of all determinations.

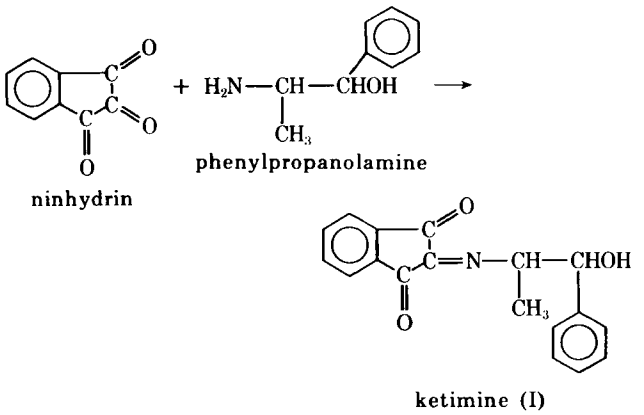
strated that aspirin does not interfere with the procedure (Table II). With formulations of this type, the adsorbate first is released with alkali before the ninhydrin reaction is performed.

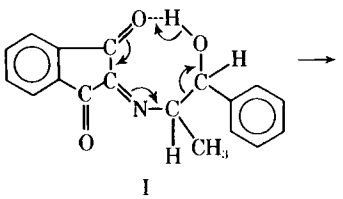
Phenylpropanolamine hydrochloride analyzed in capsule formulations contained the drug as the sole active agent. It was found that capsule diluents do not interfere with the ninhydrin reaction. The usual tablet and capsule diluents, including lactose, talc, starch, and magnesium stearate, did not contribute to the absorbance.

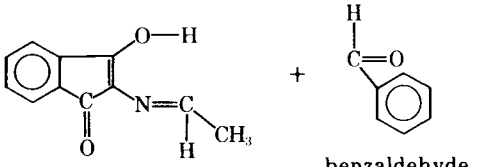
Additionally, phenylpropanolamine hydrochloride was analyzed in a tablet formulation containing acetaminophen (amide), atropine sulfate, and chlorpheniramine maleate (tertiary amine) (Table III). No interference was noted from either the amide or the tertiary amines present. As noted, the preparations are quite variable, which confirms the relative specificity of the ninhydrin reaction as given in this report. Data collected for the assay of other available formulations appear in Table IV.

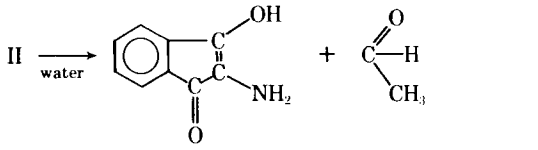
DISCUSSION

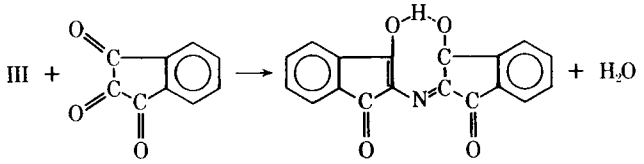
The reaction of several representative types of amino alcohols with ninhydrin was investigated in which the effect of the hy-



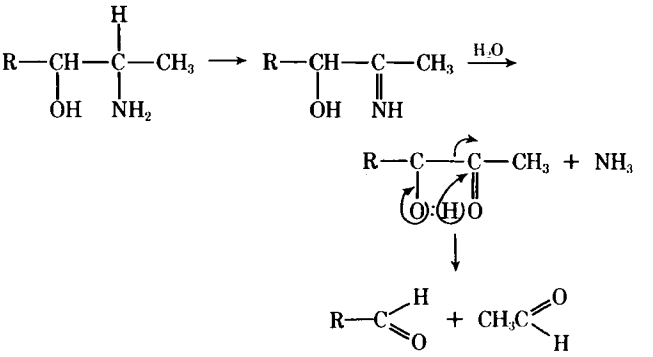








Scheme I

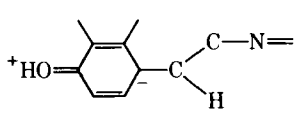


Scheme II

droxyl and the phenyl moieties on the molar absorptivity was examined. The similarity between the reaction of phenylpropanolamine hydrochloride and amino acids with ninhydrin is clearly demonstrated by a favorable comparison of their molar absorptivities as well as the similarity in the reaction conditions (15–18). This includes the fact that both the amine and amino acids give a maximum reaction with ninhydrin at about pH 5.0.

By using the procedure given for the preparation of the standard reference solution, 14 amines were analyzed in pure form to compare the absorbance (A_{570}) and molar absorptivity values. As noted in Table V, of those amino alcohols and amino acids tested, only glycine, phenylalanine, phenylpropanolamine hydrochloride, and methoxamine hydrochloride resulted in a molar absorptivity of similar magnitude (2×10^4), which is typical of a solution of Ruhemann's purple. Glycine and phenylalanine were used as reference models.

It is assumed that the mechanism of chromophore formation



V

with phenylpropanolamine hydrochloride and methoxamine hydrochloride is identical to that for glycine and phenylalanine, which follows the classic ninhydrin scheme, since the molar absorptivities are the same. A mechanism was clearly established and proposed by Neuzil *et al.* (19) for the reaction of primary amino aliphatic alcohols with ninhydrin. It was stated that primary amines having a hydroxyl group on the same carbon chain react with ninhydrin to form the Schiff base diketohydrindylidene–diketohydrindamine or Ruhemann's purple (Table V). The maximum reaction occurs at pH 5, and the maximum reaction for amino acids with ninhydrin occurs at the same pH.

Since phenylpropanolamine hydrochloride, methoxamine hydrochloride, and the amino acids tested have the same range of molar absorptivity, it can be postulated that the mechanism is similar. It also can be theorized that both compound types react *via* a Schiff-base intermediate. By comparing the molar absorptivity obtained for amphetamine sulfate (absence of hydroxyl) and ephedrine (secondary amine) with the molar absorptivity of the two adrenergic amines given, the hydroxyl group and primary amino group appear to be substituents for the proposed mode of reaction, as the carboxyl and amino groups are for the amino acids. By comparing the molar absorptivity of 2-aminopropanol with phenylpropanolamine hydrochloride, it can be seen that the addition of the phenyl group increases the molar absorptivity by a factor of about five. The inductive and conjugative effect of the phenyl group further promotes ease of formation of the aldehyde (Scheme I). Amphetamine, having a phenyl group but no hydroxyl group, has a molar absorptivity significantly less than phenylpropanolamine hydrochloride and, therefore, must be assumed to react in a manner that is not consistent with the amino acid mechanism.

Both the primary amino and alcoholic group are required for the complete reaction *via* the ketimine. Furthermore, it is necessary that cleavage of the ketimine occurs specifically to form an

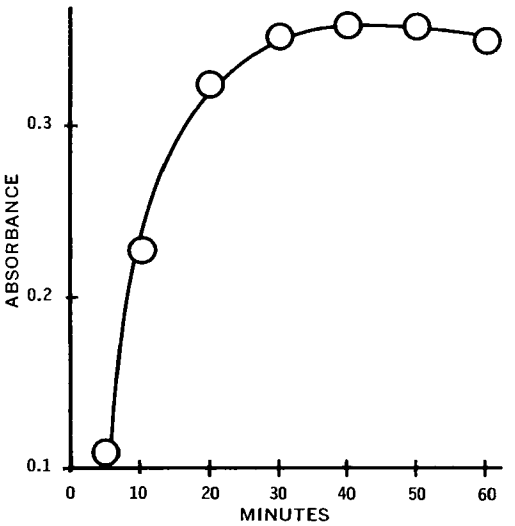


Figure 1—*Heating time–response curve for phenylpropanolamine hydrochloride–ninhydrin reaction.*

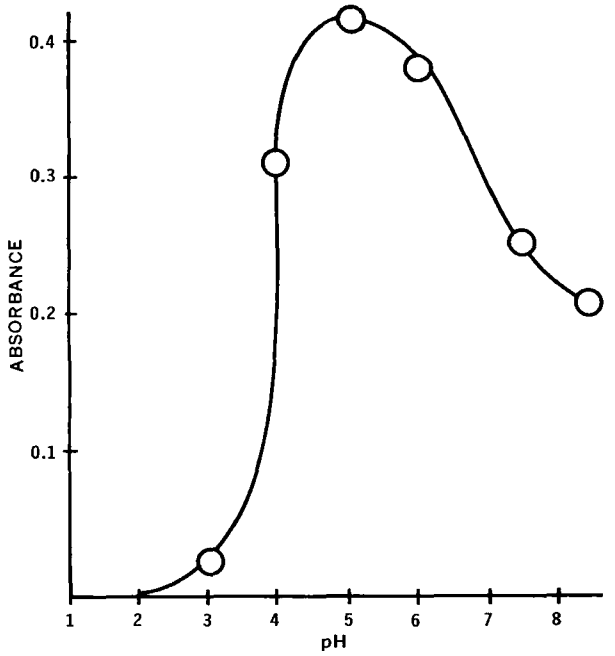


Figure 2—*The pH–response curve for phenylpropanolamine hydrochloride–ninhydrin reaction.*

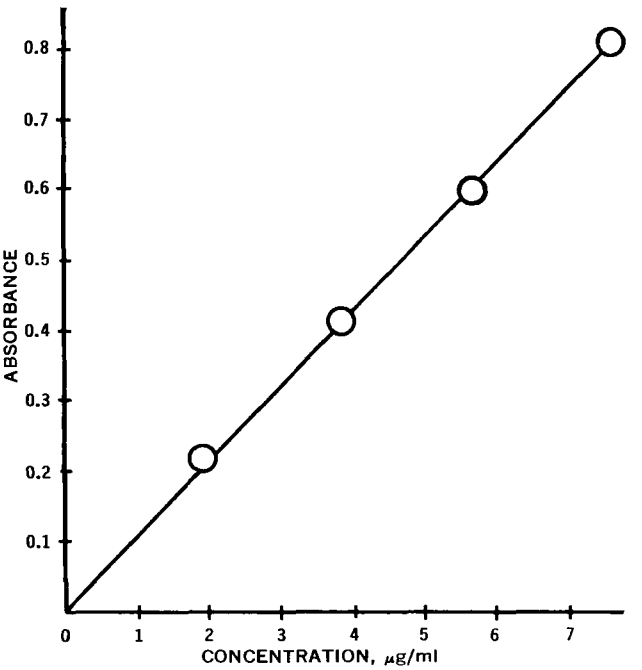


Figure 3—*Concentration–absorbance plot for phenylpropanolamine hydrochloride–ninhydrin reaction.*

aldimine and a carbonyl compound. This occurs to produce the intermediate species capable of reacting with a second mole of ninhydrin. It can be seen from the molar absorptivity values (Table V) that unless all of these conditions are met, complete conversion to Ruhemann's purple cannot occur and a molar absorptivity substantially lower than that for the amino acids is obtained. It is also apparent that the secondary amines or primary amino alcohols with strong deactivation at the aromatic apical center do not give a quantitative conversion. In the case of the secondary amines, the formation of the ketimine is retarded. In the deactivated primary amino alcohols, the deactivation retards cleavage of the ketimine to aldimine by the presence of Structure V. In aromatic secondary amino alcohols, little difference occurs regardless of the changes in nuclear substitution because the retarding influence is based upon the available amine protons.

An alternate mechanism for amino alcohols could be based upon the formation of an imino alcohol followed by formation of ammonia according to Scheme II. The liberated ammonia then reacts with ninhydrin to form III which then follows the latter part of Scheme I.

This alternative is logical since secondary amines (such as ephedrine) do not readily form imino derivatives and thus would not enjoy facile oxidative deamination. In addition, these would not release ammonia and, therefore, would necessarily yield lower molar absorptivity values.

During a study of the variables of the method, it was established that reproducibility largely depended upon heating time and pH. It was found that 30–35 min was required for the reaction to reach its maximum level (Fig. 1) and that the reaction had to be immediately quenched by cooling for 5 min. It also was established that the maximum reaction occurred at about pH 5 (Fig. 2).

The concentration–absorbance plot (Fig. 3) shows that the colorimetric response is linear with concentration in the range of 1.91–7.64 μg/ml. This result is consistent with that originally described by Yem and Cocking (15) for the amino acid reaction with ninhydrin.

SUMMARY

A specific reaction for the determination of phenylpropanolamine hydrochloride is reported. This method can be used to determine phenylpropanolamine hydrochloride in complex mixtures without prior separation. A reaction of phenylpropanolamine hydrochloride with ninhydrin is involved. A reaction mechanism is presented and compared with the classical reaction of amino acids with ninhydrin. The molar absorptivity of a number of amines is listed to show the functional group relationship of phenylpropanolamine hydrochloride to the listed amino acids and to other amines, not yielding a large absorptivity value.

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[x] To whom inquiries should be directed.

Determination of Sodium *o*-Iodohippurate in Pharmaceutical Dosage Forms by High-Speed Liquid Chromatography

ARTHUR J. FALK

Abstract □ A method is presented for the quantitative analysis of sodium *o*-iodohippurate by high-speed liquid chromatography. Following a preliminary extraction to remove benzyl alcohol, the samples are acidified and extracted with ethyl acetate. After evaporation of the solvent, the residue is dissolved in an aliquot of ethyl acetate containing an internal standard and assayed by comparison of peak heights after liquid chromatography. This method is independent of the amount of radioactively tagged material present, thus permitting analysis of the total sodium *o*-iodohippurate present.

Keyphrases □ Sodium *o*-iodohippurate injection—analysis, high-speed liquid chromatography □ High-speed liquid chromatography—analysis, sodium *o*-iodohippurate injection

Sodium *o*-iodohippurate, radioactively tagged with ^{131}I, is referred to in USP XVIII (1) as a diagnostic aid for renal function determinations. Recent interest in such determinations has been stimulated by its use for early postoperative evaluation of the status of transplant patients (2) and for the prediction of function in renal failure (3).

Previous analytical methods have relied on paper chromatography (1), or paper chromatography in combination with electrophoresis (4), followed by detection with autoradiography, β-ray scanning, or scintillation measurements. However, these methods of detection are limited to radioactive material and will not measure the total amount of sodium *o*-iodohippurate present. In addition, Høye (5) indicated that nonradioactive and ^{131}I-tagged sodium *o*-iodohippurate behave differently when chromatographed on paper.

An alternative approach was sought for the quantitation of sodium *o*-iodohippurate exclusive of its radioactivity. GLC and TLC techniques were tried, but difficulty was encountered in the preparation of stable volatile derivatives in the former and in recovery in the latter, which resulted in poor precision. Recently, high-speed liquid chromatography has been applied successfully to pharmaceutical analysis (6–8). This article describes the application of high-speed liquid chromatography to the estimation of sodium *o*-iodohippurate in sterile solution for injection.

EXPERIMENTAL

Apparatus—A high-pressure liquid chromatograph[1], equipped with a UV detector (254 nm) capable of operating at inlet pressures up to 1200 psig, was used.

Column—The column was purchased from the instrument manufacturer and was a 1-m × 2.1-mm i.d. × 0.6-cm o.d., precision bore stainless steel tube. The column packing was an adsorption material[2].

Reagents—Ethyl acetate, chloroform, hydrochloric acid, sodium hydroxide, acetic acid, dibasic sodium phosphate, and anhydrous sodium sulfate were reagent grade and required no further purification. Methyl 2,5-dihydroxybenzoate was prepared by methylation of 3,5-dihydroxybenzoic acid[3] in the usual manner (9). Sodium *o*-iodohippurate[4], *o*-iodobenzoic acid[5], and water for injection, bacteriostatic, with 1% benzyl alcohol[6], were used without further purification.

A simulated solution for injection[7] was prepared in this laboratory. It contained 2 mg/ml sodium *o*-iodohippurate and 0.5 mg/ml dibasic sodium phosphate in water for injection, bacteriostatic, with 1% benzyl alcohol at pH 7.5.

Acetic Acid in Chloroform (1% v/v)—Dilute 5.0 ml of acetic acid to 500 ml with chloroform.

Internal Standard Solution—Dissolve approximately 200 mg of methyl 3,5-dihydroxybenzoate in 200 ml of ethyl acetate to yield a final concentration of approximately 1 mg/ml.

Standard Reference Solution—Accurately weigh 30 mg of sodium *o*-iodohippurate into a screw-cap vial large enough to hold 20 ml. Pipet 10.0 ml of the internal standard solution into the vial, followed by approximately 0.5 ml of acetic acid, and shake to dissolve all solids.

Sample Preparation—Pipet 15.0 ml of the sample solution (*i.e.*, 30 mg of sodium *o*-iodohippurate) into a 125-ml separator

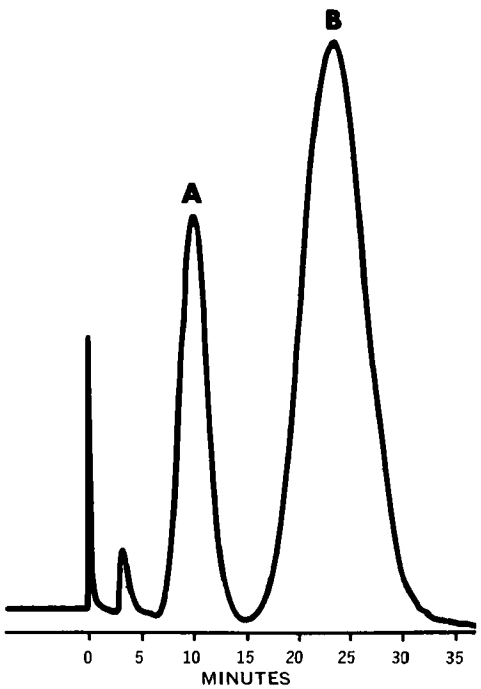

Figure 1—*High-speed liquid chromatogram of a sample extract with internal standard. Key: A, o-iodohippuric acid; and B, methyl 3,5-dihydroxybenzoate (internal standard). The two earlier peaks are artifacts caused by injection and a solvent peak, respectively.*

[1] DuPont model 820, duPont de Nemours and Co., Wilmington, Del.
[2] Sil-X, Nester/Faust Mfg. Corp., Newark, Del.
[3] Naftone, Inc., New York, N.Y.
[4] Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Eastman Kodak Co., Rochester, N.Y.
[6] Abbott Laboratories, North Chicago, Ill.
[7] Hippuran, Abbott Laboratories, was prepared for this experiment with nonradioactive materials.

Table I—Standard Addition

Sodium *o*-Iodohippurate Added[a], mg	Sodium *o*-Iodohippurate Found, mg	Percent Recovered of Amount Added
0.00	19.62	
5.65	25.32	100.9
11.30	30.40	95.6
16.95	36.40	99.0

[a] Milligrams added to 10.0 ml of a solution for injection containing 2.0 mg/ml of sodium *o*-iodohippurate.

and extract twice with 50-ml portions of ethyl acetate, saving both layers. Combine the organic layers and extract with 15 ml of 0.001 *N* sodium hydroxide, discarding the organic phase containing the benzyl alcohol. Combine the remaining aqueous phases; adjust the pH of the solution, using a pH meter, to 2.0 with 1.2 *N* hydrochloric acid; and extract the aqueous phase three times with 50-ml portions of ethyl acetate, discarding the aqueous layer after the final extraction. Filter the combined ethyl acetate layers over approximately 40 g of anhydrous sodium sulfate, and rinse the filter with approximately 20 ml of ethyl acetate, being careful that no water remains in the organic phase after filtration. Evaporate the ethyl acetate to dryness in a current of air (no heat). Pipet 10.0 ml of the internal standard solution into the evaporating dish, followed by approximately 0.5 ml acetic acid, and swirl to dissolve all of the residue. The solution may now be chromatographed or transferred to a screw-cap vial for storage.

Chromatographic Procedure—The analytical column was already described. The mobile phase was 1% acetic acid in chloroform, the temperature was ambient, and the solvent flow was approximately 0.8 ml/min (at an inlet pressure of 1200 psig). The precision photometer detector (254 nm) was set at a sensitivity of 0.04 absorbance unit full-scale.

Samples and standards of 3 μl (approximately 9 μg of sodium *o*-iodohippurate) were injected with the flow stopped. A standard was injected for every four sample injections, and the peak heights of *o*-iodohippuric acid and the internal standard were measured.

Calculations—The response factor, *F*, is the ratio of the internal standard peak height to the *o*-iodohippuric acid peak height in the standard reference solution, multiplied by the weight of sodium *o*-iodohippurate in the standard. The concentration of sodium *o*-iodohippurate in milligrams per milliliter in the sample is then calculated:

$$\text{mg/ml sodium } o\text{-iodohippurate} = (F)\left(\frac{H_1}{H_2}\right)\left(\frac{1}{15}\right) \quad \text{(Eq. 1)}$$

where H_1 and H_2 are the peak heights of *o*-iodohippuric acid and the internal standard in the sample, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the chromatogram of a typical sample extract containing the internal standard. If present, *o*-iodobenzoic acid, the major degradation product, elutes with the solvent and does not interfere with the analysis. Ethyl acetate was chosen as the solvent for extraction because of the success previously shown for the extraction of hippuric acid (10). In addition, the first extraction from a basic aqueous phase serves to remove benzyl alcohol which would interfere with the assay.

The linearity of response (Table I) has been shown for sample solutions containing 1.0–4.0 mg/ml of sodium *o*-iodohippurate.

The precision of the method (Table II) was determined from 10 replicate determinations of sodium *o*-iodohippurate in a solution for injection performed by three analysts over 4 days.

Table II—Precision and Accuracy

Trial	Sodium *o*-Iodohippurate Found[a], mg/ml	Percent Label Claim
1	1.97	98.0
2	1.94	96.4
3	1.98	98.5
4	2.00	99.6
5	1.96	97.7
6	1.97	98.0
7	1.96	97.7
8	1.99	99.2
9	2.02	100.6
10	2.05	102.0
	Mean	98.8%
	Relative standard deviation	1.6%

[a] Theory: 2.008 mg/ml sodium *o*-iodohippurate.

Peak height rather than peak area was chosen as the method of measurement because, as observed in this laboratory and as previously reported (6), peak height varies less with fluctuations in inlet pressure (flow rate) than does peak area.

CONCLUSION

This is a stability-indicating assay because the excipients are separated by the formation of *o*-iodohippuric acid and its subsequent extraction, and the major degradation product, *o*-iodobenzoic acid, is separated chromatographically.

The method, as written, is applicable to nonradioactive material. Therefore, it may be used to determine the concentration of sodium *o*-hippurate in solutions regardless of activity. However, for use with highly radioactive material, the sample preparation procedure may be scaled down accordingly to avoid exposure to radiation.

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Formulation of a Stable Pilocarpine Hydrochloride Solution

IRWIN S. GIBBS* and MURRAY M. TUCKERMAN^x

Abstract □ A stable 1% pilocarpine hydrochloride solution was formulated without added buffers which requires a minimum amount of alkali for neutralization. The solution has an equilibrium pH of about 3.8, contains about 93% of the original pilocarpine used in the formulation, and is self-buffered by a pilocarpine–pilocarpic acid system formed at equilibrium. A solution of pilocarpine hydrochloride is adjusted to about pH 5, and then equilibrium conditions are attained involving a slow, acid-catalyzed resynthesis of pilocarpine from its hydrolysis products.

Keyphrases □ Pilocarpine hydrochloride—formulation in stable solution without added buffers □ Ophthalmic formulations—stable pilocarpine hydrochloride solution without added buffers

Pilocarpine, as the hydrochloride or nitrate salt, enjoys a prominent position in ophthalmic therapeutics because of efficacy in the treatment of chronic wide-angle glaucoma (1–4). Although alkaloids, such as pilocarpine, are thought to be best absorbed through the cornea from an alkaline solution (5–7), the marked chemical instability of these drugs at pH values above 7 precludes formulation of a basic solution for distribution and use over a long period.

Unlike the decomposition of alkaloids containing an ester functional group, the hydrolytic decomposition of pilocarpine, a lactone, is unique in that ring closure of the base-catalyzed hydrolysis product takes place on acidification (8–10). This reversibility of the hydrolysis of pilocarpine was overlooked by several authors writing about pilocarpine instability (11–14).

Baeschlin and Etter (15) performed a kinetic study on the reversible decomposition of pilocarpine in buffered solutions. Using a published assay method (16), they determined that the hydrolysis of pilocarpine hydrochloride is a pseudo-first-order reaction at pH 6.0 in the presence of phosphate buffer. The equilibrium concentration of pilocarpine hydrochloride was found to be dependent on the pH value in solution but independent of temperature between 50 and 70°.

The kinetics of pilocarpine hydrolysis in aqueous solution utilizing pH-stat titrimetry and polarimetry were also studied (17). A cyclic mechanism for the hydrogen-ion- and hydroxyl-ion-catalyzed hydrolysis was proposed. Rate constants, equilibrium constants, and the energy of activation for the hydroxyl-ion-catalyzed hydrolysis were calculated.

This research was designed to study the stability of 1% (4.09×10^{-2} *M*) pilocarpine hydrochloride in unbuffered aqueous solutions stored in glass (Pyrex) and polyethylene containers at elevated temperatures utilizing an optimal ferric hydroxamate assay (18) for the determination of intact pilocarpine. Solutions found to exhibit suitable stability were compared with commercially available solutions using an *in vitro* model of expected irritation upon ocular instillation.

EXPERIMENTAL[1]

Stability Study Procedures—One percent (4.09×10^{-2} *M*) pilocarpine hydrochloride solutions were prepared by dissolving pilocarpine hydrochloride USP in water and adjusting the pH to the desired value with 70% perchloric acid, 4 *M* NaOH solution, or 18 *M* sulfuric acid. After sterilization by bacteriological filtration, aliquots of the solutions were transferred into previously sterilized and weighed 100-ml volumetric flasks (Pyrex) or 120-ml (4-oz) polyethylene[2] bottles. The filled containers were weighed and stored at 45 ± 2 and 65 ± 2°.

From the unused filtered solution in each batch, samples were taken for the initial pilocarpine assay by the method of Gibbs and Tuckerman (18), performed in triplicate, and for measurement of the pH value. Additional samples of each batch were placed in thioglycollate medium[3] for testing sterility. One sample solution in each batch at each temperature was maintained as a weight control. This container was weighed periodically, and the percentage of water lost by evaporation was calculated. This percentage factor, when larger than 1%, was used to determine the proportional volume of solvent necessary to reconstitute the other solutions of the batch at a given temperature based upon their weight at the time at reconstitution. The solvent employed for reconstitution was water adjusted to the pH value exhibited by the solution on the previous sampling by the addition of sodium hydroxide or perchloric acid. This solvent was sterilized by bacteriological filtration.

From the other solutions held at a given temperature, samples of approximately 8 ml each were removed on days subsequent to preparation. After allowing the sample to cool at room temperature, one 2.00-ml aliquot from each sample was assayed and the pH value of the residual sample was measured. Concomitant with removal of samples for assay and pH measurement, samples for sterility testing were aseptically taken from each container.

All solutions were maintained in a sterile state throughout the study.

The slope and absorbance intercept (ordinate) for the set of standards run each day were calculated by linear regression analysis. The pilocarpine content of the samples assayed on that day was obtained from the linear equation.

Graphical representations of typical concentration–pH profiles obtained in glass (Pyrex) and polyethylene containers are shown in Figs. 1 and 2. On each day of sampling, three samples of each batch were assayed. Where a range of assay or pH values was obtained, the range is represented by a vertical line with an intersecting horizontal line at the average value.

Titration with Sodium Hydroxide Solution as a Measure of Expected Ocular Irritation upon Instillation of Pilocarpine Hydrochloride Solutions—The amount of alkali needed to ti-

[1] The equipment used consisted of a Gilford Instrument Laboratories model 2000 automatic spectrophotometer with a Beckman Instruments model DUR monochromator, a Cary Instruments model 15 recording spectrophotometer, and a Fisher Scientific Co. model 210 pH meter with a Beckman Instruments combination electrode, No. 39013.

[2] Alathon 20, Dupont.

[3] Bacto Fluid Thioglycollate Medium, Difco Laboratories.

Table I—Pilocarpine Hydrochloride Content at Time of Last Assay of Titrated Solutions, Initial pH Values, and Volume of Titrant (0.1 *N* Sodium Hydroxide Solution) Necessary to Attain pH 7.40 in the Solutions

Solution	Mean Pilocarpine[a] Hydrochloride Concentration in Titrate, moles liter^{-1} × 10^2	Mean Initial pH Value before Titration	Mean Volume of Titrant to Attain pH 7.40 in Mixture, ml
A	3.54 ± 0.07	4.77 ± 0.03	3.81 ± 0.09
M	3.82 ± 0.02	4.71 ± 0.02	3.96 ± 0.04
I	—	4.87 ± 0.00	3.48 ± 0.04
R	3.78 ± 0.01	3.91 ± 0.01	2.97 ± 0.03
S	3.71 ± 0.01	3.90 ± 0.01	2.91 ± 0.01
T	3.88 ± 0.05	3.50 ± 0.01	3.11 ± 0.04
U	3.88 ± 0.02	3.48 ± 0.01	3.04 ± 0.01

[a] Obtained by ferric hydroxamate assay procedure.

trate the stable solutions to the pH of lacrimal fluid (pH 7.4) was compared with that needed for commercial preparations. The solutions titrated and their compositions are: Solution A, a sterile buffered solution of 1% pilocarpine hydrochloride with 0.01% benzalkonium chloride, boric acid, potassium chloride, and sodium carbonate; Solution M, a sterile buffered solution of 1% pilocarpine hydrochloride with 0.01% benzalkonium chloride, 0.01% disodium edetate, sodium phosphate, sodium biphosphate, and sodium chloride; Solution I, a sterile buffered solution of 1% pilocarpine hydrochloride with 0.5% hydroxypropyl methylcellulose (4000 cps), benzalkonium chloride (1:25,000), phenylmercuric nitrate (1:75,000), boric acid, sodium chloride, and sodium citrate; Solution R, 1% pilocarpine hydrochloride solutions prepared at pH 5.11 and stored at 65° in flasks (Pyrex); Solution S, 1% pilocarpine hydrochloride solutions prepared at pH 5.08 and stored at 45° in flasks (Pyrex); Solution T, 1% pilocarpine hydrochloride solutions prepared at pH 4.15 and stored at 65° in flasks (Pyrex); and Solution U, 1% pilocarpine hydrochloride solutions prepared at pH 4.08 and stored at 45° in flasks (Pyrex).

Commercial Solutions A and M and all experimental solutions titrated were assayed by the ferric hydroxamate method within 5 days of the titrimetric studies. Commercial Solution I was not assayed because of the presence of the thickening agent, hydroxypropyl methylcellulose. The effect of this cellulose derivative upon the results of the assay procedure is not known.

From each sample of experimental or commercial solutions employed, a 10.00-ml aliquot was removed and placed in a small beaker. A combination electrode was inserted into the solution, the pH value of the solution was measured, and the solution was titrated with 0.1 *N* sodium hydroxide. The pH value of the titrated solution was measured after the addition of each 0.20- or 0.40-ml volume increment of titrant. The solution was stirred by hand while adding titrant and between the addition of incremental volumes of titrant. Titrant was added until the pH value of the titrated solution was slightly above pH 7.40.

The volume of 0.1 *N* sodium hydroxide necessary to attain pH 7.40 was calculated using interpolation of the titrant volume and pH value data where the addition of an exact 0.20-ml increment of titrant did not yield the pH value 7.40. Table I shows the mean values together with the standard deviations for the pilocarpine content of the solutions employed, the initial pH value of the solutions before addition of titrant, and the volume of titrant necessary to raise the pH value of each solution to 7.40.

RESULTS

Only those solutions prepared at pH 6.80 and stored in polyethylene bottles at 45 and 65° showed significant degradation with time (Figs. 3 and 4). Actual assay values are given in Tables II and III. In all cases, however, the pH values decreased and, in glass (Pyrex), finally approximated a stable value. The rate of approach to equilibrium was increased by increasing the temper-

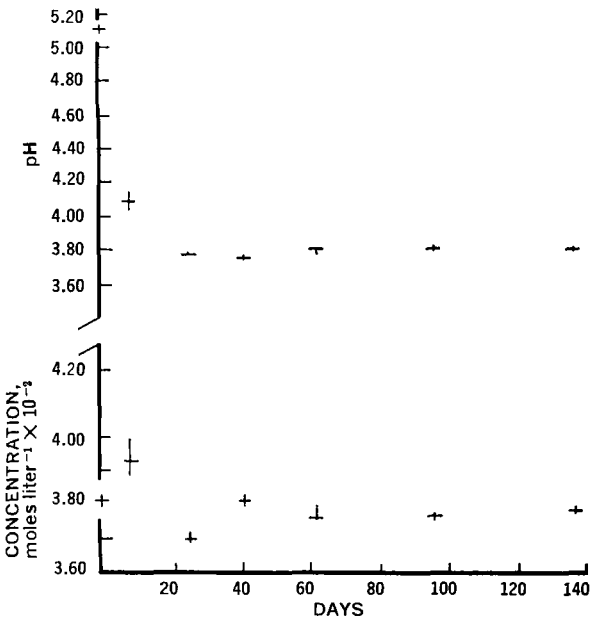

Figure 1—*Concentration–pH profile for pilocarpine hydrochloride solution prepared at pH 5.11 and stored at 65° in flasks (Pyrex).*

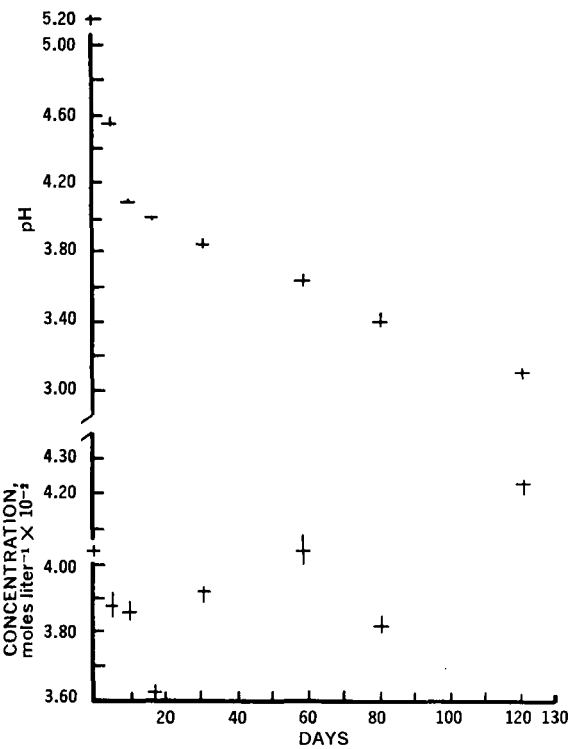

Figure 2—*Concentration–pH profile for pilocarpine hydrochloride solution prepared at pH 5.15 and stored at 65° in polyethylene bottles.*

Table II—Mean Assay Values for Unbuffered Pilocarpine Hydrochloride Solutions Stored in Flasks (Pyrex) at 45 and 65° and Percent Recovery Based on Initial Concentration (1% w/v, 4.09×10^{-2} mole liter^{-1})

Solution							
Initial pH	Final (Equilibrium) pH	Storage Temperature	Time of Study, days	Total Number of Assays	Mean Assay Value, moles liter$^{-1} \times 10^{2}$	Standard Deviation	Percent Recovery Based on Mean Assay Value and Initial Concentration of 4.09×10^{-2} mole liter^{-1}
5.11	3.80	65°	137	21	3.79	0.07	92.7
5.08	3.80	45°	118	18	3.80	0.13	92.9
4.15	3.40	65°	137	21	3.86	0.09	94.4
4.08	3.40	45°	118	18	3.92	0.13	95.8

Table III—Mean Assay Values for Unbuffered Pilocarpine Hydrochloride Solutions Stored in Polyethylene Containers at 45 and 65°

Solution						
Initial pH	Final pH	Storage Temperature	Time of Study, days	Total Number of Assays	Mean Assay Value, moles liter$^{-1} \times 10^{2}$	Standard Deviation
5.15	3.15	65°	121	24	3.93	0.17
5.15	3.85	45°	131	24	3.89	0.10
4.00	3.00	65°	111	18	3.93	0.13
4.00	3.32	45°	104	17	3.96	0.10
5.17	3.14	65°	148	15	3.75	0.12
5.17	3.77	45°	148	15	3.84	0.12
4.10	3.00	65°	147	15	4.00	0.11
4.10	3.40	45°	147	15	3.99	0.09
3.72	2.95	65°	147	15	3.94	0.18
3.72	3.24	45°	147	15	3.94	0.16

ature, but the final value obtained depended only on the initial pH at preparation, not on the temperature of storage.

The solution prepared at pH 5.11 had a final pH of 3.80 and an intact pilocarpine content of 92.7% of the original value. The solution prepared at pH 4.15 had a final pH of 3.40 and an intact pilocarpine content of 94.4% of the original value.

The solutions stored in polyethylene, although showing the decrease in pH, did not approach a constant value; the final pH was below the equilibrium value found in glass (Pyrex). In addition, the mean final pH value for solutions held at 65° was uniformly lower than for those held at 45°.

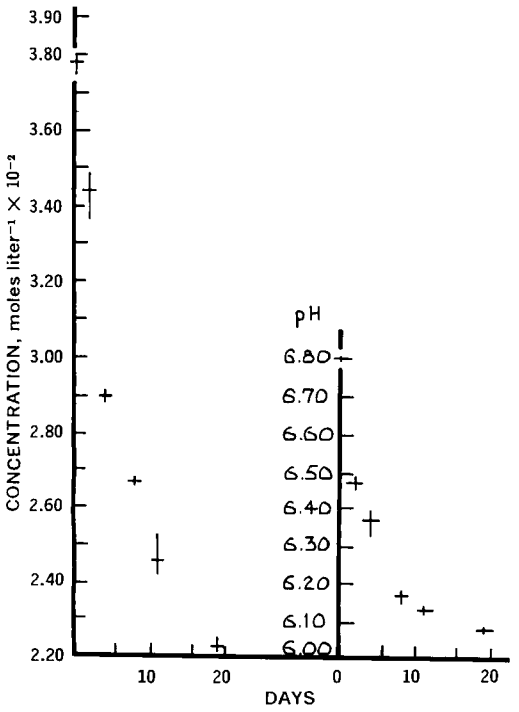

Figure 3—*Concentration–pH profile for pilocarpine hydrochloride solution prepared at pH 6.80 and stored at 65° in polyethylene bottles.*

The titrimetric studies (Table I) showed that Solutions R, S, T, and U, the solutions prepared without added buffer, require substantially less alkali for neutralization than the commercial preparations. If the amount of alkali consumed is taken as a measure of potential irritation upon ocular instillation, Solution S must be considered as an optimum formulation.

DISCUSSION

In the past, because of the use of lime-glass containers that leached alkali which catalyzed the hydrolysis of pilocarpine, it was desirable to buffer pilocarpine solutions. The availability of glass containers that leach very little alkali and, more recently, the availability of plastic containers[4] (20) for dispensing ophthalmic solutions obviate this need for a buffered formulation.

It was found (21) previously that there is little need to apply a buffered pilocarpine hydrochloride solution of near physiological pH and that a somewhat more acid, but unbuffered, solution will suffice. Furthermore, an isotonic solution of pH 3.0 hydrochloric acid is not irritating while isotonic phosphate and acetate buffers of approximately 0.1 *M* and pH 5.0 are distinctly irritating upon ocular instillation (22). Adjustment of the pH value of a solution to the acid range using a buffer may actually have the deleterious effect of providing resistance to normal change toward neutrality upon ocular instillation, thus resulting in a prolongation of irritation and decreased drug absorption (23).

The final pH values for the solutions prepared at pH 4.15 and 4.08 and stored in glass (Pyrex) at 45 and 68° agree within 0.1 pH unit with the values obtained (12) with unbuffered pilocarpine hydrochloride solutions containing sodium chloride and phenylmercuric nitrate stored for 12 months at room temperature. The decrease of pH in solutions stored in polyethylene containers to values below the equilibrium values in glass (Pyrex) is attributed by the authors to the leaching of oxidative degradation products

[4] Drop-Tainers.

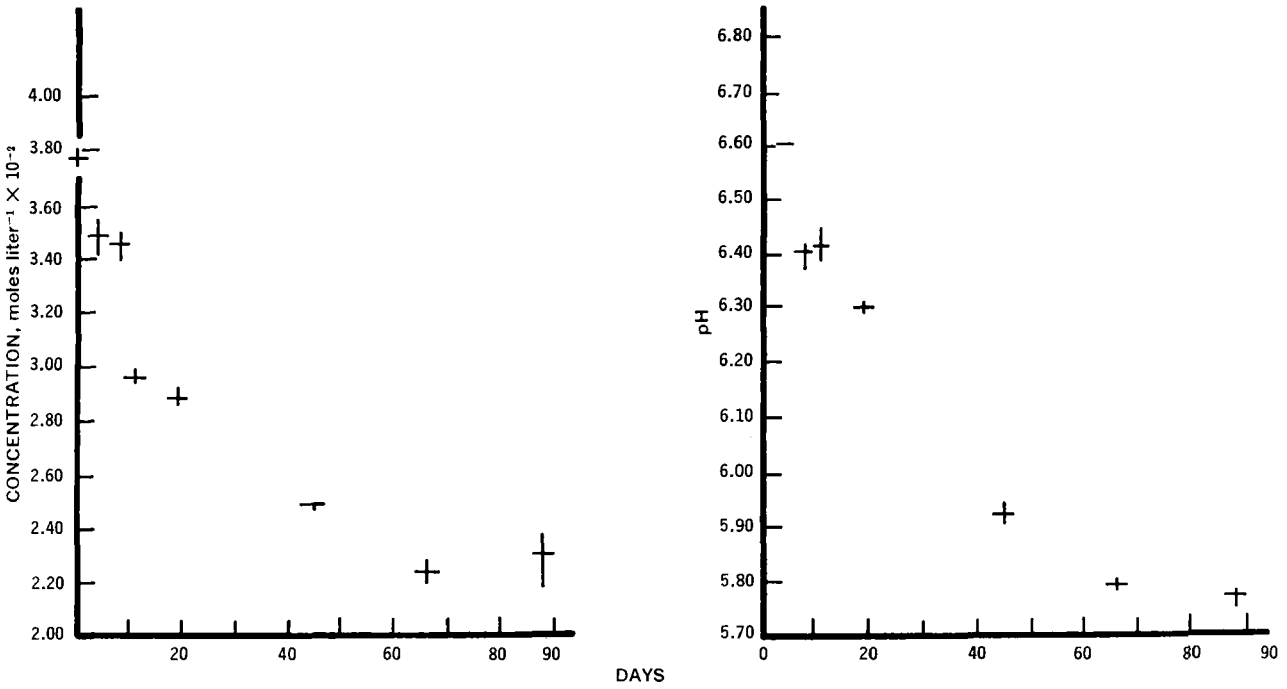

Figure 4—*Concentration–pH profile for pilocarpine hydrochloride solution prepared at pH 6.80 and stored at 45° in polyethylene bottles.*

formed in the polyethylene by the prolonged heating of the polyethylene at the elevated temperatures. The observation that the final mean pH value of solutions stored at 65° is lower than those stored at 45° is in accord with this explanation.

The amount of alkali needed to neutralize any of the commercial solutions is larger than that needed for any of the experimental solutions. The results are explainable since the commercial solutions contain additional buffer systems (observed as differences in the shape of the titration curves obtained for the commercial pilocarpine hydrochloride solutions and the experimental solutions) which account for the increased volume of titrant required to elevate the pH value of the solution to 7.40.

With the commercial solutions, the alkalimetric titration involves neutralization of the adjuvant buffer system, of any pilocarpic acid in solution, and of the less acidic protonated nitrogen of the imidazole moiety of the pilocarpine molecule (pKb = 7.15, pKa = 6.85). The alkalimetric titration of unbuffered Solutions R, S, T, and U, however, involves only neutralization of the pilocarpic acid and the protonated imidazole nitrogen.

CONCLUSION

An especially stable ophthalmic solution of 1% pilocarpine hydrochloride was prepared. This solution is expected to give minimal irritation and good drug absorption when instilled into the eye. This expectation is based upon the amount of alkali necessary to raise the pH value of the solution to the approximate pH value of the lacrimal fluid. The solution can be prepared by dissolving pilocarpine hydrochloride in distilled water, adjusting the pH value to about 5, sterilizing by bacteriological filtration or autoclaving, and packaging the solution in a sterile container made of an inert material.

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* Present address: Squibb Institute for Medical Research, New Brunswick, N.J.

x To whom inquiries should be directed.

Microencapsulation of a Waxy Solid: Wall Thickness and Surface Appearance Studies

P. L. MADAN, L. A. LUZZI, and J. C. PRICE x

Abstract □ Microencapsulation of solid stearyl alcohol particles by complex coacervation was studied. Spherical particles of solid stearyl alcohol were prepared by the vibrating capillary method. Various size fractions of these particles were encapsulated by a modified technique described in an earlier report. Particle concentration and particle-size studies revealed that only a small fraction of the total amount of colloid was used in the formation of the capsule wall. Wall thickness decreased with increasing ratios of solid particles and increased as the particle diameters increased. Scanning electron micrograph observations of the surface showed that acacia was retained on the surface of particles melted and congealed in acacia solution and that the final encapsulated particles had a scaly surface appearance. The studies tended to confirm indications from earlier work that gelatin molecules interact directly with acacia on the surface of the stearyl alcohol particles to form the capsule.

Keyphrases □ Microencapsulation—acacia-coated solid stearyl alcohol particles with gelatin solution, wall thickness, surface appearance □ Encapsulation, micro—acacia-coated solid stearyl alcohol particles with gelatin solution, wall thickness, surface appearance □ Stearyl alcohol particles, acacia coated in gelatin solution—microencapsulation, wall thickness determined, surface appearance evaluated □ Gelatin—interaction with acacia on stearyl alcohol particle surface, microencapsulation, wall thickness, surface appearance □ Acacia—interaction with gelatin on stearyl alcohol particle surface, microencapsulation, wall thickness, surface appearance

There have been a number of reports concerning encapsulation of various substances but few concerning the extent or nature of encapsulation. Undoubtedly the characteristics of the product depend, not only on the extent of encapsulation, but also on the final thickness and integrity of the deposited encapsulating material. The gelatin–sulfamerazine ratio was used (1) as an indication of wall thickness in studying encapsulation of sulfamerazine by simple coacervation. Apparently few attempts have been made to evaluate the wall thickness of microcapsules made by complex coacervation. A recent report (2) considered wall thickness and diffusion from microcapsules containing a liquid organic base.

The purposes of this investigation were to: *(a)* encapsulate spherical solid particles by complex coacervation, *(b)* evaluate the wall thickness of the microcapsules, *(c)* study the effect of particle concentration and particle size on the wall thickness of microcapsules, and *(d)* study the surface appearance of the particles at several stages of the process.

EXPERIMENTAL

Materials—Acacia USP, stearyl alcohol USP, and pigskin gelatin[1] (isoelectric point at pH 8.0) were used.

Selection of Material to Be Encapsulated—To achieve uniformity of the particles to be encapsulated, it was desirable to produce spherical particles, preferably monodisperse. The selection of the material to be encapsulated was, therefore, quickly narrowed to materials that could be easily melted and rapidly congealed to fulfill requirements of available methods of spherical particle production. Wax-like materials most nearly fulfill these requirements; stearyl alcohol was chosen, although a number of other wax-like substances, *e.g.*, beeswax and paraffin, could have been used with little difficulty.

Selection of Particle Size of Core Material—The following reasons predetermined the use of particles larger than 250 μm in diameter: *(a)* convenience, ease in handling, and microscopic observation; *(b)* the discovery that the larger wax particles when encapsulated by a modified procedure seemed to give thin, uniform capsule walls (3) from which thicknesses could be more easily estimated; and *(c)* a desire to explain the formation of the thin, uniform walls around the waxy particles.

Preparation of Particles to Be Encapsulated—The vibrating capillary method of production of spherical particles, similar to that described previously (3, 4), was used. A nest of sieves was employed for the separation of particles in various size ranges. Particles passing through one sieve and retained on the next finer sieve were assigned the arithmetic mean size of the two screens. Sieves 60, 40, 30, and 20 were used to obtain particles having average diameters of 335, 505, 715, and greater than 840 μm, respectively. The particles obtained were spherical in shape and had a predictable size distribution.

Microencapsulation Procedure (Scheme I)—All experiments were carried out under identical experimental conditions. In all experiments, gelatin and acacia solutions were prepared by dissolving, separately, equal quantities of gelatin and acacia in 20 ml of distilled water. These solutions were allowed to hydrate for at least 12 hr before being used. Coacervation was carried out at 40° using a water bath maintained at 40 ± 1°.

A known weight of the solid stearyl alcohol particles was dispersed in acacia solution with gentle stirring, and the dispersion was heated to 60°. At this temperature, stearyl alcohol particles melted and apparently became coated with acacia. The dispersion was then cooled quickly to congeal the particles, and the acacia-coated particles were encapsulated by the procedure reported previously (3).

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[1] American Agricultural Co., Detroit, Mich.

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Table I—Effect of Particle Concentration on Encapsulation and Wall Thickness[a]

Weight of Particles, g	Calculated Surface Area of Particles, cm^2	Number of Particles (n) × 10^{-4}	Stearyl Alcohol Encapsulated, %	Wall Thickness[b] (T), μm	$T\sqrt{n} \times 10^{-2}$
1.00	208	5.9	91.5	0.44 ± 0.02	107
3.00	624	17.7	86.6	0.26 ± 0.02	109
6.00	1248	35.4	81.6	0.17 ± 0.01	102
10.00	2080	59.0	65.6	0.15 ± 0.01	115

[a] Using 335-μm diameter particles in 40 ml mixture of 5% total colloid concentration. [b] Each value represents the average of at least three experiments; ± values indicate the maximum range of values obtained.

on the wall thickness of the encapsulated particles. Particles having an average diameter of 335 μm were encapsulated in a system consisting of 1.00 g of acacia and 1.00 g of gelatin dissolved separately in 20 ml of distilled water. A series of experiments was conducted using 1.00, 3.00, 6.00, and 10.00 g of particles in each experiment while keeping all other conditions constant.

The extent of particles encapsulated was determined by briefly washing the dried encapsulated particles with toluene. Stearyl alcohol particles not encapsulated or only partially encapsulated were dissolved by the toluene, leaving encapsulated particles relatively untouched. The percent of particles encapsulated was then calculated from the original weight of particles and the dried weight remaining after the brief toluene wash.

Particle-Size Studies—Particle-size studies were conducted to determine the effect of particle size on the wall thickness of the encapsulated particles. Particles having average diameters of 335, 505, 715, and greater than 840 μm were encapsulated as described. All experiments were conducted by encapsulating 3.00 g of the particles in a system consisting of 1.00 g of acacia and 1.00 g of gelatin dissolved separately in 20 ml of distilled water. Wall material was recovered from the encapsulated particles, and wall thickness was computed as described in the following sections.

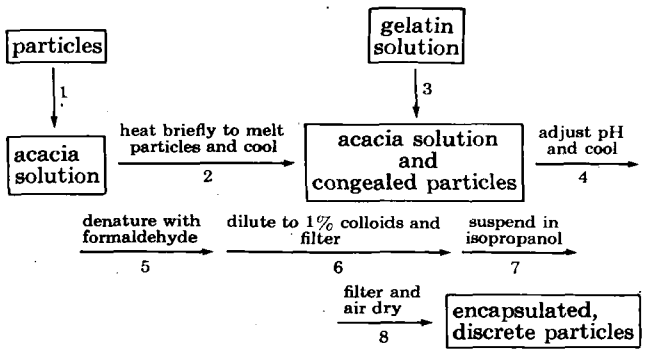
Encapsulation of Acacia-Coated Particles with Gelatin Solution—To obtain evidence concerning whether gelatin can interact directly with acacia at the particle surface to encapsulate the particles, acacia-coated particles were encapsulated by placing the pretreated particles in a 5% gelatin dispersion and proceeding as outlined previously, except that no further acacia solution was used. Encapsulation was followed microscopically, but wall thickness was not determined for these particles.

Wall Recovery Procedure—The wall material was recovered from the encapsulated particles by extraction of stearyl alcohol with toluene as follows. The dried, encapsulated material was treated briefly with toluene to dissolve any uncoated or partially coated particles. The washed and dried microcapsules were carefully separated mechanically from any adhering debris or excessive encapsulating material with the aid of a low power microscope and microspatula. A portion of these microcapsules was then carefully dried, and a known weight was refluxed with toluene for at least 30 min. Forty milliliters of toluene was used for each gram of microcapsules. This treatment completely extracted and dissolved the stearyl alcohol, leaving undissolved wall material. After separation by filtration and further washing with toluene, the wall material was dried and weighed. As a check on completeness of extraction, the weight of the stearyl alcohol extracted was determined after evaporation of the toluene washings and filtrate.

Determination of Wall Thickness—When assuming uniform, smooth, spherical particles, the average wall thickness (for thin-walled capsules) is given by Eq 1:

$$\text{wall thickness} = \frac{\text{volume of wall material recovered}}{\text{surface area of particles encapsulated}} \quad \text{(Eq. 1)}$$

If W = weight of microcapsules taken, W_w = weight of wall material collected, ρ_w = density of wall material, ρ = density of stearyl alcohol particles, and d = diameter of stearyl alcohol particles, then:



Scheme I—Modified encapsulation procedure

$$\text{volume of wall material recovered} = W_w/\rho_w \quad \text{(Eq. 2)}$$

The surface area, s, of a single spherical particle is:

$$s = \pi d^2 \quad \text{(Eq. 3)}$$

The number of particles per gram, N, is given by:

$$N = \frac{6}{\pi d^3 \rho} \quad \text{(Eq. 4)}$$

The total surface area, S, of N particles in a unit weight is, therefore:

$$S = (\pi d^2)\left(\frac{6}{\pi d^3 \rho}\right) \quad \text{(Eq. 5)}$$

or $S = 6/d\rho$ per unit weight of particles.

Since the weight of particles encapsulated is $W - W_w$:

$$\text{surface area of particles encapsulated} = (W - W_w)\left(\frac{6}{d\rho}\right) \quad \text{(Eq. 6)}$$

Therefore:

$$\text{wall thickness} = \left(\frac{W_w}{W - W_w}\right)\left(\frac{\rho}{\rho_w}\right)\left(\frac{d}{6}\right) \quad \text{(Eq. 7)}$$

The density of the wall material was calculated from the displacement volume of a known weight of wall material using toluene as the displacement fluid. The density of solid stearyl alcohol was determined in a similar manner using water as the displacement fluid. The calculated values are 1.341 g/cm^3 for the wall material and 0.861 g/cm^3 for the solid stearyl alcohol at 25°.

For thick-walled capsules, an encapsulated particle can be considered as two concentric spheres of volumes V_1 and V_2 having radii of r_1 and r_2, respectively. The thickness can then be determined from the volume relationships of the concentric spheres.

Optical and Electron Scanning Microscopic Studies of Capsule Wall—Photomicrographs, using an optical microscope[2], were made of small particles encapsulated by a reported procedure (5) for purposes of comparison with the large particles encapsulated by the procedure reported here. Photomicrographs of the wall of the large particles could not be made by the usual method because of the thinness of the walls. The walls of these particles were, therefore, shown by slow extraction of the wax with toluene, leaving the capsule wall suspended in the toluene. Photomicrographs were then taken, at various stages of the dissolution of the wax, while the capsules were still suspended in the toluene.

Scanning electron micrographs[3] were made of particles taken

[2] Bausch & Lomb photobinocular flat field microscope, Bausch & Lomb Optical Co., Rochester, N.Y.

[3] Cambridge Stereoscan, type 96113-2A, Cambridge Instrument Co., London, England.

Table II—Effect of Particle Size on Wall Thickness[a]

Particle Diameter, μm	Calculated Surface Area of Particles, cm^2	Number of Particles $(n) \times 10^{-4}$	Wall Thickness[b] (T), μm	$T\sqrt{n} \times 10^{-2}$
335	624	17.7	0.26 ± 0.02	109
505	414	5.17	0.49 ± 0.03	111
715	292	1.82	0.64 ± 0.02	86
840	249	1.12	1.31 ± 0.13	139

[a] Using 3 g of particles in 40 ml mixture of 5% total colloid concentration. [b] Each value represents the average of at least three experiments; ± values indicate the maximum range of values obtained.

from various stages of the process to show surface features of the particles. These particles were all shadowed with palladium-gold alloy before the micrographs were made.

RESULTS AND DISCUSSION

Effect of Particle Concentration—The effect of particle concentration on the extent of encapsulation and on wall thickness is shown in Table I. The percent of particles encapsulated decreased slightly as the weight of stearyl alcohol particles was increased, showing about a 10% decrease as the concentration of particles was changed from 20 to 75% (1–6 g in Table I) of the total solids present. However, a further increase in particles to 80% of the total solids resulted in an additional 16% drop in the ratio of particles encapsulated. Although the percent of particles decreased, the total quantity of particles encapsulated and encapsulated area increased.

In contrast, wall thickness of the encapsulated particles showed over a 60% drop (0.44–0.17 μm) when particle concentration was increased from 20 to 75% of total solids but showed an insignificant drop (to 0.15 μm) when the particle concentration was further increased to 80% of total solids. In all cases, only a small fraction of the total colloids available was incorporated into the walls of the microcapsules. The maximum colloid used amounted to about 2% of that available, and the internal or encapsulated phase exceeded 98% in all cases.

Effect of Particle Size—Table II shows the effect of particle size on wall thickness of the encapsulated particles. Results indicate that the wall thickness of the capsules increased as the diameter of the particles encapsulated was increased and the surface area of the particles decreased.

Although the results in both Tables I and II show an increase in wall thickness as the surface area of the particles decrease, correlation between the two experimental techniques on the basis of surface area does not appear to be useful. This is true, since wall thickness appears to show a different relationship to surface area in each reported experiment. However, the number of particles present appears to correlate more closely. It is not possible to draw any firm conclusions on the basis of these data, but the wall thickness times the square root of the number of particles present gives a reasonably constant number (about 110), indicating that wall thickness in these experiments is inversely related to the square root of the number of particles present when either the particle size or the weight of particles present is varied (Fig. 1).

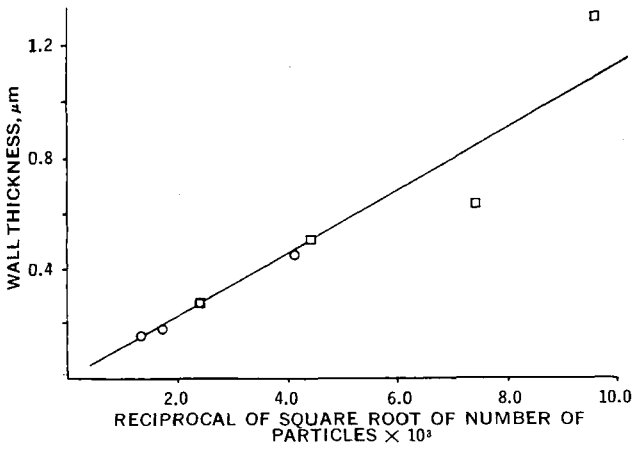


Figure 1—*Wall thickness of microcapsules as a function of reciprocal of square root of number of stearyl alcohol particles. Key: ○, particle size constant, weight of particles varied; and □, weight of particles constant, particle size varied.*

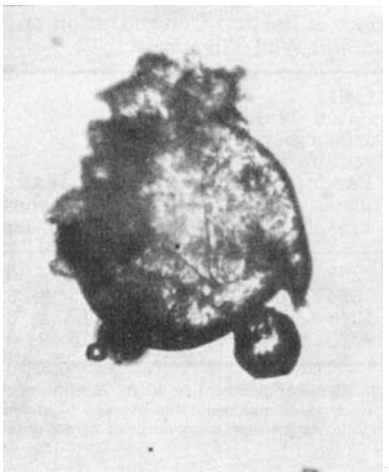


Figure 2—*Partially crushed encapsulated particle, encapsulated by the method reported by Luzzi and Gerraughty (5) (magnification 150×).*

The implication is that in this system, where only a small part of the total colloid present is used in the coating of the particle, the coating thickness is influenced by the total number of particles present. Further experiments are necessary to determine the validity of this relationship and to discern whether it applies to other systems.

Microscopic Studies—Figure 2 shows the nature of the coating on microcapsules encapsulated by the conventional method (3). In this photograph, the wall material is surrounding a 200-μm diameter particle. The wall was cracked open slightly at one end to expose a portion of the particle. The coating on these particles appears to be thick (relatively speaking), confirming the belief that encapsulation of smaller particles takes place by coalescence of one or more coacervate droplets around the particle.

In contrast, the larger particles encapsulated by the modified method reported here were observed to have much thinner coatings than those on the small 200-μm diameter particles (Fig. 2). In fact, the coating was very difficult to see by the microscopic techniques used for the smaller particles referred to previously. To obtain visual evidence of evenness and completeness of coating, it was necessary to go to an extraction technique where the stearyl alcohol was slowly extracted with toluene at room temperature, leaving the wall material undisturbed. Figures 3*a*–3*c* were made during the extraction of stearyl alcohol from an encapsulated particle suspended in toluene. The dissolution of the stearyl alcohol in toluene seemed to leave the capsule wall undisturbed except for some initial shrinkage. These photographs, plus observation with a stereomicroscope, indicated that the wall material was uniformly distributed on the particle and that the coating on these particles was much thinner than that found on the smaller particles of stearyl alcohol encapsulated by the unmodified method. Previous experiments in these laboratories showed that wall thickness of microcapsules of other substances (such as phenobarbital) tend to be much thicker for similar sized particles.

A number of particles were treated similarly and studied microscopically to get an indication of the uniformity of the coating. It was found that almost all the particles examined were covered with a smooth thin wall over the entire surface.

It was expected that encapsulation of relatively larger particles by the modified method would probably be due to a mechanism where a number of individual coacervate droplets adsorb to and coalesce about the particles. If true, then the wall material should show a somewhat uneven coating and the coating on the particles

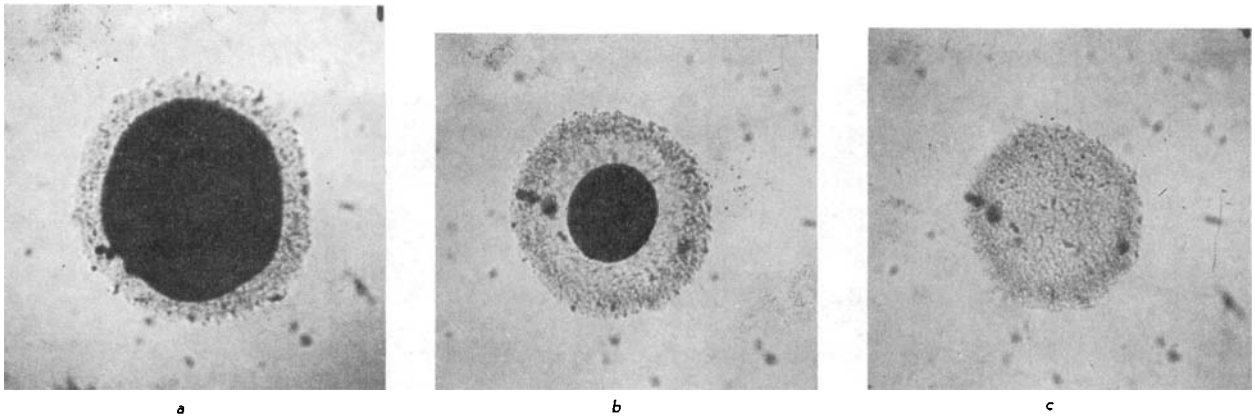


Figure 3—(a) *Encapsulated particle shortly after beginning extraction with toluene, using modified method of encapsulation and 335-μm particles.* (b) *Same particle after several minutes of extraction with toluene.* (c) *Extraction complete; only unsupported wall remains. Small round dark spots are air bubbles trapped within the wall.*

would be comparable in thickness to the one on particles shown in Fig. 2. The observation that the coating was very thin and uniform and had no adhering modules, together with a high rate of encapsulation, supports the supposition that there is a third mechanism responsible for encapsulation of the larger particles. Experiments where only gelatin was included in the encapsulation mixture using acacia-coated particles also gave a coating sufficiently stable to remain intact while the stearyl alcohol was extracted with toluene. It is probable that by first coating the particles with acacia as described, the hydrophobic portions of the acacia molecule are oriented to the relatively hydrophobic stearyl alcohol molecules, leaving the hydrophilic portion of the acacia open for reaction with gelatin. If this is true, then the initial coating on the particles is essentially a reaction of colloidal gelatin with the acacia film around the particle. Subsequently, adsorption of coacervate droplets may occur, but microscopic observation of a large number of finished particles did not indicate that it did. A few adhering droplets were observed at some stages of the process but they may have been loosely attached and were washed off during the recovery process.

To elucidate further what was happening at the surface of the particle, electron micrographs of the particles were taken at several points during the encapsulation process. These results are shown in Figs. 4–7. Figure 4 shows the surface of an uncoated stearyl alcohol particle. The surface shows some fissuring, probably because of the crystallization of the stearyl alcohol upon cooling from the molten state.

Figure 5 shows a similar particle which has been melted and quickly recongealed in water. The surface is somewhat rougher than with a particle congealed in air. Particles so treated show no improvement in tendency to be encapsulated over air-congealed particles.

A particle that had been melted and recongealed in 5% acacia solution is shown in Fig. 6. This particle shows areas that are smoother than the uncoated particles interspersed by surface crack lines. The particle appears to be coated with a thin film of acacia and thus gives visual evidence for earlier suppositions. Such acacia-coated particles do not appear to be retarded in their dissolution in toluene, and no intact film can be seen when the dissolution is observed with an optical microscope. Since acacia films are rather brittle, it is probable that the coating breaks up as soon as a small amount of the supporting wax is dissolved from beneath the coating film.

The surface of a particle coated by the modified coacervation procedure is shown in Fig. 7. In this photograph, the coating has a flaky or scale-like surface. This appearance was not expected since observation with the optical microscope seemed to indicate a smooth surface. The depth of focus of the optical microscope may have been insufficient at high power to reveal significant details of the surface, or the coating may have been raised to some extent by the vacuum during the metal shadowing treatment. If it is assumed that the scale-like surface is not an artifact caused by the metallizing process, it is possible that it is caused either by a brief toluene wash of the particles used to remove any remaining uncoated stearyl alcohol or by the alcohol treatment after encapsulation.

The calculated wall thickness (Tables I and II) is liable to certain experimental variations, including weight fluctuations encountered in weighing gelatinous material due to absorption of moisture, variations in particle diameter during coating with

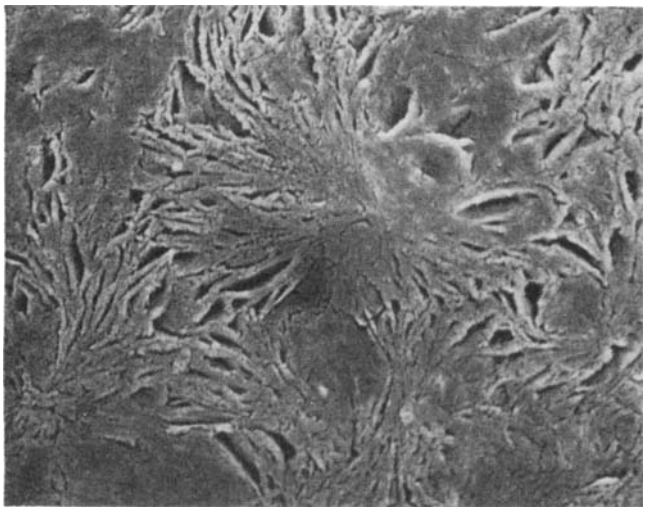


Figure 4—*Electron scanning micrograph of the surface of an uncoated stearyl alcohol particle (magnification about 475×).*

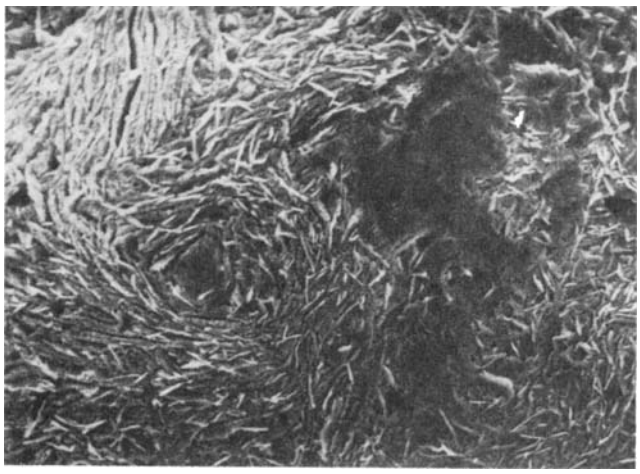


Figure 5—*Electron scanning micrograph of the surface of a stearyl alcohol particle melted and recongealed in water (magnification about 480×).*

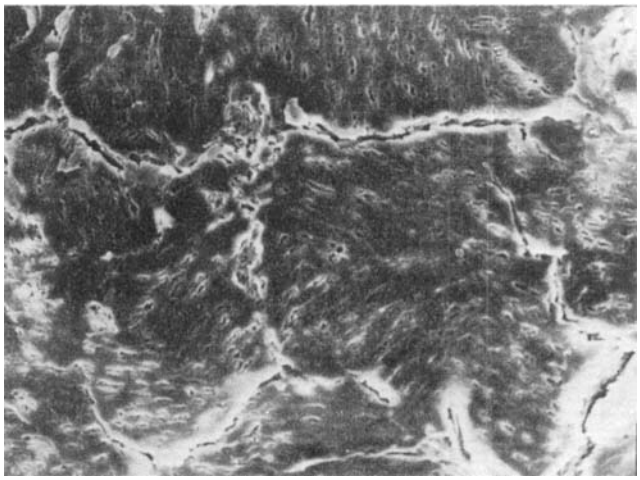


Figure 6—*Electron scanning micrograph of the surface of a stearyl alcohol particle melted and recongealed in acacia solution (magnification about 550×).*

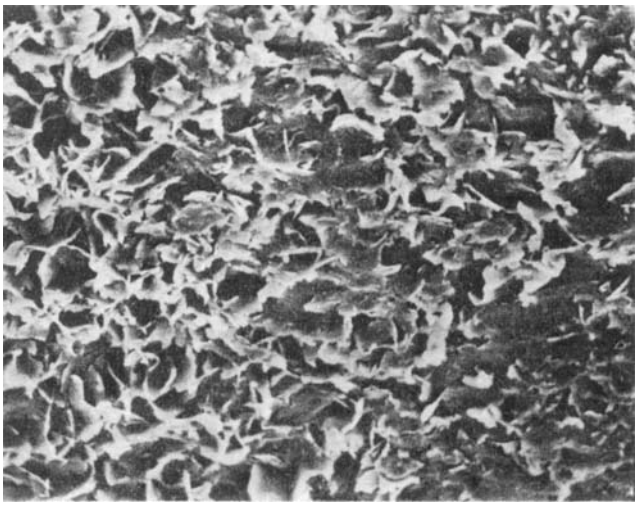


Figure 7—*Electron scanning micrograph of the surface of a stearyl alcohol particle encapsulated by the modified method (magnification about 500×).*

acacia solution in preparing particles for encapsulation by the modified method, use of average particle diameter in a distribution of sizes, and small errors in the determination of density of the wall material. Nevertheless, it seems logical to believe that the thin capsule walls formed on particles encapsulated by the modified method reported here strongly indicate that the initial wall was formed by molecular interaction between gelatin and acacia previously attached to the stearyl alcohol during the brief melting in acacia solution rather than by coalescence of preformed coacervate droplets around the particle.

CONCLUSIONS

From the results of this study, the following conclusions can be drawn.

1. Only a small fraction of the total amount of colloid was used in the formation of the capsule wall.

2. The extent of encapsulation (percent particles encapsulated) was affected only slightly up to the point where the particles constituted 75% of the total solids but decreased somewhat at higher concentrations.

3. The wall thickness decreased with increasing ratios of solid particles.

4. The wall thickness increased as particle diameters increased and total surface areas of the particles decreased.

5. An inverse relationship between the square root of the number of particles present and the wall thickness was noted.

6. Optical and electron scanning microscopic observations of the encapsulated particles indicated that treatment by melting in acacia solution left a thin fragile acacia coating on the particle and that the final coated particle had a scale-like appearance. The final coating was sufficiently strong to remain intact during dissolution of the wax in toluene.

7. The evidence presented strongly supported earlier conclusions (3) that the capsule wall can be formed as a result of a molecular interaction between colloidal gelatin in the coacervate mixture and acacia held at the surface of the particles.

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[x] To whom inquiries should be directed.

Effects of Spheronization Process Variables on Selected Tablet Properties

HENRY J. MALINOWSKI* and WILLIAM E. SMITH[x]

Abstract □ This study evaluated the effects of certain process variables on the properties of tablets prepared from granulations composed of essentially spherical, free-flowing pellets. A complete factorial experimental design was used to indicate the significant main effects and interactions related to water content, extruder speed, extruder screen size, spheronizer speed, and spheronizer residence time on tablet hardness and dissolution rate. This series of experiments demonstrated that changes in process variables, particularly water content, can result in significant differences in the dissolution rate and hardness of tablets composed of 80% drug and 20% binder. The factorial experimental design proved useful in predicting the effects of such changes between the limits producing usable pelletized granulations.

Keyphrases □ Spheronization process variables—effects of water content, extruder speed, extruder screen size, spheronizer speed, and spheronizer residence time on tablet properties □ Tablets—effects of spheronization process variables on dissolution rate and hardness □ Dissolution—effects of spheronization process variables □ Hardness, tablets—effects of spheronization process variables

The spheronization process using the equipment[1] involved in this study was previously described (1-4). This paper deals with the use of a complete factorial experimental design (5-9) to determine the effects of five process variables on tablet hardness and dissolution rate. The variables studied were water content, extruder speed, extruder screen size, spheronizer speed, and spheronizer residence time. Each variable was studied at two levels, resulting in 32 different experiments (Table I). The method of analysis was analysis of variance (10).

All 32 experimental granulations were prepared in random order. The relationship between the experiment number and variable levels associated with that experiment is shown in Table II.

To describe easily the levels of variables used in a factorial experiment, a commonly employed system uses lower case letters to denote the presence of a particular variable at the high level of that variable. For example, Experiment ade would imply that variables A, D, and E were present at the high level whereas variables B and C were at the low level. Since the experiment where all variables were at the low level would require no lower case letter notation, the symbol (1) is used to describe that experiment.

In analysis of variance tables, upper case letters are used to denote the effects of variables and interactions between or among variables. For example, A denotes the effect of variable A, and AD denotes the first-order interaction between variables A and D.

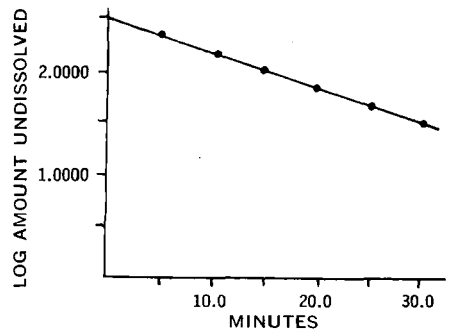

Figure 1—*Determination of dissolution rate from acetaminophen tablets.*

Table I—Values of Levels for Variables Investigated

Variable	Low Level	High Level
Water content (A)	250 ml	325 ml
Extruder speed (B)	39 rpm	59 rpm
Screen size (C)	0.8 mm	1.5 mm
Spheronizer speed (D)	700 rpm	1010 rpm
Spheronizer time (E)	1.0 min	3.0 min

EXPERIMENTAL

Materials—Microcrystalline cellulose[2] and acetaminophen NF[3] were used.

Manufacturing and Testing Procedure—Eight hundred grams of acetaminophen and 200 g of microcrystalline cellulose were weighed separately. The two materials were blended for 10 min[4]. While blending, material was scraped from the outer edge of the bowl after 5 min. Purified water was added slowly over 30 sec, and mixing was continued for 2 min for a total of 12 min of blending. The wetted material was placed into the extruder feed chamber and processed at the prescribed conditions. The first material out of the extruder screens was discarded. The extrudate was placed into the spheronizer in 200-g batches, removed after the required residence time, and tray dried at 40° for 7 hr. After drying, the spheronized material was stored in sealed, plastic bags.

The analytical procedure for the determination of acetaminophen was based on that of Walters (11). Solutions of NF acetaminophen reference standard were prepared in triplicate, containing 15.00, 11.25, 7.50, 3.75, 1.875, and 1.40 μg/ml, and the absorbance was measured at 242 nm. The wavelength of maximum absorption was determined by a scan from 220 to 270 nm. Identical standard curves were obtained[5].

The acetaminophen used was assayed in triplicate by placing 325.0 mg in a 1-liter volumetric flask, bringing to volume with purified water, stirring for 60.0 min using a magnetic stirrer, and then diluting a 1.0-ml sample to 100.0 ml in a volumetric flask.

Granulations were assayed after dissolving the acetaminophen by placing 390.0 mg of granulation in a 1-liter volumetric flask, bringing to volume with purified water, stirring for 60.0 min using a magnetic stirrer, and then centrifuging a 5.0-ml sample for 15 min. A 1.0-ml sample was diluted to 100.0 ml and the absorbance

[1] Extruder type EXDS-60 and Marumerizer type Q-230, Elanco Products Co., a division of Eli Lilly & Co., Indianapolis, Ind.

[2] Avicel RC-581, FMC Corp., Marcus Hook, Pa.

[3] S. B. Penick and Co., New York, N.Y.

[4] In a Day Pony Mill.

[5] Both the Beckman model DU and Hitachi Perkin-Elmer model 139 spectrophotometers were used.

Table II—Relationship between Experiment Number and Variable Levels

Experiment	Variable Levels	Experiment	Variable Levels
1	a	17	bde
2	ae	18	bce
3	ac	19	abd
4	ce	20	de
5	ade	21	e
6	(1)	22	abe
7	acd	23	ad
8	b	24	bcde
9	cde	25	abcde
10	d	26	ace
11	abcd	27	cd
12	be	28	abc
13	abce	29	bc
14	ab	30	abde
15	c	31	acde
16	bcd	32	bd

was read. Assays were performed in triplicate, using random samples obtained by standard sampling techniques (Table III). Tablets were compressed on a laboratory press[6] using 0.031-cm (13/32-in.) standard curvature punches. Applied pressure was 3410 kg, and tablet weight was 390.0 mg.

Tablet Hardness—The hardness of tablets compressed from each granulation was determined[7]. The values used in calculations represent the means of four readings for each experiment.

Tablet Disintegration Time—When using the USP disintegration apparatus, tablet disintegration was less than 10 sec in all cases.

Tablet Dissolution Rate—The dissolution of acetaminophen from tablets compressed from each granulation was determined using the USP XVIII basket apparatus at 50 rpm. Each experiment was repeated four times, and mean values were used in calculations.

Samples were removed at 0, 5, 10, 15, 20, 25, and 30 min using a 5.0-ml pipet. Five milliliters of dissolution medium was added to the beaker after each sample was removed. Each 5-ml sample

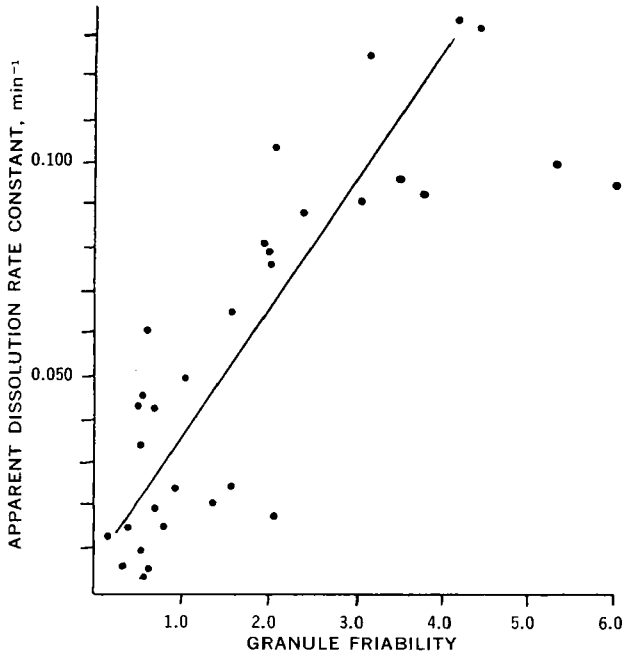

Figure 2—*Correlation between granule friability and dissolution rate.*

[6] Fred S. Carver, Inc., Summit, N.J.
[7] Pfizer hardness tester.

Table III—Tablet Data

Experiment	Apparent Dissolution Rate Constant[a], min^{-1}	Granulation Assay, mg[b]	Tablet Hardness, kg (Carver Press Tablets)
1	0.082	312	4.2
2	0.016	312	3.0
3	0.021	312	4.8
4	0.125	315	4.2
5	0.006	312	2.5
6	0.095	307	6.1
7	0.005	307	3.7
8	0.097	311	6.4
9	0.025	309	3.4
10	0.079	307	4.7
11	0.010	314	3.7
12	0.091	309	3.8
13	0.006	312	3.4
14	0.025	312	4.4
15	0.131	311	6.5
16	0.066	307	6.6
17	0.061	309	3.0
18	0.093	315	4.6
19	0.035	319	3.4
20	0.089	322	3.0
21	0.089	317	5.8
22	0.046	317	2.9
23	0.020	317	3.9
24	0.104	317	4.0
25	0.014	314	3.1
26	0.043	319	3.0
27	0.133	317	6.7
28	0.077	320	5.4
29	0.100	317	8.3
30	0.044	315	3.1
31	0.017	315	3.0
32	0.018	319	6.3

[a] Mean of four values. [b] Milligrams acetaminophen, mean of three assays.

was centrifuged for 15 min in a centrifuge[8] at 3400 rpm to precipitate suspended microcrystalline cellulose and sodium carboxymethylcellulose. A 1.0-ml aliquot from each sample was diluted to 100.0 ml with purified water, and the absorbance was read.

A blank dissolution test was run, using a sample containing no drug. No absorbance was observed with this blank sample.

A standard solution of 10.0 μg/ml was read daily as a check on the operation of the spectrophotometer.

During the dissolution test, 5.0-ml samples were removed at 5, 10, 15, 20, 25, and 30 min; 5.0 ml of purified water was added each time a sample was removed. A correction factor was added to each "weight dissolved" term except, of course, the 5-min sample. This correction procedure may be explained mathematically by the equations:

$$W_r = \frac{5}{900} W_{tn} \qquad \text{(Eq. 1)}$$

$$W_{Ctn} = W_{Mtn} + \sum_{1}^{n-1} W_r \qquad \text{(Eq. 2)}$$

where:

W_r = weight of dissolved drug removed in a particular sample
W_{tn} = uncorrected weight dissolved at time t
W_{Ctn} = corrected weight dissolved at time t
W_{Mtn} = spectrophotometrically measured weight dissolved at time t

This procedure was used by Wurster and Taylor (12) using concentration, rather than mass, as the basis for the correction.

The dissolution rate (Table IV) was determined by a plot of log undissolved drug *versus* time (13, 14). A polynomial regression computer program (15) was used to determine the slope of the

[8] International clinical centrifuge.

Table IV—Log Weight of Acetaminophen Undissolved (Milligrams)[a]

Experiment	Minutes					
	5	10	15	20	25	30
1	2.3026	2.1182	1.8800	1.7370	1.5320	1.4639
2	2.4140	2.3699	2.3337	2.3114	2.2927	2.2778
3	2.3682	2.3032	2.2686	2.2402	2.2067	2.1887
4	2.3495	2.1263	1.6869	1.4127	1.1620	0.9916
5	2.4339	2.4218	2.4141	2.4093	2.3994	2.3905
6	2.3536	2.2054	1.9889	1.7045	1.4663	1.3087
7	2.4426	2.4359	2.4327	2.4259	2.4177	2.4112
8	2.4045	2.2673	2.0858	1.7552	1.4828	1.3143
9	2.3982	2.3179	2.2589	2.2178	2.1862	2.1541
10	2.3672	2.2007	2.0138	1.7978	1.6270	1.5090
11	2.4294	2.4087	2.3914	2.3761	2.3644	2.3521
12	2.3591	2.1824	1.9265	1.7305	1.5575	1.3333
13	2.4572	2.4434	2.4357	2.4269	2.4195	2.4105
14	2.3765	2.2932	2.2333	2.2013	2.1725	2.1447
15	2.3468	2.1239	1.8339	1.5240	1.2787	0.7467
16	2.3683	2.2375	2.0731	1.8977	1.7534	1.6702
17	2.3432	2.1449	1.9945	1.8768	1.8015	1.7093
18	2.4219	2.2535	1.9849	1.7330	1.4977	1.3918
19	2.3855	2.2644	2.1888	2.1291	2.0853	2.0488
20	2.3614	2.2216	2.0576	1.9882	1.9153	1.8703
21	2.3805	2.2497	2.0990	1.8077	1.5789	1.3814
22	2.3823	2.2384	2.0961	2.0195	1.9631	1.9231
23	2.4111	2.3137	2.2851	2.2662	2.2454	2.2209
24	2.3481	2.1751	1.9492	1.6751	1.3708	—[b]
25	2.4343	2.3999	2.3664	2.3396	2.3258	2.3017
26	2.4061	2.3030	2.1837	2.0864	2.0229	1.9603
27	2.3350	2.1839	1.9184	1.2655	—[b]	—[b]
28	2.3379	2.1826	1.9745	1.8099	1.6771	1.5127
29	2.3531	2.2221	1.9828	1.6798	1.4545	1.2508
30	2.4180	2.2710	2.1440	2.0712	2.0043	1.9525
31	2.4017	2.3455	2.3174	2.2931	2.2723	2.2511
32	2.3937	2.2779	2.1154	1.8975	1.5902	1.3607

[a] Each value is the mean of four readings. [b] All drug was dissolved.

Table V—Analysis of Variance, Tablet Hardness (Carver Press Tablets)

Source of Variation	Sum of Squares	Degrees of Freedom	Variance	$F_{1,6}$
Water content (A)	21.66	1	21.66	108.3[a]
Extruder speed (B)	0.51	1	0.51	2.6
Screen size (C)	1.97	1	1.97	9.9[b]
Spheronizer speed (D)	5.02	1	5.02	25.1[c]
Spheronizer time (E)	26.63	1	26.63	133.2[a]
AB	0.08	1	0.08	0.4
AC	0.20	1	0.20	1.0
AD	0.28	1	0.28	1.4
AE	3.36	1	3.36	16.8[c]
BC	0.41	1	0.41	2.1
BD	0.02	1	0.02	0.1
BE	0.44	1	0.44	2.2
CD	0.02	1	0.02	0.1
CE	0.65	1	0.65	3.3
DE	0.05	1	0.05	0.3
ABC	0.08	1	0.08	0.4
ABD	0.17	1	0.17	0.9
ABE	0.83	1	0.83	4.2
ACD	0.43	1	0.43	2.2
ACE	0.33	1	0.33	1.7
ADE	0.94	1	0.94	4.7
BCD	1.17	1	1.17	5.9
BCE	0.17	1	0.17	0.9
BDE	0.61	1	0.61	3.1
CDE	0.36	1	0.36	1.8
Higher order	1.23	6	0.20	—

[a] Significant at $p < 0.001$. [b] Significant at $p < 0.05$. [c] Significant at $p < 0.01$.

best straight line through the data points. A representative example is shown in Fig. 1. In all cases, the significance of the linear regression lines was greater than 99%.

Granule Friability—A measure of granule friability was determined by rotating 10.0 g of 20–30-mesh granules along with 200 5-mm glass spheres in a friability tester[9] for 30 min. The granules were then separated from the glass beads, placed on a No. 30 sieve, and shaken[10] for 5 min. Granule friability was determined by subtracting the weight of granules retained on the sieve after testing from 10.0.

RESULTS AND DISCUSSION

Tablet Hardness—Hardness values ranging from 2.5 to 8.3 kg were obtained. Analysis of variance results (Table V) indicated that water content, screen size, spheronizer speed, and spheronizer time were significant main effects and that a significant first-order interaction between water content and spheronizer time existed. Increasing screen size resulted in increased tablet hardness whereas increasing water content, spheronizer speed, or spheronizer time decreased tablet hardness (Table VI). The significant first-order interaction shown in Table VII indicated that increased spheronizer time at the low water content resulted in a greater decrease in tablet hardness than at the high water content. As water content increased, a greater decrease in tablet hardness occurred at the low spheronizer time, although tablet hardness was greater throughout at the low spheronizer time. In general, tablet hardness may be maximized at low water content, low spheronizer speed and time, and high screen size. The maximum increase in tablet hardness occurred at the low water content–low spheronizer time combination. These results suggest that particles lacking the cohesive properties necessary to form hard tablets may be formed as indicated by the significant process variables.

Disintegration Time—Disintegration time was excellent for all experiments, indicating that differences in dissolution rate were a result of intragranular changes due to changes in the processing variables. No correlation between disintegration time and dissolution rate would be apparent. In general, this lack of correlation would be expected (16). It has been proposed (17) that the granule friability test may provide a better indication of expected dissolution rate in cases where there is no correlation between disintegration time and dissolution rate.

Dissolution Rate—Values ranging from 0.005 to 0.113 min^{-1} were calculated. Analysis of variance results (Table VIII) indicated the only significant main effect or interaction to be the main effect of water content. Increasing water content resulted in decreased dissolution rate (Table IX). If dissolution rate is expected to be a problem, water content during extrusion should be minimized.

A more precise indicator of expected dissolution rate was the value obtained for granule friability. A plot of granule friability *versus* dissolution rate is shown in Fig. 2. The correlation coefficient is 0.816 with 99% confidence limits (range 0.582–0.925). For 30 degrees of freedom, the correlation is significant at the 99% level at the value of 0.499 or greater. For all experiments having values of less than 5 for granule friability, there was found a close relationship between tablet dissolution rate and granule friability.

This correlation was tested by preparing an experiment at mean levels of the five process variables. Granule friability was 1.4 and tablet dissolution rate was 0.043 min^{-1}. From Fig. 2, the

Table VI—Tablet Hardness (Kilograms)[a]

Significant Main Effect	Low Level	High Level
Water content	5.2	3.6
Screen size	4.2	4.7
Spheronizer speed	4.8	4.0
Spheronizer time	5.3	3.5

[a] Mean of 16 experiments.

[9] Erweka.
[10] Cenco Meinzer sieve shaker.

Table VII—Tablet Hardness (Kilograms)[a]

Spheronizer Time, min	Water Content, ml	
	250	325
1.0	6.5	4.2
3.0	4.0	3.0

[a] Mean of eight experiments.

Table VIII—Analysis of Variance, Dissolution Rate Constant

Source of Variation	Sum of Squares	Degrees of Freedom	Variance	$F_{1,6}$
Water content (A)	0.0247	1	0.0247	19.0[a]
Extruder speed (B)	0.0001	1	0.0001	0.1
Screen size (C)	0.0004	1	0.0004	0.3
Spheronizer speed (D)	0.0064	1	0.0064	4.9
Spheronizer time (E)	0.0008	1	0.0008	0.6
AB	0.0006	1	0.0006	0.5
AC	0.0024	1	0.0024	1.9
AD	0.0005	1	0.0005	0.4
AE	0.0000	1	0.0000	0.0
BC	0.0000	1	0.0000	0.0
BD	0.0002	1	0.0002	0.2
BE	0.0016	1	0.0016	1.2
CD	0.0000	1	0.0000	0.0
CE	0.0001	1	0.0001	0.1
DE	0.0002	1	0.0002	0.2
ABC	0.0000	1	0.0000	0.0
ABD	0.0001	1	0.0001	0.1
ABE	0.0014	1	0.0014	1.1
ACD	0.0002	1	0.0002	0.2
ACE	0.0003	1	0.0033	0.2
ADE	0.0006	1	0.0006	0.5
BCD	0.0000	1	0.0000	0.0
BCE	0.0006	1	0.0006	0.5
BDE	0.0018	1	0.0018	1.4
CDE	0.0001	1	0.0001	0.1
Higher order	0.0075	6	0.0013	—

[a] Significant at $p < 0.01$.

predicted tablet dissolution rate would be 0.048 min^{-1}, which indicates the validity of the correlation.

Particles resulting from this spheronization process are much less friable than conventionally processed granules. To attempt a similar correlation using conventionally processed granules, it would be necessary to make the granule friability test less severe. Otherwise, no relationship would be noted between rapid and slow tablet dissolution rates and granule friability because all granule friability values would be high.

SUMMARY

The effects of process variables on tablet hardness and dissolution rate were determined. The process variables had no effect on tablet disintegration time, which was less than 10 sec in all cases. Increasing extruder screen size resulted in increased tablet hardness, whereas increasing the amount of granulating solvent, the spheronizer speed, or the spheronizer time resulted in decreased tablet hardness. The dissolution rate was found to decrease as the amount of granulating solvent added was increased.

The results may also be used as an indication of which process variables have no significant effect on tablet hardness and dissolution rate.

A correlation between granule friability and tablet dissolution rate was found. The validity of this correlation was tested by performing an additional experiment which indicated a close agreement between predicted and experimental dissolution rates.

Table IX—Dissolution Rate Constant ($Minutes^{-1}$)[a]

Significant Main Effect	Low Level	High Level
Water content	0.085	0.030

[a] Mean of 16 experiments.

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* Present address: School of Pharmacy, University of Missouri-Kansas City, Kansas City, MO 64110

‡ To whom inquiries should be directed.

Magnesium Lauryl Sulfate in Tableting: Effect on Ejection Force and Compressibility

A. M. SALPEKAR and L. L. AUGSBURGER[x]

Abstract □ The effect of magnesium lauryl sulfate on tablet ejection force and filler compressibility was evaluated and compared to magnesium stearate in four direct compression fillers (microcrystalline cellulose, compressible starch, spray-dried lactose, and direct compression sucrose) and a typical wet granulation (terra alba) using a rotary tablet press instrumented to monitor ejection and compression forces. Generally, higher concentrations of magnesium lauryl sulfate were required to produce ejection forces similar to those obtained with magnesium stearate. However, batches employing magnesium stearate were generally less compressible than batches employing magnesium lauryl sulfate at levels sufficient to produce similar or lower ejection forces.

Keyphrases □ Magnesium lauryl sulfate—effect on ejection force and compressibility of four direct compression fillers and a wet granulation, compared to magnesium stearate □ Tableting—effect of magnesium lauryl sulfate on ejection force and compressibility of four direct compression fillers and a wet granulation, compared to magnesium stearate □ Compressibility of tablet formulations—effect of magnesium lauryl sulfate, compared to magnesium stearate □ Lubricant effect of magnesium lauryl sulfate in tableting—ejection force and compressibility parameters, compared to magnesium stearate

Lubricants are agents added in small quantities to tablet formulations to improve certain processing characteristics of direct compression powder blends and granulations. In this regard, three activities have been identified (1) with the use of the term "lubricant." These are: (*a*) preventing sticking to punch faces and the die wall (antiadherent activity), (*b*) improving flow properties of the granulation (glidant activity), and (*c*) reducing friction at the tablet–die wall interface during tablet formation and ejection (true lubricant activity). While a given lubricant may provide one or more of these actions to varying degrees, the primary function of lubricants in tableting is that of true lubricants (1, 2). Therefore, it is not surprising that hydrophobic materials are more effective than hydrophilic ones. Although such an agent would only be needed at the interface of the tablet and the die wall, it must be distributed throughout the tablet, thereby tending to make the tablet matrix hydrophobic. Because of this, lubricants as a class are known to retard moisture penetration, disintegration times, and dissolution rates (3–5).

In a search for a water-soluble lubricant, water-soluble magnesium lauryl sulfate and the most widely used lubricant, magnesium stearate, were compared (6) in four tablet and capsule formulations. The data were reported to indicate that magnesium lauryl sulfate has the lubricating activity of magnesium stearate but not its waterproofing effect. Although it was noted whether or not the formulations would run, conclusions were based on weight variation data. These data provide information on the glidant properties of these materials but give no indication of true lubricant action. Tablet weight variation was used (7) to evaluate several silica-type glidants. Most true lubricants are poor glidants and may actually retard flow properties (1). Indeed, formulators often include both true lubricants and glidants in tablet formulations.

In an earlier study (8), the true lubricant effect of magnesium lauryl sulfate (as one of 70 different compounds) in a sulfathiazole granulation was evaluated on the basis of the R value (ratio of maximum lower to maximum upper punch force) and ejection force. According to the theory, R would approach 1 with perfect lubrication. Thus, the more closely R approaches 1, the better the lubricant and the lower ejection force should be. The tabulated data indicated that magnesium stearate was the most effective true lubricant of the compounds tested and somewhat more efficient than magnesium lauryl sulfate (magnesium stearate: $R = 0.95$, ejection force = 8 kg; magnesium lauryl sulfate: $R = 0.88$, ejection force = 24 kg; unlubricated control: $R = 0.67$, ejection force = 110–170 kg). Since these data were based on a single compression force and lubricant concentration (2%), they give no indication of the effect of compression pressure or lubricant concentration on lubricant efficiency. Both R values and ejection force are known to vary with compression pressure (8, 9). The fact that these data were generated on a single-punch, eccentric-type machine may limit their applicability because the rotary tablet press is more representative of the type of press used in the industry. Furthermore, no studies were conducted on the newer, direct compression filler systems.

The present study was designed to provide a more definitive evaluation of the true lubricant effect of magnesium lauryl sulfate in tableting, particularly in direct compression systems. Since lubricants such as magnesium stearate are known to interfere with interparticulate bonding in tablets and thereby reduce compressibility, one objective of this study was to evaluate the effect of magnesium lauryl sulfate on compressibility.

EXPERIMENTAL

Instrumentation—All studies were carried out on a rotary tablet press[1] instrumented to measure compression force and ejection force after the manner of Wray *et al.* (10, 11). Compression force is monitored from a remote site using a pair of metal foil resistance strain gauges[2] (in parallel circuit) bonded to either side of

[1] Stokes model RB2.
[2] Type SR-4, BLH Electronics, Waltham, Mass.

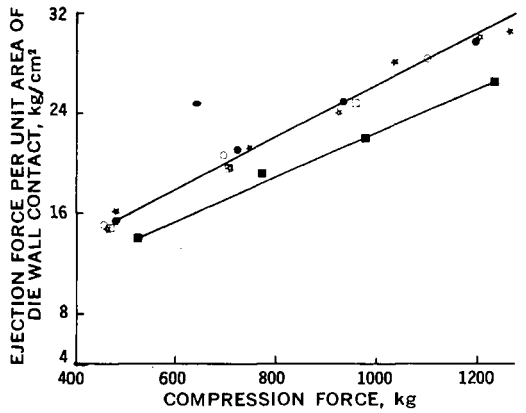

Figure 1—*Effect of magnesium lauryl sulfate (□, 2%; ○, 3%; ☆, 5%) and magnesium stearate (★, 0.5%; ●, 1%; ■, 2%) on the ejection force of spray-dried lactose tablets.*

the eye-bolt connecting the overload spring with the lower compression roller. These gauges form two active arms of a Wheatstone bridge. On tablet compression the eye-bolt is compressed, thereby changing the resistance in the gauges and unbalancing the bridge circuit. This force sensed at the eye-bolt is proportional to the tablet compression force and is monitored by measuring the bridge unbalance voltage using a carrier preamplifier[3], which also serves to activate the bridge. Ejection forces are monitored in a somewhat similar manner by replacing a portion of the ejection cam with a tool-steel cantilever beam. Metal foil resistance gauges are bonded to either side of the beam (in series) to form two active arms of a Wheatstone bridge. As the lower punch travels up the ejection cam, the force of ejection produces a deflection in the cantilever beam, thereby unbalancing the bridge.

Both the compression and ejection events were continuously recorded on an oscillographic recorder[4]. Responses were read directly in microstrains, which were converted to kilograms of force.

Preparation of Tablets—Because of its widespread use and recognized high efficiency as a true lubricant, magnesium stearate[5] was used throughout this study as a control for evaluating magnesium lauryl sulfate[6] in representative types of direct compression fillers and a typical granulation.

To promote good blending and uniform dispersion, both materials were passed through an 80-mesh screen before blending with the direct compression fillers or granulation. No other additives or active ingredients were included in these formulations.

The fillers used in the direct compression studies were microcrystalline cellulose[7], compressible starch[8], spray-dried lactose[9], and a direct compression sucrose[10]. All direct compression batches were mixed[11] for 10 min without the intensifier bar and then for 2 min with the intensifier bar running.

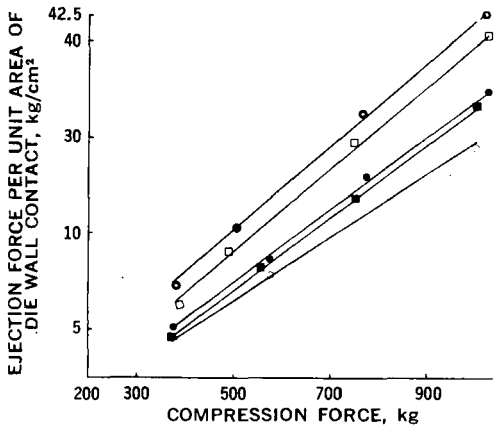

Figure 2—*Effect of magnesium lauryl sulfate (●, 3%; ○, 5%) and magnesium stearate (◘, 0.5%; □, 1%; ■, 2%) on the ejection force of direct compression sucrose tablets.*

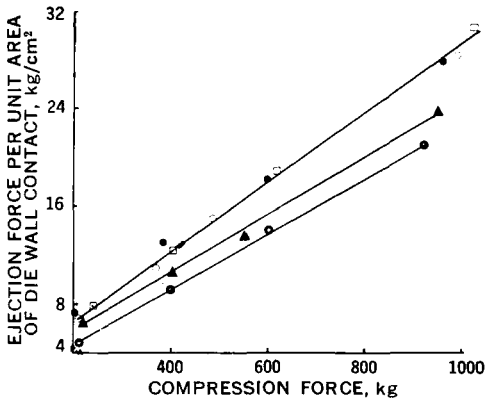

Figure 3—*Effect of magnesium lauryl sulfate (▲, 1%; ◘, 2%) and magnesium stearate (○, 0.25%; □, 0.5%; ●, 1%) on the ejection force of terra alba granulation tablets.*

For the granulation studies, a terra alba granulation was prepared using a 15% aqueous acacia solution as the binder. The wet mass was passed through a #34 plate on a laboratory wet granulating unit[12], dried 48 hr at 55°, and then passed through a 20-mesh screen by hand. All batches were blended in the mixer for 15 min. The intensifier bar was not used with these batches to avoid creating excessive fines.

The batches were of 500 g with the exception of the microcrystalline cellulose blends, which were 350 g.

All tablets were compressed using a single set of 1.11-cm (0.43-in.) flat-faced punches and die at a machine speed of 28 rpm. Tablets were compressed in a controlled humidity area (30 ± 10% relative humidity) where temperature was maintained at 26 ± 1°.

Ejection Force Study—Ejection forces were monitored at each of four compression forces to provide data over a wide range of tableting conditions. After evaluating each sample, the punches and die were thoroughly cleaned with acetone before running the next sample.

Magnesium lauryl sulfate was evaluated at the lowest concentration at which the batches appeared to run and at least one higher concentration. Magnesium stearate was evaluated at one lower concentration (usually 0.5%), at a concentration equal to the lowest effective concentration of magnesium lauryl sulfate as determined previously, and at one higher concentration, except as otherwise noted. In all cases, ejection forces were monitored for 10 min to ensure constancy of readings. To minimize variations in tablet thickness that might bias ejection force data, it was necessary to adjust tablet weight to account for the differences in density among the fillers. Thus, tablet weights were adjusted to about 500 mg for all fillers except microcrystalline cellulose and the terra alba granulation where the tablet weights were adjusted to about 350 and 750 mg, respectively. Nevertheless, small variations in tablet thickness (0.35–0.40 cm) were observed due to the various compression forces used. Although these variations were small relative to overall tablet thicknesses, ejection forces were reported in terms of kilograms force per unit apparent area of tablet-die wall contact (*i.e.*, kilograms per square centimeter) to ensure further that there would be no bias due to differences in tablet thickness. The data reported represent the means of at least 10 readings.

Compressibility—Compressibility, defined as the ease with which hard tablets are produced, was assessed by determining tablet hardness at each compression force. The mean hardness of

[3] Sanborn model 35-1100B, Hewlett-Packard Co., Palo Alto, Calif.
[4] Sanborn model 964, Hewlett-Packard Co., Palo Alto, Calif.
[5] Ruger Chemical Co., Inc., New York, N. Y.
[6] Courtesy of Alcolac Chemical Corp., Baltimore, Md.
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[9] Lactose USP, spray dried, Foremost Dairies, Inc., San Francisco, Calif.
[10] DiPac, a cocrystallization of 97% sucrose and 3% modified dextrins, Amstar Corp., New York, N.Y.
[11] Patterson Kelly V-mixer.
[12] Eureka.

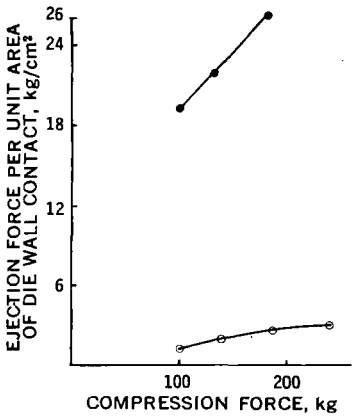

Figure 4—*Effect of magnesium lauryl sulfate on ejection forces of microcrystalline cellulose tablets. Key: ●, control (no lubricant); and ○, 0.25% magnesium lauryl sulfate.*

at least 10 tablets at each compression force was determined in Strong-Cobb units using a motorized tablet hardness tester[13].

RESULTS AND DISCUSSION

The ejection force data for the spray-dried lactose batches are summarized in Fig. 1. Although the use of 2% magnesium lauryl sulfate resulted in ejection forces comparable to the 1% magnesium stearate batch at compression forces up to 950 kg, an attempt to run this batch at a compression force of 1200 kg (average hardness of 21.4 Strong-Cobb units) was unsuccessful. Continuous monitoring showed that ejection force increased with each tablet ejection, going from 27.8 to 46.8 kg/cm² after only 300 tablets had been produced. There was no indication that an equilibrium value had been reached, and the run was terminated to prevent possible damage to the cantilever beam mechanism. Visual examination of these tablets revealed scoring of the tablet sides, and there was evidence of sticking to the die wall. This effect was not observed when magnesium lauryl sulfate was added at concentrations of 3 or 5%, but ejection forces were no lower. Increasing magnesium stearate to 2% measurably lowered ejection forces at all compression force levels. There was no evidence of sticking to the die wall in any of the magnesium stearate runs.

The ejection force results for the direct compression sucrose appear in Fig. 2. Ejection force was clearly reduced as magnesium stearate was increased to 2%. Closely similar ejection forces were observed with 3% magnesium lauryl sulfate; however, 5% of this compound resulted in ejection forces lower than any of the batches in this series, especially at the higher compression forces. There was noticeable sticking to the die wall when magnesium lauryl sulfate was employed at concentrations below 3%.

No significant differences in ejection force were noted when magnesium stearate was increased from 0.25 to 1% in the terra alba granulation, but ejection forces were higher at all compression force levels than when 1% magnesium lauryl sulfate was the

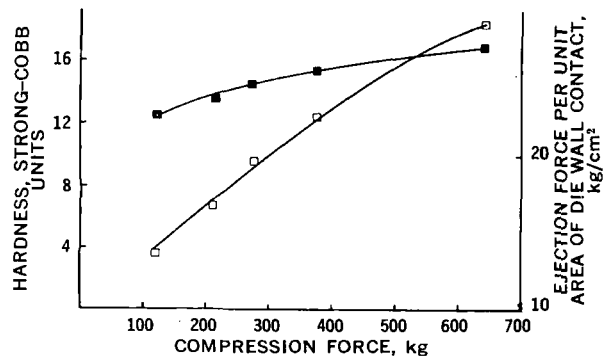

Figure 5—*Compressibility and ejection forces with unlubricated compressible starch. Key: ■, effect on ejection force; and □, effect on hardness.*

[13] Heberlein model 2E/106, Key Industries, Farmingdale, N.J.

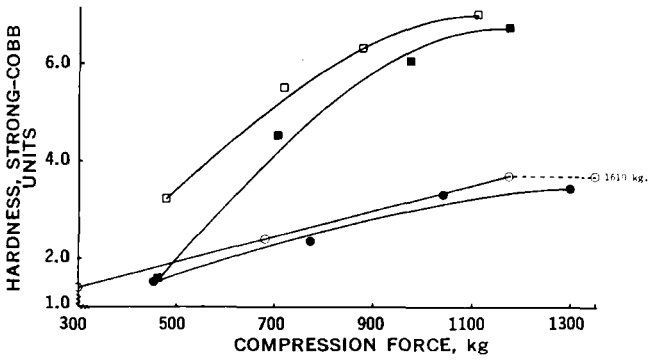

Figure 6—*Effect of magnesium lauryl sulfate (□, 0.3%; ■, 0.5%; ●, 1%) and magnesium stearate (○, 0.2%) on the compressibility of compressible starch.*

lubricant (Fig. 3). A further reduction in ejection force was noted when magnesium lauryl sulfate concentration was increased to 2%. Although these data suggest that concentrations of magnesium lauryl sulfate below 1% may still produce ejection forces below those observed here with magnesium stearate, it was not possible to compress such tablets successfully. At concentrations ranging from 0.25 to 0.75%, sticking to both the die wall and punch faces was observed. Apparently, magnesium lauryl sulfate was more efficient than magnesium stearate as a true lubricant in this system but less efficient as an antiadherent. The magnesium stearate data suggest that there would be no advantage in terms of ejection force to adding more than 0.25% of the lubricant in this system. Such a distinction could never be made in studies based on a single lubricant concentration. Similar results were reported by Wray *et al.* (10), who found no significant differences in ejection force at all compression forces tested between batches of a stock granulation using concentrations of magnesium stearate ranging between 0.1 and 1%. However, lower ejection forces were noted at the highest compression forces when 2% of the lubricant was added.

Adding 0.25% magnesium lauryl sulfate to microcrystalline cellulose dramatically reduced ejection forces to barely measurable levels (Fig. 4). At higher magnesium lauryl sulfate levels, ejection forces were too low to be measured and could not be plotted. Ejection forces were also too low to be measured when microcrystalline cellulose was lubricated with 0.25% or higher concentrations of magnesium stearate. Similarly, ejection forces too low to be measured were noted when 0.25% or greater concentrations of either magnesium lauryl sulfate or magnesium stearate were added to the compressible starch. These results were not unexpected because these fillers, by themselves, are known to be self-lubricating (12, 13). Ejection forces observed with the unlubricated compressible starch tablets (Fig. 5) were lower than those observed with the microcrystalline cellulose tablets and appeared to be less dependent on the compression force. More important than the ejection force data for these fillers are the compressibility data, particularly in the case of compressible starch. The deleterious ef-

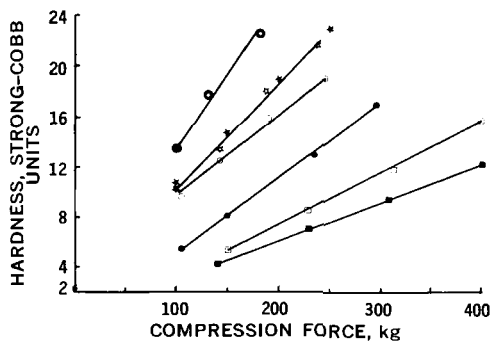

Figure 7—*Effect of magnesium lauryl sulfate (☆, 0.25%; ★, 0.5%; ○, 1.0%) and magnesium stearate (●, 0.25%; □, 0.5%; ■, 0.75%) on the compressibility of microcrystalline cellulose tablets. ✪: control (no lubricant).*

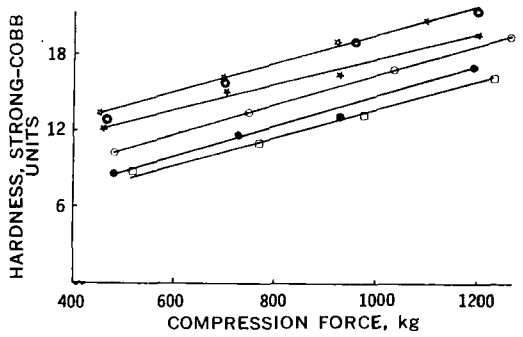

Figure 8—*Effect of magnesium lauryl sulfate* (◘, *2%*; ☆, *3%*; ★, *5%*) *and magnesium stearate* (○, *0.5%*; ●, *1%*; □, *2%*) *on the compressibility of spray-dried lactose.*

fect of magnesium stearate on the compressibility of these two fillers was previously reported (13, 14) and is confirmed here in plots of hardness *versus* compression force (Figs. 5–7).

As can be seen, the compressibility of the starch filler is dramatically reduced when only 0.2% of magnesium stearate is added. The maximum hardness that could be obtained with this formulation was 3.7 Strong-Cobb units at a compression force of 1175 kg; increasing the compression force to 1610 kg did not increase hardness. When 0.3 or 0.5% magnesium lauryl sulfate was the lubricant, compressibility was significantly greater than that observed with the magnesium stearate batch. For instance, with 0.3% magnesium lauryl sulfate, only about 550 kg compression force was required to produce tablets with a mean hardness of 3.7 Strong-Cobb units, and hardness could be increased to 7 units by increasing compression force to about 1100 kg. Compressibility was poorer than that observed with the magnesium stearate batch when 1% magnesium lauryl sulfate was used.

The compressibility of microcrystalline cellulose was also dramatically reduced with magnesium stearate (Fig. 7). However, this filler is inherently so compressible as to not negate its usefulness at the levels tested. Compressibility was also reduced when magnesium lauryl sulfate was the lubricant but to a much lesser extent. Although compressibility continued to decrease as the concentration was increased to 1%, even the 1% magnesium lauryl sulfate batches were significantly more easily compressed than the 0.25% magnesium stearate batches.

To assess the effect of these lubricants on the compressibility of spray-dried lactose, the direct compression sucrose, and the terra alba granulation, it would have been useful to first determine the compressibilities in the absence of lubricants. Although it was not possible to compress these substances without a lubricant, it may be seen in Figs. 8–10 that for a given filler the batches employing magnesium stearate are generally less compressible than the batches employing magnesium lauryl sulfate at the higher levels needed to yield similar or lower ejection forces. Although magnesium lauryl sulfate appears to have less effect on the compressibility of these fillers than magnesium stearate, both agents exert a more drastic effect on the compressibility of microcrystalline cellulose and the compressible starch than on the other three fillers. Similar results were reported (14) with magnesium stearate. A possible explanation for this phenomenon is that fracture and the

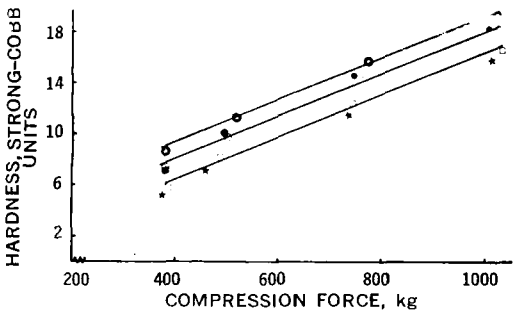

Figure 9—*Effect of magnesium lauryl sulfate* (◘, *3%*; ●, *5%*) *and magnesium stearate* (○, *0.5%*; □, *1%*; ★, *2%*) *on the compressibility of direct compression sucrose.*

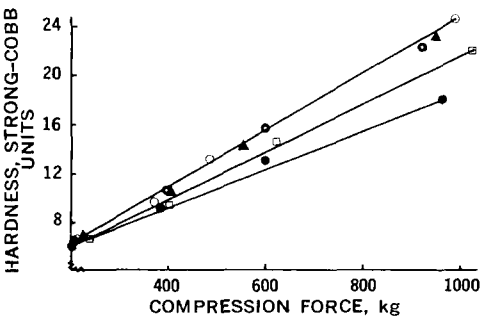

Figure 10—*Effect of magnesium lauryl sulfate* (▲, *1%*; ◘, *2%*) *and magnesium stearate* (○, *0.25%*; □, *0.5%*; ●, *1%*) *on the compressibility of terra alba granulation.*

creation of new surfaces under compression may not be major factors in the bonding of microcrystalline cellulose or starch tablets but may be important in the bonding of the other three fillers. Thus, the presence of lubricant at particle surfaces may exert a greater role in weakening the interparticulate bonds in compressible starch or microcrystalline cellulose tablets. With the granulation, it is recognized that the binder plays a major role in holding the tablet together. However, the granulation might be less sensitive to the presence of the lubricant because the granules may be expected to fracture under compression and expose fresh tablet matrix that would not be coated with lubricant.

SUMMARY AND CONCLUSIONS

The true lubricant properties of magnesium lauryl sulfate were evaluated and compared with magnesium stearate in microcrystalline cellulose, compressible starch, spray-dried lactose, direct compression sucrose, and a terra alba granulation using an instrumental rotary tablet press. With the exception of the terra alba granulation, generally higher concentrations of magnesium lauryl sulfate were required to produce similar ejection forces, and there was evidence of sticking to the punches and die at lower concentrations in the spray-dried lactose and compressible sucrose batches. In the case of the terra alba granulation, magnesium lauryl sulfate appeared to be more efficient than magnesium stearate in lowering ejection force but less efficient as an antiadherent.

There was no evidence of sticking to the punch faces or die wall in any batch employing magnesium stearate.

The effect of magnesium lauryl sulfate on the compressibility of these fillers was also studied. The results indicate that magnesium lauryl sulfate reduces compressibility to a lesser extent than magnesium stearate. These data also indicate that the compressibility of the sugars, spray-dried lactose, and direct compression sucrose, as well as that of the terra alba granulation, is affected less by lubricants than microcrystalline cellulose or compressible starch, thereby suggesting differences in the bonding mechanisms of these substances.

Because of the limited batch sizes, these results must be regarded as preliminary; the ultimate test of any lubricant would be its performance in a full-scale production run. Nevertheless, these results do clearly point up the potential of magnesium lauryl sulfate as a soluble lubricant. Furthermore, the fact that magnesium lauryl sulfate did not reduce compressibility to the same extent as magnesium stearate suggests that its use would enhance the usefulness of direct compression fillers. This was particularly evident in the compressible starch runs.

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The technology used relating to the measurement of ejection forces is protected under U.S. Patent 3,388,434, assigned to Lederle Laboratories. The authors are grateful to Lederle Laboratories for granting permission to publish research, part of which makes use of this technology.

[x] To whom inquiries should be directed.

NOTES

Metabolism of 1-Methyl-5-nitro-2-(2′-pyrimidyl)imidazole

K. M. BAKER[*], M. COEREZZA[‡], L. DEL CORONA[‡], A. FRIGERIO[*x], G. G. MASSAROLI[‡], and G. SEKULES[‡]

Abstract □ 5-Acetamido-1-methyl-2-(2′-pyrimidyl)imidazole is shown to be one metabolite of 1-methyl-5-nitro-2-(2′-pyrimidyl)-imidazole in both rats and humans. Characterization was carried out by mass spectroscopy, NMR, and IR and by synthesis of the metabolite. This first example of a metabolite reduction product of the nitro group of an imidazole is discussed in relation to previous studies.

Keyphrases □ 1-Methyl-5-nitro-2-(2′-pyrimidyl)imidazole—metabolism, identification of 5-acetamido-1-methyl-2-(2′-pyrimidyl)imidazole as metabolite □ 5-Acetamido-1-methyl-2-(2′-pyrimidyl)imidazole—identified as metabolite of 1-methyl-5-nitro-2-(2′-pyrimidyl)imidazole □ Metabolism—1-methyl-5-nitro-2-(2′-pyrimidyl)imidazole

The drug 1-methyl-5-nitro-2-(2′-pyrimidyl)imidazole (I) was recently synthesized according to Scheme I (1). The drug I shows antitrichomonal properties both *in vitro* and *in vivo* against *Trichomonas vaginalis* in mice. During studies on the absorption, excretion, and metabolism of the drug, one major metabolite of I was noticed in the urine of both rats and humans. This paper presents data on the isolation, identification, and synthesis of this metabolite and shows it to be a product of reduction of the nitro group, with the structure 5-acetamido-1-methyl-2-(2′-pyrimidyl)imidazole.

Previous work on the metabolism of the nitroimidazole class of drugs (2, 3) showed that amines may be formed as metabolic reduction products of the nitro group, but they have never been isolated due to the extreme instability of 5-aminoimidazole derivatives. Work on the nitrofurfuraldehyde derivative class of drugs has shown (4, 5) that acetylated amine derivatives can be found as metabolites, and the first example of such a metabolite from the 5-nitroimidazole series is presented here.

EXPERIMENTAL[1]

1-Methyl-5-nitro-2-(2′-pyrimidyl)imidazole (I)—This com-

[1] Melting points were determined with a Büchi capillary apparatus and are uncorrected. IR spectra were obtained on a Hilger-Watts spectrophotometer, and UV spectra were obtained on a Beckman DB-GT spectrophotometer with a Sargent-Welch SRG recorder. NMR spectra were determined with a Perkin-Elmer R12-B spectrometer using tetramethylsilane as the internal reference. Mass spectra were obtained on an LKB 9000 instrument equipped with a gas chromatograph and a 3% SE-30 column (2 m long), operating at 250°. The ion beam energy was 70 ev, the ion source temperature was 290°, the accelerating voltage was 3.5 kv, and the trap current was 60 μamp. GLC was carried out on a Carlo Erba Fractovap GI chromatograph equipped with a flame-ionization detector using a 3% SE-30 glass column (2 m long). The operating conditions were: injection port temperature, 270°; oven temperature, 250°; nitrogen (carrier gas) flow rate, 30 ml/min; hydrogen flow rate, 100 ml/min; and air flow rate, 280 ml/min. TLC was carried out on Merck F_{254} silica gel plates, and column chromatography was carried out on Florisil, 100–200 mesh (British Drug Houses).

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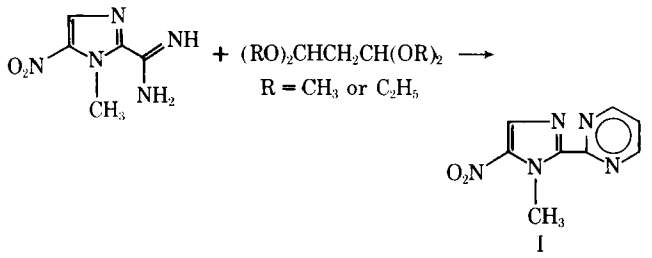


Scheme I

pound was prepared according to published procedures (1) and showed the following characteristics: mp 205–207°; UV (ethanol): λ_{max} 314 nm, $E_{1cm}^{1\%}$ 655; IR (KBr): 1570 and 1537 cm^{-1}; NMR ($CDCl_3$): δ 4.42 (s, 3, N—CH_3), 7.40 (t, 1, C-5′ pyrimidyl H), 8.12 (s, 1, imidazole H), and 8.93 (d, 2, C-4′ and C-6′ pyrimidyl H); mass spectrum: M^+ 205.

5-Acetamido-1-methyl-2-(2′-pyrimidyl)imidazole (II)—Compound I (4.1 g, 0.02 mole) in acetic acid (20 ml) was treated with zinc powder (10 g) for 10 min at 15°, and the solution was filtered and treated with 20 ml of acetic anhydride for 15 min at 60°. After the solution was brought to dryness, the residue was dissolved in 50 ml of water at 20° and successively treated with hydrogen sulfide until the complete precipitation of zinc sulfide. Then the solution was filtered and dried, and the residue was dissolved in 10 ml of ethanol and chromatographed on a 100–200-mesh magnesium silicate[2] column (20 cm high, 3 cm i.d.). The first eluant added was 350 ml of ethanol–acetone (1:1), and later 400 ml of ethanol–acetone–water (2:2:1) was added. The solution was dried and recrystallized from ethanol, yielding 160 mg II, mp 224–228°; UV (ethanol): λ_{max} 295 nm, $E_{1cm}^{1\%}$ 715; IR (KBr): 3240, 3220, and 1710 cm^{-1}; NMR ($CDCl_3$): δ 2.20 (s, 3, $COCH_3$), 3.90 (s, 3, N—CH_3), 7.10 (t, 1, C-5′ pyrimidyl H), 7.26 (s, 1, imidazole H), and 8.70 (d, 2, C-4′ and C-6′ pyrimidyl H); mass spectrum: M^+ 217.

Anal.—Calc. for $C_{10}H_{11}N_5O$: C, 55.28; H, 5.10; N, 32.25. Found: C, 54.98; H, 5.05; N, 32.26.

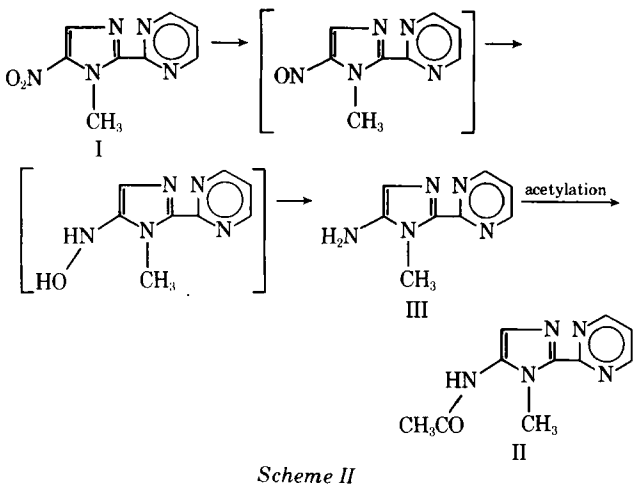
Animal Studies—Compound I was administered orally every 24 hr for 5 days (300 mg/kg/day) as a suspension in carboxymethylcellulose (0.375%) to male Wistar rats weighing 100–120 g. The animals were fasted for 24 hr prior to the experiments, but food was administered regularly during the experiments. After drug administration, the urine was collected for 5 days from both the treated group and a parallel control group of untreated rats.

TLC and GLC Studies—Urine (50 μl), concentrated 10:1 with a rotary evaporator[3] at a temperature not exceeding 50° from each treated and untreated animal, was examined on silica plates together with authentic samples of I and II. Plates were developed to 15 cm using the following solvent systems: A, *n*-propanol–ammonia (7:3); and B, acetone–ethanol–water (2:2:1).

After development, UV chromophores were located with UV light of 254 and 360 nm, and there were two major spots corresponding to the metabolite II and the unchanged drug I. The R_f values of I and II in Solvent System A were 0.94 and 0.90, respectively; in Solvent System B, they were 0.21 and 0.90, respectively.

Urine (10 ml) at pH 10 was extracted twice with 20 ml of chloroform, filtered through anhydrous sodium sulfate, and evaporated to dryness at reduced pressure. The extracts were dissolved in 100 μl chloroform–ethanol (1:1), and 3-μl samples were injected in a 5% SE-30 column. The samples were chromatographed isothermally at 200° for 6 min, programmed (10°/min) from 200 to 250°, and kept isothermally at 250° for 5 min. Two major peaks were obtained with retention times identical to those of I and II.

Isolation of I and II—Urine (400 ml) was adjusted to pH 10–11 with 0.1 *N* NaOH and extracted with chloroform as described previously. The residue was dissolved in chloroform–ethanol (1:1) and placed on a column of magnesium silicate, 100–200 mesh (2 × 15 cm). The column was washed with chloroform (100 ml), and the chloroform washings were discarded. Then the column was eluted with acetone–ethanol (1:1) to give Fraction A (150 ml) and with acetone–ethanol–water (2:2:1) to give Fraction B (150 ml).



Scheme II

Examination by TLC using the described solvent systems showed that Fraction A contained mostly unmetabolized I while Fraction B contained mostly the major metabolite II.

Both fractions were rechromatographed in the same way. The first eluate from Fraction A contained pure I while the pure metabolite II was obtained in the acetone–ethanol–water (2:2:1) eluate from the rechromatography of Fraction B.

Fraction A—The material from Fraction A was recrystallized from acetone–petroleum ether to give material, mp 205–206°, identical with Compound I.

Fraction B—The material from Fraction B was dissolved in 1 *M* hydrochloric acid. The acid solution was washed twice with chloroform that was then discarded. Then the solution was made basic with 1 *M* NaOH and extracted with chloroform, the chloroform was brought to dryness, and the residue was treated with ethanol and filtered. The filtrate was concentrated and recrystallized twice from ethanol to give material, mp 224–228°, identical to that of Compound II. The UV, IR, NMR, and mass spectra of the extracted metabolite were identical to those of synthetic II.

RESULTS AND DISCUSSION

1-Methyl-5-nitro-2-(2′-pyrimidyl)imidazole (I) was administered to rats as already described, and the urine contained only one major metabolite [R_f 0.21, acetone–ethanol–water (2:2:1)] not present in the urine of untreated rats. This metabolite was also present in the urine of human subjects[4]. The unchanged starting material and the metabolite were separated as described, and each was identified by spectral means and comparison with authentic samples.

The metabolite having R_f 0.21 in acetone–ethanol–water (2:2:1) had IR absorption bands at 3240 and 3220 cm^{-1}, indicating an amine or an amide, and 1710 cm^{-1}, indicating a carbonyl group. The bands at 1570 and 1537 cm^{-1} had disappeared, indicating that metabolic attack had occurred at the nitro group. The bands present were consistent with the presence of an acetamido group in the molecule. NMR spectroscopy showed that, in comparison to I, the compound had acquired a singlet methyl resonance at δ 2.20 attributable to an acetyl group, the *N*-methyl resonance had shifted from δ 4.42 to 3.91, and the C-4 proton of the imidazole ring had shifted from δ 8.12 to 7.26.

These data were consistent with the conversion of the nitro group to an acetamido group. The mass spectrum of the metabolite (II) was obtained using GLC–mass spectroscopy and showed (Fig. 1) a molecular ion at *m/e* 217 and a primary loss of 42 amu (CH_2=C=O) with a metastable ion to give the base peak at *m/e* 175. The loss of 42 amu confirms the presence of an acetyl group. The molecular ion at *m/e* 217 is consistent with a molecular formula $C_{10}H_{11}N_5O$ and with the structure 5-acetamido-1-methyl-2-(2′-pyrimidyl)imidazole. The mass spectrum of the drug I (Fig. 1) shows a molecular ion at *m/e* 205 and fragment ions at *m/e* 189 (M − O) and 175 (M − NO).

[2] Florisil.
[3] Büchi rotavapor.

[4] G. Sekules and M. Coerezza, unpublished results.

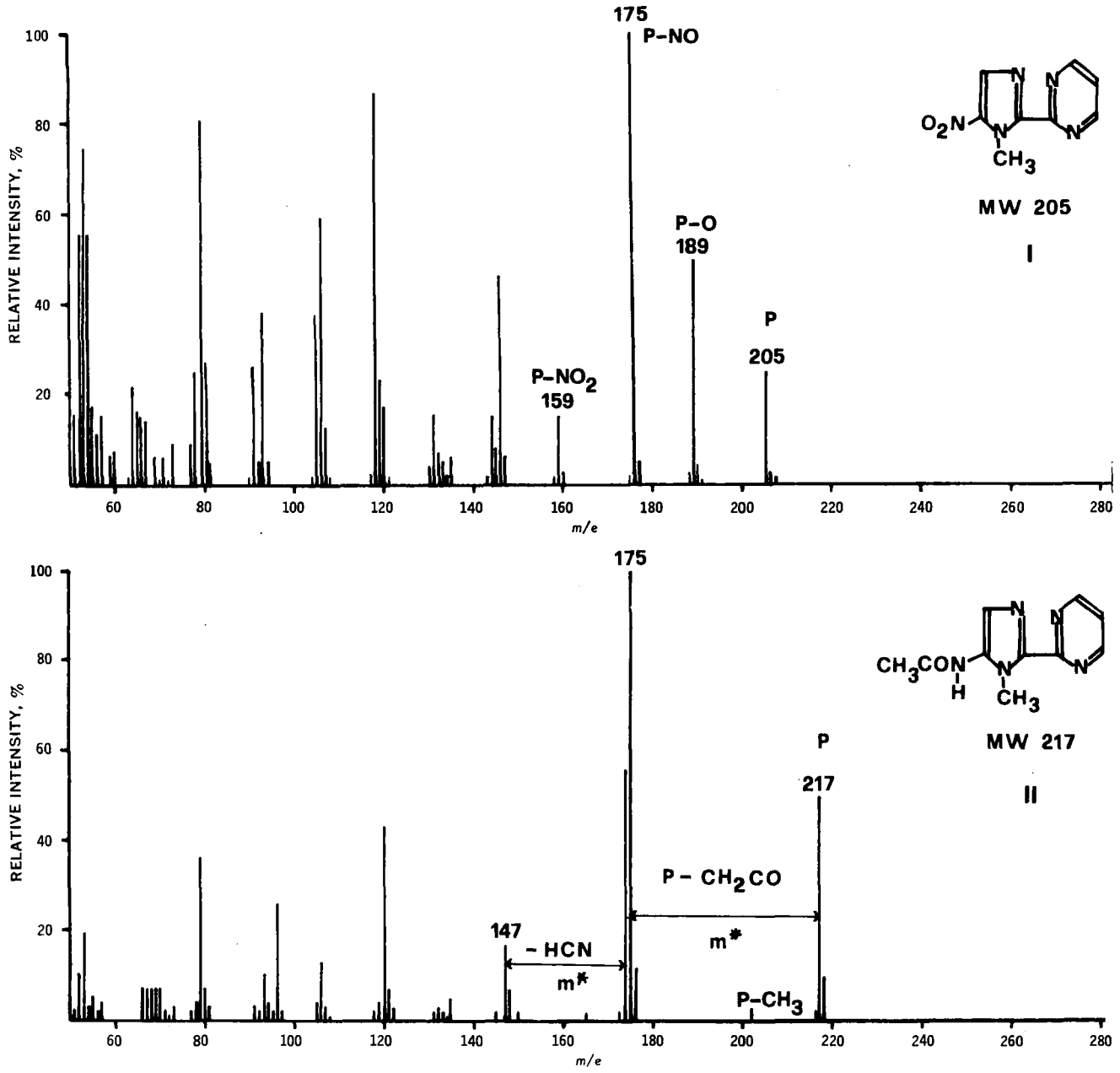


Figure 1—*Mass spectra of drug 1-methyl-5-nitro-2-(2'-pyrimidyl)imidazole (I) (upper) and metabolite 5-acetamido-1-methyl-2-(2'-pyrimidyl)imidazole (II) (lower) obtained by GLC–mass spectroscopy.*

To confirm the structure assignment, the acetamide was synthesized as already described. Reduction of the nitro group to form the amine (III) proved difficult; and once formed the amine was unstable, giving rise to a number of products. Acetylation of the amine gave the required material, II, which was identical in all its physical properties with the metabolite isolated from urine. The synthesized II also contained very minor amounts of impurities, which probably resulted from the decomposition of the amine intermediate in the synthesis. These minor impurities were also present in the urine of the treated rats, and hence it seems likely that the amine (III) is an intermediate in the metabolic pathway. A proposed scheme for the metabolic pathway is shown in Scheme II.

The *in vivo* acetylation of the amine forms a stable derivative II which allows its isolation. This is the first example of the isolation of a metabolite resulting from the reduction of the nitro group in the nitroimidazole class of drugs, and the inability to isolate amines previously was doubtless due to the extreme instability of that class of compound (3).

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* To whom inquiries should be directed.

GLC Method for Quantitative Determination of Amitriptyline in Human Plasma

HOWARD B. HUCKER[x] and SUSAN C. STAUFFER

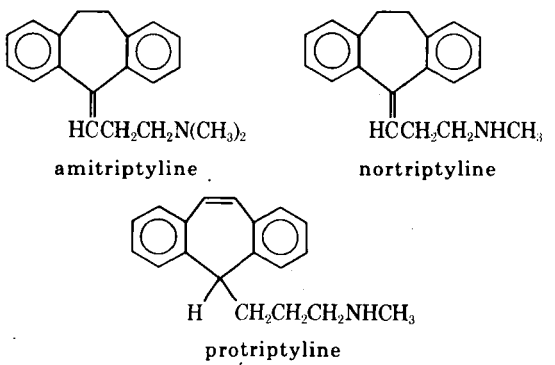
Abstract □ A GLC method was developed for the measurement of steady-state levels of amitriptyline and nortriptyline in human plasma after therapeutic doses of amitriptyline. The drugs were extracted from alkalinized samples with heptane–3% isoamyl alcohol and back-extracted into 0.1 *N* HCl. After subsequent extraction into ether, the drugs were analyzed by GLC. The method is accurate, specific, and sensitive, being capable of measuring concentrations as low as 25 ng/ml of these drugs. After 6 days of amitriptyline administration (75 mg/day), levels of amitriptyline and nortriptyline in three subjects were approximately 40–90 and 40 ng/ml, respectively, 12 hr after the last dose.

Keyphrases □ Amitriptyline—GLC determination in plasma □ Nortriptyline—GLC determination in plasma □ GLC—determination of steady-state levels of amitriptyline and nortriptyline in human plasma

Published methods for the determination of amitriptyline (1–5) lack either the sensitivity or specificity for determination of the drug in human plasma after therapeutic doses in the presence of its metabolite, nortriptyline. Recently, a GLC method for amitriptyline was published (6) with sensitivity sufficient to measure "steady state" plasma levels of the drug and its metabolite in man. However, difficulties were encountered with this method because plasma extracts produced multiple interfering peaks on the chromatograms and an unstable baseline. Accordingly, the GLC method described in the present paper was developed.

EXPERIMENTAL

Reagents—Pesticide quality *n*-heptane[1] was used without further purification. Reagent grade isoamyl alcohol[2] was washed successively with 1 *N* NaOH, 1 *N* HCl, and water before use. Ether[3] and ethyl acetate[4] were redistilled immediately before use. Double-distilled water was used to prepare 0.5 *N* NaOH and 0.1 *N* HCl. Amitriptyline, nortriptyline, and protriptyline were used as the hydrochloride salts, with all concentrations expressed in terms of the free base. Drugs were dissolved in double-distilled water.



Apparatus—Analyses were performed on a gas chromatograph[5] equipped with a flame-ionization detector. The column, 1.82 m (6 ft) × 4 mm (i.d.), was packed with 1.5% OV-17 on Gas Chrom Q[6], 80–100 mesh. The packed column was conditioned before use by heating at 250° overnight under helium flow. Chromatographic conditions were as follows: column oven temperature, 212°; detector temperature, 280°; injection port temperature, 260°; hydrogen, 50 ml/min; air, 700 ml/min; and helium carrier gas, 100 ml/min. The recorder[7] was used on range 1 (4×10^{-12} amp) and attenuation 32, 16, or 8 as required. Disposable glass tubes with constricted tips[8] were used for evaporation of the final ether extract and were cleaned by washing with detergent, rinsing thoroughly with double-distilled water, draining, and drying in a vacuum oven at 100°.

Procedure—From 3 to 5 ml of human plasma, 0.1 ml of protriptyline internal standard (1 or 2 μg/ml), 1 ml of 0.5 *N* NaOH, and 25 ml of heptane containing 3% isoamyl alcohol were combined in a 45-ml glass-stoppered centrifuge tube. The mixture was shaken for 15 min and centrifuged. Most of the organic phase was transferred to a similar tube containing 5 ml of 0.1 *N* HCl, and the mixture was shaken for 10 min. After centrifugation, the organic phase was discarded by aspiration and the aqueous phase was shaken three times with 25 ml each of *n*-heptane (to remove

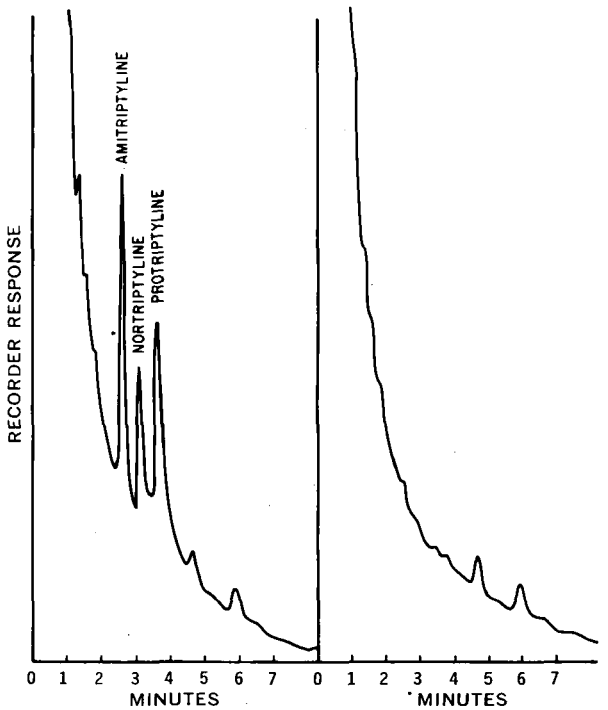


Figure 1—*Gas chromatogram (left) of an extract of 3 ml of human plasma containing 100 ng/ml of amitriptyline, 100 ng/ml of nortriptyline, and 200 ng/ml of protriptyline. The right-hand curve depicts an extract of 3 ml of control human plasma containing no drugs.*

[1] Matheson, Coleman and Bell.
[2] J. T. Baker.
[3] Anhydrous, ACS, Fisher.
[4] Fisher Certified, ACS.
[5] Hewlett-Packard (F&M) model 810.
[6] Applied Science Labs., Inc.
[7] Hewlett-Packard, model 7123A.
[8] Laboratory Research Co., Los Angeles, Calif.

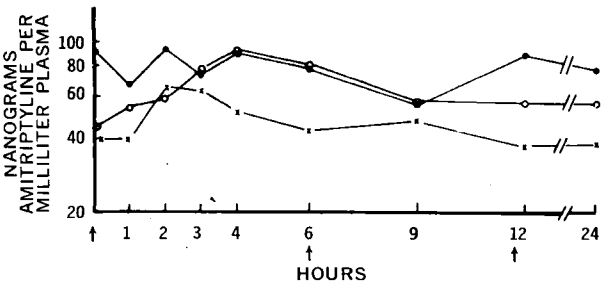


Figure 2—*Plasma levels of amitriptyline in three human subjects after 6 days of daily administration of 3 × 25-mg tablets of amitriptyline. Arrows indicate the time of administration of 1 × 25-mg tablet of amitriptyline on the day of analysis (Day 7).*

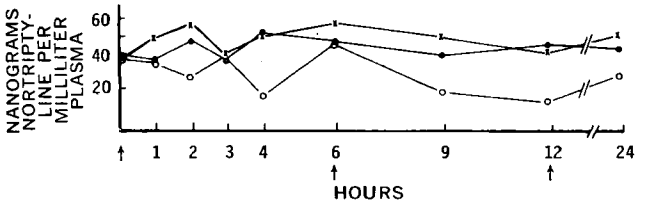


Figure 3—*Plasma levels of nortriptyline in the three human subjects described in Fig. 2.*

isoamyl alcohol). After aspirating the final heptane wash, most of the aqueous phase was transferred to a 13-ml glass-stoppered centrifuge tube. Five milliliters of freshly distilled ether and 1.5 ml of 0.5 *N* NaOH were added and the tube was shaken for 15 min. After centrifugation, as much as possible of the ether layer was transferred with a pipet (Pasteur) to a glass tube with constricted tip. The ether was evaporated in a water bath at 40°, material on the sides of the tube being washed down by periodically chilling the tube in ice water (7). The residue was dissolved in 25 μl of ethyl acetate, and 5 μl was injected into the gas chromatograph. All samples were assayed in duplicate.

Following chromatography, a baseline was drawn and peak heights of amitriptyline, nortriptyline, and protriptyline were measured. The ratio of amitriptyline and nortriptyline peak heights to that of the internal standard corrected for attenuation was calculated, and the concentration of each was obtained by reference to the standard curve.

A standard curve was constructed by analysis of samples to which known amounts of amitriptyline, nortriptyline, and internal standard had been added. When the ratio of peak heights of each compound to that of protriptyline internal standard was plotted *versus* concentration of amitriptyline and nortriptyline in nanograms per milliliter of plasma, the best-fit straight line was drawn that passed through the experimental points and origin.

RESULTS AND DISCUSSION

As shown in Fig. 1, amitriptyline, nortriptyline, and the internal standard were adequately separated under these conditions, with retention times of 2.9, 3.4, and 4.0 min, respectively. Blank plasma samples assayed in the same manner gave no significant peaks on the chromatogram. Several known metabolites of amitriptyline and nortriptyline, *i.e.*, the 10-hydroxy and 10,11-dihydroxy analogs of both drugs, were found not to interfere, having retention times of 5.3–12.6 min under the assay conditions (Table I).

The precision and accuracy of the method were demonstrated by analysis (in quadruplicate) of plasma samples containing 25, 50, 75, and 100 ng/ml each of amitriptyline and nortriptyline.

Table I—Relative Retention Times of Amitriptyline, Nortriptyline, and Their Metabolites

Compound	Relative Retention Time
Amitriptyline	1.0
Nortriptyline	1.18
10-Hydroxyamitriptyline	1.96
10-Hydroxynortriptyline	2.38
10,11-Dihydroxyamitriptyline	3.89
10,11-Dihydroxynortriptyline	4.67

The concentrations found were equal to 97 ± 8% (mean ± *SD*) of the actual values for amitriptyline and 96 ± 9% of the actual values for nortriptyline over this range of values. The lower limit of sensitivity was approximately 20 ng/ml, and the range of linearity extended to approximately 100 ng/ml for nortriptyline and was somewhat higher for amitriptyline. The absolute recoveries were approximately 100% for amitriptyline, 85% for nortriptyline, and 90% for protriptyline.

The results of analysis for the drug and its metabolite, nortriptyline, in plasma from three human subjects receiving amitriptyline are depicted in Figs. 2 and 3. Levels of amitriptyline varied between 38 and 94 ng/ml, while those of nortriptyline were somewhat lower, ranging from 26 to 57 ng/ml. These values are similar to those reported previously (6). However, the spread of plasma levels was less than the ranges of 20–303 and 20–178 ng/ml, respectively, reported by previous authors (6) for six subjects on the same amitriptyline dosage regimen (25 mg tid). Thus, it seems possible that the smaller variation between subjects with the present method is a consequence of analyzing samples with less endogenous contaminants and of the use of a mass internal standard (8) which reflects losses during extraction.

The present method appears to have a number of advantages over the procedure of Braithwaite and Widdop (6). Reproducibility is improved by the use of an internal standard which is carried through the entire procedure. There is no interference from endogenous constituents in plasma extracts, but in the previous method (6) care must be taken to avoid interference during repetitive analyses from large, slowly eluting impurity peaks.

No need was found to use silanized glassware. Preparation of the gas chromatographic column used in the present method was much simpler, and the column packing itself was of known stability, requiring no precautions for use as did the mixed phase column used by the previous authors.

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[x] To whom inquiries should be directed.

Mechanism and Stereochemistry of a Novel Reaction of Cyanogen Bromide with (+)-1-Methyl-3-benzoyl-3-hydroxypiperidine

JAMES R. SOARES* and GILBERT HITE*

Abstract □ The reaction of cyanogen bromide and (+)-1-methyl-3-benzoyl-3-hydroxypiperidine affords two unusual products, (−)-1-methyl-3-bromo-3-benzoylpiperidine hydrobromide [(−)-VII-HBr] and (+)-1-cyano-3-bromo-3-benzoylpiperidine [(+)-VIII], which are formed stereospecifically with retention of configuration. The mechanism involves initial formation of 1-methyl-3-cyanato-3-benzoylpiperidine (IX), followed by displacement of cyanate by bromide *via* a closed ion-pair.

Keyphrases □ Cyanogen bromide—mechanism and stereochemistry of reaction with (+)-1-methyl-3-benzoyl-3-hydroxypiperidine □ (+)-1-Methyl-3-benzoyl-3-hydroxypiperidine—mechanism and stereochemistry of reaction with cyanogen bromide

In contrast to the behavior of Compounds I–IV under the conditions of the von Braun demethylation of tertiary amines with cyanogen bromide (1), Compound V behaves anomalously to give VI after acid hydrolysis of the acid-insoluble, benzene-soluble fraction (2). Analysis of the reaction mixture obtained from V indicated the presence of VII and VIII (2). It was inferred that IX, an alkyl cyanate, was the precursor of VII. The unusual stereomechanistic aspects of reactions involving anchimeric assistance of amine nitrogen have long been of interest in these laboratories (3–8). Recent studies on the chemistry of alkyl cyanates revealed a "superhalogenoid" propensity for participation in ionically mediated substitutions, eliminations, and rearrangements (9–14). The novelty of this transformation prompted this reinvestigation using an optically active substrate to determine the mechanism and stereochemistry of this reaction, first noted during attempts to prepare some local anesthetic esters of X where R_1 represents various alkyl substituents.

EXPERIMENTAL[1]

Reaction of (*R*)-(+)-1-Methyl-3-benzoyl-3-hydroxypiperidine [*R*-(+)-V] with Cyanogen Bromide—A solution of 6 g (27.4 mmoles) of *R*-(+)-V in 50 ml of dry (sodium) benzene was added over 4 hr to a stirred solution of 7.2 g (68 mmoles) of freshly distilled cyanogen bromide. Nitrogen was passed through the reaction vessel, which was maintained at 50–55° during the addition. The cooled (25°) reaction mixture was stirred overnight and filtered to remove the precipitated, water-soluble salt, which was washed with three 10-ml portions of benzene. The dried solid was recrystallized from ethanol–ether to give 3.0 g (8.22 mmoles, 30%) of (*R*)-(−)-1-methyl-3-bromo-3-benzoylpiperidine hydrobromide [*R*-(−)-VII-HBr], mp 159.5–161°; NMR (D_2O): δ 8.37–7.20 (m, 5H, C_6H_5), 4.37–1.17 (m, 8H, $CH_2NCH_2CH_2CH_2$), and 3.03 (s, 3H, NCH_3) ppm; IR (mull): 1680 (C=O, ketone) cm^{-1}; $[\alpha]_D^{25}$ (ethanol) $-27.6 \pm 0.5°$ (c 0.203).

Anal.—Calc. for $C_{13}H_{17}Br_2NO$: C, 42.99; H, 4.72; Br, 44.02; N, 3.86. Found: C, 42.9; H, 4.62; Br, 44.3; N, 3.81.

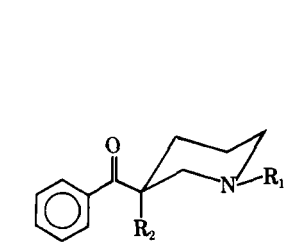
The benzene-soluble filtrate was evaporated and the residue was dissolved in 30 ml of ether. This solution was extracted with two 15-ml portions of 1.2 *N* HCl. The ether was dried, filtered, and concentrated to about 10 ml. On standing (5°), this gave 1.3 g (4.38 mmoles, 16%) of (*R*)-(+)-1-cyano-3-bromo-3-benzoylpiperidine [*R*-(+)-VIII], mp 76–77°; NMR ($CDCl_3$): δ 8.20–7.25 (m, 5H, C_6H_5), 3.75 (s, 2H, NCH_2CBr), 3.55–3.03 (m, 2H, NCH_2), and 2.57–1.35 (m, 4H, $NCCH_2CH_2$) ppm; IR (mull): 1660 (C=O, ketone) and 2220 (C≡N) cm^{-1}; $[\alpha]_D^{25}$ (ethanol) $+24.5 \pm 0.5°$ (c 0.266).

Anal.—Calc. for $C_{13}H_{13}BrN_2O$: C, 53.26; H, 4.47; Br, 27.26; N, 9.54. Found: C, 53.3; H, 4.64; Br, 27.1; N, 9.44.

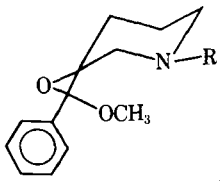
(*R*)-(+)-3-Benzoyl-3-hydroxypiperidine *p*-Toluenesulfonate [*R*-(+)-X-*p*-Toluenesulfonate]—A solution of 600 mg (2.3 mmoles) of *R*-(+)-III[2] was allowed to react with cyanogen bromide in a manner identical to that described for *R*-(+)-V. The same work-up afforded a benzene-soluble oil containing 1-cyano-3-acetoxy-3-benzoylpiperidine (XI); IR (film): 2220 (C≡N) and 1730 (C=O, acetate) cm^{-1}. This was dissolved in 10 ml of 50% water–ethanol. To the stirred solution was added a drop of phenolphthalein indicator. Over 12 hr, a 5% solution of sodium hydroxide was added dropwise until the pink color persisted. The solution was evaporated and the residue was mixed with 10 ml of ether. This mixture was extracted twice with 10-ml portions of water and was dried, filtered, and concentrated to 4–5 ml. On standing (0°), 100 mg (0.44 mmole, 19%) of (*R*)-(+)-1-cyano-3-benzoyl-3-hydroxypiperidine [*R*-(+)-XII] crystallized, mp 130–132°; $[\alpha]_D^{25}$ (methanol) $+69.0 \pm 0.5°$ (c 0.232); IR (mull): 3350 (OH), 2220 (C≡N), and 1670 (C=O, ketone) cm^{-1}. The ether filtrate yielded an oil as well. This was assigned the structure 1-carboxamido-3-hydroxy-3-benzoylpiperidine (XIII); NMR ($CDCl_3$): δ 8.32–6.97 (m, 5H, C_6H_5), 5.25 (s, 1H, OH, D_2O exchangeable), and 4.17–0.63 (m, 10H, $CH_2NCH_2CH_2CH_2$ and $CONH_2$, D_2O exchangeable) ppm; IR (film): 3400 (OH) and 1660 (C=O, urea) cm^{-1}. This was mixed with 10 ml of 12 *N* HCl. The mixture was allowed to reflux for 24 hr. The reaction mixture was extracted with ether and the extracts were discarded. The aqueous solution was evaporated and the residue was dissolved in 5 ml of water.

[1] Melting points were determined in a Thomas-Hoover Unimelt apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer model 141 photoelectric polarimeter in a 1.000-dm cell. IR spectra were determined with a Perkin-Elmer model 421 spectrophotometer using sodium chloride plates or cells. Mulls were prepared from mineral oil (Nujol). Assignments of absorption bands, believed accurate to within ±5 cm^{-1}, were made by analogy with reported values (15). Proton nuclear magnetic resonance spectra were determined with a Varian A-60D spectrometer. Assignments of absorption bands, believed accurate to within ±1 Hz, were made by analogy with reported values (16). When designation of exchangeable protons was of interest, samples were treated with D_2O. Tetramethylsilane was used as the internal reference. Drying, clarifying, and decolorizing of solutions were carried out simultaneously with anhydrous sodium sulfate and activated charcoal (Norit), followed by suction filtration through diatomaceous earth (Celite) pads in sintered-glass funnels. All solvents were evaporated in a rotary evaporator under reduced pressure. Oils and solids were dried (0.1 mm Hg, 27°, 4–12 hr) prior to spectral analysis. Petroleum ether refers to the fraction boiling from 30 to 60°. Elemental analyses were performed by Dr. F. B. Strauss, Oxford, England. (+)-1-Methyl-3-benzoyl-3-hydroxypiperidine [(+)-V], mp 72.5–73°, $[\alpha]_D^{28}$ (ethanol) $+10.9 \pm 0.5°$ (c 5.25), and (+)-1-methyl-3-benzoyl-3-acetoxypiperidine [(+)-III], mp 66–67°, $[\alpha]_D^{25}$ (ethanol) $+34.7 \pm 0.2°$ (c 10.56), were prepared as described previously (7, 17, 18). Both compounds have the *R*-configuration (19, 20).

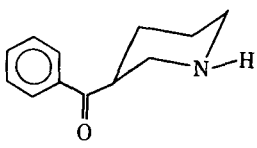
[2] NMR ($CDCl_3$): δ 8.13–7.13 (m, 5H, C_6H_5), 3.62–1.50 (m, 8H, $CH_2NCH_2CH_2CH_2$), 2.28 (s, 3H, NCH_3), and 1.98 (s, 3H, CH_3CO) ppm; IR (chloroform): 1735 (C=O, ester) and 1670 (C=O, ketone) cm^{-1}.



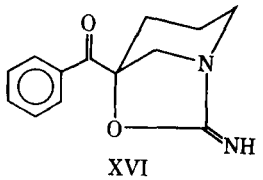
I: $R_1 = CH_3, R_2 = Cl$
II: $R_1 = CH_3, R_2 = OCH_3$
III: $R_1 = CH_3, R_2 = O_2CCH_3$
V: $R_1 = CH_3, R_2 = OH$
VII: $R_1 = CH_3, R_2 = Br$
VIII: $R_1 = CN, R_2 = Br$
IX: $R_1 = CH_3, R_2 = OCN$
X: $R_1 = H, R_2 = OH$
XI: $R_1 = CN, R_2 = O_2CCH_3$
XII: $R_1 = CN, R_2 = OH$
XIII: $R_1 = CONH_2, R_2 = OH$
XV: $R_1 = C(=NH)OCH_3, R_2 = OH$
XVII: $R_1 = CN, R_2 = OCN$



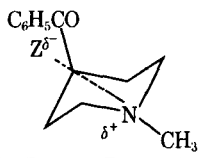
IV: $R = CH_3$
XIV: $R = C(=NH)OCH_3$



VI



XVI



XVIII: Z = Br or OCN

Pathways used to define the steric relationships between (+)-V, (−)-VII-HBr, and (+)-VIII:

(+)-V ⟶ (+)-III ⟶ XI ⟶ (+)-XII + XIII ⟶ (−)-X ↓ (+)-X-*p*-toluenesulfonate

(−)-VII-HBr ↓ (+)-VIII ⟶ XIV ⟶ XV ⟶ (+)-X ⟶ (−)-X-*p*-toluenesulfonate

Scheme I

The solution was alkalized with 10% aqueous sodium carbonate solution and was extracted with two 10-ml portions of ether. The extract was dried, filtered, and concentrated to 3–4 ml. On standing (25°), 30 mg (0.15 mmole, 6.5%) of (*R*)-(−)-3-benzoyl-3-hydroxypiperidine [*R*-(−)-X] crystallized, mp 158–160.5°; NMR ($CDCl_3$): δ 8.28–7.11 (m, 5H, C_6H_5), 3.59–2.18 (m, 6H, CH_2NCH_2 and OH, NH, D_2O exchangeable), and 2.18–1.13 (m, 4H, $NCCH_2CH_2$) ppm; $[\alpha]_D^{25}$ (chloroform) −10.8 ± 1° (c 0.315). This was converted to the *p*-toluenesulfonate salt [*R*-(+)-X-*p*-toluenesulfonate], mp 121–122° [lit. (5) mp 124–125°]; $[\alpha]_D^{25}$ (methanol) +7.9 ± 1° (c 0.260) [lit. (5) $[\alpha]_D^{25}$ (methanol) +11.4 ± 0.3° (c 2.73)]; NMR (D_2O): δ 8.28–7.03 (m, 9H, C_6H_5 and C_6H_4), 3.90–1.32 (m, 8H, $CH_2NCH_2CH_2CH_2$), and 2.38 (s, 3H, CH_3) ppm.

(*S*)-(−)-3-Benzoyl-3-hydroxypiperidine *p*-Toluenesulfonate [*S*-(−)-X-*p*-Toluenesulfonate] from *R*-(+)-VIII—A solution of 200 mg of *R*-(+)-VIII in 5 ml of anhydrous methanol was added to a solution of 20 mg of sodium in 5 ml of anhydrous methanol. The solution was stirred for 45 min and the solvent was evaporated. The residue was mixed with 10 ml of ether. This mixture was extracted twice with 10 ml of water and was dried, filtered, and evaporated to yield a halogen-free oil assigned the structure 2-methoxy-2-phenyl-5-carbiminomethoxy-1-oxo-5-azaspiro[2.5]octane (XIV); NMR ($CDCl_3$): δ 7.68–7.18 (m, 5H, C_6H_5), 4.83 (broad s, 1H, C=NH, D_2O exchangeable), 3.88–2.90 (m, 4H, CH_2NCH_2), 3.65 (s, 3H, $N{=}COCH_3$), 3.20 (s, 3H, $CH_3OCC_6H_5$), and 2.48–0.72 (m, 4H, $NCCH_2CH_2$) ppm; IR (film): 3380 (=NH) and 1620 (C=N, iminocarbamate) cm^{-1}; no absorption in the region of 3500 (OH) or 1680 (C=O, ketone) cm^{-1}. A solution of XIV in 10 ml of methanol was acidified with 10 ml of 1% HCl, and the solution was stirred for 5 min. The methanol was evaporated. The remaining, aqueous solution was mixed with 10 ml of ether and treated with solid sodium carbonate to alkalize the aqueous layer. Three subsequent 10-ml ether extracts of the aqueous fraction were combined, dried, filtered, and evaporated to yield a basic oil assigned the structure 1-carbiminomethoxy-3-benzoyl-3-hydroxypiperidine (XV); NMR (carbon tetrachloride): δ 8.25–7.00 (m, 5H, C_6H_5), 5.08 (broad s, 2H, C=NH and OH, D_2O exchangeable), 4.08–3.00 (m, 4H, CH_2NCH_2), 3.53 (s, 3H, OCH_3), and 2.17–1.10 (m, 4H, $NCCH_2CH_2$) ppm. This oil was allowed to reflux with 10 ml of 12 *N* HCl for 24 hr. The solution was extracted with ether and the extract was discarded. The aqueous solution was evaporated, and the residue was dissolved in 5 ml of water. The solution was alkalized with 10% sodium carbonate solution and extracted with two 10-ml portions of ether. The extract was dried, filtered, and concentrated to 1–2 ml. On standing, crystals of *S*-(+)-X were obtained. The NMR and IR spectra were superimposable upon those of *R*-(−)-X. The IR and NMR spectra of the *p*-toluenesulfonate salt [*S*-(−)-X-*p*-toluenesulfonate], $[\alpha]_D^{25}$ (water) −10.0 ± 1° (c 0.250), were superimposable upon those of *R*-(+)-X-*p*-toluenesulfonate.

(*R*)-(+)-1-Cyano-3-bromo-3-benzoylpiperidine [*R*-(+)-VIII] from *R*-(−)-VII-HBr—A solution of 600 mg of *R*-(−)-VII-HBr in 15 ml of water was mixed with 10 ml of chloroform and alkalized with 5% sodium carbonate solution. The chloroform fraction and two subsequent 10-ml chloroform extracts were combined, dried, filtered, and evaporated under a stream of nitrogen at 5°. The free base was dissolved in 40 ml of benzene. This was allowed to react with cyanogen bromide as described for *R*-(+)-V. Work-up of the reaction mixture, as described for *R*-(+)-V, gave *R*-(+)-VIII, mp 76–77°, $[\alpha]_D^{25}$ (ethanol) +24.1 ± 0.5° (c 0.100), from the benzene-soluble fraction. The IR and NMR spectra of *R*-(+)-VIII obtained in this way were superimposable on those of *R*-(+)-VIII obtained from *R*-(+)-V.

RESULTS AND DISCUSSION

Treatment of *R*-(+)-V with cyanogen bromide afforded a benzene-insoluble precipitate. The presence of a center of asymmetry was confirmed by its optical activity. Lack of absorption in the 3500-cm^{-1} region demonstrated that the hydroxyl group was either functionalized or absent. That the benzoyl moiety was intact was shown by carbonyl absorption at 1680 cm^{-1} and by aromatic proton absorption at 8.37–7.20 ppm. This water-soluble material gave an instantaneously positive test (silver nitrate) for halogen and was convertable to a free amine which also contained organically bound halogen. Together with the elemental analyses, this information led to the assignment of structure (−)-VII-HBr to this product in which the 3-bromo substituent has stereospecifically or stereoselectively replaced the hydroxyl group.

An optically active benzene-soluble, acid-insoluble product was also isolated. Its acid insolubility, C≡N absorption at 2220 cm^{-1}, and lack of $N{-}CH_3$ absorption in the NMR spectrum prove that this is a cyanamide. Spectral evidence established that the hydroxyl group was functionalized or absent and that the benzoyl moiety was intact. The presence of bromine was demonstrated. This information and elemental analyses are consistent with structure (+)-VIII in which a bromo substituent has stereospecifically or stereoselectively replaced the 3-hydroxyl function.

The characterization of these products corroborates an earlier inference of a unique conversion of the 3-hydroxyl to the 3-bromo analog. The stereochemistry of the transformations was established through the sequence of reactions shown in Scheme I. Under von Braun conditions, (+)-III afforded XI. Mild basic hydrolysis gave XIII and a small amount of (+)-XII. Vigorous acidic hydrolysis of XIII yielded (−)-X from which (+)-X-*p*-toluenesulfonate was prepared. Treatment of (+)-VIII with sodium methoxide in methanol gave XIV which was converted to XV in diluted hydrochloric acid. Hydrolysis of XV in 12 *N* HCl provided (+)-X from which (−)-X-*p*-toluenesulfonate was prepared. Finally, (−)-VII-HBr was converted to (+)-VIII under von Braun condi-

tions. This establishes the configurations of all compounds in Scheme I and thus the stereochemistry of the transformations of (+)-V to (−)-VII-HBr and (+)-VIII under von Braun conditions.

It is known that the conversion of (+)-VIII to XIV proceeds stereospecifically with retention of configuration and that the rest of the reactions of Scheme I do not affect the original center of asymmetry (3–7). Accordingly, the fact that (+)-V and (+)-VIII afford antipodal X-*p*-toluenesulfonates proves that they have the same configuration. The conversion of (−)-VII-HBr to (+)-VIII establishes that it, too, has the same configuration as (+)-V and (+)-VIII. This proves that the conversion of (+)-V to (−)-VII-HBr and (+)-VIII proceeds with retention of configuration. Within the limits of experimental error, the rotations of the *p*-toluenesulfonates indicate that the reaction is stereospecific (90 ± 10%).

If cyanogen bromide attacks at nitrogen to give XII, the only *direct* route to a 3-bromo analog must involve XVI, which would result in inversion of configuration. The less direct route through XVII and XII is also untenable on stereochemical grounds. Thus, the mechanism of the reaction must involve initial attack of cyanogen bromide on the hydroxyl to give IX (*cf.*, 2). Displacement of cyanate by bromide is consistent with both the chemistry of alkyl cyanates (9–14) and the formation of (+)-V from (−)-I *via* a closed ion-pair (XVII) with 15% retention of configuration under *quasi*-Favorskii conditions (4, 5, 8). Accordingly, VII is the precursor of VIII.

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* Present address: Department of Pediatrics, School of Medicine, University of California, Los Angeles, CA 90024

x To whom inquiries should be directed. Present address: School of Pharmacy, University of Connecticut, Storrs, CT 06268

COMMUNICATIONS

General Influence of Physicochemical Properties on Drug–Receptor Combination

Keyphrases □ Drug–receptor combinations—influence of physicochemical properties □ Structure–activity relationships—influence of physicochemical properties on drug-receptor combinations □ Receptor sites—influence of physicochemical properties on interaction with drugs

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COMMUNICATIONS

General Influence of Physicochemical Properties on Drug–Receptor Combination

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Previous publications (1–10) showed how the physicochemical properties of chemically and structurally related compounds, particularly homologs, can be the principal determinant of the structure–biological activity "parabolic" profile for a congeneric family by reason of the influence of the physicochemical properties on relative rates of transport. There are, of course, circumstances that preclude involvement of transport in the determination of activity profiles. Even in the absence of a significant or identifiable transport component, so-called "parabolic" dependencies are generally still observed for compounds chemically similar in polar functionality but widely dissimilar in hydrophobic property (11). Again, models founded on fundamental physicochemical relationships have been proposed to explain some of these data (12, 13), the most sophisticated treatment being that of Higuchi and Davis (13) in which the relative degree of receptor site occupation is computed using a quasiequilibrium distributional model. Among other things, the Higuchi and Davis model assumes the net receptor interaction or "occu-

pancy" at a given concentration to be a summation of: *(a)* interactions with the receptor of specific functional groups having specific positional placements, the "lock and key" effect; and *(b)* interactions influenced by the gross structure of the molecules, which in a general and often predictable manner contribute to the receptor site–receptor environment equilibrium.

The present communication suggests another mechanism whereby physicochemical properties can determine or influence structure–activity profiles. The principles outlined here apply to binding in two-dimensional phases (interfaces, surfaces, and protein surfaces) generally, but the particulars are directed to drug–receptor interactions. The concepts presented are based on relative receptor site occupation by homologs. The receptor is viewed as a homogeneous two-dimensional phase obeying a Langmuir-type isotherm, which is a common idealization of a receptor phase. Thus, the receptor "compartment" is considered to have a finite, limiting capacity, *i.e.*, full occupancy, for the active compounds.

Langmuir's isotherm can be expressed in the following form:

$$f = \frac{(k_1/k_{-1})C}{1 + (k_1/k_{-1})C} \qquad \text{(Eq. 1)}$$

where f is the fractional occupation of available sites ($1 > f > 0$); (k_1/k_{-1}) is the ratio of the adsorptive rate constant, k_1, to the desorptive rate constant, k_{-1}; and C is an arbitrarily chosen and fixed bulk equilibrium concentration. Here we are assuming that C is constant after equilibrium is established, a condition that can be obtained by titrating to a fixed C at equilibrium or by using a large bulk phase reservoir so that C_0 and C_∞ are not appreciably different. As formulated, the isotherm has f in lieu of the more standard "amount adsorbed per unit weight of adsorbent." Thus, the limiting capacity term, often designated as A, does not appear explicitly in the equation, since the limiting capacity in terms of fractional site occupation is obviously unity.

In addition to the assumptions of Higuchi and Davis, two commonly accepted assumptions regarding the relationship of biological response to receptor occupation are incorporated in the thoughts presented; namely, the biological response is proportional to the fractional occupation of receptor sites and, due to analytical sensitivity or other similar constraints, there is a finite value of f, f^*, below which response is uncertain. The f^* value is the threshold for measurable pharmacological activity. Collectively, these assumptions and Eq. 1 imply that as f approaches unity, the response approaches a maximum. Moreover, at low values of f, a linear dependency between response and concentration exists. It is tacitly assumed that the receptor environment is aqueous in nature.

To understand better the relationship between pharmacological response (or f) and the physicochemical properties of the compounds in question, it is necessary first to define physicochemical relationships related to structure for each parameter (k_1, k_{-1}, and, possibly, C) in Eq. 1. Fortunately, simple and reasonably general mathematical relationships can be drawn for such properties if the discussion is limited to homologs. It cannot be stressed too strongly that the concepts (but not the simple equations) also apply to less regularly behaving structural modifications.

Within an homologous series, relative values of the adsorption rate constant/desorption rate constant ratio are known generally to follow Traube's rule (14–16) or to conform to the mathematical relationship:

$$K_n = (k_1/k_{-1})_n = (k_1/k_{-1})_0 10^{\epsilon n} \qquad \text{(Eq. 2}a\text{)}$$

or alternatively:

$$\log (k_1/k_{-1})_n = \log (k_1/k_{-1})_0 + \epsilon n \qquad \text{(Eq. 2}b\text{)}$$

where $(k_1/k_{-1})_n$ is the adsorptive equilibrium constant at chain length n, $(k_1/k_{-1})_0$ is the Y intercept of the adsorptive equilibrium constants (K_n values) plotted semilogarithmically against chain length n (corresponding to the adsorptive equilibrium constant of the hypothetical zero chain length compound), and ϵ is the slope of the plot.

Combination of Eqs. 1 and 2a yields for fractional occupation by a given homolog, f_n, at a given concentration:

$$f_n = \frac{(k_1/k_{-1})_0 10^{\epsilon n} C}{1 + (k_1/k_{-1})_0 10^{\epsilon n} C} \qquad \text{(Eq. 3)}$$

If we arbitrarily choose and fix a value of C such that, at small n, $f_n << 1$ [alternatively, $(k_1/k_{-1})_0 10^{\epsilon n} << 1$], this equation predicts:

1. As chain length is extended from small values to intermediate values of n, the fractional occupation of the receptor phase grows exponentially.

2. Considering the exponential nature of the function, $10^{\epsilon n}$, at some value of n, the condition $(k_1/k_{-1})_0 10^{\epsilon n} C >> 1$ will develop. The fractional occupation thus will asymptotically approach 1. This is true regardless of the choice of C and may even be true for small n if C is sufficiently large. Providing there are no limitations on C, maximal response (full receptor occupation) will occur at some chain length and will be maintained thereafter with further increases in n.

There are, of course, definite limitations on concentration dictated by the solubilities of the respective homologs in the series. It has been shown that, in water, homolog solubilities generally obey the relationship (17, 18):

$$S_n = S_0 10^{-\delta n} \qquad \text{(Eq. 4}a\text{)}$$

or, alternatively:

$$\log S_n = \log S_0 - \delta n \qquad \text{(Eq. 4}b\text{)}$$

where S_n is the solubility of the homolog of chain length n, S_0 is the Y intercept (zero chain length value) of a plot of log (solubility) against chain length, and δ is the absolute value of the slope of the function as described in Eq. 4b. Equation 3 may be

reformulated for the case where adsorption from saturated solutions of the respective homologs is solely considered by incorporation of the solubility relationship for C, *i.e.*:

$$(f_n)_s = \frac{(k_1/k_{-1})_0 S_0 10^{(\epsilon-\delta)n}}{1 + (k_1/k_{-1})_0 S_0 10^{(\epsilon-\delta)n}} \qquad \text{(Eq. 5)}$$

It is obvious from Eq. 5 that the fractional occupation of sites from saturated solutions for a given homolog, $(f_n)_s$, is responsive to the extrapolated adsorptive equilibrium and solubility of the hypothetical zero chain length homolog and the relative magnitudes of ϵ and δ, which appear in exponential form as a difference. Clearly, if $\epsilon > \delta$, the fractional occupation will approach the maximum value of 1 as alkyl chain length is extended when dealing with saturated solutions. But if $\delta > \epsilon$, the reverse will ultimately be true; that is, occupancy will tend to zero with lengthening of the alkyl chain.

It is our contention that the latter situation, $\delta > \epsilon$, is invariably true. Values of the function δ were previously organized by Saracco and Marchetti (17). They analyzed data for the aqueous solubilities of a large and diverse number of homologous series and found that δ values ranged from 0.43 to about 0.75, with an average value in excess of 0.6 (the values stated here are in terms of base$_{10}$ logs). Values of ϵ have not been similarly organized, but some idea of the magnitude of ϵ can be garnered from literature data (14–16, 19–32). These data indicate that the incremental increase in the adsorptive constant (or other related adsorptive property) per methylene unit for adsorption of homologs from a water onto a solid surface has an upper limit of about 3 and ranges down to values less than 1.5. This translates into an ϵ value range of roughly 0.15–0.5. These data do not lend themselves to simple tabulation because several adsorption isotherms are involved, adsorptive capacities and adsorptive constants have generally not been factored, and, in some cases, only single-point (single-concentration) determinations have been made. It is clear, however, that δ values are larger than ϵ values.

With all of the limiting assumptions in mind, particularly the rather idealized assumption that we are dealing with a homogeneous two-dimensional phase, we can proceed in the analysis of the impact of incrementally increasing adsorptive constants and decrementally decreasing solubilities on "occupancy" profiles. Equation 5 can be restated in the following logarithmic form:

$$\log (f_n)_s = \log[(k_1/k_{-1})_0 S_0] + (\epsilon - \delta)n - \log[1 + (k_1/k_{-1})_0 S_0 10^{(\epsilon-\delta)n}] \qquad \text{(Eq. 6)}$$

Two subcases of Eq. 6 are evident. When $(k_1/k_{-1})_0 S_0 10^{(\epsilon-\delta)n} >> 1$, Eq. 6 becomes:

$$\log (f_n)_s = 0 \qquad \text{(Eq. 7a)}$$

and

$$(f_n)_s = 1 \qquad \text{(Eq. 7b)}$$

Considering that $(\epsilon - \delta)$ is less than zero, this condition will only result at small n values, if at all. In other words, the product of the solubility-determined concentration[1] and the equilibrium constant is greater at short chain lengths and diminishes as chain length is extended. Due to the diminishing solubility–adsorptive constant product, at some chain length the condition $1 >> (k_1/k_{-1})_0 S_0 10^{(\epsilon-\delta)n}$ ensues which leads to the second case:

$$\log (f_n)_s = \log[(k_1/k_{-1})_0 S_0] - (\delta - \epsilon)n \qquad \text{(Eq. 8)}$$

Since the term $\log [(k_1/k_{-1})_0 S_0]$ is constant, the log (occupancy fraction) drops by $(\delta - \epsilon)$ for each additional methylene unit added. When using hypothetical values of 0.6 and 0.3 for δ and ϵ, respectively, this corresponds to a twofold decrease in the fraction of sites occupied per unit increase in chain length in this region of the profile.

The general situation at short chain lengths is that practical experimental concentrations lie considerably under the solubility curve and adsorptivities may, in fact, be studied over the short chain length range at a fixed equilibrium concentration, C. Thus, Eq. 3 may initially describe the adsorptive trend.

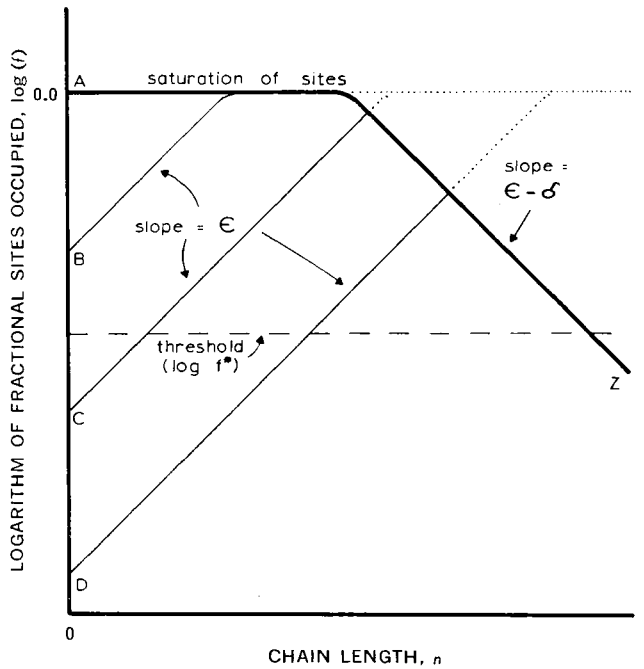


Figure 1—*Theoretical curves for the fractional occupancy of a perfect Langmuirian receptor phase as a function of alkyl chain length under the condition that* $S_0(k_1/k_{-1})_0 >> 1$. *The heavy line, line A–Z, is the expected trend for saturated solutions. Profiles initiating at points B, C, and D are idealized profiles obtained using fixed concentrations* C_1, C_2, *and* C_3, *respectively, where* $S_0 > C_1 > C_2 > C_3$. *In such cases, log* (f) *increases linearly with chain length until the saturation limitation is obtained. Thereafter, the profiles are superimposed on the saturation profile. The dotted lines represent the continuing trends expected, neglecting the solubility restriction. "Parabolic" curves can be generated at a fixed concentration. The apex of the "parabola," the chain length of maximal activity, can be flat or broad, depending on concentration. Its position on the chain length axis is also concentration dependent.*

[1] Here we assume that saturation of all homologs is at concentrations much less than those that would change the solvent character from water to that of a mixed solvent.

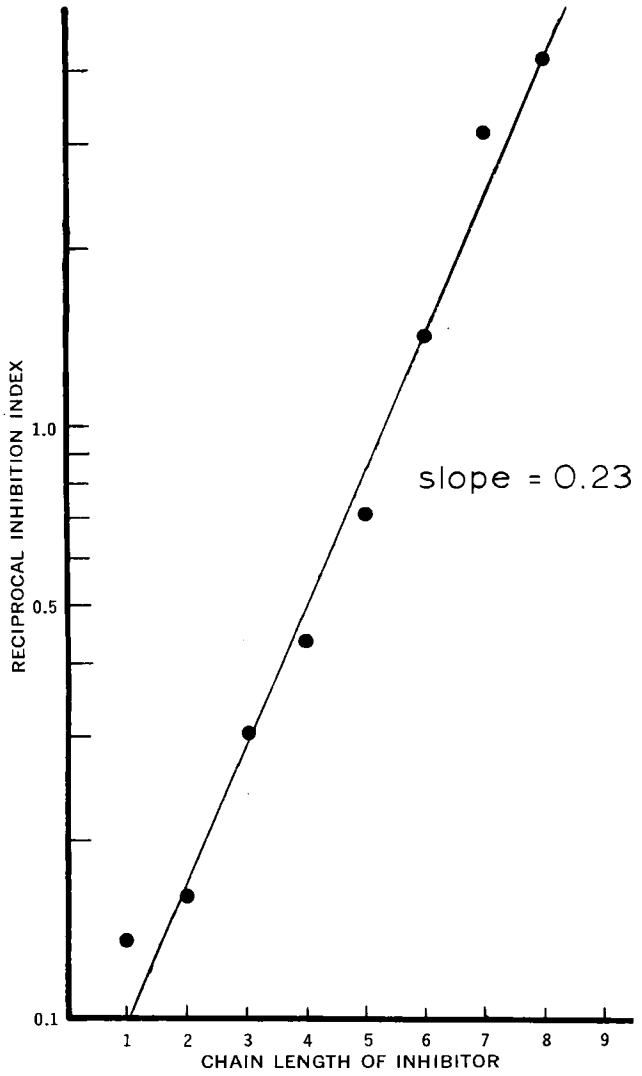


Figure 2—*Data of Schaeffer (41) for the inhibition of adenosine deaminase. The slope of the plot, 0.23, is a measure of ϵ in the system in question. Despite the exponentially increasing efficiency of inhibition, the C_9 and C_{10} compounds proved too insoluble for measurement of inhibition.*

However, irrespective of the choice of C, at some chain length the solubility limitation must enter the picture due to the exponential declivity of the solubility function; thereafter, Eq. 5 or 6 describes the adsorptive pattern. The possible homolog profiles generated by Eqs. 3 and 6 are collectively presented in Fig. 1 in terms of log (f_n) *versus* n.

To put the concepts into perspective, it seems necessary now to answer several basic questions regarding this analysis. What underlying energetic phenomena make the gross generalizations of the treatment sound or at least possible? And, considering the complexities of biological systems, are there actual instances where the presumed phenomena might be demonstrated or observed?

With regard to the first question, the adsorptive equilibrium constant, $(k_1/k_{-1})_n$, is a thermodynamically controlled parameter. It reflects a summation of all enthalpic and entropic contributions to the free energy of adsorptive binding. In other words, the adsorptive equilibrium constant is responsive to the free energy differential experienced when a homolog leaves the bulk phase and occupies an adsorption site. For active compounds, it can be assumed that the enthalpic exchange in this process is significantly large and mainly attributable to the specific "lock and key" receptor interaction. This free energy input is assumed constant throughout a homologous series. There also is a slight "binding unfavorable" entropic effect derived from pinning of the molecule to the receptor. As chain length is extended, there is enthalpic contribution to the free energy of binding which are derived from the differential of van der Waal's forces experienced in the bulk phase less those experienced on the adsorptive surface. Based on the arguments of Nemethy and Sheraga (33) and other water structure experts (34, 35), the magnitude of this enthalpic input would be small and the sign indeterminant. In other words, this effect is not of great magnitude, and it plays a small role in the free energetic scheme of events.

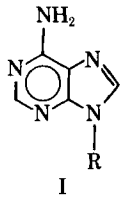
The principal effect of extending chain length is attributable to water structuring at nonpolar surfaces. A large, negative entropic contribution to free energy results when a hydrocarbon or any other low energy surface is exposed to water (33–35). This entropic factor is proportional to the net molecular surface area, which is incrementally increased as methylene units are added to the chain. Any process or factor that conserves surface area exposed to water diminishes the absolute magnitude of the hydrophobic, entropic free energy. Since the molecular surfaces of interest, particularly the peptide backbones of proteins, are relatively nonpolar (36), a low energy surface is conserved as the homolog lays itself down onto the biopolymer and a significant net increase in entropy (net decrease in free energy) proportional to the length of the hydrocarbon chain is obtained. In other words, there is less total surface exposed to the aqueous solvent in the bound situation than in the dissociated situation.

The molal free energy of binding for compound of chain length n at equilibrium follows the usual relationship $\Delta G_n = -RT \ln K_n$; thus, considering Eq. 2*b*:

$$\Delta G_n = \frac{-RT}{2.303}[\log(k_1/k_{-1})_0 + \epsilon n] \qquad \text{(Eq. 9)}$$

which highlights the linearity of the free energy relationship. The general conclusion is that there should be a linearly decreasing, free energy of site occupation with chain length extension as factors other than hydrophobic bonding are either constant or only slightly changing compared to the entropy gained in surface area conservation. Since the conservation of low energy surface area is less in an adsorption situation than in partitioning where the molecule completely escapes the aqueous phase, the effect of general hydrophobic interaction on adsorption should be less than that experienced in partitioning, and this is as observed (37–39).

In what systems might the general trends predicted here actually be observed? It is apparent upon studying the extensive review of Hansch and Coats



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(40) that these conditions can be approached in a test tube using pure enzyme, its substrate, and a homologous series of competitive inhibitors[2]. Apparently the enzyme provides, to a good approximation, the homogeneous two-dimensional phase; and its interactions with homologous competitive inhibitors follow patterns of behavior that have been outlined here, presuming, of course, that there is a true split in specific and nonspecific binding. One interesting literature case can be cited to substantiate this view. Schaeffer (41) studied the influence of homologous inhibitors on the deamination of adenosine mediated by adenosine deaminase. For inhibitors of the type indicated by Structure I, where R varied from CH_3 to CH_3—$(CH_2)_7$, the inhibition index decreased exponentially. The inhibition index as defined by Schaeffer is reciprocally related to K_n, and his observations are thus synonymous with an exponentially increasing adsorptive equilibrium. As can be seen in Fig. 2, the free energy relationship is reasonably linear over the full chain length range and the deviations appear quite random. The value of ϵ is the slope of this plot of the logarithm of the reciprocal of the inhibition index against n and is roughly 0.23 in this system. Thus, the inhibition at a given concentration apparently increases by a factor of about 1.7/methylene unit.

In apparent paradox, considering the exponentially increasing efficiency of inhibition, the 9-*n*-nonyl and 9-*n*-decyl adenines were found to be inactive. This finding was attributed to insufficient solubility by the author (41) and is the expected case considering the small magnitude of ϵ (0.23), assuming a typical magnitude for δ of about 0.6. In other words, Schaeffer's (41) results strongly suggest that the product of K_nS_n for the C_9 and C_{10} compounds is below that necessary for a threshold response. This paper thus highlights the fact that relative activities based on response data normalized to a given fixed concentration can be, and generally are, very misleading in that the normalized concentration is usually an impractical or unobtainable concentration for the compounds in question in a real or usage situation. In the last analysis, relative rates of transport (1), relative amounts distributed to various biophases in the pseudosteady state (13), and relative receptor site occupation are all functions of a phenomenological constant times a concentration term. Limitations on the magnitude of either the phenomenological constant or concentration can be the source of biological inactivity, and it is necessary when characterizing relative biological activities to consider the product term as well as its component part, the phenomenological constant.

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G. L. Flynn[x]
S. H. Yalkowsky
N. D. Weiner

College of Pharmacy
University of Michigan
Ann Arbor, MI 48104

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[x] To whom inquiries should be directed.

[2] Data concerning the influence of alkyl substituents on protein binding or enzyme inhibition are found in Refs. 16, 17, 23, 26, 30, 46, 48, 49, 50-52, and 57 of the Hansch and Coats (40) review. These data support the formulations of Eqs. 2 and 9 found in this communication.

Antagonism of Neuromuscular Actions of Hexamethonium by Choline Precursors

Keyphrases □ Hexamethonium—antagonism of neuromuscular actions by choline precursors without affecting hypotensive effects □ Choline precursors—used to antagonize neuromuscular actions of hexamethonium without affecting hypotensive actions □ Hypotensive agents—antagonism of neuromuscular actions of hexamethonium by choline precursors

Sir:

Hexamethonium has been classified as a ganglionic blocking agent (1) and is used as a hypotensive drug clinically. However, because of its numerous side effects, its use has been quite limited. Recently, it has been shown that hexamethonium possesses not only antinicotinic action but also hemicholinium-like action which inhibits acetylcholine synthesis to paralyze cholinergic neurons (2–5). The side effects of hexamethonium could be due primarily to this hemicholinium-like action, which can be antagonized by pretreatment of the animals with choline (6). In this study, we tried to determine whether or not the choline precursors, *e.g.*, dimethylaminoethanol plus methionine, will also prevent the hemicholinium-like action of hexamethonium.

Holtzman rats, 300–400 g, were anesthetized with 1.6 g/kg of urethan, and the jugular vein was cannulated for the injection of drugs. One carotid artery was cannulated for the determination of blood pressures using a pressure transducer and a polygraph. The sciatic nerve was exposed and passed through a pair of platinum electrodes. Interrupted tetanic stimulations were applied to the nerve with 250-Hz frequency, 0.5-msec duration, and a supramaximal voltage (4–8 v) for 0.1 sec every 10 sec. The tendon of the gastrocnemius muscle was tied with a thread and connected to an isometric force transducer for registering muscle twitches.

Table I—Effects of Choline Precursors on Neuromuscular Blockade and Hypotension Induced by Hexamethonium in Rats

Drug	Neuromuscular Blockade, %	Hypotensive Effect, %
Hexamethonium, 10 mg/kg	34 ± 5.3[a]	45 ± 4.2
Choline precursors[b] followed with hexamethonium, 10 mg/kg	9 ± 3.1[c]	46 ± 2.0

[a] Mean ± *SE*, $N = 6$. [b] Dimethylaminoethanol, 10 mg/kg, plus methionine, 5 mg/kg, administered three times in 20 min before hexamethonium injection. [c] $p < 0.005$ compared with hexamethonium alone.

When 10 mg/kg of hexamethonium chloride was injected *via* the jugular vein into the animal, there was an abrupt drop of blood pressure and a gradual inhibition of the neuromuscular transmission (Fig. 1A and Table I). On the other hand, when the animals were pretreated with 10 mg/kg dimethylaminoethanol plus 5 mg/kg methionine three times for 20 min before the administration of 10 mg/kg hexamethonium, the neuromuscular blockade was prevented while the hypotensive effect remained unaffected (Fig. 1B and Table I). These results clearly indicate that the paralysis of somatic nerves, which is one of the numerous side effects of hexamethonium when it is used to treat hypertension, can be prevented with choline precursors while the hypotensive effects of hexamethonium remain effective. This simple manipulation could make hexamethonium

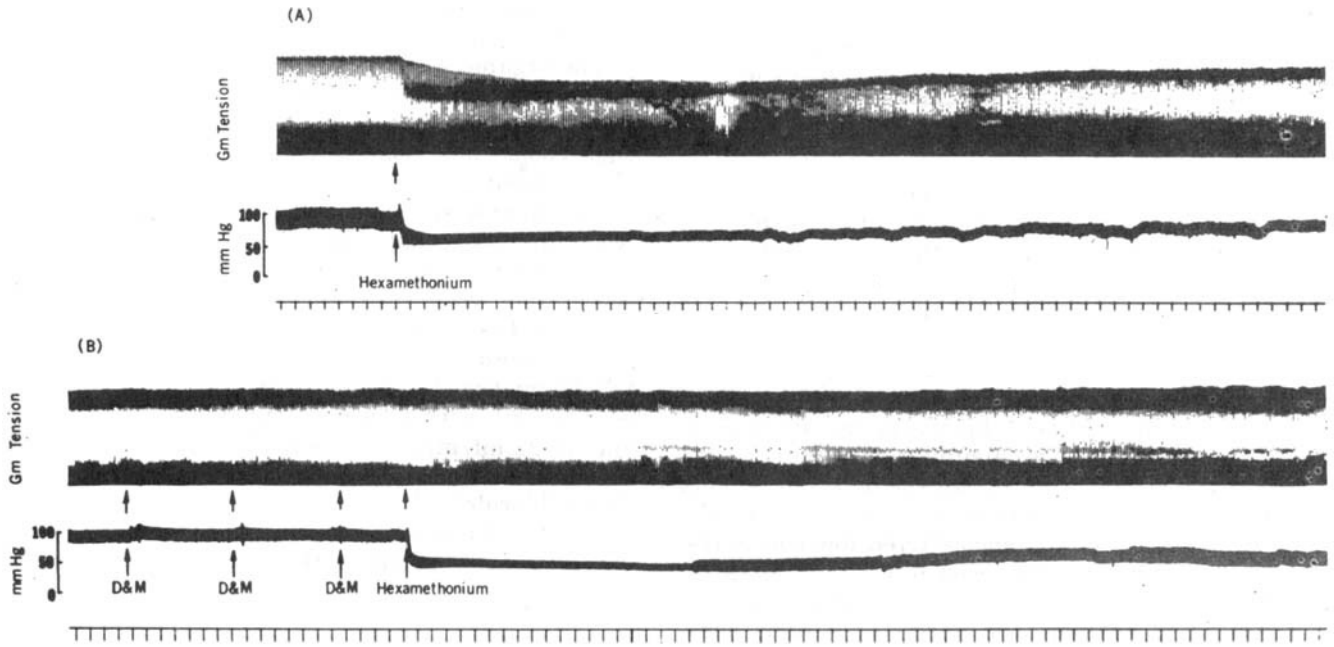

Figure 1—*Neuromuscular blockade and hypotensive effects of hexamethonium, 10 mg/kg, in rats (A) and effects of dimethylaminoethanol, 10 mg/kg, plus methionine, 5 mg/kg (D&M), on neuromuscular blockade and hypotensive effects induced by hexamethonium, 10 mg/kg, in rats (B). Each time interval represents 1 min.*

useful in treating hypertension without having side effects.

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C. Y. Chiou
Department of Pharmacology and Therapeutics
College of Medicine
University of Florida
Gainesville, FL 32610

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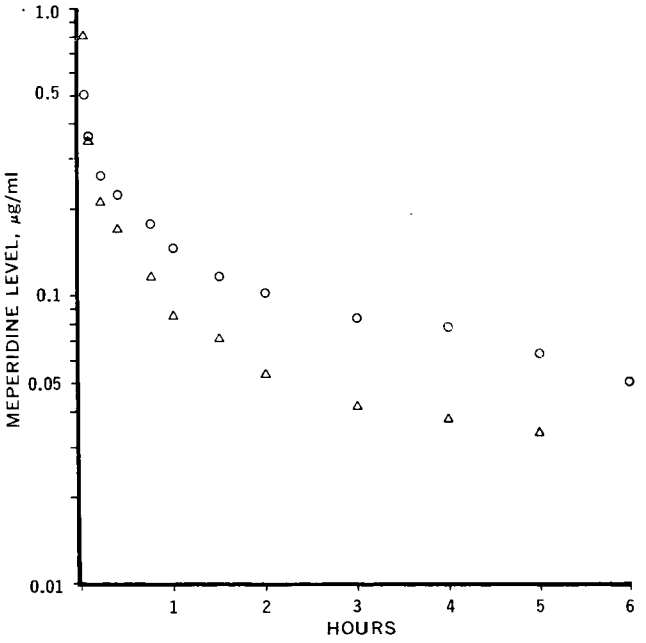

Figure 1—*Representative plasma concentration–time profiles following the intravenous injection of 50 mg meperidine hydrochloride. Key: ○, venous plasma, Subject L.M., dose corresponding to 0.74 mg/kg; and △, arterial plasma, Subject D.C., dose corresponding to 0.37 mg/kg.*

Meperidine and Other Basic Drugs: General Method for Their Determination in Plasma

Keyphrases □ Meperidine (and other basic drugs)—GLC analysis of plasma samples □ Amine drugs (basic)—GLC analysis of plasma samples □ GLC—analysis, meperidine and other basic drugs

Sir:

In a recent paper, Goehl and Davison (1) reported a GLC technique for the determination of meperidine in blood plasma. The method was used to determine plasma drug concentration *versus* time curves in dogs following administration of 15–25 mg/kg iv and yielded a limit of sensitivity of around 0.1 μg/ml.

We wish now to describe a method for the determination of meperidine, which is generally applicable to basic amine drugs. A similar procedure was reported by Hodshon *et al.* (2) for the determination of ketamine in plasma. This procedure allows the determination of clinically encountered low concentrations of meperidine for at least 6 hr following intravenous injection of 0.35–1.0 mg/kg and is currently being used to evaluate the effects of various anesthetic protocols on the kinetics of meperidine in man. Representative concentration *versus* time profiles are depicted in Fig. 1.

In the general method, the sample aliquot (usually 1.0 ml) was mixed with the internal standard solution (usually 100 μl, usually 1.0 μg), made basic with sodium hydroxide solution (100 μl, 1 *M*), extracted with ether (3 ml) in a Teflon-lined, screw-capped centrifuge tube (15 ml) by stirring on a mixer[1] (30 sec), and the phases were separated by centrifugation (2000 rpm, 2 min). Following flash freezing in an acetone–carbon dioxide bath, the organic phase was decanted into a second tube containing hydrochloric acid solution (200 μl, 1 *M*) and the phases were mixed and separated as before.

The organic phase was discarded and the aqueous phase was heated to around 60° for several minutes in a water bath to remove the residual ether. It was then cooled and transferred to a conical tube[2] with Teflon-lined screw cap (1 ml), made basic with sodium hydroxide solution (200 μl, 2 *M*), and extracted with methylene chloride (25 μl). The phases were separated as before. Aliquots (2 μl) of the organic phase were withdrawn with a microliter syringe and injected directly into the gas chromatograph[3]. The glass column, 150 cm × 3.2 mm o.d., packed with 3% OV-17 on Gas Chrom Q (100–120 mesh) was operated at 180° with helium as the carrier gas at a flow rate of 22 ml/min. For meperidine (R_T 3.8 min), the internal standard was *N*-methyl-*N*-ethyllidocaine hydrochloride[4] (R_T 5.1 min). Concentrations were calculated from a previously constructed standard curve of peak height ratio of meperidine/internal

[1] Vortex.
[2] ReactiVial, Pierce Chemical Co.
[3] Varian 1200, equipped with flame-ionization detector.
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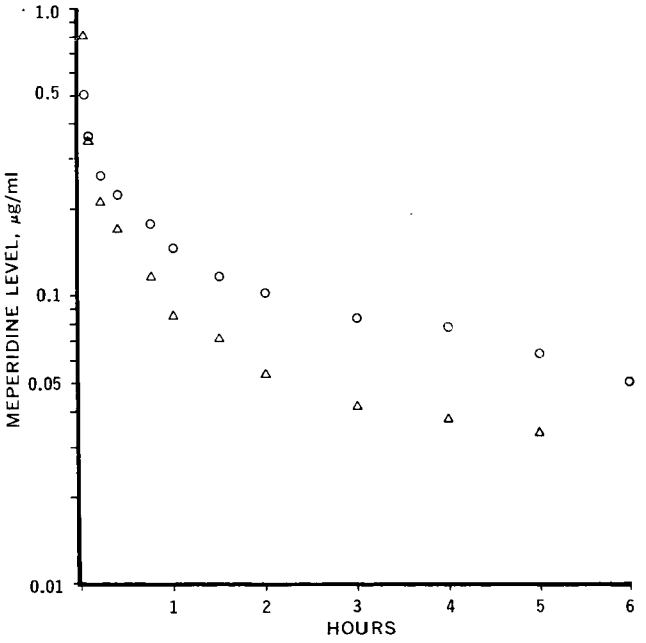

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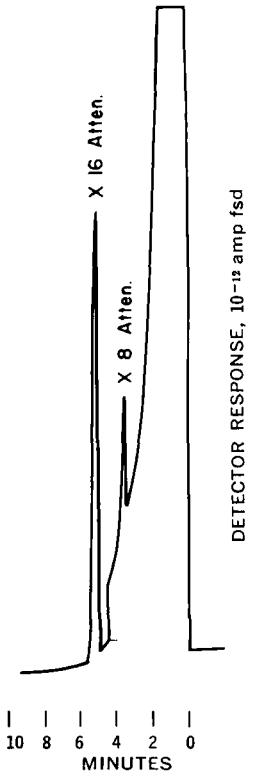

Figure 2—*Representative gas chromatogram obtained from a plasma sample containing 0.104 μg/ml meperidine (as base). Key: peak 1, meperidine (×8 attenuation); and peak 2, internal standard (×16 attenuation). Scale was expanded on first peak for more accurate measurement and to show lack of interference from other peaks.*

standard *versus* amount meperidine. The coefficient of variation of 2% was typical over the concentration range encountered. The limit of sensitivity attainable was approximately 0.005 μg/ml. A representative chromatogram is shown in Fig. 2.

Because procaine (and many ester-type local anesthetics) is susceptible to base-catalyzed hydrolysis with the general method, the following modifications were found useful. The plasma samples (previously treated with sodium arsenite to prevent enzymic hydrolysis) were made basic, where appropriate, with sodium carbonate solution (2 *M*, saturated with sodium chloride). Saturation with sodium chloride assisted the partition into the organic phase at the lower pH of the sodium carbonate (where the tertiary amine is still appreciably ionized). Final extraction was best accomplished with chloroform, which increased the partition of the basic drug.

The technique presented here offers the following advantages over that described by Goehl and Davison (1):

1. It is applicable, with appropriate choice of internal standard (*i.e.*, similar physiochemical properties to drug) and GC conditions, to most basic drugs and is routinely used in the authors' laboratories for the determination of lidocaine, mepivacaine, etidocaine, bupivacaine, ketamine, and, with small modifications, to base-labile ester-type local anesthetics such as procaine.
2. It has greater sensitivity.
3. It is an internal standard technique and hence is not dependent on volumes transferred, diluted, or injected.
4. No evaporation of solvent is required; therefore, there is no potential for drug loss by volatilization (3, 4).
5. Inclusion of hexamethyldisilazane, with its potential detector contamination problems, is not required.
6. Final solvent may be altered to chloroform for greater polarity or to carbon disulfide (centrifuged at 10,000 rpm for 10 min) for greater sensitivity (*i.e.*, reduced solvent peak size and larger injection volume permitted).
7. This technique may be combined with an alkali flame detector for even greater sensitivity[5].

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L. E. Mather[x]
G. T. Tucker
Department of Anesthesiology
University of Washington
Seattle, WA 98195

Received July 16, 1973.
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[x] To whom inquiries should be directed.

[5] L. E. Mather, unpublished observations.

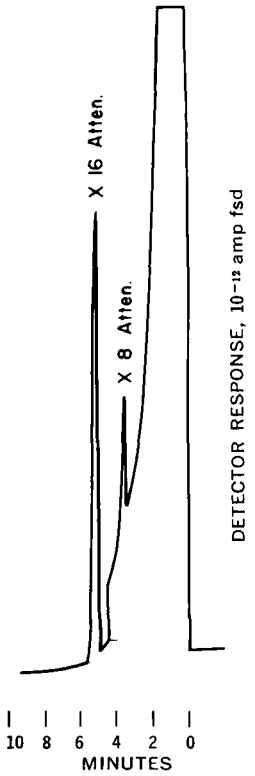
Novel Common Structural Feature among Several Classes of Antimalarial Agents

Keyphrases □ Antimalarial agents—discussion of common structural feature □ Structure-activity relationships—antimalarial agents, common structural feature of several classes discussed

Sir:

A common triangular feature among certain antimalarial cinchona alkaloids, aminoalcohols, and 2-(*p*-chlorophenyl)-2-(4-piperidyl)tetrahydrofuran was recently proposed from this laboratory (1). The components as well as parameters involved in this pharmacophore are interestingly similar to those proposed for α-adrenergic receptor features (2).

In connection with a structural modification study of some 6-aminoquinolines (I) (3–6) and 8-aminoquin-



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L. E. Mather[x]
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University of Washington
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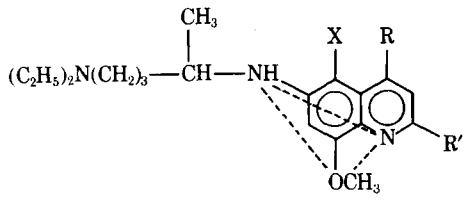

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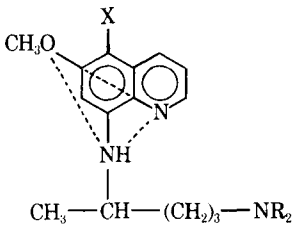

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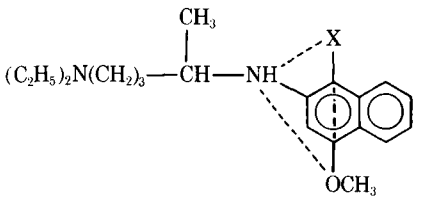
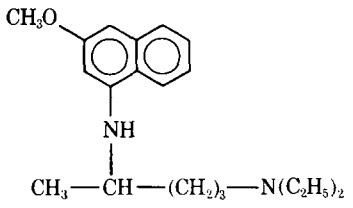


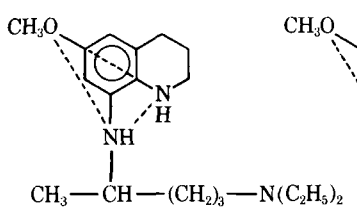
IIIa: X = H
IIIb: X = OCH_3



IV

olines (II) (3, 7–17), three deaza analogs, 3-(4-diethylamino-1-methylbutylamino)-1-methoxynaphthalene (IIIa), 2-(4-diethylamino-1-methylbutylamino)-1,4-dimethoxynaphthalene (IIIb), and 1-(4-diethylamino-1-methylbutylamino)-3-methoxynaphthalene (IV), were synthesized and their antimalarial activity was evaluated (18). Compounds IIIa and IV were devoid of antimalarial activity, but some antimalarial activity was observed with Compound IIIb (18).

A comparative study of the structures of the active *versus* the inactive compounds revealed an interesting pattern. The active compounds (I, II, and IIIb) possess *three* electronegative elements, each containing one or two lone pairs of electrons (N, O, *etc.*), substituted on a benzene (planar) ring at the 1-, 2-, and 4-positions; the inactive compounds (IIIa and IV) lack such an arrangement. One of the three substituents in the active compounds consists of a substituted aminoalkylamino side chain. The same



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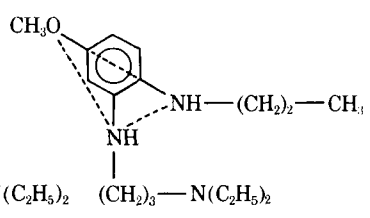

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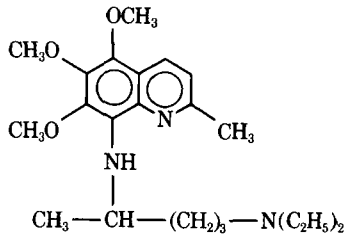
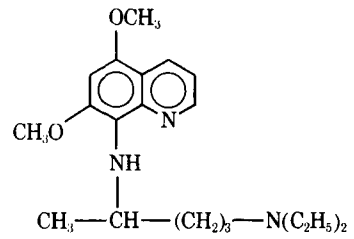


VII

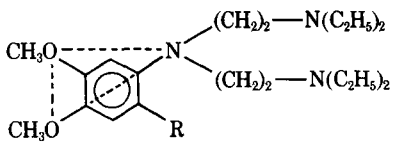


VIII

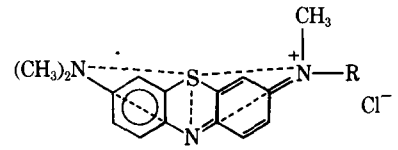
pattern can also be extended to 1,2,3,4-tetrahydro-8-aminoquinolines such as V (19–21) as well as to the corresponding open chain *o*-phenylenediamines such as VI (22, 23), both of which have demonstrated antimalarial activity.

Apparently, substitution of an additional electron-donating group, such as a methoxyl, at the 5-position of the benzene nucleus does not destroy and may even enhance the original antimalarial activity. This is illustrated by the activity exhibited by Compounds I (X = OCH_3; R, R′ = H or CH_3) (5) and II (X = OCH_3; R = H or C_2H_5) (3). However, additional substitution at the 3-position of the benzene nucleus with an electron-donating group gave deleterious results, as shown by the lack of antimalarial activity of Compounds VII and VIII (3).

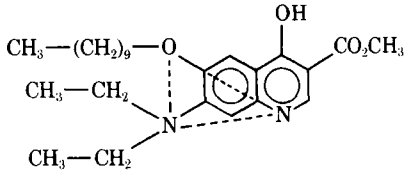
Certain aminopyrocatechol antimalarials (24–31), represented by 1,2-dimethoxy-4-[bis(diethylami-



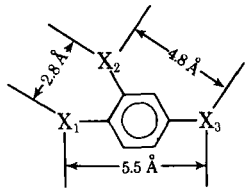
IXa: R = Br
IXb: R = H



Xa: R = CH_3
Xb: R = $(CH_2)_2$—$N(C_2H_5)_2$
Xc: R = CH—CH—CH_2—$N(C_2H_5)_2$ (CH_3 CH_3)



XI

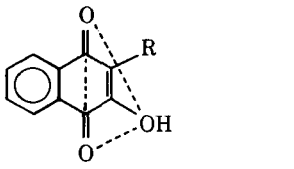


Scheme I

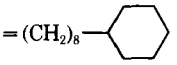
noethyl)amino]-5-bromobenzene (IX*a*, RC-12) and its debrominated derivative dimeplasmin (IX*b*), also possess a similar structural feature. Again, one substituent is a substituted aminoalkylamino side chain, which is probably necessary for secondary binding with certain biopolymers such as with mitochondria (32, 33) *in vivo*. The trisubstitution feature can also be visualized with the antimalarial methylene blue (X*a*) (34, 35) and related compounds (X*b* and X*c*) (36, 37). However, the dibasic side chain is absent in Compound X*a*. This chain is also absent in another antimalarial, methyl 6-decyloxy-7-diethylamino-4-hydroxy-3-quinolinecarboxylate (XI) (38), which does, however, possess the substitution pattern noted in other active compounds.

Thus, a common triangular feature (Scheme I) is proposed for the aforementioned antimalarials. The substituents X_1, X_2, and X_3 represent lone pair electron-containing electronegative atoms such as nitrogen, oxygen, or sulfur. Minimum distances between each of the atoms were obtained through measurements of Dreiding molecular models (39).

Lapinone (XII*a*), other 2-hydroxy-3-alkyl-1,4-naphthoquinones (XII*b* and XII*c*) (40–42), and some quinone imines (*e.g.*, XIII) and 4-amino-1,2-naphthoquinones (43–45) showed most antimalarial activity in prophylactic tests. The absence of the dibasic side chain probably necessitates different secondary structure–activity requirements. Nevertheless, introduction of an amino function at the 3-position (XII*d* and XII*e*) still led to inactive compounds (46, 47).

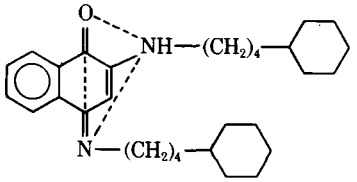


XII*a*: R = $(CH_2)_8$—C(OH)[$(CH_2)_4$—CH_3]$_2$
XII*b*: R = CH_2—CH(CH_3)—$(CH_2)_5$—CH_3



XII*d*: R = NH—NH—COR′





XIII

Although the 4-aminoquinolines (such as chloroquine) and the 9-aminoacridines (such as mepacrine) contain functional groups similar to those of 6-aminoquinolines and 8-aminoquinolines, they do not possess the proposed triangular pattern. Differences in structural features between these two types of compounds and their structural dissimilarity with the earlier reported aminoalcohols (1) may explain the variance in the mode of action (47–50) as well as the rarity in occurrence of cross-resistance between these three types of antimalarials (51, 52). Compounds possessing the presently postulated feature that may participate in biological redox reactions, such as on the energy-producing pathways of oxidative phosphorylation and the electron transport system (3, 53, 54), may have a closer relationship to causal prophylactic or radical curative agents rather than schizonticidal or suppressive agents. The antimalarial test results of the compounds were obtained from various literature sources and different test systems. The proposed structural feature serves only as a working hypothesis for classifying certain types of antimalarials without consideration of drug transport, distribution, and metabolism. Additional attention may be focused on the redox potential and partition coefficient studies (54, 55) as well as on the nature of side-chain substituents for designing more effective and less toxic antimalarial agents.

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C. C. Cheng

Midwest Research Institute
Kansas City, MO 64110

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Staff Review

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DR. JEKYLL AND MR. HYDE

Schizophrenia is a serious mental disorder disrupting the lives of many persons. But schizophrenic behavior can also creep into the operating procedures of institutions; in their sphere of influence, the effects of "institutional schizophrenia" can be equally devastating, but presumably this condition should be immensely more amenable to treatment.

The Food and Drug Administration's (FDA) schizophrenia relative to pharmacy, and specifically related to the role of the pharmacist in the health-care system, appears to have been developing for several years. Particular isolated examples might have been dismissed as petty or picky, but a recent action seems to constitute an especially acute episode.

The publication in the *Federal Register* of the "General Conditions for OTC Drugs" and "Tentative Final Order for Antacid Products," regulations related to the general review of over-the-counter (OTC) drugs and specifically the proposed antacid monograph, suggests that FDA's institutional schizophrenia has taken a decided turn for the worse.

Currently available FDA-generated consumer information repeatedly and explicitly suggests that the consumer utilize the expertise of the pharmacist. Although an entire series of comparable examples could be cited from various FDA publications, news releases, consumer-oriented information, exhibits, approved prescription drug product package inserts, and so on, for purposes of illustration we will quote from just one of these. An FDA brochure entitled *Medicines without Prescriptions* states:

"*Your pharmacist and doctor are trained in drug therapy. They know what a drug is supposed to do and what adverse effects you can expect. Whenever you are in a pharmacy buying an OTC drug, ask your pharmacist's advice....*

"*Ask your pharmacist whether he can recommend a drug that will relieve the symptoms but that will not cause the adverse reaction....*

"*Before using any combination of drugs—prescription drugs with OTC drugs, or several OTC drugs together—it is important that you ask your doctor and follow his advice. Your pharmacist can also advise whether certain drugs can safely be taken at the same time.*"

However, when a recommendation was made by the FDA's own OTC panel on antacids to include on certain product labels the statement "Do not take this product concurrently with a prescription drug except on the advice of your physician or pharmacist," the FDA Commissioner elected to drop the "or pharmacist" from the wording. While the panel's recommendation applied to antacid products containing activated charcoal, the Commissioner's rewording would apply to all instances where a statement of this nature was deemed appropriate for any category of OTC product. His accompanying statement in part stated:

"*...His [the pharmacist] precise role in clinical health care, however, is the subject of intense interest and debate as part of the larger issue of the future of the entire health care delivery system. The Commissioner concludes that such an important matter should be resolved in the context of broad health policy deliberations and not as a part of the OTC drug review, and thus that no reference should be made to pharmacists in OTC drug labeling at this time.*"

Such ambivalence on the part of FDA in promoting the pharmacist as an advisor on drugs to the consumer, while simultaneously refusing to recognize this expertise in product labeling and other professional areas, is blatantly contradictory and absurdly confusing to everyone. It may be true that the questions concerning the pharmacist as an advisor on OTC drugs, on prescription drugs, and as to his future role in the health-care system are complex, comprising economic, legal, professional, and consumer aspects.

However, it is not our purpose or interest here to comment on any of these points. Instead, our purpose is to focus on the apparent tactic chosen by FDA to present one position to the public and another position to the relevant professions. This tactic, if successful, would establish a serious precedent for the future; that is, official recognition or sanction of two dichotomous positions, on the part of a single federal regulatory agency. To us, this would not only assure continued confusion, but also present a dilemma for the regulated industry and professions—an altogether unattractive prognosis.

For FDA's schizophrenic condition, we prescribe a large dose of internal consistency, in order to overcome its multiple personalities. Any further perpetuation of the current FDA approach to the role of the pharmacist could have long-term detrimental effects on the entire health-care system.

Edward G. Feldmann

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REVIEW ARTICLE

Synthesis, Structure, and Biological Properties of Sequential Polypeptides

BRIAN J. JOHNSON

Keyphrases □ Polypeptides, sequential—review of synthesis, structure, optical integrity, enzyme models, immunochemical properties □ Collagen silk fibroin—structural models, conformation studies, and helix stability, similarity of sequential polypeptides □ Antibodies, polypeptide produced—specificity, review □ Peptides—synthesis, structure, and biological properties of sequential polypeptides □ Protecting groups, peptide synthesis—review □ Immunochemical properties—sequential polypeptides, review

Investigators have been interested in sequential polypeptides for a number of reasons. The synthesis of these materials was for a long time very difficult and time consuming. These problems have been alleviated somewhat by the techniques of modern peptide chemistry. However, even now there can arise valid questions concerning the molecular weight and optical purity of certain preparations. The field has in recent years tended to veer toward the utility of sequential polypeptides for structural and biological studies. This may be due to the maturation of this speciality or, more likely, to the reluctance of federal agencies to fund anything that is not applicable to an immediate problem. Notwithstanding the rationale of this shift in emphasis, the field of sequential polypeptides has borne, and should continue to bear, useful information about protein structure and biological functions.

SYNTHESIS

A review in 1958 (1) on polyamino acids also included a small number of synthetic sequential polypeptides which had been prepared up to that date. Since then the volume of literature concerning the synthesis of sequential polypeptides has increased tremendously, and this information is collected in Table I.

In simplistic terms, the synthesis of sequential polypeptides involves the self-condensation of a preformed monomer as exemplified in Scheme I, where

$$HX\cdot H—AA_1—AA_2\cdots AA_x—OY \xrightarrow{TEA} poly(AA_1—AA_2\cdots AA_x)$$

Scheme I

TEA = triethylamine, HX = strong acid salt, AA = amino acid residue, and Y = ester group. Essentially, this method of synthesis was first introduced by Fischer in 1906 (2). Fischer used alkyl esters for his C-terminal activation, so naturally his polymerization conditions required heat and long reaction times to produce only low molecular weight polypeptides. Today the most popular method for the synthesis of sequential polypeptides is still through the use of preformed peptide monomers, but they possess C-terminal esters which are highly activated toward aminolysis. By these means, polymerization can be

performed under very mild conditions. The synthesis of such monomers is achieved by the normal techniques of peptide synthesis, which were reviewed by Kapoor (3).

Historically, Wieland and Schafer (4) in 1952 reported that amino acid thiophenyl esters were capable of forming peptide linkages in aqueous solution. These esters were soon applied to the synthesis of sequential polypeptides (5), and their success prompted other workers to look for activated esters that also possess easier handling, crystallization, and storage characteristics. Of the numerous activated esters available, the pentachlorophenyl (6), the *p*-nitrophenyl (7), and the *N*-hydroxysuccinimido (8) esters are the most widely used. The application of the *p*-nitrophenyl activated ester for the synthesis of sequential polypeptides was described by Kovacs *et al.* (9) who prepared [β-Asp(OBzl)] for structural studies. Almost simultaneously, DeTar *et al.* (10), using the self-condensation of a tripeptide *p*-nitrophenyl ester, prepared the sequential polypeptide poly[Asp(OMe)-Gly_2]. This procedure has been used extensively by a number of workers; DeTar and coworkers (11–16) further explored the utility of the *p*-nitrophenyl activated ester method for the preparation of models for enzyme active sites (15, 16) and also for investigations into the optical integrity of the polymers produced by this polymerization method. Stewart and coworkers (17–25) used the *p*-nitrophenyl ester method extensively for the synthesis of polypeptides for structural studies. Other examples also were demonstrated (26–37), and some workers (28–37) have produced a wealth of literature using this activated ester for polymerizing their polymers which were designed principally as models of silk fibroin and collagen. Many investigators (38–46) have used the *p*-nitrophenyl activated ester method for the synthesis of their sequential polypeptides.

Pentachlorophenyl activated esters were first introduced by Kupryszewski and coworkers (6, 47) for peptide synthesis. In 1965, Kovacs and coworkers (48, 49) began investigating the use of pentachlorophenyl esters for the synthesis of sequential polypeptides. The pentachlorophenyl derivatives frequently have higher melting points (50), better recrystallization characteristics, and higher rates of aminolysis than the corresponding *p*-nitrophenyl derivatives. For these reasons, several workers in this field prefer this activated ester for the synthesis of high molecular weight polypeptides. The utility of this method was extended (50–53), and this method was successfully applied to prepare a series of polytetrapeptides (54–70) for studies on the antigenicity of proteins and peptides and for investigating antibody specificity. Other examples are illustrated in the literature (43, 71–74). The pentachlorophenyl ester method of polymerization was used extensively for the synthesis of a large number of collagen models (34, 36, 75–86).

Along with the pentachlorophenyl activated esters, the pentafluorophenyl and the trichlorophenyl esters have received some attention. The pentafluorophenyl esters are considerably more reactive than the pentachlorophenyl esters, whereas the trichlorophenyl esters are slightly less so (87, 88). The increased reactivity of the pentafluorophenyl esters makes them useful as alternatives for the backing-off procedure of Goodman and Stueben (89); however, their low melting points and ease of hydrolysis can be unfavorable characteristics (90, 91). Both the pentafluorophenyl (92) and the trichlorophenyl (28, 32, 34, 36, 93–98) activated esters were used for the synthesis of sequential polypeptides. Sometimes, because of the low solubility of many polypeptide active ester monomers possessing relatively large molecular weights, the desirability of the improved solubility of these trichlorophenyl esters may make them valuable intermediates for the synthesis of sequential polypeptides.

In 1965, Katchalski (99) reported the use of the *N*-hydroxysuccinimido ester for the synthesis of poly(Tyr-Ala-Glu). This ester is highly reactive and can be used in aqueous media; the by-product of its reaction, *N*-hydroxysuccinimide, is water soluble and easily removable. The average molecular weight of the polypeptide, poly(Tyr-Ala-Glu), was found to be 20,000 (99), but subsequent preparations have increased this value to over 60,000 (100). This activated ester has been gaining popularity for the synthesis of sequential polypeptides (46, 101–108).

In 1968, Johnson and Jacobs (109) reported the use of a new amino acid carboxyl protecting group, the 4-(methylthio)phenyl ester. These esters are easily prepared by the dicyclohexylcarbodiimide method (110). The attractive feature of this protective ester is its facile conversion, by oxidation and without racemization (111), to the activated 4-(methylsulfonyl)phenyl ester. This method of protection and then subsequent activation was applied to the synthesis of the linear polypeptide poly(Lys-Gly)Gly (112).

In 1968, the use of the *o*-hydroxyphenyl esters for peptide synthesis was reported (113), and this activated ester was applied to the preparation of sequential polypeptides (114–118). The reactivity of this ester is due to intramolecular base catalysis, which accelerates aminolysis without, it is claimed, the occurrence of racemization. An advantage of this method is that it can be used as a protective ester in the unreactive, *o*-benzyloxyphenyl ester form. Activation is achieved by fission of the benzyl ether by acidolysis (114–116) or hydrogenolysis (117).

All of these methods of preparing sequential polypeptides employ a high concentration of the polymerizing unit in a polar solvent and in the presence of an organic base. Such conditions minimize cyclization of the polymerizing unit and favor linear intermolecular polymerization. However, these conditions can also favor early precipitation of the polypeptide from solution. Some polypeptides prepared by this method have also shown discrepancies between end-group assays and molecular weights obtained by other methods (119). This deficiency of free amino groups may be due to the formation of very large cyclic polypeptides since the growing polypeptide chain always carries the activating moiety. To circumvent these possible defects in the normal method of pre-

paring sequential polypeptides, it has been shown that polymerization can be conducted in dilute solution if a partially blocked monomer is added. This monomer, the C-terminal end of which is blocked, can act as a nucleus for growth of the polymer chain. The resulting polymer does not carry the activating moiety and thereby eliminates the possibility of cyclization of the polypeptide. The dilute conditions of polymerization allow greater solution of the polypeptide as it is formed, which should allow higher molecular weight polymers to be obtained when compared to the conventional procedure (54–70, 120, 121).

Although the activated ester method is by far the most popular procedure for the synthesis of sequential polypeptides, it is by no means the exclusive procedure for the preparation of such materials. Some investigators still use the tetraethyl pyrophosphite procedure for polymerization as illustrated in Scheme II, where TEPP = tetraethyl pyrophosphite, and AA = amino acid residue.

$$H—AA_1—AA_2\cdots AA_x—OH \xrightarrow{TEPP} poly(AA_1—AA_2\cdots AA_x)$$

Scheme II

The advantage of this process resides in the comparative ease of synthesis of the monomer to be polymerized since the extra step of forming the activated ester is eliminated. However, it was reported (122) that this procedure produces phosphorus-containing polymers. Reservations are also held concerning the optical integrity of those amino acid residues activated by this method, provided they are not glycyl or prolyl moieties. Notwithstanding these criticisms, the method has been employed successfully (28, 123–130).

Dicyclohexylcarbodiimide and the mixed anhydride methods have also found application in the preparation of sequential polymers (123, 131–134). However, due to their undesirable side reactions (135), which can lead to early termination of polymerization, these two methods have not been as widely adopted as the activated ester method. Before this last mentioned method was developed, the acid chloride (136, 137), azide (138), the later hydrazide oxidation (139, 140) methods were also used to prepare polypeptides of low molecular weight. The acid chloride method was recently resurrected by Ridge *et al.* (141) for the preparation of polydepsipeptides. Reasonable molecular weights were reported in this paper.

Application of the Merrifield (142) method of solid phase peptide synthesis has been applied to the preparation of sequential polypeptides (143–145). In these cases, the repeated coupling of blocks of peptides to an insoluble support has shown promise. Although time consuming, this method is most probably the best for the synthesis of polymers of a narrowly defined molecular weight.

The molecular weight of the polypeptides prepared by the solution method has been of concern to many workers (34, 52). In general, the pentachlorophenyl activated ester method is superior to the other methods of activation. Kovacs *et al.* (52) compared various activated esters by the synthesis of the polymers poly(γ-D-Glu-Gly) and poly(γ-Glu-Gly) through the mixed anhydride, carbodiimide, the pentachlorophenyl ester methods. This last method proved to be the best. Similarly, Shibnev *et al.* (34) made a comparison of several of the activated esters in the preparation of the polymers poly(Gly-Hyp_2), poly(Gly-Pro-Hyp), and poly(Gly-Ala-Hyp). It was concluded that the molecular weight of the polypeptides obtained varied with the method of activation. It was found that the pentachlorophenyl ester produced polypeptides with the highest molecular weights, followed in the order of: *N*-hydroxysuccinimido, 2,4,5-trichlorophenyl, 2,4,6-trichlorophenyl, *p*-nitrophenyl, pentafluorophenyl, and 8-hydroxyquinolyl esters. Another factor that plays a large role in the molecular weight of the resulting polymer is the purity of the polymerizing unit. In the experience of the author, it has been necessary to use extensive chromatography to ensure an analytically pure polymerizing unit before polymerization is considered. Also the solubility of the resulting polypeptide in the medium in which it is being produced is another important parameter affecting the molecular weight of the polymer obtained.

Optical integrity is one main concern in peptide chemistry. There is a risk of racemization upon polymerization with the majority of activated esters (12–16, 90, 97, 146–149) mentioned in this review if the C-terminal amino acid of the polymerizing unit is not a glycine or an imino acid. Even small amounts of racemization can have far-reaching effects on the behavior of polypeptides. The backing-off procedure overcomes some of these difficulties by allowing information of the *N*-blocked peptide activated esters without racemization of the C-terminal amino acid residue (89, 150–154). Use of the *N*-hydroxysuccinimido ester (155–157) would appear to be racemization free, as exemplified by the synthesis of poly(Tyr-Ala-Glu) (99, 100); however, one report (158) throws doubt on this assertion. A similar claim of racemization-free synthesis is made for the *o*-hydroxyphenyl esters (114–118). However, the detection of small quantities of racemate is extremely difficult with present methods. Thus, to substantiate this claim, this method will have to be scrutinized fully by a number of investigators.

STRUCTURE

Sequential polypeptides have been used extensively for structural studies, and this is a continuing process. Since collagen possesses a high content of glycyl, prolyl, and hydroxyprolyl residues, it has prompted the synthesis of sequential polypeptides as models for this protein. These models possess, like the protein (159–161), a glycine moiety at every third residue as well as one or both of the imino acids proline and hydroxyproline.

It has been shown that poly(Gly-Pro-Hyp) (124, 162–167), poly(Gly-Pro_2) (168–173), poly(Gly-Pro-

Ala) (169, 171–178), poly(Gly-Hyp_2) (126, 171, 178), poly(Gly-Hyp-Pro) (170, 178), and poly(Gly-Pro-Lys) (178) possess collagen-like X-ray patterns. It would appear that all of these polymers have helical parameters close to those of collagen, and they possibly have a similar conformation with three polypeptide chains wound about a common axis. It has been suggested that the structure of poly(Gly-Pro_2) probably represents some 30–50% of those regions of collagen that consist of the sequence (Gly-Pro-X), where X can be an amino or imino acid (170, 179, 180). In general, it would appear that polytripeptides of the form poly(Gly-$Imino_2$) or poly(Gly-Imino-Amino) readily assume the triple-helix conformation (181). However, polytripeptides of the form poly(Gly-Amino-Imino), including poly(Gly_2-Pro) (182), poly(Gly-Ser-Pro) (183), and poly(Gly-Ala-Pro) (101), have been found to form structures of a different type in which the chains, rather than being wound around each other, are hydrogen bonded together to form sheet-like aggregates. In contrast, X-ray studies (178) have shown that poly(Gly-Ala-Hyp) has a collagen-like conformation. It is suggested that the sequence (Gly-Amino) is a source of instability in the triple-helix structure of collagen. Further support is given to such a conclusion by the deuterium–hydrogen exchange rate of a number of sequential polypeptides (184). Results showed that the extent of exchange in the solid state in the sequences (Gly-Ala-Pro) and (Pro-Gly-Ala) is higher than in (Gly-Pro-Ala). Thus, this latter sequence possesses a more stable structure than the former two sequences.

The molecular conformation of collagen has every third residue near the axis of the triple helix where there is room only for the smallest of the amino acids, namely glycine. Thus, it is not too surprising that the sequential polymers poly(Ala-Pro_2) and poly(Ala-Hyp_2) do not form collagen-like structures (127). Indeed, appreciable amounts of imino acid residues are required to prevent these sequential polymers from assuming α-helix or β-pleated sheet conformations as have been found for poly(Gly-Ala_2), poly(Gly-Ala-Phe), and poly[Gly-Ala-Glu(OEt)]. The four polyhexapeptides poly(Gly-Ala-Pro-Gly-Pro_2), poly(Gly-Pro-Ala-Gly-Pro_2), poly(Gly-Ala-Pro-Gly-Pro-Ala), and poly(Gly-Ala_2-Gly-Pro_2) were shown (102, 185) to possess collagen-like X-ray patterns. These polyhexapeptides all contain alanine and are, therefore, sterically less restricted than poly(Gly-Pro_2). By comparing the denaturation temperatures of poly(Gly-Ala-Pro-Gly-Pro_2), poly(Gly-Pro-Ala-Gly-Pro_2), and poly(Gly-Pro_2), Segal (102) and Segal *et al.* (185) suggested that substitution of an alanyl for a prolyl residue in position 2 has a greater destabilizing effect than substitution in position 3. This tends to be confirmed by the observations that poly(Gly-Ala-Pro) and poly(Gly-Ser-Pro) do not give collagen-like X-ray patterns when prepared by evaporation from aqueous solution whereas poly(Gly-Pro-Ala) and poly(Gly-Pro-Ser) do. Segal *et al.* (185) suggested that the amino acid residue in position 2 may be able to twist outward and thereby facilitate hydrogen bonding to water, thus accounting for the observed instability of (Gly-Amino-Pro) sequences compared with (Gly-Pro-Amino).

Several studies (162, 186) suggested that hydroxyprolyl residues do not play any special role in stabilizing the molecular conformation of collagen. However, this suggestion was questioned by Sakakibara *et al.* (145) who compared the solution properties of the homogeneous polypeptides (Pro-Hyp-Gly)$_n$ and (Pro_2-Gly)$_n$, where in each case n = 5 and 10. It was found that all four of the polytripeptides form the triple-helix conformation, which is analogous to that found in collagen. However, a comparison of their maximum rotation temperatures showed that these values were considerably higher for each hydroxyproline polypeptide than for the corresponding proline analog. From these results it has been concluded that the hydroxyprolyl residues stabilize the triple-helix conformation of collagen. To resolve these conflicting reports on the role of the hydroxyproline residues in collagen, it has been suggested (145) that previous workers (162, 186) were using polymers that were far larger and more heterogeneous in size. It is known (161) that the stability of the triple helix of a synthetic polytripeptide is dependent upon its length. Thus, the use of heterogeneous polymers gave only qualitative results.

Anderson *et al.* (187) investigated some model tripeptides which resemble the polar regions of collagen; preliminary characterization by X-ray diffraction, IR spectrometry, circular dichroism measurements, and optical rotatory dispersion measurements indicated that poly[Ala-Glu(OEt)-Gly] as obtained from an aqueous suspension has an antiparallel β-sheet conformation while poly(Ala-Gly_2) and poly-[Glu(OEt)-Gly_2] possess random conformations.

All stereoisomers of poly(Ala-Gly-Pro), as well as a number of closely related polymers containing modifications to the prolyl residue, have been synthesized (73). Thus, proline has been replaced by thiazolidine-4-carboxylic acid, piperidine-2-carboxylic acid, and azetidine-2-carboxylic acid (74). The conformation of these materials has not been reported so far, however; it will be of interest to learn of their stabilities and to note any correlations with the conclusions obtained from previous work (102, 185).

Evidence for the mode of chain association in poly(Gly-Pro_2) sequences was obtained by Kobayashi *et al.* (144) who used polymers of defined molecular weight. The molecular weight determined for the polypeptides (Gly-Pro_2)$_{10}$, (Gly-Pro_2)$_{15}$, and (Gly-Pro_2)$_{20}$ are in good agreement with values calculated for the corresponding trimers. Berg *et al.* (188) concluded from the titration behavior of such homologous polymers that the three *N*-terminal ends are close to each other and at the same end of the molecule. Thus, it appears that the three strands of the molecule are aggregated parallel to each other with little overlap. A more impressive demonstration of the triple-helix structure of a small synthetic collagen model was shown by Sakakibara *et al.* (189). It was shown by X-ray diffraction that the unit cell of (Pro_2-Gly)$_{10}$ consisted of 12 chains of the oligopep-

tide arranged on four triple-helix structures.

Silk fibroin is characterized by the high composition of glycine, alanine, and serine. This has prompted the synthesis of a number of sequential polypeptides as models of silk fibroin (20, 32, 36, 38, 92, 190, 191). The polypeptides poly(Ala-Gly) (36, 38, 92, 190), poly(Ala-Gly_2) (38), and poly(Gly-Ser-Gly) (20) were all found to form β-structures similar to that found in silk fibroin.

Homopolymers and random copolymers can exist in well-recognized conformations such as the α-helical. Similar studies have been conducted on the molecular conformation of sequential polypeptides. The nature of these polymers lends itself to the process of modifying one or more residues within the repeating chain and thereby observing the effects of the substitution on the conformation of the molecule. A similar process is used for locating the active sites of the biological functions of these materials (as illustrated in the next section). Fraser *et al.* (192) extensively studied the effects of nonhelix-forming amino acid residues on the α-helix conformation adopted by the γ-ethyl ester of polyglutamic acid. For this purpose, a number of sequential polypeptides were synthesized in which valyl (17, 18), S-benzyl-cysteinyl (17, 19), glycyl (22), and *O*-acetyl-seryl (24) residues were incorporated at regular intervals and in varying proportions in the primary structure of poly[Glu(OEt)]. It was found that although valyl and S-benzyl-cysteinyl residues reduce the stability of the α-helix, they do not preclude α-helix formation even when present in considerable proportions (193). However, large molar concentrations of glycyl and *O*-acetyl-seryl residues completely inhibited α-helix formation (194). Another study, investigating the effects of the outer portions of the side chains on the helicity of a polymer, was conducted with homopolypeptides related to poly-γ-benzylglutamate (195). It was shown that interactions between the outer portions of residues well removed from the main chain can also have an important influence on the stability of the α-helix.

In other secondary structural studies, poly[Glu(OBzl)-Tyr(OAc)] (71) was shown to have a right-handed helix with some deviation, which may be caused by the acetyl group, and poly(Ala-Phe-Gly) (43) was shown to have a possible α-helix; NMR measurements on this material did not reveal any side-chain interactions involving the phenylalanyl residues. Poly(Met_2-Ala) (40, 41) possesses a β-structure but tends to form an α-helix with increasing molecular weight. A series of sequential polypeptides of general molecular formula poly[Ala-$(Gly)_{1-3}$] (72) showed varying structures depending upon their workup, such as precipitation or evaporation from various solvents. These structures were reminiscent of the β-structure, or polyglycine II, or a new and not fully elucidated conformation as characterized by IR and X-ray diffraction. When two-thirds of the lysyl residues in the α-helix formed by polylysine are replaced by alanine such as in poly($Lys-Ala_2$) (107), a polymer results that forms a more stable α-helix when fully ionized (107). Presumably there are fewer side-chain interactions in this latter polymer than in polylysine. The structure of the antigen poly(Tyr-Glu-Ala-Gly)Gly (57, 59) has been shown to be of the β-turn conformation (196) and is most definitely not α-helical. The conformation of this antigen is stable to temperatures ranging from 25 to 56° and in pH from 2.5 to 10.0 (197), thus indicating the very stable nature of this particular conformation. The mechanism of hydrogen-bonding collapse has been investigated for some time (198, 199). Ridge *et al.* (141) synthesized the sequential polypeptide of leucine and L-2-hydroxy-4-methylpentanoic acid and came to the conclusion that one in three or four peptide linkages was protonated before the α-helix conformation collapsed to a random coil.

From the preceding comments, it is apparent that sequential polypeptides can be of great use for the elucidation of new conformations occurring in proteins. Possibly polymers of well-defined and narrow molecular weight distributions may offer even more information for structural elucidation.

BIOLOGICAL

The use of synthetic sequential polypeptides for biological studies is advantageous, because once the biological activity of one synthetic material has unequivocally been demonstrated, many analogs of varying size, charge, and conformation can be prepared and tested to elucidate the molecular aspects of the biological phenomenon. The low antigenicity of collagen has been an obstacle to many investigators in studying the antigenic sites of this protein. In spite of this deterrent, Schmitt *et al.* (200) and Davison *et al.* (201) demonstrated that the antigenic sites of this protein lay in regions that are open to proteolytic attack and that these regions are high in tyrosine content. These areas were most probably the nonhelical portions of the *N*-terminal and C-terminal regions of collagen. Michaeli *et al.* (202) found that rat or guinea pig skin collagen produced antibodies in the rabbit which were directed primarily against the α-2-chain. Further evidence for this was found when an *N*-terminal fragment of this chain, produced by cyanogen bromide treatment, was a very effective inhibitor of the antigen–antibody reaction. Similarly other investigators (203–205), using peptides, located antigenic determinants at the C-terminal ends of the α-1- and α-2-chains of calf and rat skin collagen. Furthmayer and Timpl (206) found that the antigenic determinants of the α-1-chain of human skin collagen were located at both the *N*- and C-terminal ends of this particular chain. When denatured collagen is used as the antigen, additional antigenic sites can be demonstrated, thus supporting the conclusion that only the nonhelical regions of native collagen are significantly antigenic. It is thus not too surprising that many synthetic sequential polypeptides used for structural studies are usually very weakly antigenic and do not cross-react with collagen. However, notwithstanding these pitfalls, the polymers poly(Pro-Gly) and poly[Pro-Gly-Hyp(Ac)] have been shown (207) to be immunogenic

and their antibodies appear to be exclusively directed against determinants that predominantly consist of proline. Poly(Pro-Gly-Pro) was also found (208) to be immunogenic in guinea pigs and rabbits. In a passive cutaneous anaphylaxis test, cross-reactions were observed between poly(Pro-Gly-Pro) and fish, rat, and guinea pig collagen. It was suggested (208) that the sequential polymer cross-reacts immunologically with collagen by virtue of the triple-helix conformation common to both substances.

Sequential polypeptides were used as enzyme models by DeTar and Vajda (15), who synthesized the polytripeptide poly(Gly-Ser-Gly) which contains those amino acid residues that are found at the active site of hydrolytic enzymes. However, this polymer did not show any detectable catalytic activity toward the hydrolysis of *p*-nitrophenyl acetate (15). Poly(Asp-Ser-Gly) (16) also contains the sequence that occurs at the active site of several hydrolytic enzymes, and the acetylated polymer poly[Asp-Ser(Ac)-Gly] (16) is a model of the acetylated enzymes which are intermediates in the hydrolysis process. The rate of hydrolysis was found to be comparable to that of certain simple serine derivatives and to be much slower than that of the acylated enzymes (16).

Another enzyme system that has been studied is the protocollagen proline hydroxylase system. This enzyme hydroxylates the peptidyl proline in collagen to hydroxyproline. To ascertain the specificity of this enzyme's activity, sequential polypeptides have been used as the substrate. Kivirikko and Prockop (209) showed that this enzyme does not hydroxylate free proline, the simple peptide Gly-Pro-Pro, or polyproline. However, a small rate of hydroxylation was observed with the tripeptide Pro-Pro-Gly (210), and a far larger rate of hydroxylation was observed with the sequential polymer poly(Pro-Gly-Pro) (209). It was speculated that protocollagen proline hydroxylase is specific for prolyl residues that follow glycyl moieties. This concurs with the positions in which hydroxyproline is found in collagen. Further evidence supports this conclusion (211) in that protocollagen proline hydroxylase did not hydroxylate (Glu-Pro-Gly)$_2$, (Glu-Pro-Gly)$_3$, [Glu(OMe)-Pro-Gly]$_4$-OMe, or Pro-Pro-Gly-[Glu(OMe)-Pro-Gly]$_2$-OMe. Indeed, the Glu-Pro-Gly sequence, which is not hydroxylated, did not inhibit hydroxylation of the other proline residues in Glu-Pro-Gly-Leu-Pro-Gly-Pro-Pro-Gly.

Continuing the investigation of hydroxylation by protocollagen proline hydroxylase, Kivirikko *et al.* (212) investigated a series of peptides of the general formula X-Pro-Gly to study the effect of chain length, conformation, and amino acid sequence in the substrate. A series of peptides containing the sequence X-Pro-Gly was synthesized using the Merrifield (142) method, which produced essentially homogeneous products. When using the random coil forms of the peptides (Pro$_2$-Gly)$_n$, where n = 5, 10, 15, and 20, it was found that all the peptides were hydroxylated; however, their Michaelis constants, K_m, decreased markedly with increasing chain length. Hydroxylation of the polypeptide (Pro$_2$-Gly)$_{10}$ in its triple-helix conformation (189) did not prevent hydroxylation. Although the reaction was slower than the random coil form, both conformations had similar K_m values when expressed in molar concentrations. The hydroxylation of (Pro$_2$-Gly)$_{10}$ in a triple-helix conformation is consistent with previous results, which suggested that protocollagen can be hydroxylated in both the native and random coil forms.

Sequential polypeptides have found great use in investigations concerning the molecular aspects of antigenicity and antibody specificity. The capsular polypeptide obtained from *Bacillus anthracis* is composed exclusively of residues of D-glutamic acid linked by γ-peptide bonds (213). This material precipitates antibodies elicited by the encapsulated organism. A specificity for the residues of D-glutamic acid was demonstrated by the inability of a sample of poly-α-L-glutamic acid either to precipitate with antiserum or to inhibit precipitation of antiserum with the homologous anthrax polypeptide (214). To investigate the role of the carboxyl group of the native polyglutamic acid in the precipitin reaction with the immune serum against anthrax, the following polymers were synthesized: poly(γ-D-Glu-Gly) (52), poly(γ-Glu-Gly) (52), poly(γ-D-Glu-β-Ala) (50), poly(γ-Glu-β-Ala) (50), poly(γ-D-Glu-γ-Abu) (215), and poly(γ-Glu-γ-Abu) (215). When antiserum to the encapsulated organism was cross-reacted with poly(γ-D-Glu-Gly), poly(γ-Glu-Gly), poly(γ-D-Glu-β-Ala), and poly(γ-Glu-β-Ala), no precipitation was observed (214). However, inhibition of the precipitin reaction was observed with all of these polypeptides, the best inhibitors being those polymers containing D-glutamyl residues. Interestingly, it was found that the polymers poly(γ-D-Glu-γ-Abu) and poly(γ-Glu-γ-Abu) both produced precipitates with antiserum to the capsular material of *B. anthracis*. The ability of the L-isomers of the polymer poly(γ-Glu-γ-Abu) to produce such a precipitin reaction was unexpected (215).

The protective effect of glutathione against radiation prompted the synthesis of polyglutathione, polyasparthione, and related sequential polypeptides (53). The antiradiation abilities of such materials have yet to be reported. However, such materials would be considered to have a high probability to be antigenic.

The synthetic polypeptide poly(Tyr-Ala-Glu) has been used to investigate the role of the conformation in immunogenicity and antigenic specificity (216). It was shown that this polymer possesses an α-helix structure under physiological conditions and that antibodies produced against it do not cross-react with a branched polymer consisting of the tripeptide (Tyr-Ala-Glu) attached to the backbone polymer poly(DL-Ala)...Lys. This latter branched polymer exists as a random coil conformation. It was shown that the antigenic determinants of the α-helix polymer were conformation dependent, whereas the branched polymer possessed sequential determinants (106, 216–221).

The molecular aspects of antigenicity have also

been studied using synthetic polypeptides of a known primary structure. It has been found that poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C-OEt (57, 59) gives a precipitin reaction with its antiserum (222). The role of the aromatic moiety on the antigenicity of this material has been studied by the use of various analogs of this antigen. It has been found that the polymer deficient in tyrosine, *i.e.*, poly(Glu-Ala-Gly)Gly-OEt (54), is very weakly antigenic (223) and that its antigenicity is enhanced by the inclusion of the tyrosyl residue, confirming the observation made some time ago that the tyrosyl residue enhances the antigenicity of a molecule. The role of the aromatic residue also has been studied by use of the polymers poly(Phe-Glu-Ala-Gly)Gly-1-^{14}C-OEt (60), poly[Tyr(Me)-Glu-Ala-Gly]Gly-1-^{14}C-OEt (64), and poly(Ala-Glu-Ala-Gly)Gly-1-^{14}C-OEt (65). Both polymers containing an aromatic moiety have been shown to be antigenic (60, 64), whereas the polymer devoid in such a residue has not (65).

Sequential polypeptides have been used to study the specificity of antibodies produced by an antigen. Thus, using the antigen poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C-OEt (57, 59) as a model, it has been shown that the antigenic determinants are most probably conformation dependent since the small peptides $(\text{Tyr-Glu-Ala-Gly})_n\text{Gly}$, where n = 1-3, do not cross-react with these antibodies nor inhibit the precipitate of these antibodies with their homologous antigen. Further evidence for the conformational dependency of the antigenic determinants was shown by the use of the polymer poly(Tyr-γ-Glu-Ala-Gly)Gly-OMe (66). By maintaining the same order of amino acids as the antigen but producing a polymer with a different conformation due to the γ-peptide linkage, it was shown that there was no cross-reaction with the anti-poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C-OEt serum, nor was there any inhibition of the precipitin reaction of this antiserum and its homologous antigen (66). Further work on the specificity of antibody to poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C-OEt has been conducted with respect to the alanyl residue. The polypeptides poly(Tyr-Glu-Gly_2)Gly-OMe (61) and poly(Tyr-Glu-Val-Gly)Gly-1-^{14}C-OEt (58) were used for this purpose. It was found that the former material did not cross-react with the antiserum; however, the latter material removed all of the antibody as shown by absorption experiments. It is thought that the polymer poly(Tyr-Glu-Gly_2)Gly-OMe does not have the same conformation as the antigen and so its determinants are not correctly oriented for cross-reaction. However, it is capable of covering part of the active site on the antibodies since it can cause inhibition of the precipitin reaction of the antiserum and its homologous antigen. The polymer poly(Tyr-Glu-Val-Gly)Gly-1-^{14}C-OEt (58) has the alanyl residue replaced by the sterically larger valyl residue. Thus, it could be expected that if the alanyl moiety is part of the antigenic determinant, then the antibody receptor site would be too small to accommodate the valyl residue and some change in the precipitating ability of this valyl polymer should be observable. However, poly(Tyr-Glu-Val-Gly)Gly-1-^{14}C-OEt (58) causes complete precipitation of antibody, thus indicating that this polymer has the same conformation as the antigen and that the alanyl residue, although important for maintaining the conformation of the molecule, is not part of the active site.

The role of the tyrosyl residue and the specificity of the antibodies for this residue have been investigated. For this purpose the polypeptides poly(Phe-Glu-Ala-Gly)Gly-1-^{14}C-OEt (60), poly[Tyr(Me)-Glu-Ala-Gly]Gly-1-^{14}C-OEt (64), poly(Trp-Glu-Ala-Gly)Gly-OMe (67), and poly(Ala-Glu-Ala-Gly)Gly-1-^{14}C-OEt (65) have been used, using absorption and cross-reaction techniques (67). It was found that the first three polymers cross-react with antiserum to poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C-OEt; however, none precipitated all of the antibodies. Modification of the tyrosyl residue to *O*-methyltyrosyl precipitated 75% of the antibody, modification to phenylalanyl precipitated 54%, and modification to tryptophanyl precipitated 40% of antibodies, whereas the alanyl moiety gave no precipitate. This latter polymer did cause inhibition of the precipitin reaction (224).

The role of the glutamyl residue in the specificity of antibodies produced by the antigen poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C ethyl ester was studied with the use of the polymers poly(Tyr-Asp-Ala-Gly)Gly-OMe (69) and poly[Tyr-(γ-Gly)-Glu-Ala-Gly]Gly-OMe (70). Both polymers represent changes in the distance of the side-chain carboxyl groups from the backbone polypeptide. It was observed that both polymers gave precipitin reactions with antiserum to poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C-OEt; however, in both cases the amount precipitated was markedly reduced. From these experiments it has been concluded that antibodies to the antigen poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C-OEt are dependent upon the conformation of the antigen and they also possess specificities for the phenolic hydroxyl group of the tyrosyl moiety, the aromatic ring of the same amino acid residue, and the γ-carboxyl group of the glutamyl residue. Similar investigations have concerned the specificity of the antibodies produced by the antigens poly(Phe-Glu-Ala-Gly)Gly-1-^{14}C-OEt (60, 225) and poly(Tyr-Glu-Val-Gly)Gly-1-^{14}C-OEt (226, 227).

It is evident that polypeptides possessing a known repeating sequence of amino acids have been useful as structural models for proteins, and some can also imitate their biological properties as well. In this latter respect, these sequential polypeptides may possibly find their greatest use as vehicles to study the molecular aspects of various biological phenomena. This undoubtedly will continue to include the use of polymers for enzyme and immunochemical studies. However, in the next few years it could also include the use of polymers for studies on at least one of the components of complement and on the chemistry of membranes. This latter area could provide a wealth of information concerning the selective transport of ions, *etc.*, across membranes and the peptidyl requirements of the membrane itself to allow such a process to occur.

Table I contains, hopefully, all of the sequential polypeptides that have been reported since the review by Katchalski and Sela in 1958 (1). The long hours required to prepare these sequential polypeptides is readily recognized, so apologies are extended to anyone whose work has inadvertently been omitted. All of the compounds listed are of the L-configuration unless otherwise designated. The abbreviated nomenclature follows the IUPAC-IUB Commission on Biochemical Nomenclature Revised Recommendations on Abbreviated Nomenclature of Synthetic Polypeptides (Polymerized Amino Acids) (228), as well as the tentative Abbreviated Designation of Amino-acid Derivatives and Peptides (229). Additional abbreviations which appear are: Glycol = glycolic acid, 2-Hy-4-mepent = L-2-hydroxy-4-methylpentanoic acid, Azet = L-azetidine, Pipec = piperidine, Thz = thiazolidine, OPib = *p*-iodobenzyl, OPcp = pentachlorophenyl, OPfp = pentafluorophenyl, *o*-ONp = *o*-nitrophenyl, TEPP = tetraethyl pyrophosphite, OPyr = 3-hydroxypyridine, S.P. = solid phase method, OHp = o-hydroxyphenyl, M.A. = mixed anhydride, OTcp = trichlorophenyl, $OMSO_2P$ = 4-methylsulfonylphenyl, [O] = oxidative method, Cl = acyl chloride method, ODnp = dinitrophenyl, and Res = residues.

Table I—Synopsis of Synthetic Sequential Polypeptides

Polypeptide	Method of Preparation	Average Molecular Weight[a]	Yield, %	Purpose of Preparation[b]	Reference
(D-Ala-Ala)$_n$	ONp	—	78	A	17
(Ala-Gly)$_n$	ONp	12,000[4]	80	A, C	17, 32, 36, 190
(Ala-Gly)$_n$	OTcp	—	60	C	32, 36
(Ala-Gly)$_n$	OPfp	12,000	30–40	A, C	92
(Ala-Gly)$_n$	S.P.	n = 5, 10, 20	—	A, C	230
(Ala-Gly)$_n$	OPcp	14,000	—	A, D	72, 231
(Ala-Gly)$_n$	DCC	—	—	A	232
(β-Ala-Phe)$_n$	OHp	3400[4]	39	A	116
(β-Asp)$_n$	ONp	8800[4]	—	A	9
(β-Asp)$_n$	OPcp	—	—	A	49
[Asp(OBzl)-Glu(OBzl)]$_n$	ONp	<100 Res[4]	57	A, D	23
[Cys(Bzl)]$_n$	ONp	60–80 Res[4]	44	A, D	21
[Cys(Bzl)-Glu(OEt)]$_n$	ONp	—	68	A	17, 19, 193
[Cys(Bzl)-Gly]$_n$	ONp	60–80 Res[4]	65	A, D	21
(Gln-Ala)$_n$	OHp	10,000	—	A, C	233
(γ-D-Glu-β-Ala)$_n$	OPcp	10,000[3]	48.5	A, E	50, 214
(γ-Glu-β-Ala)$_n$	OPcp	10,000[3]	74	A, E	50, 214
(γ-Glu-γ-Abu)$_n$	OPCp	—	49	A, E	215
(D-Glu-γ-Abu)$_n$	OPcp	12,300[3]	64	A, E	215
(Glu-γ-Abu)$_n$	OPcp	12,500[3]	62	A, E	215
(γ-D-Glu)$_n$	OPcp	8000[3]	89	A	234
(γ-Glu)$_n$	OPcp	9000[3]	89	A	234
[γ-Glu(OMe)-Ala]$_n$	OHp	10,000	—	A, C	233
[Glu(OBzl)]$_n$	ONp	—	27	A	15
[Glu(OBzl)-D-Glu(OBzl)]$_n$	ONSu	—	42	D	105
[Glu(OBzl)-D-Glu(OBzl)]$_n$	ONp	12,000–23,000	—	A, D	235
[Glu(OBzlNO_2)-Glu(OBzl)]$_n$	ONp	<100 Res[4]	93	A, D	23
[Glu(OBu^t)-D-Glu(OBu^t)]$_n$	DCC	12,000	69	A, D	105, 236
[Glu(OMe)-Glu(OEt)]$_n$	ONp	70–80 Res[4]	68	A, D	21
[Glu(OMe)-γ-Glu(OMe)]$_n$	DCC	—	—	A, D	131
[Glu(OPib)-Glu(OBzl)]$_n$	ONp	<100 Res	88	A, D	23
[Glu(OBzl)-Gly]$_n$	ONp	<100 Res	90	A, E, D	15, 23, 39
[Glu(OEt)-Gly]$_n$	ONp	—	87	A, D	17, 194
[Glu(OMe)-Gly)$_n$	OPcp	—	—	A	85
[Glu(OMe)-Gly]$_n$	OTcp	—	—	A	85
(Glu-Gly)$_n$	ONp	5000–10,000[4]	55	A, D, E	15, 39
(γ-D-Glu-Gly)$_n$	M.A.	1100[3]	97	A, E	52, 214
(γ-D-Glu-Gly)$_n$	DCC	1100[3]	84	A, E	52, 214
(γ-D-Glu-Gly)$_n$	OPcp	11,500[3]	86	A, E	52, 214
(γ-Glu-Gly)$_n$	M.A.	1100[3]	81	A, E	52, 214
(γ-Glu-Gly)$_n$	DCC	1100	48	A, E	52, 214
(γ-Glu-Gly)$_n$	OPcp	2800[3]	84	A, E	52, 214
[Glu-(OBzl)-Glycol)$_n$	ONp	—	16	D	25
[D-Glu(OBzl)-Leu]$_n$	ONSu	13,500[2]	51	A, D	158
[Glu(OBzl)-Lys(Z)]$_n$	ONp	<100 Res[4]	78	A, D	23
[Glu(OBzl)-Tyr(Ac)]$_n$	OPcp	—	13	D	71
(Gly-Ala)$_n$	ONp	—	—	C	32
(Gly-Ala)$_n$	OTcp	—	48	C	32, 36
(Gly-Ala)$_n$	OHp	12,000	—	A	116
[Gly-Glu(OBzl)]$_n$	OPcp	—	—	A	85
[Gly-Glu(OBzl)]$_n$	OTcp	—	—	A	85
(Gly-Glycol)$_n$	ONp	—	71	D	25
[Gly-Lys(Tos)]$_n$	OTcp	—	—	A	78, 85
[Gly-Lys(Tos)]$_n$	OPcp	—	—	A	85
(Hyp-Gly)$_n$	ONp	8000[3]	—	A	11, 13, 237
(Leu-2Hy-mepent)$_n$	Cl	24,000[4]	38	A, D	141
(Lys-Ala)$_n$	ONp	19,000	—	A, D	43
(Lys-Gly)$_n$Gly-1-^{14}C-OEt	$OMSO_2P$	11,000[2]	16	A	112
[Lys(Z)-Glu(OBzl)]$_n$	ONp	<100 Res[4]	93	A, D	23
[Lys(Tos)-Gly]$_n$	OTcp	—	—	A	85
[Lys(Tos)-Gly]$_n$	OPcp	—	—	A	85

Table I—(*Continued*)

Polypeptide	Method of Preparation	Average Molecular Weight[a]	Yield, %	Purpose of Preparation[b]	Reference
[Lys(Z)-Gly]$_n$	ONp	<100 Res	97	A, D	23
[Lys(Tos)]$_n$	ONp	—	—	E	31
(Met-Ala)$_n$	ONp	15,000	—	A, D	42
(Phe-Gly)$_n$	ONp	—	—	A	10
(Pro-Ala)$_n$	ONp	3000	—	A, D	238
(Pro-Gly)$_n$	ONp	13,200	—	A, D, E	11, 13, 207, 238, 237, 239, 232
(Pro-Gly)$_n$	TEPP	—	—	A	232
(Pro-Gly)$_n$	ODnp	—	—	A	232
(Pro-Gly)$_n$	[O]	—	—	A	232
(Sar-Gly)$_n$	ONp	—	67	A	240
[Ser(Ac)-Glu(OMe)]$_n$	ONp	14 Res[4]	61	A, D	24
[Tyr(Me)-Glu(OEt)]$_n$	ONp	70–80 Res[4]	84	A, D	21
[Val-Glu(OEt)]$_n$	ONp	70–80 Res[4]	75	A, D	21
[Val-Glu(OMe)]$_n$	ONp	—	86	A	17, 18
(Ala$_2$-Gly)$_n$	ONSu	2200–4500[3]	92	A, B	103
(Ala$_2$-Gly)$_n$	ONp	35,000[1]	40	A, C	38, 241, 242
(Ala$_2$-Gly)$_n$	OPcp	14,000	—	A, D	72, 231
(Ala$_2$-Glycol)$_n$	ONp	—	68	D	25
[Ala-Glu(OEt)-Gly]$_n$	ONp	9000[4]	—	A, D	187
(DL-Ala-Gly$_2$)$_n$	ONp	30,000[1]	87	A	17
(Ala-Gly$_2$)$_n$	ONp	30,000[1]	40	A, C, D	38, 46, 187, 241
(Ala-Gly$_2$)$_n$	ONSu	40 Res	—	A, D	46
(Ala-Gly$_2$)$_n$	OPcp	14,000	—	A, D, C	72, 231
(Ala-Gly$_2$)$_n$	*o*-ONp	40 Res	—	A	242
(Ala-Gly-Pro)$_n$	OPcp	9000	70	A	73
(D-Ala-Gly-D-Pro)$_n$	OPcp	12,700	53	A	73
(Ala-Gly-D-Pro)$_n$	OPcp	3700	41.5	A	73
(D-Ala-Gly-Pro)$_n$	OPcp	4300	30	A	73
(Ala-Gly-Pro)$_n$	TEPP	12,000[4]	—	A, B	28, 127
(Ala-Gly-Lys)$_n$	OPcp	n = 10	—	A	82
[Ala-Gly-Lys(Z)]$_n$	OPcp	—	64	A	74
(Ala-Gly-Thz)$_n$	OPcp	10,000[1]	61.5	A, D	74
(Ala-Hyp$_2$)$_n$	TEPP	6000[3]	—	A, B	127
(Ala-Phe-Gly)$_n$	OPcp	—	—	D	43
(Ala-Pro-Gly)$_n$	ONSu	5000–14,000[3]	81	A, B	101, 104, 174
(Ala-Pro-Gly)$_n$	ONp	5000–14,000[3]	60	A, B	11, 13, 104
(Ala-Pro-Gly)$_n$	TEPP	—	—	A	232
(Ala-Pro$_2$)$_n$	TEPP	9450[4]	—	A, B	127
(Asp-Cys-Gly)$_n$	OPcp	6000[3]	60	A, E	53
(β-Asp-Cys-Gly)$_n$	OPcp	7000[3]	60	A, E	53
[Asp(OMe)-Gly$_2$]$_n$	ONp	19,000[1]	70	A	10, 14
[Asp(Im)-Gly$_2$]$_n$	ONp	5000–10,000[1]	55	A	14
[Asp(OMe)-Phe-Gly]$_n$	ONp	—	—	A	10
[Asp(OMe)-Ser-Gly]$_n$	ONp	3000–11,000	84	A	16
(Asp-Ser-Gly)$_n$	ONp	3000–11,000[3]	—	A, E	16
[Asp-Ser(OAc)-Gly]$_n$	ONp	3000–11,000[4]	—	A, E	16
{Cys(Bzl)-[Glu(OEt)]$_2$}$_n$	ONp	90 Res[4]	92	A, D	19, 21, 193
[Cys(Bzl)-Gly$_2$]$_n$	ONp	—	40	A	17
(Glu-Ala$_2$)$_n$	OHp	3000[1]	62	A	117
(Glu-Ala-Glu)$_n$	OPcp	20,000[4]	94	A	48, 51
(Glu-Ala-Gly)$_n$	OPcp	10,000	75	A	54
(γ-Glu-Ala-Gly)$_n$	OPcp	13,500[3]	44	A	54
[Glu(OEt)-Cys(Bzl)-Glu(OEt)]$_n$	ONp	60 Res[4]	92	A, D	17, 19, 193
(γ-Glu-Cys-Gly)$_n$	OPcp	9000[3]	59	A, E	53
(Glu-Cys-Gly)$_n$	OPcp	16,000[3]	58	A, E	53
[Glu(OBzl)]$_n$	OHp	11,000[1]	50	A, D	118
{[Glu(OEt)]$_2$-Gly}$_n$	ONp	50–100 Res[4]	95	A, D	22, 194
{[D-Glu(OBzl)]$_2$-Leu}$_n$	ONSu	12,100–21,600[2]	—	A	158
(Glu-Gly-Ala)$_n$	ONp	8700[4]	—	A	35
(Glu-Gly-Ala)$_n$	OPcp	6780[4]	—	A	35
[Glu(OBzl)-Gly-Ala]$_n$	ONp	—	35	A	37
[Glu(OEt)-Gly$_2$]$_n$	ONp	7000[4]	72	A, D	17, 187
[Glu(OMe)-Gly-Lys(Tos)]$_n$	OTcp	—	—	A	78
[Glu(OBzl)-Gly-Lys(Tos)]$_n$	ONp	—	—	A	78
[Glu(OBzl)-Gly-Lys(Tos)]$_n$	OPcp	—	—	A	78
(Glu-Ser-Gly)$_n$	ONp	5000–10,000[4]	45	A, D, E	15, 39
[Glu(OBzl)-Ser-Gly]$_n$	ONp	—	—	A, D, E	15, 39
[Glu(OMe)-Ser(Ac)-Glu(OMe)]$_n$	ONp	40–100 Res[4]	100	A, D	24
[Glu(OMe)-Val-Glu(OMe)]$_n$	ONp	—	92	A	17, 18
(Gly-Ala-Hyp)$_n$	TEPP	13,500[4]	—	A, B	127, 178
(Gly-Ala-Hyp)$_n$	OPcp	25,000	—	A	34
(Gly-Ala-Pro)$_n$	TEPP	10,000[4]	—	A, B	127
(Gly-Azet-Ala)$_n$	OHp	2000[2]	27	A, D	74
[Gly-Glu(OBzl)-Lys(Tos)]$_n$	OPcp	3528	—	A	81
(Gly$_2$-Lys)$_n$	OTcp	n = 9	—	A, E	98
(Gly$_2$-Phe)$_n$	OPcp	20,000[3]	49	A, E	48, 51
(Gly$_2$-Phe)$_n$	ONp	—	—	A	12, 13

(*continued*)

Table I—(*Continued*)

Polypeptide	Method of Preparation	Average Molecular Weight[a]	Yield, %	Purpose of Preparation[b]	Reference
$(Gly_2\text{-}Phe)_n$	OHp	5000[4]	45	A	116
$(Gly_2\text{-}Pro)_n$	TEPP	—	—	A, B	127
$[Gly_2\text{-}Lys(Tos)]_n$	ONp	10,000	—	A	31
$(Gly\text{-}His\text{-}Gly)_n$	OTcp	—	—	A	93
$(Gly\text{-}Hyp\text{-}Gly)_n$	OPyr	4000	39	A	243
$(Gly\text{-}Hyp_2)_n$	OPcp	157,800[1]	89	A, B	34, 75, 76, 171, 178
$(Gly\text{-}Hyp_2)_n$	ONSu	—	—	B	34
$(Gly\text{-}Hyp_2)_n$	OTcp	16,000	48	A, B	34, 96
$(Gly\text{-}Hyp_2)_n$	TEPP	25,000[4]	—	A, B	126, 127
$(Gly\text{-}Hyp\text{-}Gly)_n$	TEPP	—	—	A	127
$[Gly\text{-}Lys(Tos)\text{-}Gly]_n$	OTcp	<11,000[4]	66	A	86
$[Gly\text{-}Lys(Tos)\text{-}Gly]_n$	OPcp	5000[4]	40	A	86
$\{Gly\text{-}[Lys(Tos)]_2\}_n$	OTcp	21,000	—	A	84, 94
$\{Gly\text{-}[Lys(Tos)]_2\}_n$	OPcp	21,000	—	A	84
$\{Gly\text{-}[Lys(Tos)]_2\}_n$	ONp	3000	—	A	31, 94
$\{Gly\text{-}[Lys(Z)]_2\}_n$	OTcp	—	—	A	244
$(Gly\text{-}Orn\text{-}Gly)_n$	DCC	10,800[2]	55	A	245
$(Gly\text{-}Orn_2)_n$	[O]	1500[2]	22	A	245
$(Gly\text{-}Orn_2)_n$	OTcp	2100[2]	25	A	245
$(Gly\text{-}Pipec\text{-}Ala)_n$	OHp	2000[2]	15	A, D	74
$(Gly\text{-}Pro\text{-}Ala)_n$	OHp	12,000[7]	66	A	114
$(Gly\text{-}Pro\text{-}Ala)_n$	ONp	15,000[3]	—	A	26, 172, 175
$(Gly\text{-}Pro\text{-}Ala)_n$	ONSu	20,000	—	A	108
$(Gly\text{-}Pro\text{-}Ala)_n$	OTcp	4300[4]	23	A	28
$(Gly\text{-}Pro\text{-}Ala)_n$	TEPP	2000	—	A	127
$[Gly\text{-}Pro\text{-}Glu(OBzl)]_n$	ONp	2500	30	A, B	33
$(Gly\text{-}Pro\text{-}Gly)_n$	OTcp	9000	53	A, B	28, 34, 96
$(Gly\text{-}Pro\text{-}Gly)_n$	ONp	6000[3]	31	A, B	28, 34, 26, 172
$(Gly\text{-}Pro\text{-}Gly)_n$	ONSu	11,000	—	A, B	34, 108
$(Gly\text{-}Pro\text{-}Gly)_n$	OPfp	—	—	B	34
$(Gly\text{-}Pro\text{-}Gly)_n$	OQu	3200	—	A	246
$(Gly\text{-}Pro\text{-}Hyp)_n$	OTcp	20,000	—	A, B	34, 96
$(Gly\text{-}Pro\text{-}Hyp)_n$	OPcp	100,000	57	A, B	34, 79
$(Gly\text{-}Pro\text{-}Hyp)_n$	TEPP	36,000	—	A, B	124, 125, 127,162 167, 232
$(Gly\text{-}Pro\text{-}Hyp)_n$	OPfp	—	—	B	34
$(Gly\text{-}Pro\text{-}Hyp)_n$	ONSu	—	—	B	34
$(Gly\text{-}Pro\text{-}Hyp)_n$	ODnp	—	—	A	232
$(Gly\text{-}Pro\text{-}Leu)_n$	TEPP	6000	—	A, B	123, 127
$(Gly\text{-}Pro\text{-}Leu)_n$	ONSu	4000–6000	81	A, B	108
$(Gly\text{-}Pro\text{-}Lys)_n$	TEPP	—	—	B	127
$[Gly\text{-}Pro\text{-}Lys(Tos)]_n$	ONp	7000–10,000	—	A, B, E	30, 94
$[Gly\text{-}Pro\text{-}Lys(Tos)]_n$	OTcp	7000–10,000	—	A, E	94
$[Gly\text{-}Pro\text{-}Lys(Z)]_n$	ONp	—	—	A	244
$(Gly\text{-}Pro_2)_n$	ONp	3000	—	A, B	29
$(Gly\text{-}Pro_2)_n$	TEPP	11,700[4]	—	A, B	127, 168
$(Gly\text{-}Pro_2)_n$	OPcp	100,000	—	A, B	83
$(Gly\text{-}Pro\text{-}Ser)_n$	TEPP	3000	45	A, B	129
$(Gly\text{-}Pro\text{-}Tyr)_n$	TEPP	3500[4]	—	A, B	127
$(Gly\text{-}Ser\text{-}Ala)_n$	[O]	3000[3]	50	A	140
$(Gly\text{-}Ser\text{-}Gly)_n$	ONp	4700[3]	—	A, E	16
$(Gly\text{-}Ser\text{-}Gly)_n$	OPcp	3000	50	A, C	36, 77
$(Gly\text{-}Ser\text{-}Hyp)_n$	OPcp	6000	—	A, B	80
$(Gly\text{-}Ser\text{-}Pro)_n$	TEPP	3000[2]	60	A	129
$(Gly\text{-}Ser\text{-}Pro)_n$	OPcp	2500	—	A, B	80
$(His\text{-}Gly_2)_n$	ONp	4000[4]	—	A	10, 13
$[His(ImZ)\text{-}Gly_2]_n$	OTcp	—	—	A	93
$(Hyp\text{-}Glu\text{-}Gly)_n$	OTcp	8500	—	A, B, E	95, 97
$[Hyp\text{-}Glu(OBzl)Gly]_n$	OTcp	8500	90	A, B	97
$(Hyp\text{-}Pro\text{-}Gly)_n$	ONp	10,400	—	A, D	238
$(Hyp\text{-}Ser\text{-}Gly)_n$	OTcp	6130	35.3	B, E	95, 97
$(DL\text{-}Leu\text{-}Gly_2)_n$	−Cl	$n = 5$	—	A	247
$(Leu\text{-}Gly\text{-}Pro)_n$	TEPP	8700[4]	—	A, B	127
$[Leu_2\text{-}Asp(OBzl)]_n$	ONp	—	—	A, D	248
$(Leu_2\text{-}Asp)_n$	ONp	—	—	A, D	249
$(Leu_2\text{-}2Hy\text{-}4\text{-}mepent)_n$	−Cl	32,000[4]	45	A, D	141
$(Leu\text{-}Orn\text{-}Leu)_n$	ONSu	4000–10,000[3]	90	A, E	250
$(Leu\text{-}Orn\text{-}Leu)_n$	TEPP	2700[3]	90	A, E	250
$(Lys\text{-}Ala_2)_n$	OHp	3000[1]	62	A	115, 117
$(Lys\text{-}Ala_2)_n$	ONSu	13,000	—	A, D	251
$(Lys\text{-}Ala\text{-}Gly)_n$	OPcp	13,100[5]	81	A	55
$(Lys\text{-}Arg\text{-}Ala)_n$	OTcp	5300	—	A	252
$(Lys\text{-}Arg\text{-}Gly)_n$	OTcp	7000	—	A	252
$[Lys(Z)\text{-}Gly\text{-}Ala]_n$	OPcp	—	55	A	82
$(Lys\text{-}Gly\text{-}Ala)_n$	OPcp	$n = 9$	—	A	82
$(Met_2\text{-}Ala)_n$	ONp	6000	—	A, D	40, 41
$(Phe\text{-}Pro\text{-}Gly)_n$	ONp	—	70	D	44
$(Pipec\text{-}Ala\text{-}Gly)_n$	OPcp	—	16	D	74
$(Pro\text{-}Ala\text{-}Gly)_n$	OPcp	—	34	A	73

(*continued*)

Table I—(*Continued*)

Polypeptide	Method of Preparation	Average Molecular Weight[a]	Yield, %	Purpose of Preparation[b]	Reference
(Pro-Ala-Gly)$_n$	ONSu	—	16	A	73
(Pro-Ala-Gly)$_n$	TEPP	15,000[2]	—	A, B	128, 173, 232
(Pro-Ala-Gly)$_n$	ONp	12,400[4]	81	A ,B	27, 73, 253
(Pro-Ala-Glycol)$_n$	ONp	1400	90	A, B, D	25
(Pro-Gly-Ala)$_n$	TEPP	—	—	D	184
[Pro-Gly-Hyp(Ac)]$_n$	TEPP	2700	—	A, B, E	170, 207, 239
(Pro-Gly$_2$)$_n$	ONp	—	—	A	17
(Pro-Gly$_2$)$_n$	TEPP	3500[4]	—	A, B	170, 182
(Pro-Gly$_2$)$_n$	[O]	1400[4]	—	A	139
(Pro-Gly-Pro)$_n$	TEPP	1000–12,000[3]	64	A, B, E	99, 122, 170, 179, 208, 209, 253
(Pro-Gly-Ser)$_n$	TEPP	2800[2]	50	A	129
(Pro-Hyp-Gly)$_n$	ONp	40,000[3]	—	A	11, 13
(Pro-Hyp-Gly)$_n$	S.P.	n = 5, 10	—	A, B	145
(Pro-Leu-Gly)$_n$	TEPP	6600	—	A	123
(Pro-Leu-Gly)$_n$	DCC	6600	—	A	123
(Pro-Phe-Gly)$_n$	ONp	—	74	D	44
(Pro$_2$-Gly)$_n$	S.P.	n = 10, 15, 20	—	A, B	143, 144, 189, 212
(Pro-Ser-Gly)$_n$	ONp	16,000[4]	65	A, B	27, 253
(Pro-Ser-Gly)$_n$	TEPP	5000[2]	60	A	129
(Pro-Ser-Gly)$_n$	ONSu	5100[2]	32	A	129
(Pro-Ser-Gly)$_n$	OPcp	4000	50	A, B	183
(Ser-Gly-Pro)$_n$	TEPP	2500[2]	40	A	129
(Ser-Pro-Gly)$_n$	TEPP	3900[2]	54	A	129
(Ser-Pro-Gly)$_n$	ONp	10,000[3]	—	A	11, 13
(Tyr-Ala-Glu)$_n$	ONSu	60,000[3]	63	A, D, E	99, 100, 106, 216–221, 254
[Tyr(Me)-Ala-Gly]$_n$	ONp	70–80 Res[4]	84	A, D	21
(Val-Pro-Gly)$_n$	ODnp	109,000[3]	—	A, B	232
(Val-Pro-Gly)$_n$	[O]	—	—	A	232
(Ala$_3$-Gly)$_n$	ONp	20,000[1]	40	A, C	38, 241, 242
(Ala$_2$-Gly$_2$)$_n$	OPcp	10,500	—	A, D	231
(Ala-Glu-Ala-Gly)$_n$Gly-1-^{14}C-OEt	OPcp	<100,000[2]	14	A, E	65
(Ala-Gly$_3$)	OPcp	14,000	—	A, D	72, 231
[Glu(OEt)-Cys(Bzl)-(Glu(OEt))$_2$]$_n$	ONp	60 Res[4]	82	A, D	19, 21
(Glu$_2$-Ala-Gly)$_n$Gly-1-^{14}C-OEt	OPcp	260,000[5]	78	A	56
{[Glu(OEt)$_2$-Cys(Bzl)-Glu(OEt)]}$_n$	ONp	48 Res[4]	92	A, D	19, 21, 193
{[Glu(OEt)]$_3$-Gly}$_n$	ONp	50–100 Res[4]	95	A, D	22, 194
{[D-Glu(OBzl)]$_3$-Leu}$_n$	ONSu	11,400–23,000[2]	56	A	158
{[Glu(OMe)]$_2$-Ser(Ac)-Glu(OMe)}$_n$	ONp	40–100 Res[4]	100	A, D	24
{[Glu(OMe)]$_2$-Val-Glu(OMe)}$_n$	ONp	—	93	A, D	17, 18
(Glu-His-Lys-Tyr)$_n$	DCC	15,500[4]	53	A	132, 133
(Glu-Tyr-Ala-Gly)$_n$Gly-1-^{14}C-OEt	OPcp	100,000[2]	32	A, E	63
(Gly$_2$-Hyp-Gly)$_n$	ONp	17,000[3]	—	A, D	11, 13, 237, 238
(Gly$_2$-Pro-Gly)$_n$	ONp	6700	—	A, D	237, 238
{Gly$_2$[Lys(Tos)]$_2$}$_n$	ONp	10,000	—	A, E	31
(Leu$_3$-2-Hy-4-mepent)$_n$	Cl	46,000[3]	42	A, D	141
(Phe-Asp-Val-Gly)$_n$Gly-OMe	OPcp	1–5 × 10,000[6]	6	A, E	255
(Phe-Glu-Ala-Gly)$_n$Gly-1-^{14}C-OEt	OPcp	<100,000[2]	41	A, E	60, 62, 225
(Phe-Glu-Val-Gly)$_n$Gly-OMe	OPcp	1–5 × 10,000[6]	21	A, E	255
[Pro-Gly-Hyp(OAc)-Pro]$_n$	ONp	—	—	E	239
(Trp-Glu-Ala-Gly)$_n$Gly-OMe	OPcp	3000[6]	22	A, E	67
(Tyr-Asp-Ala-Gly)$_n$Gly-OMe	OPcp	<100,000[6]	27	A, E	69
(Tyr-Glu-Ala-β-Ala)$_n$Gly-OMe	OPcp	<100,000[6]	30	A, E	224
(Tyr-γ-Glu-Ala-Gly)$_n$Gly-OMe	OPcp	20,000[2]	27.8	A, E	66
[Tyr(Me)-Glu-Ala-Gly]$_n$-Gly-1-^{14}C-OEt	OPcp	100,000[2]	24	A, E	64
(Tyr-Glu-Ala-Gly)$_n$Gly-1-^{14}C-OEt	OPcp	100,000[5]	78.6	A, E	57, 59, 222
(Tyr-Glu-Gly$_2$)$_n$Gly-OMe	OPcp	100,000[2]	58	A, E	61
[Tyr-(γ-Gly)-Glu-Ala-Gly]Gly-OMe	OPcp	<100,000[6]	27	A, E	70
(Tyr-Glu-Val-Gly)$_n$Gly-1-^{14}C-OEt	OPcp	155,000[2]	82	A, E	58, 226, 227
[Tyr-(ϵ-Glu)-Lys-Ala-Gly]$_n$Gly-OMe	OPcp	20,000[2]	28	A, E	68
(Ala$_2$-Gly$_3$)	OPcp	40 Res	—	A, D	231
{Glu(OEt)-[Cys(Bzl)]$_2$-[Glu(OEt)]$_2$}$_n$	ONp	60–80 Res[4]	100	A, D	19, 21, 193
[Glu(OEt)-Cys(Bzl)-Glu(OEt)-Cys(Bzl)-Glu(OEt)]$_n$	ONp	60–80 Res[4]	94	A, D	19, 21, 193
{[Glu(OEt)]$_3$-Cys(Bzl)-Glu(OEt)}$_n$	ONp	60–80 Res[4]	100	A, D	19, 21, 193
{[Glu(OEt)]$_4$-Gly}$_n$	ONp	50–100 Res[4]	96	A, D	22, 194
{[D-Glu(OBzl)]$_4$-Leu}$_n$	ONSu	31,500[2]	57	A	158
{[Glu(OMe)]$_3$-Ser(Ac)-Glu(OMe)}$_n$	ONp	40–100 Res[4]	100	A, D	24
{[Glu(OMe)]$_3$-Val-Glu(OMe)}$_n$	ONp	—	94	A, D	17, 18
(Ala$_3$-Gly$_2$)	OPcp	40 Res	—	A, D	231
(Ala$_2$-Gly-Pro$_2$-Gly)$_n$	ONSu	2000–12,000[3]	—	A, B	102, 185
(Ala-Gly-Ala-Gly-Ser-Gly)$_n$	ONp	16,000[4]	96	A, C	20, 191
(Ala-Pro-Gly-Pro-Ala-Gly)$_n$	ONSu	2000–12,000[3]	—	A, B	102, 185
[Cys(Bzl)-Gly$_2$-Glu(OMe)$_n$-Val-Glu(OMe)]$_n$	ONp	—	65	A	17
{[Glu(OMe)]$_3$-Val-Val-Glu(OMe)}$_n$	ONp	70–80 Res[4]	89	A, D	21

(*continued*)

Table I—(*Continued*)

Polypeptide	Method of Preparation	Average Molecular Weight[a]	Yield, %	Purpose of Preparation[b]	Reference
$\{[Glu(OEt)]_2$-Gly-Glu$(OEt)_3\}_n$	ONp	50–100 Res[4]	98	A, D	22, 194
$(Pro$-Ala-Gly-Pro_2-$Gly)_n$	ONSu	2000–12,000[4]	—	A, B	102, 185
$(Leu$-Glu-Lys-Ala-Glu-Ala-$Gly)_n$	ONp	6000[2]	33	A	256
$(Leu$-Glu-Lys-Ala-Glu-Ser-$Gly)_n$	ONp	11,000[2]	34	A	256

[a] Method of molecular weight estimation is given by superscript number as follows: [1], viscosity; [2], gel filtration; [3], ultracentrifugation; [4], end-group assay; [5], osmometry; and [6], Diaflo membrane. [b] A = synthetic study, B = collagen model, C = silk fibroin model, D = conformation study, and E = biological study.

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RESEARCH ARTICLES

Cholesterol Solubility in Model Bile Systems: Implications in Cholelithiasis

DANIEL MUFSON[x], KRISNA TRIYANOND, JOHN E. ZAREMBO, and LOUIS J. RAVIN

Abstract □ Studies of cholesterol solubility in a physiologically realistic model bile system as a function of its (crystalline) form revealed significant behaviorial differences in the rate of solution and the approach to equilibrium. The solubility of the equilibrium species, hydrated cholesterol, and of fresh human gallstones was found to be approximately 5 mole %. Anhydrous and coprecipitated cholesterol samples attained metastable solubility values. The lag time for nucleation and growth to occur in these systems could account for lab-to-lab variations in previous studies and could be of extreme importance *in vivo* when lithogenic bile is present. These studies were extended to measurement of the solubility as a function of the bile salt–lecithin ratio. Evaluation of these data with respect to available clinical data reveals that many normal individuals have bile that is supersaturated with respect to cholesterol. The principles governing the maintenance of supersaturation in these persons are discussed and suggestions for future studies are offered.

Keyphrases □ Cholesterol solubility in model bile systems—relationship to solid-state (crystalline) properties and bile salt–lecithin ratio, implications in cholelithiasis □ Gallstones and cholesterol-supersaturated bile in man—implications of cholesterol solubility in model bile systems, solution rate and equilibrium considerations □ Bile salt–lecithin ratio—relationship to cholesterol solubility, model

Cholesterol is the major component of gallstones found in western man. This water-insoluble lipid is primarily excreted in the bile. The nature of the interactions between cholesterol and the other biliary lipids, namely bile salts and phospholipids, has been the subject of many investigations. Transport of the cholesterol has been shown to be mediated by a lecithin–bile salt micelle system. Overloading this system can result in cholelithiasis, which is the deposition of cholesterol as gallstones, a disease state that has been estimated to affect some 15 million Americans. "More than 300,000 gallbladders are removed annually because of cholelithiasis while medical, surgical, and hospitalization expenses ascribed to gallstone disease total almost 1 billion dollars a year" (1).

The functions of bile almost seem paradoxical; on the one hand it is involved with intestinal digestion and absorption of lipids, and on the other it is responsible for the excretion of the otherwise water-

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RESEARCH ARTICLES

Cholesterol Solubility in Model Bile Systems: Implications in Cholelithiasis

DANIEL MUFSON[x], KRISNA TRIYANOND, JOHN E. ZAREMBO, and LOUIS J. RAVIN

Abstract □ Studies of cholesterol solubility in a physiologically realistic model bile system as a function of its (crystalline) form revealed significant behaviorial differences in the rate of solution and the approach to equilibrium. The solubility of the equilibrium species, hydrated cholesterol, and of fresh human gallstones was found to be approximately 5 mole %. Anhydrous and coprecipitated cholesterol samples attained metastable solubility values. The lag time for nucleation and growth to occur in these systems could account for lab-to-lab variations in previous studies and could be of extreme importance *in vivo* when lithogenic bile is present. These studies were extended to measurement of the solubility as a function of the bile salt–lecithin ratio. Evaluation of these data with respect to available clinical data reveals that many normal individuals have bile that is supersaturated with respect to cholesterol. The principles governing the maintenance of supersaturation in these persons are discussed and suggestions for future studies are offered.

Keyphrases □ Cholesterol solubility in model bile systems—relationship to solid-state (crystalline) properties and bile salt–lecithin ratio, implications in cholelithiasis □ Gallstones and cholesterol-supersaturated bile in man—implications of cholesterol solubility in model bile systems, solution rate and equilibrium considerations □ Bile salt–lecithin ratio—relationship to cholesterol solubility, model

Cholesterol is the major component of gallstones found in western man. This water-insoluble lipid is primarily excreted in the bile. The nature of the interactions between cholesterol and the other biliary lipids, namely bile salts and phospholipids, has been the subject of many investigations. Transport of the cholesterol has been shown to be mediated by a lecithin–bile salt micelle system. Overloading this system can result in cholelithiasis, which is the deposition of cholesterol as gallstones, a disease state that has been estimated to affect some 15 million Americans. "More than 300,000 gallbladders are removed annually because of cholelithiasis while medical, surgical, and hospitalization expenses ascribed to gallstone disease total almost 1 billion dollars a year" (1).

The functions of bile almost seem paradoxical; on the one hand it is involved with intestinal digestion and absorption of lipids, and on the other it is responsible for the excretion of the otherwise water-

insoluble cholesterol. Bile is a complex mixture, secreted by the liver, consisting of bile salts, phospholipids, cholesterol, pigment, proteins, and mucus. During nonmeal periods, the bile is held in a reservoir, the gallbladder. Upon hormonal stimulus due to a meal, the gallbladder slowly contracts, releasing the bile into the duodenum. Here the bile salts facilitate the digestion and absorption of lipids. The majority of these bile salts are actively reabsorbed in the ileum and then are rapidly picked up by the liver for resecretion into the bile. Because of its many components, numerous theories as to bile's mode of biliary cholesterol transport were proposed.

DISCUSSION

Isaksson's (2) *in vitro* studies served to establish the importance of both bile salts and lecithin in the solubilization of cholesterol. Above a critical concentration, bile salts in aqueous media spontaneously form micelles. These aggregates have little capacity to solubilize cholesterol. However, the addition of lecithin to micellar bile salt results in micelles with the capacity to solubilize substantial quantities of cholesterol. Normal human bile contains approximately 10% by weight of bile salts and lecithin.

Several different disease processes, such as inproportionate secretion of cholesterol by the liver, infection, or absorption of bile acids through the gallbladder wall, may act to overwhelm the ability of bile to carry cholesterol, resulting in the production of bile supersaturated with cholesterol. Relief of the supersaturation by cholesterol precipitation can proceed to the point of filling the gallbladder and/or blocking the bile ducts. Such blockage generally necessitates surgical removal of the deposit, a gallstone. Autopsy data (3) indicate that 10% of the world's population has gallstones. To study rationally the development of such stones, the solubility of cholesterol in bile must be well defined.

With advances in analytical and preparative methods, it has been possible to prepare model systems for systematic studies on the pathogenesis and therapy of cholelithiasis. Through the use of such systems the mechanism of cholesterol's solubilization has been investigated. While the chemical identity and purity of these lipids are generally well documented, the same cannot be said for their physical properties. Although there have been many investigations on this subject, the literature is confusing, no one value has been universally accepted. "Obviously, establishment of a solubility limit to which everybody can agree is called for" (4).

Table I is a compilation of the solubility methods and equilibrium times employed in representative studies. These studies generally suffer from a lack of definition of the physical state of the cholesterol employed and/or from ill-defined equilibrium states. The most widely utilized procedure of sample preparation was that of simultaneous (evaporation) precipitation or coprecipitation of the lecithin, bile salt, and cholesterol from an organic solvent system. Upon evaporation, a suitable aqueous phase is added. It is a very convenient method to use for ease in quantifying addition of each component and to ensure mixing. However, while this coprecipitate system offers much in the way of facilitating laboratory manipulation[1], it can alter the physical properties of the lipids. This is not implicitly bad; it is simply a fact that must be recognized in the interpretation of the data. Where the use of crystalline cholesterol was reported, its exact nature was generally not specified; different methods and solvents of crystallization were used. Hydrated cholesterol has been reported as the physiologically stable form of cholesterol (15–17). Upon storage at low humidities, the hydrate becomes anhydrous (16, 18).

The solubility of cholesterol in these studies was judged by methods varying from gross determination of a vial for the presence of cholesterol to actual assay of micellar cholesterol. The temperature of these studies was not always physiological. Furthermore, the solubility data were reported in various ways, *i.e.*, milligrams per milliliter or molar ratio of bile salts to cholesterol, which makes an overall assessment difficult. The presentation of such data was put on a rational basis by application of phase diagrams (6). These diagrams emphasize the molecular interactions between the four species involved in the solubilization: cholesterol, bile salt, lecithin, and water. Additional information regarding phase behavior may be gleaned from such diagrams; systematic analysis of the effects of composition on the physical-chemical properties of the system revealed the presence of regions containing crystalline cholesterol, liquid crystalline material, or both.

Later papers attempted to correlate these model data with *in vivo* findings. Admirand and Small (19) were able to show that the bile composition of patients with cholelithiasis fell outside the micellar region while normal patients were generally within the micellar boundaries. However, while the use of the quaternary phase diagram has greatly increased comprehension of the interactions of these lipid materials, it does not seem to be accurately distinguishing between the biles of normal or stone-forming human (20–24) or animal (25) subjects as originally reported. In one study, the control population was found to possess a greater mean cholesterol level than the gallstone patients (24). The poor correlation in these studies might be attributed to errors in bile sample collection and manipulation (26) or to deficiencies in the data used to establish the micellar region (27). A discussion of this latter point is the subject of this report.

Studies in these laboratories indicated that the experimental methods employed to produce these diagrams may have acted to bias the results in favor of supersaturated cholesterol levels. This bias is due in part to the slow rates at which these systems move to equilibrium. Experiments were designed to monitor the solubility of cholesterol in model bile systems as a function of the solid-state properties of cholesterol and of the bile salt–lecithin ratio.

EXPERIMENTAL

Cholesterol—Cholesterol[2] was recrystallized from 95% ethanol. TLC in systems designed to reveal autoxidative products and other common impurities showed the sample to be equivalent to National Bureau of Standards reference cholesterol. The cholesterol was stored at 4° under nitrogen.

Cholesterol-4-^{14}C[3] was tested for radiopurity upon receipt and found to be >99%.

Prior to the production of different physical forms of cholesterol, the radioactive ^{14}C-cholesterol was mixed with chemically pure cholesterol. The benzene solution of ^{14}C-cholesterol as received from the manufacturer was carefully added to a hot solution of cholesterol in 95% ethanol. The solution was brought to boiling and then allowed to cool, and the solvent was removed by vacuum. The specific activity was then determined. This cholesterol was used to prepare the following samples.

Hydrated Crystal—Hydrated cholesterol has been reported to exist as a monohydrated species. Some difficulty was experienced in producing and maintaining this species. Samples of cholesterol gain and lose water readily at room temperature. Hydrated cholesterol was prepared by crystallization from 95% ethanol (17). However, it is difficult to estimate its water composition by thermal methods because large quantities of residual ethanol remain in the sample. Attempts to remove the ethanol selectively did not meet with success. Although titrimetric analysis for water could be employed, it was not considered desirable to utilize crystals containing ethanol in these studies. In the method finally selected, the hydrate is precipitated from 95% ethanol, dried under vacuum, and then allowed to equilibrate in an atmosphere saturated with water vapor.

Anhydrous Crystal—Anhydrous cholesterol was prepared by dissolving cholesterol in an excess of dry ether. The solvent was then evaporated *in vacuo*, and the resulting dry powder was ground and stored at 4°.

Coprecipitated—Appropriate quantities of stock solutions of the lipids [10% bile salts in methanol, 10% lecithin in methanol, and 10% cholesterol in methanol–benzene(1:1)] were accurately mixed in a round-bottom flask. The solvent was removed under vacuum.

[1] Woodford reported that upon adoption of coprecipitation he was able to solubilize 10 times more cholesterol in his taurocholate micelles (9).

[2] Eastman Organic Chemicals, Rochester, N.Y.

[3] New England Nuclear, Boston, Mass.

Table I—Summary of Literature Data Pertaining to the Solubility of Cholesterol

Reference	Method	Equilibration Time
2	Cholesterol, lecithin, and bile salts coprecipitated	1 hr
5	Cholesterol adsorbed onto Hyflo Super-Cel added to human or dog bile	4 hr
6	Cholesterol, lecithin, and bile salts coprecipitated	2–7 days
7	Crystalline cholesterol added to lecithin–bile salt solution	6 days
8	Cholesterol and phospholipid coprecipitated, added to aqueous bile salt solution	4 hr
9	(*a*) Aqueous taurocholate and crystalline cholesterol; (*b*) coprecipitated taurocholate and cholesterol	1–3 days? Immediate?
10	Cholesterol, lecithin, and bile salts coprecipitated	3 days
11	Crystalline cholesterol added to lecithin–bile salt solution	6 days
12	Cholesterol, lecithin, and bile salts coprecipitated	3 days
13	Crystalline cholesterol added to canine bile	5-min sonication or longer unspecified times
14	Cholesterol, lecithin, and bile salts coprecipitated	3 days

Human Gallstones—Upon their surgical removal, the gallstones[4] were placed in saline for storage. The stones used for this study were all from a single patient who yielded 127 stones totaling 12.6 g. Aliquots were ground and sized immediately prior to their use. TLC was used to show the presence of cholesterol in these stones. Sectioning revealed the presence of bile pigments.

Diffractograms were obtained on the powdered cholesterol samples using a diffractometer[5] (CuKα radiation).

Bile Salts—Conjugated bovine bile salts[6] were purified following the method of Small *et al.* (28). TLC revealed the ratio of trihydroxy to dihydroxy bile salts to be about 1.5:1 with about equal quantities of taurine and glycine conjugates.

Lecithin—Lecithin was prepared from fresh chicken egg yolks by the procedure of Small *et al.* (28) with the following additions. Following alumina chromatography, the lecithin-containing fractions were dried under vacuum. The residue was dissolved in petroleum ether and then slowly added to acetone, thereby precipitating white, waxy lecithin. The precipitate was collected and dried under vacuum, and the dry lecithin was stored at 4° under nitrogen.

The fatty acid composition of the lecithin determined by GLC compared favorably to literature values (11, 28). The phosphorous–nitrogen ratio was 1.04. Thin-layer silica gel chromatography revealed no nonphospholipid impurities.

Lecithin–Bile Salt Solution—Literature values for the composition of normal human bile show wide variations. The values presented (135.2 ± 64.6 m*M* bile salt and 38.0 ± 19.6 m*M* lecithin) by Admirand and Small (19) for normal human gallbladder bile were chosen for the preparation of the model dissolution system because they appeared to be representative. It is necessary to know the relative composition of the biliary lipids for interpretation of the phase behavior. It is also important to know the actual quantity of such lipids. These are usually expressed in terms of percent solids. Unfortunately, this term has been used in the cholelithiasis literature to express two different groups of solids: the total solids remaining after evaporation of water from whole bile or the quantity of lecithin, bile salt, and cholesterol. From a solubilization point of view, the most meaningful description would be that of the quantity of solids present in bile capable of solubilizing cholesterol: lecithin and bile salt expressed as a weight/volume percentage of the bile (percent solids as lecithin–bile salt). One might then assume that the greater the percentage of such solids the greater the actual cholesterol-holding capability of bile. However, there may not be a strictly linear function between the two. Ekwall *et al.* (29) presented data showing changes in bile salt micelles as a function of concentration and defined three limits of solubility. It is possible that such discontinuities could occur in the more complex lecithin–bile salt–cholesterol system. The experience of the authors and of others (30, 31) has been that changes in micellar behavior are observed as the percentage of solids and the intramicellar solution are altered by dilution. Similar alterations in electrolyte composition could be expected during storage of hepatic bile in the gallbladder. It is also possible that variations in hormonal levels due to the menstrual cycle, oral contraceptive agents, or pregnancy and their subsequent effects on electrolyte balance (32, 33), biliary components (34, 35), and biliary function (36, 37) could play a role in cholelithiasis by modifying the cholesterol carrying capacity of bile.

Phase work to date (19) has generally been done at 10% solids (calculated as lecithin, bile salt, and cholesterol), the approximate level of biliary solids. Extrapolations of phase boundaries from one well-defined system to a system of differing percentage solids might not be correct. For instance, the phase diagram approximating hepatic bile may not accurately serve to describe the phase behavior of the more concentrated gallbladder bile. Ideally, authors would state molar (or weight) biliary compositions—from this, anyone could calculate mole percent if so desired. The percent solids, weight-to-volume (as lecithin–bile salt), employed in these studies was 10.3 ± 0.7. The formula used to prepare the lecithin–bile salt model system for dissolution studies was 135 m*M* conjugated bovine bile salts and 38 m*M* egg lecithin in a 0.05 *M* phosphate buffer, pH 7.4.

Preparation of Lecithin–Bile Salt Solutions—Stock solutions of lecithin and bile salt were mixed to the required ratio in a round-bottom flask. The solvent was evaporated under vacuum, and buffer solution was added to the dried powder to form the micellar system. Initial solubility experiments were adversely influenced by the presence of microorganisms, necessitating the application and design of aseptic techniques and equipment; the reconstituted lecithin–bile salt was cold sterilized and stored in a sterile incubator-stirrer (38).

Determination of Cholesterol Solubility—*Crystalline Cholesterols*—An excess (20 mg/ml) of the crystalline cholesterol was added to the lecithin–bile salt system contained in glass vials. The vials were sealed and placed in a ferris-wheel-type rotating thermostated apparatus. Equisized fractions of all cholesterol samples were utilized for dissolution experiments.

Coprecipitated Cholesterol—The coprecipitate consisting of cholesterol (to supply 20 mg/ml of reconstituted system), lecithin, and bile salts was carefully weighed into glass vials and warmed to 37°. At time zero, appropriate quantities of prewarmed buffer were added and the vials were placed in the rotating apparatus.

Assay Method—The solubility of the ^{14}C-cholesterol in the lecithin–bile salt system was determined by liquid scintillation counting of an aliquot of the filtrate obtained by passage of the system through a glass-fiber prefilter and a cellulose ester membrane filter[7]. Preliminary experiments showed no significant loss of cholesterol by this filtration technique. The membrane, its stainless steel "Swinney" holder, sampling syringe, and cannula were all prewarmed to 37° to prevent precipitation of cholesterol during sampling. A scintillation counting solution was developed, following Bray (39), to accept both aqueous and organic additives as follows: naphthalene, 100 g; 2,5-diphenyloxazole, 7 g; 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, 0.3 g; toluene, 200 ml;

[4] Obtained through the courtesy of Dr. R. T. Holzbach, St. Luke's Hospital, Cleveland, Ohio.
[5] General Electric XRD-5.
[6] General Biochemicals, Chagrin Falls, Ohio.
[7] Type AP20 and HA, Millipore Corp., Bedford, Mass.

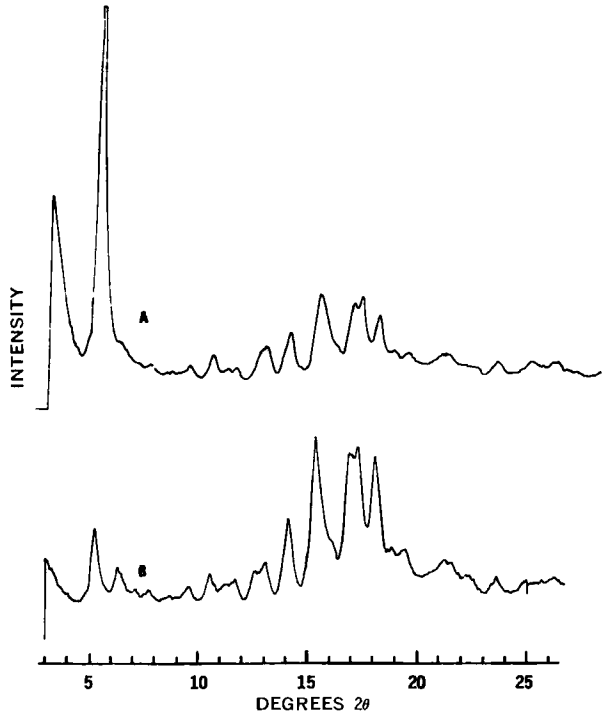

Figure 1—*Diffractogram of anhydrous (A) cholesterol and National Bureau of Standards anhydrous (B) cholesterol.*

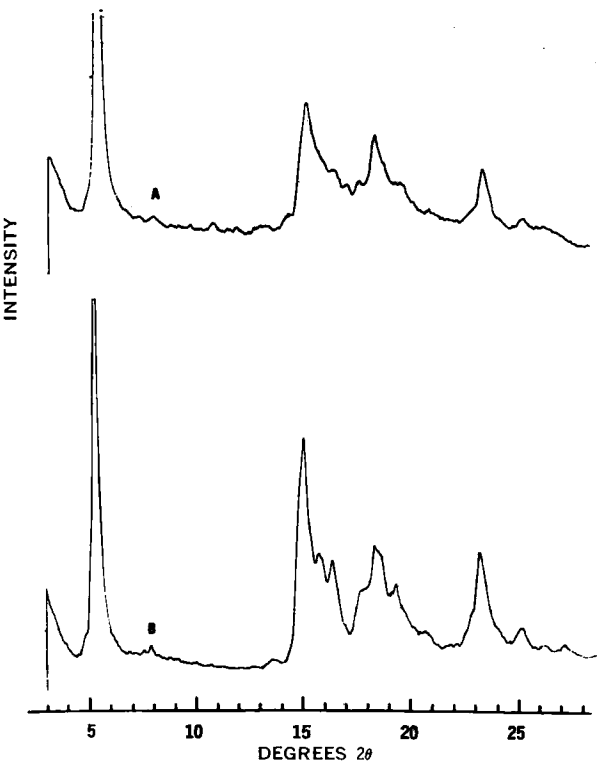

Figure 2—*Diffractogram of human gallstone (A) and of hydrous (B) cholesterol.*

methanol, 100 ml; and *p*-dioxane, *qs* to 1.0 liter. The sample was counted[8] employing an automatic external standardization procedure to account for quenching. The total count collected was $>10^4$.

RESULTS

X-Ray Diffractometry—X-ray diffractometry was utilized to provide information about the crystallographic nature of the cholesterol samples. In Fig. 1 the diffractogram of the anhydrous sample is plotted along with that of National Bureau of Standards reference cholesterol. Good agreement between these and reported data (16) for anhydrous cholesterol was obtained. The hydrated cholesterol and the human gallstone samples yielded similar diffractograms (Fig. 2), which compare favorably with literature values (16). When care is taken to prevent water loss, the hydrated form of cholesterol is observed in pathological deposits. The diffractogram of the coprecipitated sample (Fig. 3) is devoid of well-defined peaks, indicating that the cholesterol in this sample exists as extremely small crystallites or that it is molecularly dispersed with the lecithin and bile salts in a noncrystalline, amorphous matrix.

Dissolution Behavior—The dissolution behavior of cholesterol as a function of its physical form in a model bile system is shown in Fig. 4, where the amount of cholesterol in solution (in mg/ml) is plotted as a function of time[9]. It is useful to express the solubility of cholesterol in moles as a percentage of the molar quantities of cholesterol, lecithin, and bile salt present (in units of mole percent). The solubility expressed in this manner can be thought of as defining the boundary between the cholesterol–lecithin–bile salt micellar phase and the heterogeneous phases possible when excess cholesterol and/or phospholipid are present. The solubility of hydrated cholesterol in this model bile system can be expressed as 5 mole %. The rate of dissolution of the hydrate is in marked contrast to the rapidity of dissolution observed with the anhydrous and coprecipitated forms and even the powdered human gallstone sample. The anhydrous species dissolved quickly and appeared to reach an "equilibrium" solubility of 7 mole % within several hours. The driving force for the dissolution of the anhydrous species must therefore be greater than that of the hydrate, since all other factors relevant to dissolution were maintained constant, *i.e.*, rate of agitation and particle size. The quantity of cholesterol in solution in the "anhydrous system" was monitored over many days and found to remain constant. After a week on this plateau, however, the apparent cholesterol solubility slowly began to fall, eventually converging with that of the hydrated species. Samples of the excess cholesterol removed from the system at this time were found to be hydrated.

The amount of cholesterol in solution following addition of the buffer to the coprecipitate sample reached a plateau within 20 min. This value, 7.5 mole %, higher than that obtained with either of the two crystalline forms of cholesterol, was undoubtedly a result of the ease with which the solvent was able to effect solution of the amorphous cholesterol. The solvent (aqueous buffer) must first act to dissolve the bile salts and lecithin. These materials dissolve instantaneously (observed microscopically) and form micelles. The cholesterol, being essentially molecularly dispersed with the bile salts and lecithin in the coprecipitate, can be rapidly incorporated within the micelles as they form. The coprecipitation process has been employed to induce rapid dissolution and the attainment of high solubility values as an aid to the bioavailability of pharmaceuticals.

A human gallstone sample dissolved more rapidly than the hydrate sample; but unlike the anhydrous or coprecipitated sample, it directly equilibrated at the solubility value of the hydrate. This would seem to imply that its crystalline properties as related to solubility were identical to those of the hydrated system. The more rapid rate of dissolution may be ascribed to particle-size phenomena. Although all samples were screened to provide similar size distributions, it is possible that the gallstone particles containing a mixture of cholesterol and stone constituents actually provided cholesterol with a greater degree of subdivision to the dissolution media.

As with the anhydrous form, the amount of cholesterol in solution following dissolution of the coprecipitate was monitored *versus* time. The time lag for the relief of the supersaturation was not as long for the coprecipitate as for the anhydrous material. This could be due to the greater degree of supersaturation (as compared to the equilibrium hydrated state) in the coprecipitate system. The major driving force in crystal nucleation and growth processes is the degree of supersaturation.

The basic relationships pertaining to the transformation of a

[8] Packard Tri-Carb.
[9] A portion of these data appeared in a preliminary report (27).

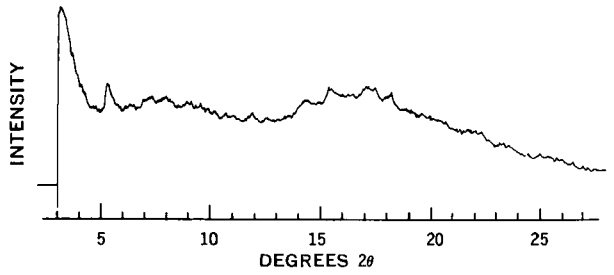

Figure 3—*Diffractogram of coprecipitated cholesterol.*

high activity, supersaturated state to an equilibrium state can be explained by use of Fig. 5. In the upper curve, the quantity of solute is steadily increased above the saturation solubility. A point is eventually reached, where the number of solute molecules is great enough to aggregate spontaneously into a stable cluster, the nucleus, on which crystal growth can proceed. At this point, the critical supersaturation, spontaneous homogeneous nucleation is said to begin, with the system rapidly moving toward equilibrium. Another case can be imagined where the quantity of solute present in a system exceeds the saturation solubility but is below the critical saturation level. Although thermodynamic principles predict that such a system should adjust to the lower energy state, during long periods of observation no such changes may be found; kinetic barriers may be operative which retard the transformation. Such a system is termed metastable. Upon the addition of a suitable catalyst, however, the barriers are lowered and the chemical potential of the system decreases as nucleation and growth commence. Nucleation in this case is termed heterogeneous; in a complex system like bile, nucleation is undoubtedly of this type. A specialized case of heterogeneous nucleation known as epitaxy has been proposed as a mechanism for cholesterol gallstone development (40).

Considering the cholesterol–lecithin–bile salt system, it can be seen that a critical supersaturation must exist above which almost instantaneous precipitation will occur, with lesser supersaturation ratios being potentially lithogenic. Metzger *et al.* (41) suggested the use of a "lithogenic index" as a measure of the relative supersaturation of biles. Metastable systems will be strongly influenced by the presence of "foreign" materials which could act as nucleating sites or conversely as crystal growth inhibitors (42, 43). It is intriguing that in the present model system, where thermodynamic considerations forecast precipitation of cholesterol levels greater than 5 mole %, a supersaturated state could exist for days. It is interesting to speculate that perhaps this time lag to deposition is beneficial in that it gives the body a chance to rid itself of any lithogenic bile during the normal emptying cycles of the gallbladder. However, extended bile residency times such as are present during stasis due to fasting, pregnancy, or vagotomy or as induced physically by viscosity-producing agents in microareas of the gallbladder (44–46) may allow sufficient residence time for the pathogenic bile to deposit cholesterol. During this time, other components can further alter bile composition, increasing the possibilities of deposition (47).

Solubility as Function of Bile Salt–Lecithin Ratio—The finding that physical differences in cholesterol could explain solubility variations between laboratories prompted a more complete study of cholesterol solubility as a function of the bile salt–lecithin ratio. The data are plotted in Fig. 6 on a mole percentage basis. The cross-hatched area within the triangular diagram serves as a guide to the position of the system under study; the rectangular diagram represents the area of primary physiological concern. The tie-line connecting the data points describes the boundary between the clear micellar phase and a multiphase (micellar plus crystalline and/or liquid crystalline) system. The micellar limit in the human physiological range of the bile salt and lecithin can be seen to be about 5 mole %. The significance of this value lies in the fact that many persons considered normal because no crystals of cholesterol or gallstones can be demonstrated in their bile have been found to possess far greater quantities (>5 mole %) of cholesterol in their bile (14, 20, 22–24, 48, 49). In a recent paper (25), the gallbladder bile composition of normal subjects was compared to that of patients with cholesterol gallstones. The control population had no (clinical or radiological)

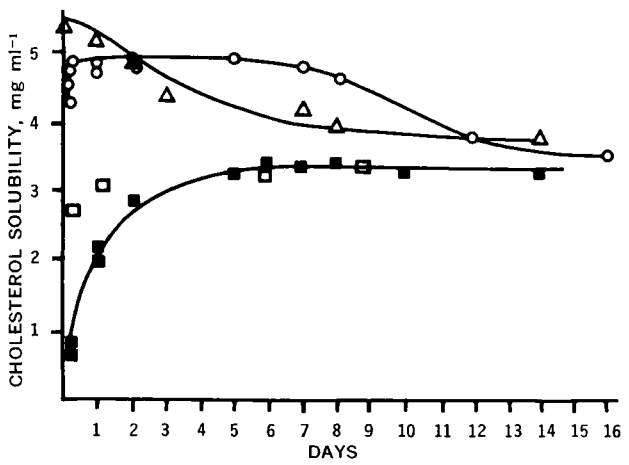

Figure 4—*Dissolution behavior of coprecipitated* (△), *anhydrous* (○), *and hydrated* (■) *cholesterol and of human gallstone* (□) *in model bile system.*

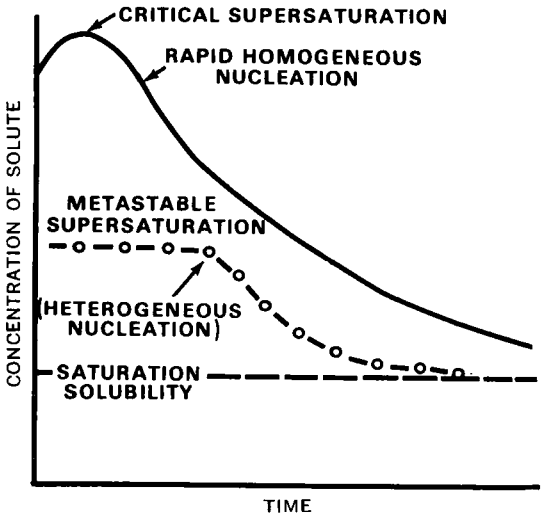

Figure 5—*Plot describing the relationship between degree of supersaturation and precipitation behavior.*

evidence of gallbladder disease. The bile composition of two out of the 12 (16%) control patients was found to be out of the micellar region (>10 mole % cholesterol) as defined by Admirand and Small (19). If the micellar boundary defined in Fig. 6 is used, 75% of these normal patients are found to possess bile supersaturated with respect to cholesterol. Of the patients with cholesterol gallstones, nine out of the 16 (56%) had a bile composition within the micellar zone of Admirand and Small (19) while only one of the 16 (6%) was below the boundary of Fig. 6. Recently, it was reported that normal persons began to secret a lithogenic bile during fasting (50). Thus, lithogenic or metastable bile in man would seem to be the rule rather than the exception. The definition of this lower value for cholesterol solubility prompts some thought into what factors might be responsible for the actual precipitation of cholesterol. The phase diagram serves to inform us as to the value of cholesterol's solubility in a given milieu at equilibrium. It does not take into account the role of other (nonsolubilizing) biliary components which can act to modify the rate of attainment of equilibrium. The importance of such materials on the precipitation behavior of cholesterol awaits further study.

During these studies, the authors became aware of other investigators who were employing similar methodologies (use of crystalline anhydrous cholesterol in model or whole bile systems) and arriving at similar values for the cholesterol-holding capacity of bile. Unlike earlier workers who also employed anhydrous cholesterol, Hegardt and Dam (11) and Holzbach *et al.* (51) presented data that essentially coincide with this study's data. Discrepancies with earlier literature values are undoubtedly due to labora-

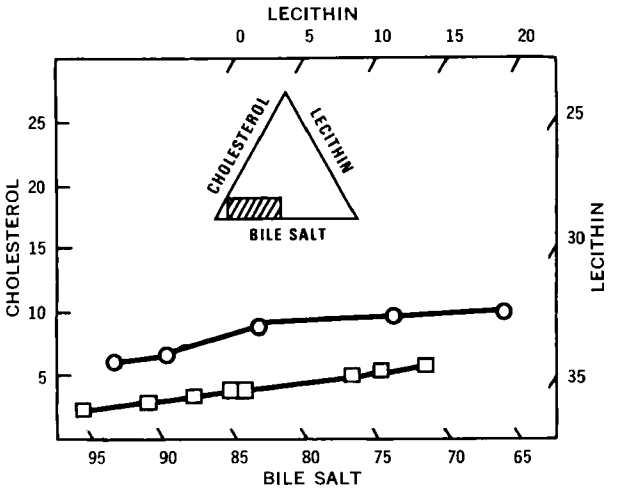

Figure 6—*Equilibrium solubility of cholesterol as a function of lecithin–bile salt ratio* (□) *and the data from Admirand and Small* (*19*) (○).

tory variation in those factors responsible for rate of attainment of equilibrium, *e.g.*, intensity and extent of agitation. It is of importance that three laboratories have independently arrived at similar values.

The application of physical-chemical concepts and methodology to the study of this disease state has been most rewarding. The mechanisms by which gallstones may dissolve was recently reported (52). Chemotherapy of cholelithiasis is becoming a reality (24, 53). Agreement on a value for the equilibrium solubility of cholesterol in bile coupled with the observation that many (most?) nonstone-bearing individuals have a bile that is supersaturated with respect to cholesterol, at least part of the time, points the way for further application of physical-chemical (kinetic) methods to clarify the factors involved in the maintenance of this metastable condition or, additionally, the factors influencing the nucleation and growth of cholesterol crystallites and their aggregation into stones.

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[x] To whom inquiries should be directed.

Drop Size and Initial Dosing Frequency Problems of Topically Applied Ophthalmic Drugs

SUKHBIR S. CHRAI, MICHAEL C. MAKOID, STUART P. ERIKSEN*, and JOSEPH R. ROBINSON×

Abstract □ The amount and rate of drug loss through drainage, for a single drop of topically applied ophthalmic solution, increase with increasing volume of instilled solution. However, the concentration of drug in the precorneal tear film immediately after instillation of drug is higher with larger instilled volumes. Multiple drops administered at short time intervals have the advantage of increasing drug concentration in the precorneal film but the disadvantage of considerable drug loss through drainage, which for potent drugs can lead to systemic toxicity. When using radioactive technetium (^{99m}Tc) as the test substance in unanesthetized albino rabbits, it was shown that a 5-min interval between drops minimizes drainage loss of drug and the drug concentration buildup in the precorneal film as compared to the corresponding dosage regimen of drops administered at shorter time intervals. As the time interval between drops shortens to the point of one drop followed immediately by another drop, both the amount of drug lost through drainage and the tear film concentration of drug increase. Separate administration of two drugs presents a great problem as to dosage regimen. It was shown by radioactive technetium dilution and drainage studies, as well as by aqueous humor drug concentration and miosis studies, that the order of addition of drug to the eye, the volume of solution instilled, and the time of instillation of the first and second drugs all influence the ultimate activity of each drug. In general terms, the first drug administered always suffers a greater loss than the second drug and the degree of loss is proportional to the volume of each drug instilled and the time interval between drops. The implications of the present study to clinical practice are discussed. The multidrug data presented in this report constitute a strong argument for combination drug products whenever multidrug therapy is indicated.

Keyphrases □ Ophthalmic drugs, topically applied—drop size and initial dosing frequency problems, relevance to ophthalmic combination products discussed □ Drop size—considerations relative to optimum size and frequency discussed, topically applied ophthalmic drugs, albino rabbits □ Dosing frequency—considerations relative to topically applied ophthalmic drugs, drop size, relevance to ophthalmic combination products discussed

Dosing schedules for ophthalmic drugs should be based on such factors as intrinsic drug activity, pharmacokinetics of drug disposition, the disease being treated, and the type and method of drug delivery (1–5). The clinician can greatly influence the dosing schedule and, therefore, drug therapy through his or her choice of the drug delivery system and the method of drug delivery. However, understanding of both of these areas for ophthalmic drug delivery systems is minimal so that the clinician must rely on clinical experience to decide on a particular type and method of drug delivery. Unfortunately, this approach may not reveal an optimum drug treatment schedule since it is not possible to examine all variations, particularly when multiple-drug therapy is involved.

The purpose of the present report is to examine some aspects of the ophthalmic dosing regimen for topically applied, single- or multidrug systems. Specifically, it was of interest to investigate the influence of the volume of drug instilled and the time interval between drops on the resulting precorneal tear film concentration of drug as well as the extent of drug loss through solution drainage and tear turnover.

Chrai *et al.* (6) examined the kinetics of instilled solution drainage from the eye of rabbits. They showed that the rate of drainage of an instilled solution is linearly related to the volume instilled. Based on this work, it is reasonable to assume that instillation of multiple drops will result in an increased drainage rate due to a volume buildup. Therefore, excessive loss of instilled drug will occur unless the drops are instilled at time intervals that allow the volume in the eye to return to normal. Drugs instilled at this interval, 5–6 min in the rabbit (6), will reduce the drug loss but should also reduce the resultant tear film concentration of drug. Shorter dosing intervals should result in a reversal of this trend. The dosing picture should become considerably more complicated when two separate drugs are used. The concentration of each drug in the precorneal film as well as the rate of drainage is expected to be a function of the order of drug addition, the volume instilled for each drug, the time between drops, and the initial concentration of each drug.

EXPERIMENTAL

Materials—Water was doubly distilled from alkaline permanganate in an all-glass distillation apparatus. Radioactive technetium (^{99m}Tc) sulfur colloid suspensions were freshly prepared as previously described (6). Pilocarpine nitrate, epinephrine, ^{3}H-pilocarpine nitrate[1] (specific activity 0.44 mCi/mg), and ^{14}C-epinephrine[1] (specific activity 0.32 mCi/mg) were obtained from commercial sources. All other chemicals were reagent or analytical grade.

Methods—Unanesthetized, New Zealand, male, albino rabbits, weighing between 1.8 and 2.4 kg, were employed in all experiments. Experimental animals were placed in restraining boxes, with their heads in a natural position, during the experiments to minimize movement. All animals were conditioned to the experimental procedure by being kept in restraining boxes several hours each day for 7 days prior to an experiment and instilling normal saline into their eyes daily. Each restraining box was a simple wooden receptacle, just large enough to hold the rabbit, with an opening through which the animal's head protruded. All solutions were prepared fresh as outlined earlier (6, 7) and were discarded immediately after the experiment.

Drug or a tracer substance was instilled into the rabbit eye using a microliter syringe[2]. Solutions were instilled against the cornea and collected in the lower cul-de-sac. During instillation, the lower eyelid was extended slightly away from the globe of the eye to form a pouch in which to collect the instilled fluid. Immediately after instillation, the lower eyelid was returned to its normal position and natural movement of the eye and lid was used to mix the instilled solution with tear fluid. Experiments were conducted

[1] New England Nuclear, Boston, Mass.
[2] Hamilton Co., Whittier, Calif.

Table I—Concentration of Drug in the Tear Film after Dilution with Tears as a Function of Volume Instilled

Volume Instilled, μl	Concentration as Percent of Instilled Concentration[a]
5	40
10	57
25	77
50	87
75	91

[a] Assuming the same concentration in all cases.

with either eye of the test animal since earlier studies (6) had not shown any difference in drainage rates between right or left eyes.

Radioactive Technetium Dilution and Drainage Studies—After instillation of the radioactive technetium colloid, the concentration and/or amount of isotope remaining as a function of time were measured as described earlier (6). A minimum of four eyes was used for each experiment, and no animal was used more than once.

Miosis-Time Studies—Methods for the miosis-time studies were described earlier (7). However, in the present study, only unanesthetized animals were used. Twenty-five microliters of 2.5 × 10^{-2} *M* pilocarpine nitrate solution was instilled into the rabbit eye followed by 25 μl of normal saline at an appropriate time interval. A minimum of four eyes in four different experimental animals was used for each time interval. The second eye of each animal served as a control because no animal was used more than once.

Aqueous Humor Drug Concentration Studies—Separate solutions containing 1 × 10^{-2} *M* pilocarpine nitrate and epinephrine hydrochloride were prepared, both with and without the appropriate radioisotope, by addition of drug to a pH 5.5 Sørensen buffer. In addition, two combination drug solutions were prepared in which one drug was radioactive and the other was nonradioactive. For example, a solution containing tritiated pilocarpine nitrate, prepared as already described, was added to a nonradioactive epinephrine hydrochloride solution so that the concentration of each drug was 1 × 10^{-2} *M*. Similarly, a solution containing ^{14}C-epinephrine hydrochloride and nonradioactive pilocarpine nitrate was also prepared. Solution pH was checked and adjusted where necessary (6).

Fifty microliters of a radioisotopic drug solution was instilled into the eye followed in 1 min by 50 μl of the nonisotopic solution containing the other drug. The combination drug solutions were also instilled using 50 μl as the dose volume. By this approach, either tritiated pilocarpine nitrate or ^{14}C-epinephrine hydrochloride could be studied in the presence of the other drug.

Twenty-five minutes postinstillation, the test animal was anesthetized with a solution of sodium pentobarbital. Aqueous humor was withdrawn 30 min after the last drug was instilled. The aqueous humor samples were placed in vials containing Aquasol[1] and were stored for 48 hr to eliminate chemiluminescence. Drug concentrations in aqueous humor were determined by scintillation detection[3], and appropriate blank corrections were made.

Pilocarpine-nitrate-tritium exchange was determined not to be a problem in these experiments. Whether metabolized or intact drug was being measured in aqueous humor was not established.

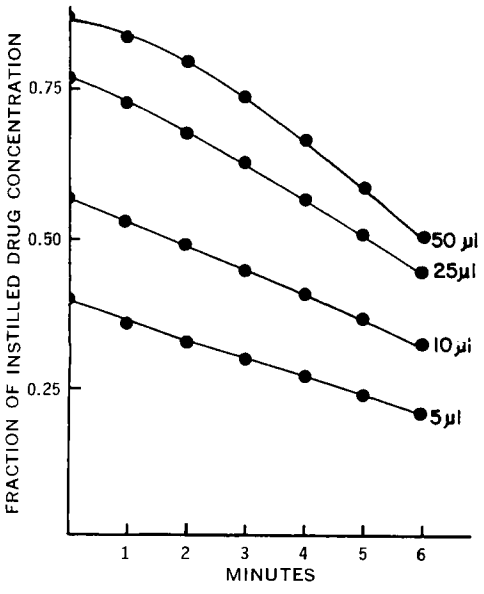

Figure 1—*Calculated concentration of drug in the precorneal film as a function of time for various instilled volumes. Absorption of drug into eye tissues is neglected in these calculations. For calculations, see Footnote a of Table II.*

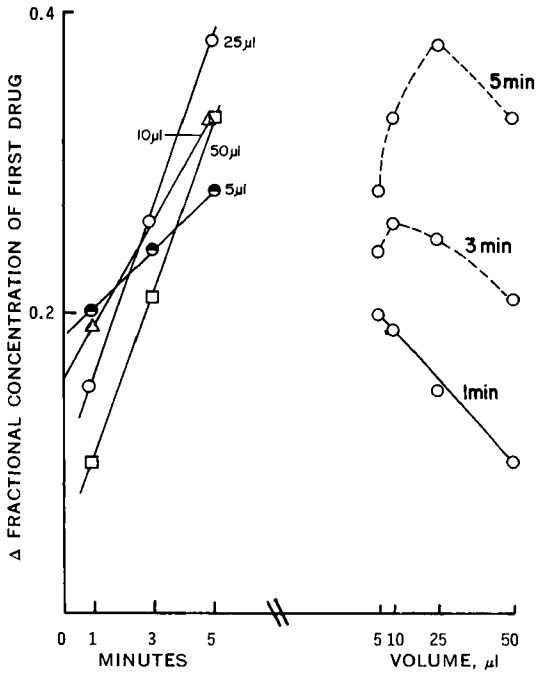

Figure 2—*Gain in fractional concentration of drug in the tear film after instillation of a second drop of drug as a function of time of instillation and volume of the drop instilled. Gain in fractional concentration of the drug is the difference between the fractional concentration before and after the instillation of the second drop.*

RESULTS AND DISCUSSION

Effect of Single and Multiple Drops on Concentration of Drug in Tear Film for a Single Drug—After instillation of a single drop of a drug into the eye, there will be a change in the concentration of the drug due to dilution of the instilled solution by the tears (Table I). The values in Table I are calculated values based on a tear volume of 7.5 μl (6) and neglect the small initial loss of unmixed drug which will occur with the larger instilled volumes (6) and which will cause a further lowering of drug concentration. As expected, the smaller the instilled volume, the greater is the dilution effect when the drug solution mixes with tears.

The tear-drug solution shown in Table I will undergo a further decrease in concentration as time progresses due to drug absorption into various eye tissues and normal tear turnover. Complicating the concentration change picture somewhat is the drainage of instilled solution that commences immediately after instillation of the drug solution into the eye. When assuming that absorption of drug into various eye tissues is negligible and that tear turnover in rabbits is 0.5 μl/min (6), the change in concentration of drug in the precorneal film can be calculated as a function of time. This change is depicted in Fig. 1 for the first 6 min postin-

[3] Packard model 2002 scintillation detector, Packard Instruments, Downers Grove, Ill.

Table II—Calculated Concentration of Drug in the Precorneal Film after Instillation of a Second Drop of Drug Administered at Various Times after the First Drop

Instilled Volume, μl	Time of Instillation of Second Drop, min	Fractional Concentration at Time of Instillation of Second Drop[a,b]	Volume Remaining Immediately after Instillation of Second Drop[c]	Final Fractional Concentration[d]	Percentage Improvement in Concentration
5	0.167[e]	0.40	17.5	0.57	—
	0.25[f]	0.394	17.2	0.57	—
	0.50[g]	0.387	16.9	0.57	—
	1	0.360	15.9	0.56	56
	3	0.30	14.5	0.54	90
	5	0.24	13.6	0.52	117
10	1	0.53	24.8	0.72	38
	3	0.45	21.0	0.71	57
	5	0.37	18.7	0.70	74
25	1	0.73	45.0	0.88	19
	3	0.63	36.9	0.88	39
	5	0.51	33.8	0.87	68
50	0.167[e]	0.87	107.5	0.93	—
	0.25[f]	0.87	101.2	0.93	—
	0.50[g]	0.86	91.7	0.94	—
	1	0.84	78.2	0.94	12
	3	0.74	62.4	0.95	29
	5	0.59	59.4	0.92	55

[a] $C_t = [(V_r - 0.5)C_{t-1}]/(V_r + 0.5)$, for $t \geq 1$, where C_t is the concentration at time t and V_r is the residual volume at time t. [b] Assuming lacrimal turnover rate to be equal to 0.5 μl/min (6). [c] Calculated assuming lacrimal fluid volume to be 7.5 μl (6). [d] $C_f = [(C_t \times V_r) + (C_0 \times V_i)]/(V_r + V_i)$. [e] $C_{0.167} \cong C_0$, where C_0 is the concentration at time zero. [f] $C_{0.25} = [(V_r - 0.125)C_0]/(V_r + 0.125)$. [g] $C_{0.5} = [(V_r - 0.125)C_{0.25}]/(V_r + 0.125)$.

Table III—Concentration of Two Drugs in the Precorneal Film from Administration of Either a Combined Solution or Separate Solutions at Various Times

Time of Instillation of the Second Drug, min	Volume of Drop Instilled, μl	Fractional Concentration of First Drug before Instillation of Second Drug[a]	Fractional Concentration of First Drug after Instillation of Second Drug[b]	Fractional Concentration of Second Drug[c]	Percentage Loss of Concentration of First Drug due to Instillation of Second Drug	Percentage Loss of Concentration of Second Drug due to Presence of First Drug
Both drugs together (same drop)	50	0.87	0.87	0.87	0	0
0		0.87	0.47	0.47	46.0	46.0
1		0.84	0.30	0.64	64.5	26.5
3		0.74	0.15	0.80	80.0	8.5
5		0.59	0.085	0.86	98.5	1.2
Both drugs together	5	0.40	0.40	0.40	0	0
0		0.40	0.29	0.29	27.5	27.5
1		0.36	0.25	0.32	30.6	20.0
3		0.30	0.20	0.35	33.4	12.5
5		0.24	0.15	0.37	37.5	7.5

[a] See Footnote *a* of Table II. [b] $C_f = (C_t \times V_r)/(V_r + V_i)$. [c] $C_f\ (C_0 \times V_i)/(V_r + V_i)$.

stillation. The calculated values were based in part on data reported elsewhere (6).

As can be seen in Fig. 1, the change in drug concentration as a function of time appears to be much larger in the case of larger instilled volumes than with smaller volumes, particularly in the 3–6-min period postinstillation. This observation is opposite to what is expected since tear turnover should have a larger influence on drug concentration in smaller volumes than in larger volumes. If the fraction of drug concentration at zero time for all of the instilled volumes were normalized to one, the influence of tear turnover would appear greater for the smaller instilled volumes. Thus, smaller volumes of drug solution are not only diluted more than larger volumes when placed in the eye but, as expected, they are influenced most by tear turnover.

The precorneal film drug concentration after instillation of two or more drops is a function of the drop size and the time interval between instillations. Calculated drug concentrations in the tear film after the administration of a second drop of drug solution at various times after the first drop are shown in Table II. The values are based on the various rate constants for instilled solution drainage (6), and drug absorption into various eye tissues is assumed negligible for calculation purposes.

Table I shows that there is a smaller dilution effect with larger instilled volumes as compared to smaller volumes. This appears to be the case also with administration of a second drop, as can be seen from the next to last column in Table II, since a higher concentration is generated for the 50-μl drop as compared to the 5-μl drop. However, the percentage improvement in drug concentration, by administering a second drop, is greater for smaller instilled volumes than for larger volumes, as shown in the last column of this table.

The time interval between the first and the second drops influences the final concentration of drug in the precorneal film. This point can be illustrated by plotting the data from Table II as shown in Fig. 2. The left side of this figure, where the improvement in drug concentration from the first to the second drop is plotted *versus* the time interval between drops, shows that the improvement is linearly related to the time interval and that the time of instillation of the second drop for the 5-μl case seems to have a relatively small influence on the final concentration

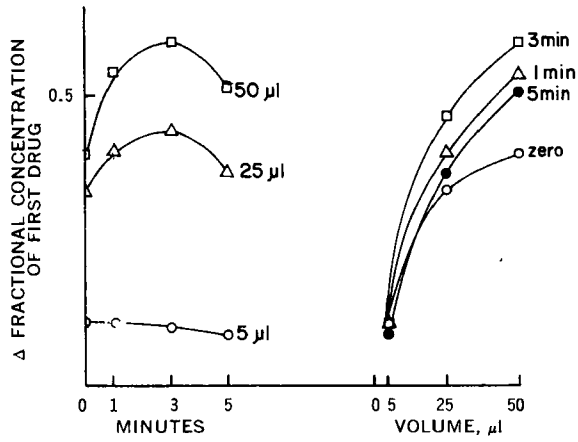

Figure 3—*Loss in fractional concentration of first drug in the precorneal tear film after instillation of a second drug as a function of time of instillation and volume of the drop instilled. Loss in fractional concentration is the difference between the fractional concentration of the first drug before and after instillation of the second drug.*

achieved as compared to the 50-μl drop. The increasing rate of drainage of instilled solution with larger volumes accounts for this change in sensitivity from small to large drops. The maximum improvement observed is a function of both the volume of instillation and the time at which the second drop is instilled, as shown in the right portion of Fig. 2 where improvement in drug concentration is plotted *versus* volume of drug instilled. The maximum improvement, when the second drop is instilled at 5 min, is with a 25-μl drop whereas the 10-μl drop is optimum for a 3-min interval.

It is apparent that two drops are preferable to one drop if elevated precorneal drug concentrations are desired and that large drops, even at a 5-min interval, will generate higher levels than will smaller drops at shorter time intervals. However, a more acceptable approach, based on amount of drug lost to drainage, is to administer smaller drops with a higher initial drug concentration rather than multiple large drops of lower concentration. A severe disadvantage to the multidrop approach is the variation in precorneal concentration that occurs with different time intervals between drops, a problem that exists when the drug solution is administered by the patient and that can lead to variation in efficacy for a particular drug.

Effect of Single and Multiple Drops on Concentration of Drugs in Tear Film for Two Drugs—When two drugs are to be administered in the form of topically applied drops, the concentration of each drug in the precorneal film will follow the results presented earlier for a single drug for the system where both drugs are in the same solution (combination product). The situation becomes considerably more complicated when the drugs are administered as separate solutions.

The concentration of drug in the precorneal film, after instillation of a second drop, was shown in the previous section to be related to the residual volume and concentration of the first drop. It would be expected, therefore, that the order of instillation of two drugs will affect the final concentration of each that is obtained. This can be seen in Table III for a hypothetical drug concentration, using 5- and 50-μl drop sizes and a variable time between instillation of the first and second drugs.

Clearly, the first drug administered will always show a larger decrease in concentration than the second drug. The extent of decrease is a function of the time interval between drops and the volume of solution instilled. In Fig. 3, the fractional concentration loss of the first drug due to instillation of the second drug is plotted as a function of instillation time and instillation volume. As can be seen from the right-hand portion of Fig. 3, the time of instillation of the second drug plays a significant role, particularly for larger instilled volumes. At first glance, it would seem that the extent of loss in fractional concentration is greatest with longer time periods between instillation. However, this is not the case since the line for the 5-min time period is between the 0- and 1-min time lines. The maximum loss in concentration is seen when the second drop is instilled at 3 min. This parabolic relationship is due to the fact that the rate of loss of instilled volume and rate of drug loss are not parallel. The loss in fractional concentration at zero time and 5 min is due principally to dilution, whereas the loss in fractional concentration at time periods between zero and 5 min is due to drainage and dilution.

It can be seen from Table III that smaller drops undergo less of a change in concentration upon instillation of the second drug than do larger drops, in concert with earlier observations on single drugs. Also apparent is the fact that, for any time interval between drops, the percentage loss of concentration of the first drug instilled is larger with larger drops than with smaller drops. The percentage loss of concentration of the second drug instilled is a function of the time interval between drops (Table III). Naturally, this situation does not exist when both drugs are administered together in the same solution.

To verify that changes in precorneal drug concentration can be calculated *a priori*, a study was initiated using radioactive technetium and normal saline to represent two different drugs. The change in radioactive technetium concentration was monitored for the system where 25 μl of technetium solution was instilled into the eye followed by 25 μl of normal saline at different time intervals (Table IV). Experimentally determined values are in good agreement with calculated values, showing that it is possible to calculate concentrations of drug in the precorneal film of unanesthetized rabbits with a good degree of confidence. Naturally, absorption of drug into various eye tissues adds a degree of com-

Table IV—Effect of Instillation of Second Drop of Different Drug on Precorneal Drug Concentration[a]

Time Interval between Drops, min	Fractional Concentration of Technetium (^{99m}Tc)[b]				
	Prior to Instillation of Normal Saline		Postinstillation of Normal Saline		
	Calculated[c]	Calculated[d]	Calculated[c]	Calculated[d]	Experimental[e]
0	0.77	0.66	0.44	0.37	—
1	0.73	0.61	0.32	0.29	0.26
2	0.68	0.55	0.26	0.21	0.22
3	0.63	0.51	0.20	0.18	—
4	0.57	0.47	0.16	0.16	0.18
5	0.51	0.43	0.13	0.15	0.15

[a] Twenty-five-microliter drops were used throughout. [b] Given as fraction of instilled concentration. [c] Calculated on basis of a lacrimal volume of 7.5 μl with tear turnover of 0.5 μl/min and drainage rate of 0.545 min^{-1} (6). [d] Calculated on basis of a lacrimal volume of 13.0 μl as consistent with instillation of 25 μl, as previously explained by Chrai *et al.* (6), with tear turnover of 0.5 μl/min and drainage rate of 0.545 min^{-1}. [e] Minimum of four separate determinations. Standard deviations of reported values were less than 7%. Student's *t* test failed to show a significant difference between calculated and experimental values at the 95% confidence level.

Table V—Calculated Amount of Drug Lost from the Precorneal Portion of the Eye for a Single Drop as a Function of Instilled Volume[a]

Instilled Volume, μl	Time Postinstillation, min	Initial Amount	Amount Lost, units	Percent of Drug Lost
5	1	5	0.625	12.5
	3	5	1.20	24.0
	5	5	1.55	31.0
10	1	10	1.50	15.0
	3	10	3.05	30.5
	5	10	3.95	39.5
25	1	25	12.25	49.0
	3	25	16.00	64.0
	5	25	18.25	73.0
50	1	50	27.0	55.0
	3	50	41.0	82.0
	5	50	43.75	87.5

[a] Assuming no absorption into eye tissues occurs, turnover rate of tears is 0.5 μl/min, and rate constants for drainage are as reported earlier (6).

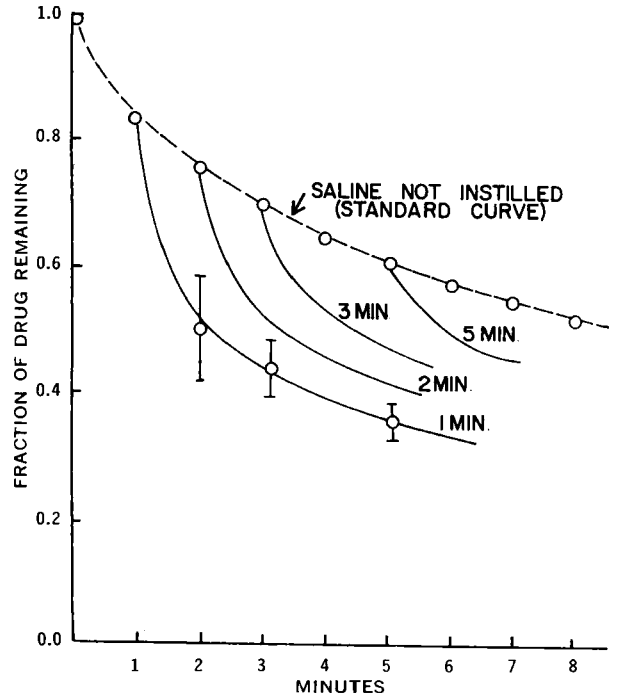

Figure 4—*Change in fractional amount of technetium in the precorneal tear film following instillation of a second drop of normal saline at various times. Volume of the technetium suspension and the normal saline instilled into the eye was 10 μl. The dotted line represents the change in amount of technetium when a second drop has not been administered, and the solid lines represent the change in amount when a drop of normal saline has been instilled. The solid lines are continuous tracings of the change in amount of technetium with time. Representative standard deviations are given at a few points for the 1-min run for the sake of clarity. A minimum of four experiments, in four separate animals, was conducted for each time interval.*

plexity to the calculation but can be accommodated if penetration rates are known.

Effect of Single and Multiple Drops on Amount of Drug in Precorneal Tear Film for Single and Multiple Drugs—As indicated earlier (6), the amount of drug lost from the eye due to drainage increases with increasing instilled volume. Table V gives a comparative loss of drug as a function of instilled volume. The considerable amount of drug lost for the larger instilled volumes can be an important factor for systemic side effects attendant with the use of some ophthalmic drugs (8, 9).

Instillation of a second drop of the same drug or of a different drug increases the volume present in the eye, which will result in a more rapid drainage of the instilled solution. The greater drainage results in a greater loss of drug from the precorneal area. Figure 4 shows the change in amount of technetium in the tear film following instillation of a drop of normal saline at various time intervals.

The rate of loss of drug from the rabbit eye is related to the volume of solution present in the eye at any time. For two drops, the volume present is the residual volume of the first drop plus the volume of the second drop instilled. The rate at which drug is lost from the eye is predictable, irrespective of the number of drops added as long as the volume of each drop and the time interval between drops is known. This is shown in Table VI for the technetium–normal saline system described in Fig. 3. The agreement between calculated and observed drainage rate constants is excellent (Table VI).

Earlier portions of this report described precorneal concentrations of drug from different dosing regimens and concluded that it was preferable to instill large drops rather than small drops to minimize the initial dilution effect and that two drops of a drug solution would give a larger precorneal concentration of drug than would one drop. However, based on the amount of drug lost, when

Table VI—Effect of Instillation of a Second Drop on Drainage Rate Constant[a]

Time	Extra Volume in Eye upon Instillation of Second Drop, μl[a]	Drainage Rate Constant, min^{-1}	
		Calculated[a,b]	Experimental[b,c]
0	20	0.486	0.486
15 sec	19.1	0.475	
30 sec	18.3	0.466	0.475
1 min	16.9	0.449	
2 min	14.8	0.424	0.425
3 min	13.3	0.406	
4 min	12.3	0.395	
5 min	11.6	0.386	0.378
∞ min	10.0	0.368	0.368

[a] Ten microliters of technetium colloid followed by 10 μl of normal saline at some appropriate time interval. [b] Calculated on the basis of a tear volume of 7.5 μl and a turnover rate of 0.5 μl/min (6). [c] Experimental values are based on at least four separate determinations and are given with a standard deviation of 7% or less. Student's *t* test failed to show a significant difference between calculated and experimental values at the 95% confidence level.

large volumes or multiple volumes are used, it would seem preferable to use a larger concentration of drug in the instilled solution as well as a small volume for instillation, particularly if systemic toxicity is a problem. For example, if the concentration of an ophthalmic solution was raised from 1 to approximately 2.2 units/μl and 5 μl was instilled into the eye, the resulting precorneal drug concentration would be the same as if 50 μl of the 1-unit/μl solution had been used. From a drug loss viewpoint, the amount of drug lost from the 5-μl drop over a 5-min period is 3.4 units whereas the 50-μl drop loses 43.75 units over this same time period.

Aqueous Humor Concentration of Two Drugs Administered Together and Separately as Topical Solution—The driving force for corneal penetration of drug is the concentration existing in the precorneal tear film. If two drugs are administered separately, with a time interval between drops, dilution limits the extent of absorption of both drugs as compared to a combined drug solution.

To verify this, a study was conducted whereby tritiated pilocarpine nitrate and ^{14}C-epinephrine hydrochloride were instilled into rabbit eyes and aqueous humor drug levels were determined

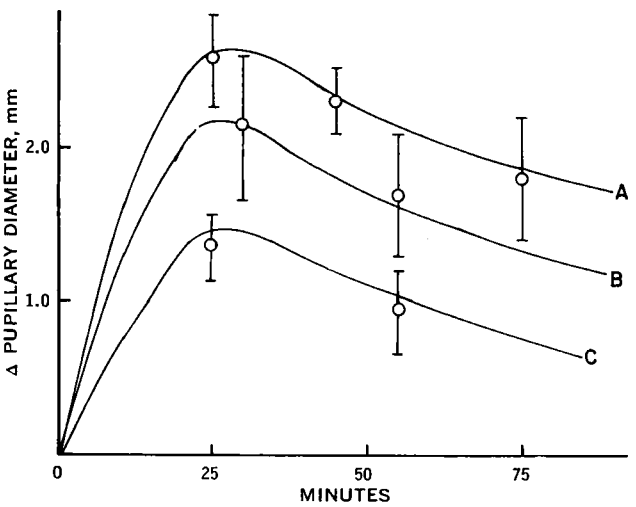

Figure 5—*Change in pupillary diameter as a function of time after instillation of 25 μl of 2.5 × 10^{-2} M pilocarpine nitrate followed by 25 μl of normal saline at various time intervals. The data represent the sum of five separate runs. Vertical lines indicate representative standard deviations at random points on the curve, and each curve was drawn on the basis of 35–40 experimental points. Key: A, drug alone; B, drug followed in 2 min by normal saline; and C, drug followed in 30 sec by normal saline.*

Table VII—Aqueous Humor Concentration of Pilocarpine Nitrate and Epinephrine Hydrochloride Determined 30 min Postinstillation of Combined or Separate Solutions

Dosing Regimen	Pilocarpine Concentration, μg/ml[a]	Relative Bioavailability	Epinephrine Concentration, μg/ml[a]	Relative Bioavailability
Monitoring first drug in series[b]	0.388	0.64	0.016	0.61
Monitoring second drug in series[b]	0.573	0.94	0.024	0.96
Monitoring drug in combination, 1 drop	0.707	1.16	0.026	1.02
Monitoring drug as single drop	0.611	1.00	0.025	1.00

[a] Minimum of three separate determinations with a standard deviation of 8% or less. [b] Fifty microliters of drug solution was instilled 1 min apart; see *Experimental* for details.

30 min postinstillation (Table VII). As expected, the combined product gives considerably higher levels of both drugs as compared to administration of separate solutions. The order of drug instillation, as described earlier and shown in Table III, is important since the first drug administered always suffers a greater loss than the second drug; this is verified in Table VII.

A 1-min time interval between drops was arbitrarily chosen in this study, and it is expected that shorter or longer time intervals would influence the aqueous humor drug levels obtained.

Not shown in Table VII, because only one determination was made, is the schedule where two drops of the combination drug solution were administered with a 1-min time interval between drops. As expected, the aqueous humor level of drug obtained from this treatment is higher than with the administration of one drop of the combination product. This expected behavior was described earlier and was shown in Table II, in which a higher precorneal concentration of drug was obtained by administration of two drops.

Miosis-Time Studies—To illustrate the effect of separate drops of two drugs on the bioavailability of drug, pilocarpine nitrate and normal saline were selected to represent the two drugs and the miosis-time effect of pilocarpine nitrate was monitored (10). Pilocarpine nitrate solution was instilled into rabbit eyes, followed at appropriate times by instillation of normal saline. Measurements of pupillary diameter as a function of time were made (Fig. 5).

As already discussed, concurrent administration of two solutions into the eye reduces the precorneal drug concentration of both drugs and, hence, reduces the apparent biological activity of the drugs. Curves B and C in Fig. 5 illustrate this effect. For time intervals between drops longer than 5 min, there was no modifying influence on miotic activity of pilocarpine nitrate. Since the resident volume from the first drop returns to normal within 5 min in the rabbit (6), this effect was expected.

All the work presented in this report dealt with albino rabbits as the experimental animal. Understanding of the physicochemical processes that occur in the precorneal portion of the rabbit eye is admittedly minimal, and thus extrapolation of the present data is done with caution. Nevertheless, it is presumed that tear and instilled fluid dynamics of the rabbit are proportional to humans, with humans having a larger tear turnover rate (11) and more rapid drainage rate of instilled solutions. This would mean that the various effects discussed in this report would be magnified when applied to humans.

With regard to qualitative application of these results to clinical therapy, several observations can be made. The use of a large as opposed to a small drop will result in a higher precorneal concentration of drug; however, toxicity can be a problem. It would appear more advisable to raise the concentration of the drug in the instilling solution and use a much smaller drop. At present, most commercial ophthalmic droppers deliver from 40 to 70 μl of drug solution and it is suggested that a 5- or 10-μl drop containing a larger concentration of drug would be more appropriate.

Two drops of a single drug will raise the precorneal concentration over that of a single drop. This effect, however, does not justify the use of two or more drops for the simple reason that the increase in precorneal concentration due to two or more drops will never exceed the precorneal concentration due to a single drop of a correspondingly higher concentration, while at the same time it will always exceed the single drop in amount of drug lost through drainage or spillage on the cheek. It would also appear that for large drops the use of three or more drops given consecutively or at short time intervals serves little purpose since most of the drug solution will either spill onto the cheek or be rapidly drained. The slight increase in precorneal drug concentration achieved by the third of fourth drop is minimal.

Spacing of drops by 5 min in rabbits and presumably a slightly shorter period in humans will reduce the drug loss problem but will also reduce the precorneal drug concentration over this time period. A more acceptable approach, as indicated earlier, is to reduce drop size and raise the concentration of drug in the drop to be instilled.

When two or more drugs are indicated for therapy, the results presented in this report constitute a strong argument for a single combination product rather than separate solutions.

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* Present address: Allergan Pharmaceuticals, Irvine, CA 92664

x To whom inquiries should be directed.

Influence of Dispersion Method on Dissolution Rate and Bioavailability of Digoxin from Triturations and Compressed Tablets II

NAVNIT SHAH, RICHARD PYTELEWSKI, HENRY EISEN, and CHARLES I. JAROWSKI[x]

Abstract □ Ball milling or muller milling digoxin with a 20-fold excess of lactose, sucrose, calcium phosphate dibasic, or microcrystalline cellulose significantly enhanced its dissolution rate. Tablets prepared with such triturations also exhibited superior rates of dissolution. Those prepared with the digoxin-lactose trituration gave plasma levels in rats that were 89.6% of the values from an oral solution of digoxin. A simple blend of unmilled digoxin with lactose yielded tablets producing plasma levels only 80% as high. Excellent content uniformity was characteristic for all triturations and tablets prepared by ball or muller milling.

Keyphrases □ Digoxin—effects of ball milling and muller milling on dissolution and bioavailability from triturations and compressed tablets □ Dissolution rates—digoxin triturations and compressed tablets prepared by ball milling or muller milling □ Bioavailability—digoxin triturations and compressed tablets prepared by ball milling or muller milling □ Dispersion methods—effects of ball milling and muller milling on dissolution and bioavailability of digoxin triturations and compressed tablets □ Milling—effects on dissolution and bioavailability of digoxin triturations and compressed tablets

The variation in bioavailability of digoxin from tablet dosage forms was recently reported (1–5). Such variations were also found in different lots of the formulated product made by the same manufacturer (1, 6, 7). Several poorly absorbed preparations were reported (1–4) to be therapeutically ineffective or associated with digoxin levels below the therapeutic range. The hydrophobicity and poor aqueous solubility of digoxin account to a significant degree for these variations.

Excellent correlation of digoxin tablet dissolution rate with biological availability in humans was demonstrated by Lindenbaum *et al.* (8). These authors recommended that a lower limit of acceptability for the dissolution rate be established for digoxin to be used in patients.

In an earlier report (9), it was shown that the application of frictional force to digoxin–lactose or hydrocortisone–lactose blends produced 1:20 triturations with markedly improved dissolution rates. Thus, for example, at the end of 60 min, 91.7 ± 3.5% of digoxin had dissolved in simulated gastric fluid whereas a simple blend of 325-mesh digoxin and powdered lactose exhibited 65.4 ± 5.2% dissolution at the end of 120 min. The application of frictional force was carried out with a mortar and pestle.

This paper describes the use of a ball mill and a muller to prepare 1:20 lactose, sucrose, calcium phosphate dibasic, and microcrystalline cellulose triturations possessing excellent dissolution rates. Tablets prepared from the four triturations by wet and dry methods also exhibited rapid rates of digoxin dissolution. Excellent correlation of digoxin dissolution from the tablet dosage form and bioavailability is demonstrated. Plasma digoxin levels of fasting rats after oral administration of tablets and a solution were used as the basis for comparison.

EXPERIMENTAL

Materials—The following were obtained from commercial sources: digoxin[1,2] USP; lactose[3] USP; lactose[4], spray-dried; sucrose[3] USP; calcium phosphate[5] NF, dibasic, anhydrous; microcrystalline cellulose[6] NF; sodium chloride[7] USP; hydrochloric acid[5], reagent grade; ethanol[5], absolute; acetone[5], reagent grade; chloroform[3], reagent grade; potato starch[8]; sodium lauryl sulfate[8]; magnesium stearate[7] USP; digoxin radioimmunoassay kit[9]; and ion-free serum[10].

Equipment—The following pieces of equipment were used: USP XVIII dissolution test basket assembly[11]; temperature-controlled water bath[12]; jarmill, 10.16-cm (4-in.) diameter with 1.27-cm (0.5-in.) diameter porcelain balls[13]; muller, laboratory model[14]; double-beam spectrophotometer[15]; immersion filter, 1.57 × 5.08 cm (0.62 × 2 in.), medium porosity[16]; Swinny adapter (13 mm)[17]; filter paper, 0.45-μm porosity[17]; tablet press, single punch[18]; Carver press[19]; tablet hardness tester[20]; gyrotory shaker[21]; and U.S. standard sieves[22].

Preparation of Drug Triturations—Powdered digoxin[23] had a particle-size range of 40–180 μm (microscopic examination, average of 10 determinations). A portion of the powdered digoxin was reduced in particle size using a mortar and pestle to a 3–45-μm range (microscopic examination).

Manual bottle tumbling of the unmilled digoxin with a 20-fold excess of diluent for 15 min was carried out in preparing the simple blend. The diluents used were: powdered lactose; powdered sucrose; calcium phosphate dibasic, anhydrous; and microcrystalline cellulose. A simple blend of micronized digoxin[24] with powdered lactose was also prepared. The micronized digoxin had a particle-size range of 1–25 μm (microscopic examination).

Solvent deposition consisted of dissolving the digoxin in sufficient 80% ethanol and uniformly wetting a 20-fold excess of powdered diluent contained in a beaker. The mixture was stirred with a magnetic stirrer, and the solvent was evaporated in a stream of air. The powder was then dried at 37° for 24 hr. Passage through a 60-mesh screen to break up any agglomerates and bottle blending to ensure homogeneity completed the operation.

[1] Barrows Pharmacal Co., Inwood, N.Y.
[2] Roussel Corp., New York, N.Y.
[3] Matheson, Coleman and Bell, Norwood, Ohio.
[4] McKesson Foremost, Inc., Bridgeport, Conn.
[5] J. T. Baker Chemical Co., Phillipsburg, N.J.
[6] Avicel, F. M. C. Corp., Newark, Del.
[7] Mallinckrodt Chemical Works, St. Louis, Mo.
[8] Fisher Scientific Co., Fairlawn, N.J.
[9] Schwarz/Mann, Orangeburg, N.Y.
[10] Chem-Varion, Los Angeles, Calif.
[11] Hanson Research Corp., Northridge, Calif.
[12] Precision Scientific Co., Chicago, Ill.
[13] Paul O. Abbe, Inc., Little Falls, N.J.
[14] Science Machine Shop, St. John's University, Jamaica, N.Y.
[15] Coleman Hitachi 124, Coleman Instrument Corp., Maywood, Ill.
[16] Arthur H. Thomas Co., Philadelphia, Pa.
[17] Millipore Corp., Bedford, Mass.
[18] Stokes, Pennsalt Chemicals Corp., Union, N.J.
[19] Fred S. Carver Inc., Summit, N.J.
[20] Strong Cobb Arner Inc., Cleveland, Ohio.
[21] New Brunswick Scientific Co., New Brunswick, N.J.
[22] Dual Manufacturing Co., Chicago, Ill.
[23] Barrows.
[24] Roussel.

Table I—Dissolution of Digoxin and Digoxin Triturations in Simulated Gastric Fluid at 37°

Minutes	Sample (Processing Method)	Percentage Dissolved				
		No Diluent	Lactose	Sucrose	Calcium Phosphate Dibasic	Microcrystalline Cellulose
15	Pure drug, unmilled	26.6	—	—	—	—
	Pure drug, pestle ground	28.5	—	—	—	—
	Pure drug, micronized (simple blend)	—	50.0	—	—	—
	Trituration (simple blend)	—	32.0	30.0	29.3	26.6
	Trituration (solvent deposited)	—	45.8	42.8	40.1	40.1
	Trituration (ball milled)	—	85.0	67.5	66.5	60.5
	Trituration (muller milled)	—	72.0	64.0	58.5	45.5
30	Pure drug, unmilled	31.8				
	Pure drug, pestle ground	35.3	—	—	—	—
	Pure drug, micronized (simple blend)	—	59.0	—	—	—
	Trituration (simple blend)	—	38.3	43.0	35.2	32.6
	Trituration (solvent deposited)	—	49.2	48.0	43.3	43.3
	Trituration (ball milled)	—	90.0	78.8	73.0	70.8
	Trituration (muller milled)	—	86.5	72.0	70.7	59.5
45	Pure drug, unmilled	34.4	—	—	—	—
	Pure drug, pestle ground	41.5	—	—	—	—
	Pure drug, micronized (simple blend)	—	64.0	—	—	—
	Trituration (simple blend)	—	44.0	47.0	38.7	36.0
	Trituration (solvent deposited)	—	58.1	52.9	50.0	50.0
	Trituration (ball milled)	—	94.5	86.0	82.0	75.0
	Trituration (muller milled)	—	92.0	81.0	75.0	69.5
60	Pure drug, unmilled	39.4	—	—	—	—
	Pure drug, pestle ground	45.3	—	—	—	—
	Pure drug, micronized (simple blend)	—	66.0	—	—	—
	Trituration (simple blend)	—	50.5	51.0	44.9	42.2
	Trituration (solvent deposited)	—	62.3	59.6	56.6	54.0
	Trituration (ball milled)	—	97.0	90.9	87.5	81.0
	Trituration (muller milled)	—	95.0	85.5	85.0	76.0
90	Pure drug, unmilled	44.5	—	—	—	—
	Pure drug, pestle ground	59.5	—	—	—	—
	Pure drug, micronized (simple blend)	—	68.0	—	—	—
	Trituration (simple blend)	—	56.0	54.8	51.3	50.8
	Trituration (solvent deposited)	—	66.6	66.3	63.3	58.0
	Trituration (ball milled)	—	99.0	95.7	92.0	88.7
	Trituration (muller milled)	—	98.5	92.5	90.0	89.0
120	Pure drug, unmilled	52.0	—	—	—	—
	Pure drug, pestle ground	66.0	—	—	—	—
	Pure drug, micronized (simple blend)	—	68.0	—	—	—
	Trituration (simple blend)	—	62.5	58.5	58.0	57.7
	Trituration (solvent deposited)	—	70.9	70.5	67.3	67.3

Table I—(*Continued*)

Minutes	Sample (Processing Method)	Percentage Dissolved				
		No Diluent	Lactose	Sucrose	Calcium Phosphate Dibasic	Microcrystalline Cellulose
	Trituration (ball milled)	—	100.3	100.3	97.5	95.0
	Trituration (muller milled)	—	99.5	97.0	94.5	93.5

Ball-milled triturations were prepared as follows. Digoxin powder and a 20-fold excess of the various diluents were blended and transferred to a ball mill jar. The jar was half filled with 1.27-cm (0.5-in.) porcelain balls. The sealed jar was set in motion on motor-driven rollers. Samples were removed at various time intervals to establish the optimal grinding time. Ball milling for 48 hr was found to be ideal, since no improvement in digoxin dissolution rate was obtained after an additional 12 hr of milling.

Muller-milled triturations were prepared in a similar manner. The laboratory muller was more efficient than the ball mill, since only 6 hr of muller milling was required to duplicate the dissolution rate of digoxin–lactose triturations ball milled for 48 hr.

Preparation of Digoxin Tablets (0.25 mg) Using Various Triturations—Wet and dry methods were used in preparing the following tablet (quantities for one tablet): *(a)* digoxin trituration, 5.25 mg; *(b)* spray-dried lactose, 55 mg; *(c)* sucrose powder, 55 mg; *(d)* potato starch, 8.5 mg; *(e)* magnesium stearate, 1.125 mg; and *(f)* sodium lauryl sulfate, 0.125 mg. The weight of one tablet was 125 mg.

The triturations (5.25 mg) were prepared by simple blending, solvent deposition, ball milling, and muller milling. They consisted of 0.25 mg of digoxin and 5 mg of diluent (lactose; sucrose; calcium phosphate dibasic, anhydrous; or microcrystalline cellulose). By the dry method, a blend of *(b)*, *(c)*, and *(d)* was prepared; *(a)* was diluted with the blend by geometric dilution. A mixture of *(e)* and *(f)* was separately prepared, and an appropriate quantity was added to the remaining ingredients. After blending to ensure homogeneity, the powder mixture was compressed on a single-punch tablet machine using 0.48-cm (0.19-in.) square punches.

Chloroform was used as the granulating solvent in converting the entire series of triturations into tablets by the wet method. Ingredients *(b)*, *(c)*, and *(d)* were blended; *(a)* was then incorporated by geometric dilution. Sufficient chloroform was added to wet the mixture of powders. The wet mass was passed through a 10-mesh sieve to form granules, and the granules were dried at 37° for 24 hr. The dried granules were passed through a 40-mesh sieve, and the mixed lubricant [blend of *(e)* and *(f)*] was added. After manual bottle tumbling the mixture for 10 min, the blend of powders was compressed into tablets.

Absolute ethanol was also used as a granulating solvent in converting the ball-milled lactose trituration into tablets using the standard formula already described.

Tablet hardness, by the wet or dry methods, ranged from 4 to 6. The disintegration rates were less than 5 min.

Preparation of Digoxin Tablets (0.067 mg) for Blood Level Studies in Rats—Smaller digoxin tablets, weighing 0.0283 ± 0.002 g [0.32-cm (0.125-in.) diameter], were prepared to facilitate oral administration to rats. They were prepared with a Carver press by direct compression at a force of 509.4 kg (1100 lb). These tablets represented an aliquot of the blend for the 125-mg tablet. Their content uniformity did not deviate outside the range of 100 ± 5%.

One batch of tablets used the 48-hr ball-milled digoxin–lactose trituration. The second batch used a trituration prepared as a simple blend of powdered digoxin and lactose. Tablet hardness for either batch ranged from 4 to 7. Their disintegration rates were uniformly less than 3 min.

Spectrophotometric Absorption and Calibration Curves for Digoxin in Simulated Gastric Fluid—Twenty milligrams of digoxin was dissolved in 10 ml of hot 80% ethanol. Sufficient simulated gastric fluid without pepsin was added to make 1 liter. This solution was used to determine absorption spectral and calibration curves. The concentrations of digoxin in the dissolution study obey Beer's law at 224 nm. Incorporation of a 20-fold excess of the four diluents did not alter the absorbance values for digoxin.

Dissolution Studies—A modification of the procedure of Monkhouse and Lach (10) was used to determine the dissolution rates of the various samples of digoxin and its triturations. This procedure was described in an earlier publication (9). It was established that a saturated solution of digoxin in simulated gastric fluid contained 188.8 mg/200 ml (shaking for 13 days at 37°). Sink conditions were maintained throughout the dissolution test since only 4 mg of digoxin was added to 200 ml of simulated gastric fluid.

The procedure for determining the dissolution rate of digoxin from the various tablet formulas was identical to that used for the triturations with two exceptions. First, accurately weighed tablets equivalent to 4 mg of digoxin were placed into the USP dissolution basket at zero time. Second, it was not always possible to use simulated gastric fluid as the blank, since the large excess (greater than 20-fold) of inert ingredients affected the digoxin absorbance values. In such instances, placebo tablets containing an identical quantity of inert ingredients were added to simulated gastric fluid. The filtrate was used as the blank.

The dissolution data are summarized in Tables I and II.

Plasma Levels of Digoxin in Sprague-Dawley Rats—Three groups of six 10-week-old, female Sprague-Dawley rats (weight range 214 ± 8.4 g) were fasted overnight and orally dosed with 0.067 mg of digoxin. The drug was administered as an aqueous solution (1 ml) or as compressed tablets. One milliliter of water was administered after each tablet.

Blood samples were drawn into heparinized glass capillaries from the rats' tails after 0.25, 0.50, 1, and 2 hr. A 4-hr sample was drawn into a heparinized syringe after cardiac puncture. After centrifugation to remove the red blood cells, the samples were diluted with ion-free serum to ensure that the readings would be in the linear portion of the standard curve. Digoxin plasma levels were determined by a radioimmunoassay procedure using ^{125}I as the marker (11). The assay data are summarized in Table III.

RESULTS AND DISCUSSION

In Table I, the digoxin dissolution from triturations is summarized. After 120 min, the extent of dissolution of milled and unmilled digoxin is 66 and 52%, respectively. A simple blend of micronized digoxin and lactose shows 68% dissolution after 120 min. The poor performance of the milled and micronized drug is not unexpected. Lin *et al.* (12) demonstrated that the *in vitro* dissolution of micronized griseofulvin and glutethimide was slower than that of coarser particles. Poor wettability and the aggregation of fine particles most likely account for these observations.

The dissolution of digoxin from solvent-deposited triturations was not much faster. Thus, the digoxin–lactose trituration prepared by solvent deposition was only 70.9% dissolved at 120 min. The superiority of ball-milled or muller-milled triturations is clearly evident. All four diluents exhibit greater than 93.5% drug dissolution within 120 min. The soluble diluents, lactose and sucrose, release the digoxin more rapidly than the insoluble ones. Of the four, powdered lactose proved to be the best. Within 15 min, 85% of the digoxin from the ball-milled lactose trituration had dissolved.

From the data in Table II, a number of generalizations can be made on the dissolution of digoxin from tablets prepared from the various triturations.

Tablets Prepared from Triturations Made by Simple Blending—The dissolution rates of digoxin from tablets prepared by di-

Table II—Dissolution Rate of Digoxin in Simulated Gastric Fluid at 37° from Tablet Dosage Forms

Minutes	Trituration Sample and Processing Method	Percentage Dissolved			
		Lactose	Sucrose	Calcium Phosphate Dibasic	Microcrystalline Cellulose
15	Simple blend, dry	32.0	37.2	32.1	29.3
	Solvent deposition, dry	48.1	44.2	40.1	40.1
	Ball milled, dry	77.5	72.1	69.5	62.0
	Muller milled, dry	64.1	58.8	48.1	42.7
	Simple blend, wet (chloroform)	37.4	34.7	40.1	32.1
	Solvent deposition, wet (chloroform)	53.4	48.2	45.7	42.7
	Ball milled, wet (chloroform)	64.2	63.6	53.4	53.4
	Ball milled, wet (ethanol)	64.0	61.0	53.4	53.4
	Muller milled, wet (chloroform)	64.0	58.8	48.1	42.7
30	Simple blend, dry	35.2	40.9	35.2	35.2
	Solvent deposition, dry	54.5	46.5	46.5	43.2
	Ball milled, dry	87.4	84.2	79.1	74.0
	Muller milled, dry	78.6	70.1	56.9	67.0
	Simple blend, wet (chloroform)	43.5	40.9	41.5	35.5
	Solvent deposition, wet (chloroform)	60.2	49.1	49.1	46.5
	Ball milled, wet (chloroform)	73.7	76.4	78.8	62.8
	Ball milled, wet (ethanol)	79.2	76.2	76.2	76.0
	Muller milled, wet (chloroform)	79.0	68.1	67.9	65.5
45	Simple blend, dry	41.7	44.6	41.5	38.7
	Solvent deposition, dry	58.5	50.0	50.0	47.2
	Ball milled, dry	92.4	90.0	86.2	80.4
	Muller milled, dry	85.7	80.2	69.2	74.8
	Simple blend, wet (chloroform)	47.3	44.6	46.3	39.0
	Solvent deposition, wet (chloroform)	64.2	58.2	58.1	50.0
	Ball milled, wet (chloroform)	78.1	81.0	83.4	77.5
	Ball milled, wet (ethanol)	83.6	83.4	80.7	83.2
	Muller milled, wet (chloroform)	83.5	80.5	74.8	72.4
60	Simple blend, dry	50.8	48.3	45.2	44.9
	Solvent deposition, dry	65.5	54.0	53.7	54.0
	Ball milled, dry	96.2	94.1	91.1	85.2
	Muller milled, dry	93.0	85.0	73.5	81.8
	Simple blend, wet (chloroform)	51.5	48.3	48.6	42.5
	Solvent deposition, wet (chloroform)	68.4	64.9	62.2	56.6
	Ball milled, wet (chloroform)	85.6	90.0	87.9	82.1
	Ball milled, wet (ethanol)	85.5	85.5	85.2	85.2
	Muller milled, wet (chloroform)	84.9	95.2	82.1	79.6
90	Simple blend, dry	54.5	52.1	49.3	51.3
	Solvent deposition, dry	69.2	62.5	60.9	60.5
	Ball milled, dry	97.5	96.5	95.9	92.7
	Muller milled, dry	98.0	92.4	80.4	86.6
	Simple blend, wet (chloroform)	52.4	52.1	49.7	46.2
	Solvent deposition, wet (chloroform)	72.7	71.8	66.5	60.6
	Ball milled, wet (chloroform)	92.7	90.1	92.7	86.6
	Ball milled, wet (ethanol)	87.4	87.4	87.1	87.0
	Muller milled, wet (chloroform)	90.3	89.8	86.6	84.2
120	Simple blend, dry	58.5	58.5	50.0	51.6
	Solvent deposition, dry	72.7	70.1	67.5	67.5

Table II—(*Continued*)

Minutes	Trituration Sample and Processing Method	Percentage Dissolved			
		Lactose	Sucrose	Calcium Phosphate Dibasic	Microcrystalline Cellulose
	Ball milled, dry	100.8	98.5	97.5	95.0
	Muller milled, dry	98.0	94.5	91.5	91.1
	Simple blend, wet (chloroform)	53.5	53.2	50.8	47.2
	Solvent deposition, wet (chloroform)	74.0	72.0	70.8	66.3
	Ball milled, wet (chloroform)	97.5	95.1	94.9	91.5
	Ball milled, wet (ethanol)	89.5	89.5	89.4	89.3
	Muller milled, wet (chloroform)	98.0	94.6	91.4	91.4

Table III—Digoxin Plasma Levels in Sprague–Dawley Rats after Oral Administration of an Aqueous Solution and Tablets[a]

Hours	Concentration, ng/ml		
	Aqueous Solution (1 ml)	Tablet Prepared from Simple Blend Trituration	Tablet Prepared from Ball-Milled Tritur tion
0.25	34.8 ± 2.2	22.6 ± 2.3	25.1 ± 2.2
0.50	46.2 ± 3.1	37.1 ± 0.6	41.4 ± 3.1
1	29.4 ± 2.0	20.9 ± 2.1	25.5 ± 2.1
2	25.4 ± 2.9	18.2 ± 1.2	20.9 ± 1.7
4	21.9 ± 2.2	16.5 ± 1.0	19.5 ± 1.2

[a] Dose administered was 0.067 mg.

rect compression were slightly faster than from triturations prepared by simple blending. Digoxin tablets prepared by the wet method exhibited faster dissolution of drug than those prepared by the dry procedure.

Tablets Prepared from Solvent-Deposited Triturations—Tablets prepared by the wet or dry procedures exhibited digoxin dissolution rates as good as those for the four solvent-deposited triturations used in their preparation. Within 120 min, the percentage of digoxin dissolved was similar for the four triturations and the eight tablets.

Tablets Prepared from Ball-Milled and Muller-Milled Triturations—Digoxin tablets prepared from ball-milled and muller-milled triturations were clearly superior to those using triturations prepared by simple blending or solvent deposition. At each time interval, tablets prepared with ball-milled or muller-milled triturations exhibited greater dissolution of drug. As might be expected, the soluble diluents, lactose and sucrose, yielded ball-milled and muller-milled triturations and compressed tablets with the fastest dissolution rates.

The content uniformity of the various tablets and triturations was never a problem. Replicate assays rarely fell outside the range of 100 ± 5%.

The drug plasma levels attained in fasting rats after oral administration of 0.067 mg of digoxin as an aqueous solution or as compressed tablets are summarized in Table III. Figure 1 presents a logarithmic plot of the data in Table I. The points are experimental, whereas the lines are drawn from predicted values obtained by the least-squares method. Peak plasma levels for the aqueous solution and the two tablet formulas occurred at 0.5 hr. In addition, the slopes of the lines are similar: solution, −0.041; tablet prepared from a trituration prepared by simple blending, −0.032; and tablet prepared from a ball-milled trituration, −0.035. As a consequence of these similarities, oral absorption efficiency of the tablets can be determined from peak plasma levels with a fair degree of accuracy (9). Thus, the oral absorption efficiency of the digoxin tablet containing the simple blend trituration is only 80% that of the aqueous solution, whereas the oral absorption efficiency of the tablet employing the ball-milled trituration is 89.6%. These differences in peak plasma levels were significant in the analyses of variance (solution *versus* tablet with ball-milled trituration, $p < 0.02$; tablet with ball-milled trituration *versus* tablet with simple blend trituration, $p < 0.01$).

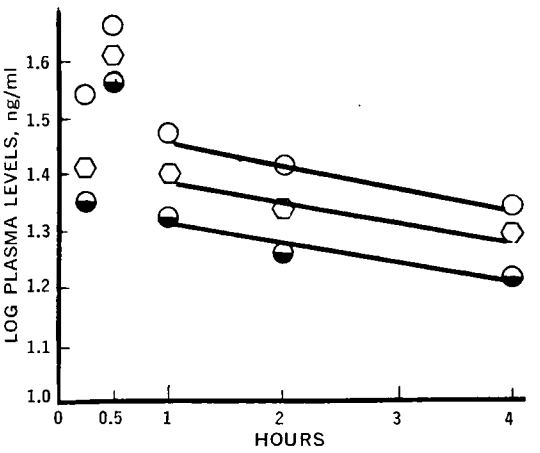

Figure 1—*Digoxin plasma levels in Sprague–Dawley rats after oral administration of an aqueous solution* (○), *tablet prepared from a simple blend of digoxin* (◒), *and tablet prepared from ball-milled digoxin–lactose (1:20)* (○).

Thus, it has been demonstrated in rats that oral absorption efficiency follows *in vitro* dissolution for the two digoxin tablet preparations. The concept of compressing an aliquot of a blend designed for a larger tablet in assessing bioavailability in animals deserves wider application. The excellent content uniformity of triturations and tablets prepared by ball milling a 1:20 blend of digoxin and lactose should simplify the manufacture of digoxin tablets.

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Dissolution Profile of Log-Normal Powders II: Dissolution before Critical Time

DANA BROOKE

Abstract □ The dissolution of log-normal powders, particularly in that period before the smallest particles disappear, was examined. An approximation for the slope of a cube-root law plot was developed for the dissolution before the critical time of ideally distributed powders. Such an approximation does not hold, however, for severely truncated log-normal distributions. The work of previous investigators in this area appears to be incorrect and is discussed.

Keyphrases □ Dissolution profiles—log-normal powders, dissolution before critical time, cube-root law plot approximation □ Powders, dissolution—log-normal distribution profile, dissolution before critical time, cube-root law plot approximation □ Particle dissolution—log-normally distributed powders, dissolution before critical time, cube-root law plot approximation

In their paper on the dissolution of powders that follow the log-normal distribution, Carstensen and Musa (1) attempted to find correlations between the well-known "cube-root law" (2) and their computer-generated dissolution patterns. They offered two approximations to the cube-root law wherein the slopes were stated as functions of the means and standard deviations of the log-normal distributions. One equation dealt with dissolution before the time when the smallest particles disappeared, *i.e.*, before the "critical time." Another equation dealt with dissolution after the critical time.

Brooke (3), in a discussion of exactly calculated dissolution profiles for log-normally distributed powders, reported that the Carstensen and Musa approximation for dissolution before the critical time gave excellent results. Further examination of this subject, however, shows that the approximation is incorrect. The test of the approximation (3) was, in fact, inconclusive.

The present paper examines the time course of dissolution, particularly in that period before a significant number of particles disappear, for log-normally distributed powders varying in standard deviation and extent of truncation. As expected, for narrow distributions the cube-root law holds for dissolution before the critical time. However, one approximation of the cube-root law slope will not apply for all log-normal distributions. An approximation is offered that does hold for powders obeying the ideal log-normal distribution.

THEORETICAL AND CALCULATIONS

The complete derivation of an expression that exactly describes the dissolution of log-normal powders was given by Brooke (3). Briefly, if there is a powder containing spherical particles of diameters a_0 which are distributed, on a numbers basis, so that $\ln a_0$ is normal with mean μ and standard deviation σ, then the probability frequency f of log diameters is:

$$f = (1/\sigma\sqrt{2\pi})e^{-(\ln a_0 - \mu)^2/2\sigma^2} \qquad \text{(Eq. 1)}$$

If such particles dissolve isotropically under sink conditions and if the solubility C_s is independent of particle size, then the diameters a_τ of particles at some function τ of time can be written (1) as:

$$a_\tau = a_0 - \tau \qquad \text{(Eq. 2)}$$

Here τ is $2kC_st/\rho$, where k is a proportionality constant, ρ is particle density, and t is time. The weight w_τ of a powder remaining at any τ is found by the integration:

$$w_\tau = \int(\pi\rho N/6)(a_0 - \tau)^3 f \, d \ln a_0 \qquad \text{(Eq. 3)}$$

where N is the number of particles at time zero. This is somewhat different from the integral in Eq. 6 of the Carstensen and Musa (1) paper.

The limits of integration in Eq. 3 depend on τ and on the original powder distribution. Let i and j be numbers that reflect the extent of truncation at the small particle end and the large particle end, respectively. If the powder is such that all $\ln a_0$ are found between $\mu - i\sigma$ and $\mu + j\sigma$, then for $\ln \tau \leq \mu - i\sigma$, the integration would be from $\mu - i\sigma$ to $\mu + j\sigma$. For $\ln \tau > \mu - i\sigma$, the integration would be from $\ln \tau$ to $\mu + j\sigma$. If $i = j = \infty$, the solution to Eq. 3 becomes Eq. 13 of the previous paper (3).

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Table I—Exactly Calculated Dissolution Profiles for Various Log-Normal Powders[a]

		Ideal Distributions, $i = 10, j = 10$							Truncated Distributions: $i = 2, j = 10$			Truncated Distributions: $i = 1, j = 10$		Truncated Distributions: $i = 2, j = 2$		
τ/M	$\sigma = 0$	0.03	0.05	0.1	0.2	0.3	0.5	0.7	0.03	0.1	0.3	0.1	0.3	0.03	0.1	0.3
0.1	72.9	73.0	73.1	73.5	75.3	78.1	85.0	91.6	73.0	73.6	78.1	74.0	78.6	72.9	73.4	76.8
0.2	51.2	51.3	51.5	52.2	55.3	59.9	72.0	84.0	51.3	52.4	60.0	53.0	60.7	51.3	52.0	57.8
0.3	34.3	34.4	34.6	35.6	39.3	45.2	60.9	77.1	34.5	35.7	45.2	36.4	46.0	34.4	35.3	42.4
0.4	21.6	21.7	21.9	23.0	27.0	33.4	51.4	70.8	21.8	23.1	33.5	23.8	34.2	21.7	22.6	30.3
0.5	12.5	12.6	12.8	13.8	17.7	24.2	43.3	65.2	12.7	13.9	24.3	14.5	24.9	12.6	13.5	21.1
0.6	6.4	6.5	6.7	7.6	11.1	17.3	36.6	60.1	6.5	7.6	17.3	8.1	17.7	6.5	7.3	14.2
0.7	—	—	—	—	6.7	12.2	31.0	55.6	—	—	12.2	—	12.4	—	—	9.4
0.8	—	—	—	—	—	8.7	26.5	51.5	—	—	8.7	—	8.7	—	—	—
1.0	—	—	—	—	—	—	19.6	44.5	—	—	—	—	—	—	—	—
1.5	—	—	—	—	—	—	10.5	32.3	—	—	—	—	—	—	—	—
2.0	—	—	—	—	—	—	—	24.8	—	—	—	—	—	—	—	—
3.0	—	—	—	—	—	—	—	16.5	—	—	—	—	—	—	—	—
4.0	—	—	—	—	—	—	—	12.2	—	—	—	—	—	—	—	—

[a] The tabled values are ($w_\tau \times 100/w_0$).

Table II—Slopes (β) for the Cube-Root Law, Eq. 6, for Various Log-Normal Distributions[a]

σ	i 10 j 10	2 10	1 10	2 2	10 2	10 3	3 3
0.03	0.997	0.996	0.989	0.998	0.999	0.997	0.997
0.05	0.993	0.991	0.981	0.994	0.997	0.993	0.993
0.1	0.974	0.971	0.954	0.980	0.983	0.975	0.974
0.2	0.901	0.898	0.877	0.924	0.927	0.904	0.904
0.3	0.792	0.790	0.772	0.841	0.843	0.800	0.800
0.5	0.526	0.526	0.519	0.643	0.643	0.552	0.552
0.7	0.288	0.288	0.286	0.456	0.456	0.334	0.334

[a] The values of the table are β; i and j indicate extent of truncation.

The general expression for weight fraction undissolved, w_τ/w_0, is based on the derivations of the previous report (3) and the above discussion. If it is remembered (3) that $\mu = \ln M$, where M is the geometric mean diameter, then it can be shown that w_τ/w_0 is:

$$1 - 3(\tau/M)e^{-5\sigma^2/2}\left[\frac{F(j-2\sigma) - F((T-\mu-2\sigma^2)/\sigma)}{F(j-3\sigma) - F(-i-3\sigma)}\right] + 3(\tau/M)^2 e^{-4\sigma^2}\left[\frac{F(j-\sigma) - F((T-\mu-\sigma^2)/\sigma)}{F(j-3\sigma) - F(-i-3\sigma)}\right] - (\tau/M)^3 e^{-9\sigma^2/2}\left[\frac{F(j) - F((T-\mu)/\sigma)}{F(j-3\sigma) - F(-i-3\sigma)}\right] \quad \text{(Eq. 4)}$$

where $F(\ )$ is the area under a standard normal curve from $-\infty$ to the stated argument, and T is defined by $T = \mu - i\sigma$ when $\ln \tau \leq \mu - i\sigma$, or $T = \ln \tau$ when $\ln \tau > \mu - i\sigma$.

For any set σ, i, and j, all dissolution profiles will be identical if considered on a w_τ/w_0 *versus* τ/M basis. When T in Eq. 4 is $\mu - i\sigma$ the term μ disappears from the equation. When $T = \ln \tau$, then every $T - \mu$ can be expressed as $\ln(\tau/M)$.

Dissolution profiles for different log-normally distributed powders were calculated according to Eq. 4 using a computer[1]. Each $F(\ )$ term was evaluated by numerical methods. Values so calculated compared with tabled values for the standard normal distribution to the fourth significant figure. Several computer results for w_τ/w_0 were checked for correctness by independent calculations. All values w_τ/w_0 were calculated to four significant figures and rounded to three figures in this report to conserve space.

RESULTS AND DISCUSSION

Exactly calculated dissolution profiles for hypothetical log-normal powders variously truncated and having standard deviations ranging from 0.03 to 0.7 are presented in Table I as weight percent undissolved ($100 \times w_\tau/w_0$) *versus* τ/M. In the strictest sense, no profiles were calculated for "ideal" distributions, *i.e.*, where $-\infty < \ln a_0 < +\infty$. Practically speaking, however, profiles for distributions where $i = j = 10$ should be considered ideal.

[1] IBM 1800.

In the previous paper (3), Brooke indicated how to calculate w_τ/w_0 for distributions truncated at the large particle end. Instead of treating a truncation at the small particle end, Brooke suggested that truncation effects at the small end could, in many cases, be ignored without introducing large errors. Comparisons of dissolution patterns (Table I) of distributions that are "ideal" at the small end ($i = 10$) with those for similar distributions truncated at the small end ($i = 1, 2, \ldots$) show that this is generally true. The difference between values of w_τ/w_0 for an ideal distribution and for a truncated ($i = 2$) distribution is less than 1% through 95% of the profile. The differences between w_τ/w_0 for an ideal *versus* a truncated distribution with $i = 1$ runs to about 6.6%. For $\sigma \geq 0.3$, differences tend to be negligible. Distributions truncated so that $i = 3$ could be treated as ideal at the small end with errors much smaller than 1%.

The dissolution pattern for a log-normal distribution with $\sigma = 0$ is given in Table I. For $\sigma = 0$, Eq. 4 becomes:

$$w_\tau/w_0 = [1 - (\tau/M)]^3 \quad \text{(Eq. 5)}$$

which is the equation for a monosized distribution of particles of diameter M. The profile so calculated comes close to, but slightly underestimates, the weight fraction remaining through 80 or 90% of the profile for powders having standard deviations as large as 0.1. Thus, for many log-normal distributions with small σ, including all of those treated by Carstensen and Musa (1), it may be more desirable to approximate dissolution patterns quickly by Eq. 5 and to accept the generally modest errors that result than to calculate tediously such profiles exactly.

To treat the dissolution before the critical time of log-normal powders, the initial portions of profiles like those of Table I were fitted to a modified cube-root law equation given by:

$$1 - (w_\tau/w_0)^{1/3} = \beta(\tau/M) \quad \text{(Eq. 6)}$$

Here β is the slope of a linear plot of $1 - (w_\tau/w_0)^{1/3}$ *versus* (τ/M). Carstensen and Musa (1) used the more classical form of the cube-root law shown below:

$$w_0^{1/3} - w_\tau^{1/3} = \alpha\tau \quad \text{(Eq. 7)}$$

Table III—Least-Squares Regression Data for Plots of ln β *versus* σ^2 Based on Values in Table II[a]

	i 10 j 10	2 10	1 10	2 2	10 2	10 3	3 3
−slope	2.539	2.533	2.519	1.610	1.616	2.244	2.244
−intercept	0.0023	0.0047	0.0210	0.0119	0.0100	0.0095	0.0095
r^2	0.99997	0.99997	0.9996	0.9970	0.9970	0.9990	0.9990

[a] Extent of truncation is indicated by i and j.

where $w_0 = 100$ and α is a constant. It is seen that α and β are related by $\alpha = (w_0^{1/3}\beta/\mathrm{M})$. Equations 6 and 7 really apply only for the narrowest of distributions (4).

Values for β for numerous distributions are given in Table II. In every case, the value was calculated from the portion of the profile given for $0 < \tau/\mathrm{M} < 0.1$. If the critical time τ_c is taken as that τ when the smallest particles disappear, then no such time exists for ideal distributions since $0 \leq a_0 \leq \infty$. However, if one defines the critical time by $\ln \tau_c = \mu - 3\sigma$, then only small errors are introduced into the calculations. Every β in Table II was calculated for a time shorter than that defined by $\ln \tau_c = \mu - 3\sigma$.

By the following reasoning, it was found that, for ideal or nearly ideal distributions, a good approximation for β would have the form $e^{-c\sigma^2}$. For ideal distributions or for not severely truncated distributions (*e.g.*, where $j - 3\sigma$ and $i + \sigma$ are greater than about 3), Eq. 4 for $\tau < \tau_c$ can be approximated by:

$$w_\tau/w_0 = 1 - 3(\tau/\mathrm{M})e^{-5\sigma^2/2} + 3(\tau/\mathrm{M})^2e^{-4\sigma^2} - (\tau/\mathrm{M})^3e^{-9\sigma^2/2} \quad \text{(Eq. 8)}$$

Although the right side of the equation is not a perfect cube, it can be treated as a cubic of the form:

$$w_\tau/w_0 = [1 - (\tau/\mathrm{M})e^{-c\sigma^2}]^3 \quad \text{(Eq. 9)}$$

since the terms $3(\tau/\mathrm{M})^2e^{-4\sigma^2}$ and $(\tau/\mathrm{M})^3e^{-9\sigma^2/2}$ for small (τ/M) are relatively unimportant and can be manipulated without seriously affecting the equation. If this is true, then a plot of ln β *versus* σ^2 will be a straight line with a slope of $-c$ and an intercept of zero. Figure 1 shows such a plot for a series of ideal ($i = j = 10$) distributions and for a series of truncated ($i = j = 2$) distributions.

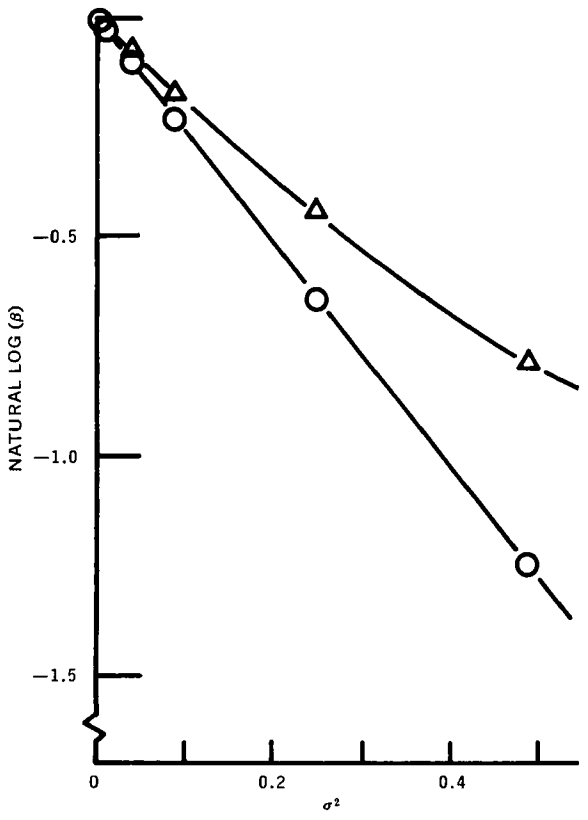

Figure 1—*Plot of ln* β versus σ^2 *for a series of ideal* (i = j = *10) distributions* (○) *and for a series of truncated* (i = j = *2) distributions* (△). *The symbols refer to calculated slopes given in Table II.*

The plot in Fig. 1 for the ideal distributions appears to be straight. Linear regression techniques (Table III) indicate a slope of −2.54 and a near zero intercept. The linearity, as judged by a coefficient of determination (r^2) of 0.99997, is excellent. Thus, for ideal or nearly ideal distributions, β can be approximated by $e^{-2.54\sigma^2}$ and the dissolution before the critical time can be approximated by:

$$1 - (w_\tau/w_0)^{1/3} = e^{-2.54\sigma^2}(\tau/\mathrm{M}) \quad \text{(Eq. 10)}$$

For distributions like those where $i = j = 2$, the effect of truncation becomes more and more important as σ is increased. A convenient approximation such as Eq. 10 is not acceptable with severely truncated distributions.

It is intuitively satisfying that in the approximation $\beta = e^{-2.54\sigma^2}$ the coefficient of σ^2 is close to −2.5. It is also satisfying that as $\sigma \to 0$, $\beta \to 1$, a result required by Eq. 5. Furthermore, it is satisfying that the approximation holds over a wide range of σ, although this might not have been expected since the cube-root law is to be applied only for narrow distributions.

In contrast, the Carstensen and Musa (1) approximation, given in the notation of this report by $\alpha = 50/\mathrm{M}(\sigma/2.303)^{1.05}$, predicts that as $\sigma \to 0$, $\alpha \to \infty$. Furthermore, the form of the approximation does not hold over a wide range of σ. In addition, the slopes of cube-root plots for times before the critical time presented in the Carstensen and Musa table cannot be calculated by their approximation. The tabled slopes do appear to be correct and are calculable by the approximation used in Eq. 10. By using the slopes in the Carstensen and Musa (1) paper and linear regression techniques, it can be determined that α should be given by:

$$\alpha = 4.26/\mathrm{M}(\sigma/2.303)^{0.0195} \quad \text{(Eq. 11)}$$

In the previous paper, Brooke (3) calculated α from the incorrect approximation, chose a point $(\tau^*, w_\tau^*/w_0)$ from the exactly calculated profile, and then calculated a $w_0^{1/3}$ to fit the following expression:

$$w_\tau^*/w_0 = [1 - (\alpha/w_0^{1/3})\tau^*]^3 \quad \text{(Eq. 12)}$$

Obviously, the ratio $\alpha/w_0^{1/3}$ obtained was correct, even though the separate values α and $w_0^{1/3}$ were not. Thus, the test of the approximation α offered by Brooke was inconclusive.

This report has dealt with approximations for the slopes of cube-root law plots for the dissolution of log-normal powders before the critical time. Although such approximations may seem useful, a convenient approximation for all log-normal distributions has not been found. To apply an approximation like that of Eq. 10 for the dissolution of ideal log-normal powders before the critical time, one must know all of the parameters that would allow exact calculations to be made. Such approximations might, however, be useful in curve-fitting experimental data to determine one or several basic parameters of dissolution profiles.

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(3) D. Brooke, *J. Pharm. Sci.*, **62**, 795(1973).

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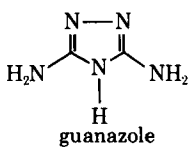
Guanazole (3,5-diamino-1,2,4-triazole, NSC-1895, mol. wt. 99.1), reportedly synthesized by Pellizzari in 1894 (1), is a new antileukemic agent which is therapeutically effective against mouse leukemia L-1210 (2) and human acute myelocytic leukemia (3, 4). Its antileukemic actions appear to be related to its ability to inhibit ribonucleoside diphosphate reductase, a key enzyme involved in deoxynucleotide synthesis during the DNA-synthetic phase of the cell cycle (5).

A sensitive colorimetric procedure was developed to study the pharmacokinetics of guanazole in patients with acute myelocytic leukemia (6). The rationale for the present methodology is based upon the fact that the primary amino groups of 1,2,4-triazole are diazotizable (7). The diazotized product in the case of guanazole was coupled with diphenylamine, resulting in the formation of a red dye which could be readily extracted with isopentyl alcohol.



guanazole

EXPERIMENTAL

Chemicals and Reagents[1]—The purity of labeled and unlabeled guanazole was determined by TLC on silica gel precoated plastic sheets without fluorescent indicator[2] using the following three solvent systems (v/v): (*a*) methanol–dioxane (2:1), (*b*) water–dioxane (1:10), and (*c*) water–methanol (1:10).

Chromatograms were developed in a TLC chamber[3] for 30–45 min. Spots were visualized by placing the dried chromatogram in iodine vapors, and the R_f values for guanazole were found to be 0.31, 0.73, and 0.61. Both labeled and unlabeled compounds migrated as a single spot on the chromatogram with each solvent system.

For a standard colorimetric assay, the following reagents were prepared: (*a*) acid mixture, 1.5% (v/v) sulfuric acid in acetic acid; (*b*) nitrite reagent, 1% (w/v) $NaNO_2$ in water; and (*c*) diphenylamine reagent, 10% (w/v) diphenylamine in the acid mixture reagent.

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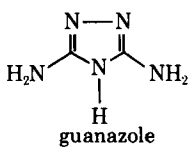

guanazole

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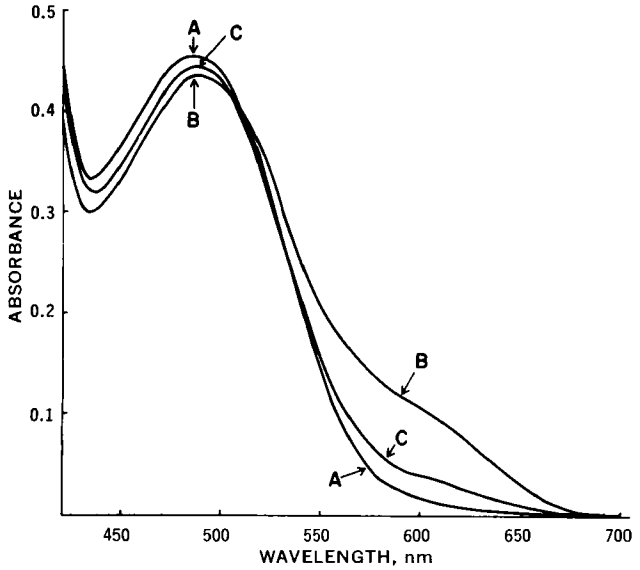

Figure 1—*Spectrum of azo dye product from guanazole. Azo dye solution in isopentyl alcohol was obtained from 4.5 μg guanazole dissolved in 2 ml water (A), from 2 ml of 5% trichloroacetic acid (B), or from 2 ml of 5% trichloroacetic acid extract of 0.5 ml fetal calf serum containing 9 μg/ml guanazole (C). The spectra A, B, and C were recorded on a dual-beam spectrophotometer (Cary model 14) using the isopentyl alcohol layer from the corresponding blank reagent mixtures in the reference cell.*

gave blank readings similar to the ones obtained with 5% trichloroacetic acid alone, the absorbance values of the samples were read against reagent blanks obtained with 2.0 ml of 5% trichloroacetic acid alone.

Blood Levels of Guanazole in Rat—Charles River female rats (200–250 g) were injected intravenously with 60 mg guanazole containing 0.5 μCi of ^{14}C-labeled compound in 0.5 ml water. At various time intervals after guanazole administration, 0.1 ml blood was withdrawn from the tail vein into heparinized graduated capillary tubes[5] through a surgical incision which was then sealed

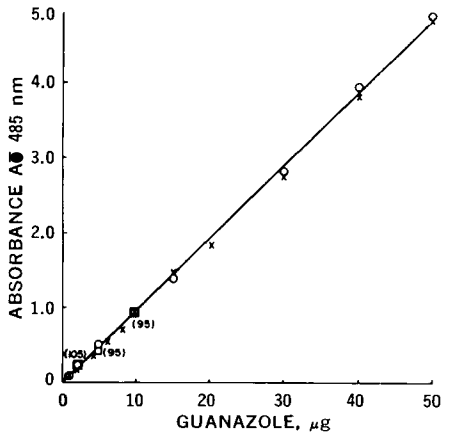

Figure 2—*Standard curve for determination of guanazole. The color of the azo dye from guanazole in 2 ml water (○) or 5% trichloroacetic acid (×) was extracted in 3 ml isopentyl alcohol as described in the text. Extracts with absorbance greater than 1.0 were diluted sufficiently with isopentyl alcohol. Absorbances of color from 0.2-, 0.5-, and 1.0-ml samples of dog plasma containing 10 μg/ml guanazole are also plotted (□), and the percentage recoveries are indicated by the corresponding numbers in parentheses.*

[5] Clay-Adams, Inc., N.J.

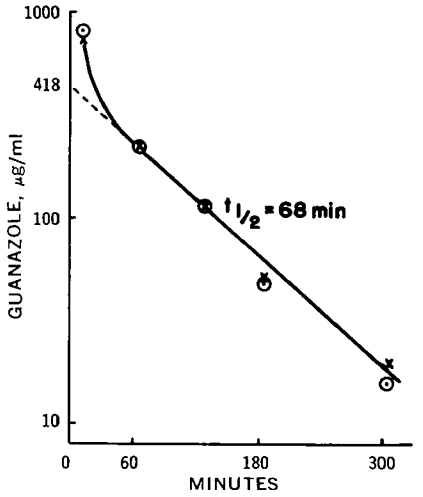

Figure 3—*Levels of guanazole in rat blood. After a single intravenous injection of 0.5 μCi/60 mg of ^{14}C-labeled guanazole, blood samples were withdrawn and analyzed for guanazole by the colorimetric procedure (○) and by radioactivity measurements (×).*

with flexible collodion. For subsequent samples, it was usually possible to reopen the same wound by removing the collodion seal. The content of the capillary tube was emptied in nine volumes of 5% trichloroacetic acid and centrifuged at 1000×*g* for 5 min. Aliquots from the supernate were analyzed by the colorimetric procedure and by measuring radioactivity.

Determination of ^{14}C-Radioactivity—Aliquots of trichloroacetic acid supernate from plasma or blood were directly transferred to glass counting vials, and water was added to make 1 ml. To a glass vial, 10 ml of toluene counting fluid containing 0.6% 2,5-diphenyloxazole, 0.06% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 15% (v/v) Biosolv-3[6] was added and mixed, and the vial was counted in a liquid scintillation counter[7]. The counts were corrected for quenching by automatic external standard ratio method (8, 9).

RESULTS

Spectrum and Standard Curve—As shown in Fig. 1, a spectrum with a symmetrical peak at 485 nm was obtained with azo dye derived from guanazole in the absence of trichloroacetic acid. In the presence of 5% trichloroacetic acid, a shoulder appeared at 590 nm in addition to the peak at 485 nm. In the case of trichloroacetic acid extract obtained from fetal calf serum, the 590-nm shoulder was reduced considerably, and the spectrum of the resulting azo dye was essentially similar to that obtained in the absence of trichloroacetic acid.

A typical linear regression curve passing through the origin (correlation coefficient = 0.9999) was obtained for the colorimetric assay of guanazole as shown in Fig. 2. This was in excellent agreement with Beer's law at least up to about 1.5 absorbance units. For absorbance greater than 1.5, the azo dye solution was diluted sufficiently with isopentyl alcohol and read at 485 nm, and the absorbance value of the original solution was calculated from the absorbance value of the dilute solution and the dilution factor. It is also evident from Fig. 2 that trichloroacetic acid did not noticeably affect the color intensity at 485 nm, the λ_{max} of the azo dye. The recovery of guanazole from dog plasma was also in excellent agreement with the different amounts of guanazole added to each sample as indicated in Fig. 2. Similar recoveries (95–105%) were also obtained with fetal calf serum (Fig. 1), rat blood (Fig. 3), and human plasma (data not shown).

Data obtained for colorimetric assay of guanazole over several months were statistically analyzed on a programmable calculator[8] using the least-squares method (Table I). Values of reagent blank

[6] Beckman Instruments Inc.
[7] Packard.
[8] Wang 700B.

Table I—Reproducibility of the Colorimetric Assay of Guanazole

Experiment	Range Analyzed, μg	Reagent Blank[a], A	Regression Reaction[b], $Y = SX + C$	Correlation Coefficient
1	0–14 (8)[c]	0.018	$= 0.0949X - 0.0033$	0.9999
2	0–12 (7)	0.009	$= 0.1032X - 0.0065$	0.9996
3	0–12 (6)	0.018	$= 0.1033X - 0.0078$	0.9999
4	0–50 (9)	0.022	$= 0.0977X - 0.0384$	0.9998
5	0–15 (7)	0.013	$= 0.0958X + 0.0090$	1.0000
	Mean ± SE	0.016 ± 0.002	$= 0.0989\ (\pm 0.0018)X - 0.0094\ (\pm 0.0078)$	

[a] Obtained with 5% trichloroacetic acid. [b] Y = absorbance, S = slope, X = amount of guanazole in micrograms, and C = constant intercept to y-axis. [c] Numbers in parentheses denote number of samples containing different amounts of guanazole analyzed.

with 5% trichloroacetic acid are around 0.02 absorbance unit in most cases. With 10% trichloroacetic acid as blank, this value nearly doubled; whereas in the absence of trichloroacetic acid, the value was below 0.01 absorbance unit. Values obtained from the intercept were nearly zero in all the cases, in agreement with Beer's law. Values for the slope and the correlation coefficient obtained over time were also in agreement with each other (Table I).

Conditions for Reaction with Nitrite Reagent—As shown in Fig. 4, the optimal diazotization, as monitored by azo dye formation, occurred between 10 and 15 min at 0–4° in the presence of 0.1 ml nitrite reagent to 3.0 ml of reaction mixture containing guanazole. With a lower concentration of nitrite reagent (0.075–3.0 ml), it took longer to develop color of equal intensity. At higher concentrations of nitrite reagent (from 0.2–0.4 to 3.0 ml), diazotization occurred very rapidly, although the product appeared unstable.

Condition for Reaction with Diphenylamine Reagent—As shown in Fig. 5, the color intensity of the azo dye obtained with 1–5 μg guanazole increased with increasing concentration of diphenylamine and reached a plateau value at a ratio of about 0.1 ml of 10% diphenylamine to 3.1 ml of the diazotized mixture. Although the color intensity obtained with 15% diphenylamine solution was slightly higher, for practical purposes 0.1 ml of diphenylamine reagent was used for the color development. As shown in Table II, the color intensity reached a plateau value after 10 min; variation of temperature between 0 and 37° did not significantly affect the intensity of color. Therefore, the coupling reaction with diphenylamine was carried out at room temperature (23°) for 15 min.

Color Reaction with Other Congeners—When 100 μg 3-amino-1,2,4-triazole was tested for color development under the conditions described for the standard procedure, an orange-red-colored product was obtained which turned yellow on extraction with isopentyl alcohol and gave an absorption peak (0.67 absorbance unit) at 450 nm (Fig. 6) instead of the 485-nm peak for guanazole. At 485 nm, the dye from 3-amino-1,2,4-triazole had a color intensity 60% that of its own at 450 nm and about 5% of that obtained with guanazole. Again, in similarity with the product from guanazole, this dye was also found to be very stable for at least 2 days and the development of color was quite reproducible. The parent compound, 1*H*-1,2,4-triazole, did not give any absorbance in the visible spectrum.

Interference with Other Chemicals—The standard procedure was run in the presence and absence of 5 μg guanazole with 2 mg urea, 100 mg sucrose, or 1 ml tissue[9] culture medium (10) containing, among other nutrients, about 1 mg amino acids/ml. In all cases, the blank readings were less than 0.03 absorbance unit and the recovery of guanazole was within 5% the actual amount added.

Levels of Guanazole in Rat Blood—A typical time course of guanazole level in rat blood is shown in Fig. 3 after a single intravenous injection of the drug. The blood levels determined with the ^{14}C-radioactivity and colorimetric procedures tallied reasonably well. An initial rapid distribution phase was followed by a slower first-order elimination process with a half-life ($t_{1/2}$) of 68 min regardless of the technique used.

DISCUSSION

The newly developed colorimetric procedure was found to be sensitive and specific for guanazole and was adapted to drug level determination in physiological fluids. While this manuscript was in preparation, another method utilizing the Bratton-Marshall (11) diazotization reaction and coupling was reported (12) for guanazole determination. Among the advantages of the present procedure are its reproducibility, its sensitivity, and the relative lack of interference by constituents in the blood or tissue culture media containing fetal calf serum. The high solubility of the azo

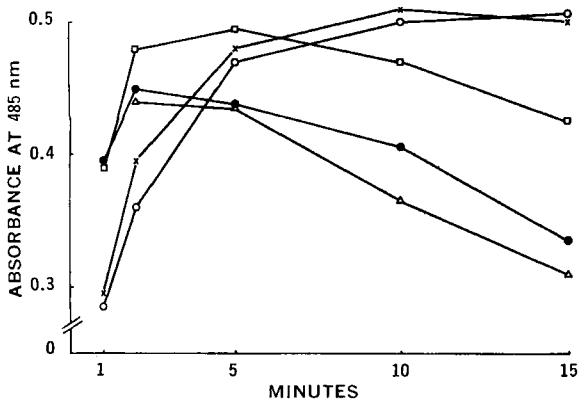

Figure 4—*Effect of time for diazotization and of concentration of nitrite reagent on azo dye formation. At time = 0, 0.075 (○), 0.10 (×), 0.20 (□), 0.3 (●), or 0.4 (△) ml of nitrite reagent was added to an acidified solution of guanazole (5.0 μg in 3.0 ml) on ice. At different time intervals thereafter, the color was developed with diphenylamine reagent, extracted, and read as described in the text.*

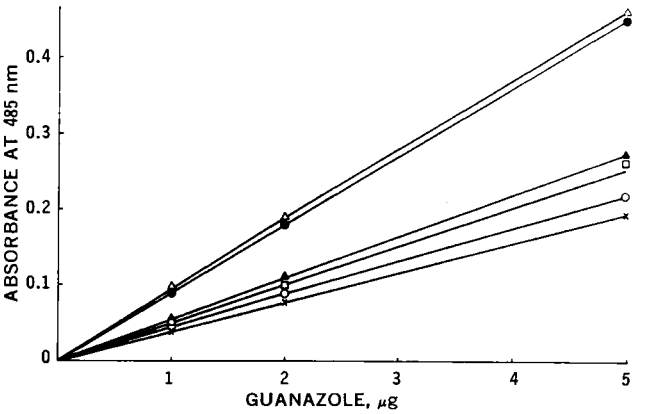

Figure 5—*Effect of varying concentration of diphenylamine on intensity of color. To the 3.1-ml diazotized mixture, prepared as described in the text, 0.1 ml of 1 (×), 2 (○), 3 (□), 4 (▲), 10 (●), or 15 (△) % diphenylamine in the acid mixture was added, kept for 15 min at 23°, extracted, and read as described in the text.*

[9] RPMI 1640.

Table II—Effect of Time and Temperature on the Coupling Reaction between Diazotized Guanazole and Diphenylamine[a]

Amount of Guanazole, μg	Reaction with Diphenylamine for					
	5	10	15 min	15 min		
	at 23°			at 0°	23°	37°
5	0.469	0.500	0.500	0.485	0.500	0.520
10	—	—	—	0.980	1.000	1.030

[a] Absorbance of blank = 0.018. All values are in absorbance units. Diazotization was carried out with nitrite reagent as described in the text.

dye into isopentyl alcohol permits the use of a small volume of solvent for dye extraction, thereby increasing the sensitivity of the assay. As has been shown, there is an excellent agreement between the colorimetric method and that using ^{14}C-radioactivity.

The optimization of the diazotization reaction with nitrous acid was a crucial step in the colorimetric reaction for guanazole, this being consistent with similar findings reported for 3-amino-1,2,4-triazole (13). The choice of diphenylamine as a coupling agent was based upon the rationale that coupling of one or two diphenylamine residues with hydrophobic aromatic rings to a guanazole molecule would render the azo dye lipid soluble and extractable with water-immiscible hydrophobic solvents. The azo product obtained using 1-aminonaphthol-2,4-disulfonic acid was not extractable in isopentyl alcohol.

Since the isopentyl alcohol–water partition coefficient of the azo dye appears to be high and since the miscibility of isopentyl alcohol and water is quite low, the intensity of color in the organic phase is dependent on the total guanazole content or color produced in the aqueous mixture rather than on the drug concentration *per se*. Thus, by altering the proportions of isopentyl alcohol to the aqueous mixture, it should be possible to increase the sensitivity of the test. Indeed, when 1 ml of isopentyl alcohol was used to extract the color obtained with 2.5 μg guanazole in 3.2 ml aqueous mixture, an absorbance of 0.710 was obtained, using 1-cm path length narrow cells, instead of the expected 0.729 or 0.243 × 3 when 3 ml of the solvent was used for extraction. Extraction of the azo dye with organic solvent may be responsible not only for a decreasing interference by other nonspecific absorbing materials in the reaction mixture but may also impart remarkable stability to the color developed. Different plasma or serum proteins were found to prevent to a variable extent the optimal diazotization with nitrous acid as well as the coupling with diphenylamine. Thus, it was necessary to remove proteins with agents like trichloroacetic acid. Other protein-denaturing agents were not tested. Normal acid-soluble constituents in plasma or serum having amino groups, *i.e.*, amino acids and urea, did not interfere at the concentrations tested, either with blank readings or with the test samples.

Since guanazole is excreted unchanged in mice, rat, dog (14), and man (6, 12), no interference is expected from metabolites during the colorimetric determination of the drug in plasma or serum from patients undergoing treatment. Indeed, the procedure was already applied satisfactorily to the determination of the levels of guanazole in patients undergoing therapy (6). The same procedure was also satisfactorily applied to the determination of guanazole in rat tissues[10].

The fact that the color of the azo product of guanazole is distinct from that of 3-amino-1,2,4-triazole indicates some degree of specificity of the reaction with guanazole. The lack of reactivity of 1*H*-1,2,4-triazole is consistent with the expectation that a primary amino group is indeed required for the color reaction to occur. It seems from the results that both 3- and 5-primary amino groups of guanazole participate in diazotization reaction. In contrast, 3-amino-1,2,4-triazole, having only one primary amino group, can form an azo dye with only one diphenylamine molecule. This being a smaller system of conjugated olefinic bonds, it would form a smaller chromophore-absorbing maximum at 450 nm than that seen with azo dye from guanazole (λ_{max} 480 nm).

[10] Unpublished data of the authors.

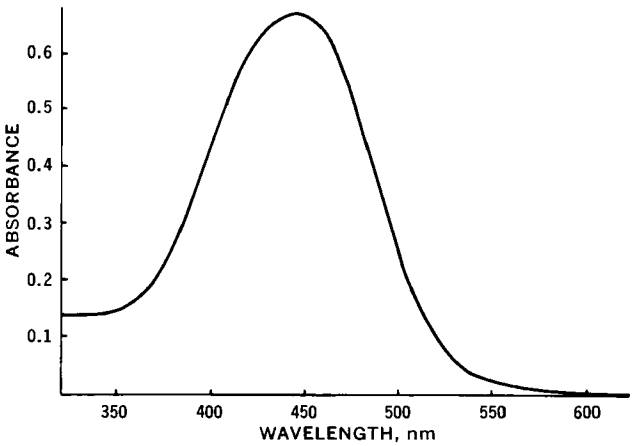

Figure 6—*Spectrum of azo dye product from 3-amino-1,2,4-triazole. The azo dye solution obtained from 3-amino-1,2,4-triazole (100 μg in 2 ml water) was recorded on a spectrophotometer (Cary model 14).*

This postulate is consistent with the observed ability of the primary amino group at 3- and 5-positions in 1,2,4-triazole to form diazonium compounds (7).

3-Amino-1,2,4-triazole, a herbicide (15, 16), and an inhibitor of the enzyme catalase (13, 17), could be estimated by several methods (13, 15) including the one involving azo dye formation (13). Without any modification, the presently described procedure could also be utilized for the determination of 3-amino-1,2,4-triazole at 450 nm with a sensitivity of 50–100 nmoles. This sensitivity is comparable to that of a recently described procedure (13). Optimization of diazotization and coupling reactions, however, might further increase the sensitivity of the color reaction for 3-amino-1,2,4-triazole using the present method.

In conclusion, a sensitive colorimetric procedure has been described for the determination of guanazole in plasma and blood. The assay may be useful for monitoring drug levels in plasma as well as in other tissue fluids.

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Evaluation of Mathematical Models for Diffusion from Semisolids

JAMES W. AYRES and PAUL A. LASKAR[x]

Abstract □ Models used in the description of diffusion processes were applied to drug release from semisolids. Two models, an exponential model for finite systems and a nonexponential model for semi-infinite and infinite systems, are evaluated for their suitability in describing literature data for release of substances from semisolids. The regression lines calculated for these data are evaluated using analysis of variance and examination of residuals. Evaluation of these models using these data indicates that the exponential model derived for finite systems is applicable in all the literature cases while the nonexponential model has restricted applicability.

Keyphrases □ Diffusion, drugs from semisolids—exponential and monoexponential models evaluated using literature data, regression lines calculated □ Semisolids—evaluation of mathematical models used to describe drug diffusion □ Ointments—evaluation of mathematical models used to describe drug diffusion □ Drug diffusion from semisolids—exponential and monoexponential models evaluated using literature data, regression lines calculated

Diffusion of drug agents from different solid and semisolid vehicles has been the subject of numerous reported investigations. During work in this laboratory with the diffusion of local anesthetics through semisolid dosage forms, it became necessary to evaluate mathematical models commonly used to describe diffusion. Several authors (1–3) described diffusion models which quantitate the diffusion process under various experimental conditions. For example, point, plane, and spherical sources of diffusion in finite, semi-infinite, and infinite systems in one, two, or three dimensions have been described. The terms finite, semi-infinite, and infinite are descriptive terms which locate the boundaries to diffusion in the x direction when a drug diffuses from a dosage form bounded between the planes $x = 0$ and $x = j$, while the semi-infinite system extends from a bounding plane at $x = 0$ to $x = +\infty$ and the infinite svstem extends to infinity in the $+x$ and $-x$ directions (3). In the case of diffusion in one dimension for a finite system, the desired solution for the amount diffused is given by the equations (4, 5):

$$M_t = M_\infty\left[1 - \frac{8}{\pi^2}\sum_{m=0}^{\infty}\frac{1}{(2m+1)}\exp\left(-(2m+1)^2 D\pi^2 t/l^2\right)\right] \quad \text{(Eq. 1)}$$

where:

M_t = amount diffused up to time t
M_∞ = amount diffused to time ∞
D = diffusion coefficient
t = time
l = thickness of membrane or layer

$$Q_{0,t} = C_0AH\left\{1 - \frac{8}{\pi^2}\sum_{m=0}^{\infty}\left[\frac{1}{(2m+1)^2}\exp\left(-\frac{(2m+1)^2\pi^2 Dt}{4H^2}\right)\right]\right\} = C_0AH\left\{1 - \frac{8}{\pi^2}\left[\exp\left(-\frac{\pi^2 Dt}{4H^2}\right) + \frac{1}{9}\exp\left(\frac{-9\pi^2 Dt}{4H^2}\right) + \ldots\right]\right\} \quad \text{(Eq. 2)}$$

where:

$Q_{0,t}$ = amount diffused between time = 0 and t
C_0 = initial concentration in phase from which the substance diffuses
A = area over which diffusion occurs
H = thickness of diffusing region
D = diffusion coefficient
t = time

It should be noted that $1 + (1/9) + (1/25) + \ldots = 8/\pi^2$. A model for semi-infinite and infinite systems (6) was suggested by Higuchi (7) as a simplified model for percutaneous absorption from an ointment. This model has the form:

$$Q = (2A - C_s)\left[\frac{Dt}{1 + 2(A - C_s)/C_s}\right]^{1/2} \quad \text{(Eq. 3)}$$

where:

Q = amount absorbed at time t per unit area of exposure
A = concentration of drug expressed in units per cubic centimeters
C_s = solubility of drug in units per cubic centimeter in external phase of ointment
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Equation 3 may take the form (6) used in the analysis of diffusion of medicaments from semisolid dosage forms (8–11):

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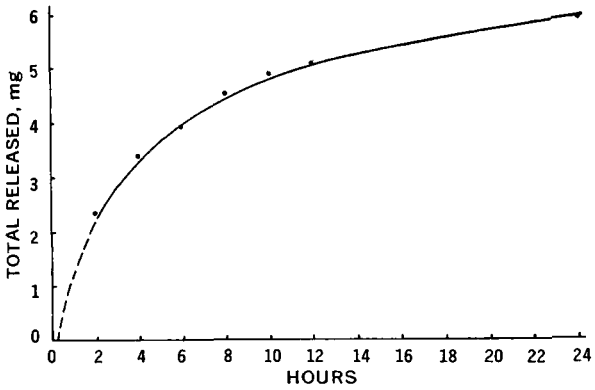


Figure 1—*Release of 0.025% fluocinolone acetonide from a water-soluble gel.*

$$Q_{0,t} = 2C_0A\sqrt{\frac{Dt}{\pi}} \qquad \text{(Eq. 4)}$$

where:

$Q_{0,t}$ = amount diffused between time = 0 and t
C_0 = initial concentration in semisolid dosage form
A = area for diffusion
D = diffusion coefficient
t = time

A similar form is (12):

$$M_t = \frac{4M_\infty}{l}\left(\frac{Dt}{\pi}\right)^{1/2} \qquad \text{(Eq. 5)}$$

The purposes of this report are: *(a)* to compare these models of diffusion, various forms of which are used in the analysis of diffusion experiments; *(b)* to use these models to describe various literature data; and *(c)* to evaluate these two models for statistical goodness of fit of the data.

MODEL THEORY AND GENERAL DESCRIPTION

Development of a model for diffusion should have some applicability in quantitating physical phenomena as well as in estimating a future course of diffusion under conditions like those under which the diffusion parameters were determined. Parameters that can be determined include the ultimate concentration or amount diffused, the thickness of the region from which diffusion is taking place, and the diffusion coefficient.

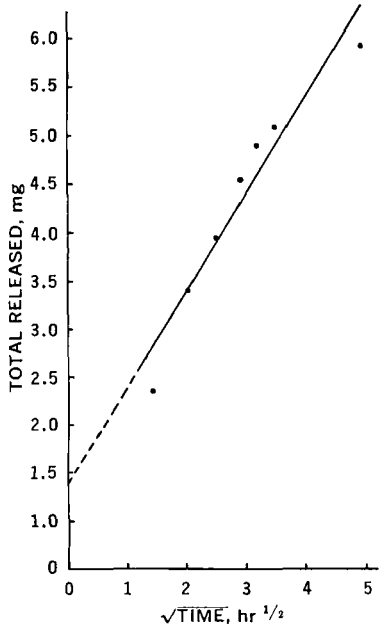


Figure 2—*Release of fluocinolone acetonide from a water-soluble gel.*

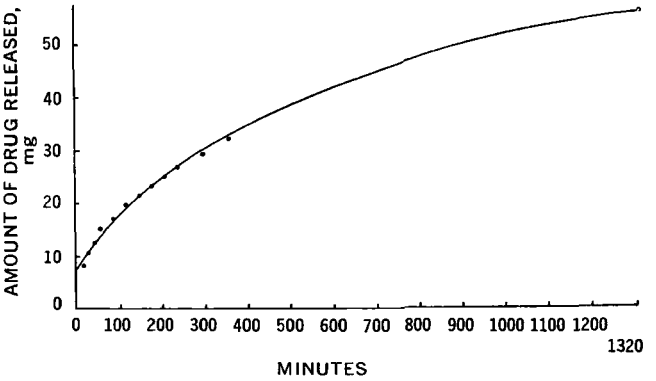


Figure 3—*Release of 0.126* M *pyridine from water/oil emulsion.*

The use of the nonexponential model (Eqs. 4 and 5) is of primary utility in the estimation of D from the initial gradient of sorption or diffusion due to the approximately linear behavior of diffusion *versus* square root of time up to $M_t/M_\infty \approx \frac{1}{2}$ for most systems (13). The total amount diffused at infinite time (M_∞) and the diffusion coefficient (D) can be obtained from Eq. 5 by noting that:

$$M_\infty = \frac{b_1 l \pi^{1/2}}{4D^{1/2}} \qquad \text{(Eq. 6)}$$

where b_1 = slope of the calculated line.

The diffusion coefficient may then be found by rearranging Eq. 5 or 4 to yield:

$$D = \frac{b_1 l \pi}{16M_\infty^2} \qquad \text{(Eq. 7)}$$

or:

$$D = \frac{b_1^2 \pi}{4C_0^2A^2} \qquad \text{(Eq. 8)}$$

where b_1 = slope of the line.

Problems associated with obtaining diffusion parameters by this method include lack of knowledge concerning, or the necessity of determining experimentally, M_∞. The linear model is useful only for the period from the end of the lag time to that time when $M_t \approx \frac{1}{2}M_\infty$, which is difficult to ascertain if M_∞ is unknown. In some cases, this relationship has been extended beyond $M_t \approx \frac{1}{2}M_\infty$ (14, 15).

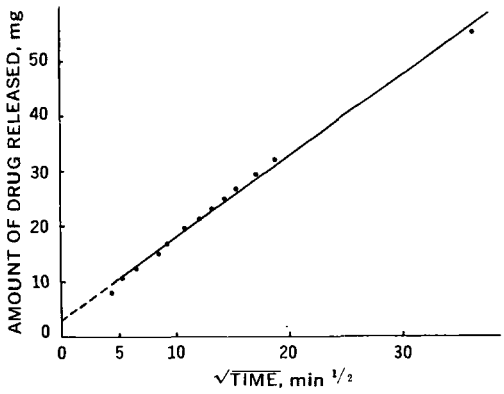


Figure 4—*Release of 0.126* M *pyridine from water/oil emulsion.*

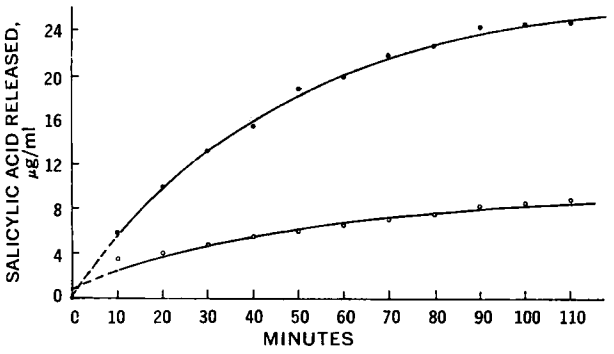


Figure 5—In vitro *release of salicylic acid from ointment bases. Key (ointment base): ●, hydrophilic cream; and ○, white petrolatum USP.*

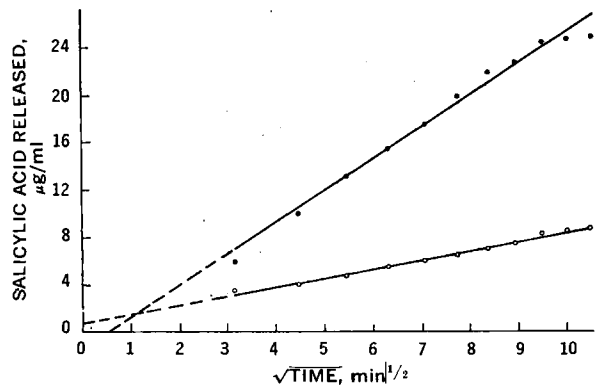
Another error that may be made is the assumption that the curve must pass through the origin, *i.e.*, neglecting the effect of a lag time. Depending on the plotting procedure used, this results in a significant curvature in the straight line or a systematic negative deviation, assuming lag phase is complete. If this relationship is used prior to the end of the lag phase, the error appears as a curvature or a systematic positive deviation.

Several nonlinear curve-fitting procedures are available which readily permit estimation of the necessary parameters to describe the diffusion process using the exponential model. In practical use of Eqs. 1 and 2, summation beyond $m = 0$ may be ignored unless the term $(\pi^2 Dt/l^2)$ is sufficiently small that a significant contribution of this exponential term is made in the region(s) of interest (16, 17). In cases where terms with $m > 0$ must be included, inclusion of only $m = 0$ and $m = 1$ is sufficient for adequate estimation of the parameters. If terms beyond $m > 0$ are ignored, the diffusion model reduces to:

$$M_t = M_\infty - \frac{8M_\infty}{\pi^2} \exp\left(-\frac{D\pi^2 t}{A}\right) \qquad \text{(Eq. 9)}$$

Use of the single exponential term results not only from the lack of necessity for use of further members of the series but also from the frequent lack of sufficient experimental data for adequate elucidation of the parameters in the second and greater terms. It can be seen that the second term of the series is $(8M_\infty/\pi^2)(1/9)\ \exp\{-9\pi^2 Dt/l^2\}$. The preexponential term is one-ninth the previous term and the exponent has a ninefold greater absolute magnitude. Therefore, the contribution of this term is negligible, except at small times at which data are usually lacking. If a fit using the second exponential term is forced upon a data set with insufficient values at small times, the estimated parameters: *(a)* do not have their theoretical relationship, *(b)* are very "volatile," or *(c)* cannot be calculated due to singular or ill-conditioned normal equations (18).

Regardless of the number of terms retained in Eqs. 1 and 2,



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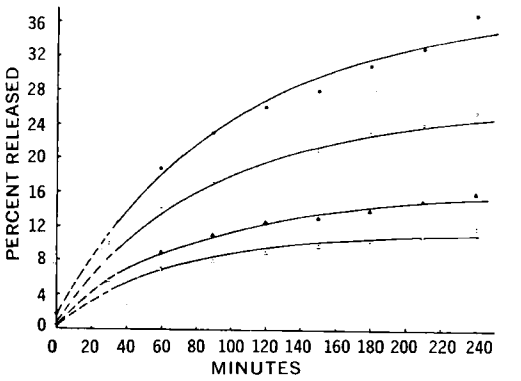

Figure 7—*Release of sodium radioiodide from ointment bases. Ointment bases containing: sodium salt of lauryl ether sulfate 1% (●); sodium salt of lauryl ether sulfate 2% (○); triethanolamine lauryl sulfate 3% (▲); and stearamidopropyl dimethyl β-hydroxyethylammonium nitrate 3% (△).*

M_∞ or C_0AH is estimated as the asymptote of the exponential equation. The term C_0AH implies that no equilibrium distribution of material that is diffusing will be attained but all will diffuse out. The term M_∞, however, is the equilibrium amount that diffuses out, which includes the above-mentioned terms as well as those terms giving the equilibrium conditions. It is also apparent that estimates of D may be found from each exponent portion of the series used in Eqs. 1 and 2. From the first term of the series, $D = B_3 l^2/\pi^2$ or $4B_3H^2/\pi^2$, where B_3 = estimated exponential parameter and is estimated or known independently. If further terms of the series are used, D may be estimated from each term using $D = B_i l^2$ (or $4H^2)/\pi^2\ (2m + 1)^2$, where B_i = estimated exponential parameter and $m = 1, 2, \ldots, \infty$. If several exponential terms are used, a weighted average using a term such as some function of $1/m$ or of the number of observations contributing to the estimate of D would be appropriate. Some indication of the constancy of D may be obtained by inspection or formal regression of the time dependency of D.

The lag time prior to establishment of steady-state diffusion may be estimated from both models. The presence of a lag time results in nonconformance of the preexponential factor(s) to theory in the exponential model and in a nonzero intercept for the square root of time model. Barrer (19) and Jacobs (20) noted that lag time is proportional to D and membrane thickness. This expression for lag time is sufficient if the diffusing substance is dis-

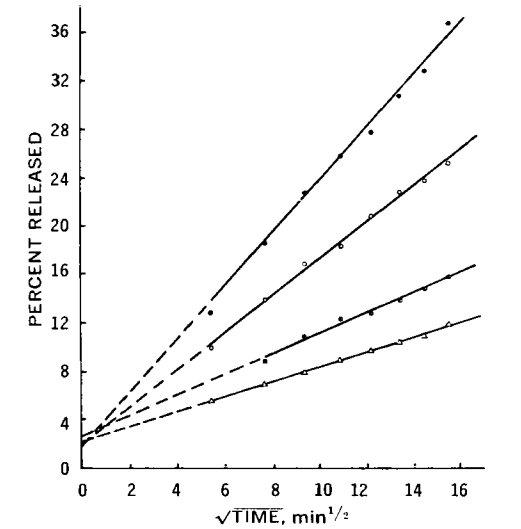

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Table I—Statistical Parameters for the Linear Model

Diffusion System	Intercept	Standard Error of Intercept	Slope	Standard Error of Slope	Sum of Squares Linear Regression (*df*)	Sum of Squares Quadratic Deviation (*df*)	Sum of Squares Residual (*df*)	Sum of Squares Cubic Deviation (*df*)	Sum of Squares Combined Quadratic and Cubic (*df*)	Sum of Squares Residual (*df*)
Fluocinolone acetonide[a]	1.370	0.390	1.019	0.127	7.906 (1)	0.600 (1)	0.013 (4)	0.001 (1)	0.600 (2)	0.013 (3)
Pyridine[b]	3.186	0.474	1.473	0.031	1778.378 (1)	6.874 (1)	1.569 (10)			
Salicylic acid, ●[c]	−1.589	1.033	2.689	0.133	401.978 (1)	6.956 (1)	1.934 (8)	0.897 (1)	7.853 (2)	1.037 (7)
Salicylic acid, ○[c]	0.705	0.199	0.767	0.026	32.704 (1)	0.198 (1)	0.132 (8)	0.043 (1)	0.242 (2)	0.088 (7)
Sodium radioiodide ($Na^{-131}I$), ●[d]	1.821	0.825	2.197	0.071	400.857 (1)	0.505 (1)	2.003 (5)	0.898 (1)	1.398 (2)	1.105 (4)
$Na^{-131}I$, ○[d]	1.937	0.469	1.540	0.040	196.566 (1)	0.311 (1)	0.498 (5)	0.002 (1)	0.313 (2)	0.496 (3)
$Na^{-131}I$, ▲[d]	2.657	0.509	0.858	0.042	33.818 (1)	0.062 (1)	0.335 (4)	0.247 (1)	0.309 (2)	0.876 (3)
$Na^{-131}I$, △[d]	2.194	0.112	0.619	0.010	31.734 (1)	0.002 (1)	0.044 (5)	0.002 (1)	0.004 (2)	0.043 (4)
Radioiodide from rectum[e]	0.034	5.096	9.280	0.840	4126.682 (1)	278.141 (1)	26.087 (8)	0.072 (1)	278.213 (2)	26.015 (7)

[a] Figure 2. [b] Figure 4. [c] Figure 6. [d] Figure 8. [e] Figure 10.

solved and in contact with the membrane. If, however, dissolution or some physical change, such as disintegration or dissolution of a dosage form, is necessary prior to diffusion, a more complex proportionality between lag time and D and the membrane thickness exists. This is apparent since the surface area for diffusion is changing. For adherence of the model to theory, a transformation of the independent variable, time or square root of time, to (time − lag time) or (time − lag time)$^{1/2}$ would be required.

MODEL EVALUATION

Sets of literature data for drug movement across a membrane, real or virtual, were evaluated[1] using Eqs. 1 and 4.

Experimental data reported for diffusion experiments were analyzed for goodness of fit to the mathematical models described by Eqs. 1 and 4. Examination of the parameters estimated from the experimental data for the two models can be compared using accepted statistical methods such as analysis of variance (ANOVA) and analysis of residuals. In using ANOVA methods, both linear regression and deviations from linear regression, calculated as the quadratic and cubic regression components, were examined. Analysis of residuals examines the deviations from regression for a nonrandom distribution about the calculated line. In the determination of the significance of lack of fit, the F-ratio of the mean square of interest to the residual mean square is examined. Significant lack of fit is defined when the F-ratio, as described, is greater than the appropriate tabular value at the $p < 0.05$ level. That these deviations from linearity are often not due solely to the quadratic (second-order) term would indicate that the choice of region of fit is not completely responsible for this lack of fit but also that a deficiency in the model is likely at fault. From an examination of the F-ratios generated by the mean squares computable from the sums of squares tabulated, significant lack of fit of the data to the linear model is apparent for most data sets. Indeed, some data reported by Higuchi (8) are the only ones that do not exhibit either significant or highly suggestive lack of fit.

Lack of fit or model deficiencies for the exponential model can be estimated only when an estimate of pure error, obtained from replications, is available. In all systems reported here, only a single determination was available for each point because the data were taken from graphs presented in the literature.

The effect of vehicle composition on the *in vitro* release of fluocinolone acetonide from a water-soluble[2] gel in direct contact with isopropyl myristate was reported (21) and subsequently (22) found to correlate with diffusion through human abdominal skin *(in vitro)* and vasoconstriction in the forearm *(in vivo)*. The data points for diffusion into isopropyl myristate are shown in Fig. 1. The solid line is that predicted from Eq. 1, when the values of $m = 0, 1$ are included. This line is $M_t = 7.102 - 3.701 \exp [0.2114 (\pm 0.0175)t] - 2.471 \exp (-0.0322t)$. The determination of lack of fit for the nonlinear estimation is not obvious from examination of the graph. Examination of residuals shows no evidence of nonrandom distribution of residuals about the estimated line. Figure 2 shows the data points for this study and the solid line predicted by using Eq. 4. The data points in the graph exhibit a sigmoid-shaped scatter about the estimated line. By using ANOVA, this observation is substantiated in that significant deviation from linear regression is observed (Table I).

Diffusion of pyridine from a water-in-oil emulsion through a cellophane membrane into an aqueous sink was measured (14), and the data are shown in Figs. 3 and 4. The solid line in Fig. 3 was generated with Eq. 9, and random scatter about the line can be observed. In Fig. 4, a significant deviation from linearity, determined by ANOVA as the quadratic regression component, is evident (Table I). The calculated line was determined using Eq. 4.

Figures 5 and 6 show the data (23) and calculated lines for the dialysis of salicylic acid from white petrolatum and a hydrophilic cream[3]. Figures 7 and 8 show the data (24) and predicted lines for the diffusion of sodium radioiodide ($Na^{-131}I$) from various surfac-

[1] All calculations were performed using library computer programs of the OS-3 system at Oregon State University. In particular, the programs SIPS (Statistical Interactive Programming System) and CURVFIT (Non-Linear Curve-Fitting Program) were utilized.

[2] Carbopol 934.

[3] Acid Mantle Creme.

Table II—Statistical Parameters for the Exponential Model

Diffusion System	M_∞ (C_0AH)	Standard Error of M_∞ (C_0AH)	Preexponential Factor	Standard Error of Preexponential Factor	Exponent	Standard Error of Exponent
Pyridine[a]	59.570	1.815	−51.182	1.689	−0.0019	0.0001
Salicylic acid, ○[b]	27.736	0.573	−27.552	0.534	−0.0216	0.0012
Salicylic acid, ●[b]	9.464	0.845	−8.630	0.766	−0.0200	0.0048
Sodium radioiodide ($Na^{-131}I$), ●[c]	37.690	2.934	−35.955	2.824	−0.0101	0.0022
$Na^{-131}I$, ○[c]	26.112	1.320	−25.170	1.372	−0.0116	0.0018
$Na^{-131}I$, ▲[c]	16.1525	0.578	−16.022	0.688	−0.0124	0.0014
$Na^{-131}I$, △[c]	11.450	0.639	−10.889	0.801	−0.0145	0.0029
Radioiodide from rectum[d]	76.918	1.188	−81.613	1.864	−0.0468	0.0072

[a] Figure 3. [b] Figure 5. [c] Figure 7. [d] Figure 9.

tant-containing ointment bases[4]. In each case, the drug was diffused through a cellophane membrane into an aqueous sink. A systematic sigmoidal deviation can be seen for one product in Fig. 6, but the data points all exhibit a random scatter in Figs. 5, 7, and 8. In the cases with random distribution of the residuals, the data appear to be equally well described by Eq. 1 or 4.

Riegelman and Crowell (25) described an *in vivo* method for investigating the rectal absorption of drugs in rats and reported the percent of the dose remaining in the rectum *versus* time. Those data were converted to amount diffused from the rectum and are plotted in Figs. 9 and 10. The sigmoidal deviation of the data points from the predicted line is clearly evident in Fig. 10 and exhibits a significant quadratic regression component (Table I).

CONCLUSION

Models describing diffusion have been discussed and compared. Their advantages and usefulness in various experimental conditions including *in vitro* diffusion with no membrane present, *in vitro* diffusion through cellophane membranes, and *in vivo* absorption from the rat rectum have been described. The advantages of the linear model (Eq. 4) include the ease of analysis and interpretation of linear relationships. The usefulness is in the region up to $M_t \approx \frac{1}{2}M_\infty$ for time greater than the lag time. A disadvantage lies in the necessity of estimating, if M_∞ is not known, when $\frac{1}{2}M_\infty$ is achieved. Furthermore, a high degree of dependence of estimation of M_∞ and D must occur since these are calculated from the same parameter. The nonlinear model, however, is useful over the entire diffusion time from the end of lag time to infinite time since, if sufficient terms of the series are retained, it is an exact physical model for a finite system. The errors inherent in the application of the linear relationship (Eqs. 4 and 5) appropriate for infinite and semi-infinite systems to systems more properly viewed as finite are avoided. The primary advantage in the use of the nonlinear model is that the parameters M_∞ and D are estimated as separate terms in the fitting procedure. Disadvantages lie in the greater unfamiliarity with nonlinear estimation procedures and in the iterative fitting techniques. These disadvantages can be overcome utilizing established estimation methods and the computer. Estimation of goodness of fit is somewhat more difficult since the statistical procedures are not rigorous but do provide a measure of comparison. However, examination of residuals can provide rigorous quantitative information concerning goodness of fit, if desired, or qualitative indications regarding suitability of the model. Models are often developed with the hope that they can be used beyond the end-points of experimental data and with some concern for the accuracy of estimation of the parameters of the physical process that the model describes. The asymptotic nature of the diffusion process is considered in the nonlinear model and is, therefore, useful in these

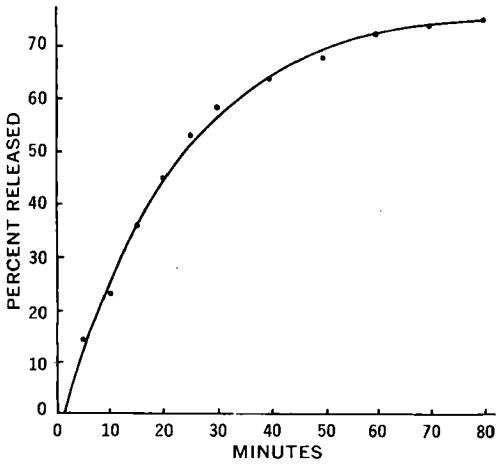

Figure 9—*Diffusion of radioiodide from rectum.*

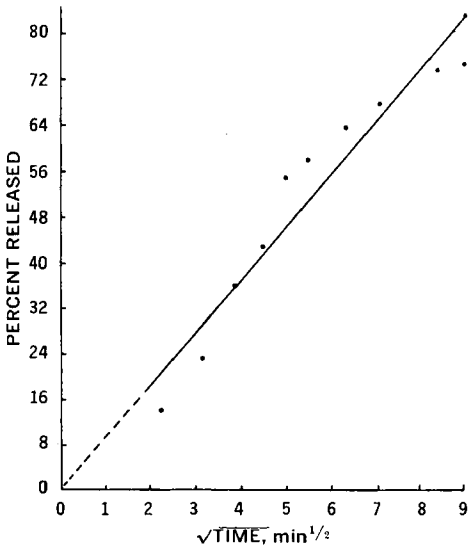

Figure 10—*Diffusion of radioiodide from rectum.*

[4] The bases used were the sodium salt of lauryl ether sulfate (Sipon ES), triethanolamine lauryl sulfate (Maprofix TLS), and stearamidopropyl dimethyl β-hydroxyethylammonium nitrate (Catanac SN).

respects. That all the data *generated* are used in parameter estimation, rather than merely that up to about $\frac{1}{2}M_\infty$, strengthens the parameter estimates.

It is anticipated that this evaluation will provide a basis for the use of the described models when the use of a physically exact or a general model is contemplated.

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[x] To whom inquiries should be directed. Present address: Department of Pharmacy Administration, Office of the Dean for Educational Development, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

Substituted Piperazinoquinazolones: Relationship between Selective Inhibition of Nicotinamide Adenine Dinucleotide-Dependent Oxidations and Anticonvulsant Activity

SURENDRA S. PARMAR*‡[x], A. K. CHATURVEDI‡, A. CHAUDHARY‡, and STANLEY J. BRUMLEVE*

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Keyphrases □ Piperazinoquinazolones—synthesis, anticonvulsant activity, and relationship to NAD-dependent oxidations □ Structure-activity relationships—piperazinoquinazolones, anticonvulsant activity, rats □ Inhibition of respiratory activity, NAD-dependent oxidations—synthesis and evaluation of piperazinoquinazolones □ Oxidation of pyruvate, citrate, DL-isocitrate, β-hydroxybutyrate, α-ketoglutarate, NADH, and succinate—effect of piperazinoquinazolones

Piperazinoureas possessing central nervous system (CNS)-depressant activity were recently shown to inhibit the oxidation of pyruvic acid by rat brain homogenate (1). Adrenergic, hypotensive, and antihistaminic activities were also reported for several 2-methyl-3-alkyl or piperazinoalkyl-4-quinazolones (2). Furthermore, selective inhibition of nicotinamide adenine dinucleotide (NAD)-dependent oxidation of pyruvic acid and other substrates of the tricarboxylic acid cycle by 2-methyl-3-*o*-tolyl-4-quinazolone (3–5) possessing hypnotic (6) and anticonvulsant properties (7) led to the synthesis of substituted pipera-

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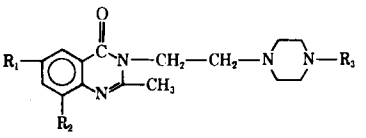
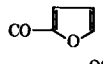
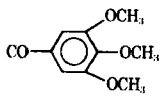
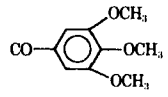


Table I—Substituted Piperazinoquinazolones

Compound	R_1	R_2	R_5	Crystallization Solvent	Yield, %	Melting Point[a]	Molecular Formula	Analysis, % Calc.	Found
I	H	H	H	Absolute ethanol	40	245°	$C_{15}H_{20}N_4O \cdot 2HCl$	C 52.17	52.45
								H 5.79	6.0
								N 16.23	16.20
II	Cl	H	H	Ethanol	75	128°	$C_{15}H_{19}ClN_4O$	C 58.72	59.08
								H 6.19	5.88
								N 18.27	18.12
III	Br	H	H	*n*-Propanol	68	190°	$C_{15}H_{19}BrN_4O$	C 51.28	50.75
								H 5.41	5.67
								N 15.95	15.67
IV	I	H	H	Ethanol	75	210°	$C_{15}H_{19}IN_4O$	C 45.22	45.33
								H 4.77	4.87
								N 14.03	14.25
V	Cl	Cl	H	Benzene–ethanol	70	245°	$C_{15}N_{18}Cl_2N_4O$	C 52.78	52.57
								H 5.27	5.66
								N 16.42	16.30
VI	Br	H	$COCH_3$	Ethyl acetate	60	105°	$C_{17}N_{21}BrN_4O_2$	C 51.90	52.00
								H 5.34	4.90
								N 14.24	13.98
VII	Cl	Cl	$COCH_3$	Methanol	62	190°	$C_{17}H_{20}Cl_2N_4O_2$	C 53.26	52.97
								H 5.22	4.82
								N 14.62	14.26
VIII	Cl	H	COC_6H_5	Acetonitrile	70	255°	$C_{22}N_{23}ClN_4O_2$	C 64.31	64.71
								H 5.60	6.00
								N 11.20	11.54
IX	Br	H	COC_6H_5	Ethyl acetate	60	200°	$C_{22}H_{23}BrN_4O_2$	C 58.02	58.13
								H 5.05	4.95
								N 12.30	12.45
X	Cl	Cl	COC_6H_5	Ethanol	70	270°	$C_{22}H_{22}Cl_2N_4O_2$	C 59.34	59.52
								H 4.94	4.72
								N 12.58	12.78
XI	Cl	Cl		Dioxane	75	270°	$C_{20}H_{20}Cl_2N_4O_3$	C 55.17	55.00
								H 4.59	4.32
								N 12.87	12.60
XII	Br	H		Ethanol	82	144°	$C_{25}H_{29}BrN_4O_5$	C 55.04	54.95
								H 5.32	5.72
								N 10.27	10.57
XIII	Cl	Cl		Ethanol	77	265°	$C_{25}H_{28}Cl_2N_4O_5$	C 56.07	55.97
								H 5.23	5.46
								N 10.46	10.73

[a] Melting points were taken in open capillary tubes and are uncorrected.

zinoquinazolones and to an investigation of their ability to inhibit respiratory activity of rat brain homogenate, with a view to studying their biochemical mechanism of action. In the present study, attempts also were made to correlate the anticonvulsant activity possessed by these piperazinoquinazolones with their enzyme inhibitory effectiveness.

CHEMISTRY

The various piperazinoquinazolones (Table I) were synthesized by following the methods outlined in Scheme I. Acetanthranils (II) were synthesized by refluxing 1 mole of the appropriate anthranilic acid (I) with 2 moles of acetic anhydride. Piperazinoquinazolones (IV) were synthesized by heating equimolar quantities of appropriate acetanthranils (II) and *N*-(2-aminoethyl)piperazine (III).

EXPERIMENTAL[1]

Anthranilic Acids (I)—The method of Endicott *et al.* (8) was used for the synthesis of 5-chloro- and 3,5-dichloroanthranilic acids. 5-Bromoanthranilic acid was prepared by the method of Wheeler and Oats (9), while the method of Klemme and Hunter (10) was used for the synthesis of 5-iodoanthranilic acid.

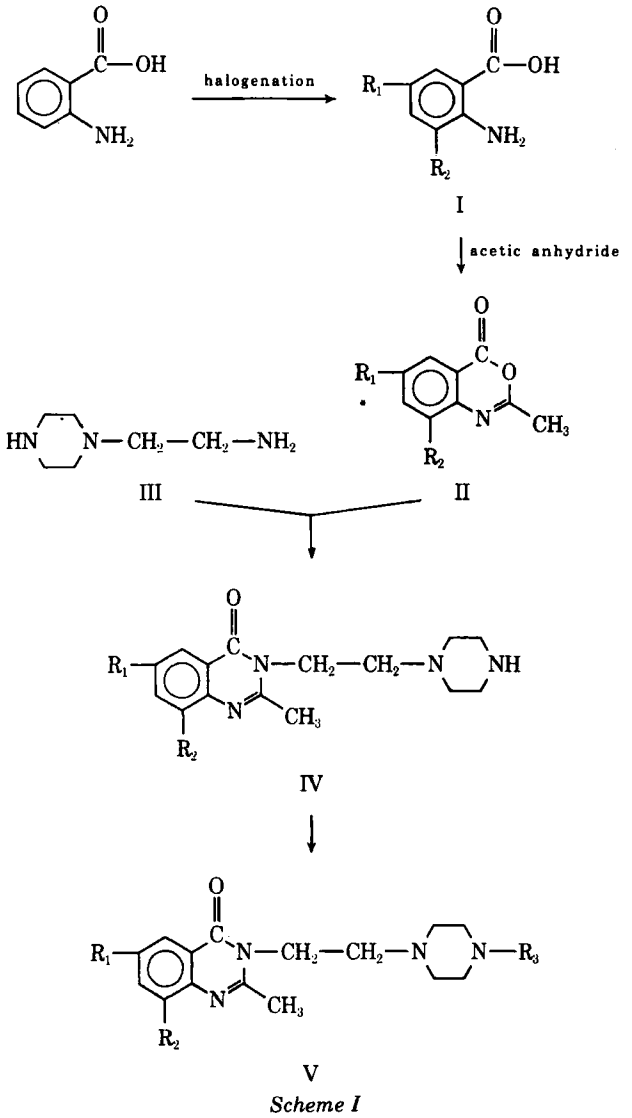
Acetanthranils (II)—The appropriate anthranilic acid (1 mole) was refluxed with acetic anhydride (2 moles) for 1 hr. After excess acetic anhydride was distilled off, the acetanthranil that separated out as a solid mass was dried quickly and used without further purification (II).

Piperazinoquinazolones (IV)—Equimolar quantities (1 mole) of the appropriate acetanthranil and *N*-(2-aminoethyl)piperazine were heated directly to yield the desired piperazinoquinazolones (11), which were recrystallized from suitable solvents and characterized by their sharp melting points and elemental analyses (Table I, Compounds I–V).

Substituted 3-[2-*N*-(*N'*-Acetylpiperazino)ethyl]quinazolones (V)—Appropriate piperazinoquinazolones (0.01 mole) were refluxed with acetic anhydride (0.02 mole) and anhydrous sodium acetate (0.02 mole) for 2 hr. The oily products thus obtained were added slowly into crushed ice, and the mixture was stirred for 1 hr. The crude products that separated were filtered, washed, dried, and recrystallized from suitable solvents. The various piperazinoquinazolone derivatives were characterized by their sharp melting points and elemental analyses (Table I, Compounds VI and VII).

Substituted 3-[2-*N*-(*N'*-Benzoyl/2-furoyl/3,4,5-trimethoxybenzoyl/piperazino)ethyl]quinazolones (V)—Piperazinoquinazolones (1 mole) and appropriate acid chloride (1 mole) were re-

[1] Melting points were taken in open capillary tubes and are uncorrected.



Scheme I

fluxed in dry benzene on a steam bath for 6–8 hr. The mixture was cooled and washed with 5% HCl, 5% Na_2CO_3 solution, and finally water. After distilling off the excess of benzene under reduced pressure, the solid mass that separated was filtered and recrystallized from suitable solvent. The various piperazinoquinazolone derivatives were characterized by their sharp melting points and elemental analyses (Table I, Compounds VIII–XIII).

BIOCHEMICAL[2] AND PHARMACOLOGICAL

Determination of Respiratory Activity of Rat Brain Homogenate—Male albino rats, kept on *ad libitum* diet, were used in all experiments. Rat brains isolated from decapitated animals were immediately homogenized in ice-cold 0.25 *M* sucrose in a homogenizer (Potter-Elvehjem) in a ratio of 1:9 (w/v). All incubations were carried out at 37°, and the oxygen uptake was measured by the conventional Warburg manometric technique with air as the gas phase (5). Fresh rat brain homogenate (1 ml) equivalent to 100 mg wet weight was added to chilled Warburg vessels containing 6.7 m*M* magnesium sulfate, 20 m*M* sodium hydrogen phosphate buffer solution (pH 7.4), 1 m*M* adenosine monophosphate (sodium salt), 33 m*M* potassium chloride, and 500 μg of cytochrome c in a final volume of 3 ml unless otherwise stated. The central well contained 0.2 ml of 20% KOH solution. Pyruvate, succinate, α-ketoglutarate, DL-isocitrate, β-hydroxybutyrate, and citrate were used at a final concentration of 10 m*M*. NADH and NAD were used at a final concentration of 0.5 m*M*. It was presumed that the endogenous NAD, present in this homogenate, was sufficient for these oxidative processes. All compounds under assay were dissolved in propylene glycol (100%), and an equal volume of the solvent was added to the control vessels.

Table II—Inhibition of Oxidation of Pyruvic Acid in Rat Brain Homogenate by Substituted Piperazinoquinazolones

Compound	Percent Inhibition[a] Without Added NAD, 1 m*M*[b]	Without Added NAD, 2 m*M*[b]	With Added NAD, 2 m*M*[b]
I	14.3 ± 1.2	28.0 ± 1.2	22.9 ± 1.4
II	25.1 ± 2.2	37.6 ± 1.2	34.6 ± 0.9
III	27.2 ± 1.9	55.3 ± 2.4	47.8 ± 1.1
IV	30.5 ± 2.0	68.2 ± 1.4	56.6 ± 0.4
V	40.4 ± 2.3	77.6 ± 2.6	68.0 ± 2.4
VI	27.7 ± 2.6	51.2 ± 1.3	43.4 ± 1.3
VII	12.4 ± 2.0	28.4 ± 2.0	22.8 ± 1.9
VIII	16.1 ± 3.8	42.7 ± 1.0	35.2 ± 1.5
IX	27.3 ± 3.4	44.8 ± 1.0	39.9 ± 0.9
X	21.3 ± 1.0	37.0 ± 2.0	29.6 ± 1.5
XI	17.0 ± 0.9	26.7 ± 1.5	11.9 ± 2.0
XII	25.0 ± 3.4	43.1 ± 2.0	37.3 ± 1.0
XIII	9.3 ± 0.8	22.5 ± 1.0	13.3 ± 1.8

[a] Vessel contents and assay procedures were as described in the text. Each experiment was done in duplicate, and the values are the mean values of three separate experiments with ± standard error of the mean. The oxygen uptake was measured at 5-min intervals during a 1-hr incubation. The percentage inhibition was calculated from the decrease in the oxygen uptake per 100 mg wet brain weight. The final concentrations of pyruvic acid and NAD were 10 and 0.5 m*M*, respectively. [b] Indicates final concentration of substituted piperazinoquinazolones.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined in albino mice, 20–25 g, of either sex (12). The mice were divided into groups of 10, keeping the group weights as near the same as possible. Each piperazinoquinazolone was suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compound was injected intraperitoneally, 100 mg/kg, in a group of 10 animals. Four hours after the administration of the test compounds, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol was shown to produce convulsions in almost all untreated mice, and the mice were also found to exhibit 100% mortality during 24 hr. On the other hand, no mortality was observed during 24 hr in animals treated with 100 mg/kg of the test compounds alone.

The mice were observed 60 min for the occurrence of seizures. An episode of clonic spasm persisting for at least 5 sec was considered a threshold convulsion. Transient intermittent jerks of tremulousness were not counted. Animals devoid of threshold convulsions during the 60-min period were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of these piperazinoquinazolones was represented as percent protection. The animals were then observed for 24 hr and their mortality was recorded.

RESULTS AND DISCUSSION

Inhibition of certain metabolic processes in the brain was shown to be the mechanism of various CNS depressants (12, 13) where a parallelism exists between *in vitro* and *in vivo* effects, since greater hypnotic activity of some agents was reflected by greater *in vitro* inhibition of respiration (14, 15).

The inhibitory effects of piperazinoquinazolones on the oxidation of pyruvic acid by rat brain homogenate are presented in Table II. All piperazinoquinazolones inhibited oxidation of pyruvic acid, and this inhibition was concentration dependent since an increase in inhibition was observed with a simultaneous increase in concentration. The presence of added NAD was found not only to increase the respiratory activity of rat brain homogenate but also to decrease the inhibitory effectiveness of these piperazinoquinazolones. These results thus provide evidence for a possible competition between the piperazinoquinazolones and

[2] Commercial chemicals were used. Sodium pyruvate, adenosine monophosphate (sodium salt), and cytochrome c were obtained from Sigma Chemical Co., St. Louis, Mo. Other common chemicals were obtained from British Drug House, Bombay, India.

Table III—Inhibition of Respiratory Activity of Rat Brain Homogenate by Substituted Piperazinoquinazolones

Compound	Percent Inhibition[a]					
	Citrate	DL-Isocitrate	β-Hydroxybutyrate	α-Ketoglutarate	NADH	Succinate
I	21.2 ± 1.4	11.3 ± 0.8	34.4 ± 1.2	53.1 ± 1.3	10.1 ± 0.9	Nil
II	36.4 ± 1.0	12.5 ± 0.9	37.2 ± 1.4	45.1 ± 2.0	21.2 ± 1.0	Nil
III	57.6 ± 1.2	59.5 ± 1.2	61.3 ± 1.5	78.8 ± 4.3	62.3 ± 2.3	Nil
IV	44.1 ± 1.1	12.7 ± 1.0	37.4 ± 1.3	47.0 ± 2.0	41.1 ± 1.0	Nil
V	89.4 ± 3.5	72.0 ± 2.3	73.8 ± 2.9	79.4 ± 3.0	72.0 ± 3.3	Nil
VI	31.5 ± 1.0	59.9 ± 2.3	37.2 ± 1.1	66.3 ± 3.0	42.1 ± 2.1	Nil
VII	57.1 ± 1.7	36.0 ± 1.0	45.9 ± 1.2	67.7 ± 2.0	13.2 ± 1.7	Nil
VIII	38.1 ± 1.3	51.3 ± 1.7	41.0 ± 1.3	61.0 ± 1.9	44.1 ± 2.3	Nil
IX	43.3 ± 1.7	35.1 ± 1.6	41.9 ± 2.0	48.0 ± 1.0	13.6 ± 2.1	Nil
X	53.0 ± 1.3	51.4 ± 1.3	54.2 ± 1.5	70.8 ± 2.1	29.6 ± 0.8	Nil
XI	59.5 ± 2.3	45.6 ± 1.2	46.3 ± 1.3	54.2 ± 1.8	28.9 ± 1.2	Nil
XII	57.6 ± 3.1	17.4 ± 1.1	30.3 ± 1.7	44.9 ± 1.6	40.0 ± 1.4	Nil
XIII	57.7 ± 1.2	50.2 ± 1.4	49.3 ± 1.7	58.9 ± 1.3	15.8 ± 1.3	Nil

[a] Vessel contents and assay procedures were as described in the text and in Table II. The final concentrations of the various substrates, NADH, and substituted piperazinoquinazolones were 10, 0.5, and 2 m*M*, respectively.

Table IV—Anticonvulsant Activity of Substituted Piperazinoquinazolones

Compound	Anticonvulsant Activity[a], % Protection	Percent Pentylenetetrazol Mortality
I	Nil	100
II	70	10
III	50	50
IV	30	30
V	30	80
VI	10	80
VII	50	20
VIII	Nil	80
IX	Nil	80
X	40	60
XI	20	50
XII	30	20
XIII	20	30

[a] Screening procedures for the determination of anticonvulsant activity were described in the text. Substituted piperazinoquinazolones were administered intraperitoneally (100 mg/kg) 4 hr before the administration of pentylenetetrazol (90 mg/kg) subcutaneously. Mortality in pentylenetetrazol-treated animals was observed for 24 hr.

NAD for the active site(s) on the enzyme molecule. This was further supported by the ability of the piperazinoquinazolones to inhibit the oxidation of NADH. As is evident from Table III, all piperazinoquinazolones selectively inhibited NAD-dependent oxidations of citrate, DL-isocitrate, β-hydroxybutyrate, α-ketoglutarate, and NADH while the NAD-independent oxidation of succinate remained unaltered. These results exhibiting selective inhibition of NAD-dependent oxidations are in agreement with earlier studies with 2-methyl-3-*o*-tolyl-4-quinazolone (4, 5). Inhibition of the oxidation of NADH has indicated possible inactivation of the electron-transport chain by piperazinoquinazolones, presumably at the site of the transfer of the electrons from NADH to flavine adenine dinucleotide.

Inhibitory effects of piperazinoquinazolones were not found to be related to their chemical structure, so a definite structure–activity relationship was not exhibited. Introduction of the halogen substituents at the 6-position or the 6- and 8-positions caused a significant increase in their inhibitory effects. A decrease in the effectiveness of these compounds to inhibit oxidation of pyruvic acid because of the attachment of an acetyl, benzoyl, 2-furoyl, or 3,4,5-trimethoxybenzoyl substituent at the R_3-position indicated that the presence of a free amine group at the piperazino nucleus is essential for the inhibitory effects of piperazinoquinazolones. In the present study, maximum inhibition was observed with 6,8-dichloro-2-methyl-3-(2-*N*-piperazinoethyl)-4-quinazolone (Compound V), having chloro substituents at both the 6- and 8-positions.

Results presented in Table IV represent anticonvulsant activity exhibited by some piperazinoquinazolones against pentylenetetrazol-induced seizures. Anticonvulsant activity of these piperazinoquinazolones ranged from 10 to 70%; 6-chloro-2-methyl-3-(2-*N*-piperazinoethyl)-4-quinazolone (Compound II) was found to exhibit maximum protection in a dose of 100 mg/kg. Two compounds (Compounds VIII and IX) were found to be devoid of anticonvulsant activity. These studies failed to provide any definite correlation between the anticonvulsant property exhibited by these piperazinoquinazolones and their ability to inhibit the respiratory activity of the rat brain homogenate as reflected by the inhibition of NAD-dependent oxidations. Data on anticonvulsant activity of these compounds and 24-hr pentylenetetrazol-induced mortality (Table IV) did not indicate an association between increased protection from convulsions and decreased pentylenetetrazol mortality in experimental animals. None of these piperazinoquinazolones exhibited any appreciable sedative or CNS-depressant effect or 24-hr mortality in the dose of 100 mg/kg used in the present investigation. It is hoped that detailed pharmacological and toxicological studies and investigations of the effects of these piperazinoquinazolones on other enzyme systems may reflect a biochemical basis for their anticonvulsant activity.

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Determination of Clindamycin 2-Palmitate in Clinical Human Serum Samples

T. F. BRODASKY ˣ and F. F. SUN

Abstract □ Clindamycin 2-palmitate, used in pediatric formulations, is readily hydrolyzed to clindamycin. During clinical investigations of this antibiotic, it was necessary to determine if unhydrolyzed clindamycin palmitate was present in the serum of clinical subjects. Chemical and enzymatic hydrolyses were unsuitable and instrumental techniques were ultimately developed. Two methods are described, one based on a combination of GLC and single-ion focused mass spectroscopy and a second using TLC and GLC. Examination of human serum from subjects receiving 300–600 mg clindamycin base equivalents of clindamycin palmitate hydrochloride revealed no statistically significant quantities of unhydrolyzed clindamycin palmitate.

Keyphrases □ Clindamycin 2-palmitate—determination in human serum using GLC and GLC–mass spectroscopy □ GLC—determination, clindamycin 2-palmitate in human serum □ GLC–mass spectroscopy—determination, clindamycin 2-palmitate in human serum

Clindamycin palmitate hydrochloride[1] (II) is the derivative of clindamycin used in pediatric formulations. The presence of the palmitate group markedly improves the taste characteristics of the derivative compared to the parent compound. Although the ester is not biologically active, it is readily hydrolyzed *in vivo* to clindamycin (I). During clinical studies, the question arose as to the presence of unhydrolyzed clindamycin 2-palmitate in human serum. Initially, attempts were made to hydrolyze the palmitate using specific esterases; although this approach was successful in urine samples, it failed in serum. Chemical hydrolysis in serum was unsuitable since the reaction, in addition to hydrolyzing clindamycin 2-palmitate, destroyed other biologically active metabolites of clindamycin (1). In some cases, this led to less biological activity after hydrolysis than was observed before.

At this point, studies were initiated which ultimately led to instrumental methods of detecting clindamycin 2-palmitate. Although the techniques do not require hydrolysis of the palmitate ester, it is necessary to prepare trimethylsilyl ether derivatives to impart volatility. Two independent studies will be described, one based on a GLC–mass spectroscopy technique and a subsequent method based on GLC only. Because of the need to extract the ester from serum samples prior to derivatization, poorer quantitation was achieved than had been expected. It was possible, however, to demonstrate the absence of unhydrolyzed clindamycin palmitate in clinical blood samples.

EXPERIMENTAL

GLC–Mass Spectroscopy Method—*Clindamycin 2-Palmitate Trimethylsilyl Ether (IV)*—A stock solution of II containing 10 μg/ml in analytical reagent grade pyridine (dried over sodium hydroxide pellets) was prepared. Then 100 μl of the II stock solution and 900 μl of the trimethylsilylation reagent[2] (V) [trimethylsilylimidazole–bis(trimethylsilyl)acetamide–trimethylchlorosilane (3:3:2)] were mixed in a dry, evacuated, 1-ml tube[3] and heated on a hot plate for 5 min at 75°. These solutions were used for initial GLC and GLC–mass spectroscopy studies. Solutions for structural correlation work were prepared in a similar manner from 20 mg of solid II and 1 ml of V.

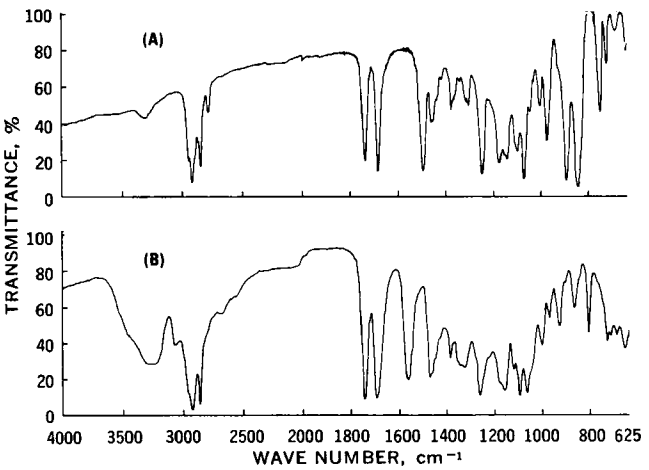

Figure 1—*Total reflectance IR spectra of II (B) and IV (A).*

[1] Cleocin Pediatric is The Upjohn Co. trademark for a product containing clindamycin palmitate hydrochloride (clindamycin 2-palmitate hydrochloride).

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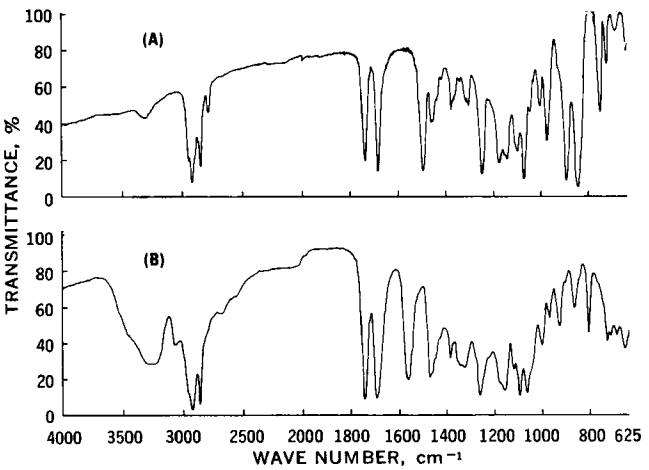

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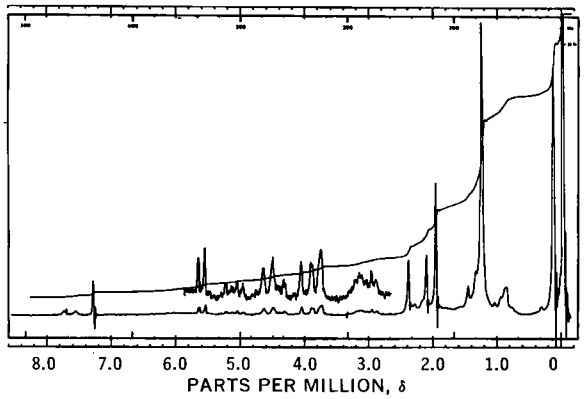


Figure 2—*NMR spectrum of IV (isolated solid).*

The solutions were stored at −10° when not in use. Under these conditions, IV was stable for several weeks.

Isolation and Characterization of IV—About 500 mg of II was treated with 3 ml of V as already described. After cooling, 50 ml of water was added to the reaction solution, which was then extracted with 75 ml of chloroform. The chloroform solution was evaporated to dryness under vacuum, and the resulting residue was characterized by IR, mass spectroscopy, NMR, and GLC.

Experimental Serum Samples—A solution containing 5 μg/ml II in 100 ml of pooled human serum[4] was prepared. After agitation, 4 volumes of acetone was added and the resulting precipitate was filtered and discarded. The filtrate was reduced to an aqueous solution under vacuum at 50°, and the pH was adjusted to 10 using potassium hydroxide. After extraction with hexane–methylene chloride (50:50), the mixture was centrifuged and the phases were separated. The solvent phase was dried with anhydrous sodium sulfate and, after filtering, was evaporated to dryness at 50° under vacuum. The entire residue was treated with 1 ml of V as previously described.

Clinical Serum Samples—Approximately 300 ml of blood was collected from each of four subjects prior to receiving 988 mg (600-mg base equivalents of clindamycin) of II (predrug blood) and another 300 ml each 1.5 hr after drug administration. The blood was allowed to stand at room temperature for 30 min, after which it was centrifuged for 30 min at 2000 rpm and the serum was collected. The predrug serums were pooled, but the postdrug serums were treated separately. By using the procedures described under *Experimental Serum Samples*, 100 ml of pooled predrug serum was prepared as a blank (no II added), 100 ml was spiked with 5 μg/ml of II, and another 100 ml was spiked with 1 μg/ml of II and derivatized as described. Each subject's postdrug serum (100–115 ml each) was treated as described for the predrug serum blank.

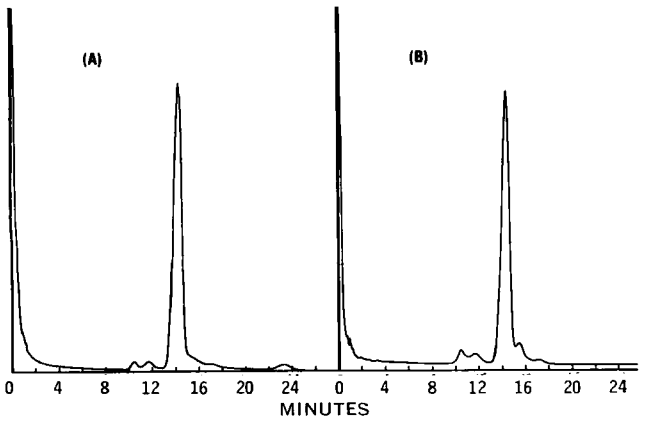
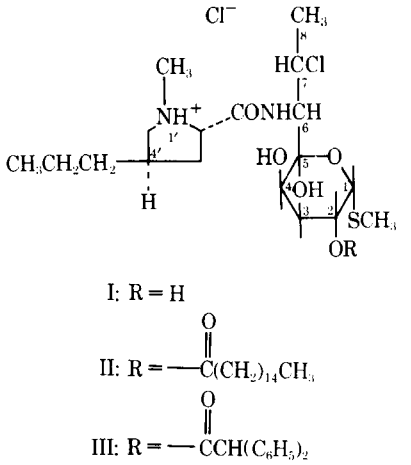


Figure 3—*Gas–liquid chromatogram of IV: (A) isolated solid, and (B) 20 mg/ml II and V reaction solution.*

[4] Grand Island Biological.



I: R = H

II: R = —C(CH₂)₁₄CH₃ (O)

III: R = —CCH(C₆H₅)₂ (O)

IR Spectroscopy—All IR spectra were recorded on a double-grating instrument[5] using double-beam total reflectance optics[6] and 50 × 2 mm KRS-5 plates.

NMR Spectroscopy—NMR spectra were recorded on a 60-MHz instrument[7] in deuterochloroform using tetramethylsilane as a reference. A sweep time of 250 sec and a sweep width of 500 Hz at an amplitude of 10 were used.

GLC—The gas-liquid chromatograms were recorded on a high efficiency, biomedical unit[8] using a 1.21-m (4-ft) × 0.63-cm (0.25-in.) glass column packed with a 3.8% UCW 98 on Diatoport S, 80–100 mesh. The column was operated in the isothermal mode at 300° and was monitored with a flame detector.

GLC–Mass Spectroscopy—The GLC-mass spectroscopy spectra were recorded[9] using a GLC column as already described. The column was limited to operation at 280° with the separator at 290°. The instrument was operated at 70 ev, and the source and detector slits were opened to three times normal. The detector was focused at a constant mass of *m/e* 126.4, and all other conditions were as described in the instrument manual specifications.

GLC Method—*Serum Extraction*—Five milliliters of serum in a 15-ml centrifuge tube was adjusted to pH 8 using 0.2 *M* KOH. After adding 0.25 ml of pH 8, 0.1 *M* tromethamine buffer, and 5 ml benzene, the tube was agitated for 15–30 min and then centrifuged for 10 min at 2000 rpm. The benzene layer was transferred to a 45-ml centrifuge tube, and the extraction of the serum was repeated twice more. The extracts were combined and evaporated to dryness under nitrogen.

TLC—A 20 × 20-cm silica gel plate was sectioned into five 4 × 20-cm strips. Serum extracts were streaked 0.5 cm from one end. The extraction tube was washed twice with 0.2-ml aliquots of chloroform, and the combined washes were also applied to the plate. Four strips were used for serum samples and the fifth for the II standard. The plates were irrigated in either of two mobile phases: *(a)* chloroform–ethyl acetate (3:1), and *(b)* hexane–ether–methyl ethyl ketone–methanol–ammonium hydroxide (70:15:15:3:0.25).

The best resolution was obtained by two unidimensional irrigations in the same system. After development, the strip containing the reference sample (II) was sprayed with permanganate–periodate reagent. The silica gel in the sample strips in the region of the R_f of the II standard was removed, and the ester was eluted into centrifuge tubes with five 5-ml portions of chloroform. The solvent was evaporated to dryness, and the vials were rinsed with a small amount of chloroform. The solvent was evaporated to dryness, and the vials were rinsed with a small amount of chloroform which was then transferred to a second centrifuge tube and evaporated to dryness for derivatization.

Trimethylsilylation—Clindamycin 2-diphenylacetate hydrochloride (III) was used as an internal standard for GLC analysis. One-half microgram was added to sample and standard extracts and unextracted standards with 0.1 ml of V, after which each

[5] Perkin-Elmer model 257.
[6] Wilks.
[7] Varian model T-60.
[8] Hewlett-Packard model 402.
[9] On an LKB 9000 GLC–mass spectroscopy unit.

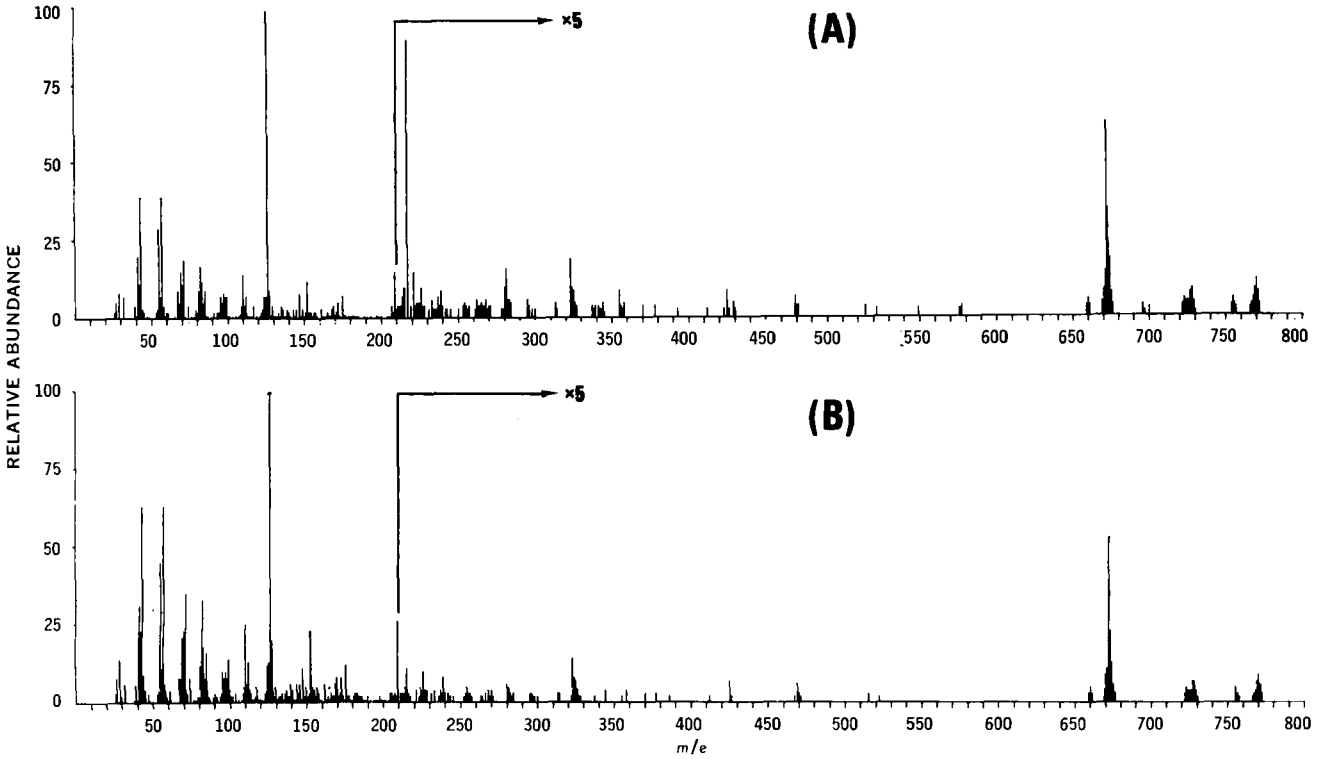


Figure 4—*Mass spectra of IV (using GLC–mass spectroscopic technique): (A) isolated solid, and (B) 20 mg/ml II and V reaction solution.*

tube was stoppered and shaken at room temperature for 20 min. The derivatives were extracted by adding 2 ml of cyclohexane and 1 ml of water to the tubes and shaking for 15 min or until no cloudiness was present in either phase. After separation, the aqueous layer was extracted with an additional 2 ml of cyclohexane, which was combined with the first extract and evaporated to dryness. The residues were dissolved in 2 ml of chloroform for analysis.

GLC Analysis—The analysis of IV was carried out on a GLC unit[8] using a 0.61-m (2-ft) × 0.63-cm (0.25-in.) o.d. glass column packed with a mixture of 1.5% (w/w) lexan and 1.5% (w/w) polysulfone on 80–100-mesh Gas Chrom Q. The column, operated isothermally at 295°, was monitored with a flame-ionization detector. Flow rates and other temperatures were consistent with manufacturer's manual specifications. The standard of IV was characterized[9] using the same column (temperature 290°). The mass spectrometer was operated as previously described.

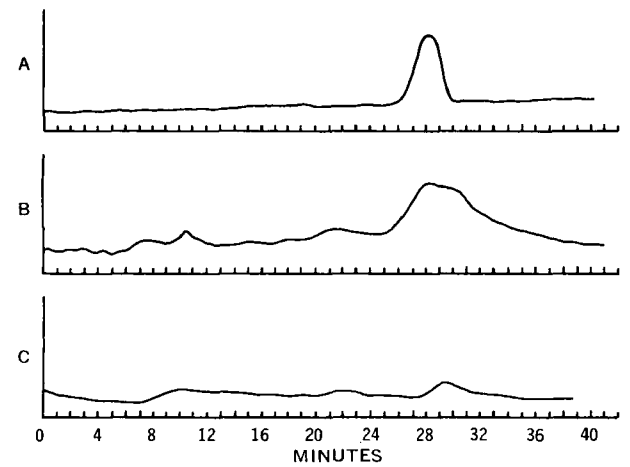


Figure 5—*Single-ion focused GLC–mass spectroscopy spectra of: (A) 1 μl, 1 mg/ml IV reaction solution (amplification ×1); (B) 2 μl, trimethylsilyl ether derivatized extract of 5 mg/ml II in serum (amplification ×10); and (C) 2 μl, trimethylsilyl ether derivatized extract of 1 mg/ml II in serum (amplification ×10).*

RESULTS AND DISCUSSION

GLC–Mass Spectroscopy Method—The basis of the GLC–mass

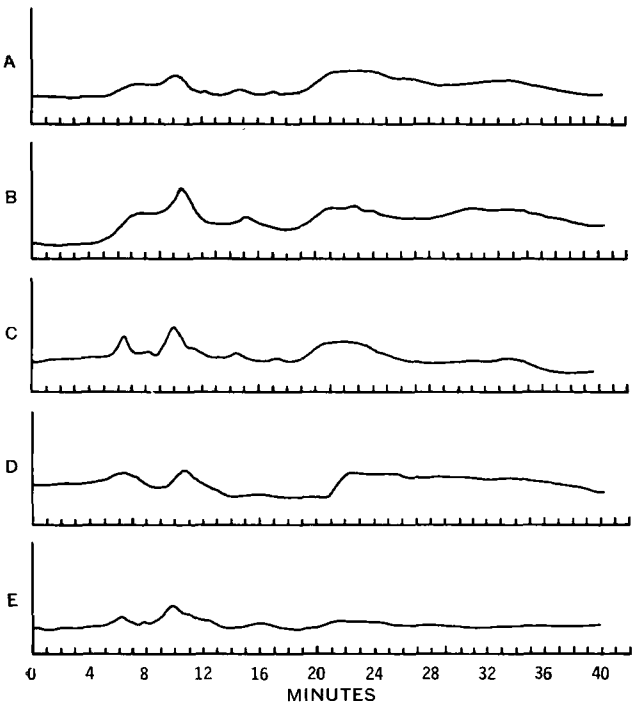


Figure 6—*Single-ion focused GLC–mass spectroscopy spectra of: (A–D) 2 μl, Subjects 1–4, derivatized serum extracts (amplification ×10); and (E) 2 μl, serum blank (amplification ×10).*

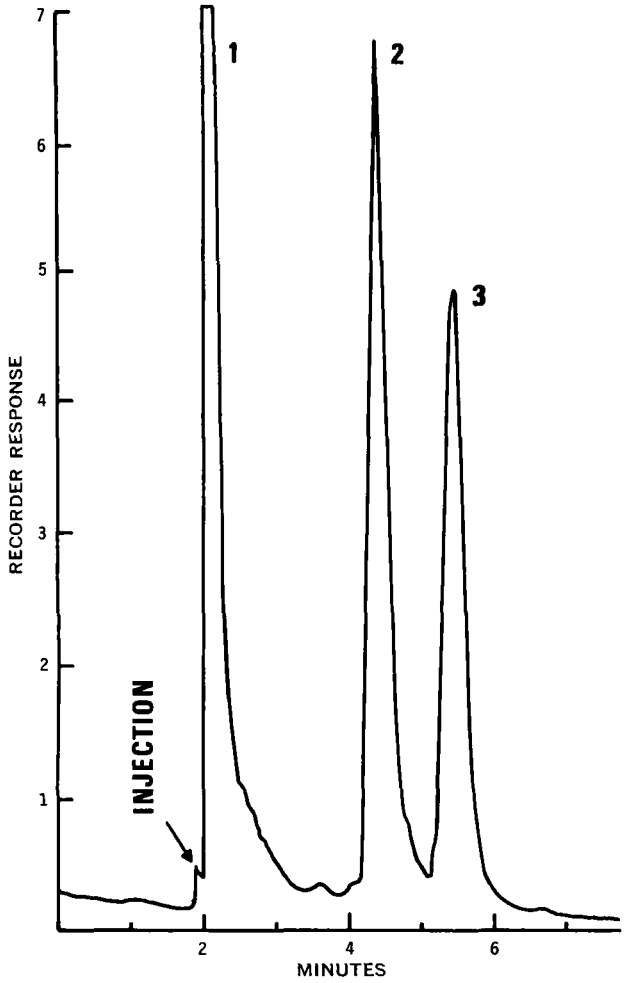
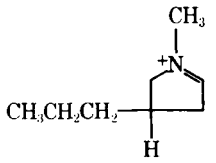


Figure 7—*Gas–liquid chromatogram of serum extract: (1) solvent, (2) IV, and (3) III silyl ether.*

spectroscopy method for the detection and quantitation of clindamycin palmitate is the observation of a peak (*m/e* 126) at a GLC retention time of 27–29 min in trimethylsilylated human serum extracts. In this single-ion monitoring technique (2), *m/e* 126 was chosen because it is the base peak of all clindamycin derivatives and results from a fragmentation reaction yielding the ion:



which carries 99% of the charge of the reaction. The inherently small yields of intact ester obtained from spiked serum samples and the nature of serum lyophilates and extracts precluded any direct characterization of IV in the samples except by comparison of GLC retention times and generation of the *m/e* 126 peak. To test the derivatization procedures and establish the nature of the derivatives, IV was prepared, isolated, and characterized by several methods. The internal reflectance IR spectra of clindamycin palmitate hydrochloride and its trimethylsilyl ether are shown in Fig. 1. The spectrum of the silyl ether is consistent with spectra of other similar derivatives prepared in this laboratory. Of particular significance is the lack of OH absorption and the presence of strong bands at 1250, 895, and 840 cm^{-1} [associated with O—$Si(CH_3)_3$ vibrations]. The bathochromic shift of the amide II band in clindamycin palmitate (1560 cm^{-1}) to 1500 cm^{-1} in IV is in keeping with a change in the ionic character of the compounds, clindamycin palmitate being the hydrochloride salt and IV a free base.

The NMR spectrum of IV is shown in Fig. 2. The spectrum is in agreement with the basic structure of clindamycin palmitate, the anomeric proton showing a doublet at 5.6 ppm and the NCH_3 and SCH_3 groups giving rise to singlets at 2.4 and 2.1 ppm, respectively.

The upfield shift of NCH_3 (normally at 3.0 ppm in clindamycin hydrochloride) agrees with the nonionic character of 1′-nitrogen in the free base. The intense peak at 1.24 ppm accounts for the 28 CH_2 protons in the palmitate ester group and two CH_2 protons in the 4′-propyl group (I). Using the integral for the 30 protons described, the peak at 0.15 ppm [—$Si(CH_3)_3$] integrates to 18 protons or two trimethylsilyl groups.

The gas–liquid chromatograms of isolated IV and a 20-mg/ml standard reaction solution of II and V are shown in Fig. 3. The retention times (14.5 min) are identical, thus indicating that the compound emerging from the column at this time is IV. The 20-mg/ml solution is typical of standard solutions used to establish retention times in this GLC–mass spectroscopy unit.

The mass spectra of both isolated IV and the 20-mg/ml standard solutions are shown in Fig. 4. The spectra were recorded after passage of the compound through the GLC column under the conditions described under *Experimental.* The retention time for both compounds is about 29 min. The molecular ion mass of 770 represents the disilyl ether of clindamycin palmitate minus *m/e* 36, which results from loss of hydrogen chloride through dehydrohalogenation of the 7,8- or 6,7-positions (see I). This reac-

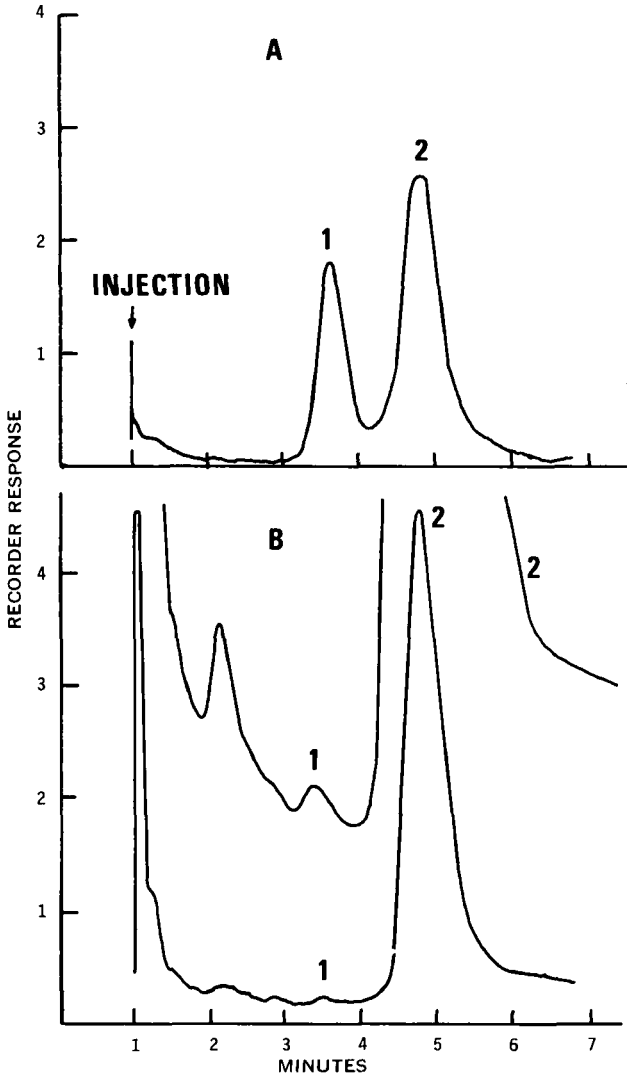


Figure 8—*Single-ion focused GLC–mass spectroscopy spectra of: (A) standard: 1, IV; 2, III silyl ether (amplification ×10); and (B) subject serum extract: 1, IV; 2, III silyl ether (upper amplification ×100, lower amplification ×10).*

Table I—Serum Concentrations of Free Clindamycin (Bioassay) and Clindamycin 2-Palmitate (GLC Assay) in Human Subjects following a Single Oral Dose of 300 mg Drug

Subject	Sampling Time, min	Clindamycin Bioactivity, μg/ml[a]	Estimate of Intact Clindamycin 2-Palmitate, μg/ml	Standard Deviation	95% Confidence Limits	
1	15	—[b]	0.1	0.3	−0.6	0.8
	30	1.99	0.2	0.3	−0.5	0.8
	45	—[b]	0.1	0.3	−0.6	0.8
	60	3.18	0.1	0.3	−0.6	0.8
2	15	—[b]	—[c]	—	—	—
	30	2.24	—[c]	—	—	—
	45	—[b]	—[c]	—	—	—
	60	2.29	—[c]	—	—	—
3	15	—[b]	0.2	0.3	−0.5	0.8
	30	1.32	—[c]	—	—	—
	45	—[b]	—[c]	—	—	—
	60	2.08	—[c]	—	—	—
4	15	—[b]	0.1	0.3	−0.7	0.7
	30	0.94	0.1	0.3	−0.6	0.7
	45	—[b]	0.1	0.3	−0.7	0.8
	60	1.57	—[c]	—	—	—
5	15	—[b]	0.1	0.3	−0.6	0.8
	30	1.29	—[c]	—	—	—
	45	—[b]	—[c]	—	—	—
	60	2.26	—[c]	—	—	—
6	15	—[b]	—[c]	—	—	—
	30	0.90	0.1	0.3	−0.6	0.8
	45	—[b]	—[c]	—	—	—
	60	1.17	—[c]	—	—	—
7	15	—[b]	0.2	0.2	−0.3	0.7
	30	1.71	0.4	0.3	−0.3	1.0
	45	—[b]	0.5	0.3	−0.2	1.2
	60	2.83	0.3	0.3	−0.4	0.9
8	15	—[b]	—[c]	—	—	—
	30	1.11	—[c]	—	—	—
	45	—[b]	0.1	0.3	−0.6	0.8
	60	1.52	0.1	0.3	−0.6	0.7

[a] By independent analysis. [b] No data available. [c] No measurable peak for clindamycin 2-palmitate observed in gas–liquid chromatogram.

tion occurs in all clindamycin compounds analyzed by mass spectroscopy. These data establish that the compound generated in the clindamycin palmitate silylation reactions is the disilyl ether.

The GLC–mass spectroscopy recordings for 1 μg of IV from a standard reaction solution and the derivatized 1- and 5-μg/ml clindamycin palmitate spiked predrug serums are shown in Fig. 5. The retention time for the 1-μg/ml spiked serum extract is slightly higher than that for other samples and reflects variation in the retention time associated with running samples on different days. Although actual quantity of II extracted from the spiked predrug serum was not determined, the mass chromatograms do indicate that it is possible to detect levels of 1 μg/ml (2 ng in 2-μl samples). The GLC–mass spectroscopy recordings for extracts of serum from clinical subjects are shown in Fig. 6. A predrug serum blank extract is included as a control.

Although there is considerable variation in the baselines of the serum extracts (most probably due to instrument variability and extracted blood solids), all subject serums showed minima in the region of 28–29 min. On the basis of this evidence, it was concluded that no more than 1 μg/ml clindamycin palmitate exists in the serum of these four subjects. The sensitivity of the method is based on the response of the 1-μg/ml spiked predrug serum extract. No statistically significant lower limit was placed on the sensitivity because of the lack of sufficient sampling. This was precluded by the inherent nature of the GLC–mass spectroscopy system to diminish in sensitivity on repetitive sampling of blood extracts due to source fouling. The presence of clindamycin in these serums had been established independently. Quantitation of samples of the same subject serums as used in this study indicated an average level of 4 μg/ml of clindamycin at the time the blood was drawn (1.5 hr).

GLC Method—This method, developed independently, is based on benzene extraction of serum samples, separation of the clindamycin palmitate from lipids by TLC, and subsequent quantitation of the trimethylsilyl ether derivatives by GLC. Experiments with various solvents and pH conditions revealed that benzene and pH 8 were optimum. Recovery of clindamycin palmitate from spiked serum samples, including the TLC step, varied between 23 and 49%, depending upon the subject serum used. Consistent recoveries were obtained for levels above 0.5 μg/ml of clindamycin palmitate, but recoveries varied considerably below this level. The clinical protocol used for subjects in this study differed from that used for the GLC–mass spectroscopy study in both dose and sampling times. Single oral doses of 494 mg II (300-mg base equivalents of clindamycin) with blood samplings at 0.25, 0.50, 0.75, and 1 hr were used for the GLC method, while 988-mg doses with blood samplings at 1.5 hr only were used for the GLC–mass spectroscopy technique. The latter was chosen since the peak serum levels of clindamycin after administration of clindamycin palmitate hydrochloride occur at 1–1.5 hr (3). The former conditions were chosen to cover shorter time periods in the event that clindamycin palmitate, if present, was being rapidly hydrolyzed.

Prior to analyzing all of the subject serums, IV (Fig. 7) was compared with one of the subject's serum by single-ion focused mass spectroscopy as previously described. The derivative had a fragment at m/e 126 and a retention time of 3.6 min and corresponded to a weak but observable peak in the serum sample (Fig. 8). The results of subsequent GLC analysis of all subjects' serums are given in Table I. Analysis of these results indicates that the concentration of clindamycin palmitate was below the level of assay sensitivity. This was determined by least-squares regression analysis of the standard curves which resulted in a lower limit of sensitivity, for which the 95% confidence limits did not include zero, of 0.66 μg IV/ml of serum. Therefore, after administration of a single oral dose of 494 mg II, the serum concentration of intact clindamycin palmitate in the blood could not be differentiated statistically from zero. Combined with the results

from the GLC–mass spectroscopy method, it is apparent that no significantly measurable quantities of clindamycin palmitate can be found in the serum of subjects receiving 494–988 mg of clindamycin palmitate hydrochloride 0.25–1.5 hr after administration.

Although GLC methods have been used extensively for the detection and quantitation of antibiotics and other pharmaceuticals, the single-ion focused mass spectroscopy method represents the application of a new mass spectroscopy technique to antibiotic research. With suitable sample extraction procedures, single-ion focusing can be a very specific and highly sensitive means of characterizing and quantitating antibiotics.

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[x] To whom inquiries should be directed.

Controlled Drug Release from Polymeric Devices I: Technique for Rapid *In Vitro* Release Studies

YIE W. CHIEN[x], HOWARD J. LAMBERT, and DONALD E. GRANT

Abstract □ A simple system was developed which allows the rapid and reproducible quantification of parameters influencing the release of drug by solid polymeric devices. The apparatus utilizes only 150 ml of elution medium and maintains "sink" conditions with water-miscible (nonaqueous) cosolvent combinations. Constant diffusion layer thickness is controlled by constant stirring and fixed temperature conditions. Factors influencing the rate of release of ethynodiol diacetate, a synthetic progestin, from solid silicone polymer vaginal devices were evaluated. The cumulative amount of drug released was proportional to the square root of time. The drug diffusivity was calculated and found in excellent agreement with previously reported data for similar compounds in silicone matrixes. The presence of a matrix-controlled process was confirmed by the independence of the steady-state diffusion rate on drug solubility in the eluant.

Keyphrases □ Drug release, controlled—method for measuring rapid *in vitro* release from polymer devices, ethynodiol diacetate from silicone polymers □ Transport, drug—rapid *in vitro* method for measuring steady-state drug release flux, ethynodiol diacetate from solid silicone polymer vaginal devices □ Permeation, drug—ethynodiol diacetate through solid silicone matrix, rapid *in vitro* method for measuring steady-state drug release flux □ Ethynodiol diacetate—release from solid silicone vaginal devices, effect of concentration and solubility of drug, rapid *in vitro* method for measuring steady-state drug release flux □ Contraceptives—rapid *in vitro* method for measuring ethynodiol diacetate release from solid silicone vaginal devices □ Vaginal devices—method for measuring drug release from polymer matrix

Recent interest has centered on the idea of replacing daily administration of a drug with delivery devices that release a constant effective dose to target tissues *via* a controlled-release mechanism (1–10). The high permeability of silicone polymer to steroids has been applied to the development of drug-filled and drug-impregnated silicone devices for long-acting hormonal contraception (11–24).

It is apparent from the literature that the development of a suitable measurement system is needed to understand drug release mechanisms in *in vivo* systems and also to correlate *in vitro* and *in vivo* drug release rate profiles accurately. An *in vitro* apparatus for studying the release of medroxyprogesterone acetate from a silicone device was designed (25). Because of the low aqueous solubility of medroxyprogesterone acetate, a large quantity (60 liters/day) of distilled water was used for dissolution to approximate a sink condition. The sensitivity of the diffusion cell was ignored (26–28). The drug release rate was determined weekly by assaying the residual drug content or measuring the thickness of the depletion zone in the devices. The same mechanical concepts were also applied in constructing a diffusion system for studying the release of progesterone from a polyethylene matrix (29).

One prime concern in making permeability measurements is the ability to determine the rate of drug release reliably in as short a time as possible (26–28). The development of such a technique and system should also make possible the direct measurement of drug release flux. With these considerations in mind, a relatively simple and easily constructed drug release system was designed, and a rapid, reproducible technique was developed. The ring-shaped polymeric device was mounted in a holder and rotated at constant speed in the elution medium so that constant hydrodynamics were maintained. This procedure allowed for a constant thickness of diffusion layer on the immediate surface of the device and a homogeneous drug concentration in the elution medium. Additionally, a polymer-compatible, drug-stable,

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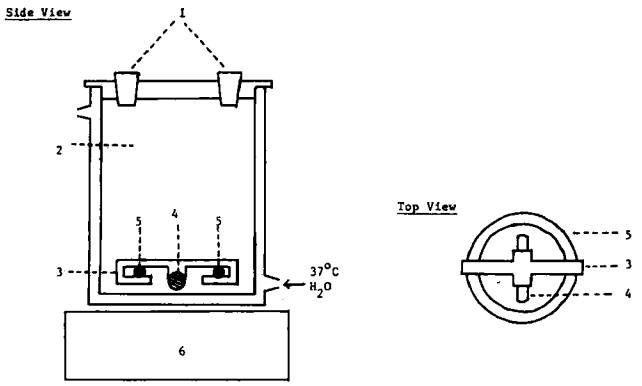

Figure 1—*Schematic representation of the* in vitro *system used to measure the release of drug from silicone devices. Key: 1, sampling holes with Teflon stopcocks; 2, water-jacketed cell; 3, Plexiglas holder for ring-shaped silicone devices; 4, Teflon-coated spin bar; 5, drug-impregnated silicone devices; and 6, magnetic stirrer. Up to six cells were connected in series and thermostated at 37°.*

and water-miscible macromolecular substance was mixed with the distilled water to increase drug solubility. A perfect sink condition was maintained throughout the experiment. The cell sensitivity was sufficiently high that hourly or daily measurements of drug release flux were possible. The drug release profile was followed closely by directly assaying the drug concentration in the elution medium. The developed system and technique provide a rapid methodology for reliably characterizing the pattern and rate of drug release from polymeric devices. This report defines the system and technique developed and compares the *in vitro* drug release profiles obtained with the results taken from the literature.

EXPERIMENTAL

Drug Release System—The system developed for studying the pattern of drug release is shown schematically in Fig. 1. A ring-shaped device was mounted in the arms of a Plexiglas holder. The holder was then rotated at a constant speed (360 rpm) in 150 ml of elution medium at 37°. At scheduled intervals, a given volume (25–150 ml) of elution medium was sampled and replaced with the same quantity of drug-free elution medium maintained at 37°. The sample was then assayed.

Drug Delivery Devices—The delivery devices were prepared[1] by mixing the required amount of ethynodiol diacetate or other steroid into a silicone elastomer[2] and then polymerizing with catalyst in a mold at room temperature to form a ring (6 × 50 mm to 6 × 63 mm).

Elution Medium—The elution medium was prepared by mixing a given volume of reagent grade polyethylene glycol 400[3] with distilled water. The drug solubility–polyethylene glycol 400 concentration profile, as expected from the following theoretical expression, is shown in Fig. 2:

$$\log C_s = \log C_{H_2O} + ef \qquad \text{(Eq. 1)}$$

where C_s and C_{H_2O} denote the solubilities of drug in the polyethylene glycol 400–water mixture and in the pure water system, respectively, e is the slope of solubility–concentration profile, and f is the volume fraction of polyethylene glycol 400 (% v/v) added. The solubility of ethynodiol diacetate (C_{H_2O} = 1.37 mg/100 ml) was enhanced remarkably with the addition of polyethylene glycol 400, so perfect sink condition was maintained.

[1] By Dow Corning Corp., Midland, Mich.
[2] Silastic.
[3] Matheson, Coleman and Bell.

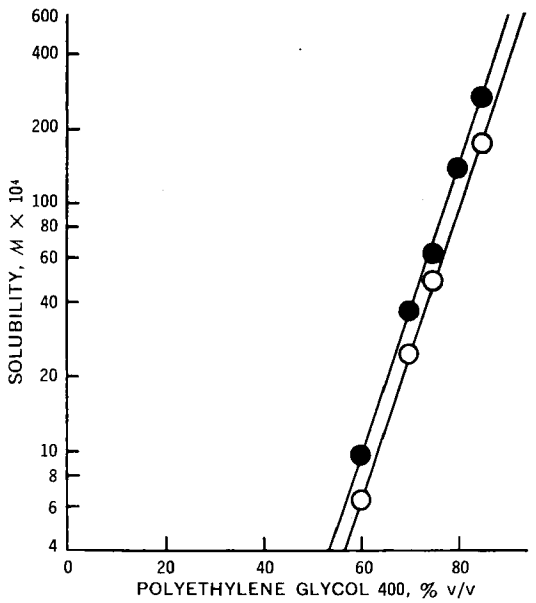

Figure 2—*Semilogarithmic relationship between the solubility of ethynodiol diacetate and the volume fraction of polyethylene glycol 400 in the elution medium at 37° (●) and 25° (○).*

The stability of ethynodiol diacetate and its compatibility with the silicone polymer also were examined for an observation period of 3 weeks. Both were stable and compatible.

Analytical Method—From 1 to 5 ml of sample solution was mixed with 10 ml of methanol, spectrophotometric grade, and 1 ml of 6 *M* hydrochloric acid solution. The mixture was boiled for exactly 5 min and then cooled to room temperature, and methanol was added to 25 ml. The drug concentration in such a solution was measured spectrophotometrically against a reference prepared from the same sample solution without acidic hydrolysis. The peak absorbance (at λ_{max} 236 nm) was recorded. No absorption peak was observed for the solution without acidic hydrolysis. The molar absorptivity determined was 19,140 M^{-1} for the acidic product (3,5-diene derivative) of ethynodiol diacetate. The analytical procedure for medroxyprogesterone acetate was the same as that for ethynodiol diacetate, except that no acidic hydrolysis was required and the same elution medium without drug was used as the reference. The λ_{max} and ϵ values for medroxyprogesterone acetate are 241 nm and 17,520 M^{-1}, respectively.

Determination of Drug Solubility—The solubility of ethynodiol diacetate at the study temperature (25 or 37°) was determined by vigorously mixing an excess amount of powdered drug

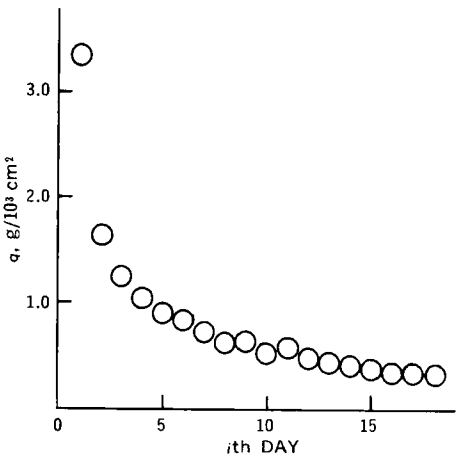

Figure 3—*Daily amount of drug release per unit area of device* (q) *on the ith day for a silicone device containing 12.79 g/10² cm³ of ethynodiol diacetate.*

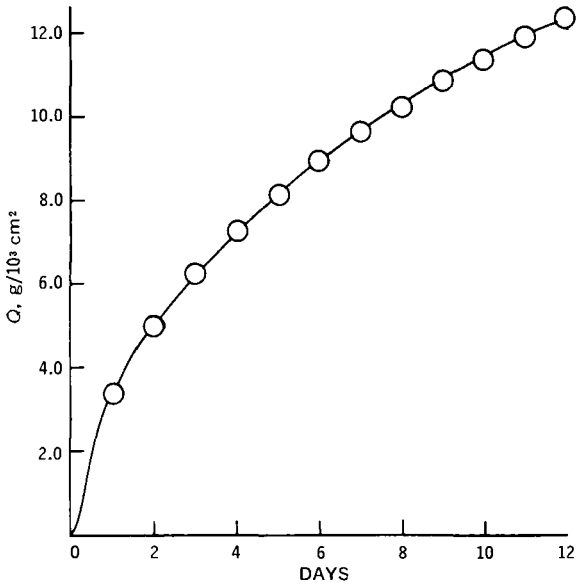

Figure 4—*Cumulative amount of drug release* (Q) *per unit area of device* versus *time for a silicone device containing 12.79 g/10² cm³ of ethynodiol diacetate.*

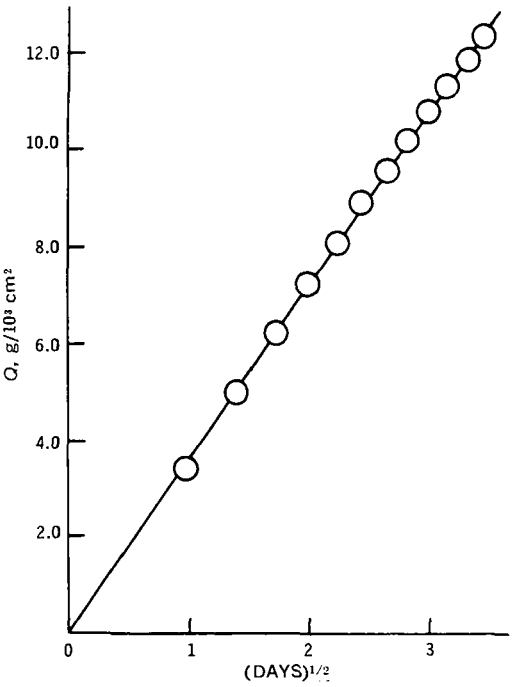

Figure 5—*Linear relationship between the cumulative amount of drug released per unit area of device* (Q) *and the square root of time* ($t^{1/2}$). *The rate of drug release calculated from the slope is 3.57 g/10³ cm²/day$^{1/2}$.*

in 10 ml of elution medium. At equilibrium (48 hr), samples were rapidly filtered through syringes equipped with a filter holder[4] containing a glass fiber membrane[5]. For the 37° measurements, the whole filtration apparatus was preheated. The clear filtrate was then assayed as previously described.

The solubility of ethynodiol diacetate in the silicone polymer was determined in the same manner as already described, except that a silicone liquid with 24 dimethylsiloxyl units was used. After filtration, one part of the clear filtrate was extracted with 10 parts of methanol with shaking at 37° for 24 hr. The solubility of ethynodiol diacetate in the silicone polymer at 37° was 1.4791 mg/ml.

RESULTS AND DISCUSSION

In the present study, a speed of 360 rpm for the silicone elastomer[2] was satisfactory. At this speed, the thickness of the hydrodynamic diffusion layer on the immediate surface of the device (26) was calculated, according to Eq. 2, to be from 63×10^{-4} to 70×10^{-4} cm, depending upon the kinematic viscosity of the elution medium used:

$$\delta_D = 1.62 D^{1/3} \omega^{-1/2} \nu^{1/6} \qquad \text{(Eq. 2)}$$

where δ_D is the thickness of the hydrodynamic diffusion layer around the device, D is the diffusion coefficient of the drug species examined, ω is the angular rotation speed, and ν is the kinematic viscosity of the elution medium. Apparently, the magnitude of δ_D may be decreased by the increase of the angular rotation speed. The effect of ω on the thickness of the hydrodynamic diffusion layer has been well established (26). The magnitude of δ_D should be held constant for an investigation of the real mechanism of matrix-controlled permeation and for a meaningful comparison between experimental observations.

The drug release profile obtained from such a system is shown in Fig. 3, where the amount of drug release daily *(q)* from the ring-shaped drug-impregnated silicone matrix is plotted against time. A large quantity of drug was released initially, and the daily amount of drug released then decreased with time. A plot of the cumulative amount of drug *(Q)* released from a unit area of matrix with time yields a curved *Q–t* profile (Fig. 4). The same type of observation was reported previously for both *in vitro* (23, 25, 29–31) and *in vivo* (12, 29) studies. The higher initial drug release has been attributed to release of drug molecules at the matrix surface (*i.e.*, burst effect) (32).

Theoretical treatment of the data obtained indicated that the release of drug from an insoluble, inert polymeric matrix, (*e.g.*, the silicone polymer in the present investigation) is best described by the equation developed by Higuchi (30) if diffusion is the rate-determining factor for the drug release process:

$$Q = \sqrt{D_m(2A - C_s)C_p t} \qquad \text{(Eq. 3)}$$

where Q is the cumulative amount of drug released per unit area of device (g/cm²); A is the amount of solid drug impregnated in a unit volume of device (g/cm³); C_s and C_p are the solubilities of the drug in the elution medium and the polymer phase, respectively; t is time; and D_m is the diffusivity of the drug species in the matrix.

The theoretical model defined in Eq. 3 indicates that the cumulative amount of drug released *(Q)* from a unit area of matrix

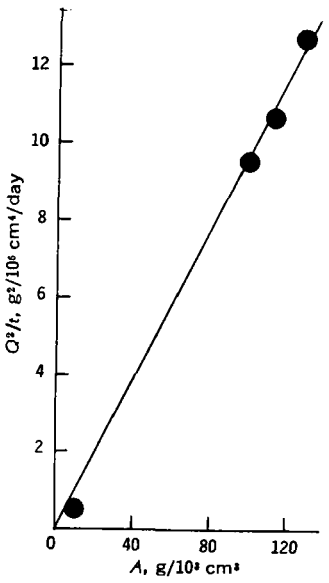

Figure 6—*Effect of the drug concentration* (A) *impregnated in the silicone devices on the square of the drug release rate* $(Q/t^{1/2})^2$. *The slope, defined as* $2D_mC_p$ *in Eq. 6, is calculated to be 96.67 g/10⁶ cm/day.*

[4] Millipore.
[5] Reeve Angel.

Table I—Effect of Drug Solubilities (C_s) on Rate of Drug Release from Silicone Devices Containing 98.8 g/10³ cm³ Ethynodiol Diacetate

Volume Fraction of Polyethylene Glycol 400, %	C_s, g/10³ cm³	Rate of Drug Release ($Q/t^{1/2}$) Initial[a], g/10³ cm²/day$^{1/2}$	Steady State[b], g/10³ cm²/day$^{1/2}$
65	0.78	1.597	2.99
70	1.39	2.607	3.115
75	2.43	3.024	3.09
80	4.45	3.478	3.04
85	8.00	3.651	3.05

[a] Initial rate of drug release is calculated from the 1st day drug release profile. [b] Steady-state rate of drug release is estimated from the slope of Q–$t^{1/2}$ profile up to 18 days.

should be a linear function of the square root of time ($t^{1/2}$). As demonstrated in Fig. 5, this linear relationship was obeyed when the drug release data in Fig. 4 were plotted following Eq. 3. The slope of the Q–$t^{1/2}$ profile is defined as:

$$Q/t^{1/2} = \sqrt{D_m(2A - C_s)C_p} \quad \text{(Eq. 4)}$$

Experimentally, the system was so designed that the amount of solid drug (A) in the matrix was much larger than the solubility of drug (C_s) in the elution medium ($A \geq 98.8$ g/10³ cm³ and $C_s \leq 8$ g/10³ cm³). Therefore, $2A \gg C_s$ may be established, and Eq. 4 may be reduced to Eq. 5 without inducing any significant error:

$$Q/t^{1/2} = \sqrt{2D_mAC_p} \quad \text{(Eq. 5)}$$

Taking the square of both sides of Eq. 5 results in:

$$Q^2/t = 2D_mC_pA \quad \text{(Eq. 6)}$$

On the basis of Eq. 6, linearity should exist between Q^2/t and A. Experimentally, this linearity was found to follow (Fig. 6); the slope of this line is $2D_mC_p$. When the solubility of drug in the polymer (C_p) is known, the magnitude of D_m, the diffusivity of drug in the matrix, may be estimated from this slope. In the present study, D_m was calculated to be 3.79×10^{-7} cm²/sec for ethynodiol diacetate. This result is very close to the 3.36×10^{-7} cm²/sec for medroxyprogesterone acetate (31) and the $3.0 \sim 3.3 \times 10^{-7}$ cm²/sec for chlormadinone acetate (23) determined by the lag time method using a similar silicone matrix. All three compounds are progestins. By using the semilogarithmic relationship between the self-diffusion coefficients and the number of the dimethylsiloxyl monomer units reported by McCall *et al.* (33), it is interesting to note that these steroids have diffusion coefficients in the silicone polymer approximately equal to the self-diffusion coefficient of a dimethyl silicone molecule containing 10 silicon atoms. One could interpret this finding to indicate that an opening in the polymer sufficiently large to permit a steroid molecule to jump from one hole to another would require the motion of a silicone chain segment of the order of 10 monomer units in length. By the same approach, Robb (34) concluded that the permeation of oxygen, nitrogen, and carbon dioxide required the motion of three silicon atoms.

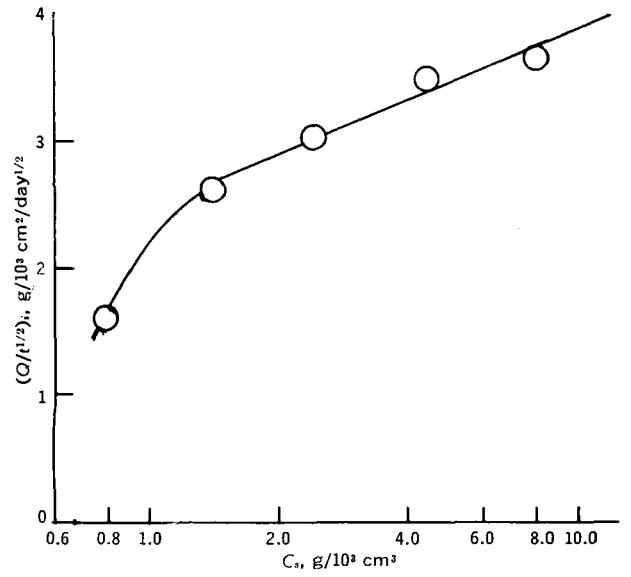

Figure 7—*Effect of the drug solubility* (C_s) *in the elution medium on the initial (1st day) rate of drug release.*

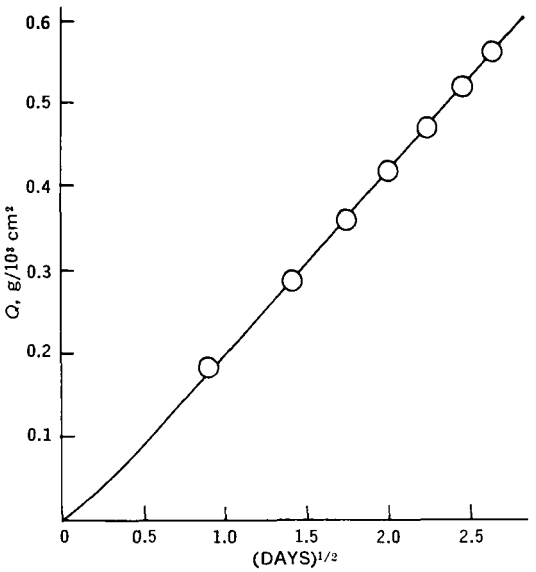

Figure 8—*Drug release profiles for medroxyprogesterone acetate measured in the present system.*

In developing Eq. 5, the C_s term in Eq. 4 was neglected on the basis of $2A \gg C_s$. Experimentally, a series of drug release studies were carried out in the elution medium with a wide range of drug solubilities (0.78 ~ 8.00 g/10³ cm³) to determine the effect on drug release profile (Table I). The data indicate that only the initial rate of drug release (the 1st day drug release) is dependent on the solubility of the drug in the elution medium (C_s). On the other hand, the steady-state rate of drug release is constant although the magnitude of C_s is enhanced 10-fold. The proportionality of initial drug release rate to drug solubility is also illustrated in Fig. 7, where a linear relationship is established after $C_s \geq 1.39$ g/10³ cm³. The linearity may be correlated with the dissolution kinetics of the drug concentration on the matrix surface.

The validity of Eqs. 4 and 5 and their correlation to the experimental data are further analyzed in Table II. Apparently, the omission of the C_s term results in a maximum error of only 2%. The experimental results correlate perfectly with the values calculated from the theoretical model of either Eq. 4 or 5.

The drug release pattern of medroxyprogesterone acetate from the silicone matrix was also examined in the same system. The reasons for including this drug in this investigation are simply that it has been studied extensively and literature reports are available for comparison. As seen in Fig. 8, the drug release profile for medroxyprogesterone acetate also followed the Q–$t^{1/2}$ relationship as did the ethynodiol diacetate reported in the present studies. Roseman and Higuchi (25) reported the same observation. The release of chlormadinone acetate was also described by the same theoretical model (23).

For comparison, the rate of drug release for medroxyprogesterone acetate, reported by Roseman and Higuchi (25), was cited in Table II. The data on drug solubility in the silicone polymer (C_p) and the drug diffusivity in the polymeric device (D_m) available in the literature (31) were also applied to estimate the theoretical rate of drug release (Table II). Both of these literature values are close to the experimental result (0.218 g/10³ cm²/day$^{1/2}$) and demonstrate the applicability of the drug release system. The comparisons point out that this system allows the rapid and direct characterization of the mechanism and rate of drug release from polymeric drug devices.

Table II—Comparison of Theoretical Rates of Drug Release with Experimental Data

Drug	Volume Fraction of Polyethylene Glycol 400, %	Rate of Drug Release, $g/10^3\ cm^2/day^{1/2}$			
		Theoretical[a]			
		A	*B*	Experimental	Ratio[b]
Ethynodiol diacetate	65	3.085	3.09	2.99	0.97
	70	3.081	3.09	3.115	1.01
	75	3.073	3.09	3.09	1.00
	80	3.056	3.09	3.04	0.99
	85	3.028	3.09	3.05	0.99
Medroxyprogesterone acetate	75	0.282[c]	0.241[d]	0.218	0.905

[a] *A* and *B* values are calculated from Eqs. 4 and 5, respectively, where $D_m = 3.27 \times 10^{-2}$ cm²/day, $A = 98.8\ g/10^3\ cm^3$, and $C_p = 1.4791\ g/10^3\ cm^3$. [b] Ratio of the experimental data over the theoretical *B* values. [c] Calculated from the drug release profile reported in Ref. 25. [d] Estimated from the data reported in Ref. 31 following Eq. 5.

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[x] To whom inquiries should be directed.

Experiments in Powder Blending and Unblending

J. C. SAMYN and K. S. MURTHY[x]

Abstract □ The effects of blending and segregation procedures on the uniformity of aspirin–lactose (1:99) blends were studied. The blending was conducted in a twin-shell blender with and without a high shear attrition bar and evaluated based on the mean and the coefficient of variation of 20 samples. In the mixing of the dry powders, the particle sizes were varied; preblending and the use of silica were also studied. The best blend was obtained with milled aspirin and powdered lactose when the liquid feed bar was used. Without the bar, a restriction often necessary for direct compression and heat-sensitive materials, the milled aspirin proved difficult to distribute. Segregation studies carried out in a two-dimensional segregation cell suggested that materials with good flow show unmixing tendencies, whereas blends with poor flow are less apt to separate. In the second part of the study, the drug was added as a solution in ethanol. This method yielded homogeneous blends when limited amounts of solvents were used. However, with a large quantity of solvent, drug migration occurred during drying. Subsequent milling of the dried material did produce good drug distribution, but the powder blend retained the potential to segregate.

Keyphrases □ Blending, powders—influence of attrition bar, particle size, preblending, solvents, and use of silica on uniformity of aspirin–lactose blends □ Segregation, powders—effects of processing variables on uniformity of aspirin–lactose blends □ Drugs, powder blends—effects of processing variables on uniformity of aspirin–lactose blends

One basic requirement in solid formulations is the preparation of a uniform blend prior to final tableting or encapsulation. The importance of uniform drug distribution in the final dosage form is recognized by the official compendia (1, 2) which require a content uniformity check on all dosage units containing 50 mg or less of active component. Recently, instances of excessive intertablet dose variation in commercial pharmaceutical products was brought to light (3, 4), and many drug recalls have occurred because of unsatisfactory drug distribution. Problems of excessive dose variation generally appear to be confined to high potency, low dose drugs where a substantial portion of the weight of the final product is due to excipients employed as fillers, lubricants, disintegrants, *etc.*

Several published reviews dealt with various aspects of mixing (including theories of mixing), methods of sampling, mixture and mixer evaluations, and related statistical techniques (5–7). Some articles also were published on mixing operations in V-type mixers (8–10). These studies focused their attention primarily on the rates of mixing, the operative mechanisms involved, and performance evaluations comparing V-mixers with other types of mixing equipment.

There is, however, a lack of information on methods to optimize the process, including the use of high speed attrition devices commonly available for pharmaceutical mixing operations. In addition, most published work involves the use of well-defined materials of narrow size distribution as opposed to actual industrial materials.

In pharmaceutical mixing operations where materials of different particle characteristics (size, shape, size distribution, density, *etc.*) are encountered, preparation of a uniform blend involves not only successful mixing but also the avoidance of segregation in any subsequent handling. It is generally felt that segregation is a potentially greater problem with free-flowing materials than with poor flowing blends. This is based on one criterion for mixing: powders must have independent motion or flow to mix or unmix (5).

In an attempt to understand the mechanism of segregation in idealized particulate systems, the various factors influencing the rate and extent of segregation were examined (11, 12) using steel and glass spheres. Williams (13) discussed different types of segregation which occur in the process of pouring, vibrating, and stirring and gave explanations of the properties that cause these phenomena. It was suggested that segregation resulting from small differences in physical properties of the components is better detected by a systematic sampling scheme, where samples are removed from selected sites, than from random or spot sampling schemes (14). One major drawback in this area is a lack of suitable procedures to assess the segregating tendencies of powders based on evaluations of small samples of materials.

The distribution of low dose drugs *via* solvent was suggested by previous workers (15). Colorants have commonly been added to tablet granulations in this manner. Migration of colors has long been recognized

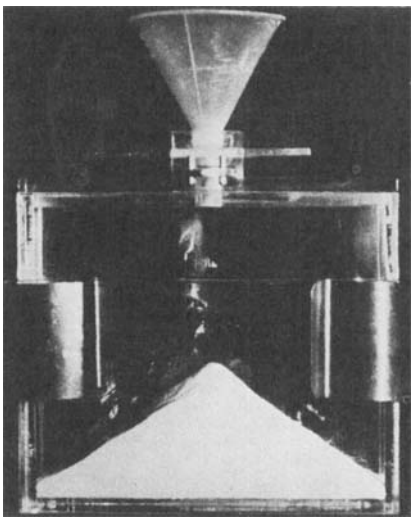

Figure 1—*Segregation cell during pile formation.*

as a problem and, more recently, drug migration (16) and binder migration (17) during drying of wet granulations were reported.

The current investigation was designed to study several different methods of producing a good blend using a twin-shell blender. The twin-shell blender was selected because of its widespread use in the pharmaceutical industry. Powdered or spray-dried lactose at a 99% level was used as the excipient; 1% aspirin, powdered or milled, was used as the active ingredient. The choice of aspirin was partly based on the relatively simple assay procedure for this drug, an important consideration in view of the large number of samples to be analyzed. In addition, unblending was also investigated. First attempts at unblending involved vibration of the powder beds. This invariably led to bed consolidation and no segregation. A segregation cell was devised which permitted segregation to occur in an "open bed" system. The cell, the method of segregation, the sampling procedure, and data are presented.

Finally, in addition to dry blending, blends were produced through the use of a solvent. The uniformity of the resultant blends before and after drying was evaluated. The volume of solvent was shown to affect the potential drug migration markedly during the drying operation. The segregation of these blends was also investigated.

EXPERIMENTAL

Materials—Lactose USP powder[1], lactose USP spray dried[2], aspirin USP powder[3], and silica[4] were used as received. Milled aspirin was obtained by milling aspirin through a hammer mill[5] using a 0.15-cm (0.062-in.) diameter perforated screen.

Equipment and Methods—The blending operations were carried out in 4.7-liter (5-qt)[6] and 15-liter (16-qt)[7] working capacity twin-shell blenders, equipped with liquid feed bars. Sieve analyses of the different materials were carried out in an oscillating air column particle-size analysis instrument[8]. Chemical assays of the mixtures were performed using a spectrophotometer[9].

In the mixing studies, 7 kg of blend [equal to the recommended working capacity of the 15-liter (16-qt) blender] was used for each run. Depending on the type of experiment, the blender was run with or without a liquid feed bar. At the specified time intervals, the blender was stopped and each leg of the blender was sampled with plunges of the sample thief parallel to the shell walls. The sample thief was a modification of a reported design (18). It consisted of a brass rod, 1.57 cm (0.62 in.) o.d. and 38.1 cm (15 in.) long, and contained six capsule-shaped cavities, each with a capacity of 200–250 mg drilled at regular intervals. This rod was fitted inside an outer brass sleeve. Rotation of the two members would either cover or expose the cavities. In actual practice, the sample thief was introduced into the powder mixture with the cavities covered; then the brass rod was rotated, and the powder was taken into the exposed cavities. The rod was rotated again to cover the cavities and then withdrawn from the blend. These samples were deposited on a sheet of paper for direct weighing or stored in capsules. The sample size in all experiments was 100 mg.

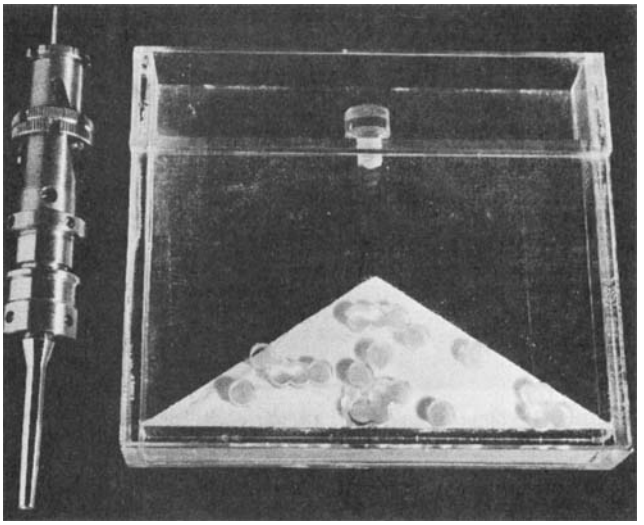

Figure 2—*Segregation cell at the sampling stage along with the dosator.*

The fabrication of the two-dimensional segregation cell was based on the concept originally proposed by Williams (19) to demonstrate visually the segregation of colored materials. The unit designed here was reduced in size, so that a suitable powder pile could be formed with about 70 g of blend. The dimensions of the box are 15.2 × 13.3 × 2.54 cm (6 × 5.25 × 1 in.). A portion of the front face of the unit is removable for sampling. Fitted to the top of the unit is a slide assembly patterned after that suggested previously (20). Variable orifice openings are available as well as a slide plate to initiate and stop powder flow. The fully assembled unit is shown in Fig. 1 during powder pile formation.

Following pile formation, the upper slide assembly is removed, and a solid Plexiglas wedge cut with an inverted "V" (45°) is inserted into the box to immobilize the powder pile. The wedge fits snugly to the inside dimensions of the two-dimensional box. A slight distortion in the actual pile occurs when the wedge is inserted, but it does not materially change the segregation accomplished by the flow onto the pile.

With the wedge in place, the box is placed flat on a tabletop and the solid front panel of the box is removed. A templet drilled with randomly positioned holes is placed over the open face of the box. The positions of the drilled holes were selected by numbering all of the possible positions available for a vertical sampling (approximately 70 samples) and selecting 20 consecutive numbers from a random number table.

The unit at this stage is shown in Fig. 2 along with a No. 1 dosator from a capsule filler[10] which was used to sample the powder bed. The dosator consists of a filling or dosing chamber which collects and discharges the desired quantity of powder and a piston which slides into the filling head and controls the depth of the dosing chamber. Samples of about 110 mg were removed at each of the 20 random positions, and 100 mg of powder was weighed and assayed as before.

In actual operation of the segregation cell, a freely flowing material such as spray-dried lactose will easily flow from the funnel through the various orifices selected. For nonflowing materials such as powdered lactose, the funnel must be hand fed with the powder to form the pile satisfactorily. The inability to compare segregation at the same flow rates is a serious limitation of the apparatus. However, the data obtainable are still viewed to be useful in a comparative manner.

No attempt was made to control the humidity in the segregation experiments, except the area was air conditioned and the relative humidity was in the 40–70% range. Extremes in humidity have been shown to affect segregation.

In the liquid addition experiments, a drying cell is employed to

[1] McKesson Chemicals, Detroit, Mich.
[2] McKesson & Robbins, Highland Park, Mich.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Cab-O-Sil, G. L. Cabot Corp., Boston, Mass.
[5] Raymond Laboratory Mill, Combustion Engineering, Inc., Chicago, Ill.
[6] Change-shell lab blender, Patterson Industries, Inc., East Liverpool, Ohio.
[7] Patterson-Kelley twin-shell liquid-solids blender, Patterson-Kelley Co., East Stroudsburg, Pa.
[8] Allen-Bradley sonic shifter, model L3P, Allen-Bradley Co., Milwaukee, Wis.
[9] Beckman model DU spectrophotometer, Beckman Instruments, Fullerton, Calif.
[10] Automatic capsule-filling machine, model LZ/64, Fratelli Zanasi S.p.A., Ozano Emilia-Bologna, Italy.

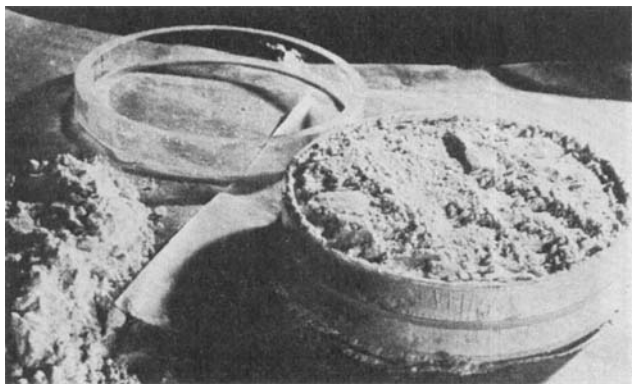

Figure 3—*Drying cell used in the study of drug migration shown after the top ring has been sampled and removed. The second level is now exposed and ready to be sampled.*

facilitate the study of drug migration. This Plexiglas unit consists of three concentric, interlocking rings, 13.9 cm (5.5 in.) i.d., 0.45 cm (0.18 in.) wall thickness, and a total assembled height of 5.08 cm (2 in.). The wetted powder is filled into the cell in a manner similar to that employed in loading trays for oven drying. After drying, the top layer of the dried bed is sampled in the four quadrants. At this point, the top ring is removed and the powder above the next ring is scalped off with a spatula. The exposed bed of powder is again sampled at this level in the four quadrants. This operation is repeated for the next layer. These samples are worked up in the same manner as previous samples. The partially assembled cell during sampling is shown in Fig. 3.

The assay of aspirin was carried out as follows. One hundred milligrams of blend, accurately weighed to the nearest 0.5 mg, was dissolved in 0.1 *M* H_2SO_4 and brought to volume in a 10-ml volumetric flask. Absorbance readings were made at 275 nm, 0.3-mm slit width, against 0.1 *M* H_2SO_4, with additional dilution if necessary.

RESULTS AND DISCUSSION

The degree of mixing achieved was evaluated based on the mean assay value ($\bar{X}$) and percentage coefficient of variation (% *CV*), calculated from the experimental data. The theoretical maximum degree of mixedness is based on the relationship developed by Stange (21) as modified by Poole *et al.* (22). For binary mixtures where each component has a size range, the standard deviation for a completely random mixture is given by the expression (23):

$$\sigma_R = \left[(xy) \Big/ \left(\frac{M}{y(\Sigma fw)_x + x(\Sigma fw)_y}\right)\right]^{1/2} \qquad \text{(Eq. 1)}$$

where:

x = proportion of one component
y = proportion of second component
M = sample size in grams
f = weight fraction of each component within a given particle-size range
w = mean particle weight of the given size fraction
Σfw = effective mean particle weight of a component

As an example, the calculated standard deviation for a completely random mixture for 1% powdered aspirin in powdered lactose with a sample size of 100 mg would proceed as follows. The mean particle weights, $(\Sigma fw)_x$ and $(\Sigma fw)_y$, are calculated based on the particle-size data in Table I for these materials; $(\Sigma fw)_x$ is shown in Table II for powdered aspirin.

The mean particle sizes calculated are 118 μm [$(\Sigma fw)_x = 1.18 \times 10^{-6}$ g] for aspirin powder and 87 μm [$(\Sigma fw)_y = 0.53 \times 10^{-6}$ g] for lactose powder. From these values, the theoretical standard deviation and coefficient of variation are calculated to be 3.4×10^{-4} and 3.4%, respectively.

Finally, because of the limited sampling (20 samples in this study), the measured variability (% *CV*) is only an estimate of the true variability of the population. The chi-square distribution may be used with the experimentally determined values to establish a range in which the theoretical value should fall. Alternatively, the range around the theoretical value can be calculated in which experimental values would be expected. The latter procedure was carried out. The theoretical percentage coefficient of variation and range values based on 20 samples with a 95% confidence interval are shown in Table III for the four possible blends.

As shown in Table III, the theoretical variation attainable is dependent on the particle size of the aspirin and essentially independent of the size of the lactose. This is to be expected in view of the fact that the standard deviation of a random mixture is primarily dependent on the mean particle size of the minor component.

Since the calculated variance from a group of sample assays contains the contribution due to weighing and analytical errors, the magnitude of the latter was independently determined and subtracted in arriving at the data presented in the tables. The analytical and weighing error makes a significant contribution to the measured variance only when the latter is relatively small (*CV* <2.0%), *i.e.*, as the blend approaches a random state.

The results obtained are shown in Tables IV–XII. In Table IV, the data are presented for the blending of powdered aspirin and powdered lactose in the twin-shell blender with and without the use of the high speed bar. The $\bar{X}$ (expressed as percent of theory) is at the theoretical value for all sampling times. The coefficient of variation is within the theoretical range for 20 samples throughout both blending trials. The changes in $\bar{X}$ or coefficient of variation appear random with time in these two studies. The blend approaches a random state within 10 min of mixing without the bar and within 5 min of mixing with the bar. These findings are in accord with what was reported previously by others—*viz.*, with this equipment, long blending times are not needed (10). Moreover, no segregation occurred on prolonged blending. The use of the bar did not result in a better blend. As shown later, however, the bar does have an effect where agglomerates are present.

Table V shows the data for the blending of powdered aspirin and spray-dried lactose in the 15-liter (16-qt) twin-shell blender without the use of the high speed bar. The use of the bar in this case is counter-indicated due to the friable nature of the spray-dried lactose. Experimental values for $\bar{X}$ and coefficient of varia-

Table I—Particle-Size Analysis by Sieving

Sieve Number	Size, μm	Cumulative Percent Retained: Powered Aspirin[a]	Milled Aspirin[b]	Powdered Lactose[b]	Spray-Dried Lactose[b]
60	250	1.0	0.2	0.5	0.8
100	149	10.0	4.8	3.1	22.7
120	125	24.9	7.2	6.9	35.4
170	88	62.1	19.0	18.9	67.2
270	53	88.8	44.5	43.0	89.7
325	44	96.0	61.1	53.7	95.6

[a] Mean of three determinations. [b] Mean of two determinations.

Table II—Calculation of Mean Particle Weights $(\Sigma fw)_x$ for Powdered Aspirin[a]

Average Sieve Opening, μm	Mean Particle Weight, μg	Fraction (f) of Sample in Each Range	Mean Particle Weight in Each Range (fw), μg
274	14.55	0.001	0.01455
200	5.66	0.090	0.5091
137	1.82	0.129	0.2352
107	0.867	0.391	0.3390
71	0.254	0.268	0.0681
49	0.0834	0.072	0.0060
25	0.0111	0.040	0.0044
		$(\Sigma fw)_x$ =	1.18 μg or 1.18×10^{-6} g

[a] Based on data in Table I, density = 1.35 g/cm³.

Table III—Theoretical Coefficients of Variation and Range Intervals[a]

Blends $(x:y):(1:99)$	CV, %	Range
Powdered aspirin–powdered lactose	3.4	2.6–5.0
Powdered aspirin–spray-dried lactose	3.4	2.6–5.0
Milled aspirin–powdered lactose	2.3	1.6–3.2
Milled aspirin–spray-dried lactose	2.3	1.6–3.2

[a] Calculated from equation of Poole *et al.* (22). Based on 20 samples with 95% confidence.

tion approximate calculated values at all sampling times without the use of a bar.

Table VI shows the segregation data on the two blends prepared without a bar (described in Tables IV and V). These data indicate the increase in variability resulting from the segregation of the powder as it is allowed to flow through an orifice and form a pile. Difficulties with the flow of the powdered lactose are indicated, particularly with the 0.32-cm (0.125-in.) orifice. The relative segregating tendency of these two blends is probably best compared at an equal flow rate. Although sufficient data are not available to do this with great accuracy, comparison of the 0.63-cm (0.25-in.) orifice data for the powdered lactose with the 0.32-cm (0.125-in.) orifice spray-dried lactose data indicates the greater tendency for the spray-dried preparation to segregate. At the same flow rates, it is reasonable to presume that the difference would be still greater.

The $\bar{X}$'s obtained for the most segregated samples are seen to depart significantly from theory. This is not too unexpected when one considers that the population being evaluated with only 20 samples is no longer normally distributed. These data and other data on nonuniform blends suggest that the departure of the average value from theory may possibly be related to the formation of a bimodal or multimodal distribution. In such situations, the possibility exists that high concentrations of drug are localized in a relatively small proportion of the total blend. To obtain powder from such areas with only 20 samples would appear to be a difficult task.

The next set of experiments describes the mixing behavior of 1% milled aspirin with powdered lactose (Table VII). The theoretical $\bar{X}$ is still, of course, 100%, but the theoretical coefficient of variation for a random mixture has decreased to 2.3% because of the fine size of the milled aspirin. With 20 samples, the 95% confidence interval for the theoretical coefficient of variation is 1.6–3.2%.

The blends produced in the twin-shell blender without the bar are poor, as demonstrated by a low experimental mean value and a high coefficient of variation. The data are clearly poorer than those reported earlier for the unmilled aspirin blended with the powdered lactose. Therefore, under these mixing conditions, the fundamentally finer particle size of the milled aspirin does not aid blending because of the agglomeration present in this powder.

The use of the high speed bar produces totally different data. The $\bar{X}$ and coefficient of variation values in the theoretical range are obtained within 5 min of mixing. Further blending with the bar, up through 15 min, produces no further noticeable changes.

Table IV—Results of Blending of 1% Aspirin Powder with Powdered Lactose with and without Use of Liquid Feed Bar

Time of Mixing, min	Without Bar		With Bar	
	$\bar{X}$, % Theory	CV, %	$\bar{X}$, % Theory	CV, %
5	—	—	99.2	3.4
10	100.5	3.4	100.0	4.2
15	—	—	99.1	3.9
20	100.0	4.3	—	—
30	101.4	4.0	—	—
60	100.2	3.5	—	—

Table V—Results of Blending of 1% Aspirin Powder with Spray-Dried Lactose and without Use of Liquid Feed Bar

Time of Mixing, min	$\bar{X}$, % Theory	CV, %
10	99.4	3.4
20	99.4	3.1
30	99.1	3.6
60	100.2	3.5

Table VI—Segregation of 1% Aspirin Powder in Powdered and Spray-Dried Lactose

Orifice Diameter, cm (in.)	Flow Time for 70 g	$\bar{X}$, % Theory	CV, %
Powdered Lactose			
0.63 (0.25)	190 sec[a]	99.1	5.4
0.31 (0.125)	26 min[a]	93.4	8.4
Spray-Dried Lactose			
0.63 (0.25)	23 sec	99.2	5.4
0.31 (0.125)	120 sec	93.7	7.8

[a] Hand feeding, with interruption; particularly difficult with 0.31-cm (0.125-in.) orifice.

The blending of the milled aspirin with the spray-dried lactose presents a particular difficulty. The use of the high speed bar is known to alter the flow and compressing characteristics of a direct-compression material such as spray-dried lactose. In the earlier experiment in which powdered aspirin was blended with spray-dried lactose (Table V), a good blend was obtainable without a bar. However, the milled aspirin with powdered lactose experiment failed to produce a good blend until the bar was employed. The blending of the milled aspirin with spray-dried lactose was, therefore, attempted in three ways, each designed to avoid or minimize high shear attrition of the spray-dried lactose. The data for the blending without a bar, with and without pretreatment of the milled aspirin with silica (1% of the aspirin level), are shown in Table VIII. A poor blend, low $\bar{X}$ and high coefficient of variation, was obtained without the bar and without pretreatment with silica. The blend is quite comparable to that obtained when the milled aspirin was blended with powdered lactose without a bar.

Pretreatment of the milled aspirin appears to have a definite beneficial effect on the blending of these materials. However, the 3.0–3.5% coefficients of variation are at, or above, the calculated upper limit. The use of a bar with milled aspirin–powdered lactose produced approximately a coefficient of variation of 2%.

A further attempt was made to accomplish the blending of the milled aspirin and spray-dried lactose by first preparing a preblend. The preblend used about one-third of the total spray-dried lactose and was carried out in a 4.7-liter (5-qt) blender fitted with a high speed bar. This preblend was then transferred to the larger blender, the remaining two-thirds of spray-dried lactose was added, and the blending was continued without the bar. The data are shown in Table IX.

A good preblend was obtained in 3–5 min with the use of the

Table VII—Blending of 1% Milled Aspirin with Powdered Lactose with and without Use of Liquid Feed Bar

Time of Mixing, min	Without Bar		With Bar	
	$\bar{X}$, % Theory	CV, %	$\bar{X}$, % Theory	CV, %
5	—	—	100.1	2.0
10	—	—	100.3	1.6
15	—	—	101.2	2.2
20	95.4	6.5	—	—
30	96.3	5.8	—	—
60	95.4	6.2	—	—

Table VIII—Blending of 1% Milled Aspirin with Spray-Dried Lactose without Use of Liquid Feed Bar

Time of Mixing, min	Without Additive		With Aspirin Pretreated with Silica	
	$\bar{X}$, % Theory	*CV*, %	$\bar{X}$, % Theory	*CV*, %
10	—	—	102.7	3.4
20	96.5	5.3	101.7	3.5
30	96.2	5.5	102.4	3.0
60	95.4	6.1	100.8	3.1

Table IX—Preblend with High Speed Bar and Final Blend without Bar for 1% Milled Aspirin in Spray-Dried Lactose

Time of Blending, min	Preblend with Bar		Final Blend without Bar	
	$\bar{X}$, % Theory	*CV*, %	$\bar{X}$, % Theory	*CV*, %
1	88.9	6.0	—	—
3	98.6	2.3	—	—
5	99.5	2.6	—	—
30	—	—	92.2	5.4
60	—	—	94.8	5.1

high speed bar. Dilution of this blend with additional spray-dried lactose failed to produce a satisfactory blend without a bar, as measured by the $\bar{X}$ and percentage coefficient of variation. It would appear that a carefully restricted use of the high speed bar on the total blend would have provided a good blend, if changes in the structure of the spray-dried lactose did not result in this time interval. Recently, capping and low hardness were experienced by the authors for tablets compressed from spray-dried lactose subjected to 5 min with a bar. A control material compressed satisfactorily at the same time.

The segregating tendencies of the milled aspirin from the powdered lactose prepared with a bar and the spray-dried lactose prepared without a bar (aspirin pretreated with silica) are shown by the data in Table X. These data illustrate the ready segregation of the spray-dried lactose blend and the nonsegregating nature of the powdered lactose blend, even under extremely slow hand feeding of the latter powder. The segregation of the milled aspirin (pretreated with silica) was also greater than the powdered aspirin from the spray-dried excipient (Table VI). The increased difference in particle masses due to the milling could account for part of this effect. The use of the silica, which aided blending to some extent, may also facilitate segregation. The blend of the milled aspirin, without silica, accomplished with the liquid feed bar showed no tendency to segregation. This could in part be due to the cohesive/adhesive nature of the milled aspirin.

The distribution of low concentrations of active ingredients can be, and often is, carried out by dissolving the drug in a liquid and mixing with the excipient, usually with the use of high shear. This is conveniently accomplished with the twin-shell blender

Table X—Segregation of 1% Milled Aspirin in a Powdered Lactose Blend (Prepared with Bar) and Spray-Dried Lactose (without Bar) with Aspirin Pretreated with Silica

Orifice Diameter, cm (in.)	Flow Time for 70 g	$\bar{X}$, % Theory	*CV*, %
Powdered Lactose			
0.63 (0.25)	180 sec	99.9	1.2
0.31 (0.125)	30 min[a]	99.8	1.1
Spray-Dried Lactose			
0.63 (0.25)	23 sec	99.8	8.3
0.31 (0.125)	110 sec	89.9	15.7

[a] Hand feeding, interrupted flow.

Table XI—Blending by Liquid Addition, 1% Aspirin in Powdered Lactose[a]

State of Processing	$\bar{X}$, % Theory	*CV*, %
Wet blend	99.9	2.2
Dried cake		
Individual $\bar{X}$ Values, % Theory		
Top layer	146, 167, 263, 177	
Second layer	56, 81, 77, 42	
Third layer	37, 35, 39, 45	
Bottom layer	55, 81, 51, 31	
Dried cake		
Screened-minimal Blending	101.2	11.7
Total blend		
After screening and blending	99.8	0.9
Segregated blend		
0.63-cm (0.25-in.) orifice, 55 sec	99.7	2.2
0.31-cm (0.125-in.) orifice, 6 min	101.5	5.6

[a] Seventy grams aspirin, 6.93 kg lactose, and 1500 ml ethanol.

equipped with the high speed liquid addition bar. On some occasions, where a wet granulating procedure is employed in the work-up of the blend for tableting, it appears logical to use this liquid to distribute the drug as well as the binder throughout the excipient. The first liquid addition experiment described employed 1500 ml of absolute ethanol to distribute 70 g of aspirin on the 6.93 kg of powdered lactose. This volume of solvent, greater than necessary to dissolve the drug, was selected since it approaches the quantity that would be used if wet granulation was being accomplished. Experimentally, the aspirin was added in 500 ml ethanol followed by the remaining liter of ethanol, which served as wash and would normally contain the binder. The total liquid addition time was 5 min. Five additional minutes of blending with the bar then followed. The blend was sampled wet for content uniformity at this stage of processing. A portion of the wetted powder was placed in the drying cell described previously and was then dried at 48° (120°F). The remainder of the material was also dried at the same time in open trays. The dried cake in the Plexiglas frame was sampled in each of four quadrants at the top level, with one section removed, with two sections removed, and at the bottom of the bed. A portion of the dried cake was carefully size reduced through a No. 40 screen, avoiding as much blending as possible. This material was also sampled. Finally, the bulk of the dried material was handled in a more normal way by screening through a No. 40 screen with no attempt to minimize blending at this point. The results of this work are shown in Table XI.

The wet blend is shown to be well blended. However, the drying operation caused extensive migration and, therefore, un-

Table XII—Liquid Addition, 1% Aspirin in Powdered Lactose[a]

State of Processing	$\bar{X}$, % Theory	*CV*, %
Wet blend:		
1 min to add liquid	99.6	2.6
5 min added use of bar	99.8	0.9
10 min added use of bar	100.1	1.1
Dried cake		
Individual $\bar{X}$ Values, % Theory		
Top layer	99.7, 101.1, 101.4, 101.5	
Second layer	102.3, 98.8, 99.7, 99.4	
Third layer	100.4, 97.1, 97.4, 97.0	
Bottom layer	101.5, 100.3, 97.8, 97.2	
Final blend[b]	98.8	1.5
Segregated blend, 0.31-cm (0.125-in.) orifice, 5 min	101.3	1.1

[a] Seventy grams aspirin, 6930 g lactose, and 450 ml ethanol. [b] After screening and blending.

blending of the sample. This is shown by the data for the various layers of the undisturbed dried powder bed as well as for the screened material where mixing was avoided. The total blend, following screening and blending, is seen to be quite uniform. Finally, this blend was shown to have a slight segregating tendency, more than was observed for the milled aspirin–powdered lactose blend in Table X. This tendency to segregate is understandable in light of the migration data on the intact dried cake.

The last experiment repeated the above work but restricted the total solvent used for the liquid addition to that needed to dissolve the drug (350 ml) and wash the feed system (100 ml). The wet mix is seen to be quite uniform with 5 min of shear using the bar. The dried cake was sampled at various locations, and no evidence of migration was found.

The final powder blend after drying and mixing was not sampled, since there was no evidence of migration during drying. In addition, the subsequent attempt to segregate the blend also showed no tendency for migration to occur.

Similar data were obtained in the authors' laboratories in mixing studies involving salicylic acid and lactose (1:499). A good blend was obtained with a limited amount of solvent; drug migration occurred with a large amount of solvent.

In the two liquid addition experiments, 450- and 1500-ml total alcohol volumes, both gave good blends at the wet stage and the final dry stage (screened and remixed). However, significant unblending due to migration occurred with the use of a large amount of solvent. In addition, one sample did show a tendency to segregate. The restricted use of solvent provided a good wet and dry blend with no evidence of migration or segregation.

SUMMARY

The blending of 1% aspirin with lactose was experimentally studied. Changes in particle size of the active component as well as the excipient were included. The best dry mix was obtained with milled aspirin and powdered lactose with the use of a high speed bar. There was no segregating tendency for this mix. The blends with spray-dried lactose were more difficult to accomplish and more readily separated by flow.

Distribution of the aspirin *via* solution could produce excellent blends. However, problems of drug migration in the drying operation were observed. Segregation was again a problem for samples where migration had occurred.

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[x] To whom inquiries should be directed.

Toxicological and Pharmacological Actions of Methacrylate Monomers III: Effects on Respiratory and Cardiovascular Functions of Anesthetized Dogs

G. N. MIR*, W. H. LAWRENCE, and J. AUTIAN[x]

Abstract □ Methacrylic acid and 12 methacrylate esters were evaluated for their effects upon blood pressure, heart rate, electrocardiogram, and respiration in anesthetized dogs following intravenous administration. All of these compounds increased respiratory rate, decreased heart rate, and produced electrocardiographic changes. The compounds could be divided into three types according to their effects on blood pressure: I, those producing a biphasic response, *i.e.*, an abrupt fall followed by a secondary rise; II, those producing only a hypotensive effect; and III, one producing only a hypertensive response. The compounds comprising Type I were methacrylic acid; methyl, ethyl, *n*-propyl, *n*-butyl, isobutyl, and hydroxyethyl methacrylates; and 1,3-butylene dimethacrylate. Type II compounds were 2-ethylhexyl, isodecyl, lauryl, and *tert*-butylaminoethyl methacrylates. Dimethylaminoethyl methacrylate produced the Type III response.

Keyphrases □ Methacrylate monomers—effects on respiratory and cardiovascular functions, anesthetized dog □ Biomaterials, methacrylate monomers—effects on respiratory and cardiovascular functions, anesthetized dog □ Toxicity—methacrylate monomers, effects on respiratory and cardiovascular functions, anesthetized dog

The use of acrylic polymers as biomedical materials has been well established over the past several years. Typical applications include dentures, bone substitutes, contact lenses, and, experimentally, artificial "implantable" teeth. The completely polymerized material appears to be reasonably well tolerated by various tissues *in vivo*, although there may be some degradation and/or solubilization of the polymer by the body's fluids or mechanical "wearing" of a device such as a Judet prosthesis (1, 2). The use of self-curing acrylics, however, may present problems associated with a residual monomer, an activator, an initiator, heat generated from the exothermic reaction, or a combination of these.

Some early toxicity studies (3, 4) were conducted on the methacrylate monomers, and the chronic oral toxicity of the methyl ester was studied in rats and dogs (5). Strain (6) suggested that the methyl methacrylate monomer was responsible for certain cases of hypersensitivity reactions to self-cured acrylics. Cohen and Smith (7) attributed the fall in blood pressure frequently seen from orthopedic use of a bone cement (self-curing methyl methacrylate cement) and occasional cardiac arrests to the methyl methacrylate monomer and/or small particles of the polymer, either as the primary or a contributory cause. Deichmann (3) reported a fall of blood pressure in the rabbit from the methyl methacrylate monomer and, more recently, a similar transient, hypotensive response in dogs was reported (8).

In previous articles of this series, it was reported that the methacrylate monomers tend to reduce rate and force of contraction of the isolated rabbit heart (9), to inhibit spontaneous contraction of the guinea pig ileum, and to antagonize the stimulant effect of acetylcholine and barium chloride upon the ileum (10). (Three of the higher members of the series were not active on the isolated ileum, while dimethylaminoethyl methacrylate produced an effect opposite to that described.)

The use of self-curing methyl methacrylate bone cement in fitting orthopedic prostheses has been associated with a significant number of transient hypotensive reactions in patients (7, 11–14), with a small incidence of cardiac arrests. Some investigators (7, 11) suggested that the monomer is responsible for, or at least a contributory factor in, the observed hypotensive states and cardiac arrests. Others (12, 13, 15–17) hypothesized that these result from factors other than the monomer such as fat emboli and temperature of reaction. The description by Deichmann (3) concerning the effect of the methacrylate monomer upon blood pressure of the rabbit, "When injected intravenously in doses of 0.03 or 0.04 cc./kg. body weight, the compounds produced a prompt and sudden fall in the arterial pressure (from about 90 to 50 mm. Hg.) followed by recovery during the following 3 to 4 minutes," is quite similar to that by Frost (13) concerning clinical use of bone cement. Frost commented: "In these cases the insertion of the cement into the femoral component was followed after approximately one minute by a precipitous fall in blood pressure to about 70 mm. Hg. In all cases recovery was rapid, unassisted by transfusion or vasopressors, and normal levels of blood pressure were regained in five to six minutes."

The following studies were undertaken in anesthetized dogs with a number of methacrylate monomers to supplement earlier data (3, 8) for methyl methacrylate and to extend the information to a number of its homologs.

EXPERIMENTAL

Methacrylic acid and 12 of its esters were included in this study[1]; these were: methyl, ethyl, *n*-propyl, *n*-butyl, isobutyl, 2-ethylhexyl, isodecyl, lauryl (dodecyl), hydroxyethyl, *tert*-butylaminoethyl, and dimethylaminoethyl methacrylates and 1,3-butylene dimethacrylate.

The methacrylates were prepared for testing by dissolving the more soluble ones (methacrylic acid and hydroxyethyl and di-

[1] All of these compounds were obtained from Rohm & Haas, Philadelphia, Pa., except for *n*-propyl and 2-ethylhexyl methacrylates, which were obtained from K & K Labs, Plainview, N.Y.

methylaminoethyl methacrylates) in normal saline (5% v/v) and preparing a suspension of the others in normal saline with 0.1% gum acacia. Satisfactory suspensions of 5% (v/v) methacrylates were prepared with a blender[2].

Male mongrel dogs (9–12 kg) were anesthetized with 35–45 mg/kg ip of sodium pentobarbital which was supplemented, if needed, by small intravenous doses. By using standard techniques, the right common carotid artery was surgically exposed, cannulated, and attached to a pressure transducer[3] for determining systemic blood pressure; the trachea was cannulated and attached to another pressure transducer[4] to record respiratory pressure changes in the airway. The right femoral vein was cannulated to facilitate intravenous injections. Needle electrodes were placed subdermally in the four limbs of the dog for recording the electrocardiogram. All of these were connected to the appropriate preamplifiers and calibrated, and data were recorded using a polygraph[5].

Four dose levels of each methacrylate were tested; the highest was rapidly fatal to the dog, while the other three produced measurable changes in one or more of the vital functions being recorded.

RESULTS AND DISCUSSION

Blood Pressure—Based upon the pattern of blood pressure responses observed, these 13 compounds can be divided into three categories: Type I, those producing a biphasic response (an abrupt fall, followed by a more sustained rise); Type II, those producing a sustained hypotensive response; and Type III, one compound producing a sustained hypertensive effect. The compounds included in Type I were methacrylic acid; methyl, ethyl, *n*-propyl, *n*-butyl, isobutyl, and hydroxyethyl methacrylates; and 1,3-butylene dimethacrylate. A Type II response was produced by isodecyl, 2-ethylhexyl, lauryl, and *tert*-butylaminoethyl methacrylates. Only dimethylaminoethyl methacrylate produced a Type III response. In an earlier report, using the isolated guinea pig ileum, dimethylaminoethyl methacrylate was also found to elicit an atypical type of response when compared to the other members of this series (10). These data are presented in Table I, which shows the mean values obtained as well as the range of responses noted. Test solutions and suspensions were prepared on a volume-to-volume basis; both the volume and moles of compound contained in the dose are presented in this table.

Type I Compounds—This group comprised the greatest number of compounds in the series tested. The responses observed were qualitatively similar, differing only quantitatively. Following an injection of the methacrylate, there was an abrupt fall in systemic pressure, ranging from 18 to 42% for the lowest doses used and from 39 to 79% for the highest doses. This hypotensive effect generally persisted for 2–4 min. Pressure then began to rise slowly until it reached a plateau somewhat higher than control values. The hypertensive phase was sustained for about 10–15 min at about 3.2–17.2% above control values for the low doses and at about 11.5–41.5% above control values for the highest of the three dose levels evaluated.

Type II Compounds—Four compounds in this series elicited only a hypotensive response. They also showed variation among compounds as to effective dose levels, with lauryl methacrylate being the least active and the *tert*-butylaminoethyl derivative being the most potent. The three dose levels of isodecyl and lauryl methacrylates reduced blood pressure by 5–25%, with a duration of 2–4 min. 2-Ethylhexyl methacrylate produced a more sustained hypotensive effect, lasting 20–30 min, with a 17–41% decrease in blood pressure. Much smaller doses of *tert*-butylaminoethyl methacrylate produced a 20–50% reduction in blood pressure, with a duration of 30–40 min.

Type III Compound—Only one of the compounds tested, dimethylaminoethyl methacrylate, elicited a hypertensive effect without a preceding fall in pressure. It was also the most potent compound, producing a 28–67% increase in pressure with smaller doses than required for the others. The hypertensive effect persisted for 30–40 min. Thus, this compound seems to be an atypical member of the series, both qualitatively and quantitatively.

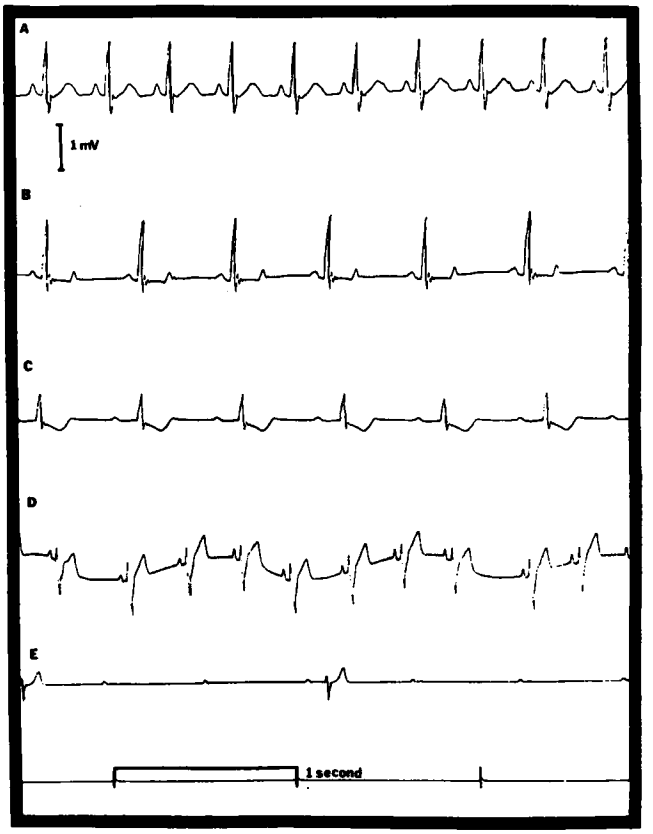

Figure 1—*Typical electrocardiographic changes produced by Type I methacrylate compounds. Key: A, control electrocardiogram; B, electrocardiogram after administering lowest dose of methacrylate; C, electrocardiogram after administering next higher dose of methacrylate; D, electrocardiogram after administering high dose of methacrylate; and E, electrocardiogram after administering highest (lethal) dose of methacrylate (mv = millivolt).*

Although the doses varied for the individual compounds, a dose-response relationship for each compound was observed within the three-dose range employed. Quantitatively, lauryl methacrylate was the least potent compound of the series in its effect upon blood pressure, while dimethylaminoethyl methacrylate was the most potent.

Heart Rate—The changes in heart rate following methacrylate administration are shown in Table I. All compounds produced a decrease in heart rate within the dosage range tested; however, the magnitude of change was usually less than noted for blood pressure. Propyl and isobutyl methacrylates appeared to reduce heart rate the most, while lauryl and isodecyl methacrylates seemed to cause the least reduction. The duration of effect upon heart rate was similar to that observed for blood pressure. Although the reduction in heart rate was dose related for each test agent, the magnitude of effect upon rate did not necessarily correlate with the magnitude of change in blood pressure.

Respiration—All tested compounds increased the respiratory rate (Table I). This increase was seen with all three dose levels for each compound, except with the two lowest doses of lauryl methacrylate, which produced no change. The magnitude of this increase ranged from 7 to 356% over control values. The duration of this effect was rather short for lauryl and isodecyl methacrylates; however, most compounds produced an effect lasting up to 20 min. Butyl and isobutyl methacrylates showed the most dramatic increase in respiratory rate.

Electrocardiogram—The effects of methacrylate injection upon the electrocardiogram of the dog fell into three patterns, as did the effects upon blood pressure.

Type I Compounds—The lower doses produced a slight increase

[2] Waring.
[3] Statham P23AC.
[4] Statham P23Dc.
[5] Grass, model 7.

Table I—Pharmacological Effects of Methacrylate Monomers

Dose per Kilogram	Test Sample	Number of Dogs	Blood Pressure					Heart Rate			Respiratory Rate		
			Control, mm Hg	Initial Response, mm Hg	Percent Change	Secondary Response, mm Hg	Percent Change	Control, beats/min	After Treatment, beats/min	Percent Change	Control, rate/min	After Treatment, rate/min	Percent Change
112×10^{-6} *M* (0.0095 ml)	Methacrylic acid	3	158.33[a] (155–160)[b]	101.66 (100–105)	−35.79	168.33 (165–170)	+6.31	104.00 (98–110)	102.66 (98–108)	−1.28	6.66 (6–8)	9.66 (9–10)	+45.40
224×10^{-6} *M* (0.0190 ml)		3	158.33 (155–160)	63.33 (60–65)	−60.00	173.33 (170–175)	+9.47	104.00 (98–110)	89.33 (86–96)	−14.10	6.66 (6–8)	12.66 (11–15)	+90.00
562×10^{-6} *M* (0.0476 ml)		3	158.33 (155–160)	33.33 (30–35)	−78.94	181.66 (175–185)	+14.74	102.33 (98–105)	80.00 (72–86)	−21.82	6.33 (6–7)	16.33 (12–18)	+157.97
1124×10^{-6} *M* (0.0952 ml)		(Lethal dose)											
140×10^{-6} *M* (0.0149 ml)	Methyl methacrylate	3	168.33 (165–175)	96.66 (95–100)	−42.57	181.66 (175–185)	+7.91	135.33 (130–142)	122.33 (120–125)	−9.61	9.66 (9–10)	16.00 (15–18)	+65.66
280×10^{-6} *M* (0.0299 ml)		3	168.33 (165–175)	76.66 (75–80)	−54.45	208.33 (200–215)	+23.76	134.00 (130–140)	120.00 (116–122)	−10.44	9.66 (9–10)	21.66 (21–22)	+124.22
560×10^{-6} *M* (0.0599 ml)		3	166.66 (160–175)	35.00 (30–40)	−78.99	216.66 (205–225)	+30.00	133.33 (130–140)	111.66 (105–120)	−16.25	10.00 (10–10)	23.66 (22–25)	+136.60
1120×10^{-6} *M* (0.1198 ml)		(Lethal dose)											
135×10^{-6} *M* (0.0171 ml)	Ethyl methacrylate	3	161.66 (160–165)	110.00 (105–115)	−31.95	173.33 (170–175)	+7.21	146.66 (142–152)	130.33 (125–136)	−11.13	14.66 (12–20)	19.66 (16–25)	+34.10
270×10^{-6} *M* (0.0342 ml)		3	161.66 (160–165)	88.33 (85–95)	−45.36	181.66 (180–185)	+12.37	145.33 (140–150)	115.66 (112–120)	−20.41	11.33 (11–20)	29.00 (25–36)	+155.95
540×10^{-6} *M* (0.3684 ml)		3	161.66 (160–165)	66.66 (65–70)	−58.66	201.66 (195–210)	+24.74	146.00 (142–148)	109.33 (104–116)	−25.11	13.66 (11–18)	33.66 (32–36)	+146.41
1080×10^{-6} *M* (0.1368 ml)		(Lethal dose)											
195×10^{-6} *M* (0.0280 ml)	Propyl methacrylate	3	170.00 (160–180)	113.33 (110–120)	−33.33	181.66 (170–190)	+6.85	140.00 (136–144)	122.00 (118–124)	−12.85	11.66 (10–15)	19.00 (15–26)	+62.95
390×10^{-6} *M* (0.0560 ml)			170.00 (160–180)	95.00 (95–100)	−44.11	208.33 (195–220)	+22.54	140.00 (135–145)	106.66 (105–115)	−23.81	11.66 (10–15)	26.00 (22–32)	+122.98
780×10^{-6} *M* (0.1120 ml)			168.33 (160–175)	55.00 (50–60)	−67.32	228.33 (225–230)	+35.64	140.00 (135–144)	93.33 (90–100)	−33.33	11.66 (10–15)	35.00 (30–40)	+200.17
1560×10^{-6} *M* (0.2240 ml)		(Lethal dose)											
135×10^{-6} *M* (0.0207 ml)	Butyl methacrylate	3	158.33 (150–165)	130.00 (120–135)	−17.89	163.33 (155–170)	+3.15	134.33 (125–145)	117.33 (100–130)	−12.65	13.00 (10–17)	34.33 (32–37)	+164.07
270×10^{-6} *M* (0.0415 ml)			158.33 (150–165)	111.66 (100–120)	−29.47	171.66 (165–180)	+8.41	134.00 (125–145)	112.00 (95–126)	−16.41	13.00 (10–17)	46.00 (45–48)	+253.84
540×10^{-6} *M* (0.0830 ml)			158.33 (150–165)	96.66 (95–100)	−38.95	176.66 (170–185)	+11.57	134.00 (125–145)	98.33 (80–115)	−26.61	13.00 (10–17)	52.33 (50–55)	+302.53
1080×10^{-6} *M* (0.1660 ml)		(Lethal dose)											
104×10^{-6} *M* (0.0167 ml)	Isobutyl methacrylate	3	155.00 (150–160)	103.33 (100–110)	−33.33	181.66 (175–190)	+17.20	127.33 (118–138)	114.66 (102–126)	−9.95	9.66 (8–10)	25.33 (25–26)	+162.21
208×10^{-6} *M* (0.0334 ml)			155.00 (150–160)	86.66 (85–90)	−44.09	196.66 (190–205)	+26.87	131.00 (115–140)	101.66 (90–115)	−22.39	9.33 (8–10)	40.33 (35–50)	+332.26
416×10^{-6} *M* (0.0668 ml)			151.66 (145–160)	61.66 (60–65)	−59.34	205.00 (200–210)	+35.17	125.33 (110–134)	85.66 (62–102)	−31.65	11.33 (10–14)	51.66 (45–62)	+355.95
832×10^{-6} *M* (0.1336 ml)		(Lethal dose)											
101×10^{-6} *M* (0.0124 ml)	Hydroxyethyl methacrylate	3	143.33 (135–150)	101.66 (100–105)	−29.07	160.00 (155–170)	+11.63	146.33 (135–164)	134.00 (130–144)	−8.42	12.33 (10–14)	17.33 (15–19)	+40.55
202×10^{-6} *M* (0.0248 ml)			140.00 (130–150)	81.66 (80–85)	−41.67	166.66 (155–180)	+19.04	143.33 (135–160)	127.00 (120–135)	−11.39	12.33 (10–14)	24.66 (22–26)	+100.00
404×10^{-6} *M*			136.66	63.33	−53.65	183.33	+34.15	138.33	115.33	−16.62	11.33	31.33	+176.52

Dose per Kilogram	Test Sample	Number of Dogs	Blood Pressure					Heart Rate			Respiratory Rate		
			Control, mm Hg	Initial Response, mm Hg	Percent Change	Secondary Response, mm Hg	Percent Change	Control, beats/min	After Treatment, beats/min	Percent Change	Control, rate/min	After Treatment, rate/min	Percent Change
(0.0496 ml)			(130–145)	(60–65)		(170–190)		(130–150)	(110–120)		(10–12)	(28–34)	
808×10^{-6} *M*			(Lethal dose)										
(0.0992 ml)													
200×10^{-6} *M*	1,3-Butylene dimethacrylate	3	143.33	101.66	−29.07	163.33	+13.95	113.33	103.66	−9.32	11.66	25.00	+114.40
(0.0449 ml)			(130–160)	(95–110)		(155–185)		(92–150)	(88–135)		(11–12)	(20–30)	
400×10^{-6} *M*			141.66	91.66	−35.29	173.33	+22.35	116.66	94.33	−23.67	10.66	30.33	+184.52
(0.0898 ml)			(130–165)	(80–100)		(155–200)		(90–145)	(76–125)		(10–12)	(24–25)	
800×10^{-6} *M*			136.66	63.33	−53.65	193.33	+41.46	108.66	85.66	−26.85	10.66	37.66	+253.28
(0.1796 ml)			(125–160)	(60–65)		(180–210)		(86–145)	(64–115)		(10–12)	(35–40)	
1600×10^{-6} *M*			(Lethal dose)										
(0.3592 ml)													
144×10^{-6} *M*	2-Ethylhexyl methacrylate	3	156.66	133.33	−14.89			145.33	132.00	−10.09	12.00	28.00	+133.33
(0.0326 ml)			(150–160)	(125–140)				(136–164)	(120–148)		(6–18)	(18–38)	
288×10^{-6} *M*			153.33	123.33	−19.56			142.33	122.33	−16.34	16.33	33.00	+102.08
(0.0652 ml)			(145–160)	(115–130)				(132–164)	(114–135)		(8–20)	(22–45)	
576×10^{-6} *M*			153.33	108.33	−29.34			139.33	112.00	−24.40	14.00	39.33	+180.92
(0.1304 ml)			(145–155)	(100–115)				(130–158)	(102–122)		(10–20)	(28–52)	
1152×10^{-6} *M*			(Lethal dose)										
(0.2608 ml)													
178×10^{-6} *M*	Isodecyl methacrylate	3	161.66	153.33	−5.15			149.33	141.33	−5.66	9.33	10.00	+7.18
(0.0461 ml)			(148–170)	(150–165)				(142–158)	(136–152)		(8–12)	(8–12)	
356×10^{-6} *M*			161.66	146.66	−9.27			147.66	135.66	−8.84	9.33	16.66	+78.56
(0.0922 ml)			(150–175)	(140–155)				(155–175)	(130–145)		(8–12)	(14–18)	
712×10^{-6} *M*			158.33	126.66	−20.00			146.33	130.33	−12.27	10.00	23.66	+136.60
(0.1844 ml)			(150–170)	(120–135)				(144–155)	(128–135)		(8–12)	(22–25)	
1424×10^{-6} *M*			(Lethal dose)										
(0.3688 ml)													
418×10^{-6} *M*	Lauryl methacrylate	2	147.50	140.00	−5.08			120.00	118.00	−1.66	11.00	11.00	0
(0.1550 ml)			(145–150)	(135–145)				(120–120)	(116–120)		(10–12)	(10–12)	
836×10^{-6} *M*			145.00	127.50	−12.06			120.00	115.50	−3.75	11.00	11.00	0
(0.3100 ml)			(140–150)	(120–135)				(120–120)	(115–116)		(10–12)	(10–12)	
1672×10^{-6} *M*			145.00	117.50	−18.96			120.00	107.50	−10.41	11.00	15.50	+40.90
(0.6200 ml)			(140–150)	(105–130)				(120–120)	(105–110)		(10–12)	(15–16)	
3344×10^{-6} *M*			(Lethal dose)										
(1.2400 ml)													
45×10^{-6} *M*	*tert*-Butylaminoethyl methacrylate	2	167.50	132.50	−20.89			153.00	138.00	−9.80	17.00	21.50	+26.47
(0.0095 ml)			(165–170)	(130–134)				(148–158)	(134–142)		(14–20)	(18–25)	
90×10^{-6} *M*			165.00	117.50	−28.78			151.00	128.00	−15.23	17.00	24.00	+14.17
(0.0190 ml)			(160–170)	(115–120)				(146–156)	(124–132)		(14–20)	(22–26)	
180×10^{-6} *M*			162.50	80.00	−50.76			150.50	116.00	−22.92	17.00	27.00	+58.82
(0.0380 ml)			(160–165)	(80–80)				(146–155)	(112–120)		(14–20)	(26–28)	
360×10^{-6} *M*			(Lethal dose)										
(0.0760 ml)													
15×10^{-6} *M*	Dimethylaminoethyl methacrylate	3	150.00	191.66	+27.77			143.33	123.33	−13.95	15.33	35.33	+130.26
(0.0026 ml)			(145–155)	(190–195)				(138–148)	(120–128)		(12–18)	(34–38)	
30×10^{-6} *M*			150.00	218.33	+45.55			140.66	112.66	−19.90	16.00	44.00	+175.00
(0.0052 ml)			(135–155)	(215–220)				(135–145)	(105–118)		(12–20)	(38–48)	
60×10^{-6} *M*			150.00	253.33	+66.88			140.00	111.66	−20.24	16.00	59.00	+268.75
(0.0104 ml)			(145–155)	(250–260)				(135–145)	(105–115)		(12–20)	(55–62)	
120×10^{-6} *M*			(Lethal dose)										
(0.0208 ml)													

[a] Mean value. [b] Range of responses noted.

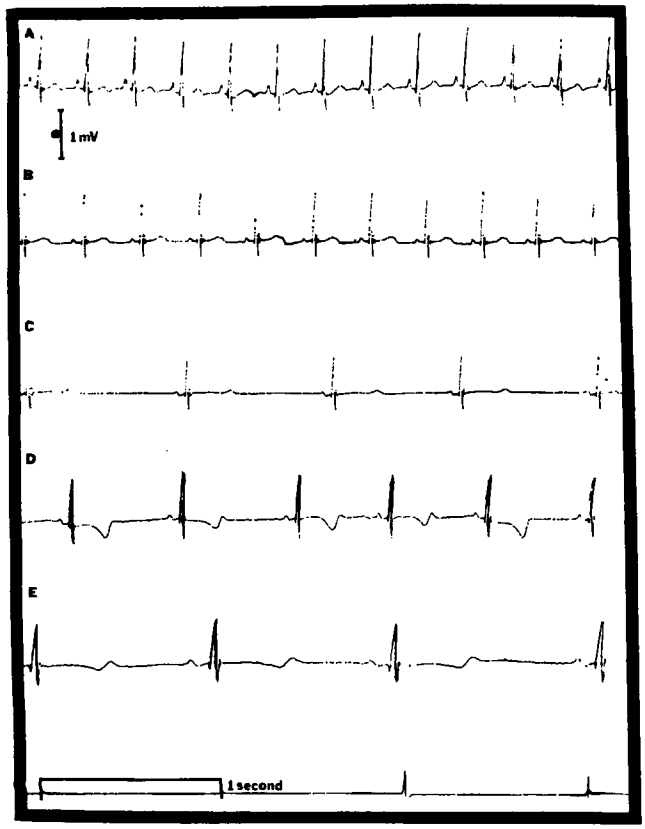

Figure 2—*Typical electrocardiographic changes produced by Type II methacrylate compounds. For key, see Fig. 1.*

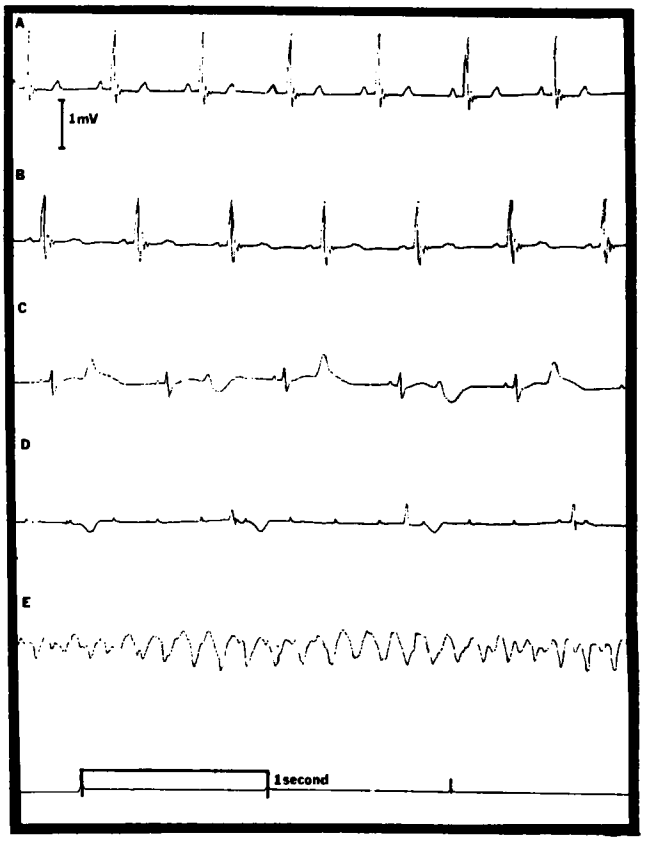

Figure 3—*Typical electrocardiographic changes produced by Type III methacrylate compound. For key, see Fig. 1.*

in the PR interval, an increase in the RR interval, prolongation of the ST segment, and a depression of the T wave (Fig. 1B). The median doses depressed and caused a marked sagging of the ST segment, inversion of the T wave, and further prolongation of the PR interval (Fig. 1C). Higher doses reduced the height of the R wave, increased the negativity of the S wave, and produced a prominent upright T wave (Fig. 1D). Premature ventricular contractions were frequently seen. With still higher (lethal) doses, the ventricular rate became very slow with a depressed QRS complex, an upright T wave, and a pattern of three P waves per QRS-T complex (Fig. 1E).

Type II Compounds—The lowest doses employed produced depression of the P wave, an increase in the RR interval, prolongation of the ST segment, and a slight depression of the T wave (Fig. 2B). The next higher doses produced a further increase in PR and RR intervals, with further depression of the T wave (Fig. 2C). Higher doses produced an increase in the PR interval, an increase in the RR interval, prolongation of the ST segment, and an inversion of the T wave (Fig. 2D). With the highest (lethal) doses, atrial–ventricular function was preserved in its proper sequence, although there was pronounced bradycardia and marked prolongation of the PR interval and ST segment (Fig. 2E). There was a suggestion of some electrical instability following these two highest doses, but it did not appear to be well-organized flutter or fibrillation (Figs. 2D and 2E).

Type III Compound—The lower doses produced a depression of the P wave, an increase in the PR interval, and a decrease in the amplitude of the R and T waves (Fig. 3B). The next higher dose produced a marked distortion of the electrocardiogram; the P wave was small and the ST segment was prolonged. The T wave appeared enlarged, upright, and "domed" in the first, third, and fifth cycles (Fig. 3C), while it was biphasic in the second and fourth cycles shown. With larger doses, there was a complete dissociation of the P waves from the QRS-T complexes (complete heart block), with the atrial rate approximately three times the ventricular rate (Fig. 3D). With the highest (lethal) dose, electrocardiographic abnormalities may be noted along with superimposed skeletal muscular contractions (Fig. 3E).

Thus, the electrocardiographic changes may be summarized as follows. Type I compounds showed a dose-related response in which there is bradycardia, a reduced rate of impulse transmission through the A-V node (increased PR interval), and possible acute cardiac ischemia. Higher doses produced premature ventricular contractions and incomplete A-V block.

The methacrylate esters with Type II action produced bradycardia, as well as a marked effect upon ventricular repolarization. As the dosage was increased, the T wave was reduced and then became inverted or biphasic with a considerable increase in the ST segment; the PR interval also became prolonged.

Dimethylaminoethyl methacrylate, the only Type III compound in this series, produced bradycardia, apparently also of sinus origin; as the dose was increased, abnormalities of the T wave were observed. In the next higher dose (Fig. 3D), complete A-V block was noted.

Administering suspensions by the intravenous route logically poses a question of whether the observed effects on blood pressure, heart rate, and the electrocardiogram are due to an intrinsic action of the compound injected or, secondarily, from physical blockage of small vessels by the suspension. The data obtained in this study suggest that the observed effects are due to the former and not to physical blockage of the coronary or other circulatory systems. Lauryl methacrylate was the least potent of these compounds in producing changes in these parameters; at the same time, it was the least water soluble and was injected in the largest quantities. Thus, if the effects were of a physical nature, this compound should have produced the greatest changes in these parameters.

Several authors (7, 11–14) reported cases of an abrupt fall in blood pressure in their patients immediately after the use of a bone cement containing methyl methacrylate monomer. Some authors (7, 11, 17) encountered cardiac arrest in a few patients. Homsy *et al.* (8) reported hypotensive effects of methyl methacrylate monomer (a member of the Type I series) when administered to dogs; however, they did not mention a secondary rise in pressure. Their figure depicting the blood pressure response of the dog to the monomer covered only the first 2 min postinjection.

during which we, too, observed only a hypotensive (initial) response to the monomer. In addition, a secondary rise in blood pressure, above control values, which occurred a few minutes after injection of the methacrylate, was observed in the present study.

Powell *et al.* (11) suggested that the hypotensive effect may result from a vasodilatory action of the methacrylate. In a previous study on the isolated guinea pig ileum (10), these methacrylates (Types I and II) induced relaxation of the smooth muscle of the intestine; thus Powell's postulate that such an effect may also be produced on the smooth musculature of the vascular system would not be inconsistent with the reported effects of these methacrylate monomers upon intestinal smooth muscle.

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* Present address: William H. Rorer, Inc., Fort Washington, PA 19034

ˣ To whom inquiries should be directed.

Direct Estimation of Hexadecyltrimethylammonium-Ion Adsorption at Liquid Interfaces by a Radioisotope Technique I: Specific Ion Effects at Air–Water Interface

JAY WEISS*, GEORGE ZOGRAFIˣ, and ANTHONY P. SIMONELLI‡

Abstract □ The adsorption of the surfactant ^{14}C-hexadecyltrimethylammonium ion at the air-water interface was measured directly by detecting radioactivity emitted by adsorbed molecules. This was accomplished by placing a gas flow detector above the surface containing surfactant of known specific activity. Adsorption in the presence of potassium halide ions was found to change in the order bromide > chloride > fluoride, indicating specific ion interactions at the surface. Tetraalkylammonium halides produced the same order of effect for halide ions, but an inhibitory effect on adsorption was noted with increasing alkyl chain length. This effect appears to be related to their salting-in properties. Large hydrophobic anions, such as some alkyl- and arylsulfonates, produce two effects, depending on the concentration of surfactant and sulfonate. Ion association at the surface occurs at low sulfonate concentrations, whereas at higher sulfonate concentrations the ion association in bulk solution occurs, leading to less adsorption.

Keyphrases □ Hexadecyltrimethylammonium ion, adsorption—radioisotope technique for direct estimation at air–water interface, specific ion effects □ Surfactants, adsorption—radioisotope technique for direct estimation of hexadecyltrimethylammonium ion at air–water interface, specific ion effects □ Adsorption—radioisotope technique for direct estimation of hexadecyltrimethylammonium ion at air–water interface, specific ion effects □ Radiolabeling—used to measure hexadecyltrimethylammonium-ion adsorption at air–water interface

In recent years it has become increasingly apparent that the therapeutic potency of many drug molecules is related to their hydrophobic behavior or their tendency to leave an aqueous environment for one that is more nonpolar (1). At the molecular level, a number of processes may be responsible for this behavior. For example, *(a)* the drug may interact with a specific receptor or enzyme with a considerable contribution from hydrophobic interactions; *(b)* a rate-limiting barrier to the site of action may exist which is lipoidal and hence more limiting to less lipoidal molecules; and *(c)* there may be an interaction of the drug directly with biological membranes, causing a change in membrane permeability either by direct interaction with membrane molecules or by an indirect effect due to competition or

during which we, too, observed only a hypotensive (initial) response to the monomer. In addition, a secondary rise in blood pressure, above control values, which occurred a few minutes after injection of the methacrylate, was observed in the present study.

Powell *et al.* (11) suggested that the hypotensive effect may result from a vasodilatory action of the methacrylate. In a previous study on the isolated guinea pig ileum (10), these methacrylates (Types I and II) induced relaxation of the smooth muscle of the intestine; thus Powell's postulate that such an effect may also be produced on the smooth musculature of the vascular system would not be inconsistent with the reported effects of these methacrylate monomers upon intestinal smooth muscle.

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In recent years it has become increasingly apparent that the therapeutic potency of many drug molecules is related to their hydrophobic behavior or their tendency to leave an aqueous environment for one that is more nonpolar (1). At the molecular level, a number of processes may be responsible for this behavior. For example, *(a)* the drug may interact with a specific receptor or enzyme with a considerable contribution from hydrophobic interactions; *(b)* a rate-limiting barrier to the site of action may exist which is lipoidal and hence more limiting to less lipoidal molecules; and *(c)* there may be an interaction of the drug directly with biological membranes, causing a change in membrane permeability either by direct interaction with membrane molecules or by an indirect effect due to competition or

interaction with substances normally membrane bound, *e.g.*, calcium or some enzymes.

Previous studies from this laboratory were concerned with the interfacial activity of various drugs at the air–solution interface and at interfaces covered with insoluble monomolecular films, with particular interest in the substituted phenothiazines (2–6). The position has been taken that studies at the air–water interface can give useful information about factors influencing hydrophobic behavior of drugs in aqueous solution. Studies using the monomolecular film technique, on the other hand, provide the opportunity to present a well-defined array of molecules placed in such a position that microenvironmental factors at the surface can be controlled. For example, by judicious choice of film substances and by compression of the monomolecular film, one can alter the chemical composition, molecular orientation, charge density, and, perhaps, interfacial water structure in such a way as to produce different drug–"film molecule" interactions. In this way, one can simulate the conditions a drug will encounter as it acts on a biological membrane. Some studies involving the surface activity of drugs recently were reviewed (7, 8).

Of major interest is an understanding of the kinetic and equilibrium relationship between molecules at an interface and those in the bulk aqueous solution. To obtain such information, one must be able to measure the surface concentration of drug molecules, *i.e.*, molecules per unit area in excess of the bulk drug concentration. An approach traditionally used at various liquid interfaces is to measure surface tension change with increasing drug concentration with the assumption that reduction in surface tension reflects increasing surface concentration. The more correct approach involves the use of the Gibbs (9) equation:

$$-d\gamma = \sum \Gamma_i \, d\mu_i \qquad \text{(Eq. 1)}$$

where Γ_i and μ_i are the surface concentration and chemical potential, respectively, of each species present. For a system containing a solute of activity a_s and an insoluble film having activity a_f, one can write:

$$-d\gamma = RT\Gamma_s \, d\ln a_s + RT\Gamma_f \, d\ln a_f \qquad \text{(Eq. 2)}$$

where R is the gas constant, and T is the absolute temperature.

In the absence of an insoluble film, the second term is missing and the major problem is to account properly for the activity change, $d \ln a_s$. For dilute solutions of nonelectrolyte, one can utilize the solute concentration; but for higher concentrations where solution activity is not equal to concentration or for electrolytes, there is a fair degree of uncertainty in the use of concentration (10, 11). In the case of drug molecules, such as the phenothiazines, there is good evidence to suggest self-association at relatively low concentrations, even below an apparent critical micelle concentration (CMC) (12), so that a great deal of uncertainty exists in such cases when attempting to apply the Gibbs equation.

To begin to apply Eq. 2 to the case where film molecules are present, one is forced to assume that no perturbation of the film occurs with a change in solute activity or bulk concentration and that only solute concentration changes influence the surface tension. Pethica (13) suggested that this assumption was only approached for penetration of a solute into a highly compressed film, where the number of penetrating molecules is small and where no change in the area per film molecule (the reciprocal of Γ_f) can occur. Consequently, much work that only measures surface tension change when adding drugs beneath monomolecular films is of little direct use in the estimation of drug–monolayer interaction. Another major problem encountered specifically when studying cationic substances is the difficulty of measuring surface tension accurately because of strong adsorption on the glass or platinum surfaces used in various surface tension measuring devices.

To obviate these difficulties, a technique was needed that allowed for the direct estimation of molecules adsorbed to a liquid surface. One attractive approach, first reported in 1949 (14–16), involves the use of radiolabeled compounds and the detection of weak β-particles emitted from the solution surface. This technique was used at the air–water interface to confirm the Gibbs equation with sodium lauryl sulfate (17, 18), as well as with multicomponent systems where different species compete for the surface (19, 20). A few studies with monomolecular films also were reported, but these were confined to the detection of some ^{14}C-labeled proteins or $^{45}Ca^{+2}$ at surfaces covered with phospholipid films (21–23).

To develop this technique for studying drug absorption to various interfaces, it was decided to use ^{14}C-labeled hexadecyltrimethylammonium ion, a highly surface-active molecule. In this first report, studies concerned with adsorption at the air–water interface are presented. In a subsequent report, adsorption at interfaces covered with lipid monomolecular films will be considered.

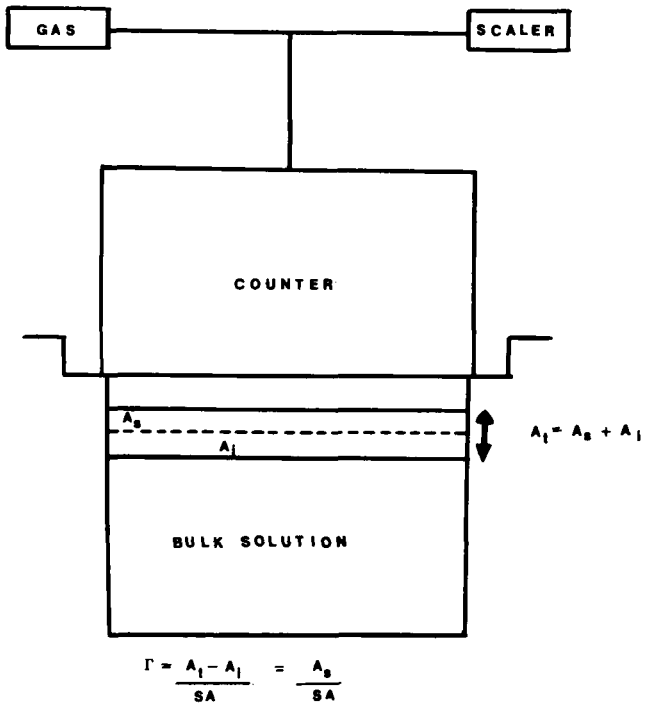
EXPERIMENTAL

Chemicals—The ^{14}C-labeled compounds[1] used were: hexadecyl-1-^{14}C-trimethylammonium bromide, specific activity 11.3 mCi/mmole; urea-^{14}C, specific activity 58.5 mCi/mmole; and glycerin, specific activity 11.7 mCi/mmole, uniformly ^{14}C-labeled. Radiochromatograms confirmed that each compound was chemically pure. Reagent grade urea and glycerin and chemically pure hexadecyltrimethylammonium bromide[2] served as the unlabeled carriers. All electrolytes used were of reagent grade, and double-distilled water was utilized as the solvent.

Radioactivity Detection—The radiation detector consisted of a thin formvar–ethylene dichloride end window (less than 150 $\mu g/cm^2$), gas flow detector connected to a series decade scaler[3]. The flow gas was commercial "Q-Gas"[4]; the flow rate was 50 ml/min.

All solutions to be counted were placed into a Teflon dish (subsequently referred to as a cuve), which was 6.0 mm deep and 5.7 cm in diameter. The counting area exposed to the detector was

[1] All of the labeled compounds were supplied by Amersham Searle, Arlington Heights, Ill.
[2] Calbiochem, La Jolla, Calif.
[3] Nuclear-Chicago 8703, Nuclear Chicago, Des Plaines, Ill.
[4] Nuclear Chicago, Des Plaines, Ill.



Scheme I—Counting apparatus used to detect radiolabeled molecules at a solution surface

25.9 cm². A constant solution volume of 10.0 ml was used throughout the study, and the detector was placed over the solution at a fixed distance from the surface (about 2 mm). Thus, constant counting geometry was maintained. All studies were conducted at a room temperature of 23 ± 1°. A schematic representation of this setup is given in Scheme I.

Determination of Surface Concentration—Because of the relatively large range of ^{14}C-β-particles with respect to the thickness of the surface region, when the detector is placed over the surface of a solution containing ^{14}C-labeled solute, the total counts per minute recorded represent the molecules present down to some depth within the solution. Thus, a certain proportion of the total counts, A_T, is due to molecules counted merely because of bulk concentration of solute; this is expressed as A_l (Fig. 1). To be able to measure counts due to those molecules present at the surface in excess of the bulk concentration A_s, A_T and A_l must be determined independently and A_s must be obtained by their difference. Values of A_l are determined by using solutions of a nonsurface-active radiolabeled compound, having the same specific activity and molar concentration as the surfactant solution. In this way the difference between A_l and A_T has to be due only to the presence of adsorbed hexadecyltrimethylammonium ions. Radiolabeled solutions of urea and/or glycerin were used to determine A_l for all concentrations of surfactant and all solution conditions considered. Since various solution conditions were to be used, *e.g.*, different electrolytes, varying ionic strength, and pH, a systematic check on the effect of such changes on A_l was made. Under no conditions used was the value of A_l altered by varying solution conditions.

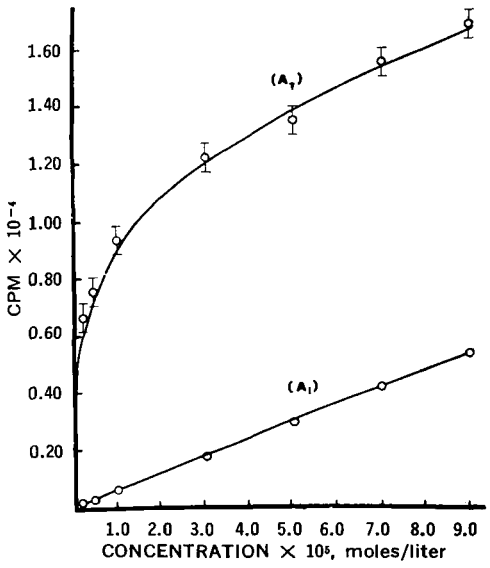


Figure 1—*Counts per minute* versus *solute concentration. Ionic strength = 0.10, ammonium bromide.*

Table I—Hexadecyltrimethylammonium–Urea System in the Presence of 0.10 *M* Ammonium Bromide

Hexadecyltrimethylammonium Concentration, moles/liter	$\Gamma \times 10^{-10}$, moles/cm²	Å²/Ion
1.0×10^{-6}	1.05 ± 0.03	158.0
3.0×10^{-6}	1.99 ± 0.06	83.4
5.0×10^{-6}	2.22 ± 0.01	74.8
7.0×10^{-6}	2.59 ± 0.04	64.1
1.0×10^{-5}	2.63 ± 0.03	63.1
3.0×10^{-5}	3.19 ± 0.05 (48.9)[a]	52.1
5.0×10^{-5}	3.32 ± 0.05 (46.4)	50.0
7.0×10^{-5}	3.49 ± 0.04 (43.3)	47.6
9.0×10^{-6}	3.56 ± 0.06 (42.1)	46.7

[a] Numbers in parentheses obtained from parabolic fit (Fig. 2).

Once the counts per minute due to adsorbed molecules, A_s, have been determined, the surface concentration of surfactant, Γ, in moles per square centimeter, can be estimated by the following expression:

$$\Gamma = \frac{A_s}{SA} \qquad \text{(Eq. 3)}$$

where A equals total surface area of the solution in square centimeters, and S represents the specific activity of surfactant molecules at the surface in units of counts per minute per mole.

To evaluate S in Eq. 3, known amounts of labeled hexadecyltrimethylammonium ion were spread as an insoluble monomolecular film on the surface of an almost saturated solution of ammonium bromide (76.2 g/100 ml); this was done to ensure that the surfactant molecules remained on the surface and did not dissolve. Approximately 5 min elapsed before counting was initiated to allow

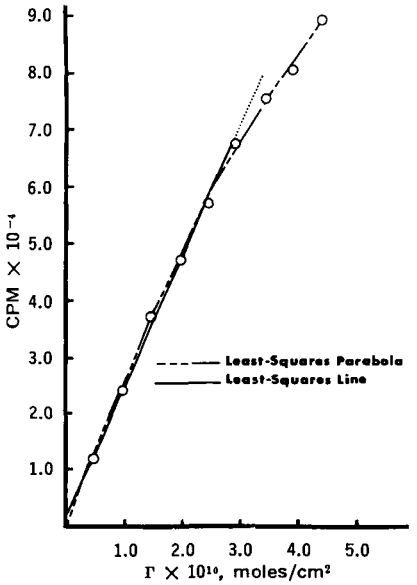


Figure 2—*Counts per minute* versus *amount of hexadecyltrimethylammonium ion applied to the surface of an almost saturated solution of ammonium bromide.*

Table II—Surface Concentration × 10^{10} (Moles per Square Centimeter) for Hexadecyltrimethylammonium Adsorption in Aqueous Solutions of 0.10 *M* Tetraalkylammonium Bromide, and Data for Hexadecyltrimethylammonium Adsorption in 0.10 *M* KBr

Hexadecyltrimethylammonium Concentration, moles/liter	Potassium Bromide	Tetramethyl	Tetrapropyl	Tetrabutyl
7.0×10^{-6}	2.09 ± 0.04	2.11 ± 0.02	2.03 ± 0.05	1.43 ± 0.02
1.0×10^{-5}	2.27 ± 0.03	2.22 ± 0.02	2.19 ± 0.03	1.58 ± 0.02
3.0×10^{-5}	2.77 ± 0.04	2.62 ± 0.02	2.54 ± 0.03	2.07 ± 0.04
5.0×10^{-5}	2.92 ± 0.06	2.78 ± 0.04	2.77 ± 0.03	2.27 ± 0.03

Table III—Surface Concentration × 10^{10} (Moles per Square Centimeter) for Hexadecyltrimethylammonium Adsorption in Aqueous Solutions of Tetraalkylammonium Chloride, and Data for Hexadecyltrimethylammonium Adsorption in 0.10 *M* KCl

Hexadecyltrimethylammonium Concentration, moles/liter	Potassium Chloride	Tetramethyl	Tetrapropyl	Tetrabutyl
7.0×10^{-6}	1.63 ± 0.02	1.24 ± 0.02	1.22 ± 0.04	1.21 ± 0.03
1.0×10^{-5}	1.87 ± 0.03	1.67 ± 0.04	1.38 ± 0.03	1.37 ± 0.05
3.0×10^{-5}	2.22 ± 0.03	1.92• ± 0.03	1.70 ± 0.03	1.79 ± 0.03
5.0×10^{-5}	2.42 ± 0.06	2.12 ± 0.03	1.88 ± 0.04	1.88 ± 0.04

time for the spreading solvent, chloroform, to evaporate. Values were constant over 15 min and did not change by more than 5% over 1 hr. The value of S was obtained from the slope of a plot of the surface radioactivity, A_s, *versus* the moles per square centimeters of surfactant spread after appropriate correction for nonradioactive species present. As later results indicated, a possible difference in counting geometry because of the saturated solution used was not an important factor. Also, no significant difference in S was found using a nearly saturated potassium bromide solution rather than one containing ammonium bromide.

RESULTS

Preliminary Studies—To demonstrate the suitability of this technique, initial measurements were made on a system consisting of hexadecyltrimethylammonium ion in 0.1 *M* NH_4Br solution. The concentration of the surfactant ranged from 1×10^{-6} to 9.0×10^{-5} *M*; the CMC of this surfactant under these conditions is 8.0×10^{-5} *M* (24). Unless otherwise specified, urea was used to determine A_i.

Figure 1 illustrates plots of A_T and A_i *versus* bulk surfactant concentration and urea concentration, respectively. These counts represent the average of ten 1-min counts run on at least duplicate solutions, after correcting for background and detector deadtime. The error bars represent three standard deviations. From these data, A_s was obtained for each molar concentration by taking the difference between A_T and A_i. Essentially identical results were obtained when glycerin was substituted for urea.

Figure 2 contains the "standard plot" used to convert A_s to Γ. The counts per minute of spread surfactant increased proportionally to the amount on the surface up to about 3.0×10^{-10} mole/cm²; beyond this value a significant decrease in slope occurred. Similar results were observed by others working with sodium lauryl sulfate and were attributed to dissolution of film molecules into the underlying solution (17). To obtain a good estimate of the slope, a least-squares line was fitted to the data up to about 3.0×10^{-10} mole/cm² while a parabola was used to include all of the data points.

Table I summarizes the results of preliminary analyses of data presented in Fig. 1, utilizing the linear standard curve. The data are presented as ±1 *SD*. Note that the values of area per molecule (reciprocal of Γ) in parenthesis were obtained by parabolic fit and that reasonable agreement is seen up to 3.5×10^{-10} mole/cm². The absolute values of area per molecule obtained at the higher concentrations (40-50 Å²) are in good agreement with those obtained for shorter chain alkylammonium ions using surface tension measurements and applying the Gibbs equation (25, 26).

Effect of Ionic Strength—To test the applicability of this technique further, the influence of ionic strength on hexadecyltrimethylammonium ion adsorption was considered. As seen in Fig.

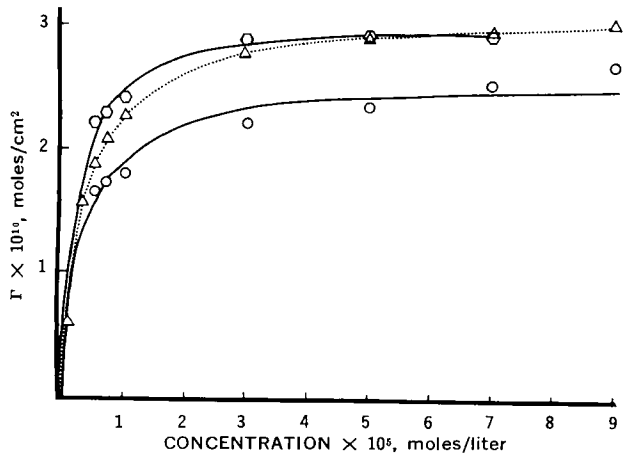


Figure 3—*Surface concentration* versus *bulk concentration as a function of ionic strength. Key:* ○, *0.01* M *KBr;* △, *0.10* M *KBr; and* ○, *0.20* M *KBr.*

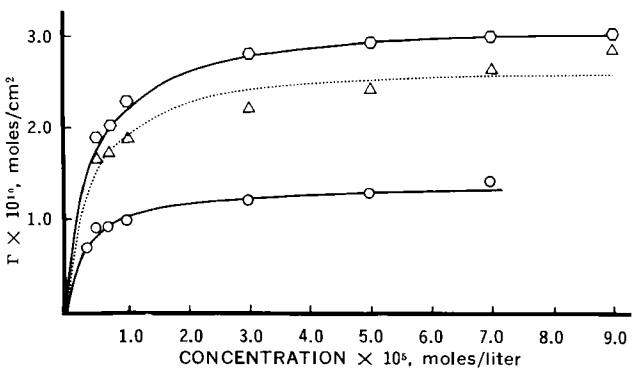


Figure 4—*Surface concentration* versus *bulk concentration in the presence of 0.10* M *halide counterions. Key:* ○, *potassium fluoride;* △, *potassium chloride; and* ○, *potassium bromide.*

Table IV—Surface Concentration × 10^{10} (Moles per Square Centimeter) for Hexadecyltrimethylammonium Adsorption in Aqueous Solutions of 0.10 *M* Sodium Benzenesulfonate and 0.10 *M* Sodium Naphthalenesulfonate

Hexadecyltrimethylammonium Concentration, moles/liter	0.01 *M* Sodium Benzenesulfonate	0.01 *M* Sodium Naphthalenesulfonate	0.1 *M* Sodium Benzenesulfonate	0.1 *M* Sodium Naphthalenesulfonate
1.0×10^{-6}	0.926 ± 0.01	0.484 ± 0.01	0.873 ± 0.01	0.469 ± 0.01
3.0×10^{-6}	1.32 ± 0.01	1.26 ± 0.02	1.45 ± 0.03	0.999 ± 0.02
5.0×10^{-6}	1.40 ± 0.02	1.32 ± 0.03	1.56 ± 0.02	1.21 ± 0.04
1.0×10^{-5}	1.37 ± 0.02	1.48 ± 0.02	1.68 ± 0.02	1.33 ± 0.03
3.0×10^{-5}	1.53 ± 0.02	2.73 ± 0.02	1.95 ± 0.02	1.51 ± 0.02
5.0×10^{-5}	1.58 ± 0.03	3.06 ± 0.03	1.86 ± 0.04	1.64 ± 0.03

Table V—Surface Concentration × 10^{10} (Moles per Square Centimeter) for Hexadecyltrimethylammonium Adsorption in Aqueous Arylsulfonate–Potassium Fluoride Solutions[a]

Hexadecyltrimethylammonium Concentration, moles/liter	Potassium Fluoride	Benzenesulfonate Ion		
		10^{-5} *M*	10^{-4} *M*	10^{-3} *M*
1.0×10^{-6}	0.646 ± 0.02	0.529 ± 0.02	0.649 ± 0.01	0.560 ± 0.02
5.0×10^{-6}	0.894 ± 0.02	0.922 ± 0.02	0.977 ± 0.03	1.28 ± 0.03
3.0×10^{-5}	1.19 ± 0.03	1.24 ± 0.02	1.36 ± 0.03	1.52 ± 0.03
		Naphthalenesulfonate Ion		
		10^{-5} *M*	10^{-4} *M*	10^{-3} *M*
1.0×10^{-6}	0.646 ± 0.02	0.560 ± 0.01	0.709 ± 0.01	1.07 ± 0.02
5.0×10^{-6}	0.894 ± 0.02	1.11 ± 0.02	1.40 ± 0.03	1.49 ± 0.03
3.0×10^{-5}	1.19 ± 0.03	1.26 ± 0.02	1.68 ± 0.03	2.04 ± 0.03

[a] A constant ionic strength of 0.10 is maintained with fluoride ion while varying the concentration of arylsulfonate.

3, the amount of adsorption increased with increasing ionic strength, going from 0.01 to 0.1 *M* KBr, and then changed much less above 0.1 *M*. This is expected if one considers that the major effect of the electrolyte on the adsorption of a surface-active cation is due to the screening of repulsive interactions between the ions already adsorbed at the surface. At lower ionic strength and particularly in the absence of an electrolyte, a significantly increased time period for the attainment of an apparent equilibrium value was found. Such slow rates, again, are expected because of the repulsion of the like-charged surfactant coming to the surface. In all subsequent studies, therefore, with a few exceptions, an ionic strength of 0.1 was used, and consideration of kinetic aspects of adsorption was not necessary.

Specific Ion Effects—Earlier work from this laboratory had demonstrated that the surface activity of some substituted phenothiazines was significantly influenced by the presence of specific ions in solution (4, 5). The present technique appeared to offer an opportunity to get a more direct estimate of specific ion effects on the number of molecules adsorbing and, at the same time, to see whether the technique was sensitive enough to see such changes. Based upon the earlier studies, therefore, the effects of fluoride, chloride, bromide, several tetraalkylammonium halides, and several alkyl- and arylsulfonates on the adsorption of hexadecyltrimethylammonium ion were measured.

Figure 4 illustrates the effect of specific halide ions at 0.1 *M*, with the order of adsorption being bromine > chlorine > fluorine. Unfortunately, at all practical concentration levels, the iodide ion produced a very insoluble salt with the surfactant (CTA^+) and thus it could not be included in this part of the study. Tables II and III indicate for four surfactant concentrations the effect of adding tetraalkylammonium ions instead of potassium. In general, one can see a retardation of adsorption relative to potassium with increasing tetraalkyl chain length. However, the patterns differ when either chloride or bromide is present. This will be discussed more fully later, but significant retardation occurs only with tetrabutyl ion in the presence of bromide ion, whereas retardation is seen with tetramethyl ion in the presence of chloride ion and this retardation levels off at the tetrapropyl ion.

Previous studies of the surface tension of substituted phenothiazine solutions indicated that some alkyl- and arylsulfonates change the concentration of drug required to produce a given degree of surface tension lowering (5). It seemed of interest to quantitate these effects by direct measurement of surface concentration. In the first series of experiments, counting studies were conducted with a series of 0.1 *M* sodium alkylsulfonates, including methane-, ethane-, 2-propane-, butane-, pentane-, hexane-, and benzenesulfonates. Figure 5 depicts the results of this experiment, indicating reduction in all cases relative to potassium bromide, little difference between the various alkylsulfonates, and more adsorption in the presence of benzenesulfonate than with the alkylsulfonates. In Table IV, the adsorption of surfactant in the presence of benzenesulfonate and naphthalenesulfonate at 0.01 and 0.1 *M* levels is compared. Note that 0.1 *M* naphthalenesulfonate produces less adsorption than 0.1 *M* benzenesulfonate, but at 0.01 *M* this order of effect is seen only at lower surfactant concentrations. At higher surfactant concentrations, naphthalenesulfonate produces more adsorption than benzenesulfonate. Further comparison of the two arylsulfonates was made by using concen-

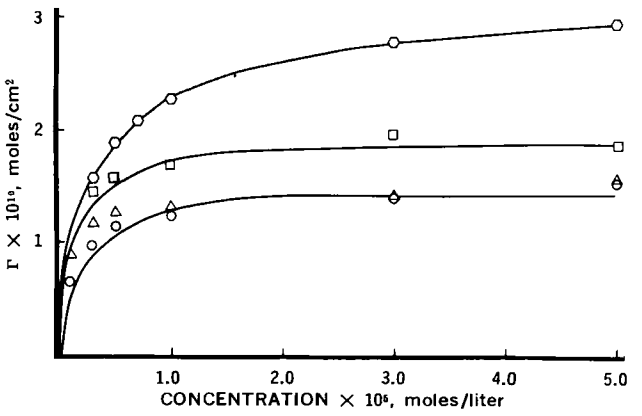


Figure 5—*Surface concentration* versus *bulk concentration in solutions of 0.10* M *sulfonate ion. Also included for comparison are adsorption data in 0.10* M *KBr. Key:* ○, *methanesulfonate;* △, *hexanesulfonate;* □, *benzenesulfonate; and* ○, *potassium bromide.*

Table VI—Surface Concentration × 10^{10} (Moles per Square Centimeter) for Hexadecyltrimethylammonium Adsorption in Aqueous Solutions of 0.10 *M* Sodium Naphthalenesulfonate and 0.10 *M* Orange II

Hexadecyltrimethylammonium Concentration, moles/liter	0.01 *M* Sodium Naphthalenesulfonate	0.01 *M* Orange II
1.0×10^{-6}	0.484 ± 0.01	0.199 ± 0.01
3.0×10^{-6}	1.26 ± 0.02	0.793 ± 0.02
5.0×10^{-6}	1.32 ± 0.03	1.13 ± 0.01
1.0×10^{-5}	1.48 ± 0.02	1.31 ± 0.02
3.0×10^{-5}	2.73 ± 0.02	1.44 ± 0.02
5.0×10^{-5}	3.06 ± 0.03	1.47 ± 0.03

trations lower than 0.01 *M* but maintaining the ionic strength at 0.1 with potassium fluoride. In all of these cases, increasing levels of sulfonate increased adsorption of surfactant, and the naphthalenesulfonate always produced more adsorption than the benzenesulfonate (Table V). It appears from these results that a critical excess of naphthalenesulfonate over hexadecyltrimethylammonium ion causes a reduction in adsorption, but as the ratio of surfactant to sulfonate gets closer to 1, a marked increase in adsorption occurs.

To check out this behavior further, it was decided to study a sulfonate of large molecular weight, the dye Orange II [*p*-(2-hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt]. Its ability to interact with long chain quaternary ammonium ions was studied previously (27). Because of the combined salt's low solubility product, a concentration only up to 0.01 *M* Orange II could be used in this study. Table VI compares the effect of naphthalenesulfonate and Orange II at a 0.01 *M* level. At all concentrations, adsorption was significantly reduced by Orange II relative to naphthalenesulfonate. Table VII lists the results of using lower amounts of Orange II at constant ionic strength. Once again one can see increased adsorption relative to no sulfonate (all potassium fluoride) but a definite *decrease* with increasing Orange II, except for higher surfactant concentrations where lower Orange II produced increased adsorption. Clearly, one is observing the same trend of retarded adsorption when the sulfonate is in excess of surfactant but a reversal if the ratio is closer to 1. A comparison of the three arylsulfonates reveals that the higher the molecular weight the greater is the effect when both retardation and accentuation of surfactant adsorption occur.

DISCUSSION

General Considerations—The results obtained in this study indicate that with the radioisotope technique one can measure directly the adsorption of hexadecyltrimethylammonium ion at the air-water interface as well as the effect of specific ions on this process. It must be kept in mind that experimental variation in these measurements is on the order of 10%, with the most serious problems occurring at low concentrations because of low counting rates. At high concentrations, there is also some error because of a greater contribution percentagewise to total counts by unadsorbed molecules. However, the differences reported are significant and they do indicate that one can study some effects specific ions produce on the adsorption of hexadecyltrimethylammonium ion to liquid interfaces.

Table VIII—Association Constants for Some Cation–Anion Interactions in Aqueous Solution Taken from Data of Packter and Donbrow (40)[a]

$ArSO_3 \ldots N(CH_3)_3R$		
Ar	R	K_a, liters/mole
Benzene	$C_{10}H_{21}$	7.2
Naphthalene-1	C_8H_{17}	7.2
Naphthalene-1	$C_{10}H_{21}$	20
Anthraquinone-1	C_6H_{13}	9.7
Anthraquinone-1	C_8H_{17}	25
Anthraquinone-1	$C_{10}H_{21}$	263
Azobenzene-4	C_4H_9	5.5
Azobenzene-4	C_6H_{13}	19.6
Azobenzene-4	C_8H_{17}	102
Azobenzene-4	$C_{10}H_{21}$	4000

[a] K_a was calculated by taking the reciprocal of instability constants given in this study (40).

To understand how the various ions can influence the adsorption of hexadecyltrimethylammonium ion to the air-water interface, it is first convenient to have an expression that relates surface concentration to bulk concentration. Davies (28) derived such an expression for the adsorption of charged surfactants to liquid interfaces, taking into account the influence of the electrical double layer produced as charged molecules are adsorbed, which is expressed as:

$$\Gamma = \frac{k'c \exp\left(\frac{W - Z_1 e\psi_0}{kT}\right)}{1 + k'\Gamma_0 c \exp\left(\frac{W - Ze\psi_0}{kT}\right)} \qquad \text{(Eq. 4)}$$

where Γ is the number of molecules adsorbed per square centimeter, c is the bulk concentration, Γ_0 is the maximum number of molecules per square centimeter that can be adsorbed, k' is a constant related to hydrodynamic factors, W is the free energy of desorption (minus free energy of adsorption) due essentially to the alkyl chain, Z is the valency of the long chain ion, e is the charge of an electron, ψ_0 is the electrical potential of the surface, k is the Boltzmann constant, and T is the absolute temperature. Basically, the equation indicates that a long chain compound with a large negative free energy of adsorption, W, will exhibit a large tendency to adsorb but that an ionic species will produce a surface potential, ψ_0, that will retard further adsorption due to repulsive effects. Consequently, it is not surprising that excess electrolyte or specific counterion interactions that reduce the effect of ψ_0 provide enhanced adsorption of surfactant at a given bulk concentration, c. At the present time with the data collected, it is very difficult to test this equation quantitatively because of a number of factors. First, the technique prevents working at low enough concentrations to simplify the expression (surface concentrations $<10^{14}$ molecules/cm^2) (28). Second, the potential term, $Ze\psi_0$, is difficult to evaluate because of limitations in the Guoy-Chapman model at higher potentials (28). Third, this equation does not take into account the very specific interactions involving counterions which are being observed. Further compli-

Table VII—Surface Concentration × 10^{10} (Moles per Square Centimeter) for Hexadecyltrimethylammonium in Aqueous Orange II–Potassium Fluoride Solutions[a]

Hexadecyltrimethylammonium Concentration, moles/liter	Potassium Fluoride	Orange II		
		10^{-5} *M*	10^{-4} *M*	10^{-3} *M*
1.0×10^{-6}	0.646 ± 0.02	0.919 ± 0.02	0.656 ± 0.01	0.393 ± 0.01
3.0×10^{-6}	0.812 ± 0.02	1.52 ± 0.03	1.33 ± 0.01	1.10 ± 0.03
5.0×10^{-6}	0.894 ± 0.02	1.58 ± 0.02	1.51 ± 0.02	1.30 ± 0.02
1.0×10^{-5}	0.994 ± 0.03	1.51 ± 0.02	1.46 ± 0.02	1.29 ± 0.02
3.0×10^{-5}	1.19 ± 0.03	1.64 ± 0.03	2.69 ± 0.02	1.47 ± 0.04
5.0×10^{-5}	1.21 ± 0.03	1.74 ± 0.03	2.70 ± 0.04	1.71 ± 0.03

[a] A constant ionic strength of 0.10 is maintained with fluoride ion while varying the concentration of Orange II. Included are data for hexadecyltrimethylammonium adsorption in 0.10 *M* KF.

cations come from some uncertainties related to the state of the hexadecyltrimethylammonium ion in bulk solution so that concentration is not necessarily related to activity. For example, there is good evidence for dimerization of such surfactants below the CMC (29), as well as some "ion-pairing" in aqueous solution when relatively large hydrophobic counterions are present (30). Such evidence will be presented when discussing the various specific ion effects.

Specific Effect of Halide Ions—The influence of halide ions on the adsorption of surfactant, as measured directly in this study, is consistent with earlier results of surface tension measurement using quaternary dodecylammonium salts (31) and chlorpromazine ion (5). These results also agree with those obtained for micelle formation (32) and for oil-water partitioning of protonated amine ion-pairs (33). When hexadecyltrimethylammonium ion is adsorbed to the interface, it must have counterions in its vicinity. If these counterions tend to interact with surfactant at the surface so as to reduce effectively the positive charge there, the free energy term in Eq. 4 must be reduced, leading to enhanced adsorption.

A number of factors can determine the extent of the interaction between ions at an interface, including a lower local dielectric constant relative to water (34), a tendency of anions to leave an aqueous environment for one that is more nonpolar (hydrophobic properties, *i.e.*, the state of hydration (35), and the tendency of the ion to be polarized and to interact specifically by noncoulombic forces of attraction (32). Indeed, halide ions have a tendency to accumulate at the air-water interface, even in the absence of an opposite charge, in the order bromine > chlorine > fluorine; this is the order of increasing hydration and decreasing size (35). Thus, one can conclude that, whereas simple ions such as the halides have no tendency to pair with hexadecyltrimethylammonium ion in aqueous solution, the nature of the air-water interface is such that it favors such ion-pairing. With the radioisotope technique, one is able to detect differences between the three halides studied and it now remains to quantitate this in future work by working at lower levels of adsorption where Eq. 2 is applicable.

In the series of experiments with the tetraalkylammonium bromides and chlorides, there appears to be an effect due to the coion as well as the counterion. The specific effect of bromine *versus* chlorine, noted earlier, is apparent from a comparison of surfactant adsorption in the presence of the same coion but different counterions; bromine favors adsorption over chlorine (Tables II and III). The second effect at constant counterion is a reduction in surfactant adsorption relative to that seen in the presence of potassium, with an increased effect at higher tetraalkylammonium-ion chain length. Some general questions one can ask first are how does an ion of similar charge cause a reduction in adsorption of surfactant and why does the effect increase with increasing alkyl chain length. One also can ask why the relative effect noted for the series of tetraalkylammonium ions does not occur to the same extent for bromine and chlorine.

First, it is possible that some competition exists for the surface between the surfactant and the other tetraalkylammonium ions. Indeed, tetrabutylammonium ion in the presence of chloride and bromide does produce a 10- and 13-dynes/cm change, respectively, in the surface tension of water (5). However, the tetramethylammonium chloride system suppresses adsorption quite significantly, yet it exhibits little surface activity (5). Further evidence against simple competition is seen from work on the effect of these coions on the oil-water partitioning of protonated amine ion-pairs (33). It was shown by direct measurement that these tetraalkylammonium ions reduced the partitioning of chlorpromazine ion into *n*-octanol in the same order noted here, but that no tetraalkylammonium ion partitioned into the oil phase.

In view of these observations, it seems most probable that inhibition of adsorption occurs in the bulk aqueous solution. The most likely possibility would seem to be an effect on water structure by the tetraalkylammonium ions such that the hydrophobic properties of the surfactant are reduced. Steigman *et al.* (36) measured the effect of these tetraalkylammonium ions on the CMC of hexadecyltrimethylammonium ion with the same general result seen here, namely, increased CMC with increasing alkyl chain length and thus reduced hydrophobic behavior. They attributed this effect to the presence of alkyl groups in addition to those of the surfactant which organize water structures in such a way as to reduce the tendency for nonpolar groups to leave an aqueous environment. Apparently, this structuring of water offsets the favorable entropy change ordinarily occurring when water structure breaks down after a long chain group goes to a micelle or interface. This would explain the enhanced effect when increasing alkyl chain length. Lindenbaum (37) measured increased hydrogen bonding of water in the presence of tetraalkylammonium salts.

The other question centers on the different patterns of chain length effect with bromine and chlorine. If it is assumed that both counterion and coion effects act in opposite directions, one might expect such differences. For example, bromine produces ion-pairing to a greater extent than chlorine and, hence, one might have to go to tetrabutylammonium bromide before seeing a significant reduction. On the other hand, tetramethylammonium chloride is able to offset these ion-pairing effects to some extent.

Effects of Alkyl- and Arylsulfonates—The results of experiments involving the organic sulfonates support an earlier conclusion that adsorption of hexadecyltrimethylammonium ion is enhanced by ion-pairing at the surface but retarded by conditions in the bulk aqueous phase. Whereas the most likely explanation for the retarding effect of the tetraalkylammonium ions is their effect on water structure, an additional possibility exists when considering the effect of sulfonates, *i.e.*, the formation of ion-pairs in water as a competitive equilibrium to the adsorption process. Ordinarily, ion-pairs do not form in water because of the high dielectric constant and hydrating capacity of water which offsets coulombic attraction of opposite charges. However, when these ions both contain hydrophobic groups, there is an enhanced possibility of interaction. A number of factors are probably responsible for this, all centering on hydrophobic interactions. Diamond (38) suggested that a major factor is the reduced extent of the bulk water structuring brought about by having two closely placed hydrophobic groups rather than two hydrophobic ions, resulting in a favorable increase in entropy to the system. He referred to this as water-structure-enforced ion-pairing. Since increased hydrophobicity would also favor such association at the surface, ion-pairing at the surface and bulk aqueous solution would be favored with increasing size of the sulfonate ion, possibly producing competitive effects on surfactant adsorption.

A few studies included association constants in aqueous solution for systems related to those used in the present study. Mukerjee and Mysels (30) determined association constants for tetraalkylammonium ions, up to propyl, with lauryl sulfate ion in the range of 12.5-21 at 25°; Modin and Schill (39) measured association constants for these cations with toluenesulfonate and naphthalenesulfonate, ranging from 5.0 to 30. Table VIII presents some association constants for systems of much closer relationship to the present work; these were obtained by Packter and Donbrow (40). In general, one observes that increasing the size of either ion has a significant effect on association; for systems similar to hexadecyltrimethylammonium ion and Orange II, one can expect a relatively high association constant on the order of 10^5–10^6. This upper limit is believed to be reasonable based on a value reported for octadecyltrimethylammonium ion and the dye methyl orange of about 10^6–10^7 (41).

Assuming that there is good evidence for association in aqueous solution as well as at the surface, how does this explain the results of this study? First, one can consider the situation where going from methanesulfonate to hexanesulfonate has little effect on adsorption of surfactant. There is always the possibility that these effects are too small to be separated by the present technique. However, it is also highly probable that compensating effects due to surface and bulk ionic association are occurring. More revealing is the effect of going from hexanesulfonate to benzenesulfonate (Fig. 5). It is suggested here that association in solution and/or other water-structuring effects are greater for the hexanesulfonate ion because of greater hydrophobicity. Thus, a greater adsorption of surfactant with benzenesulfonate occurs.

Following this idea, one can see similar patterns with the other systems. For example, in Tables IV–VII, with naphthalenesulfonate, benzenesulfonate, and Orange II, a situation exists where the larger, more hydrophobic ion always retards surfactant adsorption if the concentration of anion is in large enough excess of the surfactant. However, at lower anion concentrations, the reverse, enhancing effect is noted. Assuming association to be described by a simple expression, with CTA^+ representing the surfactant:

$$CTA^+ + A^- = CTA^+ \cdots {}^-A \qquad \text{(Eq. 5)}$$

and considering the relatively low concentration of hexadecyltrimethylammonium ion used, a significant excess of anion would be needed to influence the surfactant monomer concentration enough to reduce "normal" adsorption, hence the dependency of extent of retardation on anion concentration or ratio of surfactant to anion.

That ion association of hexadecyltrimethylammonium ion and Orange II can occur in aqueous solution and can interfere with "extraction" of both species to a nonpolar phase was shown in the work of Scott (42). He measured the percent of this surfactant extracted into chloroform in the presence of Orange II (not soluble in chloroform) and found an inefficiency of extraction when less than a 50% excess of Orange II was present. The inefficiency was attributed to association of surfactant and Orange II in aqueous solution, as suggested here. Unfortunately, to quantitate this picture completely, it will be necessary to get a better measure of the equilibrium constants describing what occurs at the surface. In addition, one must somehow account for the many equilibria involving ion-pairing in water, as well as the self-association of hexadecyltrimethylammonium ion and anions such as Orange II (29, 43). It is clear, however, that this method in conjunction with other techniques and with more refinement can be used to answer these questions.

CONCLUSIONS

1. In this study the adsorption of a long chain ion, hexadecyltrimethylammonium, was measured at the air-water interface using a radiotracer technique. The applicability of this technique was tested by observing changes in adsorption due to the presence of various ionic species.

2. Adsorption of surfactant was shown to be dependent on the type of halide ion present; the halide ion apparently forms an ion-pair at the surface, thus increasing the extent of adsorption in the order bromine > chlorine > fluorine. A secondary retarding effect was noted with the use of tetraalkylammonium halides up to the tetrabutyl derivative. This effect is best explained by changes due to water structuring in conjunction with surface ion-pairing.

3. Adsorption of surfactant is greatly influenced by hydrophobic sulfonate ions. Here, the problem is complicated apparently by a combination of enhanced adsorption due to ion-pairing at the surface and reduced adsorption due to association of oppositely charged ions in aqueous solution.

4. This radioisotope technique can detect subtle changes in adsorption at the air-water interface due to specific ions and it can be used with other hydrophobic molecules, including many drugs, to evaluate their fundamental properties at interfaces.

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* Present address: Wyeth Laboratories, Philadelphia, Pa.

‡ Present address: School of Pharmacy, University of Connecticut, Storrs, Conn.

ˣ To whom inquiries should be directed.

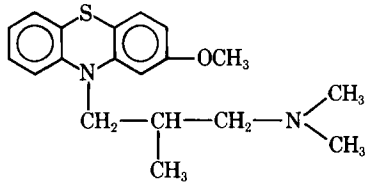
Identification and Quantitative Determination of Phenothiazine Drugs in Urine Samples of Psychiatric Patients

A. P. De LEENHEER

Abstract □ A GLC method for the specific identification and quantitation of phenothiazine drugs excreted in urine at the microgram level is described. The phenothiazine drugs and less polar metabolites are isolated by two-phase extraction with ether as an organic extractant. Quantitative determination at the microgram level is done on an acetylated fraction of the alcoholic extract by GLC using a suitable internal standard. The compounds are also identified by examining an aliquot of the final alcoholic extract by TLC for supporting evidence of the GLC method's specificity.

Keyphrases □ Phenothiazines—identification and determination in urine of psychiatric patients, GLC □ Antipsychotic agents—GLC identification and determination of phenothiazine drugs in urine samples from psychiatric patients □ GLC—identification and determination, phenothiazine drugs in urine, psychiatric patients

Medicinal chemistry has led to the development of phenothiazine drugs containing a piperidyl or piperazinyl group in their structure. Because these more potent drugs are administered in lower amounts, the analyst faces the problem of their specific identification and quantitation at the microgram level. This report describes a GLC method for detecting and assaying propericiazine[1], dixyrazine[2], perazine[3], trifluoperazine[4], prothipendyl[5], and analogs in urine samples taken from psychiatric patients under controlled drug therapy.



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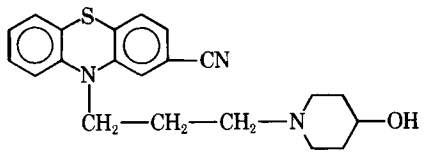
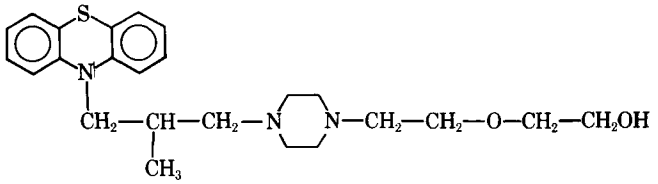


propericiazine



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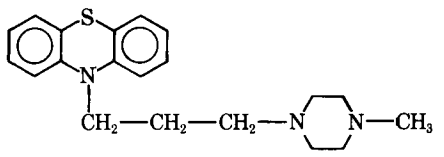


perazine

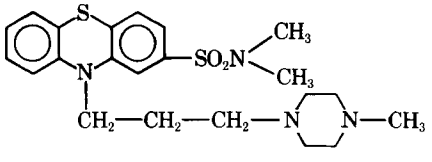
EXPERIMENTAL

Collection of Urine Samples—Twenty-four-hour urine outputs were collected separately from seven male patients under drug therapy (Table I) over 4 successive days following drug dosage. The samples were kept in the refrigerator at 4° to the moment extraction was started.

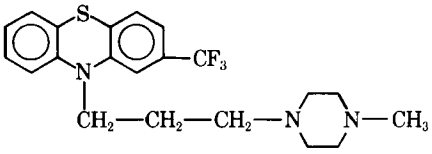
Extraction Procedure—A 200-ml volume of urine sample was acidified with 2 *N* HCl and extracted under a nitrogen atmosphere with 400- and 200-ml aliquots of ether (peroxide free, freshly distilled over hydroquinone, reagent grade) which was discarded.

The acidic urine layer was made alkaline with 10 *N* NaOH, saturated with nitrogen, and extracted twice with 400 ml of ether. The collected ether phases were washed with 5-ml fractions of 0.001 *N* NaOH until the aqueous layer remained colorless. The ether extract was evaporated at 40° in a rotory evaporator to a 100-ml final volume. The 100-ml ether phase was washed again with 5-ml fractions of 0.001 *N* NaOH, and the ether phase was then extracted twice with 10 ml of 0.1 *N* HCl.

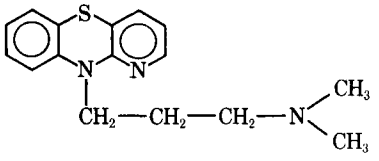
The acidic extract was made basic with 1 ml of 10 *N* NaOH



thioproperazine



trifluoperazine



prothipendyl

[1] Also known as periciazine, the chemical name is cyano-2[(hydroxy-4''-piperidino)-3'-propyl]-10-phenothiazine.

[2] The chemical name is [(B-hydroxyethoxyethylpiperazinyl)-3'-methyl-2'-propyl]-10-phenothiazine.

[3] The chemical name is *N*-{[methyl-4'-piperazinyl-(1')]-3-propyl}-phenothiazine.

[4] The chemical name is trifluoromethyl-3{[methyl-4''-piperazinyl-(1'')]-3'-*N*-propyl}-10-phenothiazine.

[5] The chemical name is (dimethylamino-3-*N*-propyl)-10-aza-1-phenothiazine.

Table I—Psychiatric Patients Receiving Phenothiazine Drug Medication

Patient	Age, Years	Drug Treatment: Type	Drug Treatment: Daily Dose Administered Orally, mg	Time Medication Went on before First Urine Collection
1	31	Propericiazine	10	3 years
2	42	Dixyrazine	3 × 25	3 weeks
3	25	Perazine	5 × 100	2.25 years
4	30	Trifluoperazine	2 × 20	7 months
5	44	Prothipendyl	3 × 20	1.5 years
6	44	Propericiazine	20	3 years
		Carbamazepine	2 × 200	
		Diphenylhydantoin[b]	100	
		Phenobarbital[c]	200	
7	60	Dixyrazine (levomepromazine)	3 × 25 —[a]	8 months

[a] Uncontrolled self-medication. [b] Diphantoine. [c] Luminal.

Table II—GLC Conditions, Retention Times (R_t), and Calibration Data ($\bar{k}$) of Phenothiazine Drugs

Phenothiazine Drug	Internal Standard	Oven Temperature	R_t (Drug), min	R_t (Standard), min	$\bar{k}$	Linearity over Weight Ratio Range, $\Delta(w_x/w_s)$
Column System: 5% OV-1 on Diatoport S, 80–100 mesh						
Propericiazine	Dixyrazine	260°	12.7	19.6	1.94	0.25/1–1.00/1
Dixyrazine	Thioproperazine	260°	18.5	21.4	1.06	0.25/1–0.67/1
Perazine	Trifluoperazine	240°	7.7	5.8	0.69	1.00/1–4.00/1
		240°	6.0	4.5	0.67	1.00/1–4.00/1
Trifluoperazine	Perazine	230°	6.9	9.3	1.28	0.11/1–0.67/1
Trifluoperazine sulfoxide	Perazine	230°	15.6	9.3	0.91	0.11/1–0.67/1
Prothipendyl	Levomepromazine	230°	2.7	4.6	0.92	0.43/1–1.50/1
Column System: 2% FFAP on Diatoport S, 80–100 mesh						
Perazine	Trifluoperazine	240°	18.5	8.0	0.55	1.00/1–4.00/1
		240°	17.8	8.0	0.53	1.00/1–4.00/1
Trifluoperazine	Perazine	230°	13.6	30.0	1.60	0.11/1–0.67/1
Prothipendyl	Levomepromazine	230°	6.1	10.6	0.75	0.43/1–1.50/1

and twice extracted with 40 ml of ether. The combined ether layers were dried on sodium sulfate, and the ether was evaporated with a nitrogen stream in a water bath at 40°.

The residue obtained was dissolved in 10.0 ml of ethanol, and the alcoholic extract was kept in a deep freeze for further analysis.

GLC—A research gas chromatograph[6] equipped with two dif-

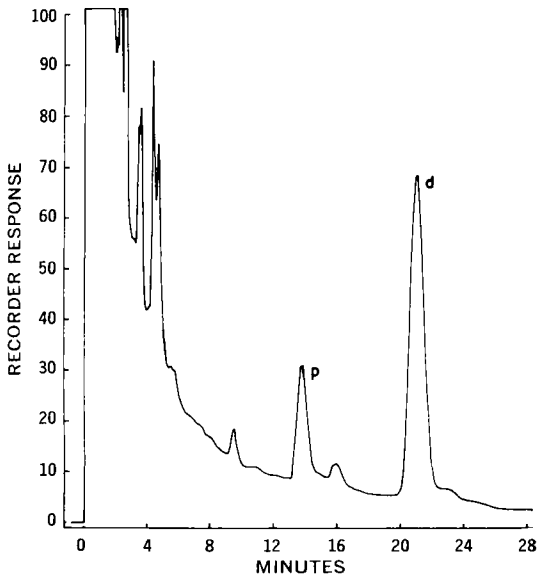


Figure 1—*Gas chromatogram of a urine extract from Patient 1. The column was 5% OV-1; quantitative determination (acetylation) of propericiazine (p) with internal standard dixyrazine (d).*

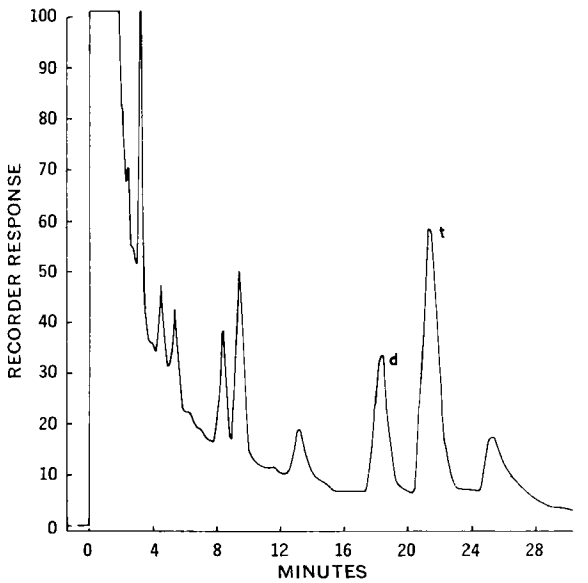


Figure 2—*Gas chromatogram of a urine extract from Patient 7. The column was 5% OV-1; quantitative determination (acetylation) of dixyrazine (d) with internal standard thioproperazine (t).*

[6] Hewlett-Packard 5750.

Table III—Recovery Data of Phenothiazine Drugs Added to Blank Urine Samples and Determined by Quantitative GLC[a]

Phenothiazine Drug	Amount Added to 200 ml of Blank Urine Sample, μg	Recovery of Drug							
		5% OV-1				2% FFAP			
		First Sample		Second Sample		First Sample		Second Sample	
		μg	%	μg	%	μg	%	μg	%
Propericiazine	400	344	86	352	88	—	—	—	—
Dixyrazine	400	376	94	376	94	—	—	—	—
Perazine	248	241	97	223	90	245	99	223	90
		233	94	226	91	241	97	236	95
Trifluoperazine	339	315	93	325	96	315	93	319	94
Prothipendyl	336	319	95	329	98	323	96	323	96

[a] Identical operating conditions as indicated in Table II.

Table IV—Quantitative Results of GLC Determination of Phenothiazine Drugs in Urine Samples Collected from Psychiatric Patients Receiving Drug Therapy

Patient	Phenothiazine Drug	Sample	$F(U)$[a], ml (ml)	I[b], μg	v[c], μl	f[d], μl	C_u, μg/100 ml Urine		Urinary Excretion[e], %
							5% OV-1	2% FFAP	
1	Propericiazine	1	2.0 (40)	20	40	0.9	4.2		1.01
		2	2.0 (40)	20	40	1.3	7.1		3.12
		3	2.0 (40)	20	40	1.4	7.5		1.80
		4	2.0 (40)	20	40	1.4	7.3		0.70
2	Dixyrazine	1	1.8 (36)	15	15	1.4	6.2		0.10
		2	1.8 (36)	15	15	1.4	4.7		0.12
		3	1.8 (36)	15	15	1.5	5.9		0.15
		4	1.8 (36)	15	15	1.4	3.5		0.08
3	Perazine	1 (a)	1.0 (20)	10	160	0.5	190		1.33
					40	0.9		216	1.51
		(b)	1.0 (20)	10	40	1.0	208		1.46
					40	1.0		211	1.48
		2 (a)	1.0 (20)	10	200	0.4	313		1.50
			1.0 (20)	10	40	0.6		307	1.47
		(b)	1.0 (20)	10	120	0.7	307		1.47
			1.0 (20)	10	40	0.9		306	1.47
		3 (a)	1.0 (20)	10	120	0.5	55.2		0.22
			1.0 (20)	10	20	0.6		50.2	0.20
		(b)	1.0 (20)	10	40	0.9	55.9		0.22
			1.0 (20)	10	40	1.4		50.0	0.20
		4 (a)	1.0 (20)	10	200	1.0	98.3		0.24
			1.0 (20)	10	20	1.0		104	0.25
		(b)	1.0 (20)	10	80	0.9	96.8		0.23
			1.0 (20)	10	40	1.0		107	0.26
4	Trifluoperazine (sulfoxide)	1	2.0 (40)	20	40	0.7	5.0 (6.8)		0.09 (0.15)
			2.0 (40)	20	20	1.9		5.4	0.10
		2	2.0 (39)	20	40	0.7	8.8 (18.3)		0.24 (0.57)
			2.0 (39)	20	20	1.4		8.1	0.22
		3	2.0 (40)	20	40	0.8	—(−)		
			2.0 (40)	20	20	1.4		—	
		4	2.0 (40)	20	40	0.8	16.3 (27.5)		0.45 (0.88)
			2.0 (40)	20	20	1.4		14.5	0.40
5	Prothipendyl	1	3.0 (60)	10	40	0.8	4.2		0.24
			3.0 (60)	10	20	0.6		4.1	0.24
		2	3.0 (60)	10	40	0.7	2.3		0.24
			3.0 (60)	10	20	0.8		1.7	0.18
		3	3.0 (50)	10	40	0.5	1.3		0.22
			3.0 (50)	10	20	0.9		0.9	0.15
		4	3.0 (60)	10	40	0.7	1.7		0.22
			3.0 (60)	10	20	0.8		1.4	0.18
6	Propericiazine	1	2.0 (40)	30	60	1.5	19.7		1.87
		2	2.0 (40)	30	60	1.5	19.8		1.58
		3	2.0 (40)	30	60	1.4	13.6		0.75
		4	2.0 (40)	30	60	1.5	4.9		0.37
7	Dixyrazine	1	1.7 (34)	20	20	1.3	24.0		0.51
		2	2.0 (40)	20	20	1.5	27.0		0.68
		3	1.8 (36)	20	20	1.4	18.9		0.07
		4	2.0 (40)	20	20	1.3	5.9		0.12

[a] F = aliquot of alcoholic extract in milliliters taken for evaporation; (U) = corresponding volume in milliliters of original urine sample. [b] I = amount of internal standard in micrograms added to fraction F of alcoholic extract for evaporation. [c] v = volume in microliters of ethyl acetate in which residue was dissolved. [d] f = fraction in microliters of end volume v injected on top of GLC column. [e] Urinary excretion or percent of administered dose recovered as unchanged drug.

Table V—TLC Results of Reference Substances and Extracts of Urine Samples Collected from Psychiatric Patients Receiving Drug Therapy[a]

Patient	Material Spotted (Sample and Reference Substance)	Number of Samples Analyzed	$F(U)$[b]	Rel. R_f[c]	(R_f)[d]	Detection[e]
1	Propericiazine			1.00	(0.41)	I, orange
	Propericiazine sulfoxide			0.46		I, orange
		1	2.0 (40)	0.46		I, orange
				0.90		I, orange
				1.22		I, orange
				1.48		I, orange
2	Dixyrazine			1.00	(0.44)	I, pink
	Dixyrazine sulfoxide			0.39		I, pink
		4	2.0 (40)	0.35		I, pink
				0.86		I, blue
				1.00		I, pink
3	Perazine dimalonate			1.00	(0.39)	I, pink
	Perazine sulfoxide			0.28		I, pink
		3	1.0 (20)	0.18		I, pink
				0.31		I, pink
				0.49		I, pink
				1.02		I, pink
				1.64		I, pink
4	Trifluoperazine dihydrochloride			1.00	(0.49)	I + III, brown
	Trifluoperazine sulfoxide			0.39		I + III, brown
		4	2.0 (40)	0.18		I + III, brown
				0.41		I + III, brown
				1.00		I + III, brown
				1.23		I + III, brown
5	Prothipendyl hydrochloride monohydrate			1.00	(0.50)	I + II, yellow-brown
	Prothipendyl sulfoxide			0.52		I + II, yellow
		1	2.0 (40)	—		
		2	2.0 (40)	—		
		3	2.0 (40)	—		
		4	2.0 (40)	—		
6	Propericiazine			1.00	(0.38)	I, pink
	Propericiazine sulfoxide			0.50		I, pink
		2	1.0 (20)	0.50		I, pink
				1.08		I, pink
				1.74		I, yellow
				2.02		I, yellow
7	Dixyrazine			1.00	(0.53)	I, pink
	Dixyrazine sulfoxide			0.38		I, pink
		1	2.0 (40)	0.09		I, pink
				0.38		I, pink
				0.98		I, pink
		2	2.0 (40)		(0.11)	I, blue
					(0.55)	I, blue

[a] Drug therapy as described in Table I. [b] F = aliquot of alcoholic extract in milliliters taken for evaporation and spotting on the plate; (U) = corresponding volume in milliliters of original urine sample. [c] Rel. R_f = R_f of compound/R_f of reference substance. [d] (R_f) = R_f of reference substance as measured on chromatogram. [e] I = ferri reagent; II = vanadium reagent; III = iodoplatinate reagent.

ferent columns for simultaneous single-column operation was used. The columns consisted of 5% OV-1 (methyl silicone polymer, apolar) and 2% FFAP (reaction product of Carbowax 20M and 2-nitroterephthalic acid, polar) coated on Diatoport S[7], 80–100 mesh (acid washed and silane treated), and packed in 1.80-m × 4-mm i.d. spiralized Pyrex columns.

Nitrogen was used as the carrier gas at 60–80 ml/min (bubble flowmeter), and a double flame-ionization detector was used as the detection system. The hydrogen flow rate was 50 ml/min; air was optimized for good sensitivity and stability.

Both columns were conditioned for 72 hr, at 280° for the 5% OV-1 and at 245° for the 2% FFAP column. Analyses were done isothermally with 20° higher temperature settings for the injector and detector blocks and at an electrometer setting range of ×1 and an attenuation of ×32.

Fixed volumes of alcoholic solutions of the compound to be determined and of internal standard were transferred with a 50-μl syringe[8] into conical, silylated (left in contact for 1 hr with 5% dichlorodimethylsilane in benzene, rinsed with benzene, washed three times with water, and dried in the air) centrifuge tubes of 15-ml capacity and thoroughly mixed. For each calibration series, four or five different solutions of equal weight ratio increments ($\Delta w_x/w_s$) were prepared. After the mixtures were evaporated in a water bath at 40° with the aid of a slow stream of nitrogen, acetylation was performed by dissolving residues in 0.2 ml of pyridine (refluxed and redistilled from potassium hydroxide) and 0.2 ml of acetic acid anhydride (refluxed and redistilled from calcium carbide), mixing thoroughly with a mixer[9], and leaving for a 3-hr reaction time in a phosphorus pentoxide desiccator. Reagents were evaporated at 40° with the aid of a nitrogen stream, and the residue was redissolved in a small volume of ethyl acetate. Volumes varying from 1 to 3 μl were injected with a 10-μl syringe[10] on top of the column(s).

An appropriate aliquot of the alcoholic extract of the urine sample was mixed with a known amount of internal standard and treated in a manner similar to the calibration solutions. Accord-

[7] Hewlett-Packard.
[8] Hamilton 705N.
[9] Vortex.
[10] Hamilton 701N.

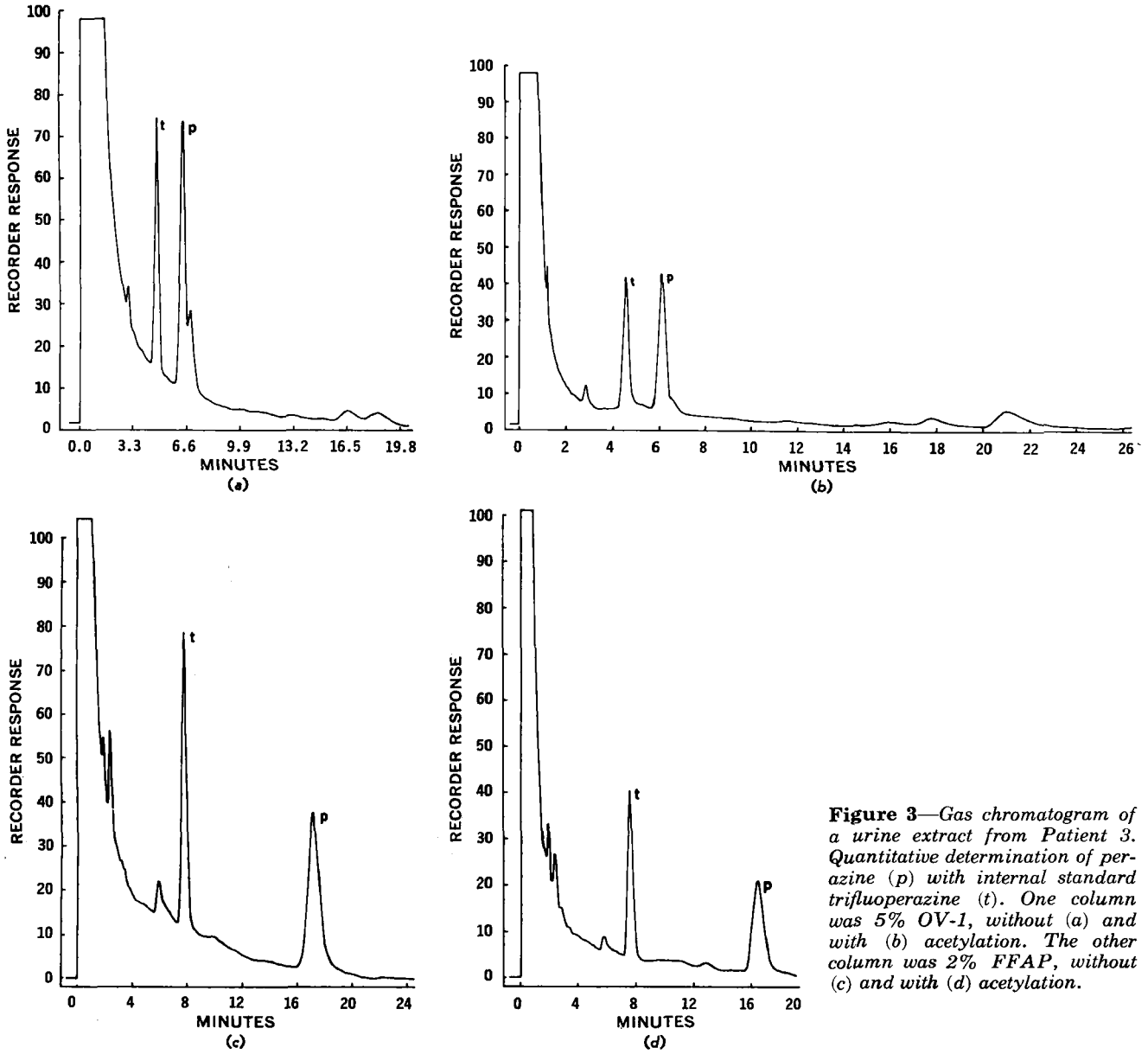


Figure 3—*Gas chromatogram of a urine extract from Patient 3. Quantitative determination of perazine (p) with internal standard trifluoperazine (t). One column was 5% OV-1, without (a) and with (b) acetylation. The other column was 2% FFAP, without (c) and with (d) acetylation.*

ing to their chemical structure relationship and retention properties, dixyrazine, thioproperazine, trifluoperazine, perazine, and levomepromazine served as suitable internal standards.

GLC Calculations—Calibration factors k_i were determined by measuring the corresponding peak areas on chromatograms from the calibration series:

$$k_i = A_x w_s / A_s w_x \qquad \text{(Eq. 1)}$$

where A_x and A_s and w_x and w_s are peak areas and weights of the compound to be determined and the internal standard, respectively. Peak areas are measured by the method of peak height times peak width at half-height.

For each calibration series, the mean calibration factor $\bar{k}$ was calculated using four or five measurements.

Results for urine samples C_u (in μg/100 ml) were found by the equation:

$$C_u = \frac{5}{v} \frac{A_x'}{A_s'} w_s \qquad \text{(Eq. 2)}$$

where $5/v$ is the conversion factor for the volume v of the alcoholic extract recalculated for 100 ml of original urine sample; A_x' and A_s' are peak areas of the compound to be determined as present in the urine extract and the internal standard, respectively; and w_s is the weight of the internal standard added to a fraction of the alcoholic extract prior to evaporation.

TLC—TLC plates were prepared by mixing 12.5 g of silica gel HF_{254}[11] and 12.5 g of cellulose MN300[12] with 90 ml of water and using the slurry for five 20 × 20-cm plates of 250-μm thickness. The plates were air dried and used without further temperature activation.

A 1–2-ml aliquot of the alcoholic extract was transferred into conical centrifuge tubes of 15-ml capacity and evaporated with a nitrogen stream in a water bath at 40°. The residue was dissolved in 10 μl of ethanol and quantitatively spotted with a micropipet in a 2-cm line on 3 cm of the lower edge of a TLC plate. A 10-μl volume of a solution containing 10 mg of a reference substance in 10 ml ethanol was applied in the same way on each plate.

Elution was performed over a 10-cm distance taken from the point of the applied spots and using a mixture of chloroform, acetone, and 25% ammonia (50:50:1 ml).

Substances were visualized by inspection under 254- and 350-nm UV light and by spraying with ferri, vanadium, and iodoplatinate reagents. After spraying with one of the reagents, stains were intensified by blowing a hot air stream over the TLC plate for a few moments.

For the ferri reagent (1), 500 mg of ferric nitrate was dissolved in 80 ml of concentrated sulfuric acid and the solution was diluted with water to 1000 ml. For the vanadium reagent, 500 mg of

[11] Merck.
[12] Macherey, Nagel and Co.

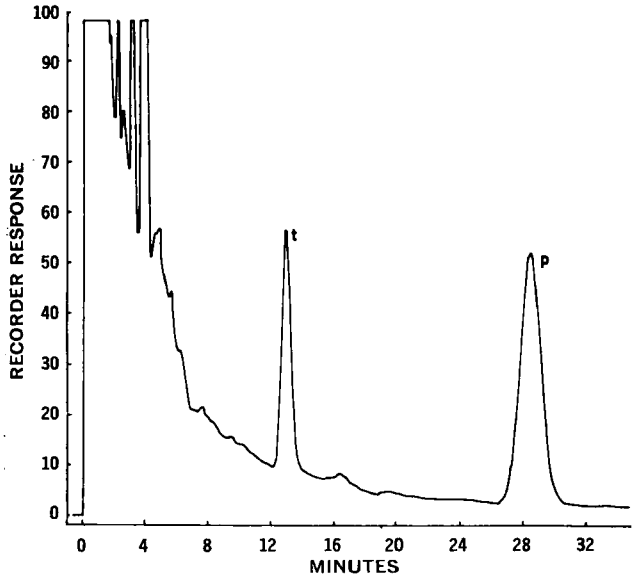


Figure 4—*Gas chromatogram of a urine extract from Patient 4. The column was 2% FFAP; quantitative determination of trifluoperazine (t) with internal standard perazine (p).*

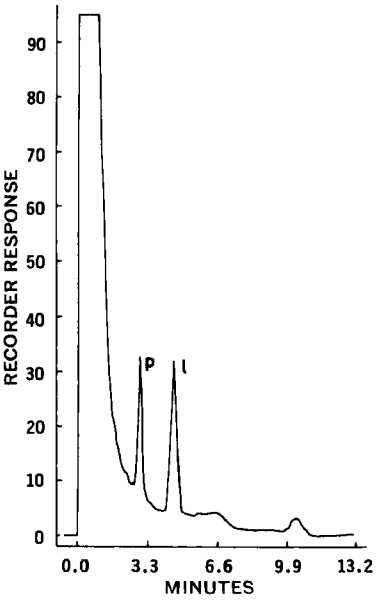


Figure 5—*Gas chromatogram of a urine extract from Patient 5. The column was 5% OV-1; quantitative determination of prothipendyl (p) with internal standard levomepromazine (l).*

vanadium pentoxide was dissolved in 80 ml of concentrated sulfuric acid and the solution was diluted with water to 1000 ml. For the iodoplatinate reagent (2), 5 ml of 5% platinum chloride was mixed with 45 ml of 10% KI and 100 ml water was added.

Recovery Experiments—To fractions of 200 ml of urine, collected from persons under no drug medication, propericiazine, dixyrazine, perazine, trifluoperazine, and prothipendyl were added in amounts of 400, 400, 248, 339, and 336 μg, respectively, and taken through the complete procedure.

RESULTS AND DISCUSSION

GLC operating conditions, retention times (R_t) of phenothiazine compounds and internal standards, calibration data ($\bar{k}$), and linear ranges of standard curves [$\Delta(w_x/w_s)$] are presented in Table II. Substances with a free polar alcohol group in their structure, such as propericiazine and dixyrazine, had first to be derivatized to the corresponding acetyl esters. In this way, excessive tailing effects were eliminated while separation efficiency and analytical precision increased. Calibration mixtures of perazine–trifluoperazine were examined with and without acetylation treatment. From the results, it appeared that no selective loss of one of the compounds mentioned occurred during the derivatization step. Acetyl esters of propericiazine, dixyrazine, and their sulfoxide metabolites are high molecular weight compounds and showed long retention times on apolar liquid phases. They could not have been chromatographed on the more polar and selective 2% FFAP column system within a reasonable time.

Results for recovery experiments of the phenothiazine compounds added to blank urine samples are given in Table III. Figures obtained on two different samples using both GLC columns correlate well. These data indicate an extraction efficiency of 86–99% and a method reproducibility of about 3%. They also are considered representative over the entire range of the standard curves. The described procedure for these phenothiazine drugs has a low detection limit of 5–10 ng/ml urine and an overall precision of about 7%.

Actual concentrations of drugs excreted in urine and expressed as μg/100 ml or percent of administered dose recovered as unchanged drug are tabulated in Table IV. Some examples of quantitative chromatograms are given in Figs. 1–5. Chromatographic patterns of urine extracts determined on both GLC columns show extraneous peaks produced by unidentified metabolites, which were also detected by TLC. Only small amounts or rather low percentages of the phenothiazine drugs were excreted in urine; regardless of the compound, the absolute dose administered, and the physiological condition of the patient, urinary excretion varied from 0.1 to 1.9%. These data demonstrate that this GLC method is very selective and sensitive for the quantitation of phenothiazine drugs in urine following the administration of low therapeutic doses of these drugs. Furthermore, as might be evaluated from the retention characteristics described in Table II, this GLC procedure should enable one to separate and measure more than one phenothiazine drug from the urine of a patient receiving a combination of these drugs.

TLC results were expressed as Rel. R_f values, *i.e.*, ratio of actual R_f to R_f of phenothiazine compound, and some typical examples are given in Table V. As indicated by underlined figures, the presence of free drug administered, sulfoxides, and other unidentified, structurally related metabolites was established in urine extracts of all patients except Patient 5. In this case, negative results were obtained because of the low dose of administered drug. Both separation efficiency and a high sensitivity of 25–50 ng/ml urine make TLC a valuable tool for following low therapeutic levels of phenothiazine drugs and apolar metabolites as excreted in urine for the verification of the specificity of the GLC procedure used for the quantitative measurement of these drugs.

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Synthesis and Screening of Cyclic Benzylhydrazine Congeners as Antidepressants

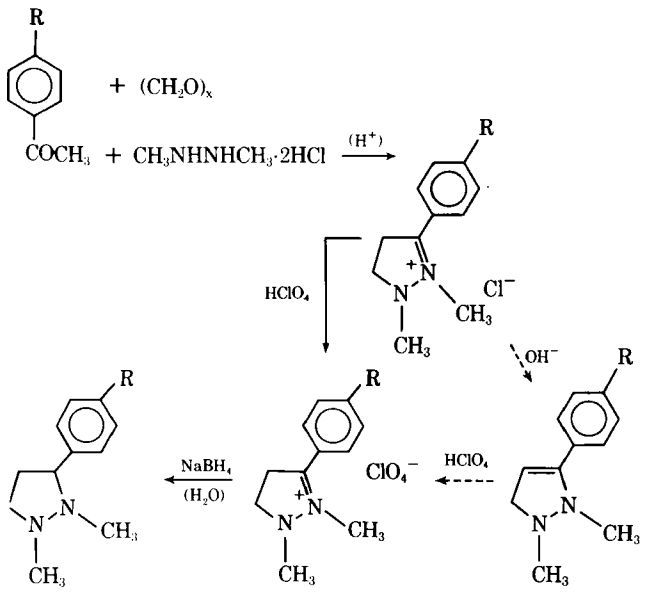
A. M. TAHA*x, N. M. OMAR*, S. M. BAYOMI*, E. M. AMMAR‡, and A. AFIFI‡

Abstract □ Several 2-pyrazolinium salts and their corresponding pyrazolidines, congeners of a cyclic benzylhydrazine structure, were synthesized and their pharmacological activity as antidepressant agents was evaluated. The 2-pyrazolinium salts were obtained through the interaction of 1,2-dimethylhydrazine dihydrochloride, paraformaldehyde, and the appropriately substituted aralkyl ketone *via* a Mannich reaction. The corresponding pyrazolidines were synthesized by reduction of the pyrazolinium perchlorates with sodium borohydride in aqueous medium. The antidepressant activity of both series was evaluated in mice in comparison with nialamide. As indicators of antidepressant action, use was made of the antagonism of reserpine-induced ptosis and the antagonism of reserpine protection against amphetamine lethality in mice. The possible correlations between structure and antidepressant activity are suggested.

Keyphrases □ Benzylhydrazine congeners (cyclic)—synthesized and screened as antidepressants, structure-activity relationships □ 2-Pyrazolinium perchlorates—synthesized and screened as antidepressants, structure-activity relationships □ Antidepressants, potential—synthesis and screening of cyclic benzylhydrazine congeners

The established monoamine oxidase inhibitory activity of iproniazid was demonstrated clinically as an antidepressant effect (1). This led to the synthesis and screening of several hydrazines in search of analogous antidepressant agents useful in treating psychic disturbances associated with overactivity of monoamine oxidase (2, 3).

Of the several hydrazines tested, the aralkyl derivatives were found to possess marked activity (4), a fact that justified the clinical adoption of such monoamine oxidase inhibitors as phenelzine[1] (phenethylhydrazine) and pheniprazine[2] (β-phenylisopropylhydrazine) as well as the unsubstituted benzylhydrazine (5).



Scheme I

Although the 3-aryl pyrazolines may be regarded as the cyclic analogs of benzylhydrazine, a recent survey of the diverse monoamine oxidase inhibitor hydrazines revealed that all derivatives cited belong to the class of open-chain hydrazines (6).

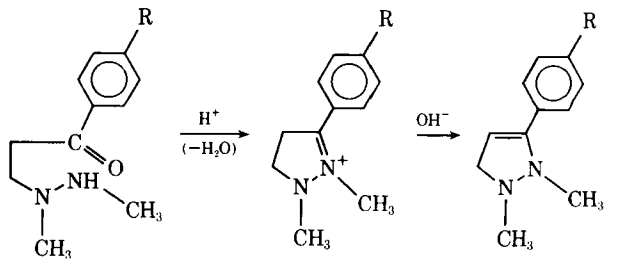
The work presented is a preliminary report on the synthesis and pharmacological evaluation of a series of new 2-pyrazolinium perchlorates and some of their corresponding pyrazolidines incorporating the basic benzylhydrazine skeleton —C_6H_4—C—N—N— in a cyclic form.

CHEMISTRY

The substituted 2-pyrazolinium perchlorates and the corresponding pyrazolidinium perchlorates, synthesized by the route outlined in Scheme I, are recorded in Tables I and II, respectively.

The procedure employed for the synthesis of the new 2-pyrazolinium perchlorates was that of Omar and Eltsov (7), which is a modification of the original method of Hinman *et al.* (8). It involves a one-step Mannich reaction of 1,2-dimethylhydrazine dihydrochloride, paraformaldehyde, and a substituted acetophenone. In support of the previous findings of Omar and Eltsov (7), which were based on spectrophotometric monitoring of the reactions, a 2-hr reflux period and a hydrazine–aldehyde–acetophenone mole ratio of 1:1:1 were again found adequate for completion of the reaction. These findings contradict some recent reports (9) where other authors used a 24-hr reflux time and a 1:2:2 mole ratio of reactants. The latter specifications were originally reported by Hinman *et al.* (8) for a different product, which is disubstituted.

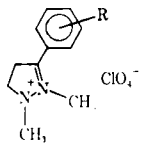
In previous reports (7–9), it was the common practice to isolate the 3-pyrazolines as end-products of the Mannich reaction. From the 3-pyrazoline bases, which are usually oils, the 2-pyrazolinium perchlorates were prepared. In this work the isolation of the base was found unnecessary. Direct workup of the reaction mixture with perchloric acid afforded the desired salts in good yields and relatively pure state, and without the need of the nitrogen atmosphere recommended previously (9). This may be taken to suggest that cyclization of the intermediate Mannich base to give rise to



Scheme II

[1] Nardil.
[2] Catron.

Table I—Physical Constants of 3-Aryl Pyrazolinium Perchlorates



Compound	R	Melting Point	Yield, %		Analysis, % Calc.	Found
I	H	134°	65	C	48.09	48.09
				H	5.50	4.97
				N	10.19	9.88
II	*p*-CH_3	97°	55	C	49.91	49.66
				H	5.93	5.89
				N	9.70	9.68
III	*p*-OCH_3	154°	60	C	47.29	47.53
				H	5.62	6.36
				N	9.19	8.93
IV	*p*-OC_2H_5	85–86°	72	C	48.98	49.37
				H	6.01	6.60
				N	8.78	8.37
V	*p*-Cl	152°	55	C	42.73	42.36
				H	4.56	4.96
				N	9.06	8.94
VI	*p*-Br	169°	40	C	37.36	37.46
				H	3.99	4.35
				N	7.92	7.34
VII	*m*-NO_2	163°	40	C	41.32	41.38
				H	4.41	4.80
				N	13.14	12.65

2-pyrazolinium salts precedes the formation of the 3-pyrazolines (Scheme II) under the influence of the acid catalyst.

In the preparation of the pyrazolidines by metal hydride reduction, it was found that replacement of the popularly used (7, 9, 10) lithium aluminium hydride–ether system by a sodium borohydride–aqueous system leads to an improvement in yields and a significant reduction in reaction time—from 12 (7) or 18 (9) hr to 15 min. These improvements may be attributed to the homogeneous phase reduction system of the aqueous borohydride, as contrasted to the lithium aluminium hydride–ether system in which the perchlorate salts are insoluble.

PHARMACOLOGY

Antagonism of reserpine-induced ptosis in mice has been employed frequently as an initial procedure for testing antidepressant activity. Under various experimental conditions, all current antidepressant drugs will give this antireserpine effect (11).

Antidepressant agents are fairly capable of counteracting the sedative effect of reserpine. This sedative effect is expressed as the protective action of reserpine against amphetamine-induced lethality in mice (12). The antagonism of this effect by antidepressants is specific for monoamine oxidase inhibitors and is parallel to inhibition of brain monoamine oxidase as well as reserpine-induced hypothermia and ptosis (13).

Using these techniques, the cyclic aralkyl hydrazine congeners synthesized were found to possess antidepressant activity comparable to that of nialamide.

EXPERIMENTAL[3]

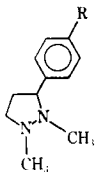
Aralkyl Ketones—Substituted acetophenones were synthesized according to reported methods (14, 15).

1,2-Dimethyl-3-arylpyrazolinium Perchlorates (I–VII)—As a general procedure, a mixture of equimolar amounts (0.1 mole) of 1,2-dimethylhydrazine, paraformaldehyde, and the appropriately substituted acetophenone was heated under reflux in 100 ml absolute ethanol for 2 hr. The reaction mixture was concentrated *in vacuo* to near dryness and triturated with 20-ml portions of ether; then the ether was removed and the residue was dissolved in 50 ml water. The clear aqueous solution was treated with 5 ml of 40% perchloric acid while stirring and cooling. The separated crystalline salts were filtered, washed with ether, and purified by recrystallization from an absolute ethanol–ether mixture. The perchlorate salts were characterized by sharp melting points, elemental analyses, and IR spectra. The spectra showed a moderate to strong absorption of a conjugated C=N$^+$ stretching vibration in the 1600–1640-cm^{-1} range and, more significantly, the absence of any absorption at or near 2200 cm^{-1} (N—H$^+$) (16).

1,2-Dimethyl-3-arylpyrazolidines and Their Perchlorates (VIII–XIII)—As a general procedure, an excess of sodium borohydride (0.075 mole) was added to the appropriate pyrazolinium perchlorate (0.025 mole) in 50 ml water with continuous stirring. The vigorous exothermic reaction subsided within 10 min, and continuous stirring was performed for 5 min longer at 60–65° on a water bath. The reaction mixture was cooled and made strongly alkaline by the addition of 10 ml of 30% sodium hydroxide solution, and the light-yellow oily layer that separated was taken up with 25-ml portions of ether. The washed ethereal extract was dried and the ether was distilled, followed by distillation of the oily residue *in vacuo*. The title pyrazolidines were obtained as colorless to pale-yellow liquids in almost quantitative yields. They were characterized by boiling points, specific gravity values, and refractive indexes. The latter values were used to compute the molecular refraction (MR_D) values, which were compared with calculated values from atomic refraction (Table II).

The perchlorate salts were obtained by mixing equimolar amounts (0.005 mole) of the pyrazolidines in absolute ethanol and 50% perchloric acid with cooling. The corresponding salts separated and were filtered, washed with ether, and recrystallized from an absolute ethanol–ether mixture. They were characterized

Table II—Physical Constants of 3-Aryl Pyrazolidines and Perchlorates



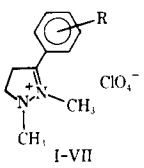
Compound	R	Physical Constants	Yield, %			
		Pyrazolidines				MR_D[b]
VIII	H	bp 83°/2 mm[a]	90.8			55.08
		n_D^{20} 1.5290[a]				55.31
		Specific gravity, d_4^{20} 0.9815				
IX	CH_3	bp 82°/2 mm	92.3			
		n_D^{20} 1.5255				59.69
		Specific gravity, d_4^{20} 0.9721				59.80
X	Cl	bp 98°/2 mm	91.6			
		n_D^{20} 1.5415				59.94
		Specific gravity, d_4^{20} 1.0962				60.33
		Perchlorates			Analysis, % Calc.	Found
XI	H	mp 106°		C	47.77	47.25
				H	6.19	5.71
				N	10.12	10.07
XII	CH_3	mp 152°		C	49.57	50.07
				H	6.59	6.50
				N	9.63	9.46
XIII	Cl	mp 172°		C	42.45	42.22
				H	5.18	5.47
				N	9.00	8.72

[a] Lit. (8) bp 72° (1.2 mm), n_D^{21} 1.5318. [b] Molecular refraction found from the equation:

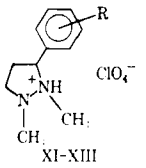
$$MR_D = \frac{n^{2-1}}{n^{2+2}/d_4^{20}} \times \text{molecular weight}$$

and calculated from atomic refraction after Eisenlor (18).

[3] Melting points were taken in an open capillary and are uncorrected; IR spectra were recorded by a Beckman IR-20 spectrophotometer.



I-VII



XI-XIII

Table III—Antidepressant Activity of Cyclic Benzylhydrazine Congeners

Preparation	R	Mortality %	p	Antagonism of Reserpine-Induced Ptosis	Percent Antagonism[a]
Amphetamine sulfate	—	50	<0.005	—	—
Reserpine + amphetamine sulfate	—	0	—	—	—
Nialamide + reserpine + amphetamine sulfate	—	50	<0.005	++++	100
I + reserpine + amphetamine sulfate	H	30	<0.005	++++	90
II + reserpine + amphetamine sulfate	p-CH_3	40	<0.005	++++	95
III + reserpine + amphetamine sulfate	p-OCH_3	40	<0.005	++++	100
IV + reserpine + amphetamine sulfate	p-OC_2H_5	40	<0.005	++++	100
V + reserpine + amphetamine sulfate	p-Cl	20	<0.05	++	45
VI + reserpine + amphetamine sulfate	p-Br	30	<0.01	+++	75
VII + reserpine + amphetamine sulfate	m-NO_2	20	<0.05	++	50
XI + reserpine + amphetamine sulfate	H	30	<0.01	+++	70
XII + reserpine + amphetamine sulfate	p-CH_3	30	>0.01	+++	75
XIII + reserpine + amphetamine sulfate	p-Cl	10	>0.25	+	20

[a] Expressed as percent of animals protected, taking nialamide protection as 100%. All preparations are dissolved in "water for injection."

by sharp melting points, elemental analyses, and IR spectra (strong absorption at 2200 cm^{-1} of the $N—H^+$).

Pharmacological Examination—The antidepressant activity of the perchlorate salts was compared with that of nialamide in mice of either sex weighing 18–26 g. The antagonism of reserpine-induced ptosis (11, 12) and the antagonism of reserpine protection against amphetamine lethality in mice (12) were used for evaluating the antidepressant action.

In a group of 20 mice, amphetamine sulfate (30 mg/kg) was injected intraperitoneally and the percent mortality was determined within the first 24 hr.

In another group of 20 mice, reserpine (1.5 mg/kg) was injected intraperitoneally 2 hr prior to the above-mentioned dose of amphetamine. The degree of reserpine-induced ptosis was noted, and the mortality percentage was calculated after amphetamine injection.

Other groups of 20 mice each were used for evaluating the different compounds under investigation in comparison with nialamide as a reference compound. Nialamide and the new compounds were injected intraperitoneally in doses of 30 mg/kg, followed after 2 hr with 1.5 mg/kg ip reserpine. The degree of ptosis was noted and, 3 hr after reserpine administration, 30 mg/kg amphetamine was injected. The percent mortality within 24 hr was determined.

In another set of experiments, groups of 10 mice were used as the control for injecting the compounds under investigation in the same dose (30 mg/kg) used in the experiment. Any lethality due to the administration of the new compounds was noted.

The significance of the results was statistically evaluated by calculation of χ^2 and the corresponding p values.

RESULTS AND DISCUSSION

Amphetamine sulfate (30 mg/kg) was found to cause 50% mortality in mice within 24 hr of the intraperitoneal injection. Pretreatment of mice with reserpine (1.5 mg/kg ip) 2 hr prior to the administration of amphetamine protected mice against lethality and reduced the mortality to 0%. Reserpine at this dose caused marked ptosis. Nialamide (30 mg/kg), which was used as a reference compound possessing antidepressant activity, was found to counteract the protective action of reserpine; 50% of the animals injected with nialamide, reserpine, and amphetamine died within 24 hr. Furthermore, nialamide prevented the ptosis caused by reserpine administration. The compounds under investigation were found to possess an activity similar to nialamide when administered in 30-mg/kg doses (Table III). The activity varied according to variation in the chemical structure. Control experiments showed that the investigated compounds, in the doses used, were devoid of any lethal effect.

Data in Table III reveal that pyrazolinium and pyrazolidinium perchlorates, as cyclic congeners of benzylhydrazine, possess an antidepressant activity comparable to that of nialamide.

Regarding the structure–activity relationships, certain suggestions may be advanced as follows.

1. Pyrazolinium compounds substituted in the 3-phenyl moiety by electron-donating groups (II, III, and IV) are more potent than those substituted by electron-attracting centers (V, VI, and VII). Such a variation in activity may be attributed to differences in oxidation–reduction potentials of these systems. Pyrazolinium compounds (I–VII), particularly I–IV, were characterized by strong reducing activity as demonstrated by the reduction of an alcoholic solution of 1,3,5-triphenyltetrazolium chloride as well as the rapid reduction of silver nitrate solution at room temperature. Quantitative differences in the reducing power were observed in the polarographic half-wave potentials of these compounds[4].

2. The pyrazolidinium compounds (XI–XIII) may be regarded as having generally weaker activity as antidepressants than the corresponding pyrazolinium salts (I, II, and V). This could be a direct result of saturation of the former compounds, thus bringing about a marked decrease in the electron density on the nitrogen atoms of the free base. This is in accord with the fact that amines are less basic than eneamines (17) with the overall result of a decrease in the reducing power of the carrier compounds. This was demonstrated practically in the finding that the pyrazolidines (VIII–XIII) slowly reduced silver nitrate at room temperature but could not affect an alcoholic solution of 1,3,5-triphenyltetrazolium chloride even after prolonged boiling.

3. It may be inferred that the monoamine oxidase inhibitory activity of the compounds synthesized is dependent mainly upon their reducing potentials.

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[x] To whom inquiries should be directed.

Sodium–Calcium-Ion Exchange in Phosphatidyl Serine Monolayer

A. W. MALICK, N. D. WEINER[x], and A. FELMEISTER*

Abstract □ The effect of replacement of sodium chloride subphase solution with calcium chloride subphase solution and the effect of replacement of calcium chloride subphase solution with sodium chloride subphase solution were studied on monolayers of phosphatidyl serine. When the sodium chloride subphase was replaced with calcium chloride solution, the π–A curve shifted to almost the same position as the π–A plot of a film spread on calcium chloride without subphase replacement. This showed that almost all sodium ions had been replaced by calcium ions in the film. When the calcium chloride subphase was replaced by a sodium chloride solution, the film expanded somewhat, showing that some calcium ions had been replaced by sodium ions. This study gives evidence for the existence of cation exchange in phosphatidyl serine films, and sodium seems to be in competition with calcium for the negatively charged sites in the film. It is possible that this competition is concentration dependent.

Keyphrases □ Sodium-calcium subphase exchange—effects on phosphatidyl serine monolayers □ Calcium-sodium subphase exchange—effects on phosphatidyl serine monolayers □ Phosphatidyl serine monolayers—effects of sodium-calcium and calcium-sodium subphase exchange, sodium-calcium competition, cation-exchange evidence □ Monolayers, phosphatidyl serine—effects of sodium-calcium and calcium-sodium subphase exchange, sodium-calcium competition, cation-exchange evidence

A previous article (1) reported an apparatus and technique for studying monomolecular films by which the subphase can be exchanged without disturbing the film. Data were presented to demonstrate the utility of this system for studying protein and enzyme monolayers. This article will show the applicability of the system to the study of ion-exchange properties of phospholipid monolayers.

DISCUSSION

Binding of cations by phospholipids and fatty acids has been studied extensively (2–26). Calcium ions were found (3) to produce little reduction in area per molecule of cephalin, spread at an air–water interface at a given surface pressure. Studies (4, 5) on the dependence on surface pressure of the penetration rate of water molecules through lipid monolayers found that calcium ions, at low concentration, decreased such penetration. The association of synthetic lecithin with ions was studied (6), showing indirectly that, with dilute solutions, there is no binding of sodium, potassium, or lithium. However, the same study reported that magnesium seemed to be adsorbed from dilute solutions. Vilallonga *et al.* (7) observed that the presence of monovalent ions increases the area per molecule, the surface potential, and the surface dipole moment of dipalmitoyl lecithin monolayers in the order sodium > potassium ≈ lithium > water. It was reported (8) that lecithin monolayers bind calcium (0.1 mM) and that sodium or potassium ions (112 mM) each displace the same fraction of this calcium (about 50%). Many workers reported interaction of divalent and monovalent cations with phosphatidyl serine (2, 9–13).

Shah and Schulman (14) reported that the π–A curves of lecithins, phosphatidyl choline, and dicetyl phosphate are not affected by the presence of divalent metal ions. However, dipalmitoyl lecithin shows a higher surface potential in the presence of divalent metal ions than in the presence of monovalent ions; the increase is greatest for the fully saturated dipalmitoyl lecithin and the smallest for a highly unsaturated lecithin. They suggested that the divalent metal ion–lecithin monolayer interaction is dependent on the packing of the hydrocarbon chains. The large intermolecular separation permits water, hydrated monovalent ions, and nitrogen ion of the same molecule to associate with the phosphate ion; this prevents the calcium interaction with the phosphate ion. On compression, some water molecules and hydrated ions are squeezed out and the conditions become more favorable for calcium, because one calcium ion between two phosphate groups is smaller than two monovalent ions. These authors also postulated a position for calcium in dicetyl phosphate monolayers that would not affect the area of the film but would increase the surface potential.

In a later publication, Shah and Schulman (15) suggested that there is an internal salt linkage between the phosphate and trimethylammonium group on the same lecithin molecule, which prevents the interaction of the phosphate group with calcium. An increase in unsaturation of the fatty acyl chains increases the intermolecular spacing, which reduces the ionic repulsion between polar groups and hence strengthens the internal salt linkage. Thus, increasing unsaturation decreases calcium binding in lecithin monolayers. They suggested a vertical rather than a coplanar orientation of the phosphoryl choline group with respect to the interface and proposed a position for calcium in the lecithin films. It was suggested, based on surface potential studies, that calcium forms an ionic dipole ($Ca^{+2} \leftrightarrow O^{-}$).

Colacicco (16) showed that the reported (14, 15) surface poten-

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DISCUSSION

Binding of cations by phospholipids and fatty acids has been studied extensively (2–26). Calcium ions were found (3) to produce little reduction in area per molecule of cephalin, spread at an air–water interface at a given surface pressure. Studies (4, 5) on the dependence on surface pressure of the penetration rate of water molecules through lipid monolayers found that calcium ions, at low concentration, decreased such penetration. The association of synthetic lecithin with ions was studied (6), showing indirectly that, with dilute solutions, there is no binding of sodium, potassium, or lithium. However, the same study reported that magnesium seemed to be adsorbed from dilute solutions. Vilallonga *et al.* (7) observed that the presence of monovalent ions increases the area per molecule, the surface potential, and the surface dipole moment of dipalmitoyl lecithin monolayers in the order sodium > potassium ≈ lithium > water. It was reported (8) that lecithin monolayers bind calcium (0.1 mM) and that sodium or potassium ions (112 mM) each displace the same fraction of this calcium (about 50%). Many workers reported interaction of divalent and monovalent cations with phosphatidyl serine (2, 9–13).

Shah and Schulman (14) reported that the π–A curves of lecithins, phosphatidyl choline, and dicetyl phosphate are not affected by the presence of divalent metal ions. However, dipalmitoyl lecithin shows a higher surface potential in the presence of divalent metal ions than in the presence of monovalent ions; the increase is greatest for the fully saturated dipalmitoyl lecithin and the smallest for a highly unsaturated lecithin. They suggested that the divalent metal ion–lecithin monolayer interaction is dependent on the packing of the hydrocarbon chains. The large intermolecular separation permits water, hydrated monovalent ions, and nitrogen ion of the same molecule to associate with the phosphate ion; this prevents the calcium interaction with the phosphate ion. On compression, some water molecules and hydrated ions are squeezed out and the conditions become more favorable for calcium, because one calcium ion between two phosphate groups is smaller than two monovalent ions. These authors also postulated a position for calcium in dicetyl phosphate monolayers that would not affect the area of the film but would increase the surface potential.

In a later publication, Shah and Schulman (15) suggested that there is an internal salt linkage between the phosphate and trimethylammonium group on the same lecithin molecule, which prevents the interaction of the phosphate group with calcium. An increase in unsaturation of the fatty acyl chains increases the intermolecular spacing, which reduces the ionic repulsion between polar groups and hence strengthens the internal salt linkage. Thus, increasing unsaturation decreases calcium binding in lecithin monolayers. They suggested a vertical rather than a coplanar orientation of the phosphoryl choline group with respect to the interface and proposed a position for calcium in the lecithin films. It was suggested, based on surface potential studies, that calcium forms an ionic dipole ($Ca^{+2} \leftrightarrow O^{-}$).

Colacicco (16) showed that the reported (14, 15) surface poten-

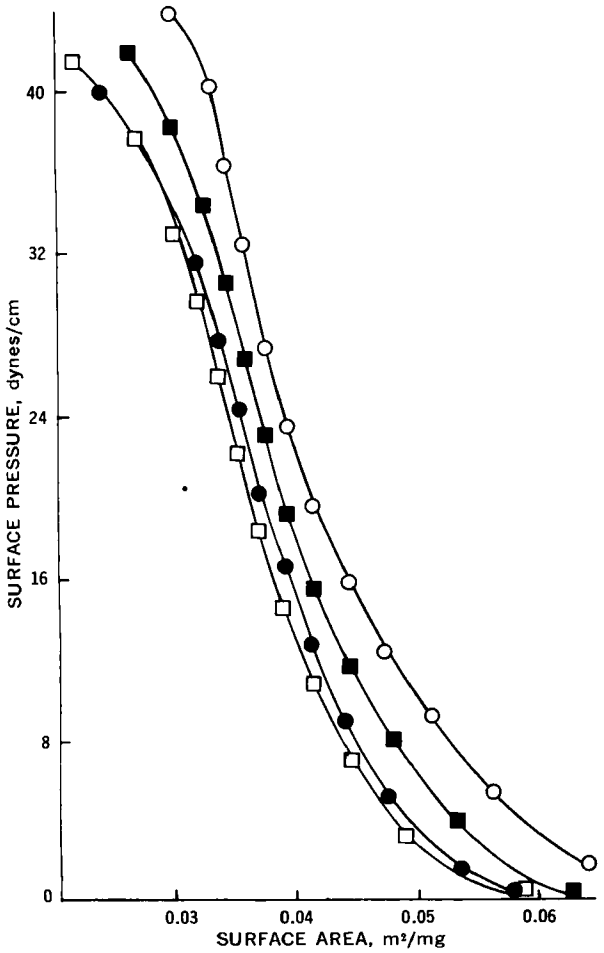

Figure 1—*The π–A curves of phosphatidyl serine films. Key:* ○, *spread on 0.1* M *sodium chloride solution;* □, *spread on 0.033* M *calcium chloride solution;* ●, *spread on 0.1* M *sodium chloride solution and subphase replaced with 1500 ml of 0.033* M *calcium chloride solution; and* ■, *spread on 0.033* M *calcium chloride solution and subphase replaced with 1500 ml of 0.1* M *sodium chloride solution.*

tial increase in dipalmitoyl lecithin monolayers due to calcium ions was actually due to acidic contaminants in the impure lecithins. Thus, the increased potentials of calcium chloride solutions may be due to the effects of interaction of calcium with fixed charges of the acidic contaminants. The potentials that the previous workers attributed to fixed dipoles of the lipid may not exist, and the vertical orientation proposed (14, 15) for the phosphoryl choline is questionable. From studies of electrokinetic potentials, Hanai *et al.* (17, 18) concluded that the phosphatidyl choline head groups of lecithin in bimolecular leaflets are probably oriented so that the trimethylammonium and phosphate ionic groups are both in a plane parallel to the leaflet. Measurements of surface dipole moments and surface pK values (19–21) also indicate that this configuration exists in monolayers of lecithins and phosphatidyl ethanolamine. However, on the grounds of X-ray long-spacing studies (22), it was suggested that the preferred orientation of the polar group in lecithin has the zwitterion extended normal to the bilayer.

Many workers believe that acidic phospholipids provide negatively charged sites for cation exchange. Blaustein (2) demonstrated ion exchange in phosphatidyl serine by using distribution ratios between a chloroform–methanol phase as the criterion. Abramson *et al.* (11) showed interaction of cations with aqueous dispersions of phosphatidyl serine. Phosphatidyl serine and phosphatidyl glycerol have been shown to exhibit discrimination between sodium and potassium (12). Antagonistic effects of calcium and local anesthetics on the permeability of phosphatidyl serine vesicles have been reported (23). Nielson (24) reported that lipids show evidence of cation exchange when eluted down a silicic acid column. Association of inorganic cations with acidic phospholipid monolayers has been studied by many workers (9–13).

Rojas and Tobias (9) reported that calcium condenses phosphatidyl serine monolayers and monovalent cations (sodium, potassium, and hydrogen) are in competition with calcium ions for the negatively charged sites in the film. The extent of this competition depends on the competing ion concentration. More calcium is displaced by either sodium or potassium ions at low surface pressure than at high pressures; this they interpreted in terms of peripolar group volume available for ion intrusion. By isotope techniques, they revealed that there is one calcium ion adsorbed per phosphatidyl serine and 0.35 calcium ion adsorbed per phosphatidyl ethanolamine molecule in their monolayers at pH 7. At this pH, the phosphoric groups of phosphatidyl serine and phosphatidyl ethanolamine and the carboxyl group of phosphatidyl serine seem to be ionized, thus generating monolayers with negatively charged sites for cation exchange. The cations, of course, condense the phosphatidyl serine and phosphatidyl ethanolamine monolayers. In striking contrast, however, the π–A curves of phosphatidyl choline were independent of hypophase sodium-, potassium-, or calcium-ion concentration. It was concluded that within the pH range used, the phosphatidyl choline monolayers were not negatively charged. However, as previously mentioned, some workers have reported condensation of lecithin monolayers in the presence of calcium. Hauser and Dawson (13) also reported the displacement of adsorbed calcium from phosphatidyl serine monolayers by sodium and potassium.

Papahadjopoulos (10) reported that acidic phospholipids interact strongly with bivalent metal ions at low concentrations, the interaction being accompanied by a condensation of the monolayer and an increase in the surface potential. Phosphatidyl serine and phosphatidic acid films exhibit the highest affinity above pH 7, since both possess two negative groups per molecule at these pH values. The relative affinity for the different bivalent cations was calcium > barium > magnesium. Based on these studies, a model was proposed for the complex of phosphatidyl serine and calcium whereby the phosphatidyl serine molecules are packed in such a way in the film that the calcium can form six coordination bonds with four phosphatidyl serine molecules to form a linear polymeric arrangement. Addition of bivalent ions under neutral phospholipid films (phosphatidyl choline and phosphatidyl ethanolamine) had very little effect on π or ΔV. These studies were carried out in the presence of physiological concentrations (10^{-1} M) of univalent salts, sodium chloride or potassium chloride.

The importance of acidic lipids as tissue ion-exchange agents has been emphasized by a number of workers. In addition, there is evidence that these lipids may play a role in transmembrane cation transport (27–29). Reported sodium–potassium discrimination by phosphatidyl serine and phosphatidyl glycerol (12) seems highly relevant to the physiological mechanism of transmembrane ion movement. Phosphatidyl serine constitutes 10–20% of the total phosphatidyl content of most cell membranes (30) and phospholipids may contain the ion-exchange sites controlling neuronal excitation (31). Therefore, ionic binding and ion-exchange capabilities of the purified acidic lipids are very important.

There does not seem to be general agreement on how cations bind to phospholipid monolayers. Whether or not the acidic phospholipid films (*e.g.*, phosphatidyl serine) exhibit ion exchange between divalent cations and monovalent cations has not yet been fully established.

The purpose of this investigation was to examine the subphase exchange system (1) as a means of studying the sodium–calcium-ion exchange characteristics of phosphatidyl serine films.

EXPERIMENTAL

Bovine phosphatidyl serine (25 mg/ml in chloroform) was purchased[1]. TLC analysis yielded a single spot. Spreading solutions of phosphatidyl serine were prepared with hexane to yield a solution containing 0.625 mg/ml. Then 0.08 ml of this solution was spread on either a 0.1 M NaCl tromethamine–hydrochloric acid-buffered (pH 7.4) subphase or a 0.033 M $CaCl_2$ tromethamine–hydrochloric acid-buffered (pH 7.4) subphase. A pH value of 7.4

[1] Applied Science Laboratories, State College, Pa.

was chosen since the π–A curves of phosphatidyl serine are independent of pH in the 6–8 pH range (10).

The π–A plots were then constructed for phosphatidyl serine films on the sodium chloride and calcium chloride subphases. In addition, the sodium chloride subphase was exchanged with calcium chloride, while the calcium chloride subphase was exchanged with sodium chloride. The ionic strength of the subphase remained constant throughout the exchange process. The π–A plots were also constructed for each system. In all experiments, the π–A plots were reproducible to within 0.1 dyne/cm.

The details for the apparatus used and the method of subphase exchange were given previously (1).

RESULTS

The π–A plots of phosphatidyl serine films spread on buffer solution containing 0.1 M sodium chloride or 0.033 M calcium chloride are shown in Fig. 1. The two π–A plots are almost parallel, but the film on calcium chloride is much more condensed.

Figure 1 also shows the π–A plots of phosphatidyl serine when it was spread on 0.1 M sodium chloride subphase and the subphase was replaced by 1500 ml of 0.033 M calcium chloride solution. The π–A plot of the film has shifted very close to the π–A plot of phosphatidyl serine film spread on calcium chloride solution. Therefore, by replacing the sodium chloride subphase with calcium chloride solution of the same ionic strength, almost all of the sodium is replaced by calcium in the film. The replacement of sodium by calcium in the film is expected in light of the many reports that affinity of phosphatidyl serine for calcium is much greater than for sodium (9, 13).

Figure 1 also shows the π–A plot of phosphatidyl serine when it was spread on calcium chloride solution and the subphase was replaced with 1500 ml of sodium chloride solution of the same ionic strength. The π–A plot has shifted midway between the plot for a film spread on calcium chloride solution and a film spread on sodium chloride solution. This suggests that some calcium in the film has been replaced by sodium as a result of the subphase exchange. This demonstrates that cation exchange between sodium and calcium does occur in phosphatidyl serine films. If the phosphatidyl serine film interacted with calcium so as to form very stable complexes, as suggested (10), the observed replacement of calcium by sodium would not be expected.

As the subphase exchange proceeds, the calcium-ion concentration in the subphase is decreasing while that of sodium is increasing. The exchange in the film may also depend on the rate of diffusion of the sodium ions from the bulk to the interfacial region. If the competition was not concentration dependent, it would be expected that the π–A plot would shift completely to the π–A plot of the film spread on sodium chloride solution, as was observed in the replacement of the sodium chloride subphase solution with calcium chloride solution. It can be seen clearly from the results that calcium interacts preferentially and more strongly than sodium with phosphatidyl serine films. However, this study also gives definite evidence for the existence of sodium–calcium exchange in phosphatidyl serine films in which sodium, at relatively high concentrations, competes with calcium for the negatively charged sites in the film.

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* Present address: College of Pharmacy, Rutgers—The State University, New Brunswick, NJ 08903

ˣ To whom inquiries should be directed.

Substituted Benzoxazole/Benzthiazole-2-thiones: Interrelationship between Anticonvulsant Activity and Inhibition of Nicotinamide Adenine Dinucleotide-Dependent Oxidations and Monoamine Oxidase

RADHEY S. MISRA*, JAYANTI P. BARTHWAL*, SURENDRA S. PARMAR*‡ˣ, and STANLEY J. BRUMLEVE‡

Abstract □ 3-Alkyl benzoate/benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones were synthesized and evaluated for their ability to affect the respiratory activity of rat brain homogenate. All compounds were found to inhibit selectively the nicotinamide adenine dinucleotide (NAD)-dependent oxidation of DL-isocitrate, α-ketoglutarate, and pyruvate while NAD-independent oxidation of succinate remained unaltered. Inhibition of monoamine oxidase by 3-benzhydrazide aminomethyl benzoxazole/benzthiazole was greater with benzthiazoles as compared to the corresponding benzoxazoles. Anticonvulsant activity exhibited by these benzoxazole/benzthiazole derivatives against pentylenetetrazol-induced seizures was found to be unrelated to their enzyme inhibitory properties.

Keyphrases □ 3-Alkyl benzoate/benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones—synthesis, anticonvulsant activity and relationship to inhibition of NAD-dependent oxidations and monoamine oxidase □ Benzoxazole/benzthiazole-2-thiones, 3-alkyl benzoate/benzhydrazide aminomethyl—synthesis, anticonvulsant activity and relationship to inhibition of NAD-dependent oxidations and monoamine oxidase □ Inhibition of NAD-dependent oxidations and monoamine oxidase—synthesis and evaluation of 3-alkyl benzoate/benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones

Diverse psychopharmacological effects exhibited by benzoxazoles (1) and benzthiazole (2, 3) led to the synthesis of 3-alkyl benzoate/benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones. In the present study, their ability to inhibit nicotinamide adenine dinucleotide (NAD)-dependent oxidation of DL-isocitrate, α-ketoglutarate, and pyruvate and the ability of 3-benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones to inhibit monoamine oxidase activity were investigated. Attempts also were made to determine the anticonvulsant activity of these compounds to correlate their enzyme inhibitory properties with their ability to afford protection against pentylenetetrazol-induced seizures.

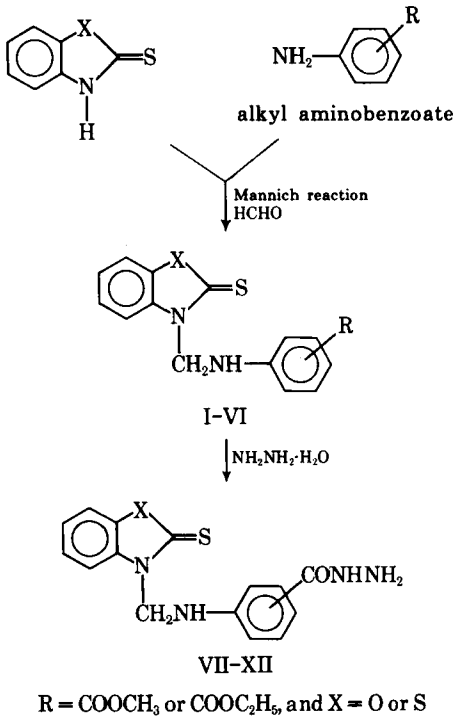
EXPERIMENTAL[1]

Alkyl Aminobenzoates—These were prepared by treating aminobenzoic acids with appropriate alcohols according to the method reported earlier (4).

3-Alkyl Benzoate Aminomethyl Benzoxazole/Benzthiazole-2-thiones (I–VI) (Scheme I)—A mixture of an appropriate alkyl aminobenzoate (0.011 mole) and 2-mercaptobenzoxazole/2-mercaptobenzthiazole (0.01 mole) in ethanol with 37% formalin (0.012 mole) was stirred on an ice bath for 3 hr. The reaction mixture was refrigerated overnight, and the solid mass which separated out was filtered, washed with water, dried, and recrystallized from ethanol. The various 3-alkyl benzoate aminomethyl benzoxazole/benzthiazole-2-thiones were characterized by their sharp melting points and elemental analyses (Table I). The presence of characteristic bands for $COOC_2H_5$ (1730 cm^{-1}), NH (3320 cm^{-1}), and CH_2 (2930 cm^{-1}) groups in their IR spectra provided further support for their structure.

3-Benzhydrazide Aminomethyl Benzoxazole/Benzthiazole-2-thiones (VII–XII)—3-Alkyl benzoate aminomethyl benzoxazole/benzthiazole-2-thione (0.01 mole) dissolved in absolute ethanol was heated under reflux in a water bath with 0.02 mole of 99–100% hydrazine hydrate for 10–12 hr. Excess solvent was removed by distillation. The hydrazides which separated out on cooling were collected by filtration, dried, and recrystallized from ethanol. The various 3-benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones thus obtained were characterized by their sharp melting points and elemental analyses (Table II). These hydrazides were further characterized by the presence of the characteristic bands for CONH (1660 cm^{-1}), NH_2 (3350 cm^{-1}), NH (3200 cm^{-1}), and CH_2 (2840 cm^{-1}) in their IR spectra.

Assay of Respiratory Activity of Rat Brain Homogenate—Male albino rats, allowed food and water *ad libitum*, were used in all experiments. Rats weighing 100–150 g were sacrificed by decapitation. The brains were removed immediately and homogenized[2] in the ratio of 1:9 (w/v) in cold 0.25 *M* sucrose. All incubations were carried out in the conventional Warburg manometric apparatus at 37° with air as the gas phase. The oxygen uptake



Scheme I

[1] All compounds were analyzed for their carbon, hydrogen, and nitrogen content. Melting points were taken in open capillary tubes and are corrected. Commercial chemicals were used in the present study. DL-Isocitrate, α-ketoglutarate, pyruvate, succinate, kynuramine, AMP (sodium salt), and cytochrome c were obtained from Sigma Chemical Co., St. Louis, Mo.

[2] Potter-Elvehjem homogenizer.

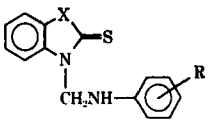


Table I—3-Alkyl Benzoate Aminomethyl Benzoxazole/Benzthiazole-2-thiones

Compound	R	X	Yield, %	Melting Point	Molecular Formula	Analysis, % Calc.	Found
I	2-$COOCH_3$	O	66	150°	$C_{16}H_{14}N_2O_3S$	C 61.14 H 4.46 N 8.92	60.96 4.62 8.70
II	3-$COOC_2H_5$	O	72	180°	$C_{17}H_{16}N_2O_3S$	C 62.19 H 4.88 N 8.54	62.40 4.76 8.42
III	4-$COOC_2H_5$	O	83	169°	$C_{17}H_{16}N_2O_3S$	C 62.19 H 4.88 N 8.54	62.34 4.70 8.38
IV	2-$COOCH_3$	S	68	172°	$C_{16}H_{14}N_2O_2S_2$	C 58.18 H 4.24 N 8.48	58.46 4.08 8.60
V	3-$COOC_2H_5$	S	76	135°	$C_{17}H_{16}N_2O_2S_2$	C 59.30 H 4.65 N 8.14	59.52 4.50 7.98
VI	4-$COOC_2H_5$	S	80	155°	$C_{17}H_{16}N_2O_2S_2$	C 59.30 H 4.65 N 8.14	59.12 4.74 8.00

[a] Melting points were taken in open capillary tubes and are corrected.

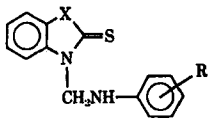


Table II—3-Benzhydrazide Aminomethyl Benzoxazole/Benzthiazole-2-thiones

Compound	R	X	Yield, %	Melting Point[a]	Molecular Formula	Analysis, % Calc.	Found
VII	2-$CONHNH_2$	O	44	248°	$C_{15}H_{14}N_4O_2S$	C 57.32 H 4.46 N 17.83	57.60 4.28 17.62
VIII	3-$CONHNH_2$	O	52	172°	$C_{15}H_{14}N_4O_2S$	C 57.32 H 4.46 N 17.83	57.14 4.60 17.73
IX	4-$CONHNH_2$	O	57	140°	$C_{15}H_{14}N_4O_2S$	C 57.32 H 4.46 N 17.83	57.10 4.62 17.78
X	2-$CONHNH_2$	S	50	200°	$C_{15}H_{14}N_4OS_2$	C 54.54 H 4.24 N 16.96	54.78 4.10 16.65
XI	3-$CONHNH_2$	S	54	197°	$C_{15}H_{14}N_4OS_2$	C 54.54 H 4.24 N 16.96	54.66 4.02 16.70
XII	4-$CONHNH_2$	S	60	203°	$C_{15}H_{14}N_4OS_2$	C 54.54 H 4.24 N 16.96	54.32 4.40 16.74

[a] Melting points were taken in open capillary tubes and are corrected.

was measured at 10-min intervals. Fresh rat brain homogenate, equivalent to 125 mg wet weight, was added to the chilled Warburg vessels containing 6.7 m*M* $MgSO_4$, 20 m*M* Na_2HPO_4, 1 m*M* adenosine monophosphate (AMP) (sodium salt), 33 m*M* KCl, buffer solution (pH 7.4), and 500 μg of cytochrome c in a final volume of 3 ml. The central well contained 0.2 ml of 20% KOH solution. All substrates were used at a final concentration of 10 m*M* while the final concentration of the various benzoxazole/benzthiazole derivatives was 2 m*M*. The compounds were dissolved in propylene glycol (100%), and an equivalent amount of the solvent was added to the control vessels.

Determination of Monoamine Oxidase Activity—A spectrophotofluorometric method was used for the determination of monoamine oxidase activity of the rat brain homogenate, using kynuramine as the substrate (5). The 4-hydroxyquinoline formed during oxidative deamination of kynuramine was measured fluorometrically in a spectrophotofluorometer[3], using activating light of 315 nm and measuring fluorescence at the maximum of 380 nm. The reaction mixture in a final concentration consisted of 0.5 ml of phosphate buffer (0.2 *M*, pH 7.5), 1×10^{-4} *M* kynuramine, and 0.5 ml of brain homogenate (equivalent to 10 mg of wet weight of the tissue). The monoamine oxidase activity was determined by incubation at 37° in air for 30 min. The various 3-benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones were added to the brain homogenate to produce a final concentration of 1×10^{-4} *M* and then were incubated for 10 min before the addition of kynuramine. The mixture was then incubated for an additional 30 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (w/v), and the precipitated proteins

[3] Aminco Bowman.

Table III—Biochemical and Pharmacological Properties of 3-Alkyl Benzoate Aminomethyl Benzoxazole/Benzthiazole-2-thiones

Compound	Inhibition[a], %				Anticonvulsant Activity[b], % Protection	Mortality after 24 hr, %
	DL-Isocitrate	α-Ketoglutarate	Pyruvate	Succinate		
I	66.1 ± 0.8	53.5 ± 0.9	49.2 ± 0.4	Nil	40	20
II	55.2 ± 0.6	31.4 ± 0.5	60.9 ± 0.8	Nil	20	30
III	59.1 ± 0.7	24.9 ± 0.4	68.0 ± 1.0	Nil	30	30
IV	63.8 ± 0.9	42.8 ± 0.5	54.8 ± 0.7	Nil	40	10
V	70.1 ± 1.2	47.9 ± 0.6	61.5 ± 0.9	Nil	10	50
VI	61.7 ± 0.9	38.2 ± 0.4	77.4 ± 1.3	Nil	20	40

[a] Each experiment was done in duplicate. Assay conditions and vessel contents were as reported in the text. All values represent mean values of percent inhibition with ±standard error calculated from three separate experiments. Inhibition was determined by the decrease in the oxygen uptake with 125 mg wet tissue weight/hr. The final concentrations of the compounds and the substrates were 2 and 10 m*M*, respectively. The oxygen uptake in the control experiments, in the absence of added 3-alkyl benzoate aminomethyl benzoxazole/benzthiazole-2-thiones, was 142.0 ± 7.6, 125.0 ± 8.8, 113.0 ± 6.5, and 210.0 ± 11.4 μl/125 mg wet tissue weight/hr using DL-isocitrate, α-ketoglutarate, pyruvate, and succinate, respectively, as substrates. [b] Compounds were administered at the dose of 100 mg/kg ip 4 hr before administration of pentylenetetrazol (90 mg/kg sc).

Table IV—Biochemical and Pharmacological Properties of 3-Benzhydrazide Aminomethyl Benzoxazole/Benzthiazole-2-thiones

Compound	Inhibition[a], %					Anticonvulsant Activity[a], % Protection	Mortality after 24 hr, %
	DL-Isocitrate	α-Ketoglutarate	Pyruvate	Succinate	Monoamine Oxidase[b]		
VII	14.7 ± 0.3	56.5 ± 0.8	18.8 ± 0.2	Nil	37.2 ± 0.4	20	60
VIII	22.4 ± 0.3	42.7 ± 0.7	21.6 ± 0.3	Nil	46.3 ± 0.5	Nil	90
IX	40.1 ± 0.4	49.1 ± 0.5	37.3 ± 0.7	Nil	58.4 ± 0.7	30	40
X	35.9 ± 0.6	55.8 ± 0.7	38.5 ± 0.8	Nil	54.1 ± 0.7	10	80
XI	52.1 ± 0.8	60.4 ± 0.8	32.2 ± 0.4	Nil	60.4 ± 1.0	20	70
XII	38.5 ± 0.5	64.1 ± 1.0	44.1 ± 0.6	Nil	70.2 ± 1.1	10	80

[a] Assay conditions were as described in Table III. [b] Each experiment was done in duplicate, and the values indicate mean values with ±standard error of three separate experiments. Final concentrations of kynuramine and the various 3-benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones were 1×10^{-4} *M*.

were removed by centrifugation. Suitable 1-ml aliquots of the supernate were taken in 2 ml of 1 *N* NaOH solution and were assayed for 4-hydroxyquinoline. An increase in absorbance provided a direct measurement of 4-hydroxyquinoline formation, which was taken as an index of the monoamine oxidase activity. The percentage inhibition was calculated from the decrease observed in absorbance, and this provided an index of the inhibitory property of these 3-benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined against pentylenetetrazol-induced seizures in mice of either sex weighing between 25 and 30 g. The mice were divided into groups of 10, keeping the group weights as near the same as possible. All compounds (100 mg/kg) were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). A 100-mg/kg ip dose of the test compound was administered to 10 animals. Four hours after the administration of these compounds, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol has been shown to produce convulsions in almost all untreated animals and to exhibit 100% mortality in mice during 24 hr. On the other hand, no mortality was observed during 24 hr in animals treated with 100 mg/kg of the test compounds alone. The mice were observed for 60 min for the occurrence of seizures. An episode of clonic spasm persisting for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks or tremulousness were not counted. Animals devoid of threshold convulsions during 60 min were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of these compounds was represented as percentage protection. The animals were then observed for 24 hr and the mortality was recorded.

RESULTS AND DISCUSSION

Selective *in vitro* inhibition of NAD-dependent oxidation of DL-isocitrate, α-ketoglutarate, and pyruvate by rat brain homogenate was observed in the present study with all 3-alkyl benzoate aminomethyl benzoxazole/benzthiazole-2-thiones (Table III). However, NAD-independent oxidation of succinate was found to be unaffected. Maximum inhibition of DL-isocitrate oxidation was observed with Compound V, while maximum inhibition of α-ketoglutarate oxidation was observed with Compound I. Compound V produced maximum inhibition of the oxidation of pyruvate. Anticonvulsant activity exhibited by 3-alkyl benzoate aminomethyl benzoxazole/benzthiazole-2-thiones is recorded in Table III. All compounds, when used at the dose of 100 mg/kg, possessed low anticonvulsant activity, as evidenced by their ability to afford only 10–40% protection against pentylenetetrazol-induced seizures. Maximum protection was observed with I and IV, while V was the least effective compound. 3-Alkyl benzoate aminomethyl benzoxazole/benzthiazole-2-thiones, possessing low anticonvulsant activity, showed higher mortality in experimental mice treated with pentylenetetrazol during 24 hr.

The selective inhibitory effect of 3-benzhydrazide aminomethyl benzoxazole/benzthiazole-2-thiones on NAD-dependent oxidation of DL-isocitrate, α-ketoglutarate, and pyruvate by rat brain homogenate is recorded in Table IV. As was observed with their precursors, 3-alkyl benzoate aminomethyl benzoxazole/benzthiazole-2-thiones, these compounds were devoid of an inhibitory effect on the oxidation of succinate. Maximum inhibition of the oxidation of DL-isocitrate was observed with Compound XI, while maximum inhibition of the oxidation of α-ketoglutarate and pyruvate was observed with Compound XII. On the other hand, all compounds inhibited monoamine oxidase activity in the 37.2–

70.2% range when used at a final concentration of 1×10^{-4} *M* during oxidative deamination of kynuramine. Compound XII produced maximum inhibition. The degree of monoamine oxidase inhibition was influenced by the position of the hydrazide group on the phenyl nucleus attached at the aminomethyl portion of the benzoxazole or benzthiazole moiety. The presence of the hydrazide group at position 4 of the phenyl nucleus produced greater inhibition of monoamine oxidase in benzoxazole or benzthiazole derivatives. The ability of these hydrazides to inhibit monoamine oxidase was greater with 3-benzhydrazide aminomethyl benzthiazole-2-thiones as compared to their corresponding benzoxazole derivatives. All of these hydrazides possessed low anticonvulsant activity, lower than that of their precursor esters (I–VI). The low anticonvulsant activity of these hydrazides corresponded with high pentylenetetrazol mortality, which ranged from 40 to 90% in experimental mice treated with hydrazide derivatives during 24 hr. None of these compounds exhibited any appreciable sedative or central nervous system (CNS) depressant effect nor 24-hr mortality at 100 mg/kg. These observations failed to provide any correlation between *in vitro* enzyme inhibitory activity of these compounds and their low anticonvulsant activity.

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Mechanism for Riboflavin Enhancement of Bilirubin Photodecomposition *In Vitro*

DILIP R. SANVORDEKER* and H. B. KOSTENBAUDERˣ

Abstract □ Riboflavin increases the rate of bilirubin photodecomposition as much as 25-fold under aerobic conditions. In the absence of oxygen, bilirubin photodecomposition is virtually arrested, with or without riboflavin. It is proposed that singlet oxygen generated by the riboflavin triplet species is primarily responsible for the observed rate enhancement. The occurrence of similar photochemical reactions *in vivo* might provide a means for improving the efficiency of bilirubin phototherapy through systemic administration of appropriate agents.

Keyphrases □ Riboflavin—enhancement of bilirubin photodecomposition *in vitro*, mechanism □ Bilirubin, photodecomposition—mechanism for riboflavin enhancement *in vitro* □ Photodecomposition, bilirubin—mechanism of enhanced rates under aerobic conditions with riboflavin *in vitro*

Bilirubin is a tetrapyrrolic dicarboxylic acid produced *in vivo*, mainly by the hepatic and splenic reticuloendothelial systems, from hemoglobin released from senescent erythrocytes (1). Nonhemolytic neonatal hyperbilirubinemia is ascribed to deficient glucuronidation of bilirubin arising from either capacity-limited hepatic uptake of bilirubin (2, 3) or deficiency of the hepatic enzyme bilirubin uridine diphosphate glucuronyltransferase (4), which catalyzes the conjugation of lipophilic bilirubin to its hydrophilic diglucuronide. Facile elimination of this diglucuronide occurs *via* biliary and urinary excretion.

Phototherapy has become a common procedure in neonatal hyperbilirubinemia (5–8). Despite the well-established efficacy of light therapy in reducing plasma bilirubin levels, reservations have been expressed with respect to exposing infants to light intensities of 300–500 foot-candles, often continuously for several days. Conceivably, an enhancement of bilirubin phototherapy may be accomplished by administration of suitable agents which would promote bilirubin photodecomposition *in vivo*. An enhancement of bilirubin phototherapy could mean either a reduction of exposure time or a reduction of light intensity, thereby reducing some potential hazards (9) of this treatment.

In view of its clinical importance, several investigators have studied bilirubin photochemistry in the presence and absence of a complexing polymeric molecule such as a serum albumin (10–12). Recent studies (13) showed that oxygen is essential for bilirubin photodecomposition. In the presence of oxygen, bilirubin undergoes irreversible photooxidation to a variety of products (14–18), including methylvinylmaleimide, biliverdin, and water- or methanol-propentdyopent adducts. Furthermore, under nitrogen and in the presence of a suitable nucleophilic substrate such as *N*-acetyl-1-cysteine, bilirubin undergoes an irreversible reaction with the substrate to give hydrophilic photoaddition products (19, 20). In the presence of oxygen, light-excited bilirubin sensitizes the formation of singlet oxygen which then attacks ground-state bilirubin with resultant bilirubin photooxidation (21). The same author (21) also showed that singlet oxygen-generating compounds

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such as methylene blue enhance the rate of bilirubin photodecomposition *in vitro*.

The photochemistry of riboflavin has been extensively studied (22–26). It has been recognized that riboflavin can promote photodecomposition of other compounds in fluid solution *via* several mechanisms, including energy transfer, coupled oxidation–reduction, and sensitized photooxygenation. Energy transfer involves absorption of light by riboflavin and excitation to a higher energy state (triplet) which may, upon collision with an acceptor molecule, decay to the ground state and transfer the electronic excitation energy to the acceptor. Examples of riboflavin promotion of substrate photodecomposition through triplet–triplet energy transfer include photodecomposition of the polyene fungicide pimaricin (27), isomerization of stilbene-4-carboxylic acid and cinnamic acid (28), and photooxidation of the acridan drug clomacran to its acridine derivative (29). Riboflavin might also be expected to promote photodecomposition of a substrate through a coupled oxidation–reduction process involving hydrogen abstraction by riboflavin triplet. The latter mechanism has been used to explain the flavin-sensitized photooxidation of amino acids (30) and of indoleacetic acid (31). Additional mechanisms that have been described for flavin-promoted photooxidation include singlet oxygen-mediated reactions such as riboflavin-sensitized photooxidation of limonene (32) and reactions involving excited flavin–substrate complexes such as the lumiflavin-sensitized photooxidation of nucleotides (33).

It appeared possible that an *in vitro* enhancement of bilirubin photodecomposition might be achieved through use of a suitable promoter and that such a process might be of clinical value. This report constitutes the investigation on *in vitro* enhancement of bilirubin photodecomposition by riboflavin. Evidence that riboflavin can indeed increase the efficiency of bilirubin phototherapy in an animal model was presented previously (34).

EXPERIMENTAL

Reagents—All reagents except nitrogen gas were used as received from the supplier. The following were used: bilirubin[1]; β-carotene[2]; dimethylformamide[3], 99% pure; potassium iodide USP; sodium carbonate, reagent grade; sodium chloride, reagent grade; trichloroacetic acid, reagent grade; bovine serum albumin, fraction V[4]; and vanadyl sulfate, reagent grade.

Nitrogen gas was purified by passing it through a solution of vanadyl sulfate and sulfuric acid containing zinc–mercury amalgam (35) and then through a 10% solution of pyrogallol in dimethylformamide to remove traces of oxygen.

Preparation of Solutions—Stock solutions for photochemical studies were prepared in subdued light. The bilirubin solution (6.6×10^{-5} *M*), the riboflavin monosodium-5′-phosphate solution (1.94×10^{-4} *M*), and the potassium iodide solution (10^{-3} *M*) were prepared in 0.05 *M*, pH 7.4, phosphate buffer. For the nonaqueous study, the bilirubin solution (6.84×10^{-5} *M*), the β-carotene solution (10^{-3} *M*), the riboflavin solution (1.33×10^{-4} *M*), and the potassium iodide solution (10^{-3} *M*) were prepared in dimethylformamide.

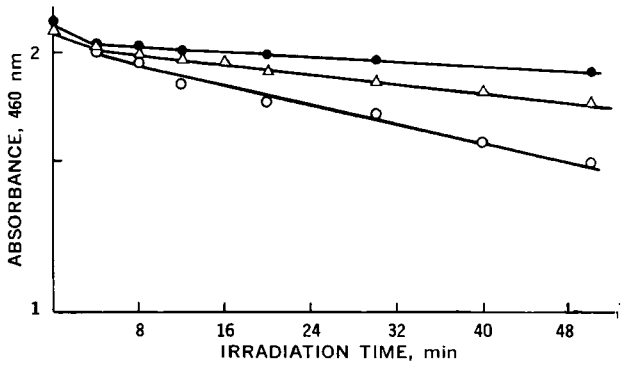

Figure 1—*Effect of oxygen on bilirubin photodecomposition in 1% aqueous serum albumin solution, pH 7.4. Initial bilirubin concentration was approximately 3.4 × 10⁻⁵ M (2 mg %). Key: ●, anaerobic; △, air equilibrated; and ○, oxygen bubbled.*

A stock bilirubin solution in phosphate buffer, pH 7.4, was prepared by dissolving 4 mg of bilirubin in 10 ml of a 1% sodium carbonate solution and then quickly transferring this solution, with constant stirring, to a 150-ml beaker containing a solution of 4 g bovine serum albumin, fraction V, in 60 ml phosphate buffer. The pH of this solution was adjusted to 7.4 with approximately 0.4 ml of 1 *N* hydrochloric acid and the solution was diluted to 100 ml with standard phosphate buffer solution. These solutions were diluted appropriately and used as needed for photochemical studies.

Irradiation Setup and Procedure for Analysis—Photochemical studies were conducted at a room temperature of 22–26°. Solutions were irradiated intermittently in 1-cm quartz cells located 61 cm from a projector equipped with a 500-w tungsten lamp[5]. Studies were conducted both with unfiltered light and with light passed through a filter[6] to exclude wavelengths outside the 360–480-nm range.

Anaerobic studies were conducted in Thunburg and standard spectrophotometric cells fitted with ground-glass stoppers. Aqueous solutions were degassed by a freeze, thaw, and vacuum procedure in three to four cycles. Dimethylformamide solutions were purged for at least 40 min with purified nitrogen prior to irradiation. Absorption spectra were recorded *versus* an appropriate blank solution[7].

To permit determination of riboflavin concentration in aqueous solutions containing unknown quantities of bilirubin, the aqueous solutions were treated with 5% trichloroacetic acid to precipitate the bilirubin–albumin complex. Upon centrifugation, the clear supernatant liquid was diluted and analyzed for riboflavin by a standard fluorometric method (36). Anaerobic solutions of riboflavin containing bilirubin could be analyzed for riboflavin by difference absorption spectrophotometry, since the bilirubin concentration in these solutions remained constant under anaerobic conditions.

To determine the effect of bilirubin on riboflavin photodecomposition under anaerobic conditions, riboflavin solutions in dimethylformamide with and without bilirubin were placed in fluorometric cells and purged with purified nitrogen for at least 40 min. The cells were then quickly stoppered and placed in the spectrophotofluorometer[8]. These solutions were irradiated intermittently with the excitation wavelength set at 440 nm and the slit facing the cell holder removed. The riboflavin concentration in these solutions was monitored by difference absorption spectrophotometry as described previously.

Treatment of Rate Data—The effect of flavin on the rate of bilirubin photobleaching was evaluated by plotting the logarithm of bilirubin absorbance *versus* irradiation time and determining the rate constant for each individual run from the slope of the re-

[1] Lot No. 9324, Nutritional Biochemicals Corp.
[2] Sigma Chemical Co.
[3] Fisher Scientific Co.
[4] Lot No. F32402, Armour Pharmaceutical Co.
[5] Type DAK, Sylvania Electric Products.
[6] CS 560, Corning Glass Works.
[7] Cary 15 spectrophotometer, Cary Instruments Co.
[8] Aminco-Bowman spectrophotofluorometer, American Instrument Co.

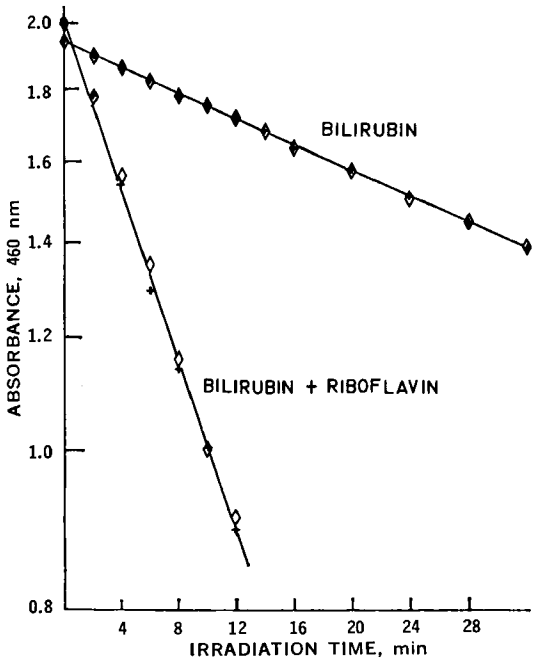

Figure 2—*Effect of 5.3 × 10⁻⁶ M riboflavin on rate of photodecomposition of bilirubin in dimethylformamide solution exposed to unfiltered visible light. Initial bilirubin concentration was approximately 3.4 × 10⁻⁵ M (2 mg %). Key: ◇ and + represent points from duplicate studies.*

sulting linear plot. Control bilirubin solutions containing no flavin were run along with each study of the effect of flavin, and data are reported as the ratio of the rate of bilirubin photodecomposition with flavin to the rate without flavin to compensate for any variation in light intensity from study to study. Each series of studies designed to illustrate the effect of variables such as oxygen concentration was run at the same light intensity.

RESULTS AND DISCUSSION

Figure 1 illustrates the effect of oxygen concentration on the rate of loss of bilirubin absorbance. In both the aqueous serum albumin solution and in dimethylformamide, bilirubin exhibits an increased rate of photofading with increasing concentration of oxygen. The mechanism proposed by McDonagh (21) for bilirubin photooxidation (Scheme I) involves generation of singlet oxygen, *via* light-excited bilirubin, followed by attack of singlet oxygen on ground-state bilirubin. Data obtained in the present study are consistent with such a mechanism.

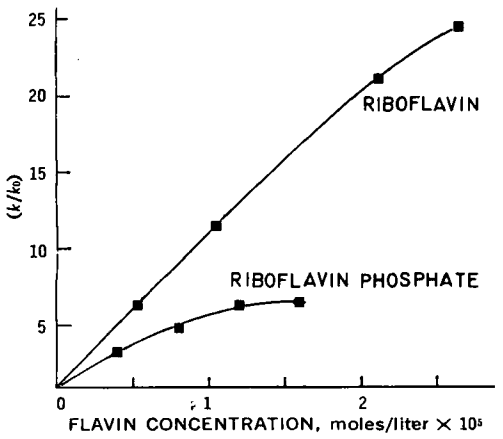

Figure 3—*Influence of flavin concentration on rate of bilirubin photodecomposition in solutions exposed to unfiltered visible light.*

$$B^0 + h\nu \longrightarrow B^1 \text{ (bilirubin in singlet state)}$$
$$B^1 \longrightarrow B^3 \text{ (bilirubin in triplet state)}$$
$$B^3 + O_2^3 \longrightarrow B^0 + O_2^1 \text{ (singlet oxygen)}$$
$$B^0 + O_2^1 \longrightarrow \text{photooxidation products}$$

Scheme I

Figure 2 is a typical plot illustrating the effect of riboflavin on bilirubin photodecomposition in air-equilibrated solutions. At least a 25-fold rate increase may be achieved by riboflavin in dimethylformamide solution, and at least a sixfold rate enhancement may be achieved from riboflavin phosphate in aqueous 1% serum albumin. Potassium iodide, at a concentration of 10^{-4} *M*, reduced the riboflavin enhancement of bilirubin photodecomposition while showing no effect on the rate of bilirubin photodecomposition in the absence of riboflavin. Since potassium iodide at such a concentration is known to be an effective quencher of the flavin triplet state (37, 38), this observation implicates the flavin triplet in the overall enhancement of bilirubin photodecomposition. The lack of any effect of 10^{-4} *M* potassium iodide on the rate of bilirubin photodecomposition suggests that the bilirubin triplet state may be too short lived to collide with potassium iodide at these concentration conditions.

Figure 3 shows a plot of the ratio of first-order rate constants for bilirubin decomposition in the presence of flavin to the rate constant for bilirubin photodecomposition in the absence of flavin under otherwise identical conditions. These data were collected for air-equilibrated solutions exposed to unfiltered visible light. All rate constants were obtained from linear plots of the logarithm of bilirubin absorbance *versus* time. The magnitude of the rate enhancement by flavin varied with light intensity and with wavelength of the light source, but all results were qualitatively similar to those illustrated in Fig. 3. At low flavin concentration, the rate of bilirubin photodecomposition is first order with respect to flavin and becomes independent of change in flavin concentration at high flavin concentration. Such a profile is to be expected for a flavin-sensitized photochemical reaction.

It has become increasingly recognized that many photooxidation reactions proceed *via* singlet oxygen. Kearns *et al.* (39) suggested that the triplet energy of the sensitizers of singlet oxygen should be in the range of 37 kcal. In view of the reported triplet energy level of riboflavin at 46 kcal (40), it is evident that an efficient collisional energy transfer from riboflavin triplet to oxygen triplet (ground state) is possible on energetic grounds. Since riboflavin is capable of generating singlet oxygen (ΣO_2^1 or ΔO_2^1) *via* its excited triplet state (32, 41–43), it is possible that riboflavin might promote bilirubin photodecomposition by this mechanism. β-Carotene, an efficient singlet oxygen quencher at 10^{-4} *M* concentration (43), completely quenched bilirubin photodecomposition in the absence of flavin and partially inhibited the promotion of bilirubin photodecomposition by riboflavin. This observation is consistent with a mechanism (Scheme II) involving generation of singlet oxygen by riboflavin.

As shown in Figs. 1 and 4, absence of oxygen virtually arrests bilirubin photodecomposition, with or without riboflavin. Since there was no evidence for any photodecomposition of bilirubin by riboflavin under anaerobic conditions, the possibility of a coupled oxidation–reduction process involving bilirubin and riboflavin can be rejected.

Figure 5 shows that the presence of bilirubin decreases riboflavin fluorescence. The decrease in riboflavin fluorescence is obtained under both aerobic and anaerobic conditions. Such fluorescence quenching might be attributed either to an energy transfer from light-excited flavin to bilirubin or to an "inner filter" effect (44) arising from the bilirubin absorption spectrum overlapping that of riboflavin from 350 to 550 nm. Without knowledge of the triplet level of bilirubin, the possibility of a triplet–triplet energy

$$Rf^0 + h\nu \longrightarrow Rf^1 \text{ (riboflavin singlet)}$$
$$Rf^1 \longrightarrow Rf^3 \text{ (riboflavin triplet)}$$
$$Rf^3 + O_2^3 \longrightarrow Rf^0 + O_2^1 \text{ (singlet oxygen)}$$
$$B^0 + O_2^1 \longrightarrow \text{photooxidation products}$$

Scheme II

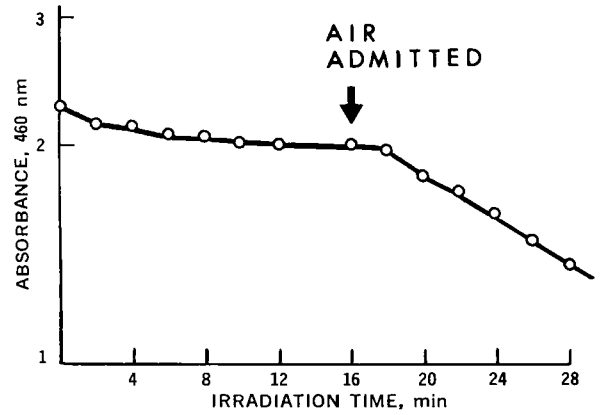

Figure 4—*Effect of oxygen on bilirubin photodecomposition under blue light in the presence of 9.6 × 10^{-6} M riboflavin phosphate in 1% aqueous serum albumin, pH 7.4. Initial bilirubin concentration was approximately 3.4 × 10^{-5} M.*

transfer could not be excluded on energetic grounds. However, if such an energy transfer were to occur, it would be anticipated that it would serve to depopulate the riboflavin triplet and thus retard the photobleaching of riboflavin. As shown in Fig. 6, the presence of bilirubin retards riboflavin photobleaching, but it can be shown that the extent of inhibition does not exceed that to be expected simply from the inner filter effect of overlapping absorption spectra of bilirubin and riboflavin.

To determine whether the effect of bilirubin on flavin fluorescence and photofading is attributable solely to an inner filter effect, a quantitative comparison was made between the effect of bilirubin on the rate of anaerobic photofading of riboflavin and the relative amounts of light absorbed by riboflavin alone and in the presence of bilirubin. If the effect of bilirubin is attributable solely to an inner filter effect, the rate of riboflavin photofading in the presence of bilirubin should decrease in direct proportion to the decrease in the quanta of light absorbed by riboflavin[9]. Based on determination of the absorbances of riboflavin alone and of a solution containing both riboflavin and bilirubin, it can be shown that 1.32 × 10^{-4} *M* riboflavin in the presence of 1.44 × 10^{-5} *M* bilirubin absorbs only 57% as much of the incident light as in the absence of bilirubin. After 30% of the riboflavin is decomposed (range over which the study was conducted), there would be 52% as much light absorbed by riboflavin in presence of 1.44 × 10^{-5} *M* bilirubin. It might, therefore, be anticipated that the rate of photofading of riboflavin, in decreasing the concentration from 1.32 × 10^{-4} to 0.92 × 10^{-4} *M* in the presence of 1.44 × 10^{-5} *M*

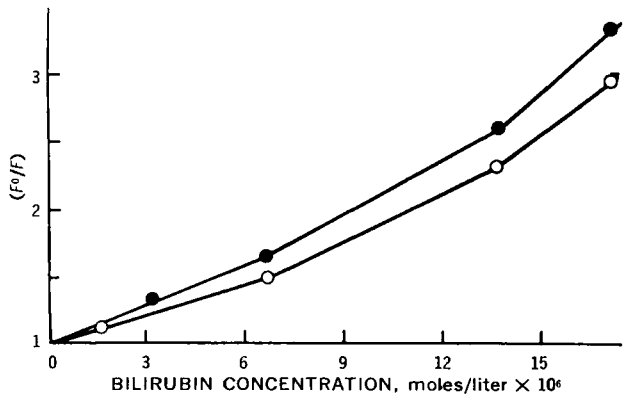

Figure 5—*Effect of bilirubin on flavin fluorescence intensity, where F°/F is the ratio of flavin fluorescence in absence of bilirubin to that in the presence of bilirubin. Key: ●, riboflavin phosphate, 9.6 × 10^{-6} M in 0.2% aqueous serum albumin; and ○, riboflavin, 1.3 × 10^{-5} M in dimethylformamide.*

[9] The comparison is made for the anaerobic data since under these conditions the bilirubin concentration remains constant.

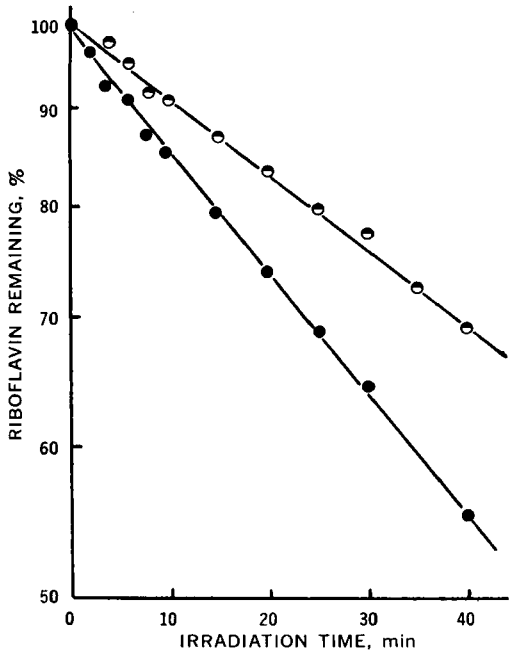

Figure 6—*Effect of bilirubin on rate of riboflavin photodecomposition in deoxygenated dimethylformamide solution. Initial riboflavin concentration was 1.32 × 10^{-4} M, and bilirubin concentration was 1.44 × 10^{-5} M. First-order rate constant is 0.0144 min^{-1} for riboflavin alone and 0.0092 min^{-1} in the presence of bilirubin. Key: ◓, riboflavin plus bilirubin; and ●, riboflavin alone.*

bilirubin, would be between 57 and 52% of the rate observed in the absence of bilirubin. In fact, the comparison of the rates shown in Fig. 6 indicates that the apparent first-order rate of riboflavin photofading in the presence of 1.44 × 10^{-5} *M* bilirubin is approximately 64% of the rate observed in the absence of bilirubin. Within experimental limitations, it appears that the inhibition of riboflavin photofading by bilirubin may be attributed solely to an inner filter effect. Since the observed rate is slightly greater than, if not equal to, the predicted rate in the presence of bilirubin, the possibility of an energy transfer (triplet-triplet or singlet-singlet) from riboflavin to bilirubin, existence of which would be expected to depopulate the riboflavin triplet state and further slow riboflavin photofading, can be ignored.

It is also evident from these studies that serum albumin-bound bilirubin must be capable of participation in photochemical reaction in the bound state. For singlet oxygen, with a lifetime of the order of 10^{-5} sec, to collide with a reactive substrate, the concentration of that substrate in solution should be above or in the range of 10^{-4}–10^{-5} *M* (41). Since the saturation solubility of unbound bilirubin at pH 7.4 is reported as 1.71 × 10^{-6} *M* (45), it is evident that *in vitro*, and perhaps *in vivo*, photooxidation of bilirubin *via* singlet oxygen can involve attack on bilirubin in the bound state as well as on free bilirubin.

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* Present address: G. D. Searle & Co., Chicago, IL 60680

ˣ To whom inquiries should be directed.

Homogeneity of Multicomponent Powder Mixtures

JOHN A. HERSEY*ˣ, PETER COOK‡, MAX SMYTH‡, ELIZABETH A. BISHOP*, and ELIZABETH A. CLARKE*

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The operation of powder mixing is common to the manufacture of many formulations in the pharmaceutical industry. While the problems involved with powder mixtures were elucidated previously (1), further investigations have been concerned with the relatively simple system of binary mixtures (2, 3). In practice, such binary mixtures are of academic importance only, since the mixing of either a single drug or a number of drugs with a number of excipients is a more frequent occurrence in pharmacy. Two such multicomponent powder mixing operations were recently investigated (4, 5).

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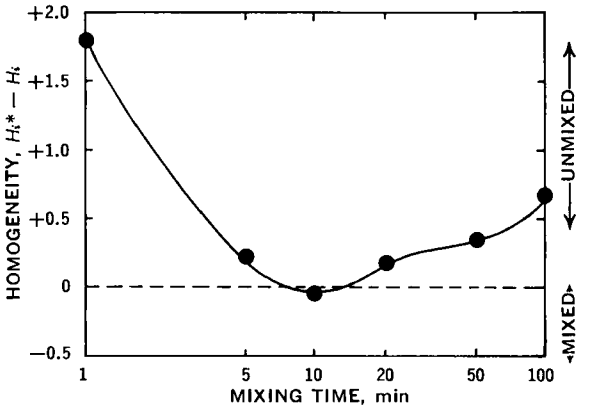

Figure 1—*Mixing of fenfluramine hydrochloride in Mixture A. Requirement for* H_i^* *is that 99.7% of the results (assuming a normal distribution) lie within 10% of the mean value.*

$$\sigma_R = (xy/N)^{1/2} \qquad \text{(Eq. 1)}$$

where x and y are the proportions of the two ingredients X and Y, and N is the number of particles per sample taken. Hersey (8–10) criticized such indexes for use in pharmacy since they are dependent upon the particle size and proportions of the components and are not related to the homogeneity of the systems under investigation. A more useful index for use in pharmacy was proposed, using as its criterion the desired degree of homogeneity as, for example, laid down by a pharmacopeial standard. An example of such a requirement would be that tablets containing 1% of an active ingredient should have contents within ±10% in 99.7% of the samples, assuming a normal distribution. The standard deviation (σ_A) corresponding to this requirement is given by:

$$\pm 3\sigma_A = \pm 10 \times 0.01 = (\text{tolerance} \times \text{mean}) \qquad \text{(Eq. 2)}$$

Such criteria can be used to assess the degree of powder mixing to a desired level [by plotting the standard deviation of an ingredient *(s)* for a number of samples taken from the mix] as a ratio of σ_A or to assess the homogeneity of a number of tablets taken after production from the batch.

Buslik (11) recently introduced a new concept of homogeneity of binary systems based on the weight of sample (W_1) necessary to give a fixed variation (*e.g.*, a standard deviation of 1%) between samples. The homogeneity *(H)* is then defined as the reciprocal of W_1:

$$H = \frac{1}{W_1} \qquad \text{(Eq. 3)}$$

The negative logarithm of W_1 is used to overcome the large range of values of homogeneity that could be found for H:

$$H_i = \log H = -\log W_1 \qquad \text{(Eq. 4)}$$

The percentage standard deviation (σ_B) of an ingredient *(G)* of particle weight w found from randomly mixed samples of weight W is given by (Eq. 1):

$$\sigma_B = [g(100 - g)w/W]^{1/2} \qquad \text{(Eq. 5)}$$

where g is the percentage weight of ingredient G, and w is the weight of all particles present in the mixture when σ_B = 1%; then $W = W_1$ and:

$$W_1 = g(100 - g)w \qquad \text{(Eq. 6)}$$

Substituting from Eq. 4:

$$H_i = -\log [g(100 - g)w] \qquad \text{(Eq. 7)}$$

By calculation of the required standard deviation according to Eq. 2 and assuming randomized mixing by analogy with Eq. 5:

$$\sigma_A = [g(100 - g)w/W]^{1/2} \qquad \text{(Eq. 8)}$$

Substituting in Eq. 7:

$$H_i^* = -\log [\sigma_A^2 W] \qquad \text{(Eq. 9)}$$

where H_i^* is the degree of homogeneity required by the pharmacopeial standard. By plotting the difference between H_i^* and H_t [the homogeneity during mixing found by evaluating the standard deviation *(s)* from a series of samples of weight W]:

$$H_i^* - H_i = -\log \sigma_A^2 W + \log s^2 W \qquad \text{(Eq. 10)}$$
$$= \log s^2 W/\sigma^2 W$$
$$= 2 \log (s/\sigma_A)$$

where s/σ_A is the same index of homogeneity previously proposed (8).

Equation 9 may also be used to calculate the desired degree of homogeneity of a number of pharmaceutical disperse systems and to calculate, using Eq. 8, the particle size *(w)* necessary to achieve that homogeneity.

The problem of dealing with real systems consisting of a particle distribution and of more than two ingredients complicates many of the previous findings. Work has been done to enable the calculation of the standard deviation of the completely randomized mixture for components having a distribution of particle sizes (12–14) and for multicomponent mixtures (15, 16), but the homogeneity of such systems has not been described.

Theory—The standard deviation (σ_P) of an ingredient *(P)* in a completely randomized mixture was calculated by Stange (15) to be given by:

$$\sigma_p^2 = \frac{p^2}{W}\left[\left(\frac{1-p}{p}\right)^2 p\sum(fw)_p + q\sum(fw)_q + r\sum(fw)_r \ldots\right] \qquad \text{(Eq. 11)}$$

where p, q, are r are the proportions of the ingredients P, Q, and R; $\Sigma(fw)$ is the effective mean particle weight of that ingredient denoted by the subscript and is calculated from the formula of Poole *et al.* (14); and W is the sample weight as before.

Assuming that the components must be reduced to the same particle-size level prior to mixing:

$$\sum(fw)_p = \sum(fw)_q = \sum(fw)_r = w \qquad \text{(Eq. 12)}$$

where w is the effective mean weight of all particles in the mixture.

Since $Q + R = 1 - P$ for the three-component systems, Eq. 11 reduces to:

$$\sigma_p^2 = wp(1 - p)/W \qquad \text{(Eq. 13)}$$

which is identical to Eq. 5 in percent terminology:

$$\sigma_{B_{(P)}} = [P(100 - P)w/W]^{1/2} \qquad \text{(Eq. 14)}$$

Similarly, for the other ingredients:

$$\sigma_{B_{(Q)}} = [Q(100 - Q)w/W]^{1/2} \qquad \text{(Eq. 15a)}$$

$$\sigma_{B_{(R)}} = [R(100 - R)w/W]^{1/2} \qquad \text{(Eq. 15b)}$$

When σ_B = 1%, then $W = W_1$ gives a different equation for the homogeneity of each of the four ingredients in the multicomponent mixture:

$$H_{i(p)} = -\log [P(100 - P)w] \qquad \text{(Eq. 16)}$$

etc. The equations can be used to follow the course of mixing of a single component in a mixing operation or to compare the various requirements of powders necessary for mixing to a desired degree of homogeneity in a multicomponent mixing operation.

Table I—Mixing of Fenfluramine Hydrochloride in Mixture A

Time (t), min	Standard Deviation of Fenfluramine Hydrochloride
1	0.0524
5	0.0086
10	0.0064
20	0.0089
50	0.0101
100	0.0147

Table II—Mixing of Barbiturates in Mixture B

Time (t), min	Standard Deviation		
	Phenobarbital	Butethal	Secobarbital
1	0.0310	0.0054	0.0042
2	0.0157	0.0017	0.0034
4	0.0060	0.0004	0.0002
8	0.0045	0.0006	0.0004
16	0.0044	0.0006	0.0008
32	0.0033	0.0005	0.0002
64	0.0058	0.0006	0.0004

Table III—Limiting Values of Homogeneity

Ingredient	Mixture	$H_{i\,\mathrm{lim}}$
Fenfluramine hydrochloride	A	2.53
Phenobarbital	B	3.00
Butethal	B	3.82
Secobarbital	B	3.84

Content Uniformity of a Compound Tablet—This is a problem of required homogeneity in a multicomponent system. Consider the three components P, Q, and R present in a 100-mg tablet at the 50, 5, and 0.5% levels, respectively. It is required that these percentages be present to within ±10% at the 99.7% level, assuming a normal distribution. From Eq. 2 for ingredient P:

$$\pm 3\sigma[A_{(P)}] = \pm 10 \times 0.05 \qquad \text{(Eq. 17}a\text{)}$$

$$3\sigma[A_{(P)}] = 0.5 \qquad \text{(Eq. 17}b\text{)}$$

Similarly, $3\sigma[A_{(Q)}] = 0.05$ and $3\sigma[A_{(R)}] = 0.005$. Substituting these values in Eq. 8 allows calculation of the effective mean particle weight (w) and, assuming spherical particles of density 1.2 g cm^{-3}, the calculation of the particle diameter:

$$d_{(P)} = 120.9\ \mu m \qquad \text{(Eq. 18}a\text{)}$$

$$d_{(Q)} = 26.1\ \mu m \qquad \text{(Eq. 18}b\text{)}$$

$$d_{(R)} = 5.6\ \mu m \qquad \text{(Eq. 18}c\text{)}$$

To achieve the required homogeneity, it would be necessary to reduce the particle sizes of the components to these levels. In practice, it would be difficult to mix particles of differing particle size and an overall reduction to 5 μm would be necessary. This would bring the assumption implicit in Eq. 12 into order and would result in greater homogeneity than necessary for components P and Q in the resulting tablets, providing the particles were randomly dispersed and did not show a tendency toward segregation.

Multicomponent Powder Mixing—Two examples of multicomponent powder mixing were recently described (4, 5). In each case the particle size of each component was determined by sieving, the densities were determined, and the powders were mixed in a mixer[1]. Mixture A consisted of 20% fenfluramine hydrochloride in a 100-mg sample containing a number of tablet excipients. Samples were removed from the mixer at various intervals and assayed spectrophotometrically. Mixture B consisted of three barbiturates (10% phenobarbital, 1% secobarbital, and 1% butethal) blended with lactose as the diluent in a 100-mg tablet preblend. Samples were removed as previously described and assayed using GLC with hexobarbital as an internal standard. The standard deviations *(SD)* calculated for each drug in the two mixtures are given in Tables I and II.

From a consideration of the particle-size data and densities, the effective mean particle diameters for each component in the two mixtures were calculated. Since in these practical situations the powders for each mixture are reduced to similar dimensions, it is possible using Eq. 16 to calculate the value of H_i for each drug. Substituting the desired degree of homogeneity (σ_A) and the standard deviation *(SD)* found in practice, it is then possible to trace the homogeneity of each drug as it is mixed throughout the mixture using $H_i^* - H_i$. Figure 1 shows this relationship for fenfluramine hydrochloride in Mixture A. In this case, limits of ±10% for 99.7% (*i.e.*, at the 3σ level of a normal distribution) were used. Figure 2 shows this same relationship for each barbiturate in Mixture B, where limits of ±15% for 95% (*i.e.*, at the 1.96σ level of a normal distribution) were chosen. When $H_i = H_i^*$, the position of desired homogeneity is attained. When $H_i < H_i^*$, the term $H_i^* - H_i$ becomes negative and the drug is mixed within specification—a desirable state since further processing (*e.g.*, tableting) may cause some degree of segregation (loss of homogeneity).

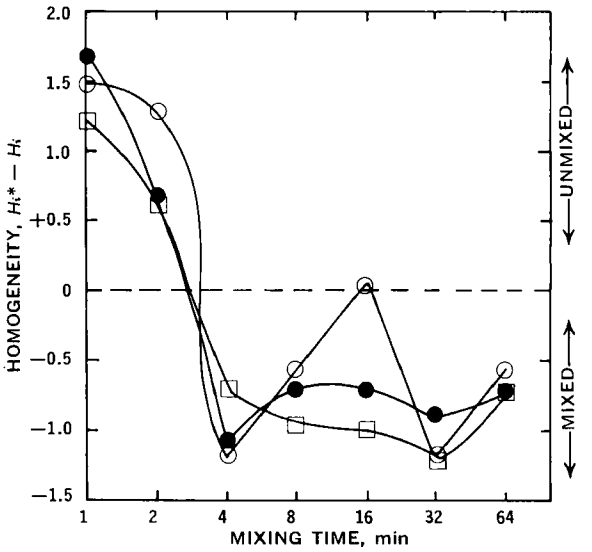

Figure 2—*Mixing of barbiturates in Mixture B. Requirement for* H_i^* *is that 95% of the results (assuming a normal distribution) lie within 15% of the mean. Key:* ●, *butethal;* ○, *secobarbital; and* □, *phenobarbital.*

According to the proportion of powder present in a mixture and its particle size, there will be a limiting homogeneity value which is possible to attain ($H_{i\,\mathrm{lim}}$), corresponding to the standard deviation of the randomized mixture (σ_R). It is essential if a powder is to be mixed within specification that $\sigma_A > \sigma_R$ or $H_i^* < H_{i\,\mathrm{lim}}$. Table III lists the $H_{i\,\mathrm{lim}}$ values for each active ingredient in the two mixtures. Figure 2 shows that the homogeneity of the different components does not follow the same pattern during the mixing process. Care should be taken when attempting to predict the homogeneity of a component from data on the homogeneity of another component in the same mixture.

CONCLUSION

The concept of homogeneity is useful for following the mixing of a drug or drugs in a multicomponent mixture. It also allows calculation of the particle size necessary to produce the desired degree of homogeneity (*i.e.*, within tolerance specifications) for powders in multicomponent mixtures.

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ˣ To whom inquiries should be directed.

Evidence for Variable Digoxin Absorption as Estimated by Pharmacological Response Intensities

R. D. SCHOENWALD

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Keyphrases □ Digoxin, variable absorption—estimated by pharmacological response intensities, dose–effect curves, ventricular rate slowing, response–time curves □ Absorption, digoxin—evidence for variability estimated by ventricular rate slowing, dose–effect and response–time curves constructed □ Ventricular rate slowing—used as pharmacological response intensity parameter for studying variable digoxin absorption □ Enterohepatic cycling, digoxin—evidence suggesting new significance

Recent reports (1–3) focused on the variation in bioavailability of digoxin following oral dosing. Blood level curves representing dosing of different brands as well as different lots of the same brand have shown variations in the values[1] of C_{max}, t_{max}, and areas under plasma curves, encouraging authors to conclude that various tablets of digoxin are not uniform. Formulation defects in the drug products administered were cited as probable causes for the differences in bioavailability. From computer simulations of central and peripheral compartment digoxin levels, Sorby and Tozer (4) concluded that bioavailability differences reported for commercial tablets of digoxin could additionally be a consequence of a variable absorption rate. Although variations in tablet formulation and/or method of manufacture are quite often responsible for the observed differences in drug blood level patterns, a variation in absorption rate must also be considered. The purposes of this report are to illustrate the variability in apparent absorption rates of digoxin following oral dosing and to demonstrate the sensitivity of pharmacological response intensities in obtaining this information.

[1] The C_{max} is defined as the maximum concentration of digoxin in plasma corresponding to the time t_{max}.

THEORETICAL

Experimental results often suggest that dose–effect relationships can be mathematically expressed according to drug–receptor (biophasic) occupation theory assuming a single type of receptor:

$$F_I = \frac{I}{I_{max}} = \frac{Q_B}{K_d + Q_B} \qquad \text{(Eq. 1)}$$

where F_I is the fraction of the maximum intensity attainable, Q_B is the quantity of drug in the biophase responsible for eliciting the response, and K_d is a constant which, under the conditions of one-half maximum intensity, equals the quantity of drug in the biophase. Although the relationship is not linear, it is possible to rearrange Eq. 1 to produce Eq. 2 so that dose is directly proportional to the transformed response intensities, f (5):

$$Q_B = K_d f \qquad \text{(Eq. 2)}$$

where:

$$f = \frac{F_I}{1 - F_I} \qquad \text{(Eq. 3)}$$

If pharmacological response is a direct consequence of biophasic drug levels and if drug disposition adheres to first-order kinetics, then it follows that Q_B can be described by Eq. 4:

$$Q_B = K_d f = D\sum_{i=1}^{n} A_i e^{-m_i t} \qquad \text{(Eq. 4)}$$

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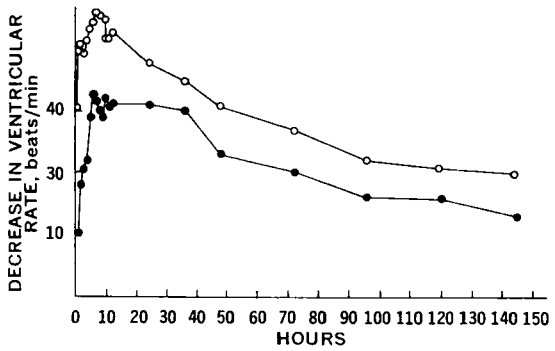


Figure 1—*Time course of ventricular rate slowing for intravenous* (○) *and oral* (●) *doses of 1.2 mg of digoxin administered to patients hospitalized with auricular fibrillation. Each data point represents a mean of 17 patients. Original data were taken from Ref. 7. Fluctuations occurring at approximately* t_{max} *are suggestive of enterohepatic cycling.*

or:

$$\frac{Q_B}{K_d} = f = \frac{D}{K_d}\sum_{i=1}^{n} A_i e^{-m_i t} \qquad \text{(Eq. 5)}$$

where A_i and m_i are equation parameters evaluated from the data, and D denotes the dose absorbed. Under the conditions of Eq. 5, a plot of f *versus* time gives a graphic representation for the disposition of proportionate biophasic drug levels with time; however, because of the inclusion of K_d in Eqs. 4 and 5, one too many parameters is present so that all of the individual model parameters (*i.e.*, k_{ij}'s or transfer constants between compartments i and j) may not be calculated.

By assuming Eq. 2 to hold at all times following dosing, it is possible to eliminate K_d by dividing it by the maximum transformed response intensity, f_{max}, occurring at t_{max} and corresponding to $(Q_B)_{max}$ to yield:

$$\frac{Q_B}{(Q_B)_{max}} = \frac{f}{f_{max}} \qquad \text{(Eq. 6)}$$

Under the conditions of Eq. 6, experimental f values recorded at each time and divided by the value[2] of f_{max} can be plotted against time to represent the time course of fractional biophasic drug levels.

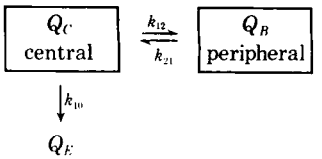
If K_d can be obtained, then Q_B may be plotted *versus* time according to Eq. 4 to yield the appropriate model parameters. Experimentally, this would require: (*a*) identification of the specific anatomical site associated with the biophase, which for drugs acting on a cellular level may not be known; and (*b*) development of a sensitive assay for drug and/or metabolite, whichever is responsible for eliciting a response. This would obviously not be practically feasible for many drugs. As will be subsequently shown, it is not always necessary to calculate values for the model parameters or, for that matter, for all equation parameters to estimate apparent absorption rates; only the equation parameters m_i need be available.

Provided the biophase is kinetically associated within a peripheral compartment and drug first enters the central compartment, it then becomes possible to calculate A_t, the cumulative amount of drug absorbed up to time t, using Eq. 7:

$$A_t = \frac{1}{k_{12}}\frac{dQ_B}{dt} + \left(\frac{k_{21} + k_{12} + k_{10}}{k_{12}}\right)Q_B + \frac{k_{21}k_{10}}{k_{12}}\int_0^t Q_B\,dt \qquad \text{(Eq. 7)}$$

Equation 7 is derived (*Appendix*) for the simplest case, in which the biophase is contained within a peripheral compartment and is communicating directly with the central compartment from which drug is eliminated, *i.e.*, the familiar two-compartment model (Scheme I).

Equation 7, divided by A_∞, can be expressed totally in terms of



Scheme I

equation parameters, obviating the necessity for calculating model parameters when absorption rate analyses are to be performed (6):

$$\frac{A_t}{A_\infty} = \frac{\dfrac{dQ_B}{dt} + (m_1 + m_2)Q_B + m_1m_2\displaystyle\int_0^t Q_B\,dt}{m_1m_2\displaystyle\int_0^\infty Q_B\,dt} \qquad \text{(Eq. 8)}$$

The m_i's are related to the model parameters by:

$$m_1m_2 = k_{10} + k_{12} + k_{21} \qquad \text{(Eq. 9)}$$

$$m_1m_2 = k_{10}k_{21} \qquad \text{(Eq. 10)}$$

Equation 8 is valid regardless of the compartment to which elimination is assigned. By substituting $K_d f$ from Eq. 2 for Q_B in Eq. 8, canceling the unknown K_d from the numerator and the denominator, and subtracting the resulting value from unity, Eq. 11 results. Equation 11 describes the fractional amount remaining to be absorbed as a function of time, calculated wholly from pharmacological response intensities:

$$1 - \frac{A_t}{A_\infty} = \frac{\dfrac{df}{dt} + (m_1 + m_2)f + m_1m_2\displaystyle\int_0^t f\,dt}{m_1m_2\displaystyle\int_0^\infty f\,dt} \qquad \text{(Eq. 11)}$$

The corresponding equation for a three-exponential model is given by Eq. 12:

$$1 - \frac{A_t}{A_\infty} = \frac{\dfrac{d^2f}{dt^2} + (m_1 + m_2 + m_3)\dfrac{df}{dt} + (m_1m_2 + m_2m_3 + m_1m_3)f + m_1m_2m_3\displaystyle\int_0^t f\,dt}{m_1m_2m_3\displaystyle\int_0^\infty f\,dt} \qquad \text{(Eq. 12)}$$

METHODS

Gold *et al.* (7) reported on the decrease in ventricular heart rate in hospitalized patients with auricular fibrillation following oral and intravenous dosing of 1.2 mg of digoxin. Subsequent data pertaining to digoxin and retreated in this article were taken from that report. Computerized fits of the dose–effect curve and biophasic drug levels as a function of time were obtained[3]. Beginning with an initial set of parameter values, the program obtains a least-squares fit to experimental data using stepwise Gauss–Newton iterations (8). To obtain initial estimates, the response intensities (Eq. 5) were plotted as a function of time on semilogarithmic paper. By using back-extrapolation procedures (9), the curve was resolved into the appropriate number of exponential components to yield values for the A_i's and m_i's. A Scatchard-type plot representing a linear transformation of Eq. 1 enabled its initial estimates to be obtained for use in the nonlinear curve-fitting program. Other necessary calculations were also performed by computer[3].

RESULTS

Figure 1 shows the time course of ventricular rate slowing for the oral and intravenous doses. Each point represents a mean of 17 subjects; zero time values were determined following a control period of several days in which patients were put to bed in an at-

[2] The f_{max} is a function of dose and the time course of drug at the active site, whereas I_{max} is independent of time and represents the maximum theoretical response obtainable according to Eq. 1.

[3] Using the BMDX.85 FORTRAN IV computer program and a CDC 6400 digital computer, Computer Science Center, University of Washington, Seattle, Wash.

Table I—Mean Ventricular Rates (Beats per Minute) and Standard Deviations for Two Time Determinations following Dosing of 1.2 mg of Digoxin to 17 Human Subjects[a]

Time of Determination	Mean	Standard Deviation	Coefficient of Variation
	Oral[b]		
Zero	107.7	26.0	24.1
Peak	74.6	11.2	15.0
	Intravenous[c]		
Zero	107.4	30.8	28.7
Peak	62.9	10.6	16.8

[a] Data calculated from Fig. 2 of Ref. 7. [b] $n = 18$. [c] $n = 16$.

tempt to establish a stable baseline, a prerequisite in quantifying pharmacological response intensities. Table I lists the standard deviations calculated for zero and peak time response values corresponding to the 1.2-mg oral and intravenous doses. For digoxin, a perceptible graded response, a slowing of ventricular rate, was measured through 168 hr. It is necessary that drug and not metabolite be responsible for eliciting a response and that the rate-determining step is the time required for drug to reach the biophase. The closest evidence for the latter comes from a report (10) in which the temporal change in left ventricle ejection time and Q-S_2 intervals were closely associated with computer-generated amounts of drug in the tissue compartment of a two-compartment open model. Additional reports have shown a relationship between myocardial digoxin concentration and hemodynamic effects (11) and the selective affinity that myocardial tissue exhibits for digoxin as compared to serum and other organs (12). Moreover, the biochemical activity of digoxin, namely, its role in the inhibition of adenosine triphosphatase to promote myocardial contraction, suggests an intracellular biophase which would be expected to behave kinetically distinguishable from the rapidly perfused heart muscle.

Assuming that occupation theory (Eq. 1) correctly describes response as a function of Q_B, values of K_d and I_{max} must be determined to obtain estimates for Q_B as a function of time following dosing (Eq. 4). If Eq. 1 contains a dose term in place of Q_B, the value of K_d under the conditions of one-half maximum intensity does not equal Q_B but equals Q_B times an unknown proportionality factor and, therefore, cannot be used in Eq. 4. The I_{max} value, however, remains the same regardless of whether Q_B or dose is plotted *versus* response intensities because the abscissa values, but not the ordinate values, are multiplied by the proportionality factor. The proportionality factor equals the summation terms on the right-hand side of Eq. 4 relating Q_B to dose where t equals a fixed time. Its value need not be known since the use of K_d is obviated by the utilization of Eqs. 6 and 11.

The dose-effect curve presented as Fig. 2 represents a steady-state response to a particular daily oral maintenance dose. Its use in this report serves to establish that occupation theory describes the interaction between drug and receptor and to obtain an estimate of I_{max} for calculation of f. The pharmacological response intensity, I, is represented by a decrease in ventricular rate measured in reference to an average controlled baseline of 108 beats/min. Each point is an average of response intensities obtained from 30 ambulatory patients with auricular fibrillation, to whom a digitalizing dose was first administered to produce a ventricular heart rate of approximately 70 beats/min followed by 4 weeks of daily maintenance dosing. It is assumed that due to the large number of subjects studied, the fraction absorbed for each dose level is constant so that the I_{max} value is not significantly altered. Weekly monitoring of ventricular rate provided the steady-state response for the particular dose level. When the dose was adjusted to another maintenance level, a week elapsed before measurements were resumed so that a steady-state response representative of the new dosing level would be attained. Under the conditions of steady state, the daily oral maintenance dose would be expected to be directly related to Q_B; consequently, a plot of maintenance dose *versus* steady-state response intensities would give the same I_{max} value as a plot of Q_B *versus* the corresponding response intensity following the administration of single doses, intravenous or oral. As long as dose, either intravenous or oral, remains proportional to Q_B, the abscissa values of a dose-effect curve can be multiplied by any constant factor without influencing I_{max}.

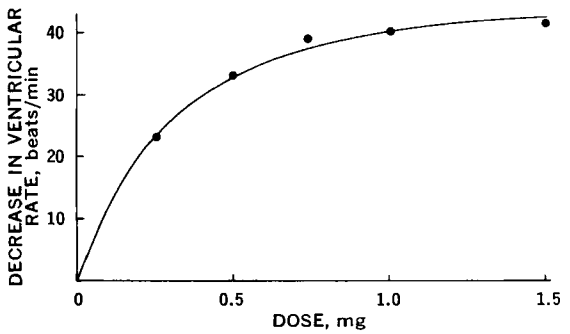


Figure 2—*Relationship of steady-state ventricular rate slowing to oral maintenance dosing of digoxin. Each patient was digitalized prior to a 4-week period of daily maintenance dosing. Each circle (●) denotes an average of four determinations taken weekly from 30 ambulatory patients with auricular fibrillation. Data were taken from Ref. 7.*

The hyperbolic shape of Fig. 2 is characteristic of what one might expect of a drug adhering to the tenets of occupation theory. Evidence for assuming occupation theory to describe drug-receptor interaction at biophasic levels correctly comes from the good least-squares nonlinear fit of the experimental dose-effect data, yielding an I_{max} value of 51.3 beats/min. From this value, the fraction of the maximum response attainable for the 1.2-mg intravenous and oral doses was calculated to be 0.906 and 0.633, respectively. Although these values are somewhat higher than anticipated for a drug with a low margin of safety such as digoxin, nausea and vomiting were experienced by 37.5% of those taking the 1.2-mg oral maintenance dose.

Values of f were calculated according to Eq. 3 following a single intravenous injection to 17 patients (7). Reciprocal weighting was used to provide a biexponential and a triexponential least-squares fit of the data to Eq. 5 as a function of time, yielding the following expressions with accompanying mean sum of squares (SS):

$$f = 2.842e^{-0.0144t} - 3.134e^{-1.200t} \quad \text{(Eq. 13)}$$

$$SS = 1.25 \times 10^{-2}$$

$$f = 1.708e^{-0.0086t} + 2.138e^{-0.073t} - 3.478e^{-0.500t} \quad \text{(Eq. 14)}$$

$$SS = 1.03 \times 10^{-2}$$

The regression line corresponding to the triexponential fit only slightly improved the fit as compared to the biexponential as judged by the similar SS values. A closer fit would normally be expected on the basis of more degrees of freedom associated with the triexponential case. Systematic deviation was not excessively apparent with either fit. According to Scheme I, the initial conditions of the system dictate that at time zero no drug has distributed to the biophasic compartment; therefore, a sum of the A_i's should equal zero. Initially, this constraint was added to the two-exponential case but a poor fit resulted. The added constraint to the three-exponential case produced equation parameters that gave identical absorption rate results as those obtained from the unconstrained biexponential fit.

Although the curve-fitting method followed by most investigators is to use the simplest model consistent with the experimental data, an attempt was made to constrain each case to a known criterion to choose between bi- and triexponential fits more confidently. It is reasoned that the cumulative amount of drug absorbed at all times following a rapid intravenous dose of drug should approximate the dose. In terms of Eq. 11 for the biexponential case and of Eq. 12 for the triexponential case, the fractional amount of drug remaining to be absorbed at all times should correspondingly approximate zero. The mean ($n = 24$) and the standard deviation of fractional amounts unabsorbed for the biexponential case, 0.030 ± 0.164, as compared to the widely varying values calculated for the triexponential case, 0.676 ± 28.8, indicated the biexponential case to approximate physiological con-

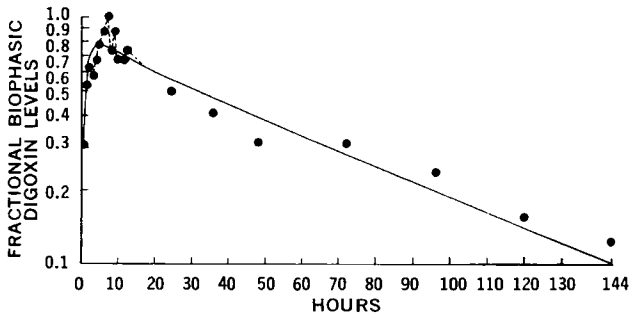


Figure 3—*Semilogarithmic plot of fractional amount of drug in biophase* versus *time after intravenous dosing of 1.2 mg of digoxin to 17 patients hospitalized with auricular fibrillation. Each point (●) represents an average calculated from ventricular rate slowing. The smooth line represents a least sum of squares, biexponential, computerized fit to the data taken from Ref. 7. Fluctuations about the theoretical line occurring at approximately* t_{max} *are suggestive of enterohepatic cycling.*

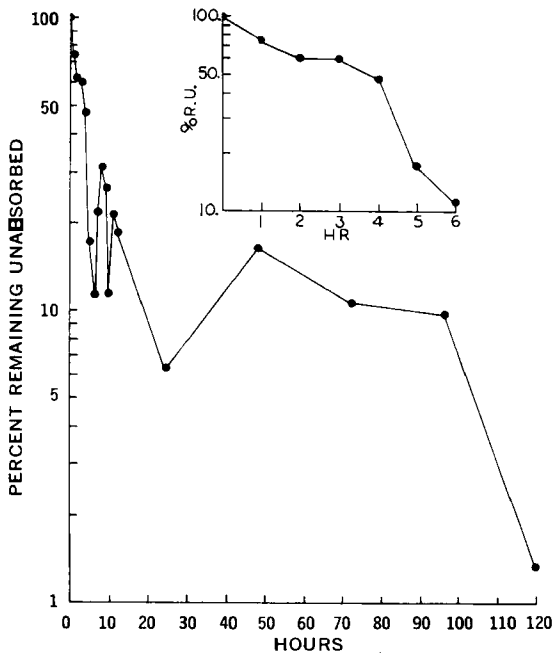


Figure 4—*Semilogarithmic plot of percent digoxin remaining unabsorbed following oral dosing of 1.2 mg to 17 patients hospitalized with auricular fibrillation. Each point (●) represents an average calculated for a two-compartment model utilizing ventricular rate slowing, taken from Ref. 7, as input data. The insert represents the same data but with an expanded time scale and shown over the first 6 hr only.*

ditions more closely. The unconstrained biexponential fit to the experimental data, denoted by Eq. 13, is shown in Fig. 3. The *f* values were normalized according to Eq. 6 and, as such, represent the fractional amount of digoxin present in the biophase with time.

By repeated administration of oral and intravenous doses, Gold *et al.* (7) found that an oral dose[4] 1.5 times the intravenous dose produced approximately the same maximum response intensity; however, Gold *et al.* (7) did not mention whether aqueous solutions, alcoholic solutions, or tablets were administered. From these oral dosing studies, it was concluded that the extent of absorption for an oral dose approximated 50–60%. From the same data and assuming first-pass effects to be negligible, 57.9% of the oral dose reached the biophase and elicited a response as calculated from the ratio of the total areas under the average *f versus* time curves for the oral and intravenous doses. Doherty *et al.* (13), on the other hand, reported values of 85% for the absolute (including recycling) absorption of digoxin based upon studies of tritiated digoxin serum levels as well as urine and stool excretion of patients with surgically induced biliary fistula. The drug was administered orally as an alcoholic solution. Wagner *et al.* (14) also found that the average absolute absorption was 80% of the dose calculated from areas under plasma–time curves when digoxin was administered orally in 5% dextrose solution, but when administered as commercially available tablets only 56.7 and 30.7% of the dose was absorbed. The extent of bioavailability, therefore, appears to be very dependent upon the choice of oral dosage form used as well as upon formulation factors varying within a dosage form.

The fractional amount of digoxin remaining to be absorbed following oral dosing was calculated according to Eq. 11 and plotted logarithmically *versus* time. Inspection of Fig. 4 reveals that absorption is 89% complete over the first 6 hr[5]. A log linear relationship would indicate simple first-order absorption; however, the insert shown in Fig. 4 indicates that this is not the case. From 6 through 24 hr, the absorption pattern is quite variable, fluctuating between 67.1% at 8 hr and 93.7% at 24 hr. Analysis of the absorption rate calculated from the cumulative absorption data illustrates the relatively rapid rate of absorption occurring over the first 5 hr (Fig. 5). The absorption rate becomes negative (net drug leaving body) at 6 hr and, with the exception of the 10-hr rate, continues negative until 12 hr. A positive absorption rate, although small, continues throughout the entire time course of 168 hr.

DISCUSSION

Significant absorption of digoxin appears to be occurring through 24 hr at progressively decreasing, but noticeably variable, rates until 120 hr when only 1.3 of the percent unabsorbed remains to be absorbed. This is evident from Figs. 4 and 5 and also from Fig. 1 in which a decrease in ventricular rate for the intravenous and oral curves returns to normal in a parallel fashion only after 24 hr following dosing. Although a peak response for the oral data is observed at 11 hr in Fig. 1, a general plateau effect is evident through 36 hr. The same trend was reported previously (14); following a slow intravenous infusion of digoxin, plasma levels maintained a plateau from about 2 to 7 hr.

One possible explanation is that absorption is occurring continuously along the various segments of the small intestine, duodenum, jejunum, and ileum but at decreasing rates as drug encounters less effective surface area per unit length while traversing the small intestine. Enterohepatic cycling could also account for the long duration associated with digoxin absorption and, along with variable gallbladder emptying time, could additionally explain the variable absorption rate observed between 5 and 12 hr for the oral dose. Oscillations occurring immediately before and after t_{max} in the intravenous and oral response–time curves of Fig. 1 and the corresponding Q_B–time curve of Fig. 3 for the intravenous data were observed between 1 and 12 hr. Fluctuations in data might have been detected over larger segments of the curves if more frequent determinations had been made beyond 12 hr. However, if the fluctuations in data were a result of the error associated with the determination of f/f_{max}, then the variation of experimental points in Fig. 1 and of the derived Q_B values for the intravenous data in Fig. 3 would be expected to be similar along the entire curve instead of occurring at approximately peak times. Moreover, the variations between subjects (expressed in Table I as coefficients of variation for peak and zero times) would indicate scattering about the theoretical curve to be less at t_{max} than at other times, gradually increasing as f/f_{max} returns to predosing values. Although no direct proof is available that significant intermittent recycling is occurring, the medical literature suggests the possibility. Similar trends can be observed in the plasma curves of digoxin reported (3) for patients taking 0.5 mg of digoxin 0.5 hr after food daily. Plasma curves for fasting subjects did not exhibit these fluctuations. It is known that the release of gallbladder contents occurs when food present in the stomach and intestine stimulates the release of GI hormones,

[4] Prepared from material supplied by Burroughs Wellcome & Co., Inc., as the pure form which served as the USP reference standard of digoxin.

[5] The 89% complete refers to the percentage of drug absorbed that will ultimately be absorbed.

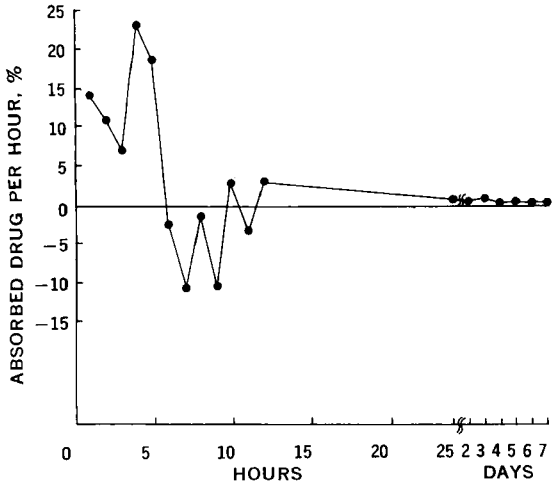
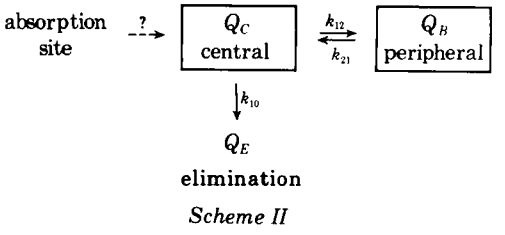


Figure 5—*Absorption rate of 1.2-mg oral dose of digoxin* versus *time calculated according to a two-compartment model. Each data point* (●) *calculated from ventricular rate slowing, taken from Ref. 7, represents a mean of 17 patients hospitalized with auricular fibrillation.*

which causes the coordinated contraction of the gallbladder and dilation of the sphincter of Oddi. Bile containing digoxin is subsequently released into the duodenum. Bile, although continuously secreted by the liver, does not continuously enter the duodenum but is stored along the common bile duct and in the gallbladder prior to relaxation of the sphincter of Oddi. The total evacuation period of the gallbladder varies from 15 min to several hours (15) but can be considered intermittent over the long disposition time of digoxin (16, 17).

According to Doherty *et al.* (13), a relatively small fraction of digoxin, 6.5%, is involved in enterohepatic cycling. This value was reported for human subjects with surgically induced biliary fistula. The bile was found to contain about 9% of the administered digoxin, which approximated the usual total stool excretion of parenteral digoxin. The serum half-life of these patients did not differ from those without biliary fistula, indicating to these authors that this factor is of minor importance to the drug's pharmacokinetic behavior. Half-life determinations, however, are calculated from widely spaced plasma concentrations which are approximately 4% or less of peak concentration levels. In view of the deviations observed at peak times for response, biophasic digoxin levels, and plasma levels of digoxin, it is conceivable that a small fraction of peak circulating concentrations of digoxin could be stored in the gallbladder in high enough concentrations such that its release and subsequent absorption would be in large enough quantities to account for the observed trends. Additionally, if the actual volume of the biophase is small in comparison to the circulating volume and if affinity of digoxin for biophasic receptors was great, the small fluctuations in plasma concentrations, particularly at peak times, could extrapolate to large changes in biophasic drug levels. Fluctuations in the normalized f values of Fig. 3 immediately before and after t_{max}, at 3 and 7 hr, deviated by 23.4 and 35.5%, respectively, from the computer-generated line of best fit for the intravenous dose. However, a compensating factor exists to nullify partially these large differences in terms of pharmacological response. Because of the hyperbolic shape of the dose–effect curve, large changes in biophasic drug levels translate into disproportionately smaller changes in response as higher biophasic drug levels are reached. This is exemplified by the pharmacological data; maximum response for the intravenous dose was estimated from the dose–effect curve to be 90.6% of the theoretical maximum. Within this range on the dose–effect curve, large deviations calculated for fractional biophasic drug levels represent a theoretical difference in response of 5.6 beats/min; experimentally, a difference of 4.5 beats/min was observed. It is likely that dose–effect curves representing various electromechanical events occurring in the heart and collectively referred to as systolic time intervals (18) would show greater sensitivity to digitalis intoxication than dose–effect curves constructed from ventricular rate slowing. This is indicated from a



Scheme II

study (19) in which systolic time intervals as well as heart rate were measured in newborn infants before and again at 4 and 8 hr after oral administration of digoxin. Varying degrees of statistically significant differences were found for all measurements after dosing as compared to predosing values except for heart rate; however, a relatively small pediatric dose (30 μg/kg) was used. In addition, the maximal inotropic effect, as measured by preejection changes, occurred with smaller doses of digoxin. If toxicity was apparent along lower segments of the dose–effect curve, smaller changes in fractional biophasic drug levels would translate into larger changes in response.

From the observed trends in the response data, it is apparent that the significance of enterohepatic cycling as it relates to the kinetics of pharmacolological effect warrants further study.

APPENDIX

The derivation of A_t for an open two-compartment system (Scheme II) as a function of time is based on a material balance which accounts for drug absorbed at all times. For this particular system, material balance dictates that A_t at any time is the sum of the quantities of drug present in the biophase, Q_B (a component of the peripheral compartment), central compartment, Q_C, and that which has been eliminated, Q_E:

$$A_t = Q_B + Q_C + Q_E \qquad \text{(Eq. A1)}$$

Equation A1 can be written in differential form:

$$dA_t = dQ_B + dQ_C + dQ_E \qquad \text{(Eq. A2)}$$

By referring to Scheme II, the following relationships can be derived:

$$\frac{dQ_B}{dt} = k_{12}Q_C - k_{21}Q_B \qquad \text{(Eq. A3)}$$

$$\frac{dQ_E}{dt} = k_{10}Q_C \qquad \text{(Eq. A4)}$$

Equation A5 is obtained by solving Eq. A3 for Q_C, differentiating with respect to time, and expressing in terms of dQ_C:

$$dQ_C = \left(\frac{1}{k_{12}}\frac{d^2Q_C}{dt^2} + \frac{k_{21}}{k_{12}}\frac{dQ_B}{dt}\right)dt \qquad \text{(Eq. A5)}$$

Solving Eq. A3 for Q_C, substituting into Eq. A4, and simplifying yield:

$$dQ_E = \left(\frac{k_{10}}{k_{12}}\frac{dQ_B}{dt} + \frac{k_{10}k_{21}}{k_{12}}Q_B\right)dt \qquad \text{(Eq. A6)}$$

Substituting Eqs. A5 and A6 for the appropriate terms in Eq. A2 and simplifying give the expression:

$$dA_t = \frac{1}{k_{12}}\left(\frac{d^2Q_B}{dt^2} + (k_{12} + k_{21} + k_{10})\frac{dQ_B}{dt} + k_{21}k_{10}Q_B\right)dt \qquad \text{(Eq. A7)}$$

Upon integration between limits 0 and t, Eq. A7 becomes:

$$A_t = \frac{1}{k_{12}}\left(\frac{dQ_B}{dt} + (k_{12} + k_{21} + k_{10})Q_B + k_{21}k_{10}\int_0^t Q_B\,dt\right) \qquad \text{(Eq. A8)}$$

Equation A8 is Eq. 7 in the *Theoretical* section. In the limit when

$t = \infty$, Eq. A8 reduces to Eq. A9:

$$A_\infty = \frac{k_{21}k_{10}}{k_{12}}\int_0^{t_\infty} Q_B\,dt \qquad \text{(Eq. A9)}$$

By dividing Eq. A8 by Eq. A9 and canceling like terms, an expression results which permits the evaluation of the fractional amount of drug ultimately absorbed, A_t/A_∞, at any time:

$$\frac{A_t}{A_\infty} = \frac{\frac{dQ_B}{dt} + (k_{12} + k_{21} + k_{10})Q_B + k_{21}k_{10}\int_0^t Q_B\,dt}{k_{21}k_{10}\int_0^t Q_B\,dt} \qquad \text{(Eq. A10)}$$

The validity of Eq. A10 is upheld providing drug first enters the central systemic compartment before being distributed to the biophasic compartment. It is also required that the differential equation written for Q_B does not contain a term representing the absorption process. The rate constants in Eq. A10 can be written in terms of the m_i's shown in Eqs. 9 and 10 such that:

$$\frac{A_t}{A_\infty} = \frac{\frac{dQ_B}{dt} + (m_1 + m_2)Q_B + m_1m_2\int_0^t Q_B\,dt}{m_1m_2\int_0^{t_\infty} Q_B\,dt} \qquad \text{(Eq. A11)}$$

Equation A11 is the same as Eq. 8. In a similar manner, absorption equations can be derived for two-compartment open models in which either $k_{10} = 0$ or $k_{10} \neq k_{20} \neq 0$. The relationships between the m_i's and k_{ij}'s are not altered, and each equation becomes identical in form to Eq. A11; only the numerical values of the m_i's vary. Therefore, Eq. A11 corresponds to a generalized two-compartment model in which the existence of a specified elimination rate constant need not be established. A general equation for A_t representing a three-compartment model can be similarly derived in which elimination also need not be assigned as a particular compartment; only the m_i's are required (20, 21).

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Assay Procedures for Thioridazine, Trifluoperazine, and Their Sulfoxides and Determination of Urinary Excretion of These Compounds in Mental Patients

NORMAN R. WEST*, MARCUS P. ROSENBLUM‡, HERBERT SPRINCE‡, SIMON GOLD‡, DIETHELM H. BOEHME§, and WOLFGANG H. VOGEL*x

Abstract □ Assay procedures for thioridazine, trifluoperazine, and their sulfoxides are described which are rapid, simple, and relatively specific (about 0.5 μg of drug or sulfoxide/ml). Chlorpromazine interferes with the determination of these drugs whereas imipramine, desipramine, biperidin, and haloperidol do not. The urinary concentrations of these four compounds were determined in 24-hr samples and timed-interval collections obtained from patients with schizophrenic reactions. Thioridazine and trifluoperazine seem to be extensively metabolized, and less than 1% of the unchanged drugs and 2–6% of the corresponding sulfoxides were observed in 24-hr urine samples. The ratio between the parent drug and its respective sulfoxide was fairly constant. Urinary excretions of thioridazine and its sulfoxide were dose independent, whereas those of trifluoperazine and its sulfoxide showed a strong dose dependency. Timed-interval collections during an 8-hr period showed rather consistent excretions of unchanged trifluoperazine and thioridazine but wide intra- and interpatient variations in the sulfoxide excretions.

Keyphrases □ Thioridazine, trifluoperazine, and their sulfoxides —spectrophotofluorometric analysis in patient urine, interference of chlorpromazine, imipramine, desipramine, biperidin, and haloperidol considered □ Trifluoperazine, thioridazine, and their sulfoxides—spectrophotofluorometric analysis in patient urine, interference of chlorpromazine, imipramine, desipramine, biperidin, and haloperidol considered □ Spectrophotofluorometry—analysis, thioridazine, trifluoperazine, and their sulfoxides in patient urine

Phenothiazines such as chlorpromazine, thioridazine (I), and trifluoperazine (III) find wide clinical use, but most pharmacological studies on the fate of phenothiazines in humans have been limited to chlorpromazine (1–3). A possible reason is the lack of rapid and simple, yet relatively sensitive and specific, methods for measuring trifluoperazine, thioridazine, and some of their metabolites. Therefore, assay procedures were developed for trifluoperazine, thioridazine, and their sulfoxides, and the 24-hr and timed-interval urinary excretion of these drugs and metabolites from mental patients was determined.

EXPERIMENTAL

Materials—Standards of chlorpromazine[1], chlorpromazine sulfoxide[1], trifluoperazine[1], and thioridazine[2] and its sulfoxide[2] were received from commercial sources. Trifluoperazine sulfoxide was synthesized according to the method of Huang and Bhansali (4) and found to be identical to trifluoperazine sulfoxide obtained from these investigators. All reagents used were of the highest purity commercially available.

Patients—Urinary excretion studies were performed with male schizophrenic patients who had been hospitalized for at least 1 year at the Veteran's Administration Hospital in Coatesville, Pa. All urines were collected, stored at 4°, and frozen after the collection. Assays were performed within 4 weeks.

[1] Smith Kline and French.

[2] Sandoz Pharmaceuticals.

Table I—Fluorescence of Thioridazine (I), Thioridazine Sulfoxide (II), Trifluoperazine (III), and Trifluoperazine Sulfoxide (IV) as a Function of Concentration of the Compound during the Fluorometric Determinations[a]

Amount Added, μg	Fluorescence, Relative Units	
I and II	I	II
0	10 ± 5	15 ± 5
0.5	35 ± 5	40 ± 5
1.0	60 ± 10	80 ± 15
10.0	600 ± 100	750 ± 110
50.0	3200 ± 450	4000 ± 590
III and IV	III	IV
0	30 ± 10	10 ± 5
0.1	45 ± 15	20 ± 5
0.5	90 ± 15	60 ± 10
1.0	120 ± 25	105 ± 20
10.0	1000 ± 75	950 ± 100
50.0	5500 ± 600	5000 ± 450

[a] Values represent the mean ± standard deviations of at least three determinations.

Determination of Chlorpromazine and Its Sulfoxide—Chlorpromazine and its sulfoxide were determined according to the method of Salzman and Brodie (5), and creatinine values were obtained by routine clinical laboratory procedures.

Determination of Thioridazine (I) and Its Sulfoxide (II)—A 10-ml sample of urine was adjusted to pH 12 by the addition of 10 *N* NaOH and extracted with 25 ml of heptane containing 1.5% isoamyl alcohol. After shaking for 5 min and centrifugation for 5 min, 22.5 ml of the heptane phase was transferred to a second tube containing 2.0 ml of 1 *M* acetate buffer (pH 5.6), shaken, and centrifuged. The buffer contained II. Twenty-one milliliters of the heptane phase was washed with 2.0 ml of the same acetate buffer. After shaking and centrifugation, 20 ml of heptane was extracted with 2.0 ml of 0.1 *N* HCl; the acid fraction contained I. For quantitation of both compounds, 0.15 ml of 10 *N* H_2SO_4 was added to 1.5 ml of the first buffer and 1.5 ml of the acid fraction was combined with 0.02 ml of 10 *N* H_2SO_4. Both samples received 0.1 ml of 0.1% potassium permanganate and were then mixed and allowed to stand for 5 min at room temperature. The reactions were stopped by the addition of 0.1 ml of 0.6% hydrogen peroxide. The samples were read within 30 min on a spectrophotofluorometer[3] using excitation and emission wavelengths of 350 and 440 nm, respectively. Urine from individuals who did not receive drug treatment served as "blank" specimens; after addition of a specified amount of thioridazine or thioridazine sulfoxide, they served as "internal" standards.

The recovery by this method was approximately 80% (70–85%) for both compounds, and standard curves of extracted thioridazine and thioridazine sulfoxide were linear from 0.5 to 50 μg (Table I).

Separation and specificity of the methods were checked as follows. Addition of thioridazine (20 μg) and thioridazine sulfoxide (20 μg) to control urine resulted in complete separation of both compounds by the extraction method described. Fluorescent spectra of extracts from control urines with added thioridazine or thioridazine sulfoxide were identical to those seen with the corresponding fractions from urines of patients receiving thioridazine.

[3] Aminco-Bowman.

Table II—Concentrations of Chlorpromazine (V), Thioridazine (I), Trifluoperazine (III), and Their Sulfoxides (II and IV) in 24-hr Urine Samples Obtained from Schizophrenic Patients

Compound	Patient	Dose, mg	Volume, ml[a]	Drug		Sulfoxide		Ratio[c]
				μg/24 hr	%[b]	μg/24 hr	%[b]	
V	B	100	1520	486	0.4	2,736	2.7	5.6
	M	500	1310	4323	0.8	12,838	2.6	3.0
	H	600	3220	1030	0.2	3,542	0.6	3.4
	D	800	830	2822	0.4	7,636	1.0	2.7
I	T	100	1305	76	0.1	1,005	1.0	13.0
	F	100	1330	1205	1.2	12,600	12.6	10.5
	R	200	1110	1232	0.6	9,350	4.7	7.6
	S	400	1010	1120	0.2	9,560	2.4	8.6
	M	400	1465	1465	0.4	9,950	2.5	6.8
	W	600	470	1280	0.2	1,805	0.3	1.4
	B	600	1550	1220	0.2	10,200	1.7	8.4
	S	600	1060	1520	0.3	15,700	2.6	11.2
III	H	5	825	12[d]	0.3	84[d]	1.7	6.8
	R	5	1670	16	0.3	215	4.3	12.9
	H	10	1240	34	0.4	127	1.3	3.6
	D	10	3980	83	0.8	374	3.7	4.5
	J	15	2375	135	1.0	505	3.4	3.8
	F	20	780	100	0.5	660	3.3	6.6
	B	30	1045	196	0.6	1,870	6.2	9.5
	M	40	1025	205	0.5	1,670	4.2	8.2
	L	50	4000	300	0.6	1,970	3.9	6.6

[a] Urine volume of 24-hr collection. [b] Percent of total dose. [c] Ratio of sulfoxide to unchanged drug. [d] Excretion of III and IV was found to be significantly dose dependent using a Student t test ($p < 0.001$).

Urine extracts from patients receiving thioridazine were subjected to TLC on 250 μm silica gel G in two solvent systems [acetic acid–ethanol–water (30:50:20) or methanol–25% ammonia (95:5)]. Methods of visualization were fluorescence under 253- and 320-nm UV light and color reactions with sprays of iodoplatinate, 2% ferric chloride, or 50% sulfuric acid in ethanol (4:1) (2, 6). The thioridazine fraction contained only one spot corresponding to standard thioridazine. The thioridazine sulfoxide fraction contained three spots; the major spot corresponded to standard thioridazine sulfoxide whereas the two minor spots fluoresced at different wavelengths and did not react with the sprays. Thus, it can be concluded that the thioridazine and thioridazine sulfoxide fractions were essentially free of interfering compounds. Nevertheless, the metabolism of phenothiazines is extensive; it is possible that small quantities of minor metabolites might still be present, but they should not significantly interfere with the determination of thioridazine or thioridazine sulfoxide.

The addition of various drugs (amounts equal to thioridazine) frequently used in combination with thioridazine medication showed that chlorpromazine and trifluoperazine interfered with the determination of thioridazine and thioridazine sulfoxide, whereas imipramine, desipramine, biperidin, and haloperidol did not. Thioridazine and thioridazine sulfoxide were stable when added to normal urine, left at room temperature for up to 24 hr, frozen, and stored for at least 4 weeks.

Determination of Trifluoperazine (III) and Its Sulfoxide (IV)—A 10-ml sample of urine was adjusted with 10 N NaOH to pH 12 and extracted with 25 ml of heptane containing 1.5% isoamyl alcohol. The mixture was shaken for 5 min and then centrifuged for 5 min. Twenty-two milliliters of the heptane was transferred to a tube containing 1.5 ml of 1 M acetate buffer, pH 5.6. After shaking and centrifuging, the buffer phase contained the trifluoperazine sulfoxide and was subjected to fluorescence analysis as described later. Twenty milliliters of the remaining heptane was extracted with 1 ml of 0.1 N HCl. After shaking and centrifuging, 0.8 ml of the hydrochloric acid phase containing trifluoperazine was combined with 0.6 ml of 1 M sodium acetate and 0.05 ml of 0.1% potassium permanganate at room temperature. After mixing, the oxidation was stopped after 30 sec with 0.05 ml of 0.6% hydrogen peroxide. Both samples were read within 30 min on a spectrophotofluorometer[3] using excitation and emission wavelengths of 350 and 405 nm, respectively. Again, urines from drug-free individuals served as blanks or, after addition of trifluoperazine or trifluoperazine sulfoxide, as internal standards. The recovery of the method is approximately 75% (68–80%), and standard curves of extracted drug and its sulfoxide were linear from 0.5 to 50 μg (Table I).

Separation and specificity of the assay procedure were checked as follows. Addition of trifluoperazine (10 μg) and its sulfoxide (10 μg) to normal urine was quantitatively separated by the extraction procedure. Spectral scans of both fractions obtained from urines of patients receiving trifluoperazine were identical to those obtained from blank urines to which either trifluoperazine or trifluoperazine sulfoxide had been added. TLC of the trifluoperazine fraction obtained from the urine of patients receiving the drug and standard trifluoperazine on 250-μm silica gel G plates [acetic acid–ethanol–water (30:50:20) or methanol–25% ammonia (95:5)] produced identical results. The trifluoperazine sulfoxide fraction showed three fluorescent spots, one major spot corresponding to trifluoperazine sulfoxide and two very faint spots which fluoresced at the same wavelength (less than 10% of the total fluorescence after elution from the plate) but did not respond to iodoplatinate spray, 2% ferric chloride, or 50% sulfuric acid in ethanol

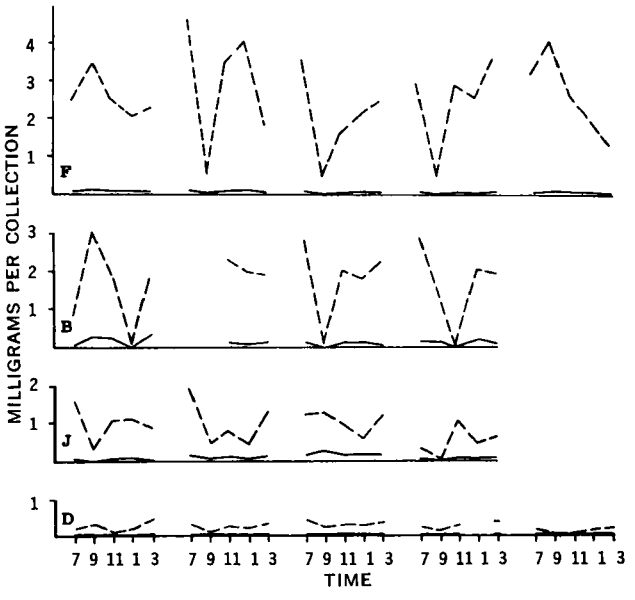

Figure 1—*Timed-interval urinary excretion of thioridazine (—) and its sulfoxide (- - -) in patients taking 100 mg (D), 600 mg (J and B), and 800 mg (F) of thioridazine daily. The abscissa indicates the time of day collection periods ended.*

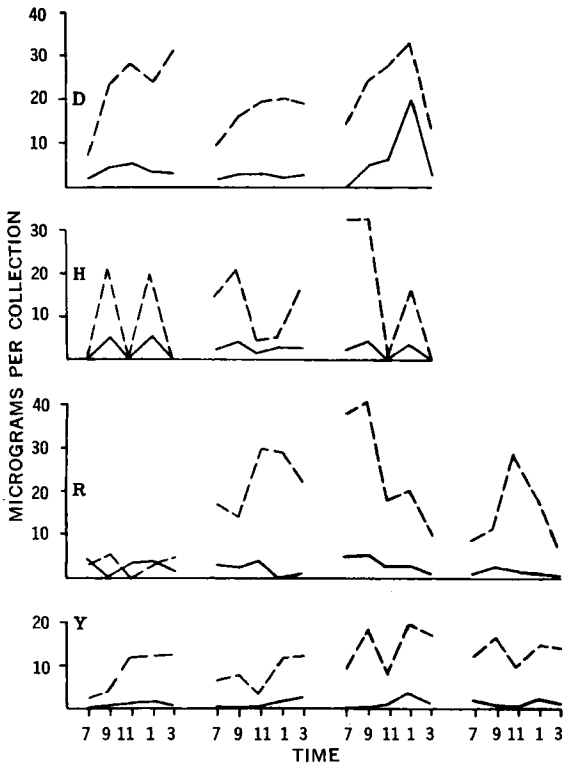

Figure 2—*Timed-interval urinary excretion of trifluoperazine (—) and its sulfoxide (- - -) in patients taking 5 mg (Y and R) and 10 mg (H and D) of trifluoperazine daily. The abscissa indicates the time of day collection periods ended.*

(4:1) (8). Since the concentrations of trifluoperazine and its sulfoxide had been measured in rat plasma and brain with a sensitive and specific radioisotope procedure (9), these experiments were repeated using the extraction and determination procedure. Results obtained for both plasma and brain levels of trifluoperazine and trifluoperazine sulfoxide were in excellent agreement with reported values. Thus, the trifluoperazine fraction seems to be free of interfering compounds, whereas the trifluoperazine sulfoxide fraction might contain some interfering metabolites which, however, do not account for more than 10%. Again, trifluoperazine is probably extensively metabolized in the body, and the presence of minor amounts of other metabolites in both fractions is likely.

The addition of equal amounts of chlorpromazine and thioridazine interfered strongly with the determination of trifluoperazine, whereas imipramine, desipramine, biperidin, and haloperidol did not. Again, trifluoperazine and its sulfoxide remained unchanged when added to normal urine, kept at room temperature for 24 hr, frozen, and stored for at least 4 weeks.

RESULTS AND DISCUSSION

Analytical Procedures—Methods based on published analytical procedures (5, 7, 8) were developed for the determination of thioridazine, trifluoperazine, and their sulfoxides. The objective was to find relatively specific, rapid, and easy methods, so that they could be performed in the average clinical laboratory. The developed methods do fulfill these criteria and are indeed quite specific for the particular drug and its sulfoxide; impurities resulting from the extensive metabolism of phenothiazines are either absent or present in negligible concentrations, except in the case of trifluoperazine sulfoxide fraction which contained an impurity accounting for 5–10% of the reading. The procedures are easy and rapid, and a laboratory technician can readily determine 20 samples in 4 hr. Preliminary results indicate that the assay procedures can also be used to determine the drugs and their sulfoxides in plasma or biological tissues.

Twenty-Four Hour Urine Concentrations of Thioridazine, Trifluoperazine, and Their Sulfoxides—The patients studied were males who had been taking either thioridazine or trifluoperazine for at least 4 months. For comparison, a few patients taking chlorpromazine were also included. Collections were made under the supervision of the particular ward physician, and several urine samples were assayed for creatinine to ensure proper 24-hr specimens; values obtained for creatinine were as expected for 24-hr specimens.

Table II shows the results of the 24-hr collection. As reported previously (2), unchanged chlorpromazine was found only in small, dose-unrelated quantities in the urine and the sulfoxide accounted for 1–3% of the total dose. The ratio of both compounds in this study was fairly constant, ranging between 2.7 and 5.6.

Thioridazine also was metabolized extensively, and a similar small, dose-unrelated quantity was found in the urine. The sulfoxide accounted for approximately 1–5% of the dose, with one patient excreting 12%. The ratio of the sulfoxide and thioridazine was fairly constant and, with one exception, ranged between 7 and 13.

Unchanged trifluoperazine accounted for less than 1% of the dose, indicating extensive metabolism of the drug. The sulfoxide was 1–6% of the total dose, and the ratio of sulfoxide to drug was between 4 and 13. In contrast to the other two drugs, the excretion of this drug and its sulfoxide was strongly dose dependent.

Timed-Interval Urine Concentrations of Thioridazine, Trifluoperazine, and Their Sulfoxides—The patients studied were males who had been taking thioridazine or trifluoperazine for at least 4 months. Urine was collected in the morning (7:00 am), and then the medication was given by mouth and urine was collected until 9:00 am and from 9:00 to 11:00 am, 11:00 am to 1:00 pm, and 1:00 to 3:00 pm. Another dose of the medication was given in the evening.

The timed-interval urinary excretion of thioridazine of four patients proved to be rather consistent and constant (Fig. 1). The excretion of its sulfoxide varied among patients and within the same patient on different days; it was rather uniform in Patient D but varied widely in each of the other three patients. The ratios between sulfoxide and unchanged drug were markedly higher than those found for 24-hr samples. In the 8-hr collection period, the patients excreted about 30% of I and about 75% of II of the expected daily excretion of the morning dose. No correlation between urine volume and drug excretion was noted.

The timed-interval urinary excretion of unchanged trifluoperazine was rather constant among the four patients studied and on different days within one particular patient, whereas the sulfoxide excretion showed wide inter- and intrapatient variations (Fig. 2). During the collection periods, the patients excreted 30–50% of the expected 24-hr amounts of both the parent compound and its sulfoxide. No correlation between drug excretion and urine volume was noted.

The methods and the urinary excretion data reported, particularly for trifluoperazine excretion which is dose dependent, should be useful in screening patient populations as to the proper intake of the prescribed doses and in studying possible correlations between the fate of thioridazine or trifluoperazine and the clinical response of the patient.

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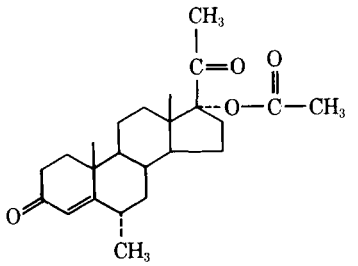
Abstract □ To study the absorption, metabolism, and excretion of medroxyprogesterone acetate in animals and man, a method was developed for measurement of the intact drug in plasma based on: *(a)* cyclohexane extraction of the specimen, *(b)* formation of the 3-enol heptafluorobutyrate ester, and *(c)* quantification *via* GLC using an electron-capture detector. The assay is quantitative above 1 ng medroxyprogesterone acetate/ml of plasma, and overall precision is approximately ±10% *(SD)* in the range of 5–20 ng/ml. The procedure was successfully applied to absorption studies in dogs after intramuscular drug administration. Plasma drug concentrations, as measured by radioimmunoassay, were five to 10 times greater as compared to the GLC assay, indicating that drug-related materials in addition to intact medroxyprogesterone acetate were in peripheral circulation.

Keyphrases □ Medroxyprogesterone acetate—GLC analysis in plasma, compared to radioimmunoassay technique, absorption in dogs after intramuscular administration □ GLC—analysis, medroxyprogesterone acetate in plasma, compared to radioimmunoassay technique

Medroxyprogesterone acetate[1] (17α-acetoxy-6α-methylprogesterone, I), a synthetic progestogen (1, 2), is an effective agent for the control of fertility (3) and for the treatment of malignancies. Investigations in animals (4, 5) and humans (6–8) indicated that, after subcutaneous or intramuscular drug administration, I and drug-related materials were slowly absorbed from the injection site, resulting in prolonged activity.

To study the absorption, metabolism, and excretion of I in animals and man as well as to aid in determining the specificity of the radioimmunoassay (7) for measurement of I, a simple, sensitive, and specific GLC method of analysis was developed for the intact drug in plasma.



I

EXPERIMENTAL

Reagents and Materials—Compound I and the methyl ester of 3β-hydroxy-5-cholenic acid[2] (II), used as internal standard, were synthesized. Acetone, benzene, and cyclohexane, distilled in glass, were used as supplied[3]. Spectral grade acetonitrile[4] and heptafluorobutyric anhydride (1-ml ampuls)[5] were used without further purification. Aqueous stock solutions of I (10 μg/ml) were prepared by dissolving 1 mg of drug in 1 ml of acetone. The solution was diluted to a final volume of 100 ml with distilled water. Aliquots were diluted with distilled water to final concentrations of 12.5, 25, 50, and 100 ng/ml. The stock solution of II (10 μg/ml) was prepared in 50% (v/v) acetonitrile in acetone. All stock solutions were stored in glass containers. Silicone gum rubber (OV-17) on 80–100-mesh Gas Chrom Q (10% w/w) was used as supplied[6].

Instrumentation—A two-speed reciprocating shaker[7] was used for shaking the samples in the horizontal position. A mixer[8] was used to aid in preparing the heptafluorobutyrate esters. GLC measurements were made with a gas chromatograph[9] equipped with a hydrogen flame-ionization detector, a nickel-63 electron-capture detector, and a −0.2 to 1.0-mv recorder[10]. All cylinders of gases for chromatography (*i.e.*, helium, hydrogen, oxygen, and 10% methane in argon) were fitted with filters containing molecular sieve 4A.

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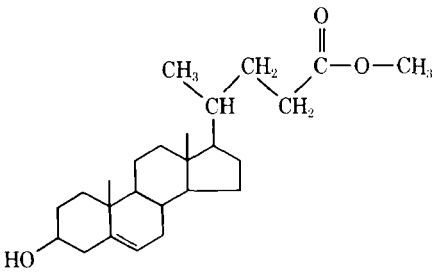

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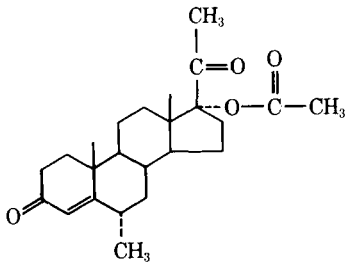

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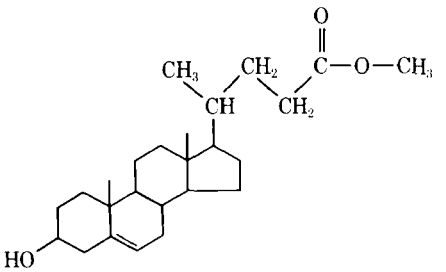

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Table I—Effect of pH on the Extraction of Medroxyprogesterone Acetate-7-^{3}H from Water and Dog Plasma Using Cyclohexane as the Solvent for Extraction[a]

Matrix	Solution pH	Amount Added[b], ng	Amount Found, ng	Recovery, %
Water	1.5	200.0	195.8	97.9
	7.2	200.0	185.0	92.5
	10.0	200.0	204.0	102.0
Plasma	1.5	200.0	179.8	89.9
	7.2	200.0	190.6	95.3
	10.0	200.0	185.6	92.8

[a] Ratio of organic solvent to biological specimen was 2:1 (v/v). [b] Total amount of medroxyprogesterone acetate-7-^{3}H added to 5-ml sample (equivalent to 40 ng/ml).

prepared columns were preconditioned at 275° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and electron-capture detector were maintained isothermally at 250, 270, and 310°, respectively. Flow rates of 10% methane in argon, used as carrier and purge gas, were maintained at 60 ml/min. The pulse interval for the electron-capture detector was 150 μsec. Under these conditions, the heptafluorobutyrate esters of I and II had retention times of 9.6 and 13.6 min, respectively (Fig. 1).

In ancillary studies, using the flame-ionization detector, chromatography was conducted on a U-shaped glass column [0.61-m (2-ft) × 3-mm i.d.] of 1% (w/w) OV-17 on 80–100-mesh Gas Chrom Q. The column, injection port, and detector block were maintained isothermally at 240, 245, and 245°, respectively. Helium, hydrogen, and oxygen flow rates were 60, 40, and 400 ml/min, respectively. The retention times for I-heptafluorobutyrate ester, 5α-cholestan-3-one (utilized as GLC standard), and I were 1.6, 3.4, and 5.4 min, respectively (Fig. 2).

Synthesis of Standard Materials—Standard materials were prepared for identification by dissolving 200 mg of I or II in 5 ml

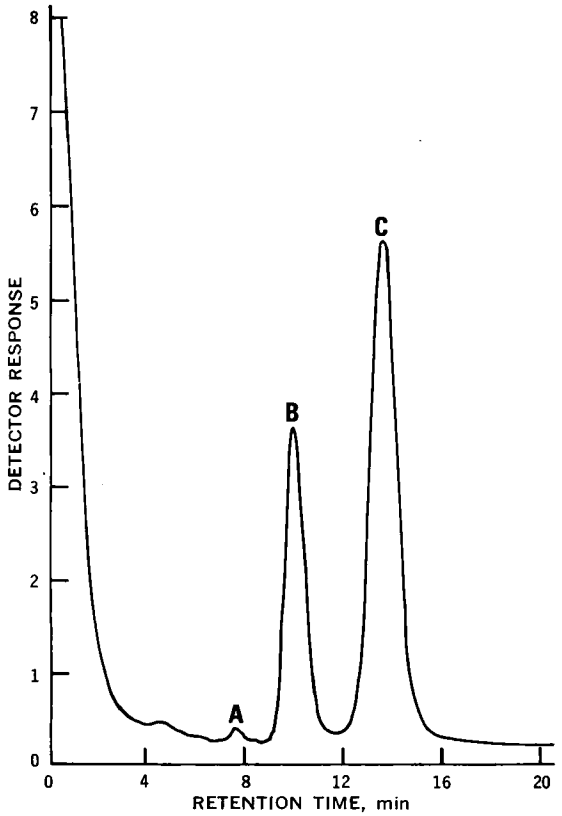

Figure 1—*Gas–liquid chromatogram (electron-capture detector) of medroxyprogesterone acetate and internal standard heptafluorobutyrates. Key: A, III-heptafluorobutyrate; B, I-heptafluorobutyrate; and C, II-heptafluorobutyrate.*

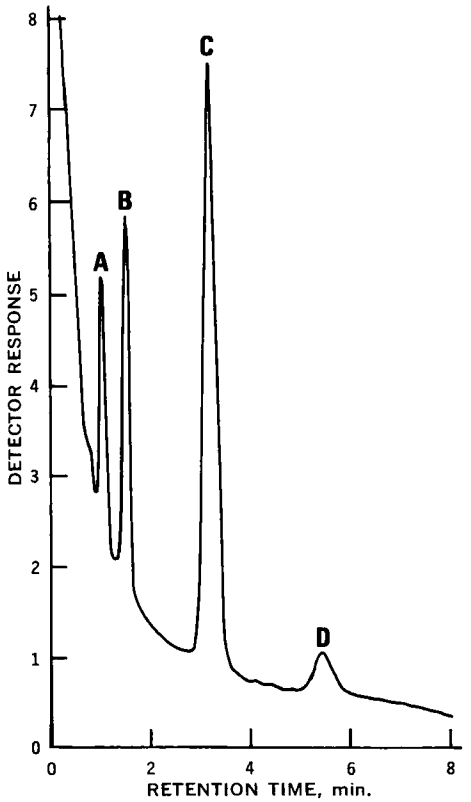

Figure 2—*Gas–liquid chromatogram (flame-ionization detector) of medroxyprogesterone acetate-3-enol-heptafluorobutyrate reaction mixture (reaction temperature = 90°, and reaction solvent = benzene). Key: A, III-heptafluorobutyrate; B, I-heptafluorobutyrate; C, 5α-cholestan-3-one; and D, I.*

50% (v/v) acetonitrile in acetone. Heptafluorobutyric anhydride (1 ml) was added, and the mixture was allowed to react at 25° for 1 hr. The reaction mixture was evaporated to dryness under a gentle stream of nitrogen gas. The I residue was reconstituted in 5 ml ether and washed with 10 ml water. The resulting material was filtered, dried, and crystallized from acetone–commercial hexane[11]. The residue from reaction of II was dissolved in ether, washed with water, and decolorized with activated charcoal. The resulting material was filtered, dried, and crystallized from methanol (9).

Assay Procedure—*Preparation of Plasma Standards*—Pipet 1.0-ml aliquots of the I aqueous stock solutions equivalent to 12.5, 25, 50, and 100 ng into glass-stoppered centrifuge tubes. Add 4.0 ml of control plasma to each centrifuge tube and mix well with the mixer. Prepare an appropriate blank. Extract all standards in the same manner as described below for the plasma specimens.

Preparation of Samples—Place 5.0 ml of plasma in a glass-stoppered centrifuge tube. For plasma specimens less than 5 ml, dilute to a final volume of 5.0 ml with distilled water. Add 10.0 ml cyclohexane and shake in the horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm. Transfer a 9.0-ml aliquot of the cyclohexane layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness under a gentle stream of nitrogen gas. Wash down the walls of the centrifuge tube with 0.5 ml cyclohexane and evaporate to dryness with nitrogen. Add 0.2 ml of the II stock solution and 50 μl heptafluorobutyric anhydride. Mix thoroughly and allow to react at 25° for 60 min. Evaporate to dryness with a gentle stream of nitrogen gas. Reconstitute the residue in 50 μl benzene. Inject a 1-μl aliquot of the solution for analysis into the chromatograph.

Calculations—The peak heights for the heptafluorobutyrate esters of I and II are measured. Peak height ratios are obtained by dividing the peak height of I-heptafluorobutyrate by the peak height of II-heptafluorobutyrate. Calibration curves for known concentrations of I in plasma are prepared by plotting peak

[11] Skellysolve B.

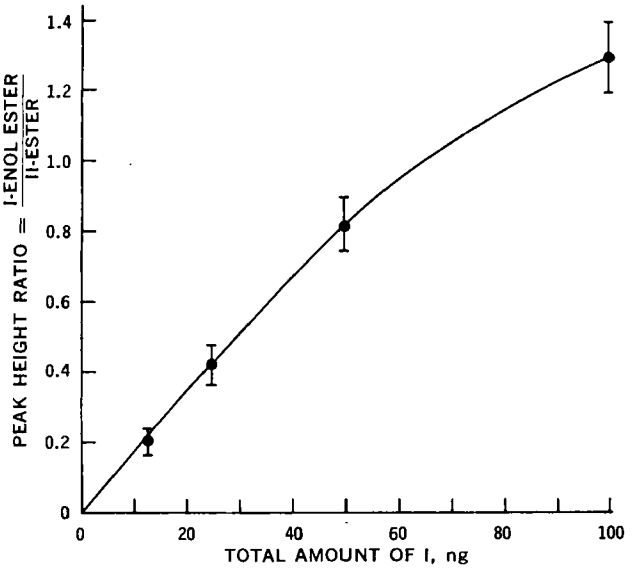

Figure 3—*Calibration curve; relationship between ratio of peak height of medroxyprogesterone acetate-3-enol-heptafluorobutyrate/internal standard heptafluorobutyrate and amount of medroxyprogesterone acetate in plasma. Each point represents the mean* ± SD *for eight determinations.*

height ratios *versus* total free I in the standard, expressed as nanograms. Values for unknown concentrations of I in plasma specimens, calculated in the same manner, are then read directly from the nonlinear standard curve (Fig. 3). The total I is adjusted for the initial sample volume and expressed as nanograms I per milliliter plasma.

Preparation of Animals—In studies designed to determine the utility of the analytical methodology, two female beagle dogs (E-102 and G-101) weighing 8.25 and 8.10 kg, respectively, received a single intramuscular dose of 75 mg I/kg body weight. The drug was administered as two separate 2-ml injections of an aqueous suspension (150 mg I/ml)[12] into the gluteus muscle of each animal. The animals were housed in holding cages with free access to food[13] (300 g/day) and water. All blood specimens (10 ml) were withdrawn in heparinized syringes from the jugular veins at preselected time intervals from 0 to 59 days postdrug administration. The plasma was harvested and stored at −18°. All plasma specimens were analyzed for I using the GLC assay.

In a second study, the aqueous suspension of I was administered (75 mg/kg im) to two female beagle dogs (G-403 and K-202) weighing 9.8 and 11.8 kg, respectively. An equivalent volume of physiological saline (~5 ml) was administered intramuscularly to a third female beagle dog (G-303) weighing 10.6 kg, which served as an untreated control. Blood specimens were withdrawn *(vide supra)* at predetermined time intervals from 0 to 108 days postdrug administration. All plasma specimens were analyzed using the GLC assay and the radioimmunoassay (7).

RESULTS AND DISCUSSION

Selection of Solvent for Extraction of Plasma Specimens—Studies designed to determine the extraction efficiency of known amounts of tritium-labeled I (specific activity, 32 μCi/μg)[14] from simple aqueous solution and dog plasma showed that the compound was quantitatively extracted with cyclohexane, ether, or methyl ethyl ketone. Cyclohexane was selected as the solvent of choice, since: *(a)* no volume changes were observed during the extraction of I from plasma or water, *(b)* it evaporated rapidly under a gentle stream of nitrogen gas, and *(c)* less endogenous background materials (*viz.*, cholesterol), as measured by GLC using an electron-capture detector, were extracted from the plasma specimens.

Table II—Recovery of Medroxyprogesterone Acetate from Dog Plasma

Added, ng	Found[a], ng	Recovery[b], %
5.0	4.57	91.4 ± 14.6
12.5	12.00	96.0 ± 12.1
25.0	23.55	94.2 ± 10.0
50.0	47.65	95.3 ± 7.4
75.0	74.02	98.7 ± 9.5
100.0	102.70	102.7 ± 5.8

[a] Means of five replicate samples. [b] Mean ± *SD*.

Selection of pH for Extraction of Plasma Specimens—A series of plasma and aqueous specimens (5 ml) containing known amounts of tritium-labeled I were adjusted to pH 1.5, 7.2, and 10.0 with 0.1 *N* aqueous sulfuric acid or sodium hydroxide. All samples were extracted with 10 ml cyclohexane. The results (Table I) showed that the pH of the samples had little, if any, effect on the extraction efficiency of I. GLC of the extract residues, after reaction with heptafluorobutyric anhydride, indicated that less endogenous materials were extracted from the plasma specimens at neutral pH (*i.e.*, 7.2–7.4) as compared to acidic or basic pH.

Selection of Internal Standard—Ideally, an internal standard should have physical and chemical properties similar to the compound to be analyzed. The two materials should be adequately separated from each other by GLC. During this study, 5α-cholestan-3-one and the monoheptafluorobutyrate derivatives of various steroids were investigated as potential internal standards. As the monoheptafluorobutyrate derivative, only II was adequately separated from I and the endogenous background materials present in plasma extracts. Since II was not efficiently extracted from plasma into cyclohexane, it was added to the extract residue prior to derivative formation. Consequently, II served only as a quantitative measure of heptafluorobutyrate derivative formation and the amount of material injected into the chromatograph.

Ester Formation with Heptafluorobutyric Anhydride—The reaction of steroid 4-en-3-ones with heptafluorobutyric anhydride, in an equal volume of benzene, was reported (10, 11) to produce nearly quantitative yields of the 3-enol heptafluorobutyrate es-

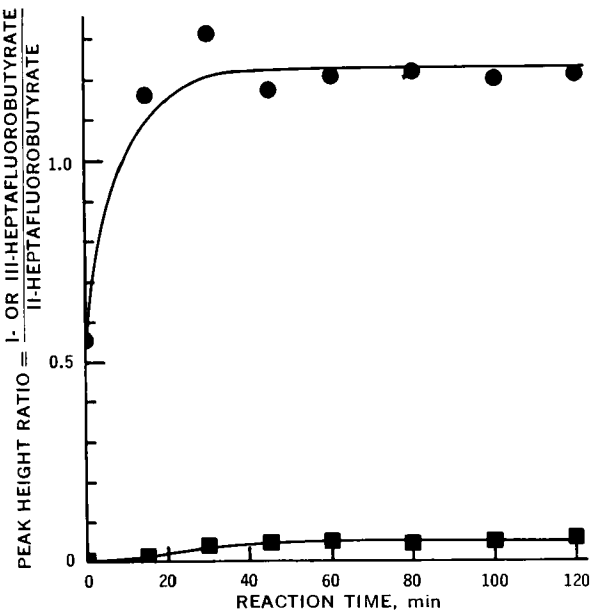

Figure 4—*Effect of reaction time on formation of medroxyprogesterone acetate-3-enol-heptafluorobutyrate [reaction temperature = 25°, and reaction solvent = 50% (v/v) acetone in acetonitrile]. Key:* ●—●, *I-heptafluorobutyrate; and* ■—■, *III-heptafluorobutyrate.*

[12] Depo-Provera, The Upjohn Co., is a sterile aqueous suspension of medroxyprogesterone acetate.
[13] Purina Lab Chow, Ralston Purina Co., St. Louis, Mo.
[14] Medroxyprogesterone acetate-7-^{3}H, prepared by Dr. R. C. Thomas, The Upjohn Co., Kalamazoo, Mich.

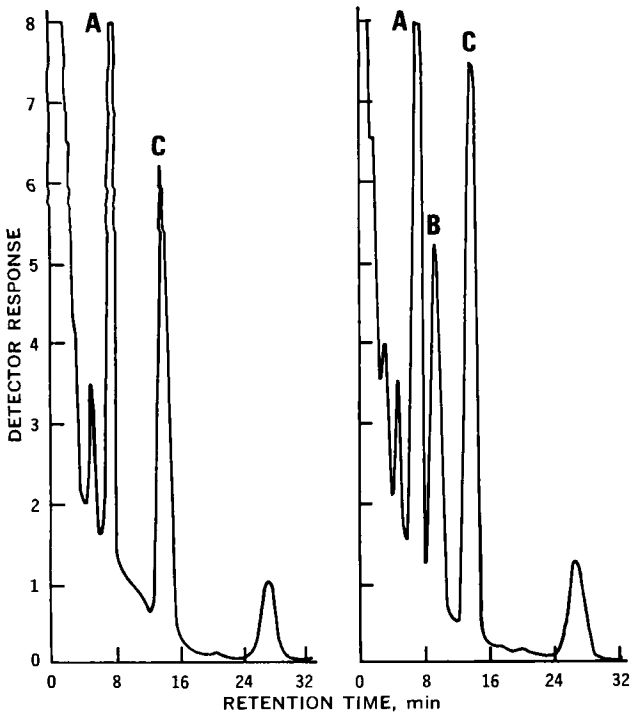

Figure 5—*Gas–liquid chromatogram (electron-capture detector) of dog plasma extracts. Left: normal plasma specimen. Right: plasma specimen from dog at 24 days after single-dose intramuscular administration of 75 mg medroxyprogesterone/kg body weight. Key: A, cholesterol heptafluorobutyrate; B, I-heptafluorobutyrate; and C, II-heptafluorobutyrate.*

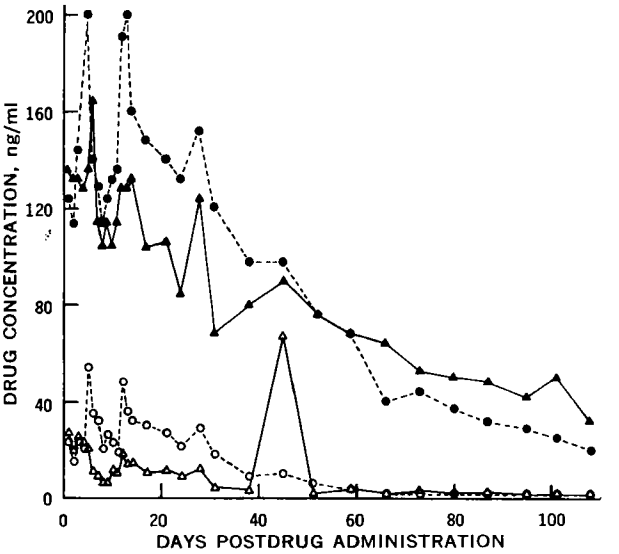

Figure 6—*Plasma drug concentrations* versus *time in dogs after single-dose intramuscular administration of 75 mg medroxyprogesterone acetate/kg body weight. Key: ▲—▲, Dog G-403, radioimmunoassay; △—△, Dog G-403, GLC; ●—●, Dog K-202, radioimmunoassay; and ○—○, Dog K-202, GLC.*

ters. Studies of the formation of I-heptafluorobutyrate *versus* time, using GLC with a flame-ionization detector and microgram amounts of I, indicated that at least two products were formed (Fig. 2). At 90°, the half-time for disappearance of I starting material (R_T = 5.4 min) from the reaction mixture was estimated to be less than 10 min. The I-enol-heptafluorobutyrate (R_T = 1.6 min) appeared very rapidly, reached a maximum concentration at about 1–2 hr, and then decreased with additional reaction time. An initial lag time was observed in the formation of the second reaction product (R_T = 1.2 min), designated III-heptafluorobutyrate. A maximum concentration was found at 3 hr, after which it decreased until termination of the experiment (*i.e.*, 6 hr). Results from studies conducted at reaction temperatures of 70 and 50° indicated that a single reaction product (as measured by GLC) could not be obtained.

Subsequent studies, using GLC in conjunction with mass spectrometry, confirmed that I-enol-heptafluorobutyrate was being chromatographed intact and provided evidence suggesting that the second reaction product (*viz.*, III-heptafluorobutyrate) was an isomer of I-enol-heptafluorobutyrate formed by migration of double bonds. Intact I-enol-heptafluorobutyrate and III-heptafluorobutyrate had identical molecular ions (*m/e* 582) and similar fragmentation patterns. The data provided no indication that diheptafluorobutyration or thermal cleavage (10, 12) at C-17 had taken place. These results were consistent with the studies described for heptafluorobutyration of progesterone (13) and testosterone-17β-acetate (14).

Tetrahydrofuran, methylene chloride, acetone, acetonitrile, and a mixture of 50% (v/v) acetonitrile in acetone were evaluated as potential solvents to replace benzene in the esterification. All reactions were conducted for 1 hr at 25°, using 4:1 ratio of organic solvent to heptafluorobutyric anhydride. Based upon *(a)* volatility of the solvent, *(b)* solubility of the internal standard, and *(c)* minimum formation of III-heptafluorobutyrate, 50% (v/v) acetonitrile in acetone was selected. Studies of the formation of I-heptafluorobutyrate *versus* time indicated that the reaction was completed within 60 min at 25° (Fig. 4). Less than 5% of III-heptafluorobutyrate was formed during the reaction, based upon peak area ratios. Similar studies with II showed that heptafluorobutyration was complete in less than 15 min. These results were consistent with the milder conditions required for esterification of free hydroxyl groups (15).

Synthesis of standard material indicated that the heptafluorobutyrate ester of I was a crystalline material (mp 138–140°) at room temperature. GLC (using a solid sample injector and flame-ionization detector) indicated that the material submitted for elemental analysis was greater than 98% pure.

Anal.—Calc. for $C_{28}H_{33}F_7O_5$: C, 57.73; H, 5.71; F, 22.83. Found: C, 57.87; H, 5.96; F, 23.36.

The heptafluorobutyrate ester of II was isolated as fine white crystals (mp 81–83°). GLC (using a solid sample injector and flame-ionization detector) indicated that the material submitted for elemental analysis was greater than 99% pure.

Anal.—Calc. for $C_{29}H_{39}F_7O_4$: C, 59.58; H, 6.72; F, 22.75. Found: C, 59.59; H, 6.75; F, 23.21.

IR and mass spectrometric analyses, before and after GLC, supported the proposed structures and confirmed that the heptafluorobutyrate esters of I and II chromatographed as the intact molecules.

Assay Sensitivity and Specificity—At a sensitivity of 3.2×10^{-10} amp/mv, 0.0036 μg of I-heptafluorobutyrate produced a full-scale response. Under the assay conditions described, the lower limit of detection sensitivity for I in extracts of plasma is 0.001 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Using peak height ratios or peak area ratios, a nonlinear relationship between detector response and concentration is obtained from I over the range of 0–0.1 μg/ml. Peak height ratios are obtained facilely, and quantification from a standard curve is adequate. Analysis of plasma specimens from drug-treated animals, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay is identical to known I-heptafluorobutyrate.

Recovery Experiments—Known amounts of I in aqueous solution were placed in centrifuge tubes, and water or plasma was added. The samples were thoroughly mixed and extracted with cyclohexane. All extract residues were esterified and analyzed chromatographically. The results (Table II) indicated that recovery of I from plasma ranged from 91.4 ± 14.6 to 102.7 ± 5.8% as compared to simple aqueous samples. In the range of 5–20 ng I/ml plasma, overall precision was approximately ±10% *(SD)*.

Identification of Cholesterol—During analysis of dog plasma extracts for I, a major peak (R_T = 7.5 min) was observed in the gas–liquid chromatograms of specimens from both drug-treated and control animals (Fig. 5). Mass spectrometric analysis of the material(s) in the chromatographic peak suggested that the major component was the heptafluorobutyrate ester of cholesterol. GLC

and mass spectrometric analyses of aqueous and plasma specimens, containing known amounts of cholesterol, indicated that nanogram amounts of cholesterol were extracted and measured as the heptafluorobutyrate ester. Additional studies, based upon measurement of the free sterol, confirmed the identification of the endogenous material as cholesterol. These results were consistent with the serum cholesterol concentrations observed in normal dogs (*i.e.*, 1.4–2.2 mg cholesterol/ml) (16, 17).

Plasma Levels of Medroxyprogesterone Acetate in Dogs—Results from the measurement of plasma I concentrations in female beagle dogs, after single-dose intramuscular administration of 75 mg I/kg body weight, demonstrated the utility of the analytical methodology. In the first study, peak plasma I concentrations of 29 and 14 ng/ml were observed at 11 and 14 days postdrug administration, respectively, indicating slow drug release from the injection site. Measurable amounts of intact I (1.7–6.0 ng/ml) were found in peripheral circulation on Day 59.

In the second study, peak plasma I concentrations of 27 and 54 ng/ml (as measured by GLC) were observed at 1–5 days postdrug administration in Dogs G-403 and K-202, respectively (Fig. 6). Beyond Day 95, plasma I concentrations were below the level of assay detection sensitivity (*i.e.*, less than 1 ng/ml). As measured by radioimmunoassay, peak plasma drug concentrations of 200 and 164 ng I equivalents/ml were found at 5 and 6 days, respectively, in Dogs K-202 and G-403. Measurable amounts of drug-related materials (*i.e.*, 20–30 ng I equivalents/ml) were found in peripheral circulation on Day 108. Using either GLC or radioimmunoassay, the observed plasma drug concentrations showed approximately a two- to threefold variation between animals and a cyclic variation within each animal. Plasma drug concentrations, as measured by radioimmunoassay, were five to 10 times greater as compared to GLC. The radioimmunoassay–GLC ratio increased with time, suggesting that drug-related materials other than intact I were circulating in plasma. Since the plasma specimens analyzed by radioimmunoassay were not extracted or fractionated, it is concluded that drug-related materials in addition to intact I were being measured.

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[x] To whom inquiries should be directed.

NMR Analysis of Pharmaceuticals XII: Determination of Amantadine Hydrochloride in Soft Gelatin Capsules and Syrups

JOHN W. TURCZAN[x] and THOMAS MEDWICK*

Abstract □ An NMR procedure is described by which amantadine hydrochloride is determined in soft gelatin capsules and syrup preparations. A standard deviation of 0.8% was obtained on eight synthetic mixtures. Analysis of capsule and syrup preparations by NMR and NF XIII procedures indicates a maximum difference of 1.6%, but generally a difference of less than 1.0% was obtained. The NMR spectrum provides a very specific means of identification of amantadine hydrochloride.

Keyphrases □ Amantadine hydrochloride—NMR analysis of soft gelatin capsules and syrups □ NMR spectroscopy—analysis, amantadine hydrochloride in soft gelatin capsules and syrups

Amantadine (1-adamantanamine) (I) and its hydrochloride salt (I · HCl), whose structure is shown in Fig. 1, are synthetic chemicals unrelated to other chemotherapeutic anti-infective drugs that represent a new approach to the prevention of respiratory infection caused by the influenza A_2 (Asian) virus strain as well as to the treatment of Parkinson's disease.

Some analytical methods for this drug have been reported. Indirect procedures carried out after derivative formation (1) include a GLC determination, which is based on the conversion of I into 1-(chloroacetamido)adamantate by reaction with chloroacetic anhydride, and a spectrophotometric method, which consists of measuring the UV absorption of 1-(*p*-nitrobenzylidineamino)adamantane obtained by the reaction of I with *p*-nitrobenzaldehyde. Other procedures are direct and are based on reaction with coulometrically generated bromine (2) in a buffered medium, titrimetry (3) in which I is determined by residual base titration of a distillate collected in 0.1 *N* HCl, and potentiometric titration (4) of I in a nonaqueous medium with perchloric acid. In addition to quantitative methods, a few qualitative procedures are described, including GLC (5) and NMR spectrometry (6), in the characterization of I and its derivatives. The official NF XIII procedure (7) for the determination of I in soft gelatin capsules and in syrup involves the solvent extraction of I with benzene and titration of the free base with perchloric acid.

The procedure for I · HCl proposed here uses NMR spectroscopy, a very specific measurement technique which avoids difficulties such as those inherent in titration of a basic center or complete conversion to a desired derivative. The isolation scheme developed in this work is based on the NF method (7) with the addition of one more liquid–liquid partitioning step, a necessary extension to avoid interferences. Briefly, the scheme involves the addition of an internal standard followed by the solvent extraction, NMR spectral scan, and integration. Good quantitative results can be obtained together with a positive identification of the active component in terms of the NMR spectrum.

EXPERIMENTAL[1]

Materials—The following were used: standard, amantadine hydrochloride[2]; internal standard, succinimide[3] (II); and reference standard, Tiers' salt (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) (III).

Compound I capsules and syrup, used as the samples, were obtained from a commercial source.

Procedures—*Capsules*—Dissolve a number of soft gelatin capsules, equivalent to 200 mg of I · HCl, in 10 ml of water by warming on a steam bath with intermittent shaking. Cool, transfer the sample quantitatively with water into a 125-ml separator, make basic with 30% sodium hydroxide solution, add exactly 50 ml of benzene, stopper, and shake gently for a few minutes. Transfer a 25-ml aliquot to a second 60-ml separator; add approximately 200 mg of II, accurately weighed, 4 ml of deuterium oxide, and 4 drops of concentrated hydrochloric acid; stopper; and shake. Allow the layers to separate and transfer about 0.4 ml of the bottom layer to an analytical NMR tube. Place into an NMR spec-

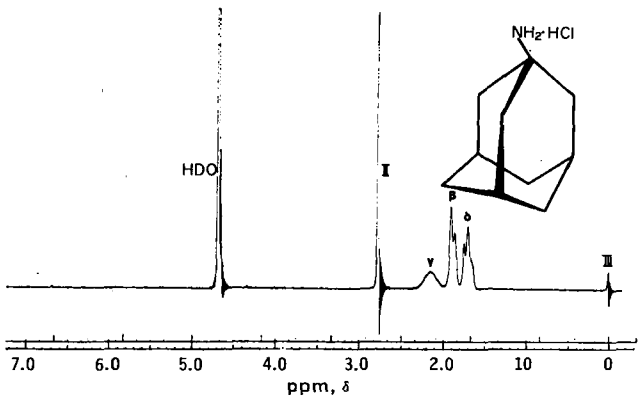

Figure 1—*NMR spectrum of amantadine hydrochloride in acidified deuterium oxide. Key: II, succinimide; and III, Tiers' salt.*

[1] A Varian A-60 NMR spectrometer, equipped with a V-6031 variable temperature probe having a six-turn insert, was used. All spectra were scanned at a probe temperature of 42°.

[2] DuPont, Lot QCD 1-156.

[3] Eastman Kodak Co., Rochester, NY 14650

trometer and obtain the spectrum, adjusting the spin rate to about 30 Hz. All peak field positions are referred to III at 0 ppm using the δ-scale. Integrate the peaks of interest at least five times and average the results.

Syrup—Pipet a portion of the sample solution, equivalent to 200 mg of I · HCl, into a 125-ml separator. Make basic with 30% sodium hydroxide solution and proceed as in the procedure for capsules, beginning with "... add exactly 50 ml of benzene, stopper, ..."

For the procedures given here, the amount of I · HCl may then be calculated as follows:

$$\frac{\text{mg I}}{\text{capsule}} = \frac{A_I}{A_{II}} \times \frac{EW_I}{EW_{II}} \times \frac{\text{mg II}}{C} \times \frac{50}{25} \qquad \text{(Eq. 1)}$$

$$\frac{\text{mg I}}{\text{ml of syrup}} = \frac{A_I}{A_{II}} \times \frac{EW_I}{EW_{II}} \times \frac{\text{mg II}}{V} \times \frac{50}{25} \qquad \text{(Eq. 2)}$$

where:

A_I = integral value of the signal representing I·HCl
A_{II} = integral value of the signal representing II
EW_I = formula weight of I·HCl/15 = 12.514
EW_{II} = formula weight of II/4 = 24.773
C = number of capsules taken initially
V = volume of syrup taken initially

Any variations introduced into the described procedure may introduce changes into Eqs. 1 and 2. The required changes may be simply applied.

RESULTS AND DISCUSSION

When the choice of suitable solvents for NMR is considered, the complex nature of dosage forms sometimes complicates the particular system to be used. Although water is an obvious and excellent solvent for the dissolution and analysis of I · HCl, this solvent cannot be used in a simple analytical scheme for I · HCl in various dosage forms because both the soft gelatin capsules and the syrups encountered in this work have some water-soluble excipients with proton resonance in the vicinity where the I protons resonate, thereby interfering with the integration of the peaks ascribable to I · HCl. As a result, a simple selective dissolution could not be used to isolate I · HCl in a suitable fashion.

To overcome this difficulty, a solvent distribution procedure was evolved. I · HCl was neutralized to yield I by pH adjustment (making the solution alkaline) followed by extraction into benzene and then a second extraction of the benzene with acidified deuterium oxide.

The second partitioning—*viz.*, from benzene into acidified deuterium oxide, is necessary to eliminate obvious benzene-soluble interferences that were in evidence in the NMR spectrum.

The choice of deuterium oxide eliminates the integration interference arising from the strong water resonance that would otherwise extend to the nearby region where the four methylene protons of II resonate. Although the slight amount of residual HDO present in deuterated water is further increased by the two exchangeable amino protons of I, the imide proton of II *(vide infra)*, and the small amount of hydrochloric acid used, the increase in the intensity of this resonance and its side bands is fortunately not great enough to interfere at any time (Fig. 1) with the integration and subsequent quantitation of I · HCl.

The choice of an internal standard requires the compound to be a pure chemical, preferably with a single, strong (large number of equivalent protons) signal at an appropriate field position. Compound II satisfies these requirements and, advantageously, is soluble in water but not benzene. This solubility behavior is important since II is introduced at a point where partitioning between deuterium oxide and benzene is taking place. The results indicate that II does, in fact, behave well in the described scheme.

A 60-MHz NMR spectrum of I obtained in the analysis is exhibited in Fig. 1. The resonance signals used in the actual calculations are the multiplet between 1.5 and 2.4 ppm due to the six β and the six δ methylene protons and the three γ methine protons of I · HCl and the singlet at about 2.75 ppm ascribable to the four methylene protons of II (internal standard).

The unambiguous assignment of chemical shifts for substituted adamantane hydrogens is not a straightforward task. No problem is presented in the case of the three γ hydrogens since the peak ascribable to them at 2.16 ppm is distinct from other spectral features and gives rise to three relative area units. The difficulty, however, arises in the differentiation of the peaks ascribable to the β and δ hydrogens. Fort and Schleyer (6) pointed out that the δ proton multiplet is broader than the β multiplet because the two methylene protons in the δ position are not equivalent. Although there is some reservation about the magnitude of the effect of a charge transmitted from the protonated amine (in the α position) to the β hydrogens in a rigid constrained diamondoid system such as adamantane, there is no question that this effect should be greater than the effect on the δ hydrogens. Thus, on this basis, the multiplet at 1.9 ppm is assigned to the β hydrogens whereas the δ hydrogens give rise to the somewhat broader multiplet at 1.7 ppm. This assignment is consistent with that made previously (8) in work with the 1-adamantyl carbonium ion.

In addition to the I peaks in Fig. 1, other spectral components are observed, some of which can prove troublesome in the analytical scheme. The singlet at about 4.7 ppm is due to the HDO present as a solvent impurity as well as to exchangeable protons from the solutes, whereas the singlet at 0 ppm arises from the nine methyl protons of Tiers' salt (III). Although the resonance signals ascribable to the six methylene protons α, β, and γ to the sulfonyl group in the III reference standard are not visually detectable in the spectrum, they are present in the analytical region and contribute to the integration, thereby interfering with measurement of I. Consequently, the use of III was restricted to the assignment of the chemical shifts seen in Fig. 1; in the actual analysis, no reference standard (III) is used or needed to carry out satisfactory measurements. Furthermore, since Fig. 1 is a spectrum that includes the reference standard (III) for the sake of completeness, no integration of the peaks of interest is made; the spectrum is not one that would be used for actual quantitative analysis.

Table I—Determination of Amantadine Hydrochloride in Standard Mixtures by NMR

Standard Mixture	Succinimide Internal Standard, mg	Amantadine Hydrochloride Added, mg	Found, mg	Recovery, %
1	155.7	80.4	80.8	100.5
2	102.2	101.8	101.2	99.4
3	200.0	101.0	100.5	99.5
4	100.3	101.1	100.2	99.1
5	300.2	99.8	100.9	101.1
6	200.8	102.4	101.3	98.9
7	201.5	99.9	99.3	99.4
8	200.1	102.9	103.4	100.5
			Average =	99.8
			SD =	0.8
			NF procedure =	99.3

Table II—Determination of Amantadine Hydrochloride in Capsules and Syrups by NMR

Sample	Declared Dosage, mg/unit[a]	By NMR Procedure mg/unit[a]	By NMR Procedure %	By Official Procedure mg/unit[a]	By Official Procedure %
Capsules					
1	100	99.7	99.7	98.9	98.9
2	100	98.7	98.7	99.2	99.2
3	100	101.5	101.5	99.9	99.9
4	100	99.3	99.3	100.1	100.1
5	100	99.0	99.0	99.4	99.4
Syrups					
1	50	49.95	99.9	50.5	101.0
2	50	49.8	99.5	49.9	99.7
3	50	49.8	99.6	50.3	100.5
4	50	50.5	101.0	50.4	100.8
5	50	49.9	99.8	50.3	100.6

[a] For syrup (mg/5 ml).

The accuracy and precision of the NMR procedure were studied by analyzing solutions of pure I · HCl in the range from about 80 to 100 mg in 3–4 ml of solution together with varying amounts of II internal standard. Five integrations were run on each of eight samples prepared by using the double-extraction procedure described (Table I). Since the sample taken was a pure chemical, the average value is close to 100% as expected. The standard deviation obtained for the eight known mixtures was 0.8%. The agreement between the NMR and NF procedures is excellent; both results show the measurements to be accurate. The relative proportions of I to II, as noted in Table I, have no significant bearing on the accuracy of the determination for the range of proportions shown.

The suitability of this procedure for the analysis of actual samples was established since 15 trials involving commercial I · HCl soft gelatin capsules and syrups were analyzed by NMR with no evidence of interference from excipients present. For comparison, 10 of these samples were also analyzed by the NF XIII procedure. The results (Table II) indicate good agreement between the NMR and official procedures, the average values being within at most 0.5% of each other. The reproducibility of the NMR measurement is 0.9% *SD*.

As previously demonstrated, the use of NMR for quantitative analysis offers a number of advantages, experienced in this case as well. Thus far, monograph specifications for the identification and purity of pharmaceutical substances drawn up by books of standards do not use NMR spectroscopy. There can be little doubt, however, that NMR has an important role to play in the area of good drug standards. This situation arises probably because many people are unaware of the full potential of the NMR technique and hesitate before allowing a relatively new, and apparently expensive, method to take its rightful place alongside the established methods such as IR, UV, and visible spectroscopy.

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* Present address: College of Pharmacy, Rutgers University, New Brunswick, NJ 08903

[x] To whom inquiries should be directed.

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Quantitative Analytical Method for Determination of Drugs Dispersed in Polymers Using Differential Scanning Calorimetry

FELIX THEEUWES*[x], ANWAR HUSSAIN[‡], and TAKERU HIGUCHI[‡]

Abstract □ A differential scanning calorimeter was used to determine quantitatively the concentration of dispersed progesterone and cholesterol in a silicone rubber matrix. With this technique, the heat of mixing and the solubility of the drug at the drug melting point also were obtained. In the progesterone study, four polymorphic forms in silicone rubber were observed.

Keyphrases □ Polymers, silicone rubber matrix—determination of dispersed progesterone and cholesterol, differential scanning calorimetry □ Progesterone—determination of concentration dispersed in silicone rubber matrix, four dispersed polymorphic forms observed, heat of mixing and solubility at drug melting point determined, differential scanning calorimetry □ Cholesterol—determination of concentration dispersed in silicone rubber matrix, differential scanning calorimetry □ Silicone rubber—determination of dispersed progesterone and cholesterol, differential scanning calorimetry □ Polydimethylsiloxane—determination of dispersed progesterone and cholesterol, differential scanning calorimetry □ Differential scanning calorimetry—analysis, progesterone and cholesterol dispersed in silicone rubber matrix

Differential scanning calorimetry has been employed for semiempirical determinations of purities of drug substances (1, 2) and for kinetic studies of polymorphic phase transitions (3). In the present study, the technique was used to determine quantitatively the concentration of solid drugs dispersed in a polymeric matrix. Polydimethylsiloxane (silicone rubber) was selected as the matrix material since it has been widely used as a rate-controlling membrane in sustained-release drug delivery devices (4–12). With these devices, it is important to know in which physical state the drug is present; in quantitative work, determination of the total amount of drug remaining in the matrix is a frequent analytical problem. Conventionally, radiolabeled drug can be used or the matrix can be extracted and the drug determined spectrophotometrically or by GLC. These techniques are often time consuming and yield no in-

The accuracy and precision of the NMR procedure were studied by analyzing solutions of pure I · HCl in the range from about 80 to 100 mg in 3–4 ml of solution together with varying amounts of II internal standard. Five integrations were run on each of eight samples prepared by using the double-extraction procedure described (Table I). Since the sample taken was a pure chemical, the average value is close to 100% as expected. The standard deviation obtained for the eight known mixtures was 0.8%. The agreement between the NMR and NF procedures is excellent; both results show the measurements to be accurate. The relative proportions of I to II, as noted in Table I, have no significant bearing on the accuracy of the determination for the range of proportions shown.

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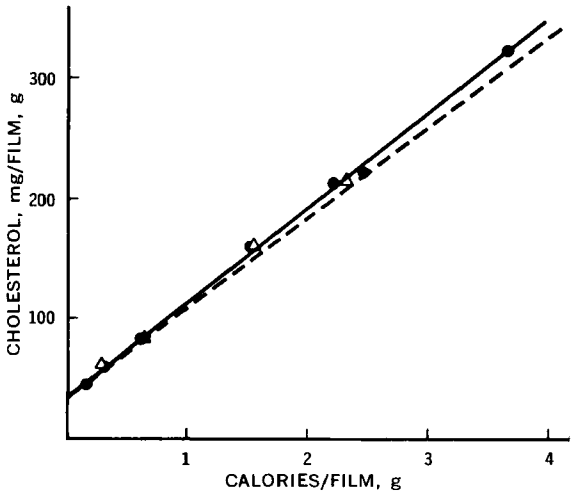

Figure 1—*Cholesterol concentration of the film samples as a function of the observed heat. Key: ●, observed heats at first melting; △, observed heats at second melting; —, average line through experimental data; and – – –, calculated line from drug solubility intercept and heat of melting of the pure drug.*

formation as to the physical state of the drug. Differential scanning calorimetry, on the other hand, is rapid and simple. In addition, it provides information about several useful thermodynamic properties of the drug polymer dispersion such as the heat of mixing of the drug in the polymer, the presence of various polymorphic forms of the drug in the polymer, the solubility of the drug in the polymer at the drug melting point, and the fraction of the drug present in the crystalline form.

EXPERIMENTAL

Materials—Cholesterol[1] and progesterone[2] were used without further purification. Polydimethylsiloxane[3] was the polymer used.

Sample Preparation—Polydimethylsiloxane was mixed with a known weight of steroid at room temperature. To obtain a workable viscosity of the mix, small quantities of silicone oil were added in quantities less than 3% of the total weight of the blend. Stannous octoate (≃0.04 wt%), a room temperature polymerization catalyst, was then added and the dispersion was cast as a film approximately 1 mm thick between two glass plates.

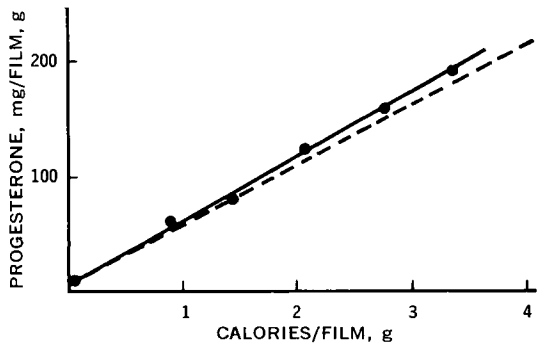

Figure 2—*Progesterone concentration of the film samples as a function of the observed heat. Key: ●, observed heat at first melting; —, average line through experimental data; and – – –, calculated line from drug solubility intercept and heat of melting of the pure drug.*

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Calbiochem, Los Angeles, Calif.
[3] Medical grade Silastic 382, Dow Corning Co.

Table I—Observed Endothermic Heats on Silicone Rubber Samples Containing Dispersed Cholesterol

Concentration of Cholesterol in the Film, mg/g of Film	Observed Endotherm (First Heating), cal/g	Observed Endotherm (Second Heating), cal/g
7.96	Not observed	—
43.7	0.088	—
61.2	0.328	0.287
83.6	0.62	0.66
160.0	1.54	1.56
213.8	2.21	2.34
222.5	2.47	—
322.9	3.65	—

Differential Scanning Calorimetry Analysis—The pure steroids in about 2-mg samples and sections of the films in about 20-mg samples are analyzed in aluminum pans on a differential scanning calorimeter[4]. A scanning speed of 10°/min is used. Temperature is calibrated with an indium standard with the melting point at 430°K.

The heats of melting are calculated based on a gallium standard with heat of melting taken to be 19.15 cal/g. Upon heating of a film sample in the differential scanning calorimeter, one observes an endothermic peak at the melting temperature of the steroid. The endotherm observed at a scanning speed of 10°/min was found to be the same for an identical sample when the scan was made starting from room temperature or when the sample was held for several minutes a few degrees below the melting point of the steroid. Because of this and because of the presence of the drug in the homogeneous fine dispersions, it was assumed that the polymer was continuously in equilibrium with the excess steroid at this scanning speed of 10°/min. Thus, at each temperature, the equilibrium amount of drug is dissolved in the matrix. At the melting temperature, the observed endotherm then corresponds to the melting of the residual undissolved drug. After melting, the samples are cooled to room temperature and then reheated at 10°/min.

RESULTS AND DISCUSSION

Cholesterol Samples—For pure cholesterol, the heat of melting was found to be 13.4 cal/g at 404°K. The melting of cholesterol in the film samples was observed at the same temperature, and the endothermic heats for films containing different concentrations of cholesterol are shown in Fig. 1 and listed in Table I.

After one heating cycle, the samples were cooled to room temperature for about 5 min and then slowly reheated. Upon reheating, melting occurred at the same temperature and the observed

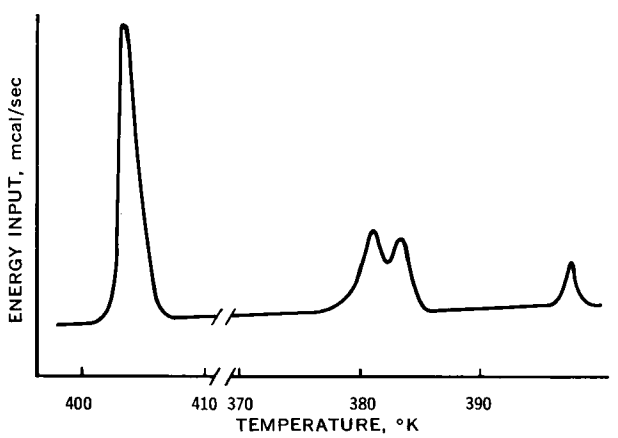

Figure 3—*Differential scanning calorimeter thermogram on the first and second heating of a progesterone sample in polydimethylsiloxane.*

[4] Perkin-Elmer DSC-1B.

Table II—Observed Endothermic Heats on Silicone Rubber Samples Containing Dispersed Progesterone

Concentration of Progesterone in the Film, mg/g of Film	Observed Endotherm (First Heating), cal/g
9.36	0.056
62.61	0.907
80.95	1.44
125.2	2.08
160.0	2.77
191.8	3.36

heats were essentially identical, indicating that the drug was in the same crystal form. By using these calibration data, the concentration of cholesterol in any other sample of silicone rubber can be determined to within ±10 mg/g of polymer by simply measuring the endothermic heat. The intercept of the line in Fig. 1 at 35 mg cholesterol/g of film is the solubility of the drug at the melting temperature. It follows that concentrations below 35 mg/g of film cannot be detected with differential scanning calorimetry.

From the solubility of cholesterol of 35 mg/g in polydimethylsiloxane and from the heat of melting of pure cholesterol, the expected endothermic heat for each film sample can be calculated from the composition when other caloric processes have negligible heats (Fig. 1). The expected values (Fig. 1) fall outside the limit of experimental error, indicating that an exothermic mixing process is also occurring at melting. The heat of mixing is calculated from Eq. 1:

$$q_o = (m_t - m_s)q_m - (m_t - m_s)q_d \qquad \text{(Eq. 1)}$$

where q_o is the observed heat per unit film weight, q_m is the heat of melting of the drug, q_d is the heat of mixing, m_t is the total mass of drug per unit film weight, and m_s is the dissolved mass of drug per unit film weight. The heat of mixing is found to be 0.6 cal/g by taking the q_o as the average observed heat from Fig. 1.

Progesterone Samples—The heat of fusion of pure (α) progesterone was found to be 19.1 cal/g at the melting temperature of 402°K. From the literature, two crystal forms are known: the α-form melting at 402°K and the β-form melting at 395°K. Melting and cooling of normal α-progesterone produce progesterone which, on reheating, melts at 395°K, indicating that the drug has crystallized from the melt in the β-form.

The polydimethylsiloxane samples containing the dispersed progesterone showed endothermic peaks at 402°K at the first heating. These heats (Table II) are plotted in Fig. 2 against the concentration of progesterone in the film. As before, the intercept gives the solubility of the drug in the matrix at the melting point. The solubility is found to be 6 mg/g film. The calculated values (Fig. 2) represent the expected heat calculated from Eq. 1, neglecting q_d, the heat of mixing. The difference between the lines gives the heat of mixing calculated from Eq. 1 and is found to be 1.3 cal/g.

After heating to about 410°K, the film samples were cooled to room temperature for 5 min and then reheated. Upon reheating, a complete new picture was obtained (Fig. 3). The α-progesterone peak previously found completely disappeared and was replaced by three new peaks due to different polymorphs of progesterone. Two peaks were close together, one at about 378°K and the second one at 381°K, while the third at 396°K was presumably due to the β-form. That all three peaks were due to progesterone was shown by varying the mode of cooling which changed the relative magnitude of the peak areas.

SUMMARY

From the differential scanning calorimetry study of dispersed drugs in polymer matrixes, one can obtain the total concentration of drug in the polymer, the solubility of the drug in the polymer at the melting point of the drug, and the heat of mixing of the drug in the polymer. At the same time, different polymorphic forms of the drug present in the polymer can be detected.

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*To whom inquiries should be directed.

Rapid Quantitative Determination of Three Active Ingredients in Liquid Antitussive Preparations by High-Speed Liquid Chromatography

A. MENYHARTH, F. P. MAHN, and J. E. HEVERAN[x]

Abstract □ Three active ingredients in liquid antitussive preparations, acetaminophen, chlorpheniramine maleate, and dextromethorphan hydrobromide, were separated by high-speed liquid chromatography on an octadecylsilane (permanently bonded to a controlled surface porosity support) column with a buffered, pH 8.8, alcohol–water mobile phase and measured with a UV photometer. Due to the relatively large excess of acetaminophen and the fixed detector wavelength (254 nm), the analysis was performed sequentially in two steps. Nonetheless, results were obtained in one-third the time required for conventional methods and instrument flexibility was maintained, thereby permitting its use for a variety of analyses. The relative standard deviations for the three components were 1.8, 0.76, and 2.0%, respectively.

Keyphrases □ Acetaminophen, chlorpheniramine maleate, and dextromethorphan hydrobromide mixtures—high-speed liquid chromatographic separation and UV analysis □ Chlorpheniramine maleate, acetaminophen, and dextromethorphan hydrobromide mixtures—high-speed liquid chromatographic separation and UV analysis □ Dextromethorphan hydrobromide, acetaminophen, and chlorpheniramine maleate mixtures—high-speed liquid chromatographic separation and UV analysis □ Antitussive mixtures (acetaminophen, chlorpheniramine maleate, and dextromethorphan hydrobromide)—separation, analysis □ High-speed liquid chromatography—separation, UV analysis, acetaminophen, chlorpheniramine maleate, and dextromethorphan hydrobromide mixtures □ UV spectrophotometry—analysis, acetaminophen, chlorpheniramine maleate, and dextromethorphan hydrobromide mixtures after high-speed liquid chromatographic separation

Chromatography, which originated with Day (1) and Tswett (2), first was reported in 1900–1903. Until recently, liquid chromatography was of limited utility to the analytical chemist because of lengthy analysis time and relatively poor column efficiency. The potential for rapid analysis of single- and multicomponent formulations was attained when commercial instrumentation and new column materials became available (3–8). Although optimum operating conditions, maximum efficiency, and qualitative separation of various components have been described (9–14), reports of quantitative determinations, specifically on pharmaceutical preparations, are lacking.

The analyses of acetaminophen, chlorpheniramine maleate, dextromethorphan hydrobromide, and similar components have been performed utilizing partition (15, 16), ion-exchange[1] (17), and gas (18) chromatography. The objective of this study was to develop, on a practical basis, a more rapid and precise method for these three compounds. This report describes the successful application of high-speed liquid chromatography to the quantitative determination of these components, an amide and two amines, in commercial antitussive preparations.

[1] Quality control procedure, Hoffmann-La Roche Inc., Nutley, NJ 07110

EXPERIMENTAL[2]

Instrumentation—A liquid chromatograph[3], having a pressure capability of 3000 psig, was equipped with a UV precision photometer and a differential refractometer for detection, a fraction collection valve, a degassing unit, a thermostatically controlled oven, and a gradient elution accessory. Two recorders[4] were employed. The UV detector had a fixed measuring wavelength at 254 nm. The sample cell volume was approximately 7 μl, and the cell path was 8.6 mm. Samples were injected with a 5-μl syringe[5]. UV spectra were obtained with a recording spectrophotometer[6].

Reagents and Chemicals—The following were used: alcohol[7] (anhydrous 3A), alcohol[7] USP, chloroform[8], boric acid[8], potassium chloride[8], sodium chloride[8], sodium sulfate[9] (anhydrous granular), and ammonium hydroxide[10].

A pellicular cation-exchange column packing[11] was employed for preliminary experimental studies.

Determination of Acetaminophen—*Column and Conditions*—Stainless steel, precision bore tubing, 100 cm × 6.35 mm o.d. × 2.1 mm i.d., was packed with octadecylsilane[12] chemically bonded to the surface of a controlled surface porosity support[13] and containing approximately 1% stationary phase (octadecylsilane) by weight. The instrument operating conditions for this column (Column A) were: pressure, ~1800 psig; flow rate, ~0.65 ml/min; oven temperature, ambient; injection volume, 5 μl; and chart speed, 0.75 in./min.

Preparation of Mobile Phase—Weigh accurately 6.184 g of boric acid into a 1-liter volumetric flask and dissolve in 297 ml of water. Add 0.5 ml of concentrated hydrochloric acid and 2.5 ml of concentrated ammonium hydroxide. Dilute with approximately 650 ml of alcohol USP, mix well, adjust to pH 9.0 with concentrated hydrochloric acid or ammonium hydroxide, and bring to the final mark with alcohol USP.

Preparation of Standard Solutions—Internal Standard: Weigh accurately 10 mg of reference amitriptyline hydrochloride into a 10-ml volumetric flask. Dissolve and dilute to volume with the mobile phase.

Reference Standard: Weigh accurately 25 mg of acetaminophen reference standard into a 10-ml volumetric flask. Dissolve and dilute to volume with water (Standard Solution I). Dilute 4 ml of Standard Solution I to 100 ml with the mobile phase (Standard Solution II).

Working Standard Solution: Into a small vial, pipet 1.0 ml of reference Standard Solution II and 1.0 ml of the internal standard solution. Stopper and mix thoroughly.

Sample Preparation—Accurately transfer a sample aliquot equivalent to approximately 50 mg of acetaminophen into a 100-ml volumetric flask. Dilute to volume with distilled water and mix thoroughly (Sample Solution I).

Transfer a 10-ml aliquot of Sample Solution I into a 50-ml volumetric flask, dilute to volume with the mobile phase, and mix thoroughly (Sample Solution II).

Into a small vial, pipet 1.0 ml of Sample Solution II and 1.0 ml

[2] All reference standards were obtained from Hoffmann-La Roche Inc., Nutley, NJ 07110

[3] Du Pont Instruments, model 820.

[4] Honeywell Electronic 194 and Texas Instrument Servo-Riter, 0–1 mv.

[5] Hamilton High-Pressure, model HP 305 N.

[6] Cary model 14.

[7] Publicker Industries Inc.

[8] Merck reagent grade.

[9] Mallinckrodt.

[10] J. T. Baker Chemical Co.

[11] Pellex CP 122, Northgate Laboratories, Hamden, Conn.

[12] Permaphase ODS (Catalog No. 820951001), E. I. du Pont & Co.

[13] Zipax, E. I. du Pont & Co.

Table I—Reproducibility of the Response Ratios for Multiple Injections of a Single Solution

Component	Response Ratio	Mean	Standard Deviation	Relative Standard Deviation
Acetaminophen	3.533 3.645 3.557 3.633 3.592 3.542	3.584	±0.0475	±1.3%
Chlorpheniramine maleate	0.2870 0.2997 0.2880 0.2915 0.2987 0.2961	0.2935	±0.0054	±1.8%
Dextromethorphan hydrobromide	0.01666 0.01655 0.01627 0.01641 0.01671 0.01630	0.01648	±0.00018	±1.1%

Table II—Comparative Analyses of Three Active Ingredients in a Single Cough Preparation[a]

	Acetaminophen[b]		Chlorpheniramine Maleate[c]		Dextromethorphan Hydrobromide[d]	
Sample	HSLC[e]	Ion-Exchange Method[f]	HSLC[e]	Ion-Exchange Method[f]	HSLC[e]	Ion-Exchange Method[f]
1	126	123	1.00	0.98	16.0	15.1
2	120	121	1.01	1.00	15.8	15.1
3	124	122	1.03	1.01	16.3	15.1
4	126	124	1.00	1.05	14.7	15.5
5	126	124	1.03	1.04	15.2	15.3

[a] Results in mg/5 ml are the average of duplicate determinations. [b] Label claim: 120 mg/5 ml. [c] Label claim: 1.00 mg/5 ml. [d] Label claim: 15.0 mg/5 ml. [e] High-speed liquid chromatography. [f] Data obtained in a separate laboratory using the manual ion-exchange method.

of the internal standard solution. Stopper and mix thoroughly (working sample solution).

Chromatographic Analysis—Equilibrate the column with the mobile phase at ambient temperature and inject 5 μl of the working standard solution. Acetaminophen is eluted first, followed by amitriptyline. A typical chromatogram for acetaminophen on the octadecylsilane-bonded column is shown in Fig. 1. After the amitriptyline is eluted, the column is ready for another injection. (The septum should be changed after approximately six injections.) All peak areas are obtained from an electromechanical (disk) or electronic digital integrator. Repeat the injection of the standard solution until a reproducible response ratio is obtained and then chromatograph the working sample solution.

Calculations—For the determination of the response ratio of acetaminophen:

$$R_N \text{ (acetaminophen response ratio)} = \frac{A_N \text{ (standard)} \times C_A}{A_A \text{ (standard)} \times C_N} \quad \text{(Eq. 1)}$$

where:

A_N = acetaminophen peak area of the standard
A_A = amitriptyline peak area of the standard
C_N = concentration of acetaminophen in milligrams per milliliter of working standard solution
C_A = concentration of amitriptyline hydrochloride in milligrams per milliliter of working standard

For the analysis of acetaminophen in the antitussive preparation:

$$\frac{A_N \text{ (sample)} \times C_A \times 5000}{A_A \text{ (sample)} \times R_N \times S} = \text{mg acetaminophen/5 ml antitussive preparation} \quad \text{(Eq. 2)}$$

where A designates area, C is concentration, and R_N is the response ratio as calculated in Eq. 1; 5000 = dilution factor and conversion to 5 ml; and S = sample aliquot taken for analysis.

Determination of Dextromethorphan Hydrobromide and Chlorpheniramine Maleate—*Column Conditions*—The following conditions were employed: column, octadecylsilane bonded to a controlled surface porosity support (Column A); pressure, ~1900 psig; flow rate, ~1.5 ml/min; oven temperature, 50° (and reservoir); injection volume, 5 μl; and chart speed, 0.75 in./min.

Preparation of Mobile Phase—Buffer Solution (0.2 *M* potassium chloride and 0.2 *M* boric acid): Weigh accurately 12.37 g of boric acid and 14.91 g of potassium chloride into a 1-liter volumetric flask. Dissolve and dilute to volume with water.

Mobile Phase: Transfer 100 ml of buffer solution into a 1-liter volumetric flask. Add 399 ml of distilled water and 0.2 ml of concentrated ammonium hydroxide. Dilute with approximately 450 ml of 3A alcohol, mix well, adjust to pH 8.8, and bring to the final mark with 3A alcohol.

Preparation of Standard Solution—Internal Standard: Weigh accurately 45 mg of chlorprothixene into a 100-ml volumetric flask. Dissolve and dilute to volume with alcohol USP.

Reference Standard: Weigh accurately 100 mg of reference chlorpheniramine maleate and 1.50 g of reference dextromethorphan hydrobromide into a 100-ml volumetric flask. Dissolve and dilute to volume with water.

When chlorpheniramine maleate is not present in the sample, the standard should be prepared by weighing 750 mg of reference dextromethorphan hydrobromide into a 100-ml volumetric flask and dissolving and diluting to volume with water.

Working Standard Solution: Transfer 2.0 ml of reference standard solution into a 125-ml separator. Add 28 ml of water and mix thoroughly. From this point, handle exactly as for the *Extraction Procedure* of the sample solution.

Sample Preparation—For products containing only dextromethorphan hydrobromide, quantitatively transfer, with the aid of 20 ml of water and using a suitable volumetric flask, an aliquot of antitussive preparation equivalent to approximately 15 mg of dextromethorphan hydrobromide into a 125-ml separator.

For products containing both chlorpheniramine maleate and dextromethorphan hydrobromide, quantitatively transfer, with

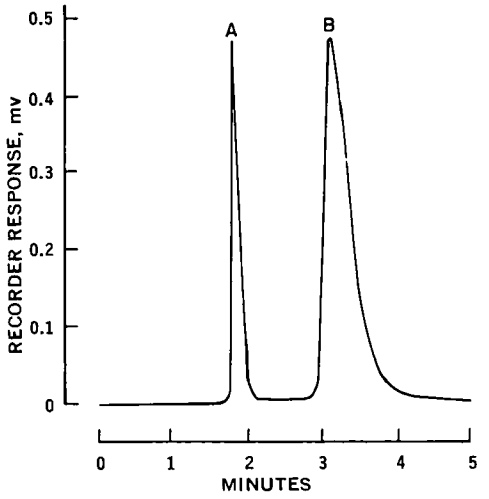

Figure 1—*Separation of acetaminophen (A) and amitriptyline (B) on the permanently bonded octadecylsilane column at an attenuation of 32 × (0.32 absorbance unit full-scale).*

the aid of 20 ml of water and using a suitable volumetric flask, an aliquot of antitussive preparation equivalent to approximately 2 mg of chlorpheniramine maleate and 30 mg of dextromethorphan hydrobromide into a 125-ml separator.

The amount of water used for the transfer will depend on the quantity of the sample taken for analysis, and the alcohol content of the preparation.

Extraction Procedure—Add 3 g of sodium chloride to the separator, dissolve, and make the solution strongly basic with 10 ml of concentrated ammonium hydroxide. Extract with 3 × 40 ml of chloroform (if emulsions are encountered, the quantity of chloroform can be increased). Transfer the chloroform layers into a 250-ml separator, combine, and wash until neutral with 3 × 50 ml of distilled water. Pass the chloroform layer through a funnel containing 25 g of anhydrous granular sodium sulfate into a 250-ml erlenmeyer flask, and then wash the sodium sulfate with an additional 15 ml of chloroform. Evaporate the chloroform to dryness under a flow of nitrogen on a steam bath. Dissolve a residue in 2.0 ml of the internal standard solution (working sample solution).

Chromatographic Analysis—Equilibrate the column with the mobile phase at 50° (oven and reservoir temperature). A temperature of 60° should be used for analysis of products containing only

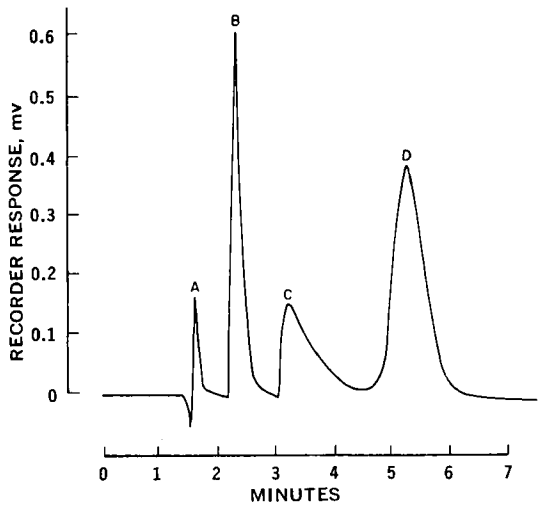

Figure 2—*Separation of chlorpheniramine, dextromethorphan, and chlorprothixene at an oven temperature of 50° and attenuation of 16× (0.16 absorbance unit full-scale) on the permanently bonded octadecylsilane column. Key: A, instrument dead volume; B, chlorpheniramine; C, dextromethorphan; and D, chlorprothixene.*

Table III—Comparative Precision Data

Compound	Standard Deviation, mg: Ion-Exchange Method[a]	Standard Deviation, mg: HSLC[b]	Relative Standard Deviation, %: Ion-Exchange Method[a]	Relative Standard Deviation, %: HSLC[c]
Acetaminophen	±1.3	±2.3	±1.05	±1.83
Dextromethorphan hydrobromide	±0.27	±0.31	±1.72	±1.99
Chlorpheniramine maleate	±0.009	±0.008	±0.93	±0.76

[a] Data obtained in a separate laboratory using the manual ion-exchange method. [b] High-speed liquid chromatography. Pooled standard deviation using results from Table II. [c] High-speed liquid chromatography. Pooled coefficient of variation.

dextromethorphan. Adjust the pressure to obtain a flow rate of 1.5 ml/min and inject 5 μl of the working standard solution. Chlorpheniramine, which is eluted after the dead volume, is followed by dextromethorphan and chlorprothixene (Fig. 2).

After the chlorprothixene is eluted completely, the column is ready for another injection. (The septum should be changed after approximately six injections.) All peak areas are obtained from an electromechanical (disk) or electronic digital integrator. Repeat the standard solution until a reproducible response ratio is obtained and then chromatograph the working sample solution.

Calculations—For the determination of the response ratio for dextromethorphan hydrobromide and chlorpheniramine maleate:

$$R_D = \frac{A_D \text{ (standard)} \times C_T}{A_T \text{ (standard)} \times C_D} \qquad \text{(Eq. 3)}$$

$$R_C = \frac{A_C \text{ (standard)} \times C_T}{A_T \text{ (standard)} \times C_C} \qquad \text{(Eq. 4)}$$

where:

A_D or A_C = dextromethorphan or chlorpheniramine peak area of the standard
A_T = chlorprothixene peak area of the standard
C_D or C_C = concentration of dextromethorphan hydrobromide or chlorpheniramine maleate in milligrams per milliliter of the working standard solution
R_D or R_C = dextromethorphan hydrobromide or chlorpheniramine maleate response ratio
C_T = concentration of chlorprothixene in milligrams per milliliter of the working standard solution

For the analysis of dextromethorphan hydrobromide or chlorpheniramine maleate in the antitussive preparation:

$$\frac{A_D \text{ (sample)} \times C_T \times 10}{A_T \text{ (sample)} \times R_D \times S} = \text{mg dextromethorphan hydrobromide/5 ml antitussive preparation} \qquad \text{(Eq. 5)}$$

$$\frac{A_C \text{ (sample)} \times C_T \times 10}{A_T \text{ (sample)} \times R_C \times S} = \text{mg chlorpheniramine maleate/5 ml antitussive preparation} \qquad \text{(Eq. 6)}$$

where A designates area, C is concentration, and R is the respective response ratio as calculated in Eqs. 3 and 4; 10 = dilution factor and conversion to 5 ml; and S = sample aliquot taken for analysis.

RESULTS AND DISCUSSION

Column and Mobile Phase Selection—Preliminary experiments indicated that qualitative separation of the three components could be obtained on a pellicular cationic column, which is a logical choice for these types of compounds. Reasonable peak

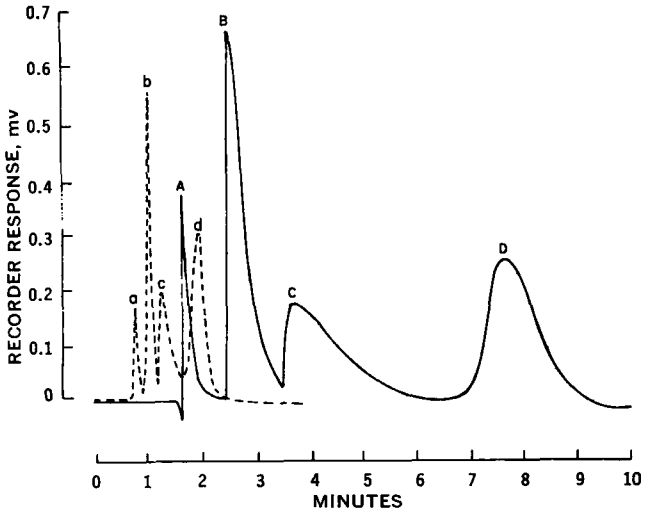

Figure 3—*Separation of chlorpheniramine, dextromethorphan, and chlorprothixene at an oven temperature of 30° (16×, 0.16 absorbance unit full-scale) and 70° (32×, 0.32 absorbance unit full-scale) on the permanently bonded octadecylsilane column. Key (30°, 70°): A, a, instrument dead volume; B, b, chlorpheniramine; C, c, dextromethorphan; and D, d, chlorprothixene.*

shapes for the three compounds in the salt form were achieved during a single chromatographic analysis but, due to the relatively large excess of acetaminophen, complete resolution from the other two components was not obtained.

Direct injection of the syrups was also not feasible since it resulted in interferences and unreproducible chromatograms. Therefore, it was necessary to determine acetaminophen after dilution of the sample with water, while the other two components were analyzed following extraction from an alkaline solution. Quantitative determinations for acetaminophen were carried out, and results equal to the label claim were obtained. When assays were performed for the other two components in their base form on the pellicular cation-exchange column, poor reproducibility, approximately ±10%, was obtained; consequently, a partition chromatographic approach was evaluated.

The behavior of the three components was investigated on an octadecylsilane, permanently bonded to a controlled surface po-

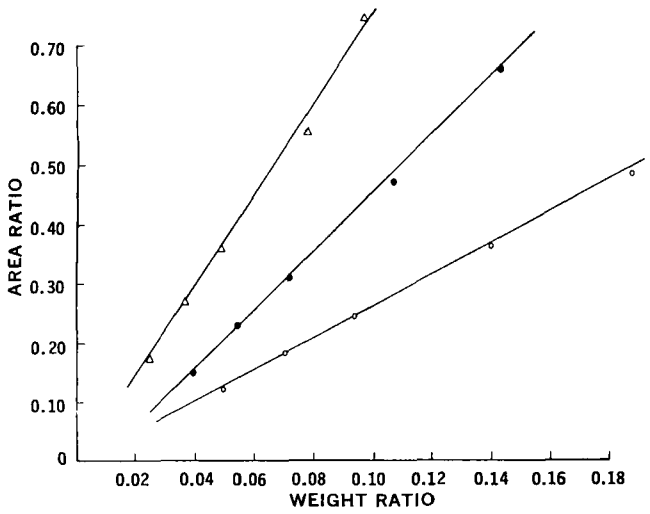

Figure 4—*Standard curves for acetaminophen with amitriptyline as the internal standard, chlorpheniramine with chlorprothixene as the internal standard, and dextromethorphan with chlorprothixene as the internal standard. Key: ○, acetaminophen; △, chlorpheniramine (area ratio × 2:weight ratio × 5); and ●, dextromethorphan (area ratio × 2:weight ratio × 500).*

Table IV—Concentration of the Components in the Analyzed Antitussive Preparations as Shown in the Tables

Product	Components	Claim
A	Dextromethorphan hydrobromide	15 mg/5 ml
	Chlorpheniramine maleate	1 mg/5 ml
	Acetaminophen	120 mg/5 ml
	Fluidextract of ipecac	0.005 ml/5 ml
	Chloroform	0.25%
	Alcohol	10%
B	Dextromethorphan hydrobromide	7.5 mg/5 ml
	Sodium citrate	
	Menthol	
C	Dextromethorphan hydrobromide	5 mg/5 ml
	Chlorpheniramine maleate	0.34 mg/5 ml
	Acetaminophen	100 mg/5 ml
	Phenylephrine hydrochloride	1.7 mg/5 ml
	Alcohol	25%
D	Dextromethorphan hydrobromide	Quantitative statement not available
	Chlorpheniramine maleate	
	Acetaminophen	
	Ephedrine sulfate	
	Alcohol	25%

rosity, solid-core microbead column which had the following advantages: a nonextractable chemically bonded organic polymeric stationary phase and commercial availability of uniformly packed columns (8).

Permanently bonded octadecylsilane is nonpolar and, therefore, a polar mobile phase (alcohol–water) was the appropriate choice. While the mobile phase previously used for acetaminophen on the pellicular cation-exchange column also was suitable for acetaminophen on the octadecylsilane-bonded column, it did not yield a good resolution for chlorpheniramine maleate and dextromethorphan hydrobromide. Since previous studies did not indicate the mechanism of separation (9) and since a minor alteration of the mobile phase could result in changes in selectivity, a study of suitable mobile phases was initiated. Experiments indicated that a slightly buffered solution was preferable and confirmed, as reported elsewhere (13), that an elevated temperature improved the separation. The elevated temperature decreased solvent viscosity, decreased band broadening, and provided more symmetrical peak shapes by apparently increasing the rate of mass transfer (Fig. 3). Considering these factors, the desirable mobile phase was a mixture of 0.02 *M* potassium chloride and 0.02 *M* boric acid in 50% 3A alcohol, adjusted to pH 8.8 with ammonium hydroxide.

Internal Standard and Retention Times—To minimize apparatus and injection errors, a study was performed to select an internal standard. After screening 35 compounds, amitriptyline hydrochloride was found to be suitable as the internal standard for the analysis of acetaminophen. The retention times were 1.9 min for acetaminophen and 3.2 min for amitriptyline. While acetaminophen has the same retention time as the dead volume, interference was not encountered from any excipient. In addition, the use of the mobile phase for sample preparation minimized any contribution from the solvent to the total area at this retention time. Chloride ion was necessary in the mobile phase for reproducible elution of the amitriptyline.

The most suitable internal standard for the analysis of chlorpheniramine maleate and dextromethorphan hydrobromide was chlorprothixene, which exhibited a retention time of 5.2 min compared to chlorpheniramine at 2.3 min and dextromethorphan at 3.2 min. The initial peak at 1.7 min was attributed to the instrumental response for any unretained component. Ephedrine, if present, will elute between chlorpheniramine and dextromethorphan at a retention time of 2.7 min. Phenylephrine did not interfere.

Response and Linearity—To ascertain the coincidence of the UV absorption maxima of the measured compounds with the instrument setting of 254 nm and to determine the effect of the mo-

Table V—Determination of Acetaminophen, Chlorpheniramine Maleate, and Dextromethorphan Hydrobromide in Liquid Antitussive Preparations

Product	Component	Found, mg/5 ml	Percent Claim
A	Dextromethorphan hydrobromide	15.3	102
	Chlorpheniramine maleate	1.04	104
	Acetaminophen	124	103
B	Dextromethorphan hydrobromide	7.6	101
C	Dextromethorphan hydrobromide	5.0	100
	Chlorpheniramine maleate	0.37	109
	Acetaminophen	101.5	101.5
D	Dextromethorphan hydrobromide	3.0	—[a]
	Chlorpheniramine maleate	0.42	—[a]
	Acetaminophen	105	—[a]

[a] Quantitative statement not available.

bile phase on the spectra, complete UV curves were recorded. The peak maxima for all three compounds did not coincide with 254 nm. The total absorption at 254 nm for dextromethorphan hydrobromide was approximately 10% of the peak maximum value at 280 nm, which accounted for the necessity to overload the column and the resultant tailing peak. The other two compounds exhibited UV absorptions at 254 nm equivalent to approximately 85% of their maximum intensity values.

Response *versus* concentration curves for each compound demonstrated that sample concentration was linear with area and that elution times were reproducible (within 1–2% relative to the respective average retention times).

Five different concentrations of acetaminophen, ranging from 0.12 to 0.48 μg, were injected. Figure 4 shows varying ratios of acetaminophen standard solutions *versus* amitriptyline, plotted as relative area per relative concentration. The symmetrical peaks permitted accurate measurements of area, and the data fitted a straight-line plot.

The same procedure was followed for chlorpheniramine maleate and dextromethorphan hydrobromide. An area *versus* concentration curve for each compound employing chlorprothixene as the internal standard is shown in Fig. 4, and the data fitted a straight-line plot in the concentration range studies.

Instrument Repeatability and Recovery—After column conditions were defined, instrument precision was evaluated. Multiple injections of a single solution of each compound indicated that the relative standard deviation of the response ratio was between 1 and 2% (Table I).

Chloroform proved to be the best extraction solvent for these compounds (19). Sodium chloride was added because, in the presence of chloride ion, chloroform would extract dextromethorphan readily regardless of the pH (15). Extraction recoveries carried out for the two amines, with standards analyzed in exactly the same manner to minimize any extraction loss due to handling, yielded results comparable to the label claim. When the extracted samples were compared to unextracted standards, lower levels (90–95%) were obtained.

Sample Analysis—Duplicate assays were performed, as described under the *Experimental* section, on six lots of one antitussive preparation. Results agreed with the label claims for all three components (Table II). The relative standard deviations were comparable to those reported for the manual ion-exchange method (Table III). In addition, four commercial antitussive preparations were analyzed using this method. The concentration of the components and the results are listed in Tables IV and V.

CONCLUSION

Although all possible combinations of instrument and column conditions were not thoroughly explored, an efficient separation of three active ingredients in commercial antitussive preparations was realized. Interference was not observed due to the presence of excipients or other active ingredients such as phenylephrine hydrochloride and ipecac fluidextract. Ephedrine sulfate, after extraction as ephedrine from an alkaline medium, was eluted between chlorpheniramine and dextromethorphan; but at the level present in the preparation studied, it did not interfere with the measurement of the other peaks.

As observed in this study and as previously reported (10), high-speed liquid chromatography is restricted by the limited peak capacity which results from the noncoincidence of the UV absorption maximum of the component and the available detector wavelength.

Although the liquid chromatographic method did not completely eliminate the sample preparation steps, the results of the analysis of six samples of one antitussive preparation showed that operator analysis time can be reduced from approximately 12 hr/lot for the manual ion-exchange method[1] to approximately 4 hr/lot. In conjunction with the reduction in analysis time, instrument flexibility was maintained, thereby permitting its use for a variety of analyses.

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[x] To whom inquiries should be directed.

GLC Analysis of Chloramphenicol: A Collaborative Study

MICHEL MARGOSIS

Abstract □ A GLC method for the assay of chloramphenicol was subjected to a rigorous collaborative study. An overall recovery of 100.1 ± 2.97% for three samples from 14 participating laboratories was obtained.

Keyphrases □ Chloramphenicol—collaborative GLC analysis in 17 laboratories □ GLC—analysis, chloramphenicol, collaborative study in 17 laboratories

The GLC method for the assay of chloramphenicol in pharmaceutical products reported previously (1) included the separation of the drug and was shown to be more specific, precise, accurate, and reliable than the current official UV and microbial assay methods (2). However, to demonstrate and validate its usefulness in other laboratories, it appeared advisable to subject this method to a wide collaborative study. The intent was to devise an analytical system that would maintain control while allowing maximum flexibility so that the method could be performed anywhere by any competent analyst.

COLLABORATIVE STUDY

Separate vials containing three "unknown" samples, two bulk materials, and one internal standard (*m*-phenylene dibenzoate) were prepared and sent to 22 laboratories[1]. The first unknown sample contained a dried mixture of 91.83% chloramphenicol and 8% of chloramphenicol palmitate; unknown samples 2 and 3 were commercial capsule granulations containing 71.90 and 64.89% chloramphenicol, respectively. The nominal values of the chloramphenicol concentration in these mixtures were obtained from the manufacturer and verified independently by this GLC procedure and by the official UV method. One bulk material was provided for use as a reference standard, and the other was provided to allow the analyst to become familiar with the method and to optimize the instrumental conditions.

EXPERIMENTAL

A final protocol of analysis for chloramphenicol by GLC was sent to each laboratory.

Materials—*Gas Chromatograph*—The gas chromatograph, capable of accommodating a glass column and attaining temperatures of 350°, was equipped with a flame-ionization detector. The injection and detector temperatures should be the same as or above that of the column (ranging from 190 to 240°, depending upon the variables in individual instrument systems) but not exceed it by more than 15°.

Column—A glass column, 0.9–1.9 m (3–6 ft), 4 mm i.d., was packed with 1–5% (w/w) low polarity methyl silicone gum or fluid on 80–100- or 100–120-mesh silanized, acid-washed, flux-calcined diatomite[2]. Apply carrier gas at ambient temperature for about 15 min; then condition with no flow for 1 hr at 340° and then with a flow of carrier gas at isothermal operating temperature until stable. It may be further sample-conditioned with a silanizing and column conditioning agent[3] to minimize adsorption. On-column injection is recommended if available. *Caution:* Use a minimum amount of silanized glass wool for plugging the ends of the column, because it may have a deleterious effect on the analysis.

Subsequent to column curing and conditioning, perform a suitability test for inertness of support by injecting cholesterol. A single, symmetrical peak with no evidence of decomposition should be obtained. Typically, the 0.9-m (3-ft) column with 3% OV-1 on 100–120-mesh Gas Chrom Q used for the analysis of these collaborative samples gave about 1200 apparent plates (or about 1350 plates/m), with a tailing factor of 1.29 for cholesterol.

The use of a low polarity dimethyl silicone polymer such as OV-1, OV-101, or SE-30 is recommended. The relative retention time factor for chloramphenicol *versus* *m*-phenylene dibenzoate (internal standard) on OV-1 (0% phenyl) is 2, whereas it is 4 on OV-17 (50% phenyl) and about 11 on PPE-20 (poly-M-phenyl ether).

Detector—Flame ionization, with air and hydrogen flow rates adjusted so as to obtain maximal response, is used. The flow rate of the carrier gas, helium, is about 60 ml/min. Adjust the column temperature and carrier gas flow rate so as to offer complete resolution of the peaks (about 5 min from solvent to chloramphenicol). Set current at 2×10^{-9} amp full-scale deflection (fsd), or adjust to obtain peak heights greater than 50% fsd, depending upon sharpness of peak.

Reagent Solution (Prepare in Hood)—Dissolve about 200 mg of *m*-phenylene dibenzoate in about 6 ml of acetonitrile. Add 1 ml of *N,O*-bis(trimethylsilyl)acetamide, and dilute to 10 ml with acetonitrile with shaking until a uniform phase is obtained. (See *Discussion.*)

Sample Preparation—*Bulk and Standard*—Accurately weigh 5–10 mg of the specimen directly into a reaction tube (conical centrifuge tube), or make a stock solution in ethyl acetate, transfer an aliquot equivalent to 5–10 mg to the tube, and evaporate to dryness under a current of dry air.

Dosage Forms—Accurately weigh the sample and make a solution in ethyl acetate. Allow insoluble excipients to settle, transfer an aliquot equivalent to 5–10 mg, and evaporate to dryness.

Procedure—To each dried sample, add 1.00 ml of reagent solution and stir vigorously[4] to obtain a single uniform phase. Inject 1 μl (equivalent to 5–10 μg of chloramphenicol) into the gas chromatograph. Retention time for bis(trimethylsiloxy)-chloramphenicol should be no less than 4–5 min. Measure the *area* of each peak by a suitable nondestructive technique.

Calculations—Equation 1 was used:

$$\%\ (w/w)\ \text{chloramphenicol} = \frac{R_{sa}}{R_{std}} \times \frac{W_{std}}{W_{sa}} \times 100 \quad \text{(Eq. 1)}$$

where R = ratio of bis(trimethylsiloxy)-chloramphenicol to inter-

[1] The 17 that actually participated are listed here. The author acknowledges the gracious participation of the following collaborators: D. H. Calam, National Institute for Medical Research, London, England; Vernon G. Davies, Parke-Davis Laboratories, Detroit, Mich.; Roberta Hartman and Joy Tagle, McKesson Labs., Bridgeport, Conn.; Gerard Janssen and Hubert Vanderhaeghe, Katholieke Universiteit te Leuven, Belgium; Siv Johannson and Bengt Ohrner, Apotekens Centrallaboratorium, Solna, Sweden; Ernest J. Kubiak and Marvin Grostic, The Upjohn Co., Kalamazoo, Mich.; Dr. Lettenbauer and Dr. Futscher, Boehringer Mannheim Gmbh, Germany; A. M. Monard, Association Pharmaceutique Belge, Service de Controle des Medicaments, Brussels, Belgium; Ida Mortensen and Jørgen Bang, Statens Serumsinstitut, Copenhagen, Denmark; A. Pacheco, Zenith Laboratories, Northvale, N.J.; Robert Puchalski, S. B. Penick and Co., Lyndhurst, N.J.; Bruce A. Ross, Food and Drug Administration, Office of Pharmaceutical Research and Testing, Division of Drug Chemistry, Washington, D.C.; Don Rowe, Food and Drug Administration, Office of Pharmaceutical Research and Testing, National Center for Antibiotic Analysis, Washington, D.C.; H. Van der Veen, Vrije Universiteit, Amsterdam, The Netherlands; Clyde E. Wells, Food and Drug Administration, Office of Pharmaceutical Research and Testing, National Center for Drug Analysis, St. Louis, Mo.; William L. Wilson, Food and Drug Directorate, Ottawa, Ontario, Canada; and Dorothy K. Wyatt, Drug Standards Laboratory, Washington, D.C.

[2] A 0.91-m (3-ft) × 4-mm column with 3% OV-1 on 100–120-mesh Gas Chrom Q (Applied Science Laboratories, Inc.) is recommended.

[3] Silyl-8, Pierce Chemical Co., Rockford, Ill.

[4] With Vortex mixer.

Table I—Results for Chloramphenicol Bulk (Sample 1)

Laboratory	Number of Runs	Mean Recovery, %	Coefficient of Variation
1	4	100.07	1.07
2	4	106.09	2.56
3	8	102.70	3.10
4	4	101.34	1.14
5	4	99.16	0.48
6	4	100.92	0.83
7	2	98.11	0.37
8	2	100.94	1.14
9	4	100.81	4.33
10	4	106.04	8.39
11	2	101.33	0.08
12	4	102.22	1.53
13	2	102.47	1.35
14	2	102.20	0.51
15	2	99.19	1.12
16	4	100.42	1.31
17	8	99.26	2.74
WD Mean	64	101.44	3.43
Mean	17	101.37	2.16
Corr Mean	14	100.85	1.35

Table II—Results for Chloramphenicol Capsule Granulation (Sample 2)

Laboratory	Number of Runs	Mean Recovery, %	Coefficient of Variation
1	8	100.59	0.96
2	4	104.24	1.26
3	8	105.41	2.10
4	4	101.32	1.14
5	4	99.22	0.97
6	4	88.94	6.49
7	2	97.28	0.78
8	2	99.06	3.58
9	4	97.73	2.28
10	4	101.82	8.28
11	2	101.39	0.58
12	4	101.67	1.01
13	2	102.71	1.24
14	2	103.67	0.08
15	2	100.54	1.54
16	4	97.84	1.20
17	8	106.29	4.18
WD Mean	68	101.16	4.98
Mean	17	100.57	3.96
Corr Mean	14	99.81	3.91

nal standard peak, and W = weight of standard or sample (std or sa, respectively).

Collaborators were requested to run duplicate injections of two separate weighings of each material and to compare the values to the average value obtained on the standard; they were also requested to determine the coefficient of variation of five injections of each standard. To ascertain the suitability of experimental conditions of the chromatographic system, a measure of efficiency, resolution factors, and tailing factors was also requested:

$$\text{efficiency} = 16(tr/Wb)^2 \qquad \text{(Eq. 2)}$$

where tr = adjusted retention time, and Wb = peak width (width at base cut by the two tangents drawn to the inflection point of the peak);

$$\text{resolution factor} = \frac{2D}{A + B} \qquad \text{(Eq. 3)}$$

where D = distance between peak maxima, A = Wb for peak 1, and B = Wb for peak 2; and:

$$\text{tailing factor} = \frac{A + B}{2A} \qquad \text{(Eq. 4)}$$

measured at 5% of peak height where A = distance from time point of peak maximum to ascending slope, and B = distance from time point of peak maximum to descending slope.

RESULTS AND DISCUSSION

Statistics—All calculations, data processing, and statistical analyses were performed through the APL time-share system of a computer[5]. Calculations and results from each collaborator were verified when sufficient data were available. The final results were normalized to the nominal concentration of the sample as described initially and are summarized in Tables I–IV for each sample and for the sum of the three samples. In each of the four tables, "WD Mean" is that obtained through the weighted analysis of all observations from each collaborator, inclusive of "within sample" errors; "Mean" is that obtained from the calculated mean from each collaborator, thus attributing equal weight to each collaborator. As shown in these tables, mean percent recoveries of 101.37 ± 2.16, 100.57 ± 3.96, and 100.4 ± 3.68 were obtained for Samples 1, 2, and 3, respectively, with an overall recovery for all samples from all 17 collaborators of 100.67 ± 3.32%. Although these recoveries amply validate the method and demonstrate its reliability, results from three collaborators were rejected outright. Those of two collaborators (10 and 17) were rejected because a precision exceeding 8% in the replications of the standard was obtained; results from Collaborator 2 were rejected because they exceeded an acceptable sum rank limit by the Youden (3) test. The final overall recovery for all samples from 14 collaborators was 100.1 ± 2.97%.

A computer plot of all available values *versus* the frequency of observations, numbering 190, yielded the typical S-shaped curve. A chi-square test fitted these data to a normal distribution as expected. Data obtained by electronic and disk integration were analyzed by Bartlett's test; it showed that the variances within each sample were homogeneous, indicating similar reliability from each collaborator. Data obtained by the peak height method most adversely affected this homogeneity of variances.

Duncan's multiple-range test was used to determine which collaborators differed from each other at a particular level of significance. At $p < 0.05$, overlapping random groups of three to seven collaborators showed no significant difference *within* groups of collaborators, but significant differences were noted among these same groups. With Sample 2, results of Collaborator 6 were most significantly different from all others; with Sample 3, results of Collaborators 3 and 6 were significantly different from each other and from those of all other collaborators.

A one-way analysis of variance was performed on the results calculated from each sample. At $p < 0.05$, significant differences between collaborators were detected for Samples 2 and 3 but not for Sample 1. Variations between replicate samples in all cases were fairly small or actually negligible. Differences among laboratories were found to be largely attributable to results derived by manual techniques of peak measurements. A two-way analysis of variance of the means (for each sample from each collaborator) showed no statistical differences between samples at $p < 0.05$ but confirmed the differences among laboratories as noted previously.

Methodology—Thirteen different models of GLC instruments were used, with a flame-ionization detector being about the only common denominator among these. Deviations between analysts could be ascribed largely to differences in such characteristics as nature (type and density) of packing, carrier gas, pressure drop, column material, length, diameter, and efficiency. The columns ranged in length from 0.91 to 1.83 m (from 3 to 6 ft), and various supports were employed under different conditions of temperature and carrier gas flow rates. Most used helium, but some used nitrogen.

To maintain control of this method, it is essential to ascertain that a minimum performance requirement be met *a priori* for the entire analytical system whatever components it may include. In the case of inert supports, such as the silicones, a curing, conditioning, and suitability test similar to that described in USP XVIII should become mandatory. A failure of this test probably indicates an inadequate packing material. In all cases, a mini-

[5] IBM 360.

Table III—Results for Chloramphenicol Capsule Granulation (Sample 3)

Laboratory	Number of Runs	Mean Recovery, %	Coefficient of Variation
1	4	100.72	1.35
2	4	108.53	1.34
3	6	105.56	1.82
4	4	101.41	0.33
5	4	99.07	0.20
6	4	93.00	0.10
7	2	97.48	0.28
8	2	99.70	0.80
9	4	96.00	1.22
10	—	—	—
11	2	102.10	0.11
12	4	99.45	0.84
13	2	101.86	0.86
14	2	100.14	0.50
15	2	97.67	0.98
16	4	96.86	1.74
17	8	101.04	1.99
WD Mean	58	100.34	3.98
Mean	16	100.04	3.68
Corr Mean	14	99.36	3.10

Table IV—Combined Results for Three Chloramphenicol Samples

Laboratory	Number of Runs	Mean Recovery, %	Coefficient of Variation
1	16	100.49	1.09
2	12	106.29	2.38
3	22	104.46	2.67
4	12	101.36	0.86
5	12	99.15	0.58
6	12	94.29	6.39
7	6	97.62	0.57
8	6	99.90	1.91
9	12	98.18	3.41
10	8	103.93	8.02
11	6	101.61	0.46
12	12	101.11	1.63
13	6	102.35	0.98
14	6	102.00	1.59
15	6	99.13	1.61
16	12	98.37	2.06
17	24	102.20	4.24
WD Mean	190	101.00	4.21
Mean	50	100.67	3.32
Corr Mean	42	100.01	2.97

mum efficiency, resolution, and symmetry factor should be specified, as well as a maximum coefficient of variation of a certain number of replicate injections of a standard, before the system is deemed acceptable for actual analysis.

The efficiencies of the columns used in this study ranged from 840 to 1580 theoretical plates/m, with a mean of 1140 ± 20% theoretical plates/m. The symmetry or tailing factor ranged between 1.04 and 1.3, with most at about 1.1. The retention time of the bis(trimethylsiloxy)-chloramphenicol relative to the internal standard is also used as a qualitative tool and is particularly valid when related to an authentic sample. In this study, the relative retention times of bis(trimethylsiloxy)-chloramphenicol ranged between 1.76 and 2.04, with a mean of 1.88 ± 3.3%.

Derivatization—This was probably the most common source of difficulty in the analysis. Several collaborators reported the presence of one or two extraneous peaks when using *N,O*-bis(trimethylsilyl)acetamide as the reagent as specified. These peaks have been ascribed to the monotrimethylsilyl and tris(trimethylsilyl) derivatives (5) and were recently corroborated in this laboratory with an OV-17 column, which allows greater resolution than the nonpolar OV-1 phase prescribed for this analysis. Furthermore, it was noted that not only did *N,O*-bis(trimethylsilyl)-acetamide behave in this manner but so did the trifluoro reagent. These occurrences were not constant; they appeared with some lots of reagents and not others. The causal factors, whether they be due to extraneous silyl donors present, the age of the lot, storage, and/or reaction temperatures, *etc.*, could not be ascertained even by the manufacturer of this reagent. Several users, however, tend to ascribe this difficulty to the age of the reagent.

It is advised, therefore, that should the reagent yield more than one major peak, alternative reagents, such as hexamethyldisilazane combined with trimethylchlorosilane, Tri-Sil, or trimethylsilylimidazole (specific for hydroxyl functions), that will result in obtaining the proper bis(trimethylsilyl) derivative be employed.

GENERAL NOTES

Comments were received from collaborators; some are useful in optimizing the method for more widespread applicability. Some, discussed randomly here, have been incorporated into the method.

1. Although helium was specified as carrier gas, other gases (*e.g.*, nitrogen) may be used if proven to be satisfactory.

2. *m*-Phenylene dibenzoate may be slow in dissolving initially or in redissolving from low temperature storage; however, this is easily remedied with gentle heating and mixing.

3. It is preferable to complete the analysis within the day of silylation to minimize any change of configuration.

4. It is easier and more accurate to take an aliquot of 1.00 ml of reagent than the 0.5 ml prescribed in the procedure.

5. The final concentration and amount injected could be reduced to avoid overloading the column. The amount of bis(trimethylsiloxy)-chloramphenicol can easily be reduced two- to 10-fold without adversely affecting the accuracy and precision of the system; in fact, this should improve the efficiency, resolution, and symmetry factors. Naturally, this also depends to some extent upon the quality of the instrument employed.

CONCLUSION

These results indeed prove the merits of this method. Because of its demonstrated superiority, it has been proposed for inclusion into the *Code of Federal Regulations* and is recommended for primary compendial usage.

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Comparison of Availability of Ions from Sodium Salicylate and Salicylic Acid Tablets

T. E. NEEDHAM, Jr.[x], L. A. LUZZI, and W. D. MASON*

Abstract □ The *in vitro* release of the drug components from salicylic acid and sodium salicylate tablets was studied. Comparisons were made concerning the availability of sodium ions and salicylate ions from typically formulated tablets containing the salt of the drug. Similarly formulated salicylic acid tablets were also compared. The availability of sodium from the sodium salicylate tablets, while unaffected by pH, was substantially different from that of the salicylate ion and would represent the inherent disintegration-deaggregation-dissolution behavior due to the tablet formulation itself.

Keyphrases □ Sodium salicylate and salicylic acid tablets—*in vitro* drug release compared, pH effect □ Salicylic acid and sodium salicylate tablets—*in vitro* drug release compared, pH effect □ Dissolution, sodium salicylate and salicylic acid tablets—percentage of drug released *in vitro*, pH effect

The *in vitro* release of drug from the tablet dosage form has been studied in numerous ways to ascertain the efficacy of certain formulations, manufacturing techniques, and even the physical and chemical form of the drug to be used. Disintegration was adopted as the primary means of testing drug release but has been replaced by dissolution. Many different dissolution methods have been developed including the hanging pellet (1) and various beaker (2), basket (3), and flowing-stream apparatus (4).

Several of these dissolution methods have been used under special circumstances and with specially formulated tablets to elucidate some of the mechanisms involved in drug dissolution. Nelson (1) compared the dissolution of several weak acids and their sodium salts from pellets. These pellets, containing only the pure drug, were suspended from a balance and the dissolution was recorded as the loss of weight per unit time. For all drugs and at all hydrogen-ion concentrations tested, the rate of dissolution of the salt form of the drug far exceeded that of the acid form. Later, Higuchi *et al.* (5) developed the theoretical basis of dissolution from pure drug tablets. This work was expanded to include the theoretical and actual dissolution of several drugs from compressed disks (6, 7).

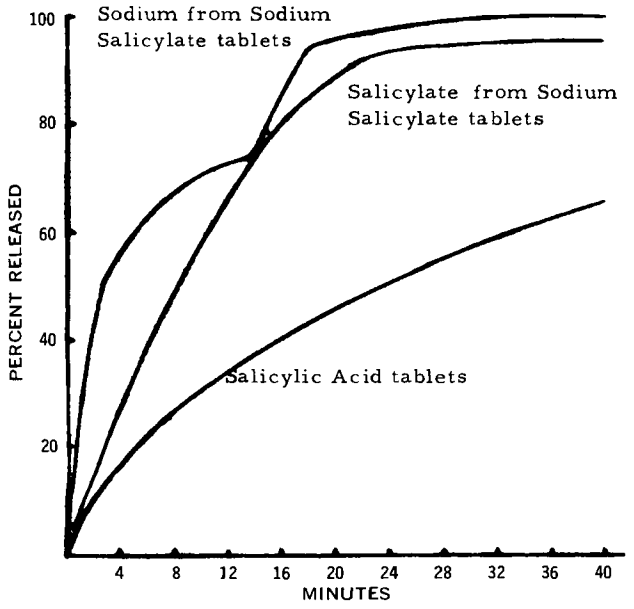

Figure 1—*Comparison of the dissolution rates of salicylic acid and sodium salicylate tablets at pH 2.0.*

Most reports have been concerned with dissolution from disks consisting of drug only. The purposes of this work were to follow the appearance of each drug component as it dissolved from more typical formulations of compressed tablets and to compare the dissolution of the sodium salt with that of the free acid at several hydrogen-ion concentrations.

EXPERIMENTAL

Materials—Salicylic acid[1], sodium salicylate[1], fast flow lactose[2], microcrystalline cellulose[3], starch[4], and stearic acid[5] were obtained from commercial sources. The dissolution medium at pH 2 consisted of a buffer mixture of hydrochloric acid and potassium chloride; at pH 8, it was made up of a 0.2 *M* buffering solution of monobasic potassium phosphate and potassium hydroxide (8).

Equipment—The magnetic basket (3) was used as the dissolution apparatus.

Tablets were compressed using a 16-station rotary tablet press[6] equipped with an induced die feeder. Standard concave punches, 0.95 cm (0.37 in.) diameter, were used. An electronic hardness tester[7] was employed to determine hardness.

Tablet Formulation—Tablets were compressed from the following formulations (in milligrams) to contain the same molar concentration of sodium salicylate and salicylic acid:

sodium salicylate	50.0	salicylic acid	43.13
stearic acid	3.6	stearic acid	3.6
lactose	203.3	lactose	228.27
starch	35.0	starch	35.0
microcrystalline cellulose	50.0	microcrystalline cellulose	50.0

All tablets were weighed, and 50% of those tablets meeting

[1] J. T. Baker Chemical Co., Phillipsburg, N.J.
[2] Foremost Dairy, San Francisco, Calif.
[3] Avicel, FMS Corp., Newark, Del.
[4] Ruger Chemical Co., Inc., Irving, N.J.
[5] Fisher Chemicals, Fairlawn, N.J.
[6] Model 216-RP Cherry Burrell.
[7] Erweka Electronic.

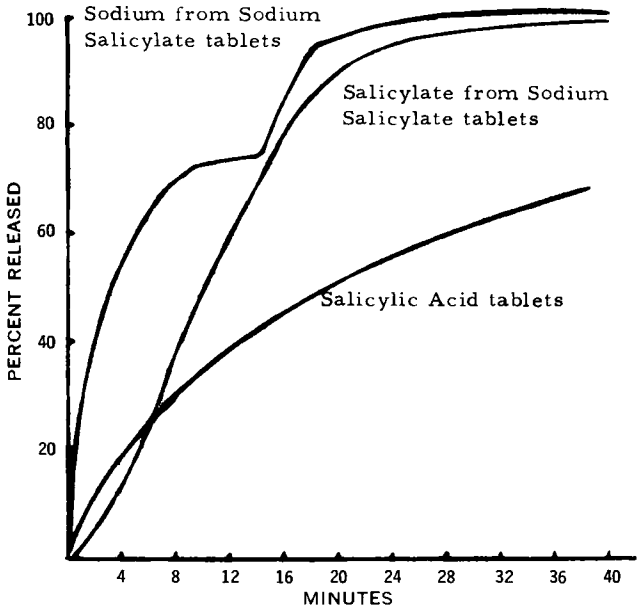

Figure 2—*Comparison of the dissolution rates of salicylic acid and sodium salicylate tablets at pH 8.0.*

specifications were examined for hardness. Although tablets of varying hardnesses were prepared, tablets of only one specific hardness for each formulation were used in this work. The salicylic acid tablets showed an average hardness of 0.8 unit with a range of 0.5–1.1, and the sodium salicylate tablets had an average hardness of 1.1 units with a range of 0.8–1.3.

Analysis of Tablets—The weight of each tablet was determined prior to dissolution. The amount dissolved at any time *t*, reported as a percent of the total active ingredient, was determined spectrophotometrically at 293 nm in the pH 8 buffer and at 301 nm in the pH 2 buffer using the appropriate blanks. The appearance of sodium was assayed using an atomic absorption spectrophotometer[8] on those samples previously assayed in the UV spectrum. All sodium concentrations reported have been corrected for the small amount of extraneous sodium present in the formulation. Samples were taken at 2-min intervals for the first 14 min, at 4-min intervals through 30 min, and then at 5-min intervals until dissolution was complete. Each dissolution profile is the average of at least five tablets determined separately in the magnetic basket dissolution apparatus (3) at 37 ± 1° with an impeller speed of 60 rpm.

RESULTS AND DISCUSSION

In this study the dissolution of three components of the tablet was followed at two different hydrogen-ion concentrations. The tablets were prepared to contain a lubricant, binder, disintegrant, and the drug to provide *in vitro* drug release patterns of typical compressed tablet formulations. Figure 1 illustrates the dissolution of the drug at pH 2 from identically formulated and manufactured tablets containing sodium salicylate and salicylic acid, respectively. Faster dissolution of the salicylate ion from the sodium salicylate tablets as compared to the dissolution of the salicylate ion from the salicylic acid tablets is evident, although the magnitude of difference is somewhat less than that reported for the dissolution of these two moieties from tablets containing only pure drug (1).

The release of the sodium ion from the sodium salicylate tablets at pH 2 provides an interesting contrast to that witnessed for the salicylate ion from the same tablets. As can be seen from Fig. 1, the concentration of sodium ion increases much more rapidly than that of the salicylate ion, but then its rate of dissolution decreases until a similar amount of release is found for both species at 14 min. After this point, the sodium ion again dissolves faster than the salicylate ion. This behavior of the sodium-ion concentration can be correlated to the visual observation of tablet dissolution. During the first phase, the tablet is disintegrating and falling from the basket to form a mound at the bottom of the beaker. Thereafter the release of sodium ions takes place from this mound. So in essence the dissolution profile formed by the sodium ions dissolving into the solution seems to represent the inherent disintegration–deaggregation–dissolution process due to the tablet formulation itself. The profile formed by following the salicylate ion should, therefore, represent the appearance of the drug from the tablet. This availability of the salicylate ion would be dependent on the dissolution media and the solubility of the drug. Two processes have been reported to occur which would affect the appearance of the salicylate ion during dissolution: (*a*) the initial formation of an acid coat around the tablet and its deaggregated particles (5) which, when combined with the diffusion coefficient of that species, will slow the dissolution of salicylate ion; and (*b*) the precipitation of the free acid into microsized particles (1) which, in turn, must dissolve before their concentration can be recorded (2). The sum total of these two processes shows (Figs. 1 and 2) a slower dissolution of the salicylate ion as compared to that of the sodium ion.

Figure 2 represents the dissolution of the same three species at pH 8. Although there seems to be little difference in the profiles as the pH is increased, Tables I and II present several interesting comparisons among the concentrations of sodium ions, salicylate ions, and the salicylate from the salicylic acid tablets. In comparing the appearance of salicylic acid at the two different hydrogen-ion concentrations, the expected slight increase in the rate of dissolution can be seen at pH 8. The salicylate ion at the higher pH lags slightly behind the amount dissolved at pH 2, while the availability of sodium ions at both pH's are almost identical.

Table I—Percentage of Drug Released from Salicylic Acid and Sodium Salicylate Tablets at pH 2.0

Minutes	Drug Released[a], %		
	Salicylic Acid Tablets	Sodium Salicylate Tablets	
		Sodium Ion	Salicylate Ion
2	9.6	46.8	12.2
4	15.0	53.9	25.3
6	21.4	62.0	38.3
8	24.8	66.7	50.0
10	31.6	66.9	57.6
12	34.4	72.4	66.1
14	35.8	72.2	73.1
18	42.0	96.2	88.5
22	47.3	96.9	92.1
26	55.3	100.0	93.8
30	57.0	100.0	94.1
35	60.8	100.0	94.6
40	65.9	100.0	95.1

[a] Each value is the average of five tablets.

Table II—Percentage of Drug Released from Salicylic Acid and Sodium Salicylate Tablets at pH 8.0

Minutes	Drug Released[a], %		
	Salicylic Acid Tablets	Sodium Salicylate Tablets	
		Sodium Ion	Salicylate Ion
2	12.2	48.9	5.7
4	19.8	51.2	12.1
6	23.6	65.1	26.2
8	28.7	70.2	44.9
10	33.0	73.1	61.8
12	37.6	72.9	59.7
14	40.3	74.3	71.2
18	47.9	94.8	87.2
22	53.2	96.7	93.7
26	55.8	100.0	96.5
30	58.1	100.0	97.5
35	64.7	100.0	98.5
40	69.5	100.0	98.9

[a] Each value is the average of five tablets.

[8] Perkin-Elmer 290 B.

This similarity witnessed for the appearance of sodium ions indicates that the inherent disintegration–deaggregation–dissolution process for the tablet formulation itself is not affected by pH.

In conclusion, tablets containing binders, lubricants, and disintegrants as well as drug itself dissolve in much the same manner as tablets containing only drug. The inclusion of these additional ingredients into the formulation may show changes in the dissolution profiles, but the overall relativity of behavior seems to remain the same. By following the appearance of sodium in the dissolution of a tablet containing the salt of a drug as well as the drug, two processes may be defined: (*a*) the disintegration–deaggregation–dissolution procedure, which is dependent on the tablet formulation; and (*b*) the solubility–dissolution behavior of the drug itself. Consequently, the inherent dissolution characteristics of the tablet formulation may be seen and changes in dissolution caused by various formulation ingredients may be adjusted. Furthermore, availability of the drug itself can be analyzed.

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* Present address: School of Pharmacy, University of Missouri at Kansas City, Kansas City, Mo.

ˣ To whom inquiries should be directed.

Suspension Polymerization for Preparation of Timed-Release Dosage Forms

ROGER W. CROSWELL and CHARLES H. BECKER ˣ

Abstract □ An investigation was undertaken to develop a process, utilizing suspension polymerization, for the production of timed-release medicated dosage forms. The effects of production variables upon dissolution of acetaminophen from these dosage forms were studied. Nonexpanded spherical polystyrene beads produced in the presence of acetaminophen exhibited no timed release of medication. However, timed release of medicament was observed when spherical polystyrene beads produced in the absence of acetaminophen were expanded with the aid of a blowing agent and subsequently allowed to absorb medicament. In general, 64% of the available drug was released from the free expanded polystyrene beads within the 1st hr of dissolution, with an additional 29% being released by the conclusion of dissolution testing.

Keyphrases □ Suspension polymerization—formation of timed-release dosage forms, polystyrene beads containing acetaminophen □ Polymerization, suspension—formation of timed-release dosage forms, polystyrene beads containing acetaminophen □ Acetaminophen timed-release dosage forms—prepared by suspension polymerization of polystyrene □ Polystyrene beads—prepared by suspension polymerization for timed release of drugs (acetaminophen) □ Timed-release dosage forms—preparation, suspension polymerization

Although the technique of suspension polymerization has been in use for more than 40 years, its application to the production of a pharmaceutical dosage form had not been studied until very recently. One of the first pharmaceutical investigations involving suspension polymerization was undertaken by Khanna *et al.* (1). In their work, methyl methacrylate, vinyl acetate, and divinylbenzene were polymerized in suspension to produce spherical beads containing medicament. The variables studied included type of medicament, speed of agitation, length of polymerization, and concentration of suspension stabilizers added to the aqueous phase.

An investigation into the release of medicament from spherical beads produced by suspension polymerization has been undertaken. Styrene monomer, alone or in the presence of medicament, was polymerized to produce spherical beads. A portion of these beads was then expanded with the aid of a blowing agent, and dissolution studies were conducted on both the nonexpanded and expanded polystyrene beads.

Suspension polymerization is synonymous with bead or pearl polymerization. In this process, the monomer is dispersed by vigorous mechanical agitation into small droplets. These droplets are suspended in a second liquid phase in which both the monomer and polymer are insoluble. The droplets of monomer are polymerized with the aid of a catalyst and heat while dispersion is maintained. Agents that hinder the coalescence of these droplets during polymerization are added to the suspending liquid.

Depending upon the particular monomer treated, hard or soft spheres, beads, or, less often, irregularly shaped granules are formed. The intense heat of polymerization, common to most monomers, is dissipated rapidly; easily filtered products, which may then be dried, can be obtained from many types of monomers.

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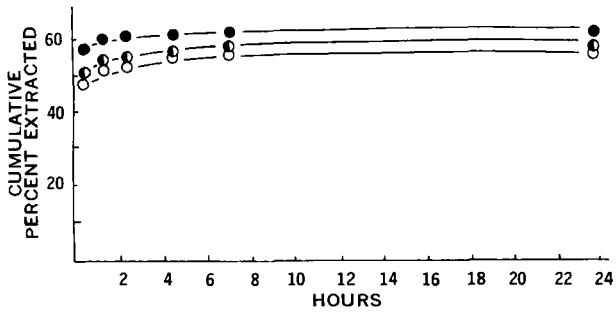

Figure 1—*Dissolution patterns of acetaminophen from non-expanded polystyrene beads. Key:* ●, $N_3S_2P_1$; ◐, $N_3S_2P_2$; *and* ○, $N_3S_2P_3$.

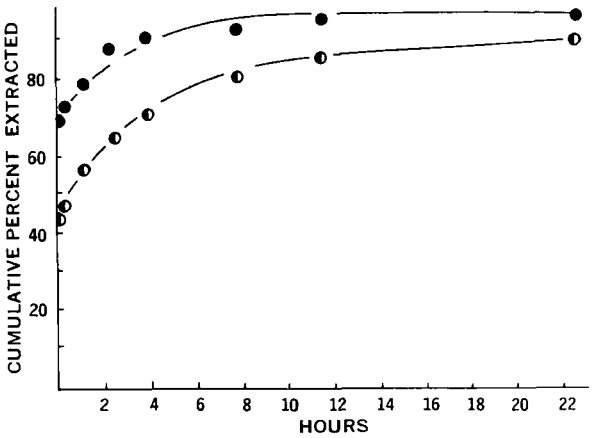

Figure 2—*Dissolution patterns of acetaminophen from expanded polystryrene bead formulations. Key:* ●, *Formulation 1; and* ◐, *Formulation II.*

EXPERIMENTAL

Materials—The following were used:

Monomers—Styrene[1], divinylbenzene[1], ethylvinylbenzene[2], and methyl methacrylate[1].

Catalysts—Benzoyl peroxide[1] and 2,2′-azobisisobutyronitrile[1].

Suspension Stabilizers—Reagent grade sodium sulfate, sodium polyacrylate[3], polyvinyl alcohol[1], and reagent grade dibasic calcium phosphate.

Drugs—Acetaminophen and sulfaethidole, drug grade.

Suspension Polymerization Apparatus—*Principle of Operation*—An apparatus similar to that used by Khanna *et al.* (1) was employed. Bead size was controlled by carefully regulating the rate of agitation and by varying the concentration of suspension stabilizers added to the aqueous phase. For this investigation, a bead size of approximately 30 mesh was desired.

Production Procedures—Polymerization with styrene as the only monomer was conducted in the presence of 0.4 g of calcium phosphate and 0.1 g of polyvinyl alcohol. Polymerization of styrene–divinylbenzene–ethylvinylbenzene terpolymer was conducted in the presence of 2.4 g of sodium carboxymethylcellulose. Three speeds of agitation, 400, 450, and 500 rpm, were used to obtain dispersion of the monomer; two dispersion stabilizers, sodium polyacrylate (in concentrations of 0.05, 0.10, and 0.15%) and sodium sulfate (in concentrations of 0.50, 1.00, and 1.50%), were incorporated in the formulations. To designate individual samples, the following system of nomenclature was used for the non-expanded polystyrene beads:

Speed of Agitation	Sodium Polyacrylate Concentration	Sodium Sulfate Concentration
N_3 = 400 rpm	P_1 = 0.05%	S_1 = 0.50%
N_4 = 450 rpm	P_2 = 0.10%	S_2 = 1.00%
N_5 = 500 rpm	P_3 = 0.15%	S_3 = 1.50%

All formulations of nonexpanded beads were prepared in 600 ml of water using 100 g of monomer and 1 g of benzoyl peroxide as catalyst. To produce beads, the water was added to a 1000-ml, three-necked, round-bottom flask and then placed in an 84° water bath. While stirring, the dispersion stabilizers were added to the flask through the center neck and the flask was flushed with nitrogen. When the contents of the flask were well dispersed, with continuous stirring, the monomer, catalyst, and medicament, previously mixed, were added through the center neck. The reaction was allowed to continue for 24 hr at the specified temperature and rate of agitation. Following polymerization, the beads were filtered, washed with several portions of water, and allowed to dry at 70°.

Preparation of Expanded Beads—To manufacture expanded beads, nonexpanded beads were first produced in the absence of drug according to the previous procedure using 450 rpm, 3 g of sodium sulfate, and 0.9 g of sodium polyacrylate. The beads were then sieved and those beads retained on a 30-mesh screen were placed in *n*-pentane and vigorously agitated for various times. When agitation was discontinued, the beads were filtered and added slowly to boiling water. Four formulations of expanded beads were prepared. All formulations were identical except for the length of time they were exposed to *n*-pentane. Formulations I, II, and III were exposed to *n*-pentane for 24 hr while Formulation IV was exposed to *n*-pentane for only 12 hr. After expansion, the beads were placed in a concentrated alcoholic solution of acetaminophen (1 g of acetaminophen/10 ml of 95% ethanol) and agitated for 24 hr.

Preparation of Tablets from Expanded Beads—Several samples of the dried expanded polystyrene beads containing acetaminophen were accurately weighed. Accurately weighed portions of potassium chloride were then added in specific proportions so that a mixture weighing exactly 0.5000 g would be obtained. Potassium chloride was added to demonstrate the effect of a channeling agent upon dissolution of acetaminophen from the compressed tablets. These mixtures were then compressed at each of two different pressures (3000 and 6000 psi) using a press[4] and a 1.27-cm (0.5-in.) test cylinder. The finished tablets weighed 0.5000 ± 0.005 g. Tablets were made to contain 0, 20, and 40% potassium chloride. No binders or lubricants were added.

Test Methods—*Assay Procedure*—A modified spectrophotometric assay based upon the NF assay for acetaminophen (2), using a grating spectrophotometer[5], was used to determine the acetaminophen content of the various formulations. A standard curve was constructed using five different concentrations of acetaminophen in absolute methanol, and absorbances were determined at 250 nm. Using the method of least squares, a plot of concentration *versus* absorbance was made to establish the existence of a linear relationship which agreed with Beer's law.

To assay for drug content, the polymer beads were pulverized using a wedgewood mortar and pestle to a fine (approximately 50-mesh) powder and accurately weighed portions were placed into 100-ml volumetric flasks containing 1.0 ml of 0.1 *N* hydrochloric acid and made to volume with absolute methanol. The flasks were allowed to stand for 1 hr with occasional shaking. At the end of this time, the contents of the flasks were filtered and the insoluble polymer removed. Specified quantities of the filtrate were added to each of two 50-ml volumetric flasks containing 0.5 ml of 0.1 *N* hydrochloric acid and made to volume with absolute methanol. Absorbances of the samples were then determined against a reference standard consisting of 1.0 ml of 0.1 *N* hydrochloric acid in enough methanol to make 100 ml. Samples of polystyrene beads containing no acetaminophen were treated in a similar manner to determine if any impurities had been extracted that might interfere with the assay. No interference was noticed.

Density—Bulk density of the various formulations was determined by placing a known quantity of the polymerized beads into a 100-ml cylindrical graduate. The graduate was then dropped,

[1] Matheson, Coleman and Bell, Norwood, Ohio. Monomers were washed with 1 *N* sodium hydroxide before use to remove stabilizer.
[2] Evisol, Foster Grant, Leominster, Mass.
[3] Acrysol, Rohm and Haas, Philadelphia, Pa.
[4] Carver laboratory press model C.
[5] Beckman model DB-GT.

from a height of 5.08 cm (2 in.), five times onto a desk top. The bulk density was then calculated by dividing the weight of beads added to the graduate by the volume occupied by the beads after tamping.

True density was determined using a pycnometer and filling it with 95% ethanol. Subsequent calculations provided the true density.

Dissolution—Dissolution studies were conducted using the apparatus described by Souder and Ellenbogen (3).

Approximately 2-g samples of beads, accurately weighed and containing drug, were placed into eight 90-ml, glass, screw-cap vials. To each vial was added 60 ml of prewarmed simulated gastric fluid USP. The glass vials were then rotated, end-over-end, at 40 rpm in a water bath maintained at 37°. One vial was removed at each of the following time periods: 0.25, 0.50, and 1.50 hr. The five vials remaining after 1.50 hr were also removed and filtered to allow replacement of gastric fluids by prewarmed simulated intestinal fluid USP. The five vials were returned to the dissolution apparatus, and one vial was then removed at 2.33, 4.50, 7.00, and 24 hr from the start of dissolution testing.

A similar procedure was followed for samples of tablets compressed from the expanded beads.

Typical release patterns from nonexpanded and exanded bead formulations are presented in Figs. 1 and 2. The effect that slight changes in porosity has upon dissolution is very evident in Fig. 2. Although Formulations I, II, and III were exposed to *n*-pentane for the same time, they each exhibited slightly different values for porosity, thus leading to different rates of dissolution.

RESULTS AND DISCUSSION

All beads produced by the suspension polymerization procedure employed in this investigation were of a white opaque nature and were free flowing. The nonexpanded polystyrene beads of all formulations were almost perfect spheres containing several areas of entrapped gas. Closer examination indicated that these areas of entrapped gas were not interconnecting. Expansion of the polystyrene beads produced particles which were distorted; however, they still possessed spheroidal characteristics. These beads possessed many internal interconnecting channels and considerable external pore openings. Examination of tablets compressed from the expanded beads revealed that most individual beads retained their original shape, but some were deformed to various degrees. Channels between the individual beads in the tablet were also in abundance.

A major preliminary problem encountered in this investigation was the instability of the medicament in the reaction medium during the polymerization period. Initially, sulfaethidole was the test drug chosen for incorporation. However, it underwent considerable chain transfer and was, therefore, unsuitable for use in the polymerization process. Acetaminophen was selected on the basis of its compatibility with the reactants.

Polymerizations involving the terpolymer, styrene–divinylbenzene–ethylvinylbenzene, did not yield beads suitable for this investigation. Considerable agglomeration of the polymer and degradation of the medicament were observed. It was, therefore, decided to limit this study to styrene.

Initially, styrene was polymerized in the presence of acetaminophen. The average percent acetaminophen in the 27 different formulations of the nonexpanded beads was found to be 1.30%. Dissolution studies revealed that the nonexpanded polystyrene beads released an average of 64% of the available medicament within the first 30 min of dissolution. No further increase in the amount of drug released was noticed throughout 24 hr of dissolution testing. This appears to indicate that 64% of the dissoluted drug was drug located on or near the surface of the bead; the 36% not available for use was bound internally by the plastic matrix.

To overcome this problem, several samples of spherical polystyrene beads were produced in the absence of drug. These beads were then expanded with the aid of a blowing agent. After expansion they were exposed to a concentrated alcoholic solution of acetaminophen (1 g of acetaminophen/10 ml of 95% ethanol) for 24 hr. Drug content of these formulations was dependent on the porosity of each formulation. Those beads exposed to *n*-pentane for 24 hr (Formulations I, II, and III) exhibited an average porosity of 92.4% and contained an average of 20.4% acetaminophen. Formulation IV, exposed to *n*-pentane for only 12 hr, showed considerably less porosity, 40.5%, and a greatly diminished drug content, 5.78%. Dissolution studies indicated that the expanded polystyrene beads released 64% of the available medicament within the first 30 min of dissolution. However, they also provided for an exponential release of an additional 29% throughout the 24 hr of dissolution testing.

Tablets compressed from the expanded beads, containing a channeling agent, released 86% of the drug during the first 30 min and released an additional 12% of available acetaminophen over 12 hr. The expanded polystyrene beads employed were found to exhibit two distinct phases of drug release. The first phase occurs very rapidly and is believed to be due to dissolution of the drug from the surface of the bead. The second phase occurs at a much slower rate and is attributed to leaching of the drug through the channels present in the plastic matrix of the expanded beads.

The investigation was limited to the study of suspension polymerization using initiators which decompose when heated to form free radicals. Most of these initiators are strong oxidizers and, therefore, limit the types of medicaments that may be added to the polymerization reaction. The process of expanding the polystyrene beads and incorporating the medicament after bead expansion allows for an unlimited choice of medicaments.

SUMMARY

The technique of suspension polymerization for incorporation of drugs into a polymer was studied. Nonexpanded polystyrene beads exhibited no timed release of medicament. Expansion of the beads produced a large number of interconnecting channels and allowed the medicament to be released from the bead in a timed-release manner. Drug incorporation into the nonexpanded beads was limited to 1.30% while that of the expanded, more porous beads was about 20.4%.

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[x] To whom inquiries should be directed.

Semisolid Oleaginous Ointment Bases for Ophthalmic Use

RAYMOND W. JURGENS, Jr.[x], and CHARLES H. BECKER

Abstract □ A high molecular weight nonemulsifiable polymer, a fused micronized silica, and sodium stearate were each evaluated for their gel-forming characteristics with hexadecyl alcohol and other liquid vehicles. Selected formulations of these were studied as possible oleaginous, semisolid bases for use as ophthalmic ointments over 0 to 50° and compared to two commonly used ointment bases for physical characteristics and drug release.

Keyphrases □ Ointment bases for ophthalmic use—formulation of semisolid oleaginous, water-free bases, physical properties and pilocarpine hydrochloride release □ Ophthalmic ointment bases—formulation of semisolid oleaginous, water-free bases, physical properties and pilocarpine hydrochloride release □ Oleaginous semisolids—evaluated for use as ophthalmic ointment bases, physical properties and pilocarpine hydrochloride release

Little research has been done on ophthalmic ointment bases. Until recently, if a water-free, oleaginous base was desired, petrolatum was the base most frequently employed. Because petrolatum is of natural origin, however, it may be expected to vary with its source and its method of refinement (1). Due to this variability, a base with a more uniform consistency would be desirable. Also, under cold weather conditions, petrolatum base ointments are difficult to extrude from an ointment tube and, when instilled into the eye by application to the lower eyelid, they exhibit poor release of the medicament (2). Water-removable or water-containing bases sometimes are not desirable carriers of medicaments unstable in the presence of water; at freezing temperatures, the water may crystallize, causing separation of the vehicle into two phases.

Recently the gelling of various nonpolar solvents from new pharmaceutical materials has brought about many applications for oleaginous-type bases (3). The versatility and applicability of recent pharmaceutical materials have provided many new opportunities for the formulation of oleaginous bases. The stability of these bases has been improved as has, in some cases, their activity due to the increased release of an incorporated medicament of the base.

In this study, a high molecular weight nonemulsifiable polymer[1], a food grade sodium stearate, and a fused micronized silica[2], when gelled with different nonpolar solvents, were investigated as to their possible effectiveness for use as oleaginous ophthalmic ointment bases. An evaluation of their physical properties included a comparison with two commonly used oleaginous bases, petrolatum and a hydrocarbon oil and wax gel combination[3].

The objectives of this investigation were to formulate by reproducible methods and to evaluate several properties by different parameters water-free, oleaginous, water-insoluble, gel-type pharmaceutical bases which are spreadable or extrudable over 0–50°. The ingredients of the bases were chosen to be compatible with the eye and surrounding areas, although no attempt was made to perform an *in vivo* eye irritation study.

[1] Epolene C-10, Eastman Kodak.
[2] Cab-O-Sil, Cabot Corp.
[3] Plastibase, Squibb.

Table I—Description of Bases Employed

Base Description	Percent	Method of Preparation
1. White petrolatum		
2. Hydrocarbon oil and wax gel combination[a]		
3. Micronized fumed silica[b]	12.0	A
Stearyl alcohol USP	2.0	
Hexadecyl alcohol[c]	86.0	
4. Nonemulsifiable polymer, mol. wt. 8003[d]	10.0	B
Light mineral oil USP	90.0	
5. Sodium stearate, food grade	10.0	C
Hexadecyl alcohol[c]	90.0	

[a] Plastibase, Squibb. [b] Cab-O-Sil, Cabot Corp. [c] Enjay Corp. [d] Epolene C-10, Eastman Kodak.

EXPERIMENTAL

Preparation of Ointment Bases—New materials introduced as gelling agents in different vehicles were investigated in preliminary studies to determine if the gelled products would be applicable for evaluation as ophthalmic ointment bases. The bases (Table I) were chosen as a result of their apparent stability over 1 month at 0–50°.

Method A—The liquid vehicle was placed in a beaker and heated to 90° on a water bath while stirred[4] at medium speed. The matrix polymer or solid was then added with continued stirring and heating until either a homogeneous dispersion or gelation resulted. The resulting semisolid was then allowed to cool to room temperature.

Method B—The vehicle was heated to 110–120° on a hot plate. The matrix and any modifiers were added to the vehicle with moderate stirring until thoroughly dispersed or dissolved. The mixture was then shock cooled by pouring it as a thin-layered film over precooled aluminum foil stretched on an acetone–ice bath at approximately −5° until gelation resulted.

Method C—The matrix and any modifiers were suspended or dissolved in a small amount of hot (85–90°) distilled water. This mixture was then added to the previously heated vehicle in an evaporating dish, and the vehicle–mixture combination was heated until *all* water was removed by evaporation. The resulting material, after being allowed to cool to room temperature, was levigated thoroughly with a spatula until a smooth semisolid formed.

Softness Point—Determination of the temperature at which the base became soft was accomplished by immersing three-quarters of a 10-ml beaker containing approximately 3.0 g of the base to be tested in a controlled-temperature water bath. A 1.1-g stainless steel ball bearing was then placed on the surface of the base. When the ball bearing became completely covered with the

[4] With a Lightnin' Mixer, Model F.

Table II—Water Numbers of Bases Stored in Type I Glass Containers at Different Time Intervals at Selected Temperatures

Base	Weeks[a]	Storage Temperature 0°	30°	40°	50°
1	0[a]	17.0[b]	17.0	17.0	17.0
	2	19.0	15.0	12.6	16.2
	4	16.9	15.4	13.5	15.4
	12	16.7	15.6	21.0	16.4
	24	15.0	22.0	20.0	18.0
2	0	24.8	24.8	24.8	24.8
	2	20.6	20.7	20.7	19.2
	4	18.6	17.2	14.5	20.6
	12	18.7	18.6	15.0	16.8
	24	20.0	20.9	16.7	16.7
3	0	76.8	76.8	76.8	76.8
	2	33.6	78.0	68.0	77.8
	4	32.8	64.5	54.5	72.0
	12	35.0	41.7	37.3	20.0
	24	49.2	39.2	37.2	20.0
4	0	21.0	21.0	21.0	21.0
	2	18.5	16.4	19.2	20.8
	4	16.3	16.3	18.1	16.8
	12	16.7	16.1	23.8	16.9
	24	18.5	17.1	20.1	20.6
5	0	42.6	42.6	42.6	42.6
	2	46.6	40.8	47.8	43.8
	4	48.7	45.9	48.0	38.8
	12	48.9	50.0	49.0	32.3
	24	50.1	50.0	54.0	34.1

[a] Zero time is defined as the 24-hr period necessary to prepare and to test the ointment base initially. [b] All values have an allowable variance of ±3.

ointment from increasing the temperature of the water bath, the temperature was then recorded as the base's softness point.

Water Absorption—In this study, a "water number" was used as a parameter for the percentage of water absorbed by a given base. The percentage of water absorption was determined similarly to a method described by Halpern and Zopf (4). The base was first weighed to the nearest 0.01 g and placed in an evaporating dish; then the ointment-containing dish was heated on a hot plate to a temperature at which the particular base became very soft. Distilled water was added in increments of 0.5 ml and thoroughly mixed in the base. The base was then removed from the heat source and allowed to cool to room temperature. This process was continued until the water extruded from the base after cooling. The quantity of water absorbed per amount of base was used to calculate the water number according to Eq. 1:

$$\text{water number} = \frac{\text{milliliters of water absorbed}}{\text{grams of base used}} \quad \text{(Eq. 1)}$$

Viscosity Determination—Viscosity studies of the bases over a 6-month period were conducted using a rotational viscometer[5]. Yield values before and after storage over 6 months were determined by a viscometer[6], and the shearing stress was applied by a weight hanger with various slotted weights. The bases were stored in Pyrex beakers tightly sealed[7] at 0, 30, 40, and 50°. To observe any changes in flow rates of each base over the specified time, rheograms of shearing stress *versus* rate of shear at the different temperatures were plotted.

Release Rate Observations—To compare the release rates of the different bases after storage, a frequently employed ophthalmic drug (pilocarpine hydrochloride) was incorporated into the ointment. This incorporation was accomplished by levigating the finely powdered pilocarpine hydrochloride USP[8], 4%, into approximately 30.0 g of base. Each ointment base with the incorporated drug was then suspended in a dissolution apparatus, and the quantity of the drug released from the base into the normal saline dissolution medium was assayed on a grating spectrophotometer[9] by analyzing 5-ml aliquots of the normal saline dissolution medium at the specified intervals. A standard curve was constructed using five different concentrations of pilocarpine hydrochloride in normal saline. Absorbances of these solutions were determined at 216 nm. A plot of concentration *versus* absorbance was then made to establish a linear relationship in conformity with Beer's law.

The dissolution apparatus consisted of placing the drug-incorporated ointment in one side of a disposable laboratory petri dish, 100 × 15 mm, and inverting and suspending it in 800 ml of normal saline solution (0.9%), using a stainless steel sieve[10] affixed to the open end to prevent the ointment from falling out of the container. A water bath held at 30°, surrounding the container of normal saline, kept the saline dissolution medium at constant temperature.

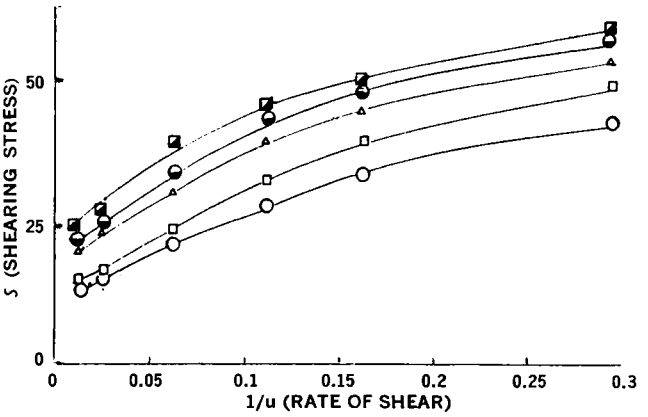

Figure 1—*Characteristic rheogram for Base 4 at different storage times at 40°. Curves represent relative flow patterns from initially prepared sample and are not indicative of exact viscosity values. Key:* ○, S_0 *(initial reading);* □, S_1 *(2 weeks of storage);* △, S_2 *(4 weeks of storage);* ◒, S_3 *(12 weeks of storage); and* ◪, S_4 *(24 weeks of storage).*

RESULTS AND DISCUSSION

This investigation was conducted to develop an oleaginous water-free ointment base intended for ophthalmic use that would maintain its consistency over 0–50°. Three experimental bases (Bases 3, 4, and 5, Table I) were chosen from a large number of exploratory bases because of their apparent physical stability over 0–50°. Quantities of these experimental bases were manufactured and compared to two standard bases (Bases 1 and 2) in relation to possible changes in viscosity over 6 months, drug release studies over 1 month, and water absorption and softness temperature alterations over 6 months. All bases were stored at 0, 30, 40, and 50° in Type I glass vials and Pyrex-type containers to observe any alterations in these physical characteristics.

Water numbers were calculated to give an indication of the aqueous absorptivity of the various bases and of release of the drug from the base. Their initial values decreased in the following order: Base 3 < Base 5 < Base 2 < Base 4 < Base 1. Base 3 decreased its water-absorbing capacity significantly after initial storage at 40 and 50°. All of the remaining bases increased or maintained their water absorptivity after storage at all temperatures (Table II). The increase in water absorption of Bases 1, 2, and 4 appeared independent of the temperature at which the base was stored. It is possible that the base may have slightly altered its physical molecular arrangement upon standing at various temperatures or some impurity in the base might have caused a slight emulsification. The tendency of emulsification then could have increased water absorption by the base yielding higher values after an extended time.

The initial softness temperatures or points of the various bases stored in Type I glass vial containers decreased in the following order: Base 5 < Base 2 < Base 4 < Base 1 < Base 3. After 6

[5] Haake Rotovisco (67-369) with a six-bladed star-shaped paddle (FL-10).
[6] Stormer.
[7] With Parafilm (American Can Co.) and aluminum foil.
[8] S. B. Penick.
[9] Beckman model DG-GT.
[10] Fisher No. 4-6.

Table III—Release of Pilocarpine Hydrochloride from Freshly Prepared Bases at Selected Time Intervals

Base	Concentration (moles/liter) × 10^{-5} (Cumulative Percent Extracted)					
	Hours					
	0.25	0.50	1.00	3.00	6.00	12.00
1	1.00 (0.140)	1.00 (0.140)	1.30 (0.183)	1.50 (0.212)	1.50 (0.212)	1.80 (0.254)
2	3.10 (0.459)	3.20 (0.474)	4.00 (0.592)	4.10 (0.607)	4.40 (0.651)	5.00 (0.740)
3	39.00 (5.790)	80.00 (11.880)	160.00 (23.760)	300.00 (44.550)	350.00 (51.970)	500.00 (74.242)
4	12.50 (1.672)	13.00 (1.740)	13.50 (1.809)	13.50 (1.809)	14.00 (1.874)	15.00 (2.008)
5	34.00 (5.049)	40.00 (5.939)	45.00 (6.685)	60.00 (8.909)	80.00 (11.879)	120.00 (17.818)

Table IV—Release of Pilocarpine Hydrochloride from Different Bases at Selected Time Intervals after 1 Month of Storage at 30°

Base	Concentration (moles/liter) × 10^{-5} (Cumulative Percent Extracted)					
	Hours					
	0.25	0.50	1.00	3.00	6.00	12.00
1	1.50 (0.210)	1.70 (0.238)	1.80 (0.252)	2.00 (0.280)	2.20 (0.308)	2.40 (0.336)
2	3.50 (0.490)	4.00 (0.560)	4.00 (0.560)	4.50 (0.630)	4.70 (0.658)	5.00 (0.770)
3	20.00 (3.182)	28.00 (4.455)	35.00 (5.568)	50.00 (7.955)	120.00 (19.091)	380.00 (60.455)
4	7.00 (1.039)	9.00 (1.364)	10.00 (1.485)	10.00 (1.485)	12.00 (1.782)	14.00 (2.079)
5	60.00 (9.671)	80.00 (12.895)	100.00 (16.118)	120.00 (19.342)	130.00 (20.954)	180.00 (29.013)

months of storage at various temperatures, Base 3 increased its initial softness temperature and Bases 1, 2, and 4 decreased their softness point. An increase or decrease of the softness temperature of a base was independent of the storage temperature. The time of storage, however, was significant. An increase or decrease from the original softness value of the base always occurred after a minimum of 1 month of storage in any container. This may have been due to a "settling" phenomenon of the base giving more rigidity or compactness after no disturbance of the base occurred (as in its production, packaging, *etc.*). From the data available, however, it was not possible to explain the increase in softness values of Base 3 after storage.

Viscosity rheograms were constructed for the various bases stored at 0, 30, 40, and 50° for 6 months from rotational viscometer measurements to show possible alterations in flow behavior upon storage of the ointment. All bases showed a plastic flow behavior with various yield values measured with a viscometer. The gelled matrix-type bases, 2 and 4, showed insignificant changes in flow characteristics, altering little from their initial stress *(S)* values after storage at the selected temperature (Fig. 1). The remaining bases increased their stress values significantly after storage at all temperatures, with 0° readings showing the greatest increases from the initial readings.

The flow behavior was not considered significant in any case; no base was unspreadable or unmanagable to work with after storage. Base 5, after storage for more than 1 week, lacked spreadability and became too wax-like and hard to construct a rheogram.

Incorporated pilocarpine hydrochloride released from the bases into a normal saline solution had the following order of release: Base 3 > Base 5 > Base 4 > Base 2 > Base 1 (Tables III and IV). A dissolution model was developed to describe *in vitro* the release that may occur if the ointment is instilled into the eye *via* application to the lower eyelid. Bases 3 and 5 showed the best release of the bases although Base 3 started to disintegrate in the dissolution medium after 6 hr of exposure. Bases 4 and 2 released between 1 and 2% of their medicament after the conclusion (12 hr) of dissolution. Base 1 showed an increased release of the drug after 1 month of storage at 30 and 50°, but a decrease occurred at 0° after the same storage period.

Of the bases investigated using the experimental parameters, Base 4 had the most overall desirable properties. However, this investigation was limited to a study of certain *in vitro* parameters that may be used to develop and select a desirable ophthalmic ointment base. *In vivo* irritation studies of the bases on eye surfaces would be the next useful step in determining which of the bases studied may be applicable for further testing.

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[x] To whom inquiries should be directed. Present address: School of Pharmacy, Southwestern State College, Weatherford, OK 73096

Oxyphenbutazone and Phenylbutazone Determination in Plasma and Urine by GLC

ROBERT B. BRUCE[x], WILLIAM R. MAYNARD, Jr., and LYNDA K. DUNNING

Abstract □ A method was developed for the determination of phenylbutazone and oxyphenbutazone in biological fluids using GLC. Plasma levels and urinary excretion following doses of 150 mg of phenylbutazone to dogs and 8.9 mg/kg to horses are presented. The average plasma half-lifes of phenylbutazone found were 2.5 hr in dogs and 4 hr in horses. No measurable hydroxy derivative could be detected in the plasma. The hydroxy derivative was present in horse urine for at least 48 hr but not after 72 hr.

Keyphrases □ Phenylbutazone—GLC determination in plasma and urine, compared to UV method □ Oxyphenbutazone—GLC determination in plasma and urine, compared to UV method □ GLC—determination, phenylbutazone and oxyphenbutazone in plasma and urine

The present study was undertaken to determine the bioavailability of new formulations of phenylbutazone, an anti-inflammatory and antiarthritic agent. Previously reported blood level and excretion studies were based on estimation methods using UV spectrophotometry (1, 2). In this study, GLC methods were developed and used. Such methods are more specific and easier to use than those involving spectrophotometry. Another GLC method using different conditions was reported (3) while this study was in progress. However, no results were presented in that study, and a method for the primary metabolite, oxyphenbutazone, was not given.

EXPERIMENTAL

Analytical Methods—Phenylbutazone was determined directly by GLC. However, the primary metabolite, oxyphenbutazone, had to be derivatized before it could be determined.

Materials—The tablets (Formulations I[1] and II[2]) contained 100 mg phenylbutazone/tablet. Some tablets from each preparation were weighed, and one-half tablet (within ±4%) was used so that 150-mg doses of phenylbutazone could be administered to the dogs.

Two tablets and a granulation of phenylbutazone were compared with a comparable dose of a solution in the horse studies. The tablets contained 1 g phenylbutazone/tablet, and the granules contained 2 g phenylbutazone/7.7 g. The solution was prepared as a 6% solution (w/v) in 5% $NaHCO_3$.

***In Vivo* Testing**—All animals were fasted overnight before dosing. Six male mongrel dogs were used to study Formulation I and the solution. Four dogs were given Formulation II. Six mares were used in the horse studies. The dogs were given doses of 150 mg of phenylbutazone as a single oral dose, and horses were given a single oral dose of 8.9 mg/kg body weight. Two weeks were allowed between studies to overcome enzyme induction effects.

Blood samples were taken at the intervals shown in the tables. Urine from dogs was pooled for 0–24 and 24–48 hr. Since it was not practical to collect 24-hr samples of urine from the horses, spot samples were collected at intervals as shown in the tables.

To 2.0 ml of plasma or 5.0 ml of urine, 5.0 ml of water and 0.5 ml of 2 *N* H_2SO_4 were added. This mixture was extracted three times with 20-ml portions of nanograde benzene, centrifuging after each extraction and drying the benzene through a layer of anhydrous sodium sulfate. The sodium sulfate was washed with an additional 5 ml of benzene, and the pooled benzene extracts were evaporated to dryness under a stream of nitrogen. Heptafluorobutyric anhydride (0.20 ml) was added to the residue, mixed, and allowed to stand for 10 min. The excess reagent was evaporated with a stream of nitrogen, and the residue was dissolved in 0.5 ml of methylene chloride. The methylene chloride was evaporated to 100 μl, and 5.0 μl was immediately injected into the gas chromatograph[3]. Delays may cause hydrolysis of the esterified products. The conditions for GLC were: flame-ionization detector temperature, 280°; column, 1.21 m (4 ft), stainless steel with 3% OV-210 on Gas Chrom Q at 185°; injector temperature, 300°; and flow rate, 40 ml nitrogen/min. The retention times were 5 min for phenylbutazone and 11 min for the heptafluorobutyrate of oxyphenbutazone.

If it were desired to determine only unchanged phenylbutazone, the esterification step was omitted and the residue was taken up in ether and injected.

UV Method—The results from the GLC method were compared with those obtained by a slight modification of the UV method originally described by Burns *et al.* (2). A baseline modification was used by measuring the maximum absorbance (265 nm) from a baseline drawn between the recorded absorbances at 255 and 280 nm. The background absorbance was eliminated by this procedure. A linear relationship, passing through the origin, between the amount of phenylbutazone added to blood and absorbance was obtained. The slope of the line was 0.231 μg/mm.

Table I—GLC Determination of Known Quantities of Phenylbutazone and Oxyphenbutazone Added to 5.0 ml of Control Sample

Phenylbutazone, μg		Oxyphenbutazone, μg	
Added	Found	Added	Found
	Plasma		
2.5	2.3	10	9.5
5.0	4.9	15	14.0
10.0	10.2	20	19.0
15.0	15.1	40	44.0
15.0	14.7	40	42.0
	Urine		
10	10.8	10	10.2
20	19.2	20	19.0
30	30.8	30	28.6
40	42.1	40	39.0
50	50.0	50	44.9

RESULTS AND DISCUSSION

The recoveries of phenylbutazone and oxyphenbutazone when known amounts were added to plasma or urine are shown in

[1] A. H. Robins Co.
[2] Veterinary formulation purchased on the open market.
[3] Barber Coleman Series 5000.

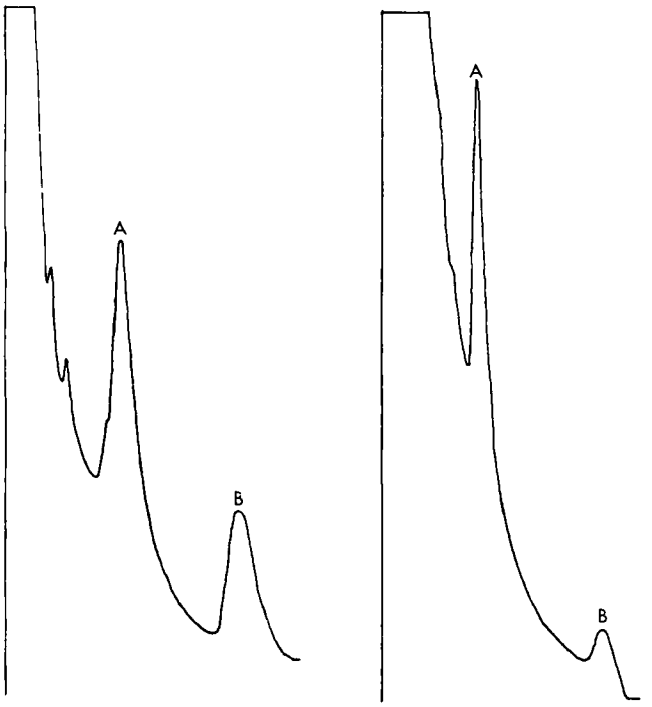

Figure 1—*Typical chromatograms found for plasma (left) and urine (right) samples. Key: A, phenylbutazone; and B, oxyphenbutazone heptafluorobutyrate.*

Table I. Chromatograms shown in Fig. 1 are typical of those obtained during the analyses. Sensitivities of the UV and GLC methods are approximately the same.

Dogs—Results obtained by the UV and GLC methods using plasma from the individual dogs that had received the solution are shown in Table II. In general, the values found by the UV method were considerably higher than those found by GLC. Mean values for the six dogs gave a maximum plasma concentration of 50.5 μg/ml by the UV method, whereas those found by the GLC method (Fig. 2) gave 30.5 μg/ml. The half-lives obtained from the mean values were approximately the same, being about 2.5 hr, and the mean maximum plasma level in each case occurred at 1 hr.

The difference in results obtained by the two methods warrants some discussion. Accuracy and precision as measured by the percent recovery and standard deviation were approximately the same, and the average percent recovery was 100.8% in each case. The standard deviations for (10 μg) 10 determinations were ±0.46 for GLC and ±0.67 for the UV method. Therefore, the difference must be due to the presence of some phenylbutazone-related material. The remaining plasma from one dog was pooled and extracted in the usual manner for the UV procedure. The solvent was evaporated to a small volume and subjected to TLC, using ethyl acetate–benzene (1:9) on silica gel. This procedure revealed three UV-absorbing spots at R_f 0.26, 0.68, and 0.89 in addition to phenylbutazone, which may have interfered with the UV method of analysis. These were not investigated further for lack of sufficient material.

Phenylbutazone appeared in the first samples of plasma taken after dosing (30 min) when the solution was administered, and the maximum plasma level occurred at this time or at 1 hr. When Formulation I was given, the first appearance of measurable plasma concentration was at 30 min for three dogs, at 1 hr for two dogs, and at 2 hr for the other dog. The maximum plasma concentration occurred at 2 hr in all dogs except one, where it occurred at 4 hr. With Formulation II, none of the dogs showed measurable plasma levels at 30 min; in two dogs the drug appeared at 1 hr, and in the other two dogs it occurred at 2 hr. The maximum plasma levels appeared only after 4 hr in all cases with Formulation II.

The phenylbutazone dose was prepared as an aqueous solution

Table II—Phenylbutazone Plasma Levels in Six Dogs following Oral Administration of 150 mg of Phenylbutazone in Solution

	Phenylbutazone, μg/ml					
Hours	Dog 1 (15.2 kg)	Dog 2 (11.1 kg)	Dog 3 (8.0 kg)	Dog 4 (10.9 kg)	Dog 5 (8.3 kg)	Dog 6 (8.2 kg)
			GLC			
0	0	0	0	0	0	0
0.5	30.0	33.2	23.4	25.3	26.6	37.1
1.0	31.4	18.3	24.7	36.4	30.6	47.8
2.0	16.9	14.3	20.8	14.1	14.8	23.2
4.0	12.2	7.8	13.8	9.6	6.2	10.7
6.0	9.7	2.8	7.4	7.8	2.0	5.4
8.0	6.1	0.7	2.8	5.8	0.4	4.9
24	0	0	0	0	0	0
			UV			
0	0	0	0	0	0	0
0.5	45.9	54.0	34.0	31.6	49.3	60.7
1.0	35.5	32.6	59.2	68.3	50.6	56.9
2.0	41.6	32.6	63.6	32.9	29.1	49.3
4.0	32.6	18.5	42.2	20.2	14.6	15.2
6.0	18.5	5.9	19.2	13.9	5.1	15.2
8.0	14.1	3.7	10.4	13.9	0	10.1
24	3.7	2.2	2.6	0	0	0

Table III—Excretion of Phenylbutazone and Oxyphenbutazone in Urine by Dogs Receiving 150 mg as Measured by GLC Analysis

	Phenylbutazone, mg			
	Dog 1	Dog 2	Dog 3	Dog 5
Formulation II:				
0–24 hr	0.009	0.69	1.18	9.30
24–48 hr	2.43	1.12	4.15	2.37
Solution:				
0–24 hr	0.56	0.27	0.33	0.52
24–48 hr	1.53	1.57	0.32	0.87
Formulation I:				
0–24 hr	2.40	5.43	0.76	2.43
24–48 hr	0	1.37	0.58	Lost
	Oxyphenbutazone, mg			
	Dog 1	Dog 2	Dog 3	Dog 5
Formulation II:				
0–24 hr	0	3.06	3.87	8.84
24–48 hr	4.25	8.10	3.01	30.60
Solution:				
0–24 hr	0.54	0.40	0.23	0.67
24–48 hr	4.65	1.39	1.88	6.08
Formulation I:				
0–24 hr	0.95	8.05	1.35	8.47
24–48 hr	3.68	6.01	3.15	Lost

Table IV—Concentrations (Micrograms per Milliliter) of Phenylbutazone in Plasma after Orally Dosing 8.9 mg of Phenylbutazone/kg as a Solution to Horses

Hours after Dosing	Horse					
	1	2	3	4	5	6
0	0	0	0	0	0	0
0.5	3.5	3.1	14.9	4.8	5.4	7.1
1.0	4.8	4.0	19.3	6.3	6.3	15.8
2.0	3.6	4.4	11.5	11.5	8.2	9.5
4.0	2.5	3.9	4.1	12.0	8.3	4.8
6.0	2.1	2.4	3.9	12.2	6.5	5.4
8.0	1.8	1.8	3.7	5.8	4.2	3.7
12.0	0.9	0.6	1.0	1.3	3.4	1.6
24.0	0	0	1.1	0.8	0.9	0.8
36.0	0	0	0.9	0.6	0	0
48.0	0	0	0.6	0.4	0	0

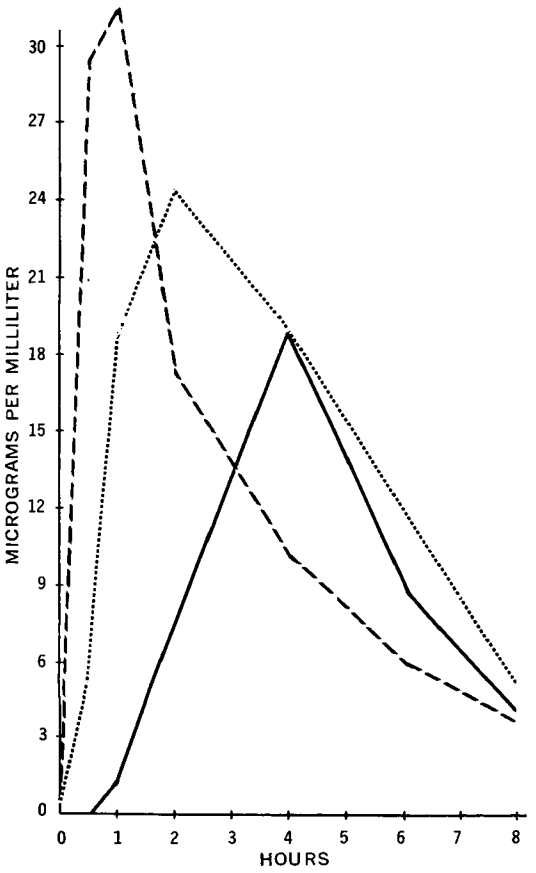

Figure 2—*Average phenylbutazone plasma levels found by GLC analysis after the administration of phenylbutazone to dogs. Key: – –, solution; - - -, Formulation I; and ——, Formulation II.*

in sodium bicarbonate because of its insolubility in water. This results in the formation of a sodium salt of the drug which is more rapidly absorbed than the free drug (4). The results presented in Fig. 2 show this effect. Perhaps a better comparison in the rates of plasma level rise and decrease would have been shown by the administration of the drug in a capsule. However, this procedure also has drawbacks since gelatin capsules often disintegrate slowly. A comparison of the total bioavailability can still be made from the results obtained by the methods used here.

The average values obtained by GLC analysis are shown in Fig. 2. These curves reflect the observations made previously. As another estimation of the relative bioavailability, the areas under the curves for the first 8 hr were calculated by triangulation, yielding the values of 71.8, 100.0, and 120.5 for Formulation II, the solution, and Formulation I, respectively. Each plasma sample obtained from the dogs after administration of the solution was analyzed for oxyphenbutazone, but none could be detected. Therefore, the remaining plasma samples were not analyzed for this compound.

The excretions of phenylbutazone and oxyphenbutazone are shown in Table III. The total amount of these compounds excreted is similar for various doses, but larger amounts of the hydroxy derivative were excreted from the solution and Formulation I than from Formulation II.

Horses—The plasma levels of phenylbutazone in horses (Table IV) show much greater variation than those found in dogs. No

Table V—Concentrations of Phenylbutazone and Oxyphenbutazone Found in Horse Urine after Dosing with 8.9 mg/kg as a Solution

Hours after Dosing	Horse 1	2	3	4	5	6
Phenylbutazone, μg/ml						
4.0	3.8	12.8	13.0	1.3	7.0	0
8.0	9.5	11.5	6.0	0	2.5	1.0
12.0	7.0	3.5	8.0	0	2.3	0
24.0	1.5	2.0	1.8	2.0	0	1.0
36.0	0	0	0	0	0	0
48.0	0	0	0	0	0	0
72.0	0	0	0	0	0	0
Oxyphenbutazone, μg/ml						
4.0	5.0	16.0	31.5	8.0	54.8	0
8.0	15.5	14.5	35.3	7.3	62.8	1.8
12.0	21.0	8.5	63.0	21.5	90.3	0
24.0	13.5	18.0	51.0	2.0	29.8	4.5
36.0	11.3	26.5	13.5	7.3	2.8	0
48.0	7.3	4.5	2.8	2.5	5.5	2.3
72.0	0	0	0	0	0	0

correlations could be found between plasma levels with either dosage form or with different animals. The estimated half-life of the drug using the average plasma level values found when the solution was given was about 4 hr. Measurable concentrations of phenylbutazone were present in the plasma of two of the horses for 48 hr. As in the case of the dogs, no measurable hydroxy metabolite could be determined in the plasma of horses.

The total excretion or excretion rates of phenylbutazone and oxyphenbutazone could not be determined in the horses because a suitable method of continuous collection was not available. Disappearance of the two compounds from the analyses of intermittent samples is shown in Table V. These figures indicate that, following a single oral dose of 8.9 mg/kg, oxyphenbutazone persists in the urine for at least 48 hr as measured by the described methods. The 72-hr samples gave negative results in all cases, and the unchanged drug could not be detected in the 36-hr samples.

The results of these studies indicate that GLC analysis of blood and urine for phenylbutazone and oxyphenbutazone is simple and specific. The method should be adaptable to the analysis of race horse urine in cases of suspected doping. Methods involving UV spectrophotometry have recently come under criticism (5) for such use.

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[x] To whom inquiries should be directed.

CNS Depressant Effects of Schiff Bases Derived from 4,6-Dichloro-5-pyrimidinecarboxaldehydes and Substituted Anilines

ARTHUR A. SANTILLI[x], DONG HAN KIM, RICHARD A. FIEBER, and STEPHEN V. WANSER

Abstract □ Several Schiff bases were prepared by the reaction of 4,6-dichloro-5-pyrimidinecarboxaldehydes with substituted anilines. When the aniline was substituted at the *ortho*-position by a mercapto group, cyclization to the corresponding benzothiazoline resulted. A representative number of the new compounds showed CNS depressant activity in mice at doses ranging from 12.7 to 400 mg/kg ip.

Keyphrases □ Schiff bases—prepared from 4,6-dichloro-5-pyrimidinecarboxaldehydes and substituted anilines, CNS depressant activity □ 4,6-Dichloro-5-pyrimidinecarboxaldehydes—reacted with substituted anilines to form Schiff bases, CNS depressant activity □ Anilines, substituted—reacted with 4,6-dichloro-5-pyrimidinecarboxaldehydes to form Schiff bases, CNS depressant activity □ CNS depressant activity—Schiff bases formed from 4,6-dichloro-5-pyrimidinecarboxaldehydes and substituted anilines

Anils and related isosteres such as stilbenes and diazo-type compounds are known to have varying chemotherapeutic effects (1). Certain Schiff bases derived from pyridoxal have been implicated in biological mechanisms involving transamination and oxidation (2). Relatively little has been reported, however, about the biological effects of Schiff bases derived from halogenated pyrimidinecarboxaldehydes (3). During an investigation of the synthesis of fused pyrimidine systems, Schiff bases were prepared from 4,6-dichloro-5-pyrimidinecarboxaldehyde intermediates (4) and substituted anilines. A representative number of these compounds were found to be central nervous system (CNS) depressants. In view of the paucity of information available for these types of derivatives, their preparation and observed biological responses are now reported.

CHEMISTRY

The Schiff bases II*a*–II*k* (Table I) were prepared by treating various substituted anilines in acetic acid solution with one equivalent of the pyrimidinecarboxaldehydes I*a* and I*b* at room temperature (Scheme I). In certain instances, chloroform was added to the acetic acid solution of the amine to improve solubility. Yields varied from 30 to 85%.

In one experiment, heating *p*-chloroaniline in acetic acid for a few minutes on a steam bath with one equivalent of I*a* resulted not only in Schiff-base formation but also in displacement of the 6-chloro group by the amine. The product thus afforded was 4-chloro-6-(*p*-chloroanilino)-5-[*N*-(*p*-chlorophenyl)formimidoyl]-2-(methylthio)pyrimidine (III*a*). Similarly, heating *p*-chloroaniline with I*b* gave 4-chloro-6-(*p*-chloroanilino)-5-[*N*-(*p*-chlorophenyl)-formimidoyl]-2-phenylpyrimidine (III*b*). The preparation of similar types of Schiff bases was recently reported (5); in that report the reaction of 2,4-diamino-6-chloro-5-pyrimidinecarboxaldehyde with two equivalents of various substituted anilines, in boiling ethanol containing hydrochloric acid, resulted in Schiff-base formation with concomitant displacement of the chloro group by the amine.

When *o*-mercaptoaniline was allowed to react with I*a* in acetic acid at room temperature, the product obtained was 2-(4,6-dichloro-2-methylthio-5-pyrimidinyl)benzothiazoline (IV*a*). The Schiff base initially formed presumably must then undergo a cyclo-addition process initiated by attack of the mercapto group on the azomethine linkage. In identical fashion, reaction of I*b* with *o*-mercaptoaniline afforded 2-(4,6-dichloro-2-phenyl-5-pyrimidinyl)benzothiazoline (IV*b*). Hull (6) also observed thiazoline formation in the reaction of 4-hydroxy-6-methyl-2-piperidinopyrimidine-5-carboxaldehyde with *o*-mercaptoaniline.

PHARMACOLOGY

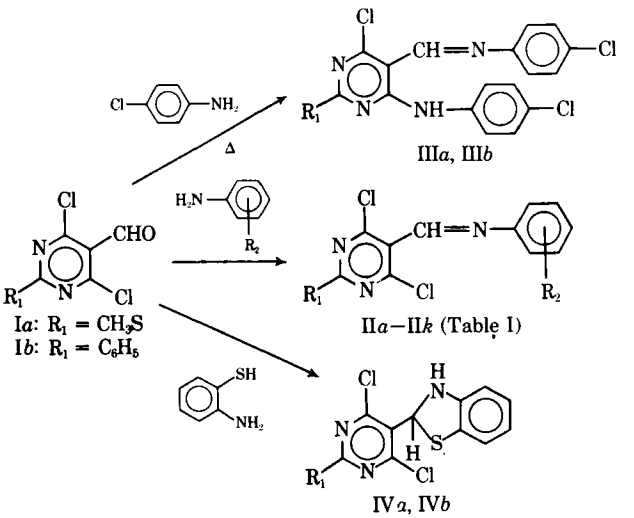
The title compounds were administered orally or intraperitoneally to each of three mice at four separate doses: 400, 127, 40, and 12.7 mg/kg. The animals were observed for a minimum of 2 hr during which time signs of stimulation (*i.e.*, increased spontaneous motor activity, hyperactivity on tactile stimulation, and twitching) or depression (*i.e.*, decreased spontaneous motor activity and decreased respiration) were noted.

Compounds II*a*, II*c*, II*d*, II*f*, II*g*, II*h*, II*i*, and II*k* as well as IV*a* and IV*b* were found to have CNS depressant activity when administered in doses ranging from 40 to 400 mg/kg ip, as manifested by decreased motor activity and respiration. In addition to depressing the CNS, IV*a* moderately lowered blood pressure in hypertensive rats when administered orally at a dose of 100 mg/kg.

Compounds III*a* and III*b* decreased the motor activity and respiration of mice when they were administered at a dose of 12.7 mg/kg ip.

EXPERIMENTAL[1]

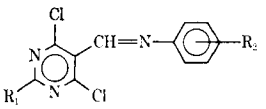
4,6-Dichloro-5-[*N*-(*o*-chlorophenyl)formimidoyl]-2-methylthiopyrimidine (IIc)—This example typifies the method used to



Scheme I

[1] Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. The IR spectra were determined in potassium bromide disks using a Perkin-Elmer model 21 spectrophotometer. NMR spectra were determined on a Varian A-60 spectrometer in deuterochloroform, using tetramethylsilane as the internal reference. The intermediate pyrimidinecarboxaldehydes (I*a* and I*b*) were previously described (Ref. 4). The various substituted anilines were all commercially available. The nomenclature used is recommended by *Chemical Abstracts*.

Table I—Properties of Schiff Bases of 4,6-Dichloropyrimidine-5-carboxaldehydes



Compound	R_1	R_2	Yield, %	Melting Point	Crystallization Solvent	Formula	Analysis, % Calc.	Found
II*a*	CH_3S	4-Cl	30	103–105°	Pentane	$C_{12}H_8Cl_3N_3S$	C 43.33 H 2.42 Cl 31.98 N 12.64	43.70 2.34 31.78 12.40
II*b*	CH_3S	3-Cl; 4-OH; 6-Cl	67	139–141°	Cyclohexane	$C_{12}H_7Cl_4N_3OS$	C 37.62 H 1.84 Cl 37.02 N 10.97	37.65 2.20 37.07 10.91
II*c*	CH_3S	2-Cl	45	112–115°	Heptane	$C_{12}H_8Cl_3N_3S$	C 43.33 H 2.42 Cl 31.98 N 12.64	43.61 2.57 31.91 12.62
II*d*	CH_3S	2-CH_3; 5-Cl	85	145–147°	Ethanol	$C_{13}H_{10}Cl_3N_3S$	C 45.04 H 2.91 Cl 30.68 N 12.12	45.17 2.65 30.57 12.05
II*e*	CH_3S	4-$OCH_2C_6H_5$	62	105–108°	Heptane	$C_{19}H_{15}Cl_2N_3OS$	C 56.44 H 3.74 Cl 17.54 N 10.39	56.66 3.87 17.26 10.52
II*f*	C_6H_5	4-Cl	70	190–193°	Chloroform	$C_{17}H_{10}Cl_3N_3$	C 56.30 H 2.78 Cl 29.33 N 11.59	56.00 2.50 29.38 11.40
II*g*	C_6H_5	2-Cl	70	157–159°	Ethyl acetate	$C_{17}H_{10}Cl_3N_3$	C 56.30 H 2.78 Cl 29.33 N 11.59	56.00 2.81 29.20 11.52
II*h*	C_6H_5	4-OCH_3	39	142–144°	Cyclohexane	$C_{18}H_{13}Cl_2N_3O$	C 60.35 H 3.66 Cl 19.79 N 11.73	60.31 3.54 19.53 11.72
II*i*	C_6H_5	4-$OCH_2C_6H_5$	69	168–171°	Cyclohexane	$C_{24}H_{17}Cl_2N_3O$	C 66.37 H 3.94 Cl 16.33 N 9.67	66.54 3.64 16.01 9.90
II*j*	C_6H_5	2-$CONH_2$	65	159–161°	Ethanol	$C_{18}H_{12}Cl_2N_4O$	C 58.24 H 3.26 Cl 19.10 N 15.09	58.24 3.52 19.15 15.05
II*k*	C_6H_5	4-$NHCOCH_3$	39	215–218°	Ethanol	$C_{19}H_{14}Cl_2N_4O$	C 59.24 H 3.66 Cl 18.40 N 14.54	59.56 3.78 18.17 14.44

prepare II*a*–II*k* (Table I). To 2.6 g of *o*-chloroaniline in 50 ml of acetic acid was added 4.5 g of 4,6-dichloro-2-methylthio-5-pyrimidinecarboxaldehyde (I*a*). The reaction mixture was allowed to stand at room temperature for a few minutes, during which time a crystalline product was deposited. This material was collected on a filter (6.5 g) and recrystallized from heptane, giving 3 g of product, mp 112–115°; IR (KBr): 6.17 (C=N) μm.

2-(4,6-Dichloro-2-methylthio-5-pyrimidinyl)benzothiazoline (IV*a*)—This compound was prepared from 2.5 g of *o*-mercaptoaniline and 4.6 g of I*a* in 50 ml of acetic acid. In a few minutes, a yellow precipitate was formed. After 0.5 hr at room temperature, the reaction mixture was filtered and the collected solid was recrystallized from cyclohexane, giving 1.1 g of product, mp 116–118°; IR: 3.05 (NH) μm; NMR: δ 4.04 (s, 1H, NH, disappears on deuteration) and 7.11 (s, 1H, H—C(—S)—N) ppm.

Anal.—Calc. for $C_{12}H_9Cl_2N_3S_2$: C, 43.64; H, 2.75; Cl, 21.47; N, 12.72. Found: C, 43.75; H, 2.77; Cl, 21.29; N, 12.59.

2-(4,6-Dichloro-2-phenyl-5-pyrimidinyl)benzothiazoline (IV*b*)—This compound was prepared in similar fashion from 2.5 g of *o*-mercaptoaniline and 5.0 g of I*b*. Recrystallization from cyclohexane gave 1.5 g of product, mp 156–158°; IR: 3.02 (NH) μm; NMR: δ 3.95 (s, 1H, NH, disappears on deuteration) and 7.23 (s, 1H, H—C(—S)—N) ppm.

Anal.—Calc. for $C_{17}H_{11}Cl_2N_3S$: C, 56.68; H, 3.08; Cl, 19.68; N, 11.66. Found: C, 56.94; H, 2.90; Cl, 19.39; N, 11.71.

4-Chloro-6-(*p*-chloroanilino)-5-[*N*-(*p*-chlorophenyl)formimidoyl]-2-(methylthio)pyrimidine (III*a*)—To a solution of 2.5 g of *p*-chloroaniline in 50 ml of acetic acid was added 4.46 g of I*a*. The reaction mixture was heated on a steam bath for approximately 10 min and then cooled to room temperature, and 25 ml of water was added. The precipitate which formed was collected on a filter. Recrystallization from benzene–pentane gave 1.1 g of product, mp 204–206°; IR: 6.08 (C=N) μm.

Anal.—Calc. for $C_{18}H_{13}Cl_3N_4S$: C, 51.02, H, 3.09; Cl, 25.10; N, 13.20. Found: C, 51.25; H, 3.09; Cl, 25.30; N, 13.20.

4-Chloro-6-(*p*-chloroanilino)-5-[*N*-(*p*-chlorophenyl)formimidoyl]-2-phenylpyrimidine (III*b*)—Compound III*b* was prepared in similar fashion from 3.8 g of *p*-chloroaniline and 6.6 g of I*b* in 100 ml of chloroform containing 40 ml of acetic acid. After heating on a steam bath for 0.5 hr, the reaction mixture was chilled in ice. The yellow precipitate which was formed amounted to 3.6 g after recrystallization from chloroform, mp 235–237°; IR: 6.12 (C=N) μm.

Anal.—Calc. for $C_{23}H_{15}Cl_3N_4$: C, 60.88; H, 3.33; Cl, 23.44; N, 12.35. Found: C, 61.03; H, 3.35; Cl, 23.40; N, 12.47.

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Thiazine Dye Antagonism of Opioid Lethality in Mice

DAVID H. BURKE[x] and DAVID E. MANN, Jr.[‡]

Abstract □ The thiazine dyes, methylene blue and tolonium chloride, were administered subcutaneously alone and in combination to adult male mice in low (0.1 mg) and high (1.0 mg) doses 5 min prior to lethal intraperitoneal doses ($LD_{99.5}$) of morphine, codeine, meperidine, levorphanol, methadone, levopropoxyphene, and propoxyphene. Survivors were recorded at 2- and 24-hr intervals after each opioid challenge. Tolonium pretreatment (0.1 mg) significantly increased the number of survivors that had received lethal doses of morphine and propoxyphene in the 2-hr phase in contrast to methylene blue pretreatment at either dosage level which failed to protect animals against opioid lethality in either the 2- or 24-hr phase. At both the 2- and 24-hr intervals, the low-dosage dye combination (0.1 mg of each dye) significantly protected the animals from lethal doses of morphine, levorphanol, and methadone. The low-dosage dye combination shortened the duration of sleep induced by hexobarbital (100 mg/kg), presumably through inhibition of CNS depression.

Keyphrases □ Thiazine dyes—antagonism of opioid lethality, mice □ Methylene blue—antagonism of opioid lethality, mice □ Tolonium chloride—antagonism of opioid lethality, mice □ Opioids, lethal doses—protection by thiazine dyes

Earlier observations by Harpel and Mann (1, 2) revealed that certain thiazine dyes, methylene blue or tolonium chloride, when administered prophylactically in conjunction with either nalorphine or levallorphan, enhanced the antidotal capabilities of these specific inhibitors against toxic doses of propoxyphene hydrochloride in mice. Similarly, in a pilot study employing methadone hydrochloride in mice, the preadministration of these thiazine dyes alone reduced opioid lethality. Accordingly, the purpose of this investigation was to ascertain the antidotal effectiveness of low (0.1 mg) and high (1.0 mg) doses of methylene blue and tolonium chloride, when administered singly or in an equal dosage combination, prior to lethal doses of several opioid[1] agents in mice. It was also anticipated that insight into the mechanism and extent of protection might be realized by testing the influence of these thiazine dyes upon hexobarbital sleeping time.

EXPERIMENTAL

Adult male mice[2], weighing between 20 and 25 g, were used. Before treatment, the animals were caged in groups of 50 for several days with free access to laboratory chow[3] and water. Immediately prior to experimentation, the animals were placed singly in stainless steel, wire-mesh cages without food and water. In the toxicity studies, however, food and water were provided for mice that were alive 2 hr after opioid injections.

Immediately before each day's testing, drug solutions[4] were prepared with water distilled in this laboratory, with the exception of methadone hydrochloride, which was procured from the manufacturer in vials (10 mg/ml, calculated as the salt).

The opioid drugs and their concentrations were: morphine sulfate, 6.0%; codeine sulfate, 2.0%; meperidine hydrochloride, 2.0%; levorphanol tartrate, 1.6%; methadone hydrochloride, 1.0%; levopropoxyphene hydrochloride, 2.0%; and propoxyphene hydrochloride, 2.0%. The thiazine dyes, methylene blue and tolonium chloride, each were prepared as 0.1 and 1.0% solutions. Sodium hexobarbital was prepared as a 1.0% solution.

A precision timer was used to determine survival and sleeping times, with all measurements being recorded to the nearest minute.

To determine the effects of thiazine dyes, alone and in combination, on opioid lethality and hexobarbital sleeping time, the following pretreatment regimen was employed: (*a*) single-dye pretreatment—methylene blue (0.1 mg), methylene blue (1.0 mg), tolonium chloride (0.1 mg), and tolonium chloride (1.0 mg); and (*b*) multiple-dye pretreatment—methylene blue (0.1 mg) + tolonium chloride (0.1 mg), and methylene blue (1.0 mg) + tolonium chloride (1.0 mg).

Toxicity Studies—The intraperitoneal LD_{50}'s were determined for each opioid drug (Table I) according to the method of Litchfield and Wilcoxon (3). Based upon the data obtained, doses corresponding to 99.5% lethality were selected for use in experimentation.

Each test animal was weighed[5] to the nearest gram, and the proposed pretreatment regimen[6] was performed subcutaneously in the upper left abdomen. When two dyes were administered, the upper right abdomen was used as the second site of injection.

[1] For the purpose of this study, the following agents are designated as opioid drugs: morphine sulfate, codeine sulfate, meperidine hydrochloride, methadone hydrochloride, levorphanol tartrate, propoxyphene hydrochloride, and levopropoxyphene hydrochloride.

[2] Huntingdon Farms, HTF strain.
[3] Purina.
[4] In each case, the salt form was used for the calculation of drug concentration and dose.
[5] Triple-beam Ohaus balance, model 730.
[6] Pretreatment injections were each administered in a volume of 0.1 ml as fixed doses.

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Abstract □ The thiazine dyes, methylene blue and tolonium chloride, were administered subcutaneously alone and in combination to adult male mice in low (0.1 mg) and high (1.0 mg) doses 5 min prior to lethal intraperitoneal doses ($LD_{99.5}$) of morphine, codeine, meperidine, levorphanol, methadone, levopropoxyphene, and propoxyphene. Survivors were recorded at 2- and 24-hr intervals after each opioid challenge. Tolonium pretreatment (0.1 mg) significantly increased the number of survivors that had received lethal doses of morphine and propoxyphene in the 2-hr phase in contrast to methylene blue pretreatment at either dosage level which failed to protect animals against opioid lethality in either the 2- or 24-hr phase. At both the 2- and 24-hr intervals, the low-dosage dye combination (0.1 mg of each dye) significantly protected the animals from lethal doses of morphine, levorphanol, and methadone. The low-dosage dye combination shortened the duration of sleep induced by hexobarbital (100 mg/kg), presumably through inhibition of CNS depression.

Keyphrases □ Thiazine dyes—antagonism of opioid lethality, mice □ Methylene blue—antagonism of opioid lethality, mice □ Tolonium chloride—antagonism of opioid lethality, mice □ Opioids, lethal doses—protection by thiazine dyes

Earlier observations by Harpel and Mann (1, 2) revealed that certain thiazine dyes, methylene blue or tolonium chloride, when administered prophylactically in conjunction with either nalorphine or levallorphan, enhanced the antidotal capabilities of these specific inhibitors against toxic doses of propoxyphene hydrochloride in mice. Similarly, in a pilot study employing methadone hydrochloride in mice, the preadministration of these thiazine dyes alone reduced opioid lethality. Accordingly, the purpose of this investigation was to ascertain the antidotal effectiveness of low (0.1 mg) and high (1.0 mg) doses of methylene blue and tolonium chloride, when administered singly or in an equal dosage combination, prior to lethal doses of several opioid[1] agents in mice. It was also anticipated that insight into the mechanism and extent of protection might be realized by testing the influence of these thiazine dyes upon hexobarbital sleeping time.

EXPERIMENTAL

Adult male mice[2], weighing between 20 and 25 g, were used. Before treatment, the animals were caged in groups of 50 for several days with free access to laboratory chow[3] and water. Immediately prior to experimentation, the animals were placed singly in stainless steel, wire-mesh cages without food and water. In the toxicity studies, however, food and water were provided for mice that were alive 2 hr after opioid injections.

Immediately before each day's testing, drug solutions[4] were prepared with water distilled in this laboratory, with the exception of methadone hydrochloride, which was procured from the manufacturer in vials (10 mg/ml, calculated as the salt).

The opioid drugs and their concentrations were: morphine sulfate, 6.0%; codeine sulfate, 2.0%; meperidine hydrochloride, 2.0%; levorphanol tartrate, 1.6%; methadone hydrochloride, 1.0%; levopropoxyphene hydrochloride, 2.0%; and propoxyphene hydrochloride, 2.0%. The thiazine dyes, methylene blue and tolonium chloride, each were prepared as 0.1 and 1.0% solutions. Sodium hexobarbital was prepared as a 1.0% solution.

A precision timer was used to determine survival and sleeping times, with all measurements being recorded to the nearest minute.

To determine the effects of thiazine dyes, alone and in combination, on opioid lethality and hexobarbital sleeping time, the following pretreatment regimen was employed: (*a*) single-dye pretreatment—methylene blue (0.1 mg), methylene blue (1.0 mg), tolonium chloride (0.1 mg), and tolonium chloride (1.0 mg); and (*b*) multiple-dye pretreatment—methylene blue (0.1 mg) + tolonium chloride (0.1 mg), and methylene blue (1.0 mg) + tolonium chloride (1.0 mg).

Toxicity Studies—The intraperitoneal LD_{50}'s were determined for each opioid drug (Table I) according to the method of Litchfield and Wilcoxon (3). Based upon the data obtained, doses corresponding to 99.5% lethality were selected for use in experimentation.

Each test animal was weighed[5] to the nearest gram, and the proposed pretreatment regimen[6] was performed subcutaneously in the upper left abdomen. When two dyes were administered, the upper right abdomen was used as the second site of injection.

[1] For the purpose of this study, the following agents are designated as opioid drugs: morphine sulfate, codeine sulfate, meperidine hydrochloride, methadone hydrochloride, levorphanol tartrate, propoxyphene hydrochloride, and levopropoxyphene hydrochloride.

[2] Huntingdon Farms, HTF strain.
[3] Purina.
[4] In each case, the salt form was used for the calculation of drug concentration and dose.
[5] Triple-beam Ohaus balance, model 730.
[6] Pretreatment injections were each administered in a volume of 0.1 ml as fixed doses.

Table I—Lethal Dosages of Several Narcotic Agents in Mice after Intraperitoneal Injection

Compound	LD_{50}, mg/kg	$LD_{99.5}$, mg/kg	Slope Function[a]
Morphine sulfate	400	820	1.32
Codeine sulfate	143	245	1.23
Meperidine hydrochloride	104	230	1.36
Levorphanol tartrate	118	209	1.25
Methadone hydrochloride	29	62	1.34
Levopropoxyphene hydrochloride	114	185	1.21
Propoxyphene hydrochloride	118	200	1.24

[a] The fold change in dose required to produce a unit change in standard deviation in response along the line.

After 5 min, the animal was injected intraperitoneally with a lethal dose of the opioid and the time of injection was recorded. The survivors, defined as animals that were alive at 2 and 24 hr, were appropriately recorded.

To maintain the 5-min interval between dye and opioid treatments, the number of animals dosed at one time never exceeded 12; at least two of the mice received subcutaneous injection(s) of water (0.1 ml) instead of the dye injection(s) and served as controls.

Animal groups receiving dye–opioid treatments were compared with the proper water-treated controls with respect to incidence of survival. Statistical significance was determined by the use of the χ-square distribution (corrected for continuity). Probability values of 0.05 or less were considered to be significant.

Sleeping Time Studies—Each mouse was weighed and pretreated according to the previously described procedure. Sodium hexobarbital (100 mg/kg ip) was administered 5 min later and the time of injection was noted. The animal was returned to its individual cage and the time of loss of the righting reflex, as well as its subsequent return, was recorded. Sleeping time was considered to be the interval between loss and return of the righting reflex.

To monitor sleeping time properly, the number of animals under observation at a given time never exceeded eight, half of which received injection(s) of water instead of dye(s) and served as controls. In every case, a dye-treated animal was associated with a control. Only control animals that were obtained during the accumulation of a particular dye-treated group were used for comparison with the treated group.

Dye-treated groups were compared with their corresponding controls with respect to duration of sleep. Significance was determined by use of the Student t distribution at the 95% confidence level.

RESULTS

Toxicity Studies—The 2- and 24-hr survival values for each opioid control group are listed in Table II. In each tabulation, the incidence of survival was not greater than 11.1%. The onset of symptoms developed within 5 min from the start of opioid treatment and was characterized by ataxia, a Straub-tail reaction, intermittent convulsions, and prostration, followed by the cessation of respiration. The convulsant episodes were strychnine-like, because they occurred spontaneously and could be initiated by tactile stimuli. The evaluated agents that are considered to be the most potent analgesics clinically (morphine, methadone, and levorphanol) showed less convulsant activity than the weaker analgesics (levopropoxyphene, propoxyphene, meperidine, and codeine). Preadministration with low (0.1 mg) or high (1.0 mg) doses of methylene blue was incapable of protecting the animals from mortality in both the 2- and 24-hr phases (Table II).

Subcutaneous pretreatment with tolonium chloride (0.1 mg) increased the incidence of survival at the 2-hr interval in mice subjected to lethal amounts of either morphine or propoxyphene (Table II). This regimen, however, failed to protect mice against the lethality caused by codeine, meperidine, levorphanol, methadone, and levopropoxyphene after 2-hr.

In the more stringent 24-hr survival phase, tolonium chloride (0.1 mg) was unable to alter significantly the lethality produced by any opioid. Pretreatment with this dye appeared to antagonize morphine lethality (survival = 20%), but comparison with the water-treated controls showed the difference to be nonsignificant ($p = 0.08$).

Opioid antagonism did not occur upon administration of high doses (1.0 mg) of tolonium chloride in the 2- and 24-hr phases.

In both the 2- and 24-hr intervals, the low-dosage dye combination (0.1 mg each) was capable of protecting against fatal doses of morphine, levorphanol, and methadone (Table II). No significant antidotal activity was demonstrated by the multiple-dye pretreatment in the high dosages at either interval.

Sleeping Time Studies—From a total of 216 mice, data were compiled to give mean sleeping time values for each of the six control groups obtained during the accumulation of the various dye-treated groups. The administration of sodium hexobarbital (100 mg/kg ip) elicited hypnosis which lasted approximately 40–60 min.

The preadministration of methylene blue and tolonium chloride (0.1 mg each) was found to decrease significantly the duration of sleep induced by sodium hexobarbital (Table III). The high dose of methylene blue appeared to prolong sleeping time; but when compared with the water-treated controls, the difference was nonsignificant ($p = 0.08$).

DISCUSSION

Isomerism appears to be a factor in determining whether tolonium chloride can antagonize opioid lethality. At the 2-hr inter-

Table II—Effect of Subcutaneous Pretreatment with Methylene Blue and Tolonium Chloride, Administered Singly or in Combination, on Incidence of Survival at 2- and 24-hr Intervals following Intraperitoneal Injection of Lethal Amounts of Opioid Agents

	Survival, %															
	Water[a], 0.1 ml		Methylene Blue[b]				Tolonium Chloride[b]				Water and Water[a], 0.1 ml Each		Methylene Blue and Tolonium Chloride[b]			
			0.1 mg		1.0 mg		0.1 mg		1.0 mg				0.1 mg Each		1.0 mg Each	
Opioid	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr	2 hr	24 hr
Morphine	5.6	2.8	10.0	6.7	13.3	13.3	36.7[c]	20.0	13.3	13.3	2.8	0.0	40.0[c]	16.7[c]	6.7	0.0
Codeine	2.8	2.8	6.7	6.7	0.0	0.0	6.7	6.7	3.3	3.3	0.0	0.0	6.7	3.3	10.0	6.7
Meperidine	0.0	0.0	0.0	0.0	10.0	0.0	0.0	0.0	3.3	0.0	5.6	0.0	13.3	3.3	6.7	3.3
Levorphanol	5.6	5.6	6.7	0.0	0.0	0.0	13.3	13.3	10.0	6.7	11.1	2.8	36.7[c]	23.3[c]	6.7	3.3
Methadone	8.3	5.6	10.0	6.7	3.3	0.0	6.7	6.7	20.0	10.0	8.3	0.0	26.7[c]	20.0[c]	0.0	0.0
Propoxyphene	5.6	0.0	10.0	6.7	13.3	6.7	30.0[c]	6.7	3.3	3.3	8.3	2.8	13.3	0.0	10.0	3.3
Levopropoxyphene	8.3	0.0	0.0	0.0	6.7	0.0	3.3	0.0	13.3	3.3	5.6	0.0	3.3	0.0	0.0	0.0

[a] Groups of 36 animals. [b] Groups of 30 animals. [c] Compared to water or water and water, χ-square test (corrected for continuity), $p < 0.05$.

Table III—Effect of Subcutaneous Pretreatment with Methylene Blue and Tolonium Chloride, Administered Singly or in Combination, on Duration of Sleep following Intraperitoneal Injection of Sodium Hexobarbital

Treatment[a]	Dose, mg	Mean Sleeping Time, min	*SEM*
Water	—	56.6	2.59
Methylene blue	0.1	52.8	3.00
Water	—	51.5	3.10
Methylene blue	1.0	58.4	2.73
Water	—	54.4	2.19
Tolonium chloride	0.1	52.4	3.68
Water	—	50.0	2.84
Tolonium chloride	1.0	49.9	2.26
Water (two injections)	—	51.6	3.63
Methylene blue and tolonium chloride	0.1 (each)	40.5[b]	2.39
Water (two injections)	—	47.8	2.52
Methylene blue and tolonium chloride	1.0 (each)	46.9	2.19

[a] Water (one or two injections) or thiazine dyes (alone or in combination) administered to groups of 36 animals 5 min prior to sodium hexobarbital (100 mg/kg ip). [b] Compared to water, Student t distribution, $p < 0.05$.

val, lethality from propoxyphene was inhibited, whereas no such effect was seen against levopropoxyphene. This distinction between tolonium antagonism against the *d*- and *l*-isomers of propoxyphene was not apparent at 24 hr. Therefore, tolonium might possess early or transient antagonistic activity that operates mainly against one of a pair of isomers.

Lethal amounts of morphine, methadone, and levorphanol appeared to produce less convulsant activity than the weaker analgesics studied. This observation is in complete agreement with that of Johannesson (4), who noted that toxic doses of morphine in rats caused a flaccid type of respiratory paralysis while death by codeine was always associated with convulsions. Therefore, the possibility was entertained that the thiazine dyes may act in combating narcotic poisoning through the inhibition of opioid-induced depression of the central nervous system (CNS).

In the sleeping time studies, only the low-dosage dye combination significantly reduced the duration of hexobarbital hypnosis. Based upon this finding, it is suggested that protection against the lethality of the potent narcotic analgesics occurred principally through dye interference with opioid-induced CNS depression.

When administered alone or in combination at the high-dosage level, methylene blue and tolonium chloride were unable to prevent opioid lethality. High doses of tolonium chloride are known to decrease the survival time in mice following lethal amounts of propoxyphene, presumably through enhancement of convulsant activity (2). The failure of methylene blue to antagonize opioid lethality may be due to the ability of the dye to produce CNS depression in high doses (5).

Because the precise way in which the opioid drugs act to produce their lethality still is unknown, an attempt to propose a mechanism of inhibition by the dyes becomes futile. Nevertheless, it is of considerable interest to note that morphine, the prototype of narcotic analgesics, and the thiazine dyes, methylene blue and tolonium chloride, show certain dissimilarities with respect to their actions within the CNS. For example, the inhibition of oxidative deamination and the enhancement of dehydrogenase enzymes represent dye actions that are exactly opposite to those of morphine (6, 7). These actions, either of a specific or nonspecific nature, may play a part in the inhibition demonstrated by the "Janus"-type dyes.

SUMMARY

1. The subcutaneous administration of tolonium chloride at the lower dosage level (0.1 mg) significantly reduced the lethality of the opioids, morphine and propoxyphene, at 2 hr after treatment.

2. At the 24-hr interval, toxicity by the clinically potent narcotic analgesics (morphine, levorphanol, and methadone) was antagonized by the low-dosage combination of methylene blue and tolonium chloride (0.1 mg each).

3. Hexobarbital sleeping time was significantly decreased by the low-dosage, multiple-dye pretreatment, presumably through interference with CNS depression.

4. The mechanism by which the thiazine dyes antagonize opioid lethality remains unknown.

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ˣ To whom inquiries should be directed.

In Vitro Assessment of *In Vivo* Absorption of Drug Complexes

S. FELDMAN[x], J. RICHARDSON, C. FREEMAN, S. POLLOCK, J. BERGER, F. KAPLAN, and G. RHOA

Abstract □ This study determined if *in vitro* transfer of drug complexes across the everted rat small intestine would reflect the *in vivo* absorption characteristics of the complex. The *in vitro* transfer of prednisolone in the presence of dimethyl-, diethyl-, dipropyl-, and dibutylpropionamide was in rank-order agreement with the increase in absorption reported previously from an *in situ* preparation. Excellent agreement was found between the *in vitro* and *in situ* absorption of prednisolone in the presence of various concentrations of dipropylpropionamide. The interaction of atropine with eosin-B, which has been reported not to increase the transfer of the dye across the everted rat intestine, was found to have no effect on absorption from an *in situ* preparation. The difference in effect of complexation on drug transfer across the everted rat intestine may be related to a difference in rate-limiting barriers for prednisolone and eosin-B.

Keyphrases □ Drug complexes—*in vitro* transfer as an indicator of *in vivo* absorption characteristics □ Dialkylpropionamides—effect on absorption of prednisolone, everted rat intestine, *in vitro* preparation as an indicator of absorption-enhancing characteristics of complex □ Complexes (dialkylpropionamides-prednisolone)—*in vitro* transfer as an indicator of *in vivo* absorption characteristics

The isolated everted small intestine has been used extensively to study the effects of drug interactions on GI absorption (1–5) as well as to study drug permeability across the intestinal membrane (6–8). More recently, it has been used as a model to classify drugs that may present potential absorption problems in the development of pharmaceutical dosage forms (9). The use of the everted rat intestine has also been evaluated as an animal model to predict antibiotic absorption in man (10).

The everted rat intestinal preparation has not been well studied as to whether the technique could be used to predict the *in vivo* absorption characteristics of the drug complex. Hayton and coworkers (11–13) examined the enhancement of the intestinal absorption of prednisone and prednisolone by dialkylpropionamides in rats using an *in situ* technique. It was therefore of interest to investigate the effect of the dialkylpropionamides on the absorption of prednisolone across the everted rat intestine to determine if the *in vitro* preparation would reflect the absorption-enhancing characteristics of the complex found *in vivo*.

EXPERIMENTAL

Materials—The propionamides[1], prednisolone[2], eosin-B[3], and atropine[2] as well as all other chemicals were used as received.

Drug Solutions—Prednisolone and the propionamides were dissolved in 0.9% NaCl solution with the addition of 1% ethanol. Eosin-B and atropine were dissolved in a Krebs-Ringer solution buffered to pH 7.0 with phosphate (1).

Test Animals—Male, Sprague-Dawley descent rats[4], weighing 250–350 g and fasted for 20–24 hr, were used in all experiments. Drinking water was readily accessible.

***In Vitro* Absorption Studies**—The animals were anesthetized with ether and the small intestine was excised. The first 15 cm was discarded, and the intestine was everted over a glass rod as described previously (4). Two consecutive 10-cm segments were taken, attached to a cannula as described previously (4), and suspended in 80 ml of a mucosal solution consisting of 0.9% NaCl containing prednisolone alone or prednisolone in the presence of the dialkylpropionamides. Two-milliliter samples of the serosal fluid (0.9% NaCl) were withdrawn and replaced at appropriate intervals over 2 hr and assayed for drug content as previously described (4). In all cases, one segment served as the control (mucosal solution contained only prednisolone) while the other segment contained the steroid and dialkylpropionamide. No distinction was made as to the segments, and they were designated as the control or the complex segments on a random basis.

Stripping Studies—The experiment was set up as described in the preceding section with the following modifications. One intestinal segment was stripped of the mucosal layer by running forceps up and down its length. This resulted in essentially total removal of the mucosal layer. Identical solutions of 0.5 m*M* prednisolone or 0.5 m*M* prednisolone plus 28 m*M* dipropylpropionamide were used as the mucosal solution, and the amount transferred with time was measured.

***In Situ* Studies**—Each animal was anesthetized with ethyl carbamate (1.5 g/kg ip) and prepared as previously described (12, 14). A sufficient volume of drug solution at 37° containing either 1 m*M* eosin-B or 1 m*M* eosin-B and 5 m*M* atropine was passed from the duodenal syringe through the gut into the ileal syringe to yield a volume of 5 ml in the latter. The solution in the ileal syringe was then returned to the intestine. At appropriate intervals, the drug was transferred from the intestine into one of the syringes and a 0.1-ml sample was removed for assay. Prior to removing a sample, the volume of the drug solution in the syringe was adjusted to 5 ml with pH 7.0 Krebs-Ringer phosphate buffer.

Assay Procedures—Prednisolone concentrations were determined spectrophotometrically[5] by the colorimetric method of Porter and Silber (15). "Blank" serosal fluid (obtained from experiments where no drug was placed in the mucosal fluid) and the dialkylpropionamides did not interfere with the assay procedure.

Eosin-B concentrations were determined spectrophotometrical-

Table I—Effect of Dialkylpropionamides on the Transfer of 0.5 m*M* Prednisolone across the Everted Rat Small Intestine

Amide	Number	Ratio of Amount Prednisolone Transferred in 2 hr (with Amide/Control)[a]
Dimethyl, 28 m*M*	5	1.05 ± 0.07
Diethyl, 28 m*M*	5	1.32 ± 0.17
Dipropyl, 28 m*M*	5	1.55 ± 0.18
Dibutyl, 5.6 m*M*	5	2.08 ± 0.15

[a] Represents the average and standard deviation of the mean.

[1] Eastman Organic Chemicals, Rochester, N.Y.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] J. T. Baker Co.
[4] Huntingdon Farms, Conshohocken, Pa.
[5] Coleman 124 spectrophotometer.

Table II—Effect of Various Concentrations of Dipropylpropionamide on the Transfer of 0.5 m*M* Prednisolone across the Everted Rat Small Intestine in 2 hr

Concentration Amide, %	Number	Ratio (Amide/Control)[a]
0.096	6	1.24 ± 0.08
0.24	5	1.39 ± 0.10
0.37	6	1.53 ± 0.10
0.44	5	1.55 ± 0.18

[a] Represents the average and standard deviation of the mean.

Table III—Effect of Stripping Mucosa on Amount of Prednisolone Transferred across the Everted Rat Small Intestine in 2 hr

Drug Solution	Number	Ratio of Amount Transferred, Stripped/Intact[a]
0.5 m*M* Prednisolone	3	1.024 ± 0.071
0.5 m*M* Prednisolone + 28 m*M* dipropylpropionamide	3	0.998 ± 0.029

[a] Represents the average and standard deviation of the mean.

ly[5] at 516 nm after appropriate dilution of the samples with pH 7.0 phosphate buffer. Atropine did not interfere with the assay procedure.

RESULTS AND DISCUSSION

The effects of dimethyl-, diethyl-, dipropyl-, and dibutylpropionamide on the transfer of prednisolone across the everted rat small intestine are presented in Table I. The initial amide concentration in each case was 28 m*M* except for the butyl-amide concentration which was limited by solubility considerations to 5.6 m*M*. The total amount of steroid transferred in 2 hr was not significantly increased by the methyl-amide but was significantly affected by the ethyl-, propyl-, and butyl-amides. The results are qualitatively identical to those of Hayton and Levy (12) who examined the interaction of steroid and amide in an *in situ* preparation. A comparison of the *in vivo* and *in vitro* experiments is presented in Fig. 1. This figure illustrates the ratio of the amount of prednisolone transferred across the isolated gut in 2 hr in the presence of the amides to that in the absence of each amide *versus* the ratio of the percent absorbed in 1 hr from the *in situ* preparation. The *in situ* data were obtained from Hayton and Levy (12). As can be seen from the figure, the rank-order agreement between the two sets of data is good and the isolated preparation gives an adequate rank-order prediction as to the *in vivo* effect of the prednisolone–amide interaction on GI absorption.

The effect of various concentrations of dipropylpropionamide on the absorption of prednisolone from the *in situ* rat intestine was studied previously (13). To determine if the everted rat small intestine would predict the *in vivo* results, experiments were carried out using the *in vitro* preparation with various concentrations of the propyl-amide present in the mucosal solution (Table II). Increasing the concentration of propyl-amide in the mucosal solution results in an increase in the total amount of prednisolone transferred in 2 hr across the everted intestinal segments. The relationship between the effect of the complexing agent on the transfer of prednisolone both *in vitro* and *in vivo* is quite apparent in Fig. 2. The plot of the ratio of the amount of steroid transferred in 2 hr in the presence and absence of the amide *in vitro versus* the apparent absorption rate constant of prednisolone *in situ* is apparently linear, and an excellent quantitative prediction of the *in vivo* results can be obtained from the *in vitro* experiments.

In view of the excellent agreement between the *in vitro* and *in vivo* absorption results with the prednisolone–amide complexes, it was decided to examine the *in vivo* case where *in vitro* studies did not indicate increased intestinal transfer in the presence of a complexing agent. The data provided by Levy and Matsuzawa (1) on the absorption of water-soluble dye–drug complexes across the everted rat small intestine were examined as to the *in vivo* absorption characteristics of the dye and lipid-soluble complex. As representative of the group of dye–drug complexes, the complexation of eosin-B and atropine was examined using the *in situ* rat gut technique of Doluisio *et al.* (14). The *in vitro* data (1) showed no increase in the absorption rate of the eosin-B–atropine complex as compared to eosin-B alone, despite the fact that the chloroform–water partition coefficient of eosin-B increased 240-fold by the addition of atropine. The results from this part of the experiment appear in Fig. 3. After a rapid drop in the concentration of eosin-B in the intestinal lumen for the initial 10 min in the presence of atropine, both the esin-B and eosin-B complex had essentially identical half-lives for the disappearance of drug from the intestinal lumen. In each case, the half-life for "absorption" was approximately 40 min. No increase in the intestinal absorption rate of eosin-B was observed in the presence of atropine except at the initial 10-min period. This finding was in agreement with the *in vitro* results of Levy and Matsuzawa (1).

It is of interest to speculate as to why a lipid-soluble complex results in increased absorption in the case of prednisolone but has

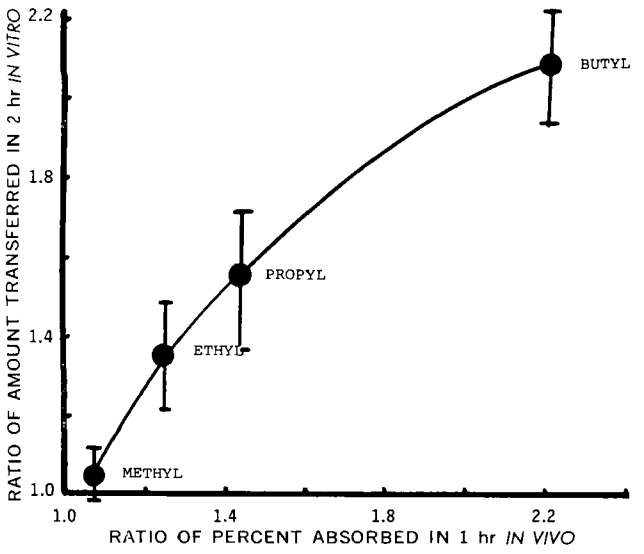

Figure 1—*Plot of the ratio (amide/control) of the amount of prednisolone transferred in 2 hr* versus *the ratio (amide/control) of the percent absorbed in 1 hr* in vivo. In vivo *data are from Ref. 12.*

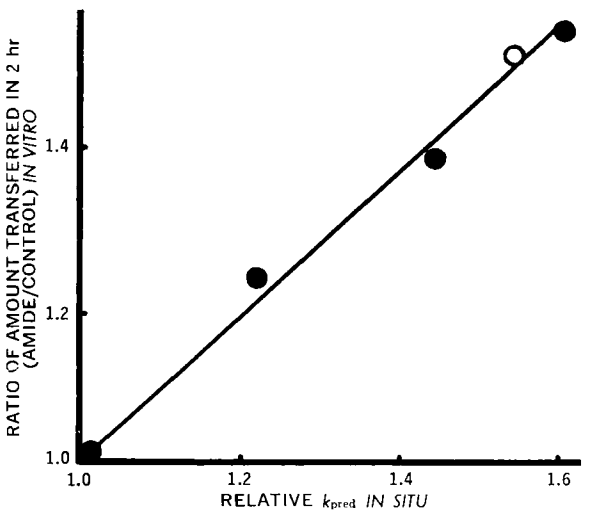

Figure 2—*Plot of the ratio (dipropylamide/control) transferred in 2 hr* in vitro versus *the relative absorption rate constant* (k_{pred}) in vivo. *The open circle represents an extrapolated point from Fig. 6 of Ref. 13.* In vivo *data are from Ref. 13.*

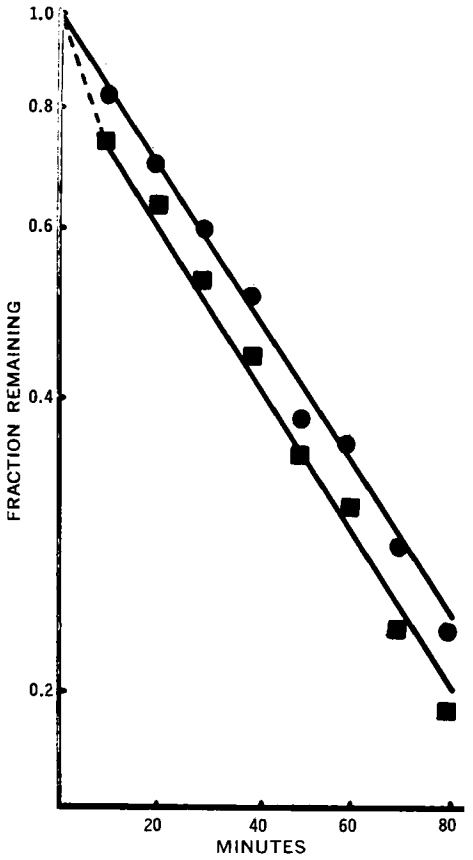

Figure 3—*Plot of fraction eosin-B remaining* versus *time for absorption from the* in situ *rat intestine. Key:* ●, *1 mM eosin-B; and* ■, *1 mM eosin-B and 5 mM atropine.*

no effect in the case of eosin-B. Prednisolone is a drug that has a relatively high clearance across the everted rat intestine (approximately 1 ml/hr for the initial 30 min). The clearance of eosin-B is much lower, around 0.04 ml/hr (16). Gibaldi and Grundhofer (16, 17) showed that a difference exists in the nature of the rate-limiting barrier to passive diffusion of poorly lipid-soluble and freely lipid-soluble compounds across the intestinal epithelium. Compounds with initial clearance values of >1 ml/hr fell into the category of rapidly absorbed drugs while those with less than 1 ml/hr clearance were the polar, more slowly absorbed drugs. It was found (17) that destruction of the epithelial barrier resulted in an increased rate of clearance for the poorly absorbed drugs while essentially no change occurred with the lipid-soluble compounds. This is presumably due to a difference in the rate-limiting barrier for the two different classes of drugs. To determine if this were the case with prednisolone and the amide complex, the stripping experiment was set up to examine the effect of removing the mucosa on the transfer of prednisolone and the prednisolone–amide complex across the everted rat intestine. The ratio of the amount transferred in 2 hr of the stripped to intact intestine was calculated (Table III). The ratio of unity suggests that the rate-limiting barrier to prednisolone transfer across the everted intestinal preparation is not the epithelial barrier but some other anatomical barrier in the preparation.

From the preceding discussion, it is obvious that in the cases of the prednisolone–amide and eosin-B–atropine complexes the results of the *in vitro* experiments utilizing the isolated everted rat small intestine would be a valid indicator of the *in vivo* intestinal absorption properties of the drug complex. The reason for the difference in absorption characteristics of the two drug complexes needs further investigation, but in the examples studied it is possible to predict the *in vivo* absorption of drug complexes from *in vitro* experiments utilizing the everted rat small intestine.

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[x] To whom inquiries should be directed.

Modern Approaches for Selecting Biologically Active Plants I: CNS Depressants

N. R. FARNSWORTH[x], J. P. BEDERKA, Jr., and M. MOSES

Abstract □ Based on literature reports and other arbitrarily assigned criteria, a method is suggested with which one can select plants with high theoretical probability for CNS depressant activity. Their extracts experimentally demonstrate this activity. The method was tested by selecting 11 high scoring plants and 16 low scoring plants from a list of 575 plants which theoretically should contain CNS depressant principles. These 27 plants were screened for CNS depressant activity utilizing the activity cage. A statistically significant difference was found between plants selected from the top of the list and those selected from the bottom of the list.

Keyphrases □ Medicinal plants—novel approach for selecting plants with CNS activity □ Phytological screening—novel approach for selecting biologically active plants, CNS activity □ Screening, biologically active plants—novel approach for selection using literature and assigned criteria

Most useful drugs obtained from plants have been discovered following investigations initiated on the basis of their medicinal folkloric descriptions. One can cite digitoxin *(Digitalis purpurea)*, reserpine *(Rauvolfia serpentina)*, ergonovine and ergotamine *(Claviceps purpurea)*, khellin *(Ammi visnaga)*, atropine *(Atropa belladonna)*, and scopolamine *(Datura stramonium)* as major examples. On the other hand, many investigations of plants that were designed on the basis of their folkloric reputations have not yielded biologically active compounds; indeed, extracts of many such plants could not be shown to have the folkloric biological activity when evaluated in laboratory animals. Therefore, many investigators discount altogether the folkloric approach as a means of discovering new biologically active entities.

A second approach to discovering plants having useful biologically active agents has been to screen large numbers of randomly selected plants for one or more types of biological activity (1–5). The clinically important antitumor agents vincristine (6) and vinblastine (6) were discovered in this way. A number of other promising antitumor agents have likewise been discovered through this approach, including acronycine (7), camptothecin (8), thalicarpine (9), fagaronine (10), *Acer*-saponin-P (11), tetrandrine (12), maytansine (13), and bruceantin (14). But critics of this mass screening approach point out that it is expensive and unscientific.

It appeared that some other means should be available for selecting a few candidates for evaluation, for a specific and desired type of biological activity, from the more than 750,000 estimated species of higher plants. About a 3-year period from the scientific literature together with an arbitrary scoring system was used to rank a list of plants that might be expected to exert a sedative effect in laboratory animals. This ranking was followed by the testing of extracts of plants from this list for central nervous system (CNS) depressant activity in mice to evaluate the effectiveness of the method.

Table I—Criteria for Selection of Potential Sedative-Containing Plants

Points Assigned	Criteria
10	Plant reported in the literature as having some degree of CNS depressant activity as a result of biological evaluation in laboratory animals
6	Plant for which credible folkloric references were found in the literature, in which some type of sedative activity was mentioned for the plant
5	Plant taxonomically related to a group of plants that was well known to yield effective sedative principles (at either the family or genus level)
4	Plant for which literature reports indicated that alkaloids were present in the plant or had been isolated from the plant [Two recent compilations covering the world literature on this subject were consulted (16, 17)]
3	Plant classified in a group that had received little or no chemical or biological investigation, as evidenced from literature files or general knowledge
2	Plant for which two or more species of the genus in question had been assigned both 10 and 6 points as indicated above
−5	Plant for which literature sources directly indicated any type of toxicity ascribed to the plant, its extracts, or isolated compounds, either experimentally or on the basis of folklore

EXPERIMENTAL

Plant Selection—A literature search[1] from the years 1970–1972 and sporadically prior to 1970 revealed a total of 575 plants that had either been reported as folkloric sedative remedies or whose extracts had been reported to show sedative or CNS depressant activity when tested in laboratory animals. Each of the 575 plants was assigned points according to the arbitrarily appointed criteria presented in Table I. By using this system, a maximum of 30 points or a minimum of −5 points could be assigned to any given plant. It was found that 75 plants were assigned 15 or more points, which was an arbitrarily selected cut-off point for further consideration of plants in this study. By inspection, a number of plants were eliminated from this list of 75 on the following grounds. Plants that were well known for their sedative or CNS depressant properties or for which the active principle(s) was (were) known were removed from the list, *i.e.*, *R. serpentina*, *Cannabis sativa*, and *Papaver somniferum*. Also, plants that contained uncharacterized alkaloids on the basis of literature reports and that were in such taxa as the Leguminosae were removed from the list. The rationale here was that many Leguminosae alkaloids are of the pyrrolizidine type, which are usually very toxic. Similarly, plants containing coumarins, a group of compounds usually toxic to the liver, were discounted.

Plants were eliminated from the list of 75 for the reasons given until a final list of 20 plants was achieved. It was hoped to acquire samples of all 20 plants and to evaluate them in animals for

[1] *Chemical Abstracts* and *Biological Abstracts*.

Table II—ED_{50} Values for Plant Extracts from the Upper 20 Selections

Plant	ED_{50}, mg/kg ip
SP-01	28.0
SP-02	56.0
SP-03	66.0
SP-04	70.0
SP-05	70.0
SP-06	72.0
SP-07	76.0
SP-08	110.0
SP-09	118.0
SP-10	120.0
SP-11	250.0
Sodium pentobarbital	27.0

CNS depressant activity. However, it was only possible to acquire 11 of the 20 plants, but this number was considered a representative sample.

For comparative purposes, and to test the validity of the criteria used for the selection of plants, an attempt was made to obtain the plants with the lowest number of points on the list of 575. It was possible to obtain 16 of the lowest scoring 20 plants.

The arbitrary criterion assigning four points to plants containing alkaloids seemed to bear a distinct relationship to the 575 plants initially selected. Seventy-five percent of the 575 plants selected were reported in the literature to contain alkaloids, but the normal distribution of alkaloids in plants on a random-selection basis is only 5–10% (15). Thus, alkaloids in general could be considered as a type of marker for CNS depressant activity.

Preparation of Extracts for Testing—Each plant sample was milled to a coarse powder, and 200 g was added to 1.0 liter of boiling distilled water. The mixture was allowed to cool to room temperature and then was filtered through a Büchner funnel. The filtrate was then frozen and lyophilized. All extracts were prepared in an identical manner, and subsequent biological tests could be compared on a weight basis. All extracts were soluble in water at the concentrations tested. Immediately prior to use, the dry powder was dissolved in a convenient volume of distilled water. This method of sample preparation was utilized since one criterion employed for plant selection was that the plant had been described in the literature as having been used as a folkloric remedy to induce sleep, to act as a tranquilizer or narcotic, or for some other type of CNS depressant activity. The method of preparation of test extracts was similar to the method that would have been used by natives, or by the laity, in preparing such an infusion, both in procedure and in amounts used.

Evaluation for CNS Depressant Activity—Pharmacological evaluation of the potential sedative activity of the plant extracts was determined in female albino mice[2] weighing 16–25 g. Mice were initially divided into five groups of five animals each for preliminary screening of the extracts. All extracts were administered in graded doses of 1–1600 mg/kg ip in amounts not exceeding 0.5 ml/mouse. Different groups of control animals received intraperitoneal injections of water in amounts that varied between 0.1 and 0.5 ml. No effect of volume of injection on gross motor activity was found in the controls. Sodium pentobarbital was used as the sedative reference compound.

Four- or five-dose levels of each extract or of sodium pentobarbital were tested, and dose–response curves were determined.

Two naive control groups were employed on each day (morning and afternoon), and the data from the extract-treated groups were compared with their respective morning and afternoon control data. Naive mice were used since previous exposure to the counting chamber and retesting showed approximately a 50% reduction in activity. Each point on the final dose–response curves represented a mean of either duplicate or triplicate consecutive determinations with five mice in each treatment group. The percent decrease in motor activity in most cases ranged from 20 to 80% of controls.

Spontaneous motor activity was measured with an activity cage[3] equipped with photoelectric cells for recording gross movements of the mice by a mechanical counter. Counting was performed for 30 min following injection of the test solutions. The data obtained were plotted graphically as the percent decrease in motor activity against the logarithms of the doses used. The ED_{50} values[4] for sodium pentobarbital and each plant extract were obtained from graphical extrapolation of the 50% point on the ordinate to the regression line and then to the corresponding dose on the abscissa.

The mean ED_{50} values, calculated for the 11 plants selected from the top 20 plants on the list of 575, are presented in Table II. Similar data for the 16 plants selected from the lower 20 of the 575 are presented in Table III.

Table III—ED_{50} Values for Plant Extracts from the Lower 20 Selections

Plant	ED_{50}, mg/kg ip
SP-12	1.8
SP-13	9.0
SP-14	38.0
SP-15	67.0
SP-16	92.0
SP-17	94.0
SP-18	138.0
SP-19	205.0
SP-20	230.0
SP-21	220.0
SP-22	370.0
SP-23	520.0
SP-24	610.0
SP-25	665.0
SP-26	1000.0
SP-27	1360.0
Sodium pentobarbital	27.0

DISCUSSION

The purpose of this study was to test a hypothesis that a few plants could be selected from many that have either a folkloric reputation as sedatives or for which extracts may have shown some sedative activity in laboratory animals. The criteria presented (Table I) are in part highly subjective and rely to some degree on the depth of knowledge of the natural product literature possessed by the person assigning the scores; nevertheless, the results seem to establish that the method has some merit. The names of specific plants are not listed since they are irrelevant to what is being reported, *i.e.*, a method for selecting plants having a high degree of sedative activity.

The method has been shown to be effective since the mean ED_{50} values of the two groups of extracts were significantly different. The ED_{50} of the 11 tested plants from the 20 plants that scored highest was 87 mg/kg, with a standard error of ±18 mg/kg. In contrast, for the 16 evaluated plants from the 20 lowest scoring plants, the ED_{50} was 351 mg/kg, with a standard error of ±97.5 mg/kg. The difference between these two mean doses was significant at the $p = 0.02$ level (Student t comparison).

The accurate results for selecting sedative plants were achieved by utilizing the equivalent of only about 3 years of the available scientific literature. If a larger data base had been employed, it is reasonable to assume that the results might have been improved.

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Mechanisms of Hydrolysis of Salicylanilide *N*-Methylcarbamates

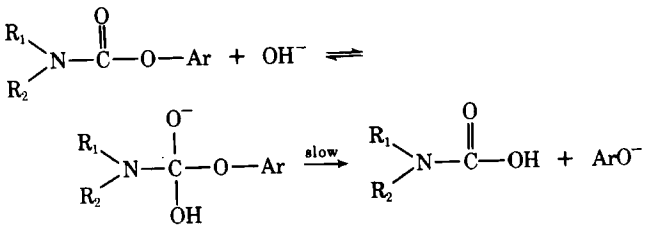
LEO W. BROWN, RICHARD S. P. HSI, and ARLINGTON A. FORIST[x]

Abstract □ The facile hydroxide-ion-catalyzed hydrolysis of the carbamoyl group in salicylanilide *N*-methylcarbamate (I) and analogs unsubstituted on the anilide nitrogen proceeds by a reaction mechanism involving anchimeric assistance by the *o*-carboxanilide group. Substitution of an alkyl or aryl group for the anilide proton greatly reduces the hydrolysis rate; the reaction rate constant for I is ~7 × 10^2 times that for *N*-methylsalicylanilide *N*-methylcarbamate (II). To characterize the mechanism for II, comparative parameters of activation were determined for I and II. The entropy of activation ($\Delta S\ddagger$) for II was 8.6 eu more negative than that for I, suggesting steric hindrance in the transition state for II. The observed reaction rate constant for II was consistent with estimates based on the electronic *ortho*-substituent effect of the carboxanilide group. It is concluded that II is hydrolyzed *via* the established mechanism for simple aryl *N*-monosubstituted carbamates (rapid proton extraction followed by a rate-determining conversion of the carbamate anion to an isocyanate) rather than by the mechanism followed by I.

Keyphrases □ Salicylanilide *N*-methylcarbamates—mechanisms of hydrolysis □ Hydrolysis mechanisms—salicylanilide *N*-methylcarbamates □ Carbamoyl group hydrolysis—mechanisms of salicylanilide *N*-methylcarbamate hydrolysis

A growing body of literature (1–7) has established a duality of mechanism for the alkaline hydrolysis of aryl carbamates. In the absence of a proton on the nitrogen atom (*N,N*-disubstituted carbamates), the reaction follows Scheme I, characteristic of simple ester hydrolysis. However, for compounds containing a proton on the nitrogen atom (*N*-monosubstituted carbamates), hydrolysis occurs *via* Scheme II (the ElcB mechanism).

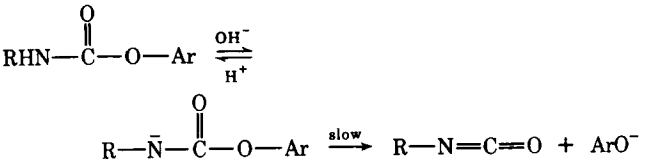
Reports from these laboratories described the kinetics of hydrolysis of a number of aryl carbamates possessing anti-inflammatory activity (8–10). Hsi *et al.* (9) showed that the extremely rapid hydroxide-ion-catalyzed hydrolysis of the carbamoyl group of



Scheme I

salicylanilide *N*-methylcarbamate (I) and a number of analogs with *para*-substituents in the phenyl ring of the anilide moiety involved participation by the neighboring *o*-carboxanilide group. Reaction rate constants, k_{OH^-}, were logarithmically related to the Hammett σ-constants (ρ = 0.46 at 37°). A special case of the mechanism shown in Scheme II, involving rapid proton extraction from the carbamate nitrogen followed by a rate-limiting intramolecular cyclization and subsequent ring opening and fragmentation, was indicated (Scheme III).

Substitution of an alkyl or aryl group for the proton on the anilide nitrogen of I produces a dramatic reduction in the carbamate hydrolysis rate (9). Reaction rate constants for *N*-methylsalicylanilide *N*-methylcarbamate (II) and *N*-phenylsalicylanilide *N*-methylcarbamate (III) are nearly identical and only about 15 times that for phenyl *N*-methylcarbamate



Scheme II

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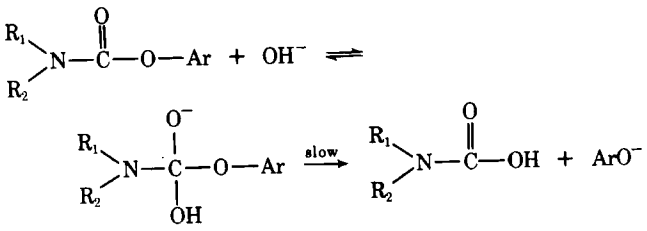

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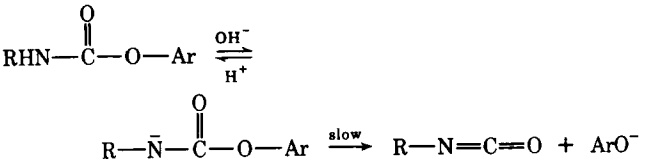


Scheme II

Table I—Reaction Conditions and Kinetic Constants for Carbamate Hydrolysis in 50% Aqueous Ethanol

Compound	Concentration, $M \times 10^5$	Apparent pH	Temperature	$k_{OH^-} \pm SD$, M^{-1} min^{-1}
I	9.26	5.4	30°	$1.29 \pm 0.01 \times 10^5$
	9.26	5.4	37°	$1.97 \pm 0.02 \times 10^5$
	8.60	5.4	47°	$4.48 \pm 0.02 \times 10^5$
II	21.9	8.6	30°	$1.82 \pm 0.01 \times 10^2$
	19.2	8.6	37°	$3.05 \pm 0.01 \times 10^2$
	18.9	8.6	47°	$7.15 \pm 0.05 \times 10^2$
III[a]	10.4	8.6	37°	$2.89 \pm 0.03 \times 10^2$
IV[a]	42.3	10.2	37°	$1.90 \pm 0.01 \times 10^1$

[a] Reference 9.

(IV) (37°, 50% aqueous ethanol). By contrast, I is hydrolyzed at a rate 10^4 times that for IV. Hydrolysis of II (and III) predominantly *via* Scheme II is suggested by these results.

Limited studies to date indicate that the entropy of activation ($\Delta S\ddagger$) for Scheme II is considerably more positive than that for Scheme I (2, 6). Parameters of activation have not been reported for the *o*-carboxanilide-assisted carbamate hydrolysis (9) or for the more recently described intramolecular nucleophilic attack on carbamates by the phenoxide ion (11) and the carboxylate ion (12). Therefore, to provide such information for Scheme III and to clarify the reaction mechanism for II, comparative parameters of activation for the hydrolysis of I and II were determined and are reported, together with estimated rate constants for nonanchimerically assisted hydrolysis based on electronic substituent effects.

EXPERIMENTAL

Solutions of I and II were prepared in 50% aqueous ethanolic acetate and phosphate (0.025 *M*) buffers, respectively, at the concentrations and apparent pH values shown in Table I. Each solution was placed in a UV spectrophotometer equipped with a constant-temperature cell compartment maintained at the temperatures indicated in Table I. Absorbances (A_t) were determined at appropriate times at the absorption maximum of the product phenols (300 and 285 nm for I and II, respectively) until no further absorbance increases occurred (A_∞). Pseudo-first-order reaction rate constants (k') were obtained from the slopes of log ($A_\infty - A_t$) *versus* time curves fitted by linear least-squares regression analysis. Based on the previously established fact that the hydrolysis of I, II, and other aryl carbamates displays first-order dependence on the hydroxide ion (8–10), specific reaction rate constants (k_{OH^-}) were calculated from the equation:

$$\log k' = \log k_{OH^-} + \text{pH} - \text{pKw} \qquad \text{(Eq. 1)}$$

Activation energies (ΔE_a) were determined from the classical Arrhenius equation by linear least-squares regression; $\Delta H\ddagger$ and $\Delta S\ddagger$ were determined similarly from the absolute reaction rate equation.

RESULTS AND DISCUSSION

Specific reaction rate constants (k_{OH^-}) for hydrolysis of the carbamoyl groups in I and II in 50% aqueous ethanol are shown in Table I; similar data for III and IV from previous work (9) are included for comparison. Derived parameters of activation for I and II are presented in Table II. Activation energies (ΔE_a) and enthalpies of activation ($\Delta H\ddagger$) are not significantly different for the two compounds. Vontor *et al.* (6) observed a ΔE_a value of 16.5 kcal mole^{-1} for the alkaline hydrolysis of 1-naphthyl *N*-methylcarbamate in aqueous solution; Christenson (2) reported a $\Delta H\ddagger$ value of 16.6 kcal mole^{-1} for phenyl *N*-phenylcarbamate hydrolysis in 5–10% ethanol. The corresponding parameters for I and II are slightly less, but direct comparisons are complicated by the different solvents employed. In studies (13) of the alkaline hydrolysis of formate esters, $\Delta H\ddagger$ in water was significantly smaller than in 85% ethanol. By analogy, predicted ΔE_a and $\Delta H\ddagger$ values for the hydrolysis of I and II in aqueous solution would be significantly smaller than the values observed in 50% ethanol and, hence, less than those reported for other aryl carbamates in aqueous solution.

Table II—Activation Parameters for the Hydrolysis of I and II in 50% Aqueous Ethanol

Parameter	I	II
ΔE_a (kcal mole^{-1})	14.2 ± 1.3	15.6 ± 0.8
$\Delta H^\ddagger$ (kcal mole^{-1})	13.6 ± 1.3	15.0 ± 0.8
$\Delta S^\ddagger$ (eu)	1.6 ± 4.3	−7.0 ± 2.7

Entropies of activation ($\Delta S\ddagger$) of 0.6 and 5.0 eu were reported for the hydrolysis of 1-naphthyl *N*-methylcarbamate (6) and phenyl *N*-phenylcarbamate (2), respectively, consistent with expectations for the mechanism shown in Scheme II (14). The value of $\Delta S\ddagger$ for I, 1.6 eu, is intermediate in magnitude and indicates that its facile hydrolysis *via* Scheme III is not the consequence of a more favorable $\Delta S\ddagger$ but rather of a reduced $\Delta H\ddagger$.

The value of $\Delta S\ddagger$ for the hydrolysis of II, −7.0 eu, is considerably more negative than for I. In addition, it is the most negative of those cited from the literature as well as those found in these laboratories[1] for various aryl *N*-methylcarbamates hydrolyzed *via* Scheme II. The $\Delta S\ddagger$ alone does not provide unequivocal proof of mechanism. However, considered with other evidence (*vide infra*), the observed negative $\Delta S\ddagger$ is consistent with steric hindrance in the transition state (14, 15) and hydrolysis of II *via* Scheme II.

For hydrolysis of II by Scheme II, it should be possible to relate k_{OH^-} to the electronic substituent effect of the *ortho*-substituted phenyl *N*-methylcarbamate. Williams (7) correlated hydrolysis rate constants for *para*- and *meta*-substituted phenyl *N*-phenylcarbamates with Hammett σ (or σ^-) constants and obtained ρ =

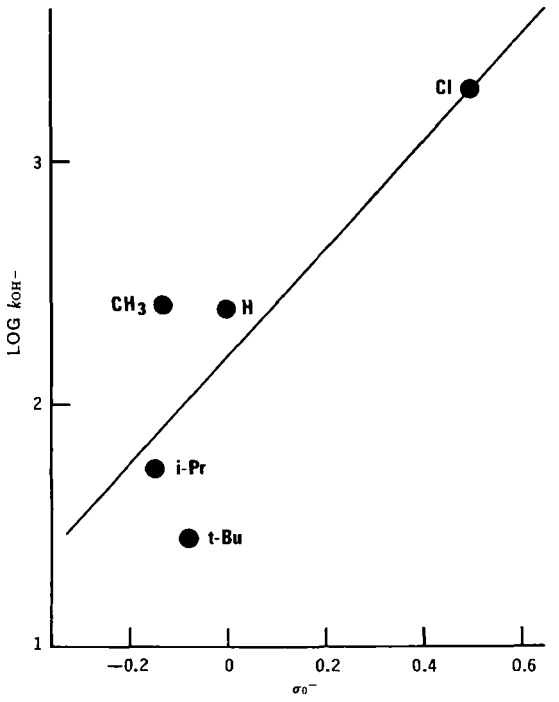
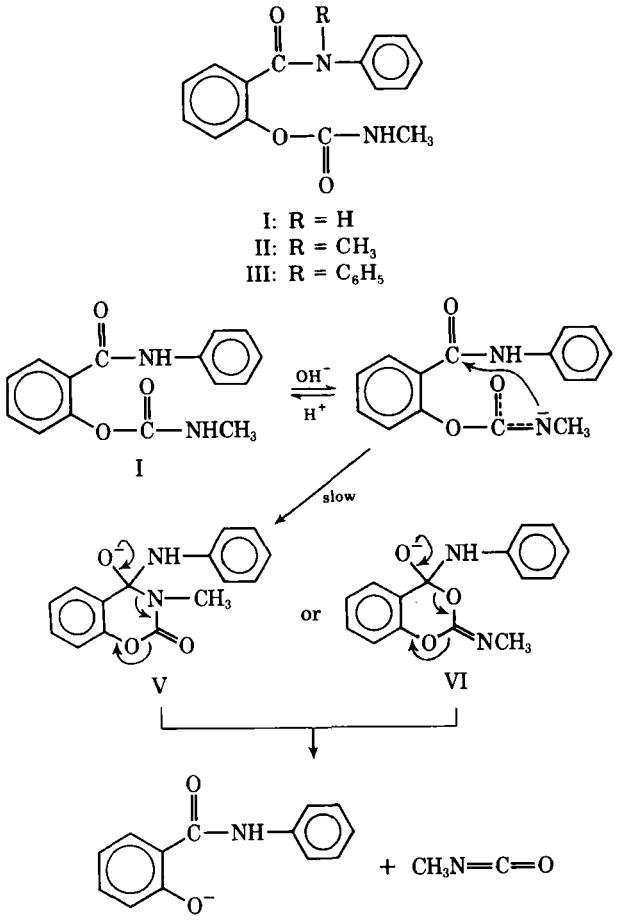


Figure 1—*Linear least-squares regression of log* k_{OH^-} *for the hydrolysis of* o*-substituted phenyl* N*-methylcarbamates (37.5°, aqueous solution) (17) versus* σ_0^- *(16).*

[1] Unpublished results.



Scheme III

2.86. No similar study of *ortho*-substituent effects has been reported.

The *ortho*-substituent constants (σ_o-) for a large number of functional groups were recently determined (16). Therefore, utilizing these σ_o- values and the k_{OH}- values (37.5°) reported (17) for five *ortho*-substituted phenyl *N*-methylcarbamates, a linear least-squares regression of log k_{OH}- *versus* σ_o- was obtained (Fig. 1). The fit was acceptable (r = 0.83), considering the limited number of data points, and gave a ρ value of +2.2, in reasonable agreement with that obtained by Williams (7) based on *para*- and *meta*-substituents.

With the k_{OH}- for IV hydrolysis (37°, 50% aqueous ethanol, Table I), σ_o- for the *N*-phenylcarboxamide group (0.79), and the previously determined ρ, k_{OH}- reflecting only *ortho*-substituent effects was calculated for I (and for II, assuming a similar σ_q- for the *N*-methyl-*N*-phenylcarboxamide group). The resulting value, $1.04 \times 10^3\ M^{-1}\ min^{-1}$, is markedly less than that observed for I ($1.97 \times 10^5\ M^{-1}\ min^{-1}$, Table I), as expected. However, the calculated k_{OH}- is actually larger (by a factor of 3.4) than that found for II ($3.05 \times 10^2\ M^{-1}\ min^{-1}$, Table I). In view of the inherent assumptions, this is considered quite reasonable agreement and argues strongly against anchimeric assistance (Scheme III) in the hydrolysis of II. Apparently, the presence of a methyl (II) or phenyl (III) group on the anilide nitrogen exerts sufficient steric influence to preclude formation of the cyclic intermediate of Scheme III. This is supported by the negative $\Delta S^{\ddagger}$ for II.

In Scheme III, V is considered the more likely intermediate by analogy to the cyclization of salicylaldehyde *N*-methylcarbamate to yield 3,4-dihydro-4-hydroxy-3-methyl-2*H*-1,3-benzoxazin-2-one (18). This intermediate is also consistent with the results (19) on intramolecular reactions involving ureido group participation.

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[x] To whom inquiries should be directed.

Cytotoxic Cardenolides from *Acokanthera longiflora* Stapf. and Related Species

DAVID G. I. KINGSTON*x and T. REICHSTEIN‡

Abstract □ An alcoholic extract of the stems and twigs of *Acokanthera longiflora* Stapf. showed inhibitory activity against the KB cell culture and was fractionated through a series of partitions and column chromatography to yield three cardenolides: acovenoside A, opposide, and acolongifloroside K. Tests of these and other cardenolides from related species indicated that nearly all of the compounds tested had high *in vitro* activity but no *in vivo* activity.

Keyphrases □ *Acokanthera longiflora* Stapf.—isolation, identification of three cytotoxic cardenolides □ Cytotoxicity—cardenolides □ Cardenolides—isolated from *Acokanthera longiflora* Stapf., cytotoxicity

A random screening of botanical sources for anticancer activity showed that the aqueous alcoholic extract of the stems and twigs of *Acokanthera longiflora* Stapf. (family Apocynaceae) gave reproducible activity against the cell culture (KB) of a human carcinoma of the nasopharynx[1]. Systematic fractionation of the extract led to the isolation of three cardenolides: acovenoside A (I), opposide (II), and acolongifloroside K (III). In view of the cytotoxicity of these compounds, tests were also carried out on a number of cardenolides from related species, and several of these were found to be cytotoxic. The more readily available compounds were tested for their *in vivo* activity against L-1210 lymphoid leukemia[2]; the results of the tests indicated that these compounds had no activity in this system.

DISCUSSION

The alcoholic residue from percolation of the plant material with ethanol was partitioned between water and chloroform and resulted in a division of the activity between the two phases. The active chloroform fraction was partitioned between petroleum ether and 10% aqueous methanol to give an inactive petroleum ether fraction and an active methanol fraction. Chromatography of this active fraction on silicic acid yielded nine main fractions. Crystallization of the major active fraction yielded directly a material identified as acovenoside A (I) (1–3). The material was identified from its spectral properties and by direct comparison with authentic material[3] (IR, melting point, and mixed melting point).

The active aqueous extract from the original chloroform–water partition was subjected to partition between chloroform–ethanol (2:1) and water, and the aqueous residue was partitioned between butanol and water. Both organic extracts showed comparable activity, while the aqueous extract was essentially inactive. Analysis of the organic extracts by paper chromatography indicated that they both consisted of essentially identical mixtures of three materials giving a positive Kedde's reaction. The butanol fraction was subjected to partition column[4] chromatography with chloroform–benzene, half saturated with water, as the eluant. Combination of fractions on the basis of paper chromatographic analysis gave three fractions, each consisting of one component giving a positive Kedde's reaction together with several other fractions containing mixtures of Kedde-positive materials. The three fractions containing only one Kedde-positive component were each purified further by chromatography on silicic acid and, in two cases, by crystallization.

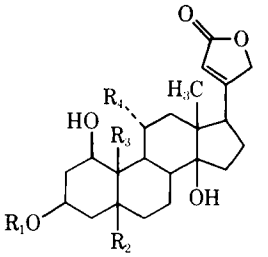
The first material was isolated in too small yield to allow complete characterization but was not identical with any of the known constituents of *A. longiflora*. Its characterization will be the subject of a subsequent report.

The second compound was identified as opposide (II) by comparison of its IR spectrum, color reaction with 84% H_2SO_4, paper chromatographic mobility, and melting point with literature values for authentic material (4, 5). This is the first reported isolation of opposide from *A. longiflora*.

The third compound was identified as acolongifloroside K (III) by paper chromatographic comparison with an authentic sample. The preparation available could not be induced to crystallize, although it was chromatographically homogeneous. Treatment with acetic anhydride and pyridine, however, yielded a crystalline acetate identical with acolongifloroside K pentaacetate (6).

The earlier investigation (7) of the cardiac glycosides of *A. longiflora* resulted in the isolation of eight compounds: acovenoside A; acolongiflorosides E, G, H, J, and K; and substances D and F. Paper chromatographic analysis of the extract of the present plant sample showed the absence of more than trace amounts of any other cardenolides; in particular, the acolongiflorosides G, H, and J were present in only trace amounts in their appropriate crude fractions. It may thus be concluded that the cytotoxicity of *A. longiflora* extracts is due largely to the presence of acovenoside A and acolongifloroside K, with lesser contributions from opposide and other cardenolides present in small quantities.

In view of the high cytotoxicity of the compounds isolated from *A. longiflora*, it was of interest to investigate the cytotoxicity of similar compounds from related species (Table I). With few exceptions, all investigated compounds had a moderately high cytotoxicity. The cytotoxicity of acovenosides A and B was previously reported (10). Other cardenolides that are cytotoxic are



I: R_1 = L-acovenose, R_2 = H, R_3 = CH_3, R_4 = H
II: R_1 = 6-desoxy-L-talose, R_2 = OH, R_3 = CH_3, R_4 = OH
III: R_1 = 6-desoxy-L-talose, R_2 = OH, R_3 = CH_2OH, R_4 = OH

[1] The cell culture assay was performed through the auspices of the Cancer Chemotherapy National Screening Center (CCNSC) according to the procedure given in *Cancer Chemother. Rep.*, **25**, 22(1962). A purified substance is considered active if, on the average of two tests, it shows an $ED_{50} \leq 4\ \mu g/ml$, which is confirmable by another testing laboratory.

[2] See Footnote 1. A purified substance is considered active if it shows a T/C ratio ≥125 on the average of two tests, which is confirmable by another testing laboratory.

[3] D. G. I. Kingston thanks Dr. G. J. Kapadia, Department of Pharmacognosy, Howard University, for an authentic sample of acovenoside A.

[4] Hyflo Supercel.

Table I—Cytotoxicity of Cardenolides from *Acokanthera* Species

Cardenolide	ED_{50}, μg/ml	Reference
Acovenoside A	0.025	This work
Acolongifloroside K	0.064, 0.40	This work
Opposide	0.045	This work
Acolongifloroside G	0.15	6
Acolongifloroside H	0.25	6
Acoschimperoside P	0.10	7
Acoschimperoside Q	0.25	7
Acoschimperoside N	>100	7
Diglucoacoschimperoside P	1.7	7
Diglucoacoschimperoside N	3.7	7
Acofrioside L	0.16	8
Acovenoside B	0.22	9

known (11-15), and a parallel appears to exist among cytotoxicity toward KB cells, a heart action, and the inhibition of the ATPase enzymes of the active transport of potassium and sodium ions (16).

Table II records the results of *in vivo* bioassays of the more readily available cardenolides in the L-1210 leukemia system. None of the compounds tested showed an *in vivo* activity high enough to suggest that it held promise as an antitumor drug, presumably because of high mammalian toxicities (3, 7).

EXPERIMENTAL[5]

Plant Material—The stems and twigs of *A. longiflora* Stapf. were collected[6] in Kenya, received air dried, and then ground in a hammermill to 0.31-cm (0.125-in.) mesh.

Extraction and Initial Separation—The powdered plant material (2.2 kg) was extracted with ethanol to give, after evaporation, 102 g of a thick residue which was partitioned between chloroform and water. The chloroform extract (43 g) was partitioned between 10% aqueous methanol and petroleum ether (bp 60–80°). The methanol phase, after removal of solvent, left a residue of 25 g.

The aqueous extract from the initial chloroform–water partitioning was extracted with chloroform–ethanol (2:1); the chloroform–ethanol extract weighed 7.5 g after evaporation of the solvent. The remaining aqueous solution was extracted with *n*-butanol; after evaporation and freeze drying, the *n*-butanol extract weighed 14 g, with the remaining aqueous extract accounting for approximately 36 g.

Adsorption Chromatography of Aqueous Methanol Fraction—Chromatography of the methanol fraction was carried out on silica gel with a gradient of chloroform to chloroform–12% methanol as eluant (8). Combination of fractions on the basis of dry weight and paper chromatography [chloroform–formamide system (4, 17-20)] yielded nine major fractions. No materials giving a purple color with the Kedde spray reagent (21) could be detected in fractions 1-5. Fraction 6 contained mainly acovenoside A, while fraction 7 contained small quantities of materials with the same R_f as authentic samples of the acolongiflorosides G and H (overlapping spots) together with other minor components. Fraction 8 showed the presence of a trace amount of Kedde-positive material near the origin, with the same R_f as acolongifloroside J.

Acovenoside A—Crystallization of fraction 6 from chloroform–benzene gave crystals, mp 220–225°. Recrystallization (three times) from acetone–petroleum ether yielded needles, mp 230–231° [lit. (1) mp 230–232°]; concentration of the mother liquors yielded rods with double melting points of 158–162° → 230–231° [lit. (1) 160–163° → 230–232°]. A mixed melting point with authentic material was undepressed, and the material had UV and IR spectra and optical rotation identical with those of authentic material.

Acetylation (acetic anhydride and pyridine at room temperature for 16 hr) of acovenoside A gave the crystalline diacetate as colorless needles from acetone–petroleum ether, mp 221–222° [lit. (1) mp 229–230°].

Partition Chromatography of Butanol Fraction—Partition column[4] chromatography was carried out on 6.0 g of the butanol fraction with benzene–butanol, half saturated with water, as eluant (4). A total of 100 fractions was obtained and combined on the basis of paper chromatography in the system of butanol–water (22).

Compound J′—Fractions 34-56 yielded a material which showed only one Kedde-positive spot on paper chromatography in butanol–water, R_f 0.54. The crude fraction was purified by adsorption chromatography on a silicic acid (80 g) column and elution with linear gradient of chloroform to chloroform–methanol (50:50). Fractions of 50 ml were collected, and fractions 18-20 yielded material homogeneous by paper chromatography (20 mg) and with a different R_f than any known constituent of *A. longiflora*.

Opposide—Fractions 62-71 showed only one Kedde-positive spot on paper chromatography. Adsorption chromatography on 90 g silicic acid with a chloroform to chloroform–methanol (50:50) gradient and collection of 50-ml fractions gave crude opposide (300 mg) in fractions 15-19. Crystallization from acetone followed by moist ether–methanol gave opposide (110 mg), mp 282–287° [lit. (4) mp 292–295°], undepressed when mixed with authentic material. The compound had an identical IR spectrum with that recorded for authentic material (5), an identical color reaction with 84% H_2SO_4, and identical chromatographic behavior to that of authentic material (4).

Acolongifloroside K—Fractions 74-87 were combined and submitted to adsorption chromatography on 100 g silicic acid. Elution with a gradient of chloroform to chloroform–methanol (50:50) and collection of 50-ml fractions gave acolongifloroside K (600 mg) in fractions 16-20. The chromatographically homogeneous material did not crystallize but behaved identically to authentic material on paper chromatography in butanol–water.

A sample was acetylated (acetic anhydride in pyridine) at room temperature for 16 hr. Usual workup gave acolongifloroside K pentaacetate, mp 290–291° [lit. (6) mp 286–292°].

Table II—*In Vivo* Activity of Various Cardenolides

Cardenolide	Tumor	Percent T/C
Acovenoside A	L-1210	111
Acolongifloroside K	L-1210	Toxic
Acovenoside C	L-1210	87

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[5] Melting points were determined on a Mel-Temp capillary melting-point apparatus and are corrected. The IR spectra were obtained as KBr disks on a Beckman IR-8 spectrophotometer. UV spectra were recorded on a Cary model 15 recording spectrophotometer. Optical rotation was recorded in methanol on a Bellingham and Stanley spectropolarimeter. Paper chromatography was carried out by the ascending technique.

[6] The authors are indebted to Dr. R. E. Perdue, Jr., of the U.S. Department of Agriculture, Beltsville, Md., for these collections received in 1969 as part of the agreement with the CCNSC. Voucher specimens are on deposit with Dr. Perdue.

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ˣ To whom inquiries should be directed.

Sterols of Marine Algae

L. M. SAFE, C. J. WONG, and R. F. CHANDLERˣ

Abstract □ The free and bound sterols of the marine algae *Laminaria saccharina*, *Ascophyllum nodosum*, and *Furcellaria fastigiata*, were isolated and identified by using a combination of TLC, GLC, and mass spectroscopic techniques. Preparative TLC enabled the fast, efficient separation of each sterol from the mixture for positive identification by GLC and mass spectroscopy. The chromatographic techniques employed permitted the detection and isolation of the more polar sterols in the presence of the more common 3β-monohydroxy sterols. To the authors' knowledge, this is the first recorded detailed sterol analysis of *L. saccharina* and the first recorded isolation of saringosterol and 24-ketocholesterol from *Laminaria*. The mass spectra for these two sterols are presented.

Keyphrases □ Sterols, free and bound—isolated and identified from marine algae (*Laminaria saccharina*, *Ascophyllum nodosum*, and *Furcellaria fastigiata*) □ Algae, marine—isolation and identification of free and bound sterols □ Marine algae—isolation and identification of free and bound sterols □ *Laminaria saccharina*—isolation and identification of free and bound sterols

Marine algae have been the subject of numerous investigations in the fields of pharmacognosy and natural product chemistry for many years (1). Sterols found in algae (2, 3) and marine invertebrates (4) have been reported to exhibit antihypercholesterolemic activity in test animals (5–7), as has β-sitosterol in humans (8).

In most studies on the sterol content of marine and other organisms, the identification has been made on the basis of GLC retention times (9), occasionally accompanied by mass spectrometry of the mixture as a whole or of the individual GLC peaks when a GLC–mass spectrometer combined unit was available (10, 11). Column chromatography has also been employed extensively to separate simple mixtures of sterols (12), and preparative GLC can be used to complete purification that has been partially achieved by column chromatography (13).

Occasionally, new compounds have appeared when the natural sterol mixtures were analyzed by GLC. It is then necessary to isolate the individual component to characterize it fully (14, 15). Many sterols can be readily isolated from mixtures by preparative TLC in sufficient quantities to obtain NMR spectra and melting points as well as mass spectra (16). These physical data are necessary to give positive identification of unknown as well as known compounds (14, 15).

The preparative TLC methods used are simple techniques that can be employed along with GLC in analytical screening of algae as well as other biological material to determine sterol, triterpenoid, or other natural product content. These or other similar TLC methods have been in use for some time by workers studying sterol metabolism (17).

In the present work, the dried algae were extracted initially with a chloroform–methanol mixture to obtain the free sterols or their esters and subsequently with aqueous methanolic potassium hydroxide to obtain the water-soluble or bound sterols (18). In the two brown algae examined, the bound and unbound sterols were similar in composition and in approximately equal quantities, while the red algae showed the bound fraction to be present in approximately one-third the amount of free sterols. Isolation and separation were achieved completely by preparative

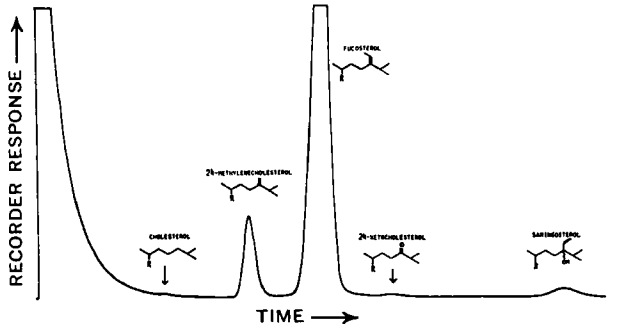

Figure 1—*GLC separation of* L. saccharina *sterols on 1.5% OV-17.*

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ˣ To whom inquiries should be directed.

Sterols of Marine Algae

L. M. SAFE, C. J. WONG, and R. F. CHANDLERˣ

Abstract □ The free and bound sterols of the marine algae *Laminaria saccharina*, *Ascophyllum nodosum*, and *Furcellaria fastigiata*, were isolated and identified by using a combination of TLC, GLC, and mass spectroscopic techniques. Preparative TLC enabled the fast, efficient separation of each sterol from the mixture for positive identification by GLC and mass spectroscopy. The chromatographic techniques employed permitted the detection and isolation of the more polar sterols in the presence of the more common 3β-monohydroxy sterols. To the authors' knowledge, this is the first recorded detailed sterol analysis of *L. saccharina* and the first recorded isolation of saringosterol and 24-ketocholesterol from *Laminaria*. The mass spectra for these two sterols are presented.

Keyphrases □ Sterols, free and bound—isolated and identified from marine algae (*Laminaria saccharina*, *Ascophyllum nodosum*, and *Furcellaria fastigiata*) □ Algae, marine—isolation and identification of free and bound sterols □ Marine algae—isolation and identification of free and bound sterols □ *Laminaria saccharina*—isolation and identification of free and bound sterols

Marine algae have been the subject of numerous investigations in the fields of pharmacognosy and natural product chemistry for many years (1). Sterols found in algae (2, 3) and marine invertebrates (4) have been reported to exhibit antihypercholesterolemic activity in test animals (5–7), as has β-sitosterol in humans (8).

In most studies on the sterol content of marine and other organisms, the identification has been made on the basis of GLC retention times (9), occasionally accompanied by mass spectrometry of the mixture as a whole or of the individual GLC peaks when a GLC–mass spectrometer combined unit was available (10, 11). Column chromatography has also been employed extensively to separate simple mixtures of sterols (12), and preparative GLC can be used to complete purification that has been partially achieved by column chromatography (13).

Occasionally, new compounds have appeared when the natural sterol mixtures were analyzed by GLC. It is then necessary to isolate the individual component to characterize it fully (14, 15). Many sterols can be readily isolated from mixtures by preparative TLC in sufficient quantities to obtain NMR spectra and melting points as well as mass spectra (16). These physical data are necessary to give positive identification of unknown as well as known compounds (14, 15).

The preparative TLC methods used are simple techniques that can be employed along with GLC in analytical screening of algae as well as other biological material to determine sterol, triterpenoid, or other natural product content. These or other similar TLC methods have been in use for some time by workers studying sterol metabolism (17).

In the present work, the dried algae were extracted initially with a chloroform–methanol mixture to obtain the free sterols or their esters and subsequently with aqueous methanolic potassium hydroxide to obtain the water-soluble or bound sterols (18). In the two brown algae examined, the bound and unbound sterols were similar in composition and in approximately equal quantities, while the red algae showed the bound fraction to be present in approximately one-third the amount of free sterols. Isolation and separation were achieved completely by preparative

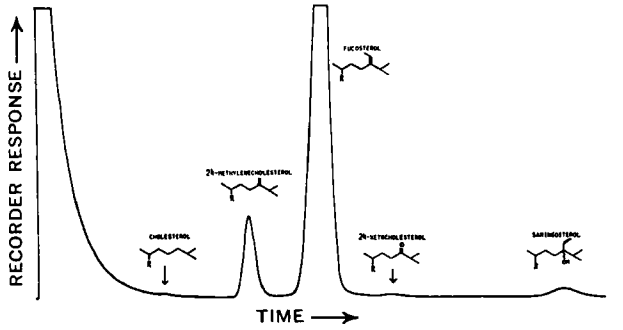


Figure 1—*GLC separation of* L. saccharina *sterols on 1.5% OV-17.*

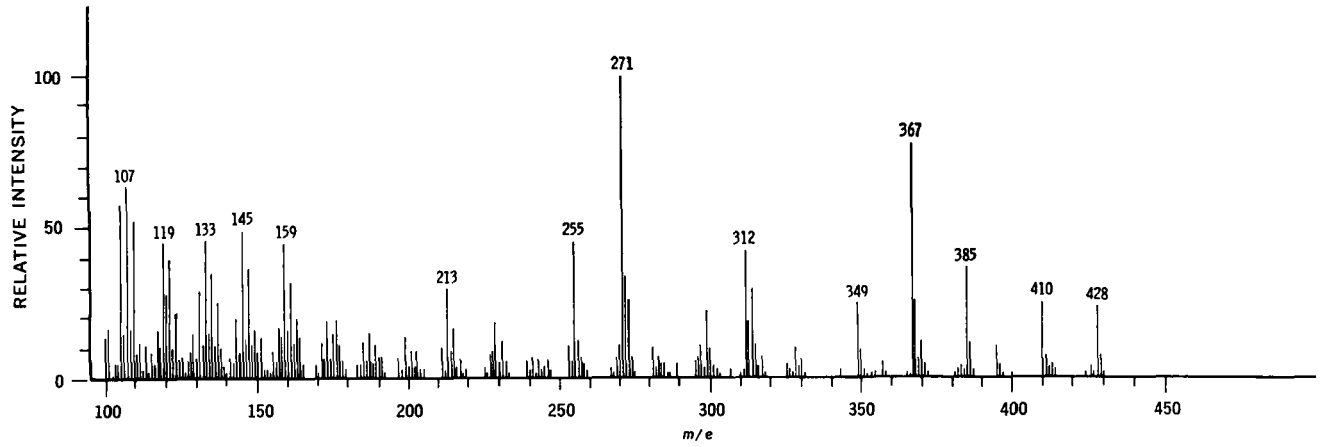


Figure 2—*Mass spectrum of saringosterol isolated from* L. saccharina.

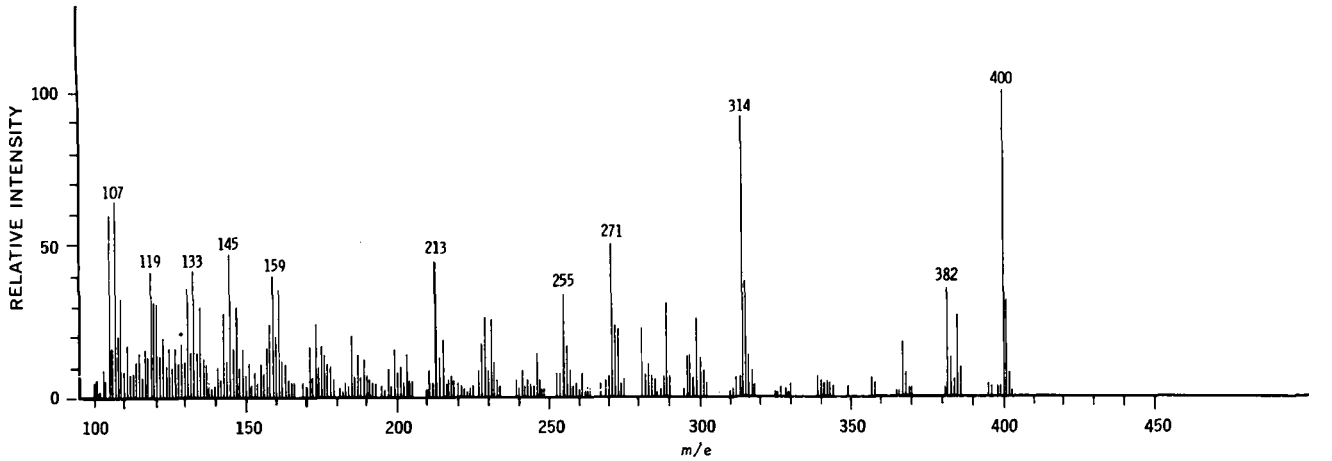


Figure 3—*Mass spectrum of 24-ketocholesterol isolated from* L. saccharina.

TLC using GLC to monitor the separation and to identify the separated sterols tentatively.

EXPERIMENTAL[1]

Chromatographic Analysis—The extraction procedure was intended to determine both free and bound sterols and included two stages with methods similar to those used in a study of *Euglena gracilis* (19). For each species studied, 100-g samples of dried, ground algae were thoroughly extracted with 400 ml of methanol–chloroform (2:1) under reflux for 1 hr. This extract was concentrated and saponified using standard procedures (20). The plant cell debris was subsequently boiled with 400 ml of potassium hydroxide (10%) in methanol-water (9:1) for 1 hr; the extract was diluted with an equal volume of water and extracted with ether.

The two extracts thus obtained were purified, following drying and evaporation of the ether, by preparative TLC utilizing 20 × 20-cm plates coated with silica gel $HF_{254\&366}$ (10 g/plate); not more than 0.25 g of extract was applied per plate. After development in hexane–ether–ethanol (80:20:4), visualization was accomplished by spraying a narrow band with concentrated sulfuric acid and warming at 60° until color appeared. The band(s) with R_f in the sterol region that turned red or violet was (were) marked by following the band(s) across the plate under longwave UV light, and the entire section (except for that sprayed) was scraped into a sintered-glass funnel and extracted with ether.

GLC analyses were then carried out on the ether solutions using cholestane as an internal standard. The chromatograph[2] was equipped with glass columns (2 m × 3 mm) and hydrogen flame detectors. It was operated isothermally at either 240° with column packing of 1.5% OV-17 on Gas Chrom Q (100–120 mesh) or 215° with a mixture of 3% XE-60 and 3% NGS on the same support. The samples were injected into the OV-17 column as free sterols or their acetates and onto the mixed phase column as the trimethylsilyl ether derivatives.

With each species analyzed, the sterol composition of the free and bound sterols was not significantly different. Therefore, the two extracts were combined before a separation of the individual components was begun.

Isolation of Individual Sterols—After GLC analysis of the total sterols, the mixture was acetylated (20 parts pyridine and 20 parts acetic anhydride) at room temperature overnight. The remaining acetic anhydride was destroyed with excess methanol, and the mixture was poured onto ice and extracted with ether. The acetates were subjected to the exact preparative TLC conditions described for obtaining the sterols from the crude lipid, except that not more than 0.025 g of the mixture was applied per plate. The steryl acetates separated into three bands. The least polar top band (R_f 0.90–0.97) contained the major portion with two minor bands below (R_f 0.75–0.79 and R_f 0.67–0.72). After scraping and extracting the adsorbent, the composition of the bands was monitored by GLC.

The top band was subjected to a further preparative TLC separation using a system in which the silica gel $HF_{254\&366}$ was impregnated with silver nitrate (50 g silica gel–10 g silver nitrate). The elution solvent mixture was hexane–benzene (5:3). The bands on these silver nitrate plates were easily visualized under longwave UV light so spraying with sulfuric acid was not re-

[1] The samples extracted were obtained from the Gulf of St. Lawrence through the courtesy of Mr. J. Claude Wallace, Marine Plants Experimental Station, Miminegash, Prince Edward Island. The plants were dried and sorted from foreign material prior to grinding and extraction.

[2] F&M 700.

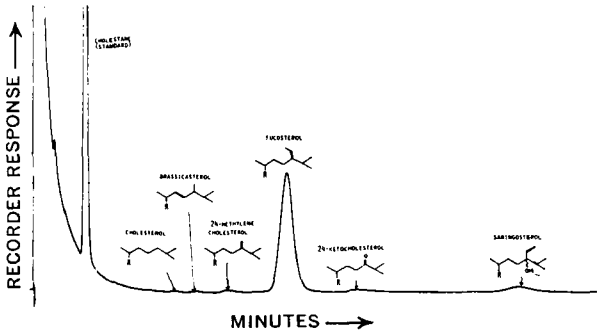


Figure 4—*GLC separation of* A. nodosum *sterols on 1.5% OV-17.*

quired. The bands were treated as already described, and GLC indicated complete separation.

Mass spectra were then recorded[3]. Melting points and IR spectra were consistent with those reported for the individual compounds.

RESULTS AND DISCUSSION

In the figures, the structure of R is as shown in Structure I, with attachment of the side chain at position 17.

Phaeophyta—*Laminaria saccharina* was found to contain 0.20% sterols. GLC showed the relative sterol composition to be: fucosterol, 87%; 24-methylenecholesterol, 11%; cholesterol, 0.05%; 24-ketocholesterol, 0.05%; and saringosterol, 1.8% (Fig. 1). Following isolation by preparative TLC, mass spectral analyses were obtained for the various sterols. The mass spectrum of the isolated saringosterol (Fig. 2) compared favorably with the previously reported fragments (11, 21). The spectrum of 24-ketocholesterol (Fig. 3) was identical to that of the authentic compound synthesized from fucosterol in this laboratory[4].

To the knowledge of the authors, this is the first detailed sterol analysis of *L. saccharina*, although fucosterol was reported previously (22). This is also the first recorded isolation of saringosterol and 24-ketocholesterol from *Laminaria*. These results are consistent with the sterol pattern found in other brown algae (10) and thus support the phylogenetic classification.

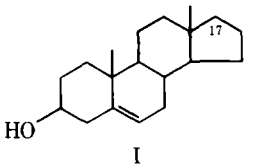
Ascophyllum nodosum was found to contain 0.10% sterols. The mixture was composed of cholesterol (0.05%), brassicasterol (<1%), 24-methylenecholesterol (2%), fucosterol (90%), 24-ketocholesterol (<1%), and saringosterol (6%) (Fig. 4). This is in good agreement with previous documentation (11).

Knights (11) suggested, upon finding 24-ketocholesterol and saringosterol in dried *A. nodosum* but not in fresh material, that these sterols might be due to aerial oxidation of fucosterol. However, 24-ketocholesterol was reported in another phaeophyte, *Pelvetia canaliculata* (23), and saringosterol was identified in several brown algae (2).

Rhodophyta—*Furcellaria fastigiata* was found to contain approximately 0.02% sterols. GLC showed cholesterol to be the major component (>99%), with minor traces of 22-*trans*-dehydrocholesterol, 24-methylenecholesterol, β-sitosterol, fucosterol, isofucosterol, and desmosterol. No further purification of the sterols was attempted because of the small amounts present.

Gibbons *et al.* (3) reported cholesterol as the only sterol present while Idler and Wiseman found 0.004% sterols, 62.7% as cholesterol and 23.2% as 24-methylenecholesterol (24). These differences may be due to the influence of seasonal collection. Significant seasonal variation in sterol content has been shown in the red alga *Rhodymenia palmata*, with varying ratios of cholesterol–desmosterol (24), and *Halosaccian*, changing from almost pure demosterol in spring to 100% cholesterol in late fall[5].

The GLC analyses of the complete sterol mixtures of the *L. saccharina* and *A. nodosum* are shown in Figs. 1 and 4. The TLC techniques employed to isolate the sterols and the GLC conditions used enabled the detection of the more polar sterols, 24-ketocholesterol and saringosterol, without the aid of column chromatography. As can be seen, the gas–liquid chromatogram readily shows the relative quantities of these rare compounds in the presence of the more common 3β-monohydroxy sterols. Preparative TLC also enabled the fast, efficient separation of each of these sterols for positive identification and possible future use.



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[3] DuPont CEC 21-110B double-focusing high-resolution mass spectrometer.

[4] Unpublished data.

[5] S. H. Safe and J. McLachlan, Atlantic Regional Laboratory, National Research Council of Canada, Halifax, Nova Scotia, personal communication, 1973.

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[x] To whom inquiries should be directed.

Identification of 2-Ethyl-2-methyl-3-hydroxysuccinimide as a Major Metabolite of Ethosuximide in Humans

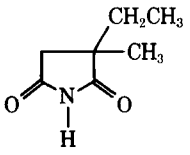
P. G. PRESTE, C. E. WESTERMAN, N. P. DAS, B. J. WILDER, and J. H. DUNCAN[x]

Abstract □ A previously unreported major urinary metabolite of the antiepileptic drug ethosuximide (I) was isolated from a patient suffering from petit mal epilepsy. The metabolite was identified as the ring-hydroxylated ethosuximide, 2-ethyl-2-methyl-3-hydroxysuccinimide (II), by GLC, low- and high-resolution electron-impact mass spectrometry, and proton NMR spectroscopy of its *N*-methyl derivative.

Keyphrases □ Ethosuximide—isolation and identification of 2-ethyl-2-methyl-3-hydroxysuccinimide as human urinary metabolite □ 2-Ethyl-2-methyl-3-hydroxysuccinimide—isolation and identification as major human urinary metabolite of ethosuximide □ Antiepileptic agents—isolation and identification of 2-ethyl-2-methyl-3-hydroxysuccinimide as major human urinary metabolite of ethosuximide

Ethosuximide (2-ethyl-2-methylsuccinimide, I) is the anticonvulsant drug of choice in the treatment of petit mal epilepsy. Two human urinary metabolites of this antiepileptic were recently (1) identified: 2-(1-hydroxyethyl)-2-methylsuccinimide (present as a diastereoisomeric pair) and 2-acetyl-2-methylsuccinimide (found only in cases of multiple dosing). Other metabolic products of I were indicated but not identified.

This paper reports the identification of a major human urinary metabolite of I, 2-ethyl-2-methyl-3-hydroxysuccinimide (II), employing the combined techniques of GLC, high- and low-resolution electron-impact mass spectrometry, and proton NMR spectroscopy[1].



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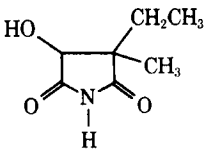

II

EXPERIMENTAL

Isolation and Chemical Pretreatment of Urinary Extracts—Twenty-four-hour urine collections were obtained from: *(a)* a 24-year-old female patient, 60 kg, receiving 1000 mg/day for 7 years, *(b)* a 20-year-old male volunteer, 80 kg, receiving a 500-mg single oral dose, and *(c)* a 30-year-old male volunteer, 70 kg, receiving a 500-mg single oral dose. Aliquots (20 ml) of urine were acidified to about pH 1 with 6 *M* HCl, saturated with sodium chloride, and extracted with three 20-ml portions of ether. The combined ether extracts were evaporated to dryness at reduced pressure[2], and the residue was dissolved in 1 ml of methanol. The resulting solution was treated (2) with excess ethereal diazomethane for about 1 hr at room temperature, after which time the sample was concentrated to near dryness under an air jet. This residue was then dissolved in 200 μl of methanol for subsequent GLC analysis.

GLC and Mass Spectrometry—Analytical separations were performed by on-column injections using a dual-column gas chromatograph[3] equipped with dual hydrogen flame-ionization detectors. Stainless steel columns, 1.82 m (6 ft) × 0.31 cm (0.125 in.), packed with either 3% SE-30 or 3% OV-17 on Chromosorb G (80–100 mesh) were employed for separations under the following conditions: injector, 225°; detector, 310°; carrier gas (helium), 30 ml/min; and column oven, 100–300° at 8°/min. Relative retention times are expressed in terms of Kovats Indices (KI) (3). Preparative GLC was performed on the same instrument under the same conditions utilizing a splitting device designed so that about 5% of the effluent from the GLC column was directed to the detector and the remainder was directed to a collection device (4) *via* a stainless steel transfer line maintained at 225°.

Combined GLC–mass spectrometry was carried out under identical GLC conditions as already reported employing a single-column gas chromatograph[4] interfaced to a low-resolution, double-focusing mass spectrometer[5]. Half of the GLC column effluent was directed to a flame-ionization detector and the other half was directed to the ion source of the mass spectrometer *via* a stainless steel transfer line (230°) and a single-stage jet separator (260°). The following electron-impact mass spectrometric conditions were employed: ionizing voltage, 70 ev; ionizing current, 100 μamp; resolution, 1100 (10% valley); and ion source temperature, 280°.

High-resolution mass measurements were performed[6] employing the direct probe insertion technique under the following electron-impact mass spectrometry conditions: ionizing voltage, 70 ev; resolution, 10,000 (10% valley); ion source temperature, 70°; and direct probe temperature, 70°.

NMR Spectrometry[7]—Approximately 100 μg of the GLC isolated samples was dissolved in a stock solution of carbon tetrachloride, which was either saturated with perfluorocyclobutane or contained 5% by volume hexafluorobenzene and 0.1% by volume tetramethylsilane. The fluorocarbons served as the lock material for spectrometer frequency stabilization, and the tetramethylsilane served as the chemical shift reference material. Spectra were obtained in 5-mm diameter cylindrical microcavity (about 100 μl) sample cells.

RESULTS AND DISCUSSION

The GLC trace (OV-17) obtained upon injection of the methylated urine extract from a patient maintained on ethosuximide is presented in Fig. 1. Comparison of this tracing to similar extracts obtained from subjects not receiving ethosuximide indicated that the peaks at KI 1480, 1595, and 1625 result from drug administration. When the urine extract shown in Fig. 1 was not treated with diazomethane or was treated with diazomethane but without the

[1] While this manuscript was in preparation, another report appeared concerning the identification of II as a metabolite of ethosuximide by GLC–mass spectrometry; see M. G. Horning, C. Stratton, J. Nowlin, D. J. Harvey, and R. M. Hill, *Drug Metab. Dis.*, 1, 569(1973). Structure II was inferred from the mass spectrum of its *N*-methyl-*O*-trimethylsilyl ether derivative, but hydroxylation at the C-2 methyl group could not be conclusively ruled out.

[2] Roto-vap.

[3] Varian model 2700.

[4] Varian model 1200.

[5] DuPont 21-491.

[6] On an AEI MS-9 mass spectrometer.

[7] Proton NMR spectra were obtained at 90 MHz on a Bruker HX-90 high-resolution spectrometer equipped with a fast Fourier transform system employing a Nicolet 1083 computer.

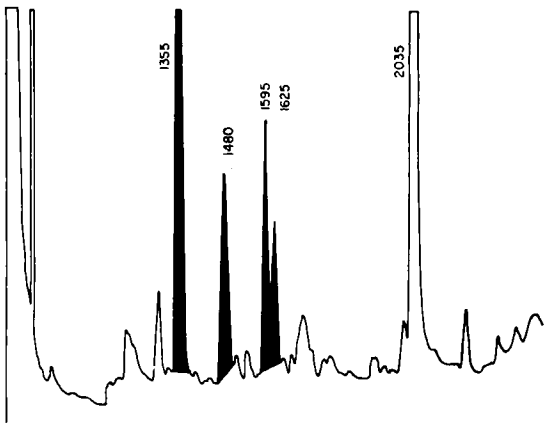

Figure 1—*Analytical GLC trace (OV-17) of methylated human urine extract from a female patient maintained on ethosuximide for 7 years. Relative retention times are expressed in terms of Kovats Indices. Shaded areas represent peaks due to ingestion of drug.*

added methanol catalyst, only one significant peak, at KI 1450, resulted upon subjection to identical GLC analysis. This peak was found to correspond to unmethylated ethosuximide by cochromatography (OV-17 and SE-30) and combined GLC-mass spectrometry.

The compound possessing KI 1355 (Fig. 1) was shown by cochromatography (OV-17 and SE-30) to be *N*-methylethosuximide. In addition, the electron-impact mass spectrum of this compound was identical to an authentic sample (Fig. 2). The spectrum is characterized by a weak but detectable molecular ion (M^+) at *m/e* 155 and a base peak (100% relative intensity) at *m/e* 127 (M − 28). Ring fission of the succinimide nucleus (5) gives rise to major fragment ions at *m/e* 70 and 55.

Figure 3 shows the mass spectrum obtained for KI 1480. Occurrence of a molecular ion (M^+) at *m/e* 171, 16 amu higher than that obtained from the methylated drug, suggests that the compound is a monohydroxylated derivative of I. The shift of 16 amu for each of the three major ions in the spectrum (*m/e* 143, 86, and 71) further supports the monohydroxyethosuximide structure. Isolation of this compound by preparative GLC and subsequent subjection to high-resolution mass spectrometry confirmed that *m/e* 171 does possess the $C_8H_{13}NO_3$ molecular formula (calc., 171.0895; found, 171.0901).

The intense peak at *m/e* 143 in the mass spectrum of this methylated metabolite was composed of a single ionic species of empirical formula $C_6H_9NO_3$ (calc., 143.0582; found, 143.0590). Formation of this ion represents loss of the elements of ethylene (C_2H_4) from the molecular ion, a fragmentation pathway favorable only if the integrity of the C-2 ethyl side chain of I is maintained. The mechanism by which this 143 ion occurs undoubtedly involves a McLafferty rearrangement, as previously proposed for similarly substituted cyclic imides (5) and barbiturate derivatives (6).

Table I—Proton NMR Chemical Shifts and Coupling Patterns of *N*-Methyl Derivatives of Ethosuximide (I) and Metabolite, KI 1480 (II)

Chemical Shift[a]		Coupling Patterns[b]
	I	
NCH_3	2.91	s
Ring CH_2	2.30 2.44	*AB*
Ethyl CH_2	1.74 1.68	pair q by 7.3 Hz
CH_3	1.26	s
Ethyl CH_3	0.87	t by 7.3 Hz
	II	
NCH_3	2.96	s
Ring CH	4.22	s
OH	2.59	bs
Ethyl CH_2	1.80 1.74	pair q by 7.3 Hz
CH_3	1.16	s
Ethyl CH_3	0.97	t by 7.3 Hz

[a] In parts per million downfield with respect to tetramethylsilane. [b] s = singlet, t = triplet, q = quartet, and b = broad.

The two major ions resulting from ring fission of the succinimide nucleus at *m/e* 86 and 71 corresponded to $C_5H_{10}O$ (calc., 86.0732; found, 86.0734) and C_4H_7O (calc., 71.0497; found, 71.0503). These ions could arise from either the *N*-methyl derivative of the ring-hydroxylated compound, II, or its positional isomer formed by hydroxylation at the C-2 methyl group. The latter structure, however, would be expected to yield a significant peak at *m/e* 141 corresponding to loss of CH_2O from M^+ *via* a McLafferty rearrangement similar to that proposed for $M^+ - C_2H_4$. Conclusive proof that KI 1480 represents the ring-hydroxylated derivative was obtained by preparative GLC isolation and subsequent proton NMR analysis.

The close correspondence of the proton spectra of the *N*-methyl-derivatives of ethosuximide (I) and KI 1480 (II) is summarized in Table I. Ring hydroxyl substitution in the methylated metabolite replaced the characteristic *AB* splitting pattern observed for the nonequivalent ring methylene protons in the methylated I with a singlet and a broad singlet at 4.33 and 2.59 ppm for the ring proton and the hydroxyl proton, respectively. Saturation of the sample solution with aqueous hydrochloric acid displaced the 2.59-ppm resonance to about 1.1 ppm, whereas all other resonances were unaffected. The quartet and triplet patterns are characteristic of the methylene and methyl protons of an ethyl group. The chain methylene protons are nonequivalent, as they are on a car-

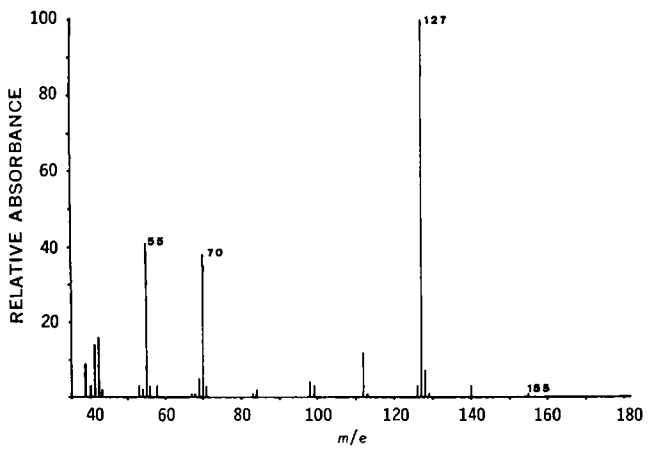

Figure 2—*Electron-impact mass spectrum of* N-*methylethosuximide (peak 1355, Fig. 1).*

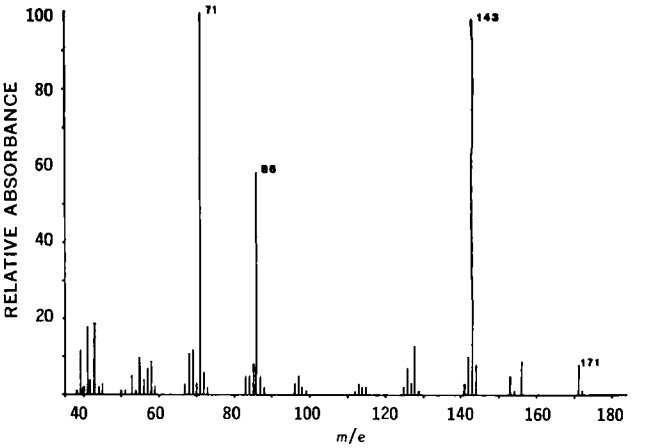

Figure 3—*Electron-impact mass spectrum of* N-*methyl-2-ethyl-2-methyl-3-hydroxysuccinimide (peak 1480, Fig. 1).*

bon bonded to an asymmetric carbon, and appear as a pair of overlapping quartets.

By combined GLC–mass spectrometry, KI 1595 and 1625 were also shown to represent *N*-methylmonohydroxyethosuximide derivatives (M^+ at m/e 171). The mass spectra of these compounds were virtually identical, indicating a stereoisomeric pair. The most intense peak in both spectra corresponded to loss of C_2H_4O from the molecular ion. In addition, the spectra were devoid of an m/e 143 peak corresponding to loss of C_2H_4 from M^+. On this basis it was concluded that these compounds represented the previously reported (1) stereoisomeric hydroxyethylethosuximides, 2-(1-hydroxyethyl)-2-methylsuccinimide.

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Synthesis and Antibacterial Properties of Substituted Decylbarbituric Acids

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Abstract □ A series of substituted decyloxybarbituric and decylthiobarbituric acids was prepared and evaluated for antibacterial activity. The acids were synthesized by condensing the appropriate disubstituted malonic ester with urea in a potassium *tert*-butoxide–dimethyl sulfoxide medium at room temperature. When tested against *Bacillus subtilis* and *Staphylococcus aureus*, the most active compound was 5-allyl-5-decylbarbituric acid. A number of other decyloxybarbituric acids showed some activity, but the decylthiobarbituric acids were completely inactive. All compounds were inactive against *Escherichia coli* and *Proteus vulgaris*.

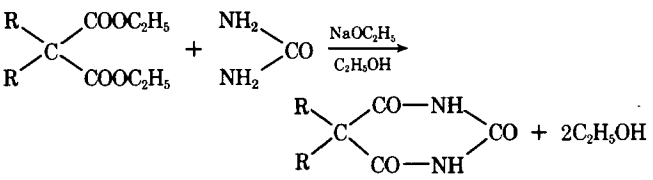
Keyphrases □ Barbituric acids, substituted decyl—synthesized and screened for antibacterial properties □ Decyloxybarbituric and decylthiobarbituric acids—synthesized and screened for antibacterial properties □ Antibacterial activity—substituted decyloxybarbituric and decylthiobarbituric acids

The most general method for the synthesis of barbituric acids has been the base-catalyzed condensation of a malonic ester with urea in refluxing ethanol (1) (Scheme I). Yields from this reaction are normally in the 50–75% range. The advantage of using a potassium *tert*-butoxide–dimethyl sulfoxide medium for this condensation was previously reported (2). This technique was used to synthesize a series of new decyl-substituted barbituric acids which were screened for antibacterial activity. Barbituric acid itself has been shown to inhibit the growth of *Escherichia coli* (3); sodium phenobarbital inhibits the growth of *E. coli*, several hemolytic streptococci, pneumococci, and *Haemophilus influenzae* (4); and several substituted quinolinium barbituric acid salts inhibit the growth of *Streptococcus* and *Staphylococcus* species (5).

RESULTS AND DISCUSSION

The disubstituted malonic esters (Table I) were prepared by condensing *n*-decyl bromide with the appropriate sodiomalonic ester in dimethylformamide according to a procedure modified from that of Zaugg *et al.* (6). The resulting diesters were then condensed with urea at room temperature in a potassium *tert*-butoxide–dimethyl sulfoxide medium to give the oxybarbituric and thiobarbituric acids (Table II). Of special note are the general improvement of the yields and the simplicity of the reaction workup as compared to the classical technique.

Compounds 6–12 were tested *in vitro* against four microorganisms (Table III): *Bacillus subtilis, Staphylococcus aureus, E. coli,* and *Proteus vulgaris*. The only activity found was against the Gram-positive organisms. All compounds were inactive against the Gram-negative organisms at 250 μg/ml, the highest concentration studied. The most active compound was the 5-allyl-5-decyl derivative, Compound 6. Substitution of sulfur for



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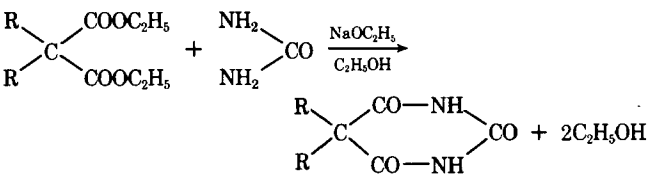
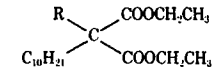


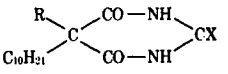
Scheme I

Table I—Substituted Decyl Malonic Esters



Compound	R	Yield, %	Boiling Point (mm)	n_D^{25}	Formula	Analysis, % Calc.	Found
1	Allyl	65	150–152° (0.3)	1.4452	$C_{20}H_{36}O_4$	C 70.55	70.83
						H 10.66	10.73
2	*n*-Butyl	63	154–156° (0.2)	1.4412	$C_{21}H_{40}O_4$	C 70.74	70.45
						H 11.31	11.35
3	Isobutyl	56	154–156° (0.3)	1.4427	$C_{21}H_{40}O_4$	C 70.74	71.15
						H 11.31	11.35
4	Benzyl	46	192–194° (0.3)	1.4695	$C_{24}H_{38}O_4$	C 73.80	73.97
						H 9.81	9.66
5	Methallyl	30	158–160° (0.4)	1.4478	$C_{21}H_{38}O_4$	C 71.12	71.24
						H 10.82	10.64

Table II—Substituted Decylbarbituric Acids



Compound	R	X	Reaction Time, hr	Yield, %[a]	Melting Point[b]	Formula	Analysis, % Calc.	Found
6	Allyl	O	24	89	88–90°	$C_{17}H_{28}N_2O_3$	C 66.21	66.18
							H 9.15	9.08
7	Allyl	S	24	91	87–89°	$C_{17}N_{28}N_2O_2S$	C 62.93	63.12
							H 8.70	8.78
8	*n*-Butyl	O	24	62	93–95°	$C_{18}H_{32}N_2O_3$	C 66.63	66.82
							H 9.94	9.84
9	Isobutyl	O	24	88	61–63°	$C_{18}H_{32}N_2O_3$	C 66.63	66.48
							H 9.94	9.74
10	Benzyl	O	48	78	144–146°	$C_{21}H_{30}N_2O_3$	C 70.36	70.28
							H 8.44	8.44
11	Benzyl	S	48	73	94–96°	$C_{21}H_{30}N_2O_2S$	C 67.34	67.75
							H 8.07	8.18
12	Methallyl	O	48	73	83–84°	$C_{18}H_{30}N_2O_3$	C 67.04	66.79
							H 9.40	9.31

[a] Crude. All compounds were recrystallized from 95% ethanol. [b] Melting points are uncorrected.

oxygen in Compound 6 produced thiobarbituric acid, Compound 7, which was completely inactive. The same substitution with the 5-benzyl-5-decyl derivatives, Compounds 10 and 11, gave a similar effect, although somewhat less dramatic owing to the decreased activity of 10. Although both 5-butyl-5-decyl derivatives were equally active against *B. subtilis*, branching in the butyl side chain, Compound 9 *versus* Compound 8, gave a slightly increased activity against *S. aureus*. Compound 12, combining both the allyl group and the branched chain, was also less active than Compound 6 against *S. aureus*.

EXPERIMENTAL[1]

The synthesis of compounds listed in Tables I and II is illustrated by the following examples.

Diethyl Allyldecylmalonate (Compound 1)—To a suspension of 0.10 mole sodium hydride (4.0 g of a 60% dispersion in mineral oil) in 50 ml dry dimethylformamide was added 20.0 g (0.10 mole) diethyl allylmalonate (slowly over 20 min). After the addition was complete, the mixture was stirred for 1 hr or until hydrogen evolution ceased. Then 23.0 g (0.10 mole) *n*-decyl bromide was added slowly, and the mixture was stirred for 1 additional hr. The reaction mixture was poured into two volumes of ice water and extracted three times with equal volumes of ether. The extracts were dried over magnesium sulfate, the solvent was removed *in vacuo*, and the residue was fractionally distilled *in vacuo* to give 22.0 g (65%) of the malonate, Compound 1, bp 150–152° (0.3 mm). The IR and NMR spectra were fully consistent with structure 1.

[1] Melting points were determined with a Fisher Mel-Temp apparatus and are uncorrected. IR spectra were recorded on an Infracord spectrophotometer, and NMR spectra were measured on a Perkin-Elmer R-20B instrument. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

5-Allyl-5-decylbarbituric Acid (Compound 6)—To a well-stirred suspension of 1.70 g (5 mmoles) Compound 1 and 1.50 g (25 mmoles) urea in 35 ml dry dimethyl sulfoxide was added slowly, with stirring, a solution of 1.22 g (11 mmoles) potassium *tert*-butoxide in 40 ml dry dimethyl sulfoxide. The resulting solution was stirred at room temperature for 24–48 hr, poured into two volumes of an ice-water slush, and acidified with dilute hydrochloric acid. The solid barbiturate (Compound 6) obtained in 89% yield was removed by suction filtration, washed with water, and dried. Recrystallization from 95% ethanol gave white crystals, mp 88–90°; λ_{max} 243 nm, ϵ 10,500 (10^{-3} *M* KOH in ethanol). The IR and NMR spectra were fully consistent with structure 6.

Biological Activity—The antibacterial activity was evaluated using a method modified from that of Waksman and Reilly (7). A stock solution of the compound in methanol was diluted into li-

Table III—Antibacterial Activity of Disubstituted Barbituric Acids

Compound	Minimum Inhibitory Concentration, μg/ml[a] *B. subtilis* ATCC 6633	*S. aureus* ATCC 6538 P	*E. coli* ATCC 10536	*P. vulgaris* ATCC 13315
6	10	10	>250	>250
7	>500	>500	>250	>250
8	10	>500	>250	>250
9	10	350	>250	>250
10	100	250	>250	>250
11	500	>500	>250	>250
12	10	250	>250	>250

[a] Minimum inhibitory concentration is the lowest concentration of compound that prevents visible growth after 48 hr of incubation at 34°.

quefied nutrient agar[2]. After hardening, the plates were inoculated with an aqueous suspension of the test organism, incubated at 34°, and examined after 48 hr.

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COMMUNICATIONS

Antiviral Activity of Triterpenoid Saponins Containing Acylated β-Amyrin Aglycones

Keyphrases □ Triterpenoid saponins containing acylated β-amyrin aglycone structure—antiviral activity □ Antiviral activity—11 triterpenoid saponins containing acylated β-amyrin aglycone, structure evaluated

Sir:

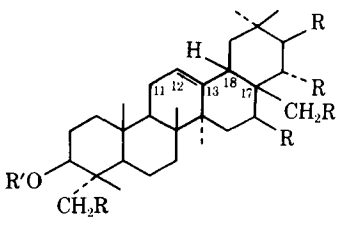
Previously we reported (1) the *in vitro* antiviral activity of gymnemic acids A and B, which were isolated from *Gymnema sylvestre* R. Br. leaves and were shown to be acylated derivatives of β-amyrin glycosides (2–5). Gymnemic acid A also exhibited significant antiviral activity against influenza A virus in mice when administered intraperitoneally (1). The wide occurrence in nature (6) of the triterpenoid saponins containing the β-amyrin skeleton (I) prompted us to evaluate the antiviral activity of this group of natural products. The present communication reports the *in vitro* anti-influenzal activity of 11 naturally occurring triterpenoid saponins structurally related to the gymnemic acids. These results, together with those obtained with gymnemic acid derivatives, permit delineation of preliminary structure–antiviral activity relationships among the triterpenoid saponins studied.

Tables I and II summarize the *in vitro* antiviral activity of gymnemic acids, their derivatives, and various triterpenoid saponins against influenza (A_2/Japan 305) virus. The antiviral activity was evaluated by measuring either the decrease in the 48-hr infectivity yield of virus in eggs from calf kidney cells inoculated with virus (1) or the inhibition of hemadsorption of guinea pig erythrocytes by virus on primary calf kidney cells (15). Based upon the preliminary antiviral results obtained with gymnemic acids (II–V), their derivatives (VI–IX), and other structurally related triterpenoid saponins (X–XX), the following remarks may be made regarding the structure–antiviral activity relationships among the triterpenoid saponins containing β-amyrin skeleton:

1. The majority of the triterpenoid saponins containing the acylated β-amyrin skeleton employed in this study exhibited anti-influenzal activity *in vitro*.

2. The presence of aldehyde (XI, XIV, and XVI) and/or carboxyl (XII, XV, XVI, and XIX) groups in place of or in addition to acyl groups in the β-amyrin skeleton of triterpenoid saponins markedly reduced their antiviral activity.

3. Alteration of the basic β-amyrin skeleton (I), *e.g.*, replacement of the 12,13-double bond with 11,13(18)-diene as in saikosaponin b (XVIII), completely abolished *in vitro* anti-influenzal activity. However, the activity is restored if the 11,12-double bond and methyleneoxy group at the 13,17-position



I: R = R′ = H
VII: R = OH, R′ = H
IX: R = $OCOCH_3$, R′ = $COCH_3$

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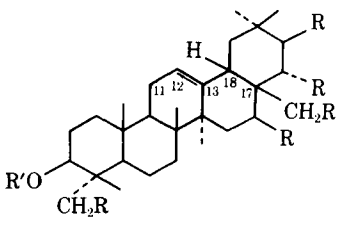

I: R = R′ = H
VII: R = OH, R′ = H
IX: R = $OCOCH_3$, R′ = $COCH_3$

Table I—Antiviral Activity of Gymnemic Acids and Their Derivatives against Influenza (A_2/Japan 305) Virus *In Vitro*

Compound Number	Compound	Reference	Infectivity[a] Concentration, μg/ml Ethanol	Infectivity[a] Treated	Infectivity[a] Control	Activity
II	Gymnemic acid A	2	200	1	5.5	Good
III	Gymnemic acid B	2	200	4.3	7.3	Moderate
IV	Gymnemic acid C	2	200	7.0	7.3	None
V	Gymnemic acid D	2	200	7.5	7.3	None
VI	Gymnemic acid A methyl ester	7	200	1	4.5	Good
VII	Gymnemagenin	7–9	200	4.5	4.3	None
VIII	Genin mixture[b]	3	200	1	5.5	Good
IX	Gymnemagenin acetate mixture[c]	7	100[d]	4.0	4.3	None

[a] This represents the ability of compounds tested to decrease the 48-hr infectivity yield of virus (reported as $\log_{10}$ of the infectious dose) in eggs from primary calf kidney cells inoculated with virus. See Ref. 1 for experimental details. [b] The genin mixture was obtained by acid hydrolysis of the crude gymnemic acid mixture and contains no sugar residues (3). [c] Acetylation of gymnemagenin gives rise to a mixture of acetate derivatives, the hexaacetate IX being the major product (7). [d] Maximum tolerated concentration.

Table II—Antiviral Activity of Triterpenoid Saponins against Influenza (A_2/Japan 305) Virus *In Vitro*

Compound Number	Compound	Reference	Plant Source	Concentration[a], μg/ml Ethanol	Maximum Tolerated Concentration, μg/ml	Inhibition[b], %
X	Aescin[c]	10	*Aesculus hippocastanum* L. (Hippocastanaceae)	12.5	12.5	92
XI	Cyclamin A	10	*Cyclamen europeum* (Primulaceae)	12.5	12.5	33
XII	Glycyrrhizin[c]	10	*Glycyrrhiza glabra* L. (Leguminosae)	100[d]	—	37
XIII	Hederocoside C	10	*Hedera helix* L. (Araliaceae)	100[d]	—	54
XIV	Primula saponin[c]	10	*Primula veris* L. (Primulaceae)	6.2	6.2	89
XV	Prosapogenin	11	*Polygala senega* L. (Polygalaceae)	100[d]	—	36
XVI	Quillaja saponin[c]	10	*Quillaja saponica* (Rosaceae)	12.5	25[e]	22
XVII	Saikosaponin a	12	*Bupleurum falcatum* L. (Umbilliferae)	50[d]	—	69
XVIII	Saikosaponin b	12	*Bupleurum falcatum* L. (Umbilliferae)	100[d]	—	0
XIX	Senegin	10	*Polygala senega* L. (Polygalaceae)	12.5	50[e]	34
XX	Theasaponin[c]	10	*Thea sinensis* L. (Theaceae)	6.2	6.2	73
II	Gymnemic acid A	2	*Gymnema sylvestre* R. Br. (Asclepiadaceae)	100	500[e]	63
VII	Gymnemagenin	7–9	*Gymnema sylvestre* R. Br. (Asclepiadaceae)	50[d]	—	0
IX	Gymnemagenin Acetate[f]	7	*Gymnema sylvestre* R. Br. (Asclepiadaceae)	100[d]	—	0
XXI	Amantadine[g]	13, 14	—	25	100	98

[a] The concentrations tested were at 100 μg/ml or at the maximum tolerated concentration if availability of samples permitted. [b] Inhibition of quantitative hemadsorption of guinea pig erythrocytes by virus on primary calf kidney cells. See Ref. 15 for experimental details. [c] Crude sample. [d] Maximum concentration tested. [e] Estimated maximum tolerated concentration. [f] Acetylation of gymnemagenin gives rise to a mixture of acetate derivatives, the hexaacetate IX being the major product (7). [g] Synthetic compound (16, 17) used as a positive control.

are present in place of the 12,13-double bond, as in saikosaponin a (XVII).

4. For *in vitro* activity, a sugar residue in the triterpenoid compounds tested does not appear to be essential since sugar-free acylated derivatives of gymnemic acids (VIII) exhibited comparable antiviral activity. Also, methylation of the glucuronic acid moiety in gymnemic acid A (VI) did not produce any significant change in *in vitro* activity against influenza virus.

5. Polyhydroxy and polyacetyl derivatives of β-amyrin, *e.g.*, gymnemagenin (VII) and gymnemagenin acetate (IX), were found to be inactive as inhibitors of influenza virus *in vitro*.

Although a number of triterpenoid saponins are known to possess antimicrobial (10) and antiparasitic (6) activities, antiviral activity has not been previously indicated for this group of natural products. Gymnemic acid A appears to be the first triterpenoid saponin reported to exhibit antiviral activity both *in vitro* and *in vivo* (1).

However, the antiviral activity of various synthetic

steroids (18) and that of antibacterial agents, fusidic acid, cephalosporin P_1, helvolic acid, and related compounds (19,20) have been reported. Cardiac glycosides[1] (21–24) and steroidal glycosides isolated from starfish (25) also possess antiviral activity. Furthermore, the triterpenoid saponins (X–XIV, XVI, XIX, and XX) found to inhibit influenza virus in the present study were reported to be antibacterial agents (10).

At least one site of action of gymnemic acid A against influenza virus is indicated to be associated with relatively early events in the virus infectious cycle which may involve inhibition of virus–host cell attachment (1). Cardiac glycosides are believed to exhibit antiviral action by competing with infectious viruses for the virus-specific receptor sites on the cell membrane and adenosine triphosphatase molecules involved in virus–host cell interactions (24). The recent observation that gymnemic acid also inhibits adenosine triphosphatase activity (26) suggests that the mechanism of antiviral action of gymnemic acid may be similar to that proposed for cardiac glycosides.

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Joseph E. Sinsheimer[x]

College of Pharmacy
University of Michigan
Ann Arbor, MI 48104

Kenneth W. Cochran

The Virus Laboratory
Department of Epidemiology
School of Public Health and
Department of Pharmacology
Medical School
University of Michigan
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Trapa bispinosa Starch as a Tablet Disintegrant

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Sir:

Trapa bispinosa (Faln. onagraceae) (1), commonly known as water caltrop or water chestnut, is an aquatic plant which has been grown in India since ancient times at negligible cost. It is widely used as a general article of food and has a palatable taste. It has been reported that the plant nut contains 73% starch. These preliminary data prompted us to study this starch[1] as a tablet disintegrant vis-à-vis other starches currently used.

Starch was isolated by the commonly used water extraction method. Preliminary physical and chemical analysis of trapa starch showed similar characteristics to those of maize starch, commonly used as a disintegrant for compressed tablets. Trapa starch passes all the tests described under the starch mono-

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Table I—Disintegration Time (Minutes) of Prepared Tablets

Substance	Starch Disintegrant	Mean Disintegration Time, 10% Starch	Mean Disintegration Time, 15% Starch
Aspirin	Potato	5.35	6.12
	Trapa	6.49	4.30
	Wheat	7.0	5.20
	Maize	6.0	4.10
Calcium carbonate	Potato	8.1	6.0
	Trapa	8.30	6.0
	Wheat	9.0	8.0
	Maize	7.0	5.0
Sulfathiazole	Potato	4.0	4.3
	Trapa	4.4	3.3
	Wheat	4.56	5.3
	Maize	6.1	4.0
Sodium bicarbonate	Potato	12.2	10.2
	Trapa	12.45	9.2
	Wheat	10.1	13.2
	Maize	9.3	8.3

graph in the India Pharmacopoeia (2). Trapa starch is tasteless, odorless, and white in color with a fine texture. The starch consists of polyhedral or rounded granules about 10–40 μm. The gelatinization temperature of the starch was found to be at 80°, as observed by the method suggested by Radley (3). Chemical studies gave the following results: total reducing sugars, 67.5%; protein, 7.58%; and crude fiber, 2.34%. Identification of sugars was carried out by a paper chromatographic method, and glucose and maltose were identified. Among the metals, iron was detected.

For the present investigation, four starches were selected as tablet disintegrants: trapa, potato, maize, and wheat. By using each of these starches separately, tablets of aspirin, sulfathiazole, calcium carbonate, and sodium bicarbonate were prepared[2]. Granules of calcium carbonate, sulfathiazole, and sodium bicarbonate were prepared by moist granulation, while aspirin granules were prepared by dry granulation. All starches were used in 10 and 15% concentrations. The tablet weight was kept constant at 0.400 g, and the tablet hardness was kept as near to 5.5 kg/cm^2 as possible by a hardness tester[3].

Appearance—All tablets prepared with trapa starch had an excellent appearance and were glossy.

Uniformity of Weight—This parameter conformed to India Pharmacopoeia (2) specifications.

Compression Ratio—The ratio was found to be nearly uniform in all the tablets (4). It ranged from 0.87 to 0.88 for aspirin, from 0.87 to 0.89 for sulfathiazole, from 1.12 to 1.13 for sodium bicarbonate, and from 1.34 to 1.37 for calcium carbonate.

Disintegration Time—The USP XV (5) apparatus and method were used to determine disintegration time. The tablets prepared with trapa starch at 15% concentration showed a remarkable decrease in disintegration time in contrast to other starches at 15% concentration as well as its own 10% concentration. The mean disintegration time of different tablets is given in Table I.

By using an analysis of variance technique, it was observed that at the 1% level of significance the 15% concentration gave lower disintegration times than the 10% concentration for all four starches. By using Scheefe's (6, 7) 95% probability interval estimate, it was observed that tablets with trapa starch had lower disintegration times than those containing potato or wheat starch but greater disintegration times than those containing maize starch.

Thus, trapa starch proved efficient as a disintegrating agent in 15% concentration. The potentialities of starch from *T. bispinosa* as a substitute for currently used starches are considerable.

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A. S. Bal[x]
S. B. Joshi

Department of Pharmaceutical Sciences
Nagpur University
Nagpur, India

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[x] To whom inquiries should be directed. Present address: c/o Principal S. D. Bal, Sydenham College, 'B' Road, Churchgate, Bombay, 400 020, India.

Binding of a New Antitumor Agent, Thalicarpine, to DNA

Keyphrases □ Thalicarpine (antineoplastic alkaloid)—binding to calf thymus DNA, N—O—O triangulation structure □ Antineoplastic alkaloids with N—O—O triangulation structure—binding of thalicarpine to calf thymus DNA □ Binding of antineoplastic alkaloids to calf thymus DNA—radiolabeled thalicarpine □ Equilibrium dialysis—determination, binding of radiolabeled thalicarpine to calf thymus DNA

Sir:

Thalicarpine (I), a new antineoplastic alkaloid with novel structure, is in initial clinical trial in our service (1).

We previously described the multiplicity of action of thalicarpine against L-1210 mouse leukemia cells in culture (2). We reported that we could not detect any binding of thalicarpine to calf thymus DNA *in vitro* by UV spectroscopy.

We now wish to present evidence for DNA binding

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A. S. Bal[x]
S. B. Joshi

Department of Pharmaceutical Sciences
Nagpur University
Nagpur, India

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[x] To whom inquiries should be directed. Present address: c/o Principal S. D. Bal, Sydenham College, 'B' Road, Churchgate, Bombay, 400 020, India.

Binding of a New Antitumor Agent, Thalicarpine, to DNA

Keyphrases □ Thalicarpine (antineoplastic alkaloid)—binding to calf thymus DNA, N—O—O triangulation structure □ Antineoplastic alkaloids with N—O—O triangulation structure—binding of thalicarpine to calf thymus DNA □ Binding of antineoplastic alkaloids to calf thymus DNA—radiolabeled thalicarpine □ Equilibrium dialysis—determination, binding of radiolabeled thalicarpine to calf thymus DNA

Sir:

Thalicarpine (I), a new antineoplastic alkaloid with novel structure, is in initial clinical trial in our service (1).

We previously described the multiplicity of action of thalicarpine against L-1210 mouse leukemia cells in culture (2). We reported that we could not detect any binding of thalicarpine to calf thymus DNA *in vitro* by UV spectroscopy.

We now wish to present evidence for DNA binding

obtained by equilibrium dialysis with tritium-labeled thalicarpine (thalicarpine-6α-7-^{3}H)[1]. This is the first report to confirm the conjecture that I, which has the necessary N—O—O triangulation for the receptor–complement feature of Zee-Cheng and Cheng (3, 4), binds to DNA.

Radioactive I (122 μCi/mg), 40 μg, was dialyzed against 40 ml of isotonic saline by placing, into 1.91 × 7.62-cm (0.75 × 3-in.) dialysis bags with 5 ml of saline, 5 ml of heat-denatured calf thymus DNA (0.7 μg/ml), 5 ml of native calf thymus DNA (0.7 μg/ml), or 5 ml of native calf thymus DNA (2.41 μg/ml). The solutions were incubated at 37° with constant shaking. At intervals of 0.25, 0.5, 1.0, 2.5, 5.0, and 18.5 hr, 100 μl was removed in duplicate and counted by liquid scintillation spectrometry. Conversions of counts per minute to disintegrations per minute were done by a computer program written by us.

It was found that the systems reached equilibrium in about 12 hr and no additional thalicarpine dialyzed from the bags into the external solution. For the same amount of DNA (3.5 μg), the binding of I was greater to denatured (3.28 nmoles/μg) than to native (0.41 nmole/μg) DNA, but the transfer rate constant for dissociation of I from DNA was less for native (3.08 hr^{-1}) than for the denatured (25.51 hr^{-1}) DNA. Therefore, native DNA was necessary for tighter binding of I.

After 24 hr, the external medium was replaced with fresh, isotonic saline and assayed as before. After an additional 24 hr, the solutions in each bag were assayed for radioactive I. The finding of persistent binding of I to native DNA suggested that this might be irreversible. Therefore, aliquots (0.2 ml) of I bound to native DNA were centrifuged in 5–20% alkaline sucrose gradients as previously described (5). Fractions from each run were analyzed for radioactivity, and the absorbance at 260 nm was determined.

In all cases, the radioactivity was found clearly separated from DNA and at the very top of the gradients. This clearly established that the binding of I to DNA was completely reversible and confirmed our previous finding that I does not lead to appreciable degradation of the DNA with which it is in contact. In similar experiments, we found that I does not bind to human serum albumin *in vitro*, but it does tightly bind to some as yet unidentified human serum component *in vivo*.

Consequently, thalicarpine is a member of a wide variety of antineoplastic compounds which bind to DNA and possess the receptor-complement feature of Zee-Cheng and Cheng (3).

Studies with DNA polymerase from *Escherichia coli* and rat liver nuclei are underway to understand further the relationship of the N—O—O triangulation feature and DNA binding by I and several of its bisbenzylisoquinoline derivatives.

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[1] Monsanto.

Larry M. Allen[x]
Patrick J. Creaven

Biochemical Pharmacology Laboratory
Oncological Pharmacology Section
NCI-VA Medical Oncology Branch
Veterans Administration Hospital
Washington, DC 20422

Received September 27, 1973.
Accepted for publication December 3, 1973.
[x] To whom inquiries should be directed.

2-Ethylpyridine Derivatives with Antitubercular Properties

Keyphrases □ 2-Ethylpyridine derivatives—antitubercular activity determined □ 2-Ethylisonicotinaldehyde thiosemicarbazone—tested for antitubercular activity □ Antitubercular agents, potential—2-ethylpyridine derivatives tested

Sir:

Treatment of tuberculosis with several recently introduced chemotherapeutic agents, such as tiocarlide, ethambutol, and rifampin, remained unsuccessful in most cases refractory to standard antitubercular drugs. Therefore, it is appropriate to continue looking for potentially active agents against resistant strains of *Mycobacterium tuberculosis*. We wish to report observation of the desired activity in a 2-ethylpyridine derivative, a member of a group previously reported as possessing tuberculostatic properties (1–3). The observation was made while screening the following types of compounds: 2-ethylisonicotinic acid thiosemicarbazide, some *N*-arylsulfonyl substituted 2-ethylpyridineisonicotinic acid hydrazides, and the thiosemicarbazone of the corresponding 2-ethylisonicotinaldehyde. The structures of these compounds and pertinent references are summarized in Table I.

Tuberculostatic activity was examined in liquid (Proskauer–Beck) and on solid (Lowenstein–Jensen) media. In one set of experiments, the compounds were tested in a series of concentrations (0.1, 1.0, 10, and 100 μg/ml medium) against a sensitive strain of *M. tuberculosis* H37 Rv. In another set, a strain resistant to standard antitubercular agents was used as the test organism and exposed to the same concentrations.

As in previous work, *in vivo* activity was routinely checked along with the bacteriostatic tests. *In vivo* activity was tested in mice infected by intravenous inoculation with *M. bovis* Ravenel Rv. Controls survived this treatment for 19–22 days. A compound

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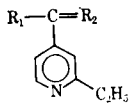
As in previous work, *in vivo* activity was routinely checked along with the bacteriostatic tests. *In vivo* activity was tested in mice infected by intravenous inoculation with *M. bovis* Ravenel Rv. Controls survived this treatment for 19–22 days. A compound

Table I—2-Ethylpyridine Derivatives Tested for Antitubercular Activity



Compound	R_1	R_2	Reference
	(*a*): —NHNH—X	=O	
1	X: —$CSNH_2$		5
2	X: —O_2S—C_6H_4-*p*-Y		4
	Y: —H		
3	Y: —CH_3		5
4	Y: —Cl		5
5	Y: —NO_2		5
6	Y: —NH_2		6
7	Y: —$NHCOCH_3$		5
8	(*b*): —H	=$NNHCSNH_2$	4, 6, 7

was considered to be active *in vivo* if a 10-mg/kg ip dose, repeated daily through 14 days, increased the mean survival period by a statistically significant increment. Statistical significance was assessed by comparing reaction time ratios according to Litchfield (8).

The results from *in vitro* experiments are shown in Table II and those from *in vivo* tests are given in Table III. The data in Table II show that the thiosemicarbazide and most *N*-arylsulfonyl hydrazides of 2-ethylisonicotinic acid (Compounds 1 and 4–7) possess rather weak tuberculostatic activities against sensitive *M. tuberculosis*, since these compounds inhibited bacterial growth only at the highest concentration level. Two hydrazides, the unsubstituted *N*-phenylsulfonyl derivative (Compound 2) and the *N*-(*p*-tolyl)sulfonyl derivative (Compound 3), were inactive in these experiments. With the resistant strain, all but one of the compounds tested were inactive. The outstanding exception was Compound 8, 2-ethylisonicotinaldehyde thiosemicarbazone. This compound is a strong tuberculostatic, since it inhibited the growth of the resistant strain at a concentration of 1.0 μg/ml and that of the sensitive microorganism at 0.1 μg/ml.

Table III—Antitubercular Action of 2-Ethylisonicotinic Acid and 2-Ethylisonicotinaldehyde Derivatives: *In Vivo* Effects

Compounds Tested[a]	ET_{50}[b], Days	*RR*[b]	*fRR*[b]
1	33.5	1.52	1.46
2	25	1.13	1.15
3	37	1.68	1.23
4	33.5	1.52	1.46
5	25	1.13	1.15
6	36	1.63	1.105
7	51	2.31	1.09
8	36.5	1.66	1.365
Streptomycin	36	1.63	1.105
Aminosalicylic acid	37	1.68	1.23
Isoniazid	51	2.31	1.09
Controls	20.5	—	—

[a] Numbers correspond to compounds listed in Table I; duration of treatment was 14 days with daily doses of 10 mg/kg. Number of animals treated was 10 per group with 10 animals as controls. [b] Statistics according to Litchfield (8); ET_{50} = median effective time, *RR* = reaction time ratio, *fRR* = factor or reaction time ratio; criterion for statistical significance of test was $RR > fRR$.

In vivo, all compounds, with two exceptions, significantly lengthened the mean survival period of mice infected with *M. bovis* (Table III). Compound 2, *N*-phenylsulfonyl hydrazide, was ineffective both *in vitro* and *in vivo;* Compound 5, *N*-(*p*-nitrophenyl)sulfonyl hydrazide, was inactive *in vivo* but was slightly active *in vitro*. Another hydrazide, the *N*-(*p*-tolyl)sulfonyl hydrazide (Compound 3) behaved in an opposite manner, with activity being observed only *in vivo*.

2-Ethylisonicotinaldehyde thiosemicarbazone was moderately effective against *M. bovis in vivo*. Its relative potency was comparable to those of streptomycin and aminosalicylic acid.

The strongest *in vivo* effect was obtained with N^1-(*p*-acetamidophenyl-N^2-(2-ethylisonicotinoyl) hydrazide (Compound 7) (about 1.4 times as potent as streptomycin), yet this compound was only weakly tuberculostatic and altogether inactive against resistant *M. tuberculosis*.

Table II—Antitubercular Action of 2-Ethylisonicotinic Acid and 2-Ethylisonicotinaldehyde Derivatives: *In Vitro* Effects

Medium	Organism[a]	Concentration of Agent, μg/ml	Effect[b] of Compounds Listed in Table I								Effect[b] of Antitubercular Drug[c]		
			1	2	3	4	5	6	7	8	S	A	I
Liquid[d]	Sensitive	0.1	−	−	−	−	−	−	−	+	+	+	+
		1.0	−	−	−	−	−	−	−	+	+	+	+
		10	−	−	−	−	−	−	−	+	+	+	+
		100	+	−	−	+	+	+	+	+	+	+	+
	Resistant[e]	0.1	−	−	−	−	−	−	−	−	−	−	−
		1.0	−	−	−	−	−	−	−	+	−	−	−
		10	−	−	−	−	−	−	−	+	−	−	−
		100	−	−	−	−	−	−	−	+	−	−	−
Solid[f]	Sensitive	0.1	−	−	−	−	−	−	−	+	+	+	+
		1.0	−	−	−	−	−	−	−	+	+	+	+
		10	−	−	−	−	−	−	−	+	+	+	+
		100	+	−	−	+	+	+	+	+	+	+	+
	Resistant[e]	0.1	−	−	−	−	−	−	−	−	−	−	−
		1.0	−	−	−	−	−	−	−	+	−	−	−
		10	−	−	−	−	−	−	−	+	−	−	−
		100	−	−	−	−	−	−	−	+	−	−	−

[a] *M. tuberculosis* H37 Rv. [b] + = growth inhibition; − = no inhibition. [c] S = streptomycin, A = aminosalicylic acid, and I = isoniazid. [d] Proskauer–Beck medium. [e] Resistant to drugs listed in Footnote *c*. [f] Lowenstein–Jensen medium.

In summary, the experiments reported in this communication revealed a strong tuberculostatic activity for 2-ethylisonicotinaldehyde thiosemicarbazone. The growth-inhibiting action of this compound extends against a strain of *M. tuberculosis* resistant to streptomycin, aminosalicylic acid, and isoniazid. In addition, 2-ethylisonicotinaldehyde thiosemicarbazone is also moderately effective in an *in vivo* test and may be considered as a promising candidate as an antitubercular drug.

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D. Korunčev[x]
Research Department
Pliva Pharm. and Chem. Works
Zagreb, Yugoslavia
I. Babić
Pediatric Clinic
Medical Faculty
University of Zagreb
Zagreb, Yugoslavia

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[x] To whom inquiries should be directed.

BOOKS

REVIEWS

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I think that this Specialist Report will appeal particularly to the pharmaceutical scientist and researcher interested in pharmacologically active and clinically useful alkaloids. It should also find a place on every library shelf as an aide to teachers and researchers.

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College of Pharmacy and Allied Health Professions
Northeastern University
Boston, MA 02115

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Staff Review

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I think that this Specialist Report will appeal particularly to the pharmaceutical scientist and researcher interested in pharmacologically active and clinically useful alkaloids. It should also find a place on every library shelf as an aide to teachers and researchers.

Reviewed by John L. Neumeyer
College of Pharmacy and Allied Health Professions
Northeastern University
Boston, MA 02115

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Staff Review

CTFA Cosmetic Ingredient Dictionary 1973. Edited by NORMAN F. ESTRIN. The Cosmetic, Toiletry, and Fragrance Association, Inc., 1625 Eye Street, N.W., Washington, DC 20006, 1973. xiv + 253 pp. 21.5 × 27.5 cm.

Information on materials utilized in the manufacture of cosmetics is compiled in this publication. To make cosmetic ingredient nomenclature more uniform, the Cosmetic, Toiletry, and Fragrance Association has designated or adopted preferred names for commonly used ingredients. For the most part, these names do not conflict with official nomenclature, *e.g.*, USAN or NF, although some deviations are found.

The bulk of the book consists of short monographs for cosmetic ingredients which include the adopted name, CAS registry number, structural formulas, reference sources, and a listing of other names. A listing of chemical/trade names referenced to the adopted name completes the book.

In summary, the experiments reported in this communication revealed a strong tuberculostatic activity for 2-ethylisonicotinaldehyde thiosemicarbazone. The growth-inhibiting action of this compound extends against a strain of *M. tuberculosis* resistant to streptomycin, aminosalicylic acid, and isoniazid. In addition, 2-ethylisonicotinaldehyde thiosemicarbazone is also moderately effective in an *in vivo* test and may be considered as a promising candidate as an antitubercular drug.

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D. Korunčev[x]
Research Department
Pliva Pharm. and Chem. Works
Zagreb, Yugoslavia
I. Babić
Pediatric Clinic
Medical Faculty
University of Zagreb
Zagreb, Yugoslavia

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[x] To whom inquiries should be directed.

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ON BEING OUR BROTHER'S KEEPER

The "golden era" of drug discovery that primarily occurred immediately following World War II resulted in explosive growth and proliferation of the entire system of producing, marketing, and distributing drug products. This dramatically rapid growth, coupled with the fact that this boom largely occurred away from the public eye, combined to create a situation which was ripe for abuse as well as for public review and investigation.

The spotlight of public scrutiny was first focused on this area in December 1959 when the late Senator Kefauver launched his congressional committee hearings; from the initial day these hearings were highlighted by a series of headline catching revelations. Since then, the spotlight has been directed at this subject on almost a continuous basis as one or another congressional committee has probed into various facets of the pharmaceutical complex. Undoubtedly, these hearings have included various doses of politics, publicity seeking, and exaggeration. On the other hand, it is undeniable that they have also served to expose many serious and diverse abuses. In some cases, mere exposure of these practices has been sufficient to prompt voluntary corrective action; in other cases, however, specific legislation has been enacted to rectify the problems brought to light.

After fifteen years of such inquiry, one might expect that there would be little more to be told. But, on the contrary, the hearings conducted in recent weeks by the Nelson Senate Subcommittee served to bring out substantial new information, and the concurrent hearings conducted by the Kennedy Senate Subcommittee brought to light a whole series of marketing abuses originating with the industry and participated in by a substantial segment of the medical and pharmacy professions.

Through much of this fifteen-year experience there has been a tendency on the part of many of us to dismiss the revelations, the abuses, even the scandals, by asserting that we have not directly fostered these practices or participated in them. However, is it enough to be innocent? Is there not some responsibility on the part of even those who are guiltless to exert a positive influence to bring about change in their area of involvement?

The Kennedy hearings publicized the prostituting of drug samples by detailmen who use them to gain favor with practitioners, by physicians who trade them to pharmacists for razor blades and toothpaste, and by pharmacists who improperly use them in dispensing to patients. The practice of many company representatives to peruse confidential prescription files, as well as the willingness of many pharmacists to open their files in this manner, also was the subject of considerable eyebrow raising on the part of the Subcommittee and the general public alike. And the free goods, gifts, prizes, and assorted "payola," as it was termed by the Senator, likewise came in for a solid round of criticism.

The public image of every drug company, of every physician, of every pharmacist was tarnished at least a little bit by these disclosures. Some certainly deserved it, but others did not. Hoffmann-La Roche, for example, does not distribute unsolicited samples in recognition of all the potential and actual abuses in the system. Likewise, a solid core of pharmacists has been responsible for the advertising code which prohibits ads for free goods and deals in APhA journals and prohibits the handout of merchandize in APhA meeting exhibits.

Unfortunately, however, enough "good people" have not protested these promotion practices as loudly and vigorously as they could. Too many pharmacists in community and hospital practice, while not approving such practices, have not actively objected either. But those who have the greatest opportunity to exert a positive influence are those who are actually employed within the industry.

In particular, many pharmaceutical scientists have long closed their eyes to the marketing and promotion practices of the companies with which they are affiliated. They have excused themselves on the grounds that such matters are outside their area of responsibility and concern. However, as suggested above, when abuses come to light the fallout touches everyone; and those who are in more of a position to exert a positive influence, but neglect to do so, will be splattered with a proportionately bigger dose.

Individually, and through their professional societies, scientists can be an effective force in fostering reforms; for example, at the very least they could commend the courage and leadership of Hoffmann-La Roche in its policy on drug samples and push other firms to adopt similar policies.

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REVIEW ARTICLE

Mass Transport Phenomena and Models: Theoretical Concepts

G. L. FLYNN*, S. H. YALKOWSKY[x], and T. J. ROSEMAN

Keyphrases □ Diffusion (free) of nonelectrolytes in the absence of bulk flow—review □ Transport and membrane permeation—review of theory and effect of permeant and barrier properties □ Membrane permeation—review of models, passive diffusion, barrier effect, partition coefficients □ Permeation, membrane—review of models, passive diffusion, barrier effect, partition coefficients

CONTENTS

Fundamentals of Membrane Permeation 480
The Modelistic Approach 480
Introduction to Membrane Characterization 481
Passive Diffusion: Kinetic and Thermodynamic Considerations 482
Diffusion in An Isotropic Medium 484
Barriers in Series: Diffusion through Laminated Structures 488
Parallel Pathways: Influence of Shunts and Pores 490
Dispersed Phases and Barrier Property 491
Time Variable Boundaries 492
Influence of Some Specific Permeant and Barrier Properties on Mass Transport 495
Diffusant Solubility as a Flux-Limiting Factor 495
Factors Determining Partition Coefficients 497
Effective Concentrations 498
Factors Affecting Diffusivity 499

"For many years I was troubled by a statement attributed to Faraday, which I have not verified, that he held his theories by his fingertips so that the least breeze of fact might blow them away. I was troubled because it seemed to me that some theories are more trustworthy and tenable than many facts. When I realized that a fact to Faraday meant something different from what it did to me, that for him a fact was not something he read in a book or a journal, but was something he observed in a laboratory and that Faraday was an exceptionally able observer, my troubles stopped until I began to study membranes. Now I need two handfuls of finger-tips; one for alleged theories and one for alleged facts .... From the first days ... the study of membranes ... has been confused by the misuse of theories. It would be so much better if we called most of them models rather than theories. Then we would not have to defend their truth but only their usefulness. Most of us do need models in order to think. Almost everyone who thinks about membranes first thinks of small holes in a plate which is very thin even compared to the size of the holes. Some biological membranes may be such diaphragms, but synthetic membranes, and many natural ones, have holes which are much smaller than the thickness of the membrane. Almost everyone takes as second choice right-circular cylindrical pores, and as a third model lets the pores curve and change in cross-section. I believe that a much more useful model resembles a pile of sand, or brush, or tangled fish-nets in that as many channels run in one direction as any other, and the channels are continually branching and coming together again. There are few if any dead-end pockets or neighbouring points connected only through long loops. The model need hardly be more specific than this .... Many of us are studying synthetic membranes with the hope that they may serve as models for natural membranes. Some-

times I am hopeful that these model studies will give a positive contribution to our knowledge of physiological membrane phenomena. Often I can only admire the methods of the physiologists, but perhaps remind them of Faraday's finger-tips and of the fact that since their phenomena are more complicated than those of the physical chemists, their thinking must be less naive . . . ." G. Scatchard (1)

Membrane transport is a subject of great breadth, diversity, and complexity. Since life itself is dependent on the normal functioning of membranes and membrane transport systems, the barrier properties of membranes are an intense concern of biologists and biologically oriented physical scientists. The essence of the permeability problem facing the bioscientist was succinctly set forth in the introductory thoughts of Scatchard (1)[1]. In particular, Scatchard pointed to the fabrication of models consistent with experimental observations and based on fundamental thermodynamic considerations as a means of furthering comprehension of the phenomena involved. Since pharmacy, defined in its broadest sense, also is concerned with a broad spectrum of physical kinetic processes, the research pharmacist's applications of membrane transport principles are not limited solely to biological areas. The development of formulations that meter predetermined dosages on exact schedules to intended surfaces of absorption is at least partially dependent on sound application of permeation theory. Selection of appropriate packaging materials is another area where barrier properties are of great importance. These areas too can be advanced measurably by a fundamental modelistic point of view. Presenting the conceptual tools to accelerate the modelistic approach is a major goal of this review.

There is an enormity of literature dealing with mass transport. Most extensive compilations (books and reviews) are highly specialized or slanted to the needs of a particular membrane transport problem area. Few have been directed to the specific needs of the medically oriented scientist. This fact has rendered difficult the selection of suitable models for drug delivery and absorption. Therefore, two important purposes of the present review are to provide meaningful criteria for typing membranes and to indicate how different macroscopic fabrics and microstructures influence membrane barrier properties. To accomplish these ends, the various possible components of barriers (*i.e.*, membrane continuum, fillers, crystalline regions, *etc.*) and shunts, pores, diffusion layers, *etc.*, are discussed separately with respect to their influence on barrier resistance and/or permeability. Emphasis is placed on integrating them into an overall barrier property; attention is especially drawn to circumstances where permeabilities and diffusional resistances are additive. By using this approach, it is often possible to characterize complex membrane structures from the dimensions, arrangements, and diffusional resistances of the component parts.

To accomplish these specified goals, the content of the review has been primarily limited to passive transport (free diffusion) of nonelectrolytes in the absence of bulk flow; therefore, electrochemical gradients, osmotic gradients, thermal gradients, facilitated transport, and active transport are not within its scope. Many excellent reviews of these topics can be found in the literature. In addition to these exclusions, laboratory methodology *per se* is only incidentally covered. Recent discussions on the design and operation of diffusional apparatus may be found in Refs. 2–8.

Before proceeding, it is worthwhile to consider in more detail what makes mastery of transport theory and its applications a valuable tool for the pharmaceutical scientist. Some of the diverse array of membranes and barriers of concern within the greater field of pharmaceutics from the standpoints of drug activity, availability, or formulation development are compiled in Table I. Interest in the membranes depicted spans considerations on structure–activity relationships within drug families and drug absorption by whole animals to controlled drug release and appropriate product packaging. Certain specific problems requiring application of transport theory are presented in Table II. Collectively, these tables give ample evidence that some of the most pressing problems pharmaceutical scientists face are in the mass transport sphere. In this respect the tables, which are representative and hardly complete, speak for themselves. A few of these membranes and problems will be discussed explicitly. Whether expressly considered or not, the theories and approach to membrane transport detailed in this review provide necessary background so that membranes can be better characterized and transport problems can be handled with greater facility.

FUNDAMENTALS OF MEMBRANE PERMEATION

The Modelistic Approach—Total characterization of complex membranes and barriers and the development of suitable models to describe their properties require characterization of each and every independent phase and subphase constituting the barrier system, including the spacial configurations and arrangements of the phases and phase interactions with each other and with the diffusing species under consideration. Based on a conceptual scheme and on information or assumptions about these relationships, the task of building a model is to develop techniques to represent all the existing interrelationships and to sort out those relevant or applicable to a given situation. Where there is a systematic approach to the construction of models, often several alternative models are formulated and the researcher's efforts may be channeled into defining the limitations of each possibility. The result is usually a keener insight into the mechanics of the transport process.

[1] From the introductory remarks at the 1956 Faraday Society Discussions on Membrane Transport (see Ref. 1).

Table I—Membranes and Barriers of Medical and Pharmaceutical Interest

Biological membranes and barriers:
Cellular membranes (bacterial and mammalian)
Mucosal barriers
GI
Buccal
Vaginal, *etc.*
Skin (epidermis and dermis)
Cornea
Whole tissues
Barriers intimately associated with dosage form:
Films, coatings
Polymeric containers, seals, stoppers, *etc.*
Packaging films and laminates
Time and system variable barriers:
Fluid diffusion layers
Dosage form boundary layers (in implants, *etc.*)
Theoretical or model membranes:
Interfacial barriers and monolayers
Bilayers
Polymeric barriers
Fluid partitioning systems

Table II—Dosage Form-Related Mass Transport Problems

Preadministration:
Leaching or sorption of drug and/or adjuvant by containers or closures
Leaching of contaminants and/or reactants from packaging materials by the product (heavy metals, plasticizers, *etc.*)
Sorption and/or permeation of containers, coatings, *etc.*, by undesirable agents from the external environment (moisture, oxygen, *etc.*)
Postadministration:
Drug delivery from the intact physical system—timed-release properties
Disintegration (permeation of water into solid matrix)
Dissolution
Absorption
Biological distribution
Elimination
Protection (*i.e.*, protective ointments)

Introduction to Membrane Characterization—It is necessary for the purposes at hand to define what constitutes a membrane and what constitutes a barrier and to indicate how these terms differ. A membrane, in the context of this review, is a sheet of solid or semisolid material of fixed dimensions which is insoluble in its surrounding medium and which separates phases that are usually (but not necessarily) fluid. A membrane transport system is created when there is passage (active, facilitated, or passive) of solute across a membrane. The term barrier is more inclusive. It is the region or group of regions within a system, contiguous or physically separated, that offers finite resistance to transport of a substance from a point in one region of a system to another point located at some distance down the diffusional field. Since it is generally convenient to think of mass transport in a unidimensional sense, one usually considers the line traversed as being the shortest path in one plane aligned parallel to the source of diffusional resistance to a similarly aligned plane on the other side of the diffusional field. In this context, the diffusional field or barrier includes unstirred fluid strata and all interfaces as well as all interposed membranes. Flowing or mechanically mixed regions generally offer no diffusional resistance. This assumption cannot be regarded as absolute, however, and each situation must be analyzed on its own merits. The total barrier property of the intervening space between the planes may be considered to be the sum of the individual resistances or barrier properties of its component strata.

The first step in delineating total barrier property is to identify all independent strata that a substance must traverse to pass from one point to another. Here we are considering, for the moment, one-dimensional diffusion, *i.e.*, diffusion along a single vector that is perpendicular to the barrier planes. Independent strata are considered to offer uniform resistance, at least to a first approximation, over their entire thickness. In the extreme, a lamina may be taken as a single molecular distance (9, 10). However, usually thick regions exist where potential energy barriers for each molecular move in the vector of the flux are of constant magnitude, and the full thickness of such regions may be considered as an individual barrier segment.

To describe a barrier in terms of the number and placement of its laminae, it is necessary to develop a convention affording both identification of each distinct segment of a particular composition and classification in terms of the relative positioning of the strata. The simple method of Barrer (11) suffices for most cases. In this notation, each laminate of a given type is given a letter designation. Furthermore, if noncontiguous strata of a given property are repeated in the series, the letter designation is repeated. As an example, consider the situation where a homogeneous membrane is interposed between two like fluid phases, *i.e.*, two aqueous compartments. The first region of potentially significant diffusional resistance is the unstirred fluid region on the high diffusant concentration side of the system. Neglecting interfaces, the membrane is a second laminate and the unstirred fluid region generated at the other membrane interface is a third. Since the fluid regions are comparable in composition, excluding considerations of diffusant concentration, this is an ABA system. If a second membrane of different composition is placed downstream and permeation of the system is considered from the first fluid compartment to a third fluid (aqueous) compartment, the system may be characterized as being ABAACA if the middle fluid compartment is stirred (this will generate a diffusion layer at each membrane interface in the internal compartment) or ABACA if the middle compartment is unstirred and the diffusional resistance is continuous across this compartment's full thickness. As in the situations cited here, it is usually a straightforward process to identify potentially significant diffusional strata. Where there are questions regarding the role of regions such as interfaces or diffusion layers, it is advantageous to include them in the treatment or model. The point where their barrier contributions are truly insignificant eventually becomes mathematically evident and derived permeability expressions are readily simplified.

The next logical step in barrier characterization is to determine the thicknesses of the identified laminae. Often the thicknesses of the membranes in the composite barrier may be measured directly. Effec-

tive diffusion layer thicknesses are a function of solvent viscosity and stirring at a given temperature (12) and also of the molecular volume of the diffusing species. Their widths are usually estimated indirectly during the permeation experiment (13, 14) or by independent experiment using comparable mechanical conditions (15). Direct measurement of diffusion layer thickness on occasion has been accomplished (16). In some cases, particularly in biological systems, barrier strata are highly interdigitated, *i.e.*, the brush border of the GI lining and the juncture of epidermis with dermis. Such cases defy exact mathematical resolution, and effective thicknesses must be assigned. Usually, the error in such approximations is no more than the expected biological variations in permeation experiments, so these approximations suffice in a modelistic approach.

Having identified all participating strata, it is then necessary to characterize each individually with respect to gross physical state (solid, semisolid, or fluid) of the material(s) from which it is constructed and its detailed structure (11, 17). Variations in these details are limitless because segments can be, and often are, multiphasic and of diverse composition. Fluid phases are, to good approximation, homogeneous and of constant diffusional property. Membranes, on the other hand, are generally complex structures requiring more precise analysis and description. Although there is no generally agreed on method of classification, most membranes can be characterized as being composed of three general types of phases: *(a)* continuous, *(b)* shunt, and *(c)* dispersed. These phase types must be further classified as primary, secondary, tertiary, *etc.*, the subclassifications depending on the spacial relationships of each phase to the other phases present.

A primary continuous phase is a phase that is uninterrupted between membrane surfaces as well as laterally or is in the plane perpendicular to the flux vector. Depending on its composition relative to the compositions of associated phases, it may provide an uninterrupted diffusional path for a diffusing species or be operationally impervious and exist as a supporting structure only. A primary shunt phase is one that passes completely through the membrane but that is laterally discontinuous, *i.e.*, a pore or channel. These too may be inert with respect to permeability, may provide parallel diffusional pathways depending on relative compositions, or may be the sole diffusional pathway. Dispersed phases are embedded in continuous phases or shunt phases. They are discontinuous along the flux vector and do not provide an uninterrupted pathway through the membrane or through any of its subphases. If they are also discontinuous laterally, they are fillers or inclusion bodies (biological cases). Fillers are commonly found in synthetic membranes because they favorably influence mechanical properties such as elasticity, permeability, and resistance to tear (18). Dispersed phases that are continuous in the plane perpendicular to the flux vector are also found in synthetic membranes. Netting is sometimes embedded in a membrane structure as a means of reinforcement. The scanning electron micrographs of the 0.45-μm and 0.4-μm membranes in Figs. 1 and 2, respectively, provide examples of the ultrastructure of real membranes[2-4]. Since there can be phases within phases and phases, in turn, within these, it is necessary to designate whether a given identified phase is contributing to the coarsest structural breakdown (primary) or is a subphase of a primary or higher order phase. In this regard, each major phase identified must then be examined for the presence of secondary phases of each type, and these in turn for tertiary phases, *etc.*, until all distinct regions are accounted. Secondary phasic structure is common in real membranes; recourse to characterization of tertiary and finer subclassifications is generally unnecessary.

Once a barrier has been segmented and its segments characterized with respect to structure and composition, it is then necessary to assess the manner in which the diffusing species interacts with the various phases and subphases it must pass through, both on an absolute and relative basis. In other words, diffusivity within a given homogeneous region must be considered, as must adsorption onto or partitioning into all other phases in contact with the particular region under consideration. Subsequent sections provide some means of coping with these aspects. In narration, the process of characterization may seem exceedingly complex; in practice, it is usually manageable, particularly with synthetic membranes. Where membrane properties are indeterminate or too complex for exact physical and chemical description, use of simplifying assumptions and/or hybrid diffusivities still allows for construction of useful models and subsequent membrane typing.

Passive Diffusion: Kinetic and Thermodynamic Considerations—*Diffusion and Probability*—Diffusion is by nature a probabilistic process involving the random movement of molecules. The following description of the classical experiment involving the diffusion of a dye solution into solvent provides a feel for the process. Consider a cylinder half filled with solvent and then layered over with dye solution. Suppose these are separated by a nonpermeable divider which can be removed instantaneously without producing mechanical or other nondiffusive mixing. The following will occur upon removal of the divider. Throughout each medium the molecules will be moving randomly among each other roughly with translational velocities of a rifle bullet, the course of each molecule, solvent and dye alike, being extremely erratic due to multiple elastic collisions with like and unlike molecules in its physical path. In any finite time and in every part of the system, molecules of solvent and solute (where dye exists) will be directed along every vector of a three-dimensional coordinate system. In reference to the plane of interest, namely the dye solution–pure solvent interface, the molecules will be moving laterally within the

[2] Millipore MF.
[3] Nucleopore.
[4] Photographs of the Millipore and Nucleopore filters are courtesy of General Electric Co.

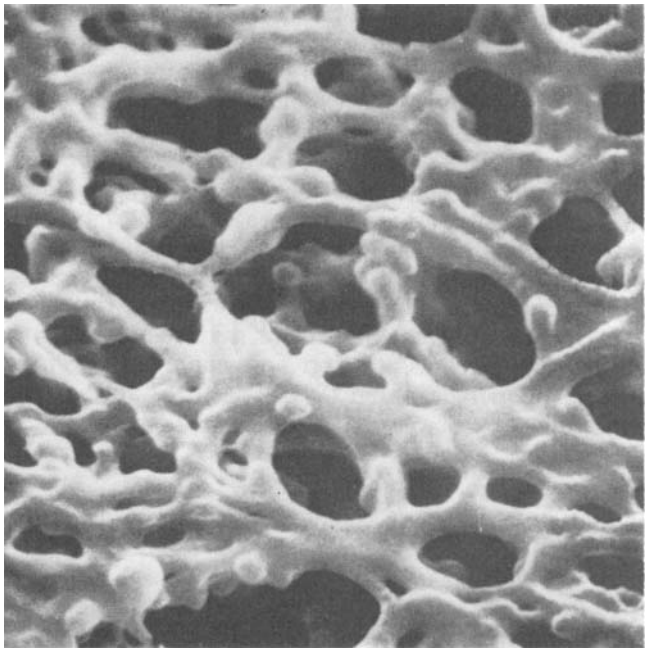


Figure 1—*Scanning electron micrograph of a filter (Millipore) rated as 0.45 μm in pore size. The highly porous nature of the structure and the nonlinearity of the "holes" are notable.*

plane as well as in all angles up to and including a right angle through the plane.

In the instant following the removal of the divider between solvent and solution, dye molecules at the interface will either be kinetically thrust back into the bulk of the solution or move laterally within the interface or penetrate the pure solvent. Due to the large numbers of molecules involved, dye molecules will instantaneously follow each of these courses. In this instant, dye molecules leaving the interface to enter the solution phase will be offset by an equal number (statistically speaking) of dye molecules moving from the depth of the solution to the interface. However, dye molecules diffusing into the pure solvent will be offset by only solvent molecules moving in the opposite direction. The random movements of these respective species thus lead to a net penetration of the dye into the pure solvent and solvent into the dye solution. In other words, the deficiency of solute molecules in the direction of pure solvent and the deficiency of solvent molecules in the direction of the solution, coupled with the fact that on the molecular level the system is in a highly dynamic state, cause the components to flow or diffuse into one another nonselectively and randomly. The net mass "movement" results from the concentration gradient of dye, initially across the interface but ultimately expanding through the entire system. In this case, an equal concentration gradient opposite in sign exists simultaneously for the solvent. The slope of the gradient is a reflection of the inequality of concentration across any plane perpendicular to the flux vector and, clearly from the example, the difference in concentration determines the net exchange of component across any chosen plane. In this manner, the rate of movement of mass across a given region is related to the concentration differential of the agent under consideration over that region. Obviously, the interdiffusion of miscible substances is a spontaneous, irreversible process. The pure components constituting a diffusively equilibrated system cannot be regenerated without doing work on the system.

Thermodynamic Considerations—The tendency toward total randomness by diffusive flow can be equated with an increase in entropy. By thermodynamic definition, a spontaneous process is one where there is an overall decrease in free energy of the system. At isothermal conditions:

$$\Delta G = \Delta H - T\Delta S \qquad \text{(Eq. 1)}$$

or the free energy change in the system is equal to the enthalpic change, ΔH, less the product of the absolute temperature, T, and the entropic change, ΔS. In the case of components that form ideal solutions, there will be no enthalpic contribution to diffusive mixing and the entire net change in free energy arises from increased entropy. In all real situations, there will be some enthalpic input into the overall free energy change; however, its magnitude in general will be relatively small and the diffusive process can be regarded as an entropically driven phenomenon.

It is hoped that at this point a picture is forming which, on one hand, describes the individual molecule's movements as being without preferred direction, a so-called random-walk process, but which at the same time, due to the large populations of molecules involved, prescribes that the direction of the total population is relentlessly toward regions of low concentration from relatively concentrated areas. Furthermore, it is implicit that interdiffusion of at least two substances is involved and there must, therefore, be multiple diffusion equations to describe the movement of each species present. For two com-

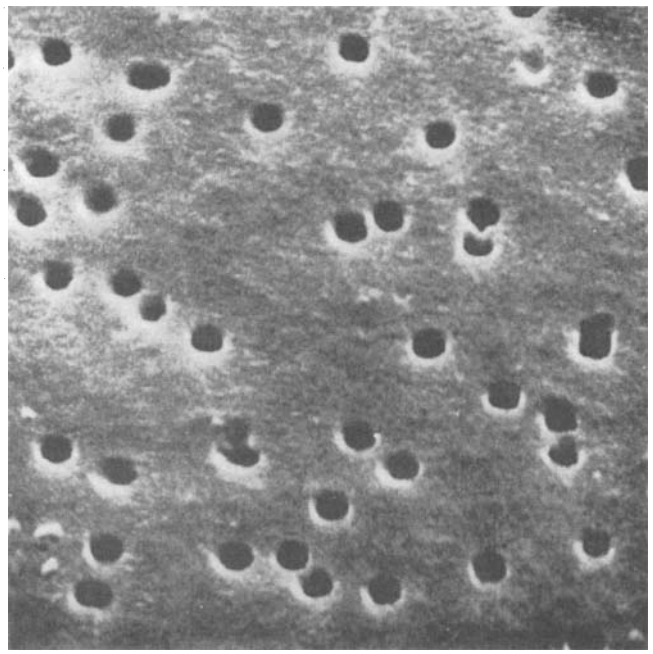


Figure 2—*Scanning electron micrograph of a 0.4-μm filter (Nucleopore). In this filter the pore density is relatively sparse but the pores are compensatingly uniform, linear, and short.*

ponents, two equations are actually necessary. However, since in the absence of a net volume change across the plane of reference the rates of interpenetration are equal in magnitude but opposite in sign, one equation suffices to describe the process and the second equation need not be considered explicitly. It is important to recognize this mutual dependency of the diffusion coefficient on each species. This is particularly true when the molecular species involved are disproportionate in size (*i.e.*, water *versus* protein) because the larger species may be limiting and set the diffusive current for each species at a low value. In the case of diffusion of a substance through a stationary solid or semisolid phase such as a membrane affixed into place in a diffusional apparatus, it is convenient to view the stationary phase as a fixed reference plane and only consider the flux of the mobile penetrant.

Diffusion in An Isotropic Medium—*Fick's First Law*—It was postulated by Bertholot (19) in the early 1800's that the diffusive flow of mass or flux is proportional to a constant times the concentration gradient of the diffusing species across the region of interest. Nearly 50 years later, Fick (20) reformulated this law by analogy to heat transfer and, most importantly, gave credence to the postulate by means of experiment. The first of the two diffusion laws that bear Fick's name is a simple statement of this principle, namely:

$$J = -D\left(\frac{dC}{dx}\right) \qquad \text{(Eq. 2)}$$

The formula explicitly says that the flux, J, of a component across a unit of area in a predetermined reference plane is proportional to the concentration differential across that plane, a conclusion presented intuitively in the preceding discussion. The term D is the proportionality constant, and the negative sign indicates mathematically that the current is in the direction of decreasing concentration. It was initially believed by Fick that D was a constant for a given system. It is now known that D is concentration sensitive, in the general case for reasons paralleling those that lead to deviations at high concentration from the ideal gas laws, osmotic pressure laws, *etc.*, and in specific cases due to the penetrant directly influencing the properties of the diffusion medium (*i.e.*, time- and concentration-dependent effects on polymeric films, *etc.*). For this reason, D is designated a coefficient, not a constant. As expressed in Eq. 2, D is a differential diffusion coefficient.

In certain experimental methods, particularly those used to characterize membranes, an averaged integral diffusion coefficient, $\bar{D}$, is obtained; this is related to the differential coefficient by:

$$\bar{D} = \frac{1}{C_0 - C_h}\int_{C_0}^{C_h} D\,dC \qquad \text{(Eq. 3)}^5$$

where the thickness of the regions is measured from $x = 0$ to $x = h$. When D is independent of concentration, it is obvious that $D = \bar{D}$. The units of D (or $\bar{D}$) are distance squared per time, preferably square centimeters per second. It is conceptually helpful to translate this to velocity by realizing that, for diffusion through a uniform field of unit thickness (*i.e.*, 1 cm), the diffusivity corresponds to the average distance the diffusing particle travels per unit of time (*i.e.*, centimeters per second) in the direction of flow.

Although the concentration differential, an experimentally easily assessable parameter, is most frequently taken as the driving force in diffusion, the chemical potential differential or activity differential is the fundamental parameter determining the direction and rate of flux. Barrer has derived the relationship between concentration and chemical potential in the following fashion (21). The force acting on a molecule at point x is:

$$F \propto -\frac{d\mu}{dx} \qquad \text{(Eq. 4)}$$

and thus the total force acting on all molecules is:

$$F_{\text{total}} \propto -C\frac{d\mu}{dx} \qquad \text{(Eq. 5)}$$

Assuming flux to be proportional to the total force, one obtains:

$$J = -BC\frac{d\mu}{dx} \qquad \text{(Eq. 6)}$$

where B is a coefficient reflecting the mobility of the diffusing species. The equation relating activity to chemical potential is:

$$\mu = \mu_0 + RT\ln a \qquad \text{(Eq. 7)}$$

where a is activity. Therefore:

$$d\mu = RT\,d[\ln a] \qquad \text{(Eq. 8)}$$

and:

$$J = -BRT\frac{C}{a}\frac{da}{dx} \qquad \text{(Eq. 9)}$$

and, by multiplying by unity in the form dC/dC:

$$J = -BRT\frac{d\ln a}{d\ln C}\frac{dC}{dx} \qquad \text{(Eq. 10)}$$

which, when compared with Fick's first law, yields for the differential diffusion coefficient:

$$D = BRT\frac{d\ln a}{d\ln C} \qquad \text{(Eq. 11)}$$

Both B and $d\ln a/d\ln C$ may be dependent on C and, as Barrer (21) further pointed out, each may in turn depend on x in an inhomogeneous medium. Having introduced these complexities, it is necessary to provide the perspective that in the majority of membrane transport situations of biological and pharmaceutical interest, concentration differentials and integral diffusivities derived therefrom suffice to characterize permeation phenomena in both absolute and relative terms. In other words, activity coefficients are usually confined within a narrow range in a given isotropic medium at the low concentrations one is limited to by the physicochemical and pharmacological natures of drug species.

[5] In the remainder of the text, the symbol D will be used interchangeably for both differential and integral diffusion coefficients.

Fick's Second Law—Although Fick's first law is a concise mathematical statement, it is not directly applicable to the solution of most permeation problems for it contains three principal variables: J, C, and x (22). Further, J itself is a multiple variable, the net amount of a substance crossing a unit of area of the diffusional reference plane, dM, per unit time, dt. The number of these variables is effectively reduced by one in Fick's second law, which is the fundamental mathematical statement of diffusion and the useful form in resolving most diffusion problems.

When D is independent of x, Fick's second law is readily derived from Fick's first law. Consider the volume element lying between two planes perpendicular to the vector of diffusive flow, x, separated by a distance, ΔX. The rate of entry of diffusant (in units of mass per time) into the volume element per unit of area from the high concentration side is:

$$\frac{dM_{in}}{dt} = -D\frac{d}{dx}\left[C - \frac{\Delta X}{2}\frac{dC}{dx}\right] \qquad \text{(Eq. 12)}$$

where C is the concentration in the plane parallel to and equidistant from the two planes prescribing the volume element. Similarly, the loss of mass of diffusant from the low concentration interface may be described by:

$$\frac{dM_{out}}{dt} = -D\frac{d}{dx}\left(C + \frac{\Delta X}{2}\frac{dC}{dx}\right) \qquad \text{(Eq. 13)}$$

The terms $C \pm (\Delta X/2)(dC/dx)$ give the concentrations at the planar boundaries of the volume element chosen. The rate of change of mass of diffusing substance in the volume element is obviously equal to the difference in the rate of entry and escape, *i.e.*:

$$\frac{dM_{in}}{dt} - \frac{dM_{out}}{dt} = D\Delta X\frac{d^2C}{dx^2} \qquad \text{(Eq. 14)}$$

but this is also equal to the rate of change in concentration within the volume element, dC/dt, times the volume of the element, ΔX (unit area is assumed), and thus:

$$\frac{\left[\frac{dM_{in}}{dt} - \frac{dM_{out}}{dt}\right]}{\Delta X} = \frac{dC}{dt} = D\frac{d^2C}{dx^2} \qquad \text{(Eq. 15)}$$

This is Fick's second law or the differential equation of diffusion for the unidimensional flow case. If more than one-dimensional diffusion is to be considered, it is necessary to assess the net change in mass along the other diffusional vectors by similar methods and, in the notation of rectangular coordinates, Fick's second law may be generalized to:

$$\frac{dC}{dt} = D\left[\frac{d^2C}{dx^2} + \frac{d^2C}{dy^2} + \frac{d^2C}{dz^2}\right] \qquad \text{(Eq. 16)}$$

A full derivation of Eq. 16 was presented by Crank (23).

In essence, Fick's second law states that the rate of change in concentration in a volume element within the diffusional field is proportional to the rate of change in concentration gradient at that point in the field, the proportionality "constant" being the diffusivity, D. There are numerous exact solutions of Eq. 15, a particular derivation depending on the boundary conditions imposed by the diffusion problem. Most mathematically tractable cases require or assume invariant diffusivity. Only those situations with obvious bearing on pharmaceutical problems will be discussed here. For more complete coverage and particularly for specifics of the derivations, the reader is referred to Refs. 22–26.

The Simple "Zero-Order" Flux Situation—The solution of Fick's second law for the commonly employed unidimensional experimental situation, where the concentration differential is maintained at a constant value during the course of a run and the diffusant receptor compartment is maintained at essentially zero concentration or a "sink" condition, was provided by Daynes (27) and later generalized by Barrer (28). Considering the diffusive current to be unidirectional, beginning at $x = 0$, the high concentration surface of the membrane, and moving toward the other membrane surface where $x = h$, the boundary conditions are in precise terms; C (concentration) is C_0 (constant) at $x = 0$ for all values of t (time), $C = 0$ at all $x > 0$ for $t = 0$ (initial condition), $C = 0$ at $x = h$ for all values of t (sink condition), and diffusivity, D, is constant. For these conditions, the concentration at any point x (or in a plane through x perpendicular to the flux vector) is given by:

$$C = C_0\frac{x}{h} + \frac{2}{\pi}\sum_{n=1}^{\infty}\frac{C_0}{n}\cos(n\pi)\sin\left(\frac{n\pi x}{h}\right)e^{-n^2\pi^2Dt/h^2} \qquad \text{(Eq. 17)}$$

This equation can be solved for the cumulative mass of diffusant per unit area, M, which passes through the membrane in time, t, by the following three steps: *(a)* differentiation with respect to x to obtain the instantaneous concentration gradient; *(b)* determination of the flux, dM/dt, at $x = h$; and *(c)* integration from $t = 0$ to $t = t$. The resulting equation is[6]:

$$M = \frac{DC_0t}{h} - \frac{hC_0}{6} - \frac{2hC_0}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}e^{-n^2\pi^2Dt/h^2} \qquad \text{(Eq. 18)}$$

which, as $t \to \infty$, approaches the straight line given by:

$$M = \frac{DC_0}{h}\left(t - \frac{h^2}{6D}\right) \qquad \text{(Eq. 19)}$$

In effect, Eq. 19 is the situation where the permeation has attained a steady state, dC/dt at all x within the field is zero, and thus the amount penetrating per unit time is constant. The steady-state flux is readily obtained from Eq. 19 by differentiation and is:

$$\frac{dM}{dt} = \frac{DC_0}{h} \qquad \text{(Eq. 20)}$$

Furthermore, if the steady-state line is extrapolated to the time axis, one obtains a value of t (t at $M = 0$) which is:

$$t_L = \frac{h^2}{6D} \qquad \text{(Eq. 21)}$$

[6] The integration constant here and in subsequent derivations is zero due to the choice of boundary conditions.

This intercept is called the lag time, t_L, and it provides estimation of D providing the diffusional field (membrane) thickness is known. The lag time, coupled with the steady-state flux, provides estimates of all the permeation controlling variables because it affords calculation of the membrane surface concentration, C_0. For an isotropic membrane, the steady state is achieved within 1% error when $Dt/h^2 \cong 0.45$, which corresponds to about 2.7 times the lag time, t_L (29). It should be noted that boundary conditions to this point apply to concentrations existing within the membrane, albeit at the surfaces of the membrane. In the typical membrane experiment, one measures concentrations in the external fluids bathing the membrane. In most instances, there is a facile equilibrium between the membrane and the phases contiguous to it. Such equilibria are expressed in terms of solubility coefficients (gaseous contiguous phases) or distribution or partition coefficients (liquid, semisolid, and solid contiguous phases). In other words, it is the usual case that $C_0 = C_0'K$, where C_0' is the applied phase concentration and K is the partition coefficient. Combining this with Eq. 19 yields:

$$M = \frac{DKC_0'}{h}\left[t - \frac{h^2}{6D}\right] \qquad \text{(Eq. 22)}$$

In the case of a gaseous applied phase, pressure or partial pressure of the diffusing gas is used in lieu of concentration and K is the solubility coefficient of the gas in the membranous material. For some materials, *e.g.*, synthetic membranes, the equilibrium coefficient can be obtained independently by equilibrating the membrane material with the applied phase and determining the relative concentrations in each. Alternatively, the distribution can be measured knowing C_0' and determining the steady-state flux and the lag time in a diffusional run (30). Both methods generally yield comparable results, attesting to the fact that the diffusant's interfacial equilibrium between membrane and applied phase is basically instantaneous. For those unusual cases where the equilibrium is not instantaneous, one has in effect an interfacial barrier of significant diffusional resistance so recourse to analysis of barriers in series is necessary.

In many situations of pharmaceutical interest (most biological cases), it is either impossible or experimentally difficult to measure distribution coefficients by either the equilibrium or kinetic (diffusional) method. In such instances, one is limited to characterizing permeability by the composite term, DK, which is often designated as the permeability coefficient, P. When only P can be measured, it is impossible to separate diffusive and gradient (partitioning) contributions to flux. This situation is particularly limiting when relative fluxes of several compounds are being compared. Relative permeabilities are determined by the influences of the structural modifications on both D and K, and one is at a loss to assign the effects to either of these specific parameters.

Since diffusive processes are often thought of and treated as kinetic problems with obvious analogy to chemical kinetic processes, it is interesting to analyze this common transport problem in these regards. The boundary conditions of constant applied phase concentration, C_0', on one side of the membrane and a receiver sink lead to a zero-order permeation process once the steady state has developed. The zero-order "rate constant" is equal to KD/h.

Short Time Approximation—As stated previously, Eq. 18 converges at large values of t to the straight line given by either Eq. 19 or 22. In circumstances where diffusivities are very small or membranes are very thick, extremely long times are required to attain steady-state conditions and diffusional runs are very lengthy. Protracted experiments are at least analytically inconvenient and, in addition, foster many ancillary but very troublesome problems of analytical origin (instrument stability, overgrowth of microbes, *etc.*). Furthermore, considering the limited solubilities of some compounds, coupled with concentration requirements for analysis, maintaining a receptor sink condition to a sufficient approximation can be difficult or impossible in some situations. All equations based on the series converging at large values of time would be dubious if not totally inapplicable under these circumstances. Rodgers *et al.* (31) and Short *et al.* (32) provided an alternative, although somewhat untested, approach to handle such cases. Collectively they derived a general expression converging at short time by means of a Fourier transformation of Eq. 18 and its integration. The final expression for small t is:

$$\log \frac{M}{\sqrt{t}} = \log\left[\frac{8C_0'K}{h^2\sqrt{\pi}}\right] + {}^3/_2 \log D - \frac{h^2}{2.3(4)Dt} \qquad \text{(Eq. 23)}$$

A plot of the log $[M/\sqrt{t}]$ *versus* $1/t$ yields a slope of $h^2/9.2D$, providing an estimate of diffusivity, D, for known membrane thickness, h. The terms M, t, and K have the same meaning as in Eqs. 18–22. This line intercepts with the $\log[M/\sqrt{t}]$ axis at log $[(8C_0'KD^{3/2})/(\pi^{1/2}h^2)]$ which, in conjunction with the slope, affords individual estimates of all the experimental variables. Rodgers *et al.* (31) indicated that there is approximately a 1% error by both short time and long time converging series in their respective approximated forms (converged forms) at 2.7 times the standard lag time, t_L. This is taken as the juncture of the validity and applicability of the two equations. In other words, the limit of use of the short time approximation is up to $2.7t_L$, which is about the onset of the steady state and the beginning point of applicability of the long time converging equation. Rodgers *et al.* (31) also showed that values obtained by the short time approximation for the diffusivity of helium in glass are comparable to those obtained by other investigators using the more conventional diffusional analysis.

Solutions Involving Quasisteady State—To this point, we have dealt with the simplest transport case in an isotropic medium. A more general solution, where there is an initial uniform concentration in the membrane and an initial concentration in the receptor phase, was developed (33). Such cases are solved by the assumption of independent streams; that is,

diffusion from the contiguous phases into the membrane and from the membrane into the external media are solved individually and summed to give the instantaneous concentration at any point, x, in the membrane slab at any arbitrary time, t. When either the applied phase and/or the receptor phase concentrations vary with time, the mathematical analysis is considerably more complicated, and utilizable solutions to Fick's laws are generally limited to conditions after establishment of the concentration gradient across the slab. In other words, after some finite time, an instantaneous or quasisteady state develops and the time course for the diffusional process is initiated and followed after the onset of the quasisteady state. Requisite to successful analysis of these situations are the following conditions:

1. The gradient within the membrane must instantaneously adjust to the external conditions (contiguous phase concentrations).
2. The amount (not concentration) of diffusant in the membrane must be negligible.

These conditions are approached closely for very thin membranes. Ultrathinness is not an absolute requirement, however, as pointed out by Barnes (34). When the conditions are such that there is a linear fall of concentration within the barrier, the instantaneous concentration gradient may be expressed by:

$$-\frac{dC}{dx} = \frac{C_0 - C_h}{h} \qquad \text{(Eq. 24)}$$

where C_0 and C_h are the respective surface concentrations of the membrane. According to Fick's first law, the instantaneous flux per unit area would be:

$$J = \frac{dM}{dt} = -D\frac{dC}{dx} = D\left[\frac{C_0 - C_h}{h}\right] \qquad \text{(Eq. 25)}$$

This can be related to concentrations in the contiguous phases by incorporation of the appropriate partition coefficients. When the amount of material within the membrane is negligibly small compared to that in the external media, the material balance change in the external phases may be represented, respectively, by $(M_D - M)/V_D$ and $(M_R + M)/V_R$, where M_D and M_R are the total amounts of diffusant initially in the high concentration (donor) and low concentration (receptor) contiguous phases, respectively; V_D and V_R are the respective volumes in these phases; and M is the net mass change in the donor phase in time, t (35). On substitution of these expressions into Eq. 25, one obtains:

$$\frac{dM}{dt} = \frac{DK}{h}\left(\frac{M_D - M}{V_D} - \frac{M_R + M}{V_D}\right) \qquad \text{(Eq. 26)}$$

which integrates to:

$$\frac{DK}{h}t = \frac{V_D V_R}{V_D + V_R}\ln\left[\frac{M_D V_R - M_R V_D}{M_D V_R - M_R V_D - (V_D + V_R)M}\right] \qquad \text{(Eq. 27)}$$

When the phase volumes are equal and M_R is zero, Eq. 27 simplifies to:

$$\frac{2DK}{hV}t = -\ln\left[\frac{M_D - 2M}{M_D}\right] \qquad \text{(Eq. 28)}$$

where V is the volume of either phase. Furthermore, since:

$$\frac{M_D - 2M}{M_D} = \frac{2(M_D - M) - M_D}{M_D} = \frac{2\left(\frac{M_D - M}{V}\right) - \frac{M_D}{V}}{(M_D/V)} \qquad \text{(Eq. 29)}$$

and $(M_D - M)/V$ and M_D/V are the donor concentrations at finite time, t, and at $t = 0$, respectively:

$$\frac{2DK}{hV}t = -\ln\left[\frac{2C' - C_0'}{C_0'}\right] \qquad \text{(Eq. 30)}$$

Therefore, a plot of the log $[(2C' - C_0')/C_0']$ against time yields a straight line with a slope of -0.87 (DK/hV). In situations where these equations are applicable, separation of the partition coefficient, K, and the diffusivity, D, is not possible without an independent measurement of one of these parameters.

A special but important case is derivable from Eq. 27 for the situation where the diffusant is collected into a receptor sink. This is to a first approximation the situation for absorption of soluble drugs from the GI tract since the body acts as an overwhelmingly large reservoir. In this circumstance, V_R may be assumed infinite (very large with respect to V_D) and Eq. 27 simplifies to:

$$\frac{DK}{hV_D}t = -\ln\left[\frac{M_D - M}{M_D}\right] \qquad \text{(Eq. 31)}$$

which, by manipulation similar to the previous case, yields:

$$\frac{DK}{hV_D}t = -\ln\frac{C'}{C_0'} \qquad \text{(Eq. 32)}$$

Diffusive phenomena obeying this relationship correspond to simple first-order processes and, for a given run, the first-order rate constant is given by (DK/hV_D). The conditions in the typical dialysis experiment are also suitable for the use of this mathematical form. In this case, the dialyzed small molecule is continuously flushed from the receptor compartment to maintain the maximum concentration differential throughout the protein purification or other separation procedure.

One further general case should be considered: the situation where a constant concentration is maintained on one side of a membrane system and the concentration on the other side is allowed to approach equilibrium. The boundary conditions are specifically $C_D = C_0$ for all t, $C_R = 0$ (or more generally $C(0_R)$ at $t = 0$, and $C_R > 0$ at t. By analogy to the previous cases, one can write for the flux per unit area:

$$\frac{dM}{dt} = V_R\left(\frac{dC_R}{dt}\right) = \frac{KD}{h}(C_0' - C_R) \qquad \text{(Eq. 33)}$$

This integrates to:

$$\frac{KD}{hV_R}t = -\ln\left[\frac{C_0' - C_R}{C_0' - C(0_R)}\right] \qquad \text{(Eq. 34)}$$

which, when $C(0_R) = 0$, leads to an obvious simplification. Geometry considerations aside, Eq. 34 approximates the cases of absorption of a drug by a small organism in a large drug reservoir (microbes in a beaker of drug solution or goldfish in a tank of

drug solution) or of gases through the lungs, the atmosphere in this latter instance acting as the constant concentration reservoir.

Temperature Effects—Regardless of the diffusional system and the mathematical model required to characterize the same, the temperature dependency in all these instances essentially resides entirely in the temperature dependency of the diffusion coefficient. Partition coefficients (but not gaseous solubility coefficients), volumes, membrane thicknesses, *etc.*, are relatively unaffected by temperature variation. The diffusion coefficient may be written in the form:

$$D = D_0 e^{-Ea/RT} \qquad \text{(Eq. 35)}$$

where D_0 is the hypothetical diffusivity at infinite temperature (from the Y intercept of a plot of log D *versus* $1/T$) and Ea is the activation energy. Ea is markedly influenced by the nature of the barrier; values range from about 5 kcal/mole for diffusion of low molecular weight nonelectrolytes in liquids to 15–20 kcal/mole for diffusion of comparable compounds in some polymeric structures (36).

Barriers in Series: Diffusion through Laminated Structures—*General Considerations*—Without exception, the systems treated to this point have dealt with situations where concentration gradients are confined entirely within a single isotropic segment of material. For many transport systems, gradients are distributed over several contiguous or noncontiguous strata in a multilayered barrier. Diffusion through a thick segment of a biological tissue like a muscle is one case in point because the diffusing substance must alternately pass through cell membranes and cell cytoplasm. Diffusion through plastic laminates used as packaging materials is another case of pharmaceutical interest. In formulating an attack on characterizing the permeability of stratified barriers, one must consider how the consecutive strata are arranged and characterize the individual physicochemical properties and dimensions of each distinct phase. Zwolinski *et al.* (9) provided a basic approach for analysis of laminates. Using rate theory and a diffusional model which treats diffusion as point-to-point jumps of the diffusing molecules, they derived equations which relate the rate of movement of matter in the steady state to the relative heights of the potential energy barriers encountered in each molecular move. The diffusion constant in a given isotropic field (layer) derived in this analysis is equated to the square of the mean molecular jump distance, λ, times a rate constant, k_i; *i.e.*:

$$D_i = \lambda_i^2 k_i \qquad \text{(Eq. 36)}$$

In extending this analysis, Scheuplein (10) simplified the original expressions, arriving at the following generalized result:

$$\frac{dM}{dt} = \frac{\lambda[C_0' - C_R]}{\frac{1}{k_{sm}} + \sum_{i=1}^{n-2}\left(\frac{1}{K_i k_i}\right) + \frac{1}{k_{sm'}}} = \frac{\Delta C}{\frac{1}{P_T}} \qquad \text{(Eq. 37)}$$

where $(n - 2)$ is the total number of barriers exclusive of the interfacial barriers at the extremes of the diffusional field, ΔC is the instantaneous concentration differential across the whole system, k_i is the rate constant in the ith layer as defined by Eq. 36, and K_i is the distribution coefficient in the ith phase with respect to the external phases, which are assumed to be of the same composition. In effect, Eq. 37 states that the reciprocal of the total, complex permeability coefficient, P_T, is equal to the sum of the diffusional resistances encountered in each lamina. In this context, diffusional resistance is equal to the sum thickness of the individual isotropic segment, $N_i\lambda$ (where N_i is the number of unit molecular "jumps" within that part of the field), divided by the diffusion coefficient. The order in which the potential energy barriers are met within the diffusional field does not influence the net steady-state flux across the complex barrier. This latter generalization must only be applied to situations where resistances in all sections of the diffusional field are independent of diffusant concentration. In this situation, the diffusional resistance, R_i, in the ith lamina can be defined by (37):

$$R_i = \frac{1}{P_i} = \frac{h_i}{D_i K_i} \qquad \text{(Eq. 38)}$$

where P_i is the thickness-weighted permeability of the ith segment; and h_i, D_i, and K_i are thickness, diffusivity, and partitional coefficient, respectively. The total diffusional resistance[7] may be computed by:

$$R_T = \frac{1}{P_T} = R_1 + R_2 + \cdots + R_n \qquad \text{(Eq. 39)}$$

or:

$$R_T = \frac{1}{P_T} = \frac{h_1}{D_1 K_1} + \frac{h_2}{D_2 K_2} + \cdots + \frac{h_n}{D_n K_n} \qquad \text{(Eq. 40)}$$

where the distribution coefficients are computed for a given phase with respect to the initial external phase in the system. The terms $h_1, h_2, \ldots, h_n$ represent the thickness of the individual strata, and $D_1, D_2, \ldots, D_n$ represent the respective diffusivities in each region. Thus, the composite permeability coefficient for stratified barriers may be obtained with a knowledge of individual thickness and diffusivities of each barrier phase plus the equilibria function between the ith phase and the initial external phase (again we assume the terminal external phase to be the same as the initial external phase). In some cases, these properties may be obtained by independent analysis of the various segments, the properties of the laminate then being formulated from the properties of its component parts. This approach would have particular utility in designing laminated packaging materials to meet predetermined specifications. These equations have been invoked to explain data for many diverse experimental situations.

[7] It may be noted that the additivity of diffusional resistances in series is analogous to the additivity of sequential electrical resistances. The K values appear as relative capacity terms.

Some particularly good examples may be found in Refs. 9, 10, and 37–42.

Three-Ply Laminate—Derivation of the composite permeability coefficient for a three-ply membrane placed between two well-stirred liquid phases (assuming insignificant diffusion layer resistance), where all membrane strata are of different composition, serves to illustrate the above method. The total resistance encountered by the permeant in traversing the composite barrier, based on Eq. 40, would be:

$$R_T = \frac{1}{P_T} = \frac{h_1}{D_1K_1} + \frac{h_2}{D_2K_2} + \frac{h_3}{D_3K_3} \qquad \text{(Eq. 41)}$$

and:

$$P_T = \frac{(D_1D_2D_3)(K_1K_2K_3)}{h_1D_2D_3K_2K_3 + h_2D_1D_3K_1K_3 + h_3D_1D_2K_1K_2} \qquad \text{(Eq. 42)}$$

If resistance in one segment is overwhelming, Eq. 42 simplifies to $P_T = (K_iD_i/h_i)$, where i equals 1, 2, or 3, and the composite barrier property is determined by the single high resistance phase. If but one layer is of insignificant diffusional resistance (arbitrarily the third), the overall permeability coefficient becomes:

$$P_T = \frac{D_1D_2K_1K_2}{h_1D_2K_2 + h_2D_1K_1} \qquad \text{(Eq. 43)}$$

The total permeability coefficient derived here differs from that derived by Barrie *et al.* (42) by the product of the total thickness, $h_1 + h_2 + \ldots + h_n$. In effect, Barrie *et al.* defined the permeability coefficient as an intensive property or averaged property per unit thickness over the total thickness. Recognizing that permeability is not uniform throughout the laminated field, we prefer to view it as the summed property irrespective of total thickness, *i.e.*, as an extensive property. In use (that is, when substituted into a flux equation), the expressions are equivalent because total thickness appears twice and as a ratio in the Barrie *et al.* case and, hence, "cancels."

It is important to realize that, regardless of boundary conditions, a diffusional profile, *i.e.*, a plot of the amount that has penetrated a barrier with respect to time, cannot be used to distinguish between a simple isotropic barrier and a series barrier. For a given experimental condition, profiles obtained with simple and series barriers will be qualitatively the same. Thus, in the absence of knowledge of the diffusional mechanism, standard treatment of the diffusional curve and, particularly, calculation of diffusivity from the Daynes and Barrer lag time relationship must be performed with caution.

Once the series barrier has been described in terms of its complex permeability coefficient, this term may be substituted for the simpler coefficient, KD, in any flux equation previously presented for steady-state or quasisteady-state conditions. The nonstationary state, on the other hand, is exceedingly more complex. This was quantitated for zero-order permeation (constant applied phase concentration and receptor sink, *etc.*) for a three-layer laminate by Barrie *et al.* (42). The lag time for a trilaminate is:

$$t_{L_{1,2,3}} = \left[\frac{h_1^2}{D_1}\left(\frac{h_1}{6D_1K_1} + \frac{h_2}{2D_2K_2} + \frac{h_3}{2D_3K_3}\right) + \frac{h_2^2}{D_2}\left(\frac{h_1}{2D_1K_1} + \frac{h_2}{6D_2K_2} + \frac{h_3}{2D_3K_3}\right) + \frac{h_3^2}{D_3}\left(\frac{h_1}{2D_1K_1} + \frac{h_2}{2D_2K_2} + \frac{h_3}{6D_3K_3}\right) + \frac{K_2h_1h_2h_3}{D_1D_3K_1K_3}\right] \Big/ \left(\frac{h_1}{D_1K_1} + \frac{h_2}{D_2K_2} + \frac{h_3}{D_3K_3}\right) \qquad \text{(Eq. 44)}$$

where h and D are lamina thickness and diffusivity, respectively; and K terms are partition coefficients with respect to the external phases. The subscripts 1, 2, and 3 refer to the order of placement of the layers with respect to the flux vector. When the partition coefficients (solubility coefficients for gases) are comparable and any h_n/D_n term is much greater than the two others, n being 1, 2, or 3, the equation reduces to the simple Daynes and Barrer expression, $h_n^2/6D_n$. In other words, when the diffusional resistance effectively lies in only one of the strata, the remaining strata are shunted out not only with respect to determining steady-state flux but also with respect to determining the duration of the nonstationary state. Numerous other simplifications of Eq. 44 are possible, depending on the relative magnitudes of the h, D, and K values. It may be noted from Eq. 44 that the outer lamina, 1 and 3, may be interchanged without affecting the lag time. However, if the middle phase is relocated in the series, the lag time will in general be altered. This is in contrast to the steady-state flux which will be independent of phase placement.

Membrane-Diffusion Layer Case—One special case involving a trilaminate is of particular importance. Often membrane permeation is from an aqueous phase, through a nonpolar (lipid) membrane, into a second aqueous phase of lesser concentration. Fluid dynamics require the generation of essentially unstirred solvent layers at any stationary surface regardless of stirring conditions (thickness of diffusion layers will depend on stirring but not the existence of the diffusion layers themselves). Thus, in the cited instance, an unstirred aqueous layer will necessarily exist at each membrane surface. From the diffusional standpoint, the system is an ABA trilaminate. This system was recently analyzed (37, 43, 44). The steady-state flux (zero-order boundary conditions) is described by:

$$\frac{dM}{dt} = \left[\frac{KD_MD_{AQ}}{h_MD_{AQ} + (h_{AQ(I)} + h_{AQ(II)})KD_M}\right](C_D' - C_R') = P_T\,\Delta C \qquad \text{(Eq. 45)}$$

where K is the membrane–water partition coefficient, and the subscripts M and AQ refer to membrane and water, respectively. The terms $h_{AQ(I)}$ and $h_{AQ(II)}$ represent the individual thickness of the two aqueous diffusion layers, and the other terms are as previously defined. In most instances, biological or otherwise, $h_MD_{AQ} \gg KD_M\Sigma h_{AQ}$ and P_T will effectively be KD_M/h_M. However, for compounds with high relative lipid solubilities (partition coefficients) and/or for very thin membranes or, in combination with either of these two factors, for the unusual sit-

uations when D_M is of the same magnitude as D_{AQ}, P_T may in effect become $D_{AQ}/\Sigma h_{AQ}$. In the first situation, the concentration gradient is confined entirely within the membrane and the flux and lag time are membrane controlled. In the latter situations, virtually the entire diffusional resistance arises in the diffusion layers and diffusion layer control of permeation is operative[8]. It may be believed by some that increasing "lipophilicity" within a drug family will always produce improved absorption of drugs. Equation 45 states, on the other hand, that there is an upper limit on partition coefficient which, when exceeded, will force the permeation process into diffusion layer control and, once in diffusion layer control, the permeation process is insensitive to partition coefficient. Furthermore, the aqueous diffusivity will decrease as molecular volume is increased, albeit gradually, and the aqueous solubility will drop exponentially with "hydrophobic" derivatization; thus overtitration of "lipophilicity" may actually decrease drug availability. These effects have been experimentally demonstrated and quantitated for the alkyl *p*-aminobenzoates permeating silicone rubber membranes (37).

Under membrane control and for the zero-order condition, the duration of the nonstationary state is given by the Daynes and Barrer relationship, *i.e.*, $h_M^2/6D_M$. For ultrathin membranes operating under diffusion layer control, the lag time will be $(\Sigma h_{AQ})^2/6D_{AQ}$. When the nonpolar membrane is thick and the partition coefficient is sufficiently large to place a permeation process in diffusion layer control, the lag time has been shown to be (44):

$$t_L = \frac{h_M h_{AQ(I)} h_{AQ(II)} K}{[h_{AQ(I)} + h_{AQ(II)}] D_{AQ}} \qquad \text{(Eq. 46)}$$

or, when the diffusion layers are of identical thickness:

$$t_L = \frac{h_M h_{AQ} K}{2D_{AQ}} \qquad \text{(Eq. 47)}$$

where h_{AQ} is the thickness of the individual aqueous layer. The appearance of the partition coefficient term in this equation indicates that, once diffusion layer control has been reached, further increases in lipophilicity within a drug series will significantly increase the time for onset of the steady state. It should be kept in mind that partition coefficients grow exponentially with alkyl chain length.

Temperature Effects in Series Barriers—The temperature dependency of the laminated system can be quite complex when the segments offer comparable diffusional resistance and the activation energies of the diffusion coefficients are very different. By substituting $D_0 e^{-Ea/RT}$ terms (see Eq. 35) for D terms in Eq. 45, the permeability coefficient for the membrane-diffusion layer system becomes:

$$P_T = \frac{K D_{0M} D_{0AQ} e^{-(Ea_{AQ} + Ea_M)/RT}}{K D_{0M} e^{-Ea_M/RT} \sum h_{AQ} + D_{0AQ} e^{-Ea_{AQ}/RT} h_M} \qquad \text{(Eq. 48)}$$

Since Ea_{AQ} for water is about 5 kcal/mole and Ea_M for many polymers will be as much as 10 or 15 kcal/mole higher, variation of temperature around the critical point where the diffusion resistances in each layer are equal will lead to a change in diffusional mechanism. As temperature is raised, starting 20–30° below the critical point and going to 20–30° above, the system will pass from membrane control to diffusion layer control of flux. A plot of log P *versus* $1/T$ will be curvilinear. At small values of $1/T$ (high temperatures), the curve will asymptotically approach a line with a slope of $-Ea_{AQ}/2.3RT$, the diffusion layer activation line. At low temperatures, the slope approached will be $-Ea_M/2.3RT$, indicating membrane control. Thus the temperature dependency in principle can provide much information about the nature of a laminate and the mechanisms of transport. If the diffusional resistance in one layer of the composite barrier dominates over the full temperature span considered, the activation energy will be a function of the diffusivity in that region only and can be used to indicate which layer is rate controlling, providing activation energies are known and are different.

Parallel Pathways: Influence of Shunts and Pores—*General Analysis*—When two or more independent diffusional pathways are linked in parallel in a given diffusional medium, the total diffusional current, J_T, through the composite is simply the sum of the individual currents through the separate routes (45). Thus, for a unit of cross-sectional area, the flux through independent parallel routes, instantaneous or steady state, may be expressed by:

$$J_T = f_1 J_1 + f_2 J_2 + \cdots + f_n J_n \qquad \text{(Eq. 49)}$$

where the f_1, f_2, *etc.*, terms give the fractional areas of each route. Therefore, the total flux where independent, parallel pathways exist may be resolved by solving Fick's laws for the operative boundary conditions of the experiment, adjusting for relative areas of the routes, summing over all pathways, and solving for the total area involved. For two parallel, linear routes directly through a membrane, the solution of the flux equations for the zero-order permeation condition with a receptor sink is:

$$M = f_1 \left[\frac{K_1 D_1 C_0'}{h} t - \frac{h C_0' K_1}{6} - \frac{2h C_0' K_1}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} e^{-n^2 \pi^2 D_1 t / h^2} \right] + f_2 \left[\frac{K_2 D_2 C_0'}{h} t - \frac{h C_0' K_2}{6} - \frac{2h C_0' K_2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} e^{-n^2 \pi^2 D_2 t / h^2} \right] \qquad \text{(Eq. 50)}$$

which, as $t \to \infty$, approaches the straight line:

$$M = f_1 \frac{K_1 D_1 C_0'}{h} \left(t - \frac{h^2}{6D_1} \right) + f_2 \frac{K_2 D_2 C_0'}{h} \left(t - \frac{h^2}{6D_2} \right) \qquad \text{(Eq. 51)}$$

[8] This may seem impossible when $D_M \gg D_{AQ}$. However, if $D_{AQ}/D_M = n$ and there are many times n molecules passing through the membrane for each molecule passing through the aqueous region, the capacity of the diffusion layers becomes flux limiting with the membranes becoming more or less a reservoir between.

The steady-state flux through the parallel routes is thus:

$$J_T = \frac{dM}{dt} = \left[\frac{f_1K_1D_1}{h} + \frac{f_2K_2D_2}{h}\right] C_0' \quad \text{(Eq. 52)}$$

In general then, for independent, parallel pathways in the steady state:

$$\frac{dM}{dt} = [f_1P_1 + f_2P_2 + \cdots + f_nP_n]C_0' \quad \text{(Eq. 53)}$$

where P_1, P_2, . . . , P_n are the individual permeability coefficients for each pathway.

Solving Eq. 52 for $M = 0$, one obtains the composite lag time:

$$t_L = \frac{h^2}{6}\left(\frac{f_1K_1 + f_2K_2}{f_1K_1D_1 + f_2K_2D_2}\right) \quad \text{(Eq. 54)}$$

If one of the two possible routes is impervious, the steady-state flux is determined by the fractional area of the permeation available pathway times the rate of permeation through this channel, and the lag time takes the simple Daynes and Barrer form. Membranes containing pores or channels in a rigid, impenetrable superstructure fall into this class.

Unlike series barriers, the diffusional profile (*M versus t*) may provide *prima facia* evidence for the existence of parallel routes. This occurs when the respective nonstationary-state periods are experimentally separable. In this situation, considering only two routes, the diffusional profile will appear "normal" until the lagging pathway is breached by the permeant, at which time there will be a surge in flux and an approach to a new steady-state line of increased slope. This exact effect has been demonstrated for steroid penetration of human skin (46).

Mechanical Effects—When dealing with pores or channels, two additional mechanical factors must be considered. If the pore is either nonlinear or if it is not oriented perpendicularly within the diffusional field, the effective diffusional path length will exceed the actual thickness of the membrane. This situation is handled by applying a tortuosity factor, τ. The effective thickness of the membrane, H, is computed from:

$$H = \tau h \quad \text{(Eq. 55)}$$

τ having been estimated considering a representative sampling of pores. The second mechanical effect becomes significant when the diffusant's radius approaches the radius of the pores. This leads to hindered diffusion (15, 47–49). This phenomenon is treated in a subsequent section on diffusion through polymers.

Temperature Effects in Parallel Pathways—The temperature dependency for parallel pathways is equally complex to that found for series barriers. Scheuplein (45) thoroughly reviewed the method of analyzing temperature effects for the case of two parallel, independent routes. As in previous analyses, individual diffusion coefficients may be replaced by their $D_0e^{-Ea/RT}$ forms (Eq. 35), and Eq. 52 becomes:

$$\frac{dM}{dt} = \left[\frac{f_1K_1D_{0_1}}{h}e^{-Ea_1/RT} + \frac{f_2K_2D_{0_2}}{h}e^{-Ea_2/RT}\right] C_0' \quad \text{(Eq. 56)}$$

Like the case of the laminated barrier, when fluxes through the parallel routes are of the same magnitude at the midpoint of the temperature range of interest and activation energies for each pathway are widely divergent, a curvilinear relationship between log [steady-state flux] and reciprocal temperature is obtained. The curve is inverted in shape from that found for series barriers. A prototype plot may be found in Scheuplein's paper (45).

Scheuplein demonstrated the temperature effects discussed for the permeation of human skin by tritiated water. He provided strong evidence for the existence of pores through the stratum corneum continuum. Activation energies found for each route are 5 kcal for aqueous filled pores, an expectable value based on free diffusion in liquid medium, and 19 kcal for the stratum corneum matrix. Scheuplein's analysis thus shows that the assessment of temperature effects can be a powerful tool in characterizing a membrane and formulating an appropriate model.

Dispersed Phases and Barrier Property—*General Considerations*—The presence of dispersed particles or bodies in the diffusional matrix is another barrier complexity commonly found in pharmaceutical systems. Higuchi and Higuchi (50) previously reviewed the problems associated with this form of heterogeneity, indicating that the theory is applicable to the design of protective ointments, to drug release from semisolid dispersed systems such as solidified emulsions and suspensions (creams and ointments), and to the passage of drugs through biological barriers. Characterization of a membrane containing a dispersed phase necessarily requires full characterization of the dispersed phase itself, including its spatial distribution, its size distribution, its orientation, its shape, its interactions with the diffusing species, and its overall concentration or relative volume. The following analyses will only consider situations where dispersions are uniform within the barrier continuum. Furthermore, the particles will be assumed to be randomly oriented and, in the case of emulsions, spherical in shape. In combination, these features lead to a diffusional field that is isotropic on the macroscopic level. More specific considerations on particle geometry and nonuniform particle distribution may be found in the Higuchi and Higuchi (50) review and in Ref. 51.

Influence of Inert Fillers—The simplest case of filler influence arises when the dispersed phase is diffusionally inactive. Such fillers (here the term is used generally, considering the filler as either solid, liquid, or gas) are referred to as being inert. The inert filler influences the time course of the diffusional profile in basically two ways:

1. It forces the diffusant to stream around the filler, thereby lengthening the average diffusional path.
2. It occupies volume which is excluded as a diffusional path.

As in the case of nonlinear pores, the average path length is adjusted with a tortuosity factor, τ (see Eq. 55). Furthermore, the relative volumes of filler and continuum may be computed in terms of their volume fractions, ϕ_2 and ϕ_1, respectively. Obviously, $\phi_1 = 1 - \phi_2$. Recognizing that the relative cross-sectional

area of the continuum phase is exactly equal to its relative volume (randomness of dispersion is assumed), the steady-state flux equation (zero-order process with a receptor sink) is readily formulated; *i.e.*:

$$J = \frac{dM}{dt} = \left[\frac{KD}{h}\,\frac{\phi_1}{\tau}\right] C_0' = \left[\frac{KD(1-\phi_2)}{H}\right] C_0' \quad \text{(Eq. 57)}$$

The lag time for the zero-order situation as defined is unaffected by the filler volume fraction but is influenced by the increased diffusional path length and is:

$$t_L = \frac{h^2\tau^2}{6D} = \frac{H^2}{6D} \quad \text{(Eq. 58)}$$

Influence of Adsorptive Fillers—When a filler participates in the diffusion process in a more direct manner, *i.e.*, by sorption of the permeant as it crosses the barrier, it is designated as being active. Sorption here implies either adsorption or absorption. In general, adsorptive influences are less complicated and more readily quantitated. When the adsorption follows Langmuir's isotherm, two distinct dependencies have been formulated: one where the amount adsorbed is directly dependent on concentration and a second representing the plateau of the isotherm where a fixed amount of permeant is adsorbed per unit of filler. The former is designated as the Case I mechanism, and the latter is Case II. The Case I relationship, originally derived by Finger *et al.* (52), was recently reexamined by Flynn and Roseman (53). For the standard zero-order permeation process, the steady-state flux and lag time equations are:

$$J = \frac{dM}{dt} = \frac{KD(1-\phi_2)}{H} C_0' \quad \text{(Eq. 59)}$$

and:

$$t_L = \frac{H^2}{6D}(1 + z\phi_2) \quad \text{(Eq. 60)}$$

where z is the adsorptive constant. The steady-state flux equation is identical to Eq. 57, indicating that the steady-state flux is unaffected by the filler short of its influences on effective area for diffusion and effective path length. The lag time is directly related to square of tortuosity, to filler volume fraction, and to filler adsorptive capacity as measured by z. For a given Case I filler, a plot of lag time divided by H^2 against V_2 will have a slope of $1/6D$ and an intercept of $z/6D$, affording in one plot estimates of both D and z. These relationships have been shown to be closely approximated for the permeation of silicone rubber membranes containing a silica filler by ethyl *p*-aminobenzoate (53, 54).

The Case II adsorptive mechanism was derived by Higuchi and Higuchi (50). The steady-state equation is the same as for the Case I mechanism. The lag time, on the other hand, is considerably different from Case I and is given by:

$$t_L = \frac{H^2}{D}\left[\frac{1}{4} + \frac{Z\phi_2}{2KC_0'}\right] \quad \text{(Eq. 61)}$$

where Z_{II} is the maximum (saturation) adsorptive capacity of the filler. The Case I and Case II mechanisms may be differentiated by the donor phase concentration dependency in Case II. When the diffusant is irreversibly bound (Case II) to the adsorbent as may occur in chemisorption, breakthrough of the barrier will be dramatically lengthened at low applied phase concentrations. Higuchi and Higuchi (50) pointed out that the lag time equation for Case II is only approximate in that it reduces to $H^2/4D$ and not to $H^2/6D$ as $Z\phi_2$ approaches zero.

Absorptive, Permeable Fillers—Situations where the filler particles themselves are permeable (*i.e.*, an emulsion) are exceedingly complex and have proven refractory to exact analysis. Such processes involve absorption of diffusant by the filler. It is intuitive that when the filler is permeable a significant flow of material will occur within the filler, and this will affect both transient and steady-state processes. Higuchi and Higuchi (50) derived the following equation for the effective permeability coefficient, P_T, in the steady state, assuming spherical filler particles:

$$P_T = \frac{2P_1^2(1-\phi_2) + P_1P_2(1+2\phi_2) - GP_1\left(\frac{P_2-P_1}{2P_1+P_2}\right)^2(2P_1+P_2)(1-\phi_2)}{P_1(2+\phi_2) + P_2(1-\phi_2) - G\left(\frac{P_2-P_1}{2P_1+P_2}\right)^2(2P_1+P_2)(1-\phi_2)} \quad \text{(Eq. 62)}$$

where P_1 and P_2 are the individual permeabilities for the planar case, and ϕ_2 is the dispersed phase volume fraction. The term $(1 - \phi_2)$ is the continuum volume fraction, and G is a constant which has a value of about 0.8 based on dielectric constant data for powders and suspensions (Higuchi and Higuchi estimated that G may range down to 0.4 in some instances).

Time Variable Boundaries—*General Considerations*—To this point, transport situations of gradually increasing complexity have been discussed. Further escalation of complexity arises when the barrier's dimensions and/or diffusion properties change in the course of an experimental run. Some systems in which boundaries advance or recede with time, so-called moving boundaries, lend themselves to quantitative analysis. Such systems are exemplified by the following processes: *(a)* drug release from inert matrixes (55), *(b)* freeze drying (the receding ice layer) (56), *(c)* film-forming chemical reactions such as tarnishing of metals (57), and *(d)* formation of a calcium fluoride layer on tooth enamel (hydroxyapatite) (58).

The first two examples require that the diffusant be transported through a region whose dimension in the flux vector is lengthening due to depletion of diffusant. The latter examples differ in that the diffusant travels through a continuously expanding region (boundary) formed by a chemical reaction of the diffusant and the substrate. With the exception of surface oxidations, there appear to be few examples of these processes outside the pharmaceutical field.

Release from Inert Matrixes—From a pharmaceutical point of view, drug release from inert matrixes represents the most studied system involving moving

boundaries. Mathematical equations have been derived for several different physical situations. With a knowledge of the mechanism of drug release and the appropriate parameters that alter the release process, the researcher is in a better position to design systems to meet a given set of predetermined requirements.

The rate of release of drugs suspended in a stationary matrix (semisolid ointment) was presented by Higuchi (59). Figure 3 depicts the physical model for a planar system. With the assumptions that: *(a)* a quasisteady state exists, *(b)* the drug particles are small compared to the average distance of diffusion, and *(c)* perfect sink conditions exist in the external media, the following equations were derived. The change in amount released per unit area, dM, corresponding to a change in the thickness of the depleted zone, dh, is:

$$dM = W\,dh - \frac{C_s}{2}\,dh \qquad \text{(Eq. 63)}$$

where W is the total amount of drug, soluble and undissolved in a unit volume of the matrix, and C_s is the saturation concentration of the drug within the matrix. According to Fick's law, dM is also equal to:

$$dM = \frac{D_s C_s}{h}\,dt \qquad \text{(Eq. 64)}$$

where D_s is the diffusion coefficient in the matrix phase. Equating Eqs. 63 and 64, integrating, and solving for h yield:

$$h = \left(\frac{2D_s C_s t}{W - C_s/2}\right)^{1/2} \qquad \text{(Eq. 65)}$$

This can be substituted into the integrated form of Eq. 63 to obtain:

$$M = [C_s D_s (2W - C_s)t]^{1/2} \qquad \text{(Eq. 66)}$$

and when $W \gg C_s$:

$$M = (2C_s D_s W t)^{1/2} \qquad \text{(Eq. 67)}$$

In other words, the amount released is a linear function of the square root of time.

The analogous equation for the release of a single drug from a granular matrix is (55):

$$M = \left(D_a C_a \frac{\epsilon}{\tau}[2W - \epsilon C_a]t\right)^{1/2} \qquad \text{(Eq. 68)}$$

where ϵ is the porosity of the matrix, τ is tortuosity, C_a is the solubility of drug in the release medium, and D_a is the diffusivity in the release medium. In this model, the drug is dissolved by a leaching action of the solvent which enters the matrix through con necting capillaries. The corresponding equations, when $W \gg C_a$, for spherical pellets (55) and cylindrical pellets of infinite length (60) are, respectively:

$$F = -\frac{3D_a \epsilon C_a t}{W \tau a_0^2} + {}^3/_2\left[1 - \left(\frac{a}{a_0}\right)^2\right] \qquad \text{(Eq. 69)}$$

and:

$$F = \frac{4D_a \epsilon C_a t}{W \tau a_0^2} - \frac{2a^2}{a_0^2}\ln\left(\frac{a}{a_0}\right) \qquad \text{(Eq. 70)}$$

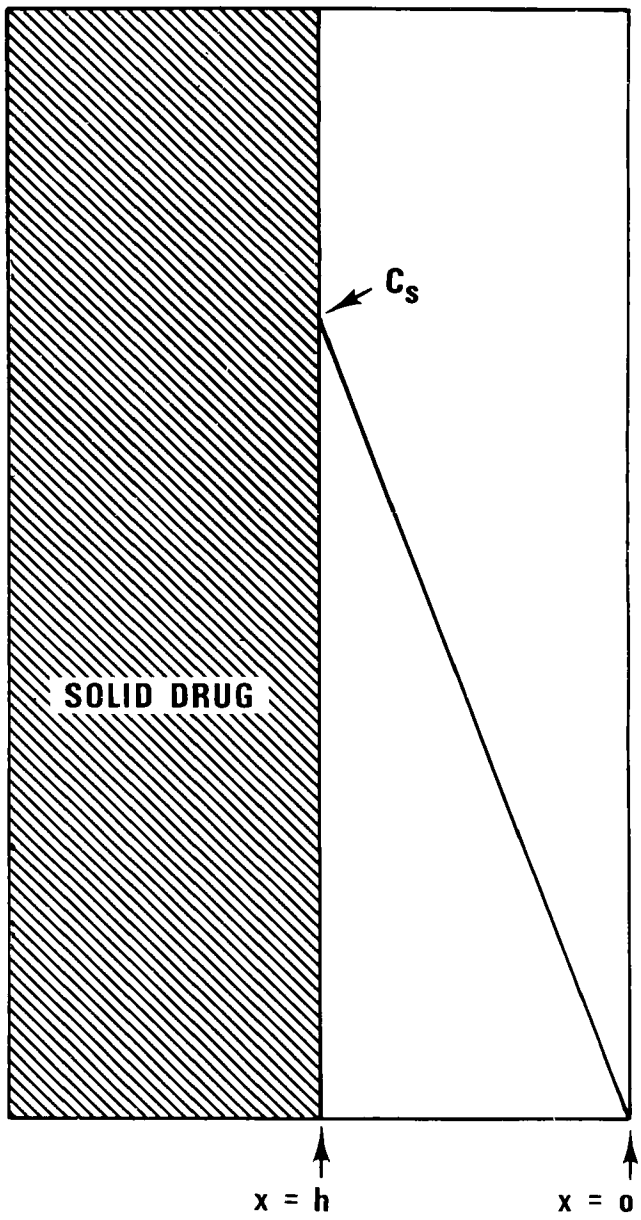


Figure 3—*Physical model for the release of drug suspended in an ointment base under perfect sink conditions (59).*

where F is the fraction of the total amount in the pellet released, a_0 is the radius of the pellet, and a is the radius of the matrix which is unextracted at time, t. When the initial porosity of the matrix is small or the fraction of the matrix volume occupied by the solute is relatively large, ϵ may be represented by W/ρ, where ρ is the density of the drug. In this situation, Eq. 68 reduces to:

$$M = W\left[\frac{D_a}{\tau\rho}\left(2 - \frac{C_a}{\rho}\right)C_a t\right]^{1/2} \qquad \text{(Eq. 71)}$$

and, hence, a linear dependency of M upon W is expected. It should also be noted that the fraction of drug released in a given time for the spherical or cylindrical cases is not appreciably different from the planar case until about 50% of the total amount is released (55, 61).

The following equation has been presented (62) for

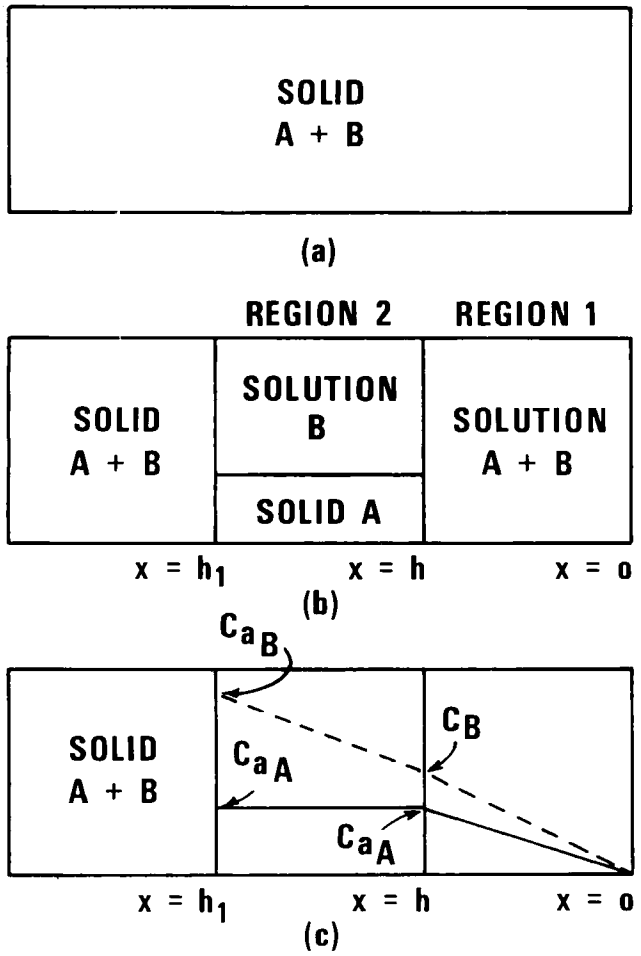


Figure 4—*Physical model for the release of a mixture of two noninteracting drugs from an inert matrix under perfect sink conditions (73). Key:* a, *conditions existing at* t = 0; b, *conditions existing at finite time* (t); *and* c, *illustration of the concentration gradients in regions 1 and 2.*

the situation where equilibrium drug binding to the matrix phase occurs but solute does not diffuse through the matrix phase:

$$M = \left[\frac{D_a \epsilon C_a}{\tau}[2W - C_a(\epsilon + K - K\epsilon)]\right]^{1/2} t^{1/2} \quad \text{(Eq. 72)}$$

where K is the equilibrium partition coefficient, *i.e.*, the drug concentration in solution in the matrix divided by the drug concentration in solution.

The applicability of Eq. 68 to the release of drugs from an inert tablet matrix of polyethylene was tested by Desai *et al.* (63) under controlled experimental conditions. As predicted by theory, a linear relationship was found experimentally when M was plotted against $t^{1/2}$ for the release of sodium salicylate. The effect of W and C_a on the release of drug was in qualitative agreement with theory. However, indirect effects of these two variables along with those of additives and leaching solvent on the porosity and tortuosity terms were noted. Subsequent studies (64) demonstrated that surface-active agents increase the rate of release of solutes by increasing the porosity of the matrix (due to the wetting of channels). When the constants in Eq. 68 were independently determined, quantitative agreement of data and theory was found. Porosity, however, was not a direct function of the percent of drug in the matrix, and a direct dependence of M upon W as suggested by Eq. 71 was not observed. Furthermore, the choice of the inert matrix strongly influences the observed M *versus* $t^{1/2}$ plots. Polyvinyl chloride releases sodium salicylate four to six times faster than polyethylene, and M *versus* $t^{1/2}$ plots are sigmoidal (65). This atypical behavior was attributed to the slow removal of air from the tablet.

The release of drugs from wax matrixes (66), methyl acrylate–methyl methacrylate copolymer matrixes (67, 68), and hydroxypropyl methylcellulose (69, 70) have also been shown to follow Eq. 68. However, using a sulfanilamide–wax system, calculated tortuosity values were extremely high ($\tau > 1000$) and a more complex model was presented where the permeability characteristics of the drug in the matrix were considered using the Bruggeman equation (71). A receding boundary model has also been proposed to describe the dissolution rates of polyvinylpyrrolidone–sulfathiazole coprecipitates from tablets (72).

The physical model for the simultaneous release of two noninteracting drugs from an inert wax matrix was developed by Singh *et al.* (73). The equations describing the release of each of the two components are based on the physical model given in Fig. 4. The slower moving component's release follows the basic relationship given by Eq. 68. The faster component's release, on the other hand, is described by:

$$M_B = \frac{2D_{a_B}\epsilon_1 W_A}{\tau_1 k_A}\left[\frac{\frac{\epsilon_2 C_{a_B}}{\tau_2}}{\frac{\epsilon_2}{\tau_2} + \frac{\epsilon_1}{\tau_1}\left[\left(\frac{k_B}{W_B} - \frac{k_A}{W_A}\right)\Big/\frac{k_A}{W_A}\right]}\right] t^{1/2} \quad \text{(Eq. 73)}$$

The subscripts A and B refer to the different drug species, the subscripts 1 and 2 refer to the regions under consideration, the k values are the slopes of the corresponding M *versus* $t^{1/2}$ plots, and C_B is the concentration of drug, B, at the h boundary. Singh *et al.* (74) also derived equations for the case where the two drugs interact to form a complex.

The release of drug from a homogeneous matrix of planar geometry is given by Eq. 66. In this case, the drug dissolves directly in the matrix phase and diffuses through this isotropic environment to the surrounding medium. When the matrix is heterogeneous, that is, when it contains an inert filler, ϕ_1 (the continuum volume fraction) and τ must be included. Equations 69 and 70 are applicable for spherical and cylindrical geometries for this case as long as $W \gg C_s$. Note that C_s, D_s, and ϕ_1 now replace C_a, D_a, and ϵ, respectively. When W approaches C_s, the amount of diffusing component dissolved in the partly extracted matrix must also be considered. The general case relating a (radius of matrix that is unextracted) to time for the spherical case was derived by Higuchi (55):

$$(1-\alpha)\left[1 + 2\left(\frac{a}{a_0}\right)^3\right] - (3-4\alpha)\left(\frac{a}{a_0}\right)^2 - \alpha\left(\frac{a}{a_0}\right) - \alpha \ln\left(\frac{a_0}{a}\right) = \frac{6D_s C_s \phi_1 t}{W a_0^2 \tau} \quad \text{(Eq. 74)}$$

where $C_s = \alpha W$. The fraction of drug remaining (F') at t is:

$$F' = \left(\frac{a}{a_0}\right)^3 + \frac{\alpha}{2}\left[\left(\frac{a}{a_0}\right) + \left(\frac{a}{a_0}\right)^2 - 2\left(\frac{a}{a_0}\right)^3\right] \quad \text{(Eq. 75)}$$

As α approaches unity, Eqs. 74 and 75 lose their validity because the quasisteady state necessitates the condition $W \gg C_s$.

Boundary Layers and Matrix Release—In the foregoing derivations involving moving boundaries, it is implicit that the release process is dependent on the properties of the matrix only and is not significantly affected by the external medium (sink) collecting the drug. Due to the presence of solvent boundary layers at surfaces, this may not always be true. Roseman and Higuchi (60) considered the more general system where the solvent (aqueous) diffusion layer is placed in series with the receding drug boundary, much as in the membrane-diffusion layer laminate situation. The expressions relating M to t when $W \gg C_s$ are:

$$M = Wh \quad \text{(Eq. 76)}$$

and:

$$\left[h^2 + \frac{2D_s\phi_1 K h_a h}{\tau D_a}\right] = \frac{2D_s\phi_1 C_s}{\tau W} t \quad \text{(Eq. 77)}$$

where, again, K is the matrix–solvent partition coefficient, and h_a is the thickness of the solvent diffusion layer. The thickness of the zone of depletion (h) is related to time by Eq. 77 and increases as a function of time as shown in Fig. 5 for the release of medroxyprogesterone acetate from a silicone rubber cylinder. These equations do not predict a linear dependence of M on $t^{1/2}$ at all times unless the condition $h \gg (2D_s\phi_1 h_a K/D_a\tau)$ is fulfilled. When this occurs, Eqs. 76 and 77 combine to yield Eq. 67, which predicts a linear relationship[9].

The applicability of Eqs. 76 and 77 have been tested by studies on the release of progesterone-type steroids from a silicone polymer (61). Nonlinearity of M *versus* $t^{1/2}$ plots was found during early stages of the release process. The duration of the nonlinear region was dependent upon the particular steroid studied. With the ratio D_s/D_a presumed to be relatively constant for a class of structurally similar compounds in a given matrix and with h_a fixed by the experimental design, the nature of the M *versus* $t^{1/2}$ plots are dependent upon the magnitude of K, which is, in turn, strongly dependent upon molecular structure. Further support for this model of drug release can be abstracted from the data of Haleblian *et al.* (75) for the release of chlormadinone acetate from a silicone polymer. When M *versus* $t^{1/2}$ plots were made for five different concentrations of micronized drug within the matrix, nonlinearity was observed at early times. In this study, the higher drug loads required a longer time to reach the linear region of the M *versus* $t^{1/2}$ curve. This finding is consistent with the notion that it requires a longer time at higher drug levels for h to be sufficiently large so that the release process is solely matrix controlled, as given by Eq. 67.

[9] In Eq. 67, ϕ_1 and τ are unity.

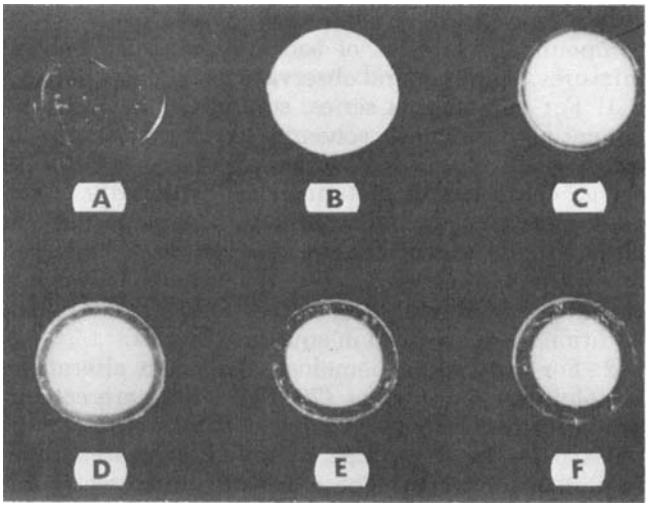


Figure 5—*Cross-sectional views of silicone rubber cylinders (60). Key: A, placebo; B, drug-filled, initial; C, 1 week; D, 2 weeks; E, 3 weeks; and F, 4 weeks.*

INFLUENCE OF SOME SPECIFIC PERMEANT AND BARRIER PROPERTIES ON MASS TRANSPORT

Diffusant Solubility as a Flux-Limiting Factor—*General Solubility Considerations*—Regardless of flux mechanism, it is clear upon examination of all of the permeability expressions that flux is proportional to the concentration differential across the total barrier. This is maximized in a given system for a given permeant when the penetrating agent is present in the applied phase in a saturated state. There are many situations of pharmaceutical interest where this solubility limitation plays a critical role in the transport process. For example, the rate of dissolution of a solid in a given solvent is dependent on its solubility, the barrier for dissolution in most cases being the solvent diffusion layer at the solid's surface. The absorption of slightly soluble drugs from suspensions or from solid dosage forms, the passage of pure gases and vapors across membranes, and the permeation of pure solvents through synthetic containers also involve saturation of the barrier interface in contact with the penetrant. Even in uptake experiments and other situations where saturation is unattained, the solubility of the diffusant is important as a measure of the maximum driving force for transport. Furthermore, since studies concerned with determinations of diffusivity or release from matrixes, *etc.*, often involve permeability from saturated phases, it is necessary to know the solubility to calculate diffusivity or otherwise to quantitate the processes.

A review of solubility theory is beyond the scope of this paper; the reader may want to consult Refs. 76–80 for information along these lines. However, several generalizations regarding chemical structure and its influence on solubility are particularly relevant and will be considered.

Most systematic studies on the solubilities of organic nonelectrolytes have been directed to congeneric solubilities in a given solvent (particularly solubilities of homologs) or to the solubilities of a single

compound in a series of solvents or binary solvent mixtures. Some general observations follow.

1. For homologous series, solubilities decrease exponentially in most solvents as chain length increases (79, 80). Negative slopes of log (solubility) *versus* chain length plots increase as polarity of solvent increases. In hydrocarbons, the slope may be close to zero and it ranges between −0.5 and −0.7 for water. In other words, somewhere between a three- to fivefold decrease in solubility per methylene addition is experienced in aqueous systems.

2. For crystalline homologs, odd–even alterations in solubility are evident (79, 81). These are concurrent with generally decreasing solubility and may be masked in the very large solubility decreases found in aqueous systems. These arise from energetic differences in crystal packing of odd and even chains which lead to odd–even alterations in heats of fusion. Moreover, the behavior of the first few homologs in a series may be aberrant with regard to both slope and odd–even alterations since the nonchain portion of the molecule can assert a disproportionate influence on crystal structure in this instance (81, 82).

3. Solubilities of nonpolar organic compounds are directly influenced by their molecular "hydrophobic" surface area (83). For example, neopentane is more water soluble than *n*-pentane because it is a more globular molecule offering less total surface to the aqueous environment. The lipid–water partition coefficients of organic homologs and analogs increase exponentially with "hydrophobic" surface area because the affinity for water decreases as the molecules are made more "hydrophobic" and *not* because the absolute lipid affinity is raised.

The emphasis here is on water because water is the principal component of the fluids found in the GI tract and elsewhere in the body as well as the solvent of choice for most liquid pharmaceuticals intended for systemic effects. While most of the comments are made with specific reference to homologs, the properties of analogs would tend to follow the same patterns, albeit less regularly or rigorously. What the solubility phenomena mean with respect to transport was summarized by Flynn and Yalkowsky (37). They combined the zero-order flux equation for the membrane-diffusion layer system with the following homolog relationships:

$$\log S_n = \log S_0 - \delta n \qquad \text{(Eq. 78)}$$

and:

$$\log K_n = \log K_0 + \pi n \qquad \text{(Eq. 79)}$$

where S_n and K_n are the solubility and partition coefficient, respectively, of the homolog of chain length n; and S_0 and K_0 are the solubility and partition coefficient, respectively, of the reference homolog of chain length equal to zero; the latter are often hypothetical values found by taking the Y intercept of the log (solubility) *versus* chain length plot for log S_0 and the Y intercept of the log (partition coefficient) *versus* chain length plot for log K_0. Using this solubility and partitioning reference point, the steady-state flux from saturated solutions for the compound of chain length n in the standard zero-order process may be expressed by:

$$\log J_n = \log S_0 + \log K_0 + (\pi - \delta)n - \log \left[\frac{h_{AQ}}{D_{AQ}} K_0 10^{\pi n} + \frac{h_M}{D_M}\right] \qquad \text{(Eq. 80)}$$

Often $h_M/D_M \gg (h_{AQ}/D_{AQ})K_0 10^{\pi n}$ for small values of n, in which case the steady-state flux is given by:

$$\log J_n = \log \frac{S_0 K_0 D_M}{h_M} + (\pi - \delta)n \qquad \text{(Eq. 81)}$$

This is the membrane control situation and, where operative, the relative flux of homologs from saturated solutions will depend almost entirely on the relative values of π and δ. Generally, $\delta > \pi$, particularly for biological systems where π is only about 0.2–0.3, and there will be a decreasing flux with increasing chain length for saturated conditions (84). When n gets large, $(h_{AQ}/D_{AQ})K_0 10^{\pi n} \gg h_M/D_M$ and the steady-state flux obtained will be related to solubility by:

$$\log J_n = \log \frac{S_0 D_{AQ}}{h_{AQ}} - \delta n \qquad \text{(Eq. 82)}$$

which, neglecting small changes in aqueous diffusivity of the compounds, indicates that the flux will drop exponentially and in exact parallel to the solubility. Thus, based on literature values of δ, which range from 0.5 to 0.7, a three- to fivefold decrease in steady-state rate of penetration per unit increase in n is experienced. From the standpoint of relative biological activity, a fourfold decrease in rate of supply of drug to the biological "receptor" will often overwhelm other activity-determining factors and the biological activity will plunge precipitously once diffusion layer control is attained, assuming, of course, application in saturated aqueous solutions. The latter assumption is eventually good in all cases because the exponentially decreasing solubility line will of necessity cross any fixed (initial) concentration line at some value of n. Thus, to generalize, there is a practical limit for the adjustment of "hydrophobicity" of homologs and analogs past which the absorption behavior is so seriously affected as to preclude biological activity. It is implicit in this generalization that the membrane–diffusion layer system serves as the model of minimum complexity for the absorption of drugs.

Solubility of Gases—The solubility of pure nonpolar gases and vapors in amorphous nonpolar polymers can generally be described by any of several relationships of the form:

$$\log S = AE + B \qquad \text{(Eq. 83)}$$

where A and B are constants that depend upon the temperature, the gas, and the polymer, respectively. For mixtures of gases, the solubility is usually replaced by the solubility constant, defined as the ratio of the concentration in the polymer phase to that in the gas phase. The heats of solution of gases and vapors are exothermal and tend to increase with solute size and eventually level off for large solutes.

In most cases, heats of solution of gases in nonpolar polymers and in nonpolar liquids can be approximated by:

$$\Delta H_s = V_1(\psi_1 - \psi_2)\Phi_2^2 \qquad \text{(Eq. 84)}$$

where V_1 is the partial molal volume of the solute, ψ_1 and ψ_2 are the solubility parameters of the solute and either the solvent or polymer, and Φ_2 is the volume fraction of solvent or amorphous polymer. Both volumes and solubility parameters for most common polymers and solutes are known (85, 86) or can be estimated by empirical calculations (82, 87). Therefore, for poorly soluble substances, where Φ_2 is approximately unity, it is possible to estimate the heats of solution. For solid dissolution, the heat of solution is dominated by the heat of melting and is thus usually endothermic.

Special Case: Dissolution of Solids—The dissolution of a solid in a fluid medium is described by the Noyes-Whitney equation (88):

$$\frac{dM}{dt} = R_{AQ}\frac{A}{V_B}(S - C_B) \qquad \text{(Eq. 85)}$$

where C_B is the bulk concentration, S is the solubility, M is the weight, A is the surface area of the solute, V_B is the volume of the solution, and R_{AQ} is the resistance of the diffusion layer as described earlier. If, as in the beginning of a dissolution experiment, $C_B \ll S$, Eq. 85 becomes:

$$\frac{dM}{dt} = R_{AQ}\frac{A}{V_B}S \qquad \text{(Eq. 86)}$$

which describes the initial rate of dissolution. The role of aqueous solubility in determining the initial dissolution rates of about 50 drugs and drug-like substances from uniform surface area pellets was illustrated by Hamlin *et al.* (89). Their data show excellent agreement with Eq. 86, the linearity extending over five orders of magnitude.

The solubility of a substrate and, thus, its dissolution rate can be increased significantly in several ways. The addition of surfactants, complexing agents, and solvents and the adjustment of pH can have profound effects on dissolution rates of drugs (89). Complete characterization of such systems must, of course, account for the diffusivity of any micellized or complexed drug and for changes in viscosity that accompany the alteration of solvent composition.

Factors Determining Partition Coefficients—The permeability of a membrane separating two aqueous phases is proportional to the product of the membrane-water partition coefficient and membrane diffusion coefficient of the solute when the membrane provides the sole source of diffusional resistance. Thus, in certain instances, the role of partitioning in determining permeability is similar to the role of absolute aqueous solubility already discussed.

The relationships between partitioning behavior and chemical structure were thoroughly reviewed (90–92). Although the discussion of Leo *et al.* (92) is aimed primarily at octanol-water partitioning, it provides regression equations which can be used to convert octanol-water data to ether-water partition coefficients, chloroform-water partition coefficients, *etc.* Presumably, if enough data were available for biological systems, it would be possible to obtain equations from which membrane-water partition coefficients could be calculated.

In the structural approach to partitioning, a given functional group or other structural component is presumed to have a constant input to the overall partition coefficient of the given whole molecule. By using a reference derivative with a known partition coefficient in the partitioning system of choice, the log (partition coefficients) of a homolog or analog can be calculated by simply summing the group contributions (π values) of the structural modifications with respect to the reference compound and adding these to the log [partition coefficient] of the reference. Fortunately, there is a great parallelism seen for these specific structural effects among differing partitioning systems. This usually makes it possible to assess, at least qualitatively, the effect a given moiety will exert on one partitioning system if its influence on another is known. This is particularly helpful for analyzing data from biological systems, where partition coefficients are often unattainable, because a convenient *in vitro* reference partitioning system may be used to correlate the data and predict optimal properties.

The permeability of many polymers to gases is often more a function of the relative concentration of the gases than their diffusivities, according to Barrer and Chio (93). For example, the diffusivity of helium is almost nine times greater than that of xenon in silicone rubber; yet the permeability of the latter is over 10 times the permeability of helium because of its 100-fold greater solubility. In the case of gases, the distribution function is referred to as the solubility coefficient.

The role of substrate partition coefficients in determining flux is conveniently illustrated by studies on alkyl homologs. The diffusivities of the members of most homologous series in water or artificial membranes generally do not change by more than a slight factor (2 or 3) as the series is ascended over a 10-unit increase in chain length. A 10-unit variation in chain length may be taken as the rough limits of practical chain length manipulation (see section on diffusivity for further conditional factors). However, by examining Eq. 79, it is obvious that over the same 10 methylene unit range, partition coefficients will increase by from 2^{10} ($\simeq$1000) to 4^{10} ($\simeq$1,000,000). These numbers correspond, respectively, to π values for a methylene unit of 0.3, which is commonly found for biological membranes (84), and 0.6, which was reported by Davis *et al.* (83) for hexane and similar apolar solvents. Obviously, changes in partitioning dominate diffusivity effects. When measured from solutions of equivalent concentration, the steady-state flux of the homologs will grow essentially as the partition coefficient grows as long as the membrane is the controlling barrier. However, for all membranes that are "lipoid" in character, a point is reached as the partition coefficient is made larger

where the system crosses over to diffusion layer control. As shown by Stehle and Higuchi (43), a plateau in flux is reached at this point for these conditions and flux remains constant upon further alkyl chain length extension, unless, of course, the solubility limitation is also attained, in which case the flux falls exponentially (see previous section).

Garrett and Chemburkar (94) and Flynn and Yalkowsky (37) showed that under membrane control of flux the permeability of alkyl *p*-aminobenzoates through a silicone rubber membrane parallels the partition coefficients of these compounds between water and a nonpolar solvent. Similarly, Nasim *et al.* (95) showed that the permeabilities of a variety of substances through polyethylene is proportional to their hexane–water partition coefficients. Other workers correlated permeability of various nonpolar membranes with different oil–water partition coefficients.

Effective Concentrations—Factors that alter compound solubility such as micelle formation, complex formation, and cosolvents also affect the permeation processes by changing the thermodynamic activity of the penetrating substance in either the applied phase or the barrier phases. Since the partition coefficient actually is a ratio of the activity coefficients in the various phases, such effects are directly reflected in measured (or effective) partitioning constants. The influence of complexation, micellization, *etc.*, on permeability of lipid (partitioning) membranes lacks predictability since these processes are complicated in multiphasic systems because all interphase equilibria are simultaneously affected.

Possibly the simplest and best characterized equilibria with respect to permeation of "lipid" membranes are dissociations of weak acids and weak bases. Generally, ionized species formed in such equilibria do not have favorable free energies for transfer to lipid phases and, for all intents and purposes, only nonionized species partition. Thus, the net membrane gradient in membrane control of flux is determined solely by the fraction of unionized drug (diffusant) in the applied phase. In such cases the pH profile for drug permeation is sensitive to the pH profile of the nonionized species[10]. This has been called the pH–partition hypothesis by some authors. The literature is replete with examples illustrating the influence of pH on the transport of both acidic and basic species.

The influence of cosolvents on membrane transport is interesting and of practical importance in the field of topical dosage forms. Garrett and Chemburkar (94) and Yalkowsky and Flynn[11] studied the effect of regularly altering aqueous solvent composition on the transport of several drugs across silicone rubber membranes. For the systems studied, it can be shown that the partition coefficient of a drug between the membrane and any solvent mixture is proportional to the reciprocal of the drug solubility in the solvent system. Furthermore, a plot of log (reciprocal solubility) *versus* log (permeability) is linear and has a slope of unity. This indicates that in the systems studied the permeability is directly proportional to the partition coefficient and that the systems are under membrane control of flux. If the permeant is sufficiently nonpolar to produce diffusion layer control, the permeability would be independent of solvent composition.

Self-association of a diffusant into micelles increases the total apparent solubility of a substrate in the aqueous phase and decreases its apparent partition coefficient, but it does not significantly alter the concentration of free drug or its true partition coefficient. Therefore, if the micelle cannot pass through the flux-controlling membrane, its effect on permeability will be small. However, if the dominant resistance is the aqueous diffusion layer, the micelle can play a significant role in the transport process (96–98).

The influence of micelles where the association colloid-forming agent and the diffusant are not the same is a very different matter. When the diffusant has no affinity for the micelle, no appreciable effects will be noted. As the micellar affinity increases, the fraction of unassociated diffusing species will be depleted and the flux will drop proportionally. In the extreme, there may be so little free drug that transport becomes limited by the rate of diffusion of the micelle or the energetics of removing the diffusant from the micelle once at the partitioning surface. The latter interfacial barrier mechanism appears to be operative in the transport of cholesterol and related sterols in the presence of bile acid–lecithin micelles into and out of a hexadecane phase (99). Such processes are orders of magnitude slower than free diffusion.

Complex formation, like micelle formation, alters the apparent solubility and partition coefficient of a substance. If complexation only occurs in the aqueous phase, complexation will influence transport in a manner analogous to micellization. However, if the complex is also stable in the membrane, transport of the complexed form effectively results in a parallel pathway for transport. A complete description must include the stability constant for the complex as well as the diffusivity of free and complexed drug in each phase and the partition coefficient of the drug and complex between phases. The relative magnitudes of these values will determine whether permeability is increased, decreased, or unchanged by complexation. These rather interesting possibilities were effectively separated from one another by Nakano and Patel (100), who studied the effects of alkylamides on the passage of *p*-nitrophenol across silicone rubber membranes. They found that dimethylacetamide produced no effect on the apparent permeability but that dimethylpropamide, diethylacetamide, and diethylpropamide increased permeability by 11, 29, and 95%, respectively. This point was further illustrated by Bates *et al.* (101), who showed that certain caffeine–drug complexes, which have lower partition coefficients than the respective free drugs, reduced

[10] The permeation profile is *superimposable* on the pH profile of the nonionized species only when the partition coefficient is equal to one.

[11] In preparation.

the apparent permeability of the drugs. The apparent permeability of caffeine, on the other hand, was increased as a result of formed complexes that were more hydrophobic than free caffeine.

Factors Affecting Diffusivity—*General Remarks*—In grossly generalized terms, diffusivity is dependent on the state of matter of the diffusing medium, *i.e.*, gas, liquid, or solid. In all theories relating diffusion to the state of matter, it is implicit that no two molecules or atoms may occupy the same point in space at the same time and, thus, diffusion must always be through void spaces in the matter under consideration. In gaseous media, the void space, or free volume as it is often referred to, is thousands of times greater than the physical space occupied by the molecules themselves and the mean free path between molecular collisions is large, leading to large diffusivities. Typical diffusivities in air are on the order of 0.05–1 cm^2/sec at ambient temperature. Thus, the average gas molecule from a large population will cross a centimeter thick barrier of air in from 1 to 20 sec. In condensed fluid phases, the free volume is but a small fraction of that found in the gaseous state; mean free paths and, thus, diffusivities are correspondingly smaller. For example, in liquid water the diffusivities of 10^{-5}–10^{-6} cm^2/sec are common and it would take a molecule somewhere between 1 and 10 years to cross a 1-cm thick, unstirred layer[12]. Solidified polymers generally have even less free volume which, in this case, is dependent on the polymer's density and degree of crystallinity. Diffusivities in polymers are orders of magnitude less than in the fluid state. For all intents and purposes, metallic and ionic crystalline solids are without free volume and are virtually impervious to small molecules at ordinary temperatures. However, small gas molecules (hydrogen, helium, *etc.*), which can "squeeze" through the atoms in the crystalline array, do often penetrate with detectable velocities at ordinary temperature.

In more specific terms, the diffusivity of a substance in a particular medium is a complex phenomenological parameter, which is dependent upon the properties and the degree of interaction between the diffusant and the diffusion medium. The diffusion coefficient, D, is related to the frictional resistance, f, that the diffusing particle experiences in moving through a medium by (26):

$$D = \frac{RT}{f} \qquad \text{(Eq. 87)}$$

The primary effect of structural modification on diffusivity is the alteration of the frictional resistance. However, since structural changes are frequently associated with changes in molecular interaction, a secondary effect is to alter the effective concentration gradient, ΔC. The sensitivity of ΔC and f to molecular modification depends upon the characteristics of both diffusant and barrier. Unfortunately, it is not possible at this time to present a single relationship describing diffusivity in terms of other known or measurable parameters. To provide a meaningful discussion of diffusivity, this section will be subdivided according to the nature (homogeneity, fluidity, and polarity) of the diffusion medium. Since all possible barriers cannot be covered, a variety of representative systems was chosen so that reasonable extrapolations can be made to media that are not specifically discussed.

Diffusivity in Homogeneous Liquids—In a homogeneous liquid, the frictional resistance that a particle experiences is dependent largely upon its size and shape and on the nature of the solvent. There is a variety of theoretical and empirical correlations of f or D in terms of solvent and solute properties (102–105), but no single equation can account for all experimental data. One of the most useful relationships for the frictional resistance of a particle in a homogeneous fluid is the Sutherland equation (103, 106):

$$f = 6\pi\eta r \left[\frac{2\eta + r\beta}{3\eta + r\beta}\right] \qquad \text{(Eq. 88)}$$

which relates f to solvent viscosity, η; solute radius, r; and a "slip" factor, β. This equation is applicable for spherical particles in sufficiently dilute solution so that solute–solute interactions can be neglected. The slip factor, β, or more properly the coefficient of sliding friction is a measure of the tendency of solvent molecules to adhere to the diffusant. For large particles, the solvent molecules tend to be dragged along with the diffusant and $\beta \rightarrow \infty$. If β is large, Eq. 88 becomes Stokes' law:

$$f = 6\pi\eta r \qquad \text{(Eq. 89)}$$

If, on the other hand, the solvent and diffusant are of similar size, the tendency to slip is large and $\beta \rightarrow 0$ (*i.e.*, there is very little resistance to slip) and Eq. 88 becomes:

$$f = 4\pi\eta r \qquad \text{(Eq. 90)}$$

Equation 90 is particularly applicable to self-diffusion where the solvent and diffusant have identical dimensions. In cases where the solute is smaller than the solvent, it is frequently observed that the frictional resistance is less than $4\pi\eta r$ (106).

It must be realized that the radius to be used in Eqs. 88–90 is not that of the bare molecule but of the hydrodynamic particle (106). The hydrodynamic particle consists of the diffusant molecule plus any solvent or solute (such as a complexing agent) that is adsorbed or bound to the surface or entrapped within the diffusant. If it is known that the diffusant hydrates or complexes, the resultant volume increase must be accounted for in the equations.

The above equations were derived for spherical particles but are applicable to ellipsoids. For oblate (saucer-shaped) and prolate (cigar-shaped) ellipsoids, the equations must be modified to account for the greater surface area and thus the greater resistance that a nonspherical particle experiences. Perrin (107) and Herzog *et al.* (108) independently extended Stokes' law to ellipsoids of revolution. Their results are frequently expressed as the ratio of the frictional resistance of an ellipsoid, f_e, to that of a

[12] These values are computed from the average molecular velocity, D/h.

sphere of equal volume f (106). For an oblate ellipsoid of major axis, a, and minor axis, b, the frictional ratio, F, is:

$$F = \frac{f_e}{f} = \frac{\sqrt{a^2/b^2 - 1}}{\left(\frac{a}{b}\right)^{2/3} \tan^{-1}\sqrt{a^2/b^2 - 1}} \qquad \text{(Eq. 91)}$$

and for a prolate ellipsoid with axes (a, b, b):

$$F = \frac{f_e}{f} = \frac{\sqrt{1 - b^2/a^2}}{\left(\frac{b}{a}\right)^{2/3} \ln\left(\frac{1+\sqrt{1-b^2/a^2}}{b/a}\right)} \qquad \text{(Eq. 92)}$$

These functions were shown graphically by Tanford (106) for axial ratios up to 20. These calculations can be extended to a cylinder by approximating the cylinder with a prolate ellipsoid of the same volume. To obtain the axial ratio of the equivalent prolate spheroid, simply multiply the length over the diameter of the cylinder by 0.815 (106). These equations have been demonstrated to be valid for rigid ellipsoidal organic ions ranging from 121 to 367 in molecular weight (109), for linear oligosaccharides containing 1–6 sugar units (110), and for cyclic oligosaccharides (111). Similar equations have also been found useful for linear oligophenylenes (112).

From the above discussion, it can be seen that for solutes whose molar volume, v, is greater than or equal to the molar volume of the solvent, the diffusivity can be expected to range from:

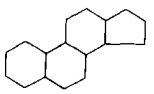
$$D = \frac{kT}{4\pi\eta}\left(\frac{4\pi}{3v}\right)^{1/3} \qquad \text{(Eq. 93)}$$

for small particles to:

$$D = \frac{kT}{6\pi\eta F}\left(\frac{4\pi}{3v}\right)^{1/3} \qquad \text{(Eq. 94)}$$

for large particles. Note the correction for nonsphericity in the large particle case. Assuming the viscosity of the solvent to be either known or readily measurable, the only parameters needed to estimate D are the molar volume and the frictional ratio of the diffusant. Although these values are frequently difficult to measure experimentally, they can be estimated with reasonable accuracy. Fortunately, the molar volume of a substance is an additive property of its constituent atoms and functional groups, and it is thus possible to estimate v from the chemical formula of the diffusant. Some workers (104) preferred to calculate the van der Waals' volume of the solute while others (102, 113–117) chose the partial or apparent molal volume; still others (15, 118) determined v from space-filling molecular models. In view of the approximate nature of atomic and group values and the possibility of solvent incorporation into the hydrodynamic particle, it is not possible to justify a theoretical preference for any of these approaches. The partial molal volume is frequently easiest to calculate and has been used extensively in the estimation of protein hydrodynamic properties (106). It has also been used successfully in the calculation of micelle surface charge density (119, 120) and in the estimation of solubility parameters (82). The partial molal volumes of some common atoms and groups are listed in Table III. Many of these values were originally determined by Traube (121) in 1899.

Table III—Partial Molal Volumes of Some Common Atoms and Groups[a]

Atom	Partial Molal Volume, cm³/mole[b]	Group	Partial Molal Volume, cm³/mole[b]
C	9.9	CH_3	19.3
H	3.1	CH_2	16.2
H^+	−4.5	NH_2	7.7
N	1.5	$N(CH_3)_3^+$	66.3
N^+	8.4	COOH	19.0
O (=O or —O—)	5.5	COO^-	11.5
O (—OH)	2.3	C_2H_5	35.3
O (diol)	0.4	C_3H_7	51.7
S	15.5	C_4H_9	67.9
P	17.0	C_6H_{13}	100.3
P^+	28.5	C_8H_{15}	132.7
Li^+	−5.2	$C_{10}H_{21}$	165.1
Na^+	−5.7	$C_{12}H_{25}$	197.5
K^+	4.5	$C_{14}H_{29}$	229.9
Cl^-	22.3	OCH_2CH_2	37.9
Br^-	29.2	One ring	−8.1
I^-	40.8	Two fused rings	−26.4
			191.0

[a] From Ref. 13. [b] To convert to Å³ per molecule, divide by 0.6023.

For nonspherical substrates, the axial ratio can be estimated from bond distances and group radii (122) or from space-filling molecular models. The frictional ratio can then be determined by solving Eqs. 91 and 92. The inclusion of the frictional ratio amounts to less than a 10% correction for all but the most elongated structures and can thus be safely ignored in obtaining a first approximation of D.

In their strictest sense, Fick's laws are valid only in highly dilute solutions where there is absolutely no interaction between solute molecules. Increasing concentration, C, can result in an alteration of the thermodynamic activity coefficient of the diffusant. In general, if the solution and solvent have the same viscosity (123), then:

$$D = D_{c=0}\left[1 + C\left(\frac{\partial \ln \gamma}{\partial C}\right)\right] \qquad \text{(Eq. 95)}$$

It is obvious from this equation that if Henry's law is obeyed, D is linearly dependent on C, and if C is small, D is independent of concentration. If the solute concentration is sufficiently high, it can alter the solvent viscosity and thus exert a secondary effect on its own diffusivity. Equation 95 can readily be modified to account for this effect:

$$D = D_{c=0}\left[1 + C\left(\frac{\partial \ln \gamma}{\partial C}\right)\right]\frac{\eta}{\eta_{c=0}} \qquad \text{(Eq. 96)}$$

Certain solutes exhibit critical concentrations at which self-aggregation begins to occur. Critical phenomena are often associated with dramatic changes in $\partial \ln \gamma/\partial C$ and viscosity, but there is another effect which deserves special attention. Once the aggregate (dimer, trimer, or micelle) is formed, the size of the diffusing particle is increased and, conse-

quently, diffusivity is decreased. Specific examples of aggregation in aqueous and nonaqueous solutions and their effects on diffusivity will be discussed in the next two sections.

The presence of materials that can interact with the diffusant will invariably result in a decrease in the diffusion coefficient. The magnitude of the decrease is related to the effective size difference of the diffusant alone and in the presence of the interactant. In general, if a 1:1 complex is formed, there will be only a slight change in D; but if a higher order interaction, such as a drug–micelle complex, is formed, there can be a very marked decrease in diffusivity. Complex and micelle formation are extremely important because of their effect on the partitioning properties of the diffusant. This effect is discussed elsewhere in this review. Specific examples of self-association and association with other solutes will be discussed in the next section.

The observed effect of temperature on diffusivity in homogeneous fluids is a combination of several factors. An increase in temperature results in an intensification of thermal motion of the diffusant. This effect of kT results in a proportional increase in D, as expected from Eq. 87. A more important effect of temperature alteration is the accompanying change in solvent viscosity. For example, a temperature increase from 20 to 30° results in a viscosity change from 1.002 to 0.7975 cps for water (124). This 20% change is much greater than the 3.4% change in the value of kT. A temperature increase could also have an indirect effect on diffusivity by virtue of its structure-breaking effect on complexes and micelles and its effects on molecular activity coefficients. If these indirect effects are small and the effects of temperature are only those predicted by Eqs. 89–94, the energy of activation for diffusion in a solvent is a constant for that solvent.

Diffusivity in Aqueous Solutions—The aqueous diffusivities calculated for some common substrates by Eqs. 93 and 94 which become:

$$D = \frac{4.95 \times 10^{-6}}{v^{1/3}} \qquad \text{(Eq. 97)}$$

and:

$$D = \frac{3.3 \times 10^{-6}}{v^{1/3} F} \qquad \text{(Eq. 98)}$$

in water at 25°, and the partial molar volumes of Table III are listed in Table IV along with the experimentally determined values. In the case of the more nonspherical substrates, the axial ratio was estimated from space-filling molecular models, and the frictional coefficient was calculated *via* Eqs. 91 and 92.

Virtually all of the experimental values of Table IV fall between the diffusivities calculated by Eqs. 97 and 98. As would be expected from the above discussion, the smaller diffusants are better approximated by Eq. 97, whereas the larger ones approach Eq. 98. As can be seen from the table, the shape factor described by Eqs. 95 and 96 is only important for certain special cases. The effect of alkyl chain length on the diffusivity is illustrated for several series in Fig. 6. For each of the six series shown, the diffusivity decreases with chain length. For homologs con-

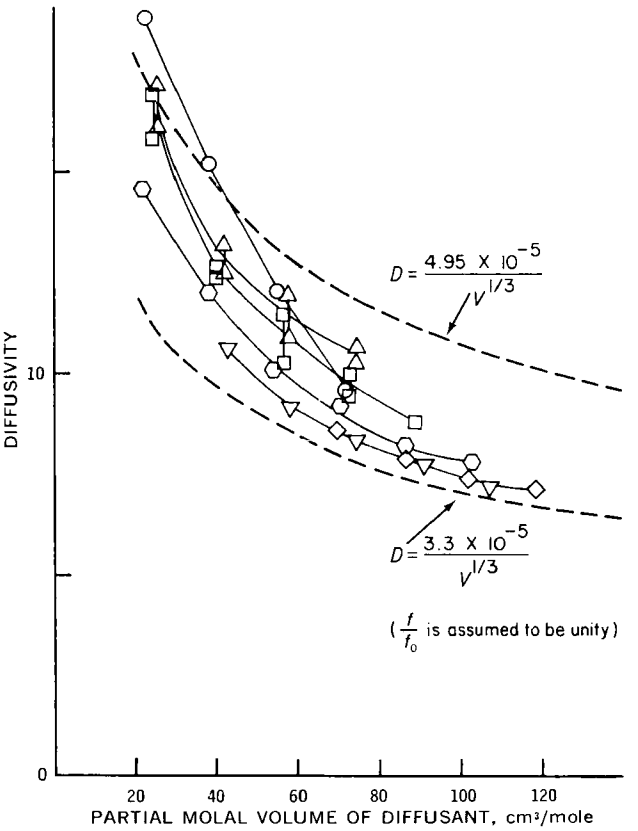


Figure 6—*Diffusivities of some simple homologous series as a function of partial molal volume. The plot indicates that polar moieties increase the hydrodynamic volume of the diffusing species, probably by forming strong hydrogen bridges to adjacent water molecules. Key:* ○, *alkanes;* □, *alcohols;* △, *amides;* ○, *acids;* ◇, *diacids; and* ▽, *α-amino acids.*

taining four or more carbons, diffusivities are within 25% of the values calculated using Eq. 98.

As would be expected from Eqs. 97 and 98, adding a methylene group to a substance decreases the diffusivity only slightly. The larger the molecule, the more insignificant is the effect of a single methyl group. For most homologous series in a partitioning system, it is probably safe to ignore the increase in diffusivity as the series is ascended because this effect is negligible compared to the change in the partition coefficient that accompanies the chain length elongation. In general, branching tends to make the molecule more compact and thus increases diffusivity.

Horowitz and Fenichel (125, 126) compared several homologous series on the basis of diffusivity at a given molecular weight and concluded that, as a class, the alcohols diffuse more rapidly than amides which, in turn, diffuse faster than alkanes. They concluded that this behavior results primarily from the stabilized aqueous domains (icebergs) that are associated with hydrophobic molecules, the effect presumed larger in hydrocarbons than in alcohols or amides. This explanation seems unlikely because the hydrophobic surface area of a molecule such as ethane is not greatly different from that of ethanol and the latter can have additional water molecules firmly associated with it *via* hydrogen bonding. Indeed, if

Table IV—Calculated and Experimental Aqueous Diffusivities at 25°

Diffusant	Partial Molal Volume, cm³/mole	D_{calc} ($\times 10^6$) $\left(\frac{4.95 \times 10^{-5}}{v^{1/3}}\right)$	f/f_0	D_{calc} ($\times 10^6$) $\left(\frac{3.36 \times 10^{-5}}{v^{1/3} f/f_0}\right)$	D_{exper} ($\times 10^6$)	Reference
Methane	22.4	17.5	1.00	11.9	18.8	184
Ethane	38.6	14.1	1.00	9.7	15.2	184
Propane	55.2	13.0	1.01	8.6	12.1	184
Butane	71.3	11.9	1.03	7.7	9.6	184
Methanol	24.7	17.0	1.00	11.3	15.8	128
					17.0	185
					13.7	165
Ethanol	40.9	14.3	1.00	9.6	12.4	128
					12.6	185
n-Propanol	57.1	12.8	1.01	8.5	10.2	128
					11.5	185
n-Butanol	73.3	11.8	1.03	7.6	9.5	128
					11.0	185
n-Pentanol	89.5	11.0	1.05	7.0	8.8	165
Isopropanol	57.1	12.8	1.00	8.6	10.2	128
					10.7	185
Isobutanol	73.3	11.8	1.01	7.8	9.3	128
sec-Butanol	73.3	11.8	1.00	7.9	9.2	128
tert-Butanol	73.3	11.8	1.00	7.9	8.8	128
					9.8	185
Formamide	26.0	16.7	1.00	11.1	17.2	185
Acetamide	42.2	14.2	1.00	9.5	13.2	185
Propionamide	58.4	12.7	1.03	8.3	12.0	185
Butyramide	74.6	11.7	1.05	7.5	10.7	185
Isobutyramide	74.6	11.7	1.03	7.6	10.2	185
Formic acid	22.1	17.6	1.00	11.7	14.6	127
Acetic acid	38.3	14.7	1.00	9.8	12.0	127
Propanoic acid	54.5	13.0	1.00	8.7	10.1	127
Butyric acid	70.9	12.0	1.07	7.9	9.2	127
Pentanoic acid	86.9	11.2	1.03	7.2	8.2	127
Hexanoic acid	103.1	10.6	1.05	6.7	7.8	127
Isobutyric acid	70.7	12.0	1.00	8.0	9.5	127
Isopentanoic acid	86.9	11.1	1.00	7.4	8.2	127
Chloroacetic acid			1.00		10.0	127
Hydroxyacetic acid	40.5	14.4	1.00	9.6	9.8	127
Oxalic acid	70.4	12.0	1.00	8.0	8.6	127
Succinic acid	86.6	11.2	1.02	7.3	7.9	127
Adipic acid	102.8	10.6	1.03	6.9	7.4	127
α,ω-Octanedioic acid	119.0	10.0	1.05	6.4	7.1	127
Pyridine	65.8	12.2	1.00	8.2	11.4	3
4-Methylpyridine	82.0	11.4	1.00	7.6	10.8	3
2-Ethylpyridine	98.2	10.7	—	—	9.8	3
4-Ethylpyridine	98.2	10.7	—	—	10.0	3
2-Propylpyridine	114.4	10.2	—	—	8.8	3
4-Propylpyridine	114.4	10.2	—	—	10.0	3
4-*tert*-Butylpyridine	130.6	9.7	—	—	9.2	3
4-*n*-Amylpyridine	46.8	9.4	—	—	9.5	3
Glycine	42.9	14.1	1.00	9.4	10.6	128
α-Alanine	59.1	12.7	1.00	8.4	9.1	128
β-Alanine	59.1	12.7	1.00	8.4	9.3	128
α-Aminobutyric acid	75.1	11.7	1.02	7.6	8.3	128
α-Aminopentanoic acid (norvaline)	91.5	11.0	1.03	7.1	7.7	128
α-Aminohexanoic acid (norleucine)	107.7	10.4	1.05	6.6	7.2	128
α-Aminoisobutyric acid	75.1	11.7	1.00	7.8	8.1	128
α-Aminoisopentanoic acid (valine)	91.5	11.0	1.02	7.2	7.7	128
α-Aminoisohexanoic acid (leucine)	107.7	10.4	1.03	6.7	7.3	128
Sererie	60.8	12.5	—	—	8.8	128
Threonine	76.9	11.6	—	—	8.0	128
Asparagine	78.0	11.3	—	—	8.3	128
Proline	81.0	11.4	—	—	8.8	128
Hydroxyproline	89.0	11.3	—	—	8.3	128
Histadine		10.7	—	—	7.3	128
Phenylalanine		10.0	—	—	7.0	128
Tryptophan		9.4	—	—	6.6	128
Sodium lauryl sulfate	235	8.1	1.07	5.0	6.2	135
Dimethyldodecylamine oxide	243	7.9	1.07	4.9	5.7	135
Dodecylbenzenesulfonate	293	7.4	1.10	4.5	6.1	140
Glucose	116[a]	10.1	1.63	6.6	6.8	110
Cellobiose	222[a]	8.3	1.00	5.2	5.2	110
Triose	326[a]	7.2	1.07	4.5	4.2	110
Tetrose	432[a]	6.5	1.09	4.0	3.8	110
Pentose	535[a]	6.1	1.19	3.4	3.2	110
Hexose	639[a]	5.7	1.24	3.1	2.9	110
α-Cyclodextrin	1090[b]	4.8	1.03	3.1	3.4	157
β-Cyclodextrin	1235[b]	4.6	1.04	3.0	3.4	157
					3.2	128

[a] Author's values. [b] From dimensions given in Ref. 15 for overall molecule including hole.

the diffusivities are compared on the basis of molecular volume rather than on molecular weight, a totally opposite conclusion is reached.

When diffusivities are compared on the basis of volume (as in Fig. 6), the alkanes become the most rapidly diffusing materials, with the relative rates being alkanes > alcohols > amides > acids > amino acids > dicarboxylic acids. This order is what would be anticipated if "tightly" hydrogen-bonded water travels with the diffusant and thus increases its hydrodynamic volume. As expected, the effect of such hydration is proportionally lower for large molecules, and the curves appear to merge at volumes greater than 100 cm^3/mole.

Albery *et al.* (127) showed that there is no significant difference between the diffusivities of nine carboxylic free acids and their anions (Table IV). However, their data for ω-dicarboxylic acids indicate a 5% decrease in diffusivity on complete ionization. This decrease is consistent with the expected repulsion of the negative charges of the dianion which results in a more fully stretched out structure. It is also possible that the di-free acids can form intramolecular hydrogen bonds, which would result in a more compact and possibly less hydrated cyclic structure.

Longsworth (128) compared the diffusivities of zwitterionic glycine and neutral glycolamide which have the same molecular formula. Due to a greater degree of solvation of the former, its diffusivity is nearly 10% less than that of the neutral compound. Wendt and Gosting (129) obtained similar results with alanine and its isomer lactamide. Likewise, Longsworth's data for the diffusion of *ortho-*, *meta-*, and *para*-aminobenzoates show that the diffusivity of the *meta*-compound, which exists as a zwitterion, is significantly less than the values obtained for the *ortho-* and *para*-isomers, which exist in solution as neutral molecules. The fact that the more slowly diffusing species of each of the two above sets of isomers interact more strongly with water is evidenced by their higher degree of electrostriction (increase in the density of neighboring solvent molecules) (130, 131).

Since most aqueous solutions show a negative deviation from Henry's law, the observed diffusivity usually decreases as solute concentration increases. This decrease is frequently enhanced by the increased viscosity that usually accompanies increased solute concentration. Certain compounds such as sucrose for which $\partial \ln \gamma/\partial C$ is positive show a decrease in diffusivity with increasing concentration because of the resultant increase in viscosity (131). In general, however, diffusivities determined at concentrations below 0.10 M are within a few percent of values determined by extrapolation to infinite dilution.

Surfactants such as sodium lauryl sulfate, which tend to form large micelles in aqueous solution, have diffusivities that are highly dependent upon concentration. Below the critical micelle concentration (CMC), the diffusivity of the monomer, D, is essentially independent of concentration. At the CMC, the apparent diffusivity begins to decrease as micelles are formed; at concentrations significantly greater than the CMC, where the number of monomers is negligible compared to the number of micellar surfactant molecules, the diffusivity begins to level off. If the apparent diffusivity, D_{app}, is plotted against reciprocal surfactant concentration, the Y intercept (reciprocal concentration equal to zero) is the diffusivity of the micelle, D_M, in the absence of monomer. This type of plot can be used to determine the CMC from diffusion coefficient data. According to the Stokes-Einstein equation, the ratio of D_M to D is proportional to the cube root of the volume ratio (assuming no shape correction for either monomer or micelle). In the absence of any significant differences in the degree of hydration of monomer and micelle, the volume ratio is equal to the aggregation number, n, of the micelle. Therefore, the diffusivities of micelles and monomer are related by:

$$D_M = r^{1/3}D \qquad \text{(Eq. 99)}$$

Deviations from this relationship have been used (132–134) to estimate various properties of micelles.

A substance that is not surface active can become associated with a surfactant micelle and thereby acquire the hydrodynamic properties of the micelle. This is possible for highly polar diffusants, such as cadmium ions, which are adsorbed on the micelle surface of nonionic surfactants (135) or for nonpolar materials such as testosterone (136), hydrocortisone (137), salicylamide (138), and salicylic and benzoic acids (139), which are incorporated within the micelle, and even for surfactants interacting with oppositely charged surfactants to form mixed micelles (140).

As stated previously, in a homogeneous liquid such as water the effect of temperature upon diffusivity can usually be calculated from Eq. 87. Longsworth (141) found that the ratios of 25° diffusivity to 1° diffusivity of 21 compounds varied between 2.049 and 2.178, in excellent agreement with the calculated value of 2.107. Similar results were obtained by other workers (113, 142) for a variety of solutes. The energies of activation calculated from aqueous diffusion data are generally between 4.5 and 5.0 kcal/mole. As expected from previous discussions, these values are in good agreement with the value of 4.2 kcal for viscous flow of water.

Diffusivity in Nonaqueous Solutions—Ideally the only difference between diffusivities of a substance in various solvents is due to the different viscosities of the solvents. In practice, however, this is not the case. A large solute such as benzoic acid, whose frictional resistance in water is described by Eq. 89, might be of comparable size to solvent molecules of carbon tetrachloride or benzene and thus its frictional resistance would be described by Eq. 90 in these solvents. Also, interactions that can occur in one solvent may not be significant in another. There are a number of compilations of diffusion coefficient data for many nonaqueous solvents (102, 104, 143) as well as a number of semiempirical methods of calculating D in terms of solvent and solute properties (102–105).

Table V—Diffusivities in Chloroform from Longsworth (143) at 25°

Diffusant	Partial Molal Volume, cm³/mole	D_{calc} ($\times 10^6$) $\left(\frac{4.95 \times 10^{-5}}{v^{1/3}}\right)$	f/f_0	D_{calc} ($\times 10^6$) $\left(\frac{3.36 \times 10^{-5}}{v^{1/3} f/f_0}\right)$	D_{exper} ($\times 10^6$)
Pentane	87	11.2	1.05	7.1	15.7
Hexane	103	10.5	1.06	6.6	15.0
Heptane	120	10.0	1.07	6.2	13.5
Octane	136	9.6	1.08	5.9	12.6
Decane	168	9.0	1.10	5.5	10.8
Dodecane	201	8.5	1.14	5.0	9.5
Hexadecane	265	7.8	1.22	4.3	7.8
Octadecane	297	7.4	1.25	4.0	6.9
Ercosane	330	7.1	1.27	3.7	6.6
Docosane	362	7.0	1.33	3.5	6.2
Octacosane	459	6.4	1.37	3.1	5.3
Dotriacontane	525	6.1	1.35	2.9	4.8
Methanol	25	16.9	1.00	11.3	26.1
Tetramer	100	10.7	1.00	7.1	11.3 0.2 *M*
Ethanol	35	15.1	1.00	10.1	19.5
Tetramer	100	9.5	1.00	6.4	7.9 0.5 *M*
Hexadecanol	268	7.8	1.22	4.3	7.4 Dilute
Tetramer	1072	4.9	1.00	3.2	3.7 0.4 *M*
Acetic acid	32	15.7	1.00	10.4	...
Dimer	64	12.4	1.05	7.9	14.2
Hexadecanoic acid	265	7.8	1.22	4.3	...
Dimer	530	6.1	1.31	2.9	4.5

Because we are primarily interested in diffusion through water or through membranes, the discussions will be confined to a single nonaqueous solvent, carbon tetrachloride. This solvent has been studied extensively and serves as a model nonpolar phase in which the aggregation behavior of polar solutes can be illustrated. The interactions which occur in carbon tetrachloride likely also occur in nonpolar membranes.

Table V shows the diffusivities obtained by Longsworth (143) for several substances in carbon tetrachloride, along with the values calculated by Eqs. 93 and 94 (coincidentally, carbon tetrachloride and water have nearly the same viscosity at 25° so that Eqs. 97 and 98 can be used directly). Because of the large molecular size of carbon tetrachloride (V = 315 cm³/mole) compared to water (V = 18 cm³/mole), the diffusivities of solutes whose volume is significantly less than 315 cm³/mole are not adequately described by either of these equations. Solutes of sizes comparable to that of carbon tetrachloride appear to be described by Eq. 93 to a satisfactory extent, and larger solutes have diffusivities between the value expected from Eqs. 93 and 94. Interactions between polar functional groups are extremely important in nonpolar solvents such as carbon tetrachloride or benzene. This is illustrated by some data in Table V. Hexadecanoic acid has nearly the same molar volume as hexadecane, yet, because it exists as a dimer in carbon tetrachloride, its diffusivity is similar to that of dotriconatane, a 32-carbon alkane. Because of the large dimerization constant of organic acids in nonpolar solvents, Longsworth (143) was unable to determine experimentally the diffusivity of the monomeric acid. The constancy of D with concentration suggests that no higher order aggregates of the acids are formed. This is in agreement with the spectral and partitioning data of Aveyard and Mitchell (144). Alcohols, on the other hand, have much smaller association constants, and Longsworth was able to illustrate dramatic concentration dependencies. The values he obtained for high concentration of hexadecanol suggest that tetramers and possibly higher order aggregates can be formed. This is again in agreement with spectral and partitioning data (145).

Gels—A gel consists of a complex three-dimensional network of polymeric material which imparts rigidity to the phase. The degree of solution structuring and, hence, the solution viscosity is dependent upon the chain length and the degree of cross-linking of the polymer. The presence of the gel substance can modify the observed diffusivity by either interacting with and adsorbing the diffusant on its surface or by mechanically blocking the path of the permeant. Highly concentrated gels can behave as a filter and show diffusional specificity on the basis of diffusant size. The case in which the gel is sufficiently concentrated to act as a filter will be treated in the next section as a porous barrier. In this and the next section, only aqueous gels and aqueous pores will be discussed. The discussion, however, can be applied directly to other systems.

Since in gelatinous and porous media the diffusion occurs only in the fluid phase, the relationship between diffusivity and the other parameters discussed for pure liquid phases are generally applicable. This and the following section will be concerned only with the alteration in diffusivity produced by the presence of the solid phase. The case in which the solid phase is also permeable to the diffusant can be treated as a parallel pathway in accordance with the principles described previously.

The mechanical blockage of diffusion in polymer solutions (obstruction effect) is related to the volume fraction of polymer and not to the degree of polymerization or cross-linking of the polymer. The latter parameters affect only bulk viscosity and not microscopic viscosity, *i.e.*, the viscosity of the entrapped fluid phase. Since the solute travels primarily

through the fluid phase and collides with individual polymer segments, the microscopic viscosity and the number of polymer segments present determine diffusivity. The lack of importance of bulk viscosity was verified by Taft and Malm (146), who showed that the diffusivity of salts through aqueous gelatin solutions before and after setting is unchanged. Lauffer (147) showed, by electrical analogy, that for randomly oriented long thin rods:

$$D_g = \frac{D_w}{1 - \frac{2}{3}\phi} \qquad \text{(Eq. 100)}$$

where D_w and D_g are the diffusion coefficients of the small solute in water and in an aqueous gel containing volume fraction, ϕ, of polymer, respectively. The effect of increasing diffusant size in a polymer solution over and above the effect of size on D_w is slightly to increase the collision diameter and thus the likelihood of an interaction between diffusant and polymer. In other words, small diffusants will be less affected by the presence of polymeric material than large ones.

If the effect of the polymer is strictly mechanical, there should be no specific structural effects (other than size as already mentioned); *i.e.*, polar and nonpolar molecules of the same size should be affected to the same extent. Horowitz and Fenichel (125) determined the diffusivities of a variety of solutes in an aqueous dextran gel. As would be expected from Eq. 100, they found that the ratio of diffusivities in water and in gel to be nearly independent of temperature and of solute size and polarity. Since the gels that they used contained 18.3 ± 0.9% dextran, the expected ratio based on Eq. 100 is 0.82. Their observed ratios of 0.65 ± 0.05 can be explained if it is assumed that about two volumes of water are associated with, or in some way immobilized by, each volume of polymer. This is consistent with the results of Friedman (148) for several nonelectrolytes in gelatin and agar gels, of Langdon and Thomas (149) for anion diffusion, and of Nakayama and Jackson (150) for water self-diffusion in agar gels, who found 3.4 and 3.6 g water immobilized/g dry gel. The amount of immobilized water is diminished at high salt concentration where hydration is reduced.

In addition to decreasing diffusivity by obstructing the permeant's path, a gel can decrease diffusivity by interacting with or adsorbing the diffusant. The adsorption of the diffusant onto the gel substance tends to retard diffusion in much the same way as the passage of a sample along a chromatographic column is slowed by adsorption onto the column. As in the case of the obstruction effect, the adsorption effect is dependent upon the volume fraction, ϕ, of polymer present and not on the viscosity of the solution. If K is the adsorption constant of the diffusant per unit volume of gel, and conditions are such that the gel is not saturated with diffusant, the diffusivity in the presence of an adsorbing polymer, D_P^{ads}, is given by (151):

$$D_P^{ads} = \frac{D_w}{1 + K\phi} \qquad \text{(Eq. 101)}$$

For the case in which the gel becomes saturated with diffusant:

$$D_P^{ads} = \frac{D_w}{1 + \frac{K\phi}{k}} \qquad \text{(Eq. 102)}$$

where k is a constant.

Unlike the obstruction effect, the adsorption effect is strongly dependent upon the chemical nature of the diffusant. The value of K is dependent upon the number of adsorptive sites for a given diffusant per unit volume of polymer and on the relative degree of solute attraction of the solvent and the adsorptive sites. In general, the number of sites available to a particular solute depends upon the solute's polar functional groups. For example, a particular polymer might have more sites that are capable of interacting with carboxylates than sites that can bind amines.

For a particular polymer and a series of compounds having the same functional groups (such as a homologous series), the value of K for each member will usually depend upon the solubility of that compound. Therefore, since solubility usually decreases exponentially with chain length (80), the value of K would be expected to increase exponentially as the series is ascended.

If the increase in diffusivity is the result of adsorption of the diffusant, it is possible to counteract this effect by the addition of a competitive adsorbent. Alhaigue *et al.* (152) showed that bovine serum albumin reduced the apparent diffusivity of chloramphenicol significantly and that it was possible to return the diffusivity nearly to its aqueous value by the addition of submicellar amounts of sodium lauryl sulfate.

Pores and Their Influence—As already mentioned, there is no clearcut distinction between diffusion through a dense gel and through a highly porous phase. In either case, when the regions available for diffusion are very large compared to the molecular dimensions of the diffusing species, the previous discussion can be applied directly. However, when ϕ is large, it is frequently more meaningful to characterize the membrane by its porosity, $\epsilon = 1 - \phi$, and a tortuosity factor, τ, as described previously. This section deals with the case in which the diffusing particle diameter approaches the pore diameter and free diffusion can no longer occur. While the discussion will be directed specifically to uniform right circular pores and to dense gels, the principles involved are more generally applicable.

It has been repeatedly observed that the flux of a solute through a small aqueous pore is less than the value calculated from the solute's aqueous diffusivity and the geometry of the pore. The magnitude of the discrepancy between diffusivity in the pore, D_P, and in free solution, D_f, has been shown (47–49) to be related to the spherical solute radius, r_s, and the cylindrical pore radius, r_p, by:

$$\frac{D_P}{D_f} = \left(1 - \frac{r_s}{r_p}\right)^2 \left[1 - 2.104\frac{r_s}{r_p} + 2.09\left(\frac{r_s}{r_p}\right)^3 - 0.95\left(\frac{r_s}{r_p}\right)^5\right] \qquad \text{(Eq. 103)}$$

The first portion of the Renkin (49) equation was originally proposed by Ferry (153) to account for the statistical likelihood of a particle entering a pore. The remainder of the equation was proposed by Lane and Perry (154) to represent the solvent drag on a solute molecule traversing a narrow pore. It should be noted that according to Eq. 15, $D_P \rightarrow D_f$ as $r_s/r_p \rightarrow 0$ and $D_P \rightarrow 0$ as $r_s \rightarrow r_p$. Equation 103 can be approximated successfully by:

$$\frac{D_P}{D_f} = \left(1-\frac{r_s}{r_p}\right)^4 \qquad \text{(Eq. 104)}$$

for $r_s/r_p < 0.2$. Beck and Schultz (15) found that these equations adequately described the passage of seven solutes with diverse molecular volumes through right cylindrical pores ranging from 45 to 300 Å in diameter.

It can be seen from Eqs. 103 and 104 that large particles are slowed to a much greater extent than are small ones in their passage through narrow pores. Consequently, substances that differ only slightly in free diffusion coefficients can have D_P values that are greatly different. The technique of differential dialysis capitalizes on this principle and has been used successfully to separate substances differing by only a factor of 2 in molecular weight. It has been shown (155) that the dialysis rates of glucose and sucrose across acetylated membranes (Visking) differ by a factor of 8.6 while the free diffusivities of these sugars differ by only a factor of 1.29. This and other examples of size segregation by dialysis membranes were reviewed (155, 156).

For nonspherical particles, some choice must be exercised in obtaining a value for r_s. Gary-Bobo *et al.* (157) stated that the minimum cross-sectional radius gives better correlation than the equivalent spherical radius $(3r/4\pi)^{1/3}$ for permeation through dense cellulosic membranes. Certainly, in systems that involve bulk flow of solvent, the diffusants can be expected to have a preferential orientation in the direction of flow. But in the absence of bulk flow, where the particles are randomly oriented, it is difficult to justify the use of the cylindrical radius over the equivalent spherical radius. The data (155, 156) for the passage of linear oligosaccharides across acetylated membranes (Visking) show much better correlation with spherical radius than minimum cylindrical radii. Craig and Pulley (155) also found similar dialysis rates for a linear hexose and a cyclohexose, which have very different minimum radii.

This relationship among pore radius, solute radius, and solute diffusivity enabled Solomon and his coworkers (158–160) to calculate an "equivalent pore radius" for a biological membrane. From the diffusivities of several solutes, they calculated an equivalent pore radius of 3.5–4.5 Å for erythrocyte membranes. Similarly, Stein (123) showed that several other biological barriers have equivalent pore radii between 4 and 6 Å. Interestingly, Solomon and Gary-Bobo (158) showed that lipid bilayers, treated with nystatin or amphotericin, also have equivalent pores between 4 and 6 Å in radius.

The validity of the equivalent radius calculation is strictly dependent on the assumption that all solutes permeate only through aqueous pores. It is inappropriate to discuss such a radius for a solute having any reasonable degree of hydrophobicity; consequently, the equivalent pore is of limited value in understanding the permeation of most drug molecules. Additionally, most drugs have radii similar to or greater than the equivalent pore radii of biological barriers and thus are incapable of pore transport.

Diffusivity in Amorphous Isotropic Polymers (Above T_g*)*—In polymers, it is not possible to relate diffusivity to molecular size by a simple theoretically valid equation such as Eqs. 93 and 94. Although there is a good deal of data available for diffusion coefficients in polymers, the theory is not sufficiently developed to provide a complete theoretical model. Several theoretical and empirical relationships have been developed (161–164) and are of value in certain instances. One major obstacle to quantitating diffusivity in polymers is the inability to describe adequately viscosity for a solid or semisolid. For this reason, most theories must rely upon free volume approaches. These theories emphasize the spaces within the polymer which are available for diffusion of a particle rather than the frictional resistance that the particle experiences. The available data are more consistent with the free volume approach than with a frictional resistance or viscosity approach.

In general, diffusivity in polymers is more sensitive to molecular size (or molecular weight) than in homogeneous liquids. In most polymers, it is possible to relate log D empirically to some function of molecular size. One commonly used relationship is:

$$\log D = -s_v \log v + k_v = -s_M \log M + k_M \qquad \text{(Eq. 105)}$$

where M is molecular weight; v is molecular volume; and s_v, s_M, k_v, and k_M are constants. Whereas s_v in fluid media is a constant equal to one-third [some workers (123, 164) allow s_M to vary between one-third and one-half], s_v can be as high as 4 for polymers (93, 164). Another frequently used relationship is:

$$\log D = -p_v(v) + q_v = -p_M(M) + q_M \qquad \text{(Eq. 106)}$$

where the p's and q's are constants for a particular polymer. Stein and Nir (165) prefer the latter equation because it can be justified on theoretical grounds. However, they also point out the fact that for small variations in molecular size the equations are mathematically similar.

In polymers the value of D is much more sensitive to molecular shape than in liquids. In a liquid, diffusivity decreases with deviation from a spherical shape (*cf.*, Eqs. 95 and 96); in polymers, a sphere usually has a much lower diffusion coefficient than an ellipsoid of the same volume. The chemical nature of the diffusant is of secondary importance to its size and shape for diffusion in nonpolar polymers. Functional groups become important in the situations where interaction can produce an aggregate whose geometry differs significantly from that of the monomer and where there is interaction with fixed sites on filler surfaces.

Table VI—Diffusivities of Alkanes in Some Polymers

Diffusant	Polymer				
	Polyisobutylene ($D \times 10^9$)	Natural Rubber 40° ($D \times 10^7$)	Linear Polyethylene 25°	Ethylcellulose 50° ($D \times 10^9$)	Silicone Rubber 50° ($D \times 10^6$)
Methane	—	14.5 (171)	—	—	—
Ethane	—	5.47 (171)	—	—	—
Propane	4.8 (173)	3.4 (171)	—	—	—
n-Butane	3.2 (173)	4.3 (172)	—	11.8 (178)	8.5 (178)
		3.4 (171)	—	—	—
Isobutane	1.5 (173)	2.8 (172)	—	2.1 (178)	7.7 (178)
n-Pentane	2.6 (173)	4.2 (172)	—	5.6 (178)	6.9 (178)
Isopentane	1.3 (173)	2.3 (172)	—	—	—
Neopentane	0.6 (173)	1.4 (172)	—	2.2 (178)	4.3 (178)
n-Hexane	—	—	4.4 (177)	—	—
Isohexane	—	—	2.4 (177)	—	—
3-Methylpentane	—	—	2.4 (177)	—	—
Neohexane	—	—	0.7 (177)	—	—
Cyclohexane	—	—	2.3 (177)	—	—
n-Heptane	3.0 (174)	—	—	—	—
n-Octane	—	—	1.8 (177)	—	—
n-Decane	—	—	0.7 (177)	—	—

The vast majority of studies of the diffusivity of a series of permeants through a polymer involve either gases or normal and branched paraffins. The gases provide a means of studying the effect of size without significantly altering diffusant shape. With the linear paraffins, it is possible to change only one dimension of the permeant while keeping its cross-sectional area constant. The isomeric paraffins enable one to keep volume constant and to study the effects of altering diffusant shape. Most studies using gases show good fit to equations of the form of Eq. 105 or, more often, Eq. 106.

The importance of shape on diffusion in polymers is illustrated by the many studies (93, 161, 166–179) on diffusion of normal isomeric alkane vapors in various polymers. The following general trend in diffusivities is observed (see Table VI): methyl > ethyl > propyl ≥ butyl ≥ pentyl ≥ octyl > isobutyl ≥ isopentyl > neopentyl. In other words, after a minimum chain length is reached (propyl), the addition of a methyl group to extend a linear chain has only a slight effect on D. However, the addition of branched methyl groups tends to reduce the diffusion coefficient significantly.

It is obvious then that neither mass nor molecular volume alone can be satisfactorily correlated with diffusivity for asymmetric solutes in polymers. Michaels and Bixler (179) found that, for purposes of correlation with Eq. 106, the square root of the ratio of molecular volume to the maximum linear dimension of the molecule can be used with satisfactory results. Another means of calculating the effective diameter, d, is to take the diameter of the smallest circle through which the molecule can pass.

The use of d instead of v is frequently less necessary when the correlation is being made by Eq. 105 rather than Eq. 106. This is because log v (or log M) is less sensitive to changes in v or M as these parameters increase. In other words, adding a methyl group to a long chain has only a small effect on log v and no effect on d.

Crank and Park (180) and Park (181, 182) found excellent correlation between log D and v for halomethanes. In their studies, the more nonspherical haloethanes and especially the halopropanes showed significantly higher values of D than would be expected on a volume basis. The use of the effective diameter improved the correlation somewhat, but best fit was obtained by fitting log D to an empirical function of v and d.

While it is evident that the above studies cannot be described by a single correlative equation, the following generalizations appear to be prevalent in these and other systems: *(a)* diffusivity decreases with molecular size; *(b)* diffusivity becomes less sensitive to size as size is increased (this is especially true for increases in length such as accompany the extension of a homologous series); and *(c)* branched compounds have lower diffusivities than their linear isomers. These findings, as well as the commonly observed temperature and concentration dependencies of D, are all consistent with the free volume theory of Eyring and its many modifications. Basically, these theories relate D to the size and shape of preexisting cavities in the polymer. The partial alignment of polymer chains would result in greater number of cylindrical holes than spherical ones and, thus, can explain the observed shape dependencies of diffusivity.

The size dependencies observed presumably result from the fact that there are a larger number of holes capable of containing a small molecule than there are holes that can accommodate a large penetrant. The different values of s_v, s_M, *etc.*, are thus the result of different distributions of hole sizes in different polymers. A complete detailed discussion of the commonly used free volume theories can be found in Ref. 161 (chap. 4).

In most instances of diffusion in polymers, the effect of temperature is given by an equation identical in form to Eq. 35, thus:

$$D = D_0 e^{-Ea/RT} \qquad \text{(Eq. 107)}$$

where D_0 and Ea are constants. The value of Ea, the energy of activation for diffusion, tends to increase linearly with diffusant size (178) until a plateau value which is characteristic of the polymer is

reached. This was nicely illustrated by Auerbach *et al.* (174) who showed that the energies of octadecane and hexatriconatane are nearly equal in polyisobutylene. This type of behavior was interpreted by Meares (178) as indicating that small solutes can diffuse without the complete rotation of the chain segments which is necessary for diffusion of larger penetrants. The independence of *Ea* from diffusant size is similar to the situation found in water and other liquids. However, *Ea* is much larger for polymers than for liquids. Values commonly range from 7 to 20 kcal/mole. As the temperature of an amorphous polymer is lowered below its glass transition point, crystallites begin to form. Since these crystallites are for all practical purposes unavailable for diffusion, they act as impermeable inclusions in the polymeric diffusional field.

Diffusion coefficients in polymers are almost invariably related to diffusant concentration by:

$$D = D_{c=0}e^{AC} \qquad \text{(Eq. 108)}$$

where A is a constant at any given temperature in a particular polymer. This equation is frequently found to be valid over several orders of magnitude of D. Aitken and Barrer (171) and others observed that A is nearly constant and independent of size for most solutes. Deviations from Eq. 108 will, of course, occur if there is a tendency for the diffusant to self-associate. Certain molecules such as fatty acids and alcohols can aggregate in nonpolar membranes just as they might in nonpolar solvents. Auerbach *et al.* (174) found the following diffusivities in polyisobutylene at 100°: octadecane, 1.97×10^{-7}; octadecanol, 1.56×10^{-7}; octadecanoic acid, 0.74×10^{-7}; and octadecanoyl octadecanoate, 0.46×10^{-7} cm^2/sec. These values have been interpreted as indicating that stearic acid exists almost exclusively as a dimer in the medium. They further suggest a weak association of octadecanol.

If the diffusant is present in sufficient concentration (>1%), it can dilute or solvate the polymer, expanding and "loosening" the polymer matrix, with the swelling producing secondary effects on the permeant's diffusivity, leading to significant deviation from Eq. 108. This effect was recently discussed by Schultz and Asunmaa (183) and will not be pursued here[13].

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* Present address: College of Pharmacy, University of Michigan, Ann Arbor, Mich.
x To whom inquiries should be directed.

Inhibition of Central Pressor Effects of Angiotensin I and II

THOMAS A. SOLOMON*, ICILIO CAVERO‡, and JOSEPH P. BUCKLEY ×

Abstract □ Two peptides which naturally occur in the venom of *Bothrops jararaca* were studied for their effects on the centrally mediated pressor responses of angiotensin I and II in α-chloralose-anesthetized cats utilizing the lateral ventricular perfusion technique. Intraventricular administration of either the pentapeptide or the nonapeptide attenuated the central pressor effects elicited by both angiotensin I and II. This action appeared to be specific to the CNS because administration of the pentapeptide or the nonapeptide peripherally did not antagonize angiotensin II but did inhibit angiotensin I. The inhibitors did not significantly affect ganglionic or neuronal transmission in nictitating membrane studies in cats and did not alter the parasympathetic components since there was no shift in the frequency-response curve obtained by vagal stimulation. In addition, they did not produce significant alterations in the blood pressure responses to acetylcholine, epinephrine, or bilateral carotid occlusion. Therefore, inhibition of the centrally elicited pressor effect of angiotensin II by these peptides apparently is selective to CNS receptor sites.

Keyphrases □ *Bothrops jararaca* venom peptides—effect on central pressor effects of angiotensin I and II □ Pyrrolidone carboxylic acid-L-lysyl-L-tryptophyl-L-alanyl-L-proline and pyrrolididone carboxylic acid-L-tryptophyl-L-prolyl-L-arginyl-L-prolyl-L-glutaminyl-L-isoleucyl-L-prolyl-L-proline—studied as inhibitors of central pressor effects of angiotensin I and II, anesthetized cat □ Angiotensin I and II—inhibition of central pressor effects by pentapeptide and nonapeptide of *Bothrops jararaca* venom, anesthetized cat □ Pressor responses—angiotensin I and II effects inhibited by pentapeptide and nonapeptide from *Bothrops jararaca* venom, anesthetized cat

In 1961, Bickerton and Buckley (1) presented evidence that angiotensin II exerted effects on the central nervous system (CNS). Utilizing the dog cross-circulation preparation, they showed that administration of this octapeptide into the vascularly isolated, neurally intact head of the recipient resulted in a pressor effect in the trunk. This action of angiotensin II has been confirmed (2–11) and shown to be mainly mediated *via* a central augmentation of sympathetic neuronal tone to the vasculature (1, 12). It has been suggested that this effect of angiotensin II is important in the central control of the cardiovascular system (13) as well as in the development of cardiovascular hypertensive disease (14). If this is true, then a possible mechanism for counteracting this pathological condition would be to block the renin-angiotensin system in the CNS. One way of achieving this could be to inhibit the synthesis of angiotensin II from angiotensin I.

Several peptides isolated from a pharmacologically active peptide fraction from *Bothrops jararaca* venom (15) have been reported to inhibit the conversion of angiotensin I to angiotensin II (16–20). Two of these peptides, a pentapeptide (pyrrolidone carboxylic acid-L-lysyl-L-tryptophyl-L-alanyl-L-proline[1]) and a nonapeptide (pyrrolididone carboxylic acid-L-tryptophyl-L-prolyl-L-arginyl-L-prolyl-L-glutaminyl-L-isoleucyl-L-prolyl-L-proline[2]), were studied to determine their effects on the centrally induced pressor activities of angiotensin I and II.

METHODS

Autonomic Function Studies—Adult cats, weighing 2.2–3.5 kg, were anesthetized with purified α-chloralose (70 mg/kg iv). Blood pressure was monitored from a femoral artery, and the ipsilateral femoral vein was catheterized for the administration of the test compounds. The left and right common carotid arteries were isolated so that the bilateral carotid occlusion reflex could be obtained. The right cervical vagus was isolated for supramaximal stimulation (v = 3–4.5, duration = 1 msec, frequency = 1.5–12 Hz, for 15 sec) by means of bipolar electrodes, and decreases in heart rate were recorded. The preganglionic sympathetic trunk to the nictitating membrane was also isolated for supramaximal stimulation in the same manner, and the developed nictitating membrane tension was measured with a force-displacement transducer. Test doses of angiotensin I and II (0.5 and 2.0 μg/kg), epinephrine (1.0 μg/kg), and acetylcholine (0.5 μg/kg) were administered intravenously prior to and during intravenous infusion of the pentapeptide or the nonapeptide (100 or 500 μg/kg/min). The inhibitors of the converting enzyme were infused for 15 min prior to administering the other compounds or stimulating the neurons, and infusion was continued for approximately 110 min. A minimum of 10 min was permitted between procedures. Only one inhibitor was administered per animal. The mean blood pressure equals diastolic pressure plus one-third the pulse pressure.

Perfused Cat Lateral Ventricle Preparation—Adult cats of either sex, weighing 2–3 kg, were anesthetized and prepared for blood pressure monitoring as already described. A tracheotomy was performed and a tracheal catheter was inserted for the establishment of artificial respiration. The animal was then affixed into a stereotaxic instrument[3], and the calvarium was surgically exposed along the sagittal suture line. A small hole was made in the skull above the right lateral ventricle and an unbeveled 22-gauge stainless steel needle, approximately 35 mm in length, was stereotaxically lowered into the ventricle according to the coordinates described by Snyder and Niemer (21). The coordinates used were: frontal, 15 mm; horizontal, 6.75 mm; and lateral, 2.5 mm. The cannula was affixed to the skull with dental acrylic cement, and the cerebral ventricles were perfused with artificial cerebral spinal fluid (22) after the method of Bhattacharya and Feldberg (23). The cerebral spinal fluid entered the lateral ventricle and passed through the third ventricle, the aqueduct of Sylvius, and the fourth ventricle and drained through a catheter inserted into the cisterna magna. The perfusion rate was kept constant at 0.1 ml/min by means of a pump[4] connected by a polyethylene tubing to a three-way stopcock placed in the stainless steel catheter. The artificial cerebral spinal fluid was maintained at 37° by passing the tubing through a heated water jacket. Injections of angioten-

[1] SQ 20,475.
[2] SQ 20,881.
[3] Trent H. Wells, Jr.
[4] Harvard Petti-pump.

Table I—Effects of the Pentapeptide and the Nonapeptide, Administered Intravenously, on Mean Blood Pressure and Blood Pressure Responses Induced by Bilateral Carotid Occlusion and Intravenously Administered Epinephrine and Acetylcholine in α-Chloralose-Anesthetized Cats[a]

	Inhibitor	Dose of Inhibitor, μg/kg/min		
		Control (n = 8)	100 (n = 4)	500 (n = 4)
Mean blood pressure, mm Hg[b]	Pentapeptide	113.3 ± 7.2	119.4 ± 6.1	112.5 ± 11.6
	Nonapeptide	106.3 ± 10.4	112.5 ± 8.6	121.6 ± 13.1
		Changes in Mean Blood Pressure, mm Hg[b]		
Epinephrine, 1.0 μg/kg	Pentapeptide	68.1 ± 7.3	61.7 ± 9.4	63.7 ± 12.5
	Nonapeptide	61.2 ± 9.1	62.0 ± 10.3	61.9 ± 8.3
Acetylcholine, 0.5 μg/kg	Pentapeptide	−44.5 ± 9.3	−46.1 ± 13.8	−45.5 ± 7.0
	Nonapeptide	−49.5 ± 5.4	−51.0 ± 5.3	−47.5 ± 6.9
Bilateral carotid occlusion	Pentapeptide	36.1 ± 5.2	35.4 ± 4.8	38.1 ± 7.3
	Nonapeptide	38.2 ± 7.1	41.4 ± 8.1	42.5 ± 10.3

[a] No significant differences. [b] $X \pm SEM$.

Table II—Mean Percent Inhibition of Pressor Responses of Angiotensin I and II by the Pentapeptide and Nonapeptide[a]

	Route of Angiotensin	Route of Pentapeptide	Dose of Pentapeptide, μg/kg/min	
			100	500
Angiotensin I	Intravenous	Intravenous	41.5	63.8
	Intraventricular	Intraventricular	58.8	74.2
Angiotensin II	Intravenous	Intravenous	4.9	9.6
	Intraventricular	Intraventricular	46.9	72.1
	Route of Angiotensin	Route of Nonapeptide	Dose of Nonapeptide, μg/kg/min	
			100	500
Angiotensin I	Intravenous	Intravenous	60.4	93.7
	Intraventricular	Intraventricular	77.6[b]	92.0[b]
Angiotensin II	Intravenous	Intravenous	3.2	11.8
	Intraventricular	Intraventricular	77.5[b]	88.2[b]

[a] All values are expressed as percent change from control. [b] Inhibitory effects significantly greater than that produced by the pentapeptide ($p < 0.05$) as determined by analysis of variance.

sin I or II (0.5–2.0 μg/kg) were made through the stopcock, without interruption of the perfusion, prior to and during the intraventricular perfusion of the pentapeptide or nonapeptide (100 or 500 μg/kg/min). The inhibitors of the converting enzyme were infused for 15 min prior to administering angiotensin I and II, and infusion was continued for approximately 90 min. A minimum of 15 min was permitted between treatments, and only one inhibitor was administered per animal.

Drugs and Chemicals—Angiotensin I[5], 94% pure; angiotensin II[6]; and the pentapeptide (pyrrolidone carboxylic acid-L-lysyl-L-tryptophyl-L-alanyl-L-proline) and the nonapeptide (pyrrolididone carboxylic acid-L-tryptophyl-L-prolyl-L-arginyl-L-prolyl-L-glutaminyl-L-isoleucyl-L-prolyl-L-proline) were dissolved in artificial cerebral spinal fluid for intraventricular administration and in saline for intravenous administration.

RESULTS

Table I summarizes the effects of intravenous administration of the enzyme inhibitors on blood pressure and the blood pressure responses induced by bilateral carotid occlusion and intravenous administration of epinephrine and acetylcholine. Neither the pentapeptide nor the nonapeptide in doses of 100 and 500 μg/kg/min altered the mean blood pressure of α-chloralose-anesthetized cats. They also had no effect on the bilateral carotid occlusion pressor reflex; the decrease in heart rate induced by vagal stimulation at frequencies of 1.5, 3.0, 6.0, and 12.0 Hz; the increased tension of the nictitating membrane due to stimulation of the preganglionic sympathetic trunk; the pressor response to epinephrine; or the depressor response to acetylcholine.

The intravenous infusion of either the pentapeptide or the nonapeptide produced significant inhibitory effects on the pressor responses to intravenously administered angiotensin I but had little effect on hypertensive responses to angiotensin II, as determined by analysis of variance. Doses of 100 and 500 μg/kg/min of the pentapeptide significantly ($p < 0.05$) attenuated the dose-response curve to angiotensin I, 41.5 and 63.8%, respectively (Fig. 1); the nonapeptide produced 60.4 and 93.7% inhibition ($p < 0.01$) in the same doses (Fig. 2). The dose required to produce approximately 60% inhibition of the angiotensin I pressor response was five times greater for the pentapeptide than for the nonapeptide. This observation is in accord with the findings reported by Schaeffer and Evans[7].

Perfused Cat Lateral Ventricle Preparation—The pentapeptide, 100 μg/kg/min, infused into the lateral ventricles did not alter the mean blood pressure significantly (control, $\bar{X} \pm SEM$, 102.5 ± 10.6 mm Hg; treated, 108.8 ± 10.2 mm Hg) but there was a significant increase ($p < 0.05$) in mean blood pressure to 123.8 ± 9.2 mm Hg with 500 μg/kg/min. Intraventricular administration of both doses of nonapeptide caused significant pressor effects ($p < 0.05$) (control, 109.4 ± 7.0; 100 μg/kg/min, 125.0 ± 7.2 mm Hg; 500 μg/kg/min, 130.6 ± 8.5 mm Hg), but these increases in mean blood pressure by the nonapeptide were not significantly greater than those resulting from intraventricular infusion of the pentapeptide.

Intraventricular administration of the converting enzyme in-

[5] Schwarz-Mann, Orangeburg, N.Y.
[6] Hypertensin, Ciba-Geigy.

[7] T. Schaeffer and D. B. Evans, Squibb Institute for Medical Research, personal communication.

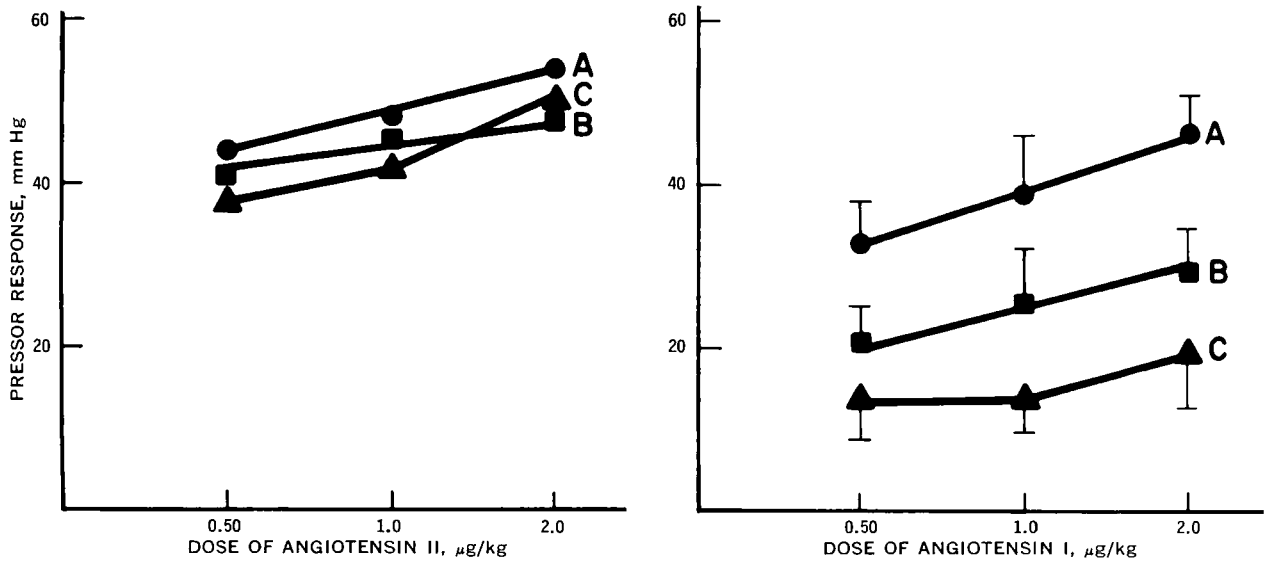

Figure 1—*Effects of the pentapeptide on the pressor response to angiotensin I and II, administered intravenously to an α-chloralose-anesthetized cat. Key: A, control,* n = *8; B, pentapeptide, intravenous, 100 μg/kg/min,* n = *4; and C, pentapeptide intravenous, 500 μg/kg/min,* n = *4. Significant differences by analysis of variance: angiotensin I, A and B,* $p < 0.05$; *A and C,* $p < 0.05$; *and angiotensin II, A and B, not significant; A and C, not significant. Vertical bars = standard error of the mean.*

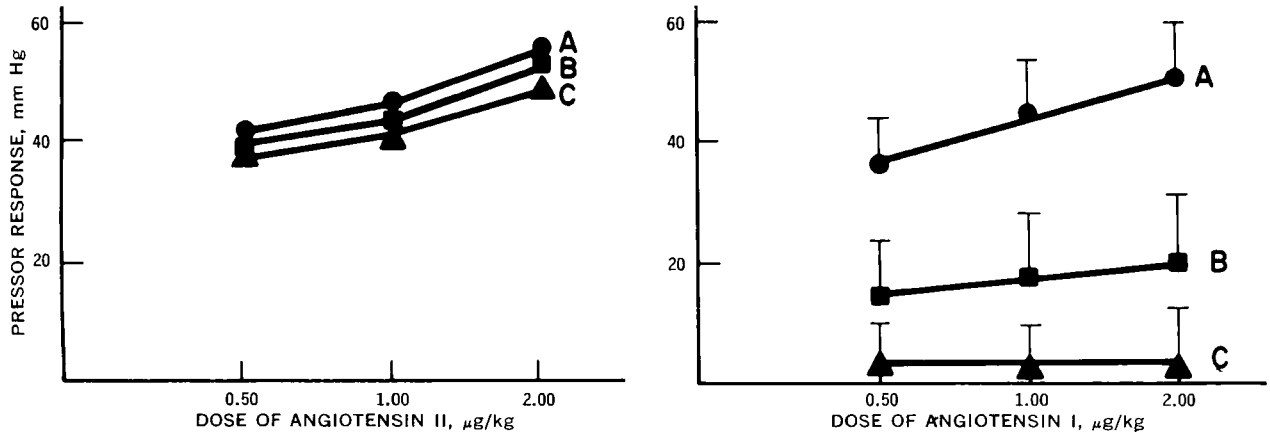

Figure 2—*Effects of the nonapeptide on the pressor response of angiotensin I and II, administered intravenously to an α-chloralose-anesthetized cat. Key: A, control,* n = *8; B, nonapeptide, intravenous, 100 μg/kg/min,* n = *4; and C, nonapeptide, intravenous, 500 μg/kg/min,* n = *4. Significant differences by analysis of variance: angiotensin I, A and B,* $p < 0.01$; *A and C,* $p < 0.01$; *and angiotensin II, A and B, not significant; A and C, not significant. Vertical bars = standard error of the mean.*

hibitors produced qualitatively similar effects on the pressor activity of angiotensin I observed in the peripheral studies. However, inhibition of angiotensin II, which was not seen on intravenous administration, was also observed. Figure 3 summarizes the effects of the pentapeptide and nonapeptide on the centrally mediated pressor response of angiotensin I. As in the peripheral studies, the attenuation of the nonapeptide was greater than that produced by the pentapeptide. The intraventricular infusion of 100 μg/kg/min of the nonapeptide inhibited the angiotensin I pressor response an average of 77.6% while the pentapeptide attenuated this response by only 58.8%; the 500-μg/kg/min dose produced a 92.0 and 74.2% inhibition, respectively. When each pair of means was subjected to an unpaired *t* test for the difference between two means, they were shown to be significant at the $p < 0.05$ level (Table II).

The centrally induced hypertensive effect of angiotensin II was also antagonized by the intraventricular perfusion of the converting enzyme inhibitors (Fig. 4). The pentapeptide decreased the activity of angiotensin II by an average of 46.9% at a dose of 100 μg/kg/min and of 72.1% at 500 μg/kg/min. Intraventricular administration of the nonapeptide had greater inhibitory effects than the pentapeptide, which is similar to the data obtained in the peripheral studies. The lower dose of the nonapeptide attenuated the pressor effect to intraventricularly administered angiotensin II 77.5% while the higher dose decreased this response by 88.2%. The inhibition produced by the nonapeptide was significantly greater than the pentapeptide at both dose levels ($p < 0.05$).

DISCUSSION

The two peptides, which have been reported to inhibit the angiotensin-converting enzyme (16–20), demonstrated a selective activity in the CNS. The data obtained with the pentapeptide and the nonapeptide intravenously agree with those reported previously (16–20); however, both peptides also inhibited the pressor response to centrally administered angiotensin II. The pressor activity induced by the intraventricular infusion of angiotensin II was antagonized to approximately the same degree as that produced by angiotensin I. Although there was a quantitative difference in the inhibition of angiotensin I by the peptides depending on the route of administration, it seems unlikely that this could account for the fact that the activity of angiotensin II is also attenuated by the converting enzyme inhibitors. Therefore, although the converting enzyme inhibitors have not been reported to have any effects other than blocking conversion of angiotensin I to angiotensin II, the data obtained in these experiments indicate that the activity of these compounds may be more complex. If angiotensin I is an active peptide and possibly important in the development of essential hypertension (24), then the two inhibi-

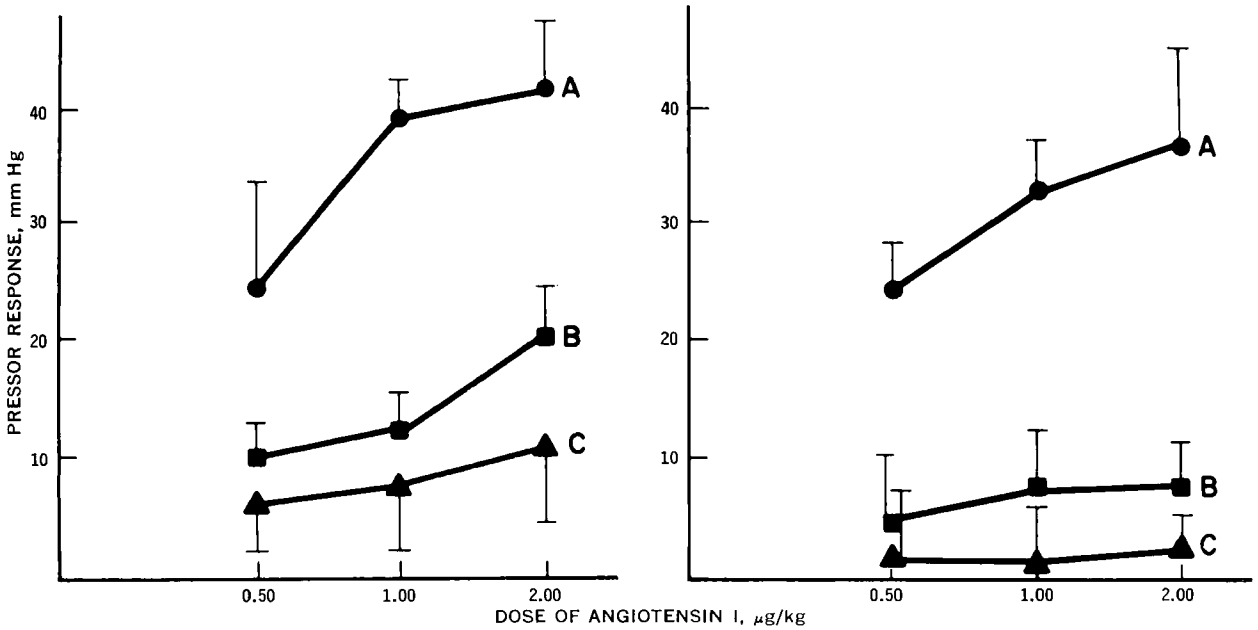

Figure 3—*Effects of intraventricular administration of the pentapeptide (left) and the nonapeptide (right) on the pressor response to intraventricular administration of angiotensin I in α-chloralose-anesthetized cats. Key: A, control,* n = *8; B, inhibitor, 100 μg/kg/min,* n = *4; and C, inhibitor, 500 μg/kg/min,* n = *4. Significant differences by analysis of variance: pentapeptide, A and B,* p < *0.01; A and C,* p < *0.01; and nonapeptide, A and B,* p < *0.05, A and C,* p < *0.01. Vertical bars = standard error of the mean.*

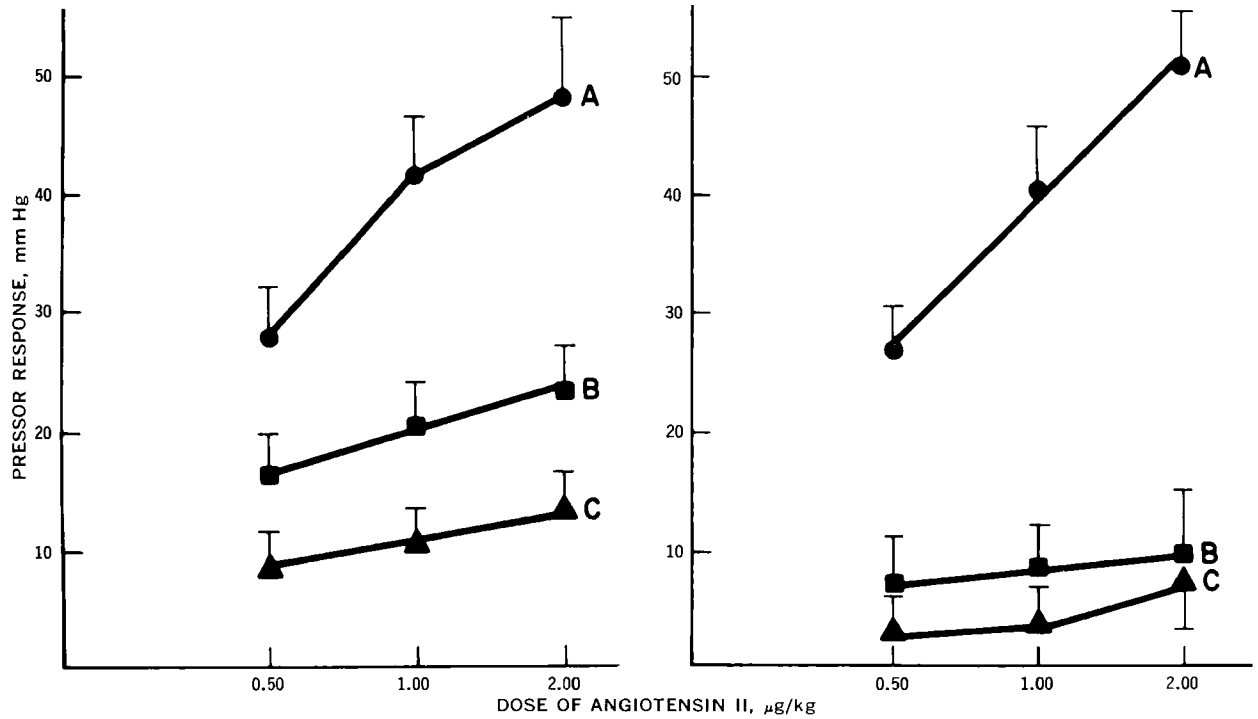

Figure 4—*Effects of intraventricular administration of the pentapeptide (left) and the nonapeptide (right) on the pressor response to intraventricular administration of angiotensin II in α-chloralose-anesthetized cats. Key: A, control,* n = *8; B, inhibitor, 100 μg/kg/min,* n = *4; and C, inhibitor, 500 μg/kg/min,* n = *4. Significant differences by analysis of variance: pentapeptide, A and B,* p < *0.05; A and C,* p < *0.01; and nonapeptide, A and B,* p < *0.01; A and C,* p < *0.01. Vertical bars = standard error of the mean.*

tors of the converting enzyme could be acting by inhibition of the "receptors" for angiotensin I and angiotensin II. If angiotensin I is, in fact, inactive, then they could be inhibiting the converting enzyme plus blocking the receptor site for angiotensin II. The antagonists had no effect on mean blood pressure, vagal stimulation, stimulation of the innervation to the nictitating membrane, the depressor response to acetylcholine, or the pressor response to bilateral carotid occlusion or epinephrine.

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* Present address: Laboratory of Cardiodynamics, School of Medicine, Johns Hopkins University, Baltimore, MD 21205

‡ Present address: Department of Experimental Medicine, F. Hoffmann-La Roche Co., Ltd., CH 4002 Basel, Switzerland.

ˣ To whom inquiries should be directed. Present address: Department of Pharmacology, College of Pharmacy, University of Houston, Houston, TX 77004

Controlled Drug Release from Polymeric Delivery Devices II: Differentiation between Partition-Controlled and Matrix-Controlled Drug Release Mechanisms

YIE W. CHIENˣ **and HOWARD J. LAMBERT**

Abstract □ The drug release pattern of micronized ethynodiol diacetate from silicone devices was thoroughly investigated in polyethylene glycol-containing elution media with a wide range of solubility and partition properties. When high drug solubility was maintained, the drug release pattern followed a $Q - t^{1/2}$ relationship (matrix controlled). Under this matrix-controlled process, the drug release profiles were independent of the variation in partition coefficient magnitude and insensitive to the change in solubility parameters. As the drug solubility in the elution medium was decreased, the drug release process shifted from matrix controlled to partition controlled, and a $Q - t$ (zero-order) relationship was observed. The drug release profile was then a function of the partition coefficient of drug from the polymer matrix to the elution medium. A transition phase was also seen between these two processes. Matrix-controlled and partition-controlled drug release processes were analyzed theoretically. The experimental rates of drug release were in perfect agreement with the values calculated from the theoretical model.

Keyphrases □ Drug release, controlled—differentiation between partition-controlled and matrix-controlled release mechanisms, ethynodiol diacetate from silicone devices in polyethylene glycol 400 media □ Permeation, drug—ethynodiol diacetate from silicone devices in polyethylene glycol 400 media, differentiation between partition- and matrix-controlled release mechanisms □ Ethynodiol diacetate—release from silicone devices in polyethylene glycol 400 media, matrix- and partition-controlled release mechanisms □ Silicone devices—release of ethynodiol diacetate

An *in vitro* drug release system, which is simple in construction and allows rapid characterization of the drug release mechanism, was introduced previously (1). The rate of drug release from silicone devices measured in such a system was found to follow current theoretical models (2–9). The application of such methodology allowed characterization of the mechanism and rate of drug release. In the studies

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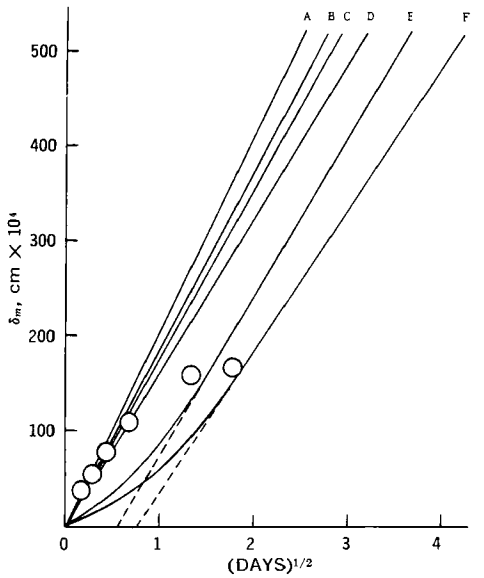

Figure 1—*Relationship between the thickness of depletion zone* (δ_m) *and the square root of time* ($t^{1/2}$) *as estimated from actual drug release data. The lines A, B, C, D, E, and F are the experimental* $\delta_m \sim t^{1/2}$ *profiles in the elution media containing 85, 80, 75, 70, 65, and 62.5% of polyethylene glycol 400, respectively. The circles are the values of* $2D_m\delta_D/KD_s$ *(calculated from the parameters of* D_m, δ_D, K, *and* D_s) *for the corresponding media used.*

(1) reported earlier, the experimental conditions were designed such that matrix-controlled drug release profiles were obtained.

Roseman and Higuchi (4) proposed that under certain conditions the rate of diffusion from the surface of the matrix to the surrounding bulk solution makes a significant contribution to the total diffusional process. Recently, Haleblian *et al.* (6) also suggested the possibility that the rate of solute transfer across the matrix–solution interface may control the release. Both reports, therefore, pointed out the possible existence of a partition-controlled model of drug release flux. Unfortunately, no experimental observations pertinent to this model were reported. By using the same drug release system developed earlier (1), the studies have been extended to the low partition coefficient region to gain an insight into the partition-controlled drug release mechanism. A completely different pattern of drug release was discovered. This paper reports these new observations and provides a theoretical analysis of the differentiation between partition-controlled and matrix-controlled drug release mechanisms.

EXPERIMENTAL

The apparatus for drug release studies, the drug-impregnated silicone devices, the polyethylene glycol 400–water cosolvent system for drug elution, and the analysis of drug samples utilized in the present investigation were essentially the same as those reported previously (1).

One additional study involved the determination of partition coefficients. A ring-shaped silicone device containing no drug was rotated in the same manner as those for drug release studies in 150 ml of elution medium containing a fixed concentration of ethynodiol diacetate at 37° for 24 hr. The drug concentration before and after the partitioning study was measured and used to calculate the partition coefficient (K) of ethynodiol diacetate from the matrix phase to the solution phase as follows:

$$K = \frac{V_p C_e}{V_s(C_i - C_e)} \qquad \text{(Eq. 1)}$$

where V_s and V_p are the volumes of elution solutions and of polymeric devices, respectively; and C_i and C_e are the initial and equilibrium drug concentrations in solution, respectively. The 24-hr period was adequate to ensure equilibrium.

For the elution media containing high volume fractions (≥60% v/v) of polyethylene glycol 400, the following relationship may also be used to calculate the pertinent high partition coefficients (5):

$$K = \frac{C_s}{C_p} \qquad \text{(Eq. 2)}$$

where C_s and C_p are the solubilities of drug in elution solutions and in polymeric devices, respectively.

THEORETICAL ANALYSIS

The mechanisms of drug release from various polymeric matrix systems have been extensively discussed (2–4). In the present study, the thickness of the hydrodynamic diffusion layer ($\leq 70 \times 10^{-4}$ cm) was much smaller than the surface area of the matrix (33.75 cm^2) available for the diffusion of drug species. Therefore, the diffusion of drug molecules to and from the matrix across the hydrodynamic diffusion layer may be treated as one-dimensional diffusion to a plane surface (10). From earlier theoretical treatments (3, 4), the following general equations describing the release of drug from a polymeric matrix are obtained:

$$\delta_m^2 + \frac{2D_m\delta_D\delta_m}{KD_s} = \frac{4C_pD_mt}{(2A - C_s)} \qquad \text{(Eq. 3)}$$

$$Q = (A - C_s/2)\delta_m \qquad \text{(Eq. 4)}$$

where δ_m and δ_D are the thicknesses of the depletion zone and the hydrodynamic diffusion layer, respectively; D_m and D_s are the diffusivities of drug in the matrix phase and the solution phase, respectively; A is the total amount of solid drug impregnated per unit volume of polymeric matrix; C_p and C_s are the drug solubilities in the polymer and in the solution, respectively; K is the partition coefficient of the drug species from the matrix phase to the elution medium; Q is the amount of drug released per unit surface area of devices; and t is time.

For a matrix-controlled process:

$$\delta_m^2 \gg \frac{2D_m\delta_D\delta_m}{KD_s} \qquad \text{(Eq. 5)}$$

Then Eq. 3 is reduced to:

$$\delta_m^2 = \frac{4C_pD_mt}{(2A - C_s)} \qquad \text{(Eq. 6}a\text{)}$$

or:

$$\delta_m = 2\sqrt{\frac{C_pD_mt}{(2A - C_s)}} \qquad \text{(Eq. 6}b\text{)}$$

Substituting Eq. 6*b* for δ_m in Eq. 4 results in:

$$Q = \sqrt{D_m(2A - C_s)C_pt} \qquad \text{(Eq. 7)}$$

Equation 7 was analyzed extensively in the first report of this series (1). Similar equations have been developed and correlated with the experimental observations in a number of studies (2–9). In matrix-controlled drug release mechanisms, Eq. 7 was followed perfectly in all investigations that were carried out in this laboratory (1).

It is obvious that Eq. 7 will be valid only under the condition that Eq. 5 is existing experimentally. The validity of Eq. 5 may be confirmed by comparing the $\delta_m \sim t^{1/2}$ profiles (estimated from the drug release data) with the magnitudes of $2D_m\delta_D/KD_s$ (calculated from the parameters of D_m, δ_D, K, and D_s) (Fig. 1). Except for the cases where 65 and 62.5% polyethylene glycol 400

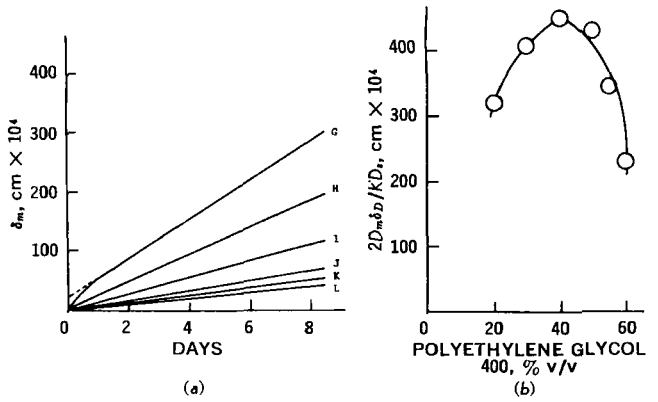

Figure 2—*Comparison of the magnitudes of* $2D_m\delta_D/KD_s$ *to those of* δ_m *in the region of low partition coefficient.* (a) *Linear relationship between the thickness of depletion zone* (δ_m) *and time* (t) *as computed from actual drug release data. The lines G, H, I, J, K, and L are the experimental* $\delta_m \sim t$ *profiles in the elution media containing 60, 55, 50, 40, 30, and 20% of polyethylene glycol 400, respectively.* (b) *Calculated values of* $2D_m\delta_D/KD_s$ *for the corresponding elution medium. The scales for* δ_m *and* $2D_m\delta_D/KD_s$ *are drawn in the same magnitude for a convenient comparison.*

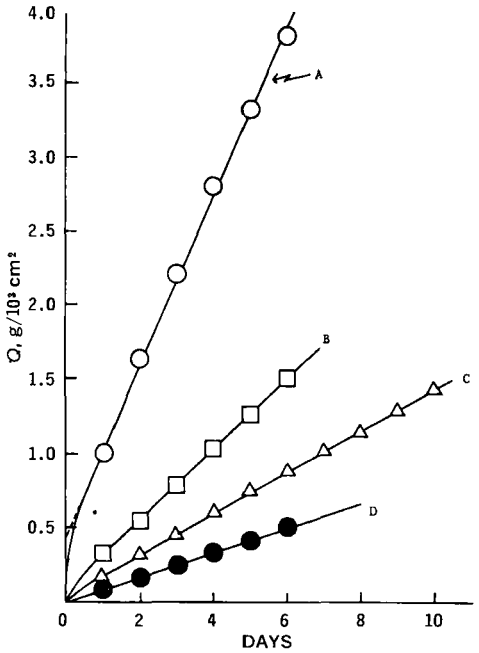

Figure 3—*Linear relationship between the cumulative amount of drug released* (Q) *from per unit area of devices and the time (days) in the elution media with partition coefficients of: (A) 0.2873, (B) 0.099, (C) 0.061, and (D) 0.036.*

were used as the elution media, all other drug release fluxes took only 1 day to satisfy Eq. 5. In the 65 and 62.5% cases, 1–2 more days was required to reach the experimental condition defined by Eq. 5. Equation 5 was apparently followed quite well in the present study.

However, situations where δ_m^2 is smaller than $2D_m\delta_D\delta_m/KD_s$ are also possible[1] when K, the partition coefficient, is very small (partition-controlled process) and/or δ_D is much larger than δ_m (diffusion layer-controlled process). The comparison made in Fig. 2 clearly demonstrates that, except for the elution medium containing 60% polyethylene glycol 400 (K = 0.287), Eq. 8 is obeyed perfectly. In the elution medium containing 60% polyethylene glycol 400, Eq. 8 is followed only before the 6th experimental day. This special case will be discussed later.

For the partition-controlled process:

$$\delta_m^2 \ll \frac{2D_m\delta_D\delta_m}{KD_s} \qquad \text{(Eq. 8)}$$

Then Eq. 3 is reduced to:

$$\frac{2D_m\delta_D\delta_m}{KD_s} = \frac{4C_pD_mt}{(2A - C_s)} \qquad \text{(Eq. 9}a\text{)}$$

or:

$$\delta_m = \frac{2KD_sC_pt}{\delta_D(2A - C_s)} \qquad \text{(Eq. 9}b\text{)}$$

Substituting Eq. 9*b* for δ_m in Eq. 4 results in:

$$Q = \frac{KD_sC_p}{\delta_D}t \qquad \text{(Eq. 10}a\text{)}$$

or:

$$Q = \frac{KD_sC_s}{\delta_D}t \qquad \text{(Eq. 10}b\text{)}$$

if the dissolution of drug species into the elution medium is a slower step than the solubilization of the solid drug into the polymer phase, *e.g.*, $C_p \gg C_s$.

It is obvious that under matrix control, the drug release profile follows a $Q - t^{1/2}$ relationship (Eq. 7) while under partition control the drug release profile shifts to follow $Q - t$ linearity (Eq. 10). According to Eq. 7, the drug solubility (C_s) in the elution medium, the partition coefficient (K) of the solution–polymer system, and the thickness of diffusion layer (δ_D) do not make any major contribution to the drug release rate. On the other hand, according to Eq. 10*b*, the parameters mentioned become highly significant in determining the magnitude of drug release fluxes.

RESULTS AND DISCUSSION

The experimental observations on matrix-controlled drug release processes were previously presented and analyzed thoroughly (1). The present investigation was devoted to partition-controlled processes and the drug release patterns were compared to those of matrix-controlled processes.

As stated earlier, the drug release studies were extended to the low polyethylene glycol 400 concentration region (≤60%) in which the magnitude of the partition coefficient is very low (≤0.287). The results on the pattern of drug release in these elution media with low partition coefficients are illustrated in Fig. 3. Obviously, a $Q - t$ relationship is obeyed perfectly, as expected from Eq. 10*b*. The drug release profiles in Fig. 3 also indicate that the magnitude of the slopes is a function of the partition coefficients of drug from the polymer phase to the elution medium. From Eq. 10*b* the slope of $Q - t$ profiles may be defined as:

$$\frac{Q}{t} = \frac{KD_sC_s}{\delta_D} \qquad \text{(Eq. 11)}$$

A linear relationship should therefore exist between the slope of the $Q - t$ profile (Q/t) and the partition coefficient (K). The data shown in Fig. 4 demonstrate this $Q/t \sim K$ linearity (bottom scale). The Q/t value for the K = 0.287 case is apparently lower than that expected from Eq. 11. This lower Q/t value is explained by the fact that the values of δ_m are close to the magnitude of $2D_m\delta_D/KD_s$ at K = 0.287 (Fig. 2). In other words, Eq. 8 is no longer well established. This deviation may indicate the approach of a transition state.

Equation 11 also points out the possible existence of another linear relationship of the rate of drug release (Q/t) to the drug solubility (C_s) in the elution medium. Experimentally, this linearity is also followed as expected in the low C_s range (Fig. 4, upper scale). As the $Q/t \sim K$ profile illustrated earlier, when the drug solubility (C_s) in the elution medium was raised from 200 to 437 μg/cm³, the $Q/t \sim C_s$ linearity was lost. As stated before, this probably indicates a transition state.

[1] T. J. Roseman, The Upjohn Co., Kalamazoo, Mich., personal communication.

Table I—Comparison of Experimental Rates of Drug Release to Theoretical Values and Their Relationship to $C_b(t)/C_s$

Polyethylene Glycol 400, %	$C_b(t)/C_s$	Rate of Drug Release, g/10⁶ cm²/day		
		$(Q/t)_T$[a]	$(Q/t)_E$[b]	$(Q/t)_T/(Q/t)_E$
20	0.183	75.5	82.6	1.09
30	0.165	103.95	109.8	1.05
40	0.146	145.2	141.3	0.97
50	0.155	237.65	245.7	1.03
55	0.187	371.31	360.0	0.97
60	0.109	1245.0	550.0	0.442
62.5	0.116	2209.3	728.8[c]	0.330

[a] Theoretical rate of drug release, $(Q/t)_T$, is estimated from Eq. 11. [b] $(Q/t)_E$, the experimentally observed rate of drug release. [c] Rate of drug release calculated from the initial 4-day drug release data.

If Eq. 11 is really the theoretical model defining the partition-controlled drug release mechanisms, then $(Q/t)_T$, the theoretical rate of drug release, calculated from the parameters of K, C_s, D_s, and δ_D following Eq. 11 should be comparable in magnitude to the values measured experimentally, $(Q/t)_E$. A comparison is made in Table I. The agreement between $(Q/t)_T$ and $(Q/t)_E$ data is remarkable except for the case in which more than 60% polyethylene glycol 400 was used as the elution medium. The lower $(Q/t)_E$ values obtained in the elution media containing 60 and 62.5% polyethylene glycol 400 solutions further demonstrate the invalidity of Eq. 8 in these two cases. As illustrated in Figs. 1 and 2 for the 60% case (G in Fig. 2), Eq. 8 is valid only earlier than 5 experimental days; for the 62.5% case (F in Fig. 1), Eq. 8 is substituted by Eq. 5 after the 3rd experimental day. Obviously, the drug release fluxes in the elution medium containing 60–62.5% polyethylene glycol 400 are close to the neighborhood of a transition phase where both Eqs. 5 and 8 do not apply.

Haleblian *et al.* (6) proposed that a constant release rate would be observed if the release of drug was controlled by either the rate of drug dissolution into the matrix or the rate of solute transfer across the matrix-solution interface. They observed that the rate of drug release from devices containing micronized drug particles was four times higher than those containing macrosized drug. In the present investigations, micronized ethynodiol diacetate particles were used. Therefore, the possibility of a dissolution-controlled release mechanism may be ruled out, and the rate of partitioning of drug species across the matrix-solution interface may be considered as rate determining.

In developing Eq. 3, the necessary condition that a perfect sink

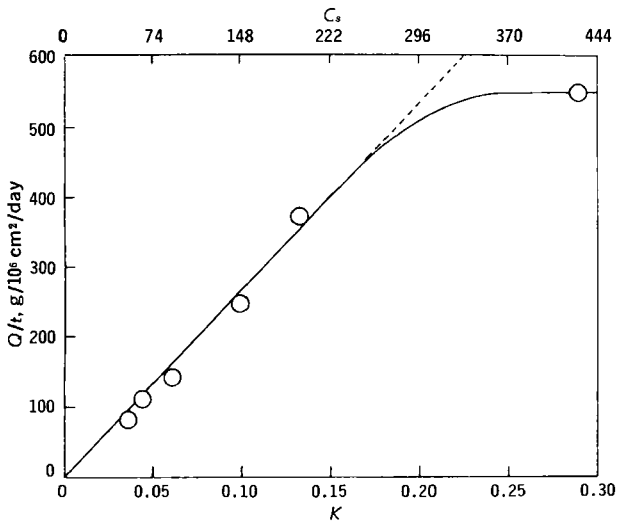

Figure 4—*Rate of drug release* (Q/t), *as a function of the partition coefficient* (K, *bottom scale*) *and the solubility* (C_s) *in the elution medium* (*upper scale*).

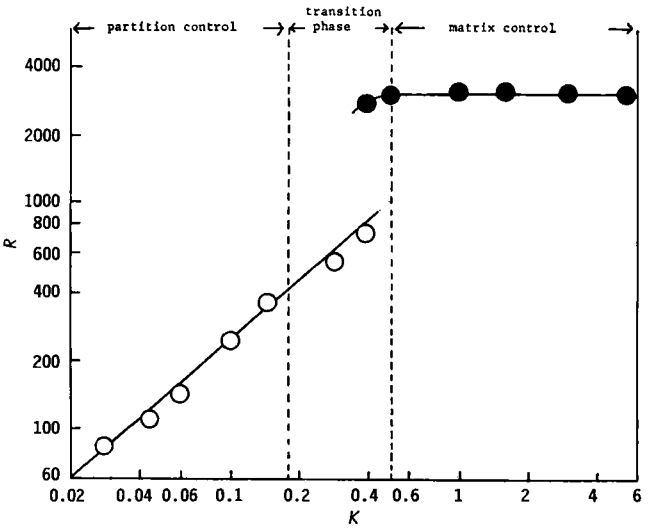

Figure 5—*Relationship between the steady-state drug release rate constant and the partition coefficient. In the region of partition control, the rate constant of drug release* (Q/t, *g/10⁶ cm²/day*) *is a linear function of the partition coefficient* (*slope = 0.88*). *In the region of matrix control, the rate constant of drug release* (Q/t$^{1/2}$, *g/10⁶ cm²/day*$^{1/2}$) *is independent of the partition coefficient* (*slope = 0*).

condition has to be maintained throughout the study was assumed (3, 4). In other words, $C_b(t)$, the drug concentration in the elution medium at a given time, has to be much smaller than C_s, the drug solubility in the elution medium. The data on $C_b(t)/C_s$ (Table I) indicate that the magnitudes of $C_b(t)$ are well below those of C_s, *i.e.*, $[C_b(t)/C_s < 0.2]$, and the requirement that $C_s \gg C_b(t)$ is satisfied. Where more than 60% polyethylene glycol 400 was used as the elution medium, the experimental rate of drug release (listed in the fourth column of Table I) reached only 33–44% of the theoretical value (calculated from Eq. 11 and shown in the third column of Table I) although a perfect sink condition, *e.g.*, $C_b(t)/C_s$ = 0.109–0.116, was maintained. This result rules out the possibility that the deviation of the drug release rate from theory might be due to the effect of nonsink conditions, *e.g.*, $C_b(t)/C_s \simeq 1$.

On the other hand, when the ratio of $C_b(t)/C_s$ becomes larger, a different type of deviation may be observed. The data in Table II demonstrate that a zero-order $(Q \sim t)$ relationship (Eq. 10b) was maintained, but the experimental Q/t values were found to be smaller than the values estimated from Eq. 11. The larger the ratio of $C_b(t)/C_s$, the greater is the deviation of experimental value from theory. The results (Tables I and II) illustrate the importance of maintaining a sink condition for all drug release studies.

The relationship of drug release fluxes in both partition-controlled and matrix-controlled processes to the partition coefficients of drug species (from the matrix to the elution medium) is analyzed further in Fig. 5. In Fig. 5, the most noticeable observa-

Table II—Deviation of Experimental Drug Release Rates from Theoretical Values as a Function of the Ratio of $C_b(t)/C_s$

Sample Renewing Speed[a], ml/day	$C_b(t)/C_s$[b]	Rate of Drug Release, g/10⁶ cm²/day		
		Theoretical[c]	Experimental	Ratio[d]
25	0.588	237.65	162.5	0.684
50	0.402	237.65	197.2	0.830
100	0.155	237.65	245.7	1.03

[a] The total volume of the elution medium is 150 ml. [b] The maximum solubility of ethynodiol diacetate in the elution medium containing 50% polyethylene glycol 400 is 156.0 μg/cm³. [c] Calculated from Eq. 11. [d] Ratio of experimental drug release rates over theoretical values.

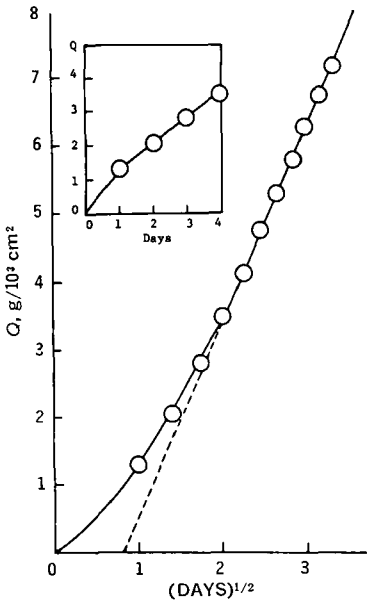

Figure 6—*Drug release profile for ethynodiol diacetate in the 62.5% polyethylene glycol 400 solution. The* Q ~ t *relationship is followed in the initial 4-day data (inserted plot) and the* Q ~ $t^{1/2}$ *relationship is followed thereafter. The slopes are 728.8* $g/10^6$ cm^2/day *and 2.79* $g/10^3$ $cm^2/day^{1/2}$, *respectively.*

tion is that steady-state drug release rates are linear with increasing partition coefficients only up to a certain point ($K \simeq 0.4$) beyond which the matrix-controlled mechanism is observed and the steady-state drug release profiles are virtually independent of variation in partition coefficient. The dependency of the drug release rate on the partition coefficients of drug from polymeric devices to the elution medium may be expressed (11) by the following relationship:

$$\text{rate constant of drug release} = R = K^{\gamma} \qquad \text{(Eq. 12)}$$

where K is the partition coefficient of the drug investigated, and γ is the slope of log $R \sim$ log K profiles. The plot in Fig. 5 indicates that γ is 0.88 for the partition-controlled process and is essentially zero for the matrix-controlled process.

Figure 5 also points out that, in between these two processes, an overlapping transition phase is seen where both conditions (Eqs. 5 and 8) do not exist and may be replaced by a new condition:

$$\delta_m^2 \approx \frac{2D_m\delta_D\delta_m}{KD_s} \qquad \text{(Eq. 13)}$$

Therefore, the general model described by Eq. 3 should be applied to define the drug release profile in the transition phase where both Eqs. 7 and 10*b* fail to give a satisfactory expression.

The drug release profile in the elution medium containing 62.5% polyethylene glycol 400 solution (with a partition coefficient of 0.387) is plotted in Fig. 6. The $Q - t$ relationship was followed in the initial 4 experimental days and the $Q - t^{1/2}$ relationship was followed thereafter. The transition point is located around the 3rd to 4th experimental day where the calculated value of $2D_m\delta_D/KD_s$ is approximately 165×10^{-4} cm (line F in Fig. 1). When δ_m is smaller than this magnitude, the $Q - t$ relationship is observed. As the value of δ_m gets larger, the $Q - t^{1/2}$ relationship becomes apparent (Figs. 1 and 6) and then overweighs the $Q - t$ relationship.

A similar relationship as that demonstrated in Fig. 5 was also reported (12) for the lag time–carbon chain length profile and was derived (13) for the steady-state flux–partition coefficient profile. The marked change in slope from the total partition-control region to the total matrix-control region observed in the present investigation was also seen in both reports (12, 13).

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[x] To whom inquiries should be directed.

Determination of Clonazepam and Flunitrazepam in Blood and Urine by Electron-Capture GLC

J. ARTHUR F. de SILVA[x], CARL V. PUGLISI, and NANCY MUNNO

Abstract □ A sensitive and specific electron-capture GLC assay was developed for the determination of clonazepam and flunitrazepam (two members of the 7-nitro-1,4-benzodiazepin-2-one class of compounds) in blood and urine. The assay for clonazepam uses flunitrazepam as a reference standard and vice versa. The assay for both compounds involves selective extraction into ether from blood or urine at pH 9.0 followed by acid hydrolysis to their respective benzophenones, which are extracted and quantitated by electron-capture GLC. Recovery from blood or urine is quantitative, and the sensitivity limits of the assay are 0.5–1.0 ng/ml of specimen analyzed. The assay was successfully applied to the disposition of clonazepam in man following single 2-mg oral doses and of flunitrazepam in the dog following intravenous and oral doses of 2 mg/kg. A differential pulse polarographic assay for the determination of the major urinary metabolites of clonazepam is also presented.

Keyphrases □ Clonazepam—electron capture GLC determination in biological specimens □ Flunitrazepam—electron-capture GLC determination in biological specimens □ Benzodiazepines, clonazepam and flunitrazepam—electron-capture GLC determination in biological specimens □ GLC, electron-capture detection—analysis, clonazepam and flunitrazepam in biological specimens

The 1,4-benzodiazepine class of compounds has shown marked anticonvulsant properties in addition to their antianxiety and muscle relaxant properties (1, 2), and these compounds are among the more widely prescribed minor tranquilizers and anticonvulsant drugs (3). The 7-nitro-1,4-benzodiazepin-2-ones (nitrazepam, clonazepam, and flunitrazepam) (4) have shown marked anticonvulsant properties in several animal species (1, 2, 5–10). Clonazepam is clinically effective in controlling minor motor seizures (petit mal) at doses of 1–2 mg/day orally (11–17), while flunitrazepam is a clinically potent hypnotic (18, 19) and anesthetic induction agent (20, 21).

Clonazepam[1] [7-nitro-5-(2-chlorophenyl)-1,3-dihydro-(2*H*)-1,4-benzodiazepin-2-(1*H*)-one, A] (Scheme I and Table I), flunitrazepam [7-nitro-5-(2-fluorophenyl)-1,3-dihydro-1-methyl-(2*H*)-1,4-benzodiazepin-2-one, I] (Scheme I and Table II), and *N*-desmethyl-flunitrazepam (Compound II in Table II) can be hydrolyzed quantitatively in acid to their benzophenones (22) (Scheme I), 2-amino-5-nitro-2′-chlorobenzophenone (Compound J in Table I), 2-methylamino-5-nitro-2′-fluorobenzophenone (Compound V in Table II), and 2-amino-5-nitro-2′-fluorobenzophenone (Compound VI in Table II), respectively. Nitrazepam[2] [7-nitro-5-phenyl-1,3-dihydro-(2*H*)-1,4-benzodiazepin-2-(1*H*)-one, G] (Scheme I and Table I) is hydrolyzed to 2-amino-5-nitrobenzophenone (Compound H in Table I and Scheme I). These benzophenones are quantitated by the highly sensitive GLC assay described.

Attempts at determining clonazepam and flunitrazepam as the intact benzodiazepin-2-ones were unsuccessful. Clonazepam gave a very broad and poorly defined peak by electron-capture GLC on the phenyl silicone phases OV-1 and OV-17, whereas flunitrazepam was eluted as a symmetrical Gaussian-shaped peak with sufficient sensitivity for quantitation in the nanogram range. However, flunitrazepam and its *N*-desmethyl metabolite (II), unlike clonazepam, are unstable in acid (Table II). Since back-extraction from an organic solvent into acid is an essential step for sample cleanup prior to electron-capture GLC analysis (23), it was necessary to convert them to their respective stable *o*-aminobenzophenones (24) by acid hydrolysis. The benzophenones of clonazepam (J) and of flunitrazepam (V) are eluted as well-resolved symmetrical peaks, using the polar phase OV-17 in the clonazepam assay and OV-225 in the flunitrazepam assay. Both compounds are quantitated in the nanogram range using the ^{63}Ni electron-capture detector.

This report describes an assay procedure for clonazepam which is also applicable with minor modifications for the determination of flunitrazepam (I), its *N*-desmethyl metabolite (II), and nitrazepam (25). The method described for the determination of clonazepam uses flunitrazepam as an internal reference standard and vice versa. Alternatively, nitrazepam can be substituted for flunitrazepam as the reference standard in the clonazepam assay, since its benzophenone (H) (Scheme I) has about the same retention time as that of V and is, therefore, resolved from J derived from clonazepam.

Studies on the biotransformation of nitrazepam (26, 27) and of clonazepam in man (28) have shown that the major pathway of elimination is by the reduction of the nitro group to the amine, which is then acetylated to the acetamide. Hydroxylation at the C-3 position also occurs, resulting in the elimination of these metabolites as their glucuronide and/or sulfate conjugates. Flunitrazepam is rapidly *N*-demethylated to yield II, the major blood metabolite in the dog, which is further metabolized by pathways analogous to that of clonazepam[3]. The 7-nitro-3-hydroxy analogs of clonazepam and flunitrazepam, which are also hydrolyzed to the same benzophenones (Scheme I), are removed by selective solvent extraction prior to acid hydrolysis to ensure the

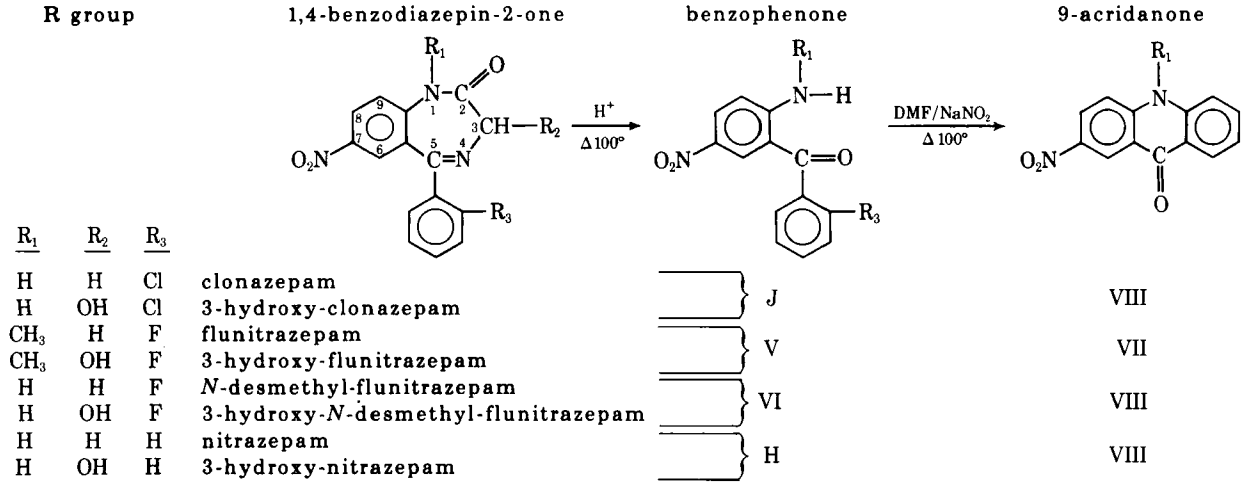
[1] Preferred USAN chemical name is (*o*-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one.

[2] Preferred USAN chemical name is 1,3-dihydro-7-nitro-5-phenyl-2*H*-1,4-benzodiazepin-2-one.

[3] M. A. Schwartz and S. Kolis, unpublished data on file, Hoffmann-La Roche Inc., Nutley, NJ 07110, 1970.

Table I—Chemical Names and Physical Properties of Clonazepam, Nitrazepam, and Their Chemical Derivatives Referred to in Scheme I and Figs. 4 and 5

Compound	Chemical Name	Molecular Weight	Melting Point
A	7-Nitro-5-(2-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-(1*H*)-one (clonazepam)	315.70	238–240°
B	7-Nitro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-(1*H*)-one hemiacetonate	361.0	159–160°
C	7-Amino-5-(2-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-(1*H*)-one	285.74	230–232°
D	7-Amino-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-(1*H*)-one	301.74	340° dec.
E	7-Acetamido-5-(2-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-(1*H*)-one	322.77	292–300°
F	7-Acetamido-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-(1*H*)-one	343.77	187–190° dec.
G	7-Nitro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-(1*H*)-one (nitrazepam)	281.0	224–226°
H	2-Amino-5-nitrobenzophenone	242.0	157–159°
J	2-Amino-5-nitro-2′-chlorobenzophenone	276.7	118–120°
K	2,5-Diamino-2′-chlorobenzophenone	246.69	30–40°



Scheme I—Chemical reactions of clonazepam, flunitrazepam, nitrazepam, and their derivatives referred to in Tables I and II

specificity of the assay for the respective parent compounds.

The methods were applied to the determination of blood levels of clonazepam in man following single 2-mg oral doses (29, 30) and of flunitrazepam in the dog following single 2-mg/kg doses by intravenous and oral routes (29).

EXPERIMENTAL

Clonazepam (A) Column—The column packing[4] was a pretested preparation containing 3% OV-17 on 60–80-mesh Gas Chrom Q packed in a U-shaped, 1.21-m (4-ft), 4-mm i.d. borosilicate glass column. The column was conditioned at 325° for 4 hr with no flow of carrier gas, followed by 12 hr at 275° with carrier flowing at 40 ml/min. This column effectively resolves the benzophenones J and V from coextracted impurities (Fig. 1) and has a useful lifespan of about 4–5 months of continuous use.

Flunitrazepam (I) Column—The column packing[4] was a pretested preparation containing 3% OV-225 on 60–80-mesh Gas Chrom Q packed in a U-shaped, 0.91-m (3-ft), 4-mm i.d. borosilicate glass column. This column was conditioned at 275 and 250° as described above. This column is preferred for the analysis of flunitrazepam (I) and its *N*-desmethyl metabolite (II), using clonazepam as the reference standard, since better resolution of their respective benzophenones V, VI, and J is achieved (Fig. 2), resulting in more precise quantitation.

Instrumental Conditions—The following conditions are common to both assays. A gas chromatograph[5] equipped with a ^{63}Ni electron-capture detector, containing a 15-mCi ^{63}Ni β-ionization source, was used. Argon–methane[6] (90:10) was used as the carrier gas, and the column head pressure was preset at 40 psig. The temperature settings were as follows: oven, 230°; injection port, 260°; and detector, 325°. The solid-state electrometer[7] input was set at 10^2, and the output attenuation was 32, giving a response of 3.2×10^{-9} amp for full-scale deflection (fsd); the chart speed was 30 in./hr, and the time constant on the 1.0-mv recorder[8] was 1 sec (fsd). The response of the ^{63}Ni electron-capture detector (operated in the pulsed dc mode) to the benzophenones J, V, and VI showed maximum sensitivity at 60 v dc at a 300-μsec pulse rate and a 10-μsec pulse width. Under these conditions, 1.0 ng of each benzophenone (V, VI, and J) gave nearly full-scale pen response (>95%) on the 1.0-mv recorder.

In Clonazepam Assay—The flow rate used was adjusted to 120 ml/min (with the detector purge gas at 20 ml/min) to obtain retention times of 4.0 min for the reference benzophenone V and of 6.5 min for the benzophenone J derived from clonazepam (Fig. 1).

In Flunitrazepam Assay—The flow rate was adjusted to 100 ml/min (with the detector purge gas at 20 ml/min) to obtain re-

[4] Applied Sciences Labs., Inc., State College, Pa.

[5] Micro-Tek, model MT-220.
[6] Matheson, oil pumped and dry.
[7] Model 8169.
[8] Honeywell, model 194.

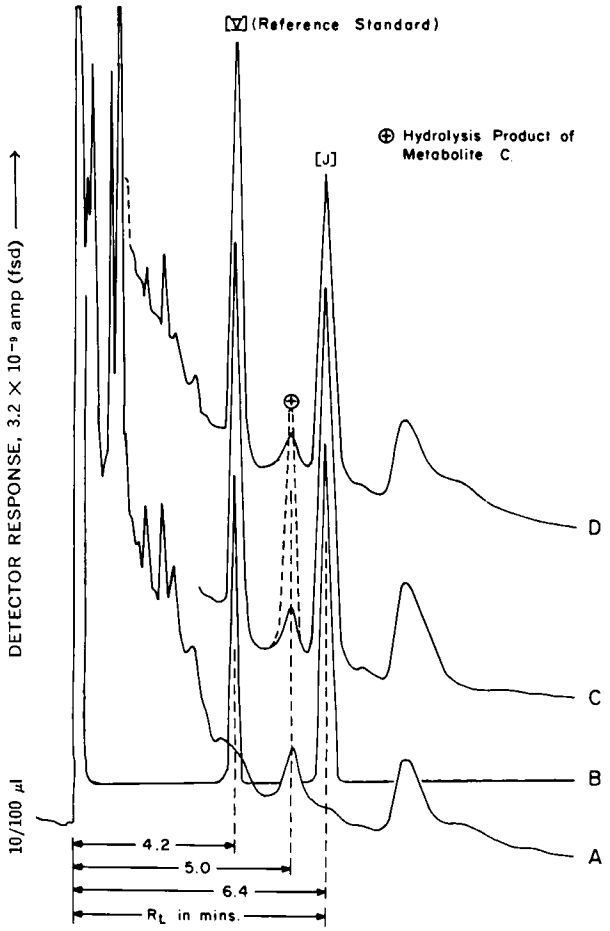


Figure 1—*Chromatograms of ether extracts of: (A) control blood, (B) authentic benzophenone standards, (C) control blood containing added clonazepam, and (D) patient blood 8 hr after 2-mg oral dose of clonazepam.*

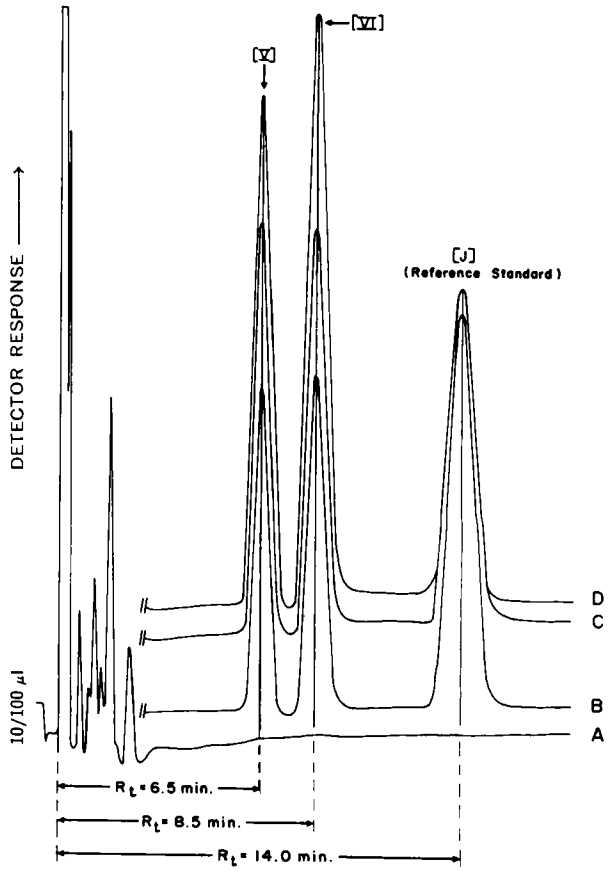


Figure 2—*Chromatograms of the ether extracts of: (A) control dog blood, (B) authentic benzophenone standards, (C) internal standards of I, II, and clonazepam (A) recovered from blood as the benzophenones after acid hydrolysis, and (D) dog blood after 2 mg/kg iv of flunitrazepam.*

tention times of 14 min for the reference benzophenone J and of 6.5 and 8.5 min for the benzophenones V and VI derived from flunitrazepam (I) and its *N*-desmethyl metabolite (II), respectively (Fig. 2). Under these conditions, the minimum detectable amount of clonazepam (A), flunitrazepam (I), and its *N*-desmethyl metabolite (II) is 0.5–1.0 ng of each/ml of blood or urine using a 2-ml specimen per analysis.

Preparation of Standard Solutions of Benzodiazepin-2-ones—The respective 7-nitro-1,4-benzodiazepin-2-ones [clonazepam (A), flunitrazepam (I), and its *N*-desmethyl metabolite (II)] that are required as internal analytical standards are listed in Tables I and II, respectively.

Weigh 10.0 mg each of clonazepam (A), flunitrazepam (I), and its *N*-desmethyl metabolite (II) (free base) into separate 10-ml volumetric flasks. Dissolve, using 1.0 ml of absolute ethanol followed by 1 ml of acetone, and then dilute to volume with 20% acetone–hexane. These stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions by suitable dilutions in 20% acetone–*n*-hexane, 100-µl aliquots of which are added to blood or urine as internal standards.

For the clonazepam assay, standard solutions containing 2.0–10.0 ng of clonazepam, each containing 10.0 ng of flunitrazepam (as the reference standard)/100 µl of 20% acetone–*n*-hexane solution, are prepared.

For the flunitrazepam assay, standard solutions containing 2.0–10.0 ng each of flunitrazepam and of its *N*-desmethyl metabolite (II), each containing 10.0 ng of clonazepam (as the reference standard)/100 µl of 20% acetone–*n*-hexane solution, are similarly prepared.

Benzophenone Standard Solutions for Electron-Capture GLC Analysis—The benzophenones J, V, and VI required as analytical standards are given in Tables I and II. Weigh 10.0 mg each of the free base of J, V, and VI into separate 10-ml volumetric flasks and prepare stock solutions analogous to those described previously for the intact benzodiazepin-2-ones. Prepare suitable dilutions of these stock solutions in 20% acetone–*n*-hexane, combining suitable aliquots of the respective dilutions to obtain the following working solutions.

For the clonazepam assay, prepare working solutions containing 2.0–10.0 ng of J, each containing 10.0 ng of V (the reference standard)/100 µl of 20% acetone–*n*-hexane.

For the flunitrazepam assay, similarly prepare working standard solutions containing 2.0–10.0 ng each of V and VI, each containing 10.0 ng of J (the reference standard)/100 µl of 20% acetone–*n*-hexane.

Ten-microliter aliquots of each solution (equivalent to 0.2–1.0 ng of compound) are injected into the gas chromatograph to establish the detector response and calibration linearity of the electron-capture GLC system for each day's analysis.

Reagents—All reagents were of analytical grade purity (>99%) and were used without further purification. The inorganic reagents were made up in double-distilled water and included: (*a*) 1.0 *M* (pH 9.0) H_3BO_3–Na_2CO_3–KCl buffer solution, prepared as described previously (23); (*b*) 4.0 *N* HCl–4.0 *N* H_2SO_4 (95:5 v/v) as the hydrolysis mixture, made fresh *daily* from the respective concentrated acids; and (*c*) 6.0 *N* NaOH.

The organic reagents used were: ether[9] (diethyl, absolute), analytical reagent grade, with a residue after evaporation and peroxide content of 0.0005% from a can opened no more than 3 days previously[10]; *n*-hexane[11], 99 mole % pure; acetone, reagent grade, and 20% acetone in *n*-hexane, both stored over anhydrous sodium

[9] Mallinckrodt.

[10] Check the purity of the ether by evaporating 20 ml to dryness, dissolve the residue in 100 µl of 20% acetone–*n*-hexane, and inject a 10-µl aliquot for electron-capture GLC. The chromatogram must be free of interfering peaks in the retention area of 2–15 min.

[11] Fisher H-301.

Table II—Chemical Names and Physical Properties of Flunitrazepam and Its Chemical Derivatives Referred to in Scheme I

Compound	Chemical Name	Molecular Weight	Melting Point
I	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (flunitrazepam)	313.29	170–171.5°
II	7-Nitro-5-(2-fluorophenyl)-3*H*-1,4-benzodiazepin-2(1*H*)-one	299.77	210–211°
III	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one	329.30	216–217°
IV	7-Nitro-5-(2-fluorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one	315.28	193–195°
V	2-Methylamino-5-nitro-2′-fluorobenzophenone	274.26	186–187°
VI	2-Amino-5-nitro-2′-fluorobenzophenone	200.23	154–158°
VII	2-Nitro-10-methyl-9-acridanone	254.3	282–284°
VIII	2-Nitro-9-acridanone	240.2	>350°

sulfate; and bromthymol blue indicator solution [0.1% in ethanol–water (50:50)].

Extraction of Clonazepam—Into a 50-ml centrifuge tube containing 10.0 ng of flunitrazepam (reference standard), add 2.0 ml of blood (or 5 ml of urine), 5 ml pH 9.0 borate buffer, and 10 ml of ether. Seal the tube with a Teflon stopper, and extract by shaking for 10 min on a reciprocating shaker. Centrifuge the samples for 10 min at 2000 rpm (preferably in a refrigerated centrifuge at 0–4°). Repeat the extraction with another 10-ml portion of ether, centrifuge, and combine the ether extracts. Along with the samples, run a specimen of control blood or urine (taken from the subject prior to medication) and four 2-ml specimens of control blood (or 5-ml specimens of urine) containing 2.0, 4.0, 8.0, and 10.0 ng of clonazepam added as internal standards, with each tube containing 10.0 ng of flunitrazepam added as the reference standard. (The sensitivity limits of detection can be increased by extracting 4 ml of blood/assay. In this event, add 6 ml of pH 9.0 buffer and extract twice with 15-ml portions of ether. Combine the ether extracts and back-extract into 5.0 ml of the hydrolysis mixture. Wash the acid layer three or four times with fresh 10-ml portions of ether to effect the complete removal of the heavy layer of lipid material present at the interphase.)

Extraction of Flunitrazepam and Its *N*-Desmethyl Metabolite—Perform the extraction procedure as described for clonazepam, using 10.0 ng of clonazepam added as the reference standard into each unknown. Process along with a set of internal standards of 2.0, 4.0, 8.0, and 10.0 ng each of flunitrazepam (I) and its *N*-desmethyl metabolite (II) added to control blood or urine, with each tube containing 10.0 ng of clonazepam added as the reference standard.

The following steps are common to both assays. To the ether extracts from the respective assays, add 5.0 ml of the hydrolysis mixture containing 4 *N* HCl–4 *N* H_2SO_4 (95:5), shake for 10 min, and centrifuge for 5 min. Carefully remove the ether phase by aspiration without removing any of the acid (aqueous) phase[12]. Wash the acid extract twice with 15 ml of ether, shaking for 10 min and centrifuging for 5 min, and remove the ether by aspiration after each washing[12]. Hydrolyze the samples in a boiling water bath (100°) for 1 hr to convert the benzodiazepin-2-ones to their respective benzophenones (Scheme I).

Cool the samples in an ice bath, add 1 drop of bromthymol blue indicator, and carefully add 3.5 ml of 6.0 *N* NaOH dropwise until the blue end-point is reached. Extract this alkaline solution twice with 10 ml of ether by shaking for 10 min and centrifuging for 5 min. The ether extracts are combined sequentially in 15-ml conical centrifuge tubes and evaporated to dryness at 35–40° in the water bath of a rotary evaporator[13]. The residues are vacuum dried (under mild vacuum) for 15–30 min to remove all traces of moisture and dissolved in 100 μl of the 20% acetone–*n*-hexane mixture, a suitable aliquot of which (5–10 μl) is analyzed by electron-capture GLC. The benzophenone peaks due to their respective benzodiazepin-2-ones are identified by their respective retention times (Figs. 1 and 2), and their respective peak areas are determined by measuring peak height (centimeters) × width at half height (centimeters) using the slope baseline technique or by electronic digital integration.

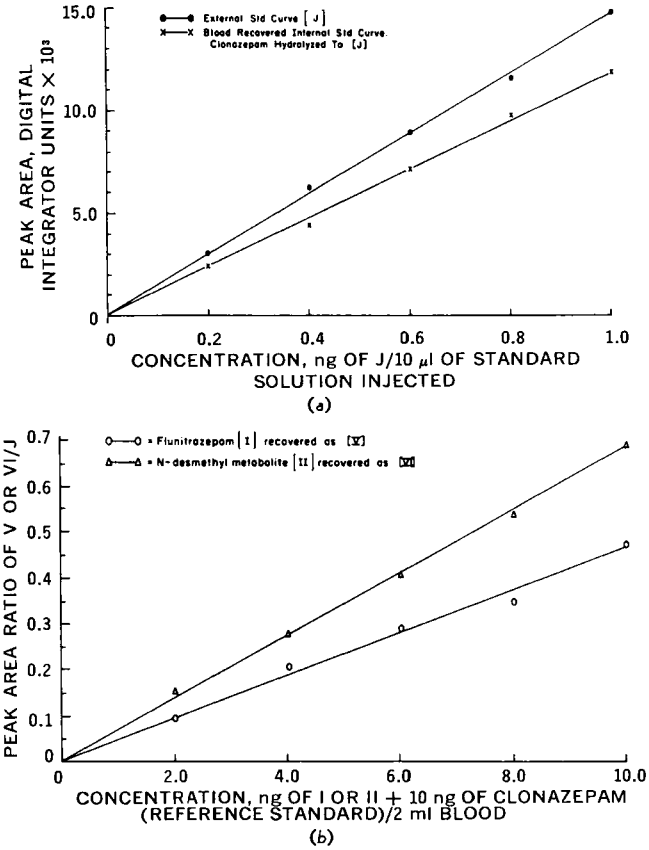


Figure 3—*Electron-capture detector calibration curves using:* (a) *the direct standard method (peak area* versus *concentration of the benzophenone (J) shown for clonazepam, and* (b) *the relative standard method (peak area ratio of V or VI/J* versus *concentration of I or II + 10 ng of clonazepam − reference standard)/2 ml of blood.*

Quantitation of Clonazepam (A), Flunitrazepam (I), and Its *N*-Desmethyl Metabolite (II) by Electron-Capture GLC Analysis—Calibration curves of the electron-capture detector response to the benzophenones J, V, and VI are made by plotting peak area *versus* concentration of the respective benzophenone, as shown for clonazepam in Fig. 3*a*. These calibration (external standard) curves are used not only to establish the linear response of the electron-capture detector and the stability of the GLC system but also to determine the percent recovery of internal standards. The internal standards are used to prepare calibration curves (Figs. 3*a* and 3*b*) for the quantitation of the concentration of clonazepam, flunitrazepam, and its *N*-desmethyl metabolite in biological specimens. These calibration curves have

[12] Save the ether phase if the determination of the 7-nitro-3-hydroxy metabolite is desired. Combine the ether washes with the original and evaporate to dryness. Dissolve the residue in 5 ml of the hydrolysis mixture, wash it twice with *n*-hexane as a cleanup step, hydrolyze, and process as for clonazepam.

[13] Buchler.

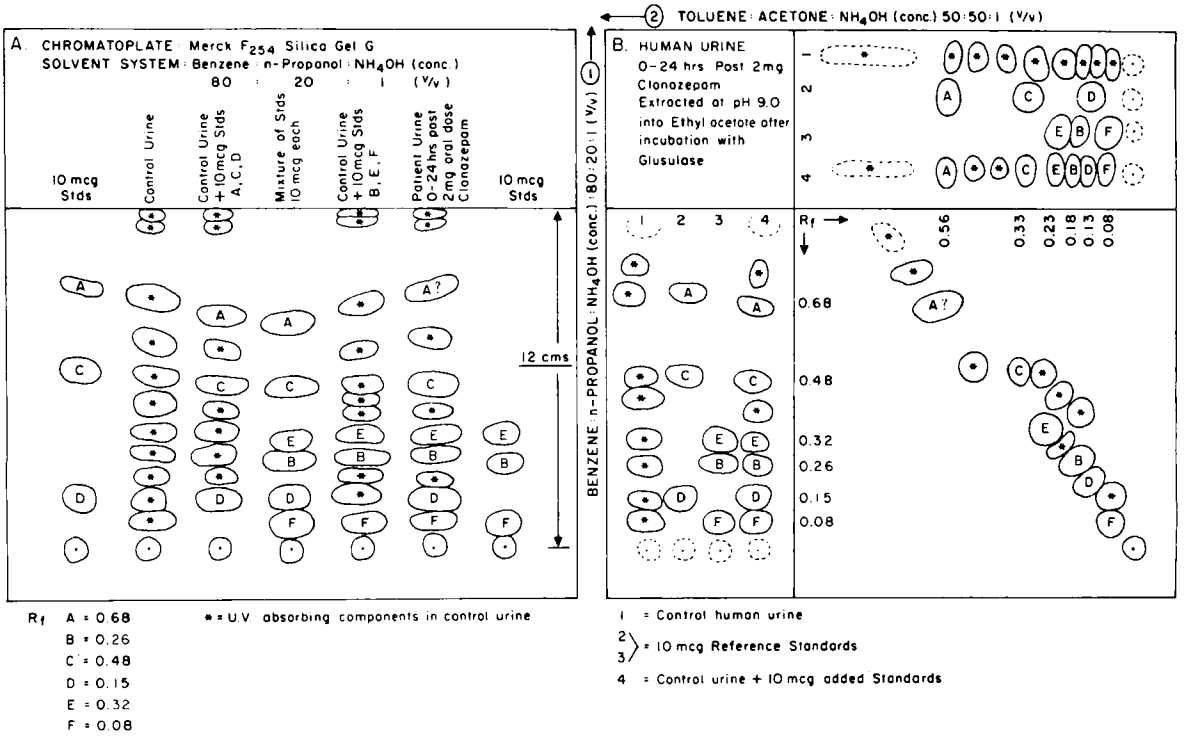


Figure 4—*Thin-layer chromatograms of human urine extracts containing clonazepam and its major metabolites developed by (A) one-dimensional and (B) two-dimensional chromatography.*

to be run each day with each set of unknowns and for each compound to be quantitated.

Calculations—Calibration curves using either the direct standard method (peak area *versus* concentration of benzophenone), as shown for clonazepam in Fig. 3*a*, or the relative calibration method (peak area ratio *versus* concentration), as shown for flunitrazepam and its metabolite (II) in Fig. 3*b*, can be used for the quantitation of either compound in the unknowns.

If the relative calibration (peak area ratio) method is used (Fig. 3*b*), the concentration in the unknowns is read directly (by interpolation) from the calibration curve because corrections for the aliquot of the total sample injected and the overall recovery of the compound measured are not required.

If the direct standard (peak area) method is used, then correction factors for the aliquot injected, the overall recovery of added standards, and the conversion of the benzophenone measured to its benzodiazepin-2-one equivalent have to be made.

The concentration of each component per milliliter of sample analyzed is calculated from the formula:

$$\frac{\text{ng of benzophenone found}}{\text{recovery factor of internal standards}} \times \frac{\text{dilution or aliquot factor}}{\text{ml of sample assayed}} \times \text{mol. wt.} \times \text{conversion factor}^{14} = \text{ng of clonazepam, flunitrazepam, or metabolite/ml of blood or urine} \quad \text{(Eq. 1)}$$

Determination of Percent Recovery—The overall recovery of clonazepam (A), flunitrazepam (I), and its *N*-desmethyl metabolite (II) was determined to be quantitative (*i.e.*, >85 ± 5.0%). When the relative calibration (peak area ratio) method (Fig. 3*b*) is used, the recovery factors of the respective compounds measured are assumed to remain constant. However, the percent recovery of added internal standards can be readily determined for either drug by using the direct calibration (peak area *versus* concentration) method (Fig. 3*a*).

Differential Pulse Polarographic Analysis of Major Urinary Metabolites of Clonazepam—The major clonazepam metabolites are the 7-amino (C), the 7-acetamide (E), and their respective 3-hydroxy analogs (D and F) (Table I) found mainly in the unconjugated form (28). Five milliliters of urine buffered to pH 9.0 is extracted with 2 × 15 ml of ethyl acetate. The residue of the combined extracts is separated by one-dimensional TLC in benzene–*n*-propanol–NH_4OH (concentrated) (80:20:1, no chamber saturation) (28) (Fig. 4A). The areas corresponding by R_f to metabolites C, D, E, and F are eluted with 2 × 5 ml of methanol, and its residue is dissolved in 4 ml of 0.1 *N* HCl and analyzed in a three-electrode cell (30) as previously described for other benzodiazepines (31). The peak produced by the reduction of the azomethine (>C_5=N_4—) group (common to all these metabolites) at around −0.600 v *versus* saturated calomel electrode (SCE) (Fig. 5) is used for their quantitation. The sensitivity limit of the assay is about 0.50–0.75 μg of each/5 ml of urine analyzed.

RESULTS AND DISCUSSION

Although electron-capture GLC methods for the determination of 1,4-benzodiazepin-2-ones as the intact compounds have been reported for medazepam, diazepam (23), and nitrazepam (32, 33), this principle was not applicable for clonazepam and flunitrazepam because of the poor electron-capture detector response of the former and the instability of the latter in acid. Extraction from blood into an organic solvent followed by back-extraction into acid and reextraction from an alkaline solution is essential for the necessary cleanup of the sample extract and for the quantitative recovery of basic 1,4-benzodiazepin-2-ones prior to electron-capture GLC analysis (23).

Therefore, it was necessary to convert them to a stable derivative, the benzophenone, by strong acid hydrolysis (Scheme I). Quantitative yields of the respective benzophenones were obtained by hydrolysis at 100° for 1 hr in a mixture of 4 *N* HCl–4 *N* H_2SO_4 (95:5 v/v). Although 4 or 6 *N* HCl also gave good yields of the benzophenones, this medium was not wholly suitable since erratic hydrolysis was obtained, resulting in a second product which affected the precision of the assay and overall recovery of the benzophenones of interest. Thus, in the case of clonazepam,

[14] Conversion factors: ng of J (benzophenone) × 1.14 = ng equivalent of clonazepam (A), ng of V × 1.14 = ng equivalent of flunitrazepam (I), and ng of VI × 1.15 = ng equivalent of *N*-desmethyl metabolite (II).

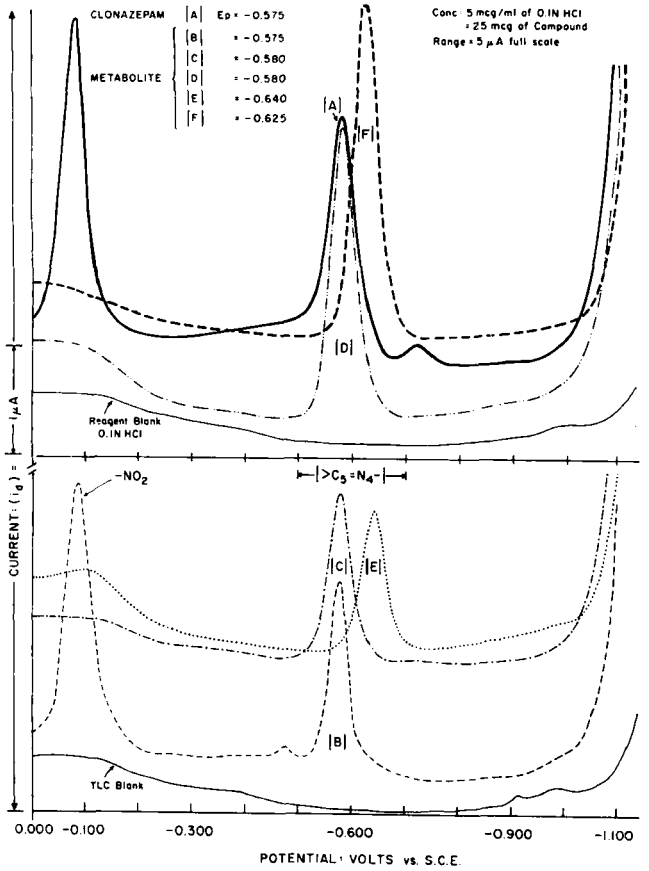


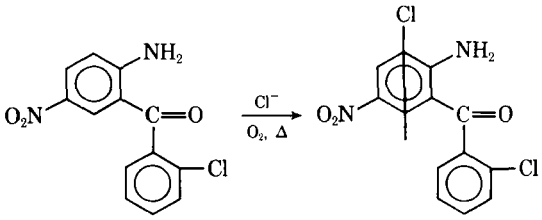
Figure 5—*Differential pulse polarograms of clonazepam and authentic standards of its major metabolites extracted from urine.*

the benzophenone J accounted for only 50–60% of the added parent compound, the losses being more acute in the lower nanogram concentration range.

The structure of this secondary product (which has a 1-min shorter retention time than J) was characterized by GLC–mass spectroscopy analysis to be a benzophenone compatible with the structure of a dichloro compound (Scheme II), which showed a molecular ion (*m/e*) at 310 amu, an increase of 34 amu over that of J (*m/e* 276 amu). This increase is compatible with the addition of a chlorine atom in the molecule.

This secondary product was more prevalent with hydrochloric acid solutions that had been standing for some time (>2–3 weeks). It may be due to an increase in the oxygen content on standing which, under the hydrolysis conditions, can either liberate or activate dissolved free chlorine in solution in the acid. The addition of the chlorine to the phenyl ring may be catalyzed by any one of the heavy metals present in trace amounts. The use of a freshly prepared mixture of 4 *N* HCl containing a small amount of 4 *N* H_2SO_4 (95:5) solved the problem and produced quantitative yields of the desired benzophenone (J). Similar problems encountered in the hydrolysis of flunitrazepam and its *N*-desmethyl metabolite to their respective benzophenones V and VI were overcome by the use of the same hydrolysis mixture. Following acid hydrolysis it is necessary to cool the sample in ice prior to neutralization with alkali to reduce the heat of neutralization evolved. This precaution is necessary because the benzophenones V and VI are readily cyclized at elevated temperatures in strongly basic media (especially in dimethylformamide containing sodium nitrite) to yield the 9-acridanone derivatives (34, 35) (Scheme I) due to the high reactivity of the fluorine group present in the 2′-position of the 5-phenyl ring. The benzophenone J does not readily cyclize to the acridanone, probably due to the greater chemical stability of the 2′-chloro group.

Assay Specificity—Since clonazepam and flunitrazepam are determined as their respective benzophenones J and V, any metabolite yielding the same benzophenone would interfere with the



Scheme II

specificity of the assay. Of the many possible metabolites, only the 7-nitro-3-hydroxy analogs (Scheme I) would yield the same benzophenones. Thus, for clonazepam, the presence of measurable amounts of Metabolite B would be a source of error. Although B is quantitatively extracted from blood at pH 9.0 into ether, it is only partially back-extracted into the hydrolysis mixture. The ether washes of the acid phase quantitatively remove any of Metabolite B present in the hydrolysis mixture prior to hydrolysis, leaving only the parent compound clonazepam (A) in the acid phase. This anomalous behavior of the 7-nitro-3-hydroxy analogs indicates that they are less basic than their parent compounds.

These ether washes are combined with the original blood ether extract (remaining after back-extraction of clonazepam into the acid hydrolysis mixture) for the subsequent determination of the 7-nitro-3-hydroxy metabolite (B) (see Footnote 12). Thus, the respective parent drugs clonazepam and flunitrazepam are the only compounds present in the hydrolysis mixture that would yield either J or V, therefore ensuring the specificity of the assay.

The 7-amino metabolite (C), if present, is also quantitatively extracted into ether from blood buffered to pH 9.0 but, on hydrolysis, it yields a product which is not the expected 2,5-diamino-2′-chlorobenzophenone (Compound K in Table I). Compound K has a retention time of about 4 min, whereas the derivative formed has a longer retention time of about 5 min. The electron-capture detector response to this product is very poor, and relatively high concentrations of the parent compound (C) are required (>200 ng) to produce a measurable peak of this derivative. Since it is resolved from the benzophenone peaks V and J, its presence even in trace amounts will not interfere with the quantitation of these benzophenones (Fig. 1). This product has not been chemically characterized as yet; hence, its overall recovery by electron-capture GLC and the efficiency of the hydrolysis of C have not been established. No measurable amounts of either Metabolite B or C have been noted in analyzed clinical blood specimens.

Recovery—The overall recovery of varying amounts of clonazepam (2.0–50 ng) added/ml of blood determined by electron-capture GLC and scintillation radiometry using ^{14}C-labeled clonazepam was 86 ± 6.5%. The recovery of the 7-nitro-3-hydroxy metabolite (B) determined by electron-capture GLC was about 70%. The overall recovery of flunitrazepam (I) and its *N*-desmethyl metabolite (II), added in varying amounts (50–250 ng/ml) to blood and determined by electron-capture GLC analysis[15], was 108.7 ± 8.0% for I and 110.6 ± 8.3% for II. This anomalous behavior is probably due to the formation of a Schiff-base "complex" with blood-extracted impurities, thereby reducing adsorption losses on the column during GLC analysis and resulting in an "apparent" enhanced recovery of compound. Since the quantitation of flunitrazepam and its metabolite (II) in the unknowns is based on their respective internal standard curves (Fig. 3*b*), the actual recovery factor is automatically compensated for yielding analytically valid data.

Differential Pulse Polarographic Assay for Urinary Metabolites of Clonazepam—Although spectrophotometric (26) and fluorometric (36) assays have been reported for the determination

[15] The development of the electron-capture GLC assay for flunitrazepam (I) and its *N*-desmethyl metabolite was originally done using an Aerograph model 205-B gas chromatograph with a ^{3}H-electron-capture detector of lower sensitivity, consequently requiring larger amounts (50–250 ng) of added standards. This instrument was adequate for measuring blood levels in animal studies at relatively high doses (2 mg/kg or greater) of administered flunitrazepam. Therapeutic doses in man are expected to be 2 mg or less and will require the use of the Micro Tek-220 chromatograph, with a 15-mCi ^{63}Ni electron-capture detector, or equivalent instrument.

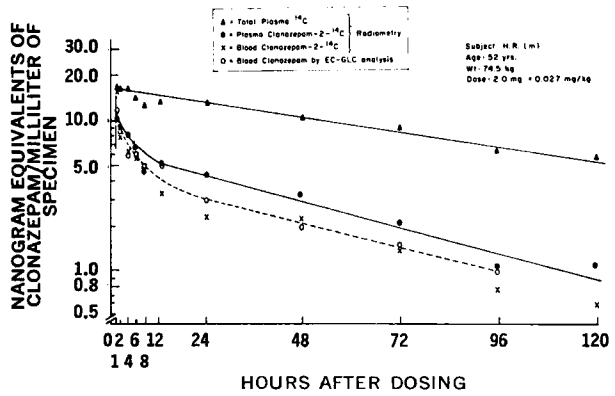


Figure 6—*Plasma levels of radioactivity and of intact clonazepam as a function of time following a 2-mg oral dose of micronized ^{14}C-clonazepam. Key: ▲, plasma total ^{14}C in nanogram equivalents of clonazepam per milliliter; ●, plasma ^{14}C-clonazepam in nanograms per milliliter; ×, blood ^{14}C-clonazepam; and ○, blood clonazepam determined by electron-capture GLC analysis in nanograms per milliliter.*

of nitrazepam and its major metabolites (which are analogous to those of clonazepam), they require chemical manipulations to produce either a suitable chromophore or a fluorophore for their determination. These assays also lack specificity, since all metabolites are quantitated as a total equivalent of the parent compound. The polarographic assay developed is specific by virtue of not only the chromatographic separation step (Fig. 4) but also of the specificity of the electrochemical reduction of the azomethine group, which produces a peak at about −0.600 v *versus* SCE (Fig. 5). Biological impurities present in the sample do not interfere with the reduction process, thereby resulting in a sensitive, specific, and precise quantitation.

The overall percent recoveries of clonazepam and its major metabolites added to urine and separated by TLC were: 88, A; 86, B; 68, C; 65, D; 93, E; and 88, F. The method was applied to the quantitation of urinary metabolites in man following a single 2-mg oral dose. Although Metabolites C, D, E, and F were detectable on the chromatoplate (under shortwave UV light), their concentrations following elution from the silica gel were at the sensitivity limit of the assay, *i.e.*, <0.5–0.75 μg/ml; hence, they are not reported. It is expected that the method will be useful in measuring urinary metabolites following chronic administration of clonazepam.

Application of the Electron-Capture GLC Method in Biological Specimens—The accuracy of the chemical assay was verified in one subject who received 2 mg of micronized labeled clonazepam-2-^{14}C administered in a gelatin capsule containing anhydrous lactose as a diluent. Duplicate whole blood specimens (10 ml, oxalated) were drawn at appropriate time intervals up to 120 hr. One set of specimens was centrifuged immediately after being drawn, and the separated plasma was analyzed for its ^{14}C-clonazepam and total ^{14}C content as previously described (37). The duplicate set was analyzed for its ^{14}C-clonazepam content following ether extraction by TLC separation, and scintillation radiometry of the separated drug[16], and direct electron-capture GLC analysis. The plasma levels of total ^{14}C and of extracted ^{14}C-clonazepam are shown in Fig. 6 and indicate measurable levels up to 120 hr postdosing. The half-life of elimination of the unchanged drug was calculated to be about 47 hr in this subject. The levels of total plasma ^{14}C indicate the presence of metabolites in plasma.

The blood levels of ^{14}C-clonazepam in this subject (Fig. 6) determined by both TLC radiometry and electron-capture GLC analysis showed good agreement, confirming the accuracy and sensitivity of the electron-capture GLC assay and its specificity for the intact drug.

Evidence that steady-state blood levels of clonazepam are achieved following chronic clonazepam therapy is presented in Table III. Blood levels monitored in a single subject administered 2 mg of clonazepam (tid) for the management of petit mal seizures showed that the blood levels of clonazepam ranged from 20 to 49 ng/ml over 4 months, suggesting steady state. No measurable blood levels of either the 7-nitro-3-hydroxy metabolite (B) or the 7-amino metabolite (C) were seen in this subject.

The electron-capture GLC assay was also used successfully for the determination of clonazepam blood levels in man following the administration of 2 mg of the micronized drug in a tablet formulation in studies on the blood level profile and the pharmacokinetics of clonazepam in man and of flunitrazepam in the dog (29).

[16] M. A. Schwartz, C. W. Williams, and A. S. Leon, unpublished data on file, Hoffmann-La Roche, Inc., Nutley, NJ 07110, 1972.

Table III—Blood Levels of Clonazepam in Human[a] following Chronic Administration

Date	Time Drawn	Blood Concentration, ng/ml
11/16/71	10:00 am	26.4
11/17/71	10:00 am	34.3
11/18/71	10:20 am	35.8
11/18/71	3:00 pm	27.2
11/18/71	1:00 pm	19.8
1/4/72	9:00 pm	49.6
1/5/72	12:30 pm	31.0
1/5/72	7:05 pm	21.7
3/9/72	9:00 pm	42.0
3/10/72	10:00 am	34.3
3/10/72	7:00 pm	25.6

[a] Subject: J. N., female, 51 kg. Dose: 2.0 mg tid taken at 7:00 am, 3:00 pm, and 9:00 pm. Total: 6 mg = 0.04 mg/kg. Data courtesy of Dr. H. J. Kupferberg, National Institute of Narcotics and Dangerous Substances, National Institutes of Health, Bethesda, Md.

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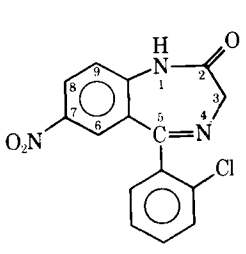
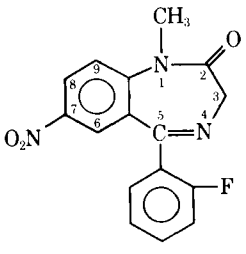
Keyphrases □ Clonazepam—blood levels and urinary excretion in dogs and humans □ Flunitrazepam—blood levels and urinary excretion in dogs □ Pharmacokinetic profiles—clonazepam in dogs and humans, flunitrazepam in dogs

The 7-nitro-1,4-benzodiazepin-2-ones, clonazepam and flunitrazepam, have shown anticonvulsant properties in several animal species (1–5). In addition, clonazepam is clinically effective in controlling minor motor seizures (petit mal) at minimal effective doses in the range of 1–2 mg/day orally (6–9). Metabolic



clonazepam



flunitrazepam

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The present study reports the pharmacokinetic profile of clonazepam in the dog and humans and of flunitrazepam in the dog based on the evaluation of blood level and urinary excretion data determined using a sensitive and specific electron-capture GLC assay procedure (15). The assay follows for the measurement of clonazepam following its administration and of flunitrazepam and *N*-desmethyl flunitrazepam following flunitrazepam administration.

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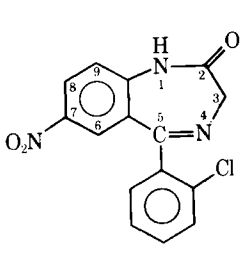

clonazepam

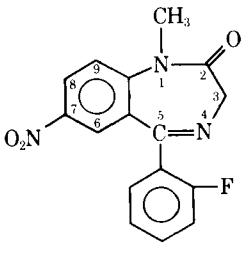

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Table I—Pharmacokinetic Parameters Associated with Physiological Disposition of Clonazepam in the Dog

Parameters	Route of Administration and Formulation			
	Intravenous Solution	Oral Solid (Nonmicronized)	Oral Solution	Oral Tablet (Micronized)
Dose, mg	30	30	30	30
Weight, kg	13.1	13.0	13.0	13.0
Dose, mg/kg	2.3	2.3	2.3	2.3
A, ng/ml	1205	—	—	—
B, ng/ml	601	—	—	—
α, hr^{-1}	30.621	—	—	—
β, hr^{-1}	0.129	0.136	0.149	0.110
Half-life, hr	5.4	5.1	4.7	6.3
C_p^0, ng/ml	1806	—	—	—
V_p, volume of central compartment, liters	16.6	—	—	—
% V_p, % of body weight	127	—	—	—
$(V_d)\beta$, total volume of distribution, liters	29.3	—	—	—
% $V_d\,\beta$, % of body weight	224	—	—	—
k_{12}, hr^{-1}	7.780	—	—	—
k_{21}, hr^{-1}	10.283	—	—	—
k_{el}, hr^{-1}	0.384	—	—	—
Area (blood level curve), ng/ml/hr	4672	61	4486	5043
Area ratio, oral/intravenous × 100	—	1.3	96	110

Table II—Blood Level Profile of Clonazepam in Humans following Oral Administration of Single 2-mg Doses

	Subject									
	1	2	3	4	5	6	7	8	9	10
Weight, kg	71.3	77.2	79.5	64.9	81.3	96.3	66.3	83.1	96.8	75.5
Hours	Blood Levels, ng/ml									
0.5	1.8	0.6	N.M.[a]	4.3	N.M.	4.0	1.2	5.7	N.M.	1.7
1	10.7	5.7	3.3	8.3	6.2	6.6	11.3	13.5	3.7	6.1
1.5	10.7	13.0	5.9	9.7	6.7	9.0	12.6	13.2	5.4	7.4
2	10.0	11.9	6.8	12.6	6.8	10.9	10.2	9.9	6.5	6.9
4	10.1	7.6	7.3	10.2	8.2	7.2	9.2	6.9	5.6	7.6
6	9.1	9.7	8.2	9.9	7.8	6.5	8.2	7.5	N.S.T.[b]	N.S.T.
8	9.6	6.5	8.6	9.4	7.2	5.6	8.6	6.6	4.2	6.6
12	7.7	7.7	6.9	7.7	6.9	4.6	7.3	5.7	4.0	5.5
24	5.8	4.8	4.0	5.4	5.2	3.2	6.4	4.9	3.2	4.4
48	3.3	2.7	2.6	3.7	2.6	1.6	3.1	1.5	N.S.T.	N.S.T.
Percent of dose in 0–24-hr urine as intact drug	0.2	0.2	0.5	0.2	0.2	0.2	0.3	0.5	0.2	0.3
Area under blood level curve[c], ng/ml/hr	297	259	229	299	249	181	297	230	162	213
Blood peak time, hr	1–1.5	1.5	8	2	4	2	1.5	1	2.0	1.5–4.0
Blood peak concentration, ng/ml	10.7	13.0	8.6	12.6	8.2	10.9	12.6	13.5	6.5	7.6
Apparent first-order elimination rate constant, hr^{-1}	0.025	0.030	0.029	0.024	0.026	0.033	0.024	0.037	0.018	0.027
Corresponding half-life, hr	27.7	23.1	23.9	28.9	26.6	21.0	28.9	18.7	39.0	25.9

[a] N.M. = not measurable, <0.5 ng/ml using a 2-ml specimen per analysis. [b] N.S.T. = no specimen taken. [c] Calculated using trapezoidal rule.

EXPERIMENTAL

Clonazepam—Clonazepam exhibits pKa values of 1.5 and 10.5. The pKa of 1.5 corresponds to the removal of the proton of the protonated nitrogen in the 4-position of the molecule, and the pKa of 10.5 corresponds to the deprotonation of the nitrogen in the 1-position. Therefore, the compound would be virtually undissociated throughout the physiological pH range. It exhibits low aqueous solubility within the physiological pH range, with a maximum of 76 μg/ml at pH 2.0 and a minimum of 19 μg/ml at pH 7.4. The permeability characteristics of clonazepam across the everted rat intestinal sac are consistent with good absorbability (16). This suggests that once in solution the permeability (absorbability) of clonazepam across the GI mucosa will not be a rate-limiting factor following oral administration of the drug.

Clonazepam in the Dog—Clonazepam was administered to a dog intravenously and orally, on separate occasions, as a 2.3-mg/kg dose in propylene glycol solution. The same dog also received a 2.3-mg/kg dose orally as a solid, administered as the nonmicronized drug, hand packed into gelatin capsules and as the micronized drug formulated into 2-mg tablets. Five-milliliter oxalated blood specimens were obtained at appropriate time intervals up to 72 hr postdosing. The total volumes of urine voided were collected at 24-hr intervals from −24 to 72 hr following each administration. All specimens were stored frozen until analyzed.

The blood level data following intravenous and oral administrations of clonazepam in a dog are presented in Fig. 1. The pharmacokinetic profile of clonazepam in the dog is summarized in Table I. The recovery of intact drug in the 0–72-hr urine was less than 0.1% of the administered dose following intravenous administration and was nonmeasurable following oral administration. These findings suggest complete biotransformation and/or alternative routes of excretion.

Following the intravenous administration of clonazepam, a

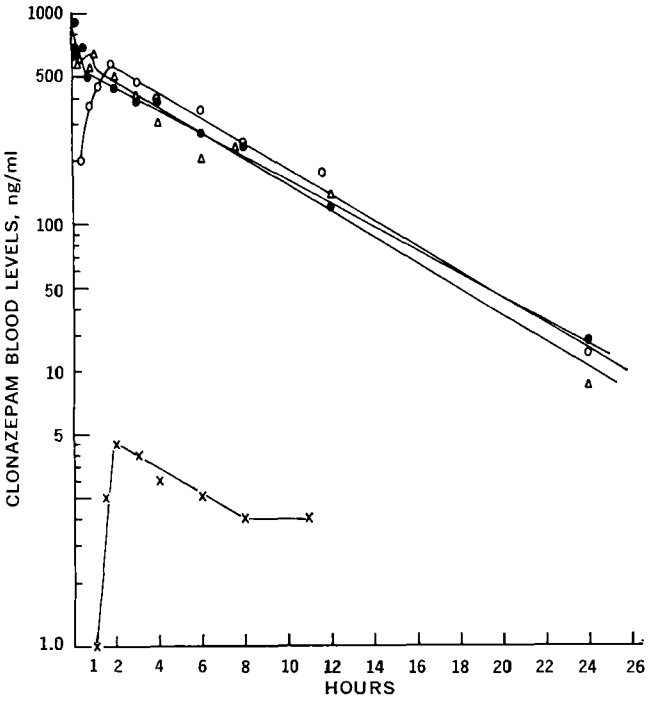


Figure 1—*Clonazepam blood level curves in a dog following intravenous and oral administration of 2.3-mg/kg doses. Key: ●, intravenous solution; △, oral solution; X, oral solid (non-micronized in gelatin capsule); and ○, oral solid (micronized in tablet formulation).*

biexponential blood level curve was observed (Fig. 1). Therefore, the pharmacokinetic evaluation of clonazepam would require a minimum of a two-compartment open-system model as previously described (17).

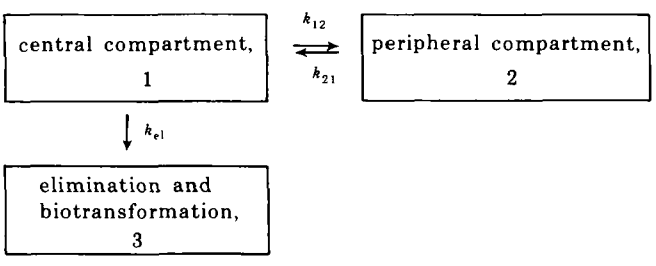
The first exponential is referred to as the fast disposition rate, with a rate constant α, and reflects the disposition of the compound from the central compartment into the body tissues. The second exponential is referred to as the slow disposition rate or "apparent" elimination rate, with a rate constant β. Both α and β are hybrid first-order rate constants which are each influenced by all of the individual processes involved in the disposition of the drug.

Solution of the differential equations for the two-compartment open-model (Scheme I) yields the following integrated equation describing the blood level–time curve following intravenous administration:

$$(C_p)_t = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where $(C_p)_t$ is the concentration of drug in the blood at time t, and A and B are the ordinate axis intercepts. The individual rate constants of the model, k_{12}, k_{21}, and k_{el}, are calculable from α and β (17).

The pharmacokinetic profile of clonazepam (Table I) indicates that the initial distribution phase, α value, is rapid. The percent volume of the central compartment was calculated to be 127% of body weight, and the corresponding overall percent volume of distribution was calculated to be 224% of body weight. The drug was eliminated at a moderately rapid rate, β value of 0.129 hr^{-1}, corresponding to a half-life of 5.4 hr.



Scheme I

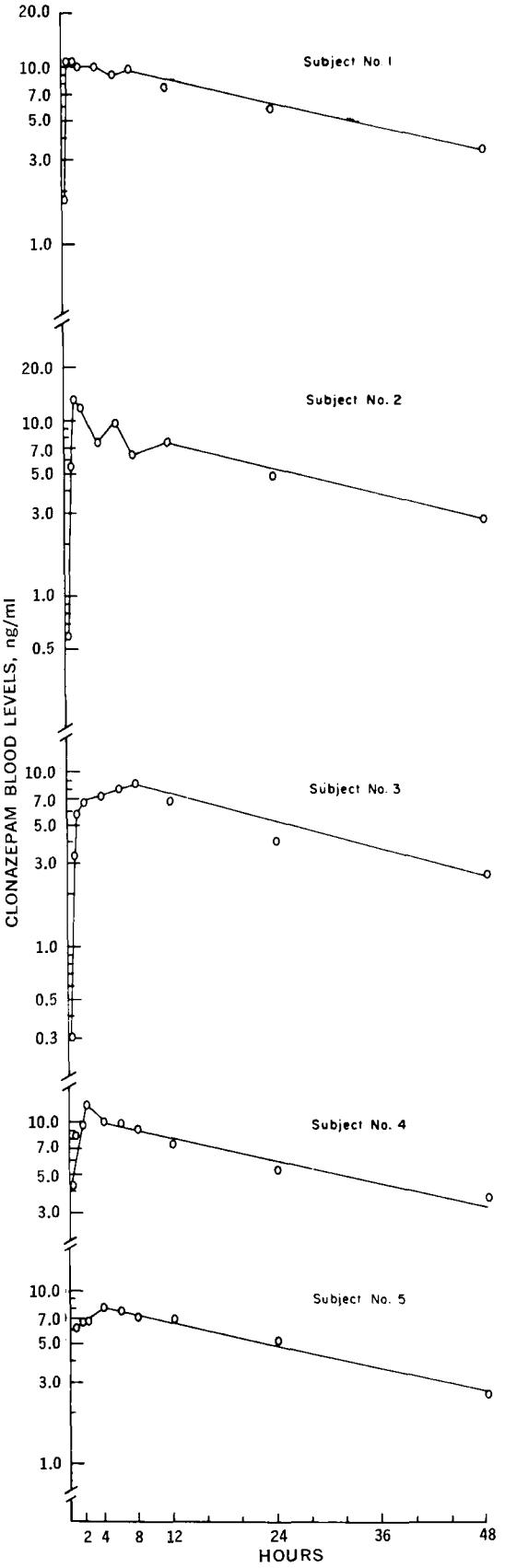


Figure 2—*Clonazepam blood level curves in humans following the administration of a single, oral 2-mg dose of the micronized drug formulated as a tablet.*

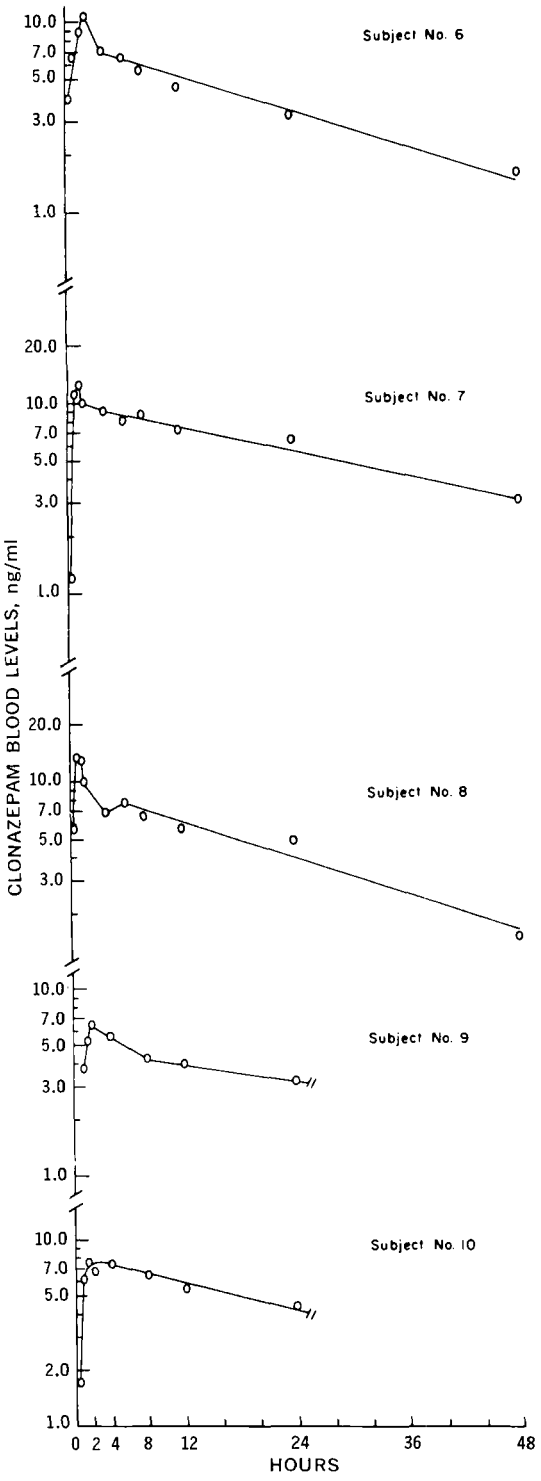


Figure 3—*Clonazepam blood level curves in humans following the administration of a single, oral 2-mg dose of the micronized drug formulated as a tablet.*

The blood level curves seen following a single oral dose of the nonmicronized drug in a gelatin capsule indicated that the absorption was slow and incomplete, with approximately 1.2% being absorbed (Fig. 1). A solution of clonazepam was prepared in propylene glycol and administered orally to circumvent the potential limitation of absorption by the dissolution rate. The blood levels showed that clonazepam administered orally in solution was absorbed rapidly and completely. The data suggest that the absorption of clonazepam can be dissolution rate limited; however, once in solution, the permeability of the drug is consistent with good absorbability. The administration of micronized clonazepam formulated as a 2-mg tablet resulted in blood levels that indicated that the micronized drug was rapidly and completely absorbed. Therefore, micronization of clonazepam succeeded in overcoming the dissolution rate-limiting characteristics of the compound in the overall absorption of the drug. Such a formulation was used in the clinical evaluation of the drug in humans.

Table III—Pharmacokinetic Parameters Associated with the Physiological Disposition of Flunitrazepam and Its *N*-Desmethyl Metabolite in the Dog

	Dog 1		Dog 2	
	Intravenous	Oral	Intravenous	Oral
Flunitrazepam Profile				
Dose, mg/kg	2	2	2	2
A, μg/ml	1.87	—	0.93	—
B, μg/ml	0.35	—	0.57	—
α, hr^{-1}	17.25	—	32.85	—
β, hr^{-1}	0.97	—	1.78	—
$t_{1/2}$, $\left(\frac{0.693}{\beta}\right)$, min	42.8	—	23.4	—
C_p^0, μg/ml	2.22	—	1.49	—
V_p, liters	11.7	—	13.4	—
% V_p, volume central compartment, % of body weight	90	—	134	—
% V_d, volume of distribution, % of body weight	345	—	242	—
k_{12}, hr^{-1}	9.96	—	13.53	—
k_{21}, hr^{-1}	3.52	—	16.78	—
k_{el}, hr^{-1}	4.73	—	4.32	—
Area under blood level curve, μg/ml/hr	0.43	0.03	0.36	—[a]
***N*-Desmethyl-Flunitrazepam Profile**				
Area under blood level curve, μg/ml/hr	4.55	2.37	3.10	1.25
Ratio of areas, oral/intravenous × 100	—	52	—	40
β, hr^{-1}	0.15	0.17	0.12	0.05
$t_{1/2}$, $\left(\frac{0.693}{\beta}\right)$, hr	4.7	4.1	6.0	13.1
Peak height, μg/ml	1.28	0.33	0.74	0.16
Peak time	5 min	4 hr	10 min	2 hr

[a] Flunitrazepam below detectable level.

Clonazepam in Humans—A clinical study was conducted with 10 adult male volunteers[1]. Each subject received a single oral 2-mg dose of micronized drug in the form of a tablet formulation in the morning following an overnight fast. Ten-milliliter oxalated blood specimens were obtained at 0 hr (control) and at 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, and 48 hr after administration. The total volumes of urine voided were collected from −24 to 0 hr and from 0 to 24 hr after administration. All specimens were stored frozen until analyzed.

The blood levels, urinary excretion, and pharmacokinetic profile of clonazepam in humans are summarized in Table II; the blood level curves are shown in Figs. 2 and 3.

Peak blood levels of clonazepam were seen in eight subjects between 1 and 2 hr after administration and at 4 and 8 hr in the other two subjects, respectively. The peak blood levels ranged from 6.5 to 13 ng/ml, and the apparent elimination rate, β, of clonazepam ranged from 0.018 to 0.037 hr^{-1}. The corresponding half-lives of elimination ranged from 18.7 to 39.0 hr, with a mean of 26.4 hr. Less than 0.5% of the dose was recovered in the urine as intact drug in the 0–24-hr excretion period, indicating extensive biotransformation and/or alternative routes of excretion.

The pharmacokinetic profile of clonazepam in the dog and in humans shows that the half-life of clonazepam in the dog is four to five times shorter than in humans. In addition, both species

[1] Conducted at the Deer Lodge Research Unit, Deer Lodge, Mont., under the supervision of Dr. J. D. Moore.

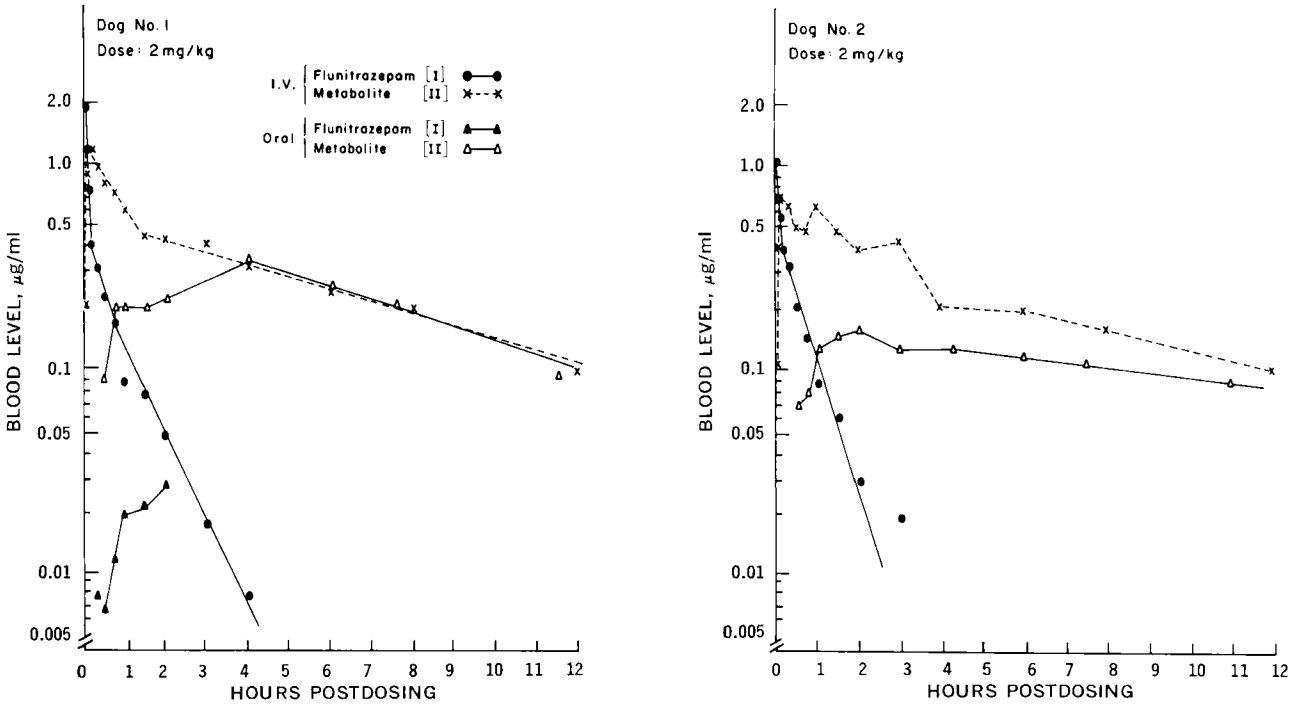


Figure 4—*Blood level curves of flunitrazepam [I] and its* N*-desmethyl metabolite [II] in two dogs following the intravenous and oral administration of 2-mg/kg doses of the drug.*

show extensive biotransformation of clonazepam, with less than 0.5% of the dose recovered in the urine as the intact drug. The data presented herein are consistent with the previously reported findings (10).

Pharmacokinetics of Flunitrazepam in Dog—Flunitrazepam exhibits a pKa of 1.8, which would allow the drug to be virtually unionized in the physiological pH range. The aqueous solubility decreases with an increase in pH, being 850 μg/ml at pH 1 and 11 μg/ml at pH 5.3. The permeability of the drug across the lumen of the everted rat intestinal sac (16) was consistent with good absorbability. Such *in vitro* parameters suggest a potential for a dissolution rate-limited absorption. However, once in solution the drug should transfer readily from the GI tract into the bloodstream.

In vivo studies were performed in which two dogs, Dogs 1 and 2, each received a 2-mg/kg single intravenous dose administered in propylene glycol and a single oral dose of micronized drug administered in a gelatin capsule 1 week apart. Oxalated blood specimens were obtained at appropriate time intervals up to 72 hr after administration by each route, and the entire volume of urine voided was collected at 24-hr intervals from −24 to 72 hr after administration.

Following intravenous administration of flunitrazepam in the two dogs, the biexponential blood level curves (Fig. 4) suggested that the pharmacokinetic evaluation would require minimally a two-compartment open-system model (17) as described for clonazepam in the dog. The pharmacokinetic parameters of flunitrazepam (Table III) show that the fast disposition rate constant, α, was 17.2 and 32.8 hr^{-1} for Dogs 1 and 2, respectively. These values correspond to an apparent half-life of 2.4 and 1.3 min, respectively. The slow disposition rate constants, β, of 0.97 and 1.78 hr^{-1} correspond to an apparent half-life of 43 and 23 min, respectively. The magnitude of the rate constants α and β indicates that the drug is distributed and eliminated at very rapid rates.

The volume of the central compartment, V_p, was 90 and 134% of body weight, whereas the total volume of distribution, V_d, was calculated to be 345 and 242% of body weight in Dogs 1 and 2, respectively, indicating extensive distribution of flunitrazepam in the dog.

Following the oral administration of the drug, only Dog 1 showed measurable blood levels of the parent drug, which were both low and erratic. No measurable blood levels of flunitrazepam were seen in Dog 2 following oral administration. This might be attributed to poor absorption, biodegradation in the GI tract, or rapid metabolism during the first pass of the drug through the liver. However, following both the intravenous and oral administrations of flunitrazepam, rapid biotransformation is evident from the levels of the *N*-desmethyl metabolite, the only metabolite detectable by the assay procedure (Table III and Fig. 4).

The areas under the blood level curve of the *N*-desmethyl flunitrazepam following the intravenous and oral administrations of flunitrazepam in each dog were compared. Their ratios indicate that at least 40–50% of the administered compound was available following oral administration but mostly as the *N*-desmethyl metabolite. Therefore, the lack of measurable parent drug in the blood after oral administration can probably be attributed to the GI or first-pass metabolism effect. The comparison of areas for the metabolite may lead to an underestimation of the extent of availability of administered drug if absorption is nonlinear due to the low solubility and slow dissolution rate in the GI tract. However, more extensive studies will be required to define the oral drug level profile. No measurable levels of either flunitrazepam or *N*-desmethyl-flunitrazepam were seen in the urine of both dogs, indicating complete and extensive biotransformation and/or alternative routes of excretion. The assay procedure did not allow for the detection of other possible metabolites.

SUMMARY

The pharmacokinetic profile of clonazepam in the dog indicated that the drug exhibited a volume of distribution of 224% body weight and that it was extensively biotransformed and rapidly eliminated. Less than 1% of the dose was detected in the 0–72-hr urine as intact drug, indicating complete biotransformation and/or alternative routes of excretion. The drug was rapidly and completely absorbed following oral administration.

In humans, a 2-mg single oral dose exhibited peak blood levels ranging from 6.5 to 13.5 ng/ml at 1–2 hr after administration. The apparent half-life of elimination ranged from 18.7 to 39.0 hr. Less than 0.5% of the dose was recovered in the urine as intact clonazepam, again indicating complete biotransformation and/or alternative routes of excretion.

The pharmacokinetic profile of flunitrazepam in the dog indicates that this drug exhibited a volume of distribution of approximately 300% body weight and was also extensively biotrans-

formed and rapidly eliminated. The virtually instantaneous biotransformation of flunitrazepam to its *N*-desmethyl metabolite, the major blood component seen following both intravenous and oral administrations of the drug, suggests a first-pass effect. The apparent half-life of elimination of the *N*-desmethyl metabolite following intravenous administration of flunitrazepam was 4.7 and 6 hr in the two dogs studied. No measurable levels of either flunitrazepam or *N*-desmethyl-flunitrazepam were seen in the urine, indicating extensive and complete biotransformation and possible alternative routes of excretion.

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[x] To whom inquiries should be directed.

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SARFARAZ NIAZI and WIN L. CHIOU[x]

Abstract □ Partition coefficients of the three most commonly used fluorocarbon propellants (trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane) in water, normal saline, cyclohexane, chloroform, human plasma, and human blood were studied over a wide range of propellant concentrations. They were all found to be independent of concentration. No salting-out effect was seen in normal saline. The partition coefficients in plasma and blood were much greater than in normal saline. The implications of the results of this study on absorption, distribution, elimination, and assay of these propellants are discussed.

Keyphrases □ Fluorocarbon aerosol propellants—partition coefficients in six solvents □ Aerosol propellants, fluorocarbon—partition coefficients in six solvents □ Partition coefficients—determined for fluorocarbon aerosol propellants, six solvents □ Dichlorodifluoromethane—partition coefficient in six solvents □ Dichlorotetrafluoroethane—partition coefficient in six solvents □ Trichloromonofluoromethane—partition coefficient in six solvents

Solubilities of many volatile or gaseous anesthetic compounds in a liquid medium such as blood and olive oil have been often defined as being equivalent to their partition coefficients between the liquid phase and gaseous phase at an equilibrium state. The importance of this parameter to the absorption, distribution, elimination, and pharmacological activities of many compounds in humans and animals has been excellently reviewed (1). Volatile fluorocarbons such as trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane have been widely used as propellants in aerosol products for cosmetic, pharmaceutical, household, and other purposes. The toxicity of these fluorocarbon propellants has been a subject of intensive research and controversy in recent years, as was clearly illustrated in an editorial (2) and a rebuttal (3) to that editorial published recently.

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each propellant in human serum, the solubilities in water, normal saline, human plasma, and human blood were not studied by them. It was felt that these data were also important for the better understanding of their pharmacokinetic properties in humans and animals. Therefore, this investigation was undertaken to study their solubilities or partition coefficients over a wide range of concentrations in water, normal saline, cyclohexane, chloroform, human plasma, and human blood.

The inclusion of cyclohexane in the study was prompted by a report (6) that this organic solvent was used to represent the lipoid phase in a partition coefficient study and the results correlated well with the biological activities of many drugs. It was also used as a solvent in this investigation for the fluorocarbon propellants to establish the standard curves for GC analyses. Only the three most widely used fluorocarbon propellants (trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane) were studied.

EXPERIMENTAL

Materials—The three high purity fluorocarbons[1], normal saline USP[2], cyclohexane[3] (99 mole %), and chloroform[4] were obtained from commercial sources. The human blood[5] used was pooled from blood samples of four healthy subjects and contained 12.5% (v/v) of anticoagulant citrate and dextrose solution USP. The plasma was obtained by centrifugation of the blood.

The standard solutions of the three fluorocarbon propellants used for GC analyses were prepared by the following method. Four milliliters of cyclohexane was transferred to a 5-ml capacity serum bottle[6] which was then quickly sealed with a flange-type rubber stopper[6,7] and an aluminum seal using a manual crimper. The bottle was weighed and care was taken to avoid wetting of the rubber stopper by cyclohexane. The trichloromonofluoromethane solution was prepared by drawing some refrigerated liquid trichloromonofluoromethane into a precooled (5°) glass syringe through a 22-gauge hypodermic needle, and a few drops were injected into the sealed bottle containing cyclohexane. Then the bottle was reweighed. A difference in weight, ranging from 20 to 80 mg, gave the amount of the propellant dissolved in cyclohexane.

The solutions of dichlorodifluoromethane and dichlorotetrafluoroethane were prepared by purging them in the gaseous state into the sealed serum bottle containing cyclohexane. This was accomplished by the following procedure. The pressure tank containing the liquid propellant was connected to a 22-gauge hypodermic needle by a rubber tubing through which the flow rate could be controlled by adjusting a clamp. Very low flow rates were used to avoid any pressure build-up inside the bottle. After purging the surface of cyclohexane with the gas for about 5 sec, the needle was withdrawn and another 22-gauge hypodermic needle was inserted just enough to pierce through the stopper to release any pressure inside the bottle. The bottle was then shaken gently and reweighed. These solutions also contained about 20–80 mg of the propellant. Every time a needle was inserted in a bottle, care was observed to avoid contacting cyclohexane to prevent its possible loss through the needle. Further dilutions were made with cyclohexane by volume. It is believed that this method of preparation of standard solutions can be more easily and accurately carried out than the method used by Shargel and Koss (7). Their method required handling at very low temperature and data on the densities of some propellants.

The concentrated solutions of dichlorodifluoromethane or dichlorotetrafluoroethane in normal saline were prepared by purging the gas slowly from the pressure tank, into a 50-ml capacity serum bottle filled with normal saline, through an 18-gauge hypodermic needle inserted well below the surface of the solution. The gas was allowed to escape the bottle during the purging through another hypodermic needle inserted just enough to pierce the rubber stopper. The concentrated solutions of trichloromonofluoromethane in normal saline were prepared by adding 1 ml of the liquid propellant to a serum bottle (15–50-ml capacity) filled with normal saline; the bottle was then sealed and shaken for 15 min and set aside for about 30 min to achieve a clear phase separation. The aqueous layer was then removed using a 50-ml glass syringe and injected into an empty serum bottle which was quickly sealed. The stock solution so prepared was kept filled to the top of the bottle to avoid any excessive loss due to partitioning into the gaseous phase. Proper dilutions of the three propellant stock solutions with normal saline were then made to give a wide range of concentrations for each propellant: about 40–1000 μg/ml for trichloromonofluoromethane, 20–200 μg/ml for dichlorodifluoromethane, and 50–100 μg/ml for dichlorotetrafluoroethane.

The exact concentrations of the propellants in normal saline were determined by injecting[8] 10 μl of the solution into an empty, sealed serum bottle of 15–50-ml capacity and analyzing the gaseous phase for the propellant concentration after shaking[9] the bottle for 5 min. The peak heights of the symmetrical gas chromatograms were then converted to amounts of the propellants injected using the standard curves prepared from the standard solutions in cyclohexane. The concentration of the original propellant concentration in normal saline was calculated by the following equation:

$$\text{concentration in normal saline} = \frac{\text{concentration in gaseous phase} \times \text{internal volume of bottle}}{\text{volume of propellant solution in normal saline added}} \quad \text{(Eq. 1)}$$

This method of calculation was accurate to at least 99.9%, because the volume of the normal saline propellant solution in the bottle was negligible compared to that of the gaseous phase and the solubilities of the propellants studied in normal saline were low. The internal volume of the serum bottle used in this study had been determined previously by the gravimetric method (weighing the sealed bottle before and after it was filled with water and then dividing the difference in weight by the water density at room temperature).

Solubility Determination—Samples for solubility studies were prepared by injecting 10 μl of concentrated propellant solutions in normal saline into 5 or 10 ml of distilled water, normal saline, plasma, or blood contained in the sealed serum bottle with a capacity of 15, 25, or 50 ml. The preparation of cyclohexane samples was the same as that described previously. The propellant solutions in chloroform were prepared by injecting 10 μl of known concentrated propellant solutions in cyclohexane into the sealed serum bottle containing chloroform.

The propellant samples were shaken[9] for 5 min. They were then placed in a constant-temperature water bath[10] maintained at 25° or, in some instances, at 37° and shaken at 100 rpm for 30 min. The concentrations of the propellants in both the liquid phase and head space were analyzed directly by the GC method. However, only the head space was analyzed in the studies of chloroform and plasma or blood in the case of dichlorotetrafluoroethane. To obtain maximum accuracy (using a larger volume for injection) and to avoid the GC detector response beyond the linear range, the high concentration gaseous samples were further diluted with air in 15–50-ml, sealed, empty bottles before analysis. Shaking[9] for 5 min was enough to reach an equilibrium state for the partitioning of the propellants between liquid and gaseous

[1] Supplied by E. I. duPont de Nemours & Co., Wilmington, Del.
[2] McGaw Laboratories, Glendale, Calif.
[3] Fisher Scientific Co., Fairlawn, N.J.
[4] Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Obtained from the University of Illinois blood bank.
[6] Wheaton Scientific, Millville, N.J.
[7] This stopper was preferred because it had a lacquer coating which could reduce or prevent sorption of propellants onto the rubber stopper. It was obtained from West Co., Phoenixville, Pa.
[8] Hamilton syringe 700, Supelco, Inc., Bellefonte, Pa.
[9] Vortex, Fisher Scientific Co., Springfield, Mass.
[10] Eberbach Corp., Ann Arbor, Mich.

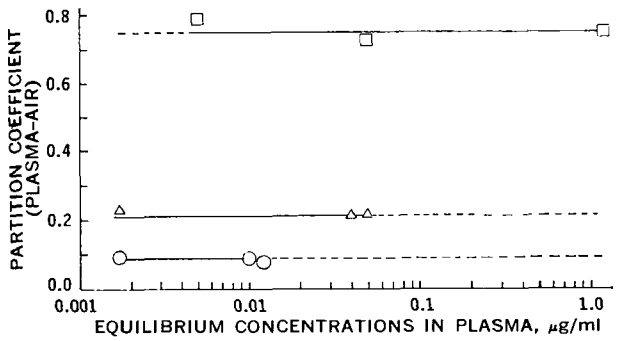

Figure 1—*Partition coefficients of fluorocarbon aerosol propellants between plasma and air as a function of concentration. Key:* □, *trichloromonofluoromethane;* ○, *dichlorodifluoromethane; and* △, *dichlorotetrafluoroethane.*

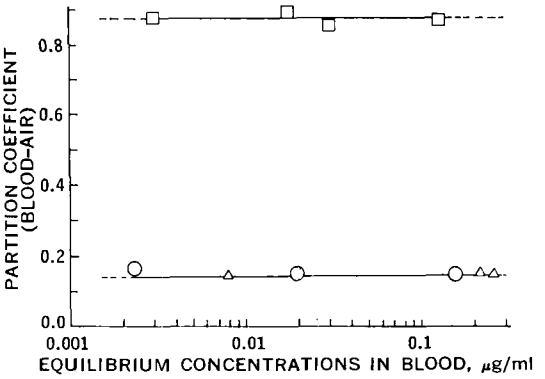

Figure 2—*Partition coefficients of fluorocarbon aerosol propellants between blood and air as a function of concentration. Key:* □, *trichloromonofluoromethane;* ○, *dichlorodifluoromethane; and* △, *dichlorotetrafluoroethane.*

phases. The concentrations of the propellants in the head space were essentially constant even after 2 hr of shaking.

The solubility, S, was calculated directly by dividing the concentration of the propellant in the liquid phase by that in the gaseous phase. It was also estimated indirectly by the following head-space method. After injecting the amount of the propellant contained in the 10 μl of normal saline, A_t, to the volume of the liquid phase, V_l, in the sealed serum bottle with a total internal volume of V_t, the following relationship based on material balance was obtained:

$$A_t = C_h(V_t - V_l) + C_lV_l \qquad \text{(Eq. 2)}$$

where C_h and C_l are the concentrations in the head space and liquid phase, respectively, at the equilibrium state. After rearranging Eq. 2:

$$S = \frac{C_l}{C_h} = \frac{A_t - C_h(V_t - V_l)}{C_hV_l} \qquad \text{(Eq. 3)}$$

Equation 3 enables one to estimate S by merely determining C_h since A_t, V_t, and V_l can be easily predetermined.

GC Analysis—A dual-column gas chromatograph[11] equipped with a tritium foil electron-capture detector was used. A 1.82-m (6-ft) U-shaped glass column (4 mm i.d.) packed with Porapak Q[12] (80–100 mesh) was used. The column temperature was 170°, and the injection port and the detector were maintained at 175°. The carrier gas (nitrogen) was used at a flow rate of 85 ml/min. For analysis of liquid samples, 1–10 μl was injected onto the column using a 10-μl syringe. For analysis of the head-space samples, 5–100 μl of the gaseous phase was injected onto the column using 50- or 100-μl gastight syringes[13]. All GC analyses were performed at least in duplicate, and reproducible results were obtained throughout the study. The retention times relative to the air peak were 0.9 min for dichlorodifluoromethane, 1.75 min for dichlorotetrafluoroethane, 3.25 min for trichloromonofluoromethane, 11 min for cyclohexane, and 8.0 min for chloroform.

RESULTS AND DISCUSSION

The average solubility data of the three fluorocarbon propellants determined at different concentrations in various media are summarized in Table I. No difference in solubility data could be found when determined either at 25 or 37°. In the past (1, 5, 8, 9), the solubilities of many anesthetic gases were often determined only at one concentration, assuming that they were independent of the concentration of the gases. Such an assumption may be wrong, for example, if the percentage of plasma protein binding of the anesthetic varies with its concentration in the plasma since the molecule in the head space is only thermodynamically in equilibrium with the unbound molecule and not with the bound molecule. The concentration-dependent protein binding of nonvolatile drugs is well known. Plots of solubilities of the propellants in human plasma and blood *versus* their equilibrium concentrations in plasma and blood are shown in Figs. 1 and 2. Within experimental errors, these results indicate that the solubilities or partition coefficients in plasma and blood were independent of concentration in the wide concentration range studied. Similar results were obtained in studies using water, normal saline, cyclohexane, and chloroform.

Identical results were obtained from the two methods of solubility determination for trichloromonofluoromethane and dichlorodifluoromethane. These findings were valuable because they showed that the simple indirect head-space method could be employed when the direct injection of the liquid phase such as plasma or blood onto the GC column was not desirable (7). Although the head-space method was used recently to study the solubilities of several volatile anesthetics in human and dog blood (9), the equilibration flask and tube used were more complicated than the serum bottle used in this study. Cowles *et al.* (9) used 50 ml of blood in each study, while 1–2 ml of the sample was enough in this study when using a 5-ml capacity bottle (currently used in this laboratory to study the solubilities of propellants in dog plasma and blood). The equilibration time used in their method was also much longer. Furthermore, the present method permits duplicate assay from the same bottle, and this is not easily feasible with their equilibration tube because some gas sample may be lost through the punctured hole of the parafilm.

The solubilities, expressed in terms of weight percent, of many fluorocarbon propellants in water at 1 atm and 25° were reported previously (10). Their solubility values as defined in this study can be theoretically calculated using the law of Gay-Lussac (11). The theoretical values for the three propellants studied are also shown in Table I. It is obvious that both the experimental and theoretical data are in good agreement. No salting-out effect was seen in normal saline, because the solubilities determined in water and normal saline were identical. This seems to indicate that hydration was not an important factor for the solution of these small and hydrophobic molecules.

The solubilities of the three propellants in plasma were all higher than those in normal saline. This was especially significant for trichloromonofluoromethane (3.5 times higher) and dichlorotetrafluoroethane (11 times higher). The enhancement of solubility was probably due to the binding of the propellants with proteins in the plasma. Further work is required to establish the mechanism for such a marked solubilization effect. The solubilities of trichloromonofluoromethane and dichlorodifluoromethane were higher in blood than in plasma, whereas the solubility of dichlorotetrafluoroethane was higher in plasma than in blood. These findings were similar to those of a previous report (9) which showed that the solubilities of ethylene and cyclopropane increased with an increase in hematocrit while halothane and ether showed an opposite effect.

The previously reported (5) solubility values for the three propellants in human serum are shown in Table I. Compared with the solubility data in plasma, it seems difficult to explain why

[11] Packard model 824, Packard Instrument Co., Downers Grove, Ill.
[12] Water Associates, Framingham, Mass.
[13] Hamilton syringe 7000, Supelco, Inc., Bellefonte, Pa.

Table I—Average Solubilities of Three Fluorocarbon Propellants in Various Media

	Water[a]	Water or Normal Saline	Human Plasma	Human Blood	Human[b] Serum	Cyclohexane	Chloroform
Trichloromonofluoromethane	0.19	0.22	0.76	0.87	0.9	75.4	73.3
Dichlorodifluoromethane	0.055	0.060	0.085	0.15	0.2	22.4	14.6
Dichlorotetrafluoroethane	0.019	0.020	0.22	0.15	0.2	35.1	19.3

[a] Values calculated from literature data. See text for calculation. [b] Obtained from Ref. 5.

Table II—Partition Coefficients of Three Fluorocarbon Propellants in Cyclohexane–Water and Chloroform–Water Systems

Fluorocarbon Propellants	*P*(Cyclohexane–Water)	*P*(Chloroform–Water)
Trichloromonofluoromethane	343	333
Dichlorodifluoromethane	373	243
Dichlorotetrafluoroethane	1755	965

the solubilities of trichloromonofluoromethane and dichlorodifluoromethane in serum were much higher when fibrinogen and other clotting factors were removed from the plasma. The data of dichlorotetrafluoroethane from the two studies, however, appear to be in reasonable agreement.

The solubility of a volatile compound in blood is one of the most important factors affecting the rate of absorption after inhalation. Both dichlorodifluoromethane and dichlorotetrafluoroethane had the same solubility in blood, which was approximately one-sixth of that of trichloromonofluoromethane. A similar relationship also existed for the early absorption rates of these propellants in humans. The data in Fig. 2 from Ref. 5 showed that both dichlorodifluoromethane and dichlorotetrafluoroethane were absorbed at the same rate and that the absorption rate of trichloromonofluoromethane in the first 40 sec was approximately five to six times higher than the other two propellants.

The low solubilities of the three propellants studied in water, normal saline, human plasma, and human blood indicate that special precautions must be exercised in handling these chemicals to prevent any loss through evaporation. This is especially important for dichlorodifluoromethane and dichlorotetrafluoroethane. Paterson *et al.* (4) attributed the low or negligible blood levels of these two propellants in humans after inhalation to the possible rapid loss of the propellants from the blood samples and recommended that special techniques of collection were needed. Although the solubilities of the three propellants in organic solvents such as chloroform and cyclohexane (Table I) are markedly higher, the possible loss to the head space or gaseous phase from these media cannot be ignored, particularly when the ratio of the volume of the gaseous phase to that of the liquid phase is high. For example, a 49% loss to the head space at equilibrium may result if 1 ml of dichlorodifluoromethane in chloroform is contained in a sealed 15-ml capacity bottle. In the GC analysis of propellants in blood, if the peak height response from the extracted fluid is compared directly with standards that have not been subjected to such an equilibration with the head space, a significant error may be anticipated. It seems that such an error might have been made in a previous study (12) using chloroform for extraction from blood samples. Unfortunately, the analytical procedure reported in that study was not detailed enough to permit one to make a definite conclusion.

The partition coefficients of the three propellants in cyclohexane-water and chloroform-water systems are shown in Table II. These were calculated by dividing the solubility in the organic solvent by that in water. The higher partition coefficients of dichlorotetrafluoroethane in both systems indicate that it may have a larger volume of distribution in the body due to its accumulation in lipoid tissues and longer biological half-life based on the terminal phase of the blood concentration–decay curve (13–15). The limited data of the human study by Morgan *et al.* (5), which showed a slightly smaller elimination rate (smaller retention decay in their Fig. 3) between 10 and 30 min after inhalation for dichlorotetrafluoroethane than for trichloromonofluoromethane, tend to support such a contention.

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[x] To whom inquiries should be directed.

Catharanthus Alkaloids XXIX: Isolation and Structure Elucidation of Vincoline

G. H. AYNILIAN*, S. G. WEISS*, G. A. CORDELL*, D. J. ABRAHAM‡, F. A. CRANE*, and N. R. FARNSWORTH* ˣ

Abstract □ Vincoline, an alkaloid of undetermined structure, previously isolated from *Catharanthus roseus, Catharanthus lanceus,* and *Vinca libanotica,* has been reisolated and its structure elucidated.

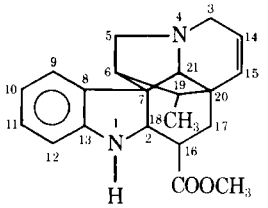
Keyphrases □ *Catharanthus* alkaloids—isolation, structure identification of vincoline □ *Vinca* alkaloids—isolation, structure identification of vincoline □ Vincoline—isolation, structure identification from *Catharanthus* and *Vinca* alkaloids

In the continuing search for novel alkaloids from Apocynaceae, the alkaloid vincoline was isolated from *Catharanthus roseus* and *Vinca libanotica.* This alkaloid was previously reported from *C. roseus* (1), from *C. lanceus* (2), and from *V. libanotica* (3); however, the structure of this base had not been deduced. In 1966, when this alkaloid was first isolated from *C. lanceus* (2), insufficient material was obtained to determine the structure. More recently, vincoline was isolated from the previncaleukoblastine fraction of *C. roseus* leaves in the same manner as described by Svoboda *et al.* (4) and simultaneously from *V. libanotica* (3). The structure elucidation studies reported at this time were made possible by the increased quantity of base available.

EXPERIMENTAL

Source and Properties of Vincoline—The vincoline used in this study was obtained from the crude previncaleukoblastine alkaloid fraction (4) of *C. roseus*[1]. It was found to have a melting point of 228–232°; UV[2] λ_{max}: 245 (log ϵ 3.91) and 299 (3.56) nm; $[\alpha]_D^{29}$ −242° (concentration 0.5% in chloroform); and important bands in the IR spectrum[3] at ν_{max} 3480 (OH), 3350 (NH), 1715 (ester), 1600 (indoline), and 740 (1,2-disubstituted benzene) cm^{-1}.

Acetylation of Vincoline—A solution of 5 mg of vincoline, 0.15 ml pyridine, and 0.2 ml acetic anhydride was heated at 45° for 4 hr. The solution was basified with 10% NH_4OH and extracted with three 5-ml portions of ether. After drying the combined ether solutions over anhydrous sodium sulfate, the solution was filtered and concentrated to afford a pure (TLC) but oily product. Mass spectral evidence (M^+ 410) indicated that monoacetylation had occurred.



I: vindolinine

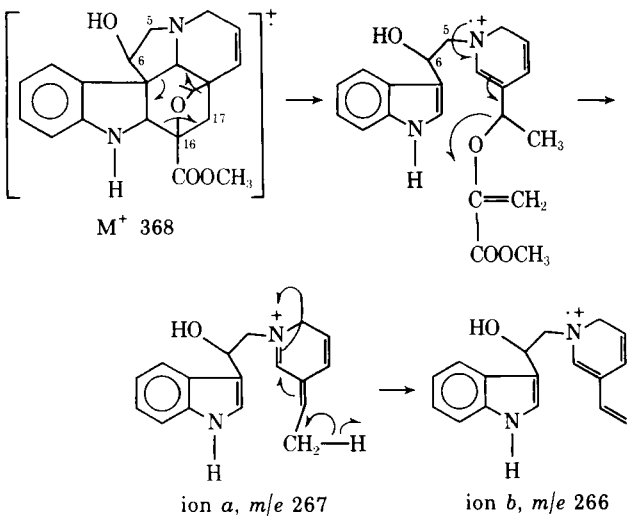
Attempted Sodium Borohydride Reduction of Vincoline—To a methanolic solution of 5 mg of vincoline, 5 mg of sodium borohydride was added. The solution was stirred for several hours at room temperature, neutralized, and extracted for alkaloid material with ethyl acetate. TLC and mass spectral data revealed that no reaction had occurred. The procedure was repeated several times under a variety of conditions, but no reaction was observed.

DISCUSSION

According to Svoboda *et al.* (1), the IR and NMR spectra of vincoline showed it to be related to vindolinine[4] (I); furthermore, the NMR spectrum showed the presence of a methoxyl group and indicated a dihydroindole moiety unsubstituted at *N*-1.

An examination of the NMR spectrum of vincoline[5] (Table I) revealed a multiplet between 7.11 and 6.44 ppm, integrating for four protons, together with a broad singlet at 5.56 ppm, indicating the presence of an indole moiety with the *N*-1 unsubstituted. Exchange of this proton with deuterium oxide confirmed this assignment. A 12-line pattern was assigned to two olefinic protons having two nonequivalent proton neighbors (C-3), while their chemical shift indicated the presence of a nearby deshielding group (*N*-4) (5). Therefore, the double bond was located at the expected position (5, 6). A singlet at 3.82 ppm indicated the presence of a methoxyl group. The presence of a methyl group on a carbon bearing one proton was indicated by a doublet (J = 6.6 Hz) at 0.60 ppm. This hinted that probably C-19 was attached to C-6, as in vindolinine (I).

The mass spectrum[6] of vincoline gave major peaks at M^+ 368 (55%), *m/e* 350 (25), 281 (25), 267 (100), 266 (62), 223 (16), 170 (12), 169 (10), 160 (68), 146 (10), 136 (14), 122 (23), 121 (44), 108 (62), 107 (10), and 93 (20). The molecular formula of vincoline,



M^+ 368

ion *a*, *m/e* 267 ion *b*, *m/e* 266

Scheme I

[1] The plants were cultivated at the Department of Pharmacognosy and Pharmacology, Drug and Horticultural Experiment Station, Downers Grove, Ill. The crude alkaloid fraction was prepared from about 1000 kg of air-dried leaves by Eli Lilly and Co., Indianapolis, Ind. The plant material was authenticated by F. A. Crane as *C. roseus* (L.) G. Don (Apocynaceae), and voucher specimens representing the collection were deposited in the Herbarium of the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, Ill.

[2] Recorded using a Beckman model DB-G grating UV spectrophotometer.

[3] Recorded using a Beckman model IR-18A IR spectrophotometer.

[4] The number system shown in the structure is that of J. LeMen and W. I. Taylor, *Experientia,* **21**, 508(1965).

[5] Recorded using a Brüker 90-MHz NMR spectrometer.

[6] Recorded using a Hitachi Perkin-Elmer model RMU-6D mass spectrometer.

Table I—NMR Spectrum of Vincoline[a]

Chemical Shifts, ppm	Integration for Protons	Pattern of Splitting	Coupling Constants
7.11–6.44 (aromatic protons)	4	Multiplet	—
5.37–5.89 (olefins)	2	Multiplet	—
5.56 (NH)	1	Broad singlet	—
3.89 (C*H*—CH_3)	1	Quartet	J = 6.6 Hz
3.82 ($COOCH_3$)	3	Singlet	—
3.81 (C—2H)	1	Singlet	—
0.60 (CH—C*H*$_3$)	3	Doublet	J = 6.6 Hz
0.6–3.7	9	Complex system which could not be interpreted	—

[a] The spectrum was recorded at 90 MHz.

Table II—High-Resolution Mass Spectral Measurements for Vincoline

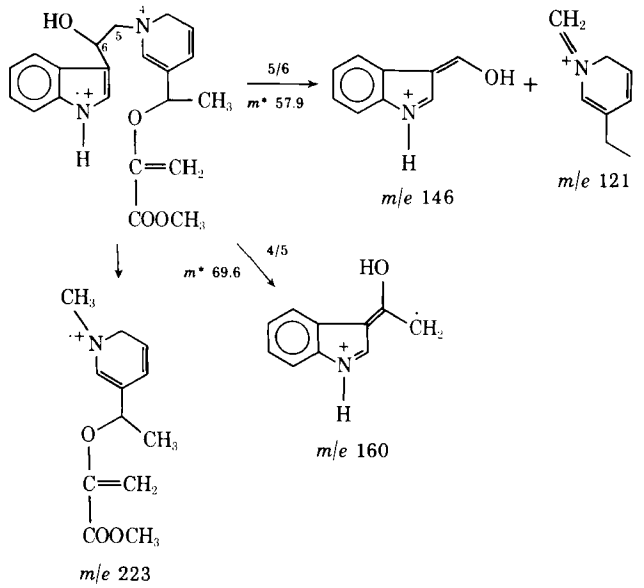
Formula	Observed m/e	Calculated m/e
C_6H_7N	93.05785	93.05820
$C_7H_{10}N$	108.08132	108.08301
$C_8H_{10}N$	120.08132	120.08265
$C_8H_{11}N$	121.08915	121.09022
$C_8H_{12}N$	122.09697	122.09737
C_9H_8NO	146.06059	146.06080
$C_{10}H_{10}NO$	160.07624	160.07852
$C_{12}H_{17}NO_3$	223.12084	223.12353
$C_{17}H_5N_2$	247.12352	246.12352
$C_{17}H_{18}N_2O$	266.14191	266.14377
$C_{17}H_{19}N_2O$	267.14526	267.14481
$C_{18}H_{21}N_2O$	281.1653	281.1654
$C_{19}H_{19}N_2O$	291.14974	291.15071
$C_{19}H_{21}N_2O_2$	309.16030	309.15999
$C_{21}H_{22}N_2O_3$	350.16304	350.16009
$C_{21}H_{24}N_2O_4$	368.17361	368.17094

$C_{21}H_{24}N_2O_4$, was established by high-resolution mass spectral measurements[7] (Table II). The mass spectral fragmentation pattern indicated the compound to have an aspidospermine-type skeleton (7). In the mass spectrum of many aspidospermine-type alkaloids, a retro-Diels–Alder reaction occurs, expelling C-16 and C-17 with any substituents (6, 8). In the case of vincoline, this process afforded the base peak in the spectrum, ion *a* at m/e 267, and a close relative, ion *b* at m/e 266 (Scheme I). High-resolution mass measurements of these two species indicated molecular formulas of $C_{17}H_{19}N_2O$ and $C_{17}H_{18}N_2O$, respectively. Since these two species contain only one oxygen atom each, the carbomethoxy group could be at either C_{16} or C_{17} and expelled by the retro-Diels–Alder reaction. In view of the overwhelming biogenetic precedence for the existence of such a carbomethoxy grouping at C_{16} in all related indole alkaloids (6), it was placed at C_{16} rather than C_{17} in vincoline.

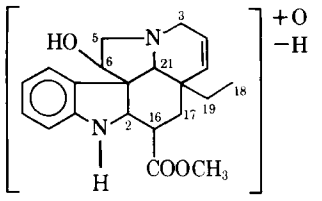
Acetylation (acetic anhydride–pyridine) afforded only a monoacetyl derivative (mol. wt. 410), suggesting that only one oxygen was in the form of an alcohol. The mass spectrum of the monoacetyl derivative showed a base peak at m/e 309, 42 mass units higher than the base peak of vincoline. It followed that the oxygen atom in the ions at m/e 267 and 266 was in the form of an alcohol. The possibility of a carbinolamine at C-2, C-3, C-5, or C-21 was ruled out when vincoline remained unchanged after treatment with sodium borohydride, suggesting that the alcohol function was at C-6. Additional evidence for this assignment was the occurrence of a fragment at m/e 160 (68%) ($C_{10}H_{10}NO$), consisting of an indole moiety with two carbon atoms, placing the hydroxyl group at C-5 or C-6. The base peak underwent 5–6 cleavage to give fragments at m/e 146 and 121 (Scheme II and Table II). Analysis of these ions showed that only the former contained oxygen. The rearranged parent ion also underwent 5–6 cleavage to give ions at m/e 146 and 223, the latter containing three of the four oxygens (Scheme II). The hydroxyl group was thus placed at C-6. This eliminated the vindolinine skeleton for vincoline; at this stage, Structure II could be written for this base.

The nature of the fourth oxygen could not be determined directly. The presence of the two peaks in the mass spectrum at m/e 267 and 266 indicated attachment of this oxygen at either C-16 or C-17. However, if it were a hydroxyl group, acetylation would have yielded a diacetyl derivative instead of a monoacetate.

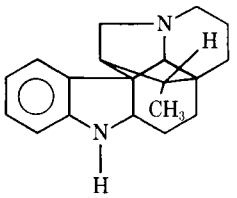
The nature of this oxygen atom was determined from NMR studies. The NMR spectrum, as mentioned earlier, showed a doublet integrating for three protons and having a chemical shift of 0.60 ppm (J = 6.6 Hz). This clearly indicated the presence of a methyl group (C-18) attached to a carbon atom (C-19) with a sin-



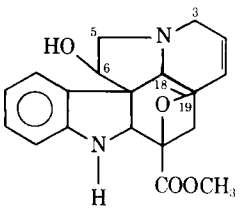
Scheme II



II

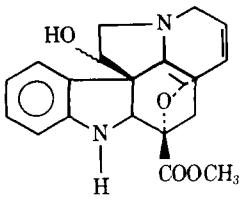


III: tuboxenine

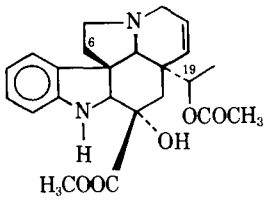


IV

[7] Recorded using a double-focusing mass spectrometer, model 110 (Consolidated Electro Dynamics Co.), operating at 70 ev.



V: vincoline



VI

gle proton. Double-resonance studies of tuboxenine (III), a vindolinine-type alkaloid, showed that this methine proton (C-19) gave a chemical shift of 1.7 ppm (5, 9), with a J value of about 7 Hz.

A double-resonance study of vincoline showed the methine proton to be a quartet (J = 6.6 Hz) centered at 3.89 ppm. However, only three lines were apparent, the fourth line being covered by the carbomethoxy signal. It was clear that C-19 was attached (in addition to C-20, C-18, and a proton) to an electronegative atom, in this case, oxygen. Therefore, the fourth oxygen moiety was in the form of an ether and, by points presented previously, was attached at C-16 or C-17. If a C-19—O—C-17 linkage existed, then the methine proton at C-17 should have been downfield at about 4.5 ppm (10). However, the region between 4.0 and 5.3 ppm was devoid of absorption. The only other possible linkage was one involving C-19—O—C-16. Structure IV was thus proposed for vincoline at this point.

Vincoline showed a negative rotation, so that the tryptamine bridge was beta (11). Molecular models indicated that stable structures could be derived if C-19, C-18 was either *cis* or *trans* to the tryptamine bridge. The chemical shift (0.60) of the methyl group at C-18 was best accounted for by the strong shielding effect of the aromatic ring current. In the structure where the tryptamine bridge and C-18, C-19 are *cis*, this possibility does not arise. However, in one of the C-19 epimers, when the tryptamine bridge and C-18, C-19 are *trans*, a close proximity of the dihydroindoline nucleus and C-18 is apparent. It is therefore suggested that vincoline has Structure V, omitting the stereochemistry of the hydroxyl group. The occurrence of a *cis*-relationship between C-18, C-19, and C-5, C-6 is rare, examples thus far only being observed in the vindolinine series. Biogenetically, vincoline may be produced from a molecule such as VI *via* nucleophilic attack at C-19 with subsequent hydroxylation at C-6. This again suggests a *trans*-relationship of these groups.

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ˣ To whom inquiries should be directed.

Metabolic Fate of *N,N*-Dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide in Rat, Dog, and Human

F. F. SUN[x] and J. E. STAFFORD

Abstract □ The patterns of urinary excretion products of *N,N*-dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide (I) in the rat, dog, and human have been examined. After oral administration, the drug was extensively metabolized in all species and the amount of intact drug excreted in the urine was negligible (<1%). The metabolic pathways were *via* hydroxylation of the phenoxy ring, *N*-demethylation, *O*-methylation, and conjugation. The following metabolites have been identified in free and/or conjugated form: *N*-methyl-*N'*-(*p*-phenoxyphenyl)sulfamide (II), *N,N*-dimethyl-*N'*-*p*-(4-hydroxyphenoxy)phenylsulfamide (III), *N,N*-dimethyl-*N'*-*p*-(2-hydroxyphenoxy)phenylsulfamide (IV), *N*-methyl-*N'*-*p*-(4-hydroxyphenoxy)phenylsulfamide (V), *N*-methyl-*N'*-*p*-(2-hydroxyphenoxy)phenylsulfamide (VI), *N,N*-dimethyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VII), and *N*-methyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VIII). The major differences in the patterns of urinary metabolites were: (*a*) the monomethyl (II) was the major metabolite in the dog and human but a minor one in the rat, (*b*) the *para*-hydroxylated compound (III) was the major metabolite in the rat, (*c*) hydroxylation of the phenoxy ring in the *ortho*-position (IV and VI) was observed in the rat and dog but not in the human, and (*d*) the methoxylated compound (VII) was found only in rat urine.

Keyphrases □ *N,N*-Dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide—urinary excretion after oral administration, metabolic pathways, rat, dog, man □ Urinary excretion—metabolites of *N,N*-dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide, oral administration, rat, dog, man □ Metabolism, *N,N*-dimethyl-*N'*-(*p*-phenoxyphenyl)sulfamide—urinary products after oral administration, rat, dog, man

N,N-Dimethyl- *N'* -(*p*-phenoxyphenyl)sulfamide[1] (I) reduces cholesterol levels significantly in both tyloxapol[2]-treated and cholesterol–cholic acid-fed rats (1). It has no significant effect on the cholesterol levels in normal rats and dogs. TLC analyses indicated that the drug was extensively metabolized. The results from studies designed to characterize the metabolism of I in the rat, dog, and human are reported here.

EXPERIMENTAL

Dosage and Collection of Samples—*Rat*—Micronized I was suspended in 0.25% methylcellulose in sterile water[3] with a glass Teflon homogenizer. The suspension was administered *via* oral intubation to six Sprague–Dawley rats (three males and three females) at a dose level of 100 mg/kg. A second group of six rats (three males and three females) that received the vehicle only served as the control. The rats were dosed daily for 21 days. Urine samples were collected daily, pooled, and kept frozen until analyzed.

Dog—Four dogs, participating in chronic toxicity studies, had received daily oral doses of 100 mg/kg I for approximately 10 months. Urine samples were collected by catheterization at 4–8 hr after administration of the drug. Urine samples were also obtained from untreated animals.

Human—Four subjects each received a single 500-mg oral dose of I in hard-filled capsules. Urine samples were collected from −12 to 0 and from 0 to 24 hr after drug administration. The postadministration urine for each subject was pooled. Urine collected prior to drug administration was used as the control.

Extraction and Purification—Aliquots of the urine samples (150–200 ml) were acidified to pH 3.5 with acetic acid and extracted six times with an equal volume of chloroform. The chloroform layers were separated, pooled, and concentrated under reduced pressure. Residual chloroform in the extracted urine specimens was removed at 45° under reduced pressure (1 hr). The pH of the urine was adjusted to 5.0 with 1 *M* sodium acetate buffer (pH 5.0), and 1/100 volume of a concentrated β-glucuronidase aryl sulfatase solution[4] was added; the mixture was then incubated at 37° for 24 hr. The hydrolysis mixture was acidified to pH 3.5 and extracted three times with an equal volume of chloroform. The extracts were pooled and concentrated under reduced pressure. The control urine samples were processed in the same manner.

The concentrated extracts were streaked at the origin across three-fourths of the width of a 2-mm thick silica gel G-254 preparative TLC plate[5]. The corresponding control urine extract was streaked on the remaining one-fourth of the width of the plate. The plate was developed in a solvent system of chloroform–methanol (95:5). To obtain good resolution, the plate was developed, dried, and redeveloped three to five times. After development was completed, the chromatogram was visualized with a 254-nm UV lamp.

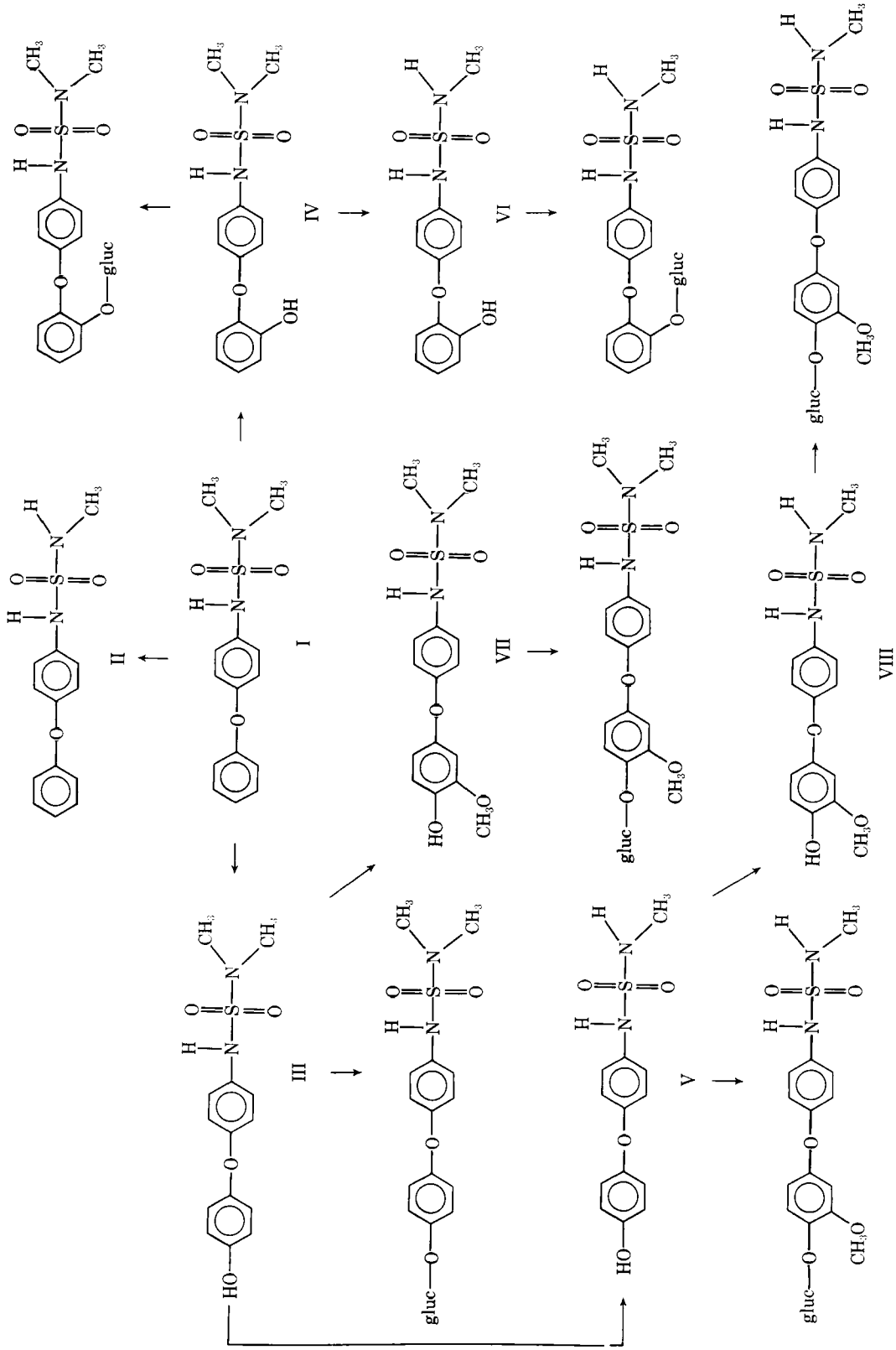
Zones that were present in the urine extracts from drug-treated subjects but not in the controls were scraped off the plate and eluted with chloroform–methanol (9:1). The purified extracts were evaporated to dryness. The residues were dissolved in a small amount of ethanol-free chloroform for introduction into the gas chromatograph[6]–mass spectrometer.

Identification of Metabolites—Identification of the urinary metabolites of I was carried out by using mass spectrometry, IR spectroscopy, and, when possible, NMR spectroscopy. Mass spectrometric analysis was routinely carried out as follows: 5 μl of a sample and 2 μl of bis(trimethylsilyl)acetamide were injected simultaneously into the gas chromatograph–mass spectrometer. A 1.21-m (4-ft) column of 1% SE-30 (methylpolysiloxy gum) on Gas Chrom Q was maintained at 210°. The mass spectrometer was operated at an ionization potential of 70 ev with an ion-source temperature of 290°. On-column silylation was selected for simplicity and conservation of material. Unless the metabolite being studied was contaminated with large amounts of urinary impurities, the on-column silylation technique generally provided a completely silylated derivative of the compound.

IR and NMR analyses were carried out without silylation. IR spectra were obtained with a spectrophotometer[7] and micro KBr pellets containing 50–100 μg of the sample. Since the NMR analysis[8] required 3–5 mg of sample, it was done only when that amount of material was available.

TLC Characterization of I Metabolites—Specific spray reagents were used to detect the phenolic and nonphenolic metabolites on the thin-layer chromatograms. Phenolic metabolites appeared immediately as a pink spot on the plate when sprayed with either of the following two reagents: (*a*) a freshly mixed 1:1 solution of 0.5% (w/v) aqueous ferric chloride and 0.5% (w/v) methanolic dipyridyl, and (*b*) a freshly mixed 1:1 solution of 0.5%

[1] V-25,030.
[2] Triton WR-1339.
[3] Upjohn Vehicle 122.
[4] Sigma type H-2 β-glucuronidase No. G-0876, 143,000 units/ml.
[5] Brinkmann.
[6] LKB-9000.
[7] Perkin-Elmer model 421.
[8] Varian XL-100.



Scheme I.—Proposed metabolic pathway of I in rat, dog, and human (gluc = glucuronide)

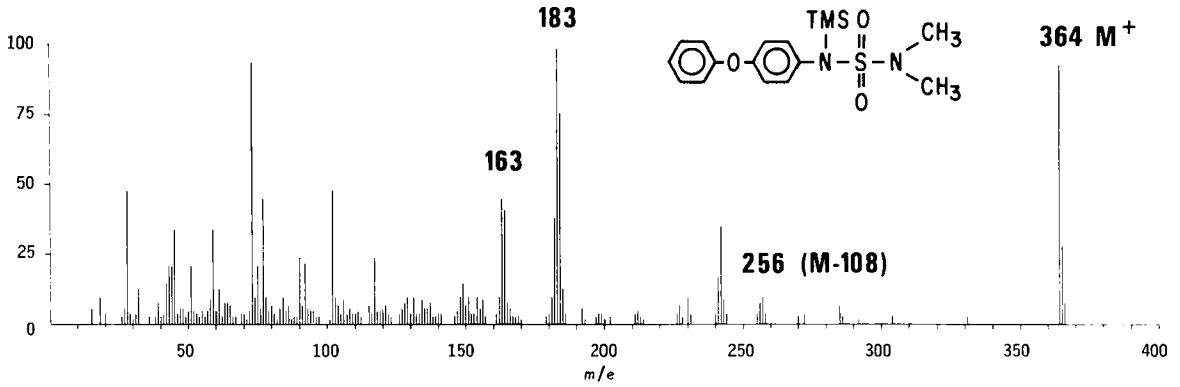


Figure 1—*Mass spectrum of trimethylsilyl derivative of I.*

benzidine in 0.17 *M* HCl and 10% aqueous sodium nitrite. These sprays were also used to detect any drug-related compounds obscured by other UV-absorbing materials present in the chromatographed urine extracts.

RESULTS AND DISCUSSION

Isolation and characterization studies of the urinary excretion products of I indicated extensive metabolism (Scheme I) of the drug in animals and humans. The metabolites were present in the urine mainly as glucuronide and/or sulfate conjugates. A summary of all detectable drug-related compounds found in the urine of I-treated rats, dogs, and humans is presented in Table I. A metabolic map, based on this information, is presented in Scheme I.

Combined GLC–mass spectrometry were used extensively to determine the structure of the metabolites. The mass spectrum of the trimethylsilyl derivative of I is presented in Fig. 1. The drug shows a strong molecular ion peak at *m/e* 364. Cleavage between the aniline and sulfamoyl group (M−108) gave another peak at 256. In the metabolism studies, the mass of these two fragments provided important information of *N*-demethylation pathways. The free phenoxyaniline ions appeared at *m/e* 183 and 184. Further fragmentation of the phenoxyaniline part of the molecule gave mass peaks at 163 and 164, which served as indicators of any metabolic modifications in the aromatic system.

Negligible amounts of unchanged drug were found in the urine specimens from animals and humans. In the rat and dog, the presence of trace amounts of the unchanged drug could be demonstrated only by GLC–mass spectrometric analysis. The relative amounts of each metabolite excreted were estimated from the size of the peaks on the gas chromatogram (Fig. 2). Evidence for the identification of the major metabolites is summarized below. The common mass peaks appearing in the mass spectra of I-related compounds are presented in Table II.

***N*-Methyl-*N'*-(*p*-phenoxyphenyl)sulfamide (II)**—This compound is the *N*-demethylation product of I found in the urine of the dog and human in unconjugated form. It was also found in the urine of the rat but only in trace amounts. The mass spectrum of the *N,N'*-bis(trimethylsilyl) derivative of II is shown in Fig. 3. The molecular ion was at *m/e* 422. Loss of the sulfamoyl group (M − 166) showed the material to be the desmethyl derivative of I. The fragmentation peaks at 163 and 164 indicated that the aromatic system was not modified. The most prominent peak in the spectrum was at *m/e* 198. Precise mass measurement gave the empirical formula of $C_{13}H_{12}NO$ (calc. 198.09189, found 198.09142). These results suggested that a methyl from one tri-

Table I—Summary of All Drug-Related Compounds Found in the Urine of Rat, Dog, and Human

Compound	Form of Excretion		Urine in which Compound Was Found		
	Free	Conjugated	Rat	Dog	Human
I	+	−	Trace	Trace	Trace
II	+	−	Trace	+	+
III	+	+	+	+	+
IV	+	+	+	+	−
V	+	+	+	+	+
VI	−	+	+	−	−
VII	−	+	+	−	−
VIII	−	+	+	+	+

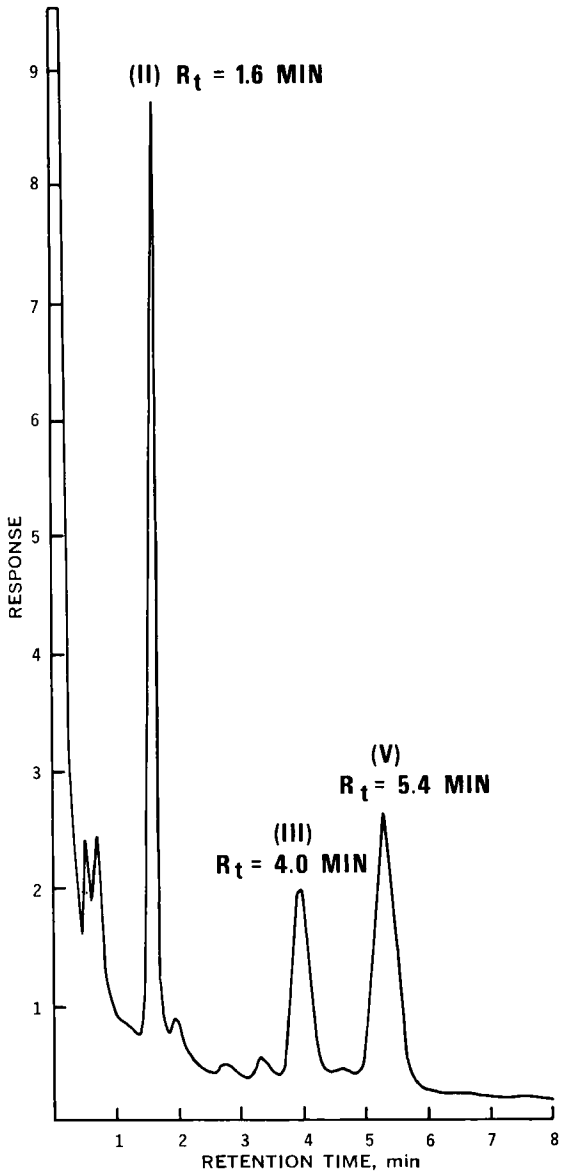


Figure 2—*Gas–liquid chromatogram of β-glucuronidase aryl sulfatase hydrolyzed (37° for 24 hr) human urine extract.*

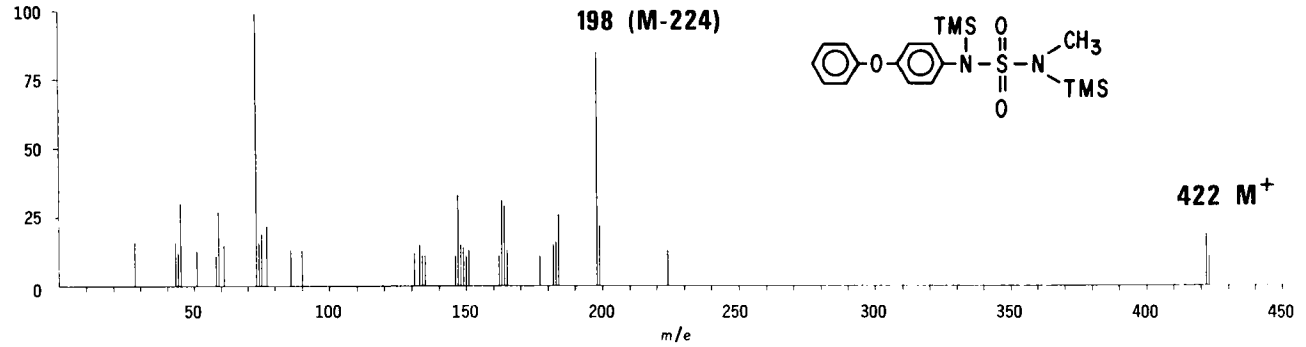


Figure 3—*Mass spectrum of bis(trimethylsilyl) derivative of II.*

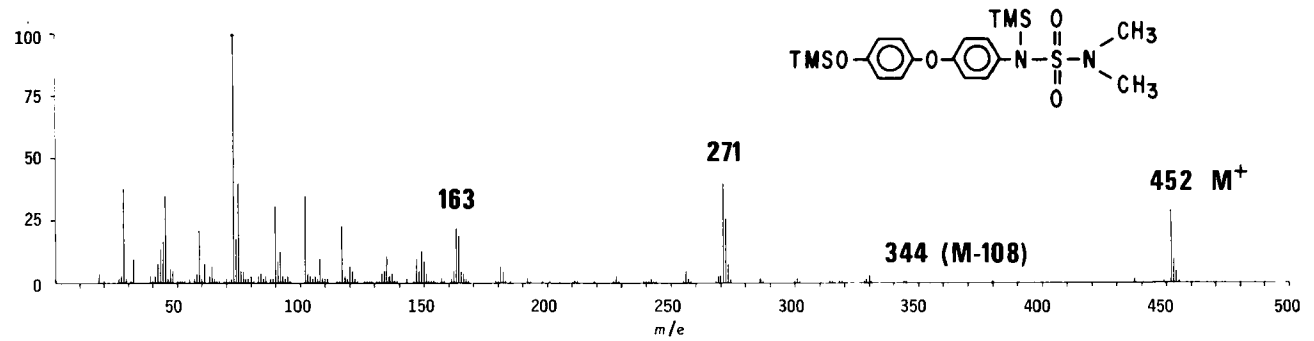


Figure 4—*Mass spectrum of bis(trimethylsilyl) derivative of III.*

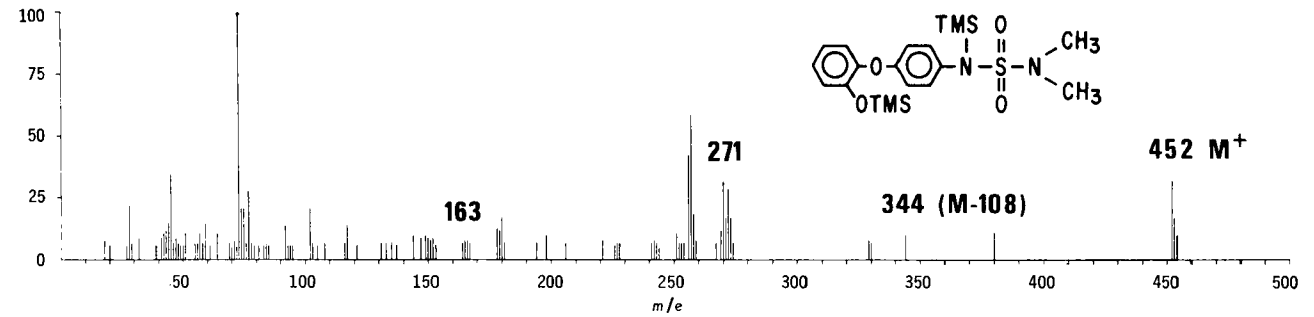


Figure 5—*Mass spectrum of bis(trimethylsilyl) derivative of IV.*

methylsilyl group migrated to the phenoxyaniline system upon electron impact. This unique property was shared by all desmethyl I-related compounds (Metabolites V, VI, and VIII).

Both IR and UV spectra of II were identical to unchanged I. The chromatographic and spectroscopic characteristics of II, synthesized independently, were identical with the isolated material. The hypolipidemic activity of II was equivalent to the parent, I.

***N,N*-Dimethyl-*N'*-*p*-(4-hydroxyphenoxy)phenylsulfamide (III)**—This metabolite was found both free and conjugated in the urine of all three species.

The mass spectrum of the bis(trimethylsilyl) derivative (Fig. 4) showed a molecular ion at *m/e* 452. Loss of 108 indicated that the sulfamoyl group was intact. The presence of the 163 and 164 pair indicated that the hydroxyl substitution did not affect the cleavage between the phenoxy oxygen and the aniline ring. Loss of the trimethylsilyl group from the aniline nitrogen and the loss of trimethylsilanol from the phenoxy ring gave strong mass peaks at *m/e* 271 and 181.

UV spectra of III were obtained in 95% ethanol and also with the addition of base (0.1 *N* KOH). In the absence of base, the spectrum showed a strong band at 238 nm and a weak band at 282 nm, which was similar to the parent compound (238, 270, and 278 nm). In the presence of 0.1 *N* KOH, the strong band was shifted to 252 nm and the weak band was shifted to 299 nm. The intensity of both bands was increased. The behavior was consistent with a monohydric phenol (4). The IR spectrum showed bands at 1295 and 1270 cm^{-1} which were phenol C—O vibrations. The features of the aromatic system and the sulfamoyl group of the parent compound were preserved.

The assignment of the hydroxyl group to the *para*-position of the phenoxy ring was confirmed by NMR. The spectrum showed two sets of *AA'BB'* multiplets, which indicated that all aromatic substitutions were *para*. On the thin-layer plate, III gave a strong positive response when sprayed with bipyridyl-ferric chloride reagent or benzidine reagent, which indicated the presence of the phenolic functional group.

Compound III, synthesized independently, had identical chromatographic and spectroscopic characteristics to the isolated material. The hypolipidemic activity of III was also equivalent to the parent, I.

***N,N*-Dimethyl-*N'*-*p*-(2-hydroxyphenoxy)phenylsulfamide (IV)**—This compound was found by its distinct chromatographic behavior both by TLC and GLC. Compound IV migrated more rapidly on silica gel G-254 than did III and had an R_f value very close to II. Compounds II and IV were distinguished from each other by spraying the plate with bipyridyl-ferric chloride reagent; IV appeared as a pink spot, whereas II remained colorless. Upon GLC, the trimethylsilyl derivative of IV (retention time 1.8 min) emerged much faster than the corresponding derivative of III (retention time 4.4 min). The mass spectrum (Fig. 5) of the bis(trimethylsilyl) derivative of IV showed the molecular ion at *m/e* 452, identical with its 4-isomer. The fragmentation pattern of IV

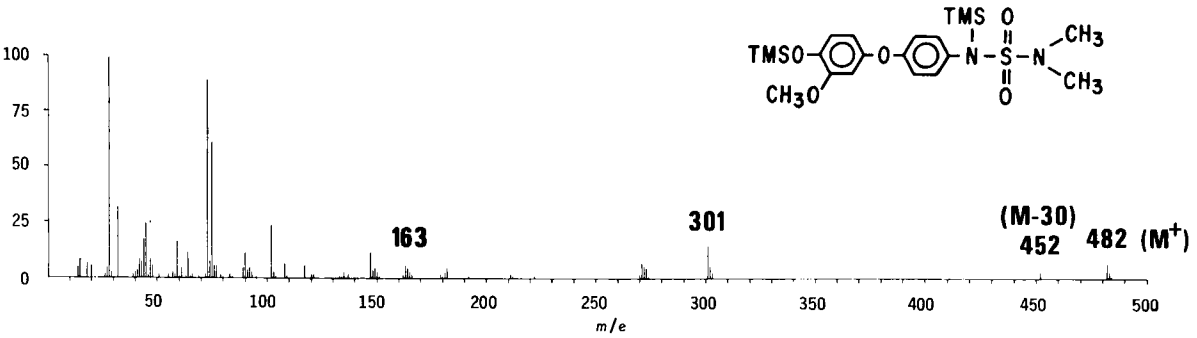


Figure 6—*Mass spectrum of bis(trimethylsilyl) derivative of VII.*

was similar to III except for the lack of the 163 and 164 pair, which indicated that a metabolic modification had occurred in the proximity of the oxygen between the two phenyl rings.

The NMR spectrum of IV could not be factored with certainty because the eight aromatic hydrogens form two highly coupled sets that were overlapping. One set of the multiplet was identified as an *AA'BB'* pattern, indicating that one phenyl ring had symmetrical *para-para* substitution. Thus, the possibility of hydroxylation on the aniline ring was ruled out. Hydroxylation in the *meta*-position relative to the phenoxy oxygen was unlikely because of the unfavorable inductive effect. Therefore, the hydroxyl group in IV was assigned to the *ortho*-position. The IR spectra of III and IV were similar.

Compound IV was found in both free and conjugated forms in the urine of the rat and dog *only*. It was not found in human urine during the 24 hr after drug administration.

***N*-Methyl-*N'*-*p*-(4-hydroxyphenoxy)phenylsulfamide (V)**—The *N*-desmethyl-4-hydroxy metabolite, both free and conjugated, was found in the urine of all three species. The mass spectrum of the trimethylsilyl derivative showed that the molecular ion was at *m/e* 510. Fragments at *m/e* 344 (M − 166) and 286 (M − 244) were characteristic of *N*-demethylated I. The presence of the 163 and 164 pair suggested that the hydroxyl substitution was not *ortho* to the phenoxy oxygen. Loss of trimethylsilanol was indicative of the relation of V with III.

The presence of a phenolic hydroxyl group in V was established by its UV spectra, reactivity with bipyridyl-ferric chloride reagent, and NMR spectrum. The UV spectrum taken in a neutral ethanol solution resembled that of the parent drug (238 and 282 nm). When the spectrum was redetermined at alkaline pH, a bathochromic shift of both bands to 252 and 298 nm, respectively, was observed, as was found for III, which suggested the presence of a monohydric phenol. NMR spectra showed two sets of *AA'BB'* multiplets, suggesting that all aromatic substitutions were *para*. The IR spectrum was also consistent with the structure assignment.

***N*-Methyl-*N'*-*p*-(2-hydroxyphenoxy)phenylsulfamide (VI)**—The metabolite was found in conjugated form in rat urine only. It was not isolated and its presence was established by GLC–mass spectrometric analysis of the partially purified extract containing III and VII. The mass spectrum of the trimethylsilyl derivative was identical to that of V except for the lack of the 163 and 164 pair. This suggested that VI was the 2-isomer of V (the *N*-demethylated analog of IV). On TLC, VI had a higher R_f than V and reacted with bipyridyl-ferric chloride reagent to give a pink-colored spot.

***N,N*-Dimethyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VII)**—Compound VII was a minor metabolite found only as a conjugate in rat urine. It had the same R_f value as III on TLC but was separated from III by repeated TLC. The mass spectrum (Fig. 6) of the trimethylsilyl derivative showed a molecular ion at *m/e* 482. Loss of 108 mass units indicated that the dimethyl sulfamoyl group was intact. Loss of the trimethylsilyl group from the aniline nitrogen (M − 72) followed by the loss of trimethylsilanol gave two prominent ions at 301 and 211. Presence of the 163 and 164 pair indicated that substitutions on the aromatic system were not in the close proximity of the diphenyl ether oxygen. Loss of 30 (CH_2O) and 31 (CH_3O) from all major peaks indicated the presence of a phenyl methyl ether linkage (5).

Since the amount of this compound in the urine was very

Table II—Common Mass Peaks Appearing in Mass Spectra of I-Related Compounds

m/e	Structure	Comments
73	$[Si(CH_3)_3]^+$	Appears in all spectra
75	$[HO{=}Si(CH_3)_2]^+$	Appears in all spectra of phenolic compounds
163	$[C_6H_5N{-}Si(CH_3)_2]^+$	Appears in all spectra except IV and VI
90	$[HO{-}Si(CH_3)_3]^+$	Appears in all phenolics
180	$[HO{-}C_6H_5N{-}Si(CH_3)_3]^+$	Appears in all phenolics
271	$[(CH_3)_3SiO{-}C_6H_5O{-}C_6H_5NH]^+$	Appears in all phenolics
256	$[C_6H_5OC_6H_5N{-}Si(CH_3)_3]^+$	Appears in I and II
185	$(C_6H_5OC_6H_5NH)^+$	Appears in I and II
344	$[(CH_3)_3SiO{-}C_6H_5OC_6H_5N{-}Si(CH_3)_3]^+$	Appears in all phenolics
198	$(C_6H_5OC_6H_5NCH_3)^+$	Appears in II
286	$[(CH_3)_3SiO{-}C_6H_5OC_6H_5NCH_3]^+$	Appears in all *N*-desmethyl hydroxylated metabolites
316	$[(CH_3)_3SiO{-}C_6H_5OC_6H_5NCH_3]^+$	Appears in VIII
M − 15	Loss of CH_3	Appears in all spectra
M − 30 or M − 31	Loss of HCHO or CH_3O	Appears in all metabolites with methoxyl group
M − 108	Loss of $SO_2N(CH_3)_2$	Appears in all compounds with sulfamide intact
M − 166	Loss of $SO_2N(CH_3)Si(CH_3)_3$	Appears in all desmethyl metabolites
M − 224	Loss of $SO_2N(CH_3)Si(CH_3)_3$ and trimethylsilyl from nitrogen	Appears in all desmethyl metabolites

small, it could not be isolated for further spectral analysis. Therefore, the positions of hydroxy and methoxy substitution on the aromatic system were assigned, based on the following information: (*a*) the presence of the 163 and 164 pair in the mass spectrum precluded the possibility of any substitution located *ortho* to the diphenyl ether oxygen; (*b*) the product of enzymatic hydroxylation of *para*-substituted monohydric phenols is catechol, which undergoes *O*-methylation yielding *para*-substituted *ortho*-methoxy phenol (6); and (*c*) catechol *O*-methyltransferase does not *O*-methylate resorcinol derivatives (7).

***N*-Methyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VIII)**—Compound VIII is the *N*-demethylation product of VII. It was found in the urine of all three species in conjugated form. The amount was very small but was separated from the crude urine extract by repeated TLC. The mass spectrum of VIII of the trimethylsilyl derivative showed that the molecular ion was at *m/e* 540. Loss of the *N*-trimethylsilyl-*N*-methylsulfamoyl group (M − 166) and the appearance of the 4-hydroxy-3-methoxy-*N*-methylphenoxyaniline ion at 316 (M − 224) indicated a monomethyl sulfamide analog. Loss of 30 or 31 mass units suggested it was a methoxy phenol. Presence of the 163 and 164 pair indicated that the hydroxyl and methoxy substitutions were not *ortho* to the phenoxy oxygen.

The NMR spectrum of VIII was difficult to interpret because of the overlapping of aromatic protons. However, a set of *AA'BB'* multiplets indicated one phenyl ring had *para-para* symmetric disubstitution. This ruled out the possibility that the aniline ring was modified. Based on the same argument as for VII, VIII was assigned the structure of the *N*-demethyl-4-hydroxy-3-methoxy analog of the parent drug.

In conclusion, the results of these experiments indicated that the metabolic fate of the parent drug was different in these three species. Rats can only demethylate the drug at reduced efficiency, as shown by the presence of only trace amounts of II (the *N*-demethylated product) and the presence of VII in rat urine. The lack of IV and VI (*ortho*-hydroxylated metabolites) in human urine may indicate that humans can only hydroxylate I in the *para*-position. However, since the human subjects in this study were only administered a single dose of the drug while the animals were administered multiple doses, this apparent species variation may be caused by enzyme induction in animals.

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[x] To whom inquiries should be directed.

Microbiological Synthesis of L-Dopa

JOHN ROSAZZA*[x], PAUL FOSS[‡], MICHAEL LEMBERGER[‡], and CHARLES J. SIH[‡]

Abstract □ The ability of microorganisms to convert *N*-carbobenzoxyl-L-tyrosine, *N*-*tert*-butyloxycarbonyl-L-tyrosine, and *N*-formyl-L-tyrosine into their respective *N*-substituted L-dopa derivatives was studied. These tyrosine derivatives were examined because of the ease with which the blocking groups may be removed. L-Tyrosine derivatives were incubated with *Aspergillus ochraceous* or *Gliocladium deliquescens*, and the resulting L-dopa products were isolated and characterized. L-Dopa was metabolized by *G. deliquescens* to 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenethyl alcohol. L-Ascorbic acid and hydrocinnamic acid increased yields of L-dopa when added to fermentation media.

Keyphrases □ L-Dopa—microbiological synthesis, L-tyrosine derivatives incubated with *Aspergillus ochraceous* and *Gliocladium deliquescens*, isolation and identification of L-dopa products □ *Aspergillus ochraceous*—used for microbiological synthesis of L-dopa products from L-tyrosine derivatives □ *Gliocladium deliquescens*—used for microbiological synthesis of L-dopa products from L-tyrosine derivatives □ Microbiology—synthesis of L-dopa from L-tyrosine by microorganisms □ Levodopa—microbiological synthesis of L-dopa from L-tyrosine derivatives

Three different types of enzymes are known to catalyze the formation of L-dopa from L-tyrosine:

1. Tyrosine hydroxylase is an enzyme associated with the biosynthesis of norepinephrine. It catalyzes the tetrahydropteridine-dependent hydroxylation of L-tyrosine to L-dopa (1).

2. Tyrosinase catalyzes the oxidation of tyrosine to melanin. It is generally accepted that the reaction proceeds through the formation of L-dopa and hallochrome, with the eventual formation of the polymeric pigment, melanin (2).

3. β-Tyrosinase from *Escherichia intermedia* catalyzes the synthesis of L-dopa from L-tyrosine and pyrocatechol *via* β-replacement (3).

In view of the therapeutic importance of L-dopa (levodopa) in the treatment of Parkinsonism, the authors wish to record in detail the examination of reactions using microbial systems for the conversion

small, it could not be isolated for further spectral analysis. Therefore, the positions of hydroxy and methoxy substitution on the aromatic system were assigned, based on the following information: (*a*) the presence of the 163 and 164 pair in the mass spectrum precluded the possibility of any substitution located *ortho* to the diphenyl ether oxygen; (*b*) the product of enzymatic hydroxylation of *para*-substituted monohydric phenols is catechol, which undergoes *O*-methylation yielding *para*-substituted *ortho*-methoxy phenol (6); and (*c*) catechol *O*-methyltransferase does not *O*-methylate resorcinol derivatives (7).

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[x] To whom inquiries should be directed.

Microbiological Synthesis of L-Dopa

JOHN ROSAZZA*[x], PAUL FOSS[‡], MICHAEL LEMBERGER[‡], and CHARLES J. SIH[‡]

Abstract □ The ability of microorganisms to convert *N*-carbobenzoxyl-L-tyrosine, *N*-*tert*-butyloxycarbonyl-L-tyrosine, and *N*-formyl-L-tyrosine into their respective *N*-substituted L-dopa derivatives was studied. These tyrosine derivatives were examined because of the ease with which the blocking groups may be removed. L-Tyrosine derivatives were incubated with *Aspergillus ochraceous* or *Gliocladium deliquescens*, and the resulting L-dopa products were isolated and characterized. L-Dopa was metabolized by *G. deliquescens* to 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenethyl alcohol. L-Ascorbic acid and hydrocinnamic acid increased yields of L-dopa when added to fermentation media.

Keyphrases □ L-Dopa—microbiological synthesis, L-tyrosine derivatives incubated with *Aspergillus ochraceous* and *Gliocladium deliquescens*, isolation and identification of L-dopa products □ *Aspergillus ochraceous*—used for microbiological synthesis of L-dopa products from L-tyrosine derivatives □ *Gliocladium deliquescens*—used for microbiological synthesis of L-dopa products from L-tyrosine derivatives □ Microbiology—synthesis of L-dopa from L-tyrosine by microorganisms □ Levodopa—microbiological synthesis of L-dopa from L-tyrosine derivatives

Three different types of enzymes are known to catalyze the formation of L-dopa from L-tyrosine:

1. Tyrosine hydroxylase is an enzyme associated with the biosynthesis of norepinephrine. It catalyzes the tetrahydropteridine-dependent hydroxylation of L-tyrosine to L-dopa (1).

2. Tyrosinase catalyzes the oxidation of tyrosine to melanin. It is generally accepted that the reaction proceeds through the formation of L-dopa and hallochrome, with the eventual formation of the polymeric pigment, melanin (2).

3. β-Tyrosinase from *Escherichia intermedia* catalyzes the synthesis of L-dopa from L-tyrosine and pyrocatechol *via* β-replacement (3).

In view of the therapeutic importance of L-dopa (levodopa) in the treatment of Parkinsonism, the authors wish to record in detail the examination of reactions using microbial systems for the conversion

of L-tyrosine to L-dopa. A preliminary account was published previously (4).

EXPERIMENTAL

Materials—The following materials were purchased: *N*-carbobenzoxyl-L-tyrosine[1]; L-dopa[1]; D-tyrosine; 3,4-dihydroxyphenylacetic acid[1]; L-tyrosine[2]; *N-tert*-butyloxycarbonyl-L-tyrosine[3]; hydrocinnamic acid[4]; L-ascorbic acid[5]; ion-exchange resin[6], 200–400 mesh; silica gel G[7]; silicic acid[8], 100 mesh; and diatomaceous earth[9].

Gliocladium deliquescens (1086) and *Aspergillus ochraceous* (398) were maintained on malt-agar[10] slants[11].

Methods—*N*-Formyl-L-tyrosine and *N*-formyl-D-tyrosine were prepared by the method of Waley and Watson (5). *N*-Carbobenzoxyl-D-tyrosine was synthesized according to the procedure of Bergmann and Zervas (6). TLC was performed using 0.25-mm thick silica gel G plates with ethyl acetate–acetic acid–isooctane–water (11:2:5:10) as the developing solvent. After development, TLC plates were sprayed with diazotized sulfanilic acid reagent (7); tyrosine derivatives appeared yellow, whereas catechols appeared orange-brown on sprayed chromatograms. Mass spectra were taken on a mass spectrometer[12], operating at an ionization of 70 ev and a block temperature of 120°. IR spectra were recorded on a double-beam recording spectrophotometer[13].

L-Dopa and L-tyrosine were assayed quantitatively after their separation on an ion-exchange column[6]. A typical elution profile obtained in the separation of these compounds is illustrated in Fig. 1. L-Tyrosine has a UV absorption maximum at 274 nm (ϵ 13,300), whereas the absorption maximum for L-dopa occurs at 279 nm (ϵ 26,600). These extinction coefficients were used in the determination of L-dopa yields and L-tyrosine recoveries as shown in the tables.

Fermentations were conducted in cotton-plugged erlenmeyer flasks in a soybean–dextrose medium of the following composition: dextrose, 20 g; soybean meal, 5 g; yeast extract, 5 g; sodium chloride, 5 g; dibasic potassium phosphate, 5 g; and distilled water, 1000 ml; it was adjusted to pH 7.0 with hydrochloric acid. The medium was sterilized in an autoclave at 121° for 15 min prior to use.

A two-stage incubation procedure was used in the cultivation of microorganisms. In a typical fermentation, the surface growth from a 1-week-old malt-agar slant was suspended in 5 ml of sterile saline (0.85%) solution. Two-milliliter portions of the resulting spore suspension were aseptically transferred to inoculate 50 ml of soybean–dextrose medium held in 250-ml erlenmeyer flasks (F-1 stage). These F-1 stage flasks were incubated at 25° on a rotary shaker operating at 250 rpm [5.08-cm (2-in.) stroke] for 24–48 hr. The resulting thick F-1 stage cultures were used to inoculate F-2 stage cultures, which were also grown in erlenmeyer flasks containing the soybean–dextrose medium. The inoculation volume used was 10% of the volume of medium contained in F-2 flasks. Usually, after 24 hr of incubation on the rotary shaker, various L-tyrosine substrates, dissolved in dimethylformamide, were added aseptically to F-2 stage flasks. L-Ascorbic acid was dissolved in 0.2 *M* phosphate buffer, pH 6.8, when it was added to cultures. In the various experiments reported, substrates containing F-2 stage cultures were incubated on the rotary shaker for an additional 12–60 hr before being harvested and analyzed for L-dopa. In separate experiments, it was determined that both microorganisms had reached the stationary phase of the growth cycle after 24 hr in the F-2 stage. Thus, the potential for contaminating microorganisms to influence the outcome of fermentations was considered slight. To avoid the possibility that microbial contaminants might have been responsible for the observed conversions of L-tyrosine derivatives to L-dopa, controls consisting of sterile medium with substrates added as usual were examined. None of the controls gave evidence of bacterial or fungal contamination or of the possible artifactual conversions of L-tyrosine to L-dopa under the incubation conditions.

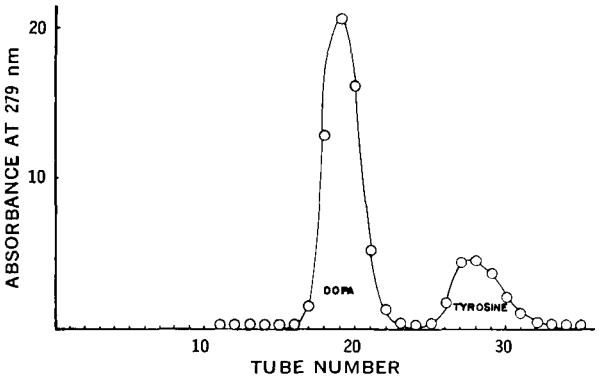

Figure 1—*Separation of* L-*tyrosine and* L-*dopa on a strong acid (H^+) ion-exchange column. The column (1.1 × 17.5 cm) was charged with 26 mg each of* L-*dopa and* L-*tyrosine and was eluted with 1.5* N *HCl at a rate of 6.4 ml/15 min. The absorbance at 279 nm was monitored as an estimation of their concentrations.*

***N*-Carbobenzoxyl-L-dopa**—*Fermentation*—*A. ochraceous* was grown according to the general fermentation procedure. The F-2 stage cultures were conducted in four 2-liter erlenmeyer flasks containing 500 ml each of the soybean–dextrose medium. After 24 hr of incubation on the rotary shaker, 250 mg of *N*-carbobenzoxyl-L-tyrosine, dissolved in 2 ml of dimethylformamide, was added to each flask. The F-2 stage flasks containing substrate were returned to the shaker and incubated for an additional 24 hr.

Isolation—Twenty-four hours after the addition of *N*-carbobenzoxyl-L-tyrosine, the mycelia were removed by filtration through cheesecloth. The filtrate was acidified to pH 2.8 with 6 *N* HCl and successively extracted with three 800-ml portions of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness *in vacuo*. The residue (1.95 g) was chromatographed on a silicic acid column, 2.54 × 20.32 cm (1 × 8 in.) in size. Elution of the column with benzene–ethyl acetate (3:1) afforded 523 mg of *N*-carbobenzoxyl-L-dopa, which was chromatographically identical to a sample prepared by acylation of L-dopa. The product gave orange-brown-colored spots on chromatograms sprayed with diazotized sulfanilic acid spray reagent and possessed a mobility on silica gel G TLC plates 0.9 that of *N*-carbobenzoxyl-L-tyrosine.

When the compound (200 mg) was dissolved in a 1:1 mixture of acetic acid and water (20 ml) and hydrogenated for 16 hr in the presence of 10% palladium-on-carbon, 63 mg of L-dopa, mp 286° dec., was obtained. The isolated compound was spectrally (IR and UV) and chromatographically identical to an authentic specimen of L-dopa.

***N-tert*-Butyloxycarbonyl-L-dopa**—*Fermentation*—*A. ochraceous* was incubated with *N-tert*-butyloxycarbonyl-L-tyrosine (500 mg) as previously described, except that the F-2 stage was incubated for a total of 60 hr after substrate addition.

Isolation—At 60 hr, the fermentation was terminated by filtration through cheesecloth to remove the mycelia. The filtrate was acidified with 6 *N* HCl to pH 2.5 and was successively extracted with three 600-ml portions of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness *in vacuo*. The residue was chromatographed over 22 g of silicic acid containing 15% diatomaceous earth. Elution of the column with 23% ethyl acetate in benzene gave 141 mg of *N-tert*-butyloxycarbonyl-L-tyrosine and 200 mg of *N-tert*-butyloxycarbonyl-L-dopa, which was identified by converting it into L-dopa by treatment with trifluoroacetic acid (8).

***N*-Formyl-L-dopa**—*Fermentation*—*G. deliquescens* was grown according to the usual fermentation procedure, and the F-2 stage was conducted in a 125-ml erlenmeyer flask containing 25 ml of

[1] Sigma.
[2] Nutritional Biochemicals.
[3] Pierce.
[4] Eastman.
[5] Pfizer.
[6] Dowex 50W X4, Baker.
[7] Brinkmann.
[8] No. 2847, Mallinckrodt.
[9] Celite, Johns Manville.
[10] Difco.
[11] The microorganisms used in this study were supplied by Professor K. B. Raper, Department of Bacteriology, University of Wisconsin, Madison, Wis.
[12] Finnigan 1015.
[13] Beckman IR5A.

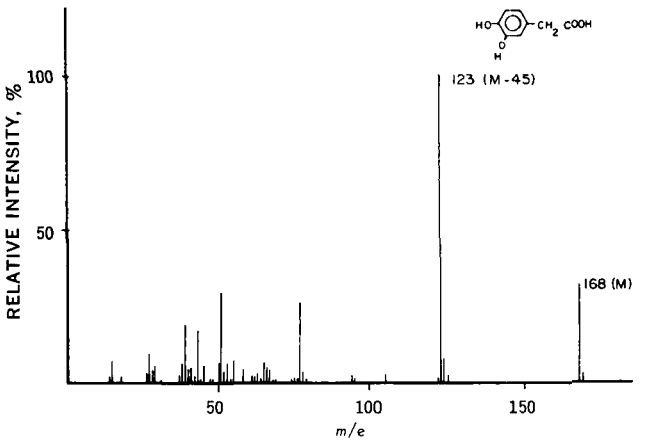

Figure 2—*Mass spectrum of Metabolite A which was isolated from* G. deliquescens *cultures containing* L-*dopa.*

the soybean-dextrose medium. After 12 hr of incubation on the shaker, 750 mg of *N*-formyl-L-tyrosine, dissolved in 2 ml of 1 *M* $KHCO_3$, was added to the flask. The substrate containing F-2 stage culture was incubated for an additional 42 hr under the prescribed conditions. L-Ascorbic acid was added intermittently to the F-2 stage flask in five separate portions at 0, 12, 18, 24, and 36 hr following substrate addition. A total of 500 mg of L-ascorbic acid was added. Because previous work had demonstrated that metabolic transformation was most active between 20 and 30 hr after substrate addition, most of the L-ascorbic acid was added during this time interval.

Isolation—Forty-two hours after the addition of the *N*-formyl-L-tyrosine, the mycelia were removed from the culture by filtration through cheesecloth. The filtrate was acidified with 6 *N* HCl to pH 2.5 and successively extracted with three 25-ml portions of *n*-butanol. The combined butanol extracts were evaporated to dryness *in vacuo*. The residue was dissolved in 20 ml of 5 *N* HCl and was allowed to stand at room temperature for 12 hr. After evaporation of the aqueous hydrochloric acid, the residue was dissolved in 5 ml of 0.75 *N* HCl and chromatographed on an ion-exchange column[6], 200–400 mesh, H^+ form, 2 × 28 cm in size. The column was eluted with 0.75 *N* HCl, and successive 9.5-ml fractions were collected. L-Dopa hydrochloride (186 mg) was found in fractions 35–46, and L-tyrosine hydrochloride (375 mg) was found in fractions 48–59. The isolated L-dopa, $[\alpha]_D^{25}$ −11° (c, 4% in 0.75 *N* HCl), was spectrally (IR) and chromatographically identical to authentic L-dopa.

Metabolism of L-Dopa by *G. deliquescens*—To a 24-hr F-2 stage culture of *G. deliquescens* in a 2-liter erlenmeyer flask containing 500 ml of the soybean-dextrose medium was added 1 g of L-dopa and 1 g of L-ascorbic acid. After incubating for 24 hr on a rotary shaker as previously described, the mycelia were removed

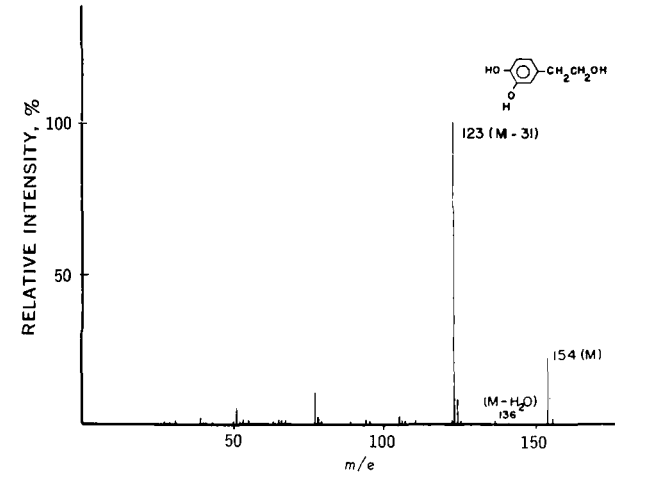

Figure 3—*Mass spectrum of Metabolite B which was isolated from* G. deliquescens *cultures containing* L-*dopa.*

Table I—Effect of L-Ascorbic Acid on Yield of L-Dopa[a]

Ascorbate, mg/ml	L-Dopa, % Yield	L-Tyrosine, % Recovered
None	10.0	13.2
2.1	11.2	14.7
5.3	23.1	63.5

[a] Fermentation conditions using *G. deliquescens* were the same as described under *Experimental*. *N*-Formyl-L-tyrosine concentration was 10 mg/ml, and incubations were terminated 12 hr after substrate addition. L-Ascorbic acid was added at the time of substrate addition.

Table II—Effect of Substrate Concentration on Yield of L-Dopa[a]

Substrate, mg/ml	L-Dopa, % Yield	L-Tyrosine, % Recovered
3	22.3	42.2
10	23.1	63.5
30	10.6	76

[a] *G. deliquescens* was incubated with substrate for 12 hr under the same conditions as described under *Experimental*.

by filtration through cheesecloth. The filtrate was acidified to pH 2.0 and extracted with *n*-butanol. The combined butanol extracts were evaporated to dryness *in vacuo*, and the residue was chromatographed on a silica gel column. The column was eluted with ethyl acetate containing 2% acetic acid. Two products were obtained from this column while residual L-dopa remained at the origin. These substances possessed R_f values of 0.5 and 0.35 on silica gel G TLC plates in a solvent system consisting of chloroform–methanol–acetic acid–water (80:17.5:2.4:0.1). The compounds were isolated by preparative TLC using this solvent system and were rechromatographed for further purification using benzene–propionic acid–water (2:2:1). The major product, A (R_f 0.5), was identified as 3,4-dihydroxyphenylacetic acid (200 mg); the minor product, B (R_f 0.35), was characterized as 3,4-dihydroxyphenylethanol (5 mg).

RESULTS AND DISCUSSION

Among the wide variety of microorganisms that metabolize L-tyrosine, the majority appears to degrade this amino acid *via* homogentisic acid with the initial formation of *p*-hydroxyphenylpyruvic acid, a route characteristic of mammals (9–12). An alternative route for the dissimilation of L-tyrosine *via* *p*-coumaric acid, *p*-hydroxybenzoic acid, and protocatechuic acid was shown to be widespread among the *Basidiomycetes* (13). Although protocatechuic acid and catechol (14) were identified as metabolites of L-tyrosine, no L-dopa was detectable, suggesting that deamination of L-tyrosine may be the first and major degradative reaction.

If deamination and aromatic hydroxylation do not follow a compulsory sequence, it should be possible to inhibit deaminase activity selectively by the introduction of suitable *N*-blocking groups, resulting in the accumulation of the desired *N*-substituted L-dopa derivatives. As additional features, *N*-substituted tyrosine substrates should be resistant to the action of racemases and also should be more soluble in organic solvents. Thus, substrates and products are more easily extracted from aqueous fermentation media. For this study, *N*-carbobenzoxyl, *N*-formyl, and *N*-*tert*-butyloxycarbonyl derivatives of L-tyrosine were selected because they are easily removed, either by acid hydrolysis or by hydrogenolytic cleavage (8).

In initial experiments, several microorganisms were screened for their capacities to convert *N*-formyl-L-tyrosine and *N*-carbobenzoxyl-L-tyrosine into their L-dopa derivatives. As previously reported (4), not every microorganism examined accomplished this conversion, but *A. ochraceous, Penicillium duclauxi, G. deliquescens, Stemphylium solani, Scopulariopsis constantini, Memnoniella echinata, Trichoderma viride, Corynespora cassicola, Fusarium solani, Stysanus fimetarius,* and others did. *A. ochraceous* and *G. deliquescens* appeared to accomplish the transformation efficiently, and these cultures were selected for further work.

Table III—Effect of Time on Yield of L-Dopa by *G. deliquescens*[a]

Hours	L-Dopa, % Yield	L-Tyrosine, % Recovered
12	10.6	76
39	16.0	51.0
46	18.5	57.3

[a] Fermentations were conducted as described under *Experimental*. L-Ascorbate (5.3 mg/ml) was added to the cultures every 12 hr.

When *N*-carbobenzoxyl-L-tyrosine and *N-tert*-butyloxycarbonyl-L-tyrosine were added to growing cultures of *A. ochraceous*, the corresponding L-dopa derivatives were formed in about 30% yield. This organism gave poor yields of *N*-formyl-L-dopa when incubated with *N*-formyl-L-tyrosine. Conversely, *G. deliquescens* preferentially converted *N*-formyl-L-tyrosine into *N*-formyl-L-dopa, while giving poorer yields with *N*-carbobenzoxyl- and *N-tert*-butyloxycarbonyl-L-tyrosine substrates. Qualitative studies in which these organisms were incubated with *N*-formyl-D-tyrosine and *N*-carbobenzoxyl-D-tyrosine resulted in the formation of the respective catechol derivatives as judged by TLC. Thus, it appears that hydroxylase activity is not dependent on the symmetry of the amino acid derivative used.

Insight into this microbial conversion was sought so that fermentation conditions might be optimized to obtain higher yields of L-dopa from various L-tyrosine derivatives. For this purpose, efforts were concentrated on *G. deliquescens* using *N*-formyl-L-tyrosine as the substrate. Initially, it was observed that the fermentation medium rapidly turned black following addition of substrate. The appearance of pigment could be correlated with the hydroxylation of *N*-formyl-L-tyrosine, but the amount of L-dopa and L-tyrosine recoverable from these fermentations was low. This observation suggested that melanin formation was taking place at the expense of L-dopa as it was being formed. To counteract this undesirable effect, L-ascorbic acid was included in the medium. Table I clearly shows that the recovery of both L-dopa and L-tyrosine was improved and that the yield of L-dopa was significantly increased when L-ascorbic acid was added.

An experiment was conducted to determine the effect of substrate concentration on the yield of L-dopa with *G. deliquescens*. The results in Table II indicate that the optimum substrate concentration level is 10 mg/ml of *N*-formyl-L-tyrosine. Higher substrate levels at 30 mg/ml appeared, on the basis of TLC, to inhibit side reactions and also delayed the formation of melanin-like pigments in the medium.

To take advantage of this finding, the kinetics of L-dopa formation were investigated utilizing 30 mg/ml of *N*-formyl-L-tyrosine as substrate. Table III shows that the maximum yield of L-dopa was obtained at 46 hr in this experiment.

The level of *N*-formyl-L-dopa decreased on prolonged incubations with *G. deliquescens*. This finding suggested that the organism may possess an enzyme capable of cleaving the formyl group, albeit slowly. When *G. deliquescens* was incubated in a medium containing L-dopa, two metabolites were detected. The major product (A), obtained in 20% yield, was identified as 3,4-dihydroxyphenylacetic acid on the basis of the following data. The mass spectrum of Metabolite A is shown in Fig. 2. The molecular ion of this metabolite occurred at *m/e* 168, while the base peak occurred at *m/e* 123, corresponding to an $M^+ - COOH$ ion. Both the IR and mass spectra of the metabolite were identical to an authentic specimen sample of 3,4-dihydroxyphenylacetic acid. The mass spectrum of the minor metabolite, B, is shown in Fig. 3. The molecular ion of this metabolite occurred at *m/e* 154, while the base peak occurred at *m/e* 123, consistent with the loss of a fragment of mass 31, or $M^+ - CH_2OH$. The mass spectral evidence suggested that Metabolite B is 3,4-dihydroxyphenylethanol.

The previous experiment demonstrated that *G. deliquescens* possesses the enzymatic capacity to degrade L-dopa to 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylethanol. The reaction sequence giving rise to these metabolites is probably analogous to the decomposition of other amino acids in yeasts and other organisms (15). On the basis of these results, it is suggested that the presence of an acylase capable of cleaving the formyl group from the formyl amino acids would lower the yields of L-dopa produced in fermentations. Hydrocinnamic acid has been reported to be a powerful inhibitor of carboxypeptidase activity (16). In an attempt to inhibit the hydrolytic activity of *G. deliquescens*, hydrocinnamate was added to the medium. The results of two experiments are shown in Table IV. An increase in the yield of L-dopa was obtained by utilizing only small quantities of hydrocinnamate. Although yields of L-dopa were improved, the exact mechanism by which hydrocinnamate exerts its influence remains uncertain. It was observed that the metabolic transformation of *N*-formyl-L-tyrosine to *N*-formyl-L-dopa proceeded at a more rapid rate in the presence of hydrocinnamic acid. Thus, it is possible that hydrocinnamate may be functioning as an inducer of aromatic hydroxylase rather than as an inhibitor of acylase activity.

Table IV—Effect of Hydrocinnamate on Yield of L-Dopa by *G. deliquescens*[a]

	L-Dopa, % Yield	L-Tyrosine, % Recovered
Experiment I (32-hr fermentation)		
Control (no hydrocinnamate)	10.6	65
1×10^{-4} *M* (hydrocinnamate)	17.0	60
Experiment II (62-hr fermentation)		
Control (no hydrocinnamate)	14.0	30.5
1×10^{-4} *M* (hydrocinnamate)	18.5	37.7

[a] Fermentation conditions are described under *Experimental*.

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* To whom inquiries should be directed.

Coacervation of Alkyltrimethylammonium Bromides by Tartrazine, Amaranth, Carmoisine, and Erythrosine

B. W. BARRY[x] and G. M. T. GRAY

Abstract □ The interaction of aqueous solutions of alkyltrimethylammonium bromides (C_{12}-C_{16}) with 0.15 and 15 mmoles/liter aqueous solutions of the anionic dyes tartrazine, amaranth, carmoisine, and erythrosine was studied by phase separation techniques and microscopy between 5 and 95°. Cetrimide–dye systems were also studied in the presence of sodium chloride, sodium sulfate, sodium citrate, and calcium chloride. Between certain surfactant concentrations and temperature limits, a coacervate phase, or an anisotropic phase, formed. Phase separation diagrams, in conjunction with coacervation theory, were used to interpret the effects of surfactant concentration and chain length, of dye concentration and chemical structure, of temperature, and of total ionic strength on the extent of interaction. Differences between the interactions of a hydrophilic dye, tartrazine, and those of amaranth, carmoisine, and erythrosine, which all possess hydrophobic as well as hydrophilic moieties, were shown. Tartrazine functioned as a simple electrolyte and interacted only with the surface charges of surfactant micelles. For the other dyes, interaction was *via* solubilization of a dye–surfactant complex within the micelle, together with ionic surface interactions. Amaranth, carmoisine, and erythrosine induced the formation of surfactant micelles at concentrations far below the CMC's of the surfactants in water. The limits of compatibility of the dyes with the surfactants were also defined.

Keyphrases □ Dyes (tartrazine, amaranth, carmoisine, and erythrosine)—coacervation by alkyltrimethylammonium bromides, phase separation techniques and microscopy, effects of salts on cetrimide–dye systems, effects of surfactant concentration and chain length, dye concentration and structure, temperature, and total ionic strength on interaction, micelle formation □ Alkyltrimethylammonium bromides—interactions with anionic dyes, phase separation techniques and microscopy □ Cetrimide–dye (anionic) systems—interactions studied by phase separation techniques and microscopy, effects of added salts □ Coacervation—tartrazine, amaranth, carmoisine, and erythrosine with alkyltrimethylammonium salts, interactions studied by phase separation techniques and microscopy, phase separation diagrams, possible mechanisms

Coacervation, the term used (1) to describe the separation of a colloidal solution into two liquid layers, one of which is rich in colloidal component and the other poor, is a well-established phenomenon. Dervichian (2) compared the coacervates produced in different systems and showed that many similarities existed between them. Voorn (3) applied a theoretical treatment to complex coacervation of polycations and polyanions to explain many previously reported observations. More recently, attention has been focused on the coacervation of aqueous solutions of cationic and anionic surfactants by inorganic salts (4–10).

In a study of the compatibility of the dye amaranth with long-chain quaternary ammonium bromides, a similar phenomenon was observed (11). In view of the extent of the literature concerning the interactions of dyes with surfactants (*e.g.*, 12–18), it is surprising that no similar findings have been reported.

In a preliminary investigation, under certain conditions other food and drug dyes coacervated aqueous solutions of alkyltrimethylammonium bromides. Furthermore, some aqueous dye–surfactant coacervates showed a different temperature dependence from those formed by interaction of the surfactants with inorganic salts (5, 7). In the latter case, coacervated solutions when warmed became homogeneous at some critical temperature, whereas in the former situation the temperature dependence was reversed.

As food and drug preparations are often subjected to thermal fluctuations, it is important to know and to understand the effects of differing temperatures on the interactions of the constituents of these products. Therefore, the dyes used in the present study were chosen on the basis of two considerations: *(a)* their importance as colorants in the food, drug, and cosmetic industry, which would thus provide further insight into the compatibility of the dyes with the pharmaceutically important long-chain quaternary ammonium bromides; and *(b)* the chemical natures of the dyes, in an attempt to elucidate structure–activity relationships with regard to their effects on coacervation.

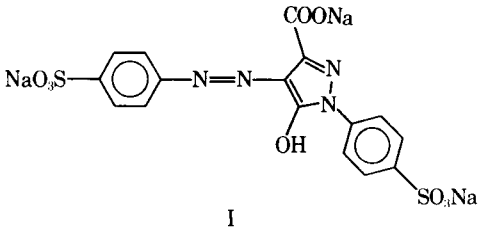
Phase diagrams for the interaction of the dyes with surfactants were constructed. These were used to illustrate the effects of concentration and chain length of the quaternary ammonium bromides, of concentration and chemical structure of the dyes, of temperature, and of added inorganic salts on the extent of coacervation. The morphology of the systems was also examined to determine the nature of the separated phase.

EXPERIMENTAL

Materials—The following dyes were used: tartrazine[1], trisodium salt of 3-carboxy-5-hydroxy-1-*p*-sulfophenyl-4-*p*-sulfophenylazopyrazole (I); amaranth BPC (1954)[1], trisodium salt of 1-(4-sulfo-1-naphthylazo)-2-naphthol-3,6-disulfonic acid (II); carmoisine[2], disodium salt of 2-(4-sulfo-1-naphthylazo)-1-naphthol-4-sulfonic acid (III); and erythrosine[1], disodium salt of 2′,4′,5′,7′-tetraiodofluorescein (IV). All samples were analyzed for their dye content according to a reported method (19). The purity of each dye with respect to its dry weight was: amaranth, 93.3%; carmoisine, 95.5%; tartrazine, 98.4%; and erythrosine, 97.7%. All dyes were used as received. Ancillary dyes used were: Sunset Yellow FCF, disodium salt of 1-(4-sulfophenylazo)-2-naphthol-6-sulfonic acid (V); and Orange II, *p*-(2-hydroxy-1-naphthylazo)-benzenesulfonic acid sodium salt (VI).

The alkyltrimethylammonium bromides used, *i.e.*, hexadecyl-, tetradecyl-, dodecyl-, and trimethylammonium bromides, and cetrimide BP were described elsewhere (11, 20). Since each surfactant was a mixture of homologs, an "average chain length" was calculated by GLC analysis of the surfactants (20). These average values were 15.68, 13.64, 12.40, and 13.56, respectively.

[1] D. F. Anstead Ltd., Billericay, England.
[2] Donated by I.C.I., Ltd., Dyestuffs Division, Manchester, England.



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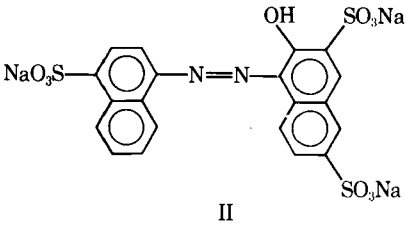

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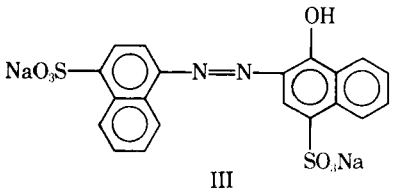

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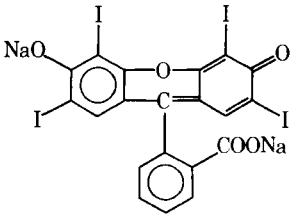

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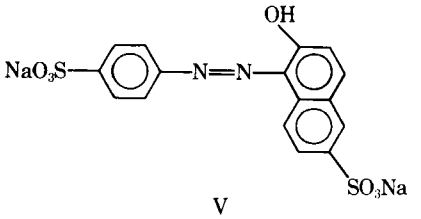

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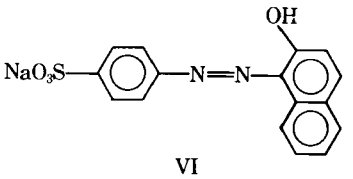

VI

The inorganic salts were analytical grade. Water, double distilled from an all-glass still, had a conductance not greater than 1.2×10^{-6} ohm^{-1} cm^{-1}.

Morphology—With a microsyringe[3], various volumes of 0.15-mmole/liter aqueous dye solutions were mixed on a cavity slide with various volumes of surfactant dissolved in 0.15-mmole/liter aqueous dye solution so that the final volume was 50 μl. A coverslip was sealed in place and the solutions were examined microscopically in normal light and between crossed polars at ×100 and ×400 on a hot-stage[4]. Changes were noted as the temperature was slowly raised. The experiment was repeated using 15-mmoles/liter dye solutions and correspondingly higher concentrations of surfactant.

When the systems were too viscous to be mixed properly on the slide, 10-ml quantities were agitated using a mixer-emulsifier[5] fitted with a microhead; portions were examined as before.

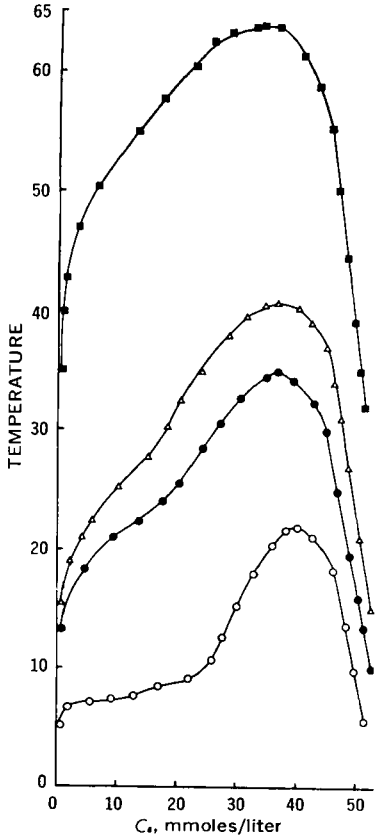

Figure 1—*Diagram showing temperatures and surfactant concentrations at which tartrazine–alkyltrimethylammonium bromide solutions separated into two or more phases. Tartrazine concentration is 15 mmoles/liter. Surfactant concentration is* C_s, *mmoles/liter. Homogeneous solutions are represented by points outside the phase separation boundaries. Key:* ■, *tartrazine–hexadecyltrimethylammonium bromide;* △, *tartrazine–tetradecyltrimethylammonium bromide;* ●, *tartrazine–cetrimide; and* ○, *tartrazine–dodecyltrimethylammonium bromide.*

Phase Diagrams—These were constructed as follows. A 10-ml aliquot of an aqueous 0.15-mmole/liter dye solution was pipeted into a covered titration vessel jacketed by a water bath to ensure uniform heating. A clear solution of a suitable concentration of surfactant (2–10 mmoles/liter) in aqueous 0.15-mmole/liter dye solution was added from a semimicroburet.

The mixture was stirred with a magnetic stirrer, and the temperature was measured using a bead thermistor[6] connected to a universal bridge[7] (required because of the small volumes used). The thermistor had previously been calibrated[8] in the range 5–95° so that the temperature could be read correctly to 0.1°.

In the neighborhood of the critical regions, the surfactant–dye solution was added dropwise at constant temperature until coacervation occurred (left-hand boundary of diagrams) or was suppressed (right-hand boundary). Conversely, the surfactant concentration was held constant and the temperature was varied until the solution coacervated. In both cases, the final surfactant concentration and the temperature of the mixture were recorded to provide data at surfactant concentrations below their critical micelle concentrations (CMC's) in water.

To check results, a 0.15-mmole/liter aqueous dye solution was titrated against aliquots of surfactant–dye solution. The relevant final surfactant concentration and the temperature were noted as before.

Phase diagrams of surfactant concentration, in mmoles/liter, *versus* temperature at constant dye concentration were drawn. Since coacervation is a thermodynamically reversible phenome-

[3] Clark Hamilton Manufacturing AG, 7402 Bonaduz GR, Switzerland.
[4] Kofler.
[5] Silverson Machines Ltd., London, England.

[6] Thermistor TH-B12, Radiospares Ltd., London, England.
[7] Wayne-Kerr.
[8] Against National Physical Laboratory thermometers.

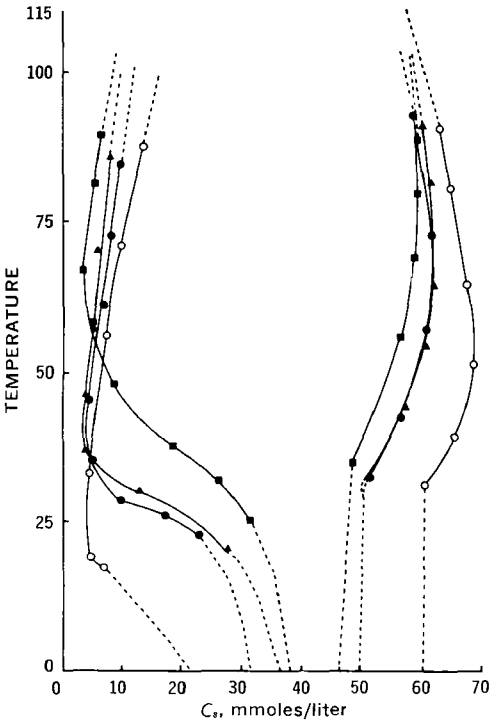

Figure 2—*Diagram showing temperatures and surfactant concentrations at which amaranth–alkyltrimethylammonium bromide solutions separated into two or more phases. Amaranth concentration is 15 mmoles/liter. Surfactant concentration is* C_s, *mmoles/liter. Homogeneous solutions are represented by points outside the phase separation boundaries. Key:* ■, *amaranth–hexadecyltrimethylammonium bromide;* ▲, *amaranth–tetradecyltrimethylammonium bromide;* ○, *amaranth–dodecyltrimethylammonium bromide; and* ●, *amaranth–cetrimide.*

non, the phase separation points obtained by these different methods all coincided on the same phase diagram. Thus, the second method was valuable in confirming the location of the phase diagram.

The procedure was repeated using aqueous 15-mmoles/liter dye solutions and clear surfactant-dye solutions containing surfactants at concentrations above their nominal CMC's (usually 200 mmoles/liter). When these mixtures became too viscous for a magnetic stirrer (mainly at ambient temperature), a mixer-emulsifier[5] provided intimate mixing.

Effect of Ionic Strength—The effect of ionic strength on the coacervation of dye–surfactant solutions was investigated. Ten milliliters of aqueous 0.15-mmole/liter dye solutions containing various amounts of sodium citrate was pipeted into the constant-temperature titration vessel. Cetrimide solutions containing 0.15 mmole/liter dye and various concentrations of sodium citrate were added, and the amount added prior to the occurrence of coacervation was noted. The titration was continued until the coacervate dissolved, and the total volume added was noted. The limits of coacervation at constant temperature and at various, constant sodium citrate concentrations were thus found. These were compared with the values found in the absence of sodium citrate; a suitable salt concentration, which had a significant effect on the limits of coacervation, was then chosen. Phase diagrams were constructed as before, using cetrimide and dye solutions containing the chosen concentration of sodium citrate. Phase diagrams were also constructed for sodium chloride, sodium sulfate, and calcium chloride at the same concentration (in milliequivalents per liter) as sodium citrate.

The experiment was repeated using 15-mmoles/liter dye solutions.

Minimum Ratio of Dye to Surfactant Required to Produce Coacervation—The concentration, y, in milliequivalents per liter, of electrolyte (in this case dye) required to produce coacervation in a surfactant system is a linear function of the surfactant concentration. It may be described by the equation:

$$y = Ax + B \qquad \text{(Eq. 1)}$$

where x is the surfactant concentration in milliequivalents per liter, and A and B are constants for any given system. A plot of y against x yields a straight line whose slope, A, provides the value of the minimum ratio of dye–surfactant required to produce coacervation and whose intercept, B, gives the equilibrium concentration of unbound electrolyte.

Bungenberg de Jong and Recourt (5) found that to calculate A for interaction of hexadecyltrimethylammonium bromide with the inorganic salts potassium thiocyanate, potassium iodide, and potassium nitrate, they had to use salt concentrations at "coacervate volumes adapted to the soap concentration," because the values of B varied at different stages of coacervation.

However, in the present work, when coacervation occurred, practically all the dye was bound to the surfactant and B was negligible. Hence, the tedious method of Bungenberg de Jong and Recourt (5) was unnecessary. The minimum ratio of dye–surfactant required for coacervation was thus found by pipeting 1- or 2-ml aliquots of surfactant in aqueous dye of various concentrations into the temperature-controlled glass vessel and agitating with a magnetic stirrer as an aqueous dye solution of the same concentration was added from a 10-ml semimicroburet until an end-point was reached, as shown by persistent turbidity of the mixture. When concordant titers were obtained, graphs of dye concentration, in milliequivalents per liter, *versus* final surfactant concentration, in milliequivalents per liter, were drawn. The experiment was repeated at several temperatures. The gradients and the intercepts of the straight lines were calculated by the method of least squares.

RESULTS

Preliminary Morphological Examinations—Under certain conditions, when aqueous solutions of tartrazine, amaranth, carmoisine, and erythrosine were mixed with the quaternary ammo-

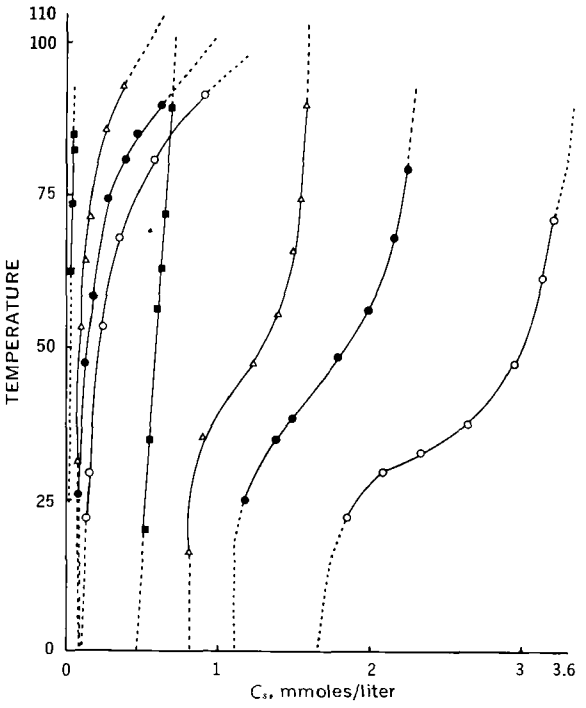

Figure 3—*Diagram showing temperatures and surfactant concentrations at which amaranth–alkyltrimethylammonium bromide solutions separated into two or more phases. Amaranth concentration is 0.15 mmole/liter. Surfactant concentration is* C_s, *mmoles/liter. Homogeneous solutions are represented by points outside the phase separation boundaries. Key:* ■, *amaranth–hexadecyltrimethylammonium bromide;* △, *amaranth–tetradecyltrimethylammonium bromide;* ●, *amaranth–cetrimide; and* ○, *amaranth–dodecyltrimethylammonium bromide.*

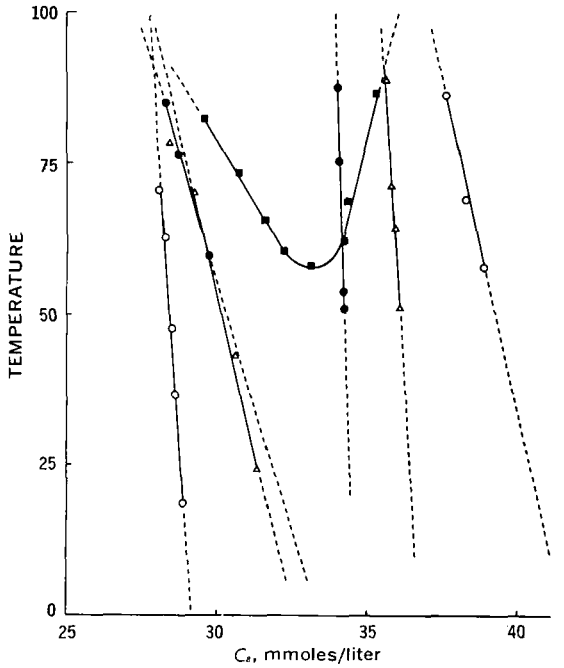

Figure 4—*Diagram showing temperatures and surfactant concentrations at which carmoisine–alkyltrimethylammonium bromide solutions separated into two or more phases. Carmoisine concentration is 15 mmoles/liter. Surfactant concentration is* C_s, *mmoles/liter. Homogeneous solutions are represented by points outside the phase separation boundaries. Key:* ■, *carmoisine–hexadecyltrimethylammonium bromide;* △, *carmoisine–tetradecyltrimethylammonium bromide;* ●, *carmoisine–cetrimide; and* ○, *carmoisine–dodecyltrimethylammonium bromide.*

nium compounds, the solutions became turbid. Microscopically, for carmoisine, above 40° the dispersed phase consisted of isotropic, oily droplets and no anisotropic phase was observed, although at temperatures below 40° viscous gels formed. For tartrazine, erythrosine, and amaranth, viscous, mesomorphic phases were often seen, which changed into isotropic, oily droplets as the temperature rose. The temperature at which the dispersed phase became totally isotropic increased with an increase in surfactant chain length. When the turbid dye-surfactant-water systems were equilibrated, two layers formed at temperatures for which the separated phase was isotropic. The bottom layer was a viscous, oily liquid, rich in dye and surfactant. The upper layer was a mobile, clear solution containing little dye or surfactant. This type of phase separation has been termed coacervation (1).

Phase Diagrams—Phase diagrams of the various aqueous dye-surfactant systems are reproduced in Figs. 1–7. (Although a faint turbidity was observed when surfactant was added to an aqueous 0.15-mmole/liter tartrazine solution, microscopic examination yielded no information on the morphology of the species causing the opacity. These dilute systems were not investigated further.) In all dye-surfactant systems studied, phase separation only occurred at certain mixing proportions and temperatures. An excess of dye or of surfactant suppressed coacervation. In some cases there was a maximum temperature for interaction, above which no coacervation occurred. Thus, Figs. 1–7 show that phase separation only took place, at any given temperature, if the system had a composition which fell on, or between, the coacervation limits. Outside the areas defined by these limits, homogeneous solutions formed.

The effect of surfactant chain length on the areas between the coacervation curves differed for each dye. For tartrazine, increasing the chain length increased the area of interaction, whereas for the other dyes the order was usually reversed.

The dye-surfactant-water systems behaved differently with respect to temperature. For the tartrazine systems, warming suppressed coacervation and a maximum temperature for coacervation in each system existed. A plot of this maximum temperature for each surfactant homolog *versus* "average chain length" (see *Materials* section for definition of average chain length) yielded a straight line (Fig. 8), suggesting a simple relationship between the two parameters.

Heating the aqueous 0.15- and 15-mmoles/liter erythrosine-surfactant systems increased the area of coacervation, but the temperature dependence of the amaranth and carmoisine systems was more complex.

The temperatures at which the dispersed amaranth-surfactant and tartrazine-surfactant phases became totally isotropic, as viewed under the polarizing microscope, proved interesting in relation to the phase diagrams. The transition temperatures (anisotropic to fully isotropic) at different mixing ratios for the cetrimide-dye dispersed phases are shown in Fig. 9. At approximately ambient temperature and below, the separated phase showed definite anisotropy and often consisted of irregularly shaped particles. As the temperature rose, these particles rounded off and myelin figures formed. On further heating, the separated phase became isotropic and oily. This anisotropic to isotropic transition occurred over a small temperature range. The starting temperature for the transition was ill defined (approximated by a broken line in Fig. 9). However, the temperature at which the separated phase became totally isotropic was readily measured under polarized light (full line in Fig. 9).

Similar phase transitions were observed when samples of aqueous amaranth or tartrazine containing the other surfactant homologs were heated. The temperature at which the anisotropic to isotropic transition occurred increased as the surfactant chain length increased.

Effect of Ionic Strength—The effects of the relevant inorganic salt concentrations on the dye-cetrimide coacervation curves are shown in Figs. 10–13. With amaranth, carmoisine, and erythrosine, only the effect of salt on the coacervation curves at a dye concentration of 0.15 mmole/liter was studied. The high concentrations of inorganic salt that would have been required to produce a significant effect on the coacervation curves at the higher dye concentration yielded solutions in which the dyes were insolu-

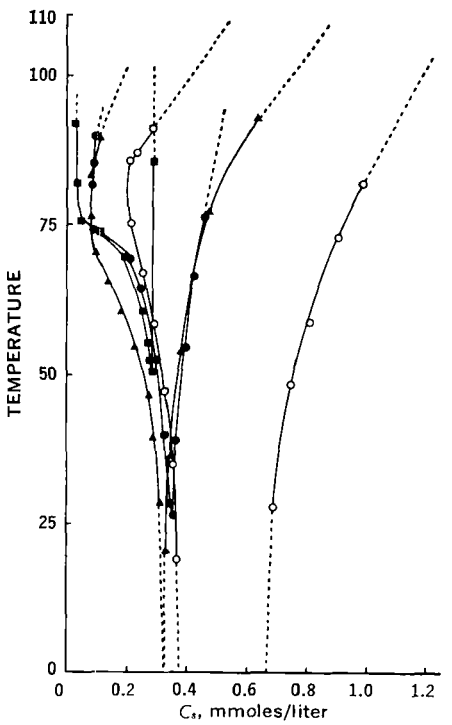

Figure 5—*Diagram showing temperatures and surfactant concentrations at which carmoisine–alkyltrimethylammonium bromide solutions separated into two or more phases. Carmoisine concentration is 0.15 mmole/liter. Surfactant concentration is* C_s, *mmoles/liter. Homogeneous solutions are represented by points outside the phase separation boundaries. Key:* ■, *carmoisine–hexadecyltrimethylammonium bromide;* ▲, *carmoisine–tetradecyltrimethylammonium bromide;* ●, *carmoisine–cetrimide; and* ○, *carmoisine–dodecyltrimethylammonium bromide.*

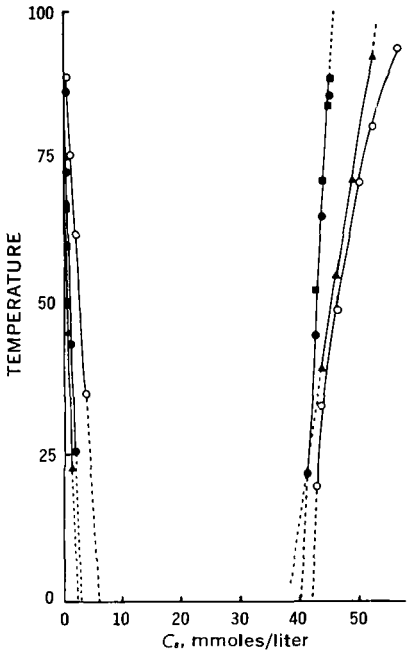

Figure 6—*Diagram showing temperatures and surfactant concentrations at which erythrosine–alkyltrimethylammonium bromide solutions separated into two or more phases. Erythrosine concentration is 15 mmoles/liter. Surfactant concentration is* C_s, *mmoles/liter. Homogeneous solutions are represented by points outside the phase separation boundaries. Key:* ■, *erythrosine–hexadecyltrimethylammonium bromide;* ▲, *erythrosine–tetradecyltrimethylammonium bromide;* ●, *erythrosine–cetrimide; and* ○, *erythrosine–dodecyltrimethylammonium bromide.*

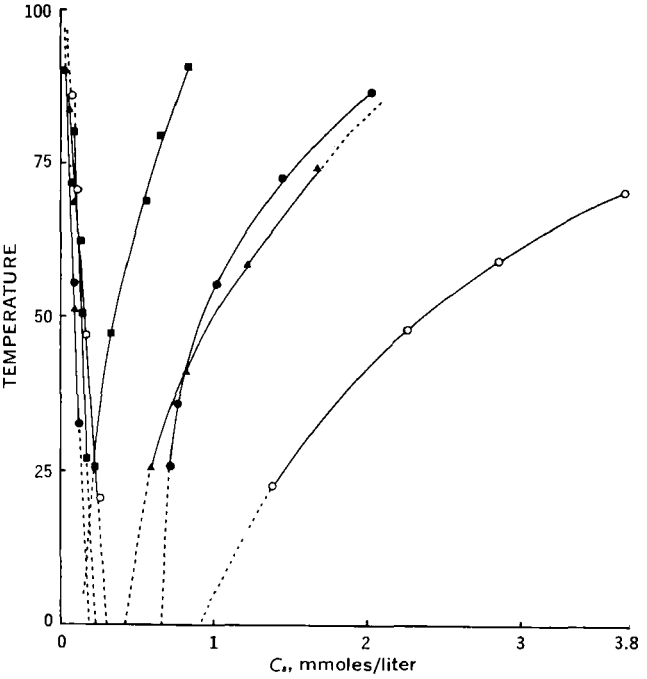

Figure 7—*Diagram showing temperatures and surfactant concentrations at which erythrosine–alkyltrimethylammonium bromide solutions separated into two or more phases. Erythrosine concentration is 0.15 mmole/liter. Surfactant concentration is* C_s, *mmoles/liter. Homogeneous solutions are represented by points outside the phase separation boundaries. Key:* ■, *erythrosine–hexadecyltrimethylammonium bromide;* ▲, *erythrosine–tetradecyltrimethylammonium bromide;* ●, *erythrosine–cetrimide; and* ○, *erythrosine–dodecyltrimethylammonium bromide.*

ble. The effects of the inorganic salts on the other systems may be summarized as follows.

Aqueous 15-mmoles/Liter Tartrazine-Cetrimide System (Fig. 10)—An inorganic salt concentration of 150 milliequivalents/liter reduced the area under the coacervation curve in the order: sodium chloride > sodium sulfate > sodium citrate: sodium chloride > calcium chloride. The position of the maximum in the coacervation curves was also affected.

Aqueous 0.15-mmole/Liter Amaranth-Cetrimide System (Fig. 11)—An inorganic salt concentration of 60 milliequivalents/liter reduced the area under the coacervation curve, and a maximum temperature for coacervation was found. In the absence of salt, no maximum was observed. The effect on the concentration of cetrimide required to induce coacervation was negligible, as shown by the fact that the left-hand limit of the coacervation curve was unaffected by the addition of salt. The order of reduction of coacervation area was: sodium citrate > sodium sulfate > sodium chloride; calcium chloride > sodium chloride.

Aqueous 0.15-mmole/Liter Carmoisine-Cetrimide System (Fig. 12)—An inorganic salt concentration of 30 milliequivalents/liter *increased* the area of coacervation. The right-hand boundary of the coacervation area when sodium citrate was present differed in shape from the corresponding curves obtained when sodium chloride, sodium sulfate, or calcium chloride was the added salt. For the systems containing sodium citrate, the coacervation area was much larger and a peak occurred at an added cetrimide concentration of 1.35 mmoles/liter. For the other systems, there was no peak. The salts had very little effect on the left-hand boundaries of the coacervation areas. The salts *increased* the coacervation area in the order: sodium citrate > sodium sulfate > sodium chloride: sodium chloride = calcium chloride.

Aqueous 0.15-mmole/Liter Erythrosine-Cetrimide System (Fig. 13)—An inorganic salt concentration of 30 milliequivalents/liter decreased the area under the coacervation curve. There was little difference in efficiency between the ions.

Minimum Ratio of Dye to Surfactant for Coacervation—Graphs of the dye concentration required to produce coacervation *versus* surfactant concentration yielded straight lines which

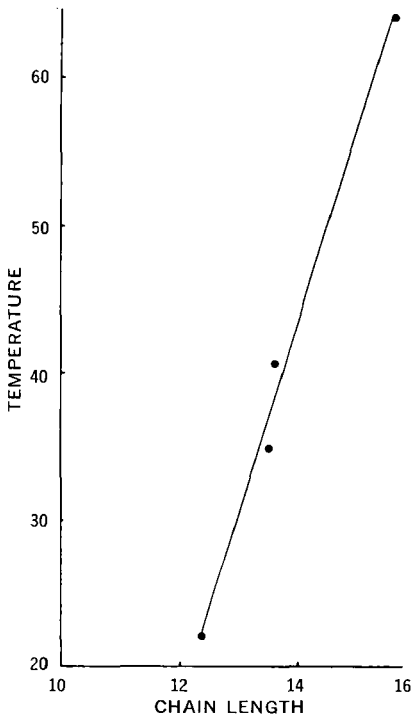

Figure 8—*Plot of maximum temperature for phase separation in tartrazine–alkyltrimethylammonium bromide systems (Fig. 1) versus chain length of alkyltrimethylammonium bromide.*

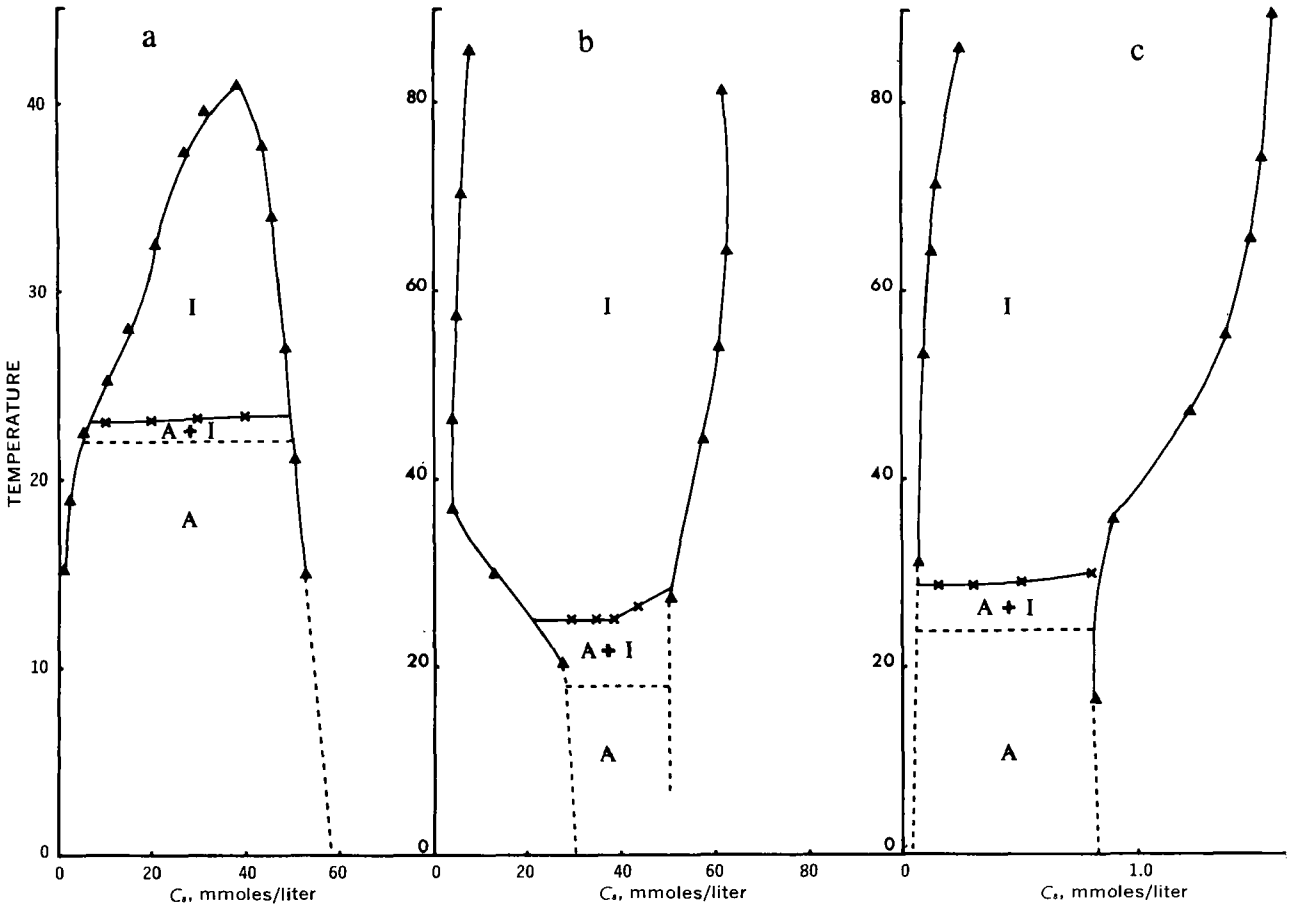

Figure 9—*Diagrams showing temperatures at which dye–tetradecyltrimethylammonium bromide interaction products changed phase, as determined by microscopy:* (a), *15 mmoles/liter tartrazine–tetradecyltrimethylammonium bromide;* (b) *15 mmoles/liter amaranth–tetradecyltrimethylammonium bromide; and* (c) *0.15 mmole/liter amaranth–tetradecyltrimethylammonium bromide. In each case the surfactant concentration is* C_s, *mmoles/liter. Key: I, separated phase is isotropic; A, separated phase is anisotropic; and A + I, separated phase is mixed anisotropic and isotropic.*

passed through, or very close to, the origin. Plots for the tartrazine–tetradecyltrimethylammonium bromide systems at various temperatures are shown in Fig. 14. Similar graphs were obtained when the alkyltrimethylammonium bromides were titrated against the other dyes. The gradients A and the intercepts B (see Eq. 1) were calculated by the method of least squares (Tables I and II). The values of B were, with a few exceptions, within ±1 milliequivalent/liter of the origin. This was within experimental error, so the values of B were assumed to be negligible (*cf.*, Ref. 5).

Two types of behavior were observed:

1. For tartrazine, increasing the chain length of the alkyltrimethylammonium bromide reduced the minimum ratio of dye-surfactant required for coacervation. Furthermore, a plot of the gradient A *versus* temperature at which the experiment was performed, for each of the homologs, yielded a straight line (Fig. 15). At temperatures close to the maxima of the coacervation curves, the minimum volume of dye that coacervated the surfactant-dye solution was not reproducible. Results obtained at these temperatures were not used. Therefore, the data for dodecyltrimethylammonium bromide (which does not coacervate in the presence of tartrazine above a temperature of 22°) cover a restricted temperature range.

2. For amaranth and carmoisine, this ratio was increased with an increase in surfactant chain length. A similar increase with chain length was observed for erythrosine at 60°, but for erythrosine at 35° the ratio increased from the dodecyl to the tetradecyl homolog and then remained constant.

DISCUSSION

When a long-chain quaternary ammonium salt is added to water such that the surfactant is at a concentration above the CMC, an equilibrium develops between surfactant monomers and the micellar species. The withdrawal of the hydrocarbon chains from an aqueous environment into the micelle favors aggregation, whereas the coulombic repulsion of the cationic head-groups at the surface of the micelle opposes aggregation. Therefore, factors that enhance the first phenomenon, or suppress the second, encourage micelle formation and growth. When conditions are favorable, the micelle may become lamellar and grow to such extreme proportions that phase separation, sometimes in the form of a coacervate, occurs. The surfactant micelles in the coacervate are in a more ordered state than in the aqueous solution, but this phase is still optically isotropic and has the appearance of an oil. A con-

Table I—Effect of Temperature on the Minimum Ratio of Tartrazine–Surfactant Required for Coacervation (A) (in Milliequivalents per Liter of Tartrazine: Milliequivalents per Liter of Surfactant) and the Equilibrium Concentration of Unbound Tartrazine (B) (in Milliequivalents per Liter Tartrazine) for Each of the Surfactant Homologs (See Eq. 1)

Temperature	Alkyltrimethylammonium Bromide					
	Dodecyl-		Tetradecyl-		Hexadecyl-	
	A	B	A	B	A	B
10°	0.611	−0.26	—	—	—	—
16°	0.637	0.10	0.578	0.10	—	—
25°	—[a]		0.611	0.14	0.565	0.20
30°	—[a]		0.625	0.42	—	—
35°	—[a]		0.643	0.64	0.594	0.06
50°	—[a]		—[a]		0.641	0.18

[a] No coacervation.

Table II—Values of the Minimum Ratio of Dye–Surfactant Required for Coacervation (*A*) (in Milliequivalents per Liter of Dye:Milliequivalents per Liter of Surfactant) and the Equilibrium Concentration of Unbound Dye (*B*) (in Milliequivalents per Liter) for the Interaction of Amaranth, Carmoisine, and Erythrosine with Each of the Surfactant Homologs at 35 and 60°

Dye	Temperature	Alkyltrimethylammonium Bromide							
		Dodecyl-		Cetrimide		Tetradecyl-		Hexadecyl-	
		A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Amaranth	35°	0.78	−1.77	0.89	−0.57	0.88	−0.27	0.92	0.03
	60°	0.72	−2.43	0.78	−0.84	0.79	−0.57	0.88	−0.06
Carmoisine	35°	0.77	−0.11	0.95	0.04	0.90	0.12	—[a]	
	60°	0.84	−0.46	0.93	−0.02	0.88	0.12	0.97	0.02
Erythrosine	35°	0.70	−1.04	0.78	−0.26	0.78	−0.20	0.78	0.12
	60°	0.64	−1.62	0.74	−0.54	0.74	−0.52	0.78	−0.02

[a] No coacervation.

sideration of the factors that enhance, or suppress, micellization is a valuable preliminary to the discussion of the coacervates produced by the interaction of tartrazine, amaranth, carmoisine, and erythrosine with the alkyltrimethylammonium bromides. The degree of micellization is affected by the nature and concentration of the surfactant, the addition of other materials, and the temperature of the system. For ionic surfactants: *(a)* The CMC must be reached before micellization occurs. *(b)* With increasing chain length in a homologous series, the CMC decreases and the micellar molecular weight increases. *(c)* Addition of ions of opposite charge quenches the coulombic interactions of the charged headgroups at the surface of the micelle. This promotes micellar growth and, in cases where the quenching is sufficient, coacervation occurs (4–10). *(d)* Compounds with hydrophobic portions such as organic ions of opposite charge (21), alcohols (22), and dyes (11, 13) markedly decrease the CMC. These compounds exert their influence not only on the coulombic forces but also on the hydrophobic interactions of the micelle. Goddard *et al.* (21) found that the effect of short-chain quaternary ammonium ions on the sodium dodecyl sulfate micelle was different from that of inorganic cations. They suggested that this was because the hydrocarbon groups of the organic cations enter the palisade layer of the micelle and affect both the hydrophobic and electrostatic interactions of the surfactants. Shinoda (22) described a relationship between the number of carbon atoms in an alcohol and the CMC-decreasing power of the alcohol. *(e)* Temperature changes may also affect micelle formation. For example, Adderson and Taylor (23) showed that increasing the temperature of long-chain quaternary ammonium bromide solutions increased the CMC. Studies on the thermodynamics of micellization of the surfactants used in the present work (20) showed this to be the situation, although in some cases an initial minimum in the CMC *versus* temperature plots occurred.

A general summary of the effects on micellization of changing the composition and temperature of the alkyltrimethylammonium bromide–dye system is provided in Table III. The variables are listed in the order shown for the sake of clarity of discussion. Since coacervation in aqueous quaternary surfactant solutions is the result of rapid micellar growth, coupled with the reduction of

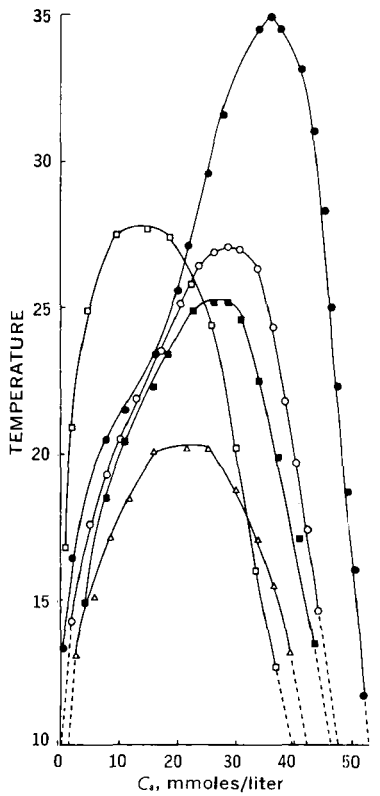

Figure 10—*Effect of inorganic salts on area of coacervation of tartrazine–cetrimide system. Tartrazine concentration is 15 mmoles/liter. Inorganic salt concentration is 120 mEq/liter. Cetrimide concentration is* C_s, *mmoles/liter. Key:* ●, *no salt;* ○, *sodium citrate;* ■, *sodium sulfate;* △, *sodium chloride; and* □, *calcium chloride.*

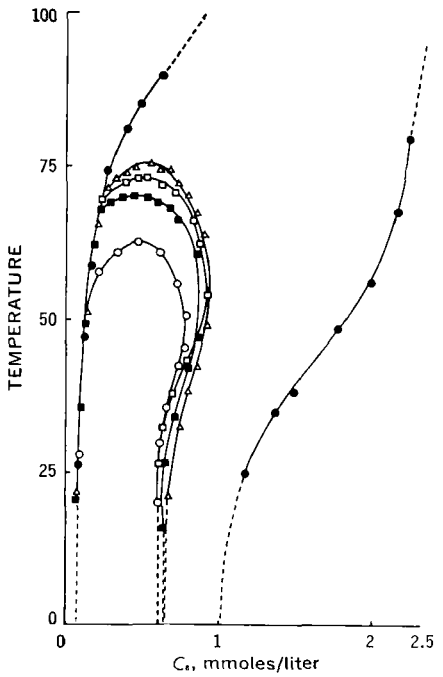

Figure 11—*Effect of inorganic salts on area of coacervation of amaranth–cetrimide system. Amaranth concentration is 0.15 mmole/liter. Inorganic salt concentration is 60 mEq/liter. Cetrimide concentration is* C_s, *mmoles/liter. Key:* ●, *no salt;* ○, *sodium citrate;* ■, *sodium sulfate;* △, *sodium chloride; and* □, *calcium chloride.*

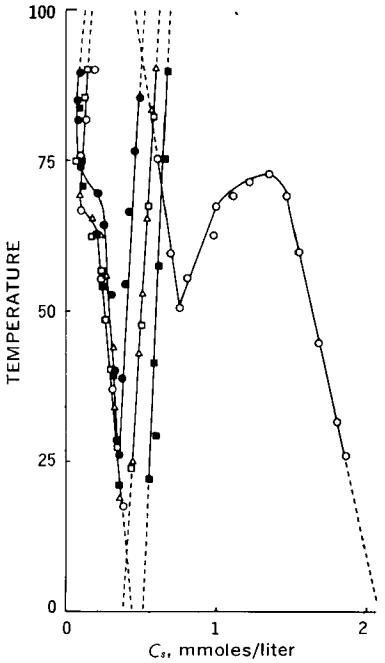

Figure 12—*Effect of inorganic salts on area of coacervation of carmoisine–cetrimide system. Carmoisine concentration is 0.15 mmole/liter. Inorganic salt concentration is 30 mEq/liter. Cetrimide concentration is* C_s, *mmoles/liter. Key;* ●, *no salt;* ○, *sodium citrate;* ■, *sodium sulfate;* △, *sodium chloride; and* □, *calcium chloride.*

the micellar surface charge (7), factors that promote micellization should also encourage coacervation. With these factors in mind, an attempt to explain the complex effects of the dyes on coacervation of alkyltrimethylammonium bromide solutions may be made.

When the composition or temperature of a coacervating system is varied by only a slight amount, the system may be profoundly changed. A sensitive indication of these changes occurs at a critical point where a homogeneous solution separates into two phases. The phase separation is readily observed (the solution becomes turbid) and is thermodynamically reversible. It can thus be used to study the effects of composition and temperature on coacervation. Figures 1–7 show the temperatures at which surfactant solutions of varying compositions become turbid in the presence of dye.

Maximum quenching of the electrostatic repulsion between the micellar cationic head-groups occurs when there is an equivalent amount of negative charges at the surface of the micelle. At this point, the volume of coacervate is at a maximum. An excess of cationic, or anionic, species results in a net positive, or negative, surface charge which tends to suppress coacervation (3). Hence, systems with compositions that fell on, or inside, the coacervation boundaries were turbid, whereas those which fell outside the boundaries were clear. Therefore, the right-hand boundaries of the coacervation areas denote the minimum ratio of surfactant–dye required to suppress coacervation or, conversely, the minimum ratio of dye–surfactant required to induce coacervation (Tables I and II). The left-hand boundaries denote the minimum ratio of dye–surfactant required to suppress coacervation.

Since more is known about the factors inducing coacervation in aqueous solutions of the alkyltrimethylammonium salts, the discussion of the different coacervation curves begins by considering the right-hand boundaries of the coacervation areas.

Tartrazine–Alkyltrimethylammonium Bromide Systems—Tartrazine (I) is a very polar molecule with no strictly hydrophobic portions. Only criteria *(a)*, *(b)*, *(c)*, and *(e)* (Table III) need be considered in the tartrazine–alkyltrimethylammonium systems. *(a)* In distilled water, tartrazine is a doubly charged anionic molecule, in which both sulfonate groups are fully dissociated. (Potentiometric titration showed that the COONa group started to dissociate at about pH 8.5.) It is very effective in quenching the coulombic interactions of the quaternary ammonium groups at

Table III—Effects on Micellization of Changing the Composition and Temperature of Alkyltrimethylammonium Bromide Systems

Change in the System	Effect on Micellization
(*a*) Increase in number of dye anions (up to stoichiometric amount) at surface of micelle	Increases
(*b*) Increase in surfactant concentration	Increases
(*c*) Increase in surfactant chain length	Increases
(*d*) Addition of dye with hydrophobic portion	Increases
(*e*) Increase in temperature	Decreases

the surface of the micelle, so that large anisotropic micelles with low surface charge may be built up. This, of course, favors coacervation. *(b)* For coacervation to occur, an adequate amount of surfactant must be present. Below the CMC, the surfactant does not aggregate into micelles and one would not expect to find coacervation of the surfactant solution. When the tartrazine concentration was 0.15 mmole/liter, turbidity was produced at surfactant concentrations that were below the CMC's of the surfactants in water. However, the turbidity was very faint and microscopic examination yielded no information on the morphology of the separated phase. When the tartrazine concentration was 15 mmoles/liter, the surfactant concentrations at the right-hand boundaries of the coacervation areas were well above the CMC. In these systems, the turbidity was easily observed and a coacervate layer was readily produced. *(c)* Table I shows that as the surfactant chain length increases, the minimum amount of tartrazine required to produce coacervation decreases. This is expected on the basis that the longer the chain length the bigger is the micelle and, therefore, less electrolyte is required to promote micellar growth to limiting proportions. *(e)* Adderson and Taylor (23) showed that the CMC of aqueous alkyltrimethylammonium bromide solutions increases as the temperature increases, due to increased thermal agitation. Therefore, to maintain coacervation, more electrolyte must be added. Table I and Fig. 15 show that the minimum ratio of dye–surfactant required for coacervation

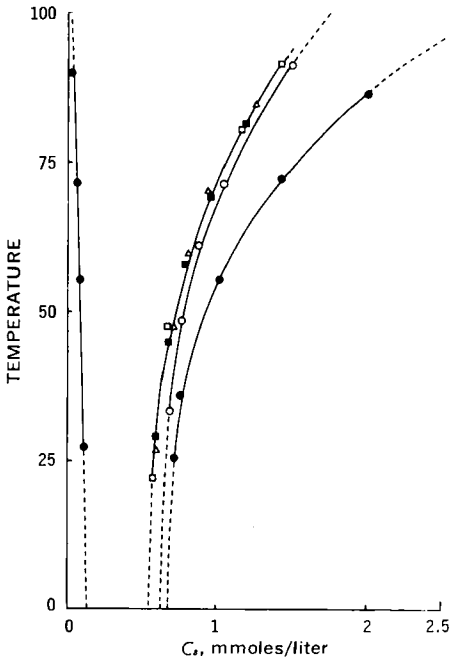

Figure 13—*Effect of inorganic salts on area of coacervation of erythrosine–cetrimide system. Erythrosine concentration is 0.15 mmole/liter. Inorganic salt concentration is 30 mEq/liter. Cetrimide concentration is* C_s, *mmoles/liter. Key:* ●, *no salt;* ○, *sodium citrate;* ■, *sodium sulfate;* △, *sodium chloride; and* □, *calcium chloride.*

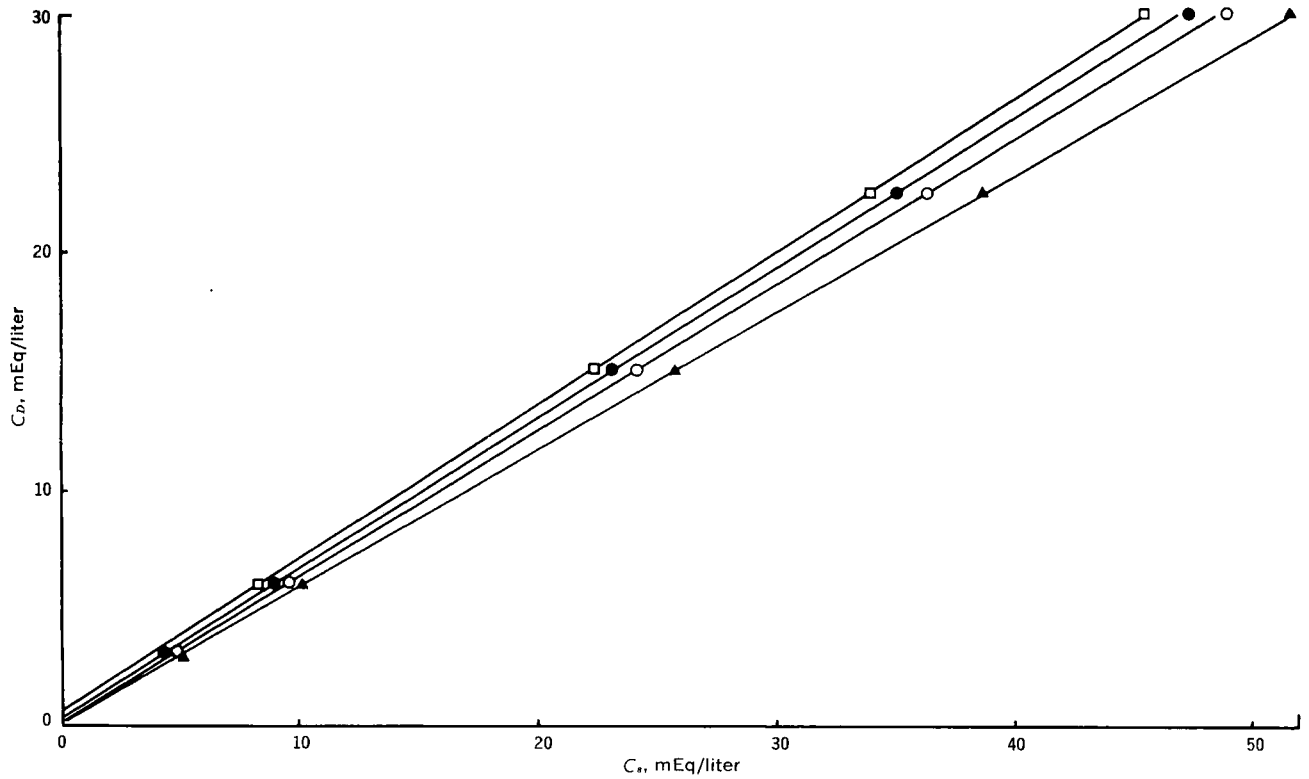

Figure 14—*Plots of tartrazine concentration* (C_D, *milliequivalents/liter*) *required to produce coacervation of tetradecyltrimethylammonium bromide solution at different temperatures* versus *tetradecyltrimethylammonium bromide concentration* (C_s, *milliequivalents/liter*). *Key:* ▲, *16°;* ○, *25°;* ●, *30°; and* □, *35°*.

increases linearly with temperature. Furthermore, Fig. 1 shows that, for each surfactant homolog, a maximum temperature for coacervation is reached. This maximum temperature decreases as the surfactant chain length decreases, as expected on the basis of *(c)*. At temperatures about 15–20° below the maximum temperature, the separated phase has such a high degree of order that it is optically anisotropic (*cf.*, Fig. 9*a*).

In summary, the factors that promote coacervation of aqueous alkyltrimethylammonium bromide systems by tartrazine are: surfactant and tartrazine concentrations above a limiting concentration, large chain length, and low temperature. These criteria are identical to those found in coacervating systems of alkyltrimethylammonium salts with inorganic electrolytes (5, 7).

Amaranth–Alkyltrimethylammonium Bromide Systems—Amaranth (II) carries three sulfonate groups that are totally dissociated in distilled water and also possesses two hydrophobic naphthalene nuclei. Therefore, it interacts with the micelle not only through the ionic groups but also through London–van der Waal's association forces. All five criteria listed in Table III are involved in the amaranth–surfactant interactions. Several authors reported the formation of mixed micelles in aqueous surfactant–dye solutions (13, 14, 24). However, for amaranth, an arrangement that permits maximum association between the naphthalene nuclei of the dye and the hydrocarbon chains of the quaternary surfactant micelle does not allow maximum interaction of the dye's sulfonate groups with the quaternary ammonium groups at the surface of the micelle. The converse is also true. Therefore, there are two possible positions for the amaranth molecule in contact with the surfactant micelle. (The values of B in Table II show that only a negligible amount of amaranth is *not* bound to the micelle.)

Position (i)—The amaranth molecule lies flat on the surface of the surfactant micelle. In this position, all three sulfonate groups are involved in the quenching of the interactions of the quaternary ammonium groups. However, this does not allow any interaction between the hydrophobic portions of the amaranth and the micelle.

Position (ii)—The amaranth molecule is partially inserted into the micelle, such that two sulfonate groups are level with the quaternary ammonium ions at the surface of the micelle and the azo-linked naphthalene rings penetrate the hydrocarbon interior of the micelle. In this position, the naphthalene nuclei and the hydrocarbon chains associate by nonpolar forces. However, one sulfonate group must penetrate the interior of the micelle. This sulfonate group is probably solubilized in the interior of the micelle as part of an ion-pair formed with a molecule of surfactant. Lindman and Ekwall (25) previously reported the formation of strongly bound ion-pairs in sodium caprylate–caprylic acid systems. Furthermore, three-dimensional atomic models[9], built to scale, show that the amaranth molecule may fit into a lamellar surfactant micelle as described, to yield a fairly ordered arrangement. Such a lattice arrangement must exist to allow liquid crystal formation at lower temperatures (Figs. 9*b* and 9*c*).

How this postulate fits the experimental observations and the criteria listed in Table III may now be examined. *(a)* The contribution of an amaranth molecule to the quenching of the electrostatic repulsions of the quaternary ions at the surface of the micelle depends upon how it associates with the micelle. In Position (i), all three sulfonate ions may interact with the surface of the micelle to provide maximum electrostatic quenching. In Position (ii), only two sulfonate groups are involved in surface interactions. Therefore, in this position an amaranth molecule is less effective in producing coacervation than it is in Position (i). *(b)* Systems containing 15 or 0.15 mmole/liter amaranth (and, therefore, surfactant at concentrations that were well above and well below their CMC's in distilled water) produced coacervates that could be readily studied (Figs. 2 and 3). This is in contrast to tartrazine where only the systems at the higher concentration could be examined; this underlines the influence of the hydrophobic portion of amaranth on the reduction of the CMC. *(c)* In the quaternary surfactant systems containing tartrazine, increasing the chain length of the surfactant decreases the amount of electrolyte required for coacervation. However, in the amaranth systems, increasing the surfactant chain length has the additional effect of increasing the solubilization of the dye–surfactant ion-pair in the interior of the micelle.

Hence, hexadecyltrimethylammonium bromide micelles bind more amaranth in Position (ii) than tetradecyl micelles which,

[9] Orbit Molecular Building Set, R.J.M. Exports Ltd., Oxford, England.

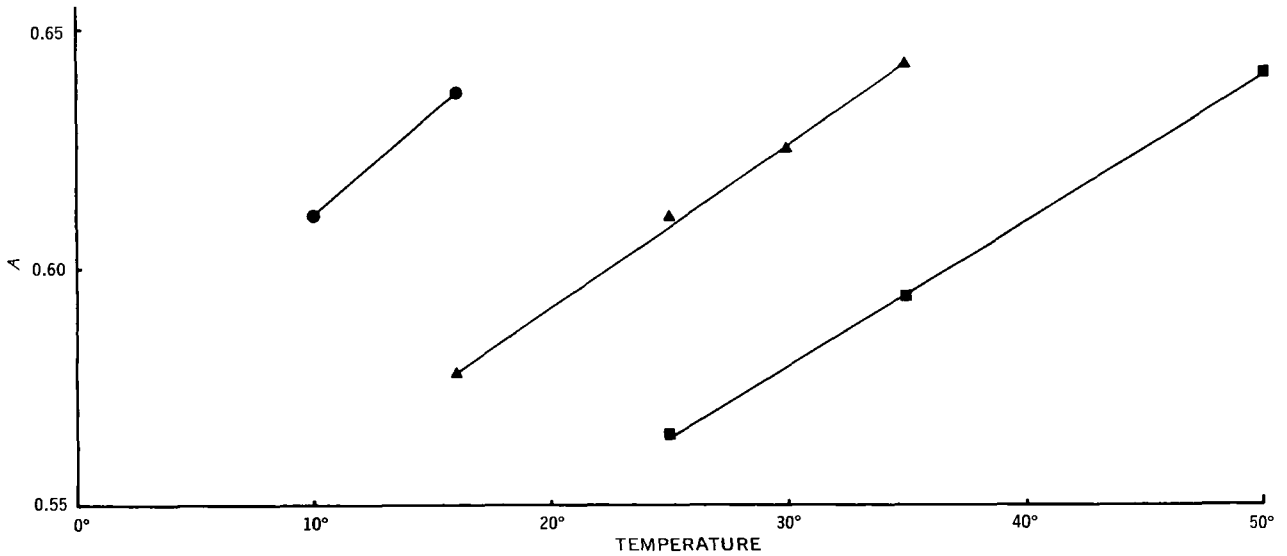

Figure 15—*Plots of gradient* A *(obtained from Table I) for the tartrazine–alkyltrimethylammonium bromide systems* versus *temperature. Key:* ■, *tartrazine–hexadecyltrimethylammonium bromide;* ▲, *tartrazine–tetradecyltrimethylammonium bromide; and* ●, *tartrazine–dodecyltrimethylammonium bromide.*

in turn, solubilize more dye than the dodecyl homolog. But dye bound in this position is less effective in producing coacervation than dye bound in Position (i). Therefore, increasing the surfactant chain length has two opposing effects. The one that predominates decides the amount of dye required to produce coacervation. *(d)* Figures 2 and 3 show how the hydrophobic portion of amaranth markedly alters the phase diagrams as compared to dyes where there is no hydrophobic group (Fig. 1). The discussion of the effects of the hydrophobic group is included in the discussion of the other criteria. *(e)* In alkyltrimethylammonium systems containing tartrazine or inorganic salts, warming tended to suppress coacervation. However, when amaranth is present, raising the temperature has a greater disruptive effect on weak van der Waal's interactions than on strong electrostatic associations. This favors the binding of amaranth to the micelle at Position (i), where it is more effective in quenching the coulombic interactions. Therefore, increasing the temperature also has two opposing effects on coacervation.

It is evident that the main differences of the amaranth systems compared to those of tartrazine arise because of the extra mode of interaction of amaranth with the micelle. An increase in the amount of amaranth solubilized in the interior of the micelle increases the ratio of dye–surfactant required for coacervation and displaces the right-hand boundaries of the coacervation areas to the left. As shown in Table II and by the right-hand boundaries of Figs. 2 and 3, the effect of increasing chain length is predominantly to increase solubilization; *i.e.*, the ratio of amaranth–surfactant required to produce coacervation increases in the order hexadecyl homolog > tetradecyl homolog; cetrimide > dodecyl homolog. Furthermore, an initial temperature increase displaces the right-hand boundaries to the right, because the decreased solubilization of amaranth overshadows the effect of the increased energy of activation for micellization. However, at higher temperatures, the latter effect becomes more important and the right-hand boundaries tend to level off with increased temperature (Figs. 2 and 3) and even to curve to the left (Fig. 2).

Carmoisine–Alkyltrimethylammonium Bromide Systems—The molecular structure of carmoisine (III) is similar to that of amaranth, except for the number of sulfonate groups, the position of the substituents, and the position of the azo-linkage. However, the carmoisine phase diagrams (Figs. 4 and 5) differ markedly from those of amaranth. In an auxiliary investigation, Sunset Yellow FCF (V), which is related to amaranth but contains a slightly different hydrophobic nucleus and has only two sulfonate groups, did not induce coacervation in solutions of tetradecyltrimethylammonium bromide. However, Orange II (VI), which has the same structure as sunset yellow but contains one less sulfonate group, brought about phase separation of solutions of this surfactant. The separated phase was optically anisotropic at room temperature but isotropic, oily globules formed at about 100°. These observations indicate that the extent of hydrophobic interaction, as well as the quenching of the electrostatic interactions, is important in determining whether phase separation takes place and the nature of the separated phase.

Carmoisine is more hydrophobic than amaranth and may be solubilized in the form of a surfactant–dye complex to a greater extent than amaranth. As a result, the right-hand boundaries of the phase diagrams are displaced to the left. The ratio of dye–surfactant required for coacervation (Table II) increases in the order hexadecyl homolog > tetradecyl homolog; cetrimide > dodecyl homolog. Furthermore, at temperatures below about 50°, so much of the complex is solubilized in the hexadecyltrimethylammonium bromide systems that the electrostatic quenching at the surface of the micelle is not sufficient to bring about coacervation.

The data for the more concentrated systems are restricted to temperatures above 50°, because at lower values the systems gelled, inhibiting complete mixing.

Erythrosine–Alkyltrimethylammonium Bromide Systems—The structure of erythrosine differs greatly from those of amaranth and carmoisine and is similar only in that it, too, has polar and nonpolar regions. A planar arrangement of the molecule is impossible because of steric hindrance between the xanthene and benzene rings, but it appears that the molecule is still partially inserted into the micelles, as shown by the fact that the right-hand boundaries of the coacervation areas (Figs. 6 and 7) are similar to those obtained with carmoisine. This suggests that a similar mechanism for interaction operates.

So far, only the right-hand boundaries of the coacervation areas have been considered. The shape and position of the left-hand boundaries provide a measure of the solubility of the dye–surfactant product in excess dye. The solubility varies with the nature and concentration of the dye, surfactant chain length, temperature, and ionic strength.

Striking differences occur between the left-hand boundaries of the coacervation areas for the three azo dyes: tartrazine, amaranth, and carmoisine (Figs. 1–5). For tartrazine, an initial large increase in solubility with increase in temperature occurs after the paracrystalline phases have melted to form a coacervate, as exemplified by the tartrazine–tetradecyltrimethylammonium bromide system (Fig. 9*a*). The solubility then continues to increase with temperature until a coacervate is no longer produced.

For the other azo dyes, amaranth and carmoisine, a different phenomenon must be present. Frank (26) found that, although micelles were not present to any appreciable extent in solutions of sulfonated azo-dyes in water, rod-like micelles formed when inorganic salts were added. These micelles were readily destroyed by warming the solutions. It is possible that the quaternary ammonium bromides also promote the formation of dye micelles, which could then solubilize the dye–surfactant complex, thus preventing the formation of a coacervate. Evidence for the existence of such

a mechanism is provided by the following: *(a)* the areas of solubility at the left-hand side of the coacervation diagrams decrease with an increase in temperature (the dye micelles are heat labile); *(b)* the areas of solubility are greater with 15-mmoles/liter dye solutions than with 0.15-mmole/liter dye solutions (presence of a CMC for formation of dye micelles); and *(c)* the areas of solubility are greater in the carmoisine systems than in the amaranth systems (carmoisine is more hydrophobic than amaranth, so it is more likely to form micelles). However, apart from what may be inferred from the phase diagrams (Figs. 2–5), there is no experimental evidence for the presence of such a phenomenon. A detailed discussion of the left-hand boundaries is, therefore, not possible.

The complex formed between erythrosine and the alkyltrimethylammonium bromides is so insoluble that solutions become turbid when only small amounts of surfactant are added to a dye solution (Figs. 6 and 7). Increasing the temperature slightly decreases the solubility of the complex.

Effect of Ionic Strength on Phase Diagrams—In general, inorganic salts suppress complex coacervation of polycations and polyanions (3, 27). This effect increases with an increase in the valency of the inorganic ions. However, exceptions to this rule are known (27). Furthermore, some inorganic ions coacervate ionic surfactant solutions (4–10). It is desirable to know the effects of ionic strength on the coacervation produced by interaction between the dyes and alkyltrimethylammonium bromides. The effects of constant concentrations of sodium chloride, sodium sulfate, sodium citrate, and calcium chloride are shown in Figs. 10–13. For tartrazine, amaranth, and erythrosine, the areas under the coacervation curves are decreased by the addition of salts at the concentrations studied, but the order differs in all three cases. For carmoisine, the area under the coacervation curve is *increased* on addition of inorganic salts and a secondary coacervation area is observed in solutions containing sodium citrate (Fig. 12).

In summary, it is evident that these dye–surfactant interactions are very complex, as emphasized by the many works published that attempt to elucidate the mechanisms involved. For example, Colichman (24) claimed that his results, obtained from surface and interfacial tension titrations of long-chain quaternary salts in bromphenol blue and bromthymol blue solutions, showed: "the micellar nature of the quaternary-dye ion-pair compound as formed in aqueous solutions." Kondo *et al.* (17) used the terms "zones of flocculation" and "deflocculation number" to define the extent of interaction of a number of acid dyes with dodecylpyridinium bromide. Zografi *et al.* (14) studied the interactions between Orange II and several long-chain quaternary ammonium salts from the point of view of the electronic and steric configuration of the surfactant. However, in all of these and in many other studies, no mechanism was proposed that could fully explain all of the observed phenomena of the dye–long-chain quaternary ammonium salt interactions.

In addition to these studies, reports (12, 13) were made concerning the interactions of anionic surfactants with cationic dyes. Again, differing theories for the mechanism of interaction were advanced.

The present work was initiated to solve some of the unanswered questions. In some aspects, this goal has been achieved, but in others the authors have succeeded only in raising further questions. The "reversibility" of the interactions in the dye–alkyltrimethylammonium bromide systems studied has been explained by showing the presence of a coacervate phase and applying the coacervation theory of Voorn (3) which Cohen and Vassiliades (7) showed was applicable to coacervation of long-chain quaternary ammonium salts by oppositely charged ions. The difference between coacervates obtained from solutions of the quaternary salts on addition of an essentially hydrophilic anion on one hand and anions containing hydrophilic *and* hydrophobic moieties on the other has also been shown. An attempt has been made to interpret the differences observed between the members of the latter category. However, the conclusions drawn are essentially inductive, and additional work must be performed before their validity can be confirmed. It has also been shown that dye induces formation of surfactant micelles at concentrations that are well below the CMC's of the surfactants in water. For example, Fig. 9*c* illustrates that at 40°, a coacervate is present at concentrations of tetradecyltrimethylammonium bromide of less than 0.1 mmole/liter. The CMC of this homolog was 3.6 mmoles/liter at 40° (28). Since coacervation in surfactant solutions is dependent on the formation of anisotropic micelles (7), this implies that the CMC has been reduced about 40-fold. However, the exact nature of the micelles formed in this region is less certain. Here, too, additional work is required.

From the practical point of view, it is obvious that when the surfactants and dyes are present in the same preparation, they must be mixed in proportions that are not close to stoichiometric amounts. It is preferable that there be an excess of surfactant over the stoichiometric amount of dye. Furthermore, the effect of the total ionic strength on the stability of the solution must be examined in detail.

SUMMARY

The interactions of the dyes tartrazine, amaranth, carmoisine, and erythrosine with alkyltrimethylammonium bromides of chain length C_{12}–C_{16} were investigated by phase separation techniques. Between certain surfactant concentrations and temperature limits, phase separation occurred. Microscopy showed that the separated phase was either a coacervate or an anisotropic phase or both. The effect of sodium chloride, sodium sulfate, sodium citrate, and calcium chloride on the extent of coacervation of cetrimide–dye systems was investigated. Phase separation diagrams for all dye–surfactant systems showed marked differences between the systems. The minimum ratio of dye to surfactant required for coacervation varied markedly with different dye–surfactant systems with respect to the effect of surfactant chain length and to temperature. Inorganic salts also produced different effects on the extent of phase separation in each system investigated.

The differences were attributed to the presence, or absence, of a hydrophobic nucleus in the dye molecule. It is suggested that when the dyes and surfactants are present in the same preparation, they be mixed in proportions that are not close to stoichiometric amounts. The effect of total ionic strength of the preparation must also be carefully investigated.

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[x] To whom inquiries should be directed.

Effect of Selected Surfactants, above and below the CMC, on Aspirin Solubility

J. K. LIM[x] and C. C. CHEN*

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Keyphrases □ Aspirin in aqueous suspension—solubilizing effect by cetylpyridinium chloride, benzalkonium chloride, polysorbates 20 and 80, and dioctyl sodium sulfosuccinate above and below their CMC's □ Solubilizing effect of surfactants above and below CMC—aspirin in aqueous suspension □ Surfactant effect—solubilization of aspirin in aqueous suspension

During the past few years, numerous high molecular weight compounds functioning as surfactants have been successfully formulated in pharmaceutical and cosmetic preparations because of micellar solubilization. These included nonionic surfactants such as polysorbates for solubilizing drugs such as aspirin, hydroxybenzoic acid, and phenobarbital (1).

In this study an attempt was made to investigate representative classes of surfactants for their influence on aspirin solubility under conditions closely simulating the human gastric environment. The objective was to search for relatively nontoxic compounds that could enhance the solubility of aspirin in the acid pH of the gastric fluids without resulting in a concomitant increase in aspirin hydrolysis. Such surfactants might potentially be formulated in soft gelatin capsule or compressed tablet dosage forms of aspirin for improved bioavailability of the drug. The poor solubility of aspirin in water is a contributing factor to its irritant effect on the gastric mucosa. Aspirin crystals could be lodged physically in the rugae of the gastric mucosa to cause serous erosion and hemorrhages in the presence of even moderate amounts of acid (2). Its rapid dissolution and consequent absorption in the body seemed desirable because of these reasons.

EXPERIMENTAL

Materials—The six surface-active compounds originally chosen for this study, representing the nonionic, cationic, and anionic classes, were: polysorbate 20[1], polysorbate 80[1], benzalkonium chloride[2], cetylpyridinium chloride[3], dioctyl sodium sulfosuccinate[4], and sodium lauryl sulfate[4], all USP, NF, or analytical reagent grade. Sodium lauryl sulfate produced a precipitate in the pH 2.4 buffer solution during preliminary tests and was therefore abandoned for further study.

CMC Determinations—The critical micelle concentration (CMC) data for the various surfactants were obtained by the surface tension method (3) employing a surface tensiometer[5]. It was preferred over other literature methods for determining the CMC because of its simplicity and reproducibility. The inflection point in the slope when the logarithm of concentration of a surfactant was plotted against the surface tension of its solution represents the CMC. The solvent was a Clark-Lubs buffer with potassium chloride and hydrochloric acid adjusted to pH 2.4.

Solubility Determinations—The solubility data for aspirin were obtained by placing 350 mg of aspirin USP crystals[4], which would be in excess of its solubility, in 25 ml of the "solvent" contained in erlenmeyer flasks. These surfactant solutions were fresh dilutions of the more concentrated stock solutions that had previously been equilibrated by stirring using a magnetic stirrer for 24 hr at room temperature. Just prior to commencing the solubility study, the diluted surfactant solutions were equilibrated at 37 ± 0.4° in a water bath for at least 1 hr. These aspirin suspensions, prepared in triplicate for each strength of aspirin, were agi-

[1] Atlas Chemical Industries.
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[3] K & K Labs.
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[5] Roller-Smith Rosano surface tensiometer.

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[x] To whom inquiries should be directed.

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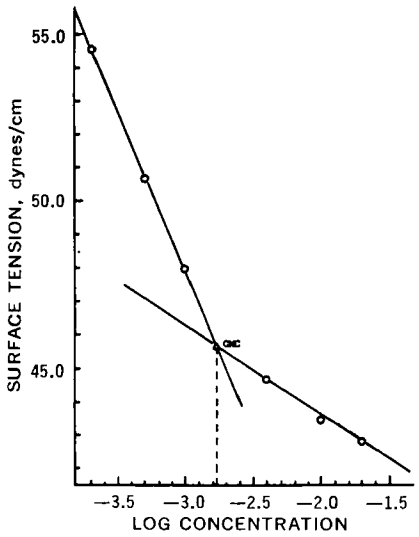

Figure 1—*Surface tension (dynes per centimeter) as a function of the logarithm of concentration (w/v %) of polysorbate 80 when determined at 25° in pH 2.4 Clark-Lubs buffer solution. Point of inflection represents the CMC.*

tated on a mechanical shaker immersed in a constant-temperature bath at the rate of 120 vibrations/min. At suitable intervals, flasks were removed and the contents were quickly filtered, with the aid of a filter[6] and syringe holder, through No. 2 Whatman filter paper. Suitable aliquots of the clear filtrate were pipeted and diluted in ice-cold pH 7.4 buffer.

The free salicylic acid referred to in this work was that produced by the hydrolysis of aspirin during the agitation period in the buffered surfactant solutions at 37 ± 0.4°. The absorbance of the sample, maintained at 5° to avoid further hydrolysis, was determined at a wavelength of 296.5 nm on a double-beam spectrophotometer[7] equipped with a constant-temperature water circulator[8]. The total salicylic acid content of each sample was determined by first hydrolyzing it with 1 *N* sodium hydroxide solution for 10 min before neutralization with 1 *N* hydrochloric acid solution and final dilution to volume in volumetric flasks with pH 7.4 buffer. The Beer's law plot was obeyed in the concentration range of 0–30 μg/ml of salicylic acid under this condition. A factor of 1.3 was applied to the total salicylic acid value obtained to compute for the total amount of aspirin dissolved.

RESULTS AND DISCUSSION

Figure 1 shows clearly the CMC inflection point of the curve obtained with polysorbate 80. This was typical of all other surfac-

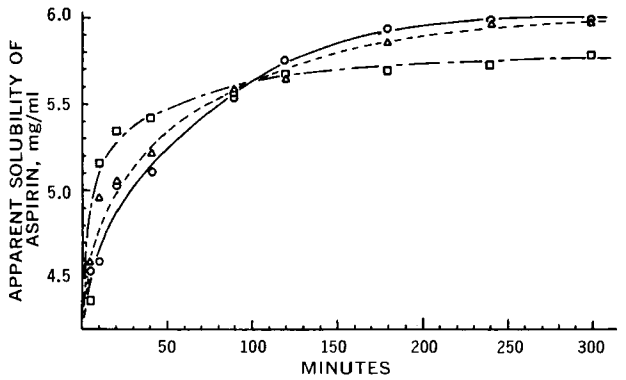

Figure 2—*Effect of polysorbate 20 on the apparent solubility of aspirin as a function of time at 37° in pH 2.4 buffer. Key: △, above CMC; ○, below CMC; and □, buffer control.*

[6] Millipore.
[7] Perkin-Elmer.
[8] Lauda-Brinkmann circulator, model K-2/R.

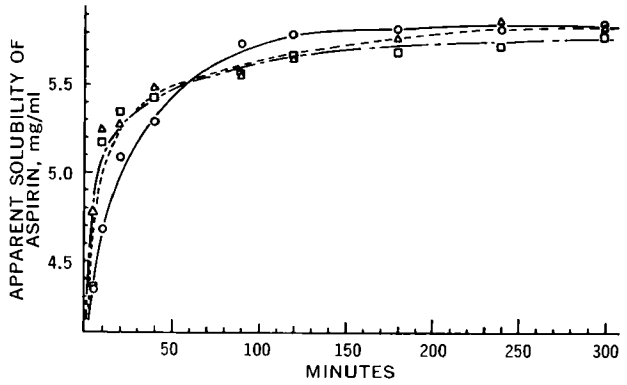

Figure 3—*Effect of polysorbate 80 on the apparent solubility of aspirin as a function of time at 37° in pH 2.4 buffer. Key: △, above CMC; ○, below CMC; and □, buffer control.*

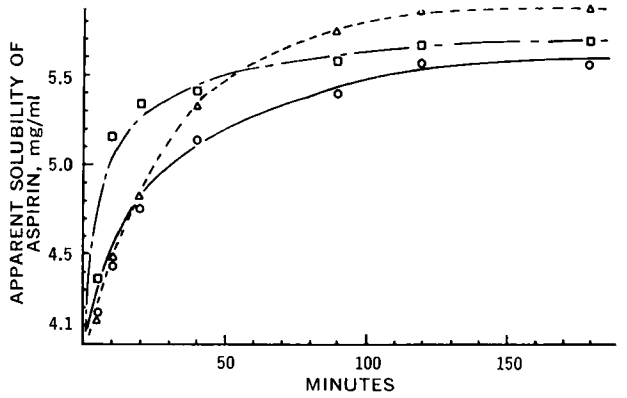

Figure 4—*Effect of benzalkonium chloride on the apparent solubility of aspirin as a function of time at 37° in pH 2.4 buffer. Key: △, above CMC; ○, below CMC; and □, buffer control.*

tants studied. These CMC values, however, differed somewhat from those reported in the literature because of differences in solvent composition (4), pH (5), and temperature. Results of the apparent solubilities of aspirin exposed to the different surfactants, based on calculations of the free and total salicylic acid determinations, are listed in Table I. Figures 2-6 show graphically these apparent solubilities of aspirin exposed to the various surfactants, above and below the CMC, and in relation to the control containing no surfactant. Since the solubility of a compound is generally dependent on the concentration of the solubilizing agent, effects by two levels of surfactant strength (arbitrarily one above the CMC and the other below the CMC) would be of interest. Studies on submicellar concentrations of the surfactant were included because of the inevitable and considerable lowering of the surfac-

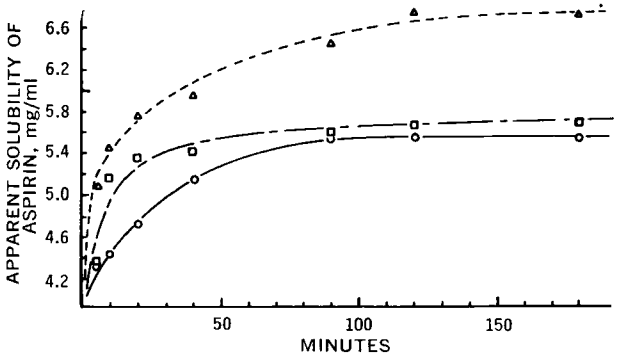

Figure 5—*Effect of cetylpyridinium chloride on the apparent solubility of aspirin as a function of time at 37° in pH 2.4 buffer. Key: △, above CMC; ○, below CMC; and □, buffer control.*

Table I—Apparent Solubilities (Milligrams per Milliliter) of Aspirin in pH 2.4 Buffer Containing Different Surfactants at 37°

Surfactant	Concentration of Surfactant	Minutes								
		5	10	20	40	90	120	180	240	300
Polysorbate 80	Above CMC	4.78 ±0.03[a]	5.24 ±0.03	5.27 ±0.05	5.48 ±0.04	5.57 ±0.02	5.64 ±0.02	5.76 ±0.07	5.86 ±0.04	5.83 ±0.04
	Below CMC	4.33 ±0.05	4.68 ±0.12	5.09 ±0.11	5.30 ±0.08	5.74 ±0.05	5.79 ±0.01	5.81 ±0.03	5.81 ±0.02	5.85 ±0.05
Polysorbate 20	Above CMC	4.59 ±0.06	4.96 ±0.03	5.05 ±0.07	5.21 ±0.03	5.59 ±0.03	5.64 ±0.04	5.85 ±0.01	5.97 ±0.02	5.98 ±0.00
	Below CMC	4.54 ±0.03	4.58 ±0.02	5.06 ±0.04	5.10 ±0.07	5.50 ±0.08	5.75 ±0.03	5.94 ±0.02	5.97 ±0.02	5.97 ±0.02
Dioctyl sodium sulfosuccinate	Above CMC	4.03 ±0.06	4.25 ±0.09	4.27 ±0.05	4.61 ±0.14	5.24 ±0.02	5.52 ±0.08	5.76 ±0.02	5.79 ±0.00	5.81 ±0.04
	Below CMC	3.98 ±0.03	4.39 ±0.04	4.74 ±0.05	5.05 ±0.03	5.48 ±0.03	5.68 ±0.03	5.79 ±0.00	5.78 ±0.06	5.79 ±0.00
Cetylpyridinium chloride	Above CMC	5.09 ±0.04	5.47 ±0.21	5.76 ±0.21	5.97 ±0.15	6.44 ±0.11	6.72 ±0.02	6.73 ±0.05		
	Below CMC	4.32 ±0.13	4.44 ±0.10	4.72 ±0.02	5.14 ±0.04	5.55 ±0.03	5.55 ±0.03	5.55 ±0.03		
Benzalkonium chloride	Above CMC	4.14 ±0.18	4.49 ±0.09	4.82 ±0.08	5.33 ±0.10	5.76 ±0.09	5.87 ±0.03	5.87 ±0.03		
	Below CMC	4.17 ±0.06	4.44 ±0.06	4.77 ±0.21	5.15 ±0.06	5.41 ±0.06	5.57 ±0.04	5.57 ±0.06		

[a] ± Standard deviation.

tant concentration after the oral administration of a drug dosage form by dilution with the gastric fluids. The attainment of isotonicity was deemed not to be an important consideration in this situation.

The graph drawn for cetylpyridinium chloride (Fig. 7) presents a typical example of the percentage hydrolysis of the aspirin suspensions due to the effects of the surfactant as averaged from three determinations. It may also be seen from Fig. 7 that the percentage hydrolysis of aspirin assumed a near linear relationship with respect to time after an initial lag period of approximately 20–25 min. This is roughly analogous to the report by James (6) for the rate of hydrolysis of an aspirin suspension at room temperature, which was shown not to follow either zero-order or first-order kinetics at any level of aspirin concentration. Comparison of Fig. 7 with the graphs obtained for the apparent solubilities of aspirin *versus* time (Figs. 2–6) shows that the onset of hydrolysis was approximately coincident with the time when about 90% of the apparent solubilities had been reached.

Figure 8 shows the extent of hydrolysis of aspirin due to either polysorbate 20 or polysorbate 80 when present above and below the CMC. A slightly reduced hydrolysis of aspirin occurred when these surfactants were present at concentrations above the CMC. This finding is in general agreement with the literature report (7) that nonionic surfactants suppressed hydrolysis of the undissociated form of aspirin. Data from the present study indicate that the presence of surfactants of any class, either above or below the CMC, did not show significant differences in the extent of hydrolysis of aspirin from the controls. This observation could well be attributed to the relatively low concentrations of surfactants involved in the study.

A statistical analysis for variance (8) developed by Fisher was performed on the apparent solubility measurements of aspirin after each solution had reached equilibrium. Following this, Fisher's least significant difference (LSD) test was applied to provide some quantitative concepts in the solubility changes of aspirin as influenced by the different surfactants. Statistically, all of the surfactants studied, except for dioctyl sodium sulfosuccinate, yielded significant differences from the controls in solubility (Table II). Both cationic surfactants, cetylpyridinium chloride and benzalkonium chloride, when present below the CMC showed significantly lower apparent solubilities compared to the controls. In contrast, cetylpyridinium chloride, above its CMC demonstrated in order of ranking the greatest significant increase in aspirin solubility. The apparent solubility of aspirin increased approximately 17% in the range above (0.2%) the CMC of this surfactant, while below the surfactant CMC the solubility of aspirin was actually lowered. Similarly, benzalkonium chloride when present above (0.1%) its CMC increased aspirin solubility but decreased the solubility when studied below its CMC.

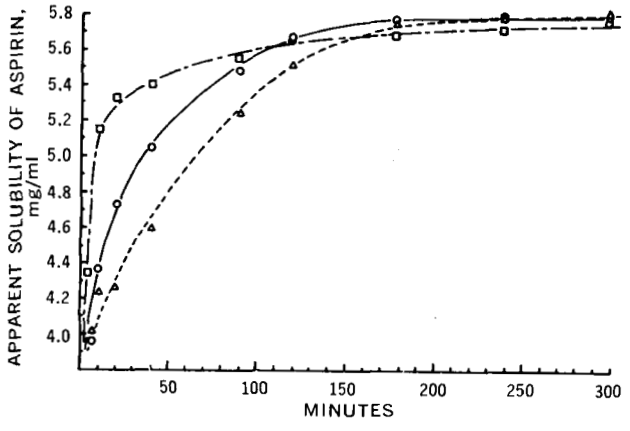

Figure 6—*Effect of dioctyl sodium sulfosuccinate on the apparent solubility of aspirin as a function of time at 37° in pH 2.4 buffer. Key: △, above CMC; ○, below CMC; and □, buffer control.*

SUMMARY

1. The solubilizing effect of the various classes of surfactants on the apparent solubility of aspirin as obtained under the experimental conditions described were, ranked in decreasing effective-

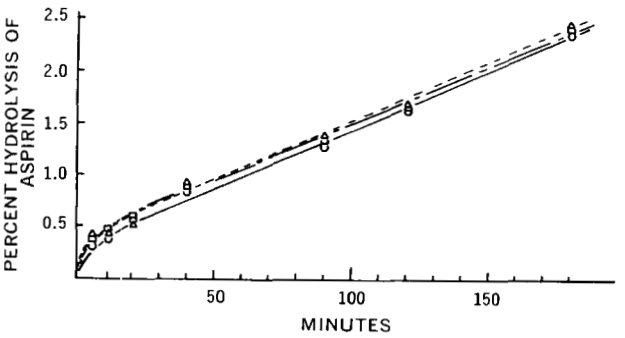

Figure 7—*Percentage hydrolysis found for aspirin as influenced by cetylpyridinium chloride strength in pH 2.4 Clark–Lubs buffer at 37°. Key: △, above CMC; ○, below CMC; and □, buffer control.*

Table II—Results of Fisher's Least-Significant Difference Test[a]

Groups[b]										
4[c]	5[c]	6	3[c]	3[d]	1[c]	1[d]	5[d]	2[c]	2[d]	4[d]
Mean Aspirin Solubility										
5.5467	5.5687	5.7527	5.7823	5.7960	5.8283	5.8448	5.8717	5.9693	5.9747	6.7223

[a] At the 0.05 level; LSD = 0.0448. [b] Group 1 = polysorbate 80, Group 2 = polysorbate 20, Group 3 = dioctyl sodium sulfosuccinate, Group 4 = cetylpyridinium chloride, Group 5 = benzalkonium chloride, and Group 6 = pH 2.4 buffer (control). [c] Below CMC. [d] Above CMC.

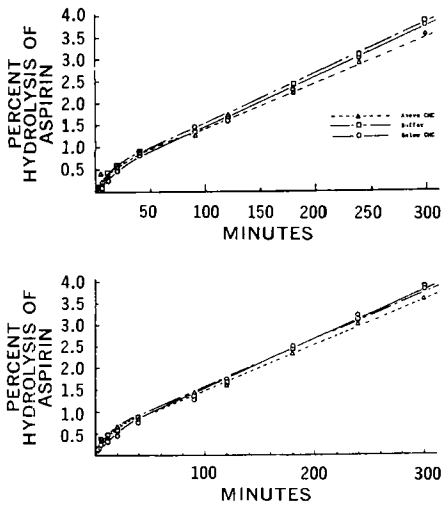

Figure 8—*Percentage hydrolysis found for aspirin as influenced by polysorbate 20 (top) and polysorbate 80 (bottom) in pH 2.4 buffer at 37°. Key: △, above CMC; ○, below CMC; and □, buffer control.*

ness, as follows: cetylpyridinium chloride (above CMC) > polysorbate 20 > benzalkonium chloride (above CMC) > polysorbate 80 > dioctyl sodium sulfosuccinate.

2. Both cationic surfactants, benzalkonium chloride and cetylpyridinium chloride, when present in concentrations less than their CMC failed to enhance the apparent solubility of aspirin. In contrast, cetylpyridinium chloride, above its CMC, showed the most significant increase in aspirin solubility.

3. The mechanism for the varying solubilizing effects of the surfactants on aspirin solubility cannot be fully explained at this time. It appears that the micellar size and shape formed with the different surfactant molecules may have played a prominent role in producing the observed solubilizing effects.

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* Present address: Department of Biochemistry, University of Southern California, Los Angeles, CA 90024

ˣ To whom inquiries should be directed.

Effect of Cadmium on Sodium and Potassium Excretion and on Action of Hydrochlorothiazide in Rats

JUDITH D. FOULKE*, WAYNE V. KESSLER^x, GORDON S. BORN, STANLEY M. SHAW, and RICHARD J. VETTER

Abstract □ Cadmium administered to rats decreased urinary sodium excretion. Single doses of 1.0 mg/kg or greater were equally effective. Doses of 0.5 mg/kg or less and 20 multiple doses of 0.5 mg/kg had no effect. A single dose was effective for only a limited time. The greatest decrease in sodium excretion occurred at a time that was 0–12 hr after injection, but an effect was still seen at 24 hr and up to 7 days. The action of hydrochlorothiazide was not altered by either a single 10-mg/kg dose or 20 0.5-mg/kg doses of cadmium. An effect of cadmium on potassium excretion was seen but was less evident than the effect on sodium excretion. The concentration of cadmium in the kidneys was not directly related to its effect on sodium excretion, indicating that the biochemical form and not the amount is the determinative factor. It is postulated that cadmium exerts its effect by an indirect mechanism and not by direct action on the nephron.

Keyphrases □ Cadmium—effect on sodium and potassium excretion and action of hydrochlorothiazide, rats, mechanism of action □ Hydrochlorothiazide—effect of single and multiple doses of cadmium, rats □ Sodium, excretion—effect of single and multiple doses of cadmium □ Potassium, excretion—effect of single and multiple doses of cadmium

Two major reviews (1, 2) within the last 2 years dealing with the heavy metal cadmium emphasized the growing hazard resulting from environmental contamination by this trace metal. Analyses of human tissues have shown that about one-third of the cadmium normally present in the body is in the kidneys (3). Although fairly high amounts must be accumulated before renal damage occurs (4–6), the question arose as to whether the presence of cadmium might alter the action of drugs that exert their primary effect on the kidneys. Since the most important of these drugs are the diuretics, this investigation was initiated to determine if single or multiple doses of cadmium would alter the action of a commonly prescribed diuretic, hydrochlorothiazide, as measured by the excretion of sodium in the urine. Since diuretics increase potassium excretion as well, this ion was also studied.

Vander (7, 8), who infused cadmium into anesthetized dogs undergoing saline diuresis, found that during the first 2 hr cadmium caused a decrease in sodium excretion and, less consistently, a decrease in potassium excretion. However, his studies were conducted over relatively short time intervals. Also, cadmium was infused directly into the kidney in a mixture containing cysteine, which is known to bind cadmium. This paper reports studies of the effect of intraperitoneally administered cadmium on sodium excretion in the intact rat. The relationship between the dose of cadmium administered and the decrease in sodium excretion produced as well as the time during which a single dose of cadmium is effective in altering sodium excretion was investigated.

EXPERIMENTAL

Animals—Adult female rats[1], descendants of the Sprague–Dawley strain, were used. The rats weighed 190–210 g at the start of the experiments. They were housed individually in metal cages in temperature- and humidity-controlled rooms having a 12-hr day/night cycle, and they were allowed free access to food[2] and tap water except during urine collection. Prior to the experiments, the animals were acclimated for at least 1 week under these conditions.

Radionuclides—Both ^{24}Na and ^{42}K were obtained as high specific activity chloride salts in water. Radionuclidic purities were checked by comparison of the γ-spectra to reference spectra and by half-life determinations. The injection solution for ^{24}Na was prepared so that 0.2 ml contained about 20 μCi of ^{24}Na and 0.0018 mg of sodium chloride as carrier at the time of injection. The injection solution for ^{42}K was prepared so that 0.2 ml contained about 100 μCi of ^{42}K and 0.01 mg of potassium chloride as carrier. For both ^{24}Na and ^{42}K, the dose was 0.2 ml, which was given intraperitoneally.

Cadmium—The cadmium injection solutions were prepared by dissolving cadmium acetate in doubly distilled water. All doses were based on the cadmium ion, not the salt, and were given intraperitoneally.

Hydrochlorothiazide—The drug[3] was obtained as a white crystalline powder. It was given as a suspension in 0.5 ml of water. The dose chosen was 220 mg/kg/day given as divided doses every 8 hr.

Counting Equipment—Polyethylene bottles containing the urine samples were counted in a large well-type NaI(Tl) scintillation crystal housed in a 15.24-cm thick steel shield lined with lead and stainless steel. All samples were counted in the integral mode for a time sufficient to obtain a counting error less than 1%.

Kidney Analysis for Cadmium—Both kidneys from each animal were digested by heating with concentrated nitric acid. Cadmium concentration was determined by atomic absorption spectrophotometry.

Effect of Amount of Cadmium Given as a Single Dose on Sodium Excretion—Forty-two rats were injected with ^{24}Na and were placed immediately in individual metal metabolism cages where neither food nor water was provided. After 12 hr equilibration, the animals were divided randomly into seven groups of six animals. The dose of cadmium given to six of the groups was 8.0, 4.0, 2.0, 1.0, 0.5, or 0.1 mg/kg. The remaining group served as a control and received doubly distilled water. Urine was collected for 24 hr following cadmium administration. All animals were hydrated with 5 ml of doubly distilled water *per os* at the time of cadmium administration and at 8 and 16 hr. After completion of the experiment, the animals were sacrificed with chloroform and their kidneys were removed for cadmium analysis. The urine samples were counted as previously described.

Time during which a Single Dose of Cadmium Is Effective in Altering Sodium Excretion—Forty-two rats were randomly assigned to seven groups of six animals. Six of the groups were injected with 2.0 mg cadmium/kg at one of the following times prior to ^{24}Na administration: 240, 48, 24, 12, 2, and 0 hr. The remaining group served as a control and received doubly distilled water at the same time as the last cadmium injection. All animals were injected with ^{24}Na and were placed immediately in individual metal metabolism cages where neither food nor water was provided. Urine was collected for 24 hr following the ^{24}Na administra-

[1] Laboratory Supply Co., Indianapolis, Ind.
[2] Wayne Lab-Blox, Allied Mills Inc., Chicago, Ill.
[3] Merck Sharp & Dohme, West Point, Pa.

Table I—Effect of Amount of Cadmium Given as a Single Dose on Sodium Excretion

Dose, mg/kg	^{24}Na Excreted, cpm[a]	Cadmium in Kidneys, ppm[a,b]
0.0	1,328,360 ±460,640	Not detectable
0.1	981,220 ±279,570	1.8 ± 0.8
0.5	804,330 ±283,930	4.6 ± 0.7
1.0	446,560 ±233,590	6.2 ± 1.8
2.0	323,630 ± 71,320	9.6 ± 2.6
4.0	387,240 ± 71,250	14.3 ± 5.9
8.0	347,310 ±110,150	25.7 ± 11.8
Newman–Keuls Comparison[c]		
Dose, mg/kg	2.0 8.0 4.0 1.0	0.5 0.1 0.0

[a] Mean ± standard deviation. [b] Wet basis. [c] Ranked according to increasing sodium excretion. Doses underlined did not produce a significantly different effect ($p = 0.01$).

tion. All animals were hydrated with 5 ml of doubly distilled water *per os* at the time of ^{24}Na administration and at 8 and 16 hr. After completion of the experiment, the animals were sacrificed and their kidneys were removed for cadmium analysis. The urine samples were counted as previously described.

This experiment was repeated with 36 rats randomly assigned to six groups of six animals. Five of the groups were injected with 2.0 mg cadmium/kg at one of the following times prior to ^{24}Na administration: 15, 10, 7, 4, and 2 days. The remaining group served as a control and received doubly distilled water just prior to the ^{24}Na injection.

Effect of Single and Multiple Doses of Cadmium on Sodium and Potassium Excretion and on Action of Hydrochlorothiazide—The sodium study and the potassium study were carried out with 48 animals each during time intervals about 2 weeks apart. The 48 animals in each study were randomly divided into eight groups of six animals, with four groups used in the cadmium single-dose study and four in the multiple-dose study. In both cases, the four groups received the following treatments: control, cadmium, hydrochlorothiazide, and cadmium plus hydrochlorothiazide.

In the single-dose study, the animals used in the ^{24}Na experiments were injected with 10.0 mg cadmium/kg. The animals in the ^{42}K experiments received 5.0 mg cadmium/kg because of toxicity observed in the preceding ^{24}Na study. In the multiple-dose study, the animals in both the ^{24}Na and ^{42}K experiments were

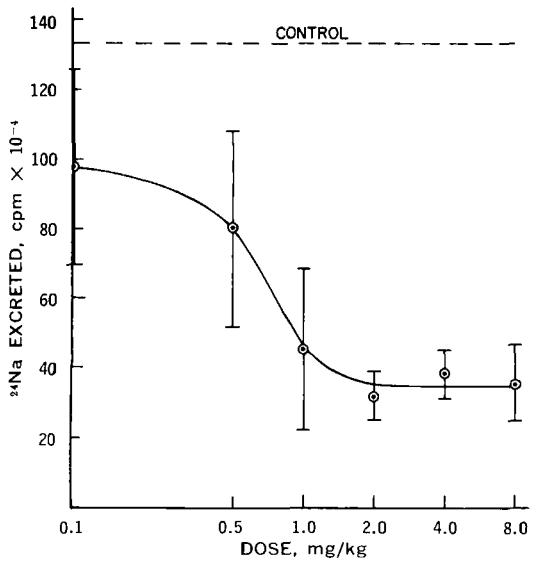

Figure 1—*Dose–response curve. Dose is plotted on a logarithmic scale. Curve was not mathematically fit.*

Table II—Time during which a Single Dose of Cadmium Is Effective in Altering Sodium Excretion

Time Interval[a] Hours	Time Interval[a] Days	^{24}Na Excreted, cpm[b]	Cadmium in Kidneys, ppm[b,c]
0		332,120 ±144,530	5.9 ± 0.8
2		354,850 ±216,070	5.6 ± 1.1
12		135,200 ±174,030	10.1 ± 1.8
24		1,005,560 ±489,840	11.6 ± 3.2
48		2,246,080 ±823,250	14.5 ± 3.1
240		982,160 ±301,550	20.0 ± 4.4
Control		2,140,495 ±997,450	Not detectable
	2	863,120 ±407,280	12.4 ± 1.5
	4	715,500 ±387,060	15.1 ± 1.8
	7	905,210 ±259,560	15.2 ± 6.8
	10	1,151,140 ±748,000	19.8 ± 5.6
	15	1,072,360 ±609,520	17.4 ± 3.6
	Control	1,654,410 ±653,240	Not detectable
Newman–Keuls Comparison for 240-hr Study[d]			
Time interval, hr	12 0 2	24 240	48

[a] Time interval between cadmium and ^{24}Na administrations. [b] Mean ± standard deviation. ^{24}Na excretion values for the two studies are not comparable because the ^{24}Na solution injected was different. [c] Wet basis. [d] Ranked according to increasing sodium excretion. Times underlined did not produce a significantly different effect ($p = 0.01$).

injected with 0.5 mg cadmium/kg every other day for 20 times. Hydrochlorothiazide was administered *per os* every 8 hr for 24 hr.

In the single-dose study, the cadmium and the first hydrochlorothiazide dose were administered 12 hr after the ^{24}Na or ^{42}K injections. In the multiple-dose study, the ^{24}Na or ^{42}K was administered 48 hr after the last dose of cadmium; the first dose of hydrochlorothiazide was given 12 hr later to allow for equilibration of ^{24}Na or ^{42}K.

Following the nuclide administration, the animals were placed in individual metal metabolism cages where neither food nor water was provided. Urine was collected for 24 hr following the first administration of hydrochlorothiazide. At 0.5 hr prior to each diuretic administration, all animals were hydrated with 5 ml of doubly distilled water *per os*. Immediately after completion of each study, all animals of the ^{42}K study and the single-dose animals of the ^{24}Na study were sacrificed and their kidneys were removed for cadmium analysis. The urine samples were counted as previously described.

RESULTS AND DISCUSSION

A preliminary study showed that the only applicable parameter for assessing the treatment effects was the ^{24}Na activity in the urine. Urine weights did not correlate with ^{24}Na activity, since cadmium had no significant effect on the weight of urine excreted while it did significantly decrease the excretion of ^{24}Na. Fasting the animals also had no significant effect on the ^{24}Na excreted by the rats receiving cadmium. However, since experiments of this kind are conventionally performed with fasted animals and since the amount of food eaten might introduce an added source of variability, it was decided to fast the animals during urine collection.

In each experiment, the net counts per minute of ^{24}Na or ^{42}K was corrected for decay to the time of nuclide administration. The data were tested for homogeneity of variance, and square root transformations were performed to achieve homogeneity.

Effect of Amount of Cadmium Given as a Single Dose on Sodium Excretion—The ^{24}Na activities of the urine samples and

Table III—Effect of Single and Multiple Doses of Cadmium on Sodium and Potassium Excretion and on Action of Hydrochlorothiazide

Single Dose				Multiple Dose			
No Diuretic		Diuretic		No Diuretic		Diuretic	
Control	Cadmium	Control	Cadmium	Control	Cadmium	Control	Cadmium
^{24}Na in Urine, cpm							
1,713,460 ±325,500	453,960 ±517,000	3,077,660 ±455,760	1,440,680 ±524,080	1,597,520 ±315,790	1,607,380 ±415,140	3,104,820 ±443,010	3,289,900 ±1,008,310
^{42}K in Urine, cpm							
1,869,750 ±623,270	1,922,970 ±641,450	1,904,140 ±360,510	2,275,140 ±364,120	1,682,910 ±120,820	1,817,060 ±358,880	2,041,400 ±445,200	2,574,700 ±306,410

the cadmium concentrations in the kidneys are shown in Table I. A one-way analysis of variance on the ^{24}Na activities showed a significant (p = 0.01) dose effect. A Newman–Keuls comparison (Table I) showed two distinct groups, with no significant differences (p = 0.01) within each group. The two lowest doses of 0.1 and 0.5 mg/kg fell in the group with the controls, indicating no cadmium effect. The other four doses fell in the second group, indicating the same degree of sodium retention for doses of 1.0 mg/kg or greater. A dose–response curve (Fig. 1) shows a sharp fall corresponding to the break between the two groups and a flat portion corresponding to the leveling off of the effect at high doses.

The concentration of cadmium in the kidneys steadily increased with the administered dose. A correlation test performed to relate the kidney concentration of cadmium to the amount of sodium excreted did not show a significant relationship even at p = 0.10. Although these kidney concentrations of cadmium were determined after the completion of the sodium excretion study and are not the concentrations that existed during the study, it is assumed that the relationship between dose level and kidney concentration was essentially the same both during and after the study.

Time during which a Single Dose of Cadmium Is Effective in Altering Sodium Excretion—The results of the two time studies are shown in Table II and Fig. 2. The data for each study were analyzed separately by a one-way analysis of variance. There was a significant time effect (p = 0.01) for the 240-hr study but no time effect (p = 0.10) for the 15-day study.

For the 240-hr study, a Newman–Keuls comparison (Table II) showed the greatest sodium retention with animals injected 0, 2, and 12 hr prior to receiving ^{24}Na and the least retention with the 48-hr animals (p = 0.01). The animals injected at 24 and 240 hr had intermediate values of sodium retention. A two-tailed t test to compare the ^{24}Na excretion of the control group with the excretions of the 24-, 48-, and 240-hr groups showed significant differences (p = 0.05) for the 24- and 240-hr groups but not for the 48-hr group. For the 15-day study, the values for the 2-, 4-, and 7-day groups differed significantly from the control, whereas the values for the 10- and 15-day groups did not.

A correlation test to relate the kidney concentration of cadmium (Table II) to the excreted sodium was run separately for both studies. No significant (p = 0.10) relationship was found for either study. Again, these kidney concentrations of cadmium were determined after the completion of the sodium excretion study. However, it was previously found (9) that the levels of cadmium in the kidney do not change appreciably between 8 and 240 hr.

Although the results of the statistical analyses are somewhat inconsistent for the two studies, it is clear that cadmium greatly affects sodium excretion within the first 12 hr after administration (Fig. 2). It appears that beyond 12 hr and up to 7 days it is still exerting an appreciable effect. Beyond 7 days, a significant effect could not be shown statistically.

Effect of Single and Multiple Doses of Cadmium on Sodium and Potassium Excretion and on Action of Hydrochlorothiazide—The ^{24}Na and ^{42}K activities of the urine samples are shown in Table III. Since the sum of the multiple doses of cadmium equaled the single dose, the ^{24}Na excretion data were analyzed by an analysis of variance for a three-factor factorial design. The three factors were: *(a)* type of dosing, single or multiple; *(b)* diuretic, presence or absence; and *(c)* cadmium, presence or absence. A Newman–Keuls comparison was made where necessary. The single dose of cadmium significantly increased (p = 0.01) the retention of sodium, whereas the multiple doses did not. This effect was obtained in spite of the fact that the levels of cadmium in the kidneys of the single-dose animals were about 30 ppm, wet weight, while those of the multiple-dose animals were about 150 ppm. The increase in sodium excretion produced by the diuretic was expected because of the known properties of the diuretic. Most important was the finding that the cadmium treatment and diuretic treatment each exerted its effect independently of the presence of the other. Therefore, the presence of cadmium in the kidney, even at the high concentrations following multiple dosing, did not alter (p = 0.01) the action of hydrochlorothiazide.

The ^{42}K excretion data were analyzed separately for the single- and multiple-dose studies by an analysis of variance for a two-factor factorial design since the sum of the multiple doses did not equal the single dose. The two factors were cadmium and diuretic each at two levels, absence or presence. For the single-dose study, neither cadmium nor diuretic had any effect on potassium excretion at any significance level. For the multiple-dose study, both cadmium (p = 0.05) and the diuretic (p = 0.01) caused increased potassium excretion.

Hydrochlorothiazide would be expected to exert at least a minimal effect on potassium excretion under the experimental conditions. The discrepancy in its action in the two studies cannot be explained.

For the multiple-dose study, the F value for the interaction between the cadmium and diuretic treatments approached significance at the 0.10 level and was significant at the 0.25 level. For this reason, a Newman-Keuls comparison of the means was performed. Only the combination of cadmium and diuretic had a significant effect (p = 0.05).

For the single-dose study, it was observed that the difference in means for the group receiving cadmium plus diuretic and for the group receiving only diuretic was about the same as the difference for the respective means in the multiple-dose study. The conclu-

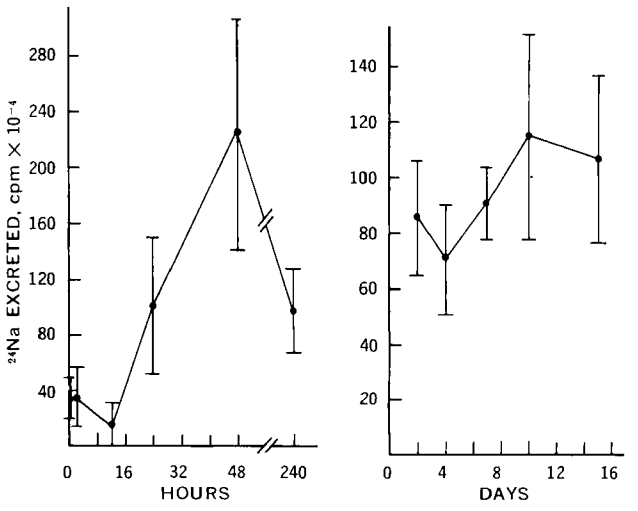

Figure 2—*Time–response curves. ^{24}Na excretion values for the two studies are not comparable because the ^{24}Na solution injected was different.*

sion drawn from these results is that both cadmium and the diuretic affect potassium excretion to a slight degree and, when both are given together, they exert an additive effect for which it may or may not be possible to show statistical significance.

The cadmium concentrations in the kidneys of the single-dose animals in the ^{42}K study were about 15 ppm, wet basis. These were about half the concentrations in the single-dose ^{24}Na study where the animals received a dose of cadmium twice as high. The concentrations for the multiple-dose animals were about 150 ppm, as previously stated.

CONCLUSIONS

The antinatriuretic effect found in the animals that received cadmium as a single dose of 10.0 mg/kg may have been due to the same mechanism, a direct effect on the proximal tubule, as that postulated by Vander (8) to explain his observations following infusion of 0.025 mg/kg into anesthetized dogs. More recent evidence would suggest, however, that cadmium may affect sodium excretion indirectly through stimulation of the renin–aldosterone system. Renin production is known to be stimulated by a fall in blood pressure, and there is evidence that cadmium may decrease blood pressure. Youkilis *et al.* (10) found that 3 mg/kg produced significant *in vivo* dilatation of venules and arterioles of innervated bat wings, and Scott and Haddy (11) found decreased peripheral resistance in canine limbs during infusion of $3\text{–}24 \times 10^{-4}$ *M* cadmium ion. The increased renin production following the fall in blood pressure would cause an increase in aldosterone production. Sodium retention would thus be increased after cadmium treatment by the action of the higher levels of aldosterone on the distal tubule.

The lack of any effect of 20 multiple cadmium doses of 0.5 mg/kg on sodium excretion is not surprising in view of the protective action of pretreatment with small doses of cadmium against the toxicity of a single large dose (12, 13). This detoxification has been attributed to the formation of a complex between cadmium ions and a low molecular weight protein known as metallothionein (14). The discovery of increased amounts of metallothionein in the livers (14) and blood (15) of animals exposed to cadmium may explain why a single dose of cadmium affects sodium excretion whereas multiple smaller doses do not, since the basal level of metallothionein is not sufficient to handle all of the ions introduced in a single large dose. Thus, even though the cadmium concentration was much higher in the kidneys of animals receiving the multiple doses, the cadmium was not physiologically free to exert its effect.

The increased excretion of potassium which was apparent but not statistically significant in the animals that received cadmium as a single dose of 5.0 mg/kg can be explained as the direct consequence of the increased reabsorption of sodium since aldosterone is known to cause exchange of sodium, which is reabsorbed, for potassium, which is excreted. The apparent increase in excretion of potassium in the animals that received multiple doses of cadmium cannot be similarly explained since no effect on sodium excretion was found in these animals.

If the proposed mechanism for the effect of cadmium on electrolyte excretion is correct, cadmium would not be expected to alter the action of any diuretic, regardless of its site of action in the nephron. However, further studies with other diuretics are necessary before such a conclusion can be drawn.

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* Present address: Directorate of Licensing, U.S. Atomic Energy Commission, Bethesda, MD 20545

ˣ To whom inquiries should be directed.

GLC Determination of *dl*-2-(2-Fluoro-4-biphenylyl)propionic Acid (Flurbiprofen) in Plasma

DAVID G. KAISER[x], S. ROBERT SHAW, and G. J. VANGIESSEN

Abstract □ To evaluate the pharmacokinetics and drug availability from various dosage formulations, a method for the determination of *dl*-2-(2-fluoro-4-biphenylyl)propionic acid (flurbiprofen) in human plasma was required. A sensitive and specific procedure, based on *(a)* benzene extraction of the acidified specimen, *(b)* TLC of the benzene extract residue, *(c)* formation of the pentafluorobenzyl esters of the materials eluted from the thin-layer chromatogram, and *(d)* quantification of the pentafluorobenzyl esters by GLC, utilizing electron-capture detection, was developed. The lower level of assay detection sensitivity for measurement of flurbiprofen in plasma is 0.05 μg/ml. Statistical analyses indicated an average recovery of 97.1% with a standard deviation of ±9.2%. IR and mass spectrometric analyses, in conjunction with GLC, confirmed the specificity of the method for the intact drug. The procedure was successfully applied to drug absorption studies in humans.

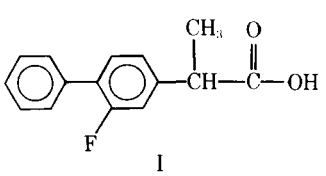
Keyphrases □ *dl*-2-(2-Fluoro-4-biphenylyl)propionic acid (flurbiprofen)—GLC analysis in plasma □ Flurbiprofen [*dl*-2-(2-fluoro-4-biphenylyl)propionic acid]—GLC analysis in plasma □ GLC—analysis, flurbiprofen in plasma

In a continuing search for nonsteroidal anti-inflammatory drugs, flurbiprofen [*dl*-2-(2-fluoro-4-biphenylyl)propionic acid] (I) was observed (1) to be one of the most potent members of a series of substituted phenylalkanoic acids, based upon a wide range of anti-inflammatory, analgesic, and antipyretic tests in various animal species. Double-blind crossover clinical trials showed I to be well tolerated, nontoxic, and orally effective in the treatment of rheumatoid arthritis in humans (2, 3).

To study the absorption, metabolism, and excretion of this agent in animals and humans at relatively low doses (*i.e.*, 10 mg), a highly sensitive and specific method for drug analysis in plasma was needed. Since earlier investigations (4) showed GLC to be a generally useful tool for measuring structurally related compounds in biological matrixes, a method utilizing GLC in conjunction with electron-capture detection was developed.

EXPERIMENTAL

Reagents and Materials—The Compound I and *dl*-2-(2-methoxy-4-biphenylyl)propionic acid (II) used in this study were synthesized[1]. Methyl ethyl ketone (III), acetone, and cyclohexane, distilled in glass, were used as supplied[2]. Stock solutions of I in III (100 μg/ml), II in III (100 μg/ml), pentafluorobenzyl bromide[3] in acetone (25 μg/ml), and aqueous sulfuric acid (1 *N*) were stored in glass containers. All other solvents were analytical reagent grade. Silicone gum rubber (OV-17) on 80–100-mesh Gas Chrom Q (3% w/w) was used as supplied[4].



I

Instrumentation—A two-speed reciprocating shaker[5] was used for shaking the samples in the horizontal position. A mixer[6] was used to aid in eluting the zones removed from thin-layer chromatograms. GLC measurements were made with a gas chromatograph[7] equipped with a hydrogen flame-ionization detector, a nickel-63 electron-capture detector, and a −0.2 to 1.0-mv recorder[8]. All cylinders of gases used for chromatography (*i.e.*, helium, hydrogen, oxygen, and 5% methane in argon) were fitted with filters containing molecular sieve 4A.

TLC—All analytical chromatography was conducted on thin layers (250 μm) of silica gel F_{254}[9], ascendingly developed in a solvent system composed of 20% (v/v) acetic acid in toluene. The separated materials were visualized by irradiation of the plates with a short wavelength (254 nm) UV lamp. Under these conditions, I and II had the same R_f values, *i.e.*, 0.45. For preparative and analytical chromatography, during the isolation of the II-pentafluorobenzyl ester, a solvent system composed of hexane–acetone–methanol (100:10:10) was utilized. The R_f values for I- and II-pentafluorobenzyl esters were identical, *i.e.*, 0.42.

GLC Conditions—All chromatography was conducted using U-shaped glass columns [0.61 m (2 ft) × 3 mm i.d.] packed with 3% (w/w) OV-17 on 80–100-mesh Gas Chrom Q. All newly prepared columns were preconditioned at 250° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and electron-capture detector were maintained isothermally at 210, 250, and 270°, respectively. Flow rates of 5% methane in argon, used as carrier and purge gas, were maintained at 50 ml/min. The pulse interval for the electron-capture detector was 150 μsec. Under these conditions, I- and II-pentafluorobenzyl esters had retention times of 4.2 and 7.4 min, respectively (Fig. 1). In ancillary studies, using the flame-ionization detector, flow rates for helium, hydrogen, and oxygen were maintained at 50, 40, and 300 ml/min, respectively.

Synthesis of Standard Materials—Place 200 mg I in a 15-ml glass-stoppered centrifuge tube. Add 8 ml acetone, 0.4 ml undiluted pentafluorobenzyl bromide, and 113 mg anhydrous potassium carbonate. Stopper the centrifuge tube and heat in a water bath at 60° for 16 hr. Evaporate to dryness with a gentle stream of nitrogen gas. Add 2 ml water and 5 ml cyclohexane. Shake for 10 min and allow the phases to separate. Transfer the cyclohexane to a fresh 15-ml centrifuge tube. Repeat the extraction with two additional 5-ml portions of cyclohexane, and evaporate the pooled cyclohexane extracts to dryness with nitrogen gas.

Reconstitute the cyclohexane extract residue in 10 ml of a solvent mixture composed of toluene–acetic acid (80:20) (v/v). Apply the solution to a silica gel column (2.8 × 50 cm) and develop at a flow rate of 3 ml/min with a solvent system composed of toluene–acetic acid (80:20) (v/v). Collect 10-ml fractions and monitor each fraction by TLC *(vide supra)*. Pool fractions 10–25 and remove the solvent under reduced pressure (15 mm).

[1] Research Division, Boots Pure Drug Co. Ltd., Nottingham, England.

[2] Burdick and Jackson Labs., Muskegon, Mich.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Applied Science Labs., State College, Pa.
[5] Eberbach and Sons, Ann Arbor, Mich.
[6] Vortex model K-500, Scientific Industries, Queen's Village, N.Y.
[7] F&M model 400, Hewlett-Packard Co., Avondale, Pa.
[8] Honeywell Elektronic 15.
[9] Brinkmann Instruments, Westbury, N.Y.

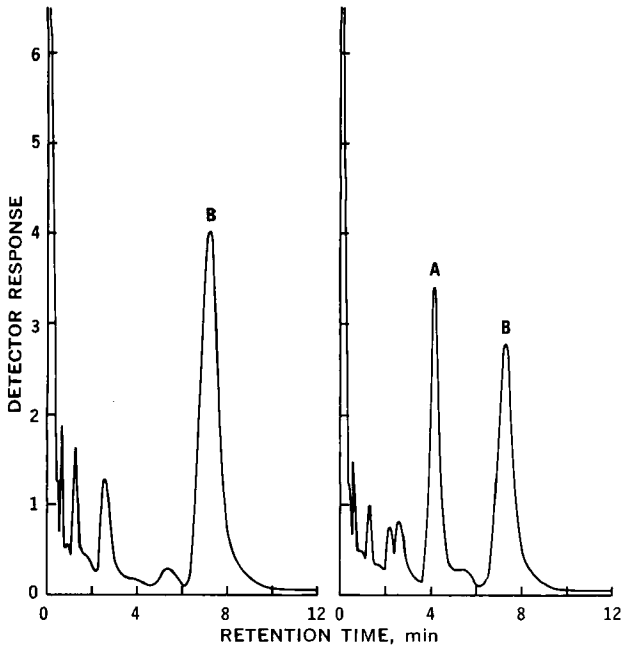


Figure 1—*Gas–liquid chromatograms (electron-capture detector) of human plasma extracts. Left: normal plasma specimen. Right: plasma specimen from subject at 1 hr after single-dose oral administration of 10 mg flurbiprofen. Key: A, flurbiprofen pentafluorobenzyl ester; and B, II-pentafluorobenzyl ester.*

Synthesize standard material for identification of II-pentafluorobenzyl ester, utilizing the same general reaction conditions as described for I. Reconstitute the cyclohexane extract residue in 10 ml of the column chromatography solvent system (*vide infra*). Apply the solution to a silica gel column (2.8 × 50 cm) and develop at a flow rate of 3 ml/min with a solvent system composed of hexane–acetone–methanol (100:10:10). Collect 10-ml fractions and monitor each fraction by TLC (silica gel F_{254}) in hexane–acetone–methanol (100:10:10). Pool fractions 20–32 and remove the solvent under reduced pressure (15 mm). Recrystallize from methanol–ether.

Assay Procedure—*Preparation of Plasma Standards*—Pipet 1-ml aliquots of the I–III stock solution, diluted to 0.1, 0.5, 1, 3, and 5 μg/ml, into glass-stoppered centrifuge tubes. Add a 1-ml aliquot of the II–III stock solution, diluted to 4 μg/ml, to each centrifuge tube. Evaporate to dryness with a gentle stream of nitrogen gas. Add 1 ml of control plasma to each centrifuge tube and mix well with the mixer. Prepare an appropriate blank. Extract all standards in the same manner as described later for the plasma specimens.

Preparation of Samples—Pipet 1-ml aliquots of the II–III stock solution, diluted to 4 μg/ml, into a series of glass-stoppered centrifuge tubes. Evaporate to dryness with a gentle stream of nitrogen gas, and place 1 ml of plasma into each centrifuge tube. Add 0.25 ml 1 *N* aqueous sulfuric acid and 5 ml benzene, and shake in the horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm. Transfer a 4-ml aliquot of the benzene layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with a gentle stream of nitrogen gas. Wash down the walls of the centrifuge tube with 0.5 ml chloroform and evaporate to dryness with nitrogen.

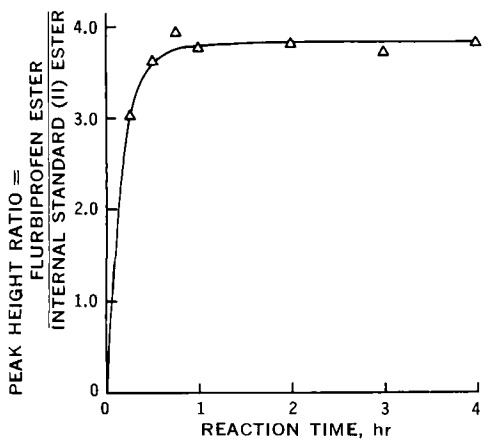


Figure 2—*Effect of reaction time on formation of flurbiprofen pentafluorobenzyl ester.*

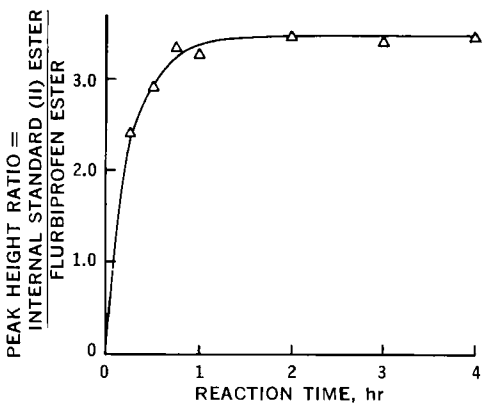


Figure 3—*Effect of reaction time on formation of internal standard pentafluorobenzyl ester (II-pentafluorobenzyl ester).*

Reconstitute the benzene extract residues from the plasma standards and samples in 50 μl chloroform. Together with appropriate standards, spot all of each sample on thin layers of silica gel F_{254} (*vide supra*). Ascendingly develop each plate for a distance of 165 mm, air dry all chromatograms thoroughly at room temperature (24°), and visualize the zones by irradiation with a 254-nm UV lamp. Scrape the zones corresponding to I into separate glass-stoppered centrifuge tubes. Add 2 ml methanol to each tube and gently agitate using the mixer. Centrifuge for 5 min at 2000 rpm. Transfer 1 ml of the methanol layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with nitrogen gas.

Add 25 mg anhydrous potassium carbonate and a 2-ml aliquot of the pentafluorobenzyl bromide stock solution to each centrifuge tube. Stopper tightly and heat in a water bath at 60° for 90 min. Evaporate to dryness with nitrogen gas. Add 1 ml water and 1 ml cyclohexane, shake manually for 1 min, and allow the phases to separate. Inject a 2-μl aliquot of the cyclohexane layer for analysis into the chromatograph (operating in the electron-capture detector mode).

Calculations—The peak heights for I- and II-pentafluorobenzyl esters are measured. Peak height ratios are obtained by dividing the peak height of the I-pentafluorobenzyl ester by the peak height of the II-pentafluorobenzyl ester. Calibration curves from known concentrations of I in plasma are prepared by plotting peak height ratios *versus* free acid concentration, expressed as micrograms per milliliter plasma. Values for unknown concentrations of I in plasma specimens, obtained in the same manner, are then read directly from the graph or calculated from the slope of the standard curve.

Drug Administration to Humans—Informed written consent was obtained from each of six normal human male volunteers prior to participation in this study. All subjects were between the ages of 21 and 55 years and ranged in body weight from 62.3 to 83.2 kg and in height from 1.73 to 1.85 m. All subjects fasted for 16 hr prior to drug administration. Each subject received a 10-mg dose of I as a compressed tablet. Food was withheld for an additional 2 hr. Blood specimens (10 ml) were withdrawn into heparinized syringes at predetermined time intervals from 0 to 24 hr after drug administration. The plasma was harvested and stored at −18°.

RESULTS AND DISCUSSION

Synthesis and Identification of Pentafluorobenzyl Esters—Investigations by Kawahara (5, 6) indicated that pentafluorobenzyl bromide was a useful reagent for the microdetermination of

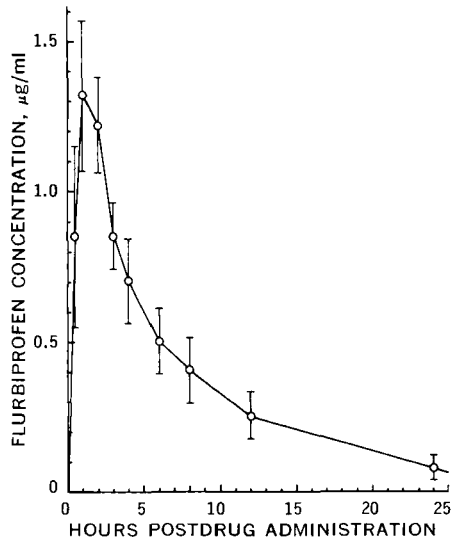


Figure 4—*Mean* (±SEM) *plasma concentrations of flurbiprofen* versus *time in humans* (n = 6) *after single-dose oral administration of 10 mg drug as a compressed tablet.*

phenols, mercaptans, and organic acids in water. The general reaction for the preparation of pentafluorobenzyl esters from carboxylic acids is presented in Scheme I. A series of samples, containing known amounts of I and II, were prepared to determine optimal reaction times for formation of the pentafluorobenzyl esters. In studies with I, known amounts of II-pentafluorobenzyl ester were added as internal standard. Similarly, in studies with II, known amounts of I-pentafluorobenzyl ester were added as internal standard. The results indicated that ester formation for both compounds was completed within 90 min (Figs. 2 and 3). In the presence of water and cyclohexane, the derivatives were stable for at least 48 hr.

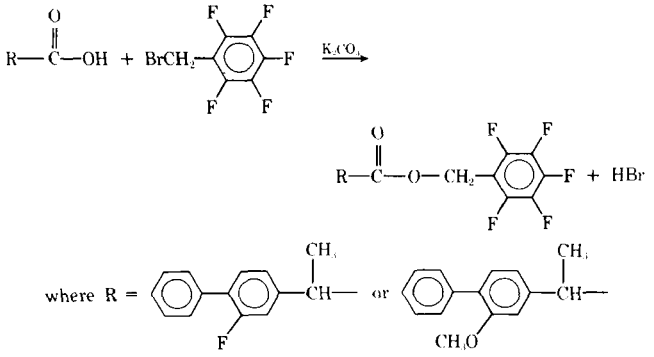
Synthesis of standard material indicated that the pentafluorobenzyl ester of I was a liquid at room temperature. Attempts to isolate crystalline material were unsuccessful. GLC, using a solid sample injector and flame-ionization detector, indicated that the material submitted for elemental analysis was greater than 99% pure.

Anal.—Calc. for $C_{22}H_{14}F_6O_2$: C, 62.27; H, 3.33; F, 26.87. Found: C, 62.26; H, 3.86; F, 26.70.

The pentafluorobenzyl ester of II was isolated as fine white crystals, mp 53–55°. GLC, using a solid sample injector and flame-ionization detector, indicated that the material submitted for elemental analysis was greater than 99% pure.

Anal.—Calc. for $C_{23}H_{17}F_5O_3$: C, 63.30; H, 3.93; F, 21.77. Found: C, 63.43; H, 3.91; F, 21.31.

IR and mass spectrometric analyses, before and after GLC, supported the proposed structures and confirmed that the pentafluorobenzyl esters of I and II chromatographed as the intact molecules.



Scheme I—Preparation of pentafluorobenzyl esters from carboxylic acids

Table I—Recovery of Flurbiprofen from Human Plasma[a]

Added, μg/ml	Found, μg/ml	Recovery, %
0.10	0.100	100.0
0.50	0.563	112.6
1.00	0.860	86.0
3.00	2.871	95.7
5.00	4.510	90.2
10.00	9.792	97.9
	Mean ± *SD*	97.1 ± 9.2

[a] *dl*-2-(2-Methoxy-4-biphenylyl)propionic acid utilized as internal standard.

TLC of Plasma Extracts—Pilot studies were conducted with various TLC solvent systems to separate materials extracted from plasma which interfered in the direct GLC measurement of II. A solvent system composed of 20% (v/v) acetic acid in toluene separated two materials from human plasma extracts, designated zone A (R_f 0.16) and zone B (R_f 0.31), from known I and II (R_f values 0.45). The materials were eluted from the silica gel and reacted with pentafluorobenzyl bromide. Based upon GLC behavior of the esters, on a column of 3% (w/w) OV-17, the material in zone A (R_f 0.16) was responsible for the interference observed in the analysis of II. No interference from zone B was observed in the GLC analysis of I or II.

Assay Sensitivity and Specificity—At a sensitivity of 3.2×10^{-10} amp/mv, 0.0028 μg of I as its pentafluorobenzyl ester produced a full-scale response. However, under the assay conditions, the lower limit of detection sensitivity of I in extracts of human plasma is 0.05 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under the assay conditions described, a linear relationship between detector response and concentration is obtained for I over the range of 0–10 μg/ml. Quantification from a standard curve was adequate. Analysis of plasma specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay was identical to known I-pentafluorobenzyl ester.

Recovery Experiments—Known amounts of I and II, in III, were evaporated to dryness in centrifuge tubes, and water or plasma was added. The samples were thoroughly mixed and extracted with benzene. All extract residues were chromatographed on thin layers of silica gel F_{254}, and the zones corresponding to I and II were eluted. The materials were esterified and analyzed *via* GLC. The results (Tables I and II) indicated that recoveries of I (97.1 ± 9.2%) and II (98.3 ± 3.9%) from plasma were essentially quantitative as compared to simple aqueous samples.

Plasma Levels of Flurbiprofen in Humans—Results from the measurement of plasma I concentrations in six normal human subjects, after single-dose oral drug administration, demonstrated the utility of the analytical methodology (Fig. 4). A peak mean *(±SEM)* level of I (1.32 ± 0.25 μg/ml) was observed at 1 hr after drug administration, indicating rapid drug absorption from the compressed tablet formulation. Only trace amounts of intact drug (0.07 ± 0.04 μg/ml) were found in the 24-hr plasma specimens, suggesting rapid drug disappearance from peripheral circulation. A plot of the logarithms of the average plasma I concentrations,

Table II—Recovery of Internal Standard (II)[a] from Human Plasma[b]

Added, μg/ml	Found, μg/ml	Recovery, %
0.10	0.094	94.0
0.50	0.479	95.8
1.00	1.015	101.5
3.00	2.838	94.6
5.00	5.095	101.9
10.00	10.200	102.0
	Mean ± *SD*	98.3 ± 3.9

[a] Internal standard (II) = *dl*-2-(2-methoxy-4-biphenylyl)propionic acid. [b] Flurbiprofen utilized as internal standard.

expressed as micrograms per milliliter, *versus* time in hours showed that the disappearance of I from plasma was biphasic. The half-life for the terminal phase, as estimated graphically from the data between 6 and 24 hr after drug administration, was 6.2 hr. The combined results from these investigations showed that the GLC method could be used for: *(a)* evaluating the pharmacokinetics, *(b)* evaluating drug availability from various dosage formulations, and *(c)* selecting an optimum dosage regimen for I administration to humans.

REFERENCES

(1) S. S. Adams and K. F. McCullough, *7th European Rheumatology Congress, London, England,* Abstract 32.2, 1971.

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(3) I. M. Chalmers, M. A. Bell, and W. W. Buchanan, *ibid.*, **32** 58(1973).

(4) D. G. Kaiser and G. J. VanGiessen, *J. Pharm. Sci.*, **63**, 219(1974).

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Abstract □ The binding of sulfonylureas to plasma proteins was studied by a fluorescence method, using 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine (III) and 1-anilinonaphthalene-8-sulfonic acid (IV) as fluorescence probes. Chlorpropamide, acetohexamide, and tolbutamide competed with IV but not with III in binding to bovine serum albumin. In the cases of glipizide (I) and glyburide (glibenclamide) (II), however, competitive binding between these drugs and III was observed. In contrast to the decrease in intensity, fluorescence enhancement of IV by I and by II was found in human serum albumin and in both human and bovine serum albumins, respectively. The fluorescence of warfarin in bovine serum albumin solution was increased by the addition of I. This may indicate the formation of ternary complexes between these drugs and the probe-protein complex; as a result, these drugs may enhance the protein binding of the probe or drugs that compete with the probe for the same binding sites. In comparison with other sulfonylureas, I and II bind to different but closely located sites on the protein with greater affinities.

Keyphrases □ Sulfonylureas—binding to plasma proteins, fluorescence probe technique □ Plasma proteins—binding of sulfonylureas, fluorescence probe technique □ Binding, sulfonylureas to plasma proteins—studied using fluorescence probe technique □ Fluorescence probe technique—used to study sulfonylurea binding to plasma proteins

Binding of drugs to plasma proteins has long been known as an important factor in drug availability, drug efficacy, and drug transport (1). It has been shown (2–4) that the unbound drug in plasma is considered to account for the drug's pharmacological activity. In other words, the correct therapeutic plasma level is not the total drug concentration in plasma but the unbound portion of the drug. The binding occurs mainly in the albumin fraction of the plasma and is reversible (5). Brand *et al.* (6) showed that drugs having low affinities in binding to albumin are at least partially dissociated from their protein binding sites by drugs with higher affinities and, to a lesser extent, by those with equal or lesser affinities. This is true provided the binding of these drugs occurs at the common or adjacent sites on the protein. The dissociation of a drug from its binding sites by a suitable competitor may cause an enhancement of activity of that drug and can otherwise alter its metabolism. The binding affinity of one drug may also be enhanced by the presence of another drug due to drug interactions and drug–protein complexations.

Chlorpropamide, acetohexamide, and tolbutamide are commonly used sulfonylureas. Protein binding of these drugs has been tentatively studied using such techniques as ultracentrifugation (7), equilibrium dialysis (8–11), ultrafiltration (9), and electrophoresis (9, 12). In the study reported here, a fluorescence probe technique (13) was used because this method provides an excellent model to show the competitive binding character between drugs and probe or possibly other drugs in binding to plasma protein.

Recently, two new hypoglycemic agents, glipizide (I) and glyburide (glibenclamide) (II), were reported (14). Metabolism and kinetics studies (14–16) showed that these drugs are extensively bound to plasma proteins and showed a limited volume of distribution. Because of the structural differences, the binding of glipizide and glyburide to plasma protein may differ from those of other sulfonylureas. Therefore, to understand further the pharmacological activities of these drugs, quantitative studies of their protein binding are needed. In this respect, the binding of glipizide and glyburide to different proteins was also studied using the fluorescence probe technique.

Two probes, 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine (III) and 1-anilinonaphthalene-8-sulfonic acid (IV), were used in this study. The synthesis and usage of III as a fluorescence probe were previously studied in this laboratory (17).

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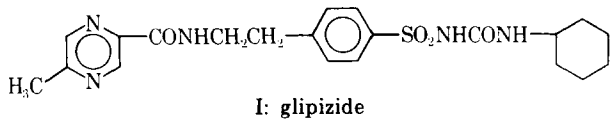
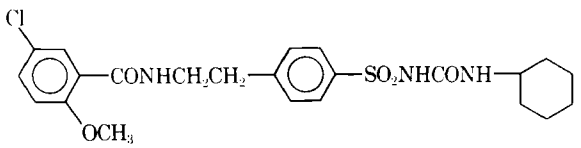


I: glipizide



II: glyburide

EXPERIMENTAL

Materials—Tolbutamide[1], chlorpropamide[2], acetohexamide[3], glipizide[4], glyburide[5], sodium warfarin[6], and 1-anilinonaphthalene-8-sulfonic acid[7] were obtained from commercial sources. The sodium salt of III was prepared in this laboratory (17). All other chemicals were reagent grade or of special purity.

Apparatus and Methods—Fluorescence measurements were made with a spectrophotofluorometer[8] equipped with a 150-w xenon lamp and a 1P21 photomultiplier tube. The relative fluorescence intensities were recorded directly from fluorometer readings. Fluorescence spectra were recorded with an *X-Y* recorder[9]. The entrance slit for the excitation light and the exit slit for the fluorescence emission were 3 and 4 mm, respectively.

Fluorescence titrations of protein solutions with probes in the absence and presence of sulfonylureas were carried out manually with microsyringes[10]. Two milliliters of each protein solution was titrated with successive additions of 2 μl of 1×10^{-3} *M* probe solution dissolved in methanol. After each titration, the fluorescence intensity was recorded as a function of probe concentration. The excitation and emission wavelengths were taken to be 420 and 485 nm for III and 375 and 475 nm for IV, respectively. The protein solutions were prepared in pH 7.4 phosphate buffer (0.05 *M*). A molecular weight of 69,000 was used for bovine serum albumin. Because of their low solubility in water and in methanol, all sulfonylureas studied were dissolved in 0.1 *N* NaOH solution. The pH and the ionic strength of all solutions were controlled at constant values by use of 0.1 *N* NaOH solution. The final pH was 7.45. The temperature of all measurements was maintained at 25 ± 0.1°.

The binding of probes and sulfonylureas to 1.38×10^{-6} *M* bovine serum albumin was determined by the methods previously used in these laboratories (18, 19). To calculate the fractions of free and bound probe concentrations, fluorescence data of the probe at a high protein concentration, which provides excess protein binding sites, are required. In this study, the fluorescence titrations were carried out at several protein concentrations, and the fluorescence data of the probe at high protein concentration were obtained by an extrapolation method. The binding parameters of the probe to the protein were calculated using the Scatchard (20) equation. Fluorescence titrations of 1.38×10^{-6} *M* bovine serum albumin with probes were also carried out in the presence of sulfonylureas. The competitive binding of these drugs to the protein were determined using the Klotz *et al.* (21) equation. All fluorescence data were the result of six separate determinations.

RESULTS AND DISCUSSION

Compounds III and IV were used as fluorescence probes in the study of the interaction of sulfonylureas to plasma proteins. The binding of III to bovine serum albumin has been studied using a fluorescence method. The Scatchard plot and the binding parameters are shown in Fig. 1 and Table I, respectively. It was found that III binds to bovine serum albumin in an approximate 1:1 molar ratio. A previous report (17) showed that the carboxylate group of III interacts with a positively charged residue on the protein molecules. Hydrophobic interactions between the protein and the compound are also expected. The fluorescence changes of III by solvents and the protein environment (17) are very similar to those of IV (22, 23) and may be interpreted as a result of the charge transfer between the probe and the protein molecules. The binding constants of these two probes are approximately the same; however, the number of binding sites for the binding of IV to bovine serum albumin is three.

Interactions of glipizide and glyburide to bovine serum albumin were studied using III as the indicating probe. It was found that these drugs compete with the probe for the same binding sites on the protein. The results are shown in Fig. 1 and Table I. Other

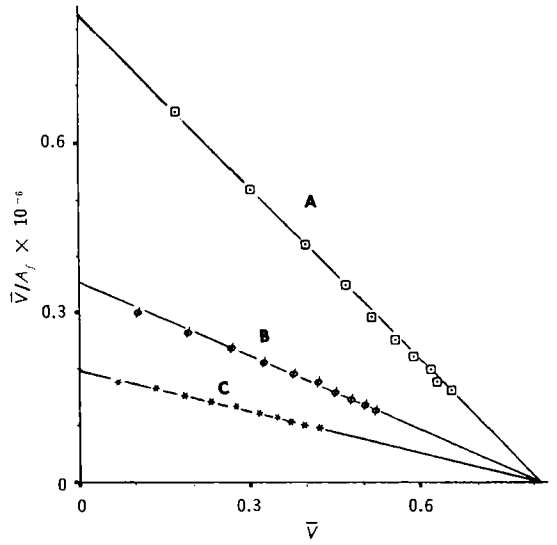


Figure 1—*Scatchard plots for the binding of III to bovine serum albumin. Key: A, in the absence of drug; B, in the presence of* 2×10^{-5} M *glipizide; and C, in the presence of* 1×10^{-5} M *glyburide.* $\bar{V}$ *is the number of moles of bound probe per mole of protein, and* A_f *is the free probe concentration.*

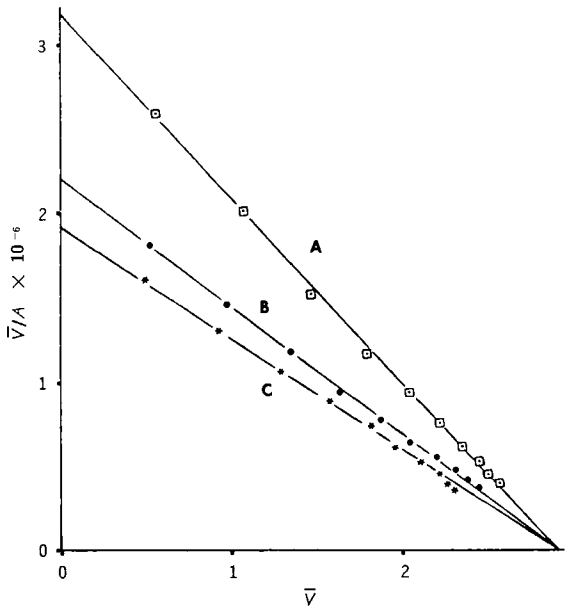


Figure 2—*Scatchard plots for the binding of the probe IV to bovine serum albumin (*1.38×10^{-6} M*). Key: A, in the absence of drug; B, in the presence of* 2×10^{-5} M *glipizide; and C, in the presence of* 2×10^{-5} M *acetohexamide.* $\bar{V}$ *is the number of moles of bound probe per mole of protein, and* A_f *is the free probe concentration.*

[1] Upjohn Co., Lot No. 342AE.
[2] Pfizer Inc., Lot No. 19559.
[3] Eli Lilly and Co., Lot No. QA 106A.
[4] Instituto Carlo Erba Per Ricerche Therapeutiche, Italy, Lot No. K4024.
[5] Upjohn Co., Lot No. 8707-KGW-2D.
[6] Abbott Laboratories, Chicago, Ill., Lot No. 833-1521.
[7] Aldrich.
[8] Aminco Bowman, American Instrument Co., Silver Spring, Md.
[9] Omnigraphic, Houston Instrument, Bellaire, Tex.
[10] Hamilton.

Table I—Binding Constants of Sulfonylureas to Bovine Serum Albumin[a]

Compound	$A(n = 2.90)$	$B(n = 0.81)$
IV	1.10×10^6	—
III	—	1.02×10^6
Chlorpropamide	2.02×10^4	—
Acetohexamide	3.76×10^4	—
Tolbutamide	9.04×10^4	—
Glipizide	2.45×10^4	6.88×10^4
Glyburide	—	3.26×10^5

[a] A = determined by the use of IV as the probe, B = determined by the use of III as the probe, and n = number of binding sites for the probes.

sulfonylureas such as chlorpropamide, acetohexamide, and tolbutamide did not affect the fluorescence or the binding of III to bovine serum albumin. When using IV as the probe, however, competitive binding between the probe and chlorpropamide, acetohexamide, and tolbutamide was observed. The results, as indicated by the Scatchard plots, are shown in Figs. 2 and 3. The binding constants, calculated from the Klotz *et al.* (21) equation, are listed in Table I. Of the three, tolbutamide and chlorpropamide have the highest and the lowest binding affinities to bovine serum albumin, respectively. The results also show that III and IV are bound to bovine serum albumin at different sites.

The binding mechanism for glipizide and glyburide to serum albumins is slightly different from that of other sulfonylureas. The fluorescence change of the protein-IV mixtures by the addition of glipizide and of glyburide is shown in Table II. Displacement of the probe by these two drugs is clearly shown in rat, rabbit, horse, and methylated bovine albumins and bovine α-globulin. However, the fluorescence change is different when the protein is human or bovine serum albumin, two proteins known to be very similar to each other. Figure 4 shows the effect on the fluorescence emission of the probe-bovine serum albumin mixture by glipizide and glyburide. An increase of the intensity accompanied by a blue shift of 10 nm was observed in the case of glyburide, whereas the addition of glipizide resulted in quenching of the fluorescence intensity. In the probe-human serum albumin system (Fig. 5), the fluorescence intensity was enhanced by both glipizide and glyburide and the emission maximum was also shifted toward the blue. Furthermore, neither glipizide nor glyburide showed fluorescence in protein solutions, nor is the fluorescence of IV changed by the addition of these drugs. Glyburide affects the binding of the probe to bovine serum albumin in a similar fashion. Binding of glipizide to bovine serum albumin, however, is somewhat different from that of glyburide. The cause of this effect is not clear; it is probably due to a slight difference in structure of glipizide and glyburide or to the structural and/or conformational differences of human and bovine serum albumins.

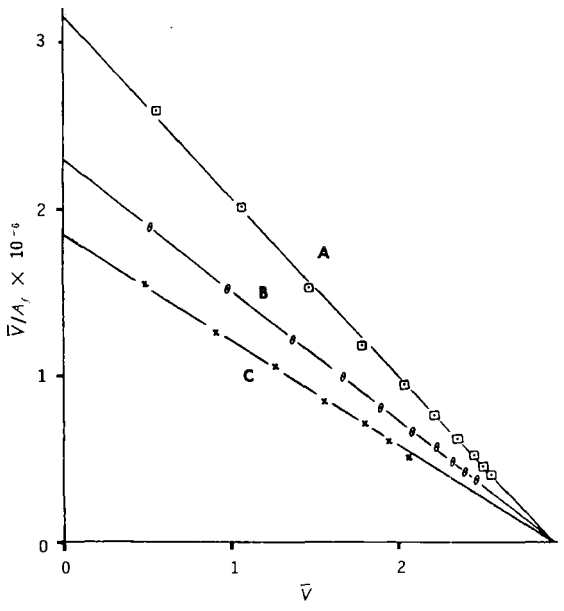


Figure 3—*Scatchard plots for the binding of the probe IV to bovine serum albumin (1.38 × 10⁻⁶ M). Key: A, in the absence of drug; B, in the presence of 2 × 10⁻⁵ M chlorpropamide; and C, in the presence of 1 × 10⁻⁵ M tolbutamide. $\bar{V}$ is the number of moles of bound probe per mole of protein, and A_f is the free probe concentration.*

Table II—Effect of Fluorescence Intensity of IV–Anilino Protein Mixtures by the Addition of Glyburide and Glipizide[a]

Protein	Addition of Glyburide	Addition of Glipizide
Bovine serum albumin	+	−
Bovine serum albumin V	+	−
Bovine serum albumin, methylated	−	−
Human serum albumin	+	+
Human serum albumin V	+	+
Horse albumin V	−	−
Rat albumin	−	−
Rabbit albumin	−	−
Bovine α-globulin	−	−
Bovine β,γ-globulin	No change	No change

[a] + = increase and − = decrease in intensity.

Drugs that are competitively interacting with IV for the same protein binding sites may also be affected by glipizide and glyburide. For example, the effect on the fluorescence of warfarin in the presence of bovine serum albumin by glipizide and glyburide is shown in Fig. 6. Warfarin has been shown (4) to compete with IV for the same sites in binding to bovine serum albumin. As shown in Fig. 6, the fluorescence effect of warfarin in bovine serum albumin solution by these drugs is very similar to that shown in Fig. 4. The pharmacological activity of a drug is greatly affected by its protein binding. The binding enhancement caused by another drug such as in the warfarin-glyburide system of bovine serum albumin may therefore reduce its drug activity.

Glipizide and glyburide do compete with IV in binding to methylated bovine serum albumin (Table II). This suggests that ionic binding, probably by means of the —CONH— groups in these drugs, also plays an important role and may be primarily responsible for the difference from other sulfonylureas in binding to plasma protein. In methylated bovine serum albumin, these methyl groups not only increase the hydrophobicity of the protein but also reduce the capacity of hydrogen bond formation between the drug and the protein. As a result, the reducing hydrogen bonding ability of bovine serum albumin alters drug interactions with the probe.

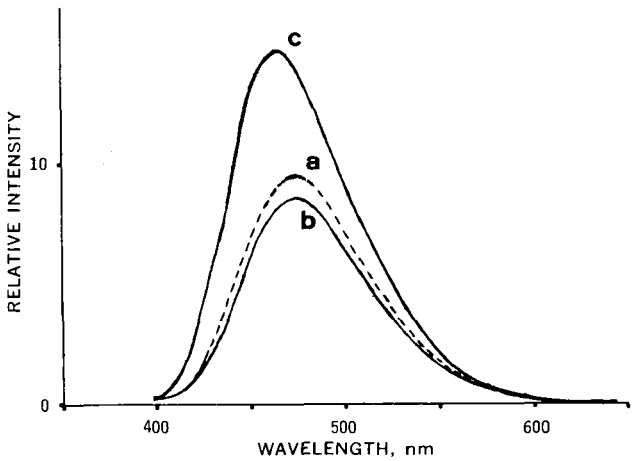


Figure 4—*Fluorescence emission spectra for solutions containing 2 × 10⁻⁶ M IV and 0.1 mg/ml bovine serum albumin (excitation 375 nm, pH 7.45). Key: a, in the absence of drug; b, in the presence of 2 × 10⁻⁵ M glipizide; and c, in the presence of 1 × 10⁻⁵ M glyburide.*

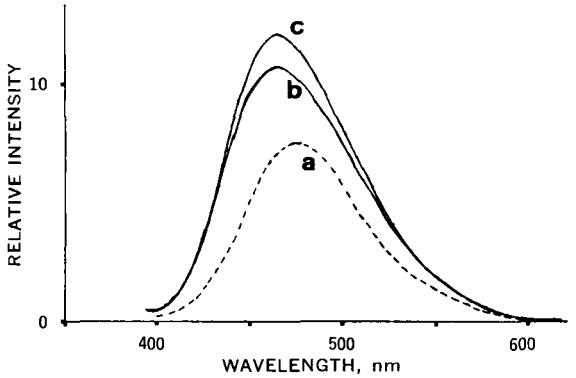


Figure 5—*Fluorescence emission spectra for solutions containing 2 × 10⁻⁶ M IV and 0.1 mg/ml human serum albumin (excitation 375 nm, pH 7.45). Key: a, in the absence of drug; b, in the presence of 2 × 10⁻⁵ M glipizide; and c, in the presence of 1 × 10⁻⁵ M glyburide.*

SUMMARY

9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine (III) and 1-anilinonaphthalene-8-sulfonic acid (IV) were used as fluorescence probes in the study of the interactions of sulfonylureas to plasma proteins. The results show that these two probes may bind to different but closely located sites on the protein. Among the sulfonylureas reported in this study, chlorpropamide, acetohexamide, and tolbutamide compete with IV but not with III for the binding sites on bovine serum albumin. On the other hand, glipizide and glyburide were found to compete with III in binding to bovine serum albumin. In contrast to the decrease in fluorescence intensity, fluorescence enhancement of IV by glipizide and by glyburide was found in human serum albumin and in both human and bovine serum albumins, respectively. These results indicate that glipizide and glyburide may bind to different but closely located sites on the protein as compared to the binding of other sulfonylureas. Glipizide and glyburide may simultaneously bind to the protein and the drug bound to closely located sites on the protein to form ternary complexes and, therefore, enhance the binding of the drug to the protein.

The binding constants of these sulfonylureas to bovine serum albumin were calculated; the results show that glyburide has the highest affinity to the protein.

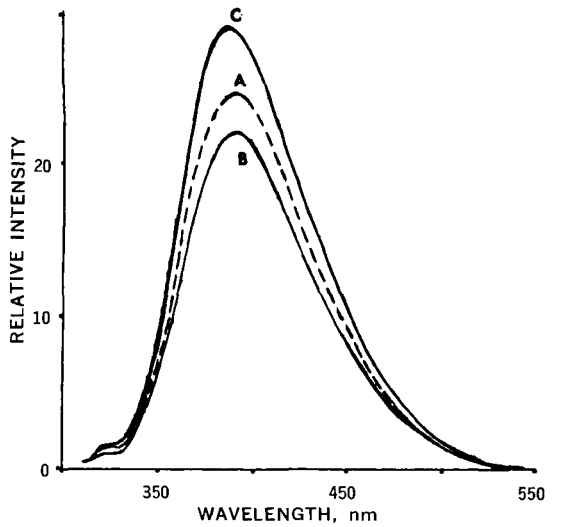


Figure 6—*Fluorescence emission spectra for solutions containing 2 × 10⁻⁵ M sodium warfarin and 0.1 mg/ml bovine serum albumin (excitation 315 nm, pH 7.45). Key: A, in the absence of drug; B, in the presence of 2 × 10⁻⁵ M glipizide; and C, in the presence of 2 × 10⁻⁵ M glyburide.*

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* Present address: School of Pharmacy, University of Georgia, Athens, GA 30601

ˣ To whom inquiries should be directed.

Cactaceae Alkaloids XVIII: Two New Alkaloids from *Coryphantha calipensis* H. Bravo

JAN G. BRUHN[x] and STIG AGURELL*

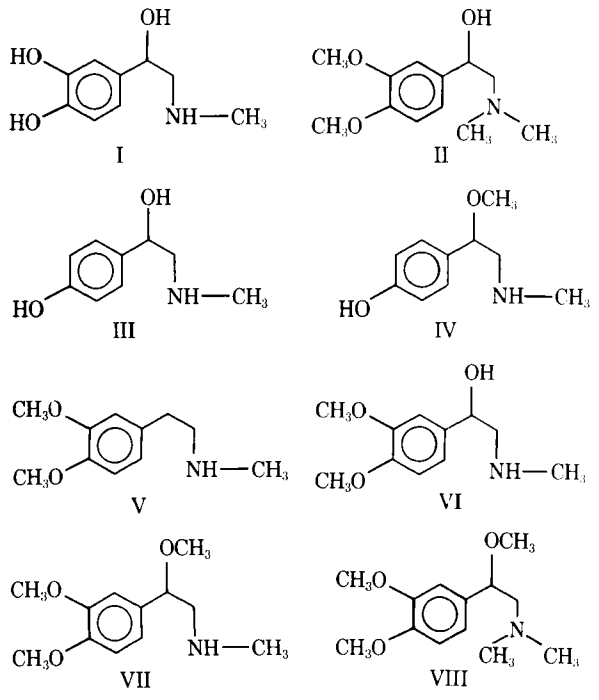
Abstract □ Two new alkaloids were isolated from the Mexican cactus *Coryphantha calipensis* H. Bravo. Based on chemical and spectroscopic data, their structures were determined as (−)-*N*-methyl-3,4-dimethoxy-β-methoxyphenethylamine [(−)-β-*O*-methylnormacromerine] and (−)-*N,N*-dimethyl-3,4-dimethoxy-β-methoxyphenethylamine [(−)-β-*O*-methylmacromerine]. The isolation of (−)-normacromerine and *N*-methyl-3,4-dimethoxyphenethylamine from this species is also reported.

Keyphrases □ Cactus alkaloids—isolation, identification of alkaloids from *Coryphantha calipensis* H. Bravo □ *Coryphantha calipensis* H. Bravo—isolation, identification of alkaloids □ (−)-*N*-Methyl-3,4-dimethoxy-β-methoxyphenethylamine [(−)-β-*O*-methylnormacromerine]—isolation, identification from *Coryphantha calipensis* H. Bravo □ (−)-*N,N*-Dimethyl-3,4-dimethoxy-β-methoxyphenethylamine [(−)-β-*O*-methylmacromerine]—isolation, identification from *Coryphantha calipensis* H. Bravo

Since the end of the 19th century Cactaceae species have been known to produce alkaloids, but not until recently has attention been drawn to the genus *Coryphantha* (2). From *Coryphantha macromeris* (Engelm.) Br. & R., Hodgkins *et al.* (3) isolated the new alkaloid macromerine (II), which was reported to cause hallucinations in test animals. The structure of macromerine is closely related to epinephrine (I) and the β-phenethylamine alkaloids found in several cacti (4, 5).

Further work on *Coryphantha* species showed the presence of macromerine in *C. runyonii* Br. & R. as well (6), and this cactus also contains several simpler phenethylamines, *e.g.*, *N*-methyl-3,4-dimethoxyphenethylamine (V) (2) and *N*-methyl-4-methoxyphenethylamine (7). Recently, the β-hydroxylated phenethylamines normacromerine (8), *N*-formylnormacromerine, metanephrine, *N*-methylmetanephrine, and synephrine (9) were found in *C. runyonii*[1].

In a field screening of cacti in Mexico (10), *C. calipensis* H. Bravo (11) was found to give a positive test for alkaloids. Plants were collected and the alkaloids were extracted and studied according to the principles already outlined (2). This paper describes the isolation of alkaloids from *C. calipensis* and the structure elucidation of these alkaloids.



EXPERIMENTAL[2]

Plant Material—*C. calipensis* H. Bravo was collected in Mexico[3]. Plants[4] were collected on April 19, 1971, not far from Calipan, on the road between Tehuacán and Ajalpan, Puebla (11).

Isolation and Separation of Alkaloids—The fresh plants (2.56 kg) were cut to pieces and immediately homogenized in twice their weight of ethanol. The slurry was left overnight at 4°. The ethanol solution was filtered and evaporated to dryness to give a crude extract. Portions (5 g) of the crude extract were dissolved in 100 ml acetic acid (3%), and the solution was extracted with 100 ml chloroform. The aqueous phase was basified to pH 10 with ammonia and extracted with 2 × 100 ml chloroform followed by 100 ml of chloroform–ethanol (3:1). The combined chloroform extracts were condensed to almost complete dryness. This alkaloid extract was dissolved in chloroform and purified by passing through an acid diatomaceous earth[5] column as earlier described (2). The total amount of crude alkaloids obtained in this manner was 3.6 g, 0.14% of the fresh plant material.

One gram of the alkaloid extract was fractionated on a 2 × 50-cm aluminum oxide column[6]. The solvents used are shown in Table I. A total of 200 ml of each solvent was passed through the column. Fractions of 10 ml each were collected and analyzed by TLC and GLC. The fractions richest in the respective alkaloids were combined, and the alkaloids were crystallized as the hydrochlorides from absolute ethanol–ether.

TLC and GLC—GLC was carried out using a flame-ionization detector[7] and glass columns [1.82 m × 0.31 cm (6 ft × 0.125 in.)] packed with 5% SE-30 and 5% XE-60 on Gas Chrom P (100–120 mesh). Mass spectra were recorded with a gas chromatograph–mass spectrometer[8]. For TLC, silica gel G plates were chromato-

[1] *C. runyonii* is sometimes considered a variety of *C. macromeris*, and these two species have also been grouped together in the genus *Lepidocoryphantha* (*cf.*, 7, 9).

[2] Melting points were determined with a Leitz Mikroskopheitztisch 350, using calibrated thermometers. IR spectra were recorded in KBr or chloroform using a Perkin-Elmer 237 IR spectrophotometer. NMR spectra were recorded with a Varian A-60 instrument in deuterochloroform or tetradeuteromethanol with tetramethylsilane as internal standard. Optical rotation was measured on a Perkin-Elmer 141 polarimeter.

[3] By J. G. Bruhn and Dr. Hernando Sánchez-Mejorada.

[4] The identification of the plant material was checked by Dra. Helia Bravo H., Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, where a herbarium specimen has been deposited.

[5] Celite.

[6] Merck, activity IV according to Brockmann.

[7] Varian 204 Aerograph.

[8] LKB 9000.

graphed with chloroform–ethanol–diethylamine (85:5:10) or chloroform–ethanol–concentrated ammonia (85:15:0.4) and sprayed with an iodoplatinate solution to locate alkaloids (12).

Characterization of (−)-*N*-Methyl-3,4-dimethoxy-β-methoxyphenethylamine (VII)—The major compound eluted from the column (Table I) was crystallized as the hydrochloride (yield 210 mg), mp 213–214°; $[\alpha]D^{25}$ − 91.7°; c 0.010 g/ml in absolute ethanol. The homogeneity of the alkaloid was shown by TLC and GLC. NMR and mass spectra indicated that the compound is VII. The mass spectrum showed major peaks at *m/e* (relative intensity): 44 (67), 151 (8), 166 (17), 181 (100), 182 (28), and 225 (M^+, 0.4). The NMR spectrum (60 MHz) of the free base in deuterochloroform showed a singlet at δ 6.91, indicating three aromatic hydrogens, and a doublet of doublets centered at δ 4.32 (J_1 = 8 Hz, J_2 = 5 Hz), indicating a benzylic proton. Two singlets at δ 3.91 and 3.88 were indicative of two methoxyl groups, and two three-proton singlets at δ 3.29 and 2.48 indicated an *O*-methyl and an *N*-methyl group, respectively.

Oxidation of VII—Alkaline permanganate oxidation of the alkaloid hydrochloride (50.0 mg) was performed as earlier described for 3,4-dimethoxyphenethylamine (13). Sublimation *in vacuo* afforded 11 mg of a crystalline derivative, mp 178–181°, which was identified as 3,4-dimethoxybenzoic acid by IR spectral comparison. The reported (13) melting point for 3,4-dimethoxybenzoic acid is 181°.

Acid Hydrolysis of VII—Compound VII-HCl (20 mg) was hydrolyzed overnight at room temperature in hydrochloric acid (6 *N*, 10 ml). The major product was purified by preparative TLC and identified as normacromerine (TLC, GLC, and IR) by comparison with an authentic sample.

Characterization of (−)-*N,N*-Dimethyl-3,4-dimethoxy-β-methoxyphenethylamine (VIII)—The hydrochloride of this alkaloid was recrystallized from absolute ethanol–ether (yield 40 mg), mp 178°; $[\alpha]D^{25}$ − 93.7°; c 0.010 g/ml in absolute ethanol. The isolated material was chromatographically homogeneous (TLC and GLC). The mass spectrum showed major peaks at *m/e* (relative intensity): 58 (100), 181 (6), 182 (0.7), and 239 (M^+, 0.2). This suggested that VIII was the *N*-methyl analog of VII. The NMR spectrum (60 MHz) of the free base in deuterochloroform confirmed this interpretation, showing a singlet at δ 6.90, indicating three aromatic protons, and a doublet of doublets centered at δ 4.30 (J_1 = 8 Hz, J_2 = 4 Hz), indicative of a benzylic hydrogen. Singlets at δ 3.92 and 3.90 indicated two methoxyl groups, and another *O*-methyl group was indicated by a three-proton singlet at δ 3.25. A six-proton singlet at δ 2.35 indicated two *N*-methyl groups.

Methylation of VII—Compound VII-HCl (50 mg) was dissolved in methanol (4 ml), and 0.2 ml of 36% formaldehyde solution was added. The mixture was stirred for 15 min and then reduced for 1 hr with sodium borohydride (0.3 g). The methanol was evaporated and the residue was taken up in 0.5 *N* HCl (3 ml). The solution was made alkaline (ammonia) and extracted with ether. The ether residue was crystallized as the hydrochloride from methanol–ether to yield VIII-HCl, 40 mg, mp 177–179°; $[\alpha]D^{25}$ − 83.7°; c 0.010 g/ml in absolute ethanol. Comparison with VIII showed the two compounds to be identical in all respects [NMR, IR (chloroform), mass spectroscopy, TLC, GLC, melting point, and mixed melting point]. (±)-*N,N*-Dimethyl-3,4-dimethoxy-β-methoxyphenethylamine was also prepared from (±)-macromerine (II) by refluxing a solution of the latter compound in 2 *N* HCl in methanol (14). The product, isolated by preparative TLC, was identical with VIII and the *N*-methyl derivative of VII [TLC, GLC, NMR, IR (chloroform), and mass spectroscopy].

Identification of (−)-Normacromerine (VI)—The chloroform–ethanol fractions (Table I) contained one major alkaloid, crystallized as the hydrochloride (yield 135 mg), mp 131–132°; $[\alpha]D^{25}$ −51.3°; c 0.010 g/ml in absolute ethanol. TLC and GLC analyses showed the homogeneity of the isolated alkaloid and also afforded a preliminary identification as normacromerine (VI). The reported (8) melting point of 132–133° and the reported (8) optical rotation of $[\alpha]D^{27}$ −47.5°, c 0.020 g/ml in absolute methanol, of natural (−)-normacromerine hydrochloride from *C. runyonii* also support this identification. Superimposable IR (chloroform) spectra were obtained with an authentic sample of (±)-normacromerine hydrochloride (8, synthetic) and the now isolated alkaloid. In addition, the melting point of VI-HCl (mp 131–132°) was not depressed by the addition of (−)-normacromerine hydrochloride isolated from *C. runyonii* (mixed mp 129–132°).

Table I—Column Chromatographic Separation of the Crude Alkaloid Extract of *C. calipensis* H. Bravo

Solvent[a]	Alkaloids Identified	Structure
Benzene	—	—
Chloroform–benzene		
1:2	—	—
1:1	(−)-*N,N*-Dimethyl-3,4-dimethoxy-β-methoxyphenethylamine	VIII
2:1	(−)-*N*-Methyl-3,4-dimethoxy-β-methoxyphenethylamine	VII
	N-Methyl-3,4-dimethoxyphenethylamine	V
Chloroform	—	—
Chloroform–ethanol		
4:1	(−)-Normacromerine	VI
Ethanol	—	—

[a] For details, see *Experimental*.

Identification of *N*-Methyl-3,4-dimethoxyphenethylamine (V)—In the fractions following VII (Table I), GLC analysis indicated the presence of small amounts of *N*-methyl-3,4-dimethoxyphenethylamine. After purification by preparative TLC in chloroform–ethanol–diethylamine (85:5:10), this compound could be crystallized as the hydrochloride from chloroform–ether (yield 3 mg), mp 134–137° [lit. (5) mp 134–136°]. The IR spectrum and chromatographic behavior (TLC and GLC) confirmed the identity of these crystals with reference V-HCl.

RESULTS AND DISCUSSION

As in previous investigations (2, 9, 15), the now isolated *Coryphantha* alkaloids are all β-phenethylamine derivatives. The major alkaloids were VII and VI. The percentage of alkaloids in fresh plants of *C. calipensis* H. Bravo was found to be 0.14%. Compounds VII and VIII have not previously been isolated from nature, although they are closely related to the known cactus alkaloids normacromerine (VI) and macromerine (II), being the β-*O*-methyl ethers of these compounds.

The identity of the alkaloids was established by spectroscopic data, chemical transformations, and comparison with authentic reference materials using TLC, GLC, IR, NMR, and mass spectroscopy. The two important fragment ions at *m/e* 44 and 181 in the mass spectrum of VII result from a benzylic cleavage.

(−)-Normacromerine earlier was isolated from *C. runyonii* (8) and recently from *Desmodium tiliaefolium* (Leguminosae) (16). *N*-Methyl-3,4-dimethoxyphenethylamine was first identified in *C. runyonii* (2) and is found in several cacti (1, 5).

The relationship of these alkaloids to human neural transmittor substances is of special interest. Compounds VI, VII, and VIII may be regarded as *O*-methylation products of epinephrine. Epinephrine has not been found in plants as yet, but its analog norepinephrine was reported in various species (17). The sympathomimetic drug (−)-synephrine (III), closely related to norepinephrine, has been isolated from citrus leaves (18) and various *Coryphantha* species (9, 15, 19). Similar in structure to the now isolated methyl ethers of macromerine and normacromerine is (−)-β-*O*-methylsynephrine (IV), found in tangerine leaves by Stewart and Wheaton (14). These researchers suggested that this compound could be an artifact from their extraction process. Compound IV was also identified together with synephrine in *C. cornifera* var. *echinus* (15) and in *C. ramillosa* (19).

The alkaloids of *C. calipensis*, except for V, are all levorotatory, as is naturally occurring macromerine[9] (20). The cold extraction of *C. calipensis* was carried out with absolute ethanol to avoid the easy formation of methyl ethers from β-hydroxylated compounds (14, *cf.*, 19). Therefore, VII and VIII are believed to be natural constituents of *C. calipensis*.

[9] Natural (−)-macromerine and natural epinephrine were recently shown to have the same absolute configuration, *R* (20).

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* Also at Central Military Pharmacy, Karolinska Hospital, S-104 01 Stockholm, Sweden.

ˣ To whom inquiries should be directed.

Entropy of Transfer of Molecular Benzoic Acid from a Pure Liquid to an Aqueous Solution

JOHN W. MAUGERˣ and ANTHONY N. PARUTA*

Abstract □ The solubility of benzoic acid in distilled, deionized water was determined over a limited temperature range. The entropy of transfer of molecular benzoic acid from a pure liquid to an aqueous solution was calculated. Data also were analyzed in terms of the hypothetical partial molal entropy of transfer of molecular benzoic acid from an ideal or a regular solution to an aqueous solution. Interpretation of the data indicates that solute–solvent interactions result in a reduction of the number of independent molecules relative to an ideal or a regular solution.

Keyphrases □ Benzoic acid—transfer entropy from pure liquid to aqueous solution, calculation of partial molal entropy □ Solutes, semipolar nonelectrolyte (benzoic acid)—transfer entropy from pure liquid to aqueous solution, equations □ Entropy of transfer from pure liquid to aqueous solution—benzoic acid, calculation of partial molal entropy

Molecular interactions involving solute (nonelectrolyte)–solvent (water) components have attracted considerable research interest (1–10). Solute substances nearly insoluble in water have provided several hypotheses about the structure of water and interactions occurring in this condensed liquid phase (1–4). To date, no one hypothesis has gained universal acceptance (8, 11–13).

Bulk properties of water, such as surface tension and the dielectric constant, have been useful for interpreting solution behavior (14, 15). The importance of the entropy of solution as an interpretive quantity also has been discussed (16, 17).

This study was undertaken to investigate the behavior of a semipolar nonelectrolyte solute, benzoic acid, in aqueous systems. Chertkoff and Martin (18) mentioned that benzoic acid serves as a prototype of relatively polar pharmaceutical solids. Solubility data at several temperatures were analyzed in terms of the entropy consequence of transferring molecular benzoic acid from a pure liquid to an aqueous solution. Equations were used which allow the calculation of the partial molal entropy of transfer from an ideal or a regular solution to an aqueous solution.

THEORETICAL

The partial molal entropy of solution, $\Delta\bar{S}_2$, developed by Hilde-

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* Also at Central Military Pharmacy, Karolinska Hospital, S-104 01 Stockholm, Sweden.

ˣ To whom inquiries should be directed.

Entropy of Transfer of Molecular Benzoic Acid from a Pure Liquid to an Aqueous Solution

JOHN W. MAUGERˣ and ANTHONY N. PARUTA*

Abstract □ The solubility of benzoic acid in distilled, deionized water was determined over a limited temperature range. The entropy of transfer of molecular benzoic acid from a pure liquid to an aqueous solution was calculated. Data also were analyzed in terms of the hypothetical partial molal entropy of transfer of molecular benzoic acid from an ideal or a regular solution to an aqueous solution. Interpretation of the data indicates that solute–solvent interactions result in a reduction of the number of independent molecules relative to an ideal or a regular solution.

Keyphrases □ Benzoic acid—transfer entropy from pure liquid to aqueous solution, calculation of partial molal entropy □ Solutes, semipolar nonelectrolyte (benzoic acid)—transfer entropy from pure liquid to aqueous solution, equations □ Entropy of transfer from pure liquid to aqueous solution—benzoic acid, calculation of partial molal entropy

Molecular interactions involving solute (nonelectrolyte)–solvent (water) components have attracted considerable research interest (1–10). Solute substances nearly insoluble in water have provided several hypotheses about the structure of water and interactions occurring in this condensed liquid phase (1–4). To date, no one hypothesis has gained universal acceptance (8, 11–13).

Bulk properties of water, such as surface tension and the dielectric constant, have been useful for interpreting solution behavior (14, 15). The importance of the entropy of solution as an interpretive quantity also has been discussed (16, 17).

This study was undertaken to investigate the behavior of a semipolar nonelectrolyte solute, benzoic acid, in aqueous systems. Chertkoff and Martin (18) mentioned that benzoic acid serves as a prototype of relatively polar pharmaceutical solids. Solubility data at several temperatures were analyzed in terms of the entropy consequence of transferring molecular benzoic acid from a pure liquid to an aqueous solution. Equations were used which allow the calculation of the partial molal entropy of transfer from an ideal or a regular solution to an aqueous solution.

THEORETICAL

The partial molal entropy of solution, $\Delta\bar{S}_2$, developed by Hilde-

brand (19) has proved useful for interpreting solute-solvent interactions (20, 21). The $\Delta\bar{S}_2$ represents the entropy change of a solid forming a solution in a liquid, $(\bar{S}_2 - S_2^s)$, and may be written as:

$$R[(\partial \ln X_2/\partial \ln T)(\partial \ln a_2/\partial \ln X_2)] = \Delta\bar{S}_2 = (\bar{S}_2 - S_2^s) \quad \text{(Eq. 1)}$$

where X_2 is the mole fraction solubility of the solute, T is the temperature (Kelvin), a_2 is the activity of the solute, and the superscript s refers to the solid state of the solute. At constant temperature and pressure:

$$\Delta\bar{S}_2 = (\bar{S}_2 - S_2^\circ)_{P,T} + (S_2^\circ - S_2^s)_{P,T} \quad \text{(Eq. 2)}$$

and the superscript ° refers to the pure liquid solute. This equation may be rearranged to:

$$(\bar{S}_2 - S_2^\circ)_{P,T} = \Delta\bar{S}_2 - (S_2^\circ - S_2^s)_{P,T} \quad \text{(Eq. 3)}$$

where $(\bar{S}_2 - S_2^\circ)$ represents the partial molal entropy of transfer from the pure liquid to a solution.

The partial molal entropy of transfer from a pure liquid to a solution $(\bar{S}_2 - S_2^\circ)$, according to ideal or regular solution theory is equal to $(-R \ln X_2)$ (22), indicating molecular randomness. For systems that do not obey ideal solution behavior:

$$(\bar{S}_2 - S_2^\circ)_{P,T} - (\bar{S}_2' - S_2^\circ)_{P,T} = (\bar{S}_2 - \bar{S}_2')_{P,T} \quad \text{(Eq. 4)}$$

and $(\bar{S}_2 - \bar{S}_2')$ may be regarded as the partial molal entropy of transfer from an ideal solution to one that does not exhibit ideal solution behavior. By definition:

$$(\bar{S}_2' - S_2^\circ)_{P,T} = (-R \ln X_2)_{P,T} \quad \text{(Eq. 5)}$$

and Eq. 4 may be rewritten as:

$$(\bar{S}_2 - S_2^\circ)_{P,T} - (-R \ln X_2)_{P,T} = (\bar{S}_2 - \bar{S}_2')_{P,T} \quad \text{(Eq. 6)}$$

EXPERIMENTAL

Reagents—Benzoic acid[1] USP was recrystallized from chloroform.

Procedure—The solubility of benzoic acid in distilled, deionized water was determined using a previously published method (23). Temperatures were maintained within ±0.05° throughout the equilibration period. Prior to analysis, all solutions were filtered using a filter holder[2] and filters[3] with a pore size of 0.22 μm. Benzoic acid concentrations were determined by spectrophotometric assay[4] at 227 nm and/or the USP method of assay for benzoic acid (24). Comparable assay results were observed with either method.

RESULTS

Calculation of Concentration of Molecular Benzoic Acid—The total molar concentration of dissolved benzoic acid $[C]$ is equal to the sum of the unionized $[U]$ and ionized $[I]$ species:

$$[C] = [U] + [I] \quad \text{(Eq. 7)}$$

According to the equilibrium expression for the dissociation of a weak acid:

$$Ka = [I]^2/[U] \quad \text{(Eq. 8)}$$

where Ka is 6.31×10^{-5} (25). When $[I]$ is small in comparison to $[C]$, then:

$$[I]^2 \cong Ka \times [C] \quad \text{(Eq. 9)}$$

$$[I] \cong (Ka \times [C])^{1/2} \quad \text{(Eq. 10)}$$

and:

$$[U] = [C] - (Ka \times [C])^{1/2} \quad \text{(Eq. 11)}$$

where $[U]$ is the molar concentration of dissolved molecular benzoic acid.

Table I—Mole Fraction Solubility of Molecular Benzoic Acid in Distilled, Deionized Water

Temperature (Kelvin)	$X_2 \times 10^4$
293.3°	3.97
298.2°	4.73
303.2°	5.62
308.2°	6.71
313.2°	7.92
318.2°	9.57

Calculation of $\Delta\bar{S}_2$—Mole fraction solubilities for molecular benzoic acid at several temperatures are presented in Table I. The entropy of solution, $\Delta\bar{S}_2$, was calculated from the slope of the solubility curve, $\ln X_2$ *versus* $\ln T$, according to the relationship:

$$\Delta\bar{S}_2 = R(d \ln X_2/d \ln T)_{\text{sat},P} \quad \text{(Eq. 12)}$$

The correlation coefficient for this linear relationship is 1.00, and $\Delta\bar{S}_2$ is 21.2 eu (sat = saturated).

The limited solubility of molecular benzoic acid placed the solution within the range of dilute solution behavior such that it could be assumed that the Henry's law factor $(\partial \ln a_2/\partial \ln X_2)$ approaches unity. The limited temperature range over which the solubilities were determined allows the assumption that $(d \ln X_2/d \ln T)$ is independent of temperature.

Calculation of $(S_2^\circ - S_2^s)_{P,T}$—The quantity $(S_2^\circ - S_2^s)$ may be approximated by the relationship given by Hildebrand and Scott (26):

$$(S_2^\circ - S_2^s)_{P,T} \cong \Delta S_m{}^F - \Delta C_p \times \ln (T_m/T) \quad \text{(Eq. 13)}$$

where $\Delta S_m{}^F$ is the entropy of fusion at the melting point, $\Delta C_p = (C_p{}^1 - C_p{}^s)$ where $C_p{}^1$ and $C_p{}^s$ are the molal heat capacities of the liquid and solid forms, respectively, T_m is the melting point, and T is the temperature at which the process takes place. The values for $\Delta S_m{}^F$, ΔC_p, and T_m were taken from previously published data (27).

Calculation of $(\bar{S}_2 - S_2^\circ)_{P,T}$—The relationship among $\Delta\bar{S}_2$, $(\bar{S}_2 - S_2^\circ)_{P,T}$, and $(-R \ln X_2)_{P,T}$ was discussed previously (16, 28). For a series of regular solutions, a plot of $\Delta\bar{S}_2$ *versus* $(-R \ln X_2)_{P,T}$ yields a linear relationship with a y intercept of $(S_2^\circ - S_2^s)_{P,T}$.

Actually, it is found that the y intercept is greater than $(S_2^\circ - S_2^s)_{P,T}$ when the data are plotted in this manner. The discrepancy results because $\Delta\bar{S}_2$ also includes the value $(-R \ln a_2)_{P,T}$, where a_2 is the activity of the pure solute. Therefore, Eq. 2 must be modified to include the quantity $(-R \ln a_2)$ and:

$$\Delta\bar{S}_2 = (\bar{S}_2 - S_2^\circ)_{P,T} + (S_2^\circ - S_2^s)_{P,T} + (-R \ln a_2)_{P,T} \quad \text{(Eq. 14)}$$

and:

$$(\bar{S}_2 - S_2^\circ)_{P,T} = \Delta\bar{S}_2 - (S_2^\circ - S_2^s)_{P,T} - (-R \ln a_2)_{P,T} \quad \text{(Eq. 15)}$$

Finally:

$$(\bar{S}_2 - S_2^\circ) = 21.2 \text{ eu} - (S_2^\circ - S_2^s)_{P,T} - (-R \ln a_2)_{P,T} \quad \text{(Eq. 16)}$$

The data for the various thermodynamic functions are summarized in Table II.

[1] Lot RK556, Ruger Chemical Co.
[2] Swinny, Millipore Corp., Bedford, Mass.
[3] Millipore filter, Catalog No. GSWP 013 00.
[4] Perkin-Elmer 124 spectrophotometer.

Table II—Summary of Thermodynamic Data for Molecular Benzoic Acid Dissolved in Distilled, Deionized Water

Temperature (Kelvin)	$(-R \ln a_2)$[a], eu	$(-R \ln X_2)$, eu	$(S_2^\circ - S_2^s)$, eu	$(\bar{S}_2 - S_2^\circ)$, eu	$(\bar{S}_2 - \bar{S}_2^i)$, eu
293.2°	3.25	15.6	6.74	11.2	−4.40
298.2°	3.07	15.2	6.97	11.2	−4.00
303.2°	2.89	14.9	7.20	11.1	−3.80
308.2°	2.72	14.5	7.42	11.1	−3.40
313.2°	2.54	14.2	7.64	11.0	−3.20
318.2°	2.37	13.8	7.86	11.0	−2.80

[a] J. H. Hildebrand, J. M. Prausnitz, and R. L. Scott, "Regular and Related Solutions," Van Nostrand Reinhold, New York, N.Y., 1970, p. 22, Eq. 2.51 ($R \ln a_2 \cong \Delta S_m^F \ln T/T_m$).

DISCUSSION

When a semipolar nonelectrolyte containing both an apolar substituent and a polar group that is capable of hydrogen bonding is introduced into an aqueous environment, two types of interaction might occur: *(a)* the polar substituent may interact with the water molecules by hydrogen bond formation, and *(b)* an interaction as a result of van der Waals' forces between water and the apolar portion of the solute may take place.

The data in Table II are somewhat uncertain in view of the approximate relationships used to calculate $(-R \ln a_2)$ and $(S_2^\circ - S_2^s)$. However, over the temperature range studied, all values for $(\bar{S}_2 - \bar{S}_2^i)$ are negative for molecular benzoic acid dissolved in distilled, deionized water. These thermodynamic data suggest that the experimental solution does not conform to a structure of maximum disorder or randomness, *i.e.*, an ideal or a regular solution.

Interpretation of the negative entropies of transfer from an ideal solution to an aqueous solution is a matter of controversy. Feldman and Gibaldi (6) discussed the thermodynamics of aqueous solutions of benzoic acid in keeping with the view of Nemethy and Scheraga (2–4). Nemethy and Scheraga (2–4) considered the negative entropy a consequence of increased water structure due to the introduction of a nonpolar molecule into the aqueous environment, a condition not thermodynamically favored. Related to this unfavorable state is the partial reversal of the solution process which is manifested by an association of the nonpolar side chains, the association being termed the hydrophobic bond. The low water solubility of hydrocarbons is said to be a consequence of this entropic relationship. Nemethy and Scheraga (2–4) also considered the influence of the polar side chains of proteins (4). It was their view that the nonpolar portion of a molecule carrying a polar group can still exert about the same influence on the energy states of water as in nonpolar solutes. They further pointed out that the exact local structure of water in the neighborhood of the polar group may differ from that found in solutions where the solute does not contain a polar portion.

In this connection, it is important to compare the aqueous solubilities of benzene and benzoic acid in order to discuss the role of the carboxyl group in the solution process. The mole fraction solubility of benzene in water (29) is reproduced in Table III, and certain comparisons are of interest.

1. The mole fraction solubilities of benzene and benzoic acid are 3.99×10^{-4} (299.2°K) and 4.73×10^{-4} (298.2°K), respectively. Therefore, at temperatures near 25°, the aqueous solubility of benzoic acid is only about 1.2 times greater than benzene. Since solubility expresses a summary of all solute-solvent interactions, it appears that the carboxyl group is not particularly influential at this temperature.

The lack of difference between the mole fraction[5] solubilities of benzene and benzoic acid is surprising based on their differing physicochemical properties: physical state at room temperature and standard pressure, molar volume, electronic characteristics, and relative polarity. Part of the answer relating to the magnitude of aromatic hydrocarbon–water interactions concerns the uniqueness of water as a solvent, a topic summarized by other authors (10). Consideration must also be given to the strength of the solute–solute interaction and its relationship to solubility. The physical state of a molecule is an indication of intermolecular bonding. Benzene is a liquid at room temperature and benzoic acid is a solid. Therefore, the presumed advantage of the carboxyl group in increasing the aqueous solubility of benzoic acid over benzene may be somewhat balanced by the propensity of benzoic acid molecules to attract each other rather than to interact with water. This argument is partly intuitive since the partial molal free energy of transfer from solid to solution contains two components: *(a)* the transfer from solid to liquid, and *(b)* the transfer from liquid to solution. On the other hand, a liquid needs only to be transferred from the liquid state to solution.

Table III—Mole Fraction Solubility of Benzene in Water[a]

Temperature (Kelvin)	$X_2, \times 10^4$
290.2°	3.95
295.2°	3.97
299.2°	3.99
302.2°	4.02
305.2°	4.12
308.2°	4.20
315.2°	4.40
317.2°	4.45
319.2°	4.57

[a] Results from Ref. 29.

Limitations to the interpretation of solubility data for two compounds in water were outlined by Hildebrand and Scott (30). In particular, it is difficult to compare a solid and a liquid solute because the solubility of the latter relates to the vapor pressure at any given temperature[6, 7]. Furthermore, steric implications have not been considered which may influence solubility interpretation.

2. As temperature increases, the mole fraction solubility of benzoic acid in water increases more rapidly than benzene. At 318°K, benzoic acid solubility is approximately two times greater than that of benzene. Evidently, the effect of temperature on the water molecule and solute favors increased interactions between water and the polar carboxyl group of benzoic acid. Regardless of the model proposed for water structure, hydrogen bonding between water molecules diminishes as temperature is increased, making water molecules available for increased hydrogen bonding with the carboxyl group.

The views of Nemethy and Scheraga (2–4) are not shared by Hildebrand (12) who criticized the term hydrophobic bond. Hildebrand's criticism centers about the idea that hydrocarbons prefer a nonpolar environment rather than be surrounded by water. He contended that: "molecules of water prefer to be hydrogen bonded together rather than separate to admit alkanes." Hildebrand (11) also questioned the iceberg concept of water, based on the relative diffusivities of methane in water and carbon tetrachloride. At 25°, methane diffuses 0.6 times as fast in water as it does in carbon tetrachloride. Diffusivity is mainly dependent upon temperature, viscosity of the solvent, and molecular cross section of the diffusant. Since the viscosities of water and carbon tetrachloride are nearly identical, it is suggested that the molecules of methane are only retarded by the hydrogen bonds in water and not by an ice-like structure. According to Hildebrand's argument, the low water solubility of molecular benzoic acid is due in large part to the difference in internal pressures between

[5] Interpretation of solubility data depends on the concentration unit utilized. For example, the aqueous solubilities of benzene and benzoic acid are different if the data are expressed as grams per milliliter. The mole fraction unit is preferred for theoretical discussions, however, because the molecular ratios of solute and solvent are immediately evident.

[6] This limitation also relates to interpretation of solubility data using thermodynamic parameters. For example, a plot of $\ln X_2$ *versus* $\ln T$ for the benzene data implies the choice of the pure liquid hydrocarbon as the standard state (29). This standard state may be criticized from two standpoints: *(a)* liquid aromatic hydrocarbons are said to have some structure of their own (29); and *(b)* Franks *et al.* (29) showed that this choice of standard state for benzene masks certain solution characteristics, such as heat capacity change, as compared with a different standard state.

[7] A comment made by the reviewer is important to a discussion of solubility and vapor pressure. The vapor pressure of benzoic acid is expected to be much lower than that of benzene at 298°K. Hence, if solubilities were compared at equal vapor pressures, the benzoic acid solubility would be much higher than that of benzene.

the solute and solvent. He noted (12) that: "Ice is wet by octane. The fact that octane is nearly insoluble in water is merely the result of the fact that this attraction is not strong enough to penetrate the high cohesion of water."

Application of regular solution theory to the molecular benzoic acid solution provides an alternative method of data interpretation. The thermodynamic consequences of the regular solution postulate (22) are as follows:

$$(\bar{F}_2 - \bar{F}_2') = RT \ln (a_2/X_2) = (\bar{H}_2 - \bar{H}_2') - T(\bar{S}_2 - \bar{S}_2') \quad \text{(Eq. 17)}$$

$$(\bar{S}_2 - \bar{S}_2') = 0 \quad \text{(Eq. 18)}$$

and:

$$(\bar{F}_2 - \bar{F}_2') = RT \ln (a_2/X_2) = (\bar{H}_2 - \bar{H}_2') \quad \text{(Eq. 19)}$$

Thus, a regular solution has a positive "excess" free energy and enthalpy and the excess free energy is essentially temperature independent. The excess free energy is a function of composition[8]. Although the data in Table II indicate positive excess free energies at all temperatures (*i.e.*, a_2 is greater than X_2), the excess entropies are less than what would be expected for a regular solution. Regular solutions of iodine are violet and those in which a specific solute–solvent interaction has occurred are brown. In the latter solutions, there is also evidence of negative excess entropies (16, 17). For solutions of molecular benzoic acid in water, an analogy would suggest that a specific interaction has occurred between the solute and solvent. The temperature effect on the excess entropies is also of interest to this suggestion. At higher temperatures, the excess entropies diminish.

The suggestion of a specific interaction does not absolutely require that the interaction of molecular benzoic acid and water is of a magnitude such that a_2 is less than X_2. Furthermore, it is even possible that the interaction is equal to or less than that of a geometric mean. Shinoda and Hildebrand (31) reported data showing that iodine forms a weak complex with 1,1-dichloroethane and 1,2-dichloroethane. Data are also reported which show that the solute–solvent interactions are less than that of a geometric mean, while at the same time the entropies are nearly regular for solutions of iodine and 1,2-dichloroethane and are slightly negative for solutions of iodine and 1,1-dichloroethane. An explanation for this situation concerns the polarity of the solvents. It is reasoned that the high polarity contributes to an increase in the energies of vaporization of the solvent without a corresponding increase in solvent power for the solute. This argument may be applied to the case at hand. Among the unusual properties of water are its high polarity, relatively large energy of vaporization, and degree of hydrogen bonding. These factors would diminish solute–solvent interactions, as evidenced by the small solubilities of molecular benzoic acid in water, and at the same time permit zero or even negative entropies to be evidenced.

[8] When X_2 is much less than X_1, the composition dependence can be neglected. Such is the case for aqueous solutions of molecular benzoic acid.

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* Present address: University of Rhode Island, Kingston, R.I.

[x] To whom inquiries should be directed.

Effect of Polysorbates on Absorption Rates of Water-Soluble, Micelle-Free Drugs Administered Intramuscularly in the Rat

HIROSHI KOBAYASHI, TAEKO NISHIMURA, KATSUHIKO OKUMURA, SHOZO MURANISHI, and HITOSHI SEZAKI ˣ

Abstract □ The effects of the polysorbates on the intramuscular absorption of water-soluble, micelle-free drugs were investigated in the rat. The presence of a low concentration of the polysorbates caused a pronounced decrease in the absorption rates of drugs, and the reduction was reflected in their plasma levels. Absorption rates of a drug in the muscle were dependent on the concentration rather than on the absolute amount of the surfactant. The delayed clearance seemed to be operative in various drugs and was caused by the polysorbates *per se*—not by the hydrolysis products.

Keyphrases □ Absorption, intramuscular—effects of surfactants, water-soluble, micelle-free drugs, absorption rates, plasma levels, rat muscle □ Surfactants—effects on intramuscular absorption rate, water-soluble, micelle-free drugs, plasma levels, rat □ Polysorbates—effects on intramuscular absorption rate, water-soluble, micelle-free drugs, plasma levels, rat □ Drugs, water soluble, micelle free—effect of surfactants on intramuscular absorption rate

Studies involving the experimental conditions and biopharmaceutical factors influencing the intramuscular absorption of drugs from parenterally administered aqueous solutions without surfactant have been reported (1–4). Despite the importance in early testing of drugs in animals, in preclinical screening, and in clinical use, little is known about the mechanism of absorption from heterogeneous parenteral preparations such as suspensions and emulsions. When discussing parenteral absorption from such dosage forms, it is important to elucidate the role of surfactants in absorption since they are often formulated in such preparations. Several reports on systemic toxicity and local irritation in general have appeared (5, 6). However, less is known about the effects of surfactants on parenteral absorption than is known about their role in absorption from the GI tract.

In this study, the effects of polysorbates on the absorption of water-soluble, micelle-free drugs were investigated in the rat to elucidate the mechanism of absorption from heterogeneous parenteral preparations.

EXPERIMENTAL

Materials—*Test Substances*—Isonicotinamide[1], methyl isonicotinate[2], isonicotinic acid[1], sulfanilamide[3], and procainamide hydrochloride[3] (JP VIII) were of analytical grade and were obtained commercially. Inulin-(carboxylic acid-^{14}C) was also purchased[4].

Additives—The nonionic surfactants used were polysorbates 20, 40, 60, and 80[5] and a polyoxyethylene derivative of hydrogenated castor oil[6]. Lauric acid (99–100%)[5] was obtained commercially. The hydrolysis products of polysorbates, sorbitan[7], and polyoxyethylene 20 sorbitan[7] also were used.

Procedure of Absorption Experiments—Male Wistar albino rats, 150–180 g, were used in all absorption experiments. The absorption experiments were almost identical to those described previously (1). An injection volume of 10 μl was delivered with a microliter syringe[8]. At various times after injection of drugs into the center of the rat thigh muscle *(musculi rectus femoris)*, the muscle was removed. The muscle was excised and then homogenized in 10 ml of distilled water, and the substance remaining at the injection site and associated area was determined. At least five rats were used at each time interval. Blood samples for plasma level analyses were obtained by cardiac puncture immediately after the muscle was removed.

Preparation of Injection Solutions—In the absorption experiments, the drugs were dissolved in isotonic NaH_2PO_4–Na_2HPO_4 buffer solution of pH 7.0. For the preparation of injection solutions having other pH values, three isotonic buffers were used: *(a)* a citric acid-Na_2HPO_4 system for acidic pH ranges, *(b)* NaH_2PO_4–Na_2HPO_4 for neutral pH ranges, and *(c)* $NaHCO_3$–Na_2CO_3 for alkaline pH ranges. Lauric acid was dissolved with equimolar sodium hydroxide in 0.1% polysorbate 80 or 1.0% polyoxyethylene derivative of hydrogenated castor oil solutions. The final pH values of the solutions injected were determined.

Drug Concentrations—In the absorption experiments, the following concentrations were maintained: 50 m*M*, in usual cases; 25 m*M*, with sulfanilamide; and 2.5 μCi/ml, with inulin-(carboxylic acid-^{14}C). In the plasma level determinations, the concentrations were: 100 m*M*, isonicotinamide; and 2.5 μCi/ml, inulin-(carboxylic acid-^{14}C).

Determination of Possible Interaction of Drugs with Polysorbate 80—Molecular sieve technique employing a cross-linked dextran gel[9] (9) was used. To prepare the swollen gel, 4 g of the dry gel was added to pH 7.0 isotonic phosphate buffer (15 ml) and the gel was allowed to swell (2 hr). The solutions containing the constant drug concentration (0.1 m*M*) in polysorbate 80 solutions of known but varying concentrations were then added along with additional phosphate buffer so that the total volume of liquid added to the gel was always 25 ml. The system was then equilibrated by shaking (1 hr) at 37°. After equilibration, the concentration of drug in the supernatant liquid was determined colorimetrically. By plotting the ratio of total (free and micellar) drug to free drug in solutions containing varying concentrations of polysorbate 80 against the polysorbate 80 concentration, the apparent distribution constants were determined.

Assay—*Isonicotinic Acid Derivatives*—Isonicotinic acid derivatives were determined by the same spectrophotometric method as described previously (1). In the case of blood samples, 2.0 ml of plasma was deproteinized by the addition of 1.5 ml of 30% trichloroacetic acid and centrifuged. After neutralization, 3.0 ml of the supernate was used for the estimation.

Procainamide Hydrochloride and Sulfanilamide—These drugs were determined spectrophotometrically for the diazotizable amines by a previously reported method (10).

Inulin-(carboxylic acid-^{14}C)—The muscle, swollen overnight in

[1] Fluka Co., Switzerland.
[2] Tokyo Kasei Kogyo Co., Japan.
[3] Dai-ichi Seiyaku Co., Japan.
[4] Japan Radio Isotope Association, Tokyo, Japan.
[5] Nihon Yushi Co., Japan.
[6] HCO-100, Nikko Chemicals Co., Japan.
[7] Nikko Chemicals Co., Japan.
[8] Hamilton.
[9] Sephadex G-25 fine grade.

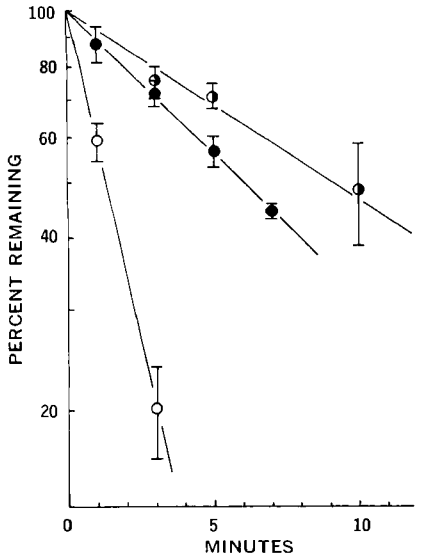

Figure 1—*Effect of polysorbate 80 on the clearance of isonicotinamide from the rat thigh muscle. Each point represents the mean value of at least five animals. Vertical bars indicate standard deviation, and straight lines are the result of least-squares regression analysis. The isonicotinamide concentration was 50 mM. Key (polysorbate 80 concentration, w/v): ○, 0.0%; ●, 1.0%; and ◑, 5.0%.*

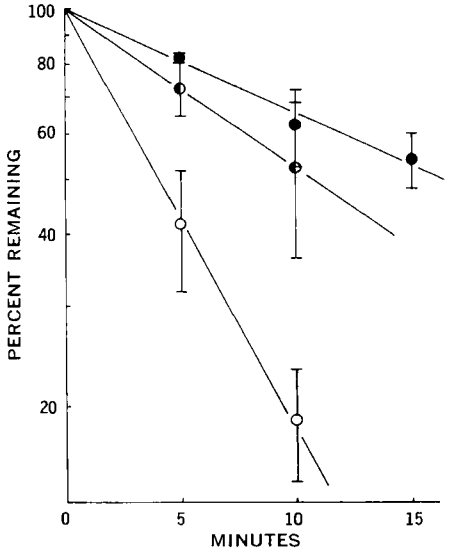

Figure 2—*Effect of polysorbate 80 on the clearance of ^{14}C-inulin from the rat thigh muscle. Each point represents the mean value of at least five animals. Vertical bars indicate standard deviation, and straight lines are the result of least-squares regression analysis. The ^{14}C-inulin concentration was 2.5 μCi/ml. Key (polysorbate 80 concentration, w/v): ○, 0.0%; ◐, 0.5%; and ●, 1.0%.*

3 ml of 0.5 *N* NaOH, was dissolved by heating to 90–95° with intermittent vigorous shaking in a glass-stoppered tube for about 2 hr. When cooled, the sample solutions were diluted to 5.0 ml with distilled water, and 2.5 ml of a 10% solution of zinc sulfate was added to precipitate the protein. After centrifugation, 0.5 ml of the supernate was taken and mixed well with 10 ml of scintillation medium[10] and 0.5 ml of 1 *N* HCl. In the case of blood samples, 1 ml plasma underwent zinc hydroxide precipitation to remove protein. After spinning, 0.5 ml clear supernate from a sample was added to 10 ml of the scintillation medium and 0.5 ml of 1 *N* HCl. The radioactivity of each sample was measured in a liquid scintillation system[11].

RESULTS AND DISCUSSION

To determine the possible effects of the polysorbates upon the kinetics of drug absorption, it was first necessary to study the time course of drug clearance from the site of injection. In earlier studies (1–4), it was found that the clearance of drugs from muscle could be described by a pseudo-first-order process. As shown in Figs. 1 and 2, clearance of isonicotinamide and ^{14}C-inulin was monoexponential, although there was a pronounced decrease in the rate of absorption. It appears that there was no change in the basic mechanism of absorption in the presence of the surfactant, and a reduced absorption might be ascribed to the effect of polysorbate 80 on some factors governing the passive diffusion of the drugs.

The results of the studies of isonicotinamide and ^{14}C-inulin plasma concentrations in rats are shown in Figs. 3 and 4, respectively. The decrease in the clearance from the site of injection is well reflected by the plasma level of the two drugs having a considerable molecular weight difference from one another (isonicotinamide, mol. wt. 122.13; inulin, mol. wt. approximately 5000). There is a pronounced decrease in the plasma concentration of isonicotinamide and ^{14}C-inulin in the presence of polysorbate 80. From this observation, together with the results shown in Figs. 1 and 2, it is evident that surfactants exert an absorption inhibitory effect through a similar mechanism not only for drugs of low molecular weight but for those of comparatively high molecular weight such as inulin.

Several formulation factors, such as the pH and the volume of injection solution, can influence the rate of intramuscular absorption from aqueous solutions. It has been shown that absorption of parenterally administered drugs can be influenced by the pH of the injected solution. Marked decreases in absorption in the acidic pH range contrary to a slight or negligible decrease in the alkaline pH range were reported (2). Figure 5 shows the effect of the pH of the injection solution on the 3-min absorption of isonicotinamide in the presence and absence of polysorbate 80. The apparent pattern of the pH–absorption profile in the presence of polysorbate 80 is somewhat similar to the one seen with the control experiment, although a general decrease in absorption is noticed

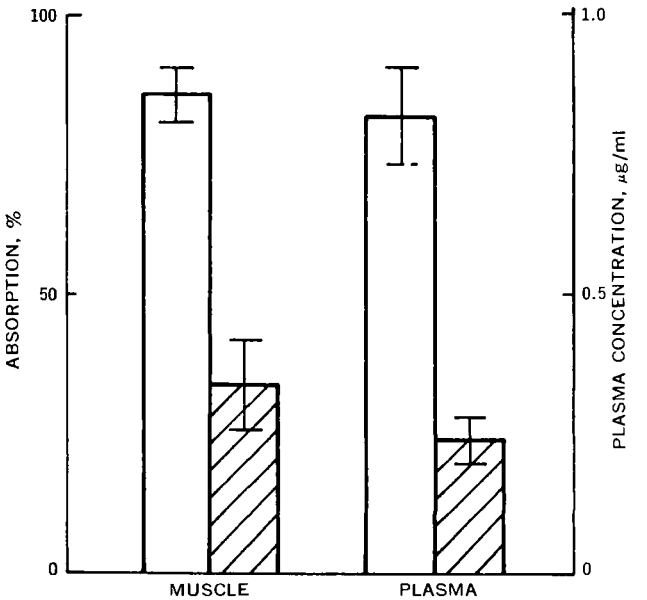

Figure 3—*Relationship between absorption and plasma concentration of isonicotinamide, and effect of polysorbate 80 at end of 3 min. Each column represents the mean of results from experiments with six animals with standard deviation. The isonicotinamide concentration was 100 mM. Key (polysorbate 80 concentration, w/v): open columns, 0.0%; and hatched columns, 5.0%.*

[10] NT-scintillation medium: a mixture of toluene (700 ml), nonylphenol-polyethoxyethanol (300 ml), and 2,5-diphenyloxazole (4 g).
[11] Beckman LS-232.

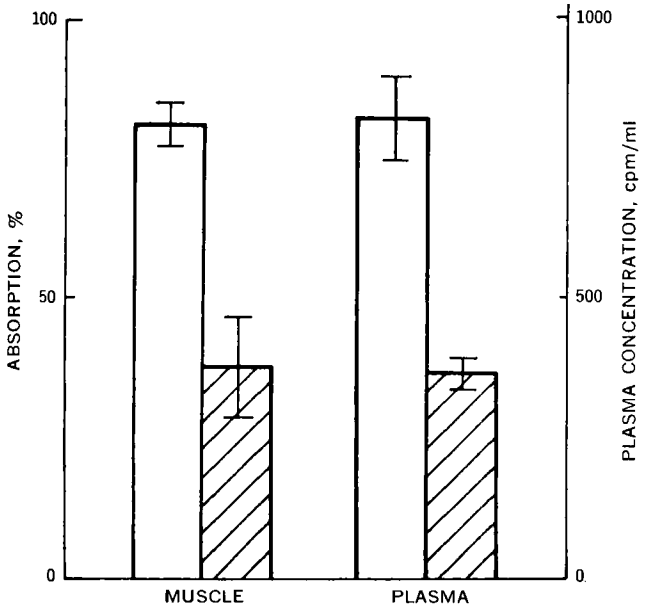

Figure 4—*Relationship between absorption and plasma concentration of ^{14}C-inulin, and effect of polysorbate 80 at end of 10 min. Each column represents the mean of results from experiments with five animals with standard deviation. The ^{14}C-inulin concentration was 2.5 μCi/ml. Key (polysorbate 80 concentration, w/v): open columns, 0.0%; and hatched columns, 1.0%.*

in the former. This supports the view that the basic kinetics of absorption in the presence of polysorbate 80 is somewhat similar to that in the absence of surfactant.

The effect on the rate of intramuscular absorption of isonicotinamide by varying the injection volume was examined in the presence of 0.1 and 1.0% polysorbate 80. For isonicotinamide (50 m*M*), the percentages of the injected dose remaining were examined for injection volumes of 5, 10, and 20 μl. The results of the 3-min absorption of isonicotinamide are shown in Table I. For comparison, the control values obtained in the absence of the surfactant are listed. Within the range of volumes investigated, the injection volumes do not particularly influence absorption rate of isonicotinamide. In contrast, the absorption rate depends markedly upon the concentration of the surfactant.

The possibility of the delayed clearance of drug in the presence of polysorbate 80 regardless of the physicochemical characteristics

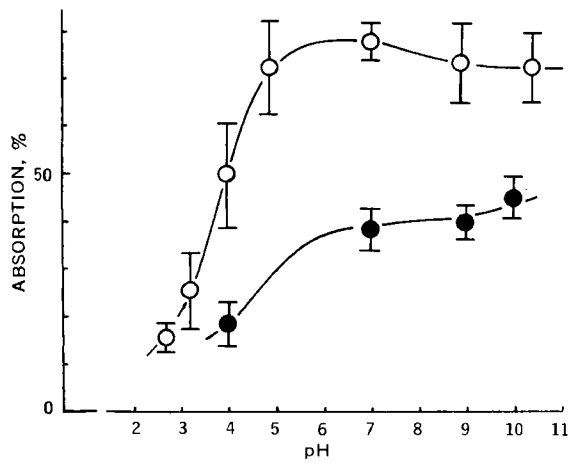

Figure 5—*Effect of pH and polysorbate 80 on the absorption of isonicotinamide at end of 3 min. Each point represents the mean values of at least five animals. Vertical bars indicate standard deviation. The isonicotinamide concentration was 50 mM. Key (polysorbate 80 concentration, w/v): ○, 0.0%; and ●, 1.0%.*

Table I—Effect of Injection Volume on the Absorption of Isonicotinamide in the Presence of Polysorbate 80[a]

Concentration of Polysorbate 80, % w/v	Absorption[b], % Injection Volume, μl		
	5	10	20
0	74.0 ± 7.8 (5)	78.0 ± 4.1 (5)	80.2 ± 6.4 (5)
0.1	52.9 ± 6.8 (4)	51.1 ± 4.9 (5)	50.8 ± 5.8 (3)
1.0	30.8 ± 12.5 (8)	28.3 ± 3.8 (9)	31.0 ± 5.9 (5)

[a] Values represent the mean ± *SD*. Figures in parentheses are the number of animals. Concentration of isonicotinamide was 50 m*M*. [b] Percent absorption was determined for 3 min.

of the drugs was tested. For that purpose, isonicotinic acid and procainamide hydrochloride were chosen as charge-bearing compounds at the pH of the absorption experiment. For nonionic drugs, three types were selected according to their relative magnitude of lipid solubility, namely isonicotinamide and ^{14}C-inulin as the least soluble, sulfanilamide as intermediate, and methyl isonicotinate as the most soluble. Figure 6 shows the results obtained through the molecular sieve technique. As can be seen, the ratios of total to free isonicotinamide, isonicotinic acid, and procainamide hydrochloride are approximately unity within the concentration range of surfactant, which implies that these drugs also are free from micellar interaction. In the case of methyl isonicotinate and sulfanilamide, however, some interaction was observed. Semilogarithmic plots of the residual amounts of drugs *versus* time yielded straight lines for solutions containing up to 10.0% of polysorbate 80. The absorption rate constants obtained are summarized in Table II. As is evident from these results, absorption of this rather diverse group of drugs is decreased as the concentration of polysorbate 80 is increased. To compare the magnitude of the delayed clearance effect on a variety of drugs, a plot of the absorption rate constant ratio *versus* the log of surfactant concentration was constructed. Absorption rate constants are expressed as a ratio relative to the rate constant observed with injection solutions containing only drug and buffer. As illustrated in Fig. 7, absorption of a variety of drugs was reduced to an almost similar extent independent of their ionogenic nature, lipophilicity, molecular size, and pharmacological class. In the case of muscular absorption of drugs, it has been reported that diffusion through pores of the capillary wall is dominant compared with the penetration process through the vascular endothelial cells, unlike the GI absorption (8, 11, 12). Also it has been pointed out that in contrast to the GI tract, absorption from muscle could not be related to the pKa's and lipid solubilities of the substances (1). Nonselectivity in the absorption-decreasing action of the polysorbate may support the foregoing view. In the following experiments, isonicotinamide was selected as a model drug because of its negligible micellar interaction as well as its well-documented absorption characteristics (1–4).

Further experiments were conducted in which the absorption inhibitory effect of other polysorbates was investigated. As in the case of polysorbate 80, first-order kinetics were observed in the absorption of isonicotinamide in the presence of polysorbates. The results of the study with polysorbate 20, 40, and 60 (Table III) are in good agreement with the results obtained with polysorbate 80. This finding may be explained by the fact that these polysorbates share the common polyol moiety and only the fatty acid portions

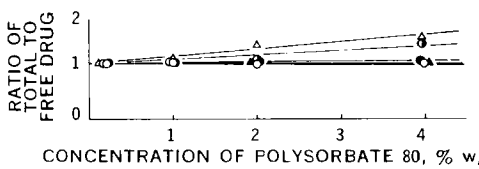

Figure 6—*Ratio of total to free drug containing various concentrations of polysorbate 80 as determined by a molecular sieve technique. Key: ●, isonicotinamide; ◑, methyl isonicotinate; ○, isonicotinic acid; ▲, procainamide hydrochloride; and △, sulfanilamide.*

Table II—Effect of Polysorbate 80 on the Absorption Rate Constants[a] of Drugs

Drug	Absorption Rate Constant, min^{-1} Concentration of Polysorbate 80, % w/v							
	0	0.01	0.05	0.1	0.5	1.0	5.0	10.0
Isonicotinamide	0.537	0.483	0.252	0.240	0.147	0.116	0.074	0.054
Methyl isonicotinate	0.613	—	—	0.256	—	0.103	—	—
Isonicotinic acid	0.463	0.430	0.220	0.140	0.100	0.073	0.038	—
Sulfanilamide	0.230	—	0.097	0.040	—	0.030	0.026	—
Procainamide hydrochloride	0.170	—	0.093	0.073	0.024	—	—	—
^{14}C-Inulin	0.171	—	—	0.065	—	0.042	—	—

[a] Absorption rate constants were calculated from clearance curves.

Table III—Effect of Polysorbates on Absorption of Isonicotinamide[a]

Concentration of Polysorbate, % w/v	Absorption, %			
	Polysorbate 80	Polysorbate 20	Polysorbate 40	Polysorbate 60
0.01	76.5 ± 7.8 (6)	80.9 ± 5.8 (5) N.S.	80.7 ± 6.8 (9) N.S.	85.1 ± 2.6 (5) $p < 0.05$
0.05	52.9 ± 4.8 (6)	51.1 ± 4.1 (5) N.S.	52.0 ± 2.2 (4) N.S.	60.1 ± 4.7 (7) $p < 0.05$
0.1	51.1 ± 4.9 (5)	34.5 ± 4.8 (7) $p < 0.01$	48.8 ± 6.2 (8) N.S.	57.8 ± 6.6 (12) N.S.
0.5	35.6 ± 3.2 (4)	17.5 ± 2.6 (5) $p < 0.001$	35.3 ± 5.7 (6) N.S.	39.7 ± 7.6 (3) N.S.
1.0	43.4 ± 3.5* (4)	12.3 ± 1.4* (4) $p < 0.001$	40.3 ± 8.9* (5) N.S.	38.8 ± 3.6* (4) N.S.
5.0	29.1 ± 3.7* (7)	12.2 ± 8.0* (3) $p < 0.01$	30.8 ± 10.1* (3) N.S.	31.8 ± 8.6* (5) N.S.
10.0	23.9 ± 4.5* (5)			

[a] Percent absorption was determined for 3 min except for items marked by asterisk which indicates 5 min. Values represent the mean ± *SD*. Figures in parentheses are the number of animals. A Student *t* test was performed among the same concentration against the value of polysorbate 80. N.S. = not significant ($p > 0.1$).

differ from each other rather than the molecular weight, hydrophilic–lipophilic balance, and other physicochemical properties. The more pronounced effect exerted by polysorbate 20 in concentrations above 0.1% could be attributed to its tendency to release the endogenous biogenic compounds or to the self-depression of absorption caused by histamine (13). In the case of polysorbate 60, at concentrations of 0.05% and below, the absorption inhibitory effect was somewhat lessened but the cause was not clear.

The polysorbates are readily hydrolyzed in the GI tract to fatty acid and polyol (polyoxyethylene 20 sorbitan) (14). Levy and Anello (15) noted the permeability-modifying effect of polysorbate hydrolysis products on secobarbital absorption in goldfish. Little is known about the biotransformation of these compounds in the muscle. But it is of interest to determine the effect of such hydrolysis products of polysorbates on the intramuscular absorption, since the accidental presence of any of the hydrolysis products as contaminants may be expected. The results of studies with lauric acid and with sorbitan and polyoxyethylene 20 sorbitan are shown in Figs. 8 and 9, respectively. Various concentrations of lauric acid, the fatty acid component of polysorbate 20, the surfactant that showed the strongest absorption-inhibiting tendency, were solubilized with 0.1% polysorbate 80 and 1.0% polyoxyethylene derivative of hydrogenated castor oil. As is evident from Fig. 8, the inhibitory effect of lauric acid on the 3-min absorption of isonicotinamide was almost negligible. In the case of sorbitan, no delayed absorption of isonicotinamide was observed even at a concentration as high as 5.0%.

The polyol moiety, at a concentration higher than 2.0%, decreased significantly the absorption of isonicotinamide, but the pattern of the curve was somewhat different from that of polysorbate 80 which exerted a much more pronounced absorption-inhibiting effect at lower concentrations. The contribution due to the possible presence of hydrolysis products in polysorbates is negligible because they are considered small in quantity. These observa-

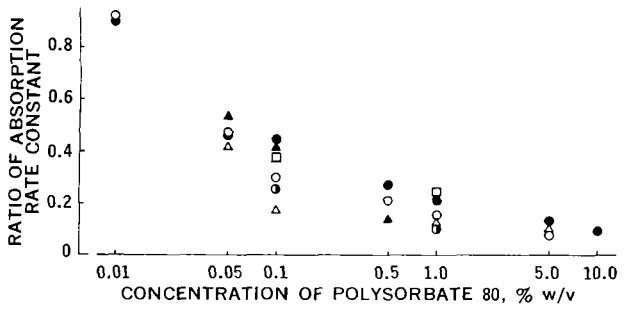

Figure 7—*Relationship between ratio of absorption rate constant and concentration of polysorbate 80. Ratios of absorption rate constants are expressed as a ratio relative to that observed with injection solutions containing only drug and buffer. There are no significant differences by the variance analysis in the ratio of absorption rate constant among the drugs at the polysorbate 80 concentrations of 0.01, 0.05, and 5.0% [0.01%, $F_{calc} = 1.85 < F(1,7; 0.05) = 5.59$; 0.05%, $F_{calc} = 0.68 < F(3,19; 0.05) = 3.13$; 5.0%, $F_{calc} = 2.06 < F(2,15; 0.05) = 3.68$]. Key: ●, isonicotinamide; ◑, methyl isonicotinate; ○, isonicotinic acid; △, sulfanilamide; ▲, procaine amide hydrochloride; and □, ^{14}C-inulin.*

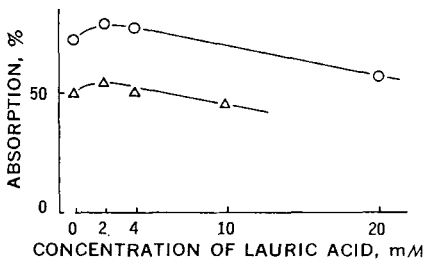

Figure 8—*Effect of lauric acid on the absorption of isonicotinamide at the end of 3 min. Each point represents the mean value of at least five animals. Key: ○, with 1.0% (w/v) polyoxyethylene derivative of hydrogenated castor oil; and △, with 0.1% (w/v) polysorbate 80.*

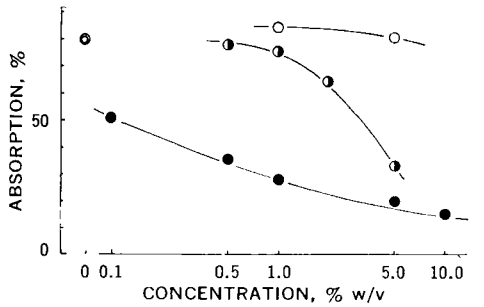

Figure 9—*Effect of sorbitan, polyoxyethylene 20 sorbitan, and polysorbate 80 on the absorption of isonicotinamide at the end of 3 min. Each point represents the mean value of at least five animals. Key: ⓪, control; ○, sorbitan; ◑, polyoxyethylene 20 sorbitan; and ●, polysorbate 80.*

tions suggest the possibility that the delayed absorption of a drug is caused by the surfactant *per se* and not by its hydrolysis products.

The results in this study indicate that: *(a)* a low concentration of the polysorbates dramatically reduces the absorption of water-soluble, micelle-free drugs and such reduction is reflected on the plasma levels of drugs; *(b)* the relative absorption rate of a drug solution in the muscle is dependent on the surfactant concentration rather than on the absolute amount; and *(c)* the delayed clearance seems to be operative in a variety of drugs and is caused by the polysorbates *per se*. The exact cause of the phenomenon is uncertain at present. Several mechanisms could operate alone or in combination to bring about the decrease of absorption observed in the presence of the polysorbates. Previous investigations concerning the absorption and viscosity of aqueous injection solutions (3) and on the membrane transport phenomena (16) in the presence of a surfactant under negligible micellar interaction ruled out the possibility that the reduction of the rate of absorption is caused mainly by the decreased diffusion rate of drugs in the injected solution containing surfactant. There is a possibility that the biological events that depend on other complex and interrelated events, which cannot be adequately explained by a simple physicochemical concept, influence the absorption.

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ˣ To whom inquiries should be directed.

PHARMACEUTICAL ANALYSIS

GLC Analysis of Ergonovine Maleate

DAVID L. SONDACK

Abstract □ A method is described for the quantitative determination of ergonovine maleate by GLC. Data are presented to show that this method provides the quantitative and semiquantitative results of the USP colorimetric assay and TLC in a single determination. Favorable quantitation is achieved by using brucine as internal standard. The sample is dried in the dark, derivatized with *N*-trimethylsilyldiethylamine and *N*-trimethylsilylimidazole in pyridine, and chromatographed on a column containing a nonpolar methyl silicone liquid phase. Degradation of the sample by preanalytical preparation (pH changes, air, heat, and light) are thereby minimized.

Keyphrases □ Ergonovine maleate—GLC analysis □ GLC—analysis, ergonovine maleate

It is generally recognized that the chemical purity of ergonovine (I) maleate is affected by oxygen, light, temperature, and extremes in pH (1–7). The chemical nature of the degradation products (6, 7) may cause falsely high values when assays are performed by the colorimetric method described in USP XVIII

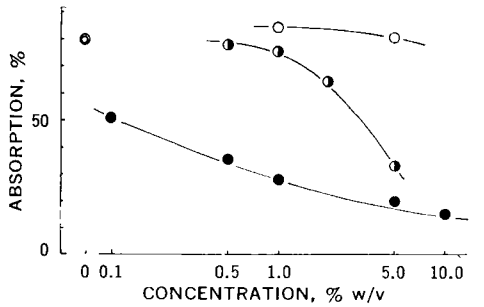

Figure 9—*Effect of sorbitan, polyoxyethylene 20 sorbitan, and polysorbate 80 on the absorption of isonicotinamide at the end of 3 min. Each point represents the mean value of at least five animals. Key: ⓪, control; ○, sorbitan; ◑, polyoxyethylene 20 sorbitan; and ●, polysorbate 80.*

tions suggest the possibility that the delayed absorption of a drug is caused by the surfactant *per se* and not by its hydrolysis products.

The results in this study indicate that: *(a)* a low concentration of the polysorbates dramatically reduces the absorption of water-soluble, micelle-free drugs and such reduction is reflected on the plasma levels of drugs; *(b)* the relative absorption rate of a drug solution in the muscle is dependent on the surfactant concentration rather than on the absolute amount; and *(c)* the delayed clearance seems to be operative in a variety of drugs and is caused by the polysorbates *per se*. The exact cause of the phenomenon is uncertain at present. Several mechanisms could operate alone or in combination to bring about the decrease of absorption observed in the presence of the polysorbates. Previous investigations concerning the absorption and viscosity of aqueous injection solutions (3) and on the membrane transport phenomena (16) in the presence of a surfactant under negligible micellar interaction ruled out the possibility that the reduction of the rate of absorption is caused mainly by the decreased diffusion rate of drugs in the injected solution containing surfactant. There is a possibility that the biological events that depend on other complex and interrelated events, which cannot be adequately explained by a simple physicochemical concept, influence the absorption.

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ˣ To whom inquiries should be directed.

PHARMACEUTICAL ANALYSIS

GLC Analysis of Ergonovine Maleate

DAVID L. SONDACK

Abstract □ A method is described for the quantitative determination of ergonovine maleate by GLC. Data are presented to show that this method provides the quantitative and semiquantitative results of the USP colorimetric assay and TLC in a single determination. Favorable quantitation is achieved by using brucine as internal standard. The sample is dried in the dark, derivatized with *N*-trimethylsilyldiethylamine and *N*-trimethylsilylimidazole in pyridine, and chromatographed on a column containing a nonpolar methyl silicone liquid phase. Degradation of the sample by preanalytical preparation (pH changes, air, heat, and light) are thereby minimized.

Keyphrases □ Ergonovine maleate—GLC analysis □ GLC—analysis, ergonovine maleate

It is generally recognized that the chemical purity of ergonovine (I) maleate is affected by oxygen, light, temperature, and extremes in pH (1–7). The chemical nature of the degradation products (6, 7) may cause falsely high values when assays are performed by the colorimetric method described in USP XVIII

Table I—Quantitative Analysis of Ergonovine Maleate by GLC

	Label	Range Found	*RSD*	*RE*[a]
Injectables[b]				
Ampuls Disposable syringe	0.2 mg/ml	0.198–0.202	±1.0%	−1%
Tablets[c]	0.2 mg/tablet	0.197–0.209	±3.0%	−2%

[a] Determined by the method of standard addition. [b] Ergotrate Maleate ampuls and disposable syringe, Lilly. [c] Ergotrate Maleate tablets, Lilly.

(8). Therefore, the sample must also be examined in a semiquantitative way by TLC to estimate the amount of degradation.

Analytical methods based on paper chromatography (9–11), TLC (12–14), gel permeation chromatography (15), ion-exchange chromatography (16), and thin-layer electrophoresis (17) have been proposed. These methods either are unable to resolve interferences or require excessive handling conducive to degradation during sample preparation.

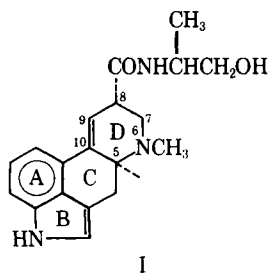
GLC has been used for the identification of narcotic drugs (18, 19) and alkaloids (20–28). A quantitative procedure for the measurement of lysergic acid diethylamide (lysergide) used ergonovine, which is lysergic acid 2-aminopropanolamide, as an internal standard (21). The method required catalytic hydrogenation of the two compounds prior to analysis and was found to be relatively insensitive and irreproducible (22). GLC of lysergic acid derivatives has been attempted, but only the amide and diethylamide produced unique peaks (28). Monosubstituted amides were pyrolyzed to the amide. Attempts to produce stable volatile derivatives were not successful.

This report describes the quantitative GLC analysis of ergonovine maleate using a trimethylsilyl derivative. The method provides the quantitative and semiquantitative results obtained by the two USP procedures in a single determination. Furthermore, evidence is presented that excessive handling, as by multistep extractions, causes degradation and premature rejection of pharmaceutically satisfactory material.

EXPERIMENTAL

Equipment—A gas chromatograph[1] equipped with a flame-ionization detector was used. The detector signal was fed to a computer[2] for peak integration and to a 1-mv recorder[3] with a chart speed of 38.1 cm (15 in.)/hr and a 1-sec full-scale response. Samples were injected with a 10-μl syringe[4].

Materials—Helium was used as a carrier gas, and electrolytic hydrogen and oxygen were used in the detector. The stationary phase was 1% OV-1 on Gas Chrom Q[5], 80–100 mesh, packed in borosilicate glass columns, 1.2 m × 0.64 cm o.d. All chemicals used were reagent grade or the best quality available.



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Operating Conditions—The column was operated isothermally at 260° with the detector block and injection port at 280°. The helium flow rate was 55 ml/min with an inlet pressure of 40 psi. The electrometer range was 10 with an attenuation of 16. Sample injections of 1 μl were made.

Preparation of Liquid Formulations—*Standard Solutions*—Solutions of ergonovine maleate reference standard, 0.2 mg/ml, were prepared by dissolving the solid in the internal standard solution. Standards were prepared daily and used immediately. One-milliliter portions were added by pipet to screw-cap vials, fitted with Teflon septa, and freeze dried.

Sample Solutions—One-milliliter aliquots, 0.2 mg/ml, of ergonovine maleate[6] were added by pipet to screw-cap vials. An equal volume of internal standard was added and the samples were freeze dried.

Internal Standard Solutions—Brucine was selected for use because of its known retention time under these GLC conditions (29) and its water solubility. Concentrations of 0.2 mg/ml were used.

Preparation of Tablet Formulations—*Standard Solutions*—Solutions of ergonovine maleate reference standard, 0.2 mg/ml, were prepared by dissolving the solid in pyridine–water (2:1), previously prepared and cooled to room temperature. One-milliliter portions were added by pipet to screw-cap vials, fitted with Teflon septa. Two milliliters of internal standard, in the same solvent, was added and the solution was evaporated under nitrogen without heat and protected from light.

Sample Solutions—A single tablet of ergonovine maleate[6] or its

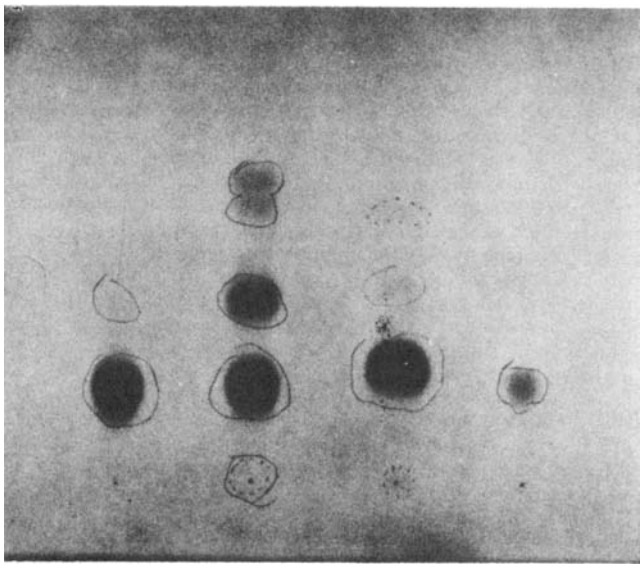

Figure 1—*TLC of ergonovine maleate performed in the system described in Ref. 8. Key (left to right): Lane 1, standard; Lane 2, sample extracted as per Ref. 8; Lane 3, sample lyophilized; and Lane 4, standard (3:100 dilution).*

[1] Hewlett-Packard, model 402.
[2] IBM 1800.
[3] Honeywell Electronik 16.
[4] Hamilton No. 701.
[5] Applied Science Laboratories, State College, Pa.
[6] Ergotrate Maleate (Eli Lilly and Co.), 0.2 mg/ml and 0.2 mg/tablet.

equivalent weight of a finely ground composite was placed in a centrifuge tube. Two milliliters of internal standard in pyridine–water solution was added by pipet. The tablet disintegrated readily and was intermittently agitated for 20 min. The solution was then centrifuged at 400 rpm for 5 min, and the clear supernate was decanted into screw-cap vials fitted with Teflon septa. The solvent was evaporated under nitrogen as previously described.

Internal Standard Solution—Pyridine–water (2:1), containing 0.1 mg/ml of brucine, was used.

Derivatization—Approximately 75 μl of *N*-trimethylsilyldiethylamine (II), 150 μl of dry pyridine, and 100 μl of *N*-trimethylsilylimidazole (III) were added to each vial through the Teflon septum using a tuberculin syringe. The liquid was then brought into contact with all of the glass surface inside the vial. Aliquots of 1 μl were taken for GLC.

Calculations—For each chromatogram, the ratio *(R)* of the area of the ergonovine peak to that of the internal standard was determined. Then the concentration of ergonovine maleate per milliliter of liquid formulations is given by:

$$\text{milligrams ergonovine maleate per milliliter} = \frac{R\ \text{sample}}{R\ \text{standard}} \times \frac{\text{milligrams standard}}{\text{milliliter standard solution}} \times \text{purity of standard} \quad \text{(Eq. 1)}$$

and:

$$\text{milligrams ergonovine maleate per tablet} = \frac{R\ \text{sample}}{R\ \text{standard}} \times \frac{\text{milligrams standard} \times \text{purity}}{\text{milliliter standard solution}} \times \frac{\text{average tablet weight}}{\text{milligram sample}} \quad \text{(Eq. 2)}$$

Colorimetric Analysis and TLC—Standards and samples were prepared and analyzed by the methods described in the USP (8).

RESULTS AND DISCUSSION

Although excellent reproducibility can be obtained with the colorimetric assay for ergonovine maleate (8), TLC repeatedly gives erratic results. To investigate this, two samples were taken. One was prepared by the USP method (8), and the other was freeze dried to eliminate the extraction procedure entirely. The latter sample was redissolved in 100 μl of ethanol and spotted for TLC along with the first sample and a standard (Fig. 1). The results clearly showed that sufficient degradation had occurred during the USP procedure to cause rejection of the material for pharmaceutical use. The freeze-dried sample showed no such effect. The USP procedure was repeated with modifications of pH, rapidity, temperature, solvent, exposure to light, and gaseous atmosphere. Each gave evidence of excessive degradation.

N,O-Bis(trimethylsilyl)acetamide was reported (30) to derivatize hydroxyl, indole, and perhaps amide groups. However, this reagent did not give a unique peak with ergonovine maleate on GLC. Compound III was reported to be a stronger reagent (31). This reagent and also II gave single peaks with ergonovine maleate. The latter reagent, however, dissolved the sample very slowly. Experimentation demonstrated that the most consistent results could be obtained using a mixture of the two reagents in pyridine as described under *Experimental*.

Degraded samples, either aged or extracted by the USP method, were treated with the silylating reagents and subjected to GLC (Fig. 2). Three peaks were clearly observed. Authentic samples of some identified degradation products (5–7) of ergonovine maleate were also subjected to derivatization and GLC. In this way, the two smaller peaks in the chromatogram can be tentatively identified as ergonovinine (peak A, Fig. 2) and lumiergonovine I (peak B, Fig. 2). Peak A was also obtained from the degradation spot observed on TLC. Ergonovinine is epimeric to ergonovine (I) at C-8. This position is made particularly sensitive to pH changes by its proximity to the carboxamide on one side and to the double bond on the other. Lumiergonovine I is the result of hydration of the 9,10-double bond with the hydroxyl in one of two possible configurations at C-10.

Attempts to obtain GLC–mass spectra of the observed peaks were unsuccessful. When the peaks were trapped and submitted to TLC, they had almost completely decomposed (presumably due to the heat and light at the column exit). The following is evidence for tentative identification of the observed peak as *N*(amide),*N*(indole),*O*-tris(trimethylsilyl)ergonovine. No lysergamide peak was observed in comparison to an authentic sample, derivatized and underivatized. Thus, the amide nitrogen is doubly substituted since underivatized monosubstituted lysergamides pyrolyze (28).

It is a certainty that the hydroxyl has been derivatized since both II and III are reactive toward alcohols (31). Treating a sample of ergonovine with II alone produced a peak with a shorter retention time than that produced by III. Compound III is reactive toward indole nitrogen (31). Therefore, the derivative with II is *N*-(amide),*O*-bis(trimethylsilyl)ergonovine and that with III is *N*-(amide),*N*(indole),*O*-tris(trimethylsilyl)ergonovine.

Solutions of differing ergonovine concentrations were prepared to establish the linearity of the procedure. The extrapolated results passed through the origin. Replicate standards and samples provided a residual standard deviation of ±1.0% and a relative error of less than 1% for injectables.

Tablets presented an additional problem of excipients. To separate ergonovine maleate from the solid, the tablet was suspended in 1 ml of aqueous internal standard and centrifuged and the

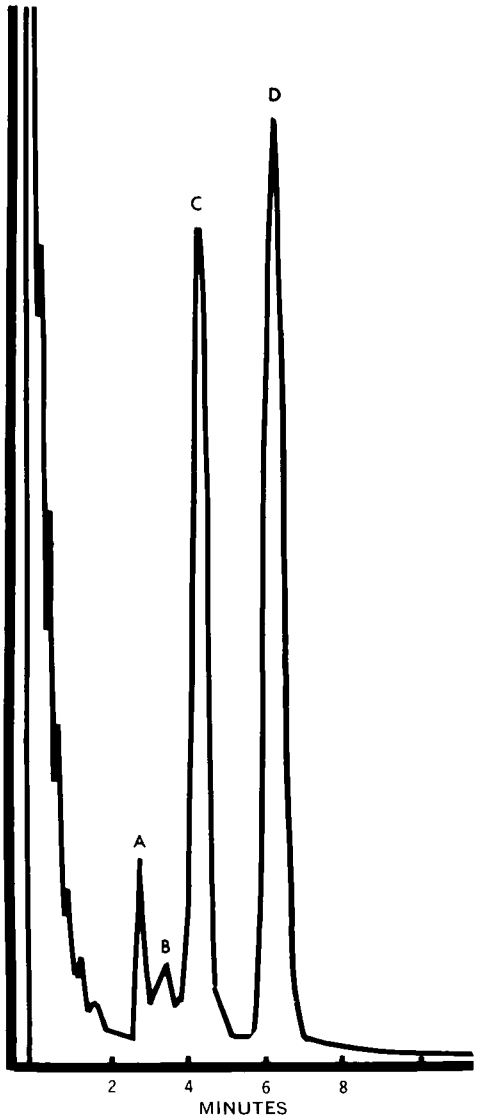

Figure 2—*GLC of derivatized ergonovine maleate (for conditions see text). Key: Peak A, derivatized ergonovinine; Peak B, derivatized lumiergonovine I; Peak C, derivatized ergonovine; and Peak D, brucine (internal standard).*

supernate was freeze dried. The residue was treated with silylating reagents but a portion remained insoluble. GLC of aliquots of the supernate did not give reproducible results. There was evidence of a large amount of degradation, thought to be due to the concentration of excipient potassium acid tartrate in the aqueous suspension. Alcohol–water mixtures were tried with poor results. Pyridine–water mixtures afforded the best results and the solution could be evaporated under nitrogen. There was little evidence of excessive degradation during sample workup, and the residue left after evaporation of the supernate was almost completely soluble in the silylating reagents. The small amount of insoluble material did not interfere with sampling for GLC. Replicate samples provided a residual standard deviation of ±3 and −2% relative error (Table I).

Some degradation always occurred during the sample workup. The sensitivity of the compound makes this unavoidable. As demonstrated in Fig. 1, however, the amount of degradation was small, and it is advisable to treat the samples and standard in the same way [unlike the USP method (8)] to compensate. This was also demonstrated by chromatography of derivatized reference standard weighed directly into the screw-cap vial and silylated. Peaks A and B (Fig. 2) were absent, while both were present in standards prepared and treated as the samples. The respective size of the peaks appeared to correlate with the age of the sample, peak A increasing faster than peak B, but this aspect was not pursued further.

This method should prove useful in the analysis of preparations of ergonovine and of methylergonovine which has a slightly longer retention time. The elimination of extraction procedures minimizes difficulties encountered due to degradation during analysis.

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Analysis of Riboflavin in Commercial Multivitamin Preparations by High-Speed Liquid Chromatography

D. WITTMER and W. G. HANEY, Jr.[x]

Abstract □ A high-speed liquid chromatographic procedure for analysis of riboflavin in multivitamin preparations was developed and evaluated. The method utilizes 2,2′-diphenic acid as the internal standard and 254-nm detection of separated constituents. This procedure was compared with that of the USP with respect to accuracy in the analysis of riboflavin in the presence of its photolytic degradation products. In addition, the procedure was applied to the analysis of several commercial multivitamin products containing riboflavin, with reproducible results ranging from 27.23 to 103.45% of the labeled amount of riboflavin.

Keyphrases □ Riboflavin—high-speed liquid chromatographic analysis in commercial multivitamin preparations, compared to official method □ High-speed liquid chromatography—analysis of riboflavin in commercial multivitamin preparations, compared to official method □ Vitamins—high-speed liquid chromatographic analysis of riboflavin in commercial multivitamin preparations

Determination of riboflavin by chemical methods has been of considerable interest to the pharmaceutical analyst. While microbiological methods of analysis have been available for some time (1), it was not until fluorometric analyses of riboflavin were developed (2) that chemical methods approached microbiological methods in sensitivity and selectivity. The fluorometric procedure has now been refined to such an apparently acceptable point that it has been adopted, in about the same form, by the USP (3), the BP (4), and the Association of Official Analytical Chemists (5).

Recently, however, Ismaiel and Yassa (6) published evidence indicating that fluorometric procedures are unsuitable for determination of riboflavin, since the degradation products of riboflavin appear to interfere with the analysis. With most official drug products, the possibility that degradation products interfere with assay results would not be critical, since other monograph requirements (description, identification, *etc.*) would probably suffice to eliminate those products having significant degradation. The complex nature of such products as decavitamin capsules USP (7), however, prohibits use of such supplementary tests, and the reliability of the assay procedure is particularly important in establishing drug quality.

As a result, a procedure was developed for the analysis of riboflavin using high-speed liquid chromatography. The effectiveness of this procedure was evaluated in determining riboflavin in the presence of its degradation products and other vitamins. Finally, this procedure was applied to the determination of riboflavin in several commercial multivitamin products and the results were compared to those obtained using a spectrophotofluorometric modification of the currently official procedure (3).

Table I—Analysis of Contrived Mixtures Containing Riboflavin

Sample	Riboflavin Added, mg	Riboflavin Found, mg	Error, %	SD^a, %
1	0.80	0.814	1.72	2.14
2	1.50	1.473	1.83	2.02
3	2.50	2.480	0.83	1.74
4	3.50	3.42	2.33	2.46
5	7.50[b]	7.41	1.20	1.47
6	10.00[b]	9.81	1.93	2.36
Mean			1.64	2.03

[a] $n = 10$. [b] High potency modification.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with a 254-nm detector was used in the study. A stainless steel column [0.31 cm (0.12 in.) i.d. and 1.22 m (4 ft) long] was packed with liquid chromatography grade silicic acid[2]. A hot-block attached to a circulating water bath heated the column. Peak areas were determined using an electronic digital integrator[3] and a planimeter. Injections into the pressurized system were made with syringes[4] (10 μl) fitted with special plunger tips. A spectrophotofluorometer[5] with excitation and emission wavelengths of 442 and 564 nm, respectively, and slit width of 0.5 mm was used for supporting studies.

Chromatographic Conditions—The chromatographic solvent was a mixture of chloroform, methanol, and acetate buffer, pH 4 (60:28:4.5 v/v). The column temperature was 42°, and the rate of flow was 0.95 ml/min. A pressure of 105.43 kg/cm^2 (1500 lb/in.^{2}) was necessary to maintain this rate of flow.

Materials and Reagents—The chromatographic internal standard was 2,2′-diphenic acid[6]. USP Riboflavin Reference Standard[7] was treated according to official procedures (3). All other reagents and solvents were of the best grade commercially available and were used without further purification.

Solutions—*Acetate Buffer Solution, pH 4*—Dissolve sodium acetate (25.0 g) in distilled water (400 ml) and adjust to pH 4 with acetic acid. Dilute the solution with distilled water to a final volume of 500 ml.

Riboflavin Standard Solution—Place 50.0 mg of USP Riboflavin Reference Standard in a light-resistant volumetric flask (100 ml) containing acetate buffer, pH 4 (60 ml). Heat the mixture until the riboflavin has dissolved, cool to room temperature, and dilute to 100 ml with acetate buffer, pH 4.

Internal Standard Solution—Dissolve the internal standard (375.0 mg) in methanol and dilute with methanol to a final volume of 250 ml.

Preparation of Standard Curve—Add internal standard solution (10.0 ml) to each of seven light-resistant containers (100 ml). To each container add the riboflavin standard solution in amounts from 1.0 to 7.0 ml in 1.0-ml increments. Then add chloroform (50 ml) and methanol (15 ml), shake the solution, and inject 4–8 μl into the chromatographic system. The ratio of areas

[1] ALC 202, Waters Associates.
[2] LiChrosorb SI 60, E. Merck.
[3] Varian model 475.
[4] Pierce Chemical Co., Rockford, Ill.
[5] SPF 125, Aminco-Bowman.
[6] Baker-Analyzed, J. T. Baker Co.
[7] USP Reference Standards, Rockville, MD 20852

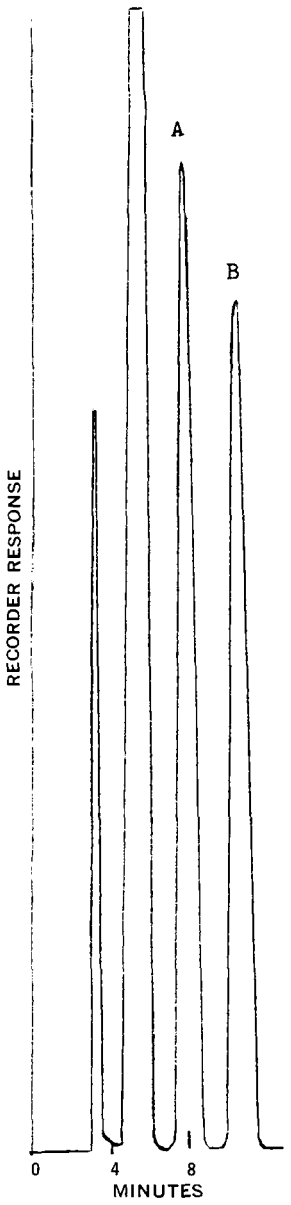

Figure 1—*Chromatographic trace of a multivitamin preparation. Key: A, riboflavin; and B, internal standard.*

under the peaks corresponding to riboflavin and the internal standard is plotted against the quantity of riboflavin in the solution.

Analysis of Riboflavin in Vitamin Products—Place 1 dosage unit in a light-resistant container containing acetate buffer, pH 4 (5 ml). Heat the mixture to boiling for 1 min, shake, and allow to cool to room temperature. If the dosage unit does not appear to be homogeneously distributed, repeat the procedure. Add internal standard solution (10.0 ml), chloroform (50 ml), and methanol (15 ml). Shake the resulting mixture and inject 4–8 μl into the chromatographic system. Calculate the ratio of the area under the peaks corresponding to riboflavin and the internal standard and determine the quantity of riboflavin in the sample graphically.

For high potency preparations (those containing more than 5 mg of riboflavin per dosage unit), the procedure is modified by the use of an additional 15.0 ml of internal standard solution and the omission of methanol.

In addition, vitamin products were analyzed for riboflavin content according to the USP procedure (3) using a spectrophotofluorometer.

Analysis of Riboflavin in Presence of Its Photolytic Degradation Products—Riboflavin standard solution (40 ml) was placed in a clear Pyrex volumetric flask and exposed to normal laboratory artificial lighting. Aliquots were taken for analysis at 20-min intervals, and the apparent riboflavin content was determined by the chromatographic method and the fluorometric method.

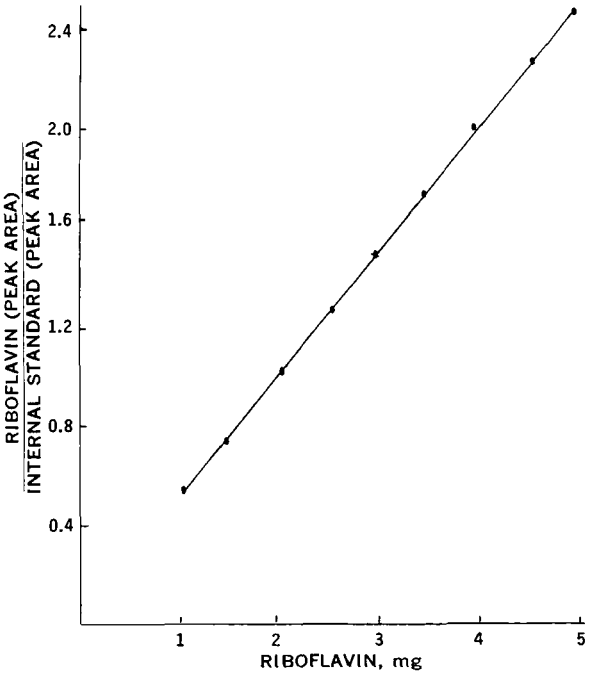

Figure 2—*Standard curve for the chromatographic analysis of riboflavin.*

Separation of Riboflavin Degradation Products by TLC—Riboflavin solutions that had been exposed to light were applied to the system developed by Ismaiel and Yassa (6). Spots on the developed chromatogram were eluted with acetate buffer, pH 4.

RESULTS AND DISCUSSION

The solvent system used in this study is a modification of one used previously in TLC studies of riboflavin (6). With this solvent system, riboflavin elutes after 8 min with a column efficiency of 750 theoretical plates. It is well resolved from other vitamins and from common constituents of vitamin preparations (Fig. 1). In the products studied, thiamine elutes closest to riboflavin (relative retention volume 0.69), but the thiamine content must exceed the riboflavin content by 60-fold before the two peaks are incompletely resolved.

Linearity of the response over the range studied is shown in Fig. 2. Standard curves were prepared daily for 10 days, and the standard deviation of the slope of the curve was found to be 1.41%. The average correlation coefficient was 0.993, and the average y-intercept value was −0.012. This indicates that the procedure is amenable to use of a single-point standard.

Accuracy and precision of the procedure were studied using contrived mixtures (Table I). Each value represents the mean of two determinations on each of 5 consecutive days. In contrast to the precision shown here, the coefficient of variation of the fluoro-

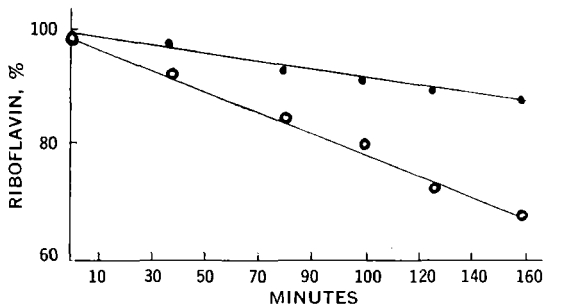

Figure 3—*Apparent percent riboflavin remaining after photolysis as determined by fluorometric procedure* (●) *and chromatographic procedure* (○).

Table II—Analysis of Riboflavin in Multivitamin Products

Sample	Dosage Form	Labeled Components	Labeled Riboflavin Content, mg	Riboflavin Found, %	
				Chromatographic Method	Fluorometric Method
1	Tablet	Iron, 10 vitamins, artificial colorings, flavorings, and sweeteners	1.5	97.64[a] (9.16)	98.47[a] (9.74)
2	Tablet	11 vitamins, natural sweeteners, artificial coloring, and flavoring	1.2	95.26 (3.70)	97.93 (4.33)
3	Tablet	10 vitamins and sodium fluoride	1.5	98.34 (5.15)	104.42 (4.86)
4	Capsule	Nine vitamins	3.0	101.18 (4.77)	109.31 (5.11)
5	Capsule	Six vitamins	10.0	103.45[b] (3.94)	105.62 (4.72)
6	Liquid	Nine vitamins, natural sweeteners, and artificial flavoring	0.7	27.23 (2.14)	89.47 (3.51)
7	Liquid	Nine vitamins and natural sweeteners	1.2	93.61 (3.14)	106.29 (4.12)

[a] Percent of labeled amount (% *SD*) with $n = 10$. [b] High potency modification.

metric procedure was shown to be as high as 15.1% in a collaborative study (8).

The results of experiments comparing the effectiveness of this procedure with that of the official procedure in determining riboflavin in the presence of its photolytic degradation products are shown in Fig. 3. Since apparent riboflavin content as determined by the chromatographic procedure drops more rapidly than that determined by the fluorometric procedure, it would appear that one or more degradation products are contributing to fluorescence at the analytical wavelengths. If a fluorometer had been used in place of the spectrophotofluorometer, it is quite possible that the broader range of excitation and emission wavelengths would have resulted in an even larger discrepancy between results of the two procedures.

In light of the number of isolated riboflavin degradation products (9), it is not surprising that one or more possess fluorescence characteristics similar to the parent compound. By using the described system, it was possible to isolate four peaks from riboflavin solutions exposed to artificial light in pH 4 buffer. At a flow rate of 0.95 ml/min, two peaks with retention volumes of 0.25 and 0.56 (relative to riboflavin) appeared after photolysis. The more rapidly eluting peak was, however, resolvable into three components when the flow rate was lowered to 0.25 ml/min.

To investigate the selectivity of the procedure further, photolytic degradation products of riboflavin were isolated by the method of Ismaiel and Yassa, eluted, and injected into the chromatographic system. Spot 3 (6) on the thin-layer chromatogram corresponds to the peak at 0.56 relative retention volume, and Spots 4, 5, and 6 correspond to the peak with relative retention volume 0.25. Spot 1 does not emerge from the chromatograph.

Results of the analysis of riboflavin in multivitamin preparations using the official and chromatographic procedures are presented in Table II. Placebo preparations were subjected to analysis where these were available (Samples 1, 2, 4, and 7, Table II), and no constituent interfered with the analysis.

The chromatographic procedure gives significantly lower values for riboflavin content in several cases, especially the liquid preparations. However, results from the two methods with respect to the riboflavin content of Liquid 1 are so disparate as to require comment. Two different lots of the same preparations were tested with approximately the same results. There are two possible explanations for this disparity. A derivative of riboflavin having similar fluorescence characteristics but different chromatographic characteristics may have been used to prepare the liquid. Information regarding this possibility was not available, but riboflavin-5′-phosphate, a likely candidate for such a substitution, does not appear in the chromatogram (relative retention volume 1.86). On the other hand, degradation of riboflavin may have occurred in the solution, generating one or a combination of products which account for the apparent riboflavin level as determined by the USP procedure. The latter possibility is supported by the fact that the chromatogram of this product has a peak at 0.25 relative retention volume, corresponding to that from riboflavin degradation products. Additionally, DeMerre and Brown (9) showed that, in certain solvent systems, photolytic degradation of riboflavin appears to cease when fluorometry indicates 60–80% of the riboflavin still remains. They accounted for these findings by theorizing that photolytic degradation products formed in these solvents systems possess similar fluorescence characteristics to the parent compound.

Results from the analysis of Tablet 1 (Table II) have relatively high standard deviations with both methods. However, when a larger number of tablets was analyzed and the results were grouped according to tablet color, the red tablets were found to contain 91.34% (2.74 *SD*) of the labeled amount of riboflavin, the blue tablets contained 104.21% (3.37 *SD*), and the green tablets contained 97.60% (3.14 *SD*). These results indicate that tablet color influences degradation or, more likely, that different formulation batches are used to make the different colored tablets.

The chromatographic method itself appears to be practical. It is possible to determine riboflavin content of seven units, each of three different products, in a 4-hr period. The life of the column under the assay conditions is at least 3 months, with no perceptible change in retention characteristics or results.

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[x] To whom inquiries should be directed.

High-Pressure Liquid Chromatographic Determination of Adrenergic and Antihistaminic Compounds in Pharmaceutical Preparations

TERRY L. SPRIECK

Abstract □ The application of high-pressure liquid chromatography to the separation and quantitative analysis of certain combinations of adrenergic and antihistaminic compounds in pharmaceutical dosage forms is demonstrated. The analysis of: *(a)* phenylpropanolamine hydrochloride and chlorpheniramine maleate, *(b)* pseudoephedrine hydrochloride and chlorpheniramine maleate, and *(c)* phenylpropanolamine hydrochloride, pheniramine maleate, and pyrilamine maleate, each with an internal standard, from pharmaceutical dosage forms was carried out using high-pressure liquid chromatography. The chromatographic conditions described were chosen to optimize the resolution and total elution time of these amine compounds.

Keyphrases □ Adrenergic-antihistaminic tablets and syrups—high-pressure liquid chromatographic analysis □ Antihistaminic-adrenergic tablets and syrups—high-pressure liquid chromatographic analysis □ Phenylpropanolamine with chlorpheniramine or pheniramine/pyrilamine dosage forms—high-pressure liquid chromatographic analysis □ Pseudoephedrine with chlorpheniramine dosage forms—high-pressure liquid chromatographic analysis

With the broad use of certain combinations of vasoconstrictors and antihistamines in pharmaceutical preparations, rapid separation and quantitative analysis of these combinations have become important. Other investigators have reported several approaches to the rapid analysis of cough–cold preparations. An automated, simultaneous determination of dextromethorphan hydrobromide, glyceryl guaiacolate, and phenylpropanolamine hydrochloride was recently developed (1), and these three compounds and chlorpheniramine were determined by GLC. Ek *et al.* (3) developed an automated procedure for the determination of total antihistamines by UV spectrophotometry and of phenylpropanolamine hydrochloride by colorimetry.

With the recent developments in high-pressure liquid chromatographic instrumentation and column packing materials, this technique of rapid quantitative analysis has proved to be quite useful. The separation from complex mixtures and quantitative analysis of several drugs of biomedically important systems have been reported (4–9). Pyrilamine maleate was separated from phenylephrine hydrochloride and naphazoline hydrochloride by high-pressure anion-exchange chromatography (10). Chlorpheniramine maleate and pyrilamine were also separated from acetaminophen, dextromethorphan hydrobromide, ephedrine sulfate, and methapyrilene by high-pressure reverse-phase chromatography (11).

This paper describes the concurrent separation and quantitative analysis of some decongestant–antihistamine combinations by high-pressure liquid chromatography. The preparation of samples is simple and does not require prior extraction of active ingredients from excipients. Either a single tablet or a ground composite sample of a group of tablets may be assayed by the described procedure. A strong cation-exchange column with an eluent of aqueous dibasic ammonium phosphate and an organic modifier, dioxane, was used to effect the separation in a reasonably short time. Slight changes in the concentration of the organic modifier and ionic strength have rather pronounced effects on the retention times and resolution of the chromatographic peaks.

EXPERIMENTAL[1]

Reagents—The mobile phases were prepared from reagent dibasic ammonium phosphate[2], specially purified dioxane[3], and distilled water. All other reagents were of USP (13) or NF (14) quality and were used as received.

Standard Stock Solutions—*Mixture I*—Standard stock solution I was prepared by weighing accurately and combining 600 mg pseudoephedrine hydrochloride and 40 mg chlorpheniramine maleate and then diluting to 100 ml with distilled water.

Mixture II—Standard stock solution II was prepared by weighing accurately and combining 250 mg phenylpropanolamine hydrochloride and 40 mg chlorpheniramine maleate and then diluting to 100 ml with distilled water.

Mixture III—Standard stock solution III was prepared by weighing accurately and combining 250 mg phenylpropanolamine hydrochloride, 125 mg pheniramine maleate, and 125 mg pyrilamine maleate and then diluting to 100 ml with distilled water.

Internal Standard Stock Solutions—*Internal Standard I*—Internal standard stock solution I was prepared by weighing accurately 400 mg of phenylpropanolamine hydrochloride and diluting to 100 ml with 0.2 *N* HCl.

Internal Standard II—Internal standard stock solution II was prepared by weighing accurately 250 mg ephedrine hydrochloride and diluting to 100 ml with 0.2 *N* HCl.

Internal Standard III—Internal standard stock solution III was prepared by weighing accurately 500 mg of ephedrine hydrochloride and diluting to 100 ml with 0.2 *N* HCl.

Preparation of Tablet Samples—A representative sample consisting of 100 tablets of a batch of tablets was weighed to determine the average tablet weight; then 20 tablets were ground to a fine powder. An accurately weighed portion equivalent to one tablet weight of the powdered sample was transferred to a 10-ml volumetric flask. Five milliliters of the appropriate internal standard stock solution and about 2 ml of distilled water were added to the flask. The sample was stirred for 45 min using a small stir bar and a magnetic stirrer. The stir bar was then removed and the sample was diluted to volume with distilled water. After mixing well, the sample was centrifuged and then filtered through a 5-μm filter[4].

Preparation of Syrup Samples—Five milliliters of the syrup

[1] A DuPont model 830 liquid chromatograph equipped with a UV photometer and a gradient elution accessory, and Autolab model System IV integrator, and a Honeywell model 194, 1-mv recorder were used. The analytical column was 0.5-m, 6.35-mm o.d., 2.10-mm i.d. stainless steel (DuPont) packed by the "modified tap fill" technique (12) with DuPont "Zipax" supported strong cation-exchange (SCX) material.

[2] Fisher Scientific Co.

[3] Burdick & Jackson Laboratories.

[4] Millipore Mitex.

Table I—Effect of Mobile Phase Molarity on Retention Time (Seconds from Injection to Chromatographic Peak)

Compound	Concentration of $(NH_4)_2HPO_4$ in 32% Dioxane–Water (1100 psig, 1.1 ml Flow Rate, Ambient Temperature)		
	0.01 *M*	0.02 *M*	0.04 *M*
Phenylpropanolamine	271	172	112
Pseudoephedrine	760	436	219
Chlorpheniramine	1320	781	367

was transferred using a pipet[5] to a 10-ml volumetric flask, and the pipet was rinsed with several small portions of the appropriate internal standard solution. The sample was then diluted to 10 ml with internal standard and mixed well.

Chromatographic Conditions—All data and chromatograms presented here were obtained under isocratic conditions.

The degassed mobile phase was passed through the strong cation-exchange column under a pressure of about 1100 psig, to obtain a flow rate of 1.0–1.1 ml/min at room temperature. The photometer was operated at an attenuation of 0.01 absorbance unit full-scale with peak attenuation of from ×1 to ×4 made at the integrator channel module. After an initial period of column and detector stabilization, the detector noise was nominally 1% or better at maximum sensitivity.

Eight-microliter injections of sample and standard solutions were made with a syringe[6]. The chromatographic peaks were electronically integrated to determine the areas relative to that of the internal standard.

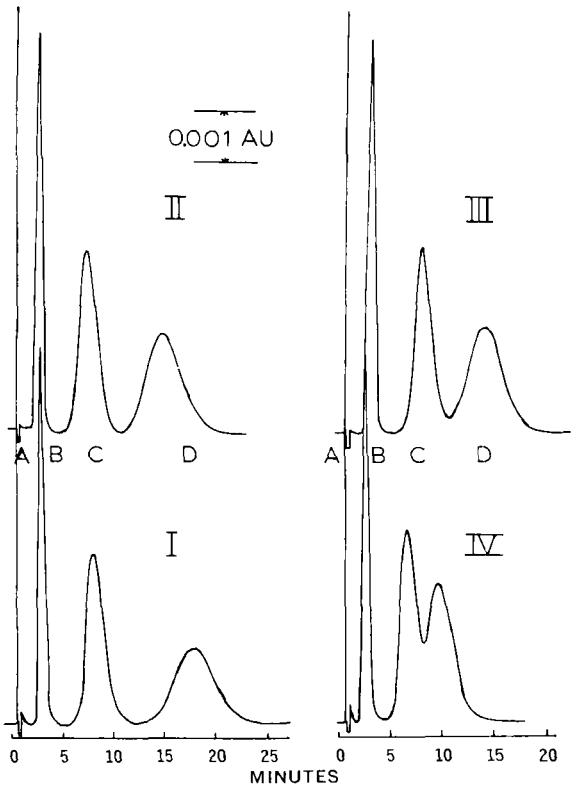

Figure 1—*Elution of Mixture I and Internal Standard I. Peak identification: A, solvent and unretained species (e.g., maleate and excipients); B, phenylpropanolamine; C, pseudoephedrine; and D, chlorpheniramine. Mobile phase was 0.02 M $(NH_4)_2HPO_4$ in 28% (I), 30% (II), 32% (III), and 36% (IV) dioxane–water (v/v) (apparent pH 8.2; 1100 psig; flow rate 1.1 ml/min; ambient temperature).*

[5] Ostwald to-contain.
[6] Hamilton 701N.

Table II—Response Factors for Standard Solution I

Compound	Area Response Factor[a]	Relative Standard Deviation[b]	Area Response Factor/ Concentration[c]	Linear Correlation Coefficient
Pseudoephedrine	1.378	1.11%	0.454	0.9992
Chlorpheniramine	1.403	3.24%	7.323	0.9733

[a] Average of six injections of Standard Solution I with Internal Standard I. Integrated areas of each amine peak divided by area of internal standard peak. [b] Relative standard deviation of the respective area response factors; indicates precision obtained from repetitive injections of the same standard solution. [c] Linearity of integrated area response, with concentration in milligrams per milliliter, is shown for five standard solutions with concentrations of 90, 95, 100, 105, and 110% of Standard Solution I.

RESULTS AND DISCUSSION

High-pressure ion-exchange chromatography of an 8-μl injection of 5 ml of Mixture I diluted with 5 ml of Internal Standard I is shown in Fig. 1. The effect of percent dioxane on the retention and resolution of this mixture is also shown in Fig. 1. As the organic modifier percentage is increased, the retention of the three amines is decreased. As in many other applications of ion-exchange chromatography, changes in ionic strength and pH are most often used to obtain the desired resolution or retention times. The effect of changes in the molar concentration of dibasic ammonium phosphate on retention time is illustrated in Table I.

Relative area precision data (Table II) for the standard solution were obtained after determining the chromatographic conditions that would optimize the resolution of the amines in a reasonably short time (Fig. 1, II). After determining linearity of area response factors (the ratio of each amine peak to the area of the internal standard) with concentration for several concentration levels, the active amine content of several different pharmaceutical preparations was determined (Table III). Response factors used for the data shown in Table III were calculated from a standard injection prior to and following the duplicate sample injections to minimize the changes in response factors caused by changes in chromatographic conditions.

The chromatograms of injections of syrups are changed slightly in the early stages of the elution. The excipients of the syrups that are unretained or very slightly retained by the strong cation-exchange column cause some broadening of the solvent peak. For the syrups sampled, baseline resolution was attained prior to the onset of the internal standard peak. If some excipients of a syrup are slightly retained and interfere with the baseline resolution of the internal standard, norpseudoephedrine hydrochloride, a diastereomer of phenylpropanolamine, may be substituted as an internal standard (Fig. 2). The peak retention time for norpseudoephedrine was about 80 sec longer than for phenylpropanolamine under the conditions described in Fig. 2. This internal standard reduces slightly the baseline resolution prior to the onset of the pseudoephedrine peak; precise analytical area measurements may, however, still be made.

The optimum chromatographic conditions and calibration data

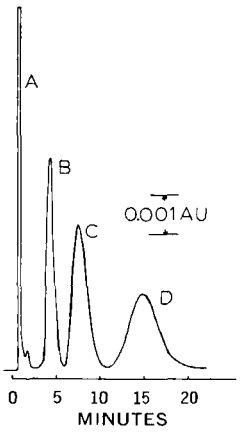

Figure 2—*Chromatogram of a syrup sample using norpseudoephedrine hydrochloride as internal standard (4 mg/ml). Mobile phase was 0.02 M $(NH_4)_2HPO_4$ in 30% dioxane–water (1100 psig; flow rate 1.1 ml/min; ambient temperature). Peak identification: B, norpseudoephedrine; and other peaks same as in Fig. 1.*

Table III—Concentration of Amines in Tablets and Syrups

Sample	Phenylpropanolamine Hydrochloride		Pseudoephedrine Hydrochloride		Chlorpheniramine Maleate		Pheniramine Maleate		Pyrilamine Maleate	
	T.C.[a]	%[b]	T.C.	%	T.C.	%	T.C.	%	T.C.	%
Tablet A	—	—	30.0	100.5	2.00	103.7	—	—	—	—
Tablet B	—	—	30.0	99.9	2.00	104.0	—	—	—	—
Tablet C	—	—	30.0	95.7	2.00	100.6	—	—	—	—
Syrup A	—	—	30.0	101.6	2.00	100.3	—	—	—	—
Syrup B	—	—	30.0	97.9	2.00	98.1	—	—	—	—
Tablet D	12.5	97.5	—	—	2.00	103.0	—	—	—	—
Tablet E[c]	25.0	100.8	—	—	4.00	103.5	—	—	—	—
Syrup C	12.5	98.1	—	—	2.00	99.5	—	—	—	—
Syrup D	12.5	98.3	—	—	2.00	101.8	—	—	—	—
Tablet F[c]	25.0	98.0	—	—	—	—	12.5	100.7	12.5	97.8
Tablet G[c]	25.0	99.8	—	—	—	—	12.5	95.1	12.5	100.5
Syrup E	12.5	101.8	—	—	—	—	6.25	101.6	6.25	99.4
Syrup F	12.5	99.9	—	—	—	—	6.25	100.8	6.25	101.2

[a] Theoretical content: milligrams per tablet or milligrams per 5 ml syrup. [b] Content of sample found by this procedure; percent of theoretical content. [c] One-half tablet weight used in sample preparation.

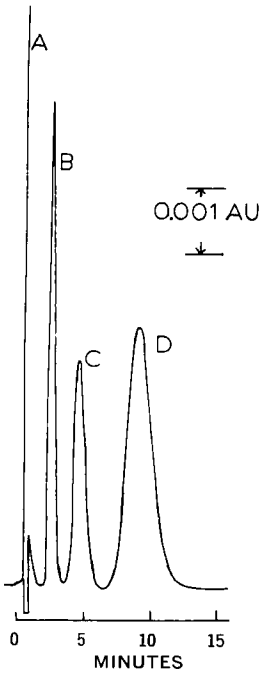

Figure 3—*Chromatogram of Tablet D sample preparation with Internal Standard II. Peak identification: A, solvent and unretained species; B, phenylpropanolamine; C, ephedrine; and D, chlorpheniramine. Mobile phase was 0.02 M $(NH_4)_2HPO_4$ in 36% dioxane–water (apparent pH 8.2; 1100 psig; flow rate 1.1 ml/min; ambient temperature).*

for the other mixtures were determined in a similar manner. Changes in the dioxane content of the mobile phase were most useful since large increases in the ionic strength reduce the resolution between sample excipients and the initial peak. An example of the chromatographic elution of a tablet sample preparation with an active ingredient composition (Tablet D, Table III) similar to Mixture II is shown in Fig. 3. An example of the chromatographic elution of a syrup sample preparation with an active ingredient composition (Syrup E, Table III) similar to Mixture III is shown in Fig. 4.

Representative analytical data obtained from several lots of tablets and syrup formulations are presented in Table III. These data demonstrate the utility of high-pressure liquid chromatography for the analysis of certain combinations of adrenergic and antihistaminic compounds in pharmaceutical dosage forms.

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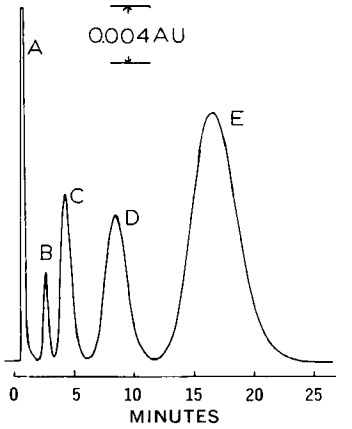

Figure 4—*Chromatogram of Syrup E sample preparation with Internal Standard III. Peak identification: A, solvent and excipients; B, phenylpropanolamine; C, ephedrine; D, pheniramine; and E, pyrilamine. Mobile phase and conditions were the same as in Fig. 3.*

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Identification and Determination of Synthetic Estrogens in Pharmaceuticals by High-Speed, Reversed-Phase Partition Chromatography

ROBERT W. ROOS

Abstract □ A high-speed, reversed-phase partition chromatographic method is described for the identification and quantitative determination of synthetic estrogens in pharmaceutical dosage forms. The method is applicable to diethylstilbestrol, dienestrol, hexestrol, benzestrol, chlorotrianisene, and the dipropionic acid esters of diethylstilbestrol and promethestrol. Prior to UV spectrophotometric detection at 254 nm, the synthetic estrogens are separated on either a chemically bonded hydrocarbon column or an ether partition column, utilizing 2-propanol–water mobile phase systems. Limits of detectability are at the nanogram level for diethylstilbestrol and dienestrol, the synthetic estrogens most commonly found in commercial preparations. Chromatographic studies were conducted after a number of the synthetic estrogens underwent photochemical change, and the synthetic estrogen 4,4′-stilbenediol esters underwent controlled ester hydrolysis. The chromatograms of the photochemical and hydrolysis degradation products were found to be useful for identification. For example, a scheme developed for the identification of diethylstilbestrol provides for the separation of three photochemical by-products: the *cis*-isomer of diethylstilbestrol, the phenanthrenedione (3,4,5,6-hexahydro-3,6-dioxo-9,10-diethylphenanthrene), and the phenanthrenediol (9,10-diethyl-3,6-phenanthrenediol). Application of the method to the analysis of synthetic estrogens in commercial preparations is described.

Keyphrases □ High-speed, reversed-phase partition chromatography—identification and determination of synthetic estrogens in pharmaceuticals □ Estrogens, synthetic—identification and determination in pharmaceuticals by high-speed, reversed-phase partition chromatography

Recent publications have shown the application of high-speed (pressure) liquid chromatography to the separation and/or quantitative determination of analgesics (1, 2), sulfonamides (3, 4), barbiturates (5, 6), steroids (7–9), benzodiazepines (10, 11), monosubstituted pyridine isomers (12), purine and strychnos alkaloids (13), and vitamins (14, 15). The technique appears most promising in providing for the separation of highly polar, nonvolatile, and thermally labile compounds. To extend the usefulness of this analytical method, this paper describes its potential application to the qualitative and quantitative determination of the synthetic estrogen family of drugs.

Various methods have been proposed for the quantitative determination of synthetic estrogens present in pharmaceutical dosage forms. Gottlieb (16), introducing a spectrophotometric method for the synthetic estrogenic 4,4′-stilbenediols—*viz.*, diethylstilbestrol, dienestrol, hexestrol, benzestrol, and promethestrol, based on the formation of their corresponding *o*-nitrosophenols, gave an excellent review of a number of conventional methods together with a discussion of their various shortcomings. The official compendia (17, 18) provide assays for the quantitative determination of five individual synthetic estrogens. These methods, although applicable to the products for which they are intended, are, in general, tedious or nonspecific and most often are not applicable when an unrelated drug, such as a sulfonamide, steroid, or barbiturate, is present in combination.

GLC has also been applied to the identification and quantitative determination of the synthetic estrogens. For example, Fricke *et al.* (19) described a method for the synthetic estrogen dienestrol when in combination with methyltestosterone; the method is based on the formation of a bis(trimethylsilyl) ether derivative. Other papers have also reported methods that use GLC, and most of these methods require the formation of derivatives.

The objectives of this paper are: *(a)* to show that the synthetic estrogens are amenable to separation, identification, and quantitative determination in pharmaceutical dosage forms, utilizing high-speed, reversed-phase partition chromatography; and *(b)* to indicate the potential use of photolysis and ester hydrolysis in synthetic estrogen identification studies.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with a UV absorption photometer, set at 254 nm, was utilized throughout this study. The instrument provides for: *(a)* a maximum absorbance unit full-scale (aufs) setting of 0.5×10^{-2}, and *(b)* mobile phase degassing by vacuum. The UV (254 nm) irradiation unit applied in qualitative studies is that described in USP XVIII (17) for the quantitative determination of diethylstilbestrol.

Columns and Mobile Phases—Two chemically bonded, reversed-phase partition columns applied in achieving synthetic estrogen separations are commercially available from the instrument manufacturer and are 1-m × 2.1-mm i.d. × 0.6-cm (0.25-in.) o.d., precision bore stainless steel tubes, containing the following stationary phases: *(a)* Column 1, an octadecylsilane (hydrocarbon) chemically bonded to the surface of a controlled porous surface[2]; and *(b)* Column 2, an ether chemically bonded to the surface of a controlled porous surface[3]. Column 2 was the most utilized and was applied in quantitative analysis. The mobile phases used were simple combinations of 2-propanol[4] and water. Prior to use, the mobile phases were degassed by vacuum.

Solvents and Standards—During chromatographic elution behavior studies, methanol was used as the solvent for the synthetic estrogens. The synthetic estrogen 4,4′-stilbenediols were readily soluble in methanol; however, heating was required to achieve solution of the 4,4′-stilbenediol esters and chlorotrianisene. For quantitative studies, three solvent systems were utilized to both dissolve and extract the synthetic estrogens from pharmaceutical dosage forms; these were: *(a)* methanol, *(b)* methanol–ether (1:1), and *(c)* dimethylformamide. The synthetic estrogen standards were of laboratory working grade, with previous assay results

[1] Du Pont model 820, equipped with a model 410 UV detector, E. I. du Pont de Nemours and Co., Wilmington, Del.
[2] ODS-Permaphase, Du Pont.
[3] ETH-Permaphase, Du Pont.
[4] Fisher Spectranalyzed (A-419).

Table I—Summarized Sample Preparation

Sample Preparation	Sample Size[a], mg	Solvent	Vessel Size, ml	Vessel Type[b]	Amount of Solvent	Heating Required	Internal Standard	Aliquot, ml	Final Vessel Size, ml	Solvent
Diethylstilbestrol and dienestrol										
Tablets	2.5	Methanol	50	V.F.	*q.s.*	No	Phenothiazine[c,d]	—	—	—
Creams	1	Methanol + ether (1:1)	100	g.s.f.	50	Yes	Phenothiazine[c,d]	—	—	—
Injection[e]	10	Dimethylformamide + methanol (1:1)	100	V.F.	50 ml	No	Phenothiazine[c,d]	—	—	—
Suppositories	0.5	Methanol + ether (1:1)	50	g.s.f.	20 ml	Yes	Phenothiazine[c,d]	—	—	—
Chlorotrianisene soft capsules	100	Dimethylformamide	100	V.F.	*q.s.*	No	Phenothiazine[c,d]	10	50	Methanol
Promethestrol dipropionate tablets	10	Dimethylformamide + methanol (1:1)	50	V.F.	20 ml then *q.s.*	No	Chlorotrianisene[d,f]	—	—	—
Hexestrol tablets	25	Methanol	50	V.F.	*q.s.*	No	Benzestrol[g]	—	—	—
Benzestrol tablets	25	Methanol	50	V.F.	*q.s.*	No	Hexestrol[g]	—	—	—

[a] Representative sample accurately weighed to contain this much active ingradient. [b] V.F. = volumetric flask; g.s.f. = glass-stoppered flask. [c] Using 0.5 mg/ml in methanol, add an aliquot to contain an amount similar to amount of active ingredient in sample. [d] In preparation of standard solution, some heating may be necessary to aid dissolution. [e] Cottonseed oil formulation. [f] Add one-tenth the amount of active ingredient using 0.5 mg/ml in methanol. [g] Add internal standard directly to volumetric flask to provide a final concentration similar to that of the sample.

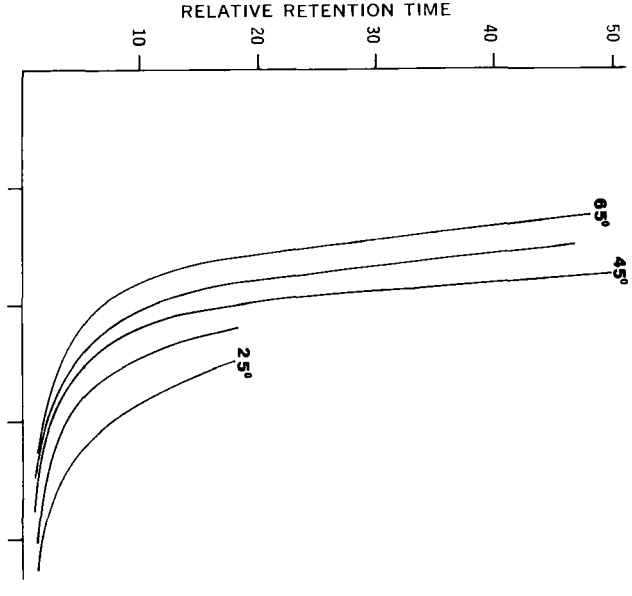

Figure 1—*Effects of mobile phase variation on the elution behavior, expressed in terms of relative retention times, of trans-diethylstilbestrol. Chromatography conditions were: Column 2; pressure at inlet, 1000 psig; flow rate, variable; column temperature, 25, 35, 45, 55, and 65°, respectively; and UV detector sensitivity, 254 nm, 0.16 aufs.*

meeting regulatory standards. Promethestrol was not available and was formed by heating promethestrol dipropionate in methanol–potassium hydroxide solution, a procedure taken from Gottlieb (16).

Qualitative Analysis—*Irradiation Studies*—Methanol solutions of *trans*-diethylstilbestrol (0.2 mg/ml), chlorotrianisene (0.2 mg/ml), diethylstilbestrol dipropionate (0.2 mg/ml), and promethestrol dipropionate (0.5 mg/ml) were irradiated with 254-nm radiation for 30–60 min. This procedure was repeated for *trans*-diethylstilbestrol using equal volumes of (*a*) methanol–1.8% aqueous dipotassium hydrogen phosphate, and (*b*) methanol–acetic acid. Further studies of *trans*-diethylstilbestrol after initial UV radiation involved the direct conversion of derived phenanthrenedione to phenanthrenediol in sodium bisulfite–hydrochloric acid solution.

Hydrolysis Studies—Hydrolysis of the 4,4′-stilbenediol esters, *e.g.*, diethylstilbestrol dipropionate and promethestrol dipropionate, was carried out by adding an equal volume of 1.8% aqueous dipotassium hydrogen phosphate solution to the methanol solution of the ester.

Quantitative Analysis—Methods of analysis were developed for six synthetic estrogens included in this study and directly applied to their quantitative determination in commercial preparations (Table I). The methods are based on extraction and solubilization of the synthetic estrogens and the use of internal standards.

Chromatographic Conditions—The following were used: Column 2; mobile phase, 25% 2-propanol in water (v/v); column temperature, 60°; pressure at inlet, 1000 psig; and flow rate, 0.55 ml/min.

Sample Preparation—The various products and dosage forms were prepared as outlined in Table I.

Standard Solution—A standard solution, containing internal standard and sample synthetic estrogen in the same quantities chosen for the sample solution, was prepared concurrently.

Chromatography—Make preliminary injections of the standard solution to determine the UV detector setting (in terms of absorbance unit full-scale) and sample volume that provide an acceptable full-scale response. When the instrumental settings and sample volume (usually 4–10 μl) have been satisfactorily determined, inject a replicate number of sample volumes for quantitative analysis. Calculate the peak areas of both the internal stan-

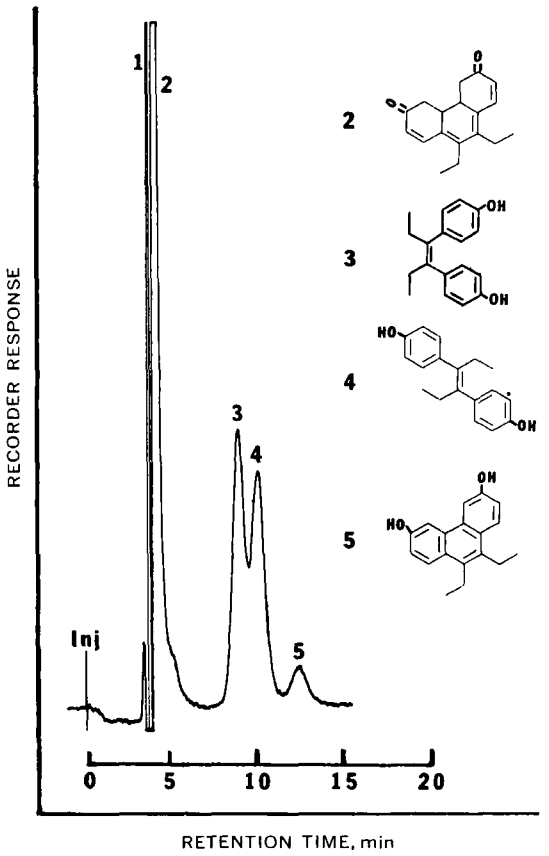

Figure 2—*Chromatogram of the photochemical by-products of* trans-*diethylstilbestrol. Key: peak 1, methanol solvent; peak 2, the phenanthrenedione (3,4,5,6-hexahydro-3,6-dioxo-9,10-diethylphenanthrene); peak 3,* cis-*diethylstilbestrol; peak 4,* trans-*diethylstilbestrol; and peak 5, the phenanthrenediol (9,10-diethyl-3,6-phenanthrenediol). Chromatography conditions were: Column 2; mobile phase, 20% 2-propanol in water; pressure at inlet and column temperature, 400 psig and 60°, respectively; and UV detector sensitivity, 254 nm, 0.04 aufs.*

dard and sample synthetic estrogen. In this work, the areas *(A)* were computed using the relationship $A = \frac{1}{2}bh$, where b = peak base, and h = peak height. Area was found proportional to concentration within ±50% of the quantity of synthetic estrogen chosen for analysis.

Calculations—The total quantity of sample synthetic estrogen taken for analysis (final volumetric or glass-stoppered flask) may be calculated using Eq. 1:

$$W_1 = \frac{A_1 A_3 W_2 W_4}{A_2 A_4 W_3} \qquad \text{(Eq. 1)}$$

where A represents peak area; W represents weight (milligrams); and subscripts 1, 2, 3, and 4 represent the sample synthetic estrogen, the standard synthetic estrogen, the internal standard in the standard solution, and the internal standard in the sample solution, respectively.

Calculation of the quantity of synthetic estrogen per dosage unit can be achieved by introduction of appropriate factors into Eq. 1.

RESULTS AND DISCUSSION

Column Characteristics and Elution Behavior—The reversed-phase partition chromatographic elution behavior on two chemically bonded columns, expressed in terms of relative retention times (methanol as 1.00) of 10 synthetic estrogens, is shown in Table II.

Both columns were utilized previously for the separation of compounds of interest in pharmaceutical analysis. For example, Column 1 was found useful for the separation of several steroids (8), and Column 2 was useful for the separation of barbiturates (6). The value of chemically bonded stationary phases in comparison with stationary fluids that are retained by physical interaction was discussed by Kirkland (20). The most apparent advantages seem to be the elimination of precolumns and presaturated mobile phases. The mobile phases selected to achieve satisfactory migrations of the synthetic estrogens are simple combinations of 2-propanol and water.

Table II—Chromatographic Behavior of Synthetic Estrogens

Synthetic Estrogens	Relative Retention Times[a]		
	Column 1[b]		Column 2[c]
	15%[d]	30%[d]	20%[d]
Methallenestril	2.53	—[e]	1.14
Dienestrol	3.03	—[e]	5.10
Diethylstilbestrol[f]	3.62	—[e]	5.42
Hexestrol	3.78	—[e]	5.19
Benzestrol	9.20	—[e]	7.28
Promethestrol	14.3	—[e]	9.10
Dienestrol diacetate	20.2	1.70	5.32
Chlorotrianisene	—[g]	4.30	20.8
Diethylstilbestrol dipropionate	—[g]	5.22	13.8
Promethestrol dipropionate	—[g]	6.64	16.7
Methanol emergence, min	1.34	1.85	1.73
Pressure at inlet, psig	1000	1000	1000
Column temperature	50°	50°	70°

[a] Relative retention times are expressed relative to the solvent methanol as 1.00. [b] Chemically bonded hydrocarbon column (ODS-Permaphase, Du Pont). [c] Chemically bonded ether column (ETH-Permaphase, Du Pont). [d] Mobile phase: percentage of 2-propanol in water (v/v). [e] Less than 1.20. [f] Refers to *trans*-diethylstilbestrol. [g] Greater than 25.0.

The retention data (Table II) indicate that both partition columns provide for synthetic estrogen separations, with Column 1 permitting the greater degree of separability. However, peak tailing on Column 1 greatly limits its potential use, particularly in quantitative analysis. Comparison of the data between columns shows some elution order variations. For example, chlorotrianisene is shown to elute before the dipropionic acid esters of diethylstilbestrol and promethestrol on Column 1, whereas it elutes later on Column 2. Another important observation is the similarity in elution behavior of dienestrol and its diacetic acid ester, *e.g.*, dienestrol diacetate, on Column 2 in contrast to the wide difference in elution behavior on Column 1.

When utilizing Column 2, the column selected for most subsequent studies and applied in quantitative analysis, a further investigation was undertaken to determine the effect of 2-propanol content variation in the mobile phase on elution behavior. Figure 1 shows the relative retention times (relative to methanol as 1.00) of *trans*-diethylstilbestrol *versus* 2-propanol concentration in the mobile phase. The study was conducted at five different column temperatures, varying in 10° intervals, and relative retention times were calculated for successive 2.5% increments of increasing 2-propanol concentration. Although other synthetic estrogens are not shown, studies have indicated a similar response. The series of curves shown in Fig. 1 indicates that the analyst can rapidly estimate a mobile phase composition and a column temperature to provide a desired relative retention time.

Although the mobile phase may be varied, some considerations must be borne in mind relating to sensitivity. For example, increases in the 2-propanol concentration of the aqueous mobile phase result in a higher mobile phase viscosity and, therefore, in a loss of column efficiency. This loss in efficiency leads to a greater peak band width and a loss in peak height response. In addition, lowering of the 2-propanol concentration of the aqueous mobile phase was found to provide a greater peak height response for the synthetic estrogen hormones diethylstilbestrol and dienestrol. This increase in peak height response is directly related to the increase in molar absorptivity values at 254 nm for these synthetic estrogens; *i.e.*, the UV absorption curves are shifting to a longer wavelength, with a corresponding increase in absorbance at 254 nm.

Qualitative Analysis—*Photochemical Behavior*—A potentially

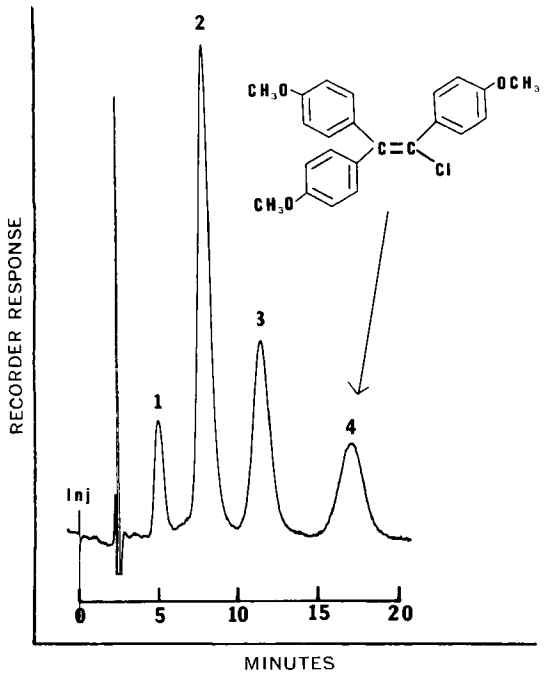

Figure 3—*Chromatogram of the photochemical by-products of chlorotrianisene. Peaks 1–3 are unidentified, and peak 4 is chlorotrianisene. Chromatography conditions were the same as in Fig. 2; however, pressure at inlet was 600 psig.*

useful approach in the rapid qualitative analysis (in addition to their normal retention time) of a number of synthetic estrogens, *e.g.*, diethylstilbestrol, chlorotrianisene, and the dipropionic acid esters of diethylstilbestrol and promethestrol, is their chromatographic behavior after undergoing photochemical change.

Figure 2 presents a chromatogram of a methanolic solution of *trans*-diethylstilbestrol which was irradiated by UV light. Five peaks are noted. The solvent methanol (peak 1) eluted very rapidly, followed closely by a tall peak (peak 2) which was found to be the phenanthrenedione (3,4,5,6-hexahydro-3,6-dioxo-9,10-diethylphenanthrene). The doublet corresponds to *cis*- (peak 3) and *trans*- (peak 4) diethylstilbestrol, and the final small peak (peak 5) is a phenanthrenediol (9,10-diethyl-3,6-phenanthrenediol).

The nature of the photochemical changes brought about by the irradiation of diethylstilbestrol in solution has been carefully studied (21, 22). In fact, formation of both the phenanthrenedione (22) and the phenanthrenediol (23) has been the basis of quantitative methods of analysis for this particular synthetic estrogen. Diethylstilbestrol, usually present as the *trans*-isomer, is induced to equilibrate with the higher energy *cis*-isomer, which then rearranges to yield phenanthrenedione and, finally, phenanthrenediol. The presence of each of these compounds in this study was corroborated as follows. The phenanthrenedione and phenanthrenediol were identified by elution behavior studies of reference standards prepared by previously reported methods (21, 23) and, in addition, by collecting each compound after chromatographic separation and performing a UV spectral comparison with the reference standards. The *cis*-diethylstilbestrol was identified by an elution behavior comparison with that of a reference standard, prepared by allowing a pure reference standard of *trans*-diethylstilbestrol in chloroform to equilibrate between the two isomers (24).

Variation in solution composition results in a different chromatographic pattern. In the system composed of equal volumes of methanol and aqueous 1.8% dipotassium hydrogen phosphate, a weakly basic medium, the chromatogram shows three peaks: a single peak for the phenanthrenedione and a doublet once again corresponding to *cis*- and *trans*-diethylstilbestrol. No peak for the phenanthrenediol is present, as expected (21). When diethylstilbestrol in acetic acid–methanol solution is irradiated, the chromatogram shows the presence of the peaks corresponding to the *cis*- and *trans*-isomers of diethylstilbestrol and a greatly increased peak height response for the phenanthrenediol. The conversion of *trans*-diethylstilbestrol to the phenanthrenediol in acetic acid was reported previously (25). Thus, in summary, it is noticed that in basic methanol solution, irradiation favors formation of the phenanthrenedione, and in an acidic medium, phenanthrenediol forms upon irradiation.

If the identification of *trans*-diethylstilbestrol at submicrograms levels is to be carried out by UV conversion to phenanthrenedione, difficulties are encountered. Since phenanthrenedione elutes relatively close to the solvent front, it will not be observed in most cases owing to interferences. However, the dione mobility may be altered and the resultant peak separated from the solvent front by using a 5–10% 2-propanol mobile phase system together with a temperature adjusted to provide for a desired retention time. In addition, a favorable change—*viz.*, stronger photometric absorption, would result if the detector was used at 280 nm since a phenanthrenedione maximum occurs near this wavelength.

A practical approach for the identification of *trans*-diethylstilbestrol at submicrogram levels is based on the preparation of the phenanthrenediol directly from the phenanthrenedione. After irradiation of a methanol or basic buffered methanol solution of *trans*-diethylstilbestrol to yield phenanthrenedione, the solution is acidified with hydrochloric acid and a quantity of sodium bisulfite (approximately 100 mg) is added. The resulting solution is heated on a steam bath until the yellow color disappears and there is no odor of sulfur dioxide (usually 10 min). Under these conditions, phenanthrenediol is formed in almost quantitative yield.

A second compound whose photochemical behavior was studied is chlorotrianisene. Figure 3 is a chromatogram of this drug after irradiation. The first peak to emerge is methanol; peaks 1, 2, and 3 represent unidentified decomposition products, and peak 4 is the parent compound which could be totally converted into other products with continued irradiation. Although no effort was made to identify the irradiation products, studies indicated that the compound contributing to peak 3 is a secondary photochemical by-product of chlorotrianisene and is formed directly from the compound contributing to peak 2. Therefore, peak 3 will be the dominant peak when chlorotrianisene is allowed to undergo con-

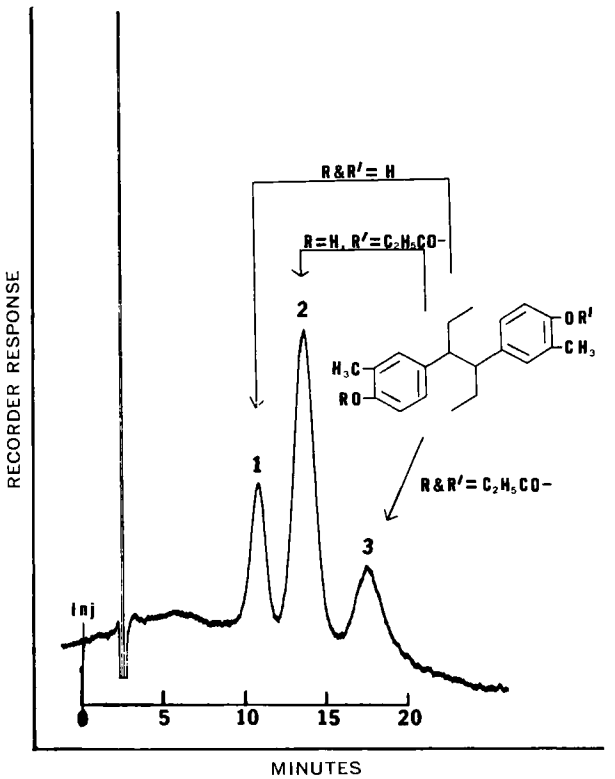

Figure 4—*Chromatogram of the ester hydrolysis decomposition products of promethestrol dipropionate. Peak at 2.5 min is the solvent methanol, and peaks 1–3 are identified as noted. Chromatography conditions were the same as in Fig. 3.*

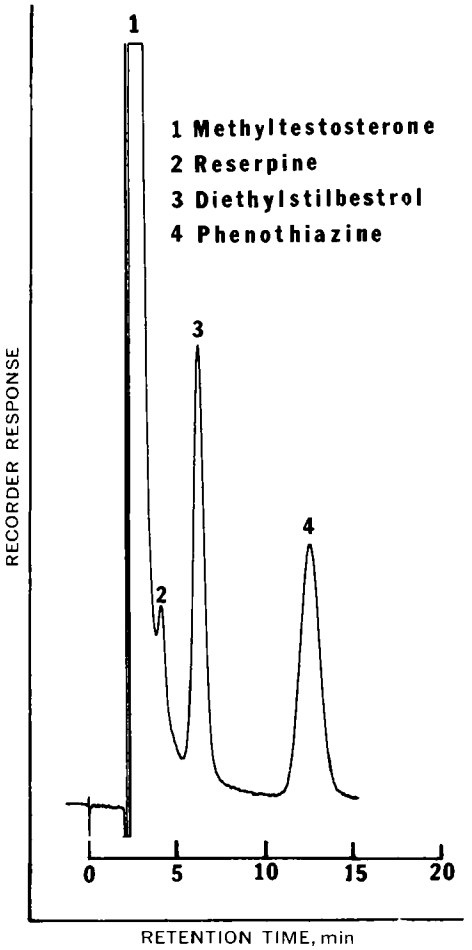

Figure 5—*Chromatogram of a methanol extract of a commercial preparation, including the internal standard phenothiazine. Chromatography conditions were: Column 2; mobile phase, 25% 2-propanol in water; pressure at inlet and column temperature, 600 psig and 50°, respectively; and UV detector sensitivity, 254 nm, 0.08 aufs.*

tinued irradiation in methanol. At any rate, the chromatographic pattern is characteristic and useful for identification.

Chromatographic studies of the dipropionic acid esters of diethylstilbestrol and promethestrol after irradiation in methanol also indicated the potential use of this approach in their identification. In each case, the synthetic estrogen underwent photochemical change and provided two additional peaks, *i.e.*, one peak immediately before and one immediately after the parent compound. Each peak provided a greater peak height response than the parent compound, therefore indicating an indirect route to higher sensitivity. Dienestrol, hexestrol, and benzestrol did not appear suitable for this type of study due to their lack of useful chromatographic change following UV exposure.

Ester Hydrolysis—In addition to the photochemical behavior studies of promethestrol dipropionate and diethylstilbestrol dipropionate, chromatographic studies of these synthetic estrogens were conducted while undergoing controlled ester degradation. Each synthetic estrogen was dissolved in methanol, and an equal volume of 1.8% aqueous dipotassium hydrogen phosphate was added to provide for partial degradation prior to chromatographic study. Figure 4 shows a chromatogram produced after promethestrol dipropionate underwent decomposition for 1 hr. In addition to some quantity of promethestrol dipropionate (peak 3), two additional compounds corresponding to promethestrol (peak 1) and the intermediate promethestrol monopropionate (peak 2) are present. Since the normal route of degradation of phenolic esters is ester hydrolysis, the detection of both of these compounds could be useful in stability studies of standard materials or dosage forms. Diethylstilbestrol dipropionate was found to undergo

Table III—Analysis of Synthetic Estrogens in Commercial Preparations

Sample	Declared	Found, % Liquid Chromatography[a]	Found, % Alternate Method
Dienestrol tablets	0.1 mg/tab	97.9	96.8[b]
Dienestrol tablets	10.0 mg/tab	98.8	98.1[c]
Dienestrol cream	0.1 mg/tab	107.0	109.0[d]
Diethylstilbestrol tablets	0.25 mg/tab	97.2	96.0[e]
Diethylstilbestrol injection	5.0 mg/ml	101.2	99.0[f]
Diethylstilbestrol lotion	0.70 mg/g	100.0	98.6[e]
Diethylstilbestrol suppositories	0.1 mg/sup	104.0	102.0[g]
Chlorotrianisene capsules	72.0 mg/cap	99.7	99.2[h]
Hexestrol tablets	3.0 mg/tab	98.1	98.7[c]
Benzestrol tablets	5.0 mg/tab	96.2	95.0[i]
Promethestrol dipropionate tablets	1.0 mg/tab	101.0	103.0[j]

[a] Chemically bonded ether column (Column 2, ETH-Permaphase); mobile phase, 25% 2-propanol in water (v/v); column temperature, 60°; pressure at inlet, 1000 psig; internal standards: (*a*) phenothiazine for dienestrol, diethylstilbestrol, and chlorotrianisene, (*b*) benzestrol for hexestrol, (*c*) hexestrol for benzestrol, and (*d*) chlorotrianisene for promethestrol dipropionate. [b] NF XIII, p. 229. [c] Extraction and UV. [d] NF XIII, p. 228. [e] USP XVIII, p. 189 (modified). [f] USP XVIII, p. 188. [g] USP XVIII, p. 188 (modified). [h] NF XIII, p. 163. [i] NF XIII, p. 76. [j] Extraction, hydrolysis, and UV.

degradation in the same solvent medium, with quantitative conversion to diethylstilbestrol in 1.3 hr. As could be expected, the intermediate diethylstilbestrol monopropionate separated. The solvent system used for the hydrolytic conversion of the diester to diethylstilbestrol is suitable for the irradiation studies previously described if further identity is desired.

Quantitative Analysis—Six different synthetic estrogens were quantitatively determined in pharmaceutical dosage forms in this study. The commercial preparations were selected to be representative of the various dosage forms currently marketed.

The results for the quantitative analysis of the various dosage forms containing synthetic estrogens are presented in Table III. In all cases except three, the liquid chromatographic results are somewhat higher than the alternative method. Considering the relatively small quantities of drug in most dosage forms, the maximum difference of 1.9% is reasonable. Where the drug dosage is large, as in chlorotrianisene capsules, the results are closer (0.5%). Area integrations were carried out manually, but an electronic integrator could minimize integration error and variation.

The most difficult experimental tasks are the extraction and solubilization of the synthetic estrogens prior to chromatographic injection. The solvent required varies and depends on both the dosage form and the individual synthetic estrogen under study. For example, dienestrol in solid dosage forms, *i.e.*, a tablet powder, could readily be extracted and placed in solution with methanol. When present in a cream, however, dienestrol required an equal mixture of ether–methanol. The ether was necessary to solubilize the cream base, thereby preventing any undissolved dienestrol from remaining in the base.

Various internal standards were used for the quantitative determination of the synthetic estrogens. For example, phenothiazine was selected for diethylstilbestrol, dienestrol, and chlorotrianisene; hexestrol for benzestrol; benzestrol for hexestrol, and chlorotrianisene for promethestrol dipropionate. Although phenothiazine is not the ideal internal standard owing to its photochemical instability, its delayed elution position relative to diethylstilbestrol and dienestrol is desirable.

Although both chromatographic columns were found useful in providing separations of the synthetic estrogens, Column 2 was selected for quantitative studies. The primary reasons for its selection were: *(a)* all of the synthetic estrogens were eluted in a reasonable time for quantitative analysis, and *(b)* the peaks in

each case were symmetrical, whereas the peaks showed some tailing on Column 1. The column selected is thermally stable at elevated temperatures and is often used at temperatures up to 75°.

Limits of detectability for the two most common synthetic estrogens, diethylstilbestrol and dienestrol, reached the nanogram level. In fact, when 1 ng of each compound was injected onto the chromatographic column, using a 20% 2-propanol–water mobile phase and a column temperature of 70°, a peak height response twice that of the noise level was observed. However, there are indications that Column 1 might prove more useful in submicrogram identification studies, since the synthetic estrogens can be eluted using a mobile phase containing a higher percentage of water, a solvent composition trend resulting in increasing photometric absorption.

Figure 5 shows an actual separation of the synthetic estrogen hormone diethylstilbestrol from two other drugs, methyltestosterone and reserpine, which are sometimes found in combination. In this particular case the methyltestosterone was present in approximately a 20-fold excess. The chromatogram shows the relative position of phenothiazine internal standard.

SUMMARY

The method proposed provides for the direct isolation of the synthetic estrogens without derivatization and appears applicable when these compounds are present in various dosage forms. Studies have indicated that compounds often present in combinations will not interfere with the method. In addition, the chromatographic patterns that result when synthetic estrogens undergo photochemical or hydrolytic change may be used for identification as an adjunct to the retention time.

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Specific Analysis for Homatropine Methylbromide in Syrups

F. F. CANTWELL ˣ, M. DOMJAN, and C. F. HISKEY

Abstract □ A sensitive, specific, and stability-indicating method is described for the analysis of homatropine methylbromide in complex pharmaceutical syrups. The procedure involves the isolation of methylhomatropinium ion on a cation-exchange resin column followed by on-column hydrolysis to mandelate ion by treatment with sodium hydroxide. The alkaline eluate is washed with chloroform and acidified. Ceric reagent is added to oxidize mandelic acid to benzaldehyde, which is then extracted into isooctane and measured by UV spectrophotometry. The procedure is routinely applied to complex syrups and is suitable for analyzing aged syrup samples.

Keyphrases □ Homatropine methylbromide—analysis in complex pharmaceutical syrups, ion-exchange separation and UV spectrophotometry □ Column chromatography, ion exchange—analysis of homatropine methylbromide in complex pharmaceutical syrups □ UV spectrophotometry—analysis, homatropine methylbromide in complex pharmaceutical syrups

The requirements of an analytical method for homatropine methylbromide (I) in pharmaceutical syrups include both sensitivity and selectivity. The drug is presently marketed in doses as low as 0.03 mg/ml and in formulations containing several other active ingredients, preservatives, buffers, flavors,

each case were symmetrical, whereas the peaks showed some tailing on Column 1. The column selected is thermally stable at elevated temperatures and is often used at temperatures up to 75°.

Limits of detectability for the two most common synthetic estrogens, diethylstilbestrol and dienestrol, reached the nanogram level. In fact, when 1 ng of each compound was injected onto the chromatographic column, using a 20% 2-propanol–water mobile phase and a column temperature of 70°, a peak height response twice that of the noise level was observed. However, there are indications that Column 1 might prove more useful in submicrogram identification studies, since the synthetic estrogens can be eluted using a mobile phase containing a higher percentage of water, a solvent composition trend resulting in increasing photometric absorption.

Figure 5 shows an actual separation of the synthetic estrogen hormone diethylstilbestrol from two other drugs, methyltestosterone and reserpine, which are sometimes found in combination. In this particular case the methyltestosterone was present in approximately a 20-fold excess. The chromatogram shows the relative position of phenothiazine internal standard.

SUMMARY

The method proposed provides for the direct isolation of the synthetic estrogens without derivatization and appears applicable when these compounds are present in various dosage forms. Studies have indicated that compounds often present in combinations will not interfere with the method. In addition, the chromatographic patterns that result when synthetic estrogens undergo photochemical or hydrolytic change may be used for identification as an adjunct to the retention time.

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Specific Analysis for Homatropine Methylbromide in Syrups

F. F. CANTWELL ˣ, M. DOMJAN, and C. F. HISKEY

Abstract □ A sensitive, specific, and stability-indicating method is described for the analysis of homatropine methylbromide in complex pharmaceutical syrups. The procedure involves the isolation of methylhomatropinium ion on a cation-exchange resin column followed by on-column hydrolysis to mandelate ion by treatment with sodium hydroxide. The alkaline eluate is washed with chloroform and acidified. Ceric reagent is added to oxidize mandelic acid to benzaldehyde, which is then extracted into isooctane and measured by UV spectrophotometry. The procedure is routinely applied to complex syrups and is suitable for analyzing aged syrup samples.

Keyphrases □ Homatropine methylbromide—analysis in complex pharmaceutical syrups, ion-exchange separation and UV spectrophotometry □ Column chromatography, ion exchange—analysis of homatropine methylbromide in complex pharmaceutical syrups □ UV spectrophotometry—analysis, homatropine methylbromide in complex pharmaceutical syrups

The requirements of an analytical method for homatropine methylbromide (I) in pharmaceutical syrups include both sensitivity and selectivity. The drug is presently marketed in doses as low as 0.03 mg/ml and in formulations containing several other active ingredients, preservatives, buffers, flavors,

Table I—Recovery of Benzaldehyde Measured by Optical Absorbance *versus* Oxidation Time[a]

Minutes	$A_{242\ nm}$	$A_{242\ nm} - A_{263\ nm}$
5	0.926	0.887
10	0.944	0.905
15	0.952	0.909
20	0.950	0.910
25	0.952	0.909
30	0.952	0.909
35	0.952	0.909

[a] Milligrams of I per sample = 0.656, milligrams of ammonium chloride per sample = 86, and milliliters of ceric reagent = 8.0.

dyes, and sweeteners. Furthermore, if an analytical method is to be stability indicating it must be specific for homatropine methylbromide in the presence of its hydrolytic degradation products, mandelic acid and tropinium methylbromide.

Classical methods employed in the analysis of homatropine methylbromide in syrups involve the precipitation of an insoluble salt of the methylhomatropinium cation (II) by anions such as the reineckate, silicotungstate, and tetraiodomercurate, followed by colorimetry (1) or turbidimetry (2, 3). These methods lack the sensitivity, selectivity, or specificity required for syrup analysis at low doses.

The recently described colorimetric procedure involving chromogenesis with Dragendorff's reagent (4, 5) is also nonspecific and, therefore, requires separation of homatropine methylbromide from other amines present to achieve specificity. Compound I may also be analyzed by conversion to the hydroxamic acid derivative of mandelic acid, followed by colorimetric measurement of a ferric hydroxamate complex (6). This method is stability indicating for II but it lacks the sensitivity of the procedure reported in the present paper. The recently published chromatographic procedure for I in tablets and elixirs (7) has been found inapplicable to the present syrup formulations.

Chafetz (8) employed a procedure for esters of mandelic acid such as II; it involves alkaline hydrolysis followed by oxidation to benzaldehyde, extraction, and spectrophotometry. This procedure is quite satisfactory for the analysis of many simple formulations. However, the large amount of oxidizable substances present in most syrups interferes with this oxidation step and produces background absorbance in the final spectrogram. Therefore, it seemed desirable to separate II from many of these substances prior to the hydrolysis and oxidation steps.

This laboratory has been using cation exchange to separate the II-ion from various formulations, and recently a procedure was reported (9) which involves ion-exchange separation of II from syrups and other liquid formulations, followed by UV spectrophotometry of the eluted II. The method is applicable only to relatively high doses of drug.

The present paper describes a sensitive, specific, and stability-indicating method of analysis for I in syrups. The method involves separation of II from noncationic syrup components by cation exchange, followed by on-column alkaline hydrolysis and elution and removal of amine components from the resulting mandelic acid by chloroform extraction. Quantitation is achieved by ceric-ion oxidation to benzaldehyde followed by salting-out extraction into isooctane and spectrophotometric determination. A wash step is included to reduce the small background absorbance occasionally present in aged samples.

Table II—Recovery of Benzaldehyde Measured by Optical Absorbance as a Function of Amount of Resin and Volume of Sodium Hydroxide Eluent[a]

Resin, ml	Cation-Exchange Capacity, mEq/Column[b]	Volume of 1 *N* NaOH Eluent, ml	$A_{242\ nm}$	$A_{242\ nm} - A_{263\ nm}$
4	1.6	2 × 5	0.930	0.888
4	1.6	2 × 5 + 3	0.930	0.888
4	1.6	5 × 5	0.938	0.893
5	2.0	5 × 5	0.930	0.888
6	2.4	5 × 5	0.930	0.888
7	2.8	5 × 5	0.927	0.887
0	—	—	0.945	0.903

[a] Oxidation time = 25 min; milligrams of I per sample = 0.662, and milliliters of ceric reagent = 8.0. [b] Exchange capacity = 0.4 mEq/ml from manufacturer's label.

EXPERIMENTAL

Apparatus—A recording spectrophotometer[1] was used to record the spectrogram of benzaldehyde in isooctane solution. Matched 1.000-cm, fused silica, bottle-type cells were used in making all spectrophotometric measurements. The ion-exchange columns used were 0.8-cm i.d. × 19-cm long glass tubing with a Teflon stopcock at the bottom and a 2.5-cm i.d. × 10-cm reservoir at the top.

Reagents—A strongly acidic cation-exchange resin[2], 50–100 mesh, H^+ form, was used. Before use it was conditioned as described later.

Ceric ammonium sulfate[3] was reagent grade, and isooctane was 99.5+% practical grade[4]. Compound I was NF grade, and the other chemicals used were either reagent grade or spectral grade. Other drugs, preservatives, dyes, and excipients used in preparing the "blank syrup" were USP or NF grade where applicable. Otherwise, they were of standard pharmaceutical grade.

The ceric reagent was approximately 0.2 *M* ceric ammonium sulfate in 6 *N* sulfuric acid. It was prepared by suspending 31.6 g of ceric ammonium sulfate dihydrate in 250 ml of 6 *N* sulfuric acid, stirring overnight, and filtering off the undissolved solid.

Preparation of Resin Column—The specific volume of resin (4–7 ml), measured in a small graduate as the settled volume under water, was quantitatively rinsed into the glass column which had a small wad of glass wool placed in the bottom. The resin was conditioned by successively passing 6 *M* hydrochloric acid, water, and 1 *M* sodium hydroxide through it. The resin is sufficiently clean when the 1 *M* sodium hydroxide effluent shows an absorbance of less than 0.025 at 242 nm when measured in a 1-cm cell against 1 *M* sodium hydroxide as the reference.

After cleaning, the resin was rinsed successively with water, 50 ml of 6 *M* hydrochloric acid, water, 50 ml of 10% ammonia, and, finally, water until the effluent was neutral to litmus. The resin is in the ammonium form at this point and is ready for use in the assay.

General Procedure—Aqueous syrup or standard solutions containing 0.012–0.033 mg I/ml were prepared by appropriately diluting an aliquot of a syrup or a stock standard solution. The solution was filtered if not clear, and an aliquot of the clear solution containing 0.66 mg of I was pipeted into the reservoir on top of the resin column and allowed to flow through the resin at a rate

[1] Coleman-Hitachi, model EPS-3T.
[2] AG-50W-X1, Bio-Rad Laboratories.
[3] G. F. Smith Co.
[4] Eastman Organic Chemicals.

Table III—Composition of "Blank Syrup"[a]

Ingredients	Milligrams per Milliliter	Milliequivalents Cation per Milliliter
Active ingredients:		
Pyrilamine maleate	2.5	0.0124
Codeine phosphate	2.0	0.0049
Dextromethorphan hydrobromide	2.0	0.0074
Hydrocodone bitartrate	1.0	0.0020
Phenylephrine hydrochloride	2.0	0.0103
Ammonium chloride	12.0	0.224
Excipients:		
Methylparaben	13.5	—
Propylparaben	1.5	—
Sodium citrate dihydrate	4.2	0.0428
Citric acid	8.4	0.131
Sorbo	7.5% v/v	—
Liquid sugar	60.0% v/v	—
Carkole brown shade, 60%	0.015	—
FD&C Yellow No. 6, 92%	0.33	—
FD&C Red No. 2, 92%	0.109	—
Minoline green	0.050	—
FD&C Casiline Orange, 92%	0.080	—
Imitation loganberry flavor	0.35% v/v	—
Imitation cherry flavor	0.20% v/v	—
Imitation wild cherry flavor	0.04% v/v	—
Caramel	0.05%	—
Total		0.435

[a] In the experiments, a 20.0-ml aliquot of this syrup in 50 ml of solution was passed through the resin column.

of 0.4 ml/min. The reservoir was rinsed with 5–10 ml of water at the same flow rate, and then the resin bed was washed with eight 25-ml portions of water at a rate of about 2 ml/min. The final few milliliters of wash water was collected and its absorbance at 242 nm was measured to ensure that all noncationic UV absorbers had been removed. In every case the absorbance was less than 0.06 at this point.

Elution was accomplished by passing two 5-ml portions and one 15-ml portion of 1 *M* sodium hydroxide solution through the resin, again at a rate of 0.4 ml/min, allowing each portion of eluent to run completely into the resin before adding the next. This hydrolyzed the II to mandelate ion on the resin bed and eluted the latter. The alkaline eluate was collected in a 125-ml separator fitted with a Teflon stopcock and extracted with two 25-ml portions of chloroform. The chloroform layers were discarded. Filtered air was then bubbled through the aqueous solution until the odor of chloroform had completely disappeared.

Next, 8.0 ml of the ceric reagent and 25.0 ml of isooctane were added and the mixture was agitated for 25 min; then 8.0 g of sodium chloride was added and the separator was shaken for about 1 min. The phases were allowed to separate, and 20.0 ml of the clear isooctane phase (centrifuge if emulsified) was pipeted into a 50-ml ground-glass-stoppered centrifuge tube. This was extracted in the centrifuge tube with 8.0 ml of 1 *M* hydrochloric acid solution, 32% in sodium chloride. The phases were allowed to separate, and a portion of the clear isooctane layer was scanned against isooctane on a recording spectrophotometer between 340 and 220 nm.

When using the proposed procedure for the analysis of syrups, the milligrams of I per milliliter of syrup was calculated by the formula:

$$\text{mg I/ml syrup} = \frac{(A_{242\,nm} - A_{263\,nm})\ \text{sample}}{(A_{242\,nm} - A_{263\,nm})\ \text{std.}} \times \frac{\text{mg I std.}}{\text{ml syrup}} \quad \text{(Eq. 1)}$$

where "mg I std." and "ml syrup" are the amounts loaded onto the resin column.

Procedure without Ion Exchange—In some experiments the ion-exchange step was eliminated. In these experiments, 25.00 ml of an aqueous solution that was 1 *M* in sodium hydroxide and contained about 0.66 g of I and 0–500 mg of ammonium chloride, as specified below, was pipeted into a 125-ml separator. After allowing it to stand for at least 30 min to ensure complete hydrolysis, the solution was extracted with two 25-ml portions of chloroform and the aqueous phase was bubbled free of the odor of chloroform as already described. Then a volume of from 6.0 to 10.0 ml of the ceric reagent was added, as specified below, along with 25.0 ml of isooctane, and the mixture was agitated for the specified time. From this point the remainder of the procedure is identical to that given under *General Procedure*.

RESULTS AND DISCUSSION

This section reports the results of experiments designed to determine the optimum conditions for analysis such as oxidation time, reagent concentrations, and elution conditions. It also includes the results of analyses performed on a synthetic syrup formulation, the results obtained when standard additions were made to commercial syrups, and typical results of analyses of commercial syrups.

Optimum Conditions of Analysis—To optimize the post-ion-exchange steps in the procedure, the influence of oxidation conditions was investigated in a simulated column eluate solution. Patel and Lemberger (10) reported a second-order rate constant of 1.11 liters $mole^{-1}$ sec^{-1} for the alkaline hydrolysis of I at 20°. Thus, the (pseudo) first-order half-time in 1 *M* sodium hydroxide is 0.62 sec. It is apparent that the minimum 30-min hydrolysis time in approximately 1 *M* sodium hydroxide, called for in the *Procedure without Ion Exchange* in the present study, is more than adequate for quantitative hydrolysis of I.

To determine the effect of oxidation time on the final benzaldehyde recovery, an experiment was performed in which the amounts of I, ammonium chloride, sodium hydroxide, and ceric oxidant were held constant while the time of oxidation was varied. Ammonium chloride was included because the resin column is used in the ammonium form and, consequently, there is ammonia in the column eluate solution in which the oxidation is performed. The amount of ammonium chloride included in this experiment is equivalent to the amount of ammonia present on 4 ml of resin. Under the experimental conditions, maximum recovery of benzaldehyde is obtained for oxidation times of 15–35 min (Table I).

The third column in Table I gives the difference between the absorbance at the benzaldehyde maximum and minimum. This figure was used for calculating assay results since it is less sensitive to background absorbance than the absorbance at the maximum alone.

To determine the effect of using different amounts of ceric reagent and the influence of ammonium chloride on the quantitation, an experiment was performed in which these quantities were varied while other conditions were held constant as described in the *Experimental* section. It was found that variation of the volume of ceric reagent from 6.0 to 10.0 ml changed the final absorbance by less than 1.5% and that variation of the amount of ammonium chloride from 0 to 500 mg in the 25.00-ml sample had no effect on the final absorbance.

The distribution coefficient of benzaldehyde between isooctane and water is about 9 and that between isooctane and 32% aqueous sodium chloride[5] is about 45. Consequently, the addition of sodium chloride is made to the separator after oxidation is complete to improve the extraction of benzaldehyde. Addition of the salt prior to complete oxidation is undesirable because it would alter the oxidation potential of the Ce (IV)–Ce (III) half-reaction (11) and prolong the oxidation step. Washing of the isooctane layer to reduce the background absorbance was done with 32% sodium chloride in dilute hydrochloric acid for the same reason. The net loss of benzaldehyde due to incomplete extraction in the oxidation and wash steps is thus expected to be about 3%.

The absolute recovery of benzaldehyde in the oxidation step may be calculated by comparing the $A_{242\,nm}$ with that predicted from a knowledge of the molar absorptivity of benzaldehyde [$\epsilon_{242\,nm}$ = 14,000 (12)] and the amount of I taken. The recovery obtained for standards not subjected to ion exchange, using 8.0 ml of ceric reagent and 25 min oxidation time, was 95%. This figure, plus the calculated loss of 3% due to incomplete extraction, yields a total absolute recovery of about 98% of the theoretical amount. This recovery is equal to 100% within the experimental

[5] Determined by T. Meites in this laboratory.

Table IV—Analysis of Simulated Syrups by the Proposed Method[a]

Blank Syrup	Syrup Passed through Resin[b], ml	I Added, mg	Mandelic Acid Added, mg	$A_{242\ nm}$	$A_{242\ nm} - A_{263\ nm}$
Room temperature sample	20.0	0.680	0	0.943, 0.933	0.898, 0.890
	20.0	0	0	0.008	0.005
	20.0	0	0.270	0.012	0.003
60° sample	20.0	0.680	0	0.987, 0.985	0.906, 0.900
	20.0	0	0	0.062	0.023
No syrup (standard), subjected to ion exchange	0	0.680	0	0.958, 0.957	0.914, 0.913

[a] Used 4.0-ml resin column; oxidation time = 25 min, milliliters of ceric reagent = 8.0. [b] The 20.0 ml syrup contained in 50.0 ml of solution passed through the resin.

Table V—Analysis of Commercial Syrups for I[a]

Syrup Sample	Label Claim, mg I/ml	Age of Syrups, years	Added I, mg/ml	Milligrams I per Milliliter Found[b]	Recovery of Added I, %
A	0.033	7.0	0.0	0.0335	—
A	0.033	7.0	0.0342	0.0680	101
B	0.055	0.5	0.0	0.056	—
C	0.11	1.0	0.0	0.108	—
D	0.165	0.2	0.0	0.170	—
E	0.33	0.7	0.0	0.342	—
F	0.33	7.1	0.0	0.274	—
F	0.33	7.1	0.342	0.614	99

[a] Oxidation time = 25 min, milliliters of resin = 4.0, and milliliters of ceric reagent = 8.0. [b] Calculated by the formula:

$$\frac{(A_{242nm} - A_{263nm})\ \text{sample}}{(A_{242nm} - A_{263nm})\ \text{std. on resin}} \times \frac{\text{mg I std. on resin}}{\text{ml syrup on resin}}$$

error of the molar absorptivity used, indicating that the oxidation of mandelate to benzaldehyde is virtually quantitative.

Conditions of Ion-Exchange Separation—The strongly acidic sulfonate resin column was used in the ammonium form rather than in the hydrogen form because it was felt, initially, that acid hydrolysis of II on the resin column would be less likely. No experiments have been done to verify this, and it is quite possible that the H^+ resin could be used in the procedure. Compound II is, in fact, rather stable toward acid hydrolysis (8).

An experiment was performed to evaluate the influence of various parameters on the ion-exchange separation of II. A constant amount of I was loaded onto columns containing various amounts of resin, washed with 200 ml of water, and eluted with different volumes of 1 *M* sodium hydroxide into 125-ml separators. Then an amount of 1 *M* sodium hydroxide was added to the separator to bring the total volume of solution to 25 ml. The analysis was performed from this point on as already described.

It is apparent from Table II that the 4.0 ml of resin and the 2 × 5 + 15 ml of sodium hydroxide eluent called for in the *General Procedure* are quite adequate to retain I quantitatively, to hydrolyze II, and to elute mandelate. The last row in Table II shows the absorbance found for a sample containing the same amount of I in 25 ml of 1 *M* sodium hydroxide but not subjected to ion exchange prior to oxidation. It can be seen from Table II that all of the ion-exchange conditions used afforded the same recovery, *i.e.*, 98–99%, compared to the standard that was not subjected to ion exchange. This slight loss of I in standards subjected to ion exchange has been found to vary between 0.5 and 2.0% through numerous analyses. This apparent loss of I in the ion-exchange step is small and reproducible within the experimental error. To eliminate any systematic bias as a result of it, the standards called for in the *General Procedure* are subjected to the ion-exchange step as are the samples.

Analysis of Simulated Syrup—To verify the quantitative character of the proposed analytical procedure, a "blank syrup" was prepared in the laboratory. It contained many common drugs, preservatives, flavors, dyes, and other excipients but no homatropine methylbromide (Table III). A portion of the syrup was stored at room temperature for about 2 weeks, and another portion was stored at 60° for the same period. Then 40.0-ml aliquots of each were pipeted into 100-ml volumetric flasks, an aliquot of an aqueous stock solution containing 1.360 mg of I was added to each, and the solutions were diluted to 100 ml with water. Then 50.0-ml aliquots were analyzed by the *General Procedure* for I. The experiment was repeated on the room temperature and 60° samples without the addition of I and also on the room temperature sample without I but with the addition of an amount of mandelic acid equivalent to 0.66 mg of I.

These last three blank samples were run to determine the background spectrum and to demonstrate that mandelic acid is not retained on the resin column under the conditions of analysis. The amount of sample taken was equal to that which would be used for a syrup containing about 0.033 mg I/ml; furthermore, the number of ingredients is considerably greater than would be found in any one pharmaceutical formulation. The third column of Table III lists the number of milliequivalents of exchangeable cationic substances in a milliliter of the prepared syrup. Since the 50.0 ml of diluted syrup solution that is passed through the resin in the loading step contains 20.0 ml of syrup, the total of exchangeable cations passed through the resin in this experiment is 8.7 mEq.

Although this figure considerably exceeds the total exchange capacity of the 4-ml resin bed, 98–99% recovery of I was obtained in the analyses of both room temperature and 60° samples when compared to the standard subjected to ion exchange (Table IV). In addition, the background absorption is small, even in the 60° sample, and is roughly constant between 263 nm, the wavelength of minimum absorbance, and 242 nm, the wavelength of maximum absorbance of benzaldehyde. Thus, by subtracting the former absorbance from the latter in all samples and standards, an adequate background correction can be made even in the analysis of samples that are several years old or that have been stored at elevated temperatures.

The blank sample containing mandelic acid, the hydrolysis

product of I, yielded no absorbance at 242 nm, indicating that the method is stability indicating with respect to hydrolytic decomposition of I.

Analysis of Commercial Syrups—Typical analyses of six commercial syrups, 0–7 years old, are presented in Table V. These syrups contained from one to three of the active ingredients listed in Table III at levels equal to or less than those listed, plus liquid sugar and various of the dyes, flavors, preservatives, and excipients listed. To verify that the conditions that prevail in old syrups do not interfere with the analysis, the standard addition technique was employed with Syrups A and F. In this technique, one aliquot of the syrup was analyzed in the regular manner while a second, equal aliquot was analyzed after the addition of an amount of I equal to that in the syrup itself. The resulting isooctane solution for this sample was diluted in half prior to scanning on the spectrophotometer. It can be seen from Table V that the recovery of added standard is quantitative.

When analyzing aged samples, emulsion formation is often encountered in the oxidation step. However, a clear isooctane layer is readily obtained in these cases upon centrifugation. In addition, old samples of syrup produce considerable darkening of the ion-exchange resin and a small amount of background in the benzaldehyde spectrum, even after washing with the hydrochloric acid-salt solution. This background is readily corrected for by using the absorbance difference between 242 and 263 nm in calculations.

Although phenylephrine is known to undergo periodate oxidation to *m*-hydroxybenzaldehyde (13), no spectral evidence of this strong UV absorber was detected in the final isooctane solution resulting from the analysis of commercial syrup or blank syrups containing phenylephrine.

SUMMARY

The proposed method of analysis for homatropine methylbromide in syrup formulations is sensitive, specific, and stability indicating. It permits the routine, accurate analysis of I in syrups at the lowest dosage levels commercially available and is suitable for the analysis of darkened, aged samples.

No attempt was made to determine the maximum sensitivity of this method. It is apparent, however, that lower doses and different formulations, containing larger amounts of other substances, could be analyzed for I by this method or by a modification of it using, for example, a larger amount of ion-exchange resin and a longer path length cell for recording the spectrogram.

The method and minor modifications of it have been used routinely for several years for the analysis of I in syrups, elixirs, and complex tablet formulations.

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[x] To whom inquiries should be directed.

Determination of Sodium Penicillin G in Disodium Carbenicillin Preparations

JAN BIRNER

Abstract □ Aqueous solutions of disodium carbenicillin containing sodium penicillin G (sodium benzylpenicillin) are chromatographed by TLC using silica gel on aluminum foil and acetone–chloroform–acetic acid–water (50:45:5:1 v/v) as a developing solvent. The location of penicillin G is determined by reference to standard strips cut from the edges of the chromatogram and visualized colorimetrically. The appropriate area is removed and penicillin is eluted from the silica with phosphate buffer at pH 7.0. The amount of penicillin is determined spectrophotometrically after formation of penicillenic mercuric mercaptide formed by heating penicillin with an imidazole reagent containing mercury.

Keyphrases □ Carbenicillin—TLC analysis of penicillin G (benzylpenicillin) content □ Disodium carbenicillin—TLC analysis of sodium penicillin G (sodium benzylpenicillin) content □ Penicillin G (sodium)—TLC analysis as impurity in disodium carbenicillin □ Sodium penicillin G—TLC analysis as impurity in disodium carbenicillin □ Benzylpenicillin—TLC analysis as impurity in carbenicillin □ TLC—analysis, sodium penicillin G (sodium benzylpenicillin) in disodium carbenicillin

Disodium carbenicillin (the disodium salt of α-carboxybenzylpenicillanic acid) is a semisynthetic penicillin prepared from monobenzylphenylmalonyl chloride and 6-aminopenicillanic acid. A subsequent reduction of this compound under specific conditions yields disodium carbenicillin.

product of I, yielded no absorbance at 242 nm, indicating that the method is stability indicating with respect to hydrolytic decomposition of I.

Analysis of Commercial Syrups—Typical analyses of six commercial syrups, 0–7 years old, are presented in Table V. These syrups contained from one to three of the active ingredients listed in Table III at levels equal to or less than those listed, plus liquid sugar and various of the dyes, flavors, preservatives, and excipients listed. To verify that the conditions that prevail in old syrups do not interfere with the analysis, the standard addition technique was employed with Syrups A and F. In this technique, one aliquot of the syrup was analyzed in the regular manner while a second, equal aliquot was analyzed after the addition of an amount of I equal to that in the syrup itself. The resulting isooctane solution for this sample was diluted in half prior to scanning on the spectrophotometer. It can be seen from Table V that the recovery of added standard is quantitative.

When analyzing aged samples, emulsion formation is often encountered in the oxidation step. However, a clear isooctane layer is readily obtained in these cases upon centrifugation. In addition, old samples of syrup produce considerable darkening of the ion-exchange resin and a small amount of background in the benzaldehyde spectrum, even after washing with the hydrochloric acid-salt solution. This background is readily corrected for by using the absorbance difference between 242 and 263 nm in calculations.

Although phenylephrine is known to undergo periodate oxidation to *m*-hydroxybenzaldehyde (13), no spectral evidence of this strong UV absorber was detected in the final isooctane solution resulting from the analysis of commercial syrup or blank syrups containing phenylephrine.

SUMMARY

The proposed method of analysis for homatropine methylbromide in syrup formulations is sensitive, specific, and stability indicating. It permits the routine, accurate analysis of I in syrups at the lowest dosage levels commercially available and is suitable for the analysis of darkened, aged samples.

No attempt was made to determine the maximum sensitivity of this method. It is apparent, however, that lower doses and different formulations, containing larger amounts of other substances, could be analyzed for I by this method or by a modification of it using, for example, a larger amount of ion-exchange resin and a longer path length cell for recording the spectrogram.

The method and minor modifications of it have been used routinely for several years for the analysis of I in syrups, elixirs, and complex tablet formulations.

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[x] To whom inquiries should be directed.

Determination of Sodium Penicillin G in Disodium Carbenicillin Preparations

JAN BIRNER

Abstract □ Aqueous solutions of disodium carbenicillin containing sodium penicillin G (sodium benzylpenicillin) are chromatographed by TLC using silica gel on aluminum foil and acetone–chloroform–acetic acid–water (50:45:5:1 v/v) as a developing solvent. The location of penicillin G is determined by reference to standard strips cut from the edges of the chromatogram and visualized colorimetrically. The appropriate area is removed and penicillin is eluted from the silica with phosphate buffer at pH 7.0. The amount of penicillin is determined spectrophotometrically after formation of penicillenic mercuric mercaptide formed by heating penicillin with an imidazole reagent containing mercury.

Keyphrases □ Carbenicillin—TLC analysis of penicillin G (benzylpenicillin) content □ Disodium carbenicillin—TLC analysis of sodium penicillin G (sodium benzylpenicillin) content □ Penicillin G (sodium)—TLC analysis as impurity in disodium carbenicillin □ Sodium penicillin G—TLC analysis as impurity in disodium carbenicillin □ Benzylpenicillin—TLC analysis as impurity in carbenicillin □ TLC—analysis, sodium penicillin G (sodium benzylpenicillin) in disodium carbenicillin

Disodium carbenicillin (the disodium salt of α-carboxybenzylpenicillanic acid) is a semisynthetic penicillin prepared from monobenzylphenylmalonyl chloride and 6-aminopenicillanic acid. A subsequent reduction of this compound under specific conditions yields disodium carbenicillin.

Table I—Estimation of Sodium Penicillin G Added to 200 μg of Commercial Disodium Carbenicillin

Added, μg	Found, μg	Recovery, %
0	9.3 ± 0.9	—
10	18.8 ± 0.4	95.0
20	28.7 ± 1.3	97.0
30	37.9 ± 0.6	95.3

Disodium carbenicillin has a different bacteriological spectrum from penicillin G (benzylpenicillin) and is active against Gram-negative organisms. Commercial preparations of disodium carbenicillin contain sodium penicillin G but the amount should not exceed 5% w/w (1).

The present BP method (2) for the determination of benzylpenicillin sodium in disodium carbenicillin is based on electrophoretic separation of the penicillins (3) and biological determination of penicillin G. As an alternative, the method reported here was developed and is based on the chromatographic separation of the penicillins, using plates of silica gel on aluminum foil and acetone–chloroform–acetic acid–water (50:45:5:1 v/v) as the developing solvent. The R_f value for penicillin G is between 0.51 and 0.53. A strip containing penicillin is removed from both edges of the plate, the penicillin is hydrolyzed in ammonia vapor, and the penicillin spot is located by starch iodine spray. By using these strips as a guide, the penicillin G zones are located on the remaining portion of the plates. The zones are cut out and the silica is transferred into test tubes and eluted with phosphate buffer. The eluted penicillin is determined spectrophotometrically as the mercuric mercaptide of penicillenic acid (4). A set of standards is run simultaneously with the samples on the same plate.

EXPERIMENTAL

Reagents, Solutions, and Materials—The following were used: acetone, analytical reagent; acetic acid, analytical reagent; chloroform BP; TLC aluminum sheets[1], silica gel, 20 × 20 cm (without fluorescent indicator), layer thickness 0.25 mm; imidazole[1], recrystallized twice from benzene; mercuric chloride, analytical reagent; hydrochloric acid, 5 *M*; sodium penicillin G, standard grade, BP; chromatographic tank for 20 × 20-cm plates; microsyringe, 30-μl capacity with 1-μl divisions; 1% soluble starch solution[2]; 0.1 *N* iodine in 4% (w/v) potassium iodide in water; ammonia, 0.880 specific gravity; and 1% phosphate buffer, pH 7.0 (91 g Na_2HPO_4, 62 g $NaH_2PO_4 \cdot 2H_2O$, and 350 ml water as stock solution; dilute 24 ml to 1 liter and autoclave).

Procedure—Prepare freshly from the test sample of disodium carbenicillin a 20-mg/ml solution in water and 0.5-, 1-, and 2-mg/ml solutions of standard sodium penicillin G in water. The sodium penicillin G used had a potency of 1655 u/mg.

Using a 20 × 20-cm silica plate on aluminum foil, divide the plate using lead pencil marking into seven 25-mm sections and a strip 12.5 mm wide on each side. At one end of the 25-mm sections, apply (in triplicate), using the microsyringe, 10 μl of the sample (200 μg carbenicillin), each in the form of a strip 12–15 mm long; then apply four standards of sodium penicillin G (5, 10, and 20 μg, the last in duplicate) in the same manner. Use two such plates. Place the plates in a suitable chromatographic tank containing the developing solvent of acetone–chloroform–acetic acid–water (50:45:5:1 v/v) and develop for a distance of about 13 cm (1.5–2 hr). Dry the plates and cut a 37.5-mm strip from both sides of each plate. Hang the cut strips in the vapor in a jar containing ammonia for 5–10 min, dry in an air current, and spray with starch iodine solution. This solution is prepared by mixing 50 ml of 1% soluble starch with 1 ml of 0.1 *N* iodine in 4% (w/v) KI and 3 ml acetic acid.

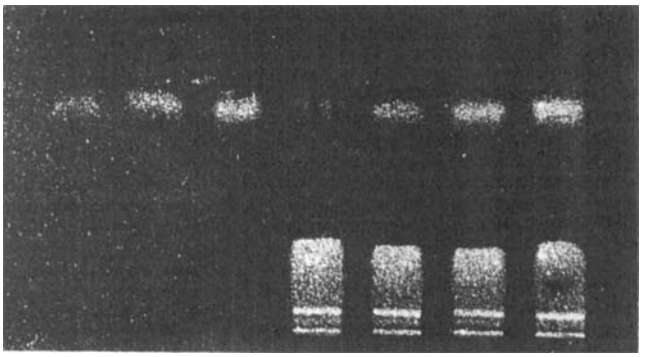

Figure 1—*Chromatogram of penicillin G standards (top) and carbenicillin (bottom) containing increasing amounts of penicillin G.*

The strips with penicillin G spots located in this manner are used as a template for locating the separated penicillin G on the remaining five sections of the developed plate. Cut out the located spots and transfer the silica quantitatively to centrifuge tubes, 11.5 × 1.5 cm. To each tube add 2.5 ml of 1% phosphate buffer, pH 7.0, and stir for 3 min on a mixer[3], placing a small glass bead into each test tube to help break up the silica particles. Centrifuge the tubes for 10 min at 2000 rpm, and assay the solutions for penicillin according to Bundgaard and Ilver (4).

Prepare 100 ml of the imidazole reagent by dissolving 8.25 g of imidazole in 60 ml of water and adding 10 ml of 5 *M* hydrochloric acid and then 10 ml of a solution of mercuric chloride (0.27 g dissolved in 100 ml of water). Adjust the pH to 6.80 ± 0.05 with 5 *M* hydrochloric acid and bring the volume to 100 ml with water. From each of the centrifuged penicillin solutions, pipet two 1-ml samples into two suitable test tubes, one serving as a sample, *A*, the other as a blank, *B*. To the *A* samples, add 5 ml of imidazole reagent; after mixing, stopper the tubes and incubate in a water bath at 60° for 25 min. After cooling to 20°, measure the *A* samples spectrophotometrically at 325 nm, using as a blank 1 ml of phosphate buffer mixed with 5 ml imidazole reagent. To the *B* samples, add 5 ml of water and measure at 325 nm, using as a blank 1 ml of phosphate buffer and 5 ml of water. The difference between *A* and *B* determines the penicillin concentration. Plot a standard reference curve from the standard values and calculate the amount of sodium penicillin G in the sample.

RESULTS AND DISCUSSION

Using the described technique, several samples of commercial carbenicillin were assayed and the amount of sodium penicillin G found was between 3.5 and 4.9%.

According to the study of Bundgaard and Ilver (4), the standard deviation in penicillin G assays is 0.45% when 20 assays are performed on the same solution. Since no pure carbenicillin was available, a commercial preparation was selected and assayed by the described procedure. The amount of sodium penicillin G found was 4.65%.

To evaluate the accuracy of the procedure, an extra amount of penicillin G was added to the sample to furnish an additional three solutions, each containing the same amount of carbenicillin (200 μg) and an additional 10, 20, and 30 μg of sodium penicillin G for each 10 μl. The original sample and three additional samples with corresponding standards were run through the same

[1] E. Merck.
[2] Analar B.D.H.
[3] Vortex J. R. mixer.

procedure, and the amount of sodium penicillin G was determined. The data from three experiments run in duplicate are given in Table I; the recoveries were 95–97%.

A photograph of a developed chromatogram containing standards of sodium penicillin G, commercial disodium carbenicillin, and commercial disodium carbenicillin with additional sodium penicillin G after ammonia treatment and spraying with starch iodine is shown in Fig. 1.

In comparison with the electrophoretic method of penicillin separation and subsequent biological assay of the separated penicillin, this method seems to be much simpler and several assays can be performed on the same day.

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PHARMACEUTICAL TECHNOLOGY

Tensile Strength of Compressed Powders and an Example of Incompatibility as End-Point on Shear Yield Locus

E. N. HIESTAND[x] and C. B. PEOT

Abstract □ Three methods of measuring tensile strengths of compacted powders are described. These methods include transverse compression using squares and disks and the direct tensile stressing of lightly pressed powder. The transverse compression of squares gave the best overall results. The tensile strength of sitosterols NF was found to be too large to be compatible with its use as the end-point of the shear yield locus. However, a Warren–Springs-type equation can be used to describe the yield locus if the tensile strength is replaced by a smaller internal cohesion. A method of estimating both the internal cohesion and the exponent of the Warren–Springs equation from shear cell data is described.

Keyphrases □ Tensile strength, compressed powders—determined by transverse compression of squares and disks and direct tensile stressing, incompatibility as end-point on shear yield locus, internal cohesion estimated, Warren–Springs-type equation, relevance to yield locus □ Powders, compressed—tensile strength determined by transverse compression of squares and disks and direct tensile stressing, incompatibility as end-point on shear yield locus, internal cohesion estimated, Warren–Springs-type equation, relevance to yield locus □ Shear cell data—method of estimating internal cohesion and exponential of Warren–Springs equation, tensile strength of compressed powders □ Yield locus—testing of compressed powders, tensile strength role reevaluated

Tensile strength measurements of powders compacted to various degrees provide a direct measurement of the bonding potential[1] of a given solid material. This information is useful to the formulator in the selection of excipients. An excessively strong bond may prevent rapid disintegration and concomitant dissolution. Very weak bonding characteristics may limit the selection and/or quantity of lubricant that may be added to a formulation. In addition to these obvious practical uses of tensile strength information, the tensile strength has been used in characterizing flow properties. Specifically, the tensile strength has been designated as: *(a)* a point on the shear failure yield locus (1–3), and *(b)* a limiting factor to the extension of the yield locus (4). The magnitude of the tensile strength played an important role in the evolution of a third position presented herein—*viz.*, the tensile strength is not a point on the yield locus and is not predictable from the yield locus of shear failure. This interpretation is a departure from commonly accepted practice and is believed to be an important conclusion of this research.

[1] Conditions in the compaction process must be controlled to produce maximum values if the true bonding potential is to be observed experimentally.

THEORY

The tensile strength of powders is an important measurement for characterizing the interaction between solid particles. The strong interactions are at the true contact areas. The extent of the true area of contact between particles after elastic recovery is dependent on the magnitude of the maximum stress applied and the amount of plastic deformation that has occurred. These concepts are basic to the discussion of the results of the experimental work reported here.

Experimentally, tensile strength is not always a readily mea-

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Experimentally, tensile strength is not always a readily mea-

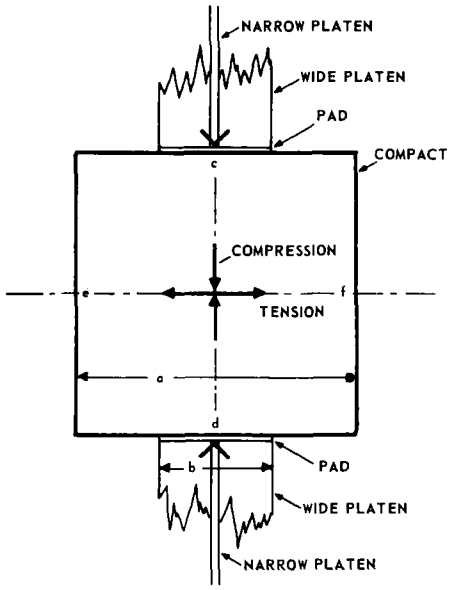

Figure 1—*Transversely compressed square. Key: a, compact width; b, platen width; c–d, vertical bisector; and e–f, horizontal bisector. Planar diagram does not show the thickness, but platen must extend across entire thickness of compact. Only one set of platens is used. The two narrow platens represent one case and the two wide ones represent another case.*

sured property because stress concentration and brittle fracture with crack propagation may cause failure at much lesser mean values of applied stress than the true tensile strength. Also, some methods of determining tensile strength produce compression, shear, and tension stresses simultaneously in different directions within the solid. In these cases, it is necessary to distinguish between shear and tensile failure to assure that the stress at failure is a measure of tensile strength.

Three methods of determining tensile strength are described. Two of these are transverse compression methods: the diametral compression and the compressed square methods. Both use intact compacts removed from the die after compression. They differ from each other only in the geometry of the compact and the relative area of the stress application. The third method is a direct tensile measurement using much smaller compressive loads than would be required to make compacts of sufficient strength to be removed from a die.

Transverse Compression Methods—It is assumed that many scientists interested in pharmaceutical formulations have not

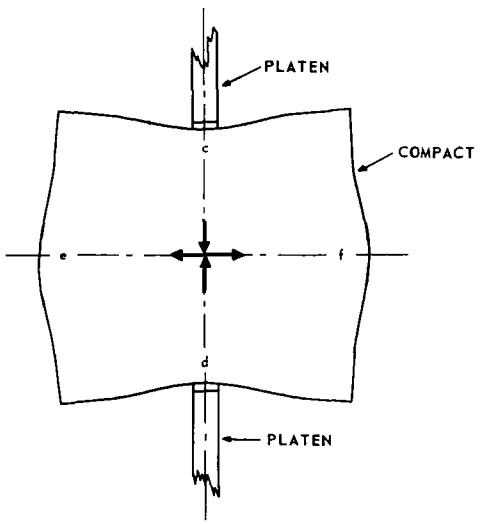

Figure 2—*Exaggerated elastic distortion of a square compact under transverse compression.*

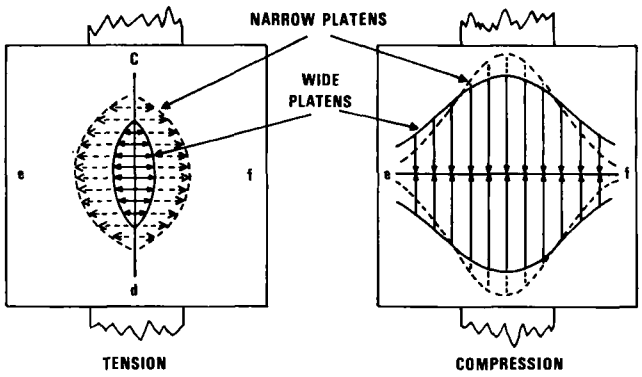

Figure 3—*Diagrammatic representation of stresses along bisectors of the compact: tension stresses along c–d on the left and compression stresses along e–f on the right. Solid lines are for case with platen widths about 0.4 of the width of the compact, and dashed lines are for platen width of about 0.1 of the width of the compact. Relative length of arrows indicates the approximate relative magnitude of the stresses.*

been involved in stress analysis problems. In the absence of such a background, the relationship between compression in one direction and a resultant tension may not be obvious, the tension being in a direction normal to the compression. Therefore, a somewhat oversimplified discussion of concepts is presented in the discussion of the transverse compression of square wafers.

Compressed Square—Figure 1 illustrates the case of the square. If a compressive load is applied at the edge of the compact through the very narrow platens (indicated by wide arrows), the shear stresses would be very large at these edges and would cause failure in shear. However, temporarily it is convenient to ignore the shear stresses and consider the compressive and tensile stress distribution within the compact.

When pressure is applied vertically across the center, elastic deformation occurs. The grossly exaggerated elastic distortion of the square after compression would be somewhat like that shown in Fig. 2. The compressive force causes displacement of molecules, and this displacement is transmitted from one molecule to the next. The resistance to compression of molecules to the left and right of the compressed bisector region results in an imbalance of forces which produces tension horizontally in the central region of the compact. The curves in Fig. 3 show diagrammatically the distribution of these stresses along the vertical and horizontal bisectors. The solid curves describe the case of wider[2] platens, and the dashed curves are for narrower platens. If the platens are as wide as the compact ($b/a = 1$), no tension develops and the compressive stress is a constant across the compact. Based on Fig. 3, the narrower platens would appear to be desirable. However, shear stresses also are present; they are not shown in Fig. 3. Berenbaum and Brodie (5) used photoelastic methods to evaluate the stresses and concluded that the maximum shear stresses occur at the edges of the platens. With very narrow platens, these shear stresses usually exceed the shear strength before the tensile stress exceeds the tensile strength at any region of the compact. Thus, failure in shear occurs instead of tensile failure. However, the shear stresses at the platen edges may be reduced by distributing the compressive load over a somewhat larger surface area. Also, stress concentration may be reduced by using padded platens[3]. The pads are sufficiently deformable to avoid excessive stress concentration at surface irregularities. Figure 4 shows examples of failure patterns. Wider platens also reduce the tensile stress that develops at the center of the compact, fortunately, at a different rate from the accompanying reduction in shear stress. Thus, with the proper choice of platen widths, the ratio of the shear stress to tensile stress can be minimized. This is the optimum condition attainable for producing tensile failure. Berenbaum and Brodie (5) reported that the ratio of shear stress

[2] The platen width will be expressed as a fraction of the compact width (fraction b/a in Fig. 1). The platens extend completely across the thickness of the compact.

[3] In the authors' laboratory, pads are made from ink blotters. Double-sided adhesive, pressure-sensitive tape is used to fasten the blotter to the platen and to laminate the blotter to prepare double-thickness pads.

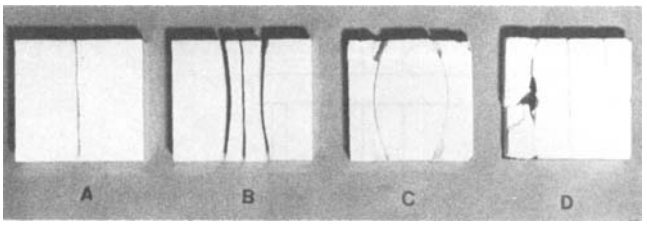

Figure 4—*Photograph of square compacts broken by transverse compression. Tensile failure occurs when compact breaks in a central vertical line. Platen width/compact width = 0.4. Key: A, tensile failure; B and D, tensile failure accompanied by secondary failures; and C, shear failure.*

at the platen edge to the tensile strength at the compact center is a minimum when the b/a ratio is about 0.4. This b/a ratio gives a maximum tensile stress equal to 0.16 times the mean compressive stress. Furthermore, if the applied stress (*i.e.*, pressure, not total load) at the platen surface is kept constant as the b/a ratio is varied, the maximum tensile stress occurs when $b/a \approx 0.4$. Therefore, optimum conditions for tensile failure exist when the value[4] of $b/a \approx 0.4$. When tensile failure occurs, the fracture initiates at the point of maximum tensile stress, the center of the compact, and propagates vertically to the platens[5].

Because compacts made by compressing powders are not of uniform density throughout, the photoelastic evaluation made with a homogeneous model material only approximates the stress distributions in the powder compact. However, in the absence of a better value, the 0.16 factor will be used to calculate the tensile strength. As noted later, this 0.16 factor, obtained by photoelastic methods, appears to give tensile strengths comparable to those obtained with circular disks. Values for disks are not dependent on the photoelastic evaluation of models.

Diametrally Compressed Disk—Much of the same discussion could be presented for the stress distribution in the disk in Fig. 5 as for the square wafer. Very narrow platens would produce shear failure. The load may be distributed by using padded platens of finite width. Sometimes limited crushing occurs under the platens. Since crushing changes the geometry of the system, the ratio of the tensile stress to the applied load also may become unknown. Some investigators recommend that the platen width be limited to 0.1 of the diameter of the compact to minimize the changes in the effective width of the platens.

Again, the experimenter must check the shape of the fragments after fracture to determine the mode of failure (8). If the compact splits into two equal halves, tensile failure has occurred. Because the relative platen width is limited by the circular geometry, the shear stresses may be too large in a given material and fracture along a shear plane often occurs[6]. (It is the authors' experience that this same material may fracture in tension when the compressed square is used. Thus, the latter is believed to be the preferred method for general application.)

The diametral compression case has yielded to mathematical evaluation. The tensile strength (8) is given by:

$$\sigma_E = \frac{2F}{\pi Dt} \qquad \text{(Eq. 1)}$$

where σ_E is the tensile strength, F is the load applied diametrally at fracture, D is the diameter of the compact, and t is the thickness of the compact.

Equation 1 applies to knife-edge loading but is not significantly

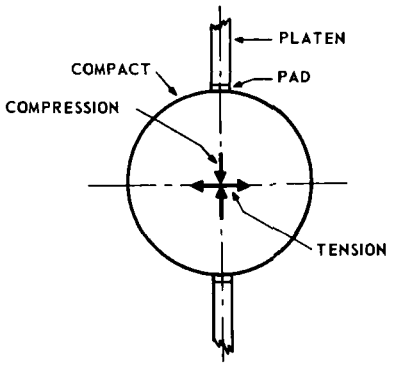

Figure 5—*Diametrally compressed disk.*

in error as long as the platen width is not larger than 0.1 of the specimen diameter (8). Again, for real compacts, the lack of uniform density introduces some error. Thus, tensile strength data, obtained by transverse compression methods, are at best good estimates of the correct value. In practice, the results may be the best estimates obtainable by methods requiring only a reasonable experimental effort.

Simple Tensile Failure Measurements—Conceptually, the simplest method of measuring tensile strength is to measure the force needed to pull an object apart. However, the practical problem proves to be very difficult because, when brittle materials are used, stress concentration and crack propagation cause premature failure. Stress concentration may occur because of the difficulty of attachment of the instrument to a compact in a manner that will pull uniformly on all parts of the compact. Also, the nonuniformity of the internal density of the compact distorts the stress pattern internally so that it is difficult to determine the point of origin of catastrophic failure. Ideally, elongated dumbbell shapes should overcome some of these difficulties. However, it is somewhat impractical to produce these shapes by the compression of powder; furthermore, uniform density may not be achieved. Objects cast from melts cannot be used to characterize the tensile strength of objects produced by a compression–elastic recovery cycle because the factors determining the true area of contact are quite different in the two processes. Therefore, a method was selected that uses compacts of simple shape and requires only very limited compression forces. This method uses forces of compression of the same order of magnitude as are used in the shear cell studies done in these laboratories.

An instrument[7] for determining the tensile strength of powders after subjecting them to small compression loads has been described (10, 11). The cell consists of two lengths of cylindrical tubing that serve as a split die (Fig. 6). These are placed end-to-end in a third, aligning cylinder which may be moved away after the loose compact is formed. The compact is compressed so that it extends across the junction of the other two cylinders. After compaction, the top punch is removed; the aligning cylinder is moved away from the top cylinder, and then the force required to lift the top cylinder and fracture the loose compact is observed. By repeating this process with different amounts of powder in the die, one may prepare a plot of the logarithm of the observed tensile strength *versus* the height of powder above the fracture plane. This semilog plot is then extrapolated to zero height. The extrapolated value is assumed to be the tensile strength at the applied load. Similar plots are prepared by using different loads and repeating the same process for each load. Since the scatter is very large, at least five values for each point on a line were needed, and several points were desired for obtaining a line. The results provide a plot of tensile strength *versus* compaction stress. Obviously, the amount of effort to obtain usable data makes this approach very unsatisfactory. Furthermore, only a few materials gave sufficiently good results to be acceptable. Sitosterols were the most satisfactory.

Obviously, the theoretical considerations are simple unless one attempts to evaluate the effects of lack of uniform powder density in the failure plane. For practical reasons, no consideration was

[4] Efforts to obtain analytical solutions for the stresses during transverse compression of squares (indentation test) have been based on various assumptions (6, 7). The results suggest that the photoelastic results give somewhat high tensile strength values when b/a is less than 0.25. The authors have elected to accept the experimentally determined values of Berenbaum and Brodie (5) instead of results calculated for a hypothetical model case.

[5] Since failure along the bisector leaves two independently stressed fragments between the platens, the stress distribution in each fragment determines whether a second failure with further fragmentation occurs. Even when secondary failure occurs, the measurement of the force required to produce tensile failure still is valid.

[6] In a sufficiently rigid apparatus, progressive shear failure may occur at least with concrete, before fragmentation occurs (9). This suggests that tensile failure may not occur if the rate of supplying energy to the compact is too slow after a critical stress has been attained.

[7] One of these instruments was built at Parke, Davis and Co. Dr. Joseph Samyn kindly loaned the cell components to the authors.

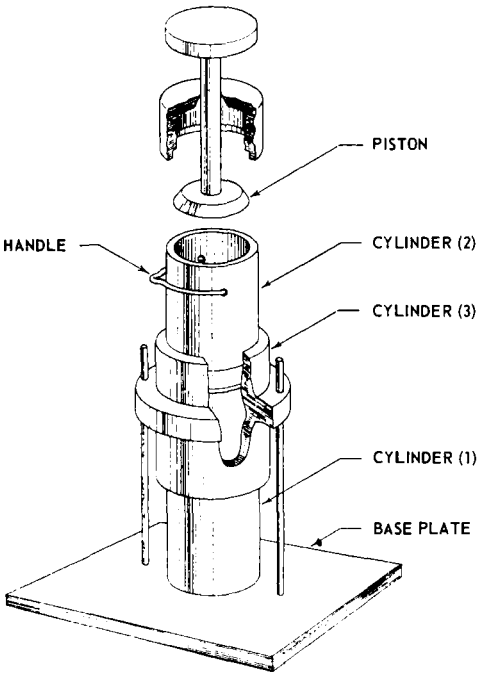

Figure 6—*Cell for direct tensile strength test. (Reproduced, with permission from the American Chemical Society, from Ref. 11.)*

given to this complication and the observed values were accepted *prima facie.*

EXPERIMENTAL

The same procedures were used for both transverse compression methods. A motorized hydraulic jack was used to apply the compressive load (Fig. 7). The lower platen is supported on a piece of brass tubing to which are bonded the wires of a strain gauge. The output of the strain gauge drives a recorder and provides the needed information for calculating the tensile strength. Interchangeable platens permit the selection of the desired platen width. The pressure surfaces of the platens are covered with a double layer of blotter paper which serves as the pad.

A compact is made in a die by compression with a hydraulic press[8]. The maximum compression force is held constant for 1 min. The compact is removed and placed on edge between the platens. The jack motor is started and the hydraulic jack pressure increases until the compact fractures. By preparing a series of compacts at various compression loads, one obtains the data for a plot of tensile strength *versus* compaction stress.

In these experiments, the round compacts were 4.29 cm in diameter and the square ones were 3.81 cm on a side. Since a constant weight of powder was used, the thickness of the compacts varied with the compression force; the thickness was estimated by averaging five values determined with a micrometer. Four measurements were made equally spaced radially near the edge and one was made in the center of the compact.

The direct tensile test apparatus shown in Fig. 6 has the general features already described. Certain modifications were made[9] in the mechanical linkage for pulling the two sections apart. The top cylinder was attached to three fine wires whose opposite ends were attached to a pivot, needle-bearing support. (This assembly replaced the bail handle shown in Fig. 6.) Thus, the tension on the three wires was equalized. The needle bearing was supported from the probe of a strain-gauge assembly[10]. The output of the strain gauge was connected to a recorder.

The base of the cell was clamped to a motor-driven table that moved up and down on ways. This permitted stress to be applied to produce fracture.

[8] Model 341-20, Loomis Engineering and Manufacturing Co., Caldwell, N.J.

[9] In the Upjohn laboratories.

[10] No. 7035, GI 8-350, 8 oz, 12V, Statham Instruments Inc.

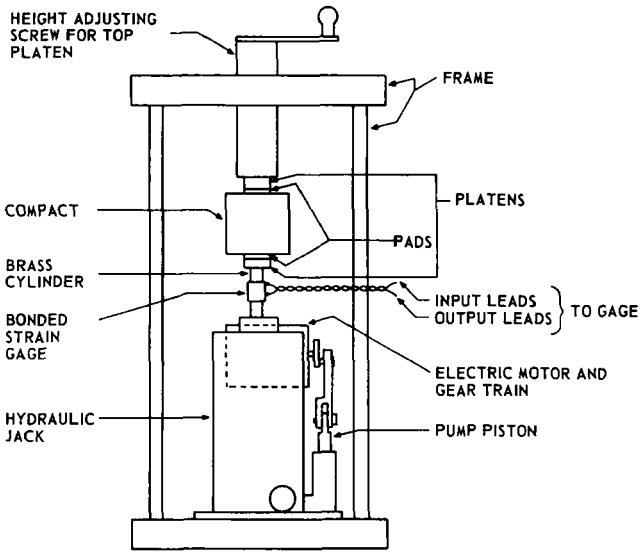

Figure 7—*Schematic diagram of instrumented jack for compression testing of tensile strength.*

RESULTS

Figure 8 compares the results of the two transverse compression tests for powdered sucrose. In both cases, the fracture clearly was tensile failure. These data indicate that the 0.16 factor is approximately correct. This comparison is the primary reason for including these data. Figure 9 shows similar results for sitosterols. With sitosterols, the diametral test was questionable because the compact disintegrated into many fragments. The square compacts made at smaller compression forces failed in tension and yielded strength values greater than those of the diametral compression case. Thus, it appears that in the diametral case, stress concentration caused shear failure to occur. When square sitosterols compacts were made at higher compression forces, the square compacts failed in shear also. This was established by the shape of the fragments.

Figure 10 and the insert in Fig. 9 show the results for sitosterols with the direct tensile method[11]. Figure 10 shows the scatter of the data used to extrapolate to zero height. The slope of the insert graph in Fig. 9 is compared to the slope of the extrapolated curve. As expected, the former is somewhat larger than one would expect from the extrapolation. Since the extrapolation is over such a large distance, the extrapolated slope is of questionable value. However, it is less than the slope experimentally determined at low relative density.

Figure 11 shows the same tensile strength data as in Figs. 8 and 9, but here the abscissa is the mean relative density of the compact, where the mean relative density is the apparent density of the compact divided by the absolute density of the pure compound. The data obtained by the direct tensile strength measurements are not included in Fig. 11 because the column of powder is far from uniform in density and the density at the fracture plane is unknown. It is obvious from Fig. 11 that the same compaction stress does not produce identical relative density compacts in both the round and square dies. These plots provide an alternative comparison of the data obtained with the round and square dies.

When considering the tensile strength and shear cell data together, Fig. 8 is of greater interest than the relative density plot in Fig. 11. This is because the relative density is not determined in the shear cell procedure used in the authors' laboratory. While relative density data are used with some other shear cells where the powder bed is much thicker, the problem of producing a known uniform density in the entire shear cell becomes formidable. The authors' cell uses a very thin layer of powder to mini-

[11] Both the highest values observed and the average values observed with the direct tensile test are shown in Figs. 9 and 10. Since errors in tensile strength observations are expected to cause less than true strength values to be observed, it was decided to show both results.

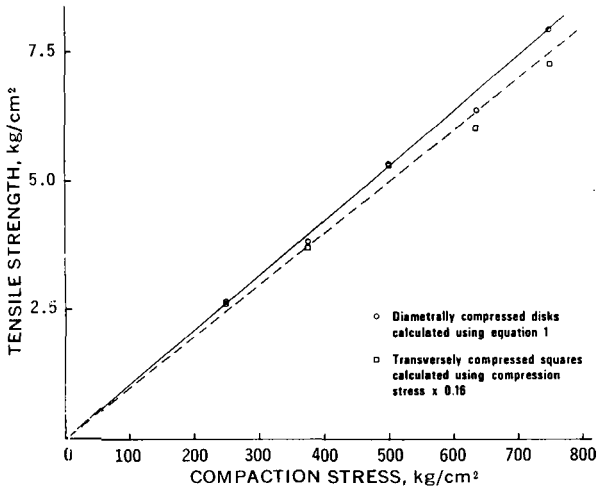

Figure 8—*Comparison of tensile strength of sucrose powder compacts as determined by diametral compression of a disk and transverse compression of a square; confirms 0.16 factor for squares.*

mize this problem. Some results of shear strength measurements are shown in Figs. 12 and 13. The experimental procedures for obtaining these data will not be described, because they are identical to the procedures described earlier (4). Tensile strength data for powders of aspirin USP and calcium carbonate USP (precipitated, extra light) are shown in Fig. 14.

DISCUSSION

Tensile Strength Measurements—Precise measurements of the tensile strengths of loosely compacted powders are difficult to achieve. Kočova and Pilpel (3) reported tensile strength values for various particle-size fractions of lactose and of calcium carbonate, which are said to be based on: ". . . a best straight line by regression analysis." However, their reported values are not always linear and no systematic variations with size fractions are obvious. These data were obtained using an apparatus of the design described by Ashton *et al.* (12), which uses a direct tensile stress method of measurement. This apparatus is accepted by various workers using the Jenike shear cell (1, 3). However, the published data obtained with this cell have not been sufficiently precise to motivate the authors to build an apparatus of this design. Apparently, no existing method using loosely packed powder beds is highly successful.

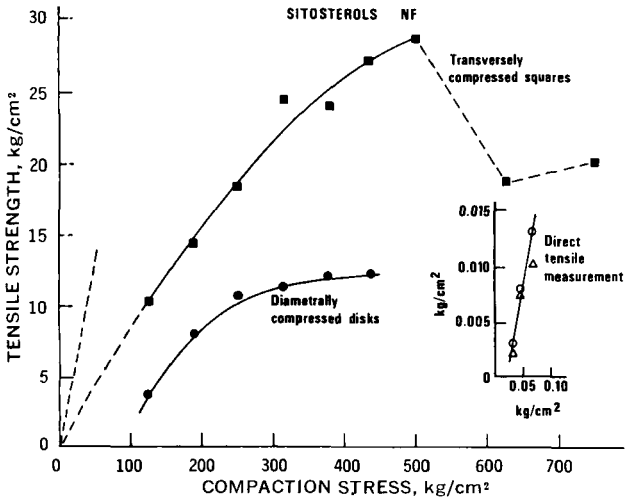

Figure 9—*Tensile strength of sitosterols NF (40 mesh) compacts determined by transversely compressed squares (■), diametrally compressed disks (●), and the Nash direct tensile apparatus (in insert graph, △ indicates average values and ○ indicates the highest values). Insert graph is a magnification of region near origin and shows direct tensile data points. Dashed line with larger slope has same slope as data in insert graph.*

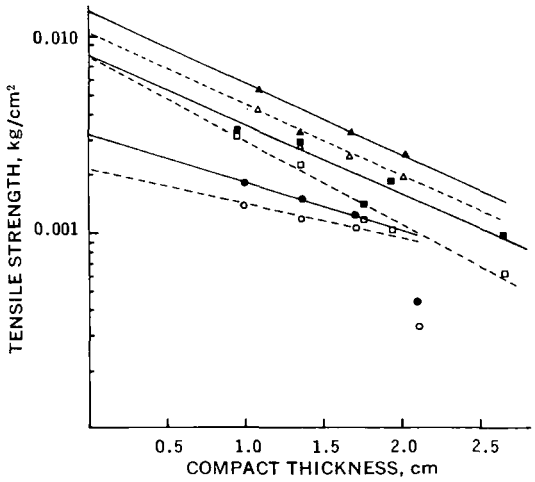

Figure 10—*Data used to obtain tensile strength values for sitosterols by extrapolation to zero compact thickness; used only with direct tensile test method. Key: ▲, 150-g load, high value; △, 150-g load, average value; ■, 100-g load, high value; □, 100-g load, average value; ●, 50-g load, high value; and ○, 50-g load, average value.*

Use of Tensile Strength as a Point on Shear Yield Locus—Figure 12 is a shear yield locus for sitosterols. Note the tensile strength[12], point X, plotted on the negative side of the σ axis. The table lists values obtained from experimental work with sitosterols. The slope based on the direct tensile measurements is about 3.4 times the slope used to estimate the magnitude of point X. Extrapolation of the insert graph would yield a value for the

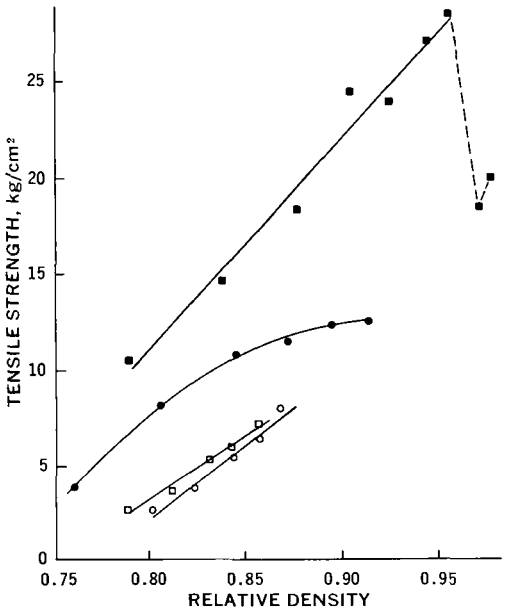

Figure 11—*Tensile strength of sitosterols and sucrose as a function of the relative density of the compact (same experimental points as in Figs. 8 and 9). Key: ■, sitosterols—transversely compressed squares; ●, sitosterols—diametrally compressed disks; □, sucrose—transversely compressed squares; and ○, sucrose—diametrally compressed disks.*

[12] The magnitude of X depends on the resultant principal compressive stress, σ_{pm}, which is the major consolidation stress produced by the normal load and the shear stress; σ_{pm} is obtained by using a Mohr diagram (4). The magnitude of X was obtained by multiplying σ_{pm} by the slope of the line between the origin and the closest point of the tensile strength *versus* compaction load plot of Fig. 9. As will be noted, this is a very conservative estimate of the tensile strength.

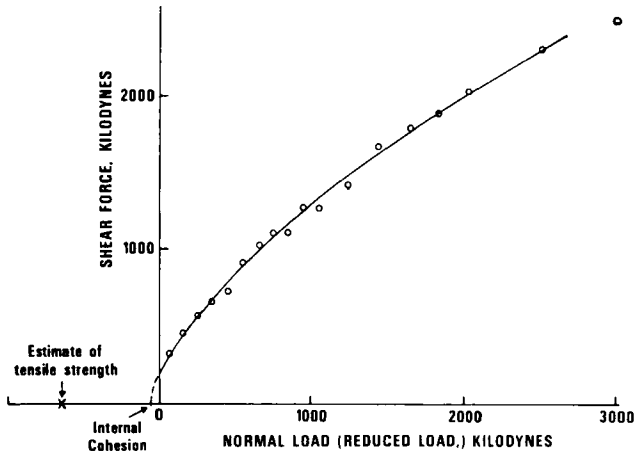

Figure 12—*Shear yield locus for sitosterols. Note that extrapolation to the normal load axis cannot be to the tensile strength point,* X, *as some have suggested (1–3). The* X *value plotted is estimated from transversely compressed square data. The* X *value based on direct tensile test would be very far from the origin on this scale.*

tensile strength of about 2100 kdynes instead of the 618 shown in Fig. 12. Even the very conservative estimate of tensile strength grossly exceeds the magnitude of any point that could be on the yield locus.

To emphasize the importance of this observation, it is necessary to discuss the shear yield locus and to develop an empirical equation to describe it. However, before proceeding, supplemental results will be considered. As may be seen in Fig. 13, the data for aspirin are similar to the data for sitosterols. Again the point marked X is estimated from the slope between the lowest transversely compressed square tensile strength value and the origin. Again the estimated value of the tensile strength is too large to fit onto the yield locus for the powder. In this case the tensile strength at low relative density was not determined. Therefore, it is not established that the tensile strength of the lower relative density powder beds would be comparable to the magnitude estimated from the transverse compression data. However, these limited observations are not in conflict with the previous case of the sitosterols. Table I lists the values obtained from shear cell measurements for both materials. The equations in the *Appendix* were used to estimate n and S from shear cell data.

The area of true contact between particles is established with the application of a shear force in the shear cell and by a compressive force in the tensile strength tests. Therefore, one may question whether the two processes may be compared. Certainly the condition of the powder in the two beds is not identical; however, some shearing occurs in the compression of a powder bed. Therefore, the two processes are not totally different and gross differences of tensile strength of the two differently prepared beds would not be expected. Shearing should increase the true contact

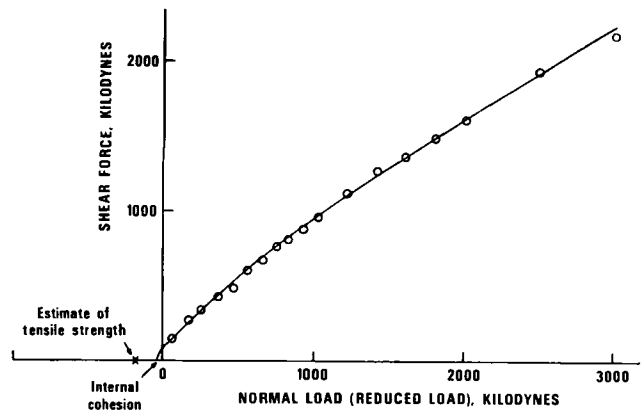

Figure 13—*Shear yield locus for aspirin. The* X *value is estimated from transversely compressed square data.*

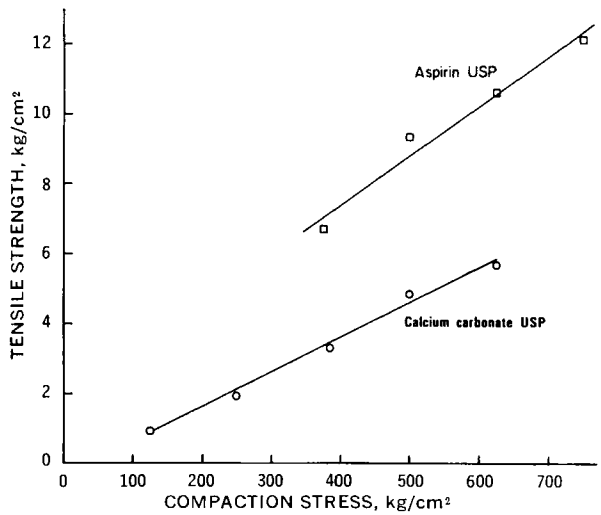

Figure 14—*Tensile strength by transversely compressed squares.*

area and would be expected to increase the tensile strength. Thus, any difference would be expected to favor a larger tensile strength for a bed prepared by the application of shearing motion. Therefore, any real differences should increase the discrepancies considered herein.

In the interest of brevity, it is assumed in the following discussion of the yield locus that the reader is familiar with shear cell methodology and terminology such as are used in Refs. 1 and 4.

In an earlier publication (4), an empirical expression was used to describe the area of true contact as the applied load stress, σ_r, of a shear cell, is reduced from its maximum value, σ_p:

$$\sigma_r + S = kA_r^n \qquad \text{(Eq. 2)}$$

where S is the internal cohesional stress and is a function of σ_p, A_r is the true contact area per unit cross section of the powder bed (a dimensionless number), k is a proportionality term, and n is a constant. Obviously at the maximum load conditions (indicated by a subscript p):

$$\sigma_p + S = kA_p^n \qquad \text{(Eq. 3)}$$

where A_r has its maximum value, A_p, when σ_r has its maximum value, σ_p. Dividing Eq. 2 by Eq. 3 gives:

$$\frac{\sigma_r + S}{\sigma_p + S} = \left(\frac{A_r}{A_p}\right)^n \qquad \text{(Eq. 4)}$$

It is reasonable to assume that the shear stress at failure is directly proportional to the area of true contact that is sheared. Therefore, Eq. 4 may be used to obtain:

$$\frac{\sigma_r + S}{\sigma_p + S} = \left(\frac{\tau_r}{\tau_p}\right)^n \qquad \text{(Eq. 5)}$$

where τ_r is the shear strength observed when the applied load is σ_r, and σ_p is the shear strength when the applied load is its maximum value[13], σ_p. Equation 5 will describe a yield locus. It is equivalent to the well-known Warren-Springs equation, except that in this derivation S is the internal cohesive strength. In the Warren-Springs equation, S is considered to be the tensile strength.

If S is assumed to be the tensile strength, some conceptual problems arise. These are indicated in the following arguments where momentarily S is identified as the tensile strength.

For convenience, a normalized term, S^*, is used where:

$$S^* = \frac{S}{\sigma_p + S} \qquad \text{(Eq. 6)}$$

[13] A_p may be estimated if the mean deformation pressure, P, is constant and known.

Table I—Tensile and Shear Failure Values of Various Powders

Material	Slopes of Tensile Strength Data: Direct[a] Tensile	Slopes of Tensile Strength Data: Compressed[b] Squares	Shear Strength Values: n^c	S^d	$\sigma_{pm}{}^e$	X^f
Sitosterols NF	0.283	0.0834	1.57	0.661	119.5	9.96
Aspirin USP		0.0179	1.30	0.372	109.8	1.96
Sucrose		0.0103	1.61	1.20	119.5	1.23
Calcium carbonate		0.0078	1.26	0.767	125.1	0.976

[a] Slope for data in insert graph of Fig. 9. If this value were used to estimate the tensile strength for the sitosterols in the shear cell, the value would be 33.8 instead of 9.96 kdynes/cm². [b] Based on straight line from origin to point at lowest compression stress used with compacts. [c] Estimated using Eq. A5. [d] Kilodynes per square centimeter; obtained by using Eqs. A4 and A6. Internal cohesion in shear cell is obtained by multiplying by the cross-sectional area of cell, 62.04 cm². [e] Kilodynes per square centimeter; major consolidation stress; estimated from a Mohr semicircle drawn tangent to the right end of the yield locus. [f] Kilodynes per square centimeter; $\sigma_{pm} \times$ slope of tensile strength (squares). (This value is not assumed to be an accurate estimate of the tensile strength for powders in the shear cell but is included for comparison. At least for sitosterols, the tensile strength is a larger value than this estimate.)

It is reasonable to assume that the tensile strength, S, is directly proportional to the zero load area of true contact, A_0, *i.e.*, when $\sigma_r = 0$, $S \propto A_0$. (A different A_0 value would be obtained for each σ_p.) Also, when permanent deformation occurs, and it must occur at any significant applied load, the area of true contact under maximum load is directly proportional to the maximum load applied. Of course, this assumes that the deformation pressure remains constant. This appears to be true for many pharmaceutical materials (13). Therefore, $\sigma_p + S \propto A_p$, and:

$$S^* \propto \frac{A_0}{A_p} \qquad \text{(Eq. 7)}$$

However, solving Eq. 4, when $\sigma_r = 0$, gives:

$$S^* = \left(\frac{A_0}{A_p}\right)^n \qquad \text{(Eq. 8)}$$

Obviously, relationships shown in Eqs. 7 and 8 are compatible only when $n = 1$.

The proponents of the Warren-Springs equation reject the case of $n = 1$ because it is argued that the yield locus (Eq. 5) must cross the σ_r axis at right angles to it. If $n = 1$, a plot of τ_r *versus* σ_r would be a straight line with a slope of $\tau_p/(\sigma_p + S)$, so it does not meet this criterion. These arguments suggest that S in Eq. 5 should not be equal to the tensile strength.

With some materials, the estimate of tensile strength obtained from the transversely compressed squares is not very different from the estimate of S. Because these are high relative density values, the significance is doubtful. The results for calcium carbonate and for sucrose are listed in Table I to illustrate that not all materials are alike. Calcium carbonate and sucrose are known to be harder, *i.e.*, have larger permanent deformation pressures, than sitosterols and aspirin. It seems reasonable to assume that the effect of this resistance to deformation would reduce the true area of contact established by compression and, thereby, account for a diminished tensile strength.

Since sitosterols were the only material for which low relative density tensile strengths were determined, only that case can be considered as strong evidence that the tensile strength is not an end-point on the yield locus. However, the data for the other materials appear useful in providing additional perspective.

Because of the apparent need to accommodate a large tensile strength value, a linear model for the yield locus was used by Hiestand (4). The tensile strength was described by a Mohr circle drawn through the origin (4). While this model was compatible with much larger values of the tensile strength, than was the curvilinear model, the linear model ignored the experimentally observed curvature of the shear failure data. As the precision of these measurements was improved and shear failure stresses were obtained at smaller reduced loads, it became obvious that the shear cell data are best described by a Warren-Springs-type equation. Therefore, it has been concluded that: (*a*) the Warren-Springs equation is suitable for describing the yield locus for failure in shear but, contrary to common usage, the S term in the Warren-Springs equation is not the tensile strength but instead is the internal cohesion; and (*b*) the magnitude of the internal cohesion may be much less than the tensile strength.

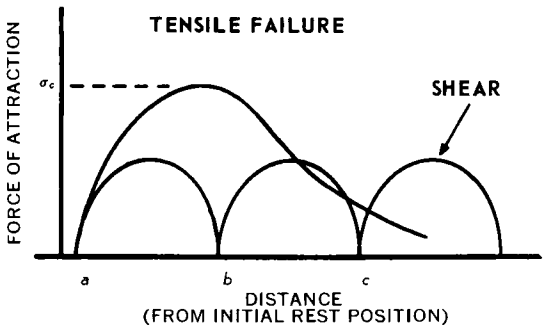

Figure 15—*Diagrammatic comparison of attraction between two atoms as they move from a potential energy well in tensile separation and in shear.* σ_c *is the critical tension at failure.* a, b, *and* c *are equal potential energy wells related to the shearing motion. Movement to a new energy well does not necessarily lead to separation.*

The following *a priori* arguments are presented to support these conclusions.

Consider a powder bed of uniform spheres. A force of attraction exists between the particles because of van der Waals' forces. These forces plus the externally applied load, σ_p, produce deformation at the regions of contact. All molecules in the individual particles contribute to the attraction between particles. However, at the regions of true contact, the molecules are closer than their equilibrium separation, where the intermolecular repulsion to the immediate neighbor would be equal to the force pushing them together. Thus, no contribution to the *net*, at rest attraction arises from attraction between immediate neighbor atoms in the interface. However, in tensile failure, the molecules in the interface are going to be the primary contributors to the strength because they must be moved out of the potential energy well in which their close contact has placed them. Thus, the tensile strength is the result of both the at rest attraction between particles and the maximum attraction produced as molecules in true contact regions are separated.

The attractive stress acting at the initiation of shear also includes the at rest portion of the attractive stress and that portion of the separation stresses necessary to permit shear motion. Since the separation in shear may not reach the critical separation distance, the magnitude may be less than the tensile strength. However, that force arising from the separation is overcome by the shear force and is not contributing to generating areas of true contact. Therefore, S should include only the internal, *i.e.*, the at rest, cohesion and not the attraction arising as a consequence of the separation of interfaces.

The comparison of tensile and shear strengths is shown diagrammatically in Fig. 15, where the relationship between force and displacement of atoms from their true contact position for the two processes, shear and tensile failure, are shown. In tensile fracture, the atoms are moved from their potential energy well until a critical value, σ_c, of attraction is exceeded. Beyond this critical separation, attraction decreases and fracture has occurred. In shear, the movement is from one potential energy well on the interface to the next (illustrated in Fig. 15 by movement from *a* to *b* to *c*, *etc.*), often without the separation distance becoming large enough to produce permanent separation or fracture. A smaller separation results in a smaller shear force. Because of the particulate nature of the shear plane across the powder, a significant fraction of the molecules will be separated completely even during shear. However, an approximately equal number of molecules at the opposite edges of the particles will be approaching each other. Therefore, the net effect should be small.

In Eq. 2, the significance of S clearly is the force that must be added to the normal load to produce the true area of contact that

must be sheared. Since S is the force added to σ_p to account for the total normal force acting during the shear motion, the magnitude of S is determined by the at rest attractive forces. Since this force is expected to be less than the tensile force, S must be less than the tensile force.

CONCLUSIONS

Transverse compression methods provide good estimates of the tensile strengths produced by the compression of powdered materials. It is the authors' experience that the method using squares provides tensile failure with more materials than the disk method.

The observed tensile strengths of a plastic material, sitosterols, was too large to be fitted as the end-point on the shear yield locus of the powder. Therefore, it is concluded that an alternative characterization of the end-point of the yield locus should be used. Conceptually, this point is an internal cohesion value and it is so designated. A method of evaluating the internal cohesion from shear cell data alone is presented.

APPENDIX: ESTIMATION OF n AND S FROM SHEAR CELL DATA

If the tensile strength is not used as the value for S, then a method of estimating both n and S is needed. A graphical method is useful. While not limited to simple powders[14], this discussion is directed to these cases.

The simple powder exhibits constant S^* values; therefore, S is a linear function of $\sigma_p + S$. Thus, for a simple powder:

$$\left(\frac{\tau_r}{\tau_p}\right)^n = (1 - S^*)\frac{\sigma_r}{\sigma_p} + S^* \qquad \text{(Eq. A1)}$$

The graphical method for the evaluation of n assumes that n is a constant for a single yield locus. A plot is made of log (τ_r/σ_p) *versus* log (σ_r/σ_p). The slope at $\sigma_r = \sigma_p$ and $\tau_r = \tau_p$ is measured from the graph and is designated $1/n'$. Mathematically at this same point[15]:

$$\frac{1}{n'} = \left.\frac{d \log(\tau_r/\sigma_p)}{d \log (\sigma_r/\sigma_p)}\right|_{\sigma_r=\sigma_p} = \frac{\sigma_p}{n(\sigma_p + S)} \qquad \text{(Eq. A2)}$$

Using Eqs. 6 and A2, one may obtain:

$$\frac{n}{n'} = \frac{\sigma_p}{\sigma_p + S} = 1 - S^* \qquad \text{(Eq. A3)}$$

Combining Eqs. 5 and 6, one obtains at $\tau_r = 0$:

$$S^* = \left(\frac{\tau_0}{\tau_p}\right)^n \qquad \text{(Eq. A4)}$$

where τ_0 is the intercept of the yield locus, *i.e.*, the shear stress when $\sigma_r = 0$.

Combining Eqs. A3 and A4 gives:

$$\left(1 - \frac{n}{n'}\right)^{1/n} = \frac{\tau_0}{\tau_p} \qquad \text{(Eq. A5)}$$

Extrapolation[16] of plots such as in Fig. 12, where τ_r is plotted against σ_r, provides an estimate of τ_0. Thus, in Eq. A5, τ_0/τ_p and n' are estimated graphically and n can be determined by an iterative procedure. Then S^* is calculated with Eq. A4.

For sitosterols, τ_0/τ_p was estimated to be 0.0635 and the n' estimate gave 1.58; $n = 1.56$ was obtained with Eq. A5 and $S^* = 0.0136$ by Eq. A4. From Eq. A3, one can obtain:

$$S = \frac{\sigma_p}{1 - S^*} - \sigma_p \qquad \text{(Eq. A6)}$$

This gives a value for the data in Fig. 12 of $S = 41$ kdynes for the internal cohesion.

Small changes in the magnitude of n cause the left side of Eq. A5 to change value markedly. Thus, the precision of the determination of n and S^* is limited only by the precision of the graphical determination of n' and τ_0/τ_p.

Some investigators (3) assumed that the log $\tau_r/(\sigma_p + S)$ *versus* log$(\sigma_r + S)/(\sigma_p + S)$ must be linear. Using this criterion, they determined n by using the measured tensile strength for S to prepare the log–log plot from which they obtained an estimate of the slope which is $1/n$. In Eq. A3, one notes that S^* is a small number even when S is relatively large. Therefore, large relative errors in the value of S^* produce only small changes for the value of n. Add to this the insensitivity of a log–log plot and it becomes obvious that the method is not very sensitive to errors in determining S. Possibly this is the reason that the use of the tensile strength for S has not been questioned previously.

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[x] To whom inquiries should be directed.

[14] Some investigators (3) gave the designation "simple powder" to materials that exhibit constant S^* and $\tau_p/(\sigma_p + S)$ values.

[15] Equation 5 is rearranged to give:

$$\frac{\tau_r}{\sigma_p} = \frac{\tau_p}{\sigma_p}\left(\frac{\sigma_r + S}{\sigma_p + S}\right)^{1/n}$$

$$d\left(\frac{\tau_r}{\sigma_p}\right) = \frac{\tau_p}{n(\sigma_p + S)}\cdot\left(\frac{\sigma_r + S}{\sigma_p + S}\right)^{(1/n)-1}\cdot d\left(\frac{\sigma_r}{\sigma_p}\right)$$

and:

$$\left(\frac{\tau_r}{\sigma_p}\right) d \log \frac{\tau_r}{\sigma_p} = \left(\frac{\tau_p}{n(\sigma_p + S)}\right)\left(\frac{\tau_r}{\sigma_p}\right)\left(\frac{\sigma_r + S}{\sigma_p + S}\right)^{-1}\left(\frac{\sigma_r}{\sigma_p}\right) d \log \frac{\sigma_r}{\sigma_p}$$

$$\left.\frac{d \log \frac{\tau_r}{\sigma_p}}{d \log \frac{\sigma_r}{\sigma_p}}\right|_{\sigma_r=\sigma_p} = \frac{\sigma_r}{n(\sigma_r + S)}\Bigg|_{\sigma_r=\sigma_p} = \frac{\sigma_p}{n(\sigma_p + S)}$$

[16] A more general form of Eq. A5 is:

$$\left[1 + \frac{n}{n'}\left(\frac{\sigma_r}{\sigma_p} - 1\right)\right]^{1/n} = \frac{\tau_r}{\tau_p}$$

This form avoids the use of the intercept value, τ_0; but the change in magnitude of the left side of the equation with change in n is less rapid when the magnitude of σ_r/σ_p becomes significant.

Tumor Inhibitors I: Preliminary Investigation of Antitumor Activity of *Sarracenia flava*

D. HOWARD MILES*x, UDOM KOKPOL*, LEON H. ZALKOW‡, STEVEN J. STEINDEL‡, and JAMES B. NABORS‡

Abstract □ The chloroform and aqueous extracts of the roots of *Sarracenia flava* showed antitumor activity against human epidermoid carcinoma of the nasopharynx (KB). Betulin was identified as one constituent responsible for this activity. Testing data are reported for betulinic acid and derivatives.

Keyphrases □ *Sarracenia flava*—chloroform and aqueous extracts examined for antitumor activity □ Betulin and betulinic acid—isolated from *Sarracenia flava*, examined for antitumor activity □ Antitumor agents, potential—isolation and screening of active constituents from *Sarracenia flava*

Reports[1] that the ethanol (moonshine) extracts of the roots of *Sarracenia flava*[2] (golden trumpet) have been used as a folk remedy by residents of the Okefenokee swamp region of southeastern Georgia prompted the examination of this and related species. The genus *Sarracenia,* one of three genera in the Sarraceniaceae, has been the subject of many investigations since the mid-19th century. The insectivorous habit, the conspicuous leaves and flowers, and the apparent ease of hybridization have led to interest in the group by botanists, biochemists, and horticulturists. Various aspects of the genus associated with its insectivorous habit were summarized (1). More recently, it was proved conclusively that nutrients from entrapped insects are absorbed into foliar tissue (2). The historical development of the genus has been discussed (1, 3, 4), and recently a chromatographic review was published (5). In spite of this great interest in *Sarracenia,* there is no evidence in the literature of a detailed examination of its chemical constituents.

The authors wish to report confirmation of antitumor activity in the roots of *S. flava* and also to comment on the activity of compounds and derivatives from this plant.

RESULTS AND DISCUSSION

Preliminary studies involving methanol extraction of the plant roots[3] yielded two fractions, a methanol-soluble oil and a methanol-insoluble, beige-colored solid. Testing of these crude fractions[4] showed that against human epidermoid carcinoma of the nasopharynx (KB) a 27-μg/ml dose was required to inhibit 50% growth of the test cells *versus* the control (ED_{50}); in the same system the solid showed an ED_{50} of 11 μg/ml. The solid was judged to warrant further study and showed in the later tests ED_{50}'s of 3.0, 2.6, and 2.6 μg/ml. These tests confirmed KB activity and further investigations were justified. Neither sample was active against L-1210 lymphoid leukemia in BDF_1 mice or Walker carcinosarcoma 256 (intramuscular) in random-bred albino rats.

Further extraction of the finely ground roots[5] was carried out

Table I—Activity of Fractionated Plant Material

Fraction[a]	Plant Material, %	ED_{50}[b]	Slope[c]
A	7.9	>100	0.0
B	13.6	26	−1.09
C	2.3	>100	0.0
I	2.0	>100	0.0
J	0.3	24	−0.51

[a] See Scheme I. [b] Activity against human epidermoid carcinoma of the nasopharynx *in vitro*, expressed as micrograms per milliliter that inhibited 50% of control dose. [c] Slope is the change of response for each one-log change of dose.

Table II—Activity of Betulinic Acid and Some Related Compounds[a]

Compound	Dose, mg/kg	T/C
Betulinic acid[b]	400	76
	200	90
	100	101
Betulinic acid[c]	200	91
	100	91
	50	101
Methyl betulinate[d]	400	91
	200	100
	100	103
Acetobetulinic acid	400	91
	200	93
	100	93
Betulonic acid	400	94
	200	96
	100	102
Methyl betulonate	400	94
	200	96
	100	91

[a] All tests on L-1210 lymphoid leukemia in BDF_1 strain of mice using intraperitoneal injections, with hydroxypropyl cellulose (HPC, Klucel) as a vehicle unless otherwise noted. T/C is the ratio of the mean survival time of the test animals to the control animals. [b] Test on Walker carcinosarcoma 256 (subcutaneous) in random-bred albino rats using intramuscular injections, with carboxymethylcellulose as a vehicle. [c] Test on P-388 lympholytic leukemia in BDF_1 strain of mice. Injections were intraperitoneal, using saline with polysorbate 80 as a vehicle. Activity against KB cell culture showed an ED_{50} of 26 and a slope of −1.16. [d] Alcohol used as a vehicle.

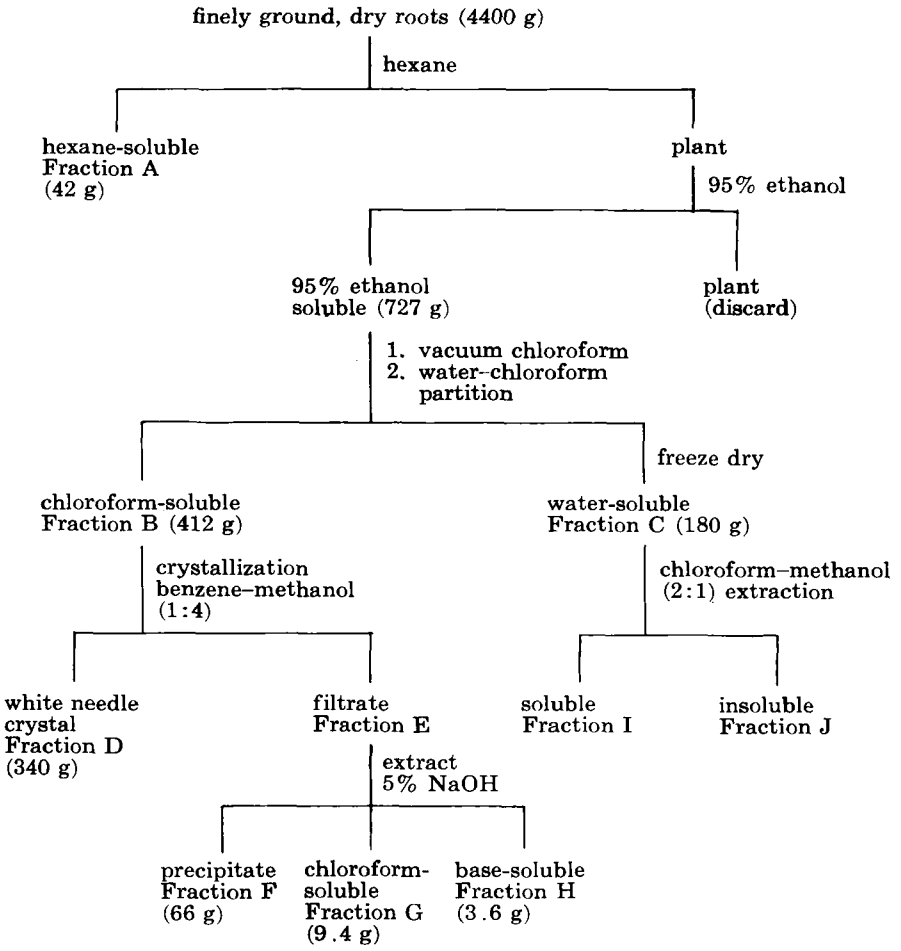
[1] Private conversation with local residents.

[2] The plant material used in this investigation was identified as *Sarracenia flava* L. (Sarraceniaceae) by Dr. Sidney McDaniel, Department of Botany, Mississippi State University. A voucher (preserved) specimen (SM-16,702) representing material collected for this investigation is available for inspection at the Herbarium of the Department of Botany, Mississippi State University.

[3] Collected in Okefenokee Swamp region during February 1968.

[4] Cancer Chemotherapy National Service Center, Bethesda, MD 20014, 1970.

[5] Collected in the Okefenokee Swamp region during May 1969.



Scheme I—Procedure for extraction of the roots of Sarracenia flava

using the Wall *et al.* (6) procedure for KB active compounds (Scheme I). Testing of the fractions derived from this procedure gave the results shown in Table I. Even though the KB activity of these fractions is less than that observed for the crude methanol extract, it is clearly concentrated in Fractions B (chloroform extract) and J (water extract).

Further fractionation by basic extraction of chloroform Fraction B gave a precipitate (Fraction E), which was recrystallized from methanol–acetone to yield a compound that did not melt even when heated to 315°. The IR spectrum of this compound showed bands characteristic of a carboxylic acid salt. Acidification and recrystallization of the salt gave a compound identified as betulinic acid by reduction to betulin with lithium aluminum hydride, followed by a direct comparison of IR, NMR, and mass spectra with those of an authentic sample of betulin. Betulinic acid was then shown to be the major constituent of the beige solid remaining after the initial methanol extraction. The presence of KB activity in this fraction suggested that betulinic acid might be the active component.

Samples of betulinic acid, its methyl ester, the acid acetate, its keto derivative (betulonic acid), and the keto ester (methyl betulonate) were prepared (8) and sent to the National Cancer Institute for testing (Table II). These data clearly indicated that betulinic acid was not the active constituent of the plant, even though the active chloroform extract is over 80% betulinic acid by weight.

Chromatography of the chloroform Fraction B after removal of betulinic acid by fractional crystallization resulted in the isolation of the known tumor inhibitor betulin (7). The mass spectrum of betulin showed an M^+ peak at 442, and the NMR spectrum indicated the appropriate signal for vinyl protons. The IR spectra of the compound and its diacetate were superimposable with authentic samples (8).

EXPERIMENTAL

Isolation of Betulinic Acid—The white needle crystals (Fraction D) were recrystallized four times from benzene–methanol (1:4) to yield a white crystalline material, mp 287–290°, which was identical to the acid from the beige solid Fraction F. Fraction F was recrystallized from hot methanol–acetone to yield a white crystalline material which did not melt when heated to 315°. The partially dissolved salt in 10% HCl was continuously extracted with chloroform. The chloroform layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo* to yield an off-white solid which could be recrystallized from ether, mp 290–292° [lit. (9) for betulinic acid mp 316–318°]. The IR spectra of both acids from Fractions D and F were identical with that of a sample of betulinic acid[6] (~90%), mp 295–298°.

Isolation of Betulin—Fraction E (80 g) was dissolved in 300 ml benzene–chloroform (8:1). The soluble part (about 30 g) was chromatographed on a neutral alumina column (1.3 kg, activity I, 7 × 27 cm) prepared in hexane. The column was eluted first with hexane and then with solvents of increasing polarity. Each fraction (2 liters) was examined by TLC (silica gel GF) using acetone–benzene–chloroform (1:2:17) as the solvent system and potassium dichromate–sulfuric acid as the developing agent. Fractions 1–13 of the chloroform–benzene (3:4) elution were combined and purified twice by preparative TLC [0.75 mm, silica gel GF, acetone–benzene–chloroform (1:2:17)], yielding betulin as a white crystalline solid (35 mg), mp 214–217°; IR (KBr pellet): 3300, 1630, and 875 cm^{-1}; NMR ($CDCl_3$): δ 0.76 (3H, s), 0.84 (3H, s), 0.99 (3H, s), 1.02 (3H, s), 1.05 (3H, s), 1.71 (3H, s), 3.58 (2H, d of d, J = 11 Hz), and 4.67 (2H, d, J = 5 Hz); mass spectroscopy: parent ion at M^+ = 442; calculated for $C_{30}H_{50}O_2$, M^+ = 442. The precise mass deter-

[6] Obtained from Aldrich Chemical Co.

mination using polyfluorokerosine as the standard gave M^+ = 442.3745 (calc. M^+ = 442.3811).

The difference in melting points [lit. (10) betulin mp 252–253°] was due to traces of betulinic acid. The IR spectra of the isolated betulin (KBr pellet) and that of an authentic sample of betulin plus traces of betulinic acid were in complete agreement.

Preparation of Betulin Diacetate—Betulin (12 mg), pyridine (0.5 ml), and acetic anhydride (0.5 ml) were refluxed for 1 hr. After cooling, the reaction mixture was poured over crushed ice. The precipitated compound was collected and washed thoroughly with water until free of acetic acid and pyridine. After drying under vacuum, the compound was recrystallized from absolute ethanol. Approximately 10 mg of solid needles of betulin diacetate was obtained, mp 218–221° [lit. (10) mp 223–224°]. The IR spectra of the prepared betulin diacetate and that of an authentic sample were in complete agreement.

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Keyphrases □ Anthranilic acid, *para*-substituted *N*-benzenesulfonyl derivatives—synthesized and screened for anti-inflammatory activity □ Anti-inflammatory activity—*para*-substituted *N*-benzenesulfonyl derivatives of anthranilic acid, synthesis and screening □ Structure–activity relationships—*para*-substituted *N*-benzenesulfonyl derivatives of anthranilic acid

Considerable effort continues to be expended in the search for effective nonsteroidal anti-inflammatory agents. Various chemical classes have been demonstrated to possess therapeutically useful properties—*viz.*, salicylates, carboxylic acid amides, arylalkanoic acids, pyrazolidinediones, and anthranilic acids. Recently, significant anti-inflammatory activity was reported for members of a series of *para*-substituted *N*-benzenesulfonyl derivatives of amino acids, most notably phenylalanine (1). While phenylalanine itself possesses anti-inflammatory activity (2), the *N*-benzenesulfonyl moiety was found to enhance this activity significantly. In view of the pronounced anti-inflammatory activity of the anthranilic acid derivatives mefenamic acid[1] and flufenamic acid[2], earlier studies were extended to the synthesis of various *N*-*para*-substituted benzenesulfonyl derivatives of anthranilic acid (I–VI) and the examination of the influence of the *para*-substituent through quantitative structure–activity correlations.

EXPERIMENTAL[3]

General Synthetic Procedure—A solution of the appropriate benzenesulfonyl chloride (1.2 moles) in dioxane was added to a solution of anthranilic acid (1.0 mole dissolved in sufficient 2 *N* NaOH) at such a rate as to maintain the pH of the mixture between 9.5 and 10.5. Additional 2 *N* NaOH was added when the

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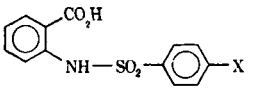
Table I—Properties of Anthranilic Acid Derivatives



Compound	X	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
I	H	80	211–214°[a]	—	—	—
II	CH_3	78	229–231°	$C_{14}H_{13}NO_4S$	C 57.72	57.77
					H 4.50	4.22
					N 4.81	4.53
III	NO_2	80	215–218°	$C_{13}H_{10}N_2O_6S$	C 48.46	48.64
					H 3.10	3.59
					N 8.69	8.56
IV	F	65	171–173°	$C_{13}H_{10}FNO_4S$	C 52.89	52.87
					H 3.42	3.19
					N 4.74	4.45
V	Cl	72	201–203°	$C_{13}H_{10}ClNO_4S$	C 50.09	50.16
					H 3.24	3.17
					N 4.49	4.19
VI	Br	81	221–223°	$C_{13}H_{10}BrNO_4S$	C 43.84	43.83
					H 2.84	2.78
					N 3.93	3.79

[a] Lit. (12) mp 211–212°.

Table II—Pharmacological Results

Compound	Percent Inhibition of Heat-Induced Hemolysis Concentration, M 10^{-5}	10^{-4}	10^{-3}	Percent Inhibition of Edema Dose, mg/kg[a] 30	60	120	Estimated I_{50}[b]	LD_{50}, mg/kg[a]
I	0	0	55.9	26.3	29.2	41.0	168	540
II	18.7	37.4	31.7	23.2	−25.6	−0.1	—	890
III	22.8	26.0	73.2	16.8	14.6	47.4	125	—
IV	17.9	37.4	87.8	21.4	20.8	64.0	100	395
V	0	62.6	66.7	22.6	35.0	58.5	99	275
VI	17.0	30.2	85.7	22.6	71.5	83.8	47	385
Phenylbutazone	17.2	35.0	83.7	—	34	52	—	336[c]

[a] Administered intraperitoneally. [b] Graphically estimated dose that produces 50% inhibition of edema. [c] Ref. 13.

pH fell below 9.5. After addition of the benzenesulfonyl chloride, the resulting solution was stirred until no further pH decrease was observed. The solution was then acidified with concentrated hydrochloric acid to pH 2–3 and vigorously stirred. The resulting solid was collected and recrystallized from aqueous ethanol. Appropriate physical data are summarized in Table I.

Erythrocyte Membrane Stabilization Assay—The procedure of Brown *et al.* (3) was followed using fresh human blood from fasted Type O$^+$ donors. The compounds were tested at three dose levels and compared to phenylbutazone as a standard. Each value (Table II) is the average of 18 separate values determined as triplicates on the blood of three to five subjects.

Carrageenin Rat Paw Edema Assay—Anti-inflammatory activity was measured as inhibition of carrageenin-induced edema in the hindpaw of a Sprague–Dawley rat (150–200 g) according to the procedure of Winter *et al.* (4). Edema formation was measured 3 hr after an intraperitoneal injection of test drug suspended in saline and polysorbate 60 and 2.5 hr after carrageenin. The edema inhibition of each compound (eight rats per group) was compared with animals receiving only the vehicle and animals receiving phenylbutazone. No irritant effects were observed upon administration of any compound tested. Each value (Table II) is the average percent inhibition of paw edema measured on 32 rats.

Toxicity Data—The LD_{50} values were determined and calculated by the method of Litchfield and Wilcoxon (5) following intraperitioneal injections of four dose levels for each compound (Table II).

Quantitative Structure–Activity Correlations—The concentration of *para*-substituted *N*-benzenesulfonylanthranilic acid required for 50% inhibition of edema (I_{50}) was estimated graphically from the data reported herein. In accordance with the procedure developed by Hansch (6), the I_{50} values are measures of the concentration of anthranilic acid derivative with substitutent X required to exert a standard biological response. The structural contributions to this response are then factored in the form of the Hammett-type free energy relationship as follows:

$$\log BR_x = k\pi x + k'\sigma x + k'' Es_x + k''' \qquad \text{(Eq. 1)}$$

where BR_x is the biological response, $1/I_{50}$, for a molecule with substituent X; and the constants k, k', k'', and k''' are fixed for a given system and are determined by computerized multiparameter regression analysis. The π values for substituents were determined from the apparent log P values for the molecules where $\pi = \log P_x - \log P_H$, where P_x is the octanol-water partition coefficient for *N*-benzenesulfonylanthranilic acid with substituent X and P_H is the partition coefficient for unsubstituted *N*-benzenesulfonylanthranilic acid. The partition coefficients were measured in octanol *versus* 0.13 M phosphate buffer, pH 8.0, and are the average of five or more determinations for each substituent. The π values are used here instead of log P since the partition coefficients measured were apparent values and not true partition coefficients. Also, the substituent constant, π, is more useful in emphasizing the effect of substituents changes at a single position. The Hammett σ values (7) and the Taft E_s values (8) were taken from the sources indicated. Table III contains the data on substituent changes and biological activities. The correlations were derived using a computer[4] with a multiparameter regression analysis program.

RESULTS AND DISCUSSION

The syntheses of the target compounds were achieved in a straightforward manner (Table I). Optimum yields were obtained when pH of the basic solution of anthranilic acid was maintained

[4] IBM 360/65.

Table III—Edema Inhibition by Anthranilic Acid Derivatives

Compound	π^a	E_s^b	σp^c	Obs. log $1/I_{50}^d$	Calc. log $1/I_{50}^e$
I	0	1.24	0.0	0.22	0.22
II	0.51	0.0	−0.17	—	—
III	0.43	−1.28	0.78	0.41	0.40
IV	0.49	0.78	0.06	0.47	0.43
V	1.00	0.27	0.23	0.50	0.65
VI	1.29	0.08	0.23	0.88	0.78

[a] Calculated from measured log P values; apparent log P for Compound I is −0.46. [b] From Ref. 14. [c] From Ref. 15. [d] Estimated graphically from data in Table II; I_{50} value for Compound II could not be derived; I_{50} is in millimoles. [e] Calculated *via* Eq. 2.

between 9.5 and 10.5 during the addition of the benzenesulfonyl chloride.

Pharmacological results are presented in Table II. The carrageenin rat paw edema model of acute inflammation has been widely used as an accurate model for the detection of potential anti-inflammatory agents (9). The ability of a compound to inhibit heat-induced hemolysis of red blood cells has been suggested as a rapid *in vitro* technique for screening potential anti-inflammatory agents (3), and this procedure was followed. The reasonable correlation observed between the carrageenin rat paw edema assay and the erythrocyte membrane stabilization assay in Table II substantiates the proposal (3) that the erythrocyte membrane stabilization assay can serve as a rapid *in vitro* screening procedure for this class of pharmacological agents. The halogenated derivatives (IV, V, and VI) possessed significant activity in both assays and were more active than the unsubstituted parent compound (I). The nitro derivative (III) possessed greater activity in the erythrocyte membrane stabilization assay than in the carrageenin rat paw edema assay. The methyl derivative (II) gave interesting results. While weak activity was observed in the erythrocyte membrane stabilization assay, II induced edema formation at higher doses in the carrageenin rat paw edema assay. The *p*-bromo derivative possessed the greatest activity in the carrageenin rat paw edema assay. In previous studies of *N-para*-substituted benzenesulfonyl derivatives of phenylalanine (1), the *N-p*-bromobenzenesulfonyl derivative was also the most potent member of the series and was more active than phenylbutazone (74% *versus* 52% inhibition of carrageenin rat paw edema at 120 mg/kg, respectively). Unfortunately, while VI possesses significant activity in the carrageenin rat paw edema assay, it is only slightly less toxic than phenylbutazone based upon LD_{50} data.

In an attempt to correlate edema I_{50} values with the substituent constants in Table III, all possible combinations of the parameters were investigated including potential parabolic relationships. The best relationship was Eq. 2 involving π alone:

$$\log 1/I_{50} = 0.44\,(\pm 0.34)\,\pi + 0.22\,(\pm 0.27)$$
$$n = 5 \quad s = 0.11 \quad r = 0.92 \qquad \text{(Eq. 2)}$$

where the values in parentheses are the 95% confidence intervals for the coefficients, n is the number of data points, s is the standard deviation, and r is the correlation coefficient. An equation involving a combination of steric and electronic parameters (r = 0.96) must be discounted because of the extremely high degree of covariance between E_s and σ, as indicated by a squared correlation coefficient of 0.97 for E_s *versus* σ. Neither term alone can account for the variation in biological activity, nor does addition of either parameter to π significantly improve Eq. 2.

The positive coefficient associated with π in Eq. 2 suggests that increases in lipophilic character will result in increases in biological activity. The relative size of this coefficient places it in the intermediate range of lipophilic sensitivity, 0.40–0.85 (10). While no similar biological test system was correlated, Eq. 2 is similar to that derived for the ability of aromatic acids to induce fibrinolysis of hanging blood clots (10, 11). This may suggest that the biological effect of the anthranilic acid derivatives results from an analogous interaction with some protein at the site of action.

Since the biological test employed here is an *in vivo* system, one might anticipate a parabolic relationship to arise between lipophilic character and biological action as a result of the "random-walk" process (6). It is highly probable that the substituents evaluated all lie on a linear portion of the parabola since the substituent variations examined were primarily designed to delineate electronic effects and do not cover a wide range in lipophilicity.

Attempts to correlate the effects of I–VI on heat-induced hemolysis were unsuccessful.

These preliminary studies provide some valuable information. The biological activity of these derivatives, at least against edema, solely depends on lipophilic character with respect to variations on the benzenesulfonyl ring. More lipophilic substituents could provide valuable information about the limits of this dependency.

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¶ National Science Foundation Undergraduate Research Participant, 1973.

ˣ To whom inquiries should be directed.

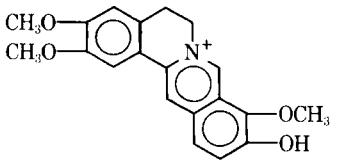
Chemical Constituents of *Legnephora moorei* Miers (Menispermaceae)

S. CAMPOS FLOR*, N. J. DOORENBOS*, G. H. SVOBODA‡§, J. E. KNAPP‡, and P. L. SCHIFF, Jr.‡ˣ

Abstract □ A phytochemical investigation of an ethanolic extract of the whole plant of *Legnephora moorei* Miers (Menispermaceae) resulted in the isolation and characterization of the alkaloids dehydrocorydalmine, magnoflorine, laurifoline, and stepharine; the pentacyclic triterpene friedelin; and the sterol mixture campesterol, stigmasterol, and sitosterol.

Keyphrases □ *Legnephora moorei* Miers (Menispermaceae)—isolation and identification of dehydrocorydalmine, magnoflorine, laurifoline, stepharine, friedelin, campesterol, stigmasterol, and sitosterol ■ Column chromatography—separation of *Legnephora moorei* constituents ■ GLC-mass spectrometry—identification of campesterol, stigmasterol, and sitosterol mixture from *Legnephora moorei*

Legnephora moorei is a vine indigenous to Australia and New Guinea. An initial survey showed the presence of alkaloids in the genus (1) and, in particular, in the species *L. moorei* (2). A preliminary phytochemical study (3) resulted in the isolation of the cyclitol (+)-quercitol and the quaternary aporphine alkaloid isocorydine methiodide. The relative paucity of knowledge of the constituents of *Legnephora* species plus the established toxicity of many Menispermaceous species prompted the phytochemical investigation of this plant. The isolation and identification of the alkaloids dehydrocorydalmine (I), magnoflorine (II), laurifoline (III), and stepharine (IV); the pentacyclic triterpene friedelin; and the sterol mixture campesterol, stigmasterol, and sitosterol are reported.



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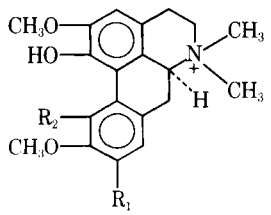
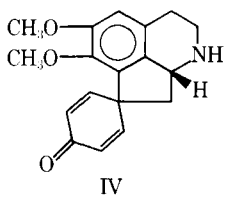


II: R_1 = H, R_2 = OH
III: R_1 = OH, R_2 = H



IV

DISCUSSION

The plant material was moistened with dilute ammonium hydroxide and extracted by percolation to exhaustion with ethanol. The extract residue was partitioned between dilute hydrochloric acid and ether. The ether extract containing the nonbasic substances was designated Fraction A. Chromatography of Fraction A over neutral alumina afforded the pentacyclic triterpene friedelin and the sterol mixture campesterol, stigmasterol, and sitosterol. The acid extract was basified with ammonium hydroxide and extracted with ether. The ether extract, containing the nonquaternary bases, was separated into nonphenolic (Fraction B) and phenolic (Fraction C) portions by treatment with dilute sodium hydroxide. Chromatography of Fraction B over neutral alumina afforded the proaporphine alkaloid stepharine. Fraction C was reserved for future investigation. The ammonium hydroxide layer was acidified with dilute hydrochloric acid, and the quaternary bases were precipitated by treatment with ammonium reineckate solution. Treatment of the reineckate complex with an anion-exchange resin in the chloride form afforded a quaternary alkaloid chloride fraction (Fraction D). Chromatography of Fraction D over silicic acid afforded the quaternary alkaloids dehydrocorydalmine (I), magnoflorine (II), and laurifoline (III).

The occurrence of proaporphine, aporphine, and protoberberine alkaloids in members of the plant family Menispermaceae has been firmly established (4). However, this is the first reported occurrence of dehydrocorydalmine, magnoflorine, laurifoline, and stepharine in the genus *Legnephora*. Since the occurrence of sterols and triterpenes in higher plants is rather common (5), the isolation of the pentacyclic triterpene friedelin and the sterol mixture campesterol, stigmasterol, and sitosterol was not unexpected.

EXPERIMENTAL[1]

Plant Material—*Legnephora moorei* Miers (Menispermaceae)[2] was received from Australia after identification at the point of collection. The whole plant was air dried and ground to a fine powder.

Extraction and Initial Fractionation—The plant material (10 kg) was moistened with aqueous ammonium hydroxide (10%) and extracted by percolation to exhaustion with ethanol. The ethanol extract was concentrated *in vacuo* at 40° to a brown syrup (464 g) which was stirred with 1% hydrochloric acid (1.5 liters). The acid solution was filtered and extracted with ether (3 × 1.5 liters). The combined ether extracts were dried (anhydrous sodium sulfate) and evaporated to leave a residue (44.8 g) of acidic and neutral compounds (Fraction A). The aqueous acidic solution was basified to pH 8 with concentrated ammonium hydroxide and extracted with ether (3 × 1.5 liters). The combined ether extracts were dried (anhydrous sodium sulfate) and evaporated to leave a residue (9.5 g) of crude bases. This residue was dissolved in ether

[1] Melting points were taken on a Thomas-Hoover Uni-Melt capillary apparatus and are corrected. The IR spectra were determined on a Perkin-Elmer model 257 or 137 spectrometer in KBr pellets. The UV spectra were obtained on a Perkin-Elmer model 202 recording spectrometer. Optical rotations were measured in a Rudolph polarimeter. Mass spectra were taken with a LKB-9000 or DuPont 21-492 mass spectrometer. For GLC analysis, an F&M Biomedical gas chromatograph, model 400, was operated isothermally at 255°. The column was 0.31 cm (0.125 in.), 1.84 m (6 ft) stainless steel, packed with 0.8% OV-17 (phenyl methyl silicone) on 80–100 mesh Gas Chrom Q. Helium was the carrier gas at a flow of 30 ml/min. Peak area calculations were made by the method of peak height × width at half-height.

[2] Collected in Casino Forestry District by the Forestry Commission of New South Wales, Australia (Lot 43653). Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, University of Mississippi.

(500 ml) and extracted with 2% sodium hydroxide (3 × 500 ml). The ether layer was washed with water, dried, and evaporated to afford a residue (1.4 g) of nonphenolic bases (Fraction B). The sodium hydroxide solution was acidified to pH 5 with 1% hydrochloric acid, alkalinized with ammonium hydroxide to pH 9, extracted with ether (3 × 1.5 liters), and evaporated to yield a residue (1.7 g) of crude phenolic bases (Fraction C).

The ammoniacal solution from which the bases had been extracted was reacidified to pH 3 with 1% hydrochloric acid and treated with a 2% ammonium reineckate solution until precipitation was complete. The precipitate was filtered by suction, suspended in dilute hydrochloric acid solution (pH 5), and stirred for 12 hr with 200 g of anion-exchange resin[3]. The resulting suspension was filtered and the filtrate was evaporated *in vacuo* to afford a mixture of crude quaternary chlorides (20 g) (Fraction D).

Chromatography of Quaternary Chloride Salts (Fraction D)—Fraction D (20 g) was chromatographed over a column (6.3 × 45 cm) of silicic acid[4]-diatomaceous earth[5] (4:1) (1.3 kg) with chloroform (1 liter) and chloroform–methanol mixtures (1 liter) of increasing polarity (1, 2, 4, 8, 16, and 32% methanol).

Isolation of Dehydrocorydalmine (I)—Elution with 16% methanol in chloroform afforded a fraction (200 mg), a portion of which (80 mg) was rechromatographed on preparative silica gel G plates[6] [methanol–ammonium hydroxide–water (8:1:3)]. Elution with methanol and addition of saturated methanolic potassium iodide (0.1 ml) afforded needles of dehydrocorydalmine iodide (I) (13 mg); mp 239–241° dec. [lit. (6) mp 228–230° dec.]; λ_{max} (ethanol): 228 (log ϵ 4.51), 278 (4.44), 348 (4.40), and 435 nm (3.65). The alkaloid was identified as I by direct comparison (IR, UV, and melting point) with an authentic sample.

Isolation of Magnoflorine (II)—An aliquot (1 g) of the fraction eluted with 32% methanol in chloroform was rechromatographed over a column (2.5 × 60 cm) of polyamide[7] (90 g) in ammonium hydroxide–water (1:10) (1 liter) and then over a column (3.5 × 60 cm) of acidic alumina[7] (Grade IV) (195 g). Elution with 32% methanol in chloroform (500 ml) followed by treatment with methanolic potassium iodide yielded magnoflorine iodide (II) (30 mg), mp 248–249° dec.; [lit. (7) mp 248–249° dec.]; $[\alpha]_D^{25}$ + 202.0° (c 0.70, methanol), λ_{max} (ethanol): 220 (log ϵ 4.55), 273 (3.90), and 311 nm (3.99). A direct comparison (IR, UV, melting point, and mixed melting point) with an authentic sample of II confirmed the identity.

Isolation of Laurifoline (III)—Another aliquot of the fraction eluted with 32% methanol in chloroform (1 g) was chromatographed over a column (2.5 × 60 cm) of acidic alumina[7] (Grade V) (140 g). Elution with 16% methanol in chloroform (250 ml) followed by treatment with methanolic potassium iodide (0.1 ml) afforded laurifoline iodide (III) (15 mg), mp 241.5–242° dec.; λ_{max} (ethanol): 224 (log ϵ 4.60), 280 (4.12), and 305 nm (4.22). A direct comparison (IR, UV, and melting point) with an authentic sample of III confirmed the identity. Although the optical rotation of the alkaloid was positive (confirming the configuration as *S*), a sufficient quantity of base was not available to determine accurately the specific rotation.

Isolation of Stepharine (IV)—Chromatography of a portion of Fraction B (1.1 g) over a column (2.5 × 60 cm) of neutral alumina (Grade V) (106 g) and elution with chloroform (500 ml) afforded IV (12 mg), mp 177–179° [lit. (8) mp 179–181°]; $[\alpha]_D^{25}$ + 110° (c, 0.10, chloroform); λ_{max} (ethanol): 218 (log ϵ 4.37), 233 (4.30), and 286 nm (3.52). The mass spectrum showed a molecular ion at *m/e* 297 (100%) corresponding to $C_{18}H_{19}NO_3$ and significant peaks at *m/e* 296 (43), 268 (65), 253 (15), 237 (13), and 225 (13). The alkaloid was identical with an authentic sample by direct comparison (IR, UV, mass spectroscopy, melting point, and mixed melting point).

Chromatography of Fraction A—An aliquot of Fraction A (6.0 g) was chromatographed over a column (3.75 × 60 cm) over neutral alumina[7] (Grade I) (500 g) in petroleum ether.

Isolation of Friedelin—Elution with petroleum ether–benzene (4:1) (1 liter) gave a fraction (605 mg) which was further purified by preparative TLC on silica gel G utilizing a solvent system of methanol–ammonium hydroxide–water (8:1:3). Elution of the band at R_f 0.72 with 2% methanol in chloroform afforded a residue, which on crystallization from ether gave friedelin (6 mg), mp 251–253° [lit. (9) mp 248–252°]; ν_{max} (KBr): 2920, 1718, and 1390 cm^{-1}. The mass spectrum showed a molecular ion at *m/e* 426 (35%) for $C_{30}H_{50}O$ and important fragment ions at *m/e* 411 (12), 341 (7), 302 (23), and 273 (40). The compound was identical with an authentic sample of friedelin by direct comparison (IR, mass spectroscopy, melting point, and mixed melting point).

Isolation of Sterol Mixture—Continued elution with petroleum ether–benzene (4:1) (1 liter) afforded a fraction (820 mg) which gave a positive Liebermann-Burchard test. This fraction was rechromatographed on a column (2.5 × 60 cm) of silicic acid[4] –diatomaceous earth[5] (4:1) (150 g). Elution with petroleum ether–benzene (1:1) (500 ml) afforded a sterol mixture which crystallized from ethanol (14 mg); ν_{max} (KBr): 3410, 2940, 1380, and 1360 cm^{-1}. GLC[1] showed the mixture to be composed of sitosterol (48%), stigmasterol (39%), and campesterol (13%). The identity was confirmed by GLC–mass spectroscopy. The spectra were consistent with those of authentic samples.

[3] IRA-400 (Cl), Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Celite, supplied as Celite Analytical Filter Aid by Johns-Manville Co., New York, N.Y.
[6] PF-254, Brinkmann Instruments, Westbury, N.Y.
[7] Woelm, Alupharm Chemicals, New Orleans, La.

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§ Present address: Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46206

ˣ To whom inquiries should be directed.

Synthesis and Evaluation of Antimicrobial Properties of Amidinoazaandrostanes and Guanidinoazaandrostanes

NORMAN J. DOORENBOS and JACK C. KIM[x]

Abstract □ The intermediate, 17α-methyl-4-aza-5α-androstan-17β-ol (III) required for the synthesis of 4-amidino-17α-methyl-4-aza-5α-androstan-17β-ol (VII) and 4-(β-guanidinoethyl)-17α-methyl-4-aza-5α-androstan-17β-ol (VI) was obtained through a reaction of 17α-methyl-3,5-*seco*-4-norandrostan-17β-ol-5-on-3-oic acid with ammonium hydroxide followed by two reductions (platinum dioxide and lithium aluminum hydride). Condensation of III with chloroacetonitrile under anhydrous condition, followed by reduction of the nitrile with lithium aluminum hydride, gave 4-(β-aminoethyl)-17α-methyl-4-aza-5α-androstan-17β-ol (V). The reaction of V with 2-methyl-2-thiopseudourea sulfate and of III-hydrochloride with cyanamide provided the title compounds, VI-sulfate and VII-hydrochloride, respectively. A serial dilution assay showed the title compounds to have antimicrobial activity against *Saccharomyces cerevisiae*. This activity is especially significant since this is the greatest activity thus far obtained in a 4-azaandrostane with a hydroxyl group at the 17β-position.

Keyphrases □ 4-Amidino-17α-methyl-4-aza-5α-androstan-17β-ol and 4-(β-guanidinoethyl)-17α-methyl-4-aza-5α-androstan-17β-ol —synthesis, antimicrobial activity □ 4-Azasteroids—synthesis, antimicrobial activity of amidino- and guanidinoazaandrostanes □ Antimicrobial activity—4-amidino-17α-methyl-4-aza-5α-androstan-17β-ol and 4-(β-guanidinoethyl)-17α-methyl-4-aza-5α-androstan-17β-ol

Doorenbos and coworkers (1–5) synthesized 4-azasteroids which have a wide variety of pharmaceutical properties such as coronary dilatory, hypocholesterolemic, hypercholesterolemic, hypotensive, and antimicrobial. The active azasteroids inhibited the growth of various Gram-positive bacteria, yeasts, and molds (3, 6). 4-Methyl-4-aza-5α-cholestane has quite a high antimicrobial activity, *i.e.*, approximately 1 μg/ml is bactericidal (1) against Gram-positive bacteria, yeast, and molds.

Previous studies indicated that 4-aza-5α-androstanes possessing a free 17β-hydroxyl (2) are devoid of antimicrobial activity except at very high concentrations. Explanation for the lack of activity in this molecule may be microbial degradation enhanced by hydroxyl substitution. It is commonly known that testosterone (17β-hydroxyl) is inactive orally but that deactivation is blocked by a 17α-methyl group. The objectives of this investigation were to test this hypothesis by synthesizing additional azasteroids through the introduction of the "antimicrobially active moiety," amidine or guanidine functions to azasteroids, namely a 4-azaandrostane, and to evaluate the resulting antimicrobial activity (7–9). Guanidine or amidine derivatives of azasteroids have not been prepared previously, however.

DISCUSSION

Synthesis—17α-Methyltestosterone was used as starting material for the synthesis of 4-amidino-17α-methyl-4-aza-5α-androstan-17β-ol (VII) and 4-(β-guanidinoethyl)-17α-methyl-4-aza-5α-androstan-17β-ol (VI) (Scheme I). Catalytic hydrogenation of the double bond of 17α-methyl-4-aza-5-androsten-17β-ol-3-one (I) (10) was accomplished with platinum dioxide in acetic acid (11). The course of the reaction was monitored by the disappearance of UV absorption at 233 nm, and the reduction product, 17α-methyl-4-aza-5α-androstan-17β-ol-3-one (II), gave a disappearance of a weak shoulder at 840 cm^{-1} which is characteristic of the carbon–carbon double bond in this lactam. 17α-Methyl-4-aza-5α-androstan-17β-ol (III), obtained from lithium aluminum hydride reduction of II, was condensed with chloroacetonitrile under anhydrous acetone to obtain 4-(β-cyanoethyl)-17α-methyl-4-aza-5α-androstan-17β-ol (IV). The condensation failed to occur when such solvents as anhydrous benzene, dioxane, and dimethyl sulfoxide were used. The nitrile function of IV was reduced by refluxing lithium aluminum hydride in tetrahydrofuran, and 4-(β-aminoethyl)-17α-methyl-4-aza-5α-androstan-17β-ol (V) was obtained.

For the preparation of VI, V was reacted with 2-methyl-2-thiopseudourea salt (12). The reaction of cyanamide with III gave VII. The 2-methyl-2-thiopseudourea method was unsuccessful in the preparation of VI. Only the sulfate salt of the starting amine was isolated (13).

Biological[1]—An antimicrobial screen, using a reported method (4), was employed to determine the approximate antimicrobial potency of VII and VI. As a standard, 4-methyl-4-aza-5α-cholestane (2), a 4-azasteroid of known activity, was tested by the same procedure. The biological data are presented in Table I.

The first tubes of each series contained 100 μg of sample; succeeding tubes contained 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 μg. The level of activity of the compounds was determined by choosing the last tube in which there was no noticeable growth of the organism. No statistical analyses were employed. To ensure that the cells were viable under test conditions, tubes containing steroid were used as blanks. The assay was duplicated. The following media were utilized: tryptic soy broth for bacteria and mycophil broth for molds and yeasts.

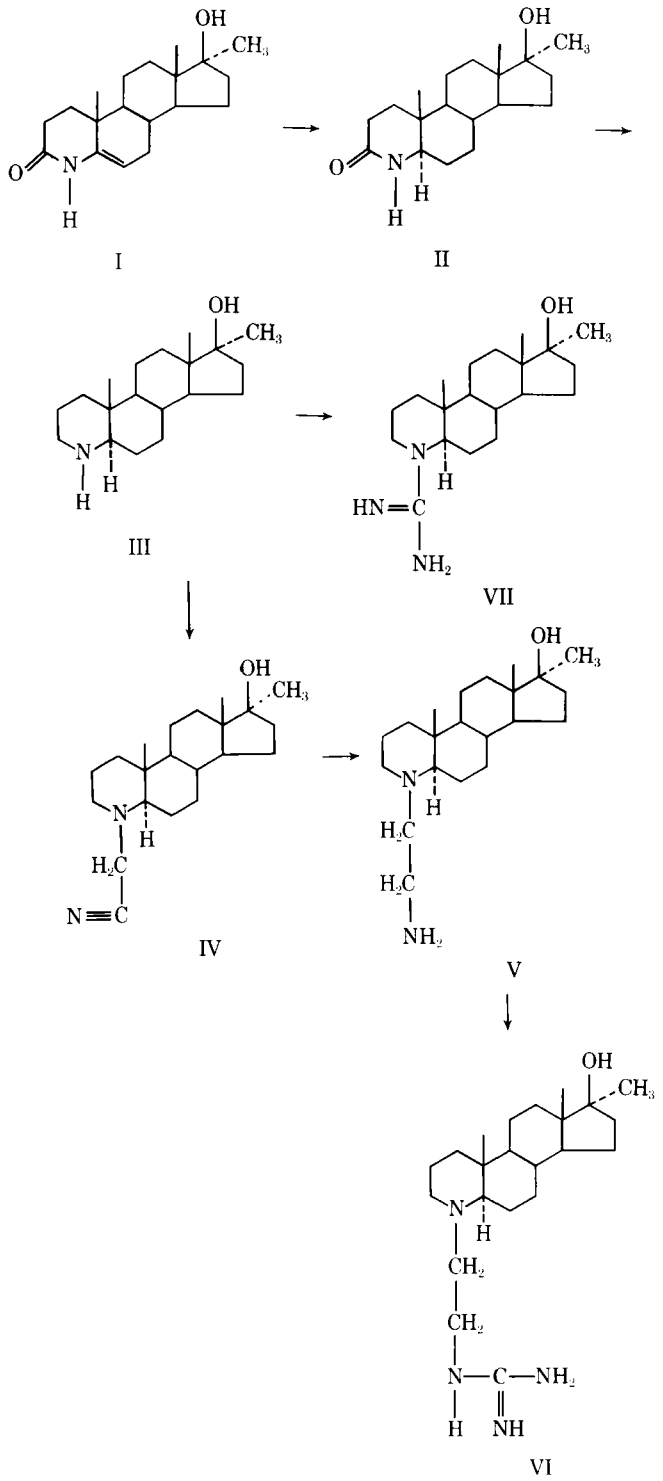
The activity of VI against *Saccharomyces cerevisiae* is especially significant since this is the greatest degree of activity thus far obtained in 4-azaandrostanes with a free hydroxyl group at the 17β-position. Previous studies indicated that 4-aza-5α-androstanes possessing a free 17β-hydroxyl (2) are detrimental to the antimicrobial activity.

EXPERIMENTAL[2]

Compound I—A solution of 20.00 g (0.0062 mole) of 17α-methyl-3,5-*seco*-4-norandrostan-17β-ol-5-on-3-oic acid in 200 ml of concentrated aqueous ammonium hydroxide was heated in a pressure vessel under a nitrogen atmosphere at 180° for 7 hr. The mixture was cooled, and the white solid was separated by filtration to obtain 17.47 g (92%). Crystallization from acetone gave 16.83 g (89%) of white needles, mp 256–258° [lit. (10) mp 256–258°]; IR (KBr): 3090 and 3210 (N—H), 1673 (lactam C=O), and

[1] The cell cultures used were obtained from the University of Mississippi, Department of Biology. For *Candida albicans*, *Staphylococcus aureus*, and *Escherichia coli*, the strains are identified by the numbers ATCC 10231, K-257, and ATCC 4157, respectively. *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Bacillus cereus* have been part of University stock cultures for many years.

[2] Melting points were taken in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus unless otherwise indicated, and the elemental analyses were obtained from Galbraith Laboratories, Knoxville, Tenn. A Perkin-Elmer 202 UV-visible spectrophotometer was used to record the UV spectra. The R_f values were determined by applying a methanolic solution of the steroid at a rate of 50–100 μg of the steroid as a spot on a silica gel TLC plate (Eastman chromatogram sheet) and developing in a Brinkmann jar in the following solvent systems: Solvent A, benzene–methanol–ethyl acetate (85:10:5); and Solvent B, chloroform–methanol–ammonia (85:14:1). The steroids were detected by iodine vapor.



Scheme I

840 and 1630 (C=C) cm^{-1}; λ_{max}: 233 nm; R_f 0.16 and 0.71 in Solvents A and B, respectively.

Compound II—To a solution of 150 ml acetic acid were added 4.00 g (0.013 mole) of I and 0.35 g of platinum dioxide. The reaction mixture was hydrogenated at 46 psi and 55° for 3 days. After removing the catalyst, evaporating the solvent *in vacuo*, and recrystallizing the residue from acetone, 3.65 g (82%) of white needles was obtained, mp 245–247°; IR (KBr): 1673 (lactam C=O) cm^{-1} and absence of a shoulder at 840 cm^{-1}; R_f 0.11 and 0.67 in Solvents A and B, respectively.

Anal.—Calc. for $C_{19}H_{32}NO_2$: C, 74.71; H, 10.23; N, 4.59. Found: C, 74.92; H, 10.23; N, 4.55.

Compound III—To a solution of 2.00 g (0.006 mole) of II in 250 ml of dry tetrahydrofuran was added, in small portions, 0.97 g (0.026 mole) of lithium aluminum hydride. The mixture was refluxed with stirring for 24 hr. Excess hydride was destroyed with tetrahydrofuran saturated with water. The mixture was filtered and the filtrate was evaporated *in vacuo* to yield 1.35 g of a yellow oily residue. Crystallization from acetone gave 1.21 g (75%) of white needles, mp 200–202°; IR (KBr): disappearance of lactam C=O at 1673 cm^{-1}; R_f 0.73 in Solvent B.

Anal.—Calc. for $C_{19}H_{33}NO$: C, 78.29; H, 11.41; N, 4.81. Found: C, 78.02; H, 11.22; N, 4.86.

Compound IV—To a stirred solution of 5.00 g (0.016 mole) of III and 2.43 g (0.032 mole) of chloroacetonitrile in 750 ml of anhydrous acetone was added 8.00 g of anhydrous sodium carbonate. The mixture was refluxed with vigorous stirring for 17 hr and filtered when hot. The filtrate was evaporated *in vacuo* to obtain a light-yellow oily residue. Crystallization from ethanol gave 3.29 g (64%) of a white solid, mp 183–185°; IR (KBr): 2260 (C≡N) cm^{-1}; R_f 0.72 in Solvent B.

Anal.—Calc. for $C_{21}H_{34}N_2O$: C, 76.31; H, 10.37; N, 8.51. Found: C, 76.27; H, 10.26; N, 8.64.

Compound V-Dihydrochloride—To a stirred solution of 1.00 g (0.003 mole) of IV in 150 ml of dry tetrahydrofuran was added, in small portions, 0.57 g (0.015 mole) of lithium aluminum hydride. The mixture was refluxed with stirring for 17 hr. After decomposing excess lithium aluminum hydride with methanol and then water, the mixture was filtered and the filtrate was evaporated *in vacuo* to obtain a yellow oily residue. All attempts to obtain crystals were unsuccessful; IR (neat): 3090 and 3210 (N—H) cm^{-1} and disappearance of the nitrile peak at 2260 cm^{-1}. The yellow oily residue, V, was dissolved in 100 ml of anhydrous ether and was treated with hydrogen chloride until a gummy solid formed. After chilling, the solid was separated by filtration and the filtrate was evaporated *in vacuo* to obtain a light-yellow solid. Crystallization from a mixture of ethanol and ether gave 0.92 g (77%) of white solid, mp 281–284°; IR (KBr): absence of nitrile peak at 2260 cm^{-1}; R_f 0.74 in Solvent B.

Anal.—Calc. for $C_{21}H_{40}Cl_2N_2O$: C, 61.90; H, 9.90; N, 6.88. Found: C, 61.59; H, 9.79; N, 6.78.

Compound VI-Sulfate—One gram (0.0028 mole) of V and 0.45 g (0.0058 mole) of 2-methyl-2-thiopseudourea sulfate were dissolved in 50 ml of 50% aqueous ethanol and heated under reflux with vigorous stirring for 1 hr (13). After chilling, the reaction mixture was filtered to obtain a gray solid. Crystallization from 50% aqueous acetone gave 2.02 g (82%) of a cream-colored solid, mp >300°; IR (KBr): 3100 and 3300 (guanidine absorption) and 1620 and 1660 (C=N) cm^{-1}; R_f 0.30 in Solvent B.

Anal.—Calc. for $C_{44}H_{82}N_8O_6S$: C, 62.08; H, 9.71; N, 13.13. Found: C, 61.89; H, 9.79; N, 13.08.

Compound VII-Hydrochloride—III-Hydrochloride (III-HCl) was made in the usual manner. Equal molar amounts of III-HCl (0.8 g, 0.0024 mole) and cyanamide (0.15 g, 0.0024 mole) were added to 20 ml of water and refluxed with vigorous stirring for 3 days. The reaction mixture was evaporated *in vacuo* to obtain a yellow oily residue. Crystallization from a mixture of ethanol and ether gave 0.62 g (70%) of white needles, mp 274–275°; IR (KBr): 3100 and 3300 (N—H) and 1620 and 1660 (C=N) cm^{-1}; R_f 0.47 in Solvent B.

Anal.—Calc. for $C_{20}H_{36}ClN_3O$: C, 64.93; H, 9.81; Cl, 9.58; N, 11.36. Found: C, 65.11; H, 9.91; Cl, 9.51; N, 11.21.

Table I—Minimum Inhibitory Concentrations of Steroids (Expressed in Micrograms per Milliliter)

Microorganisms	VI	VII	ND-502[a]
Bacillus cereus	—[b]	—[b]	3.12
Escherichia coli	—[b]	—[b]	—[b]
Staphylococcus aureus	—[b]	—[b]	6.25
Saccharomyces cerevisiae	6.53	25	3.12
Aspergillus niger	—[b]	—[b]	3.12
Candida albicans	—[b]	—[b]	6.25

[a] 4-Methyl-4-aza-5α-cholestane. [b] No inhibition.

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[x] To whom inquiries should be directed. Present address: Department of Biochemistry, Purdue University, Lafayette, IN 47907

Oscillopolarography of Mercaptopurine and Its Synthesis Intermediates Using a Commercial Oscilloscope and an Adapter Circuit

VOJTECH PARRAK[*] and MURRAY M. TUCKERMAN[x]

Abstract □ Details are given of an adapter circuit to be used with a storage oscilloscope fitted with a camera back for the recording of oscillopolarograms with the dropping mercury electrode. The satisfactory operation of the unit was shown by studies with zinc, cadmium, cupric, and plumbous ions in various supporting electrolytes. The unit was also used for an oscillopolarographic study of 6-purinethiol (mercaptopurine) and its synthesis intermediates: purine-6(1*H*)-one (hypoxanthine), 4,5-diamino-6-hydroxypyrimidine (4,5-diaminohypoxanthine), 4,5-diamino-6-hydroxy-2-thiopyrimidine (4,5-diamino-2-thiouracil), and 4-amino-6-hydroxy-2-thiopyrimidine (4-amino-2-thiouracil).

Keyphrases □ Oscillopolarography—mercaptopurine and synthesis intermediates, using commercial oscilloscope and adapter circuit □ Mercaptopurine and synthesis intermediates—oscillopolarographic study

The first experiments in oscillopolarography (1, 2) used alternating current to investigate the electrode processes in polarography and followed the changes in electrode potential on the oscilloscope. Since the commercial introduction[1] of the polaroscope, papers have shown that the method is applicable to qualitative and quantitative analyses of inorganic and organic compounds, to the determination of decomposition kinetics, and to following fast electrode processes. Many substances that are inactive in dc polarography give characteristic ac oscillopolarograms. The technique supplements classical polarography but has its own established theory and instrumentation (3–6).

This study was an investigation of 6-purinethiol (mercaptopurine) (I), an antitumor agent, and four synthesis intermediates which might be present in mercaptopurine: purine-6(1*H*)-one (hypoxanthine) (II), 4,5-diamino-6-hydroxypyrimidine (4,5-diaminohypoxanthine) (III), 4,5-diamino-6-hydroxy-2-thiopyrimidine (4,5-diamino-2-thiouracil) (IV), and 4-amino-6-hydroxy-2-thiopyrimidine (4-amino-2-thiouracil) (V) (7).

EXPERIMENTAL

Equipment—The following were used: adapter circuit to give the desired function, $dE/dt = f(E)$ as illustrated in Fig. 1 (any oscillator capable of a 10-v peak-to-peak output can be used in the circuit); type 546 B storage oscilloscope with type 2B67 time base and 3A3 dual-trace differential amplifier, fitted with an oscilloscope camera[2] having a film pack holder[3]; dropping mercury electrode[4], 3-sec drop time; and refrigerated, heated water bath and circulator[5].

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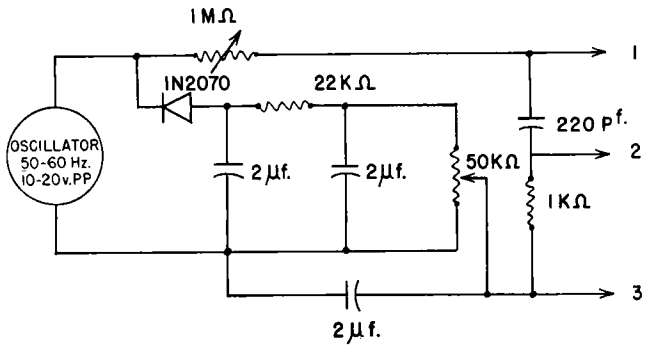

Figure 1—*Oscillopolarographic adapter circuit. To record* dE/dt = f(E), *connect 1 to mercury pool reference and oscilloscope horizontal, 2 to oscilloscope vertical, and 3 to mercury cathode and oscilloscope ground.*

at pH 1.0, 2.5, 4.5, 6.2, 7.3, 8.4, 9.0, 11.0, and 13.5. The best separations for Q values were found in 1 N H_2SO_4 (Table II). Oscillopolarograms of the five substances are shown in Figs. 4 and 5.

DISCUSSION

The qualitative and quantitative results for the inorganic ions are in agreement with the literature (8, 9). This indicates that the unit is performing as intended.

In the classical polarography of mercaptopurine, pyrimidine, and 2-thiopyrimidine, only a catalytic wave is observed (10). The forte of oscillopolarography is its capability of detecting substances that are neither oxidizable nor reducible but that form capacitive incisions due to adsorption on the surface of the mercury. Figures 4 and 5 show that all five compounds are oscillopolarographically active and show different behavior.

Mercaptopurine shows a sharp, irreversible, capacitive indentation at Q = 0.45. Hypoxanthine and Compound III have extremely negative values of 0.95 and 0.94, respectively. Compound IV has Q = 0.46 and Compound V has Q = 0.47, lobe-like rather than sharp. The results suggest that both the sulfhydryl group and the pyrimidine ring are polarographically active. All compounds are characterized by irreversible electrode processes.

Even though there are sufficient differences in Q values to allow quantitative determination of hypoxanthine in a mixture with mercaptopurine, the relative depth of the incisions indicates

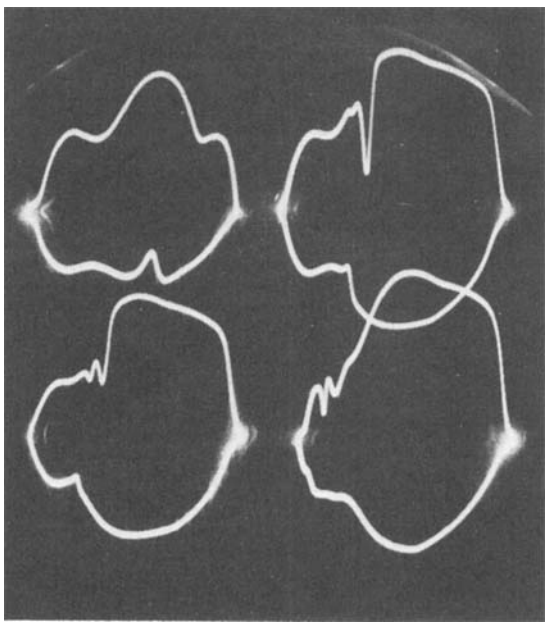

Figure 2—*Oscillopolarograms in 1* N *LiCl, 4.5* $\times$ 10^{-4} M *solutions. Key: top left, zinc; bottom left, plumbous; top right, cadmium; and bottom right, cupric.*

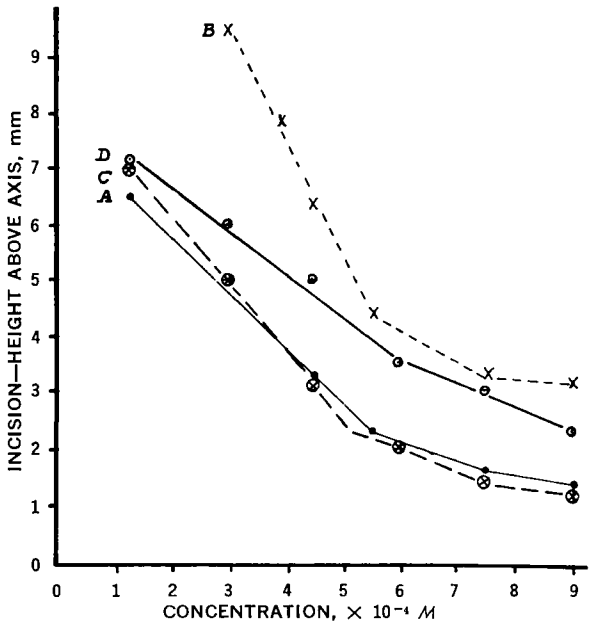

Figure 3—*Relation between concentration and depth of incision. Key: A, zinc,* Q = *0.60, anodic; B, plumbous,* Q = *0.35, cathodic; C, cadmium,* Q = *0.40, cathodic; and D, cupric,* Q = *0.15, cathodic.*

that it is unlikely that the determination can be made when the hypoxanthine-mercaptopurine ratio is smaller than 1:10.

CONCLUSIONS

1. The adapter circuit suggested was shown to be functional. The sensitivity of the apparatus is such that it is expected to be useful for solutions at a concentration of 1-5 $\times$ 10^{-4} M.

2. Mercaptopurine, which yields only a catalytic wave in classical polarography, gives a characteristic oscillopolarogram, different from that of four possible contaminants.

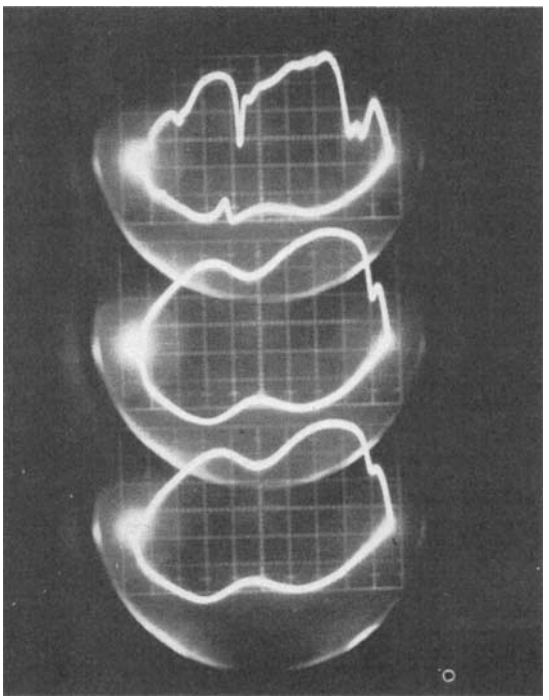

Figure 4—*Oscillopolarograms of (from top to bottom) Compound I, Compound II, and Compound III;* dE/dt = f(E) *(3×10^{-4} M in 1* N H_2SO_4, *dropping mercury electrode).*

Table I—Q Values in 1 N Lithium Chloride

Ions	Q Cathodic	Q Anodic
Zinc	0.85	0.60
Plumbous	0.30	0.20[a]
	0.35	
Cadmium	0.35	0.30[a]
	0.40	
Cupric	0.15	—
	0.25	

[a] Lobe-like, a flat indentation instead of a sharp one.

Table II—Q Values in 1 N H_2SO_4

Compound	Cathodic	Anodic
I	0.20	0.35
	2.45	
	0.85	
	0.90	
II	0.95	—
III	0.94	—
IV	0.46	—
V	0.20	0.20
	0.47	

3. Because of the differences in Q values shown by mercaptopurine and the possible contaminants, it might be possible to develop a quantitative measure of mercaptopurine in the presence of these contaminants. No advantage is foreseen over the more conventional spectrophotometric determination.

4. Because of the relative depth of the incisions, the determination by oscillopolarography of the contaminants at trace levels in mercaptopurine does not seem possible.

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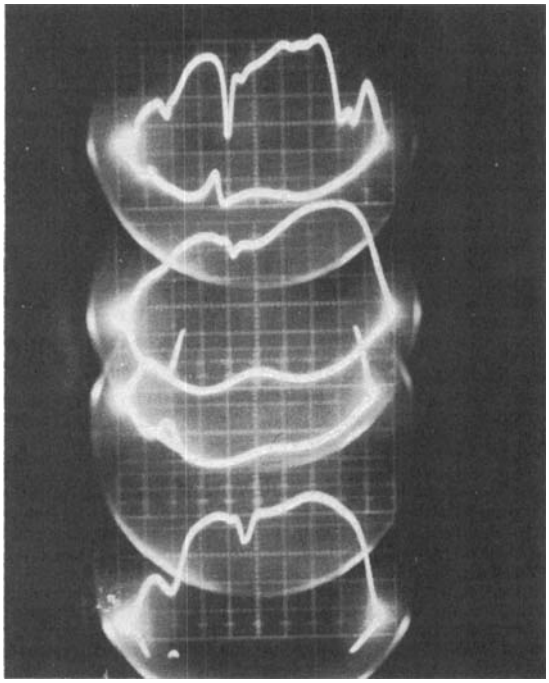

Figure 5—*Oscillopolarograms of (from top to bottom) Compound I, Compound IV, Compound V (anodic branch), and Compound V (cathodic branch); $dE/dt = f(E)$ (3×10^{-4} M in 1 N H_2SO_4, dropping mercury electrode).*

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* Present address: Statny Ustav pre Kontrolu Lieciv, Kvetna 11, Bratislava, Czechoslovakia.

x To whom inquiries should be directed.

Role of Deoxyribonuclease in Cancer Chemotherapy

IRIS H. HALL[x], K. S. ISHAQ, and C. PIANTADOSI

Abstract □ It has been postulated that the lysosomal enzyme deoxyribonuclease is responsible for nicks in both strands of deoxyribonucleic acid so that alkylating agents can cross-link the nucleotides of the two strands. Administration of an alkylating agent, *p*-di(2-chloroethyl)amino-L-phenylamine, to CF_1 mice results in elevated activities of free deoxyribonuclease in Ehrlich ascites tumor cells and normal liver homogenates and isolated lysosomal fractions. The drug acts specifically on the release of the hydrolytic enzyme deoxyribonuclease rather than nonspecific rupture of the lysosomal membrane. Deoxyribonuclease activity increases in cancer tissue as cell proliferation continues.

Keyphrases □ Deoxyribonuclease—role in cancer chemotherapy, effect of *p*-di(2-chloroethyl)amino-L-phenylamine on activity □ Cancer chemotherapy—role of deoxyribonuclease, effect of alkylating agent on activity □ *p*-Di(2-chloroethyl)amino-L-phenylamine—effect on deoxyribonuclease activity

The nitrogen mustard *p*-di(2-chloroethyl)amino-L-phenylamine[1] (I) is a phenylalanine derivative. Compound I is used in the treatment of multiple myeloma (1) and Burkitt's lymphoma (2). The accepted mechanism of action of a nitrogen mustard containing two functional centers is the cross-linking of twin strands of deoxyribonucleic acid at the 7-position of guanine and, to a lesser extent, at the 3- and 1-positions of adenine (3). This prevents deoxyribonucleic acid replication, and cell proliferation is halted probably in the S or G_2 period of the cell cycle of tumor cells (4). Furthermore, labilization of the alkylated guanine occurs with the formation of apurinic acid, which is unstable and decomposes with the breakage of the backbone of the deoxyribonucleic acid strand (5). For the nitrogen mustard to induce cross-linking in the nucleotide strands, the nucleotide strands must be broken initially. Allison (6) reported that all cells have substantial amounts of deoxyribonuclease in the lysosomes and that this enzyme is capable of breaking both strands of a double helix of the nucleic acids. Consequently, this study was designed to determine if Compound I treatment results in elevated deoxyribonuclease activity in normal and cancerous tissue.

EXPERIMENTAL

Maintenance of Tumor—CF_1 male mice were transplanted intraperitoneally with 0.1 ml of 7-day Ehrlich ascites tumor cells from infected mice. The average life expectancy for these mice was 14.9 days after injection. Transplantation was normally carried out once a week to maintain the tumor cell line in these laboratories.

Cytotoxicity of Compound I—A single dose (20 mg/kg) of Compound I in 1% carboxymethylcellulose was administered orally on Day 3 or 7 after tumor transplant. Twenty hours after dosing, the animals were sacrificed by cervical dislocation and the ascites tumor was drained from the peritoneal cavity. A microcrit and the total volume of the tumor were recorded for each animal.

[1] Alkeran brand Melphalan, Burroughs Wellcome Co.

Table I—Percent Free Deoxyribonuclease Activity of Ascites Tumor Cells

	N	$\bar{x} \pm SD$, %	*p*
Day 4 control	8	13 ± 5	—
20 mg/kg I	8	23 ± 2	0.001
Day 8 control	18	33 ± 3	—
20 mg/kg I	18	37 ± 4	—

Table II—Percent Free Deoxyribonuclease Activity of CF_1 Mouse Liver Homogenate

	N	$\bar{x} \pm SD$, %	*p*
Control	19	19 ± 3	—
I (*in vivo*):			
1 hr after 1 mg	12	28 ± 6	0.001
20 hr after 5 mg	8	24 ± 6	0.010
20 hr after 10 mg	18	26 ± 5	0.001
20 hr after 20 mg	12	23 ± 5	0.010

Free and Total Deoxyribonuclease Activity—*In Vivo*—Free and total acid deoxyribonuclease activity was determined according to the method of deDuve *et al.* (7). The detergent octoxynol[2] (0.2%) was used to release all bound lysosomal enzyme. Deoxyribonuclease was determined on the livers of normal animals and on the livers of animals treated with 20 mg/kg of I 20 hr previously. Deoxyribonuclease activity was also determined on ascites tumor fluid on Day 4 or 8 for controls and on animals 20 hr after treatment with 20 mg/kg of Compound I.

Free and total acid phosphatase activity of Ehrlich ascites tumor and mouse liver was determined by the Gianetto and deDuve method (8). The inorganic phosphate released was measured by the method of Chen *et al.* (9).

In Vitro—Free and total deoxyribonuclease activity was determined on liver after incubating at 37° for 1 hr in a metabolic shaker[3], using 1 ml of a 20% homogenate in 0.25 *M* sucrose + 0.001 *M* ethylenedinitrilotetraacetic acid (pH 7.2) and 5 ml of Hanke's basic salt medium reinforced with glucose (pH 7.2). Zero, 0.5, 1, and 2 mg of I were added to the medium.

Lysosomal Preparation—A highly purified liver lysosomal fraction was obtained from normal mice and from mice 20 hr after treatment with 20 mg/kg of I using the method of Sawant *et al.* (10). Fraction IV (17,000×*g* for 20 min) was used to determine the deoxyribonuclease enzymatic activity of purified lysosomes.

Statistical Analysis—Tables I–V list the number of animals in the group, expressed as *N*, and the mean and standard deviation, expressed as $\bar{x} \pm SD$. The probable significant level (*p*) between each test group and the control group was determined by the Student *t* test (11).

RESULTS

Compound I caused a 96% inhibition ($N = 10$) of ascites tumor growth of mice administered the drug on Day 3 and a 71% inhibition when administered on Day 7. The deoxyribonuclease activity of Ehrlich ascites tumor fluid increased significantly from Day 4 to Day 8. Compound I treatment at 20 mg/kg ip resulted in an accelerated deoxyribonuclease activity above that of the normal cancer cell on the given day examined (Table I). Treatment with Compound I also resulted in elevation in mouse liver deoxyribonuclease activity both *in vivo* and *in vitro* (Tables II and III). *In vitro*, high concentrations of the drug inhibited the free deoxy-

[2] Triton X-100, Rohm & Haas Co.
[3] Dubnoff.

Table III—*In Vitro* 1 hr Percent Free Incubation Deoxyribonuclease Activity of CF_1 Mouse Liver Homogenate

	N	$\bar{x} \pm SD$, %	p
Control	19	31 ± 4	—
0.5 mg I	8	44 ± 12	0.001
1.0 mg I	10	19 ± 4	0.001
2.0 mg I	5	17 ± 9	0.001

Table IV—Deoxyribonuclease Activity of Lysosomes Isolated from CF_1 Mouse Liver

	N	$\bar{x} \pm SD$, %	p
1% carboxymethylcellulose, control	8	52 ± 3	—
20 mg/kg I	8	60 ± 6	0.01

ribonuclease activity. When the lysosomes were isolated from normal and treated liver, it was observed that treatment with Compound I resulted in a significant elevation of lysosomal hydrolytic deoxyribonuclease activity of the lysosomal preparation (Table IV). Acid phosphatase is a well-known hydrolytic lysosomal enzyme; when its activity was measured in the liver of mice treated with I, no increase in activity was noticed but an inhibition of enzymatic activity was observed. The total lysosomal activity of deoxyribonuclease and acid phosphatase was unaltered by treatment with I.

DISCUSSION

Compound I causes an increase in percent free acid deoxyribonuclease activity of liver and Ehrlich tumor cells. The deoxyribonuclease hydrolytic enzyme is released into the cell sap by the drug from the lysosomes, where it is normally bound and inert. Purified lysosomal deoxyribonuclease can cause breaks in chromatin material (6). Davidson (12) claimed that nucleases are probably involved in maturation of ribonucleic acid, in the synthesis of deoxyribonucleic acid, and in the recombination events of proliferating cells. According to the Kornberg (13) theory, replication begins with the introduction of a nick in one strand, possibly by the action of an endonuclease. Deoxyribonucleic acid polymerase then binds to the nick and begins to extend the 3′-hydroxyl end of the nicked strand. The endonuclease can also cleave the newly formed strands at forks, resulting in short segments of the nucleic acid which are ligated together enzymatically. Thus, it is not surprising that a 2.5-fold increase of deoxyribonuclease activity was observed in the Ehrlich tumor cells as the cells proliferated over 8 days.

This study supports the idea that chemotherapy drugs, once introduced into the cell, react with lysosomal hydrolases to cause their release in cancer cells and in normal cells and direct the drug to its cellular site of action, *i.e.*, the nucleic acid (14–16). The action of I is specific for the enzyme deoxyribonuclease, which is needed for the cross-linking of the alkylating drug in the deoxyribonucleic acid strands. Since the total lysosomal enzymatic activity of acid phosphatase and deoxyribonuclease was normal after treatment with I, the drug did not lyze the lysosomal membrane as a detergent would. Furthermore, treatment with I does not cause an increase in free activity of the enzyme acid phosphatase, a known marker of hydrolytic activity. Thus, it appears that the action of I on lysosomes is specific for the release

Table V—Percent Free Acid Phosphatase Activity of CF_1 Mouse Liver Homogenate

	N	$\bar{x} \pm SD$, %	p
Control	20	59 ± 7	—
I (*in vivo*):			
1 hr after 1 mg	12	4 ± 4	0.001
2.5 hr after 1 mg	8	2 ± 2	0.001
20 hr after 20 mg	16	13 ± 11	0.001

of deoxyribonuclease and does not involve a general rupture of the lysosomal membrane. Since alkylating agents are capable of electrophilic reactions with functional groups of proteins (*i.e.*, ionized thiol, amine, ionized phosphate, and ionized carboxylic acid), it can be postulated that the polypeptide chains of the deoxyribonuclease enzyme are attached and then the enzyme acts as a carrier of the drug to the nucleus, resulting in cross-linking of the deoxyribonucleic acid strands.

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[x] To whom inquiries should be directed.

Kinetics of Hydrolysis of Acylals of Aspirin: Hydrolysis of (1′-Ethoxy)ethyl 2-Acetoxybenzoate

ANWAR HUSSAIN ˣ, M. YAMASAKI, and J. E. TRUELOVE

Abstract □ A derivative of aspirin, (1′-ethoxy)ethyl 2-acetoxybenzoate, was synthesized and its hydrolysis was studied in aqueous solution. The rate of hydrolysis was followed spectrophotometrically and found to be: *(a)* first order with respect to the compound, *(b)* independent of pH, *(c)* very sensitive to solvent polarity, and *(d)* an isotope effect (k_{D_2O}/k_{H_2O}) near unity. Based on these results, the hydrolysis of the derivative appears to proceed by a classical S_N1-type mechanism.

Keyphrases □ Aspirin derivatives, (1′-ethoxy)ethyl 2-acetoxybenzoate—synthesis and hydrolysis □ (1′-Ethoxy)ethyl 2-acetoxybenzoate (aspirin derivative)—synthesis and hydrolysis

The oral administration of aspirin has been shown to induce gastric irritation and bleeding (1–3). It is believed (1) that this effect is due to a local irritation of the mucous membranes by the very acidic aspirin particles.

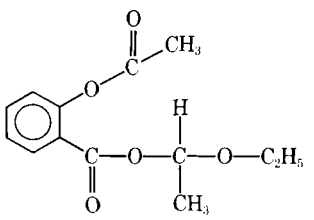
It was indicated (1) that present aspirin products containing alkaline additives to reduce the gastric irritation have the disadvantages of either high sodium content or limited buffer capacity.

In an attempt to mask reversibly the acidic carboxylic group of aspirin and hopefully to reduce its local irritation, (1′-ethoxy)ethyl 2-acetoxybenzoate (I) was synthesized and its rate of hydrolysis was studied. In aqueous solution, Compound I cleaves extremely rapidly, generating aspirin.

EXPERIMENTAL

Compound I was prepared according to the following procedure. To 3.0 g of aspirin in a 100-ml stoppered, round-bottom flask, 50 ml of freshly distilled dichloromethane, 30 ml of freshly distilled ethyl vinyl ether[1], and 10 ml of dichloromethane saturated with hydrogen chloride gas were added. The reaction mixture was stirred at room temperature for 2 hr. The solvents were then removed at 40° using a rotary evaporator, leaving a viscous oil.

Alternatively, and more conveniently, the hydrogen chloride gas was replaced with 0.1 ml of a 0.4% solution of *p*-toluenesulfonic acid in benzene. At the completion of the reaction, the remaining p-toluenesulfonic acid was neutralized with 1 equivalent of pyridine. The solvents were then removed at ≤40° using a rotary evaporator, leaving a viscous oil. The NMR and IR spectra and the elemental analysis were consistent with the structure of I; NMR ($CDCl_3$): δ 6.93–8.15 (m, 4, ArH), 6.13 [q, 1, —O—C*H*(CH_3)—O—], 3.35–3.95 (m, 2, —O—C*H*$_2$—CH_3), 2.31 (s, 3, —CO—CH_3), 1.46 [d, 3, —O—CH(C*H*$_3$)—O], and 1.20 (t, 3, —CH_2C*H*$_3$); IR (neat): 3050 (ArH), 2920 (aliphatic C—H), 1770 [CH_3—C(=O)—O—], 1720 [Ar—C(=O)—O—], 1300 and 1190 (C—O, ester), 1070 and 1030 (C—O, ether), and 750 and 700 (ArH) cm^{-1}.



I

Anal.—Calc. for $C_{13}H_{16}O_5$: C, 61.85; H, 6.39. Found: C, 61.58; H, 6.11.

The rate of hydrolysis was measured spectrophotometrically at 285 nm by measuring the formation of aspirin and was found to be first order with respect to the compound over the pH range studied.

Table I—Half-Lives of Hydrolysis of I at 25° and Ionic Strength of 0.1

pH	$t_{1/2}$, sec[a]
3.25	3.2
4.00	3.1
5.23	3.5
6.28	3.5

[a] Each half-life is an average of four determinations.

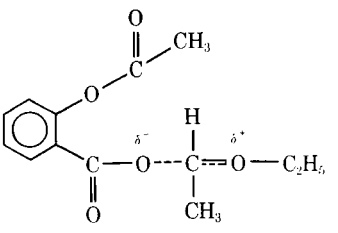
Table II—Dependency of First-Order Rate Constants for Hydrolysis of I on the Dielectric Constant of the Solution

Dioxane, %	Dielectric Constant (ε)	k_{obs}, sec^{-1}
0	78.6	0.23
5	74	—
10	70	0.111
25	56.3	0.046
50	34.3	0.0027

RESULTS AND DISCUSSION

The facts that the rate of hydrolysis is independent of pH (Table I) and markedly dependent on the dielectric constant (Table II) and that the ratio of k_{D_2O}/k_{H_2O} is unity[2] would strongly suggest that the hydrolysis of I proceeds by an S_N1-type mechanism (4, 5). The rate-limiting step in the hydrolysis reaction is probably the C—O bond cleavage as shown in Scheme I.

The presented data show that *transient* blocking of the very



Scheme I

[1] Aldrich Chemical Co.

[2] The half-lives in seconds for the hydrolysis of I in deuterium oxide and water are 3.2, 3.3, and 3.2 and 3.2, 3.1, and 3.2, respectively.

acidic carboxylic group of aspirin may be achieved by making derivatives similar to Compound I. One possible advantage of a compound such as I is that the mucous membranes of the stomach are in contact with a neutral molecule. As I dissolves, however, it will rapidly hydrolyze, generating aspirin in solution. The mechanism of hydrolysis of I was shown to proceed by a classical S_N1-type reaction.

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[x] To whom inquiries should be directed. Present address: College of Pharmacy, University of Kentucky, Lexington, KY 40506

Selenium Heterocycles X: Synthesis and Antibacterial Activity of Pyridyl-1,2,3-thiadiazoles and Pyridyl-1,2,3-selenadiazoles

I. LALEZARI[x], A. SHAFIEE, and S. YAZDANY

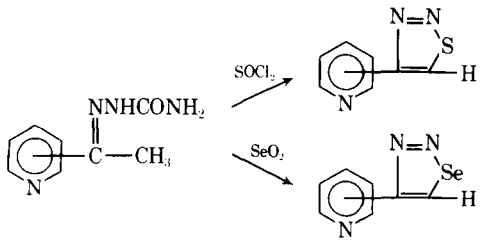
Abstract □ Three isomeric 4-pyridyl-1,2,3-selenadiazoles and their corresponding diselenafulvines were prepared. 4-(3-Pyridyl)-1,2,3-thiadiazole and its dithiafulvene were also synthesized. The hydrochloride salts of all compounds prepared showed significant antibacterial activity.

Keyphrases □ Selenium heterocycles—synthesis and antibacterial activity of pyridyl-1,2,3-thiadiazoles and pyridyl-1,2,3-selenadiazoles □ Pyridyl-1,2,3-thiadiazoles and selenadiazoles—synthesized and screened as antibacterial agents □ Selenadiazoles, pyridyl-1,2,3—synthesized and screened as antibacterial agents □ Antibacterial agents, potential—synthesis and screening of pyridyl-1,2,3-thiadiazoles and pyridyl-1,2,3-selenadiazoles

Recently, the synthesis of 1,2,3-selenadiazoles by selenium dioxide oxidation of aldehyde or ketone semicarbazones having an α-methyl or methylene group was reported (1–4). It was also shown that 1,2,3-selenadiazoles and base afforded 1,4-diselenafulvenes (5).

DISCUSSION

In the present work, the three isomeric 4-pyridyl-1,2,3-selenadiazoles were prepared. Also, 4-(3-pyridyl)-1,2,3-thiadiazole was prepared in high yield by reaction of thionyl chloride and 3-acetylpyridine semicarbazone (Scheme I). All thia- and selenadiazoles prepared (Tables I and II) were converted to the corresponding dithia- and diselenafulvenes by reaction with potassium hydroxide in ethanol (Scheme II).



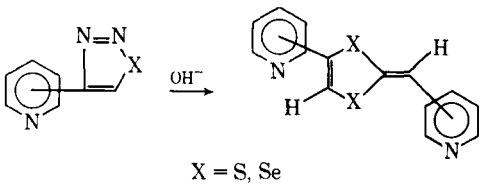
Scheme I

Some of the 1,3,4-thiadiazole derivatives have shown antibacterial and antiviral activities (6, 7). Also, 1,2,3-selenadiazole and some of its derivatives were found to have significant antibacterial activity (5). In the search for new potent antibacterial agents, it was of interest to study the antibacterial activity of the pyridyl thia- and selenadiazoles closely related to these compounds. All compounds (Tables I and II) were tested against *Bacillus subtilis* NCTC 3610, *Staphylococcus aureus* ATCC 6538, *Klebsiella pneumoniae* ATCC 10031, and *Sarcina lutea* ATCC 9341. Nitrofurazone was used as a control. Standard paper disks of 6 mm diameter were immersed in solution and placed on inoculated assay medium surface[1].

The antibacterial activity of all compounds that were dissolved in acetone at the concentration 0.5% were insignificant. However, the hydrochloride salts (prepared by dissolving the base in dilute hydrochloric acid and drying at room temperature in the dark) at a concentration of 0.5% in distilled water (and nitrofurazone dissolved in dimethylformamide at the same concentration) were active (Table III).

EXPERIMENTAL[2]

4-(2-Pyridyl)-1,2,3-selenadiazole (I)—2-Acetylpyridine semicarbazone (4.4 g, 0.025 mole) was dissolved in 50 ml of boiling



Scheme II

[1] Antibiotic Assay Medium; British Pharmacopoeia, 1968.

[2] Melting points were taken on a Kofler hot-stage microscope and are uncorrected. The IR spectra were recorded using a Leitz spectrograph. UV spectra were obtained with a Varian Techtron 635 instrument. The mass spectra were recorded on a Varian Mat 111 instrument.

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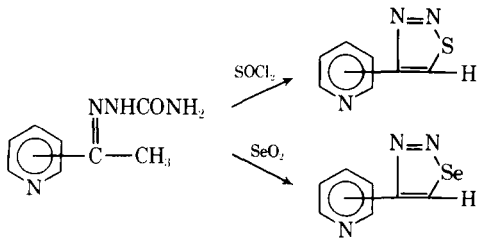

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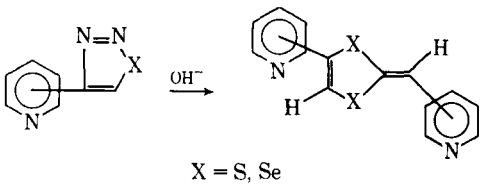
The antibacterial activity of all compounds that were dissolved in acetone at the concentration 0.5% were insignificant. However, the hydrochloride salts (prepared by dissolving the base in dilute hydrochloric acid and drying at room temperature in the dark) at a concentration of 0.5% in distilled water (and nitrofurazone dissolved in dimethylformamide at the same concentration) were active (Table III).

EXPERIMENTAL[2]

4-(2-Pyridyl)-1,2,3-selenadiazole (I)—2-Acetylpyridine semicarbazone (4.4 g, 0.025 mole) was dissolved in 50 ml of boiling

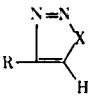


Scheme II

[1] Antibiotic Assay Medium; British Pharmacopoeia, 1968.

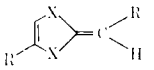
[2] Melting points were taken on a Kofler hot-stage microscope and are uncorrected. The IR spectra were recorded using a Leitz spectrograph. UV spectra were obtained with a Varian Techtron 635 instrument. The mass spectra were recorded on a Varian Mat 111 instrument.

Table I—1,2,3-Thia- and Selenadiazoles



Compound	R	X	Melting Point	Yield, %	Formula	Analysis, % Calc.	Found
I	2-Pyridyl	Se	79–81°	65	$C_7H_5N_3Se$	C 39.81	39.99
						H 2.37	2.39
II	3-Pyridyl	Se	85°	52	$C_7H_5N_3Se$	C 39.81	39.86
						H 2.37	2.29
III	4-Pyridyl	Se	132°	48	$C_7H_5N_3Se$	C 39.81	39.82
						H 2.37	2.40
IV	3-Pyridyl	S	88–90°	82	$C_7H_5N_3S$	C 51.53	51.44
						H 3.07	3.06

Table II—*trans*-Dithiafulvenes and *trans*-Diselenafulvenes



Compound	R	X	Melting Point	Yield, %	Formula	Analysis, % Calc.	Found
V	2-Pyridyl	Se	258–260°	85	$C_{14}H_{10}N_2Se_2$	C 45.90	45.92
						H 2.73	2.80
VI	3-Pyridyl	Se	170–175°[a]	92	$C_{14}H_{10}N_2Se_2$	C 45.90	46.01
						H 2.73	2.72
VII	4-Pyridyl	Se	250°[b]	86	$C_{14}H_{10}N_2Se_2$	C 45.90	45.99
						H 2.73	2.70
VIII	3-Pyridyl	S	188–190°	83	$C_{14}H_{10}N_2S_2$	C 62.22	62.26
						H 3.70	3.78

[a] *cis*-Isomer, mp 130–132°. [b] *cis*-Isomer, mp 183–185°.

acetic acid. To the hot solution, 2.75 g (0.025 mole) of selenium dioxide was added and the reaction mixture was stirred until the vigorous reaction ceased. Heat was applied for 0.5 hr to complete the reaction. Charcoal was added and the solution was filtered hot, diluted with 250 ml of water, and extracted with 50 ml of chloroform. The organic layer separated and was washed with 10% sodium bicarbonate solution and then with water. After evaporation of the solvent, the crystalline residue was recrystallized from benzene containing 25% petroleum ether to give 3.3 g of I (65%), mp 79–81°; molecular weight (by mass spectroscopy): 211; UV: λ_{max} 269 and 306 nm; IR: ν_{max} 3330, 3130, 1587, 1493, 1417, 1344, 1293, 1235, 1055, 1017, 960, 916, 818, 799, 777, 740, and 727 cm^{-1}.

4-(3-Pyridyl)-1,2,3-selenadiazole and 4-(4-pyridyl)-1,2,3-selenadiazole were prepared similarly.

4-(3-Pyridyl)-1,2,3-thiadiazole (IV)—3-Acetylpyridine semicarbazone (5.34 g, 0.03 mole) was added portionwise to 15 ml of thionyl chloride at ice bath temperature and kept 0.5 hr at room temperature. Chloroform, 50 ml, was added and the mixture was decomposed with ice-cold concentrated sodium carbonate solution. The organic layer was washed with water and dried with anhydrous sodium sulfate. After evaporation of the solvent, the crystalline mass was recrystallized from aqueous ethanol to give 4 g (82%) of IV, mp 88–90°; molecular weight (by mass spectroscopy): 163.

2,ω-Di(2-pyridyl)-1,4-diselenafulvene (V)—4-(2-Pyridyl)-1,2,3-selenadiazole (1.0 g, 5 mmoles) was dissolved in 20 ml of 95% ethanol and a pellet of potassium hydroxide was added. After gas evolution ceased, water was added and the crystals were filtered, washed with water, and recrystallized from ethanol to give 0.77 g (85%) of V, mp 258–260°; molecular weight (by mass spectroscopy): 366; UV: λ_{max} 245 and 385 nm; IR: ν_{max} 3305, 1582, 1518, 1455, 1400, 1380, 1215, 1145, 1052, 990, 908, 880, 823, 780, and 743 cm^{-1}.

Table III—Antibacterial Activity[a]

Compound[b]	Average Inhibition Zone Diameter, mm *B. subtilis*	*K. pneumoniae*	*Staph. aureus*	*S. lutea*
I	14	15*	8*	14
II	10	15	10*	8
III	10	18	16	10
IV	12	–	–	12
V	15	–	14	25
VI	16	15	17	18
VII	14	15	17	13
VIII	14	8	18*	17
Nitrofurazone	18	21	23	20

[a] – = inactive; * = inhibition zones were hazy. [b] As the hydrochloride.

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× To whom inquiries should be directed.

Simple Modified Colorimetric Method for Total Salicylate Assay in Urine after Salicylate Administration

WIN L. CHIOU[x] and IJEOMA ONYEMELUKWE

Abstract □ A simpler and yet sensitive colorimetric method, modified after Levy's method for the assay of total urinary salicylate, is presented. The values of salicylate recovery after oral administration in humans, based on the present method and Trinder's reagent method, are compared.

Keyphrases □ Salicylates, total—modified colorimetric method for assay in urine □ Colorimetry—analysis, total salicylates in urine

Measurements of urinary salicylate excretion have been used to study the bioavailability of salicylates such as aspirin and sodium salicylate from their dosage forms. Two analytical methods are most often employed: *(a)* Trinder's reagent method (1–4), and *(b)* Levy's modification (5–9) of the method of Smith *et al.* (10).

The first method is simpler than the second. It merely involves the measurement of absorbance at 540 nm of the mixture of 1 ml of urine sample and 5 ml of Trinder's reagent. The absorbance is then corrected for the value due to blank urine taken before drug administration. However, it suffers from two major drawbacks. First, the urinary blank contribution not due to absorbed salicylates may vary markedly during the day (11). As a result, this analytical method may underestimate or overestimate the salicylate excretion. For example, one subject was estimated to excrete 159 mg of sodium salicylate in 24 hr even if no salicylate was ingested. Second, Trinder's reagent does not react with the glucuronide metabolites of salicylic acid and underreacts with salicyluric acid, a major metabolite, when compared with salicylic acid on an equimolar basis (10). Consequently, this method tends to underestimate the total salicylate excretion if the blank contribution remains constant during the study. The method is further complicated by the fact that the relative composition of excreted aspirin metabolites may vary markedly with dose and time during the study (8).

In Levy's modified method, 3 ml of the urine sample and 3 ml of concentrated hydrochloric acid are heated in a sealed 50-ml (5) or 20-ml (6) glass ampul at 100° for 16 hr to hydrolyze completely all metabolites to salicylic acid. An aliquot of the hydrolyzed sample is then accurately removed, acidified, and extracted with 30 ml of ethylene chloride (5) or carbon tetrachloride (6) in a glass-stoppered bottle. The salicylic acid from an aliquot of the organic solvent phase was assayed colorimetrically after complexing with ferric ion in the acidic medium. In this method, the total salicylate, which includes aspirin, salicylic acid, and all other metabolites, is analyzed. Since essentially all absorbed aspirin or sodium salicylate can be recovered from the urine after oral administration (6, 8, 12), this method is obviously better than the Trinder's reagent method for studying the total bioavailability of salicylates from dosage forms.

This article reports a simpler and yet sensitive modified procedure for assaying the total salicylate in urine specimens. The values of urinary salicylate recovery after oral administration of aspirin based on the Trinder's reagent method and the presently proposed method also are compared. The reported studies are relevant, especially in light of the facts that the salicylates are the most widely used drugs today and that their adequate bioavailability from dosage forms is critical, particularly for patients on extensive and chronic medication (13).

EXPERIMENTAL

Chemicals—The following reagent grade chemicals were used: sodium salicylate[1], hydrochloric acid[2], chloroform[3], mercuric chloride[3], and ferric nitrate[4].

Analytical Procedure—Concentrated hydrochloric acid (2 ml) was added to 3 ml of urine samples in a screw-top Pyrex culture tube[5] or screw-top disposable culture tube[6]. After sealing the tubes with plastic caps[6], they were incubated in an oven at 100° for 17 hr or 65° for 1 week.

After cooling, 0.5 ml of approximately 6 *N* hydrochloric acid (prepared by dilution of concentrated hydrochloric acid with an equal volume of distilled water) and 6 ml of chloroform were added. The tubes were shaken[7] for 10 min. They were then centrifuged for 5 min in a regular clinical centrifuge[8], and the aqueous phase was aspirated. Three milliliters of the chloroform layer was then accurately transferred to another screw-top culture tube. Six milliliters of Trinder's reagent (1) without mercuric chloride (11) was added. The tubes were shaken for 10 min and centrifuged. The chloroform phase was aspirated, and the absorbance of the aqueous layer was measured[9] at 540 nm. Trinder's reagent without mercuric chloride was used for 100% transmittance adjustment. To calculate the concentration of total salicylate in urine specimens, a standard curve was constructed using known concentrations of sodium salicylate (0.125, 0.25, 0.5, 1.0, and 2 mg/ml) in distilled water and the same incubation and extraction procedure. All assays were run in duplicate. Reproducible results were obtained throughout the study.

Blank Urine Analysis—The intrasubject variation of the urine blank value was studied in six healthy subjects between 22 and 34 years old. No drugs were taken for at least 1 week before the

[1] Fisher.
[2] J. T. Baker.
[3] Mallinckrodt.
[4] Matheson, Coleman and Bell.
[5] Fisher No. 14-932A, 125 × 16 mm, 16-ml size.
[6] Fisher No. 14-959-36A.
[7] Using an Eberbach shaker.
[8] Centricone, G. C. A. Corp., Chicago, Ill.
[9] Using a Beckman DBGT spectrophotometer.

Table I—Absorbances of Blank Urine Samples at 540 nm

Sample	Subject 1	2	3	4	5	6
			Day 1			
Before breakfast	0.015	0.000	0.006	0.000	0.000	0.019
Breakfast–lunch	0.010	0.005	0.005	0.000	0.006	0.017
Lunch–supper	0.008	0.010	0.005	0.000	0.004	0.015
Supper–bedtime	0.010	0.005	0.005	0.000	0.002	0.015
			Day 2			
Before breakfast	0.010	0.004	0.005	0.000	0.000	0.015
Supper–bedtime	0.010	0.010	0.01	0.025	0.004	0.005
			Day 3			
Before breakfast	0.009	0.001	0.005	0.01	0.000	0.01
Supper–bedtime	0.008	0.008	0.004	0.01	0.01	0.015
Mean	0.010	0.005	0.006	0.01	0.003	0.014
$\pm SEM$	0.001	0.001	0.001	0.003	0.001	0.002
Mean as milligrams equivalent aspirin per milligram urine sample	0.009	0.005	0.006	0.009	0.003	0.014

study and during the study. On the 1st day, urine samples were collected before breakfast, between breakfast and lunch, between lunch and supper, and between supper and bedtime. On the 2nd and 3rd days, only the urine samples before breakfast and between supper and bedtime were collected for analysis. The subjects were instructed to have normal food and fluid intake. The urine specimens were kept at about 5° until analyzed (within 1 week).

Aspirin Absorption Study in Humans—Two commercial 300-mg (5-gr) buffered aspirin tablets were administered before breakfast with 200 ml of lukewarm water to two healthy subjects (ages 22 and 34). Urine samples were collected at 3, 6, 12, and 24 hr after administration. The volumes were recorded and portions of samples were stored in culture tubes. The samples were analyzed for salicylate content by both the proposed method and the Trinder's reagent method. In both methods, correction was made for the contribution due to blank urine collected before drug administration.

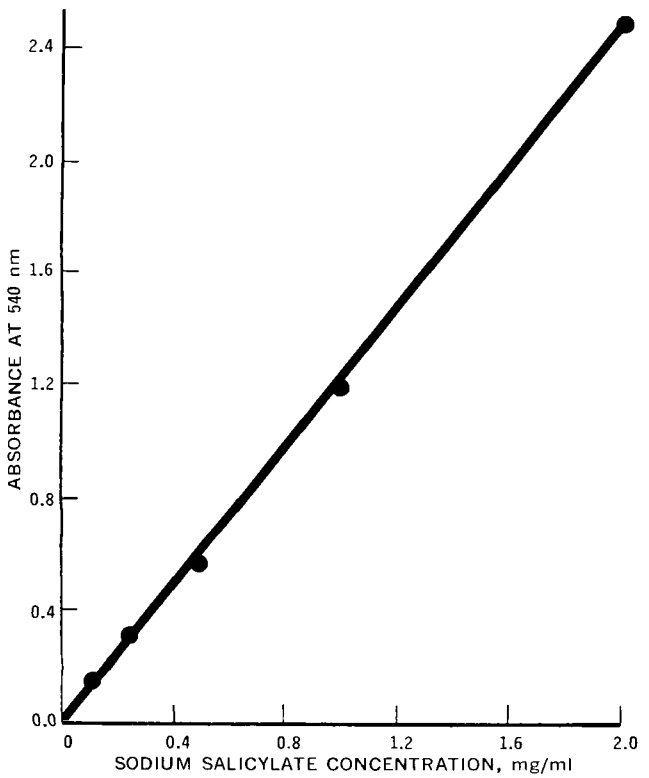

Figure 1—*Standard curve of sodium salicylate solutions.*

RESULTS AND DISCUSSION

The standard curve using the proposed procedure is shown in Fig. 1. The absorbances of the final solutions were proportional to the original concentrations of sodium salicylate. It can be calculated that 1 unit of absorbance corresponds to 0.84 mg/ml of sodium salicylate or 0.945 mg/ml of aspirin in the original sample. Based on the salicylate dose usually administered, this assay sensitivity is believed adequate to quantitate accurately the total amount of salicylate excreted in the urine. Although Levy's modified method has been used successfully in many studies (5–9), its sensitivity does not appear to have been reported. It must be emphasized that this investigation was not intended to challenge its accuracy or sensitivity but merely to explore another simpler and yet sensitive procedure for the assay of total salicylate in urine.

As reported previously (1), the absorbance of the salicylic acid–ferric-ion complex resulting from the reaction of salicylic acid with Trinder's reagent could vary markedly with the pH of the solution. Therefore, some precaution should be exercised in preparing Trinder's reagent (without mercuric chloride) and a standard curve should be established for each lot of the reagent prepared. (The prepared reagent was found to be stable for several months when stored at room temperature in a light-resistant, tight container.) Although significantly less of the organic solvent, chloroform, was used in the present method as compared to the ethylene chloride or carbon tetrachloride used in the previous methods (5, 6), the extraction efficiency of salicylic acid from the aqueous phase to the organic phase was quite high (91%). Moreover, chloroform is more commonly available in most laboratories and is probably less toxic. Therefore, it appears that this solvent should be preferred. The extraction of salicylic acid from the chloroform to the Trinder's reagent phase was found to be 65% complete, which is reasonably satisfactory in view of its high solubility in the organic solvent.

In developing an analytical method, the blank contribution should be of major concern. The results of the blank urine study on six subjects are shown in Table I. It is evident that the intra- and intersubject variation in blank readings was consistently low

Table II—Urinary Salicylate Excretion (Milligrams) in Terms of Equivalent Aspirin Analyzed by Two Methods

Time Interval, hr	Trinder's Reagent Method		Proposed Method	
	Subject 1	Subject 2	Subject 1	Subject 2
0–3	74.6	156.0	129.4	178.7
3–6	100.0	145.7	135.3	194.4
6–12	115.8	152.2	182.9	216.9
12–24	74.6	0	113.1	13.2
Total	365.0	453.9	560.7	603.2

as compared to the results obtained from the Trinder's reagent method (11). One can estimate that the average blank contribution from the subjects is equivalent to 7.7 mg aspirin/day, assuming 1 liter of daily urine output. This value is extremely low when compared to the usual dose of salicylates ingested. The individual blank values using Levy's modified method were reported to be less than 24 mg salicylic acid/day (5). The inter- and intrasubject variation in blank contribution, however, was not reported previously.

The screw-top culture tubes used in the present method are obviously more convenient and easier to use than the ampuls used in the previous methods. The sealing of the ampuls requires extra work, facility, and skill. Since the filled ampuls have to resist pressure when heated at 100° for 17 hr, incomplete sealing results in the loss of sample and makes the study invalid. On the other hand, tightening of a screw-cap on a culture tube can be accomplished in seconds without failure and requires no training and extra facility. Furthermore, the culture tubes and caps can be reused. Although there was some darkening of the rubber liner in the screw-cap after repeated use, it did not interfere with the assay sensitivity. The sorption, if any occurred, of aspirin or salicylic acid onto the screw-cap was found to be negligible. The evaporation of samples from the sealed tubes was also negligible, even after several days of incubation at 100°. Identical results were obtained with screw-caps with liners coated with Teflon, which were much more expensive than the regular caps. The presently proposed method also eliminates one step of the transfer of an aliquot of the hydrolyzed sample. Moreover, the sample in the ampul would presumably have to be poured into another container before the quantitative transferring of the hydrolyzed sample, and this would require additional work.

In the previous methods, an extraction process involving 30 ml of organic solvent and subsequent centrifugation for phase separation in a large enough glass-stopper bottle requires more careful manipulation than the extraction process performed in a small culture tube with a screw cap. Furthermore, the centrifugation in the present method can be carried out by the more common and less expensive clinical centrifuge, making the study possible in a laboratory with only a small budget. Due to its simplicity and sensitivity and the need for less facility, the present method was adopted in the undergraduate biopharmacy laboratory experiment for the bioavailability evaluation of commercial aspirin dosage forms. The preliminary results of bioavailability study on five commercial buffered aspirin dosage forms in human subjects employing the proposed assay method were published previously (12).

The urinary salicylate excretion in terms of equivalent aspirin after dosing of two 300-mg (5-gr) aspirin tablets in two subjects is shown in Table II. The ratios of values obtained from the Trinder's reagent method and proposed method were found to vary with time and subjects. In Subject 1, the value of estimated salicylate recovery in 24 hr based on the Trinder's reagent method was only 65% of that based on this method. In Subject 2 the relative recovery was 75.2%. These data indicate that the Trinder's reagent method, while simple, may significantly underestimate the extent of salicylate absorption from dosage forms. They also confirmed the pitfalls of using this method as already discussed.

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[x] To whom inquiries should be directed.

Ophthalmic Bioavailability I: Corneal Penetration of Aceclidine (3-Acetoxyquinuclidine) into the Rabbit Eye Using a Perfusion Technique

B. BRIAN, J. J. BOLTRALIK[x], L. THOM, and L. D. ZELEZNICK

Abstract □ A rapid, efficient, and sensitive method was developed for extraction and subsequent quantitation of aceclidine (3-acetoxyquinuclidine) hydrochloride and 3-quinuclidinol hydrochloride from biological fluid. After adjustment of pH and salt concentration, chloroform extracts of serum, urine, or aqueous fluid could be quantitated by GLC without derivative formation. The analytical procedure was used to determine the corneal absorption of aceclidine in the rabbit eye. Aceclidine, a drug used topically in glaucoma treatment, was found to enter the anterior chamber of the eye by penetration exclusively through the cornea. In experiments on conjunctival absorption, the amount found in the anterior chamber was less than 50 ng. The corneal absorption experiments gave cumulated absorption of from 1 to 8.5 μg in 30 min.

Keyphrases □ Aceclidine—ophthalmic bioavailability, corneal penetration into rabbit eye, perfusion technique, analytical determination □ Bioavailability, ophthalmic—aceclidine analysis, rabbit eye, perfusion technique, corneal penetration □ Ophthalmic drugs, aceclidine—bioavailability, corneal penetration, rabbit eye, perfusion technique, analysis □ 3-Quinuclidinol hydrochloride—analysis, biological fluids, ophthalmic bioavailability of aceclidine, corneal penetration

For a drug to be effective in topical glaucoma therapy, it should have good penetrability into the anterior chamber so that direct action on the parasympathetic end-organs in the iris, the trabecular meshwork, and ciliary body is accomplished to influence intraocular pressure. Since aceclidine hydrochloride (I) is a cholinomimetic drug and has properties in common with acetylcholine, it was of interest to determine the amount of absorption of the drug through the cornea and/or conjunctival membrane into the anterior chamber of the eye.

These absorption studies necessitated the development of a rapid, efficient, and sensitive method for the extraction and quantitative analysis of drug from aqueous fluid or alternative biological fluid such as serum and urine. Since deacetylation of I by the biological system to yield 3-quinuclidinol (II) seemed probable, the analytical method required a selective procedure with equal efficiency for both I and II. Such a method is reported in this paper[1].

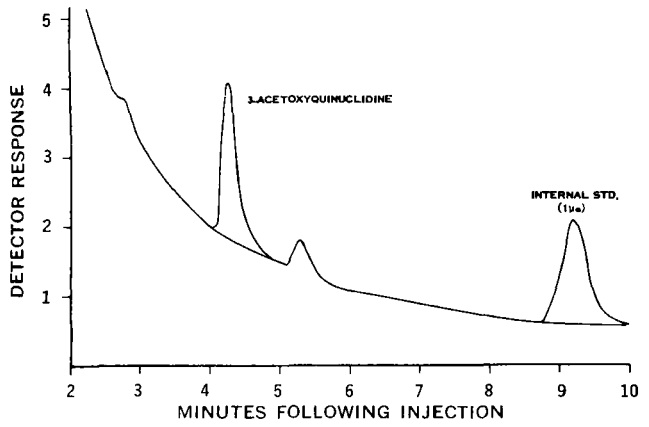

Figure 1—*GLC scan of a 4-ml serum sample containing 1 μg aceclidine (3-acetoxyquinuclidine).*

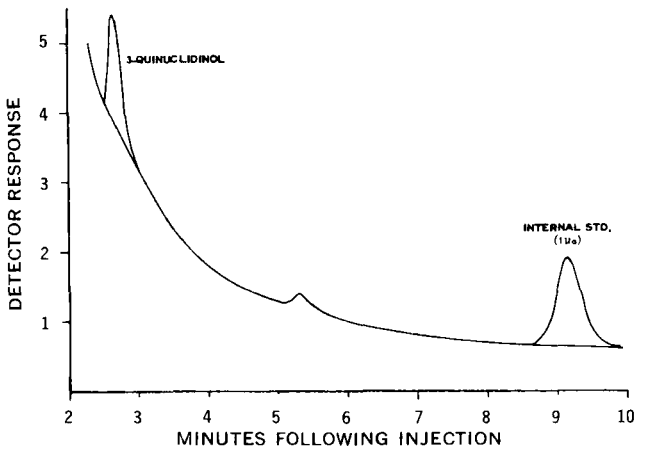

Figure 2—*GLC scan of a 4-ml serum sample containing 1 μg 3-quinuclidinol.*

Consideration was given to which of the two topical routes tested, conjunctival or corneal, would produce a measurable amount of drug in the anterior chamber of the rabbit eye and to the correlation, if possible, of the presence of drug with cholinomimetic symptoms.

EXPERIMENTAL[2]

Quantitation of I in Aqueous Fluid, Serum, or Urine—Aceclidine hydrochloride[3] (I) and 3-quinuclidinol[4] (II) were employed. The GLC internal standard was 2,4-dichloro-3-methyl-5-ethylphenol[4]. All solvents were spectrophotometric grade.

Serum or aqueous fluid containing I was adjusted to pH 8–9 and extracted three times with 1.5 volumes of chloroform in a separator. The chloroform extracts were combined and evaporated to dryness under reduced pressure. The internal standard was added prior to dissolving the residue in 30 μl chloroform.

Urine samples containing I were first acidified to pH 2–3 with hydrochloric acid and then were washed three times with 1.5 vol-

[1] A preliminary report appeared in Abstracts of the 9th National Meeting, APhA Academy of Pharmaceutical Sciences, Nov. 15–18, 1970, p. 61.

[2] GLC was performed with 0.82-m × 0.31-cm (6-ft × 0.12-in.) stainless steel columns packed with 10% UCC-W-982 (methyl vinyl silicone gum rubber) on Diataport S (80–100 mesh) operated at 170°. The injector ports were at 230°, and hydrogen flame detectors were at 250°. Helium carrier flow was 30 ml/min, hydrogen was at 40 ml/min, and air was at 500 ml/min. Peak areas were quantitated by multiplication of peak height and width at one-half peak height.

[3] Laboratories Chibret, France.

[4] Aldrich Chemical Co.

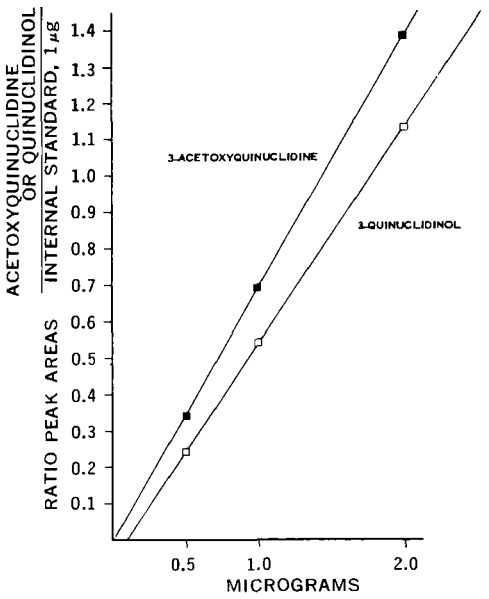

Figure 3—*Response curves of aceclidine (3-acetoxyquinuclidine) hydrochloride and 3-quinuclidinol (0.5–2 μg) relative to 1 μg internal standard using GLC flame detector.*

umes of chloroform. The chloroform extracts were discarded, and the urine was adjusted to pH 8–9 with ammonium hydroxide. The alkaline urine was then treated identically to the serum.

To extract II, solid sodium chloride was added to make a 12.5% solution in the chloroform-extracted serum and the solution was shaken vigorously for 1 min. One volume of sodium chloride-saturated concentrated ammonium hydroxide was added, and the mixture was extracted three times with 1.5 volumes of chloroform. The chloroform was removed, the internal standard was added, and the residue was taken up in 30 μl chloroform for injection into the gas chromatograph.

The extraction of II from human urine requires that 4 ml chloroform-extracted urine, 1 g sodium chloride, and 1 g potassium carbonate be added prior to addition of the sodium chloride-saturated ammonium hydroxide. From this point, the procedure was the same for the urine as for the serum or aqueous fluids.

A typical chromatographic scan for a spiked serum sample is depicted in Figs. 1 and 2. Recovery of I and II from biological samples averaged 91 and 83%, respectively.

Prior to analysis of extracted compounds, response curves for I and II (0.5, 1, and 2 μg each) were determined relative to 1 μg internal standard (Fig. 3).

Perfusion Method—Rabbits weighing 2 kg were anesthetized with 50% urethan solution intravenously, and then two cannulas (No. 20 needles) were inserted at the limbus carefully into the anterior chamber of the eye. The input was under a hydrostatic head of pressure of 20 mm Hg, and the outflow was at atmospheric pressure. The anterior chamber perfusate was collected in a graduated tube to determine volume per unit time. Heparin (1 unit/ml) was added to 0.15 *M* NaCl input solution to prevent coagulation in the outflow tube if bleeding occurred.

Corneal Absorption—Eye retractors were placed under the eyelid to expose the eyeball fully. A low viscosity petrolatum vehicle was placed into the eye socket using a syringe until the petrolatum was a few millimeters over the limbus. The "eye well," a modified plastic contact lens having a 4.5-mm hole and to which was cemented a plastic cylinder having a volume of approximately 0.1 mm^3, was set on top of the cornea. A high viscosity petrolatum was used to seal the well to the cornea. The well prepared in this manner was "leakproof" for 30–50 min. One hundred microliters of 2% I in 0.15 *M* NaCl was added to the well, and the collection of perfusate was started. The perfusion flow rate under these conditions varied from 0.1 to 0.3 ml/min.

Conjunctival Absorption—The rabbit was anesthetized and the eyeball was exposed with retractors. The head of the rabbit was positioned so that the lachrimal duct was away from the area where drug was to be placed. The eyeball could be shifted slightly using sutures under the inferior and superior latis rectus muscles to prevent the cornea from being immersed in the drug. The scleral conjunctiva was bathed in 100 μl drug solution.

Table I—Correlation of Aceclidine in the Anterior Chamber with Salivation Resulting from Topical Administration

Type of Absorption Experiment[a]	Minutes	
	First Trace of Drug	Salivation
Corneal	2	4
Corneal	10	45
Corneal	2	16
Corneal	2	14
Corneal	2	30
Corneal	7	—
Conjunctival	16	6
Conjunctival	25	5
Conjunctival	10	4
Conjunctival	None	31
Conjunctival	None	14
Conjunctival	None	26

[a] No miosis was noted in the opposite eye.

RESULTS AND DISCUSSION

GLC of 3-quinuclidinol and aceclidine was performed without derivative formation of either compound. Some peak tailing occurred with both compounds but was more pronounced with 3-quinuclidinol. Columns containing a higher percentage of stationary phase (10%) prevented the formation of active sites caused by injection of entrained acid or base in the chloroform extracts, and both periodic silylation[5] and conditioning of the column overnight at 250° were required to obtain linear response curves with high sensitivity.

Compound I was extracted efficiently at low concentrations with chloroform from aqueous solution at pH 8 but remained in the aqueous phase under acidic conditions (pH < 6) for both serum and urine. It was necessary to extract chloroform-soluble materials from urine under acidic conditions before extraction of I due to the many peaks observed on the chromatogram as chloroform-soluble compounds. Acid extraction of serum was not necessary because the major serum peak occurred at 25 min.

Sternbach and Kaiser (1) required the use of a very alkaline pH and high salt concentration to extract 3-quinuclidinol from aqueous solutions. For this study, a procedure for extraction required compatibility of the final sample with GLC analysis as well as ability to extract very small amounts of compound (1 μg or less) efficiently.

Sodium chloride was an effective salting-out agent in the extraction of 3-quinuclidinol. Additionally, ammonium hydroxide was an ideal base for controlling the required pH during the extraction. Neither sodium chloride nor ammonium hydroxide affected the GLC analysis. Both I and II may be extracted together by using the procedure for extraction of II. This alternative is feasible when large numbers of samples are to be analyzed. This method was used to analyze samples obtained in the topical absorption studies.

Figure 4 describes the cumulative absorption of I into the anterior chamber. During the first 15–20 min, there is a rapid penetration of the drug which then decreases proportionally with time. Cumulative absorption of I can range from 1 to 8.5 μg in the first 30 min. Conjunctival absorption experiments did not give a measurable amount of I (<50 ng) in the anterior chamber. In preliminary experiments, some drug was detected in the anterior chamber due to exposure of drug to the corneal surface.

The rate of penetration of I into the anterior chamber has been assessed as a function of miosis (2). Whether cholinomimetic symptoms such as miosis, peristalsis, and sialismus are directly related or can be used as an index of corneal penetration is arguable.

Table I shows one cholinomimetic reaction, salivation, as a

[5] Silyl 8, Pierce Chemical Co.

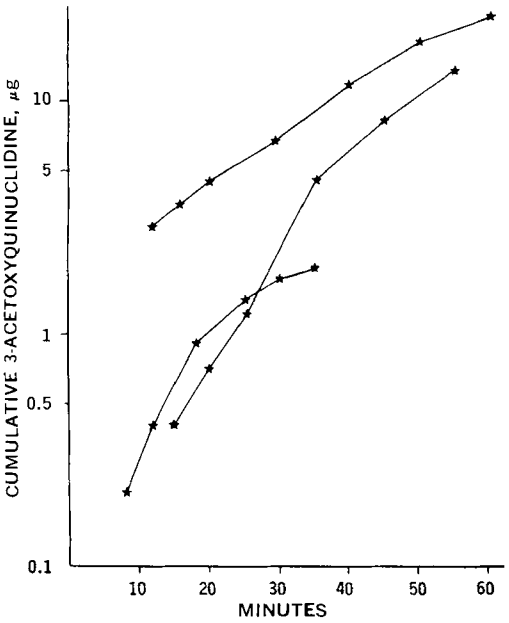

Figure 4—*Cumulative absorption of aceclidine (3-acetoxyquinuclidine) into the anterior chamber of the rabbit eye.*

function of the time of drug detection in the anterior chamber. Statistical analysis of the significance of the means between the times for onset of salivation resulting from corneal or conjunctival absorption was found to be insignificant at the 95% level. It has been assumed (2) that salivation is an index of corneal penetration of I. This assumption is not warranted from the observations reported here. Support for the lack of correlation is found in Table I (corneal absorption) where it can be seen that drug in the anterior chamber can precede salivation from 2 to 35 min. It is conceivable that pharmacological assay with sensitivities considerably greater than reported here would show the different symptomatic relationships to be significant. The drawback in either chemical or pharmacological assay was the subjectivity involved in defining the onset of symptoms as a function of drug intake. For example, observation of salivation was partially subjective since the end result and not the initial action was observed.

In these experiments, there was no evidence for deacetylation of I to II. Therefore, as would be predicted, the intact drug (I) penetrates primarily through the cornea into the anterior chamber where it can act to influence intraocular pressure. The experiments indicated that the scleral conjunctival membrane was not a major absorbing surface for I into the anterior chamber.

SUMMARY

Aceclidine (I), after topical administration, was detected in the anterior chamber. The penetration was primarily, if not exclusively, through the cornea and not the conjunctiva. In a sensitive GLC assay, only I was detected. There was no evidence of hydrolysis to the corresponding 3-quinuclidinol (II).

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Effect of Washing on Physicochemical Properties of Aluminum Hydroxide Gel

ROBERT H. GREEN and STANLEY L. HEM[x]

Abstract □ The negative electrophoretic mobility of aluminum hydroxide gel approaches zero logarithmically as the gel is washed and is related to the elution of chloride ion. Chloride ion is eluted at a constant rate and appears to be associated with the gel by a single mechanism. The viscosity of the gel increases with increased washing.

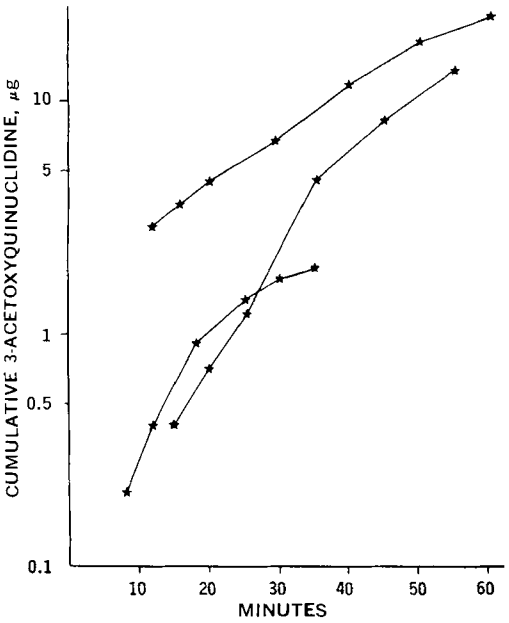
Keyphrases □ Aluminum hydroxide gel—effect of washing on physicochemical properties, loss of electrophoretic mobility, elution of chloride ion □ Gels, aluminum hydroxide—effect of washing on physicochemical properties, loss of electrophoretic mobility, elution of chloride ion □ Electrophoresis—loss of mobility of aluminum hydroxide gel, effect of washing, elution of chloride ion

An important step in the preparation of aluminum hydroxide gel is washing the gel free of reactants and soluble components following precipitation. The USP (1) places a limit on the chloride and sulfate content of aluminum hydroxide gel. The effect of washing on the physicochemical properties of aluminum hydroxide gel has not been extensively reported, although precipitation factors such as pH (2), temperature (3, 4), and order of addition and concentration of reactants (4–6) have been studied. The effect of washing on such dosage form-related properties as electrophoretic mobility and viscosity is examined in this report.

EXPERIMENTAL

Materials—All chemicals used were either official or reagent grade.

Preparation of Aluminum Hydroxide Gel—Aluminum hydroxide was prepared by the reaction of aluminum chloride, sodium carbonate USP, and sodium bicarbonate USP at pH 6.5 (2). Doubly distilled water was used for both precipitation and washing. Fractions of the wash water were collected for conductivity and chloride determinations. The extent of washing is expressed as the volumes of wash water eluted per volume of finished gel.



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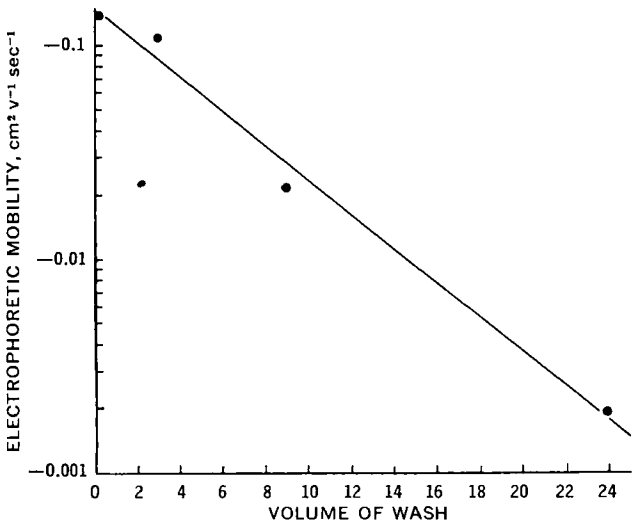

Figure 1—*Effect of washing on electrophoretic mobility.*

Thus, 1 liter of wash water eluted from 1 liter of gel is 1 volume.

Analytical Procedures—The aluminum oxide content of each aluminum hydroxide gel was determined by ethylenediaminetetraacetic acid titration (1).

The electrophoretic mobility was determined using an electrophoretic mass-transport analyzer[1] with the collection chamber containing the anode. The electrode was replaced whenever pitting was observed. The suitability of this instrument for the determination of the electrophoretic mobility of aluminum hydroxide gel was demonstrated by performing four replicate determinations of a gel having a mean electrophoretic mobility of −0.173 (cm/sec)/(v/cm). The standard deviation was ±0.006. Six replicates of a second gel had a mean electrophoretic mobility of −0.133 (cm/sec)/(v/cm) and a standard deviation of ±0.006. The precision in both trials was within ±5% *SD*.

The conductivity of the wash water was determined by using the electrophoretic mass-transport analyzer[1] as a Wheatstone bridge. The conductivity cell constant was 1.1760 cm^{-1}.

The concentration of chloride ion in each fraction of wash water was determined directly with a chloride-selective electrode[2] calibrated with a sodium chloride standardizing solution. A pH meter[3] was used to measure the voltages developed.

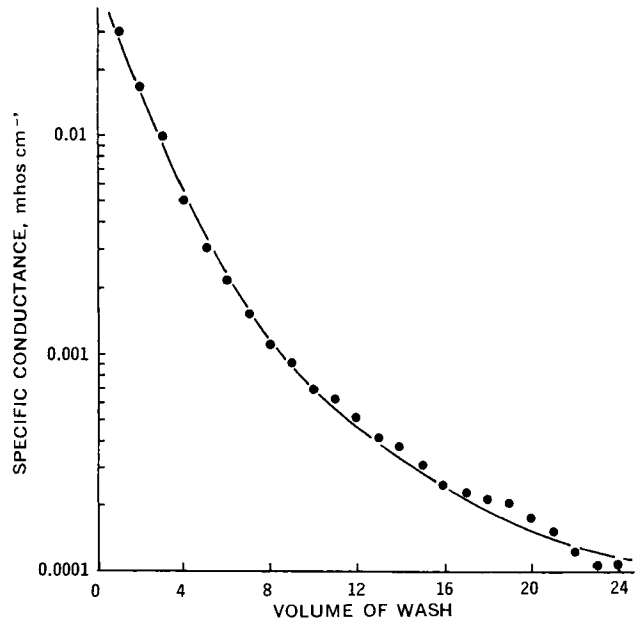

Figure 2—*Specific conductance of wash water.*

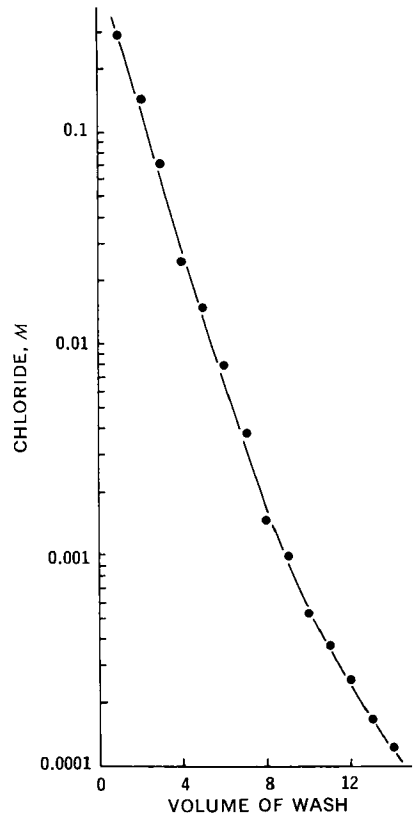

Figure 3—*Chloride content of wash water*

A portion of each gel was adjusted in volume to contain 5% Al_2O_3, and the viscosity of this sample was measured using a viscometer[4]. The reported results were determined using spindle 4 at 10 rpm.

RESULTS AND DISCUSSION

The electrophoretic mobility of aluminum hydroxide gel is negative, but the negative charge diminishes logarithmically as the gel is washed (Fig. 1). The electrophoretic mobility of the gel de-

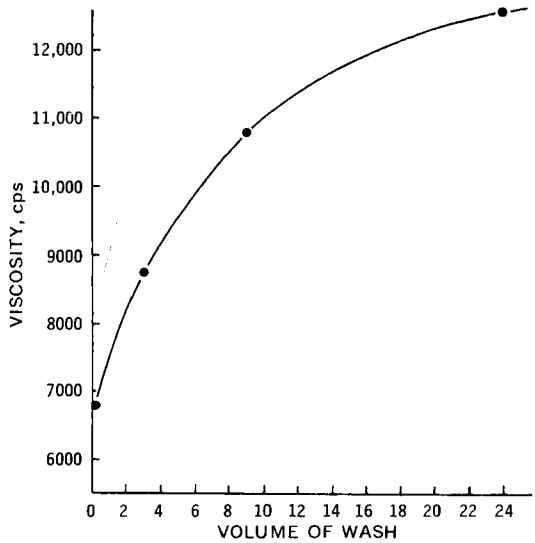

Figure 4—*Effect of washing on viscosity.*

[1] Model MIC 1201, Numec Instruments and Controls Corp., Monroeville, Pa.

[2] Model 94-17, Orion Research Inc., Cambridge, Mass.

[3] Model NX, Sargent-Welch Scientific Co., Skokie, Ill.

[4] Brookfield model RVT, Brookfield Engineering Labs., Stoughton, Mass.

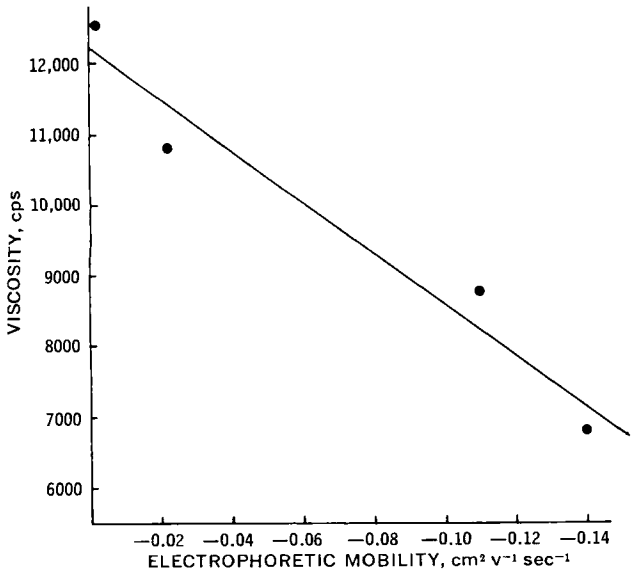

Figure 5—*Relationship between electrophoretic mobility and viscosity.*

creased from −0.140 (cm/sec)/(v/cm) to essentially zero as the gel was washed with 24 volumes of water. This decrease in electrophoretic mobility is due to the removal of water-soluble components as demonstrated by the decrease in specific conductance of the wash water (Fig. 2). The specific conductance reached a low, essentially constant value after 22 volumes of wash water had been eluted.

The loss of the gel's negative electrophoretic mobility during washing suggests that chloride ion present at the time of precipitation may be responsible for the negative surface charge. This is confirmed by determining the concentration of chloride ion in the wash water. Figure 3 shows that chloride ion was present in the wash water in decreasing amounts during washing. Chloride ion was eluted at a constant rate of 0.76 volume^{-1} during the first 8 volumes of wash, during which the chloride-ion content of the wash water decreased from 3.0×10^{-1} to 1.5×10^{-3} *M*. Further elution of chloride ion occurred more slowly as the chloride content of the wash water fell to 1×10^{-4} *M*, the lower limit of sensitivity for the chloride-selective electrode, during the 15th volume of wash. The sum of the chloride ion present in the first 15 volumes of wash water plus the amount present in the initial draining totals 2.11 moles. This amount accounts for virtually all of the 2.25 moles of chloride ion contributed by the aluminum chloride. The removal of chloride ion from the gel was demonstrated further by dissolving 10 g of the gel that had been washed with 24 volumes of water in 1.5 ml of nitric acid and 20 ml of doubly distilled water. No precipitate formed when several drops of 0.1 *N* silver nitrate were added.

The decrease in electrophoretic mobility during washing thus appears to be directly related to the elution of chloride ion. The constant rate of elution suggests that chloride ion is associated with the gel by a single mechansim which allows the chloride ion to be completely eluted.

The viscosity of the gel is also related to the degree of washing. The viscosity increases as the gel is washed, but the viscosity appears to be reaching a limiting value as chloride ion is eluted and the electrophoretic mobility approaches zero (Fig. 4). The increased viscosity is inversely related to electrophoretic mobility (Fig. 5) and may be due to greater interaction occurring between colloidal gel particles possessing low surface charge.

The washing operation is an important part of the preparation of aluminum hydroxide gel because properties such as electrophoretic mobility and viscosity, which are significant in the design of suspension dosage forms, are directly related to washing.

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Improvements in the Synthesis of Pentylenetetrazol-10-^{14}C

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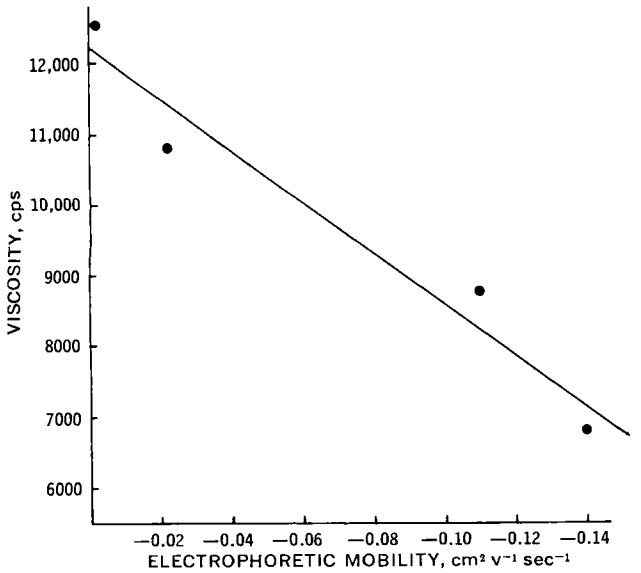
Keyphrases □ Pentylenetetrazol, radiolabeled (10-^{14}C)—improvements in synthesis □ Radiolabeled compounds—improved synthesis of pentylenetetrazol-10-^{14}C

Pentylenetetrazol has been synthesized in a number of ways. One of the most productive and yet simple synthesis developed (1) involves reacting cyclohexanone with hydrazoic acid. Modifications of this method have been utilized to produce pentylenetetrazol-5,9-^{14}C (2) and pentylenetetrazol-10-^{14}C (3).



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Close examination of the reported syntheses (1–3) revealed large variations in the yields obtained. Ronzio and Murray (2) reported a 22% yield of pentylenetetrazol-5,9-^{14}C. Stiver (4) reported a 47% yield of pentylenetetrazol-10-^{14}C with a radiochemical purity of 94.4% after four sublimations and a 36% yield with a radiochemical purity greater than 99% after thick-layer chromatography. In contrast, Chapman *et al.* (1) reported a 70% yield of pentylenetetrazol after distillation and a 50% yield after crystallization. This paper describes improvements made in the radiolabeled synthesis of Stiver *et al.* (3).

EXPERIMENTAL

Hydrazoic Acid (5)—*Caution:* "Since hydrazoic acid is very poisonous, all reactions involving it should be carried out under a good hood" (6). Sodium azide (16.58 g or 0.255 mole) was placed in a 250-ml, three-necked, round-bottom flask fitted with a mechanical stirrer, thermometer, and dropping funnel. An equal weight of water was added to the sodium azide, forming a paste. To the reaction vessel was added 100 ml of a solvent consisting of benzene–*n*-heptane (85:15). The solution was cooled to 0°, using rapid stirring (650 rpm), in a bath consisting of dry ice-2-ethoxyethanol. While maintaining the desired temperature, 6.78 ml (0.127 mole) of sulfuric acid was added dropwise (1 mole of sulfuric acid for each 2 moles of sodium azide).

When acid addition was complete, stirring was stopped and the resultant semisolid mass was thoroughly broken up. The reaction was then allowed to continue for 3 hr more; the 0° temperature and vigorous stirring were maintained. At the end of 3 hr, most of the organic layer was decanted into a 500-ml erlenmeyer flask. The inorganic salts were filtered from the remaining organic layer using a büchner funnel and water aspirator. The combined organic layer was dried over anhydrous sodium sulfate for 2 hr.

Pentylenetetrazol-10-^{14}C (3)—The dried hydrazoic acid obtained was placed in a 250-ml, three-necked, round-bottom flask fitted with a mechanical stirrer, dropping funnel, and three-way connecting tube. Anhydrous ferric chloride was added (1 g for each gram of ketone) to the reaction flask, and the mixture was cooled to 0° with a dry ice-2-ethoxyethanol bath. Five grams of cyclohexanone containing 0.033 g of cyclohexanone-1-^{14}C[1] (2.36 mCi) and 4.967 g of unlabeled cyclohexanone was dissolved in 50 ml of benzene. This solution was added dropwise to the reaction flask. The reaction was stirred for 4 hr while the temperature was allowed to rise slowly to room temperature.

At the end of the prescribed time, the organic layer was decanted into a 500-ml separator to which was added 35 ml of a 30% sodium hydroxide solution. The separator was shaken well. The lower alkaline aqueous layer was removed and placed in the three-necked reaction flask which still contained the black, tarry pentylenetetrazol–ferric chloride complex. The resulting mixture was stirred for about 2 min, and the aqueous suspension was centrifuged at 3000 rpm for 6 min. The centrifugate was poured into a 150-ml beaker, and the residue from the centrifugation was resuspended in another 35-ml portion of sodium hydroxide. The suspension was shaken on a mixer for 2 min and centrifuged again at 3000 rpm for 6 min. The centrifugate was added to the first centrifugate. A third 35-ml portion of 30% sodium hydroxide was added to the centrifugation residue, and the extraction was completed as described previously. The combined aqueous sodium hydroxide solution was saturated with ammonium sulfate. The undissolved salt was filtered from the alkaline solution using a medium porosity fritted-glass büchner funnel and a filtering flask attached to a water aspirator. The undissolved salt remaining in the büchner funnel was washed with two 20-ml portions of benzene to remove any pentylenetetrazol-10-^{14}C remaining with the salt.

The ammonium sulfate-saturated sodium hydroxide solution, approximately 100 ml, was placed in the 500-ml separator containing the original organic reaction layer decanted earlier. The labeled pentylenetetrazol was extracted from the solution in the separator with six 100-ml portions of benzene. The combined extract was dried for 12 hr over anhydrous sodium sulfate and the benzene was removed under reduced pressure, leaving the impure pentylenetetrazol. An 81.8% chemical yield was obtained.

Purification by Crystallization—The impure product was placed in a 50-ml erlenmeyer flask in a 70° water bath and was dissolved in the smallest possible quantity of absolute ethanol. *n*-Heptane was then added slowly to the point of cloudiness. Absolute ethanol was added dropwise until the cloudiness just disappeared. The warm solution was placed in a refrigerator and allowed to cool. When a temperature of approximately 40° was observed, a small crystal of authentic pentylenetetrazol was placed in the flask to seed the saturated solution, and the container was again placed in the refrigerator for further cooling. Upon further cooling, well-defined crystals of pentylenetetrazol-10-^{14}C were obtained. Crystallization was complete within 12 hr. After purification, a 62.6% chemical yield of pentylenetetrazol-10-^{14}C was obtained. The radiochemical yield was found to be 62.7%.

Radiochemical purity was determined by TLC, autoradiography, and liquid scintillation counting. A study of the TLC separation of pentylenetetrazol, cyclohexanone, cyclohexanone oxime, and ϵ-caprolactam revealed that pentylenetetrazol and cyclohexanone oxime were chromatographically inseparable in the two solvent systems reported previously (3). GLC showed that cyclohexanone oxime, which was present during the synthesis, was removed during the crystallization procedure. The results of radiochemical determinations revealed that the synthesized pentylenetetrazol-10-^{14}C had a radiochemical purity greater than 99.7%. The specific activity was 0.326 mCi/g. The melting point was 57.7–58.9°; the reported melting point is 57–59° (7). A mixed melting-point determination showed no depression.

This procedure was used five times with unlabeled cyclohexanone. The chemical yields of purified pentylenetetrazol ranged from 57.0 to 63.1%.

SUMMARY

The improved synthesis reported in this paper resulted in a 62.6% yield as compared to reported yields for the radiolabeled synthesis of 22% (2) and 36% (3). Specifically, the following improvements were made in the synthetic procedure reported by Stiver *et al.* (3).

1. The cold bath used was dry ice–2-ethoxyethanol as compared to the ice-acetone (not dry ice) bath reported previously. This improvement was needed to maintain the 0° temperature necessary for the reactants in the synthesis.

2. Preliminary syntheses revealed that the reaction was complete in approximately 2 hr and that the longer time periods reported (1, 3) produced no increase in yield. In this study, a 4-hr reaction time was utilized for convenience and to ensure completion of the synthesis.

3. Improved yields were realized by improvements in the extraction of the product from the reaction mixture. Specifically, a study revealed that three sodium hydroxide washes were essential for removal of the product from the pentylenetetrazol–ferric chloride complex. Also, this study revealed that two benzene washes were necessary to remove the product from the undissolved ammonium sulfate salts.

4. Crystallization from a suitable solvent was shown to be superior to sublimation and thick-layer chromatography as a method for purification of pentylenetetrazol-10-^{14}C. A greater yield was achieved with considerably less expenditure of time and no sacrifice in either chemical or radiochemical purity.

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* Present address: Nuclear Medicine, Medical Center, University of Nebraska, Omaha, NB 68105

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Sorption Rates of Water-Soluble Dyes on Soft Gelatin Capsules

HAROLD L. SMITH

Abstract □ A method was developed for determining sorption rates of water-soluble dyes by soft gelatin capsules. This method was used for determining the diffusivities and activation energies of five FD&C water-soluble dyes. The activation energies of these dyes were in either of two groups which had ranges of 25.1–25.7 and 34.8–36.0 kcal/mole.

Keyphrases □ Dyes, water soluble—determination of sorption rates on soft gelatin capsules □ Gelatin capsules, soft—determination of sorption rates of water-soluble dyes □ FD&C water-soluble dyes—determination of sorption rates on soft gelatin capsules □ Surface dyeing—determination of sorption rates of five FD&C water-soluble dyes on soft gelatin capsules

Several FD&C dyes were removed from the Food and Drug Administration's food additives list because they exhibited carcinogenic activity in animals (1). As a result, various methods for reducing the quantity of dyes in pharmaceutical dosage forms have become a subject of considerable interest. For soft shell gelatin capsules, such dye reductions have been achieved by surface dyeing untinted capsules instead of incorporating the dye into the gelatin shell formulation prior to preparing the capsules. The use of surface dyeing methods for this purpose is predicated upon the dyes diffusing into the capsule surface at rates slow enough to obtain a desired color and fast enough to be a reasonable processing step in the manufacture of the dosage form. A survey of the literature reveals that a method suitable for studying dyeing rates of this type of product has not been reported.

The purpose of this article is to describe a method for determining the sorption rates of dyes by intact untinted soft gelatin capsules under conditions similar to those used in production for surface dyeing. Data obtained using this method are presented for five FD&C water-soluble dyes: Yellow No. 5 (Tartrazine), Yellow No. 6 (Sunset Yellow FCF), Red No. 2 (Amaranth), Red No. 4 (Ponceau SX), and Green No. 3 (Fast Green FCF).

Table I—Diffusivities for the Sorption of FD&C Water-Soluble Dyes by Soft Gelatin Capsules

FD&C Dye	Temperature	Diffusivity $\times 10^7$, cm^2 sec^{-1}	Correlation Coefficient
Yellow No. 5	3.8°	1.11	0.994
Yellow No. 5	8.4°	1.79	0.997
Yellow No. 5	12.9°	4.70	0.997
Yellow No. 5	15.6°	6.66	0.994
Yellow No. 5	20.0°	13.6	0.999
Yellow No. 6	6.0°	1.35	0.995
Yellow No. 6	11.2°	2.88	0.999
Yellow No. 6	15.0°	4.75	0.994
Yellow No. 6	20.3°	12.6	0.999
Yellow No. 6	25.0°	24.5	0.999
Red No. 4	4.3°	0.657	0.989
Red No. 4	7.8°	1.28	0.999
Red No. 4	12.4°	2.34	0.995
Red No. 4	16.1°	4.85	0.999
Red No. 4	20.0°	7.70	0.999
Red No. 2	4.0°	0.865	0.998
Red No. 2	7.6°	1.65	0.996
Red No. 2	13.0°	5.94	0.997
Red No. 2	16.2°	12.4	0.999
Red No. 2	20.0°	25.2	0.999
Green No. 3	4.0°	0.323	0.998
Green No. 3	8.3°	0.805	0.995
Green No. 3	12.9°	2.31	0.999
Green No. 3	15.6°	5.05	0.999
Green No. 3	20.0°	10.8	0.999

EXPERIMENTAL

The capsules used were prepared with no fill material (air-fills) on a conventional soft gelatin encapsulating machine. The dried capsules contained approximately 66% gelatin[1] (125 Bloom strength), 30% glycerol, 3% water, and 1% parabens [methylparaben-propylparaben (1:3)]. All dye solutions contained 0.5% of one of the FD&C soluble dyes[2] in 50% (v/v) isopropyl alcohol and water.

Three capsules were mounted near the end of each of five sample holders (8 × 1 × 0.1 cm) with a space of approximately 1.0 cm left between capsules. Each capsule was mounted perpendicular to the holder with a small amount of silicon rubber[3]. The

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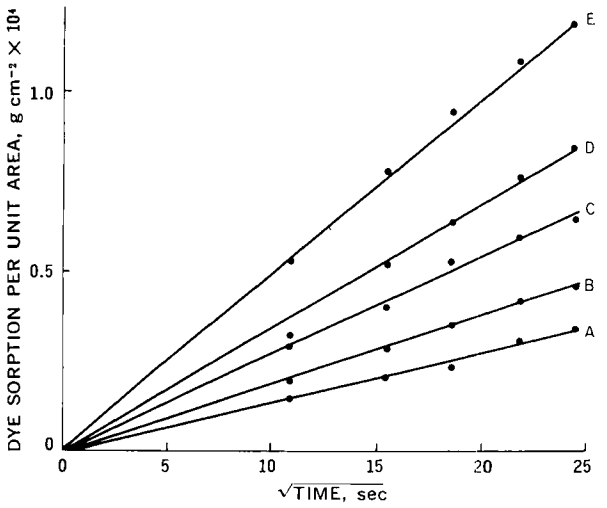

Figure 1—*Temperature dependency of the sorption of FD&C Red No. 4 by soft gelatin capsules as a function of the square root of time. Key: A, 4.3°; B, 7.8°; C, 12.4°; D, 16.1°; and E, 20.0°.*

sample holders were mounted near the top of a 1.0-liter water-jacketed beaker located on a magnetic stirrer. The temperature was controlled (±0.1°) by connecting the jacketed beaker to a circulating water bath. The holders were positioned around the inside of this beaker in a manner that would maintain the capsules below the surface of 500 ml of dye solution. The opposite ends of the holders projected from the water-jacketed beaker for easy removal. The magnetic stirrer was started and set at a predetermined rate for moderate stirring of the dye solution. Five hundred milliliters of dye solution was added and a stopwatch was started. Samples (holders) were withdrawn at 2, 4, 6, 8, and 10 min. Upon withdrawal, each sample was immediately dipped into isopropyl alcohol and water (50:50 v/v) and then into isopropyl alcohol.

The samples were dried for 2 hr to reestablish the original moisture content of the capsules. A 54.5-mm² disk was cut from one surface of each capsule. The three disks obtained from the capsules for each sample time were dissolved in sufficient water to provide a volume of 100 ml. These solutions were assayed spectrophotometically at the appropriate wavelengths.

Table II—Activation Energies for the Sorption of FD&C Water-Soluble Dyes by Soft Gelatin Capsules

FD&C Dye	Activation Energy, kcal/mole	Sulfonate Groups per Dye Molecule	Correlation Coefficient
Yellow No. 5	25.7	2	0.994
Yellow No. 6	25.1	2	0.998
Red No. 4	25.4	2	0.998
Red No. 2	34.8	3	0.999
Green No. 3	36.0	3	0.999

RESULTS AND DISCUSSION

Diffusivities were reported (2) for the rates of dye uptake by gelatin films using the following equation, which is an approximate solution for Fick's law:

$$x = 2C_0(kt/\pi)^{1/2} \qquad \text{(Eq. 1)}$$

where x is the amount of dye sorbed per unit area of surface at any time t, C_0 is the initial concentration of the dyeing solution, and k is the diffusivity. Several reports (3–5) indicated that good agreement is usually obtained with this equation when it is used for describing diffusion rates into infinite solids for low time periods.

Diffusivities were determined for all five dyes at various temperatures between 3.8 and 25.0° by linear regression analysis of this equation (Table I). A typical plot of the dye uptake per unit area as a function of the square root of time is presented for FD&C Red No. 4 at several temperatures in Fig. 1. Inspection of the correlation coefficients listed in Table I and the lines plotted in Fig. 1 indicates that data collected using this method fit this equation. Activation energies, obtained from the linear regression analysis of the Arrhenius equation, were calculated from the diffusivities for each dye studied (Table II). Arrhenius plots for FD&C Red No. 4, Yellow No. 5, and Yellow No. 6 are presented in Fig. 2. Similar plots for Red No. 2 and Green No. 3 are presented in Fig. 3.

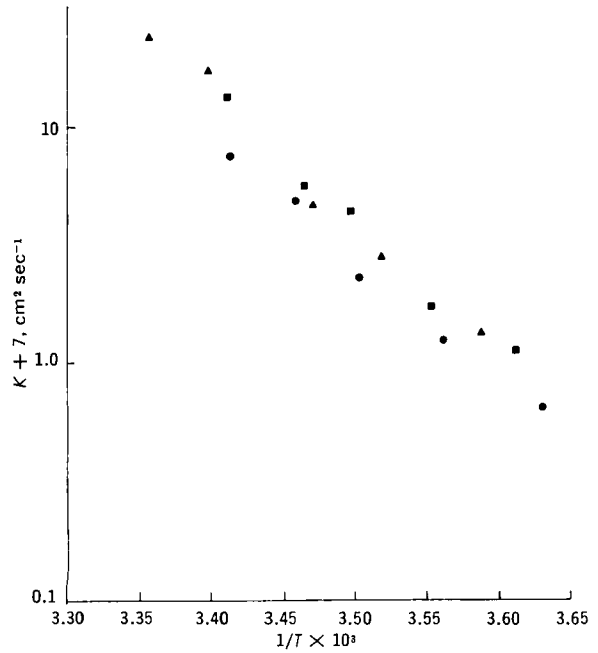

Figure 2—*Arrhenius-type plots for sorption rates of water-soluble dyes with two sulfonate groups per dye molecule. Key: ■, FD&C Yellow No. 5; ▲, FD&C Yellow No. 6; and ●, FD&C Red No. 4.*

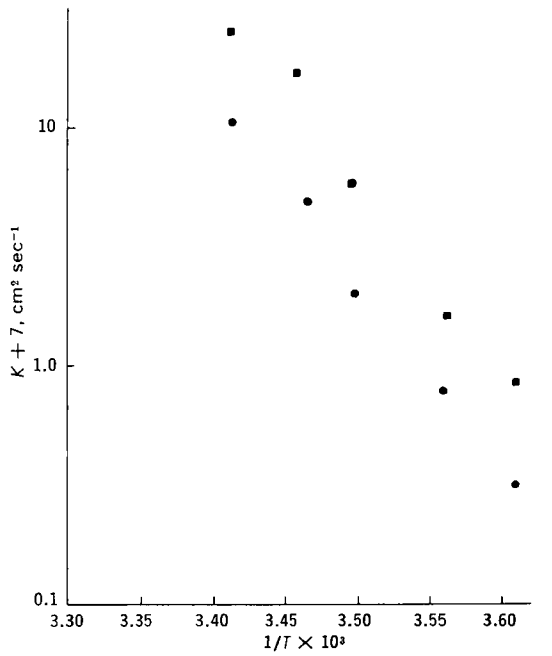

Figure 3—*Arrhenius-type plots for sorption rates of water-soluble dyes with three sulfonate groups per dye molecule. Key: ■, FD&C Red No. 2; and ●, FD&C Green No. 3.*

The method reported here is simple and reliable and could be easily modified for studying all gelatin capsule dosage forms. The diffusivities and activation energies calculated from the data obtained by this method are consistent with those obtained from diffusion studies of similar systems (6, 7). The activation energies calculated for the five water-soluble dyes studied fall into two distinct groups (Table II). The first group includes Yellow No. 5, Yellow No. 6, and Red No. 4, which have similar activation energies ranging from 25.1 to 25.7 kcal/mole. The energies for these three dyes were found to be statistically indistinguishable by testing the homogeneity of the regression coefficients for their Arrhenius slopes. The second group includes Red No. 2 and Green No. 3 which have activation energies of 34.8 and 36.0 kcal/mole, respectively. The activation energies for these two dyes were also statistically indistinguishable.

The dyes in the first group, with lower activation energies, contain two sulfonate groups per molecule; those in the second group, with higher activation energies, contain three sulfonate groups per molecule. It will be necessary, however, to conduct various additional studies on a larger number of dyes before attempting to establish a relationship between the activation energies of these compounds and their structural variations.

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Differential Pulse Polarographic Analysis of Everninomicin Complex in Fermentation Broth

PETER KABASAKALIAN[x], SAMI KALLINEY, and ANITA WESTCOTT

Abstract □ A direct, sensitive, and fast (15 min) method for following the laboratory fermentation course of everninomicins is described. It replaces the 20-hr elapsed-time agar-diffusion bioassay method.

Keyphrases □ Everninomicin complex—differential pulse polarographic analysis in fermentation broth □ Polarography, differential pulse—analysis, everninomicin complex in fermentation broth □ Fermentation broths—differential pulse polarographic analysis of everninomicin complex

The production of everninomicin complexes by fermentation was described previously (1, 2). To optimize the yield, a fast, reliable analytical method should be used to follow the course of fermentation. Since the components of an everninomicin complex (*e.g.*, B, C, and D) possess an electroactive group, the nitro group (3), they are amenable to polarographic analysis (4). A more recent advance, differential pulse polarography, would be ideally suited for this purpose due to its sensitivity (5) and larger linear range.

EXPERIMENTAL

The broth (5.00 ml) was transferred to a 50-ml volumetric flask and diluted to volume with acetonitrile. The suspension was mixed well and left for a few minutes to allow the insoluble matter to settle, thus permitting the antibiotic to be separated from the mycelium. The supernate (5.00 ml) was mixed with an equal volume of 0.01 *M* phosphate buffer of pH 7.0. This solution was placed in the sample compartment of a small polarographic H-cell along with the dropping mercury electrode; in the other compartment was a normal calomel electrode, used both as a reference and as a counter-electrode. The cell compartments were separated by an agar plug and a fritted-glass diaphragm. The chloride ion in both the calomel cell and the agar plug was furnished by 1 *N* tetramethylammonium chloride[1]. Both compartments of the H-cell were sealed with rubber stoppers. A small annulus was made around the dropping mercury electrode to allow the capillary to move to dislodge the mercury drops and to permit the escape of nitrogen.

The solution to be analyzed was deoxygenated using nitrogen. Before being passed through the cell, the nitrogen was passed through a gas washing bottle containing a solvent similar to that of the solution. While polarograms[2] were being run, nitrogen was passed over the solution.

A drop time of 1 sec, a scan rate of 2 mv/sec, and a modulation amplitude of 100 mv were kept constant throughout the analysis. Potentials were scanned from −0.6 to −1.4 v (normal calomel electrode). The current scale was 2 μamp.

RESULTS AND DISCUSSION

A typical differential pulse polarogram of the broth yielded peaks at a potential corresponding to the reduction of everninomicin D. No other peak was observed in the vicinity (Fig. 1). The peak currents of the broth samples as a function of the fermentation time are shown in Fig. 2. These results are in good agreement with the everninomicin production curve shown in Fig. 4 of Ref. 2, which was determined by the bioassay method.

This assay lacks specificity since it is essentially a functional group assay responding to the nitro group present in everninomicin complex. This lack of specificity is also present in the bioas-

[1] Southwestern Analytical Chemicals, Austin, TX 78767

[2] A Princeton Applied Research model 170 electrochemistry system in conjunction with a model 172A drop timer (Princeton Applied Research Corp., Princeton, NJ 08540) was used to obtain the differential pulse polarograms.

The method reported here is simple and reliable and could be easily modified for studying all gelatin capsule dosage forms. The diffusivities and activation energies calculated from the data obtained by this method are consistent with those obtained from diffusion studies of similar systems (6, 7). The activation energies calculated for the five water-soluble dyes studied fall into two distinct groups (Table II). The first group includes Yellow No. 5, Yellow No. 6, and Red No. 4, which have similar activation energies ranging from 25.1 to 25.7 kcal/mole. The energies for these three dyes were found to be statistically indistinguishable by testing the homogeneity of the regression coefficients for their Arrhenius slopes. The second group includes Red No. 2 and Green No. 3 which have activation energies of 34.8 and 36.0 kcal/mole, respectively. The activation energies for these two dyes were also statistically indistinguishable.

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EXPERIMENTAL

The broth (5.00 ml) was transferred to a 50-ml volumetric flask and diluted to volume with acetonitrile. The suspension was mixed well and left for a few minutes to allow the insoluble matter to settle, thus permitting the antibiotic to be separated from the mycelium. The supernate (5.00 ml) was mixed with an equal volume of 0.01 *M* phosphate buffer of pH 7.0. This solution was placed in the sample compartment of a small polarographic H-cell along with the dropping mercury electrode; in the other compartment was a normal calomel electrode, used both as a reference and as a counter-electrode. The cell compartments were separated by an agar plug and a fritted-glass diaphragm. The chloride ion in both the calomel cell and the agar plug was furnished by 1 *N* tetramethylammonium chloride[1]. Both compartments of the H-cell were sealed with rubber stoppers. A small annulus was made around the dropping mercury electrode to allow the capillary to move to dislodge the mercury drops and to permit the escape of nitrogen.

The solution to be analyzed was deoxygenated using nitrogen. Before being passed through the cell, the nitrogen was passed through a gas washing bottle containing a solvent similar to that of the solution. While polarograms[2] were being run, nitrogen was passed over the solution.

A drop time of 1 sec, a scan rate of 2 mv/sec, and a modulation amplitude of 100 mv were kept constant throughout the analysis. Potentials were scanned from −0.6 to −1.4 v (normal calomel electrode). The current scale was 2 μamp.

RESULTS AND DISCUSSION

A typical differential pulse polarogram of the broth yielded peaks at a potential corresponding to the reduction of everninomicin D. No other peak was observed in the vicinity (Fig. 1). The peak currents of the broth samples as a function of the fermentation time are shown in Fig. 2. These results are in good agreement with the everninomicin production curve shown in Fig. 4 of Ref. 2, which was determined by the bioassay method.

This assay lacks specificity since it is essentially a functional group assay responding to the nitro group present in everninomicin complex. This lack of specificity is also present in the bioas-

[1] Southwestern Analytical Chemicals, Austin, TX 78767

[2] A Princeton Applied Research model 170 electrochemistry system in conjunction with a model 172A drop timer (Princeton Applied Research Corp., Princeton, NJ 08540) was used to obtain the differential pulse polarograms.

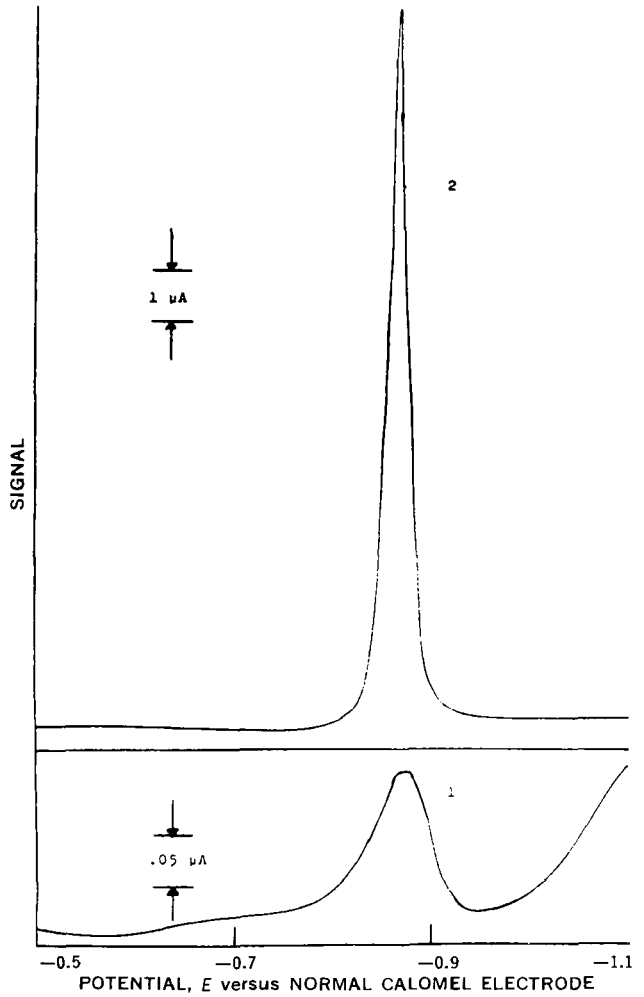

Figure 1—*Differential pulse polarograms of: (1) a 140-hr fermented broth sample, and (2) everninomicin D standard (0.099 mg/ml). The following conditions were employed: acetonitrile–phosphate buffer solution (0.005 M, pH 7.0); drop time, 1 sec; modulation amplitude, 100 mv; and scan rate, 2 mv/sec.*

say procedure (2), which cannot differentiate the various everninomicins. However, the linear relationship between the polarographic current and everninomicin concentration makes the polarographic method a more precise one. The polarographic method is the most efficient way to follow the fermentation. However, a bioassay of the final product is still essential to assure the potency of the product.

The polarographic current determined can be converted into equivalent units of everninomicin D (the predominant everninomicin) bioactivity per milliliter by running an assay with the appropriate concentration of everninomicin D in the unfermented broth. With the experimental conditions given, 1.00 μamp of current represents 150 units of everninomicin D per milliliter. The

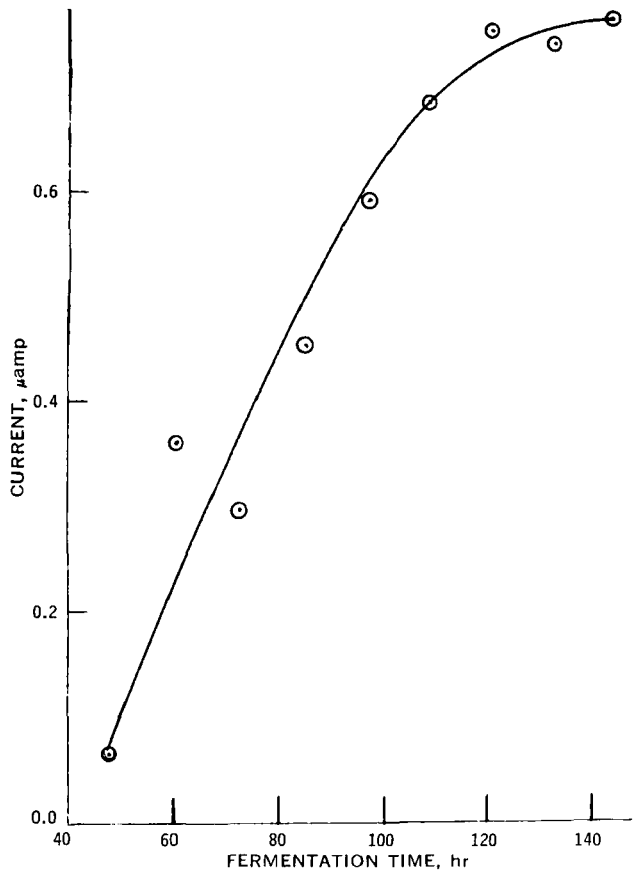

Figure 2—*Differential pulse polarogram of the fermentation of everninomicin. The following conditions were employed: acetonitrile–phosphate buffer solution (0.005 M, pH 7.0); drop time, 1 sec; modulation amplitude, 100 mv; scan rate, 2 mv/sec, and* $E_{peak} = -0.85$ *v (normal calomel electrode).*

everninomicin D unit here is the same as that given by Wagman *et al.* (2).

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[x] To whom inquiries should be directed.

pH-Dependent Dissolution Rate of Nitrofurantoin from Commercial Suspensions, Tablets, and Capsules

THEODORE R. BATES[x], JERRY M. YOUNG*, CAMILLA M. WU*, and HOWARD A. ROSENBERG

Abstract □ The dissolution rate of nitrofurantoin from commercial suspensions and tablets containing microcrystalline drug particles and from capsules containing macrocrystalline drug particles was determined at 37° in simulated gastric (pH 1.12) and intestinal (pH 7.20) fluids using the stirrer-flask method. As expected from solubility considerations alone, the dissolution rate of nitrofurantoin (pKa 7.2) from the suspension and capsule dosage forms at pH 7.20 was significantly faster than at pH 1.12. In contrast, the drug from the tablet dosage form dissolved in the pH 1.12 dissolution medium at a rate twice that observed at pH 7.20. This unusual pH effect could be explained satisfactorily on the basis of observed differences in the physical characteristics of the tablet when exposed to the two media. The pH 7.20 phosphate buffer medium suggested by USP XVIII was unable to discern inherent, particle-size-dependent differences in the rate of solution and the reported incidence of side effects of nitrofurantoin from the microcrystalline drug tablet and macrocrystalline drug capsule. The reported existence of a relationship between the dissolution rate of nitrofurantoin from these two dosage forms in simulated gastric fluid and their reported toxicities suggests that the official USP XVIII dissolution rate specification for products of this drug should be modified.

Keyphrases □ Nitrofurantoin—pH-dependent dissolution rate from commercial dosage forms □ Dissolution—nitrofurantoin from commercial dosage forms □ Bioavailability—pH-dependent dissolution rate of nitrofurantoin from commercial suspensions, tablets, and capsules

The dissolution rate (1), bioavailability in humans (1–4), and incidence of side effects (5) of the poorly soluble, weakly acidic antibacterial agent nitrofurantoin (pKa 7.2) are significantly affected by its particle size.

Presumably, in an attempt to ensure a minimal incidence of drug-induced side effects (nausea and emesis), the USP XVIII monograph for nitrofurantoin tablets (6) requires that 60% of the labeled amount of drug dissolve in pH 7.2 phosphate buffer in *not less* than 1 hr. Based on the lack of a dissolution rate specification for nitrofurantoin oral suspension USP, coupled with the markedly faster dissolution rate of microcrystalline nitrofurantoin from commercial suspension compared to commercial tablet dosage forms, the consistency underlying the official requirement of an unusually slow dissolution rate of nitrofurantoin from tablets has been questioned (7).

Table I—Effect of pH on the Dissolution Rate of Nitrofurantoin from Commercial Dosage Forms at 37°

Commercial Dosage Form	Mean Dissolution Rate Half-Life (T_{50})[a], min	
	pH 1.12	pH 7.20
Aqueous suspension[b]	12.5 (1.21)[c]	2.64 (0.336)
Compressed tablet[d]	77.9 (19.0)	167 (35.8)
Gelatin capsule[e]	212 (44.5)	160 (24.7)

[a] Determined from log-normal probability plots of individual dissolution rate data. Mean of five determinations. [b] Furadantin suspension containing microcrystalline (<10 μm) drug. [c] Standard deviation in parentheses. [d] Furadantin tablets containing microcrystalline (<10 μm) drug. [e] Macrodantin capsules containing macrocrystalline (80–200 mesh) drug.

Upon oral ingestion, nitrofurantoin dosage forms are exposed first to the highly acidic (pH 1–2) gastric fluid. As a result, the drug contained therein is afforded the opportunity to be released and dissolve in this region of the GI tract. The amount of nitrofurantoin capable of dissolving in this region would depend primarily on the residence time of the dosage form in the gastric pouch and on the release and dissolution rate of the drug from the dosage form.

To reduce the incidence of nitrofurantoin-induced nausea and emesis, all dosage forms of this drug are commonly prescribed with food and/or milk. A recent report (8), related to the effect of food on nitrofurantoin absorption in humans, indicated that the presence of food in the stomach appreciably delays gastric emptying. A marked enhancement in the bioavailability of both macro- and microcrystalline nitrofurantoin from commercial capsule and tablet dosage forms in nonfasting as compared to fasting subjects was also observed (8). These findings are consistent with the argument that a significant fraction of drug from both dosage forms dissolves in the stomach prior to being emptied into the duodenal region of the small intestine where absorption is optimal (9). Hence, to design the most appropriate dissolution rate specification for official and nonofficial dosage forms of nitrofurantoin, which could potentially correlate best with the absorption characteristics and toxicity of the drug from these products, it is important to examine their dissolution rate profiles under pH conditions simulating both gastric and intestinal fluids. The official USP dissolution rate test for tablet dosage forms of nitrofurantoin presently utilizes only a pH 7.2 phosphate buffer medium (simulated intestinal fluid). As a result, the specification does not consider the possibility that some physicochemical property (*e.g.*, solubility) of an "inert" ingredient(s) in a particular tablet formulation may be pH dependent and, thus, affect the release, dissolution, and absorption of the drug. This shortcoming may be partly responsible for the inability of the USP dissolution rate test to reflect the *in vivo* absorption characteristics of the drug from 19 different commercial tablet formulations (10).

The present investigation was designed to characterize the pH-dissolution rate behavior of nitrofuran-

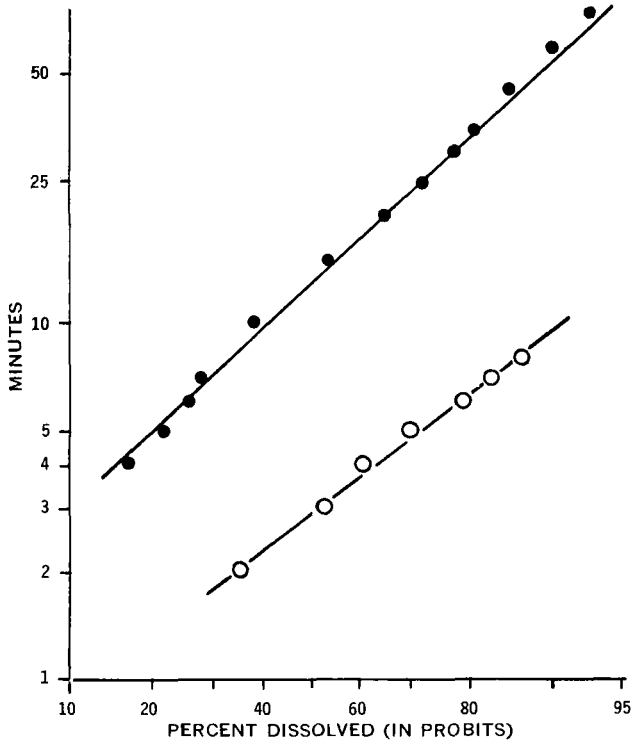

Figure 1—*Representative log-normal probability plots of dissolution rate data for nitrofurantoin from a commercial suspension at 37°. Key: ●, pH 1.12; and ○, pH 7.20.*

toin from official commercial suspension and tablet dosage forms containing microcrystalline drug, as well as from a nonofficial commercial capsule dosage form containing macrocrystalline drug.

EXPERIMENTAL

Materials—The commercial suspension of microcrystalline (<10 μm) nitrofurantoin[1], the compressed tablets of microcrystalline (<10 μm) nitrofurantoin[2], and the capsules of macrocrystalline (80–200 mesh) nitrofurantoin[3] employed in this investigation were purchased on the open market and were assayed for drug content prior to use. All other materials were reagent grade and were used as received.

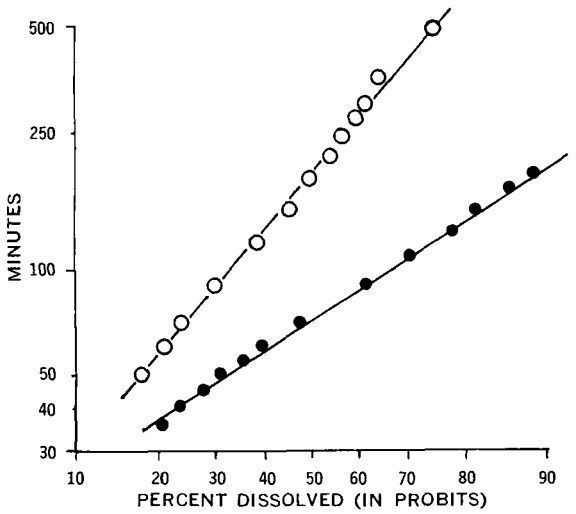

Figure 2—*Representative log-normal probability plots of dissolution rate data for nitrofurantoin from a commercial compressed tablet at 37°. Key: ●, pH 1.12; and ○, pH 7.20.*

Equilibrium Solubility Determinations—The equilibrium solubility of nitrofurantoin at 37° was determined in pH 1.12 hydrochloric acid and pH 7.20 phosphate buffer media. Excess quantities of nitrofurantoin were placed into 50-ml glass-stoppered flasks together with 25.0-ml portions of either buffer solution. The flasks were closed securely, protected from light, and shaken mechanically in a water bath maintained at 37 ± 0.25° until equilibrium was attained. The equilibrated samples were subjected to filtration[4] (0.45-μm pore size) at 37°, the filtrates were diluted and adjusted to pH 7.20 with phosphate buffer when necessary, and the concentration of drug in solution was determined spectrophotometrically at 383 nm (1).

Dissolution Rate Determinations—The dissolution apparatus consisted of a 1-liter, three-necked, round-bottom flask containing 880 (suspension studies) or 900 (tablet and capsule studies) ml of pH 1.12 hydrochloric acid or pH 7.20 phosphate buffer solution. The dissolution medium was maintained at 37 ± 0.25° and agitated at 200 rpm by means of a Teflon stir blade (70 mm diameter) connected to a constant-speed stirring mechanism[5].

At time zero, one tablet, one capsule, or a weight of suspension corresponding to 100 mg of nitrofurantoin was introduced into the dissolution medium. Periodically, 5-ml samples were withdrawn from the flask, filtered, adjusted to pH 7.20 when necessary, and assayed spectrophotometrically at 383 nm using a recording spectrophotometer[6]. Immediately following the removal of each sample, a 5-ml quantity of fresh dissolution medium was added to the flask. All dissolution data were corrected appropriately for this dilution effect (11).

Both the aqueous, gel-like suspension and tablet dosage forms were sufficiently dense to sink immediately to the bottom of the flask and occupy a central position, 5 cm below the stir blade. To accomplish this with the capsule dosage form, it was necessary to employ a specially constructed, nonmetal holding device. The device made minimal contact with the capsule dosage form and, thus, had an insignificant effect on the release characteristics of this dosage form.

Five dissolution rate determinations were made with each dosage form, and the data obtained were interpreted by the log-probit method suggested by Wagner (12).

RESULTS AND DISCUSSION

The equilibrium solubility of nitrofurantoin at 37° was determined to be 104 and 272 mg/liter under pH conditions simulating those of gastric (pH 1.12) and intestinal (pH 7.20) fluids, respectively.

Representative dissolution rate profiles of nitrofurantoin from the commercial aqueous suspension, tablet, and capsule dosage forms are depicted in Figs. 1–3, and the dissolution half-lives (T_{50}), determined from plots of this nature, are summarized in Table I. Inspection of the data reveals an interesting interrelationship among the pH of the dissolution medium, the solubility of the drug, the release and/or solubility characteristics of some "inert" component(s) of the dosage forms, and the resultant dissolution rate profile of the drug. The significantly slower dissolution rate of nitrofurantoin from either the aqueous suspension or capsule dosage form at pH 1.12 as compared to that at pH 7.20 is consistent with the pH-dependent solubility characteristics of the drug. In contrast, the drug from the tablet dosage form dissolved in the pH 1.12 dissolution medium at a rate approximately *twice* that observed at pH 7.20. This unusual pH effect is contrary to that expected based on drug solubility considerations alone, but it can be correlated satisfactorily with observed differences in the physical release characteristics of the tablet dosage form when exposed to acidic and slightly alkaline dissolution media. Unlike physical characteristics of the tablet at pH 1.12, when this dosage

[1] Furadantin suspension, 25 mg/5 ml, Eaton Laboratories, Control No. 700364.
[2] Furadantin tablets, 100 mg, Eaton Laboratories, Control No. 694673.
[3] Macrodantin capsules, 100 mg, Eaton Laboratories, Control No. 695242.
[4] Millipore.
[5] Servodyne laboratory stirrer, Cole-Parmer Instrument Co., Chicago, IL 60648
[6] Beckman DB-G, Beckman Instruments Inc., Fullerton, CA 92634

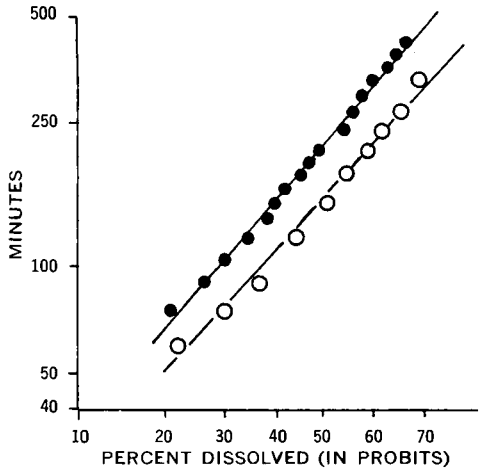

Figure 3—*Representative log-normal probability plots of dissolution rate data for nitrofurantoin from a commercial capsule at 37°. Key: ●, pH 1.12; and ○, pH 7.20.*

form was introduced into the pH 7.20 medium, it displayed poor disintegration properties, produced relatively large granules, and maintained most of its shape during the greater portion of the dissolution run.

Hailey and Glascock (5) demonstrated, in patients with known intolerance to nitrofurantoin, that the incidence of undesirable side effects is markedly reduced upon oral administration of a commercial capsule containing macrocrystalline drug particles[3] as compared to a commercial tablet containing microcrystalline drug particles[2]. In view of these findings, the lack of official recognition for this larger particle-size fraction of drug or its capsule dosage form seems surprising. When dissolution rate experiments were conducted at pH 7.2, the pH suggested by the USP (6), the dissolution rate of macrocrystalline nitrofurantoin from the commercial capsule was identical to that of microcrystalline drug from the tablet dosage form (Table I). These results are inconsistent with the anticipated slower dissolution rate of nitrofurantoin from the macrocrystalline drug capsule (1) and its lower toxicity (5). However, when the same two dosage forms were exposed to simulated gastric fluid (pH 1.12), an interrelationship was found to exist among the particle size of drug contained within the dosage form, its dissolution rate (Table I), and its reported toxicity (5).

The results of the present investigation provide evidence that the dissolution rate of nitrofurantoin from three different pharmaceutical dosage forms can be markedly affected by pH. The dissolution medium (pH 7.2 phosphate buffer) recommended by the USP (6) is unable to discern inherent, particle-size-dependent differences in the rate of solution and incidence of side effects of nitrofurantoin from commercial, microcrystalline drug tablet and macrocrystalline drug capsule dosage forms. The dissolution rate characteristics of nitrofurantoin from these two dosage forms in simulated gastric fluid (pH 1.12) and the existence of a relationship between these *in vitro* results and reported toxicity data suggest that the USP XVIII dissolution rate specification for nitrofurantoin products should be modified.

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* Undergraduate research participant.

ˣ To whom inquiries should be directed.

COMMUNICATIONS

Effect of γ-Radiation on Intestinal Absorption of Sulfanilamide

Keyphrases □ Radiation, gamma—effect on intestinal absorption of sulfanilamide, rats □ Sulfanilamide—effect of γ-radiation on intestinal absorption, rats □ Absorption, intestinal—effect of γ-radiation on rate of sulfanilamide absorption, rats

To the Editor:

Following sublethal irradiation of the mammalian small intestine, cell division in the intestinal epithelium slows. The mass of the epithelium declines temporarily and then becomes abnormally large before the intestine appears histologically normal again (1). Since the barrier characteristics of the intestinal epithelium are thought to control the absorption of many orally administered drugs, exposure of the intestine to ionizing radiation could affect drug absorption.

Although the results of a number of studies on the influence of radiation on the intestinal absorption of vitamins, minerals, water, and nutrients have been reported (1, 2, and references cited therein), there is very little published information on the effects of ra-

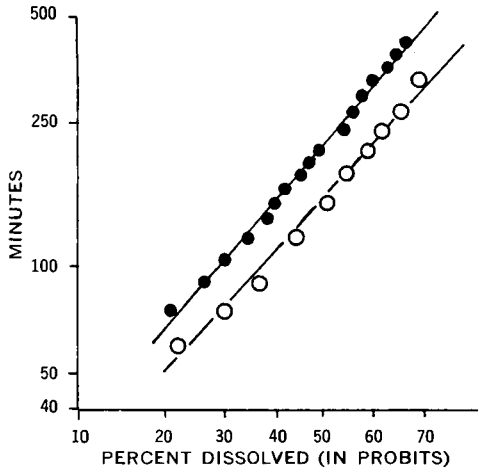

Figure 3—*Representative log-normal probability plots of dissolution rate data for nitrofurantoin from a commercial capsule at 37°. Key: ●, pH 1.12; and ○, pH 7.20.*

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Although the results of a number of studies on the influence of radiation on the intestinal absorption of vitamins, minerals, water, and nutrients have been reported (1, 2, and references cited therein), there is very little published information on the effects of ra-

Table I—Effect of 850 Rad of ^{60}Co γ-Radiation on Absorption of Sulfanilamide from Rat Intestine

	Sham[a]	850 Rad[a]	p
Absorption half-life, min	17.1 (2.24)	26.8 (5.49)	<0.01
pH of drug solution[b]	6.3 (0.12)	6.4 (0.15)	>0.30
Gut length, cm	90 (6.3)	87 (4.9)	>0.30

[a] Mean of six animals; standard deviation in parentheses. [b] Measured at the end of the experiment; initial pH was 6.5.

diation on drug absorption. The results of studies on the effects of 700 rad of ^{60}Co whole-body γ-radiation on the transfer of drugs across the *in vitro* rat small intestine were recently reported (3). Four days following irradiation, the transfer of salicylate and ethionamide was increased, the transfer of mecamylamine, aminopyrine, and isoniazid was unchanged, the transfer of phenobarbital and diphenylhydantoin was decreased slightly, and the transfer of butylscopolamine was completely blocked (3, 4). Since the GI tract is exposed to radiation during the treatment of some cancers, it is important to know how such exposure affects the absorption of orally administered drugs.

This preliminary communication describes the effect of whole-body ionizing radiation on the absorption of sulfanilamide from the *in situ* small intestine of the rat. Male Sprague–Dawley rats, weighing 180–250 g, were either exposed to 850 rad of ^{60}Co γ-radiation delivered over 5 min or sham irradiated (5). Irradiated and sham-irradiated animals underwent identical manipulation, except the sham-irradiated animals were shielded from the radiation. The animals were housed in cages with wide-mesh floors and had access to food and water *ad libitum* prior to and following the irradiation procedure. Food was withdrawn on the 3rd day after irradiation, and on the 4th day[1] the animals were prepared as described previously (6) for measuring the rate of drug absorption from solution by the *in situ* small intestine. The rectal temperature of each animal was monitored throughout the absorption experiment and maintained at 37° with a rheostatically controlled heating pad placed under the animal. Seven milliliters of 0.4 m*M* sulfanilamide dissolved in 67 m*M* sodium phosphate buffer made isotonic with sodium chloride was placed in the intestine, and 0.2-ml samples were removed at 5-min intervals for 30 min. The volume of the drug solution was maintained constant by adding 0.9% NaCl solution immediately prior to sample removal. The concentration of sulfanilamide in each sample was determined by the colorimetric procedure of Bratton and Marshall (7). Plots of log sulfanilamide concentration *versus* time appeared linear for both irradiated and sham-irradiated rats. Absorption rate constants were determined from the slopes of lines fitted to the plots by least squares. The rate constants were corrected for drug removed due to sampling by subtracting 0.0057 min^{-1} and were converted to half-lives. At the conclusion of each experiment, the pH of the intestinal perfusate and the length of the intestine lying between the cannulas were measured.

The absorption rate of sulfanilamide was significantly reduced in irradiated animals compared to the sham-irradiated controls (Table I). The data in Table I also indicate that the radiation-induced reduction in absorption rate cannot be attributed to differences between irradiated and control animals in the pH of the drug solution nor to differences in the length of intestine used in the absorption experiment.

Although the reduced rate of sulfanilamide absorption in irradiated animals could result from a decrease in the surface area of the intestinal mucosa, other mechanisms may be involved. For example, irradiation of the intestine could directly alter the permeability of the intestinal mucosa and it could alter blood flow to the mucosa. Additional experiments are underway to determine the effects of radiation on the absorption of other drugs and the mechanisms by which radiation alters drug absorption.

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William L. Hayton
College of Pharmacy
Washington State University
Pullman, WA 99163

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[1] Preliminary experiments and the results of Mattila *et al.* (3) indicate that the acute effect of radiation on the rate of intestinal drug absorption in the rat is greatest 4 days following whole-body irradiation.

Tablet Strength Testing

The paper of Bavitz *et al.*[1], comparing different instruments which determine the strength of tablets, demonstrates the use of statistical methods to draw conclusions from experiments in which the basic principles of the system are unknown. Such a paper would be valuable if nothing were known about the failure of tablets when subjected to the diametral crushing of these instruments. It has been, however, clearly established that the mechanism of failure depends on the loading conditions[2]. Tablets can fail by shear, compression, tension, or a mixture of these mechanisms. Under the correct loading condition, it can be ensured that tension is the major mechanism of failure[2]. If the correct conditions are not provided, failure occurs by a complex mixture of the three mechanisms. With sensitive instruments such as the Instron, the failure in tension results in values of the breaking load which have greater reproducibility than when failure occurs by mixed mechanisms[2].

No mention is made by Bavitz *et al.*[1] as to the mechanism of failure of the tablets. The instruments tested invariably do not ensure tensile failure; hence, one might expect to obtain poor reproducibility. The limited variation reported by Bavitz *et al.*[1] appears to contradict this concept. It is my experience that the reproducibility reflects the insensitivity of the tablet strength testers. Thus the test systems are inadequate on two counts. I suggest, therefore, that the work of Bavitz *et al.*[1] is misplaced. Greater benefit could be achieved by placing the effort behind the design and testing of an instrument that would ensure that the tablets failed in tension and provided an accurate estimate of the breaking load. Comparisons of instruments would then be unnecessary.

J. M. Newton
Pharmacy Department
The University
Nottingham, England

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BRINGING DRUG COMBINATIONS INTO THE FOLD

On at least two previous occasions in this column, we discussed the subject of drug names, and in particular nonproprietary drug names. In September 1961, we called attention to certain little-recognized features of such names, and specifically the clues that often can be conveyed in the name as to the category and classification of the drug. Subsequently, in June of 1966, we saluted the United States Adopted Names (USAN) Council on the effective strides it had been making to meet the needs of practitioners—those who actually use the drug names in the daily course of their professional pursuits—by coining shorter and easier-to-use names which still embody the desired informative elements.

Despite all the progress made by the USAN Council, however, one major area had been virtually untouched by this group as of 1966, and it has continued to be the object of neglect since then. We refer to drug combinations, and specifically the desirability—and downright *need*—to have useful nonproprietary names for rational drug combinations.

The job of coining and adopting satisfactory names for these combination drugs is admittedly not a simple task—there are a number of inherent difficulties, several of which are truly substantial. But, in addition, there have been signs that clearly indicate that at least some of the difficulty has not been scientific, medical, or linguistic. Rather, it has been due to obstacles or hurdles which are economically based. Such opposition stems from the rather obvious fact that a single short trade name will always be more convenient to say, write, and use than a series of nonproprietary names. By the same token, however, a practitioner's decision to use a trade name in place of a nonproprietary name, or the choice *vice versa*, should not be dictated by relative ease or difficulty in use of such terminology.

Consequently, the USAN Council recently was asked again to review this subject and to make a serious effort to develop suitable nonproprietary names for rational drug combinations. FDA's approval last year to market an anti-infective agent consisting of a one-to-five ratio of trimethoprim and sulfamethoxazole served to bring this issue back into the spotlight. Namely, there are some, albeit a relatively few, rational drug combinations, and in the case of these articles, practitioners should not be forced to use the catchy, easy trade names of "Bactrim" and "Septra" as the only alternative to the full "trimethoprim and sulfamethoxazole."

It seems equally evident that it is the USAN Council's responsibility to take the necessary action to provide practitioners with a satisfactory alternative, and to take such action without further delays. In contrast, for example, the British Pharmacopoeia Commission faced this same problem several years ago, and the BP Commission adopted the name "co-trimoxazole" for the above-mentioned drug combination in August 1971.

We are confident the USAN Council can and will do the job—particularly if the Council receives all the support it deserves from its respective sponsoring organizations and the professions and public which these sponsoring organizations are intended to represent. Therefore, in pursuing this effort the USAN Council must not permit itself to be deterred by pressures from the drug industry lobbyists, or any of their self-serving interest groups.

Edward G. Feldmann

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REVIEW ARTICLE

Survey of Radiopharmaceuticals and Their Current Status

ALBERT H. SOLOWAY* and MICHAEL A. DAVIS‡ˣ

Keyphrases □ Radiopharmaceuticals—survey and review of development, selection, availability, and mechanism of action as diagnostic and therapeutic agents □ Diagnostic radiopharmaceuticals—survey and review of development, selection, availability, and mechanism of action □ Scanning agents—survey and review of radiopharmaceuticals

The term radiopharmaceutical may be considered as encompassing *(a)* drugs that are specifically used by reason of their radioactivity for supplying fundamental information of the normal and pathological processes in mammalian systems and *(b)* compounds that are radioactive *per se* or may be converted *in situ* to radioactive substances whose radiation is then effective in destroying tissue. This presentation[1] will mainly examine scanning agents and other radioactive compounds that have proved to be useful diagnostic substances and therapeutic drugs that are capable of delivering intense ionizing radiation for tissue destruction. Usually, although not invariably, the latter group involves the eradication of malignant processes.

The nuclear chemistry associated with the formation, production, and purification of the radionuclides and the instrumentation and detection devices used to measure the emitted photons will be mentioned only briefly. Since this is a survey, what we shall discuss in a cursory fashion are the radiopharmaceuticals in current use, the methods for their development and selection, the mechanisms by which they function if known, and future trends in the formation of more effective drugs for both diagnostic and therapeutic uses. This article will serve as an overview of radiopharmaceuticals and will offer a useful beginning for an in-depth examination of certain radionuclides and those compounds that contain them.

The development of radiopharmaceuticals has largely evolved from the useful collaboration of the clinician with the nuclear physicist. As a consequence, the physical and preparative characteristics of the radionuclides have been of prime consideration, namely physical half-life, methods for production and separation, and type of emission including the percentage and energies of those particles in the decay processes. Development of such drugs on the basis of their chemical, biochemical, physiological, and pharmacological properties has certainly been of subordinate consideration. In fact, drug selection has been arrived at frequently in a pragmatic way, and only subsequently have efforts been expended to elucidate the underlying biochemical factors involved in the utility of a particular radiopharmaceutical drug. Examples of this development process are numerous in nuclear medicine. This emphasis on physical characteristics is readily understandable since isotopic half-life and emission characteristics are of prime importance in any diagnostic and therapeutic maneuver. Also, with few exceptions, the underlying biochemical and physiological differences between normal and pathological processes have not been fully understood, and there is little knowledge concerning drugs that specifically localize in various dis-

[1] A previous review article entitled "Radiopharmaceuticals," by W. Wolf and M. Tubis was published in *J. Pharm. Sci.*, **56**, 1(1967).

ease processes. Such information is important and will continue to unfold, but clinicians have rightly not waited for such a complete understanding prior to using radiopharmaceuticals. However, these major deficiencies in our knowledge of drug distribution and related factors have limited the rational development of new radiopharmaceuticals. As the biochemistry of various diseases becomes clearer, radioactive drugs will be developed to utilize these physiological and metabolic differences between normal and pathological tissues as a means for diagnosis and treatment. For the purpose of this presentation, drugs in each category will be considered separately.

DIAGNOSTIC DRUGS

Diagnostic drugs may be divided into two categories. In the first are those that may be classed as *in vivo* drugs and must be administered to the patient to obtain diagnostic information. In the second are those substances that are used in *in vitro* procedures and no radioactivity is injected or ingested. Radioactive drugs for diagnostic purposes are in a higher state of development than are compounds suggested for therapeutic usage. The reasons for this difference are readily apparent when we consider what we are trying to achieve by each technique. In the case of *in vivo* diagnostics, we may wish to show either differences between normal or abnormal physiological processes or the delineation between normal and diseased tissues. In either case, we wish to visualize or determine by scanning and camera devices or to quantitate by external probes such differences. Ideally, the dosage of the radiopharmaceutical for such a purpose is miniscule, and the amount of radiation delivered by such drugs to the whole body or to a particular tissue or organ is inconsequential, even to the tissue being scanned. Therefore, the entire procedure is relatively innocuous from a radiation standpoint as well as other toxicological considerations. However, there have been instances where a radiopharmaceutical has an extremely long half-life for a particular tissue; under these conditions, radiation delivered to the organ in question may be sufficiently great so that procedures are required to minimize this radiation dose. But this is not the usual problem encountered in diagnosis, and because of the benign nature of the procedure as well as the minimal trauma to the patient, radiopharmaceuticals have achieved widespread use as *in vivo* diagnostic agents. It is, of course, apparent that *in vitro* procedures are even more attractive since the patient receives no radioactive drugs *per se*. It is a sample of the patient's body fluids or tissues that is removed and exposed to the radiopharmaceuticals under *in vitro* conditions.

The problem in the development of therapeutic compounds is totally different. In this case, the attempt is to utilize the radiation emitted by the drug to effect tissue destruction. For this reason, this procedure is usually confined to the eradication of malignant processes. Obviously, we are not attempting to destroy all tissues but only those responsible for the pathology. Consequently, a concentration differential between the diseased and normal tissues must be achieved that is orders of magnitude greater than would be needed for diagnostic purposes. This accounts for the limited usefulness of such tissue-destroying drugs since, generally, one cannot achieve the high selective localization of the radiopharmaceutical in the neoplastic lesion or other uneconomic tissue. This selectivity is important for therapy since the dose administered is of such an order of magnitude that it must be confined, for all practical purposes, to the target tissue. Even moderate to low percentages of the dose administered appearing in normal tissues may have a deleterious effect upon organ function. This requirement for therapeutic drugs is so stringent that the use of radiopharmaceuticals for therapy is greatly limited and not of major importance. Therefore, these drugs primarily are used for diagnostic purposes.

In the use of an *in vivo* drug, whether for diagnosis or therapy, one wants the drug in the system ideally only for the time required to complete the procedure. This applies whether one is considering anesthetics, analgesics, or radiopharmaceuticals. The question that presents itself is how a radiopharmaceutical can be localized and its time duration be limited in a particular target tissue. Before examining the various organs and tissues and the drugs currently in use that function effectively as *in vivo* diagnostic agents, let us consider in general the ways of assessing the duration of drugs in humans and, more specifically, these methods as applied to radiopharmaceuticals and factors that may affect this duration. If the drug were stable, we would simply determine its biological half-life, which is a measure of its duration in humans, by examining both excretion and metabolism. However, since a radioactive drug is constantly decomposing, we must also be concerned with its physical half-life as well as the products derived from it.

The nuclide produced as the result of the radioactive decay of another nuclide (referred to as the daughter and parent, respectively) must be considered when evaluating the parent and its products as a potential radiopharmaceutical. The ideal situation occurs when the daughter product is stable or has an extremely long half-life so that, when compared to the human lifespan, it can be considered stable. ^{99m}Tc falls into this category as a parent, since its daughter product ^{99}Tc has a $T_{1/2}$ of 2×10^5 years. Another acceptable condition is when the daughter product has a half-life shorter than, equal to, or only slightly longer than the parent and has photons of similar energy with the absence of β-rays and positron emission. The unacceptable occurs when the daughter is relatively long lived compared to the parent and has α-, β-, positron, or high-energy γ-rays.

To determine the effective half-life of such a drug, its physical decay scheme, including those of the daughter products, and its biological clearance or metabolism must be combined. The effective half-life, T_e, can therefore be expressed in terms of both its biological, T_b, and physical, T_p, half-lives as

shown in Eqs. 1*a*–1*c*:

$$\frac{1}{T_e} = \frac{1}{T_b} + \frac{1}{T_p} \qquad \text{(Eq. 1a)}$$

$$\frac{1}{T_e} = \frac{T_p + T_b}{T_b T_p} \qquad \text{(Eq. 1b)}$$

$$\frac{1}{T_e} = \frac{T_b T_p}{T_p + T_b} \qquad \text{(Eq. 1c)}$$

As instrumentation and, more specifically, the scintillation cameras, together with the requisite data processing methodology, have improved and the diagnostic procedure times have been progressively reduced, it has become highly desirable to match the effective half-life to this procedure time. If we have a drug with a particular biological half-life, we can strive to decrease T_e by incorporating a radionuclide with a shorter T_p. However, what is important is the time for optimum localization of the drug in the tissue to be visualized. Wagner's (1) criterion is that the ideal effective $T_{1/2}$ for a radionuclide totally retained by the mammalian system is equal to the product of 0.693 and the time at which scanning is to be performed. The matching of the scanning time with the drug's effective half-life is one reason for the development of shorter half-life radionuclides since the optimum localization time may then be sufficiently short. Certainly, the ability to administer larger quantities of radioactive compounds with a minimal radiation dose and a concomitant improvement in target visualization are important factors as well. When considering the use of isotopes with half-lives in the hour and minute range, it was necessary in the past that there be close proximity to reactors and cyclotrons. In many instances, this is still the case [*i.e.*, use of $^{15}O_2$ (2 min), use of ^{13}N (10 min), and use of ^{11}C-labeled carbon dioxide (20 min) in pulmonary studies]. However, the development of nuclide generators and the availability of nuclides from commercial sources have provided a convenient means for using short-lived materials at great distances from the source of production. ^{99m}Tc, probably the single most important nuclide in radiopharmacy, is an example of a radionuclide produced by such a generator system (Scheme I) (2). Other such nuclide generators may become of increasing importance in the future in nuclear medicine (3). These generator systems are presented in Table I.

Just as the optimum effective half-life should be matched to the scanning time, likewise very little energy, aside from the emission needed for detection or destruction, should be produced within or absorbed by the individual involved in the procedure. The desired γ-ray energies for use in external scanning or camera detection systems are roughly in the 150–400-kev range, with a possible optimum value of about 250 kev. Appreciably lower energies in the 30-kev range result in total absorption; in the 80–100-kev range, interaction with tissue occurs, resulting in

$$^{98}Mo(n,\gamma) \longrightarrow {}^{99}Mo \xrightarrow{\beta^-,\gamma} {}^{99m}Tc \xrightarrow[\gamma\text{-ray}]{140\ kev} {}^{99}Tc$$

$$(T_{1/2} = 67\ hr)\ (T_{1/2} = 6\ hr)$$

Scheme I—^{99m}Tc generator

Table I—Nuclide Generator Systems

Parent Isotope	Half-Life	Daughter Isotope	Half-Life
	γ-Emitters		
^{81}Rb	4.7 hr	^{81m}Kr	13 sec
^{68}Ge	280 days	^{68}Ga	68 min
^{113}Sn	118 days	^{113m}In	1.7 hr
^{132}Te	3.2 days	^{132}I	2.3 hr
^{87}Y	80 hr	^{87m}Sr	2.8 hr
^{99}Mo	67 hr	^{99m}Tc	6 hr
^{90}Sr	28 years	^{90}Y	64 hr
^{137}Cs	30 years	^{137m}Ba	2.6 min
^{44}Ti	10^3 years	^{44}Sc	3.9 hr
^{103}Pd	17 days	^{103m}Rh	57 min
^{77}Br	58 hr	^{77m}Se	18 sec
^{191}Os	16 days	^{191}Ir	4.9 sec
^{28}Mg	21 hr	^{109m}Al	2.3 min
^{140}Ba	12.8 days	^{140}La	40 hr
^{72}Se	8.4 days	^{72}As	26 hr
^{42}A	3.5 years	^{42}K	12.4 hr
	β-Emitters		
^{144}Ce	284 days	^{144}Pr	17.3 min

the scattering of these photons and obscuring of the tissue visualization. Nuclides on the opposite end of the energy spectrum with γ-ray energies above 500 kev are extremely difficult to collimate and result in poor scan quality from the degraded resolution.

These physical decay characteristics are constants, and the given generalizations may be applied to all *in vivo* radiopharmaceuticals. The biological factors are, however, quite diverse, and the chemical composition and formulation are directly dependent upon such factors. In other words, understanding the biochemistry and physiology of both the normal and the abnormal or diseased tissue may permit the tailor-making of radiopharmaceuticals and their more selective localization in the abnormal structures. This would be the ideal.

It is certainly easier to visualize an elevated concentration of a radionuclide in a target organ than to observe the so-called "cold spots" areas of decreased isotopic concentrations in a matrix of high radioactive concentration. The problem, unfortunately, is the paucity of information regarding both biochemical and physiological processes which would permit the design of a radiopharmaceutical that could utilize such differences between normal and abnormal structures. In some instances, there is a great deal of information relating to normal function. But how the abnormal or diseased tissues differ in the first place frequently is unknown; and how this information, when it is known, may be used to develop a radiopharmaceutical may not be readily apparent.

Some important agents used for individual organs and the mechanisms for localization will now be discussed.

In Vivo *Radiopharmaceuticals*

Radiopharmaceuticals for Imaging—*Liver Agents*—The liver is one major organ for which scanning agents have been developed (4). These agents comprise two major categories: *(a)* those that are phagocytized by the Küpffer cells, and *(b)* those that are taken up by the polygonal cells and subsequently

excreted through the biliary system into the GI tract (Table II). Radiopharmaceuticals developed for the former are based on the fact that colloidal particles are phagocytized by the reticuloendothelial system; included in this system are the Küpffer cells of the liver (5). As will be apparent, this property of the reticuloendothelial cells is also utilized to scan the spleen and bone marrow. The uptake by these Küpffer cells is based primarily on particle size, a physical attribute of a particular material, and to a less extent upon any chemical property (6).

A number of colloidal agents have been considered and used at various times. Initially, clinicians used ^{131}I-labeled human serum albumin and colloidal ^{198}Au but now, with the availability of useful colloids containing the shorter T_p radionuclides ^{99m}Tc and ^{113m}In, the latter substances have become the drugs of choice, especially those containing ^{99m}Tc. Their use is dependent not only on half-life but also on suitable emission characteristics. In this regard, ^{99m}Tc is a particularly attractive radionuclide. Efforts have been directed toward more facile incorporation of these radioactive isotopes into colloidal particles or into substances from which colloidal particles may be generated (7, 8). In addition, attempts have been made to produce particles with a more uniform and predetermined particle size, as is essential in view of the short T_p of these isotopes.

Polygonal cells of the liver do absorb certain dyes and various organic and inorganic compounds in large amounts, and these are then excreted *via* the gallbladder into the GI system (9). This is the basis for the use of ^{131}I-rose bengal (sodium rose bengal I^{131}) and ^{131}I-sodium iodipamide (sodium iodipamide I^{131}). The facile incorporation of iodine into various organic structures had led to the widespread use of iodine-labeled radiopharmaceuticals. The physical characteristics of ^{131}I are not as suitable as other more current nuclides, but ^{123}I is highly desirable from a physical standpoint and its incorporation into organic structures may result in the more favorable competition of such compounds with ^{99m}Tc-labeled products. The key limitation has been the availability and cost of ^{123}I relative to other nuclides; but should compounds be developed having superior clinical properties, these restrictions will disappear (10). Other iodine isotopes have been considered and used in the past, but unfavorable half-life, decay pattern, and method of production have limited the use of ^{124}I, ^{125}I, ^{126}I, ^{130}I, ^{132}I, ^{133}I, and ^{135}I.

A most interesting technique which may have application to other organs in the future is the measure of liver metabolism by use of ^{75}Se-selenous acid (H_2SeO_3). In the liver, this compound is converted to the volatile gas $(CH_3)_2Se$, which is excreted by the lung. Hepatectomies or ligatures of the portal vein produce a significant diminution in the percentage of dimethyl selenide formed and excreted. It should be interesting to determine the effect of hepatomas on the metabolism of selenous acid. This procedure offers the opportunity of studying the metabolic function of an organ under *in vivo* conditions and is based upon the selection of drugs that are altered in specific ways by a single organ producing volatile products. It remains to be seen how general and widespread the applications of this technique will be in nuclear medicine.

Table II—Compounds for Liver Scanning and Function

Colloidal:
^{99m}Tc-sulfur colloid (Te_2S_7) (technetium Tc 99m sulfur colloid)
Human serum albumin (^{99m}Tc-labeled and ^{131}I-labeled)
^{113m}In-hydroxide gelatin
^{198}Au
^{99m}Tc-stannous phytate
Polygonal cells and biliary system excretors:
^{131}I-rose bengal (sodium rose bengal I 131)
^{131}I-sodium iodipamide (sodium iodipamide I 131)
^{99m}Tc(SCN)
^{99m}Tc-penicillamine and penicillamine–acetazolamide complex
^{99m}Tc-tetracycline
^{75}Se-selenous acid

Spleen and Bone Marrow Agents—Two other tissues that also have cells comprising the reticuloendothelial system are the spleen and bone marrow (Table III). Consequently, both tissues can be scanned by use of colloidal agents (11). As in the case of the liver, these drugs are concentrated by viable, normal tissue and the basis for using them as scanning agents is the possibility that they will be excluded from abnormal or pathological structures. The spleen also functions as a scavenger for damaged red blood cells, and it is on this basis that ^{51}Cr-labeled red blood cells, slightly damaged by heat treatment, are filtered out by the spleen and thereby serve as the means for scanning this organ (12). Once again, the normal functioning tissue will concentrate the radionuclide, and abnormal or pathological areas should show areas of decreased uptake. This procedure would also delineate any enlargement of this organ. ^{197}Hg-Hydroxy(2-hydroxypropyl)mercury[2] functions in a somewhat similar fashion (13). This drug, on intravenous administration, both labels and damages red blood cells; as a result, these cells are sequestered by the spleen. More recently, researchers have labeled red blood cells with ^{99m}Tc, which looks like a promising agent for scintigraphy of the spleen (14).

Bone marrow has been scanned by use of a colloid of ^{157}Dy-gluconate; 40–48% of the administered dose of this agent is concentrated by this tissue (8.1 hr $T_{1/2}$ ^{157}Dy-326 kev monoenergetic γ) (15). ^{111}In-Chloride has also been used for this same purpose (16). This cyclotron-produced radionuclide, with a half-life of 67 hr, has a γ-ray emission spectra that is ideal for use with gamma cameras and rectilinear scanners. These properties have resulted in increased consideration for the incorporation of ^{111}In into various compounds.

In addition to the reticuloendothelial structure, the bone marrow also contains cells with a hematopoietic function. This is the normal site for the production of erythrocytes; and since iron is an important component of red blood cells, erythrocyte incorporators such as ^{52}Fe and ^{59}Fe may serve as a means

[2] Merisoprol Hg 197.

Table III—Scanning Agents for the Spleen and Bone Marrow

Spleen scanning agents:
Colloidal agents
^{51}Cr-labeled red blood cells
^{197}Hg-hydroxy(2-hydroxypropyl)mercury (merisoprol Hg 197)
Bone marrow scanning agents:
Colloidal agents–reticuloendothelial system
Erythrocyte incorporators (^{52}Fe and ^{59}Fe)
^{157}Dy-gluconate
^{111}In-chloride

for delineating the functioning bone marrow elements (17).

Bone Scanning Agents—For the bone itself, several radionuclides have been used (18): strontium salts (as ^{85}Sr or ^{87m}Sr), gallium citrate (^{67}Ga and ^{68}Ga), ^{18}fluoride, barium salts (as ^{131}Ba, ^{135m}Ba, and ^{140}Ba), ^{47}calcium^{+2}, ^{99m}Tc-polyphosphates and ^{99m}Tc-diphosphonates, and ^{157}Dy-ethylenediaminetetraacetic acid chelate. Since the skeleton comprises 99% of the calcium of the body and its mineral composition is in a dynamic metabolic state, elements related chemically to calcium and other bone components have been used to visualize normal and diseased tissues. Two isotopes of calcium have been used, ^{45}Ca and ^{47}Ca. Also, in view of their chemical similarities to calcium, the other alkaline earth elements have been effective as bone scanning agents including two radionuclides of strontium and several of barium (19). Two of these, ^{135m}Ba with a single γ 268 kev ($T_{1/2}$ = 29 hr) and ^{131}Ba ($T_{1/2}$ = 11.6 days), have been proposed to be suitable for imaging with both the gamma camera and the rectilinear scanner. The bone uptake of ^{135m}Ba is very rapid; 60% of the dose is absorbed within 2 hr. Such isotopes are useful in the early detection of bone tumors, the visualization of fracture sites and osteoarthritic lesions, and the determination of metabolic bone diseases. These agents appear to have significant advantages over the strontium nuclides.

In addition to the alkaline earth elements, compounds of gallium have shown a propensity for bone (20). This observation has been the basis for the use of gallium citrate as ^{68}Ga and ^{67}Ga for the localization in this tissue, and these compounds are useful in delineating the extent of malignant processes (21). Recently, ^{157}Dy as its ethylenediaminetetraacetic acid chelate was reported to be an excellent bone scanning agent ($T_{1/2}$ = 8.1 hr, 100% electron capture with monoenergetic γ-ray 326 kev) (15). Approximately 50% of the injected dose appears in bone in 3 hr, with the remainder being excreted by the kidney. Based upon its physical and biological properties, this chelate is preferable with conventional gamma cameras and scanners compared to the other isotopes that have been considered heretofore. Not only have cations been used, but in the past ^{32}P-phosphate was found to appear even in the dense portions of the shaft of large bones. These findings led to the development of ^{99m}Tc-polyphosphate and various ^{99m}Tc-phosphonates as new drugs for skeletal imaging (22–25).

Fluoride, by reason of its facile ability to substitute in the hydroxyapatite crystals of bone, rapidly accumulates 10–20 times more in bone and skull than in the soft tissues of humans (26, 27). Tumors of the bone, with their increased metabolism, may attain 10 times the normal bone concentration. Consequently, even with a T_p of less than 2 hr, ^{18}F appears to be a useful isotope for this purpose. It has been applied in imaging radium-induced bone pathology and is especially useful in detecting active *versus* inactive lesions. This radionuclide also has been used recently in detecting lesions related to lymphoma metastases (28).

Kidney Agents—Kidney scanning agents take advantage of the biochemical and physiological properties of the renal system for their use in visualizing this structure (29). These agents include: organomercurials—^{197}Hg- and ^{203}Hg-chlormerodrin (chlormerodrin Hg 197 and Hg 203), ^{99m}Tc-iron–ascorbate complex, ^{125}I- and ^{131}I-sodium iodohippurate (sodium iodohippurate I 125 and I 131), and ^{99m}Tc-penthanil and other ^{99m}Tc-labeled agents which are rapidly excreted.

The development of mercury-containing drugs as kidney scanning agents arose from the initial use of certain mercurials as diuretics. It was observed that such compounds are rapidly taken up by the renal cortex and are also temporarily fixed by renal tubular cells. The thioactivated enzyme systems in this tissue, essential for renal tubular transport, are inactivated. As a consequence, these compounds labeled with ^{197}Hg have been useful in detecting the parenchymal structures (30). ^{203}Hg was used initially, but the high radiation dose delivered to the kidney by this nuclide favored the use of ^{197}Hg. Now more promising drugs are also used. Another compound which has been used is ^{131}I-sodium iodohippurate (31). It has utility because such compounds are urographic contrast agents and, in this particular case, the drug is rapidly excreted *via* the kidney. In this way, visualization of the kidney and the excretion process may be accomplished.

The need for improved renal imaging recently led to the synthesis and use of ^{123}I-sodium iodohippurate. ^{123}I has emission characteristics that are preferable to those of ^{131}I (32). Another drug which functions in a similar way is ^{99m}Tc-penthanil (diethylenetriaminepentaacetic acid) (33). It is rapidly cleared by the kidney *via* glomerular filtration and makes use of the desirable physical characteristics of ^{99m}Tc. Other ^{99m}Tc compounds have been prepared as possible renal imaging agents. These include a complex of ^{99m}Tc-penicillamine–acetazolamine (34), a ^{99m}Tc-iron–ascorbate complex (35), ^{99m}Tc-labeled meglumine diatrizoate (36), ^{99m}Tc-mannitol, and ^{99m}Tc-tetracycline (37).

Lung Agents—Particle size has been important to the development of lung scanning agents, and it will be useful to consider the rationale behind the use of such compounds (38). Radioactive colloidal particles, as already discussed, have been useful for visualizing the reticuloendothelial system. When particle size increases sufficiently, capillary trapping action re-

sults and the particular organ that acts as the filtering device may be visualized. This is the basis for the development of aggregated proteins and inorganic particulate materials, both labeled with those radionuclides having desirable physical properties. Such important characteristics are half-life and the energetics of the emitted radiation. Initially, ^{131}I-human serum albumin was macroaggregated. This compound has been used effectively in estimating regional pulmonary function, especially prior to preoperative lobectomy, and in locating nonfunctional and avascular regions (40). Subsequent research emphasized the preparation of materials with appreciably reduced pulmonary toxicity, greater uniformity in particle shape and size, autoclavability, and more suitable radionuclides from a standpoint of half-life and emission characteristics (Table IV). The desirable requirements have led to the preparation of metabolizable microspheres with greater uniformity (40–43) of human serum albumin containing ^{125}I, ^{99m}Tc, and ^{113m}In. The goal of uniform particle size was in part the basis for synthesizing ^{113m}In-, ^{111}In-, and ^{99m}Tc-labeled ferric hydroxide particles. Additionally, these can be autoclaved following their formation. The potential difficulty with these latter entities is that they are not completely biodegradable (44–49).

Radioactive gases also have been used to determine regions of decreased ventilation and perfusion (50). These agents include some very short-lived positron emitters: $^{15}O_2$ ($T_{1/2}$ = 2 min), ^{11}C (20 min), and ^{13}N (10 min) (51). In addition there are the noble gases, which are relatively inert biochemically and rapidly excreted *via* the pulmonary system. These include ^{85}Kr (10.3 years) and, more recently, ^{133}Xe (5.3 days), ^{135}Xe (9.2 hr), and ^{81m}Kr (13 sec) (52), which can be used to distinguish between pulmonary embolisms and chronic obstructive lung disease. Radioaerosols have been developed as inhalation scanning agents (50). ^{99m}Tc-labeled albumin, ^{99m}Tc-sulfur colloid, and ^{113m}In- and ^{111}In-hydroxides have been nebulized for this purpose. They have been used in studying obstructive pulmonary disease and are reported to have advantages over radioxenon diagnostic procedures in that they provide clinically useful information regarding precise sites of partial and complete bronchial obstruction. Their slow rate of removal has the distinct advantage of not requiring rapid imaging devices or procedures.

Circulatory Agents—Labeled proteins are also used in the measurement and/or visualization of the various circulatory systems (53–56). Other uses are the measurement of plasma volume, cardiac output, and cerebral blood flow (Table V). The labeling radionuclides for these proteins are ^{123}I, ^{125}I, ^{131}I, ^{99m}Tc, ^{113m}In, and ^{111}In (57). The latter two radionuclides are rapidly bound under *in vivo* conditions to plasma proteins (particularly transferrin) and in this way may be used for both circulation and blood scanning. Also, ^{129}Cs ($T_{1/2}$ = 32 hr) and ^{43}K ($T_{1/2}$ = 22.4 hr) have been suggested as suitable nuclides for myocardial uptake studies (58–60). These monovalent cations are rapidly accumulated by striated muscle and

Table IV—Lung Scanning Agents

Macroaggregated albumin and other proteins:
^{99m}Tc
^{131}I and other iodine isotopes
^{113m}In
Albumin microspheres:
^{99m}Tc
^{125}I
Ferric hydroxide aggregates:
^{113m}In
^{99m}Tc
^{68}Ga
Other macroaggregates:
Tc-sulfur colloid
Tc-labeled-glutaraldehyde
Radioactive gases:
^{11}C as CO and CO_2
$^{15}O_2$ as CO and CO_2
^{13}N
^{133}Xe and ^{135}Xe
^{81m}Kr and ^{85}Kr
Radioaerosols:
^{113m}In- and ^{111}In-hydroxides
^{99m}Tc-sulfur colloid (Tc_2S_7) (technetium Tc 99m sulfur colloid)
^{99m}Tc-albumin

myocardium. In an attempt to distinguish between normal and ischemic myocardium, ^{131}I-pyrazole was developed and shows a concentration gradient between normal and infarcted myocardium of 25 (61). Additional drugs which have been considered are ^{201}Tl, ^{13}N-ammonia, and ^{99m}Tc-tetracycline (62–64).

Several other tissues (Table V) that have been imaged by use of radiopharmaceuticals are the thyroid, pancreas, and parathyroid. Thyroid scanning was the initial basis for the development of radiopharmaceuticals (65). The propensity for the thyroid to sequester iodine and to incorporate it into hormones is well established. Therefore, thyroid scanning is on a firm biochemical basis, and uptake studies of iodine by this gland supply useful information to the clinician. Several iodine isotopes have been tried, including ^{131}I (8 days), ^{132}I (2.3 hr), ^{124}I (4.5 days), ^{125}I (60 days), ^{130}I (12.6 hr), and ^{123}I (13.3 hr). Of these, the two most widely used are ^{131}I and ^{125}I, due in large part to their availability, cost, and shelflife. From the standpoint of physical half-life and emission characteristics, ^{123}I is the more desirable γ-ray scanning agent. For diagnostic uses, it delivers only 1% of the radiation dose of ^{131}I and gives a clearer radiograph. A major limitation has been the difficulty to prepare ^{123}I free of undesirable impurities and in sufficient amounts. These obstacles now appear to be overcome with recent cyclotron techniques. This development presents the possibility of using this radionuclide as iodide itself for thyroid scanning and also to incorporate it into other carriers such as proteins for external visualization of certain tissues. Pertechnetate ($^{99m}TcO_4^-$) also has been used for thyroid scanning. It is trapped by the thyroid in a manner similar to iodide. However, iodide is preferable since the ratio of thyroid to nonthyroid tissue is appreciably greater for iodide vis-à-vis pertechnetate.

The pancreas incorporates certain amino acids with a high degree of localization. Since none of the elements in the structure of naturally occurring amino acids has suitable γ-emitters for diagnostic

Table V—Miscellaneous Scanning Agents

Circulation and blood-pool scanning:
Labeled human serum albumin (^{99m}Tc, ^{131}I, and ^{125}I)
^{113m}In-complexes
Radioactive gases (^{133}Xe and ^{81m}Kr)
^{129}Cs
^{99m}Tc-labeled red blood cells
Myocardial agents:
^{129}Cs salts
^{43}K salts
^{131}I-pyrazole
^{201}Tl salts
^{99m}Tc-tetracycline
^{131}I-oleic acid
Thyroid scanning:
Radioactive iodides (^{131}I, ^{125}I, and ^{123}I)
^{99m}Tc-pertechnetate (sodium pertechnetate Tc 99m)
Pancreas and parathyroid scanning:
^{75}Se-Selenomethionine (selenomethionine Se 75)
^{18}F-labeled amino acids
^{11}C-amino acids

Table VI—Biological Basis of Localization

Active transport:
Thyroid scanning with iodide or pertechnetate
Renal scanning with chlormerodrin
Liver scanning with rose bengal
Pancreatic scanning with selenomethionine
Thrombus scanning with fibrinogen or urokinase
Bone scanning with ^{18}F, ^{85}Sr, ^{87m}Sr, or Tc-polyphosphate
Phagocytosis/pinocytosis:
Liver scanning with colloidal particles
Spleen scanning with colloidal particles
Bone marrow scanning with colloidal particles
Other (tumor, abscess, and lymphnodes) with colloid or labeled white blood cells
Cell sequestration:
Spleen scanning with heat damaged ^{51}Se-labeled, ^{99m}Tc-labeled, or ^{197}Hg-hydroxy(2-hydroxypropyl)mercury (merisoprol)
Capillary blockage:
Lung scanning with macroaggregate of albumin or ferric hydroxide labeled with ^{131}I, ^{113m}In, or ^{99m}Tc
Renal, liver, and brain scanning with the same aggregates injected intraarterially
Thrombophlebitis scanning with sulfur colloid or labeled white blood cells
Simple diffusion:
Brain tumor scanning with pertechnetate or penthanil chelates
Tumor scanning with ^{67}Ga-citrate
Compartmental localization:
Cardiac scanning with ^{99m}Tc-albumin, ^{99m}Tc-labeled, or ^{113m}In-transferrin
Placental and blood pool scanning with ^{99m}Tc-labeled or ^{113m}In-transferrin
Cisternography with ^{131}I-albumin, ^{169}Yb-penthanil, or ^{111}In-penthanil
Lymphatic scanning with colloidal ^{198}Au or ^{131}I-fats

purposes, tagging of these amino acids has become an important goal. As a result of replacement of the sulfur in the essential amino acid, methionine, by selenium, an isostere is produced which functions sufficiently like the natural amino acid to be rapidly incorporated into the pancreas. ^{75}Se ($T_{1/2}$ = 127 days) is a radionuclide which has been used for this purpose. This same compound has also been reported as being useful for localizing parathyroid adenomas and lymphomas (66). Such fraudulent amino acids as ^{75}Se-methionine are incorporated facilely into tissues which are rapidly synthesizing proteins. It is for this purpose as well that the incorporation of ^{18}F into amino acids has been tried (67, 68). Also the formation of complexes of ^{99m}Tc with amino acids and peptides and the development of synthetic procedures for creating ^{11}C-amino acids have been undertaken. These compounds would also be useful in detecting processes, such as a malignancy, where extensive *de novo* protein synthesis is occurring. For example, L-aspartic acid has achieved elevated concentrations in tumor vis-à-vis surrounding normal tissues. As a result, ^{11}C-labeled amino acid may have direct clinical usefulness (69). However, it would be essential that the synthetic and clinical studies be very convenient to the cyclotron producing ^{11}C in view of its 20-min half-life (70).

Photoscanning of the adrenal gland and related tumors has also been undertaken. Radioiodinated compounds, which do show a predilection for this tissue, have served as the basis for such scanning procedures (71).

Brain Scanning Agents—In general, the basis on which brain scanning drugs have been developed stems largely from the difference in permeability between normal and abnormal brain to a wide variety of substances (72). Neoplastic brain is certainly not as selective or restrictive as normal brain of the compounds that are able to penetrate and concentrate within it. The so-called blood–brain barrier is either absent or in an appreciably altered form in tumor compared with normal brain. As one researcher remarked, most anything will achieve a higher concentration in brain tumor than in normal parenchyma of the brain and, as such, is a brain scanning agent. This is obviously not totally true since, in the scanning process of the central nervous system (CNS), one is visualizing more than just brain and any brain pathology. The scan is a composite of other tissues including blood, muscle, skull, and scalp. Consequently, other factors come into play in addition to the drug's exclusion by the normal brain; these factors may include persistently high blood concentrations, rapid accretion by skull or scalp, and elevated amounts in muscle compared with tumor.

Brain scanning agents that have been or are still being widely used in various diagnostic centers include: sodium pertechnetate ($^{99m}TcO_4^-$), ^{197}Hg-chlormerodrin [$NH_2CONHCH_2CH(OCH_3)CH_2$-HgCl], labeled human serum albumin (^{131}I, ^{99m}Tc, ^{111}In, and ^{97}Ru), chelates of penthanil (^{64}Cu, ^{68}Ga, ^{113m}In, ^{99m}Tc, and ^{169}Yb), and miscellaneous substances [^{74}As (arsenate–arsenite), ^{204}Bi, ^{206}Bi, ^{131}I-polyvinylpyrrolidone, ^{111}In-bleomycin, and ^{67}Ga-citrate].

Sodium pertechnetate (Tc 99m) is in general use now as a brain scanning substance (73). This use results not so much from increased tumor specificity, but from its exclusion by normal brain, its reduced scanning time compared with longer half-life radionuclides, and the improved precision arising from the larger doses that may be administered. However, preliminary studies have indicated that cellular incorporation of ^{99m}Tc into brain tumors may occur. Binding of pertechnetate into albumin is not the *in*

vivo mechanism by which this agent delineates brain tumors, since *in vitro* incorporation of technetium into various blood proteins produced compounds that were completely unsatisfactory as cerebral imaging agents. Thus, the mechanism of how pertechnetate functions in delineating brain lesions still remains obscure.

The second agent ^{197}Hg-chlormerodrin is also a very effective brain scanning drug (74). This drug attaches itself rapidly and to a great extent (85%) to plasma proteins and, more specifically, to serum albumin. In view of the fact that the label is deposited on or within a wide variety of cerebral neoplasms, it is conceivable that protein binding *via* sulfhydryl groups is directly related to this incorporation. A pinocytotic mechanism may be involved by which the mercurated protein attaches itself to the pinocytotic vesicles (which are appreciably larger in human gliomas than in normal glial cells) and thereby is discharged into the intracytoplasmic compartment of the neoplastic cell. More extensive studies have been performed with radioiodinated human serum albumin. The findings support a pinocytotic mechanism or at least a pathway that preserves the unity of the protein with its tag during the intracellular localization of the drug. In addition to pertechnetate and proteins or drugs which become bound to proteins, a number of chelates containing radioactive nuclides have been used as scanning agents (75–78). Two widely used chelates are edetate (EDTA) and penthanil. As a result of the desirable physical properties of ^{99m}Tc, chelates such as penthanil have also been examined and found to have promise (79). ^{99m}Tc-Penthanil for brain scanning has advantages over pertechnetate because it does not accumulate in the choroid plexus, frontal sinuses, and salivary glands.

A few of the nuclides which have been tried in addition to ^{99m}Tc are ^{64}Cu, ^{68}Ga, ^{169}Yb ($T_{1/2}$ = 32 days), and ^{113m}In. ^{169}Yb has the advantage of a longer shelflife than the others, and its rapid excretion has permitted safe multimillicurie doses. As a result of these properties, it has also been considered as an agent for radiocisternography (80). More recently, however, ^{111}In-penthanil has become available and is considered to be superior to Yb-chelates for cisternography (81). The mechanism of how these chelates function is unclear. Studies using ^{64}Cu and ^{14}C-labeled edetate demonstrated that the two components do not remain as an entity under biological conditions[3]. Evidence has accumulated that different metals of the same chelate have significantly different biological properties, from the standpoints of renal excretion and of tumor localization. Thus, the mechanism of localization remains to be elaborated.

With regard to the miscellaneous agents, it has been observed that with ^{206}Bi as its nitrate, the radionuclide becomes bound to globulins and probably in this form enters the cytoplasm of the neoplastic cells (82). Certainly major amounts are attached to cytoplasmic proteins. This interesting property of bismuth compounds has led to the development of ^{206}Bi for diagnosis of brain tumors. In contrast, however, ^{74}As (as arsenate–arsenite) shows a negligible tendency to combine with proteins but has a high affinity for erythrocytes and undoubtedly is transported to the neoplasm in this form. From this information, it is apparent that much remains to be done to elucidate tumor cell localization and that this must be done for each chemical form of every nuclide.

Biological Basis of Localization—The mechanism of radionuclide localization is very important in enhancing differences between normal and pathological processes and in the design of more effective agents. In the foregoing, cursory consideration has been given to the mechanism of localization.

At this point a summary of the main biological mechanisms is pertinent. Six mechanisms are operative in the localization of radiopharmaceuticals in various organs and physiological compartments: active transport, phagocytosis and pinocytosis, cell sequestration, capillary blockade, simple diffusion, and compartmental localization (Table VI).

Active transport, from this standpoint, can be defined as the ability of a particular organ or tissue to concentrate selectively and to bind tightly a specific species within the pool of chemicals passing through the organ. In most instances, the blood serves as the transport vehicle, and it is the affinity of a particular organ for a certain type of reagent that produces this focal concentration known as active transport. The best known example utilizing this mechanism is the thyroid gland, as has been discussed, and its affinity for iodine in the form of iodide ion (I^-). Other tissues such as salivary glands, lactating mammary glands, gastric glands, and the choroid plexus also have the ability to concentrate iodide ions. In these glands, however, the radioiodide is simply concentrated for a short time and then excreted; in the thyroid gland, the iodide is bound covalently into the thyroid hormones. Therefore, the residence time of the iodide in the thyroid is significantly longer than in the organs that have the ability only to concentrate the anion and not to incorporate it chemically.

In the initial accumulation or trapping of radioiodide by the thyroid, the iodine first passes through the follicular cells of the thyroid gland and is concentrated within the follicles. This selective accumulation is an active metabolic process and, unless bound promptly, the trapped iodide quickly diffuses back into the blood.

Another example of active transport is the uptake of chlormerodrin [chloro(2-methoxy-3-ureidopropyl)-mercury] by the kidneys. As already noted, organic mercurial compounds form mercaptides with sulfhydryl-containing enzymes. These enzymes are found in abundance in the renal tubular cells, and both ^{203}Hg- and ^{197}Hg-chlormerodrin bind to the cytoplasmic components of the renal proximal tubular cells. They are eventually excreted into the tubular lumen and out through the remainder of the collecting system. Chlormerodrin has been shown to have the highest accumulation in the outer renal cortex, less in the medulla, and the lowest in the renal papillary tissue. The presence of the sulfhydryl en-

[3] A. H. Soloway and C. Carlson, unpublished data.

zymes and the subsequent formation of the sulfide linkage between these enzymes and the mercurial compounds lead to selective accumulation of these mercurials in the kidney and this accumulation leads to the active transport.

Other examples are the uptake of certain dyes by the liver and the uptake of fluoride, calcium, and barium by bone (83, 84). The foregoing are merely a few of the more prominent examples of the utilization of active transport in imaging radiopharmaceuticals.

Phagocytosis/pinocytosis is another biological mechanism of localization. Certain mononuclear cells that line the sinusoids of the liver and spleen and, to a lesser degree, the blood vessels of certain other regions of the body (bone marrow) have a special ability to remove particulate matter from the circulation by the process of phagocytosis. Phagocytosis can be described as the engulfment and eventual ingestion of foreign particles by cells. These cells constitute the reticuloendothelial system and the use of this system in liver, spleen, and bone marrow imaging was already discussed. After intravenous injection, particles in the millimicron to 1-μm range are phagocytized with great efficiency. Most inorganic particles remain in phagocytic cells almost indefinitely because they are not metabolized. Organic particles, such as bacteria, effete blood cells, and protein aggregates are metabolized after ingestion.

The distribution of radiocolloids within the reticuloendothelial system is dependent upon several physical factors including size of the colloid, chemical composition, and surface properties. In general, very small colloids in the millimicron range tend to concentrate primarily in liver and bone marrow; larger colloids, in the 0.1–1-μm range, tend to localize in the liver and spleen. Pinocytosis is a less specific process than phagocytosis, and the material need not be particulate to be ingested by the cell. This latter process has been implicated in the localization of proteins in tumor cells. Attachment of such structures to the surface of such cells followed by invagination is the mechanism by which such structures do achieve intracellular localization (85).

Cell sequestration is a third mechanism used in imaging. Chemical or thermal denaturation of radiolabeled erythrocytes results in their being actively sequestered by the spleen and is the mechanism by which ^{51}Cr, ^{99m}Tc, and ^{197}Hg-labeled red blood cells serve as a means for visualization of this organ.

A fourth mechanism for organ visualization and function measurement is *via* capillary blockade. This mechanism is utilized in assessing lung function and arterial organ perfusion. The smallest vessels in the vasculature are the capillaries, ranging from 7 to 10 μm in diameter. Thus, labeled macroaggregates greater than 10 μm in diameter are trapped in this capillary bed. When such particles are injected intravenously, the first arteriolar capillary bed contacted by the administered macroaggregates is that of the lung. It is such a blockade that gives rise to the feasibility of lung imaging. The lungs are not the only organs that may be visualized in this manner. By placing a catheter in the appropriate vessel, the macroaggregates can be selectively delivered to numerous organs. Studies aimed at determining regional blood flow and perfusion defects using the particle distribution technique have been performed in organs such as the kidneys, liver, brain, and myocardium. ^{131}I-Labeled macroaggregated human serum albumin, ^{99m}Tc-macroaggregated human serum albumin, and ^{113m}I- and ^{99m}Tc-ferric hydroxide macroaggregates all function by capillary blockade.

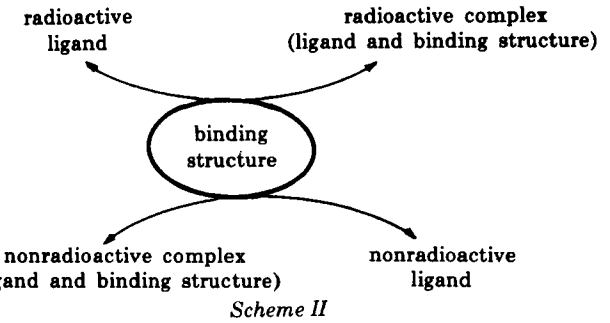
Simple diffusion is another mechanism applicable to the study of brain pathology. The diffusion of various organic and inorganic structures into those areas where the normal blood–brain barrier mechanism is no longer operative is the basis for the visualization of these pathological processes. These nuclides and their compounds were previously discussed.

Another mechanism of localization within the body is termed compartmental localization. The strict definition of this mechanism is the retention of the radiopharmaceutical in the administered tissue or body fluid space for a significant time. Thus, radioisotopic accumulation in the oral cavity, the GI tract, the vascular supply, the lymphatic system, and the cerebrospinal fluid compartment would be evidence for compartmental localization. Examples of drugs that function by this mechanism are ^{131}I-, ^{123}I-, ^{113m}In-, and ^{99m}Tc-labeled proteins for cardiac scanning, radioiodinated fats for lymphatic visualization, and ^{111}In and its compounds for cerebrospinal canal delineation (86).

Radiopharmaceuticals for Function—In addition to imaging radiopharmaceuticals, a number of drugs are injected for measuring physiological function and for determining the biochemical status of various organs. Such drugs may be classified as *in vivo* nonimaging radiopharmaceuticals. They are administered to the patient but are not used for structure visualization. Among these compounds are: *(a)* radiocobalt-labeled (^{60}Co and ^{61}Co) cyanocobalamin (vitamin B_{12}), used in the diagnosis of pernicious anemia and the malabsorption syndromes; *(b)* ^{55}Fe- and ^{59}Fe-citrate, used in a study of iron metabolism and ferrokinetics; *(c)* ^{51}Cr-labeled red blood cells, used for measuring erythrocyte mass, total circulating blood volume, red cell survival time, and GI blood loss; *(d)* ^{22}Na, ^{24}Na, ^{82}Br, and ^{35}S, used for the measurement of extracellular water; *(e)* radioactive sodium and ^{42}K, used for the determination of total exchangeable sodium and potassium; *(f)* radioiodinated human serum albumin and other labeled proteins, used for plasma volume measurement; *(g)* ^{45}Ca and ^{47}Ca, used in the assessment of calcium metabolism; and *(h)* radioiodinated fat and fatty acids, used for the study of fat absorption defects (87). The use of nonimaging drugs as radiopharmaceuticals was described in a previous review article (88).

In Vitro *Radiopharmaceuticals*

The diagnostic drugs considered so far must be administered directly to obtain meaningful information by scanning scintigraphy and excretion studies. In



Scheme II

all cases, a definitive radiation dose is delivered to the patient, even though this level may be of minimal importance now with the reduction in physical half-lives of a number of drugs. Ideally, one would prefer administering no radioactive material to a patient and yet obtain useful diagnostic data. The *in vitro* radiopharmaceutical drug meets this objective; as a consequence, use of this category of diagnostic agents is increasing at a rapid rate (87, 89–92). It offers as an ultimate goal the capability of measuring the levels of every hormone or other biochemical structure that is of importance in metabolism as well as determining the concentrations of exogenous compounds and their products which may be absorbed and/or generated. This procedure will be referred to here as competitive radioassay. The literature, however, is replete with a variety of terms that are very closely related and comprise the category of *in vitro* radiopharmaceuticals. Several of these will be used in this presentation, and they include radioimmunoassay, saturation analysis, radiostereoassay, competitive inhibition, and binding radioassay.

Essentially, this procedure involves the competition generally between a small molecule which can be considered a ligand and the radioactive form of this same ligand for some binding structure which has a demonstrable affinity for this ligand. The basis for this method of detecting analytically the ligand in biological fluids is that the radioactive and nonradioactive forms are readily exchanged and compete for those attaching sites on the binding structure. In this manner, the amount of unbound radioactive ligand is directly dependent upon the level of nonradioactive ligand in the fluid analyzed. This competitive binding may be visualized as in Scheme II.

Implicit in this assay method is a second important requirement, namely a capability for separating the complex from the ligand. In this way, it is totally feasible to measure the amount of radioactivity in each entity and to extrapolate to the concentration of the ligand in body fluids. From this brief description, it is apparent that no radioactivity is administered to the patient and that a sample of blood, urine, or other body fluid isolated from the patient may serve as the source for the nonradioactive ligand and sometimes even the binding structure. This technique is an extremely sensitive assay, comparable at least to and generally having greater sensitivity than photofluorometric and bioassay procedures.

In general, the ligand to be radioassayed is a small molecule such as a simple organic drug, its metabolites, a vitamin, hormone, or biochemical precursor. The radioactive form of the ligand should be totally comparable in its binding constant and mechanism of attachment to the binding structure as the nonradioactive ligand. For this reason, tritium may be used for hydrogen, ^{14}C for ^{12}C, ^{18}F for ^{19}F, ^{125}I or ^{131}I for ^{127}I, ^{32}P for ^{31}P, and ^{35}S for ^{32}S. Such ligands would behave identically to their nonradioactive counterparts. Alternatively, radioactive halogens could be incorporated into a ligand containing no halogen with the important proviso that this chemical modification should not modify the way the two ligands are complexed to the binding structure.

The third component in this triangle is the binding structure. Generally, this is a much larger component from a molecular weight basis than is the ligand. This structure may be a protein molecule such as an antibody, an enzyme, a plasma protein, or structural protein; it may also be a cell membrane or component thereof or other macromolecular entities such as certain resins, which show an affinity for the ligand regardless of whether it is specific or nonspecific. This three-component system comprises the essentials for a competitive binding assay procedure.

For the present purposes, with stress on the more widely used procedures, this subject of *in vitro* radiopharmaceuticals can be divided into two broad categories: *(a)* nonimmune systems, and *(b)* immune systems. Included in the former system are methods for measuring a variety of hormones and nonhormonal compounds. Among these are the hormones triiodothyronine, thyroxine, cortisone, cortisol, aldosterone, progesterone, estradiol, testosterone, ACTH, and vitamins and other important biochemicals such as vitamin B_{12}, folic acid, and cyclic AMP. The second category includes those procedures that may be called radioimmunoassay. Many of the same compounds measured by the nonimmune system have also been assayed by this method. These include triiodothyronine, aldosterone, estradiol, testosterone, ACTH, folic acid, and cyclic AMP. Additionally, a variety of other hormones and nonhormonal substances of biochemical importance have been determined, including human chorionic gonadotropin, follicle-stimulating hormone, oxytocin, thyrotropin, insulin, angiotensin, human growth hormone, glucagon, human luteinizing hormone, vasopressin, thyroglobulin, parathyroid hormone, bradykinin, calcitonin, and the prostaglandins (93). From this broad spectrum of compounds, it is apparent that there is a great scope for this assay procedure (94). It will permit ultimately a determination of their importance and the concentrations necessary for the maintenance of normal homeostatic conditions. An obvious corollary is that these techniques will furnish important diagnostic information as to the presence of pathological conditions. Such changes may actually precede a frank clinical manifestation. The structures considered to this point are those that are naturally occurring in mammalian systems. A variety of drugs, toxicants, and their metabolites can now be measured by radioimmunoassay. Examples are aspirin; morphine and its congeners (95); digitalis glyco-

sides such as digoxin, digitoxin, and digitoxigenin; and lysergide (LSD).

For the successful utilization of competitive radioassay by either method, a number of criteria must be considered and satisfactorily met. First, it is essential that the ligand to be assayed be prepared pure in both labeled and unlabeled forms. The unlabeled structure may serve as the starting material for synthesizing the radioactive ligand. For example, incorporating radioactive halogens into such a compound would yield the labeled ligand. Additionally, the unlabeled substance would serve as the standard for calibrating the radioassay procedure. A second requirement is that there must be a binding structure with sufficient specificity for the ligand so that other compounds will not displace it or interfere in any way with its complexation to the binding structure. This specific reagent may be a naturally occurring binding protein related in some respects to the biological activity of the ligand, an antibody prepared against this hapten, or an enzyme with specificity for this ligand. It is essential that there be no lack of specificity. For this reason, purification may pose difficulties since there may be similarities in physical and chemical properties between such structures and closely related biopolymers with much less avidity for the ligand. The degree of purification will depend upon the sensitivity, precision, and accuracy required by the assay. Similar purification problems may apply as well for antibodies and enzymes. Since these are proteins also, the whole gamut of protein purification technology may be utilized. Third, and of crucial importance, is the capability of separating the free ligands from the ligand-binding structure complex. Since many of these macromolecular structures are proteins or have a protein component, the techniques for separation are frequently those utilized in protein chemistry. Among those used are solvent partitions, chromatographic techniques, electrophoresis, dialysis, ultrafiltration and centrifugation, adsorptive methodologies, antigen–antibody precipitation, enzyme–substrate interaction, and various combinations of these.

To appreciate the potentialities as well as the current uses of competitive radioassay, it is pertinent to consider selected examples of the use of this technique and the advantages it offers for diagnosis. A more exhaustive treatment of *in vitro* diagnostics appears inappropriate and would negate the overview characteristics of this review.

One of the earliest and one of continuing importance is the use of *in vitro* procedures for thyroid function determination. Two small hormones produced by thyroid gland from iodide are thyroxine and triiodothyronine. They are released into the circulation and are largely transported by a specific glycoprotein, thyroxine-binding globulin to peripheral tissues where they mediate metabolic action and rate. Measurement of both of these hormones in free and bound states in the circulation provides important data as to thyroid function. The Murphy–Pattee method serves as a means for measuring total thyroxine in the serum. The technique involves the addition of known amounts of stable thyroxine to a system consisting of thyroxine-binding globulin and bound radioactive thyroxine (^{125}I or ^{131}I). As equilibrium is attained, some labeled thyroxine is displaced from the thyroxine-binding globulin binding sites by the stable ligand. By adding increasing amounts of this compound, increased amounts of radioactivity are displaced. A determination may then be made of the ratio between the free and protein-bound radioactive thyroxine. By plotting this ratio against the amount of nonradioactive thyroxine added, a standard curve is obtained. This curve may be utilized to measure the amount of endogenous thyroxine in serum. Thyroxine may be extracted from serum by use of ethanol; when this amount is added to the competitive radioassay system, labeled thyroxine is once again displaced. Use of the standard curve permits a direct readout of the concentration of endogenous serum thyroxine. This information is important in determining conditions where excessive amounts of thyroxine are secreted or where increased levels of thyroxine can be attributed to higher concentrations of thyroxine-binding globulin. The former is indicative of hyperthyroidism; the latter may result from greater estrogen levels, liver dysfunction, congenitally elevated thyroxine-binding globulin, or pregnancy. Lower serum thyroxine values may concomitantly be associated with hypothyroidism due to decreased thyroxine secretion or to a lower concentration of thyroxine-binding globulin. The latter may be a direct result of severe liver or kidney disease, androgen or steroid therapy, or congenital factors.

In addition to this measurement of bound thyroxine, free thyroxine concentrations have also been determined. Many of the methods used involve equilibrium dialysis. Dialysis of serum containing added radioactive thyroxine is performed, and the level of radioactive thyroxine in the dialysate is proportional to the concentration of unbound thyroxine in the serum. Abnormally high values of free thyroxine may be related to its increased secretion associated with hyperthyroidism or with patients experiencing sepsis or cardiac arrhythmias or following heparin therapy. Concomitantly, low values may be attributed to hypothyroidism. These diagnostic studies with thyroxine involve the use of nonimmune protein systems in which the radioactive and nonradioactive ligands compete for binding sites on the naturally occurring plasma protein carrier.

The second thyroid hormone, triiodothyronine, also plays an important role in normal human physiology and in patients with thyroid dysfunction. The development of analytical techniques utilizing *in vitro* radiopharmaceuticals has been of great importance in determining thyroid dysfunction. Difficulties have been encountered in the quantitation of this hormone, stemming in part from the fact that this compound has only one-sixtieth the concentration of thyroxine in serum and is not as tightly bound to thyroxine-binding globulin as thyroxine. The technique widely accepted as necessary for assessing thyroid function is the uptake test of ^{131}I- or

^{125}I-labeled triiodothyronine by cation-exchange resins. This *in vitro* test is carried out by the incubation of radioactive triiodothyronine with the patient's serum in the presence of the resin in sponge form. Effectively, the labeled triiodothyronine is being competed for by the resin and the thyroxine-binding globulin in the serum. Normal and abnormal ranges have been established for the amount of radioactive triiodothyronine bound by the resin. Excessive levels of thyroxine-binding globulin in the serum results in lower percentages bound to the resin; in a corresponding way, decreased amounts of thyroxine-binding globulin produce a higher percentage attached to the resin. Without a total consideration of the utilization of these data and those derived from thyroxine, it is sufficient to state that they assist materially in a determination of thyroid function. This triiodothyronine test illustrates another *in vitro* technique in which a naturally occurring protein and a resin are competing for the radioactive hormone. Both these tests for triiodothyronine and thyroxine fall into the category of nonimmune systems.

Of greater scope and potential are the immune system *in vitro* assays. For this presentation, two will be examined as examples of the potentiality of this analytical procedure. The first deals with the measurement of a naturally occurring protein hormone. In this respect, a consideration of the levels in serum of the pituitary protein, thyrotropin, seems pertinent in view of the previous examination of triiodothyronine and thyroxine. Thyrotropin concentration is most useful in diagnosing and determining the type of hypothyroidism, detecting early decreased thyroid reserve, and monitoring the effect of therapy upon function using exogenous thyroid hormones. From current studies, it is apparent that an elevated thyrotropin level may be the first and most subtle indicator of thyroid hormone deficiency. The development of radioimmunoassay for this hormone has provided a most sensitive test for thyroid bioactivity (96, 97). The labeled ligand, in this case, is ^{125}I- or ^{131}I-human thyrotropin. The chloramine-T method of radioiodination or other appropriate mild procedures which do not alter the native structural features of the protein molecule may be used for the synthesis of labeled thyrotropin. Separation of the protein bound and nonprotein bound radioactive iodine may be accomplished by column chromatography (Sephadex). The binding agent for the system is guinea pig or rabbit antihuman thyrotropin. Standard immunological procedures are followed for the production of high antibody titers, and their qualitative detection may be accomplished by immunoelectrophoresis, neutralization through antiserum dilutions, and radioimmunoassay. For actual quantitation, a system involving the competitive binding of labeled ligand, unlabeled ligand in serum, and the antibody preparation is used. In essence, there is a competition for the antibody by both the labeled and unlabeled ligands. Several methods have been employed for the separation of bound and free hormones and these have included ethanol–sodium chloride differential precipitation, chromatoelectrophoresis, and double-antibody precipitation using guinea pig antirabbit γ-globulin. From this brief description, it is apparent that the radioimmunoassay procedure in general has the capability of measuring every naturally occurring hormone or biochemical structure; with the widespread use of this technique, fluctuation in body levels of various components may be the first indicator of pathological changes.

Not only may normally occurring biochemicals be assayed but there is the feasibility of measuring concentrations of drugs, their metabolites, and other exogenous compounds as well in various body fluids. One of the present, more important uses of radioimmunoassay in monitoring effective drug therapy involves the use of digitalis glycosides (98). These drugs are prescribed in virtually all instances of heart failure, especially in the treatment of congestive heart disease and various arrhythmias. They increase the strength of contraction and thereby cardiac output. However, overdosage for the individual results in intoxication as shown by anorexia, nausea, vomiting, and diarrhea. These early symptoms lead ultimately to ventricular extrasystoles, hypokalemia, and cardiac conduction disturbances. The range between the effective dose and the toxic one is narrow and highly variable. In effect, what is essential is a determination of the correct dosage for the individual patient. Radioimmunoassay has become an important procedure in assisting the clinician to determine proper blood concentrations. The labeled ligand, in this case, depending upon the drug used, may be ^{3}H-labeled digoxin, ^{3}H-digitoxin, or ^{125}I-iodotyrosine methyl ester succinyl-3-*O*-digitoxigenin. The binding agent is rabbit antidigoxin antibody. This same antibody may be used for either digoxin or digitoxin in view of their structural commonality. A standard curve needs to be established for each drug with this same protein. Several techniques have been used for the separation of the free ligand from the protein-bound form of the drug. These include absorption chromatography with dextran-coated charcoal and double-antibody precipitation with goat antirabbit γ-globulin. By this procedure, the detection limit of these cardioactive drugs is in the nanogram range and even below. By a similar method, morphine, lysergide, and other exogenous compounds may also be assayed. From this broad range of drugs that can be measured, it is apparent that this procedure has great versatility.

THERAPEUTIC DRUGS

This second section relates to therapeutic radioactive drugs. Although of minor clinical importance by comparison, they do provide the radiotherapist with a useful tool for tissue destruction.

Therapeutic drugs may be divided into two separate categories: *(a)* drugs that are radioactive *per se* when administered, and *(b)* drugs that are nonradioactive but with *in situ* activation produce intense ionizing radiation. The major limitation to the use of therapeutic radiopharmaceuticals is that they must have a deleterious effect solely upon the uneconomic species to be destroyed. The corollary, of course, is

that normal tissues must be unaffected. To achieve total cellular destruction, doses on the order of 4000–5000 rads must be delivered to the target tissue and less than 1% of this dose may be tolerated by certain radiosensitive tissues without demonstrating alteration in function. This poses an extremely stringent requirement on the drug differentials between economic and uneconomic tissues.

Radioactive Therapeutic Agents—For the purpose of considering different radioactive therapeutic agents, these drugs can be divided into two categories: *(a)* those whose functions are to moderate and to modify abnormal metabolic function, and *(b)* those that will destroy malignant processes (Table VII). Since there are very limiting requirements for the drugs that can be used, both from a distributive pattern and the radiant energy emitted, first approaches involved the use of radionuclides demonstrating a frank biochemical basis for their specific localization.

Abnormal Tissue-Destroying Agents—The early use of ^{128}I-iodide ($T_{1/2}$ = 25 min) as an indicator of thyroid physiology foreshadowed the radiotherapy of the thyroid gland with radioactive nuclides of iodine. Initially, ^{130}I ($T_{1/2}$ = 12 hr) and then ^{131}I ($T_{1/2}$ = 8 days) were used. The latter nuclide supplanted ^{130}I as the isotope for the treatment of diffuse toxic goiter (Graves' disease). The normal dose administered is 5 ± 3 mCi, which deposits 8000 ± 2000 rads within the thyroid gland. More recently, it has been suggested that 1–2 mCi (2000 rads) may be adequate to moderate beneficially hyperthyroidism. The major undesirable long-term effect of radioiodide appears to be hypothyroidism and, in the use of ^{130}I, frank myxedema occurred in a significant number of patients.

The treatment of nodular goiters associated with hyperthyroidism (Plummer's disease) has been tried extensively. The response to ^{131}I is less satisfactory than that of diffuse goiter, and considerably larger doses (10–75 mCi) are required. Repeated doses are often needed, and worrisome nodules frequently persist. One important reason for the frequent therapeutic failure in toxic nodular goiter is the inability of the nodule to retain enough of the administered ^{131}I to accomplish the self-destruction.

Recently, considerable attention has been given to the possibility of treating thyrotoxicosis with ^{125}I in place of ^{131}I (99). The physical characteristics of ^{125}I theoretically allow one to deliver three to four times the dose to the area responsible for the hyperthyroidism (the colloid-follicle cell interphase) compared to the same amount of ^{131}I while reducing the dose to the cell nucleus. This reduction in dose to the cell nucleus could decrease the amount of hypothyroidism associated with the aftermath of radioiodine therapy. Initial clinical trials have been encouraging, but the relapse rate has been substantially higher than expected. The most promising results have been achieved by the administration of a mixed dose of ^{125}I and ^{131}I.

There are few contraindications to the use of ^{131}I in the therapy of hyperthyroidism. Most radiotherapists prefer not to give ^{131}I to patients under age 20 due to the potential danger of radiation-induced leukemia or carcinoma. Nevertheless, this is not a strict rule. However, pregnancy is considered to be the one absolute contraindicator to the use of radioiodine; the rapidly growing fetus is considered to be sensitive to small amounts of radiation. In addition to the increased possibility of congenital malformation, there is the fear of ablating the fetal thyroid. Since iodine is concentrated in breast milk and, therefore, can be ingested by the newborn, lactating women should not be given radioiodine therapy except in extenuating circumstances. Also, there is a close relationship between circulating thyroid hormones and coronary function; patients with recent myocardial infarction should not be given radioiodine therapy unless thyroid hormone levels are carefully monitored and offsetting drugs are given.

Table VII—Therapeutic Radiopharmaceuticals

Abnormal metabolism:
Hyperthyroidism (^{131}I and other iodine isotopes)
Polycythemia vera (^{32}P)
Malignant diseases:
Radioactive iodides (^{131}I and ^{125}I)
Radioactive colloids [^{198}Au, chromic phosphate (^{32}P), and ^{90}Y]
Implanted preparations (^{192}Ir and ^{182}Ta)

Radioactive Agents for Malignant Disease—A second major use of therapeutic radioactive drugs is in the management of malignant diseases (100). In view of the clinical usefulness of radioactive iodide in the treatment of hyperthyroidism, its utilization in the treatment of thyroid cancer would seem to be a normal extension. However, clinical utility has been quite limited. The more malignant and anaplastic the thyroid carcinoma is, the less ability it has to sequester iodide from the circulation. Therefore, the normal thyroid tissue receives the maximum radiation dose and not the malignancy. For this reason, radioiodine therapy for such tumors and their metastases has not achieved widespread clinical usefulness.

From this observation it is apparent that sufficient localization of the radioactive drug in the target tissue is essential for therapy. An example utilizing this requirement is the treatment of polycythemia vera, thrombocythemia vera, and myelogenous and lymphocytic leukemias by ^{32}P-labeled sodium phosphate. Initial dosages of 3–4 mCi have been used successfully. The basis for this therapy is the deposition of phosphate in bone marrow and the utilization of the emitted β-radiation to control the abnormal and unphysiological multiplication of formed elements of the hematopoietic system. This therapeutic maneuver has been shown to be of distinct value in the treatment of lympho- and myeloproliferative diseases.

Use of tumor-seeking carriers as a means of concentrating radionuclides in malignant cells has been tried for more effective localization of neoplasms, *e.g.*, ^{111}In-bleomycin (101). This same type of approach also has been considered for tumor therapy. The rationale is that the carrier structure has a proclivity for the neoplasm by reason of its chemical composition; incorporation of a radionuclide into the

compound may offer a means of preparing a tumor-seeking radioactive substance. This was the basis for the preparation of ^{3}H-labeled menadiol diphosphate[4]. This radiosensitizing compound was reported to achieve high selective concentrations in tumors, and tritium incorporation was considered as a means for selective eradication. Although this approach has not achieved general widespread use, the basic concept still remains attractive.

Another approach to therapy is the use of radioactive colloids as a means of delivering controlled amounts of radiation to discrete sites. The colloidal particle is selected for its insolubility and particle size and, consequently, it functions not as a pharmacological agent but as an inert internal radiation source. Ideally, the radiotherapeutic drug for these purposes should be a pure β-emitter whose energy would be totally absorbed within the tissue to be destroyed. The radiant energy of suitable β sources would not be confined to very limited areas as would be expected for α sources, nor would the energy be distributed over large areas, including normal structures, as would be expected for energetic γ-nuclides. Radiocolloids that have been prepared and used are ^{198}Au, ^{32}P-chromic phosphate, ^{90}Y, and ^{177}Lu. They have been used clinically largely for two purposes: *(a)* intracavitary irradiation, and *(b)* interstitial therapy. Intracavitary radiocolloid therapy has been used for the irradiation of pleural and peritoneal cavities where invasive tumorous processes have interfered with normal fluid absorption and secretion. This form of treatment is an accepted one for the palliation of such malignant effusions. It is to be recommended for patients who have failed to respond to chemotherapy or conventional radiation therapy. There is no specific tissue selectivity afforded by this procedure; that is, normal tissues in this cavity would likewise be irradiated.

Infusion of radiocolloids into tumors directly has also been used. This interstitial administration of radioisotopes offers the advantage of delivering high dosages of radiation to the neoplasm and relatively little radiation to normal structures. An important proviso for this selectivity is based upon the radiation characteristics of the nuclide; those that are weak γ-, β-, and α-emitters are certainly preferable. The key problem for useful therapy is the uniform distribution of this colloidal suspension throughout the neoplasm. Unfortunately, the tumor process is usually not discrete but is an infiltrating one; consequently, the intratumor distribution pattern can be extremely variable. Another important problem encountered with such radiocolloids is their possible transport to the reticuloendothelial system. In this way, damaging radiation may be delivered to normal tissues such as bone marrow.

In addition to the use of radiocolloids for malignant tissue destruction, external and implanted radioactive sources have been utilized. Since the concern here is with radiopharmaceuticals, only implanted sources will be considered. These may be subdivided into two categories: *(a)* removable implants, and *(b)* permanent implants. With the removable implants, the half-lives of the encapsulated radioactive sources are such that permanent insertion is impossible. The function of this implant is to deliver the desired calculated radiation dose to the specific area; once this has been achieved the source is removed. The key advantage of this technique is good control of the radiation dosage to the discrete site. However, dose distribution cannot be readily altered after the implant has once been made. These sources are used principally in the therapy of accessible tumors where long-term palliation and even a complete cure may be feasible. Intraoral lesions and superficial cancers of the skin, breast, and neck may be subjected to this form of therapy. The following radionuclides have been used for such implants: ^{60}Co ($T_{1/2}$ = 5.3 years), ^{137}Cs ($T_{1/2}$ = 30 years), ^{182}Ta ($T_{1/2}$ = 118 days), ^{192}Ir ($T_{1/2}$ = 74 days), ^{198}Au ($T_{1/2}$ = 2.7 days), and ^{226}Ra ($T_{1/2}$ = 1600 years).

Three of these nuclides (^{182}Ta, ^{192}Ir, and ^{198}Au) have also been used in permanent implants. Other radionuclides that have also been used for such purposes are ^{125}I ($T_{1/2}$ = 60 days), ^{131}Cs ($T_{1/2}$ = 9.7 days), ^{133}Xe ($T_{1/2}$ = 5.3 days), and ^{222}Rn ($T_{1/2}$ = 3.8 days). This approach generally makes use of shorter half-life nuclides. The advantage of this method vis-à-vis temporary implants is that the procedure is simple and safer with less trauma; moreover, unresectable, deep neoplasms may be treated in this way at a surgical exploration without the concern for prolonged destructive radiation being delivered to those vital normal structures that may be in close proximity.

Radioactive-Induced Therapeutic Agents—The major limitation presented by radioactive therapeutic drugs is the fact that there must be a high differential between the target tissue and normal tissues, especially those that are radiosensitive. The ability to induce ionizing radiation in a discrete target tissue possibly remote from radiosensitive structures and in a highly selective way is the basis for attempts to utilize neutron capture radiation as a tissue-destructive maneuver. Although this technique has not achieved clinical utility, it does have desirable potential. The rationale for this therapy rests upon the capability of certain nonradioactive nuclides to absorb thermal neutrons and then emit intense ionizing radiation which may be largely confined to specific loci. Thermal neutrons and these nuclides are relatively innocuous alone, but their interaction produces a nuclear reaction capable of destroying cells. The key requirement is that the absorbing isotopes have large cross-section capture values for such slow neutrons compared with those values for normal tissue constituents (Table VIII) (102). The transuranic nuclides shown are radioactive *per se* and therefore possess some of the main drawbacks inherent with the radioactive therapeutic drugs.

The major research effort in developing this procedure clinically has involved the use of boron compounds (103). The basis for this is that the particles

[4] Synkavite

Table VIII—Thermal Neutrons and Potential Absorbers

Cross-Section Values for Thermal Neutrons Tissue Constituents (values in barns)			
H	0.32	N	1.7
Na	0.45	P	0.15
K	2.0	O	0.001
Mg	0.07	S	0.5
Ca	0.42	Cl	32.5
C	0.0045	Fe	2.43
Potential Therapeutic Neutron Absorbers			
^{6}Li	930	^{157}Gd	200,000
^{10}B	3990	^{164}Dy	2620
^{113}Cd	19,500	^{199}Hg	2500
^{149}Sn	46,000	^{235}U	687
^{155}Eu	14,000	^{242}Am	8000

emitted upon the capture reaction are large by nuclear standards ($_2{}^4He$ and $_3{}^7Li$) and are, therefore, restricted to those cells and their immediate neighbors that contain the neutron absorber. This technique has been largely concerned with the destruction of malignancies, but it also has been considered as a means for pituitary ablation. The major factor that has prevented the use of neutron capture therapy as a viable cancer therapeutic procedure has been the inability to produce boron compounds that will be largely restricted to the neoplastic area and not be distributed to normal structures contiguous to it. Thus, the important problem with these potential therapeutic agents, as with the radioactive therapeutics, is the need for adequate selective localization in the cancerous tissue. However, the requirements are not as stringent as for the radioactive therapeutics since the ionization radiation is only produced when the two components interact. In this manner, for example, high concentrations of the nuclide in normal liver, kidney, or bone marrow do not preclude the use of this technique for the treatment of brain tumors. These other tissues will not be exposed to neutron levels that will impair their physiological functions by neutron capture. This two-component technique for producing ionization radiation is certainly attractive. When compounds are developed that do show preferential localization in neoplasms, this type of procedure will have clinical utility.

NEW DIRECTIONS IN RADIOPHARMACEUTICAL DEVELOPMENT

There are multifaceted approaches in the development of new radiopharmaceutical diagnostics. These stem from the fact that nuclides with widely divergent half-lives may now be readily prepared by generators, reactors, and cyclotrons. The problems of preparing and using ultrashort-lived radioactive isotopes have been met (Table IX), and their increasing use, especially in examining the dynamics of pulmonary and cardiac function, can be expected. Some agents that are clinically useful for organ imaging are presented in Table X.

Another main development will be the continuing effort to produce agents with a biochemical rationale for their specific localization in certain target tissues, whether they be malignant or have other abnormal features. The present availability of nuclides with

Table IX—Directions in Radiopharmaceutical Development

Ultrashort-lived nuclides:
^{11}C, ^{13}N, ^{15}O, ^{81m}Kr, and ^{144}Pr
Short-lived nuclides:
^{18}F, ^{68}Ga, and ^{99m}Tc
Intermediate-lived nuclides:
^{67}Ga, ^{97}Ru, ^{111}In, ^{131}Ba, ^{135m}Ba, ^{169}Yb, and ^{201}Tl
Therapeutic agents

suitable emission characteristics and desirable half-lives will permit the emphasis being placed upon the tissue-localizing moieties into which the isotopes may be incorporated. Frequently, the basis for the selection of these localizing structures is their chemotherapeutic use. However, in many instances, knowledge as to the degree of localization in a specific target tissue is unknown; even when this information is available, the time course for this concentration, especially compared with other adjacent and normal tissues, remains undetermined. This is readily understandable because such information may not be pertinent to the compound's therapeutic utility, but it certainly is germane if the compound is being used as a scanning agent. Utilization of the bleomycin antibiotics is an example of the types of agents to which radionuclides may be attached. These compounds are rapidly incorporated into tumor cells and apparently to a high extent. They have shown very desirable cancer chemotherapeutic effects *per se;* on this basis, the incorporation of ^{111}In and ^{99m}Tc into the antibiotic has produced agents with desirable scanning features. Thus, there needs to be an assessment of the distributive properties and time course for a particular compound prior to any consideration of radionuclide incorporation. With the facile methods available now for binding radioisotopes to various compounds, incorporation is no longer a problem. Much, however, remains to be done in the development of discrete target-oriented drugs to which these radionuclides may be attached.

A major advance will be attained once immunochemistry is refined to the point of complete purification of tumor and organ antibodies. Then radiopharmacy will have reached a new plateau. The ready incorporation of ^{123}I, ^{99m}Tc, or ^{111}In into such proteins may produce the ultimate in organ and tumor visualization. The basis for this view is that the incorporation of these nuclides can now be performed in such a manner as to prevent major conformational changes in the protein. Certainly this requirement is a *sine qua non* for the attainment of such highly selective *in vivo* radiopharmaceuticals. The integrity of such proteins and proof of their lack of conformational alteration can be readily assessed by their viability in antigen–antibody reactions and their failure to cross-react with similar structural proteins. From these comments, it is apparent that important advances in immunochemistry may be a necessary prelude to major improvements in the development of *in vivo* radiodiagnostics.

The increasing use of *in vitro* radiopharmaceuticals both for the measurement of hormones and

Table X—Some Clinically Useful Organ Imaging Agents

Organ	Radiopharmaceutical	Mechanism of Localization	Administered Dose, mCi	Comments
Brain	$^{99m}TcO_4^-$ (pertechnetate)	Simple diffusion	10–20	In universal use
	^{99m}Tc-tin-penthanil	Simple diffusion	10–20	Choroid plexus blocking with ClO_4^- not necessary
	^{197}Hg- or ^{203}Hg-chlormerodrin	Diffusion and protein binding	0.5–1.0	Increased tumor uptake over TcO_4^- offset by physical decay characteristics and dosimetry
	^{131}I-human serum albumin	Diffusion and pinocytosis	0.1–0.5	24–48-hr wait for diffusion into tumor
Cerebrospinal fluid	^{111}In-penthanil	Compartmental localization	0.5	Current "agent of choice"
	^{169}Yb-penthanil	Compartmental localization	1	In use when ^{111}In is not routinely available
	^{131}I-human serum albumin	Compartmental localization	0.1–0.2	Was first and only agent for many years; now being replaced by penthanil chelates
Salivary glands	$^{99m}TcO_4^-$	Active transport	10–15	
Thyroid	^{131}I	Active transport	0.2–0.5	Most widely used radionuclide for thyroid diagnosis
	^{123}I	Active transport	0.1–0.2	Dosimetry 50–100× less than ^{131}I but expensive and not generally available
	$^{99m}TcO_4^-$	Active transport	1–2	Useful in certain types of thyroid disease as an imaging agent
Lung	^{99m}Tc-macroaggregated albumin	Capillary blockade	2–4	Instant one-step commercial kits just introduced; an excellent agent
	^{99m}Tc-albumin microspheres	Capillary blockade	2–4	Particle-size range a big factor in favor of this product; expensive, moderately complicated preparation procedure
	^{99m}Tc-ferric hydroxide aggregates	Capillary blockade	2–4	Kits are simple and rapid to prepare; question concerning clearance of iron particles still lingers
	^{131}I-macroaggregated albumin	Capillary blockade	0.2–0.4	Was most widely used agent, but physical properties of ^{131}I are a detriment; will be totally replaced by a ^{99m}Tc agent
	^{113m}In-ferric hydroxide aggregates	Capillary blockade	1–3	First iron hydroxide aggregate used; not used much due to decline in the use of ^{113m}In
	^{133}Xe, ^{127}Xe	Compartmental localization	10–25	^{133}Xe widely used for ventilation studies; physical characteristics of ^{127}Xe are excellent but as yet it is not available
	$^{11}CO_2$, $^{15}CO_2$	Compartmental localization	0.1–1	Requires "on-site" cyclotron and positron camera for good imaging
Liver	^{99m}Tc-sulfur colloid	Phagocytosis	2–4	Most widely used liver scanning agent at present
	^{99m}Tc-stannous phytate	Phagocytosis	2–4	Newest agent; forms colloidal particles *in vivo*
	^{113m}In-gelatin colloid	Phagocytosis	1–3	Used extensively from 1967–1971; not used much now
	^{198}Au-colloid	Phagocytosis	0.2	Almost completely replaced by Tc-sulfur colloid
	^{131}I-microaggregates	Phagocytosis	0.4	Short $T_{1/2}$ in liver; never achieved widespread clinical use
	^{131}I-rose bengal	Active transport	0.4	Used for determining patency of biliary tree
Spleen	^{99m}Tc-sulfur colloid	Phagocytosis	2–4	Gives a liver–spleen scan
	^{113m}In-gelatin colloid	Phagocytosis	1–3	Not used much at present
	^{99m}Tc-labeled red blood cells	Cell sequestration	2–4	Just introduced; spleen specific agent
	^{51}Cr-labeled red blood cells	Cell sequestration	0.3	Standard spleen scanning agent; should be replaced shortly by Tc-labeled red blood cells
	^{197}Hg-hydroxy(2-hydroxypropyl)mercury-labeled red blood cells	Cell sequestration	0.3–0.5	Used clinically in the 1960's but dosimetry always a problem
Kidney	^{99m}Tc-tin-penthanil	Compartmental localization	1–10	Excellent imaging and GFR agent; rapidly leaves kidneys and requires camera
	^{99m}Tc-iron ascorbate	Compartmental localization and active transport	1–10	Good agent; binds to the tubules for several hours

Table X—(*Continued*)

Organ	Radiopharmaceutical	Mechanism of Localization	Administered Dose, mCi	Comments
	^{99m}Tc-Sn-tetracycline	Compartmental localization	1–10	New agent, not in general use; gives twice as much activity in kidneys
	^{99m}Tc-Sn-glucoheptanate	Compartmental localization	1–10	New agent, currently in clinical trials
	^{99m}Tc-Sn-casedein	Compartmental localization	1–10	Small clinical study showed some clinical utility
	^{131}I-hippurate	Compartmental and active transport	0.2–0.4	Most widely used function and imaging agent
	^{197}Hg-chlormerodrin	Compartmental localization and protein binding	0.1–0.3	Still used in many centers despite high dose to kidneys
	^{113m}In-iron ascorbate or penthanil	Compartmental localization and active transport	0.1–1	Has been replaced by the Tc-counterparts
Bone	^{99m}Tc-Sn-polyphosphate	Exchange diffusion	15	Excellent bone imaging agent when proper polyphosphate is used
	^{99m}Tc-Sn-pyrophosphate	Exchange diffusion	15	Good bone scanning agent; lower kidney and bladder activity
	^{99m}Tc-Sn-EHDP	Chemisorption	15	Organic compound; also very good for skeletal imaging
	^{18}F	Exchange diffusion	2–4	Excellent physiological properties, but high cost and physical decay characteristics of ^{18}F are distinct disadvantages
	^{87m}Sr, ^{85}Sr	Exchange diffusion	2, 0.2	Being rapidly replaced by one of the three Tc-labeled products
Adrenal	^{131}I-cholesterol	Compartmental localization and active transport	1–2	New agent; only agent available for adrenal imaging; far from ideal physiologically but nevertheless useful presently
Bone marrow	^{99m}Tc-sulfur colloid	Phagocytosis	6–8	Used clinically but of limited value
	^{113m}In-gelatin colloid	Phagocytosis	3–4	Not used much any more
	^{198}Au-colloid	Phagocytosis	1–2	Not used at all any more
	^{52}Fe-citrate	Active transport	0.1–0.3	Just available; a positron emitter
	^{111}In-chloride	Active transport	3	Recently introduced; has already achieved moderate utility
Blood pool (static)	^{99m}Tc-human serum albumin	Compartmental localization	2–4	Used for pericardial effusion and placentography
	^{113m}In-chloride	Compartmental localization	1–3	Preferred by many for placentography due to decrease in bladder activity
	^{99m}Tc-labeled red blood cells	Compartmental localization	2–4	Just introduced; main use appears to be application in isotope angiocardiography
	^{131}I-human serum albumin	Compartmental localization	0.1–0.3	Seldom used
	^{123}I-human serum albumin	Compartmental localization	1–2	Expensive and presently not available
	^{51}Cr-labeled red blood cells	Compartmental localization		Still used in certain clinics but rapidly being replaced by Tc or In agents
Myocardium	^{43}K-chloride	Active transport	1–2	Concentrates in normal myocardium; not in widespread use
	^{129}Cs-chloride	Active transport	0.5–1	Similar to ^{43}K
	^{201}Tl-nitrate	Active transport	1–2	Appears to have physiological and physical decay parameters superior to ^{43}K or ^{129}Cs
	^{13}N-ammonium	Active transport	10	Currently in clinical trials; short-lived positron emitter
	^{99m}Tc-tetracycline	Diffusion and protein binding	10–20	Currently in clinical trials; localizes in the infarct
Tumor	^{67}Ga-citrate		3	In general use but of limited utility
	^{111}In-chloride		3	Not quite as good as ^{67}Ga in animal and human models; forms ^{111}In-transferrin
	^{111}In-bleomycin		3	Newly introduced; not clear if it converts to the transferrin complex *in vivo*
	^{99m}Tc-bleomycin		5–10	Recent clinical trials look encouraging
	^{99m}Tc-tetracycline		10	In clinical trials; preliminary results are encouraging

(*continued*)

Table X—(*Continued*)

Organ	Radiopharmaceutical	Mechanism of Localization	Administered Dose, mCi	Comments
Pancreas	^{75}Se-methionine	Active transport	0.2–0.3	In general use for many years; not a very satisfactory agent; active search is on to find a replacement

other biochemicals and for the analysis of drug concentrations in body fluids can be readily predicted. The former will be used both in the prevention and early diagnosis of disease; the latter will be used in the correlation of disease amelioration with drug concentrations. The great differences and vagaries among patients and even for the individual depending upon physiological status would make it very desirable to determine drug concentrations on a continuing basis. Here, once again, the field of immunology is of direct importance since the ability of purifying antihapten antibodies will assist materially in the development and use of highly specific radioimmunoassay procedures.

The therapeutic area has an even greater dependence upon immunology for the development of therapeutic radionuclides. Radiopharmaceuticals for such purposes have certainly lagged and although this is readily understandable, the need is there and is of great importance. However, until discrete localizing agents are obtained, whether they are antibodies, antibiotics, or other structures, successful radiotherapy by *in vivo* radiopharmaceuticals will remain simply a desired goal. Expected advances in immunochemistry will certainly foreshadow important developments in the field of radiopharmaceuticals.

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ˣ To whom inquiries should be directed.

Influence of Hydrophilic–Lipophilic Balance Values of Surfactants on Ephedrine Absorption and Release from Emulsified Systems after Oral Administration to Dogs

GUEY-MEEI LIN, FARID SADIK ˣ, W. FRANKLIN GILMORE*, and JULIAN H. FINCHER

Abstract □ Ephedrine release and availability characteristics *in vivo* were investigated from liquid emulsion systems as they are affected by the hydrophilic-lipophilic nature of the emulsifiers present. Three control and five test groups were used; the latter were all emulsified systems made with surfactants having hydrophilic-lipophilic balance (HLB) values from 10 to 14. All experiments were performed under an acidic urine control, and all urine samples were assayed by GLC to detect the excretion of unchanged drug. The urinary excretion data were best described by the two-compartment model with a zero-order absorption process. Correlations of availabilities between *in vivo* and *in vitro* data were determined, where appropriate. Correlations of ephedrine present in urine *in vivo* with the total amount dialyzed *in vitro* were significant at each HLB value. Poor correlation of absorption rate with dialysis rate was noted at an HLB of 10.

Keyphrases □ Ephedrine—effect of hydrophilic-lipophilic balance values of surfactants on absorption and release from emulsified system, oral administration, dogs, *in vivo-in vitro* correlations □ Surfactants—influence of hydrophilic-lipophilic balance values on ephedrine absorption and release from emulsified systems, oral administration, dogs, *in vivo-in vitro* correlations □ Hydrophilic-lipophilic balance values, surfactants—influence on ephedrine absorption and release from emulsified systems, oral administration, dogs, *in vivo-in vitro* availabilities correlated □ Emulsions—influence of hydrophilic-lipophilic balance values of surfactants on ephedrine absorption and release from emulsified systems

In 1971, Fincher and Waggoner (1) studied the influence of hydrophilic-lipophilic balance (HLB) values of surfactants on ephedrine release rates from emulsified liquid systems. The release rate of ephedrine increased in direct proportion between HLB values of 10 and 14.9. Since this work was conducted *in vitro,* it was desirable to determine if similar results would be observed *in vivo.*

EXPERIMENTAL

The emulsifiers used were polysorbate 60[1] with an HLB value of 14.9 and sorbitan monostearate[2] with an HLB value of 4.7. The amount of the emulsifiers required to obtain the desired HLB values of 10, 11, 12, 13, and 14 was calculated by simple allegation. The drug ephedrine[3] was selected because it was used in previous *in vitro* studies (1). The experimental animals were male beagle dogs, weighing from 9.1 to 13.6 kg (20 to 30 lb), with an average age of 18 months.

Phase I: Preliminary Preparations—To ensure uniformity, all emulsion systems and controls were prepared on a weight basis, with the drug supplying 1%, the surfactant supplying 5%, and the mineral oil[4] and water each supplying 47%, according to the methods previously described (1). An acidic urine was induced and maintained in the dogs by administration of ammonium chloride. One capsule containing 1 g ammonium chloride was given orally every 4 hr on the day before the drug systems were given; one capsule was given 1 hr before administering the drug, and one capsule was given every 4 hr thereafter throughout each experiment to control the urine pH between 4 and 5, thus enhancing drug elimination (2).

Urine samples were collected by catheterization at 1, 2, 4, 8, 12, 24, 36, and 48 hr. The volume and the pH of each sample were

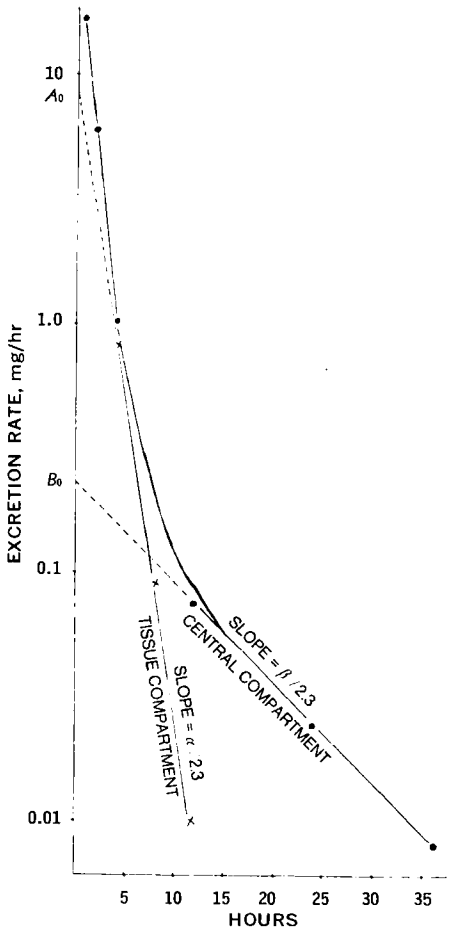

Figure 1—*Log milligrams ephedrine per hour* versus *time after intravenous injection of 20 mg ephedrine aqueous solution to dogs (eight dogs mean data). Curve is fitted by the following equation: excretion rate* $= 9.6e^{-0.58t} + 0.23e^{-0.092t}$.

[1] Tween 60, Atlas Chemical Corp., Wilmington, Del.
[2] Span 60, Atlas Chemical Corp., Wilmington, Del.
[3] Ephedrine NF (hydrous), Merck & Co., Rahway, N.J.
[4] Mineral oil, Sargent-Welch Scientific Co., Chicago, Ill.

Table I—Mean Cumulative Amounts of Ephedrine Excreted in Urine and Total Percent of Recovery from 0 to 48 hr[a]

Hours	Control, mg			Test, mg				
	I (8)	II (6)	III (6)	IV (6)	V (6)	VI (6)	VII (6)	VIII (6)
1	1.60	1.50	0.89	1.68	2.43	1.39	1.58	2.75
2	6.40	3.80	1.03	2.95	4.24	2.96	3.78	4.47
4	14.40	6.20	2.10	4.42	5.57	4.30	5.01	7.04
8	15.60	8.80	5.40	6.06	6.83	6.28	6.95	8.39
12	17.60	10.5	7.30	7.26	6.62	7.62	7.79	10.10
24	17.80	12.90	8.80	8.51	8.20	8.96	8.53	10.66
36	18.20	14.80	9.80	9.56	9.14	9.32	8.74	11.27
48	18.50	15.28	9.96	9.85	9.31	10.03	9.54	11.51
Percent of recovery, 0–48 hr	92.5	76.4	49.8	49.3	46.6	50.2	47.7	57.6

[a] Control: I = intravenous injection of 20 mg ephedrine in aqueous solution, II = oral administration of 20 mg ephedrine in aqueous solution, and III = oral administration of 20 mg ephedrine in oil solution. Test: oral administration of 20 mg ephedrine in an emulsified system at: IV, HLB of 10; V, HLB of 11; VI, HLB of 12; VII, HLB of 13; and VIII, HLB of 14. The number in parenthesis after control or test group indicates the number of dogs utilized in determining the mean.

measured, and the samples were stored at 4° until the time of the assay.

Phase II: Assay Method—The content of ephedrine in urine was determined using a modified GLC method previously reported (3). A gas chromatograph[5] equipped with a flame-ionization detector was used. The chromatographic column was aluminum tubing, 0.635 cm in diameter and 2 m in length. It was packed with 80–100-mesh Anakrom ABS coated with 5% 20M Carbowax. Prior to coating, the support was suspended in 1 *N* NaOH and then was collected by filtration. The column temperature was 180° and the injection temperature was about 200°. The nitrogen carrier flow rate was 36 ml/min.

Urine (10 ml) was added to 5 *N* HCl (0.5 ml) in a 45-ml centrifuge tube fitted with a glass cap. The mixture was extracted with 3 × 5 ml ether, and the ether layers were discarded. To the urine was then added 5 *N* NaOH (1.0 ml) and exactly 1 ml of a 0.1-mg/ml solution of internal standard (α-diethylpropion as the hydrochloride) in water. The mixture was extracted with 3 × 5 ml ether. The extracts were combined and the ether was evaporated under a nitrogen stream to about 1 ml. Approximately 0.5 μl of this solution was injected into the column. The amount of ephedrine was obtained by calculating the ratio of the peak area of ephedrine to that of the internal standard and relating this value to a relative calibration curve.

Blank urine samples of 10 ml each were spiked with 0.1, 0.25, 0.50, 1.0, 1.5, and 2.0 ml of a 1-mg/ml solution of pure ephedrine hydrochloride to give six sets of ephedrine solution of definite concentration. One milliliter of 1 mg/ml internal standard (α-diethylpropion hydrochloride) was added in each set. Then quantitative analyses of these six sets of samples were performed by the described assay procedure. From the chromatogram, the ratio of peak area of ephedrine to internal standard was measured. Then the relative calibration curve of ephedrine to internal standard was obtained. The area was estimated by multiplying the height times the width at half-height.

Phase III: Group Classification—Control groups were established to aid in the interpretation of the emulsion results.

Control I—This control consisted of the intravenous injection of 20 mg ephedrine in aqueous solution to dogs. The aqueous solution of ephedrine was prepared by dissolving 1 g ephedrine in 99 g water and heating to about 60° with stirring.

Control II—In this control, 20 mg of ephedrine in aqueous solu-

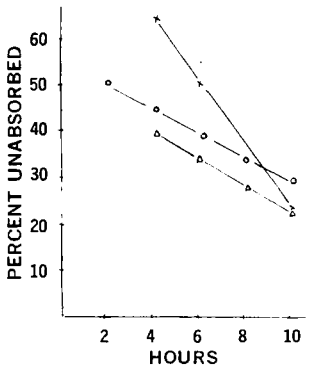

Figure 2—*Oral absorption of ephedrine from emulsion systems made with surfactant having an HLB value of 10. Key: Δ, ephedrine in emulsion system at HLB value of 10; ×, ephedrine in oil preparation; and ○, ephedrine in aqueous preparation.*

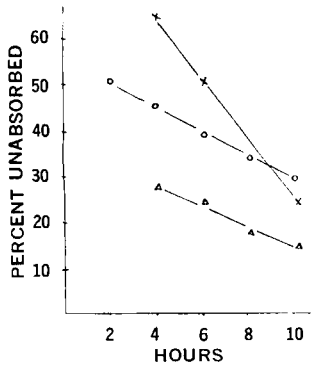

Figure 3—*Oral absorption of ephedrine from emulsion systems made with surfactant having an HLB value of 14. Key: Δ, ephedrine in emulsion system at HLB value of 14; ×, ephedrine in oil preparation; and ○, ephedrine in aqueous preparation.*

[5] Model 900, Perkin-Elmer Corp., Norwalk, Conn.

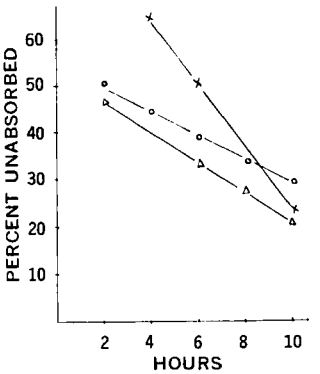

Figure 4—*Oral absorption of ephedrine from emulsion systems made with surfactant having an HLB value of 12. Key: Δ, ephedrine in emulsion system at HLB value of 12; ×, ephedrine in oil preparation; and ○, ephedrine in aqueous preparation.*

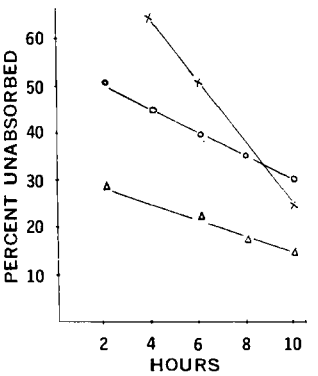

Figure 5—*Oral absorption of ephedrine from emulsion systems made with surfactant having an HLB value of 13. Key: Δ, ephedrine in emulsion system at HLB value of 13; ×, ephedrine in oil preparation; and ○, ephedrine in aqueous preparation.*

Table II—Estimated Parameters Fitting Two-Compartment Open Model at Different Doses to Dogs

Parameters[a]	Intravenous Ephedrine Injection		
	10 mg	20 mg	40 mg
K_d, hr^{-1}	0.4465	0.5159	0.4522
$K_{1,2}$, hr^{-1},	0.0450	0.0526	0.0453
$K_{2,1}$, hr^{-1}	0.1485	0.1034	0.1725
α	0.51	0.58	0.52
β	0.13	0.092	0.15
$T_{1/2}$, hr	5.3	7.5	4.6

[a] $K_d = \frac{\alpha\beta(A + B)}{A\beta + B\alpha}$

$K_{1,2} = \frac{AB(\beta-\alpha)^2}{(A + B)(A\beta + B\alpha)}$

$K_{2,1} = \frac{A\beta + B\alpha}{A + B}$

$t^1/_2 = 0.693/\beta$

$\alpha/2.3$ = slope of tissue compartment line

$\beta/2.3$ = slope of central compartment line

tion was administered orally to dogs. This aqueous solution of ephedrine was prepared by the same way as Control I.

Control III—Oral administration of 20 mg of ephedrine in oil solution to dogs was utilized. This oil solution of ephedrine was prepared by dissolving 1 g ephedrine in 99 g mineral oil and heating to about 60° with stirring.

Test Groups—There were five test groups; each dog separately and orally received 20 mg ephedrine in an emulsion made from the surfactants having HLB values of 10, 11, 12, 13, and 14, respectively. The emulsion was gently shaken by hand before it was administered.

Phase IV: Pharmacokinetic Study—Separately, single doses of 10, 20, and 40 mg ephedrine in aqueous solution were administered by intravenous injection to dogs. Acidic urine control, collection of urine, and assay of urine samples were described previously. This study was necessary to determine if the kinetic parameters (*e.g.*, volumes of distribution and rate constants) are changed with dose (4).

Phase V: Treatment of Data—The average urinary excretion rates with respect to time were plotted on semilogarithmic graph paper and then the best fit biexponential equation:

$$\text{excretion rate} = A_0e^{-\alpha t} + B_0e^{-\beta t} \qquad \text{(Eq.1)}$$

was obtained (Fig. 1). The value of α was calculated from the slope of the tissue compartment equilibration line obtained by subtracting the extrapolated portion of the terminal linear portion of the curve from the beginning nonlinear portion; $\alpha/2.3$ is equal to the slope. The value of β was calculated from the slope of the terminal linear portion of the central compartment line; $\beta/2.3$ is equal to the slope. The values of A_0 and B_0 were estimated from the Y intercepts of the tissue and central compartment lines, respectively.

RESULTS AND DISCUSSION

Kinetic Studies—Cumulative amounts of unchanged ephedrine excreted in urine and the total percentages of ephedrine recovery

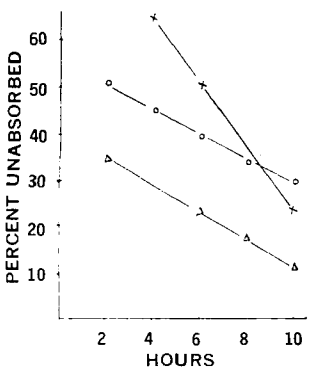

Figure 6—*Oral absorption of ephedrine from emulsion systems made with surfactant having an HLB value of 11. Key: △, ephedrine in emulsion system at HLB value of 11; ×, ephedrine in oil preparation; and ○, ephedrine in aqueous preparation.*

Table III—Ephedrine Absorption Rate Constants (in Dogs) and Dialysis Rate Constants

System[a]	Absorption Rate Constant ($-K_a$), hr^{-1}	Dialysis Rate Constant ($-m$), min^{-1}
1	2.60	0.279
2	6.80	0.101
3	2.90	0.055
4	2.30	0.077
5	3.20	0.089
6	1.90	0.061
7	2.90	0.116

[a] 1 = aqueous preparation system, and 2 = oil preparation system. The emulsified systems were: 3, HLB 10; 4, HLB 11; 5, HLB 12; 6, HLB 13; and 7, HLB 14.

from 0 to 48 hr are given in Table I. In the control groups, the aqueous intravenous injection (I) yielded the highest percentage of drug recovery, followed by the aqueous oral solution (II), and the oil oral solution (III) in order of decreasing yields. In five test groups, it was found that there was a stairwise fluctuation of percent of ephedrine recovery from 0 to 48 hr in the emulsified systems which had HLB values from 10 to 14. Higher percentages were obtained at HLB values of 10, 12, and 14, with the highest at 14. This indicated that the release of ephedrine from the emulsified systems was greatest when the emulsified system had an HLB value of 14. In no case did the percent drug recovery from the emulsified systems exceed that of Controls I and II which were aqueous solutions administered intravenously and orally. However, when the five test groups were compared to Control III (the oil solution administered orally), the emulsified systems having surfactant with HLB values of 12 and 14 yielded a higher percent recovery than that of Control III by 0.8 and 15.7%, respectively. The emulsified systems having emulsifiers with HLB values of 10, 11, and 13 were lower than the Control III by 1.0, 6.4, and 4.2%, respectively. These results are partially in agreement with the *in vitro* data (1) where the emulsions containing a surfactant with the high HLB values released the ephedrine faster than those of lower HLB values.

By utilizing the two-compartment model and excretion data after single intravenous doses, some kinetic parameters were estimated (Table II). The kinetic parameters were not dependent on dose, and the $t_{1/2}$ based on $0.693/\beta$ was in the range of 4.6–7.5 hr, which was near the value of 5.99 hr (5.01–7.46 hr) found using the two-compartment model and biexponential fitting (5).

By utilizing the two-compartment model and measuring unchanged drug excreted in the urine, the absorption rate constant, K_a, was estimated by a digital computer using Wagner's (4) equations:

$$f(A)_{tn} = 1/K_d(dAe/dt)_{tn} + (Ae)_{tn} + T_n \qquad \text{(Eq.2)}$$

where:

$$T_n = 1/K_d[(dAe/dt)_{tn-1}(K_{1,2}/K_{2,1})(1 - e^{-K_{2,1}\Delta t})] + 1/K_d[(dAe/dt)_{tn} - (dAe/dt)_{tn-1}]K_{1,2}\,\Delta_{t/2} + T_{n-1}e^{-K_{2,1}\Delta t} \qquad \text{(Eq.3)}$$

and K_d is the overall elimination rate constant, $K_{1,2}$ is the transfer rate constant going from the central to the tissue compartment, $K_{2,1}$ is the transfer rate constant going from the tissue to the central compartment, Ae is the cumulative amount of drug excreted in the urine at time t, f is the fraction of drug absorbed, A is the amount of drug absorbed, t is the time, Δt is the time interval, and n is a positive integer, *i.e.*, $n = 0, 1, 2 \ldots$. Then:

$$\text{percent absorbed} = \frac{(f)(A)_{tn}}{(f)(A)_{\max}} \times 100 \qquad \text{(Eq.4)}$$

The value of Eq. 2 progressively increases until it reaches a maximum or asymptotic value. When the values are expressed as percentage of this asymptotic value, the desired percents absorbed are obtained; *i.e.*, percent unabsorbed = 100% − percent absorbed.

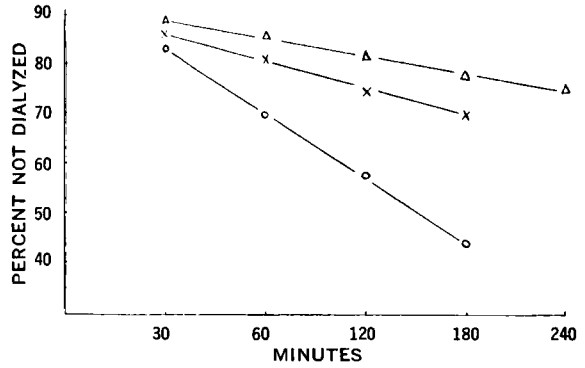

Figure 7—*Dialysis of ephedrine from emulsion systems made with surfactant having an HLB value of 12. Key: △, emulsion system at HLB 12; ×, oil preparation; and ○, aqueous preparation.*

By plotting the percent unabsorbed *versus* time on linear graph paper, a linear relationship (Figs. 2–6) was obtained and the zero-order absorption rate constant (K_a) was determined from the slope of the line. In a similar manner, to correlate these data with *in vitro* work (1), the dialysis rate constant, m, was determined by calculating the slope of the percent not dialyzed *versus* time plots (Figs. 7–11). Data for K_a and m are given in Table III.

All emulsified systems were unabsorbed at lesser percentages (more drug was absorbed) than that of the controls, both aqueous and oil systems. Similar treatment of the *in vitro* data (1) is given in Figs. 7–11. All emulsified systems were not dialyzed to the same extent as were the aqueous and oil controls, except the emulsified system having the surfactant with an HLB value of 14.

Correlations of *In Vivo* and *In Vitro* Studies—Correlation of the rate of availability for *in vivo* and *in vitro* studies at each HLB value of the emulsified system was obtained. There was poor correlation at HLB values of 11, 12, 13, and 14 (Fig. 12). The correlation coefficient was $r = 0.45$ and the regression coefficient was $b = 0.021$ for all HLB values. There was no correlation at an HLB value of 10.

Correlations of the amount of drug recovered between *in vivo* and *in vitro* studies are shown in Fig. 13. *In vivo*, the percent of total amount recovered was calculated from 0 to 48 hr using the intravenous injection control as a standard; *in vitro*, the percent of total amount dialyzed was determined from 0 to 240 min. The correlation coefficient was $r = 0.92$ and the regression coefficient was $b = 1.14$. It can be concluded from these correlations that there was poor correlation between the rate of dialysis and the rate of absorption at four HLB values studied, and no correlation was obtained at an HLB value of 10; there was good correlation between the extent of drug dialyzed and the extent of ephedrine recovered in the urine of dogs after oral administration at each HLB value.

In vitro work demonstrated that the ephedrine was dialyzed in two phases. There was an initial fast release pattern followed by a slower secondary release pattern. The slope of each percent not dialyzed *versus* time line was based on the secondary release phase, and this did not correlate well with the overall *in vivo* absorption rates obtained. The initial phase release of ephedrine from the emulsion systems was probably not observed *in vivo* because ephedrine is not absorbed from the stomach in the protonated form. The delay in absorption time, due to a mixing of the emulsion with stomach contents and stomach emptying rate, probably obliterated the potential effects of the fast initial release observed *in vitro* but not *in vivo*.

The extent of drug dialyzed did correlate well with the extent of drug recovered from the urine and at each HLB value. When consideration is given to the physiological system, the latter is a more valid correlation and demonstrates that *in vitro* work will

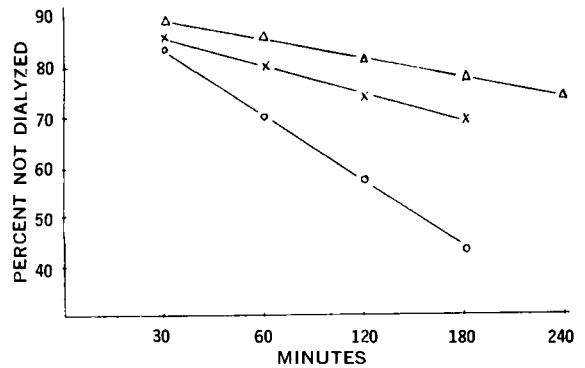

Figure 8—*Dialysis of ephedrine from emulsion systems made with surfactant having an HLB value of 10. Key: △, emulsion system at HLB 10; ×, oil preparation; and ○, aqueous preparation.*

Figure 9—*Dialysis of ephedrine from emulsion systems made with surfactant having an HLB value of 11. Key: △, emulsion system at HLB 11; ×, oil preparation; and ○, aqueous preparation.*

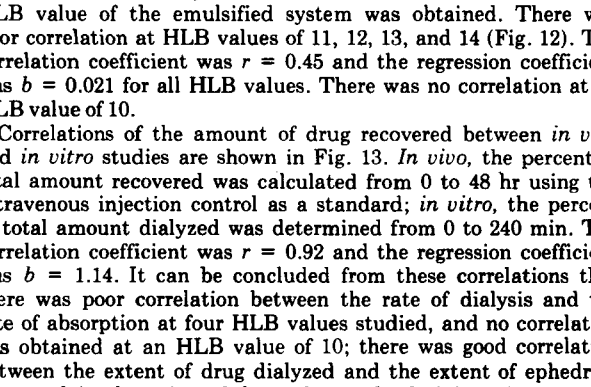

Figure 10—*Dialysis of ephedrine from emulsion systems made with surfactant having an HLB value of 13. Key: △, emulsion system at HLB 13; ×, oil preparation; and ○, aqueous preparation.*

Figure 11—*Dialysis of ephedrine from emulsion systems made with surfactant having an HLB value of 14. Key: △, emulsion system at HLB 14; ×, oil preparation; and ○, aqueous preparation.*

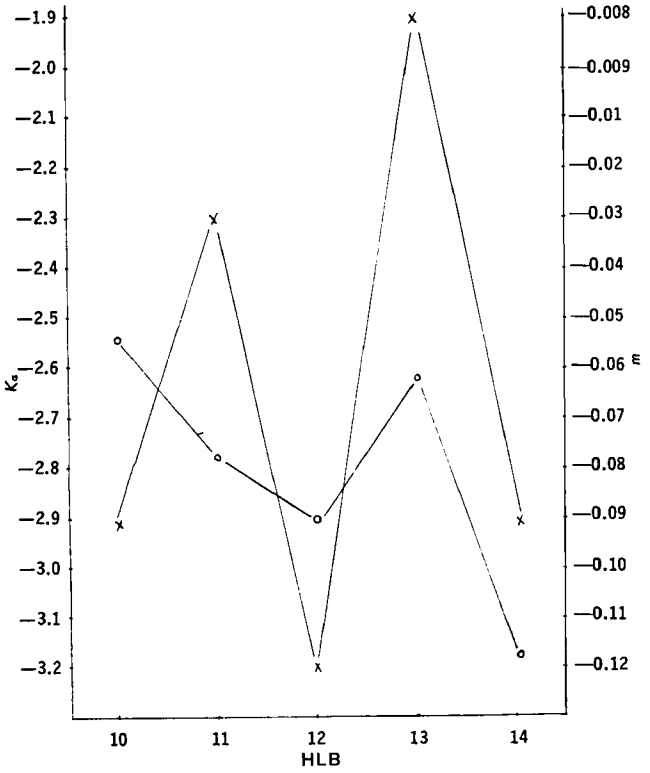

Figure 12—*Correlation of* in vivo *absorption rate constant* (K_a) *and* in vitro *dialysis rate constant* (m) *from the emulsion systems having HLB values from 10 to 14. Key:* ×, in vivo; *and* ○, in vitro.

correlate well with *in vivo* work if an appropriate method of correlation can be found.

CONCLUSIONS

1. The urinary excretion data obtained with ephedrine were adequately described by the two-compartment model with a zero-order absorption process.

2. The transfer rate constants, K_d, $K_{1,2}$, and $K_{2,1}$, for ephedrine in dogs were not dose dependent in the range tested.

3. The HLB value of the surfactant affected the absorption rate and the extent of ephedrine recovery from the orally administered mineral oil-water emulsified system.

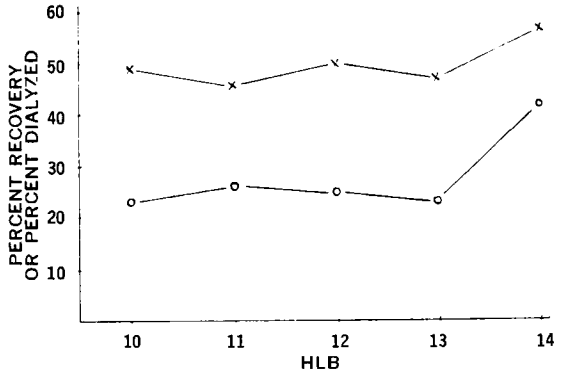

Figure 13—*Correlation of ephedrine recovery from urine and quantity dialyzed from 0 to 240 min. Key:* ×, *ephedrine recovery from urine; and* ○, *ephedrine quantity dialyzed from 0 to 240 min.*

4. *In vivo* correlations of the amount of ephedrine recovered from the urine (0–48 hr) with the total dialyzed *in vitro* (0–240 min) were significant at each HLB value.

5. Poor correlations of rates of availability with the rates of dialysis at all HLB values were noted. The correlations mentioned in Conclusion 4 are more valid considering the nature of the systems involved.

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* Present address: Department of Medicinal Chemistry, University of Mississippi, University, MS 38677

ˣ To whom inquiries should be directed.

Drug Permeation through Membranes III: Effect of pH and Various Substances on Permeation of Phenylbutazone through Everted Rat Intestine and Polydimethylsiloxane

EDWARD G. LOVERING ˣ and DONALD B. BLACK

Abstract □ The permeation rate of phenylbutazone through membranes of polydimethylsiloxane and everted rat intestine was measured over a range of pH values. The classical pH-partition hypothesis was not obeyed. Permeability coefficients were calculated using a modification of Higuchi's general equation relating pH and permeation rate. The effect of various substances on the permeability coefficient was determined. Phenylbutazone permeation rates in one or both membranes decrease in the presence of gelatin, methylcellulose, polyvinylpyrrolidone, skim milk, albumin, sodium lauryl sulfate, polysorbate 80, and cetrimonium bromide. Permeation rates through everted intestine were enhanced by bile salt concentrations below the CMC and by porcine mucin, while rates through polydimethylsiloxane were unaffected. Substances having no apparent effect were: lactose, starch, talc, kaolin, calcium chloride, acacia, calcium sulfate, sucrose, sodium saccharin, cholesterol, ferrous sulfate, and magnesium stearate.

Keyphrases □ Permeation—phenylbutazone through polydimethylsiloxane and everted rat intestine, effect of pH and formulation excipients □ Phenylbutazone—permeation through polydimethylsiloxane and everted rat intestine, effect of pH and formulation excipients □ Membranes, polydimethylsiloxane and everted rat intestine—permeation of phenylbutazone, effect of pH and formulation excipients on permeation rate and coefficient

It is well known that the choice of excipients and the manufacturing process can have a profound effect on the bioavailability of drugs in solid oral dosage forms. Formulation changes in established drugs may lead to important changes in their physiological behavior, sometimes with serious consequences, as shown by recent events concerning diphenylhydantoin (1) and digoxin (2). As far as it can be controlled by the drug manufacturer, bioavailability depends primarily on the dissolution characteristics of the formulation and on the possible interaction of excipients with the drug or the GI membranes.

Part of the bioavailability program being carried out in these laboratories concerns both dissolution behavior and the possible interaction of drugs with excipients and other materials. This paper deals with the effect of various excipients, nutrients, and endogenous materials on the permeation of phenylbutazone through polydimethylsiloxane and everted rat intestine. The work is aimed at detecting possible interactions which would have an adverse effect on bioavailability and is a continuation of the previous permeability studies of amobarbital (3).

EXPERIMENTAL

Permeation through Polydimethylsiloxane—Steady-state permeation rates were measured at 37° using cells similar to those described by Garrett and Chemburkar (4). The cells were equipped with polydimethylsiloxane membranes 0.014 cm thick. In use, they were filled with borate buffer, pH 10, and immersed in a beaker of phenylbutazone solution. The solutions both inside and outside the cell were stirred magnetically. The drug solutions were buffered with citrate or phosphate and ranged in concentration from 2×10^{-5} to 5×10^{-4} mole liter^{-1}. These conditions lead to steady-state permeation because the total amount of drug in the beaker is large compared to that permeating into the cell and the pH of 10 within the cell completely ionizes the drug, thereby preventing back-permeation. Excipients and other materials were added to the drug solution to form either a solution or a slurry. The phenylbutazone content of the desorbing borate buffer was measured by pumping the solution continuously through a spectrometer set at 265 nm and taking readings at appropriate time intervals. A Beer's law calibration curve was prepared from USP phenylbutazone reference standard, yielding a molar extinction coefficient of 21,050.

Everted Rat Intestine Permeation—Male Sprague–Dawley rats, weighing 180–250 g, were fasted 24 hr prior to the experiment, with water allowed as desired. The rats were sacrificed with ether, a method reported to prolong the structural integrity of the intestine (5). The entire small intestine was removed; after discarding the first 15 cm of the proximal end, two intestinal segments, 12 cm in length, were cut and everted on a glass rod according to the method of Crane and Wilson (6). Each everted intestinal segment (A in Fig. 1) was ligated to glass cannulas (B and C), mounted in an adjustable clamp (D) and extended until kink-free. The assembly was placed in a test tube (E) containing 50 ml of mucosal drug solution and a sintered-glass plug (F) through which a 95% oxygen–5% carbon dioxide stream was bubbled during the experiment. The entire apparatus was controlled at 37° in a bath (K). The desorbing serosal solution at pH 7.2 was placed in a 50-ml syringe (G) from which, after passing through a preheater (H), it entered the everted segment at point B. During operation, the drug was allowed to perfuse into the serosal fluid for 5 min. The sample was removed from the segment and collected at point J by pushing it through the segment with fresh serosal fluid from the syringe. A volume of 2.5 ml was sufficient to empty and rinse the segment.

The apparatus was used to measure permeation rates from mucosal solutions at different pH's and the ratio of drug permeation rates with and without an excipient or other material (7). This was done by measuring the perfusion rate of the drug for 50 min, after which the drug solution in the test tube (E) was replaced by a solution of drug and excipient and the rate measurements were continued. Two segments from each rat were run simultaneously. The order in which the mucosal solutions were presented to the intestinal segments was reversed in the case of the second segment. This allowed detection of nonreversible membrane changes brought on by substances added to the drug solution or by deterioration of the membrane. All solutions were prepared in isotonic sodium phosphate buffers (8). The 2.5-ml serosal samples taken at 5-min intervals were diluted to 5 ml and analyzed spectroscopically for phenylbutazone at 265 nm. Correction for background absorption was established by five experiments with mucosal buffer containing no drug. Compounds leached from the membrane give an average absorbance of 0.028 ± 0.004 for a 5-min period of contact with the membrane.

Materials—The following were used: phenylbutazone[1], dibasic calcium phosphate[2], calcium chloride[2], cholesterol[2], polysorbate

[1] Butazolidin, Ciba-Geigy.
[2] Fisher Scientific.

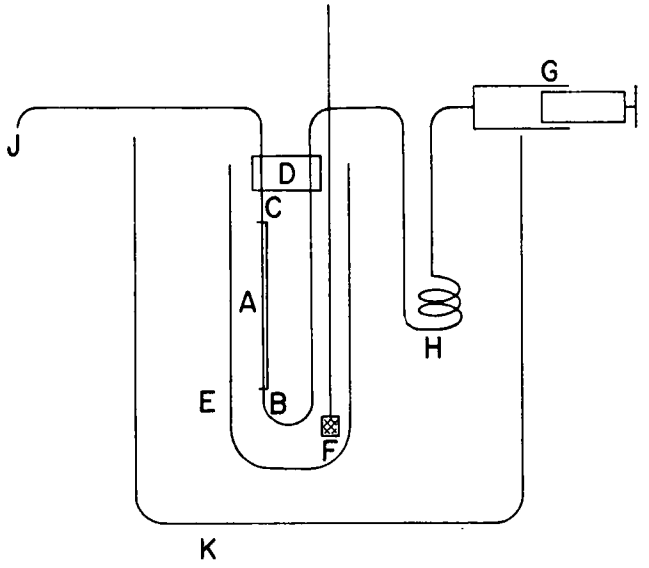

Figure 1—*Schematic diagram of the everted intestine permeation apparatus. Key: A, the everted segment; B and C, glass cannulas; D, clamp; E, 50-ml test tube; F, sintered-glass bubbler; G, syringe; H, mucosal solution preheater; J, sample collecting point; and K, thermostated bath.*

80[2], acacia USP[2], sodium lauryl sulfate USP[2], magnesium stearate USP[2], starch USP[2], ferrous sulfate[3], calcium sulfate dihydrate[3], bovine albumin[3] (fraction V), egg lecithin[3] (95–100%), polyvinylpyrrolidone[3], methylcellulose 400 cps[3], gelatin[3], sodium riboflavin-5′-phosphate dihydrate[3], talc[3] (fine powdered, acid purified), sodium taurocholate[3], sodium tauroglycocholate[3], sodium deoxycholate[3], cetrimonium bromide[3], porcine mucin[4], sodium cholate[4], lactose USP[5], skim milk (household), sodium glycocholate[6], egg albumin[7], silica[8], lecithin[9] (90% bovine), and activated charcoal[10]. The polydimethysiloxane membranes[11], 0.014 cm thick, contained a silica filler.

RESULTS AND DISCUSSION

Permeation through Polydimethylsiloxane—The permeability coefficient, P, is a measure of drug transfer from bulk solution on one side of the membrane to bulk solution on the other side. In a well-defined system, P is the product of the drug partition coefficient, K_p, and the diffusion coefficient, D_m, within the membrane. The polydimethylsiloxane membranes used here, however, contain silica filler which affects both K_p and D_m. Furthermore, $P = K_pD_m$ only if drug diffusion through the aqueous diffusion layers on either side of the membrane is not rate limiting. For these reasons, the P value reported here is not a characteristic of the phenylbutazone–polydimethylsiloxane system. It is valid only for the particular membrane and apparatus used in the experiments described in this paper.

Under steady-state conditions, P can be calculated from:

$$P = \frac{(dq/dt)l}{AC} \qquad \text{(Eq. 1)}$$

where dq/dt is the rate at which drug permeates the membrane under steady-state conditions; l and A are membrane thickness and surface area, respectively; and C is the concentration of unionized drug outside the permeation cell. C is relatively large and remains essentially unchanged throughout the experiment, while the unionized drug concentration within the diffusion cell is held at zero by suitable choice of pH. This choice of conditions permits steady-state diffusion.

Effect of pH on Permeation through Polydimethylsiloxane—The permeation rate of ionizable drugs is highly dependent on the pH of the drug solution. Before permeability coefficients obtained from experiments at different pH's can be calculated and compared, the relationship between pH and the coefficient must be known. The concentration of unionized drug was obtained from:

$$C = C_0[1 + \exp 2.303(\text{pH} - \text{pKa})]^{-1} \qquad \text{(Eq. 2)}$$

where C_0 is the total drug concentration and pKa = 4.5. Permeation rates were measured over the 2.30–8.04 pH range, and P was calculated from Eqs. 1 and 2. The results (Fig. 2) show that P, labeled the apparent permeability coefficient, is pH dependent contrary to the pH-partition principle (9) and previous experience with this technique (3). The pH dependence of P may be due to use of an incorrect pKa value or to more complex pH effects than are implied by the pH-partition principle. It is highly improbable that ionic permeation occurs in polydimethylsiloxane rubber or that its properties are pH dependent because of the very low solubility of ions in the membrane.

The pKa of phenylbutazone, determined by the spectroscopic method, is reported to be 4.5 (10). In addition, the solubility behavior of phenylbutazone calculated from this pKa (11) is in agreement with the experimentally determined solubility (12, 13). The spectroscopic determination of the pKa was repeated, giving substantially the same result as that already reported. In this study, therefore, the pKa was taken as 4.5.

According to some reports (14–16), the pH-partition theory is a special case of a more general theory of pH effects on drug permeation. From Eqs. 19, 35a, and 36 in the Suzuki *et al.* (14) paper, for an acidic drug:

$$\frac{dq}{dt} = -V\frac{dC_0}{dt} = \frac{AD_aC_0}{h}\left[\frac{1}{\left(1 + \frac{K_a}{[H^+]_m}\right)\frac{D_al}{hD_mK_p} + 1}\right] \qquad \text{(Eq. 3)}$$

where V is the volume of the bulk drug solution; D_a and D_m are the diffusion coefficients of the drug in water and in the membrane, respectively; h is the thickness of the aqueous diffusion layer; $[H^+]_m$ is the hydrogen-ion concentration at the surface of the membrane; K_p is the partition coefficient of the unionized drug between the membrane and the aqueous solution; and t is time.

Rearranging Eq. 3 yields:

$$P = D_mK_p = \frac{(dq/dt)l}{AC_0}\left\{[1 + \exp 2.303(\text{pH} - \text{pKa})] + \frac{1}{T}\right\} \qquad \text{(Eq. 4)}$$

where:

$$T = \frac{D_al}{K_phD_m} \qquad \text{(Eq. 5)}$$

In a well-buffered solution, the pH at the membrane surface would not differ substantially from that in the bulk of the solution. Hence, $[H^+]_m = [H^+]$. If T is large, the T^{-1} term in Eq. 4 becomes negligible and Eq. 4 reverts to the relationship predicted by the pH-partition hypothesis (Eqs. 1 and 2). The permeation of amobarbital and other drugs (3) was described by the pH-partition hypothesis. If T is not large, as was the case in this work, it cannot be ignored and Eq. 4 must be used to describe the effect of pH on the permeability coefficient. If T is taken as 0.063, permeability coefficients calculated from the data in Fig. 2 are independent of pH at the 95% level of confidence. On the basis of 29 experiments, $P = (4.88 \pm 0.35) \times 10^{-5}$ cm^2 sec^{-1}. The term T was estimated by writing Eq. 4 for two independent experiments, assuming P to be the same in each case, and solving for T. The fact that T^{-1} is not negligible indicates that the permeation rate is being influenced by the aqueous diffusion layer at the membrane surface.

Permeation through Everted Intestine—One difficulty encountered in permeation work in animal membranes is the large variability among individuals. Meaningful results are obtained only by repeating each experiment several times. For example,

[3] British Drug House.
[4] Sigma Chemical.
[5] Merck.
[6] K & K Laboratories.
[7] Miles.
[8] Cabosil M-5, Cabot Carbon.
[9] Nutritional Biochemicals.
[10] Darco G-60, Anachemia.
[11] Dow-Corning.

Table I—Effect of Excipients on Phenylbutazone Permeation

Excipient	Polydimethylsiloxane Membrane: Excipient Concentration, g liter^{-1}	Polydimethylsiloxane Membrane: Permeability Coefficient, P, (cm^2 sec^{-1}) × 10^5	Everted Rat Intestine: Excipient Concentration, g liter^{-1}	Everted Rat Intestine: Permeability Factor Ratio[a], P_f
Lactose	10.1	5.54	30	1.01, 0.97
	25.6	5.41	—	1.15
	53.0	5.33	—	—
Starch	4.5	5.18	30	0.98, 0.91
	22.6	5.26	—	—
Talc	1.9	5.24	—	—
	19.7	5.10	—	—
Kaolin	10.6	4.97	—	—
Magnesium stearate	2.1	5.40	10.6	0.92, 0.93
	16.4	4.75		—
Calcium chloride	0.6	4.94	—	—
	2.1	4.64	—	—
Acacia	2.8	4.82	—	—
	19.6	5.01	—	—
Fumed silica	0.5	4.62	—	—
	4.2	4.33	—	—
Gelatin	4.0	5.21	30	0.98, 0.91
	19.8	4.90	—	—
	40.0	3.67	30	1.04, 0.81
Methylcellulose	3.8	4.59	19.8	0.86, 0.74
	11.6	4.39	—	—
	18.9	3.52	—	—
Polyvinylpyrrolidone	5.5	3.72	19.1	0.58, 0.59
	12.5	3.36	—	—
	21.7	2.53	—	—
Calcium sulfate	10.1	5.10	18.7	1.01, 1.04
	40.2	4.58	—	—

[a] The permeability factor for drug from solutions of drug divided by the factor from solutions of drug and excipient.

Kaplan and Cotler (17), studying absorbability in a series of drugs, found it necessary to do from three to 41 runs, depending upon the drug, to obtain a satisfactory measurement of drug absorption. To reduce this source of variability, two rate measurements in the same intestinal segment were carried out and the ratio of the permeation rates was calculated (7). To determine the reproducibility of the ratio technique, two drug solutions, one half as concentrated as the other, were used. The rate of permeation of one solution was measured over an initial 50-min period followed by rate measurement of the second solution over the second 50 min. For eight segments the mean of the ratio was 0.51 ± 0.04. A ratio of 0.50 is predicted by Eq. 1. On the basis of these results, drug additive interactions were considered significant if the rate ratios were less than 0.90 or greater than 1.10. A ratio of unity indicates no interaction.

Effect of pH on Intestinal Permeation—The permeation of phenylbutazone through the everted rat intestine does not obey the classical pH-partition hypothesis, as is also the case with other drugs. Hogben *et al.* (18) attributed noncompliance with the pH-partition theory to the existence of an acidic zone at the lumen-epithelial border, with the acidity being maintained as long as the membrane cells live. Nogami and Matsuzawa (19, 20), on the other hand, attributed noncompliance to permeation of the intestine by drug ions. The concept of the pH static epithelial border was developed in connection with *in vivo* work using weak buffers and its applicability to *in vitro* work is questionable. Although the *in vitro* results of the present study can be interpreted on the basis of ion permeation, yielding a ratio of ionized to unionized permeation rates of about 0.48, this approach was not adopted because the results can be described more simply in terms of the general theory of pH effects of Suzuki *et al.* (14). This is not to say, however, that some permeation of ionized drug does not take place *in vitro*.

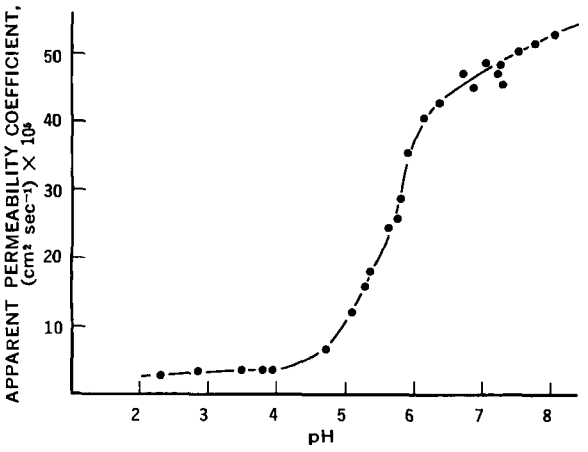

Figure 2—*Plot of apparent permeability coefficient,* P, versus *pH for phenylbutazone solutions.* P *was calculated from the pH-partition theory (Eqs. 1 and 2).*

The effect of varying pH over the 5.00–7.40 range on the permeation rate was measured in a number of intestinal segments. Two solutions were presented consecutively to the mucosal side of each segment; the solutions were usually of different drug concentration and pH, and the permeation rate was measured. Ratios of the rates were not calculated in the pH experiments. A permeability factor, P_f, defined as the permeability coefficient multiplied by the membrane area and divided by its thickness, was calculated from Eq. 4 by taking the area and the thickness as unity and $T = 1.26 \times 10^{-3}$. Other than cutting the segments to the same length for each experiment, no attempt was made to measure surface area or thickness of the intestinal segments. The factor T was found by the same procedure used for the polydimethylsiloxane membranes. Based on 22 experiments carried out on 11 intestinal segments, P_f was found to be 3.79 ± 1.00 cm^3 sec^{-1} with a coefficient of variation of 26%. Coefficients of this magnitude are typical of those observed in work with animal membranes. If ionic permeation can be neglected and if the pH at the membrane surface is the same as in the bulk solution, the value of T, small compared to that found for the polydimethylsiloxane cells, indicates that the permeation rate is controlled by the aqueous-mucus layer at the membrane surface.

Effect of Excipients—Excipients were dissolved or slurried in

Table II—Effect of Nutrients on Phenylbutazone Permeation

Nutrient	Polydimethylsiloxane Membrane: Nutrient Concentration, g liter^{-1}	Polydimethylsiloxane Membrane: Permeability Coefficient, P, (cm^2 sec^{-1}) × 10^5	Everted Rat Intestine: Nutrient Concentration, g liter^{-1}	Everted Rat Intestine: Permeability Factor Ratio[a], P_f
Sucrose	80.0	4.88	80.4	0.96, 0.99
Sodium saccharin	12.0	4.71	—	—
Skim milk powder	23.0	5.24	99.2	1.09, 0.76
	43.6	4.82	100.0	1.10, 0.83
	79.3	3.52	—	—
	100.0	2.70	—	—
Cholesterol	1.6	5.05	—	—
Egg lecithin	3.0	4.93	—	—
Egg albumin	0.5	4.70	—	—
Bovine albumin	2.1	3.43	5.0	0.74
	5.2	1.83	—	—
	8.0	1.14	—	—
Porcine mucin	3.5	4.79	19.8	1.39, 1.54
Ferrous sulfate			0.8	0.96, 0.96

[a] The permeability factor for drug from solutions of drug divided by the factor from solutions of drug and nutrient.

Table III—Effect of Surfactants on Phenylbutazone Permeation through Polydimethylsiloxane

Total Quantity of Drug, Q, (moles liter^{-1}) × 10^4	pH of Drug Solution	Weight of Surfactant, W_m, g liter^{-1}	Apparent Permeability Coefficient, P_a, (cm^2 sec^{-1}) × 10^5
	Sodium Lauryl Sulfate		
3.912	7.19	4.9	4.26
3.946	7.14	10.4	3.61
3.850	7.15	19.9	3.66
3.978	7.17	30.3	3.24
3.954	7.00	50.0	2.09
3.967	7.00	80.8	1.72
	Polysorbate 80		
4.017	7.26	2.1	4.60
4.011	7.28	11.7	3.04
4.297	7.27	25.5	2.03
3.935	7.13	33.4	1.27
	Cetrimonium Bromide		
4.497	7.30	0.104	4.21
3.746	7.35	0.200	2.51
4.284	7.29	0.297	1.72
3.863	7.28	0.477	0.78
3.978	7.29	0.903	0.00

phenylbutazone solutions, and the permeation rates through polydimethylsiloxane were measured (Table I). The permeability coefficients were compared to that of drug alone, (4.88 ± 0.35) × 10^{-5} cm^2 sec^{-1}, and any change greater than two standard deviations, *i.e.*, $p < \sim 0.05$, was considered to indicate a significant interaction between the drug and the excipient. The effect of excipient on the permeation rate through everted intestine was also evaluated. This was done by measuring the permeation rate of drug alone through a segment and then the permeation rate of the drug in the presence of the excipient, using the same segment. The results were expressed as the ratio of the permeability factor in the presence of excipient to the factor for drug alone (Table I). Ratios less than 0.90 were taken to indicate a significant interaction between drug and excipient.

The excipients examined are among those commonly present in Canadian formulations of phenylbutazone. No interaction was found between drug and lactose, starch, talc, kaolin, magnesium stearate, calcium chloride, acacia, fumed silica, or calcium sulfate. Gelatin in polydimethylsiloxane and methylcellulose and polyvinylpyrrolidone in both membranes interfere with permeation of the drug but only at concentrations much higher than would be encountered in an actual formulation. The permeability coefficient of phenylbutazone through polydimethylsiloxane is inversely proportional to the concentration of polyvinylpyrrolidone, with a correlation coefficient of −0.99. This relationship suggests a drug–excipient interaction rather than a viscosity effect, which would lead to a more complex dependence of the coefficient on the excipient concentration.

Effect of Nutrients—Permeation of drug in the presence of various nutrients was measured using polydimethylsiloxane, everted rat gut membranes, or both (Table II). Sucrose, sodium saccharin, cholesterol, ferrous sulfate, egg lecithin, and egg albumin were without effect at the concentrations examined. In the presence of dissolved skim milk powder, the permeability coefficient of phenylbutazone through polydimethylsiloxane was inversely proportional to the concentration of dissolved powder, with a correlation coefficient of −0.99. This indicates a physical interaction, possibly adsorption or binding, since a chemical reaction would probably go to completion in the presence of the large excess of milk powder. The results obtained with the intestinal segment are less clearcut, although it is evident that an interaction occurred.

Permeation of drug through both membranes is retarded if bovine albumin is present. Again the synthetic membrane results are inversely proportional to the albumin concentration (r = −0.98), suggesting a physical interaction such as adsorption or binding. Permeation of drug through polydimethylsiloxane was unaffected by porcine mucin, indicating that no appreciable interaction takes place. The permeation rate through the everted intestine was substantially increased, however, and must be the result of an interaction between the mucin and the surface or contents of the intestine rather than an effect involving the drug,

Table IV—Effect of Bile Salts on Phenylbutazone Permeation

Bile Salt	Polydimethylsiloxane Membrane: Bile Salt Concentration, g liter^{-1}	Polydimethylsiloxane Membrane: Apparent Permeability Coefficient, P_a, (cm^2 sec^{-1}) × 10^5	Everted Rat Intestine: Bile Salt, (moles liter^{-1}) × 10^2	Everted Rat Intestine: Rate Ratio: Drug[a] First	Everted Rat Intestine: Rate Ratio: Drug and Bile Salt First[b]
Sodium deoxycholate	0.52	4.93	—	—	—
	1.93	4.19	4.01	2.22	1.05
	4.0	4.48	—	—	—
	9.8	4.60	—	—	—
	16.4	4.08	—	—	—
	41.9	4.25	—	—	—
Sodium tauroglycocholate	19.7	3.30	—	—	—
	26.2	3.75	—	—	—
	50.5	3.35	—	—	—
	79.4	3.14	—	—	—
Sodium glycocholate	2.1	4.62	3.98	2.32	1.09
	9.2	4.37	—	—	—

[a] The permeation rate was measured for drug alone, after which the rate was measured for drug in solution with bile salt. The rate ratio is the former divided by the latter. [b] The permeation rate was measured for drug in solution with bile salt, after which the rate was measured for drug alone. The rate ratio is the latter divided by the former.

since mucin had no effect on the role of permeation through polydimethylsiloxane.

Effect of Surfactants—It was shown previously that the reduction in permeation rates observed in the presence of surfactants could be attributed to a reduction in the aqueous drug concentration occasioned by drug partition into surfactant micelles (3). This approach yields the relationship:

$$\frac{Q}{C_w W_w} = \left(\frac{K_p'}{\rho_m}\right)\frac{W_m}{W_w} + \frac{1}{\rho_w} \qquad \text{(Eq. 6)}$$

where Q is the total quantity of drug in the system; C_w is the quantity of drug in the aqueous phase; W_m and W_w are the weights of the micellar and water phases, respectively; and ρ_m and ρ_w are the respective densities. K_p' is the ratio of unionized drug in the micellar phase to that in the aqueous phase. From Eq. 6, K_p' can be determined from the slope of a plot of $Q/C_w W_w$ *versus* W_m/W_w. C_w was calculated from the expression:

$$C_w = \frac{P_a Q}{P} \qquad \text{(Eq. 7)}$$

where P_a is the experimentally determined, apparent permeability coefficient in the presence of the surfactant; P was previously shown to be (4.88 ± 0.35) × 10^{-5} cm^2 sec^{-1}. Equation 7 is based on the assumption that the decrease in P is due to the reduced concentration of free drug caused by partitioning into the micellar phase. The density of the surfactant was taken as unity, and it was assumed that water and surfactant volumes were additive upon mixing.

For permeation through polydimethylsiloxane, the data (Table III) yield a slope of 2.28 × 10^{-2} liter g^{-1} and an intercept of 0.99 × 10^{-3} liter g^{-1} (r = 0.98) for sodium lauryl sulfate. The slope and intercept obtained from experiments with polysorbate 80 were 8.39 × 10^{-2} liter g^{-1} and 0.72 × 10^{-3} liter g^{-1} (r = 0.96), respectively. The main effect of cetrimonium bromide is complexation or salt formation between the phenylbutazone anion and the surfactant cation, since very low concentrations of surfactant completely remove free drug from solution. It is not possible to calculate the partition coefficient from the cetrimonium bromide data, because the analysis does not distinguish between drug lost to micelles and to reaction. The partition of phenylbutazone between water and micelles of sodium lauryl surfate and polysorbate 80 yields partition coefficients, K_p', of 23 and 84, respectively.

In the rat intestine, cetrimonium bromide exhibits the drug interaction effect previously discussed. At low concentrations, the other surfactants discussed here are without apparent effect; at high concentrations, the membranes are attacked, becoming completely transparent.

Effect of Bile Salts—Bile salts are surfactants with a weak effect on the permeation of phenylbutazone through polydimethylsiloxane membranes (Table IV). Equation 6 was applied to sodium tauroglycocholate, yielding a slope of 3.9 × 10^{-3} liter g^{-1} and an intercept of 1.33 × 10^{-3} liter g^{-1} (r = 0.82). The partition coefficient was 3.9. This value, low compared to that of nonbiological surfactants, is a manifestation of the relatively poor solubility of phenylbutazone in micelles of these compounds. Changes in the permeability coefficient observed in studies of sodium deoxycholate and sodium glycocholate were not significant.

Permeation in everted rat intestine is appreciably enhanced by bile salts. The bile salt appears to interact with the membrane since the permeation rate through the segment is permanently increased after being exposed to bile salt at concentrations approximating those *in vivo*. The rate does not decrease when the solution of drug and bile salt is replaced by one containing the drug alone. Bile salts are observed to have similar effects on the permeation of salicylate (21), salicylamide (22), and riboflavin (23) through the everted rat intestine.

The apparent relationship (24) between bile salt concentration below the critical micelle concentration (CMC) and membrane permeability suggests that an equilibrium may exist between the concentration of bile salt in solution and that taken into the intestinal segments. Bile salt dissolved in the lipoidal cell wall membranes may be plasticizing the membranes, thereby enhancing their permeability. Plasticization results from the dissolution of low molecular weight compounds in high molecular weight materials. Physical properties, including permeability, undergo substantial changes. Insufficient evidence is currently available to determine whether the observed effect of bile salts on the membrane is plasticization. It is clear, however, that bile salts interact with membranes, making them more permeable. Bile salt molecules agglomerated into micelles do not appear to have much effect on the permeability of the membrane, although they undoubtedly play an important role in other aspects of the digestive process.

CONCLUSIONS

None of the excipients examined significantly affects the permeation of phenylbutazone when present in concentrations liable to be used in formulating the drug. Interactions with a number of other materials found in the GI tract occurred but, generally speaking, these materials cannot be avoided *in vivo*. The dependence of the permeability coefficient on T^{-1} (Eq. 4) is the first example of which the authors are aware involving a widely used drug in a synthetic rubber membrane, and it demonstrates the importance of the diffusion layer at the membrane surface. Further studies of the factors comprising T and their effect on the permeation of phenylbutazone are currently in progress. Comparable results were obtained from the experiments on polydimeth-

ylsiloxane and everted intestine only when materials not interacting with the membranes were under examination. If membrane interaction occurs, it is difficult to draw conclusions about possible interactions between the drug and the substance added to the drug solution.

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[x] To whom inquiries should be directed.

Surfactant Effects on Drug Absorption III: Effects of Sodium Glycocholate and Its Mixtures with Synthetic Surfactants on Absorption of Thiamine Disulfide Compounds in Rat

I. UTSUMI[x], K. KOHNO, and Y. TAKEUCHI

Abstract □ The effects of surfactants, such as sodium glycocholate and its related compounds or mixtures, on the absorption and metabolism of the model compounds, *O*-benzoylthiamine disulfide and thiamine disulfide were evaluated by both *in situ* and *in vivo* experiments using rats. The *in situ* results indicated that, in the presence of a biosurfactant, the increasing or decreasing effect of a synthetic surfactant on drug absorption and metabolism could be canceled by a possible formation of new mixed micelles consisting of the drug and both surfactants. Results were confirmed by oral experiments using the drug-micellar solution in rats.

Keyphrases □ Surfactants (sodium glycocholate, sodium lauryl sulfate, and polysorbate 80)—effects on absorption and metabolism of thiamine disulfide compounds, rats □ Drug absorption (thiamine disulfide compounds)—effects of biosurfactants and synthetic surfactants, rats □ Absorption (thiamine disulfide compounds)—effects of biosurfactants and synthetic surfactants, rats □ Thiamine disulfide compounds—effects of biosurfactants and synthetic surfactants on absorption and metabolism, rats □ Sodium glycocholate—effects on absorption and metabolism of thiamine disulfide compounds, rats

When a drug is entrapped into micelles, the thermodynamic activity (1) of the drug is decreased and the absorption is thereby suppressed while the stability of the drug is increased. However, previous studies (2) indicated that the reaction rates of the thiol–disulfide exchange reaction between thiamine disulfide compounds and thiols were influenced (acceleration or inhibition) by sodium lauryl sulfate, polysorbate 80, and sodium glycocholate, depending on the concentrations of surfactants, the lipophilic character of the disulfide, and the thiols used. These findings suggest that the thermodynamic activity of drugs is not always decreased by the solubilization or interaction with surfactants. Another study (3), which included kinetic treatment of the drug–surfactant interaction, indicated that *O*-benzoylthiamine disulfide (I) interacted with the lauryl sulfate anion to form a 1:2 molar complex and that this complex is

ylsiloxane and everted intestine only when materials not interacting with the membranes were under examination. If membrane interaction occurs, it is difficult to draw conclusions about possible interactions between the drug and the substance added to the drug solution.

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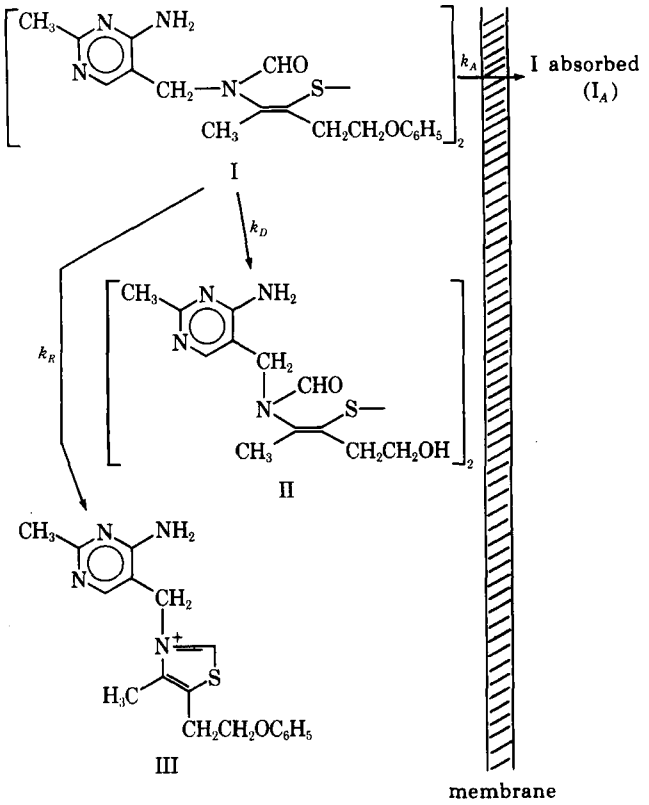
Abstract □ The effects of surfactants, such as sodium glycocholate and its related compounds or mixtures, on the absorption and metabolism of the model compounds, *O*-benzoylthiamine disulfide and thiamine disulfide were evaluated by both *in situ* and *in vivo* experiments using rats. The *in situ* results indicated that, in the presence of a biosurfactant, the increasing or decreasing effect of a synthetic surfactant on drug absorption and metabolism could be canceled by a possible formation of new mixed micelles consisting of the drug and both surfactants. Results were confirmed by oral experiments using the drug-micellar solution in rats.

Keyphrases □ Surfactants (sodium glycocholate, sodium lauryl sulfate, and polysorbate 80)—effects on absorption and metabolism of thiamine disulfide compounds, rats □ Drug absorption (thiamine disulfide compounds)—effects of biosurfactants and synthetic surfactants, rats □ Absorption (thiamine disulfide compounds)—effects of biosurfactants and synthetic surfactants, rats □ Thiamine disulfide compounds—effects of biosurfactants and synthetic surfactants on absorption and metabolism, rats □ Sodium glycocholate—effects on absorption and metabolism of thiamine disulfide compounds, rats

When a drug is entrapped into micelles, the thermodynamic activity (1) of the drug is decreased and the absorption is thereby suppressed while the stability of the drug is increased. However, previous studies (2) indicated that the reaction rates of the thiol–disulfide exchange reaction between thiamine disulfide compounds and thiols were influenced (acceleration or inhibition) by sodium lauryl sulfate, polysorbate 80, and sodium glycocholate, depending on the concentrations of surfactants, the lipophilic character of the disulfide, and the thiols used. These findings suggest that the thermodynamic activity of drugs is not always decreased by the solubilization or interaction with surfactants. Another study (3), which included kinetic treatment of the drug–surfactant interaction, indicated that *O*-benzoylthiamine disulfide (I) interacted with the lauryl sulfate anion to form a 1:2 molar complex and that this complex is



Scheme I

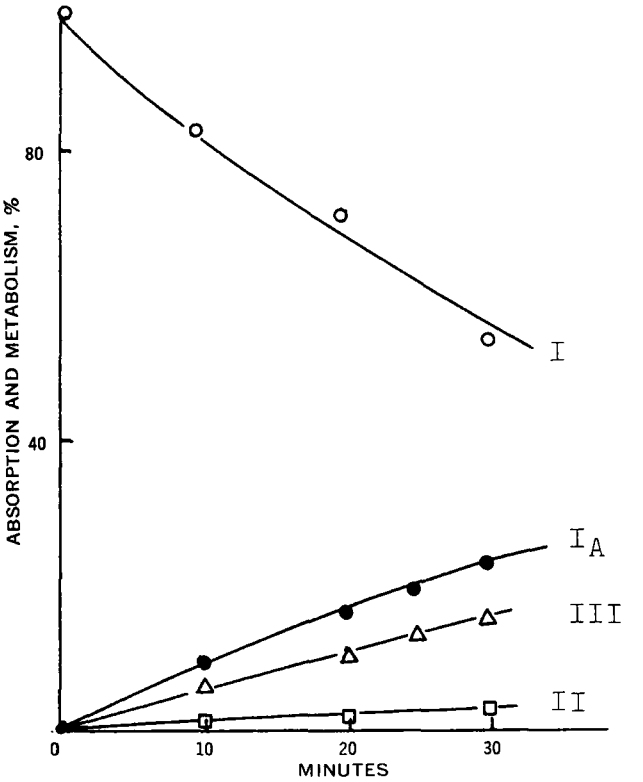


Figure 1—*Typical examples of the time course of absorption and metabolism of* O-*benzoylthiamine disulfide* (*I*).

broken up by the addition of sodium glycocholate, a biosurfactant, to produce new mixed micelles composed of the thiamine derivative and the surfactants.

On the basis of these observations, it was presumed that a biosurfactant could have a complicated effect, different from the effect of single species of the synthetic surfactant, on drug absorption and metabolism in the intestinal tract when synthetic surfactant-containing drug preparations were administered. To clarify this presumption, the effects of biosurfactants and synthetic surfactants on drug absorption were examined *in situ* and *in vivo* in rats. The results of the *in vitro* (2), *in situ*, and *in vivo* experiments also were compared to obtain a correlation of surfactant effects among the experiments.

EXPERIMENTAL

Materials—The drugs, *O*-benzoylthiamine disulfide (I) and thiamine disulfide (II), were the same preparations as in previous studies (2, 3). Sodium lauryl sulfate[1] was recrystallized from 85% acetone solution for use. Polysorbate 80[1] was used without purification. Sodium glycocholate, a chromatographically pure sample, was synthesized by the method introduced by Norman (4).

Measurements of Absorption and Metabolism—Apparent first-order rate constants of absorption and metabolism (hydrolysis and reduction) of the materials were determined by an *in situ* circulation method. The perfusate (pH 6.4) consisted of 10 μg/ml of I or II (equivalent to thiamine hydrochloride) and the isotonic phosphate buffer solution, with or without surfactant (2). The volume of the circulated solution (perfusate) was 50 ml. The small intestine of an anesthetized rat was ligated at the lower portion of the duodenum to prevent the bile fluid from entering the perfusate and at the upper portion of the ileocaecum by cannulation with a glass tube connected to a silicone tube. Both tubes were introduced into the reservoir flask containing the perfusate, thereby making a closed system through which the perfusate was circulated. The solution was kept at 37°; before circulation the intestinal tract was washed to expel the intestinal contents with 70 ml of the buffer solution free from thiamine compound and surfactant. The circulation through the intestine was done by a miniflow pump[2] at a speed of 5 ml/min. Constant agitation of the perfusate was accomplished by directing air into the reservoir flask. One milliliter of each sample was taken at a certain interval and immediately put into 4 ml of 25% KCl solution containing 0.1 *N* HCl to inhibit the enzymatic hydrolysis and reduction of I. The concentrations of I and its metabolites in each sample were assayed by means of the thiochrome and acylthiochrome methods (5). Phenol red was used as an indicator for the correction of the water absorption, since the total recovery of the phenol red added into the circulation fluid was not influenced by the surfactant after circulation for 30 min.

Measurement of Urinary Excretion of Thiamine—A rat was fixed on a board and anesthetized at the femoral area by a local anesthetic. A needle for subcutaneous injection, connected to a silicone tube (2φ), was introduced into the femoral vein and the animal was continuously infused with 0.9% NaCl solution (37°) at the speed of 0.067 ml/min using the miniflow pump. About 3–10 ml of urine was easily collected. The animals received 2 ml of a micellar solution of sodium lauryl sulfate, polysorbate 80, or sodium glycocholate containing I (100 μg/ml) by stomach tube. Excreted urine was then collected for 7 hr after administration, and the amount of thiamine excreted was determined.

Kinetic Model for Absorption and Metabolism—In the intestinal tract, I is absorbed in an intact form but is partially metabolized by two processes (Scheme I) (6). One process is a reduction, being considered nonenzymatic, to form *O*-benzoylthiamine (III); the other is an enzymatic hydrolysis of ester linkage (deacylation) to form the less absorbable II.

If it is assumed that the absorption and metabolism of I are

[1] Nikko Chemicals Co. Ltd.

[2] Sharp Co. Ltd.

Table I—Effects of Sodium Lauryl Sulfate on Rate Constants for Absorption and Metabolism of *O*-Benzoylthiamine Disulfide[a] (I)

System	Number of Rats	Rate Constants, hr^{-1}		
		k_A	k_R	k_D
Control[b]	3	0.40 ± 0.03	0.37 ± 0.05	0.68 ± 0.03
0.015% sodium lauryl sulfate	3	0.34 ± 0.08	0.79 ± 0.09	0.12 ± 0.12
0.1% sodium lauryl sulfate	3	0.18 ± 0.03	0.76 ± 0.14	0.10 ± 0.08

[a] I in perfusate: 10 μg/ml (50 ml), pH 6.4, 37°. [b] Without surfactant.

described as a first-order process and the absorption of the metabolites is negligible because of their low concentration in the circulation fluid, initial rate constants for the absorption and metabolism can be calculated. The disappearance rate of I from the *in situ* circulation fluid can be expressed by Eq. 1:

$$d[I]/dt = -(k_A + k_R + k_D)[I] \quad \text{(Eq. 1a)}$$

$$= -k[I] \quad \text{(Eq. 1b)}$$

where k_A, k_R, and k_D are the apparent first-order rate constants for absorption, reduction, and deacylation, respectively. Brackets show molar concentrations. The rates of absorption and metabolism are defined in Eqs. 2–4, from which Eqs. 5–7 are derived:

$$d[I_A]/dt = k_A[I] \quad \text{(Eq. 2)}$$

$$d[II]/dt = k_D[I] \quad \text{(Eq. 3)}$$

$$d[III]/dt = k_R[I] \quad \text{(Eq. 4)}$$

$$[I_A]/[II] = k_A/k_D \quad \text{(Eq. 5)}$$

$$[I_A]/[III] = k_A/k_R \quad \text{(Eq. 6)}$$

$$[III]/[II] = k_R/k_D \quad \text{(Eq. 7)}$$

where I_A refers to I absorbed in an intact form. According to Eqs. 5–7, the ratios of I_A and metabolites at time t can be replaced by the ratios of the rate constants. By combining these equations with Eq. 1, the rate constants were calculated. These were further corrected by fitting the analog computer-generated curves to the experimental data points at time intervals. The analog computer program for the kinetic model is shown in Scheme II.

Figure 1 shows a typical example of the data, in which it is observed that the theoretical line correlates well with the experimental data calculated.

RESULTS AND DISCUSSION

Effect of Sodium Lauryl Sulfate *(In Situ)*—Table I represents the effect of sodium lauryl sulfate, in concentrations below and above the critical micelle concentration (CMC), on the absorption and metabolism of I in the small intestine of rats. Sodium lauryl sulfate decreased the absorption rate constant with the increase of concentration in the perfusate. This retardation is considered due to the same mechanism that results in a suppression of the reaction rate between I and glutathione or cysteine in the solution of sodium lauryl sulfate *in vitro*, namely, the formation of a 1:2 complex of I with the lauryl sulfate anion in the circulation fluid (3). This consideration is also supported by the reported observation that the absorption of the entrapped drug into surfactant micelles is negligible (7, 8).

Sodium lauryl sulfate also decreased the rate constant for the enzymatic deacylation of I in concentrations below and above the CMC. This effect can be explained by the same idea of complexation as applied to retardation of the absorption rate. Another possible explanation of the suppression of hydrolysis is a denaturative inactivation of the intestinal esterase by sodium lauryl sulfate. In an experiment with an intestinal homogenate, however, practically no inactivation of the esterase activity was observed for a commonly used substrate, methyl butyrate, in the presence of sodium lauryl sulfate below the CMC under similar condition[3].

In contrast to the inhibition of absorption and hydrolysis, the reduction rates were found to increase about two times as much as the control value in the presence of sodium lauryl sulfate with its concentrations below and above the CMC (Table I). Such a reducing-accelerating action of sodium lauryl sulfate was also observed in the *in vitro* experiment, in which sodium lauryl sulfate accelerated the reaction between I and cysteamine (2).

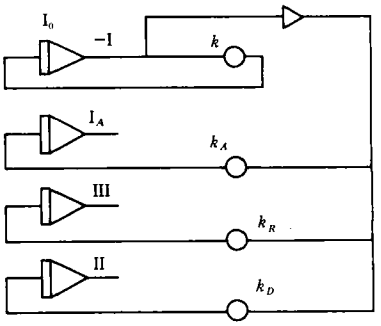
A reducing substance of low molecular weight was detected in the fluid that was circulated without I in the small intestine of rats using a gel filtration technique[3]. Thus, it may be feasible that the reductive cleavage of I in the intestinal tract *in situ* is carried out by a cysteamine-like substance.

Table II data show the effects of sodium lauryl sulfate on the absorption and metabolism of II. The intestinal behavior of II is expected to be affected to a lesser degree than I in the presence of sodium lauryl sulfate since it has a looser interaction with the lauryl sulfate anion (2). In fact, sodium lauryl sulfate produced little decrease on the rate constants for the absorption and reduction of II, consistent with the previous *in vitro* results (2).

From the observations, it is clear that sodium lauryl sulfate has similar characteristics in its effect on the absorption and the metabolism of I and II as observed in the *in vitro* experiment in the reactions of thiamine disulfide compounds with thiols (2, 3).

Effect of Sodium Glycocholate *(In Situ)*—Table III represents the rate constants for the absorption and metabolism of I in the circulation fluid with or without sodium glycocholate. Sodium glycocholate did not influence the absorption rate at the concentration of 0.015% (below the CMC) but raised it by approximately 50% at a higher concentration than 0.1%, where it formed micelles including some portion of I.

Based on these findings, it is suggested that sodium glycocho-



Scheme II—Analog computer program for kinetic model of absorption and metabolism of O*-benzoylthiamine disulfide (I)*

Table II—Effect of Sodium Lauryl Sulfate on Rate Constants for Absorption and Metabolism of Thiamine Disulfide[a] (II)

System	Number of Rats	Rate Constants, hr^{-1}	
		k_A	k_R
Control[b]	3	0.27 ± 0.01	0.25 ± 0.05
0.015% sodium lauryl sulfate	3	0.30 ± 0.07	0.26 ± 0.03
0.1% sodium lauryl sulfate	3	0.22 ± 0.04	0.25 ± 0.08

[a] II in perfusate: 10 μg/ml (50 ml), pH 6.4, 37°. [b] Without surfactant.

[3] Unpublished data.

Table III—Effect of Concentration of Sodium Glycocholate on Rate Constants for Absorption and Metabolism of *O*-Benzoylthiamine Disulfide[a] (I)

System	Number of Rats	Rate Constants, hr^{-1}		
		k_A	k_R	k_D
Control[b]	3	0.40 ± 0.03	0.37 ± 0.05	0.68 ± 0.03
0.015% sodium glycocholate	3	0.38 ± 0.05	0.36 ± 0.07	0.20 ± 0.06
0.1% sodium glycocholate	3	0.38 ± 0.02	0.32 ± 0.01	0.22 ± 0.04
0.17% sodium glycocholate	3	0.62 ± 0.04	0.36 ± 0.03	0.18 ± 0.02
0.25% sodium glycocholate	3	0.57 ± 0.06	0.40 ± 0.01	0.08 ± 0.07
0.34% sodium glycocholate	3	0.55 ± 0.02	0.32 ± 0.01	0.15 ± 0.03
0.5% sodium glycocholate	3	0.62 ± 0.07	0.23 ± 0.07	0.12 ± 0.02

[a] I in perfusate: 10 μg/ml (50 ml), pH 6.4, 37°. [b] Without surfactant.

Table IV—Effect of Polysorbate 80 on the Rate Constants for the Absorption and Metabolism of *O*-Benzoylthiamine Disulfide[a] (I)

System	Number of Rats	Rate Constants, hr^{-1}		
		k_A	k_R	k_D
Control[b]	3	0.40 ± 0.03	0.37 ± 0.05	0.68 ± 0.03
0.015% polysorbate 80	3	0.37 ± 0.05	0.32 ± 0.04	0.67 ± 0.05
0.5% polysorbate 80	3	0.17 ± 0.01	0.15 ± 0.05	0.26 ± 0.03

[a] I in perfusate: 10 μg/ml (50 ml), pH 6.4, 37°. [b] Without surfactant.

late, a main component of bile salts, does not decrease the absorption of I in concentrations above the CMC but, instead, increases the absorption. This enhancing effect above the CMC is quite the reverse of that observed with sodium lauryl sulfate. As reviewed by Gibaldi (9), it is known that bile acid salts alter membrane permeability toward certain drugs; therefore, the absorption-enhancing effect of sodium glycocholate is attributable to a membrane-altering action of sodium glycocholate.

Unabsorbed I underwent a lesser deacylation in the presence of sodium glycocholate than in the solution free from surfactant. Increased stability was also observed in the reduction process. The stabilizing action of sodium glycocholate possibly enhances the absorption of I because of a persistently high concentration of I at the absorption site.

It may be concluded that sodium glycocholate is an effective surfactant for increasing the absorption of I, enhancing the absorption rate at the same time as it suppresses hydrolysis.

Effect of Polysorbate 80 *(In Situ)*—The influence of polysorbate 80 on the absorption and metabolism of I is shown in Table IV.

Polysorbate 80 decreased the absorption rate constant at the 0.5% concentration (above the CMC) where the micelles are formed but did not decrease the rate constant at the 0.015% concentration (below the CMC). These results suggest that polysorbate 80 inhibits absorption by means of the uptake of I into micelles. Polysorbate 80 also decreased the rates of hydrolysis and reduction, but only at the 0.5% concentration. The inhibition is considered to result from the same factor suggested for the absorption-inhibiting effect. The immobilization was reported previously with other drugs (1, 7, 8, 10–12).

Effect of Binary Surfactant Mixtures *(In Situ)*—Because of the interesting effects of the mixture of sodium lauryl sulfate and sodium glycocholate on the reaction of I with glutathione noted in the previous *in vitro* experiment (3), the effect of the binary mixtures of sodium glycocholate with either sodium lauryl sulfate or polysorbate 80 on the intestinal behavior of I *in situ* was investigated.

Table V presents the values for the rate constants for the absorption and metabolism of I in solution with and without binary mixtures. The rate constant for absorption was 0.38 ± 0.03 hr^{-1} in the presence of 0.1% sodium lauryl sulfate and 0.5% sodium glycocholate, so there was no decrease as compared with the control value of 0.40 ± 0.03 hr^{-1}. Since sodium lauryl sulfate strongly decreases the absorption rate of I (Table I), it is apparent that sodium glycocholate reduces the absorption-inhibiting effect of sodium lauryl sulfate. This antagonistic effect was also observed in the reduction process, in which sodium glycocholate reduced the rate-increasing effect of sodium lauryl sulfate. On the other hand, sodium glycocholate did not influence the absorption-inhibiting action of polysorbate 80. The difference in the effect of sodium glycocholate on the absorption between the two binary systems suggests that the nonionic surfactant, polysorbate 80, differs from the ionic one, sodium lauryl sulfate, in its way of forming mixed micelles.

The detailed study on the absorption rate of I, which was determined to be a function of concentration of sodium glycocholate in a medium with 0.1% sodium lauryl sulfate, indicated that sodium glycocholate at a concentration above 0.25%, at which the molar ratio of sodium glycocholate to sodium lauryl sulfate was 1.5, was able to inhibit the action of sodium lauryl sulfate and thereby increase the decreased rate of absorption up to approximately the control level (Table VI). In an identical experiment (Table VI), sodium glycocholate reduced the increased reduction rate at the minimum concentration of 0.17% which was approximately the same as that observed for the absorption rate.

A similar antagonistic phenomenon was observed in the *in vitro* experiment (3) concerning the reaction of I with glutathione, in which sodium glycocholate reduced the rate-increasing effect of I. As predicted previously (3), the role of sodium glycocholate in binary surfactant mixtures is explained in terms of a possible formation of mixed micelles composed of I, sodium lauryl sulfate, and sodium glycocholate. Similarly, the absorption and reduction of I in the present investigation can be controlled by at least mixed micelles possibly increasing the mobility of I into the membrane surface.

***In Vivo* Studies**—The evaluation of the absorption of I by the rat was made by measuring the amount of urinary excretion of thiamine, which was ascribed to administered I. Urinary excretion data are shown in Table VII. When a control solution containing 200 μg of I was administered, 18.1 ± 1.8% of the dose was recovered from the urine during 7 hr after administration. When I was given in the micellar solution of 0.1% sodium lauryl sulfate, 30.4 ± 7.7% was recovered. It was evident that the absorption increased about two times as much as that in the control solution. Thus, sodium lauryl sulfate apparently had an enhancing action on the absorption of I *in vivo*, whereas it decreased the rate of absorption in the circulation experiment *in situ*. The reversed situation suggests that the effect of sodium lauryl sulfate *in vivo* is ex-

Table V—Effect of the Binary Surfactant Mixtures on the Rate Constants for the Absorption and Metabolism of *O*-Benzoylthiamine Disulfide[a] (I)

System	Number of Rats	Rate Constants, hr^{-1}		
		k_A	k_R	k_D
Control[b]	3	0.40 ± 0.03	0.37 ± 0.05	0.68 ± 0.03
0.1% sodium lauryl sulfate–0.5% sodium glycocholate	3	0.38 ± 0.07	0.30 ± 0.07	0.04 ± 0.08
0.5% polysorbate 80–0.5% sodium glycocholate	3	0.20 ± 0.02	0.13 ± 0.04	0.06 ± 0.01

[a] I in perfusate: 10 μg/ml (50 ml), pH 6.4, 37°. [b] Without surfactant.

Table VI—Effect of Concentration of Sodium Glycocholate on the Absorption of *O*-Benzoylthiamine Disulfide (I) from Mixtures of Sodium Lauryl Sulfate and Sodium Glycocholate[a]

System	Number of Rats	Rate Constants, hr^{-1}	
		k_A	k_R
Control[b]	3	0.40 ± 0.03	0.37 ± 0.05
0.1% sodium lauryl sulfate	3	0.18 ± 0.03	0.76 ± 0.14
0.1% sodium lauryl sulfate–0.1% sodium glycocholate	3	0.22 ± 0.03	0.50 ± 0.07
0.1% sodium lauryl sulfate–0.17% sodium glycocholate	3	0.23 ± 0.01	0.39 ± 0.03
0.1% sodium lauryl sulfate–0.25% sodium glycocholate	3	0.37 ± 0.05	0.36 ± 0.06
0.1% sodium lauryl sulfate–0.34% sodium glycocholate	3	0.43 ± 0.07	0.39 ± 0.03
0.1% sodium lauryl sulfate–0.5% sodium glycocholate	3	0.38 ± 0.07	0.38 ± 0.07

[a] I in perfusate: 10 μg/ml, pH 6.4, 37°. [b] Without surfactant.

Table VII—Urinary Excretion of Thiamine after Administration of *O*-Benzoylthiamine Disulfide in Micellar Solutions[a]

Micellar Solution	Urinary Excretion of Thiamine, %
Control[b]	18.1 ± 1.8
0.1% sodium lauryl sulfate	30.4 ± 7.7
0.5% polysorbate 80	14.4 ± 3.5
0.5% sodium glycocholate	24.7 ± 4.2

[a] Dose: 200 μg/2 ml (pH 6.4). [b] Without surfactant.

plained by at least two mechanisms. These may both be inferred from the *in situ* results of the surfactant effect on the absorption of I. First, there may be a breakdown of the micellar complex between I and the lauryl sulfate anion, formation of which is the cause of the decrease in the absorbability of I, due to the occurrence of a biosurfactant such as bile salts in the rat intestine. A possible biosurfactant effect, *i.e.*, canceling the action of sodium lauryl sulfate, is demonstrated by the *in situ* experiment (Tables V and VI). Second, in the intestinal tract *in vivo*, the biosurfactant as well as the synthetic one may act to protect I from its enzymatic deacylation and may increase the absorption. Unlike the case of sodium lauryl sulfate, polysorbate 80 decreased the absorbability of I *in vivo*, and this is consistent with the result observed in the *in situ* experiment.

When I was given in the micellar solution of 0.5% sodium glycocholate, the urinary excretion of thiamine was 24.7 ± 4.2% and little increase in absorbability was observed. Since sodium glycocholate showed an increasing effect on the absorption of I *in situ*, the *in vivo* effect is acceptable.

SUMMARY AND CONCLUSION

The role of biosurfactants and synthetic surfactants in the absorption of thiamine derivatives was investigated by both *in situ* and *in vivo* experiments using rats.

In the *in situ* experiment, sodium lauryl sulfate at concentrations above and below the CMC decreased the rates of absorption and hydrolysis of I but accelerated the rate of the reduction. Polysorbate 80 decreased all of these rates. In contrast to these two synthetic surfactants, sodium glycocholate produced a considerable increase in the absorption rate of I. The addition of a 0.1% sodium lauryl sulfate–0.5% sodium glycocholate mixture did not influence the absorption rate constant of I, indicating that sodium glycocholate inhibited the absorption-inhibiting effect of sodium lauryl sulfate. This antagonistic effect was not observable in the mixed system of 0.5% polysorbate 80 and 0.5% sodium glycocholate.

In the *in vivo* studies, sodium lauryl sulfate in 0.1% micellar solution showed an increase in the absorbability of I about twice that of the solution without surfactant. Such an increase was the reverse of the *in situ* effect of sodium lauryl sulfate on the absorption of I. It follows that endogenous bile salts in the intestine participate in the formation of mixed micelles including I and sodium lauryl sulfate. This kind of mechanism is acceptable according to the *in situ* results of the absorption and metabolism of I in binary mixtures of sodium lauryl sulfate and sodium glycocholate and in consideration of the stabilizing action of their mixtures on the hydrolysis of I. Sodium glycocholate in 0.5% micellar solution showed the enhancing action on the absorption of I *in vivo*. Administration of I in micellar solution of 0.5% polysorbate 80 resulted in lowered absorption, and this finding agreed with the results of the *in situ* experiment.

The observations of the effects of sodium lauryl sulfate, sodium glycocholate, and polysorbate 80 on the absorption of I indicate that special biosurfactant effects should be considered when evaluating the individual effect of the surfactant administered with the drug.

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Antibacterial Efficiency of Mercurials

A. E. ELKHOULY and R. T. YOUSEF[x]

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Keyphrases □ Mercurial compounds—antibacterial efficiency against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, effect of sodium thioglycolate in nutrient medium, role of sulfhydryl group □ Antibacterial efficiency—five mercurial compounds tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, effect of sodium thioglycolate in nutrient medium □ Thioglycolate (sodium)—effect on antibacterial efficiency of five mercurial compounds

The antibacterial action of mercurial compounds has been widely reported (1–7). Their mode of action was due to interference with essential metabolites and enzymes having sulfhydryl groups (8–11) or to reaction with the DNA (12) and RNA (13) of the cell. However, the antibacterial activity of these agents was found to be influenced by: *(a)* the chemical structure of the compound (14), *(b)* the type of test organism used [Gram-positive bacteria were reported to be more sensitive to mercurials than were Gram-negative ones (15)], and *(c)* the type of the quenching agent used to stop their effect, *e.g.*, sodium thioglycolate was a more efficient antagonizing agent than glutathione (11). Furthermore, previous work (16) with phenylmercuric nitrate showed that its antibacterial activity was greatly reduced by sodium thioglycolate when used in the diluent and at an optimum concentration. In this same study, thioglycolate was involved in the recovery of the damaged bacteria previously exposed to the effect of the mercurial.

Table I—Minimum Inhibitory Concentrations (MIC) of Different Mercurials against *Staph. aureus* and in the Presence of Sodium Thioglycolate

Mercurial Compound	Thioglycolate Concentration 0% MIC[a]	0.001% MIC	0.001% Hg to —SH[b]	0.003% MIC	0.003% Hg to —SH
Mercuric chloride	8.0	15	1:3	30	1:2.5
Thimerosal	1.0	4	1:12	7	1:14
Phenylmercuric acetate	0.5	4	1:8	7	1:13
Phenylmercuric borate	0.5	4	1:8	7	1:13
Phenylmercuric nitrate	0.5	4	1:8	7	1:13

[a] Micrograms per milliliter, average of six replicates. [b] Ratio of inactivated mercury molecules to thioglycolate molecules as given by: inactivated mercury molecules = (MIC of mercurial with thioglycolate − MIC without thioglycolate)/molecular weight of mercury compound.

In the present investigation, five commonly used mercurial compounds, one of which is inorganic, were tested for their antibacterial efficiencies on *Staphylococcus aureus* and *Pseudomonas aeruginosa*, using sodium thioglycolate solution at different concentrations as the antagonizing agent. The purposes of this investigation were to study the effect of thioglycolate concentration on the antibacterial activity of mercurial compounds and to evaluate the antibacterial efficiency of these agents at the thioglycolate concentrations tested.

EXPERIMENTAL

Reagents—The following were used: mercuric chloride[1], phenylmercuric acetate[2], phenylmercuric nitrate[2], thimerosal[2], phenylmercuric borate[3], and thioglycollic acid[2] [neutralized aseptically with 1 *N* NaOH to prepare the stock sodium thioglycolate solution (16)].

Organisms—*Staphylococcus aureus* (NCTC 4163) and *Pseudomonas aeruginosa* (NCTC 7244) were used.

[1] Analar, British Drug Houses.
[2] Laboratory reagent grade, British Drug Houses.
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EXPERIMENTAL

Reagents—The following were used: mercuric chloride[1], phenylmercuric acetate[2], phenylmercuric nitrate[2], thimerosal[2], phenylmercuric borate[3], and thioglycollic acid[2] [neutralized aseptically with 1 *N* NaOH to prepare the stock sodium thioglycolate solution (16)].

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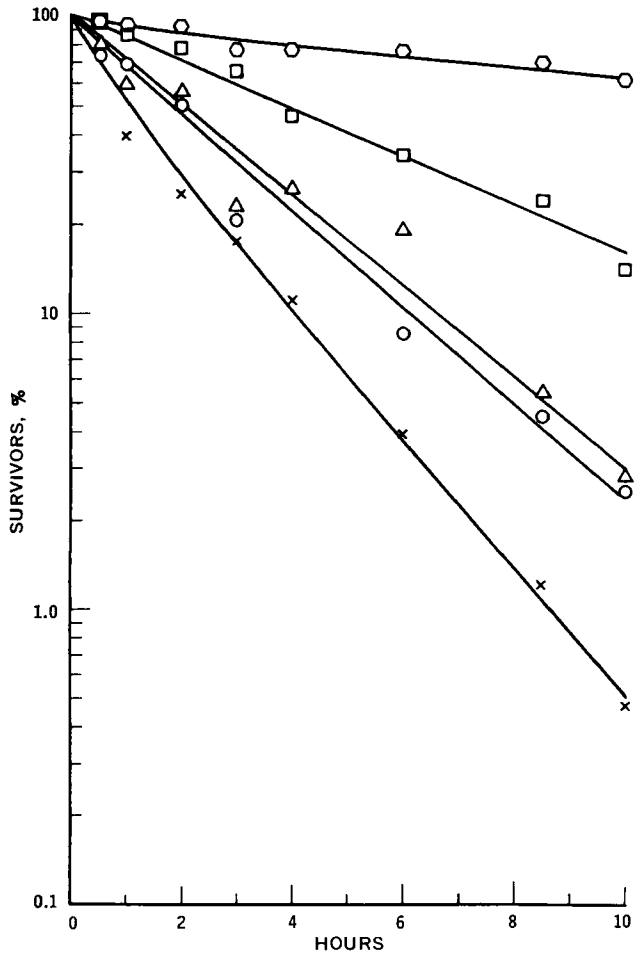

Figure 1—*Logarithm survivors–time curves of* Staph. aureus *in different solutions of mercurials when using sodium thioglycolate at 0.4%. Key:* ×, *mercuric chloride, 0.002%;* ○, *thimerosal, 0.5%;* ○, *phenylmercuric acetate, 0.02%;* □, *phenylmercuric borate, 0.02%; and* △, *phenylmercuric nitrate, 0.02%.*

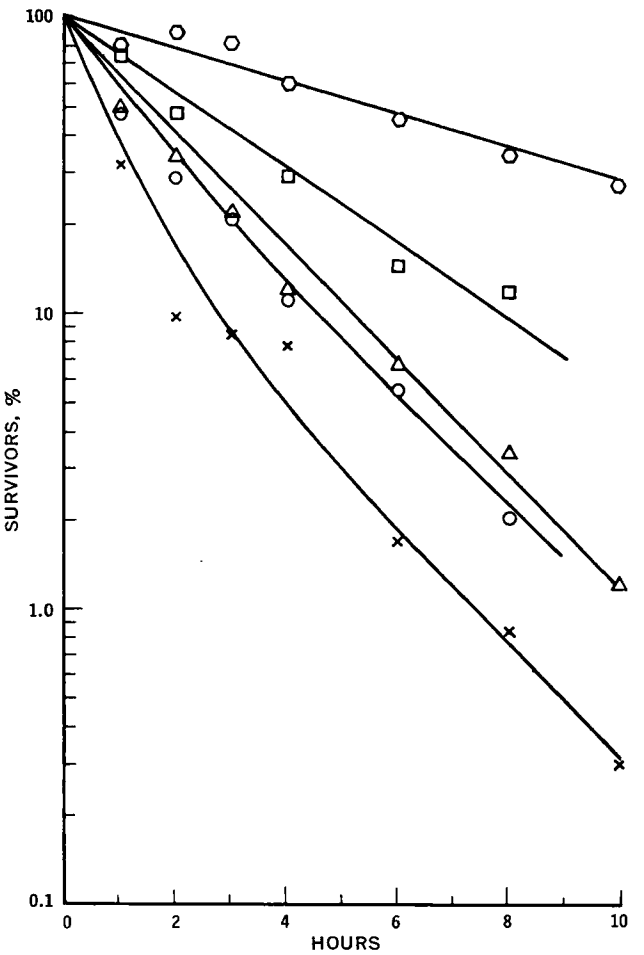

Figure 2—*Logarithm survivors–time curves of* Staph. aureus *in different solutions of mercurials when using sodium thioglycolate at 1.0%. Key:* ×, *mercuric chloride, 0.002%;* ○, *thimerosal, 0.5%;* ○, *phenylmercuric acetate, 0.02%;* □, *phenylmercuric borate, 0.02%; and* △, *phenylmercuric nitrate, 0.02%.*

Media—Nutrient broth[4] (16 g/liter), solidified when necessary with 15 g/liter of agar[5], was prepared.

Determination of Minimum Inhibitory Concentration (MIC)—The broth dilution method was adopted using as inoculum 0.1 ml (2×10^5 cells) of 24-hr broth culture. When sodium thioglycolate was included, 1 ml of thioglycolate solution at the required concentration was added aseptically to the broth before inoculation.

Determination of Bactericidal Activity—Bacterial activity was measured for each organism after treatment with the mercurial compound at appropriate concentrations for different time intervals, and the number of cells surviving treatments in each case was determined. The technique used for preparing bacterial suspensions and bactericidal reactions was the same as that used previously (16). Sodium thioglycolate solutions were used in all dilutions for the treated cells.

RESULTS AND DISCUSSION

Effect of Sodium Thioglycolate on MIC—It was previously shown that the bacteriostatic action of mercuric chloride could be eliminated by sulfhydryl-containing compounds in amounts close to the theoretical stoichiometric relation (17, 18). Therefore, the MIC's of these mercurials were determined alone and in the presence of different concentrations of sodium thioglycolate (Tables I and II) to see whether the same correlations apply. Sodium thioglycolate inactivates the mercurials since higher MIC's were obtained. However, the extent of inactivation varies with the mercurial agent and test organism, but it does not always follow the stoichiometric relation of mercury to sulfhydryl of 1:2. With *Staph. aureus*, the stoichiometric relation could be applied in the case of mercuric chloride. The ratio of inactivated mercury to sulfhydryl was 1:2.5. This result agreed with those reported on *Staph. au-*

Table II—Minimum Inhibitory Concentrations (MIC) of Different Mercurials against *Ps. aeruginosa* and in the Presence of Sodium Thioglycolate

Mercurial Compound	Thioglycolate Concentration 0% MIC[a]	0.001% MIC	0.001% Hg to —SH[b]	0.003% MIC	0.003% Hg to —SH
Mercuric chloride	6	8	1:12	15	1:8
Thimerosal	6	15	1:4	30	1:4
Phenylmercuric acetate	12	30	1:2	45	1:2.5
Phenylmercuric borate	12	30	1:2	45	1:2.5
Phenylmercuric nitrate	12	30	1:2	45	1:2.5

[a] Micrograms per milliliter, average of six replicates. [b] Ratio of inactivated mercury molecules to thioglycolate molecules as given by: inactivated mercury molecules = (MIC of mercurial with thioglycolate − MIC without thioglycolate)/molecular weight of mercury compound.

[4] Oxoid CM 15.
[5] New Zealand agar, Davis Gelatin Ltd., Warwick, England.

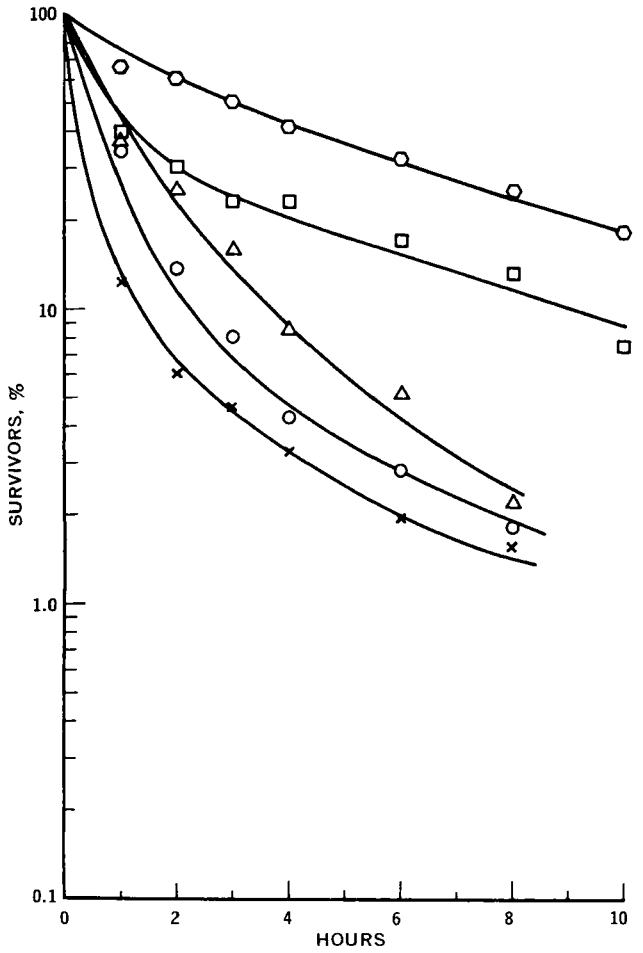

Figure 3—*Logarithm survivors–time curves of* Staph. aureus *in different solutions of mercurials when using sodium thioglycolate at 3.0%. Key: ×, mercuric chloride, 0.002%; ○, thimerosal, 0.5%; ○, phenylmercuric acetate, 0.02%; □, phenylmercuric borate, 0.02%; and △, phenylmercuric nitrate, 0.02%.*

reus (17) and *Escherichia coli* (18). However, with organic mercurials, ratios of 1:8 or higher were obtained. In the case of *Ps. aeruginosa*, the ratios were different. The mercury to sulfhydryl ratios were close to the stoichiometric relation with organic mercurials, while a higher ratio was obtained with mercuric chloride (Table II). The difference noticed in the mercury to sulfhydryl ratios of each mercurial was due to the type of organism used. This could indicate that, when the microorganism was present, the removal of the inhibitory action of the mercurial by the sulfhydryl compound was not solely due to simple chemical complexes between the free mercury molecules and sulfhydryl molecules but that there were other biological effects as well.

The organic mercurials (Table I) were found to be more inhibitory to *Staph. aureus* than to *Ps. aeruginosa*. These results agreed with similar, previously reported work (19, 20). Such findings are, however, different from those obtained with the bactericidal activity measurements; *Staph. aureus* was more resistant than *Ps. aeruginosa* to the effect of the tested mercurials. This might be attributed to a difference in the site of bacteriostatic and bactericidal action of the mercurial. As suggested previously (21, 22), when the action of the mercurial is bacteriostatic, it acts by physical adsorption on the cell surface; the bactericidal action is produced after the mercurial penetrates the cell and reacts with essential metabolites, causing structural changes in the cell.

Bactericidal Activity of Mercurials—*Staph. aureus*—The survivors-time curves of *Staph. aureus* bacteria treated with 0.002% $HgCl_2$, 0.5% thimerosal, and 0.02% phenylmercuric salts in 0.4, 1.0, and 3.0% sodium thioglycolate solutions are presented in Figs. 1–3, respectively. Even after 10 hr of treatment with such high concentrations of mercurials, viable cells were recovered; when 3% sodium thioglycolate was used, the level of mortality did not reach 99% (Fig. 3). The figures show the actual bactericidal efficiency of these agents when using high thioglycolate concentrations. The most active agent tested was mercuric chloride, although it was used at 0.002% concentration, and the least effective was thimerosal in spite of its high concentration (0.5%). The phenylmercuric salts occupied an intermediate position; however, the borate was slightly less active than the acetate and the nitrate.

The trend of reaction in Figs. 1 and 2 was logarithmic with all tested mercurials, which meant a first-order reaction. However, deviation from linearity occurred in Fig. 3, and the relation became more like a curve than a straight line. Although the bactericidal efficiencies of these compounds were different, it appeared from the shape of the survivors–time curves that their mechanism of action was the same. Difference in the bactericidal efficiency between mercurials was probably due to a difference in the rate by which the mercurial reached the vital molecules of the bacterial cell and became lethal. The results obtained are not in agreement with previous work (15) which showed that mercuric chloride was less active than organic mercurials. Such controversy is possibly due to the effect of sodium thioglycolate on the recovery of the damaged cells, particularly when the proper thioglycolate concentrations producing the highest revival were used. Sodium thioglycolate solution at 3% gave the highest number of survivors (Fig. 3).

Ps. aeruginosa—This organism was found to be much more

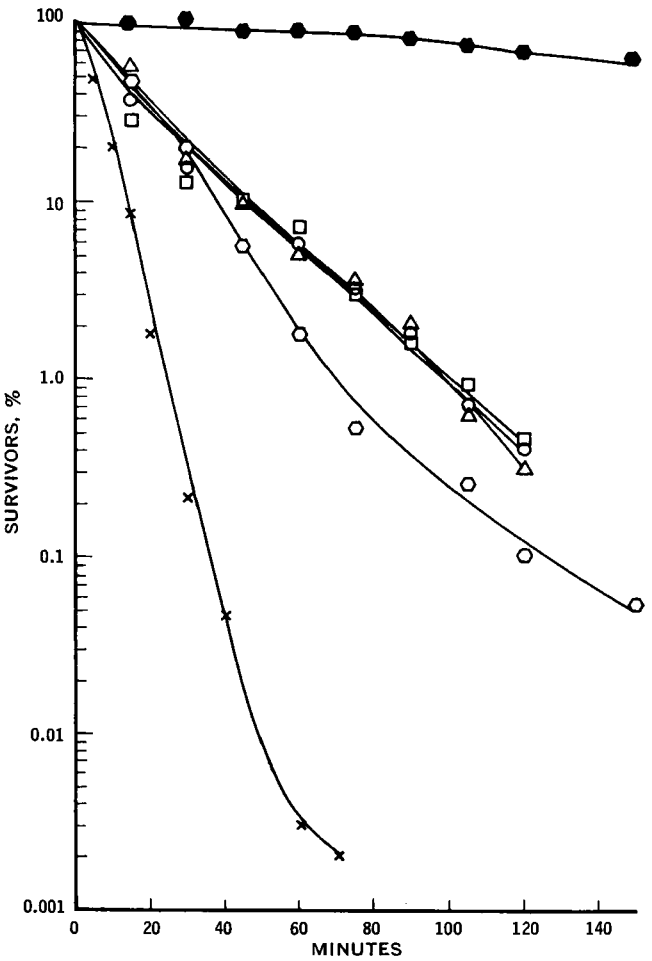

Figure 4—*Logarithm survivors–time curves of* Ps. aeruginosa *in different solutions of mercurials when using sodium thioglycolate at 0.04%. Key: ×, mercuric chloride, 0.002%; ○, thimerosal, 0.05%; ○, phenylmercuric acetate, 0.002%; □, phenylmercuric borate, 0.002%; △, phenylmercuric nitrate, 0.002%; and ●, thimerosal, 0.002%.*

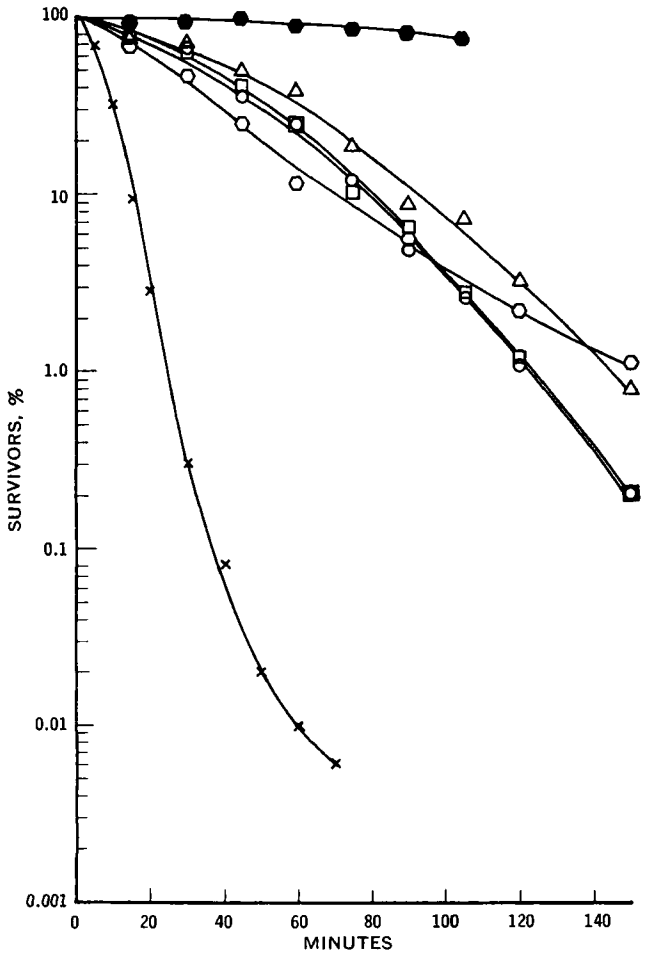

Figure 5—*Logarithm survivors–time curves of* Ps. aeruginosa *in different solutions of mercurials when using sodium thioglycolate at 0.2%. Key:* ×, *mercuric chloride, 0.002%;* ○, *thimerosal, 0.05%;* ○, *phenylmercuric acetate, 0.002%;* □, *phenylmercuric borate, 0.002%;* △, *phenylmercuric nitrate, 0.002%; and* ○, *thimerosal, 0.002%.*

sensitive to the effect of mercurials than was *Staph. aureus*. This finding is in agreement with those using phenylmercuric borate (21) and phenylmercuric nitrate (16). Consequently, to obtain reasonable survivors-time curves, the concentrations used were 0.05% for thimerosal and 0.002% for mercuric chloride and phenylmercuric salts (Figs. 4–6). The shape of the survivors curves did not follow a linear relation as it did with *Staph. aureus*. The results could indicate that the mode of action of mercurials on the two tested organisms was not the same. Furthermore, the order of bactericidal activities of these compounds differed from that obtained with *Staph. aureus*. Thimerosal at the 0.05% level appeared to be more active than the phenylmercuric salts. However, thimerosal at a concentration similar to that of the phenylmercuric salts (0.002%) proved to be less effective against *Ps. aeruginosa* (Figs. 4 and 5). The phenylmercuric salts behaved more or less similarly toward *Ps. aeruginosa* with respect to bactericidal activities. Mercuric chloride was still the most active bactericide among the tested mercurials.

With both microorganisms and under the test conditions, mercuric chloride was a remarkably efficient bactericide compared to the organic mercurials. The difference in bactericidal activity could be due to a difference in the nature of the interactions of these compounds with the cellular structures, such that the mercuric chloride interaction was not affected by the thioglycolate concentration to the same extent as the organic mercurial compounds. The sensitivity of *Ps. aeruginosa* toward the mercurials might be attributed to its relatively high lipid content of the cell wall (23, 24) as compared with *Staph. aureus* (25). The difference in the lipid content of each organism may play a role in the antibacterial activities of these agents as it affects the solubility of the mercurials in the cell membranes (22). In addition, it has been reported that the increased resistance of *Staph. aureus* to mercurials was due to a rise in its sulfhydryl content (26, 27).

Effect of Thioglycolate Concentration on Bactericidal Activity—The effect of sodium thioglycolate concentration on the number of cells surviving treatment with mercurials is shown quite clearly in Figs. 1–6. The number of survivors obtained with both tested organisms at any thioglycolate concentration used was remarkably higher than that obtained when no sodium thioglycolate was used or when the stoichiometric concentration was used. When sterile distilled water was used instead of thioglycolate, the 99% mortality of mercurial-treated bacteria was reached in <5 min with *Ps. aeruginosa* and in <10 min with *Staph. aureus* (Table III). With thimerosal-treated *Staph. aureus*, the 99% mortality was reached in 2 hr. Such significantly high survivors in the presence of thioglycolate could indicate that thioglycolate was an essential factor for the recovery and revival of the damaged bacteria. To obtain the highest level of survivors, sodium thioglycolate has to be used at an optimum concentration range which varied according to the test organism. With all tested mercurials, the optimal thioglycolate concentration for the recovery of *Ps. aeruginosa* was 0.2%; for *Staph. aureus*, it was 2–3%. Prolongation of bacterial treatments with mercurials affects the response of the damaged cells to thioglycolate concentration. The more severe the treatment the greater would be the response (16). These optimal thioglycolate concentrations are much higher than those customarily used in media for sterility testing. Since sterilization in the presence of mercurials results in severe damage to any ex-

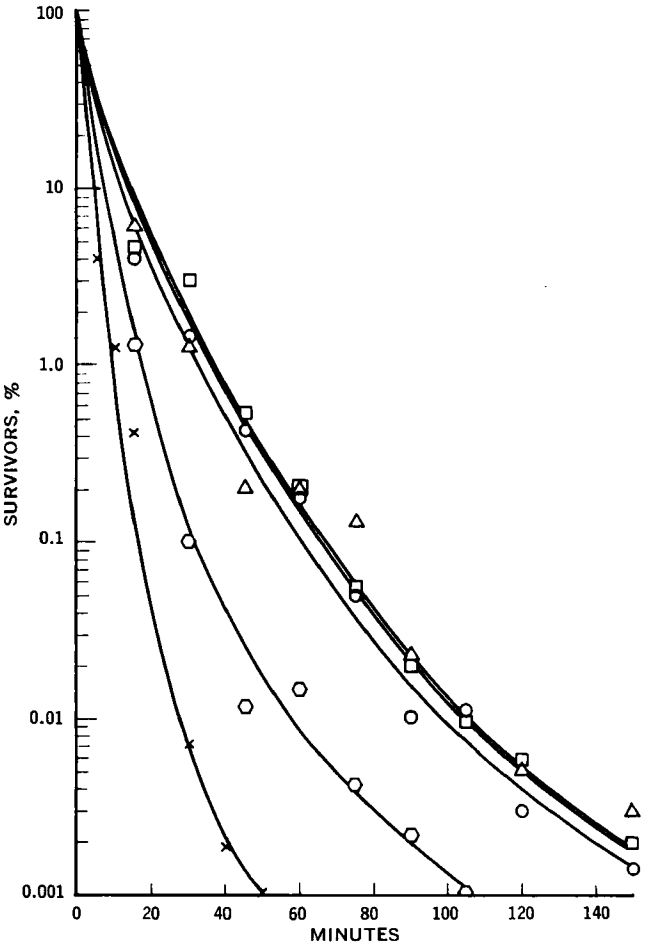

Figure 6—*Logarithm survivors–time curves of* Ps. aeruginosa *in different solutions of mercurials when using sodium thioglycolate at 1.0%. Key:* ×, *mercuric chloride, 0.002%;* ○, *thimerosal, 0.05%;* ○, *phenylmercuric acetate, 0.002%;* □, *phenylmercuric borate, 0.002%; and* △, *phenylmercuric nitrate, 0.002%.*

Table III—Effect of Sodium Thioglycolate Concentration on Bactericidal Efficiency of Mercurials against *Staph. aureus* and *Ps. aeruginosa*

Mercurial Compound		Thioglycolate Concentration			
		0%	0.4%	1.0%	3.0%
		Staph. aureus			
Mercuric chloride, 0.002%	A[a]	2 min	4 hr	3 hr	2.5 hr
	B[b]	10 min	8 hr	9 hr	10.0 hr
Thimerosal, 0.5%	A	90 min	>24 hr	>24 hr	>24.0 hr
	B	99 min	—	—	—
Phenylmercuric acetate, 0.02%	A	<2 min	6 hr	4 hr	2.5 hr
	B	2 min	11 hr	12 hr	14.0 hr
Phenylmercuric borate, 0.02%	A	5 min	11 hr	9 hr	9.0 hr
	B	10 min	21 hr	24 hr	>24.0 hr
Phenylmercuric nitrate, 0.02%	A	<2 min	7 hr	4 hr	3.5 hr
	B	2 min	11 hr	12 hr	13.0 hr
		Ps. aeruginosa			
		0%	0.04%	0.2%	1.0%
Mercuric chloride, 0.002%	A	<2 min	15 min	15 min	10 min
	B	5 min	25 min	25 min	15 min
Thimerosal, 0.05%	A	5 min	40 min	70 min	20 min
	B	10 min	70 min	155 min	30 min
Phenylmercuric acetate, 0.002%	A	<2 min	45 min	80 min	30 min
	B		100 min	125 min	60 min
Phenylmercuric borate, 0.002%	A		45 min	80 min	35 min
	B		100 min	125 min	65 min
Phenylmercuric nitrate, 0.002%	A		45 min	85 min	35 min
	B		100 min	125 min	60 min

[a] A = time to reach 90% mortality. [b] B = time to reach 99% mortality.

isting bacteria, the thioglycolate concentration becomes a critical factor for the revival of these damaged bacteria.

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[x] To whom inquiries should be directed.

Theoretical Model Studies of Intestinal Drug Absorption IV: Bile Acid Transport at Premicellar Concentrations across Diffusion Layer–Membrane Barrier

N. F. H. HO[x] and W. I. HIGUCHI

Abstract □ A general physical model is described for the simultaneous active and passive transport of conjugated and unconjugated bile acids from nonmicellar solutions in the ileum. It reduces to the passive absorption mechanism in the duodenum and jejunum. The model provides the basis for the experimental design of *in situ* studies and the quantification of physically meaningful and experimentally accessible transport parameters and of the effect of functional groups on bile acids. The interaction and factorization of *(a)* the permeability of the stagnant water layer, the lipoidal and aqueous channel pathway of the membrane according to the diffusion mechanisms, *(b)* the permeability of the membrane according to the active mechanism, *(c)* the bile acid concentration dependence, and *(d)* the pH and pKa are mathematically explicit. Theoretical computations indicated that the stagnant water layer may be the rate-determining barrier. A model is described for the first-pass absorption of bile acids from dilute solutions in the small intestine.

Keyphrases □ Drug absorption, theoretical models—bile acid transport at premicellar concentrations across diffusion layer-membrane □ Bile acids—general physical model described for active and passive transport of conjugated and unconjugated bile acids from nonmicellar solutions □ Absorption, intestinal drug—theoretical model described, simultaneous active and passive transport of conjugated and unconjugated bile acids, premicellar concentrations, diffusion layer-membrane barrier □ Diffusion layer-membrane—physical model described for bile acid transport, equations □ Membranes, diffusion layer—physical model described for bile acid transport, equations

According to recent reviews (1, 2), bile acids are absorbed passively *in vivo* in the duodenum and jejunum but are absorbed both passively and actively in the ileum. Because the nondissociated species are transported by simple diffusion across the lipoidal pathway of the intestinal membrane and the anionic species are transported passively across the aqueous channels of the intestinal membrane and actively through the ileal membrane, the total transport rate across any segment (*i.e.*, duodenum, jejunum, and ileum) depends effectively on the pKa of the acid and the pH of the solution. For this reason, the taurine-conjugated bile acids (pKa ~ 2.0) are absorbed principally within the ileal segment of the small intestine. For the glycine-conjugated bile acids (pKa 4–5) and the unconjugated bile acids (pKa 5–6), there will be a more significant contribution of the passive mechanism to the total transport rate in the ileum.

The circumstances of the concentration dependence of the total transport rate are recognized. Since the active transport rate *per se* in the ileum increases with the increasing concentration of anionic species until a maximum rate is reached, the total concentration of bile acid and the pKa and pH are important considerations. However, in the limit where the activity of the bile acid has drastically changed beyond a critical concentration, one must now also consider the transport of bile acids from micellar solutions. Here, the total transport rate decreases since micelles are not absorbed.

Many investigations on bile acid absorption have been cited in the reviews (1, 2). However, some recent studies (3–7) involve the application of physical models to the quantitation of various mechanistic transport parameters (active and passive) and the assessment of functional groups on bile acids affecting the transport across the intestinal brush border of rats. While the importance of the unstirred water layer on the mucosal side to the total transport rate of bile acids from micellar and nonmicellar solutions has been recognized (5, 6), this transport barrier admittedly has not been accounted for in determining the intrinsic active and passive flux of the bile acid across the intestinal membrane (3, 4). Although the intrinsic permeability coefficients of the membrane according to the active and passive mechanism, after factoring out the effect of the unstirred water layer and the pH of the solution relative to the pKa of the acid, have not been determined, a knowledge of these parameters would appear to be extremely useful. It has been the authors' experience, both theoretically (8, 9) and experimentally[1,2], that the unstirred water layer can significantly affect the total transport rate of solute molecules and the proper quantification of the factors involved in intestinal absorption.

The purpose of this paper is to derive a general physical model for the intestinal transport of bile acids from nonmicellar solutions. The model is based on the first principles of transport, thermodynamics, and various biological and physicochemical relationships known about the transport of bile acids in dilute solutions. As will be seen, the mathematical expressions are explicit in describing the interaction of the permeabilities of the physically relevant transport barriers, the pH of the solution at the membrane surface, and the characteristic pKa of the bile acid. Furthermore, the physical model and the accompanying mathematics are presented in a manner accessible to experimental evaluation and determi-

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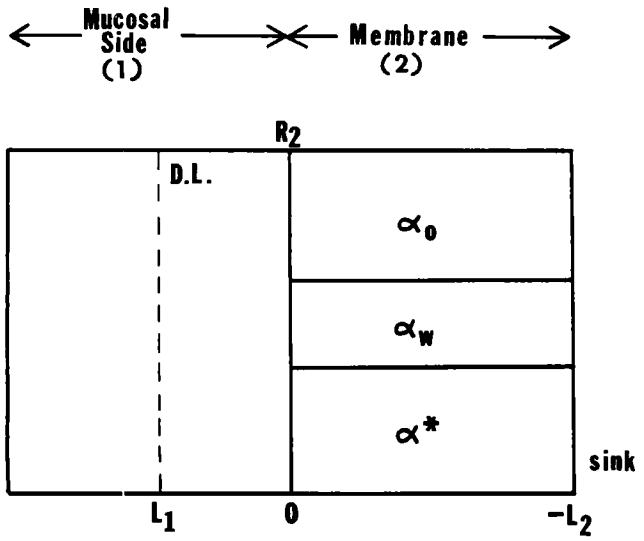
[2] K. J. Desai, N. F. H. Ho, and W. I. Higuchi, "*In Vivo* Drug Absorption II: Aqueous Diffusion Layer in the Rat Intestinal Tract," *J. Lipid Res.*, submitted for publication.



Scheme I—Physical model for the simultaneous active and passive transport of bile acids from nonmicellar solutions in the intestinal tract. The bulk aqueous solution with an aqueous diffusion layer on the mucosal side is in series with a heterogeneous membrane consisting of parallel lipoidal (α_o) and aqueous channel (α_w) pathways for passive transport and an active transport pathway (α^*). Thereafter, a sink on the serosal side follows.

nation whereby the permeability of the stagnant water layer, the permeability of the lipoidal pathway and the aqueous channels of the membrane according to the passive diffusional mechanism, the active transport kinetic parameters, and the effects of the molecular structure of the bile acids to active and passive transport can be quantified.

THEORY

General Description of Model—The first compartment of the model (Scheme I) (mucosal side) consists of the bulk aqueous phase and a stagnant water layer, *i.e.*, an aqueous diffusion layer, of thickness L_1. The second compartment of thickness L_2 provides for a heterogeneous phase to simulate the ideal membrane consisting of a lipoidal phase of volume fraction α_o and a hydrophilic phase α_w. Thereafter, sink conditions on the serosal side are assumed to prevail.

In this model, the concentration of the conjugated or nonconjugated bile acid solution is below the critical micelle concentration (CMC). All molecular species (anionic and undissociated) existing in the aqueous diffusion layer are able to permeate through the heterogeneous compartment. The aqueous pathway is accessible to all molecular species. It is assumed that only the nondissociated species can partition into and diffuse through the lipid phase. The anionic species are transported passively across the aqueous channels of the membrane, α_w, as well as actively across the α^* part of the membrane. The terms for the volume fractions of the membrane (α_w, α_o, and α^*) denote the distinct parallel pathways for transport and their relative quantitative importance.

Total Steady-State Flux Equations—The total steady-state flux in the aqueous diffusion layer, J_1, is the sum of the fluxes of the individual bile acid species. Assuming that the diffusion coefficients are nearly the same, the total flux is:

$$J_1 = \frac{AD_{w,1}}{L_1}(C_b - C_s) \qquad \text{(Eq. 1)}$$

where C_b and C_s are the total bile acid concentrations in the bulk solution and at the interface of the membrane, respectively; A is the geometric surface area; and $D_{w,1}$ is the diffusion coefficient in the stagnant water layer.

The total flux across the membrane, J_2, is expressed by:

$$J_2 = \frac{AR_2}{L_2}\{\alpha_o D_o (R_o^0)_{-o} + \alpha_w D_{w,2}[(R_w^0)_{-o} + (R_w^-)_{+o}]\} + \frac{AR_2\alpha^* J_{max}(R_w^-)_{+o}}{K_m^* + (R_w^-)_{+o}} \qquad \text{(Eq. 2)}$$

The first term on the right side of the equation represents the passive flux of all species in the membrane, where R_2 is the ratio of the interfacial area to the geometrical surface area such that $(A)(R_2)$ is the true surface area, D_o is the lipid diffusion coefficient of the nondissociated species whose concentration at the interface on the membrane side is $(R_o^0)_{-o}$, and $D_{w,2}$ is the aqueous diffusion coefficient of both the anionic and nondissociated species whose concentrations on the membrane side are $(R_w^-)_{-o}$ and $(R_w^0)_{-o}$, respectively. The superscripts (0 and −) denote the nondissociated and anionic species. The second term on the right side of the equation has the same form as the Michaelis-Menten expression for enzymatic reactions and represents the flux for the active membrane transport of the anionic species at the interface on the aqueous diffusion layer side, *i.e.*, $(R_w^-)_{+o}$. Here, J_{max} is the intrinsic maximum flux for active transport in units of moles centimeters^{-2} seconds^{-1} and K_m^* is the Michaelis constant for active transport in units of moles centimeters^{-3}.

The intrinsic lipid-aqueous partition coefficient, K, is defined as:

$$K = \frac{(R_o^0)_{-o}}{(R_w^0)_{-o}} = \frac{(R_o^0)_{-o}}{(R_w^0)_{-o}} \qquad \text{(Eq. 3)}$$

When the pH's at the interface of the membrane on the aqueous diffusion layer side and the aqueous pore side are assumed to be the same, it follows that:

$$(R_w^0)_{+o} = (R_w^0)_{-o} \qquad \text{(Eq. 4a)}$$

$$(R_w^-)_{+o} = (R_w^-)_{-o} \qquad \text{(Eq. 4b)}$$

The mole fraction of nondissociated species at the membrane interface, X_s^0, is:

$$X_s^0 = \frac{(R_w^0)_{+o}}{(R_w^0)_{+o} + (R_w^-)_{+o}} = \frac{(R_w^0)_{+o}}{C_s} \qquad \text{(Eq. 5)}$$

and, correspondingly, the mole fraction of anionic species, X_s^-, is:

$$X_s^- = \frac{(R_w^-)_{+o}}{(R_w^0)_{+o} + (R_w^-)_{+o}} = \frac{(R_w^-)_{+o}}{C_s} \qquad \text{(Eq. 6)}$$

With Eqs. 3–6 and $J_{max}^* = \alpha^* R_2 J_{max}$, the total flux in the membrane becomes:

$$J_2 = \frac{AR_2}{L_2}(\alpha_o D_o K X_s^0 + \alpha_w D_{w,2})C_s + \frac{AJ_{max}^* X_s^- C_s}{K_m^* + X_s^- C_s} \qquad \text{(Eq. 7)}$$

Furthermore, defining the permeability coefficient of the nondissociated species for the passive diffusion across the lipoidal membrane as:

$$P_o = \frac{\alpha_o R_2 D_o K}{L_2} \qquad \text{(Eq. 8)}$$

and the permeability coefficient of the anionic and nondissociated species for the passive diffusion across the aqueous channels or pores as:

$$P_p = \frac{\alpha_w R_2 D_{w,2}}{L_2} \qquad \text{(Eq. 9)}$$

and the permeability of the aqueous diffusion layer as:

$$P_{aq} = \frac{D_{w,1}}{L_1} \qquad \text{(Eq. 10)}$$

Equations 1 and 7 are rewritten accordingly:

$$J_1 = AP_{aq}(C_b - C_s) \qquad \text{(Eq. 11)}$$

$$J_2 = A(P_o X_s^0 + P_p)C_s + \frac{AJ_{max}^* X_s^- C_s}{K_m^* + X_s^- C_s} \qquad \text{(Eq. 12)}$$

Equations 11 and 12 comprise the primary set of equations that describe the rate of change in the concentration of bile acid in the

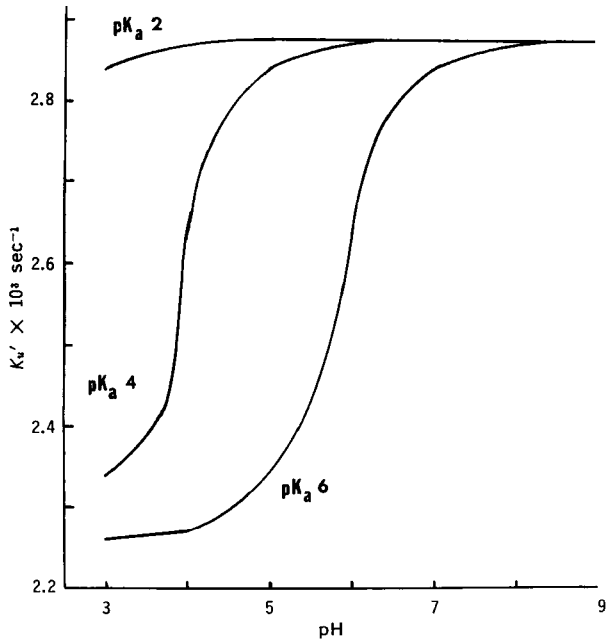


Figure 1—*Theoretical profiles of* K_u' versus *pH for bile acids with different pKa's based on Eq. 18 for the simultaneous passive and active transport in the ileum. Computations are based on Table I, and the permeability of the stagnant water layer equals* 4×10^{-4} *cm sec*$^{-1}$.

lumen of the ileum. Because Eq. 12 is nonlinear, the set of equations is more readily solved by numerical methods. To gain an explicit and helpful insight into the immediate treatment and interpretation of experimental data, approximate, but analytic, solutions to this primary set of equations are sought.

Case I: Linear Approximation of the Flux for Active Transport—In this case where $K_m^* \gg X_s^- C_s$ (concentration of bile acid anion at the mucosal surface), the total flux in the membrane is linearly related to the total concentration at the membrane surface:

$$J_2 = A(P_o X_s^0 + P_m^* X_s^- + P_p)C_s \qquad \text{(Eq. 13)}$$

where the permeability coefficient for active transport in centimeters seconds^{-1} is:

$$P_m^* = \frac{J_{max}^*}{K_m^*} \qquad \text{(Eq. 14)}$$

The continuity of flow through the interface is given by $J_1 = J_2$ from which, with Eqs. 11 and 13, one readily finds:

$$C_s = \frac{P_{aq} C_b}{P_o X_s^0 + P_m^* X_s^- + P_p + P_{aq}} \qquad \text{(Eq. 15)}$$

Combining Eqs. 11 and 15 and recognizing that:

$$J_1 = -V \frac{dC_b}{dt} \qquad \text{(Eq. 16)}$$

where V is the volume of the aqueous solution, one can then write the first-order expression:

$$\frac{dC_b}{dt} = -K_u' C_b \qquad \text{(Eq. 17)}$$

where:

$$K_u' = \left(\frac{A}{V}\right)(P_{aq})\left(\frac{1}{1 + \dfrac{P_{aq}}{P_o X_s^0 + P_m^* X_s^- + P_p}}\right) \qquad \text{(Eq. 18)}$$

Integrating between the limits of $C_b = C_b(0)$ and $C_b(t)$, one gets:

$$\ln C_b = \ln C_b(0) - K_u' t \qquad \text{(Eq. 19)}$$

Table I—Numerical Dimensions of Constants[a] Used for Computations

Constant	Value Used
Surface area to volume	$A/V = 10$
pKa	2.0, 4.0, and 6.0
Permeability coefficients:	
Aqueous diffusion layer	$P_{aq} = 2 \times 10^{-4}$; 4×10^{-4} cm sec^{-1}
Lipoidal pathway	$P_o = 5 \times 10^{-4}$ cm sec^{-1}
Aqueous pores	$P_p = 2 \times 10^{-5}$ cm sec^{-1}
Active transport	$P_m^* = 1 \times 10^{-3}$ cm sec^{-1}
Active transport parameters	$J_{max}^* = 5 \times 10^{-10}$ mole cm^{-2} sec^{-1}
	$K_m^* = 5 \times 10^{-7}$ mole cm^{-3}

[a] The values of the constants represent reasonable estimates from some studies in this laboratory and various literature sources.

There are several interesting limiting situations of Case I. Taking, for example, the absorption of taurocholic acid (pKa = 2.0) in the ileum at pH ~ 6–7, Eq. 18 reduces to:

$$K_u' = \left(\frac{A}{V}\right)(P_{aq})\left(\frac{1}{1 + \dfrac{P_{aq}}{P_m^* + P_p}}\right) \qquad \text{(Eq. 20)}$$

since the fraction of nondissociated acid is negligible at pH $\gg$ pKa. This will not apply to the other bile acids whose pKa ~ 5–6. However, in the duodenum and jejunum where all the bile acids undergo only passive transport, Eq. 18 reduces to an already familiar expression (9):

$$K_u' = \left(\frac{A}{V}\right)(P_{aq})\left(\frac{1}{1 + \dfrac{P_{aq}}{P_o X_s^0 + P_p}}\right) \qquad \text{(Eq. 21)}$$

From an experimental viewpoint, the permeability coefficients, P_o, P_m^*, and P_p can be determined simultaneously by applying Eq. 18 to the first-order rate constants obtained under three pH conditions, for example, pH $\ll$ pKa, pH = 6.0, and pH $\gg$ pKa. This is readily feasible for bile acids with pKa ~ 5–6. The permeability coefficient of the stagnant aqueous layer, P_{aq}, can be found from independent experiments using the *n*-alkanoic acid homologous series[1,2] and Eq. 21. The contribution of functional groups on the different bile acids to membrane transport can be evaluated by comparing the permeability coefficients of the bile acids to that of a reference bile acid. Thus, for the passive transport across the lipoidal pathway:

$$n = \frac{P_{o(\text{bile acid})}}{P_{o(\text{reference bile acid})}} \qquad \text{(Eq. 22)}$$

and for active transport:

$$n^* = \frac{P_{m\,(\text{bile acid})}^*}{P_{m\,(\text{reference bile acid})}^*} \qquad \text{(Eq. 23)}$$

Figure 1 shows a plot of the apparent first-order rate constant K_u' *versus* pH for bile acids with pKa 2.0, 4.0, and 6.0. The numerical dimensions of the constants used for the calculations are found in Table I. The intrinsic permeability coefficient of the membrane for active transport is taken to be larger than the permeability coefficient for passive transport. The curves are indicative of the kind of data that might be expected from the *in situ* rat intestinal absorption in the ileum of conjugated and nonconjugated bile acids in solution much below their CMC's. While it is assumed in these sample calculations that the permeability coefficients of the membrane for active and passive transport are the same among the bile acids, it may not necessarily be true (3, 4). In the pH $\ll$ pKa region, the transport across the membrane is primarily passive. As the pH becomes increasingly greater than the pKa, the passive transport becomes effectively less significant while the active transport mechanism becomes more significant;

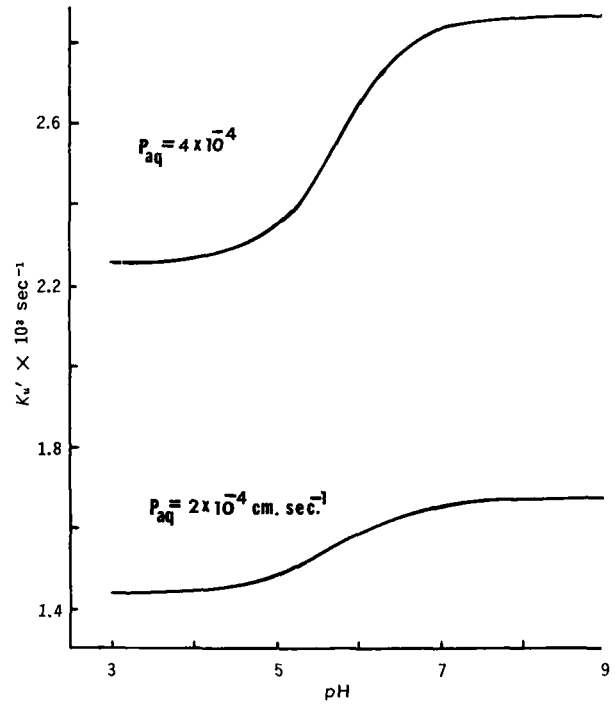


Figure 2—*Effect of the thickness of the stagnant water layer on the transport of a bile acid of pKa 6.0 in the ileum.*

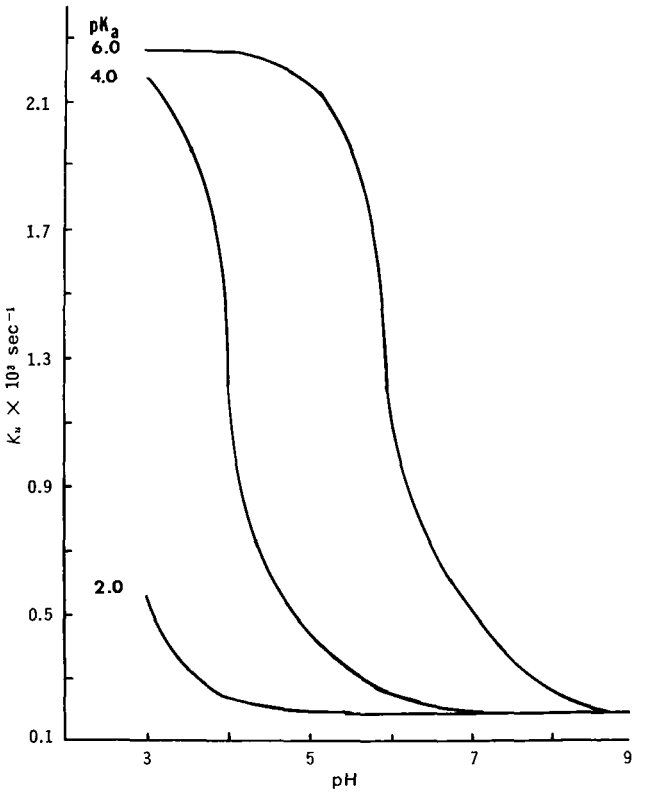


Figure 3—*Theoretical profiles of* K_u *versus pH for bile acids with different pKa based on Eq. 21 for passive transport in the duodenum and jejunum. The permeability of the stagnant water layer is* 4×10^{-4} *cm sec*$^{-1}$.

thus, the rate constant increases until it reaches a maximum. In the ileum where the pH is normally about 7.0, the active transport of the bile acids with pKa of 2.0-5.0 is dominant.

It is worthwhile to point out an important observation of the maximum rate constant of 2.87×10^{-3} sec^{-1} at pH $\gg$ pKa in Fig. 1. When the rate is aqueous diffusion controlled (Eq. 20), *i.e.*, the permeability of the stagnant aqueous layer is rate controlling, $K_u' = AP_{aq}/V = 4 \times 10^{-3}$ sec^{-1}. Here, the effective thickness of the aqueous diffusion layer is about 125 μm. Consequently, the maximum rate in Fig. 1 is about 75% aqueous diffusion controlled. At pH $\ll$ pKa, it is about 60% aqueous diffusion controlled.

Figure 2 shows a typical plot of K_u' *versus* pH at different permeability coefficients of the aqueous diffusion layer, *i.e.*, different thicknesses of the stagnant water layer, for a bile acid with pKa 6.0. By increasing P_{aq}, the rate constant increases. At $P_{aq} = 2 \times 10^{-4}$ cm sec^{-1}, corresponding to a diffusion layer of 250 μm when a diffusion coefficient of 5×10^{-6} cm^2 sec^{-1} is chosen, the maximum rate constant is then about 85% aqueous diffusion controlled.

Theoretical plots on the pH-passive transport profiles of the bile acids in the duodenum and jejunum are found in Fig. 3. The bile acid with pKa 6.0 is better absorbed at physiological pH 5-7 than is an acid with a smaller pKa. At higher pH, the rate constants converge since this is indicative of the transport across the aqueous channels of the membrane.

Case II: Maximum Flux for Active Transport—In this special case where $K_m^* \ll X_s^- C_s$, the flux for active transport is maximum and the total flux across the membrane is:

$$J_2 = A(P_o X_s^0 + P_p)C_s + AJ_{max}^* \qquad \text{(Eq. 24)}$$

In a similar procedure of derivation as in Case I, it can be shown that:

$$\frac{dC_b}{dt} = -K_u(B + C_b) \qquad \text{(Eq. 25)}$$

where:

$$K_u = \left(\frac{A}{V}\right)(P_{aq})\left(\frac{1}{1 + \dfrac{P_{aq}}{P_o X_s^0 + P_p}}\right) \qquad \text{(Eq. 26)}$$

$$B = \frac{J_{max}^*}{P_o X_s^0 + P_p} \qquad \text{(Eq. 27)}$$

The solution is:

$$\ln \frac{B + C_b}{B + C_b(0)} = -K_u t \qquad \text{(Eq. 28)}$$

The rate constant K_u is descriptive of the effective permeability coefficient for diffusional transport across the aqueous diffusion layer and the membrane. The specific permeability coefficients in Eq. 26 are readily determined by appropriate experiments based on Eq. 18. In turn, this permits the calculation of K_u for Case II. It also follows that an estimate of the constant B can be obtained from the experimental data by taking the initial slope of a log C_b *versus* t plot. Thus:

$$\left.\frac{d \log C_b}{dt}\right|_{t=0} = \frac{-K_u[B + C_b(0)]}{2.303 C_b(0)} \qquad \text{(Eq. 29)}$$

or:

$$B = -C_b(0)\left[1 + \left(\frac{2.303}{K_u}\right)\left.\frac{d \log C_b}{dt}\right|_{t=0}\right] \qquad \text{(Eq. 30)}$$

whereupon J_{max}^* can be calculated. In turn, with Eq. 14, the K_m^* is found by:

$$K_m^* = \frac{J_{max}^*}{P_m^*} \qquad \text{(Eq. 31)}$$

Case III: General Treatment of Flux for Active Transport[3]—Let us assume that: *(a)* the pH is much greater than the pKa so that $X_s^0 \sim 0$ and $X_s^- \sim 1.0$, and *(b)* the flux for active transport is much greater than the flux across the aqueous channels of the membrane. Accordingly, Eq. 12 becomes:

[3] At the time of submitting this manuscript, the authors became aware of the recently published work of Winne (10) with respect to this particular section of the manuscript. The same conclusions are arrived. However, it is believed here that the mathematical description of this special case is experimentally more accessible.

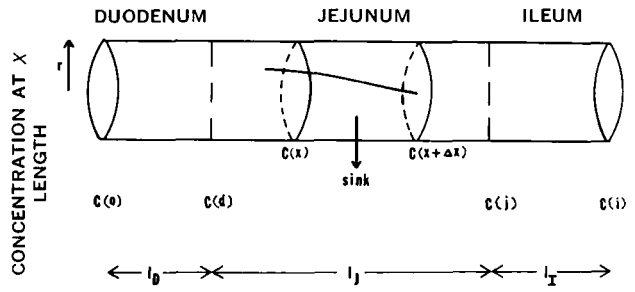


Figure 4—*Model for first-pass absorption of bile acid in the small intestine.*

$$J_2 = \frac{AJ_{max}{}^*C_s}{K_m{}^* + C_s} \qquad \text{(Eq. 32)}$$

or:

$$C_s = \frac{J_2K_m{}^*}{AJ_{max}{}^* - J_2} \qquad \text{(Eq. 33)}$$

The substitution of Eq. 33 and the expression for the continuity of flow through the membrane surface, $J_1 = J_2$, into Eq. 11 give:

$$J_1{}^2 - (\alpha + \beta C_b)J_1 + \delta C_b = 0 \qquad \text{(Eq. 34)}$$

where:

$$\alpha = A(J_{max}{}^* + K_m{}^*P_{aq}) \qquad \text{(Eq. 35)}$$

$$\beta = AP_{aq} \qquad \text{(Eq. 36)}$$

$$\delta = A^2P_{aq}J_{max}{}^* \qquad \text{(Eq. 37)}$$

The solution for J_1 by the quadratic formula is:

$$J_1 = \frac{\alpha + \beta C_b - \sqrt{(\alpha + \beta C_b)^2 - 4\delta C_b}}{2} \qquad \text{(Eq. 38)}$$

where the negative of the square root function is taken in relation to physical significance.

Although an analytic solution for C_b as a function of time is available, it is quite complicated and is not explicitly useful in the quantitative assessment of the experimental data. A plot of the total flux, J_1 *versus* C_b, at initial concentrations will be more useful since it provides several limiting situations from which various transport parameters can be quantified. For example, utilizing Eq. 38:

$$\lim_{C_b \to 0} \frac{dJ_1}{dC_b} = \frac{\delta}{\alpha} = \frac{AP_{aq}P_m{}^*}{P_{aq} + P_m{}^*} \qquad \text{(Eq. 39)}$$

$$\lim_{C_b \to \infty} J_1 = \frac{\alpha}{2} = \frac{AK_m{}^*}{2}(P_{aq} + P_m{}^*) \qquad \text{(Eq. 40)}$$

As one can readily observe in Eq. 39, if $P_m{}^* \gg P_{aq}$, the initial slope will be determined by the permeability of the stagnant water layer. The opposite is also true.

Several points should be made about the special situation indicated by Eq. 40. First, the C_b should be sufficiently large but within the limit of the CMC. Second, this situation is probably never reached in *in situ* animal absorption experiments in the ileum.

Single-Pass Intestinal Absorption Model—An idealized model is shown in Fig. 4 for the simultaneous flow of a dilute nonmicellar solution of bile acid in the intestinal lumen, taken as a cylinder, and transport of the bile acid across the membrane into the sink on the serosal side. According to mass balance considerations, the difference in the concentration of solution entering into a cylindrical element at x and leaving at $x + \Delta x$ is equal to the transverse flux of the bile acid across the membrane within the element; thus:

$$Q[C_b(x) - C_b(x + \Delta x)] = (J_1')(2\pi r\,\Delta x) \qquad \text{(Eq. 41)}$$

where Q is the bulk fluid flow rate in cubic centimeters seconds^{-1}, r is the radius of the cylinder, and J_1' is the flux of the total bile acid per unit area. Taking the limit as $\Delta x \to 0$ and noting that:

$$J_1' = P_eC_b(x) \qquad \text{(Eq. 42)}$$

where P_e is the effective permeability coefficient, one gets:

$$\frac{dC_b}{dx} = -\frac{2\pi rP_eC_b}{Q} \qquad \text{(Eq. 43)}$$

After applying the boundary conditions to each section of the small intestine, that is, the duodenum *(d)*, the jejunum *(j)*, and the ileum *(i)*, the corresponding solutions to Eq. 43 become Eqs. 44–46:

$$C_b(d) = C_b(o)[\exp(-2\pi r_d l_d P_{e,d}/Q)] \qquad \text{(Eq. 44)}$$

$$C_b(j) = C_b(d)[\exp(-2\pi r_j l_j P_{e,j}/Q)] \qquad \text{(Eq. 45)}$$

$$C_b(i) = C_b(j)[\exp(-2\pi r_i l_i P_{e,i}/Q)] \qquad \text{(Eq. 46)}$$

The final concentration of bile acid appearing at the distal end of the ileum with respect to the initial concentration at the proximal end of the duodenum is:

$$C_b(i) = C_b(o)\left\{\sum_k \exp\left[-\frac{2\pi}{Q}(l_kr_kP_{e,k})\right]\right\} \qquad \text{(Eq. 47)}$$

where k = duodenum, jejunum, and ileum.

In general, the effective permeability coefficient for passive diffusion in the duodenum and jejunum may be expressed by:

$$P_e = (P_{aq})\left(\frac{1}{1 + \dfrac{P_{aq}}{P_oX_s{}^0 + P_p}}\right) \qquad \text{(Eq. 48)}$$

while that for both the passive and active transport of the bile acid in the ileum is:

$$P_e = (P_{aq})\left(\frac{1}{1 + \dfrac{P_{aq}}{P_oX_s{}^0 + P_m{}^*X_s{}^- + P_p}}\right) \qquad \text{(Eq. 49)}$$

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[x] To whom inquiries should be directed.

Effects of Alkyl Chain Length on Biological Activity: Alkyl *p*-Aminobenzoate-Induced Narcosis in Goldfish

S. H. YALKOWSKY [x], T. G. SLUNICK, and G. L. FLYNN

Abstract □ On the basis of a diffusional model and certain well-known physical-chemical relationships, equations were derived which describe the relative biological activities of alkyl homologs. These equations were applied to the goldfish turnover time produced by both saturated solutions and equimolar solutions of eight *n*-alkyl esters of *p*-aminobenzoic acid. The results show that the apparant parabolic structure–activity curve can be described quite well by a simple solubility-limited diffusional model.

Keyphrases □ *p*-Aminobenzoic acid ester-induced narcosis in goldfish—chain length effect, solubility-limited diffusional model, equations □ Chain length effect—alkyl *p*-aminobenzoate-induced narcosis in goldfish, solubility-limited diffusional model, equations □ Goldfish turnover—chain-length effect of alkyl *p*-aminobenzoates, diffusion model, equations

The role of the lipid–water partition coefficients of a series of compounds in determining their relative biological activities has long been recognized (1–5). It is also well known that linear relationships between the logarithms of the partition coefficient and biological activity break down at high partition coefficients (6). In certain instances (7–9), this is due to the fact that the solubilities of highly lipoidal substances are not sufficient to elicit the desired responses. However, there are many instances of deviations from linearity occurring at concentrations that are well below saturation. One of the first theoretical models capable of explaining these deviations was presented by Zwolinski *et al.* (10), who pointed out that diffusion layers can play an important role in the penetration of biological barriers. These diffusion layers have been repeatedly demonstrated on *in vitro* systems (11–13) and recently were shown to be present *in situ* (14) and *in vivo* (15, 16).

Equations accounting for diffusion layers have been built upon (13, 17) to describe the transport properties of *n*-alkyl *p*-aminobenzoates across silicone rubber membranes. It was shown that certain chain length–activity relationships that were predicted and observed in synthetic membrane systems are also observed in biological systems. Several specific examples of the various expected relationships were described in the literature, but no single example of all of the predicted dependencies could be found.

In this report, an attempt is made to verify all of the authors' earlier predictions on a single biological system. As in previous studies, the approach here involves the incremental changing of physical–chemical properties of the permeating species. This approach, which is implemented by restricting the study to the members of a homologous series, greatly simplifies the analysis and interpretation of the data. A literature analysis (13) strongly suggested that the relative degrees of narcosis produced by alkyl *p*-aminobenzoates in goldfish were in accord with the predictions of a diffusion layer membrane model. Thus, this system was chosen as a model system to demonstrate the hypotheses. Some additional practical reasons for choosing the goldfish system are the relative simplicity of experimental design, the low cost of the fish, the clearly defined end-point, and the ease of controlling experimental variables.

THEORETICAL

In the field of structure–activity relationships, it is generally true that the complete description of a model consists of: *(a)* the statement of the underlying assumptions (in mathematical form, if possible), and *(b)* the arithmetic manipulation of the postulated equations to give new equations which can be used to explain and hopefully to predict biological results. The model advanced in this report, to explain and to predict the relative narcotic activity of alkyl homologs in goldfish, is based on the following five postulates:

1. The differences in the observed biological activities of each member of the series, α_n, is directly related to that compound's ability to permeate biological barriers and to reach the receptor site, *i.e.*:

$$\alpha_n = \gamma F_n \qquad \text{(Eq. 1)}$$

where γ is a constant relating the rate of transport of the homolog to the site or flux, F_n, to its activity. The constancy of γ precludes the situation where there are differences in the metabolism or binding of the homologs. In other words, since the intrinsic activity, γ, is a constant, the observed differences in activity are due solely to differences in flux. Although this supposition is somewhat speculative, it is intuitively reasonable and is generally accepted. The four remaining criteria have been demonstrated numerous times by the authors and others in both *in vivo* and *in vitro* studies.

2. The flux, F, of a substance across a region consisting of aqueous and lipid barriers is given by (12):

$$\log F = \log C - \log (R_{aq} + R_m/PC) \qquad \text{(Eq. 2)}$$

where C is the concentration differential across the region; R_{aq} and R_m are the sums of the resistances of the aqueous and lipid regions, respectively; and PC is the lipid–water partition coefficient of the substance. All lipid regions involved are regarded as identical in polarity, and all aqueous regions are assumed to be of the same polarity. Factors such as thickness, porosity, tortuosity, diffusivity, and cross-sectional area are included in the overall resistance and need not be considered separately.

3. The maximum flux of a particular series of barriers is obtained when the donor region is saturated and the receptor region is at a negligible concentration, *i.e.*, when the concentration differential, C, is replaced by the solubility, S. Thus:

$$\log F^{max} = \log S - \log (R_{aq} + R_m/PC) \qquad \text{(Eq. 3)}$$

4. The partition coefficients of the members of a homologous

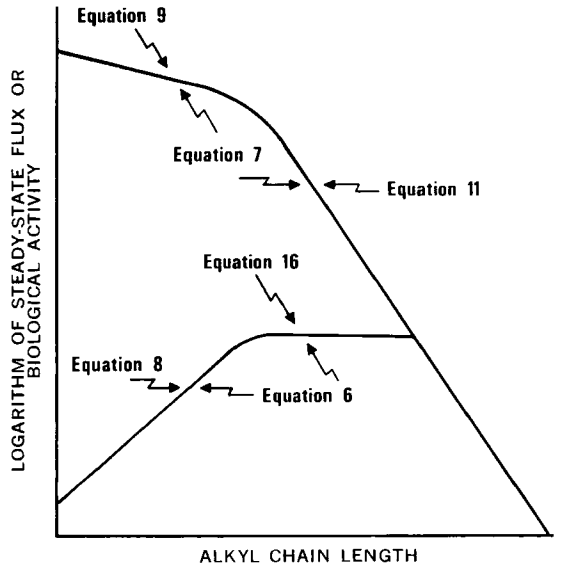

Figure 1—*Hypothetical chain length–activity relationships.*

series are described by:

$$\log PC_n = \log PC_0 + \pi n \qquad \text{(Eq. 4)}$$

where PC_n is the lipid–water partition coefficient of the homolog having chain length n, PC_0 is the extrapolated partition coefficient of the reference compound of the series, and π is a constant dependent on the polarity of the lipid.

5. The aqueous solubility of the homologs can be represented by:

$$\log S_n = \log S_0 - \delta n \qquad \text{(Eq. 5)}$$

where S_n and S_0 are the solubilities of the nth member of the series and the reference compound, respectively, and δ is a constant which is independent of the biological system.

On the basis of these equations, it is possible to derive equations that describe the observed biological activity in terms of alkyl chain length. Substituting Eqs. 4 and 5 into Eqs. 2 and 3 and the resulting equations into Eq. 1 gives[1]:

$$\log \alpha^c = \log \gamma + \log C + \log PC_0 + \pi n - \log (R_m + R_{aq}PC_0 10^{\pi n}) \qquad \text{(Eq. 6)}$$

and:

$$\log \alpha^s = \log \gamma + \log S_0 + \log PC_0 + (\pi - \delta)n - \log (R_m + R_{aq}PC_0 10^{\pi n}) \qquad \text{(Eq. 7)}$$

which describe the relative activities of the members of the series studied at a particular concentration, α^c, or the relative activities of saturated solutions of the homologs, α^s. These equations are depicted in Fig. 1 for arbitrary values of the constants. It can be seen that both curves undergo a change in slope at the same value of n. This change is due to the fact that when n is small, R_m can dominate the last term of Eqs. 6 and 7 so that they are approximated by:

$$\log \alpha^c = \log \gamma + \log C + \log PC_0 + \pi n - \log R_m \qquad \text{(Eq. 8)}$$

and:

$$\log \alpha^s = \log \gamma + \log S_0 + PC_0 + (\pi - \delta)n - \log R_m \qquad \text{(Eq. 9)}$$

But when n is large, $-\log (R_m + R_{aq}PC_0 10^{\pi n})$ approaches $-\log R_{aq} - \log PC_0 - \pi n$, and Eqs. 6 and 7 become, respectively:

$$\log \alpha^c = \log \gamma + \log C - \log R_{aq} \qquad \text{(Eq. 10)}$$

and:

$$\log \alpha^s = \log \gamma + \log S_0 - \delta n - \log R_{aq} \qquad \text{(Eq. 11)}$$

[1] A more complete derivation of Eqs. 6–11 can be found in Ref. 17.

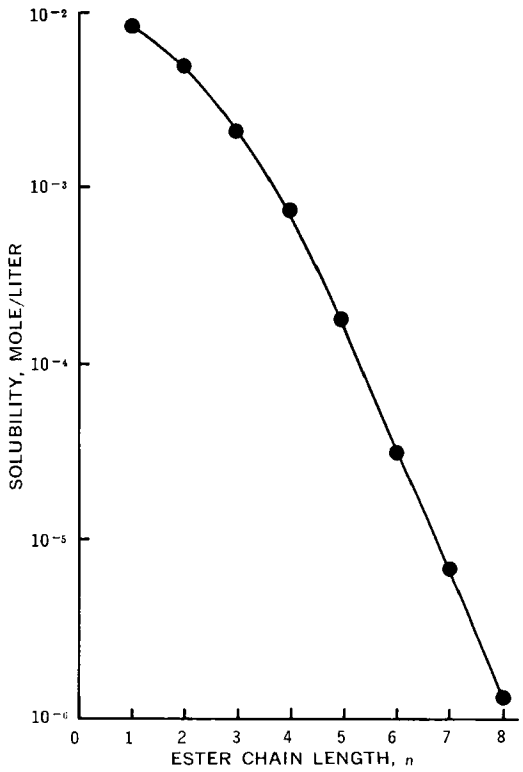

Figure 2—*Solubilities of* n*-alkyl* p*-aminobenzoates in water at 24 ± 1°.*

Equations 8 and 9, in which the only resistance appearing is the lipid or membrane resistance, describe the conditions most commonly encountered. This condition is referred to as membrane or lipid control of activity. The last two equations contain only the aqueous resistance and describe the condition of diffusion layer or aqueous control of activity. The meanings of these equations[2] in terms of chain length–activity relationships are evident from Fig. 1.

Basically, only four distinct conditions are described by the equations. Properties dependent upon flux from solutions of uniform concentration are characterized by Eqs. 6, 8, and 10. Equation 8 applies only to the situation in which there is membrane control of flux, while Eq. 10 applies when the diffusion layers offer the major resistance to transport. The special case in which the applied solutions are saturated is described by Eq. 7. Equations 9 and 11 delineate the subcases of membrane and diffusion layer control, respectively.

Linearity of the logarithms of both PC_n and S_n with chain length is not vital to this treatment. Although this simple case was used to facilitate deriving the necessary equations, any single-valued function that expresses log S_n or log PC_n as a function of n could have been used. In fact, as pointed out later, solubility is not a simple exponential function of chain length for the alkyl *p*-aminobenzoates.

EXPERIMENTAL

Materials—The alkyl *p*-aminobenzoates used in this study were either purchased or synthesized from *p*-nitrobenzoyl chloride and the appropiate alcohol[3]. Deionized water was used to prepare all solutions.

Goldfish—Common variety goldfish *(Carassius auratus)* weighing between 2 and 4 g were purchased locally.

[2] They and their implications are described extensively in Refs. 13 and 17.

[3] The sources were: *p*-nitrobenzoyl chloride, Matheson, Coleman and Bell; methyl *p*-aminobenzoate, Eastman; ethyl *p*-aminobenzoate, Aldrich; *n*-propanol, Eastman; *n*-butanol, source unknown; *n*-pentanol, Aldrich; *n*-hexanol, Aldrich; *n*-heptanol, Aldrich; *n*-octanol, Eastman; *n*-nonanol, Aldrich; *n*-dodecanol, Aldrich; *n*-hexadecanol, Eastman; and *p*-aminobenzoic acid, Eastman.

Table I—Average Turnover Times Produced by Various Concentrations of Alkyl *p*-Aminobenzoates

Ester	Fractional Saturation	Concentration, mole/liter	Number of Fish	Mean Turnover Time, min	Standard Error of Mean	Mean Reciprocal Turnover Time
Methyl	Saturated	8.3×10^{-3}	5	0.320	0.051	3.15
	2/3	5.52×10^{-3}	10	0.410	0.030	2.44
	1/2	4.15×10^{-3}	10	0.640	0.098	1.52
	1/3	2.76×10^{-3}	10	0.933	0.088	1.07
	1/4	2.07×10^{-3}	10	1.230	0.076	0.813
	1/6	1.40×10^{-3}	10	2.270	0.183	0.44
	1/10	8.3×10^{-4}	10	4.390	0.189	0.23
	1/20	4.15×10^{-4}	10	45.0	15.7	0.022
	1/40	2.07×10^{-4}	10	—[a]	—	—
	1/100	8.3×10^{-5}	10	—[a]	—	—
Ethyl	Saturated	—	5	0.340	0.062	2.95
	2/3	4.83×10^{-3}	10	0.270	0.015	3.70
	1/2	2.37×10^{-3}	10	0.460	0.040	2.17
	1/3	1.58×10^{-3}	10	0.550	0.045	1.82
	1/4	1.18×10^{-3}	10	0.990	0.095	1.01
	1/6	7.8×10^{-4}	10	1.210	0.159	0.83
	1/10	4.83×10^{-4}	10	2.800	0.226	0.36
	1/20	2.37×10^{-4}	10	6.50	0.64	0.15
	1/40	1.18×10^{-4}	10	47	9.3	0.021
	1/100	4.83×10^{-5}	10	—[a]	—	—
Propyl	Saturated	2.03×10^{-3}	6	0.333	0.042	3.0
	2/3	1.3×10^{-3}	10	0.450	0.037	2.22
	1/2	9.8×10^{-4}	10	0.510	0.038	1.96
	1/3	6.5×10^{-4}	10	0.680	0.039	1.47
	1/4	4.9×10^{-4}	10	0.900	0.097	1.11
	1/6	3.3×10^{-4}	10	1.150	0.097	0.87
	1/10	2.03×10^{-4}	10	3.150	0.442	0.32
	1/20	9.8×10^{-5}	10	8.250	0.720	0.12
	1/40	4.9×10^{-5}	10	30.50	3.06	0.03
	1/100	2.03×10^{-5}	10	—[a]	—	—
Butyl	Saturated	7.35×10^{-4}	5	0.340	0.025	2.94
	2/3	4.7×10^{-4}	10	0.640	0.072	1.56
	1/2	3.6×10^{-4}	10	0.730	0.070	1.37
	1/3	2.4×10^{-4}	10	1.110	0.094	0.90
	1/4	1.8×10^{-4}	10	1.510	0.157	0.66
	1/6	1.2×10^{-4}	10	2.600	0.256	0.39
	1/10	7.35×10^{-5}	10	4.650	0.289	0.22
	1/20	3.6×10^{-5}	10	9.950	0.664	0.10
	1/40	1.8×10^{-5}	10	70.60	18.44	0.014
	1/100	7.35×10^{-6}	10	—[a]	—	—
Pentyl	Saturated	1.79×10^{-4}	10	1.370	0.104	0.73
	3/4	1.34×10^{-4}	10	1.830	0.146	0.55
	2/3	1.29×10^{-4}	10	2.380	0.133	0.42
	1/2	9.0×10^{-5}	10	3.310	0.293	0.30
	1/3	5.95×10^{-5}	10	4.500	0.632	0.22
	1/4	4.5×10^{-5}	10	6.380	0.495	0.16
	1/6	2.98×10^{-5}	10	8.850	0.311	0.11
	1/10	1.79×10^{-5}	10	15.50	1.06	0.065
	1/20	9.0×10^{-5}	10	43.33	3.67	0.023
	1/40	4.5×10^{-5}	6	63.60	5.05	0.016
	1/100	1.79×11^{-6}	5	—[a]	—	—
Hexyl	Saturated	3.1×10^{-5}	10	6.70	0.26	0.15
	3/4	2.4×10^{-5}	10	9.20	0.41	0.11
	2/3	2.0×10^{-5}	10	13.00	0.45	0.078
	1/2	1.5×10^{-5}	10	14.30	1.68	0.070
	1/3	1.0×10^{-5}	10	25.00	2.17	0.040
	1/4	7.5×10^{-6}	10	31.20	5.88	0.032
	1/6	5.0×10^{-6}	10	—[a]	—	—
Heptyl	Saturated	7.2×10^{-6}	6	30.00	3.72	0.033
	1/2	3.6×10^{-6}				
Octyl	Saturated	—	2	—[a]	—	—

[a] Inactive.

Solubility Determination—The solubilities of the esters studied were determined by equilibrating excess drug with deionized water for at least 48 hr at 24°. The solutions were then filtered, diluted, and read at 285 nm on a spectrophotometer[4]. All esters had molar absorptivities of 20,700.

Turnover Time Measurements—The time required for a goldfish to lose its righting reflex after being placed in a specific solution at 24° was taken as the turnover time. Most values reported are the average of 10 individual experiments. The experimental details were described previously (18).

Extrapolation of Data—As explained previously (18), it is experimentally impossible to measure accurately turnover times of less than about 20 sec. Because the observed turnover times produced by saturated solutions of the lower esters were in this range, it was felt that they might be artifactual. Furthermore, these values were not in agreement with the rest of the concentration–turnover time data for these esters. More meaningful, al-

[4] Zeiss.

Table II—Dose–Response Equations

Ester	Equation
Methyl	$1/t = -0.164 + 4.76 \times 10^{-2} \times C$
Ethyl	$1/t = -0.105 + 1.11 \times 10^{-3} \times C$
Propyl	$1/t = -0.742 + 2.17 \times 10^{-3} \times C$
Butyl	$1/t = -0.040 + 3.76 \times 10^{-3} \times C$
Pentyl	$1/t = -0.003 + 3.78 \times 10^{-3} \times C$
Hexyl	$1/t = -0.0006 + 4.35 \times 10^{-3} \times C$

though hypothetical, indications of the activities of these esters were obtained by extrapolating the dose-response data for the remaining concentrations to saturation.

Extrapolations of the equations:

$$\frac{1}{t} = A\frac{C}{S} + B \qquad \text{(Eq. 12)}$$

and:

$$\log t = A' \log \frac{C}{S} + B' \qquad \text{(Eq. 13)}$$

where A, A', B, and B' are constants, to $C/S = 1$ give similar values for turnover time, t, at saturation. The averages of the extrapolated values determined by each equation for each lower ester are used in the remainder of this report in lieu of experimental values.

RESULTS

Solubility—The solubilities of the esters at 24° are plotted semilogarithmically *versus* chain length in Fig. 2. Above $C = 4$, log S is a linear function of n and there is significant deviation from this linearity for the lower esters. An explanation for this deviation in terms of changes in crystal packing with increasing chain length was already presented (19).

Turnover Time—The reciprocal mean turnover times produced by various concentrations of the esters studied are listed in Table I. An interpretation of these data in terms of absorption kinetics was recently given (18).

The data in Table I can be described satisfactorily by the equations listed in Table II. With the exception of the above-mentioned extrapolated points, all data discussed in this paper were obtained by interpolation of these equations. Figure 3 shows the results of calculations of the minimum concentration of each ester required to produce turnover in the following specified periods: 1, 3, 10, and 30 min. The vertical bars at the end of each line indicate that the next homolog is incapable of producing turnover in the designated period. The fact that there is no single, most active homolog is readily apparent from the figure. The lower the degree of activity measured, the higher is the chain length of the most active member of the series. The role of solubility in producing this relationship was first noted by Ferguson in 1939 (8) but has been largely ignored by many workers currently involved in structure-activity theory.

As stated by Ferguson (7, 8) and by the authors (9, 17), it is generally better to measure the biological end-point produced by a given concentration of drug than to measure the amount of drug required to produce a given end-point. This fact will become apparent in the discussion.

DISCUSSION

Equimolar Studies—To begin testing the model and evaluating the important parameters, one can consider the equimolar data at various concentrations. These data (Fig. 4) were obtained from the equations of Table II.

The value of the constant, π, can be determined from the initial slopes of the lines of either Fig. 3 or 4 or from the data in the last column of Table II. The best value of π appears to be 0.40, which is in good agreement with the value of 0.413 determined from the data of Adams *et al.* (20) for the lower alkyl *p*-aminobenzoates. It is also in agreement with other data for goldfish (21, 22).

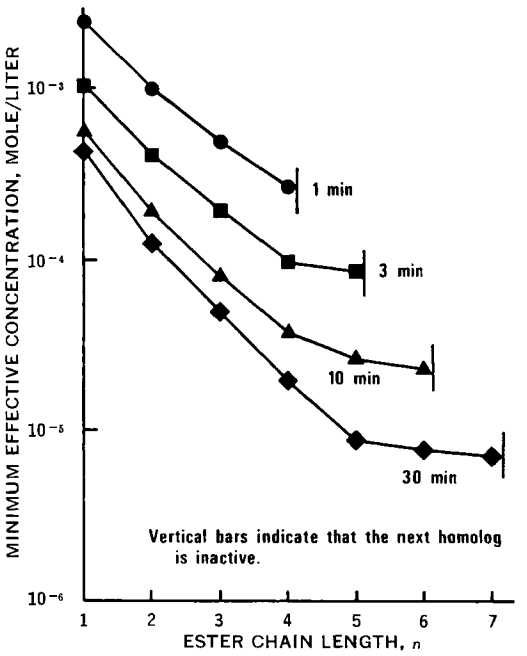

Figure 3—*Minimum concentrations of* n-*alkyl* p-*aminobenzoates required to produce goldfish turnover in specified time periods. Data were interpolated from the equations of Table II.*

According to Eqs. 8 and 10, the intercept at $n = 0$ divided by the concentration can be used to calculate log $[\gamma(PC_0/R_m)]$, and the plateau value divided by the concentration can be used to determine log (γ/R_{aq}).

The curves in Fig. 4 that fit the equimolar data quite well were generated from Eq. 2 with $R_{aq}/\gamma = 2 \times 10^{-3}$, $R_m/\gamma PC_0 = 6 \times 10^{-4}$, and $\pi = 0.40$. The factor γ is not important since it would appear in each term. In all cases, the data (not shown) for the lower concentrations, where the bulk concentration of drug approached the threshold concentration required for activity, fell considerably below the calculated lines.

Saturated Solutions—As mentioned, the logarithms of the solubilities of the homologs are not a linear function of chain length. The solubility data can, however, be adequately described by:

$$\log S_n = -2.042 + 0.342n - 0.837n^2 + 0.000303n^4 \qquad \text{(Eq. 14)}$$

To make Eqs. 7, 9, and 11 applicable to the alkyl *p*-aminobenzoates, log S must be replaced by Eq. 14, which approximates all of the data with a standard error of 9%. The incorporation of the higher order terms presents no difficulties other than the elimination of the expected linearity of log α with n for saturated solutions. The function does, however, approach linearity for $n > 4$.

Although log S is not a linear function of n, it is assumed that log PC increases linearly with alkyl chain length. The nonlinearity of the solubility data is due to alterations in crystal packing with chain length, which were shown to be inapplicable to partitioning (19). The log PC's of the esters of this series were shown to be linear functions of n in four different partitioning systems (19, 23).

To test the model further, Eq. 7 can be solved with Eq. 14 and the determined values of π, $R_m/\gamma PC_0$, and R_{aq}/γ. As shown in Fig. 4, the data for the longer chain lengths and Eq. 7 both have a limiting slope of about −0.6. This slope would be expected most frequently because the value of δ is usually about −0.6 for most homologous series in water.

All data points of Fig. 4 are in excellent agreement with the theoretical lines. These lines are based on only three biological parameters (π, $R_m/\gamma PC_0$, and R_{aq}/γ) and the aqueous concentration or solubility. In changing to another series, the only parameter (other than solubility which can easily be measured) that will change is PC_0. Therefore, if a solvent can be found that truely mimics the partitioning behavior of the diffusion-limiting membrane of the goldfish, it would be possible to characterize

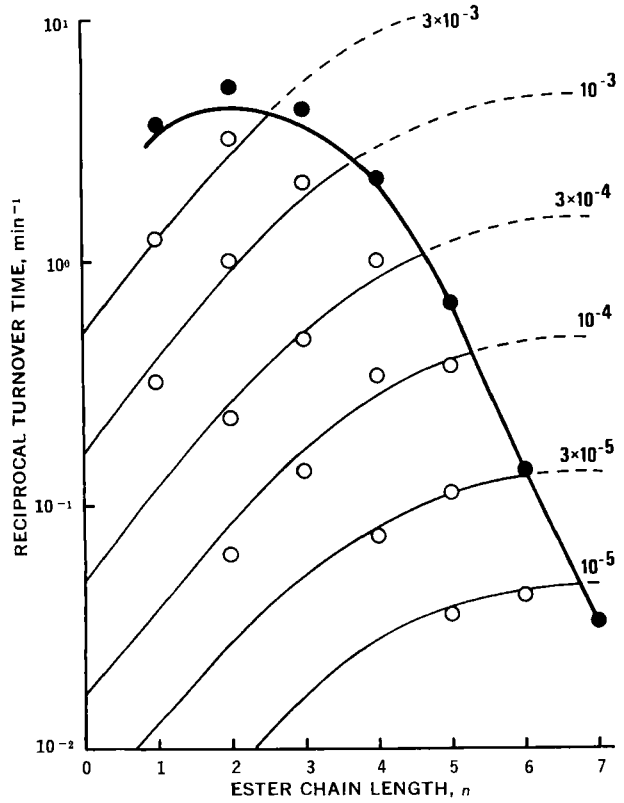

Figure 4—*Turnover times produced by various concentrations and saturated solutions of* n-*alkyl* p-*aminobenzoates. Points are experimental* (○, *unsaturated; and* ●, *saturated*), *and lines are theoretical* (——, *unsaturated; and* ——, *saturated*).

physically all important parameters. Then, since π and γ are constant and R_m and R_{aq} only change slightly, it should be possible to predict completely the relative activities of the members of any group of structurally related, nonspecifically acting compounds.

It is also clear from Fig. 4 that when the compounds are studied on an equimolar basis, rather than on an equitoxic basis as in Fig. 3, there is a definite descending portion of the curve. The term equimolar as used here refers to equivalent amounts of the homolog being present, even if the amounts in solution are not equivalent. The term is used in this manner because frequently a series of drugs is tested by noting the effect of the addition of equal amounts of each member. In many instances, it is not known whether all of the added drug is dissolved or not. The dosing of lower animals with very high milligram per kilogram levels of drug dissolved in an oil or surfactant can easily result in precipitation and diminished activity, which would not occur if the dose were comparable on a milligram per kilogram basis to that given in humans.

The effect of the choice of concentration studied is illustrated by the curves of Fig. 4. The most dilute concentration, 10^{-5} *M*, showed an optimum activity for the hexyl ester. The same compounds tested on the same biological system at 10^{-3} *M* showed maximal activity for the butyl ester while saturated solutions of the ethyl ester were the most active. These curves can be regarded as indicating the expectable error in determining the most active member of a series in humans from rat data when significantly different milligram per kilogram doses are given.

In this particular study, the decrease in activity with increasing chain length results only from the diminished solubility of the higher esters. In no way do the authors intend to suggest that the only reason for decreasing activity with chain length is solubility. Numerous phenomena can be activity limiting (17, 24). Most of these phenomena show chain-length dependencies similar to those described by Eq. 5 or 14 and can be treated in the same manner as solubility has been treated here. In fact, Higuchi and Davis (25) showed that partitioning into a hydrocarbon-like lipid phase also results in an ultimate slope of −0.6 for log α *versus n*.

The equations presented here were derived on the basis of Eq. 2, which describes the transport of a substance across a series of aqueous and lipid barriers. Other models have the same mathematical form as Eq. 2, such as the partitioning model of Wagner and Sedman (26). All of the presented equations could have been derived equally well on the basis of Wagner and Sedman's (26) equations. However, the diffusional model as used here offers a greater degree of simplicity and intuitive satisfaction.

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[x] To whom inquiries should be directed.

Titration of Acids and Acid Mixtures in Benzene–Acetonitrile Binary Solvent System

A. K. AMIRJAHED* and MARTIN I. BLAKE ˣ

Abstract □ In this investigation, the half-neutralization potential of a series of acids of varying pKa was determined in the benzene–acetonitrile solvent system. The half-neutralization potential value of the acids was related to the pKa in a series of benzene–acetonitrile mixtures covering a range of dielectric constants (D_m = 5–35). The slopes of these straight-line relationships were then related to the dielectric constants of the solvent mixtures. The solvent mixture showing the maximum slope (D_m = 30) was used as a differentiating solvent for titrating binary and ternary sulfonamide mixtures.

Keyphrases □ Solvent systems, binary—benzene–acetonitrile system used for determination of half-neutralization potential of acids, effect of dielectric constant □ Benzene–acetonitrile binary solvent system—used for determination of half-neutralization potential of acids, effect of dielectric constant □ Acetonitrile–benzene binary solvent system—used for determination of half-neutralization potential of acids, effect of dielectric constant □ Dielectric constant—series of benzene–acetonitrile mixtures used to determine half-neutralization potential of acids □ Titrimetry—benzene–acetonitrile mixtures of varying dielectric constant used to determine half-neutralization potential of acids

Fritz (1) titrated certain amines potentiometrically in acetonitrile and observed that the millivoltage reading at the half-neutralization point could be correlated with the base strength of the amine. For the alkylamines titrated in certain nonaqueous media, the observed order of base strength was similar to that in alcohol or water (2). Consequently, when data obtained for the nonaqueous solvents were plotted against the equivalent data obtained for water, straight-line relationships were obtained.

The potentiometric titration method was used in studying the relationship between the half-neutralization potential and the pKb (3). When basic amines and phenothiazines were studied in five organic solvents, a linear relationship existed between the pKb in water and the half-neutralization potential values determined in the organic solvents.

A linear relationship between the Δhnp (difference in half-neutralization potential from a standard acid) and the pKa on one hand and between the Δhnp and Hammett's σ value on the other was reported (4). The comparative strengths of some acids in benzene were studied and linear relationships were observed (5). A similar study was reported (6) in which the relative acidity strengths of *meta*- and *para*-substituted benzoic acids and aliphatic monocarboxylic acids in pyridine and water were investigated. Linear relationships were again observed. Organic bases in nitromethane have been studied and a linear relationship between the relative strength of these bases in nitromethane and water was found (7). The factors that may influence the half-neutralization potential of acids and bases in different solvents have been outlined (8). In all these investigations, a straight-line relationship was noted between the relative strengths of acids in water and in nonaqueous media. Thus, the literature indicates that when acids are transferred from water to a nonaqueous medium, the order of their relative strengths generally remains the same. Consequently, if the half-neutralization potential is plotted against the pKa, the relationship is best represented by a straight line. Deviations from linearity are usually explained on the basis of the structural differences among the acids.

THEORETICAL

The dissociation of an acid is represented by Scheme I:

$$HA \rightleftarrows H^+ + A^-$$

Scheme I

The dissociation constant, K_a, is given by:

$$K_a = \frac{[H^+][A^-]}{[HA]} \qquad \text{(Eq. 1)}$$

This equation can be rewritten as:

$$-\log K_a = -\log[H^+] - \log\frac{[A^-]}{[HA]} \qquad \text{(Eq. 2)}$$

and thus:

$$pH = pKa + \log\frac{[A^-]}{[HA]} \qquad \text{(Eq. 3)}$$

When the acid HA is dissolved in water and titrated with a base, at the half-neutralization point the concentration of the base $[A^-]$ is equal to the concentration of the acid [HA] and log $[A^-]/[HA]$ is equal to zero. Therefore, pH = pKa at the half-neutralization point. For a glass–calomel electrode system in an aqueous medium, Delahay (9) used the following equation:

$$V = b + 1.983 \times 10^{-4} T\,pH \qquad \text{(Eq. 4)}$$

where V is the corresponding voltage, b is a constant, and T is the temperature in absolute degrees (Kelvin). This equation indicates that there is an electrical potential (voltage) corresponding to the pH of the aqueous solution and it varies linearly with the pH. One can readily deduce that the pKa of an acid dissolved in an aqueous medium can also be expressed in terms of the electrical potential (millivolts) existing between the two electrodes of the calomel–glass electrode system.

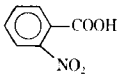
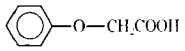
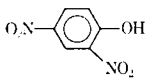
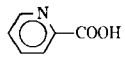
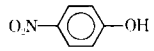
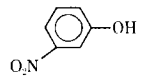
EXPERIMENTAL

Titrimeter—The titrimeter[1] used for the potentiometric titrations was equipped with a microtitration assembly[2] using a saturated calomel and glass electrode system. The titrimeter has an "input range" of ±1400 mv and a "zero stability" of 2 mv/hr after a warmup of no longer than 10 min. The sensitivity is 2 mv and the scale readability is ±2 mv. The accuracy is ±0.5%.

Materials—Tenth normal sodium methoxide in benzene–methanol was prepared and standardized as described previously (10).

[1] Fisher model 35.
[2] Fisher, catalog No. 9-313-280.

Table I—Seven Randomly Selected Acids Used

Acid	Structure	Molecular Weight	pKa at 25°	Reference
o-Nitrobenzoic acid		167.12	2.22	13
Phenoxyacetic acid		152.14	3.12	12
2,4-Dinitrophenol		184.11	4.09	13
Picolinic acid		123.11	5.30	14
4-Nitrophenol		139.11	7.16	13
m-Nitrophenol		139.11	8.34[a]	12
γ-Aminobutyric acid	$NH_2—CH_2CH_2CH_2COOH$	103.12	10.43	12

[a] The only pKa value available to the authors for this acid was 8.34 at 18°.

All other reagents and solvents including benzene, acetonitrile, the different acids, and the sulfonamides were of the highest purity grades available and were used without further purification.

Selection and Preparation of Solvent System—Acetonitrile was selected as the polar component of the binary solvent system to be investigated and benzene was selected as the nonpolar component. Solvent mixtures having dielectric constants of 5, 10, 15, 20, 25, 30, and 35 were prepared according to the procedures described previously (11).

Determination of Half-Neutralization Potentials of Acids—Acids were selected on the basis of their acidic strength in water (pKa) and their physical properties, such as solubility and lack of hygroscopicity, but without regard to their structure; thus the structure effect was randomly distributed. Three hundred potential acids were screened (12–16) and only 25 were found to fulfill these criteria. When these acids were arranged in the order of increasing pKa, seven pKa groups were formed, each containing one or more acids. From these groups, the seven acids listed in Table I were selected for this study.

Exactly 0.15 mEq of acid was weighed into a 20-ml beaker. Fifteen milliliters of the solvent mixture was pipeted into the beaker, and the beaker was immediately covered. The mixture was stirred from 15 to 45 min with the aid of a magnetic stirrer. Usually only 15 min of stirring was required for complete solution. The solution was then titrated potentiometrically with 0.1 *N* sodium methoxide. The titrant was delivered in 0.1-ml increments at a rate of approximately 1 ml/25 min. The beaker remained covered throughout the titration. The titration curve was constructed, and the millivolt reading corresponding to the half-neutralization point was determined and reported as the half-neutralization potential value.

The Δhnp/ΔpKa (the slope of the straight-line graphs obtained by plotting half-neutralization potential of the acids *versus* pKa) was plotted against the dielectric constants of various mixtures of the benzene–acetonitrile solvent system in which the half-neutralization potential's were determined. This curve showed a maximum value for Δhnp/ΔpKa at a dielectric constant, D_m, equal to 30. A large volume of the benzene–acetonitrile mixture having a D_m = 30 was prepared according to the procedure described earlier (11). This was used as the solvent for the differentiating titrations of synthetic powder mixtures of sulfonamides.

Titration of Sulfonamides—Various sulfonamides were selected for study based on their use in medicine and their availability in pure powder form (Table II).

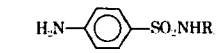
Synthetic powder mixtures of two sulfonamide components were prepared by weighing exactly 0.1 mEq of one and mixing it with exactly 0.15 mEq of the other. The mixture was dissolved in 15 ml of solvent contained in a 20-ml beaker with the aid of a magnetic stirrer. The dissolution process required from 15 to 45 min. The beaker was covered at all times. The solution was then titrated using the same apparatus and method as described previously. The titration curve was obtained by plotting millivolt readings *versus* milliliters of titrant added. The exact end-points were determined from the inflections in the titration curve. Synthetic powder mixtures of three sulfonamide components were also prepared by mixing exactly 0.1, 0.15, and 0.2 mEq, respectively, of the sulfonamides, and the mixtures were titrated in the same manner as described earlier. All titrations were run in duplicate.

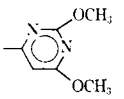
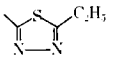
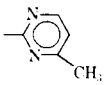
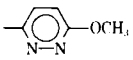
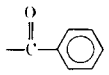
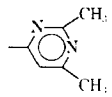
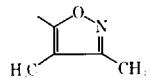
RESULTS AND DISCUSSION

Titration System—Tenth normal sodium methoxide in benzene–methanol was selected as the titrant, and the glass-saturated calomel couple was used as the electrode system. The results of this study are specific to the particular titrant and electrode combination employed. The selected solvents were inert, did not present any leveling effects, and provided a range of dielectric constants while remaining constant in terms of chemical structure. According to Kucharsky and Safarik (18), a wide range of potential measurements is also possible in these types of solvents. The mixture of benzene and acetonitrile acted, in effect, more as a nonreactive medium in which the acid molecules were dispersed and suspended as monomer, dimer, trimer, or higher polymolecular aggregates. The effect and extent of polarity and intermolecular attractions among the solvent molecules themselves are incorporated in the dielectric constant. This is an important parameter in considering a particular solvent mixture to be employed in half-neutralization potential determinations.

Selecting a binary solvent system of a polar and nonpolar component provided a range of available dielectric constants (limited by those of the pure components) out of which it was possible to select mixtures with definite compositions and specific dielectric constants. This approach was more satisfactory than using pure solvents of different dielectric constants, since in the latter case the solvent molecular structure varies with each dielectric constant. In the former case, the molecular species of the solvent system are the same and only their ratio varies with the dielectric constant. Furthermore, it is virtually impossible to find an array of solvents that will provide a range of dielectric constants with a fixed increment in dielectric constant as those of the benzene–acetonitrile solvent system used in this study. It is possible, how-

Table II—Sulfonamides Used in the Differentiating Titrations



Sulfonamide	R	Molecular Weight	pKa at 25°	Reference
Sulfacetamide	—$COCH_3$	214.24	—	—
Sulfadiazine		250.28	6.48	17
Sulfadimethoxine		310.33	—	—
Sulfaethidole		284.36	—	—
Sulfamerazine		264.30	7.06	17
Sulfamethoxypyridazine		280.32	6.7	12
Sulfanilamide	H	172.21	10.43	12
N-Sulfanilylbenzamide		276.31	4.57	12
Sulfapyridine		249.29	8.44	17
Sulfathiazole		255.32	7.12	17
Sulfisomidine		278.34	—	—
Sulfisoxazole		267.30	—	—

ever, that the dielectric constant of the environment immediately surrounding the acid molecule may not necessarily be the same as that measured for the bulk of the binary solvent system. However, any differences should not be significant since the acid molecule will interact with the components of the binary solvent system essentially in the same manner in every case.

Half-Neutralization Potential Determinations—Titration curves for a series of acids in a binary solvent system having variable dielectric constants were obtained by plotting millivolt readings *versus* titrant volume. End-points were readily determined from the inflection in the titration curve. The millivolt reading corresponding to a titrant volume of one-half that represented by the titration end-point was determined and reported as the hnp_1 (first half-neutralization potential value) for the particular acid. This procedure was repeated with the same solvent system and the same acid, and the second half-neutralization potential reading was designated as hnp_2. This process required: *(a)* making the solvent mixture of the desired dielectric constant, *(b)* weighing the exact amount of the acid, and *(c)* titrating the acid in that solvent using 0.1 *N* sodium methoxide. Seven solvent mixtures were employed (D_m = 5, 10, 15, 20, 25, 30, and 35). Seven acids were studied with two replications of the half-neutralization potential measurements for each acid. The order of operations in Steps *a*, *b*, and *c* was determined prior to experimentation by randomizing each step in itself. The two half-neutralization potential determinations on the same acid were not performed immediately. Instead, an entire set of hnp_1 determinations in the same solvent and on all seven acids was performed first; later, a second determination of hnp_2 in the same solvent and for all seven acids was performed.

According to this plan, once the same procedure was performed for each of the seven solvent mixtures, each of the seven acids had been titrated twice and two independent half-neutralization potential values were obtained for it in each of the seven solvent mixtures. The two half-neutralization potential measurements (hnp_1 and hnp_2) for the benzene–acetonitrile solvent system are arranged in Table III. In this table, the pKa's of the seven acids are arranged in an increasing order vertically and the D_m values of the seven mixtures are arranged in an increasing order horizontally. The half-neutralization potentials are recorded in millivolts. It can be seen from this table that the acid with a pKa of 2.22 was titrated in solvent mixtures of D_m = 5, 10, . . ., 35. The two sets of values (hnp_1 and hnp_2) are similar determinations which were performed independently from each other, even with respect to time. All the half-neutralization potential values given vertically under D_m = 5 were determined in a set. This was done by first preparing the solvent mixture of D_m = 5, then weighing all of the acids according to the predetermined random order, and finally titrating them according to the predetermined random order. The process was then repeated to obtain the hnp_2 values given under D_m = 5. As a result of this arrangement, the variations due to temperature fluctuations in the room (±1°), fluctuations in the instrument performance from day to day, and environmental conditions due to time and consequent change in con-

Table III—hnp_1 and hnp_2 Values Determined in Benzene–Acetonitrile Binary Solvent System

pKa		D_m 5	10	15	20	25	30	35
2.22	hnp_1[a]	42	−4	−2	2	15	20	20
	hnp_2	52	−12	−6	−5	19	11	18
3.12	hnp_1	47	42	46	52	57	50	48
	hnp_2	54	40	58	55	55	48	44
4.09	hnp_1	32	18	6	22	42	56	56
	hnp_2	30	10	12	28	38	51	58
5.30	hnp_1	−36	−73	−61	−29	−49	−55	−21
	hnp_2	−2	−76	−61	−41	−44	−38	−24
7.16	hnp_1	−58	−180	−200	−194	−227	−230	−225
	hnp_2	−132	−184	−189	−189	−217	−227	−213
8.34	hnp_1	−293	−300	−256	−294	−292	−294	−292
	hnp_2	−296	−311	−292	−314	−285	−298	−304
10.43	hnp_1	−396	−393	−419	−364	−425	−425	−390
	hnp_2	−280	−412	−433	−453	−412	−438	−408

[a] The half-neutralization potential values are recorded in millivolts.

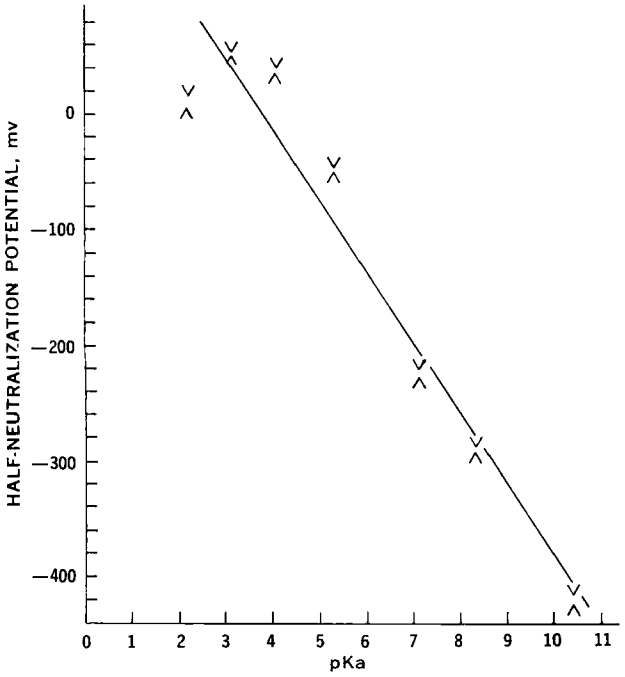


Figure 1—*Half-neutralization potential values of the seven acids measured in a benzene–acetonitrile mixture* (D_m = 25) *and plotted against the corresponding pKa values.*

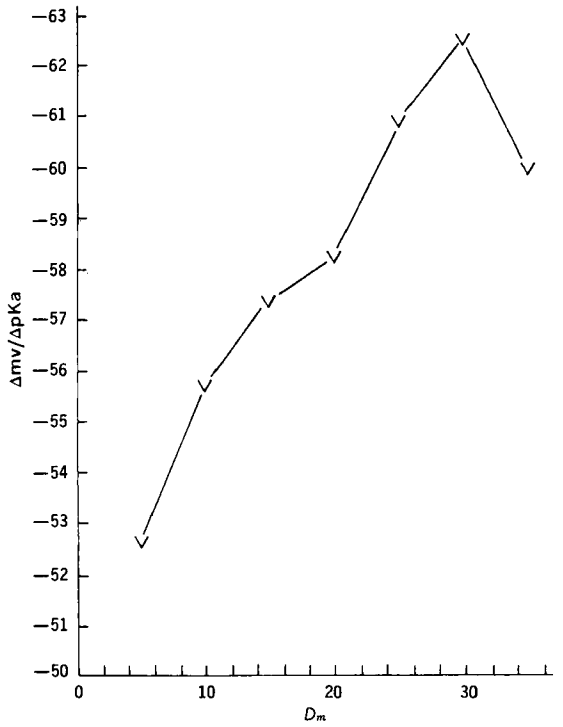


Figure 2—*Slopes of the straight-line relationships obtained in a benzene–acetonitrile binary solvent system plotted against the dielectric constant of the solvent system.*

ditions were randomly distributed among all measured half-neutralization potential values. The plotting of the necessary graphs was also randomized, since it followed the same order as the sequence of the titrations.

The hnp_1 and hnp_2 values determined for all seven acids in one solvent mixture were plotted (on the *Y*-axis) against the pKa values (on the *X*-axis). The procedure recommended by Batson (19) for the least-squares regression line analysis of a univariate normal model completely randomized design was used to determine the formula of the straight line obtained. A representative curve is shown in Fig. 1. Seven such plots were obtained, one for each benzene–acetonitrile mixture. The deviations from the straight line may be due to any one or a combination of the following factors: steric effect, electric field effect, and hydrogen bonding. The formulas of the seven straight lines are reported in Table IV. The slope of these plots is $\Delta hnp/\Delta pKa$, *i.e.*, the change in the observed half-neutralization potential of the acids per unit pKa. Seven such slopes were obtained and plotted against seven D_m values (5, 10, 15, 20, 25, 30, and 35), which represent the entire available dielectric constant range for the benzene–acetonitrile solvent system. The curve obtained (Fig. 2) shows how the change in half-neutralization potential per unit change in pKa varies with the dielectric constant of the solvent. The maximum in the curve indicates the D_m and the composition of the benzene–acetonitrile solvent mixture that may be most suitable for differentiating titrations of mixtures of acids. In differentiating titrations in nonaqueous media, it is desirable to maximize the difference in strength of the components of the mixture of acids to be titrated. This may be realized in differentiating titrations performed in a mixture of benzene–acetonitrile containing 79.50% (w/w) or 81.50% (v/v) of acetonitrile, the composition corresponding to the maximum in the curve depicted in Fig. 2.

The negative slopes of the straight lines (Table IV) indicate that the half-neutralization potential decreases as the pKa increases. In other words, as the acid strength decreases, the half-neutralization potential decreases too (approaching larger and larger negative values). The shape of the curve in Fig. 2 indicates that the acidity strength in the benzene–acetonitrile binary solvent system depends on the value of D_m. The dielectric constant of the mixture (D_m) is a physical constant that represents the total result of all intra- and intermolecular interactions in the binary solvent

Table IV—Formulas for Straight-Line Graphs of the Half-Neutralization Potentials (hnp) Plotted against pKa Values

D_m	Slope of Straight Line[a]	Formula of Straight Line
5	−52.62	hnp = −52.62 pKa + 217.3
10	−55.70	hnp = −55.70 pKa + 192.5
15	−57.36	hnp = −57.36 pKa + 204.8
20	−58.21	hnp = −58.21 pKa + 215.0
25	−60.88	hnp = −60.88 pKa + 230.4
30	−62.52	hnp = −62.52 pKa + 236.8
35	−59.96	hnp = −59.96 pKa + 231.7

[a] The slope, (Δhnp)/(ΔpKa), is in millivolts per unit pKa.

system (hydrogen bonding, induced dipole–induced dipole, permanent dipole–induced dipole, and permanent dipole–permanent dipole interactions). Figure 2 then represents the behavior of the acid molecule in the benzene–acetonitrile solvent system as the proportion of the acetonitrile molecules in the system is increased. The D_m was expressed earlier (11) as a function of X_1 and X_2 for the benzene–acetonitrile solvent system by:

$$D_m = (4.5840 \times 10^{-3}X_1 + 3.7530 \times 10^{-2}X^2)/(2.1153 \times 10^{-3}X_1 + 1.0000 \times 10^{-3}X^2) \quad \text{(Eq. 5)}$$

By using $X_1 = 1 - X_2$, the equation can be solved for X_2:

$$X_2 = (a - D_m c)/(D_m d - b + a) \quad \text{(Eq. 6)}$$

In this equation the constants a, b, c, and d correspond to the coefficients in the previous equation, respectively. When D_m is 30 (maximum of the curve), the mole fraction of acetonitrile in the binary mixture (X_2) is 0.8866. Considering the fact that $X_1 = (n_1)/(n_1 + n_2)$ and $X_2 = (n_2)/(n_1 + n_2)$, where n_1 and n_2 are the number of moles of the nonpolar and polar components, respectively, one can write $(X_2)/(X_1) = (n_2)/(n_1)$. This means that the maximum in the curve occurs when, in every 10,000 molecules of the mixture, 8866 molecules are acetonitrile and 1134 are benzene.

Differentiating Titration of Synthetic Mixtures of Sulfonamides—The principle of differentiating titrations of acids in nonaqueous solvents is to determine quantitatively each component of a mixture of acids in a single titration. The success of such a titration depends on the combination of titrant, the electrode system, and the solvent used. The usual procedure is to vary each factor and to employ all possible combinations in titrating the same mixture. The millivolt readings are plotted against the milliliters of titrant added, and the end-points are determined from the inflection of the curve. A successful differentiating titration is one in which the number of end-points is equal to the number of acid components in the mixture and each end-point accurately reflects the amount of the corresponding acid present in the mixture. This is based on the assumption that acidic impurities in the solvent or the acid mixture, as well as any other external or internal factors that would cause abnormalities resulting in the appearance of false end-points, are absent.

The most common problem in differentiating nonaqueous titrations is the proximity of the strengths of the several acids in the mixture. In other words, if the pKa's of two or more acids in the mixture are too close to each other, the mixture will produce one end-point corresponding to the total acid present in the mixture. This difficulty is especially common when the components of the acid mixture are related structurally. It becomes necessary to test different electrode–titrant–solvent combinations to find a suitable system for differentiating the acids in the mixture.

Assuming that the behavior of an acid molecule in an inert binary solvent system is, among other factors, dependent on the polarity of the solvent system and the totality of the interactions in the solvent system as represented by its dielectric constant, then a solvent mixture of the binary solvent system possessing a D_m value for which the apparent acidity strength of the acid molecule is increased will be a desirable solvent mixture for differentiating nonaqueous titrations. Such a mixture in the benzene–acetonitrile binary solvent system possessed a D_m value of 30. This mixture was represented by a maximum in Fig. 2. Several two-component

Table V—Differentiating Titrations of Synthetic Binary Sulfonamide Mixtures in the Benzene–Acetonitrile Solvent System Containing 81.50% (v/v) or 79.50% (w/w) of Acetonitrile[a]

B	Recovery of A, %	Recovery of B, %
Sulfacetamide	100.11	96.78
Sulfadimethoxine	96.61	97.11
Sulfaethidole	100.10	95.45
Sulfamerazine	100.11	95.11
Sulfapyridine	100.11	96.32
Sulfathiazole	97.61	102.66
Sulfisoxazole	102.62	98.32

[a] A = N-sulfanilylbenzamide, and B = second sulfa drug.

(Table V) synthetic mixtures of the sulfonamides listed in Table II were titrated in the binary solvent system indicated above. Each two-component mixture contained 0.10 mEq of acid A (N-sulfanilylbenzamide) and 0.15 mEq of another acid, B, which was different in each mixture. A three-component mixture containing 0.10 mEq of N-sulfanilylbenzamide, 0.15 mEq of sulfamethoxypyridazine, and 0.20 mEq of sulfanilamide was also titrated in the same solvent mixture. The differentiation of the weak acid components of the mixtures was possible in all cases. The recoveries (average of duplicate samples) for the three-component mixture were 100.11, 96.78, and 102.59%, respectively.

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* Present address: College of Pharmacy, University of Toledo, Toledo, OH 43606

ˣ To whom inquiries should be directed.

Mechanism of Histamine Binding I: Effect of Calcium on Binding of Histamine to a Plasma Peptide

JOHN R. HAZLETT*, PAUL A. KRAMER, and DANE O. KILDSIG[x]

Abstract □ A method of isolating a single polypeptide from human plasma, termed peptide H for its histaminopexic or histamine-binding capability, is described. The peptide was obtained by acid precrystallization of human plasma, followed by water extraction of the precipitate and separation on an ion-exchange column. A molecular weight of 4492 was calculated from amino acid analysis. A dynamic dialysis technique was used to study the binding of histamine to peptide H. No binding of histamine to peptide H was observed in the absence of Ca^{+2}; however, in the presence of Ca^{+2}, binding of histamine to peptide H did occur. It was determined that histamine first forms a complex with Ca^{+2}, with subsequent binding of the Ca^{+2}–histamine complex to peptide H. From the dialysis data and kinetic analysis, it was possible to demonstrate that the data are consistent with a peptide having three binding sites for the Ca^{+2}–histamine complex, two fast sites and one slow site.

Keyphrases □ Histamine binding to plasma protein—calcium effect, mechanism proposed, dialysis and kinetic data □ Plasma protein binding of histamine—calcium effect, mechanism proposed, dialysis and kinetic data □ Calcium effect—histamine binding to plasma protein, mechanism proposed □ Dialysis technique, dynamic—used to determine histamine binding to plasma protein

Histaminopexy, or the ability of serum proteins to bind histamine, was first detected by Parrot *et al.* (1) when it was noted that normal human serum reduced the effect of histamine on the isolated guinea pig ileum, the standard bioassay for histamine. Since then, Parrot and coworkers (2–4) presented several reviews relating to various aspects of histaminopexy. Following the detection of histaminopexy, Parrot *et al.* (5) described the preparation of a human serum fraction, termed plasmapexin, in which the active factor was considered to be a globulin fraction and which demonstrated an ability to bind histamine. A further fractionation was developed to yield a histamine-binding fraction termed plasmapexin I (6).

Guirgis (7) prepared a number of serum protein fractions and tested them for histaminopexic activity and protein content. He concluded that the active fraction was not a γ-globulin but was of relatively low molecular weight. Agneray *et al.* (8) also subsequently concluded that the active histaminopexic factor is probably of low molecular weight and may be associated with immunoglobulin. Several reports have appeared which differ somewhat from the findings of Guirgis (7) and Agneray *et al.* (8) in that histamine was suggested to bind to high molecular weight globulin fractions (9, 10). Gecse *et al.* (11), however, found histamine to be bound to a fraction from rat and human serum containing a polypeptide.

Other investigators found negative results with respect to biological and dialysis tests for histaminopexy. Pautrizel *et al.* (12) found that while asthmatic serum values of histaminopexy were generally lower than normal, the results were too variable to be of more than limited value. Kirtcher and Frankland (13) also indicated that histamine binding by serum is highly unselective. Yet Huriez *et al.* (14) ran over 3000 guinea pig ileum tests and confirmed Parrot's initial observation; Wodniansky (15) evaluated the ileum test on 500 serums, each run three times by three different workers in a double-blind study, and also confirmed the validity of Parrot's finding.

Binding studies using γ-globulin were also shown to produce negative results by Beall (16) and Kaplan and Davis (17). Recently, however, Cohen and Nieforth (18) demonstrated histamine binding to γ-globulin.

When the mass of literature is considered as a whole, the evidence is that when whole serum or a proper serum fraction is used low histaminopexy values are diagnostic of allergies. It is possible that variability in histaminopexy may be such that studies with small numbers of subjects may be unrewarding. One reason for variability may be that guinea pig ileum, due to endocrinological changes, may not show histaminopexy from October to March (19). A more important cause of variability in both the biological test and the binding studies involving γ-globulin and other protein fractions is that, without exception, Tyrodes solution is used as the aqueous medium. Tyrodes solution contains a number of mono- and divalent cations that, as will be seen in this and the following report, affect the binding of histamine to serum protein.

Calcium appears to play a fundamental role in both the release of histamine to the body fluids and in the binding of histamine to blood proteins. Much of the body histamine is stored in mast cells bound to a heparin–protein complex by weak electrostatic forces (20). The release of histamine from the mast cells appears to require the presence of Ca^{+2} (21).

Conversely, the binding of histamine to serum protein also appears to require the presence of Ca^{+2} (4, 22, 23). Calcium salts have been successfully used in the clinical treatment of allergic conditions. Single injections of calcium gluconate into asthmatic patients resulted in marked clinical improvement and *in vitro* tests of the patients' serums indicated histaminopexy (24). Other calcium salts have been found beneficial in the treatment of asthma and hay fever upon prolonged administration (25). Guinea pigs or rabbits on high Ca^{+2} diets or dosed orally with calcium gluconate were found resistant to the bronchoconstrictive effects of histamine (26, 27). As might be

expected, addition of edetate (EDTA) to normal human serum *in vitro* prevented the binding of histamine; however, the addition of calcium chloride restored the serum's ability to bind histamine (28).

While it is obvious that Ca^{+2} affects the binding of histamine *in vitro* and *in vivo*, the mechanism by which Ca^{+2} promotes histamine binding has not been determined. The objective of this study is to elucidate the mechanism of the Ca^{+2}-induced binding of histamine to a polypeptide isolated from human plasma. A dynamic dialysis technique was used to study the peptide–histamine interaction. This technique was developed for the study of binding to macromolecules (29, 30) and recently was thoroughly investigated (31).

EXPERIMENTAL

Materials—Histamine dihydrochloride[1], chromatographically homogeneous, and tromethamine hydrochloride[1], ultrapure, were used in this investigation. The maximum Ca^{+2} limit in the ultrapure tromethamine hydrochloride was 0.00001%. All other chemicals used were reagent grade.

The cellulose membrane[2] (average pore diameter 24 Å) was prepared for use by first rinsing with distilled water and then boiling for 15 min in a 10% acetic acid solution, followed by repeated rinsings with boiling distilled water. The distilled water used throughout was twice distilled, the second time in an all-glass still.

Preparation of Peptide Fraction—Outdated, frozen, pooled human plasma was obtained from a hospital[3] using professional donors and was used routinely. Freshly frozen and fresh pooled human plasma were used for comparisons with the outdated, frozen material. Donors were screened for allergic conditions, and the plasma used was considered to be normal human plasma. A sample of plasma was diluted with 15 times its volume of distilled water. When frozen plasma was used, it was first thawed at room temperature and strained through cotton to remove precipitated material. Hydrochloric acid (0.1 *M*) was then added to the diluted plasma with constant stirring, and the pH was continuously monitored. Any precipitate at pH 6.5 was removed by decanting and centrifugation, and 0.1 *M* HCl was again added to the supernate. At pH 5.2 the precipitate was collected by decantation and centrifugation. The precipitate was centrifuged until very dry, and the tubes were turned upside down on a towel to allow any fluid to drain off. The pellet was washed with a small volume of distilled water and again drained. The precipitate was then removed from the centrifuge tubes and mixed with a volume of distilled water equal to one-half the original plasma volume. The mixture was stirred with a magnetic stirrer for 1 hr and then allowed to settle. After centrifuging, the supernate was collected. Sodium chloride was added to make the solution 0.9% in sodium chloride. This solution was stable with refrigeration for up to 2 weeks.

Column Chromatography—The resin[4] was swollen for 2 hr on a boiling water bath in the 0.1 *M* NaCl used as eluant; it was then cooled and packed into a 35 × 2.5-cm column. A sample of the acid-precipitated fraction already described was applied by pipet, and 5-ml fractions were collected and assayed at 280 nm for protein content. A flow rate of 3 ml/min was used.

Determination of Protein Concentration—The protein concentration was determined at 280 nm, and the following equation was found to apply (32):

$$C = 1.55A \qquad \text{(Eq. 1)}$$

where C = concentration of protein in milligrams per milliliter, and A = absorbance at 280 nm. To check Eq. 1, protein solutions *having known absorbances in* 0.1 *M* NaCl were pipeted into tared aluminum foil cups and dried to constant weight. The cups were weighed and the calculated weight of sodium chloride was subtracted to allow comparison with weights calculated using Eq. 1. A maximum difference of 2.5% between concentrations determined by Eq. 1 and by weighing was observed.

Table I—Results of Amino Acid Analysis of Acid Precipitated Fraction

Amino Acid[a]	(*a*) Milligrams Percent	(*b*) Molecular Weight	(*a*)/(*b*)	(*c*) Number of Residues	(*b*) × (*c*)
Alanine	1.30	89.09	0.015	3	267.27
Valine	1.15	117.15	0.010	2	234.30
Leucine	1.95	131.17	0.015	3	393.51
Proline	0.85	115.13	0.007	2	230.26
Threonine	0.95	119.12	0.008	2	238.24
Serine	0.90	105.09	0.009	2	210.18
Phenylalanine	1.15	165.19	0.011	2	330.38
Aspartic acid	1.85	133.10	0.014	3	399.30
Glutamic acid	3.15	147.13	0.021	4	588.52
Tyrosine	0.90	181.19	0.005	1	181.19
Lysine	1.90	146.19	0.013	3	438.57
Histidine	0.80	155.16	0.005	1	155.16
Arginine	0.55	174.20	0.003	1	174.20
Cystine	0.70	240.30	0.003	1	240.30
Glycine	0.40	75.07	0.005	1	75.07
Isoleucine	0.30	131.13	0.002	1	131.13
Methionine	0.10	149.21	0.0007	0	—
					4288.00
Tryptophan[b]	—	204	—	1	204
					4492

[a] Amino acid determination by Analytical BioChemistry Laboratories, Columbia, MO 65201. [b] Tryptophan presence was confirmed by the glyoxylic acid test, and one residue is assumed.

Freezing-Point Depression Determination—The freezing-point depression of the fraction already described was determined using a freezing-point apparatus[5] with a differential thermometer and an ice–salt water bath. The freezing-point depressions of two 30-ml samples at a concentration of 0.21 mg/ml (in distilled water) were determined, as were the freezing points of two distilled water blanks.

Amino Acid Analysis—A GLC amino acid analysis was conducted on a sample of the fraction already described. Hydrolysis was effected by 6 *N* HCl, thus destroying any tryptophan present. The results (Table I) were used to calculate a molecular weight by the least-ratios method. The respective milligram percentages of each amino acid were divided by the molecular weight of the amino acid. The common divisor was 0.005, and methionine was eliminated to give the results shown in Table I.

Tryptophan Determination—Tryptophan was tested for using the Hopkins–Cole or glyoxylic acid reaction (33). One milliliter of 5% glyoxylic acid in water was mixed with 1 ml of a 0.19-mg/ml fraction in distilled water and layered over 1 ml of concentrated sulfuric acid. The appearance of a violet ring is indicative of the presence of an indole ring system. A distilled water blank was also run.

Dialysis Apparatus and Techniques—The dynamic dialysis technique used in this investigation closely follows that of Meyer and Guttman (31). The apparatus consisted of a 4-cm diameter, 7 cm high, cylindrical plastic cell suspended in a 100-ml jacketed beaker. The cellulose membrane was secured across the lower opening of the plastic cell. Seventy-five milliliters of tromethamine buffer at 37° was placed in the jacketed beaker and comprised the external solution. Ten milliliters of histamine, 9.0×10^{-4} *M*, in tromethamine buffer was pipeted into the plastic cell along with 10 ml of the peptide solution in tromethamine buffer or a blank solution of tromethamine buffer. Whenever used, the calcium chloride concentration was the same in the internal and external solutions. The plastic cell containing the internal solution was placed in the external solution and leveled with the external solution to prevent a hydrostatic head. A magnetic stirrer rotat-

[1] Schwarz/Mann, Orangeburg, NY 10962
[2] Sargent Welch, Skokie, Ill.
[3] St. Elizabeth's Hospital, Lafayette, IN 47904
[4] Sephadex G-25, *Pharmacia Fine Chemicals, Inc.*, Piscataway, N.J.
[5] Molecular weight apparatus, Beckmann, Sargent Welch Co., Skokie, Ill.

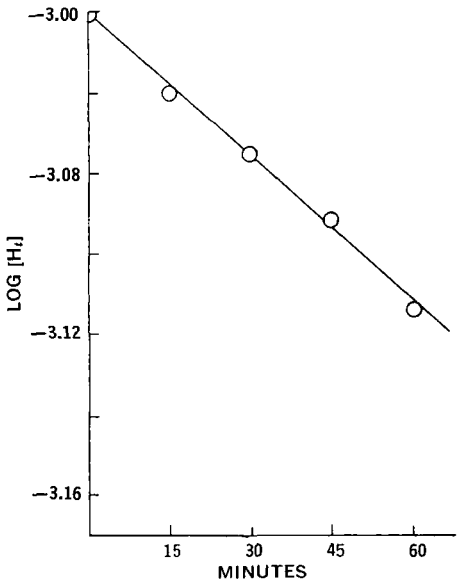


Figure 1—*Plot of log of total histamine* $[H_t]$ *remaining in the dialysis cell at time* t *as a function of time. The membrane constant,* K_m*, is determined from the slope of the line.*

ing at 200 rpm agitated the external solution. The internal solution was stirred by a twisted glass rod at 50 rpm. Temperature was maintained at 37° by pumping water through the jacketed beaker from a controlled-temperature bath.

Samples of 25 ml were taken by pipet at 15-min intervals with replacement. The sample volume maintained sink conditions in the external solution for histamine. The concentrations were corrected for the replacement volumes. Dynamic dialysis determinations were made for histamine and mixtures of histamine and Ca^{+2}. Histamine was assayed spectrophotometrically at 211 nm. There was no observable binding of histamine to the cellulose membrane or plastic container in the presence or absence of Ca^{+2} during the 1-hr dynamic dialysis determination.

Determination of Membrane Constant—The constant was determined from the dialysis of histamine through the membrane in the absence of peptide H. The basic equation for dynamic dialysis was given by Meyer and Guttman (31):

$$\frac{-d[H_t]}{dt} = K_m[H_f] \qquad \text{(Eq. 2)}$$

where $[H_f]$ is the free histamine concentration at any time, $[H_t]$ is the total histamine concentration remaining in the dialysis cell or internal solution at time t, $d[H_t]/dt$ is the dialysis rate, and K_m is the membrane constant. Plots of log $[H_t]$ *versus* time were found to be linear (Fig. 1), and K_m was calculated from the slope.

The nature and design of the experimental apparatus permitted the same cellulose membrane to be used throughout this study. Since all dialysis determinations were made through a constant area of the same membrane, there were no significant differences between replicate determinations.

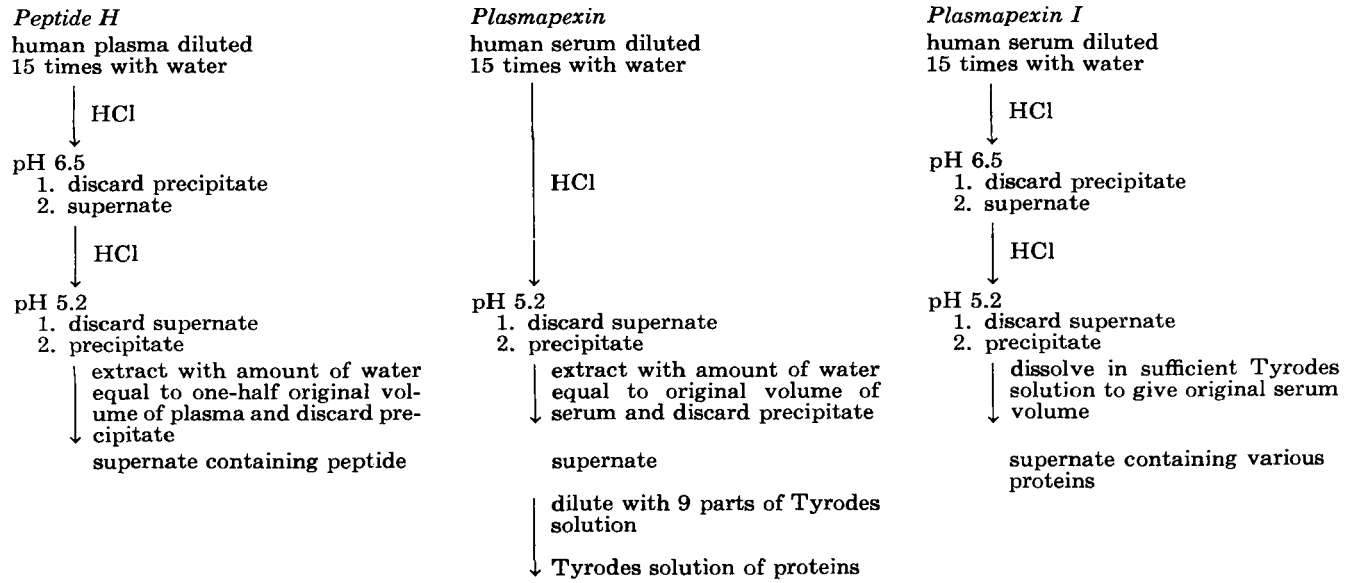
Determination of Initial Free Histamine Concentration—Solutions of histamine (4.5×10^{-4} *M*), Ca^{+2} (9×10^{-3} *M*), and peptide (8.6×10^{-5} *M*) in pH 7.4, 0.9% NaCl-tromethamine buffer were incubated in a 250-ml volumetric flask suspended in a 37° water bath. Twenty-milliliter samples were withdrawn by pipet at appropriate intervals over 24 hr. The samples were then used as the internal solutions of dynamic dialysis runs. The total histamine remaining in the cell was plotted against time, and from these plots the initial rate could be determined. By knowing the initial rate and the membrane constant, the initial free histamine concentration as a function of time was calculated by Eq. 2.

Preequilibration of Calcium Chloride with Histamine and Peptide H—Calcium chloride was equilibrated separately with histamine and peptide H for 8 hr at 37°. Solutions were in pH 7.4, 0.9% NaCl-tromethamine buffer. The calcium chloride was at twice the concentration used during the dynamic dialysis since the equilibrated solution was diluted with an equal amount of either histamine or peptide H solution prior to dialysis.

RESULTS AND DISCUSSION

Isolation of Peptide H—The method used to isolate a single polypeptide from human plasma was similar to methods previously used to isolate two histaminopexic fractions, plasmapexin and plasmapexin I, from human serum (5, 6). The isolated polypeptide was termed peptide H for reference purposes. The preparation of plasmapexin, plasmapexin I, and the acid-precipitated fraction used throughout the rest of this study is outlined in Scheme I. Plasmapexin differs from plasmapexin I in that for plasmapexin I the precipitate at pH 6.5 is discarded and the precipitate at pH 5.2 is dissolved in Tyrodes solution, not water. The fraction used in this study was prepared from plasma, not serum. The precipitate was taken only between pH 6.5 and 5.2 as for plasmapexin I but was extracted with water instead of Tyrodes solution. When Tyrodes solution was used, the entire precipitate went into solution, as it also did with pH 7.4 tromethamine buffer containing 0.9% NaCl. When water was used, however, the amount of material extracted from the precipitate was very small.

If the blood fraction responsible for histaminopexy was a polypeptide and was contained in the precipitate at pH 5.2, its water solubility should be greater than the solubility of the higher molecular weight globulins also present in the precipitate. For this reason, a selective solubilization of the pH 5.2 precipitate with



Scheme I—Isolation of plasmapexin, plasmapexin I, and peptide H

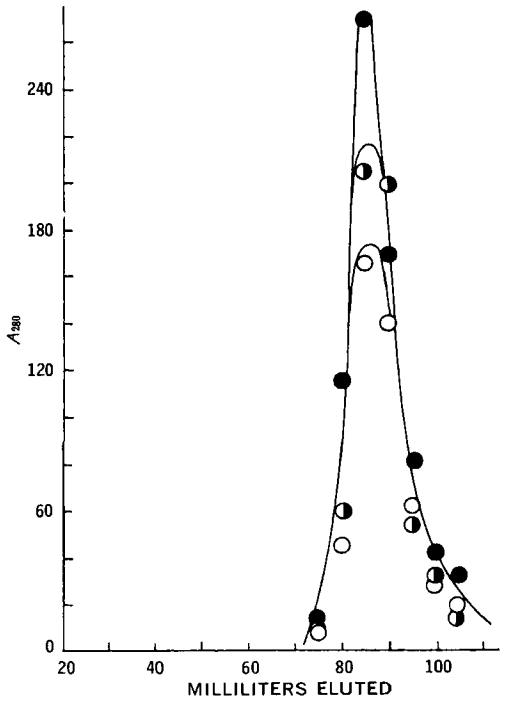


Figure 2—*Absorbance at 280 nm of the eluted peptide H from the ion-exchange column. Peptide H was prepared from fresh* (○)*, fresh frozen* (●)*, and outdated frozen* (◑) *pooled human plasma.*

distilled water, containing no salts, was used to exclude the high molecular weight proteins and isolate the polypeptide. The acid precipitation and water extraction procedure was used on fresh, pooled human plasma; fresh frozen, pooled human plasma; and outdated, frozen, pooled human plasma. In preparation it was noted that the outdated frozen plasma contained large amounts of precipitated matter when thawed. At pH 6.5, however, there was little or no precipitate present. The fresh frozen plasma contained some precipitated material when thawed and a moderate amount at pH 6.5. The fresh plasma had no precipitated material until the pH was lowered to pH 6.5 when considerable precipitate was noted. When the precipitate between pH 6.5 and 5.2 was extracted with water, most of the precipitate remained undissolved in all three cases.

When samples of these three fractions were chromatographed[4] and eluted with 0.1 *M* NaCl, only single sharp peaks with volumes of elution of 87 ml were noted (Fig. 2). The fractionation range of the resin is 1000-5000 for peptides and globular proteins.

The single sharp peak for fractions prepared from fresh, fresh frozen, and outdated frozen plasma demonstrated that the material is not a decomposition or fragmentation product due to the age or freezing of the plasma. Furthermore, it suggested that a single protein was present. The fractionation range of the resin indicated that it was actually a peptide, and the elution volume indicated that it was in the upper weight range.

Amino Acid Analysis and Molecular Weight Determination—The acid hydrolysis of peptide H, followed by amino acid analysis, resulted in the identification of 32 amino acid residues (Table I). The molecular weight of peptide H was found to be 4288. This was calculated by the least-ratios method assuming 32 amino acid residues and a milligram per molecular weight ratio of 0.005 equal to one residue.

Tryptophan was not determined in the amino acid analysis because it was destroyed by acid hydrolysis. When the peptide H-glyoxylic acid mixture was layered onto concentrated sulfuric acid, a violet ring appeared at the interface, indicating the presence of tryptophan. While the glyoxylic acid test detected tryptophan, it is a qualitative test and is not capable of quantitatively determining the number of tryptophan residues present in the peptide molecule. Therefore, the molecular weight of peptide H was also determined by measuring the freezing-point depression of a peptide solution of known concentration (the concentration was determined by Eq. 1). For two determinations the freezing points of the peptide solutions were identical, as were those of the distilled water blanks, and gave a molecular weight of 4340. If one tryptophan residue is assumed in the peptide H molecule, a molecular weight of 4492 is obtained (4288 + 204), differing from the value obtained by the freezing-point method by only 3.4% and correlating with the fractionation range of the resin. The value of 4492 was used in subsequent calculation of molar concentrations of peptide H. The agreement of the freezing-point depression molecular weight and that calculated from the amino acid analysis and added tryptophan, together with the chromatography results, substantiates the conclusion that the water extract of pH 6.5 and 5.2 acid-precipitated plasma is a single peptide.

Demonstration of Histaminopexy and Its Requirement for Divalent Calcium—From the results of dynamic dialysis it was determined that peptide H binds histamine, but only in the presence of Ca^{+2}. Figure 3, where the log of total histamine remaining in the cell is plotted *versus* time, shows that the blank run of histamine and calcium is linear following first-order kinetics. The plot for histamine in the presence of peptide H with no Ca^{+2} coincides with the blank run showing no binding. However, the plot of histamine and peptide H with Ca^{+2} shows a decreased slope, indicating binding. It also is linear over this time period, obeying apparent first-order kinetics.

There are essentially two pathways by which Ca^{+2} may promote the binding of histamine to peptide H. One pathway is through an initial Ca^{+2}-peptide H association resulting in a direct or indirect change of the charge density at the binding sites or a change in the conformation of peptide H. A conformation change for bovine serum albumin accompanying histamine binding has been reported (34). The second possible pathway is through an initial Ca^{+2}-histamine association, with subsequent binding of the Ca^{+2}-histamine complex to peptide H.

The possibility of Ca^{+2} influencing the peptide in some manner was investigated by equilibrating Ca^{+2} with peptide H prior to the addition of histamine, while the preequilibration of Ca^{+2} with histamine prior to the addition of peptide H was used to elucidate the possible requirement for the Ca^{+2}-histamine complex prior to binding. Figure 4 shows the results of the preequilibration studies. When Ca^{+2} was preequilibrated with peptide H, there was no increase in binding as compared to the binding evidenced when the Ca^{+2}, histamine, and peptide H were simultaneously mixed prior to dialysis. Thus, it is evident that a conformation change such as that reported for histamine-bovine serum albumin bind-

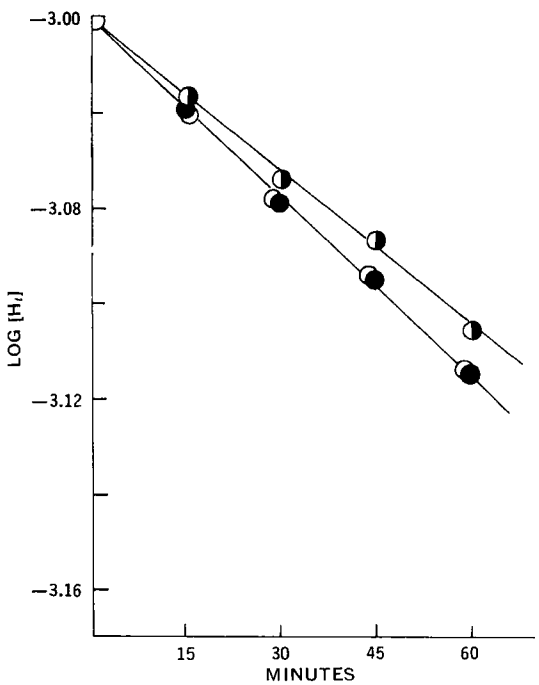


Figure 3—*Dynamic dialysis results showing the effect of Ca^{+2} on the binding of histamine to peptide H. Key:* ○*, histamine and Ca^{+2};* ●*, histamine and peptide H; and* ◑*, histamine, Ca^{+2}, and peptide H.*

ing is not involved in the mechanism of histamine binding to peptide H. However, the preequilibration of Ca^{+2} with histamine resulted in a lower dialysis rate, indicating increased histamine binding to peptide H. It is then evident that the complexation of histamine and Ca^{+2} is the essential step in binding to peptide H and that the Ca^{+2}–histamine complex is the molecular species responsible for histamine binding to peptide H.

Kinetics of Interaction of Ca^{+2}–Histamine Complex with Peptide H—The result of allowing peptide H, Ca^{+2}, and histamine to react in a volumetric flask and following the reaction using dynamic dialysis runs to determine the concentration of free histamine is shown in Fig. 5. The time scale for the disappearance of free histamine is hours for this ternary mixture of the three components. While preequilibration of Ca^{+2} and histamine was shown previously to be required for binding to peptide H, the preequilibration time was chosen as 8 hr without elucidating how much of that 8 hr might actually be required for Ca^{+2}–histamine interaction and/or conformational change and how much is a time-dependent adaptation of the peptide. It can be concluded from the data presented that the 8 hr of preequilibration of Ca^{+2} and histamine is sufficient to produce essentially maximal binding, that 8 hr of incubation of the three components is also sufficient, and that no binding is observed without preequilibration. At 24 hr, the ratio of bound histamine (2.18×10^{-4} *M*) to peptide H concentration (0.86×10^{-4} *M*) was 2.54 (Fig. 5), suggesting at least three histamine binding sites per peptide molecule.

Two kinetic mechanisms have been considered to account for the disappearance of histamine observed in Fig. 5. If the data are plotted semilogarithmically, a straight line is obtained during the initial stages of the reaction (Fig. 6). If the reaction is indeed controlled by a first-order mechanism, the final bimolecular reaction must be fast compared to a slower intermediate process. The mechanism shown in Scheme II may be proposed to account for an apparent first-order reaction:

$$Ca^{+2} + \text{histamine} \overset{\text{fast}}{\rightleftharpoons} \text{CaHi} \qquad \text{(Step 1)}$$

$$\text{CaHi} \overset{\text{slow}}{\rightleftharpoons} \text{CaHi}' \qquad \text{(Step 2)}$$

$$\text{CaHi}' + \text{peptide} \overset{\text{fast}}{\rightleftharpoons} \text{CaHi}P \qquad \text{(Step 3)}$$

Scheme II

Chawla (35) obtained an association constant of $10^3\ M^{-1}$ for Step 1 using a titrimetric method, indicating a very rapid approach to equilibrium relative to the time scale depicted in Fig. 5. The slow step, Step 2, must therefore involve a conformational change in the CaHi complex. Kier (36) reported two conformers of histamine in solution, one having an intranitrogen bond distance of 4.55 Å and the other 3.6 Å. The conformation of the CaHi complex is, however, unknown. To be consistent with a first-order mechanism, there must be a binding specificity for one conformer, with the rate-determining step being the slow conversion of the CaHi complex to the desired conformer. While such a mechanism is consistent with observed kinetics, it seems unlikely to the authors that such a rearrangement of an organic ion–metal ligand complex would occur.

It seems more likely that whatever the structure of the CaHi complex, different sites on the peptide might be available for binding at different rates. The kinetics of Fig. 5 were thus interpreted in terms of a bimolecular reaction involving sites with different rate constants for binding the CaHi complex. This is not in conflict with Fig. 6, because the linear portion of the line is over only slightly more than 2 half-lives, during which time it is difficult to distinguish between first- and second-order reactions. The following assumptions were made concerning the binding process:

1. There are three histamine binding sites per peptide H molecule (from Fig. 5).
2. The sites are independent.
3. The reaction is second order and can be treated as irreversible.
4. [Hi] is approximately equal to [CaHi] due to the high association constant, $K_{[\text{CaHi}]} = 1.18 \times 10^3\ M^{-1}$ (35).

Bimolecular reactions of the calcium-histamine complex with peptide H are depicted in Scheme III for two types of sites having different rate constants, where [CaHi] represents the concentration of the calcium–histamine complex, [*P*] represents the concentration of unoccupied peptide binding sites, and [CaHi*P*] represents the concentration of peptide-bound calcium–histamine complex, all at a given time *t*. The subscripts *f* and *s* refer to fast and slow binding sites on the peptide. In all subsequent discussion, the subscript 0 shall be used to denote the initial concentration of any one of these species; k_s and k_f are the rate constants associated with binding to the slow and fast peptide sites, respectively.

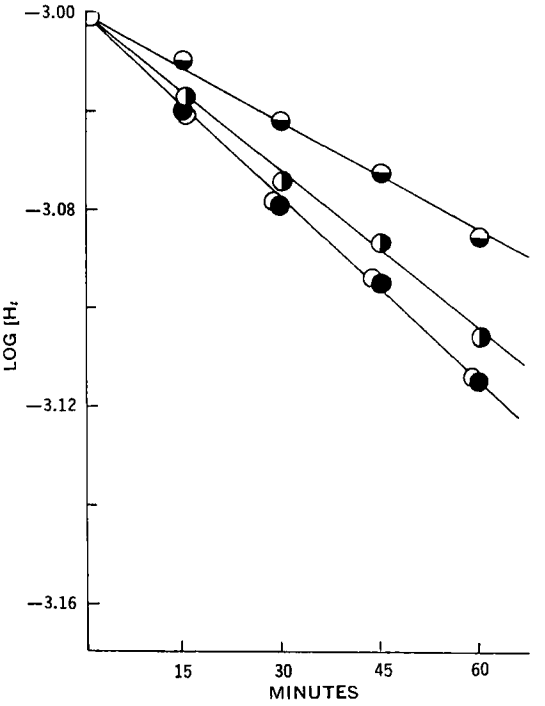


Figure 4—*Dynamic dialysis results showing the effect of preequilibration on the binding of histamine to peptide H. Key:* ○, *histamine and* Ca^{+2} *blank;* ●, *histamine and peptide H;* ◑, *histamine,* Ca^{+2}, *and peptide H (no equilibration) and histamine added to preequilibrated* Ca^{+2} *and peptide H; and* ◒, *peptide H added to preequilibrated* Ca^{+2} *and histamine.*

$$[\text{CaHi}] + [P_f] \xrightarrow{k_f} [\text{CaHi}P_f]$$

$$[\text{CaHi}] + [P_s] \xrightarrow{k_s} [\text{CaHi}P_s]$$

Scheme III

Let us consider first the second-order rate expression for the reaction of CaHi with a *single* type of peptide site, *P*:

$$\frac{d[\text{CaHi}P]}{dt} = k[\text{CaHi}][P] \qquad \text{(Eq. 3)}$$

Since:

$$[\text{CaHi}] = [\text{CaHi}]_0 - [\text{CaHi}P] \qquad \text{(Eq. 4)}$$

and:

$$[P] = [P]_0 - [\text{CaHi}P] \qquad \text{(Eq. 5)}$$

Eq. 3 can be rewritten as:

$$\frac{d[\text{CaHi}P]}{dt} = k([\text{CaHi}]_0 - [\text{CaHi}P])([P]_0 - [\text{CaHi}P]) \qquad \text{(Eq. 6)}$$

Integrating Eq. 6 between [CaHi*P*] = 0 at $t = 0$ and [CaHi*P*] = [CaHi*P*] at $t = t$ yields:

$$kt = \frac{2.303}{[\text{CaHi}]_0 - [P]_0} \log \frac{[\text{CaHi}]_0 - [\text{CaHi}P]}{[P]_0 - [\text{CaHi}P]} - K \qquad \text{(Eq. 7)}$$

A plot of $\log([\text{CaHi}]_0 - [\text{CaHi}P])/([P]_0 - [\text{CaHi}P])$ *versus* time

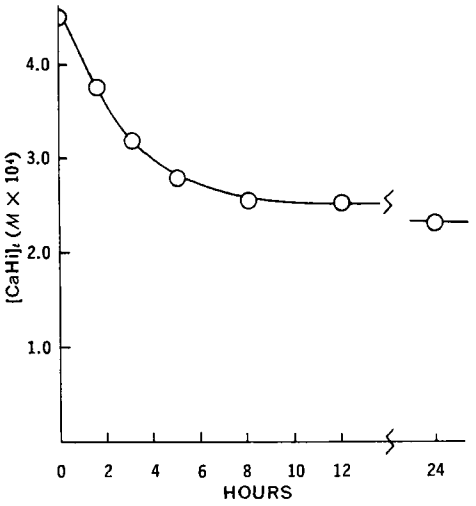


Figure 5—*Free histamine concentration as a function of time, as determined by dynamic dialysis, from which the total bound histamine can be determined.*

will, therefore, be linear for a single type of binding site with an intercept equal to K. Figure 7 shows such a plot where [CaHiP] is the difference between [CaHi]$_0$ and [CaHi] and all the peptide binding sites are considered equivalent. The plot is *not* linear, showing that an assumption of a single type of peptide site is not valid.

If one now considers the postulation of two types of peptide binding sites such that:

$$[P]_0 = [P_f]_0 + [P_s]_0 \qquad \text{(Eq. 8)}$$

then the appropriate rate constants are k_s and k_f, as given in Scheme III, and the logarithmic extrapolation method of Mark and Rechnitz (37) may be used to determine the initial number of fast and slow sites and the rate constants k_s and k_f.

The initial curved portion of Fig. 7 can now be interpreted as a time period when both fast and slow sites are involved in peptide binding of the calcium–histamine complex, and the linear portion of the curve represents a time period when all fast sites are occupied and a single type of site, namely the slow sites, is being

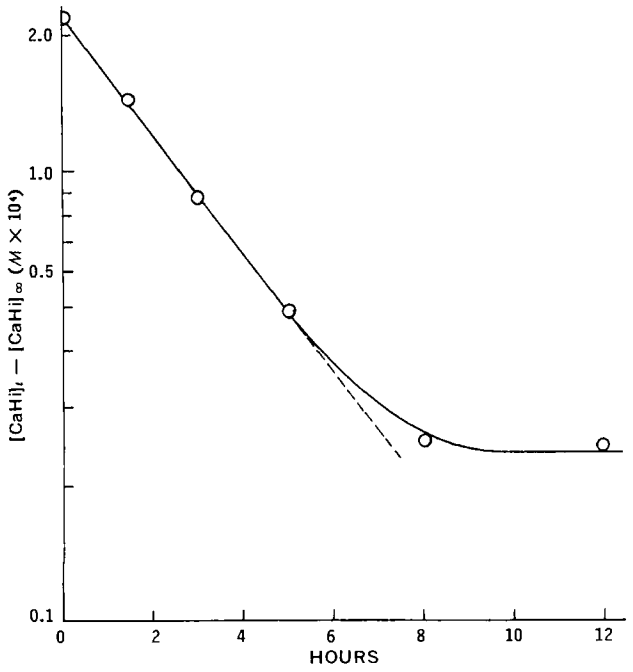


Figure 6—*Semilogarithmic plot of the data in Fig. 5 showing the apparent first-order behavior over approximately 2 half-lives.*

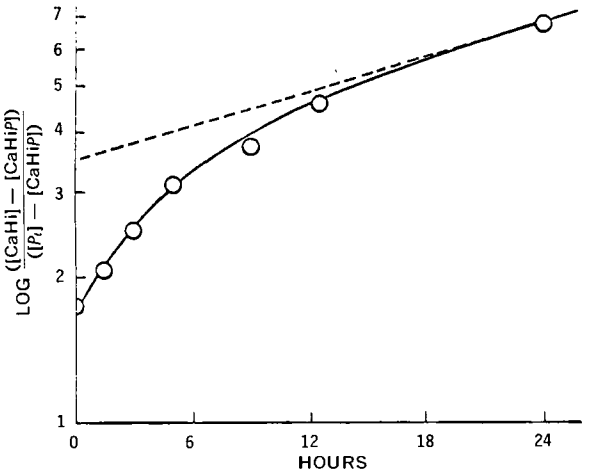


Figure 7—*Graphical representation of Eq. 8 representing the extrapolation method of kinetic analysis. The extrapolation of the linear portion of the curve* (-----) *to* t = *0 gives the numerical value of* K.

filled. For the linear portion of the curve:

$$k = k_s \text{ and } [P]_0 = [P_f]_0 + [P_s]_0 \qquad \text{(Eq. 9)}$$

$$[\text{CaHi}P] = [\text{CaHi}P_f] + [\text{CaHi}P_s] = [P_f]_0 + [\text{CaHi}P_s] \qquad \text{(Eq. 10)}$$

Equation 7 describing the linear portion of Fig. 7 can then be written:

$$k_s t = \frac{2.303}{[\text{CaHi}]_0 - [P]_0} \log \frac{[\text{CaHi}]_0 - [P_f]_0 - [\text{CaHi}P_s]}{[P]_0 - [P_f]_0 - [\text{CaHi}P_s]} - K \qquad \text{(Eq. 11)}$$

This expression enables one to calculate an intercept K for the extrapolation of the linear portion of Fig. 7 to the ordinate. At t = 0, [CaHiP_s] = 0 and Eq. 11 becomes:

$$K = 2.303 \log \frac{[\text{CaHi}]_0 - [P_f]_0}{[P]_0 - [P_f]_0} \qquad \text{(Eq. 12)}$$

From Fig. 7, an extrapolated intercept of log 3.5 is obtained; from Eq. 12, $[P_f]_0$ was calculated to be 1.80×10^{-4} M. This value is almost exactly twice the molar concentration of peptide (1.72×10^{-4} M) and is excellent evidence that there are two sites with the faster binding rate, leaving one site with the slower rate.

The rate constant for the slow reaction was determined from the slope of Fig. 7 beyond 12 hr where the two faster sites are essentially saturated, leaving only the slower site P_s. The slower constant, k_s, was calculated to be 1.19×10^2 M^{-1} hr^{-1}. To obtain the fast rate constant, k_f, the value of k_s was subtracted from the apparent constant calculated from the first linear portion of Fig. 7; k_f was calculated to be 5.08×10^2 M^{-1} hr^{-1}. To verify the accuracy of the calculated rate constants and also that there are two fast sites and one slow site, the rate of histamine-Ca^{+2} binding to peptide H was calculated by substitution of k_f and k_s into the rate equation:

$$\text{rate of binding} = \frac{d[\text{CaHi}P]}{dt} = k_f[\text{CaHi}][2P] + k_s[\text{CaHi}][P] \qquad \text{(Eq. 13)}$$

and this value for the rate of binding (5.1×10^{-5} M hr^{-1}) compared with the rate calculated by two other methods.

First the rate of binding was calculated from dynamic dialysis data, assuming that the difference between the initial rate of appearance of histamine in the external solution when no binding occurred and when binding did occur was equal to the rate at which histamine was being irreversibly bound to peptide H. This was found to be 5.9×10^{-5} M hr^{-1}. In the second method, a tangent was drawn to the initial slope of Fig. 5 and an initial rate, 6.0×10^{-5} M hr^{-1}, was calculated. These two values are in very good agreement with the rate calculated from k_f and k_s.

Recently, Gecse *et al.* (38) reported the isolation of a peptide from human serum having histaminopexic properties. They also used the same resin to isolate the peptide which must then, as they indicate, have a molecular weight between 1000 and 5000. The peptide was isolated from 30 healthy human subjects. Of significance was the fact that serum from eight allergic patients lacked the ability to bind histamine. Their isolation is in accord with the present findings.

No attempt was made in this study to determine if other plasma fractions bound histamine, and certainly other histaminopexic fractions may be isolated in further investigations. The characteristic dependence of histamine binding on the presence of Ca^{+2} is good evidence, however, that peptide H is at least in part responsible for histaminopexy. It must be realized that this conclusion is based entirely on *in vitro* evidence. Extensive additional studies will be required with allergic and nonallergic patients to evaluate the extension of this conclusion to human subjects.

While the binding of histamine to peptide H is most likely an equilibrium phenomenon, the initial assumption that the binding can be treated as irreversible appears reasonable. The excellent agreement between rates calculated using the second-order equation and those observed experimentally indicates that the rate of the back-reaction does not appreciably affect the assumption of irreversibility over the time period studied. It is important to note that the histamine binding to peptide H differs from most drug–protein interactions in that it is not a rapidly reversible process. This is evidenced by the time scale in Fig. 5 in which the concentration of unbound histamine [CaHi] is changing through 24 hr. As such, it was necessary to utilize a kinetic analysis of the data rather than the equilibrium analysis more common to drug–protein binding studies.

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*National Institutes of Health Predoctoral Fellow. Present address: Burroughs-Wellcome Company, Greenville, N.C.

x To whom inquiries should be directed.

Bioavailability of Aminosalicylic Acid and Its Various Salts in Humans III: Absorption from Tablets

SUK HAN WAN[x], PERTTI J. PENTIKÄINEN, and DANIEL L. AZARNOFF

Abstract □ The relative bioavailability of commercially available preparations of aminosalicylic acid and its sodium, potassium, and calcium salts is presented. The doses administered were 4 g for aminosalicylic acid and 2.8, 2.6, and 2.6 g for the sodium, potassium, and calcium salts, respectively. Absorption from tablets of the salts was rapid and complete, but absorption of the free acid was only 77% of the dose. Dissolution of the relatively insoluble acid is a rate-limiting factor in absorption. Although the amount of free acid administered and the absolute amount absorbed were higher, the area under the plasma concentration curve of unmetabolized drug was less than for the salts. This is attributed to capacity-limited acetylation of drug, especially during the first pass. The amount of bioavailable drug is dependent on the rate of metabolism and, hence, the rate of absorption.

Keyphrases □ Aminosalicylic acid and salts—absorption from commercial tablets compared, humans □ Bioavailability—absorption of aminosalicylic acid and salts from commercial tablets, humans □ Absorption—aminosalicylic acid and salts from commercial tablets, humans

Numerous aminosalicylic acid preparations are currently available to the practitioner. Choice of product is rendered difficult by the confusing literature on the merits of different preparations (1–4). The confusion has arisen from inadequate standardization and sampling during studies and from the previous poor understanding of the significant nonlinear pharmacokinetics of this drug.

The present report concerns absorption from tablets of aminosalicylic acid and several salts. The data were obtained as part of a series of bioavailability studies on this compound. A previous report (5) showed that the absorption of its salts in solution differed with respect to rate of absorption and the peak levels achieved. Dissolution of drug in suspension was a limiting factor in absorption. The current report describes crossover studies concerning absorption of aminosalicylic acid and the sodium, potassium, and calcium salts from commercially available tablets.

EXPERIMENTAL

Drugs—Tablets of aminosalicylic acid (I) (0.5 g), its sodium salt (II) (0.5 g), and its calcium salt (III) (0.5 g) were supplied by the Food and Drug Administration (FDA) from a single manufacturer[1]. The potassium salt (IV) (0.5 g) was purchased on the open market from a second manufacturer[2]. All tablets complied with USP specifications[3].

Subjects—Twelve healthy volunteers (nine men and three women) with no history of liver or kidney disease participated in the study. Prospective participants were screened by physical examination and laboratory tests including complete blood count, serum glutamate pyruvate transaminase, serum glutamate oxalate transaminase, urinalysis, and serum alkaline phosphatase and creatinine. Written informed consent was obtained from each volunteer.

[1] Parasal, Lot 721789; Parasal sodium, Lot 732043; and Parasal calcium, Lot 736132; Panray Division of Ormont, Englewood, N. J.
[2] Paskallium, Lot k448, Glenwood Laboratories, Tenafly, N. J.
[3] Analyses performed by FDA.

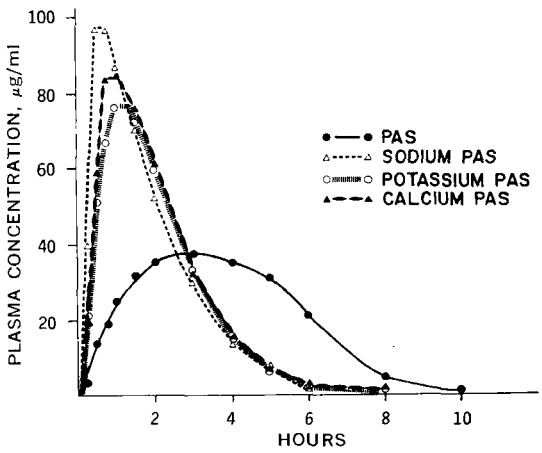

Figure 1—*Mean plasma concentrations of unchanged drug from 12 subjects following administration of four different preparations of aminosalicylic acid (PAS). Data were corrected to 70 kg body weight.*

Dose and Administration—Subjects abstained from all drugs for 2 weeks and from alcoholic beverages for 3 days prior to the study. After an overnight fast, the designated form of 4 g I, 4 g II (equivalent to 2.80 g I), 4 g III (equivalent to 2.60 g I), or 4 g IV (equivalent to 2.60 g I) was ingested with 250 ml water. The fast was continued for 5 hr, with water allowed *ad libitum*. Serial blood samples were taken from a peripheral vein at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, and 10 hr. The blood was collected in tubes containing sodium heparin and plasma was obtained by centrifugation. All urine was collected at 2-hr intervals for 10 hr and a cumulated sample was collected from 10 to 24 hr. Subjects were ambulatory during the study period. The four preparations were randomly administered in one of four sequences at intervals of at least 1 week.

Sample Analysis—Plasma and urine were stored at 0° until analyzed by the modified Marshall method described previously (5). All samples were assayed for acetylated and nonacetylated drug. The data, normalized to a 4-g dose and 70 kg body weight, were subjected to an analysis of variance for crossover experiments. Where significant differences were observed, further multiple-range analyses (6) were performed to identify the source of difference. Pharmacokinetic parameters were obtained by fitting the data to a single-compartment open model using the BMDX85 program.

RESULTS

Plasma—Mean peak plasma concentrations of nonmetabolized drug were highest with the sodium salt (II) followed by the calcium salt (III), potassium salt (IV), and free acid (I), respectively (Figs. 1 and 2). Peak concentrations after administration of I were 3.5 times lower than levels achieved for the sodium salt. Differences in peak concentration were statistically significant (Table I). Formulation I had high significant lower peak levels than II, III, or IV (Table I). High peak levels were associated with rapid absorption rates and, consequently, with short peak time values. Only peak times following I were significantly different from the other compounds (Table II). Areas under the plasma concentra-

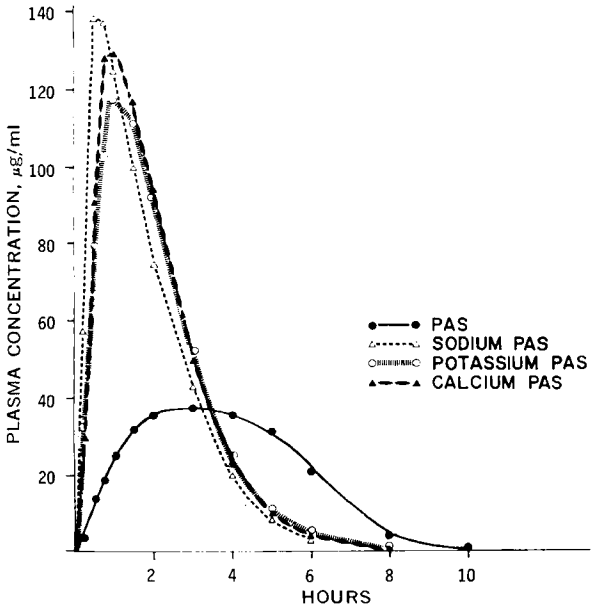

Figure 2—*Mean plasma concentrations of unchanged drug from 12 subjects following administration of four different preparations of aminosalicylic acid (PAS). Data were corrected to 70 kg body weight and to a dose equivalent to 4 g free acid.*

tion–time curve *(AUC)* were calculated using the trapezoidal rule and relative bioavailability ascertained. The *AUC* for I was significantly lower than for II, III, and IV, both with the uncorrected data and the data normalized to a 4-g dose. The analysis of the corrected data is given in Table III. Although absorption of I was markedly slower, half-lives of elimination in all cases were very similar once the absorption phase was complete. Mean half-lives were 0.94, 0.96, 0.91, and 0.91 hr for I, IV, III, and II, respectively.

Urine—Excretion of drug and metabolites was very rapid, with 50% of the dose being excreted in 2–3 hr for II, III, and IV and in 5–6 hr for I. Total recovery in urine amounted to 77% for I and was almost complete for II, III, and IV (Fig. 3 and Table IV). Drug I was the only preparation significantly different in terms of total absorption. The percentage absorption of I was somewhat lower than the absorption of the drug from suspension reported earlier (5). In that study it was found that aminosalicylic acid given in suspension was almost completely absorbed. No differences were observed in the percent of dose excreted as acetylated drug. The means for the four preparations ranged from 52 to 57% of the dose (Table V). However, significant differences were observed for the percent of dose excreted as nonacetylated drug (Table VI). Administration of I resulted in a smaller percentage of dose excreted as unchanged drug and *p*-aminosalicyluric acid (Figs. 4 and 5). The apparent discrepancy between Tables V and VI can be resolved by comparing cumulative excretion as percent of absorbed drug. Since the absolute amounts absorbed for the four preparations were different, the cumulative excretions of acetylated and nonacetylated drug were calculated as percentages of absorbed drug. When this was done, the percentages of the absorbed dose excreted as acetylated metabolite were 73.1, 54.5, 57.7 and 56.4 for I, IV, III, and II, respectively. Drug I was significant-

Table I—Peak Plasma Drug Concentrations from Tablets of Aminosalicylic Acid and Three of Its Salts

Drug[a]	I	IV	III	II
Mean Peak Concentration[b], μg/ml	49.98	121.09	139.51	155.44

Analysis of Variance

Source of Variation	*df*	*SS*	*MS*	*F*
Between individuals	11	12,273	1,115	2.14
Between drugs	3	77,903	25,967	49.76
Between periods	3	768	256	0.49
Error	30	15,654	521	
Total	47	106,598		

SE of means = 6.59
$F_{0.05}$ (3,30) = 2.92
$F_{0.05}$ (11,30) = 2.12

Multiple-Range Analysis

p	2	3	4
Shortest significant ranges	19.05	20.03	20.56

II–I = 105.46 > 20.56; significant
II–IV = 34.35 > 20.03; significant
II–III = 15.93 < 19.05; not significant
III–I = 89.53 > 20.03; significant
III–IV = 18.42 < 19.05; not significant
IV–I = 71.11 > 19.05; significant

[a] I = aminosalicylic acid, II = sodium salt, III = calcium salt, and IV = potassium salt. [b] Any two or more means not statistically different are underscored by the same straight line.

Table II—Times for Plasma Drug Concentration to Peak from Tablets of Aminosalicylic Acid and Three of Its Salts

Drug[a]	II	III	IV	I
Mean Peak Times[b], hr	0.83	1.02	1.10	3.54

Analysis of Variance

Source of Variation	*df*	*SS*	*MS*	*F*
Between individuals	11	13.14	1.19	1.89
Between drugs	3	59.18	19.73	31.20
Between periods	3	1.72	0.57	0.91
Error	30	18.97	0.63	
Total	47	93.01		

SE of means = 0.23
$F_{0.05}$ (3,30) = 2.92
$F_{0.05}$ (11,30) = 2.12

Multiple-Range Analysis

p	2	3	4
Shortest significant ranges	0.66	0.70	0.72

I–II = 2.71 > 0.72; significant
I–III = 2.52 > 0.70; significant
I–IV = 2.44 > 0.66; significant
IV–II = 0.27 < 0.70; not significant; hence,
IV–III = not significant
III–II = 0.19 < 0.66; not significant

[a] I = aminosalicylic acid, II = sodium salt, III = calcium salt, and IV = potassium salt. [b] Any two or more means not statistically different are underscored by the same straight line.

Table III—Areas under the Plasma Concentration–Time Curve *(AUC)* following Administration of Tablets of Aminosalicylic Acid and Three of Its Salts

Drug[a]	I	IV	II	III
Mean *AUC*[b], μg hr/ml	209.07	313.22	313.22	326.83

Analysis of Variance

Source of Variation	*df*	*SS*	*MS*	*F*
Between patients	11	99,499	9,045	4.75
Between drugs	3	107,807	35,935	18.87
Between periods	3	5,901	1,967	1.03
Error	30	57,124	1,904	
Total	47	270,333		

SE of means = 12.60
$F_{0.05}$ (3,30) = 2.92
$F_{0.05}$ (11,30) = 2.12

Multiple-Range Analysis

p	2	3	4
Shortest significant range	36.41	38.30	39.30

III–I = 117.76 > 39.30; significant
III–IV = 13.61 < 38.30; not significant; hence,
III–II = not significant
II–I = 104.15 > 38.30; significant
II–IV = 0 < 36.40; not significant
IV–I = 104.15 > 36.40; significant

[a] I = aminosalicylic acid, II = sodium salt, III = calcium salt, and IV = potassium salt. [b] Any two or more means not underscored by the same straight line are significantly different.

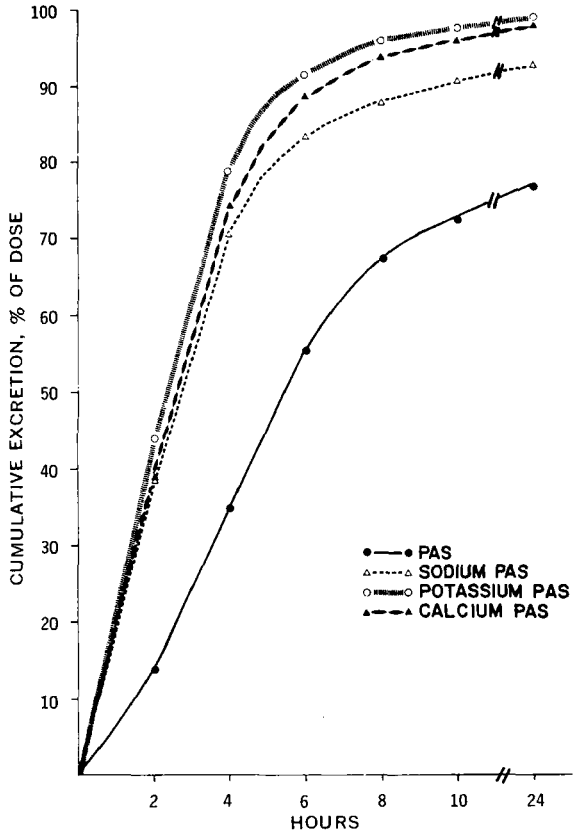

Figure 3—*Mean cumulative excretion of total drug from 12 volunteers following administration of four different preparations of aminosalicylic acid (PAS).*

ly higher. The percentages of the absorbed dose excreted as nonacetylated drug were 26.7, 45.5, 42.3, and 43.8 for the same drugs, respectively. Again, I had an inversely smaller proportion of nonacetylated drug. The ratios of acetylated to nonacetylated drug were 2.74, 1.20, 1.36, and 1.29 for I, IV, III, and II, respectively. Only I was significantly different from II, III, and IV.

DISCUSSION

Metabolism of aminosalicylic acid is mainly by conjugation. Acetylation accounts for 50–70% of the absorbed dose, and glycine conjugation to *p*-aminosalicyluric acid accounts for up to 25% of the dose. These two metabolites together constitute greater than 90% of the metabolites found in urine (7, 8). Other metabolites are of minor importance and the balance is unchanged drug. Since the analytical methods used in the present study do not differentiate between unchanged drug and the glycine conjugate, the terminology of nonacetylated drug was used in the description of drug and metabolite content in urine. Because of rapid clearance of the glycine conjugate, the nonacetylated fraction in plasma has been shown to consist of 95% unmetabolized drug, with only 5% constituting glycine-conjugated drug (9). Lauener *et al.* (10) published similar results in addition to showing that conversion of drug to the glycine conjugate at therapeutic doses is first order and independent of dose; therefore the ratio of unchanged drug to the glycine conjugate in urine is constant for an individual independent of dose. Thus, measurements of nonacetylated drug in plasma are essentially measurements of unchanged drug, and cumulative nonacetylated drug in urine will have a constant ratio of unchanged drug to the glycine conjugate.

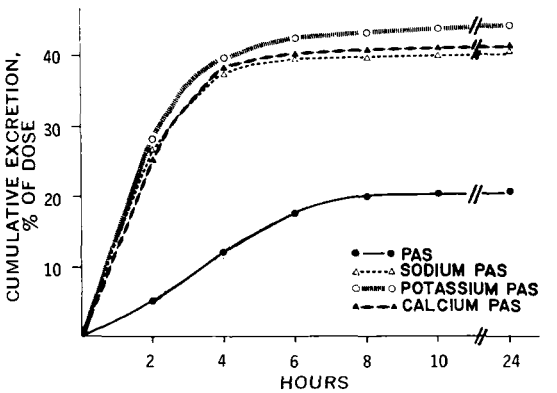

Figure 4—*Mean cumulative excretion of nonacetylated drug from 12 volunteers following administration of four different preparations of aminosalicylic acid (PAS).*

Table IV—Total Excretion of Drug and Metabolites following Administration of Tablets of Aminosalicylic Acid and Three of Its Salts

Drug[a]	I	II	III	IV
Mean Percent of Dose Excreted[b]	77.2	92.8	98.2	98.5

Analysis of Variance

Source of Variation	*df*	*SS*	*MS*	*F*
Between patients	11	1732	157	2.31
Between drugs	3	3618	1206	17.68
Between periods	3	902	300	4.41
Error	30	2047	68	
Total	47	8300		

SE of means = 2.38
$F_{0.05}$ (3,30) = 2.92
$F_{0.05}$ (11,30) = 2.12

Multiple-Range Analysis

p	2	3	4
Shortest significant range	6.88	7.24	7.43

IV–I = 21.3 > 7.43; significant
IV–II = 5.7 < 7.24; not significant; hence,
IV–III = not significant
III–I = 21.0 > 7.24; significant
III–II = 5.4 < 6.88; not significant
II–I = 15.6 > 6.88; significant

[a] I = aminosalicylic acid, II = sodium salt, III = calcium salt, and IV = potassium salt. [b] Any two or more means not underscored by the same straight line are significantly different.

The *F* values for interindividual variations were significant in three out of the six parameters measured. This was to be expected since many factors contribute to interindividual variation. In addition, there were differences between periods in the amount of drug and metabolites excreted in 24 hr. The latter differences arise from the sequence in which the drugs were administered. The reason for this difference is not known. Whatever it may be, these differences were small compared to the much greater difference between drugs.

The amounts absorbed from tablets of II, III, and IV were comparable and close to 100%, negating claims of product superiority for any specific preparation based on total absorption. When

Table V—Total Excretion of Acetylated Drug following Administration of Tablets of Aminosalicylic Acid and Three of Its Salts

Drug[a]	II	IV	I	III
Mean Percent of Dose Excreted[b]	52.3	53.7	56.4	56.7

Analysis of Variance

Source of Variation	*df*	*SS*	*MS*	*F*
Between individuals	11	1206	107	1.74
Between drugs	3	165	55	0.88
Between periods	3	506	168	2.68
Error	30	1890	63	
Total	47	3768		

$F_{0.05}$ (3,30) = 2.92
$F_{0.05}$ (11,30) = 2.12

[a] I = aminosalicylic acid, II = sodium salt, III = calcium salt, and IV = potassium salt. [b] Any two or more means not underscored by the same straight line are significantly different.

Table VI—Total Excretion of Nonacetylated Drug following Administration of Tablets of Aminosalicylic Acid and Three of Its Salts

Drug[a]	I	II	III	IV
Mean Percent of Dose Excreted[b]	20.8	40.6	41.5	44.8

Analysis of Variance

Source of Variation	*df*	*SS*	*MS*	*F*
Between individuals	11	1318	120	17.92
Between drugs	3	4275	1425	213.15
Between periods	3	749	250	37.36
Error	30	200	6.7	
Total	47	6542		

SE of means = 0.75
$F_{0.05}$ (3,30) = 2.92
$F_{0.05}$ (11,30) = 2.12

Multiple-Range Analysis

p	2	3	4
Shortest significant range	2.17	2.28	2.34

IV–I = 24.0 > 2.34; significant
IV–II = 4.2 > 2.28; significant
IV–III = 3.3 > 2.17; significant
III–I = 20.7 > 2.28; significant
III–II = 0.9 < 2.17; not significant
II–I = 19.8 > 2.17; significant

[a] I = aminosalicylic acid, II = sodium salt, III = calcium salt, and IV = potassium salt. [b] Any two or more means not underscored by the same straight line are significantly different.

using blood level as a parameter, however, differences in products may be observed. Similar percentages of absorbed drug were found in two other studies comparing other preparations of this drug[4] (11). The lower amounts of drug absorbed from Formulation I can be attributed to the relative insolubility of free acid in water. Peak times, peak plasma concentrations, and absorption rates of the three salts were different. These findings can be attributed to degree of ionization and differences in tablet formulation. A previous study (5) showed that even when the salts are given in solution, the apparent rates of absorption for the three salts are not the same, indicating absorption differences arising from degree of ionization.

Areas under the plasma concentration–time curve *(AUC)* were lowest for I and were not significantly different among II, III, and IV. However, the dose of I was also highest at 4 g. In terms of absolute amount absorbed, it was 3.09, 2.56, 2.55, and 2.6 g for I, IV, III, and II, respectively, from doses of 4, 2.6, 2.6, and 2.8 g, respectively. The discrepancy between *AUC* and amounts absorbed can be explained by rate-limited metabolism of drug. Lauener *et al.* (10) showed that while the biotransformation of aminosalicylic acid to the glycine conjugate is first order, the formation of acetylated compound is capacity-limited in humans. Rate-limited acetylation of similar compounds in humans and animals is also known (12, 13). Since the absorption rate of I was slowest, the concentrations achieved were also lowest; administration of II, III, and IV gave higher blood levels which would be more likely to approach capacity-limited metabolism. This postulation is further substantiated by the ratios of acetylated to nonacetylated drug found in urine. This ratio was 2.74 for I as compared to a mean of 1.28 for II, III, and IV. The rate constant for elimination of aminosalicylic acid was the same in all cases, suggesting that the capacity-limited phenomenon was a first-pass effect in the GI and liver tissues. During the absorption phase, concentrations of drug perfusing these tissues are high, especially with rapidly absorbed preparations, and metabolism during the first pass can reconcile the data presented here and elsewhere (5). Several drugs are known to be metabolized extensively during absorption (14–16), and acetylation of aminosalicylic acid during the first pass can now be added to that list.

Because of the dose-dependent acetylation of aminosalicylic acid, comparisons of blood levels alone are not reliable indications of its total absorption. Urine data comparisons similarly would be misleading as to the level of nonmetabolized bioavailable drug.

[4] P. Pentikäinen, S. H. Wan, and D. L. Azarnoff, in preparation.

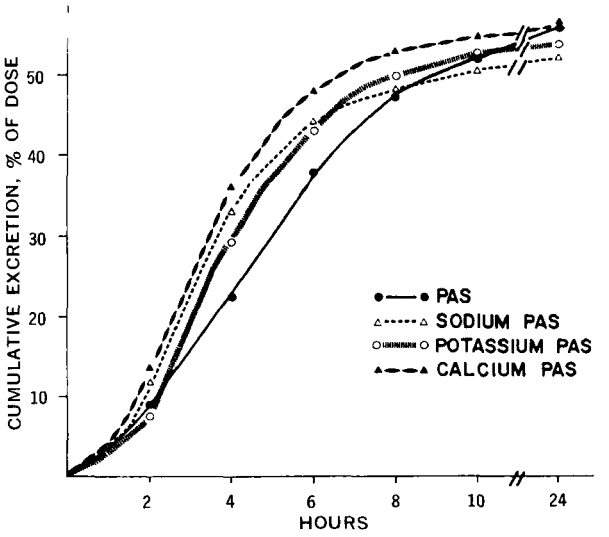

Figure 5—*Mean cumulative excretion of acetylated drug from 12 volunteers following administration of four different preparations of aminosalicylic acid (PAS).*

The confusion in past literature on aminosalicylic acid has arisen from failure to analyze blood and urine data simultaneously.

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[x] To whom inquiries should be directed.

Hydrolysis Kinetics of Benzocaine and Homologs in the Presence of a Nonionic Surfactant

GARNET G. SMITH, D. R. KENNEDY, and J. GRAHAM NAIRN[x]

Abstract □ The kinetics of alkaline hydrolysis of benzocaine (ethyl *p*-aminobenzoate), *n*-butyl *p*-aminobenzoate, and ethyl *p*-*n*-butylaminobenzoate in the presence of a nonionic surfactant (polyoxyethylene 24 monocetyl ether) were investigated. The influence of the nonionic surfactant on the solubility and on the wavelength of maximum absorbance in the UV portion of the spectrum of the three compounds was also studied. At surfactant concentrations above the CMC, the rate of hydrolysis of the esters is decreased, the solubility is enhanced, and there is a bathochromic shift in the wavelength of maximum absorbance. As a result of the investigation, the partition coefficients of the compounds between the micellar phase and the aqueous phase, the location of the compounds within the micellar phase, and the hydrolytic rate constants within the two phases were determined. It was concluded that benzocaine and the homologs are probably associated with the polyoxyethylene portion of the micelle and that the observed change in the rate of hydrolysis can be attributed to both a decrease in the rate of hydrolysis and an increase of solubility within the micellar phase.

Keyphrases □ Benzocaine and homologs—surfactant (polyoxyethylene 24 monocetyl ether) effect on alkaline hydrolysis, solubility, and UV absorbance □ *p*-Aminobenzoate esters—nonionic surfactant effect on alkaline hydrolysis, solubility, and UV absorbance □ Surfactant, nonionic, effect—benzocaine and homolog hydrolysis, solubility, and UV absorbance

Several investigations have concerned the influence of micelles on the kinetics of reaction, and some of the main types of reactions studied are those concerning hydrolysis (1) and oxidation (2). Relationships between the charge on the micelle and the charge on the reactant have been discussed (3, 4) as has secondary valence force catalysis (5). Reviews concerning the effects of surfactants on kinetics have also been presented (6, 7). Some publications relate to the present work, that is, inhibition of hydrolysis in the presence of surfactants (8–10). The significance of partition coefficients or saturation ratios on the rates of hydrolysis also has been investigated (11–13).

Due to the interest in this type of research, it was decided to study the kinetics of hydrolysis of a few compounds differing only in the number of methylene groups. The importance of the partition coefficient of the compounds between the micellar and aqueous phases as it affects the observed kinetics was also investigated. The data obtained regarding the change of wavelength of maximum absorbance (λ_{max}) of benzocaine and its homologs in the surfactant solutions were used to provide an indication of the location of the compounds within the micellar phase. The studies were conducted to determine the influence of the molecular structure of the substrates upon the observed rates of hydrolysis in the presence of surfactants. Benzocaine and its homologs were selected because the kinetics of alkaline hydrolysis have been well established (14) and the rate of hydrolysis of benzocaine is known to be altered in the presence of surfactants (15). The surfactant (polyoxyethylene 24 monocetyl ether) was used because of its resistance to hydrolysis as a result of possessing ether rather than ester linkages. Preliminary experiments indicated that the nonionic surfactant increased the solubility of benzocaine appreciably.

EXPERIMENTAL

Materials—A commercial sample[1] of ethyl *p*-aminobenzoate (benzocaine, I) was purified by recrystallization (15), and *n*-butyl *p*-aminobenzoate (II)[2] was purified in a similar manner. Ethyl *p*-*n*-butylaminobenzoate (III) was synthesized and purified (16). The characteristics of the esters were checked by their melting points and their spectral characteristics in water (wavelengths of maximum absorbance and molar absorptivity) (14, 16, 17). The nonionic surfactant polyoxyethylene 24 monocetyl ether[3], mol. wt. 1300 (18), was purified by partitioning between benzene and a saturated solution of sodium chloride in water in a manner similar to that previously reported (mp 43.5–44.5°) (19).

Kinetic Determinations—Known concentrations of I or its homologs in surfactant solutions or water were prepared by using freshly boiled distilled water. The hydrolysis was carried out in the presence of a relatively high concentration of sodium hydroxide (approximately 0.045 *N*) so that there was no significant decrease in the hydroxide-ion concentration during the reaction. The kinetic studies, in duplicate, were conducted in a spectrophotometer[4] equipped with a dual thermospacer set through which water, from a thermostatted bath, was continuously circulated to maintain the cells at a constant temperature of 30°. Hydrolysis of the esters was followed by observing the change in the absorbance at the λ_{max} of the ester in the surfactant solution. From the values of the absorbances at zero and infinite time, the first-order rate constants at 0.045 *N* NaOH were obtained by plotting the logarithm of the percentage ester remaining against time and correcting for the actual sodium hydroxide concentration.

Solubility Studies—The solubilities of the esters were determined in duplicate by shaking an excess quantity of the ester in solutions containing different surfactant concentrations. The mixtures were shaken at 30° for 5 days in a water bath shaker. An aliquot of the clear supernatant liquid was diluted with water and analyzed spectrophotometrically, employing a suitably diluted surfactant solution as a blank.

UV Absorbance Studies—The wavelength of maximum absorbance of the esters in hexane solutions, aqueous surfactant solutions, and aqueous polyethylene glycol 1000 solutions was determined by dissolving suitable amounts of the esters in the solvent and determining the absorbances at different wavelengths.

RESULTS AND DISCUSSION

The solubilities of the esters are given in Table I. There is a slight increase in the solubility of each ester in the presence of the surfactant (concentration 1×10^{-5}%) as compared to the solubility of the ester in water. This slight increase in solubility may be

[1] British Drug Houses.
[2] Matheson Coleman and Bell.
[3] Texofor A24, Glover's Chemicals Ltd.
[4] Beckman DU-2.

Table I—Effect of Surfactant Concentration on the Properties of the Esters at 30°

Ester	Surfactant Concentration, %	Solubility of Ester, %	$\frac{S_e - S_{ew}}{C_s}$ [a]	Partition Coefficient	$10^3 k$ [b], min^{-1}
I	0	0.123	—	—	11.3
	1.0×10^{-5}	0.127	—	—	11.1
	0.1	0.143	1.53	165	9.07
	0.5	0.196	1.15	121	6.85
	1.0	0.267	1.13	118	4.87
	5.0	0.825	1.10	115	1.56
II	0	2.20×10^{-2}	—	—	7.32
	1.0×10^{-5}	2.35×10^{-2}	—	—	7.77
	0.1	4.44×10^{-2}	1.49	1030	3.24
	0.5	0.126	1.39	955	1.19
	1.0	0.224	1.35	917	0.674
	5.0	1.08	1.42	965	0.138
III	0	6.48×10^{-4}	—	—	6.87
	1.0×10^{-5}	6.60×10^{-4}	—	—	7.03
	0.1	3.85×10^{-3}	0.188	4996	1.11
	0.5	1.66×10^{-2}	0.187	4971	0.288
	1.0	3.28×10^{-2}	0.188	4950	0.180
	5.0	0.16	0.186	4912	0.0407

[a] Moles of ester solubilized per mole of surfactant. [b] Pseudo-first-order rate constant in 0.045 *N* NaOH.

due to the interaction of the esters with the polyoxyethylene portion of the surfactant. Guttman and Higuchi (20) reported on the effects of macromolecules, such as polyethylene glycols, which give rise to an alteration in the solubility of a number of organic compounds. Above the critical micelle concentration (CMC), the solubilities of the ester increased significantly, as is to be expected when micelles are formed that offer a suitable medium for the dissolution of the solutes.

The results of calculating the ratio of moles of ester solubilized per mole of surfactant, $(S_e - S_{ew})/C_s$, where S_e = molar solubility of the ester, S_{ew} = molar solubility of the ester in water, and C_s = molar concentration of the surfactant, are provided in Table I. It can be seen that above the CMC the solubilization of the esters per molecule of surfactant is essentially constant, indicating that the solubilizate does not alter the composition of the micelles in such a way so as to affect the solubilizing power of the micelles. The number of molecules of II solubilized per molecule of surfactant is appreciably greater than for I as a result of the increase of the hydrocarbon portion of the molecule which makes the butyl ester more suitable for dissolution within the micellar environment. This result is similar to that reported by Patel (21), where the percentage binding to the surfactant increases with increasing hydrocarbon chain length of the solubilizate if the solubilized molecules are small. The value of $(S_e - S_{ew})/C_s$ for III is considerably smaller than are the values for the other two esters, even though the number of methylene groups has increased. It is logical to expect that I and II would be associated with the micelle in such a way that the hydrocarbon portion of the molecules would be directed toward the lipophilic interior of the micelle and that the polar portion of the molecules would be directed toward the hydrophilic polyoxyethylene part of the micelle (22). As a result of this arrangement between the solubilizate and the surfactant molecules in the micelle, the configuration of the two esters would be linear, permitting closer packing within the micelle and resulting in a relatively high value for $(S_e - S_{ew})/C_s$. In contrast to this situation, III has a nonpolar hydrocarbon group on opposite ends of the molecule and this would tend to discourage a linear configuration of the ester and the close packing arrangement if the polar portion of the ester is associated with the polar part of the micelle and the nonpolar portions of the ester are associated with the hydrocarbon interior of the micelle. The value of $(S_e - S_{ew})/C_s$ in Table I would tend to support this concept. In addition, there is a rank-order correlation between the values of $(S_e - S_{ew})/C_s$ and the difference between the λ_{max} for water and for the solution containing 5% surfactant in Table III, which indicates that both I and II have the greatest degree of interaction with the surfactant micelles.

Table II—Reaction Rate Constants of Unsolubilized (k_a) and Solubilized (k_m) Esters at 30° in 0.045 *N* NaOH

Ester	$10^3 k_a$, min^{-1}	$10^4 k_m$, min^{-1}	$10^3 k_m/k_a$
I	10.4	1.88	17.9
II	6.4	0.332	5.19
III	6.64	0.335	5.05

The partition coefficients for the distribution of the esters between the micellar and aqueous phases can be calculated from the following relationships:

$$K = \frac{C_m}{C_a} = \frac{A_m/V_m}{C_a} \qquad \text{(Eq. 1)}$$

where C_m = the concentration of the ester in the micellar phase, C_a = the concentration of the ester in the aqueous phase, A_m = the amount of ester in the micellar phase ≈ (total amount of ester present in the solution − the amount of ester in the aqueous phase), and V_m = the volume of the micellar phase ≈ the volume or weight of the surfactant corrected for the CMC, taken to be 0.001 g/100 ml (18).

The results of these calculations are given in Table I; the value of the partition coefficient increases appreciably with an increase in the length of the hydrocarbon chain of the solubilizate. The smaller the water solubility of the compound, the greater is the tendency for the ester to be partitioned in favor of the micellar phase. Consequently, even though the uptake of III per molecule of surfactant is less by a factor of almost 10, this compound is mostly in the surfactant phase due to its extremely low solubility in water.

The pseudo-first-order rate constants were averaged (Table I), and none of the individual rate constants differs from the average by more than 1%. The value of the second-order rate constant (liter $minute^{-1}$ $mole^{-1}$) for I in solution containing no surfactant (0.253) is similar to earlier studies (0.252) (23) and the value of the second-order rate constant for III (0.152) in water at 30° is comparable to the calculated value (0.183) obtained from the data of Karlen and Agren (14).

The reduction in the rate of hydrolysis of II and III in water as compared to I can be attributed to the replacement of either the ethyl group or hydrogen atom by the butyl group, which possesses greater electron-releasing properties (14, 24), thus hindering the nucleophilic attack of the hydroxide ion at the acyl carbon atom.

It can be seen from Table I that the rate of hydrolysis of each ester is reduced appreciably in high concentrations of the surfactant and that this reduction in the rate takes place when the concentration of the surfactant is above the CMC. This effect on the rate of hydrolysis can be explained from the knowledge that a considerable amount of the ester is contained within the micelle and by postulating that the rate of hydrolysis in the micellar phase is smaller than the rate in the aqueous phase.

Consequently, it is assumed that the overall rate of hydrolysis

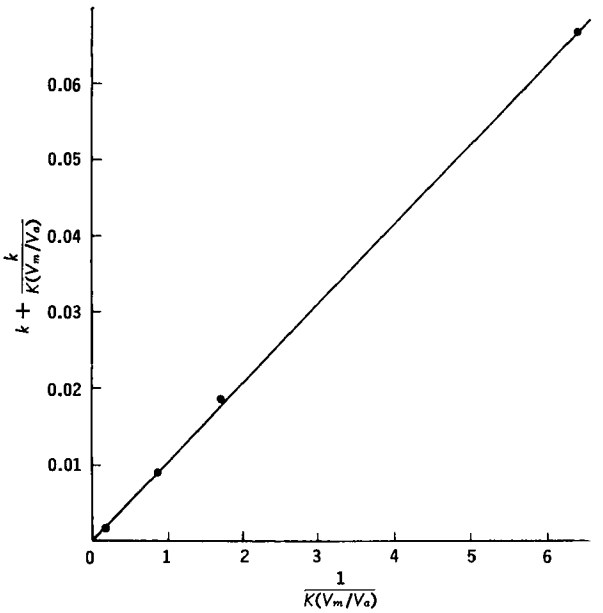

Figure 1—*Plot of Eq. 5 for I.*

can be expressed according to the following equation:

$$-(V_a + V_m)\frac{d\bar{C}}{dt} = k_a V_a C_a + k_m V_m C_m \qquad \text{(Eq. 2)}$$

where V_m = the volume of the micelles ≈ volume or the weight of surfactant added (corrected for the concentration of free molecules), V_a = the volume of aqueous alkaline solution, C_m = the concentration of ester in the micelle, C_a = the concentration of ester in the aqueous solution, k_m = the pseudo-first-order rate constant of solubilized ester, k_a = the pseudo-first-order rate constant of unsolubilized ester, and $\bar{C}$ = the average concentration of ester which is equal to $(V_a C_a + V_m C_m)/V_a + V_m$.

The partition coefficient of the ester is given by:

$$K = \frac{C_m}{C_a} \qquad \text{(Eq. 3)}$$

The combination of Eq. 2 with Eq. 3 leads to Eq. 4, where k is the measured pseudo-first-order rate constant:

$$k = -\frac{d \ln \bar{C}}{dt} = \frac{k_a - k_m}{1 + K\frac{V_m}{V_a}} + k_m \qquad \text{(Eq. 4)}$$

This mathematical treatment is similar to that reported by Tong *et al.* (11) for the decomposition of indoaniline dyes in the presence of surfactants. They assumed that the dyes were solubilized in the surfactant micelles, the dyes were distributed between the aqueous and micellar phases with a constant partition coefficient, and the rate constant for the decomposition of the solubilized dye was different in general from that of the free dye.

In the present work, it was not assumed that the partition coefficient remained constant with increasing concentrations of surfactants, since it was of particular interest to correlate the partition coefficient, the measured pseudo-first-order rate constants, and other physical properties of the system with the molecular structure of the esters. Consequently, the partition coefficients were determined by solubility experiments.

To obtain the values of k_a and k_m, Eq. 4 was rearranged as shown in Eq. 5:

$$k + \frac{k}{K\frac{V_m}{V_a}} = \frac{k_a}{K\frac{V_m}{V_a}} + k_m \qquad \text{(Eq. 5)}$$

Equation 5 was then plotted (Fig. 1) using the experimental values of k, K, V_m, and V_a; the results for k_a and k_m as determined by linear regression analysis are given in Table II.

The values of k_a should be similar to the pseudo-first-order rate constant for the hydrolysis of the ester in solutions containing no surfactant, since k_a represents the rate of hydrolysis outside of the micellar phase. It can be seen from Tables I and II, however, that the values of k_a for the three esters are somewhat smaller than the values of k at 0% surfactant concentration. While it is presumed that the surfactant is completely dissociated below the CMC, some type of complex formation may possibly exist between the ester molecules and the individual surfactant molecules. This type of interaction could account for the smaller values of k_a as compared to the rate constant in solutions containing 0% surfactant. This reduction in the rate of hydrolysis at low concentrations of surfactant is similar to the work of Lach and Pauli (25) who reported on the stabilizing and solubilizing effect of compounds, such as polyethylene glycol 4000 which does not form micelles, on benzocaine in aqueous solution. Consequently, from previous work (20, 25) and from the present work regarding both solubility and kinetics, it seems reasonable to postulate that there is some interaction between the ester and the surfactant below the CMC.

The value of k_m is the pseudo-first-order rate constant for the hydrolysis of the ester within the micellar phase. The values of k_m are considerably smaller than the values of k_a, indicating that conditions conducive to rapid hydrolysis of the ester are no longer present within the micelle. A number of factors have been suggested to explain the reduction in the rate of reactions within the micellar phase. For example, it has been shown that the rate of hydrolysis of I is reduced when the polarity of the medium is decreased by altering the ratio of organic solvent to water in the solvent system (26). Thus, it would seem logical to expect that the decrease in the rate of hydrolysis of the three esters is at least partly due to the less polar nature of the micellar phase. Another factor that may be responsible for the alteration of rates is due to enrichment of one or both of the reactants in the micelles. A catalytic effect of micelles has been observed when both reactants are enriched within the micelle. This catalytic effect has been noticed

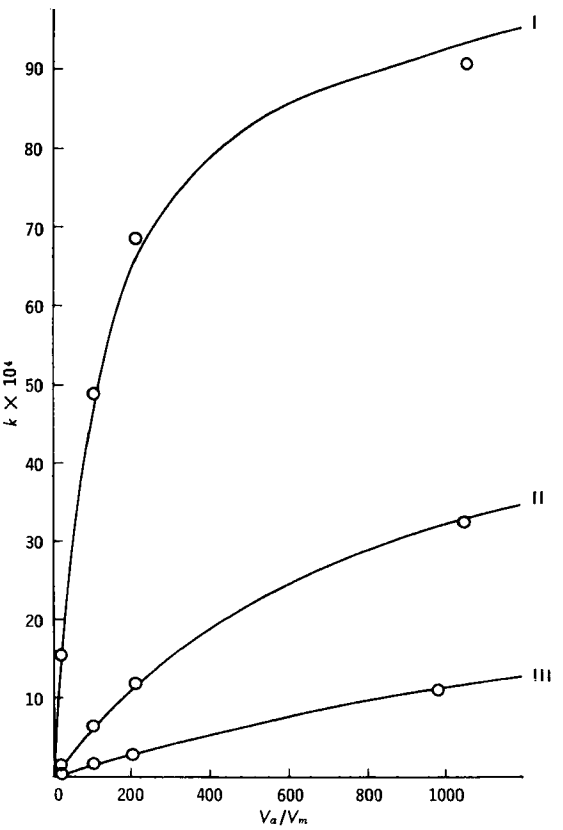

Figure 2—*Plot of observed rate constants* (○) *and calculated rate constants* (——) *from Eq. 4 as a function of* V_a/V_m *for Compounds I–III.*

Table III—Wavelength of Maximum Absorbance in Different Solvents

Solvent	I, λ_{max}, nm	II, λ_{max}, nm	III, λ_{max}, nm
Water	286	285	305
1×10^{-5}% surfactant in water	286	286	305
0.1% surfactant in water	286.2	290	307.5
0.5% surfactant in water	287	293	308
1% surfactant in water	289	294	308
5% surfactant in water	293	294	308
Hexane	270	271	291
50% polyethylene glycol 1000 in water	294.5	296	312

when, for example, both the reactants and the micelles are ionic in nature (27). Conversely, when one reactant is enriched and the other reactant is excluded, a decrease in the rate is observed. This situation exists in the present study since it has already been shown that the esters are concentrated within the micelles and it would be expected that the hydroxide ions would be excluded, at least to some extent, from the nonpolar regions of the micelle, thus leading to a decrease in the rate of reaction. A third factor that would tend to alter the rate of hydrolysis within the micellar phase is due to the interaction between the esters and the surfactant molecules (28). The complex so formed could sterically hinder the approach of the attacking agent and/or the complex could alter the electronic structure of the esters to render them less susceptible to attack by the nucleophilic reagent.

The value of the ratio of the rates of hydrolysis of the ester within the micelle phase to that within the aqueous phase should be approximately the same if the micellar environment causes a proportional decrease in the rate of hydrolysis in the two phases. It can be seen from Table II that the values of k_m/k_a are considerably smaller for II and III. This suggests that the micellar environment has a greater inhibiting effect on the rate of hydrolysis when the nonpolar portion of the substrate molecule has been increased. As a result, the values of both k_m and K depend upon the chain length of the solubilizate.

The effect of the two constants, k_m and K, on the observed rate constant is particularly important for esters with a large partition coefficient and in solutions when the ratio V_m/V_a is high since, under these conditions, nearly all of the ester is solubilized within the micelle and the observed rate constant approaches the value of k_m. This phenomenon can be seen by comparing the observed rate constants for Table I with the value of k_m in Table II.

Figure 2 illustrates the comparison of the observed and calculated rate constants in relation to the variable V_a/V_m. The calculated rate constants were plotted by using the average value for K and the values of k_m and k_a (from Table II) for each ester and by employing Eq. 4. This graph indicates that there is good agreement between the experimental results and the postulation that two main factors, namely the partition coefficient and the pseudo-first-order rate constant in the micellar phase, account for the major portion of the decrease in the observed rate when the surfactant concentration is increased for this series of compounds. It will be noted that when K is large (Compound III) in comparison with V_a/V_m, Eq. 4 becomes linear (Fig. 2).

The λ_{max} of each ester increases as the polarity of the solvent increases from hexane to water (Table III); as a result, the excited state of the esters is considered to be more polar than the ground state (29). Since the quinoid form of substituted benzene derivatives (30) contributes substantially to UV absorption, it is expected that the excited state would receive a significant contribution from the more highly polar quinoid structure (31). Such structures with separated charges are stabilized by solvent polarity in accordance with simple electrostatic theory. Hence, the polar solvent, water, facilitates excitation and a red-shift is observed as compared to the nonpolar solvent, hexane. The λ_{max} for each of the three esters shows a further red-shift in the solvent composed of polyethylene glycol 1000 (IV) and water. The composition of this solvent was selected so as to simulate the concentration of water in the polyoxyethylene portion of the micelle where there are about two or three water molecules per oxygen atom (32). This enhanced red-shift suggests that there is a further degree of interaction of the solvent with the ester molecules (33), thus encouraging the stabilization of the more polar form of the esters.

Similarly, in the presence of the surfactant, a further red-shift, compared to water, is observed, indicating a degree of interaction between the esters and the surfactant. The shift for III is not as great as it is with the two other esters, which implies that there is less interaction with the surfactant. Riegelman (15) suggested that if the surfactant concentration is increased, the characteristics of the exterior of the micelle approaches those of a mixture of IV and water. Consequently, the λ_{max} of the solubilizate at high surfactant concentrations should approach that of IV and water if the esters are located in the exterior portion of the micelle. Both I and II have λ_{max} values in the 5% surfactant solution that are close to the λ_{max} in the 50% mixture of IV and water, indicating that these two compounds are located mainly within the polyoxyethylene portion of the micelle. The relatively smaller shift for III indicates that it is located in a different portion of the micelle and that this compound is not bound to the surfactant to the same extent as the other two esters. Other evidence for the smaller degree of interaction of III compared with the other two esters was given in the discussion of $(S_e - S_{ew})/C_s$.

The UV studies thus provide some evidence for the location of the esters within the micellar phase and further evidence for interaction between the esters and the surfactant. As a result of this interaction, the ester will become more polar or quinoid in character, which will increase the electron density at the carbonyl portion of the esters. Since the alkaline hydrolysis of esters occurs as a result of a nucleophilic attack of the hydroxide ion on the carbonyl carbon atom, the rate of hydrolysis would be expected to decrease in the micelle where a more polar form of the ester exists and where there is a greater electron density at the site of attack.

In spite of the factors discussed, which tend to inhibit the rate of hydrolysis within the micelle, it seems both from a mathematical analysis and from experimental evidence that the overall kinetic rate constant approaches a limiting value, k_m, as the concentration of the surfactant is increased, which indicates that those regions of the micellar phase in which the esters are located are not impervious to penetration by the hydroxide ion.

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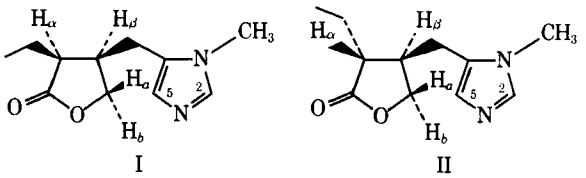
Hydrolysis and Epimerization Kinetics of Pilocarpine in Aqueous Solution

MATHEWS A. NUNES and EINAR BROCHMANN-HANSSENˣ

Abstract □ The kinetics of the hydroxide-ion-catalyzed hydrolysis and epimerization of pilocarpine in aqueous solution were investigated utilizing pH-stat titrimetry and NMR spectroscopy. The mechanism of epimerization involves formation of a carbanion stabilized by resonance with the enolate hybrid. Both hydrolysis and epimerization follow pseudo-first-order kinetics, and the appropriate rate constants and energies of activation were calculated. Epimerization was found to occur to a greater extent than previously assumed and must be considered as a major pathway of degradation and inactivation of pilocarpine. The rate of hydroxide-ion-catalyzed epimerization increases more rapidly with temperature than does the rate of hydrolysis, a fact that should be considered if ophthalmic solutions of pilocarpine are sterilized by heat. It is suggested that isopilocarpine may not be a genuine jaborandi alkaloid but an artifact produced by epimerization of pilocarpine during drying, storage, and extraction of the plant material.

Keyphrases □ Pilocarpine—kinetics of hydroxide-ion-catalyzed hydrolysis and epimerization, pH-stat titrimetry and NMR spectroscopy □ Epimerization kinetics and hydrolysis, pilocarpine—determination, pH-stat titrimetry and NMR spectroscopy □ NMR spectroscopy—determination, pilocarpine epimerization kinetics

Pilocarpine, an alkaloid obtained from various species of *Pilocarpus* (Rutaceae), was isolated independently by Gerrard (1) and Hardy (2) in 1875. Later, several closely related alkaloids were isolated, such as isopilocarpine, pilocarpidine, and pilosine. The elucidation of the chemical structures of the jaborandi alkaloids is due primarily to Jowett (3), Pinner and Kohlhammer (4), and Pinner and Schwarz (5). There are two asymmetric centers in the lactone part of the molecule, and Jowett (6) assumed the relationship between pilocarpine and isopilocarpine to be stereochemical (*cis–trans*-isomers). This assumption was supported by the work of Langenbeck (7) and proven by synthesis (8–10). However, it was not until 1966 that Hill and Barcza (11) established the absolute configuration of the two asymmetric centers as 2*S*:3*R* for pilocarpine (I) and 2*R*:3*R* for isopilocarpine (II).



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Hydrolysis of pilocarpine in aqueous solution is an equilibrium process catalyzed by hydrogen ions and hydroxide ions (14). Several investigators studied the hydrolytic decomposition of pilocarpine at various pH values and temperatures (15–20), and a detailed kinetic study was reported (14). Much less is known about the epimerization reaction. The difficulty of detecting partial epimerization has been due to the fact that there has been no simple way of separating the two diastereoisomers or of quantitating either epimer in the presence of the other. Döpke and

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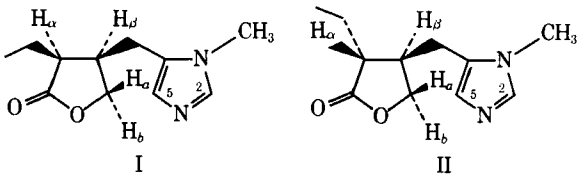

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d'Heureuse (13) showed that epimerization of pilocarpine to isopilocarpine under anhydrous alkaline conditions occurs exclusively at the α-carbon of the lactone ring. Chung *et al.* (14) and Link and Bernauer (21) found evidence that pilocarpine undergoes some epimerization in aqueous solution at alkaline pH, based on the differences in specific rotation of the two epimers.

Since the rates of both hydrolysis and epimerization are pH dependent and occur simultaneously, they may be considered as competing reactions. A study of kinetic parameters should, therefore, take into account both mechanisms. The purposes of this investigation were to develop a method for quantitating epimerization and to determine the rates of hydrolysis and epimerization of pilocarpine in aqueous solution.

EXPERIMENTAL

Reagents—The following were used: pilocarpine nitrate[1], mp 174–175° [lit. (22) mp 174–179°], $[\alpha]_D^{23}$ + 80.0° (c 2, water) [lit. (22) $[\alpha]_D$ + 79.5 to +82.0° (c 2, water)]; isopilocarpine nitrate[2], mp 158–158.5° [lit. (23) mp 159°], $[\alpha]_D^{23}$ + 39.4° (c 2, water) [lit. (24) $[\alpha]_D$ + 38.8° (water)]; deuterium oxide[3], 99.85 mole %; sodium deuteroxide[4], 40% in deuterium oxide; deuterium chloride[4], 38% in deuterium oxide; methanol-[OD][4]; and nitrogen gas, 99.996+% pure. All other chemicals were of analytical reagent grade.

Apparatus—Consumption of base was recorded by pH-stat titrimetry[5]. The reactions were carried out in a water-jacketed reaction vessel of 75-ml capacity and stirred with a magnetic stirrer. Solution temperature was maintained using a circulating water bath with proportional temperature control. The NMR spectra were prepared with a 100-MHz spectrometer[6], and the expanded spectra were integrated using a curve resolver[7].

Procedure—*NMR Spectra*—All 100-MHz NMR spectra were recorded in deuterium oxide with tetramethylsilane as the external reference. For the analysis of the proportion of epimers, the NMR spectrum of the terminal methyl group of the α-ethyl substituent was recorded with a 10-fold scale expansion and a 1.0-ppm offset. The methyl triplet of each epimer was integrated with a curve resolver.

Hydroxide-Ion-Catalyzed Reactions—The experimental procedure used for determination of the rate of base consumption was similar to that of Chung *et al.* (14), except that potassium hydroxide was used in place of sodium hydroxide to minimize the sodium effect on the electrode at high pH values. Furthermore, considerably higher concentrations of pilocarpine were used to facilitate sampling for NMR spectroscopy. Pilocarpine nitrate (2.5 g) was dissolved in 50 ml of distilled water in a water-jacketed reaction vessel surrounded by a styrofoam insulator. The vessel was continuously flushed with a stream of nitrogen which had passed through a series of three gas-washing towers containing 10% sulfuric acid, 10% sodium hydroxide, and distilled water, respectively. When the desired temperature was reached, the solution was rapidly brought to the preset pH with 2 *N* potassium hydroxide and the pH-stat recorder was returned to zero to start recording the rate of reaction. The consumption of base was recorded over 8–10 half-lives. The pseudo-first-order rate constants were obtained from Guggenheim plots (25) with the aid of a least-squares program (26) and a calculator[8]. As soon as the addition of base was discontinued, two 1-ml samples of solution were withdrawn and brought to pH < 1 with concentrated hydrochloric acid. The samples were kept at room temperature and in the dark for 1 hr, shell frozen in a dry ice–acetone bath, and lyophilized. The dry residues were dissolved in deuterium oxide (0.35 ml), and the 100-MHz NMR spectra were recorded.

[1] Sigma Chemical Co.
[2] Pierce Chemical Co.
[3] Bio-Rad Laboratories.
[4] Stohler Isotope Chemicals, Inc.
[5] Radiometer pH stat–titrimeter assembly, consisting of PHM-28 pH meter, TTT11 titrator, SBR2C titrigraph, SBU1a syringe burette, TTA3 titration assembly, GK2301C combination electrode, and B104 5-ml syringe.
[6] Joelco JNM-4H-100.
[7] Dupont model 310.
[8] Hewlett-Packard model 9100.

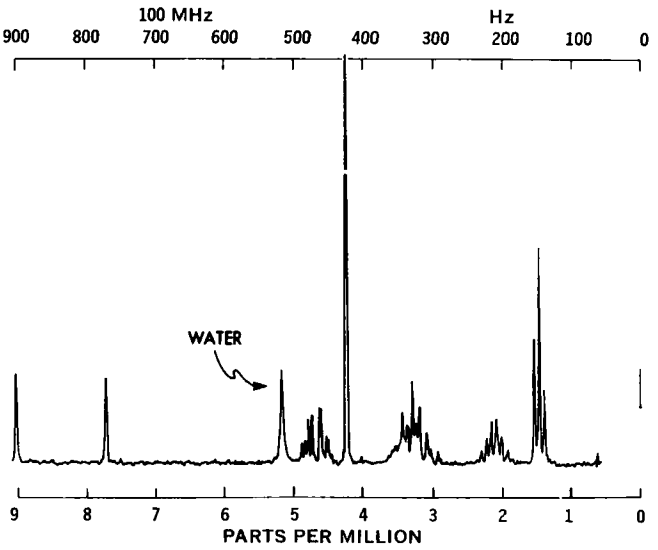


Figure 1—*NMR spectrum of pilocarpine nitrate in deuterium oxide.*

RESULTS AND DISCUSSION

The 60-MHz NMR spectrum of pilocarpine was reported (19) where NMR spectroscopy was used to determine hydrolysis of pilocarpine solutions buffered to pH 6.1. The NMR spectrum of isopilocarpine has not been reported previously, and Link and Bernauer (21) were unable to detect any change in the NMR spectrum of pilocarpine after partial epimerization.

The 100-MHz NMR spectra of pilocarpine nitrate and isopilocarpine nitrate are illustrated in Figs. 1 and 2, respectively. The principal differences in the spectra of the two epimers are caused by the γ-protons of the lactone ring and the terminal methyl protons of the α-ethyl substituent. For pilocarpine, H_a is centered at 4.55 ppm (J_{gem} = 9.0 Hz, J_v = 3.0 Hz) and H_b is at 4.80 ppm (J_{gem} = 9.0 Hz, J_v = 6.0 Hz), while isopilocarpine shows a signal for H_a at 4.48 ppm (J_{gem} = 10.0 Hz, J_v = 6.2 Hz) and H_b at 4.93 ppm (J_{gem} = 10.0 Hz, J_v = 6.3 Hz). The terminal methyl groups of the ethyl substituents appear as triplets with a coupling con-

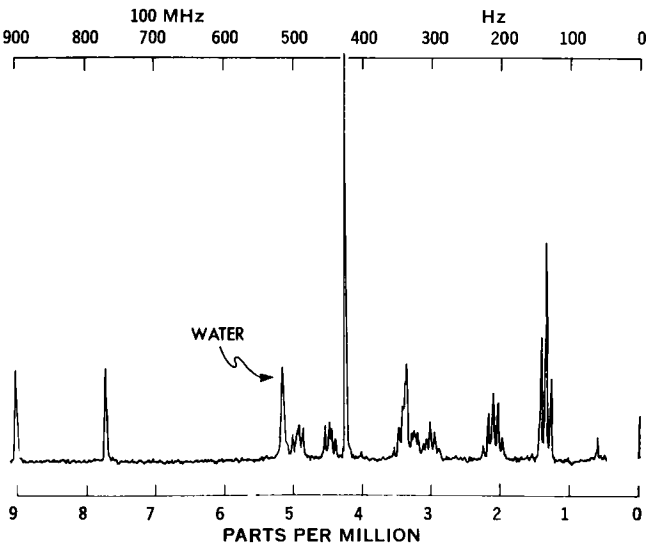


Figure 2—*NMR spectrum of isopilocarpine nitrate in deuterium oxide.*

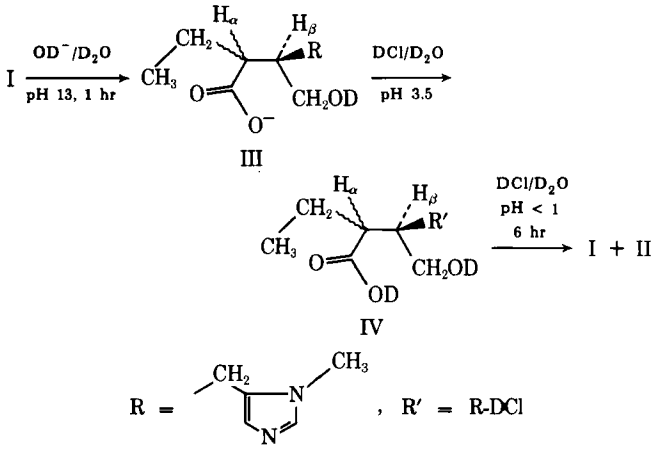
Table I—NMR Spectral Data of Pilocarpine Nitrate (I), Isopilocarpine Nitrate (II), Pilocarpate (III), Pilocarpic Acid (IV), Isopilocarpate (V), and Isopilocarpic Acid (VI) in Deuterium Oxide

	CH_3 / CH_2—	CH_3 / CH_2—	H_α	H_β	—CH_2 Bridge	N—CH_3	H_a	H_b	C-5 Proton of Imidazole	C-2 Proton of Imidazole
I	1.46 (t)	2.07 (m)	←———	3.27 (m)	———→	4.25 (s)	4.55 (dd)	4.80 (dd)	7.72 (s)	9.06 (s)
II	1.36 (t)	2.08 (m)	←———	3.21 (m)	———→	4.25 (s)	4.48 (dd)	4.93 (dd)	7.72 (s)	9.06 (s)
III	1.31 (t)	1.86 (m)	2.26 (m)	2.65 (m)	3.04 (m)	3.99 (s)	←	3.96 (m)	→	7.18 (s)	7.92 (s)
IV	1.31 (t)	2.05 (m)	←	2.71 (m)	→	3.21 (m)	4.25 (s)	←	4.04 (m)	→	7.72 (s)	9.06 (s)
V	1.31 (t)	1.98 (m)	2.36 (m)	2.72 (m)	3.12 (m)	4.02 (s)	←	3.96 (s)	→	7.18 (s)	7.92 (s)
VI	1.31 (t)	2.06 (m)	←	2.76 (m)	→	3.25 (d)	4.27 (s)	←	4.02 (d)	→	7.72 (s)	9.06 (s)

stant of 7.25 Hz; however, the chemical shifts differ by 0.1 ppm for the two epimers. The spectral data are summarized in Table I.

Pilocarpic acid and isopilocarpic acid, as well as their respective anions, are all encountered during hydrolysis and recyclization. For spectral identification of these intermediates, pilocarpine and isopilocarpine were hydrolyzed with base to the sodium salts of the γ-hydroxy acids. The solutions were then neutralized with deuterochloride to pH 3.5 to give the deuterochloride salts of the free acids[9]. At this pH, the rate of lactone formation is slow (14). Finally, acid was added to pH < 1 for cyclization of the lactones. These reactions are illustrated in Schemes I and II. Deuterated reagents and solvents were used to avoid the large water signal in the NMR spectra. This resulted in partial exchange of the α-proton of pilocarpine for deuterium as epimerization proceeded and, consequently, a reduction in the NMR signal of the α-proton relative to those of nonexchangeable protons.

When pilocarpine and isopilocarpine were hydrolyzed in alkaline solution to the anions of the γ-hydroxy acids (III and V), the NMR triplets of the terminal methyl group of the ethyl substituents shifted upfield to 1.31 ppm for both epimers. The γ-protons (H_a and H_b) also shifted upfield and overlapped the *N*-methyl group. The spectra of the hydroxy acids (IV and VI) showed that the methyl triplets of the ethyl substituents were in the same position as in the anions, whereas the γ-protons had shifted slightly downfield toward their position in the closed lactones, resonating slightly above 4 ppm. After complete hydrolysis and recyclization of pilocarpine according to Scheme I, the NMR spectrum of the product was different from that of the original pilocarpine salt. Each γ-proton still appeared as a doublet of doublets for pilocarpine; however, there were also peaks corresponding to the γ-protons of isopilocarpine. The multiplet due to H_α, H_β, and the methylene bridge between the two rings was also different from either pure pilocarpine or isopilocarpine. The signal for the methyl group of the ethyl substituent clearly showed six peaks, corresponding to the methyl triplets of pilocarpine and isopilocarpine,

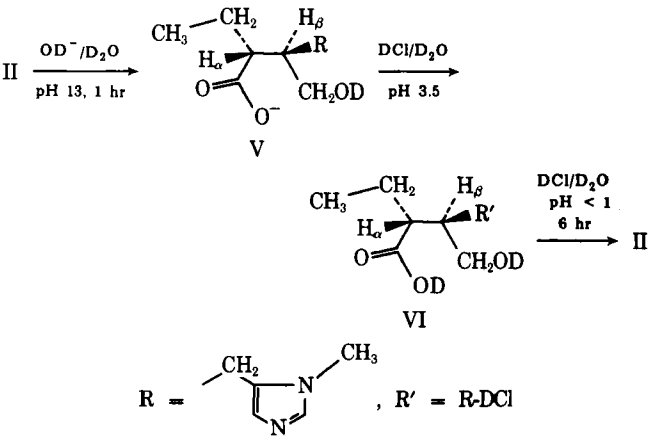


Scheme I—Effects of base on pilocarpine (I); recyclization of the lactone in acid medium

and provided the best means of quantitating epimerization. For this purpose, the triplets were recorded with a 10-fold scale expansion and an offset of 1.0 ppm (Fig. 3). Integration of the two triplets corresponding to pilocarpine and isopilocarpine showed that the mixture produced according to Scheme I at room temperature consisted of about 80% pilocarpine and about 20% isopilocarpine.

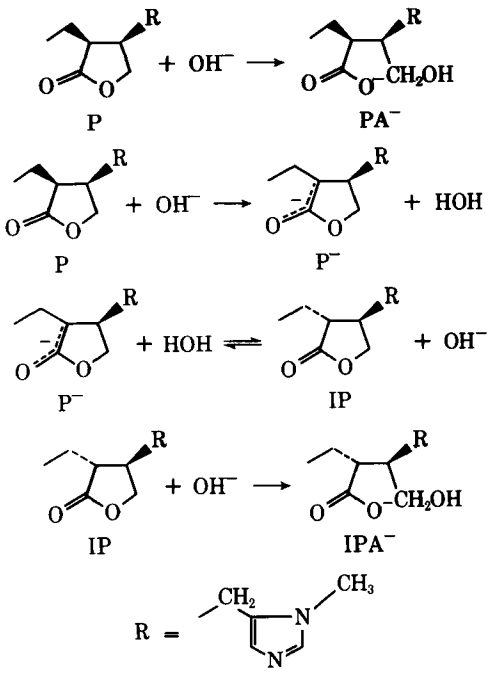
Several workers have shown that once the lactone is hydrolyzed and the hydroxy acid is in the anion form, no further epimerization takes place (12, 13). In agreement with this, NMR spectroscopy revealed that the ratio of pilocarpine to isopilocarpine was constant at constant temperature when the solutions were kept at pH 13 for various periods ranging from a few minutes to 2 days. To determine if the rate of cyclization of the lactone affected this ratio, a completely hydrolyzed solution was separated into two parts. One part was brought quickly to pH < 1 for rapid cyclization while the other was maintained at pH 3.5 for 6 hr before being brought to a pH < 1. When the methyl regions were integrated, the ratio of pilocarpine to isopilocarpine was the same in both solutions. Therefore, the spectra of III and IV in Scheme I and Table I do not represent pure pilocarpate and pilocarpic acid but mixtures of the epimers in the same ratio as that determined after recyclization of the lactone ring. When isopilocarpine was completely hydrolyzed and recyclized according to Scheme II, the spectrum of the product was identical with that of the isopilocarpine salt before hydrolysis. This indicated that isopilocarpine did not epimerize under these conditions to an extent that could be detected by NMR spectroscopy. Therefore, the equilibrium constant for epimerization is overwhelmingly in favor of the *trans*-epimer.

Epimerization requires the abstraction of the α-proton to produce a carbanion which may be stabilized by delocalization to the enolate (13). Subsequent reprotonation gives the thermodynamically more stable *trans*-epimer. The α-protons of γ-lactones are more acidic than those of open esters (27), and Döpke and d'Heureuse (13) showed the enolate to be an intermediate during the epimerization of pilocarpine in sodium ethoxide. In such anhydrous medium, the reaction proceeds smoothly without significant opening of the lactone ring. Isopilocarpine will produce the same carbanion under these conditions, as demonstrated by deuterium exchange. Isopilocarpine nitrate was refluxed with sodium



Scheme II—Effect of base on isopilocarpine (II); recyclization of the lactone in acid medium

[9] The pH measurements were performed with a pH meter standardized with aqueous buffers and expressed in pH units, ignoring the difference between the ionization products of water and deuterium oxide.



Scheme III—Hydroxide-ion-catalyzed hydrolysis and epimerization of pilocarpine (P). PA⁻ = pilocarpate, P⁻ = delocalized carbanion = IP⁻, IP = isopilocarpine, and IPA⁻ = isopilocarpate.

methoxide in methanol-[OD] for 18 hr and acidified with deuterium chloride. The solution was evaporated to dryness and the residue was dissolved in deuterium oxide. The NMR spectrum revealed that the multiplet corresponding to H_α, H_β, and the protons of the methylene bridge integrated for three protons instead of four, indicating complete exchange of the α-proton. The spectrum showed no evidence of pilocarpine salt.

The overall effect of aqueous base on pilocarpine may be considered to be the result of three principal reactions: hydrolysis to pilocarpate, epimerization to isopilocarpine, and hydrolysis to isopilocarpate. These reactions are illustrated in Scheme III, which represents the hydroxide-ion-catalyzed hydrolysis of pilocarpine to pilocarpate (P → PA⁻), the base-catalyzed epimerization of pilocarpine (P → P⁻ and P⁻ → IP), and the hydrolysis of isopilocarpine (IP → IPA⁻). The rate of disappearance of pilocarpine in alkaline solution follows pseudo-first-order kinetics (14). The rate of epimerization and subsequent hydrolysis of isopilocarpine (P → P⁻ → IP → IPA⁻) must also be governed by rate-limiting pseudo-first-order reactions. Thus, the rate of disappearance of pilocarpine is controlled by two competing pseudo-first-order reactions, and Scheme III may be abbreviated:

$$P + OH^- \xrightarrow{k_H} PA^-$$

$$P + OH^- \xrightarrow{k_E} IPA^-$$

Scheme IV

The rate law may be written in the following form:

$$\frac{-d(P)}{dt} = (k_H + k_E)(OH^-)(P) \quad \text{(Eq. 1)}$$

$$= k_{exp}(OH^-)(P) \quad \text{(Eq. 2)}$$

$$= k_{exp}'(P) \quad \text{(Eq. 3)}$$

where k_H is the second-order rate constant for the specific hydroxide-ion-catalyzed hydrolysis of pilocarpine, k_E is the second-order rate constant for the specific hydroxide-ion-catalyzed epimerization and hydrolysis of the isopilocarpine thus produced, k_{exp} is the experimentally determined second-order rate constant, and k_{exp}' is the experimentally determined pseudo-first-order rate constant. A plot of log k_{exp}' against pH yielded straight lines with slopes of one, verifying a first-order dependence on hydroxide ion. The pH profiles at various temperatures are illustrated in Fig. 4.

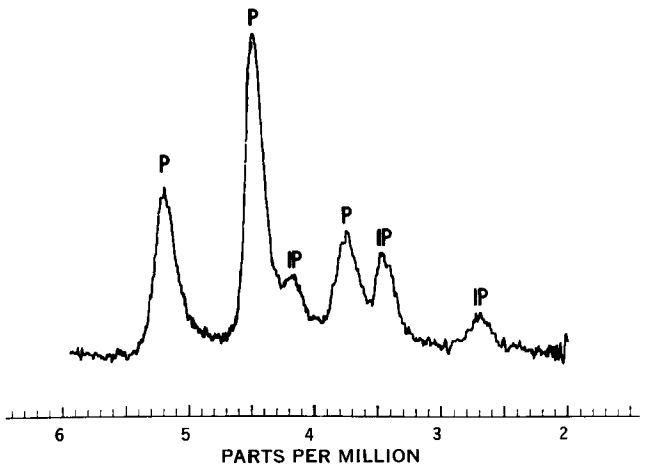


Figure 3—*Expanded NMR spectrum of the terminal methyl group of the α-ethyl substituent of pilocarpine deuterochloride after partial epimerization. P and IP designate pilocarpine and isopilocarpine, respectively.*

The experimentally determined second-order rate constant (k_{exp}) was 6.96×10^2 liters/mole/hr at 25°, or 8% lower than the value reported by Chung *et al.* (14). To determine if this difference was due to a concentration effect, control experiments were performed in which the concentration of pilocarpine nitrate ranged from 0.02 to 6%. The observed rate constants were the same over the entire concentration range.

Since the hydroxide-ion-catalyzed reactions of pilocarpine are competing pseudo-first-order reactions, the fraction of pilocarpine that disappears by each pathway during the reaction is constant as is the ratio of products from each pathway. Consequently, the ratio of pilocarpate to isopilocarpate at the end of the reaction is equal to the ratio of the rate constant for hydrolysis of pilocarpine (k_H) to the rate constant for epimerization to isopilocarpine and its hydrolysis (k_E), *i.e.*:

$$\frac{(PA^-)}{(IPA^-)} = \frac{k_H}{k_E} \quad \text{(Eq. 4)}$$

Epimerization does not occur after the lactone ring has been opened, whether in the anion or in the free acid form. Therefore, the NMR spectra recorded after complete hydrolysis and recyclization, when integrated, gave the ratio of epimers in the solution at any stage during the hydroxide-ion-catalyzed reactions. The spectra showed that the percentage of isopilocarpine formed during these reactions was independent of pH but dependent on temperature (Table II). At least three separate experiments were performed at each pH value and temperature, and two samples of

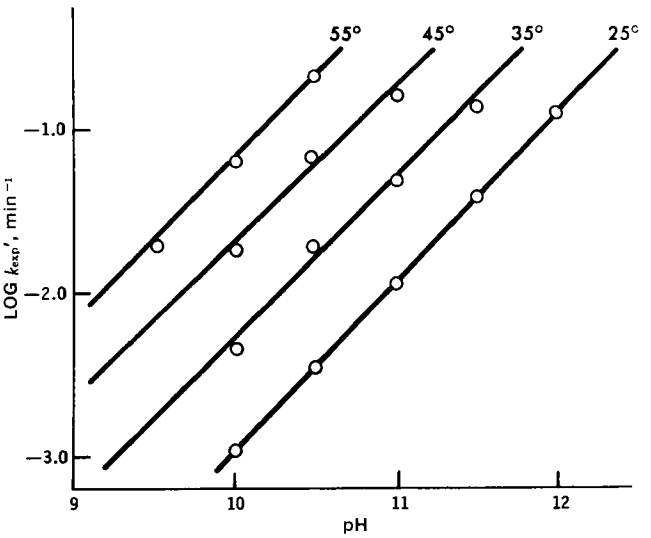


Figure 4—*The pH profile of the disappearance of pilocarpine in alkaline pH.*

Table II—Epimerization of Pilocarpine to Isopilocarpine after Complete Hydrolysis and Recyclization (Percent)

pH	Temperature 25°	35°	45°	55°
9.5	—	—	29.6	32.3
10.0	20.5	26.3	30.4	32.0
10.5	20.8	27.0	29.5	32.3
11.0	20.0	25.9	30.3	—
11.5	21.0	25.7	—	—
12.0	20.8	—	—	—

each hydrolyzed solution were analyzed for the extent of epimerization. Thus, the percentages of isopilocarpine recorded in Table II are the average values from six or more spectra. The coefficient of variation was 5.0%.

From Eq. 4 it follows that the ratio (X) of pilocarpine to isopilocarpine determined from the NMR spectra is equal to the ratio of the rate constants, *i.e.*:

$$\frac{k_H}{k_E} = X \qquad \text{(Eq. 5)}$$

Since $k_{exp} = k_H + k_E$ (Eqs. 1 and 2), substitution gives:

$$k_{exp} = k_H + \frac{k_H}{X} \qquad \text{(Eq. 6)}$$

which may be solved for k_H:

$$k_H = k_{exp}\left(\frac{X}{X+1}\right) \qquad \text{(Eq. 7)}$$

Similarly, k_E may be expressed as:

$$k_E = \frac{k_{exp}}{X+1} \qquad \text{(Eq. 8)}$$

From Eqs. 7 and 8, k_H was calculated to be 5.53×10^2 liters/mole/hr at 25°, while k_E was 1.43×10^2 liters/mole/hr at 25°. The pseudo-first-order rate constant for hydrolysis of isopilocarpine was significantly greater than that of pilocarpine. Therefore, the rate-limiting step in the conversion of pilocarpine to isopilocarpate (Scheme IV) must be epimerization to isopilocarpine. Consequently, k_E is the second-order rate constant for hydroxide-ion-catalyzed epimerization.

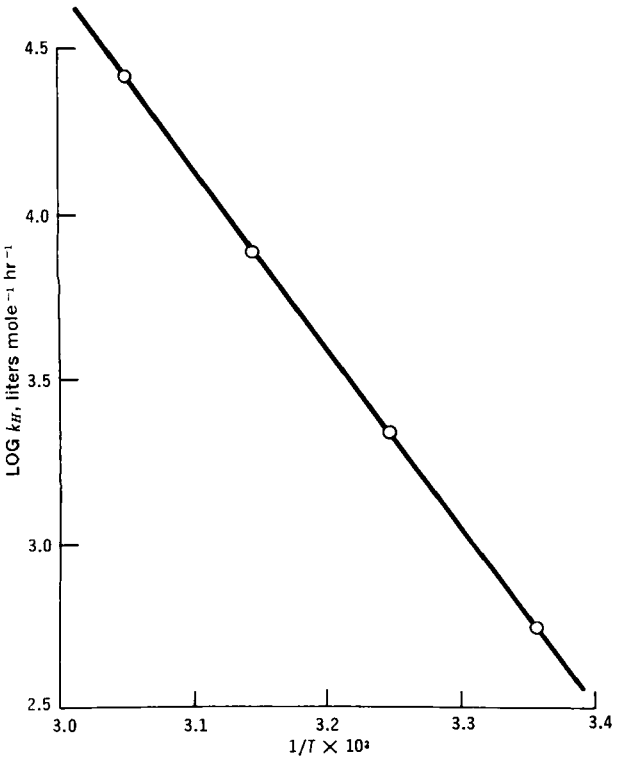


Figure 5—*Arrhenius plot of hydroxide-ion-catalyzed hydrolysis of pilocarpine.*

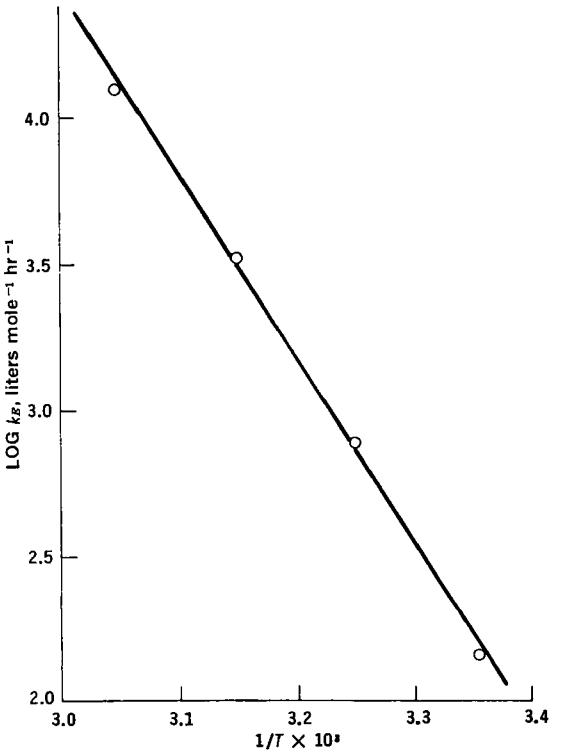


Figure 6—*Arrhenius plot of hydroxide-ion-catalyzed epimerization of pilocarpine.*

Arrhenius plots for hydrolysis of pilocarpine and for epimerization to isopilocarpine are illustrated in Figs. 5 and 6, respectively. By applying the method of least squares to each plot, the energy of activation for the hydrolysis of pilocarpine was found to be 25.02 kcal/mole (r = 0.9995), and for epimerization it was 28.48 kcal/mole (r = 0.9998). The magnitude of the epimerization rate constant, together with its comparative increase with temperature, leads to the conclusion that epimerization represents a major pathway of degradation and inactivation of pilocarpine in aqueous solution. Since the rate of hydroxide-ion-catalyzed epimerization increases more rapidly with temperature than does the rate of hydrolysis, ophthalmic solutions sterilized by heat may suffer significant loss of activity by this mechanism. It may also be surmised that it is not possible to isolate or extract pilocarpine base from its salts or from jaborandi leaves without some concurrent epimerization. It is, therefore, conceivable that isopilocarpine may not be a product of alkaloid biosynthesis in the living plants but may be an artifact produced during drying, storage, extraction of the plant material, and/or isolation of the alkaloids.

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Corneal Absorption of Ophthalmic Drugs

MINJA LEE and E. R. HAMMARLUND[x]

Abstract □ Corneal absorption of 0.0075% homatropine hydrobromide solution and 0.0025% tropicamide solution in rabbit eyes was reinforced by prebuffering the eyes with specific alkaline buffers or by adding viscolizers and a surfactant or by a combination of these measures. For 0.0075% homatropine hydrobromide solution, prebuffering with an isotonic sodium borate solution (2.6%) and two "biological" buffer solutions, 0.2 *M* cyclohexylaminopropanesulfonic acid and 0.2 *M* tris(hydroxymethyl)methylaminopropanesulfonic acid, was found to increase significantly the amount of corneal absorption of the mydriatic. Cyclohexylaminopropanesulfonic acid provided the greatest amount of increase. Two viscolizers, 0.7% hydroxypropyl methylcellulose (45 cps) and 0.375% guar gum (41 cps), increased the effect of tropicamide more than that of homatropine hydrobromide. Hydroxypropyl methylcellulose, providing slightly greater viscosity than guar gum, resulted in a slightly increased mydriatic effect with each drug. Dilute polysorbate 80 in the two mydriatic solutions gave a greater enhancement to the effect of tropicamide than to that of homatropine hydrobromide for the first 30 min, but the enhanced mydriatic effect of homatropine hydrobromide was longer lasting. The increased absorption of the mydriatics obtained by employing both prebuffer and surfactant was greater than when using either alone.

Keyphrases □ Ophthalmic (corneal) absorption of homatropine hydrobromide and tropicamide—effect of prebuffering and/or adding viscolizers and surfactant, rabbits □ Absorption, ophthalmic (corneal)—effect of prebuffering and/or adding viscolizers and surfactant, homatropine hydrobromide and tropicamide, rabbits □ Homatropine hydrobromide corneal absorption—effect of prebuffering and/or adding viscolizers and surfactant, rabbits □ Tropicamide corneal absorption—effect of prebuffering and/or adding viscolizers and surfactant, rabbits

Pretreatment of the eye with a drop of sterile, isotonic, 2.6% sodium borate solution (pH 9.2) was shown to reduce markedly the amount of an alkaloidal drug required to produce a mydriatic or miotic response in the eye (1). This technique did not affect the stability of ophthalmic solutions, since it was the eye and not the solution that was temporarily buffered.

Good *et al.* (2) introduced 12 "biological" buffers which they claimed to be suitable for biological research. In the current study the buffering effect of available biological buffers, cyclohexylaminopropanesulfonic acid[1] and tris(hydroxymethyl)methylaminopropanesulfonic acid[2], was compared with that of 2.6% sodium borate solution whose buffer action was previously known to be effective. Homatropine hydrobromide and tropicamide were the mydriatics employed for the comparisons; rabbits were used as the test subjects because the biological buffers have not been cleared by the Food and Drug Administration for use on humans.

Another objective of this study was to compare the effect of a guar gum viscolizer[3] with the routinely used viscolizer, hydroxypropyl methylcellulose, as an adjuvant for increasing the physiological effect of mydriatic eyedrops in rabbits.

Since it was reported (3–5) that agents which reduce surface tension will generally increase the permeability of certain biological membranes, another objective was to compare the mydriatic effect of homatropine hydrobromide and tropicamide solutions both with and without polysorbate 80.

[1] CAPS, Calbiochem, Los Angeles, CA 90054
[2] TAPS, Calbiochem, Los Angeles, CA 90054
[3] Jaguar J2S-1, Stein, Hall & Co., New York, N.Y.

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Keyphrases □ Ophthalmic (corneal) absorption of homatropine hydrobromide and tropicamide—effect of prebuffering and/or adding viscolizers and surfactant, rabbits □ Absorption, ophthalmic (corneal)—effect of prebuffering and/or adding viscolizers and surfactant, homatropine hydrobromide and tropicamide, rabbits □ Homatropine hydrobromide corneal absorption—effect of prebuffering and/or adding viscolizers and surfactant, rabbits □ Tropicamide corneal absorption—effect of prebuffering and/or adding viscolizers and surfactant, rabbits

Pretreatment of the eye with a drop of sterile, isotonic, 2.6% sodium borate solution (pH 9.2) was shown to reduce markedly the amount of an alkaloidal drug required to produce a mydriatic or miotic response in the eye (1). This technique did not affect the stability of ophthalmic solutions, since it was the eye and not the solution that was temporarily buffered.

Good *et al.* (2) introduced 12 "biological" buffers which they claimed to be suitable for biological research. In the current study the buffering effect of available biological buffers, cyclohexylaminopropanesulfonic acid[1] and tris(hydroxymethyl)methylaminopropanesulfonic acid[2], was compared with that of 2.6% sodium borate solution whose buffer action was previously known to be effective. Homatropine hydrobromide and tropicamide were the mydriatics employed for the comparisons; rabbits were used as the test subjects because the biological buffers have not been cleared by the Food and Drug Administration for use on humans.

Another objective of this study was to compare the effect of a guar gum viscolizer[3] with the routinely used viscolizer, hydroxypropyl methylcellulose, as an adjuvant for increasing the physiological effect of mydriatic eyedrops in rabbits.

Since it was reported (3–5) that agents which reduce surface tension will generally increase the permeability of certain biological membranes, another objective was to compare the mydriatic effect of homatropine hydrobromide and tropicamide solutions both with and without polysorbate 80.

[1] CAPS, Calbiochem, Los Angeles, CA 90054
[2] TAPS, Calbiochem, Los Angeles, CA 90054
[3] Jaguar J2S-1, Stein, Hall & Co., New York, N.Y.

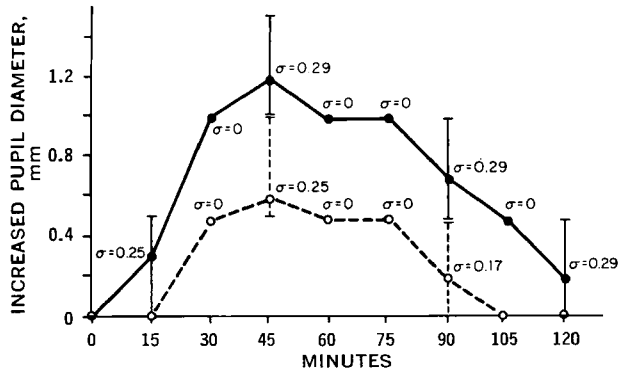

Figure 1—*Mydriatic effect of 0.0075% homatropine hydrobromide solution buffered* (●) *with 0.2* M *tris(hydroxymethyl)-methylaminopropanesulfonic acid or unbuffered* (○).

EXPERIMENTAL

Subjects and Materials—Male New Zealand white rabbits, 2.0–2.5 kg, were used. At least four rabbits were used for each experiment. The mydriatics used were homatropine hydrobromide and tropicamide; the buffers used were sodium borate decahydrate, cyclohexylaminopropanesulfonic acid, and tris(hydroxymethyl)methylaminopropanesulfonic acid. The viscolizers were guar gum and hydroxypropyl methylcellulose (4000 cps). Polysorbate 80 was used as the surface-active agent.

Preparation of Aqueous Solutions of Mydriatics and Buffers—Aqueous solutions of 0.0075% homatropine hydrobromide, 0.0025% tropicamide, and 2.6% sodium borate were prepared and sterilized by filtration using a sterile filter adapter[4] and membrane (0.45 μm)[5]. Adjustment of the tonicity of the test solutions was not considered. Aqueous solutions of 0.2 *M* cyclohexylaminopropanesulfonic acid (pKa 10.4) and 0.2 *M* tris(hydroxymethyl)-methylaminopropanesulfonic acid (pKa 8.4) were prepared with the pH of each solution adjusted to the pKa value of each buffer with 1 *M* NaOH to obtain the maximum buffer capacity in each instance. Final solutions were sterilized by filtration.

Preparation of Mydriatics in Viscous Vehicles—Solutions of hydroxypropyl methylcellulose, 0.7%, and guar gum, 0.375%, were prepared and sterilized in an autoclave at 121° for 30 min. The required amount of homatropine hydrobromide powder was then added and dissolved in the viscolizer solution. Although this method did not guarantee perfect sterility, no bacterial infections were noted in the eyes in any test animal.

Preparation of Aqueous Mydriatic Solutions Containing Surfactant—Aqueous solutions of mydriatics containing polysorbate 80 were prepared by making aqueous solutions of 0.02% polysorbate 80, 0.015% homatropine hydrobromide, and 0.005% tropicamide. The solutions were sterilized by bacterial filtration,

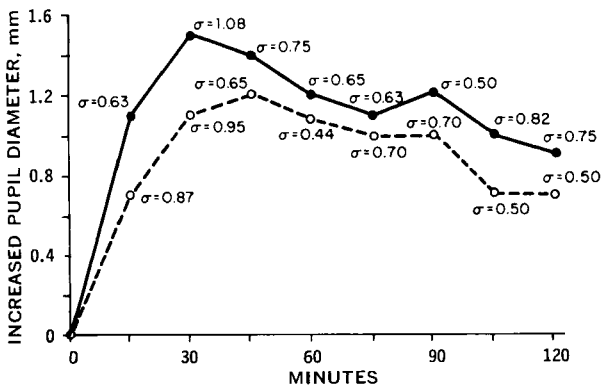

Figure 2—*Mydriatic effect of 0.0075% homatropine hydrobromide buffered with 0.2* M *cyclohexylaminopropanesulfonic acid* (●) *or 2.6% sodium borate* (○).

[4] Swinny.
[5] Type HA, Millipore Filter Corp., Bedford, Mass.

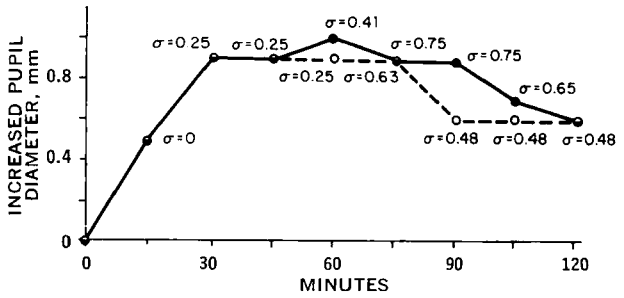

Figure 3—*Mydriatic effect of 0.0075% homatropine hydrobromide solution with 0.375% guar gum* (○) *or 0.7% hydroxypropyl methylcellulose* (●).

and equal volumes of each were mixed aseptically immediately prior to use.

Measurement of Pupil Size—The pupil sizes of the experimental rabbits were determined according to the method of Wang and Hammarlund (6). The pupil diameters were measured before the initial instillation of drops and at various intervals thereafter. The results are presented as millimeters of increased diameter of the average of at least four rabbits in each case.

Effect of Prebuffer Solution on Mydriatic Response—Aqueous 0.0075% homatropine hydrobromide was employed as the mydriatic test solution. Before any solution was instilled into the eyes, the normal pupil diameter was measured. Two drops of the buffer solution under test were dropped on the cornea of one eye, and the rabbit was allowed to blink normally several times. Any excess buffer solution on the eyelid was wiped off with soft tissue. No buffer was instilled into the other eye, which served as a control. Then 1 drop of the mydriatic solution was dropped on the cornea of each eye. The pupil diameters were measured every 15 min for 2 hr following instillation of homatropine hydrobromide. In succeeding tests comparing the relative effectiveness of two buffers on different eyes of the same rabbit at the same time, one eye of a series of four rabbits was prebuffered with 2.6% sodium borate and the other eye was prebuffered with 0.2 *M* cyclohexylaminopropanesulfonic acid prior to the instillation of homatropine hydrobromide solutions on the cornea of each eye.

Effect of Viscolizers in Mydriatic Solutions—The relative effectiveness of guar gum solution and hydroxypropyl methylcellulose solution on the corneal absorption of homatropine hydrobromide and tropicamide was tested in the eyes of four rabbits. Measurements of the pupil diameter were taken every 15 min for 2 hr following instillation of homatropine hydrobromide and every 10 min for 1 hr in the case of tropicamide.

Effect of Surfactant in Mydriatic Ophthalmic Solutions—A 0.01% aqueous solution of polysorbate 80, which is considerably greater than its critical micelle concentration (CMC), was used in this study. One rabbit's eye was treated with tropicamide in surfactant solution while the other eye was treated with a similar concentration of plain aqueous tropicamide solution. Another rabbit's eye was treated with homatropine hydrobromide in surfactant solution while the other eye was treated with plain homatropine hydrobromide aqueous solution. The pupil diameters were measured every 15 min for 2 hr for homatropine hydrobromide dilation and every 10 min for 2 hr in the case of tropicamide.

To find out if the surfactant would markedly increase the mydriatic effect given by the normal prebuffering effect of 2.6% sodium borate, each eye of a rabbit was first buffered with 2 drops of 2.6% sodium borate solution. One drop of 0.0075% homatropine hydrobromide solution was instilled into one of the two buffered eyes of a rabbit while 0.0075% homatropine hydrobromide in 0.01% polysorbate 80 solution was instilled into the other buffered eye. The pupil diameter was measured before the initial instillation and every 15 min thereafter over a 2-hr period for homatropine hydrobromide dilation.

RESULTS AND DISCUSSION

Before undertaking the mydriatic study in rabbits, it was necessary to ascertain the proper concentration of drug to give the desired response in rabbit eyes. The threshold concentration sought was the minimum concentration that would give a mea-

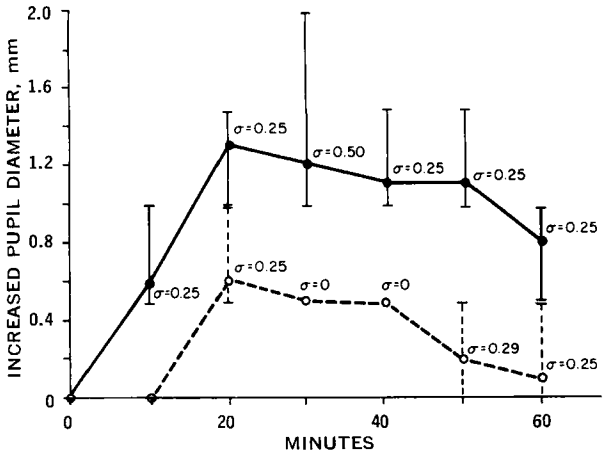

Figure 4—*Mydriatic effect of 0.0025% tropicamide solution in the presence* (●) *and absence* (○) *of 0.7% hydroxypropyl methylcellulose.*

surable mydriatic response but that was small enough for any increase in response brought about by an adjuvant to be apparent. The rabbit threshold concentration for tropicamide was found to be 0.0025%, and that for homatropine hydrobromide was reported to be 0.0075% (6).

It was observed in this investigation that the physiological effects of the ophthalmic solutions containing certain weakly basic drugs are increased by temporarily increasing the pH of the cornea with a prebuffering technique or by certain viscolizers or surfactants. The standard deviation was calculated for each set of results, and this value is indicated on each graph along with the plotted mean for four rabbits. The data for the four rabbits coincided exactly in those few cases where the standard deviation is listed as zero in the graphs. In some instances the ranges of the data from the experimental and control eyes overlapped slightly; in most instances they did not. For the experimental data in those ranges where the test and control values showed the greatest differences in the early stages of each determination, *t*-tests were performed.

The results from use of the three prebuffering solutions, aqueous 2.6% sodium borate (pH 9.2), aqueous 0.2 *M* tris(hydroxymethyl)methylaminopropanesulfonic acid (pH 8.4), and aqueous 0.2 *M* cyclohexylaminopropanesulfonic acid (pH 10.4), on rabbit corneal absorption of homatropine hydrobromide are shown in Figs. 1 and 2. In Fig. 1 the prebuffered eyes showed a markedly increased mydriatic effect over the unbuffered eyes for the entire 2-hr test duration. A *t*-test confirmed that the mydriatic effect was greater at the 5% level of significance for the period between 15 and 30 min for the prebuffered eyes as compared to the nonbuffered control eyes.

Figure 2 shows that cyclohexylaminopropanesulfonic acid prebuffer enhanced the mydriatic effect of homatropine hydrobromide more than did the sodium borate prebuffer. But a strict paired *t*-test for the period between 15 and 30 min revealed that there was essentially no difference between the effects of these

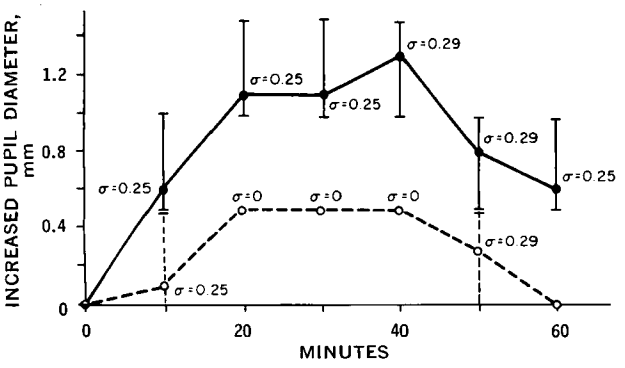

Figure 5—*Mydriatic effect of 0.0025% tropicamide solution in the presence* (●) *and absence* (○) *of 0.375% guar gum.*

two prebuffer solutions on the mydriatic action of homatropine hydrobromide. This might be because there was only a little difference in the pH values of the two prebuffers.

In comparing the enhanced mydriatic effects from the three prebuffers in Figs. 1 and 2, it is evident the amount of increase roughly parallels their differences in pH values, with the greatest increase produced by the buffer with the highest pH and the smallest increase produced by the one with the lowest pH. This is not to suggest that pH at the cornea is the only criterion affecting corneal absorption of ionizable substances; it was previously reported (7, 8) that other factors, such as buffer capacity, viscosity, surface tension, complexation, corneal contact time, and molecular size, also play a role. However, in this brief study where these other variables were fairly constant, the enhanced mydriatic activity did occur in the exact order of the increased pH of each prebuffer solution.

The amount of free base of homatropine hydrobromide available at the various pH's was calculated as follows from the pKa of each buffer:

$$\text{percentage free base} = 100\,\frac{\text{antilog (pH}-\text{pKa)}}{1 + \text{antilog (pH}-\text{pKa)}} \qquad \text{(Eq. 1)}$$

which gives an amount of free base of 0.2% at pH 7.4, 4.8% at pH 8.4, 24% at pH 9.2, and 83.3% at pH 10.4. However, the normal pH on the corneal surface is rarely as low as the theoretical value of 7.4 because some carbon dioxide is continually lost from the surface of the cornea to the air, thus increasing the actual pH of the eyes to about 8. This increase in pH varies considerably depending upon the amount of fresh tears, exposure of cornea to air, the frequency of eye blinking, *etc.*

Drop-size doses were used for the mydriatic solutions containing viscolizers as well as for the buffer solutions because the size of the drop was found not to vary enough to be significant. In fact, all of the solutions tested, whether they contained a viscolizer or not, required exactly 17 drops to measure 1 ml. The results on the effect of viscolizers on the corneal absorption of homatropine hydrobromide and tropicamide are shown in Figs. 3–5. When comparing reinforcement of corneal absorption given by two solutions containing viscolizers having quite similar viscosities, *i.e.*, 45 cps for 0.7% hydroxypropyl methylcellulose and 41 cps for 0.375% guar gum, one might expect that the presence of the viscolizers would provide a similar increase in response or that the slightly more viscous solution would have a slightly greater mydriatic effect. This was found to be the case, and Fig. 3 shows that hydroxypropyl methylcellulose produced only slightly greater mydriatic response than did guar gum for homatropine hydrobromide. But a *t*-test could not detect any difference at the 5% level of significance during the period between 60 and 105 min. Both viscolizers significantly increased ($p = 0.05$) the mydriatic effect of tropicamide about an equal amount (Figs. 4 and 5).

When the mydriatic effect of 0.0075% homatropine hydrobromide and 0.0025% tropicamide solutions in 0.01% polysorbate 80

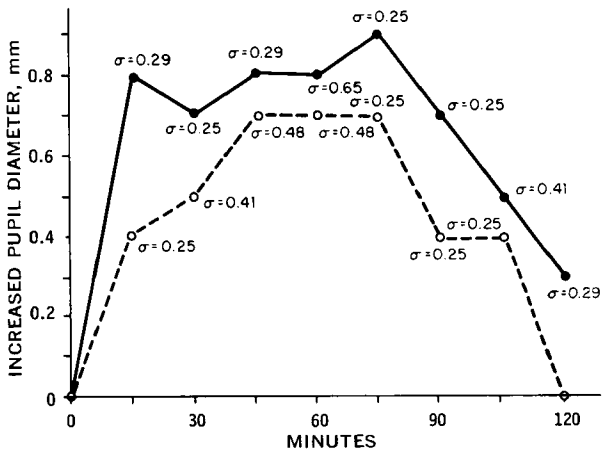

Figure 6—*Mydriatic effect of 0.0075% homatropine hydrobromide solution in the presence* (●) *and absence* (○) *of 0.01% polysorbate 80.*

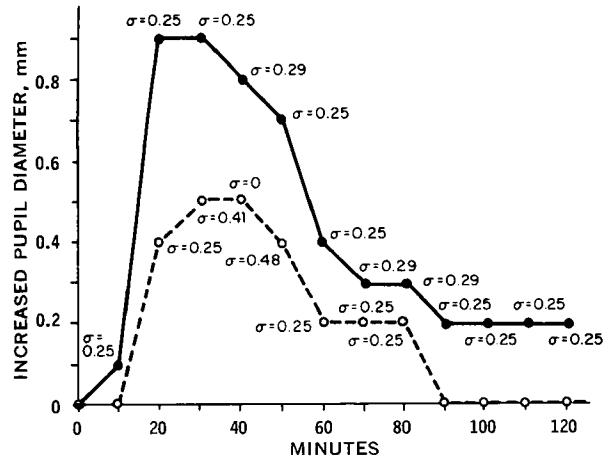

Figure 7—*Mydriatic effect of 0.0025% tropicamide solution in the presence* (●) *and absence* (○) *of 0.01% polysorbate 80.*

was compared to that given by identical but surfactant-free aqueous solutions, the mydriatic effect of both test solutions was found to have increased; *t*-tests were performed between 15 and 30 min on the data in Fig. 6 and between 20 and 40 min for Fig. 7. The increases were significant ($p = 0.05$) in both cases. The increase in mydriatic effect of both test solutions due to the presence of surfactant was much greater for tropicamide than for

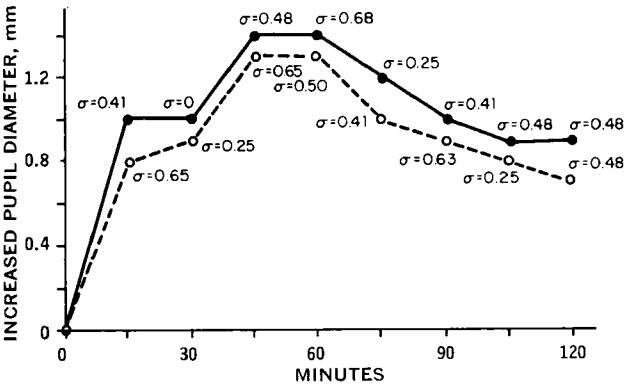

Figure 8—*Mydriatic effect of borate-prebuffered 0.0075% homatropine hydrobromide solution* (○) *and borate-prebuffered 0.0075% homatropine hydrobromide solution in 0.01% polysorbate 80* (●).

homatropine hydrobromide during the first 30 min. However, this greater effect was of shorter duration with tropicamide. To ascertain whether surfactant further enhanced the effect of borate prebuffer on the corneal absorption, 2.6% sodium borate was utilized in a similar series of experiments as a prebuffer solution. A further increase in mydriatic response was found at 15 min ($p = 0.05$) when the eye was prebuffered with sodium borate solution before instillation of the solution containing 0.0075% homatropine hydrobromide in 0.01% polysorbate 80. Therefore, it can be concluded that the effect of a mydriatic in rabbit eyes can be enhanced by employing a simple prebuffering technique for the administration of eyedrops or by adding certain adjuvants usually in addition to the prebuffer solution. This technique could make it possible to reduce the drug concentration in the drops or to give less frequent administration of drugs. It is reasonable to assume this dosage reduction would eliminate some undesirable side effects as in the case of atropine in which toxic effects have been reported to occur in patients with reduced renal function (9). The techniques studied in this investigation, if properly used, may likewise increase the efficiency of many other ophthalmic solutions normally employed in clinical practice if their pKa values are in the acceptable range.

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[x] To whom inquiries should be directed.

Influence of Shape Factors on Kinetics of Drug Release from Matrix Tablets I: Theoretical

JOHN COBBY*, MICHAEL MAYERSOHN[x], and GEORGE C. WALKER

Abstract □ An expression having a basic cubic form was derived to describe the *in vitro* release of drugs from slow-release tablets formulated with a homogeneous insoluble matrix. While the rate of drug release varies with tablet shape, rate constants that are independent of shape are included in the expression. By comparison with previously published work, it was shown that the rate constants may be described in terms of the physicochemical properties of the tablet constituents and the degree of compression. Therefore, it should be possible to evaluate the rate constants for model formulations and then, using the pertinent release expression, to predict the rate of drug release from tablets having identical compositions but varying widely in shape and size.

Keyphrases □ Tablets, slow release—*in vitro* release from homogeneous insoluble matrix, theory and equations relating release and tablet shape □ Drug release from matrix tablets—relationship between release and tablet shape, theory, equations □ Timed-release tablets—*in vitro* slow release from homogeneous insoluble matrix, effect of tablet shape, theory, equations

The advantages of timed-release medication were noted by Lazarus and Cooper (1), who stated: "The primary advantage of increasing the duration of action of a drug is to prolong its therapeutic effect, particularly where continuous action at an adequate level is essential for mitigation of the disease process itself." Attempts to meet such an aim have been numerous, giving rise to many timed-release dosage forms.

A technique often used in the manufacture of timed-release tablets is to formulate a core of material that slowly releases drug and surround it with a layer containing the same drug in a formulation designed for rapid release (2, 3). In this way, the desired therapeutic blood level of the drug should be rapidly achieved and then maintained over a suitable time. However, in some instances, the outer rapidly releasing layer is not utilized and such dosage forms become, in effect, slow-release tablets. The therapeutic efficacy of slow-release tablets has been investigated for hyoscyamine (4), alprenolol (5, 6), prednisolone (7), potassium chloride (8, 9), norepinephrine chloride (10), and lithium (11).

The purposes of this paper are to propose kinetic expressions for the rate of drug release from slow-release tablets and to relate release to tablet shape. The expressions should be equally valid when applied to the slow-release core of timed-release tablets.

REVIEW OF KINETIC TREATMENT

The release of solutes from nondisintegrating solids has been described by many kinetic theories, some of which apply specifically to slow-release matrix tablets. If it is assumed that the surface area of the solid (tablet) changes negligibly during dissolution (in effect remaining essentially equal to its initial value) and that sink conditions are operative, integration of an equation similar to that used by Nernst and Brunner (12) between time t and zero time will yield:

$$W_0 - W_t = Kt \qquad \text{(Eq. 1)}$$

where W_0 is the initial weight of solute (drug) in the solid (tablet), W_t is the weight of solute (drug) remaining in the solid (tablet) at time t, and K is a proportionality constant having the dimensions of weight per unit time. Dividing through by W_0 and simplifying yield:

$$f_t = K_0 t \qquad \text{(Eq. 2)}$$

where f_t is the fraction of solute (drug) dissolved (released) at time t, and K_0 is the zero-order release rate constant having the dimension of reciprocal time[1]. Thus, a plot of the fraction of drug released against time will be linear if sink conditions are operative and if the exposed surface area is maintained constant. Various workers (13–15) reported data that fit such a plot, either when the exposed surface area was experimentally maintained constant or assumed to be so.

Hixson and Crowell (16, 17), recognizing that the surface area of a regular particle is proportional to the two-thirds power of its volume, derived their classic "cube root law," which may be written as:

$$W_0^{1/3} - W_t^{1/3} = K_s t \qquad \text{(Eq. 4)}$$

where K_s is a constant incorporating the surface-volume proportionality and has the dimensions of the cube root of weight per unit time. This relationship applies to tablets where dissolution takes place normal to the exposed surface, and, if the tablet dimensions decrease in proportion to one another, the exact initial geometric shape of the tablet is maintained at all times. Dividing through by $W_0^{1/3}$, simplifying (see Eq. 3), and rearranging yield:

$$(1 - f_t)^{1/3} = 1 - K_\beta t \qquad \text{(Eq. 5)}$$

where K_β is the release rate constant having the dimension of reciprocal time. Thus, a plot of the cube root of the fraction of drug unreleased against time will be linear if sink conditions are operative and if the tablet dimensions decrease in proportion. Various workers (18–21) reported data that fit such a plot.

Wagner (22–24), assuming that the exposed surface area of a tablet decreased exponentially with time, suggested that drug release from most slow-release tablets could be described adequately by apparent first-order kinetics. In the symbolism of this report:

$$W_t = W_0 e^{-K_1 t} \qquad \text{(Eq. 6)}$$

where K_1 is the apparent first-order rate constant having the dimension of reciprocal time. Dividing through by W_0, simplifying (see Eq. 3), and taking logarithms yield:

$$\ln (1 - f_t) = -K_1 t \qquad \text{(Eq. 7)}$$

Thus, a plot of the logarithm of the fraction of drug unreleased

[1] One of the implied simplifying expressions:

$$f_t = 1 - \frac{W_t}{W_0} \qquad \text{(Eq. 3)}$$

will also be used in later derivations and so is stated explicitly at this juncture.

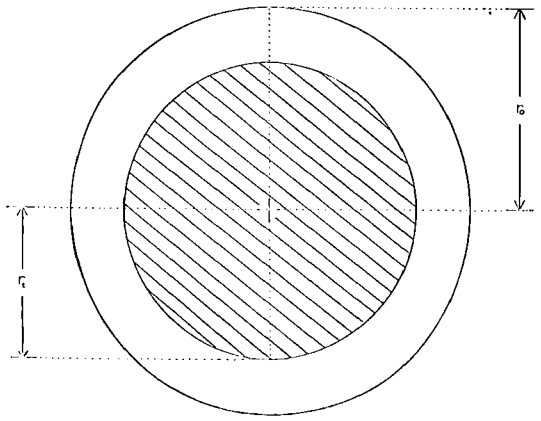

Figure 1—*Bisection of a spherical matrix tablet at time = t. Hatched area is unreleased portion; clear area is ghost portion.*

against time will be linear if sink conditions are operative. Many workers reported data that fit such a plot, and a few examples (13, 25–28) are illustrative of this commonly used plot.

In 1963, Higuchi (29) derived a kinetic theory for a spherical, slow-release pellet having a homogeneous matrix. The system discussed is illustrated in Fig. 1, which shows the situation at time t. Because of the insolubility of the matrix, the pellet still exhibits its initial radius of r_0, but leaching of the drug actually takes place at the release boundary that has a radius of r_t. Between the two radii, there is an already leached region of the sphere, the "ghost" portion, containing the matrix substance only; within the radius r_t, there remains the unreleased portion containing both drug and matrix. If it is assumed that the weight of drug in diffusive passage across the ghost portion is negligible compared to that remaining in the unreleased portion, the weight of drug remaining in the pellet is proportional to the volume (and, hence, the cube of the radius) of the unreleased portion. This assumption, which parallels that of Hixson and Crowell (16, 17), may be expressed for a regular solid as follows (see also Eq. 3):

$$\frac{W_t}{W_0} = \frac{V_t}{V_0} = \left[\frac{r_t}{r_0}\right]^3 = 1 - f_t \qquad \text{(Eq. 8)}$$

where V_t is the volume of the unreleased portion at time t, and V_0 is the initial volume of the unreleased portion and equals the volume of the pellet. By assuming further that the solubility of the drug in the dissolution fluid, C_s, is much less than the weight of drug present per initial tablet volume, A, Higuchi was able to show, in the symbolism of this report:

$$1 - 3\left[\frac{r_t}{r_0}\right]^2 + 2\left[\frac{r_t}{r_0}\right]^3 = Bt \qquad \text{(Eq. 9)}$$

where B is a release rate constant having the dimension of reciprocal time.

Substituting from Eq. 8 and rearranging give:

$$2(1 - f_t) - 3(1 - f_t)^{2/3} = Bt - 1 \qquad \text{(Eq. 10)}$$

This nonlinear equation, which applies only to spherical, slow-release pellets, is conveniently plotted using the fraction of drug released as the ordinate and the square root of time as the abscissa. Higuchi noted that data reported by Simoons (30) fit such a plot when sink conditions are operative.

Another kinetic theory was derived by Higuchi (29). By virtue of its mode of derivation, this theory, which is for tablets having a granular matrix, is limited to release of drug from planar surfaces of constant area; experimentally the area is the initial surface area, S_0. In the symbolism of this report, the equation is:

$$\frac{W_0 - W_t}{S_0} = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t} \qquad \text{(Eq. 11)}$$

where D is the diffusion coefficient of the drug in the dissolution fluid, ϵ is the porosity of the matrix (ghost portion), and τ is the tortuosity factor of the matrix. After rearranging and dividing through by W_0, the expression may be simplified (see Eq. 3) to give:

$$f_t = K_H t^{1/2} \qquad \text{(Eq. 12)}$$

where K_H may be termed the Higuchi release rate constant having the dimension of the reciprocal of the square root of time[2]. Thus, a plot of the fraction of drug released against the square root of time will be linear if sink conditions are operative. Many workers reported data that fit such a plot, some (31–40) by experimentally maintaining a constant planar surface area and others (27, 28, 41) by assuming it to be so.

THEORETICAL

Boundary Retreat Distance—Because the following derivations apply to slow-release tablets formulated with a homogeneous insoluble matrix, a ghost portion, similar to that illustrated in Fig. 1 will be present once drug release has commenced.

As drug release proceeds, the release boundary, where the actual dissolution of solid drug occurs, retreats further from the tablet edge (Fig. 2). The length of the boundary retreat distance, X_t, increases with time in a manner analogous to that shown by Higuchi (42) for the release of drugs from ointments:

$$X_t = K_b t^{1/2} \qquad \text{(Eq. 14)}$$

where K_b is the boundary retreat rate constant having the dimensions of length per unit square root of time. It may be expected that the magnitude of this constant will be dependent on the physical properties of the drug and tablet excipients and on the compaction pressure used in manufacturing the tablet; however, the constant is independent of both the shape and dimensions of the tablet.

Tablet Shapes and Boundary Retreat Distance—Two shapes predominate among commercially available tablets. The first is cylindrical; the second is biconvex, which may be visualized as a cylinder with a spherical segment added above and below.

If dissolution takes place normal to the release boundary (16, 17, 29), the boundary retreat distance, X_t, will be equal in all directions. The situation is shown in Fig. 3 for a cylindrical tablet and in Fig. 4 for a biconvex tablet. For the former, both the initial tablet radius, r_0, and half the initial tablet height, h_0, are reduced at time t to:

$$r_t = r_0 - X_t \qquad \text{(Eq. 15)}$$

and:

$$h_t = h_0 - X_t \qquad \text{(Eq. 16)}$$

where r_t and h_t are the parameters at time t. For the biconvex tablet, a geometric analysis reveals the following parameter reductions at time t:

$$r_t = r_0 - X_t \qquad \text{(Eq. 15)}$$

$$R_t = R_0 - X_t \qquad \text{(Eq. 17)}$$

where R_t and R_0 are the radii of the spherical segment:

$$HE_t = HE_0 - Y_t \qquad \text{(Eq. 18)}$$

where HE_t and HE_0 are half the heights of the tablet edge and Y_t is the dependent boundary retreat distance, and:

$$HD_t = HD_0 - (X_t - Y_t) \qquad \text{(Eq. 19)}$$

[2] The implied simplifying expression:

$$K_H = \frac{S_0}{W_0}\sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s} \qquad \text{(Eq. 13)}$$

will be useful in the further theoretical development and is stated explicitly at this juncture.

where HD_t and HD_0 are the heights of the spherical segment.

Release Rate Constant—To simplify further derivations for both tablet shapes, it is convenient to divide the boundary retreat distance (Eq. 14) by the initial tablet radius to give:

$$\frac{X_t}{r_0} = \left(\frac{K_b}{r_0}\right)t^{1/2} \quad \text{(Eq. 20)}$$

If:

$$\frac{K_b}{r_0} = K_r \quad \text{(Eq. 21)}$$

then:

$$\frac{X_t}{r_0} = K_r t^{1/2} \quad \text{(Eq. 22)}$$

where K_r is the release rate constant having only the dimension of the reciprocal of the square root of time. While its magnitude is independent of tablet shape, it is dependent on the tablet radius.

Kinetic Expression for Cylindrical Tablet—*Step 1*—The tablet parameters at time t are restated in terms of the initial tablet radius, r_0, and the boundary retreat distance, X_t. If:

$$q = \frac{r_0}{h_0} \quad \text{(Eq. 23)}$$

where q is a ratio factor, then Eq. 16 modifies to:

$$h_t = \frac{r_0}{q} - X_t \quad \text{(Eq. 24)}$$

The tablet radius at time t needs no restatement (Eq. 15).

Step 2—The volume of the cylindrical unreleased portion at time t is given by (43):

$$V_t = 2\pi r_t^2 h_t \quad \text{(Eq. 25)}$$

Substituting from Eqs. 15 and 24 gives:

$$V_t = 2\pi(r_0 - X_t)^2(r_0/q - X_t) \quad \text{(Eq. 26)}$$

Expanding gives:

$$V_t = 2\pi\left[\frac{r_0^3}{q} - r_0^2 X_t\left(1 + \frac{2}{q}\right) + r_0 X_t^2\left(2 + \frac{1}{q}\right) - X_t^3\right] \quad \text{(Eq. 27)}$$

Rearranging gives:

$$V_t = \frac{2\pi r_0^3}{q}\left[1 - (q+2)\frac{X_t}{r_0} + (2q+1)\frac{X_t^2}{r_0^2} - \frac{qX_t^3}{r_0^3}\right] \quad \text{(Eq. 28)}$$

But from Eq. 23 and a knowledge of mensuration (43):

$$\frac{2\pi r_0^3}{q} = 2\pi r_0^2 h_0 = V_0 \quad \text{(Eq. 29)}$$

Substituting into Eq. 28 and rearranging yield:

$$1 - \frac{V_t}{V_0} = (q+2)\frac{X_t}{r_0} - (2q+1)\frac{X_t^2}{r_0^2} + \frac{qX_t^3}{r_0^3} \quad \text{(Eq. 30)}$$

This expression shows the fractional loss in volume of the unreleased portion at time t.

Step 3—Substituting from Eq. 22 gives:

$$1 - \frac{V_t}{V_0} = (q+2)K_r t^{1/2} - (2q+1)(K_r t^{1/2})^2 + q(K_r t^{1/2})^3 \quad \text{(Eq. 31)}$$

If the drug is homogeneously distributed throughout the matrix tablet (29), and if the weight of drug in diffusive passage across the ghost portion is negligible compared to that remaining in the unreleased portion (29), then by substitution from Eq. 8:

$$f_t = (q+2)K_r t^{1/2} - (2q+1)(K_r t^{1/2})^2 + q(K_r t^{1/2})^3 \quad \text{(Eq. 32)}$$

This is the final kinetic expression, having a cubic form, that relates the fraction of drug released to time for a cylindrical tablet. While in theory the value of q may be infinitely variable, elegant tablets are manufactured with $q > 1$.

Kinetic Expression for Biconvex Tablet—This derivation is more complex but proceeds *via* the same steps as the cylindrical tablet derivation.

Step 1—The ratio of Y_t to X_t varies with the time of dissolution. The value of Y_t may be expressed by:

$$Y_t = \frac{Z_t}{K_r}(X_t) \quad \text{(Eq. 33)}$$

where Z_t is a simplifying factor that varies with time. The derivation of Eq. 33 is shown in the *Appendix*. If:

$$p = \frac{r_0}{HD_0} \quad \text{(Eq. 34)}$$

where p is a ratio factor, then Eq. 19 modifies to:

$$HD_t = \frac{r_0}{p} - (X_t - Y_t) \quad \text{(Eq. 35)}$$

Substituting from Eq. 33 gives:

$$HD_t = \frac{r_0}{p} - X_t(1 - Z_t/K_r) \quad \text{(Eq. 36)}$$

Consideration of Fig. 4 shows that:

$$HE_t = h_t - HD_t \quad \text{(Eq. 37)}$$

Substituting from Eqs. 24 and 36 and rearranging give:

$$HE_t = r_0(1/q - 1/p) - \frac{Z_t}{K_r}(X_t) \quad \text{(Eq. 38)}$$

Step 2—The volume of the biconvex unreleased portion at time t is given by the sum of the volume of the cylindrical portion and the volume of two spherical segments (43):

$$V_t = \pi\left[\frac{HD_t^3}{3} + r_t^2(HD_t + 2HE_t)\right] \quad \text{(Eq. 39)}$$

Substituting from Eqs. 15, 36, and 38 and expanding yield:

$$V_t = \pi\left[\frac{r_0^3}{3p^3q}\{6p^3 - 3p^2q + q\} - \frac{r_0^2 X_t}{p^2 q}\left\{4p^2 + p^2q - 2pq + q + \left(\frac{Z_t}{K_r}\right)q(p^2-1)\right\} + \frac{r_0 X_t^2}{pq}\left\{2p + 2pq + \left(\frac{Z_t}{K_r}\right)2q(p-1) + \left(\frac{Z_t^2}{K_r^2}\right)q\right\} - \frac{X_t^3}{3}\left\{4 + 3\left(\frac{Z_t^2}{K_r^2}\right) - \frac{Z_t^3}{K_r^3}\right\}\right] \quad \text{(Eq. 40)}$$

It is helpful to use five simplifying constants, C_3–C_7:

$$C_3 = 6p^3 - 3p^2q + q \quad \text{(Eq. 41)}$$

$$C_4 = 4p^2 + p^2q - 2pq + q \quad \text{(Eq. 42)}$$

$$C_5 = 2p + 2pq \quad \text{(Eq. 43)}$$

$$C_6 = q(p^2 - 1) \quad \text{(Eq. 44)}$$

$$C_7 = 2q(p-1) \quad \text{(Eq. 45)}$$

With these constants included:

$$V_t = \pi\left[C_3\frac{r_0^3}{3p^3q} - \frac{r_0^2 X_t}{p^2 q}\left\{C_4 + C_6\left(\frac{Z_t}{K_r}\right)\right\} + \frac{r_0 X_t^2}{pq}\left\{C_5 + C_7\left(\frac{Z_t}{K_r}\right) + q\left(\frac{Z_t^2}{K_r^2}\right)\right\} - \frac{X_t^3}{3}\left\{4 + 3\left(\frac{Z_t^2}{K_r^2}\right) - \frac{Z_t^3}{K_r^3}\right\}\right] \quad \text{(Eq. 46)}$$

Rearranging gives:

$$V_t = \pi r_0^3\left(\frac{C_3}{3p^3q}\right)\left[1 - \frac{3p}{C_3}\left\{C_4 + C_6\left(\frac{Z_t}{K_r}\right)\right\}\frac{X_t}{r_0} + \frac{3p^2}{C_3}\left\{C_5 + C_7\left(\frac{Z_t}{K_r}\right) + q\left(\frac{Z_t^2}{K_r^2}\right)\right\}\frac{X_t^2}{r_0^2} - \frac{p^3 q}{C_3}\left\{4 + 3\left(\frac{Z_t^2}{K_r^2}\right) - \frac{Z_t^3}{K_r^3}\right\}\frac{X_t^3}{r_0^3}\right] \quad \text{(Eq. 47)}$$

But from Eqs. 23, 34, and 41, the zero-time equivalent of Eq. 37, and a knowledge of mensuration (43):

$$\pi r_0^3\left(\frac{C_3}{3p^3q}\right) = \pi\left[\frac{HD_0^3}{3} + r_0^2(HD_0 + 2HE_0)\right] = V_0 \quad \text{(Eq. 48)}$$

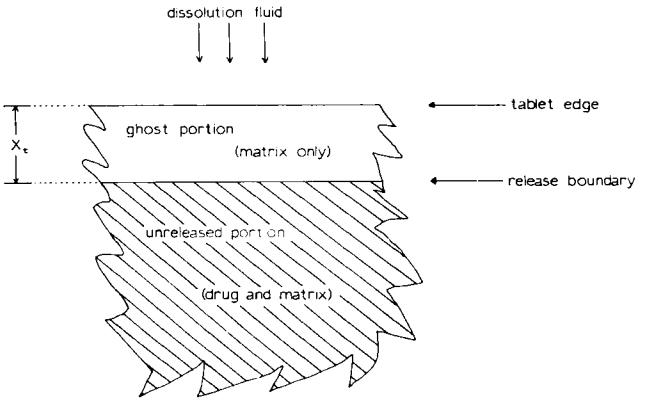

Figure 2—*Vertical bisection of a matrix tablet at time = t, showing the inward retreat of the release boundary.*

Substituting into Eq. 47 and rearranging give:

$$1-\frac{V_t}{V_0}=\frac{3p}{C_3}\left\{C_4+C_6\left(\frac{Z_t}{K_r}\right)\right\}\frac{X_t}{r_0}-\frac{3p^2}{C_3}\left\{C_5+C_7\left(\frac{Z_t}{K_r}\right)+q\left(\frac{Z_t^2}{K_r^2}\right)\right\}\frac{X_t^2}{r_0^2}+\frac{p^3q}{C_3}\left\{4+3\left(\frac{Z_t^2}{K_r^2}\right)-\frac{Z_t^3}{K_r^3}\right\}\frac{X_t^3}{r_0^3} \quad \text{(Eq. 49)}$$

Step 3—Substituting from Eq. 22, with the assumptions made previously (29), gives:

$$f_t=\frac{3p}{C_3}\left\{C_4+C_6\left(\frac{Z_t}{K_r}\right)\right\}K_rt^{1/2}-\frac{3p^2}{C_3}\left\{C_5+C_7\left(\frac{Z_t}{K_r}\right)+q\left(\frac{Z_t^2}{K_r^2}\right)\right\}(K_rt^{1/2})^2+\frac{p^3q}{C_3}\left\{4+3\left(\frac{Z_t^2}{K_r^2}\right)-\frac{Z_t^3}{K_r^3}\right\}(K_rt^{1/2})^3 \quad \text{(Eq. 50)}$$

This is the final kinetic expression, having a basic cubic form, that relates the fraction of drug released to time for a biconvex tablet. While in theory the values of q and p may be infinitely variable, elegant tablets are manufactured with $q > 1$ and $p > 1$.

Special Condition of Derived Biconvex Tablet Expression—A spherical tablet may be considered a limited form of a biconvex tablet in which the two hemispheres are joined base to base. The height of each hemisphere is then half the tablet height, which in turn is equal to the radius of each hemisphere:

$$HD_0 = h_0 = r_0 \quad \text{(Eq. 51)}$$

It thus follows from Eqs. 23 and 34 that:

$$q = p = 1 \quad \text{(Eq. 52)}$$

Furthermore, the values of C_3–C_5 (Eqs. 41–43) become:

$$C_3 = C_4 = C_5 = 4 \quad \text{(Eq. 53)}$$

For a hemisphere, it may be shown (see *Appendix*) that:

$$Z_t = 0 \quad \text{(Eq. 54)}$$

Substituting from Eqs. 52–54 in Eq. 50 yields:

$$f_t = 3K_rt^{1/2} - 3(K_rt^{1/2})^2 + (K_rt^{1/2})^3 \quad \text{(Eq. 55)}$$

This final kinetic expression for a spherical tablet (which is virtually nonexistent commercially) could also have been derived by a route analogous to that used for cylindrical and biconvex tablets.

Summarized Expressions—The three derived expressions (Eqs. 32, 50, and 55) all have a basic cubic form, so they may be summarized:

$$f_t = G_1K_rt^{1/2} - G_2(K_rt^{1/2})^2 + G_3(K_rt^{1/2})^3 \quad \text{(Eq. 56)}$$

where G_1–G_3 may be termed shape factors; their values are shown in Table I. This summarized expression, which contains the radius-dependent release rate constant, K_r, may be modified

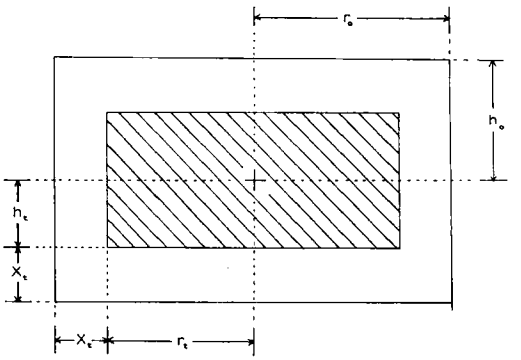

Figure 3—*Vertical bisection of a cylindrical matrix tablet at time = t. Hatched area is unreleased portion; clear area is ghost portion.*

using Eq. 21 to give an expression containing the boundary retreat rate constant, K_b, which is independent of the initial tablet radius as well as of the tablet shape:

$$f_t = \frac{G_1}{r_0}K_bt^{1/2} - \frac{G_2}{r_0^2}(K_bt^{1/2})^2 + \frac{G_3}{r_0^3}(K_bt^{1/2})^3 \quad \text{(Eq. 57)}$$

It should be recognized that, for biconvex tablets, the values of the shape factors would also require modification to replace K_r with K_b.

DISCUSSION

To compare the effect of shape alone on the release of drugs, all parameters other than shape must be standardized. Thus the tablet formulation, the initial tablet weight, the initial tablet radius, the degree of compression (and, hence, the initial tablet volume), and the dissolution test conditions should be the same for different tablet shapes. The parameters of three example tablets, identical in all respects except shape, are shown in Table II together with the times for half and complete release of drug. Their release plots are shown in Fig. 5 using the graphical coordinates analogous to those chosen by Higuchi (29): fraction of drug released and the square root of time. For a series of tablets identical in every respect but shape, a comparison of release plots indicates that the differences in rates of release are related to the shape factors.

Thus, it should be possible to prepare a pilot tablet (of any suitable shape) of a slow-release matrix formulation and determine the boundary retreat rate constant, K_b, of that formulation under the compaction load used. From this knowledge, the release rate constant, K_r, for tablets having the same formulation, but with any desired initial radius, initial volume, and shape, may be calculated providing the compaction load is the same. Furthermore, the rate of drug release from such tablets can be predicted.

Release from One Planar Surface of Cylindrical Tablet—

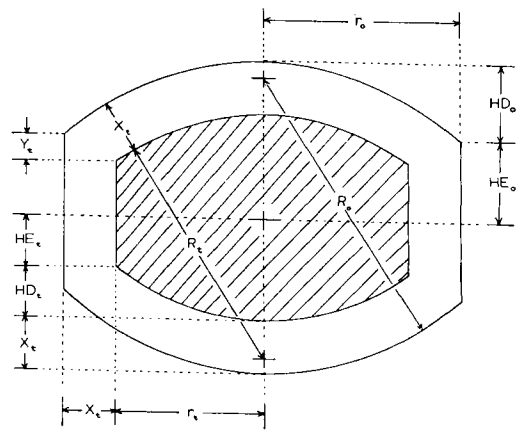

Figure 4—*Vertical bisection of a biconvex matrix tablet at time = t. Hatched area is unreleased portion; clear area is ghost portion.*

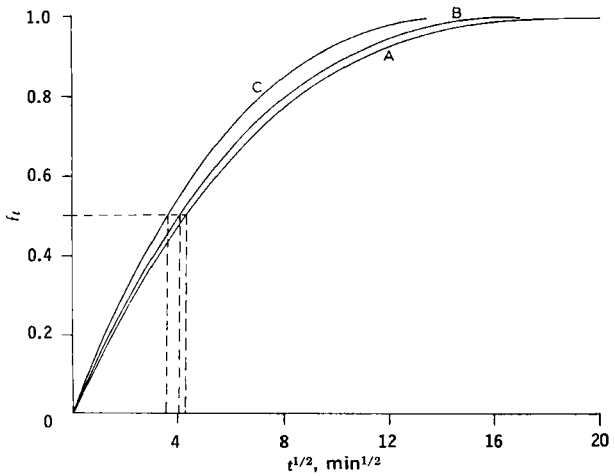

Figure 5—*Theoretical Higuchi plot of the fraction of drug released,* f_t, *with time,* t, *from matrix tablets identical in all respects except shape. Key: A, spherical; B, biconvex; and C, cylindrical. Curves were computed using the parameters listed in Table II and the release equations listed in Table I.*

The release of drugs from many cylindrical, slow-release tablets has been tested by experimentally maintaining a constant surface area (31–40). An impermeable barrier of some kind is maintained, allowing access of the dissolution fluid to one planar surface only; these dissolution conditions are shown in Fig. 6. A kinetic expression may be derived with Step 1 being identical to that for cylindrical tablets having all surfaces exposed to the dissolution fluid (Eqs. 15, 23, and 24).

Step 2—The volume of the cylindrical unreleased portion at time t is given by (43):

$$V_t = \pi r_0^2(2h_0 - X_t) \qquad \text{(Eq. 58)}$$

Substituting from Eq. 23 and manipulating as before give:

$$1 - \frac{V_t}{V_0} = \frac{q}{2}\left(\frac{X_t}{r_0}\right) \qquad \text{(Eq. 59)}$$

Step 3—Substituting from Eq. 22, with the assumptions made previously (29), gives:

$$f_t = \frac{q}{2}(K_r t^{1/2}) \qquad \text{(Eq. 60)}$$

Or, substituting from Eqs. 21 and 23 gives:

$$f_t = \frac{1}{2h_0}(K_b t^{1/2}) \qquad \text{(Eq. 61)}$$

where $2h_0$ is the initial tablet height. These are the final kinetic expressions relating the fraction of drug released to time t for a cylindrical tablet tested when only one planar surface of constant area is exposed to the dissolution fluid. Hence, it is valid to compare Eqs. 60 and 61 with Eq. 12, in which case:

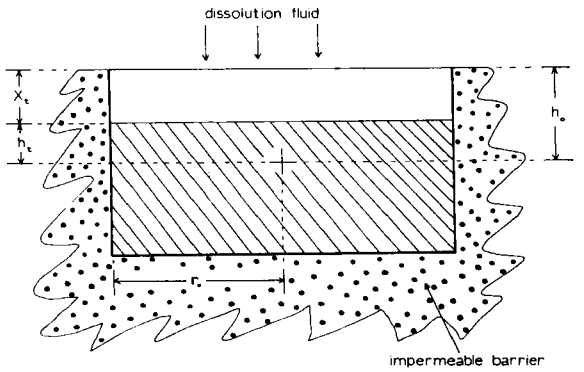

Figure 6—*Vertical bisection of a cylindrical matrix tablet at time* = t *where dissolution takes place from one constant planar surface only. Hatched area is unreleased portion; clear area is ghost portion.*

Table I—Values of Shape Factors in Release Expressions Derived for Three Shapes of Slow-Release Tablets

Shape Factor	Tablet Shape: Spherical	Cylindrical	Biconvex[a]
G_1	3	$q + 2$	$\frac{3p}{C_3}\left\{C_4 + C_6\left(\frac{Z_t}{K_r}\right)\right\}$
G_2	3	$2q + 1$	$\frac{3p^2}{C_3}\left\{C_5 + C_7\left(\frac{Z_t}{K_r}\right) + q\left(\frac{Z_t^2}{K_r^2}\right)\right\}$
G_3	1	q	$\frac{p^3q}{C_3}\left\{4 + 3\left(\frac{Z_t^2}{K_r^2}\right) - \frac{Z_t^3}{K_r^3}\right\}$
	Eq. 55	Eq. 32	Eq. 50

[a] Each value of K_r may be substituted by K_b/r_0 (see Eq. 21).

$$K_r = \frac{2K_H}{q} \qquad \text{(Eq. 62)}$$

and:

$$K_b = 2h_0K_H \qquad \text{(Eq. 63)}$$

Thus the rate constants of this paper may be related to a constant analogous to that derived by Higuchi (29). Furthermore, pilot studies of rate constants, made when only one planar surface of constant area is exposed to the dissolution fluid, may be used to predict the rates of release from tablets of the same formulation with all surfaces exposed, even though they have different shapes, initial radii, and initial volumes; however, the degree of compression must be the same.

Fundamental Parameters of Rate Constants—By definition:

$$W_0 = AV_0 \qquad \text{(Eq. 64)}$$

For a cylindrical tablet with one constant planar surface exposed to the dissolution fluid:

$$W_0 = A\left(\frac{2\pi r_0^3}{q}\right) \qquad \text{(Eq. 65)}$$

and:

$$S_0 = \pi r_0^2 \qquad \text{(Eq. 66)}$$

Substituting Eqs. 65 and 66 into Eq. 13 and simplifying yield:

$$K_H = \frac{q}{2Ar_0}\sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s} \qquad \text{(Eq. 67)}$$

Substituting into Eq. 62 and simplifying yield:

$$K_r = \frac{1}{Ar_0}\sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s} \qquad \text{(Eq. 68)}$$

Substituting from Eq. 21 gives:

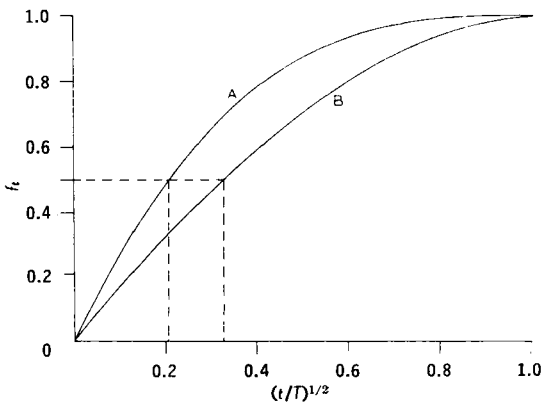

Figure 7—*Comparison of the fraction of drug released,* f_t, *with time,* t, *from spherical matrix tablets. The time is expressed as a fraction of the time for complete release* (T). *Key: A, Eq. 74; and B, Eq. 75.*

Table II—Parameters and Release Times of Drug from Three Slow-Release Tablets Identical in All Respects Except Shape

Tablet Shape	Parameters						Release Times, min	
	K_r, min^{-1}	r_0, mm	V_0, mm^3	q	p	a	Half-Release	Complete Release
Spherical (Eq. 55)	0.05	3.0	113.1	—	—	—	17.02	400.0
Cylindrical (Eq. 32)	0.05	3.0	113.1	1.500	—	—	12.37	177.8
Biconvex (Eq. 50)	0.05	3.0	113.1	1.179	2.618	1.500[a]	15.64	287.9

[a] This value of $a = R_0/r_0$ is common for "extra deep" biconvex tablets (46).

$$K_b = \frac{1}{A}\sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s} \qquad \text{(Eq. 69)}$$

Hence it is shown that the boundary retreat rate constant, K_b, depends only on the physicochemical properties of the tablet constituents and the degree of compression, whereas the release rate constant, K_r, also depends on the initial tablet radius. While it should be feasible to calculate the values of K_b and K_r from measurements of their fundamental parameters, in practical terms it is probably easier to determine the constants by direct release studies. For both rate constants to be algebraically valid, $2A > \epsilon C_s$ to comply with the assumption that the weight of drug in diffusive passage across the ghost portion is negligible compared to that remaining in the unreleased portion. Higuchi (29) noted that A should be greater than ϵC_s by a factor of three or four.

Time for Complete Release—Consideration of Figs. 3 and 4 reveals that at complete release ($f_t = 1$) for a tablet where $q > 1$:

$$X_t = h_0 \qquad \text{(Eq. 70)}$$

(where $h_0 = HE_0 + HD_0$ for biconvex tablets). Substituting in Eq. 23 gives:

$$q = \frac{r_0}{X_t} \qquad \text{(Eq. 71)}$$

Substituting into Eq. 22 gives:

$$\frac{1}{q} = K_r T^{1/2} \qquad \text{(Eq. 72)}$$

where T is the value of t when $f_t = 1$. Thus:

$$K_r = \frac{1}{qT^{1/2}} \qquad \text{(Eq. 73)}$$

Hence, Eq. 56 may be rewritten:

$$f_t = \frac{G_1}{q}\left\{\frac{t^{1/2}}{T^{1/2}}\right\} - \frac{G_2}{q^2}\left\{\frac{t^{1/2}}{T^{1/2}}\right\}^2 + \frac{G_3}{q^3}\left\{\frac{t^{1/2}}{T^{1/2}}\right\}^3 \qquad \text{(Eq. 74)}$$

For spherical tablets, where $q = 1$, it is therefore possible to prepare a standard plot of fraction released against the fraction of the square root of time for complete release. Such a plot is shown in Fig. 7, and it may be calculated that half-release occurs when $t^{1/2}/T^{1/2} = 0.2063$. In other words, for a spherical matrix tablet, the first 50% of the release occurs in approximately the first 4.5% of the complete release time.

Uniqueness of Release Expressions—To demonstrate that the expressions derived for spherical (Eq. 55), cylindrical (Eq. 32), and biconvex (Eq. 50) tablets are unique, the data used to plot Fig. 5 were also plotted on apparent first-order coordinates (Fig. 8) and Hixon–Crowell coordinates (Fig. 9). As can be seen, the data do not give linear plots.

In Fig. 7 a comparison is made between the release profiles of spherical tablets as predicted by the standard expression (Eq. 74) and as predicted from Higuchi (29); to effect comparison, Eq. 10 has been modified by setting $T = t$ when $f_t = 1$ to give finally:

$$2(1 - f_t) - 3(1 - f_t)^{2/3} = \frac{t}{T} - 1 \qquad \text{(Eq. 75)}$$

The different profiles result from different assumptions concerning the concentration gradient of the drug in passage across the ghost portion. It is possible to obtain the derived expression by use of Fick's first law (44) by assuming the concentration gradient to be uniform; Higuchi, however, considered a nonuniform concentration gradient.

Experimental work to test the derived expressions has been performed and the results are presented in a following paper (45).

APPENDIX

Relationship of Dependent Boundary Retreat Distance, Y_t, to Boundary Retreat Distance, X_t—There are two mensuration formulas (43) for the volume of a spherical segment at time t:

$$VD_t = \frac{\pi}{3}HD_t(3R_t - HD_t) \qquad \text{(Eq. A1)}$$

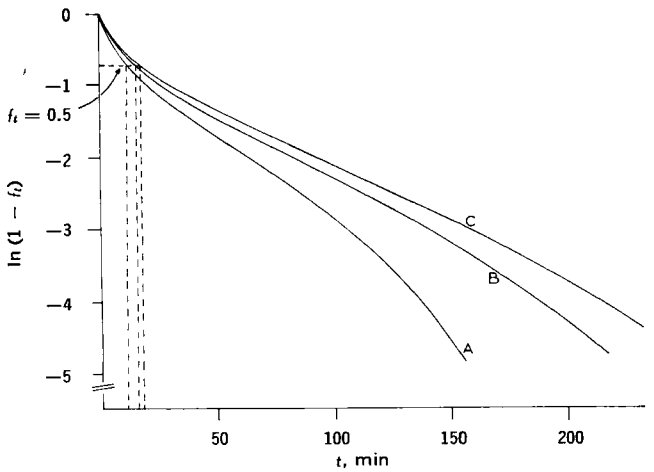

Figure 8—*Theoretical apparent first-order plot of the fraction of drug unreleased, 1 − f_t, with time, t, from matrix tablets identical in all respects except shape. Key: A, spherical; B, biconvex; and C, cylindrical.*

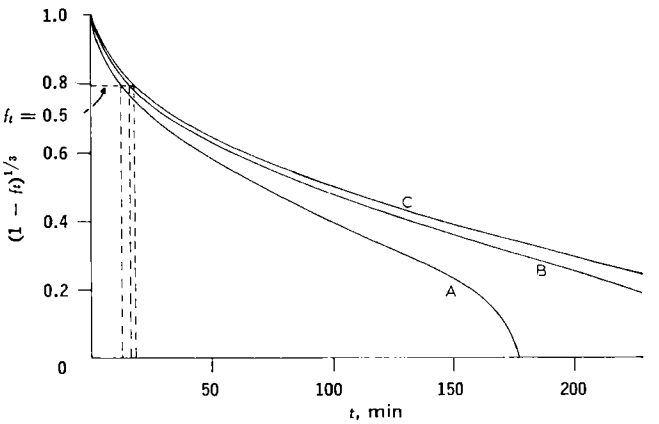

Figure 9—*Theoretical Hixson–Crowell plot of the fraction of drug unreleased, 1 − f_t, with time, t, from matrix tablets identical in all respects except shape. Key: A, spherical; B, biconvex; and C, cylindrical.*

and:

$$VD_t = \frac{\pi}{6} HD_t(HD_t^2 - 3r_t^2) \qquad \text{(Eq. A2)}$$

Equating the two:

$$HD_t^2 - 2R_tHD_t + r_t^2 = 0 \qquad \text{(Eq. A3)}$$

For a biconvex tablet, where $HD_t < R_t$, the real root of the quadratic equation is:

$$HD_t = R_t - [R_t^2 - r_t^2]^{1/2} \qquad \text{(Eq. A4)}$$

Substituting from Eqs. 15 and 17 gives:

$$HD_t = R_0 - X_t - [R_0^2 - r_0^2 - 2R_0X_t - 2r_0X_t]^{1/2} \qquad \text{(Eq. A5)}$$

If:

$$a = \frac{R_0}{r_0} \qquad \text{(Eq. A6)}$$

where a is a ratio factor, then Eq. A5 modifies to:

$$HD_t = ar_0 - X_t - [r_0^2(a^2 - 1) - X_tr_02(a - 1)]^{1/2} \qquad \text{(Eq. A7)}$$

At zero time (when $X_t = 0$), Eq. A7 becomes:

$$HD_0 = ar_0 - [r_0^2(a^2 - 1)]^{1/2} \qquad \text{(Eq. A8)}$$

Substituting from Eqs. A7 and A8 into Eq. 19 and simplifying yield:

$$Y_t = [r_0^2(a^2 - 1)]^{1/2} - [r_0^2(a^2 - 1) - X_tr_02(a - 1)]^{1/2} \qquad \text{(Eq. A9)}$$

Rearranging gives:

$$Y_t = r_0(a^2 - 1)^{1/2} - r_0\left[(a^2 - 1) - \frac{X_t}{r_0}2(a - 1)\right]^{1/2} \qquad \text{(Eq. A10)}$$

Let C_1 and C_2 be two simplifying constants:

$$C_1 = a^2 - 1 \qquad \text{(Eq. A11)}$$

$$C_2 = 2(a - 1) \qquad \text{(Eq. A12)}$$

Simplifying Eq. A10 gives:

$$Y_t = r_0[(C_1)^{1/2} - (C_1 - \left(\frac{X_t}{r_0}\right)C_2)^{1/2}] \qquad \text{(Eq. A13)}$$

Substituting from Eq. 22 for r_0 gives:

$$Y_t = \frac{X_t}{K_rt^{1/2}}[(C_1)^{1/2} - (C_1 - K_rt^{1/2}C_2)^{1/2}] \qquad \text{(Eq. A14)}$$

If:

$$Z_t = \frac{1}{t^{1/2}}\ [(C_1)^{1/2} - (C_1 - K_rt^{1/2}C_2)^{1/2}] \qquad \text{(Eq. A15)}$$

where Z_t is a simplifying factor that varies with time, then Eq. A15 modifies to:

$$Y_t = \frac{Z_t}{K_r}(X_t) \qquad \text{(Eq. A16 or 33)}$$

For a hemisphere, $a = 1$. Hence:

$$C_1 = 0 \qquad \text{(Eq. A17)}$$

$$C_2 = 0 \qquad \text{(Eq. A18)}$$

It follows that:

$$Z_t = 0 \qquad \text{(Eq. A19)}$$

Linear Representation of Spherical Tablet Release Expression—The release expression for a spherical tablet (Eq. 55) is identical to:

$$(1 - f_t)^{1/3} = 1 - K_rt^{1/2} \qquad \text{(Eq. A20)}$$

Equation A20 is analogous to Eq. 5, the Hixson–Crowell "cube root law" (16, 17). Hence, estimates of the release rate constant, K_r, and the boundary retreat rate constant, K_b, may be found from a linear plot as illustrated in Fig. 10.

Such a linear representation is not possible for the release of drug from cylindrical or biconvex tablets because the dimensions of the unreleased portions do not decrease in proportion to one another as drug release proceeds. In addition, the linear representation of nonlinear release (dissolution) data can give rise to slope inaccuracies at high f_t values[3].

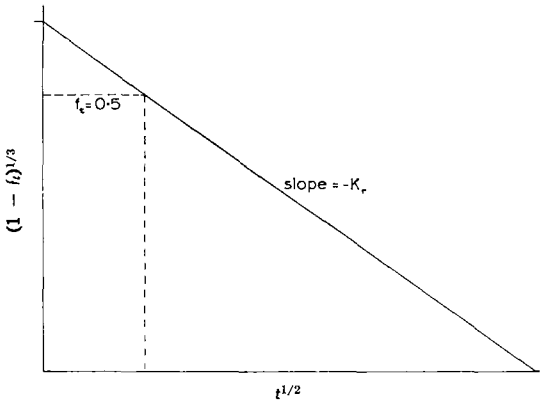

Figure 10—*Fraction of drug unreleased, 1 − f_t, with time, t, from a spherical matrix tablet, showing an analogy with Hixson–Crowell kinetics. See Eq. A20.*

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ˣ To whom inquiries should be directed.

Influence of Shape Factors on Kinetics of Drug Release from Matrix Tablets II: Experimental

JOHN COBBY*, MICHAEL MAYERSOHNˣ, and GEORGE C. WALKER

Abstract □ Tablets were prepared from two slow-release formulations, both containing stearyl alcohol as a homogeneous insoluble matrix. The release of salicylic acid and of ephedrine was measured *in vitro*. It was found that the release profiles could be described by a nonlinear expression for both cylindrical and biconvex tablets. Even though the rate of drug release varied noticeably with tablet shape, regression analysis of the release data indicated that the rate constants included in the expression did not vary significantly ($p = 0.05$) with shape for tablets of the same overall composition.

Keyphrases □ Tablets, slow release—release of salicylic acid and ephedrine from homogeneous insoluble matrix (stearyl alcohol), experimental relationship between release and tablet shape □ Drug release from matrix tablets—experimental relationship between release and tablet shape, salicylic acid and ephedrine from stearyl alcohol □ Timed-release tablets—slow release of salicylic acid and ephedrine from homogeneous insoluble matrix (stearyl alcohol), effect of tablet shape

In a previous report (1), an expression, having a cubic form, was presented describing the dissolution kinetics of a drug from a slow-release matrix tablet:

$$f_t = G_1K_rt^{1/2} - G_2(K_rt^{1/2})^2 + G_3(K_rt^{1/2})^3 \qquad \text{(Eq. 1)}$$

where f_t is the fraction of drug released to time t, K_r is the release rate constant having the dimension of the reciprocal of the square root of time, and G_1–G_3 are shape factors. The values of G_1–G_3 are dependent on the shape of the tablet under study; values for three tablet shapes—spherical, cylindrical, and biconvex—are shown in Table I. For the first two shapes, the shape factors are constants and may be obtained from measurements of the initial dimensions of the tablet. However, for a biconvex tablet, the values of the shape factors vary with time and may be obtained partially from the initial dimensions of the tablet and partially in the course of the kinetic considerations of the release process.

A plot of the fraction of drug released, f_t, against the square root of time, $t^{1/2}$, will give a nonlinear curve, the exact profile depending on the tablet shape. One purpose of this study was to determine if the proposed equation describes experimental release data for cylindrical and biconvex tablets. Values for the release rate constants, K_r, may then be obtained for both tablet shapes by nonlinear regression analysis of the curves.

The value of the release rate constant varies inversely with the initial tablet radius, r_0, as shown previously (1):

$$K_r = \frac{K_b}{r_0} \qquad \text{(Eq. 2)}$$

where K_b is a proportionality constant, termed the boundary retreat rate constant, having the dimensions of length per unit square root of time. The boundary retreat rate constant is a measure of the rate at which dissolution fluid is able to penetrate into the insoluble tablet matrix to effect drug dissolution and release; it may be expressed (1) in terms of the same fundamental parameters described by

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Table I—Values of Shape Factors for Three Shapes of Slow-Release Tablets[a]

Shape Factor	Tablet Shape		
	Spherical	Cylindrical	Biconvex
G_1	3	$q + 2$	$\frac{3p}{C_3}\left\{C_4 + C_6\left(\frac{Z_t}{K_r}\right)\right\}$
G_2	3	$2q + 1$	$\frac{3p^2}{C_3}\left\{C_5 + C_7\left(\frac{Z_t}{K_r}\right) + q\left(\frac{Z_t^2}{K_r^2}\right)\right\}$
G_3	1	q	$\frac{p^3q}{C_3}\left\{4 + 3\left(\frac{Z_t^2}{K_r^2}\right) - \frac{Z_t^3}{K_r^3}\right\}$

[a] Measurable parameters: r_0 = initial tablet radius, h_0 = half the initial tablet height, R_0 = initial radius of the spherical segment of the tablet, and HD_0 = initial height of the spherical segment of the tablet. Ratio constants: $a = R_0/r_0$, $q = r_0/h_0$, and $p = r_0/HD_0$. Simplifying factor (time dependent): $Z_t = 1/t^{1/2}\,[(C_1)^{1/2} - (C_1 - K_r t^{1/2} C_2)^{1/2}]$. Simplifying constants: $C_1 = a^2 - 1$, $C_2 = 2(a - 1)$, $C_3 = 6p^3 - 3p^2q + q$, $C_4 = 4p^2 + p^2q - 2pq + q$, $C_5 = 2p(1 + q)$, $C_6 = q(p^2 - 1)$, and $C_7 = 2q(p - 1)$.

Higuchi (2) in his derivation of the release of drugs from granular matrixes:

$$K_b = \frac{1}{A}\sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s} \qquad \text{(Eq. 3)}$$

where A is the weight of drug per unit tablet (unreleased) volume, D is the diffusion coefficient of the drug in the dissolution fluid, ϵ is the porosity of the matrix, τ is the tortuosity factor of the matrix, and C_s is the equilibrium solubility of the drug in the dissolution fluid. As noted by Higuchi (2), such an equation is essentially valid only if A exceeds ϵC_s by a factor of three or four.

Hence, it may be seen that the value of the boundary retreat rate constant depends only on the physicochemical properties of the tablet constituents and the degree of compression and, therefore, is independent of both the initial tablet radius, which partly governs the size of a tablet, and the tablet shape.

Substituting from Eq. 3 into Eq. 2 and rearranging give:

$$K_r = \frac{1}{Ar_0}\sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s} \qquad \text{(Eq. 4)}$$

While the value of the release rate constant, like that of the boundary retreat rate constant, is independent of tablet shape, it does depend on the initial tablet radius. Hence, if a pair of tablets of the same formulation (D and C_s constant), having an equal overall weight and compressed to the same degree (ϵ, τ, and A constant) is considered, the release rate constants, K_r, should not differ, providing the initial radii are equal and the test conditions are identical. Because such a pair of tablets need not be of the same shape, a second purpose of this study was to determine if the release rate constants for cylindrical and biconvex tablets having the same initial radius differ.

EXPERIMENTAL

Dissolution Fluids—Three dissolution fluids were prepared: *(a)* 0.1 *M* hydrochloric acid having a pH[1] of 1.10 ± 0.02 (mean ± *SD* of three batches), *(b)* glass-distilled water having a pH of 5.79 ± 0.09 (eight batches) after standing for at least 48 hr, and *(c)* phosphate buffer (3) having a pH of 7.52 ± 0.09 (three batches). The fluids will be referred to as the pH 1.1 fluid, the pH 5.8 fluid, and the pH 7.5 fluid, respectively.

[1] Model NX pH meter, Sargent-Welch Scientific Co., Skokie, Ill.

Table II—Formulations of Salicylic Acid Tablets (*A*) and Ephedrine Tablets (*B*) and Densities (ρ) of the Ingredients.

Ingredient	Formulations, mg/Tablet		ρ, g/ml
	A	*B*	
Salicylic acid[a] USP (drug)	90.0	—	1.70
Ephedrine[b] (BPC 1968) (drug)	—	75.0	1.42
Lactose[c] (BP 1968) (diluent)	15.0	30.0	1.45
Stearyl alcohol[d] (matrix)	37.5	37.5	0.94
Polyvinylpyrrolidone[e] (binder)	7.5	7.5	0.87

[a] Lot 721654, Fisher Scientific Co., Montreal, Quebec, Canada. [b] Lot 36410, British Drug Houses (Canada), Toronto, Ontario, Canada. [c] Lot 38450, British Drug Houses (Canada), Toronto, Ontario, Canada. [d] Lot 37434, British Drug Houses (Canada), Toronto, Ontario, Canada. [e] Plasdone C, Lot G-90509A-69, General Aniline and Film Corp., New York, N.Y.

Manufacture of Tablets—All ingredients used were of the purity supplied by the manufacturer, and solids were passed through 0.25-mm (60-mesh) stainless steel sieves prior to use. Two model formulations were prepared, one containing salicylic acid and the other ephedrine (Table II). The drug and lactose were mixed together and incorporated into a hot 50% (w/w) solution of stearyl alcohol in methylene chloride[2]. By maintaining mixing during cooling, an ointment-like mass was obtained. It was then spread thinly on paper-covered trays and allowed to solidify at room temperature. Once hardened, the mass was forced through a 0.42-mm (40-mesh) stainless steel sieve to obtain a powder.

After mixing the powder with polyvinylpyrrolidone, a quantity of distilled water equal to approximately twice the weight of the polyvinylpyrrolidone was incorporated. A wet mass was produced and granules were prepared by forcing the mass through a 1.00-mm (18-mesh) stainless steel screen. The granules were dried at room temperature, and a sample of 0.42–0.84-mm (20–40 mesh) size range was taken. Aliquots of the sample were added to each dissolution fluid and the resulting solutions, after filtration, were assayed spectrophotometrically for drug content.

If the sized granules could be compressed satisfactorily on a rotary tablet press[3], batches of tablets for experimental use were prepared using a laboratory press[4] adapted to incorporate assumed 3.175-mm (0.125-in.) radius tablet punches. For each formulation, two batches of tablets were prepared at 1600 psig. The first was compressed using flat-faced punches to give cylindrical tablets, and the second was compressed using concave punches[5] to give biconvex tablets. The weight and height of each tablet were measured before it was sealed in a No. 00 gelatin capsule and stored in a closed amber glass bottle.

Assay—Standard Beer's law curves were prepared for both drugs in each dissolution fluid. The wavelengths of measurement[6] were 294 nm (301 nm in the pH 1.1 fluid) for salicylic acid and 256 nm for ephedrine. The absorptivities of the drugs corresponded to literature values (5, 6) and remained unchanged with time and in the presence of the soluble tablet excipients.

Dissolution Test—*Apparatus*—A modified Baun and Walker (7) apparatus (Fig. 1) was used. The dissolution cell (A) consists of a glass tube (19 mm o.d. and 1 mm thick) having a screen (B) at each end. Because acidic dissolution fluids can deteriorate stainless steel (8), the screens are cut from the porous area of a polypropylene büchner funnel[7] and have a pore diameter of ap-

[2] USP reagent, Lot 707202, Fisher Scientific Co., Montreal, Quebec, Canada.

[3] Manesty Drycota (type DC500), Manesty Machines, Liverpool, England.

[4] Carver laboratory press (model B), Fred S. Carver Inc., Summit, N.J.

[5] These were Stokes "extra deep" concave punches (4) for which a = 1.500. It may be calculated that, for punches having r_0 = 3.175 mm, p = 2.618.

[6] Model DU 2400 spectrophotometer, Beckman Instruments Inc., Fullerton, Calif.

[7] Kimble Products Co., Toledo, Ohio.

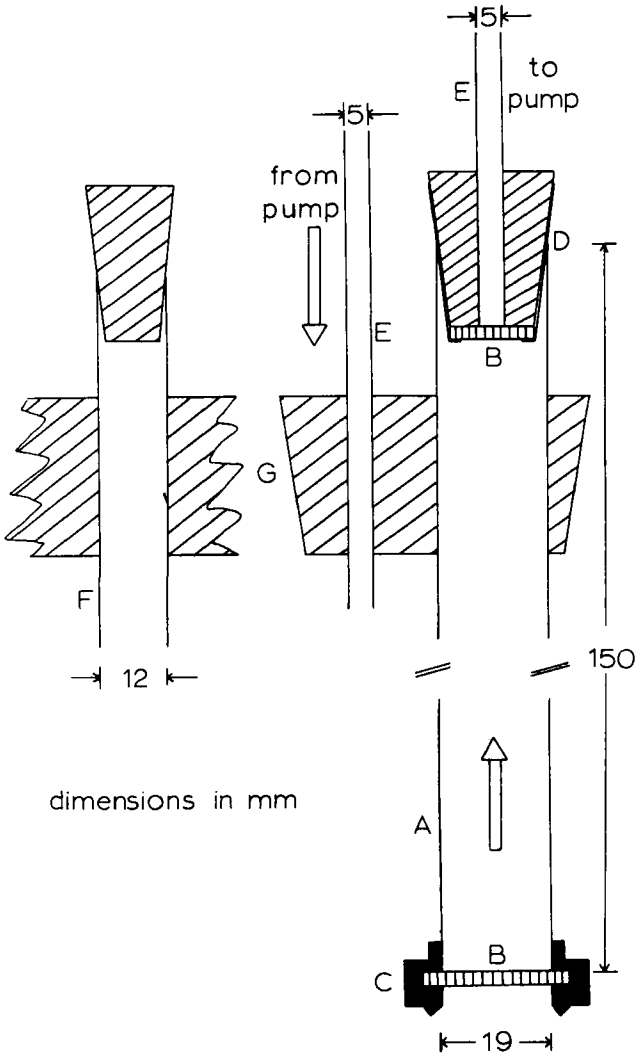

Figure 1—*The dissolution apparatus. Key: A, dissolution cell; B, screens; C, lower screen assembly; D, upper screen assembly; E, flow tubes; F, sample port; and G, reservoir stopper. (The numbers represent outside diameter in millimeters, with 1-mm thick tubes).*

proximately 0.75 mm. A lower screen assembly (C), which fits tightly onto the dissolution tube and can be removed easily, may be produced by boring a 19-mm diameter hole through both portions of a polyethylene snap cap[8] and holding the screen between them. An upper screen assembly (D) may be produced by holding the screen between a rubber stopper and a hollow polyethylene stopper[9] having a 10-mm diameter hole bored through its base. The flow of dissolution fluid is through glass tubes (E) (5 mm o.d. and 1 mm thick) and Tygon tubing[10]. A port (F), consisting of a stoppered glass tube (12 mm o.d. and 1 mm thick), is available for withdrawing samples of the dissolution fluid by pipet. The cell (A), the inlet tube (E), and the port (F) are all held in a neoprene stopper (G), which seals the reservoir containing the dissolution fluid; neither the inlet tube nor the port dips below the fluid level. The reservoir, an amber glass bottle, is suspended in a thermostatically controlled water bath.

Test Procedure—For 60 min prior to a test, the required volume of dissolution fluid, maintained at 37 ± 0.1°, was circulated through the apparatus at 50 ± 2 ml/min. This rate gave only a very slight agitation to the tablet which rested on the lower screen assembly. The pump[11] was then stopped, the upper screen assembly was removed, and the tablet was dropped into the dissolution cell. The assembly was immediately replaced and pumping was recommenced. At suitable time intervals[12], samples of the dissolution fluid were withdrawn through the port by pipet and replaced with an equal volume of the dissolution fluid. The samples were assayed spectrophotometrically to determine drug release.

Each ephedrine tablet floated, touching the upper screen assembly, before release was complete, an indication that the release of drug without any compensating reduction in overall tablet volume had lowered its density. Because fissures were noticeable in many tablets at the time of flotation (which was recorded), beyond this time all ephedrine tablets were assumed not to present an unbroken surface to the dissolution fluid as required by the theory (1).

Immediately after the conclusion of the test, the tablet was carefully removed and the final sample of the dissolution fluid was taken. The tablet was then slowly dried, weighed, and powdered, and an aliquot of the powder was dissolved in dissolution fluid. After filtration to remove insoluble particles, this solution

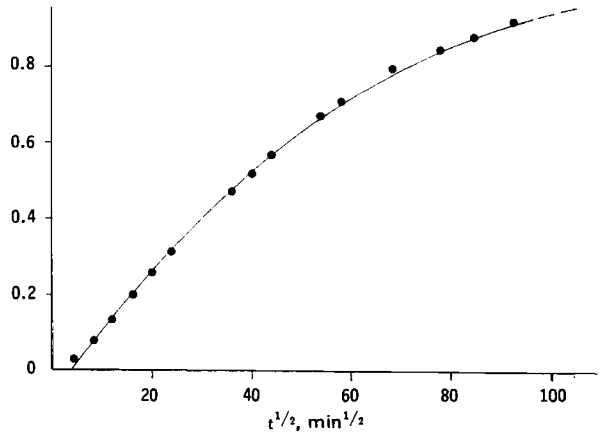

Figure 2—*Experimentally determined (●) fractions of drug released, f_t, at the sample times, t, and the optimum release curve for a cylindrical salicylic acid tablet (No. '13) in pH 1.1 fluid. Parameter values ± SE are: K_r = 0.00491 ± 0.00007 $min^{-1/2}$, and $t_0^{1/2}$ = 3.71 ± 0.38 $min^{1/2}$.*

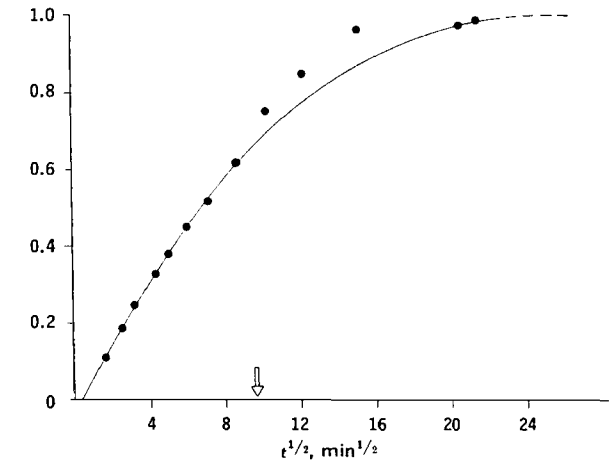

Figure 3—*Experimentally determined (●) fraction of drug released, f_t, at the sample times, t, and the optimum release curve for a cylindrical ephedrine tablet (No. 14) in pH 1.1 fluid. Parameter values ± SE are: K_r = 0.0277 ± 0.0004 $min^{-1/2}$, and $t_0^{1/2}$ = 0.422 ± 0.061 $min^{1/2}$. The arrow denotes time of tablet flotation.*

[8] Snap cap No. 3, Kimble Products Co., Toledo, Ohio.
[9] Stopper No. 1, Nalge Co., Rochester, N.Y.
[10] Tygon type R3603, Fisher Scientific Co., Montreal, Quebec, Canada.
[11] "Miniature Flow Inducer" MHRE/200, Mk. 3, Watson-Marlowe Ltd., Marlowe, England.

[12] The sampling times were arranged so that the drug released in any one time interval would be approximately equal to that in any other interval. Such a procedure precludes bias by ensuring that the release results are approximately equally spaced in the range $f_t = 0$ to $f_t = 1$.

Table III—Tablet Parameters

Parameter	Salicylic Acid Tablets		Ephedrine Tablets[b]	
	Cylindrical[a]	Biconvex[b]	Cylindrical	Biconvex
Weight, mg	150.1 ± 0.4	149.9 ± 0.3	150.0 ± 0.7	149.4 ± 0.9
Radius, r_0, mm	3.175	3.175	3.175	3.175
Height, $2h_0$, mm	3.752 ± 0.008	4.827 ± 0.011	4.108 ± 0.015	5.278 ± 0.039
$q = r_0/h_0$	1.693 ± 0.003	1.315 ± 0.003	1.546 ± 0.006	1.203 ± 0.009
Radius of spherical segment, R_0, mm	—	4.763	—	4.763
$a = R_0/r_0$	—	1.5	—	1.5
Height of spherical segment, HD_0, mm	—	1.213	—	1.213
$p = r_0/HD_0$	—	2.618	—	2.618
Volume, V_0, ml	0.1188 ± 0.0002	0.1163 ± 0.0003	0.1301 ± 0.0005	0.1306 ± 0.0012

[a] Mean ± *SD* of 11 tablets. [b] Mean ± *SD* of 12 tablets.

was assayed spectrophotometrically to determine the residual drug content of the tablet.

Evaluation Procedure—The cumulative drug released was computed[13] from the sample assays using a modified Wurster and Taylor (9) equation. The total drug content of each tablet was calculated by adding the residual drug content to the cumulative drug released at the time of the final sample. Then, knowing the total drug content, the fraction of drug released at each sample time, f_t, was computed.

In practice, Eq. 1 must be modified to account for a lag time before drug is first released or detected:

$$f_t = G_1K_r\{t^{1/2} - t_0^{1/2}\} - G_2(K_r\{t^{1/2} - t_0^{1/2}\})^2 + G_3(K_r\{t^{1/2} - t_0^{1/2}\})^3 \quad \text{(Eq. 5)}$$

where $t_0^{1/2}$ is the root lag time. If there is no lag time, $t_0^{1/2} = 0$, then Eq. 5 reduces to Eq. 1.

The experimental results were analyzed using a nonlinear regression computer program[14]. Optimum values of the release rate constant, K_r, and the root lag time, $t_0^{1/2}$, to three significant figures were found together with estimates of their standard errors.

Measurement of Some Fundamental Parameters—The density of each tablet ingredient was found using standard pycnometer techniques. The equilibrium solubility of each drug at 37°, C_s, was found by a method essentially similar to that reported by Desai *et al.* (10).

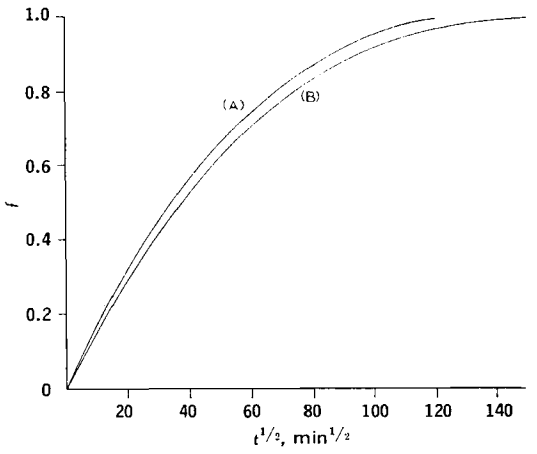

Figure 4—*Effect of shape on fraction of drug released,* f_t, *with time,* t; *mean profiles of salicylic acid tablets in pH 1.1 fluid. Key: (A), cylindrical,* $K_r = 0.00493\ min^{-1/2}$; *and (B), biconvex,* $K_r = 0.00497\ min^{-1/2}$. *For comparative purposes, the lag times are ignored.*

[13] Model 9100A calculator, Hewlett-Packard Co., Loveland, Colo.

[14] University of Toronto Computer Centre, IBM System 370-165. The program contained an iterative subroutine, GAUSHS (similar to the IBM FORTRAN IV Contributed Program 360D-13.6.007), and a model subroutine containing the appropriate release expression (Eq. 5).

RESULTS AND DISCUSSION

The drug content of the granules was found to be 91.2 ± 0.4 mg (mean ± *SD* of three results) for salicylic acid tablets and 74.0 ± 1.7 mg (14 results) for ephedrine tablets, expressed per 150.0 mg of granules. The parameters of the tablets (Table III) indicated that the salicylic acid granules were more compressible at 1600 psig than was an equal weight of ephedrine granules. However, the mean weight and volume of cylindrical and biconvex tablets containing the same drug were essentially equal. Hence the parameters ϵ, τ, and A described in Eqs. 3 and 4 could be considered constant between the two tablet shapes, a requirement for determining whether the release rate constant is a function of tablet shape.

Fitting Experimental Data to Proposed Equation—Example data of the fraction of drug released, f_t, from a tablet at various sample times, t, are shown in Table IV. Where tablet flotation was noted (ephedrine tablets), only values of f_t prior to flotation were used in computing the release rate constant, K_r, and root lag time, $t_0^{1/2}$. Figures 2 and 3 show example release profiles for single tablets, calculated from the optimum parameter values. The fit of the release curves to the experimental data points is good, deviation occurring only with ephedrine tablets after flotation. The deviation is due to fissures forming in the tablets, a factor probably resulting from an inadequate tablet formulation (possibly, in this case, too little matrix substance). The fissures permitted the dissolution fluid to flow virtually unimpeded to and from the remaining undissolved drug near the tablet center; normally the fluid would diffuse more slowly through the "ghost" region.

Further indications of the goodness of fit of each optimum curve to its corresponding experimental data may be obtained from the computed standard errors of the parameters. The standard error was 2.12 ± 0.96% (mean ± *SD* of 23 experiments) of

Table IV—Fractions of Drug Released from a Biconvex Salicylic Acid Tablet (No. 6) in pH 5.8 Fluid[a]

Minutes[b]	Fraction Released[c], f_t
24	0.042
48	0.069
92	0.116
150	0.152
243	0.209
390	0.258
610	0.335
1250	0.471
1485	0.515
1749	0.552
2155	0.591
2677	0.647
3085	0.680
4457	0.786
5589	0.851
6025	0.887

[a] Computed parameter values ± *SE* are: $K_r = 0.00562 \pm 0.00009\ min^{-1/2}$, and $t_0^{1/2} = 3.51 \pm 0.43\ min^{1/2}$. [b] Each experiment was conducted using slightly different sample times, so average values of f_t cannot be shown. [c] Based on assayed drug content.

Table V—Mean Values of Release Rate Constant, K_r, and Root Lag Time, $t_0^{1/2}$

Fluid	Cylindrical Tablets		Biconvex Tablets	
	K_r, $min^{-1/2}$	$t_0^{1/2}$, $min^{1/2}$	K_r, $min^{-1/2}$	$t_0^{1/2}$, $min^{1/2}$
	Salicylic Acid Tablets[a]			
pH 1.1	0.00493 ± 0.00007[b]	3.63 ± 0.29[b]	0.00497 ± 0.00009[c]	3.32 ± 0.06
pH 5.8	0.00530 ± 0.00012	3.93 ± 0.39	0.00550 ± 0.00009[d]	3.37 ± 0.31
pH 7.5	0.00828 ± 0.00032	2.52 ± 0.31	0.00846 ± 0.00027[c]	2.29 ± 0.09
	Ephedrine Tablets[a]			
pH 1.1	0.0275 ± 0.0012	0.61 ± 0.16	0.0292 ± 0.0016[c]	0.41 ± 0.18
pH 5.8	0.0232 ± 0.0004	1.39 ± 0.16	0.0267 ± 0.0009[d]	1.06 ± 0.16
pH 7.5	0.0224 ± 0.0006	0.98 ± 0.26	0.0225 ± 0.0004[c]	0.99 ± 0.14

[a] Mean ± *SD* of four experiments. [b] Mean ± *SD* of three experiments. [c] No significant difference ($p = 0.05$) between the mean K_r values obtained from tablets differing only in shape. [d] Significant difference ($p = 0.05$) between the mean K_r values obtained from tablets differing only in shape.

Table VI—Mean Boundary Retreat Rate Constants, K_b, of Tablets and Equilibrium Solubilities of Drugs at 37°, C_s

Fluid	K_b[a], mm × 10^2 $min^{-1/2}$		C_s[c], mg/ml	
	Salicylic Acid Tablets	Ephedrine Tablets	Salicylic Acid	Ephedrine
pH 1.1	1.58 ± 0.03[b]	9.00 ± 0.50	2.64 ± 0.02	90.6 ± 0.5
pH 5.8	1.71 ± 0.05	7.93 ± 0.63	3.64 ± 0.05	66.6 ± 0.4
pH 7.5	2.66 ± 0.09	7.14 ± 0.23	9.54 ± 0.33	60.1 ± 0.9

[a] Mean ± *SD* of eight experiments (both cylindrical and biconvex tablets). [b] Mean ± *SD* of seven experiments (both cylindrical and biconvex tablets). [c] Mean ± *SD* of three experiments.

the optimum value of the release rate constant for salicylic acid tablets and 2.79 ± 1.33% (24 experiments) for ephedrine tablets; the standard error was 12.4 ± 2.0% and 12.9 ± 6.8% of the optimum value of the root lag time, respectively. Both the statistical data and the visual curve fitting indicate that the experimental results can be closely described by the proposed expression (Eq. 5).

Values of Rate Constants K_r and K_b—The mean values of the release rate constant, K_r, and the root lag time, $t_0^{1/2}$, are shown in Table V. The K_r values obtained under identical conditions from tablets differing only in shape were not significantly different ($p = 0.05$) when an unpaired t test was applied, except for experiments conducted in the pH 5.8 fluid. Because the initial radii of both the cylindrical and biconvex tablets were identical (Table III), mean values of the boundary retreat rate constant, K_b, would also lead to the same statistical conclusions (Eq. 2).

Since Eq. 3 indicates that the boundary retreat rate constant should not vary with tablet size or shape, an explanation of the discrepancies should be sought. A possible explanation may lie with the lack of buffering capacity in the pH 5.8 fluid, which was distilled water, resulting in a measurable pH change within the fluid as released drug accumulated. In the case of salicylic acid tablets, the pH fell to 3.39 ± 0.01 (mean ± *SD* of four experiments); in the case of ephedrine tablets, the pH rose to 9.53 ± 0.19 (four experiments). Hence, the equilibrium solubility, C_s, of the drug in the fluid gradually fell during a test procedure, giving, as a consequence of Eq. 3, a gradually decreasing boundary retreat rate constant. Conceivably, this time-dependent variability of the boundary retreat rate constant may partly explain the discrepancies seen. The pH of the other dissolution fluids had not changed measurably at the conclusion of an experiment.

For experiments conducted at a constant pH, the results indicate that there is no significant difference ($p = 0.05$) between the rate constants (both K_r and K_b) obtained from tablets of different shapes. However, the actual rate of drug release varies noticeably between tablet shapes, with the biconvex tablet giving the slower release (Figs. 4 and 5). The effect of tablet shape on the release rate is thus distinguished from its lack of effect on the rate constants.

Fundamental Parameters—According to Eq. 3, the value of the boundary retreat rate constant depends on certain fundamental parameters related to the tablet composition. As defined (2), the values of the equilibrium solubility, C_s, the diffusion coeffi-

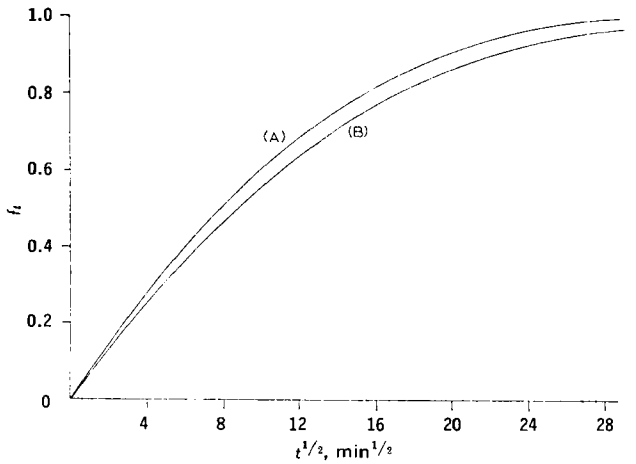

Figure 5—*Effect of shape on fraction of drug released, f_t, with time, t; mean profiles of ephedrine tablets in pH 7.5 fluid. Key: (A), cylindrical, $K_r = 0.0224\ min^{-1/2}$; and (B), biconvex, $K_r = 0.0225\ min^{-1/2}$. For comparative purposes, the lag times are ignored.*

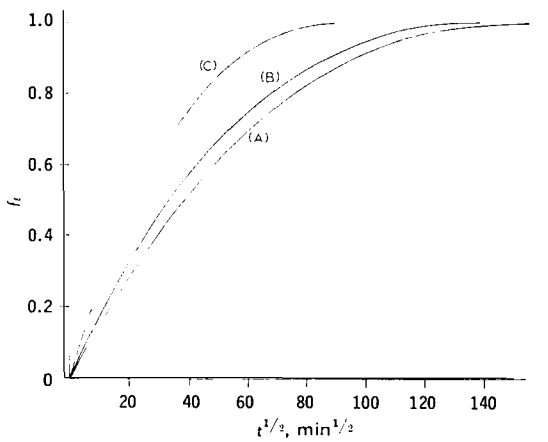

Figure 6—*Effect of fluid pH on fraction of drug released, f_t, with time, t; mean profiles of biconvex salicylic acid tablets. Key: (A), pH 1.1, $K_r = 0.00497\ min^{-1/2}$; (B), pH 5.8, $K_r = 0.00550\ min^{-1/2}$; and (C), pH 7.5, $K_r = 0.00846\ min^{-1/2}$. For comparative purposes, the lag times are ignored.*

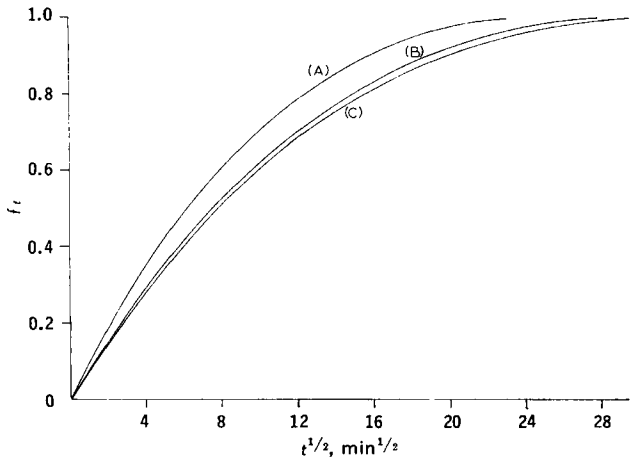

Figure 7—*Effect of fluid pH on fraction of drug released,* f_t, *with time,* t; *mean profiles of cylindrical ephedrine tablets. Key: (A), pH 1.1,* $K_r = 0.0275\ min^{-1/2}$; *(B), pH 5.8,* $K_r = 0.0232\ min^{-1/2}$; *and (C), pH 7.5,* $K_r = 0.0224\ min^{-1/2}$. *For comparative purposes, the lag times are ignored.*

cient, D, and the weight per unit tablet volume, A, apply exclusively to the drug when it is the only soluble solid distributed throughout the insoluble matrix. However, when formulating slow-release tablets, it is often necessary to include additional soluble solids as excipients, in which case the three parameters become composite values related to all soluble components. The use of lactose and polyvinylpyrrolidone, both of which are soluble in the dissolution fluids, in the formulations of the tablets used in this study was deliberate, in that it was hoped to approximate formulations applicable to large-scale manufacture. Hence, while the boundary retreat rate constant increases with the equilibrium solubility of the drug (Table VI), the dependence should be classified as a rank-order relationship in the absence of the required composite solubility data for all soluble components. The effect of changing pH on the release profiles is shown in Figs. 6 and 7.

Because Eqs. 3 and 4 are only essentially valid if A exceeds ϵC_s by a factor of three or four (2), it was felt worthwhile to ascertain whether the fissuring in the ephedrine tablets could be the physical result of a nonadherence to this requirement. By using the densities of the tablet ingredients (Table II) and employing the calculation method of Desai *et al.* (10), the mean porosity of the matrix of the salicylic acid tablets and the ephedrine tablets was 0.661 and 0.694, respectively; the mean values for A were 957 and 863 mg/ml, respectively. While the values for these two parameters (ϵ and A) have regard for all of the soluble solids in the tablets, the equilibrium solubility, C_s, values refer only to the drugs (Table VI). Hence, the calculated $A:\epsilon C_s$ ratios are only approximate, their values being 500 and 14 for salicylic acid and ephedrine tablets, respectively. Even so, the tablets used in this study apparently comply with the theoretical requirement (2), suggesting that the fissuring imperfection seen with ephedrine tablets is probably due to inadequate formulation.

In view of the complexity of the formulations, the tablets used in this study could not be suitable tools with which to predict rate constants from the direct measurement of fundamental parameters, as Eqs. 3 and 4 might suggest. Indeed, Hersey (11) suggested that predictions of the dissolution rates of drugs from fundamental parameters are hazardous, particularly given the relative ease with which direct measurements of rate constants may be made. Thus, when determining release rates of drugs from such complex dosage forms as slow-release tablets, direct experimental measurement is probably the preferred procedure.

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ˣ To whom inquiries should be directed.

Influence of Cephalin and Egg Lecithin on Pig Heart Malate Dehydrogenase Activity

SHAHID A. LODHI*, NORMAN D. WEINER*x, and ALVIN FELMEISTER‡

Abstract □ A requirement for phospholipids in the activity of a number of membrane-bound enzymes is recognized; *i.e.*, enzyme activity is reduced or lost entirely when the lipid is removed and is restored or increased when this specific lipid is added back to the system. In general, however, only the effects of relatively low lipid concentrations ($\sim 10^{-4}$ g/100 ml) have been reported. In this investigation, the influence of phospholipids (egg lecithin and bovine brain cephalin) at concentrations approaching normal human plasma levels was determined on the activity of malate dehydrogenase dispersed in water. The enzyme activity was measured by the rate of oxidation of nicotinamide adenine dinucleotide (reduced) in the presence of the substrate oxalacetic acid. On the addition of cephalin, malate dehydrogenase activity decreased with the increasing concentration of this phospholipid from 5×10^{-4} to 2×10^{-2} g/100 ml. Similarly, the addition of egg lecithin resulted in a decrease in enzyme activity. In no instance was any increase in activity observed. Moreover, at all concentrations of egg lecithin, the reduction in activity was less than that observed in the presence of equal concentrations of cephalin. Monolayer studies provided evidence that, whereas egg lecithin interacts to a significant degree with the substrate oxalacetic acid, cephalin shows relatively little interaction with that material. The difference observed in the inhibitory action of these two phospholipids may be related to the difference in their tendency to react with the substrate.

Keyphrases □ Malate dehydrogenase activity and stability—effect of phospholipids (cephalin and egg lecithin), substrate considerations □ Phospholipids (cephalin and egg lecithin)—effect on malate dehydrogenase activity and stability, substrate considerations □ Enzyme inhibition—effect of phospholipids (cephalin and egg lecithin) on malate dehydrogenase activity

The activity and stability of a number of membrane-bound enzymes have been shown to have a lipid requirement; *i.e.*, enzyme activity and stability are lost or reduced when the lipid is removed from the enzyme system. The mechanism of these effects is, in general, considered to involve either: *(a)* lipid interaction with and activation of the substrate; or *(b)* a direct lipid–enzyme hydrophobic group interaction, the result of which is a conformational change of the protein. This latter mechanism is believed to be operative with some dehydrogenases (1).

Usually, the lipid requirement exhibits some specificity. Sekuzu *et al.* (2) and Fleischer *et al.* (3) showed that phosphatidylcholine was the only lipid able to activate β-hydroxybutyrate dehydrogenase, whereas other lipids, including phosphatidylethanolamine, phosphatidylinositol, cardiolipin, sphingomyelin, and neutral lipids, were inactive. In the case of cytochrome c, it was reported that cardiolipin and phosphatidylethanolamine were the most effective activators in the mitochondrial electron-transfer system (4). Similarly, Callahan and Kosicki (5) observed an increase in activity and stability of a mitochondrial malate dehydrogenase in the presence of lipids extracted from the same tissue as the enzyme as well as an increase in enzyme activity in the presence of a series of saturated and unsaturated fatty acids. At higher concentrations of these fatty acids, however, enzyme inhibition was observed. It thus appears that both lipid specificity and lipid concentration are involved in the activity of lipid-requiring enzymes.

It was of interest, therefore, to study the activity and stability of malate dehydrogenase in the presence of phospholipids from sources different than that of the enzyme itself. Furthermore, plasma levels of phospholipids are considerably higher than those used in most previous studies on lipid-requiring enzymes. Therefore, studies were performed to determine the effect of lipids at concentrations approaching plasma levels on malate dehydrogenase activity and stability.

EXPERIMENTAL

Materials and Equipment—Malate dehydrogenase[1] (porcine heart), activity 260 units/mg, was supplied as a suspension in 70% saturated ammonium sulfate solution. The lipids used were egg lecithin[1], highly purified, and cephalin[2], bovine brain tissue. Oxalacetic acid (*cis*-enol) and nicotinamide adenine dinucleotide[1], reduced (NADH), 90%, were used to measure the activity of the enzyme. The *n*-hexane[3] was 99 mole % pure, and all other chemicals were reagent grade. The water used was first deionized by passing it through a mixed resin bed and then distilled from an all-glass distilling apparatus[4].

Spectrophotometric measurements were made using a spectrophotometer[5] equipped with a recorder[6]. Surface tension measurements were made by the Wilhelmy plate method (6) using a thin, roughened platinum plate suspended from a torsion balance[7]. Surface pressure, π, defined as surface tension of the subphase in the absence of the film minus the surface tension of the subphase in the presence of the film, was determined from these measurements. The film balance[8] consists of a 540-ml capacity Langmuir-type Teflon trough equipped with a Teflon movable barrier. The barrier could be disengaged from the lead screw for rapid sweeping of the surface. The entire film balance was enclosed in a Plexiglas cabinet to avoid possible contamination by dust and other airborne impurities.

All experiments were conducted at room temperature (25 ± 2°).

Preparation of Solutions—For the enzyme activity studies, stock solutions of oxalacetic acid were prepared each day by dissolving 20 mg of oxalacetic acid in 100 ml of a phosphate buffer (pH 7.4). The NADH, 12.4 mg, was dissolved in 25 ml of the phosphate buffer and used only for 1 hr, after which it was discarded. Both stock solutions were stored at 0° between experiments.

One gram of egg lecithin was dispersed in 10 ml of water, and the dispersion was stirred at high speed for 1 hr. Water was then added to make 100 ml, and the dispersion was stirred at high

[1] Schwarz/Mann, Orangeburg, N.Y.
[2] Natural Biochemical Laboratories.
[3] J.T. Baker Chemical Co., Phillipsburg, N.J.
[4] Model AG-1a, Corning Glass Works, Corning, N.Y.
[5] Perkin-Elmer 139, Perkin-Elmer Corp., Norwalk, Conn.
[6] Perkin-Elmer 159.
[7] Federal Pacific Electric Co., Newark, N.J.
[8] Frater Instrument Co., Corona, N.Y.

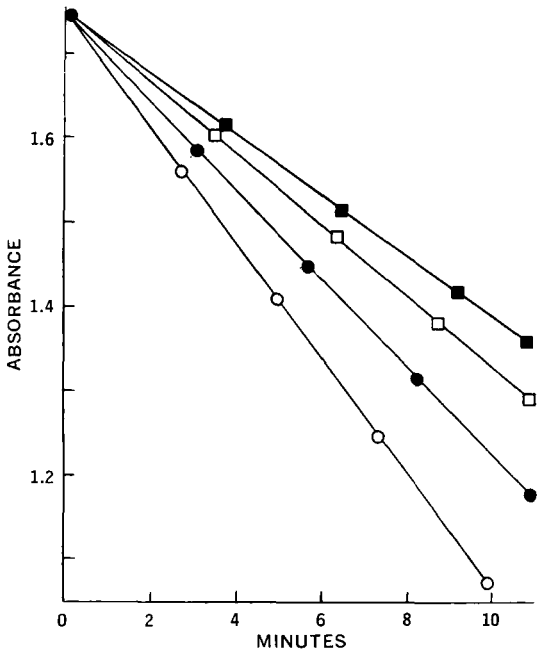

Figure 1—*Absorbance of NADH at 340 nm as a function of time in the presence of malate dehydrogenase dispersed in water and incubated for various periods at 25 ± 2°. Key (incubation times prior to assay): ○, 0 min; ●, 15 min; □, 30 min; and ■, 60 min.*

speed for another hour. The resultant stock dispersion was prepared fresh each day. Dispersions of cephalin (1 g/100 ml) were prepared in the same way as the stock dispersions of egg lecithin. These were stored in the freezer and used for a maximum of 3 days.

In all instances where stock solutions or dispersions were stored as specified, no differences were observed between experiments using the stored and the freshly prepared materials.

One-hundredth milliliter of the enzyme was measured, using a 0.1-ml disposable pipet, and dispersed in 50 ml of water contained in a 50-ml volumetric flask. The flask was inverted twice to ensure complete mixing.

Various concentrations of egg lecithin and cephalin were prepared by measuring the required amounts of the stock solution into a 50-ml volumetric flask and adding sufficient distilled water. These dispersions were stirred for 30 min. Enzyme (0.01 ml) was then added to the suspension, and the resulting dispersion was mixed for 1 min by inverting the flask twice.

Spreading solutions for the monomolecular film studies were prepared as follows: 38.8 mg of egg lecithin or 62.5 mg of cephalin was dissolved in a 1:99 (v/v) ethanol–hexane solvent mixture to make 100 ml of the respective spreading solution.

Procedure—The absorbance of various concentrations of NADH in water (from 6.25 to 25 mg/100 ml) was determined at 340 nm and a Beer's law plot was prepared.

The enzyme activity was measured by adding 0.3 ml of the stock solution of the malate dehydrogenase to 2 ml of the NADH solution and 2 ml of the oxalacetic acid solution contained in a test tube. The solution was immediately transferred to a 1-cm quartz cell, and its absorbance was recorded for 9–11 min. The slope of the absorbance–time curve, which represents the rate of enzyme-induced oxidation of NADH to NAD, was used as a measure of malate dehydrogenase activity.

In determining the effect of incubation time on enzyme stability, the enzyme dispersion, with or without lipid added, was stored for various periods at 25 ± 2° prior to measuring enzyme activity.

In the monolayer experiments, the trough was filled with a 0.1 *M* phosphate buffer (pH 7.4) alone or with varying concentrations of oxalacetic acid. The surface was then swept several times with the barrier to remove surface-active contaminants; suction was used to adjust the level of the subphase and to remove any remaining traces of dust or other contaminants. The platinum plate was then lowered into position and allowed to remain beneath the surface while the films were applied from their appropriate spreading solvents with the aid of a micrometer syringe[9]. This syringe can deliver accurately volumes as small as 0.001 ml. Generally, 0.07 ml of the spreading solution was applied onto the subphase by allowing small drops to fall from the syringe held a few millimeters away from the subphase surface. Fifteen minutes was allowed for complete evaporation of the spreading solvent from the surface before manual compression of the film was initiated.

RESULTS AND DISCUSSION

The effect of incubating the malate dehydrogenase enzyme for various time periods in water before the reaction was initiated is shown in Fig. 1. These plots represent incubation periods of 0, 15, 30, and 60 min at room temperature (25 ± 2°). As shown by the change in the slope of the absorbance *versus* time curve from 0.064 at 0 min to 0.034 at 60 min, about one-half of the activity of the enzyme was lost after 1 hr of incubation. This demonstrates that the enzyme is quite unstable when dispersed and incubated in water.

The enzyme was then dispersed in water in the presence of varying concentrations of cephalin, and its activity was determined at 0- and 60-min incubation times (Fig. 2). At concentrations of cephalin as low as 5×10^{-4} g/100 ml at zero incubation time, the activity of the enzyme decreased markedly. Increasing concentrations of cephalin under this condition resulted in further decreases in the activity of the malate dehydrogenase, reaching almost zero activity at a concentration of 2×10^{-2} g/100 ml of cephalin. Incubation of the enzyme for 60 min with all concentrations of cephalin (from 5×10^{-4} to 2×10^{-2} g/100 ml) resulted in total inactivation of the enzyme. By comparison, significant enzyme activity remained on incubation in water alone for the same period (Fig. 2).

The enzyme also showed decreasing activity at zero incubation time with increasing concentrations of egg lecithin (Fig. 3). However, the effect was not as marked as it was with cephalin. Even at high concentrations of egg lecithin (4×10^{-2} g/100 ml), the enzyme still retained about two-thirds of the activity observed in water alone. Furthermore, this reduction in enzyme activity was not observed until the concentration of egg lecithin reached 2.5 ×

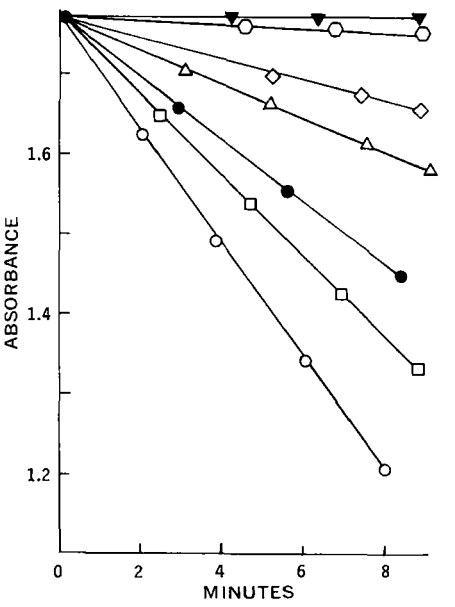

Figure 2—*Absorbance of NADH at 340 nm as a function of time in the presence of malate dehydrogenase and cephalin dispersed in water and incubated for 0 (open symbols) and 60 (closed symbols) min. Key (amount of cephalin/100 ml of dispersion): ○ and ●, 0; □, 5×10^{-4} g; △, 1×10^{-3} g; ◊, 2×10^{-3} g; ⬡, 2×10^{-2} g; and ▼, all concentrations after 60 min incubation.*

[9] Agla.

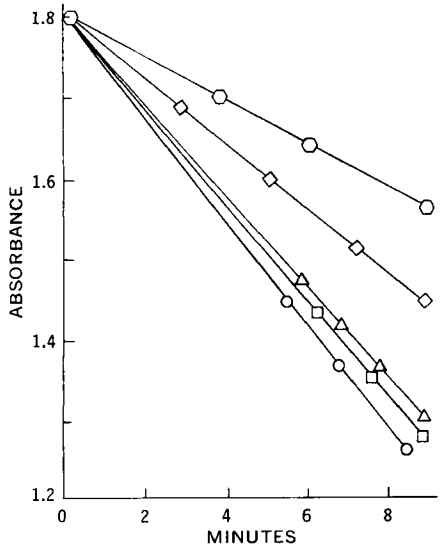

Figure 3—*Absorbance of NADH at 340 nm as a function of time in the presence of malate dehydrogenase and egg lecithin dispersed in water and incubated for 0 min. Key (amount of egg lecithin/100 ml of dispersion): ○, 0; □, 2.5 × 10^{-3} g; △, 5 × 10^{-3} g; ◊, 2 × 10^{-2} g; and ⬡, 4 × 10^{-2} g.*

10^{-3} g/100 ml as compared to 5 × 10^{-4} g/100 ml for cephalin. However, as was the case with cephalin, all enzyme activity was lost after incubation for 60 min in the presence of all concentrations of egg lecithin studied.

The difference in the concentration of these two lipids needed to decrease the enzyme activity can be seen in Fig. 4, which shows plots of lipid concentration *versus* percent of NADH oxidized per minute. These differences, as well as the difference in the shape of these two curves, suggest that the mechanism of this effect may not be the same.

Surface studies were used to determine whether these differences were due, in part, to a difference in the interaction between the lipid and the substrate. This interaction was determined by spreading the lipids as monolayers over a subphase containing oxalacetic acid and measuring changes in the surface pressure-

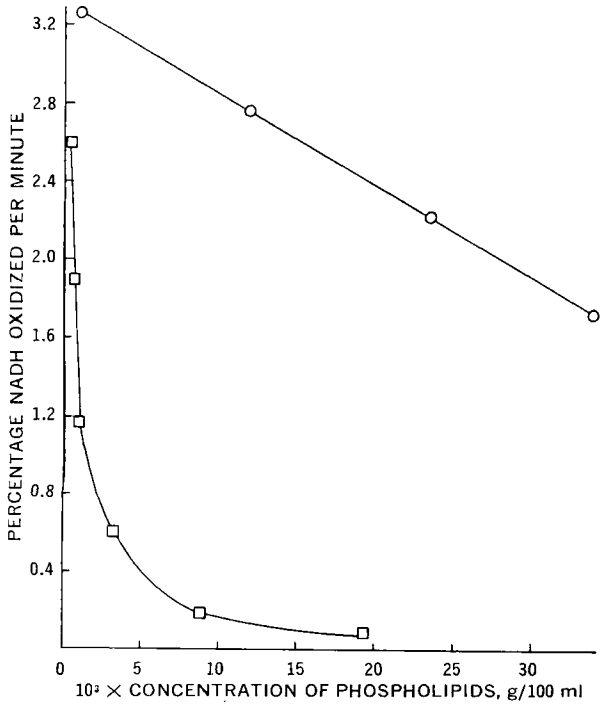

Figure 4—*Percentage of NADH oxidized per minute as a function of the concentration of phospholipid (g/100 ml). Key: □, cephalin; and ○, egg lecithin.*

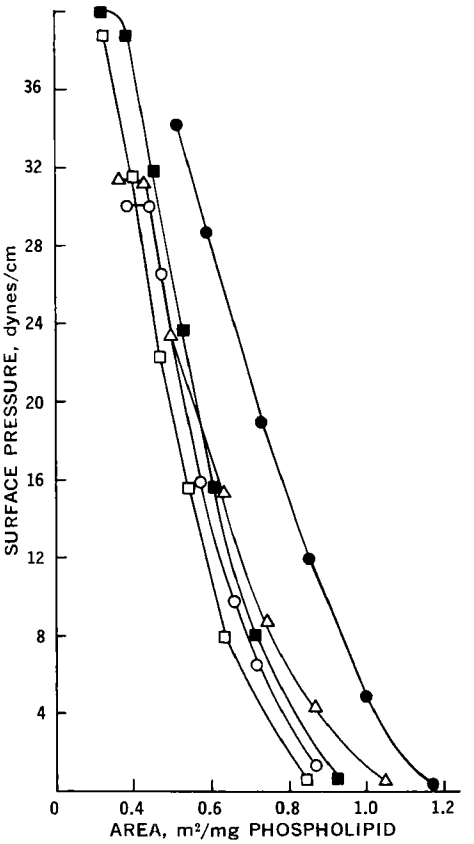

Figure 5—*Surface pressure-surface area curves of the phospholipids spread on a phosphate buffer subphase containing oxalacetic acid. Key (cephalin spread on following concentrations of oxalacetic acid): □, 0, 0.2, and 0.4%; and ■, 2.0%. Key: (egg lecithin spread on following concentrations of oxalacetic acid): ○, 0 and 0.2%; △, 0.4%; and ●, 2.0%.*

surface area (π-A) curves. Figure 5 shows the π-A curves of cephalin spread on a buffer solution and in the presence of various concentrations of oxalacetic acid. Only in the presence of very high concentrations of oxalacetic acid (10 times the amount used in the enzyme activity studies) was there a shift in the π-A curves to larger areas. Even at high concentration, the shift was small and the collapse pressure remained the same, indicating only a weak interaction between oxalacetic acid and cephalin.

In contrast, a comparison of the π-A curves of egg lecithin spread on buffer solution alone and in the presence of various concentrations of oxalacetic acid shows a significant shift in the π-A curves to larger areas (Fig. 5). This effect was observed even at low concentrations of oxalacetic acid (identical to that used in the enzyme activity studies). An increase in collapse pressure of the film also was observed at all concentrations of oxalacetic acid in the subphase. These data suggest that a strong interaction between egg lecithin and oxalacetic acid develops at the concentration levels used in the enzyme activity studies. It appears, however, that there is no significant interaction between oxalacetic acid and cephalin at similar concentrations.

Siegel and Englard (7) suggested that malate dehydrogenase activity is dependent on the oxalacetic acid concentrations and that there is an optimum concentration of oxalacetic acid at which the malate dehydrogenase activity is at a maximum. Any deviation from this optimum concentration decreases the enzyme activity. It appears then that the interaction between oxalacetic acid and egg lecithin may be the basis for the observed decrease in the reaction rates of malate dehydrogenase, while in the case of cephalin the decreased reaction rate may be due to the interaction between the enzyme and the phospholipid.

The activity of the enzyme dropped almost to zero after 30 min of incubation at room temperature (25 ± 2°) at all concentrations of cephalin and egg lecithin. This finding indicates that egg lecithin and cephalin not only do not stabilize or potentiate the activity of malate dehydrogenase but actually reduce its activity.

Callahan and Kosicki (5) observed that the use of lipids extracted from the same source as that of the malate dehydrogenase increased the activity and stability of this enzyme. They concluded that this action was due to hydrophobic interactions between the lipid and the enzyme and that any agent capable of participating in this type of interaction should serve to stabilize malate dehydrogenase.

This effect was not observed with the phospholipids (egg lecithin and cephalin) used in this study. Instead, a decrease in the activity and stability of the enzyme was observed; at concentrations approaching normal serum levels of phospholipid, total inhibition of activity resulted. This suggests that nonspecific hydrophobic interactions may not be the principal factors involved in the stability and activity of this enzyme. The enzyme probably has very specific requirements that must be met for participation in such an interaction. These requirements would be expected to be met by lipids from the same organ as the enzyme. Furthermore, high concentrations of lipid appear to be inhibitory (5).

The effect of self-association of the phospholipids, which might occur at the higher concentrations used in this study, must also be considered. By reducing the availability of interacting sites, self-association could reduce the possibility of a hydrophobic interaction between the lipid and the enzyme. This could eliminate the stabilizing effect observed by Callahan and Kosicki (5). However, it would not be expected to decrease enzyme activity below that observed in the absence of the lipids, particularly at the lower lipid concentrations where self-association would be minimal. Therefore, while this effect could be responsible for some experimental observations, self-association of the lipids could not in itself explain the reduced enzyme activity observed in this study.

In addition, it was observed that the enzyme did not lose any activity during the reaction, even after 15 min. This can be seen from the linearity of the absorbance *versus* time plots over the full course of the reaction (Figs. 1–3). But when the enzyme was incubated in water alone for 15 min prior to initiation of the reaction, the activity of the enzyme was decreased by about 20%. Furthermore, when the enzyme was incubated for 15 min in the presence of oxalacetic acid prior to the initiation of the reaction, the activity decreased to the same extent as was observed when the enzyme was incubated in water alone. When the experiment was repeated using NADH in place of oxalacetic acid, the same results were obtained. Thus, neither oxalacetic acid nor NADH alone stabilizes the enzyme in water, and the presence of both the coenzyme and the substrate is necessary for the stabilization of malate dehydrogenase. Since these two components are always present in the body along with the enzyme and the reaction is a continuous process, this interaction may be an important factor in maintaining stability of malate dehydrogenase *in vivo*. Whether high concentrations of lipid act to inhibit enzyme activity *in vivo* cannot be ascertained from this study. However, the possibility of such an effect should receive further consideration.

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Dr. Alvin Felmeister, dedicated scientist and teacher, died January 11, 1974.

ˣ To whom inquiries should be directed.

Improved Methods for Quantitative Determination of Methadone

HORACE E. HAMILTON*, JACK E. WALLACE*ˣ, and KENNETH BLUM‡

Abstract □ A spectrophotometric method is described that permits the rapid analysis of methadone in urine or tissues at concentrations corresponding to therapeutic, maintenance, or toxic doses of that drug. As little as 5 μg may be detected in a biological specimen. The procedure involves an alkaline extraction into *n*-hexane and subsequent back-extraction into a ceric sulfate–sulfuric acid solution. The acid extract is refluxed with *n*-heptane for 30 min, oxidizing methadone to benzophenone which, in contrast to the unchanged drug, has a high molar absorptivity in the UV region. A GLC method is also described. Both procedures require fewer manipulations and less analysis time than previously reported methods for determining methadone.

Keyphrases □ Methadone in urine and tissues—UV and GLC analyses □ UV spectrophotometry—analysis, methadone in urine and tissues □ GLC—analysis, methadone in urine and tissues

The low molar aborptivity of methadone renders spectrophotometric methods that determine unchanged methadone (1) unsatisfactory for analyzing the drug in biological specimens. Wallace *et al.* (2) recently developed a spectrophotometric method sufficiently sensitive to analyze therapeutic doses of methadone in urine and tissue specimens. The method is based upon the oxidation of methadone to benzophenone, a compound possessing a molar absorptivity approximately 34 times that of methadone.

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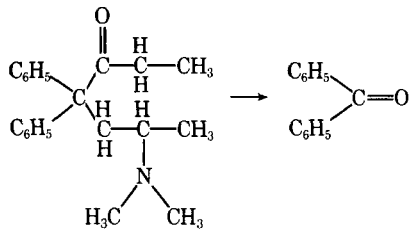

Scheme I—Chemical structure of methadone and benzophenone

proposed method employs a different oxidant, requires fewer manipulative operations, and provides greater sensitivity in less time.

EXPERIMENTAL[1]

Reagents—A 5.5 *M* sulfuric acid solution containing 25 mg of ceric sulfate[2]/ml was prepared by adding 76 ml concentrated sulfuric acid, very slowly and with constant stirring, to a large beaker containing 6.25 g ceric sulfate and 174 ml water. The solution is stable for 2 months at room temperature.

UV Spectrophotometric Procedure—Urine, 5–10 ml, or alkaline-digested (3) tissue, 5–10 g, was transferred to a separator, adjusted to pH 9–12, and extracted into spectroquality *n*-hexane in a 3-min manual extraction; the solvent volumes used in the extractions were 50 ml for urine and 200 ml for tissue specimens. The *n*-hexane layer was removed and filtered, and the recovered volume was recorded. Then 10 ml of 5.5 *M* sulfuric acid was added to the hexane, and this mixture was shaken for 3 min. Nine milliliters of the aqueous layer, 5 ml of spectrograde *n*-heptane, and 200–300 mg anhydrous ceric sulfate[2] were placed in a 250-ml round-bottom flask which was subsequently attached to a water-cooled reflux condenser. The mixture was refluxed for 30 min with constant magnetic stirring and a high reflux rate of approximately 200 drops/min. An alternative procedure was accomplished by extracting the hexane with 10 ml of a solution consisting of 25 mg ceric sulfate/ml of 5.5 *M* sulfuric acid. Nine milliliters of the aqueous acid extract was refluxed for 30 min with 5 ml heptane as already described.

After cooling, the heptane was read in the spectrophotometer from 215 to 360 nm against a similarly prepared *n*-heptane blank. Analysis at a single wavelength was achieved by determining the absorption at 247 nm. For a standard, 10 ml of an aqueous methadone solution containing 15 μg/ml was extracted and similarly determined. Alternatively, 9 ml of 5.5 *M* sulfuric acid containing 100 μg of methadone may simply be refluxed with heptane and ceric sulfate and the resulting absorbance may be adjusted for the previously determined mean recovery. The methadone concentration of an unknown is determined from the following equation:

$$\frac{\text{absorbance}_{\text{unknown}}}{\text{absorbance}_{\text{standard}}} \times \text{concentration of standard in heptane } (\mu\text{g/ml}) \times \frac{\text{ml hexane used in extraction}}{\text{ml hexane recovered}} \times \frac{\text{ml acid used for extraction}}{\text{ml acid used in reflux}} \times \frac{\text{ml heptane}}{\text{ml (g) specimen}} = \mu\text{g/ml} \quad \text{(Eq. 1)}$$

GLC Procedure—Five milliliters of urine and 1 ml of 0.5 *N* NaOH were mixed in a conical 15-ml test tube and centrifuged 10 min at 2100 rpm. Four milliliters of the supernate was transferred to a second tube, extracted with 0.25 ml of 5% isopropanol in chloroform, and again centrifuged 10 min at 2100 rpm; 5 μl of the solvent layer was injected into the chromatograph.

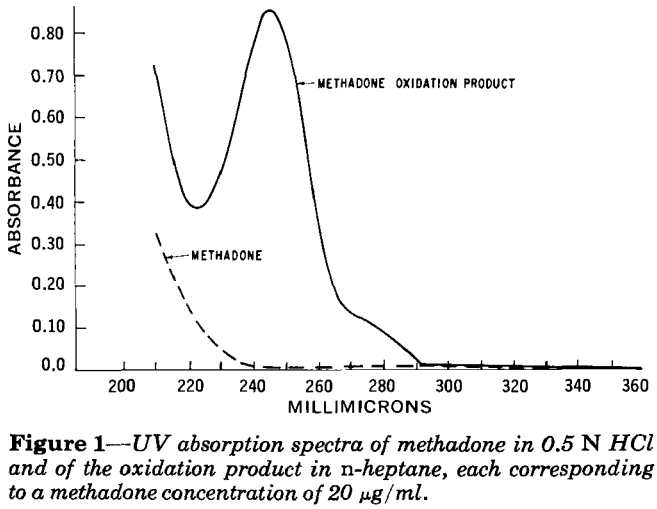

Figure 1—*UV absorption spectra of methadone in 0.5* N *HCl and of the oxidation product in* n*-heptane, each corresponding to a methadone concentration of 20 μg/ml.*

Table I—Standard Curve of Methadone Oxidation Product

Methadone Concentration, μg/ml	Absorbance[a]	Absorbance Concentration, μg/ml
5	0.282	0.056
10	0.565	0.056
15	0.816	0.054
20	1.112	0.056
30	1.662	0.055
40	2.221	0.056
60	3.173	0.053
80	4.180	0.052
120	6.132	0.051
200	10.129	0.051

[a] Absorbance, 247 nm, adjusted for blank and postreflux heptane dilution.

RESULTS

The oxidation of methadone with ceric sulfate produced benzophenone (Scheme I). Both the methadone oxidation product and benzophenone exhibited identical UV and IR spectra, GLC retention times[3], and TLC R_f values[4]. Oxidation to benzophenone results in a marked enhanced sensitivity (Fig. 1). The increase in molar absorptivity of benzophenone in *n*-heptane (ϵ = 18,713, λ = 247 nm) over that of methadone in 0.1 *N* HCl (ϵ = 554, λ = 292 nm) is approximately 34 times.

The absorption of the oxidation product adheres to the Beer-Lambert law as indicated in Table I. The data signify that the conversion of methadone to benzophenone is linear over the concentration range normally encountered in urinalysis. [In a clinical study[5] of a methadone maintenance program, 94% of the analyzed specimens (10 ml) resulted in a final heptane concentration of 4–40 μg/ml and 98.5% resulted in a concentration less than 46 μg/ml.] At concentrations less than 5 μg/ml, the wavelength of maximum absorption is displaced to slightly lower wavelengths.

[1] A Staco variable transformer was used in conjunction with a CRC Multi-lectric Outlet to deliver 60 v ac to each of six 270-w Glas-Col heating mantles positioned on magnetic stirrers. A Beckman Acta CIII ratio-recording spectrophotometer with 10-mm cells was used for UV absorption measurements. A Shimadzu 4B-M gas–liquid chromatograph, equipped with dual flame-ionization detectors and 2.0-m × 4-mm i.d. glass columns, was utilized for the chromatographic assays. The injector and detector temperatures were 260°. A column temperature of 200° and a nitrogen flow rate of 45 ml/min were used with 3% OV-1 and 3% OV-17 on Gas Chrom Q, 100–120 mesh.

[2] Fisher, purified grade.

[3] Retention time 7.85 min on a U-shaped 1.83-m (6-ft) column, 2.5% SE-30 on Gas Chrom Q, 100–120 mesh, at a nitrogen flow rate of 45 ml/min and a column temperature of 210° (Barber-Coleman model 5000 flame-ionization detector with injector and detector temperatures of 220 and 230°, respectively, and flow rates of hydrogen and air of 20 and 200 ml/min, respectively).

[4] On silica gel G, 250 μm: *(a)* R_f of 0.94 in ethyl acetate–methanol–ammonia (30:10:60), and *(b)* R_f of 0.81 in acetic acid–methanol–water (85:10:5). Spots were developed by Dragendorff spray followed by sodium nitrite.

[5] J. E. Wallace, H. E. Hamilton, and J. T. Payte, unpublished data.

Table II—Evaluation of Conditions for Reflux and Relative Yields (Adjusted to 1.00 for Suggested Conditions)

Amount of Ceric Sulfate, mg								
100		200–300		350		400		750
0.86		1.00		0.97		0.89		0.74
Concentration of Sulfuric Acid, *M*								
4.7		5.0		5.5		6.0		7.0
0.97		0.99		1.00		1.01		0.95
Duration of Reflux, min								
10	15	20	25	30	40	50	60	70
0.73	0.79	0.98	0.99	1.00	1.01	0.99	1.02	0.85
Heat of Reaction, 30-min Duration[a]								
55°	65°	75°	85°	95°	Reflux 50 v		Reflux 60 v	Reflux 70 v
0.02	0.09	0.38	0.54	0.76	0.97		1.00	0.88
Volume of Heptane, ml								
5			10				15	
0.99			1.00				0.97	
Volume of 5.5 *M* Sulfuric Acid, ml								
5–10			15				20	
1.00			0.94				0.84	

[a] Data for 55–95° obtained by immersion of flask in a heating bath. Reflux values obtained by applying the indicated voltage to heating mantles.

The extraction of methadone and its primary metabolite was effectively achieved with both *n*-hexane and 5% isopropanol in chloroform. When using GLC, it was observed that both methadone and its metabolite exhibited optimal and similar extraction characteristics over the 9–12 pH range.

The effect of variations of reflux conditions (heat and duration of reflux, volume of heptane and sulfuric acid, amount of ceric sulfate, and concentration of sulfuric acid) upon the oxidation of methadone to benzophenone were investigated (Table II).

Evaluation of Premixed Ceric Sulfate–Sulfuric Acid Solution—The separate addition of sulfuric acid and oxidant to the reflux flask was compared to the use of a previously prepared ceric sulfate–sulfuric acid solution which allows the direct extraction of methadone from the hexane. For the separate addition of reagents, the mean absorbance at 247 nm for a concentration of 20 μg methadone/ml heptane, adjusted for blank, was 1.089 ± 0.094 *SD*, and it was 1.052 ± 0.062 (n = 21) for the premixed solution. Comparisons were also made by using a blank urine pool to which 1.5 mg% methadone was added. Analysis of the urine by the two systems afforded an absorbance at 247 nm in heptane, adjusted for blank, of 0.954 ± 0.028 (n = 8) for the separate addition of reagents and of 0.928 ± 0.050 (n = 9) for the premixed solution. The absorbance obtained with the premixed oxidant solution was approximately 97% of that obtained with the separate addition of reagents for both aqueous standard solutions and extracted urines. A solution of the premixed oxidant was prepared and stored at room temperature in a clear glass flask; no attempt was made to protect the solution from normal laboratory illumi-

Table III—Interference of Compounds with the Determination of Methadone

Compound	Absorbance[a] of Ceric Sulfate	Absorbance of Compound / Absorbance of Methadone	Absorbance[a] of Barium Peroxide	Absorbance of Compound / Absorbance of Methadone	Absorbance of Ceric Sulfate / Absorbance of Barium Peroxide
Methadone	0.92	1.00	0.88	1.00	1.0
Amitriptyline	1.50	1.63	0.23	0.26	6.5
Piperidolate	0.58	0.63	0.33	0.38	1.8
Hydroxyzine	0.46	0.50	0.53	0.60	0.9
Azacyclonol	0.36	0.39	0.34	0.39	1.1
Propoxyphene	0.30	0.32	0.34	0.39	0.9
Antazoline	0.25	0.27	0.41	0.47	0.6
Pipradrol	0.25	0.27	1.21	1.37	0.2
Diphenhydramine	0.24	0.26	0.51	0.58	0.5
Dimenhydrinate	0.21	0.24	0.27	0.31	0.8
Diphenylpyraline	0.08	0.09	0.47	0.53	0.2
Mephentermine	0.05	0.05	0.47	0.53	0.1
Phenmetrazine	0.04	0.04	0.48	0.55	0.1
Chlormezanone	0.04	0.04	0.01	0.01	
Atropine	0.02	0.02			
Amphetamine	0.01	0.01	0.02	0.02	

Compounds Exhibiting ≤ 0.01 Absorbance[a]

Atropine	Chlordiazepoxide	Ephedrine	Imipramine	Nicotine
Bisacodyl	Chloroquine	Ethchlorvynol	Meperidine	Pheniramine
Caffeine	Chlorpheniramine	Ethinamate	Meprobamate	Phenobarbital
Cocaine	Chlorpromazine	Glutethimide	Methylphenidate	Tetracycline
Codeine	Diazepam	Hydantoin	Morphine	Trimethobenzamide
				Triprolidine
				Quinine

[a] Absorbance at 247 nm for 20 μg/ml, adjusted for blank.

Table IV—Recovery of Methadone after Addition to Urine, Spectrophotometric Method

Amount Added, μg/ml	Absorbance[a], 247 nm	Absorbance/ Concentration	Recovery, μg/ml
2.5	0.229	0.092	2.16
5	0.466	0.093	4.40
10	0.875	0.088	8.27
20	1.772	0.089	16.74
30	2.750	0.092	25.98
	Average percent recovery		85.5

[a] Each value represents the average of six determinations, adjusted for blank.

nation. Stability of the solution was established through periodic determinations performed over 10 weeks. There was no loss of oxidative activity during the initial 4 weeks; activity decreased to 96% of the freshly prepared reagent by the 8th week and to 83% by the 10th week, indicating the oxidant to be relatively stable for 2 months at room temperature.

Interfering Species—Several pharmacological agents were tested for interference with the spectrophotometric determination of methadone. The drugs tested included both common drugs of abuse and those having a chemical structure related to benzophenone and acetophenone. Most compounds exhibited negligible absorbance at 247 nm (Table III); several were oxidized to benzophenone or acetophenone, however, and amitriptyline was oxidized to anthraquinone. Only alkaline drugs are listed in Table III, since other compounds are eliminated in the extraction procedure. The barium peroxide and ceric sulfate reagents produced markedly different quantitative results with certain compounds, particularly amitriptyline and pipradrol. Less interference from other drugs was characteristic of ceric sulfate oxidation.

Urine Recoveries—Standard aqueous solutions of methadone hydrochloride were added to urine to provide concentrations ranging from 0.25 to 3.0 mg %. In the spectrophotometric analysis, heptane layers of the 2.0- and 3.0-mg % specimens were diluted 1:1 and 1:2, respectively, with nonrefluxed heptane prior to measurement of absorbance. Urine blanks were similarly determined, including the heptane dilution step. They were not significantly different from reagent blanks, 0.034 ± 0.011 (n = 21). The urine recovery data for the spectrophotometric analyses are shown in Table IV; urine recovery data for the GLC analyses are shown in Table V.

Application to Tissue Analysis—Methadone hydrochloride in saline solutions, adjusted to pH 7.2, was administered subcutaneously in the rear leg of adult male rats at a dose level of 20 mg/kg. One hour following administration, the animals were sacrificed by chloroform inhalation; liver tissues were immediately removed, weighed, and subjected to alkaline digestion according to the procedure of Rickards *et al.* (3).

Spectrophotometrically, tissue blanks were 0.060 ± 0.008 (n = 5), slightly higher than aqueous and urine blanks. Methadone concentrations determined by both techniques gave levels in the liver of 7.2 ± 2.0 (n = 6) μg/g wet tissue.

Urine Analysis—Urine specimens obtained from patients receiving treatment in a methadone maintenance program were extracted with hexane for spectrophotometric analysis. The extracts were divided into two aliquots, one being analyzed by barium peroxide oxidation and the other by ceric sulfate oxidation. The mean (n = 10) methadone concentrations were 0.87 and 0.86 mg %, respectively. The difference in concentration determined by the two methods was ≤0.05 mg % for eight of the 10 specimens.

Table V—Recovery of Methadone after Addition to Urine, GLC Method

Amount Added, μg/ml	Peak Height[a]	Recovery, μg/ml
2.5	1.51	2.27
5	2.88	4.32
10	5.56	8.34
20	10.01	15.02
	Average percent recovery	83.9

[a] Chromatographic conditions as given in the *Experimental* section utilizing 3% OV-17. Peak height expressed in inches at 10^3 MΩ, 0.64 v. Each value represents the average of three determinations.

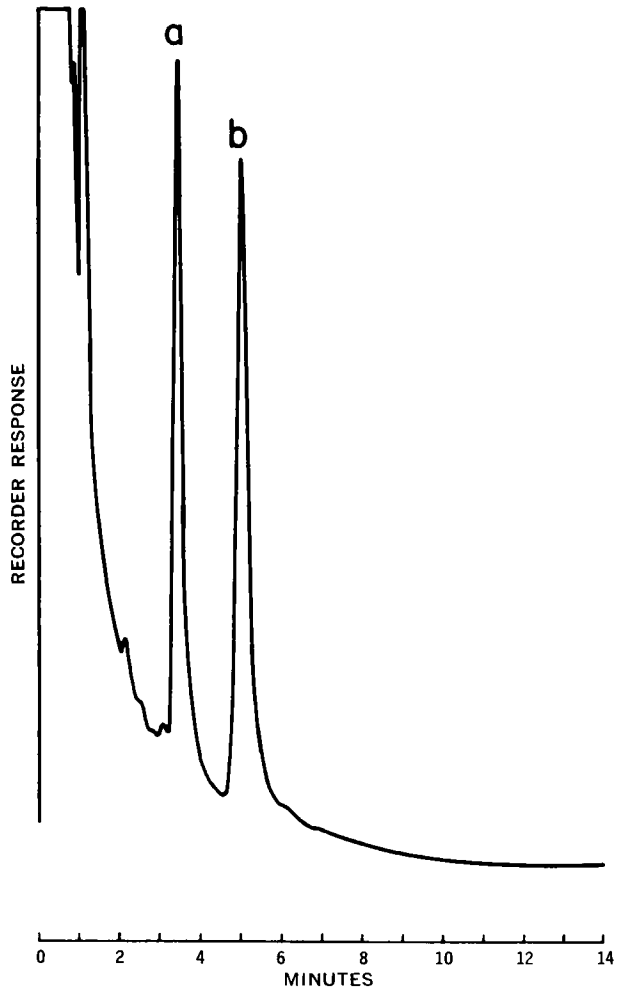

Figure 2—*Gas–liquid chromatogram of urine extract (3% OV-1 column). Key: a, 1,5-dimethyl-3,3-diphenyl-2-ethylidenepyrrolidine (methadone metabolite); and b, unchanged methadone.*

Additionally, urine specimens obtained from patients receiving methadone were divided into two aliquots and examined by the ceric sulfate spectrophotometric method and by GLC. A rapid and simple extraction procedure which did not require back-extraction, buffer washes, or solvent evaporation was utilized for the GLC study. This procedure provided well-defined chromatograms (Fig. 2) and excellent separation of the unchanged methadone and its primary metabolite, 1,5-dimethyl-3,3-diphenyl-2-ethylidenepyrrolidine (4, 5). Retention times for methadone and its metabolite were 4.9 and 3.4 min, respectively, with the OV-1 column (relative retention time of methadone/metabolite = 1.44) and 6.1 and 4.3 min, respectively, with the OV-17 column (relative retention time = 1.42).

Regression analysis of the data comparing the analysis of urine by the spectrophotometric method with the GLC technique provided a correlation of 0.6 if the combined peak areas of methadone and the metabolite from the chromatogram were used. This variation in the analysis of urine samples was the result of a lack of proper concentration of the metabolite for the standard.

Application to Methadone Metabolites—The ceric sulfate–sulfuric acid oxidation also converts the primary metabolite to benzophenone.

Methadone was extracted from urine and subjected to TLC separation according to the procedure of Wallace *et al.* (6). The positions corresponding to methadone and its major metabolite [R_f 0.59 and 0.52, respectively, in n-butanol–acetic acid–water

(4:1:2)] were scraped off, eluted into *n*-hexane, back-extracted into the ceric sulfate–sulfuric acid solution, and examined by the oxidation–UV method. The resulting UV absorption spectra from both the unchanged methadone and the metabolite were identical to that of benzophenone. GLC examination of the heptane following reflux further substantiated that benzophenone was formed from both the parent drug and the metabolite.

DISCUSSION

The extended linearity of the spectrophotometric method permits the use of a single aliquot of the specimen to encompass most urine methadone concentrations encountered in patients receiving methadone maintenance. Analysis by the barium peroxide method required varying specimen volumes since linearity was achieved up to a concentration of 25 μg/ml.

A comparison of the procedures and results of the ceric sulfate and barium peroxide methods indicates several advantages of the former in addition to the extended linearity and elimination of the required alkaline wash. Oxidation of methadone by the method of this report gives a 84.6 ± 3.2% yield of benzophenone as opposed to a 77.8 ± 3.3% yield obtained with the barium peroxide method. In addition to the time saved through the elimination of an extraction step, the proposed spectrophotometric method requires a shorter reaction time; *e.g.*, 20 min reflux with barium peroxide provided only a 48% yield of benzophenone compared to an 82% yield obtained with ceric sulfate. Additionally, reflux conditions were less critical since no significant change in percent yield was observed as the amount of primary oxidant was varied from 200 to 300 mg, the molarity of the sulfuric acid was varied from 5 to 6, or the duration of reflux was varied from 25 to 50 min. Similar variations in the barium peroxide method resulted in significantly decreased yields; changes in the amount of acid, solvent, and heat of reflux produced similar effects in both methods.

The proposed methods provide rapid and sensitive quantitative methods for determining methadone at therapeutic levels in biological specimens. Since other diphenyl-substituted drugs are susceptible to cerium sulfate, utilization of the GLC technique in conjunction with the spectrophotometric method provides a highly specific analytical methodology for methadone in biological specimens. The methods are particularly useful as a quantitative test in suspected methadone overdose cases and as a mechanism to monitor pharmacokinetic studies. In methadone maintenance programs the techniques can be used in collaboration with TLC to confirm on a relative basis that patients are receiving, within broad limits, proper dosages of the drug.

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Methionine Substitutes in Ruminant Nutrition I: Stability of Nitrogenous Compounds Related to Methionine during *In Vitro* Incubation with Rumen Microorganisms

G. A. DIGENIS*ˣ, H. E. AMOS‡, K. YANG*, G. E. MITCHELL‡, C. O. LITTLE‡, J. V. SWINTOSKY*, R. C. PARISH§, G. T. SCHELLING‡, E. M. DIETZ§, and R. E. TUCKER‡

Abstract □ Thirty-one nitrogenous compounds with possible methionine activity were included as potential nitrogen sources in media for *in vitro* incubation of rumen microorganisms. Compounds not supporting cellulose digestion were included in studies of ammonia release and specific analyses designed to confirm resistance of the compounds to microbial deamination. *N*-Acetyl-DL-methionine (IV) and DL-homocysteine thiolactone hydrochloride (XIV) were highly stable when added individually and were selected for more intensive study. When added in combination with urea, XIV was more stable than IV as indicated by cellulose digestion, ammonia release data, and specific GLC analysis for the compounds. Compound XIV was also tested in aqueous buffers at pH 6.8 and 2.3. Little destruction was observed within 24 hr at either pH. These results indicate that dietary XIV has good potential for escaping rumen destruction in a form suitable for subsequent conversion to methionine.

Keyphrases □ Methionine substitutes—ruminant nutrition, stability of 31 nitrogen compounds compared to methionine, *in vitro* incubation with rumen microorganisms □ Nitrogen sources—methionine substitutes in ruminant nutrition, 31 compounds compared to methionine, *in vitro* incubation with rumen microorganisms □ Nutrition, ruminant—31 nitrogen compounds examined to circumvent rumen microbial amino acid deamination

Ruminant animals depend on mixtures of microbial and dietary proteins for their amino acid nutrition (1–5). Dietary proteins may vary greatly in both content and availability of amino acids, and limitations

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Ruminant animals depend on mixtures of microbial and dietary proteins for their amino acid nutrition (1–5). Dietary proteins may vary greatly in both content and availability of amino acids, and limitations

have been observed in both the digestibility (6, 7) and biological value (8, 9) of microbial protein. This suggests that performance could be improved by providing additional amino acids to the postruminal digestive tract. This hypothesis is supported by responses in nitrogen balance or wool growth obtained during postruminal infusions of protein or amino acids (10–14) and parenteral administration of methionine (15). The possibilities for effecting such improvement through dietary additions of amino acids are severely limited by the instability of amino acids in the rumen (16, 17). This problem could be overcome if compounds with amino acid activity and resistance to deamination by rumen microorganisms could be discovered. Nitrogen-containing compounds related to methionine were tested for resistance to *in vitro* deamination by rumen microorganisms using procedures similar to those employed by Acord *et al.* (18) to evaluate a variety of individual nitrogen sources, including amino acids.

EXPERIMENTAL[1]

The compounds tested in this study are listed in Table I.

Compounds VIII, IX, XVIII, XXIX, and XXX were prepared according to literature procedures: VIII, mp 151–153° [lit. (19) mp 149°]; IX, mp 139–141° [lit. (20) mp 138–139°]; XVIII, mp 80–83° [lit. (21) mp 82–84°]; XXIX, mp 51–53° [lit. (22) mp 55–56°]; and XXX, bp 158° (10.8 mm), mp 49–50° [lit. (23) mp 59°]. Compounds XXIV and XXV are known (24) but testing samples contained octanoic and lauric acid impurities, respectively.

Compounds XXVI and XXVII were prepared by acylation of homocysteine thiolactone with the corresponding acid chloride in chloroform–sodium bicarbonate: XXVI, mp 84.5–85.5°, and XXVII, mp 148–149° [lit. (25) mp 134–136°]. Compound XXVIII was prepared by sulfuric acid-catalyzed esterification of XXV in methanol, mp 45–57°.

***In Vitro* Fermentation Test: Cellulose Digestion Experiment**—Rumen microorganisms were obtained 4-6 hr postprandial from a fistulated steer being maintained on 3 kg daily of a shelled corn–soybean meal concentrate mixture plus alfalfa hay *ad libitum*. The rumen ingestum (1–2 liters) was strained through four layers of cheesecloth into a Thermos bottle preheated to 39°. The microorganisms present in the liquid ingesta were centrifuged[2] at 1000×*g* for 2 min to remove any remaining feed particles. The supernate was decanted and diluted with an equal volume of carbon dioxide-saturated 39° water. The diluted material was then passed through a centrifuge[3] equipped with a continuous-flow apparatus to sediment the microbial cells. The flow rate was approximately 150 ml/min at a force of 20,000×*g*. The microorganisms were then washed from the centrifuge tubes and subsequently incubated under an atmosphere of carbon dioxide in 250 ml of a liquid medium developed by Cheng *et al.* (26). The chemical composition of this medium is shown in Table II. Urea, the only nitrogen source in the medium, was replaced by a test compound to provide 0.46 mg nitrogen/ml medium. The initial pH of the medium was adjusted to 6.86 with saturated bicarbonate. For the cellulose digestion experiments, incubations were conducted for 24 hr at 39° in 500-ml erlenmeyer flasks. At the end of the fermentation period (24 hr), the samples were immediately frozen and their cellulose content was determined by following the procedure of Cramptom and Maynard (27) as modified by Donefer *et al.* (28). For the GLC analyses of XIV and IV, the compounds were incubated with urea in 250 ml of the fermentation medium and without urea in another batch. Duplicate samples of 0.1 ml were withdrawn from each batch at various intervals and filtered through a filter[4], freeze dried, and subsequently analyzed by GLC.

***In Vitro* Ammonia–Nitrogen Release Studies**—Rumen fluid, obtained as outlined already, was centrifuged at 1000×*g* in a centrifuge[2] for 2–3 min. The supernate was decanted and diluted with an equal volume of the Cheng *et al.* (26) medium (Table II). To this incubation medium, 495.0 mg of XIV or 636.0 mg of IV/100 ml or the same quantities of the compounds plus 100 mg of urea/100 ml of medium was added. The pH of the medium was adjusted initially to 6.86 with a saturated solution of sodium carbonate and then the medium was incubated at 39°. Homocysteine or homocysteine and urea were used as control treatments (Table III). Samples were withdrawn at 1- and 2-hr intervals thereafter and the ammonia released by each sample was determined by the procedure of Conway (29).

GLC Analyses of XIV and IV in Buffers and Fermentation Fluid—A gas chromatograph[5] equipped with a flame-ionization detector and a recorder[6] was employed. Best results were obtained with a stainless steel chromatography column, 0.31-cm o.d. × 1.52 m (0.125-in. o.d. × 5 ft) packed with 3% OV-17[7] on 100–120-mesh Gas Chrom Q[7] as the solid support. Conditioning of a newly packed column was carried out at 275° with no carrier gas for the first 8 hr and then at 250° with carrier gas (nitrogen at a flow rate of 20 ml/min) for the next 24 hr. To maintain the column in good operating condition after extensive use, it was found that injection of 15 μl of a silylating agent[8] followed by heating at 200° for 30 min prior to use gave excellent results.

Silylation of XIV—The sample[9] to be silylated was filtered[4], and a 0.1-ml aliquot from the resulting clear solution was taken. The aliquot was then freeze dried in a half-dram vial and immediately covered with a rubber septum to avoid unnecessary exposure to moisture[10]. To the lyophilized sample, 0.2 ml of silylating solution[11], consisting of 0.05 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 0.15 ml of pyridine, was added and the resulting clear solution was shaken for 30 min at room temperature. A 1-μl sample was then analyzed by GLC according to the conditions described in Fig. 1.

Silylation of IV—The general procedure for the silylation of IV was similar to that used for XIV. Both *N,O*-bis(trimethylsilyl)trifluoroacetamide and *N,O*-bis(trimethylsilyl)acetamide[12] were found to silylate IV. However, best results were obtained when a 0.1-ml aliquot from the sample of IV was treated for 30 min at room temperature with 0.2 ml of a silylation solution[11] containing 0.05 ml *N,O*-bis(trimethylsilyl)acetamide and 0.15 ml of pyridine. A 1-μl sample was then analyzed by GLC according to the conditions described in Fig. 2.

Stability Studies with XIV—Stability studies were performed in 10.0-ml volumetric flasks by dissolving 0.10 g of XIV in 10.0 ml of the appropriate buffer and adjusting the pH. A phthalate buffer was used for pH 2.3, and phosphate buffer was used for pH 6.9. The flasks were shaken on a water bath shaker at 39°. Then 0.1-ml samples, withdrawn at various intervals, were immediately lyophilized and analyzed for their thiolactone content by GLC.

RESULTS AND DISCUSSION

The results reported here represent the first step in the evaluation of compounds that might be of special value for the improvement of methionine nutrition of ruminants. The nature of the ruminant digestive tract poses special problems to those interested in altering the amino acids available to the tissues (17, 39–41). Ingested feed passes immediately into the rumen and reticulum. These stomach compartments constitute a large fermentation vat

[1] Elemental analytical results were within ±0.3% of the theoretical values; the following compounds were analyzed for the elements indicated by the symbols: Compound XXX ($C_9H_{17}NO_3S$)—C, H, N, S; Compound XXVI ($C_{16}H_{29}NO_2S$)—C, H, N; Compound XXVII ($C_{11}H_{11}NO_2S$)—C, H, N; and Compound XXVIII ($C_{18}H_{35}NO_3S$)—C, H, N. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected.

[2] International model V2 centrifuge.

[3] Sorvall model SS-1.

[4] Millipore.

[5] Varian Aerograph model 1700.

[6] Varian Aerograph model 20.

[7] Applied Science Laboratories, State College, PA 16801

[8] Silyl 8, Pierce Chemical Co., Rockford, Ill.

[9] Samples from aqueous buffer solutions or fermentation media were treated similarly.

[10] The silylation reaction was found to be sensitive to moisture.

[11] The silylation solution was freshly prepared and stored in a half-dram vial covered with a rubber septum. The transfer of 0.2 ml from that solution to the lyophilized sample of XIV or IV was performed by means of a glass syringe and stainless steel hypodermic needle.

[12] Pierce Chemical Co., Rockford, Ill.

Table I—*In Vitro* Cellulose Digestion by Rumen Microorganisms Incubated with DL-Methionine Derivatives or Analogs

Compound Number	Name	Percent Cellulose Fermented
	Trial 1	
I[a]	Methionine sulfoxide	46
II[b]	DL-Methionine amide hydrochloride	95
III[a]	DL-Methionine sulfone	30
IV[a]	*N*-Acetyl-DL-methionine	0
V[a]	DL-Methionine methyl ester hydrochloride	70
VI[a]	DL-Methionine	28
	Trial 2	
VII[c]	Glycyl-DL-methionine	8
VIII	Benzoyl-L-methionine	3
IX	DL-2-Ureido-4-methylthiobutyric acid	63
X[d]	*N*-Hydroxymethyl-DL-methionine calcium salt	0
XI[d]	*N*-Acetyl-DL-homocysteine thiolactone	45
XII[d]	*N*-Phthalyl-DL-methionine	0
XIII[b]	*N*-*tert*-Butyloxycarbonyl-L-methionine dicyclohexyl ammonium salt	0
XIV[c]	DL-Homocysteine thiolactone hydrochloride	0
XV[d]	*N*-Formyl-DL-methionine	40
VI	DL-Methionine	46
XVI[a]	Urea	67
	Trial 3	
XVII[e]	*N*-Propionyl-DL-methionine	9
XVIII	*N*-Benzoyl-DL-methionine amide	45
XIX[b]	*N*-Benzoyl-DL-methionine methyl ester	45
XX[a]	*N*-*tert*-Butyloxycarbonyl-L-methionine-*p*-nitrophenyl ester	0
XXI[a]	DL-Methionine methyl sulfonium chloride	19
XXII[a]	*N*-Carbobenzoxy-DL-methionine	0
XXIII[a]	DL-Homocysteine	49
VI	DL-Methionine	21
XVI	Urea	62
	Trial 4	
XXIV	*N*-Octanoyl-DL-methionine	30
XXV	*N*-Lauryl-DL-methionine	19
XXVI	*N*-Lauryl-DL-homocysteine thiolactone	30
XXVII	*N*-Benzoyl-DL-homocysteine thiolactone	44
XXVIII	*N*-Lauryl-DL-methionine methyl ester	28
XXIX	*N*-Lauryl-DL-methionine ethyl ester	30
XXX	*N*-Acetyl-DL-methionine ethyl ester	50
XXXI	Poly-L-methionine	45
XVI	Urea	65

[a] Sigma Chemical Co. [b] Cyclo Chemical. [c] Aldrich Chemical Co. [d] Mann Research Labs. [e] Smith Kline & French. [f] Miles Laboratories.

where the feed undergoes extensive degradation by a highly complex population of anaerobic bacteria and protozoa. The contents are heavily buffered at about pH 6.5–6.8, but large amounts of organic acids are produced and pH values from 5.5 to 7.0 are not considered abnormal or unusual. Proteins, amino acids, and other nitrogenous compounds are subject to extensive, although variable, deamination to furnish ammonia, the preferred nitrogen source of much of the microbial population. Consequently, much of the protein effectively available to the animal is synthesized by the symbiotic microbial population. There is normally little absorption of amino acids at this point, and most of the free amino acids furnished in the diet or resulting from microbial digestion of protein serve mainly as nonspecific nitrogen sources for microorganisms. The mixture of microbial and feed proteins resulting from these processes eventually passes through the omasum to the abomasum, where the pH ranges from 1 to 4, and is there subjected to the first stages of protein digestion by enzymes secreted by the animal. The next compartment of the digestive tract is the small intestine where conditions are slightly alkaline, protein digestion is completed, and most amino acid absorption takes place. Thus, an effective way of increasing the amount of an amino acid available to the animal tissues might involve providing it in a form that is stable to the fermentive activity in the rumen but that can be absorbed from the lower digestive tract and is capable of conversion to the desired amino acid either before or after absorption.

The data reported here are the results of a screening procedure involving subjecting test compounds to *in vitro* fermentation by microbial populations taken from the rumen. The procedures do not provide satisfactory conditions for protozoal growth and probably result in some alteration of the bacterial population. Compounds surviving this test would logically be tested next for stability to the microbial population in the animal. Determining absorbability, establishing methionine potency in monogastric laboratory animals, and demonstrating improved methionine status in ruminants under laboratory and practical conditions would follow.

In the present study, a multidisciplinary effort was made to develop an *in vitro* test that would permit the screening of several compounds as methionine substitutes in ruminant nutrition. Since microbial destruction of methionine in the rumen is a major problem, an attempt was made to select methionine analogs that would permit correlation between structural characteristics and their resistance to microbial attack.

Table II—Composition of Nutrient Medium

Constituent	Amount, g/liter
Cellulose[a]	14.0
Urea	2.0
KH_2PO_4	0.6
Na_2HPO_4	0.64
$NaHCO_3$	3.5
KCl	4.0
NaCl	4.0
$CaCl_2$	0.55
$MgSO_4 \cdot 7H_2O$	0.29
$CuSO_4 \cdot 5H_2O$	0.002
$MnSO_4 \cdot H_2O$	0.00046
$ZnSO_4 \cdot 7H_2O$	0.00008
$FeSO_4 \cdot 7H_2O$	0.075
$CoCl_2 \cdot 6H_2O$	0.002

[a] SolKa-floc BW-200, a purified wood cellulose, Brown Co., Berlin, N.H.

Table III—Ammonia–Nitrogen Released during Fermentation (Milligrams per Milliliter)

Treatment	Hours							
	0	1	2	4	6	8	10	12
XIV	0.074	—	—	—	—	0.002	—	—
XIV and VI	0.221	0.353	0.290	0.647	0.440	0.618	0.601	0.597
IV	—	0.031	—	0.118	0.059	—	0.059	0.031
IV and VI	—	0.619	0.542	0.706	0.901	0.901	1.190	1.000
XXIII	—	—	—	—	0.357	0.266	0.308	0.294
XXIII and VI	0.147	0.588	0.470	0.470	0.706	0.266	0.322	0.294

Table II shows the composition of the nutrient medium developed by Cheng *et al.* (26) used for the *in vitro* fermentation experiment. In this medium, rumen microorganisms, obtained from fistulated steers, were incubated. The only nitrogen source in the fermentation fluid was urea or one of the methionine analogs being tested. In the fermentation test, the extent of cellulose digestion was utilized as an index of the susceptibility of the test compounds to microbial destruction (30). Thus, compounds supporting a high level of cellulose digestion (*i.e.*, an indication of good microbial growth) were considered easily degradable by rumen microorganisms; compounds not supporting *in vitro* cellulose destruction were considered as *potentially* stable to microbial destruction.

Thirty-one known methionine derivatives were subjected to the test, and the results of four different trials are shown in Table I. DL-Methionine and/or urea was used in each trial as a control. Of the compounds tested, *N*-acetyl-DL-methionine (IV), glycyl-DL-methionine (VII), benzoyl-DL-methionine (VIII), *N*-hydroxymethyl-DL-methionine calcium salt (X), *N*-phthalyl-DL-methionine (XII), *N-tert*-butyloxycarbonyl-L-methionine dicyclohexyl ammonium salt (XIII), DL-homocysteine thiolactone hydrochloride (XIV), *N*-propionyl-DL-methionine (XVII), *N-tert*-butyloxycarbonyl-L-methionine *p*-nitrophenyl ester (XX), and *N*-carbobenzoxy-DL-methionine (XXII), did not support *in vitro* cellulose digestion. The rest of the methionine analogs exhibited a wide range in ability to support *in vitro* cellulose fermentation (Table I).

Examination of the structural characteristics of the 31 methionine derivatives tested for resistance to rumen microbial degradation, evidenced by increased cellulose digestion *in vitro*, revealed the following:

1. Various acyl groups, by blockade of the amino function, may or may not have the ability to impart desirable resistance.
2. Esterification of the methionine carboxyl function does not appear to give products with enhanced resistance.
3. Conversion of the thioether linkage of methionine to sulfoxide, sulfone, or a methylsulfonium salt does not seem to increase resistance.

Belasco (30) showed that excellent bacterial growth resulted in the *in vitro* artificial rumen fermentation test, as indicated by high levels of cellulose digestion, when the medium was provided with certain nitrogenous compounds. More recently, Acord *et al.* (18, 31) found that arginine, glutamic acid, lysine, methionine, serine, and valine were poorly utilized as single nitrogen sources for *in vitro* starch digestion by mixed rumen bacteria. Aspartic acid was found to be inferior to urea as a nitrogen source for the microorganisms. However, three-way and four-way combinations of urea, valine, aspartic acid, and lysine significantly increased starch digestion. These results show that enrichment of the fermentation medium with a combination of various nitrogenous sources results in enhanced microbial growth with subsequently increased cellulose or starch digestion. It, therefore, follows that compounds that appear to be degraded under artificial rumen conditions, especially in the absence of other nitrogenous sources, would almost certainly be degraded in the rumen, in which a great variety of nitrogenous substances, enzymes, and protozoa is present. Thus, potential methionine substitutes supporting cellulose digestion were discarded (Table I). Conversely, a compound's apparent resistance to *in vitro* degradation, as indicated by the extent of cellulolytic activity of the rumen microorganisms, should not be considered as an indicator of resistance to rumen degradation. Furthermore, lack of ability of a compound to support cellulose digestion is not unequivocable proof of resistance to microbial attack. Thus, further evaluation of the stability of

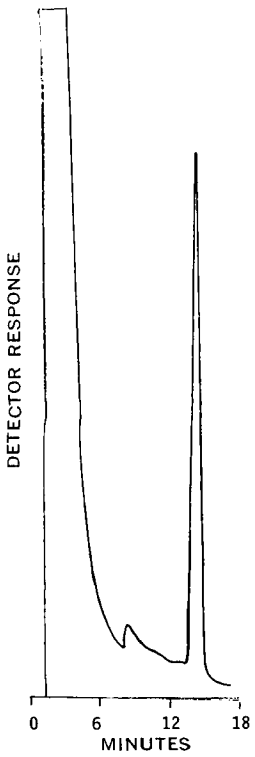


Figure 1—*GLC chromatogram obtained with a 1-μl sample from a solution of XIV in fermentation fluid (5 mg/ml). Compound XIV was silylated with* N,O-*bis(trimethylsilyl)trifluoroacetamide and its chromatography with a 1.52-m × 0.31-cm (5-ft × 0.125-in.) OV-17 column was performed under the following conditions: column temperature, 125°; injection block temperature, 225°; detector temperature, 250°; nitrogen flow rate, 20 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 300 ml/min. The retention time of XIV was 15 min. The electrometer attenuation was at ×32, and the chart speed was kept at 0.17 in./min.*

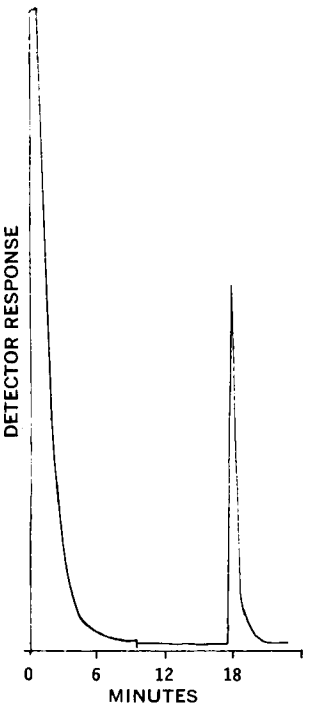


Figure 2—*GLC chromatogram obtained with a 1-μl sample from a solution of IV in fermentation fluid (0.5 mg/ml). Compound IV was silylated with* N,O-*bis(trimethylsilyl)acetamide and its chromatography with a 1.52-m × 0.31-cm (5-ft × 0.125-in.) OV-17 column was performed under the following conditions: column temperature, 155°; injection block temperature, 250°; detector temperature, 275°; nitrogen flow rate, 20 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 300 ml/min. The retention time of IV was 19.0 min. The electrometer attenuation was at ×64, and the chart speed was kept at 0.17 in./min.*

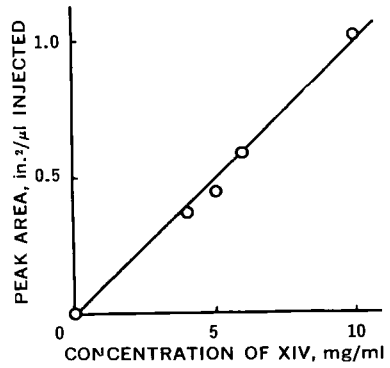


Figure 3—*Calibration curve for the GLC assay of XIV in fermentation fluid.*

these compounds is necessary before animal experimentation begins.

Of the 10 compounds that appeared to exhibit a considerable degree of resistance to microbial attack (Table I), IV and XIV were chosen for further testing. These choices were made because the compounds fulfilled the following criteria: (*a*) both compounds are relatively easily synthesized, (*b*) they are commercially available and relatively inexpensive, an important criterion in view of the large quantities involved in cattle feeds; and (*c*) IV has been shown to replace methionine in the diet of growing chicks (32). Compound XIV is well known. Its synthesis has long been reported (33), and the compound has been disclosed as a pharmaceutical. Compound XIV exhibits protection against irradiation and nitrogen mustards (34) and detoxification against tetanus toxin (35). The compound supports growth of methionine-dependent mutants of *Pseudomonas tabaci* (36). Homogenized, lyophilized, or sliced rat liver methylates XIV to methionine (37). *In vivo* experiments on young rats fed a diet of highly purified amino acids showed synthesis of methionine from XIV if the diet is properly constituted otherwise (38). This fact has been verified in these laboratories, and the details of this study will be reported.

More information on the stability of IV and XIV under the *in vitro* fermentation conditions was obtained from the ammonia release experiment. The data in Table III show net increases in ammonia concentrations over control fermentations with no nitrogen added. Such values represent a balance between the release of ammonia from nitrogenous compounds and the use of ammonia by active microorganisms. Total release of ammonia from the experimental compounds used alone without any uptake by microorganisms would have yielded an ammonia concentration of 0.46 mg/ml. With urea added, the potential concentration became 0.92 mg/ml (0.46 mg/ml from the experimental compound plus 0.46 mg/ml from urea). Decomposition of proteins from dead microorganisms would provide additional potential for ammonia release. Ammonia uptake varies greatly with activity of the microbial population. Consequently, instantaneous ammonia concentrations may provide indications of compound stability but are subject to considerable variation and are by nature likely to be somewhat inconclusive. The data for single additions of XIV and IV provide little indication of ammonia release from either compound. This observation is consistent with the lack of support of cellulose digestion by these compounds in previous experiments. Assuming no ammonia uptake and no other source of ammonia, it can be calculated that 0.0, 7.8, and 62.0% of XIV, IV, and homocysteine, respectively, were degraded. When urea was added, there was considerable evidence of compound degradation (levels above the 0.46-mg/ml potential contribution of urea), and a suggestion of ammonia release from bacterial protein (levels above the 0.92-mg/ml potential contribution of the added nitrogen source) in the *N*-acetyl methionine treatment. Similar degradation might have occurred on other treatments without being detected due to the masking effect of factors such as use of ammonia by the microorganisms.

Because of the above-mentioned difficulties with the ammonia release experiment, more definitive data, on the stability of IV and XIV toward microbial attack, were sought. Thus, GLC assays were developed for both in aqueous solutions and fermenta-

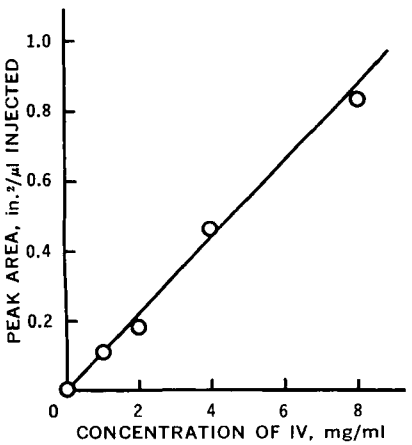


Figure 4—*Calibration curve for GLC assay of IV in fermentation fluid.*

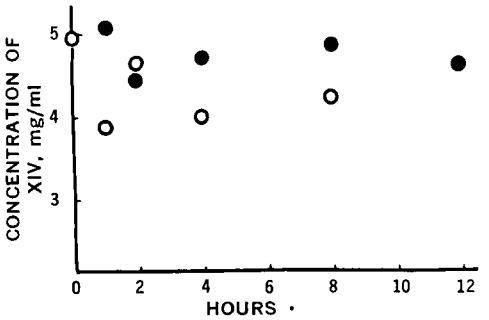


Figure 5—In vitro *microbial stability of XIV. Key:* ○, *4.95 mg XIV/ml fermentation fluid; and* ●, *4.05 mg XIV and 0.99 mg urea/ml fermentation medium.*

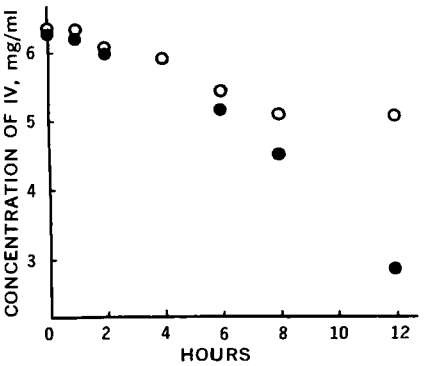


Figure 6—In vitro *microbial destruction of IV. Key:* ○, *6.36 mg IV/ml fermentation fluid; and* ●, *6.36 mg IV and 0.99 mg urea/ml fermentation medium.*

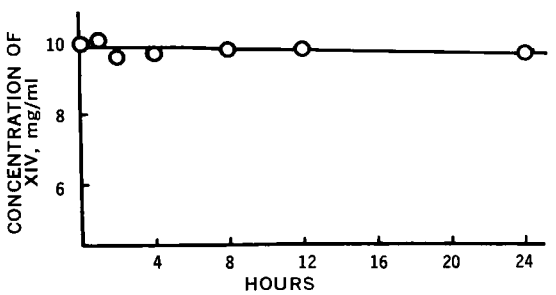


Figure 7—In vitro *stability of XIV at pH 2.3 (phthalate buffer) at 50°.*

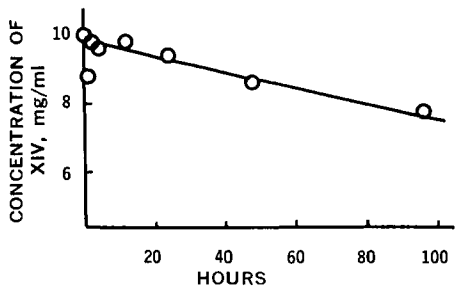


Figure 8—In vitro *stability of XIV at pH 6.8 (phosphate buffer) at 39°.*

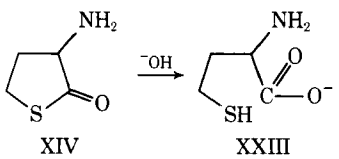
tion media. The compounds exhibited sharp, symmetrical peaks in their GLC chromatograms after silylation in pyridine (Figs. 1 and 2). Figure 1 shows a GLC chromatogram obtained from a 1-μl aliquot of a solution containing 5 mg XIV/ml fermentation fluid.

Calibration curves were obtained with various solutions of IV and XIV in aqueous solutions and fermentation media. These were found to be linear over the range of 0.5–10 mg/ml of solution for both compounds (Figs. 3 and 4). No attempt was made to determine the lowest possible sensitivity limits of the GLC assays since the concentrations of XIV and IV in the fermentation fluid were 4.95 and 6.36 mg/ml, respectively, at the start of the experiment and since good linearity was obtained with their calibration curves around these concentrations.

The extent of microbial destruction of XIV was followed by analyzing for the compound in the fermentation fluid by GLC. Compound XIV was highly stable in either the presence or the absence of urea (Fig. 5). Likewise, IV was fairly stable in the fermentation medium that contained no urea. However, when 0.99 mg of urea was added per milliliter of the medium as a nitrogen source, a considerable destruction of IV was observed (Fig. 6). This is not too surprising in view of what has been discussed earlier on the effect of nitrogen-containing substances on bacterial growth.

The potential of XIV as a methionine substitute for ruminants was further investigated by studying its *in vitro* chemical stability at pH 2.3 and 6.8. These studies were conducted by analyzing for XIV using the GLC assay. Figure 6 shows that XIV is stable at pH 2.3, even at 50°, suggesting that the compound would survive degradation due to pH in the abomasum. The stability of XIV at pH 6.8 and 39° is shown in Fig. 8. These data (Figs. 7 and 8) suggest that XIV is fairly stable at pH 2.3 and 6.8. However, the stability of the compound appears to decrease as the pH of the solution is increased, and this finding is in agreement with what is known about the stability of thiolactones. These are cyclic thioesters that are known to be hydrolyzed in basic media (42–44). Thus, XIV is expected to yield XXIII in an alkaline medium (Scheme I). The conversion of XIV to XXIII is probably catalyzed by esterolytic enzymes. This is substantiated by the fact that homogenized rat liver converts XIV to VI (37). This is probably achieved by conversion of XIV to XXIII, with subsequent methylation to produce VI.

The chemical characteristics of XIV suggest that it could be considered as a potential precursor "prodrug" of methionine in ruminants (45, 46). This means that XIV and its analogs could perhaps pass through the rumen and abomasum unchanged and subsequently be absorbed from the intestines where most absorption occurs. After absorption, the compound could be biotransformed to methionine as previously mentioned. Encouraging preliminary results on the *in situ* absorption of XIV in rats were recently obtained. By using the *in situ* rat gut absorption studies described by Doluisio *et al.* (47), it was possible to show that the compound is absorbed more readily than VI, exhibiting a half-life of absorption of about 20 min. Details on these findings will be reported.



XIV XXIII

Scheme I

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Methionine Substitutes in Ruminant Nutrition II: Stability of Nonnitrogenous Compounds Related to Methionine during *In Vitro* Incubation with Rumen Microorganisms

G. A. DIGENIS*ˣ, H. E. AMOS‡, G. E. MITCHELL‡, J. V. SWINTOSKY*, K. YANG*, G. T. SCHELLING‡, and R. C. PARISH§

Abstract □ Four new α-hydroxy derivatives related to methionine were prepared and tested for resistance to *in vitro* destruction by rumen microorganisms. 3-Hydroxydihydro-2(3*H*)-thiophenone was rapidly degraded by the microorganisms while its benzoate ester, 3-benzoyloxydihydro-2(3*H*)-thiophenone, escaped destruction as indicated by specific GLC analysis for the compounds. Two esters, methyl γ-methylmercapto-α-hydroxybutyrate and phenyl γ-methylmercapto-α-hydroxybutyrate, were found to be extensively destroyed upon incubation with rumen microorganisms.

Keyphrases □ Methionine substitutes—ruminant nutrition, stability of nonnitrogenous compounds compared to methionine, *in vitro* incubation with rumen microorganisms □ Nutrition, ruminant—stability of nonnitrogenous compounds compared to methionine, *in vitro* incubation with rumen microorganisms, effect of rumen microbial amino acid deamination □ Hydroxymethionine derivatives—synthesis, tested for resistance to *in vitro* destruction by rumen microorganisms

In ruminants, ingested feed passes immediately into the rumen and reticulum where it undergoes extensive degradation by a highly complex population of anaerobic bacteria and protozoa. The contents of these compartments are buffered at about pH 6.5–6.8; but since large amounts of organic acids are produced, it is not uncommon to observe pH values between 5.0 and 6.0. Proteins, amino acids, and other nitrogenous compounds are subjected to extensive deamination (1–3) to furnish ammonia, which is the preferred nitrogen source of much of the microbial population. Thus, much of the protein effectively available to the animal is that synthesized by the symbiotic microbial population (4). The mixture of microbial and feed proteins eventually passes from the rumen through the omasum to the abomasum where the pH is from 1 to 4. Here the proteins are subjected to enzymatic digestion by enzymes secreted by the animal. The next compartment of the digestive tract is the small intestine where conditions are slightly alkaline, protein digestion is completed, and most amino acid absorption takes place.

Normally, there is little absorption of amino acids from the rumen, and rumen microorganisms have been shown to degrade supplementary dietary amino acids (1–3). Furthermore, several studies have shown that nitrogen utilization has been improved by infusing amino acids postruminally (5–7). Thus, the development, through structural manipulation, of select amino acid preparations that resist microbial breakdown in the rumen could possibly prove useful for increasing nitrogen utilization in ruminants.

Thirty-one nitrogenous compounds related to methionine were previously examined (8) for their resistance to *in vitro* deamination by rumen microorganisms. DL-Homocysteine thiolactone hydrochloride (Ia) was found to be highly stable toward microbial destruction. Since an α-hydroxy analog of methionine (IIa) has been reported to produce a beneficial effect in ruminants (9), it was of interest to prepare α-hydroxyl derivatives of methionine and homocysteine thiolactone (Ia).

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In the present study, four new nonnitrogenous compounds (α-hydroxy derivatives) related to methionine were tested for resistance to *in vitro* destruction by rumen microorganisms.

EXPERIMENTAL[1]

3-Hydroxydihydro-2(3*H*)-thiophenone (I*b*)—One hundred milliliters of concentrated hydrochloric acid was added to a solution of 50 g of homocysteine thiolactone (I*a*)[2] in 100 ml of distilled water, and the solution was cooled to ice bath temperature. A solution of 69 g of sodium nitrite in 300 ml of distilled water was then added dropwise with vigorous stirring. The resulting brownish-red oily liquid was separated from the aqueous layer. The water layer was then extracted with chloroform (3 × 150 ml). The chloroform fractions, combined with the oily liquid, were dried, and solvent was removed *in vacuo*. Distillation at 85° (0.05 mm) yielded 8.3 g (21%) of I*b*. Further distillation over a spinning band column afforded an analytical sample.

Anal.—Calc. for $C_4H_6O_2S$: C, 40.66; H, 5.12. Found: C, 40.56; H, 5.12.

3-Benzoyloxydihydro-2(3*H*)-thiophenone (Ic)—To a solution of 1.2 g of I*b*, 20 ml of methylene dichloride, and 1 ml of triethylamine was added dropwise 2 g of benzoyl chloride with stirring at room temperature. Stirring was continued for 30 min. To the reaction mixture was then added 50 ml of cold 10% NaOH solution. The resulting solution was extracted with chloroform (3 × 30 ml) and the combined extracts were dried. Removal of solvent *in vacuo* yielded Ic (1.9 g, 85%). Two recrystallizations from chloroform–methanol afforded white leaflets, mp 121–122°.

Anal.—Calc. for $C_{11}H_{10}O_3S$: C, 59.44; H, 4.54; S, 14.42. Found: C, 59.59; H, 4.61; S, 14.40.

Methyl γ-Methylmercapto-α-hydroxybutyrate (II*b*)—Fifteen grams of γ-methylmercapto-α-hydroxybutyric acid (II*a*)[3] was refluxed with 100 ml of 3% H_2SO_4 in methanol for 3 hr. The pH of the solution was then adjusted to 7.0 with 10% KOH in methanol, the solution was filtered, and the filtrate was evaporated to dryness under reduced pressure. Then 30 ml of water was added to the residue and the mixture was extracted with ether (3 × 50 ml). The combined ether extracts were washed with 10% aqueous KOH (3 × 20 ml) and then with water (2 × 20 ml). The dried ether extract was evaporated *in vacuo*. The oily residue was distilled, bp 85–90° (0.15 mm), to afford II*b* (8.5 g, 52%). Further distillation at 80° (0.1 mm) yielded an analytical sample.

Anal.—Calc. for $C_6H_{12}O_3S$: C, 43.88; H, 7.37. Found: C, 44.02; H, 7.31.

Phenyl γ-Methylmercapto-α-hydroxybutyrate (IIc)—For 2 hr, 7.5 g of γ-methylmercapto-α-hydroxybutyric acid (II*a*)[3], 5.5 g of phenol, and 0.5 g of *p*-toluenesulfonic acid in 100 ml of benzene were refluxed (Dean–Starke trap). The mixture was shaken with 20 ml of 0.5 *N* NaOH, and the benzene layer was subsequently dried and concentrated *in vacuo*. Distillation of the residue at 190–200° (0.15 mm) afforded IIc (6 g, 54%).

Anal.—Calc. for $C_{11}H_{14}O_3S$: C, 58.39; H, 6.24. Found: C, 58.06; H, 6.24.

***In Vitro* Fermentation with Rumen Microorganisms**—Rumen microorganisms, obtained from a fistulated steer according to a reported method (8), were washed and incubated under an atmosphere of carbon dioxide in 250 ml of a liquid medium developed by Cheng *et al.* (10). This medium was composed of inorganic salts, cellulose (14 mg/ml), and urea (0.5 mg/ml) as the sole nitrogen source. The concentrations of the test compounds in the fermentation medium were as follows (mg/ml): I*b*, 13.75; Ic, 5.95; II*b*, 8.2; and IIc, 11.3. The initial pH of the medium was adjusted to 6.86 with saturated sodium bicarbonate solution. Incubation experiments were conducted for 24 hr at 39° in 500-ml erlenmeyer flasks. Duplicate samples (2.0 ml), withdrawn from the fermentation mixture, were filtered through a filter[4], freeze dried, and subsequently analyzed by GLC. At the end of the fermentation period (24 hr), the samples were immediately frozen and their cellulose content was determined by the procedure of Crampton and Maynard (11) as modified by Donefer *et al.* (12).

GLC Analysis of I*b* and Ic—GLC analyses were conducted

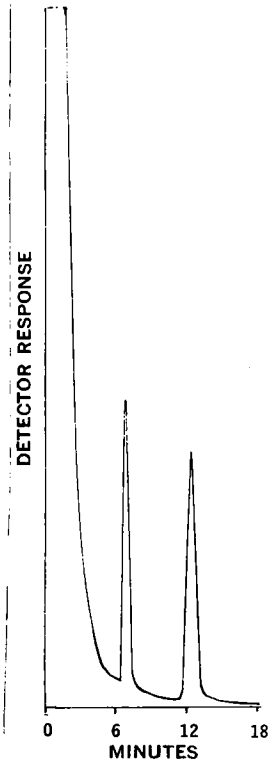


Figure 1—*GLC chromatogram obtained with a 2-μl sample from a solution of* I*b in fermentation fluid (10 mg/ml). Each sample was spiked with acetophenone (peak on the left), which exhibited a retention time of 6.9 min while that of* I*b (peak on the right) was 12.6 min. Chromatography of* I*b with a 1.52-m × 0.31-cm (5-ft × 0.125-in.) OV-17 column was performed under the following conditions: column temperature, 105°; injection block temperature, 200°; detector temperature, 200°; nitrogen flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 60 ml/min. The electrometer attenuation was at ×32, and the chart speed was kept at 0.17 in./min.*

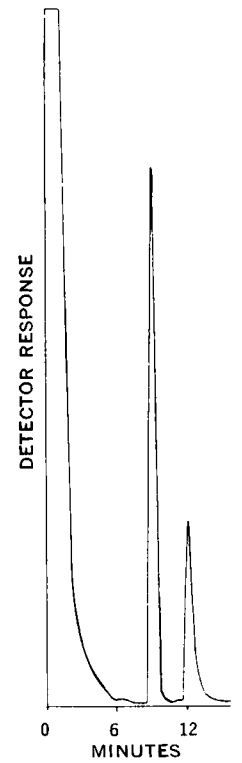


Figure 2—*GLC chromatogram obtained with 1-μl sample from a solution of* Ic *in fermentation fluid (5 mg/ml). Each sample was spiked with phenobarbital (peak on the right), which exhibited a retention time of 12.0 min while that of* Ic *(peak on the left) was 9.6 min. Chromatography of* Ic *with a 1.52-m × 0.31-cm (5-ft × 0.125-in.) OV-17 column was performed under the following conditions: column temperature, 225°; injection block temperature, 250°; detector temperature, 260°; nitrogen flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 300 ml/min. The electrometer attenuation was at ×32, and the chart speed was kept at 0.17 in./min.*

[1] Melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. Elemental microanalyses were performed by Microanalysis, Inc., Wilmington, DE 19808. The structures of all compounds were supported by IR and NMR spectra determined on Beckman IR-8 and Varian A-60 spectrometers, respectively. UV spectra were determined on a Cary 15 spectrophotometer in methanol.

[2] Compound I*a* was obtained from Aldrich Chemical Co., Milwaukee, Wis.

[3] Compound II*a* was obtained from its calcium salt preparation (Hydan, Nutritional Biochemical Corp., Cleveland, Ohio) by removal of calcium with 10% H_2SO_4 in methanol, extraction of II*a* with 10% aqueous KOH, and subsequent acidification and extraction with chloroform. The obtained II*a* was distilled, bp 135–140° (0.25 mm).

[4] Millipore.

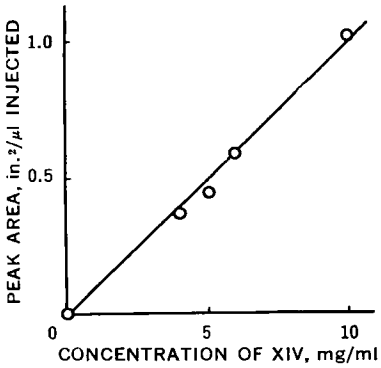


Figure 3—*Calibration curve for the GLC assay of XIV in fermentation fluid.*

with a gas chromatograph[5] equipped with a flame-ionization detector, using a 1.52-m × 0.31-cm (5-ft × 0.125-in.) stainless steel column, packed with 3% OV-17[6] on 100–120-mesh Gas Chrom Q[6] as the solid support. Conditioning of newly packed columns was carried out as previously described (8). Solutions of varying concentrations in fermentation medium were utilized for the preparation of calibration curves (Figs. 3 and 4). The concentrations ranged from 1.0 to 20.0 mg/ml for I*b* and from 0.4 to 7.0 mg/ml for Ic. Samples of 2.0 ml each were extracted twice with 5-ml increments of ether. The combined ether extracts were dried over 0.2 g of anhydrous sodium sulfate and subsequently evaporated to dryness. Then 1 ml of an internal standard was added to each sample. Acetophenone in chloroform (1 mg/ml) was used as the internal standard for I*b*, while phenobarbital in ethanol (1 mg/ml) was utilized for Ic. Samples of I*b* and Ic were then analyzed by GLC according to the conditions described in Figs. 1 and 2, respectively.

GLC Analysis of II*b* and IIc—Samples (2.0 ml) from fermentation medium containing II*b* and IIc were extracted four times with 2-ml increments of ether. The extracts were reduced to approximately 1.5 ml under reduced pressure and their volume was adjusted to 2.0 ml. A 5-μl sample was then analyzed on a 1.52-m × 0.31-cm (5-ft × 0.125-in.) stainless steel column, packed with 3% SE-30 on 100–120-mesh Varaport[7] 30. A gas chromatograph[5], equipped with a flame-ionization detector and a linear temperature programmer, was utilized. The GLC analyses of II*b* and IIc were performed under the following conditions, respectively: initial column temperature, 110°, 107°; final column temperature, 140°, 140° at a rate of 12°/min; injection block temperature, 220°, 215°; detector temperature, 220° (II*b* and IIc); nitrogen flow rate, 14 ml/min (II*b* and IIc); hydrogen flow rate, 30 ml/min (II*b* and IIc); air flow rate, 300 ml/min (II*b* and IIc). The retention times were 6 min for II*b* and 8 min for IIc. The electrometer attenuation was at ×32, and the chart speed was kept at 0.17 in./min.

RESULTS AND DISCUSSION

In a previous publication (8) and in the introductory remarks,

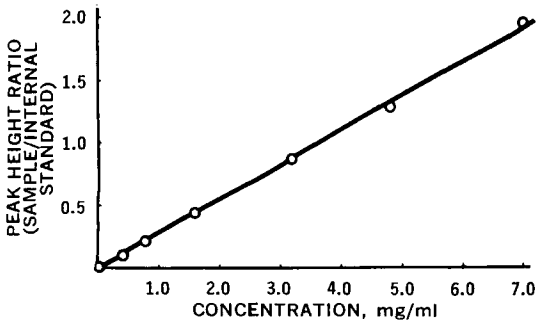


Figure 4—*Calibration curve for the GLC assay of Ic in fermentation fluid.*

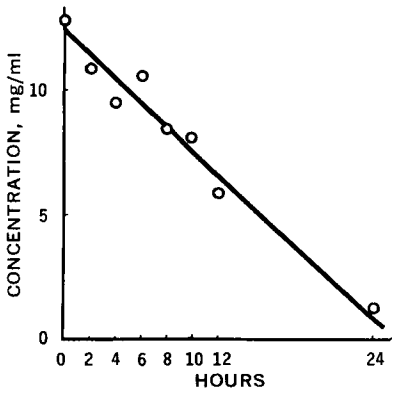


Figure 5—In vitro *microbial destruction of* Ib (*13.75 mg of* Ib*/ml of fermentation fluid*).

it was mentioned that proteins, amino acids, and other nitrogenous compounds are subjected in ruminants to extensive chemical degradation by rumen microorganisms. It appears that a large portion of the protein effectively available to these animals is that synthesized by the symbiotic microbial population. Recent research indicates that rumen microbial protein may be deficient in methionine, lysine, and threonine for supporting maximum protein utilization by growing lambs (13). Supplementary dietary amino acids are also degraded by rumen microorganisms (1–3). However, postruminal infusion of amino acids appears to improve nitrogen utilization (5–7). Thus, it is clear that resistance of an amino acid preparation toward microbial breakdown in the rumen could possibly find utility for increasing nitrogen utilization in the ruminant.

Recently it was reported (9) that an α-hydroxy analog of methionine[8] (II*a*) improved milk production in dairy cows. DL-Homocysteine thiolactone hydrochloride (I*a*) was found (8) to resist rumen microbial destruction *in vitro* and was stable in aqueous buffers at pH 2.3 and 6.8, typical pH values for the abomasum and small intestine, respectively. Furthermore, I*a* has been shown to support growth of methionine-dependent mutants of *Pseudomonas tabaci* (14), and sliced rat livers have been shown to convert the thiolactone to methionine (15). *In vivo* experiments on young rats fed a diet of highly purified amino acids showed synthesis of methionine from DL-homocysteine thiolactone (I*a*) (16). This dietary property of I*a* was also observed in growing rats[9]. In addition, evidence has been obtained that the compound is absorbed more readily than methionine by using an *in situ* rat gut absorption technique described by Doluisio *et al.* (17).

These findings suggested that α-hydroxy analogs of DL-homocysteine thiolactone (I*a*) would have good potential for escaping rumen destruction and act as good methionine precursors. Thus, 3-hydroxydihydro-2(3*H*)-thiophenone (I*b*) was synthesized by diazotization of I*a*. Subsequent esterification of I*b* with benzoyl chloride led to the synthesis of Ic.

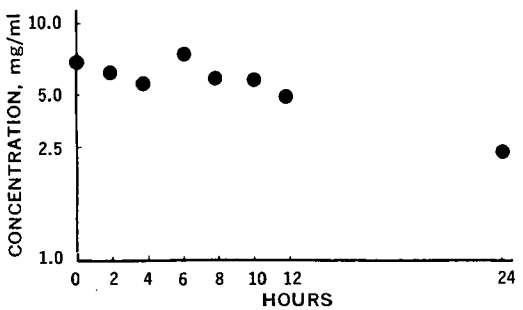
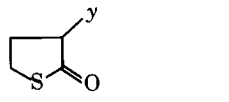


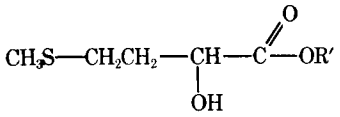
Figure 6—In vitro *stability of* Ic (*5.95 mg of* Ic*/ml of fermentation fluid*).

[5] Varian Aerograph model 1700.
[6] Applied Science Laboratories, State College, PA 16801
[7] Varian Instrument Division, Palo Alto, CA 94303

[8] Hydan.
[9] H. E. Amos, G. T. Schelling, G. A. Digenis, J. V. Swintosky, C. O. Little, and G. E. Mitchell, Jr., to be published.



Ia: $y = NH_2 \cdot HCl$
Ib: $y = OH$
Ic: $y = OCOC_6H_5$



IIa: $R' = H$
IIb: $R' = CH_3$
IIc: $R' = C_6H_5$

To investigate the resistance of I*b* and I*c* toward microbial degradation, a specific GLC analysis was developed for the compounds. Samples from the fermentation experiments were extracted with ether, and an internal standard was added to the residues obtained from the extracts. Acetophenone was used as a marker for I*b*, and phenobarbital was used for I*c* (Figs. 1 and 2). Figures 3 and 4 illustrate that an excellent linear relationship could be obtained for the calibration curves by measuring the peak height ratios of the unknown compounds with their respective markers.

When I*b* and I*c* were incubated *in vitro* with rumen microorganisms, according to the previously described technique (8), it was found that I*b* was quickly degraded by the microorganisms (Fig. 5). In contrast, however, the ester I*c* appeared to be considerably resistant to microbial destruction (Fig. 6). The results obtained with I*b* are somewhat surprising in view of the fact that I*a* was previously shown to escape microbial destruction under identical conditions (8).

As shown in Figs. 7 and 8, both II*b* and II*c* were extensively destroyed during *in vitro* incubation. The rapid rates of destruction observed during the first few hours declined considerably and permitted 10–20% of the added materials to remain after 24 hr. If a similar pattern applies to *in vivo* degradation, a sufficient quantity of these compounds might escape destruction in the rumen to permit significant passage to the lower digestive tract.

Experiments are now underway to assess the value of these compounds as dietary methionine substitutes in ruminants. The results of these studies will be reported elsewhere.

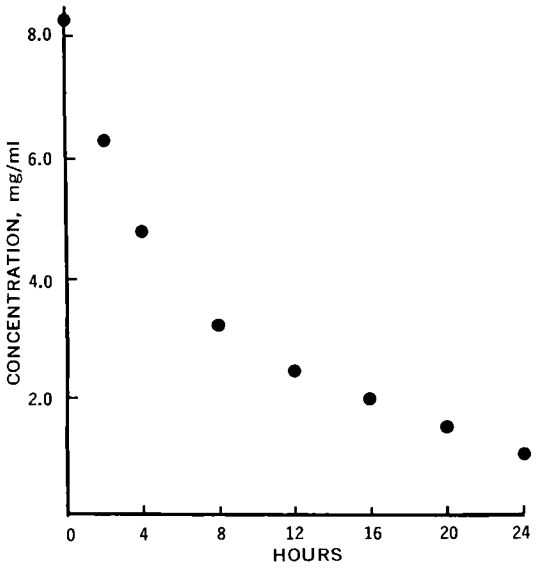


Figure 7—In vitro *microbial destruction of II*b (*8.2 mg of II*b*/ml of fermentation fluid*).

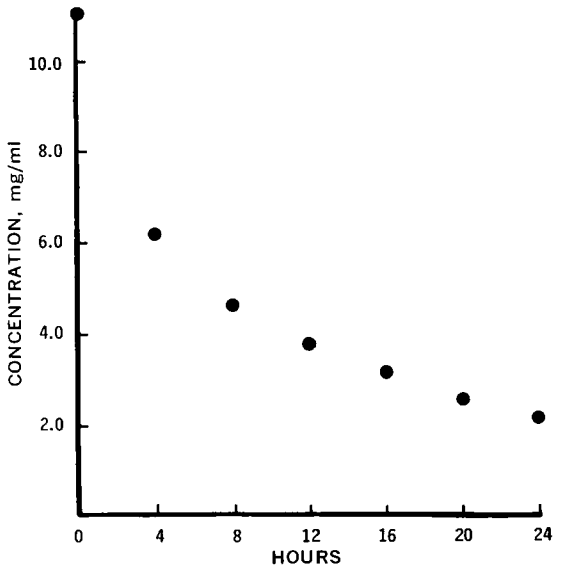


Figure 8—In vitro *microbial destruction of IIc* (*11.3 mg of IIc/ml of fermentation fluid*).

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ˣ To whom inquiries should be directed.

Topochemical Decomposition Patterns of Aspirin

ELWYN NELSON, DAVID EPPICH, and J. T. CARSTENSEN[x]

Abstract □ Aspirin in alkaline environment in a solid dosage form is shown to decompose by topochemical reaction patterns. At lower temperatures, the rate-determining step is diffusion controlled, whereas at higher temperatures it is kinetically controlled.

Keyphrases □ Aspirin—degradation in alkaline media, topochemical reactions □ Degradation, aspirin—in alkaline media, topochemical reactions

Some major reviews of solid substances have appeared in the past (1-4). The emphasis in general has been on stability prediction, although some work relating to mechanisms has also been considered (1, 3). In most reported specific cases, reaction orders were first (5) or zero (6) order; however, in a few cases (1, 7-10), broad, general (physical and chemical) bases for the degradations were arrived at.

Horikoshi and Himuro (11) reported the only topochemical reaction scheme in the pharmaceutical literature, although it was pointed out indirectly (3) that this type of reaction is probably the reason for most degradations in the solid state that appear to be first order. The purpose of the present study was to demonstrate the existence of topochemical reactions in competition with one another in the degradation of aspirin in an alkaline environment.

THEORY

If a cylinder nucleates on the curved surface but not on the ends, then the linear decomposition rate will be given by:

$$r = r_0 - kt \qquad \text{(Eq. 1)}$$

in which r and r_0 are the radii as illustrated in Fig. 1*a*, k is a rate constant, and t is time. By referring to Fig. 1*a*, it is easily seen that the mole fraction α decomposed at time t is given by:

$$1 - \alpha = \frac{h\pi r^2}{h\pi r_0^2} = [1 - (k/r_0)t]^2 \qquad \text{(Eq. 2)}$$

in which h is the height, or:

$$\sqrt{1 - \alpha} = 1 - (k/r_0)t \qquad \text{(Eq. 3)}$$

If a small amount of (spherical) material, B, is embedded in a matrix of A, and if $A + B \rightarrow C$ is the decomposition reaction, then at time t, A must diffuse through a layer of C which is equal to $r_0 - r$ in thickness. It is here assumed that A is the mobile species and that the reaction is diffusion controlled, *i.e.*, A reacts instantaneously with B, once it has diffused through the C layer. Loss of A causes a proportional increase in the C layer by a factor of γ, so one may write:

$$-\frac{dA}{dt} = \gamma\left(\frac{dl}{dt}\right) = \frac{q}{l} \qquad \text{(Eq. 4)}$$

in which $l = r_0 - r$ is the thickness of the C layer and q is a diffusional coefficient. The latter part of the equality in Eq. 4 is obtained from Fick's law.

Equation 4 may be integrated to:

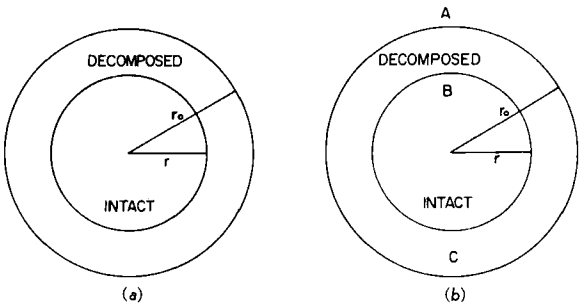

Figure 1—*Situation of* (a) *contracting cylinder and* (b) *contracting sphere, where* B *is intact drug separated from a catalyzing substance* (A) *by a layer of decomposition product.*

$$l^2 = (2q/\gamma)t = k't \qquad \text{(Eq. 5)}$$

or:

$$r_0 - r = \sqrt{k't} \qquad \text{(Eq. 6)}$$

It is seen that:

$$1 - \alpha = \frac{\pi r^3}{\pi r_0^3} = [1 - \sqrt{k''t}]^3 \qquad \text{(Eq. 7)}$$

the latter step being accomplished through Eq. 6. Here:

$$k'' = k'/r_0^2 = (2q)/(\gamma r_0^2) \qquad \text{(Eq. 8)}$$

Equation 7 is most conveniently written in the form:

$$[1 - \sqrt[3]{(1 - \alpha)}]^2 = kt \qquad \text{(Eq. 9)}$$

EXPERIMENTAL AND RESULTS

It is well known that aspirin decomposition is catalyzed by al-

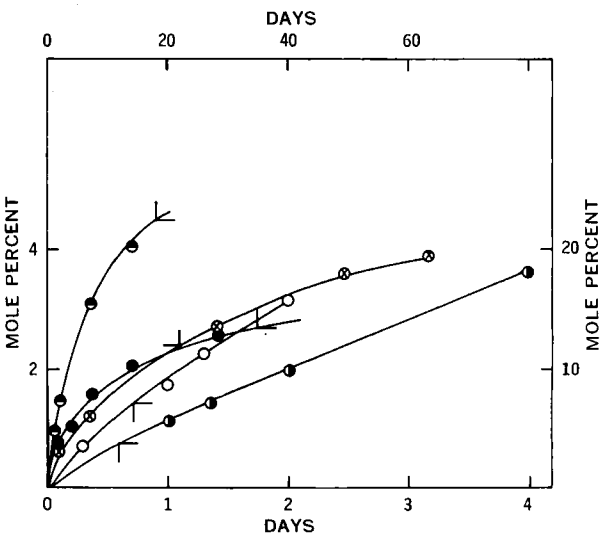

Figure 2—*Decomposition patterns of aspirin in alkaline environment. Key:* ○, *50°;* ◑, *47.5°;* ◓, *45°;* ●, *40°; and* ⊗, *25°.*

Table I—Salicylic Acid Content of Two Batches of Tablets

	2 Weeks of Storage at 40°		4 Weeks of Storage at 40°		12 Weeks of Storage at 25°	
	10.7	9.8	12.2	11.7	6.03	5.69
	10.9	9.5	12.3	12.3	6.54	5.23
	10.5	9.7	12.9	11.9	6.78	5.43
	11.2	9.9	12.8	11.7	6.21	5.66
	11.2	9.7	13.4	12.3	6.73	5.54
	11.0	9.7	12.6	12.3	6.03	5.58
Average	10.92	9.72	12.90	12.00	6.39	5.52
Standard deviation	0.28	0.13	0.40	0.30	0.34	0.17
$1 - \sqrt[3]{1 - \alpha}$	0.0378	0.0335	0.0449	0.0417	0.0217	0.0187
$\sqrt{t}$, $(\text{week})^{1/2}$	1.41	1.41	2	2	3.46	3.46
k	0.027	0.024	0.023	0.024	0.0062	0.0054

kali (12); therefore, tablets were made containing 324 mg of aspirin *(B)* in a total tablet weight of 3.5 g. The excipient was sodium bicarbonate *(A)*, pH 8.3 [0.1% (w/v) in water at 25°]. Five different batches of this composition were prepared.

Tablets were stored (four per container) in hermetically tight containers in stability ovens set at 25, 35, 40, 47.5, and 50°. Bottles were withdrawn from the ovens at the intervals indicated in Fig. 2. The four tablets were dissolved in 200 ml of water, the inside of the bottle was rinsed with water, and the volume was adjusted to 250 ml. The solution was then assayed for salicylic acid by UV absorption at 295 nm and adjusted for the contribution from aspirin at this wavelength. Prepared mixtures of salicylic acid and aspirin with 0.5–20 mole % of salicylic acid gave a standard deviation of 3–1 relative % when groups of four were assayed. Hence, the coefficient of variation of averages of four is of the order of 1.5–0.5 relative %. Six bottles from each batch were assayed from each stability point. The coefficient of variation of samples of four ranged from 1 to 2.5 relative % (Table I), the increase over the expected being the (expected) contribution of oven temperature variation (±0.5°) and tablet-to-tablet variation.

The data in Table II and in Fig. 2 are the stability patterns obtained. The figures are averages of five batches. If no batch-to-batch variation existed, one would expect the coefficient of variation to be $2.5/\sqrt{5}$–$1/\sqrt{5}$ or 1.1–0.45 relative %. In actuality, the data exhibit coefficients of variation from 1 to 2 relative % on the averages shown in the table, so that, for example, a salicylic acid content of 5.0% should have $\pm 2\sigma$ limits of about 0.2%.

DISCUSSION

The data in Fig. 2 at 47.5 and 50° adhere to Eq. 3 but not to Eq. 9 nor to a contracting sphere equation, derived in a fashion similar to the one leading to Eq. 3:

$$\sqrt[3]{1 - \alpha} = 1 - \left(\frac{k_0}{r_0}\right) \cdot t \qquad \text{(Eq. 10)}$$

The adherence to Eq. 3 is demonstrated in Fig. 3. On the other hand, the data in Fig. 2 at the lower temperatures adhere to Eq. 9 but not to Eq. 10 nor 3 (Fig. 4). The rate constants from Fig. 4 adhere well to an Arrhenius equation (Fig. 5).

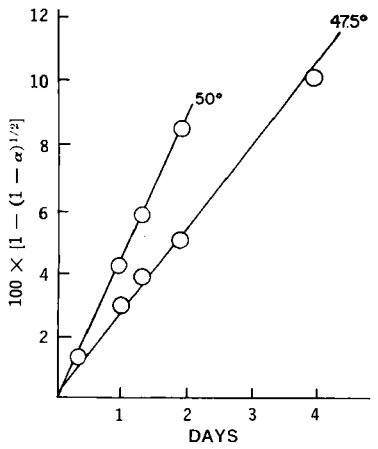

Figure 3—*Data from 50 and 47.5° treated according to Eq. 3.*

It has been pointed out (3) that topochemical reactions may *appear* first order. Figure 6 shows that the data in Fig. 2 can be made to fit an equilibrium scheme:

$$S = S_\infty(1 - e^{-Kt}) \qquad \text{(Eq. 11)}$$

As, however, the data are followed for longer times, S_∞ increases and, in essence, Eq. 11 only approximates Eq. 9 in a time interval up to $2 \times t'$, where t' is the largest time value in the study.

The inconsistency shows up in Arrhenius and Van't Hoff plots (which are only linear in fortuitous choices of t'). This is particularly important in the following context. Stability studies are usually thought of as a development tool in formulation choices and in the setting of ultimate overages in dosage forms. They are, however, *of great value in quality control as early warning systems, i.e.,* in monitoring whether a particular batch of a product has a suitable room temperature stability (*i.e.*, whether it falls into the limits projected from many other batches of the same product). For this purpose, a correlation test is usually necessary; in the cited case, *(a)* accelerated checks at temperatures above 45° would be meaningless and correlation to room temperature data would be unsound; and *(b)* in establishing correlation between behavior *at* 45° with patterns at room temperature, k and

Table II—Salicylic Acid Formation as a Function of Time at Various Temperatures

Temperature	Days (t)	Salicylic Acid, mole % (100)	$1 - \sqrt{1 - \alpha}$	$1 - \sqrt[3]{1 - \alpha}$	$\sqrt{t}$
25°	2	0.59	—	0.00197	1.41
	7	1.18	—	0.00394	2.65
	28	2.70	—	0.00908	5.29
	49	3.60	—	0.01214	7.00
	63	3.90	—	0.01317	7.94
35°	1	1.60	—	0.00536	1.00
	2	2.08	—	0.00698	1.41
	4	2.97	—	0.01002	2.00
40°	1	3.08	—	0.01037	1.00
	2	4.40	—	0.01489	1.41
	7	8.09	—	0.02772	2.65
	14	10.40	—	0.03594	3.74
	28	13.04	—	0.04550	5.29
45°	1	4.79	—	0.01622	1.00
	2	7.27	—	0.02484	1.41
	7	15.46	—	0.05444	2.65
	14	20.48	—	0.07354	3.74
47.5°	1	5.48	0.02778		
	1.33	7.26	0.03699		
	2	9.64	0.0443		
	4	18.08	0.09446		
50°	0.33	3.48	0.01755		
	1	8.7	0.04448		
	1.33	11.55	0.05952		
	2	16.93	0.08857		

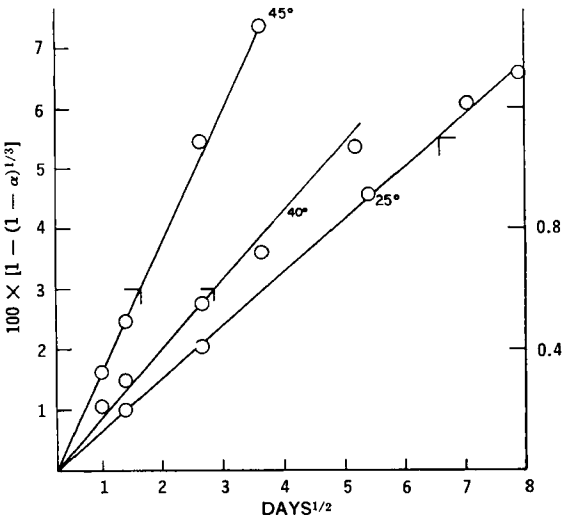

Figure 4—*Data from the remaining temperatures treated according to Eq. 10.*

E_a from Eq. 9 would be the parameters to use to get such correlations as "x days at 45° correspond to y days at 25°."

The explanation for the change in mechanism is fairly apparent. Let A = alkali, B = aspirin, and C = salicylic acid. Then $B \rightarrow C$ is catalyzed by A, so that:

$$-\frac{dB}{dt} = k_A[A] + k_B \qquad \text{(Eq. 12)}$$

where k_B is a spontaneous decomposition rate constant; $k_A \gg k_B$, but γ and k_B are such that at $T \geq 45°$ there is always A supplied to the interface between B and C, so that the first term in Eq. 12 is dominating. At $T > 47.5°$, k_B is sufficiently large so the interface proceeds more rapidly than the rate with which A can reach it. Hence, in the low temperature region the reaction is diffusion controlled, and in the higher temperature region it is kinetically controlled.

The considerations leading to Eqs. 3, 9, and 10 hold for a monodisperse powder. Carstensen and Musa (13) showed that if a cube root law (Eq. 10) holds for a monodisperse population, then it also holds for a log-normally distributed polydisperse population.

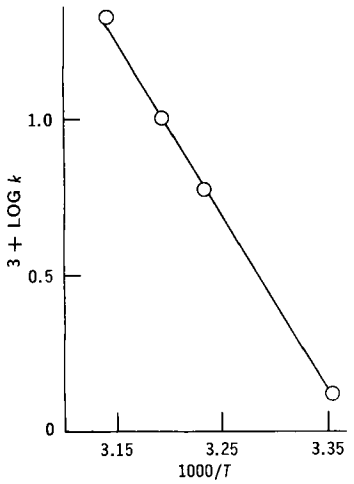

Figure 5—*Rate constants from Fig. 4 treated according to an Arrhenius equation.*

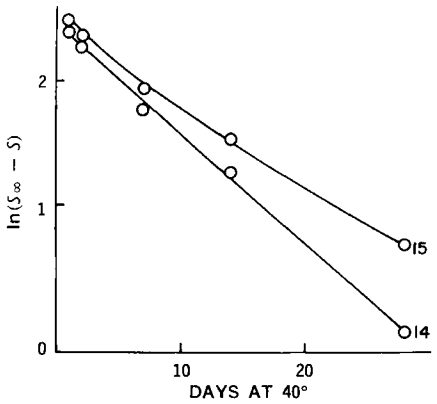

Figure 6—*Data for 40° treated by a pseudo-first-order scheme with equilibrium. Data seemingly linearize with an equilibrium level of 14 mole % salicylic acid. For limitations to this approach, see text.*

The value of slope k^*, however, would not be the same as k_0/r_0. The same type of consideration holds true for Eqs. 3 and 9 and k^* would be different from k/r_0 and k, respectively. This prohibits direct testing of the model *via* variation of r_0 (fineness of powder) in the actual situation where powders are polydisperse (as they are by necessity in tablet manufacture). Since the smallest particle decomposes completely before larger particles, it should be possible to see biphasic decompositions in cases such as these. However, in situations where the standard deviation of the particle-size distributions was not excessively large, these transitions would take place at times of high level of decomposition and were not observed in this study.

The effect of particle size on the decomposition was not a subject of systematic study in this investigation.

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[x] To whom inquiries should be directed. Present address: School of Pharmacy, University of Wisconsin, Madison, WI 53706

Sterilization of Selected Colorants by Cobalt-60

BER-LIN CHANG, WILLIAM G. HANEY, Jr., and NOEL O. NUESSLE[x]

Abstract □ A series of selected colorants composed of eight FD&C dyes, seven FD&C lakes, and four D&C dyes were subjected to 2.5 Mrads of γ-irradiation from a cobalt-60 source over approximately 10 hr. The soluble colorants so irradiated exhibited no changes when examined by visible, UV, and IR spectrophotometry, nor did they show any measurable radioactivity above that shown before irradiation. Color lakes were examined by IR spectroscopy, and the spectra of the dyes separated from the lakes in the visible and UV regions were obtained. These showed no changes. All colorants were examined by TLC using four different solvent systems, with no additional spots being obtained from the irradiated colorants. All colorants before irradiation were shown to contain microorganisms, and all were shown to be sterile after irradiation by a membrane filter technique or a direct plating method.

Keyphrases □ Colorants, certified dyes and lakes—sterilization by ^{60}Co, determination of possible irradiation-induced degradation □ Dyes, FD&C and D&C—sterilization by ^{60}Co □ Sterilization—FD&C dyes, FD&C lakes, and D&C dyes by γ-irradiation from ^{60}Co □ Irradiation—used to sterilize pharmaceutical colorants

The recent literature reflects a surge of interest in microbial contamination of cosmetics and nonsterile pharmaceuticals prompted by a concern for the safety of the product user. Numerous studies have appeared detailing microbial contamination of these products, both used and unused (1–8), as well as discussing the significance of the contaminating organism (1, 2, 9, 10). Legal aspects of the question were explored (11, 12), and limits on the acceptable (13) number and type of organisms were suggested. The types of microbial tests to be used on raw materials and finished products used topically or orally were outlined (14–17), and possible modifications of the USP microbial limits and tests were proposed previously (18).

Attention was focused on the preservatives used in these products (19–28). Testing methods and programs used to evaluate the effectiveness of preservatives were examined (29–32), as were interactions of the preservatives (33–35). Sources of contamination were indicated (36, 37) to be raw materials, equipment, environment, and personnel. Concern with microbial contamination of raw materials (38–41) led to a discussion of the use of ethylene oxide for sterilization of such materials (42, 43). Certified colorants were reported to show from 10 to 100% of samples contaminated with 10–1000 microorganisms/g (44). Controlling or eliminating troublesome microbes during the manufacturing and packaging operations has also received attention (45–51).

The use of ionizing radiation for killing microbes is widely recognized (52). An electron beam is currently used to sterilize sutures (53), and cobalt-60 (^{60}Co) has been shown to sterilize tubing, gloves (54), and various medical equipment (55) and to inactivate bacterial spores in penicillin (56). Many pharmaceuticals have been reported to be safely sterilized by a 2.5-Mrad dose of ^{60}Co (57). These include multivitamins, ascorbic acid, heparin, pregnenolone, cortisone acetate, potassium penicillin G, chlortetracycline, oxytetracycline, ergonovine maleate, and morphine sulfate. While the dose of ^{60}Co irradiation required for sterilization is still a subject of discussion,

Table I—Selected Water-Soluble Colorants

Colorant	Lot Number	Pure Dye Content, %
FD&C Blue No. 1[a]	Y8839	96
FD&C Blue No. 2[a]	Y7518	90
FD&C Green No. 3[a]	Y8944	89
FD&C Red No. 2[a]	X6945	90
FD&C Red No. 3[a]	X3699	92
FD&C Red No. 40[a]	Y9048	91
FD&C Yellow No. 5[a]	Y9021	91
FD&C Yellow No. 6[a]	Y8743	92
D&C Green No. 5[b]	X0110	85
D&C Yellow No. 10[b]	X0653	92

[a] Warner-Jenkinson Manufacturing Co., St. Louis, Mo. [b] H. Kohnstamm and Co., New York, N.Y.

Table II—Selected Water-Insoluble Colorants

Colorant	Lot Number	Pure Dye Content, %
FD&C Blue No. 1 Lake[a]	W8468	13
FD&C Blue No. 2 Lake[a]	W5126	12
FD&C Red No. 2 Lake[a]	W4868	24
FD&C Red No. 3 Lake[a]	W8904	16
FD&C Red No. 4 Lake[ab]	W6297	35
FD&C Yellow No. 5 Lake[a]	W8447	24
FD&C Yellow No. 6 Lake[a]	W9287	18
D&C Blue No. 6[c]	W9360	95
D&C Yellow No. 11[d]	—	—

[a] Warner-Jenkinson Manufacturing Co., St. Louis, Mo. [b] No longer certified. [c] H. Kohnstamm and Co., New York, N.Y. [d] American Cyanamid Co., New York, N.Y.

Table III—Sterility Test Results of Water-Soluble Colorants

Colorant	Irradiated	Nonirradiated
FD&C Blue No. 1	Sterile	Contaminated
FD&C Blue No. 2	Sterile	Contaminated
FD&C Green No. 3	Sterile	Contaminated
FD&C Red No. 2	Sterile	Contaminated
FD&C Red No. 3	Sterile	Contaminated
FD&C Red No. 40	Sterile	Contaminated
FD&C Yellow No. 5	Sterile	Contaminated
FD&C Yellow No. 6	Sterile	Contaminated
D&C Green No. 5	Sterile	Contaminated
D&C Yellow No. 10	Sterile	Contaminated

Table IV—Sterility Test Results of Water-Insoluble Colorants

Colorant	Filter Method		Direct Plating Method	
	Irradiated	Nonirradiated	Irradiated	Nonirradiated
FD&C Blue No. 1 Lake	Sterile	Contaminated	Sterile	Contaminated
FD&C Blue No. 2 Lake	Sterile	Sterile	Sterile	Contaminated
FD&C Red No. 2 Lake	Sterile	Sterile	Sterile	Contaminated
FD&C Red No. 3 Lake	Sterile	Sterile	Sterile	Contaminated
FD&C Red No. 4 Lake[a]	Sterile	Sterile	Sterile	Contaminated
FD&C Yellow No. 5 Lake	Sterile	Sterile	Sterile	Contaminated
FD&C Yellow No. 6 Lake	Sterile	Contaminated	Sterile	Contaminated
D&C Blue No. 6	Sterile	Contaminated	Sterile	Contaminated
D&C Yellow No. 11	Sterile	Contaminated	Sterile	Sterile

[a] No longer certified.

Table V—Dye Content of Irradiated Water-Soluble Colorants

Colorant	λ_{max}[a], nm	Concentration of Solution[b], mg/100 ml	Absorbance		Pure Dye, %	
			Non-irradiated	Irradiated	Non-irradiated[c]	Irradiated[d]
FD&C Blue No. 1	620	0.2	0.368	0.364	96	95.0
FD&C Blue No. 2	604	1	0.432	0.436	90	90.8
FD&C Green No. 3	618	0.2	0.348	0.349	89	89.3
FD&C Red No. 2	520	1	0.367	0.369	90	90.5
FD&C Red No. 3	524	0.4	0.456	0.452	92	91.2
FD&C Red No. 40	505	1	0.466	0.468	91	91.4
FD&C Yellow No. 5	428	1	0.459	0.458	91	90.8
FD&C Yellow No. 6	480	0.4	0.378	0.377	92	91.8
D&C Green No. 5	602	2	0.398	0.401	85	85.6
D&C Yellow No. 10	410	0.4	0.359	0.360	92	92.3

[a] Uncorrected. [b] Aqueous solution. [c] Labeled strength. [d] No significant difference compared to nonirradiated colorants.

it is generally agreed that 2.5 Mrads is sufficient (58).

Not all pharmaceuticals can be irradiated with no loss of potency. In aqueous solution, ascorbic acid, multivitamins, and heparin show a loss of activity. Insulin and atropine sulfate also suffer severe loss (57). Grosswiner and coworkers (59–61) found that fluorescein and eosin were subject to radiolysis in aqueous solution, and Kimura *et al.* (62) also found eosin to be subject to radiolysis in neutral and alkaline solutions. Prince and Welt (63) irradiated red D&C azo pigment (GBL-70-219) in the dry state and FD&C Red No. 3 in powder and in aqueous solution. They found that γ-irradiation up to 5 Mrads produced no change in the D&C pigment by several types of visual tests using trained observers. Solutions of FD&C Red No. 3 showed marked degradation of colorant at 0.2 Mrad; the same colorant, irradiated at a dose of 0.2–1.5 Mrads in the dry powder state, showed no change as judged by the absorbance of its aqueous solutions. The Food and Drug Administration has data suggesting the partial destruction of FD&C Red No. 3 and FD&C Yellow No. 5 Lake irradiated in the solid state at a 5-Mrad dose[1]. It was decided that in this experiment the selected colorants would be subjected to a 2.5-Mrad dose of γ-radiation from a ^{60}Co source, their microbial contamination would be examined, and changes in their pure dye content would be analyzed.

[1] J. T. Walters, Food and Drug Administration, personal communication, 1973.

EXPERIMENTAL

Materials—The colorants selected for γ-irradiation sterilization and their respective suppliers are given in Tables I and II. The lakes represent those dyes that have been adsorbed from solution onto alumina. Other materials used in this study were as follows: polyethylene vials, 4 dram with snap cap[2]; membrane filter, cellulose ester, 0.45-μm porosity, with hydrophobic edge[3]; Whatman No. 1 filter paper[4]; trypticase soy agar[5]; polysorbate 80[6]; pH 4 buffer solution[7]; TLC plates, silica gel, 0.25 mm thick[8]; and aseptic filtration apparatus[9].

Irradiation—Colorants were placed in polyethylene bottles previously cleaned ultrasonically. The vials were completely filled with colorant to eliminate excessive head space. Some of these vials were then placed into a cardboard mailing tube, 8.0 cm in diameter and 15.5 cm in height, and were subjected to γ-irradiation in this container. The irradiation facility[10] was so constructed that the ^{60}Co source was stored in a water well; when in use, the cobalt was raised toward or above the surface of the water to give the desired rate of irradiation. Since the maximum rate was desired, the cobalt source was raised until it surrounded the cardboard mailing tube. Three batches of colorants were irradiated, two at a dose rate of 2.53×10^5 rads/hr and one at 2.70×10^5 rads/hr. Approximately 10 hr was required to obtain a total applied dose of 2.5 Mrads for each batch.

Sterility Testing—For sterility testing, colorants were separated according to their water solubility. Those regarded as water soluble appear in Table I. A 300-mg portion of each of these colorants was aseptically obtained and dissolved in 30 ml sterile saline solution (0.9% w/v). This solution was vacuum filtered through a sterile, 0.45-μm porosity membrane filter in a sterile fil-

[2] Polyvials, Olympic Plastics, Los Angeles, Calif.
[3] Millipore filter, HAEG047A0, Millipore Corp., Bedford, Mass.
[4] W. and R. Balston, Ltd., England.
[5] Baltimore Biological Laboratory, Baltimore, Md.
[6] Tween 20, ICI America, Inc., Wilmington, Del.
[7] Catalog No. 29905-04, Matheson Scientific, Chicago, Ill.
[8] E. Merck Ag., Darmstadt, Germany.
[9] Sterifil, Millipore Corp., Bedford, Mass.
[10] Research Reactor Facility, University of Missouri, Columbia, Mo.

Table VI—Dye Content of Irradiated Water-Insoluble Colorants

Colorant	λ_{max}[a], nm	Concentration of Solution, mg/100 ml	Absorbance		Pure Dye, %	
			Nonirradiated	Irradiated	Non-irradiated[b]	Irradiated[c]
FD&C Blue No. 1 Lake	624	2	0.314	0.314	13	13.0
FD&C Blue No. 2 Lake	608	5	0.261	0.263	12	12.1
FD&C Red No. 2 Lake	520	2.5	0.317	0.319	24	24.2
FD&C Red No. 3 Lake	485	15[e]	0.381	0.381	16	16.0
FD&C Red No. 4 Lake[d]	500	2	0.383	0.382	35	34.9
FD&C Yellow No. 5 Lake	430	2.5	0.305	0.306	24	24.1
FD&C Yellow No. 6 Lake	480	2.5	0.314	0.318	18	18.2
D&C Blue No. 6	600	5	0.290	0.290	95	95.0
D&C Yellow No. 11	410	0.2[f]	0.267	0.268	—[g]	

[a] Uncorrected. [b] Labeled strength. [c] No significant difference compared to nonirradiated colorants. [d] No longer certified. [e] Ethanol solution. [f] Methanol solution. [g] Not labeled.

Table VII—R_f Values of Components of Water-Soluble Colorants

Colorant	Solvent	Visualization Method		
		Incandescent Light	UV Light	Iodine
FD&C Blue No. 1	A	0.627[a], 0.0	0.627[a], 0.0	0.627[a], 0.0
	B	1.00, 0.600[a]	0.733, 0.600[a]	1.00, 0.733, 0.600[a]
	C	0.379[a], 0.0345, 0.0	0.379[a], 0.0345, 0.0	0.379[a], 0.0345, 0.0
	D	0.128, 0.0567[a], 0.0	0.582, 0.163, 0.128	0.582, 0.163, 0.128
			0.0567[a], 0.0	0.0567[a], 0.0
FD&C Blue No. 2	A	0.797[a], 0.641, 0.0	0.797[a], 0.641, 0.0	0.797[a], 0.641, 0.0
	B	0.0[a]	0.715, 0.0[a]	0.715, 0.0[a]
	C	0.886[a], 0.725, 0.0	0.886[a], 0.725	0.886[a], 0.725, 0.0
	D	0.0[a]	0.324, 0.0[a]	0.324, 0.0[a]
FD&C Red No. 2	A	0.519[a], 0.356	0.519[a], 0.356	0.519[a], 0.356, 0.0741
	B	0.654[a], 0.615	0.654[a]	0.654[a], 0.615
	C	0.174[a]	0.174[a], 0.0	0.174[a], 0.0
	D	0.585[a], 0.363	0.585[a], 0.363	0.585[a], 0.363, 0.0
FD&C Red No. 3	A	0.414[a], 0.223	0.414[a], 0.223	0.414[a], 0.223, 0.0
	B	0.267[a]	0.267[a]	0.345, 0.267[a]
	C	0.388[a], 0.102	0.388[a], 0.133, 0.102	0.388[a], 0.133, 0.102
	D	0.0863[a]	0.0863[a]	0.130, 0.0863[a]
FD&C Red No. 40	A	0.855[a]	0.855[a]	0.855[a], 0.0
	B	0.672[a]	0.672[a]	0.672[a], 0.0
	C	0.419[a]	0.419[a]	0.419[a], 0.0
	D	0.0550[a]	0.587, 0.0734, 0.0550[a]	0.587, 0.0734, 0.0550[a]
FD&C Green No. 3	A	0.831[a]	0.831[a]	0.831[a]
	B	0.833[a]	0.833[a]	0.833[a]
	C	0.0150[a]	0.0150[a]	0.1203, 0.0150[a]
	D	0.527[a]	0.527[a]	0.527[a], 0.0388
FD&C Yellow No. 5	A	0.135[a]	0.460, 0.135[a]	0.460, 0.294, 0.135[a]
	B	0.0678[a]	0.0678[a]	0.153, 0.0678[a]
	C	0.687[a]	0.687[a], 0.084	0.687[a], 0.084
	D	0.783[a]	0.783[a]	0.783[a], 0.444, 0.0
FD&C Yellow No. 6	A	0.258[a]	0.356, 0.258[a]	0.455, 0.356, 0.258[a], 0.0
	B	0.860[a]	0.860[a], 0.174	0.860[a], 0.174
	C	0.712[a]	0.712[a]	0.712[a], 0.0
	D	0.424[a]	0.743, 0.424[a]	0.743, 0.424[a], 0.368, 0.0
D&C Green No. 5	A	0.630[a]	0.630[a], 0.243	0.630[a], 0.243
	B	0.861[a]	0.861[a]	0.861[a], 0.0
	C	0.642[a], 0.585	0.642[a], 0.585	0.642[a], 0.585, 0.100
	D	0.217[a], 0.0	0.269, 0.217[a], 0.0	0.269, 0.217,[a] 0.0
D&C Yellow No. 10	A	0.835[a], 0.671	0.835[a], 0.671	0.835[a], 0.671
	B	0.862[a]	0.862[a], 0.750, 0.0	0.862[a], 0.750, 0.658, 0.0
	C	0.792[a], 0.436, 0.0	0.792[a], 0.436, 0.0	0.792[a], 0.436, 0.0
	D	0.659[a], 0.0	0.659[a], 0.0	0.659[a], 0.477, 0.0

[a] Represents the major component.

tration apparatus. The filter was transferred to a petri dish containing trypticase soy agar and incubated at 28–32° for up to 14 days. The plates were read every 48 hr.

The water-insoluble colorants (Table II) were tested by two methods, one of which was a filtration method. A 300-mg portion of each water-insoluble colorant was aseptically weighed and transferred to 30 ml sterile saline solution (0.9% w/v). This was vacuum filtered through a sterile Whatman No. 1 filter paper in a sterile filtration apparatus, and the insoluble material and the filter were washed with 100 ml of sterile saline solution. The filtrate thus obtained was then filtered through a sterile membrane filter in a second sterile filtration apparatus. The receiver of the first apparatus and the membrane and funnel of the second were rinsed with an additional 100 ml of sterile saline solution. The membrane filter was transferred to a petri dish containing trypticase soy agar and incubated at 28–32° for up to 14 days. The plates were read every 48 hr.

The second method used for water-insoluble colorants was a direct plating method. A 1.8-g sample of the colorant was aseptically weighed and transferred to 30 ml of sterile saline solution.

Table VIII—R_f Values of Components of Water-Insoluble Colorants

Colorant	Solvent	Visualization Method		
		Incandescent Light	UV Light	Iodine
FD&C Blue No. 1 Lake[a]	A	0.546[c]	0.546[c], 0.0987	0.546[c], 0.0987, 0.0
	B	0.517[c]	0.517[c], 0.0464, 0.0	0.702, 0.517[c], 0.0464, 0.0
	C	0.603[c]	0.603[c]	0.851, 0.603[c], 0.0
	D	0.104[c]	0.104[c], 0.0	0.104[c], 0.0
FD&C Blue No. 2 Lake[a]	A	0.231[c]	0.884, 0.231[c], 0.0	0.884, 0.231[c], 0.0
	B	0.459[c]	0.753, 0.459[c], 0.178	0.753, 0.459[c], 0.178, 0.0
	C	0.0[c]	0.707, 0.188, 0.0[c]	0.707, 0.188, 0.0[c]
	D	0.0[c]	0.694, 0.167, 0.0[c]	0.694, 0.167, 0.0[c]
FD&C Red No. 2 Lake[a]	A	0.0816[c]	0.565, 0.0816[c]	0.565, 0.170, 0.0816[c]
	B	0.455[c]	0.455[c], 0.177, 0.0	0.600, 0.455[c], 0.117, 0.0
	C	0.157[c]	0.607, 0.157[c], 0.0857	0.607, 0.157[c], 0.0857
	D	0.129[c]	0.129[c]	0.457, 0.200, 0.129[c], 0.0
FD&C Red No. 3 Lake[a]	A	0.468[c]	0.468[c], 0.0851, 0.0	0.468[c], 0.0851, 0.0
	B	0.299[c]	0.299[c], 0.104, 0.0	0.667, 0.299[c], 0.104, 0.0
	C	0.451[c]	0.451[c], 0.120, 0.0	0.451[c], 0.120, 0.0
	D	0.551[c]	0.551[c], 0.118	0.551[c], 0.118, 0.0
FD&C Red No. 4 Lake[a,b]	A	0.409[c]	0.409[c], 0.0657	0.577, 0.409[c], 0.0657, 0.0
	B	0.452[c]	0.452[c], 0.167, 0.0476	0.452[c], 0.167, 0.0476
	C	0.541[c]	0.541[c], 0.0764, 0.0	0.541[c], 0.0764, 0.0
	D	0.0709[c]	0.0709[c]	0.688, 0.0709[c], 0.0
FD&C Yellow No. 5 Lake[a]	A	0.270[c]	0.688, 0.468, 0.270[c]	0.688, 0.468, 0.270[c], 0.0
	B	0.659[c]	0.659[c], 0.0741, 0.0	0.659[c], 0.0741, 0.0
	C	0.462[c]	0.462[c], 0.0690, 0.0	0.462[c], 0.0690, 0.0
	D	0.0984[c]	0.0984[c], 0.0	0.0984[c], 0.0
FD&C Yellow No. 6 Lake[a]	A	0.512[c]	0.512[c], 0.0	0.512[c], 0.0930, 0.0
	B	0.604[c]	0.604[c], 0.0719, 0.0	0.604[c], 0.0719, 0.0
	C	0.441[c]	0.441[c]	0.441[c], 0.0882
	D	0.0876[c]	0.0876[c], 0.0	0.0876[c], 0.0
D&C Blue No. 6	A	0.0[c]	0.115, 0.0[c]	0.162, 0.115, 0.0[c]
	B	0.0[c]	0.0[c]	0.192, 0.0923, 0.0[c]
	C	0.0[c]	0.116, 0.0[c]	0.116, 0.0[c]
	D	0.0[c]	0.125, 0.0[c]	0.125, 0.0[c]
D&C Yellow No. 11	A	0.694[c], 0.0750	0.694[c]	0.694[c], 0.0750
	B	0.724[c], 0.0414	0.724[c]	0.724[c], 0.0414
	C	0.608[c]	0.608[c], 0.0	0.608[c], 0.0
	D	0.587[c], 0.247	0.587[c], 0.247	0.587[c], 0.247

[a] The dye separated from the lake. [b] No longer certified. [c] Major component.

The tube was shaken to suspend the colorant, and 1- and 5-ml portions of the suspension were placed on trypticase soy agar plates. For the D&C colorants, this procedure was modified by the use of a solution of polysorbate 80 in water (1% w/v) in place of the sterile saline to obtain a more uniform suspension. The plates were incubated at 28–32° and read after 48 hr. If the result was sterile or questionable at the end of the 48-hr period, transfers from both the 1- and 5-ml plates were made by streaking a 1-mm loop across the surface of the original and then across a fresh trypticase soy agar plate. This procedure was repeated five times for each plate, and the loop was flamed between each streak to reduce accidental contamination. The transfer plates and the original plates were incubated at 28–32°, and the plates were read every 48 hr up to 14 days. All manipulations for sterility testing were performed within a laminar flow hood[11].

Spectra—Visible and UV spectra of the colorants were obtained on a spectrophotometer[12] equipped with a linear-log recorder[13]. IR spectra were obtained[14] by a potassium bromide pellet technique. The visible and UV spectra of the dyes separated from the lakes were obtained after the dyes had been removed from the aluminum oxide by sulfuric acid and alcohol (64). Further dilutions were made with water. The same method was used to solubilize D&C Blue No. 6.

TLC—The dyes were spotted on silica gel plates in quantities of 50, 10, and 1 μg. Four different solvent systems were used for development in an ascending method: A, 0.1 *N* hydrochloric acid solution and methanol (1:50); B, 10% (w/v) sodium hydroxide solution and methanol (1:50); C, pH 4 buffer solution and 95% ethanol (1:20); and D, phosphate buffer solution USP, pH 7, and isopropanol (1:10).

Components of the dyes were examined visually, under UV light, after visualization with iodine vapors and ammonium hydroxide solution (5% w/v). Dyes separated from the lakes were spotted from the sulfuric acid–ethanol solution in quantities representing 50, 20, and 2 μg of the lake. Further treatment was the same as for the other dyes.

Residual Radiation—Radiation measurements were collected using a scaler and an ultrathin window tube[15]. The coloring material (0.50 g of the dyes or 2.5 g of the lakes) was placed on a Plexiglas holder 10 mm from the tube window. The counting period was 15 min, and each dye was counted three times before and after irradiation. Samples were also scanned before and after irradiation using a single-channel analyzer[16].

RESULTS AND DISCUSSION

Sterility Tests—It is obvious that the test result "sterile" does not refer to viruses, nor has any special medium or enrichment been employed to produce conditions favorable to growth of certain species. All test results (Tables III and IV) are based on a sample size of 300 mg, as recommended by USP XVIII (65). While results with water-soluble colorants are straightforward, conflicting results obtained for the nonirradiated water-insoluble colorants require some discussion. Before the filtration method was used to test the colorants, *Aspergillus niger* and *Bacillus cereus* were added to portions of the original colorant suspension. It was proved that the microbes could be separated from the insolu-

[11] Primaire, Dexon, Inc., Minneapolis, Minn.
[12] Beckman model DB.
[13] Beckman Industries, Inc., Fullerton, Calif.
[14] Perkin-Elmer model 457 IR spectrophotometer, Norwalk, Conn.
[15] Nuclear-Chicago model 1620A, Nuclear-Chicago, Chicago, Ill.
[16] Nuclear-Chicago model 1810.

ble color by Whatman No. 1 filter paper and washed through to be collected on the membrane filter. Apparently, however, this finding is related to the number of microorganisms present, as evidenced by comparison with test results of the direct plating method.

With the direct plating method, it was usually difficult to read the results; thus, transfer of some original material to fresh plates was made after 48 hr. To reduce errors caused by accidental contamination during the manipulations, transfer plates of the nonirradiated colorants were regarded as having positive growth only if at least four of the five streaks on the plate showed growth. The apparent antimicrobial effect of D&C Yellow No. 11 prevents it from being tested by the direct plating method.

IR Spectra—Examination of the IR spectrum from 250 to 1750 cm^{-1} of each colorant showed no new peaks appearing as a result of γ-irradiation.

Visible and UV Spectra—Examination of the spectrum from 200 to 800 nm of each colorant before and after ^{60}Co radiation showed no discernible differences. Tables V and VI list the wavelengths of the major absorption peaks of the colorants in the visible range and the change in percent of pure dye. The percent pure dye of the irradiated colorants was calculated at the λ_{max} according to the following equation (66):

$$\text{pure dye} = \frac{A_{\text{sample/conc. sample}}}{A_{\text{standard/conc. standard}}} \times \%\ \text{purity of standard} \quad \text{(Eq. 1)}$$

Each absorbance represents the mean of five determinations. The absorbance values were subjected to the t test, which showed no statistically significant differences between the irradiated and nonirradiated colorants.

TLC—The R_f values of the components of the colorants are shown in Tables VII and VIII. There was no discernible difference in the chromatograms of irradiated and nonirradiated colors. The visualization by means of ammonium hydroxide solution is omitted from these tables since it did not visualize any more spots than did iodine vapor.

Residual Radiation—In no case was the radioactivity of the colorant increased by the ^{60}Co irradiation process.

SUMMARY AND CONCLUSION

Selected colorants, 10 water soluble and nine water insoluble including seven lakes, were irradiated by ^{60}Co to a total dose of 2.5 Mrads and the following conclusions were reached:

1. Colorants can be sterilized by 2.5 Mrads of γ-irradiation from ^{60}Co.
2. There is no increase in residual radioactivity.
3. Examination of IR, visible, and UV spectra showed no physical-chemical changes. Therefore, these materials may be regarded as being colorfast to γ-irradiation under these conditions.
4. TLC in four solvent systems showed no difference between irradiated and nonirradiated colorants.

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PHARMACEUTICAL ANALYSIS

Analysis of Sodium Levothyroxine or Sodium Liothyronine in Tablets

JOSEPH H. GRAHAM[x], DANIEL BANES*, M. E. BIESEMEYER, and ASHOK NADKARNI‡

Abstract □ A new procedure for the analysis of sodium levothyroxine tablets and sodium liothyronine tablets is proposed. The active ingredient is isolated by partition chromatography. The thyronine is degraded by bromine oxidation, directly converting its iodine content to iodate, which is subsequently reduced by excess iodide and determined spectrophotometrically as the triiodide ion. The method is extremely sensitive and may be applied conveniently to dosage levels as low as 5 μg of sodium liothyronine. A suitable procedure for individual tablet analysis is also presented.

Keyphrases □ Sodium levothyroxine or sodium liothyronine tablets—analysis, partition chromatography and triiodide spectrophotometry □ Levothyroxine or liothyronine tablets—analysis, partition chromatography and triiodide spectrophotometry □ Liothyronine or levothyroxine tablets—analysis, partition chromatography and triiodide spectrophotometry

The USP XVIII (1) monographs for sodium levothyroxine, sodium levothyroxine tablets, sodium liothyronine, and sodium liothyronine tablets incorporate assays that require an alkali fusion of the drugs and subsequent conversion of the alkali iodide thus produced to elemental iodine. The latter is determined titrimetrically with sodium thiosulfate.

This analytical scheme is satisfactory for the drug substances themselves, but difficulties arise when it is applied to the dosage form, particularly those containing less than 100 μg of drug. The assays stipulate that a portion of powdered tablet composite equivalent to 3 mg of sodium levothyroxine or 1 mg of sodium liothyronine be ignited; these amounts theoretically yield 0.09 and 0.03 mEq of iodine, respectively. When these quantities of iodine are titrated with 0.01 *N* sodium thiosulfate, the true end-point is extremely difficult to judge and thus the accuracy of the analysis is doubtful. Furthermore, the great mass of the charge that must be ignited may itself lead to serious errors. The analysis of tablets containing 5 μg of sodium liothyronine would require 200 tablets and about 33 g of potassium carbonate as the fusion mixture. The mass of carbonized material often generated in such an ignition interferes with the quantitative recovery of inorganic iodides produced in the fusion. These difficulties strongly emphasize the need for a more accurate and sensitive assay procedure for tablets of both sodium levothyroxine and sodium liothyronine.

In an investigation of possible new methods, particularly for the assay of sodium levothyroxine, an attempt was made to utilize the reaction between ceric ion and arsenious acid under the powerful catalysis of submicrogram quantities of thyroxine. However, this reaction is subject to so many variables that it was abandoned as an analytical approach. The isolation of thyroxine from tablets by forming an extractable ion-pair with bis(2-ethylhexyl) hydrogen phosphate gave a final sample preparation that could be analyzed by UV spectrophotometry, but the procedure lacked the necessary sensitivity. A colorimetric method utilizing the chromophore produced by oxidizing the product of the reaction between thyroxine and 3-methyl-2-benzothiazolinone hydrazone hydrochloride was successful in determining as little as 80 μg of thyroxine. This procedure also afforded a degree of specificity in that the related compounds, diiodothyronine and diiodotyrosine, did not undergo the reaction and liothyronine yielded a color about one-fifth as intense as that obtained from thyroxine under the same conditions. Unfortunately, erratic results frequently occurred, probably because of variations in tablet excipients, traces of

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organic solvents, and the pH of the reaction medium. Although this approach lacked the ruggedness desired for a standard assay procedure for sodium levothyroxine tablets, it may prove to be useful as an auxiliary test in examining thyroxine specimens directly.

In contrast, the reactions that form the basis for the official assay are rugged, reliable, and well understood. The reactions in the sequence leading to the production of elemental iodine from organically bound iodine are stoichiometric and can be conducted with ease. Therefore, the sensitivity ranges of these reactions were investigated when applied in conveniently performed quantitation procedures adaptable to the problem.

In an earlier report from this laboratory, Moody *et al.* (2) utilized the absorption of iodine in benzene solution at 295 nm for spectrophotometric determination of the free element. Custer and Natelson (3) had shown earlier that the triiodide ion in potassium iodide solution absorbed UV and visible light even more intensely than the iodine–benzene complex. It was determined that microgram quantities of thyroxine and liothyronine can be degraded to iodate by treatment with boiling, dilute acetic acid–sodium acetate–bromine solution. The iodate thus produced can be stoichiometrically converted to the triiodide ion in acetate buffer with excess potassium iodide.

These observations form the foundation for the method described here for the determination of either sodium levothyroxine or sodium liothyronine when each compound appears as the sole iodine-containing substance in tablets.

EXPERIMENTAL

Tablet Composite Assay

Reagents—*Standard Potassium Iodate*—Dilute 0.05 *M* potassium iodate (4) stepwise to 1 in 2500 to obtain a solution equivalent to 2.538 μg I_2/ml.

Bromine-Sodium Acetate Test Solution—Prepare as directed in USP XVIII (5).

Column Eluant—Dilute a solution of 30 ml of acetic acid and 200 ml of 2-propanol to 1 liter with chloroform.

In addition, the following reagents are also required: acetate buffer, pH 3.9 (1.0 *M* sodium acetate in 5.5 *M* acetic acid); potassium iodide, 15% (w/v) aqueous (freshly prepared); aqueous 0.1 *M* sodium hydroxide; methanolic 0.1 *M* sodium hydroxide; 5 *M* acetic acid (29.8 ml of acetic acid/100 ml); silicon carbide boiling chips; and chromatographic siliceous earth.

Sample Preparation—Thoroughly wet, with warming on a steam bath, an amount of powdered tablet composite (not exceeding 1.1 g) equivalent to 25–500 μg of sodium liothyronine or sodium levothyroxine with 4 ml of 5 *M* acetic acid in a 150-ml beaker. Mix thoroughly with 6 g of chromatographic siliceous earth and transfer quantitatively to a glass chromatographic column containing a glass wool plug. Compress the mass moderately tight. Dry rinse the beaker with 1–2 g of chromatographic siliceous earth, transfer it to the column, and hold the mass in place with glass wool. Elute the column into a 125-ml conical flask with 120 ml of column eluant, using a portion of it to rinse the sample beaker. Evaporate the eluate to dryness with an air jet on a steam bath. Rinse the walls of the flask with 5 ml of methanolic sodium hydroxide and reevaporate to dryness. Add 15 ml of aqueous sodium hydroxide to the residue and warm to effect complete solution. Add a few coarse silicon carbide boiling chips, chill the solution in an ice bath, and swirl vigorously to disperse finely any gelatinous precipitate. Quantitatively transfer the supernate through a filter into a 50-ml volumetric flask, completing the transfer with cool water. Equilibrate to room temperature and dilute to volume with water.

Oxidation—Pipet an aliquot of the sample preparation equivalent to 25–50 μg of sodium levothyroxine or sodium liothyronine to a 125-ml conical flask. Add 1 ml of bromine–sodium acetate test solution, water to a total of 40–50 ml, and silicon carbide boiling chips. Boil the solution, avoiding spattering, until its volume is reduced to about 10 ml. Cool the solution to room temperature and transfer quantitatively (through a small filter if the solution is hazy) to a 25-ml volumetric flask with 10 ml of acetate buffer, completing the transfer with water. Do not dilute to volume; leave space for the addition of 1 ml of potassium iodide solution.

Potassium Iodide–Potassium Iodate Color Development—Into separate 25-ml volumetric flasks containing 10 ml of acetate buffer, pipet a 10-ml aliquot of the standard preparation and 10 ml of water to serve as a blank. Add 1 ml of 15% potassium iodide to each flask (sample, standard, and blank), dilute to volume with water, and mix well. Record the spectrum of each solution relative to the blank in 1-cm cells from 460 to 340 nm. Correct the absorbance at about 352 nm by subtracting the absorbance at 450 nm. Calculate the quantity of anhydrous sodium levothyroxine or sodium liothyronine in micrograms per tablet by Eq. 1:

$$(A_u/A_{std}) \times C \times V_{std} \times (50/Q) \times (\text{average weight/weight}) \times GF \quad \text{(Eq. 1)}$$

where A_u is the corrected absorbance of the sample at about 352 nm, A_{std} is the corrected absorbance of the standard at about 352 nm, C is the equivalent iodine (I_2) concentration of standard potassium iodate (KIO_3) (micrograms per milliliter), V_{std} is the volume of the standard aliquot, average weight is the average weight per tablet (grams), GF is the gravimetric factor (1.574 for sodium levothyroxine and 1.768 for sodium liothyronine), Q is the aliquot volume of sample, and weight is the weight of sample (grams).

Shake-Out Procedure for Individual Tablet Assay

Reagents—In addition to the reagents described under *Tablet Composite Assay*, sodium sulfate, 10% (w/v) aqueous solution, is required.

Sample Preparation—Disperse the tablet in a 125-ml separator containing 10 ml of 5 *M* acetic acid. Extract vigorously for about 1 min with 20 ml of column eluant. Quantitatively transfer the lower phase to a second separator containing 30 ml of sodium sulfate solution. (Add a few milliliters of column eluant to the separator and drain it into the second separator.) Rinse the stem of the separator with water. Shake the second separator vigorously for about 30 sec, allow the phases to separate completely, and quantitatively transfer to a 50-ml volumetric flask[1]. Rinse the stem with column eluant. Repeat these serial extractions and transfers, using a second 20-ml portion of column eluant. Add a few drops of column eluant to the second separator and draw off into the flask. Rinse the stem, dilute the solution to volume with the column eluant, and mix well.

Oxidation—Pipet an aliquot of the sample preparation equivalent to 20–50 μg of sodium levothyroxine or sodium liothyronine into a 125-ml conical flask. Evaporate to dryness on a steam bath with the aid of a current of air. Rinse the walls of the flask with 5 ml of methanolic sodium hydroxide and evaporate to dryness. Add 10 ml of aqueous sodium hydroxide and warm to effect complete solution. Chill the solution in an ice bath. If a precipitate appears, filter quantitatively into another 125-ml flask, using water to complete the transfer. Add 2 ml of bromine–sodium acetate test solution, silicon carbide boiling chips, and sufficient water to make about 50 ml. Continue as directed under "Oxidation" in the *Tablet Composite Assay* procedure, beginning with "Boil the solution . . ." to the conclusion of the procedure.

Special Procedure for 5-μg Tablets: Potassium Iodide–Potassium Iodate Color Development

Proceed as directed in the *Tablet Composite Assay* procedure, but use a 1.0-ml aliquot of the standard preparation and record the spectrum using 5-cm cells. Calculate the quantity of sodium levothyroxine or sodium liothyronine in micrograms per tablet by Eq. 2:

[1] In the case of tablets containing 50 μg or less, transfer the extract to a 125-ml conical flask and use the entire extract for the determination.

Table I—Assays of Sodium Levothyroxine Tablets by Proposed Procedures

Sample	Manufacturer	Declared, μg/Tablet	Tablet Composite Assay, Column Procedure		Individual Tablet Assay, Shake-Out Procedure	
			Found, % of Declared[a]	Average, % of Declared	Found, % of Declared	Average, % of Declared
1	A	25	81.4, 81.1	81.3	—	—
2	A	25	83.4, 84.4, 84.3	84.0	84.7, 80.7, 83.3, 82.1, 80.5	82.3
3	A	100	91.5, 93.3	92.4	91.3, 90.9, 89.0, 89.8	90.3
4	A	100	91.8, 91.1	91.5	—	—
5	A	150	92.6, 94.4	93.5	94.5, 91.2, 98.1, 96.6	95.1
6	A	200	95.7, 97.5	96.6	—	—
7	A	500	91.3, 93.2	92.3	91.7, 95.6, 92.8, 93.0, 96.7	94.0
8	B	25	97.4, 99.6	98.5	97.1, 97.6, 94.8, 96.8	96.6
9	B	100	94.0, 91.0	92.5	97.3, 98.1, 101.7, 104.0	100.3
10	B	300	94.5, 96.7	95.6	106.3, 100.6, 101.7	102.9
11	B	500	101.2, 101.4	101.3	—	—
12	C	100	91.3, 91.2	91.3	—	—
13	C	200	79.3, 79.1	79.2	—	—
14	C	200	90.6, 89.4	90.0	—	—
15	C	200	87.6, 89.6	88.6	—	—
16	D	200	98.7, 103.1, 102.3, 103.2, 100.7	101.6	113.7, 116.8, 114.4, 111.3, 116.1	114.5

[a] Each entry represents a complete, separate assay.

Table II—Assays of Sodium Liothyronine Tablets by Proposed Procedures

Sample	Declared, μg/Tablet	Tablet Composite Assay, Column Procedure		Individual Tablet Assay, Shake-Out Procedure	
		Found, % of Declared[a]	Average, % of Declared	Found, % of Declared	Average, % of Declared
1	5	99.8, 96.2, 102.4	99.5	—	—
2	5	99.7, 99.9	99.8	101.2, 93.0, 116.0, 100.0, 96.0, 98.6, 116.4	103.0
3	25	105.3, 99.9, 98.0	101.1	—	—
4	25	91.0, 93.1	92.1	115.6, 95.2, 126.4 91.2	107.1
5	50	99.9, 97.1	98.5	93.2, 91.2, 93.2	92.5

[a] Each entry represents a complete, separate assay.

$$(A_u/A_{std}) \times C \times V_{std} \times (50/Q) \times GF \qquad \text{(Eq. 2)}$$

The factors have the same function ascribed in Eq. 1.

RESULTS AND DISCUSSION

Table I gives the assay results for sodium levothyroxine tablet composites and individual tablets, as obtained by the column extraction technique and the shake-out procedure, respectively, followed by bromine oxidation. Results of the assay of tablet composites indicated an overall precision of ±2.1%.

Results of the assays on various dosage levels of a single brand of sodium liothyronine tablets are given in Table II. The procedures were the same as those applied to the assay of sodium levothyroxine tablets except that the appropriate gravimetric factor, 1.768, was used in the calculations. The sodium liothyronine tablets had considerably more excipient material than the sodium levothyroxine samples analyzed; this material appeared as a gelatinous substance and was somewhat difficult to rinse thoroughly after the filtration step. The overall precision of the assays of composite tablets was ±1.7%.

During the development of the proposed method, several approaches to the extraction of thyroxine from tablets were studied to investigate the convenience of application and the effect on the iodine determination of tablet excipients entering the extract. Experiments with pure thyroxine showed that iodine can be quantitatively recovered after a 30-min fusion with alkali at 450–500° prior to oxidation with bromine and that fusion above 600° resulted in losses of iodine. Extracts of thyroxine from tablets that were essentially filtrates of their aqueous suspensions contained large amounts of carbonizable excipient material and were frequently difficult to handle, since they were incompletely carbonized at 450–500°. Butanol extracts of acidified aqueous suspensions of powdered tablets in separators are somewhat tedious to prepare; they require several washes and transfers and are prone to form emulsions. However, they do yield residues of thyroxine that are sufficiently free of interfering excipient material to be amenable to the low temperature fusion. The mechanical deficiencies of the shake-out technique are largely eliminated by the proposed column procedure (although filtration of a gelatinous precipitate is necessary), and the final assay preparation is free of interfering substances. The fact that boiling thyroxine or liothyronine in a dilute acetic acid–sodium acetate–bromine solution quantitatively yields iodate was the basis for replacement of the fusion technique.

The linearity of the spectrophotometric determination was established using a series of aliquots of a standard potassium iodate solution equivalent to 2.565 μg I_2/ml. A computer-calculated least-squares treatment of the eight data points gave the following: *(a)* linear range from 0.0 to at least 1.5 μg I_2/ml, *(b)* intercept −0.009, *(c)* slope 0.567, *(d)* standard error 0.003, and *(e)* correlation 0.999. The absorbance of the triiodide ion generated from an aliquot of the standard diluted to an equivalence of 1.026 μg I_2/ml was 0.573 at 352 nm. It was necessary, for the purpose of

this study, to buffer the iodate–iodide reaction in the 3.4–4.0 pH range; in this range the reduction of iodate is almost instantaneous and no oxidation of the excess iodide by atmospheric oxygen occurs.

The ratio, thyroxine/$2I_2$, was determined on a series of six aliquots of a standard thyroxine solution ranging from 50 to 200 μg with the proposed oxidation and spectrophotometric procedures. The average (1.5131) of the determinations was 98.7% of the theoretical value (1.5304), and the standard deviation of the series was 0.0074.

Sixteen sodium levothyroxine tablet composites were extracted with butanol in separators, and aliquots of the extracts were analyzed by the low temperature ignition and the bromine oxidation procedures. The data obtained established that the two procedures yield concordant results; the greatest discrepancy between the two procedures (3.7%) was of the same order of magnitude as the greatest discrepancy (4.0%) within either individual procedure. On this basis, the bromine oxidation procedure was concluded to be as reliable as the ignition procedure and it is more convenient and simpler.

The scheme presented here for individual tablet analyses of sodium levothyroxine or sodium liothyronine tablets is an outgrowth of the more elaborate butanol shake-out procedure developed earlier in this investigation. By substituting the chloroform–2-propanol–acetic acid system for butanol and eliminating several washes, the procedure was shortened considerably and in no case did an emulsion form even after prolonged vigorous extractions in separators. However, because of attempts to keep the procedure as short as possible, difficulties were encountered during development of the individual tablet assay. The averages of the individual tablet analyses compared satisfactorily with those obtained by using the column assay of composites and weighed amounts of these composites equivalent to single tablets for all dosage levels of sodium levothyroxine tablets from Manufacturer A, the one brand of sodium liothyronine assayed, and, fortuitously, the two lots of sodium levothyroxine tablets from Manufacturer B that were initially assayed. The one dosage level of sodium levothyroxine tablets from Manufacturer D gave a substantial discrepancy between the averaged individual tablet analyses and the column assay of the composite sample. When additional dosage levels and lots from Manufacturer B were studied, more discrepancies were noted. Several attempts to remedy the situation were made without success until a sodium hydroxide treatment of an aliquot of the extract was included, as in the column assay. Since all of the samples analyzed produced some quantity of a gelatinous precipitate after sodium hydroxide treatment in the column assay, the discrepancies may have arisen as the effect of another excipient which is denatured by the sodium hydroxide treatment. In only two cases was there sufficient precipitate from the aliquot taken to warrant filtration in the individual tablet analyses.

This procedure, coupled with the extraction and spectrophotometric techniques, provides a very convenient method feasible for the determination of sodium levothyroxine or sodium liothyronine in individual tablets or composites in the range of 5–500 μg. The essential advantages of this procedure over the USP XVIII procedure (1) are realized in its sensitivity, precision, accuracy, and simplicity. Preliminary studies indicated that the proposed oxidation technique may be advantageous for the assay of certain other organic iodo compounds, and more definitive studies are in progress.

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(3) J. J. Custer and S. Natelson, *Anal. Chem.*, **21**, 1005(1949).

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(5) *Ibid.*, p. 1024.

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* Present address: The United States Pharmacopeia, 12601 Twinbrook Parkway, Rockville, MD 20852

‡ Present address: Drugs Control Laboratory, Bombay 51, India.

ˣ To whom inquiries should be directed.

Liquid Chromatography in Pharmaceutical Analysis: Determination of Cough–Cold Mixtures

I. L. HONIGBERG ˣ, J. T. STEWART, and A. P. SMITH

Abstract □ Operating conditions are described for the qualitative and quantitative analysis of antihistaminic, antitussive, and analgesic compounds in cough–cold mixtures by high-pressure liquid chromatography. Twenty-one drugs were investigated. Determinations can be made in less than 30 min with an accuracy of 1–5%.

Keyphrases □ Antihistaminic, antitussive, and analgesic drugs—retention times, high-pressure liquid chromatography, application to analysis of cough–cold mixtures □ Analgesic, antihistaminic, and antitussive drugs—retention times, high-pressure liquid chromatography, application to analysis of cough–cold mixtures □ Antitussive, antihistaminic, and analgesic drugs—retention times, high-pressure liquid chromatography, application to analysis of cough–cold mixtures □ Cough–cold mixtures—analysis, high-pressure liquid chromatographic retention times of drug components □ High-pressure liquid chromatography—analysis, cough–cold mixtures, retention times of various antihistaminic, antitussive, and analgesic drugs

The recent introduction of commerically available, high-pressure liquid chromatography (HPLC) systems suggested a reinvestigation of the analysis of multicomponent pharmaceutical dosage forms. The cough–cold preparations which contain several therapeutic classes are exemplary of this type. The separation and quantitative analysis of these products present difficult problems to the pharmaceutical analyst. Mario and Meehan (1) reviewed the problems involved and used an all-glass GLC system because

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Table I—Effect of Mobile Phase Composition on Retention Times[a]

Compound	Mobile Phase Composition[b] A Octadecyl	A Phenyl	B Octadecyl	B Phenyl	C Octadecyl	C Phenyl	D Octadecyl	D Phenyl	E Octadecyl	E Phenyl	F Octadecyl	F Phenyl
1. Pyrilamine maleate	91	88	105	94	97	88	114	106	113	91	99	106
	[c]	102	[c]	694	113	101	[c]	[c]	340	210	125	177
					602	425					215	
2. Aspirin	91	90	96	91	90	90	109	106	97	94	92	111
					118	109					128	
3. Pseudoephedrine hydrochloride	[c]	142	105	99	113	106	[c]	142	132	123	158	106
												134
4. Phenylpropanolamine hydrochloride	[c]	106	105	99	[c]	106	[c]	118	114	113	128	118
		196	273	215		277						
5. Isopropamide iodide	[c]	90	[c]	91	90	90	[c]	[c]	99	94	85	94
		106			113	106			189	177	120	156
											146	
6. Hydrocodone bitartrate	[c]	113	[c]	101	[c]	111	[c]	[c]	257	227	243	109
		466		338		300						224
7. Chlorpheniramine maleate	91	106	96	91	94	90	[c]	94	110	92	130	94
	[c]	[c]	[c]	[c]	113	104		[c]	708	225	514	248
						881						
8. Caramiphen edisylate	[c]	118	[c]	307	[c]	109	[c]	[c]	[c]	179	111	109
		520				168					239	142
9. Diphenhydramine hydrochloride	[c]	106	[c]	101	100	99	[c]	[c]	302	182	129	111
		118		606	421	101					198	161
		850			[c]	314						
10. Triprolidine hydrochloride	[c]	118	141	101	116	101	[c]	[c]	902	310	122	106
		[c]		[c]		104					648	277
						850						
11. Salicylamide	99		105				156	118	106			
12. Promethazine hydrochloride	170								1044			
13. Phenyltoloxamine citrate							[c]		109			
									535			
14. Bromodiphenhydramine hydrochloride									142	208	116	
									261		293	
									503			
15. Ephedrine sulfate									132			
16. Homatropine methylbromide									142			
17. Pheniramine maleate									112			
									272			
									436			
18. Brompheniramine maleate									109			
									768			
19. Acetaminophen									102			
20. Phenylephrine hydrochloride									107			
21. Codeine phosphate										180		

[a] Expressed as number of seconds elapsed between injection and attainment of the chromatographic peak maximum. [b] Solvent composition: A, 50 parts acetonitrile, 50 parts 0.1% ammonium carbonate, pH 8.50; B, 60 parts acetonitrile, 40 parts 0.1% ammonium carbonate, pH 8.60; C, 90 parts acetonitrile, 10 parts 0.1% ammonium carbonate, pH 8.90; D, 20 parts acetonitrile, 80 parts 1.0% ammonium acetate, pH 7.04; E, 60 parts acetonitrile, 40 parts 1.0% ammonium acetate, pH 7.40; F, 80 parts acetonitrile, 20 parts 1.0% ammonium acetate, pH 7.58. [c] Retention time greater than 1200 sec.

the free base form of the drugs interacted with the surface of metal columns. GLC was also used by Cometti *et al.* (2), but they encountered problems in finding suitable inert solid supports. Many of their columns were somewhat cumbersome to reproduce. Other investigators (3–5) reported problems with the use of GLC in the analysis of these preparations.

Onishi *et al.* (6) used countercurrent distribution in the determination of anticold preparations, but 50 transfers were needed to obtain quantitative separations. Other methods involved nonaqueous titrations (4), conductometric titrations (7), spectrophotometry (8), and column and thin-layer chromatography (4, 9). Difficulties in end-point perception and incomplete separations of drugs were disadvantages of these techniques.

Determination of drugs in cough–cold mixtures by HPLC overcomes or circumvents many shortcomings in the previously reported methods. In this paper, development of separations by HPLC of antihistaminic, antitussive, and analgesic drugs is reported. A quantitative study is described for a typical mixture. The preparation of samples is simple and rapid since the drugs can be analyzed in their salt form. Separation and analysis times require less than 30 min.

EXPERIMENTAL[1]

Reagents and Chemicals—Powdered samples (50 mg/10 ml) of pyrilamine maleate[2], phenylpropanolamine hydrochloride[3], chlor-

[1] A Waters associates liquid chromatograph (model ALC 202), equipped with an M-6000 pump, a UV monitor, a Infotronics integrator (model CRS-204) with digital printout, and Waters packed columns, 1.22 m long × 2.3 mm i.d., was used.

[2] Dorsey Laboratories, Lincoln, Neb.

[3] Aldrich Chemical Co., Milwaukee, Wis.

Table II—Calibration Data for Standard Drug Solutions

Compound	Concentration, mg/10 ml	*D/IS* Ratio[a]	Slope	Intercept	$r \pm s$
Pseudoephedrine hydrochloride	57.65	0.2542 ± 0.0017	0.00438	0.00401	0.9999 ± 0.002
	96.80	0.4306 ± 0.0046			
	201.90	0.8869 ± 0.0029			
Codeine phosphate	10.00	0.3284 ± 0.009			
	20.00	0.6417 ± 0.011	0.03269	−0.00400	0.9999 ± 0.0059
	40.00	1.3065 ± 0.003			
Triprolidine hydrochloride	2.50	0.4001 ± 0.0010	0.16695	−0.00985	0.9998 ± 0.008
	5.00	0.8360 ± 0.0035			
	10.00	1.6559 ± 0.0398			

[a] Data represent three replicate injections of standard solutions. *D/IS* is the ratio of the integrated area of the drug at some concentration divided by the integrated area of strychnine sulfate at a concentration of 5 mg/10 ml.

pheniramine maleate[4], diphenhydramine hydrochloride[5], phenindamine maleate[2], bromodiphenhydramine hydrochloride[5], phenyltoloxamine citrate[6], phenylephrine hydrochloride[7], brompheniramine maleate[8], ephedrine sulfate[9], aspirin[10], salicylamide[11], acetaminophen[11], hydrocodone bitartrate[12], isopropamide iodide[13], caramiphen edisylate[13], homatropine methylbromide[12], and promethazine hydrochloride[14] were used in the analytical procedure. In addition, pseudoephedrine hydrochloride[15], codeine phosphate[10], and triprolidine hydrochloride[15] were used in the analytical procedure and in the preparation of standard curves. All other chemicals and solvents utilized were the highest grade of commercially available materials.

Mobile Phases—The mobile phases, consisting of various percentages of acetonitrile and aqueous solutions of ammonium carbonate or ammonium acetate, were prepared fresh daily.

Internal Standard Solution—The stock internal standard solution (5 mg/ml) was prepared by dissolving strychnine sulfate in the appropriate mobile phase. Strychnine sulfate powder was recrystallized once from ethanol before use.

Standard Solutions for Calibration Curves—Into individual 10-ml volumetric flasks were added accurately weighed quantities of 57.65, 96.80, and 201.90 mg of pseudoephedrine hydrochloride. Stock solutions of codeine phosphate (250 mg/25 ml) and triprolidine hydrochloride (250 mg/100 ml) were prepared with the appropriate mobile phase [acetonitrile–aqueous 1% ammonium acetate (60:40)]. Volumes of 1, 2, and 4 ml of each stock solution were placed in 10-ml volumetric flasks. One milliliter of the internal standard stock solution in the same solvent was added to each flask. Additional mobile phase was then removed from the solvent reservoir and added to volume.

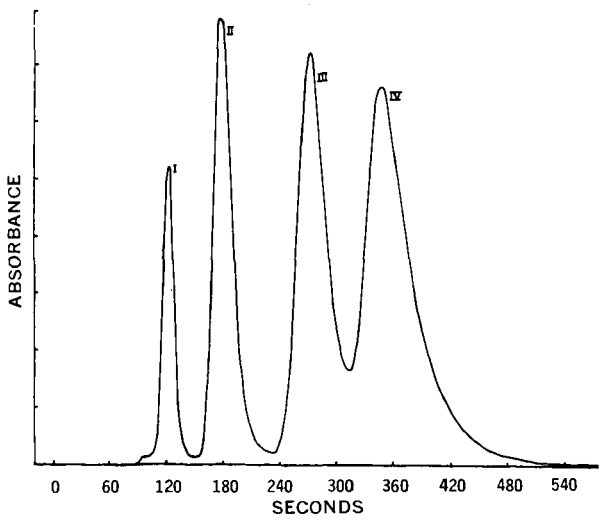

Figure 1—*Liquid chromatogram of a typical cough–cold preparation on diphenyldichlorosilane (phenyl) column with 60:40 acetonitrile–1% ammonium acetate. Key: I, pseudoephedrine hydrochloride; II, codeine phosphate; III, triprolidine hydrochloride; and IV, strychnine sulfate.*

Conditions for Chromatographic Separation and Quantification—The degassed mobile phase was pumped through columns containing monomolecular layers of octadecyltrichlorosilane (octadecyl) or diphenyldichlorosilane (phenyl) chemically bonded to a high efficiency pellicular packing, consisting of solid glass cores with a porous silica surface[16], at a flow rate of 1.4 ml/min (1000–1500 psig) at room temperature until a stable baseline was obtained. Replicate 10-μl injections of sample and standard solutions were made using a 25-μl syringe[17]. The chart recorder provided a record of drug elution from the column as peaks on a chromatogram. In all cases, the solute was measured by digital integration of the peak area[1].

RESULTS AND DISCUSSION

The purposes of this study were to determine the operating conditions for HPLC that would optimize the resolution of several therapeutic classes of heterogeneous compounds commonly found in cough–cold preparations in a reasonable time and to quantitate the analysis with a suitable level of precision.

The operating parameters studied were limited to solvent composition with respect to changing concentrations of acetonitrile and aqueous solutions of either 0.1% ammonium carbonate or 1% ammonium acetate, variations in the polarity of the reversed stationary phase, pH, and flow rate. The effect of the various parameters on the separation of the drugs studied is shown in Table I. Complete data are listed for Compounds 1–10, which were selected as representative structures of the 21 drugs studied.

Most compounds were separated best on the phenyl column using a 60:40 mixture (pH 7.4) of acetonitrile and aqueous 1% ammonium acetate. An increase in water concentration resulted in longer retention times on both columns with increased band spreading. Increases in acetonitrile concentration usually gave shorter retention times accompanied by multiple peaks on the chromatogram. Retention times were generally longer with the octadecyl column. In almost all cases, the compounds were eluted faster and the peaks were more symmetrical on the phenyl column when equivalent mobile phases were employed. A pH of 7.4 seemed essential for peak sharpness. Variations in pH had a marked effect on the number of species of the acidic or basic drugs that traveled through the column. This phenomenon is well documented in TLC. A flow rate of 1.4 ml/min, which resulted in pressures of 1000–1500 psig at room temperature, was the most satisfactory flow found for both octadecyl and phenyl columns since separations could be obtained with the various phases in less than 1200 sec.

[4] Schering Corp., Bloomfield, N.J.
[5] Parke-Davis and Co., Detroit, Mich.
[6] Bristol Laboratories, Syracuse, N.Y.
[7] Winthrop Laboratories, New York, N.Y.
[8] A. H. Robins and Co., Richmond, Va.
[9] Matheson, Coleman and Bell, East Rutherford, N.J.
[10] Merck Chemicals, Rahway, N.J.
[11] Eastman Organic Chemicals, Rochester, N.Y.
[12] Endo Laboratories, Garden City, N.Y.
[13] Smith Kline and French, Philadelphia, Pa.
[14] Wyeth Laboratories, Philadelphia, Pa.
[15] Burroughs Wellcome Co., Research Triangle Park, N.C.

[16] Corasil/C_{18} and Corasil/phenyl, 37–50 μm, Waters Associates, Milford, Mass.
[17] Model B-110, Precision Sampling Corp., Baton Rouge, La.

Table III—Analysis of Pseudoephedrine Hydrochloride, Codeine Phosphate, and Triprolidine Hydrochloride in Known Mixtures

Mixture	Added, mg	Found[a], mg	Accuracy, %
1. Pseudoephedrine hydrochloride	120.00	125.35 ± 4.47[b]	4.46
Codeine phosphate	20.00	20.01 ± 0.53	0.05
Triprolidine hydrochloride	5.00	5.14 ± 0.14	2.79
2. Pseudoephedrine hydrochloride	40.38	41.18 ± 1.89	1.98
Codeine phosphate	16.00	15.77 ± 0.17	1.46
Tripolidine hydrochloride	4.00	4.06 ± 0.09	1.50

[a] Based on three to five replicate determinations of known mixtures. [b] Confidence limits at $p = 0.05$.

A mixture of pseudoephedrine hydrochloride, codeine phosphate, and triprolidine hydrochloride was selected to demonstrate the utility of the separation and quantification method[18]. Figure 1 illustrates a chromatogram of the drugs being assayed. Various concentrations of standard solutions of each drug dissolved in the 60:40 acetonitrile–aqueous 1% ammonium acetate mixture were chromatographed using the phenyl column. Strychnine sulfate was added to each solution as the internal standard. The area under the curve for each peak on the chromatograms was integrated digitally. The ratio of each peak area to the area of the internal standard was calculated for each chromatogram. A linear regression line of these data at three concentrations of each drug gave the slope, intercept, and correlation coefficient for each calibration curve (Table II).

Known mixtures containing varied quantities of each drug were chromatographed, and the ratios of drug peak areas/internal standard peak areas *(D/IS)* were calculated. The overlap of the triprolidine and internal standard peaks in the mixture (Fig. 1) resulted in a smaller integrated area for the internal standard in the mixture *versus* its area in the calibration curves for codeine phosphate and pseudoephedrine. A suitable correction factor was applied to these two *D/IS* ratios in the mixture[19]. The *D/IS* ratios were then fed into a programmable calculator[20] along with slope and intercept data (see Table II) for each drug to obtain the concentration of the drugs in the mixture.

The data in Table III demonstrate the quantitative results obtained for two known mixtures. The utility of HPLC in the analysis of complex heterogeneous drug mixtures found in cough–cold dosage forms is clearly demonstrated. The accuracy range is from 1 to 5%.

[18] These three drugs are components of a commercially available dosage form, Actifed-C, marketed by Burroughs Wellcome Co. The expectorant, glyceryl guaiacolate, was not included as a part of this study.

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[x] To whom inquiries should be directed.

[19] For example:

$$\frac{D_{\text{pseudoephedrine}}}{IS_T} \times \frac{IS_T}{IS_0} = \text{corrected } D/IS \text{ ratio for pseudoephedrine}$$

where $D_{\text{pseudoephedrine}}$ is the area of the pseudoephedrine in the mixture, IS_0 is the area of 10 μl of a 0.5-mg/ml solution of strychnine sulfate without triprolidine overlap, and IS_T is the area of 10 μl of a 0.5-mg/ml solution of strychnine sulfate in the presence of triprolidine.

[20] Olivetti-Underwood Programma 101.

Dissolution of Lithium and Magnesium from Lithium Carbonate Capsules Containing Magnesium Stearate

HENRY C. CALDWELL

Abstract □ A lithium carbonate capsule mix designed for an automatic capsule-filling machine used about 0.25% magnesium stearate as a lubricant. While the formulation ran well, it had an undesirably long disintegration time and a slow dissolution rate due to the waterproofing effect of the lubricant. Studies on the addition of sodium lauryl sulfate separately to both the formulation and the dissolution medium showed that 2 mg (0.002%) added to the capsule (12 mg/600 ml of 0.3% HCl) was more effective in improving lithium dissolution than 6 g (1%) added directly to 600 ml of medium. Furthermore, while the addition of 0.02% sodium lauryl sulfate to the dissolution medium reduced the surface tension of the medium by more than 50%, it had no effect on dissolution rate; increasing the amount of sodium lauryl sulfate from 0.03 to 0.5% in the dissolution medium had a pronounced effect on dissolution rate. Magnesium dissolution from magnesium stearate and lithium dissolution from lithium carbonate were directly related.

Keyphrases □ Lithium carbonate capsules formulated with magnesium stearate—direct relationship between dissolution of magnesium and lithium □ Magnesium stearate as lubricant in lithium carbonate capsules—direct relationship between dissolution of magnesium and lithium □ Dissolution, lithium carbonate capsules formulated with magnesium stearate—direct relationship between dissolution of magnesium and lithium

Surface effects should be considered in the design of solid dosage forms, especially powder-filled capsules because they usually float. When the gelatin shell dissolves, liquid must displace air and spread over the powder surface before it can be immersed, dispersed, and dissolved. If wetting does not occur rapidly, the powder will float on the surface and agglomerate to minimize air–liquid contact and impede dissolution (1–5). Therefore, the practice of including surfactants in capsule and tablet formulations to ensure wetting and subsequent rapid disintegration and dissolution is widely followed (6–20). The lack of wetting properties of many formulations is not caused by the active material but by the widely used lubricant magnesium stearate, an excellent waterproofing agent (21–23). This does not imply that wetting is the only important consideration in capsule disintegration and dissolution. Packing, porosity, filling method, type and concentration of diluents, lubricants, and surfactants have effects (18, 24–28). Other variables also have been cited (29).

Surfactants have also been added to dissolution fluids in attempts to study wetting, disintegration, and dissolution. A direct correlation for phenacetin powder dissolution rate and surface tension of 0.1 *N* HCl solution with different amounts of polysorbate 80 was found (3). Phenobarbital powder and aspirin powder dissolution rates in 0.1 *N* HCl solution were also found to increase with increasing content of polysorbate 80 (30). Dilute aqueous lysolecithin solutions exhibited micellar solubilizing properties on powdered griseofulvin, glutethimide, and a diuretic compound. This solution dissolved these relatively water-insoluble drugs faster and more completely than water did (31). Benzoic acid pellets with large pores dissolved faster in a dilute sodium lauryl sulfate solution than in water (32). Hydrochlorothiazide tablets dissolved at equivalent rates in distilled water, 0.1 *N* HCl, and simulated intestinal fluid. The addition of 0.03% polysorbate 80 to these media did not change these rates (33).

The purposes of this study were to: *(a)* compare the relative effectiveness of surfactant in a formulation and in the dissolution fluid, *(b)* determine the effect of surface tension on the dissolution of slowly wettable capsules, and *(c)* compare dissolution rates of lithium and magnesium from lithium carbonate capsules containing magnesium stearate.

Table I—Capsules with Different Lubricants

Lubricant, mg/Capsule	Number of Dissolution Runs	Minutes for 50% Lithium Dissolution in 0.3% HCl by NF Method II ($\pm\sigma$)	Coefficient of Variation of Capsule Weight, %
None	3	3.4 (0.3)	3.9
1.0 Magnesium stearate	6	44.8 (4.3)	2.2
1.0 Magnesium stearate and 2.0 sodium lauryl sulfate	6	3.1 (0.3)	1.9
1.0 Magnesium lauryl sulfate	3	2.7 (0.03)	2.4
1.0 Stearic acid	3	3.0 (0.3)	1.3
1.0 Sodium stearate	3	2.7 (0.5)	1.2

EXPERIMENTAL

Preparation of Capsules—Lithium carbonate (0.9 kg), spray-dried lactose (0.27 kg), and magnesium stearate (3.0 g) were mixed on a small paddle mixer[1] for 10 min, passed through a No. 8 screen on a hammer mill[2] at high speed with impact forward, remixed for 10 min, and filled into No. 1 capsules on an automatic capsule-filling machine[3]. Theoretical fill for each capsule was

[1] Kitchen Aid.
[2] Fitzpatrick comminuting.
[3] Zanasi model LZ 164.

Table II—Effect of External Surfactant on Dissolution Rate of Lithium Carbonate from Capsules with Magnesium Stearate

Percent Sodium Lauryl Sulfate in 0.3% HCl	Number of Dissolution Runs	Minutes for 50% Dissolution in 0.3% HCl by NF Method II ($\pm\sigma$)	Student's t Test on T_{50} Values[a]	Dissolution Fluid Surface Tension, dynes/cm ($\pm\sigma$)
0	6	44.8 (4.3)	Control	71.9 (0.1)
0.0054	6	41.3 (3.2)	NS	36.6 (0.4)
0.02	9	44.8 (5.1)	NS	31.1 (0.2)
0.0316	6	23.4 (3.8)	S[b]	29.2 (0.2)
0.04325	3	16.8 (0.8)	S	28.9 (0.3)
0.115	3	10.2 (2.5)	S	25.8 (0.1)
0.173	3	10.1 (1.8)	S	26.4 (0.2)
0.5	3	5.9 (0.9)	S	30.9 (0.3)
1.0	3	5.9 (0.4)	S	30.7 (0.2)

[a] NS = not significant. S = significant at $p < 0.05$ level. [b] $p < 0.05$.

391 mg, equivalent to 300 mg of lithium carbonate, 90 mg of spray-dried lactose, and 1 mg of magnesium stearate. Other capsules were prepared as indicated in Table I.

Magnesium and Lithium Dissolution Rates—Dissolution testing was done using NF XIII dissolution method II in 600 ml of fluid (34). All glassware was washed with dilute acid and rinsed with distilled water to remove magnesium salts. Six capsules were used in each test, and tests were repeated at least three times. Samples (20 ml) were removed at some or all of the following intervals: 2, 4, 5, 6, 8, 10, 15, 20, 25, 30, 35, and 60 min. Fresh fluid was added at each interval to replace the withdrawn volume. Samples were immediately filtered[4]. Undiluted samples were analyzed for magnesium on an atomic absorption spectrophotometer[5] equipped with a magnesium lamp. Magnesium content of gelatin capsules and capsule contents with no magnesium stearate were subtracted from these assays to give magnesium dissolution from magnesium stearate. Samples were diluted 2 to 200 and analyzed for lithium by atomic absorption with a lithium lamp. Corrections were made for removed samples, and the data were plotted on logarithmic normal probability paper to obtain T_{50} values.

Surface Tension—A tensiometer[6] was used to determine surface tension of dissolution fluids. Parenteral water run as a control had a typical surface tension of 72.5 (±0.2) dynes/cm.

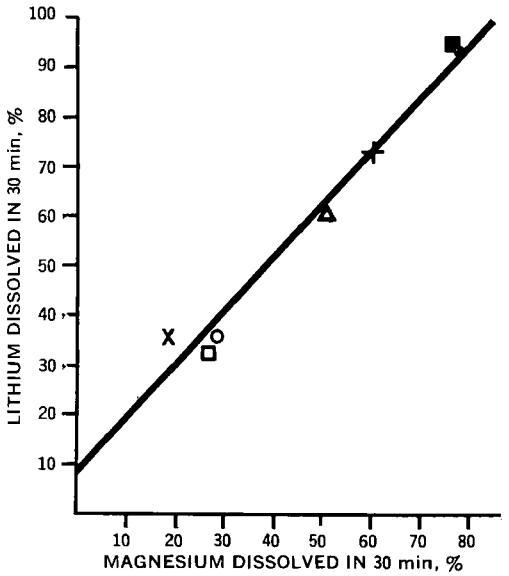

Figure 1—*Dissolution of magnesium from magnesium stearate and lithium from lithium carbonate in 0.3% HCl with added sodium lauryl sulfate in percent indicated. Key: ○, 0%; ×, 0.0054%; □, 0.02%; △, 0.0316%; +, 0.04325%; ●, 0.115%; and ■, 0.173%.*

[4] Through a 0.45-μm Millipore pad in a Swinnex 25 filter.
[5] Techtron type AA4.
[6] Du Nouy.

Lubricant Particle Size—Lubricant particle size was determined on a particle analyzer[7] as described previously (35). Mass median diameter values for magnesium stearate, sodium lauryl sulfate, and magnesium lauryl sulfate particles were between 11.1 and 18.2 μm. Stearic acid particles were slightly larger, with a mass median diameter of 37.7 (9.7–145.5) μm.

RESULTS AND DISCUSSION

Several batches of capsules were made with the same amounts of lithium carbonate and spray-dried lactose but with different lubricants (Table I). Capsules with no lubricant dissolved rapidly by NF XIII method II in 600 ml of 0.3% HCl, with effervescence, but they did not have satisfactory weight control; a lubricant was needed. In contrast, capsules with magnesium stearate (1 mg) had satisfactory weight control but dissolved slowly (T_{50} = 44.8 min); the capsules floated and when the gelatin shell dissolved, the powder continued to float. These results clearly show the dramatic effects of magnesium stearate as a lubricant and as a waterproofing agent.

Capsules with both magnesium stearate (1 mg) and the surfactant sodium lauryl sulfate (2 mg) dissolved rapidly and had satisfactory weight control. Thus, sodium lauryl sulfate counteracted the waterproofing effect without counteracting the lubricant effect of magnesium stearate. Capsules with sodium lauryl sulfate (2.5 mg) alone added as surfactant-lubricant dissolved rapidly

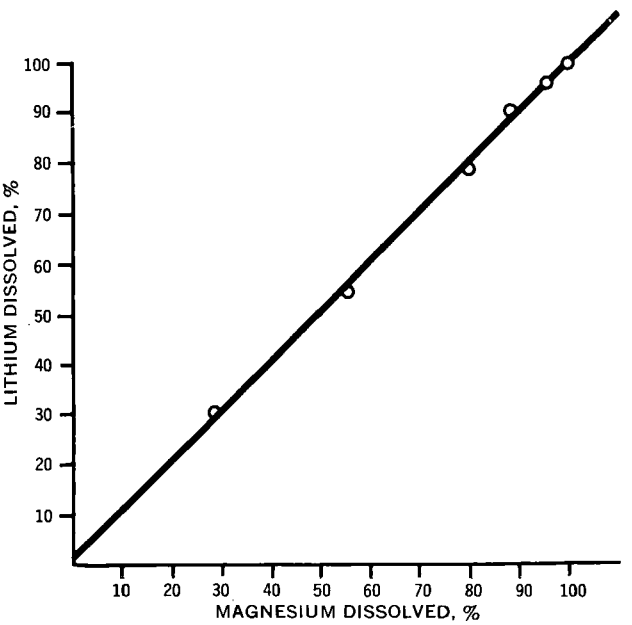

Figure 2—*Dissolution of magnesium from magnesium stearate and lithium from lithium carbonate in 3% HCl.*

[7] Coulter counter, model B.

Table III—Dissolution of Lithium and Magnesium from Lithium Carbonate Capsules Containing Magnesium Stearate

Solvent	Time for 50% Dissolution ($\pm\sigma$) Li	Mg	Slope	Intercept	r
3.0% HCl	13.4 ± 0.9[a]	13.6 ± 1.18[a]	0.968	2.132	0.998
0.5% Sodium lauryl sulfate	14.4 ± 0.8[b]	13.8 ± 0.7[b]	1.014	−6.252	0.998

[a] Not significant by t test. [b] Not significant by t test.

but did not have satisfactory weight control. Finally, capsules with the surfactant-lubricant magnesium lauryl sulfate dissolved rapidly and had satisfactory weight control. These studies show that a lubricant is necessary in these lithium carbonate capsules and that 2 mg of sodium lauryl sulfate added to the capsule formulation is able to counteract the waterproofing effect of 1 mg of magnesium stearate.

The capsules with 1 mg of magnesium stearate were studied further in an attempt to explain why they dissolved so slowly in 0.3% HCl. Low concentrations (0.0054 and 0.02%) of sodium lauryl sulfate in the 0.3% HCl dissolution fluid sharply reduced surface tension, from 72 to 37 and 31 dynes/cm, respectively, but had no effect on dissolution rate. Higher sodium lauryl sulfate concentrations, from 0.03 to 0.5%, had a pronounced effect on dissolution rates but did not further reduce surface tension (Table II). Thus, 12 mg of sodium lauryl sulfate in six capsules was more effective in increasing lithium carbonate dissolution than was 6 g (1%) of sodium lauryl sulfate added directly to 600 ml of media[8].

The ability of higher sodium lauryl sulfate concentrations to accelerate dissolution is not easily explained in view of the observation of Levy and Gumtow (20), who reported that the dissolution rate-enhancing effect of sodium lauryl sulfate is not due to any modification of microenvironmental pH or solubilization by micelles but rather to the better penetration of solvent into tablets and their component granules and the resulting greater availability of drug surface (20). The critical micelle concentration (CMC) for sodium lauryl sulfate in 0.3% HCl is about 0.009% when measured by the surface tension method. The apparent lack of dissolution rate and surface tension correlation was unexpected in view of the excellent correlation reported by Finholt and Solvang (3) for phenacetin powder and polysorbate 80.

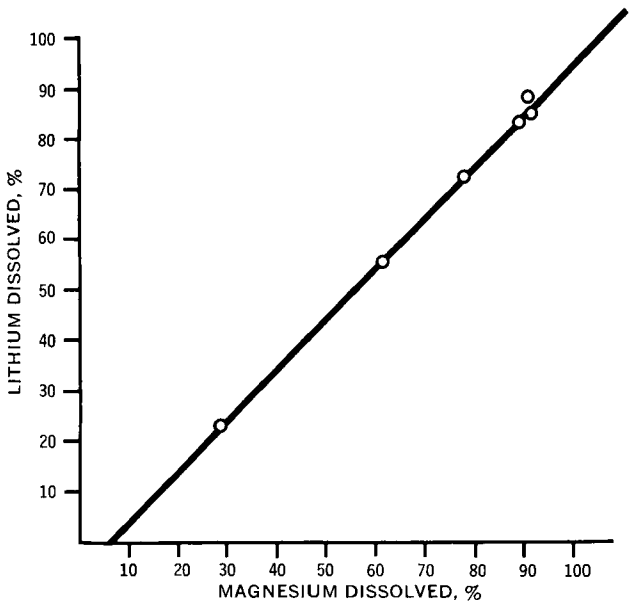

Figure 3—*Dissolution of magnesium from magnesium stearate and lithium from lithium carbonate in 0.5% sodium lauryl sulfate.*

The studies with sodium lauryl sulfate added to the 0.3% HCl solution were repeated with samples collected at 30 min. Lithium dissolution from lithium carbonate and magnesium dissolution from magnesium stearate were determined. Regression analysis of these data gave a slope of 1.076, an intercept of 8.326, and r of 0.991, which show that magnesium and lithium dissolutions are directly related (Fig. 1).

Dissolutions were also run in distilled water and 0.3, 1.0, 3.0, and 10.0% HCl. Again magnesium and lithium dissolutions were directly related. These data gave a slope of 1.053, an intercept of 5.216, and r of 0.992.

Dissolution rates were determined in 3% HCl and in 0.5% sodium lauryl sulfate. Samples were removed at 10, 15, 20, 25, 30, and 35 min. In both cases, magnesium and lithium dissolutions were directly related (Figs. 2 and 3 and Table III).

Thus in this formulation, which floats even after the gelatin shell dissolves, dissolution *of magnesium from magnesium stearate* is the rate-determining step in the dissolution of lithium from lithium carbonate in hydrochloric (3%) acid solution, dilute hydrochloric acid (0.3%) plus sodium lauryl sulfate solution, and sodium lauryl sulfate solution. Postulated mechanisms for magnesium dissolution are double-decomposition chemical reactions: *(a)* magnesium stearate + excess HCl → magnesium chloride + stearic acid, and *(b)* magnesium stearate + excess sodium lauryl sulfate → magnesium lauryl sulfate + sodium stearate.

To test these postulated mechanisms, additional batches of lithium carbonate capsules with stearic acid and sodium stearate as lubricants were prepared; magnesium lauryl sulfate has already been shown to give a rapidly dissolving formulation. Both stearic acid and sodium stearate gave formulations that ran well and had rapid dissolution rates (Table I). These results are in agreement with the postulated mechanisms.

Note that some of the surface tension values in Table II are below 30 dynes/cm. These lower values are due to the presence of impurities formed when sodium lauryl sulfate hydrolyzed in the acid solution to lauryl alcohol and sulfate (36). Motsavage and Kostenbauder (37) studied this hydrolysis in detail and found that sodium lauryl sulfate was hydrolyzed about 30 times faster above than below the CMC. Table IV shows that two solutions give lower surface tension values upon aging.

SUMMARY

Dissolution of magnesium from magnesium stearate is the rate-

Table IV—Surface Tension Changes of 0.3% HCl Solutions with Sodium Lauryl Sulfate

Sodium Lauryl Sulfate Concentration, %	Time	Surface Tension, dynes/cm ($\pm\sigma$)
0.115	Original	31.3 (0.2)
	3 days[a]	28.6 (0.2)
	8 days	25.3 (0.2)
	9 days	25.8 (0.1)
	43 days	25.4 (0.2)
0.173	Original	31.5 (0.2)
	18 days	27.8 (0.3)
	40 days	26.4 (0.3)

[a] Stored 3 days at 37°; the rest was stored at room temperature, 24°.

[8] The referee says this, in his experience, is a common observation which, based on the following arguments, appears to be reasonable. The volume of a No. 1 capsule is approximately 0.48 cm³; and the volume occupied by the 391 mg of lithium carbonate–lactose, *etc.*, assuming a weighed average density of 1.97 g/cm³, is 0.20 cm³. Therefore, the void space within the capsule is 0.28 cm³. This void space is also equal to the maximum volume of water, which will theoretically first mix with the powder blend. If the 2 mg (concentration of sodium lauryl sulfate per capsule) is considered to dissolve in this volume of water (0.28 cm³), the effective surfactant concentration, 2 mg/0.28 cm³, is approximately 7 mg/ml or 0.7%. Note from Table II that this is in the range, 0.5–1%, where greatly improved dissolution occurs.

determining step in the dissolution of lithium from slow-dissolving lithium carbonate capsules in three solvent systems. Postulated mechanisms for magnesium dissolution are double-decomposition reactions of magnesium stearate with hydrochloric acid to yield stearic acid and with sodium lauryl sulfate to yield sodium stearate and magnesium lauryl sulfate. These three suggested products were formulated into lithium carbonate capsules that exhibited rapid dissolution rates.

Sodium lauryl sulfate was much more efficient in aiding lithium dissolution when added to the formulation than when added to the dissolution medium.

Low concentrations of sodium lauryl sulfate in the dissolution fluid sharply reduced surface tension but had no effect on dissolution rate.

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Log-Normal Distribution of Tablet Pore Diameters

JOSEPH B. SCHWARTZ

Abstract □ The use of the log-normal distribution applied to the pore characteristics of tablets is discussed. An analytical method for the determination of the geometric mean pore diameter is presented and applied to several tablet samples. The method eliminates the necessity of plotting the pore volume data, eliminates subjective evaluation, and is amenable to computerized calculations.

Keyphrases □ Pore diameters, tablets—analytical method using log-normal distribution, advantages discussed □ Log-normal distribution—applied to pore characteristics of tablets, analytical method developed □ Tablets—analytical method presented to determine pore characteristics using log-normal distribution

Interest in the porous nature of pharmaceutical tablets has been growing steadily in recent years. As early as 1952, Higuchi *et al.* (1) stated: "... it seems reasonable to assume that the volume of void space in a given tablet would greatly influence its disintegrating property."

Two major parameters are associated with the porous nature of a tablet: *(a)* the volume of the void space designated as the porosity or pore volume, and *(b)* the size of those pores characterized by their diameters. This report deals with the second parameter.

Mean pore diameter has been used to characterize tablet pore size; one method of measurement used (2, 3) is air permeametry and the Kozeny–Carman equation. Adsorption isotherms have also been used (4–6). Another reported method (7) involved the mercury intrusion porosimeter. These last two methods generate pore distributions from which a mean value can be derived, although, if desirable, one may discuss the entire distribution.

The mean pore diameter is of interest in pharmacy because it is a single number that can be used to compare one tablet with another. As the level of sophistication increases and correlations are found, the total distribution will certainly tell a more complete story.

DISCUSSION

One method used to characterize porosity and pore size of materials is the mercury intrusion porosimeter. To make a measurement by a porosimeter, mercury is forced into the pores of the sample (*e.g.*, a tablet) under hydraulic pressure. The size of the pore penetrated is a function of this applied pressure, P, and the two are related by Eq. 1 (8):

$$Pr = -2\gamma \cos\theta \qquad \text{(Eq. 1)}$$

where r is the pore radius, γ is the surface tension of mercury, and θ is the contact angle between the mercury and the pore surface. The value of γ for mercury at 25° is 474 dynes/cm and, assuming a contact angle of 130°, the pore diameter, d, and the applied pressure are related as shown in Eq. 2 (8, 9):

$$d = \frac{175}{P} \qquad \text{(Eq. 2)}$$

where the pore diameter is expressed in microns and the pressure in psi (absolute).

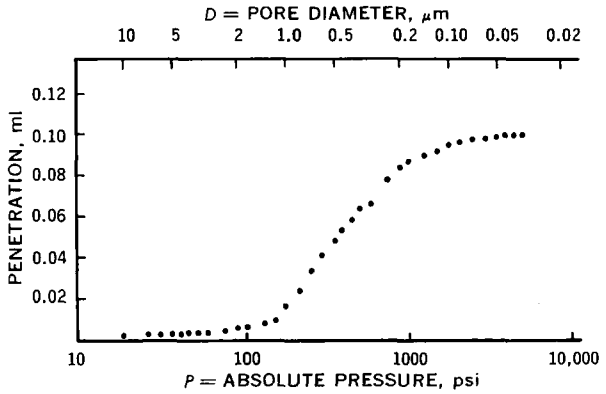

Figure 1—*Cumulative pore volume as a function of the logarithm of pressure or the logarithm of pore diameter.*

To illustrate the pore-size distribution, the cumulative pore volume is normally plotted *versus* the logarithm of the pore diameter. Alternatively, one may plot a frequency distribution. When the frequency distribution of a variable exhibits positive skewness, this distribution can be approximately described by a logarithmic normal distribution, which is shown in Eq. 3 for the variable X:

$$f(\ln X) = \frac{1}{\sigma_L\sqrt{2\pi}} \exp[-(\ln X - \ln \mu)^2/2\sigma_L{}^2] \qquad 0 < X < \infty \qquad \text{(Eq. 3)}$$

where μ is the mean value, and σ_L is the logarithm of the standard deviation. This equation is an extension of the equation for the normal distribution:

$$f(X) = \frac{1}{\sigma\sqrt{2\pi}} \exp[-(X-\mu)^2/2\sigma^2] \qquad \text{(Eq. 4)}$$

where the variable, X, is normally distributed, and μ and σ are the location and the scalar parameters of the normal curve, respectively (10). In Eq. 3, it is the logarithm of X, rather than X itself, that is normally distributed.

The log-normal or log-probability relationship has been used most frequently in pharmacy to describe the size distribution of particulate matter (11, 12), and it was suggested (13) that it be considered in connection with dissolution data. Recently for the first time, this distribution was applied to pore sizes in the hydrated cement paste system (14). That is, the parameter that is normally distributed is the logarithm of the diameter rather than the diameter itself.

This article shows that the properties of the log-normal distribution are adequate to describe the pore structure for tablets of pharmaceutical interest and presents an analytical technique for the computations.

EXPERIMENTAL

Pore-size distribution was determined on several tablet samples (four sets of data) containing 22% hydrochlorothiazide USP. Qualitatively, the formulas for the samples discussed are the same. The manufacturing process involves a starch-gelatin granulation of the active ingredient with dicalcium phosphate and lactose. Starch (corn), talc, and magnesium stearate are added before compression. The tablets are compressed on a rotary press at the same weight; since the level of active ingredient remained constant, the changes noted were due to the level of inert ingredients or to compression pressures (Table I).

The pore-size distributions were determined by a mercury in-

Table I—Formulation Differences

Data Set	Calcium Phosphate, mg	Lactose, mg	Gelatin, mg	Starch, mg	Magnesium Stearate, mg	Pressure, tons
I	50.0	30.0	0.2	6.6	0.2	1.1
II	30.0	50.0	0.5	4.6	0.5	1.5
III	55.5	24.5	1.0	5.6	1.0	1.0
IV	40.0	40.0	0.5	6.0	0.3	1.0

trusion porosimeter[1]. Vacuum was drawn by a vacuum pump[2] attached to the porosimeter, and hydraulic pressures up to 351.5 kg/cm² (5000 psi) were used in the measurements.

RESULTS

The usual graphical representation of porosimeter data (Fig. 1) shows the volume of mercury penetration into the sample *versus* the logarithm of the pressure applied. The mean pore diameter corresponding to a given pressure may also be read directly from the graph supplied with the instrument by calculating 50% of the maximum penetration and selecting the corresponding diameter (0.48 μm in this case).

When cumulative data plot out as a sigmoid curve, it suggests that the distribution is normal, in this case log-normal since the abscissa is a logarithmic scale.

Instead of the cumulative frequency plot in Fig. 1, one may generate the cumulative normal curve by simply normalizing the data. This was done for the data in Fig. 1 by using Eq. 5:

$$P = \frac{\text{cumulative frequency}}{\text{total pore volume}} \times 100 \qquad \text{(Eq. 5)}$$

where P is the percent probability, and the cumulative frequency is the cumulative volume of pores up to any diameter. As shown in Fig. 2, the sigmoid shape is retained, but probabilities have been introduced by normalizing; the total probability, or area under the normal curve, is unity.

To convert the sigmoid curve to a straight line, one makes use of probability paper. When the data shown in Figs. 1 and 2 are plotted in this manner, Fig. 3 results. If the frequency distribution is approximately normal, the plotted points lie very nearly on a straight line.

The advantage of the probability plot is that the characteristic parameter, μ, can be read directly from the graph, and the other characteristic, σ, can be easily calculated from the appropriate points.

In practice, probability plots are not really linear. The points near the extremities of the distribution are not considered very significant (15). These data points at the extremities, especially the large sizes, tend to weight the data and thus prohibit the use of an arithmetic mean.

In Fig. 3, the ends of the distribution will certainly not be on any straight line which may be drawn but the decision must be made as to which points can be considered to be on the ends. This question is considered in the following analytical method.

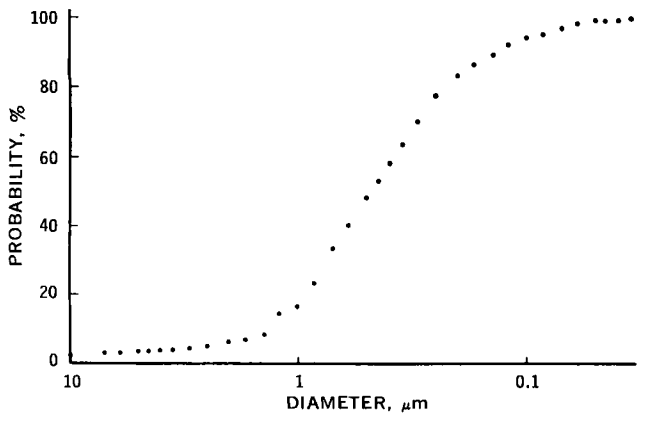

Figure 2—*Cumulative log-normal plot: percentage cumulative frequencies* versus *log pore diameter.*

Table II—Variation in Mean Pore Diameter as a Function of Proportion of Data Included

Probabilities[a], %	n^b	R^2, %[c]	D_m, μm	D_m, μm, Graphical Method
30–70	6	99.98	0.4821	0.48
25–75	7	99.97	0.4815	
20–80	9	99.90	0.4785	
15–85	11	99.60	0.4731	
10–90	14	99.60	0.4618	

[a] Points falling between these probabilities were used in the calculation. [b] Number of data points. [c] Index of determination.

ANALYTICAL

Although the graphical technique discussed will yield these values for the geometric mean pore diameter[3] and the geometric standard deviation, the method of plotting such data is time consuming. To provide a method allowing computerization of the calculation, an analytical method was developed.

The log-probability plot (Fig. 3) should linearize those data from a cumulative log-normal plot (Fig. 2), but in practice it does not do so completely. The cumulative log-normal plot is sigmoidal, but the center portion does approximate a straight line.

The parameters of interest can be read off the graph (Fig. 2); but since this set of data, or at least the straight-line portion, is amenable to regression analysis by calculator or computer, the parameters can also be calculated. The question then becomes how much of the data to include in the approximation.

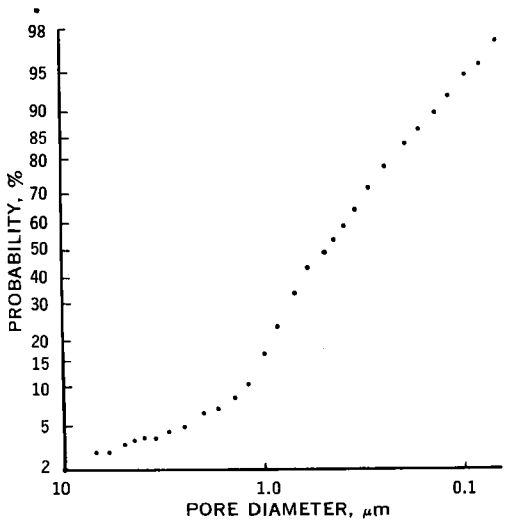

Figure 3—*Log–probability plot.*

[1] Aminco, American Instrument Co., Bethesda, Md.
[2] Cenco, Central Scientific Co., Chicago, Ill.

[3] The geometric mean diameter is equivalent, if the distribution is normalized in terms of the logarithmic variate, to the median.

Table III—Variation of Mean Pore Diameter as a Function of Proportion of Data Included

Probabilities[a], %	n^b	R^2, %[c]	D_m, μm	D_m, μm, Graphical Method
Data set 2:				0.18
30–70	6	99.40	0.1764	
25–75	8	99.64	0.1779	
20–80	10	99.72	0.1792	
15–85	12	99.83	0.1795	
10–90	15	99.72	0.1813	
Data set 3:				0.16
30–70	5	99.63	0.1610	
25–75	6	99.77	0.1613	
20–80	9	99.43	0.1637	
15–85	10	99.26	0.1649	
10–90	13	99.44	0.1651	
Data set 4:				0.16
20–80	4	99.70	0.1618	
15–85	5	96.21	0.1626	
10–90	6	85.90	0.1535	

[a] Points falling between these probabilities were used in the calculation. [b] Number of data points. [c] Index of determination.

Regression analysis was performed on these data, and Table II shows the effect of including various sections of the data in the calculations. The parameter of interest, mean pore diameter, D_m, does not change significantly as data are added or subtracted. Moreover, the index of determination for the regression[4] shows that the straight line does approximate the data satisfactorily. The same results are shown in Table III for additional sets of data yielding different mean diameters, and the diameters calculated by this method are in excellent agreement with those obtained by graphical methods in all cases.

For the calculation of the geometric standard deviation, the points at 15.87 and 84.13% probability are important[5]. It is recommended that probabilities between 15 and 85% be routinely included in the calculations. The geometric standard deviation, σ, can then be calculated by the following equations (11, 14, 16):

$$\sigma = \frac{\text{diameter at 84.13\% probability}}{\text{diameter at 50.0\% probability}} \quad \text{(Eq. 6)}$$

or:

$$\sigma = \frac{\text{diameter at 50.0\% probability}}{\text{diameter at 15.87\% probability}} \quad \text{(Eq. 7)}$$

[4] The index of determination is the square of the correlation coefficient.
[5] σ = 34.13% when probability is used.

The index of determination that determines the fit to the straight lines remains the criterion by which one can judge whether further analysis is necessary.

CONCLUSIONS

By using this method, a geometric value can be obtained for the mean pore diameter of tablets from pore distribution data generated by the porosimeter. It is not necessary to plot the data, which eliminates subjective evaluation; an additional parameter, the geometric standard deviation of the distribution, is also easily available. These parameters can be utilized as another method of comparing one tablet with another and of correlating other tablet properties or formulation parameters. The interpretation of differences in pore characteristics will be the subject of subsequent reports.

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Integral Approach to Nonisothermal Estimation of Activation Energies

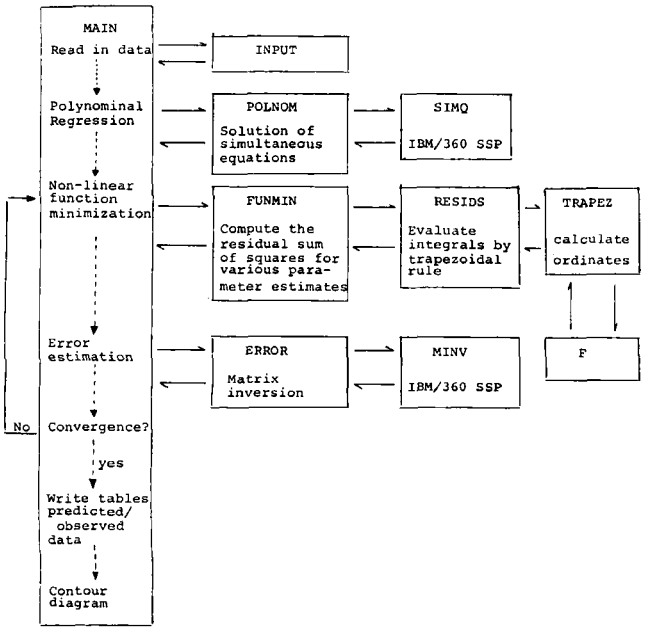
B. W. MADSEN*, R. A. ANDERSON*x, D. HERBISON-EVANS‡, and W. SNEDDON*

Abstract □ A simple experimental procedure is described, enabling estimates of the activation energy and preexponential factor for drug decomposition to be obtained from a single experiment. A computer program was written which bypasses the problem of slope determination and the consequent lack of final errors associated with graphical methods. It uses numerical integration and an iterative nonlinear least-squares treatment to ensure reliable and consistent parameter estimates and provides information on parameter uncertainties. The method is quite sensitive to deviations from the mathematical model and has been illustrated with the decomposition of riboflavin in alkaline solution.

Keyphrases □ Computer estimations, activation energies—integral approach to nonisothermal estimation, numerical integration and iterative nonlinear least-squares treatment □ Activation energies—integral approach to nonisothermal estimation, computer program, example from literature □ Decomposition—computer program for estimation of activation energy and preexponential factor of drug decomposition

The recent literature reflects a growing interest in nonisothermal methods for estimation of activation energies and the accelerated prediction of the stability of pharmaceuticals (1–4). Compared to the isothermal approach, these methods have been a compromise, reducing experimental effort but decreasing the accuracy of the final estimates.

The purpose of this paper is to show that the activation energy describing decomposition of a drug is a highly defined parameter. Provided computational facilities are available, the data obtained from a simple experimental procedure can be processed to give good activation energy estimates and, if required, rate constants at selected temperatures.



Scheme I—Computer flowchart of the program showing all subroutines

THEORETICAL

Decomposition of a drug is commonly represented by Eq. 1:

$$-\left(\frac{dc}{dt}\right)_i = kc_i^n \qquad \text{(Eq. 1)}$$

where c_i = concentration at time t_i, k = observed rate constant, and n = order of the reaction.

If the Arrhenius equation applies, then:

$$k_i = Ae^{-Ea/RT_i} \qquad \text{(Eq. 2)}$$

where A = preexponential factor, Ea = activation energy, R = universal gas constant, and T_i = absolute temperature at time t_i.

For the first-order case (n = 1), Eq. 1 can be rearranged; substituting for k gives Eq. 3:

$$-\frac{dc}{c_i} = Ae^{-Ea/RT_i}dt \qquad \text{(Eq. 3)}$$

To integrate Eq. 3, the series of temperature readings is first represented as a function of time. A polynomial is usually adequate for this, enabling Eqs. 4–6 to be formed:

$$-\int\frac{1}{c_i}dc = A\int e^{-Ea/RT(t)}dt \qquad \text{(Eq. 4)}$$

$$-\ln c_i = AI_i + \text{constant} \qquad \text{(Eq. 5)}$$

$$I_i = \int_{t_0}^{t_i} e^{-Ea/RT(t)}dt \qquad \text{(Eq. 6)}$$

Substitution for time t_0 in Eq. 5 shows that the constant term is $-\ln c_0$.

Then Eq. 5 can be seen to give:

$$c_i = c_0e^{-AI_i} \qquad \text{(Eq. 7)}$$

The concentration data (c_i) are nonlinear with respect to the two unknown parameters A and Ea. With initial estimates, an iterative least-squares regression can be used to find the best estimates, minimizing Eq. 8 until convergence:

$$SS = \sum_{i=1}^{N}(c_{i_{obs}} - c_{i_{pred}})^2 \qquad \text{(Eq. 8)}$$

Equations similar to Eq. 7 can be derived for the zero- and second-order cases (Eqs. 9 and 10, respectively) and the data may be treated using the same nonlinear approach:

$$c_i = c_0 - AI_i \qquad \text{(Eq. 9)}$$

$$c_i = (AI_i + c_0^{-1})^{-1} \qquad \text{(Eq. 10)}$$

PROGRAMMING

A flow chart of the program is shown in Scheme I. The various subroutines perform the following functions.

INPUT: Data read in by INPUT include the number of data points (N); the order of the reaction (ORDER); initial estimates for the two unknown parameters (X_1 and X_2); various experimental information pertinent to a given run (HEAD); the dependent

Table I—Source Data for Nonisothermal Treatment of Riboflavin Decomposition in 0.1 *N* NaOH

Absorbance	Minutes	Temperature
0.640	0	21.0°
0.637	10	25.7°
0.633	20	29.4°
0.622	30	33.4°
0.618	40	37.7°
0.589	51.5	43.0°
0.568	60	46.0°
0.534	70	49.2°
0.488	80	53.5°
0.423	90	57.0°
0.351	100	61.0°
0.191	120	66.6°
0.124	130	70.5°

variable, in this case aqueous absorbance (AQABS); and time and temperature (TIME and TEMP, respectively). Time is changed from minutes to hours to minimize overflow in the subsequent polynomial regression, and the temperature is changed to absolute, as required by Eq. 2.

POLNOM: Initially, a fourth-order polynomial for the temperature on time regression was found adequate for simple monotonic temperature changes, with all of the absolute residual values less than 0.4% of the predicted values. To allow additional experimental flexibility in the time–temperature relationship, a seventh-order polynomial was used, giving residuals usually less than 0.3%. The number of data points in this regression was always greater than 12, making the occurrence of spurious stationary points impossible. An 8 × 8 matrix of simultaneous equation coefficients was fed into SIMQ[1], together with eight constant terms for an analytical solution. Then the observed and predicted data, residuals, and ratios were printed out for visual inspection.

FUNMIN: The Adaptive Simplex approach (5) to function minimization was programmed as a subroutine FUNMIN. This involves a direct search based on the sum of squares, making no assumptions about the function or its derivatives, so that initial estimates are not critical to successful execution. The only consequence of poor initial estimates is a longer processing time. FUNMIN calls on a function subroutine (RESIDS) which computes the residual sum of squares for any pair of parameter estimates (X_1 and X_2). It was sufficient to set the increments *(DX)* for the initial simplex at 50 and 10% for *A* and *Ea*, respectively. FUN-

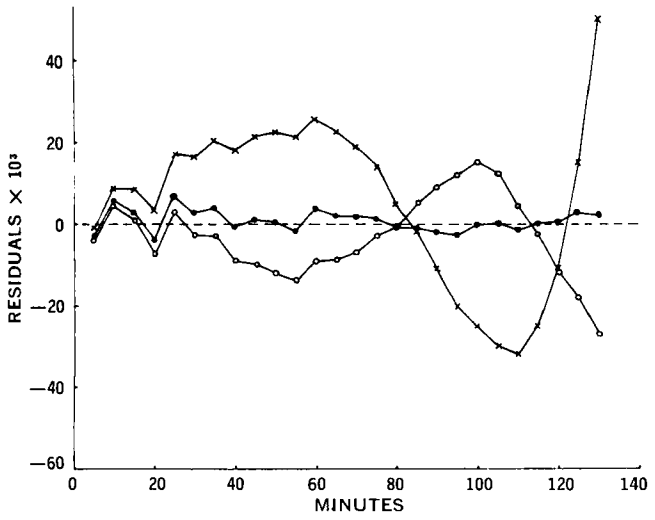


Figure 1—*Data processed according to zero-* (X), *first-* (●), *and second-* (○) *order kinetics. Along with other factors, an analysis of the residuals makes a decision on the best model simple.*

[1] IBM/360 Scientific Subroutine Package, 1967.

Table II—Polynomial Regression of Experimental Temperature Values on Time[a]

Hours	Observed Temperature	Calculated Temperature	Residuals	Ratios
0.	294.16°	294.29°	−0.131	0.9995
0.1667	298.86°	298.50°	0.361	1.0012
0.3334	302.56°	302.66°	−0.099	0.9997
0.5000	306.56°	306.87°	−0.306	0.9990
0.6667	310.86°	311.02°	−0.163	0.9995
0.8583	316.16°	315.64°	0.515	1.0016
1.0000	319.16°	318.96°	0.200	1.0006
1.1667	322.36°	322.81°	−0.446	0.9985
1.3334	326.66°	326.62°	0.037	1.0001
1.5000	330.16°	330.35°	−0.193	0.9994
1.6667	334.16°	333.84°	0.315	1.0009
2.0000	339.76°	339.89°	−0.134	0.9996
2.1667	343.66°	343.62°	0.044	1.0001

[a] Regression coefficients: 294.29, 26.33, −11.74, 41.28, −66.72, 52.69, −20.14, 29.71. Coefficients are listed in increasing order, *i.e.*, the constant term first, then the linear coefficient, *etc.*

MIN performs 24 iterations before returning to MAIN, and a maximum of 12 calls on FUNMIN is allowed.

RESIDS: This function subroutine is called by FUNMIN for each set of parameter estimates. To find the sum of squares, the right-hand side of Eq. 6 has to be evaluated. Using the trapezoidal rule, the integrals for each interval t_i to t_{i+1} are estimated separately since this involves less processing time. Then the total integral from zero time to any time t_i is obtained by summation. Then, for a chosen reaction order, a series of predicted concentrations can be calculated using Eq. 7, 9, or 10. Finally, a value for the sum of squares is computed using Eq. 8 and control returns to FUNMIN.

TRAPEZ and F: Subroutine TRAPEZ numerically estimates the integrals using the trapezoidal rule. That is, the interval is successively halved until the change in the integral is below some acceptable level (TEST). This criterion has a large effect on processing time, so it is important to find an acceptably accurate level. In the present work, concentrations were determined by measurement of absorbance values, and the error standard deviation of these readings was usually not greater than 0.003, so that in the worst case the relative error in primary absorbance data might be 0.003/0.1 = 3%. For a first-order reaction, this would correspond to an uncertainty of approximately 2% in the integral (I_i in Eq. 7). Since there is probably no point in performing subsequent calculations at better than a tenth of the error in the primary data, TEST was set at 0.2%. This value ensured fast processing, and reduction to a lower level did not alter the parameter estimates.

ERROR: The standard deviations of the derived parameters were estimated by matrix inversion using the sums of the cross-products of the partial derivatives (6). Normalization of this 2 × 2 matrix ensured that the off-diagonal elements of the unit matrix were never greater than 1×10^{-6}.

MAIN: After the errors have been computed, control returns to MAIN. If the difference between the final sum of squares from two successive calls on FUNMIN is less than 1%, then the program has converged at what most likely will be the true minimum. If not, then FUNMIN is called again, setting the new increments equal to the computed standard deviations.

When convergence has been achieved, it remains to print out a table of observed and predicted absorbance readings, residuals, and the residuals expressed as error standard deviations. If any data point is outside 3 *SD* (for 12–30 points), then a caution is printed out. The predicted rate constants for the various experimental temperatures also are listed in this table.

As a final aid in confirming convergence or in suggesting the direction of better initial estimates, a contour diagram is printed, showing the sum of squares for the two variables ±4 *SD*.

The program was written in FORTRAN IV[2], and all calculations were done on an automatic computer[3]. For average data

[2] A listing is available on request from the authors.
[3] 32K IBM 7040.

Table III—Summary of the Convergence History

	Iteration Number	Preexponential Factor, $\times 10^{13}$ hr^{-1}	Activation Energy, kcal mole^{-1}	Sum of Squares
Initial estimates		2.0	21.0	
Current value	1	2.903	20.370	5.655×10^{-5}
SD		0.273	0.063	
Final value	2	2.876	20.364	5.653×10^{-5}
SD		0.270	0.063	

and fair initial estimates, the usual execution time was 2–4 min.

EXPERIMENTAL

The degradation of riboflavin under aqueous alkaline conditions was chosen to illustrate the technique. Generally, the method followed the procedures used to study the reaction between octatomic sulfur and triphenylphosphine in benzene (7) and to follow the acid-catalyzed hydrolysis of ethyl acetate (8).

A solution of riboflavin (10^{-4} *M*) and sodium hydroxide (0.05 or 0.1 *N*) was heated so that the temperature rose conveniently over the temperature range of interest. At selected times, samples were taken, quenched in acetic acid, and assayed spectrophotometrically as previously described (9). The temperature of the solution was noted each time a sample was taken for analysis, giving three corresponding sets of primary data: concentration, time, and temperature.

To obtain the best combined parameter estimates from different experiments, the weighted means and standard deviations were calculated according to Eqs. 11–13:

$$w_i = \frac{1}{s_i^2} \qquad \text{(Eq. 11)}$$

$$\bar{x} = \frac{\sum w_i x_i}{\sum w_i} \qquad \text{(Eq. 12)}$$

$$\bar{s} = \frac{1}{\sqrt{\sum w_i}} \qquad \text{(Eq. 13)}$$

where s_i = standard deviation of an individual point x_i, w_i = weight for the point x_i, $\bar{x}$ = weighted mean, and $\bar{s}$ = weighted standard deviation.

The uncertainty in the predicted rate constant at a given temperature was computed using Eq. 14:

$$S_k = \sqrt{\left(S_A \frac{\partial k}{\partial A}\right)^2 + \left(S_{Ea} \frac{\partial k}{\partial Ea}\right)^2} \qquad \text{(Eq. 14)}$$

where S_k, S_A, and S_{Ea} are the uncertainties in the predicted rate constant and estimated preexponential factor and activation energy, respectively.

The nonisothermal estimates were confirmed by designing an isothermal experiment using the same assay procedure and 50 data points at temperatures of 20, 35, and 50°. Final parameter estimates were computed by weighted least squares (10).

RESULTS AND DISCUSSION

Table I lists typical experimental results for input to the program, and Tables II–V summarize the results of computations on this data set. Provided the reaction is followed through at least two half-lives and that the data are spread uniformly over the whole range, only 12 points are needed to define the two unknown parameters sufficiently. If additional experimental effort is indicated, it is probably better spent in repeating the whole run than in gathering more data points for the given experiment. This approach represents a distinct advance in experimental effort over the more standard isothermal procedure. Adjustment of the heating rate is completely flexible, and temperature may be increased at a rate chosen to achieve the desired degree of decomposition in a convenient experimental time. This ensures that estimates can be gained from the first experiment.

One assumption made in this method is that c_0 (the first sample taken) is known without error. Since the temperature is changing least rapidly at the commencement of a run, it should be the most certain of the assay results. By taking a duplicate and averaging the results, the error incurred should not be great. At the cost of increased processing time, the alternative is to have three variables in the regression; the program is sufficiently general to be easily modified when this is needed.

Table IV—Comparison of Experimental and Predicted Data

Observed Absorbance	Predicted Absorbance	Residuals, $\times 10^{-3}$	Scatter as *SD*	Predicted Rate Constants, hr^{-1}
0.637	0.6370	−0.02	−0.01	0.0367
0.633	0.6322	0.75	0.33	0.0558
0.622	0.6247	−2.71	−1.19	0.0869
0.618	0.6130	5.00	2.21	0.138
0.589	0.5921	−3.08	−1.36	0.240
0.568	0.5697	−1.74	−0.77	0.325
0.534	0.5339	0.10	0.05	0.447
0.488	0.4857	2.33	1.03	0.680
0.423	0.4239	−0.89	−0.39	0.948
0.351	0.3502	0.80	0.35	1.37
0.191	0.1927	−1.72	−0.76	2.28
0.124	0.1229	1.10	0.49	3.21

The fact that the predicted temperature readings from the polynomial regression do not fit the observed temperatures exactly (absolute residual values not greater than 0.2% in Table II) is not a problem, since positive and negative fluctuations are averaged out during integration. If the regression was so bad that all of the residuals were positive and +0.1%, an average error of +0.1% would be introduced in the computed integrals, which is well within the experimental level of ±2% discussed previously. Pragmatically, as reported previously (11), the parameter estimates are fairly insensitive to temperature errors with such an integral nonisothermal approach. For example, if all the temperature readings from the data in Table I are lowered by 1°, then the computed activation energy changes from 20.36 ± 0.06 to 20.24 ± 0.06[4] kcal mole^{-1}, and the preexponential factor changes from $(2.9 \pm 0.3) \times 10^{13}$ to $(2.6 \pm 0.2) \times 10^{13}$ hr^{-1}.

The final results for the two systems studied (0.05 and 0.1 *N* sodium hydroxide) are shown in Table VI, together with the isothermal results. Parameter variation between runs is probably better than the normally tolerated levels, and agreement with the isothermal method is good. However, previous workers (1, 9) quoted slightly lower values for the activation energy (Table VII). Hughes (11) commented that higher results may be obtained with nonisothermal techniques, and part of this discrepancy may be due to faulty data treatment in the classical isothermal approach. When the same isothermal data in Table VI were processed by taking log absorbance, linearly regressing on time, and then making an unweighted linear regression of log *k* on reciprocal temperature, the parameter estimates were slightly depressed (Table VII).

The approach presented here is sensitive to deviations from the expected order decomposition, as could be due to change of mechanism or approach to equilibrium. Attainable experimental preci-

[4] All calculated uncertainties in this report are given as standard deviations.

Table V—Total Sum of Squares Dependence on the Two Nonlinear Variables about the Best Estimates

Pre-exponential Factor, $\times 10^{13}$ hr^{-1}	Activation Energy, kcal mole^{-1}								
	20.12	20.18	20.24	20.30	20.36	20.42	20.48	20.54	20.60
3.96	0.108E-0	0.799E-1	0.562E-1	0.368E-1	0.215E-1	0.104E-1	0.330E-2	0.229E-3	0.101E-2
3.69	0.872E-1	0.624E-1	0.417E-1	0.253E-1	0.130E-1	0.482E-2	0.671E-3	0.423E-3	0.391E-2
3.42	0.675E-1	0.459E-1	0.285E-1	0.153E-1	0.624E-2	0.123E-2	0.153E-3	0.286E-2	0.914E-2
3.15	0.490E-1	0.309E-1	0.171E-1	0.736E-2	0.173E-2	0.674E-4	0.223E-2	0.800E-2	0.172E-1
2.88	0.322E-1	0.180E-1	0.795E-2	0.201E-2	0.565E-4	0.195E-2	0.748E-2	0.164E-1	0.285E-1
2.61	0.178E-1	0.783E-2	0.194E-2	0.580E-4	0.202E-2	0.764E-2	0.167E-1	0.288E-1	0.437E-1
2.34	0.687E-2	0.148E-2	0.103E-3	0.256E-2	0.865E-2	0.181E-1	0.307E-1	0.460E-1	0.637E-1
2.06	0.732E-3	0.401E-3	0.387E-2	0.109E-1	0.213E-1	0.346E-1	0.507E-1	0.690E-1	0.893E-1
1.79	0.151E-2	0.663E-2	0.152E-1	0.270E-1	0.416E-1	0.588E-1	0.781E-1	0.991E-1	0.122E-0

Table VI—Best Parameter Estimates for Riboflavin Decomposition

	Experiment Number	Number of Data Points	A, $\times 10^{13}$ hr^{-1}	Ea, kcal mole^{-1}
0.05 N NaOH	1	12	1.2282 ± 1.241	20.017 ± 0.717
	2	13	2.1104 ± 0.383	20.366 ± 0.124
	3	26	1.341 ± 0.0980	20.0872 ± 0.0490
Weighted mean and SD			1.39 ± 0.09	20.12 ± 0.04
Weighted least-squares isothermal estimates			1.21 ± 0.98	20.0 ± 0.5
0.1 N NaOH	4	12	2.8793 ± 0.270	20.3637 ± 0.0626
	5	12	1.9194 ± 0.451	20.0851 ± 0.161
	6	14	4.0999 ± 0.594	20.565 ± 0.0963
	7	20	1.4734 ± 1.70	19.8780 ± 0.8005
Weighted mean and SD			2.8 ± 0.2	20.39 ± 0.05
Weighted least-squares isothermal estimates			3.3 ± 4.2	20.3 ± 0.8

Table VII—Comparison of Results with Previous Literature Reports on the Decomposition of Riboflavin in 0.05 N NaOH

	Nonisothermal	Rogers (1)	Guttman (9)	Isothermal Unweighted Least Squares
Preexponential factor $\times 10^{13}$, hr^{-1}	1.6 ± 0.4	—	—	0.8 ± 0.75
Activation energy, kcal mole^{-1}	20.09 ± 0.05	17.85	19.2	19.7 ± 0.4
Rate constant at 20°, hr^{-1}	0.014 ± 0.002	0.018	0.016	—

Table VIII—Simulation of an Equilibrium Situation[a]

Observed Absorbance	Predicted Absorbance	Residuals, $\times 10^{-3}$	Scatter as Error SD
0.637	0.6365	0.44	0.09
0.633	0.6312	1.80	0.37
0.622	0.6320	−0.97	−0.20
0.618	0.6105	7.48	1.53
0.589	0.5890	0.03	0.00
0.568	0.5665	1.48	0.30
0.534	0.5312	2.75	0.56
0.488	0.4848	3.24	0.66
0.423	0.4262	−3.19	−0.65
0.351	0.3570	−5.99	−1.22
0.202	0.2082	−6.17	−1.26
0.150	0.1400	10.00	2.04

Sum of squares = 2.65×10^{-4}
Absorbance error = ±0.0049
A ($\times 10^{13}$ hr^{-1}) = 0.4 ± 0.10
Ea (kcal mole^{-1}) = 19.1 ± 0.2

[a] This table should be compared to Table IV.

Table IX—Effect of Using Absolute Absorbance or Log Absorbance Values as Dependent Variable in Nonlinear Regression

Dependent Variable (c_i)	0.05 N NaOH	0.1 N NaOH
Absolute absorbance:		
A, $\times 10^{13}$ hr^{-1}	1.3 ± 0.1	2.9 ± 0.3
Ea, kcal mole^{-1}	20.09 ± 0.05	20.36 ± 0.06
Log absorbance:		
A, $\times 10^{13}$ hr^{-1}	0.8 ± 0.1	2.6 ± 0.6
Ea, kcal mole^{-1}	19.7 ± 0.1	20.3 ± 0.2
Number of data points	26	12

sion is readily established (in this work, not greater than ±0.003 absorbance unit) and if the breakdown does not follow such a simple integer order, the program will make the best fit possible and return a large error standard deviation. Together with non-normality of residuals, this should serve as a warning that all is not well.

If an equilibrium situation applies, then the greatest deviation will occur in the later time readings. Sensitivity to such a system was tested by simulation and the results are shown in Table VIII. The last two absorbance readings in Table I were increased from 0.191 to 0.202 and from 0.124 to 0.150, and then this new data set was fed into the program. It will be seen that the uncertainty in the absorbance readings increases from ±0.0023 to ±0.0049, the residuals are no longer randomly distributed, and the last point is accounting for approximately 40% of the total sum of squares.

Weighting of data points should also be considered. In this

Table X—Effect of Treating Decomposition Data According to Different Mathematical Models, Namely Zero, First, and Second Order[a]

	Order		
	Zero	First	Second
A, hr^{-1}	$(7.0 \pm 7.0) \times 10^6$	$(1.3 \pm 0.1) \times 10^{13}$	$(1.6 \pm 0.5) \times 10^{19}$
Ea, kcal $mole^{-1}$	11.2 ± 0.7	20.09 ± 0.05	28.7 ± 0.2
Absorbance error	± 0.021	± 0.0029	± 0.010

[a] In this case, the data confirm literature reports stating the decomposition is first order.

case, the dependent variable (c_i) is measured using a UV spectrophotometer. The reported uncertainty in such an instrument is ±0.005 absorbance unit[5], which represents a constant absolute error. Therefore, it is more correct to use the absolute absorbance values than the log absorbance. In a least-squares procedure, the former approach would minimize as far as possible to a constant absolute error, whereas the latter minimizes toward a constant relative error. Log weighting of the absorbance readings invariably returned a slightly lower value for the activation energy than when the absolute values were used and often produced nonrandom residuals. The biggest factor against using log absorbance in this nonisothermal technique is the comparatively larger relative standard deviations for the two parameters (Table IX).

The nonisothermal approach is very sensitive to the order of the reaction, so prior isothermal experiments need not be made to establish order. Data can be submitted for analysis, and the consequences of treating a reaction by the wrong mathematical model are clearly pointed out (see Fig. 1 and Table X). In this instance, an examination of the residuals clearly shows the reaction is first order, and this is substantiated by the smaller parameter and absorbance error standard deviations.

[5] Manual, Perkin-Elmer model 124 double-beam spectrophotometer.

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NOTES

Quantitative GLC Analysis of Plasma Cholesterol

P. A. HARRIS[x] and K. L. HARRIS

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Keyphrases □ Cholesterol—quantitative GLC analysis in plasma, compared to colorimetric method □ GLC—analysis, cholesterol in plasma, compared to colorimetric method

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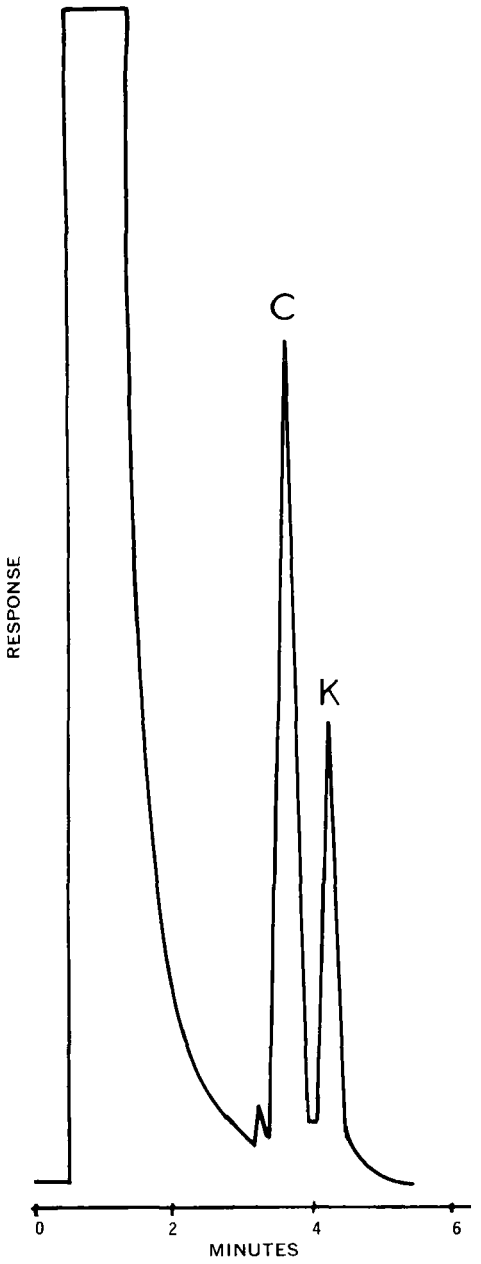

Figure 1—*Typical chromatogram resulting from an injection of an extracted plasma sample. Key: C, cholesterol; and K, internal standard, 11-ketoprogesterone. See text for details.*

tent, that is, both the nonesterified and esterified cholesterol, unless prior isolation of nonesterified cholesterol is carried out by precipitation with substances such as digitonin or tomatin. These methods, as well as others, have been discussed in detail (1). Most methods lack specificity, do not distinguish between esterified and nonesterified cholesterol, or do not have a high degree of sensitivity. For many purposes, of course, these limitations are not important, particularly when the simplicity and automation of some methods are considered. In this report, a GLC method for cholesterol analysis is described. Since the completion of this work, another publication has described a new improved GLC method for serum cholesterol (2). The present method contains some details and different conditions that may be advantageous or supplementary to this earlier report (2). The method has particular applicability in laboratories conducting cholesterol analysis occasionally, when a high degree of sensitivity is required, or when cholesterol is in the presence of significant quantities of very similar compounds such as desmosterol or lanosterol.

EXPERIMENTAL

Chemicals—All solvents used were reagent grade. The petroleum ether was the 30–60° bp fraction. Alcoholic potassium hydroxide was prepared by adding absolute alcohol to 60 ml of a 33% aqueous potassium hydroxide solution to make 1 liter. Cholesterol[1], 11-ketoprogesterone[2], cholesteryl acetate[3], lanosterol[3], and desmosterol[4] were used.

Instrumentation—Chromatography was performed with a gas chromatograph[5] equipped with a hydrogen flame-ionization detector. Prepurified dry nitrogen, 30 ml/min, was used as the carrier gas. A 1.8-m × 0.63-cm (6 ft × 0.25-in.) o.d. coiled glass column was packed with 3% OV-17 on Gas Chrom Q[6], 100–120 mesh. The column was initially conditioned at 375° for 30 min without carrier gas flow and then overnight at 325° with carrier gas flow and was subsequently operated at 300°. The temperature of both the detector and the injection port was 325°. When not in use, the column temperature was maintained at 200° to prolong the effective life of the column. Samples were injected with a 10-μl syringe[7].

Standard Solutions—A stock solution of cholesterol was prepared by dissolving 50 mg in 50 ml petroleum ether, bp 30–60°. Working standards were prepared by diluting the stock solution to concentrations of 2.5, 5, 10, and 20 mg/100 ml. An internal standard solution was prepared by dissolving 20 mg 11-ketoprogesterone in 50 ml acetone and adding petroleum ether to make 100 ml. Acetone was used because 11-ketoprogesterone is not sufficiently soluble in petroleum ether at this concentration.

Procedures—For the determination of total cholesterol, 1.0 ml of plasma was placed in a 70-ml culture tube with a Teflon-lined screw-cap, and the cholesteryl esters were saponified by adding 10 ml of alcoholic potassium hydroxide and incubating for 60 min at 45° in a slowly shaking water bath. After cooling, extraction was performed by adding 20 ml of petroleum ether. The tubes were shaken in a mechanical shaker for 5 min. To this solution, 10 ml of water was added and the mixture was shaken for 1–2 min. The mixture was then allowed to separate into two phases. Five milliliters of the petroleum ether fraction was transferred to a suitable glass tube and evaporated to dryness in a 75° water bath. The residue was dissolved in 2 ml of the internal standard solution, and approximately 1 μl of this solution was injected into the gas chromatograph. Peak height ratios of cholesterol–internal standard were used for quantitation.

Peak height ratios for known quantities of cholesterol were determined by pipeting 5 ml of each diluted standard solution into a suitable glass tube, evaporating this sample to dryness in a 75° water bath, and then dissolving the residue in 2 ml of the internal standard solution. These samples were injected in the same manner as the unknown samples. A standard curve was constructed by graphing peak height ratios of cholesterol–internal standard *versus* cholesterol concentration.

Variations of the procedure were developed for use with smaller plasma samples or biological samples containing smaller amounts of cholesterol. One such procedure consisted of removal of 15 ml of the petroleum ether extract. An appropriate quantity of internal standard was added and the entire volume was evaporated to dryness. The residue was dissolved in 100 μl of carbon disulfide and 1 μl was injected into the chromatograph. As little as 1 μg per sample may be quantitated with this procedure.

For the determination of nonesterified cholesterol, it is pro-

[1] Hormel Institute, Austin, Minn.
[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Supplied by Dr. D. Dvornik, Ayerst Laboratories, St. Laurent, Quebec, Canada.
[5] Varian model 1800.
[6] Applied Science Laboratories, State College, Pa.
[7] Hamilton Co., Whittier, Calif.

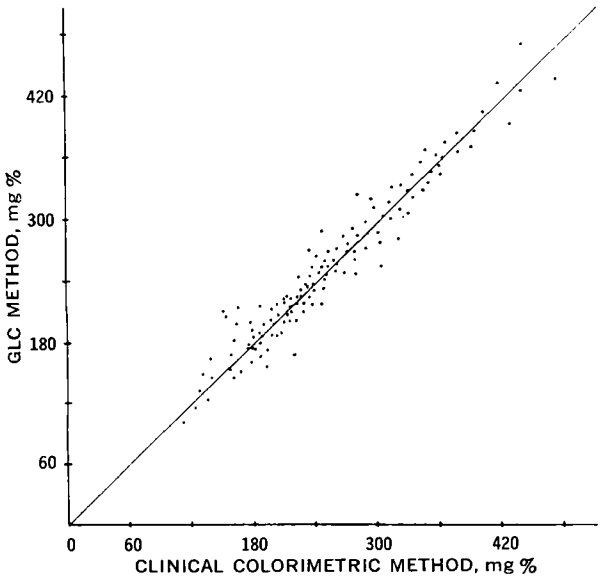

Figure 2—*Plot of the values of total plasma cholesterol performed by a clinical colorimetric method and the GLC method. The line represents a perfect correlation between the two methods. See text for details and statistical evaluation.*

posed that 95% alcohol be substituted for the alcoholic potassium hydroxide solution in the extraction and that the alcohol solution not be heated. Otherwise the procedure would be the same as described. To determine the stability of a cholesteryl ester with this procedure, cholesteryl acetate was dissolved in 95% alcohol in a concentration of 0.2 mg/ml, 10 ml of this solution and 1 ml of water were extracted with 20 ml of petroleum ether, and then 10 ml of water was added as before. Five milliliters of the petroleum ether layer was evaporated to dryness, and then 2 ml of acetone–petroleum ether (1:1) was added. One microliter was injected into the chromatograph to determine if any cholesterol was freed by hydrolysis of the cholesteryl acetate in the procedure.

Solutions of desmosterol and lanosterol were prepared in petroleum ether in concentrations of approximately 0.2 mg/ml. One microliter of these solutions was injected into the chromatograph to determine retention times relative to cholesterol.

Blood samples were obtained from 152 patients[8] for whom plasma cholesterol analysis had been ordered as part of a regular clinical workup. Venous blood samples were obtained and immediately heparinized. The sample was centrifuged, and the plasma was removed and subjected to analysis for cholesterol. Part of the plasma was sent to the clinical laboratory for total cholesterol analysis[9]. The rest of the plasma was subjected to the GLC method for total cholesterol analysis already described.

Three human subjects volunteered 40 ml of blood each from which the plasma was separated. Nineteen determinations of total cholesterol were performed on each volunteer's plasma, using 1 ml of plasma for each determination with the GLC method.

RESULTS AND DISCUSSION

A linear relationship was obtained when the peak height ratio of cholesterol-11-ketoprogesterone was plotted against cholesterol concentration over a cholesterol concentration range of 2.5–20 mg/100 ml in petroleum ether. These concentrations corresponded to total cholesterol plasma levels of 50–400 mg/100 ml with the extraction procedure described. The correlation coefficient of the peak height ratio to cholesterol concentration was 0.997, the standard error of the estimate was ±0.111, and the intercept was 0.034 with 16 determinations, four at each of four concentrations.

Similar linear standard curves were obtained with other ranges of cholesterol concentration in petroleum ether, including one range as low as 0.05–0.4 μg/ml. In this low range, 15 ml of standard petroleum ether solutions of cholesterol was evaporated along with an appropriate amount of internal standard solution and the residue was taken up in 100 μl of carbon disulfide by vortex agitation. Carbon disulfide was used as the solvent for injection into the chromatograph because of its low response in the flame-ionization detector. This allows detection of the very small quantities of cholesterol and internal standard injected (7.5–60 ng) at very sensitive electrometer settings. As little as 1 μl of plasma can be the sample size if this procedure is used. The sensitivity of this procedure compares quite well with those utilizing fluorometric techniques (3–5).

Table I—Reproducibility of Total Cholesterol by GLC

Plasma sample	1	2	3
Number of determinations	19	19	19
$\bar{X}$, mg %	161	172	184
SEM	±0.9	±1.0	±1.1
Range, mg %	153–170	162–181	176–192

A single peak with a retention time of 3.6 min was obtained for cholesterol in the gas chromatogram under the conditions stated. The internal standard, 11-ketoprogesterone, gave a single peak with a retention time of 4.2 min. A typical chromatogram from a plasma sample is shown in Fig. 1. Both peaks are quite symmetrical, and little interference in the plasma appears in the chromatogram. Progesterone has also been used successfully as an internal standard in the procedure with similar results to those obtained with 11-ketoprogesterone. It had a retention time of 3.1 min. Under certain circumstances it may be desirable to use progesterone instead of 11-ketoprogesterone for the internal standard.

Cholesteryl acetate had a retention time of 4.3 min under the GLC conditions described for cholesterol. No detectable cholesterol was liberated from the cholesteryl acetate during the extraction, evaporation, and injection procedures. Other endogenous cholesteryl esters should be as stable as the acetate and have even longer retention times. Therefore, the assay conditions described for nonesterified cholesterol appear to be reliable. Progesterone should be used as an internal standard rather than 11-ketoprogesterone since cholesteryl acetate has a similar retention time to 11-ketoprogesterone.

Desmosterol produced one major peak, with a retention time of 4.2 min, and two smaller peaks at 4.9 and 6.5 min. Obviously, progesterone is the internal standard to use rather than 11-ketoprogesterone in cholesterol assays where significant quantities of desmosterol may be encountered. Lanosterol injection gave two peaks of similar magnitude at 6.1 and 6.3 min. All peaks resulting from desmosterol and lanosterol injection are clearly separated and have longer retention times than cholesterol. Other GLC methods reported (2, 6–9) utilize SE-30 as the liquid phase. Utilization of this phase requires a much lower operating temperature, which leads to a lengthy retention time for cholesterol. A shorter column or a lower percent of liquid phase may be used to overcome this disadvantage, but resolving capacity is seriously lost. In this study, OV-17 was superior to SE-30 as a liquid phase for resolving cholesterol-related compounds. The multiple peaks seen after injection of desmosterol and lanosterol may have been due to impurities or thermal degradation.

The accuracy of the total cholesterol method reported here was assessed by comparing values with those obtained by a standard clinical laboratory method[9] on 152 human plasma samples. The values ranged from 100 to 472 mg % by the colorimetric procedure (Fig. 2). The line represents a would-be perfect correlation. The sample correlation coefficient obtained by a linear regression analysis of the two methods was 0.969, the standard error of the estimate was ±15.2, and the intercept was −7.14. The accuracy of the GLC total cholesterol method appears to be excellent, assuming that the colorimetric procedure measures a true value of total cholesterol.

To test the precision of the method, the GLC total cholesterol procedure was performed repetitively on each of three large plasma samples obtained from volunteers (Table I). The high degree of reproducibility indicates an adequate level of precision of the method for most purposes.

[8] Los Angeles County-University of Southern California Medical Center.

[9] By the Technicon N-24a procedure, an AutoAnalyzer procedure which utilizes a ferric chloride reagent.

The high temperatures used in the GLC method may lead to thermal degradation of cholesterol, especially since the 3β-hydroxyl group is not derivatized. However, no evidence for thermal degradation was observed in this study. A saturable adsorptive phenomenon for cholesterol in the column was not observed. Others reported this to be a problem (2, 6, 9). If loss on the column occurs in the method reported here, it does not manifest itself in poor accuracy or precision (Fig. 2 and Table I).

A technician may, starting with whole blood, obtain the results for total cholesterol on at least 40 samples in a working day. This assumes that manual techniques are utilized and only one column of a gas chromatograph is available. One column in these laboratories has been satisfactorily utilized for more than 1 year under the conditions specified.

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[x] To whom inquiries should be directed.

Correlation of Plasma Ibuprofen Levels with Biological Activity

DAVID G. KAISER [x] and E. MYLES GLENN

Abstract □ Investigations with ibuprofen [(±)-*p*-isobutylhydratropic acid], a well-tolerated, orally active, anti-inflammatory drug, were undertaken to: *(a)* determine the relationship among plasma drug concentrations, administered dose, and anti-inflammatory activity in developing and established polyarthritis in rats; and *(b)* compare the plasma drug disappearance half-lives in normal and polyarthritic rats. The results indicated that plasma drug concentrations in normal and polyarthritic rats were dose related. The logarithm of biological activity expressed as [% IPA/(100 − % IPA)], where % IPA is the mean percent inhibition of developing or established polyarthritis as measured by plasma inflammation units, was related to the logarithms of: *(a)* administered dose (mg kg^{-1}), *(b)* plasma drug concentrations (μg ml^{-1}) at 2 hr postadministration, and *(c)* average plasma drug concentrations (μg ml^{-1}) in a dosage interval at the equilibrium state. Half-lives for elimination of ibuprofen from the plasma of normal rats (after single-dose oral drug administration) and polyarthritic rats (after 29 doses) were essentially identical.

Keyphrases □ Ibuprofen—plasma levels correlated with biological activity □ Biological activity—correlated with ibuprofen plasma levels □ Anti-inflammatory agents—ibuprofen plasma levels correlated with biological activity

Ibuprofen[1] [(±)-*p*-isobutylhydratropic acid] (I) is a well-tolerated, orally active, anti-inflammatory agent utilized for the treatment of rheumatoid arthritis (1–3). The pharmacology, toxicology, and aspects of the absorption, distribution, and metabolism of I were reported previously (4–6). Recently, a sensitive and specific GLC procedure was described (7) for the determination of I in plasma. The present studies were conducted to: *(a)* determine the relationships among plasma I concentrations, orally administered dose, and anti-inflammatory activity in developing and established polyarthritis in rats; and *(b)* compare the elimination half-lives of I after single-dose administration to normal rats and after multiple-dose administration to rats with established polyarthritis.

EXPERIMENTAL

Rats with Developing Polyarthritis—Sixty Badger male rats (~235 g) were made polyarthritic by the intradermal injection into the tail of 0.5 mg of dead *Mycobacterium butyricum*[2] in 0.1 ml of mineral oil on Day 0. On Day 1, the animals were divided into six groups of 10 each. The groups received 0, 4.2, 9.0, 17.2, 33.6, or 67.2 mg I/kg, respectively, as a 1-ml aqueous suspension orally, bid for 14 days. On Day 15 all animals were weighed and scored. Five rats from each group received an additional (29th) oral dose of I, 2 hr prior to sacrifice. All animals were exsanguinated, and plasma specimens were obtained for the measurement of plasma inflammation units. Plasma aliquots from the animals receiving the 29th dose were stored at −18° until analyzed for I.

Rats with Established Polyarthritis—A series (180) of Badger male rats (~230 g) was made polyarthritic as described. On Day 14, the animals were visually scored, weighed, and divided into four groups of 45 each. Beginning on Day 15, the groups received 0, 16.4, 34.6, or 72.4 mg I/kg, respectively, as a 1-ml aqueous suspension orally, bid for 14 days. After 28 doses (Day 28), animals were visually scored and weighed. Beginning with the 29th dose (Day 29), two to five rats per group were sacrificed and exsanguinated at 0, 1, 2, 3, 4, 6, 8, 12, and 24 hr. Plasma specimens were

[1] Motrin, The Upjohn Co.; Brufen, The Boots Co. Ltd.

[2] Difco.

The high temperatures used in the GLC method may lead to thermal degradation of cholesterol, especially since the 3β-hydroxyl group is not derivatized. However, no evidence for thermal degradation was observed in this study. A saturable adsorptive phenomenon for cholesterol in the column was not observed. Others reported this to be a problem (2, 6, 9). If loss on the column occurs in the method reported here, it does not manifest itself in poor accuracy or precision (Fig. 2 and Table I).

A technician may, starting with whole blood, obtain the results for total cholesterol on at least 40 samples in a working day. This assumes that manual techniques are utilized and only one column of a gas chromatograph is available. One column in these laboratories has been satisfactorily utilized for more than 1 year under the conditions specified.

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[2] Difco.

Table I—Plasma Inflammation Units and Plasma I Concentrations in Rats with Developing Adjuvant-Induced Polyarthritis 2 hr after the Last Dose of a Multiple-Dose Regimen

Dose of I, Orally, bid, mg kg^{-1}	Plasma Inflammation Units[a]	Percent IPA[b]	Plasma I Concentrations[c], μg ml^{-1}
0	60.0 ± 4.6	0	0
4.2	46.0 ± 3.0	23	3.50 ± 0.27
9.0	46.0 ± 2.8	23	9.81 ± 0.95
17.2	32.0 ± 3.7	47	17.02 ± 3.11
33.6	27.0 ± 2.4	55	29.36 ± 5.61
67.2	21.0 ± 4.0	65	50.27 ± 10.56

[a] Mean ± *SEM* for 10 rats per group. [b] Percent inhibition of polyarthritis. [c] Mean ± *SEM* for five rats per group.

obtained for the measurement of plasma inflammation units and the remaining portions were stored at −18°.

Normal Rats—Forty-five Carworth male rats (~255 g), housed in screen-bottomed holding cages, were fasted for 16 hr prior to oral administration of 19.6 mg I/kg as a 1-ml aqueous suspension. Animals were fasted an additional 4 hr, after which food was returned *ad libitum*. Groups of five rats each were sacrificed and exsanguinated at 0, 1, 2, 3, 4, 6, 8, 12, and 24 hr. Plasma specimens were collected and stored at −18°.

Determination of Plasma Inflammation Units—The procedure for the determination of plasma inflammation units in the adjuvant-induced polyarthritic rat described previously (8) was employed.

Determination of Plasma I Concentrations—The GLC procedure for the determination of I in plasma reported previously (7) was used.

RESULTS AND DISCUSSION

Observed plasma inflammation units and plasma I concentrations 2 hr after the last (29th) dose in rats with developing polyarthritis are summarized in Table I. Table II shows observed plasma inflammation units and plasma I concentrations over the 0–24-hr interval after the last (29th) dose in rats with established polyarthritis, as well as plasma I concentrations after single-dose administration in normal rats.

Figure 1 indicates that both the 2-hr plasma I concentrations and the mean plasma I concentrations over the 0–12-hr dosage interval were linearly related to dose. Similar observations were reported previously for the anti-inflammatory agents indoxole (9) and 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (10) in polyarthritic rats. In the present study, plasma I concentrations in developing and established polyarthritic rats after multiple-dose administration were not different from those in normal rats after a single dose (Fig. 1), indicating no effect of the polyarthritis or of the chronic dosage regimen on the pharmacokinetics of I *(vide infra)*.

Earlier studies with indoxole (9) and 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (10) showed that inhibition of polyarthritis in chronically treated rats was linearly related to log dose and log mean circulating drug levels over the dosage interval. Similar treatment of the data for I (Tables I and II), covering a wide range of doses and plasma I concentrations, gave sigmoid curves. Wagner (11) suggested linearization of such data by the use of Eq. 1:

$$\log\left[\frac{\%\ \text{IPA}}{100 - \%\ \text{IPA}}\right] = s \log A + \log Q \qquad \text{(Eq. 1)}$$

where % IPA is percent inhibition of polyarthritis, A is dose or circulating drug concentration, and Q is [% IPA/(100 − % IPA)] at unit value of A. Linear least-squares regression analysis of the data from Tables I and II according to Eq. 1 is shown in Fig. 2. Separate analyses of the data from rats with developing and with established polyarthritis gave slopes and intercepts not significantly different, permitting the data to be pooled. From Fig. 2, it may be calculated that 50% inhibition of polyarthritis was produced by an oral dose of 28.3 mg kg^{-1} bid, by a 2-hr plasma I concentration at equilibrium of 21.7 μg ml^{-1}, and by a mean plasma I concentration over the dosage interval of 6.95 μg ml^{-1}. Of considerable interest is the fact that I was equipotent when given during the development of polyarthritis or following establishment of the polyarthritis.

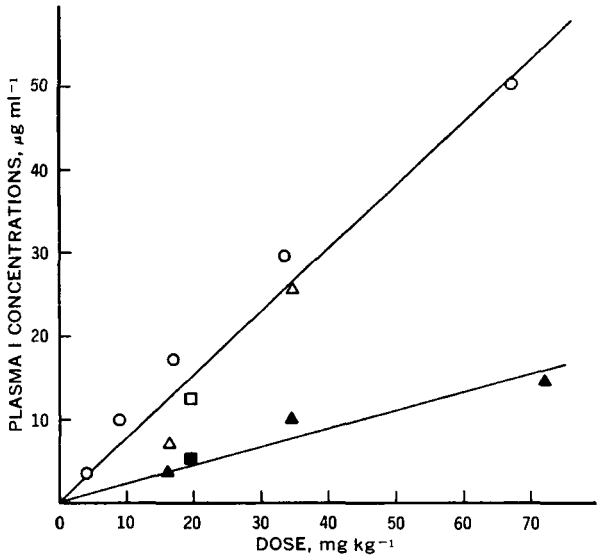

Figure 1—*Linear least-squares regressions of 2-hr plasma I concentrations (open symbols) and mean plasma I concentrations, 0–12 hr (closed symbols),* versus *dose. Key: ○, rats with developing polyarthritis; △,▲, rats with established polyarthritis; and □, ■, normal rats.*

Recent publications (12–16) have shown that marked reductions in liver microsomal drug-metabolizing enzyme systems occur during the development of adjuvant-induced polyarthritic lesions in rats. Quevauviller *et al.* (12) observed a prolongation of sleeping times after the administration of phenobarbital, pentobarbital, and thiopental. Morton and Chatfield (13) found that liver microsomal *N*-demethylase and $NADPH_2$-oxidase activities as well as P-450 levels were greatly reduced. The maximum tolerated oral dose of phenobarbital was lower than in normal rats. In addition, in rats with established polyarthritis, decreased amounts of urinary β-glucuronide and sulfate conjugates, and increased amounts of unconjugated drug were observed following

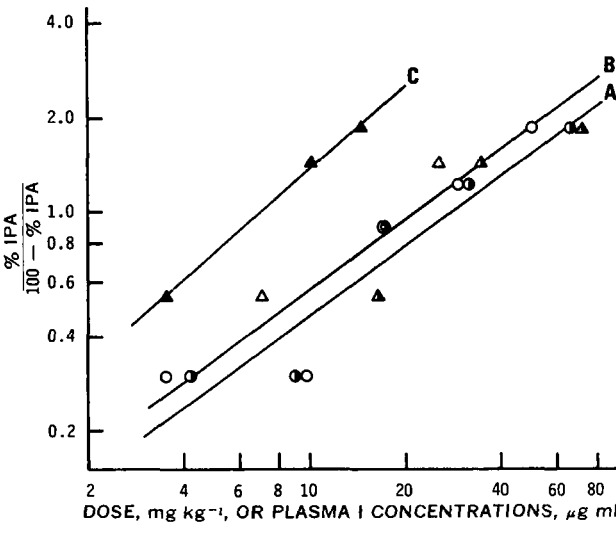

Figure 2—*Linear least-squares regressions of log [% IPA/(100 − % IPA)]* versus *log dose (mg kg^{-1}, hatched symbols, A), log 2-hr plasma I concentrations (μg ml^{-1}, open symbols, B), and log mean plasma I concentrations, 0–12 hr (μg ml^{-1}, closed symbols, C) after multiple-dose administration in rats with developing polyarthritis (circles) and with established polyarthritis (triangles).*

Table II—Plasma Inflammation Units and Plasma I Concentrations in Rats with Established Adjuvant-Induced Polyarthritis after the Last Dose of a Multiple-Dose Regimen and in Normal Rats after a Single Dose

	Polyarthritic Rats[a]			Normal Rats
Dose, mg kg^{-1}	16.4	34.6	72.4	19.6
Plasma Inflammation Units[b]	32.1 ± 3.4	20.1 ± 1.5	17.4 ± 2.4[c]	—
Percent IPA[d]	35	59	65	—
Hours	Plasma I Concentrations, μg ml^{-1}[e,f]			
0	0	0	1.88	0
1	14.99 ± 2.68	63.62 ± 16.50	49.20	30.88 ± 1.97
2	7.00 ± 1.07	25.51 ± 4.11	82.20	12.46 ± 1.94
3	5.19 ± 0.38	15.49 ± 4.06	15.08	5.96 ± 0.43
4	4.62 ± 1.20	5.48 ± 1.26	5.52	6.59 ± 1.55
6	2.03 ± 0.37	2.25 ± 0.60	6.24	1.35 ± 0.27
8	1.41 ± 0.79	1.15 ± 0.54	2.06	0.67 ± 0.17
12	0	0	0	0
24	0	0	0	0
$\int_0^{12} C\,dt$, μg ml^{-1} hr[g]	42.40	120.8	174.4	63.90
$\bar{C}$, μg ml^{-1}[h]	3.53	10.07	14.53	5.33
$T_{1/2}$, hr[i]	2.47	1.40	2.09	1.41

[a] I administered orally, bid for total of 29 doses. [b] Mean ± *SEM* for 45 rats per group except as noted. [c] Mean ± *SEM* for 17 surviving rats. At high dosage level (72.4 mg kg^{-1}), 28 of 45 animals died before Day 29. [d] Percent inhibition of polyarthritis. [e] Mean ± *SEM* for five rats per sampling except at highest dose where values are mean for two rats at each time. [f] Values <0.5 μg ml^{-1} are reported as 0. [g] Integrated area under the plasma I concentration curve over the 0–12-hr dosage interval calculated by the trapezoidal rule. [h] Mean plasma I concentration over the 0–12-hr interval. [i] Half-life for elimination of I from terminal portion (3–8 hr) of plasma concentration curves.

oral administration of acetaminophen (paracetamol). Zak *et al.* (14) observed a greatly prolonged hexobarbital sleeping time and reduced liver microsomal benzpyrene hydroxylase activity. These effects were reversed by treatment with the anti-inflammatory drugs, phenylbutazone, indomethacin, and flufenamic acid, which also improved the arthritic conditions of the animals. Treatment with phenobarbital reversed the enzymatic effects but did not affect the arthritis. Beck and Whitehouse (16) reported depressed demethylation of *N,N*-dimethylaniline or aminopyrine as well as impaired metabolism of cyclophosphamide.

To determine the possible effects of polyarthritis as well as multiple-dose administration of I on the rate of metabolism of I, drug elimination half-lives were determined from the data in Table II by linear least-squares regression analysis of the terminal (3–8-hr) portions of the log plasma I concentration *versus* time curves. Half-lives for elimination of I in normal rats and in polyarthritic rats after 29 doses were essentially identical. It may be concluded that if metabolism of I was inhibited by the adjuvant-induced arthritis, the inhibition was reversed by the anti-inflammatory effects of the drug. Multiple-dose administration of I did not appear to induce its own metabolism.

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[x] To whom inquiries should be directed.

Intestinal Motor Effects of a Unique Analgesic Agent

GARY R. JACOBS and THOMAS F. BURKS[x]

Abstract □ The effects of 2,3,5,6,11,11b-hexahydro-11,11b-dimethyl-14-indolizino(8,7,b)indole hydrochloride on intestinal motility were investigated. The indole derivative altered contractile activity in each preparation studied. Intestinal contractions *in situ* in anesthetized dogs were stimulated, but variable mild stimulatory or inhibitory effects occurred in cats and monkeys. Spontaneous contractions of monkey intestine were inhibited *in vitro*. Dose-related increases in motility were produced in dog intestine *in vitro*, although the experimental compound was considerably less potent than morphine as an intestinal stimulant. The stimulatory effects of the indole derivative in dog intestine were antagonized by atropine and by cyproheptadine. Indolazinoindole also inhibited propulsion of a test meal in mice *in vivo*.

Keyphrases □ 2,3,5,6,11,11b-Hexahydro-11,11b-dimethyl-14-indolizino(8,7,b)indole hydrochloride—intestinal motor effects studied in cats, dogs, and monkeys □ Analgesics, investigational indolazinoindole—effects on intestinal motility investigated □ Indole derivatives—intestinal motility effects of investigational indolazinoindole studied in cats, dogs, and monkeys □ Intestinal motility—effects of indolazinoindole analgesic in cats, dogs, and monkeys

The indole derivative 2,3,5,6,11,11b-hexahydro-11,11b-dimethyl-14-indolizino(8,7,b)indole hydrochloride[1], referred to in this article as indolazinoindole, has been investigated as an analgesic agent in rats and monkeys (1). Administered orally to rats, indolazinoindole was more potent than morphine as an analgesic. Administered intramuscularly to monkeys, it was again more potent than morphine. Animal studies thus indicate that this compound is a potent analgesic agent of potential value in the management of pain. A major side effect associated with virtually all potent analgesic agents is constipation. The constipating effects of the narcotic drugs appear to be secondary to stimulation of intestinal smooth muscle: an increase in nonpropulsive, phasic contractions which diminish the aboral pressure gradient required for effective propulsion of intestinal contents (2, 3).

A potent analgesic agent that, in therapeutic doses, does not stimulate intestinal smooth muscle would be desirable for clinical use. The intestinal effects of indolazinoindole were examined in four species, its intestinal stimulating potency was compared with that of morphine, and initial studies of its mechanism of intestinal actions in dog isolated intestine were performed.

EXPERIMENTAL

Propulsion of Test Meal in Mice—The effects of indolazinoindole on intestinal propulsion were tested in young adult male Swiss-Webster white mice weighing 24–28 g (4). The mice were divided into two groups of six and were administered 1 ml of a suspension of powdered charcoal in glycerin using a gastric tube. After 10 min, the mice were given intraperitoneal injections of saline (control group) or the test compound. The dose of indolazinoindole was 10 mg/kg; both it and saline were administered in volumes of 0.01 ml/g. The animals were sacrificed by cervical dislocation 20 min after drug or saline administration. The small bowel from the stomach to the cecum was removed and measured, and the percent of the bowel traveled by the charcoal marker was calculated.

Table I—Effects of Indolazinoindole on Passage of a Charcoal Meal in Mice

Treatment	Percent Passage	Confidence Limits (95%)
Saline	74.1	62.2–86.0
Indolazinoindole (10 mg/kg)	49.5	41.2–57.9

Intestinal Motility *In Situ*—Dogs, cats, and rhesus monkeys were anesthetized with sodium thiopental (15 mg/kg) and sodium barbital (250 mg/kg) administered intravenously. Femoral venous catheters were inserted for drug administration. Small and large bowel was exposed through a short midline abdominal incision, and the bowel was traced from the duodenal bulb to the cecum for accurate selection of recording sites. Latex balloons (undistended volume was 9 ml) attached to 8 Fr. nasogastric tubes were placed in ascending colon, ileum, jejunum, and duodenum. Each balloon was inserted through a 5-mm incision in the antimesenteric border of the bowel and threaded 8–12 cm from the site of the incision. A pursestring suture was employed to close the bowel incision and to secure the tube to the gut wall. Each balloon was filled with either 2 ml (in dogs) or 1 ml (in cats and monkeys) of water. The water-filled tubes were attached to pressure transducers[2], and intraluminal pressures were recorded on an oscillographic recorder[3]. The abdominal wound was closed with wound clips. All drugs were administered intravenously.

Intestinal Motility *In Vitro*—Dogs and monkeys were anesthetized as already described with thiopental and barbital. Cannulated mesenteric arteries of 8–10-cm sections of small intestine were perfused by means of a peristaltic perfusion pump[4]. The perfusion solution was warmed Krebs bicarbonate bubbled with 95% oxygen–5% carbon dioxide. Ligatures were placed around the intestine on both sides of the perfused arcade, and the segment was removed from the animal. Intraluminal pressure was measured from a latex balloon containing 2 ml (in dogs) or 1 ml (in monkeys) of water and was connected to a pressure transducer[2] by a water-filled 8 Fr. nasogastric tube. Perfusion pressure was measured by a pressure transducer[2] from a T-connection between the pump and the artery. Intraluminal pressure and perfusion pressure were recorded by an oscillographic recorder[3]. The rate of the constant-flow perfusion pump was set to produce a mean perfusion pressure of 60–70 mm Hg. Agonist drugs (indolazinoindole and morphine) were administered *via* the arterial cannula as boluses in volumes of 0.01–0.1 ml. Antagonist drugs (atropine and cyproheptadine) were added to the reservoir which contained the Krebs perfusion solution. Contractions of the isolated intestinal segments were measured as the increase in intraluminal pressure produced in response to the agonist drug.

Drugs and Chemicals—Drugs employed were indolazinoindole as the hydrochloride[5], morphine sulfate[6], atropine sulfate[7], and cyproheptadine hydrochloride[8]. Doses of all agents refer to the

[1] WY-12, 157.

[2] Statham P23Db.
[3] Beckman type RM dynograph.
[4] Sigmamotor type T-8.
[5] Wyeth.
[6] Merck.
[7] Baker.
[8] Merck, Sharp and Dohme.

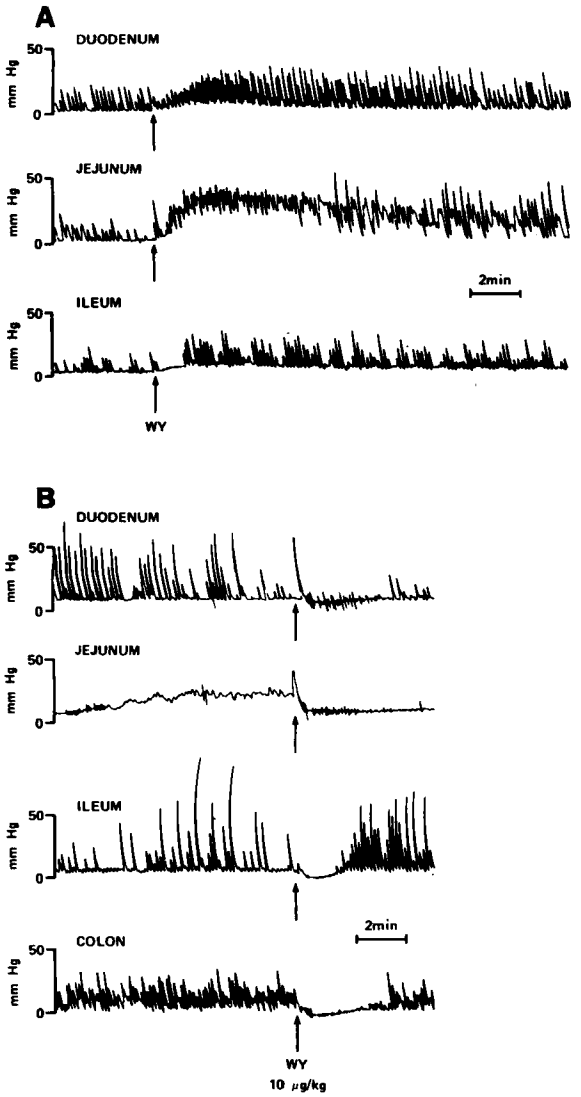

Figure 1—*Effects of indolazinoindole (WY) on intraluminal pressure of cat intestine* in situ. *Indolazinoindole was administered intravenously (10 μg/kg) at the arrows. In the cat illustrated in A, the analgesic agent stimulated motility in the duodenum, jejunum, and ileum. There was no effect on tone or contractions in the colon. In the cat illustrated in B, indolazinoindole produced inhibition of motility.*

salt forms. The composition of the Krebs bicarbonate solution was as follows (in millimoles): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.1; $MgSO_4$, 0.5; $NaHCO_3$, 25.0; and glucose, 10.0. Due to apparent instability of indolazinoindole in solution, it was made up fresh daily in aqueous solution.

RESULTS

Propulsion of Test Meal in Mice—In a dose of 10 mg/kg, indolazinoindole reduced transit of a charcoal meal in mice to approximately 67% of control (Table I). With this dose, no gross behavioral effects in mice were observed.

Intestinal Motility *In Situ*—*Cat*—In doses of 10–100 μg/kg, the test compound produced either inhibition or mild stimulation of intestinal motility in different cats. Three of the five cats tested responded with increases in motility, two with primarily inhibitory responses. Examples of stimulatory and inhibitory motility responses are illustrated in Fig. 1. Initial doses of indolazinoindole also tended to block the intestinal effects of subsequent doses. Thus, when administered first, the lowest dose (10 μg) often produced larger responses than higher doses (30 or 100 μg). For this reason, meaningful dose–response curves could not be established for this compound in individual cats. Comparison of initial doses, however, indicated that the larger doses produced relatively greater effects than did smaller doses.

Monkey—In doses of 10 and 100 μg/kg, indolazinoindole often, but not always, produced brief tonic increases in intraluminal pressure (range of 5–56 mm Hg) in the ileum and colon, but usually produced no increases in pressure in the duodenum or jejunum. Stomach motility was measured in one monkey and was not affected by indolazinoindole. When spontaneous motility in the bowel was pronounced, the test compound often produced a mild inhibition of ongoing contractions. An excitatory response to indolazinoindole in ileum is illustrated in Fig. 2.

Dog—Indolazinoindole stimulated the upper regions of the bowel *in vivo*. In the doses employed (10, 20, and 100 μg/kg), it rarely caused significant contractions in the ileum or colon (range of 2–6 mm Hg). The duodenal and jejunal responses were characterized by transient, small decreases in intraluminal pressure followed immediately by small to pronounced phasic contractions (Fig. 3). The baseline intraluminal pressure was often elevated slightly during periods of maximal phasic contractions. The phasic contractions tended to occur in bursts lasting 2–4 min, with in-

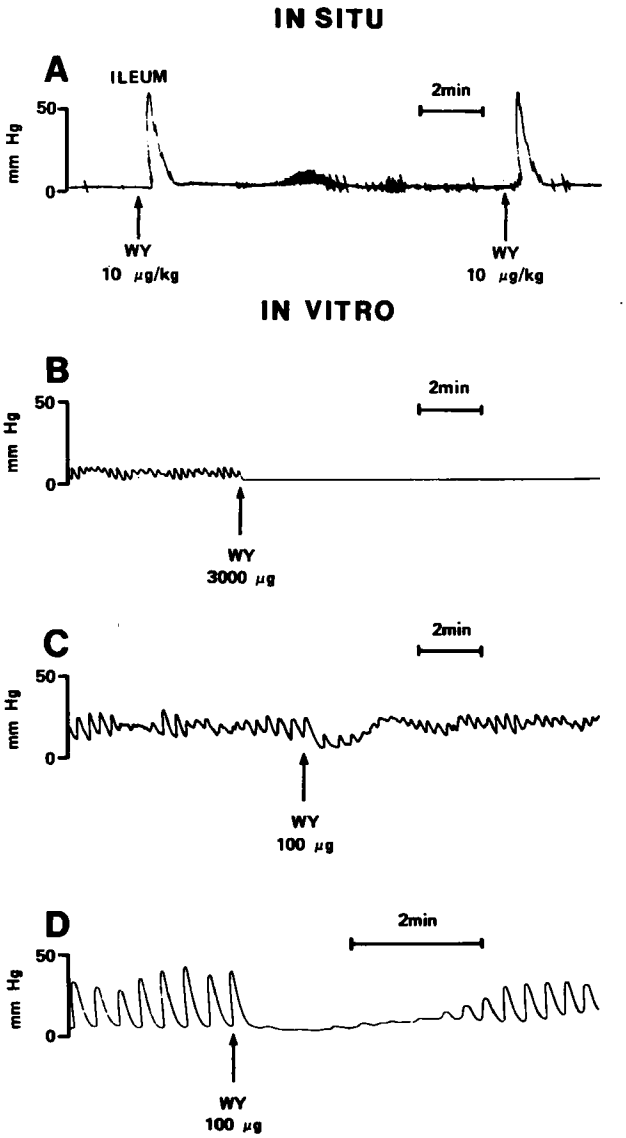

Figure 2—*Effects of indolazinoindole (WY) on intraluminal pressure of rhesus monkey small intestine* in situ *and* in vitro. *Record A illustrates the ability of indolazinoindole to produce brief tonic contractions of the lower small bowel. In this monkey, it had no effect on the duodenum or jejunum. Records B, C, and D illustrate responses of monkey isolated vascularly perfused small intestine to indolazinoindole.*

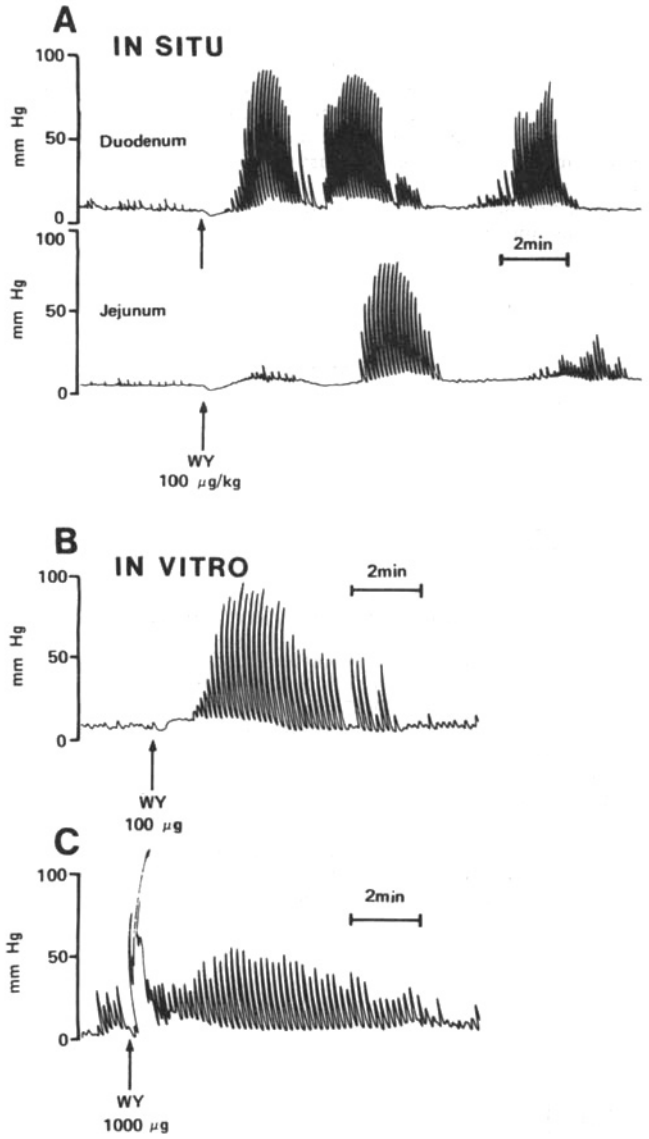

Figure 3—*Effects of indolazinoindole (WY) on intraluminal pressure of dog small intestine* in situ *and* in vitro. *Record A illustrates responses in the upper small bowel to indolazinoindole administered intravenously. In this dog, it had no effect on the ileum or colon. Records B and C illustrate responses of dog isolated vascularly perfused small intestine to two different doses of indolazinoindole.*

tervening periods of relative quiesence. These responses are similar to those produced in the dog by morphine.

Intestinal Motility *In Vitro*—*Monkey*—Indolazinoindole exerted primarily inhibitory effects on isolated gut segments; this effect was particularly prominent with doses of 100 μg or larger (Fig. 2). Except with doses of 1000 or 3000 μg, the period of inhibition was usually followed by recovery of intestinal tone and motility and was sometimes followed by enhanced rhythmic activity.

Dog—Indolazinoindole was studied most extensively in intestinal segments isolated from dogs. In bolus intraarterial doses of 0.1-3000 μg, the experimental agent produced dose-related increases in intraluminal pressure (Fig. 4). Responses to smaller doses (0.1-100 μg) consisted primarily of increases in the amplitude of rhythmic contractions. Larger doses (100–3000 μg) often produced tonic or sustained increases in intraluminal pressure, which were always followed by rhythmic contractions. These responses resembled those reported previously for morphine (5). On a weight basis, the test compound was found to be considerably less potent than morphine as an intestinal spasmogen, but the dose-response curves for the two drugs were of similar shape and the maximal responses were of equal magnitude (Fig. 4).

To obtain preliminary information about the mechanism of the intestinal stimulatory effect of indolazinoindole in dog intestine, studies were carried out with atropine and cyproheptadine. These were performed as paired experiments. Two intestinal segments were taken for each experiment; one was perfused with plain Krebs solution and the other with Krebs solution plus an antagonist. Dose-response curves for indolazinoindole were thus obtained in the absence and in the presence of atropine and cyproheptadine. Doses of indolazinoindole were administered in random order to rule out order effects. Intestinal stimulatory responses to the experimental analgesic were significantly reduced (Student's *t* test, paired comparison) in gut segments during perfusion with atropine or cyproheptadine (Fig. 4).

DISCUSSION

Effective separation of analgesic from smooth muscle actions of potent analgesic drugs is a highly desirable goal. The experimental indole derivative shows some promise in this regard. In both the monkey and the cat, the analgesic agent produced either mild stimulation or inhibition of intestinal motility. Its duration of intestinal action was quite limited in both species. The study in mice demonstrated that at fairly high dose levels (10 mg/kg), indolazinoindole can, however, inhibit intestinal propulsive activity. At this dose level, the analgesic could be expected to exert constipating effects. The intestinal stimulatory effects of indolaz-

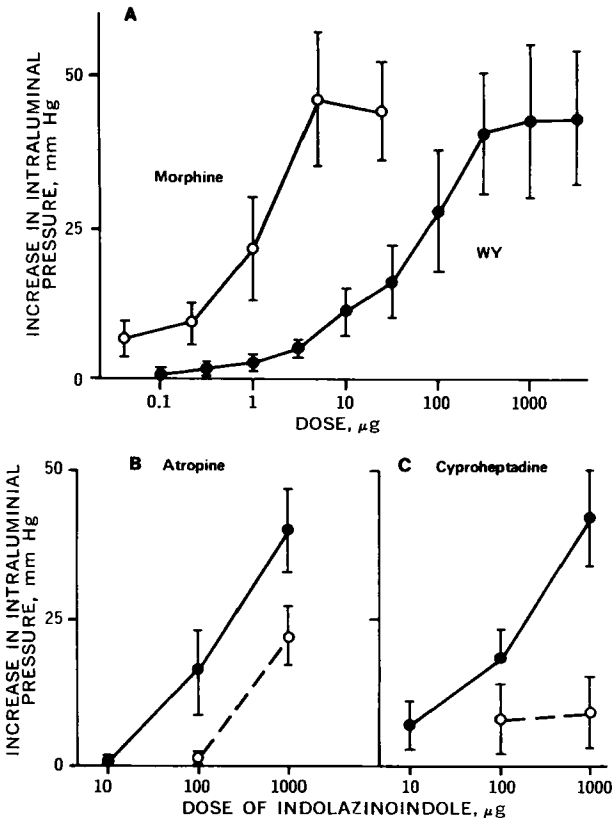

Figure 4—*Effects of indolazinoindole (WY) on intraluminal pressure of dog small intestine* in vitro. *Panel A shows dose–response curves for morphine and for indolazinoindole in dog isolated vascularly perfused intestinal segments. Each point is the mean (±SEM) of responses in preparations from six dogs. The potency ratio (with 95% confidence limits) of indolazinoindole in relation to morphine was 0.0195 (0.0136–0.1742). Panel B shows dose–response curves for indolazinoindole during perfusion with control Krebs solution (●) and with Krebs solution containing 0.1 μg/ml atropine (○). Panel C shows dose–response curves for indolazinoindole during perfusion with control Krebs solution (●) and with Krebs solution containing 1 μg/ml cyproheptadine (○).*

inoindole were more prominent in the dog. Large magnitude phasic contractions of the intestine are related to failure of intestinal propulsion, especially if the increase in nonpropulsive motility persists (2). However, indolazinoindole is less than one-fiftieth as potent as morphine as a stimulant of dog intestine. Since it may be approximately equipotent with morphine as an analgesic, it may offer significant advantages over morphine in terms of selectivity of action. It does possess the potential of causing constipation due to failure of propulsion, particularly at higher dose levels.

The relatively weak intestinal stimulatory property of indolazinoindole as compared to morphine may reflect its more pronounced inhibitory component of action. In cat and monkey intestine *in situ* and particularly in monkey intestinal preparations *in vitro*, inhibition of motility was often the primary response to indolazinoindole. Its actions in dog intestine may reflect the algebraic sum of morphine-like stimulatory effects and inhibitory effects as seen in monkey intestine. The inhibitory component of action could, therefore, account for the approximately 50-fold shift to the right in the dose–response curve in relation to morphine. In the cat intestine, the inhibitory effects of indolazinoindole sometimes outweighed its stimulatory effects. In the three species in which motility was measured, there was a spectrum of effects: primarily stimulatory in dogs, mixed in cats, and primarily inhibitory in monkeys. The decrease in meal propulsion in mice could possibly result either from stimulation of nonpropulsive contractions or from inhibition of propulsive intestinal contractions.

The ability of atropine and cyproheptadine to antagonize the intestinal stimulatory effects of indolazinoindole is significant. The narcotic analgesics are thought to produce their intestinal stimulatory effects by means of release of local 5-hydroxytryptamine from stores in the intestine (3, 5). The 5-hydroxytryptamine mobilized by the narcotics stimulates intestinal smooth muscle in two ways: by direct excitatory effects on smooth muscle 5-hydroxytryptamine receptors and indirectly by stimulation of cholinergic nerve elements in the wall of the intestine. The direct smooth muscle effect of 5-hydroxytryptamine can be reduced by 5-hydroxytryptamine receptor antagonists such as cyproheptadine (5). Since the indirect effect of 5-hydroxytryptamine is mediated in acetylcholine, the neural component of 5-hydroxytryptamine action can be antagonized by atropine (5). Cyproheptadine and atropine antagonized the intestinal stimulatory effects of indolazinoindole, and the intestinal motor effects of this compound may be mediated, at least in part, by 5-hydroxytryptamine.

Indolazinoindole thus appears to share with the narcotic analgesics the ability to affect intestinal smooth muscle, but its ratio of analgesic to intestinal stimulatory actions seems quite favorable. Potential contributions to constipation of the central components of indolazinoindole action remain to be determined.

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Abstract □ The bisquaternary ammonium drug decamethonium bromide was studied in bile salt solutions in regard to partitioning *in vitro* and lethality when absorbed from duodenal and ileal segments of rats. Sodium glycocholate, when present below or above its CMC, had little effect on the partitioning of decamethonium from a phosphate buffer into *n*-octanol. *In vivo*, no significant change in lethality (absorption) could be attributed to either the absence of endogenous bile (ligated bile duct) or the presence of normal amounts of bile (unligated bile duct). Bile salts do not appear to alter lethality (absorption) of this bisquaternary drug when given to rats *via* the enteral route.

Keyphrases □ Decamethonium bromide—effect of bile salts on partitioning and oral toxicity, rats □ Bile salts—effect on partitioning and oral toxicity of decamethonium bromide, rats □ Partitioning—effect of bile salts on decamethonium bromide in water–*n*-octanol system □ Toxicity, oral—effect of bile salts on decamethonium bromide lethality in rats

Surfactants influence the GI absorption of various drugs and nutrients (1). Polysorbate 80, above its critical micelle concentration (CMC), inhibits salicylamide absorption from the small intestine of the rat (2). A similar inhibitory effect was shown for nitrobenzene in a micellar sodium lauryl sulfate system (3). Bile salts, being endogenous surfactants, also affect GI absorption of drugs, but the properties of bile salt–drug combinations vary, depending upon the state of aggregation of the bile salts. For example, the absorption of imipramine from the rat jejunum and ileum can be reduced in the presence of

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micellar (aggregated) sodium taurocholate (4). Similar effects have been observed with a diethylaminoethylbenzamide compound (5) and with isopropamide iodide (6).

Some bile salts, below their CMC's, complex with various monoquaternary compounds to give products that partition into ethylene dichloride (7, 8) or *n*-octanol (6). In *in vivo* studies, cholic acid was shown to enhance the pharmacological response to a quaternary hypotensive agent when the two were administered concurrently (9).

Previously, micellar solutions were reported to inhibit partitioning of a monoquaternary compound (isopropamide iodide) from an aqueous to an organic phase (6). GI absorption of this drug is reduced, but not markedly, in the presence of micellar sodium glycocholate. The bisquaternary ammonium drug decamethonium bromide was chosen to be studied in relation to its tendency to interact in solution below and above bile salt CMC values. Thus, the ability of the doubly charged bisquaternary to interact like the monoquaternary can be evaluated. Oral (enteral) toxicity of the erratically absorbed (10) decamethonium was studied since the quantal response of death was readily measurable as a definite end-point of this drug's action. Water–octanol partitioning in the presence of various bile salts was also studied to test if an aggregate was present that could reduce transfer of decamethonium from an aqueous to a lipid phase.

EXPERIMENTAL

Materials—The following bile salts were used as received: the sodium salts of glycocholic and taurocholic acids[1] and sodium cholate[2], deoxycholate[2], and dehydrocholate[2]. *n*-Octanol[2] (Grade I) was used as the organic phase in the partitioning studies because of its potential to model cellular membranes (11). Aqueous solutions for partitioning with or without bile salts were prepared in pH 7.4 phosphate buffer composed of 0.022 *M* $NaH_2PO_4 \cdot H_2O$ and 0.104 *M* Na_2HPO_4.

Partitioning of decamethonium bromide (8×10^{-4} *M*) between the aqueous phase and an equal volume of *n*-octanol was carried out for 24 hr at room temperature using ^{14}C-labeled decamethonium[3]. A sample from both phases was counted by liquid scintillation[4]. Sampling at various times past 24 hr indicated equilibrium had been attained.

Solutions for the *in vivo* investigations were in the same buffer. Osmolarity was checked with an osmometer[5].

Decamethonium Administration—Male albino rats[6], weighing 200 ± 25 g and fasted overnight, were used. Following ether anesthesia, a midline incision was made. A segment of either duodenum or ileum was identified and ligated with two (00) silk sutures placed approximately 20 cm apart. These two areas were selected because bile salts are passively absorbed in all areas of the intestine and are actively transported in the ileum (12).

Drug solutions were coded and animals were assigned to treatment groups according to a completely random design. With a 27-gauge (0.5-in.) needle, 1.0 ml of the appropriate drug solution (with or without bile salt) was then injected into the segment. Gentle digital pressure over the injection site assured no solution leakage. All animals recovered from anesthesia within a few minutes. In some experiments, bile duct ligations were performed 48 hr prior to administration of the drug solutions to eliminate any possible influence of endogenous bile salts. The ligations were placed on the common bile duct at its exit from the liver, using (00) silk suture. Ligating at this level ensured that pancreatic secretions would not be interfered with. Sham operations were performed on animals with unligated common bile ducts.

Decamethonium was used at a standard dose of 25, 50, or 75 mg/animal. This amount is equivalent to 125, 250, and 375 mg/kg for a rat with an average weight of 200 g. Animals were observed for symptoms of toxicity; when death occurred, the time difference between drug injection and death was recorded as the "death time."

In one set of experiments, neomycin sulfate was used to disperse mixed micelles of bile salt, fatty acids, and decamethonium that may have been formed in the intestinal lumen.

[1] Mann Research Labs., New York, N.Y.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Amersham-Searle.
[4] Model 2002, Packard Instruments, Downers Grove, Ill.
[5] Osmette-S Automatic Osmometer, Precision Systems, Waltham, Mass.
[6] Rolfsmeyer Farms, Madison, Wis.

Table I—Partitioning of Decamethonium Bromide (8×10^{-4} *M*) into *n*-Octanol at pH 7.4 and 25°

Bile Salt Concentration[a], m*M*	Decamethonium Partitioned, %
None (control)	0.35
0.1	0.55
1.0	0.81
10.0	3.31
100.0	5.52

[a] Sodium glycocholate.

RESULTS AND DISCUSSION

Essentially no partitioning (<0.35%) of the decamethonium bromide from the pH 7.4 buffer to the *n*-octanol phase occurred in the absence of bile salts (Table I). Concentrations of sodium glycocholate below its CMC (<10 m*M*) also had little effect. If lipid-soluble decamethonium–bile salt ion-pairs or complexes were present, they should have been extractable with some organic solvent system. Ethyl acetate, ethylene dichloride, ether, butanol, and triolein instead of octanol were used for this purpose, but no decamethonium partitioned. These results agree with those found for hexamethonium (13) with ethylene dichloride as the organic phase. Bile salt concentrations above the CMC allowed about 5.5% of the decamethonium to partition into the octanol. Slight emulsification may account for this small amount transferred. This is unlike the effects seen with the monoquaternary isopropamide iodide where the presence of sodium glycocholate and other bile salts enhanced partitioning from the aqueous phase into *n*-octanol (6).

Micellar sodium glycocholate had no significant effect on the toxicity of decamethonium (Table II). Likewise, other micellar bile salt solutions and neomycin had no significant effects (Table III).

One might expect that micellar bile salt solutions would reduce the degree of absorption of decamethonium *via* mixed aggregation or "micelloid" formation (14), as has been shown for a system of sodium taurocholate and a C_{12} quaternary ammonium salt (15). These interactions would be extremely dynamic, however, and the *in situ* presence of mucin or residual food particles might also contribute to the variability of the response (16).

Neomycin sulfate has been shown to disrupt mixed micelles in solutions containing bile salts (17). If decamethonium interacts with aggregated bilt salts, then neomycin sulfate should destroy these aggregates and provide more free drug in solution to be absorbed by the intestinal epithelium. Pretreatment with neomycin sulfate had no effect on toxicity of decamethonium, suggesting little or no involvement of bile salt aggregates to retard absorption of this drug.

Decamethonium does not appear to form a lipid-soluble ion-pair or complex that will partition from an aqueous buffer *in vitro* or that will alter the oral toxicity of this drug in rats. The extraction of the monoquaternary isopropamide molecule was shown (6) to be enhanced by the presence of bile salts at concentrations below their CMC's but not at higher concentrations; however, bile salts at all concentrations appeared to diminish the absorp-

Table II—Death Times (Minutes) following Administration of Decamethonium Bromide to Rats with or without Common Bile Duct Ligation[a]

Average Dose of Decamethonium Bromide, mg/kg	Ligated Bile Duct		Unligated (Intact) Bile Duct	
	Duodenum	Ileum	Duodenum	Ileum
Control[b]	No death	No death	No death	No death
125	29.5 ± 4.0 (6)[c]	35.2 ± 9.4 (6)	18.0 ± 4.5 (3)[d]	32.5 ± 18.1 (6)
250	18.8 ± 5.9 (6)	28.0 ± 6.7 (6)	9.6 ± 1.4 (5)[e]	31.5 ± 13.8 (6)
375	18.0 ± 4.0 (6)	23.6 ± 6.7 (6)	24.3 ± 8.8 (6)[f]	17.0 ± 8.5 (6)

[a] All comparisons between conditions at each dose level and over all doses were shown to be not significant at $p = 0.05$, using a Wilcoxon rank method for determining significance between two treatments with unpaired replicates (18). [b] Control = 1.0 ml of pH 7.4 sodium phosphate buffer; six animals were used. [c] Numbers in parentheses indicate the number of animals used to calculate the average death time listed (±*SEM*) and the number of animals that died out of the six given this treatment. [d] Average weight of the three animals that died was 222.3 g; average weight of the three that survived was 217.3 g. [e] Average weight of the five animals that died was 204.8 g. [f] These six animals had an average weight of 219.3 g. Correlations were attempted between body weight and death time, as well as between exact intestinal length or the presence of residual food particles and death time. No correlations existed between any given parameter and death time in any group.

Table III—Effects of Micellar Bile Salt Solutions[a] and Neomycin Sulfate on the Oral (Duodenal) Toxicity of Decamethonium Bromide[b] in Rats

Micelle Forming or Dispersing Agent	Average Time of Death[c] (±*SEM*), min
None (control)	12.5 ± 4.9 (6)
Neomycin sulfate, 30 mg[d]	10.7 ± 3.7 (6)
Sodium cholate	23.4 ± 11.4 (5)
Sodium deoxycholate	12.5 ± 1.0 (6)
Sodium taurocholate	15.6 ± 4.9 (5)
Sodium dehydrocholate[e]	29.5 ± 7.9 (6)

[a] Concentration = 10 m*M*. [b] Average dose = 150 mg/kg. [c] Numbers in parentheses represent the number of animals that died out of six used per experiment. [d] Volume of 1.0 ml of solution in pH 7.4 sodium phosphate buffer. Six nonfasted rats (200 ± 25 g) were given decamethonium 5 min after neomycin treatment. [e] Reported not to form micelles (19).

tion of the isopropamide from the small intestine of the rat. The results from the two investigations suggest that bile salts are not of physiological importance in the absorption mechanism of the highly water-soluble and protonated quaternary ammonium compounds.

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[x] To whom inquiries should be directed.

Effects of Dimethyl Sulfoxide and Trimethylphosphine Oxide on Percutaneous Absorption of Corticosteroids in the Rat

M. B. CRAMER[x] and L. A. CATES

Abstract □ Dimethyl sulfoxide and trimethylphosphine oxide facilitate absorption of a polar steroid (hydrocortisone sodium succinate) through the rat skin. Neither agent significantly increases the rate at which a highly lipid-soluble steroid (cortisone acetate) can penetrate rat skin.

Keyphrases □ Dimethyl sulfoxide—effects on percutaneous absorption of corticosteroids, rat □ Trimethylphosphine oxide—effects on percutaneous absorption of corticosteroids, rat □ Corticosteroids (hydrocortisone sodium succinate and cortisone acetate), percutaneous absorption—effects of dimethyl sulfoxide and trimethylphosphine oxide, rat □ Absorption, percutaneous—effects of dimethyl sulfoxide and trimethylphosphine oxide on corticosteroid absorption, rat

The percutaneous absorption of steroids is influenced by many factors, including the vehicle in which the steroid is dispersed (1) and the polarity of the steroid (2, 3). The use of dimethyl sulfoxide as a vehicle has been reported to increase percutaneous absorption of many chemicals, including steroids (4–7). Tjan and Gunberg (8) reported that cortisone acetate in dimethyl sulfoxide caused reductions in total body weight, spleen weight, thymus weight, and adrenal gland weight after topical application to immature female rats. These effects paralleled those seen after subcutaneous injections of the same doses of cortisone acetate.

Since phosphine oxides are similar chemically to sulfoxides, it seemed possible that the ability of dimethyl sulfoxide to enhance percutaneous absorption of other chemicals might be shared by trimethylphosphine oxide. Both possess solubilities of the same order in polar solvents, but dimethyl sulfoxide is more soluble in nonpolar solvents. Dimethyl sulfoxide and trimethylphosphine oxide have the high, closely related dipole moment values of 3.96 and 4.39 D, respectively (9, 10).

The purpose of this study was to compare the effects of dimethyl sulfoxide and trimethylphosphine oxide on the biological activity of a lipophilic steroid (cortisone acetate) and a hydrophilic steroid (hydrocortisone sodium succinate) after topical application. This investigation was patterned after two reports of a similar nature involving dimethyl sulfoxide and cortisone acetate (8) and dimethyl sulfoxide and a water-soluble ester salt of prednisolone (11). The former study, however, did not include a control group, leaving the speculation that cortisone acetate might penetrate the skin just as well without incorporation of dimethyl sulfoxide. The latter report indicated that the steroid was applied almost daily for 5.5 weeks, a period that seems excessively long since similar reductions in organ size can be observed within 3–4 days.

Table I—Dosage Schedule for Rats Treated Topically with Cortisone Acetate or Hydrocortisone Sodium Succinate

Group	Treatment	Number of Rats
I	Chloroform (control)	5
	6 *M* Dimethyl sulfoxide in chloroform	5
	6 *M* Trimethylphosphine oxide in chloroform	5
II	Cortisone acetate in chloroform (control)	5
	Cortisone acetate and 6 *M* dimethyl sulfoxide in chloroform	5
	Cortisone acetate and 6 *M* trimethylphosphine oxide in chloroform	5
III	Ethanol (control)	4
	6 *M* Dimethyl sulfoxide in ethanol	5
	6 *M* Trimethylphosphine oxide in ethanol	5
IV	Hydrocortisone sodium succinate in ethanol (control)	5
	Hydrocortisone sodium succinate and 6 *M* dimethyl sulfoxide in ethanol	5
	Hydrocortisone sodium succinate and 6 *M* trimethylphosphine oxide in ethanol	5

EXPERIMENTAL

Male Sprague–Dawley albino rats[1] (mean weight 162 ± 2.1 g) were prepared by shaving, under light ether anesthesia, an area on the back of the neck 72 hr prior to the initiation of drug treatment. Animals were housed individually in wire-bottom cages after shaving and were provided with laboratory chow and water *ad libitum* throughout the experiment.

Preparations of cortisone acetate and hydrocortisone sodium succinate were made such that a 10-mg dose was contained in 100 μl. In preparations containing dimethyl sulfoxide or trimethylphosphine oxide, the steroid was present as a fine suspension. All other preparations were solutions. Details of the dosage schedule are summarized in Table I. One hundred microliters of the appropriate preparations was applied once daily for 4 days to the shaved area with the animal under light ether anesthesia. Twenty-four hours after the fourth dose, the rats were sacrificed by chloroform inhalation. The spleen, thymus, and adrenal glands from each rat were removed, cleaned, and weighed on a tissue balance[2]. Involution of these organs was taken as a measure of the systemic effects of topically applied corticosteroid.

Data from each group were compared by analysis of variance and, where the variance ratio indicated the existence of a statistically significant difference ($p < 0.05$), individual means were compared using the Newman–Keuls test (12).

RESULTS AND DISCUSSION

Results of this study are summarized in Table II. Topical application of either cortisone acetate or hydrocortisone sodium succinate was associated with reductions in total body weight as well as in the relative weights of the spleen, thymus, and adrenal

[1] ARS/Sprague–Dawley, Madison, Wis.
[2] Roller-Smith.

Table II—Effects of Dimethyl Sulfoxide or Trimethylphosphine Oxide on Biological Activities of Topically Applied Cortisone Acetate or Hydrocortisone Sodium Succinate

Group	Treatment[a]	Body Weight Change[b], g	Spleen Weight[b], mg/100 g Body Weight	Thymus Weight[b], mg/100 g Body Weight	Adrenal Weight[b], mg/100 g Body Weight
I	Chloroform (control)	+34 ± 6.0	380.3 ± 28.7	293.1 ± 26.8	32.7 ± 2.0
	Dimethyl sulfoxide in chloroform	+26 ± 2.4	442.0 ± 18.8	326.4 ± 26.1	27.5 ± 1.1
	Trimethylphosphine oxide in chloroform	+22 ± 1.2	396.2 ± 24.4	312.5 ± 13.9	32.3 ± 1.5
II	Cortisone acetate in chloroform (control)	−6 ± 2.4	228.3 ± 11.4	127.6 ± 15.0	20.3 ± 0.9
	Cortisone acetate + dimethyl sulfoxide in chloroform	−15 ± 9.5	258.8 ± 9.2	107.9 ± 10.3	25.7 ± 2.8
	Cortisone acetate + trimethylphosphine oxide in chloroform	+3 ± 1.2	256.4 ± 14.4	113.5 ± 7.8	22.6 ± 1.6
III	Ethanol (control)	+28 ± 3.2	314.7 ± 15.7	296.1 ± 19.9	28.2 ± 2.6
	Dimethyl sulfoxide in ethanol	+28 ± 3.0	292.6 ± 7.9	267.4 ± 17.2	25.5 ± 1.4
	Trimethylphosphine oxide in ethanol	+25 ± 4.5	320.8 ± 10.6	297.7 ± 8.8	24.2 ± 1.7
IV	Hydrocortisone sodium succinate in ethanol (control)	−7 ± 4.9	209.2 ± 11.6	96.9 ± 22.5	27.9 ± 2.0
	Hydrocortisone sodium succinate + dimethyl sulfoxide in ethanol	−3 ± 2.0	209.7 ± 10.6	100.5 ± 13.1	17.6 ± 0.8[c]
	Hydrocortisone sodium succinate + trimethylphosphine oxide in ethanol	−18 ± 2.5[c]	197.0 ± 13.5	87.6 ± 11.0	18.6 ± 0.8[c]

[a] All corresponding treatments and measures between Groups I and II and between Groups III and IV differed significantly, except hydrocortisone sodium succinate in ethanol *versus* ethanol alone for adrenal weight. [b] Mean ± standard error; see Table I for number of animals in each treatment group. [c] Significantly different ($p < 0.05$) from group control.

glands. This was true whether the steroids were applied in solvent only (chloroform for cortisone acetate, ethanol for hydrocortisone sodium succinate) or with the penetrating aid dimethyl sulfoxide or trimethylphosphine oxide.

For cortisone acetate the changes in groups treated with dimethyl sulfoxide or trimethylphosphine oxide were not significantly different from those in groups treated with the steroid in chloroform alone. Thus, cortisone acetate in chloroform readily penetrated the rat skin and the incorporation of dimethyl sulfoxide or trimethylphosphine oxide did not significantly enhance the biological effects of this penetration. It has not been ascertained whether the chloroform serves as a penetrating aid or evaporates too rapidly from the application site to act in any such capacity. In either case, the effect of the nonpolar solvent was no different from those produced by highly water-soluble dimethyl sulfoxide and trimethylphosphine oxide. It appears that cortisone acetate may penetrate as a result of its inherent lipid solubility.

Hydrocortisone sodium succinate also penetrated the skin from each of the three vehicles, as indicated by the decrease in total body weight and relative weights of the spleen and thymus. However, no appreciable decrease in adrenal weight was seen when the drug was applied in ethanol alone. In the presence of dimethyl sulfoxide, adrenal weight was significantly lower than that of controls. In the presence of trimethylphosphine oxide, both total body weight and relative adrenal weight were reduced to a significantly greater extent than in control animals. These data suggest that trimethylphosphine oxide, like dimethyl sulfoxide, may be effective in increasing the rate at which hydrophilic drugs can penetrate the skin.

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[x] To whom inquiries should be directed.

Thermal Stability of Biological Lipids I: Differential Scanning Calorimetry of Mixtures of Cholesteryl Myristate and Cholesteryl Palmitate

SYLVAN G. FRANK

Abstract □ Differential scanning calorimetry was used to study phase transitions in binary mixtures of mesomorphic (liquid crystalline) saturated cholesteryl esters. The solid to mesomorphic phase transition temperatures for binary mixtures of cholesteryl myristate and cholesteryl palmitate are lower than the phase transition temperature for either pure compound. An apparent eutectic phase formation was found for all compositions of the two components. The mesomorphic phase consists initially of a smectic phase, which converts to a cholesteric phase as the temperature is increased. The cholesteric phase exists over a narrow temperature range, above which the system becomes an isotropic liquid. The phase transition temperatures of the smectic to cholesteric and the cholesteric to isotropic phase transitions are virtually linear functions of the composition of the mixtures. The ΔH values for each phase transition were determined. The major portion of the overall ΔH for the solid to isotropic phase transitions for these mixtures occurs at the solid to mesomorphic phase transition. The behavior of these mixtures is of interest since mesomorphic mixtures of these cholesteryl esters along with other cholesteryl esters are components of the lipid deposits of atheromatous lesions.

Keyphrases □ Cholesteryl myristate and palmitate—differential scanning calorimetry of binary mixtures, phase diagrams □ Phase transitions—binary mixtures of cholesteryl myristate and palmitate studied by differential scanning calorimetry □ Differential scanning calorimetry—used to study phase transitions of binary mixtures of cholesteryl esters, phase diagrams □ Lipids, biological—differential scanning calorimetry of binary mixtures of cholesteryl esters

The significant biological importance of cholesterol and its esters is due to their wide distribution in the body and their role in normal biological chemistry as well as in certain pathological conditions such as atherosclerosis (1–3). Atherosclerosis is the major cause of death in the United States and in many other countries. This disease is characterized by atheromas or atheromatous plaques, which are soft fatty deposits that cause a thickening of the inner layer of large- and medium-sized arteries. The major components of the atheromatous plaque are cholesteryl oleate and cholesteryl linoleate with lesser amounts of cholesterol, cholesteryl palmitate, cholesteryl myristate, and numerous other saturated and unsaturated cholesteryl esters in addition to triglycerides, phospholipids, lipoproteins, and other substances (4).

Cholesteryl esters belong to a class of compounds that do not melt directly from a crystalline solid to an isotropic liquid; instead, as the temperature is increased, they pass through an intermediate phase termed the mesomorphic or liquid crystalline phase. The liquid crystalline phase has the mobility of a liquid, while the molecules remain in domains arranged in an anisotropic ordered array (1). Depending on the chain length of the ester, this phase may consist of a smectic or cholesteric structural arrangement. In certain compounds, the smectic phase may be converted to the cholesteric phase by heating to a particular temperature.

The phase behavior of the cholesteryl ester mixtures is of particular interest because the lipid deposits of atheromatous lesions occur as droplets within cells and in the extracellular spaces of the arterial intima and as streaks on the walls of the arterial lumen (4, 5). These streaks are mesomorphic at body temperature (2), and analysis of the deposits has indicated a large number of cholesteryl esters, many of which have their mesomorphic phase well above body temperature.

The thermal behavior of individual cholesteryl esters has received much attention (6–8). Although the cholesteryl ester deposits in atheromatous lesions are known to exist as mixtures, determination of the physical-chemical properties of such mixtures has only recently begun (6, 9, 10). Visual thermal microscopy of the phase transitions in binary mixtures of saturated cholesteryl esters (cholesteryl myristate and cholesteryl palmitate) has indicated that the transitions from the solid phase to the mesomorphic phase occurred at temperatures below the phase transition temperature for either pure compound (9). However, the mesomorphic to isotropic phase transition occurred at temperatures that were virtually linear functions of the composition of the mixtures, consistent with the liquid nature of the components and, therefore, their expected low degree of intermolecular interaction.

Differential scanning calorimetry (DSC) was used in the present work to study both the qualitative and quantitative thermal properties of binary mixtures of cholesteryl myristate and cholesteryl palmitate.

EXPERIMENTAL

Materials—Samples of cholesteryl myristate and cholesteryl palmitate and mixtures of these two esters were taken from the materials used in the previous visual thermal microscopy studies of their phase transition behavior (9).

Instrumentation—All samples were analyzed on a thermal analyzer[1]. The calorimeter was calibrated with gallium, indium, and tin. Sample weights ranged from 5.25 to 5.80 mg for the various cholesteryl ester mixtures. The samples were contained in aluminum pans with unsealed covers and were maintained under a positive flow of nitrogen at atmospheric pressure. All DSC traces were recorded at a heating rate of 10°/min with a temperature axis scale of 20°/in. as a function of ΔT measured at 0.5°/in. Due to the variation in sample weights, ΔH calculations were normalized to compare the data on an equivalent basis. All samples were

[1] DuPont 900 equipped with a DuPont pressure DSC cell, model 900605.

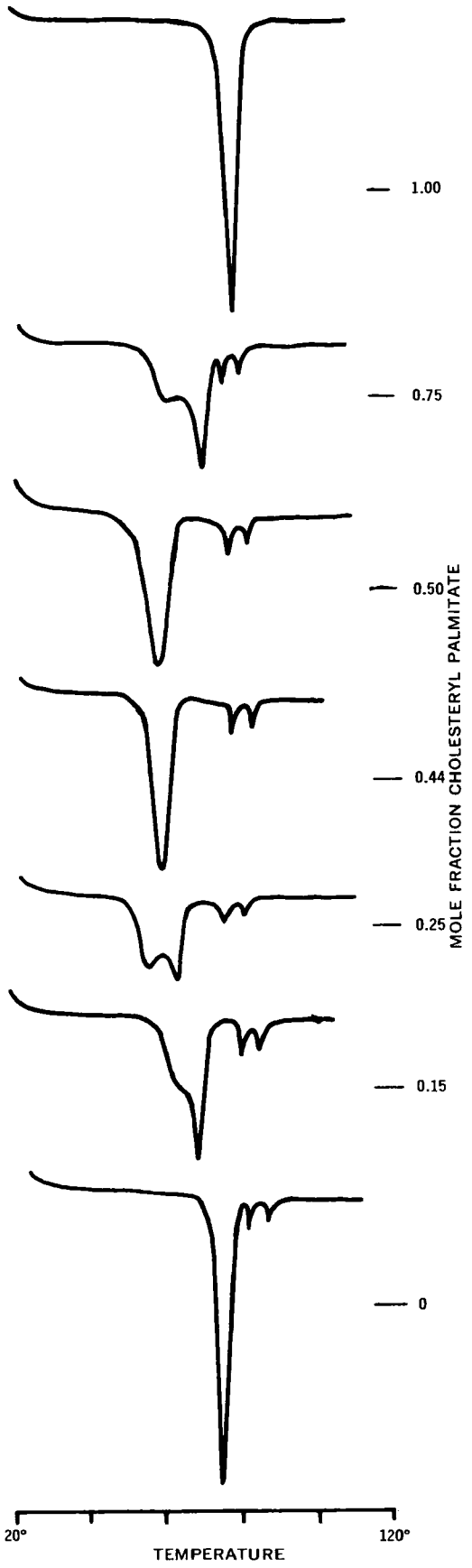

Figure 1—*DSC curves of cholesteryl myristate and cholesteryl palmitate, separately and in mixtures.*

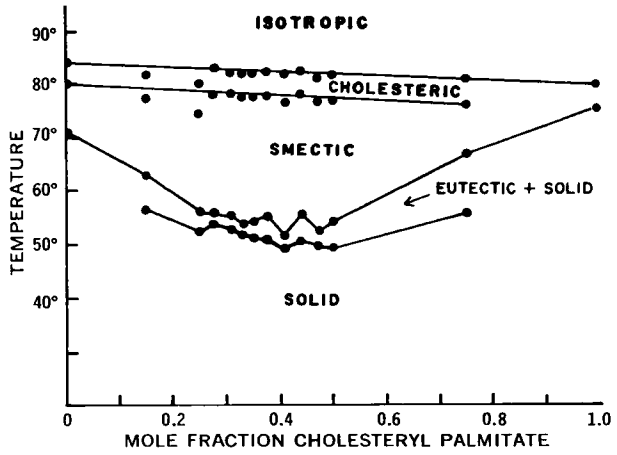

Figure 2—*Phase diagram for mixtures of cholesteryl myristate and cholesteryl palmitate as determined by DSC.*

run in duplicate. Multiple peaks corresponding to the eutectic mixture and to the melting of the excess pure compound were observed in certain cases. The areas of the respective endotherms were arbitrarily assigned by dropping a line perpendicular to the baseline to the maximum point separating the overlapping endotherms. A planimeter[2] was used to measure the areas of the endotherms.

RESULTS AND DISCUSSION

DSC was performed on cholesteryl myristate and cholesteryl palmitate and on mixtures of these materials (Fig. 1). As can be seen in Fig. 1, from the endotherm for cholesteryl myristate (bottom trace), cholesteryl myristate undergoes three endothermic transitions representing from left to right: *(a)* crystalline solid to smectic phase, *(b)* smectic phase to cholesteric phase, and *(c)* cholesteric phase to isotropic liquid. The two mesomorphic phase transitions, smectic to cholesteric and cholesteric to isotropic liquid, are enantiotropic or completely reversible upon heating and cooling (6). Cholesteryl palmitate has, however, only one enantiotropic mesomorphic transition, the transition from the cholesteric phase to the isotropic liquid phase. In this case the smectic phase appears only upon cooling the cholesteric phase and, therefore, would not appear on the endotherm in Fig. 1. The crystalline solid to cholesteric phase and the cholesteric to isotropic liquid phase transitions occur over too narrow a range for the DSC to detect individually, resulting in a single large endotherm.

The major endotherm for cholesteryl myristate (the crystalline solid to mesomorphic phase transition) represents approximately 91% of the total heat of transition from the crystalline solid to the isotropic liquid, and the second and third endotherms account for approximately 6 and 3% of the total transition heat, respectively. These results are in good agreement with those reported by others (8). The transition temperatures are also in general agreement with previously reported values (6, 8).

The DSC traces for the cholesteryl myristate–cholesteryl palmitate mixtures are shifted to lower temperatures from that of either pure compound and have certain distinctly different shape characteristics (Fig. 1). At 0.75 mole fraction of cholesteryl palmitate, four peaks are seen, corresponding, from left to right, to: *(a)* an apparent eutectic mixture appearing as a shoulder on the peak due to excess cholesteryl palmitate, *(b)* excess cholesteryl palmitate, *(c)* the smectic to cholesteric phase transition, and *(d)* the cholesteric to isotropic liquid phase transition. The latter endotherm most likely incorporates the cholesteric to isotropic liquid phase transition of cholesteryl myristate. The shift of the endotherm of the excess cholesteryl palmitate and of the two mesomorphic phase transitions to lower temperatures would be expected in the presence of the eutectic mixture.

At 0.5 mole fraction of each cholesteryl ester, a single large endotherm appears followed by two smaller ones. The large endo-

[2] Gelman Instrument Co.

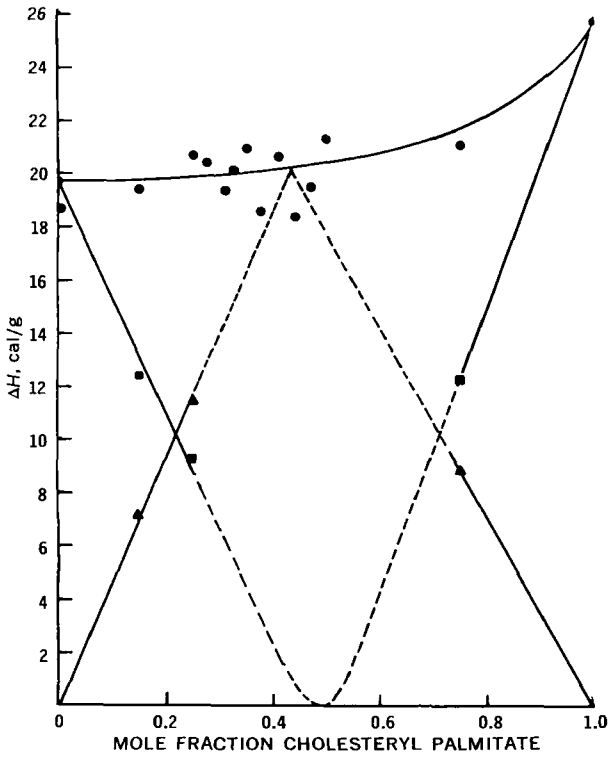

Figure 3—*Crystalline solid to mesomorphic phase transition heats for cholesteryl myristate–cholesteryl palmitate mixtures. Key: ▲, eutectic heat; ■, heat of melting of the excess pure component; and ●, total enthalpy for the phase transition.*

therm corresponds to the eutectic mixture, and the two small endotherms correspond to the smectic to cholesteric and cholesteric to isotropic liquid phase transitions. This pattern of endotherms is also seen at 0.44 mole fraction of cholesteryl palmitate. From the lack of a discernible shoulder and the near symmetry of the large endotherm, it appears that the eutectic mixture occurs close to equimolar quantities of each component. The behavior of the 0.25 mole fraction cholesteryl palmitate mixture is similar to that of the 0.75 mole fraction cholesteryl palmitate mixture. A large double-peaked endotherm appears, corresponding to the eutectic mixture on the left and the excess cholesteryl myristate on the right. At 0.15 mole fraction there is a shoulder, corresponding to the eutectic mixture, on the large endotherm of the excess cholesteryl myristate. As can be expected, it is very difficult to differentiate quantitatively the relative magnitudes of the shoulder on a peak and the peak itself.

The data from the DSC traces in Fig. 1 and from DSC measurements on other mixtures are summarized as a phase diagram in Fig. 2, in which the phase transition temperatures of the cholesteryl myristate–cholesteryl palmitate mixtures are presented as a function of the composition of the mixtures. The data in Fig. 2 correlate favorably with those obtained for these same systems by visual thermal microscopy (9). The appearance of the eutectic mixture and of the smectic to cholesteric phase transition was not determined in the microscopy study. The linearity of the smectic to cholesteric and the cholesteric to isotropic liquid phase transitions indicates that these mesomorphic phases are behaving as ideal liquid mixtures. This would be expected due to the small difference in the ester chain length and the small heat of transition.

In Fig. 3, the eutectic heats, the heat of melting of the excess pure component, and the total heat of the crystalline solid to mesomorphic phase transitions are plotted as a function of the mole fraction of cholesteryl palmitate (10). Extrapolation of the eutectic heats indicates the formation of a eutectic mixture at approximately 0.43 mole fraction of cholesteryl palmitate, whereas the extrapolation of the heats of melting of the pure component indicates that the eutectic mixture composition is about 0.49 mole fraction cholesteryl palmitate. The average of these two values gives an approximate eutectic composition of 0.46 mole fraction of cholesteryl palmitate and 0.54 mole fraction of cholesteryl myristate, or very nearly equimolar quantities of each component.

It may be concluded that binary mixtures of these saturated cholesteryl esters form a eutectic mixture of virtually equimolar composition. Although these substances are not the major components of the lipid deposits of atheromatous lesions, their physical behavior suggests that eutexia may play an important role in the formation and stabilization of the complex mixtures characteristic of these deposits.

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Prodrug Approach to Enhancement of Rate of Dissolution of Allopurinol

ANWAR HUSSAIN* § and J. HOWARD RYTTING‡ x

Abstract □ The synthesis and physicochemical characteristics of 1-ethoxyethyl-4-allopurinyl ether and 2-tetrahydropyranyl-4-allopurinyl ether are described. These compounds have lower melting points, higher solubilities, and, consequently, considerably greater rates of dissolution than does allopurinol. The rate of hydrolysis of the ethyl vinyl ether derivative was examined at 37° in 1 and 0.1 *N* HCl, yielding half-lives of 13 sec and 7.7 min, respectively, for the pseudo-first-order hydrolysis to form allopurinol. The rate of hydrolysis of the tetrahydropyran derivative was somewhat slower; *i.e.*, the half-lives were 35 sec and 12 min, respectively, under the same conditions.

Keyphrases □ Allopurinol—prodrug approach used to enhance dissolution □ Dissolution—prodrug approach to increase rate of allopurinol dissolution □ Prodrugs—synthesis and physicochemical characteristics of 1-ethoxyethyl-4-allopurinyl ether and 2-tetrahydropyranyl-4-allopurinyl ether, enhanced rate of allopurinol dissolution □ Hydrolysis—allopurinol prodrugs, synthesis, dissolution rates determined

Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) (I) is widely used in the treatment of gout and hyperuricemia. However, the compound is relatively water insoluble (solubility = 0.78 mg/ml) and, consequently, it has been reported that about 20–25% of the usual dose is excreted in the stool unchanged (1). It was felt that the prodrug approach might be utilized to increase the solubility of allopurinol and, therefore, its rate of dissolution and bioavailability.

Generally, the term "prodrug" is used for compounds that undergo some chemical or biological transformation prior to exhibiting their pharmacological effect (2). In this paper, a prodrug is defined as a drug that is prepared by chemically modifying a pharmacologically active compound to form a new compound which reverts to the parent compound after administration. The modification alters the physicochemical properties and affects the absorption, distribution, or metabolism of the drug in some desirable manner. This concept also is known as "drug latentiation" (3–5). Several problems associated with formulation (*e.g.*, solubility and stability) have been solved by preparing prodrugs (6).

The absorption of a drug from a tablet depends on tablet disintegration, dissolution, diffusion, and absorption into the blood. Each step is essential to obtain blood levels adequate for therapeutic effectiveness, and the rate at which a drug appears in the bloodstream depends on the slowest step. For a slightly soluble drug, dissolution is often rate determining and may be conveniently considered in terms of a modified form of the Noyes–Whitney equation (7, 8):

$$\frac{dC}{dt} = \left(\frac{kDA}{Vh}\right)(C_s - C) \qquad \text{(Eq. 1)}$$

where C is the concentration of the drug in the bulk solution, k is a proportionality constant, D is the diffusion coefficient of the drug in the solvent, A is the surface area of the undissolved drug, V is the volume of the solution, h is the thickness of the diffusion layer around a particle, and C_s is the solubility of the drug in the solvent.

If one assumes that dissolution is rate limiting, this equation may be simplified to:

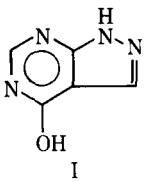
$$\frac{dC}{dt} = k'AC_s \qquad \text{(Eq. 2)}$$

The dissolution rate then becomes proportional to the surface area of the drug and its solubility.

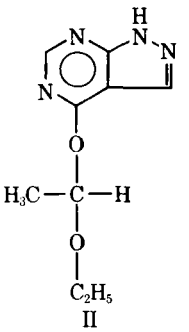
Methods for increasing surface area and solubility are well known, but the preparation of a prodrug that has increased solubility and reverts to its original form after dissolution is a relatively new approach (6). The use of this approach for allopurinol is now reported. The high melting point of allopurinol (365°) suggested that transient blocking of some polar groups in the molecule might decrease intermolecular hydrogen bonding and enhance the solubility. This paper describes two prodrugs prepared on the basis of this concept.

EXPERIMENTAL

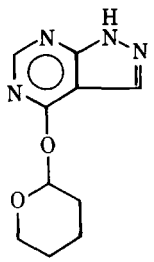
Preparation of 1-Ethoxyethyl-4-allopurinyl Ether (II) and 2-Tetrahydropyran-4-allopurinyl Ether (III)—One gram of allopurinol, contained in a stoppered, 150-ml, round-bottom flask, was dissolved in 50 ml of reagent grade dimethylformamide. Twenty milliliters of either ethyl vinyl ether or dihydropyran and 4 ml of 0.4% *p*-toluenesulfonic acid in dry benzene were added. After 4–5 hr at room temperature, 0.5 ml of 4% pyridine in benzene was added. The solution was filtered and solvent removed at 40° *in vacuo*. The resulting viscous liquid was dissolved in 100 ml of petroleum ether and 2–3 ml of benzene and allowed to recrystallize overnight in a refrigerator. The dihydropyran derivative mp 203°) and the ethyl vinyl ether (mp 185°) were obtained by



I



II



III

Table I—Rate of Hydrolysis of II and III

Hydrolytic Medium	Temperature	$t_{1/2}$, sec: Ethyl Vinyl Ether	$t_{1/2}$, sec: Tetrahydropyran
0.1 *N* HCl	37°	462	720
1 *N* HCl	37°	13	35
1 *N* HCl	25°	77	112

recrystallization from ethyl acetate–acetone and were further characterized by NMR, differential scanning calorimetry, TLC, and elemental analysis.

Dihydropyran—

Anal.—Calc. for $C_{10}H_{12}N_4O_2$: C, 54.5; H, 5.5; N, 25.4. Found: C, 53.8; H, 5.0; N, 25.0.

Ethyl Vinyl Ether—

Anal.—Calc. for $C_9H_{12}N_4O_2$: C, 51.9; H, 5.8; N, 26.9. Found: C, 51.6; H, 5.8; N, 27.2.

Solubility—The solubilities of allopurinol (0.78 mg/ml), the tetrahydropyran derivative (3.64 mg/ml), and the ethyl vinyl ether derivative (1.91 mg/ml) were measured at 247 nm, using an equilibrium spectrophotometric technique (9).

Dissolution Rates—The dissolution rates of the three test compounds were determined by the method of Poole (10), using approximately 100-mg tablets compressed[1] at 18,000 psi. Dissolution was measured in 300 ml of 0.1 *N* HCl at a stirring rate of 55 rpm. At 30-min intervals, the dissolution medium was replaced with 300 ml of fresh solvent to maintain sink conditions and the solution was assayed. In the assay procedure, 10% HCl was added and allowed to react for at least 10 min with an aliquot of each sample to hydrolyze the derivatives to allopurinol; the concentration of allopurinol was then determined spectrophotometrically at 247 nm.

Rate of Hydrolysis—An effective prodrug must revert to the active drug of interest within a reasonable time. Therefore, the hydrolysis of the two derivatives was examined.

The rates of formation of allopurinol from II and III in acid solution were monitored[2] at 25 and 37° at 277 nm.

In the case of allopurinol ethyl vinyl ether (II) in 0.1 *N* HCl, the two species had approximately the same absorbance and, thus, the absorbance changes with time were very small. Consequently, a liquid chromatograph[3] (0.5-m column) was used. The chromatograph was operated at 600 lb pressure, and the mobile phase consisted of an aqueous solution of 0.04 *M* NaH_2PO_4 and 0.05 *M* KNO_3 having a pH of 4.5. Using these conditions, the disappearance of the peak for Compound II was followed.

RESULTS AND DISCUSSION

The increase in solubility observed for the ethyl vinyl ether and tetrahydropyran derivatives compared with the parent compound is probably due to the decrease in the solute–solute intermolecular forces in the crystal form caused by blocking the hydroxyl group in allopurinol, as reflected in the decrease in melting points (from 365 to ~200°).

The results of the dissolution study are shown in Fig. 1. The tetrahydropyran derivative showed the greatest rate of dissolution, followed by the ethyl vinyl ether derivative and finally by the parent compound allopurinol. The rates appear to be roughly in proportion to the solubilities of the compounds studied, although allopurinol dissolves somewhat more slowly than expected.

The half-lives for the pseudo-first-order hydrolysis are reported in Table I.

The tetrahydropyran dissolves more rapidly than the ethyl vinyl ether, but it hydrolyzes more slowly. The acid-catalyzed hydrolyses are similar to those previously reported for acylals (11, 12) and indicate adequate allopurinol release, despite the less acidic conditions encountered in the stomach. The increased solubility, more rapid dissolution, and adequate reconversion to allopurinol imply a potential usefulness of the tetrahydropyran and ethyl vinyl ether derivatives as prodrugs.

[1] Carver press.
[2] Thermostated Cary 16 recording spectrophotometer.
[3] Dupont 840 with Dupont SCX support.

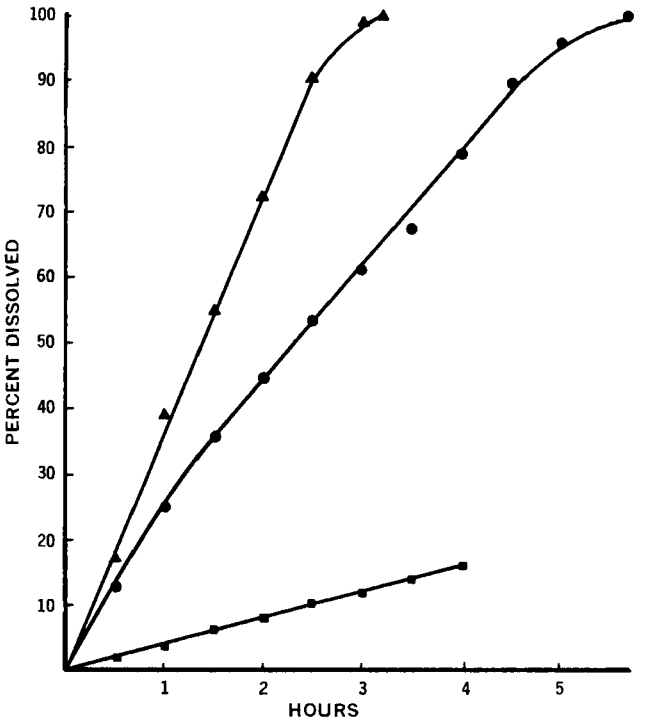


Figure 1—*Rate of dissolution of tablets containing allopurinol* (■), *1-ethoxyethyl-4-allopurinyl ether* (●), *and 2-tetrahydropyranyl-4-allopurinyl ether* (▲) *at 25°.*

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§ Present address: College of Pharmacy, University of Kentucky, Lexington, KY 40506

ˣ To whom inquiries should be directed.

Separation of Prostaglandin A_2 and Prostaglandin B_2 by Ion-Exchange Liquid Chromatography

WALTER MOROZOWICH

Abstract □ Separation of prostaglandin A_2 (PGA_2) and prostaglandin B_2 (PGB_2) was achieved by liquid chromatography on triethylaminoethyl cellulose as well as on a strong anion-exchange pellicular support. The stability of prostaglandin E_2 (PGE_2) can be monitored easily by following the appearance of the UV active compounds PGA_2 and PGB_2 using the peak height method.

Keyphrases □ Prostaglandins—separation of PGA_2 and PGB_2 by ion-exchange liquid chromatography, comparison of two columns □ Liquid chromatography—separation of prostaglandin A_2 and B_2, comparison of two columns □ Ion-exchange liquid chromatography—separation of prostaglandins A_2 and B_2, comparison of two columns

High-resolution chromatographic systems are required for separation of many closely related prostaglandins. The isomeric pair prostaglandin A_2 (PGA_2) and prostaglandin B_2 (PGB_2) show little resolution using TLC, although separation is complete with GLC (1, 2). Prostaglandin E_2 (PGE_2) undergoes rapid dehydration to yield PGA_2 and PGB_2 in consecutive fashion (3, 4) (Scheme I). GLC is not generally used to monitor the stability of PGE_2 since protection of the C_9-carbonyl group is required and the commonly used methoxime derivative gives rise to two peaks for the oximino *syn-anti*-isomers (5). PGA_2 and PGB_2 also undergo methoximation with formation of *syn-anti*-isomers and, as a result, degraded samples of PGE_2 show a complicated GLC pattern.

This work shows that single peaks are obtained for PGA_2 and PGB_2 using ion-exchange liquid chromatography without protective derivatization.

EXPERIMENTAL

Equipment—Liquid chromatography was conducted on a liquid chromatograph[1] (254-nm detector) using 2.1-mm (i.d.) × 1-m stainless steel columns.

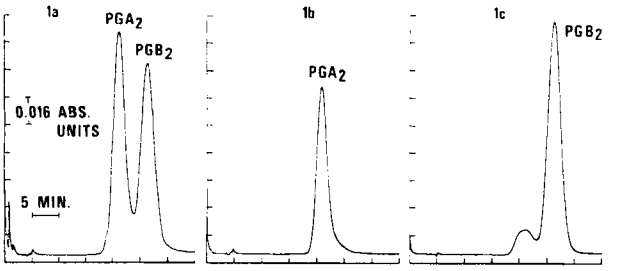


Figure 1—*Liquid chromatograms on triethylaminoethyl cellulose using aqueous 0.05* M *tromethamine acetate (pH 7.20) at 2100 psi and 0.45 ml/min. Key: 1a, mixture of* PGA_2 *(40 μg) and* PGB_2 *(7 μg); 1b,* PGA_2 *(35 μg); and 1c,* PGB_2 *(9 μg).*

[1] DuPont model 820.

Triethylaminoethyl Cellulose Columns—Triethylaminoethyl cellulose[2] (0.58 mEq/g) was freed of fines by slurrying 1–4% suspensions in water and allowing the material to settle for 1–1.5 hr. The supernate was discarded, and the slurry-settling process was repeated six times. The purified support was isolated by filtration, washed with acetone, and air dried for 24 hr.

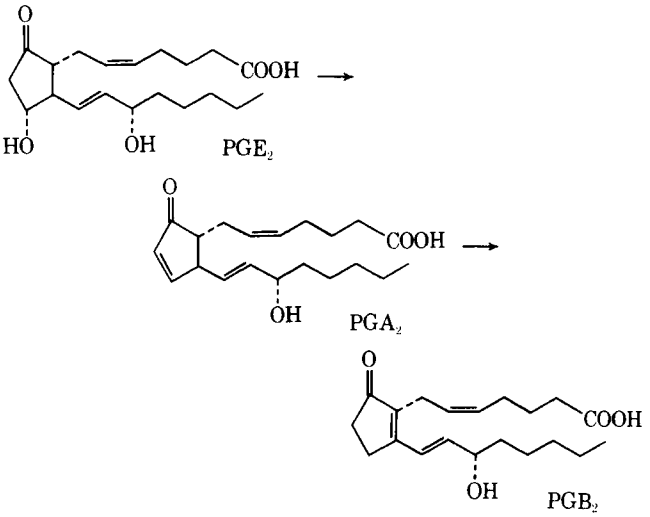
A funnel was attached to the column, and 5–10 mg of triethylaminoethyl cellulose was added from an inverted container supported on a 50-mesh screen by brief mechanical vibration of the screen. The side of the column was vibrated for a few seconds, and the material within was compacted with a loosely fitting stainless steel rod (1.8 mm) with an applied hand force of 0.454–0.681 kg (1–1.5 lb). The tamping rod was turned 120°, and three such compressions were made after each addition of support. Excessive compaction force results in columns with poor flow rates, so practice is required to produce columns with adequate flow (0.4–1.5 ml/min using 2000 psi at room temperature).

The fully packed columns were washed in succession with water, 0.5 *M* sodium acetate, and, finally, 0.5 *M* tromethamine adjusted to pH 7.20 with acetic acid. The mobile phase employed for chromatography of the prostaglandins was aqueous 0.05 *M* tromethamine adjusted to pH 7.20 with acetic acid. The operating condition was 0.45 ml/min at 2100–2300 psi (room temperature).

Pellicular Strong Ion-Exchange Columns—Columns of the strong anion-exchange support[3] were packed by the usual procedure (6). The mobile phase employed was 0.025 *M* tromethamine in acetonitrile–water (1:4) adjusted to pH 7.30 ± 0.02 with acetic acid. The operating condition was 0.50 ml/min at 350–400 psi (room temperature).

Peak Identification—Identity of the peaks was established by fraction collection followed by determination of the λ_{max} (PGA_2, 220 nm; PGB_2, 282 nm).

Compounds—PGB_2 was generated by treatment of either PGE_2[4] or PGA_2[4] with aqueous 0.05 *N* potassium hydroxide at 80° for 30 min (7). After cooling to room temperature, the solution was acidified to pH 3 with citric acid and extracted with ethyl acetate. The organic phase was dried with sodium sulfate, and



Scheme I—Conversion of PGE_2 *to* PGB_2 via PGA_2

[2] Cellex-T, BioRad Laboratories, Richmond, Calif.
[3] AS-Pellionex-SAX, Reeve-Angel, Clifton, N.J.
[4] Research Laboratories, The Upjohn Co.

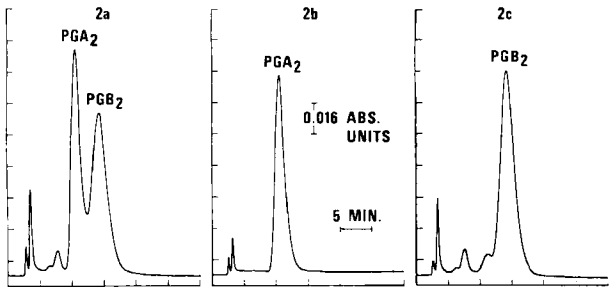


Figure 2—*Liquid chromatograms on the pellicular strong anion-exchange column using 0.025* M *tromethamine acetate (pH 7.30) in acetonitrile–water (1:4) at 350 psi and 0.5 ml/min. Key: 2a, mixture of* PGA_2 *(20 μg) and* PGB_2 *(7 μg); 2b,* PGA_2 *(17 μg); and 2c,* PGB_2 *(9 μg).*

the solvent was removed under vacuum at 45°. The oily residue was dissolved in methanol to provide a stock solution of PGB_2.

Sample Injection—Samples were injected under normal flow conditions using 1–10-μl charges of the prostaglandins dissolved in either methanol or aqueous potassium hydroxide.

RESULTS AND DISCUSSION

PGE_2 could not be detected by liquid chromatography using the 254-nm monitor due to the low molar absorptivity at this wavelength. PGA_2 and PGB_2 are both strongly UV absorbing and were detected easily at 254 nm. Figure 1*a* shows the separation of PGA_2 and PGB_2 on the triethylaminoethyl cellulose column using 0.05 *M* tromethamine acetate, pH 7.20. The separation is estimated to be 98% complete according to the method of Snyder (8). PGA_2 and PGB_2, when chromatographed separately (Figs. 1*b* and 1*c*), showed rather symmetrical peaks with little evidence of tailing.

Figure 2*a* shows the separation of PGA_2 and PGB_2 on the pellicular strong anion-exchange column using a mobile phase of 0.025 *M* tromethamine acetate, pH 7.30, in acetonitrile–water (1:4). Evidence of slight tailing was seen for the compounds when chromatographed separately (Figs. 2*b* and 2*c*). In purely aqueous systems, marked tailing occurred with this column; however, with aqueous–organic solvent systems, the tailing was minimized.

In Figs. 1*c* and 2*c*, the sample of PGB_2 was generated from PGA_2 with base. The extraneous peaks are probably due to the impurities in the initial sample of PGA_2 since generation of PGB_2 from PGE_2 in base shows very little extraneous peak formation.

Excellent linear relationships between peak heights and amount chromatographed were observed for PGA_2 and PGB_2 on both the triethylaminoethyl cellulose and the strong anion-exchange columns. Eight samples of PGA_2 were chromatographed on each column, with a peak height response range of 10–95% of full-scale (0.16 absorbance unit). A plot of micrograms injected *versus* peak height showed the following for the triethylaminoethyl cellulose and the pellicular strong anion-exchange columns, respectively: correlation coefficient 0.998, 0.999; slope 2.019, 2.307; and intercept 0.92, 1.03.

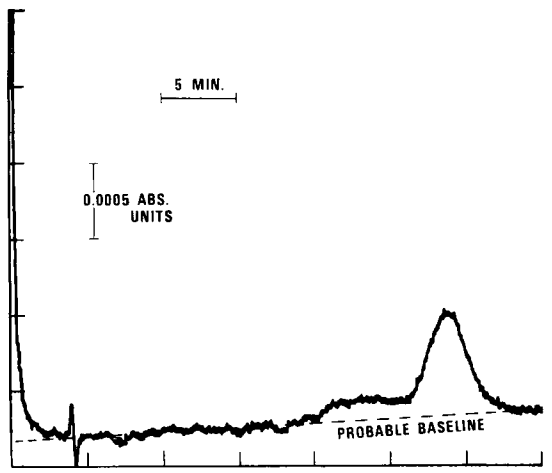


Figure 3—*Liquid chromatogram of 87 ng* PGB_2 *on the triethylaminoethyl cellulose column. Conditions were the same as in Fig. 1.*

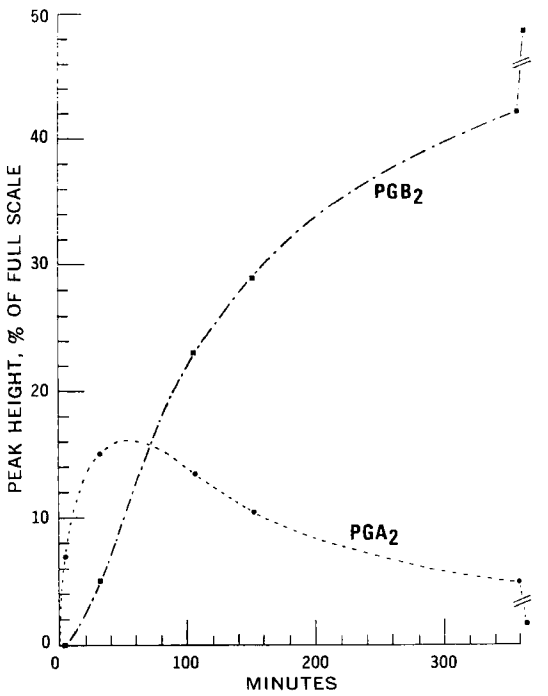


Figure 4—*Peak heights of* PGA_2 *and* PGB_2 versus *time as produced by treatment of* PGE_2 *(12.40 mg) with 1 ml aqueous 0.05* N *potassium hydroxide. Aliquots were injected directly onto the triethylaminoethyl cellulose column. The last data point on both curves was obtained at 1020 min.*

The lower limit of detection of PGB_2 was somewhat less than 87 ng on the triethylaminoethyl cellulose column (Fig. 3). A higher electrolyte concentration would permit detection of even lower levels of PGB_2.

The ease of data acquisition was demonstrated by monitoring the base-catalyzed conversion of PGE_2 to PGA_2 and PGB_2. A solution of PGE_2 (12.40 mg/ml) in 0.05 *N* potassium hydroxide at room temperature was periodically subjected to liquid chromatography by direct injection of a sample of the alkaline mixture onto the triethylaminoethyl cellulose column. The composite peak height *versus* time plot is shown in Fig. 4.

The amount of PGB_2 continually increased, whereas the amount of PGA_2 increased and then decreased as expected from the consecutive first-order reactions involved (Scheme I) (4). A small amount of 8-iso-PGA_2 is formed upon treatment of PGA_2 with base (9); since these two are inseparable by liquid chromatography, the peak heights in Fig. 4 represent a summation of these two compounds.

In conclusion, both columns are quite stable and can be used to monitor the stability of PGE_2 by following the appearance of PGA_2 and PGB_2 using the peak height method. Triethylaminoethyl cellulose gave somewhat better resolution of PGA_2 and PGB_2 as compared with the pellicular strong anion-exchange column, although this advantage is offset by the tedium required to pack columns of the former.

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Effects of γ-Irradiation on 2-Chloroethanol Formation in Ethylene Oxide-Sterilized Polyvinyl Chloride

S. BOGDANSKY ˣ and P. JEFFERY LEHN

Abstract □ γ-Irradiation prior to sterilization with ethylene oxide is shown to enhance 2-chloroethanol formation in a surgical, hospital grade, polyvinyl chloride tubing. The site of formation and the insignificantly low 2-chloroethanol levels produced in this manner are discussed.

Keyphrases □ Ethylene oxide, use as sterilant of polyvinyl chloride—effects of γ-irradiation on 2-chloroethanol formation □ 2-Chloroethanol—effects of γ-irradiation on formation in ethylene oxide-sterilized polyvinyl chloride □ Irradiation, gamma—effects on 2-chloroethanol formation in ethylene oxide-sterilized polyvinyl chloride □ Polyvinyl chloride, ethylene oxide sterilized—effects of γ-irradiation on formation of 2-chloroethanol

When the formation of 2-chloroethanol from the ethylene oxide resterilization of previously γ-irradiated polyvinyl chloride was first reported (1), no data were presented and none have been reported supporting this observation, although references to these findings have been made (2, 3). This study was undertaken because of the lack of definitive data on the effects of γ-irradiation on 2-chloroethanol formation in polyvinyl chloride and the growing concern over the biological impact of this residue in sterilized polyvinyl chloride medical devices.

EXPERIMENTAL

Materials—Surgical, hospital grade, polyvinyl chloride tubing was used[1].

Procedure—The tubing was cut into samples approximately 1 cm long, weighing 120 ± 3 mg. The samples were divided into four groups and placed in glassine envelopes. One group received 2.5 Mrads of γ-irradiation from a ^{60}Co source and one received 5.0 Mrads. The two irradiated groups and one nonirradiated group were then exposed to a 1099-mg/liter concentration of ethylene oxide (12/88, ethylene oxide/freon 12 mixture) for 4.5 hr. A temperature of 55° and relative humidity of 50 ± 5% were maintained under a sterilization pressure of 15 psig. The fourth group remained as the untreated control. Immediately following sterilization with ethylene oxide, samples for 2-chloroethanol analysis from each group were placed in glass vials with rubber septa and crimped metal caps and extracted in 5 ml distilled water for 48 hr at 72°. The same number of treated samples were also extracted after 2 and 4 days of ambient aeration.

Additional samples from the group that was only sterilized with ethylene oxide were placed in vials for ethylene oxide analysis, extracted, and analyzed by the head-space technique (4). These samples were extracted at the same time as the 2-chloroethanol samples to compare the concentrations of both residues.

A separate group of samples was soaked in 2-chloroethanol[2] for 2.5 hr and heated for 3.5 hr at 72°. This material was sampled every 2–3 days over a 28-day ambient aeration period to assess 2-chloroethanol desorption.

Apparatus—Analysis of 2-chloroethanol was accomplished using a published GLC method (5) with slight modifications.

A gas chromatograph[3], equipped with a hydrogen flame-ionization detector connected to a 1-mv strip-chart recorder[4], was employed.

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The column was initially conditioned for 24 hr at 200° with a nitrogen flow rate of 30 ml/min. The column was then connected to the detector, and 2.5-μl injections of distilled water were made approximately every 15 min for several hours at a column temperature of 180°.

The instrument was operated isothermally at a column temperature of 170°, an injector temperature of 195°, and a detector tem-

Table I—Concentration of 2-Chloroethanol and Ethylene Oxide as a Function of Aeration Time (Mean ± *SD*)

Aeration, days	2-Chloroethanol, ppm: Ethylene Oxide Sterilized Only[a]	2-Chloroethanol, ppm: 2.5 Mrads + Ethylene Oxide Sterilized	2-Chloroethanol, ppm: 5.0 Mrads + Ethylene Oxide Sterilized	Ethylene[a] Oxide, ppm: Ethylene Oxide Sterilized Only
0	35 ± 2	329 ± 5	354 ± 7	8954 ± 438
2	n.d.[b]	19 ± 1	21 ± 1	33 ± 2
4	n.d.	n.d.	n.d.	n.d.

[a] Samples from the same ethylene oxide sterilization cycle were analyzed for both residues. [b] Not detected: detection limit 10 ppm.

[1] Tygon, S-50-HL, 4.762 mm (0.1875 in.) o.d. × 3.175 mm (0.125 in.) i.d., Norton Plastics and Synthetics Division, Akron, Ohio.

[2] Baker grade (anhydrous), J. T. Baker Chemical Co.
[3] Varian model 2100.
[4] Varian model 20.
[5] Carbowax 20M, Union Carbide Corp.
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Table II—Levels of 2-Chloroethanol in Soaked Samples

Aeration, days	2-Chloroethanol, ppm	Aeration, days	2-Chloroethanol, ppm
0	44,132	14	569
3	17,747	17	235
5	9,825	19	159
7	4,866	21	91
10	2,442	28	65
12	1,364	—	—

perature of 220°. Nitrogen was used as the carrier gas with a flow rate of 30 ml/min. The hydrogen flame was maintained with an air flow of 350 ml/min and a hydrogen flow of 40 ml/min. The strip-chart recorder was operated at a speed of 50.8 cm (20 in.)/hr.

Standards, prepared by dissolving 2-chloroethanol by weight in distilled water, contained 515 (0.51), 87 (0.08), 44 (0.04), and 8.8 ppm (0.008 μg/μl) 2-chloroethanol. The linearity of the response of the detector to 2-chloroethanol concentration was established. All calculations were based on peak height comparisons between 3-μl injections of the unknowns and appropriate standards.

RESULTS AND DISCUSSION

Table I shows that polyvinyl chloride samples that were γ-irradiated, ethylene oxide sterilized, and not aerated produced about 350 ppm of 2-chloroethanol. Contrary to previous findings (1), about 35 ppm of this residue was found in samples that were only ethylene oxide sterilized; this was presumably due to small amounts of free chloride in the polymer. Although there seems to be an increase in 2-chloroethanol formation in samples that received 5.0 Mrads as compared to 2.5 Mrads, the difference is small and is not considered meaningful. Table I also shows the corresponding 2-chloroethanol concentrations for each aeration period.

While the effect of preirradiation is clear, the question of where the 2-chloroethanol is formed remains open. As discussed previously (3), it is possible that 2-chloroethanol is formed during the water extraction by the reaction of ethylene oxide with chloride generated during ^{60}Co sterilization[7]. The elevated temperature of the extraction could accelerate such a reaction. The data in Table I support this hypothesis; as the concentration of ethylene oxide decreases, the concentration of 2-chloroethanol also decreases. Alternatively, as pointed out previously (3), 2-chloroethanol might be formed in the polymer during gas sterilization by the reaction of ethylene oxide and polyvinyl chloride and may desorb during sample aeration. This would account for the drop in 2-chloroethanol as a function of aeration time (Table I). To test this hypothesis, 2-chloroethanol was incorporated into polyvinyl chloride tubing by a soaking technique and its desorption from the polymer was followed. Table II shows that 2-chloroethanol desorbs from this polyvinyl chloride at concentrations comparable to those present in the doubly sterilized samples (*i.e.*, between 325 and 360 ppm). However, this relatively slow rate of desorption is insufficient to account for the large decrease in 2-chloroethanol concentration observed for the doubly sterilized samples. This decrease seems to suggest that desorption is not a significant factor and that 2-chloroethanol is probably formed during the extraction, although some formation during sterilization is also possible.

[7] For discussion of the effects of irradiation in polyvinyl chloride, see R. Salovey, in "The Radiation Chemistry of Macromolecules," vol. II, M. Dole, Ed., Academic, New York, N.Y., 1973, p. 37.

CONCLUSIONS

While the data show that irradiation prior to ethylene oxide sterilization produces 350 ppm of 2-chloroethanol in unaerated, surgical, hospital grade, polyvinyl chloride tubing, published toxicology data have shown this relatively low level to be nonirritating and nontoxic. McDonald *et al.* (6) found the maximum nontoxic concentrations to be 10,000 ppm for topical application and 5000 ppm for intraocular application in rabbits. In a study to assess the extent and nature of tissue damage elicited by 2-chloroethanol, Bruckner and Guess (2) concluded that concentrations of 50,000 ppm or less when injected intracutaneously in rabbits did not produce cellular damage or necrosis. Guess (7) found no damage to cells in culture at levels of 20,000 ppm and no intradermal irritation in rabbits at 10,000 ppm. Unpublished data[8] showed levels up to 100,000 ppm to be nonirritating to rats when introduced *via* subcutaneous polyvinyl chloride implants.

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[x] To whom inquiries should be directed.

[8] Toxicology Section, Ethicon Research Foundation.

Relationship between pH and Antibacterial Activity of Glutaraldehyde

Keyphrases □ Glutaraldehyde—relationship between pH and antibacterial activity □ Antibacterial activity—relationship to pH, glutaraldehyde □ Bactericidal activity—aldehyde groups, relationship between pH and antibacterial activity of glutaraldehyde

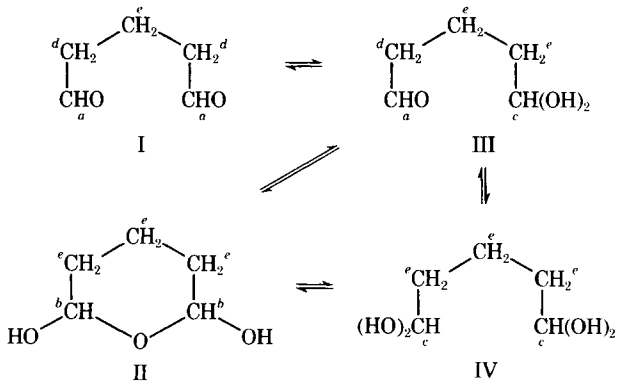
To the Editor:

Considerably enhanced bactericidal activity is achieved by adjusting glutaraldehyde solutions to pH 7.5–8.5 (1–5). In aqueous solution, glutaraldehyde undergoes rapid hydration, the free dialdehyde (I) being in equilibrium with the hydrates II–IV (Scheme I). Using PMR spectroscopy, Hardy *et al.* (6) studied this equilibrium in D_2O solution and reported signals at τ 0.28, 4.7, 5.0, 7.4, and 8.3, corresponding to protons *a*, *b*, *c*, *d*, and *e*, respectively. On the assumption that there was no free dialdehyde (I) present in aqueous solution, these authors described the equilibrium by examination of the ratio of the *b*, *c*, and *d* signal integrals after correction to an equivalent number of protons. Their results indicated that the equilibrium was not significantly modified by concentration.

Rubbo *et al.* (4) concluded, from their study on the bactericidal activities of a series of aldehydes, that the presence of free aldehyde groups was a prerequisite for activity.

Munton and Russell (7) suggested that the enhanced bactericidal activity in alkaline solution probably resulted from a modification of the glutaraldehyde molecule, the bacterial cell, or a combination of both. These workers (8) later suggested that the effect at increased pH was primarily on the outer layers of the cell, since increased polymerization, which is associated with increased free aldehyde concentration, was not observed with mild alkalinization.

No conclusive experimental data have yet been presented to show whether the enhanced bactericidal activity of glutaraldehyde in alkaline solution is a result of: (*a*) change in the free aldehyde–hydrated aldehyde equilibrium, (*b*) modification of the outer layers of the bacterial cell, or (*c*) a combination of both of these effects.



Scheme I

Table I—Effect of pH on Percentage of Free Aldehyde in Aqueous Glutaraldehyde Solutions

Glutaraldehyde Concentration, % w/v	pH	Free Aldehyde in Equilibrium Mixture[a], %
50	2.9	47.00
25	2.9	47.02
10	3.2	47.02
10	6.0	47.03
10	7.9	47.01
5	3.4	46.08

[a] Mean value calculated from corrected τ 8.3/τ 0.25 and τ 8.3/τ 7.3 integral ratios.

The PMR spectrum of aqueous glutaraldehyde was determined[1] (internal standard 4,4-dimethyl-4-silapentane sulfonate, 1%) and exhibited signals at τ 0.28, 0.73, and 0.83 (protons *a*, *d*, and *e*, respectively). Signals due to protons *b* and *c* were not observed due to overlap with the water signal (τ 4.7–5.0).

When assuming the absence of free dialdehyde (I) in aqueous solution (6), examination of the ratios of the integral of the signal at τ 8.3 (protons *c*) with that at τ 7.3 (protons *d*) or τ 0.28 (protons *a*) after correction to an equivalent number of protons (9) gives the ratio of hydrated aldehyde (II and IV) to free aldehyde (III) in the equilibrium mixture. The results (Table I) indicate that the free aldehyde–hydrated aldehyde equilibrium is not significantly modified by adjustment of the glutaraldehyde solution from pH 2.9 to 7.9.

Results of a study undertaken by Rubbo *et al.* (4) suggested that the bactericidal activities of glutaraldehyde and β-methylglutaraldehyde are related to the proportion of free aldehyde in the equilibrium mixture. Glutaraldehyde is less readily hydrated than β-methylglutaraldehyde and exhibits superior bactericidal activity. Thus, it appears that a shift of the free aldehyde–hydrated aldehyde equilibrium in favor of the hydrated aldehyde may result in a reduction in bactericidal activity.

Since no significant change in the free aldehyde–hydrated aldehyde equilibrium over the pH 2.9–7.9 range was observed (Table I), clearly the enhanced bactericidal activity of glutaraldehyde in alkaline solution is not a result of modification of the existing equilibrium. Therefore, it would appear that the alkali exerts its effect solely on the bacterial surface.

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J. A. King
W. Woodside[x]
P. V. McGucken

Department of Pharmacy
The Queen's University of Belfast
Belfast BT9 7BL
Northern Ireland

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Accepted for publication February 14, 1974.
[x] To whom inquiries should be directed.

Effect of Plasma Protein Binding on Elimination of Warfarin

Keyphrases □ Warfarin—effect of plasma protein binding on elimination, pharmacokinetics □ Protein binding, plasma—effect on elimination of warfarin, pharmacokinetics □ Elimination, warfarin—effect of plasma protein binding, pharmacokinetics

To the Editor:

Studies in humans and rats have shown pronounced intersubject variation in the elimination of the coumarin anticoagulants dicumarol and warfarin (1–4). Detailed investigations in rats revealed that this intersubject variation may be associated with large differences in the distribution of these drugs in the body (3, 4). Similar changes in distribution and elimination were produced in perfused isolated rat liver preparations by varying the concentration of plasma proteins in the perfusion solution (5). These findings raised the possibility that some intersubject variation in the elimination of coumarin anticoagulant drugs may be related to differences in the binding of these very extensively protein bound drugs to plasma proteins. It is well recognized that differences in the activity of coumarin drug-metabolizing enzyme(s), particularly due to enzyme induction or inhibition, can also account for some intersubject variation in the elimination of these drugs (6, 7). The purposes of this communication are: *(a)* to present a theoretical pharmacokinetic formulation of the relationship between plasma protein binding and the kinetics of elimination of drugs such as dicumarol and warfarin, and *(b)* to describe, preliminary to a more detailed report, experimental data in support of the pharmacokinetic theory.

Let it be assumed that: *(a)* the kinetics of elimination are apparent first order, *(b)* elimination occurs from the central compartment, *(c)* the driving force of the rate-limiting steps of the elimination processes is the concentration of free (nonprotein bound) drug, *(d)* elimination is rate limited by these processes rather than by organ perfusion rate, *etc.*, and *(e)* the extent of plasma protein binding is essentially constant over the concentration range of therapeutic or experimental interest. The last assumption is realistic for drug concentrations at which only a small fraction of the binding sites on plasma proteins is occupied (8).

The sites of drug elimination (in the liver and elsewhere in the central compartment of the body) may be viewed as being surrounded by an aqueous solution of free drug. This aqueous solution represents a physiological space consisting of plasma water and other water exclusive of dissolved macromolecules capable of binding the drug. The volume of this space is designated as V_w. The rate of elimination, $-dA/dt$, of the drug is then proportional to the amount of drug[1] in V_w:

$$-dA/dt = k'fC_pV_w \qquad \text{(Eq. 1)}$$

where k' is a first-order elimination rate constant (which may be the sum of several rate constants for different elimination processes), which relates the amount of drug[1] in V_w to the rate of elimination[2]; C_p is the concentration of total (free and protein bound drug) in the plasma; and f is the fraction of free drug in the plasma. Designating the product of k' and V_w as k'' (which may be viewed as an intrinsic clearance constant) and converting $-dA/dt$ to a concentration term yield:

$$-(dC_p/dt)V_{area} = k''fC_p \qquad \text{(Eq. 2)}$$

where V_{area} is the volume of distribution of the drug in the body as previously defined (9). Unlike V_w, which is considered to be a physiological constant, V_{area} is a parameter that is affected by changes in protein binding. Rearrangement of Eq. 2 yields:

$$-(dC_p/dt)V_{area}C_p^{-1} = k''f \qquad \text{(Eq. 3)}$$

or:

$$\text{total plasma clearance} = k''f \qquad \text{(Eq. 4)}$$

Thus, a plot of the total plasma clearance of a drug *versus* the fraction of nonprotein bound drug in the plasma should be linear and go through the origin.

The elimination rate constant and the apparent volume of distribution of warfarin were determined in a selected group of 13 adult male Sprague–Dawley rats as previously described (4), except that the drug was administered intravenously. Serum protein binding[3] was determined by equilibrium dialysis using ^{14}C-warfarin. The fraction of free drug differed widely between animals (from 0.002 to 0.015), but it

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J. A. King
W. Woodside[x]
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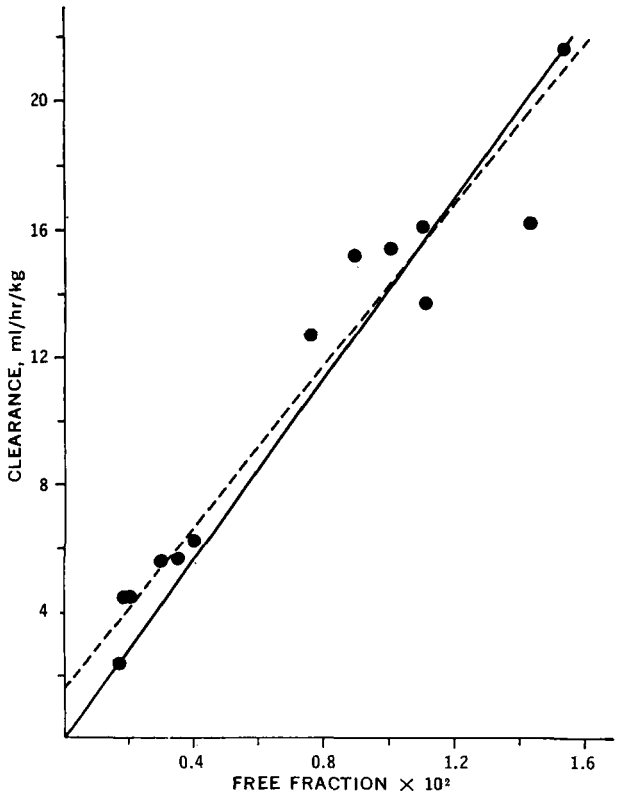

Figure 1—*Relationship between total plasma clearance of warfarin and fraction of free (not protein bound) drug in the serum of individual rats. These rats received intravenous doses of ^{14}C-warfarin, 0.6 mg/kg. Least-squares fit of the data yielded the stippled regression line; the continuous regression line was forced through the origin. The correlation coefficient is 0.97, $p < 0.001$.*

was essentially independent of total concentration for any one animal under the experimental conditions (total concentration, 0.3–4 μg/ml). Total plasma clearance[4] of warfarin ranged from about 2 to 22 ml hr^{-1} kg^{-1}. A plot of total clearance *versus* the fraction of free drug in the serum is linear, and the least-squares regression line intersects close to the origin, as predicted by Eq. 4 (Fig. 1).

In summary, our studies showed that individual differences in serum protein binding of warfarin (from 98.5 to 99.8%, equivalent to a range in the free fraction of from 0.002 to 0.015) can cause pronounced variations in the elimination rate constant (from 0.017 to 0.117 hr^{-1}) and apparent volume of distribution (from 137 to 206 ml/kg) of warfarin and that the effect of plasma protein binding on warfarin elimination can be quantified and predicted on the basis of a linear relationship between the total plasma clearance of warfarin and the free fraction of this drug in the serum. These observations may have clinical relevance in that *in vitro* protein binding studies with serum samples obtained from patients *prior to drug administration* may be useful for predicting the possibility of quantitatively unusual distribution, elimination, and pharmacological effect (4) characteristics of certain highly plasma protein bound drugs such as warfarin in individual patients.

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Gerhard Levy[x]
Avraham Yacobi
Department of Pharmaceutics
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214

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[x] To whom inquiries should be directed.

GLC Microdetermination of Plasma Anticonvulsant Levels

Keyphrases □ Anticonvulsants—plasma levels, GLC microdetermination □ GLC—microdetermination, anticonvulsant plasma levels

To the Editor:

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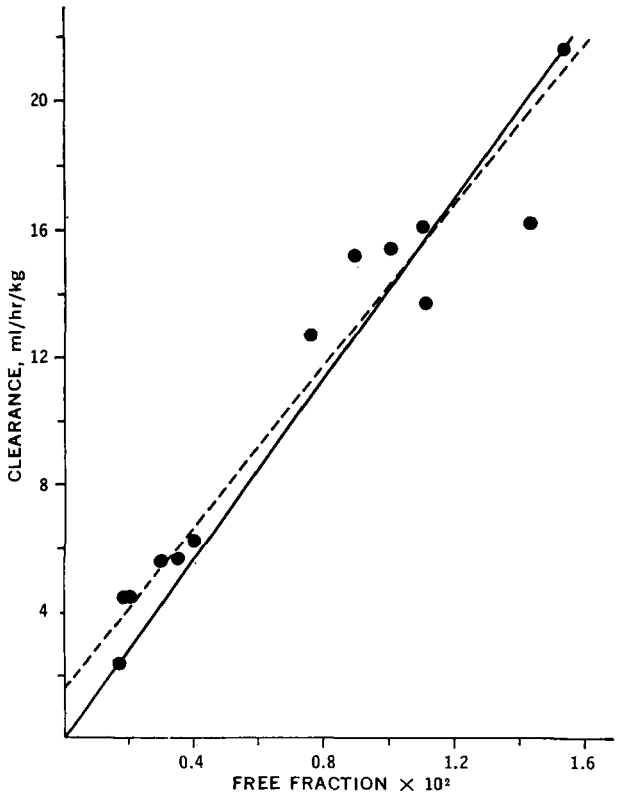

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Gerhard Levy[x]
Avraham Yacobi
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School of Pharmacy
State University of New York at Buffalo
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degraded phenobarbital to phenylethylmalonamide even at low injector temperatures (230°).

A method developed in this laboratory (3) has proven applicable to the analysis of small plasma samples. For the analysis of a 100-μl sample of plasma, the internal standard for phenobarbital, primidone, and diphenylhydantoin is 0.50 ml of a solution of 3 mg 5-(*p*-methylphenyl)-5-phenylhydantoin/500 ml 0.1 *N* NaOH. If ethosuximide is to be determined in the sample, 0.50 ml of a solution of 1 mg of 2,2,3-trimethylsuccinimide in 100 ml of distilled water is also added to the plasma. After the addition of 0.5 ml of a 1.0 *M* phosphate buffer (pH 2.7), the aqueous phase is extracted with 10 ml of ether by shaking for 10 min. The sample is centrifuged and the ether is transferred to another tube[2].

If ethosuximide is present, amyl acetate (50 μl) is added to the ether before evaporation (4). After the ether has been evaporated at 55° under nitrogen, 1 μl of the amyl acetate residue is injected into the gas chromatograph.

To determine phenobarbital, primidone, and diphenylhydantoin in this sample, the amyl acetate or ether residue is dissolved in 0.5 ml of toluene. The drugs are then extracted from the toluene, as previously reported (3), with 12 μl of 2 *M* tetramethylammonium hydroxide[3], and the sample is withdrawn from the lower phase for chromatography.

The column, a 91.5-cm × 2-mm (i.d.) glass U-tube, is packed with 7% OV-17 on 80–100-mesh Chromosorb W-HP. The amyl acetate fraction is run isothermally at 125° for ethosuximide. The tetramethylammonium hydroxide fraction is programmed from 150 to 240° at 10°/min. The carrier gas flow rate is adjusted to around 10 ml/min to give a retention time of 4.0 ± 0.2 min for diphenylhydantoin at 240°.

The limits of detection for phenobarbital, primidone, diphenylhydantoin, and ethosuximide are well below therapeutic levels. Reproducibility is comparable to that of the macrodetermination, and samples analyzed by both methods give good agreement.

Preliminary findings indicate that the anticonvulsants trimethadione, its metabolite dimethadione, and paramethadione can also be quantitated in the amyl acetate fraction with ethosuximide. With this extraction, it is not necessary to use the salting-out technique of Booker and Darcey (5) to get significant recovery of dimethadione. While trimethadione can be seen if chromatographed at 125°, it is better resolved from the solvent front if the temperature is programmed from 100 to 125° at 4°/min. The other succinimide, hydantoin, and barbiturate anticonvulsants can be determined in the tetramethylammonium hydroxide fraction as previously reported (3).

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Robert J. Perchalski[x]

Medical Research
Veterans Administration Hospital
Gainesville, FL 32602

B. J. Wilder

Medical Research
Veterans Administration Hospital, and
Department of Medicine
University of Florida
College of Medicine
Gainesville, FL 32610

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[x] To whom inquiries should be directed.

[2] Concentratube, Laboratory Research Co., Los Angeles, CA 90036

[3] Southwestern Analytical Chemicals, Inc., Austin, TX 78767

Enhancement of Optical Rotation of Levodopa by Cyclization

Keyphrases □ Levodopa—enhanced optical rotation using cyclization, purity determination □ Optical rotation, levodopa—enhanced using cyclization, purity determination

To the Editor:

The optical rotation of levodopa is an important purity criterion, since it provides the only means to control the presence of the allegedly toxic and therapeutically inactive D-isomer. The specific rotation, $[\alpha]_D^{25}$, of levodopa is only about −12°, which is insufficient to control optical purity. Barooshian *et al.* (1) proposed a TLC limit test for D-dopa in levodopa, involving conversion of the enantiomers to diastereomeric dipeptides. Jouin and Saias (2) and Coppi *et al.* (3) described methods based on stereospecific enzymatic destruction of levodopa. The BP 1973 (4) provides requirements of $[\alpha]_{365}^{20}$ −119 to −125° for levodopa drug substance and $[\alpha]_D^{20}$ −38.5 to −41.5° for its dosage forms, the rotation being increased by addition of aluminum sulfate and sodium acetate.

Pesez and Fabre (5) reported that addition of 2.5 g of aluminum chloride and 2 g of methenamine to a solution of 200 mg of levodopa in 5 ml 1 *N* hydrochloric acid, dissolution, and addition of acid to 10 ml afforded a specific rotation at the sodium D-line of −142° after the solution was allowed to stand in the dark at room temperature for 3 hr. They attributed this large increase in optical rotation to an effect of methenamine on the aluminum complex of levodopa. The chemistry of their procedure was investigated; it was found that the use of aluminum chloride is supererogatory, that the enhancement of optical rotation obtained is most probably due to pheno-

degraded phenobarbital to phenylethylmalonamide even at low injector temperatures (230°).

A method developed in this laboratory (3) has proven applicable to the analysis of small plasma samples. For the analysis of a 100-μl sample of plasma, the internal standard for phenobarbital, primidone, and diphenylhydantoin is 0.50 ml of a solution of 3 mg 5-(*p*-methylphenyl)-5-phenylhydantoin/500 ml 0.1 *N* NaOH. If ethosuximide is to be determined in the sample, 0.50 ml of a solution of 1 mg of 2,2,3-trimethylsuccinimide in 100 ml of distilled water is also added to the plasma. After the addition of 0.5 ml of a 1.0 *M* phosphate buffer (pH 2.7), the aqueous phase is extracted with 10 ml of ether by shaking for 10 min. The sample is centrifuged and the ether is transferred to another tube[2].

If ethosuximide is present, amyl acetate (50 μl) is added to the ether before evaporation (4). After the ether has been evaporated at 55° under nitrogen, 1 μl of the amyl acetate residue is injected into the gas chromatograph.

To determine phenobarbital, primidone, and diphenylhydantoin in this sample, the amyl acetate or ether residue is dissolved in 0.5 ml of toluene. The drugs are then extracted from the toluene, as previously reported (3), with 12 μl of 2 *M* tetramethylammonium hydroxide[3], and the sample is withdrawn from the lower phase for chromatography.

The column, a 91.5-cm × 2-mm (i.d.) glass U-tube, is packed with 7% OV-17 on 80–100-mesh Chromosorb W-HP. The amyl acetate fraction is run isothermally at 125° for ethosuximide. The tetramethylammonium hydroxide fraction is programmed from 150 to 240° at 10°/min. The carrier gas flow rate is adjusted to around 10 ml/min to give a retention time of 4.0 ± 0.2 min for diphenylhydantoin at 240°.

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Table I—Relationship of Optical Rotation and Concentration for Levodopa with Methenamine and Hydrochloric Acid

Levodopa, %	0.212	0.499	1.013	1.521	2.000
α	−0.349°	−0.822°	−1.672°	−2.527°	−3.312°
$[\alpha]_D^{25}$	−164.5°	−164.7°	−165.0°	−166.1°	−165.6°

lic cyclization of levodopa (I) with formaldehyde, liberated from methenamine, to form L-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (II) (Scheme I). Evidence supporting this hypothesis and a modified procedure are presented.

Methenamine hydrolyzes in acid solution to formaldehyde and ammonium ion. The 2 g used in the Pesez and Fabre procedure is 14.3 mmoles, which on complete hydrolysis would form about 86 mmoles of formaldehyde and 57 mmoles of ammonium ion, far in excess of the 1 mmole of levodopa and less than 10 mEq of acid present in the reaction mixture. Repeating their procedure[1] gave $[\alpha]_D^{25}$ −146°, and the pH of the solution was 3.2. When the aluminum chloride was omitted, the $[\alpha]_D^{25}$ was −165.6° and the pH was 5.6. Compound II was synthesized from I and formaldehyde according to the directions given by Bell *et al.* (6). It gave a melting range of 287–290° [lit. (6) mp 286–288°] and UV maxima at 282 and 210 nm as reported. Its specific rotation (c 0.14) in 20% hydrochloric acid was 122.2°, about 10% higher than the literature value of −110.5° for a 1.67% solution in this medium. However, it was found that acid concentration had a significant effect on rotation, the values for $[\alpha]_D^{25}$ (c 0.14) being −127.8° in 1 *N* hydrochloric acid and −131.3° at pH 1.04. At pH greater than 7, solutions turned red due to catechol moiety oxidation. The specific rotation of II, determined at pH 5.6 (c 0.2), was −151°. This is equivalent to a molar rotation $[\Phi]_D$ of −315.9°, which is about 3% lower than the $[\Phi]_D$ of −324.4° calculated for levodopa at the same concentration and in the same medium.

It was found that the reaction requires about 2.5 hr for completion, using the Pesez–Fabre conditions without aluminum chloride, making 3 hr a reasonable time specification. A plot of levodopa concentration *versus* observed rotation was rectilinear for amounts between 0.2 and 2.0% (w/v) as indicated in Table I. The data suggest that the method might be useful for quantitative estimation of the drug.

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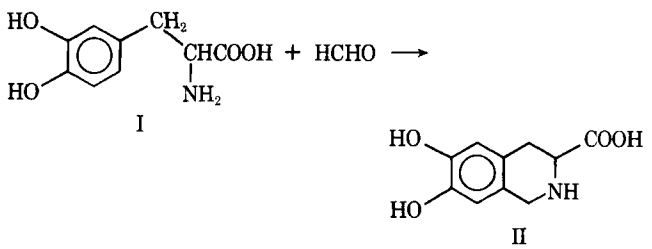

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Lester Chafetz[x]
Tsun-Ming Chen

Pharmaceutical Research and Development Laboratories
Warner-Lambert Research Institute
Morris Plains, NJ 07950

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Slide Test as a Micromethod of a Modified *Limulus* Endotoxin Test

Keyphrases □ *Limulus* endotoxin test—slide test as a micromethod □ Bacterial endotoxin—slide test as a micromethod of modified *Limulus* endotoxin test □ Endotoxin test, modified *Limulus*—slide test as a micromethod

To the Editor:

A cell lysate prepared from amebocytes of *Limulus polyphemus* (the horseshoe crab) has been described as a sensitive reagent for the *in vitro* detection of bacterial endotoxin and as a comparable test to the official pyrogen bioassay using rabbits (1, 2). The test is based upon the fact that the addition of a solution containing endotoxin to a suitable *Limulus* amebocyte lysate preparation and the subsequent incubation at 37° result in a solid gel or an increase in viscosity. Unfortunately, the determination of the end-point presents some difficulties when the described test tube method (3) is used so an objective interpretation of the results is prevented.

The slide test proposed here offers two major advantages: easier determination of the end-point and significant reduction in the amount of reagent needed.

Ten microliters of *Limulus* reagent[1] is placed on the surface of a slide (*e.g.*, glass plate used for the Latex test with a black backside[2]) using a calibrated glass capillary tube. (Slide and capillary tubes must

[1] E-Toxate, Sigma Chemical Co., St. Louis, Mo., or Pyrogent, Mallinckrodt Chemical Works, St. Louis, Mo.

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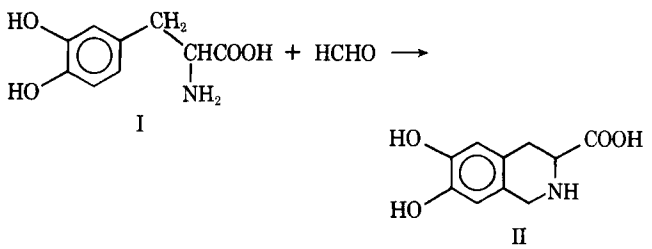

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be free of endotoxin, *e.g.*, by treating this carefully cleaned equipment at 180° for several hours.) Ten microliters of the sample is added and mixed with the aid of the capillary tube (a special pipet holder with a punched rubber cap is recommended[3]). A negative control and a positive control are placed in the same manner on two separate test fields. Air bubbles should be prevented or eliminated using a needle, which has been previously heated to red heat and then allowed to cool. All of the small droplets placed on a slide must show some slight movement when the plate is gently vibrated. The slide is then placed in a "moist chamber" consisting, for example, of a flat plastic box, the bottom of which has been covered with a piece of moistened cellulose. Two glass rods serve as support of the slide.

The whole assembly is incubated at 37° for 30 min. After 30 min, the slide is observed against a bright background. The slide is always held in a horizontal position and may be gently tilted or vibrated through an angle of a few degrees only. A solid gel or an increase in viscosity is easily detected. The incubation may be prolonged, but usually the result remains unchanged. Very hygroscopic solutions tend to increase the volume of the droplets upon prolonged standing and, therefore, to reduce the sensitivity of the test. A nonsaturated moist chamber might be used in these cases.

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P. Frauch
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CH 7000 Chur
Switzerland

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Rapidly Dissolving Forms of Digoxin: Hydroquinone Complex

Keyphrases □ Digoxin—rapidly dissolving digoxin-hydroquinone complex, bioavailability characteristics □ Hydroquinone-digoxin complex—dissolution, bioavailability characteristics □ Bioavailability—digoxin from digoxin-hydroquinone complex

To the Editor:

Numerous recent publications attested to the wide variance in bioavailability found among commercial digoxin tablets (1–5). The main cause of unsatisfactory performance seems to be directly related to the extremely low water solubility of the active substance. Although significant improvements in the efficiency of absorption from oral dosage forms have been obtained through formulation technology, it appears that intrinsically more rapidly dissolving forms of digoxin would provide greater assurance of more reproducible and more bioavailable digoxin products.

In this preliminary communication, we report the development of such forms of digoxin. The approach is based on an earlier concept reported by our group, which utilized free energy of dissolution of molecular complexes to elicit substantially faster dissolution of relatively insoluble solids (6, 7).

Figures 1 and 2 show phase solubility diagrams in water of hydroquinone in the presence of digoxin at

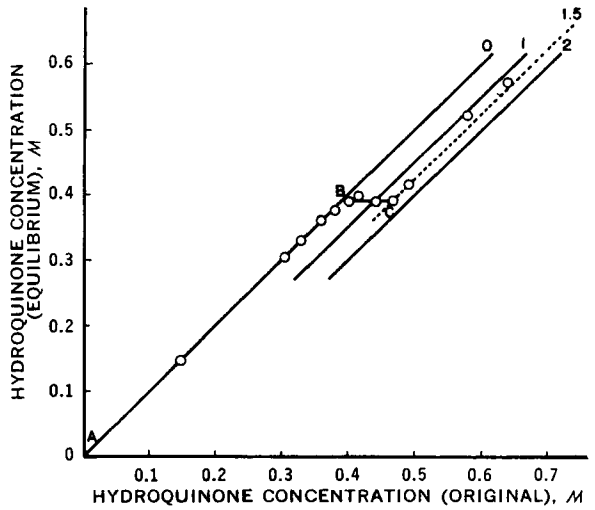

Figure 1—*Plot of hydroquinone concentrations in solution at equilibrium in the presence of 0.05 mole solid digoxin/liter against original concentrations of hydroquinone. The system was equilibrated at 25°. Point B corresponds to the triple point. Line 1 corresponds to the formation of a 1:1 complex, line 1.5 to a 2:3 complex, and line 2 to a 1:2 complex.*

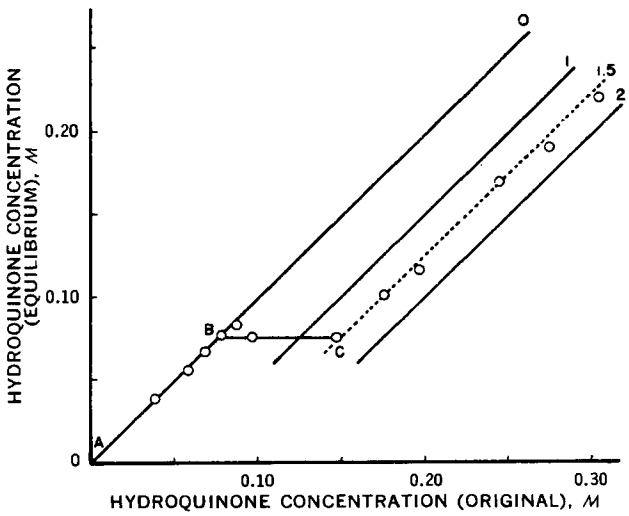

Figure 2—*Plot of hydroquinone concentrations at equilibrium* versus *original concentrations equilibrated at 1.5°. All samples initially contained 0.05* M *(39 mg/ml) of digoxin.*

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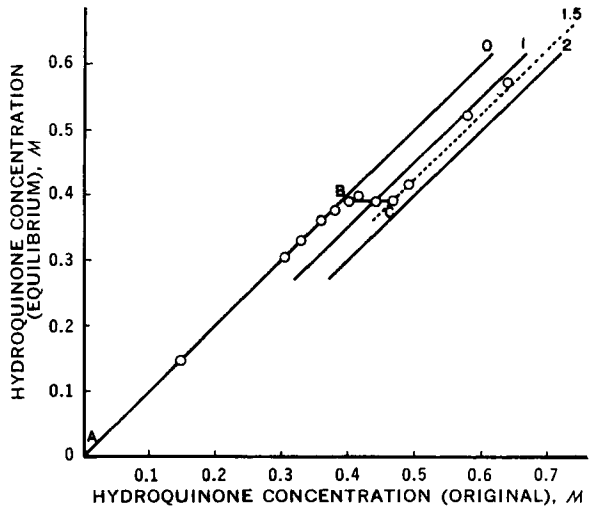

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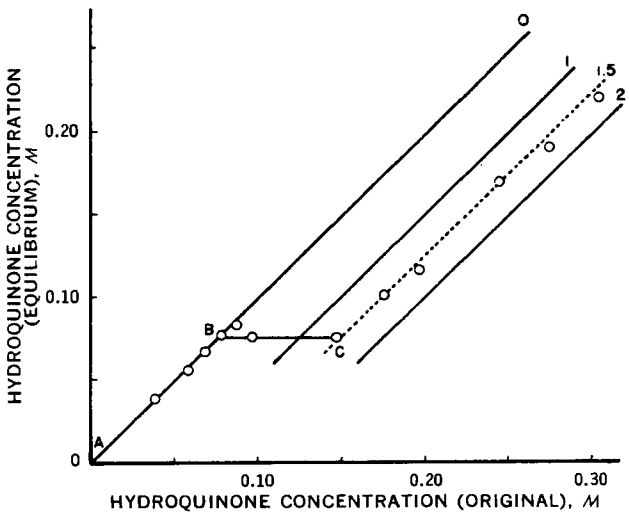

Figure 2—*Plot of hydroquinone concentrations at equilibrium* versus *original concentrations equilibrated at 1.5°. All samples initially contained 0.05* M *(39 mg/ml) of digoxin.*

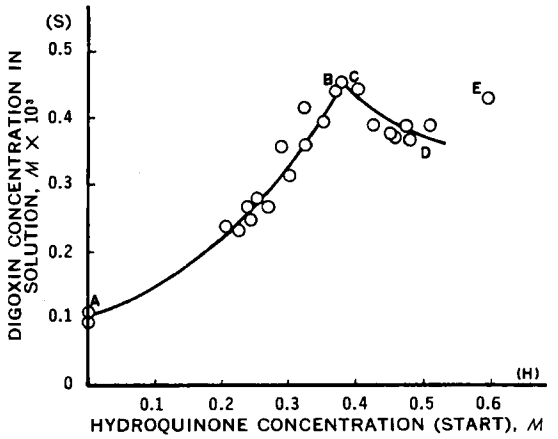

Figure 3—*Apparent solubility of digoxin as a function of hydroquinone concentration at 25°. The system contained 5.12 × 10⁻³ mole digoxin/liter.*

25 and 1.5°, respectively. The more conventional type of plot used to follow complexation reactions of organic species (Fig. 3) is less meaningful in these instances because of the extremely low water solubility of digoxin and the very high solubility of hydroquinone. In both Figs. 1 and 2, the initial total concentration of hydroquinone in solution increases linearly with a slope of one, corresponding exactly to the amount of hydroquinone originally present. At a critical triple point (solution, solid digoxin, and solid complex) corresponding to a solution concentration of approximately 0.38 *M* at 25° and 0.08 *M* at 1.5°, the plots flatten. Following the break, the curves appear to blend into straight lines, again with slopes equal to one.

The total amount of hydroquinone incorporated into digoxin corresponds to the distance between the two parallel lines shown in Figs. 1 and 2. In both instances, the values correspond very closely to formation of a complex species containing two molecules of digoxin and three of hydroquinone. In our studies, variation of conditions led to stoichiometry ranging from 1:1 to 1:2. The usual difficulty of isolating such complexes from their mother liquor without incorporating into the final recovered solid significant amounts of the more concentrated species contained in the adhering mother liquor prevents exact analytical studies on the pure complex. It is, of course, impractical to attempt recrystallization or washing to remove the adhering liquid, since the complex is much more soluble than one of the constituents. Analysis of the unwashed complex corresponded very closely to the indicated chemical compositions.

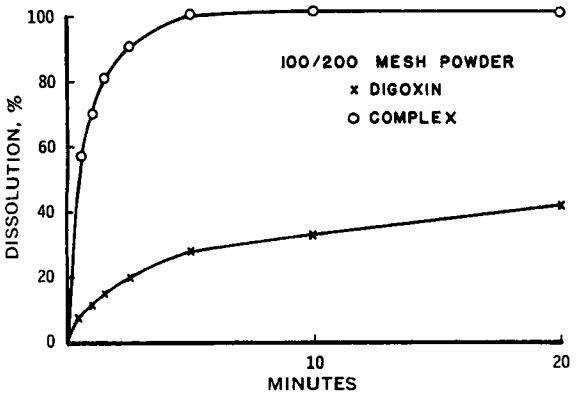

Figure 4—*Dissolution rate profiles obtained for the hydroquinone-digoxin complex and for digoxin itself under comparable conditions.*

The mathematical analysis of dissolution behaviors of complexes of this type was already treated in depth (6, 7). The general equation that applies to dissolution rates of molecular complexes cannot be solved exactly if the diffusivities of the component molecules are significantly different and for other than 1:1 complexes. The form of the equation indicates, however, that for 2 digoxin:3 hydroquinone complex the rate of dissolution per unit area under given stirring conditions will be greater than $(K_{sp})^{1/5}(S_{DG})^{-1}$ than that of pure digoxin, where $K_{sp} = [DG]^2[HQ]^3$, where both concentrations are those of free species at the triple points, and where S_{DG} is the solubility of digoxin alone in water. For the 3:2 complex, $(K_{sp})^{1/5}(S_{DG})^{-1}$ is approximately 140 at 25°, which strongly suggests that its intrinsic rate of dissolution may be as large as several hundred times that of digoxin itself.

In Fig. 4, the relative rates of dissolution of digoxin powder and digoxin-hydroquinone complex are shown[1]. The dissolution measurements were made on the equivalent of 5 mg of digoxin as a 100–200-mesh powder in 500 ml water containing two drops of polysorbate 80[2]. The dissolution medium was maintained at 25° and stirred at 100 rpm. Under these conditions, it is evident that the complexed form of digoxin dissolved much more rapidly, as expected, than digoxin itself. The observed increase in rate has been shown to carry over into tablet formulation.

Since the improved dissolution characteristic is the property of the new form of digoxin and the overall release behavior would be less subject to minor variation in processing, the complex may prove to be the answer to the current problems associated with digoxin solid dosage forms. Moreover, since the complex exists as such only in the solid state and is essentially totally dissociated when dissolved, it only serves as a delivery mechanism. Any toxicity attributable to the amount of hydroquinone present in the final dosage form is, of course, negligible.

We have also formed and isolated a resorcinol complex, but our studies have been quite limited.

(1) *Fed. Regist.*, **39**, 2471(1974).

(2) J. Lindenbaum, M. G. Mellow, M. D. Blackstone, and V. P. Butler, *N. Engl. J. Med.*, **285**, 1344(1971).

(3) T. G. Vitti, D. Banes, and T. E. Byers, *ibid.*, **285**, 1433(1971).

(4) J. G. Wagner, M. Christensen, E. Sakmar, D. Blair, J. D. Yates, P. W. Willis, III, A. J. Sedman, and R. G. Stoll, *J. Amer. Med. Ass.*, **224**, 199(1973).

(5) J. Skelly and G. Knapp, *ibid.*, **224**, 243(1973).

(6) T. Higuchi, "Teacher's Seminar of American Association of Colleges of Pharmacy," 1961, p. 119.

(7) E. Shefter and T. Higuchi, *J. Pharm. Sci.*, **52**, 781(1963).

[1] These plots were obtained for us by Dr. Richard Shaffer of INTERx Research Corp., Lawrence, Kan.

[2] Tween 80.

Takeru Higuchi[x]
Masaru Ikeda
Pharmaceutical Chemistry Laboratory
University of Kansas
Lawrence, KS 66044

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Southern Research Institute
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May 22
Rutgers College of Pharmacy Annual Pharmaceutical Conference, Rutgers University, New Brunswick, N.J. (Contact D. A. Swanson, Professional Relations Committee, Rutgers Pharmaceutical Conference, Sandoz Pharmaceuticals, East Hanover, NJ 07936)

May 22–24
Workshop/Symposium on Compliance with Therapeutic Regimens, McMaster University, Hamilton, Ontario, Canada L8S 4J9 (Contact David L. Sackett, Room 3H2, McMaster University Medical Centre, 1200 Main St. W., Hamilton, Ontario, Canada L8S 4J9)

June 10–14
Annual National Industrial Pharmaceutical Research Conference, The Land O'Lakes Conferences, Extension Services in Pharmacy, University of Wisconsin—Extension and the School of Pharmacy, University of Wisconsin, Madison, Wis.

June 12–14
Annual Summer Symposium on On-Line Analytical Chemistry, Division of Analytical Chemistry, American Chemical Society, Ft. Collins, Colo. (Contact R. A. Hagstrom, Olin Mathieson Chemical Corp., 275 Winchester Ave., New Haven, CT 06504)

June 16–20
National Medicinal Chemistry Symposium, Division of Medicinal Chemistry, American Chemical Society, University of New Hampshire, Durham, N.H. (Contact Dr. Robert E. Lyle, Department of Chemistry, Parsons Hall, University of New Hampshire, Durham, NH 03824)

June 17–20
European Congress on Thermography, The European Thermographic Association and The American Thermographic Society, Amsterdam, The Netherlands (Contact Secretariat, 1st European Congress on Thermography, Organisatic Bureau Amsterdam, N.V., P.O. Box 7205, Amsterdam, The Netherlands)

June 17–21
National Workshop in Teratogenesis and Mutagenesis, Food and Drug Administration, Teratology Society, and Jefferson Medical College (Contact R. P. Jensh, Jefferson Medical College, 920 Chancellor St., Philadelphia, PA 19107)

June 19–20
National Biomedical Plastics Conference, Chicago, Ill. (Contact W. A. Kulok, 600 Third Ave., 37NR, New York, NY 10016)

June 23–27
American Medical Association, Annual Convention, Palmer House, Chicago, Ill.

June 24–26
International Symposium on Mass Spectrometry in Biochemistry and Medicine, Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy (Contact Alberto Frigerio, Istituto di Ricerche Farmacologiche, 20157 Milan-Via Eritrea, 62, Italy)

June 24–28
Symposium on Chemistry of Natural Products, National Research Council of Canada and IUPAC, Carleton University, Ottawa, Ontario, Canada (Contact M. K. Ward, NRCC, Ottawa K1A OR6, Ontario, Canada)

June 25–28
Canadian Federation of Biological Sciences, Annual Meeting, Hamilton, Ontario, Canada (Contact D. T. Armstrong, Department of Physiology, University of Western Ontario, London 72, Ontario, Canada)

June 26–28
Sources of Drug Information for the Health Professions, Drug Information Association, Annual Meeting, Palmer House, Chicago, Ill.

July 1–5
World Congress of Environmental Medicine and Biology, Unesco House, Paris, France (Contact General and Scientific Secretariat, Dr. Richard Abbou, 115 Rue de la Pompe, 75116, Paris, France)

July 29–August 1
Annual Conference on Pharmaceutical Analysis, The Land O'Lakes Conferences, Extension Services in Pharmacy, University of Wisconsin—Extension and the School of Pharmacy, University of Wisconsin, Madison, Wis.

August 3–8
American Pharmaceutical Association, Annual Meeting, Chicago, Ill.

August 5–9
Gordon Research Conference on Medicinal Chemistry, New London, N.H. (Contact A. M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881)

August 8–13
American Podiatry Association, Annual Meeting, Marriott Motor Hotel, Atlanta, Ga.

September 2–7
General Assembly of the International Pharmaceutical Federation, Rome, Italy

September 8–13
American Chemical Society, National Meeting, Atlantic City, N.J. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

September 8–13
International Conference on Alcohol, Drugs, and Traffic Safety, Toronto, Canada (Contact Dr. W. S. Schmidt, 33 Russell St., Toronto, M5S 2S1, Canada)

September 9–13
International Symposium on Medicinal Chemistry, Medicinal Chemistry Section, Royal Netherlands Chemical Society, Noordwijkerhout, The Netherlands (Contact Secretariat, Merck Sharp and Dohme B. V., External Relations, Waarder-weg 39, P.O. Box 581, Haarlem, The Netherlands)

September 19–21
Seminar on the Relevance of Animal Models to Human Dermatopharmacological and Dermatotoxicity, Medical Sciences Building, University of California, San Francisco, Calif. (Contact Continuing Education in Health Sciences, Room 570-U, University of California, San Francisco, Calif.)

September 24–27
National Congress of Pharmaceutical Sciences, Hungarian Pharmaceutical Association, Hungarian Academy of Sciences, Budapest, Hungary [Contact Office for Conference Organization (Motesz), H-1361 Budapest, POB 32, Hungary]

October 8–12
American Society of Hospital Pharmacists, Midyear Meeting, Hollywood, Fla.

October 13–15
St. Louis Society of Pharmacists in Industry, Annual Seminar, St. Louis Marriott, St. Louis, Mo. (Contact Cecil W. Propst, Lewis-Howe Co., 319 S. 4th St., St. Louis, MO 63102)

October 14–17
Association of Official Analytical Chemists, Annual Meeting, Marriott Hotel, Twin Bridges, Washington, D.C. (Contact L. G. Ensminger, AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20037)

October 20–24
American Public Health Association, New Orleans, La.

October 25
APhA Academy of Pharmaceutical Sciences, Industrial Pharmaceutical Technology Section, Cherry Hill Inn, Cherry Hill, N.J. (Contact A. J. Scarpone, Publicity Committee, Lederle Laboratories, Pearl River, NY 10965)

October 31–November 3
American College of Apothecaries, Annual Convention, Alameda Plaza Hotel, Kansas City, Mo.

November 10–14
APhA Academy of Pharmaceutical Sciences, National Meeting, Fairmont Hotel, New Orleans, La.

November 10–14
American Dental Association, Annual Meeting, Washington, D.C.

Items to be considered for inclusion or corrections should be addressed to:

J. Pharm. Sci. Calendar
2215 Constitution Avenue, N.W.
Washington, DC 20037

Cell as Receptor System

Until relatively recently, the cell membrane has been conceptualized as a fixed structure surrounding the liquid cytoplasm. A growing literature now maintains that the cell membrane is also a liquid but that its degree of viscosity exceeds that of the cytoplasm. It would follow from this reasoning that the total cell may thus be considered to be a solution, subject to the physical-chemical laws that govern such states[1]. If one views the total cell as a solution, then the cytoplasm can be conceptualized in terms of a coacervate[2].

The proposal of the total cell as a solution has a number of immediate applications to cell-drug interactions. First, the model we advocate is consonant with Ferguson's thesis for those drugs whose action is a function of the chemical potential of the drug in solution, the cell in this instance being the solution. The extremely fine tuned specificity implicit in our model of the cell is verified by the facts pertaining to geometric and optical isomers. Thus, geometric isomers are known to have different solubility values. Even optical isomers, in the optically active media of the total solution of the cell, have different physical-chemical properties, one of which is different solubility values.

An important clinical and theoretical application involving drug activity is immediately evident. The conventional view of drug-receptor interaction presupposes a fixed, highly stereospecific similarity between the drug and the receptor surfaces of the cell. However, if the cell is to be considered a solution, as we maintain, then one may speak of different degrees of drug-solvent solubility. Thus, drugs with subtle differences in activity would be expected to have correspondingly subtle differences in their cell solution solubility values. If one considers the fact that each of the thousands of drugs now available has a different saturation point, it becomes clear that the apparently amorphous solvent system is in reality a continuum consisting of an equilibrium of an infinite number of points contributing to solvent specificity.

The classic "lock and key" concept carried to its logical conclusion implies that a universe of a thousand drugs infers a corresponding universe of a thousand receptor sites. By contrast, totality of the cell solution as a single receptor system means that it can assume a configuration specific to the slightly varying configuration of each innumerable, individual drug.

Other facts also support our proposed model. Present concepts of the cell do not satisfactorily explain paradoxical and biphasic drug dose response. Solution theory as applied to the cell does. Thus, in solution theory, it is recognized that as a point of saturation in the solution is reached and exceeded, aggregation of the drug molecules occurs. Depending on the structure of the aggregate, the drug response can level off, reverse itself, or be paradoxical, *i.e.*, contrary to expectation.

In our view, these facts and others argue for the proposition that the cell as a solution acts as a single receptor system.

Bernard Ecanow
Bernard H. Gold
College of Pharmacy
University of Illinois
Chicago, Ill.
and
Department of Anesthesiology
Rush Medical Center
Chicago, Ill.

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[1] B. Ecanow and H. L. Klawans, in "Physical Chemical Models of Membranes in Models of Human Neurological Disease," 1st ed., H. L. Klawans, Ed., Excerpta Medica, Amsterdam, The Netherlands, 1974.
[2] B. Ecanow and B. Gold, *N. Engl. J. Med.*, in press.

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OPEN SYSTEM IN NAME ONLY?

Industry traditionally has been depicted as rather conservative regarding disclosure and release of information. This approach of "holding one's cards close to the chest" is characteristic of industry in general and not just the drug industry. Trade secrets, competition, and an ingrained concern about disclosure have all contributed to this general attitude.

Government agencies, on the other hand, have not assumed a comparable public image. On the contrary, most citizens have felt that the democratic process in itself assures an openness toward the availability of information.

However, those who have had direct dealings with government, and who have felt the need to obtain information from government agencies, have often found that these agencies can be as difficult and uncooperative in releasing information as the most obstinate of industrial concerns. It was such experiences that cumulatively motivated Congress to pass the Freedom of Information Act. While this legislation has now been on the books for a couple of years, the regulations pertaining to just how it is to be implemented have dribbled along in a painfully slow process.

The concept of freedom of information covers both written documents as well as meetings at which positions and data would be considered as a basis for formulating policy.

The *Federal Register* is the daily chronicle whereby all of the various agencies within the executive branch of the federal government announce various proposed rules, issue notices, and proclaim finalized rules and regulations. The difficulty with which various agencies adapted to the new approaches brought about by the Freedom of Information Act was typified by the meeting announcements which appeared during the first few months of this new practice. Very few such announcements appeared as much as a week before the date of the meeting itself, and frequently they were not announced until after the meeting had actually been held. Probably as a result of the criticism which ensued, it appears that this problem has been largely eliminated, and from our observation, most meetings are now adequately announced in advance, to enable interested persons to attend.

All too often, however, significant proposed regulations are put forward by federal agencies with unduly brief time permitted the public, or interested parties, to consider the proposal and to submit comments. Indeed, the time allowed is occasionally completely absurd to the point where issuance in "proposed rule" form is nothing more than a sham.

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The use of such tactics by government agencies is no less reprehensible than comparable efforts on the part of industry to manipulate matters of interest to the industrial sector—which is a frequently heard criticism of industry. Moreover, such transparent maneuvering to ramrod through new regulations simply invites needless and unnecessary court challenges which introduce even greater delays, besides frustrating all parties involved. We urge that the agencies involved give due consideration to a realistic timetable, in order that the public can truly have the opportunity to participate in the governmental process as Congress has intended.

Edward G. Feldmann

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Edward G. Feldmann

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REVIEW ARTICLE

Milling of Pharmaceutical Solids

EUGENE L. PARROTT

Keyphrases □ Milling of pharmaceutical solids—review of theory, energy relationships, size distribution, rate, and time considerations, equipment □ Comminution—review of theory, energy relationships, size distribution, rate, and time considerations, equipment, application to pharmaceutical solids □ Pharmaceutical technology—mechanical size reduction of solids (milling), review □ Solids, size reduction—review of milling theory, energy relationships, size distribution, rate, and time considerations, equipment □ Size reduction of pharmaceutical solids—review

Perhaps due to its complex nature and to the inadequacy of quantitative theories of size reduction, there is little pharmaceutical literature on the mechanical operation of size reduction, which is a factor at some stage in the production of most dosage forms. Comminution is a generic term for size reduction. Many other terms—grinding, disintegration, pulverization, and dispersion—have been used synonymously for comminution. In this review, mechanical size reduction of solids will be referred to as milling.

A real particulate system always consists of a mixture of particles in a range from the minimum to the maximum particle size. The size characteristics of a real particulate system are completely expressed by a size-frequency curve, which is a plot of the quantity or percentage, by number or weight, that is within a certain size range against particle size. The distribution of many milled or chemically precipitated particles is asymmetrical or skewed (1, 2). Many asymmetrical frequency curves have a log-probability distribution and can be made symmetrical if the logarithms of size are used rather than size (3, 4). Size distribution may also be represented by a plot of the cumulative percent larger (or smaller) than a stated size against particle size. The immediate objective of milling is to satisfy the size limiting specifications, which may be expressed as a minimum and maximum size, a size-frequency distribution curve, or a specific surface.

As an arbitrary classification for pharmaceutical milling, coarse milling produces particles larger than 20 mesh, intermediate milling produces particles from 200 to 20 mesh, and fine milling produces particles smaller than 200 mesh. Ultrafine refers to sizes approaching 1 μm. A mill may operate satisfactorily in more than one case; *e.g.*, a comminuting machine[1] may be used to prepare a 20-mesh tablet granulation and to mill a crystalline material to a 200-mesh powder.

The importance of size reduction and its control has been recognized for many years, but there seems to be no way to relate the coefficients describing the physical properties of a solid to the physical process that occurs during milling. The ability to resist size reduction depends on hardness, which is the resistance to crushing, and is an indication of abrasiveness or mill wear. Since values of the Moh hardness index (5) are available for minerals but not for drugs, by analogy a medicinal compound is qualitatively

[1] Fitzpatrick.

said to be soft, intermediate, or hard. A grindability index has not been generally accepted for materials other than coal, because of the wide variety of grinding equipment that may be used and the dissimilar physicochemical properties of materials. There does not seem to be a quantitative relationship of grindability to hardness (6). Grindability depends not only on the properties of the material but also on the manner in which size reduction is achieved.

In pharmacy the extent of size reduction is determined by the improvement of clinical efficacy and product characteristics and by the facilitation of production rather than cost of the milling operation.

A 0.5-g dose of micronized griseofulvin administered orally produced serum levels indistinguishable from those produced by a 1.0-g dose of nonmicronized griseofulvin (7). Other examples of the effect of particle size of drugs and its relationship to absorption and clinical activity have been cited (8).

Particle-size distribution may affect texture, taste, and rheology of oral suspensions in addition to absorption (9). The effects of specific surface and particle-size distribution on rheology and product injectability and on the effectiveness of intramuscular suspensions of procaine penicillin G were reported (10). Solvolytic decomposition of solids initially occurs at surface irregularities and is increased by the presence of solvates or moisture. Micronization and subsequent drying increase the stability since the occluded solvent is removed (11).

The flowability of powders and granulations in high-speed filling equipment and in tablet machines affects product uniformity. Relationships between flow rate and particle size have been reported (12–14). In solid–solid blending, large and small particles tend to segregate; this may be countered by milling the components to a small size with a narrow size range (15). When small quantities of a liquid are to be blended into a powder, particle size should be uniform to obtain batch-to-batch uniformity (16). Extraction of animal glands and crude vegetable drugs is facilitated by milling to an optimum size. The drying of wet masses, *e.g.*, tablet granulations, is facilitated by milling, which increases the surface and reduces the distance the solvent must diffuse to the outer surface. The role of particle-size reduction in tablet manufacturing operations has been discussed (17).

THEORY OF COMMINUTION

Solids under stress are strained and deformed. Mechanical behavior can be illustrated in the stress–strain curve shown in Fig. 1. The initial linear portion of the curve is defined by Hooke's law, *i.e.*, stress is proportional to strain, and Young's modulus, *i.e.*, the ratio of stress to strain expresses the softness or stiffness in dynes per square centimeter. The curve becomes nonlinear at the yield point, which measures the resistance to permanent deformation. With further stress the region of irreversible plastic deformation is reached. The total area under the curve represents the energy of fracture and is an approximate measure of the impact strength of the material.

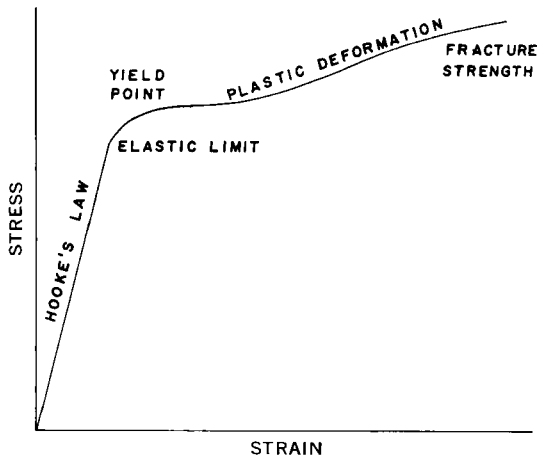

Figure 1—*Stress–strain behavior, with the area under the curve representing the energy required to fracture the solid.*

For most metals the stress–strain curve in the elastic region of Hooke's law is reversible, but for polymeric materials hysteresis is frequent. If a force is released and applied to a polymeric material, an elastic loop or hysteresis occurs in the stress–strain cycle; the area of the loop represents the dissipation of stress energy (usually as heat).

When the application and release of a force cause permanent deformation, energy is expended and cracks may be initiated. The furthest extension of a crack is the point of greatest stress. Since the surface of a particle is irregular, the force is initially applied to the higher portion of the surface with the result that high stresses and temperatures exist locally in the material. A force that exceeds the elastic limit fractures the particle. As fracture occurs, the points of application of force are shifted. Stress waves released by the initial fracture release energy, producing other regions of high stress from which new cracks or fractures may occur. If force is rapidly applied by impact, cracks may form before the strain energy has reached equilibrium in the particle and thereby decrease the total work input required for fracture.

Crystalline materials fracture along crystal cleavage planes; noncrystalline materials fracture at random. If an ideal crystal were pressed with an increasing force, the force would be distributed uniformly through its structure until the crystal disintegrated into its individual units. A real crystal fractures under much less force into a few relatively large particles and a number of fine particles with relatively few particles of intermediate size. It seems that the size of the larger particles is related to the size reduction process and that the size of the finer particles is related to the structure of the material. Crystals of pure substances have internal weaknesses due to missing atoms or ions in their lattice structures and flaws arising from mechanical or thermal stress (6).

The Griffith theory (18, 19) of cracks and flaws assumes that all solids contain flaws and microscopic

cracks, which increase the applied force according to the crack length and focus the stress at the atomic bond of the crack apex. The Griffith theory may be expressed (20, 21) as:

$$T = \sqrt{\frac{Y\epsilon}{c}} \qquad \text{(Eq. 1)}$$

where T is tensile stress, Y is Young's modulus, ϵ is the surface energy of the wall of the crack, and c is the critical crack depth required for fracture. A linear relationship between the square of tensile strength of minerals and the critical height for drop weight impact suggests that the square of tensile strength is a useful criterion of impact fracture (22).

Thermodynamic treatment of the milling process has been attempted, but there is confusion about the meaning of surface tension, surface stress, and surface energy of solids, and there is some question as to whether or not a reversible path may be devised for a milling process. Thermodynamics have shown that the work to fracture a particle is dependent on surface energy (23) and that the yield stress is dependent on the rate of strain and temperature of the fluid filling the particle pores (24). Fracture is predicted to be more efficient at elevated temperature (25).

The weakest flaw in a particle determines its fracture strength and controls the number of particles produced by fracture. Although particles with the weakest flaws fracture most easily and produce the largest particles, they are not necessarily the easiest to mill to a desired size because they may require several more stages of fracture than particles of the same size whose weakest flaw is stronger.

The goal of milling is to form cracks that propagate through the deformed particles at the expense of strain energy and produce fracture. The useful work is directly proportional to the new surface area; since the crack length is proportional to the square root of the new surface formed, the useful work is proportional to the square root of the diameter of the milled particles minus the initial diameter (26). The useful energy, E^*, expended is:

$$E^* = E\left(\frac{\sqrt{D_1}}{\sqrt{D_1} - \sqrt{D_2}}\right) \qquad \text{(Eq. 2)}$$

where D_1 is the diameter of the material to be milled, D_2 is the diameter of the milled material, and E is the energy input.

Size reduction begins with crack fracture, which requires a certain minimal energy; however, milling conditions are so random that many particles receive impacts that are insufficient to fracture them and some particles are fractured by excessively forceful impact. Hence, milling is an inefficient operation. The energy not used in fracture is dissipated in the milling equipment as heat, vibration, sound, and inefficiency of transmission; it is dissipated in the material as interparticulate friction, friction between the material and the mill, and elastic deformation of unfractured particles.

The fracture mechanism is impact when a force of sufficient magnitude is rapidly applied normal to the surface, is directed toward its center, and results in the formation of two or more fragments. If the force is slowly applied, the fracture mechanism is compression. Attrition is the fracture mechanism by which force is applied parallel to the surface of the solid and results in extremely fine particles.

ENERGY FOR COMMINUTION

The energy required to reduce the size of particles is inversely proportional to the size raised to some power. This may be expressed mathematically as (27):

$$\frac{dE}{dD} = -\frac{C}{D^n} \qquad \text{(Eq. 3)}$$

where dE is the amount of energy required to produce a change in size, dD, of unit mass of material, and C and n are constants.

In 1885, Kick (28) proposed that the energy expenditure, E, for size reduction is directly related to the reduction ratio, D_1/D_2, where D_1 and D_2 are the diameters of the material to be milled and the milled material, respectively. Thus, if a given quantity of energy is required to mill a given weight of material from 500 to 250 μm, the same energy would be required to reduce the size from 50 to 25 μm. Kick's proposal may be expressed as:

$$E = C \ln \frac{D_1}{D_2} \qquad \text{(Eq. 4)}$$

where C is a reciprocal efficiency coefficient. In the engineering literature, $C = K_k f_c$, where f_c is the crushing strength of the material, and K_k is known as Kick's constant. If $n = 1$, the general differential equation reduces to Kick's equation. Kick's proposal was developed on a stress-strain diagram for cubes under compression and represents the energy required to effect elastic deformation before fracture occurs. It is most applicable to coarse grinding (29).

In 1867, von Rittinger (30) proposed that the energy required for size reduction is directly proportional to the increase in surface as expressed by the relationship:

$$E = k_1(S_2 - S_1) \qquad \text{(Eq. 5)}$$

where k_1 includes the relationship between the particle surface and diameter, and S_1 and S_2 are the specific surfaces before and after milling, respectively. In terms of particle diameters:

$$E = C'\left(\frac{1}{D_2} - \frac{1}{D_1}\right) \qquad \text{(Eq. 6)}$$

where D_1 and D_2 are the diameters before and after milling, respectively. In the engineering literature, $C' = K_r f_c$, where K_r is known as Rittinger's constant. If $n = 2$ (because the surface is proportional to the square of the diameter), the solution of the general differential equation yields Rittinger's equation. Rittinger's relationship is most applicable to fine grinding (29).

In 1952, Bond (26, 31) suggested that the energy required for size reduction is inversely proportional to the square root of the diameter of the milled ma-

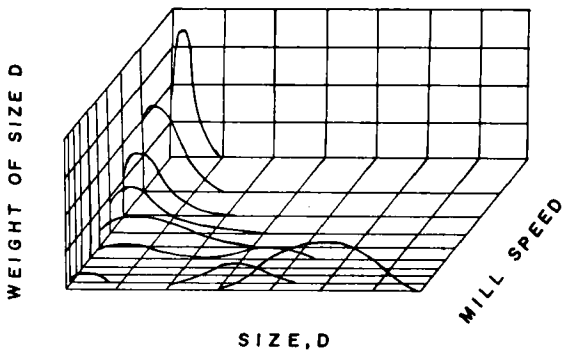

Figure 2—*Diagrammatic representation of changes in particle-size–frequency distribution as milling progresses (34).*

terial. Mathematically, this may be expressed:

$$E = 2C' \left(\frac{1}{\sqrt{D_2}} - \frac{1}{\sqrt{D_1}}\right) \qquad \text{(Eq. 7)}$$

If $n = 1.5$, the solution of the general differential equation yields Bond's equation.

Bond expressed the energy expended in milling in terms of a work index, W_i. The work index is the amount of energy required to reduce a unit mass of material from a theoretically infinite particle size to such a size that 80% passes through a 100-μm screen. It is calculated by Eq. 8:

$$W_i = \left(W \sqrt{\frac{D_2}{100}}\right) \left(\frac{\sqrt{D_1/D_2}}{\sqrt{D_1/D_2} - 1}\right) \qquad \text{(Eq. 8)}$$

where W is the work input. The work index is not a true constant because the value changes with a shift in particle-size distribution and with structural characteristics of the material. It is probably the best available method for comparing the efficiency of milling operations, and its most useful value is determined under conditions of the final operation. The dry grind work index is usually 1.3 times the wet grind value.

If n is replaced by $(1 + n)$ in the general differential equation and the equation is integrated (32, 33):

$$W = W_i \left[1 - \left(\frac{1}{D_1/D_2}\right)^n\right] \left(\frac{100}{D_2}\right)^n \qquad \text{(Eq. 9)}$$

The n may be considered as an exponent expressing variation in resistance to fracture with size change and with change in efficiency which may be influenced by particle size.

SIZE DISTRIBUTION

When a material is milled, the particles have a variety of sizes as determined by the flaw structure. The variation in size is usually expressed as a size–frequency distribution curve, which is a plot of the percentage frequencies of the various particles against size. With progressive milling, the particle size-frequency distribution has a narrower range and a finer mean size. Changes in size distribution as milling proceeds are illustrated in Fig. 2. If the initial material has a monomodal size distribution, as milling proceeds it develops a bimodal size distribution. The primary component gradually decreases in weight as milling progresses, and the secondary component increases in weight. This reduction in weight is accompanied by a decrease in modal size of the primary component and is caused by preferential fracture of the larger particles. The modal size of the secondary component remains constant. Further milling tends to eliminate the primary component. If the material is transferred to a second mill for finer size reduction, the process is repeated (34). As the particle-size distribution changes, milling characteristics, *e.g.*, work index, change because the abundance or shortage of flaws varies at different sizes.

At least two specifications are required to characterize a specific size distribution, *e.g.*, 85% through a 60-mesh screen and 5% through a 325-mesh screen. In the simplest case, one number establishes the limits of particle sizes involved and another determines the weight relationships in the various size ranges. Schuhmann (35) experimentally verified the empirical equation:

$$y = 100(D/k)^a \qquad \text{(Eq. 10)}$$

where y is cumulative weight percent smaller than size D, k is a size modulus for a given distribution, and a is a distribution modulus. The Schuhmann equation describes the size relationships of many fractured homogeneous materials. As shown in Fig. 3, when the cumulative weight percent less than stated size is plotted on log–log paper against size, a straight line is obtained with a slope of a, and it intersects the 100% ordinate line at the theoretical maximum-sized particle equal to k (36). For pure impact grinding, $a \rightarrow 1$; for abrasion grinding, $a \rightarrow 0$.

As a general rule, milled powders have a log-probability distribution (37). The method of size distribution analysis favored by the author involves the log-normal distribution equation:

$$\Delta y = \frac{1}{\sqrt{2\pi} \ln \sigma} \int_{\ln D_1}^{\ln D_2} \exp\left\{-\left[\ln \frac{D}{D_g} \Big/ (\sqrt{2} \ln \sigma)\right]^2\right\} d(\ln D) \qquad \text{(Eq. 11)}$$

where Δy is the weight fraction of particles between diameters D_1 and D_2, D_g is the geometric mean diameter, and σ is the geometric standard deviation (38).

The manipulations of these equations are tedious, and calculations are simplified by the use of log-probability paper. Particle size is plotted on the log grid, and cumulative percent smaller than the stated size is plotted on the probability grid. The best straight line is drawn through the experimental points, and the geometric mean diameter is the 50% value on this line. The range of particle sizes is indicated by the standard deviation, which is equal to the 84.13% size read from the straight line divided by the 50% size (or 50% size divided by the 15.87% size). Once the geometric mean diameter and the standard deviation are evaluated, other diameters may be easily calculated (39–42). Depending on the particulate system used, the most relevant diameter is calculated. For example, by calculating the mean

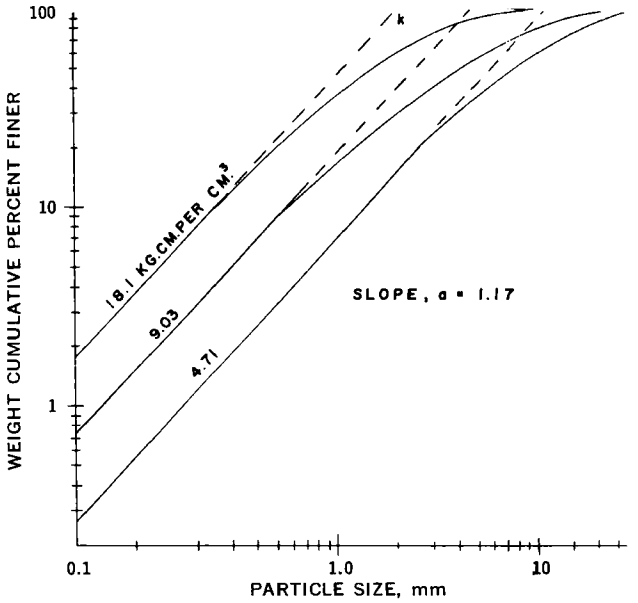

Figure 3—*Size distributions resulting from impact of lumps of salt at various levels of impact energy (36).*

volume-surface diameter, D_{vs}, and using Eq. 12:

$$S = \frac{6}{D_{vs}\rho} \qquad \text{(Eq. 12)}$$

one may find the specific surface, S, for a powder with a density ρ.

Experimental proof of the relationship between energy and size reduction is difficult because it is experimentally impossible to fracture particles of one uniform size to particles of a smaller and still uniform size as required by Eqs. 4, 6, and 7. It is also impossible to describe accurately the weight relationships of sizes in a given material by a single number since, for a given size modulus, there is theoretically an infinite number of size distributions to which the modulus may refer. In addition, the energies required to produce each size distribution from a given size will be different.

A second difficulty in verifying energy-size reduction relationships is the definition of energy actually causing fracture and other forms of energy in the milling process. Since fracture is dependent on strain energy of elastic deformation, it seems likely that the energy is related to size reduction. Strain energy absorbed by slow compression is easy to measure; strain energy absorbed by impact is very difficult to measure due to the complex milling process involving translatory motion, vibratory motion, plastic deformation, and sound.

A third difficulty in establishing energy–size reduction relationships arises from the fact that each equation implies that n is a constant independent of the mechanism of size reduction and independent of the amount of size reduction. Probably n is not a constant but is a variable depending on the material and the manner in which it is fractured. The following derivation illustrates a general method by which n and C may be calculated using a single distribution plot for any milling test.

When Eqs. 3 and 10 are combined, the energy, E, required to reduce an element of weight of material, dy, from size D_m to size D is:

$$E = \int_{D_0}^{k}\int_{D_m}^{D} (-C\, dD_1/D_1^{n})\, dy \qquad \text{(Eq. 13)}$$

As size distribution is described by the Schuhmann equation:

$$dy = \left[\frac{100a}{k^a} D^{a-1}\right] dD \qquad \text{(Eq. 14)}$$

Thus:

$$E = \int_{D_0}^{k}\int_{D_m}^{D} (-C\, dD_1/D_1^{n}) \left(\frac{100a}{k^a} D^{a-1}\, dD\right) \qquad \text{(Eq. 15)}$$

$$E = \frac{Ca}{(n-1)k^a}\left[\frac{k^{a-n+1}}{a-n+1} - \frac{k^a}{aD_m^{n-1}} - \frac{D_0^{a-n+1}}{a-n+1} + \frac{D_0^{a}}{aD_m^{n-1}}\right] \qquad \text{(Eq. 16)}$$

For materials following the Schuhmann distribution, $D_0 \to 0$. Thus:

$$E = \frac{Ca}{n-1}\left[\frac{k^{1-n}}{a-n+1} - \frac{1}{aD_m^{n-1}}\right] \qquad \text{(Eq. 17)}$$

If D_m is large compared to k, then:

$$E = \frac{Ca}{(n-1)(a-n+1)} k^{1-n} \qquad \text{(Eq. 18)}$$

When plotted on log-log paper, as shown in Fig. 4 for impact fracture of sodium chloride, Eq. 18 yields a straight line, where a and C are constant and the slope is $(1 - n)$. Thus, if several milling experiments are conducted in which the energy inputs and the size distributions are measured, n, the exponent in fundamental energy–size reduction equations, may be determined. The sole restriction is that for both size distributions the shapes of the size distribution curves remain the same and are displaced only laterally with a change in energy input.

Equation 18 is real only if n is greater than 1 and less than $(a + 1)$. From measured size distributions, a can be as great as 1.5 and is usually approximately 0.8. Thus, the values of n range from 1 to 2.5. This range was empirically chosen for Eqs. 4, 6, and 7. For hard, brittle material, $(a - n + 1)$ approaches zero, and its real value cannot be accurately determined. Consequently, the constant C cannot be determined accurately; however, for any specific mill-

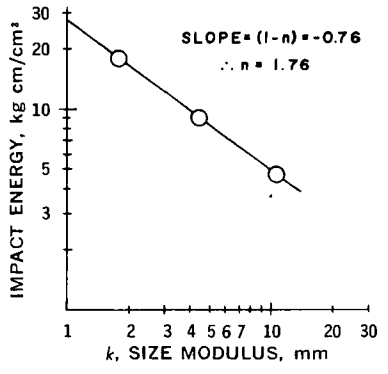

Figure 4—*Energy–size reduction relationship for salt (36).*

ing conditions, the term:

$$A = \frac{Ca}{(n-1)(a-n+1)} \qquad \text{(Eq. 19)}$$

is constant. The general energy-size reduction equation may then be expressed as:

$$E = Ak^{(1-n)} \qquad \text{(Eq. 20)}$$

where A is a mill constant, which can be determined easily for any size reduction test when the exponent n has been determined as discussed.

In many industries, the relationship of energy input to accomplished size reduction is an important economical consideration. In pharmacy, where relatively small quantities of materials are milled and the influence of size is paramount to the efficacy or physical stability of a pharmaceutical, energy consumption is not a major factor. The proposed theories of size reduction are suitable for specific applications and are best used in a qualitative manner, but the only reliable means for determining the size reduction provided by a given mill is to make experimental tests with the actual material.

LIMIT OF COMMINUTION

The general differential equation of size reduction and its special cases predict that given sufficient time a material may be reduced to unlimited fineness. Unfortunately in these equations the size and energy input are inadequately defined. If the ultimate size reduction by mechanical means were attributed to the unit of the crystal lattice, the limit of size reduction would be approximately 10^{-3} μm (or a specific surface of approximately 6×10^7 cm^2/cm^3). Milling limit refers to the size distribution to which a milling operation tends as a result of material characteristics, mill characteristics, and operating conditions when given sufficient time.

Time affects the milling process. When the resident time in a mill is a few minutes, the material is subjected to a relatively constant fracture-producing environment. Changes in milling conditions that are insignificant for short milling periods may be controlling factors in prolonged milling. In prolonged milling, the milling environment is not necessarily constant.

At 10 μm, aggregation due to van der Waals' force begins; at 1 μm, it is intense as crystals fracture across cleavage planes involving a valence type of force (43, 44).

With prolonged milling, the probability that an individual particle would be involved in a fracture diminishes as the particle becomes smaller. In general, as size reduction proceeds, the mean stress required to cause fracture increases through the depletion of cracks while the magnitude of local stress available decreases. As size reduction proceeds because of the diminishing local stress and increasing aggregation, increases in energy expenditures are fruitless, and size reduction ceases at some practical milling limit (45).

Harris (46) proposed an empirical equation expressing a limiting specific surface, S_m:

$$S = S_m[1 - \exp(-KE^n)] \qquad \text{(Eq. 21)}$$

where E is energy input, and K is a constant depending on conditions of milling. As shown in Fig. 5, Kaneniwa *et al.* (47) demonstrated that after 5 hr of ball milling the size reduction of sulfadimethoxine reached a limiting value. The rate of increase in surface area decreased gradually with the lapse of milling time and approached zero where the minimum diameter or maximum surface area was obtained. The data fitted the equation:

$$\frac{dS}{dt} = k_1 \exp(-k_2 S) \qquad \text{(Eq. 22)}$$

where k_1 and k_2 are parameters depending on physical properties of the material and coherency, respectively.

The mill and its operation influence the mill limit. Excessive clearance between the impacting surfaces limits size reduction. In wet milling, the fineness decreases with increased viscosity, which depends on the dispersion medium, the size and concentration of particles, and shear rate. In tumbling mills, as the particles become smaller and more numerous, friction diminishes and the material behaves as a semisolid. Large particles may arch and protect smaller particles from impact. As a result of surface energy, fine particles may coat the grinding medium and cushion larger particles from impact.

RATE OF MILLING

Rate of milling involves the mass and size of particles and the time in the mill. There is experimental evidence that batch milling of brittle materials in small mills follows a first-order law (48, 49). The initial particles are fractured, producing first-generation particles which are fractured to produce second-generation particles, which are also fractured and so on. Since this process is analogous to radioactive decay processes, some radiochemical terminology has been introduced into milling technology. The decay constant, λ, is a function of particle size, so with the numerous equations involved it becomes necessary to utilize computers. The decay constant varies with the size of the material introduced into the mill (50) and the size of the grinding medium in a ball mill (51).

With impact milling following a first-order rate, the number, N, of particles that survive fracture is the product of the initial number, N_0, and the probable fraction surviving fracture at time t:

$$N = N_0 \exp(-\lambda t) \qquad \text{(Eq. 23)}$$

If the average mass of a particle in a given size range is constant, then N/N_0 is equivalent to M/M_0, where M is the mass of particles yet unfractured and M_0 is the initial mass. The milling rate is:

$$\frac{dM}{dt} = -\lambda M \qquad \text{(Eq. 24)}$$

When milling time is brief, the survival probability

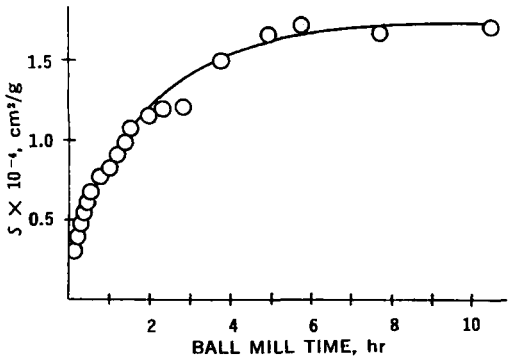

Figure 5—*Increase of surface area of sulfadimethoxine with lapse of ball-milling time (47).*

is approximately $(1 - \lambda t)$ and the most probable fraction of initial material fractured is $1 - (1 - \lambda t)$ or λt. The weight of material that has been reduced in size is:

$$M_0 - M = M_0 \lambda t \qquad \text{(Eq. 25)}$$

The total mass of material in a size range smaller than the largest size may increase, although the amount of original material in that size range must always decrease. Because particles resulting from fracture of larger particles may enter the size range of interest faster than the original material is fractured to a smaller size, the total mass of the size range of interest is increased. Sedlatschek and Bass (52) proposed a set of first-order differential equations to describe the milling process and verified them over a 30-min milling period. The weight percent of particles, M_i, having a size range between D_{i-1} and D_i, has a rate change, dM_i/dt, given by:

$$-\frac{dM_i}{dt} = \lambda_{ii} M_i - \sum_{k=i+1}^{n} \lambda_{ki} M_k \qquad \text{(Eq. 26)}$$

where the rate of change, dM_1/dt, is:

$$-\frac{dM_1}{dt} = -\sum_{k=2}^{n} \lambda_{k1} M_k \qquad \text{(Eq. 27)}$$

In general, $\lambda_{ii} = \Sigma^{i}_{k=i-1} \lambda_{ik}$ while $\Sigma^{n}_{k=1} M_k = 100\%$.

Consider particles of a material that has been milled into four arbitrary groups, $i = 1, 2, 3$, and 4, with size limits 0.0–0.1, 0.1–0.2, 0.2–0.3, and 0.3–0.4 mm, respectively. For these limits, Eqs. 26 and 27 have the following forms. For Group 4, the largest size:

$$-\frac{dM_4}{dt} = \lambda_{44} M_4 \qquad \text{(Eq. 28)}$$

For Group 3:

$$-\frac{dM_3}{dt} = \lambda_{33} M_3 - \lambda_{43} M_4 \qquad \text{(Eq. 29)}$$

For Group 2:

$$-\frac{dM_2}{dt} = \lambda_{22} M_2 - \lambda_{32} M_3 - \lambda_{42} M_4 \qquad \text{(Eq. 30)}$$

For Group 1:

$$-\frac{dM_1}{dt} = \lambda_{11} M_4 - \lambda_{31} M_3 - \lambda_{21} M_2 \qquad \text{(Eq. 31)}$$

For a mass balance:

$$\lambda_{44} = \lambda_{43} + \lambda_{42} + \lambda_{41} \qquad \text{(Eq. 32)}$$

$$\lambda_{33} = \lambda_{32} + \lambda_{31} \qquad \text{(Eq. 33)}$$

$$\lambda_{22} = \lambda_{21} \qquad \text{(Eq. 34)}$$

These equations express the rate at which the largest size Group 4 decays; the rate at which Group 3, the next size smaller, decays as modified by the contributions from Group 4; the rate at which Group 2 decays as modified by the contributions from Groups 4 and 3; and the rate at which the smallest size of material decays as modified by the contributions of all groups.

The process has also been described by use of an integro-differential equation (53). Matrix algebra methods requiring computer solution have been suggested (54).

At present, there is no theory by which one may take a given weight–size distribution and calculate the mill size and milling rate required to produce a desired weight–size distribution of milled material.

EQUIPMENT

Ball Mill—Tumbling mill is a generic term used to refer to ball, compartment, pebble, rod, and tube mills (48, 55). These mills have a cylindrical or conical shell, rotating on a horizontal axis, while partly filled with a grinding medium such as balls of steel, flint, or porcelain or in a shell lined with a nonmetallic liner. The rod mill uses a rod-shaped grinding medium. The impact of rods is probably received by the largest particle between two rods; therefore, the rod mill tends to produce a narrower particle-size distribution than the ball or tube mill. A tube mill is a tumbling mill in which the length is several times greater than its diameter. The compartment mill consists of a cylinder or cone divided into several sections by perforated partitions in which the particle size of the material being milled becomes progressively smaller as the material approaches the discharge end. Tumbling mills are available as batch-operated or continuous types.

Only the ball mill is discussed in this review because in pharmacy it is the most commonly used type of tumbling mill. A ball mill consists of a horizontally rotating ceramic jar or metallic cylindrical-shaped vessel with its length slightly greater than its diameter and a charge of porcelain or steel balls. Metallic jar mills with a one piece cover are commercially available in sizes ranging from 8.25 × 9.52 cm (3.25 × 3.75 in.) with a batch capacity of approximately 150 g to jars with a capacity of 15 kg (56). For large-scale operations, huge mills, such as are used in the ore and fuel industry, are readily available.

A ball mill grinds by impact and attrition. As a ball mill rotates, a cascading and cataracting motion is imparted to the balls. When the mill is operating properly, the load nearest the wall of the cylinder breaks free and is quickly followed by other sections so that the top layers of balls travel at a faster speed than the lower layers, causing attrition of the mate-

rial between them (cascading). Simultaneously, balls are cataracted or fall into the air from the part of the load that is highest on the wall onto the lower part of the load with sufficient impact to fragment the larger particles there.

To attain the proper motion, the mill must be operated at the proper speed. Critical speed is that speed at which the centrifugal force on the balls in contact with the mill wall at the height of their path is equal to the gravitational force. At critical speed, no size reduction occurs because the balls are held against the wall and revolve with the mill. Critical speed, n_c, may be related to mill size by (48):

$$n_c = \frac{76.65}{D} \qquad \text{(Eq. 35)}$$

where D is the diameter of the mill in feet. A larger mill attains its critical speed at a slower revolution rate than a small mill; *e.g.*, a 228.6-cm (90-in.) ball mill and a 11.4-cm (4.5-in.) jar mill may have critical speeds of 28 and 125 rpm, respectively (57). In practice, mills are operated at speeds varying from 60 to 85% of the critical speed.

Commercial grinding media are made of flint (Belgian and domestic currstone), porcelain, and various alloys and steels. Mills may be lined with flint, porcelain, rubber, and metal. In pharmacy, porcelain and stainless steel balls and jars are most commonly used.

The charge or quantity of grinding medium is expressed in terms of the percent of volume of the mill. For optimum results the mill should be 50% filled with grinding medium. If restricted by the size of a mill and the batch, with steel balls the charge of balls may be reduced to as low as 30% of the volume of the mill; however, the milling time will be increased.

The amount of material to be milled should fill the void within and just cover the grinding medium. More material than 25% of the total volume of the mill causes the balls to spread through the material so they cannot make effective contact with each other. If small batches must be milled in a given mill, the grinding medium should be removed so that the material to be milled is just covered.

Small balls facilitate fine grinding. The smaller sized balls provide smaller voids than larger balls; consequently, the void through which material can flow without being struck by a ball is less and the number of impacts per unit weight of material is greater. Void volume could be reduced by mixing ball sizes; however, the packing may then be too close and interfere with ball movements. Thus, the smallest size of grinding medium that fragments the particles is suggested. The optimum size should not change appreciably with mill size. Stainless steel balls from 1.27 to 5.08 cm (0.5 to 2 in.), increasing in size by 0.31-cm (0.125-in.) increments, are commercially available. Balls of 1.27 cm (0.5 in.) are usually the smallest size available in other grinding media.

Excessively large balls produce excess grinding energy which is expressed as heat. The relationship of size in inches of the balls, D_b, to the size, D, of the material to be milled is (27):

$$D_b^2 = KD \qquad \text{(Eq. 36)}$$

where K is a constant, which is 55 for hard materials and 35 for soft materials.

Increasing the total weight of balls of a specific size increases the efficiency of milling. Since optimum milling conditions are generally obtained when the bulk volume of the balls is 50% of the volume of the mill, the weight of the balls is increased by using a grinding medium of a greater density. Thus, steel balls grind faster than porcelain balls because they are three times more dense.

In selecting a grinding medium, consideration must be given to any interaction it may have with the drug to be milled. In production the grinding medium should be examined for wear, and the drug should be tested for contamination by components of the mill. When using glass or porcelain balls in the production of ophthalmic and parenteral products, problems with particulate matter may arise if attrition of the glass or porcelain occurs.

To discharge dry-milled material from the larger mills, the grinding cover is removed and replaced by a slotted discharge grate. Dry mills may be enclosed in discharge housings to control dust and cross-contamination. Other special features may be added to the ball mill (57). An air vent plug is useful to relieve pressure that may develop during milling, to eliminate a partial vacuum that may form during discharge, and to allow sampling during the milling cycle. The mill may be jacketed so that it may be cooled when milling waxy or low melting materials or warmed to maintain a fluid consistency if the entire formula for an ointment is to be milled.

Slip refers to the relative motion between the layer of balls and the surface of the wall. Slip is caused by the smooth surface of the cylinder wall, low viscosity of the material, material with a low coefficient of friction or a light charge of balls and material. In addition to decreasing the efficiency of milling, slip allows most of the weight of the balls to fall on the cylinder wall, resulting in wear and contamination. Slip is most prevalent in steel ball mills. Slip may be reduced by increasing the viscosity or by adding horizontal baffle bars.

Ball milling may be facilitated by the addition of liquid. As shown in Fig. 6, with all other factors being constant, as more water is present, ball milling becomes more efficient (58). The difference in the means is a measure of the degree of skew of the size-frequency curve. As the concentration of the liquid is increased, the difference among the means is decreased, indicating that the size-frequency distribution has become more symmetrical.

In milling wet and adhesive materials, a somewhat slower speed is used than in dry milling to prevent the mass from being carried around with the mill. A low viscosity allows the grinding medium to move with excess speed; this, combined with the thin protective film around the medium, may cause excess wear, contamination, and heat. A high viscosity re-

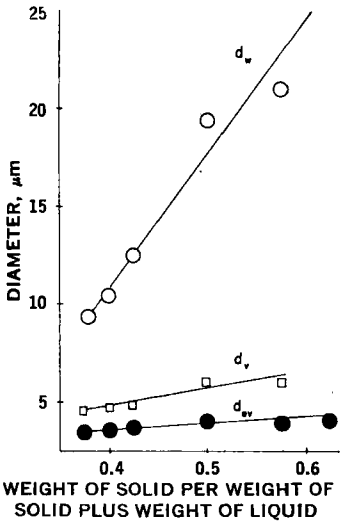

Figure 6—*Effect of moisture on wet ball milling (58). Key: ○, mean weight diameter; □, mean volume diameter; and ●, arithmetic diameter.*

stricts the motion of the grinding medium, and the impact component of milling is reduced. With 1.27-cm (0.5-in.) steel balls, a viscosity from 1000 to 2400 cps is optimum for wet milling.

Wetting agents and some electrolytes may increase the efficiency of milling and the physical stability of the product by nullifying electrostatic forces produced, when bonds are broken, and by preventing rewelding of cracks and particles. Physiological and toxicological considerations prohibit the indiscriminate use of wetting agents in pharmaceuticals; however, some formulations contain wetting agents that could be incorporated at the milling stage to facilitate size reduction and to reduce aggregation.

The vibrating ball mill uses a horizontal or vertical eccentric-activated drive mechanism which vibrates the mill 1500–2500 cpm with an amplitude of several millimeters (59). This energy for size reduction is transmitted through vibration of the mill. The acceleration of the balls is often greater than the acceleration due to gravity in a conventional ball mill, and the short, sharp impacts result in faster milling. The rate of milling is proportional to the cube of the frequency and to the amplitude of the vibration; however, most commercial vibrating mills operate at one frequency and one amplitude. The charge of balls is approximately 80% of the volume of the mill. In general, the size of the material to be milled should not exceed 60 mesh.

Experimental determination of grindability in an open-circuit ball mill is performed on a standard weight of material in a standard mill, with sieve analyses being performed on the material sampled at regular intervals (60). The samples are returned to the mill. Grindabilities are obtained from size distribution plots. Grindability, G_i, is related to the mesh of the grind, D_i, by the expression (61):

$$G_i = \frac{M_i - M_f}{\theta} = \frac{K''}{\omega} D_i^a \qquad \text{(Eq. 37)}$$

where M_f is the cumulative weight smaller than the reference mesh, D_i, in the material at the start of milling, M_i is the corresponding value in the milled material after θ revolutions of the ball mill, K'' is a constant, ω is mill speed, and a is the same constant as in Eq. 10. Therefore, if grindability is known at size D_i, it may be determined graphically at size D_1 if the Schuhmann distribution of the material is known.

The ball mill may mill materials to a 1-μm diameter, and it is unsurpassed for milling hard, abrasive materials. For general use the ball mill has the advantages that it may be used for dry or wet milling, for batch or continuous operation, and for grinding and blending simultaneously at low labor costs. Batch operation of ball mills is especially useful in pharmaceutical operations. The ball mill can be closed tightly so that dust and associated toxic hazards to employees are controlled. Unstable drugs may be sealed with an inert atmosphere and milled. Not only is the ball mill easily sanitized, but it can be sterilized and sealed for sterile milling in the preparation of ophthalmic and parenteral products.

Hammer Mill—Size reduction in an impact mill is based on a high velocity impact by which most solids act as brittle materials to form particles that are more nearly cubical than those formed by other mills (62). The most familiar impact mill is the rotary hammer mill. A hammer mill has a high speed, usually horizontal, rotor to which are mounted stationary or swinging hammers rotating in a cylindrical casing which may be lined with breaker plates. Hammer mills operate up to 10,000 rpm, and the tip of the hammer may reach a velocity of 7620 m (25,000 ft)/min. The clearance between the housing and the hammers contributes to size reduction. The material to be milled is gravity fed at the top or center, thrown out centrifugally, and fractured by impact of the hammers or breaker plates around the periphery of the casing. The bottom of the casing is formed by a cylindrical grating or retaining screen, which retains the material within the milling chamber until it has been reduced to a size; then the material is discharged through the openings in the screen. Because inertial forces vary with mass as the inverse cube of the diameter, with a constant velocity small particles impact with much less kinetic energy than larger ones, and the probability of particles less than a certain size fracturing decreases rapidly. Thus, the impact mill tends to produce a narrow size distribution. This effect is reinforced by the small particles passing through the screen almost as fast as they are formed. For these reasons the hammer mill tends to produce a minimum of fines in a granulation process.

The wide variety of hammer mills in sizes from 5 to 500 hp provides a selection for almost any type of size reduction (63–69). Hammer mills are used in the pharmaceutical industry to process dry materials, wet filter-press cakes, ointments, and suspensions. Hammer mills are not satisfactory for milling abrasive, elastic, or oily materials. Solids with a high moisture content, *e.g.*, 8%, often clog a mill. Crystalline and brittle materials are best fractured by impact from a blunt hammer; fibrous materials are best reduced in size by the cutting edge of a hammer.

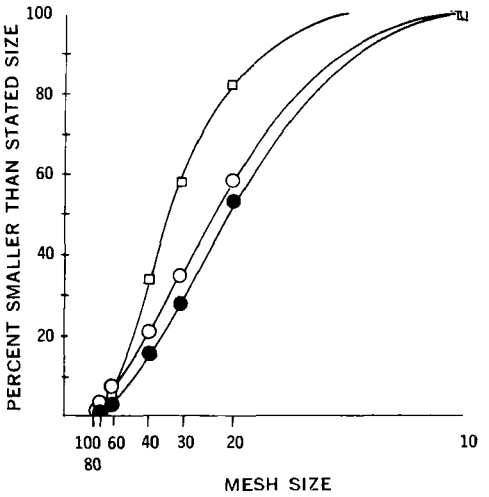

Figure 7—*Effect of speed on the size–frequency distribution of boric acid flakes milled with Mill I operating with impact forward and fitted with a round hole No. 4 screen (hole size 6.35 mm). Key: ●, 1000 rpm; ○, 2450 rpm; and □, 4600 rpm.*

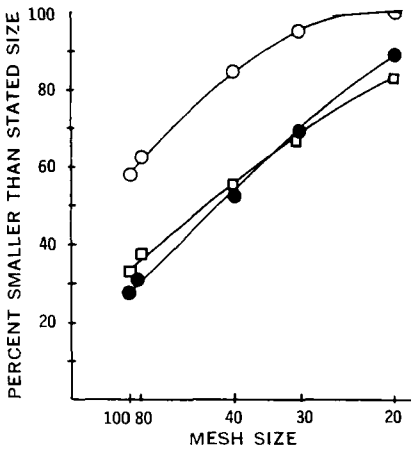

Figure 8—*Effect of screen size on the size–frequency distribution of a terra alba granulation milled with Mill I operating at 2450 rpm, and a comparison of the granulation after milling with Mill I and Mill II. Key: ○, Mill I, 0.84 mm; ●, Mill I, 1.65 mm; and □, Mill II, No. 10 screen.*

Mill I[2] (70) has a rotor which may be turned 180° so that the blunt edge can be used for pulverizing or emulsifying and the knife edge can be used for cutting or granulating. In the preparation of compressed tablets by the wet granulation method, it is operated at 2450 rpm with knife edges, using circular or square holes with a size determined by what will pass without clogging [usually 1.9- or 2.54-cm (0.75- or 1-in.) holes]. In processing a dry granulation, the material may be milled at 1000 or 2450 rpm with knife edges and circular holes in the screen [usually 0.23- or 0.27-cm (0.093- or 0.109-in.) holes]. In granulating, the rate of introduction of material should not be excessive as this produces an excess of fines and sticking.

For a given hammer mill, the size of the milled product may be regulated by rotor speed, screen size, and the rate of introduction of material. The speed of the rotor is the major factor in regulating size reduction. The effect of rotor speed on particle-size distribution is illustrated in Fig. 7, which shows the size-frequency curves for boric acid granules milled at 1000, 2450, and 4600 rpm in Mill I fitted with a No. 4 screen having 6.35-mm circular holes. At very slow speeds, the rotor turns so slowly that instead of size reduction a blending action is obtained with an overload and a rise of temperature. The presence of spheroidal particles indicates that the mill is operating with an attrition action and that a faster speed should be used to obtain an impact action. At very high speeds, it is possible that there is insufficient time between hammers for the material to fall for impact. In the wet milling of emulsions and suspensions with higher speeds, the swing hammers may lay back with an increase in clearance; in such cases, it may be more effective to use fixed hammers.

Screw feeders may be added to hoppers to provide a uniform rate of introduction. Lack of uniform introduction produces a wide size-frequency distribution, because the packed material passes radially and the loose, open material passes tangentially. With a heavy introduction, there are more fines due to attrition between particles. The location of the hopper inlet may affect the amount of fines. A horizontal inlet produces the finest grinding because it provides the maximum metal surface for impact. The forward, vertical inlet is best when fines are not wanted, because the product is immediately exposed to the screen and the amount of solid surface for impact is minimized. This tends to give a narrow size-frequency distribution. The material may be introduced into a hammer mill by air injection which provides more impact, increases the capacity, and permits cooler operation.

Many types and sizes of openings exist in screens, but the influence of the screen on particle size is secondary to the speed of the rotor. The effect of the screen size on the size-frequency distribution of a tablet granulation passed through a 4-mesh screen, after wet granulating with acacia a blend of terra alba and two active ingredients comprising 4.2% of the formulation, is shown in Fig. 8. The dried granulation was milled at 2450 rpm through a Type A plate, having 1.65-mm openings, and a Type B screen, having 0.84-mm openings[3]. The granulation was also milled at medium speed with Mill II[4] fitted with a No. 10 screen. The size-frequency distributions produced by milling with Mills I and II are compared in Fig. 8. Perforated retaining screens are preferred to woven screens because of the greater mechanical strength. The size of the milled material is smaller than the openings of the screen because the particle passes through the opening on a path approximately tangential to the rotor (71).

The circular perforated screen is the strongest and least likely to clog; it is suggested for milling fibrous materials. The herringbone screen consists of slotted holes repeated across the surface of the screen at 45°

[2] Fitzpatrick model D comminuting machine.

[3] R. J. Braun, Smith Kline & French Canada, Ltd., personal communication.

[4] Tornado.

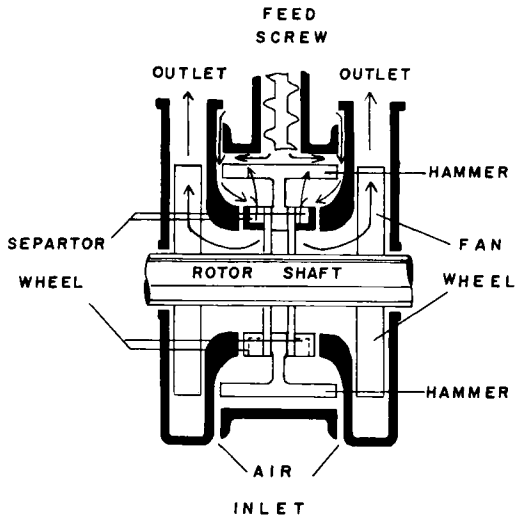

Figure 9—*Mill III, an example of a high-speed hammer mill with an internal classifier (72).*

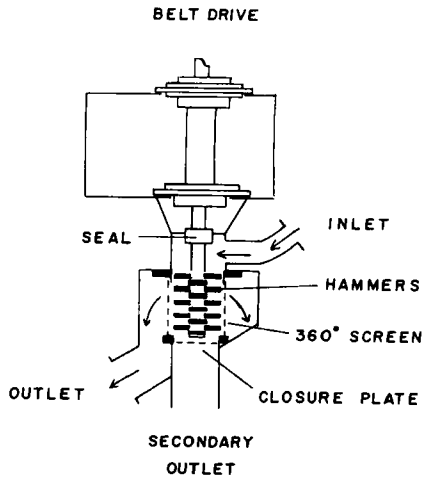

Figure 10—*Mill IV (73) and Mill II (65), examples of a vertical rotor with hammers operating inside of a 360° circular screen.*

to the length of the screen. The herringbone screen is recommended for the milling of crystalline materials and for continuous operation. A herringbone screen with the width of the slot equal to the diameter of a circular hole mills coarser than the circular hole. A cross slot at right angles to the path of the hammer is not used in fine milling because it clogs easily; it is recommended for milling suspensions. The jump-gap screen consists of bars arranged so that the particle approaches a ramp that deflects it into the chamber away from the opening of the screen; it is used with abrasive and clogging materials.

Hammer mills are easy to install and operate. Simplicity of design permits rapid and easy cleaning, so a mill may be used for several products. The speed of the rotor and the retaining screen can be rapidly changed. Size reduction of 20–40 μm may be anticipated. The simple hammer mill is generally not capable of producing the particle size that is required for injectables. For producing ultrafine material, a hammer mill should be operated with internal or external classification.

Several high-speed classifier mills of individual design are marketed. They do not have screens and use some type of pickup fan to remove the acceptable material from the milling chamber, and they require dust collectors to collect the particles in the micron range. Air or an inert gas may be used to convey the material through the system. The temperature of the milled product is lower than in screen hammer mills because the increased volume of air removes some of the heat of milling.

Mill III[5] (72) (Fig. 9) is an example of a high-speed hammer mill with an internal classifier, and it is capable of milling from 1 to 20 μm. Its horizontal rotor disk is fitted with swing hammers and fan wheels which draw air into and through the mill and into the ducts leading to the collectors. The material to be milled is introduced by a screw feed, comes in contact with the hammers, and is diverted into two high velocity air streams with a spiral motion to either side of the hammers as size reduction occurs between the hammers and the ridged liner. The air stream carries the particles toward the classifier wheels, which consist of short, radial vanes at the periphery of the rotor disk. The particles are subjected to the opposing forces of centrifugal force and aerodynamic drag, which separate the oversized particles from the fine particles. For the oversized particles, the centrifugal force exceeds the drag, and they are thrown toward the wall of the milling chamber where they enter the impact zone for further reduction. The air stream carries the fine particles inward from the grinding zone toward the shaft, through the vanes of the classifier wheels into the fans, and out the discharge outlet to a dust collector. Size reduction is controlled by the speed of the rotor and by the size and number of vanes on the classifier.

Mill IV[6] (73) and Mill II (65) (Fig. 10) consist of a vertical or angular rotor fitted with fixed or swing hammers, and it is run at close clearance inside of a 360° screen. The material to be milled enters parallel to the axis rather than tangentially as in most hammer mills, and it is discharged radially through the cylindrical screen. The disintegrator combines attrition, cutting, impact, and screen effect. Tough and elastic materials may be milled by a disintegrator. Its major advantage is the capability of milling suspensions with 40–80% solids and possessing a stickiness and resistance to flow which clogs screens.

Thermolabile, volatile, and low melting materials may be milled by passing refrigerated air or dry ice through the hammer mill with the material (74). Cryogenic techniques chill the material to a low temperature at which it generally is more brittle. Hammer mills may be equipped with a jacket for somewhat limited temperature control and to reduce sticking to the mill parts. To avoid oxidation, a mill may be equipped with a cover manifold so that milling occurs in an inert atmosphere of carbon dioxide or nitrogen. Since air-borne particles provide a po-

[5] Mikro-Atomizer.

[6] Rietz disintegrator.

tential for an explosion, it is advisable for all electrics to be explosion proof.

Fluid-Energy Mill—Most mechanical mills are limited to a size reduction of approximately 325 mesh; fluid-energy mills are used for ultrafine milling to a size less than 5 μm. In the fluid-energy mill, the material is suspended and conveyed at high velocity by a compressed gaseous fluid through an extreme turbulence, which causes size reduction because of interparticular impact and attrition. Although any gaseous fluid may be used, air compressed at 100 psi is generally used because most pharmaceuticals have a relatively low melting point or are thermolabile. The air is introduced so as much as possible of the pressure is converted to kinetic energy. All of the particles are not reduced to the desired size, and internal classification returns the oversize particles for further milling. Most fluid-energy mills use the energy of the fluid stream to effect a centrifugal classification, although a mechanical device may be used.

Mill V[7] (75) (Fig. 11) consists of a shallow circular grinding chamber, from 5.08 to 121.9 cm (2 to 48 in.) in diameter, in which the material to be milled is acted upon by gaseous fluid jets issuing from several orifices spaced around the periphery of the chamber so that fluid enters tangentially to produce a vortex. When the compressed gaseous fluid enters the small grinding chamber, it expands and imparts a high-velocity rotation to the material. Since most fluid energy is expended near the point of entry, the movement and impact of the particles are violent. The coarse particles are centrifuged to the periphery and subjected to further size reduction. The fine particles spiral inward by centripetal force and pass through a central outlet to a collector.

Mill VI[8] (76) consists of a vertical, elongated cylinder constructed in an elliptical shape. The material to be milled enters at the bottom of the mill and is subjected to high-velocity jet streams, which create rapidly circulating flow and impact of the particles. As the gaseous fluid flows around the upper bend of the casing, the large particles are thrown toward the periphery of the cylinder by centrifugal force and are returned with some gaseous fluid down the stack for further size reduction. The fluid being displaced carries the fine particles inward through an outlet to a collector.

In Mill VII[9] (77) (Fig. 12), two opposing jet streams provide head-on impact of particles. The 2.54-cm (1-in.) research model is the smallest fluid-energy mill marketed; it uses approximately 6.5 ft^3/min of air at 100 psi and can mill up to 1000 g/hr.

A finer particle size is obtained by slower rates of introduction of the material into the mill and by using higher pressures. Expansion of the compressed air results in cooling, which avoids temperature rise of the material. An inert gas may be used as the fluid if the medicinal compound is susceptible to atmospheric oxidation. To facilitate ultrafine milling, the material should be reduced to approximately 100-mesh size before processing in a fluid-energy mill.

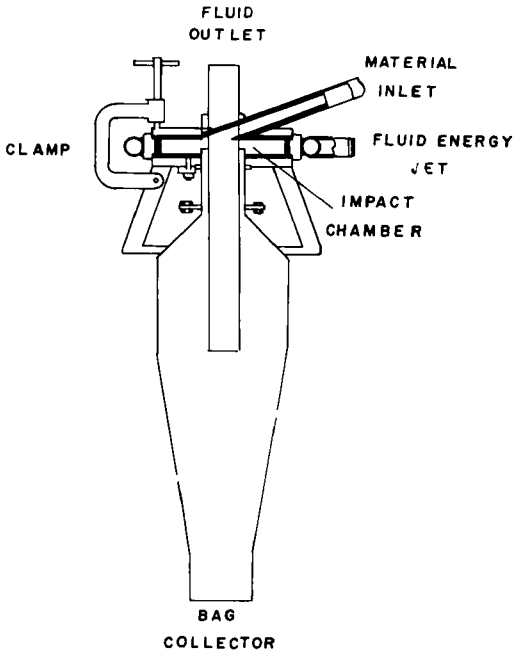

Figure 11—*Mill V, 5.08-cm (2-in.) laboratory model, a fluid-energy mill using a fine high-velocity stream at an angle around a portion of the impact-classifying chamber (75).*

Fluid-energy mills have the advantage that they may be used to mill abrasive materials. Moreover, since there are no moving parts, there is no contamination of the product due to wear. In addition to pulverization, fluid-energy mills may be used to blend, dry, or remove water of hydration. In pharmaceutical milling the fluid-energy mill has the advantage of being easily sterilized and operated by use of sterile air; it also provides low product contamination (78). Agglomeration and packing of material may present problems.

Disk Attrition Mill and Pin Mill—A single-runner, disk attrition mill consists of a vertical or horizontal steel plate, which rotates at relatively high velocity in close proximity to a similar stationary plate to produce a shearing force which reduces particle size. The clearance between the plates is adjustable to allow control of size. In a double-runner, disk attrition mill, both disks rotate at high speeds in opposite directions.

Mills with different patterns of grooves, corrugations, or teeth on the disks perform various operations such as grinding, tearing, shredding, and blending (79–82). A single-runner attrition mill with plates with concentric circular rows of projecting spikes on the rotating plate meshing with those on the stationary plate acts much as a hammer mill. A pin mill, such as Mill VIII[10] (Fig. 13), is a high-speed mill having pin breakers instead of hammers. These may be on a rotor with stator pins between circular rows of pins on the rotor disks or they may be on rotors operating in opposite directions to secure an in-

[7] Micronizer.
[8] Jet-O-Mizer.
[9] Air Impact Pulverizer.
[10] Contraplex.

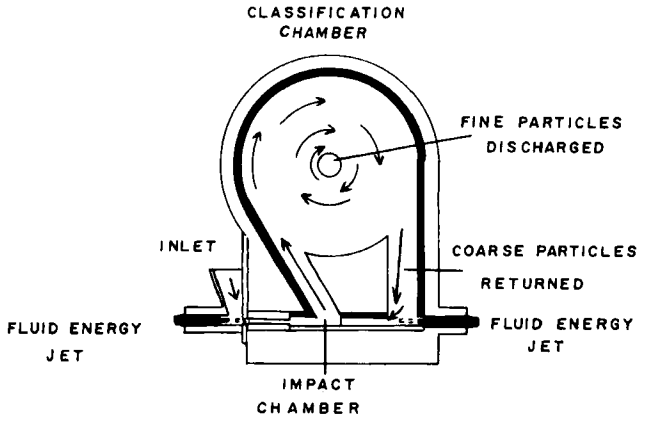

Figure 12—*Mill VII, another type of fluid-energy mill using fluid streams to convey particles at high velocity into a chamber where the two streams impact upon each other (77).*

creased differential of speed. Soft material may be milled so it passes through a 200-mesh screen. For heat-sensitive materials, the disks should be cooled.

Rotary Cutter—A rotary cutter consists of a horizontal rotor, with two to 12 knives spaced uniformly on its periphery, turning from 200 to 900 rpm, with a clearance of several thousandths of an inch, in a cylindrical casing with several stationary knives. The bottom of the casing has a screen which controls the size of the material discharged from the mill. In some models the stationary knives are alternated with screens around the casing to provide maximum discharge area and to keep the fines to a minimum.

Rotary cutters are used with fibrous and resilient materials, such as crude drugs, where successive shearing action is more effective than compression, impact, or attrition. The material to be milled should not exceed the length of the knife and be less than 2.54 cm (1 in.) thick. The size limit is approximately 80 mesh. If a size less than 20 mesh is desired, a pneumatic collection system is required. Rotary cutters and granulators are similarly designed. A granulator yields more or less irregular shaped particles; a cutter may yield cubic or square-shaped particles.

Roller Mill—Roller mills consist of two to five smooth rollers operating at different speeds. The plastic material, *e.g.*, ointment or paste, is introduced between the first two or low-speed rollers and is discharged from the final or high-speed roller by a scraper blade. The material passes from the surface of one roller to that of the next because of the differential speed, which also applies shear to the film of material passing between the rollers.

Roller mills differ from ring-roller mills. Ring-roller mills have rollers that operate in conjunction with grinding rings, and pressure or centrifugal force on the rollers comminutes the material between the ring and roller. Ring-roller mills are rarely used in pharmaceutical milling.

Roller mills may be used in the final step in the production of ointments and pastes. The clearance between adjacent rollers may be adjusted to as little as 0.002 cm (0.001 in.) to ensure smoothness and uniformity of a topical preparation. When milling

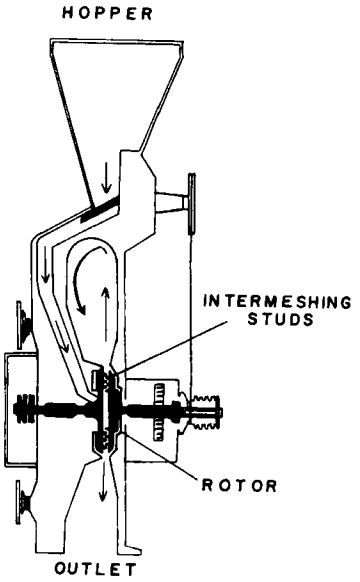

Figure 13—*Mill VIII, an example of a pin mill with concentric, intermeshing rows of pins or studs on the rotor and stator (66).*

with a three-roller mill, the rollers are adjusted so that the layer of material on the middle roller is two to three times the thickness of the layer on the final roller. Thus, the finer milling is accomplished between the middle and final rollers. When using good manufacturing procedures, the insoluble drugs are reduced in size before incorporation into the ointment vehicle and then the entire formulation is blended. Thus, passage through a roller mill is not the major procedure of size reduction, but the shearing and crushing action between the rollers breaks up any aggregated particles within the vehicle. The production rate is determined by the clearance between the rollers, the roller speed, and the size of the roller mill. Production rates vary from 22.7 kg (50 lb)/hr for a 10.2 × 20.3-cm (4 × 8-in.) laboratory mill to 317.8 kg (700 lb)/hr for a 35.5 × 76.2-cm (14 × 30-in.) production roller mill. After the mill has been operating and the rollers have become warm and expanded, an adjustment of the initial roller settings may be required. The temperature may be controlled by circulating water through the rollers.

Colloid Mill—Precisely defined, a colloid mill is not a mill, *i.e.*, a mechanical device for size reduction of solids by impact, cutting, or attrition. A colloid mill does not reduce the size of hard, crystalline particles by fracturing them across crystal planes; however, it does disperse aggregated solids into their individual particles. Homogenizers, viscolizers, dispersators, and specifically designed mixers are used to disperse and blend (83, 84), but they are not discussed because their prime function is emulsification. Although colloid mills are widely used in the emulsification operation, they are discussed from the viewpoint of the dispersion of solids in a liquid vehicle.

As shown in Fig. 14, a colloid mill consists of a high-speed rotor and stator with conical milling surfaces between which there is an adjustable clearance of 0.002–0.25 cm (0.001–0.1 in.). A clearance greater

Table I—Factors in Selection of a Mill

Material
Physical property: hard, soft, fibrous, elastic, hygroscopic, solvated
Size
Moisture content
Melting point
Flammability
Thermolability
Subsequent processing
Operation
Size specification of milled material
Ease of sanitization
Ease of sterilization
Ease of adjustments during operation
Contamination of milled material
Versatility
Capacity
Batch or continuous
Wet or dry
Rate of introduction of material
Space occupied
Labor cost
Auxiliary Equipment
Dust collector
Mechanical introduction of material
Temperature control: jacket, refrigerated air, liquid nitrogen, dry ice
Inert atmosphere: carbon dioxide, nitrogen
Air sweep
Safety
Explosivity
Irritativity
Toxicity
Safety features incorporated into mill

than 0.05 cm (0.02 in.) is seldom used in pharmaceutical operations. Rotor speeds range from 3000 to 20,000 rpm and are often greater for small mills than for production models. Mill speed refers to the peripheral speed of the rotor, *i.e.*, product of the operating speed and the circumference of the rotor. The characteristics of a colloid mill depend on the design of the rotor and stator, the horsepower of the drive motor, operating speed, diameter of the rotor, clearance, and time in the milling zone.

A colloid mill is not used to process dry materials. The individual components of a product are premixed with a liquid vehicle before being introduced into the colloid mill. Solids to be dispersed should be fine enough, *i.e.*, 100 mesh, to prevent damage to the mill. The material is generally introduced by gravity to the center of the rotor. Interfacial tension causes part of the material to adhere to, and to rotate with, the rotor. Centrifugal force throws part of the material across the rotor onto the stator. At a point between the rotor and stator, the motion imparted by the rotor ceases, and hydraulic shearing force exceeds the particle–particle attractive forces holding the individual particles in an aggregate. The particle size of milled particles is usually smaller than the clearance, because the high shear is the dispersing force. In emulsification a clearance of 75 μm may produce a dispersion with an average particle size of 3 μm. The milled liquid is discharged through an outlet in the periphery of the housing and may be recycled.

Production rate varies inversely with the viscosity of the dispersion. Widening the clearance increases

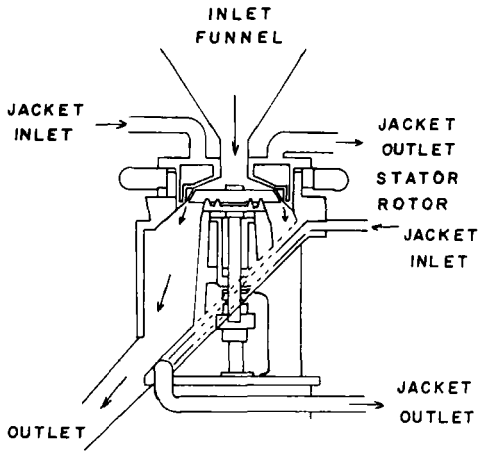

Figure 14—*Mill IX, an example of a jacketed colloid mill with a smooth-surfaced rotor shaped like the frustum of a cone (85).*

the production rate, but it provides a poorer dispersion. Colloid mills, in which the liquid flow is concurrent with the centrifugal force of the rotor or countercurrent, are marketed. Materials with a viscosity as great as 10,000 cps can be processed in a colloid mill having concurrent flow if the mill operates at 1524–2286 m (5000–7500 ft)/min. A countercurrent flow at a maximum speed of 3048 m (10,000 ft)/min is common for systems with a viscosity less than 2000 cps. Dilatant systems, which exhibit an increase in viscosity as the shearing stress is increased, may overload and damage colloid mills.

Colloid mills may have smooth-surfaced or rough-surfaced rotors and stators. In Mill IX[11] (85), the rotor and stator are smooth faced so that the thin film of material between them will have a uniform cross section and will be subjected to the maximum amount of shear. A laboratory model of Mill IX with a 5.08-cm (2-in.) rotor is marketed; it operates at 13,500 rpm and has a production rate from 2 to 25 gallons/hr. If the peripheral speed of the rotor is not high enough, insufficient hydraulic shear is developed. Larger mills have rotors that are large enough to provide high linear speeds at the rotor edge; however, with small mills it is advisable to have a belting system or a variable speed drive that ensures adequate peripheral speed.

Mill X[12] (86) has a grooved conical rotor and stator operating at 3600 rpm. The rough-surfaced mill adds intense eddy currents, turbulence, and impaction of the particles to the shearing action. Rough-surfaced mills are useful with fibrous materials because fibers tend to interlock and clog smooth-faced mills. Mill XI[13] (87) operates at a peripheral speed of 3048 m (10,000 ft)/min and may be fitted with a three-way valve and by-pass tube. The production rate of milk of magnesia is 80, 250, and 660 gallons/hr in colloid Mill XI with a rotor diameter of 5.08, 10.16, and 20.32 cm (2, 4, and 8 in.), respectively.

A colloid mill tends to incorporate air into a suspension. Aeration may be reduced by the use of a

[11] Premier.
[12] Charlotte.
[13] Gaulin.

vertical rotor, which seals the point where the rotor shaft enters the housing and maintains the rotor and stator always in contact with the liquid. Because milling operations waste energy as heat, the temperature of a product may rise as much as 40° during milling. By passing cooling water through the mill jacket, the temperature of the dispersion may be reduced as much as 20°. Sanitary design mills, which may be sterilized, are available, from a laboratory model with a capacity of 1–50 gallons/hr to a production model with a production rate exceeding 1000 gallons/hr. Colloid mills may be used in-line to prevent clogging of highly instrumented handling systems transporting suspensions. Colloid mills may be used in spray-drying operations in-line immediately preceding the spray nozzle to prevent clogging and to produce a more uniform product.

OPERATION

Fine materials, *e.g.*, dextrin, starch, and sulfur, suspended in air are potential explosion hazards. If the material is known to be combustible, it should be milled in an inert atmosphere. All electric switches should be explosionproof. To avoid static electricity, which may ignite dusts, mills should be grounded.

The mill room should have an adequate ventilation system, which removes dust so that air-borne contamination of other areas of the laboratory does not occur. Batch tickets for irritating and toxic drugs should bear warning statements. Personnel must be impressed with safety precautions of respirators and goggles, and they should have periodical medical examinations.

The rate at which the material to be milled is introduced should be constant to avoid fluctuations of particle size–frequency distribution. Frequently, overhead delivery is controlled by rotary or slide-gate valves to provide continuous and uniform flow to the milling chamber. If the rate is relatively slow, the milled material is readily discharged and the quantity of fines is minimized. If the rate is fast, *i.e.*, choke feed, the material is in the milling chamber for a longer time because its discharge is impeded by the mass of material and a greater size reduction is obtained although the capacity of the mill is reduced. Choke feed may be used when a small amount of material is to be milled in one operation, but it may cause an increase in temperature of the material.

The rate of discharge should be equal to the rate at which the material is introduced. Many mills are designed so that the force of gravity provides free discharge through the bottom of the mill. For ultrafine milling, a fluid carrier is used to convey the material from the milling chamber to the collector. A milling system in which the material from the grinding chamber is passed through a size separation device and the large particles are returned to the grinding chamber for further size reduction is known as a closed-circuit mill.

Since milling is not an efficient process, heat is produced and is mainly removed by the air and the milled material leaving the mill because the heavy construction of the mill restricts radiation loss. Most properly used mills operate below 100° (88). If thermolabile material or low melting material is to be milled, the milling chamber should be cooled. Jacketed hammer mills are of little value because of the small area, short retention time, and poor heat transfer coefficient. Air inlets may help somewhat. An airswept hammer mill or pin mill has large air inlets, and the milled material is conveyed by air from the mill to the collector. Approximately a 20° drop in temperature may be achieved by the use of dehumidified and refrigerated air. Its use is limited to the temperature at which condensation occurs.

Stainless steel equipment is routinely used to minimize contamination and reaction with drugs. To comply with regulations of the Food and Drug Administration and the U.S. Department of Agriculture, the stainless steel should be Type 304 with No. 4 finish; where specific corrosion or sterile considerations are involved, the stainless steel should be Type 316. If coolants such as dry ice or liquid nitrogen are used, the mill must be constructed of stainless steel because cast iron becomes brittle and may shatter at low temperature. Waxy materials, *e.g.*, stearic acid and beeswax, may be chilled and milled with dry ice in a hammer mill to a 100-mesh size. The fluid-energy mill may be used to mill waxy materials to an ultrafine size. The air enters the fluid-energy mill at approximately 20° and 100 psi so the mill is cooled by the expansion of the air.

Table I lists factors to be considered in the selection of a mill (89). When a tentative selection has been made, a test milling should be conducted on the material to be milled. Most mill manufacturers have pilot laboratories for milling tests. The industrial pharmacist should evaluate the pilot study personally to observe the temperature of the inlet and outlet air, the temperature of the milled material, and the size reduction performance at different rates of introduction of the material, at different mill speeds, and with various classifier settings. Samples should be collected from each, and a size–frequency analysis should be made for comparison. The final milling should be under the most severe conditions, with recycling for several minutes to determine if there is a gradual buildup in the milling chamber. The development of milling specifications is primarily a pilot responsibility because laboratory procedures of size reduction do not duplicate milling conditions in production mills.

In addition to size reduction, milling may alter the crystalline structure and cause chemical changes in some materials (37). Wet milling may be useful in the production of a suspension containing a metastable form that causes crystal growth and caking. If cortisone acetate crystals are allowed to equilibrate with the aqueous vehicle, subsequent wet milling will yield a satisfactory suspension (90). By means of a vibratory mill, starch, amylose, and amylopectin can be broken down mechanically to a wide molecular weight range but not to monomeric units (91). Powdered polyvinylpyrrolidone breaks down into lower molecular weight polymers during ball milling.

The rate of decrease of the molecular weight of the polyvinylpyrrolidone is influenced by the atmosphere (92). Pure C_{12}- and C_{16}-fatty acids may be decarboxylated and converted to the hydrocarbon containing one less carbon atom by ball milling with wet sand (93). When well-dried microcrystalline cellulose[14] was milled from 1 to 25 hr, the crystallinity decreased with milling but remained relatively high due to aggregation of microcrystals (94). In using a colloid mill to disperse solids in a suspension containing polymeric suspending agents, it should be realized that excessive shearing may damage polymeric materials so that there is a loss of viscosity (95).

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RESEARCH ARTICLES

Effect of Ethanol on Intestinal Absorption of Theophylline

RENU KOYSOOKO and GERHARD LEVY ˣ

Abstract □ The purpose of this investigation was to determine directly the effect of ethanol on the absorption of theophylline from the small intestine of the rat. In the first part of the investigation, a 50-mg % solution of theophylline containing 0, 5, 10, or 20% ethanol was placed in a ligated segment of small intestine of anesthetized rats and sampled at intervals. Theophylline absorption was significantly increased by 5% ethanol and decreased by 20% ethanol. There was a positive rank-order correlation between theophylline absorption rate from the four solutions and water net flux. In the second part of the investigation, a segment of small intestine of anesthetized rats was perfused with theophylline solutions containing 0 or 2% ethanol. The concentration of ethanol was kept constant by continuous infusion of ethanol into the circulating perfusion solution. Theophylline absorption and water net flux were significantly increased by 2% ethanol. Intravenous infusion of ethanol at a rate sufficient to produce the same plasma concentration of ethanol as was observed upon intestinal perfusion with a solution containing 2% ethanol had no effect on theophylline absorption from the small intestine. The intestinal clearance of theophylline was independent of concentration in the 10–200-mg % range. Ethanol solutions without theophylline produced the same changes in water net flux as did the theophylline–ethanol solutions. It is concluded that the effect of ethanol on the intestinal absorption of theophylline is probably due to solvent drag, secondary to a change in water flux induced by alcohol.

Keyphrases □ Ethanol—effect on intestinal absorption of theophylline, rat small intestine □ Theophylline—effect of ethanol on intestinal absorption, rat small intestine □ Absorption—effect of ethanol on theophylline intestinal absorption, rat small intestine

The relatively slow and erratic absorption of theophylline from solid oral dosage forms has led to the development of various liquid dosage forms of this drug, including hydroalcoholic solutions (1). It has been claimed that the ethanol in hydroalcoholic solutions of theophylline enhances the absorption of the drug, but there have been conflicting reports.

A solution of theophylline containing 20% ethanol produced a significantly larger increase in the vital capacity of asthmatic patients than did the same dose of theophylline in aqueous solution (2). On the other hand, in a study of theophylline concentrations in the blood of two subjects, after oral administration of theophylline in solution, a more rapid absorption was found from aqueous solution by one subject and a more rapid absorption from the hydroalcoholic solution by the other (3). Flora (4) observed no significant differences in plasma theophylline concentrations of human volunteers receiving theophylline in aqueous solutions containing either 1.43 or 8.44 ml ethanol [15 ml of a 9.5% (v/v) and 42.2 ml of 20% (v/v) solution, respectively]. Studies on rabbits (5) showed more rapid absorption of theophylline from a solution containing 20% ethanol than from an aqueous solution without ethanol.

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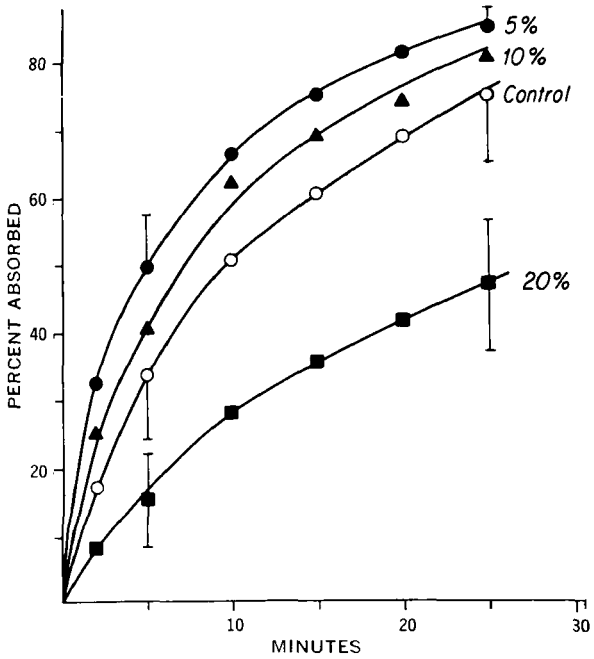

Figure 1—*Effect of 0, 5, 10, and 20% (v/v) ethanol on the absorption of theophylline from a 50-mg % solution instilled into a cannulated segment of small intestine of anesthetized rats (average of six animals per group). Vertical bars indicate 1* SD *in each direction.*

mine directly the effect of various concentrations of ethanol on the absorption of theophylline from the small intestine of the rat and to establish the mechanism by which ethanol might modify theophylline absorption.

EXPERIMENTAL

Male Sprague-Dawley rats, weighing 220–350 g and fasted for about 15 hr, were used. Each animal was anesthetized with urethan, 1.5 g/kg ip.

***In Vivo* Intestinal Loop**—Drug absorption was investigated by the *in situ* intestinal loop technique of Doluisio *et al.* (6) with certain modifications as described by Hayton and Levy (7). A solution of 50 mg theophylline in 100 ml Sørensen buffer, pH 6.4, made isotonic with sodium chloride and containing 0, 5, 10, or 20% (v/v) ethanol, was instilled into the lumen of the cannulated segment of small intestine[1], and 0.2-ml samples were removed periodically for assay. The volume of drug solution in the intestine was kept constant by addition of 0.9% sodium chloride solution before each sample removal. The difference between the total volume of saline solution added during the experiment and the volume of solution removed for assay served as a measure of net water absorption and will be referred to as water net flux. Positive flux refers to net absorption from the intestine; negative flux designates net flow into the intestinal lumen. The percent of drug remaining in the lumen of the intestine segment was determined as a function of time. All experiments were carried out in randomized order.

To confirm the effect of ethanol on the absorption of theophylline, the drug concentration in the plasma was determined in one group of rats receiving 50 mg % theophylline in buffer solution and in another group receiving a solution of 50 mg % theophylline and 5% (v/v) ethanol in Sørensen buffer. Eight milliliters of drug solution/275 g body weight of animal was instilled into the cannulated segment of small intestine, and blood was taken from the aorta 5 min later. Plasma theophylline concentrations were determined in duplicate.

[1] The length of the cannulated segment of intestine was 100.6 ± 7.0 cm (mean ± *SD*, $n = 47$).

Solutions of 0, 5, and 20% (v/v) ethanol in Sørensen buffer (but without theophylline) were used to determine the effect of ethanol alone on water net flux.

Perfusion Method with Constant Concentration of Ethanol in Perfusion Solution—The intestinal perfusion setup and other pertinent experimental details of the surgical and perfusion procedure were described previously (8). The control group of animals was perfused with a solution of 50 mg theophylline in 100 ml isotonic Sørensen buffer, pH 6.4, at a rate of 2 ml/min. The other group was perfused at the same rate with the theophylline solution also containing 2% (v/v) ethanol. An infusion pump delivered 23% (v/v) ethanol in normal saline solution into the reservoir of the perfusion setup at a rate of 0.0216 ml/min. The concentration of ethanol in this solution required to maintain the initial concentration of ethanol in the perfusion solution was calculated from its absorption rate constant (determined in preliminary experiments) and the amount of ethanol in the intestine at the beginning of the experiment, for the particular infusion rate used.

The perfusion and infusion pumps were started simultaneously; additional drug solution was then added to the reservoir, which was immersed in a 37° water bath, until the system (pump, rat intestine, reservoir, and tubing) was filled to capacity, approximately 9.0 ml. The drug solution was circulated through the intestine for 5–10 min before the zero-time sample was removed. From then on, 0.2-ml samples were obtained periodically from the reservoir for theophylline assay and 0.1-ml samples were obtained for ethanol assay. The volume of the perfusion solution was kept constant throughout the experiment by adding 0.9% sodium chloride solution intermittently to the reservoir from a buret to replace fluid loss due to sampling and water absorption from the intestine.

The apparent first-order absorption rate constant for theophylline was determined from the slope of the line, fitted by the method of least squares, of semilogarithmic plots of the percent of theophylline remaining in the intestine *versus* time. The rate con-

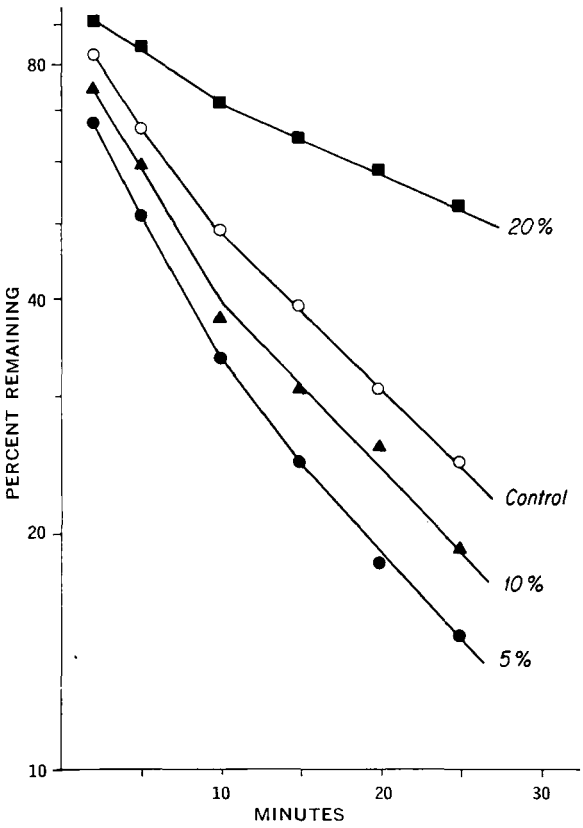

Figure 2—*Semilogarithmic plot of the time course of theophylline absorption from a 50-mg % solution also containing 0–20% (v/v) ethanol, which was instilled into a cannulated segment of small intestine of anesthetized rats (average of six animals per group).*

Table I—Statistical Analysis of Effect of Ethanol on Theophylline Absorption

Condition	Ethanol Concentration, %	Statistically[a] Significantly Different from 5 min	Statistically[a] Significantly Different from 20 min
A	0	B, D	B, D
B	5	A, C, D	A, C, D
C	10	B, D	B, D
D	20	A, B, C	A, B, C

[a] $p < 0.05$ by t test.

Table II—Effect of Ethanol on Theophylline Absorption from Rat Small Intestine as Reflected by Plasma Theophylline Concentrations[a]

50 mg % Theophylline		50 mg % Theophylline and 5% Ethanol	
Rat Number	Plasma Concentration, μg/ml	Rat Number	Plasma Concentration, μg/ml
113	16.42	114	24.25
116	16.35	115	27.14
117	17.80	120	19.06
119	17.60	121	25.08
122	14.49	123	24.72
125	17.56	124	29.60
Mean	16.70		24.98
SD	1.25		3.51
Statistical difference	$p < 0.001$		

[a] Five minutes after instillation of drug solution into the intestinal tract. The body weight of the control animals was 288 ± 31 g (mean ± *SD*) and that of the animals in the ethanol group was 304 ± 16 g.

stant was corrected for drug removed in the samples and was converted to clearance per centimeter by multiplying the rate constant by the volume of drug solution and dividing it by the length of perfused intestine.

To determine the effect of concentration on theophylline absorption and water net flux, solutions of 10 and 200 mg % theophylline in Sørensen buffer were circulated through the rat intestine and sampled as already described.

Perfusion Method with Concomitant Intravenous Infusion of Ethanol—In this series of experiments, a concentration of approximately 130 mg % ethanol was maintained in the plasma. Based on preliminary experiments, a loading dose of 1.05 g ethanol/kg body weight was given intravenously over 5 min as a 19% (v/v) solution. Then an ethanol infusion of 0.5–0.8 g/kg/hr into the tail vein was started at a rate of 0.0216 ml/min of a solution containing 19% (v/v) ethanol in normal saline. The control animals received normal saline solution at the same rate. After 30 min of intravenous infusion to attain steady state, perfusion of the intestine with 50 mg % theophylline in buffer solution at a rate of 2 ml/min was started. Samples of perfusate were obtained periodically from the reservoir, and the volume of solution was kept constant as already described. A blood sample for determination of ethanol concentration was obtained at the end of the experiment.

Assay—Theophylline concentrations in the perfusion solution and in plasma were determined by the UV spectrophotometric procedure of Schack and Waxler (9). Recovery of theophylline averaged 91% from plasma and 97% from perfusion solution. The blank apparent theophylline concentration was 2 μg/ml in plasma and 1.1 mg % in the perfusion solution.

Ethanol concentrations were determined by chromatography, using a modification of the method of Cooper (10). A gas chromatograph[2] with a flame-ionization detector and a column packed with Parapak Q[3], 80–100 mesh, was employed. The column temperature was 160°, and the injection port and detector temperatures were approximately 210°. *n*-Propyl alcohol was added to the samples as an internal standard. Helium was used as the carrier gas at a flow rate of 30 ml/min; the flow rates of hydrogen and air were 30 and 350 ml/min, respectively. A plot of the area of the ethanol peak divided by the area of the internal standard peak as a function of ethanol concentration was linear and passed through the origin. Protein in the plasma samples was precipitated by the method of Cooper (10) after addition of the internal standard. The average recovery of ethanol from plasma was 100%.

[2] Perkin-Elmer model 811.

[3] Waters Associates, Inc., Framingham, Mass. The metal column was 1.83 m (6 ft) long and 0.31 cm (0.125 in.) in diameter.

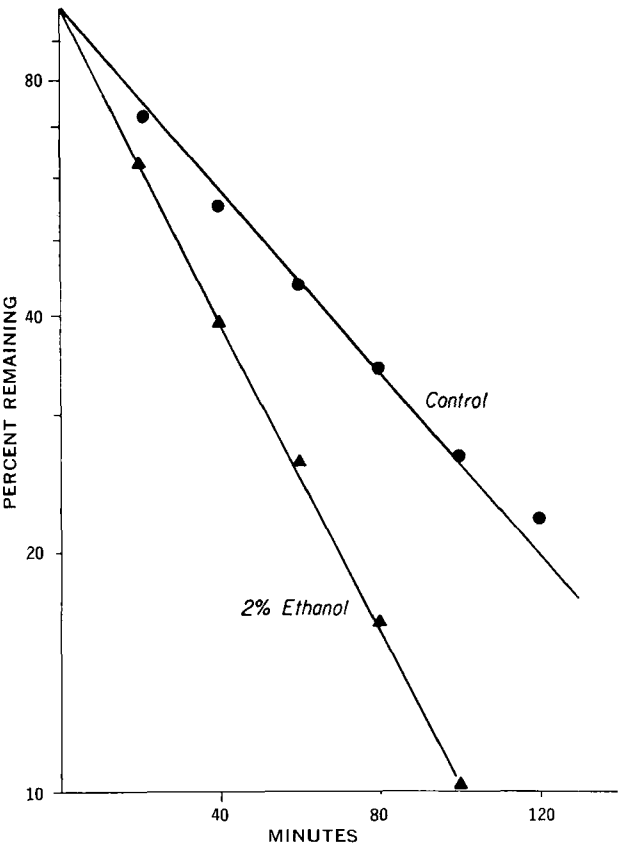

Figure 3—*Semilogarithmic plot of the time course of theophylline absorption from a perfused segment of small intestine of anesthetized rats (data from one animal each). The perfusion solution contained 50 mg % theophylline and 0 or 2% (v/v) ethanol. The concentration of ethanol was maintained constant by continuous infusion of ethanol into the circulating perfusion solution.*

RESULTS

The effect of ethanol on the absorption of theophylline from a solution instilled into a cannulated segment of small intestine of anesthetized rats is shown in Figs. 1 and 2. Solutions containing an initial concentration of 5% ethanol enhanced theophylline absorption significantly, while solutions that initially contained 20% ethanol had a significant retarding effect on theophylline absorption (Table I). The absorption-enhancing effect of 5% ethanol was verified by determining the concentration of theophylline in the plasma of additional groups of rats 5 min after instillation of theophylline solution, with and without ethanol, into the segment of cannulated intestine. The results obtained (Table II) are in excellent quantitative agreement with the results obtained from measurements of theophylline disappearance from the intestinal solution (Fig. 1). There were significant differences in intestinal water net flux observed with the different solutions. Theophylline solutions with 5% ethanol significantly increased water absorption from the intestinal lumen, while solutions containing 20% ethanol actually caused net flow of water into the intestinal lumen (Table III). There is a perfect positive rank-order correlation between the water net flux associated with the instillation of

Table III—Effect of Ethanol on Water Net Flux in Rat Small Intestine

Composition of Solution	Water Flux[a], ml/cm/25 min	Statistical Difference	Body Weight, Mean ± *SD*, g
50 mg % theophylline (control)	+ 0.012 (0.008)[b]	—	304 ± 36
50 mg % theophylline and 5% ethanol	+ 0.050 (0.010)	$p < 0.001$	276 ± 17
50 mg % theophylline and 10% ethanol	+ 0.015 (0.013)	N.S.	291 ± 26
50 mg % theophylline and 20% ethanol	−0.016 (0.012)	$p < 0.005$	279 ± 42

[a] Mean of six rats. Positive sign: water net flux from intestinal lumen; negative sign: flux into intestinal lumen. [b] Standard deviation in parentheses.

Table IV—Effect of Ethanol in Perfusion Solution on Theophylline Absorption from *In Situ* Perfused Rat Small Intestine

	Control		2% Ethanol
Theophylline clearance, ml cm^{-1} min^{-1} × 10^3	5.27 (0.62)[a]	$p < 0.001$	8.33 (1.09)
Water net flux, ml cm^{-1} hr^{-1}	0.048 (0.017)	$p < 0.001$	0.116 (0.013)
Ethanol concentration in perfusion solution, % v/v	—		1. 93 (0.35)
Ethanol concentration in plasma, mg %	—		131 (19)
Body weight, g	297 (41)	N.S.	296 (29)

[a] Mean of six animals each. Standard deviation in parentheses.

Table V—Effect of Intravenous Infusion of Ethanol on Theophylline Absorption from *In Situ* Perfused Rat Small Intestine

	Control		Ethanol
Theophylline clearance, ml cm^{-1} min^{-1} × 10^3	5.92 (0.90)[a]	$p > 0.1$	6.92 (1.32)
Water net flux, ml cm^{-1} hr^{-1}	0.085 (0.012)	$p > 0.6$	0.082 (0.016)
Ethanol concentration in plasma, mg %			125 (27)[b]
Body weight, g	282 (25)	N.S.	281 (21)

[a] Mean of six animals each. Standard deviation in parentheses. [b] Not significantly different ($p > 0.5$) from ethanol-perfused animals.

the four different theophylline solutions into the rat intestine (Table III) and the rate of theophylline absorption from the intestine (Figs. 1 and 2).

Another set of experiments was carried out by perfusing a segment of small intestine with a recirculating solution of theophylline or theophylline and 2% ethanol, with the ethanol concentration being kept constant during the 120-min experiments by infusing additional ethanol at a constant rate, equal to the rate of ethanol absorption, into the system. Unlike the results obtained in the previous experiments (Fig. 2), these experiments yielded data that could be described by first-order kinetics (Fig. 3). The presence of 2% ethanol caused a significant increase in the absorption of theophylline and also in water net flux (Table IV). Similar experiments with 5% ethanol could not be carried out for 120 min since the animals expired after 80–105 min, apparently due to ethanol intoxication; the average intestinal clearance of theophylline in three such experiments was 8.10 (range 7.60–9.00) ml cm^{-1} min^{-1} × 10^3.

Six rats were each perfused intestinally with theophylline solution and intravenously with either saline solution or ethanol in saline at a rate sufficient to produce the same plasma concentration of ethanol as was observed upon intestinal perfusion with solutions containing 2% ethanol. Intravenous ethanol had no statistically significant effect on theophylline absorption and water net flux (Table V).

Perfusion of rat small intestine segments with solutions containing 10 and 200 mg % of theophylline showed no difference in absorption kinetics and yielded almost identical clearance values to those obtained previously with solutions containing 50 mg % theophylline (Table VI). There was also no difference in water net flux in experiments with 10- and 200-mg % theophylline solutions.

The effect of ethanol alone on intestinal water net flux was determined by instilling solutions of 0, 5, and 20% (v/v) ethanol in Sørensen buffer into cannulated small intestine segments of anesthetized rats. As observed previously with theophylline solutions containing the same concentrations of ethanol (Table III), water net flux was significantly increased by 5% ethanol and was actually reversed (*i.e.*, there was net flow of water into the intestinal lumen) when the initial ethanol concentration was 20% (Table VII).

DISCUSSION

The results of this investigation show directly that low concentrations (<10%) of ethanol can increase the rate of absorption of theophylline from the small intestine. The usual therapeutic dose of theophylline hydroalcoholic solution contains about 15 ml ethanol (2). Israel *et al.* (11) found that a 40-g oral dose of ethanol yielded concentrations of up to 3% in the small intestine of normal human subjects and that ethanol concentrations above 1% were maintained there for 30–60 min. The concentrations of ethanol that enhanced theophylline absorption in this investigation are, therefore, similar to those that may be expected in the small intestine fluids of patients who take therapeutic doses of theophylline in hydroalcoholic solution.

As in a previous study on the effect of amides on steroid absorption (7), a decreasing effect of ethanol on theophylline absorption with time was encountered during experiments involving instillation of drug solutions into cannulated *in situ* small intestine segments (Fig. 2). This effect was due to the decrease of ethanol concentrations with time as a result of ethanol absorption. When the ethanol concentration was maintained constant and

Table VI—Effect of Concentration on Theophylline Absorption from *In Situ* Perfused Rat Small Intestine

Theophylline Concentration, mg %	Clearance, ml cm^{-1} $min^{-1} \times 10^3$	Water Net Flux, ml cm^{-1} hr^{-1}	Number of Animals	Body Weight, g
10	5.35 (1.02)[a]	0.069 (0.010)	7	270 (32)
50	5.27 (0.62)	0.048 (0.017)	6	297 (41)
200	5.33 (0.47)	0.062 (0.024)	3	279 (10)

[a] Standard deviation in parentheses.

Table VII—Effect of Ethanol in Buffer Solution on Water Net Flux in Rat Small Intestine

Ethanol Concentration, % v/v	Water Flux[a], ml/cm/25 min	Statistical Difference from Control	Body Weight, g
0	+0.021 (0.002)[b]	—	278 (18)
5	+0.054 (0.006)	$p < 0.001$	266 (35)
20	−0.027 (0.004)	$p < 0.001$	277 (23)

[a] Mean of four rats. Positive sign: water net flux from intestinal lumen; negative sign: flux into intestinal lumen. [b] Standard deviation in parentheses.

the system was preequilibrated in perfusion-intestinal infusion experiments, the kinetics of theophylline absorption became apparent first order (Fig. 3). The theophylline absorption-enhancing effect of ethanol could be demonstrated with both techniques. It was not possible to use ethanol concentrations above 5% in the perfusion-intestinal infusion experiments due to toxic effects resulting from absorption of large amounts of the alcohol. In the intestinal loop experiments, a high concentration (20%) of ethanol actually retarded theophylline absorption and also reversed water net flux so that water was actually flowing into the intestinal lumen. This was presumably due to the osmotic pressure of the 20% ethanol solution. The fact that intravenous infusion of ethanol had no significant effect on theophylline absorption shows that the absorption-enhancing action of ethanol is not due to a systemic mechanism.

There is a perfect positive rank-order correlation between the absorption rate of theophylline (Fig. 1) and water net flux (Table III). This suggests, but does not prove, a cause-effect relationship. A close association between solute absorption and water flux has been noted in a number of reports, summarized recently by Ochsenfahrt and Winne (12); these investigators also pointed out that changes in water net flux can affect solute concentration at the surface of the intestinal mucosa and modify blood flow rate in the capillaries near the intestinal epithelium in addition to causing solvent drag effects in a more restricted sense. Negative water net flux produced by hypertonic solutions in the intestinal lumen decreased the absorption of urea, antipyrine, and aminopyrine while hypotonic solutions had the opposite effect (12, 13). Kinetic analysis revealed that this was not due to changes in blood flow rate through capillaries near the epithelium but only to water–solute interactions within the epithelial membranes (12). Other investigators (14) demonstrated similar effects of changes in intestinal water flux, due to changes in osmotic pressure, on the absorption of salicylic acid. Kojima *et al.* (15) noted that the effect of hypotonicity on sulfaethidole absorption could be accounted for by correcting the absorption rate constant for changes in solution volume and intestinal surface area but that these changes did not account for the effect of hypertonic solutions.

It is necessary to consider certain possible alternative or additional mechanisms for the absorption-enhancing effect of ethanol. Thakkar *et al.* (16) and Kirschbaum (17) found that theophylline can undergo self-association in aqueous solution, forming dimers, trimers, and tetramers. If diffusion of theophylline to the epithelial surface were absorption rate limiting or influencing under the conditions of the present experiments, and if ethanol were to inhibit self-association of theophylline, then an absorption-enhancing effect of ethanol could be rationalized on physical-chemical grounds.

Another possibility involves a pharmacological interaction of theophylline and ethanol. Pierce *et al.* (18) found that superior mesenteric artery infusion of theophylline induced net secretion of water and electrolytes from the jejunum of dogs. They speculated that this could be a systemic effect, perhaps due to elevation of cyclic AMP levels resulting from inhibition of phosphodiesterase, or a direct effect on the intestinal mucosa. The latter possibility is supported by observations that 10 m*M* theophylline applied directly to viable isolated stripped rabbit ileal mucosa produces inhibition of sodium absorption (19). Ethanol also inhibits phosphodiesterase, but in addition it inhibits adenyl cyclase, the enzyme responsible for the formation of cyclic AMP (20). An antagonistic effect of ethanol on theophylline with respect to intestinal water flux (which, in turn, affects theophylline absorption) is, therefore, a possibility.

The assumptions underlying both of these possible mechanisms, physical-chemical and pharmacological, have been explored by determining the concentration dependence of theophylline absorption over a range of 10–200 mg % (Table VI). Kirschbaum's (17) studies showed that monomers of theophylline predominate at the lower concentration while dimers predominate at a concentration of 200 mg %. It may also be expected that pharmacological effects, such as those affecting water flux, should vary in intensity over such a wide concentration range. The lack of any effect of theophylline concentration on the absorption of this drug and on water net flux (Table VI) suggests that the two mechanisms are not operative and that a direct effect of ethanol, rather than a physical-chemical or pharmacological interaction of ethanol with theophylline, may be involved.

Evidence for a direct effect of ethanol was obtained by determining the relationship between ethanol concentration in the intestinal lumen and water net flux (Table VII). As with the solutions containing both ethanol and theophylline, 5% ethanol increased and 20% reversed intestinal water net flux. It appears, therefore, that low concentrations of ethanol enhance theophylline absorption by increasing water net flux which, in turn, increases theophylline absorption by a solvent drag effect. The mechanism by which ethanol increases water flux is not determinable from this investigation. It is known that ethanol inhibits several specialized intestinal absorption processes (11, 21), possibly by lowering the ATP content of the small intestine. Ethanol in concentrations above 0.5–1 *M* has also been shown to increase gastric mucosal permeability in dogs (22). Interestingly, ethanol in concentrations of 1–10% enhanced the absorption of barbiturates from the rat stomach, but 0.5–2% ethanol had no effect on the absorption of barbiturates and some other drugs from the perfused small intestine of the rat (23). Magnussen (23) also stated that the volume correction factor (a reflection of water net flux) was not affected by ethanol concentration, but no data were given. His study differed from the present one in some technical details, including the ionic composition of the perfusion solution, and additional studies have been initiated in this laboratory to resolve this question. The specificity of the ethanol effect as well as the effect of other agents capable of affecting intestinal water flux is also being investigated.

Finally, in considering the relationship of the results of this investigation to the clinical situation, it must be recognized that this investigation was designed to determine directly the effect of ethanol on theophylline absorption from the intestine. Ethanol also inhibits gastric emptying; 120 ml (4 oz) of whiskey 15 min before a test meal increased the half-life of gastric emptying from a mean of 105 to 204 min in eight healthy men (24). It is possible, therefore, that, under clinical conditions, the enhancing effect of ethanol on the intestinal absorption of theophylline may be modified by the effect of ethanol on gastric emptying.

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[x] To whom inquiries should be directed.

GLC Determination of Meprobamate in Water, Plasma, and Urine

LEO MARTIS and RENÉ H. LEVY[x]

Abstract □ A GLC method for the determination of meprobamate in water, plasma, and urine is described. The procedure is based on alkaline hydrolysis of meprobamate followed by silylation of the hydrolysis product to give a trimethylsilyl derivative. Quantitation of the drug was effected using a homolog of the hydrolysis product as an internal standard, which is also silylated during the procedure. The method is specific, sensitive, and reproducible and has been used for the routine analysis of biological samples obtained from meprobamate-treated dogs and humans.

Keyphrases □ Meprobamate—GLC analysis in water, plasma, and urine □ GLC—analysis, meprobamate in water, plasma, and urine

The quantitative determination of meprobamate (2-methyl-2-propyl-1,3-propanediol dicarbamate) is of interest from a toxicological standpoint because of its continued widespread use as a tranquilizing agent and as a muscle relaxant. The availability of meprobamate in a large number of generic forms as well as recent awareness of questionable bioavailability (1) has increased the amount of effort devoted to determining the quantity of product in solution following dissolution studies (2) as well as the quantity found in plasma and urine. Several spectrophotometric methods (3–9) and GLC procedures (10–21) were reported for the qualitative and quantitative analysis of this drug. Many reported colorimetric methods are adequate for the determination of the drug in dissolution studies, but they lack either specificity or sensitivity for the measurement of meprobamate levels following therapeutic drug doses. Most GLC methods presently available were developed for toxicological purposes and involve direct GLC, with the inherent problem of thermal decomposition at the injection port (20). The need for an analytical procedure useful in pharmacokinetic studies of meprobamate in humans and animals treated with therapeutic or subtherapeutic doses led to the development of the method presented in this report.

EXPERIMENTAL

Reagents—The following reagents were used: stock solutions of meprobamate[1] containing 5, 10, 15, 20, and 25 μg/100 μl in water; stock solution of internal standard (2-methyl-2-ethyl-1,3-propanediol[2], I) containing 75 μg/ml in water; sodium acetate buffer (pH 5.0) (22); 0.25 *M* K_2HPO_4 (pH 7.2); β-glucuronidase[3]; *N,O*-bis-(trimethylsilyl)acetamide[4]; and reagent grade anhydrous ether.

Instrumentation—A gas chromatograph[5] equipped with a hydrogen flame-ionization detector, a recorder[6], and a glass column [1.5 m × 0.63 cm (5 ft × 0.25 in.)] packed with a 3% SE-30 on 80–100-mesh Chromosorb W[7] was employed. A gas chromatograph peak identifier[8] coupled to a recorder[9] was employed for peak identification by mass spectroscopy.

The GLC operating conditions were: column temperature, 115°; injector temperature, 175°; and detector temperature, 175°. The gas flow rates were: carrier gas (helium), 50 ml/min; hydrogen, 30 ml/min; and air, 300 ml/min.

Procedure—*Water Standards*—To 1 ml of water contained in a 15-ml Pyrex test tube, fitted with a Teflon-lined screw cap, were added 100 μl of aqueous meprobamate stock solution, 100 μl of stock solution of I, and 1 ml of 50% KOH. The hydrolysis of meprobamate was carried out at 100° for 10 min. The tubes were cooled under running water, and 3 ml of ether was added. The

[1] Wyeth Laboratories, Philadelphia, Pa.
[2] K & K Laboratories, Hollywood, Calif.
[3] Calbiochem, Los Angeles, Calif.
[4] Tri-sil/BSA, Pierce Chemical Co., Rockford, Ill.
[5] Varian model 1400, Varian Aerograph, Walnut Creek, Calif.
[6] Varian A-25, Varian Aerograph, Walnut Creek, Calif.
[7] Varian Aerograph, Walnut Creek, Calif.
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EXPERIMENTAL

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[7] Varian Aerograph, Walnut Creek, Calif.
[8] Finnigan Corp., Sunnyvale, Calif.
[9] Visicorder, Honeywell, Denver, Colo.

contents were shaken for 1 min on a mixer[10] and centrifuged at 2000 rpm for 5 min. About 1.9 ml of the ether layer was transferred to a 12-ml glass-stoppered centrifuge tube. The ether was evaporated at 40°, and the residue was reacted with 25 μl of silylating reagent for 10 min at 60°. About 1–2 μl was injected into the gas chromatograph.

Plasma Standards—To 1 ml of plasma were added 100 μl of aqueous meprobamate solution and 5 ml of ether. The contents were shaken for 1 min on the mixer and centrifuged at 2000 rpm for 5 min. Exactly 4 ml of the organic layer was pipeted into a clean tube, and the ether was evaporated at 40°. To the dry tubes, 100 μl of stock solution of internal standard and 1 ml of 50% KOH were added. The hydrolysis of meprobamate and the remaining steps were the same as described for the water standards.

Urine Standards—To 1 ml of urine were added 100 μl of aqueous meprobamate solution, 1 ml of 0.25 *M* K_2HPO_4 buffer, and 5 ml of ether. The drug was extracted with ether, and the remaining steps were the same as described for the plasma standards.

Biological Samples from Drug-Treated Subjects—Appropriate aliquots of samples were pipeted into 15-ml tubes. The quantitative analysis of the samples was conducted as described under their respective standards except for the addition of meprobamate stock solution.

RESULTS AND DISCUSSION

The method described in this report involves extraction of unchanged meprobamate from the biological fluid and alkaline hydrolysis of the drug to 2-methyl-2-propyl-1,3-propanediol (II), followed by silylation of II with *N,O*-bis(trimethylsilyl)acetamide. Compound I is added directly to the sample prior to the hydrolysis. After hydrolysis, ether extraction, and evaporation, the residue is reacted with *N,O*-bis(trimethylsilyl)acetamide whereby I and II are converted to the corresponding trimethylsilyl ethers, III and IV. The latter yield symmetrical peaks as shown in Fig. 1, where peak A corresponds to III and peak B to IV. Peaks C, D, and E are foreign peaks, probably due to traces of moisture present in the sample. The height of the foreign peaks is time dependent. When the samples were allowed to stand at room temperature for about 3–5 hr after the addition of silylating reagent, peaks C and D decreased and finally disappeared without any measurable change in peak height ratio of IV to III.

Determination of Meprobamate: GLC *versus* Colorimetric Methods—Various methods have been reported for the determination of meprobamate, and most of them are either spectrophotometric (3–9) or GLC (10–12) procedures. Many reported colorimetric methods seem adequate for the determination of the drug in dissolution studies, but they are either not sensitive or not specific when applied to biological fluids. Agranoff *et al.* (4) determined the amount of meprobamate by measuring the color resulting from the treatment of an extract of urine with sulfuric acid. Bedson (6) adopted this method to measure the concentration of the drug in blood. However, quantitation at therapeutic concentrations would be difficult using this method since the lowest measurable concentration shown in the calibration curves is 30 μg/ml. Furthermore, the method is not specific because compounds related to meprobamate, such as II and 2-methyl-2-*n*-propyl-3-hydroxypropyl carbamate, also react with sulfuric acid and would thus interfere (4). Hoffman and Ludwig (3) described a colorimetric procedure based on the coloration effected by treatment with *p*-dimethylaminobenzaldehyde and antimony trichloride in acetic anhydride. Several investigators used this method with success for the determination of meprobamate in plasma samples (23, 24). However, when applied to urine samples, the method is probably not specific since a major metabolite of meprobamate, 2-methyl-2-(2-hydroxypropyl)-1,3-propanediol dicarbamate, would interfere with the color reaction. Recently, a colorimetric procedure was reported (9) as an analytical method for the determination of meprobamate in dissolution studies. As pointed out in that report, the method is nonspecific since any N—H-containing substances would interfere.

Although colorimetric methods can be simple, rapid, and sensitive, they are not always specific due to interference of compounds with cognate absorption characteristics. In such instances, interfering compounds may be separated by using procedures such as TLC or solvent partition. These separation procedures can sometimes be tedious and lengthy, in which case the method becomes inadequate for routine analysis of biological samples. Hence, GLC procedures are becoming more popular for quantitative analysis of drugs in biological fluids. Several reports can be found (10–14, 16) where direct GLC was used for the measurement of meprobamate levels in biological fluids. As pointed out previously (20), thermal decomposition of meproba-

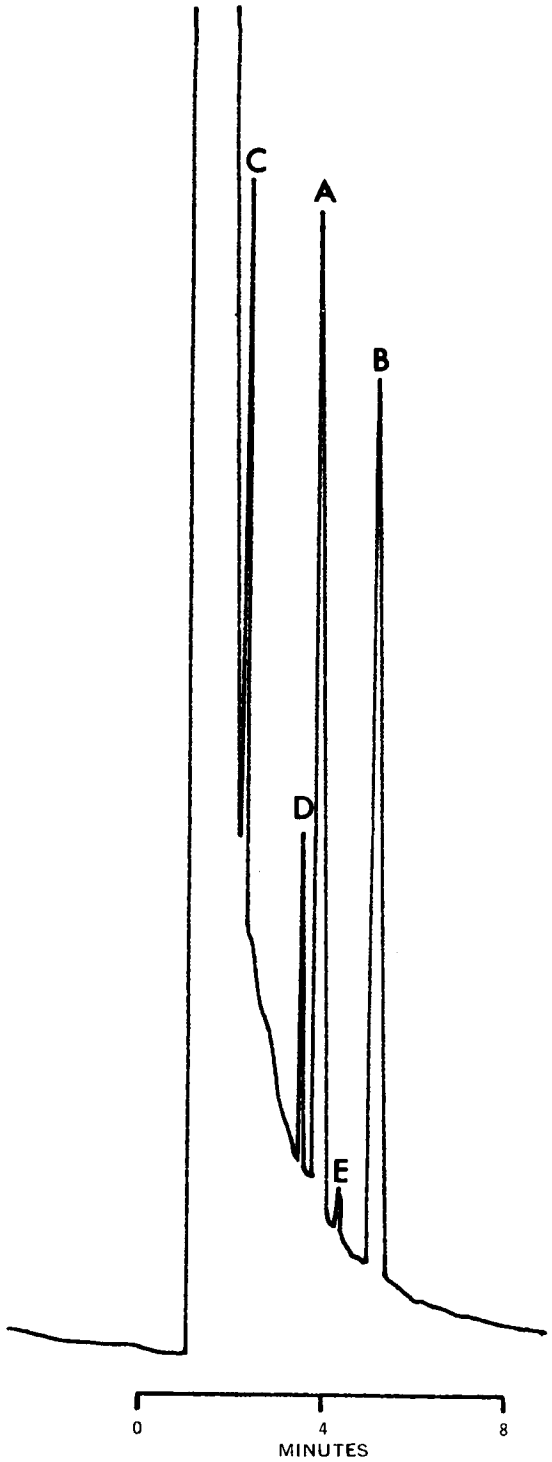

Figure 1—*Chromatogram of a plasma sample (containing 10.5 μg of meprobamate) obtained after administration of 30 mg/kg iv of meprobamate to a dog. Key: peak A, internal standard; peak B, meprobamate; and C, D, and E, foreign peaks.*

[10] Vortex.

Table I—Formation of Trimethylsilyl Derivatives of I and II at 25 and 60°

Minutes after Addition of Silylating Reagent	IV:III Peak Height Ratio Amount of Meprobamate		
	10 μg (25°)	10 μg (60°)	20 μg (60°)
5	0.791	1.00	1.810
10	0.894	1.038	1.997
20	0.993	1.040	2.031
45	1.001	1.040	2.030
120	1.003	1.037	2.020

mate at the injection port presents several problems, the lack of precision being the major drawback. Numerous reports (11, 12, 16–18, 25) document variable decomposition of the drug during direct GLC. Holch and Gjaldbaek (16) found that the main breakdown products of meprobamate are II and 2-methyl-2-propyl-1,3-propanediol monocarbamate. Hence, the decomposition of meprobamate can be overcome if it is converted to II before injecting into the gas chromatograph. However, II is a very polar compound and, when chromatographed directly, variable losses occur due to adsorption. Such losses were found in several glass columns packed with 3.8% UC-98 on Chrom W, 5% UCW-98 on Chrom W, 3% Carbowax on Chrom W, and 3% SE-30 on Chrom W. In every case, the coefficient of variation on repeated injections (five) of the same sample varied from 5 to 8%. The method proposed by Skinner (17) involves direct hydrolysis of meprobamate without extraction of unchanged drug from the biological sample. Therefore, any metabolite of meprobamate that, upon hydrolysis (as discussed later), gives II will interfere with the assay. The hydrolysis approach was also used (18), but those studies were limited to determination in water at relatively high concentrations.

Specificity of Method—Since the proposed method involves the formation of derivatives of meprobamate and the internal standard (to overcome problems associated with direct GLC), it is necessary to test for the specificity of the method. In the present method, any metabolite of meprobamate that undergoes a change in the carbamate moiety without any change in the rest of the molecule could interfere with the assay. Of the known metabolites of meprobamate in dog, rabbit (26, 27), and man (28, 29), the *N*-glucuronide of meprobamate is the only species that could thus interfere with the determination of unchanged drug in biological samples since it yields II upon alkaline hydrolysis. It was, therefore, necessary to introduce an ether extraction step before alkaline hydrolysis to remove meprobamate, leaving the *N*-glucuronide of meprobamate in the aqueous phase.

To test the specificity of the ether extraction step, the following experiment was performed. An oral dose of 800 mg of meprobamate in solution was administered to a male volunteer, and eight venous blood samples were collected at predetermined intervals. The blood samples were centrifuged for 5 min at 2000 rpm, and 3 ml of plasma was harvested from each sample. Each of the eight plasma samples was divided into three 1-ml portions, thus yielding three identical lots of Samples A, B, and C. To test whether II was present as a metabolite in drug-treated man, the plasma samples of Lot A were extracted with ether and the residue obtained after evaporation of ether was reacted with silylating reagent. Neither II nor I was present *in vivo*. The plasma samples of Lot B were assayed as described under *Plasma Standards*, except for the addition of meprobamate stock solution and the initial ether extraction step. The plasma samples of Lot C were analyzed as described under *Biological Samples from Drug-Treated Subjects* where the initial ether extraction step is included. Plasma samples collected later than 60 min after drug administration yielded higher values in the samples of Lot B than in those of Lot C. These results suggest that the *N*-glucuronide of meprobamate at least in part is not extracted by ether and that it can interfere with the determination of unchanged drug if selective separation is not achieved.

Table II—Determination of Meprobamate: Peak Height Ratios of IV to III at Five Meprobamate Levels

Sample	Amount of Meprobamate, μg	Number of Determinations	IV:III Peak Height Ratio, Mean ± *SD*
Water	5	6	1.042 ± 0.028 (2.7)[a]
Water	10	2	2.100
Water	15	6	3.133 ± 0.051 (1.6)[a]
Water	20	2	4.104
Water	25	6	5.047 ± 0.0781 (1.5)[a]
Urine	5	6	0.872 ± 0.0133 (1.5)[a]
Urine	10	2	1.836
Urine	15	6	2.535 ± 0.0237 (0.9)[a]
Urine	20	2	3.337
Urine	25	6	4.243 ± 0.0986 (2.3)[a]

[a] Corresponding coefficient of variation in percent.

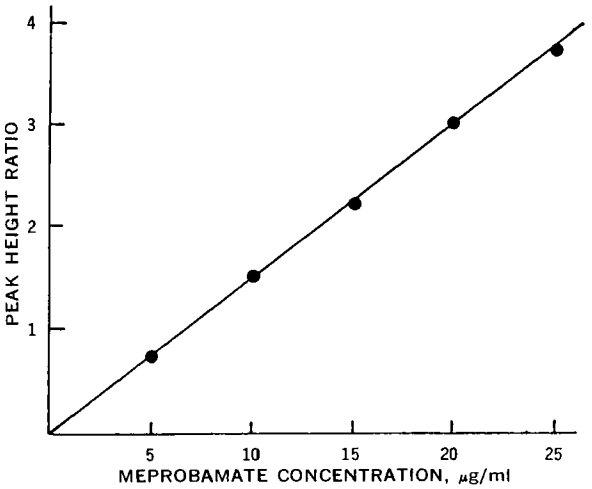

Figure 2—*Calibration curve for meprobamate extracted from rabbit plasma.*

To test whether any *N*-glucuronide at all is extracted into ether, the following experiment was performed. An oral dose of 800 mg of meprobamate was administered to a male subject and urine was collected for 48 hr. The hydrolysis of the *N*-glucuronide of meprobamate in urine samples was carried out with β-glucuronidase according to the procedure reported by Beer and Gallagher (22). The time required for maximum hydrolysis was obtained from preliminary experiments where it was found that enzymatic hydrolysis was complete in 5 days. β-Glucuronidase was then allowed to react for 5 days on four samples of evaporated ether extracts of urine which were then carried through the assay. Controls not containing β-glucuronidase were also run through this procedure. Peak height ratios obtained for samples and controls were compared using a paired *t*-test and found to be not significantly different at $p \pm 0.05$. The same experiment was repeated using a chloroform–carbon tetrachloride mixture as the extraction solvent. This solvent mixture was used previously (3) for extracting unchanged meprobamate from biological samples in that colorimetric method. This solvent mixture did not extract any *N*-glucuronide of meprobamate either. It was found, however, that when used as the extracting medium, ether removes approximately 30% more meprobamate than does a chloroform–carbon tetrachloride mixture.

Mass Spectra of Trimethylsilyl Derivative of II—Although the described experiments demonstrated that the *N*-glucuronide of meprobamate does not interfere with the assay, it is still possible that an unknown metabolite of meprobamate could yield a peak having identical retention characteristics as those of IV. To test for this possibility, the trimethylsilyl derivative of II prepared from an aliquot of a 48-hr urine sample obtained from a human subject (after oral administration of 800 mg of meprobamate) and the one prepared from pure meprobamate were studied by a combination of GLC–mass spectroscopy. The mass spectra of

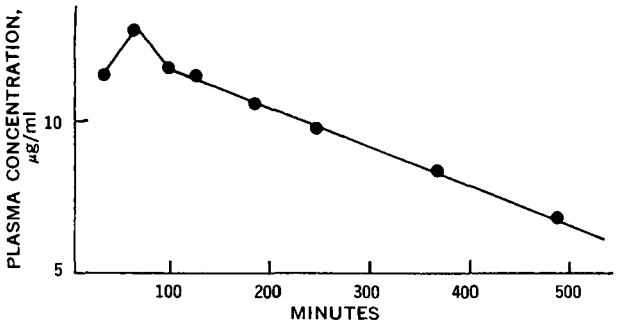

Figure 3—*Plasma levels of meprobamate following oral administration of 800 mg (in solution) to a human subject.*

IV obtained from the urine sample and the one resulting from reacting pure meprobamate were identical. Thus, it can be concluded that peak B corresponds only to meprobamate and that there is no interference from any other metabolite.

Formation of Trimethylsilyl Derivatives of I and II—To eliminate errors due to possible losses in preparation of the sample or to inaccurate injection, a known amount of an internal standard is added to the sample. Compounds to be used for this purpose should closely resemble the drug to be measured in physicochemical properties, and they should not occur spontaneously in the sample. The internal standard used in the present method is a compound very similar to II. The internal standard is added to the sample before hydrolysis of the drug, and it is carried through the steps of ether extraction and silylation. The peak due to the trimethylsilyl derivative of this internal standard (III) is symmetrical, is well resolved, and has a retention time close to that of IV. Formation of trimethylsilyl derivatives of I and II was required to overcome adsorption of these compounds on the column. Table I gives peak height ratios of IV to III as a function of time following the addition of silylating reagent at two temperatures and at two meprobamate concentrations. The peak height ratio reached the maximum value within 10 min at 60° and remained constant for at least 2 hr; subsequent experiments indicated that the peak height ratio did not change for at least 15 hr when the samples were kept in the refrigerator. At the operating conditions of the present method, III and IV were found to be stable.

Accuracy and Precision—Peak height ratios were calculated by dividing the height of the peak due to meprobamate (IV) by the height of the peak due to the internal standard (III). Calibration curves were constructed for "spiked" water, plasma, and urine samples by plotting the amount of meprobamate against the peak height ratio. A typical calibration curve obtained from spiked plasma samples is shown in Fig. 2. Table II gives peak height ratios of IV to III for various amounts of meprobamate in water and urine. In all three instances, a plot of the data (peak height ratio *versus* amount of meprobamate) yields a straight line passing through the origin, with correlation coefficients of 0.999, 0.999, and 0.998 for plasma, water, and urine, respectively. The small coefficients of variation indicate that the reproducibility of the method is adequate. The amount of drug present in biological samples obtained from a drug-treated subject is read from the calibration curve constructed simultaneously with the sample to be assayed.

Application—The proposed method was used in the determination of the biological half-life of meprobamate in a human volunteer following the oral administration of 800 mg in an aqueous solution. A semilogarithmic plot of the plasma concentration–time data is shown in Fig. 3. The half-life (7 hr) calculated from this plot is in agreement with reported half-lives for meprobamate in humans (23).

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[x] To whom inquiries should be directed.

Synthesis and CNS Activity of 3-Substituted 7-Chloro-5-phenyl-1,3,4-benzotriazepin-2-ones

H. KOHL[x], P. D. DESAI, A. N. DOHADWALLA, and N. J. de SOUZA

Abstract □ Several new 3-substituted 7-chloro-5-phenyl-1,3,4-benzotriazepin-2-ones were synthesized from appropriate 5-chloro-2-aminobenzophenone hydrazones by condensation with phosgene or treatment with chloroformic acid esters and subsequent cyclization. In an evaluation of their CNS depressant properties, all of the compounds exhibited mild depressant and anticonvulsant activity which was only marginally influenced by the 3-substituent.

Keyphrases □ 7-Chloro-5-phenyl-1,3,4-benzotriazepin-2-ones, 3-substituted—synthesis and evaluation of CNS activity □ Benzotriazepinones—synthesis of 3-substituted 7-chloro-5-phenyl-1,3,4-benzotriazepin-2-ones, evaluation of CNS activity □ CNS activity—synthesis and evaluation of 3-substituted 7-chloro-5-phenyl-1,3,4-benzotriazepin-2-ones

Interest (1, 2) in 5-chloro-2-aminobenzophenone-derived heterocyclic compounds related to chlordiazepoxide and diazepam prompted the synthesis of some 3-substituted 7-chloro-5-phenyl-1,3,4-benzotriazepin-2-ones (I) for evaluation as central nervous system (CNS) depressants. Anticonvulsant, antispasmodic, analgesic, and muscle relaxant properties have been attributed to some previously reported 7-chloro-5-phenyl-1,3,4-benzotriazepin-2-ones (3–6). Only one reported compound, however, has a substituent, a phenyl group, in the 3-position (4), and no report is available concerning the influence of the substituent on the biological properties of the compound.

DISCUSSION

The desired 3-substituted benzotriazepinones (I*a*–I*f*) were readily synthesized in yields of 75–82% by treatment of appropriate 5-chloro-2-aminobenzophenone hydrazones (II) with phosgene (Scheme I), using minor modifications of previously reported procedures (4, 7).

Physical data on newly prepared hydrazones (II*b*, II*c*, II*e*, II*f*) and on the benzotriazepinones (I*a*–I*f*) are provided in Tables I and II, respectively. Spectral data (mass spectroscopy, UV, IR, and NMR) for I*a*–I*f* were consistent with the assigned structures (4).

An alternative route, previously adopted for the synthesis of benzotriazepinones (7), was investigated for the synthesis of the compounds. The hydrazones, II*a*–II*f*, were treated with chloroformic acid esters, and the products were cyclized in refluxing collidine or diphenyl ether–diphenyl (3:1) (Scheme II). In the reaction with chloroformic acid esters, the hydrazones produced the known carbalkoxyhydrazone (IV*a*) (7) when R_1 = H, urethanes (V*d*–V*f*) when R_1 = aryl, and a mixture of both types of compounds (IV*b*, IV*c* and V*b*, V*c*) when R_1 = alkyl (Table III). The carbalkoxyhydrazones were distinguished from the urethanes through their NMR

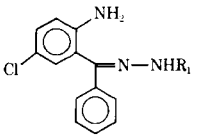


Table I—5-Chloro-2-aminobenzophenone *N*-Substituted Hydrazones (II)

Compound	R_1	Melting Point	Yield, %	Formula[a]	Analysis, %	Calc.	Found
II*a*[b]	H	133°	85	$C_{13}H_{12}ClN_3$	C	63.53	63.40
					H	4.92	5.0
					Cl	14.43	14.41
					N	17.10	17.22
II*b*	CH_3	112°	82	$C_{14}H_{14}ClN_3$	C	64.74	64.39
					H	5.43	5.64
					Cl	13.65	14.01
					N	16.17	16.41
II*c*	iso-C_3H_7	123°	64	$C_{16}H_{18}ClN_3$	C	66.79	66.80
					H	6.30	6.24
					Cl	12.32	12.07
					N	14.60	14.46
II*d*[c]	C_6H_5	107°	80	$C_{19}H_{16}ClN_3$	C	70.91	70.97
					H	5.01	5.15
					Cl	11.03	11.23
					N	13.05	12.78
II*e*	*p*-ClC_6H_4	140°	50	$C_{19}H_{15}Cl_2N_3$	C	64.07	64.26
					H	4.24	4.29
					Cl	19.91	19.77
					N	11.80	11.91
II*f*	*p*-$OCH_3C_6H_4$	106°	73	$C_{20}H_{18}ClN_3O$	C	68.28	68.16
					H	5.15	5.14
					Cl	10.08	9.88
					N	11.95	12.00

[a] IR, NMR, and mass spectra are in agreement with assigned structures. [b] Lit. (8, 9) mp 134–135°. [c] Lit. (4) melting point not reported.

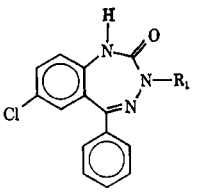


Table II—3-Substituted 7-Chloro-5-phenyl-1,3-dihydro-2*H*-1,3,4-benzotriazepin-2-ones (I)

Compound	R_1	R_2	Melting Point	Yield, %	Formula[a]	Analysis, %	Calc.	Found
I*a*[b]	H	H	242–243°	75	$C_{14}H_{10}ClN_3O$	C	61.89	62.00
						H	3.71	3.77
						N	15.46	15.72
I*b*	CH_3	H	215–218°	80	$C_{15}H_{12}ClN_3O$	C	63.05	62.92
						H	4.23	4.20
						N	14.71	14.93
I*c*	iso-C_3H_7	H	226°	78	$C_{17}H_{16}ClN_3O$	C	65.58	65.74
						H	5.18	5.11
						N	13.50	13.80
I*d*[c]	C_6H_5	H	237–238° dec.	82	$C_{20}H_{14}ClN_3O$	C	69.07	69.31
						H	4.05	4.23
						N	12.08	11.93
I*e*	*p*-ClC_6H_4	H	235–236°	76	$C_{20}H_{13}Cl_2N_3O$	C	62.85	63.00
						H	3.42	3.57
						N	10.99	11.04
I*f*	*p*-$OCH_3C_6H_4$	H	246°	78	$C_{21}H_{16}ClN_3O_2$	C	66.76	66.41
						H	4.27	4.42
						N	11.13	11.30
I*g*	CH_3	CH_3	226–228°	85	$C_{16}H_{14}ClN_3O$	C	64.12	63.92
						H	4.70	4.40
						N	14.02	14.26
I*h*	CH_3	$CH_2PO(CH_3)_2$	196–198°	90	$C_{18}H_{19}ClN_3O_2P$[d]	C	57.53	57.36
						H	5.09	5.12
						N	11.18	11.00
I*i*	C_6H_5	$CH_2PO(CH_3)_2$	245°	87	$C_{23}H_{21}ClN_3O_2P$[e]	C	63.10	62.83
						H	4.83	4.97
						N	9.59	9.81

[a] UV, IR, NMR, and mass spectra are in agreement with the assigned structures. For typical spectral data, refer to the *Experimental* section. [b] Lit. (4, 7) mp 235–237° and 246–248°, respectively. [c] Lit. (4) mp 243–244°. [d] *Anal.*—Calc.: P, 8.24. Found: P, 8.13. [e] *Anal.*—Calc.: Cl, 8.09: P, 7.07. Found: Cl, 8.01; P, 7.35.

spectra, in which predictable differences were observed for the —CONH—, $-NH_2$, and =NNH— protons (spectral data under *Experimental*). On cyclization, the carbalkoxyhydrazones formed only the benzotriazepinones (I*a*–I*c*), the urethanes in which R_1 = alkyl formed mixtures of the corresponding benzotriazepinones (I*b*, I*c*) and the known quinazolinone (VI) (4, 10), and the urethanes in which R_1 = aryl produced only the quinazolinone VI. The nature of the results provide new contributions to the chemistry of a previously little studied reaction sequence (4, 7).

Representative benzotriazepinones (I*b*, I*d*) were easily alkylated at the *N*-1 position by treatment with an alkyl halide and sodium hydride to give I*g*–I*i* (Table II).

EXPERIMENTAL[1]

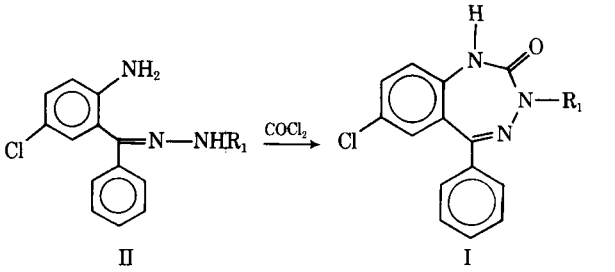
5-Chloro-2-aminobenzophenone *N*-Substituted Hydrazones (II*a*–II*f*)—The benzophenone (0.21 mole) and the appropriate hydrazine (0.42 mole) were added to a solution of potassium (9.0 g) in *tert*-butanol (200 ml). The mixture was refluxed for 6 hr and the solvent was distilled *in vacuo*. The residue was treated with water–ice, acidified with acetic acid, and extracted with methylene chloride. The extract was washed with water and 10% aqueous $NaHCO_3$, dried over anhydrous sodium sulfate, and evaporated to give the desired compounds which were recrystallized from ligroin–cyclohexane (Table I).

3-Substituted 7-Chloro-5-phenyl-1,3-dihydro-2*H*-1,3,4-benzotriazepin-2-ones (I*a*–I*f*) (4, 7)—A 15% solution of phosgene (0.1 mole) in toluene was added dropwise to a solution of the hydrazone (0.05 mole) in benzene (150 ml) at 20°. The reaction mixture was stirred for 3 hr at room temperature and then at reflux for 3 hr more. The solvents were removed *in vacuo* and the residue was triturated with methanol. Recrystallization from monomethylethylene glycol gave the desired products (Table II). Spectral data for a typical product are as follows.

*I*a—Mass spectroscopy (70 ev, 170°): *m/e* 271 (M^+); UV: λ_{max} (ethanol) ($\epsilon \times 10^{-4}$) 224 (4.61), 250 sh (2.50), and 302 (0.92) nm; IR: 1710 (CO), 1660, 1600, 1550, 1510, and 1490 cm^{-1}; NMR (dimethyl sulfoxide): 7.5 (m, 8H), 9.4 (s, NH), and 9.7 (s, NH) ppm.

*I*b—Mass spectroscopy (70 ev, 170°): *m/e* 285 (M^+); UV: λ_{max} (ethanol) ($\epsilon \times 10^{-4}$) 224 (4.24), 249 sh (2.21), and 305 (0.78) nm; IR: 1710 (CO), 1660, 1600, 1550, 1510, and 1490 cm^{-1}; NMR (dimethyl sulfoxide): 3.4 (s, 3H, $-NCH_3$) and 7.4 (m, 8H, aromatic protons) ppm.

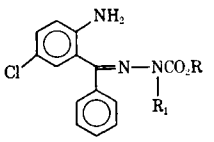
5-Chloro-2-aminobenzophenone Carbalkoxyhydrazones (IV*a*–IV*c*) and 5-Chloro-2-alkoxycarbonylaminobenzophenone *N*-Substituted Hydrazones (V*b*–V*f*)—The appropriate alkyl chloroformate (0.07 mole) was added dropwise to a well-stirred



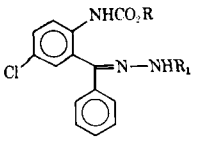
II I

Scheme I

[1] Melting points were taken on a Kofler heating bench, type 7841, and are uncorrected. UV spectra were obtained on a Pye-Unicam spectrophotometer, model SP500. The IR spectra were recorded on a Perkin-Elmer spectrophotometer, model 521, using KBr pellets. NMR spectra were obtained on Varian A-60 and T-60 spectrometers, using tetramethylsilane as the internal standard. The mass spectra were recorded on an MS9 (AEI).



IV



V

Table III—5-Chloro-2-aminobenzophenone Carbalkoxyhydrazones (IV) and 5-Chloro-2-alkoxycarbonylaminobenzophenone *N*-Substituted Hydrazones (V)

Compound	R_1	R	Yield, %	Melting Point	Formula[a]	Analysis, %	Calc.	Found
IV*a*	H	C_2H_5	85	208°[b] dec.	$C_{16}H_{16}ClN_3O_2$	C	60.47	60.41
						H	5.07	5.10
						Cl	11.15	10.97
						N	13.22	13.52
IV*b*	CH_3	C_2H_5	50	122°	$C_{17}H_{18}ClN_3O_2$	C	61.55	61.60
						H	5.46	5.63
+			+			Cl	10.70	10.65
						N	12.67	12.82
V*b*	CH_3	C_2H_5	30	117°	$C_{17}H_{18}ClN_3O_2$	C	61.55	61.50
						H	5.46	5.57
						Cl	10.70	10.61
						N	12.67	12.61
IV*c*	iso-C_3H_7	CH_3	30[c]	—	$C_{18}H_{20}ClN_3O_2$	C	62.53	62.37
						H	5.83	5.92
+			+			Cl	10.25	10.29
						N	12.15	12.08
V*c*	iso-C_3H_7	CH_3	26[c]	—	$C_{18}H_{20}ClN_3O_2$	C	62.53	62.35
						H	5.83	5.71
						Cl	10.25	10.06
						N	12.15	12.10
V*d*	C_6H_5	CH_3	60	205°	$C_{21}H_{18}ClN_3O_2$	C	66.40	66.26
						H	4.77	4.79
						Cl	9.33	9.24
						N	11.06	11.40
V*e*	*p*-ClC_6H_4	C_2H_5	65	175–178°	$C_{22}H_{19}Cl_2N_3O_2$	C	61.70	61.94
						H	4.47	4.65
						Cl	16.55	16.41
						N	9.81	10.01
V*f*	*p*-$OCH_3C_6H_4$	CH_3	42	134°	$C_{22}H_{20}ClN_3O_3$	C	64.48	64.62
						H	4.91	4.79
						Cl	8.65	8.93
						N	10.25	10.68

[a] IR, NMR, and mass spectra are in agreement with the assigned structures. For relevant NMR spectral data, refer to the *Experimental* section. [b] Lit (7) mp 209°. [c] The products were oils which resisted all attempts at crystallization.

solution of the hydrazone II (0.06 mole) in chloroform (200 ml)–pyridine (15 ml) at 10°. After allowing the reaction mixture to stand overnight, a further amount of alkyl chloroformate (0.04 mole) was added and stirring was continued at room temperature for 2 hr. In the experiments in which the *N*-aryl-substituted hydrazones (II*d*–II*f*) were used, the reaction mixture was refluxed for 3–6 hr more. The mixture was poured on ice-chilled water and the organic layer was separated, washed with water and 10% aqueous $NaHCO_3$, and dried over anhydrous sodium sulfate. The extract was evaporated *in vacuo* and the residue was separated into IV and V by column chromatography on silica gel (eluants: benzene, ethyl acetate, methanol); crystallization from ligroin–cyclohexane yielded the pure products (Table III). NMR spectral data (dimethyl sulfoxide/ppm) that help distinguish between IV and V are as follows.

*IV*a—3.4 (s, 2H, $-NH_2$) and 9.02 (s, 1H, —NNHCO); IR and NMR spectra were identical with those of the compound prepared as described previously (7).

*IV*b—3.5 (s, 2H, NH_2) and 2.94 [s, 3H, $NN(CH_3)CO$].

*V*b—2.68 (s, 3H, $NNHCH_3$) and 9.55 (s, 1H, NHCO).

*IV*c—3.5 (s, 2H, NH_2).

*V*c—9.57 (s, 1H, NHCO).

*V*d—11.7 (s, 1H, $NNHC_6H_5$) and 9.58 (s, 1H, NHCO).

*V*e—11.7 (s, 1H, NNHAr) and 9.60 (s, 1H, NHCO).

*V*f—11.7 (s, 1H, NNHAr) and 9.59 (s, 1H, NHCO).

Cyclization of IV and V—Compound IV or V (0.1 mole) was added to collidine (150 ml) or to diphenyl ether–diphenyl (3:1) and refluxed for 1–2 hr. When collidine was used, the reaction mixture was distilled *in vacuo* and the residue was triturated with ligroin–acetone (1:1, 100 ml). When diphenyl ether–diphenyl was used, ligroin–acetone (1:1, 500 ml) was added. A solid precipitated and was filtered. Crystallization from monomethylethylene glycol (using charcoal) gave different proportions of I*a*–I*c* and VI, mp 313–315° [lit. mp 310–312° (10) and 256–258° (4)], depending on the R_1 substituent (*cf.*, text). Compound VI was identified by the following spectral data: mass spectroscopy (70 ev/210°): *m/e* 256 (M^+); IR: 1640 (C=O) and 1620 (C=N) cm^{-1}; NMR (dimethyl sulfoxide): 7.7 (m, 8H, aromatic protons) ppm.

***N*-1 Alkylation of I*b* and I*d* (I*g*–I*i*)**—Compound I*b* or I*d* (0.06 mole) in xylene (150 ml) was treated with sodium hydride (55% in paraffin oil) under reflux conditions for 5 hr. A solution of methyl bromide (0.063 mole, 6.0 g) or of dimethylchloromethylphosphine oxide (0.063 mole, 8.0 g) in xylene (40 ml) was added with stirring. The mixture was refluxed for 3 hr, then cooled to 20°, and filtered. The filtrate was evaporated *in vacuo*, and the residue was crystallized from toluene. When dimethylchloromethylphosphine oxide was used, the residue was extracted with hot water. The water extract was filtered through charcoal-diatomaceous earth[2]. The filtrate was evaporated *in vacuo*, and the residue was recrystallized from toluene (Table II). The spectral data for I*h* follows: mass spectroscopy (70 ev, 240°): *m/e* 375 (M^+) and 298 [M^+, $-PO(CH_3)_2$]; IR: 1675 (C=O) and 1170 (broad, P=O) cm^{-1}; NMR ($CDCl_3$): 1.4 and 1.62 [2d, 3H, 3H, J = 14 Hz, $PO(CH_3)_2$], 3.82 (s, 3H, $N-CH_3$), 4.3 (pseudo t, 2H, J = 7 Hz, $N-CH_2-P$), and 7.6 (m, 8H, aromatic protons) ppm.

PHARMACOLOGICAL EVALUATION

Compounds I*a*–I*i* were tested in mice (11–19) to evaluate their potential as CNS depressants, using chlordiazepoxide and diazepam as reference compounds. Details of biological procedures

[2] Celite.

Table IV—CNS Depressant Activity of Some 7-Chloro-5-phenyl-1,3,4-benzotriazepin-2-ones

Compound	LD_{50}[a], mg/kg ip	Behavioral Screening[bc]: CNS Depressant Activity, mg/kg ip	Anticonvulsant Activity[d]: Pentylenetetrazol Infusion, ED_{50}, mg/kg ip	Potentiation of Hexobarbital Narcosis[e]: $ED_{+300\%}$, mg/kg ip	Methamphetamine: ED_{50} (Mouse), mg/kg po
I*a*	≫1000	++ 400	>400	50	220
I*b*	≫1000	+ 400	280	≫400	≫300
I*d*	≫1000	++ 400	>400	>400	≫300
Chlordiazepoxide	390	++++ 6	15	22	20
Diazepam	560	++++ 3	7	3.6	15

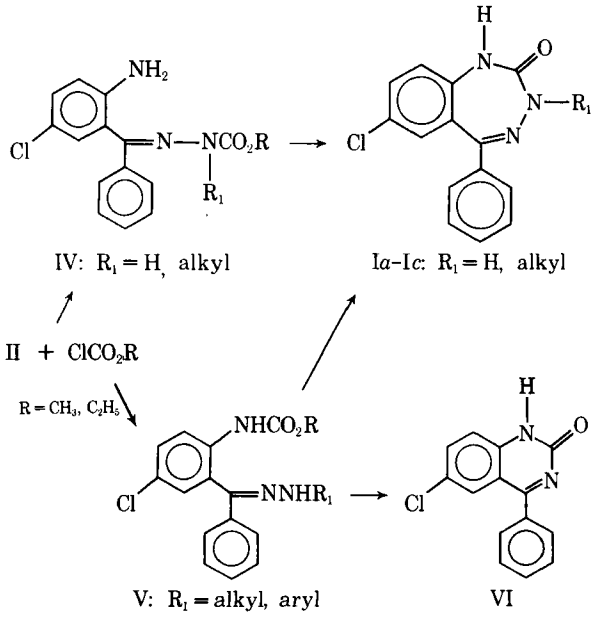
[a] References 1 and 11. [b] References 1 and 12. [c] Rating of CNS depressant activity: −, nil; +, very mild; ++, mild; +++, good; and ++++, very good. [d] Reference 13, six animals per dose. [e] References 1, 15, and 16. [f] Reference 17. Dose of methamphetamine = 0.5 mg/kg sc; 2 × 6 mice per dose.

used were described previously (1). Table IV summarizes only the most interesting results.

All compounds had LD_{50} values ≫ 1000 mg/kg (1, 11). In a primary Irwin neuropharmacological mouse profile (1, 12), all compounds caused CNS depression, as measured by a series of parameters including mild sedation, reduction in activity, relaxation of the muscle tonus, and diminution of the holding reflex. Mild anticonvulsant activity was exhibited against pentylenetetrazol-induced convulsions in mice (13) by all of the compounds, of which I*b* was the most active with an ED_{50} value of 280 mg/kg. None of the compounds, however, offered any protection at 400 mg/kg against maximal electroshock seizures (14). Only I*a* significantly potentiated hexobarbital-induced narcosis (1, 15, 16) and reduced the exploration and motility of a mouse given methamphetamine (17) (Table IV). None of the compounds at doses of 400 mg/kg exhibited a significant taming effect of Syrian hamsters (18) and stimulation of apomorphine-induced gnawing (19).

CONCLUSIONS

All benzotriazepinones described exhibited some mild CNS depressant and anticonvulsant activity. In comparison with the standard drugs, chlordiazepoxide and diazepam, the compounds were active only at relatively high doses. Introduction of alkyl or aryl substituents at the 3-position, as well as at the 1-position, did not significantly alter the CNS depressant activity of the parent 1,3-unsubstituted benzotriazepinone, and no structure–activity correlations were discernible.



Scheme II

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[x] To whom inquiries should be directed.

Chemical Modification of Erythromycin: Synthesis and Preliminary Bioactivity of Selected Amides and Esters

A. A. SINKULA

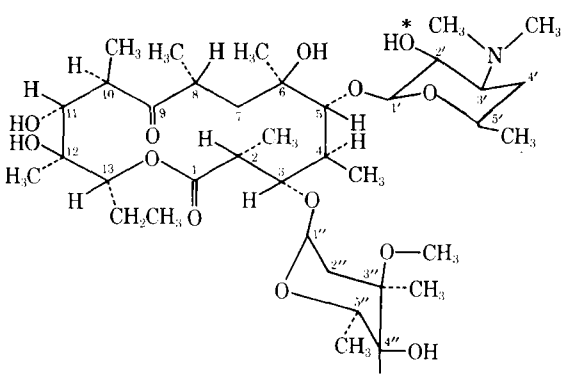
Abstract □ Erythromycin was chemically modified for the purpose of providing *in vivo* reversible tasteless derivatives of this antibiotic that might afford serum levels equivalent to erythromycin base. For improvement of taste properties and bioavailability, a series of erythromycin 2′-*N*-alkylsuccinamate and 2′-*N*-alkylglutaramate derivatives and several erythromycin 2′-alkylthiolsuccinate and 2′-alkylthiolglutarate derivatives were synthesized. The necessary chemical synthetic technology was developed to produce these derivatives and, in certain cases, alternative synthetic routes were utilized to improve yields. Most derivatives exhibited bioactivity comparable to erythromycin base in the mouse CD_{50} bioassay, and several derivatives produced serum levels of erythromycin equal to or greater than serum levels of animals dosed with erythromycin base. Several derivatives were tasteless and acceptable as candidates for pediatric formulation.

Keyphrases □ Erythromycin amides and esters—synthesis as tasteless derivatives, mouse CD_{50} bioassay and rat serum levels □ Tasteless erythromycin derivatives—synthesis, bioactivity □ Pediatric formulations, potential—synthesis and bioactivity of erythromycin amide and ester derivatives

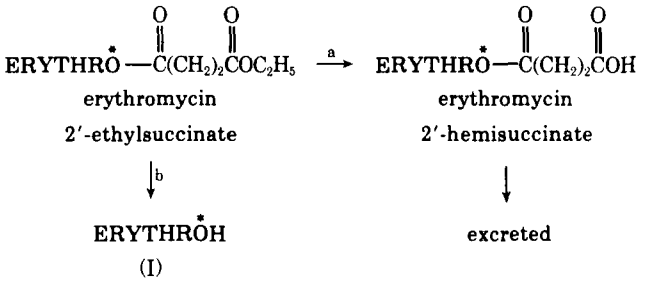
The purpose of this project was to obtain a tasteless pediatric form of erythromycin (I; ERYTHROH) that provided serum levels comparable to those of erythromycin base dosage forms. The results yielded several specially designed derivatives of erythromycin. These derivatives took the form of *N*-alkylsuccinamates and *N*-alkylglutaramates and, in four cases, erythromycin 2′-alkylthiolsuccinates and 2′-alkylthiolglutarates. These derivatives met the following requirements: *(a)* lack of bitterness (tastelessness), *(b)* bioactivity comparable to erythromycin base in the mouse CD_{50} bioassay, and *(c)* total whole blood levels (rat) equivalent to or greater than those of erythromycin base.

RESULTS AND DISCUSSION

The rationale for synthesizing a 2′-*N*-alkylglutaramate or 2′-*N*-alkylsuccinamate of erythromycin is that chemically (1–3) and, in many cases, enzymatically (4–8), amide linkages are more stable than ester linkages. Erythromycin 2′-ethylsuccinate (an ester) affords blood levels of erythromycin base consistently lower in human subjects than those of erythromycin base (9, 10). Similarly, erythromycin 2′-propionate lauryl sulfate salt affords low human blood levels of erythromycin base (11–14). This report, however, concerns itself only with comparisons to erythromycin base.



I: erythromycin (ERYTHROH)



Scheme I

Erythromycin 2′-ethylsuccinate has two ester linkages at which hydrolysis can occur (Scheme I): *(a)* hydrolysis at the terminal ester linkage of the ethylsuccinate chain yields erythromycin 2′-succinate hemiester, and *(b)* hydrolysis at the 2′-ester position yields erythromycin base.

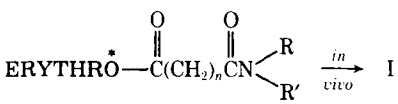
It appears that hydrolysis by route *(a)* should predominate since this ester is the least sterically hindered and would be most susceptible to enzymatic hydrolysis. If *(a)* is the predominant route of metabolism, then two factors become important:

1. The erythromycin succinate hemiester, being more polar than erythromycin ethylsuccinate or erythromycin base, would be more rapidly eliminated from the body and would tend to lower serum levels of this antibiotic. Monosubstituted succinic acid esters are known to be readily metabolized and excreted *in vivo* (15).

2. Erythromycin 2′-succinate hemiester possessed about 0.5 times the antibacterial activity as did erythromycin base in the mouse CD_{50} bioassay, implying that this derivative is less active than the free base (Table I).

In vitro results indicate about 50% base activity for erythromycin 2′-succinate hemiester (16). Thus, by all standards, it appears that erythromycin 2′-succinate hemiester is significantly less bioactive than erythromycin base itself.

It was previously stated that amide linkages in many cases are more resistant to hydrolysis than are ester linkages. Replacing the terminal ester linkage on an erythromycin 2′-alkyl hemiester with an amide linkage would enhance the stability of this portion of the molecule. If hydrolysis does occur, it should do so preferentially at the 2′-ester linkage rather than at the terminal amide linkage (Scheme II). This would ensure *in vivo* hydrolysis to erythromycin base rather than to the intermediate and less bioactive hemiester. Chemical modification would thus allow a one-step hydrolysis to the parent antibiotic erythromycin. Another important factor would be the slower rate of excretion of this amide derivative since it is not hydrolyzed to a polar intermediate. This would allow a longer residence time in the body and



$n = 2, 3$; R = H; R′ = C_2H_5 to $C_{12}H_{25}$

Scheme II

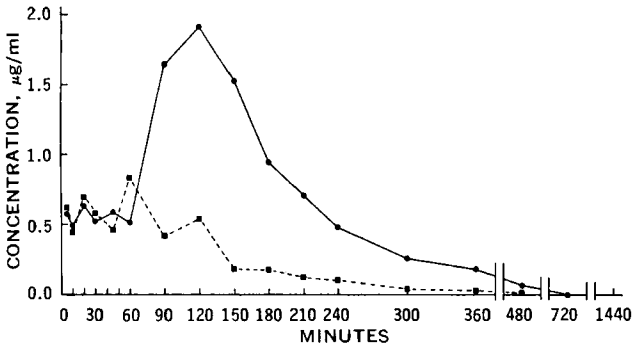


Figure 1—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-ethylsuccinate* (■).

permit the necessary esterases a longer time in which to hydrolyze this derivative to erythromycin base.

Hydrolysis of Erythromycin 2′-*N*-Alkylamides—The results of the antibacterial CD_{50} mouse bioassay illustrate some interesting points. All synthesized derivatives, with three exceptions, exhibited full antibacterial activity (compared to erythromycin base) in the mouse CD_{50} bioassay. This indicated that *in vivo* these ester-amides were being hydrolyzed to erythromycin base, affording subsequent antibacterial protection (Table I). The three exceptions to this generalization were erythromycin 2′-ethylthiolsuccinate, erythromycin 2′-ethylthiolglutarate, and erythromycin 2′-hexylthiolsuccinate. This trend is to be expected, however, since these compounds resemble erythromycin 2′-ethylsuccinate in all respects except that one is a "normal" ester (RCOOR′) and the three derivatives are thiol esters (RCOSR′). Based on what has been stated previously concerning erythromycin 2′-ethylsuccinate, this phenomenon is not unusual. The erythromycin 2′-ethylsuccinate oral CD_{50} value was on the lower end of the antibacterial bioactivity range (0.67)[1].

A more graphic illustration of the differences between erythromycin 2′-ethylsuccinate, erythromycin 2′-ethylthiolsuccinate, erythromycin 2′-ethylthiolglutarate, and the erythromycin 2′-amide derivatives is seen by a comparison of their respective blood level curves (Figs. 1–3)[2]. For one of the parameters measured (area under curve ± standard error), the ethylsuccinate, the ethylthiolsuccinate, and the ethylthiolglutarate derivatives exhibited decreased bioavailability when compared to erythromycin base (Table II). On the other hand, the ethyl- and hexylglutaramate and the dodecylsuccinamate derivatives showed bioavailability comparable to erythromycin base (Figs. 4–6). Erythromycin 2′-dodecylglutaramate and 2′-glutaryl-*N*-dicyclohexylurea exhibited bioavailability approximately 75% that of erythromycin base (Figs. 7 and 8). These blood level data thus support the contention that the amide terminal linkage in these derivatives stabilizes the molecule so that hydrolysis occurs preferentially at the 2′-ester linkage rather than at the terminal amide linkage (good hydrolysis was reflected in the oral CD_{50} values) and that these derivatives have a slower excretion rate, thus allowing a longer residence time which permits greater opportunity for hydrolysis. Extended T_{50} values reflected slow excretion of drug and bioactivity.

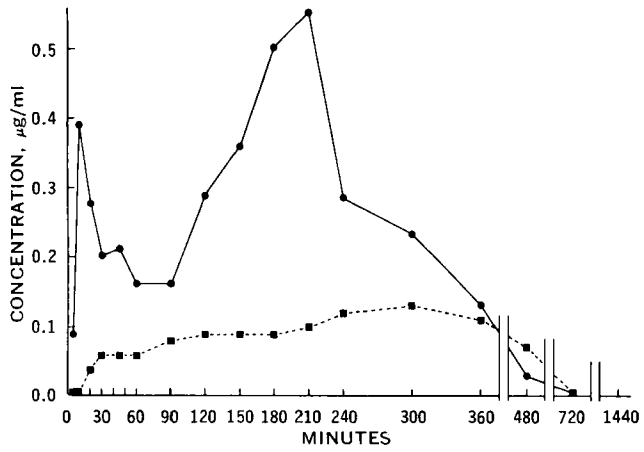


Figure 2—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-ethylthiolsuccinate* (■).

[1] Ratio of activity: CD_{50} erythromycin base/CD_{50} erythromycin 2′-ethylsuccinate.

[2] In certain figures, the concentration profile of erythromycin base is not consistently reproducible due to the variable absorption pattern of erythromycin in the rat. This phenomenon has also been noted in human subjects (15, 17). Comparison of the plasma level-time profiles between erythromycin base and ester is only valid for the series of rats dosed with a given derivative. The plasma concentrations of erythromycin are not necessarily therapeutic levels but rather reflect the amount of drug absorbed in a particular group of rats.

Table I—Antibacterial Oral Activity in *Staphylococcus aureus* (UC-76) Infected Mice

Compound	Oral CD_{50}, mg/kg[a]	Ratio of Activity[b]
Erythromycin 2′-*N*-ethylglutaramate	9.7 (7.0–12)	0.95
Erythromycin 2′-*N*-hexylglutaramate	11 (7.2–17)	0.82
Erythromycin 2′-*N*-dodecylglutaramate	14 (10–19)	2.14
Erythromycin 2′-*N*-dodecylsuccinamate	6.7 (4.6–9.7)	1.64
Erythromycin 2′-ethylthiolsuccinate	60 (45–77)	0.27
Erythromycin 2′-ethylthiolglutarate	36 (27–47)	0.44
Erythromycin 2′-hexylthiolsuccinate	40 (29–56)	0.42
Erythromycin 2′-hexylthiolglutarate	23 (17–31)	0.74
Erythromycin 2′-glutaryl-*N*-dicyclohexylurea	28 (21–38)	1.07
Erythromycin 2′-ethylsuccinate	45 (31–64)	0.67
Erythromycin 2′-succinate hemiester	19 (13–29)	0.48
Erythromycin 2′-glutarate hemiester	29 (21–42)	0.37

[a] Corrected to erythromycin base equivalent; numbers in parentheses indicate range. [b] CD_{50} erythromycin base/CD_{50} erythromycin 2′-ester.

Erythromycin 2′-hexylthiolsuccinate paralleled the time-concentration curve of erythromycin base and afforded serum levels equivalent to the base (Fig. 9). Erythromycin 2′-hexylthiolglutarate afforded levels of erythromycin base approximately twice that of erythromycin base (Fig. 10). No apparent explanation is available for the great differences in bioavailability between these hexylthiol esters and erythromycin base.

Chemistry—Erythromycin 2′-*N*-dodecylglutaramate (III) was synthesized by three different synthetic procedures, all active ester-mixed anhydride methods.

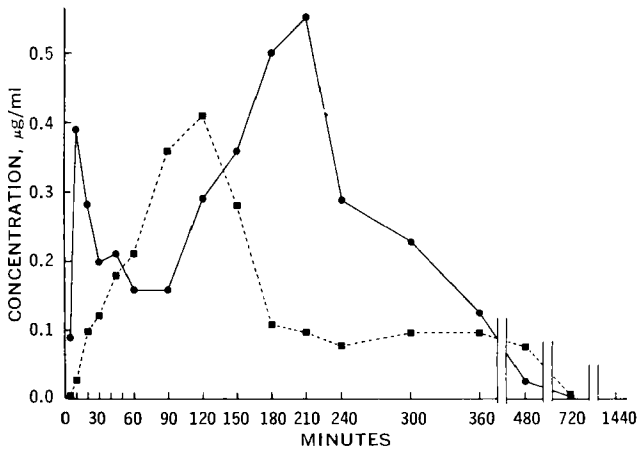


Figure 3—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-ethylthiolglutarate* (■).

Table II—Oral Blood Levels in Fasted Rats[a]

Compound	Area under Curve[b] ± *SE*	T_{50} of Area ± *SE*	Maximum Concentration, μg/ml	T_{max}, min	Ratio: Area–Control
Erythromycin 2′-*N*-ethylglutaramate	114 ± 1.4	171 ± 5.2	0.47	150	0.992
Erythromycin base	115 ± 33	193 ± 17	0.55	210	
Erythromycin 2′-*N*-hexylglutaramate	433 ± 32	278 ± 13	1.33	150	1.341
Erythromycin base	323 ± 144	180 ± 79	2.14	30	
Erythromycin 2′-*N*-dodecylglutaramate	228 ± 25	231 ± 28	0.80	60	0.77
Erythromycin base	304 ± 149	163 ± 29	1.90	120	
Erythromycin 2′-*N*-dodecylsuccinamate	103 ± 36	228 ± 18	0.35	120	0.925
Erythromycin base	112 ± 26	183 ± 22	0.42	150	
Erythromycin 2′-ethylthiolsuccinate	52.9 ± 7.6	298 ± 33	0.13	300	0.460
Erythromycin 2′-ethylthiolglutarate	80 ± 20	22 ± 60	0.41	120	0.698
Erythromycin base	115 ± 33	193 ± 17	0.55	210	
Erythromycin 2′-hexylthiolsuccinate	69 ± 17	102 ± 9	0.45	30	1.033
Erythromycin 2′-hexylthiolglutarate	144 ± 25	155 ± 3	0.61	45	2.155
Erythromycin base	67 ± 10	138 ± 9	0.40	90	
Erythromycin 2′-glutaryl-*N*-dicyclohexylurea	224 ± 124	270 ± 35	0.59	150	0.736
Erythromycin base	304 ± 149	163 ± 58	1.90	120	
Erythromycin 2′-ethylsuccinate	104 ± 23	90 ± 23	0.83	60	0.343
Erythromycin base	304 ± 149	163 ± 58	1.90	120	

[a] Single oral dose of 100 mg/kg of base equivalent. [b] Mean area from three rats.

Method I—The first method involved the use of erythromycin 2′-glutarate hemiester (II), dodecylamine, and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline[3] (IV) (18) (Scheme III). This reaction afforded crude material that was subjected to column chromatography for purification. The overall yield of pure derivative was low (43%).

Method II—The second method required the use of erythromycin 2′-glutarate hemiester, isobutyl chloroformate, triethylamine, and dodecylamine as starting materials (19–21) (Scheme IV). This method also required the use of column chromatography for purification. Again, overall yields of pure material were low (33%).

Method III—The third method involved the condensation of erythromycin base and *N*-dodecylglutaramic acid in the presence of isobutyl chloroformate (Scheme V). Column chromatography was again necessary for purification. Yields were slightly improved (~50%). Erythromycin 2′-*N*-dodecylsuccinamate was also synthesized by the third method (yield ~50%).

Erythromycin 2′-*N*-ethylglutaramate and erythromycin 2′-*N*-hexylglutaramate were prepared by Method II using ethylamine and hexylamine, respectively. Isobutyl chloroformate was again used as the condensing agent. Erythromycin 2′-*N*-ethylglutaramate was crystalline but difficult to purify because of its high water solubility. Erythromycin 2′-*N*-hexylglutaramate was also crystalline and purification posed no problem.

Erythromycin 2′-ethylthiolglutarate was synthesized by the reaction of erythromycin 2′-glutarate hemiester with ethanethiol in the presence of isobutyl chloroformate and triethylamine (Scheme VI). This material was completely crystalline and its R_f value[4] was comparable to that of erythromycin 2′-ethylsuccinate,

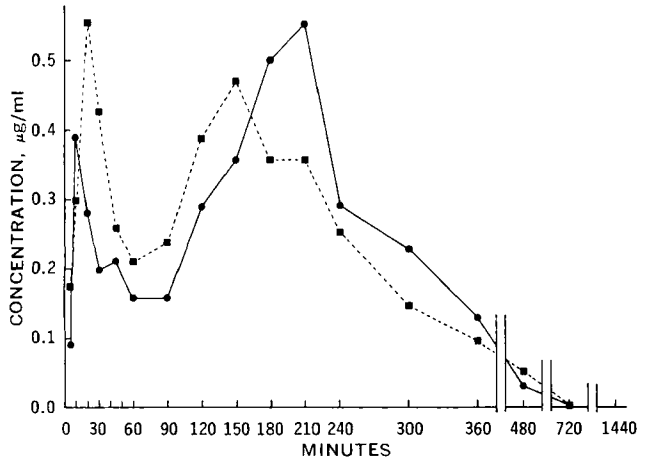


Figure 4—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-*N*-ethylglutaramate* (■).

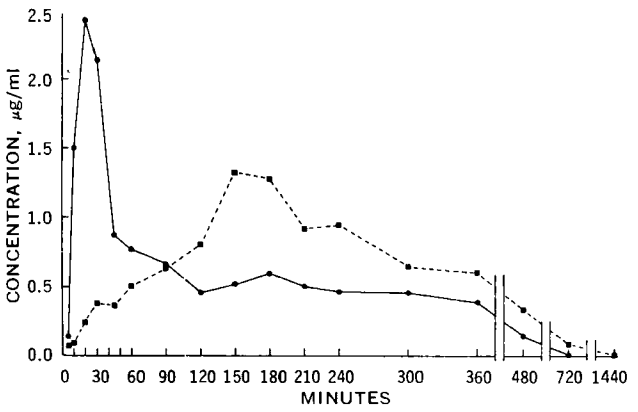


Figure 5—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-*N*-hexylglutaramate* (■).

[3] Aldrich Chemical Co., Milwaukee, Wis.

[4] Silica gel GF precoated TLC plates (Analtech, Inc., Newark, Del.), using acetone as developing solvent.

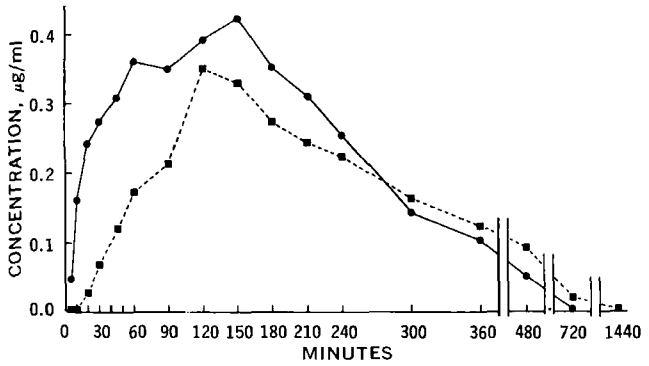


Figure 6—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-*N*-dodecylsuccinamate* (■).

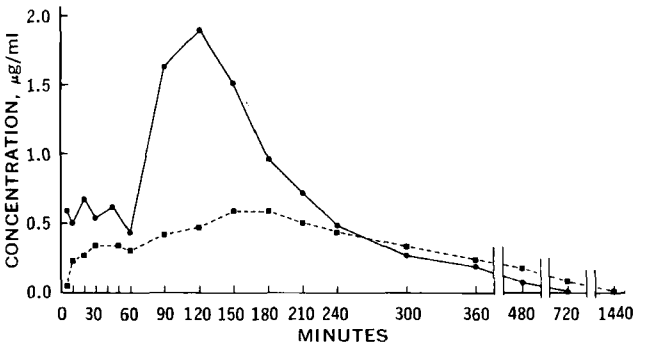


Figure 8—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-glutaryl-*N*-dicyclohexylurea* (■).

suggesting that the polarity of these substances may be similar[5].

Erythromycin 2′-ethylthiolsuccinate was synthesized in a similar manner using erythromycin 2′-succinate hemiester. Its R_f value was also similar to that of erythromycin 2′-ethylsuccinate, again suggesting a marked similarity in properties[4]. This material crystallized easily. The yield of these two thiol esters was approximately 20%.

The hexylthiolsuccinate and hexylthiolglutarate esters were synthesized similarly using hexanethiol. These esters were also highly crystalline and required minimal purification. The overall yield of the hexylthiol esters averaged 20%.

Erythromycin 2′-glutaryl-*N*-dicyclohexylurea was synthesized by condensation of erythromycin 2′-glutarate hemiester with dicyclohexylcarbodiimide. The reaction proceeded through the formation of the erythromycin 2′-glutaryl-*o*-dicyclohexylisourea intermediate, which rearranged to the urea (22–25) (Scheme VII). The overall yield of the pure derivative was about 40%.

EXPERIMENTAL

Biological

In Vitro—*In vitro* bioactivity was determined using the standard curve plate bioassay (26, 27). The sensitivity of the assay was 0.25 μg/ml using *Sarcina lutea* as the test organism. Erythromycin free base had an assigned potency of 1000 μg/mg, and the activities of the erythromycin derivatives were calculated and reported as the free base equivalents per milligram of antibiotic. All erythromycin derivatives tested showed less than 10% activity compared to erythromycin base (similar to other erythromycin 2′-esters) (28).

In Vivo—The *in vivo* protection studies were undertaken using CF-1 male albino mice randomly selected from an animal pool; they were experimentally infected using *Staphylococcus aureus* (UC-76). Experimental details were previously described (26, 27). Oral CD_{50} values obtained with the erythromycin derivatives are listed in Table I.

Blood Level Studies—The comparative absorption rates of erythromycin from the various erythromycin thiol esters and amides were determined using Sprague–Dawley (Upjohn strain) white, male rats weighing approximately 150 g. The rats were fasted for 24 hr and dosed orally by gavage with 0.5 ml of 0.25% methylcellulose[6] containing 100 mg/kg erythromycin base equivalent of derivative. The procedures for determining the quantity of erythromycin in the blood were described previously (29). In the blood level determinations, the parameters evaluated were: *(a)* total area under the curve ± standard error, *(b)* time in minutes at which 50% of the total area appeared under the curve (T_{50}) ± standard error, *(c)* maximum concentration (micrograms per milliliter) of erythromycin in the blood at time T (minutes), and *(d)* ratio of total areas of erythromycin base derived from derivative–erythromycin base control.

Synthetic

Typical examples of reaction conditions for erythromycin 2′-*N*-alkylamides and erythromycin 2′-alkylthiol esters are illustrated. Elemental analyses are listed in Table III.

Erythromycin 2′-*N*-Dodecylglutaramate (Method I)—*Erythromycin 2′-Glutarate Hemiester (16, 30)*—Erythromycin base (146.8 g, 0.2 mole) was dissolved in enough (1 liter) methylene chloride at 30° to effect complete solution. A filtered methylene chloride solution containing 24 g of glutaric anhydride was added to the solution of erythromycin base and stirred for 1 hr. The resulting crystals were collected, dried *in vacuo* at 60° for 48 hr, and used without further purification.

*Erythromycin 2′-*N*-Dodecylglutaramate*—Erythromycin 2′-glutarate hemiester (4.24 g, 0.005 mole) was dissolved in 100 ml of acetone (previously dried with 3A molecular sieve). A solution of 0.193 g (0.005 mole) of *n*-dodecylamine was dissolved in 15 ml of anhydrous acetone. A solution of 1.24 g (0.005 mole) of IV was

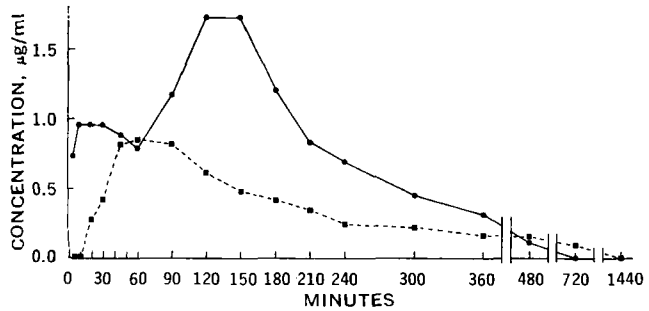


Figure 7—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-*N*-dodecylglutaramate* (■).

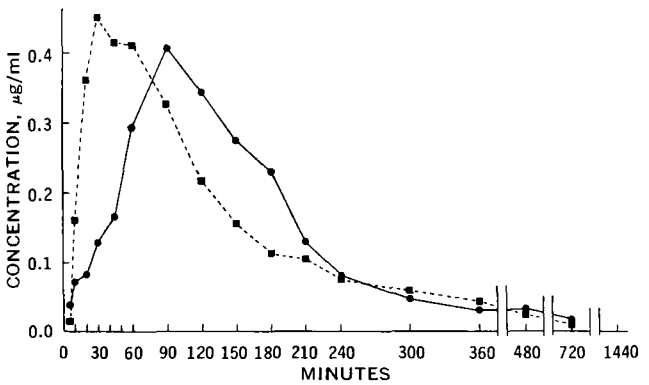


Figure 9—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-hexylthiolsuccinate* (■).

[5] Erythromycin 2′-ethylthiolglutarate, erythromycin 2′-ethylthiolsuccinate, and erythromycin 2′-ethylsuccinate were less polar in aqueous media (*via* comparison of R_f values) than any of the other derivatives synthesized in this study. The low rat blood level data suggested that the dissolution rates *in vivo* of these derivatives may be insufficient to provide adequate drug for absorption.

[6] Methocel HG, Dow Chemical Co., Midland, Mich.

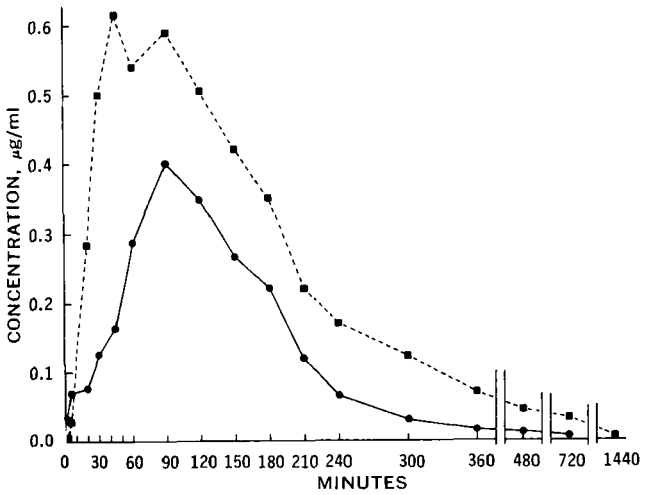
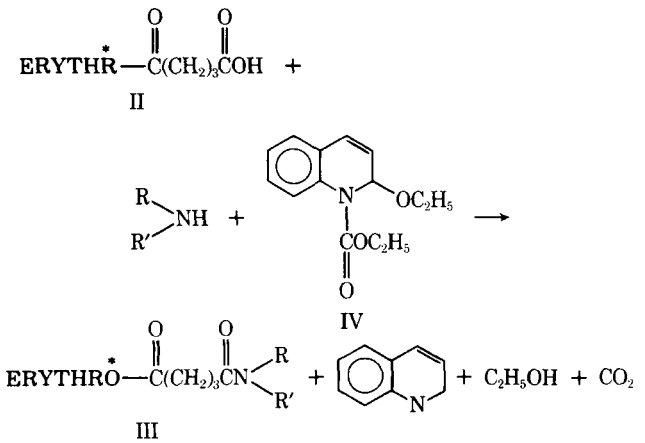


Figure 10—*Whole blood levels in rats dosed orally with erythromycin base* (●) *or erythromycin 2′-hexylthiolglutarate* (■).

dissolved in 10 ml of acetone, and the three solutions were combined at room temperature for 72 hr. The solvent was then removed *in vacuo*, and the yellow syrup was swirled with 50 ml of anhydrous ether. The resulting precipitate was discarded, and the ether was removed *in vacuo*.

One gram of the crude product was dissolved in 5 ml of anhydrous acetone, filtered, and placed on the top of a glass column containing 50 g of silica gel[7] (70–325 mesh) as an acetone slurry. The column was eluted with anhydrous acetone, and the fractions containing pure erythromycin 2′-*N*-dodecylglutaramate were collected. The solvent was removed at room temperature, and the resulting powder was dried *in vacuo* at 45°.

Erythromycin 2′-*N*-Dodecylglutaramate (Method II)—Erythromycin 2′-glutarate hemiester (33.92 g, 0.04 mole) was dissolved in acetone (200 ml) and cooled to −20°. Isobutyl chloroformate (5.44 g, 0.04 mole) and triethylamine (4.04 g, 0.04 mole) were added. *n*-Dodecylamine (7.4 g, 0.04 mole) was dissolved in 250 ml of acetone and added dropwise from a dropping funnel over 1 hr, keeping the reaction mixture at −20° until 15 min after the addition was completed. Then the reaction mixture was allowed to come to room temperature and was stirred for 24 hr. The solvent was removed *in vacuo*. Six grams of the dried powder was dissolved in 25 ml acetone, filtered, and placed on the top of a glass column containing 427 g silica gel (70–325 mesh) as an acetone slurry. The column was eluted with anhydrous acetone, the fractions containing pure erythromycin 2′-*N*-dodecylglutaramate were collected, the solvent was removed at room temperature, and the resulting powder was dried *in vacuo* at 45°.



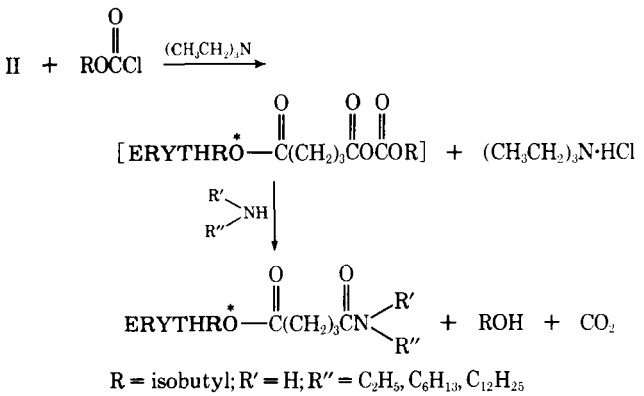
R = H; R′ = $C_{12}H_{25}$

Scheme III

Erythromycin 2′-*N*-Dodecylglutaramate (Method III)—*N-Dodecylglutaramic Acid*—Glutaric anhydride (11.41 g, 0.01 mole) was dissolved in 50 ml of acetone. *n*-Dodecylamine (16.68 g, 0.009 mole) was dissolved in 150 ml of acetone and warmed to 40°. The two solutions were thoroughly mixed and allowed to stand at room temperature for 1 hr. Then the resulting crystals were collected and dried.

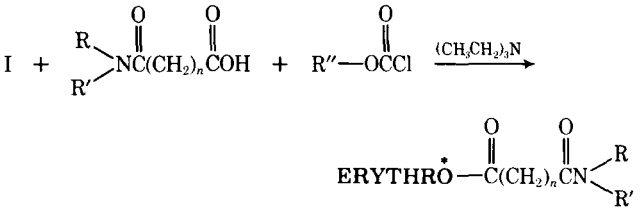
Erythromycin 2′-N-Dodecylglutaramate—Fifteen grams (0.05 mole) of *N*-dodecylglutaramic acid was dissolved in 750 ml of chloroform and cooled to −10°. To this solution were added 6.8 g (6.5 ml, 0.05 mole) of isobutyl chloroformate and 5.05 g (7 ml, 0.05 mole) of triethylamine, and the mixture was stirred well. To this mixture was added dropwise a solution of 36.65 g (0.05 mole) of erythromycin base in 250 ml. of chloroform. During addition, the temperature was maintained at −10°. The reaction mixture was warmed to room temperature and stirred for 24 hr. The solvent was then removed *in vacuo*, and the residue was dissolved in anhydrous acetone. The resulting precipitate of triethylamine hydrochloride was filtered, and the solvent was again removed *in vacuo*.

Sixty-two grams of crude erythromycin 2′-*N*-dodecylglutaramate, dissolved in 150 ml of anhydrous acetone, was introduced to a 183 × 10-cm glass column containing 6 kg of previously treated silica gel[7] (70–325 mesh) for column chromatography. The material was eluted with anhydrous acetone, and the column was run at a flow rate of 17 ml/min. One thousand-milliliter cuts were



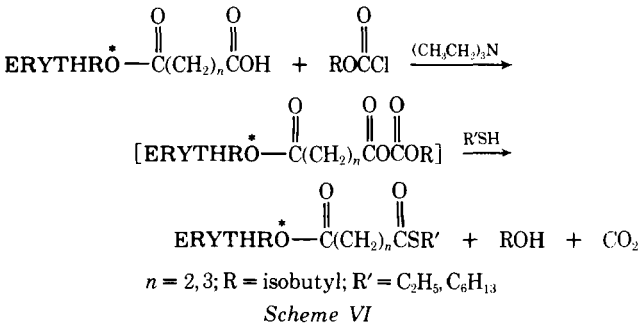
R = isobutyl; R′ = H; R″ = C_2H_5, C_6H_{13}, $C_{12}H_{25}$

Scheme IV



n = 2, 3; R = H; R′ = $C_{12}H_{25}$; R″ = isobutyl

Scheme V



n = 2, 3; R = isobutyl; R′ = C_2H_5, C_6H_{13}

Scheme VI

[7] This silica gel (Merck), prior to placement in the column, was suspended in anhydrous acetone and the "fines" were decanted. This procedure was repeated several times. The silica gel was then isolated by vacuum filtration and washed five times with 200-ml portions of chloroform (AR). The silica gel was then dried on a vacuum filter prior to suspension in anhydrous acetone.

Table III—Analytical Data for Erythromycin 2′-Esters[a]

Erythromycin Ester	Empirical Formula	Molecular Weight	Analysis, % Calc.	Analysis, % Found	Melting Point	Yield, %
2′-*N*-Ethylglutaramate	$C_{44}H_{78}N_2O_{15}$	875.11	C 60.39 H 8.98 N 3.20	C 60.03 H 9.05 N 3.15	138–141°	53
2′-*N*-Hexylglutaramate	$C_{48}H_{86}N_2O_{15}$	931.22	C 61.91 H 9.31 N 3.01	C 62.19 H 9.17 N 3.34	110–113°	54
2′-*N*-Dodecylglutaramate	$C_{54}H_{98}N_2O_{15}$	1015.34	C 63.89 H 9.73 N 2.76	C 63.74 H 9.88 N 2.70	83–85°	50
2′-*N*-Dodecylsuccinamate	$C_{53}H_{96}N_2O_{15}$	1001.35	C 63.57 H 9.66 N 2.80	C 63.10 H 9.69 N 2.72	90–93°	50
2′-Ethylthiolsuccinate	$C_{43}H_{75}NO_{15}S$	878.13	C 58.81 H 8.61 N 1.59 S 3.65	C 59.29 H 8.45 N 1.58 S 3.31	138–141°	21
2′-Ethylthiolglutarate	$C_{44}H_{77}NO_{15}S$	892.16	C 59.24 H 8.70 N 1.57 S 3.59	C 59.39 H 8.79 N 1.60 S 3.49	128–132°	20
2′-Hexylthiolsuccinate	$C_{47}H_{83}NO_{15}S$	934.24	C 60.43 H 8.96 N 1.50 S 3.43	C 60.10 H 8.99 N 1.49 S 3.13	95–96°	20
2′-Hexylthiolglutarate	$C_{48}H_{85}NO_{15}S$	948.27	C 60.80 H 9.04 N 1.48 S 3.38	C 61.17 H 9.04 N 1.36 S 3.45	91–93°	20
2′-Glutaryl-*N*-dicyclohexylurea	$C_{55}H_{95}N_3O_{16}$	1054.38	C 62.25 H 9.08 N 3.99	C 62.54 H 8.55 N 3.71	143–146°	40
N-Dodecylsuccinamic acid	$C_{16}H_{31}NO_3$	285.42	C 67.33 H 10.95 N 4.91	C 67.55 H 11.00 N 4.86	117–119°	94
N-Dodecylglutaramic acid	$C_{17}H_{33}NO_3$	299.45	C 68.17 H 11.11 N 4.70	C 68.13 H 11.03 N 4.46	95–96°	93

[a] Melting points are of the hydrated samples. They were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. The elemental analysis values are corrected for water content.

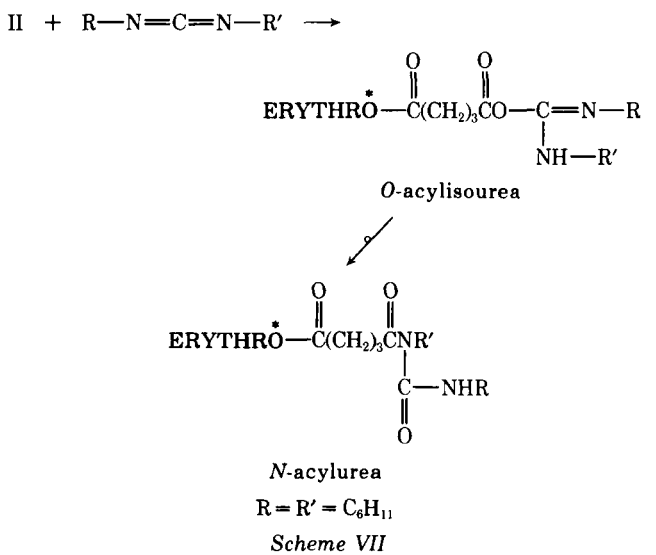
collected, fractions 7–12 were collected and pooled, and the solvent was removed *in vacuo*.

Erythromycin 2′-Thiol Esters—*Erythromycin 2′-Ethylthiolsuccinate*—Erythromycin base (146.8 g, 0.2 mole) was dissolved in 700 ml of anhydrous acetone. Succinic anhydride (22.01 g, 0.22 mole) was dissolved in 300 ml of anhydrous acetone. The two solutions were combined and stirred for 2 hr. Precipitation began after 30 min and the yield was 155 g (92%). This compound was used without further purification (16, 30).

Erythromycin 2′-succinate hemiester (50.04 g, 0.06 mole) was dissolved in 500 ml of anhydrous acetone, and 7.8 g (10.7 ml, 0.067 mole) of triethylamine was added to this solution. The mixture was cooled to −10°, 9.15 g (8.7 ml, 0.067 mole) of isobutyl chloroformate was added dropwise, and the reaction mixture was allowed to warm to room temperature. The mixture was stirred at room temperature for 12 hr, and the solvent was removed *in vacuo*. Ten grams of the resulting white powder was dissolved in 250 ml of acetonitrile, 75 g of silica gel GF (TLC grade) was added, and the mixture was swirled and warmed to 50°. The filtrate was added to 1 liter of distilled water, resulting in the precipitation of fine, white crystals. The material was pure by TLC (acetone, silica gel).

Erythromycin 2′-Hexylthiolglutarate—Erythromycin 2′-glutarate hemiester (50.88 g, 0.06 mole) was dissolved in 500 ml of anhydrous acetone. Seven and eight-tenths grams (0.067 mole) of triethylamine was added to this solution. The mixture was cooled to −10°, 9.15 g (8.7 ml, 0.067 mole) of isobutyl chloroformate was added dropwise, and the reaction mixture warmed to room temperature. The mixture was recooled to −10°, and 7.1 g (0.067 mole) of hexanethiol was added. The mixture was stirred at room temperature for 48 hr, recooled to −10°, an additional 4 g of hexanethiol was added, and the mixture was rewarmed to room temperature. Then the mixture was stirred for 12 hr and the solvent was evaporated. Ten grams of the resulting impure mixture was treated with 3 g of TLC grade silica gel in 100 ml of acetonitrile. The silica gel was removed by filtration, and the solvent was removed *in vacuo*, resulting in 2 g of pure erythromycin 2′-hexylthiolglutarate.

Erythromycin 2′-Glutaryl-N-dicyclohexylurea—Twelve and seven-tenths grams (0.015 mole) of erythromycin 2′-glutarate hemiester was dissolved in 100 ml of anhydrous acetone. Three and eight-tenths grams (0.018 mole) of dicyclohexylcarbodiimide was dissolved in 75 ml of anhydrous acetone. The two solutions were combined and stirred for 3 hr. The resulting mixture was fil-



Scheme VII

tered, and the solvent was removed *in vacuo*. The powder was pulverized and dried *in vacuo* at 40° for 2 hr. The yield was essentially quantitative by TLC.

Six grams of impure erythromycin 2′-glutaryl-*N*-dicyclohexylurea was dissolved in 25 ml of anhydrous acetone, and the solution was filtered and placed on a column packed by acetone slurry with 427 g silica gel[7] (70–325 mesh). The column was eluted with anhydrous acetone at a rate of 12 ml/min, and tubes numbering 30–79 were collected and pooled. The solvent was removed at room temperature, and 2.4 g of pure erythromycin 2′-glutaryl-*N*-dicyclohexylurea was collected.

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Preparation and Pharmacological Screening of Indanethylamines Related to Tryptamine

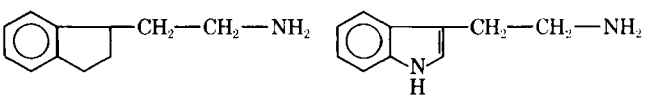
JOHN B. DATA[x], MICHAEL L. TAYLOR, and MARIO FORCIONE

Abstract □ A series of mono- and di-*N*-substituted 2-(1-indanyl)-ethylamines was prepared and screened for monoamine oxidase inhibition and serotonin antagonism. Synthetic methods for their preparation are described, and the procedures used for their screening along with the activity found are discussed.

Keyphrases □ Indanethylamines, related to tryptamine—synthesis and pharmacological screening for monoamine oxidase inhibition and serotonin antagonism □ Tryptamine-related compounds—synthesis and screening of indanethylamines as monoamine oxidase inhibitors and serotonin antagonists □ Monoamine oxidase inhibition—synthesis and screening of indanethylamines □ Serotonin antagonism—synthesis and screening of indanethylamines

During an investigation in these laboratories directed toward the synthesis of compounds related to reserpine, the occasion arose to prepare 2-(1-indanyl)-ethylamine (I). The close isosteric relationship of I to tryptamine (II) aroused interest with reference to its pharmacological activity.

A review of the literature indicated that a biological investigation of I had not been published and only recently was its preparation reported (1). Further search revealed that pharmacological studies with 1-, 2-, and 3-indanylmethyl-, indanylethyl-, and indanylpropylamines have been confined chiefly to phenyl-substituted indanamines (2–8). Very few studies concerning these amines with substituents on the cyclopentano moiety of indan used substituents other than a phenyl group (9, 10). Since most studies



I II

tered, and the solvent was removed *in vacuo*. The powder was pulverized and dried *in vacuo* at 40° for 2 hr. The yield was essentially quantitative by TLC.

Six grams of impure erythromycin 2′-glutaryl-*N*-dicyclohexylurea was dissolved in 25 ml of anhydrous acetone, and the solution was filtered and placed on a column packed by acetone slurry with 427 g silica gel[7] (70–325 mesh). The column was eluted with anhydrous acetone at a rate of 12 ml/min, and tubes numbering 30–79 were collected and pooled. The solvent was removed at room temperature, and 2.4 g of pure erythromycin 2′-glutaryl-*N*-dicyclohexylurea was collected.

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Preparation and Pharmacological Screening of Indanethylamines Related to Tryptamine

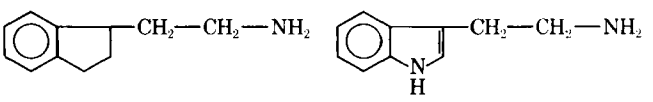
JOHN B. DATA[x], MICHAEL L. TAYLOR, and MARIO FORCIONE

Abstract □ A series of mono- and di-*N*-substituted 2-(1-indanyl)-ethylamines was prepared and screened for monoamine oxidase inhibition and serotonin antagonism. Synthetic methods for their preparation are described, and the procedures used for their screening along with the activity found are discussed.

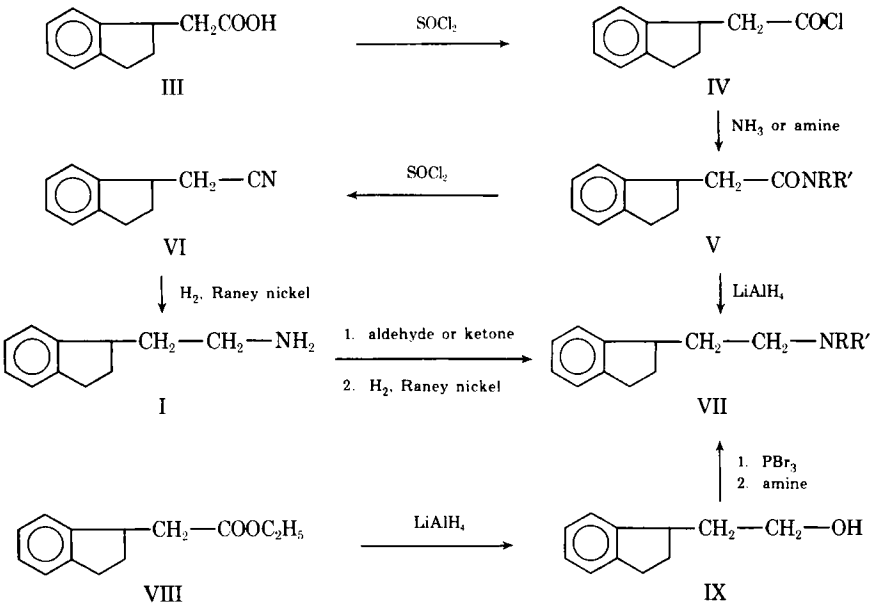
Keyphrases □ Indanethylamines, related to tryptamine—synthesis and pharmacological screening for monoamine oxidase inhibition and serotonin antagonism □ Tryptamine-related compounds—synthesis and screening of indanethylamines as monoamine oxidase inhibitors and serotonin antagonists □ Monoamine oxidase inhibition—synthesis and screening of indanethylamines □ Serotonin antagonism—synthesis and screening of indanethylamines

During an investigation in these laboratories directed toward the synthesis of compounds related to reserpine, the occasion arose to prepare 2-(1-indanyl)-ethylamine (I). The close isosteric relationship of I to tryptamine (II) aroused interest with reference to its pharmacological activity.

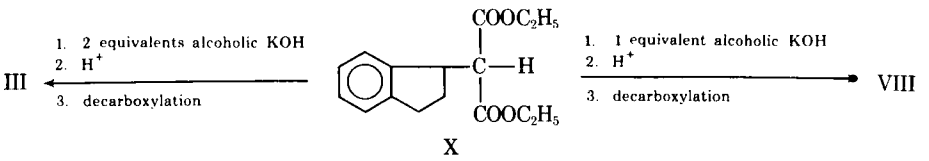
A review of the literature indicated that a biological investigation of I had not been published and only recently was its preparation reported (1). Further search revealed that pharmacological studies with 1-, 2-, and 3-indanylmethyl-, indanylethyl-, and indanylpropylamines have been confined chiefly to phenyl-substituted indanamines (2–8). Very few studies concerning these amines with substituents on the cyclopentano moiety of indan used substituents other than a phenyl group (9, 10). Since most studies



I II



Scheme I—(Structure for R and R' given in Table I)



Scheme II

have been done with amines influenced by the high electron density of the phenyl ring, the question arose as to what pharmacological response would be evident when this factor was absent. This fact, coupled with the isosteric relationship, prompted the preparation of a series of secondary and tertiary 2-(1-indanyl)ethylamines for preliminary pharmacological examination for monoamine oxidase inhibition and serotonin antagonism.

While preparation of these compounds was in progress, a closely related study was announced (11, 12), which made a comparison of the contractile effects on the rat fundus of some 2-indenealkylamines and other heterocyclic isosteres. Shortly after completion of the present study, the hypoglycemic activity of a series of indanamines was reported (13); five amines listed in Table I were a part of that study. Since most of the chemistry (see *Chemistry* section) and the pharmacological studies differ from that of the latter authors, and because of the limited pharmacological data recorded in the literature on compounds of the type reported herein, it is appropriate that this study be reported.

CHEMISTRY

The amines (Table I) were prepared by one of the following three methods (Scheme I). Lahiri and De (13) reported the preparation of five compounds (VIIc, VII*e*, VII*g*, VII*m*, and VII*n*) listed in Table I by lithium aluminum hydride reduction of the corresponding amides to the amines as exemplified by Method C *via* the acid, to the acid chloride, to the amides, and to the amines.

Method A—1-Indanylacetic acid (III) was converted with thionyl chloride to the acid chloride (IV), which was then aminated with aqueous ammonia or with an anhydrous ethereal solution of IV with gaseous ammonia to give the amide V. Dehydration of the amide with thionyl chloride gave 1-indanylacetonitrile (VI), which was reduced with hydrogen in the presence of Raney nickel to 2-(1-indanyl)ethylamine (I). Compound I was allowed to react with either an aldehyde or a ketone to give the Schiff base, which was then reduced *in situ* with hydrogen using Raney nickel as catalyst to the desired amines (VII).

Method B—Ethyl 1-indanylacetate (VIII) was reduced with lithium aluminum hydride to give 2-(1-indanyl)ethanol (IX). The alcohol IX was treated with phosphorus tribromide to give the halide, which was allowed to react with an appropriate amine to give the desired products (VII).

Method C—The substituted amides (V) were obtained from the reaction of IV with an amine. Reduction of the substituted amides with lithium aluminum hydride in tetrahydrofuran gave the desired amines (VII).

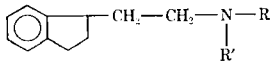
Ethyl 1-indanylacetate (VIII) or the corresponding acid (III) was obtained by selective base hydrolysis of the diethyl 1-indanylmalonate (X) (Scheme II), prepared from sodio diethyl malonate and chlorindan in dry benzene, followed by decarboxylation of the mono- or dicarboxylic acid resulting from acidification of the corresponding potassium salt obtained in the hydrolysis.

EXPERIMENTAL[1]

Diethyl 1-Indanylmalonate—Moffett and Hart (14) reported the preparation of the title compound, but a procedure for its synthesis was not described.

To a stirred suspension of 35.0 g (1.52 g-atom) of finely powdered sodium in 1.5 liters of dry benzene was added dropwise 240.3 g (1.5 moles) of diethyl malonate, and the mixture was then gent-

[1] Melting points were determined by the capillary tube method using a Buchi capillary melting-point apparatus. All melting and boiling points are uncorrected. The phrase "reduced pressure," "*in vacuo*," or the equivalent indicates water aspiration at 12–20 mm. IR data were recorded on a Beckman IR-33 spectrophotometer. NMR spectra were determined with a Jeol MH 60 spectrometer using tetramethylsilane as the internal reference. All benzyl triplets would not be expected to be first-order triplets but give this appearance in the spectra. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.



VII

Table I—Mono- and Di-*N*-substituted 2-(1-Indanyl)ethylamines

Compound	R	R′	Method	Boiling Point (mm)	Yield, %	Hydrochloride Melting Point	Formula	Analysis, %	Calc.	Found
VII*b*	CH_3	H	C	82–83° (0.25)	84	193–194°	$C_{12}H_{18}ClN$	C	68.08	67.94
								H	8.51	8.40
VII*c*	CH_3	CH_3	B	68–70° (0.17)[a]	82	154–155°[a]	$C_{13}H_{20}ClN$	C	69.17	69.19
								H	8.86	8.93
VII*d*	—CH_2—CH_2—CH_2—CH_2—CH_2—		B	117–118° (0.45)	92	219–220°	$C_{16}H_{24}ClN$	C	72.31	72.64
								H	9.03	9.12
VII*e*	CH_3—CH_2—CH_2	H	A	81–82° (0.2)[b]	76	178–179°[b]	$C_{14}H_{22}ClN$	C	70.14	69.86
								H	9.18	8.94
VII*f*	$(CH_3)_2$—CH	H	A	85–90° (0.15)	90	179–180°	$C_{14}H_{22}ClN$	C	70.14	70.13
								H	9.18	9.21
VII*g*	CH_3—$(CH_2)_2$—CH_2	H	A	113–115° (0.25)[c]	98	195–196°	$C_{15}H_{24}ClN$	C	71.00	71.57
								H	9.46	9.72
VII*h*	$(CH_3)_2$—CH—CH_2	H	A	89–90° (0.25)	80	189–190°	$C_{15}H_{24}ClN$	C	71.00	70.91
								H	9.46	9.53
VII*i*	CH_3—CH_2—CH(CH_3)	H	A	100–101° (0.45)	97	158–159°	$C_{15}H_{24}ClN$	C	71.00	70.86
								H	9.46	9.53
VII*j*	$CH_3(CH_2)_5$—CH_2	H	A	90–91° (0.22)	98	196–197°	$C_{18}H_{30}ClN$	C	73.09	73.11
								H	10.15	10.01
VII*k*		H	A	125–126° (0.35)	98	182–183°	$C_{16}H_{24}ClN$	C	72.31	72.53
								H	9.03	9.08
VII*l*	$(CH_3)_3$—C	H	B	101–103° (0.25)	60	199–200°	$C_{15}H_{24}ClN$	C	71.00	71.22
								H	9.46	9.25
VII*m*	CH_3—CH_2	CH_3—CH_2	B	108–110° (0.3)[d]	72	—	$C_{15}H_{23}N$	C	82.94	82.67
								H	10.59	10.49
VII*n*	CH_3—$(CH_2)_2$—CH_2	CH_3—$(CH_2)_2$—CH_2	B	144–146° (0.3)[e]	65	—	$C_{19}H_{31}N$	C	83.51	83.37
								H	11.35	11.32

[a] bp 120–122° (3 mm), mp 153–155° (13). [b] bp 125–127° (1.0 mm), mp 133–134° (13). [c] bp 140–142° (1.1 mm), mp 145–146° (13). [d] bp 108–110° (0.3 mm) (13). [e] bp 144–146° (0.3 mm) (13).

ly refluxed for 15 hr. Freshly prepared 1-chlorindan, 259.3 g (1.7 moles), was added dropwise to the gently refluxing, stirred mixture. After the last addition, the stirring and refluxing were continued for an additional 10 hr. Water, 500 ml, was added to the cold solution to dissolve the solid, the organic layer was separated, and the aqueous solution was extracted once with 200 ml of ether. The combined organic solutions were washed with 100 ml of hydrochloric acid solution (5%) followed by 100 ml of water. The solution was dried with fused sodium sulfate, filtered, and concentrated *in vacuo*, and the residue was distilled. The fraction distilling from about 110 to 165° at 0.1 mm was collected. The distillate was refractionated to give an average yield of 289.0 g (69.8%) of product, bp 132–142° at 0.42 mm, n_{25D} 1.5030 [lit. (14) bp 112° at 0.03 mm, n_{25D} 1.5025]; IR ($CHCl_3$): 5.78 (ester C=O) μm; NMR (CCl_4): δ 7.03 (s, 4, ArH), 4.05 (q, 2, OCH_2), 4.01 (q, 2, OCH_2), 3.25–3.79 (m, 2, two CH), 2.60–3.00 (m, 2, benzylic t at 2.78), 1.78–2.37 (m, 2, CH_2), 1.14 (t, 3, CH_3), and 1.11 (t, 3, CH_3).

Ethyl 1-Indanylacetate—Diethyl 1-indanylmalonate, 138.0 g (0.5 mole), was combined with 33.1 g (0.5 mole) of potassium hydroxide (85%) in 1.0 liter of ethanol (95%), and the solution was stirred at room temperature for 18 hr. The reaction mixture was concentrated under reduced pressure, and 100 ml of water was added to the residue. The solution was extracted with two 50-ml portions of ether and then acidified with hydrochloric acid (37%). The oily layer was separated off, the aqueous layer was extracted exhaustively with ether, and the combined organic fractions were dried over fused sodium sulfate. The dry, ethereal solution was concentrated under reduced pressure, and the residue was flash distilled. The fraction that distilled up to about 170° (5–10 mm) was collected as decarboxylation occurred. The distillate was fractionated to yield 65.0 g (64.0%) of product, bp 100–105° (0.55 mm). The analytical sample was collected at 100° (0.55 mm); n_{20D} 1.5158; IR (film): 5.77 (ester C=O) μm; NMR (CCl_4): δ 7.02 (s, 4, ArH), 4.03 (q, 2, OCH_2), 3.17–3.66 (m, 1, CH), 1.40–3.00 (m, 5, including benzylic t at 2.78), and 1.17 (t, 3, CH_3).

Anal.—Calc. for $C_{13}H_{16}O$: C, 76.44; H, 7.90. Found: C, 76.24; H, 7.80.

1-Indanylacetic Acid—To a gently refluxing, stirred solution of 46.3 g (0.7 mole) of potassium hydroxide (85%) in 300 ml of ethanol (75%), 82.8 g (0.3 mole) of diethyl 1-indanylmalonate was added at a rate to maintain refluxing and the refluxing and stirring were continued for an additional 3 hr. To the cold solution was added 200 ml of water, and the solution was concentrated *in vacuo* to a volume of about 300 ml. The solution was extracted once with 200 ml of ether and acidified with hydrochloric acid (37%) while being kept cold, and then the organic acid was removed by exhaustive extraction with ether. The dried, ethereal solution was concentrated under reduced pressure, a small amount of powdered glass was added to the residue, and the mixture was heated in a Wood's metal bath at 110–130° (internal) for 2 hr and then at 150–160° for 1 hr. The reaction mixture was fractionally distilled to give the title compound in average yields of 45.0 g (87.0%), bp 128–130° (0.45 mm) or 148–152° (3.0 mm). A small sample of the solid was recrystallized from *n*-heptane to give a white product, mp 60–61° [lit. (19) mp 60–61°]; IR (NaBr): 5.92 (acid C=O) μm; NMR (CCl_4): δ 12.29 (s, 1, COOH, H^+ eliminated by D_2O shake), 7.03 (s, 4, ArH), 3.17–3.68 (m, 1, CH), and 1.47–3.03 (m, 7, including benzylic t at 2.78).

2-(1-Indanyl)ethanol—To a stirred suspension of 13.7 g (0.36 mole) of powdered lithium aluminum hydride in 500 ml of anhydrous ether was added, at a rate to maintain vigorous reflux, 61.2 g (0.3 mole) of ethyl 1-indanylacetate in 200 ml of anhydrous ether. After the last addition the mixture was stirred at gentle refluxing for 6 hr and cooled. Then 14.0 ml of water, 14.0 ml of sodium hydroxide solution (15%), and 42 ml of water were added consecutively with caution and rapid stirring. Stirring was continued for 20 min, the solid was filtered off, and the precipitate was washed with ether. The combined ethereal solution was concentrated to a thick syrup to which was added 200 ml of ethanol (75%) and 10.0 g of potassium hydroxide (85%). After refluxing the solution for 2 hr, most of the alcohol was removed *in vacuo*, 75 ml of water was added, and the mixture was exhaustively extracted with ether. The combined extracts were washed twice with 50 ml of water, dried with fused sodium sulfate, and filtered, and the solution was concentrated under vacuum. The residue was rapidly distilled under 1–4 mm pressure to give 45–50 g of distillate which, when fractionated, gave an average yield of 40.0 g (82.0%) of product distilling at 107–108° (1.1 mm). The analytical sample was collected at 107° (1.1 mm); n_{20D} 1.5500; IR (film): 2.75–3.25 (broad) and 6.77 (primary alcohol OH) μm; NMR (CCl_4): δ 6.93 (s, 4, ArH), 4.20 (s, 1, OH), 3.57 (t, 2, CH_2), 2.88–3.27 (m, 1, CH), 2.68 (t, 2, benzylic), and 1.17–2.50 (m, 4, cyclic CH_2 and CH_2O).

Anal.—Calc. for $C_{11}H_{14}O$: C, 81.44; H, 8.70. Found: C, 81.79; H, 9.13.

2-(1-Indanyl)ethyl Bromide—To a stirred solution of 54.1 g (0.2 mole) of phosphorus tribromide in 200 ml of carbon tetrachloride at 0–5° was added dropwise 48.6 g (0.3 mole) of 2-(1-indanyl)ethanol; then the reaction mixture was removed from the ice bath. After standing for 24 hr the solution was poured onto 500 g of cracked ice, the organic layer separated, the aqueous layer was exhaustively extracted with carbon tetrachloride, and the combined extractions were washed with two 50-ml portions of sodium hydroxide (5%) followed by two 50-ml portions of water. The organic layer was dried with fused sodium sulfate, filtered, concentrated, and fractionated to yield 40.5 g (60.0%) of the bromide, bp 100–104° (0.3 mm); n_{20D} 1.5675; NMR (CCl_4): δ 6.93 (s, 4, ArH), 2.50–3.67 (m, 5, including benzylic t at 2.70 and CH_2 at 3.23), and 1.00–2.50 (m, 4, $-CH_2CH_2Br$).

Anal.—Calc. for $C_{11}H_{13}Br$: C, 58.68; H, 5.82. Found: C, 58.92; H, 6.21.

Acetamides—These compounds were synthesized from freshly prepared, crude or distilled, 1-indanylacetyl chloride and aqueous ammonia or an amine.

1-Indanylacetyl Chloride—To 52.8 g (0.3 mole) of 1-indanylacetic acid in 300 ml of benzene was added 54.0 g (0.45 mole) of thionyl chloride, and the solution was very gently refluxed for 5 hr. The benzene and excess thionyl chloride were removed under vacuum from a warm (35–40°) water bath, the residue was dissolved in 100 ml of dry benzene, and the solution was concentrated as before. This crude product may be used to prepare the amide or purified further by fractionation of the residue, bp 142–145° (10 mm) [lit. (15) bp 146° (11 mm)]; IR ($CHCl_3$): 5.50 (acetyl C=O) μm; NMR (CCl_4): δ 6.97 (s, 4, ArH), 3.10–3.66 (m, 1, CH), and 1.00–3.09 (m, 6, including benzylic t at 2.78).

1-Indanylacetamide—The crude 1-indanylacetyl chloride obtained above was added dropwise with stirring into 300 ml of ammonium hydroxide (28% NH_3). The solid was filtered off, transferred to a container, and then washed with 150 ml of potassium hydroxide (4%). The mixture was allowed to stand 2 hr, and the solid was filtered off and air dried. The average yields of crude product melting at 95–96° were 50.9 g (96.9%). A small sample was recrystallized several times from carbon tetrachloride to give a pure white product, mp 98–99° [lit. (15) mp 90°]; IR (KBr): 6.16 (amide C=O) μm; NMR (CF_3COOH): δ 7.13 (s, 4, ArH), 3.30–3.77 (m, 1, CH), and 1.50–3.16 (m, 6, including benzylic t at 2.80).

N-Methyl-1-indanylacetamide—To prepare the amide, anhydrous methylamine was bubbled into 600 ml of vigorously stirred anhydrous ether maintained at 10–15° while 35.4 g (0.18 mole) of freshly distilled 1-indanylacetyl chloride was added dropwise. The solid amide was filtered with suction and washed with water and then ether. The ethereal filtrate was removed by concentration *in vacuo*, and the solid residue was washed with water and combined with the main product to yield 31.3 g (92.0%) of crude product. After one recrystallization from water–methanol, the title compound was obtained, mp 72–73°; IR (NaBr): 5.87 (amide C=O) μm; NMR (CCl_4): δ 7.63 (broad s, 1, NH), 7.00 (s, 4, ArH), 3.24–3.60 (m, 1, CH), and 1.33–2.97 (m, 9, including d, CH_3 at 2.60).

Anal.—Calc. for $C_{12}H_{15}NO$: C, 76.19; H, 7.93. Found: C, 76.29; H, 8.00.

1-Indanylacetonitrile—To a suspension of 35.0 g of crude 1-indanylacetamide (mp 95–96°) in 300 ml of benzene was added 36.0 g (0.3 mole) of thionyl chloride, and the mixture was gently refluxed for 5 hr. Excess thionyl chloride and benzene were removed under vacuum, 150 ml of ether was added, and the ethereal solution was shaken with 75 ml of water followed by 150 ml of sodium hydroxide (3%). The organic layer was dried over fused sodium sulfate, filtered, and concentrated. The residue was fractionated to give 28.2–30.1 g or 90–96% yields (based on 0.2 mole of pure amide) of product, bp 96–98° (0.14 mm); n_{20D} 1.5411; IR (film): 4.43 (CN) μm; NMR (CCl_4): δ 6.98 (s, 4, ArH) and 1.17–3.67 (m, 7 including 1, CH at 3.15).

Anal.—Calc. for $C_{11}H_{11}N$: C, 84.07; H, 7.00. Found: C, 84.64; H, 7.32.

2-(1-Indanyl)ethylamine (VII*a*)—A mixture of 78.5 g (0.5 mole) of 1-indanylacetonitrile, 300 ml of liquid ammonia, 200 ml of absolute alcohol, a teaspoonful of Raney nickel, and hydrogen at an initial pressure of 1500 psi was heated to 85–90° in a Parr high pressure hydrogenator for 20 hr. After filtering off the catalyst, the solution was concentrated *in vacuo* and the residue was fractionated to give an average yield of 72.5 g (90.0%) of the title compound, bp 85–87° (0.5 mm).

This product was also prepared from 1-indanylacetamide in 87.0% yield by Method C described later. An analytical sample of the amine so prepared, taken at 79° (0.24 mm), gave n_{20D} 1.5472; IR (film): 3.10 (amino NH_2) μm; NMR (CCl_4): δ 7.01 (s, 4, ArH), 0.93 (s, 2, NH_2, $2H^+$ eliminated by D_2O shake), and 1.13–3.35 (complex m, 9).

Anal.—Calc. for $C_{11}H_{15}N$: C, 81.94; H, 9.38. Found: C, 81.58; H, 9.69.

The hydrochloride salt, prepared as described later, was recrystallized from ethyl acetate–absolute alcohol to give a pure white product, mp 215–216°.

Anal.—Calc. for $C_{11}H_{16}ClN$: C, 66.82; H, 8.18. Found: C, 66.87; H, 8.45.

Secondary and Tertiary Amines—These amines, listed in Table I, were prepared by one of three methods. Method A involved the formation of a Schiff base from an aldehyde or ketone with 2-(1-indanyl)ethylamine and the reduction of this base *in situ* with Raney nickel and hydrogen under pressure. Method B entailed the reaction of 2-(1-indanyl)ethyl bromide with a primary or secondary amine. Method C utilized the lithium aluminum hydride reduction of 1-indanylacetamide or an *N*-substituted derivative in dry tetrahydrofuran.

In those cases where the amines formed solid hydrochloride salts readily, these were prepared in the usual way from gaseous hydrogen chloride and the amine in anhydrous ether. All salts were recrystallized from an ethyl acetate–absolute alcohol mixture as a solvent.

Method A—A mixture of 16.1 g (0.1 mole) of 2-(1-indanyl)ethylamine, 0.15 mole of ketone or aldehyde, 150 ml of absolute alcohol, and a teaspoonful of Raney nickel was heated to 80–90° for 1 hr in a Parr high pressure hydrogenator. Hydrogen was added to 1500 psi, and the reaction mixture was rocked for 20 hr at 90–100°. After filtering off the catalyst, the filtrate was concentrated under vacuum and the residue was fractionated.

Method B—To 19.1 g (0.085 mole) of 2-(1-indanyl)ethyl bromide in 150 ml of absolute alcohol (solution cooled to 0.5° for low boiling amines) was added 0.3 mole of the required amine, and the solution was heated to 70–90° for 5 hr in a stirring autoclave. The cold reaction mixture was acidified with excess hydrochloric acid solution (10%), the alcohol was removed *in vacuo*, the mixture was extracted several times with ether, and the amine was liberated from its salt with excess solid sodium hydroxide. The base was obtained by exhaustive extraction with ether; the ethereal solutions were dried with fused sodium sulfate, filtered, and concentrated; and the residue was fractionated.

Method C—To a stirred suspension of 18.6 g (0.5 mole) of lithium aluminum hydride in 200 ml of gently refluxing, dry tetrahydrofuran was added 0.14 mole of amide in about 250 ml of dry tetrahydrofuran during 2 hr. After the last addition, refluxing and stirring were continued for 4 hr. To the cold mixture was added consecutively 18.6 ml of water, 18.6 ml of sodium hydroxide (15%), and 57 ml of water. The solid was filtered and washed with ether, the combined dry organic fraction was concentrated under vacuum, and the residue was fractionated.

IR and NMR Spectra—The structures of all amines were supported by IR and NMR spectra. The IR spectra (film) for all secondary amines gave a weak peak between 6.19 and 6.24 μm for the N—H bending vibration and a moderate peak between 3.00 and 3.03 μm for the C—N vibration. All tertiary amines gave a moderate peak between 8.36 and 8.46 μm and also at 9.73 μm for the C—N vibration.

The NMR spectra (CCl_4) for all amines gave absorption peaks within the values expressed (in δ) as follow: *(a)* protons (s, ArH), 7.00–7.09; *(b)* two protons (t, benzylic), 2.78–2.80; *(c)* one proton (m, CH), 2.93–3.50; *(d)* one proton for secondary amines (s, N—H), 0.46–0.55, except for VII*k* which occurred at 0.33; all such protons could be eliminated by D_2O shake; *(e)* all $-CH_3$ groups one or more carbons removed from the amino nitrogen gave singlets or doublets as required, 0.90–1.0; for VII*b* and VII*c*, 2.25 and 2.13, respectively; and *(f)* the $-CH_2-$ groups appeared as complex multiples at 1.00–3.50 (including benzylic and CH indicated above) which varied in position with the nature of the *N*-substitutions and which integrated for the total number of protons required.

Table II—Monoamine Oxidase Inhibition Studies and Mouse Head Twitch Response

Compound	Name	Monoamine Oxidase Inhibition	Total Number of Head Twitches
VII*a*	2-(1-Indanyl)ethylamine	325	7
VII*b*	*N*-Methyl-2-(indanyl)-ethylamine	350	5
VII*c*	*N,N*-Dimethyl-2-(indanyl)ethylamine	145	21
VII*d*	*N*-[2-(1-Indanyl)ethyl]-piperidine	61	5
VII*e*	*N*-Propyl-2-(1-indanyl)-ethylamine	181	16
VII*f*	*N*-Isopropyl-2-(1-indanyl)-ethylamine	48	5
VII*g*	*N*-Butyl-2-(1-indanyl)-ethylamine	164	29
VII*h*	*N*-Isobutyl-2-(1-indanyl)-ethylamine	0	2
VII*i*	*N*-*sec*-Butyl-2-(1-indanyl)-ethylamine	73	5
VII*j*	*N*-Heptyl-2-(1-indanyl)-ethylamine	40	32
VII*k*	*N*-Cyclopentyl-2-(1-indanyl)ethylamine	0	2
XII	1-Methyl-D-lysergic acid butanolamide dimaleate	—	0
XIII	Iproniazid phosphate	100	87
	Control	0	11

PHARMACOLOGY

All compounds except VII*l*, VII*m*, and VII*n* were examined following the *in vitro* procedure of Wurtman and Axelrod (16) for monoamine oxidase inhibition and the *in vivo* mouse head twitch method of Corne *et al.* (17) for both monoamine oxidase inhibition and serotonin antagonism.

Monoamine Oxidase Inhibition (*In Vitro*)[2]—This method consists of incubating a mixture of radioactive tryptamine, rat liver homogenate, and a monoamine oxidase inhibitor for a suitable length of time, extracting the labeled indoleacetic acid formed, and then determining the level of radioactivity in the liquid *via* liquid scintillation spectrometry.

A male Holtzman rat was sacrificed by cervical dislocation, and the liver was rapidly excised and placed in crushed ice moistened with isotonic potassium chloride. A coaxial homogenizer was used to homogenize 200 mg of liver in 10 ml of cold isotonic potassium chloride. A 1.0-ml aliquot of the homogenate was withdrawn and homogenized with 9.0 ml of cold isotonic potassium chloride so that the final concentration of 2 mg/ml was obtained. To a 15-ml centrifuge tube containing 0.5 ml of 0.1 *M* phosphate buffer (pH 7.9), 0.05 ml of radiolabeled tryptamine (0.01 μCi/10^{-8} mole), 1.0 ml of inhibitor solution, and 4.2 ml of distilled water was added 0.25 ml of the rat liver homogenate. A blank was prepared containing all reagents but with 1.0 ml of water substituted for the inhibitor solution and 0.25 ml of distilled water substituted for the homogenate. A standard was prepared containing the homogenate and all reagents but with 1.0 ml of distilled water substituted for the inhibitor. The tubes were incubated in air for 20 min and then 2.0 ml of 2 *N* hydrochloric acid was added fol-

[2] All reagents used were analytical grade. Tryptamine-2-^{14}C was obtained from New England Nuclear Corp. Iproniazid phosphate was provided through the courtesy of Hoffmann-La Roche. The liquid scintillator solution was composed of 0.05% 1,4-bis-2-(5-phenyloxazolyl)benzene and 0.4% 2,5-diphenyloxazole in toluene. All test compounds were examined in the form of the hydrochloride salts. Counting vials were low potassium glass crystallite (A-3305), T. C. Wheaton Co. Samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3003.

lowed by 6.0 ml of toluene. The tubes were shaken by hand to facilitate extraction and then centrifuged for 10 min. A 4.0-ml aliquot of the organic layer was withdrawn and transferred to a counting vial containing 15 ml of liquid scintillation solution. The samples were counted for either 10 min or 10,000 counts, whichever was obtained first. The results obtained at 1×10^{-5} *M* concentration of inhibitor are listed in Table II.

Mouse Head Twitch (*In Vivo*)—5-Hydroxytryptophan[3] (X) produces a characteristic head twitch when injected into mice. The head twitch resembles a strong pinna reflex involving the whole head of the mouse but, unlike the pinna reflex, occurs without tactile stimulation (17). The twitch is due to a central action of 5-hydroxytryptamine (XI) formed by decarboxylation of X. The head twitch response in mice provides a simple and valuable preliminary response to assess the potency of potentiators and antagonists of the central action of XI.

Male albino mice weighing 20–25 g were used. Doses, given on the basis of milligrams of free base per kilogram of body weight, were as follow: 5 mg of 1-methyl-D-lysergic acid butanolamide dimaleate[4] (XII), 50 mg of iproniazid phosphate (XIII), 200 mg of X, and 15 mg of all test compounds.

Five mice were weighed and each was placed in an individual metal box with a wire mesh top. The five mice were given subcutaneous injections of the test compound, spacing the injections 3 min apart. Thirty minutes after giving the test compound, each animal was given X intraperitoneally; 24 min later each mouse was observed over 2 min and the number of head twitches exhibited was counted and recorded. Table II lists the total number of twitches observed for a group of five mice treated with the test compound. Three groups of five mice each received only X, and the total number of head twitches for all of these was averaged.

RESULTS

Several test compounds gave evidence of monoamine oxidase inhibition *in vitro* at concentrations of 1×10^{-5} *M*. Compounds VII*a* and VII*b* exhibited the greatest activity, being about 3.5 times as active as XIII, the reference compound. Only VII*h* and VII*k* gave evidence of being inactive. The remaining test compounds exhibited varying degrees of activity between these two extremes.

Several test compounds gave evidence of monoamine oxidase inhibition *in vivo* at 15 mg/kg. Increases in the number of twitches of 21, 29, and 32 for VII*c*, VII*d*, and VII*j*, respectively, as compared to the control value of 11 reflect monoamine oxidase inhibition by the procedure employed. Thus, these compounds are two or three times as active as the reference compound, XII. A decrease in the number of twitches in relation to the control (X only with no pretreatment gave 11 twitches/2 min/five mice) is indicative of serotonin antagonism; only VII*h* and VII*k* exhibited marked decreases in the number of twitches per mouse observed in the 2-min period as evidenced by two twitches for each compound; these were the most active compounds. Compounds VII*b*, VII*f*, and VII*g* gave only about half as many twitches as the control or five twitches for each of these compounds. These are only about half as active as the latter compounds.

The compounds listed in Table II plus Compound VII*l* were examined by Sobiski (18) for general activity on the central nervous system of mice. A brief summary of this study is given here. Preliminary evaluation suggested that several compounds possessed central myorelaxant and ataractic properties of the type exhibited by the minor tranquilizers. Compounds VII*d*, VII*h*, VII*k*, and VII*l* were active in preventing hindleg extension following intravenously administered pentylenetetrazol; evaluation of these compounds against diphenylhydantoin in the maximal electroshock test showed that VII*h*, VII*k*, and VII*l* were about equiactive with diphenylhydantoin. Compounds VII*a*, VII*d*, and VII*k* exhibited the ability to check hydrochloric acid-induced writhing when compared to morphine sulfate and aspirin, suggesting that they possess analgesic activity. It was not possible to reverse ptosis with any of these indan derivatives, indicating that the marked monoamine oxidase inhibition seen with these compounds *in vitro* was not reflected in this *in vivo* test. Details of the pharmacological evaluation of these compounds are described in Ref. 18.

[3] Sigma Chemical Co.
[4] Sandoz Pharmaceuticals.

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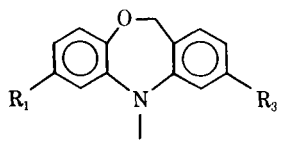
Effect of Substituted Dibenzoxazepines on Levels of Reduced Glutathione and Potassium Ions in Lenses of Rabbits *In Vitro* and of Rats *In Vivo*

KEITH K. WONG, GENG MEI WANG, JACQUES DREYFUSS[x], and ERIC C. SCHREIBER

Abstract □ Levels of reduced glutathione and potassium ion were determined in rabbit lenses after incubation *in vitro* in the presence of various compounds. After rabbit lenses had been incubated *in vitro* with 4-[3-(7-chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepin-5-yl)propyl]-1-piperazineethanol hydrochloride (I), the levels of reduced glutathione and potassium ion were lower than after incubation with the corresponding 3-chloro analog (II). Compound I was also more potent than II in producing the hemolysis of rabbit erythrocytes. The loss of reduced glutathione and potassium ion from the lens, as mediated by I and 5-[(2-dimethylamino)ethyl]-5,11-dihydrodibenz[*b,e*][1,4]oxazepine maleate (IV), was correlated with the tendency of these compounds to bind to erythrocyte ghosts. Levels of reduced glutathione were also examined in the lenses of rats that had been given chronic doses in the diet of either of two substituted dibenzoxazepines (I or its trifluoromethyl analog, III). After being dosed with either compound, the lowest levels of reduced glutathione were found in the lenses of those rats that had developed morphological changes of the greatest severity. ^{14}C-Labeled compounds, both tricyclic and nontricyclic, were given orally to rabbits, and all of these compounds, whether or not they were cataractogenic, were found to be distributed in various portions of the eye. No chemical interaction was found between the substituted dibenzoxazepines and reduced glutathione when studies were conducted *in vitro*.

Keyphrases □ Dibenzoxazepines, substituted—effect on reduced glutathione and potassium-ion levels in rabbit and rat lenses, relationship to cataractogenicity □ Glutathione, reduced—possible interaction with substituted dibenzoxazepines, significance to cataractogenicity □ Cataractogenicity—effect of substituted dibenzoxazepines on levels of reduced glutathione and potassium ions in lenses of rabbits *in vitro* and of rats *in vivo* □ Potassium ions—effect of substituted dibenzoxazepines, rabbit and rat lenses

The isolated lens has been cultured successfully *in vitro* for considerable lengths of time (1). Conditions of culture employing rabbit lenses were described previously (2), and cataracts were produced by incubating rabbit lenses for 22 hr at 37° in a culture medium containing tyrosine and tyrosinase (2). The loss of reduced glutathione (GSH) from the lens has been found to occur during the formation of most types of cataracts, including those induced by drugs (1). 4-[3-(7-Chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepin-5-yl)propyl]-1-piperazineethanol hydrochloride (I), a substituted dibenzoxazepine, was reported to be cataractogenic in rats (3). In attempts to understand the mechanism of some dibenzoxazepine-induced cataracts, the loss of reduced glutathione and potassium ion from rabbit lenses was studied *in vitro*, as was the loss from the lenses of rats that had been fed chronic doses of some dibenzoxazepines. The results suggest that the measurement of reduced glutathione and potassium-ion concentrations in the lens may be useful in the preliminary evaluation of the potential cataractogenicity of substituted dibenzoxazepines (I–VII).



Compound	R_1	R_2	R_3
I	Cl	$-(CH_2)_3N\langle\rangle N(CH_2)_2OH$	H
II	H		Cl
III	CF_3		H
IV	H	$-(CH_2)_2N(CH_3)_2$	H
V	Cl	$-CONH_2$	H
VI	Cl	$-(CH_2)_2N(CH_3)_2$	H
VII	H		Cl

EXPERIMENTAL

Incubation of Rabbit Lenses *In Vitro* and Analysis of Rat Lenses—Female New Zealand albino rabbits (1.9–2.8 kg) were sacrificed by air embolism. Both eyes were excised, and the lens of each, with the capsule intact, was excised by a posterior approach. When possible, the two lenses from one rabbit were used in comparing the effects of two drugs or of one drug relative to an untreated control lens. The lenses were incubated individually in 10 ml of medium (2), which was oxygenated by bubbling a mixture of 5% CO_2 and 95% O_2 into the medium for 5 min after the drug had been added. The drugs were dissolved in saline at a concentration of 2×10^{-3} *M* and were added to the incubation medium to give, unless noted otherwise, a final concentration of 2×10^{-4} *M*. The medium contained: $MgSO_4$, 0.46 m*M*; Na_2HPO_4, 0.24 m*M*; KH_2PO_4, 0.25 m*M*; KCl, 3.33 m*M*; $NaHCO_3$, 23.1 m*M*; $KHCO_3$, 0.84 m*M*; glucose, 5.0 m*M*; NaCl, 129 m*M*; $CaCl_2$, 1.25 m*M*; and, for each 10 ml of medium, 1000 units of penicillin and 1 mg of streptomycin sulfate.

The incubation was carried out at 37° for 18 hr under an atmosphere of 5% CO_2 and 95% O_2. After incubation, the lenses were examined grossly for any damage to the epithelium or capsule. Intact lenses were then rinsed with distilled water (adhering fluid was removed by blotting with paper) and homogenized in 4.0 ml of water, using a Teflon–glass homogenizer. The protein of the lens homogenate (1.0 ml) was precipitated with 2 ml of 10% trichloroacetic acid. An aliquot of the supernate was used for the determination of reduced glutathione by the method of Ellman (4). The remaining supernate was used for the determination of the sodium-ion and potassium-ion contents with a flame photometer[1]. The data are expressed as percent loss of reduced glutathione relative to the control lenses. The relative sodium-ion and potassium-ion concentrations were calculated by normalizing the cation concentrations of the control lens to 1.0.

Sprague–Dawley rats[2] of both sexes were fed drug added to a diet of laboratory chow[3] from the time of weaning. The animals were sacrificed after they had developed cataracts of varying degrees of severity. Both lenses from one rat were analyzed in each single determination of reduced glutathione; the samples were

[1] Model 143, Instrumentation Laboratory, Inc.
[2] Charles River CD.
[3] Rockland rat chow.

Table I—Levels of Reduced Glutathione in Rabbit Lenses as a Function of Age after Treatment with I *In Vitro*

Body Weight, kg	Reduced Glutathione Content, μg/Lens ± *SE*: Incubated without Drug	Incubated with I[a]	Decrease from Control, %
1.9	972 (1)[b]	538 ± 22 (3)[b]	44.6
2.0	886 ± 52 (3)	422 ± 52 (4)	52.4
2.1	865 ± 26 (3)	402 ± 37 (2)	53.5
2.2	995 ± 67 (7)	498 ± 28 (3)	49.9
2.3	973 ± 7 (2)	787 ± 29 (3)	19.1
2.5	1141 ± 38 (3)	848 ± 34 (2)	25.7
2.6	1245 ± 39 (2)	751 ± 5 (2)	39.7
2.8	1340 (1)	944 (1)	29.6

[a] Final concentration of 2×10^{-4} *M*. [b] The number of animals is indicated in parentheses.

processed in the same manner as were rabbit lenses that had been incubated *in vitro*.

Purity and Specific Activity of ^{14}C-Labeled Compounds—Each of two compounds, administered to rabbits to study their distribution in the eye, was labeled with ^{14}C in some portion of the side chain. The chemical names, radiochemical purities, and specific activities, respectively, were: Compound I, 4-[3-(7-chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepin-5-yl)propyl]-1-piperazine-^{14}C-ethanol hydrochloride, 96%, 21.6 μCi/mg; and Compound IV, 5-[(2-dimethylamino)ethyl-1-2-^{14}C$_2$]-5,11-dihydrodibenz-[*b,e*][1,4]oxazepine maleate, 99%, 1.77 μCi/mg.

Distribution of Drugs in Rabbit Eye—Female New Zealand albino rabbits (2.5–2.6 kg) were given by gavage a 10-mg/kg dose of either I-^{14}C or IV-^{14}C. Blood was removed from an ear vein, and the radioactivity was determined by digesting 0.2 ml of sample in 0.5 ml of 1 *N* NaOH at 80° overnight. The sample was then bleached with 30% H_2O_2, neutralized with 0.2 ml of 2-ethylhexanoic acid, and added to 15 ml of scintillator (5).

The eye was excised at the time of sacrifice, and aqueous humor was aspirated from it with a syringe fitted with a 25-gauge needle. The entire lens, a portion of the cornea or of the combined retina, choroid, and sclera, and some of the aqueous or vitreous humor were analyzed by solubilizing each sample in sufficient solubilizer[4] and counting it in 15 ml of toluene scintillator; the latter preparation contained 5 g 2,5-diphenyloxazole and 0.3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene/liter toluene.

Interaction *In Vitro* between Drugs and Reduced Glutathione—All drugs were added to phosphate-buffered saline, pH 7.4, at a concentration of 3×10^{-3} *M*. The incubation of compounds with reduced glutathione was carried out for 80–90 min at 3–6° to reduce the autoxidation of reduced glutathione. *N*-Ethylmaleimide and *p*-chloromercuriphenylsulfonic acid were chosen as reference compounds, since both react directly with —SH groups. At the end of the incubation, the reduced glutathione content of each sample was determined. The results obtained with samples containing drug were compared with those of a control sample that contained reduced glutathione but no drug.

Assay for Hemolysis—Fresh heparinized erythrocytes were obtained from female New Zealand albino rabbits (1.5–2.0 kg). The packed erythrocytes were washed three times with an equal volume of saline and were finally resuspended in an equal volume of saline. The incubation mixture contained 1.0 ml of a 50% suspension of erythrocytes and drug dissolved in 9 ml of saline buffered with 0.15 *M* phosphate at pH 7.4. All samples were incubated at 37° for 2 hr with intermittent shaking. At the end of the incubation, the erythrocytes were sedimented and the supernate was assayed spectrophotometrically for the amount of hemoglobin released (6). The results were expressed as percent hemolysis, using as 100% the absorbance obtained after having added erythrocytes to water at a final concentration of 5%.

Binding to Erythrocyte Ghosts—Rabbit erythrocyte ghosts were prepared by the method of Dodge *et al.* (7). Binding to erythrocyte ghosts was carried out by following spectrophotometrically the disappearance of drug from an incubation medium consisting of a 10% suspension of erythrocyte ghosts in 9.0 ml of 0.033 *M* sodium phosphate buffer, pH 6.0, and 1.0 ml of an aqueous solution of drug. Incubation was carried out, with shaking, at 25° for 30 min. After incubation, the erythrocyte ghosts were sedimented at 18,000×*g* for 30 min and the supernate was assayed spectrophotometrically at the wavelength of maximal absorption for each compound, *i.e.*, 212 nm for IV and 223 nm for I; the amount of each drug bound was expressed as nanomoles bound per milligram dry weight of ghost preparation.

[4] NCS Amersham/Searle.

Table II—Effect of Substituted Dibenzoxazepines on Loss of Reduced Glutathione from Rabbit Lenses *In Vitro*

Compound	Mean Reduced Glutathione Level, μg/Lens ± *SE*	Decrease of Reduced Glutathione[a], %
I	400 ± 43	58 ($p < 0.001$)[b]
IV	568 ± 47	41 ($p < 0.001$)
V	891 ± 68	7 (N.S.)[c]
Control (no drug)	963 ± 48	(0)

[a] *N* is equal to 5 for each compound. The rabbits weighed 1.9–2.2 kg. [b] Concentrations of reduced glutathione in the lenses after treatment with I were significantly lower ($p < 0.05$) than after treatment with IV. [c] N.S. = not significant.

RESULTS

The levels of reduced glutathione in rabbit lenses that had been incubated *in vitro* were found to be dependent on age (Table I). The lenses of female rabbits that weighed 1.9–2.2 kg (about 8 weeks old) contained lower levels of reduced glutathione than did those of female rabbits that weighed 2.5–2.8 kg (about 10 weeks old). In addition, the loss of reduced glutathione found after incubation *in vitro* with I was considerably greater in the lenses of younger rabbits than in those of older rabbits (Table I); the percent decrease from the control value changed abruptly in female rabbits that had attained the weight of 2.3 kg (about 9 weeks old).

Several dibenzoxazepines (I, IV, and V) were examined for their ability to affect the levels of reduced glutathione in rabbit lenses incubated *in vitro* (Table II). Levels of reduced glutathione in lenses that had been incubated with I or IV were significantly lower than those of the control lenses incubated without any drug. However, the loss of reduced glutathione in lenses incubated with I was greater than those incubated with IV. Incubation of lenses with V did not result in a loss of reduced glutathione.

To determine whether membranes of the lens had been affected by incubation with the dibenzoxazepines, the loss of potassium ion was determined in rabbit lenses incubated with the same compounds that had been employed in the experiment of Table II. The results (Table III) indicate that I, the compound that had produced the greater loss of reduced glutathione, also produced the greater loss of potassium ion. Levels of sodium ion were simultaneously determined in the same samples. The corresponding data for sodium ion (not shown) demonstrated a consistent elevation of sodium ion inside the lens in opposition to the outflow of potassium ion.

The binding of I and IV to rabbit erythrocyte ghosts, a well-characterized biological membrane, was also studied (Table IV). The results show that I, which produced a greater loss of reduced glutathione from rabbit lenses *in vitro* than did IV, was also bound more strongly to erythrocyte ghosts than was IV.

Two halogenated dibenzoxazepines were chosen for a more detailed study of the effect of tricyclic agents on the loss of reduced

Table III—Relative Concentrations of Potassium Ion in Rabbit Lenses after Incubation with Substituted Dibenzoxazepines *In Vitro*

Compound[a]	Experiment 1	Experiment 2	Mean
I	0.54	0.46	0.50
IV	0.73	0.52	0.63
V	0.94	0.89	0.92
Control (no drug)	(1.0)	(1.0)	(1.0)

[a] Drugs were present at 2×10^{-4} *M*. The rabbits weighed 2.2–2.8 kg.

Table IV—Binding of I and IV to Rabbit Erythrocyte Ghosts

Compound	Experiment	Nanomoles Bound per Milligram Dry Weight at 1.5×10^{-4} M	3.0×10^{-4} M	6.0×10^{-4} M
I	1	81.7	177	281
	2	81.7	190	327
IV	1	13.1	45.8	101
	2	0.0	35.9	88.2

glutathione and potassium ion from rabbit lenses *in vitro*. The two compounds, I and II, differ from each other in that the former contains a chloro substituent in the 7-position whereas the latter has it in the 3-position. Rabbit lenses were incubated *in vitro* with each compound at several concentrations (Fig. 1). The results show that both I and II stimulated the loss of reduced glutathione and potassium ion from the lenses; however, for both parameters, I, the 7-chloro-substituted compound, produced the greater effect. It is noteworthy that: *(a)* the curves for both reduced glutathione and potassium ion are sigmoidal; *(b)* the percent decrease of reduced glutathione *and* potassium ion is greater, at a given concentration, for I than it is for II; and *(c)* the loss of both reduced glutathione and potassium ion occurred initially in the presence of approximately the same concentrations of the two tricyclic compounds tested, I or II.

As an indication of the surface-active properties of some substituted dibenzoxazepines, the test compounds were incubated with rabbit erythrocytes (Fig. 2). Hemolytic activity was particularly notable for the piperazine-containing compounds, I and II, with the former being more potent than the latter. The dose-response curve for I is clearly sigmoidal. Studies with 7-chloro-5-[2-(dimethylamino)ethyl]-5,11-dihydrodibenz[*b,e*][1,4]oxazepine hydrochloride (VI) and the corresponding 3-chloro analog (VII) indicate that the 7-chloro-substituted compound was more hemolytic than the 3-chloro-substituted compound. However, here the difference was not as great as that observed in studies with the piperazine-containing compounds, because VI and VII are much less hemolytic than I and II.

The loss of reduced glutathione from rabbit lenses incubated *in vitro* with I may be correlated with a similar effect on the lenses of rats fed chronic doses of I or its trifluoromethyl analog (III) (Table V). In general, the amount of reduced glutathione lost from the lenses of these rats was proportional to the extent of the morphological change toward opacity.

Compound I is cataractogenic in rats (3) whereas IV is not[5]. To determine whether a compound that did not induce the formation of cataracts had, nevertheless, been distributed to various parts of the eye, the radioactive dibenzoxazepines (I and IV)

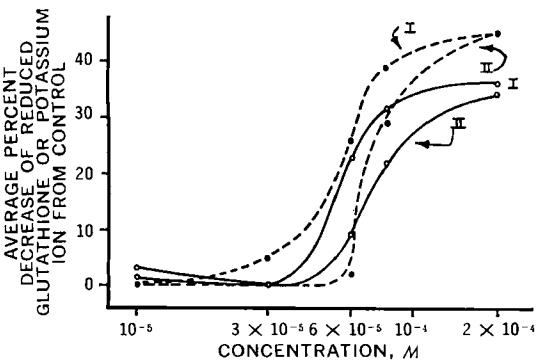


Figure 1—*Levels of reduced glutathione* (—) *and potassium ion* (- - -) *in rabbit lenses that had been incubated* in vitro *with various concentrations of a substituted dibenzoxazepine (I or II). Each point represents the average of values obtained from the individual incubation of three lenses.*

[5] Unpublished data, Toxicology Department, Squibb Institute.

Table V—Effects of I and III on Loss of Reduced Glutathione from Rat Lenses

Rat Number	Reduced Glutathione, μg/Lens	Decrease of Reduced Glutathione, %	Morphological Change
		I[a]	
10 Control rats (no drug)	79.0 ± 3.9	(0)	—
2	78.7	0	Faint anterior cortical striations
132	53.4	32	
54	39.9	50	Anterior and posterior cortical striations and/or pinpoint cataract
57	59.9	24	
93	54.4	31	
48	70.4	11	
83	37.1	53	$^1/_2$–$^3/_4$ opacity
120	15.6	80	
129	0.0	100	Entire lens opaque
146	20.0	75	
41	8.0	89	
		III[a]	
Five control rats (no drug)	68.6 ± 8.7	(0)	—
362	61.8	10	None
374	53.2	22	
358R[b]	28.9	58	$^1/_2$–$^3/_4$ opacity
348	20.9	70	Entire lens opaque

[a] Rats received 65 mg/kg/day in the diet. [b] Animal did not receive the drug for 18 weeks before it was sacrificed and its lenses were analyzed.

(Table VI) were administered to rabbits by gavage. Each compound appeared to be well absorbed, as shown by the levels of radioactivity in the blood. Furthermore, each compound and/or its metabolites were present in all portions of the eye. Similar results (not shown) were obtained in studies with two other nontricyclic compounds, 2-ethoxy-*N*-[2-(methylphenethylamino)-ethyl]-2,2-diphenylacetamide hydrochloride and 5-(2-dimethylaminoethyl)-1,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one; like IV, these latter compounds do not produce cataracts in rats[5]. Thus, it appears that the distribution *per se* of I or its metabolites in various portions of the eye is not the only factor responsible for producing a cataract.

One way in which reduced glutathione could have been lost from the lens might be by means of the sodium-ion- and potassium-ion-dependent adenosine triphosphatase cation pump. It is known that gradients of sodium and potassium ions are maintained between the lens and its surroundings by the sodium-potassium-ion-dependent adenosine triphosphatase (8). Moreover,

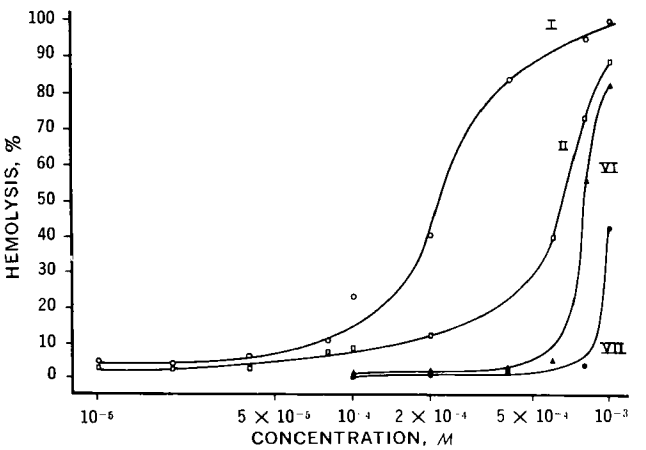


Figure 2—*Hemolytic activity of 7-chloro- and 3-chloro-substituted dibenzoxazepines as a function of their concentrations.*

Table VI—Concentrations of I and IV, Their Metabolites, or Both in Blood of Rabbits and Their Distribution in Various Parts of the Eye[a]

Concentration in Blood, hr	Compound, μg Equivalents/ml I	IV
1st	1.64	2.18
2nd	2.12	8.73
4th	2.24	1.57
Concentration in Tissue	**Compound, μg Equivalents/g I**	**IV**
Vitreous humor	0.124	0.101
Aqueous humor	0.056	0.164
Lens	0.079	0.089
Cornea	0.241	0.450
Combined retina, choroid, and sclera	0.739	0.853

[a] Each female rabbit (2.5–2.6 kg) received 10 mg/kg of drug in aqueous solution by gavage. The rabbits were sacrificed 4 hr after dosing.

rabbit lenses cultured *in vitro* are able to transport potassium ion (9). The present studies *in vitro* with rabbit lenses showed that ouabain, a specific inhibitor of the cation pump (10), did not produce any loss of reduced glutathione whereas it did produce an outflow of potassium ion (data not shown). Other studies have shown that reduced glutathione is lost from the lens by a prior conversion to oxidized glutathione, which is transported unidirectionally to the outside of the lens[6]. Thus, on this basis it appears that the cation pump is not responsible for producing the loss of reduced glutathione from the lens.

Another way in which reduced glutathione could have been lost from rabbit lenses *in vitro* might be by a direct chemical interaction between reduced glutathione and the substituted dibenzoxazepines. Accordingly, I, IV, or V was mixed with reduced glutathione in the molar proportions of 1:1 or 2:1. None of the incubations containing either a dibenzoxazepine and reduced glutathione or reduced glutathione alone showed the disappearance of any reduced glutathione. By contrast, in control experiments, none of the sulfhydryl-reactive material was recovered after reduced glutathione was incubated with *N*-ethylmaleimide or *p*-chloromercuribenzenesulfonic acid.

DISCUSSION

A number of investigations have shown that alterations in morphology or permeability of the lens epithelium can affect reduced glutathione or cation levels in the lens. In X-ray-induced cataracts, a progressive loss of reduced glutathione has been observed (11). X-irradiation is also known to produce morphological lesions of the lens epithelium (12) and an increase in permeability to cations in rabbit lenses either *in vitro* or *in vivo* (13). Kinsey and Reddy (14) reported that the lens epithelium actively transports potassium ion into the lens. More recently, Reddy *et al.* (15) demonstrated that the epithelium of cultured rabbit lenses, but apparently not the capsule, serves as the barrier to the efflux of reduced glutathione from the lens. In addition, a process for the degradation and synthesis of reduced glutathione in the lens was also indicated.

Studies with rabbit erythrocytes have shown that some dibenzoxazepines have surface-active properties. A similar phenomenon appears to be occurring in the rabbit lens *in vitro* since *(a) both* reduced glutathione and potassium ion are lost from the lens, and *(b)* those dibenzoxazepines that produce the greatest loss of reduced glutathione also produce the greatest loss of potassium ion. The sigmoidal dose-response curve for the loss of reduced glutathione and potassium ion from rabbit lenses and for the hemolysis of rat erythrocytes *in vitro* indicates that a threshold concentration of a compound is necessary before an effect on the lens epithelium or erythrocyte membrane becomes manifest. The failure to find any evidence for a chemical interaction of reduced glutathione and the substituted dibenzoxazepines and the apparent noninvolvement of the sodium–potassium-ion-dependent adenosine triphosphatase in maintaining endogenous levels of reduced glutathione are consistent with the suggested role of the lens epithelium. Thus, it is concluded that the surface-active properties of some substituted dibenzoxazepines are probably responsible for the loss of reduced glutathione and potassium ion from rabbit lenses *in vitro* and of reduced glutathione from rat lenses *in vivo*.

In view of Harding's (16) observation on the variability of endogenous levels of lens reduced glutathione in humans, rats, rabbits, and cattle as a function of age, the observation that rabbit lenses differ in their response to I as a function of age does not seem so surprising. Since these studies employed only the lenses of female rabbits, it is not known whether the lenses of male rabbits respond in a similar manner.

The change in the reduced glutathione levels of rat lenses produced by substituted dibenzoxazepines *in vivo* apparently does not precede a morphological change. Nevertheless, the test described, employing rabbit lenses *in vitro*, may be useful in screening compounds for their potential cataractogenicity. An additional parameter such as the water content of the lens, which was not measured in these studies, also could be included as a part of the assessment. The fact that a compound produces a loss of reduced glutathione and potassium ion from rabbit lenses *in vitro* does not imply that it must give rise to cataracts *in vivo*. However, a positive result in a test *in vitro* can alert the veterinarian and clinician to the need for routine examination of the eye for lenticular changes.

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[x] To whom inquiries should be directed.

Condensing Effect of Cholesterol: A Physical Model

JOEL L. ZATZ

Abstract □ A physical model is proposed to account for the "condensing effect" of cholesterol in mixed monolayers. If the other component of the monolayer has its polar groups in contact, a cholesterol molecule may interact sufficiently with the hydrocarbon tails to orient them vertically, thus creating free space for itself. In cholesterol-rich films, the polar groups of the phospholipid do not contribute to the area occupied. Both effects lead to a reduction in the observed mean molecular area.

Keyphrases □ Cholesterol—condensing effect in mixed monolayers, physical model, equations, π–A plots □ Membranes—physical model proposed for condensing effect of cholesterol in mixed monolayers, equations, π–A plots □ Films, monolayer—physical model proposed for condensing effect of cholesterol □ Monomolecular films—physical model proposed for condensing effect of cholesterol

Monomolecular films provide a useful model for studying the properties of biological membranes. Of particular interest are mixed monolayers containing cholesterol and a phospholipid (1–8). In most cases, the behavior of these mixed systems has been analyzed by plotting the mean area per molecule as a function of monolayer composition at constant surface pressure. If the molecular area occupied by each component in the mixed film is the same as in a monolayer of the pure material, the mean area plot is linear. This type of behavior is often observed in mixed monolayers containing cholesterol, but, more frequently, the mean area plot exhibits negative deviations from linearity. The apparent area occupied by the phospholipid in the mixed monolayer is less than that in a pure monolayer of the same material, so a "condensing effect" has been ascribed to cholesterol.

There is general agreement that cholesterol will cause condensation of another lipid only if that substance exhibits an expanded phase in its π–A (area) isotherm. But some practical problems cloud interpretation of the published work. Working with ostensibly the same "pure" materials, investigators have reported π–A isotherms and mean molecular area plots that disagree with other published reports. Furthermore, the experimental error involved in determination of the mean molecular area may be of the order of ± 2Å^2 (5, 8). Perhaps some "breaks" in published mean molecular area plots are due to impurities and experimental error rather than specific geometrical arrangements at particular mole ratios.

Some workers have attributed the condensation in mixed films largely to van der Waals' interactions between the hydrocarbon chains of the components (2, 6). On the other hand, Shah and Schulman (4) proposed that certain lipids form cavities due to steric effects (unsaturated fatty chains) or thermal motion (saturated fatty chains) which are capable of including cholesterol molecules. Neither theory is quantitative or capable of predicting what type of behavior will be encountered in untested systems.

In this paper, a quantitative model (the heads-or-tails model) is proposed to explain the effect of cholesterol in mixed monolayers.

THEORY

From its force–area curve, the cholesterol molecule at an air–water interface may be visualized as a rigid structure, oriented so that its single polar group is in contact with the subphase. At nearly all values of surface pressure, these molecules are close packed; the nonpolar portions are in contact and determine the area occupied by each molecule. Long-chain lipids (in a monolayer of the pure material) may be in "tail contact," in which case the molecules are close packed and vertically oriented. In this state, the area per molecule corresponds very nearly to that of the hydrocarbon portion of the molecule. This description applies to substances that form condensed monolayers (9). Some materials that exhibit π–A curves characterized as "expanded" may also be in tail contact at higher values of surface pressure, *e.g.*, oleic acid. Although the π–A curve of oleic acid is of the expanded type, it is generally agreed that the area per molecule at high surface pressures is that of the hydrocarbon portion of the molecule, since further reduction in molecular area is prevented by the "kink" in the hydrocarbon chain due to the *cis*-double bond (10).

It is also possible, in the case of a coherent film, that the hydrocarbon tails are not closely packed. The molecules may be in "head contact." The effective area occupied by each molecule is then determined by the size of the polar group and associated water molecules (11).

Some substances, as shown by their π–A curves, are in a state of tail contact at nearly all values of surface pressure, *e.g.*, cholesterol and dipalmitoyl phosphatidyl ethanolamine at 22° (2). Other lipids such as L-α-dipalmitoyl lecithin at 25° are in tail contact at high values of π but in head contact at lower surface pressures where the film is liquid (12). When the hydrated polar heads fix the surface area, the hydrocarbon tails are free to assume a random configuration (13). If a molecule of cholesterol is added, there may be sufficient intermolecular interaction (due to van der Waals' forces) to cause the hydrocarbon tails to orient vertically, thereby allowing the cholesterol molecule to fit into some free space in the surface.

In the following discussion, it is assumed that: *(a)* the two components of the mixed monolayer are miscible at the surface, *(b)* there are no specific polar interactions between the two components, and *(c)* hydrocarbon chains are vertically oriented when the two components are in contact.

The mean molecular area, A_m, in a mixed monolayer at constant surface pressure is given by:

$$A_m = \sum A_i X_i \qquad \text{(Eq. 1)}$$

where A_i is the effective area per molecule of the ith component, and X_i is the mole fraction of the ith component in the mixture. Furthermore, let A_i^0 represent the area per molecule in a monolayer containing only the ith component under the same surface pressure as that of the mixed monolayer. For a two-component system, one may write:

$$A_m = A_1 X_1 + A_2 X_2 \qquad \text{(Eq. 2)}$$

in which the subscripts identify the components. Let 1 represent cholesterol and 2 a phospholipid. The dependence of the mean molecular area on monolayer composition will be a function of the properties of the pure phospholipid monolayer, as described in the following four cases.

Case 1—The phospholipid molecules are in tail contact.

At the surface pressure studied, the molecules in a monolayer of pure phospholipid are in tail contact. In a mixed monolayer where the mole fraction of cholesterol equals or exceeds 0.5, each phospholipid molecule occupies the same area as it did in the single-component monolayer (Fig. 1*a*). This is also true when the mole fraction of cholesterol is less than 0.5 (Fig. 1*b*). Therefore, regardless of the composition of the mixed film, $A_1 = A_1^0$ and $A_2 = A_2^0$. Equation 2 becomes:

$$A_m = A_1^0 X_1 + A_2^0 X_2 \qquad \text{(Eq. 3)}$$

In other words, the mean molecular area plot will be linear and no "condensation" will be evident (Fig. 2, curve 1).

Case 2—Phospholipid molecules are in head contact, but there is insufficient space for a cholesterol molecule to fit between the chains.

In the monolayer of pure phospholipid, the molecules are in head contact at the surface pressure of interest. If cholesterol is added, the two species are put into chain contact with each other (Fig. 1*c*). Since the area occupied by the hydrocarbon portion of the phospholipid is less than that occupied by the polar groups, there is an apparent decrease in the total area needed to accommodate the cholesterol. Each molecule of the phospholipid in contact with cholesterol occupies an effective area equal to the area of its hydrocarbon tail, A_{HC}. In cholesterol-rich mixtures, the molecules are always in chain contact as in Fig. 1*a*. The polar groups of the phospholipid do not contribute to the area, so that the effective area of each phospholipid molecule is again A_{HC}. Regardless of composition, $A_1 = A_1^0$. Therefore, Eq. 2 becomes for $X_1 \geq 0.5$:

$$A_m = A_1^0 X_1 + A_{HC} X_2 \qquad \text{(Eq. 4)}$$

and for $X_1 \leq 0.5$:

$$A_m = A_1^0 X_1 + A_{HC} X_1 + A_2^0(X_2 - X_1) = (A_1^0 + A_{HC})X_1 + A_2^0(X_2 - X_1) \qquad \text{(Eq. 5)}$$

The mean molecular area plot for this case is illustrated by Fig. 2, curve 2.

Case 3—The phospholipid molecules are in head contact, and there is just enough space for a cholesterol molecule to fit between the chains.

Now, added cholesterol molecules fit between the chains of the molecules of the other component without requiring additional area (Fig. 1*d*). Effectively, then, the cholesterol molecules appear to require no space as long as the mole fraction of cholesterol does not exceed 0.5. In cholesterol-rich films, the arrangement is once again as shown by Fig. 1*a*. Therefore, for $X_1 \geq 0.5$, Eq. 4 holds. For $X_1 \leq 0.5$:

$$A_m = A_2^0 X_2 \qquad \text{(Eq. 6)}$$

The mean molecular area plot appropriate for this case is illustrated by Fig. 2, curve 3.

Case 4—The phospholipid molecules are in head contact, and there is more than enough space for one cholesterol molecule to fit between the chains but not enough space for two.

As long as the mole fraction of cholesterol does not exceed 0.5, the cholesterol molecules appear to occupy no additional space (Fig. 1*e*). Addition of cholesterol above a mole fraction of 0.5 puts those molecules in chain contact, and the monolayer contains a mixture of molecules in chain contact and head contact (Fig. 1*f*) until the mole fraction of cholesterol reaches 0.667, at which point all of the molecules are in chain contact, as in Fig. 1*a*.

For $X_1 \geq 0.667$, Eq. 4 applies. For $X_1 \leq 0.5$, Eq. 6 applies. For $0.667 \geq X_1 \geq 0.5$:

$$A_m = 2A_1^0(X_1 - X_2) + A_{HC}(X_1 - X_2) + A_2^0(2X_2 - X_1) = (2A_1^0 + A_{HC})(X_1 - X_2) + A_2^0(2X_2 - X_1) \qquad \text{(Eq. 7)}$$

The mean molecular area plot appropriate to this case is shown in Fig. 2, curve 4.

Cases 1-4 cover the situations of practical interest encountered in mixed monolayers containing two components. All theoretical curves exhibit a "break" at a mole fraction of 0.5. One consequence of the model is that, in cholesterol-rich mixed films, the mean molecular area plots for a given system at various surface pressures ought to be practically superimposable. This effect (apparent in Fig. 2) is due to the incompressibility of the cholesterol monolayer and the nearly constant area of the vertical hydrocarbon tail of the phospholipid.

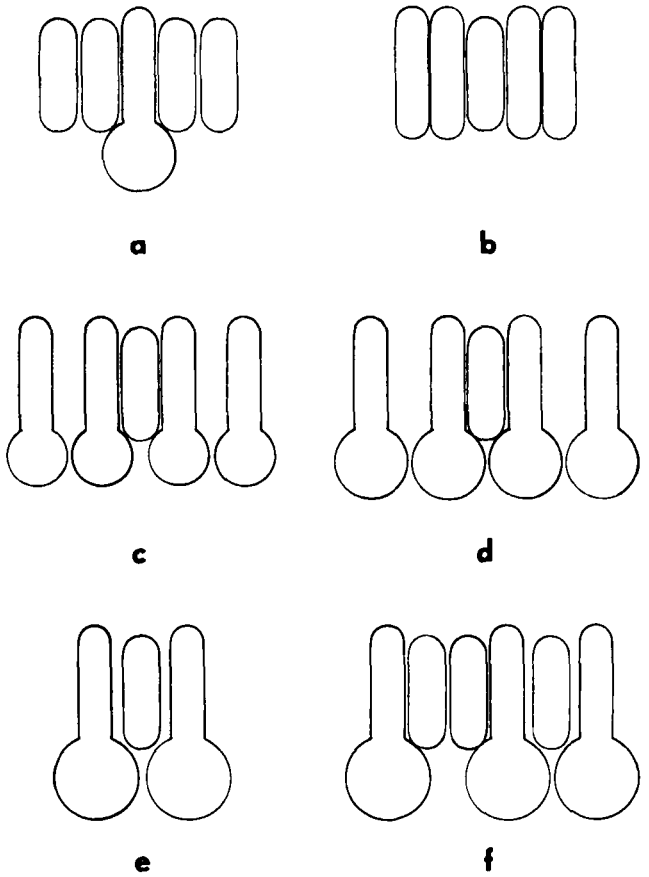

Figure 1—*Representation of molecules in mixed monolayers. The short rod is cholesterol. The other molecule is phospholipid, whose exact shape depends on its state in the surface. (See text for details.)*

DISCUSSION

In applying the heads-or-tails model, the values for X_1, X_2, A_1^0, and A_2^0 are provided by experiment. The cross-sectional area of the hydrocarbon tail of the phospholipid, A_{HC}, may be estimated from molecular models and appropriate surface balance experiments. The value of A_2^0 of a lipid at a particular surface pressure may vary a great deal with changes in environmental conditions, *e.g.*, temperature. Less variation is anticipated in A_{HC}.

If, at a particular surface pressure A_2^0 equals A_{HC}, Case 1 of the theoretical section applies. If the difference between A_2^0 and A_{HC} is significant but less than the value of A_1^0, Case 2 applies. The equations for Case 3 are used when the difference between A_2^0 and A_{HC} equals A_1^0. Case 4 applies when the difference between A_2^0 and A_{HC} exceeds A_1^0.

If the phospholipid is in a condensed state, its molecules will be in tail contact in the pure monolayer and in the mixed films. The mean molecular area plot should be linear (Case 1 of the theoretical section). In accord with the model, mixed films of cholesterol and dipalmitoyl phosphatidyl ethanolamine, distearoyl phosphatidyl ethanolamine, or distearoyl lecithin yield linear mean area curves (2, 3). Dipalmitoyl lecithin, which is in tail contact only at high surface pressures, exhibits a linear plot only at high pressures (4).

At low surface pressure, dipalmitoyl lecithin molecules are in head contact. By taking the area per hydrocarbon chain to be 20

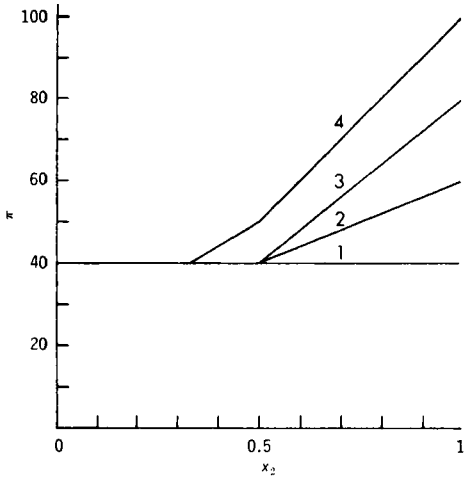

Figure 2—*Mean molecular area plots for hypothetical mixed films. A molecular area of 40 Å²/molecule is assumed for cholesterol in all cases. The hydrocarbon tails of the phospholipid have a cross-sectional area of 40 Å². Numbers correspond to the cases discussed in the text.*

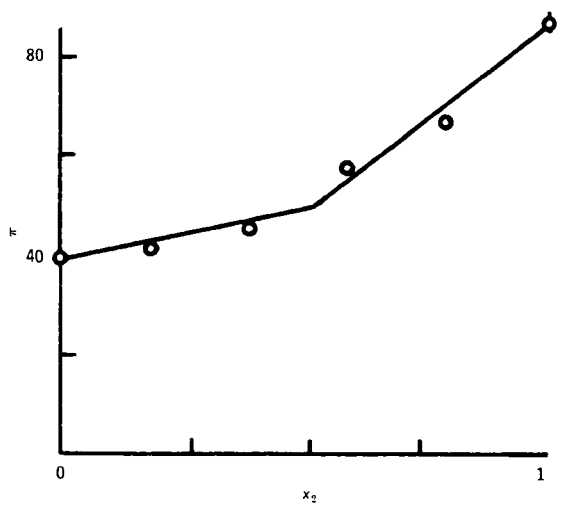

Figure 4—*Mean molecular area plot for cholesterol and* L-*stearoyl-linoleoyl lecithin at 10 dynes/cm. Key:* ○, *experimental data (Ref. 7); and* ——, *theoretical curve.*

Å² (9), A_{HC} is 40 Å², a value that agrees with the close-packed area per molecule obtained from the π–A curve of this phospholipid at 25° (4, 12). At this temperature, under a surface pressure of 5 dynes/cm, mixed monolayers of dipalmitoyl lecithin and cholesterol provide an example of Case 2 behavior. In Fig. 3, the theoretical curve is plotted and compared with the experimental results of Shah and Schulman (4). The agreement is quite good.

Many studies of mixed monolayers have included unsaturated phospholipids. Tinoco and McIntosh (7) published mean molecular area plots for several mixed films, including the combination of cholesterol with L-stearoyl-linoleoyl lecithin at 10 dynes/cm. By assuming an area of 20 Å² for the stearoyl moiety and 40 Å² for the unsaturated chain (14), a value for A_{HC} of 60 Å²/molecule is determined, close to the area per molecule occupied by this lipid under high surface pressure (7). As shown in Fig. 4, there is good agreement between theory and experiment. This is another Case 2 situation.

The mean molecular area plot for mixtures of cholesterol and dioleoyl lecithin at 12 dynes/cm was reported (2). At this surface pressure, A_2^0 exceeds A_{HC} [obtained from the π–A curve for this lecithin (2)] by only about 10 Å². The relatively small deviation observed in the mean area plot is, therefore, in accord with the heads-or-tails model but not with the cavity model of Shah and Schulman (4).

Although the equations presented were derived with the intent of describing the π–A characteristics of mixed monolayers of cholesterol and phospholipids, they may be applied to other mixed systems. The derived relationships should hold if one surface-active material forms, like cholesterol, a condensed monolayer in which the molecules are in tail contact and if the other has a long hydrocarbon chain and is in head contact on the surface. These conditions appear to be satisfied in mixed monolayers of cetyl alcohol and sodium cetyl sulfate at room temperature (15). At this temperature, cetyl alcohol forms condensed monolayers with an average area per molecule of about 20 Å². This value is approximately the cross-sectional area of a hydrocarbon chain (9) and suggests that the molecules are in tail contact. Therefore, cetyl alcohol corresponds to Component 1 of the theoretical section. Sodium cetyl sulfate, although somewhat soluble in water, was prevented from dissolving by the inclusion of 5% sodium chloride in the subphase (15). Sodium cetyl sulfate has a long, saturated hydrocarbon chain and forms expanded monolayers. It is likely that the molecules are in head contact, so sodium cetyl sulfate corresponds to Component 2 of the theoretical section. A mean molecular area plot at 10 dynes/cm permits a test of the equations describing Case 4 behavior. At this surface pressure, A_1^0 is 20 Å²/molecule, A_2^0 is 54 Å²/molecule, and A_{HC} is taken to be 20 Å². The experimental data (15) are plotted in Fig. 5. The solid line in Fig. 5 was calculated from Eqs. 4, 6, and 7 and is in reasonably good agreement with the experimental points.

The heads-or-tails model is based on the assumption that the hydrocarbon chains of the phospholipid are vertically oriented in mixed films with cholesterol. At very high temperatures or in the case of relatively short hydrocarbon chains, the kinetic energy of the phospholipid tails may be sufficient to preclude vertical orientation. In such cases, the heads-or-tails model is not expected to describe the system. However, the phospholipids found in biological membranes have sufficiently long hydrocarbon chains to satisfy the assumptions made in the model (16). Hopefully, appli-

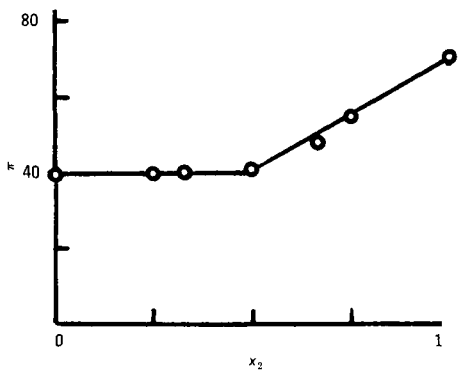

Figure 3—*Mean molecular area plot for cholesterol and dipalmitoyl lecithin at 5 dynes/cm. Key:* ○, *experimental data (Ref. 4); and* ——, *theoretical curve.*

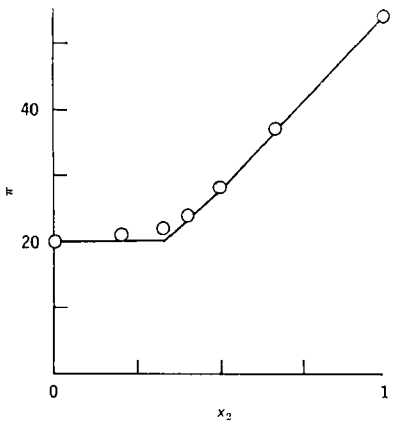

Figure 5—*Mean molecular area plot for cetyl alcohol and sodium cetyl sulfate at 10 dynes/cm. Key:* ○, *experimental data (Ref. 15); and* ——, *theoretical curve.*

cation of this framework will help remove some of the mystery surrounding the role of cholesterol in biological membranes.

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Bioavailability Determination of Two Crystal Forms of Sulfameter in Humans from Urinary Excretion Data

NAWAL KHALAFALLAH, SAID A. KHALIL[x], and MAMDOUH A. MOUSTAFA

Abstract □ Urinary excretion data were used to determine the bioavailability of crystal Forms II and III of sulfameter in humans. Agreement was observed between the ratio of absorption parameters of the two forms determined in the present study and those previously obtained from blood level data. Although the urine data revealed a significant difference in the rate of absorption of the two forms, no significant difference was observed in the extent of absorption of both forms as indicated by the 72-hr urinary excretion data. Urinary excretion rates during the absorption phase, without further mathematical treatment, were statistically shown to be adequate means for comparing the bioavailability of sulfameter crystal forms. The use of urinary excretion data of sulfameter as an alternative to the use of blood level data is discussed.

Keyphrases □ Sulfameter—bioavailability of two crystal forms, human urinary excretion data □ Sulfonamides—bioavailability of two crystal forms of sulfameter, human urinary excretion data □ Bioavailability—two crystal forms of sulfameter, human urinary excretion data □ Polymorphism—bioavailability of two crystal forms of sulfameter, human urinary excretion data

The polymorphism of sulfameter (1), as well as the GI absorption of its crystal Forms II and III (2), was recently reported. The *in vitro* dissolution behavior of the two crystal forms was shown to be reflected in their bioavailability as determined from blood level data (2). The use of urinary excretion data, however, was thought of as a simpler and perhaps more accurate (3) alternative to the use of blood level data in bioavailability determination. Reduction of costs and the elimination of venipunctures are also obvious advantages. Urinary excretion data have been successfully used to evaluate the bioavailability of various drugs including aspirin (4), riboflavin (5), chloramphenicol (6), tetracycline products (7), and sulfamethizole (8).

Several mathematical treatments have been developed for bioavailability determination using urinary excretion data. The direct proportionality between excretion rate and blood level of free unchanged drug, measured at the mean time of the urine collection period (4, 9), is a common prerequisite to such treatments. Nelson (10) developed an equation for determining the amount of drug absorbed at a certain time. Later, Wagner and Nelson (9), using a one-compartment open model, simplified the Nelson equation for calculating the percentage of drug absorbed from urinary excretion data. A two-compartment open model for data treatment was proposed by Loo and Riegelman (11), who introduced terms describing the tissue distribution phase into the Wagner–Nelson equation. Recently, Perrier and Gibaldi (12) pointed out the possibility of overestimating the absorption rate constant, using either of the previous treatments, in cases of drugs with incomplete availability.

The mechanism of drug excretion may inflict certain complications on the linear relationship between excretion rate and blood level of free unchanged drug and, consequently, upset the fundamental assumption on which the application of urine data is based. A previous report (13) showed that the above-mentioned relationship could be affected by active tubular secretion, passive reabsorption, and protein binding. It was also shown (13) that when the extent of drug protein binding is constant and the passive reabsorption is not affected by urine pH, the urinary

cation of this framework will help remove some of the mystery surrounding the role of cholesterol in biological membranes.

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Bioavailability Determination of Two Crystal Forms of Sulfameter in Humans from Urinary Excretion Data

NAWAL KHALAFALLAH, SAID A. KHALIL[x], and MAMDOUH A. MOUSTAFA

Abstract □ Urinary excretion data were used to determine the bioavailability of crystal Forms II and III of sulfameter in humans. Agreement was observed between the ratio of absorption parameters of the two forms determined in the present study and those previously obtained from blood level data. Although the urine data revealed a significant difference in the rate of absorption of the two forms, no significant difference was observed in the extent of absorption of both forms as indicated by the 72-hr urinary excretion data. Urinary excretion rates during the absorption phase, without further mathematical treatment, were statistically shown to be adequate means for comparing the bioavailability of sulfameter crystal forms. The use of urinary excretion data of sulfameter as an alternative to the use of blood level data is discussed.

Keyphrases □ Sulfameter—bioavailability of two crystal forms, human urinary excretion data □ Sulfonamides—bioavailability of two crystal forms of sulfameter, human urinary excretion data □ Bioavailability—two crystal forms of sulfameter, human urinary excretion data □ Polymorphism—bioavailability of two crystal forms of sulfameter, human urinary excretion data

The polymorphism of sulfameter (1), as well as the GI absorption of its crystal Forms II and III (2), was recently reported. The *in vitro* dissolution behavior of the two crystal forms was shown to be reflected in their bioavailability as determined from blood level data (2). The use of urinary excretion data, however, was thought of as a simpler and perhaps more accurate (3) alternative to the use of blood level data in bioavailability determination. Reduction of costs and the elimination of venipunctures are also obvious advantages. Urinary excretion data have been successfully used to evaluate the bioavailability of various drugs including aspirin (4), riboflavin (5), chloramphenicol (6), tetracycline products (7), and sulfamethizole (8).

Several mathematical treatments have been developed for bioavailability determination using urinary excretion data. The direct proportionality between excretion rate and blood level of free unchanged drug, measured at the mean time of the urine collection period (4, 9), is a common prerequisite to such treatments. Nelson (10) developed an equation for determining the amount of drug absorbed at a certain time. Later, Wagner and Nelson (9), using a one-compartment open model, simplified the Nelson equation for calculating the percentage of drug absorbed from urinary excretion data. A two-compartment open model for data treatment was proposed by Loo and Riegelman (11), who introduced terms describing the tissue distribution phase into the Wagner–Nelson equation. Recently, Perrier and Gibaldi (12) pointed out the possibility of overestimating the absorption rate constant, using either of the previous treatments, in cases of drugs with incomplete availability.

The mechanism of drug excretion may inflict certain complications on the linear relationship between excretion rate and blood level of free unchanged drug and, consequently, upset the fundamental assumption on which the application of urine data is based. A previous report (13) showed that the above-mentioned relationship could be affected by active tubular secretion, passive reabsorption, and protein binding. It was also shown (13) that when the extent of drug protein binding is constant and the passive reabsorption is not affected by urine pH, the urinary

Table I—Cumulative Urinary Excretion Data of Sulfameter Forms II and III

Subject	Crystal Form	Cumulative Amount of Sulfameter Excreted, mg										
		2 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr
M.A.	II	9.2	24.2	44.1	65.5	87.8	110.8	230	375	457	528	590
	III	4.8	14.8	30	49	70	91	200	321	402	475	537
S.K.[a]	II	14.4	40	85	142	196	242	426	583	668	736	790
	III	8	22	46	81	123	163	357	520	618	695	757
S.K.	II	16	38	71	118	162	208	413	479	572	649	712
	III	8	26	49	81	120	160	381	520	617	694	756
O.A.	II	16	35	60	89	117	145	299	422	521	606	674
	III	10	23	39	60	85	112	262	380	474	557	627
E.S.	II	2.5	11	27	47	70	98	235	353	447	—[b]	—[b]
	III	3.5	11	25	42	61	84	209	318	406	485	551
N.K.	II	4	15	36	68	100	128	253	344	422	492	551
	III	3.5	12	28	48	73	99	238	344	434	516	577
M.M.	II	11	24	42	62	84	108	212	324	425	511	583
	III	11	24	42	64	90	117	287	411	513	594	665
A.R.	II	12	30	54	90	132	169	353	448	518	585	642
	III	12	25	42	63	87	114	257	359	448	525	591
H.B.[c]	II	—	—	—	—	—	—	—	—	—	—	—
	III	9	22	41	72	108	146	314	427	523	602	667

[a] Subject S.K. in another trial, 2 months earlier. [b] Uncollected urine samples. [c] Subject H.B. did not take Form II.

excretion rate should directly reflect the blood level. Sulfameter, similar to other sulfonamides, was reported (14) to be excreted by glomerular filtration and passive tubular reabsorption. The extent of protein binding of sulfonamides was reported (14, 15) to be constant in the dosage range adopted (1 g). Furthermore, the pKa value of sulfameter (6.8) (16) is high enough to minimize the effect of urine pH on passive reabsorption (17). Therefore, urinary excretion data of sulfameter were thought of as a possible substitute to blood level data in availability determination.

The present study is concerned with the determination of the absorption parameters of sulfameter Forms II and III from urinary excretion data. Comparison of such data with previously determined (through blood level data) absorption parameters (2) and *in vitro* dissolution data (1) is also considered.

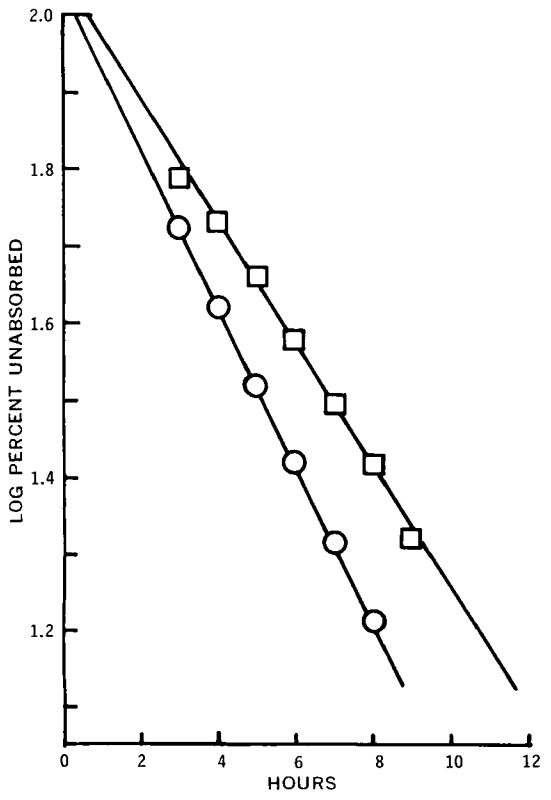

Figure 1—*Log percent sulfameter unabsorbed* versus *time. Mean of Subjects M.A., O.A., E.S., M.M., and A.R. Key:* ○, *Form II; and* □, *Form III.*

EXPERIMENTAL

Materials and Apparatus—Sulfameter[1] crystal Forms II and III were prepared (1), screened to a particle size of 80–90 μm, and identified by IR spectrophotometry[2] immediately before use.

Absorption Study—One gram of either crystal form was suspended in a mixture of 25 ml of 20% mucilage of acacia and 25 ml of simple syrup, a mixture previously shown to inhibit polymorphic transformation (18). This mixture was immediately administered, after an overnight fast, to eight healthy volunteers (six males and two females; age, 25–40 years with an average of 32 years; weight, 50–90 kg with an average of 68 kg). This administration was followed by 50 ml of water used to rinse the containing vessel. No food was permitted for 4 hr after drug administration. Urine samples were collected at 0, 2, 4, 6, 8, 12, 24, 36, 48, 60, and 72 hr after administration. Each urine sample was refrigerated for not more than 24 hr, appropriately diluted with 0.1 *N* HCl, and acid hydrolyzed, and the total (drug and metabolites) sulfameter content was determined colorimetrically[3] according to Bratton and Marshall (19).

A crossover study was performed, with the same volunteers, 1 month later.

RESULTS AND DISCUSSION

Urinary excretion data of sulfameter crystal Forms II and III are shown in Table I. Application of the Wagner–Nelson (9) treatment to the amounts excreted during absorption resulted in the absorption parameters[4] given in Table II and Fig. 1. Both the availability rate constant, K_A (for subjects showing first-order ab-

[1] Supplied through the courtesy of Alexandria Co. for Pharmaceutical and Chemical Industries, Egypt.
[2] Perkin-Elmer model 237-B spectrophotometer.
[3] Unicam SP 500 spectrophotometer.
[4] The elimination rate constant, K_E, required for the application of the Wagner–Nelson treatment was calculated for each subject from the slope of the semilog urinary excretion rate–time plot from 24 to 70 hr; K_E was found to vary among subjects from 0.014 to 0.019 hr^{-1}.

Table II—Absorption Parameters of Sulfameter Forms II and III

Subject	Crystal Form	K_A[a], hr^{-1}	$t_{50\%}$, hr	Lag Time, hr	$\frac{K_{AII}}{K_{AIII}}$
M.A.	II	0.262	2.63	—	1.34
	III	0.195	3.58	—	
S.K.[b]	II	152[c]	3.35	—	1.35
	III	113	4.49	—	
S.K.	II	140[c]	3.53	—	1.46
	III	96	5.15	—	
O.A.	II	0.295	2.38	—	1.77
	III	0.167	4.12	—	
E.S.	II	0.201	4.50	1.10	1.35
	III	0.149	5.65	1.10	
N.K.	II	165[c]	4.02	1.00	1.51
	III	109	4.62	—	
M.M.	II	0.193	4.00	0.35	1.00
	III	0.193	4.00	0.35	
A.R.	II	0.237	3.00	—	1.40
	III	0.169	4.30	—	
H.B.	II	—	—	—	—
	III	126[c]	4.75	0.80	
Mean	II	0.237[d]	3.44	—	1.40[f]
	III	0.175	4.49[e]	—	
Paired t test, significance level greater than		97.5%	99.5%		
Blood level data[g]	II	0.595	—	—	1.42
	III	0.422	—	—	

[a] Availability rate constant. [b] Subject S.K. in another trial, 2 months earlier. The values given are not included in the ratio and mean calculations. [c] Zero-order availability rate constant, milligrams per hour. [d] Mean of Subjects M.A., O.A., E.S., M.M., and A.R. [e] Excluding Subject H.B. [f] Mean of ratios for different volunteers. [g] See Ref. 2.

sorption), and the $t_{50\%}$ absorption (time required for absorption of 50% of the drug) of sulfameter Form II were significantly different from those of Form III (Table II). The ratio of the absorption rate constant (K_A) for Forms II and III is in agreement with that previously obtained from blood level data (2). The ratio of the *in vitro* apparent solubilities of Forms II and III (1) (1.6) is also in fair agreement with the absorption rate constants. Although the rate of absorption differed between the two forms, the extent of absorption, as indicated by the 72-hr urinary excretion data (Table I), did not differ significantly. Since the biological half-life of sulfameter ($t_{1/2}$) is more than 48 hr (20), determination of the total amount excreted would involve urine collection for at least 2 weeks (9). The 72-hr urinary excretion data were considered a good indication of the amount eventually excreted, since K_E (which may be used to calculate total amount excreted[4]) did not differ, for each subject, between the two forms.

The absorption of sulfameter from suspension was observed to follow zero-order kinetics with some volunteers; with others, a

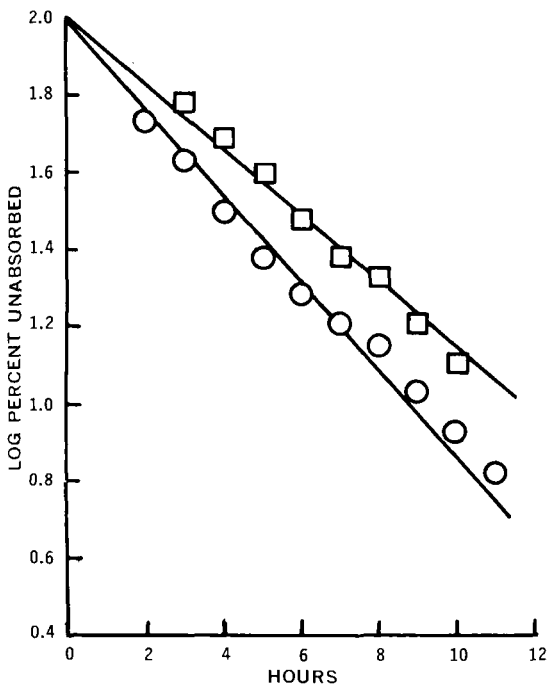

Figure 2—*Log percent sulfameter unabsorbed* versus *time for Subject M.A. showing first-order absorption. Key:* ○, *Form II; and* □, *Form III.*

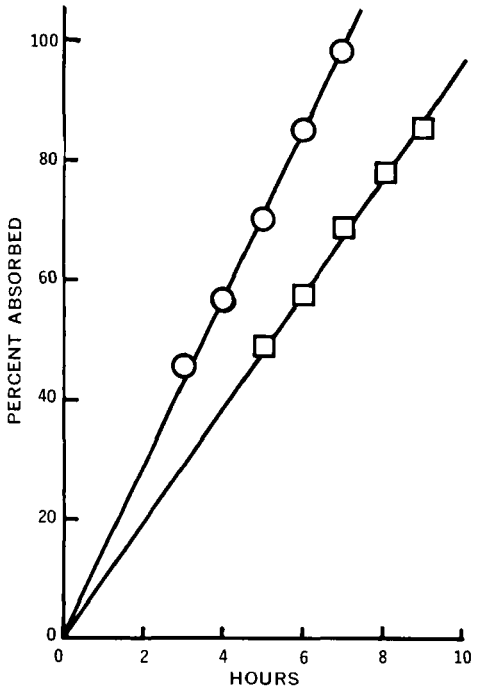

Figure 3—*Percent sulfameter absorbed* versus *time for Subject S.K. showing zero-order absorption. Key:* ○, *Form II; and* □, *Form III.*

Table III—Mean Urinary Excretion Rates of Sulfameter Forms II and III

	Hours								
	2	3	4	6	8	12	24	36	60
					II				
Mean urinary excretion rate[a], mg/hr (standard deviation)	6.33 (2.48)	7.96 (2.50)	9.40 (2.53)	12.94 (3.85)	14.95 (4.77)	11.88 (3.09)	10.58 (1.13)	8.14 (1.23)	5.82 (0.54)
					III				
	4.85 (1.63)	6.07 (1.48)	7.22 (1.58)	9.70 (1.93)	11.80 (2.81)	10.92 (2.53)	11.16 (1.93)	8.70 (0.64)	6.00 (0.27)
Paired t test, significance level greater than	97.5%	97.5%	99.0%	97.5%	95.0%				
Ratio of mean excretion rates of Forms II and III	1.31	1.31	1.30	1.34	1.27				

[a] Excluding data for Subjects H.B. and S.K. (2-month earlier trial).

better fit to first-order kinetics was observed (Table II and Figs. 2 and 3). The zero-order absorption pattern is not uncommon with oral dosage forms (tablets and suspensions), which are limited in their availability by a dissolution step, and has been attributed in some cases to such factors as low drug solubility, limited gastric fluid volume, and high viscosity at the site of absorption (15, 21), all of which prevailed to some extent in the present study. The lag time observed with some volunteers in the Fig. 1-type plot (Table II) resulted from fitting a straight line to a slightly curvilinear relationship. This phenomenon has been reported to be due to dissolution, gastric emptying, *etc.* (12, 22).

The results given in Table II show that urinary excretion data are a useful alternative to blood level data in providing information of a comparative nature concerning the absorption of sulfameter crystal Forms II and III. However, absolute absorption rate constants obtained in the present study differed from those obtained from blood level data (2). The discrepancy might be attributed to between-study variability, bearing in mind that two different groups of subjects participated in both studies.

Excretion rates also could be used as an additional absorption parameter (4) without having to resort to any further mathematical treatment. Table III shows that the mean excretion rates of sulfameter Form II during the absorption phase are significantly different from those of Form III. Furthermore, the ratios of the mean excretion rates of Forms II and III during absorption (Table III) are very close to the ratios reported in Table II and Ref. 2.

The present study revealed some intersubject variation in excretion rates. The extent of variation, as indicated by the standard deviation (Table III), was observed to be greater during the absorption phase (up to 8 hr). Excretion rates in the postabsorptive phase were close to each other. This is perhaps an indication that excretion rates of different volunteers are independent of urinary pH, which was not controlled in the present study. Only slight intrasubject variations were observed (Subject S.K., Table I).

In conclusion, it is suggested that different crystal forms (or dosage forms) of sulfameter may be compared as to their bioavailability rates by comparing their excretion rates during the absorption phase. Such a procedure would save considerable time and effort. Urinary excretion data are also suggested as a simpler alternative to blood level data in studying the kinetics of absorption and deriving absorption parameters that enable the comparison of different formulations of sulfameter.

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[x] To whom inquiries should be directed.

Chemical Equilibria in Solutions of Bisulfite Salts

DAVID W. A. BOURNE, T. HIGUCHI, and IAN H. PITMAN[x]

Abstract □ The equilibrium between bisulfite ion and pyrosulfite ion in aqueous solutions of different ionic strengths was investigated spectrophotometrically. Values for the equilibrium constant at ionic strengths of 2.0, 0.9, 0.5, 0.1, and 0.01 *M* were found to be 0.34, 0.22, 0.175, 0.115, and 0.086 M^{-1}, respectively. The extrapolated thermodynamic equilibrium constant was calculated to be 0.076 ± 0.010 M^{-1}, and the molar absorptivity of pyrosulfite ion at 255 nm was calculated to be 1980. These values differ significantly from the values in the chemical literature. No evidence was found for the formation of complexes between bisulfite and citrate ions in aqueous solution.

Keyphrases □ Bisulfite ion-pyrosulfite ion—equilibrium constants, complex formation with citrate ions □ Equilibrium constants—bisulfite-ion-pyrosulfite-ion equilibrium □ Complex formation—bisulfite ion, citrate □ Citrate ions—complex formation with bisulfite ions

When sulfur dioxide or salts of sulfurous acid (hydrated SO_2, H_2SO_3) are dissolved in water, the following equilibria are rapidly established[1]:

$$H_2SO_3 \overset{K_a^1}{\rightleftharpoons} HSO_3^- + H^+$$

$$HSO_3^- \overset{K_a^2}{\rightleftharpoons} SO_3^{-2} + H^+$$

$$2HSO_3^- \overset{K}{\rightleftharpoons} S_2O_5^{-2} + H_2O$$

Scheme I

Therefore, accurate values of the equilibrium constants K_a^1, K_a^2, and K must be known to calculate the relative concentrations of sulfurous acid, bisulfite ion (HSO_3^-), sulfite ion (SO_3^{-2}), and pyrosulfite ion ($S_2O_5^{-2}$) in any given solution. Even though bisulfite salts have been used for many years in the pharmaceutical, food, and beverage industries, and the chemical reactivity of bisulfite-sulfite salt solutions has been widely studied[2], the current literature (1, 3, 4) contains conflicting values for the equilibrium constant K (Table I).

One aim of the present study was to determine values of concentration equilibrium constants for the dimerization of bisulfite ion at various ionic strengths and to use this information to calculate a thermodynamic equilibrium constant for this reaction.

In addition, an investigation was undertaken to see whether citrate ions reduce the thermodynamic activity of bisulfite ion when the two are included in aqueous solutions. It was reported (5) that citrate and bisulfite ions may form a complex in solution. If this was true, the reactivity of bisulfite ion in such solutions would be changed, and this may have serious consequences on its effectiveness as an antioxidant or as a participant in other reactions.

Table I—Equilibrium Constants for the Reaction:

$$2HSO_3^- \overset{K}{\rightleftharpoons} S_2O_5^{-2} + H_2O$$

and Spectral Characteristics of Pyrosulfite Ion

K, M^{-1}	ϵ^a	λ_{max}	T	$K\epsilon$	Ionic Strength, M	Reference
0.07	4000	255	20°	288	—[b]	1
2.0	142.9	256–258	22°	286	0.3	3
0.07[c]	2527	255	25°	177	—[b]	4
0.34	1980	255	25°	673	2.0	This work
0.076	1980	255	25°	139	0.0	This work

[a] Molar absorptivity of pyrosulfite ion at λ_{max}. [b] Ionic strength not indicated. [c] From Ref. 1.

EXPERIMENTAL

Materials and Apparatus—Sodium bisulfite, acetic acid, sodium chloride, hydrochloric acid, sodium hydroxide, citric acid, and sodium sulfite were all reagent grade. 1,2-Dihydro-2-imino-1-methylpyrimidine hydrochloride was prepared as described previously (6), mp 277–278°. All solutions were made with double-distilled water through which oxygen-free nitrogen had been bubbled for 1 hr. One percent of methanol was added to stabilize sodium bisulfite against oxidation. Final bisulfite concentrations were determined by iodometric titration.

Spectrophotometric measurements were made on three spectrophotometers[3]. Quartz cells of path length 1, 2, 10, 20, 50, and 100 mm were used for UV measurements. Temperature was maintained for UV readings, determinations of ionization constants, and kinetic runs at 25.0 ± 0.1°. The pH values were measured using a pH meter[4] standardized with U.S. National Bureau of Standards quality buffers at pH 4.00 and 7.00.

Ionization Constants—The two pKa values of sulfurous acid were measured by potentiometric titration using a method described previously (7). For pK_a^1, a 0.1 *M* solution of sulfurous acid was titrated with 1.0 *M* NaOH solution. The second ionization constant was obtained by titrating a 0.01 *M* solution of sodium bisulfite with 0.2 *M* NaOH. The ionic strength was maintained at 2.0 *M* with sodium chloride.

Molar Absorptivity—The molar absorptivity of H_2SO_3 was determined in 1 *M* HCl with 1 *M* NaCl added to maintain ionic strength at 2.0 *M*. The molar absorptivity of SO_3^{-2} was determined using solutions of sodium sulfite of 2.0 *M* total ionic strength.

Kinetic Method—The reaction of 1,2-dihydro-2-imino-1-methylpyrimidine hydrochloride with bisulfite ion (0.005 *M*) was followed at 302 nm for 2–3 half-lives and found to follow first-order kinetics. The pH was maintained at 4.14 ± 0.05 with 0.2 *M* acetate buffer or with 0.1, 0.2, or 0.3 *M* citrate buffer. Ionic strength was maintained at 0.5 *M* with sodium chloride.

Iodometric Titration—Five milliliters of a sodium bisulfite solution (0.055 *M*) was added to 10 ml of 0.1 *N* iodine solution and

[1] The presence of a further species, $HO{\cdot}SO_2^-$, was suggested (1) and refuted (2); since the extent of the reaction $HSO_3^- \rightleftarrows HO{\cdot}SO_2^-$ is independent of pH and concentration and neither species absorbs light strongly at the wavelengths of interest in the present study (above 230 nm), this species is not discussed in this report.

[2] The chemistry of sulfur dioxide was comprehensively reviewed by L. C. Schroeter in "Sulfur Dioxide," Pergamon, New York, N.Y., 1966.

[3] Cary 14, 15, and 16.

[4] Radiometer model 26.

Table II—Molar Absorptivity of Sulfur Species in Aqueous Sodium Bisulfite Solutions

Species	ϵ_{255}
H_2SO_3	450
HSO_3^-	<1
SO_3^{-2}	9.36
$S_2O_5^{-2}$	1980

titrated with 0.1 *N* sodium thiosulfate. In some experiments, 1, 5, or 15 ml of a 1 *M* sodium citrate solution, pH ≃ 4.0, was added to the sodium bisulfite solution before the addition of the iodine solution.

RESULTS AND DISCUSSION

Bisulfite-Pyrosulfite Equilibrium—The species present in aqueous solutions of sodium bisulfite (HSO_3^-, H_2SO_3, and SO_3^{-2}) all absorb UV light but different amounts at different wavelengths. This difference at 255 nm (Table II) permits one to investigate the composition of aqueous solutions of sodium bisulfite spectrophotometrically. Since bisulfite ion itself absorbs very little light at 255 nm, the absorbance at this wavelength is due almost entirely to pyrosulfite ion, sulfurous acid, and sulfite ion. Hence:

$$A_T \simeq \epsilon_{255}(S_2O_5^{-2})[S_2O_5^{-2}]_e + \epsilon_{255}(H_2SO_3)[H_2SO_3]_e + \epsilon_{255}(SO_3^{-2})[SO_3^{-2}]_e \quad \text{(Eq. 1)}$$

where A_T is the total absorbance at 255 nm; ϵ_{255} is the molar absorptivity at 255 nm of the species indicated; and $[S_2O_5^{-2}]_e$, $[H_2SO_3]_e$, and $[SO_3^{-2}]_e$ are the equilibrium concentrations of $S_2O_5^{-2}$, H_2SO_3, and SO_3^{-2}, respectively. If S_T is the total sulfur concentration as determined iodometrically, then:

$$S_T = [H_2SO_3]_e + [SO_3^-]_e + [SO_3^{-2}]_e + 2[S_2O_5^{-2}]_e \quad \text{(Eq. 2)}$$

Using the equations defining the equilibria in Scheme I:

$$K_a^1 = [H^+][HSO_3^-]/[H_2SO_3] \quad \text{(Eq. 3)}$$

$$K_a^2 = [H^+][SO_3^{-2}]/[HSO_3^-] \quad \text{(Eq. 4)}$$

$$K = [S_2O_5^{-2}]/[HSO_3^-]^2 \quad \text{(Eq. 5)}$$

and substituting in Eq. 2 give:

$$S_T = [HSO_3^-]_e \frac{[H^+]^2 + K_a^1[H^+] + K_a^1K_a^2}{K_a^1[H^+]} + 2K[HSO_3^-]_e^2 \quad \text{(Eq. 6)}$$

This equation can then be solved for $[HSO_3^-]_e$ if a value of K is assumed. Values of $[S_2O_5^{-2}]_e$, $[SO_3^{-2}]_e$, and $[H_2SO_3]_e$ can be calculated from Eqs. 3–5, and a value of A_T can be calculated from Eq. 1 by assuming a value of ϵ_{255} ($S_2O_5^{-2}$). The experimental results in Table III were obtained using a wide range of values of S_T

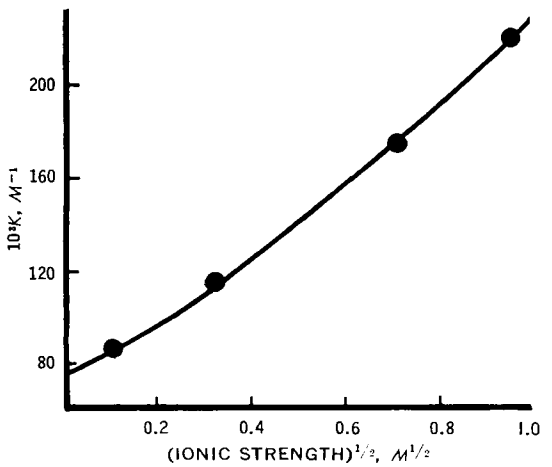

Figure 1—*Plot of values of* K *at 25° against (ionic strength)*$^{1/2}$.

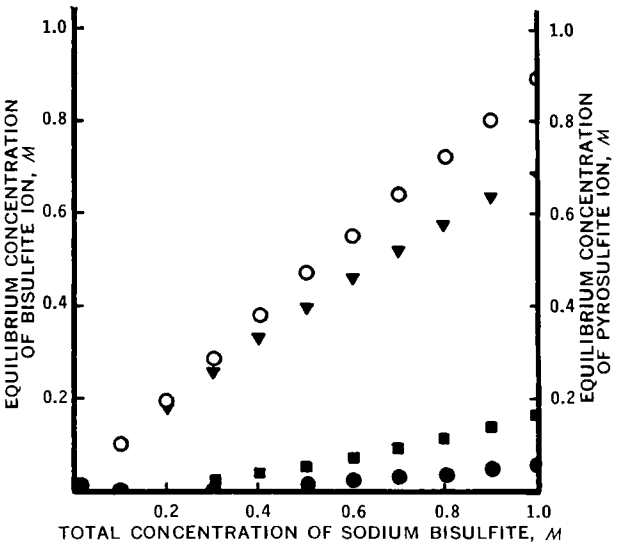

Figure 2—*Plots against total sodium bisulfite concentrations of the equilibrium concentrations of bisulfite ion and pyrosulfite ion that would be calculated if it were assumed that* K = *0.076* M^{-1} *and* K = *0.34* M^{-1}. *Key:* ○, $[HSO_3^-]_e$, *when* K = *0.076* M^{-1}; ▼, $[HSO_3^-]_e$, *when* K = *0.34* M^{-1}; ●, $[S_2O_5^{-2}]_e$, *when* K = *0.076* M^{-1}; *and* ■, $[S_2O_5^{-2}]_e$, *when* K = *0.34* M^{-1}.

(0.005–0.16 *M*) and pH values from 3.0 to 6.0. Total ionic strength was maintained constant at 2.0 *M* with sodium chloride.

The values of K_a^1, K_a^2, ϵ_{255} (SO_3^{-2}), and ϵ_{255} (H_2SO_3) were obtained independently, and values of K and ϵ_{255} ($S_2O_5^{-2}$) were changed by trial and error to obtain a minimum value for the sum of the square of the deviations between calculated absorbance and measured absorbance. At an ionic strength of 2.0 *M*, values of K = 0.34 M^{-1} and ϵ_{255} ($S_2O_5^{-2}$) = 1980 gave the best fit.

In dilute solutions of sodium bisulfite at pH values where there is little sulfurous acid or sulfite ion, about pH 4.0, the concentration of bisulfite ion is approximately equal to S_T and Eq. 1 simplifies to:

$$A_T = K\epsilon_{255}(S_2O_5^{-2})S_T^2 \quad \text{(Eq. 7)}$$

Values of $K\epsilon_{255}$ ($S_2O_5^{-2}$) can thus be obtained by plotting A_T *versus* S_T^2. In this way, values of $K\epsilon_{255}$ ($S_2O_5^{-2}$) at pH 4.00 ± 0.01 and ionic strengths (μ) from 0.01 to 0.9 *M* were calculated. Because the value of ϵ_{255} ($S_2O_5^{-2}$) is independent of ionic strength, values of K at these ionic strengths could then be calculated. Results are shown in Fig. 1 as a plot of K against $\sqrt{\mu}$. From this figure, the value of K at zero ionic strength was obtained by extrapolation. This value was 0.076 ± 0.010 M^{-1}.

Previously calculated values for K are shown in Table I. The values of Golding (1) and Eriksen and Lind (4) suffer from the fact that ionic strength was apparently not maintained constant.

Golding (1) assumed $[H_2SO_3]_e$ and $[SO_3^{-2}]_e$ to be approximately zero under his experimental conditions and substituted Eq. 5 into Eq. 2 to derive Eq. 8:

$$S_T/A_T = X[A_T^{-1/2}] + Y \quad \text{(Eq. 8)}$$

where $X^2 = [\epsilon_{255}\ (S_2O_5^{-2})\ (d)\ (K)]^{-1}$, and $Y = 2/[\epsilon_{255}\ (S_2O_5^{-2})\ (d)]$.

In these identities, d (in centimeters) was the path length of the spectrophotometer cell. Hence, plots of S_T/A_T against $A_T^{-1/2}$ were used to calculate a value of $K\epsilon_{255}$ ($S_2O_5^{-2}$) and ϵ_{255} ($S_2O_5^{-2}$). Because the value of the intercept was very small, the error in the value of ϵ_{255} ($S_2O_5^{-2}$) was large. Since K was obtained from the term $K\epsilon_{255}$ ($S_2O_5^{-2}$), an error of equal magnitude was thus carried into the value determined for K. The value of K of Arkhipova and Chistyakova (3) at an ionic strength of 0.3 was computed by trial-and-error minimizing of deviations between experimental and calculated values of absorbance. However, these workers used quite a narrow range (0.045–0.06 *M*) of S_T values, so considerable error could be involved in the value of K they obtained.

Table III—Absorbance [Experimental[a] (A_{exp}) and Calculated[b] (A_{calc})] of Aqueous Sodium Bisulfite $[Bis]_T$ Solutions with Ionic Strength 2.0 M Adjusted with Sodium Chloride

$[Bis]_T$ = 0.160 M				Cell Length 1 mm				
pH	5.90	5.64	5.34	4.92	4.48	4.05	3.60	3.16
A_{exp}	0.735	0.910	1.108	1.260	1.355	1.455	1.520	1.602
A_{calc}	0.694	0.919	1.124	1.292	1.370	1.404	1.430	1.473
$[Bis]_T$ = 0.079 M				Cell Length 2 mm				
pH	5.95	5.68	5.36	4.94	4.50	4.04	3.53	3.12
A_{exp}	0.340	0.450	0.515	0.630	0.665	0.720	0.780	0.850
A_{calc}	0.356	0.474	0.590	0.682	0.728	0.752	0.782	0.836
$[Bis]_T$ = 0.400 M				Cell Length 10 mm				
pH	5.86	5.65	5.34	4.93	4.41	3.92	3.42	
A_{exp}	0.560	0.635	0.758	0.855	0.960	1.020	1.125	
A_{calc}	0.567	0.670	0.799	0.907	0.973	1.017	1.110	
$[Bis]_T$ = 0.019 M				Cell Length 20 mm				
pH	5.81	5.72	5.42	4.99	4.51	4.01	3.50	3.03
A_{exp}	0.310	0.328	0.358	0.412	0.448	0.493	0.598	0.834
A_{calc}	0.330	0.342	0.384	0.428	0.458	0.488	0.564	0.768
$[Bis]_T$ = 0.011 M				Cell Length 50 mm				
pH	5.69	5.56	5.39	5.00	4.52	4.00	3.51	3.05
A_{exp}	0.308	0.308	0.310	0.334	0.371	0.410	0.521	0.852
A_{calc}	0.327	0.312	0.337	0.353	0.371	0.408	0.504	0.785
$[Bis]_T$ = 0.005 M				Cell Length 100 mm				
pH	5.66	5.50	5.30	4.97	4.46	3.94	3.47	3.00
A_{exp}	0.208	0.190	0.162	0.157	0.137	0.196	0.320	0.672
A_{calc}	0.204	0.191	0.181	0.173	0.177	0.212	0.315	0.612

[a] Absorbance at 255 nm. [b] Absorbance calculated using K = 0.34 M^{-1} and ϵ_{255} ($S_2O_5^{-2}$) = 1980.

The importance of having a reliable value of K when estimating the effective concentration of bisulfite ion or pyrosulfite ion in a solution of sodium bisulfite is illustrated in Fig. 2. This figure displays plots of $[HSO_3^-]_e$ and $[S_2O_5^{-2}]_e$ against $[HSO_3^-]_T$ that would be calculated if K values of 0.076 M^{-1} (the actual value at zero ionic strength) or 0.34 M^{-1} (the actual value at an ionic strength of 2.9 M) were used in the calculation.

The ionic strength dependence of K values indicates that, although dilute solutions of sodium bisulfite contain very little pyrosulfite ion if the ionic strength is low, the concentration of pyrosulfite ion can become appreciable if the ionic strength is increased.

Bisulfite-Citrate-Ion Interaction—Formation of a bisulfite-citrate-ion complex has been suggested as a reason for the greater effectiveness of citrate buffers, at pH values near 4, in removing sulfur dioxide from waste gases (5) compared to other inorganic and mixed organic and inorganic solutions. However, no evidence to support this suggestion was found in the present study. As shown earlier, the UV spectrum of pyrosulfite ion is a sensitive measure of the bisulfite-ion activity in solution, and its intensity should change as the concentration of citrate is increased if a bisulfite-citrate complex is forming in solution. However, the absorbance at 255 nm of a sodium bisulfite solution (0.01 M) at constant pH (4.0) and ionic strength (1.5 M with sodium chloride) was unaffected by the addition of sodium citrate (0.05–0.5 M) when the contribution of the citrate ion itself was taken into account. The citrate ion apparently did not affect the activity of bisulfite ion as measured spectrophotometrically.

If a stable complex between citrate and bisulfite ion is formed and the rate of its dissociation is slow, then rapid iodometric titration of bisulfite-ion solutions containing citrate ion should give results different from those obtained in the absence of citrate ion. This was not found when a solution of sodium bisulfite (0.055 M) at pH 4.0 was titrated iodometrically in the presence of different amounts of citrate ion (0.05–1.0 M). Hence, a stable, slowly dissociating complex is not likely.

The reaction of bisulfite ion with 1,2-dihydro-2-imino-1-methylpyrimidine has been extensively studied (8), and the rate of reaction was found to be first order in bisulfite ion. If a complex is formed with citrate ion, the activity of bisulfite ion would be reduced, and its rate of reaction with 1,2-dihydro-2-amino-1-methylpyrimidine would be accordingly reduced.

This reaction was investigated in solutions of constant ionic strength (0.5 M), pH (4.0), and total bisulfite concentration (0.01 M) and different citrate-ion concentrations (0.1–0.3 M). The rate of reaction was found to be independent of citrate concentration. This kinetic result further indicates that a complex between citrate and bisulfite ion is unlikely.

The efficiency of citrate buffers in the removal of sulfur dioxide from waste gases could be due to citric acid being a good buffer, because it is polyprotic, having three ionizable hydrogens. As sulfur dioxide dissolves, it is rapidly hydrated to H_2SO_3, which can ionize to HSO_3^-; since HSO_3^- has a much higher solubility than H_2SO_3, the overall solubility of sulfur dioxide is increased. Since this ionization of H_2SO_3 is pH dependent, the increased buffer capacity of the citrate buffer will maintain a higher amount of the sulfur dioxide in the form of bisulfite ion, thereby increasing the overall solubility of sulfur dioxide. Also, because citric acid is polyprotic, its solutions at pH values around 4 would have relatively high ionic strengths compared to those of monoprotic acids. Consequently, the formation of pyrosulfite from bisulfite would be favored and the effective solubility of SO_2 would increase. Therefore, the efficiency of the citrate process can be explained without having a bisulfite-citrate-ion complex.

CONCLUSION

The literature values for the equilibrium constant for the dimerization reaction of bisulfite ion to pyrosulfite ion were conflicting. A study has been completed to clarify the situation by providing a reliable value of K, the ionic strength dependence of K, and the extinction coefficient of pyrosulfite ion at 255 nm. A thermodynamic equilibrium constant of 0.076 ± 0.010 M^{-1} at 25° and a molar absorptivity for $S_2O_5^{-2}$ at 255 nm of 1980 have been determined.

Studies on aqueous solutions of bisulfite ion and citrate ions have shown that a stable bisulfite-ion–citrate-ion complex is unlikely to be formed in appreciable amounts and that the thermodynamic activity of bisulfite ion is unaffected by the presence of citrate ions.

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[x] To whom inquiries should be directed.

Comparative Pharmacokinetics of Coumarin Anticoagulants X: Relationship between Distribution, Elimination, and Anticoagulant Action of Warfarin

AVRAHAM YACOBI, L. B. WINGARD, Jr.*, and GERHARD LEVY[x]

Abstract □ The biological half-life of warfarin in a group of male Sprague–Dawley rats varied from 5 to 28 hr, and the apparent volume of distribution varied from 102 to 320 ml/kg body weight. There was a strong and statistically highly significant positive correlation between the elimination rate constant and the apparent volume of distribution of warfarin in individual rats. There was no relationship between the elimination rate constant for warfarin and either the normal (prewarfarin) prothrombin complex activity or the rate constant for decline of this activity when the synthesis of vitamin K-dependent clotting factors was blocked. The concentration of warfarin in plasma at which the synthesis rate of prothrombin complex activity was inhibited by 50% varied from 0.05 to 1.37 μg/ml and showed a strong and highly statistically significant positive correlation with the biological half-life of warfarin in individual animals. These results demonstrate a pronounced association among the apparent volume of distribution, the elimination rate constant, and the plasma concentration–anticoagulant effect relationship for warfarin which may have to be considered in the design of clinical dosage regimens.

Keyphrases □ Anticoagulants, coumarin—comparative pharmacokinetics of warfarin, relationships between distribution, elimination, and activity, biological half-life, volume of distribution □ Coumarin anticoagulants—comparative pharmacokinetics of warfarin, relationships between distribution, elimination, and activity, biological half-life, volume of distribution □ Warfarin—comparative pharmacokinetics, relationships between distribution, elimination, and activity, biological half-life, volume of distribution □ Pharmacokinetics—warfarin distribution, elimination, and activity

Warfarin is probably the most widely used oral anticoagulant. Its biological half-life varies markedly between subjects. For example, the biological half-life of warfarin in 20 healthy adult human subjects was reported to range from 15 to 80 hr (1, 2), and there are case reports of patients with a warfarin half-life of only 5.5 and 6.1 hr (3, 4). In a study (5) of warfarin elimination by mongrel dogs, half-lives of 21–48 hr were found. In random-bred Sprague–Dawley rats, warfarin half-lives from <5 to 70 hr in males and from 10 to 90 hr in females have been observed (6).

It is generally believed that intersubject differences in pharmacological response to a particular dosage regimen of a drug due to differences in the rate of elimination of that drug may be overcome by adjusting the dosage regimen to attain a given plasma concentration, *i.e.*, the therapeutic concentration. This assumes, for example, that individuals who differ with respect to the half-life for warfarin elimination nevertheless respond equally (at least in the absence of certain other drugs and diseases) to a particular concentration of that drug in the plasma[1]. This assumption would be incorrect if the differences in the elimination kinetics of warfarin are caused by, or associated with, differences in the distribution of that drug in the body. In this study, the apparent volume of distribution, the biological half-life, and the plasma concentration–anticoagulant effect curve for warfarin in individual rats were determined and the relationships between these characteristics were examined.

EXPERIMENTAL

Male Sprague–Dawley rats[2], weighing 305–400 g, were used. They had unrestricted access to food[3] and water before and during the experiments. The animals received 0.6 mg ^{14}C-warfarin/kg body weight ip. Blood samples (0.36 ml) were obtained repeatedly from the tail artery (9) for up to 96 hr after drug administration. Each blood sample was mixed with 0.04 ml sodium oxalate

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Table I—Effect of Different Separation Methods on the Assay of Warfarin in Plasma of Rats after Injection of a Single Dose of ^{14}C-Warfarin[a]

Rat	Time after Injection, hr	Total Radioactivity, μg Apparent Warfarin/ml	Concentration in Plasma, μg/ml					
			O'Reilly Method		Lewis Method		O'Reilly and Lewis Methods Combined	
			Spec.[b]	Scint.[c]	Spec.	Scint.	Spec.	Scint.
A	21.0	4.78	3.31	3.33	3.36	3.56	3.30	3.46
B	24.2	16.4	15.8	15.0	14.2	13.5	15.7	16.3
C	12.0	23.9	21.0	19.6	19.4	19.8	21.4	22.4
D	7.2	36.7	34.6	32.7	35.0	35.6	33.6	33.2

[a] Dose of 12 mg/kg body weight intraperitoneally, including 3.5 μCi ^{14}C-warfarin per rat. [b] Assayed by spectrophotometry. [c] Assayed by scintillation spectrometry.

solution (0.1 *M* in 0.7% sodium chloride), and the hematocrit and prothrombin complex activity (PCA, expressed as percent of normal) were determined as described by Wingard and Levy (9).

The synthesis rate (R_{syn}) and apparent first-order degradation rate constant (k_d) for the PCA were calculated by a previously developed method (10). The k_d was determined following injection of sodium warfarin, 12 mg/kg ip, about 1 month after the study with ^{14}C-warfarin. The biological half-life and apparent first-order elimination rate constant for warfarin were determined from the least-squares slope of plots of the logarithm of the warfarin concentrations in the plasma *versus* time. The apparent volume of distribution was calculated by dividing the injected dose by the extrapolated zero-time plasma warfarin concentration ($V_{intercept}$) or by the product of the elimination rate constant and the area under the plasma concentration *versus* time curve starting at zero concentration at zero time (V_{area}).

The injection solution was prepared within 2 hr before use by dissolving 2.17 mg ^{14}C-warfarin[4] (23 μCi/mg) in about 0.2 ml 0.001 *M* sodium hydroxide solution and immediately adding sufficient normal saline solution to yield a total volume of 10 ml. An aliquot of this solution showed only one peak by radiochromatogram scanning and yielded a concentration of 101% of theory by radioactivity assay.

Warfarin in the plasma was separated from its metabolites by TLC (11), and the eluted warfarin was assayed by scintillation counting. For this purpose, 0.1 ml of plasma was acidified with 0.5 ml 0.3 *N* hydrochloric acid and extracted with 5 ml ethylene dichloride. The phases were separated by centrifuging after 15–20 min of shaking; 4 ml of the organic phase was transferred to a 5-ml capacity vial[5] and evaporated under nitrogen (no heat).

Residual solid material on the walls of the vial was washed to the bottom with acetone and the solvent was evaporated again under nitrogen; this washing and evaporation procedure was repeated twice. The drug in the vial was then dissolved in 30–50 μl acetone and the solution was applied to a TLC sheet. The vial was washed three times with 30-μl portions of acetone and these were also applied to the TLC sheet. During all these procedures, care was taken to protect the drug from light. Chromatography was carried out on silica gel with fluorescent indicator[6] with ethylene dichloride–acetone (9:1). The ^{14}C-warfarin spot was located by a radiochromatogram scanner[7] or was visualized under UV light, using the spot obtained from a solution of warfarin in acetone as a reference. The portion of the chromatogram with the warfarin spot was cut out and dropped into a vial containing 15 ml scintillation fluid (100 g naphthalene, 7 g 2,5-diphenyloxazole, 0.3 g 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 1,4-dioxane to yield 1 liter of solution). Scintillation counting[8] was carried out at room temperature with an internal standard. The counting efficiency averaged 89%. The recovery of warfarin from plasma samples containing 0.05–5.0 μg/ml was 89.7 ± 2.7% (mean ± *SD*, *n* = 14) and showed no dependence on concentration.

In an experiment to compare the results of plasma warfarin assays by the TLC method of Lewis *et al.* (11) with those of the

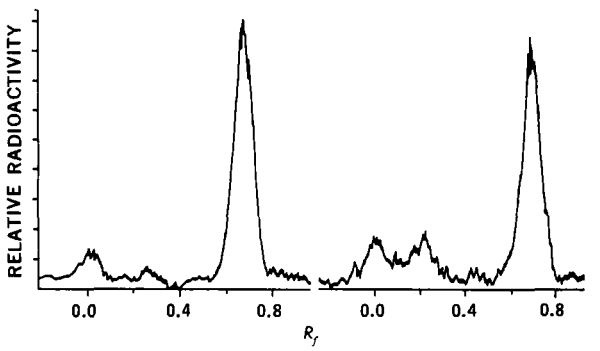

Figure 1—*Radiochromatogram scans of a plasma sample obtained from a rat 21 hr after intraperitoneal injection of ^{14}C-warfarin, 12 mg/kg body weight (Rat A in Table I). The scan on the right was obtained from a thin-layer chromatogram prepared by the method of Lewis* et al. *(11). The scan on the left was obtained by first extracting the plasma sample by the method of O'Reilly* et al. *(12) and then chromatographing the extract by the method of Lewis* et al. *The highest peak in each scan represents the warfarin spot.*

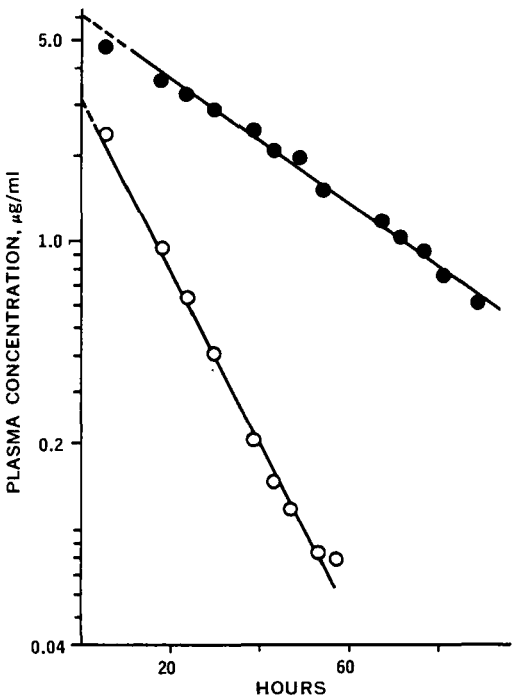

Figure 2—*Warfarin concentration in the plasma of two rats as a function of time after intraperitoneal injection of ^{14}C-warfarin, 0.6 mg/kg body weight. Key: ●, Rat 2; and ○, Rat 5.*

[4] 3-[α-Acetonyl(benzyl-α-^{14}C)]-4-hydroxycoumarin, Amersham-Searle Corp., Arlington Heights, Ill.
[5] Reacti-Vial, Pierce Chemical Co., Rockford, Ill.
[6] Silica gel F-254 TLC sheets, EM Laboratories, Inc., Elmsford, N.Y.
[7] Packard model 7201.
[8] Packard Tri-Carb liquid scintillation counter, model 2002.

Table II—Biological Half-Life, Estimated Apparent Volume of Distribution, and Body Clearance of Warfarin in Rats[a]

Rat	Half-Life, hr	Apparent Volume of Distribution, ml/kg		Total Clearance[d], ml/(kg hr)
		$V_{intercept}$[b]	V_{area}[c]	
1	28.1	104	115	2.83
2	27.7	98	104	2.60
3	26.6	122	133	3.46
4	20.8	94	102	3.40
5	10.1	188	206	14.1
6	8.0	283	320	27.7
7	6.1	248	304	34.5
8	5.1	314	317	43.0

[a] With 0.6 mg warfarin/kg body weight intraperitoneally. [b] Dose divided by the extrapolated zero-time plasma concentration. [c] Dose divided by the product of elimination rate constant and area under the plasma concentration–time curve. [d] Product of V_{area} and elimination rate constant.

spectrophotometric method of O'Reilly *et al.* (12), four rats received warfarin[9], 12 mg/kg ip, and ^{14}C-warfarin, about 3.5 μCi/rat. The four animals were exsanguinated at 7, 12, 21, and 24 hr, respectively. Sufficient blood was obtained from the aorta to yield 4–6 ml plasma, and 0.5-ml portions of each plasma sample were analyzed by both methods. To determine the specificity of the spectrophotometric method, the ethylene dichloride extract of an acidified plasma sample was washed with pH 7.25 phosphate buffer, and this extract was analyzed by the TLC procedure described in the preceding paragraph. The amount of warfarin eluted from the TLC sheet was determined both spectrophotometrically (using microcells) and by scintillation counting.

RESULTS

This investigation required a sensitive and specific method of analysis of warfarin in only 0.1 ml of plasma. The necessary sensitivity was achieved by use of ^{14}C-warfarin. The extraction method of O'Reilly *et al.* (12), the TLC separation method of Lewis *et al.* (11), and a combination of the two methods were evaluated as means of achieving assay specificity. All three methods yielded essentially similar results (Table I). The extraction method of O'Reilly *et al.* (12) separated most, but not all, of the metabolites from warfarin itself (Fig. 1). The more complete separation of metabolites achieved by the Lewis *et al.* (11) method can be important after the concentrations of warfarin have declined by more than one order of magnitude, *i.e.*, when metabolite concentrations may contribute significantly to the total radioactivity in the plasma. Therefore, this TLC method was employed in the subsequent experiments.

The concentration of warfarin in the plasma of individual animals decreased exponentially with time after injection (Fig. 2). Warfarin half-lives ranged from 5.1 to 28.1 hr (Table II). The volumes of distribution ranged from 94 to 314 ml/kg for $V_{intercept}$ and from 102 to 320 ml/kg for V_{area}. There was a strong and

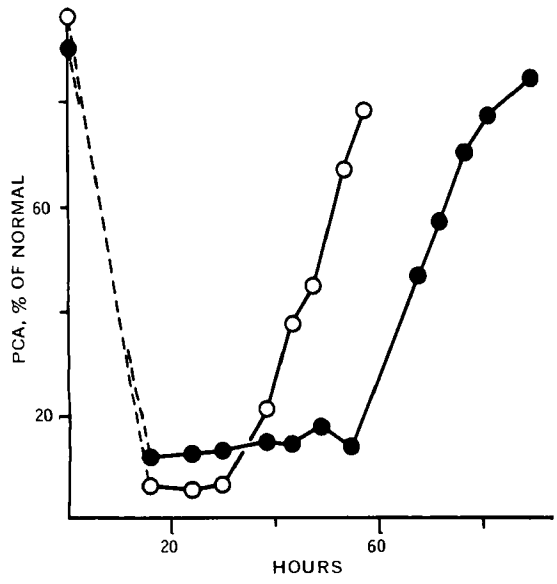

Figure 3—*PCA in the plasma of two rats, one a slow eliminator (●, Rat 2) and the other (○, Rat 5) a rapid eliminator of warfarin, as a function of time after intraperitoneal injection of ^{14}C-warfarin, 0.6 mg/kg body weight.*

[9] Endo Laboratories, Garden City, N.Y.

Table III—Relationship between Biological Half-Life and Duration of Hypoprothrombinemic Effect of Warfarin in Rats

Rat	Half-Life, hr	Time Required for Prothrombin Complex Activity to Return to 60% of Normal, hr
1	28.1	86[a]
2	27.7	72
3	26.6	84
4	20.8	81
5	10.1	50
6	8.0	36
7	6.1	43
8	5.1	34

[a] By extrapolation.

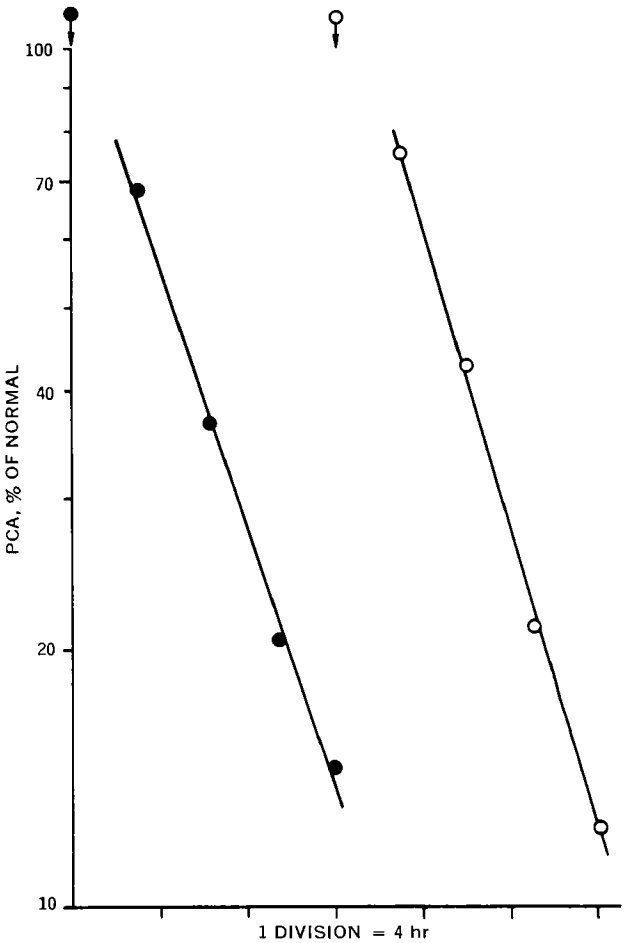

Figure 4—*Decrease in PCA as a function of time after blockade of the synthesis of vitamin K-dependent clotting factors by intraperitoneal injection of sodium warfarin, 12 mg/kg body weight. The arrows indicate the time of injection. Key: ●, Rat 2; and ○, Rat 5.*

Table IV—Normal Prothrombin Complex Activity (P^0), Rate Constant for Decline of Prothrombin Complex Activity (k_d), and Normal Synthesis Rate of Prothrombin Complex Activity (R^0_{syn}) in Rats

Rat	P^0, %	k_d, day^{-1}	R^0_{syn}, % day^{-1}
1	78.6	4.42	347
2	89.6	4.23	379
3	91.9	4.26	392
4	88.1	4.76	419
5	95.9	4.83	463
6	88.1	4.64	409
7	91.9	—[a]	—[a]
8	91.9	4.60	423
Mean	89.5	4.53	408
SD	5.1	0.24	31

[a] Rat 7 was ill at the time of this experiment.

highly statistically significant ($p < 0.001$) positive correlation between the apparent first-order rate constant for warfarin elimination (0.693/half-life) and the apparent volume of distribution of warfarin ($V_{intercept}$ or V_{area}) in individual rats.

The PCA in the plasma decreased rapidly after warfarin injection and eventually returned to normal (Fig. 3). The duration of this effect, as reflected by the time required for PCA to return to 60% of normal, increased with increasing warfarin half-life (Table III). The correlation between warfarin half-life and duration of anticoagulant effect was strong ($r = 0.946$) and highly statistically significant ($p < 0.001$). The prewarfarin PCA (P^0) in the eight rats averaged 89.5% [based on a standard curve obtained previously by determining the clotting time of serial dilutions of pooled plasma from six normal rats, with the PCA of the undiluted plasma defined as 100% of normal (9)]. The rate constant for decline of PCA (k_d) was determined from the slope of a plot (Fig. 4) of log PCA *versus* time after injection of a large dose of warfarin (*i.e.*, a dose sufficient to block the synthesis of vitamin K-dependent clotting factors) and showed very little intersubject variation (Table IV). There was no statistically significant correlation between the elimination rate constant for warfarin and either P^0, k_d, or the normal (prewarfarin) synthesis rate of PCA (R^0_{syn}).

There was an essentially linear relationship between the pharmacological effect of warfarin (expressed as the R_{syn} relative to R^0_{syn}) and the logarithm of the warfarin concentration in plasma at a given time (Fig. 5). Six of the eight animals yielded data that permitted the determination of the warfarin plasma concentration at which R_{syn} = 50% of R^0_{syn}. This concentration varied widely, from 0.052 to 1.37 μg/ml (Table V). A similar wide range was observed in the total radioactivity (reflecting the concentration of both warfarin and its metabolites) at this level of pharmacological activity. There was a strong ($r = 0.963$) and highly statistically significant ($p < 0.001$) correlation between the half-life of warfarin and the warfarin plasma concentration at which R_{syn} = 50% R^0_{syn} in individual rats. There was also a strong relationship between the half-life of warfarin and the concentration of warfarin metabolites (total radioactivity minus warfarin radioactivity) in plasma at the time when R_{syn} = 50% R^0_{syn} ($r = 0.949$, $p < 0.005$). However, it is unlikely that warfarin metabolites contribute significantly to the anticoagulant activity in rats. Of the six metabolites of warfarin identified in rats, only one (4′-hydroxywarfarin) is active, and it has only one-fourth the activity of warfarin itself (13).

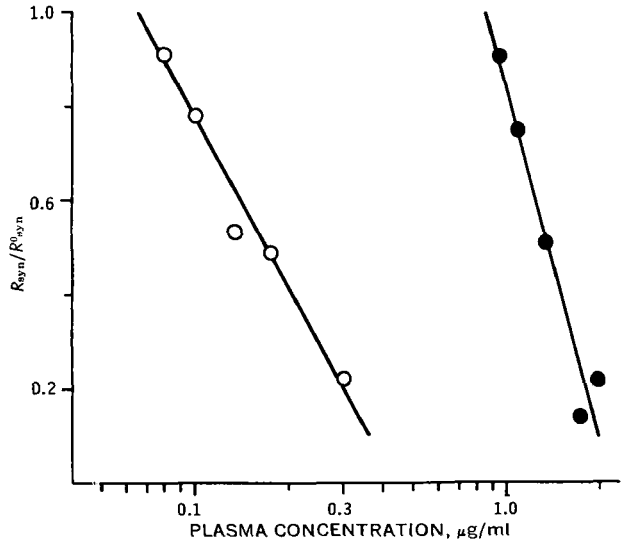

Figure 5—*Relationship between the relative synthesis rate of PCA* (R_{syn}/R^0_{syn}) *and the concentration of warfarin in the plasma of a slow eliminator* (●, *Rat 2*) *and a rapid eliminator* (○, *Rat 5*) *of warfarin after intraperitoneal injection of* ^{14}*C-warfarin, 0.6 mg/kg body weight.*

Table V—Relationship between Biological Half-Life of Warfarin and Plasma Concentration of Warfarin at which the Synthesis Rate of Prothrombin Complex Activity is Decreased by 50%

Rat[a]	Half-Life, hr	Warfarin Concentration in Plasma when R_{syn} = 50% R^0_{syn}, μg/ml[b]	Total Radioactivity in Plasma when R_{syn} = 50% R^0_{syn}, μg Apparent Warfarin/ml
2	27.7	1.37	1.48
3	26.6	0.88	0.98
4	20.8	0.78	0.84
5	10.1	0.17	0.20
6	8.0	0.16	0.20
8	5.1	0.052	0.084

[a] Timing of blood sampling was inadequate for determination of R_{syn} = 50% R^0_{syn} in Rats 1 and 7. [b] Obtained by interpolation from plots such as Fig. 5.

DISCUSSION

The results of these studies show a pronounced relationship between the distribution and elimination of warfarin in rats. This result is consistent with the results of previously reported studies on the elimination of another coumarin anticoagulant, dicumarol, by isolated perfused rat livers (14) and intact rats (15). Intersubject differences in the biological half-life of warfarin can also reflect differences in the activity of drug-metabolizing enzyme system(s), particularly due to enzyme induction, but preliminary data from ongoing experiments indicate that enzyme induction does not cause a change in the apparent volume of distribution of warfarin. It remains to be determined to what extent the activity of the warfarin-metabolizing enzyme system(s) and distributional factors each account for intersubject differences in the elimination of warfarin in humans.

An evaluation of published data on 14 healthy human subjects (1) suggests a possible relationship between the elimination rate constant and the apparent volume of distribution of warfarin in female subjects (four of five in rank order) but there is no such indication for the male subjects. However, one patient with an unusually short half-life of warfarin (6.1 hr) had an apparent volume of distribution of 14.8% of body weight (3), larger than that for 14 normal subjects (1) which ranged from 5.7 to 12.4% of body weight (by calculation of the authors) and larger than the apparent volumes reported by others (16) for nine experiments on six normal subjects (mean 11.4, range 9.0–13.5% of body weight). Pyörälä (6) reported that the mean half-life of warfarin in random-bred male Sprague–Dawley rats (18 hr) was shorter than in female rats (28 hr), and he observed that the mean estimated volume of distribution in male rats (13.2% of body weight) was statistically significantly larger than in female rats (11.4%).

The total clearance of warfarin in rats, as determined in this investigation, ranged from 2.60 to 43.0 ml kg^{-1} hr^{-1} (Table II) and may be compared to a much narrower range of 1.39–4.62 ml kg^{-1} hr^{-1} in six normal human adults (16). Because of this narrower range in humans, it is not surprising that available data from a total of only eight normal human subjects (10, 17) do not

reveal any significant relationship between the warfarin elimination rate constant and the plasma warfarin concentration required to elicit a certain degree of inhibition of R_{syn}. Such correlation is more likely to be evident in a group of human subjects consisting of individuals with widely differing warfarin half-lives not ascribable to exposure to enzyme inducers or inhibitors.

The results of this investigation demonstrate that unusually rapid or slow elimination of warfarin may be (at least in some cases) the result of, or associated with, unusually extensive or limited distribution of the drug in extravascular spaces. This, in turn, may affect markedly the relationship between warfarin concentration in the plasma and its anticoagulant effect, since this drug acts in the liver and not (like heparin) in the circulation. It is, therefore, unwise to adjust warfarin dosage regimens of patients solely on the basis of their warfarin half-life without considering the possibility that an unusually long or short warfarin half-life is associated with a change in the relationship between plasma warfarin concentration and intensity of anticoagulant effect. Subsequent reports will deal with the mechanism of the observed intersubject differences in the distribution of warfarin in the body.

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* Present address: Schools of Medicine and Engineering, University of Pittsburgh, Pittsburgh, Pa.

ˣ To whom inquiries should be directed.

Anticonvulsant Activity and Inhibition of Respiration in Rat Brain Homogenates by Substituted Oxadiazoles

SURENDRA S. PARMAR*‡ˣ, P. C. JOSHI‡, BASHEER ALI*‡, and WILLIAM E. CORNATZER*

Abstract □ Several 2,5-disubstituted 1,3,4-oxadiazoles were synthesized, characterized, and tested for their effectiveness in inhibiting the respiratory activity of rat brain homogenate. All substituted oxadiazoles and their precursors, thiosemicarbazides, were found to inhibit nicotinamide adenine dinucleotide- (NAD) dependent oxidations of pyruvate and α-ketoglutarate as well as the NAD-independent oxidation of succinate. Anticonvulsant activity, as exhibited by protection against pentylenetetrazol-induced seizures, with substituted thiosemicarbazides and the corresponding cyclized oxadiazoles ranged from 30 to 90% at a dose of 100 mg/kg. The degree of protection afforded by these compounds, however, was unrelated to their ability to inhibit oxidation of pyruvate, α-ketoglutarate, and succinate.

Keyphrases □ 1,3,4-Oxadiazoles, 2,5-disubstituted—synthesis, inhibition of NAD-dependent and NAD-independent oxidations, relationship to anticonvulsant activity □ Structure-activity relationships—2,5-disubstituted 1,3,4-oxadiazoles-anticonvulsant activity, rats □ Thiosemicarbazides, substituted—synthesis, anticonvulsant activity, inhibition of respiratory activity, rats □ Pyruvate, α-ketoglutarate, and succinate oxidation, inhibition—effects of substituted thiosemicarbazides and cyclized oxadiazoles □ Anticonvulsant activity—relationship to inhibition of respiratory activity, substituted thiosemicarbazides and oxadiazoles

Considerable interest has recently been focused on 2,5-disubstituted 1,3,4-oxadiazoles, which have been shown to possess analgesic (1, 2), central nervous system depressant (3), muscle relaxant (4–6), and tranquilizing (4, 5) properties. These observations prompted the synthesis of 2-arylamino-5-(4-*tert*-butyl-2-bromophenoxymethyl)-1,3,4-oxadiazoles. In the present study, the ability of these oxadiazoles and their precursors, thiosemicarbazides, to inhibit nicotinamide adenine dinucleotide- (NAD) depen-

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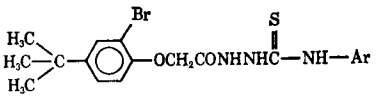
Table I—Physical Constants of 4-Aryl-1-(4-*tert*-butyl-2-bromophenoxyacetyl)thiosemicarbazides



Compound	Ar	Melting Point[a]	Yield, %	Formula	Analysis, %	Calc.	Found
I	C_6H_5	164–165°	85	$C_{19}H_{22}BrN_3O_2S$	C	52.29	52.30
					H	5.04	5.54
					N	9.63	9.34
II	*o*-CH_3-C_6H_4	85–86°	80	$C_{20}H_{24}BrN_3O_2S$	C	53.33	52.98
					H	5.33	5.30
					N	9.33	9.15
III	*m*-CH_3-C_6H_4	163°	85	$C_{20}H_{24}BrN_3O_2S$	C	53.33	53.07
					H	5.33	4.95
					N	9.33	9.30
IV	*p*-CH_3-C_6H_4	166°	90	$C_{20}H_{24}BrN_3O_2S$	C	53.33	53.56
					H	5.33	5.29
					N	9.33	9.43
V	*o*-CH_3O-C_6H_4	166°	90	$C_{20}H_{24}BrN_3O_3S$	C	51.50	51.35
					H	5.15	5.10
					N	9.01	9.23
VI	*p*-CH_3O-C_6H_4	142–143°	92	$C_{20}H_{24}BrN_3O_3S$	C	51.50	51.76
					H	5.15	5.33
					N	9.01	8.93
VII	*p*-Cl-C_6H_4	165–166°	90	$C_{19}H_{21}BrClN_3O_2S$	C	48.45	48.63
					H	4.46	4.01
					N	8.92	8.89
VIII	*p*-Br-C_6H_4	169–170°	90	$C_{19}H_{21}Br_2N_3O_2S$	C	44.26	44.03
					H	4.07	4.13
					N	8.15	8.24
IX	*p*-I-C_6H_4	170–171°	90	$C_{19}H_{21}BrIN_3O_2S$	C	40.56	40.32
					H	3.73	3.65
					N	7.47	7.33

[a] Melting points were taken in open capillary tubes and are corrected.

dent oxidation of pyruvate and α-ketoglutarate and NAD-independent oxidation of succinate was investigated. Anticonvulsant activity exhibited by thiosemicarbazides and their corresponding cyclized oxadiazoles was evaluated to correlate their pharmacological properties with their inhibitory effects on the respiratory activity of the rat brain homogenate. The various oxadiazoles were synthesized by following the methods outlined in Scheme I.

EXPERIMENTAL[1]

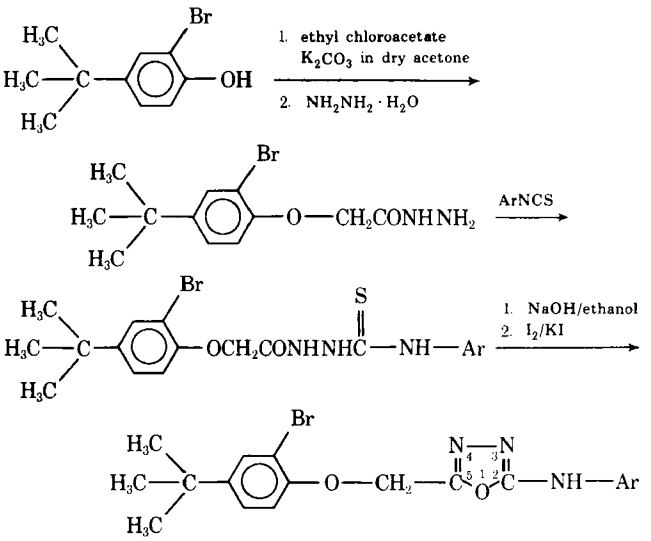
4-*tert*-Butyl-2-bromophenoxyacetylhydrazide—A mixture of 45.8 g (0.2 mole) of 4-*tert*-butyl-2-bromophenol (0.2 mole), 27.8 g of anhydrous potassium carbonate (0.2 mole), and 24.5 g of ethyl chloroacetate (0.2 mole) in 100 ml of dry acetone was refluxed on a water bath for 18–24 hr. The mixture was cooled and filtered. The excess solvent from the filtrate was removed by distilling under reduced pressure. To the residue was added 8 g of 8% $NH_2NH_2 \cdot H_2O$ (1.5 moles) and 50 ml of ethanol; this mixture was refluxed for 10–12 hr. Excess ethanol was distilled under reduced pressure. The crude product which separated on cooling was filtered and recrystallized from ethanol, yielding 36.0 g (60%), mp 122°.

Anal.—Calc. for $C_{12}H_{17}BrN_2O_2$: C, 47.84; H, 5.65. Found: C, 48.30; H, 5.70.

4-Aryl-1-(4-*tert*-butyl-2-bromophenoxyacetyl)thiosemicarbazide—The hydrazide (0.01 mole) and aryl isothiocyanate (0.01 mole) were mixed in 95% ethanol and refluxed on a water bath for 6 hr. The excess solvent was distilled under reduced pressure. The solid mass which separated out on cooling was filtered, dried, and finally recrystallized from ethanol. All thiosemicarbazides were characterized by their sharp melting points and elemental analyses (Table I).

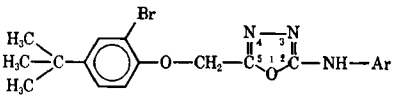
[1] Commercial chemicals were used in this study. Adenosine monophosphate, cytochrome c, α-ketoglutarate, sodium pyruvate, and sodium succinate were purchased from Sigma Chemical Co., St. Louis, Mo.

2-Arylamino-5-(4-*tert*-butyl-2-bromophenoxymethyl)-1,3,4-oxadiazoles—The thiosemicarbazides were cyclized to the corresponding 3,4-oxadiazoles by the method of Silberg and Cosma (7). To an ethanolic (300 ml) suspension of thiosemicarbazide (0.01 mole) was added sodium hydroxide (4 *N*, 5 ml) with cooling and shaking. Iodine in potassium iodide solution (5%) was added gradually with stirring to the clear solution until the color of iodine persisted at room temperature. The contents were then heated under reflux on a water bath, and more iodine solution was added carefully until a permanent color of iodine persisted. The reaction mixture was then poured into ice-cold water (500 ml), and the solid mass which separated was filtered and washed with water and then with carbon disulfide to remove the iodine. The crude product was finally recrystallized from dilute ethanol in the presence of animal charcoal (Table II).



Scheme I

Table II—Physical Constants of 2,5-Disubstituted 1,3,4-Oxadiazoles



Compound	Ar	Melting Point[a]	Yield, %	Formula	Analysis, %	Calc.	Found
X	C_6H_5	149–150°	55	$C_{19}H_{20}BrN_3O_2$	C	56.71	56.26
					H	4.97	4.29
					N	10.44	10.35
XI	o-CH_3-C_6H_4	129–130°	52	$C_{20}H_{22}BrN_3O_2$	C	57.69	57.36
					H	5.28	5.20
					N	10.09	9.80
XII	m-CH_3-C_6H_4	137–138°	60	$C_{20}H_{22}BrN_3O_2$	C	57.69	57.52
					H	5.28	5.19
					N	10.09	9.92
XIII	p-CH_3-C_6H_4	152–153°	53	$C_{20}H_{22}BrN_3O_2$	C	57.69	57.49
					H	5.28	5.33
					N	10.09	9.89
XIV	o-CH_3O-C_6H_4	157–158°	50	$C_{20}H_{22}BrN_3O_3$	C	55.55	55.15
					H	5.09	5.54
					N	9.72	10.03
XV	p-CH_3O-C_6H_4	153°	53	$C_{20}H_{22}BrN_3O_3$	C	55.55	55.32
					H	5.09	5.13
					N	9.72	9.50
XVI	p-Cl-C_6H_4	135–137°	49	$C_{19}H_{19}BrClN_3O_2$	C	52.33	52.46
					H	4.35	4.30
					N	9.62	9.89
XVII	p-Br-C_6H_4	156–157°	56	$C_{19}H_{19}Br_2N_3O_2$	C	47.40	47.40
					H	3.95	3.63
					N	8.73	8.43
XVIII	p-I-C_6H_4	158–159°	55	$C_{19}H_{19}IBrN_3O_2$	C	43.18	43.53
					H	3.59	3.58
					N	7.95	7.87

[a] Melting points were taken in open capillary tubes and are corrected.

Assay of Respiratory Activity of Rat Brain Homogenate—Male albino rats, kept on *ad libitum* diet, were used in all experiments. Rats weighing 150–200 g were sacrificed by decapitation. The brains were taken out immediately and homogenized[2] in the ratio of 1:9 (w/v) in 0.25 *M* cold sucrose. Respiratory activity was determined by measuring the oxygen consumption by the conventional Warburg manometric method at 37° with air as the gas phase. Fresh brain homogenate of healthy albino rats equivalent to 125 mg wet tissue weight was used in each flask. The reaction mixture, in a final volume of 3.0 ml, consisted of 20 m*M* Na_2HPO_4 buffer (pH 7.4), 6.7 m*M* $MgSO_4$, 1 m*M* AMP (sodium salt), 33 m*M* KCl, and 500 μg of cytochrome c. The central well contained 0.2 ml of 20% KOH solution. All thiosemicarbazides and oxadiazoles were used in the final concentration of 2 m*M* to study their effects on the oxidation of the various substrates used at a final concentration of 10 m*M*. The compounds were dissolved in propylene glycol (100%), and an equivalent amount of the solvent was added to the control vessels.

Determination of Anticonvulsant Activity—Anticonvulsant activity against pentylenetetrazol-induced seizures was determined in mice of either sex weighing 25–30 g. The mice were divided in groups of 10, keeping the group weights as near the same as possible. Various oxadiazoles and their precursor thiosemicarbazides were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compounds were injected intraperitoneally in a group of 10 animals at a dose of 100 mg/kg. Four hours after the administration of these compounds, the mice were injected with pentylenetetrazol, 90 mg/kg sc. This dose of pentylenetetrazol has been shown to produce not only convulsions in almost all untreated mice but also to exhibit 100% mortality over 24 hr. On the other hand, no mortality was observed over 24 hr in animals treated with 100 mg/kg alone of the test compounds. The mice were then observed for 60 min for the occurrence of seizures. An episode of clonic spasm persisting a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks or tremulousness was not counted. Animals devoid of threshold convulsions over 60 min were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of these oxadiazoles and their precursor thiosemicarbazides was represented as percent protection. The animals were then observed for 24 hr and their mortality was recorded.

[2] Potter-Elvehjem homogenizer.

RESULTS AND DISCUSSION

The effects of 4-aryl-1-(4-*tert*-butyl-2-bromophenoxyacetyl)-thiosemicarbazides in the *in vitro* respiratory activity using rat brain homogenate as the source of enzyme are recorded in Table III. All these thiosemicarbazides were found to inhibit NAD-dependent oxidation of pyruvic acid and α-ketoglutaric acid. These thiosemicarbazides, unlike other anticonvulsants belonging to the groups of quinazolones (8, 9), thiozolidones (10), and nitrobenzamides (11), also inhibited the NAD-independent oxidation of succinic acid, as was observed with phenothiazine (12) and salicylic acid derivatives (13).

Substituted oxadiazoles obtained by cyclization of the corresponding thiosemicarbazides exhibited inhibition (Table IV) of the respiratory activity, where such an inhibition was found to be of low order with the cyclized oxadiazoles. These results failed to provide any specific requirements in the molecular structure of the thiosemicarbazides and their corresponding cyclized oxadiazoles with respect to their ability to inhibit NAD-dependent and NAD-independent oxidations. All oxadiazoles exhibited a lower degree of inhibition of the oxidation of pyruvic acid, α-ketoglutaric acid, and succinic acid than the corresponding thiosemicarbazides, in good agreement with earlier observations (14). Protection observed against pentylenetetrazol-induced seizures reflected anticonvulsant activity of 4-aryl-1-(4-*tert*-butyl-2-bromophenoxyacetyl)thiosemicarbazides (Table III). All thiosemicarbazides possessed anticonvulsant activity, with the degree of protection ranging from 30 to 90%. Maximum protection was observed with the thiosemicarbazides having 2-methylphenyl and 4-bromophenyl substituents at position 1 of their phenyl nucleus. The cyclization of these thiosemicarbazides to corresponding oxadiazoles retained anticonvulsant effectiveness to a lesser degree, ranging from 30 to 70% (Table IV). Maximum protection of 70% was observed with 2-(4-bromophenylamino)-5-(4-*tert*-butyl-2-bromophenoxymethyl)-1,3,4-oxadiazole.

In the present study, all thiosemicarbazides and oxadiazoles

Table III—Anticonvulsant Activity and Inhibition of Respiratory Activity of Rat Brain Homogenate by 4-Aryl-1-(4-*tert*-butyl-2-bromophenoxyacetyl)thiosemicarbazides

Compound[a]	Anticonvulsant[b] Activity, % Protection	Pentylenetetrazol Mortality, %[c]	Inhibition of Respiratory Activity, %[d]		
			Pyruvate	α-Ketoglutarate	Succinate
I	70	30	81.4 ± 1.9	75.4 ± 1.8	82.6 ± 2.4
II	90	20	84.0 ± 1.9	78.5 ± 1.6	83.0 ± 2.2
III	50	20	88.4 ± 2.0	88.6 ± 2.3	94.3 ± 2.5
IV	40	20	75.2 ± 1.6	76.0 ± 1.9	84.3 ± 2.1
V	30	10	83.5 ± 1.6	74.2 ± 1.7	88.2 ± 2.0
VI	30	40	83.0 ± 1.8	76.2 ± 1.8	83.7 ± 1.8
VII	60	30	78.0 ± 1.7	73.9 + 1.6	81.3 ± 2.0
VIII	90	Nil	86.7 ± 2.0	80.5 ± 2.1	89.9 ± 2.0
IX	30	40	81.2 ± 2.0	75.9 ± 1.9	80.3 ± 1.9

[a] Compound numbers are as recorded in Table I. [b] Anticonvulsant activity was determined at a dose of 100 mg/kg as described under *Experimental*. [c] Represents mortality over 24 hr in each group of animals administered pentylenetetrazol. [d] Each experiment was done in duplicate. All values represent mean values of percent inhibition with ± standard error of the mean calculated from three separate experiments. Inhibition was determined by the decrease in the oxygen uptake/125 mg wet tissue weight/hr. Assay conditions are as indicated in the text. All compounds were used at a final concentration of 2 m*M*.

Table IV—Anticonvulsant Activity and Inhibition of Respiratory Activity of Rat Brain Homogenate by 2-Arylamino-5-(4-*tert*-butyl-2-bromophenoxymethyl)-1,3,4-oxadiazoles

Compound[a]	Anticonvulsant[b] Activity, % Protection	Pentylenetetrazol Mortality, %[c]	Inhibition of Respiratory Activity, %[d]		
			Pyruvate	α-Ketoglutarate	Succinate
X	30	20	60.9 ± 1.6	74.5 ± 1.7	84.3 ± 1.9
XI	50	Nil	56.0 ± 1.5	74.6 ± 1.7	71.0 ± 1.6
XII	50	30	49.4 ± 1.4	79.5 ± 2.0	87.8 ± 1.8
XIII	60	10	69.4 ± 1.7	85.0 ± 2.2	88.9 ± 2.1
XIV	30	30	27.1 ± 1.3	63.2 ± 1.4	39.6 ± 1.5
XV	60	50	63.6 ± 1.7	81.9 ± 2.0	72.5 ± 1.9
XVI	40	30	60.5 ± 1.8	79.5 ± 1.4	81.5 ± 2.1
XVII	70	10	54.7 ± 1.5	82.7 ± 1.6	59.2 ± 1.6
XVIII	60	10	65.8 ± 1.7	94.1 ± 2.4	57.0 ± 1.5

[a] Compound numbers are as recorded in Table II. [b] Anticonvulsant activity was determined at a dose of 100 mg/kg as described under *Experimental*. [c] Represents percent mortality over 24 hr in each group of animals administered pentylenetetrazol. [d] Each experiment was done in duplicate. All values represent mean values of percent inhibition with ± standard error of the mean calculated from three separate experiments. Inhibition was determined by the decrease in the oxygen uptake/125 mg wet tissue weight/hr. Assay conditions are as indicated in the text. All compounds were used at a final concentration of 2 m*M*.

possessing appreciable anticonvulsant activity showed low mortalities against pentylenetetrazol-induced death in the experimental animals (Tables III and IV). The unspecific inhibition of both the NAD-dependent and the NAD-independent oxidations indicates the possible sensitivity of these compounds toward both NADH-CoQ(oxido)reductase (Complex I) and succinate-CoQ(oxido)reductase (Complex II) of the electron-transport chain. It is possible that hydro CoQ-cytochrome c(oxido) reductase (Complex III) and/or cytochrome c-O_2(oxido)reductase (Complex IV) are also susceptible to these thiosemicarbazides and their cyclized oxadiazoles and may thus account for their ability to inhibit flavine adenine dinucleotide-dependent oxidation of succinate (NAD-independent) and NAD-dependent oxidation of pyruvate and α-ketoglutarate by rat brain homogenate. These studies warrant further investigations with purified enzyme preparations to elucidate the biochemical basis for the anticonvulsant activity of substituted oxadiazoles.

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[x] To whom inquiries should be directed.

Fluorescence of 6-Methoxyquinoline, Quinine, and Quinidine in Aqueous Media

STEPHEN G. SCHULMAN[x], ROSE MARIE THREATTE, ANTHONY C. CAPOMACCHIA, and W. LARRY PAUL

Abstract □ The absorption and fluorescence spectra of 6-methoxyquinoline, quinine, and quinidine were studied as a continuous function of pH. 6-Methoxyquinoline is a stronger base in the lowest excited singlet state, as reflected by the pH dependence of its fluorescence. The latter is used to calculate the rates and equilibria of proton exchange in the excited state. Quinine and quinidine, however, do not protonate during the lifetimes of their excited states, an observation attributed to the lower basicity of their aromatic heterocyclic nitrogen than that in 6-methoxyquinoline. The differences in basic properties between the alkaloids and 6-methoxyquinoline are employed to infer the ketonic character of the quinolinemethanol group of the alkaloids and the existence of an intramolecular hydrogen bond in quinine but not in quinidine.

Keyphrases □ 6-Methoxyquinoline nucleus—absorption and fluorescence spectra, pH dependence □ Quinine—absorption and fluorescence spectra, pH dependence □ Quinidine—absorption and fluorescence spectra, pH dependence □ Fluorescence spectra—6-methoxyquinoline nucleus, quinine, and quinidine, pH dependence

The cinchona alkaloids quinine and quinidine are derived from the 6-methoxyquinoline nucleus and are well known for their pharmaceutical applications (1), quinine as an antimalarial and quinidine as an antipyretic and depressant of cardiac fibrillation. These drugs are generally regarded as diastereomers of one another (1), and their pharmacological actions are thought to differ as a result of their geometric differences. The fluorescent properties of the doubly charged cation derived from quinine are well known, this species being the most widely employed as a standard for the determination of absolute fluorescence quantum yields (2–4). However, the fluorescences of other prototropic species derived from quinine as well as those of all species derived from quinidine have been less extensively investigated. At biological pH, the singly charged species derived from both quinine and quinidine are predominant in the ground electronic state. However, the species that fluoresces at biological pH may depend upon the following: the dissociation constant in the lowest excited singlet state (if prototropic equilibrium is attained during the lifetime of the lowest excited singlet state), the dissociation constant in the ground state (if proton exchange is too slow to occur during the lifetime of the excited state), and the rate constants for protonation and dissociation in the excited state (if proton exchange is comparable in rate with the fluorescence of the acid and conjugate base) (5).

Because of an interest in employing the fluorescences of quinine and quinidine as probes of the interactions of these drugs with biological macromolecules and cellular components, the present study of the pH dependences of their fluorescence spectra as well as that of the parent aromatic nucleus, 6-methoxyquinoline, was undertaken.

EXPERIMENTAL

6-Methoxyquinoline[1] was precipitated as the perchlorate salt from absolute ethanol solution and recrystallized three times from 90% ethanol. Quinine bisulfate[2] and quinidine sulfate[2] were triply recrystallized from 90% ethanol. Analytical reagent grade sulfuric acid[3] and sodium hydroxide[3] were used without further purification. Distilled, deionized water was employed to prepare solutions for spectroscopic measurement.

Absorption spectra were taken on a spectrophotometer[4]. Fluorescence spectra were taken on a fluorescence spectrophotometer[5] whose monochromators were calibrated against the xenon line emission spectrum and whose output was corrected for instrumental response by means of a rhodamine-B quantum counter. The pH measurements were made on a digital pH meter[6] employing a silver–silver chloride–glass combination pH electrode[7]. Fluorescence lifetimes were measured on a nanosecond decay time fluorometer[8], employing an 18-w pulsed deuterium lamp (pulse time 1.6 nsec) and a dual-beam oscilloscope[9] with two IAI plug-in dual-channel amplifiers. Lifetimes of excited states as short as 1.6 nsec could be measured with this apparatus. Absolute quantum yields of fluorescence were evaluated by the relative method using the quantum yield of the doubly charged quinine cation (0.55) as a standard (2).

The solutions used for determining the spectra were prepared by diluting sulfuric acid or sodium hydroxide in a 10-ml volumetric flask and then delivering 0.10 ml of about 1×10^{-3} *M* aqueous stock solution of 6-methoxyquinoline, quinine, or quinidine, with a micropipet, to the 10-ml flask. Each solution was prepared immediately prior to taking the spectra to minimize decomposition errors and errors due to pH changes of the poorly buffered solutions in the middle pH range.

Dilute sulfuric acid and sodium hydroxide solutions were employed in preference to fairly concentrated buffer solutions, because buffer ions are known to react with excited aromatic acids and bases during the lifetime of the lowest excited singlet state (6). This reaction does not, of course, affect the thermodynamics of proton exchange in the excited state but does often affect the mechanism and, therefore, the kinetics of excited-state proton transfer. It was deemed desirable, at least initially, to avoid this added complication.

RESULTS

The absorption and fluorescence spectral maxima of the various species derived from 6-methoxyquinoline, quinine, and quinidine are presented in Table I. The ground-state dissociation constants for the prototropic equilibria between the singly charged cation and neutral molecule derived from 6-methoxyquinoline and between the doubly and singly charged cations derived from

[1] K and K Laboratories, Plainview, N.Y.
[2] Merck and Co., Rahway, N.J.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Beckman DB-GT.
[5] Perkin-Elmer MPF-2A.
[6] Orion model 801.
[7] Corning.
[8] TRW.
[9] Tektronix 556.

Table I—Absorption (λ^1L_a and λ^1L_b) and Corrected Fluorescence (λ_f) Maxima of the Various Prototropic Species Derived from 6-Methoxyquinoline, Quinine, and Quinidine

Compound	$\lambda_{^1L_a}$, nm	$\epsilon_{^1L_a}$, nm	$\lambda_{^1L_b}$, nm	$\epsilon_{^1L_b}$, nm	λ_f, nm
6-Methoxyquinoline:					
Cation (pH 2.0)	314	4.10×10^3	338	3.83×10^3	438
Neutral molecule (pH 13.2)	272	2.80×10^3	325	3.65×10^3	362
Quinine:					
Dication (pH 2.0)	317	4.33×10^3	347	5.40×10^3	448
Monocation (pH 7.0)	281	3.35×10^3	331	4.93×10^3	382
Neutral molecule (pH 11.0)	281	3.68×10^3	330	4.83×10^3	380
Quinidine:					
Dication (pH 2.0)	317	4.30×10^3	347	5.39×10^3	447
Monocation (pH 7.0)	281	3.40×10^3	331	5.00×10^3	380
Neutral molecule (pH 11.0)	281	3.61×10^3	331	4.89×10^3	380

quinine and quinidine correspond to dissociation from the nitrogen atom of the quinoline ring and, therefore, affect the absorption spectra. These dissociation constants were determined spectrophotometrically (Table II) under the experimental conditions employed here and were found to be in good agreement with those previously reported (7). The equilibria between the singly charged cations and the neutral molecules derived from quinidine and quinine correspond to dissociation from the quinuclidine nitrogen atoms, which are three atoms removed from the aromatic ring and sterically prohibited from interaction with the aromatic ring. Therefore, they do not affect the electronic structure of the aromatic system or the electronic absorption spectra to an extent that permits the determination of pKa. Consequently, these dissociations were studied potentiometrically. The equilibrium constants derived from the potentiometric measurements (Table II) were also in good agreement with the literature values (7).

In each case, fluorescence was excited at an isosbestic point in the absorption spectrum of each compound to eliminate the effect of absorption on the fluorometric titrations and to make the latter correspond only to variations of fluorescence efficiency with pH.

The fluorometric titrations of the cation and neutral molecule derived from 6-methoxyquinoline are represented in Fig. 1. At pH < 4, only the blue-green emission of the cation is observed. From pH 4 to 6, the fluorescence of the cation falls to 0.41 of its maximum intensity (at pH 4) and the violet fluorescence of the neutral species appears and rises to 0.85 of its maximum value (at pH 12). From pH 6 to 10, the fluorescences of the cation and neutral molecule remain constant. However, in the interval from pH 10 to 13 the cation emission is completely quenched and the fluorescence of the neutral species rises to a maximum at pH 12 and then falls sharply with further increase in pH.

In contrast to the rather complex fluorometric titration behavior of 6-methoxyquinoline, the dependences of the relative quantum yields of fluorescence of the various species derived from quinine (Fig. 2) and quinidine upon pH are fairly straightforward. Below pH 2.5, only the fluorescences of the doubly protonated cations are apparent. These emissions fall in intensity from pH 2.5 to 6.5, vanishing completely at pH 6.5. In this interval, the fluorescences of the singly protonated cations appear and rise with increasing pH to a maximum at pH ~ 6.5. The inflection points of the fluorometric titrations of quinine and quinidine in the 2.5–6.5 pH region coincide with the ground-state pKa values for the dissociations occurring in this pH region. In the interval from pH 7 to 11, the fluorescences of the singly charged cations derived from quinine and quinidine are quenched. The inflection points in these regions of the fluorometric titration curves coincide with the ground-state pKa values for conversion of the singly protonated species to the neutral molecules. At pH 11 the maximum of the fluorescence of quinine has shifted 2 nm to a shorter wavelength relative to that at pH 7 and its quantum yield is 0.018 of the fluorescence efficiency at pH 7. The fluorescence maximum of quinidine does not change on going from pH 7 to 11, but the quantum yield of fluorescence decreases by a factor of 0.032. At pH higher than 11, the fluorescences of quinine and quinidine are quenched more gradually with increasing pH. However, quenching is not complete at pH 14.0.

The fluorescence efficiencies and lifetimes of the lowest excited singlet states of the various prototropic species derived from 6-methoxyquinoline, quinine, and quinidine were measured at pH values where the fluorescences of each species were maximal and isolated (pH 2.0 and 12.2 for 6-methoxyquinoline and pH 2.0, 7.0, and 11.0 for quinine and quinidine) (Table III). Because of the quenching of neutral 6-methoxyquinoline fluorescence by hydroxide ion at high pH, it is believed that the fluorometric titration curve of the latter emission does not correspond to only an acid–base reaction and, therefore, that the maximum emission intensity (at pH 12) from which ϕ_0 and τ_0 (Table III) were measured does not give their true values for the neutral molecule. The fluorometric titration of the 6-methoxyquinolinium cation does, however, appear to be well behaved. Consequently, the theoretical fluorometric titration curve of the neutral molecule (as if only proton exchange occurred) was constructed by plotting $1 - (\phi'/\phi_0')$ against pH, where ϕ'/ϕ_0' is the relative quantum yield of fluorescence of the 6-methoxyquinoline cation. The true values of ϕ_0 and τ_0 for the neutral species were calculated from the measured values and the ratio of the relative fluorescence intensities of the theoretical fluorometric titration to that of the actual titration at

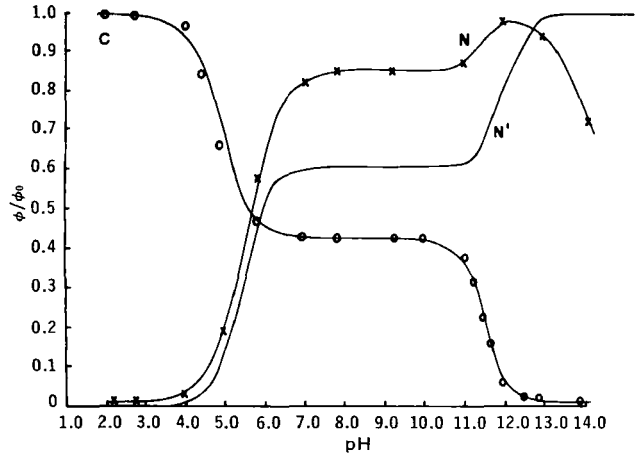


Figure 1—*The pH dependences of the relative quantum yields of fluorescence of the neutral molecule (N) and cation (C) derived from 6-methoxyquinoline. The curve represented by N is the experimental fluorometric titration curve of the neutral molecule. That represented by N′ is the theoretical fluorometric titration of the neutral molecule (in the absence of OH^- quenching at high pH).*

Table II—Dissociation Constants for the Ground-State Prototropic Equilibria between the Cation and Neutral Molecule Derived from 6-Methoxyquinoline and the Dications and Monocations Derived from Quinine and Quinidine (pKa_1) and between the Monocations and Neutral Molecules Derived from Quinine and Quinidine (pKa_2)

	pKa_1	pKa_2
6-Methoxyquinoline	5.13	
Quinine	4.34	8.43
Quinidine	4.34	8.79

Table III—Quantum Yields of Fluorescence (ϕ_0) and Lifetimes of the Fluorescing States (τ_0) of the Various Species Derived from 6-Methoxyquinoline, Quinine, and Quinidine

Compound	ϕ_0	τ_0, sec $\times 10^{-9}$
6-Methoxyquinoline:		
Cation (pH 2.0)	0.51	23.7[a]
Neutral molecule (pH 12.2)	0.16 (0.23)[b]	7.6 (10.8)[b]
Quinine:		
Dication (pH 2.0)	0.55	23.6[7]
Monocation (pH 7.0)	0.50	13.4
Neutral molecule (pH 11.0)	0.009	—[d]
Quinidine:		
Dication (pH 2.0)	0.50	24.6
Monocation (pH 7.0)	0.43	19.9
Neutral molecule (pH 11.0)	0.014	—[d]

[a] τ_0 = 22.8 nsec was reported (4) for 6-methoxyquinolinium ion. [b] Values in parentheses are corrected for OH^- quenching (see text). [c] τ_0 = 19.0 nsec was reported (4) for quinine dication. [d] The fluorescences of quinine and quinidine were too weak at pH 11.0 for their lifetimes to be determined with the apparatus.

pH 12.2. The corrected values of ϕ_0 and τ_0 are also given in Table III and were employed in all calculations.

DISCUSSION

The shifts in the long wavelength absorption and fluorescence maxima of 6-methoxyquinoline indicate that this compound is about seven orders of magnitude more basic in the lowest excited singlet state than in the ground state (6). This means that, since pKa ~5, if equilibrium is complete during the lifetime of the excited state, only the fluorescence of the cation should be observed in the 7–10 pH interval even though the neutral molecule is the only species excited in this interval. Alternatively, if proton exchange is too slow to occur at all during the lifetime of the excited state, the fluorometric titration characteristics should be identical with the ground-state titration characteristics and only the fluorescence of the neutral molecule should be observed at pH > 7. The observation of fluorescence from both the cation and the neutral molecule derived from 6-methoxyquinoline over the long pH interval from 3 to 13 is indicative of partial establishment of equilibrium in the lowest excited singlet state (8). Thus, in the 7–10 pH interval where only the neutral molecule is excited and only the cation is thermodynamically stable, a steady state is established in which a fraction (0.41) of the excited neutral molecules is converted to the cation while the remaining fraction (0.59) fluoresces from the neutral form before equilibrium in the excited state is fully established. At pH > 12, the excited cation

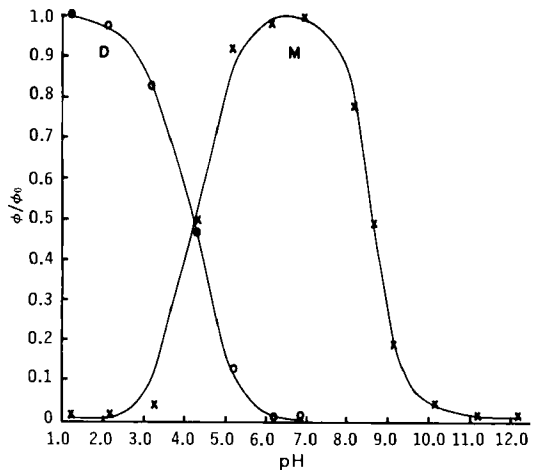
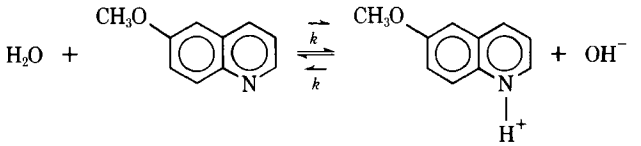


Figure 2—*The pH dependences of the relative quantum yields of fluorescence of the dication (D) and the monocation (M) derived from quinine. The fluorescence of the neutral molecule is almost identical in position with that of the monocation but much weaker in intensity.*

becomes thermodynamically unstable, so virtually all of the neutral molecules excited fluoresce as such.

Because the excited-state proton exchanges and the fluorescences of the two species derived from 6-methoxyquinoline are temporally competitive, measurement of the lifetimes of the excited singlet states (the lifetimes of the isolated species in the absence of reaction with H^+ or OH^-) permits evaluation of the kinetics of proton exchange (Scheme I and Eqs. 1 and 2) in the excited state from the fluorometric titration curve according to the steady-state approach developed by Weller (8). For the reaction:



Scheme I

$$\phi/\phi_0 = \frac{1 + \overleftarrow{k}\tau_0'[OH^-]}{1 + \vec{k}\tau_0 + \overleftarrow{k}\tau_0'[OH^-]} \quad \text{(Eq. 1)}$$

and:

$$\phi'/\phi_0' = \frac{\vec{k}\tau_0}{1 + \vec{k}\tau_0 + \overleftarrow{k}\tau_0'[OH^-]} \quad \text{(Eq. 2)}$$

where ϕ/ϕ_0 is the relative quantum yield of fluorescence of the neutral molecule at any point in the fluorometric titration (ϕ/ϕ_0 is unity at pH > 13 and zero at pH < 3 for 6-methoxyquinoline), ϕ'/ϕ_0' is the relative quantum yield of fluorescence of the cation at any point in the titration (for the 6-methoxyquinolinium cation, ϕ'/ϕ_0' = 1 when pH < 3 and ϕ'/ϕ_0' = 0 when pH > 13), $\vec{k}$ is the pseudo-first-order rate constant for protonation of the neutral molecule in the excited state by the solvent, $\overleftarrow{k}$ is the bimolecular rate constant for deprotonation of the cation by the hydroxide ion, and τ_0 and τ_0' are the lifetimes of the lowest excited singlet states when ϕ/ϕ_0 = 1 and when ϕ'/ϕ_0' = 1, respectively. The equilibrium between the neutral molecule and the cation, in the excited state, is written as a reaction of the cation with hydroxide ion rather than of the neutral molecule with hydrogen ion because in the basic region, where the reaction occurs, the hydrogen ion is in too low a concentration to be responsible for the observation of a diffusion-controlled reaction during the short lifetimes of the excited states concerned here. This, of course, does not affect the thermodynamic aspect of the reaction (pKa*) but is of mechanistic consequence.

Although Eqs. 1 and 2 indicate that ϕ/ϕ_0 and ϕ'/ϕ_0' are continuously dependent upon $[OH^-]$, both relative quantum yields are constant in the 7–10 pH interval. This is a consequence of the relative magnitudes of the terms 1 and $\overleftarrow{k}\tau_0'[OH^-]$ in Eqs. 1 and 2. In order for the relative quantum yields of fluorescence to vary with $[OH^-]$ measurably, $\overleftarrow{k}\tau_0'[OH^-]$ must be greater than or comparable in magnitude to 1. Now, τ_0 and τ_0' are generally 10^{-9}–10^{-7} sec, while $\vec{k}$ depends on the proton affinity of the excited conjugate base and $\overleftarrow{k}$ is usually 10^{11}–10^9 M^{-1} sec^{-1} for diffusion-limited protonation of bases. Consequently, at very low values of $[OH^-]$ (*i.e.*, in the mid pH range), $\overleftarrow{k}\tau_0'[OH^-]$ is usually much smaller than 1. As a result, when $[OH^-]$ is very low, Eqs. 1 and 2 reduce to:

$$(\phi/\phi_0)_{const} = \frac{1}{1 + \vec{k}\tau_0} \quad \text{(Eq. 3)}$$

and:

$$(\phi'/\phi_0')_{const} = \frac{\vec{k}\tau_0}{1 + \vec{k}\tau_0} \quad \text{(Eq. 4)}$$

which are, of course, independent of pH (*i.e.*, the appearance of cation fluorescence is governed exclusively by the forward reaction) and from which $\vec{k}$ may be immediately calculated from the value of τ_0 given for 6-methoxyquinoline (neutral) in Table I and the value of $(\phi/\phi_0)_{const}$ or $(\phi'/\phi_0')_{const}$ (0.59 and 0.41, respectively). The value of $\vec{k}$ for 6-methoxyquinoline was found to be 6.4 × 10^7 sec^{-1}. This value was then employed in Eq. 2 along with the

observed values of ϕ'/ϕ_0' at several values of [OH^-] in the pH 10–13 region (where the back-reaction as well as the forward-reaction affects the relative fluorescence efficiencies) to calculate the average value of $\bar{k}$ of $2.6 \times 10^{10}\ M^{-1}\ sec^{-1}$. From $\bar{k}$ and $\bar{k}$, one obtains the hydrolysis constant $K_b^* = (\bar{k}/\bar{k}) = 4.2 \times 10^{-3}$. Thus, pKa* = 11.40, in excellent agreement with the value of 11.5 calculated from the Förster cycle (6) employing the absorption and fluorescence maxima of Table I and the pKa value for 6-methoxyquinoline in Table II. It should be borne in mind here that the values of ϕ/ϕ_0 and ϕ'/ϕ_0' used in these calculations were taken only from the fluorometric titration curve of the 6-methoxyquinolinium cation. The fluorometric titration curve of the neutral 6-methoxyquinoline molecule shows not only an acid-base reaction but also some unspecified quenching reaction at high pH. Thus, whereas the cation emission demonstrates a clean titration curve close to the theoretical behavior predicted by Eq. 2, the fluorescence of the neutral molecule is quenched by OH^- before the acid-base titration is complete, giving apparently too short an acid-base titration interval and too high a value of ϕ/ϕ_0 (Fig. 1).

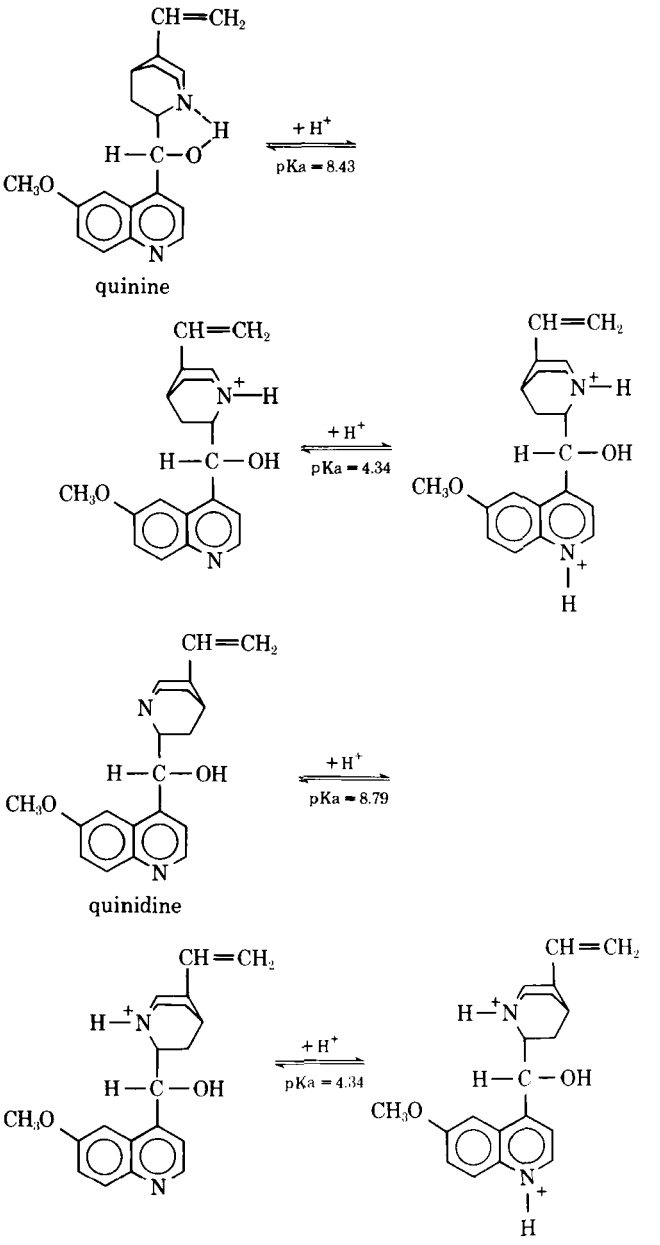
By comparison with 6-methoxyquinoline, quinine and quinidine are unusual in their ground-state chemistry and photochemistry. The ground-state pKa values of these compounds are considerably less basic than would be anticipated. The pKa of quinuclidine is 10.6 (9). However, in quinine and quinidine, the quinuclidine nitrogen atoms are 2.2 and 1.8 orders of magnitude less basic, respectively, suggesting that the quinoline methanol group is electron withdrawing. Moreover, the nitrogen atoms of the quinoline rings of quinine and quinidine are both 0.8 order of magnitude less basic than that of 6-methoxyquinoline, indicating the electron-withdrawing effect of the exocyclic group in the 4-position. The low basicities of the quinuclidine nitrogen atoms of the alkaloids are surprising, because they are three atoms removed from the quinoline ring and would not be expected to be diminished as much as two orders of magnitude in basicity solely by hyperconjugation of the methanolic carbon atom with the quinoline ring. The low basicities of the quinoline nitrogen atoms of the alkaloids are surprising, because even with the quinuclidine nitrogen atoms protonated, it is to be expected that the effect of the substituent in the 4-position of the quinoline ring would be hyperconjugative, similar to a methyl group in the same position, and should therefore be base strengthening [*e.g.*, the pKa of quinoline is 4.94 (10) but that of 4-methylquinoline is 5.34 (11)].

The anomalous basicities of the quinuclidine and quinoline nitrogen atoms of quinine and quinidine can both be explained by considering the hyperconjugation of the carbon atom attached to the 4-position of the quinoline ring as a partial oxidation. This would confer some ketonic character upon the C—O bond, which should reduce the basicity of the quinuclidine nitrogen by virtue of the electron-withdrawing influence nearby. Moreover, the polarization of the O—H bond resulting from an increase of the order of the C—O bond would enhance the hydrogen-bonding capability of the O—H proton, which is not normally strongly hydrogen bonding. The presence of a reasonably strong intramolecular hydrogen bond between the O—H proton and the quinuclidine nitrogen in the neutral molecule derived from quinine (but not quinidine) would stabilize the neutral quinine molecule, making it even more difficult to protonate, and thereby account for the substantial difference between the pKa values of the quinuclidine nitrogen atoms of quinine and quinidine (Scheme II).

Protonation of the quinuclidine nitrogen atom of quinine and quinidine introduces a positively polarizing influence on the H—C—OH group and makes it more electron withdrawing than in the uncharged species. This is transmitted with considerable strength to the quinoline nitrogen atom through the aromatic π-system with which the H—C—OH group is hyperconjugated, accounting for the reduction in basicity of singly protonated quinine and quinidine.

That the fluorescences of the doubly charged cations derived from quinine and quinidine are converted, with increasing pH, to those of the singly charged cations with ground-state titration characteristics (Fig. 2) indicates that the protonations of the singly charged cations by water in the excited state are much slower than the rates of fluorescence of the latter species. Since the fluorescence lifetimes of the monocations of quinine and quinidine are greater than those of 6-methoxyquinoline (neutral), the rates of deactivation of the former are lower than those of the latter. Thus, the rates of protonation of the excited quinine and quinidine monocations (which cannot be calculated from their fluorometric titration curves) must be substantially lower than those of the 6-methoxyquinoline molecule. This can be accounted for at least partially by the pKa* values (pKa* = 9.7 for quinine dication $\rightleftarrows$ quinine monocation and pKa* = 9.9 for quinidine dication $\rightleftarrows$ quinidine monocation) estimated from the Förster cycle, employing the corresponding ground-state pKa values and the averages of absorption and emission maxima. The Bronsted relation states that for a single proton transfer, the rate of hydrolysis of a related family of bases decreases with decreasing pKa of the conjugate acid. Thus, quinine and quinidine, which have pKa* values lower than that of 6-methoxyquinoline by 1.9 and 1.7 log units, respectively, would be expected to have hydrolysis rate constants in the excited state between 0 and 1.9 orders of magnitude lower than 6-methoxyquinoline. Whether or not this is sufficient to account completely for the failure of quinine and quinidine to demonstrate measurable excited-state ionization cannot be stated with certainty at present.

The quenching with increasing pH of the fluorescences of the singly charged cations derived from quinine and quinidine follows the ground-state titration characteristics for conversion of the singly charged cations to the respective neutral molecules. The neutral molecules, therefore, fluoresce much less intensely than



Scheme II

their singly protonated counterparts. In *n*-hexane, however, the neutral molecules and the respective singly charged cations were observed to fluoresce with about the same intensities. This suggests that the diminished quantum yields of fluorescence of the neutral molecules in water are not due to the inherent molecular structures of these species but rather to some interaction with the excited-state solvent cage, presumably hydrogen bonding at the quinuclidine nitrogen atoms. Excited-state hydrogen bonding is known to quench the fluorescences of a wide variety of molecules, presumably by coupling the molecule to the solvent quasilattice. The vibrational modes of the solvent quasilattice provide an efficient means of radiationless deactivation of the lowest excited singlet state. That the dissociations and solvent interactions of the quinuclidine nitrogen atoms have such a dramatic effect upon the fluorescent properties of quinine and quinidine indicates substantial electronic coupling in the lowest excited singlet state between the aromatic and aliphatic portions of the alkaloids. Normally, the solvent interactions and prototropic exchanges involving functional groups not directly bonded to the aromatic fluorophore or sterically free to interact directly with the fluorophore (12, 13) do not have much effect upon the fluorescence arising from the fluorophore.

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[x] To whom inquiries should be directed.

Electron Spin Resonance Studies of Free Radicals in Solution III: pH Dependence of Thiyl Free Radical of Cysteine

JEAN C. KERTESZ, MICHAEL B. WOLF, WALTER WOLF[x], and LI-YA A. CHEN

Abstract □ An electron spin resonance flow technique was utilized to follow the concentration of thiyl free radicals (RS·) generated upon the oxidation of cysteine with ceric ion over the 0.25–7.5 pH range. The thiyl free radical concentration was seen to decrease with increasing pH in a manner dependent on the extent of ionization of cysteine. The formation of thiyl free radicals was favored by the proportion of thiol present in the positively charged form. The diminution of RS· spin concentration at physiological pH was correlated with the increasing prevalence of singly and doubly negative thiolate ions. At acidic pH's below 1.5, thiyl free radical formation was depressed by solutions of high ionic strength. The utility of this technique in studying the potential role of thiyl free radicals in radioprotective processes is indicated.

Keyphrases □ Free radicals in solution—pH dependence of thiyl free radical formation from oxidation of cysteine, electron spin resonance □ Cysteine oxidation—pH dependence of thiyl free radical formation □ Electron spin resonance—monitoring, pH dependence of thiyl free radical formation from oxidation of cysteine

Recent studies in this laboratory identified and characterized thiyl (RS·) and related free radicals in aqueous solution by electron spin resonance (ESR) spectroscopy when they are generated by ceric-ion oxidation of various thiols at low pH (1–6). Since sulfhydryl compounds and thiyl free radicals exhibit roles of considerable importance in the mechanisms of radiation damage and radioprotective processes (7–12), it was of special interest to determine whether the free radicals observed at acidic pH's may also be detected and similarly characterized at physiological pH's (6.8–7.6). Since thiyl free radical signals are observed under steady-state flow conditions, it was necessary to modify the flow method so that as the ESR spectra are recorded the concomitant pH of the reaction mixture can be measured. This paper presents the results of a systematic investigation of the variation of the thiyl free radical spin concentration observed upon the oxidation of cysteine with increasing pH.

EXPERIMENTAL

The ESR continuous-flow system used was described in detail elsewhere (13). A flow rate of 1.0 ml/sec was used uniformly throughout the experiments. ESR spectra were recorded with a ESR spectrometer[1] with associated 4-in. magnet, which operated at an X-band microwave frequency of 9.5 GHz and a modulation frequency of 100 KHz. Each sample was recorded at a modulation amplitude of 1.6 gauss and a microwave power of 200 mw on 50-gauss field scans. There was no evidence of power saturation of the spectra studied in this work. A 1-sec filter was routinely used

[1] Varian model E-3.

their singly protonated counterparts. In *n*-hexane, however, the neutral molecules and the respective singly charged cations were observed to fluoresce with about the same intensities. This suggests that the diminished quantum yields of fluorescence of the neutral molecules in water are not due to the inherent molecular structures of these species but rather to some interaction with the excited-state solvent cage, presumably hydrogen bonding at the quinuclidine nitrogen atoms. Excited-state hydrogen bonding is known to quench the fluorescences of a wide variety of molecules, presumably by coupling the molecule to the solvent quasilattice. The vibrational modes of the solvent quasilattice provide an efficient means of radiationless deactivation of the lowest excited singlet state. That the dissociations and solvent interactions of the quinuclidine nitrogen atoms have such a dramatic effect upon the fluorescent properties of quinine and quinidine indicates substantial electronic coupling in the lowest excited singlet state between the aromatic and aliphatic portions of the alkaloids. Normally, the solvent interactions and prototropic exchanges involving functional groups not directly bonded to the aromatic fluorophore or sterically free to interact directly with the fluorophore (12, 13) do not have much effect upon the fluorescence arising from the fluorophore.

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[1] Varian model E-3.

for scanning experiments. All spectra were recorded when the reaction mixtures were at 23.5 ± 0.3°.

All reagent solutions were prepared in distilled water. *l*-Cysteine hydrochloride[2] and the ceric salt, ceric potassium nitrate[3] [$CeK_2(NO_3)_6$], were used without further purification. A stock solution of 1 *N* HCl was prepared from 37% hydrochloric acid[4] for subsequent dilutions. Analytical reagent grade sodium hydroxide[4] was used.

The ceric potassium nitrate was readily water soluble, yielding a pH of 2.0 at a concentration of 0.005 *M*, the acidity due to hydrolysis of the salt. The pH of ceric-ion solutions could not be raised above 4 upon addition of sodium hydroxide prior to placement in the flow system, since a flocculent precipitate of cerium hydroxides resulted. Thus, the pH of the resultant reaction mixture could be adjusted only by addition of the appropriate concentrations of sodium hydroxide or hydrochloric acid to the initial cysteine solutions. Since, in neutral or basic aqueous solutions, thiols are oxidized by the air quite readily to the disulfides (14) or higher oxidation products (15), it was important that the ESR spectra be recorded as soon as possible after dissolution of the reagents. For this reason, the hydrochloride salt of cysteine was used routinely throughout the experiments, rather than the free base, because the latter dissolves much more slowly in neutral solutions. The concentration of sodium hydroxide required for the basic pH's studied was adjusted to neutralize the additional acid introduced by the hydrochloride salt form of cysteine. Thus, upon dissolution of reagents the solutions were rapidly removed to the flow system to avoid autoxidation of thiol and autoreduction of ceric ion which is catalyzed by glass surfaces upon standing (16, 17).

In a few experiments, a ceric-citrate complex was used which prevented the ceric ion from precipitating at pH 5. The intensity of the RS· triplet signals observed at this pH was equivalent to those obtained in the absence of the citrate anions. This confirmed that the strength of the ceric-ion solutions at the time of reaction in the flat cell at the higher pH's was not diminished by the subsequent formation of hydroxide precipitates as the reaction mixture flowed from the cavity.

Vanadyl sulfate double hydrate[5] crystals were purified (18) by repetitive washings with methanol and drying under vacuum. Standard vanadyl sulfate solutions were prepared from the purified crystals dissolved in 1 *M* H_2SO_4, which prevents oxidation of the VO^{+2} ion, and were used as ESR spin concentration primary standards. The procedure for estimating free radical concentrations based on double integration of the ESR first derivative signal, adapted from the numerical method of Poole (19), will be published in detail elsewhere. At high pH values, the ESR spectra were somewhat noisy, and integration of the signals led to more ambiguous results. However, simultaneous recording of the signals with a multichannel analyzer[6] allowed suitable intensity enhancement.

All pH measurements were made with a pH meter[7], calibrated with pH 4 and 7 buffer solutions[8]. A flow method was devised to determine the pH of the reaction mixtures exiting from the ESR flat cell. Initially, a method utilizing a pH microblood assembly[8] was tested. This equipment bypassed fluids from the flat cell exit through a narrow polyethylene tube leading to a stream directly across the electrodes. A narrow diameter tubing was used to reduce the dead volume, so that pH readings as close as possible to those at the actual time of reaction in the flow cell could be recorded. Unfortunately, this arrangement obviated consistent determinations at the higher pH values, since cerium oxide precipitates obstructed the path of flow. This reduced the flow rate, affected the stability of the electrodes, and altered the true proportions of the reaction mixtures. An alternative method in which the flow path was unrestricted was then used. The pH was determined by flowing the reaction mixture exiting from a 0.63-cm (0.25-in.) i.d. tube over a semimicrocombination electrode with triple-purpose glass membrane immersed in a filter flask. This electrode reaches a stable reading within 30 sec, and the pH is monitored while the ESR readings are recorded over the entire range of pH studied, albeit at a dead time of 154 sec after reaction has occurred in the ESR flat cell.

[2] Fox Chemical Co., lot F-1011.
[3] K & K Laboratories.
[4] Mallinckrodt analytical reagents.
[5] Fischer Scientific Co.
[6] Hewlett Packard 5400 A.
[7] Corning model 10.
[8] Beckman.

Table I—Dependence of RS· Spin Concentration on pH and Ionic Strength

pH	Concentration of Acid or Base in Reaction Mixture	RS· Spins/mole × 10^7 *M*	Ionic Strength, *M*
0.25	3.00000 *N* HCl	10.92	3.040
0.60	1.50000 *N* HCl	13.42	1.540
0.80	1.00000 *N* HCl	15.24	1.040
1.50	0.25000 *N* HCl	17.86	0.290
2.10	0.00000 *N* HCl	14.62	0.0375
3.00	0.01000 *N* NaOH	11.43	0.0425
3.60	0.01500 *N* NaOH	9.95	0.0525
4.60	0.01625 *N* NaOH	9.04	0.05375
5.00	0.01650 *N* NaOH	8.13	0.0540
6.10	0.01875 *N* NaOH	6.81	0.0563
6.70	0.02500 *N* NaOH	4.52	0.063
7.50	0.03000 *N* NaOH	0.17	0.0675

RESULTS AND DISCUSSION

The thiyl free radical ESR signal observed upon the oxidation of cysteine with ceric ion is a 1:2:1 triplet at[9] g = 2.0106 ± 0.0002. The minimum concentrations of thiol and ceric ion necessary to produce a reasonable RS· signal had previously (2) been determined to be 0.02 *M* thiol and 0.005 *M* Ce^{+4} ion. Since higher concentrations of ceric ion produce more acidic solutions, the minimum concentrations proved to offer the greatest flexibility for variation of pH over a wide range without excessive precipitation of ceric ion at the higher pH's. The pH was adjusted by addition of the appropriate concentration of hydrochloric acid or sodium hydroxide to the cysteine solution before mixing. The resultant concentrations of acid or base in the flow cell reaction mixture at the time of ESR observation and the concomitant spin concentrations are given in Table I. The relationship between the total intensity of the RS· signal and increasing pH is depicted in Fig. 1. This plot, which appears to have the shape of a "titration" curve in the 1.5–7.5 pH range, represents the steady-state concentration of RS· free radicals generated in the one-electron transfer (20) reaction of Scheme I.

$$Ce^{+4} + RSH \longrightarrow Ce^{+3} + RS\cdot + H^+$$

Scheme I

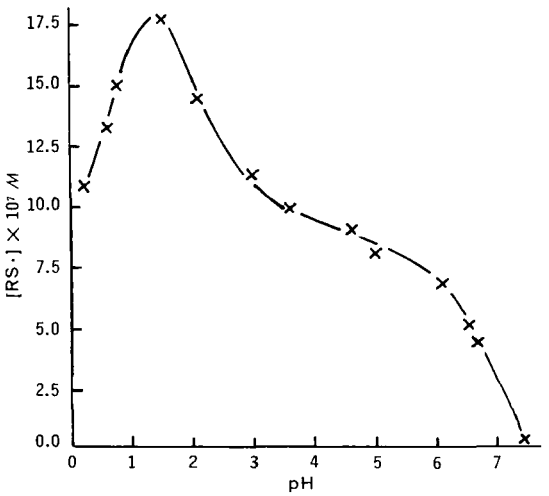

Figure 1—*pH dependency of thiyl free radicals generated upon oxidation of 0.02* M *cysteine hydrochloride with 0.005* M $CeK_2(NO_3)_6$.

[9] The g-value for a free radical is a dimensionless constant; it is also called the spectroscopic splitting factor.

The disappearance of the thiyl free radical may then occur either by dimerization:

$$RS\cdot + RS\cdot \longrightarrow RSSR$$

Scheme II

or interaction with other species:

$$RS\cdot + X \longrightarrow Y$$

Scheme III

where the possible identity of X includes molecular oxygen or RSH. The nature of Schemes II and III, determined in kinetic studies, will be discussed elsewhere.

Amino acids are present in various ionic states depending on the pH of a solution. The form of the variation of RS· concentration with pH illustrated in Fig. 1 suggests that the rate of Scheme I is markedly dependent upon the state of protonation of the various functional groups of cysteine. Cysteine has three dissociable protons. The ionization of these protons may be represented by the steps shown in Scheme IV.

$$HSCH_2CH(NH_3^+)COOH$$

$$\Downarrow pK_1$$

$$HSCH_2CH(NH_3^+)COO^-$$

$$\Downarrow pK_2$$

$$\left.\begin{array}{c} {}^-SCH_2CH(NH_3^+)COO^- \\ + \\ HSCH_2CH(NH_2)COO^- \end{array}\right\} \text{equivalent forms}$$

$$\Downarrow pK_3$$

$${}^-SCH_2CH(NH_2)COO^-$$

Scheme IV

The concentration of RS· radicals is seen to reach a maximum at pH 1.5. At lower pH's, RS· concentration is depressed, apparently due to ionic strength factors which will be discussed later. As the pH rises above 1.5, the RS· concentration decrease parallels the first dissociation constant of cysteine. The rate of decrease in the pH 3–6 region is slower; beyond pH 7.5, there is no longer a steady-state concentration of RS· radicals sufficient for ESR detection.

Correlation with Ionic Species—The ability to generate the thiyl free radical is apparently strongly influenced by the ionic form of the various substituents of cysteine. To determine whether the ease of formation of the thiyl free radical could be correlated with the presence of a specific charged parent molecule, the concentrations of ionic species as a function of pH were calculated (21, 22) utilizing the reported (23) pKa values of $pK_1 = 1.71$, $pK_2 = 8.33$, and $pK_3 = 10.78$ for cysteine. Due to the alternative ways of ionization of the —SH and $—NH_3^+$ groups, there is considerable divergence in the literature as to the exact values of these pKa's. Various pKa values, as reported for cysteine by different workers (23–27), range from 1.71 to 1.96 for pK_1, from 8.15 to 8.7 for pK_2, and from 10.28 to 10.78 for pK_3. These differences may be responsible for a slight shift in the concentration of ionic species in relation to that of the thiyl free radical. The log of each ionic species concentration as a function of pH is plotted in Fig. 2, along with the log of the observed number of spins of the thiyl free radical for comparison. These curves indicate that formation of the RS· radical is greatly favored by the presence of the positively charged $HSCH_2CH(NH_3^+)COOH$ species, which is predominant at pH's below 1.5. The RS· radical concentration begins to fall as the zwitterion, $HSCH_2CH(NH_3^+)COO^-$, becomes predominant and nearly parallels the concentration of this species past the isoionic point (pI 5.02), falling gradually as the negatively charged ${}^-SCH_2CH(NH_3^+)COO^-$ species increases with pH. As the pH rises beyond 6.7, the thiyl free radical concentration begins to fall rapidly. At this range, the zwitterion is still prevalent, while the concentrations of both the singly and doubly negative charged thiolate ions are both rapidly increasing; beyond pH 7.5 the RS· signal is no longer detectable at the initial concentrations of cysteine and ceric ion employed in this study. This correlation suggests that the rate of formation (Scheme I) decreases with increasing pH. Studies on the rate of disappearance of the thiyl free radical generated by pulse radiolysis techniques (28) suggest that decay of RS· by dimerization (Scheme II) also decreases with increasing pH. (At pH 1.0, a K_d of $1.0 \times 10^{10}\ M^{-1}\ sec^{-1}$ was indicated; at pH 6.4, a K_d of $3.4 \times 10^9\ M^{-1}\ sec^{-1}$ was indicated.)

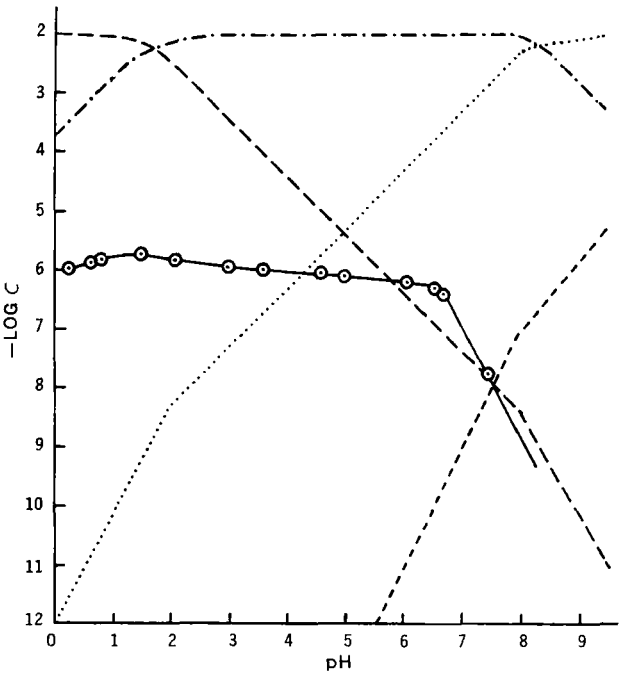

Figure 2—*Relation of the thiyl free radical concentration observed during ceric-ion oxidation of cysteine hydrochloride to the concentration of the different ionic forms of cysteine as a function of pH. Key:* C, *concentration of free radical or ionic species:* ——, *RS·;* – – –, $HSCH_2CH(NH_3^+)COOH$; –·–·–, $HSCH_2CH(NH_3^+)COO^-$; . . . . . , $^-SCH_2CH(NH_3^+)COO^-$; *and* - - - - - -, $^-SCH_2CH(NH_2)COO^-$.

Effect of Ionic Strength—The decrease in RS· spin concentration at low pH's from 1.5 to 0.25 is apparently due to the influence of increased ionic strength on the rate of formation, although the high H_3O^+ concentration may also shift Scheme I to the left, according to the law of mass action. The ionic strength represents the contribution to the electrostatic forces of the ions of all types. In this pH range the prevalent form of cysteine is the positively charged species. The oxidation potential of the Ce^{+4}–Ce^{+3} couple is known (29) to be ligand dependent. Oxidation potentials of −1.44 and −1.28 have been measured when ceric ion is in 1 *N* H_2SO_4 or 1 *N* HCl, respectively. The chloride ion forms a complex (17) with ceric ion, which lowers the oxidation potential. A greater complexation of this nature in 3 *N* HCl, the highest acid concentration studied, may thus contribute to diminution of the observed RS· signal. The behavior of both ceric ion and cysteinyl species is affected significantly by the nature of their solvation shells at high ionic strength. The relationship between the ionic strength of the reaction mixtures and RS· concentration is illustrated in Fig. 3. The thiyl radical concentration rises rapidly at ionic strengths between 0.0675 and 0.0375 at the higher pH's and reaches a maximum at 0.29 at the optimum pH of 1.5. At higher ionic strengths up to 3.04 *M*, thiyl radical concentration gradually declines.

The influence of ionic strength on the rates of reactions between ions has been described by the Brönsted–Bjerrum equation:

$$\log k = \log k_0 + 1.02\, z_A z_B \mu^{1/2} \qquad \text{(Eq. 1)}$$

where k_0 is the rate constant in an infinitely dilute solution. Unfortunately, predictions made from this expression are not valid above an ionic strength of 0.01 (30), which is below that of the solutions of this study. Other electrolyte theories have been devel-

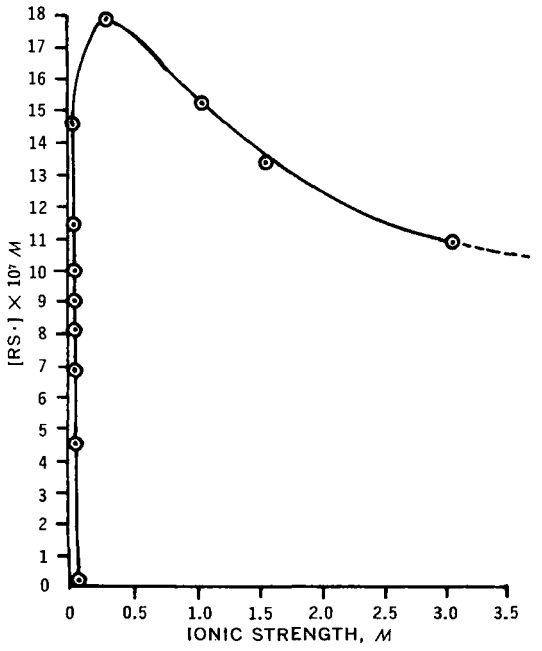

Figure 3—*Concentration of thiyl free radicals observed upon oxidation of 0.02* M *cysteine hydrochloride by 0.005* M $CeK_2(NO_3)_6$ *as a function of ionic strength.*

oped which include higher terms and additional parameters for ion sizes and repulsive forces, but they are not yet capable of making *a priori* predictions of ion–ion interactions for the higher valence ions or in the more concentrated solutions (31).

The present system may best be explained in terms of interactions between the ion clusters formed about the two reacting ions. The extent of this interaction is determined by the concentrations and charges of all the ions in solution. At high ionic strength the ceric ions are surrounded by clouds of many counterions, including nitrate ions and solvent molecules, and associate with chloride ions similarly surrounded by potassium-ion and hydronium-ion clouds. As the ionic strength increases, this association becomes greater and electrostatic repulsion between cationic groups increases, so the approach of ceric ions toward the —SH function to affect reaction is increasingly deterred. In the pH 2–7 region, where the ionic strength is much lower, ranging from 0.0375 to 0.0675, electrostatic forces still appear to predominate over the inductive effect of the —COOH group, which would enhance reaction with ceric ion. As the cysteinyl zwitterion is prevalent in this range, the $—COO^-$ function tends to attract the ceric ions away from —SH, thus lowering the rate of reaction and the observed RS· spin concentration.

CONCLUSIONS

This study verified that thiyl free radicals observed at acidic pH's can also be produced and observed at physiological pH. The steady-state level of radicals necessary for ESR detection is governed by the rates of formation and decay of the species. When cysteine is oxidized by ceric ion, the rate of appearance of the thiyl free radical is strongly dependent on pH, which determines the ionic form of the parent molecule. The sensitivity of the production of spin concentration to the ionization of acidic functional groups makes ESR a direct method for studying acid–base chemistry at the molecular level. This reaction is most favored by a positive charge on the thiol. The RS· concentration is depressed by solutions of ionic strength greater than 0.29, due to ionic associations. Since thiyl free radicals are important intermediates in radiobiological processes, a comparison of the behavior of cysteinyl free radicals in the physiological pH range and at ionic strengths similar to those of biological fluids with that of structurally related radioprotective or sensitizing thiols would be particularly valuable in assessing their role in these processes.

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[x] To whom inquiries should be directed.

Kinetics and Mechanisms of Drug Action on Microorganisms XX: Integrated Receptor Site Model Rationalizing Observed Microbial Rate Dependencies on Drug Concentration and Microbial Kinetics Affected by Sodium Fusidate

EDWARD R. GARRETT[x] and A. J. RICHARD*

Abstract □ The steady-state generation of *Escherichia coli* (ATCC 12407) was inhibited by the antibiotic sodium fusidate in the drug concentration range of 0–100 μg/ml. There was no significant change in drug activity when the initial inoculum size was varied between 5×10^5 and 2×10^6 *E. coli*/ml. The drug activity was unaffected by varying the concentrations of ammonium ions, magnesium ions, amino acids, dextrose, or citrate components of the nutrient broth in Anton's medium over a fourfold concentration range and did not implicate binding to the components. The variation of drug activity with pH could be assigned completely to the action of the unionized species. Sodium fusidate was bacteriostatic up to 500 μg/ml, about five times the minimum inhibitory concentration, and the action of the drug was readily reversible on dilution. Resistant mutants of *E. coli* appeared after exposure of the organisms to sodium fusidate, and the numbers of such resistant organisms appeared to be a function of the inoculum size at the time of drug addition. No significant inactivation of the drug by resistant organisms was observed. The dependence of the apparent generation constant, k_{app}, on drug concentration in the medium is an S-shaped curve, which can be rationalized on the premise of a saturable enzymatic degradation of drug in the biophase of the microorganism. The same model, which considers the steady-state microbial generation as a consequence of rates of drug permeation into the biophase of the organism, the saturable biophasic degradation of the drug, and the reaction of drug with saturable receptor sites that leads to generation inhibition, can serve as a proper rationalization of all previously observed various generation rate dependencies on drug concentrations for different drugs. The action of sodium fusidate in combination with tetracycline, clindamycin, and 1′-demethyl-4′-depropyl-4′-[*(R)* and *(S)*]-*n*-pentylclindamycin is indifferent; with erythromycin and oleandomycin, it is mildly synergistic; and with novobiocin, it is antagonistic.

Keyphrases □ Sodium fusidate—quantitative kinetic effects on microbial generation of *E. coli*, broth constituents, broth pH, and inoculum size considerations □ *Escherichia coli* generation—quantitative kinetic inhibition by sodium fusidate, broth constituents, broth pH, and inoculum size considerations, theory of bacteriostatic inhibition developed □ Antibiotic activity—kinetics and mechanisms of sodium fusidate inhibition of *E. coli* generation □ Microorganisms, inhibition of *E. coli*—sodium fusidate, kinetics and mechanism

Sodium fusidate is a clinically effective antibiotic (1–5) that inhibits protein synthesis (6–9) and binds extensively to plasma proteins (1).

"Synergism" was reported for sodium fusidate (1) in combinations with erythromycin and in combination with penicillin against penicillinase-producing strains of *Staphylococcus aureus*.

This paper reports on the quantitative kinetic effects of sodium fusidate on the microbial generation of *Escherichia coli* (ATCC 12407). The effects of broth constituents, broth pH, and inoculum size on the drug activity were measured. Combinations of sodium fusidate and several other bacteriostatic drugs were studied by the method of equipotent mixtures (10) to gain insight into the possible mechanism of action of such combinations on bacterial growth rates.

Previously (10), the steady-state microbial generation due to the inhibition of protein synthesis by such bacteriostatic drugs was rationalized by the postulation of equilibration of drug between the medium and the biophase wherein the drug competes for receptor sites and the degree of inhibition was presumed to be related to the fraction of occupied sites. However, the steady-state generation may not be a consequence of true equilibration but of conditions where the rate of enzymatic drug inactivation in the biophase comes into balance with the rate of drug permeation into the biophase. Such a phenomenon, when saturable, may explain the observed unusual S-shaped dependency of the apparent generation rate constant, k_{app}, on drug concentration for sodium fusidate in the medium. Equations are developed to manifest this possible mechanism and to permit the analysis of the data for the pharmacokinetic constants of such processes.

EXPERIMENTAL

Test Organism—Replicate slants of *E. coli* (ATCC 12407), previously referred to as strain B/r (11), were used in all experiments. This nonclumping strain is particularly well suited for colony counts and for particle counts[1]. The slants were prepared from a single colony and were stored in the refrigerator at 4°.

Culture Medium—Only Anton's medium (12) was used. It has a pH of 6.9 and consists of 7.00 g K_2HPO_4, 3.00 g KH_2PO_4, 2.00 g dextrose, 1.00 g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 0.10 g $MgSO_4 \cdot 7H_2O$, and 10.00 g casamino acid[2] plus distilled water to make 1 liter of solution. Since there is no protein in the broth, kinetic effects were studied without the added variable of protein binding. The broth was filtered through a 0.45-μm filter[3], autoclaved at 120° for 12 min, and then stored in an incubator overnight at 37.5° before using to ensure the absence of contaminating microorganisms. The pH of the broth was varied by changing the ratio of K_2HPO_4 to KH_2PO_4 while maintaining a total weight of 10 g/liter of phosphates. The effects of magnesium ions (Mg^{+2}), ammonium ions (NH_4^+), citrate, dextrose, and casamino acid concentrations were studied by first doubling and then halving these constituents. Dextrose and casamino acids were also studied at one-fourth the normal concentration in Anton's medium.

Antibiotics—The antibiotics used in combination experiments were all assayed bacteriostatic drugs and included sodium fusid-

[1] Coulter counter monitoring.
[2] Difco Laboratories, Detroit, Mich.
[3] HA Millipore.

ate[4], pure; erythromycin lactobionate[5], 670 μg/mg; chloramphenicol[6], purity grade; tetracycline hydrochloride[7] USP; novobiocin[7], 951 μg/mg; 1′-demethyl-4′-depropyl-4′-[(*R*) and (*S*)]-*n*-pentylclindamycin hydrochloride[7], pure; clindamycin hydrochloride[7], 860 μg/mg; lincomycin hydrochloride[7], 895 μg/mg; and oleandomycin phosphate[8], 775 μg/mg. The purities are given in terms of base equivalents.

Bacterial Generation and Monitoring—Methods used in this laboratory for bacterial culture, total count monitoring, viable counting, and size frequency distribution studies were previously described (13–15). The average background counts[9] have since been reduced to about 200/50 μl sample. The coincidence count corrections were made with a program on a calculator[10] in accordance with the coincidence correction chart supplied by the manufacturer[11].

Reversibility of Drug Action—Reversibility of drug action can be shown by taking aliquots of organism cultures containing about 10^7 *E. coli*/ml during the drug-affected steady-state growth at 37.5° and diluting into fresh broth with no added drug and also into fresh broth containing predetermined drug concentrations (14). Reversibility of drug action is established if the diluted cultures grow at the control rate in the fresh broth and at the predicted rates when drug is present in the diluent. Reversibility was studied at 0, 40, and 80 μg/ml of sodium fusidate at pH 6.9 and also at 0, 10, and 20 μg/ml of sodium fusidate at pH 6.4.

Effect of Equipotent Mixtures of Sodium Fusidate and Other Growth Inhibitors—Replicate 49.5-ml volumes of cultures containing 5×10^5 *E. coli*/ml, generating in the logarithmic phase at pH 6.9 and 37.5°, were treated with aliquots (0.5 ml) of equipotent mixtures of antibiotics (10, 14). The mixtures consisted of 100, 80, 60, 40, 20, and 0% of the equipotent sodium fusidate and the residual percentage of the other antibiotic to maintain equipotency at two different dosage levels. If the drugs precipitated on mixing, the mixture was diluted with Anton's medium until the precipitate dissolved; the appropriate volume was withdrawn from each culture flask before the addition of the drug solutions.

Prolonged Exposure of *E. coli* to Sodium Fusidate—*E. coli* was grown for 24 hr at 37.5° in Anton's medium with 100 μg/ml of sodium fusidate added. An aliquot of the culture was then diluted 1:100 into a second volume of broth with 100 μg/ml of sodium fusidate added. Following an additional 24 hr of culture, the organisms were grown as usual in the presence of graded sodium fusidate concentrations. To determine whether the sodium fusidate would be degraded significantly in the medium, the culture with 50 μg/ml of sodium fusidate was filtered after 200 min of growth on a 0.45-μm filter[3]. The bacteria were resuspended into broth and cultured, and the filtrate was seeded with *E. coli* that had not been exposed to sodium fusidate.

RESULTS

Effect of Drug Concentration on Generation Rates—The generation rates of *E. coli* in graded sodium fusidate concentrations, measured by the total count and the viable count methods in Anton's medium at pH 6.9, were coincident (Fig. 1); no bactericidal activity was observed up to 500 μg/ml of sodium fusidate, about five times the minimum inhibitory concentration (MIC). Thus, the use of total counts to determine the generation rates of organisms affected by subinhibitory concentrations of sodium fusidate is justified. Significant time elapsed before a logarithmic growth phase appeared and indicated a slow rate of equilibration of drug between the medium and the biophase of the organism. The apparent generation rate constants, k_{app}, in accordance with $\log N = k_{app}t$ + constant, where N is the number of organisms at time t, were obtained from the slopes of the terminal linear plots that characterized steady-state generation (10, 11) (Fig. 1). Plots of k_{app} against concentrations of sodium fusidate (Fig. 2) resulted in a sigmoid curve. Daily variability in generation rates could be

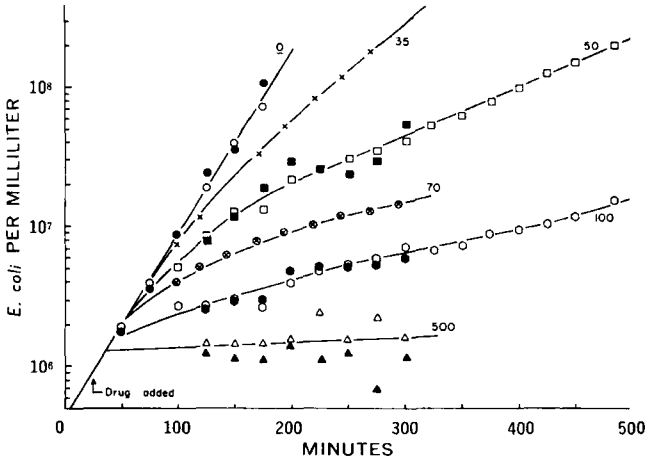


Figure 1—*Typical generation curves of* E. coli *in Anton's medium in the absence and presence of various concentrations of sodium fusidate, obtained by total (open symbols) and viable (dark symbols) counts. The curves are labeled according to the sodium fusidate concentrations in micrograms per milliliter.*

minimized by plotting $(k_0 - k_{app})$ against concentration (Fig. 3), where k_0 was the generation rate constant in the absence of drug.

Effect of Organism Population on Drug-Affected Generation Rates—In preliminary experiments on the effects of sodium fusidate on *E. coli* generation, the drug was added when the bacterial count was $1\text{–}3 \times 10^6$ *E. coli*/ml. In general, after these generation curves achieved steady state, they remained linear for about 250 min but, subsequently, the drug-uninhibited generation of resistant strains was observed since the apparent generation rate increased. This effect was eliminated when the drug was added at a lower organism population to the cultures—*viz.*, less than 5×10^5/ml.

No significant variations were observed (Fig. 2) in the drug-affected steady-state generation rate constants, k_{app}, for the addition of drug to bacterial populations that varied within $10^5\text{–}10^6$ *E. coli*/ml.

Effect of pH and Nutrient Broth Components on Drug-Affected Generation Rates—The generation rates and rate constants, k_{app}, of *E. coli* in the presence of equal concentrations of sodium fusidate were markedly reduced with decreased pH values of the medium (Fig. 3). When the total concentrations of sodium fusidate at all pH values (pKa 5.35) (1) were converted to con-

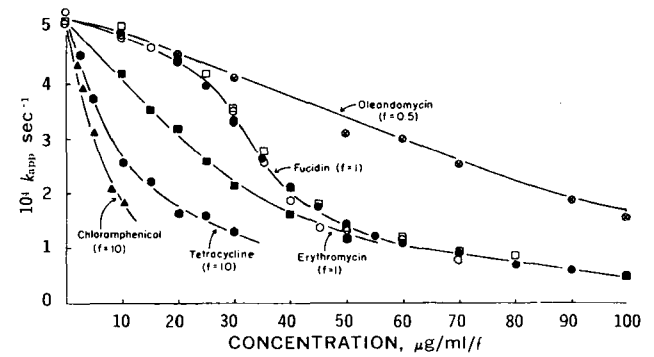


Figure 2—*The dependency of the apparent generation rate constant,* k_{app}, *of* E. coli *on drug concentration, plotted against concentration in micrograms per milliliter/*f *where* f = *10 for chloramphenicol* (C), f = *10 for tetracycline hydrochloride* (T), f = *1 for erythromycin lactobionate,* f = *1 for sodium fusidate (fucidin sodium)* (F) *where the data for several complete studies are given, and* f = *0.5 for oleandomycin phosphate* (O). *The lines drawn through the data are calculated on the premise of adherence to the saturable enzymic degradation of drug and receptor site action of drug in the biophase during the drug-affected steady state in accordance with Scheme I, the parameters of Table I, and Eqs. 59 and 61.*

[4] Leo Pharmaceuticals, Copenhagen, Denmark.
[5] Abbott Laboratories, North Chicago, Ill.
[6] Thomae GMBH, Germany.
[7] The Upjohn Co., Kalamazoo, Mich.
[8] Pfizer Inc., Brooklyn, N.Y.
[9] In the Coulter counter.
[10] Wang 700.
[11] Coulter counter model ZBI in conjunction with Channelyzer and *X–Y* plotter, Coulter Electronics, Inc., Hialeah, Fla.

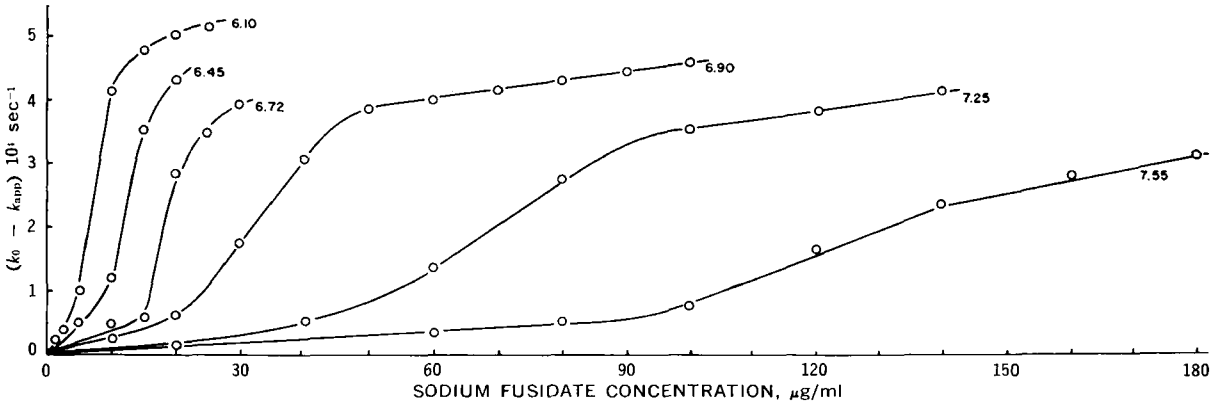


Figure 3—*Plots of the differences between the generation rate constants of* E. coli *in the absence of drug,* k_0, *and the apparent generation rate constants,* k_{app}, *at a given sodium fusidate concentration for the different pH values of the medium specified.*

centrations of unionized drug by multiplying by the fraction of total drug unionized at a given hydrogen-ion concentration (10, 11), $[H^+]/([H^+] + K_a)$, the plots of $(k_0 - k_{app})$ against the unionized drug concentrations showed that all data were coincident (Fig. 4). It may be concluded that only the unionized fraction of sodium fusidate penetrates the cell wall of *E. coli* to inhibit growth and that the MIC of unionized drug is about 2 μg/ml against *E. coli*. Such effects have been observed frequently (10).

The studied variations of the other components of Anton's broth medium had no observable effect on sodium fusidate-affected generation rate constants of *E. coli*.

Reversibility of Drug Action—The effects of sodium fusidate on *E. coli* generation were reversible after exposure of the organisms to 80 μg/ml of drug for 300 min at pH 6.9 and 6.4. When aliquots of medium were diluted 10-fold into fresh broth containing 0, 40, and 80 μg/ml of drug, the generation rates obtained were those for the new drug levels with fresh organisms (Fig. 2).

Prolonged exposure to sodium fusidate, *e.g.*, 100 μg/ml for 48 hr, permitted the emergence of resistant organisms. The generation rate of the filtered bacteria approached the generation rate in the absence of drug even when 100 μg/ml of sodium fusidate was present. When this resistant strain was grown for 200 min in the presence of 50 μg/ml of sodium fusidate and the organisms filtered from the medium, the *E. coli* showed continued resistance to 50 μg/ml but the filtrate inoculated with naive *E. coli* grew at the rate predicted (Fig. 2). Sodium fusidate, therefore, was not significantly degraded to inactive metabolite(s) by exposure to resistant *E. coli*.

Drug Effects on Size of *E. coli*—There was no significant difference between the cell size–frequency distribution of generating *E. coli* untreated with drug (14, 15) and of generating organisms treated with sodium fusidate concentrations up to 90 μg/ml at pH 6.9 at 37.5°. However, at and above the apparent MIC under these conditions of 100 μg/ml, the median size of the drug-affected cells throughout the experiment was twice that of the *E. coli* generating in the absence of sodium fusidate.

Effects of Drug Combinations with Sodium Fusidate—The observed k_{app} values for various combinations of sodium fusidate with other drugs are plotted in Fig. 5 against the volume fraction of sodium fusidate in the mixtures of equipotent concentrations of sodium fusidate and another drug.

THEORETICAL

Models (Schemes I and II) consistent with the variously observed dependencies (10) of the steady-state rate constants, k_{app}, for microbial generation on the concentration of a bacteriostatic drug in the nutrient medium can be constructed. They would be based on the premise that the drug of concentration C in the nutrient medium permeates the cell membrane into the biophase of drug concentration C' which competes with the reaction of a metabolite precursor, M, for those receptor sites, R. This latter process can result in a product, G, vital for microbial generation. Steady-state generation can result when the concentration C' of effective drug in the biophase is a constant. The accompanying postulates are that the loss of drug by binding to the receptor sites or inactivated by saturable or nonsaturable processes in the biophase does not significantly deplete the total concentration, C or C_T, in the nutrient medium.

These models differ from those given previously (10) in that they do not make it necessary to assume true equilibrium between the drug concentrations in the medium and biophase. They also afford a proper explanation for the observed differences in microbial sensitivity among Gram-positive and Gram-negative

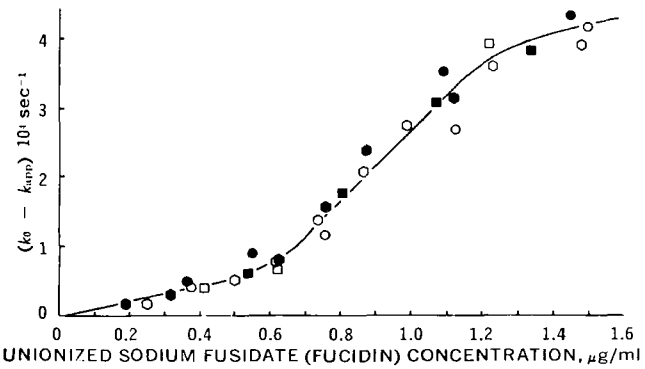
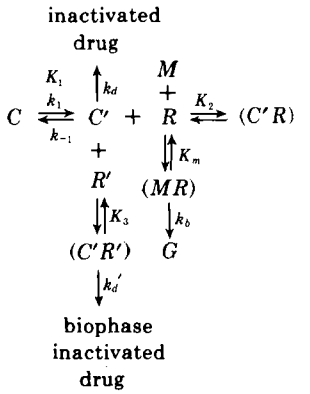


Figure 4—*Plots of the differences between the generation rate constants of* E. coli *in the absence of drug,* k_0, *and the apparent generation rate constants,* k_{app}, *against the micrograms per milliliter concentration of unionized sodium fusidate (fucidin) (sodium fusidate concentration in micrograms per milliliter times fraction of drug that is unionized). The data were obtained at different pH values of the media ranging between 6.10 and 7.55.*



Scheme I—Steady-state model for permeation of drug of concentration C *in nutrient medium into the biophase of the microorganism of concentration* C′ *with simultaneous saturable enzymic,* R′, *inactivation,* k_d', *and first-order inactivation,* k_d, *where the competition of drug* C′ *with substrate,* M, *for receptor sites,* R, *results in the observed inhibition of microbial generation rates, which is postulated to be proportional to the production of* G

microorganisms, which would not be anticipated if steady-state microbial generation is solely a consequence of true equilibrium. It follows that, in addition to the possible variable affinities of the biophase receptor sites of such organisms to a given drug and to the possible differences in metabolic processes, the differences in drug activities among microbial species may be assigned to wide differences in the interacting processes of rates of permeation into, and rates of degradation in, the biophase.

On the basis of Schemes I and II, it can be stated that:

$$dG/dt = k_b(MR) = k_bK_mRM \qquad \text{(Eq. 1)}$$

If the concentration of the necessary metabolite, M, and the total numbers of receptor sites, R_T, whether reacted or unreacted with drug, are invariant, then:

$$dG/dt = k_bK_mMR_t(1-\theta) = k_m(1-\theta) \qquad \text{(Eq. 2)}$$

where $k_m = k_bK_mMR_t$, and θ is the fraction of these sites reacted with drug. Since the equilibrium constant, K_2, for the drug-site interaction of Schemes I and II may be defined by:

$$K_2 = \frac{k_2}{k_{-2}} = \frac{(C'R)}{(R)(C')} = \frac{(C'R)}{[R_t-(C'R)](C')} \qquad \text{(Eq. 3)}$$

where $(C'R)$ is the number of drug-reacted sites, the fraction $\theta = (C'R)/R_t$ of sites reacted with drug may be obtained from rearrangement of Eq. 3:

$$\theta = (C'R)/R_t = K_2C'/(1+K_2C') \qquad \text{(Eq. 4)}$$

Thus, Eq. 2 on substitution of Eq. 4 becomes:

$$dG/dt = k_m - k_mK_2C'/(1+K_2C') \qquad \text{(Eq. 5)}$$

If it is postulated that the rate of population increase of N organisms is proportional to this rate of metabolism or product formation and to the number of organisms, N, in the balanced growth culture, it follows that:

$$dN/dt = q(dG/dt)N \qquad \text{(Eq. 6)}$$

$$dN/dt = \left[qk_m - \frac{qk_mK_2C'}{1+K_2C'}\right]N = k_{app}N \qquad \text{(Eq. 7)}$$

and if qk_m is defined as k_0, the generation rate constant in the absence of drug concentration in the biophase:

$$(k_0-k_{app})/k_{app} = K_2C' \qquad \text{(Eq. 8)}$$

where k_{app} is the apparent first-order steady-state generation rate constant in the presence of a constant drug concentration, C', in the biophase. The necessary prerequisite for the validity of a steady-state generation of rate constant, k_{app}, for the models of Schemes I and II is that ln N against time is linear and of a constant slope, k_{app}, at some time after drug addition.

Presence of Saturable and Nonsaturable Degradation of Drug in Biophase for Drug Saturable Receptor Sites (Scheme I)—When the steady-state generation characterized by Eqs. 7 and 8 holds, it must be postulated that the biophase concentration, C', of drug unbound to the receptor site in Scheme I must be constant in accordance with:

$$dC'/dt = k_1C - [k_{-1}+k_d+k_d'(1-\theta')]C' = 0, C' = \frac{k_1C}{k_{-1}+k_d+k_d'(1-\theta')} \qquad \text{(Eq. 9)}$$

where the concentration of the biophase, C', tends to increase at a rate proportional to the concentration—*viz.*, k_1C, where it is presumed that the concentration, C, or unbound drug C_u, in the nutrient medium is constant throughout the steady state, and tends to decrease by the back-diffusion and the nonsaturable and saturable degradation in the biophase characterized by the rate constants k_{-1}, k_d, and k_d', respectively. The fraction of drug-unreacted enzyme is $1-\theta'$, and the constant $k_d' = (k_d')_0R_T'$ is the product of the intrinsic rate of drug-enzyme degradation, $(k_d')_0$, and the total number of enzymes, R_T'.

The value of $\theta' = (C'R')/R_t'$ can be estimated from the rearrangement of:

$$K_3 = \frac{(C'R')}{(R')(C')} = \frac{(C'R')}{[R_t'-(C'R')]C'} \qquad \text{(Eq. 10)}$$

where $(C'R')$ is the steady-state constant amount of drug-reacted enzyme in the biophase drug and, analogous to Eq. 4:

$$\theta' = K_3C'/(1+K_3C') \qquad \text{(Eq. 11)}$$

so that:

$$(1-\theta') = 1/(1+K_3C') \qquad \text{(Eq. 12)}$$

Substitution of Eq. 12 into Eq. 9 results in:

$$C' = \frac{k_1C}{k_{-1}+k_d+k_d'/(1+K_3C')} \qquad \text{(Eq. 13)}$$

where, from Eqs. 7 and 13:

$$k_{app} = k_0 - \frac{k_0k_1K_2C/[k_{-1}+k_d+k_d'/(1+K_3C')]}{1+k_1K_2C/[k_{-1}+k_d+k_d'/(1+K_3C')]} = k_0 - \frac{k_0K_2C/[1/K_1'+k_d'/k_1(1+K_3C')]}{1+K_2C/[1/K_1'+k_d'/k_1(1+K_3C')]} \qquad \text{(Eq. 14)}$$

and from Eqs. 8 and 13:

$$(k_0-k_{app})/k_{app} = k_1K_2C/[k_{-1}+k_d+k_d'/(1+K_3C'] = K_2C/[1/K_1'+k_d'/k_1(1+K_3C')] \qquad \text{(Eq. 15)}$$

Rearrangement of Eq. 13 permits the determination of an explicit solution for C':

$$C' = \frac{k_1K_3C-(k_d+k_{-1}+k_d')\pm\sqrt{k_1^2K_3^2C^2+(k_{-1}-k_d'+k_d)2k_1K_3C+(k_d+k_{-1}+k_d')^2}}{2(k_{-1}+k_d)K_3} \qquad \text{(Eq. 16)}$$

At low values of C and C', it is apparent from Eq. 13 that:

$$\lim_{C'\to 0} C' = \frac{k_1C}{k_{-1}+k_d+k_d'} = K_1''C \qquad \text{(Eq. 17)}$$

and apparent from Eq. 15 that:

$$\lim_{C'\to 0} (k_0-k_{app})/k_{app} = K_1''K_2C \qquad \text{(Eq. 18)}$$

At very high values, when unity is very much less than K_3C' and when the degradation of C' is essentially zero order:

$$\lim_{C'\to\infty} C' = \frac{k_1C}{k_{-1}+k_d} - \frac{k_d'}{K_3(k_{-1}+k_d)} = K_1'C - \frac{k_d'}{K_3(k_{-1}+k_d)} \qquad \text{(Eq. 19)}$$

and:

$$\lim_{C'\to\infty} (k_0-k_{app})/k_{app} = K_1'K_2C - \frac{k_d'K_2}{K_3(k_{-1}+k_d)} \qquad \text{(Eq. 20)}$$

and $K_1'' < K_1'$.

In the particular case of nonsaturable inactivation of drug in the biophase, where the inactivation process is first order for all C' values, $k_d' = 0$ in Eq. 15 and:

$$(k_0-k_{app})/k_{app} = K_1'K_2C \qquad \text{(Eq. 21)}$$

where $K_1' = k_1/(k_{-1}+k_d)$. The $K_1'K_2$ values may be determined from the slope, S_2, of $(k_0-k_{app})/k_{app}$ against concentration, C, in accordance with Eq. 20 (Fig. 1*b*, curve A), where $k_d' = 0$ and thus K_1'' reduces to K_1'.

If there is no biophase inactivation in the model of Scheme I, $k_d = 0$ and K_1' reduces to K_1 where the slope, S_2, of such a plot is K_1K_2, where $K_1 = k_1/k_{-1}$ as was discussed previously (10).

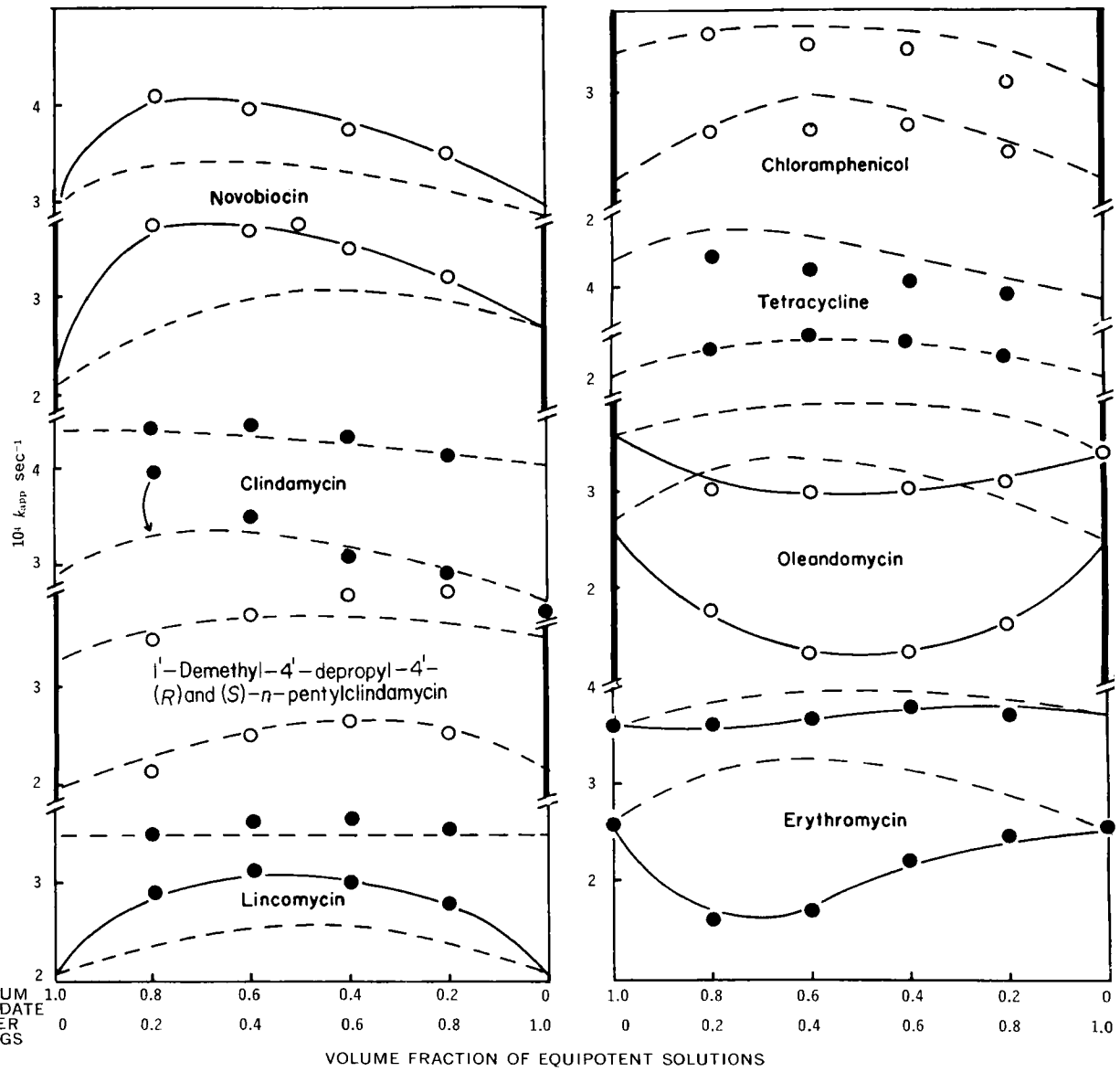


Figure 5—*Effect of varied volume fraction of sodium fusidate solution in various mixtures of sodium fusidate solution and equipotent solution of the specified drug on the apparent generation rate constant,* k_{app}, *observed for the combination at two different potency levels. Because of the nonsuperimposability of the* $(k_{app})_D$ versus *concentration curves (Fig. 2) for all of these drugs with that for sodium fusidate,* $(k_{app})_F$, *the mixtures are not expected to give the same* k_{app} *values at all volume fractions of the sodium fusidate solution. The expected* k_{app} *values for the combinations on the basis of the additivity of actions of both drugs is* $k_0 - (k_{app})_F - (k_{app})_D$ *and are indicated by the dotted lines.*

However, for both cases where there is or is not first-order biophase inactivation, the plot of k_{app} against drug concentration, C, shows a continuously decreasing rate of change of k_{app} with concentration (Fig. 6*a*, curve A). However, there is no *kinetic* method of determining the existence of a first-order inactivation process of constant k_d or of separating the quotients $K_1'K_2$ and K_1K_2. Plots in accordance with Eq. 21 (Fig. 6*b*, curve A) are completely linear under these circumstances.

In the more general case of a saturable process of degradation of drug in the biophase (Eqs. 14 and 15), the rate of decrease of k_{app} with concentration initially increases with increasing saturation of the degrading enzyme or catalyst. Subsequently, as the receptor sites necessary for microbial generation are lessened, the rate of decrease of k_{app} decreases with concentration. An S-shaped curve of k_{app} against concentration, C, would be anticipated (Fig. 6*a*, curve B, and Fig. 7*a*).

At low values of C or C', C' can approach the values expressed by Eq. 17, so that from Eq. 18, the initial linear slope, S_1, of $(k_0 - k_{app})/k_{app}$ against C (Fig. 7*b*, insert) estimates $K_1''K_2$. With increasing concentrations, C, of drug, the inactivation of C' in the biophase is essentially zero order and of maximal rate k_d'/K_3, and C' can be expressed explicitly by Eq. 19 so that Eq. 20 holds at high values of C or C', and the terminal linear slope, S_2, of $(k_0 - k_{app})/k_{app}$ against C (Fig. 6*b*, curve B, and Fig. 7*b*) estimates $K_1'K_2$, and the extrapolated ordinate intercept I of the terminal straight line of such plots (Fig. 6*b*, curve B, and Fig. 7*b*) estimates $-[k_d'K_2/K_3(k_{-1}+k_d)]$.

Concentration C_1 exists (the abscissa intercept of the terminal straight line as shown in Figs. 6*b* and 7*b*) where Eq. 20 equals zero, $C = C_1$, and:

$$C_1 = k_d'/K_1'K_3(k_{-1}+k_d) = k_d'/k_1K_3 \qquad \text{(Eq. 22)}$$

where k_d'/K_3 is the maximal zero-order rate of drug degradation in the biophase.

If the affinity constant, K_3, of drug for enzyme in the biophase is extremely high, K_3C' in Eq. 13 is very much greater than unity and C' can be defined explicitly as:

$$C' = \frac{k_1C}{k_{-1}+k_d} - \frac{k_d'}{K_3(k_{-1}+k_d)} = K_1'C - \frac{k_d'}{K_3(k_{-1}+k_d)} \qquad \text{(Eq. 23)}$$

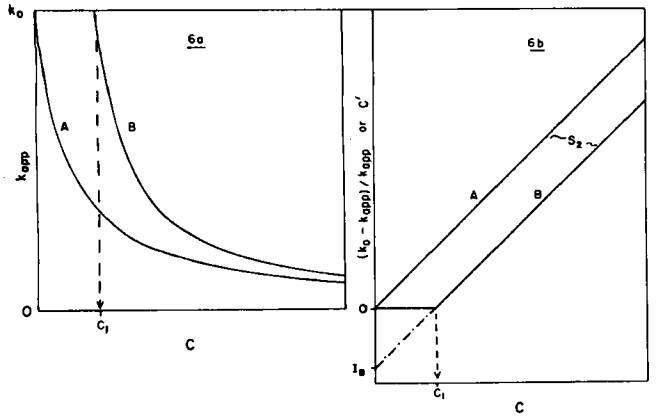
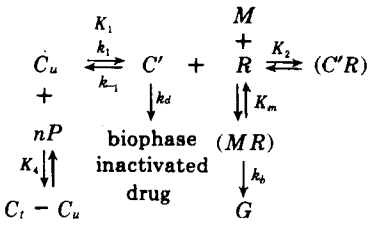


Figure 6—*Plots for the cases for a saturable receptor site model where the degree of inhibition of microbial generation depends on the fraction of receptor sites in the microbial biophase reacted with drug.* (a) *Anticipated shapes of plots of the steady-state drug-affected microbial generation rate constants,* k_{app}, *against drug concentration,* C, *in the medium for the case (curve A) when the drug may or may not be degraded by a first-order process in the biophase and for the case (curve B) when either the drug has an additional zero-order degradation in the biophase or is tightly bound to saturable binding sites in the nutrient medium, where* k_0 *is the generation rate constant in the absence of drug.* (b) *Anticipated dependencies for these cases of the concentration of free unbound drug in the biophase,* C′, *and the function* $(k_0 - k_{app})/k_{app}$ *on the concentration of drug in the medium,* C. *For* C′ versus C, *the slopes* S_2 *are* $K_1' = k_1/(k_{-1} + k_d)$, *where* k_1 *and* k_{-1} *are the rate constants for inflow and outflow with respect to the biophase, respectively, and* k_d *is the first-order rate constant for degradation in the biophase. For* $(k_0 - k_{app})/k_{app}$ versus C, *the slopes* S_2 *are* $K_1'K_2$, *where* K_2 *is the affinity constant of drug for receptor sites. In the case where there are no saturable binding sites or a saturable degradation process (curve A), the intercept is zero. In the cases of an additional saturable degradation or inactivation process of high affinity of drug for degrading enzyme or binding sites in medium, where either the rate of degradation in the biophase is constant and apparent zero order or the drug is effectively unavailable as* C′ *in the biophase, there is no apparent* C′ *or change in* k_{app} *and* $(k_0 - k_{app})/k_{app}$ *until* $C = C_1'$. *In the case of* C′ versus C *and for a saturable enzyme degradation of drug in the biophase, the extrapolated intercept* I_B *of curve B is* $-k_d'/K_3(k_{-1} + k_d) = -K_1'C_1$, *where* k_d *is the first-order rate constant for drug degradation that is nonsaturable,* k_d' *is the first-order rate constant for the saturable degradation of enzyme-reacted drug, and* k_d'/K_3 *is the maximal zero-order rate of drug degradation by this process where* K_3 *is the affinity constant of drug for enzyme; for a saturable binding in the medium, the extrapolated intercept* I_B *is* $-K_1'n[P] = -K_1'C_1$, *where the concentration of binding molecules is* [P] *with* n *sites per molecule. In the case of* $(k_0 - k_{app})/k_{app}$ versus C *and for a saturable enzyme degradation of drug in the biophase, the extrapolated intercept* I_B *is* $-k_d'K_2/K_3(k_{-1} + k_d) = -K_1'K_2C_1$; *for a saturable binding in the medium, the extrapolated intercept* I_B *is* $-K_1'K_2n[P] = -K_1'K_2C_1$.

so that from Eq. 8 at all concentrations of C:

$$(k_0 - k_{app})/k_{app} = K_1'K_2C - \frac{k_d'K_2}{K_3(k_{-1}+k_d)} \qquad \text{(Eq. 24)}$$

Thus, there is essentially a zero-order degradation of drug in the biophase and k_{app} remains constant at k_0 (Fig. 6*a*, curve B) and $(k_0 - k_{app})/k_{app}$ is zero (Fig. 6*b*, curve B) for an initial range of drug concentration, C, from 0 to C_1 since the rate of degradation, k_d'/K_3, exceeds the rate of diffusion, k_1C, of drug into the biophase for that concentration range. Subsequent to this concentration, C_1, k_{app} decreases and the rate of decrease decreases with concentration as the availability of the number of receptor sites necessary for microbial generation is lessened.



Scheme II—Steady-state model for permeation of drug of concentration C *in nutrient medium into the biophase of the microorganism of concentration* C′, *where significant binding occurs to components of the medium, and the net result is the observed inhibition of the microbial generation rate, which is postulated to be proportional to the production of* G

If the special conditions are considered for a saturable process of enzymatic degradation (Scheme I) so that the drug concentration in the biophase, C', is exceedingly small when the rate of saturable degradation is extremely large and the reactivity, K_2, of the drug for the receptor site that results in inhibition of microbial generation is great, Eq. 13 may be simplified to:

$$C' = \frac{k_1C}{k_d'/(1+K_3C')} \qquad \text{(Eq. 25)}$$

since $k_d'C'/(1 + K_3C')$ may be considered very much greater than $(k_d + k_{-1})C'$. Thus, C' can be defined explicitly as:

$$\lim_{C'\to 0} C' = k_1C/(k_d' - k_1K_3C) \qquad \text{(Eq. 26)}$$

when C is small and C' is negligible. Equations 19 and 20 can still hold as C' and C approach infinity.

However, from Eqs. 8 and 26:

$$\lim_{C'\to 0} (k_0 - k_{app})/k_{app} = K_2C' = k_1K_2C/(k_d' - k_1K_3C) \qquad \text{(Eq. 27)}$$

Then:

$$\lim_{\substack{C'\to 0 \\ 1/C\to\infty}} \frac{k_{app}}{k_0 - k_{app}} = \frac{k_d'}{k_1K_2}\frac{1}{C} - \frac{K_3}{K_2} \qquad \text{(Eq. 28)}$$

Thus, at high values of $1/C$, the slope, S_2', of the terminal straight line should estimate k_d'/k_1K_2 and the extrapolated ordinate intercept, I', of the terminal line should estimate $-K_3/K_2$.

Of course, the same information can be obtained from the slopes and intercepts of the appropriately modified Eq. 28:

$$\lim_{C'\to 0} \frac{k_{app}C}{k_0 - k_{app}} = \frac{k_d'}{k_1K_2} - \frac{K_3}{K_2}C \qquad \text{(Eq. 29)}$$

Presence of Saturable Drug Binding Sites in Medium for Drug Saturable Receptor Sites in Biophase—If the antibacterial agent binds to components of the medium, the effective concentration in the medium that establishes the concentration gradient into the biophase is that which is unbound, C_u. It is related to the total concentration in the medium by:

$$C_u = C_t\Big/\left[1 + \frac{n[P]}{K_4 + C_u}\right] \qquad \text{(Eq. 30)}$$

where n is the number of binding sites per molecule of binding agent P, and K_4 is the dissociation constant of the complex. Such values can be determined from separate binding studies (16). The explicit definition of the concentration of free drug is for $C = C_t$:

$$C_u = \frac{C - K_4 - n[P] \pm \sqrt{(K_4 + C + n[P])^2 - 4n[P]/C}}{2} = \frac{C - K_4 - n[P] \pm \sqrt{(K_4 + C - n[P])^2 + 4n[P]K_4}}{2} \qquad \text{(Eq. 31)}$$

A general expression relating the potential saturation of receptor

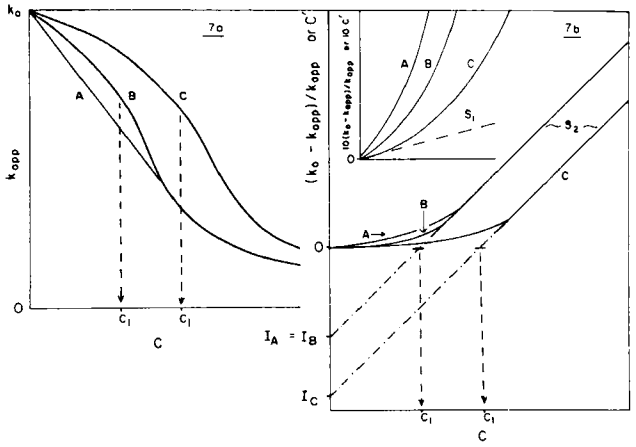


Figure 7—*Plots for the cases for a saturable receptor site model where the degree of inhibition of microbial generation depends on the fraction of receptor sites in the microbial biophase reacted with drug and the drug is degraded in the biophase by a saturable enzyme system or is bound to saturable binding sites in the nutrient medium.* (a) *Anticipated shapes of plots of the steady-state drug-affected microbial generation constants,* k_{app}, *against drug concentration,* C, *in the medium when the diffusion constants* k_1 *and* k_{-1}, *additional first-order degradation constant* k_d, *and the affinity constant* K_2 *for drug–receptor site interaction are the same. Curves A and B are drawn for the case when the maximal zero-order rate of degradation in the biophase,* k_d'/K_3, *as evaluated from the extrapolated intercepts of Fig. 7b, is the same. The first-order rate constant for the rate of degradation of enzyme-reacted drug,* k_d', *is smaller for the case of curve A than for the case of curve B. It follows that the affinity constant,* K_3, *is smaller for the case of curve A. Curves B and C are drawn for the case when the maximal zero-order rates of degradation in the biophase,* $k_d'/K_3 = (k_d')_0 R_T'/K_3$, *differ. The specific rates of degradation of an enzyme-reacted drug complex,* $(k_d')_0$, *may be the same but the amount of enzyme in the biophase,* R_T', *would be greater in the case of curve C. The first-order rate constant for the rate of degradation of enzyme-reacted drug,* k_d', *thus would differ and be greater in the case of curve C, but the affinity constant* K_3 *of the enzyme for the drug may be the same. Alternative explanations for these cases are that the drug is saturably bound to binding sites in the medium and, in the case of curves A and B, the total numbers of binding sites are equal but the dissociation constant,* K_4, *for binding in the medium are smaller in the case of curve B. Curves B and C can represent the case when the dissociation constants of the drug-bound complexes are equal, but the total numbers of binding sites are less in the case of curve B.* (b) *Anticipated dependencies for these cases of the concentration of free unbound drug in the biophase,* C′, *and the function* $(k_0 - k_{app})/k_{app}$ *on the concentration of drug in the medium,* C. *The significances of the slopes,* S_2, *of the terminal lines, and of the extrapolated intercepts of these lines were given in detail for Fig. 6b. The insert of Fig. 7b has a 10-fold expansion of the ordinate values. In the case of saturable enzymic degradation of drug in the biophase, the initial slopes,* S_1, *of these plots of* C′ versus C *approach* $K_1'' = k_1/(k_{-1} + k_d + k_d')$ *and of* $(k_0 - k_{app})/k_{app}$ versus C *approach* $K_1''K_2$. *In the case of saturable drug binding in the medium, the initial slopes,* S_1, *of these plots of* C′ versus C *approach* $k_1/(k_{-1} + k_d)\ (1 + n[P]/K_4) = K_1'/(1 + n[P]/K_4)$ *and the initial slopes of these plots of* $(k_0 - k_{app})/k_{app}$ versus C *approach* $K_1'K_2/(1 + n[P]/K_4)$.

sites and concentration C_u of diffusible drug in the medium in the absence of a saturable degradation in the biophase is, from Eq. 21:

$$(k_0 - k_{app})/k_{app} = K_1'K_2C_u \qquad \text{(Eq. 32)}$$

In the special case when drug concentration is large enough to saturate the available binding sites and there is a high affinity of drug for binding sites, K_4 is reasonably small and $C_u \gg K_4$. Thus, the latter may be neglected in Eq. 29 and:

$$\lim_{C_u \to \infty} C_u = C_t - n[P] \qquad \text{(Eq. 33)}$$

Thus, from Eqs. 32 and 33:

$$\lim_{C_u \to \infty} (k_0 - k_{app})/k_{app} = K_1'K_2C_t - K_1'K_2n[P] \qquad \text{(Eq. 34)}$$

In the general case when the concentration of drug is small and/or there is a low affinity of drug for binding sites, K_4 is reasonably large and $K_4 \gg C_u$. Thus, the latter may be neglected in Eq. 30 and:

$$\lim_{C_u \to 0} C_u = C_t/(1 + n[P]/K_4) \qquad \text{(Eq. 35)}$$

Thus, from Eqs. 32 and 35:

$$\lim_{C_u \to 0} (k_0 - k_{app})/k_{app} = \frac{K_1'K_2C_t}{1 + n[P]/K_4} \qquad \text{(Eq. 36)}$$

It follows that a plot of k_{app} against concentration C will be an S-shaped curve where the initial rate of change will increase as a greater fraction of the added drug becomes available to diffuse into the organism's biophase as the medium binding has approached saturation (Fig. 6*a*, curve B, and Fig. 7*a*).

Thus, plots of $(k_0 - k_{app})/k_{app}$ against C would show an initial slope, S_1 (Fig. 7*b*, insert), at low values of C that approaches $K_1'K_2/(1 + n[P]/K_4)$. This slope is essentially zero if the affinity of drug for binding sites, $1/K_4$, is extremely high (Fig. 6*b*, curve B). At high values of C, the slope, S_2, approached by the plot should estimate $K_1'K_2$ and the extrapolated intercept, I (Fig. 6*b*, curve B, and Fig. 7*b*), of the terminal line should be $-K_1'K_2n[P]$.

Concentration C_1 exists (the abscissa intercept of the extrapolated terminal straight line as shown in Figs. 6*b* and 7*b*) where Eq. 33 equals zero, $C = C_1 = n[P]$.

Both the binding of drug to saturable binding sites in the medium and the saturable enzymatic degradation of drug in the biophase can give the same S-shaped curve for plots of k_{app} against drug concentrations and similarly shaped plots for $(k_0 - k_{app})/k_{app}$ against concentration. The former process may be denied if there are no significant changes in plots when concentrations of nutrients in the medium are varied and when the inoculum size of organisms is varied.

Presence of Saturable and Nonsaturable Degradation of Drug in Biophase for Nonsaturable Drug Binding to Receptor Sites—It has been shown in the cases of tetracycline and chloramphenicol that k_{app} is linearly related to drug concentration. This can be rationalized on the premise of a receptor site model (10) where: *(a)* a drug-bound receptor inhibits or blocks a substrate attack on adjacent receptors so that a single drug-bound receptor inactivates multiple receptors from being effective in microbial generation, *(b)* there are multiple binding sites of equivalent affinity where the attack of a drug molecule on any one of them inhibits the reaction of substrate with a receptor site that results in microbial generation, or (*c*) it is only necessary to react the drug with a small fraction of the total number of receptor sites to obtain complete inhibition of microbial generation. If any of these factors are extent, the k_{app} implicitly defined in Eq. 7 may be approximated by:

$$k_{app} = k_0 - k_0K_2C' \qquad \text{(Eq. 37)}$$

and:

$$(k_0 - k_{app})/k_0 = K_2C' \qquad \text{(Eq. 38)}$$

since $K_2C' \ll 1$.

If there is first-order degradation in the biophase, $C' = K_1'C$ and plots of steady-state generation rate constants k_{app} or of $(k_0 - k_{app})/k_0$ against concentration C are linear with slopes of $-k_0K_1'K_2$ and $K_1'K_2$ and intercepts of k_0 and zero, respectively. Plots of $1/(k_0 - k_{app})$ against $1/C$ are linear with slopes of $1/k_0K_1'K_2$ and intercepts of zero, and plots of $C/(k_0 - k_{app}) = 1/k_0K_1'K_2$ against C are invariant.

If there is an additional degradation in the biophase that is saturable, the explicit value of C' is obtainable from Eq. 16 and the rate of decrease of k_{app} with concentration initially increases to

result ultimately in a constant rate where the initial slopes, S_1, from Eq. 17 for plots of k_{app} and $(k_0 - k_{app})/k_0$ approximate $-k_0K_1''K_2$ and $K_1''K_2$, respectively, since:

$$\lim_{C'\to 0} k_{app} = k_0 - k_0K_1''K_2C \qquad \text{(Eq. 39)}$$

$$\lim_{C'\to 0} (k_0 - k_{app})/k_0 = K_1''K_2C \qquad \text{(Eq. 40)}$$

and the slopes of the terminal straight lines approximate $k_0K_1'K_2$ (from Eq. 19) and $K_1'K_2$, respectively, in accordance with:

$$\lim_{C'\to \infty} k_{app} = k_0 - k_0K_1'K_2C + k_0K_2k_d'/K_3(k_{-1} + k_d) \qquad \text{(Eq. 41)}$$

and:

$$\lim_{C'\to \infty} (k_0 - k_{app})/k_0 = K_1'K_2C - K_2k_d'/K_3(k_{-1} + k_d) \qquad \text{(Eq. 42)}$$

with the extrapolated intercepts of $k_0 + k_0K_2k_d'/K_3(k_{-1} + k_d)$ and $k_0K_2k_d'/K_3(k_{-1} + k_d)$, respectively.

If the special conditions are considered for a saturable process of enzymatic degradation where the drug concentration in the biophase, C', is exceedingly small with an extremely large degradation rate constant, k_d', of saturable degradation and an extremely large drug affinity, K_2, for receptor sites, Eqs. 25, 26, and 38 hold and:

$$\lim_{C\to 0} (k_0 - k_{app})/k_0 = k_1K_2C/(k_d' - k_1K_3C) \qquad \text{(Eq. 43)}$$

Then the following expression is linear:

$$\lim_{C\to 0} \frac{k_0}{k_{app} - k_0} = \frac{k_d'}{k_1K_2}\frac{1}{C} - \frac{K_3}{K_2} \qquad \text{(Eq. 44)}$$

where the slope, S_1', of the plot of the left-hand function against the reciprocal of the concentration is k_d'/k_1K_2 and the negative of the intercept, $-I$, is K_3/K_2.

If there is saturable binding of drug to the components of the medium, Eqs. 30, 31, 33, 34, 37, and 38 hold and $C' = K_1'K_2C_u$. Thus:

$$\lim_{C\to 0} (k_0 - k_{app})/k_0 = K_1'K_2C/(1 + n[P]/K_4) \qquad \text{(Eq. 45)}$$

and the initial slope is $K_1'K_2/(1 + n[P]/K_4)$ and:

$$\lim_{C\to \infty} (k_0 - k_{app})/k_0 = K_1'K_2C - K_1'K_2n[P] \qquad \text{(Eq. 46)}$$

and the terminal slope is $K_1'K_2$ with an extrapolated intercept of the terminal line of $-K_1'K_2n[P]$.

DISCUSSION

The antibiotic sodium fusidate is bacteriostatic in its action on *E. coli* (ATCC 12407) but does permit the emergence of resistant strains that do not significantly degrade the drug in the medium. There does not appear to be any significant effect of inoculum size or concentration of the components of the nutrient medium used on the bacteriostatic action of sodium fusidate in the limited ranges studied. However, the addition of sodium fusidate to larger inoculum sizes does give rise to a greater number of resistant organisms than when the inoculum size is smaller.

There is a marked effect of pH on drug action, which is readily explained by the fact that the effective species in the medium is nonionized. The MIC of nonionized drug, which has a pKa' = 5.35 (1), is one-fiftieth of that at pH 6.9.

It has been shown (Figs. 6 and 7) that the S-shaped curve obtained (Fig. 2) for the dependence of the apparent generation rate constant, k_{app}, on sodium fusidate concentration in the medium can be assigned to saturable binding to the components of the medium (Scheme II) or to a saturable degradation of the drug in the biophase of the organism (Scheme I), where the drug action is postulated to be effective through saturable receptor site binding.

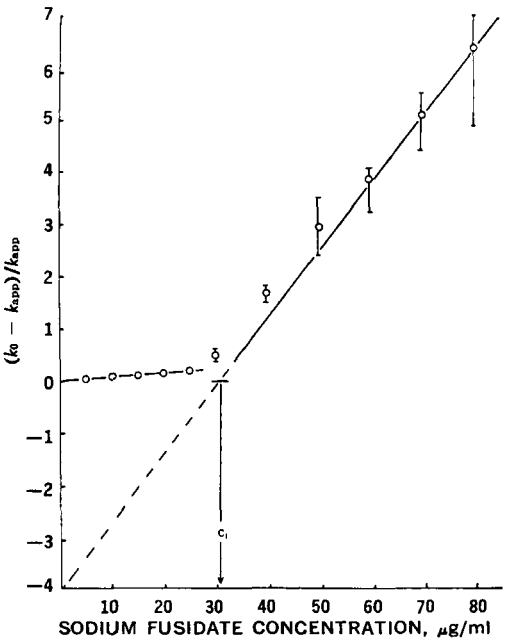


Figure 8—*Plot of* $(k_0 - k_{app})/k_{app}$ *against sodium fusidate concentration in the medium in accordance with Eqs. 15, 30, and 32 where the asymptotic terminal slope,* S_2, *is an estimate of* $K_1'K_2 = 0.13$ *ml/μg; the asymptotic initial slope,* S_1, *is an estimate of* $K_1'K_2/(1 + n[P]/K_4)$ *or* $K_1''K_2 = 0.00375$ *ml/μg; and the* C_1 *intercept on the abscissa is an estimate of* $n[P]$ *or* $k_d'/k_1K_3 = 31$ *μg/ml.*

Rationalization of Observed Generation Rate Constant, k_{app}, Dependence on Sodium Fusidate Concentration for Saturable Binding of Drug to Components of Medium—It has been shown (Figs. 6 and 7), when other evidence is lacking to the contrary, that the S-shaped curve obtained (Fig. 2) for the dependence of the apparent generation rate constant, k_{app}, on sodium fusidate concentration in the medium can be assigned to saturable binding to the components of the medium (Scheme II).

If this postulate is valid, when the data for sodium fusidate at pH 6.9 and 37.5° are plotted as $(k_0 - k_{app})/k_{app}$ against concentration C (Fig. 8) and if Eq. 32 holds, Eq. 36 may be applicable at low drug concentrations and Eq. 34 at high. The reasonable linearity of the terminal data at high sodium fusidate concentrations permits the estimation from the terminal slope, S_2, of:

$$S_2 = K_1'K_2 = 0.13 \text{ ml}/\mu\text{g} \qquad \text{(Eq. 47)}$$

The intercept of the extrapolated terminal line on the abscissa estimates:

$$C_1 = n[P] = 31 \,\mu\text{g/ml} \qquad \text{(Eq. 48)}$$

The intercept on the ordinate from Eq. 34 is:

$$I = -K_1'K_2n[P] = -4.0 \qquad \text{(Eq. 49)}$$

The initial slope, S_1, of the plot of Fig. 8 at low concentrations of drug permits the preliminary estimation, in accordance with Eq. 36, of:

$$S_1 = K_1'K_2/(1 + n[P]/K_4) = 0.00375 \text{ ml}/\mu\text{g} \qquad \text{(Eq. 50)}$$

It follows from the quotient of Eqs. 47 and 50 that:

$$S_2/S_1 = 1 + n[P]/K_4 = 34.7 \qquad \text{(Eq. 51)}$$

so that $n[P]/K_4 = 33.7$.

It also follows from Eqs. 48 and 51 that:

$$\frac{n[P]/K_4}{C_1} = 1/K_4 = 1.09 \text{ ml}/\mu\text{g} \qquad \text{(Eq. 52)}$$

or $K_4 = 0.917$ μg/ml.

For all values of C, C_u can be calculated from Eq. 31 from the evaluated parameters K_4 and $n[P]$. Thus, the values of $(k_0 -$

Table I—Evaluated Parameters at pH 6.9 and 37.5° that Fit the Observed Dependency of the Apparent Generation Rate Constant, k_{app}, on Drug Concentration for the Model of Scheme I that Includes Saturable Biophasic Enzymic Inactivation and Saturable Receptor Site Binding of Drug

Drug	Tetracycline Hydrochloride[a]	Chloramphenicol	Sodium Fusidate	Erythromycin Lactobionate	Oleandomycin Phosphate
$K_1'K_2$					
ml/μg of total drug	11.0	2.5	0.13	0.13	0.03
liters/mole of total drug	5.3×10^6	8.1×10^5	7.0×10^4	1.4×10^5	2.4×10^4
liters/mole of uncharged drug	6.1×10^6	8.1×10^5	2.6×10^6	1.13×10^7	0.96×10^6
$K_1''K_2$					
ml/μg of total drug	5.5	0.5	0.00375	0.02	0.003
liters/mole of total drug	2.6×10^6	1.61×10^5	2.0×10^3	2.2×10^4	2.40×10^3
liters/mole of uncharged drug	3.0×10^6	1.61×10^5	7.5×10^4	1.81×10^6	0.96×10^5
$C_1 = k_d'/k_1K_3$					
μg/ml (total drug)	0.15	0.28	31	31	160
mole/liter (total drug)	3.1×10^{-7}	8.7×10^{-7}	5.8×10^{-5}	2.85×10^{-5}	2.0×10^{-4}
mole/liter (uncharged drug)	2.7×10^{-7}	8.7×10^{-7}	1.6×10^{-6}	3.42×10^{-7}	5.0×10^{-6}
$K_1'K_3$					
ml/μg of total drug	66.7	14.3	1.10	0.18	0.056
liters/mole of total drug	3.2×10^7	4.6×10^6	5.9×10^5	1.96×10^5	4.38×10^4
liters/mole of uncharged drug	3.7×10^7	4.6×10^6	2.2×10^7	1.63×10^7	1.79×10^5
K_1'/K_1''	2.0	5.0	34.7	6.5	10
$k_d'/(k_{-1} + k_d)$	1.0	4.0	33.7	5.5	9
$k_d'K_2/K_3(k_{-1} + k_d) = k_d'K_1'K_2/k_1K_3$	0.20	3.8	4.0	4.6	5.6
K_2/K_3	0.20	0.95	0.12	0.84	0.62
pKa	7.7	...	5.35	8.8	8.5
Fraction uncharged at pH[a] 6.9	0.86	1.00	0.027	0.012	0.0245

[a] The pH dependency of tetracycline action is not assured. However the pKa was one chosen on the assumption that this represents the loss of a proton from the neutral zwitterion.

$k_{app})/k_{app}$ of Eq. 32 and the corresponding values of k_{app} can be calculated for all values of C from the known $K_1'K_2$ and the values of C_u estimated from Eq. 31.

In actuality, the curve of Fig. 2 for k_{app} against concentration was best fitted using the values given in Eqs. 47–52 to demonstrate that the data are consistent with the model of Scheme II for a saturable binding of drug to the components of the medium.

Although this model for saturable binding to components of the medium can be excluded in the case of sodium fusidate since varying the concentrations of the components of Anton's medium had no apparent effect on sodium fusidate activity, this procedure clearly demonstrates the applicability of microbial kinetics to the determination of the binding constants of antibacterial bacteriostatic agents to various compounds for saturable binding of drug to receptor sites. Analogous procedures can be developed to estimate the saturable binding constants to components of the medium for nonsaturable binding to receptor sites from Eqs. 45 and 46 and should be applicable to spectinomycin (17) where such a phenomenon was observed.

Rationalizations of Observed Generation Rate Constant, k_{app}, Dependence on Sodium Fusidate Concentration for Enzymic Saturable Degradation in Biophase—On the postulation of the validity of this premise for k_{app} dependence, the data for sodium fusidate at pH 6.9 and 37.5° have been plotted in Fig. 8 as $(k_0 - k_{app})/k_{app}$ against concentration C and, if Eq. 15 holds, Eq. 18 may be applicable at low drug concentrations and Eq. 20 at high. The reasonable linearity of the terminal data in accordance with Eq. 20 at high sodium fusidate concentrations permits the estimation from the terminal slope, S_2, of:

$$S_2 = K_1'K_2 = 0.13\ \text{ml}/\mu\text{g}\ (7.0 \times 10^4\ \text{liters/mole}) \quad \text{(Eq. 53)}$$

at pH 6.9. In terms of unionized drug, the value of S_2 would be 4.83 ml/μg (2.6×10^6 liters/mole). The intercept on the abscissa estimates:

$$C_1 = k_d'/K_1'K_3(k_{-1} + k_d) = k_d'/k_1K_3 = 31\ \mu\text{g/ml}\ (5.8 \times 10^{-5}\ M) \quad \text{(Eq. 54)}$$

or, for unionized drug, $C_1 = 0.84$ μg/ml (1.56×10^{-6} M), where k_d'/K_3 is the maximal rate of saturable enzymic degradation in the biophase. The value of C_1 also may be considered as the ratio of maximal zero-order degradation within the biophase to the first-order rate constant for permeation from the medium.

The intercept on the ordinate from Eq. 20 is:

$$I = -k_d'K_2/K_3(k_{-1} + k_d) = -k_d'K_1'K_2/k_1K_3 = -4.0 \quad \text{(Eq. 55)}$$

The initial slope, S_1, of the plot of Fig. 8 at low concentrations of drug permits the preliminary estimation in accordance with Eq. 18 of:

$$S_1 = K_1''K_2 = 0.00375\ \text{ml}/\mu\text{g}\ (2.02 \times 10^3\ \text{liters/mole}) \quad \text{(Eq. 56)}$$

In terms of unionized drug, the value of S_1 would be 0.139 ml/μg (7.47×10^4 liters/mole).

It follows from the quotient of Eqs. 53 and 56 that:

$$S_2/S_1 = K_1'/K_1'' = \left(\frac{k_1}{k_{-1}+k_d}\right)\Big/\left(\frac{k_1}{k_{-1}+k_d+k_d'}\right) = 1 + k_d'/(k_{-1} + k_d) = 34.7 \quad \text{(Eq. 57)}$$

so that $k_d'/(k_{-1} + k_d) = 33.7$.

It also follows from Eqs. 54 and 57 that:

$$\frac{k_d'/(k_{-1}+k_d)}{C_1} = \frac{k_d'/(k_{-1}+k_d)}{k_d'/K_1'K_3(k_{-1}+k_d)} = K_1'K_3 = 1.09\ \text{ml}/\mu\text{g}\ (5.9 \times 10^5\ \text{liters/mole}) \quad \text{(Eq. 58)}$$

In terms of unionized drug, the value of $K_1'K_3$ would be 4.04 ml/μg (2.2×10^7 liters/mole).

If the numerator and denominator of Eq. 16 are multiplied by k_1K_1' and it is realized that $K_1' = k_1/(k_{-1} + k_d)$ and $K_1'' = k_1/(k_{-1} + k_d + k_d')$, it can be shown that:

$$K_3C' = \frac{K_1'K_3C - K_1'/K_1'' \pm \sqrt{(K_1'K_3C)^2 + 2K_1'K_3C - 2K_1'K_3Ck_d'/(k_{-1}+k_d) + (K_1'/K_1'')^2}}{2} \quad \text{(Eq. 59)}$$

so that K_3C' can be calculated for all values of C from the evaluated parameters $K_1'K_3 = 1.09$ ml/μg, $k_d'/(k_{-1} + k_d) = 33.7$, and $K_1'/K_1'' = 34.7$.

If the numerator and denominator of Eq. 15 are multiplied by K_1', it can be shown that:

$$\frac{k_0 - k_{app}}{k_{app}} = \frac{K_1'K_2C}{1 + [k_d'/(k_1 + k_d)][1/(1 + K_3C')]} \quad \text{(Eq. 60)}$$

so that this function can be calculated for all values of C from the

evaluated parameters $K_1'K_2$ = 0.13 ml/μg and $k_d'/(k_{-1} + k_d)$ = 33.7 and the values of K_3C' estimated from Eq. 59.

Similarly, it can be shown from Eq. 14 that:

$$k_{app} = k_0 - \frac{k_0K_1'K_2C/\{1+[k_d'/(k_{-1}+k_d)][1/(1+K_3C')]\}}{1+K_1'K_2C/\{1+[k_d'/(k_{-1}+k_d)][1/(1+K_3C')]\}} \quad \text{(Eq. 61)}$$

In actual practice, the values given in Eqs. 53–56 were those finally resolved to give the best fit of k_{app} against C (Fig. 2) consistent with the model of Scheme I for a saturable degradation process in the biophase and saturable binding to receptor sites. The actual procedure was to vary the initial estimates of S_2 (Eq. 53), C_1 (Eq. 54), I (Eq. 55), and S_1 (Eq. 56) obtained from the plot of Fig. 8 and used to estimate $k_d'/(k_{-1} + k_d)$ from Eq. 57 and $K_1'K_3$ from Eq. 58.

Analogous procedures can be developed to estimate the parameters of a saturable degradation process in the biophase for nonsaturable binding to receptor sites from Eqs. 37–42.

If these premises are accepted, it is possible to conclude that the greater activity of sodium fusidate against Gram-positive organisms than against Gram-negative organisms such as *E. coli* (1) is due to the former's lack of a saturable degradation process in the biophase and/or a more active transport process of sodium fusidate into the biophase with lessened biophase degradation, and/or the receptor sites in the biophase of Gram-positive organisms having a higher affinity for the drug than those of Gram-negative organisms.

Compatability of Diverse k_{app} Dependencies on Drug Concentration for Various Drugs on Premise of Drug-Affected Steady-State Generation with Enzymic Saturable Degradation in Biophase—In the fluorouracil and sulfonamide cases, the saturable receptor site model appears to hold for all concentrations (10) and implies no saturable enzymic degradation in the biophase for these compounds. The form of the k_{app} dependency on drug concentration in the medium is different for several classes of drugs (10), as shown in Fig. 2. Such plots for tetracycline and chloramphenicol are usually remarkably linear (10), whereas for the macrolides and lincosaminides they are initially linear at low concentrations and the rate of change of k_{app} subsequently decreases with concentration.

However, all of these plots can be fitted to Eq. 61, obtained from Eq. 14, showing k_{app} dependency on concentration in accordance with the model of Scheme I where a saturable enzymic degradation in the biophase may be postulated. Judicious choices of values for $K_1'K_2$ (Eq. 53), $C_1 = k_d'/k_1K_3$ (Eq. 54), and K_1'/K_1'' (Eq. 57) permitted the calculation of proper values of $k_d'/(k_{-1} + k_d) = (K_1'K_1'') - 1$, $K_1'K_3$ (Eq. 58), and K_3C' (Eq. 59), so that the lines of best fit based on these values could be plotted (Fig. 2) for data obtained for sodium fusidate, tetracycline, chloramphenicol, erythromycin, and oleandomycin in Anton's medium (12) in accordance with Eq. 61.

Values of these parameters that gave the excellent fits to the data plotted in Fig. 2 are given in Table I and are for antibiotics of widely different structure. The $k_d'/(k_{-1} + k_d)$ values show the ratios of the rate constant for the loss of drug by saturable enzyme inactivation in the biophase to the loss of drug from all first-order processes. The large value assigned to sodium fusidate indicates a significant amount of saturable enzyme inactivation of this drug. The values of C_1, the ratios of the maximal rate of saturable enzyme inactivation of drug (k_d'/K_3) to the first-order rate constant for permeation of the drug into the biophase (k_1), show the largest values for those drugs of the lowest potency against the Gram-negative *E. coli*—*viz.*, sodium fusidate, erythromycin, and oleandomycin. In the case of sodium fusidate, this loss of drug to saturable enzymic inactivation explains the unique S-shaped curve observed in Fig. 2, since at lower concentrations and thus lower rates of penetration into the biophase a larger fraction of the drug that permeates the cell wall is rendered ineffective. As this enzyme becomes saturated, a larger fraction of the sodium fusidate that penetrates is available to exercise inhibition.

The parameter $K_1'K_3$ may be written $K_3(k_1/k_{-1} + k_d)$; if it were assumed that k_d were zero (no nonsaturable enzyme degradation) and that different unionized drugs had similar partition constants ($K_1 = k_1/k_{-1}$) into the biophase, then the relative affinities of the various drugs for saturable inactivating enzymes could be obtained from the ratios of the $K_1'K_3$ values. Thus, relative to sodium fusidate and in terms of equivalents of unionized drug, such relative affinities were: tetracycline, 1.6; chloramphenicol, 0.21; erythromycin, 0.74; and oleandomycin, 0.086.

Comparative Effects of *a priori* Equipotent Combinations of Other Bacteriostatic Drugs with Sodium Fusidate—The effects of *a priori* equipotent combinations of sodium fusidate predicted on the basis of the observed k_{app} values of the separate bacteriostatic drugs are shown in Fig. 5. All drug combinations were studied in Anton's medium, pH 6.9, 37.5°, at two levels of potency in the k_{app} ranges of 2.0–2.5 and 3.0–4.0. If two drugs have the same functional dependency of k_{app} on concentration and one does not affect the action of the other in combination, the k_{app} is invariant with the change in the fraction of either drug in the *a priori* equipotent combination (10). This is not true if the functional dependencies of the two drugs differ, even though the presence of the one may not affect the action of the other in the combination. For example, if a drug with an S-shaped k_{app} *versus* concentration curve is combined with a drug having a linear k_{app} *versus* concentration curve, then a constant k_{app} is not expected for combinations of various fractions of *a priori* equipotent amounts. The expected k_{app} of the combination, $(k_{app})_{comb}$, on the basis of equivalency may be calculated from the apparent generation rate constants $(k_{app})_A$ and $(k_{app})_B$, which resulted when drugs A and B were used separately (14, 18, 19):

$$(k_{app})_{comb} = k_0 - (k_{app})_A - (k_{app})_B \quad \text{(Eq. 62)}$$

The predicted k_{app} dependencies for various combinations of sodium fusidate with another drug are given in Fig. 5. Significant increases in k_{app} values above these predictions can be considered as evidence for antagonism; a significant decrease below these predictions is evidence of synergism (10). Thus, sodium fusidate and novobiocin can be interpreted to be antagonistic, oleandomycin or erythromycin with sodium fusidate is synergistic, and combinations of sodium fusidate with tetracycline, chloramphenicol, 1′-demethyl-4′-depropyl-4′-[(*R*) and (*S*)]-*n*-pentylclindamycin are indifferent (10). The apparent mild antagonism of lincomycin with sodium fusidate is not definitive since inactive complexes may be formed in solution between these drugs. Sodium fusidate precipitates both lincomycin and clindamycin at pH 6.9, although the drug-affected bacterial growth rates were measured with no obvious precipitate in the growth medium.

Erythromycin is a protein synthesis inhibitor whose mode of action requires binding to the 50S ribosomes and, in addition, a distortion of peptidyl transferase with resultant inhibition of the translocation step of peptide synthesis (9). The similarity of structure between erythromycin and oleandomycin permits the assumption of the same mode of action, an assumption that is further strengthened by the synergy both drugs show in combination with sodium fusidate. The mode of action of sodium fusidate is also inhibition of protein synthesis, but the mechanism involves a binding to, and stabilization of, an energy-releasing complex, with subsequent inhibition of translocation (6–9). Reference to Fig. 2 shows that the three drugs all exhibit saturation of action against *E. coli* in the higher concentration range, when k_{app} approaches 1.5×10^{-4} sec^{-1}. The requirement for synergistic action is that drugs in combination act on saturable receptor sites and on separate steps in sequential processes (19). This requirement appears to be met by sodium fusidate and the macrolides in the sequential steps of the translocation process.

The antagonism of sodium fusidate and novobiocin is consistent with the observed antagonism of novobiocin with other 50S binding inhibitors of protein biosynthesis such as chloramphenicol, erythromycin, and lincomycin, which has been rationalized on the premise that novobiocin inhibits the production of messenger RNA necessary for the translocation of ribosomes (18). This latter process is inhibited by the 50S binding antibiotics, and the overall rate of protein biosynthesis (and thus the dependent rate of microbial generation) is determined by whichever process is the slower with the demonstration of an apparent antagonism.

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Kinetics and Mechanisms of Drug Action on Microorganisms XXI: Effect of Quinacrine on *Escherichia coli* and Its Possible Complexation with Components of Nutrient Growth Medium

A. J. RICHARD* and EDWARD R. GARRETTˣ

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The antimalarial (1) quinacrine, 6-chloro-9-[[4-(diethylamino)-1-methylbutyl]amino]-2-methoxyacridine, has been reported to be clinically effective and synergistic *in vivo* (2) and *in vitro* (3–5) in combinations with other antibiotics to delay or prevent the emergence of antibiotic-resistant strains of Gram-negative and Gram-positive bacteria. Quinacrine, a known nucleic acid intercalating agent, inhibits DNA replication and prevents the transfer of R-factors to result in (6, 7) antibacterial activity.

This paper reports on the quantitative microbial kinetics of the Gram-negative *Escherichia coli* (ATCC 12407) as affected by quinacrine. A general theory, recently developed (8) to rationalize the steady-state microbial generation of *E. coli* affected by bacteriostatic drugs, is applied to quinacrine-affected generation rates. Specifically, it is shown how microbial kinetics may be used to quantify the protein binding or nutrient complexation of a bacteriostatic agent when such binding is a saturable process.

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Test Organism—Replicate slants of *E. coli* (ATCC 12407) were used in all experiments. This nonclumping strain is well suited for colony counts and for small particle monitoring[1].

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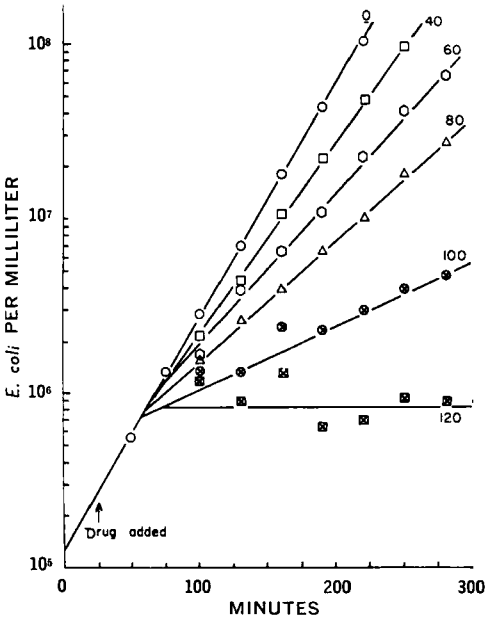


Figure 1—*Typical generation curves of* E. coli *in Anton's medium (pH 6.9) in the absence and presence of various concentrations of quinacrine, obtained by total counts. The curves are labeled according to the quinacrine concentrations in micrograms per milliliter.*

Bacterial Generation and Monitoring—Methods used in this laboratory for bacterial culture, total count monitoring, viable counting, and size frequency distribution studies were described in detail (8, 10, 11).

Reversibility of Drug Action—Reversibility of drug action can be shown by taking aliquots of organism cultures containing about 10^7 *E. coli*/ml during the drug-affected steady-state growth at 37.5° and diluting into fresh broth with no added drug and also into fresh broth containing predetermined drug concentrations (11). Reversibility of drug action is established if the diluted cultures grow at the control rate in the fresh broth and at the predicted rates when drug is present in the diluent. Reversibility was studied at 0 and 90 μg/ml of quinacrine at pH 6.9 in Anton's medium.

Effect of Equipotent Mixtures of Quinacrine and Other Growth Inhibitors—Replicate 49.5-ml volumes of cultures containing 5×10^5 *E. coli*/ml generating in the logarithmic phase at pH 6.9 and 37.5° were treated with 0.5 ml of equipotent mixtures of antibiotics (12). The mixtures consisted of 100, 80, 60, 40, 20, and 0% of the equipotent quinacrine solution and the residual percentage of the other antibiotic solution to maintain equipotency at two different dosage levels. If the drugs precipitated on mixing, the mixture was diluted with Anton's medium until the precipitate dissolved; the appropriate volume was withdrawn from each culture flask before addition of drug solutions.

RESULTS AND DISCUSSION

Effect of Drug Concentration on Generation Rates—The generation rates of *E. coli* in graded quinacrine concentrations measured by the total count and the viable count methods in Anton's medium at pH 6.9 were coincident (Fig. 1), and no bactericidal activity was observed up to 100 μg/ml of quinacrine. It was, therefore, justified to use total counts to determine generation rates up to a limiting quinacrine concentration of 100 μg/ml. At 150 μg/ml, a significant kill of bacteria was observed. The drug was rapidly equilibrated between the growth medium and the biophase, as indicated by a lag time of 25 min or less between the time of drug addition and the appearance of deviation from the control logarithmic phase growth rate. The slopes of the linear semilogarithmic plots of the drug-affected organisms obtained from the curves in Fig. 1 represent the steady-state generation rate constants (k_{app}). The plots of k_{app} values against drug concentrations are shown in Fig. 2 for three inoculum sizes: 1×10^5, 5×10^5, and 1×10^6 *E. coli*/ml at the time of drug addition. No difference in drug effectiveness was observed when the quinacrine was added over this 10-fold range in the bacterial population.

Effect of pH and Nutrient Broth Components on Drug-Affected Generation Rates—The quinacrine-affected generation rates and rate constants (k_{app}) were reduced markedly with increased pH of the growth medium (Fig. 3), whereas pH variation in the region studied had no significant effect on drug-free generation. Since the phosphate buffer of the growth medium has reduced capacity above pH 7.8, and since *E. coli* generation is significantly affected in drug-free medium above pH 8, the former is an upper limit for the pH range. Quinacrine is a dication (13) at neutral pH values with pKa's of 7.69 and 10.18. When the total quinacrine concentrations were multiplied by the fraction, *f*, of drug singly charged to obtain monocation concentrations, the various k_{app} concentration curves were coincident (Fig. 3, insert).

Variations of the ammonium ion (NH_4^+), magnesium ion (Mg^{+2}), citrate, and dextrose components of Anton's broth did not modify the drug-affected generation rates. When the broth amino acid concentration was halved, the potency of quinacrine increased; when the amino acid concentration was doubled, the potency of the quinacrine decreased (Fig. 4). This indicates significant drug inactivation by the broth amino acids of effective quinacrine in the medium. Identical effects were observed when the broth concentrations were doubled and halved. When the concentration abscissa for the doubled concentration is halved and when the concentration abscissa for the half concentration is doubled, the resultant plots are close but not exactly coincident to that for the normal amino acid concentration, indicating that the extent of inactivation is related to the amino acid concentration.

Reversibility of Drug Action—The effects of quinacrine on *E. coli* were reversible after exposure of the organisms up to concentrations of 90 μg/ml of quinacrine for 200 min at pH 6.9. A 10-fold dilution of the culture at 10^7 organisms/ml with fresh medium 225 min after the addition of 90 μg/ml of quinacrine immediately resulted in a new generation rate close to that observed in a drug-free culture. Higher drug concentrations were not studied for reversibility because of the kill effect observed at drug levels of 150 μg/ml.

Drug Effects on Size of *E. coli*—There was no difference in the size of generating *E. coli* up to quinacrine levels of 50 μg/ml as compared to organisms generating in drug-free medium. However, the average cell size was 40% greater than the control cells at 55 μg/ml of quinacrine and almost double at 95 μg/ml of quinacrine, indicating that cell division occurs at larger cell sizes with higher drug concentrations.

Effects of Drug Combinations with Quinacrine—The observed k_{app} values for variously presumed equipotent combinations of quinacrine with other bacteriostatic drugs against the volume fraction of quinacrine solution in mixtures of solutions

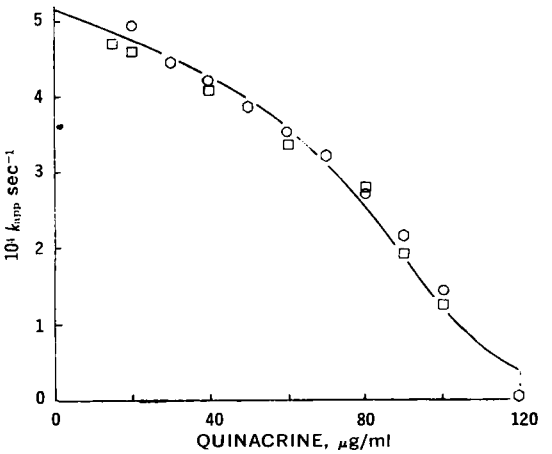


Figure 2—*Dependency of the apparent generation rate constant,* k_{app}, *at pH 6.9 of* E. coli *on drug concentration of quinacrine in micrograms per milliliter. The solid line through the data points was calculated from the theory (8) which accounts for the complexation of quinacrine with amino acids in the nutrient broth. The inoculum sizes were:* ○, 10^5 E. coli/ml; ○, 5×10^5 E. coli/ml; *and* □, 10^6 E. coli/ml.

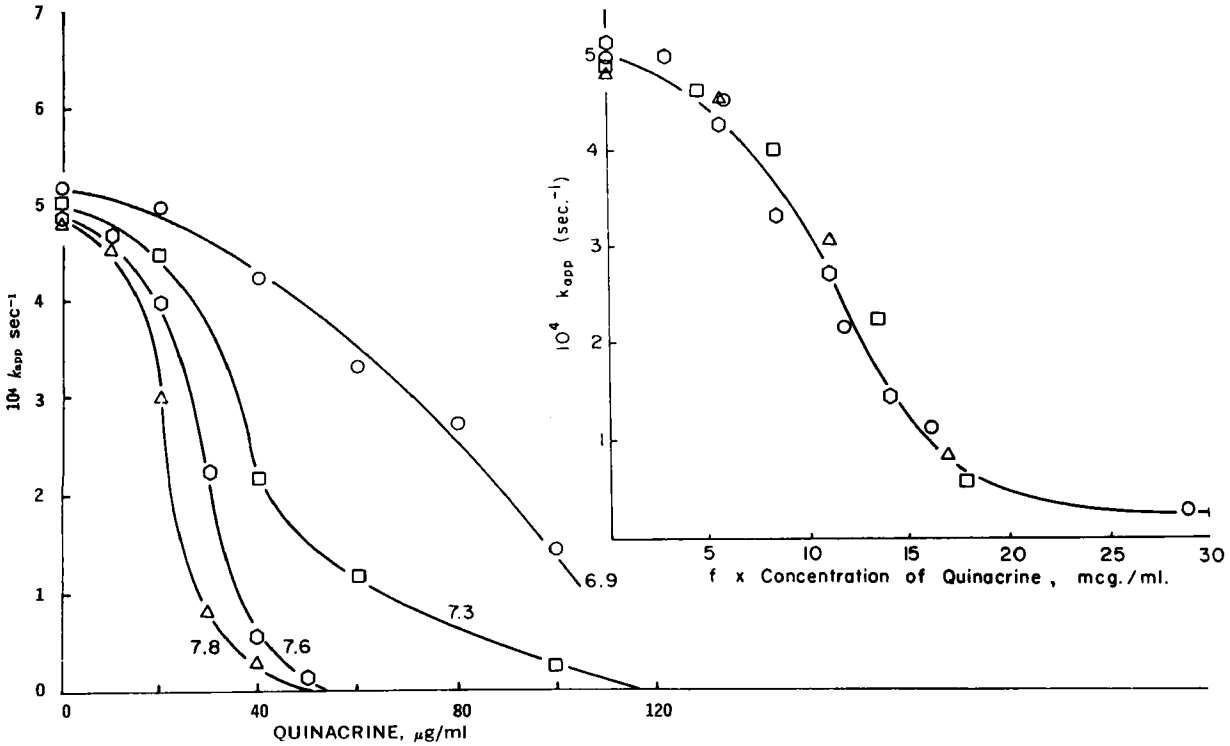


Figure 3—*Effect of pH upon the dependency of* k_{app} *on drug concentration. Only one curve (insert) is required when the quinacrine concentrations were multiplied by the fraction,* f, *of singly charged drug with a pKa' of 7.7. The curves are labeled with the pH value of the study.*

equipotent in quinacrine and tetracycline or chloramphenicol showed no significant difference in k_{app} for any mixture. Thus, it can be concluded that chloramphenicol and tetracycline act independently of the action of quinacrine.

Evaluation of Drug Inactivation in Medium by Microbial Kinetics—A significant fraction of the quinacrine is rendered inactive by the amino acid component of the nutrient broth at pH 6.9 (Fig. 4). The activity of quinacrine increases with pH (Fig. 3) and indicates improved transport of the drug across the bacterial cell wall with decreasing charge and/or decreased inactivation of drug by the broth amino acids with increasing pH.

Scheme I represents a model consistent with the observed dependency of the steady-state rate constants (k_{app}) for microbial generation on the concentration of quinacrine in the nutrient medium. This model postulates that free or effective drug, C_u, permeates the cell to result in a steady-state biophase concentration, C', which competes with the reaction of a metabolite precursor, M, for those receptor sites, R, vital to microbial generation. Steady-state generation of bacteria can result when the concentration, C', of drug in the biophase is a constant. It is assumed that biophase interactions and possible inactivation of drug do not significantly affect the external concentration of drug, C_u, and that the equilibria of M and C' with receptor sites, R, are instantaneous.

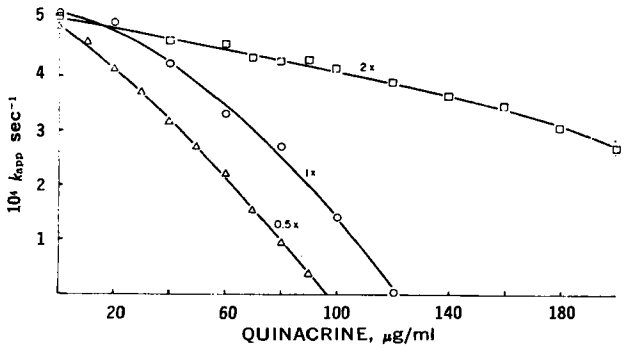


Figure 4—*Effect of varying the concentration of casamino acids of Anton's medium upon the* k_{app} *dependency on quinacrine concentration. The curves are labeled as to the multiples of casamino acid concentration. No such effects were observed when other components of Anton's medium were each varied in turn.*

An expression is available (8, 14, 15) to calculate the concentration of free or effective drug in the nutrient broth for this system when there are saturable drug complexing or reacting sites, $n[P]$, in the medium:

$$C_u = \frac{C_t}{1 + \dfrac{n[P]}{K_4 + C_u}} \qquad \text{(Eq. 1)}$$

where C_t is the total drug concentration in the medium, K_4 is the dissociation constant for complexation, and n is the number of sites per molecule of complexing agent, P. The value of C_u can be expressed explicitly as the solution of a quadratic equation when the parameters $n[P]$ and K_4 are known. It has been shown (8) that consideration of the saturable receptor site model of Scheme I with the assumption of nonsaturable inactivation of the drug in the biophase, *i.e.*, where k_d is a first-order rate constant, leads to:

$$k_{app} = k_0 - \frac{k_0 K_1' K_2 C_u}{1 + K_1' K_2 C_u} \qquad \text{(Eq. 2)}$$

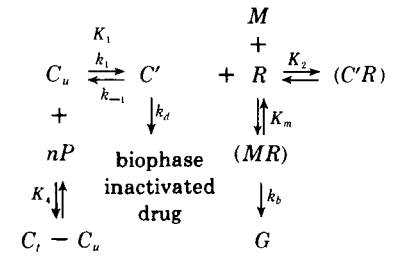
so that:

$$\lim_{C_u \to \infty} \frac{k_0 - k_{app}}{k_{app}} = K_1' K_2 C_t - K_1' K_2 n[P] \qquad \text{(Eq. 3)}$$

and:

$$\lim_{C_u \to 0} \frac{k_0 - k_{app}}{k_{app}} = \frac{K_1' K_2 C_t}{1 + \dfrac{n[P]}{K_4}} \qquad \text{(Eq. 4)}$$

where k_0 is the microbial generation rate constant in the absence of drug, and $K_1' = k_1/(k_{-1} + k_d)$ reduces to $K_1 = k_1/k_{-1}$ for the drug-affected steady-state generation if there is no biophase inactivation of drug. A plot (Fig. 5) of $(k_0 - k_{app})/k_{app}$ against drug concentration will provide a slope, S_2, at high drug concentrations to estimate preliminarily $K_1'K_2$, and an initial slope, S_1, at low



Scheme I—Steady-state model for permeation of free drug of concentration C_u *in nutrient medium into the biophase of the microorganism of concentration* C′, *and the net result is the observed inhibition of the microbial generation rate which is postulated to be proportional to the production of* G. *Capitalized* K's *represent equilibrium constants for the particular reactions; lower case* k's *are rate constants.*

drug concentrations to estimate $K_1'K_2/(1 + n[P]/K_4)$. The intercept of the extrapolated terminal linear portion of the curve on the abscissa, C_1, will provide an estimate of $n[P]$, and the ratio of the terminal slope, S_2, to the initial slope, S_1, gives $1 + n[P]/K_4$. It is then possible to calculate K_4 from the ratio:

$$\frac{C_1}{n[P]/K_4} = \frac{n[P]}{n[P]/K_4} = K_4 \qquad \text{(Eq. 5)}$$

These initial estimates of these parameters can be refined by the methods given previously (8) to give the best fit for the plot of the experimental k_{app} against drug concentration. These refined parameters of $n[P]$ and K_4 then permit the calculation (8) of C_u as a function of total drug concentration, C, by:

$$C_u = \frac{C - K_4 - n[P] \pm \sqrt{(K_4 + C + n[P])^2 - 4n[P]C}}{2} = \frac{C - K_4 - n[P] \pm \sqrt{(K_4 + C - n[P])^2 + 4n[P]K_4}}{2} \qquad \text{(Eq. 6)}$$

The obtained values for quinacrine in normal Anton's broth are $K_4 = 0.49$ μg of equivalent quinacrine/ml or 0.93×10^{-6} mole/liter and $n[P] = 105$ μg of equivalent quinacrine/ml or 2.06×10^{-4} mole/liter. The curve drawn through the points in Fig. 2 was calculated from Eq. 6 using these parameters. An appropriate plot (Fig. 6, curve A) shows a decreasing rate of change of k_{app} with the increasing calculated C_u values consistent with the saturable receptor site model (Scheme I) for drug action that was postulated for the estimates of C_u. The minimum inhibitory concentration (MIC) was about 15 μg/ml of unbound quinacrine at pH 6.9 compared to that based on total drug of about 100 μg/ml (Fig. 2). In terms of the concentration of the monoprotonated quinacrine concentration (Fig. 6, curve B), the MIC is about 2.5 μg/ml.

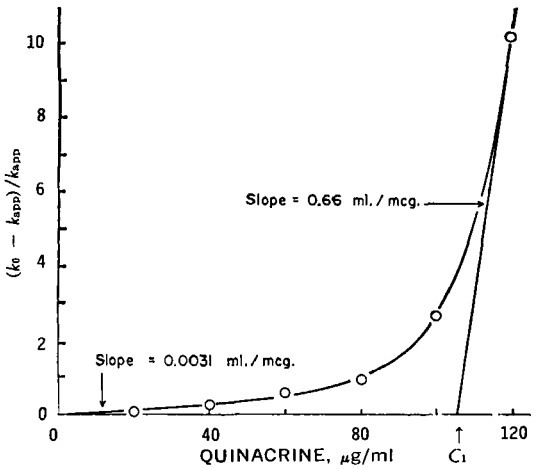


Figure 5—*Plot of* $(k_0 - k_{app})/k_{app}$ versus *concentration of quinacrine in micrograms per milliliter at pH 6.9 according to Eqs. 3 and 4, where the asymptotic terminal slope,* S_2, *is an estimate of* $K_1'K_2$, *and the initial slope estimates* $K_1'K_2/(1 + n[P]/K_4)$. *The intercept,* C_1, *on the abscissa estimates* $n[P]$.

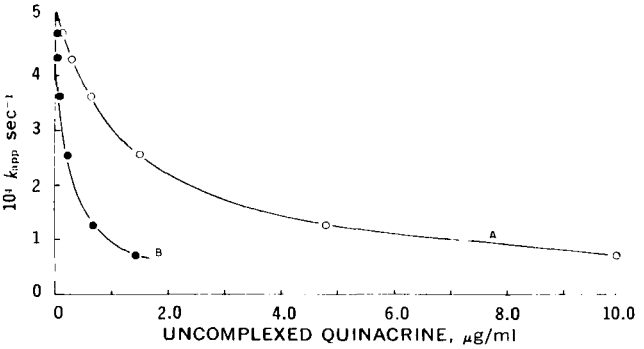


Figure 6—*Plot of* k_{app} versus *a presumed uncomplexed quinacrine concentration at pH 6.9 to the amino acids of the nutrient broth (curve A) and* versus *a presumed uncomplexed quinacrine concentration that is singly charged (curve B), i.e., the quinacrine concentrations multiplied by the fraction,* f, *of singly charged drug with a pKa′ of 7.7.*

It must be realized that these estimates of the constants for complexation of quinacrine to the media were based on Scheme I and assumed nonsaturable biophase inactivation of drug and saturable binding of drug to receptor sites so that $K_1'K_2$ of Eq. 3 for the data of Fig. 5, as deduced from such a model (8), is 0.66 ml/μg of equivalent quinacrine or 0.33 liter/mole. This may not necessarily be true since the terminal points of the k_{app} *versus* concentration plots of Figs. 2–4 also could be approximated reasonably by straight lines, which would indicate essentially nonsaturable binding of receptor sites in the biophase. The fitting of the data to a model that presumes this with saturable complexation to the components of the medium would give different values of $n[P]$ and K_4 (8).

It has been shown for a model based on such premises (8), where:

$$k_{app} = k_0 - k_0K_1'K_2C_u \qquad \text{(Eq. 7)}$$

so that $K_1'K_2C_u \ll 1$ in Eq. 2, that plots of $(k_0 - k_{app})/k_0$ against total drug concentration will provide a slope, S_2', at high drug concentrations to estimate preliminarily the $K_1'K_2$ of Eq. 7 for the data of Fig. 7, which is 77 ml/μg of equivalent quinacrine or 39 liters/mole, and an initial slope, S_1', at low drug concentrations to estimate $K_1'K_2/(1 + n[P]/K_4)$ from:

$$\lim_{C_t \to 0} (k_0 - k_{app})/k_0 = K_1'K_2C_t/(1 + n[P]/K_4) \qquad \text{(Eq. 8)}$$

$$\lim_{C_t \to \infty} (k_0 - k_{app})/k_0 = K_1'K_2C_t - K_1'K_2n[P] \qquad \text{(Eq. 9)}$$

where, when $(k_0 - k_{app})/k_0 = 0$, the intercept of the extrapolated terminal linear portion of the curve on the abscissa, C_1, will provide an estimate of $n[P]$. It would be also possible from the ratio of the terminal slope, S_2', to the initial slope, S_1', to estimate $1 + n[P]/K_4$ so that the K_4 value may be estimated by Eq. 5. Such plots (Fig. 7) in accordance with Eqs. 8 and 9 estimate $n[P] = 40$ μg of equivalent quinacrine/ml or 0.79×10^{-4} mole/liter for normal Anton's medium (Fig. 7, curve A). At half the concentration of Anton's medium, the estimated $n[P]$ is approximately half this value (Fig. 7, curve B), which is consistent with the premise that the number of complexation sites for quinacrine is proportional to the concentration of components of the nutrient medium.

The additional fact that plots of k_{app} against concentration of monoprotonated quinacrine are coincident for all studied pH values in Anton's medium (Fig. 3, insert) indicates that the extent of complexation to the components of the medium is not significantly pH dependent, that the active species in the medium are the monoprotonated and neutral molecules, and that monoprotonation of the tertiary nitrogen of the side chain (pKa′ = 10.18) has little effect on the ability of quinacrine to exercise its action, where the dication with the protonated amine of pKa′ = 7.69 (13) appears to exercise no action.

Although the inactivation of quinacrine effectiveness by amino acids can be rationalized by the postulated complexation of

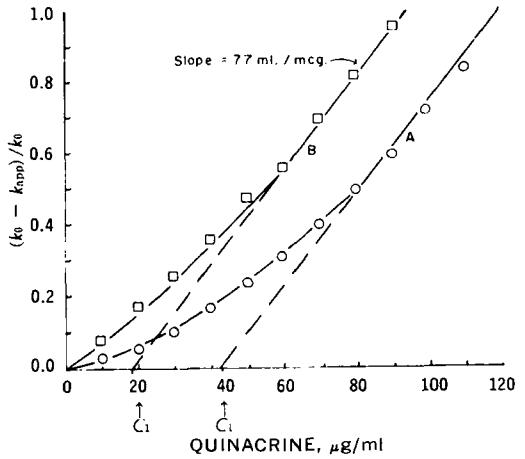


Figure 7—*Plot of* $(k_0 - k_{app})/k_0$ versus *concentration of quinacrine in micrograms per milliliter at pH 6.9 according to Eqs. 8 and 9, where the asymptotic terminal slope,* S_2, *is an estimate of* $K_1'K_2$. *The intercept,* C_1, *on the abscissa estimates* $n[P]$. *Curve A is for normal Anton's medium, and curve B is for a medium with half the concentrations of the components.*

Scheme I, where the complexed drug is presumed to be unavailable to the biophase, no direct evidence of such complexation is available as yet. Alternative explanations may be that increased amino acid concentrations decrease the rate of permeation to the biophase, characterized by the rate constant k_1 by modifying the cell wall, or increase the rate of biophase inactivation characterized by the rate constant k_d of Scheme I. However, if such processes exist, they must be saturable in that either the amino acid inhibition of the rate of permeation has a limited effect or the rate of biophase inactivation has a maximum value related to the amino acid concentration.

If the increase in concentration of a nutrient such as the amino acids has the effect of permitting a biophasic or metabolic increase in the rate of bacterial repair of damage caused by the antibacterial insult, this could also account for the observed phenomenon of amino acid antagonism of drug action. Again, such a process must be saturable and the rate of repair cannot be directly proportional to the amino acid concentration but must approach a maximum value related to the amino acid concentration.

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* On leave of absence from the School of Pharmacy, Virginia Commonwealth University, Richmond, Va.

ˣ To whom inquiries should be directed.

Kinetics and Mechanisms of Solvolysis of 5-Iodocytosine

EDWARD R. GARRETT×, TADEUSZ W. HERMANN*, and HYUK-KOO LEE‡

Abstract □ Iodocytosine is deiodinated by specific hydrogen-ion attack on the neutral species to iodouracil, which is further transformed to uracil in acid solutions. The extent of the transformation of iodouracil to uracil is dependent on its concentration and is consistent with a postulated equilibration of iodouracil and two chloride ions with uracil and the complex anion ICl_2^- and thus is a function of hydrochloric acid concentration. There are pH-independent rates of solvolysis at high acidities, indicative of negligible hydrogen-ion-catalyzed solvolysis of protonated iodocytosine. In the neutral pH region, the iodocytosine solvolysis can be rationalized by general acid–base and water catalysis to cytosine, since it was shown that phosphate and carbonate buffers served as catalysts for the deiodination although acetate buffers did not. Hydroxide ion attacked both the neutral and anionic iodocytosine and produced 6-hydroxycytosine, which further degraded on hydroxide-ion attack, probably through a fast reacting barbituric acid intermediate. Arrhenius parameters were determined.

Keyphrases □ 5-Iodocytosine—kinetics and mechanisms of solvolysis □ pH-rate profiles—5-iodocytosine solvolysis □ Solvolysis—5-iodocytosine, kinetics and mechanisms

The iodinated nucleosides have interesting antitumor and antiviral properties (1, 2). The kinetics of the solvolytic transformation of iodouridine and iodouracil were investigated (3–5) as were various pyrimidine and purine nucleosides and their derivatives (6–14). Cytosine, cytidine, and arabinocytosine undergo hydrolytic deamination to yield the corresponding uracil derivatives (12–14), and their ring system is susceptible to nucleophilic attack at the 5,6-double bond (3).

The purpose of this paper is to report the complete rate–pH profile for the solvolytic degradation of iodocytosine, its Arrhenius parameters, and the pH dependencies of the solvolytic degradative mechanisms of the compound and its initial products in acid, neutral, and alkaline solvolyses, which include iodouracil, cytosine, and 6-hydroxycytosine, where the formation of such products is pH dependent.

EXPERIMENTAL

Kinetic Measurements—Appropriate quantities of the iodocytosine[1] to produce a final concentration of 1×10^{-2} *M* were weighed into volumetric flasks and diluted to volume with nitrogen-purged distilled water. Aliquots were taken from these stock solutions and diluted with acid, alkali, or an appropriate buffer solution to produce, in general, a final nucleoside concentration of 1×10^{-4} *M*. All sample solutions were purged with nitrogen.

The fast reacting solutions in acid and alkali media were studied in 100-ml volumetric flasks. The slow reacting solutions in buffer were purged with nitrogen and sealed in 5-ml ampuls[2]. The reactions were conducted in constant-temperature oil baths controlled at selected temperatures between 70 and 90°, samples were withdrawn at suitable time intervals, and spectra were recorded[3] with time in the spectral range of 220–350 nm.

Spectrophotometric readings were also made on acidified solutions, obtained by adding 0.1–0.3 ml of concentrated hydrochloric acid to lower the pH below 1, and on alkaline solutions, obtained by adding 0.1–0.4 ml of concentrated sodium hydroxide.

pH Measurements—The pH values of the sodium hydroxide and hydrochloric acid solutions at the temperatures studied were calculated from extrapolation of the activity coefficient data in the literature (15). The pH values of buffer solutions were measured directly at the temperature of the study on a pH meter[4] equipped with a high temperature combination electrode[5], standardized with standard buffer solutions of pH 4.0, 7.0, and 10.0 at the same temperature.

TLC Studies—Solutions of iodocytosine (10^{-2} *M*) in 1 *N* HCl, distilled water, and 1 *N* NaOH were prepared and were maintained at 85° in a constant-temperature bath; aliquots were withdrawn at suitable time intervals. The acidic solutions were neutralized before spotting on TLC plates. Solutions (1×10^{-2} *M*) of cytosine, uracil, iodouracil, iodocytosine, barbituric acid, and isobarbituric acid in distilled water were prepared as standards for TLC studies.

The reaction mixtures and standard solutions were spotted on TLC plates coated with a 5-mm layer of silica gel[6] and activated at 110° overnight. The chromatograms were developed in closed tanks with a mixture of *tert*-butyl alcohol–methyl ethyl ketone–ammonium hydroxide–distilled water (4:3:2:1). The solvent front advanced 15–16 cm in approximately 4 hr. After drying, the spots were visualized under UV light. The spots of the chromatogram were extracted with 0.1 *N* HCl and 0.1 *N* NaOH, and UV spectra of the eluates were taken.

Identification of Initial Product of Alkaline Reaction, 6-Hydroxycytosine—Two grams of iodocytosine in 100 ml of 1 *N* NaOH was refluxed for 24 hr until the iodocytosine peak at λ_{max} 297 nm almost disappeared. The reacted solution was cooled and a white precipitate, which was nonchromophoric in the UV when dissolved in water, was filtered off. The filtrate was eluted with distilled water through a column of cationic resin[7] (50–100 mesh, 2.2 × 30 cm).

Aliquots of 100 ml were collected at a constant flow rate (3 ml/min). The first three fractions had no UV absorption and were discarded. Fractions 4–8 had an absorption maximum in the 256–257-nm range (9). These fractions were pale yellow and ranged in pH from 1.3 to 3.6. The fractions were combined and evaporated to dryness. The residue was washed with acetone and ether and recrystallized in methanol. The IR spectra of this compound suggested barbituric acid (16), and it is well known that 6-hydroxycytosine is readily hydrolyzed by dilute acid to this product (17).

Fractions 9 and 10, which had UV λ_{max} at 259–262 nm, were discarded. The UV absorption of fractions 10–20 had λ_{max} 264–265 nm. These were combined and evaporated to dryness, and the residue was washed with acetone and ether and recrystallized three times in water. The product exhibited UV absorption at λ_{max} (0.1 *N* HCl) 263 nm, λ_{max} (water) 265 nm, and λ_{max} (0.1 *N* NaOH) 266 nm. The UV data were in good agreement with an authentic solution of 6-hydroxycytosine and reported data (18) [λ_{max} (pH 3.0) 264 nm and λ_{max} (pH 11.0) 266 nm]. It did not melt below 310° and gave an IR spectrum identical with that of 6-hydroxycytosine. The NMR spectrum in d_6-dimethyl sulfoxide showed δ 4.4 (1, single, H-5), 6.1 (2, broad, NH_2), and 9.5 (2, broad, OH).

[1] California Corporation for Biochemical Research, Los Angeles, Calif.
[2] Kimble, neutraglass.
[3] Cary model 15 recording spectrophotometer.

* Direct-reading Beckman pH meter, Beckman Instruments Inc., South Pasadena, Calif.
[5] Corning combination electrode, Sargent-Welch Scientific Co., Birmingham, Ala.
[6] GF-254, E. Merck AG, Darmstadt, Germany.
[7] Dowex 50W-X2, J. T. Baker Chemical Co., Phillipsburg, N.J.

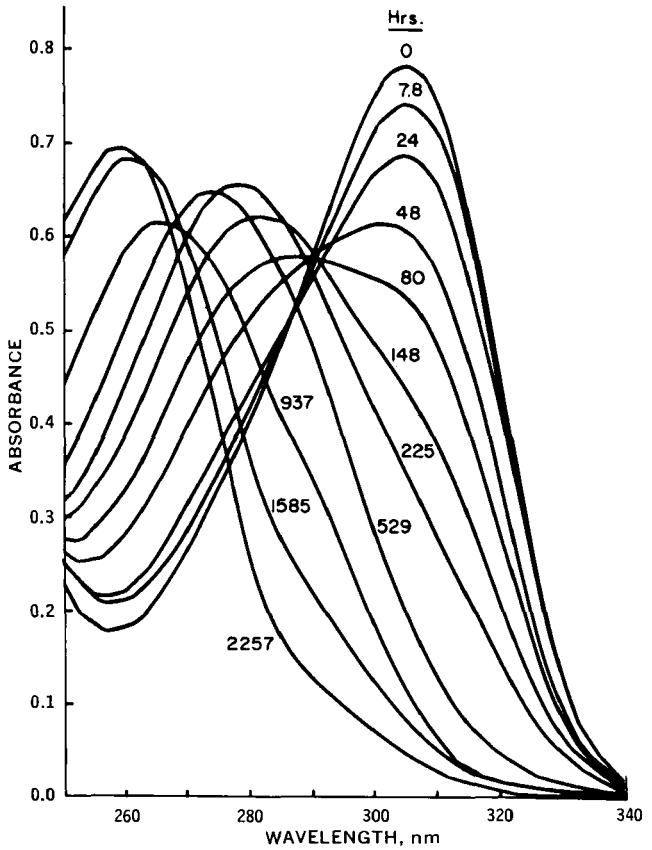


Figure 1—*Typical UV spectral changes of acidified solutions for the solvolysis of 1 × 10⁻⁴ M iodocytosine in acid and acetate buffers at 80.0°; [H₃CCOOH] = 0.1020, [H₃CCOO⁻] = 0.0090, pH 3.65. The curves are labeled as to hours after the start of reaction.*

Anal.—Calc. for $C_4H_5N_3O_2$: C, 37.8; H, 3.9; N, 33.1. Found: C, 37.41; H, 3.92; N, 32.74.

Dissociation Constant for Iodouracil—Beer's law plots for iodouracil and uracil in 1 *N* HCl solution were constructed[3] and the absorptivities were 6.08 × 10³ at 280 nm and 3.45 × 10³ at 260 nm for iodouracil and 1.88 × 10⁴ at 260 nm and 4.37 × 10² at 280 nm for uracil.

Solution of the simultaneous equations for total absorbance from a mixture of iodouracil and uracil yields expressions for the concentrations of the two compounds:

$$C_{IU} = (3.88 \times 10^{-6})A_{260} - (1.67 \times 10^{-4})A_{280} \quad \text{(Eq. 1)}$$

$$C_{U} = (3.06 \times 10^{-5})A_{280} - (5.4 \times 10^{-5})A_{260} \quad \text{(Eq. 2)}$$

Various concentrations of iodouracil in 1 *N* HCl were maintained at 80° in a constant-temperature oil bath. Spectrophotometric readings were obtained after appropriate dilution with 1 *N* HCl until final equilibrium was reached. The concentration of each component in the mixtures at equilibrium was calculated from absorbances A_{260} and A_{280} using Eqs. 1 and 2.

RESULTS AND DISCUSSION

Spectral Changes—The changes with time of typical spectra of the acidic, neutral, and alkaline samples of iodocytosine degraded in acetate buffer, phosphate buffer, and sodium hydroxide solutions, respectively, are given in Figs. 1–3.

The spectra of iodocytosine degrading in hydrochloric acid solution and in buffers up to pH 5 (after acidification) showed an initial absorbance at 305 nm, the λ_{max} of iodocytosine, which subsequently decreased with time (Fig. 1). A new band appeared at 283 nm, the λ_{max} of iodouracil, and subsequently decreased, and a new band appeared at 259 nm, λ_{max} of uracil. This information, coupled with TLC data demonstrated that iodouracil (λ_{max} 283 nm in acid, 295 nm in alkali; R_f 0.82) was the first intermediate of the acid degradation of iodocytosine and sequentially degraded to produce uracil (λ_{max} 259 nm in acid, 283 nm in alkali; R_f 0.76). The isosbestic point at 290 nm was maintained for up to 1 half-life.

The spectra of iodocytosine degrading in pH 5–9 phosphate and carbonate buffers (after acidification) showed a new band at 278 nm in 1 half-life of iodocytosine loss (Fig. 2). TLC studies on degradation in phosphate buffer at 80° showed the initial degradation product of iodocytosine to be cytosine (λ_{max} 275 nm in acid, 278 nm in alkali; R_f 0.65) and the final product to be uracil (λ_{max} 259 nm in acid, 283 nm in alkali; R_f 0.76). The isosbestic point at 285 nm was maintained for 2.5 half-lives.

The absorbance of iodocytosine in alkaline solutions above pH 9 (λ_{max} 298 nm) decreased with time, and a new band appeared with a maximum at 265 nm (λ_{max} of acidified solution 264 nm) (Fig. 3). The maintenance of an apparent isosbestic point at 275 nm was strong evidence of a one-to-one transformation. The final slow loss of absorbance at 265 nm suggested that this intermediate degrades further to a nonchromophoric product. The reaction intermediate, 6-hydroxycytosine, was the initial product of iodocytosine alkaline degradation and was separated by column chromatography and characterized by IR, NMR, and elemental analyses.

TLC Identification—The developed plates of degraded samples from acid solutions under pH 5 were visualized under UV light and indicated the presence of iodouracil (R_f 0.82) and uracil (R_f 0.76). The UV spectra of spots extracted by acid and alkali were consistent with iodouracil [λ_{max} (0.1 *N* HCl) 283 nm and λ_{max} (0.1 *N* NaOH) 295 nm] and uracil [λ_{max} (0.1 *N* HCl) 259 nm and λ_{max} (0.1 *N* NaOH) 283 nm].

TLC spots of the samples degraded in water demonstrated that the products were cytosine (R_f 0.68) and uracil (R_f 0.76). The UV

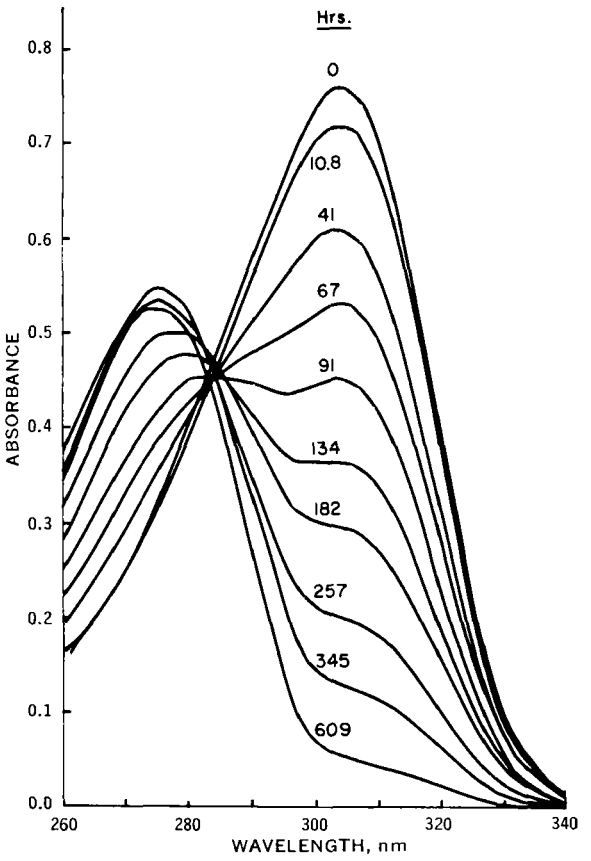


Figure 2—*Typical UV spectral changes of acidified solutions for the solvolysis of 1 × 10⁻⁴ M iodocytosine in phosphate and carbonate buffers at 80.0°; [KH₂PO₄] = 0.0314, [Na₂HPO₄] = 0.0486, pH 6.80. The curves are labeled as to hours after the start of reaction.*

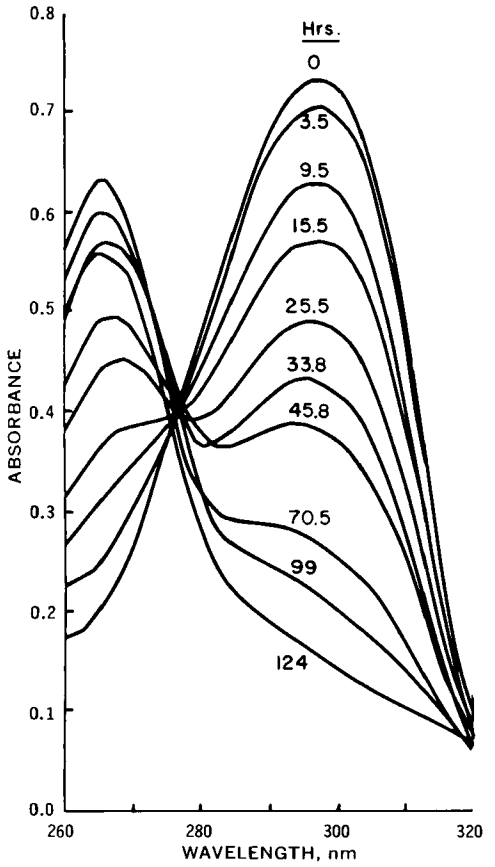


Figure 3—*Typical UV spectral changes for the solvolysis of 1 × 10⁻⁴ M iodocytosine in 0.05 N NaOH at 70°. The curves are labeled as to hours after the start of reaction.*

spectra of spots extracted by acid and alkali were in agreement with cytosine [λ_{max} (0.1 *N* HCl) 275 nm and λ_{max} (0.1 *N* NaOH) 278 nm] and uracil [λ_{max} (0.1 *N* HCl) 259 nm and λ_{max} (0.1 *N* NaOH) 283 nm]. However, the spot for the degradation intermediate in alkali (λ_{max} 263 nm in acid, 266 nm in alkali) did not correspond to any available standards of iodouracil, uracil, cytosine, and barbituric and isobarbituric acids. After isolation by column chromatography, the product was found to be 6-hydroxycytosine.

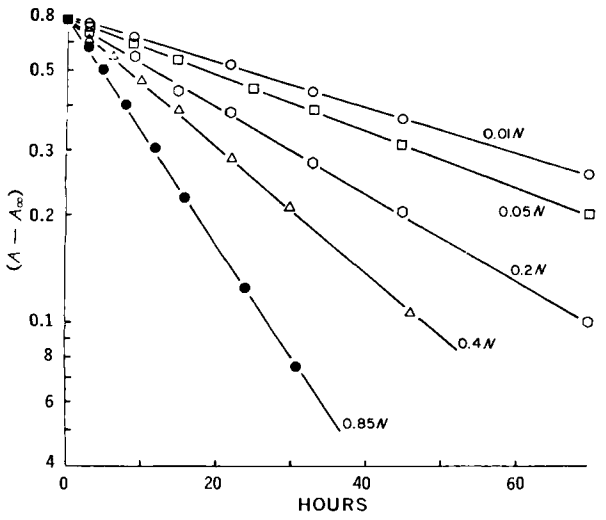


Figure 4—*Typical apparent first-order plots for the solvolysis of iodocytosine in various sodium hydroxide concentrations at 80°. The absorbance values, A, were measured at 297.5 nm, and A_∞ is the final asymptotic absorbance.*

Table I—Apparent First-Order Rate Constants, k (in Seconds^{-1}), for the Degradation of Iodocytosine at Various Temperatures and pH Values

Temperature	Buffer	pH[a]	$10^6 k$[b]
70.0°	0.85 *N* HCl	0.22	2.20
	0.05 *N* HCl	1.40	2.41
	Acetate	3.60	0.58
	Phosphate	5.85	0.14
	Carbonate	8.75	0.225
	0.05 *N* NaOH	11.4	1.93
	0.20 *N* NaOH	11.97	2.96
	0.60 *N* NaOH	12.41	5.84
	0.85 *N* NaOH	12.56	8.90
80.0°	0.85 *N* HCl	0.22	6.00
	0.40 *N* HCl	0.55	6.20
	0.10 *N* HCl	1.12	6.40
	0.05 *N* HCl	1.40	6.20
	0.01 *N* HCl	2.06	5.90
	Acetate	3.60	1.43
	Acetate	4.35	0.52
	Acetate	5.35	0.32
	Phosphate	5.85	0.44
	Phosphate	6.80	0.35
	Phosphate	8.05	0.354
	Carbonate	8.90	1.30
	Carbonate	9.50	3.40
	0.01 *N* NaOH	10.56	3.93
	0.05 *N* NaOH	11.23	4.93
	0.20 *N* NaOH	11.78	7.72
	0.40 *N* NaOH	12.05	11.3
	0.85 *N* NaOH	12.35	19.3
85.0°	0.40 *N* HCl	0.40	11.1
	Acetate	4.35	1.01
	Phosphate	6.80	0.53
	0.01 *N* NaOH	10.55	6.88
	0.10 *N* NaOH	11.41	9.63
90.0°	0.40 *N* HCl	0.40	16.0
	Acetate	5.60	8.01
	Phosphate	8.0	0.87
	0.10 *N* NaOH	11.32	16.0

[a] The pH values at various hydrochloric acid concentrations were calculated from pH = $-\log f$ [HCl] from the activity coefficient, f, data in the literature (15). The pH values of the buffer solutions were measured at the reaction temperature on the pH meter standardized at that temperature. The pH values in alkaline solutions were calculated from pKw − pOH, where pOH = $-\log f$ [NaOH]. The pKw and f values were obtained or extrapolated for the requisite temperatures from literature data (15). [b] Rate constants in the acetate, phosphate, and carbonate buffer region were determined by obtaining the intercepts from the linear plots of k *versus* buffer concentration. Ionic strengths of all buffer solutions were adjusted to 0.4 *M* with sodium chloride.

6-Hydroxycytosine, 1 × 10⁻² *M*, was heated at 80° in 0.5 *N* NaOH. The resulting degraded solution was developed on TLC plates. No spots were observed other than that of 6-hydroxycytosine, even after exposure to UV light and with the use of a spray reagent prepared with *p*-dimethylaminobenzaldehyde which visualizes barbituric acid (19).

Rate Constants—The kinetic parameters for the solvolysis of iodocytosine were determined by following the loss of absorbance, A, of the UV chromophore as a function of time to the ultimate absorbance, A_∞.

The apparent first-order rate constants (Tables I and II) for the degradation of iodocytosine at various temperatures (70, 80, 85, and 90°) were obtained from the slopes of first-order plots of absorbance at 300–320 nm for acidified solutions and at 297.5 nm for the alkaline degradations. Plots of ln $(A - A_\infty)$ *versus* time were linear for all pH values at these wavelengths. Reasonable agreement was obtained between rate constants calculated from acidified and alkaline spectra. Typical first-order plots for the degradation of iodocytosine at various alkaline concentrations are given in Fig. 4.

The apparent first-order rate constants for iodocytosine degradation determined in this manner generally represented molecular transformation to iodouracil below pH 5, to cytosine between pH 5 and 9, and to 6-hydroxycytosine above pH 9.

Kinetic studies were conducted with and without shielding from light. No significant effect on the rate constants was observed with these variations.

Table II—Apparent First-Order Rate Constants, k (in Seconds^{-1}), for the Degradation of Iodocytosine in Buffers in the 3.5–9.5 pH Region at 0.4 Ionic Strength and 80.0°

[CH_3COOH]	[CH_3COONa]	[NaCl]	pH[a]	10^6k
0.0340	0.0030	0.3960	3.55 ±	1.45
0.0426	0.0037	0.3950	0.1	1.43
0.0682	0.0059	0.3930		1.48
0.1021	0.0090	0.3900		1.48
0.0095	0.0053	0.3940	4.35	0.52
0.0017	0.0092	0.3900	5.35	0.32
[KH_2PO_4]	[Na_2HPO_4]	[NaCl]	pH[a]	10^7k
0.0175	0.0025	0.3750	5.85 ±	4.8
0.0350	0.0049	0.3500	0.05	6.4
0.0700	0.0098	0.2504		7.7
0.1230	0.017	0.2260		9.6
0.00784	0.0122	0.3560	6.80 ±	6.6
0.01568	0.0243	0.3140	0.05	9.6
0.03136	0.0486	0.2230		16.0
0.04704	0.0730	0.1340		22.6
0.00220	0.0378	0.2844	8.05 ±	7.7
0.00330	0.0567	0.2266	0.05	9.6
0.00550	0.0945	0.1100		16.0
0.00770	0.142	0.0654		19.3
[Na_2CO_3]	[$NaHCO_3$]	[NaCl]	pH[a]	10^6k
0.0032	0.0370	0.3540	8.9 ±	2.05
0.0048	0.0552	0.3300	0.1	2.45
0.0080	0.0900	0.2840		3.20
0.0112	0.1289	0.2380		3.95
0.0045	0.0055	0.3800	9.5 ±	2.62
0.0202	0.0180	0.3210	0.1	4.74
0.0404	0.0360	0.2430		5.95
0.0808	0.0720	0.0856		8.76

[a] All pH values were measured at the reaction temperature. The pH meter was standardized with standard buffers of pH 4.00, 7.00, and 10.0 at the same temperature.

Buffer Catalysis—The apparent first-order rate constant in a buffer solution is the sum of catalytic contributions from the hydrogen ion, the hydroxide ion, the acidic (HA) and basic (A^-) components of a buffer, and water.

The overall rate constant for reaction in a buffer may be expressed by (20, 21):

$$k_{app} = k_0 + k_{H^+}[H^+] + k_{HA}[HA] + k_{A^-}[A^-] + k_{OH^-}[OH^-] \quad \text{(Eq. 3)}$$

or:

$$k_{app} = k_0 + \left(k_{HA} + \frac{k_{A^-}\,Ka}{[H^+]}\right)[HA] + k_{H^+}[H^+] + k_{OH^-}[OH^-] \quad \text{(Eq. 4)}$$

or:

$$k_{app} = k_0 + k_{H^+}[H^+] + k_{OH^-}[OH^-] + \left(k_{A^-} + \frac{k_{HA}[H^+]}{Ka}\right)[A^-] \quad \text{(Eq. 5)}$$

Thus, a plot of k_{app} *versus* [HA] at a constant pH will be linear with intercept $k_0 + k_{H^+}\,[H^+] + k_{OH^-}\,[OH^-]$ and slope $S_1 = k_{HA} + k_{A^-}\,Ka\,[H^+]$. A plot of k_{app} *versus* $[A^-]$ at a constant pH will give a straight line with intercept $k_0 + k_{H^+}\,[H^+] + k_{OH^-}\,[OH^-]$ and slope $S_2 = k_{HA}\,[H^+]/Ka + k_{A^-}$. A plot of k_{app} *versus* [HA], or k_{app} *versus* $[A^-]$, at two or more pH values will permit the calculation of both catalytic constants, k_{HA} and k_{A^-}.

No significant buffer catalysis was observed in acetate buffer. However, significant buffer catalysis was observed with phosphate and carbonate buffers in the solvolysis of iodocytosine. The apparent first-order rate constants for the reactions studied at different buffer concentrations at 80.0° are given in Table II.

From the plots of k_{app} against $H_2PO_4^-$ and HPO_4^{-2} at constant pH (Fig. 5), the respective slopes of $S_1 = k_{H2PO4^-} + k_{H2PO4^-}\,Ka/[H^+]$ and $S_2 = k_{HPO4^-2} + k_{H2PO4^-2}\,[H^+]/Ka$ were obtained. The catalytic rate constants of $k_{H2PO4^-} = 1.59 \times 10^{-5}$ and $k_{HPO4^-2} = 2.53 \times 10^{-5}$ liter/mole-sec were obtained from the intercepts of S_1 against $1/[H^+]$ and S_2 against $[H^+]$ by least squares on a calculator[8], where the correlation coefficient was 0.993 in each case.

From the plots of k_{app} against $[HCO_3^-]$ and $[CO_3^{-2}]$ at constant pH (Fig. 6), the slopes of $S_1 = k_{HCO3^-} + k_{CO3^-2}Ka/[H^+]$ and $S_2 = k_{CO3^-2} + k_{HCO3^-}\,[H^+]/Ka$ were obtained. The catalytic rate constants of $k_{HCO3^-} = 2.57 \times 10^{-6}$ and $k_{CO3^-2} = 9.73 \times 10^{-6}$ liter/mole sec were obtained from the intercepts of plots of S_1 against $1/[H^+]$ and S_2 against $[H^+]$, respectively.

Log k-pH Profile—The complete log k-pH profiles for the solvolysis of iodocytosine at several temperatures are given in Fig. 7. The pH values of the highly acid and alkaline solutions were calculated from the known concentrations of hydrochloric acid and sodium hydroxide from the pKw and mean activity coefficients obtained or extrapolated from literature data (15).

The rate constants for iodocytosine are independent of pH below pH 2 where the compound is completely protonated. This implies that hydrogen-ion-catalyzed solvolysis of the protonated species is negligible. The decrease of k with increasing pH between 2 and 5 can be explained by a reduction of the fraction of the protonated species attacked by neutral solvent molecules. An alternative explanation is the kinetically equivalent hydrogen-ion attack on the neutral substrate, where the protonated species is stable with respect to deamination. The apparent first-order rate constants of iodocytosine degradation again become pH independent between 5 and 8 and indicate either water attack on the neutral species or its kinetic equivalent, hydroxide-ion attack on the protonated species. Therefore, the log k-pH profiles below pH 7 of iodocytosine were fitted (20, 21):

$$k = f_{H_2C^+}k_{H_2O} + f_{HC}k'_{H_2O} \quad \text{(Eq. 6)}$$

where k_{H2O} and k'_{H2O} are the apparent first-order rate constants (Table III) for water attack on the protonated and neutral species, respectively, and where the fraction of the protonated

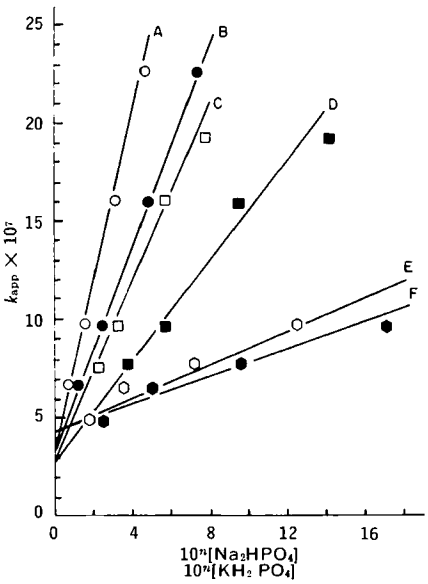


Figure 5—*Plots of apparent first-order rate constants for the solvolysis of 1 × 10^{-4} M iodocytosine at 80° in various concentrations of phosphate buffer at constant pH and 0.4 ionic strength against $[HPO_4^{-2}]$ and $[H_2PO_4^-]$. The slopes, S_1, for plots against $[H_2PO_4^-]$ were: curve A, pH 6.80, S_1 = 4.09 × 10^{-5}; curve C, pH 8.05, S_1 = 2.20 × 10^{-4}; and curve E, pH 5.85, S_1 = 4.29 × 10^{-6}. The slopes, S_2, in liters per mole seconds against $[HPO_4^{-2}]$ were: curve B, pH 6.80, S_2 = 2.64 × 10^{-5}; curve D, pH 8.05, S_2 = 1.30 × 10^{-5}; and curve F, pH 5.85, S_2 = 3.6 × 10^{-5}. The values of n were 2 except in the cases of curves C and F where they were 3.*

[8] Wang 700.

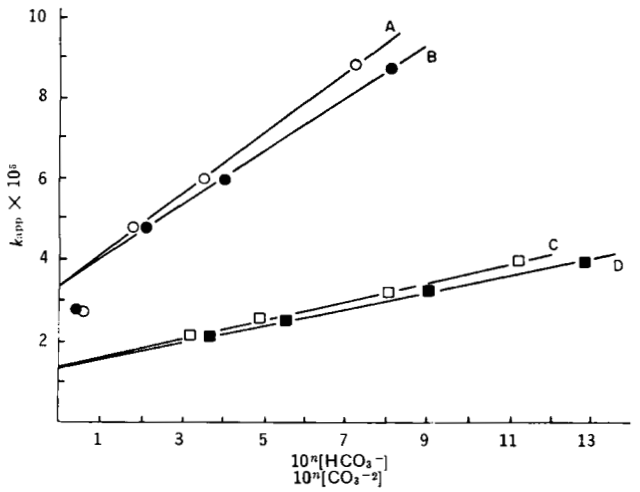


Figure 6—*Plots of apparent first-order rate constants for the solvolysis of 1 × 10⁻⁴ M iodocytosine at 80° in various concentrations of carbonate buffer at constant pH and 0.4 ionic strength against* $[HCO_3^-]$ *and* $[CO_3^{-2}]$. *The slopes,* S_1, *in liters per mole seconds for plots against* $[HCO_3^-]$ *were: curve A, pH 9.5,* $S_1 = 7.50 \times 10^{-5}$; *and curve D, pH 8.9,* $S_1 = 2.07 \times 10^{-5}$. *The slopes,* S_2, *in liters per mole seconds against* $[CO_3^{-2}]$ *were: curve B, pH 9.5,* $S_2 = 6.67 \times 10^{-5}$; *and curve C, pH 8.9,* $S_2 = 2.37 \times 10^{-4}$. *The values of* n *were 2 except in the case of curve C where it was 3.*

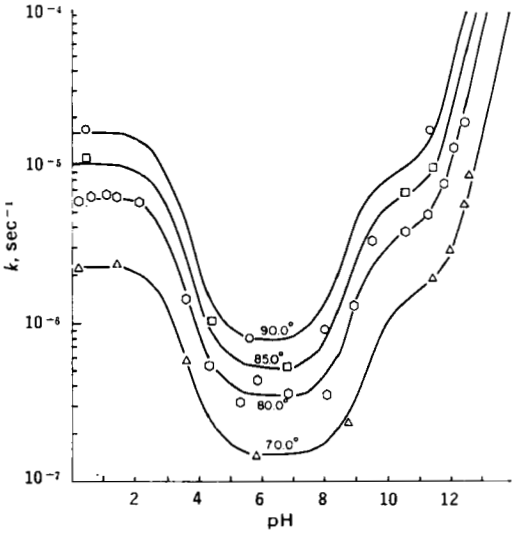


Figure 7—*Log* k–*pH profiles for the solvolysis of iodocytosine at several temperatures, where* k *is the apparent first-order rate constant for deamination to iodouracil below pH 5, for deiodination to cytosine at pH 5–9, and for hydroxylation–deiodination to 6-hydroxycytosine above pH 9.*

species is:

$$f_{H_2C^+} = \frac{[H^+]}{[H^+] + Ka_1} \qquad \text{(Eq. 7)}$$

and the fraction of the neutral species is:

$$f_{HC} = \frac{Ka}{[H^+] + Ka_1} \qquad \text{(Eq. 8)}$$

The kinetic pKa_1 values were estimated from the best fit (19) of the log k–pH curves and were also determined by the half-neutralization technique. The pKa_1 value of 3.0 was almost invariant among the temperatures studied (Table III). At room temperature, the pKa_1 value was determined spectrophotometrically to be 3.32.

The k values for iodocytosine degradation increase with pH values above 7.0. The fact that there is an inflection in the log k–pH plot (Fig. 7) in the 10–12 pH range indicates that a second acid dissociation constant, Ka_2, influences the rate of the reaction. The increase of the apparent first-order rate constant, k, with increasing pH between 8 and 10 can be explained by a hydroxide-ion attack on the neutral species or its kinetic equivalent, the attack of hydroxide ion on the monoionized molecule.

Above pH 11.5, a linear increase of log k with increasing pH to give a slope close to unity indicates specific hydroxide-ion catalysis, which can be explained by hydroxide-ion attack on the monoionized species or the kinetically equivalent water attack on the dianionic species.

The apparent first-order rate constant for iodocytosine in the alkaline region above pH 7 may be formulated (20, 21):

$$k = k_{OH^-}[OH^-]f_{HC} + k'_{OH^-}[OH^-]f_{C^-} + k'_{H_2O}f_{HC} \qquad \text{(Eq. 9)}$$

where f_{HC} is the fraction of neutral species:

$$f_{HC} = \frac{[H^+]}{[H^+] + Ka_2} \qquad \text{(Eq. 10)}$$

and f_{C^-} is the fraction of the nucleoside as the anion in the solution:

$$f_{C^-} = \frac{Ka_2}{[H^+] + Ka_2} \qquad \text{(Eq. 11)}$$

The k_{OH^-}, k'_{OH^-}, and Ka_2 values were determined from the best fit of the alkaline branch of log k–pH plots in accordance with Eq. 9, and the Ka_2 values were also determined by the half-neutralization technique (Table III). A pKa_2 value, obtained by the spectrophotometric method, was 10.59 at 25°.

Since $Kw = [H^+][OH^-]$, the components of the rate constant at any pH value from Eqs. 6–11 are:

$$k = \frac{k_{H_2O}[H^+]}{[H^+] + Ka_1} + \frac{k_{H_2O}Ka_1}{[H^+] + Ka_1} + \frac{k'_{H_2O}[H^+]}{[H^+] + Ka_2} + \frac{k_{OH^-}Kw}{[H^+] + Ka_2} + \frac{k'_{OH^-}Ka_2[OH^-]}{[H^+] + Ka_2} \qquad \text{(Eq. 12)}$$

The rate of production from iodocytosine of iodouracil is characterized by the apparent first-order rate constant given as the first term on the right-hand side of this equation. The next two terms characterize the rate of production of cytosine, and the final terms characterize the rate of production of 6-hydroxycytosine.

Dependence of Rate on Temperature—Estimates of the Arrhenius parameters for the solvolysis of iodocytosine were obtained from the slopes and intercepts of plots of the logarithm of the apparent first-order rate constants, k_{app}, *versus* the reciprocal of the absolute temperatures, T, in accordance with the expres-

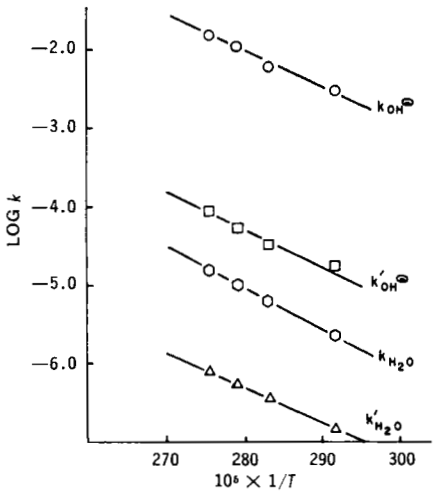


Figure 8—*Arrhenius plots for the catalytic rate constants for the solvolysis of iodocytosine.*

Table III—Microscopic Rate Constants[a] and Arrhenius Parameters for Solvolytic Degradation of Iodocytosine

Temperature	$10^6\ k_{H_2O}$	$10^7\ k'_{H_2O}$	$10^3\ k_{OH^-}$	$10^5\ k'_{OH^-}$	pKa_1[b]	pKa_2[b]
70.0°	2.20	1.40	2.98	1.77	3.0[c] 3.0[d]	9.73[c] 9.88[d]
80.0°	6.20	3.50	6.33	3.29	3.0[c] 3.0[d]	9.40[c] 9.63[d]
85.0°	10.00	5.28	11.00	5.68	3.0[c] 3.0[d]	9.24[c] 9.65[d]
90.0°	16.00	8.02	15.00	9.02	3.0[c] 3.0[d]	9.11[c] 9.55[d]
log P[e]	9.68	6.94	10.52	8.06		
ΔH_a[e], kcal/mole	24.1	21.6	20.5	20.2		
$\Delta S\ddagger$, eu[f]	−14.6	−27.1	−10.6	−22.0		

[a] Based on the kinetic expression for the overall apparent first-order rate constants, k_{app}, for the degradation of iodocytosine:

$$k_{app} = k_{H_2O}f_{H_2C^+} + (k'_{H_2O} + k_{OH^-}[OH^-])f_{HC} + k'_{OH^-}[OH^-]f_{C^-}$$

where k_{H_2O} and k'_{H_2O} are the first-order rate constants for the spontaneous decomposition of the positively and uncharged iodocytosine, respectively; k_{OH^-} is the bimolecular rate constant for the hydroxide-ion-catalyzed reaction of the neutral species; and k'_{OH^-} is for hydroxide-ion-catalyzed reaction of the monoanion of iodocytosine. The $f_{H_2C^+} = [H^+]/([H^+] + Ka_1)$ is the fraction of protonated iodocytosine; $f_{HC} = Ka_1/([H^+] + Ka_1)$ is the fraction of uncharged iodocytosine in the 0–6.5 pH range; $f_{HC} = [H^+]/(Ka_2 + [H^+])$ is the fraction of iodocytosine that is undissociated above pH 6.5; $f_{C^-} = Ka_2/([H^+] + Ka_2)$ is the fraction of iodocytosine that is monoanion at pH values above 9.5. [b] The spectrophotometrically determined values were $pKa_1 = 3.32$ and $pKa_2 = 10.6$ at 25° from the expression $pKa = pH + \log(A - A_1)/(A_2 - A)$, where A is the absorbance at any pH, and A_1 and A_2 are the absorbances of the equilibrating species. [c] Determined from the fitting of the log k–pH data. [d] Determined at the pH of half-neutralization. [e] Where $\log k_i = -\Delta H_a/2.303RT + \log P$. [f] Where $\Delta S\ddagger = [\log P - \log kT/h]\ 2.303R$ at 80°.

sion (20, 21):

$$\log k = \log P - \Delta H_a/2.303RT \qquad \text{(Eq. 13)}$$

where R is 1.987 cal deg^{-1} mole^{-1}. The energies of activation, ΔH_a, at several pH values were (kcal/mole) 24.4 (pH 2.0) and 20.8 (pH 7.0).

The Arrhenius plots for the specific rate constants are shown in Fig. 8. The ΔH_a and log P values obtained from the slope and the intercept of the Arrhenius plots for the specific rate constants are listed in Table III.

The entropies of activation, $\Delta S^{\neq}$, were calculated from the log P values using the equation:

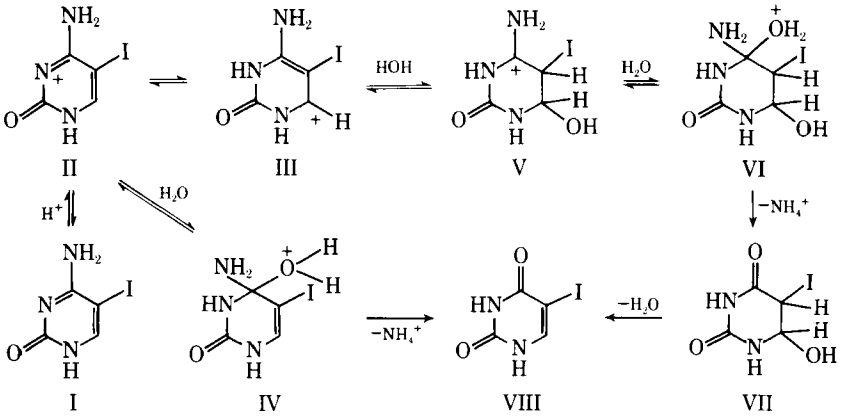
$$\Delta S^{\neq} = 2.303R\left[\log P - \log\left(\frac{kT}{h}\right)\right] \qquad \text{(Eq. 14)}$$

where k is the Boltzmann constant, 1.387×10^{-16} erg deg^{-1}, and h is the Planck constant, 6.623×10^{-27} erg sec. The calculated entropies of activation at 80° are given in Table III.

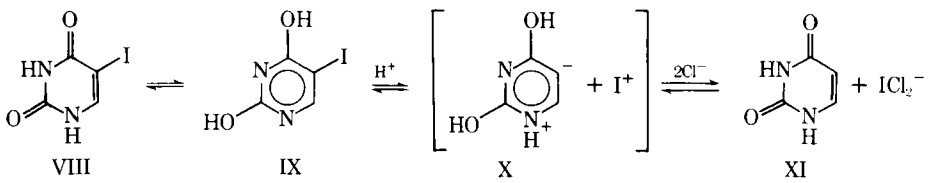
Possible Mechanisms for Solvolytic Transformations of Iodocytosine and Its Solvolytic Products—The mechanisms for the hydrolytic deamination of cytosine and its derivatives in aqueous buffer are explicable (12, 14, 22) by two kinetically equivalent processes (Scheme I). The first is analogous to the hydrolysis of an amide involving nucleophilic displacement of the amino group of the protonated cytosine, II (I → II → IV → VIII); and the second mechanism is by initial N-3 protonation of iodocytosine, I, followed by the saturation of the 5,6-double bond resulting in an electron deficiency at C-4, V, thus facilitating nucleophilic attack by water with subsequent deamination and dehydration to iodouracil (II → III → V → VI → VII → VIII). The electron-withdrawing iodo group at C-5 could help create the electron deficiency at C-4 and subsequent nucleophilic attack by water in both mechanisms. The lack of significant acetate buffer catalysis may favor the first mechanism.

In the acidic pH region, the deamination of iodocytosine occurs to give iodouracil, VIII, which subsequently produces uracil.

The deiodination mechanism of iodouracil in acidic solution was reported previously (3) and is apparently a consequence of the dissociation of iodouracil to uracil and an iodonium ion promoted by protonation of one nitrogen of the pyrimidine ring to permit the formation of an activated complex stabilized by a dipole. Facile rearrangement would give uracil (Scheme II). This is an equilibrium phenomenon, as was shown by the facile production of iodouracil from ICl and uracil in acid solution (3). The complete transformation of iodouracil to uracil occurred only in the presence of acid-degraded deoxyribose (3), which produced 5-methyl-3(2H)-furanone (23), a compound that reduced the iodon-



Scheme I



Scheme II

Table IV—Equilibration of Iodouracil with Uracil in 1 *N* HCl at 80.0°

$[IU]_0$[a]	$[IU]_e$[b]	$[U]_e$[c]	$\frac{[U]_e}{[IU]_e}$[d]	$\frac{[IU]_0 - [IU]_e}{[IU]_0} \times 100 = \%$[e]	$K_d = \frac{[U]_e^2}{[IU]_e}$[f]
4×10^{-5}	1.91×10^{-5}	2.09×10^{-5}	1.09	52.0	2.28×10^{-5}
5×10^{-5}	2.45×10^{-5}	2.58×10^{-5}	1.08	51.5	2.73×10^{-5}
1×10^{-4}	6.16×10^{-5}	3.84×10^{-5}	0.62	38.0	2.39×10^{-5}
1×10^{-3}	8.56×10^{-5}	1.44×10^{-5}	0.17	14.4	2.42×10^{-5}
				Average	2.46×10^{-5}

[a] Initial concentration of iodouracil. [b] Equilibrium concentration of iodouracil. [c] Equilibrium concentration of uracil. [d] Ratio at equilibrium of uracil to iodouracil. [e] Percent transformation of iodouracil at equilibrium. [f] Apparent dissociation constants of iodouracil may be based upon the equilibrium established in hydrochloric acid solution:

$$IU + 2Cl^- \rightleftarrows U + ICl_2^-$$

where:

$$K = [U]_e[ICl_2^-]/[IU]_e[Cl^-]^2 = [U]_e^2/[IU]_e$$

where $[ICl_2^-] = [U]_e$ and $[Cl^-] = 1.0$ in 1.0 *M* HCl.

ium ion and iodine to iodide ion and so displaced the equilibrium given in Scheme II.

These previous studies (3) were conducted at 1×10^{-3} *M* iodouracil in 1 *M* HCl at 80°, and 14.4% of iodouracil had formed the uracil product at equilibrium. In the present studies, when a 1×10^{-4} *M* concentration of iodouracil was reacted in 1 *M* HCl at 80°, 38% was transformed to uracil at equilibrium; at 5×10^{-5} *M*, it was 50%. Neither iodocytosine nor any of its degradation products, including ammonium ion, acted as a reducing agent, such as deoxyribose, to complete the transformation of iodouracil to uracil. Nor did these compounds change significantly the equilibrium between iodouracil and uracil in 1 *M* HCl solution.

However, it is apparent (Table IV) that an increase in the concentration of iodouracil in 1 *N* HCl decreases the ratio of uracil, U, to iodouracil, IU, at equilibrium. The following equilibria have been postulated (3, 24, 25) in hydrochloric acid solutions:

$$IU \rightleftarrows U + I^+$$
$$I^+ + Cl^- \rightleftarrows ICl$$
$$ICl + Cl^- \rightleftarrows ICl_2^-$$

so that the overall expression for iodouracil dissociation can be given by the sum of these equilibria:

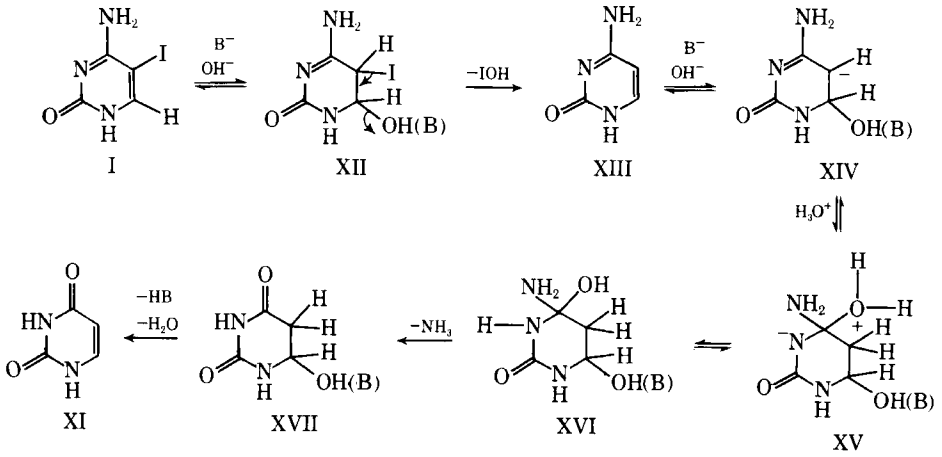
$$IU + 2Cl^- \rightleftarrows ICl_2^- + U$$

with an equilibrium constant of:

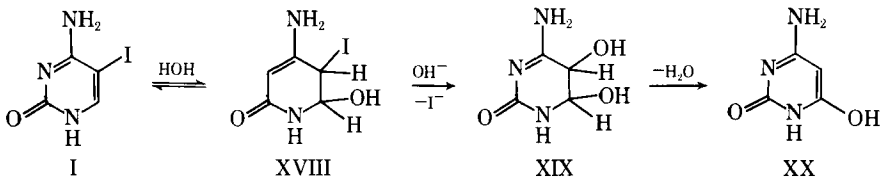
$$K = \frac{[U][ICl_2^-]}{[IU][Cl^-]^2} = \frac{[U]^2}{[IU][Cl^-]^2} \qquad \text{(Eq. 15)}$$

The premise that $[U] = [ICl_2^-]$ is based on Philbrick's (24) report that the concentration of iodine cation released in concentrated hydrochloric acid must be negligible and must be in the form of the complex anion, ICl_2^-. It also follows that, at 1.0 *M* chloride ion, the apparent dissociation constant of iodouracil is the ratio of the square of the uracil concentration to the iodouracil concentration at equilibrium. The constancy of the dissociation constant at 80.0° calculated in Table IV for various iodouracil concentrations is consistent with these relationships and the average value of K is 2.46×10^{-5} liter/mole.

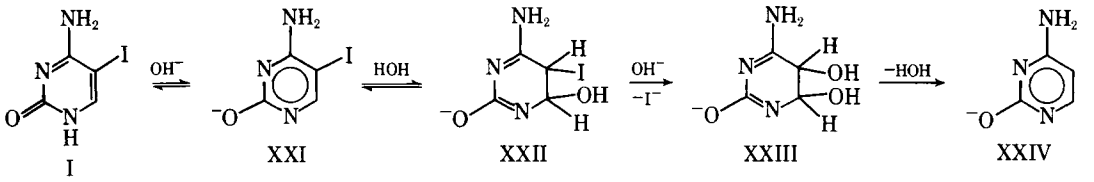
It follows from Eq. 15 that the ratio of uracil to iodouracil at equilibrium will increase at higher hydrochloric acid concentrations and decrease at higher initial concentrations of iodouracil, $[IU]_0$ (Table IV).



Scheme III



Scheme IV



Scheme V

The deiodination of iodocytosine in the 5–9 pH range produces cytosine, which then deaminates to uracil. Neutral iodocytosine could undergo a general base-catalyzed deiodination by saturation of the 5,6-double bond, XII, with subsequent regeneration of cytosine, XIII, after the elimination of hypoiodous acid, which may disproportionate to iodide ion and periodate (Scheme III). This mechanism can be rationalized with the significant phosphate and carbonate buffer catalysis observed in this pH region. The rate-determining hydroxide-ion attack on the protonated species (at N-3) is kinetically equivalent to water attack on the neutral species and can rationalize the rate constant k'_{H2O} in Eq. 6. The deamination mechanism of cytosine has been discussed previously (12, 14). This facile deamination reaction can also be explained in terms of an addition–elimination mechanism (Scheme III).

Above pH 9, the reaction intermediate isolated by column chromatographic separation was 6-hydroxycytosine. Since the product of alkaline-degraded cytosine was uracil (14), it is probable that the 6-hydroxycytosine, XX, was produced concomitantly with the deiodination process. It can be explained by saturation of the 5,6-double bond, XVIII or XXII, followed by base-catalyzed substitution of iodide ion, XIX or XXIII, with subsequent regeneration of the 5,6-double bond to give 6-hydroxycytosine, XX or XXIV (Schemes IV and V). It is possible that the final dehydration (XIX → XX, XXIII → XXIV) produced some 5-hydroxycytosine, which degraded too fast to be isolable (19).

The degradation of the 6-hydroxycytosine in alkaline range could be to barbituric acid (19) which undergoes rapid alkaline destruction of the chromophoric ring structure. This may have been indicated by the IR after concentration of degraded material separated by column chromatography, although it could have been a consequence of catalysis by H^+ (17) from the cationic-exchange resin. TLC and spectral monitoring of the reactions did not give any positive evidence. However, the difficulties inherent in the detection of barbituric acid are understandable since the rate of degradation of barbituric acid in alkali (19) is about 100–200 times faster than its probable rate of formation from 6-hydroxycytosine (Table V).

Table V—Apparent First-Order Rate Constants (in Seconds^{-1}) for the Degradation of 6-Hydroxycytosine and Barbituric Acid in Alkali at 80.0°

[NaOH]	pH	k^a Barbituric Acid	k^b 6-Hydroxycytosine
0.01	10.56	1.79×10^{-5}	6.68×10^{-8}
0.05	11.23	6.43×10^{-5}	2.59×10^{-7}
0.20	11.78	1.00×10^{-4}	6.68×10^{-7}
0.40	12.05	1.19×10^{-4}	8.02×10^{-7}

[a] Reported (19) values for apparent first-order rate constant of barbituric acid at 80°. [b] The final spectral readings, A_∞, for studies on the solvolysis of 6-hydroxycytosine in alkali were perturbed by the formation of cloudy silicate precipitates. The rate constants for 6-hydroxycytosine degradation in alkali were calculated under the assumption that the A_∞ is zero at 265 nm.

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* Present address: Institute of Chemistry and Analytics, Medical Academy, Poznan, Poland.

‡ Present address: Midwest Research Institute, Kansas City, Mo.

x To whom inquiries should be directed.

Development and Evaluation of an Inhalation Aerosol of Nitroglycerin

AVINASH M. CONTRACTOR*, JOEL M. SNYDER‡, H. PATRICK FLETCHER§, and RALPH F. SHANGRAW×

Abstract □ The disadvantages of sublingual tablets of nitroglycerin are discussed along with the need for a more stable, uniform, and rapidly acting dosage form. Data indicate that an inhalation aerosol product offers these advantages. Problems could be encountered in the choice of gasketing material in the aerosol valve and the type of activator. The onset time of an aerosol product when properly administered can be as short as 20 sec.

Keyphrases □ Nitroglycerin inhalation aerosol—formulated, compared to sublingual tablets in humans □ Aerosols, nitroglycerin—formulated, compared to sublingual tablets in humans

Many drugs have been used in the symptomatic treatment of angina pectoris, but the one that has most consistently been of benefit in relieving myocardial ischemia is nitroglycerin. Although it is often the drug of choice in such conditions, there has been no appreciable progress in the pharmaceutics involved in its dosage forms, and the sublingual administration of the drug by means of hypodermic tablets remains the only widely used method of administration.

The apparent disadvantages of sublingual tablets of nitroglycerin are: *(a)* volatility of nitroglycerin and resulting problems of stability, *(b)* tablet-to-tablet variation in weight and potency, *(c)* variability in absorption patterns, *(d)* friability of tablets, *(e)* diminutive size of tablets and difficulty in handling, and *(f)* difficulty in packaging. Many of these problems were discussed previously (1). It seems that a pressurized aerosol product would be a more convenient, stable, and accurate dosage form; it would also be faster acting, releasing the active ingredient instantly, and would surmount most of the pharmaceutical disadvantages inherent in the present commercial sublingual tablet. Several advantages of administering medicinals by inhalation as compared to other routes of administration were reported (2–6).

An aerosol of nitroglycerin[1], delivering 0.13-mg doses, is available and the manufacturer claims that it relieves anginal pain in less than half of the time taken by any other nitrate preparation. However, no scientific documentation regarding formulation or clinical effectiveness has been published. One report (7) commented that the claim of rapid onset of action rests on the results of a single, uncontrolled, and unpublished trial in which angina patients were given the product and then asked for their opinions. Consultants of this publication (7) further stated that rapid onset of effect is not required and that it was difficult to believe that use of an aerosol would be easier and more convenient. They concluded that there was no good evidence that inhalation of nitroglycerin aerosol is any better than sublingual administration.

A comparison of the effectiveness in angina of an aerosol and sublingual tablets of nitroglycerin was made by Sandler and Clayton (8). They reported that the active aerosol was no more effective than a placebo aerosol and that the tablets significantly improved ischemic changes in the ECG when given before exercise but not when given after it. However, their experiment was poorly designed. They compared two 0.13-mg doses of the aerosol with a single 0.5-mg tablet. In addition, they did not disclose the aerosol formulation, particularly in respect to stability of the drug. They did not mention how much drug was retained on the actuator, which could be more than 20% of the dose (9).

Therefore, it was decided to formulate a nitroglycerin inhalation aerosol and evaluate its stability, effectiveness, and onset of action compared to sublingual tablets.

EXPERIMENTAL

The nitroglycerin used in the study was recovered from a commercially available 10% blend in lactose by shaking with water. Nitroglycerin, being very slightly soluble and heavy, settled to the bottom of the flask. The drug was purified by repeated washings with water and was then filtered through a sodium chloride bed, using a büchner funnel to remove traces of water and insoluble impurities. The final filtrate was assayed and found to be 99.3% nitroglycerin.

Assay—The spectrophotometric method suggested by Bell (10) was selected to assay nitroglycerin because of its simplicity, rapidity, and reproducibility in assaying microgram quantities of the drug. This stability-indicating assay involves alkaline hydrolysis to nitrite ion followed by diazotization and a coupling reaction followed by subsequent colorimetric determination of the dye formed. Five actuations of aerosol were obtained by depressing the inverted container without actuator into a small beaker containing about 25 ml of water, and the volume was adjusted to 250 ml. An aliquot was used as the assay sample.

Formulation—After numerous experiments, the following formulation was selected for evaluation:

nitroglycerin	1.0% w/w
propylene glycol	3.0% w/w
ethanol (absolute)	15.0% w/w
dichlorodifluoromethane [propellant]	16.0% w/w
dichlorotetrafluoroethane [propellant]	65.0% w/w

The nitroglycerin and propylene glycol were dissolved in ethanol. The solution and propellant blend were chilled in an acetone–dry ice bath to approximately −45° and filled by weight into prechilled, 20-ml plastic-coated glass vials onto which inverted 50-μl meter valves with emptying cups were crimped. The pressure of the product was approximately 27 psig. Dose reproducibility was excellent (Table I).

[1] Cardamist, Nicholas Laboratories, Ltd., England.

Table I—Dose Reproducibility

Dose	Nitroglycerin
1	0.521
2	0.515
3	0.524
4	0.535
5	0.517
6	0.521
7	0.517
8	0.524
9	0.536
10	0.518
Mean	0.523
SD	0.0072

Since actuators are expected to retain a part of each dose, the amount remaining on the actuator must be determined and should be taken into account in the final formulation. A plastic actuator (11), 40 mm long and with a diameter of 21 mm, was used; it delivers the product at a right angle to the container. Based on a series of seven actuations, it was found that 0.13 ± 0.01 mg of nitroglycerin was retained on the actuator during each administration. This amounted to almost 25% of the average dose (0.523 mg) delivered from the meter valve and should be considered when trying to evaluate dose response.

Particle-Size Measurement—The samples for particle-size measurements were obtained by actuating the product through a camera shutter adjusted for 0.03-sec opening time and collecting the sprays from a distance of 15 cm on glass slides warmed to 37°. This was done to get the samples with as little superimposition of particles as possible. The slides were warmed to get the conditions as close to that obtained during actual use of the product.

Magnified pictures of the sample slides were taken[2], and a combination of ocular and objective lenses was used to yield 240× magnification. Illumination was provided with a heat-absorbing filter glass. The particles in the photomicrographs were classified by comparison with a standard micrometer scale picture taken at the same magnification. The measurements were made under a low power of a stereoscopic microscope[3]. About a thousand particles from eight different samples were classified in three groups. About 95% of the particles was smaller than 5 μm in diameter, about 4% fell between 5 and 10 μm, and less than 0.5% was above 10 μm in diameter.

Particle-size measurement of a solution-type aerosol product is not an absolute procedure. The three major problems encountered in the measurements of particle size of the experimental aerosol products were: *(a)* time and temperature, *(b)* sampling procedures, and *(c)* limitation of the microscope. Due to the volatile nature of the formulation, the particles decreased in size during the first minutes after spraying. This was overcome to a great extent by spraying onto a 37° surface, which caused immediate volatilization of drops and resulted in particles made up of nitroglycerin dissolved in propylene glycol. The different forces at which the particles hit the slide gave variation in degrees of flattening. Furthermore, the use of a light microscope and camera setup limited visible particles to above 1 μm. It is theorized that many particles below this size were actually produced by the aerosol.

Packaging and Stability—Three sets of test product containers were prepared. The first set contained the usual single rubber gasket to help create a seal between the rim of the glass container and the valve ferrule. The second set had three rubber gaskets placed in the product in addition to one normally used in the valve. The third set had three polyethylene gaskets placed in the product in addition to the one polyethylene gasket used with the valve. Four units each from the first two sets of containers were stored at room temperature (28 ± 1°) and at 53 ± 0.5° in a hot air oven[4]. Two units from the third set were stored at room temperature (28 ±1°).

Duplicate assays on a group of five doses from each container were carried out periodically during the stability test. It was observed, particularly with the containers stored at elevated temperature, that the assay results were showing an increase in nitroglycerin content when assayed after a long interval. This was no doubt due to the loss of propellant from the emptying cup of the valve, thereby concentrating nitroglycerin in the cup.

Table II—Experimental Design

Time, hr	Group	Treatment
Initial	1	A
	2	B
	3	C
48	1	B
	2	C
	3	A
96	1	C
	2	A
	3	B

Methods of Pharmacological Evaluation—An experiment was designed to evaluate various pharmacological effects of nitroglycerin delivered by the inhalation aerosol dosage forms and to compare these effects with those obtained from an equivalent amount of the drug in the form of sublingual tablets (nitroglycerin tablets USP[5], 0.4 mg).

Six healthy male subjects, ranging in age from 22 to 36 years (average 28 years), with normal systolic blood pressure ranging from 111 to 145 mm of mercury (average 135 mm) and with weights ranging from 57.2 to 73.5 kg (126 to 162 lb) [average 64.9 kg (143 lb)], were selected. To minimize the time, subject, dosage form, and tolerance effects, the subjects were divided into three groups of two, which were administered either the active tablet (A), active aerosol (B), or placebo aerosol (C) in a crossover random order. The subjects were not informed whether the medication given to them was active or a placebo. A 48-hr interval was allowed between each test. The exact layout of the test is shown in Table II.

Preliminary tests with placebo tablets proved to be completely negative and were not included in the study due to limitations of subjects. These preliminary tests involved the establishment and evaluation of the ECG and plethysmographic procedures. During this time, several subjects were given both active and placebo tablets. No change in either ECG or plethysmographic readings was noted upon administration of any placebo tablet.

All subjects were asked to adhere strictly to the following restrictions on their daily routine during the experiment:

1. No alcohol or drugs 24 hr prior to test.
2. No food, coffee, tea, or smoking for 3 hr prior to test.
3. No strenuous exercise on the day of test.

The experiments were carried out with the subjects in the supine position. To obtain somewhat higher response to the drug, the head of the bed was elevated by about 25°. The subjects were allowed to rest for 20 min after fixing the electrodes for ECG measurements, the plethysmographic cuff on the right arm, and the sphygmomanometer cuff on the left arm. Preliminary studies demonstrated that 20 min was sufficient to equilibrate the plethysmographic cuff with the volumetric pressure transducer[6] and to bring the blood pressure to its normal resting level. The subjects were given instructions as to the purpose of the study and were told to signify subjective responses such as headache, pounding, or flushing by gently raising the index finger of the left hand. Before, during, and after the administration of the drug, the subjects were asked to remain still, relaxed, and quiet. It was absolutely necessary to refrain from any movement to achieve dependable results.

During preliminary experiments, problems of improper self-administration of drug by the subjects were encountered, particularly with the aerosol products. The major portion of the spray was found to be striking the walls of the oral cavity instead of reaching the lungs. This gave a delayed onset of action at about 1-2 min. Moreover, the movement of the body during self-admin-

[2] A Polaroid Land Camera was mounted on an American Optical Series 682 shutter apparatus which, in turn, was mounted over a standard student model microscope (Bausch and Lomb Optical Co., Rochester, N.Y.).
[3] American Optical Spencer Cycloptic Series 58.
[4] Model A, Precision Scientific Co., Chicago, Ill.
[5] Lot 2 EG 7A, Eli Lilly & Co., Indianapolis, Ind.
[6] Model PT 5A, Grass Instruments, Quincy, Mass.

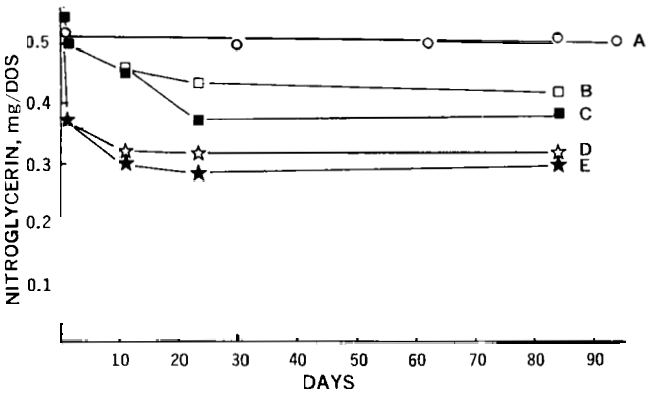

Figure 1—*Effect of time, temperature, and gasket material on the stability of nitroglycerin in aerosol dosage forms. Each point represents an average of four replicates. Key: ○, three plastic gaskets and sealing gasket at 28°; □, rubber sealing gasket only at 28°; ■, rubber sealing gasket only at 53°; ☆, three rubber gaskets and sealing gasket at 28°; and ★, three rubber gaskets and sealing gasket at 53°.*

istration created disturbances in the recording of plethysmograph. Although there would appear to be no problem of educating patients to proper use during self-administration, it was decided to administer the test drugs by a trained individual. The tablets were inserted sublingually. The aerosol preparations were administered during oral inhalation.

Measurement of Experimental Parameters—The heart rate was continuously recorded by ECG measurement on lead II using a polygraph[7]. A chart speed of 15 cm/min was used during the experiment. Recordings, at a chart speed of 50 cm/min, for each individual before the start of the experiment were made to check the normal nature of the ECG. Heart rate was calculated from the recording between 1 and 3 min before the administration of the drug and for 2 min immediately after the onset of action of the drug, and the difference was noted. Onset time was obtained both from the measurement of change in arm volume and from subjective response.

To measure change in arm volume, the right arm of the subject was elevated approximately 7 cm above the level of the heart so the venous pressure was zero (12, 13). A sphygmomanometer cuff was placed around the right upper arm and was connected to a water-filled buret to measure the amount of pressure inside the cuff; the pressure in the cuff was kept at about 2.5 cm of water. The connection with the buret was cut off, and the cuff with the pressure of 2.5 cm of water was then attached to the pressure transducer. The pressure transducer was connected to the polygraph for a continuous recording of a change in cuff pressure induced by a change in the volume of the arm. At least 20 min was allowed for equilibration before the administration of the drug. The recording was continued for 20 min after drug administration. The onset time was determined by taking the difference between the time of drug administration and the time at onset point, which was the point of intersection of the baseline and the slope due to the effect of the drug.

Fall in systolic pressure was measured as a parameter for evaluation of the test product. The blood pressure was determined by ausculatory measurements made at the brachial artery of the left arm using a mercury sphygmomanometer. Measurements were made initially and 5 min before, 5 min after, and 20 min after drug administration. The difference in systolic blood pressure between the readings taken at 5 min before and 5 min after drug administration was recorded.

RESULTS AND DISCUSSION

The stability of the nitroglycerin aerosol dosage form was investigated by employing both room temperature and accelerated aging tests, using rubber as well as plastic gaskets. Solutions containing the polyethylene plastic gaskets showed no decrease in potency for nitroglycerin on aging for more than 90 days at room temperature, as can be seen in plot A of Fig. 1. However, plots B and C, illustrating data collected from the test containers utilizing one regular rubber gasket, show a significant decrease in drug content for about a month. As would be expected, the solution at the higher temperature (plot C) showed a greater loss in potency. Plots D and E represent data from solution in which three rubber gaskets were placed in addition to the one normally found in the valve. The results are similar but more dramatic than in those containers containing only the single gasket.

[7] Model 50, Grass Instruments, Quincy, Mass.

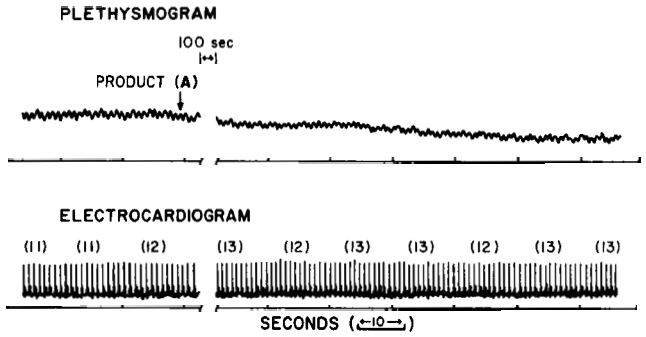

Figure 2—*Plethysmogram and ECG of a healthy male subject before, during, and after the administration of a sublingual tablet containing 0.4 mg nitroglycerin (chart speed = 15 cm/min).*

The slight increase in nitroglycerin content in the samples stored at elevated temperature was most probably due to leakage of propellant over 3 months, which caused the drug solution to become more concentrated. The fact that the decrease in potency reached a minimum and then leveled off indicates that the loss may result from a surface effect which exhibits itself rapidly but is self-limiting. However, the drop in potency from an initial reading of 0.54 mg to a reading of 0.27 mg in the case of plot E represents a 50% loss. Even the single gasket sample at room temperature exhibited a drop from 0.54 to 0.43 mg, or almost a 20% loss. In containers with a lower concentration of nitroglycerin, as in the study of Sandler and Clayton (8), this loss could be even more dramatic and could account, along with other things, for the failure to obtain significant results.

Evaluation for Effectiveness and Onset of Action—The changes in heart rate, arm volume, systolic blood pressure, and subjective responses such as headache, pounding, palpitation, and flushing were recorded to evaluate the effects of nitroglycerin in both the aerosol and the sublingual dosage forms.

Typical ECG's (lead II) of a healthy individual taken before, during, and after the administration of active tablet, active aerosol, and placebo aerosol on alternate days are shown in Figs. 2–4. From the graphs it can be seen that an increase in heart rate was obtained after administration of active medication, while no change was noticeable with placebo medication. Table III shows the increase in heart rate obtained from the records of six individuals with the three test products. The results clearly show the difference between the active and the placebo products. However,

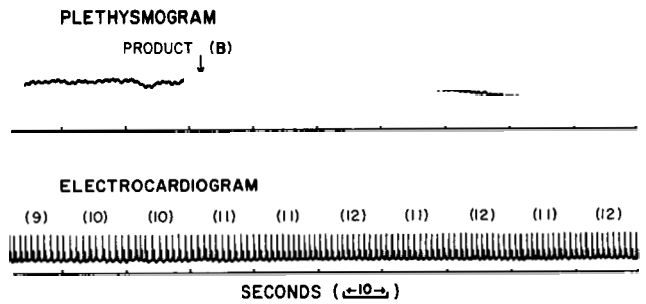

Figure 3—*Plethysmogram and ECG of a healthy male subject before, during, and after the administration of an inhalation aerosol containing 0.4 mg nitroglycerin (chart speed = 15 cm/min).*

Table III—Increase in Number of Heart Beats per Minute as Recorded by ECG in Response to Administration of Test Products[a]

Day	Test Products: Active Tablet	Active Aerosol	Placebo Aerosol	Total
1	18	12	−1	29
	12	9	−3	18
3	8	16	3	27
	14	13	−2	25
5	9	12	−1	20
	14	14	0	28
	75	76	−4	147

Analysis of Variance

	Sum of Squares	Degrees of Freedom	Mean Square	*F* Ratio	
Row means	2.33	2	1.17	0.13	$F_{0.95}(2,9) = 4.26$
Column means	702.33	2	351.17	40.04	$F_{0.95}(2,9) = 4.26$
Within groups	113.83	13	8.77		
Total	818.5	17			

[a] Three groups of two subjects on 3 different days.

Table IV—Time (Seconds) Required for Onset of Action in Response to the Administration of Test Product[a]

Day	Test Products: Active Tablet	Active Aerosol	Total
1	123	22	145
	108	72	180
3	103	19	122
	89	97	186
5	150	16	166
	68	15	83
	641	241	882

Analysis of Variance

	Sum of Squares	Degrees of Freedom	Mean Square	Mean *F* Ratio	
Row means	795.5	2	397.75	0.338	$F_{0.95}(2,6) = 5.14$
Column means	13,333.3	1	13,333.33	11.33	$F_{0.95}(1,6) = 5.99$
Within group	9,410.2	8	1,176.27		
Total	23,539	11			

[a] Three groups of two subjects on 3 different days.

a t value of −0.56 showed that there was no significant difference between the active aerosol and the active tablets.

The effects of test products on the plethysmograms of the right arm of a healthy individual are shown in Figs. 2–4. A point of onset of action can be determined by the intersection of the baseline and the slope of the plethysmograph curve due to the change in arm volume. The chart speed was fixed at 15 cm/min. It can be seen from these figures that there was no onset of action when the placebo product was administered while there was a quick onset with the active aerosol and a delayed onset with the active tablet. The data obtained regarding time required for onset of action are recorded in Table IV. No record was made for the placebo aerosol because no onset was observed with this product in any subject.

The data in Tables III and IV were subjected to analysis of variance to test for subject and time effects, if any, due to differences in results obtained on different days. The *F* values for row means were not significant at the 5% level, indicating no subject and time effects in the experiment. However, the *F* value for the column means in Table IV is significant at a 5% level, indicating that the active aerosol gave a significantly more rapid onset of action when compared with the active sublingual tablet.

The onset of action varied from patient to patient. There is a good possibility that the lengthy onset time for Patients 2 and 5 were due to improper administration, *i.e.*, failure of patient to inhale properly. Then the nitroglycerin was simply deposited on the oral mucosa and had to be absorbed, similar to the process involved in sublingual tablet administration. However, even when the slow response of these individuals is considered, the onset time for the inhalation aerosol product is significantly less than for the tablet.

A decrease in systolic blood pressure due to the administration of the test product is recorded in Table V. The data clearly indicate that the active tablet and the active aerosol caused a decrease in blood pressure while the placebo aerosol did not elicit any effect.

Subjective onset responses such as headache, pounding, palpitation, or flushing were recorded for each subject after administration of the test products. All subjects showed positive responses with active medication on the average 12 sec later than that observed in the corresponding plethysmograms. Only one subject responded positively to the placebo aerosol.

From the overall analysis of the data obtained from all experimental parameters, it can be concluded that in healthy male subjects the active aerosol is as effective as the active tablet and that the placebo aerosol is not effective when compared to either active product. The active aerosol gives a significantly more rapid onset of action.

The use of sublingual tablets of nitroglycerin in acute angina attacks has sometimes been questioned due to the delay, however short, in therapeutic effects. Most physicians feel that the pain of angina will diminish by itself within 2–3 min of onset. A dosage form that can decrease this onset time significantly as the aerosol does appears to have meaningful therapeutic advantages.

The problem of administration of an aerosol in the throes of an anginal attack certainly must be considered. This involves the re-

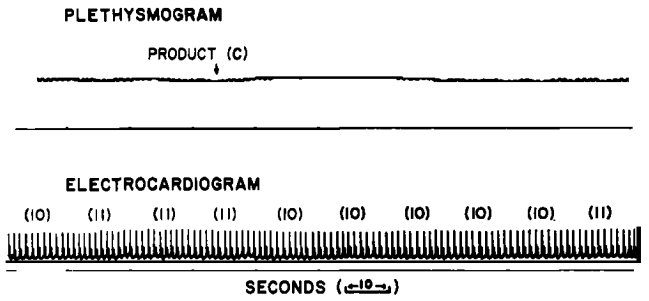

Figure 4—*Plethysmogram and ECG of a healthy male subject before, during, and after the administration of a placebo aerosol (chart speed = 15 cm/min).*

moval of the aerosol container from the pocket or pocketbook, activation of the valve, and simultaneous inhalation with valve actuation. Although these cannot be minimized, they should be surmountable with proper patient education. Even with hypodermic tablets, it is necessary for a patient to remove a screw cap from a bottle, remove a single tablet, and place the tablet beneath the tongue. It is well known that patients using nitroglycerin tablets often take more than one tablet in their anxiety to obtain relief.

The use of nitroglycerin in attacks of angina is a well-accepted treatment. Hopefully, this article will stimulate interest in the formulation of an aerosol product of nitroglycerin—if not an inhalation product, then possibly a simple sublingual one.

CONCLUSION

1. The nitroglycerin aerosol dosage form is as effective as the sublingual dosage form of the drug in healthy individuals when changes in heart rate, arm volume, and blood pressure are compared.

2. The nitroglycerin aerosol dosage form is significantly more rapid in onset of action than is the sublingual dosage form of the drug when pharmacological effects are compared in healthy male subjects.

3. The gasketing material used in aerosol meter valves offers a potential problem in formulating a stable product. Preliminary studies indicate that plastic gasketing material may be superior to traditional rubber gaskets.

4. The actuator used in inhalation products can retain significant amounts of the drug on its surface, depending upon the formulation.

5. A nitroglycerin aerosol would appear to overcome the problems of loss of potency and content uniformity associated with traditional sublingual tablets.

Table V—Decrease in Systolic Blood Pressure (Millimeters of Mercury) in Response to Administration of Test Products

Subjects	Active Tablet	Active Aerosol	Placebo Aerosol
1	5	5	−4
2	12	10	0
3	6	8	0
4	10	9	0
5	10	8	0
6	0	4	0
Average	7.1	7.3	−0.7

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* Present address. Block Drug Co., Jersey City, N.J.

‡ Present address: University of Colorado Medical Center, Denver, CO 80220

§ Present address: School of Pharmacy, Purdue University, Lafayette, Ind.

ˣ To whom inquiries should be directed.

Quantitative Determination of Carisoprodol, Phenacetin, and Caffeine in Tablets by Near-IR Spectrometry and Their Identification by TLC

LEWIS ALLEN

Abstract □ Analytical procedures for the identification and measurement of each individual component in a carisoprodol, phenacetin, and caffeine combination are reported. Identification is confirmed by TLC. Carisoprodol and phenacetin are determined simultaneously in chloroform, while caffeine is isolated from the other two components and measured in carbon tetrachloride. All determinative steps utilize near-IR spectrometry. The validity of the quantitative procedures described is substantiated by data obtained from the analysis of spiked solutions, which shows excellent accuracy and standard deviations for carisoprodol, phenacetin, and caffeine of 1.21, 0.68, and 0.74, respectively. Four commercial tablet preparations were analyzed and the standard deviations for carisoprodol, phenacetin, and caffeine were 1.38, 1.40, and 1.07, respectively. These figures are a reflection of both the manufacturing process and the reproducibility of the methodology.

Keyphrases □ Carisoprodol, phenacetin, and caffeine tablets—quantitative near-IR determination of each component, TLC identification □ Phenacetin, carisoprodol, and caffeine tablets—quantitative near-IR determination of each component, TLC identification □ Caffeine, carisoprodol, and phenacetin tablets—quantitative near-IR determination of each component, TLC identification □ IR spectrometry (near)—quantitative determination of components of carisoprodol, phenacetin, and caffeine tablets

The use of drugs in combination for a particular therapeutic advantage makes the analysis for the individual drugs in such dosage forms more complicated. This is certainly true for mixtures of carisoprodol, phenacetin, and caffeine, a useful combination when skeletal muscle relaxation is desirable.

Analytical procedures for the identification and measurement of each individual component in a carisoprodol, phenacetin, and caffeine mixture have been reported. For example, paper chromatographic (1) and TLC (2) methods have been devised for the separation and identification of mixtures of carbamates such as carisoprodol, meprobamate, mebutamate, and hydroxymeprobamate, while individual TLC methods for the identification of meprobamate (3), phenacetin (4), and caffeine (4) have been introduced. As a natural extension, quantitative TLC and GLC methods for carbamates (5–8) have been described. The measurement of phenacetin and caffeine, both individually and in mixtures, has been accomplished using various approaches. The official compendial procedure for phenacetin is a gravimetric analysis in which the unaltered drug is weighed after isolation by repeated solvent extraction and filtration (9). Quantitative IR (10) and visible and UV (11–14) methods are available to measure phenacetin and caffeine after their isolation.

This paper describes a TLC separation and identification scheme for all components in a carisoprodol, phenacetin, and caffeine mixture. It also reports a study of the measurement of these drugs in tablets using their near-IR light-absorbing properties. Although both the TLC and near-IR approaches have been used for the individual drugs, the application to the combination reported here has not been done before. The procedures described in this report are simpler, more rapid, and convenient than previous procedures.

EXPERIMENTAL

All chemicals were reagent grade unless otherwise specified.

Identification of Carisoprodol, Phenacetin, and Caffeine by TLC—Silica gel GF 254 (for TLC according to Stahl, Type 60[1]) 20 × 20-cm plates were used with a standard glass-type tank, equilibrated with ethyl acetate, reagent grade, for 30 min.

A single tablet was taken, placed into a volumetric flask containing some methanol and disintegrated by ultrasonic perturbation[2]. It was dissolved in methanol to contain 4 mg/ml carisoprodol, 3.2 mg/ml phenacetin, and 0.64 mg/ml caffeine and filtered.

Standard Solutions—Individual solutions (in methanol) of 20 mg/ml carisoprodol, 16 mg/ml phenacetin, and 3.2 mg/ml caffeine were prepared. A mixed standard solution was prepared from the individual standard solutions by dilution to contain 4 mg/ml carisoprodol, 3.2 mg/ml phenacetin, and 0.64 mg/ml caffeine.

TLC Procedure—Spot 20 μl each of sample, individual standards, and mixed standard 3–5 cm from bottom of plate and develop the plate for a distance of 15 cm. When the plate has dried, use the appropriate detection procedure.

For the detection of phenacetin and caffeine, use shortwave UV light (253.7 nm) which produces purple-colored spots.

For the detection of carisoprodol, spray the plate with 5% vanillin in concentrated sulfuric acid following the UV detection of phenacetin and caffeine. An easily seen yellow-colored spot results.

The R_f values obtained were: caffeine, 0.13; phenacetin, 0.59; and carisoprodol, 0.82.

Simultaneous Quantitation of Carisoprodol and Phenacetin—*Instruments and Reagents*—A ratio recording spectrophotometer[3] was used with the following settings: sensitivity dial, 2.00; gears, 10 nm/cm; scanning time switch, 20; time constant, 0.1; range selector, 0–100 (0–1); and photomultiplier, PbS cell mode.

Alumina[4] (basic, 80–200 mesh) was activated daily by heating for 2 hr at 300°. Chloroform, water and alcohol free (15), was prepared daily as follows. Extract the alcohol by passing about 500

[1] EM Reagents, EM Laboratories, Inc., Elmsford, NY 10523
[2] Cole-Parmer Instruments, Chicago, IL 60648
[3] Beckman DK-2A.
[4] Fisher No. A-540 or equivalent.

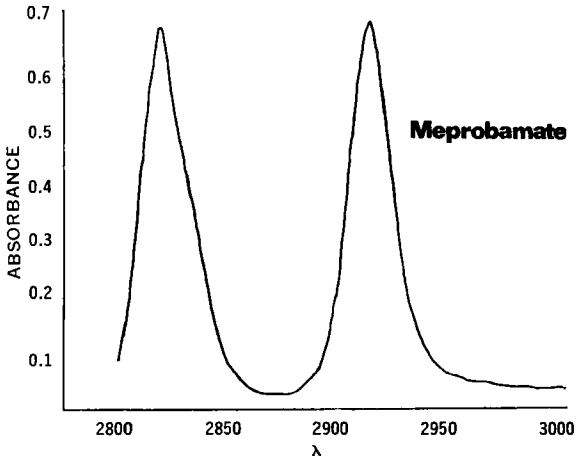

Figure 1—*Near-IR spectrum of meprobamate (64 mg/100 ml in chloroform); λ = wavelength (nanometers).*

ml of chloroform through three 1000-ml separators, each containing 50–75 ml of water. Pack two chromatographic columns (glass, 20 × 300 mm) half-full with alumina to remove the water. Mount one column above the other and pass the chloroform through both columns. If more than 500 ml of chloroform is needed, repeat the purification with fresh alumina. Since alcohol-free chloroform is prone to the formation of phosgene, it should not be stored longer than 1 week. The solvent should be kept in low-actinic glassware and transferred to avoid picking up water.

Prepare the carisoprodol standard solution as follows. Weigh about 50 mg of carisoprodol standard accurately (previously dried at 60° under vacuum for 3 hr) and transfer quantitatively to a 100-ml volumetric flask. Dissolve in and dilute to volume with alcohol-free chloroform. Filter through dry Whatman No. 1 paper containing about 2 g of anhydrous sodium sulfate. Discard the first 20–25 ml of filtrate and collect the remainder in a dry glass-stoppered erlenmeyer flask.

To prepare the phenacetin standard solution, weigh about 100 mg of phenacetin standard accurately and proceed as directed for the carisoprodol standard solution starting with "(previously dried. . . .

Preparation of Sample—Weigh and finely pulverize 20 tablets until a homogeneous powder is obtained. Accurately weigh an amount of sample composite equivalent to about 50 mg of carisoprodol and transfer quantitatively to a 100-ml volumetric flask. Dissolve in alcohol-free chloroform by shaking for 15 min and dilute to volume with the same solvent. Filter through dry Whatman No. 1 paper containing about 2 g of anhydrous sodium sulfate. Discard the first 20–25 ml of filtrate and collect the remainder in a dry glass-stoppered erlenmeyer flask.

Determination of Carisoprodol and Phenacetin—Filter a portion of alcohol-free chloroform through dry Whatman No. 1 paper containing about 2 g of granular anhydrous sodium sulfate. Discard the first few milliliters of filtrate and collect the remainder in a dry glass-stoppered erlenmeyer flask. Zero the instrument at 2914 nm with this chloroform in two matching near-IR silica cells. Scan the near-IR spectra of sample and standard solutions against chloroform between 3000 and 2750 nm. Measure the baseline absorbance values at 2910 and 2820 nm from straight lines drawn between the minima at 3000 and 2870 nm and at 2870 and 2790 nm. Subtract baseline values from peak absorbances at 2910 and 2820 nm to obtain net absorbance.

Calculate the carisoprodol concentration using absorbance at 2820 nm as follows:

$$\text{mg carisoprodol/tablet} = \frac{A_{2820}}{A_{cs2820}} \times C \times \frac{T}{W} \times 100 \quad \text{(Eq. 1)}$$

where:

A_{2820} = absorbance of sample solution at 2820 nm
A_{cs2820} = absorbance of carisoprodol standard solution at 2820 nm
C = concentration of carisoprodol standard (milligrams per milliliter)
T = average tablet weight (milligrams)
W = weight of sample composite (milligrams)

Calculate the phenacetin concentration as follows:

$$\text{mg phenacetin/tablet} = \frac{A_{2910} - A_{c2910}}{A_{p2910}} \times C \times \frac{T}{W} \times 100 \quad \text{(Eq. 2)}$$

where:

A_{2910} = absorbance at 2910 nm for carisoprodol plus phenacetin in sample solution
A_{c2910} = absorbance at 2910 nm for carisoprodol alone in sample solution
A_{p2910} = absorbance at 2910 nm for phenacetin in standard solution

and:

$$A_{c2910} = \frac{A_{c2820} A_{cs2910}}{A_{cs2820}} \quad \text{(Eq. 3)}$$

where:

A_{c2820} = absorbance at 2820 nm for carisoprodol in sample solution
A_{cs2910} = absorbance at 2910 nm for carisoprodol in standard solution

Spiked Solutions: Carisoprodol and Phenacetin—A sample composite similar to the one already described was accurately weighed and transferred quantitatively to a 100-ml volumetric flask. Then 50 mg of carisoprodol and 50 mg of phenacetin were accurately weighed and added to the sample flask. The resulting sample spike was dissolved in and diluted to volume with alcohol-free chloroform and then filtered as described previously. Then 25 ml of the resultant solution was diluted to exactly 50 ml with the same solvent. Phenacetin and carisoprodol standard solutions containing about 1 and 0.5 mg/ml, respectively, in alcohol-free chloroform were prepared as described. Near-IR spectra were obtained for the sample and standard solutions. The recoveries of carisoprodol and phenacetin were calculated based on assay values for the samples previously determined.

Quantitation of Caffeine—*Reagents*—The following were used: chloroform[5], ACS grade, water washed just prior to use; ether[6], reagent grade, water washed just prior to use; carbon tetrachloride[7], reagent grade, shaken with anhydrous sodium sulfate just prior to use; and diatomaceous earth[8], acid washed.

To prepare the caffeine standard solution, dry a caffeine reference standard for 4 hr at 80° under vacuum. Weigh about 15 mg accurately and transfer quantitatively to a 10-ml volumetric flask. Add about 7 ml of carbon tetrachloride, stopper the flask, and place in the sonic bath for 10 min to dissolve. Cool to room temperature and dilute to volume with the same solvent.

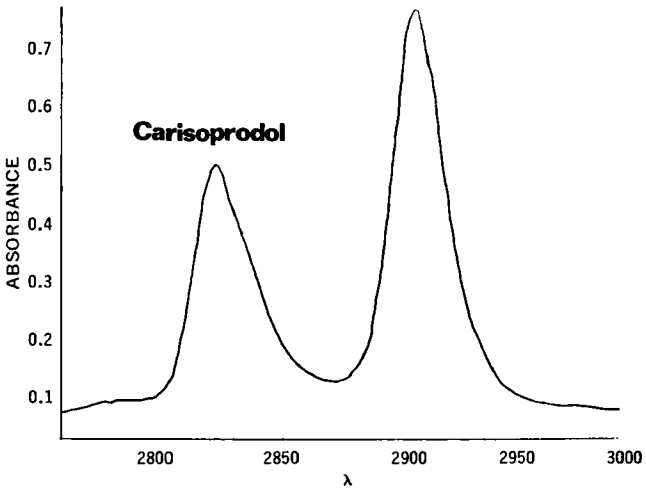

Figure 2—*Near-IR spectrum of carisoprodol standard solution (100 mg/100 ml in chloroform); λ = wavelength (nanometers).*

[5] Allied Chemical Co. or equivalent.
[6] Ethyl ether, Mallinckrodt Chemical Works or equivalent.
[7] Fisher Scientific Co. or equivalent.
[8] Celite 545, Johns-Manville.

Table I—Analysis of Mixed Carisoprodol and Phenacetin Spiked Solutions

Sample	Carisoprodol			Phenacetin		
	Added, mg	Found, mg	Recovery, %	Added, mg	Found, mg	Recovery, %
1	50.6	51.3	101.4	45.8	45.9	100.2
2	53.2	53.9	101.3	51.7	51.4	99.4
3	50.3	50.7	100.8	51.8	51.1	98.6
4	49.6	49.0	98.8	48.2	48.1	99.8
Average			100.6			99.5
Standard deviation, %			1.21			0.68
Relative standard deviation, %			1.21			0.69

Sample Preparation—Weigh an amount of sample composite equivalent to about 75 mg of caffeine into a 50-ml glass-stoppered erlenmeyer flask. Add about 25 ml of chloroform, stopper, and shake for 15 min. Filter through Whatman No. 40 paper, previously saturated with chloroform, into a 50-ml volumetric flask. Wash the erlenmeyer flask several times with chloroform and filter the washings into the volumetric flask. Wash the filter paper and dilute to volume with chloroform.

Pack a pledget of glass wool in a 24 × 240-mm glass chromatographic column. Intimately mix 2 ml of 4 *N* H_2SO_4 with 3 g of diatomaceous earth in a beaker and pack on top of the glass wool. Overlay the diatomaceous earth with a second pledget of glass wool. Pipet 10 ml of sample solution into a 100-ml beaker, dilute with 50 ml of water-washed ether, and pour onto the column. Wash the beaker with four successive 50-ml portions of water-washed ether to ensure quantitative transfer of the sample solution to the column. Wash the tip of the column with chloroform and discard the ether eluate. Place a 200-ml beaker under the column and elute the caffeine with 150 ml of water-washed chloroform. Evaporate the chloroform eluate on a steam bath under a stream of air to a volume of about 5 ml, transfer quantitatively to a 25-ml glass-stoppered erlenmeyer flask, and evaporate to dryness as before. Dry the residue for 4 hr at 80° under vacuum. Pipet 10.00 ml of carbon tetrachloride into the flask, stopper tightly, place in a sonic bath for 10 min, and cool to room temperature.

Determination of Caffeine—Zero the instrument at 3390 nm with carbon tetrachloride in two matching near-IR silica cells. Scan the near-IR spectra of sample and standard solutions against carbon tetrachloride between 3500 and 3200 nm. Measure the baseline absorbance value at 3390 nm from the straight line drawn between 3500 and 3150 nm. Subtract the baseline absorbance value from the peak absorbance at 3390 nm to obtain the net absorbance.

Calculate the caffeine concentration using the absorbances at 3390 nm as follows:

$$\text{mg caffeine/tablet} = \frac{A_{3390}}{A_{caf3390}} \times C \times \frac{T}{W} \times 50 \quad \text{(Eq. 4)}$$

where:

A_{3390} = absorbance of sample at 3390 nm
$A_{caf3390}$ = absorbance of caffeine standard solution at 3390 nm
C = concentration of caffeine standard solution (milligrams per milliliter)
T = average tablet weight (milligrams)
W = sample weight (milligrams)

Spiked Solutions: Caffeine—A sample composite equivalent to about 37.5 mg of caffeine was accurately weighed and transferred quantitatively to a 50-ml glass-stoppered erlenmeyer flask. About 37.5 mg of caffeine, 258 mg of carisoprodol, and 188 mg phenacetin were accurately weighed and transferred quantitatively to the same flask. The resultant mixture was then analyzed for caffeine as directed in the *Determination of Caffeine* section. The recoveries of caffeine were calculated based on assay values for the samples previously determined.

Table II—Analysis of Caffeine Spiked Solutions

Sample	Added, mg	Found, mg	Recovery, %
1	39.7	39.5	99.5
2	37.4	37.5	100.3
3	38.2	38.7	101.3
4	40.2	40.4	100.5
Average			100.4
Standard deviation, %			0.74
Relative standard deviation, %			0.74

RESULTS AND DISCUSSION

The described TLC system is useful in establishing the presence of each of the three therapeutic elements in the dosage form. Since the solvent is a single chemical entity, no solvent preparation is necessary. The R_f values indicate that a satisfactory resolution may be expected. Since the study was designed to find the drugs at therapeutic levels, the limits of detection of the described TLC procedure were not determined. However, these limits could easily be established if it were necessary to use the procedure to detect lower drug levels.

The success of the quantitative analysis for carisoprodol and phenacetin is dependent upon the existence of absorption bands in the 3100–2800-nm region for dilute solutions of amides in nonpolar solvents. As an example of the carbamate class, meprobamate, a dicarbamate, gives rise to two medium intensity absorption bands in the near-IR region at 2820 and 2910 nm. The band at 2820 nm is due to the asymmetric stretching mode of the NH_2 (amide) group, and the band at 2910 nm is due to the corresponding symmetric stretching mode (16).

The near-IR spectrum of meprobamate is, therefore, characterized by two equivalent intensity absorption maxima of direct importance to this work (Fig. 1). The carisoprodol molecule, which incorporates both an unsubstituted and substituted amide group, exhibits a near-IR spectrum characterized by an absorption maximum at 2820 nm due to the asymmetric stretching mode of the NH_2 (amide) group and a stronger maximum at 2910 nm ascribable to the corresponding symmetric stretching of the unsubstituted amide plus the weaker intensity vibrational change of the substituted NH (amide) group (Fig. 2). The near-IR absorption

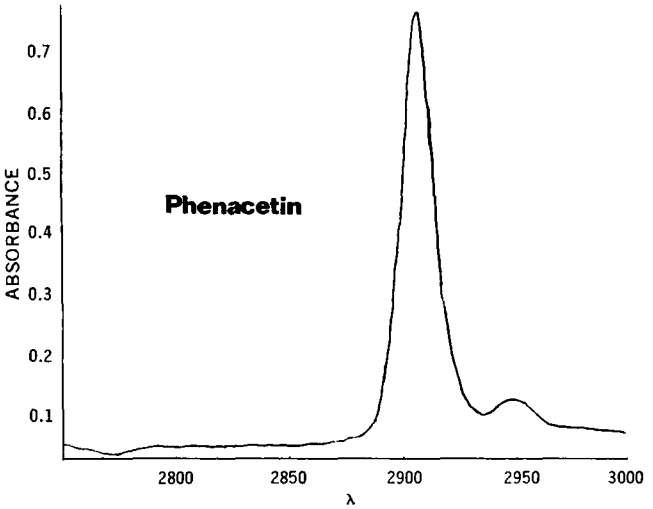

Figure 3—*Near-IR spectrum of phenacetin standard solution (150 mg/100 ml in chloroform); λ = wavelength (nanometers).*

Table III—Complete Analysis of Commercial Tablets Containing Carisoprodol, Phenacetin, and Caffeine

Sample Lots	Carisoprodol		Phenacetin		Caffeine	
	Declared, mg	Found, mg	Declared, mg	Found, mg	Declared, mg	Found, mg
1	200	188.4	160	157.9	32	30.4
2	200	186.2	160	159.0	32	30.4
3	200	185.6	160	159.3	32	32.3
4	200	188.1	160	156.2	32	32.2
Mean		187.1		158.1		31.3
Standard deviation		1.38		1.40		1.07
Relative standard deviation		0.74		0.89		3.41
Average recovery, % of declared		93.6		98.7		97.9

spectrum for phenacetin also exhibits a maximum at 2910 nm due to the vibrational transition of the NH (amide) group (Fig. 3). These absorption bands were used for the simultaneous determination of carisoprodol and phenacetin in a tablet combination (Fig. 4).

The small peak appearing at 2950 nm in the phenacetin spectrum, although not used analytically, was identified as the double-frequency overtone of the "amide I" fundamental band of —C(=O)—NHAr group (17, 18). Its presence did not affect the accuracy of the near-IR procedure.

The near-IR properties of carisoprodol and phenacetin provided a convenient method for the simultaneous spectrophotometric analysis of both drugs. Caffeine could not be measured at the same time but, fortunately, did not interfere. A caffeine standard solution did not exhibit any absorption at either 2910 or 2820 nm, the analytical wavelengths.

This system is recognized as a simple binary mixture (carisoprodol and phenacetin) with one member (phenacetin) absorbing at one analytical wavelength only. Thus, in a mixture of phenacetin and carisoprodol, the total absorbance at 2910 nm, A'_{2910}, is described by:

$$A'_{2910} = A'_{c2910} + A'_{p2910} \qquad \text{(Eq. 5)}$$

where:

A'_{c2910} = absorbance at 2910 nm for carisoprodol standard solution
A'_{p2910} = absorbance at 2910 nm for phenacetin standard solution

The value for A'_{c2910} may be calculated from the Beer's law behavior of carisoprodol at 2820 nm, whereas Eq. 6 provides the basis for evaluating A'_{p2910}:

$$A'_{p2910} = A'_{2910} - A'_{c2820}\frac{a_{c2910}}{a_{c2820}} \qquad \text{(Eq. 6)}$$

where:

A'_{c2820} = absorbance at 2820 nm for carisoprodol standard solution
a_{c2910} = absorptivity of carisoprodol at 2910 nm (0.662)
a_{c2820} = absorptivity of carisoprodol at 2820 nm (0.377)

The absorbances of a series of carisoprodol standard solutions were measured, and adherence to Beer's law was observed at both maxima (Fig. 5). A similar series of phenacetin standard solutions was also analyzed and found to obey Beer's law (Fig. 5). Finally, a series of mixed carisoprodol–phenacetin standard solutions was analyzed and exhibited linearity.

The actual analytical system is relatively simple. The 2820-nm line for carisoprodol in Fig. 5 provides the basis for carisoprodol measurement. For phenacetin determination, Eq. 6 is needed to yield A'_{p2910}. Since A'_{2910} and A'_{c2820} are direct readings and a_{c2910} and a_{c2820} are obtained as the slopes of the straight lines in Fig. 5, the calculation may be easily accomplished.

The validity of the simultaneous quantitative procedure for phenacetin and carisoprodol was substantiated by the data obtained from the analysis of spiked solutions (Table I), which show the relative standard deviations for carisoprodol and phenacetin to be 1.21 and 0.69%, respectively.

The quantitative procedure described for caffeine was inferred from an IR method reported for its determination in a multicomponent tablet mixture (19). The IR absorption spectrum of a chloroform solution of caffeine presented in the method revealed two small bands at about 3400 and 3350 nm. Chloroform is an unacceptable solvent for near-IR determinations in this region, since the radiation is absorbed by this solvent and a reference energy cannot be obtained. Carbon tetrachloride was a satisfactory substitute.

The determination of caffeine was complicated by interference from carisoprodol, a much stronger absorber at 3390 nm. The latter was removed in the chromatographic step by elution with 250 ml of ether, while caffeine was retained on the acidified column and easily eluted with 150 ml of chloroform. Although a chromatogram is not included, these volumes were found to be ideal. Evaporation of the chloroform eluate was followed by drying to remove water absorbed by caffeine from the solvents. Failure to do this would yield high results due to the light-absorbing properties of water in the near-IR region being considered.

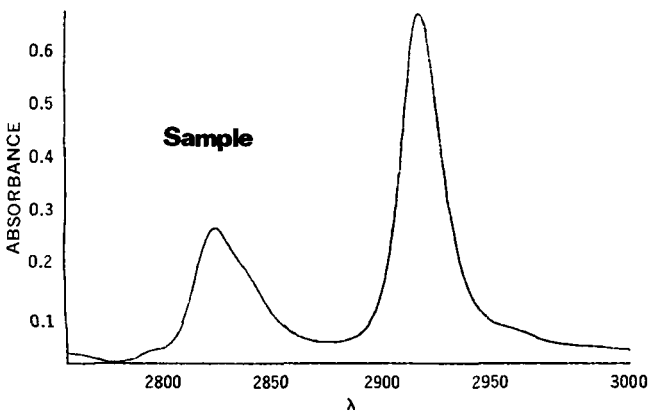

Figure 4—*Near-IR spectrum of a carisoprodol and phenacetin solution in a tablet combination (50 mg/100 ml carisoprodol and 40 mg/100 ml phenacetin in chloroform); λ = wavelength (nanometers).*

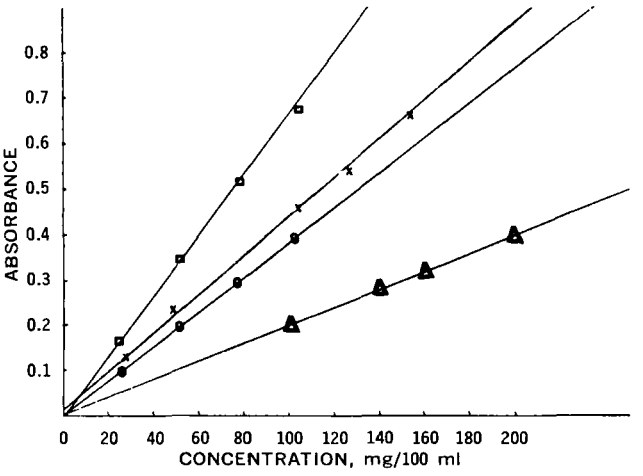

Figure 5—*Beer's law plots for individual carisoprodol, phenacetin, and caffeine standard solutions. Key: □, carisoprodol at 2910 nm in chloroform; ○, carisoprodol at 2820 nm in chloroform; x, phenacetin at 2910 nm in chloroform; and △, caffeine at 3390 nm in carbon tetrachloride.*

The need for the separation of caffeine from carisoprodol can be understood by studying the IR-absorbing properties of both molecules. The near-IR spectrum of caffeine in the 3500–3200-nm region is characterized by a strong intensity absorption maximum at 3390 nm, attributable to the asymmetric CH-stretching mode of its three methyl groups, and a weaker maximum at 3340 nm, attributable to the corresponding symmetric CH-stretching mode (21). The carisoprodol molecule, incorporating four methyl groups and four CH_2 groups, absorbs strongly at three contiguous wavelengths (3390, 3420, and 3500 nm) due to asymmetric CH-stretching and moderately at 3340 nm due to symmetric CH-stretching. These spectral similarities and, more important, the much greater absorption intensities of the carisoprodol demonstrate the need for an isolation procedure for caffeine.

A series of caffeine standard solutions in carbon tetrachloride was studied spectrophotometrically, and the Beer's law plot exhibited linearity (Fig. 5, absorptivity 0.193). The data in Table II obtained from the analysis of spiked solutions, which shows an excellent solute recovery in terms of the mean and a relative standard deviation of 0.74%, verify the validity of the quantitative procedure for caffeine.

Four different commercial lots of tablets were assayed for carisoprodol, phenacetin, and caffeine. The results (Table III) indicate that the near-IR procedures described are reproducible with standard deviations of 1.38, 1.40, and 1.07 mg for carisoprodol, phenacetin, and caffeine, respectively. Since each sample lot is a true independent sample, the uncertainties experienced reflect both the variations inherent in the manufacturing process and the statistical perturbations possessed by the proposed near-IR spectrophotometric procedure. When the recovery data in Tables I and II are compared with those of Table III, the agreement obtained is reasonable. The results presented in Table III are considered satisfactory.

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Colorimetric Analysis of Procaine Hydrochloride

HENRY S. I. TAN[x] and D. SHELTON

Abstract □ A colorimetric assay procedure was developed for the quantitative analysis of procaine hydrochloride. The method is based on the interaction of procaine hydrochloride with *p*-dimethylaminocinnamaldehyde in the presence of trichloroacetic acid in absolute methanol to form a red Schiff base which can be quantitated spectrophotometrically at 547.5 nm. The Beer–Lambert law was adhered to over the 0.1–7-μg/ml range. Best accuracy was attained for solutions containing 0.4–4 μg/ml. The color was stable for at least 70 min. The method was applied to procaine hydrochloride injections, both with and without epinephrine, without prior separation of the drug. The results were comparable to those obtained by the official procedures.

Keyphrases □ Procaine hydrochloride—colorimetric analysis using *p*-dimethylaminocinnamaldehyde □ *p*-Dimethylaminocinnamaldehyde—colorimetric reagent for determination of procaine hydrochloride □ Colorimetry—determination, procaine hydrochloride, using *p*-dimethylaminocinnamaldehyde

The USP XVIII (1) assay procedure for procaine hydrochloride is time consuming, since it is based on a diazotization titration of the compound with standard sodium nitrite at low temperatures using starch iodide paper as the external indicator. This method is subject to variations between individuals in terms of determining the end-point. The official assay method (2) for procaine hydrochloride injection is also lengthy, since a prior isolation of the compound by solvent extraction is required before the compound can be determined. These reasons prompted this laboratory to develop an assay method offering

The need for the separation of caffeine from carisoprodol can be understood by studying the IR-absorbing properties of both molecules. The near-IR spectrum of caffeine in the 3500–3200-nm region is characterized by a strong intensity absorption maximum at 3390 nm, attributable to the asymmetric CH-stretching mode of its three methyl groups, and a weaker maximum at 3340 nm, attributable to the corresponding symmetric CH-stretching mode (21). The carisoprodol molecule, incorporating four methyl groups and four CH_2 groups, absorbs strongly at three contiguous wavelengths (3390, 3420, and 3500 nm) due to asymmetric CH-stretching and moderately at 3340 nm due to symmetric CH-stretching. These spectral similarities and, more important, the much greater absorption intensities of the carisoprodol demonstrate the need for an isolation procedure for caffeine.

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Colorimetric Analysis of Procaine Hydrochloride

HENRY S. I. TAN[x] and D. SHELTON

Abstract □ A colorimetric assay procedure was developed for the quantitative analysis of procaine hydrochloride. The method is based on the interaction of procaine hydrochloride with *p*-dimethylaminocinnamaldehyde in the presence of trichloroacetic acid in absolute methanol to form a red Schiff base which can be quantitated spectrophotometrically at 547.5 nm. The Beer–Lambert law was adhered to over the 0.1–7-μg/ml range. Best accuracy was attained for solutions containing 0.4–4 μg/ml. The color was stable for at least 70 min. The method was applied to procaine hydrochloride injections, both with and without epinephrine, without prior separation of the drug. The results were comparable to those obtained by the official procedures.

Keyphrases □ Procaine hydrochloride—colorimetric analysis using *p*-dimethylaminocinnamaldehyde □ *p*-Dimethylaminocinnamaldehyde—colorimetric reagent for determination of procaine hydrochloride □ Colorimetry—determination, procaine hydrochloride, using *p*-dimethylaminocinnamaldehyde

The USP XVIII (1) assay procedure for procaine hydrochloride is time consuming, since it is based on a diazotization titration of the compound with standard sodium nitrite at low temperatures using starch iodide paper as the external indicator. This method is subject to variations between individuals in terms of determining the end-point. The official assay method (2) for procaine hydrochloride injection is also lengthy, since a prior isolation of the compound by solvent extraction is required before the compound can be determined. These reasons prompted this laboratory to develop an assay method offering

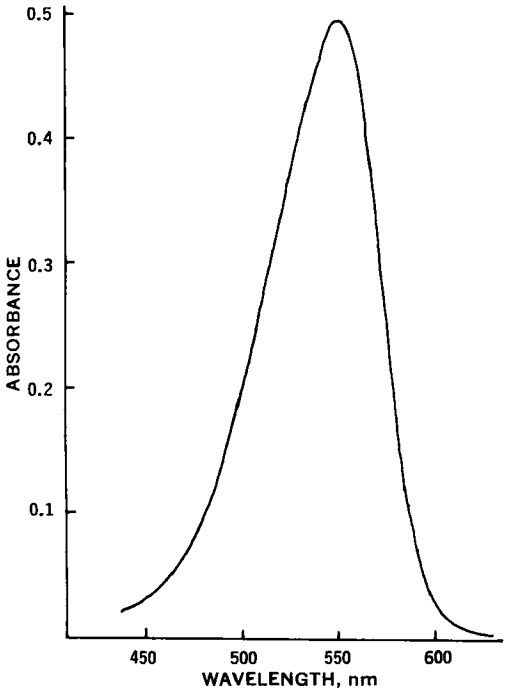

Figure 1—*Absorption spectrum of procaine hydrochloride–p-dimethylaminocinnamaldehyde reaction product at 2 μg procaine hydrochloride/ml.*

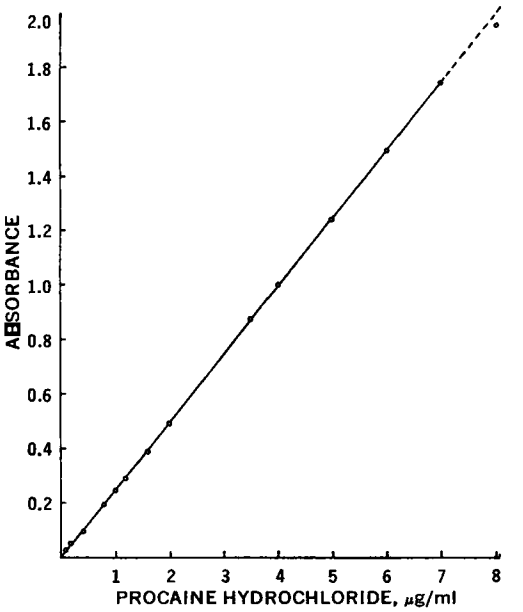

Figure 2—*Relationship between absorbance and concentration of procaine hydrochloride.*

improvements in sensitivity, speed, and ease over the official methods.

The use of *p*-dimethylaminocinnamaldehyde as a colorimetric agent for primary aromatic amines in acidic solutions was reported previously (3, 4). This reagent is advantageously employed in place of the usual aldehydes such as *p*-dimethylaminobenzaldehyde and vanillin, since the resulting Schiff bases are red. This report presents a sensitive, simple, and accurate method for the analysis of procaine hydrochloride with *p*-dimethylaminocinnamaldehyde in a nonaqueous acidic medium. The proposed procedure can be automated, which is important from the standpoint of industrial application.

EXPERIMENTAL

Instruments—The following were used: a UV-visible double-beam spectrophotometer[1] with 1-cm cells[2] and a slit width of 0.22 mm and an analytical balance[3].

Materials and Reagents—The following were used: procaine hydrochloride (ACS grade)[4]; acetone-free absolute methanol containing 0.008% water[5]; a 0.10% solution of *p*-dimethylaminocinnamaldehyde[4], mp 132–136°, in methanol (stable for at least 3 weeks if refrigerated); and a 50% solution of trichloroacetic acid[6] (ACS grade) in methanol.

Preparation of Standard Curve—A stock solution of procaine hydrochloride was prepared by dissolving 80.0 mg of procaine hydrochloride in 100 ml of methanol. Further dilutions were made to obtain standard solutions, each containing 1.0, 2.0, 4.0, 8.0, 10.0, 12.0, 16.0, 20.0, 35.0, 40.0, 50.0, 60.0, 70.0, and 80.0 μg procaine hydrochloride/ml, respectively. One milliliter of each solution was utilized for color development as described under *Assay Procedure for Procaine Hydrochloride*. The stock and standard solutions should be freshly prepared.

Assay Procedure for Procaine Hydrochloride—Pipet 1.0 ml of a methanolic solution containing 1–70 μg of procaine hydrochloride into a 10-ml volumetric flask. To this solution, add exactly 1.00 ml of *p*-dimethylaminocinnamaldehyde reagent followed by 1.0 ml of trichloroacetic acid reagent and wait for 10 min. Dilute to volume with methanol and determine the absorbance at 547.5 nm against a blank prepared similarly but omitting the procaine hydrochloride. Read the concentration of procaine hydrochloride present from a calibration curve.

Assay Procedure for Procaine Hydrochloride Injection with or without Epinephrine—Pipet 5.0 ml of a 1% procaine hydrochloride injection *or* 2.0 ml of a 2% procaine hydrochloride injection into a 100-ml volumetric flask and dilute to volume with methanol. Transfer 1.0 ml of the resulting solution into a 50-ml volumetric flask and dilute to volume with methanol. Pipet 1.0 ml of the latter solution into a 10-ml volumetric flask and proceed as described in the *Assay Procedure for Procaine Hydrochloride* beginning with "To this solution add. . . ."

RESULTS AND DISCUSSION

The interaction of procaine hydrochloride and *p*-dimethylaminocinnamaldehyde in acidic medium produced a red Schiff base showing an absorption peak at 547.5 nm in its absorption spectrum (Fig. 1). Therefore, a more sensitive and deep coloration was obtained than the yellow color resulting from the reaction of procaine hydrochloride with the commonly used aldehydes such as *p*-dimethylaminobenzaldehyde and vanillin.

As shown in Fig. 2, at 547.5 nm a linear relationship exists between concentration and absorbance throughout the region practical for absorbance measurements. Under the proposed experimental conditions the Beer–Lambert law is followed over the con-

Table I—Effect of Time on Absorbance of Procaine Hydrochloride–*p*-Dimethylaminocinnamaldehyde Color Complex[a]

Minutes	Absorbance at 547.5 nm
20	0.653
30	0.652
40	0.651
50	0.649
60	0.651
70	0.651

[a] Concentration about 2.8 μg procaine hydrochloride/ml.

[1] Beckman Acta V, Beckman Instruments, Fullerton, Calif.
[2] Bausch and Lomb.
[3] Mettler type H-18, Mettler Instrument Corp., Princeton, N.J.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] Matheson, Coleman and Bell, Norwood, Ohio.
[6] Mallinckrodt Chemical Works, New York, N.Y.

Table II—Assay of Solutions of Known Concentrations of Procaine Hydrochloride at Different Levels

Amount Weighed, mg	Analyzed at Concentration Level, μg/ml	Amount Found[a], mg		Percent Recovery		
				Proposed Method		
		Proposed Method	USP XVIII Method	Individual Samples	Average	USP XVIII Method
50.0	1.0	49.9		99.8	100.7	
	4.0	50.8		101.6		
250.0	0.5	252.2		100.9	100.4	
	5.0	249.8		99.9		
300.0	0.6	305.0		101.7	101.8	
	6.0	305.5		101.8		
300.0			302.1			100.7
500.0			500.6			100.1
Overall percent recovery					101.0	100.4
Standard deviation					0.73	

[a] Each value is the average of two replicate assays.

centration range 0.1–7 μg procaine hydrochloride/ml. However, the Ringbom (5) plot indicates that best accuracy will be obtained for solutions containing approximately 0.4–4 μg procaine hydrochloride/ml (Fig. 3). The plot also shows an inflection at 37% transmittance, indicating that the system does indeed adhere to the Beer–Lambert law (6).

The concentration optimum of the color reagent was determined by adding varying volumes of 0.10% *p*-dimethylaminocinnamaldehyde in methanol to a series of 10-ml volumetric flasks, each containing 1.0 ml of 20 μg procaine hydrochloride/ml and 1.0 ml of 50% trichloroacetic acid in methanol, and measuring the absorbance of each solution at 547.5 nm against a blank[7] after diluting each solution to volume with methanol. Increasing amounts of *p*-dimethylaminocinnamaldehyde resulted in increased absorbance readings. Since this would result in shorter ranges of usable concentrations, it was decided to use 1.0 ml of 0.10% *p*-dimethylaminocinnamaldehyde as the optimum concentration for the assay. Changing the volumes of 50% trichloroacetic acid did not appreciably affect absorbance readings. In view of the results, 1 ml of 50% trichloroacetic acid was selected as the concentration for the assay.

Water adversely affected the interaction of procaine hydrochloride with *p*-dimethylaminocinnamaldehyde. The use of methanol containing 0.02% water caused the absorbance values to decrease slowly. Higher concentrations of water caused the red color to fade in 5–15 min. However, when 0.008% water-containing methanol was used, the color appeared to be stable for at least 70 min (Table I).

The stability of the *p*-dimethylaminocinnamaldehyde reagent was evaluated by recording its UV spectrum (λ_{max} 391 nm) over an extended period. The molar absorptivity remained unchanged for at least 3 weeks if the reagent was refrigerated.

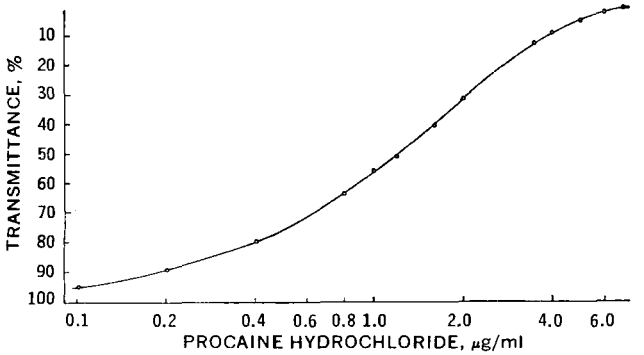

Figure 3—*Ringbom plot for the procaine hydrochloride–p-dimethylaminocinnamaldehyde reaction product.*

[7] Each blank contained the same amount of *p*-dimethylaminocinnamaldehyde and trichloroacetic acid as in the solution studied but contained no procaine hydrochloride.

Recovery data for procaine hydrochloride in the concentration range studied proved to be accurate with the proposed procedure. Recovery studies were performed in three successive experiments using 50, 250, and 300 mg of procaine hydrochloride. In each case, the accurately weighed amount of procaine hydrochloride was dissolved in 100 ml methanol. Appropriate dilutions were then prepared to afford different concentrations (Table II) which were determined by the proposed method. All results were quantitative and reproducible. The overall percent recovery for the assay samples was 101.0% with a standard deviation of 0.73. In comparison, the average percent recovery for two samples analyzed by the USP XVIII method was 100.4% (Table II).

The precision study was performed by running replication studies on nine samples of procaine hydrochloride, each containing 1 μg/ml. Each sample was then analyzed by the proposed method. The coefficient of variation for the nine replicate samples was 0.53% (Table III).

The proposed analytical method was then compared to the official method in the analysis of commercially available preparations of procaine hydrochloride injection. There is a close relationship between the results obtained by the official procedure and those obtained with the proposed analytical method (Table IV).

A favorable characteristic of the proposed procedure is the speed and ease of performing the assay. Since the *p*-dimethylaminocinnamaldehyde reagent can be stored for several weeks without any noticeable deterioration, this reagent does not have to be prepared daily. In contrast, the USP XVIII method for procaine hydrochloride requires freshly prepared sodium nitrite solutions. In the analysis of parenteral preparations, no prior isolation of the procaine hydrochloride is necessary and the drug does not have to be separated from epinephrine in epinephrine-containing formulations. Thus, it eliminates the need for extracting both the parenteral solution and the blank required by the official assay procedure. In addition, due to its speed and ease, the proposed method can be modified for automation.

Table III—Reproducibility of Color Development of Replicate Samples of Procaine Hydrochloride Containing 1 μg/ml

Solution	Absorbance at 547.5 nm
1	0.270
2	0.267
3	0.268
4	0.269
5	0.267
6	0.268
7	0.267
8	0.269
9	0.271
Average	0.268
Standard deviation	0.0014
Coefficient of variation	0.53%

Table IV—Assay of Commercially Available Procaine Hydrochloride Parenteral Solutions

Preparation	Amount of Procaine Hydrochloride Claimed, mg/ml	Amount Found[a], mg		Percent of Claim	
		Proposed Method	USP XVIII Method	Proposed Method	USP XVIII Method
Procaine, 1%	10.0	10.08	10.15	100.8	101.5
Procaine, 2%	20.0	20.25	20.40	101.3	102.0
Procaine, 2%, with 0.002% epinephrine	20.0	20.50	20.70	102.5	103.5

[a] Average of duplicate assays of samples from three manufacturers.

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(1) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 543.

(2) *Ibid.*, p. 544.

(3) S. Sakai, K. Suzuki, H. Mori, and M. Fujino, *Jap. Anal.*, **9**, 862 (1960).

(4) M. Pesez and J. Bartos, *Bull. Soc. Chim. Fr.*, **1966**, 3802.

(5) A. Ringbom, *Z. Anal. Chem.*, **115**, 332(1939).

(6) G. H. Ayres, *Anal. Chem.*, **21**, 652(1949).

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[x] To whom inquiries should be directed.

Interference by Traces of Acetic Anhydride in Nonaqueous Titrimetry of Primary Aromatic Amines: Improved Titrations for Procainamide Hydrochloride

HAROLD KADIN

Abstract □ Traces of acetic anhydride and acetaldehyde in the acetic acid titration solvent depress the nonaqueous titrimetry of primary aromatic amines. Solutions to the problem include a modified nonaqueous titration and a nitrite titration with internal ferrocyphen indicator. These alternative methods for the titration of procainamide hydrochloride were compared with the USP XVIII nonaqueous titration of procainamide hydrochloride. The nitrite titration is the best procedure.

Keyphrases □ Procainamide hydrochloride—analysis, acetic anhydride interference in nonaqueous titration, improved procedure compared with alternative nitrite and compendial titrations □ Amines, primary aromatic—interference of trace acetic anhydride in nonaqueous titrimetry, improved procedure and alternative nitrite titration □ Titrimetry, nonaqueous and nitrite—analysis, primary aromatic amines (procainamide hydrochloride) □ Acetic acid—interference of trace acetic anhydride in nonaqueous titration of primary aromatic amines (procainamide hydrochloride)

Procainamide hydrochloride[1] is an efficacious, widely prescribed agent for the control of cardiac arrhythmias. Among its purity safeguards is a USP (1) requirement that assays of the drug by nonaqueous titrimetry fall within the narrow limits of 98.0–100.5%.

In these laboratories, titrimetry of procainamide hydrochloride by the USP method (1) repeatedly yielded assays that were close to the 98% USP minimum specification. However, other evidence, including melting ranges and TLC, suggested that the purity of the samples exceeded 99%. Suspicion centered on the titration solvent, glacial acetic acid, when the use of one lot of glacial acetic acid yielded an extremely low assay for procainamide hydrochloride of 81%. When other lots of glacial acetic acid were used as the titration medium, the same batch of procainamide hydrochloride gave assay values above 98%.

Posgay (2) reported that trace amounts of acetic anhydride in glacial acetic acid can acetylate certain amines, leading to low results when these amines are titrated in acetic acid with acetous perchloric acid. Thus, the lot of glacial acetic acid whose use led to low procainamide hydrochloride assay values might have contained traces of acetic anhydride and/or acetaldehyde, which could effect acetylation or Schiff-base formation of the primary aromatic amine function. This blocked amine, being unavailable for perchloric acid titration, could be responsible for the low assay values.

Examination of the acetic acids for acetic anhydride, total carbonyls, and water indicated that the cause of the low assay values was trace amounts of acetic anhydride. Possible remedies and alternative

[1] Pronestyl, Squibb.

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Procaine, 2%, with 0.002% epinephrine	20.0	20.50	20.70	102.5	103.5

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Abstract □ Traces of acetic anhydride and acetaldehyde in the acetic acid titration solvent depress the nonaqueous titrimetry of primary aromatic amines. Solutions to the problem include a modified nonaqueous titration and a nitrite titration with internal ferrocyphen indicator. These alternative methods for the titration of procainamide hydrochloride were compared with the USP XVIII nonaqueous titration of procainamide hydrochloride. The nitrite titration is the best procedure.

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Examination of the acetic acids for acetic anhydride, total carbonyls, and water indicated that the cause of the low assay values was trace amounts of acetic anhydride. Possible remedies and alternative

[1] Pronestyl, Squibb.

titrations were then examined. The remedies included treatment or testing of the acetic acid. One alternative titration involves addition of the aromatic amine, benzocaine, to the acetic acid. The benzocaine then reacts with traces of acetic anhydride and acetaldehyde to remove these interferences. Excess benzocaine is neutralized followed by the addition and titration of the aromatic amine one wishes to measure. A highly preferable alternative is a nitrite titration with an internal indicator (3).

The frequent recurrence of marginal nonaqueous titration values suggested that the problem of trace acetic anhydride or acetaldehyde in glacial acetic acids might be widespread. Thus, the implications of these studies should be of general interest.

EXPERIMENTAL

Equipment—A conventional 50-ml buret was used for the nitrite titrations, and an automatic 10-ml buret with reservoir and drying tubes[2] was used for the nonaqueous titrations. The air vent at the top of the buret and the U-shaped drying tube on the air-inlet side arm contained dehydrating silica gel[3].

Sample dissolution was accomplished in an 80-w ultrasonic cleaner[4].

Potentiometry with the pH meter coupled to calomel and glass electrodes was used to verify the visual end-point of the nonaqueous titration. The aqueous salt bridge of the calomel electrode was replaced with 1% lithium perchlorate in glacial acetic acid. When not in use, the calomel electrode was kept in glacial acetic acid. The glass electrode was kept in distilled water and was rehydrated in distilled water for about 15 min between titrations in glacial acetic acid. Prior to its immersion in the titration mixture, excess water was removed from the glass electrode by a quick immersion in glacial acetic acid.

Reagents—For the nonaqueous titrations, the reagents included: 0.1 *N* acetous perchloric acid, USP XVIII (p. 1035); benzocaine NF; glacial acetic acid *without* adjustment of water content (by addition of acetic anhydride) as specified for USP XVIII nonaqueous titration of procainamide hydrochloride; crystal violet T.S. indicator; and mercuric acetate, 6 g in 100 ml of glacial acetic acid.

The nitrite titration required: 6 *M* hydrochloric acid; ferrocyphen [dicyanobis(1,10-phenanthroline)-iron (II)] indicator, 0.50 g dissolved *without warming* in 50 ml of concentrated sulfuric acid; 0.1 *N* sodium nitrite, and USP sulfanilamide reference standard.

Colorimetry for total carbonyls required: 2 *N* hydrochloric acid, a freshly prepared mixture of chloroform–isooctane (40:60 v/v), 0.25% 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid, and acetaldehyde.

Analysis for Carbonyls (Expressed as Acetaldehyde) in Glacial Acetic Acid—*Sample*—Transfer 20.0 ml of glacial acetic acid into a 100-ml volumetric flask. Dilute to volume with distilled water and mix.

Standards—One milliliter of refrigerated acetaldehyde (density was 0.788 g/ml) is initially diluted to 100 ml with distilled water. A secondary dilution of 2 ml of the stock standard to 100 ml of distilled water is followed by tertiary dilutions in distilled water of 5 ml to 100, 10 ml to 100, and 20 ml to 100, yielding working dilutions of 8, 16, and 32 μg acetaldehyde/ml, respectively.

Procedure—Transfer 3.0 ml of the final samples and standard dilutions into individual 60-ml separators. Use 3.0 ml of distilled water for the blank. Add 1 ml of the dinitrophenylhydrazine reagent. Swirl the mixtures and let them stand for 5 min. Add 10 ml of the 2 *N* hydrochloric acid and then 10.0 ml of the freshly prepared chloroform–isooctane mixture. Shake for 2 min. Allow layers to separate. Transfer the upper, organic solvent layers to glass-stoppered centrifuge tubes. Clarify the extracts by centrifugation. Determine absorbances in 1-cm cells *versus* the blank in a spectrophotometer at 420 nm.

Beer's Law Conformity—The standards read 0.214, 0.425, and 0.860. When these absorbances were plotted *versus* their concentrations, a straight line was obtained which intersected the origin, indicating conformity to Beer's law.

Calculations—Calculate the individual standards as ratios of micrograms of acetaldehyde to absorbance. Since Beer's law is followed, the average of the three ratios as the standard factor is used in the following calculation, in which A = absorbance of acetic acid sample and K = standard factor. Then micrograms of carbonyls (as acetaldehyde) per milliliter = $(AK \times 100)/(3 \times 20)$, which simplifies to $(AK \times 1.67)$.

Analysis for Acetic Anhydride in Glacial Acetic Acid—Accurately weigh about 0.16 g of benzocaine NF. Transfer to a 250-ml conical flask containing 100 ml of the glacial acetic acid, and seal the flask with aluminum foil. Dissolve the benzocaine in the acid by placing the flask in the ultrasonic cleaner for 2–3 min. Let stand at room temperature for 45 min. Add 2 drops of the crystal violet indicator and titrate with the acetous perchloric acid to a green end-point with absence of blue shades. Perform sample and blank titrations in duplicate.

Again, accurately weigh, in duplicate, about 0.16 g of benzocaine from the same container of benzocaine used previously. Transfer each sample individually to one of the neutralized solutions obtained from the preceding titrations. Dissolve in the acid, as indicated previously, and again titrate to a green end-point with absence of blue shades. A blank is not required for the latter two titrations.

If A = milliliters titrated for the sample after 45 min contact with acid, B = milliliters titrated for the blank, A_t = milliliters titrated for the sample added to a previously neutralized 45-min sample, N = normality of acetous perchloric acid, W_1 = weight in milligrams of the benzocaine sample used for the first titration, W_2 = weight in milligrams of the benzocaine sample added to the neutralized sample of W_1, 165.19 = equivalent weight of benzocaine, and 102.09 = equivalent weight of acetic anhydride, then micrograms of acetic anhydride per milliliter of glacial acetic acid equals:

$$102.09\left[\frac{(165.19\,NA_t/W_2)W_1 - 165.19N(A-B)}{165.19}\right]1000/100$$

which simplifies to:

$$NW_1\left[\frac{A_t}{W_2} - \frac{(A-B)}{W_1}\right] \times 1020.9$$

Nonaqueous Titration for Procainamide Hydrochloride—Weigh approximately 150–170 mg of benzocaine into a 250-ml conical flask. Transfer 100 ml of acetic acid into the flask. Dissolve the benzocaine in the acid by placing the container in an ultrasonic bath at 20–25° for 2–3 min[5].

Add 10 ml of the mercuric acetate solution, and let the flask stand for 45 min. Add 2 drops (about 0.04 ml) of the crystal violet indicator. Titrate with the 0.1 *N* perchloric acid to a green end-point with absence of blue shades. Weigh accurately about 135 mg of the procainamide hydrochloride sample into the flask containing the neutralized benzocaine solution. Dissolve the procainamide hydrochloride by placing the flask in an ultrasonic bath at 20–25° for 2–3 min. Titrate with the standardized perchloric acid to a green end-point with absence of blue shades. Do not perform a blank titration.

The percent procainamide hydrochloride = [(ml × N × 135.9)/sample wt., mg] × 10^2, where ml = acetous perchloric acid to titrate procainamide hydrochloride sample, N = normality of acetous perchloric acid, and 135.9 = equivalent weight of procainamide hydrochloride.

Nitrite Titration for Procainamide Hydrochloride—*Standardization*—Transfer an accurately weighed 0.4-g sample of USP sulfanilamide reference standard into a 250-ml conical flask. Add 100 ml of 6 *M* hydrochloric acid. Place the flask in an ultrasonic cleaner for 3–5 min to dissolve the sulfanilamide. Add 1.0 ml of

[2] No. 10-8352, Ace Scientific Supply, Linden, N.J.
[3] Tel-Tale No. 42, W. R. Grace & Co., Baltimore, Md.
[4] Model 8845-3, Cole-Parmer, Chicago, Ill.

[5] If appreciable yellowing occurs (due to Schiff-base formation of the benzocaine with excessive contaminating acetaldehyde), begin again with a new lot of glacial acetic acid. Schiff-base formation is readily visible with 0.01% (v/v) of acetaldehyde. Yellow superimposed on the blue indicator may yield premature green end-points.

the ferrocyphen solution. Titrate with the 0.1 N sodium nitrite[6] to a violet end-point that is stable for at least 3 min. Perform a blank titration on the reagents. Standardize the sodium nitrite solution daily, as needed.

Procainamide Hydrochloride Assay—Weigh accurately about 500 mg of the procainamide hydrochloride sample into a 250-ml conical flask. Add 100 ml of 6 M hydrochloric acid and place the flask in an ultrasonic water bath at 20–25° for 2–3 min to dissolve the sample. Add 1.0 ml of the ferrocyphen indicator and titrate promptly[6] with the standardized sodium nitrite until a rose end-point is observed that is stable for at least 3 min. Do a blank titration.

Calculations—

1. Standardization:

$$\text{normality of sodium nitrite solution} = \frac{\text{sulfanilamide wt., mg}}{(ml_1 - B_1) \times 172.21} \quad \text{(Eq. 1)}$$

where ml_1 = milliliters of sodium nitrite solution to titrate sulfanilamide, B_1 = milliliters to titrate blank, and 172.21 = equivalent weight of sulfanilamide.

2. Procainamide Hydrochloride Assay:

$$\text{percent procainamide hydrochloride} = \frac{(ml_2 - B_2) \times N \times 271.79}{\text{sample wt., mg}} \times 10^2 \quad \text{(Eq. 2)}$$

where ml_2 = milliliters of sodium nitrite solution to titrate procainamide hydrochloride, B_2 = average milliliters to titrate blank, N = normality of sodium nitrite, and 271.79 = equivalent weight of procainamide hydrochloride.

RESULTS AND DISCUSSION

Nonaqueous Titration—The lot (Sample 1) of acetic acid giving low procainamide hydrochloride assay values, as well as two other lots (Samples 2 and 3) of acetic acid, was analyzed by the procedures described for total carbonyls and acetic anhydride. Water was also determined with the Karl Fischer titration.

Table I summarizes the assay data and demonstrates, with experimental Modifications A–F, the effects of water, acetic anhydride, acetaldehyde, and three lots of acetic acid on the USP XVIII nonaqueous titration of procainamide hydrochloride. The three acids may be categorized as giving seriously low, slightly low, and satisfactory results in the order in which they appear in Table I from left to right.

Extremely low assay values can be obtained by adding very small amounts of acetaldehyde or acetic anhydride (Modifications D and E, respectively) to glacial acetic acids that previously gave slightly low or satisfactory titrations. Table I also compares assay results obtained with the benzocaine modification to those obtained with the USP XVIII assay, the USP assay after procainamide hydrochloride contact with the acids for 45 min, and the USP assay after the addition of water to the acid the evening prior to the assay. Again, these experimental modifications indicate that traces of acetic anhydride already present in the acetic acid titration solvent seriously lower the values obtained with the USP XVIII titration. The benzocaine method, Modification F, refers to the nonaqueous titration presented under *Experimental*. In this modification, benzocaine reacts with acetic anhydride and acetaldehyde, thus purifying the acetic acid prior to the addition of the procainamide hydrochloride.

Assays of the various glacial acetic acids (Table I) clearly implicate the acetic anhydride, rather than the acetaldehyde, as responsible for the low assay values. The evidence becomes more convincing if one calculates the theoretical effect on the titration of the concentration of acetic anhydride, 232 μg/ml, found in Sample 1. Assuming complete reaction of the acetic anhydride with the procainamide hydrochloride, a purity of 77.4% would result. This calculated value is in excellent agreement with the actual titration of 77.6% (Modification B).

Further confirmation is obtained by calculation of the effect of 300 ppm (v/v) of *added* acetic anhydride (Modification E). A theoretical value of 68.2% would be found, which is in excellent agreement with the actual value of 68.3%.

It was established that maximum acetylation of benzocaine in Sample 1 occurred after about 15 min of contact. Since no further acetylation was evident after 2 hr, it appeared that acetylation was complete. A 45-min contact period is recommended in the nonaqueous titration to provide a safety factor if the rate of acetylation should vary from acid to acid.

The low water content of 0.01% in Sample 1 (Table I) may enhance the formation and stability of the acetic anhydride. Marked, but incomplete, improvement in the assay of Sample 1 (to 91%) could be obtained by the addition of water (Modification C). However, Posgay (4) reported that trace amounts of water hydrolyze equivalent amounts of acetic anhydride in acetic acid, without a catalyst, in about 90 days. Even in the presence of 0.5% water, the hydrolysis time required would be 10–15 days (4). Therefore, the addition of water did not seem practical and was discontinued.

Glacial acetic acid T.S. is specified in USP XVIII as the titration medium for procainamide hydrochloride. The Test Solution section of USP XVIII (p. 1023) directs that if the acid contains more than 0.05% of water, one must add a few milliliters of acetic anhydride, mix, and allow to stand overnight before again determining the water content.

In view of both Posgay's (4) paper and the present report, addition of acetic anhydride to glacial acetic acid appears ill-advised.

The evidence in Table I demonstrates that the benzocaine method is a marked improvement over the USP XVIII method (1). Whereas the USP XVIII method yielded unacceptable assay variations with three different glacial acetic acids (81.0, 97.8, and 99.0%), the benzocaine modification precisely elevated these assay results to 99.5, 99.7, and 99.5%, respectively, in the same three acids.

Again, viewing Table I, it is evident that the benzocaine modification improved the nonaqueous titration in glacial acetic acid, Samples 1 and 2, but no significant advantage was observed with Sample 3. This finding suggests that, in lieu of the benzocaine modification, a simpler solution might be to set maximum specifications for acetic anhydride and acetaldehyde in acetic acids. There is presently no American Chemical Society (ACS) specification for acetic anhydride in glacial acetic acid (5). Rosin (6) described a test for acetic anhydride in acetic acid. However, this test is a qualitative color test without an expressed sensitivity limit. The frequent recurrence of the problem of low or margin-

Table I—Effects of Water, Acetic Anhydride, Acetaldehyde, and Various Acetic Acids on the Nonaqueous Titration of Procainamide Hydrochloride

	Glacial Acetic Acid		
	Sample 1[a]	Sample 2[b]	Sample 3[c]
Water content, %	0.01	—[d]	0.06
Acetic anhydride, μg/ml	232	—[d]	8
Carbonyl (as acetaldehyde), μg/ml	0.6	—[d]	None detected
Procainamide assay (percent), by experimental modification:			
A. USP XVIII method, no modification	81.0	97.8	99.0
B. USP, after 45 min contact with acid	77.6	96.9	99.4
C. USP, 0.2% water added to acid previous night	91.0	—[d]	—[d]
D. USP, 0.01% acetaldehyde added, 45 min contact	—[d]	—[d]	75.0
E. USP, 0.03% acetic anhydride added, 45 min contact	—[d]	68.3	—[d]
F. Benzocaine method, nonaqueous titration	99.5	99.7	99.5

[a] Mallinckrodt WXXK. [b] Baker 9507. [c] Mallinckrodt VMK. [d] Not tested.

[6] Prolonged interruptions during the titration or excessively slow titrations lead to overconsumption of nitrite. For example, when the titration rate was excessively slow at 0.5 ml/min, a 2% increase in nitrite consumption was obtained over that obtained when titration rates were at normal speeds (3–7 ml/min).

Table II—Percent Procainamide Hydrochloride by Nitrite, USP XVIII, and Benzocaine Modified Titrations

Lot Number	Nitrite	Nonaqueous[a]	
		USP XVIII (1)	Benzocaine
MBA97521	98.9, 98.9, 98.9	96.9, 96.7, 96.8	98.0, 98.0, 98.0
35438-029	100.0, 99.7, 99.9	99.0, 99.0 —	99.0, 99.3, 99.7
	100.2, 100.3, 99.4	— — —	99.6, 99.6, 99.4
	99.7, 99.8 —	— — —	99.4, 99.5, 99.4
	Avg. 99.9 ± *SD* 0.29		Avg. 99.4 ± *SD* 0.21
35438-028	100.5, 100.5, 100.5	98.7, 98.6 —	99.4, 99.5, 99.6
91970-543	100.3, 100.5 —	98.8, 98.8, 98.8	99.2, 99.2, 99.3
91970-506	99.1, 100.1 —	99.0, 99.0 —	— — —
91970-540	99.6, 99.4 —	99.2, 99.0 —	— — —
91970-541	99.3, 99.5, 100.2	98.5, 99.0 —	— — —
91970-542	99.4, 98.8, 99.4	98.2, 98.9 —	— — —
38974-548	99.5 — —	— — —	99.7 — —
92107-002	99.0 — —	— — —	98.5, 99.2 —
92104-001	98.9 — —	— — —	98.3 — —
40613-530	99.6 — —	— — —	99.8, 99.7 —
40613-529	99.4 — —	— — —	99.3, 99.7 —
40250-524	98.3 — —	— — —	99.7, 99.1 —
40250-523	98.3 — —	— — —	99.1, 99.5 —
39036-508	99.0 — —	— — —	99.0, 98.6 —
39035-507	98.9 — —	— — —	98.7, 99.2 —
39121-551	99.1 — —	— — —	99.0 — —

[a] These titrations were performed in various lots of glacial acetic acid other than Sample 1.

al titrations in these laboratories suggests that a quantitative specification for acetic anhydride might be desirable.

The benzocaine modification or revised ACS specifications for acetic acids may be generally applicable to titration of aromatic amines and other bases that acetylate or combine as Schiff bases with the acetic anhydride or acetaldehyde in the glacial acetic acids. However, a preferable solution to the problem is the nitrite titration with internal ferrocyphen indicator (3).

Nitrite Titration—The satisfactory precision of this titration is evident in Table II. The accuracy of the visual end-point was validated potentiometrically with a pH meter equipped with glass and platinum electrodes. Two potentiometric titrations gave purity figures of 100.4 and 100.2%, comparing favorably with the average for the visual titrations of the same lot, 99.9 ± 0.29 *SD* (Table II).

Determination of the end-point in nitrite titrations has generally been amperometric or visual, with starch iodide paper used as the external indicator (7). Although these methods have yielded satisfactory results, visual determination of the end-point by an internal indicator does, of course, offer greater convenience. Although several internal indicators had been proposed for use (7), none had been generally suitable until the advent of the stable, reversible ferrocyphen indicator (3). This investigation extends the evidence for the general suitability of ferrocyphen as an internal indicator for nitrite titrations.

A statistical comparison of the three titrations in Table II by an analysis of variance procedure (8) was made possible by averaging the replicate titrations for the single lots of procainamide hydrochloride. The means for each of the three methods were as follows: nitrite, 99.32; USP XVIII, 98.36; and benzocaine, 99.23.

The means for the nitrite titration and the benzocaine modification were very similar. This suggests that the nitrite titration is suitable as a substitute for the nonaqueous titration. Using various lots of glacial acetic acid which were not obviously suspected (as was Sample 1 in Table I), the statistical evaluation of Table II indicates that the mean for the USP XVIII titration was significantly lower than the means for both the benzocaine ($p < 0.01$) and the nitrite ($p < 0.001$) titrations. This suggests that acetic anhydride and/or possibly acetaldehyde contamination of acetic acids might be the cause of a recurrent and a not always readily apparent problem in the nonaqueous titration of primary aromatic amines.

Evaluation of Alternative Solutions—In consequence of this investigation, three solutions to this problem are evident:

1. Maintain the present USP method with a requirement that acetic acids must pass maximum acetic anhydride–acetaldehyde specifications. Abolish the present requirement for glacial acetic acid T.S. that a few milliliters of acetic anhydride be added to glacial acetic acids having water contents greater than 0.05%.
2. Substitute the benzocaine modified titration for the USP XVIII method.
3. Utilize the nitrite titration instead of the USP XVIII titration.

The first of these alternatives requires additional testing of acetic acids, which can be avoided by utilizing the second or third alternative. However, the second solution is more cumbersome and prolonged than the third. Consequently, the comparatively simple nitrite titration offers the best solution to the problem.

The nitrite titration in the presence of crushed ice and with external, visual starch iodide end-point indication is presently required by USP XVIII for procainamide hydrochloride capsules. Undoubtedly, the nitrite rather than the nonaqueous titration is used because of its greater selectivity. Indeed, the excellent selectivity of the nitrite titration has been amply demonstrated in these laboratories with simple direct titrations of procainamide hydrochloride in several experimental tablets containing such diverse ingredients as povidone (polyvinylpyrrolidone), ethylcellulose, magnesium stearate, talc, lactose, calcium silicate, stearic acid, and carboxyvinyl polymer. Since some of these excipients are titrated in glacial acetic acid with perchloric acid (9), accurate titration of procainamide hydrochloride would require time-consuming separations.

The present USP XVIII nitrite titration in the presence of crushed ice and with external, visual starch iodide end-point for procainamide hydrochloride capsules might, with advantage, be replaced by the nitrite titration at room temperature with internal ferrocyphen indicator.

The simplicity of the nitrite titration with an internal indicator likewise is recommended over the present USP XVIII analysis of procainamide hydrochloride injection. The latter requires time-consuming evaporation and heating prior to nonaqueous titration. With the nitrite titration, it is only necessary to boil a suitable aliquot of the injectable in 6 *N* hydrochloric acid for 1 min, followed by cooling to room temperature and then titrating. The boiling is required to remove the excipient bisulfite as sulfurous acid.

These considerations invalidate the present USP XVIII titration for procainamide hydrochloride and recommend its substitution by the nitrite titration at room temperature with internal ferrocyphen indicator. The latter might, for convenience and economy supplant the present nitrite titration with external indicator for procainamide hydrochloride capsules and the nonaqueous titration for procainamide hydrochloride injection.

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Colorimetric Determination of Cephradine, a Cephalosporin Antibiotic

JOEL KIRSCHBAUM

Abstract □ The quantitative colorimetric determination of microgram quantities of cephradine is detailed. An aqueous solution of this compound is reacted with sodium hydroxide, partially neutralized, and subsequently reacted with 5,5′-dithiobis(2-nitrobenzoic acid); the absorbance of the resulting yellow solution is then measured. Other cephalosporins tested give a similar color. Studies with various cephalosporins showed that the development of maximum color intensity was attributable to the presence of the R—$CHNH_2$—CO—cephalosporin nucleus, in which R is a mono-, di-, or trienyl cyclohexyl ring. Molar absorptivities of penicillin solutions tested by the same procedure were found to be 5–15% of those of the cephalosporins.

Keyphrases □ Cephradine—colorimetric analysis □ Cephalosporins and penicillins—colorimetric determination □ Penicillins and cephalosporins—colorimetric determination □ 5,5′-Dithiobis(2-nitrobenzoic acid)—reagent for colorimetric determination of cephalosporins and penicillins □ Colorimetry—analysis, cephalosporins and penicillins

This report describes a colorimetric method for the quantitative determination of cephradine (1), a cephalosporin antibiotic, utilizing treatment with base followed by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (I), a compound known to react with sulfhydryl groups. The procedure appears generally applicable to cephalosporins and penicillins.

EXPERIMENTAL

Reagents—The following were used: sodium hydroxide–sodium carbonate solution (dilute 20 ml of 0.5 *M* sodium carbonate and 8 ml of 10 *M* sodium hydroxide to 100 ml); 2 *N* sulfuric acid; 0.5 *M* sodium carbonate buffer, pH 9.2 ± 0.05; and 5,5′-dithiobis(2-nitrobenzoic acid)[1] (I) color reagent, 15.5 mg/50 ml of 95% ethanol (prepared fresh daily).

Procedure—Pipet 5 ml of an aqueous solution containing 30–600 μg of cephradine into a test tube. Then pipet 5 ml of sodium hydroxide–sodium carbonate solution. Cover the tube loosely and place it in a boiling water bath for 75 min. Rapidly cool the test tube to room temperature by immersing it in an ice bath, and then add 2 *N* sulfuric acid to adjust the contents to pH 9.2 ± 0.1 (pH meter). Transfer the solution to a 25-ml volumetric flask. Into the same flask, pipet first 10 ml of 0.5 *M* sodium carbonate buffer and then 3 ml of I color reagent. Dilute to volume with water and mix. Determine spectrophotometrically[2] the absorbance of the 412-nm peak between 5 and 20 min after addition of the color reagent, using a reagent blank for comparison.

RESULTS AND DISCUSSION

Beer's law is followed by solutions containing 1–30 μg of cephradine/ml (25–750 μg/25-ml flask). The sensitivity of this assay of 0.2 μg/ml indicates its utility, although the error of quantitation is inversely proportional to cephradine concentration. Base treatment with acetone, as described by Marrelli (2) for the estimation of cephalexin, another cephalosporin antibiotic, has a sensitivity of 0.1–2 mg/ml and does not follow Beer's law. Under the experimental conditions employed here, I is as sensitive to sulfide and sulfite (3) and to such mercaptans as cysteine and mercaptoethanol as it is to base-degraded cephradine. The thioether methionine does not interfere with the assay.

Related reagents that react with base-degraded and partially neutralized cephradine are 2,2′-dithiodipyridine and 4,4′-dithiodipyridine, but their maximal absorbance is in the same UV region of the spectrum as are the absorbance peaks of possible interfering substances, and the color development cannot be followed visually.

The results of the investigation of several variables of this procedure are discussed here.

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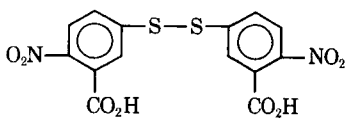

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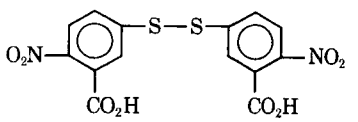

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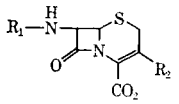
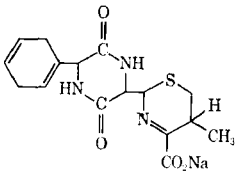
[1] Aldrich Chemical Co.

[2] A Beckman DU spectrophotometer was used.

Table I—Molar Absorptivities of Various Cephalosporins, Penicillins, and Related Compounds Carried through the Experimental Procedure

Compound	R_1	R_2	$\epsilon \times 10^{-4}$
			
Cephalosporins:			
I, cephradine	$\Delta^{1,4}$-C_6H_7—$CHNH_2$—CO—	—CH_3	1.30
II	Δ^{1}-C_6H_9—$CHNH_2$—CO—	—CH_3	1.31
III, cephalexin	C_6H_5—$CHNH_2$—CO—	—CH_3	1.29
IV, cephaloglycin	C_6H_5—$CHNH_2$—CO—	—CH_2OCOCH_3	1.28
V	C_6H_5—CH_2—CO—	—CH_3	0.53
VI, cephalothin	2(—C_4H_3S)—CH_2—CO—	—CH_2OCOCH_3	0.45
VII	C_6H_5—OCH_2—CO—	—CH_2OCOCH_3	0.39
VIII	C_6H_5—OCH_2—CO—	—CH_3	0.44
IX, 7-aminodesacetoxycephalosporanic acid	H—	—CH_3	0.11
X, 7-aminocephalosporanic acid	H—	—CH_2OCOCH_3	0.29
Penicillins:			
Methicillin			0.15
Oxacillin			0.22
Epicillin			0.08
Ampicillin			0.09
Penicillin G			0.08
Sodium cloxacillin			0.08
Phenoxymethyl penicillin			0.06
Related compounds:			
6-Aminopenicilloic acid			0.17
2-(1,4-Cyclohexadienyl)glycine			0.0
Phenylglycine			0.0
Penicillamine			0.12
XI			1.30



were less intense in color than those at pH 9.2. Above pH 9.2, the dilute carbonate interacted with the reagent to give an increased intensity of the reagent blank. When the pH remained 9.2, however, a fourfold increase in the carbonate concentration had no effect on color intensity, within the limits of 1% experimental error.

Effect of Concentration of Color Reagent—When the concentration was kept at 800 μg cephradine/25 ml, the absorbance increased as the volume of I color reagent was increased to 3 ml. Additional volumes of color reagent caused no increase in absorbance.

Effect of Time on Color Intensity—The color intensity reached a maximum within 5 min after the addition of the color reagent, remained constant for approximately 20 min, and then decreased slowly. In the single set of experiments performed, the faded colors of several samples were proportional to the diminished intensity of the standard. Waiting 3 hr after cooling the solutions, before proceeding with the remainder of the assay, also decreased the absorbance of both sample and standard, to about 60% of the original intensity.

Effect of Nitrogen—Bubbling nitrogen through the cephradine solution prior to and during degradation of the antibiotic with base had no effect on the intensity of the final color, within the limits of a 1% experimental error.

Effect of Distilled Water—The use of distilled water instead of deionized[3] distilled water resulted in a 3% reduction in absorbances.

Molar Absorptivities of Related Compounds—Table I lists the values for some cephalosporins, penicillins, and related compounds assayed as described under *Experimental*. The variables in the procedure were only investigated for cephradine.

The development of maximum color intensity appears to depend on the presence of the α-amino group in the cephalosporin side chain. This is also true for the procedure of Marrelli (2). However, subjecting cephradine to the procedure of Marrelli (base treatment in the presence of acetone) results in a peak absorbance at 500 nm, which is 40% that of cephalexin. The absorbance maximum of cephalexin is 520 nm.

The isolated base degradation product of cephradine, XI (4), also gives maximum color development. If it is assumed that the reaction with I involves a sulfhydryl group generated by the rupture of a C—S bond, the cephradine rearrangement compound, XI, most likely is an intermediate in this reaction.

This procedure is capable of producing reproducible results from day to day using bulk solids. The relative standard deviation of six samples analyzed on 2 days was 0.81%.

Two-milliliter samples of normal human serum and urine had absorbances of 0.60 and 0.75, respectively, when assayed by this procedure. Although the dilution of serum and urine samples containing cephradine would give correspondingly lower absorbances of the blanks, the extent of such dilutions would depend on the cephradine contents of the samples. The effect of such variables as diet, disease, and time of sample collection has not been determined. For these reasons, a separation of cephradine from a body fluid is recommended prior to analysis.

[3] A Barnstead-type HN cartridge demineralizer, mixed bed type, was used.

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PHARMACEUTICAL TECHNOLOGY

Comparison of Dissolution Profiles of Tablets and Capsules from the USP, Levy, and Magnetic Basket Methods

T. E. NEEDHAM, Jr.[x], and L. A. LUZZI

Abstract □ Single-batch lots of pentobarbituric acid tablets and sodium butabarbital capsules were dissolved in the USP, Levy beaker, and three different size magnetic basket dissolution apparatuses. Each method was compared using an analysis of variance and other statistical tests to ascertain if significantly different dissolution profiles were produced. Variation in terms of standard deviation of drug released by the different methods was also compared. The five different dissolution methods produced significantly different dissolution profiles at various selected times for both the tablets and capsules studied. However, differences in variations produced by the different dissolution methods upon repeated dissolution of either tablets or capsules seemed to be of the same order.

Keyphrases □ Dissolution profiles (pentobarbituric acid tablets and sodium butabarbital capsules)—comparison of USP, Levy beaker, and three different size magnetic basket apparatuses, statistical analysis □ Tablets, dissolution (pentobarbituric acid)—comparison of USP, Levy beaker, and magnetic basket apparatuses, statistical analysis □ Capsules, dissolution (sodium butabarbital)—comparison of USP, Levy beaker, and magnetic basket apparatuses, statistical analysis

The adoption by the compendia of an official dissolution apparatus (1) has produced much controversy for and against that method. Many new methods and adaptations of older methods have been reported (2). Usually each method has been presented with *in vitro* data to substantiate its effectiveness in following tablet and/or capsule dissolution as well as its ability to differentiate between common manufacturing variables. Many suggestions have been made to "improve" or replace the official method or to establish more than one official method. Some *in vitro* comparisons of the different dissolution methods also have been reported (3). However, little has been done to determine if the methods themselves actually produce significantly different dissolution profiles or if the variations seen in the dissolution apparatuses are of such magnitude as to produce overlapping curves that are essentially similar.

Table I—*F* Values for Pentobarbituric Acid Tablets at Selected Times

Minutes	*F* Value: Repeated Tablets	*F* Value: Different Dissolution Methods	$F(4,16)_{0.99}$
8	0.462	15.29	4.77
12	1.38	36.25	4.77
18	0.987	21.86	4.77
30	0.391	7.13	4.77

This study compared several different dissolution methods to determine if they produced significantly different dissolution profiles for a drug from the same dosage form. Variation in terms of standard deviation of drug released as produced by the different dissolution methods was compared, as well as the significant changes in drug availability caused by changing the impeller speed for both capsule and tablet dosage forms.

EXPERIMENTAL

Materials—Each tablet was formulated to contain 25 mg pentobarbituric acid[1], 236 mg fast-flow lactose[2], 43.5 mg microcrystalline cellulose[3], 35 mg starch[4], and 10.5 mg stearic acid[5]. Tablets were compressed using a 16-station rotary tablet press[6] equipped with an induced die feeder. Standard concave punches,

[1] Abbott Laboratories.
[2] Foremost Dairy, San Francisco, Calif.
[3] FMS Corp., Newark, Del.
[4] Ruger Chemical Co., Irving, N.J.
[5] Fisher Chemicals, Fairlawn, N.J.
[6] Model 216-RP, Cherry-Burrell.

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Table II—Average[a] Percent Dissolved and Standard Deviation of Pentobarbituric Acid Tablets for the Different Dissolution Methods at Selected Times

Dissolution Method	8 min		12 min		18 min		30 min	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Levy beaker	22.35	12.81	39.75	12.84	61.25	12.02	84.50	10.31
Small basket	24.13	5.42	32.31	4.39	42.18	4.93	59.24	5.64
Regular basket	30.55	5.85	47.22	15.36	59.36	15.61	74.08	13.27
Larger basket	50.28	16.52	59.24	4.11	74.30	3.76	91.18	2.53
USP method	70.51	14.61	91.96	3.76	92.13	1.85	95.64	3.31

[a] Each mean is the average of five tablets.

0.95 cm (0.37 in.), were used. When using an electronic hardness tester[7], the tablets were found to have a 8.6–9.0 range of hardness with an average of 8.8.

Commercially prepared capsules of sodium butabarbital[8] were purchased, since a previous publication (4) showed that capsules manufactured in an automated manner displayed less variance than hand-packed ones. All dissolution runs were done at 37° in 600 ml of a pH 2 buffer mixture consisting of hydrochloric acid and potassium chloride; unless otherwise stated, a propeller speed of 60 rpm was used.

Dissolution Methods—Tablets and capsules were dissolved in the modified Levy beaker (5–7), the official USP dissolution apparatus (1), and the magnetic basket dissolution apparatus (3, 4) using three types of magnetic baskets. The first basket (regular basket) conformed to the original specifications (4). The second (small mesh basket) conformed to the original specifications but used 16-mesh instead of the original 8-mesh stainless steel wire. The third basket (bigger basket) was constructed using 8-mesh wire but having an inner diameter of 12 mm and a length of 38 mm instead of the 11 × 25-mm dimensions of the original basket.

Analysis—The weight of each tablet or capsule was determined prior to dissolution, and the amount dissolved at any time t, reported as a percent of the total drug, was determined at 240 nm using the appropriate blanks. Each dissolution profile is the average of five tablets or capsules to provide a similar basis of comparison between methods.

RESULTS AND DISCUSSION

The dissolution rate profiles for pentobarbituric acid tablets at pH 2.0 are shown in Fig. 1. Although the same formulation and hardness were used in each case, a different dissolution pattern was found for each dissolution method, with the official USP method allowing the most rapid drug release. To evaluate these dissolution or availability profiles, the following questions must be considered:

1. Do the methods studied produce significantly different profiles?
2. In a series of tablets from the same batch, is the variation a function of the methods used or of the tablets themselves?

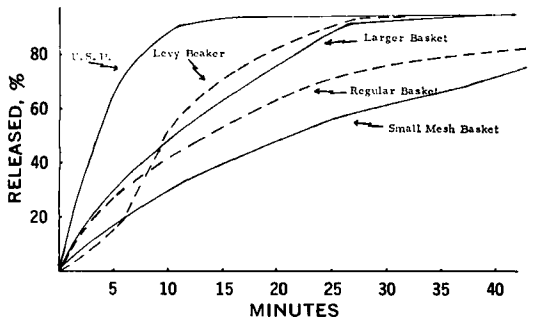

Figure 1—*Comparison of the dissolution rates of pentobarbituric acid tablets using the different dissolution apparatuses.*

[7] Erweka Electronic.
[8] McNeil Laboratories.

These factors can be partially analyzed using a two-way classification of an analysis of variance (ANOV) which, in this case, will compare at a given point in time the difference or nondifference in percent released between dissolution methods and between different tablets. By using data in the form of percent drug released for five different pentobarbituric acid tablets for each of the five different methods, an analysis of variance was calculated at 8, 12, 18, and 30 min and used as representative of the entire dissolution profile. Table I shows that at all of the times studied the calculated F value for the different dissolution methods exceeds the tabular F value at the 0.99 level. This leads to the conclusion that at each of the times studied, a statistical difference was evident between some of the dissolution methods.

Comparison of the individual dissolution methods to determine which apparatuses were producing significantly different release was accomplished using the Newman–Keuls test (8). Analysis of the values of percent pentobarbituric acid released (Table II) indicates that at 8 and 12 min the Levy beaker method was significantly different from the larger basket or the USP method but not from the regular or smaller mesh basket. The smaller mesh basket was also significantly different in the average amount of drug released from the larger basket and USP methods, while the regular basket showed a significant difference only from the USP method.

The relationship changed somewhat at 18 min, with the average amount of drug released from the smaller mesh basket differing from the Levy beaker which, in turn, showed a significantly different drug release from that of the USP method. In fact, the release from the smaller mesh basket was low enough at an average of 42.18% to be significantly different from the release seen in the Levy, larger basket, and USP methods. Again the regular basket showed a different release from that seen in the USP dissolution apparatus. At 30 min, as the tablets approached complete dissolution, a smaller difference was seen among the methods. The Levy, regular basket, larger basket, and USP methods showed no difference from each other, while the smaller mesh basket was significantly different from the Levy, larger basket, and USP methods but not from the regular basket method.

It can be seen from this analysis that tablets from the same batch will release a significantly different amount of drug at various times throughout an entire dissolution when different dissolution apparatuses are used.

The variation between different tablets from the same manufactured batch, which is usually reported as standard deviation in the percent released from a series of dissolved tablets, can be attributed to several sources. These differences can usually be

Table III—F Values for Sodium Butabarbital Capsules at Selected Times

Minutes	F Value		$F(4,16)_{0.99}$
	Repeated Capsules	Different Dissolution Methods	
8	0.608	1.23	4.77
10	0.901	0.997	4.77
30	0.680	5.80	4.77
80	0.317	6.45	4.77
100	1.05	5.66	4.77
120	0.905	3.22	4.77

Table IV—Average[a] Percent Dissolved and Standard Deviation of Sodium Butabarbital Capsules for the Different Dissolution Methods at Selected Times

Dissolution Method	8 min		10 min		30 min		80 min		100 min		120 min	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Levy beaker	24.37	16.40	27.41	5.33	52.71	10.06	77.36	13.05	84.47	14.15	88.76	13.57
Small basket	16.56	2.72	21.97	6.78	42.27	3.01	68.49	2.18	71.34	2.18	79.22	4.18
Regular basket	27.12	5.60	31.33	5.14	51.49	6.45	77.18	2.01	84.89	4.77	90.49	5.56
Larger basket	21.74	9.27	22.04	17.67	51.54	9.97	87.17	8.31	84.15	5.93	92.74	5.31
USP method	19.20	5.88	32.17	16.94	65.39	4.91	93.51	6.81	94.45	5.70	94.97	5.38

[a] Each mean is the average of five tablets.

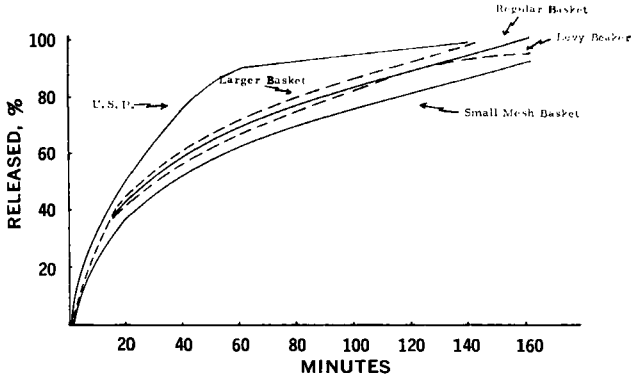

Figure 2—*Comparison of the dissolution rates of sodium butabarbital capsules using the different dissolution apparatuses.*

traced to the manufacturing procedure, variation in the sampling and analytical procedure, and variation produced by the dissolution methods. In this case, since all tablets were from the same manufactured batch and were sampled and analyzed in the same manner, this part of tablet variation should have remained constant during each method. Therefore, differences in tablet variations could be traced to that component contributed by the dissolution method.

Table I shows that the calculated F value from an analysis of variance for the repeated dissolution of tablets in all of the dissolution methods tested is significantly less than the standard F value at 0.99 level of 4.77. This leads to the acceptance of the null hypothesis that all variance between tablets is homogeneous. Further comparison can be made using the mean and standard deviation for each dissolution method at 8, 12, 18, or 30 min (Table II). At each time interval, a varying range of standard deviations was seen, which would seemingly indicate differences in variation among the methods. However, using the Cochran C test (9) to compare homogeneity of variance at each time provided slightly different values. At 8, 12, 18, and 30 min, the calculated C values were 0.2998, 0.3797, 0.4090, and 0.3786, respectively, while the tabular $C(5,4)_{0.99}$ was 0.6329. Therefore, both of these methods indicate that the compiled variation from the three sources is not significantly different in any of the five methods studied and that the variation between tablets caused by each method is quite similar.

Figure 2 illustrates the release of drug from sodium butabarbital capsules using the five dissolution methods previously described. Table III shows the results of a two-way classification of an analysis of variance at 8, 10, 30, 80, 100, and 120 min. At 8 and 10 min, the calculated F values of 1.23 and 0.997, respectively, are less than $F(4,16)_{0.99}$ of 4.77, indicating that there is no significant difference among the methods at these two times. However, the percent drug released at 30, 80, and 100 min seems to show a greater divergence among the dissolution methods. This visual observation is confirmed by an analysis of variance. At these three times, the calculated F value exceeds the tabular F in all cases. At 120 min the lines start to converge, and further calcula-

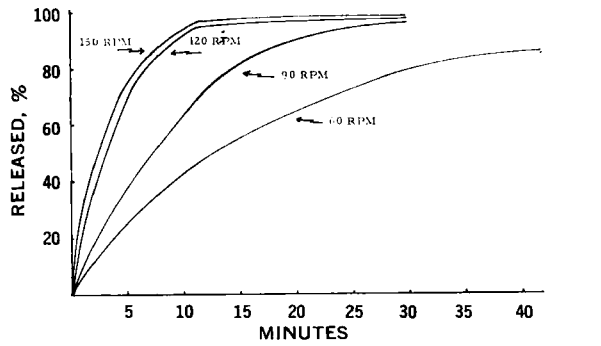

Figure 3—*Comparison of the dissolution rates of pentobarbituric acid tablets as a function of propeller speed using the regular magnetic basket dissolution apparatus.*

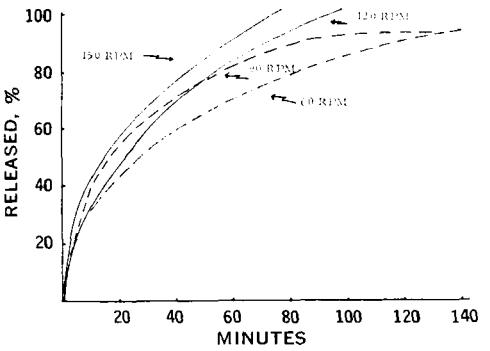

Figure 4—*Comparison of the dissolution rates of sodium butabarbital capsules as a function of propeller speed using the regular magnetic basket dissolution apparatus.*

tion reveals that the differences among the methods are no longer significant.

The average percent of drug released for each of the five dissolution methods at the three times found to be significant in the analysis of variance evaluation were compared using the Newman–Keuls sequential range test (8) to determine which dissolution methods were significantly different from the USP method while none of the other methods in any combination showed a difference in drug dissolution. With 80 min elapsed in the dissolution, the smaller mesh basket, the regular basket, and the Levy beaker methods were all significantly different from the USP method, but none of the other methods in any combination showed a difference in drug release. At 100 min, the results were similar to those seen at 80 min, with only the smaller mesh basket showing a significant difference from the USP method in the amount of drug released. Therefore, significant differences in drug dissolved from capsules from the same commercial lot number can be calculated for the five different dissolution methods studied. However, analysis of the entire dissolution profile is necessary to select the time at which these differences are significant.

The variation between the capsules due to the inherent variation in the manufacturing procedure, the assay procedure, or the dissolution methods should also be considered. In Table III the calculated F values for repeated dissolution of capsules at all of the times analyzed are definitely less than the tabular F value of 4.77. The homogeneity of variance may also be compared by using the standard deviations at each time interval from Table IV and applying the Cochran C test (9). Since at 8, 10, 30, 80, 100, and 120 min the calculated C values were 0.411, 0.341, 0.292, 0.403, 0.399, and 0.432, respectively, while the tabular value of $C(5,4)_{0.99}$ was 0.6329, all the variance between capsules was considered to be homogeneous. Since, as previously mentioned, the manufacturing, sampling, and assay variations are common to all methods, the lack of any significant difference in variation in either test indicates that the deviations due to the dissolution methods are of the same order.

Another factor that may affect the release of drug from a dosage form in each dissolution method is propeller speed. Figure 3 illustrates the differences in the dissolution profiles for the pentobarbituric acid tablets in the regular basket of the magnetic basket dissolution apparatus as a function of propeller revolutions per minute. The results agree with those of Levy (6) and Levy *et al.* (10) in that an increase in propeller speed provides an increase in drug release up to a maximum. An analysis of variance comparing the percent of drug released at 8 min *versus* revolutions per minute shows a significantly different dissolution, with the calculated F value equal to 26.74 and the tabular $F(3,16)_{0.99}$ equal to 5.29. The dissolution curves compared singly against each other using the Newman–Keuls sequential range test (8) show that the amount of drug released at 60 rpm is significantly different from that released at 120 and 150 rpm but not at 90 rpm. Furthermore, no difference can be seen between the average amount of drug released at 90, 120, and 150 rpm.

Figure 4 shows the dissolution of the sodium butabarbital capsules in the regular basket of the magnetic basket dissolution apparatus as a function of propeller speed. Upon increasing the revolutions per minute from 60 to 90 to 120 to 150, little difference in the release rate is seen. An analysis of variance at 8 min gives a calculated F value of 1.43 which, when compared to the tabular $F(3,16)_{0.99}$ of 5.29, shows no difference in the average amount of drug released at that time. If a comparison is made at 40 min where visual inspection shows a divergence of the dissolution profiles, the calculated analysis of variance increases to 3.37, which is still less than that necessary to show a significant difference in drug released at the different speeds.

In summary, the profiles produced by the five different dissolution methods for those tablets were significantly different at various selected times throughout the entire dissolution. The tablets produced a more significant difference between more of the methods at more times during the dissolution than did the capsules. The differences in variation produced by the different dissolution methods upon repeated dissolution of either tablets or capsules seemed to be of the same order.

The selection of an *in vitro* method to follow tablet and capsule dissolution should be dependent on the *in vivo* correlation desired or the quality control standard to be maintained because, although significant differences can be seen among the dissolution methods, each produces the same relative accuracy.

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* To whom inquiries should be directed.

Automated Peak Discrimination and Integration for Nonflame Atomic Absorption Analysis at Nanogram Levels

J. R. SARBECK and W. C. LANDGRAF[x]

Abstract □ A system for automated integration of nonflame atomic absorption signals is presented with improvements in the limits of detection reported for several typical elements. Theoretical considerations are developed to explain improvements in the signal-to-noise ratio as a function of high frequency noise rejection and filtering. Linearity of integration response curves is related to conventional peak height measurement.

Keyphrases □ Atomic absorption—automated integration of nonflame signals, improvements in detection limits □ Metal ions—automated integration of nonflame atomic absorption signals, improvements in detection limits for several metals

Recent studies in this laboratory indicated a good correlation between trace levels of transition metals and their influence on the degradation of chemical systems in use in the pharmaceutical industry. Frequently, one views these processes as being metal-ion catalysis of either an oxidative or a solvolytic nature, resulting in degradation of one or more compounds in that system. These conclusions resulted in a program of study for the determination of specific ions at low levels employing atomic absorption techniques. The principal objectives of this program were to simplify analytical procedures and to improve the limits of detection by atomic absorption techniques.

DISCUSSION

During the past several years, considerable effort has been expended for the improvement of higher levels of the ground-state species in atomic absorption analysis. Excellent use has been made of nonflame or graphite furnace techniques to increase significantly the population levels for ground-state atoms in atomic absorption analysis and to decrease extraneous species and other sources of noise. The use of nonflame methods for atomization has resulted in increases of two and sometimes three orders of magnitude (1-3). The improvement in radiation sources such as electrodeless discharge lamps has also resulted in better performance, especially when combined with nonflame furnace techniques (4-6).

A further improvement in overall signal-to-noise (S/N) ratios can be achieved by examining averaging techniques or perhaps by electronic data processing. A detailed discussion of possible methods including filtering, averaging, and linear transforms of signals was presented by Ernst (7). The distinction made by Ernst relates to the concept that filtering primarily affects the noise, leaving the signal undisturbed. Conversely, a transform of this signal does produce a significant change in the total signal, requiring further data processing. The accuracy of measurement of an integral for an absorption signal is limited by random noise, particularly its low frequency components (baseline stabilities). The integration process acts as a low bandpass filter and tends to eliminate high frequency components.

Noise is frequently considered to be a random function of time, $n(t)$, whose amplitude and duration are unknown. Past history of these noise components will not permit one to predict its future values; therefore, statistical methods are used to predict noise (or its components) in a signal. The probability distribution function, $P(t)$, specifies the likelihood that $n(t)$ will assume a value in the interval t_0 to $(t_0 + dt)$. A stationary random function is characterized by a probability distribution function independent of time whose amplitude values are normally distributed; then $n(t)$ is equated to a Gaussian random function whose properties are described by average values. To secure the average, a sufficiently large number of samples in time is required. The time average of the quantity Q is $\equiv Q[n(t)]$; for a random function, $n(t)$ is defined by $\langle Q[n(t)]_{av}\rangle \lim_{T\to\infty} (\frac{1}{2}T) \int_{-T}^{T} Qn(t)\,dt$. This ensemble average is obtained ideally from an infinite number of random functions, $n_1(t)$, $n_2(t)$, ... (having some common property). This ensemble average of the quantity Q at time t is defined by the average over the instantaneous values of $Q[n(t)]$ for different members, $N(t)$, of that ensemble and is expressed as follows:

$$\overline{Qn(t)} = \lim_{N\to\infty} (1/N) \sum_{i=1}^{N} Q[n_i(t)] \qquad \text{(Eq. 1)}$$

In many cases, it is permitted to replace the time average by the ensemble average or the converse. Such random functions for which time and the ensemble average can be equated are referred to as ergodic random functions. Since digital methods are considerably more accurate in the counting sense, the concept of ensemble average counting can be readily assigned to a minicomputer. Through a selection of the appropriate time windows, one can secure a good ensemble averaging of the noise and the signal. Furthermore, the selection and use of an appropriate algorithm permit discrimination against spurious signals either superimposed on the desired data or nonconcurrent with the signal.

Table I—Detection Limits (Absolute Picograms)

Element	Reference 9	Reference 10	Reference 11	This Method
Ag	0.5	0.2	0.2	0.2
Cd	0.6	0.1	0.1	0.01
Cr	—	5.0	5.0	4.0
Cu	6.0	7.0	1.0	0.5
Hg	400.0	100.0	—	40.0
Ni	—	10.0	10.0	2.0
Pb	20.0	5.0	6.0	1.0

EXPERIMENTAL

The atomic absorption analyses were performed on a spectrophotometer[1], employing standard hollow cathode lamps as wavelength sources. The atomization of the elements was performed by a carbon rod atomizer[2]. The output of the photomultiplier detector was coupled directly into the interface of a unit[3] where the A/D (analog-to-digital) conversion was completed. Since the carbon rod atomizer has three independent operating cycles, one can readily vary each independently for its optimum performance. The atomization cycle can be varied for 0-10 sec in either the pulse or ramp mode. In the present work the pulse mode of operation was used exclusively, with 2 sec being the typical operating cycle time for this parameter.

The operational sequence is as follows. Standard solutions of the metal of interest (Fisher certified atomic absorption standards) are diluted with water (previously shown to be essentially free of the metal). Dilution of the standard is such that the limiting, detectable amount is contained in 1-5 μl of solution. A 1-5-μl portion of this solution is transferred to the carbon rod using a microliter syringe equipped with an inert plastic tip. Thereafter, the automatic sequence is initiated.

[1] Perkin-Elmer model 305.
[2] Varian Techtron model 63.

Table II—S/N Improvement *versus* Wavelength

Element	Wavelength, nm	Relative Improvement
Cd	228.8	10
Ni	232.0	20
Hg	253.8	2.5
Pb	283.3	10
Cu	324.7	10
Ag	328.1	5
Cr	357.9	1.25

The resulting ground-state population is measured directly by the photomultiplier and integrated[3]. For descriptive purposes, the peak absorption due to the carbon rod atomizer can be envisaged to be triangular in shape (8) with a rapid exponential decay of this peak. Since this parameter is perhaps the most critical element in the operating sequence, it was optimized with considerable care (Fig. 1). Furthermore, the capability of precisely selecting the onset of this signal and terminating its integration was used judiciously. This ability to discriminate against spuriously generated noise and nonpertinent signals adds greatly to the increased S/N ratios of this combination. An additional feature of this unit is the ability to reject significant noise "spikes" in the signal while processing the data.

RESULTS

The limit of detection for this work is defined as the amount of analyte that produces an integrated signal area equal to twice the average area for the noise peaks occurring during the atomization step. In general, results reported are for the average of at least six S/N pairs.

For an indication of the limits for detection, a table of data was collected to show how the current method compares to previously reported data on a number of selected elements. As Table I illustrates, the lowest limit for detection is not uniformly improved by this technique; some special source of noise may be involved for these determinations. Table II gives an indication of the relative improvement in lowest detection levels permitted by this operational sequence.

Several points are clearly evident upon closer inspection of these data. With the exception of the mercury data, one sees a general inverse trend for relative improvement in S/N ratios as the wavelength for detection increases. This tail-off in sensitivity improvement may be a reflection of the increase in emission for the carbon rod at longer wavelengths. One may envisage the carbon rod as being equivalent to a blackbody where significant radiation increases beyond the 350-nm wavelength region. This type of radiation constitutes "background" or "scatter" radiation and may define a lower limit for detection of elements in the regions of longer wavelength (6)—*e.g.*, calcium, sodium, and potassium—

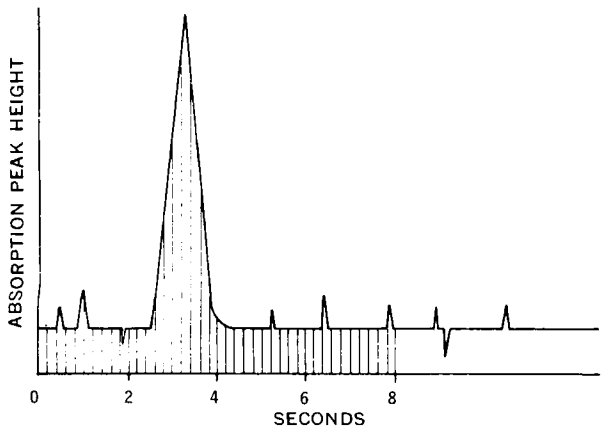

Figure 1—*Typical absorption peak; example of data bunch rate. Selected peak width = 2 sec. Bunch rate = 5/sec.*

[3] Spectra-Physics Autolab IV.

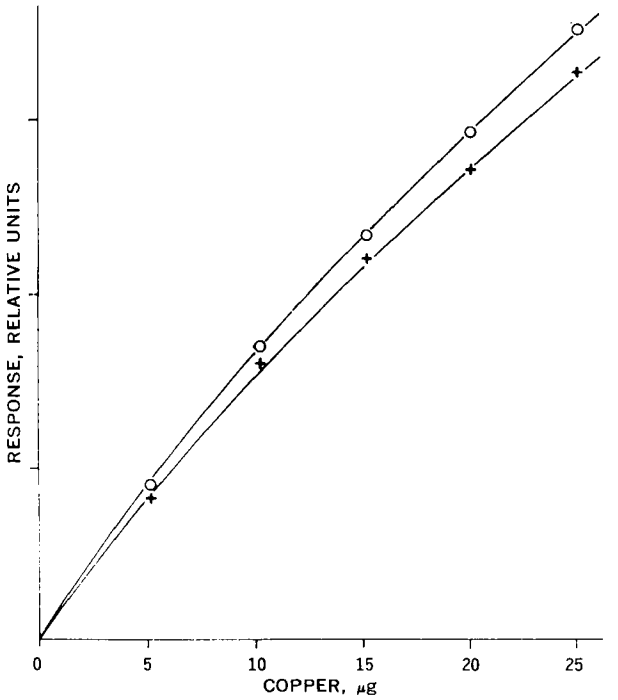

Figure 2—*Analysis of copper samples by peak area method* (O) *and peak height method* (+).

since neither integration nor peak noise rejection eliminates this condition.

Another point relates to the lower relative improvement in the current method for mercury detection. This problem may reflect the idea that with carbon rod atomization, the ground-state residence time of the mercury atom in the atomizer is relatively short and the current time window is not optimized. This parameter could be improved by choosing a lower atomization temperature by decreasing the inert gas flow (6) through an optimization of the integration time window or by selecting a faster A/D convertor where the 100-msec limit is no longer a serious handicap for integration purposes.

The aspect of linearity for the analytical curves obtained by the integration method previously described was compared to data obtained by simple peak height measurements. A series of subnanogram level samples of copper was evaluated by a comparison of integration and peak amplitude information recorded simultaneously (Fig. 2). In terms of linear response, the slight difference between the two curves is attributed to scaling factors for assigning graphical values. This similarity of the data lends support to the concept that the peaks are approximated by triangles with short and constant bases (8).

The described system has been routinely applied to the determination of iron in propylene glycol. The application is straightforward and allows for increased sensitivity as well as decreased analysis time due to the automation of peak discrimination and data acquisition.

Further studies are designed to investigate peak shapes and the factors influencing them as a means of improving S/N ratios. A study of different elements using the current technique also will be undertaken in extreme regions of the atomic absorption spectrum.

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Factors Affecting Water Vapor Transmission through Polymer Films Applied to Solid Surfaces

ANTON H. AMANN*, RICHARD E. LINDSTROM, and JAMES SWARBRICK[x]

Abstract □ The permeation of water vapor through cellulose acetate phthalate polymer films applied in a variety of ways to hygroscopic solid surfaces was determined at several different temperatures. The systems investigated consisted of films: *(a)* placed against a flat solid surface, *(b)* sprayed onto a flat solid surface, and *(c)* sprayed onto tablets in a rotating coating pan. The data indicate that the activation energy of the absorption process is determined by the film, while the method of film application affects the actual rates of permeation at a given temperature. The physical properties of the material being coated also affect the water vapor absorption rate.

Keyphrases □ Films, cellulose acetate phthalate polymer—water vapor permeation on hygroscopic solid surfaces, comparison of application methods and temperatures □ Permeation, water vapor—polymer films applied to solid surfaces, effects of temperature and application method □ Absorption, water vapor—solid dosage forms, effects of film application method and film material

A previous paper (1) presented the relative characteristics of water vapor permeation through free-standing hydrophilic and lipophilic polymer films used to coat pharmaceutical solid dosage forms. It was shown that the permeation coefficients for the lipophilic films were unaltered by the presence of water vapor on the distal surface of the film. In contrast, water vapor permeation through the hydrophilic films was dependent on whether the vapor pressure gradient resulted from water vapor on one or both sides of the film. This behavior was rationalized on the basis of plasticization of the film by the permeating water vapor. With water vapor present on only one side of the film, dehydration was postulated to occur. This would reduce permeation, an effect noted in earlier work (2).

The present report extends previous work to include the water vapor permeation through hydrophilic cellulose acetate phthalate films when applied to the surface of solid systems having a potential to absorb moisture. In this manner, it was hoped to evaluate the relationship between free-standing films and those applied to solid dosage forms in terms of their water vapor permeability properties. Several model systems were thus established to create a series of conditions intermediate between permeation through a free film and one applied to a tablet. Accordingly, moisture permeation was studied in systems in which: *(a)* a film of cellulose acetate phthalate was simply placed in contact with a flat disk containing calcium chloride; *(b)* the same polymer, in solution, was sprayed onto one surface of a flat disk containing calcium chloride; and *(c)* the polymer solution was used to spray coat the entire surface of conventionally shaped convex tablets containing calcium chloride.

Table I—Water Vapor Permeation through Various Applied Films of Cellulose Acetate Phthalate[a]

System	Temperature	Permeability Coefficient P^0, g hr^{-1} cm^{-1} mm Hg^{-1} $\times 10^{-7}$
Cast film placed onto disk	20°	4.12 ± 0.71[b]
	30°	3.54 ± 0.83
	40°	2.37 ± 0.22
Sprayed film on disk	20°	2.64 ± 0.11
	30°	1.99 ± 0.18
	40°	1.52 ± 0.13
Film-coated tablet	20°	0.31 ± 0.06
	30°	0.18 ± 0.07
	40°	0.09 ± 0.02

[a] Vapor pressure of 16.5 mm Hg. [b] A 95% confidence interval.

EXPERIMENTAL

Preparation of Disks and Tablets Containing Calcium Chloride—The powder mixture used for compression contained calcium chloride USP (33%) in microcrystalline cellulose[1]. Magnesium stearate (1%) was present as a lubricant. The compressed disks were prepared using flat-faced punches and die, 1.60 cm in diameter, and a press[2] set at 1816 kg (4000 lb). Under these conditions, the formation pressure was approximately 13,000 psi. The average weight of the disks was 1.586 ± 0.001 g.

Tablets were prepared from the same powder mixture using a single-punch tablet press[3] fitted with deep concave punches, 0.96

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[2] Carver.
[3] Stokes model E.

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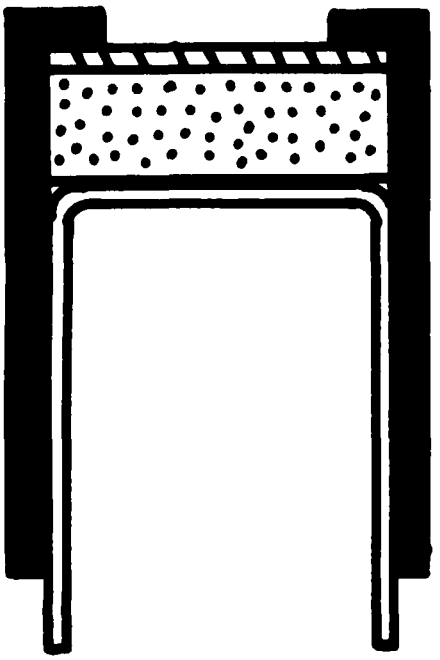

Figure 1—*Cross section of device for determining permeation through film applied to solid disk. Key:* ▧, *polymer film;* ▨, *calcium chloride disk;* ■, *rubber sleeve; and* □, *inverted glass cup.*

cm in diameter. The compression pressure was approximately 35,000 psi, the average weight was 0.281 ± 0.007 g, and the average tablet hardness was 6.2 ± 1.6 kg.

Preparation of Cellulose Acetate Phthalate Films—The compositions of the polymer solutions used in the formation of cast and sprayed films were as reported previously (1).

Spray Coating of Disks and Tablets—The flat calcium chloride disks were mounted onto glass plates by means of adhesive tape on the back of the disk. With the plate in a vertical position, the entire plate was sprayed with polymer solution from a distance of 15 cm. A forced-air spray gun was used, with the spray nozzle adjusted to give a pattern 2.54 cm wide at a distance of 15 cm when operating at a pressure of 2 psi. In this manner, a uniform film coat was applied to one surface of the disks as well as to the exposed surface of the glass plate between the disks. After drying overnight, the disks were removed.

One thousand calcium chloride tablets were placed in a 20-cm diameter coating pan, rotating at 35 rpm. Cold air was blown over the tablet bed to remove any surface dust. With the spray gun orifice set at "wide" and under a pressure of 2 psi, sufficient coating solution was sprayed on until "twinning" of the tablets was observed. Hot air was then blown into the pan to aid in the removal of solvent. This sequence was repeated until the desired thickness of film coat had been applied.

Determination of Film Thickness and Exposed Area—Film thickness was determined by means of calipers. With the disks, this was taken as the increase in thickness before and after spraying. With the tablets, half of the increase in overall thickness was held to be equivalent to the thickness of the film coat.

With the disks, the diameter of the circular aperture of the device shown in Fig. 1 was measured prior to uptake studies. The surface area of the film-coated tablets was computed from the dimensions (wheel diameter, height of curved surface, diameter, and height of flat edge) of the tablet.

Moisture Permeation of Cellulose Acetate Phthalate Films—A calcium chloride disk, with either an applied cast film or a sprayed film, was placed in the device shown in Fig. 1 and positioned in a temperature-controlled oven containing a single-pan analytical balance. Five disks were used for each run. The vapor pressure within the oven was maintained at 16.5 mm Hg. The steady-state rate of water uptake was determined as described elsewhere (1). Blank runs were undertaken using aluminum disks in place of the polymer film to confirm that weight increases were due solely to water permeation across the exposed area of the film.

Batches of tablets were also studied under similar conditions of temperature and vapor pressure over the requisite period of time. In this case, however, the tablets were simply placed in the oven.

The permeability coefficient, P, was calculated as described previously (1), as was P^0, the permeability coefficient at zero film thickness.

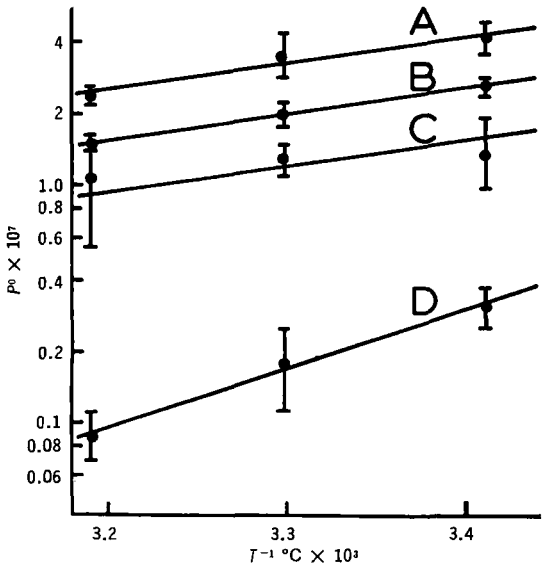

Figure 2—*Semilogarithmic plot of* P^0 versus $1/T$ *for the systems studied. Key: A, cast film placed against disk; B, film sprayed onto disk; C, free film with moisture on one side (1); and D, film sprayed onto tablets.*

Table II—Water Vapor Absorption of Uncoated Disks and Tablets Containing Calcium Chloride (33%) in Microcrystalline Cellulose[a]

System	Temperature	Rate of Water Vapor Absorption, g hr^{-1} cm^{-2} mm $Hg^{-1} \times 10^{-4}$
Uncoated disks	20°	14.8
	25°	9.8
	30°	5.3
Uncoated tablets	20°	0.29
	30°	0.25
	40°	0.18

[a] Vapor pressure of 16.5 mm Hg.

RESULTS AND DISCUSSION

The permeation results obtained with the three systems investigated are listed in Tables I and II. Permeation coefficients are plotted *versus* $1/T$ in Fig. 2. Data from free films (1) are included for comparison. Of interest in Fig. 2 is the degree of parallelism between the permeability of precast films placed against disks (A), spray-coated disks (B), and free precast films with moisture on one side (C). These Arrhenius-type plots suggest that: *(a)* the activation energy for the absorption process, *Ea*, is determined by the film, and *(b)* the actual rates of permeation at any given temperature depend on the method of film application. This latter contention is compatible with the observation made with free-standing hydrophilic polymer films, namely that the rate of water vapor permeation increases with the amount of moisture on the distal film surface (1). With systems where the film is applied to a solid surface, the distal surface is the internal one. Thus, while the permeating vapor is able to diffuse from the distal surface of the free-standing film, it is less able to do so when the film is applied to the tablet or disk surface. As a result, variations in the

method of film application may lead to differences in the steady-state accumulation of moisture at the distal surface. This, in turn, would alter the film's permeation characteristics through plasticization. The data in Fig. 2 suggest that the more intimate the contact between the film and the tablet and/or disk, the less the degree of moisture accumulation and the lower the plasticizing effect.

A second important factor relating to moisture uptake by films coated on disks or tablets is seen in Fig. 2. When contrasting the plot for the film sprayed onto the disk made with the press (B) with that for the tablet (D) made on the single-punch tablet press, it is apparent that the energy of activation varies with the character of the material being coated. Thus, the disks were prepared under approximately 13,000 psi pressure while the tablets were formed under 35,000 psi pressure. This difference is further emphasized in Table II, which gives the rate of water vapor uptake by the uncoated disks and tablets. The rate of water vapor uptake is approximately 20–50 times faster and obviously more temperature dependent for the disks than for the tablets.

Over the pressure range studied, it is reasonable to expect that the disks would have a higher porosity and specific surface than the tablets. This would account for the more rapid uptake of moisture by the disks. Assuming that the specific surface and porosity of the compressed tablet are critical and, hence, rate limiting, one would expect the moisture uptake by the disks to become related increasingly to the temperature-dependent rate of water vapor condensation. When a film is applied, however, the situation changes. The rate of water absorption by the disks, which in the uncoated state are able to absorb water considerably faster at all temperatures, is largely controlled by the film. Thus the activation energy for the spray-coated disks (B) is the same as that for the free films (C).

The coated tablets, however, present two barriers to the water permeation and absorption process. The first is due to the film. The second is the relatively low specific surface and low porosity interface of the compressed tablet. In comparison to the disk, the passage of water molecules into the tablet is restricted and the activation energy becomes a function of both factors. In support of this argument, the energy of activation for the water vapor absorption process with coated tablets is 11.9 kcal/mole, in reasonable agreement with the sum of the activation energies for permeation of the film (5.3 kcal/mole) and the rate of water uptake by the uncoated tablet (5.5 kcal/mole).

In conclusion, the results of this study indicate that the rate of water vapor absorption by solid dosage forms, film coated with a hydrophilic powder, is determined by the method of film application and the physical characteristics of the coated material. The data suggest that, with this type of film, the rate of absorption may be decreased by ensuring that the film is in intimate contact with the dosage form and by compressing the tablet to the maximum degree feasible compatible with disintegration considerations.

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* Present address: Abbott Laboratories, North Chicago, IL 60064

ˣ To whom inquiries should be directed. Present address: Sterling-Winthrop Research Institute, Rensselaer, NY 12144

Flexure Test for Determination of Tablet Tensile Strength

S. T. DAVID* and L. L. AUGSBURGERˣ

Abstract □ A new method for determining the tensile strength of tablets is proposed utilizing two fulcrums and a knife edge. The fulcrum and knife edge pieces were affixed to the platens of a motorized tablet hardness tester. The new method was found to give rapid, reproducible results which agreed well with tensile strength values obtained from diametral compression. In contradistinction to diametral compression, tablets invariably failed in tension using the new method, as evidenced by uniform splitting into halves.

Keyphrases □ Tensile strength, tablets—new flexure test, apparatus and equations □ Tablets, tensile strength—new flexure test for determination, apparatus and equations □ Flexure test—determination of tablet tensile strength, compared to diametral compression method, apparatus and equations

Several approaches have been utilized in the evaluation of tablet strength. Some of the more popular designations for values relating to the strength of tablets are bending resistance (1), crushing force (2), and tensile strength (3–7). Of these, tensile strength is of more fundamental importance because it is independent of tablet dimensions and provides a measure of the inherent strength of the compacted material (6, 7).

DISCUSSION

Fell and Newton (3, 4) determined tensile strength using a diametral compression test similar to that commonly used to determine tablet crushing strength where tablets are compressed between two flat platens. If the tablets fail in tension, tensile strength may be computed from:

$$\sigma_0 = \frac{2P}{\pi D t} \qquad \text{(Eq. 1)}$$

where σ_0 is the tensile strength, P is the applied load, D is the tablet diameter, and t is the tablet thickness. Based on elastic theory, the derivation of this equation may be found in standard texts (8–10). In general, this involves a consideration of radial pressures and concentrated loads acting along a diameter of a circular disk and an analysis of the state of stress in the circular disk in terms of rectangular stress components. The principal assumptions are that the disk obeys Hooke's law, that the disk is isotropic, and that the modulus of elasticity in tension and compression is the same. For the vertical central section, which would represent the section in a flat-faced tablet where failure takes place, compressive stresses are at a minimum at the center of the load diameter and infinite at the extremities where the

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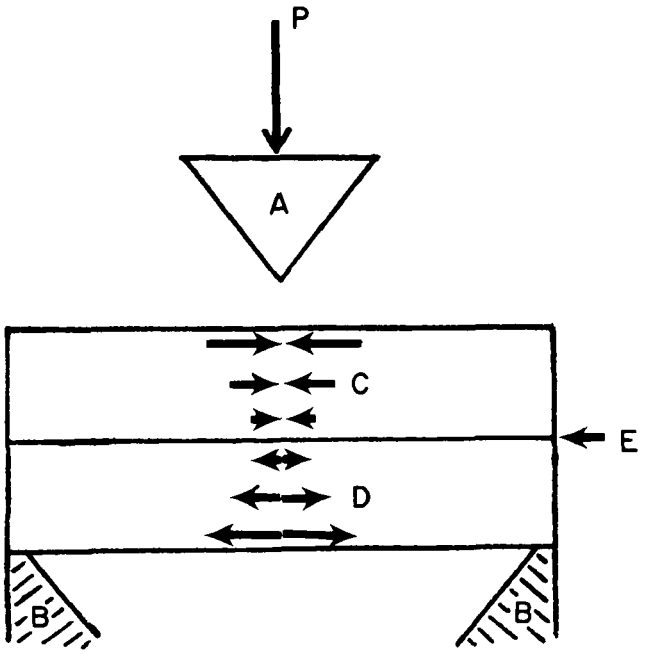

Figure 1—*Schematic drawing showing a vertical, central section of a tablet. Key: A, straight edge; B, fulcrums; C, compression; D, tension; E, neutral plane; and P, load.*

load is being applied, and a uniform tensile stress exists along the load diameter. Under the theoretical conditions of ideal line loading, these high compressive stresses and resulting high shear stresses prevent the initiation of failure in tension. In actual practice, however, failure in tension is possible because the load is distributed over an actual area of contact, thereby reducing the magnitude of the compressive and shear stresses at the extremities of the load diameter.

An alternative approach, proposed here, to the measurement of tensile strength in the plane described by Fell and Newton is to fracture the tablet by means of a straight edge in a flexure or bending test. The tablet is supported at two extreme ends of one of its flat faces and a knife edge, extending the width of the tablet on the opposite face (Fig. 1) is caused to exert an increasing load on the center of the tablet until it fractures. When a load is applied on the top face at the center and uniformly through the diameter of the tablet, the vertical, central plane feels both compression and tension (Fig. 1). The top half experiences compression while the lower half experiences tension. Therefore, an imaginary plane (E) can be drawn through the center of the tablet parallel to the tablet faces which feels neither compression nor tension. This is called the neutral plane. The neutral axis (N, Fig. 2), located at the center of the neutral plane, coincides with the tablet diameter and is parallel to the knife edge applied to the top tablet face. Compression and tension in the vertical cross section, in which the neutral axis is at the center, are assumed equal in magnitude but of opposite signs. The other main assumptions

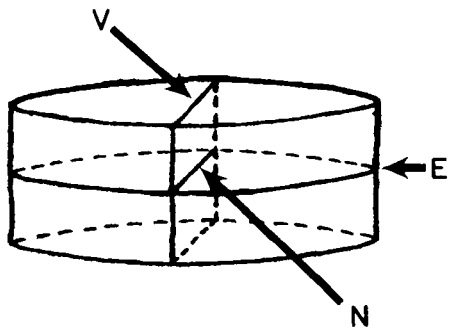

Figure 2—*Schematic drawing showing vertical, central plane and neutral axis. Key: V, vertical, central plane; E, neutral plane; and N, neutral axis.*

are the same as noted before for the diametral compression treatment.

The general form of the flexure equation, from elastic theory (9, 10), is as follows:

$$\sigma = My/I \qquad \text{(Eq. 2)}$$

where σ is bending stress, M is the bending moment, y is the distance from the neutral axis to any point or element of area in the vertical central section, and I is the moment of inertia. For a flat-faced tablet, the vertical cross section normal to the neutral plane is rectangular (unless tablet thickness is equal to tablet width and a square is generated), and its moment of inertia is given by:

$$I = \frac{bh^3}{12} \qquad \text{(Eq. 3)}$$

where I is the moment of inertia, b is tablet width (diameter), and h is tablet thickness. Since, for these purposes only, the half of the tablet that experiences tension is of interest:

$$y = \frac{h}{2} \qquad \text{(Eq. 4)}$$

By definition, maximum bending moment is:

$$M = \frac{Fb'}{4} \qquad \text{(Eq. 5)}$$

where F is the load observed when the tablet fractures, and b' is the distance from fulcrum to fulcrum. Making these substitutions in Eq. 2 results in:

$$\sigma = \frac{3Fb'}{2bh^2} \qquad \text{(Eq. 6)}$$

Since the maximum bending moment is $Fb'/4$, the tablet is loaded by a couple or resisting moment of $Fb'/8$ at each of its ends. Therefore, the equation must be divided by two, yielding:

$$\sigma_0 = \frac{3Fb'}{4bh^2} \qquad \text{(Eq. 7)}$$

where σ_0 is now the tensile strength.

EXPERIMENTAL

Comparison of Tensile Strength Measurement by Two Methods—The primary objective of this study was to compare tablet tensile strength as determined by diametral compression and by flexure. Diametral compression was carried out after the manner of Fell and Newton (3, 4) by compressing tablets between the flat platens of a constant-speed motorized tablet hardness tester[1]. Tensile strength was computed from the force required to cause failure using Eq. 1. Only the data from tablets that had obviously failed in tension, as evidenced by splitting into halves along the load diameter (11), were accepted.

For the flexure test, a knife edge and fulcrum piece machined from tool steel were affixed to the platens of the hardness tester (Fig. 3). Care was taken to align the two pieces so that the knife edge would contact the test tablets at their diameters, and the knife edge was of sufficient length to act across the entire diameter of the test tablets. The fulcrum piece was machined to an overall height and breadth equal to the diameter of the test tablets to facilitate the proper alignment of the tablets against the fulcrum piece. In this case, tensile strength was computed from the force required to cause failure using Eq. 7. The principal dimensions of the knife edge and fulcrum pieces are given in Fig. 4.

Three direct compression tablet fillers representative of different types were used. They were lactose[2] USP, compressible starch[3], and microcrystalline cellulose[4] NF. No active ingredients were included. No additives other than a lubricant were included and then only in the case of lactose, since the compressible starch and microcrystalline cellulose are known to be self-lubricating when used alone (12, 13). Lactose was lubricated with 0.75% mag-

[1] Heberlein model 2E/106, series 7203, Key Industries, Farmingdale, N.Y.
[2] Fast-Flo lactose, Foremost Dairies Inc., San Francisco, Calif.
[3] Sta-Rx 1500 starch, A. E. Staley Manufacturing Co., Decatur, Ill.
[4] Avicel PH 102, F. M. C. Corp., American Viscose Division, Newark, Del.

Table I—Comparison of Values for Tensile Strength as Determined by Flexure Test and Diametral Compression

Filler	Compression Force, kg	Tablet Dimensions, cm		Fracturing Force, kg ± SD[a]		Tensile Strength, kg/cm² ± SD[a]	t Statistic[b]
		Thickness	Diameter	Diametral Compression	Flexure Test		
Microcrystalline cellulose	208	0.201	1.118	11.8 ± 0.5	—	33.4 ± 1.4	
	208	0.201	1.118	—	2.0 ± 0	33.2 ± 0	0.428[c]
	212	0.245	1.118	14.7 ± 0.3	—	34.2 ± 0.7	
	212	0.244	1.118	—	3.1 ± 0.2	35.0 ± 2.2	1.04[c]
	254	0.214	1.118	15.3 ± 0.6	—	40.7 ± 1.6	
	254	0.213	1.118	—	2.8 ± 0.2	41.5 ± 1.1	1.10[c]
	269	0.240	1.118	18.4 ± 0.4	—	43.7 ± 0.9	
	269	0.242	1.118	—	3.7 ± 0.1	42.4 ± 1.1	2.75[d]
	316	0.190	1.118	17.8 ± 0.5	—	53.4 ± 3.5	
	316	0.190	1.118	—	2.6 ± 0.3	48.3 ± 5.6	2.23[d]
Compressible starch	1776	0.354	1.118	13.9 ± 1.3	—	22.4 ± 2.1	
	1776	0.353	1.118	—	3.7 ± 0.3	20.0 ± 1.9	2.55[d]
Lactose	810	0.304	1.118	14.4 ± 0.7	—	27.0 ± 1.3	
	810	0.303	1.118	—	3.8 ± 0.3	27.8 ± 2.2	0.940[c]

[a] Standard deviation, based on 10 determinations. [b] Student's *t* test for difference comparing the value for tensile strength determined by the flexure test to that determined by diametral compression for each run. [c] Difference not significant at 5% level. [d] Difference not significant at 1% level.

nesium stearate[5] USP which had been previously passed through an 80-mesh sieve to facilitate homogeneous blending.

The lubricated blend (500-g batch) was prepared by mixing[6] for 12 min, with the intensifier bar running for only the final 2 min. Tablets were prepared on a rotary tablet press[7] which had been instrumented with resistance strain gauges as previously described (14, 15) to monitor compression force. Press speed was 25 rpm, and all tableting was conducted in a room where temperature and relative humidity were maintained at 26 ± 1° and 35 ± 5%, respectively. Only a single station was run in order to help minimize tooling errors, and the tooling consisted of circular, flat-faced, 1.111-cm punches and die. Approximately 250-g quantities of powder were added to the hopper for each run, and tablet samples were not taken until after the press had run at least 2 min. Tensile strengths by the two methods were compared at a single compression force and tablet thickness in the cases of compressible starch and lactose. For microcrystalline cellulose, comparisons were made at five different compression forces and thicknesses because this filler makes strong tablets over a wide range of compressional force. After each tableting run, the tablets were measured for thickness and width using an inch measure[8]. Ten replicate tensile strength determinations were made using each method.

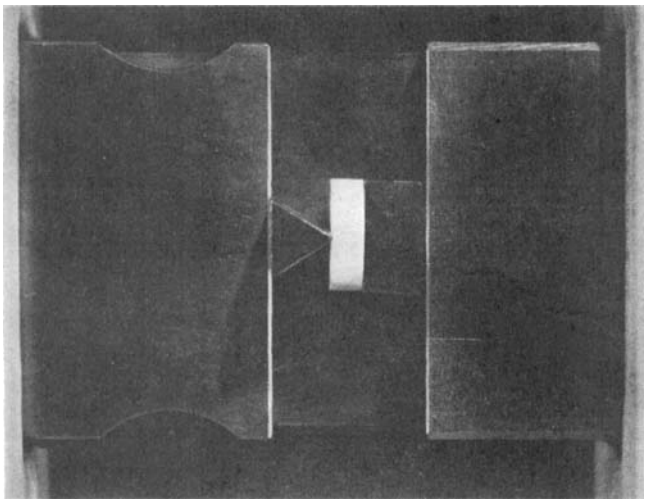

Figure 3—*Arrangement of knife edge, fulcrum piece, and tablet between the platens of the hardness tester.*

[5] Ruger Chemical Co., New York, N.Y.
[6] Patterson Kelly V-mixer.
[7] Stokes model RB-2.
[8] Federal model C815, Federal Products Corp., Wayne, Pa.

Effect of Tablet Thickness—In this study, the effect of tablet thickness on bending force and tensile strength was examined. Microcrystalline cellulose was selected for its high compressibility. Because of the very thick tablets generated, 0.3% magnesium stearate was added as a lubricant, even though, as noted previously, this filler does not normally require a lubricant when used alone. Blending and tableting were carried out as previously described. Five batches of tablets, each representing a different tablet thickness, were prepared with the same compression force of 604 ± 15 kg by changing both the die-fill and pressure settings for each run. Ten tablets from each batch were measured for thickness and diameter and fractured in the flexure test in the manner previously described.

RESULTS

From Table I it is clear that there is very good agreement in all cases between the two methods of tensile strength measurement. Application of the Student *t* test revealed that the differences in tensile strength were not significant at the 1% level in all comparisons. In four of the seven comparisons, the differences were not significant at the 5% level. Furthermore, the standard deviations for either method compare well with the variances previously reported for diametral compression (4).

The constants in Eq. 7 may be collected, and the equation may be rearranged to yield:

$$F = K\sigma_0 h^2 \qquad \text{(Eq. 8)}$$

where $K = 4b/3b'$. Thus, a plot of F against h^2 should yield a straight line passing through the origin with a slope = $K\sigma_0$, provided that tablet compression force and the force lost to the die wall are the same for the tablets at each thickness. As can be seen in Fig. 5, a straight line was produced when F was plotted against h^2 for various thicknesses of microcrystalline cellulose tablets compressed at the same compression force. The experimental data were fitted by a linear regression, least-squares plot, and the experimentally determined intercept of −0.1 kg/cm is in close agreement with the theoretically predicted intercept of zero. It is likely that the inclusion of magnesium stearate in these batches helped minimize any differences in the force lost to the die wall at the tablet thicknesses studied. However, as would be expected, the inclusion of magnesium stearate also materially weakened these tablets. From the slope of the curve, the tensile strength of these tablets was found to be 29.8 kg/cm². Comparison with Table I reveals that this figure is lower than any value found for the unlubricated microcrystalline cellulose tablets, even though they were compressed with only about one-third to one-half the compression force used to compress the lubricated batches.

In general, the flexure test was found to be a rapid, reproducible method for determining tablet tensile strength. The tensile strengths of very strong tablets can be measured more readily by this method than by diametral compression since the fracturing

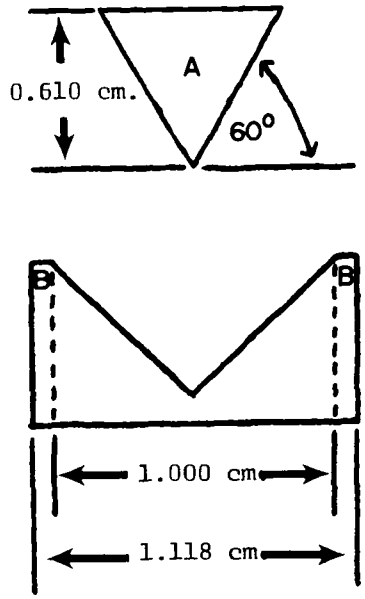

Figure 4—*Principal dimensions of straight edge and fulcrum setup. Key: A, straight edge; and B, fulcrums.*

forces obtained by the bending method were always less than those observed for fracture by diametral compression for the distance between fulcrums employed. However, the flexure test measurement has a higher coefficient of variation. If tablets are of exceptionally low strength, it may be necessary to reduce the distance between the fulcrums to get measurable fracturing forces. Tablets invariably failed in tension in the flexure test, as evidenced by uniform splitting into halves. Diametral loading can shatter the tablet and there can be noticeable shearing at the extremes of the vertical cross section where loading takes place rather than perfect diametrical splitting in one plane. To minimize this problem of the diametral compression test, Fell and Newton (4) recommended the use of soft padding material between the tablet and the platens. Also, Newton (16) noted that diametral compression does not work well if the tablets are not flat faced. The flexure test should be readily applicable to convex

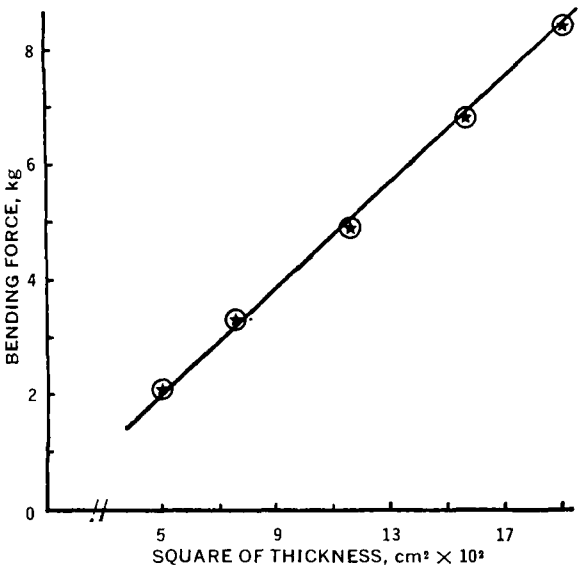

Figure 5—*Plot of bending force* versus *square of thickness for microcrystalline cellulose tablets containing 0.5% magnesium stearate. Slope = 44.3, and tensile strength = 29.8 kg/cm².*

tablets if the moment of inertia of the vertical, central plane involved in tablet fracture is known. This is presently being studied in these laboratories.

SUMMARY AND CONCLUSIONS

A flexure test for determining the tensile strength of tablets was proposed wherein tablets are compressed between two fulcrums and a knife edge. The fulcrum and knife edge pieces were affixed to the platens of a motorized tablet hardness tester. The method was rapid and the tablets invariably failed in tension, as evidenced by uniform splitting into halves. Experimental results with three different fillers (microcrystalline cellulose, lactose, and compressible starch) showed excellent agreement with tensile strength values obtained by diametral compression. In another study with microcrystalline cellulose, tensile strength was found to be independent of tablet thickness, as predicted by theory. For the distance between fulcrums employed, the fracture force in flexure was less than that required in diametral compression, thus making the new method more suitable for very strong tablets than diametral compression. For very weak tablets, it may be necessary to reduce the distance between fulcrums to get measurable fracture forces.

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* American Foundation for Pharmaceutical Education Fellow, 1971–1973. Present address: Mead Johnson Research Center, Evansville, IN 47721

ˣ To whom inquiries should be directed.

Polarographic Study of New Antimicrobial Pyrazolium Salts I: 1,2,5-Trimethyl-3-arylpyrazolium Perchlorates

NABIL M. OMAR[x] and NAWAL A. EL-RABBAT

Abstract □ The polarographic reduction of a new series of antimicrobial pyrazolium salts is described. Effects of the 3-aryl substituents on the $E_{1/2}$ values of the corresponding salts are well correlated with Hammett σ constants. The electrode reaction is shown to be mediated by a two-electron transfer and is pH independent. The irreversible electron transfer seems to be the rate-limiting factor of the electrode process. The *in vitro* minimum inhibitory concentrations of the title compounds against *Staphylococcus aureus* do not give a linear correlation with the corresponding $E_{1/2}$ values.

Keyphrases □ 1,2,5-Trimethyl-3-arylpyrazolium perchlorates—polarographic reduction, $E_{1/2}$ values correlated with antimicrobial minimum inhibitory concentrations □ Pyrazolium salts, antimicrobial—polarographic reduction of 1,2,5-trimethyl-3-arylpyrazolium perchlorates, structure-activity correlations □ Antimicrobial agents, new pyrazolium salts—polarographic study □ Polarography—reduction of new antimicrobial pyrazolium salts, effects of 3-aryl substituents on $E_{1/2}$ values

Many pyrazole derivatives have been reported to act as potent antimicrobial agents. Among these, the 1,2,5-trimethyl-3-arylpyrazolium salts (I) are characterized by marked antifungal effects (1, 2). The order of antibacterial activity of these compounds is appreciably influenced by the nature of the 3-aryl substituent. The pyrazolium ion was recently reported to undergo electrochemical reduction at the dropping mercury electrode (3); therefore, it was of interest to evaluate possible polar effects of both 3- and 5-substituents on the polarographic behavior of the pyrazolium ring and to correlate such effects with the *in vitro* antibacterial activity of these compounds.

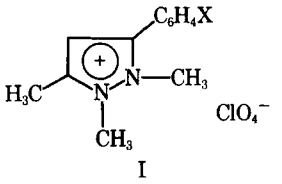
EXPERIMENTAL

1,2,5-Trimethyl-3-arylpyrazolium Perchlorates (I–VIII)—The salts were prepared according to reported methods (1) and were evaluated by UV spectrophotometry for purity.

Polarographic Reduction of Compounds I–VIII—The corresponding weight of the pyrazolium salt was dissolved in 50 ml double-distilled water containing 1.15 g polarographically pure tetraethylammonium perchlorate and 1.25 ml 0.2 % freshly prepared gelatin solution so as to produce a final concentration of 5×10^{-4} *M* depolarizer/liter. The polarograms were recorded on a polarograph[1], using an H-type cell, dropping mercury electrode, and saturated calomel electrode, after deoxygenation with oxygen-free nitrogen for 20 min at 25 ± 0.5°.

Capillary Characteristics—The drop time (*t*) was 3 sec, and the mass of the drop (*m*) was 3.5 mg/sec, measured at 30 cm height (*h*) and without applied voltage. The mercury used was of a polarographic double-distilled grade.

[1] LP-60.



I

Recording Conditions—The full-scale deflection (fsd) was 0.1 μamp, the applied voltage (v) was 0–2 in 200 mv/min, and the sensitivity shunt (S) was 1/200 fsd.

RESULTS AND DISCUSSION

The electrochemical reduction of the investigated pyrazolium salts proceeded successfully only when a supporting electrolyte of a highly negative reduction potential, like tetraethylammonium perchlorate, was used. Trials to adopt supporting electrolytes such as potassium chloride, Britton–Robinson buffer, or acetate buffer solutions resulted in the production of hydrogen waves at potentials more positive than that required for the reduction of the investigated salt. In 0.1 *M* aqueous tetraethylammonium perchlorate solution (pH 6.2–6.4), 1,2,5-trimethyl-3-phenylpyrazolium perchlorate readily affords a single cathodic wave with $E_{1/2}$ of −1.69 v (Fig. 1). As revealed from the dependence of the limiting current value, both on the concentration of the depolarizer and on the mercury column height (*h*), this wave is purely diffusion controlled. Theoretical logarithmic analysis of the wave (log $i/i_d - i$ *versus* *E*, Fig. 1) gives rise to a straight line, the slope of which corresponds, however, to a nonintegral value for *n* (α = 0.077, $\alpha n \neq 0.059$), thus denoting the irreversibility of the electrode reaction.

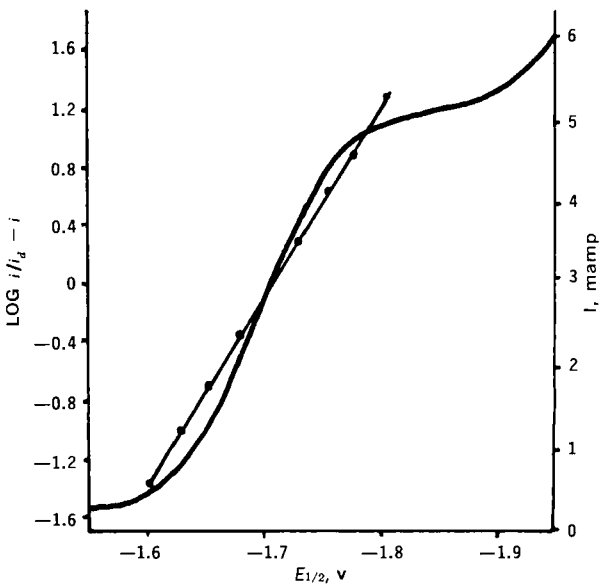


Figure 1—*Wave equation of 1,2,5-trimethyl-3-phenylpyrazolium perchlorate, 5×10^{-4} M in 0.1 M tetraethylammonium perchlorate.*

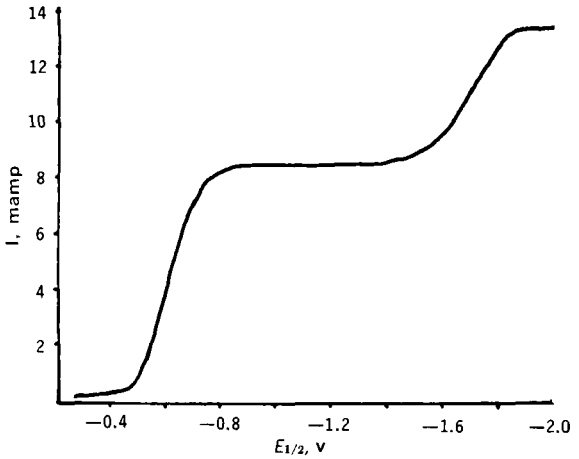


Figure 2—*Polarographic reduction of 5 × 10⁻⁴ M 1,2,5-trimethyl-3-(m-nitrophenyl)pyrazolium perchlorate in 0.1 M aqueous tetraethylammonium perchlorate.*

The number of the electrons involved in the reduction of Compound I, $n \cong 2$, is determined from the application of the Ilkovič equation (4). Essential for this determination is the diffusion coefficient of the pyrazolium ion (D_{0+}: 1.05×10^{-5} cm²/sec), computed from the limiting equivalent conductance (λ^0) of Compound I determined at 25°.

Controlled macroelectrolysis as well as chemical reduction of the 5-demethylated analogs of Compounds I–VIII afforded the corresponding 3-pyrazolines (3, 5). Accordingly, the two-electron polarographic reduction of I in protic media can be represented as shown in Scheme I.

Further study of the effect of the pH variation of the aqueous solutions of I on its polarographic behavior revealed that both the limiting current and the half-wave potential are only slightly, if at all, affected by the change of pH within a range of 6.2–13.0 (resulting from the controlled addition of sodium hydroxide). At pH values <6.2, a considerable decrease of the hydrogen overpotential is observed. Hence, in the polarographic reduction of I, protonation of the pyrazolium ion cannot be considered as the potential-determining stage.

The polarographic reduction of II–VIII is accomplished under the same exact conditions mentioned for I. The corresponding $E_{1/2}$ values (*versus* the saturated calomel electrode) are listed in Table I.

As revealed from a comparison of the wave heights of II–VIII, the electrode processes of these salts (except VIII) involve a two-electron transfer typical of that already cited. Moreover, the polarogram of VIII contains two reduction waves with $E_{1/2}$ values of −0.6 and −1.71, respectively (Fig. 2).

The peculiar polarographic behavior of VIII can be attributed to the presence of the polarographically active nitro group substituted in the 3-phenyl ring. Analysis of the corresponding heights of these two waves shows that the two-electron cathodic wave of the pyrazolium ion is preceded by a four-electron reduction of the

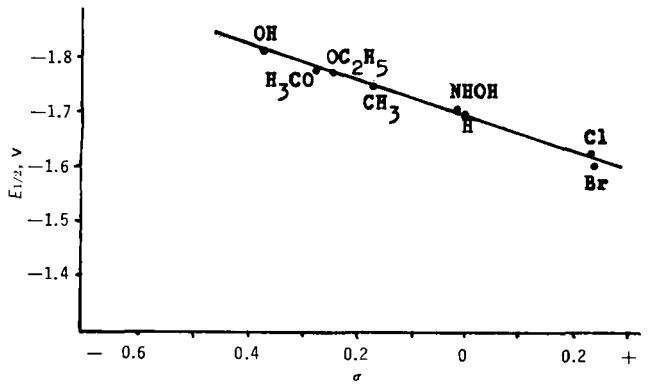


Figure 3—*Application of the Hammett equation to the half-wave potentials of I–VIII.*

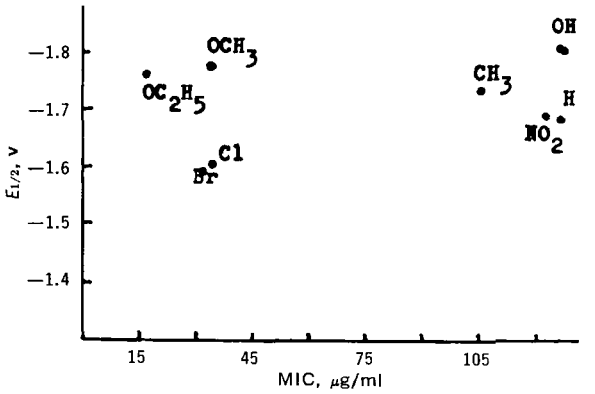


Figure 4—*Application of the Hammett equation to the MIC's of I–VIII against* S. aureus *in micrograms per milliliter.*

m-nitro group into the hydroxylamine derivative. This behavior is frequently reported for many aromatic compounds, where the nitro groups are acting principally *via* a −mesomeric effect (6–8).

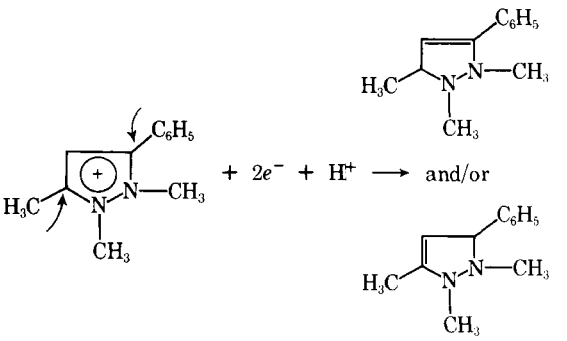
The quantitative determination of the polar effect of the different phenyl substituents of I–VIII, as well as the assignment of their electrode mechanisms, can be followed through the application of the principle of linear free energy, initially represented by the Hammett equation and recently extended to organic polarography in the form of:

$$\Delta E_{1/2} = E_{1/2} - E_{1/2}{}^0 = \rho\sigma \qquad \text{(Eq. 1)}$$

in which $E_{1/2}$ and $E_{1/2}{}^0$ are the half-wave potentials of the substituted and unsubstituted derivatives of a particular series, respectively; σ is the Hammett substitution constant; and ρ is the reaction constant (9). For the investigated pyrazolium salts, the plot of the corresponding $E_{1/2}$ values *versus* their 3-phenyl substitution constants affords a linear relationship (Fig. 3).

Statistical analysis of this linearity using the least-squares method gave a significant correlation with the following characteristics: $r = 0.99$, $\rho = 0.33$ *V*, and $s = 0.005$. The fact that values for σ, and not those for σ^+, are successfully adopted in this correlation clearly demonstrates the localization of a relatively small positive charge over the reaction center of the heterocycle. This can be accepted as a direct result of the influence of the 5-methyl group, acting by both + inductive and + mesomeric effects on the adjacent pyrazolium C-5, which can be considered as the reaction center. Moreover, the nucleophilic nature of the electrode reaction is distinctly revealed from the positive value of ρ. Thus, the rate of electron transfer is considered the potential-determining step in the reduction of the investigated ions, the electron affinity of which (reflected upon the $E_{1/2}$ values) is markedly promoted by the presence of electron-attracting substituents (10). Consequently, removal of the 5-methyl group out of the structure of I–VIII facilitated the electrochemical reduction of the corresponding demethylated pyrazolium salts (3).

Since use of the Hammett equation has also been extended to structure–activity data of some biologically active organic compounds (11), it was of interest to correlate the *in vitro* minimum inhibitory concentrations (MIC) of the studied pyrazolium salts against *Staphylococcus aureus* (Smith), determined in Ref. 1,



Scheme I

Table I—Half-Wave Potentials of 1,2,5-Trimethyl-3-arylpyrazolium Perchlorates (II–VIII)

	Compound						
	II	III	IV	V	VI	VII	VIII
X	p-CH_3	p-OCH_3	p-OC_2H_5	p-OH	p-Cl	p-Br	m-NO_2
$E_{1/2}$	−1.75	−1.77	−1.77	−1.82	−1.63	−1.60	−0.6, −1.71

with the corresponding structural parameters. As indicated from the scattered results relating the MIC of the pyrazolium salt to the $E_{1/2}$ value (Fig. 4), the microbiological data of these compounds do not correspond with their polarographic behavior. This can be probably attributed to the existence of biological pathways other than those concerned with the reduction potential of the investigated compounds. In fact, the application of the Hammett equation in such biological fields is still limited to a small group of organic compounds.

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[x] To whom inquiries should be directed.

Comparison among Four Vehicles and Four Routes for Administering Δ^9-Tetrahydrocannabinol

R. DUANE SOFIA*, ROBERT K. KUBENA‡, and HERBERT BARRY, III[x]

Abstract □ The effect of (−)-Δ^9-*trans*-tetrahydrocannabinol (I) on latency to hindlimb tonic extension produced by maximal electroconvulsive shock was studied in mice following injection by four routes of administration: oral, subcutaneous, intraperitoneal, and intravenous. For each route, four different vehicles were used: bovine serum albumin, polysorbate 80, polyvinylpyrrolidone, and propylene glycol. The anticonvulsant effect of I was strongest in the propylene glycol vehicle with subcutaneous and intraperitoneal routes and equal in the propylene glycol and polyvinylpyrrolidone vehicles with the oral route. With these three routes, bovine serum albumin and saline appeared to be inadequate vehicles for studying the anticonvulsant activity of I. When the intravenous route was used, anticonvulsant activity of I was found with all four vehicles, with propylene glycol and polyvinylpyrrolidone allowing the greatest effect of I; however, the propylene glycol vehicle itself also showed anticonvulsant activity. A further experiment showed that the duration of action of I following oral administration was substantially longer in the propylene glycol than in the polyvinylpyrrolidone vehicle.

Keyphrases □ Tetrahydrocannabinol—effect on hindlimb tonic extension, four vehicles and four routes of administration compared, mice □ Marijuana—comparison of four vehicles and four routes of administration, hindlimb tonic extension, mice □ Vehicles—effect on tetrahydrocannabinol anticonvulsant activity, comparison of four vehicles and four routes of administration, hindlimb tonic extension, mice

Recently, a report (1) from this laboratory described a study with mice which compared four vehicles for intraperitoneal administration of (−)-Δ^9-*trans*-tetrahydrocannabinol[1] (I), the apparent major psychoactive constituent of marijuana. The bioassay for an effect of I was latency to hindlimb tonic extension in mice following a maximal electroconvulsive shock. The most suitable vehicle for injection of I was found to be 10% propylene glycol–1% polysorbate 80[2]–0.9% saline. Other vehicles compared were 3% polyvinylpyrrolidone (2), 5% bovine serum albumin (3), and 1% polysorbate 80 (4), all in 0.9% sa-

[1] An alternative name for the same compound is Δ_1-tetrahydrocannabinol, using the pyran instead of the formal numbering system.

[2] Tween 80.

Table I—Half-Wave Potentials of 1,2,5-Trimethyl-3-arylpyrazolium Perchlorates (II–VIII)

	Compound						
	II	III	IV	V	VI	VII	VIII
X	p-CH_3	p-OCH_3	p-OC_2H_5	p-OH	p-Cl	p-Br	m-NO_2
$E_{1/2}$	−1.75	−1.77	−1.77	−1.82	−1.63	−1.60	−0.6, −1.71

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Recently, a report (1) from this laboratory described a study with mice which compared four vehicles for intraperitoneal administration of (−)-Δ^9-*trans*-tetrahydrocannabinol[1] (I), the apparent major psychoactive constituent of marijuana. The bioassay for an effect of I was latency to hindlimb tonic extension in mice following a maximal electroconvulsive shock. The most suitable vehicle for injection of I was found to be 10% propylene glycol–1% polysorbate 80[2]–0.9% saline. Other vehicles compared were 3% polyvinylpyrrolidone (2), 5% bovine serum albumin (3), and 1% polysorbate 80 (4), all in 0.9% sa-

[1] An alternative name for the same compound is Δ_1-tetrahydrocannabinol, using the pyran instead of the formal numbering system.

[2] Tween 80.

Table I—Effects of I on Latency to Convulsion (Mean ± *SE*) in Mice When Given by Four Different Routes of Admininistration in Four Vehicles and Tested 1 hr after Injection[a]

Vehicle	Route of Administration	Control (n = 12)	I, 20.0 mg/kg (n = 12)[b]
Bovine serum albumin (5%)	Oral	1.48 ± 0.07	1.54 ± 0.11
	Subcutaneous	1.42 ± 0.06	1.69 ± 0.10*
	Intraperitoneal	1.55 ± 0.09	1.68 ± 0.08
	Intravenous	1.53 ± 0.12	2.02 ± 0.17*
Polysorbate 80 (1%)	Oral	1.37 ± 0.08	1.55 ± 0.08
	Subcutaneous	1.42 ± 0.09	1.60 ± 0.09
	Intraperitoneal	1.56 ± 0.06	1.69 ± 0.10
	Intravenous	1.44 ± 0.08	2.28 ± 0.12***
Polyvinyl-pyrrolidone (3%)	Oral	1.49 ± 0.06	1.96 ± 0.12**
	Subcutaneous	1.40 ± 0.08	2.42 ± 0.22***
	Intraperitoneal	1.49 ± 0.11	2.75 ± 0.18***
	Intravenous	1.58 ± 0.06	3.48 ± 0.37***
Propylene glycol (10%)	Oral	1.28 ± 0.12	1.73 ± 0.10**
	Subcutaneous	1.46 ± 0.06	3.10 ± 0.21***
	Intraperitoneal	1.46 ± 0.06	3.38 ± 0.30***
	Intravenous	1.88 ± 0.09	3.88 ± 0.30***

[a] A maximum interval of 5 sec was recorded for mice that failed to convulse by that time (two with propylene glycol after intraperitoneal administration, four with propylene glycol after intravenous administration, three with polyvinylpyrrolidone after intravenous administration, and all in I-treated animals). [b]* $p < 0.05$ for difference from control. ** $p < 0.01$ for difference from control. *** $p < 0.001$ for difference from control.

line. The present report describes the results of a further investigation in which I was given by four different routes of administration in each of the same four vehicles.

EXPERIMENTAL

Two experiments were conducted on 576 male albino mice[3] weighing 17–27 g. In the first experiment, 384 mice were divided equally among the four vehicle conditions: 5% bovine serum albumin–0.9% saline, 1% polysorbate 80–0.9% saline, 3% polyvinylpyrrolidone–0.9% saline, and 10% propylene glycol–1% polysorbate 80–0.9% saline. The preparation of each vehicle has already been described in detail (1). The volume of fluid administered was 0.1 ml/10 g (10 mg/kg), containing I in a concentration of 2.0 mg/ml (20.0 mg/kg). These four vehicle groups were subdivided into groups administered the fluid by the oral, subcutaneous, intraperitoneal, or intravenous route. Half of the animals were injected with the vehicle alone (control) and the other half with I added to the vehicle. All animals were housed in the laboratory in groups of 12 for 24 hr before use and divided at the time of administration into six vehicle and six drug-treated animals with the same vehicle and route. At 1 hr following administration of I or vehicle, each mouse was tested for latency (recorded by stopwatch to the nearest 0.1 sec) to tonic convulsion, *i.e.*, complete extension of the hindlimbs, following an electroshock of 50-mamp intensity, 0.2-sec duration, applied transcorneally (5). All procedures were conducted twice, 1 week apart, so 12 mice were subjected to each of the 32 different treatments (four vehicles, each with four routes, each with vehicle and I).

In the second experiment, 192 mice were divided equally among two vehicle conditions: polyvinylpyrrolidone and propylene glycol. These two groups were subdivided into groups of 16 mice each, half of which received the vehicle alone and the other half I (20.0 mg/kg) in one of the vehicles, all by the oral route. Mice in various groups were tested at 0.25, 0.5, 1, 2, 4, and 8 hr for latency to tonic convulsion after electroconvulsive shock.

RESULTS

Table I presents the mean latencies to convulsion at 1 hr after I or control fluid in four vehicles, each administered by four routes. The 12 mice under each condition represent a combination of results obtained from two groups of six tested 1 week apart; they showed no statistically significant differences from each other. Table II summarizes the effect of I shown in Table I as a function jointly of vehicle and route of administration. In the polyvinylpyrrolidone and propylene glycol vehicles, I caused a highly reliable increase in convulsion latency with all routes of administration. In the polysorbate 80 vehicle, I had a reliable effect only with the intravenous route. In the bovine serum albumin vehicle, the effect of I was small with all four routes, reaching statistical significance only with subcutaneous and intravenous administrations. Comparison among the routes shows the largest effect of I after intravenous administration and the smallest effect after oral administration; the intraperitoneal and subcutaneous routes were intermediate and similar to each other.

In the absence of I, the median latency to convulsion showed no statistically significant differences among the groups with different vehicles and routes except for the elevated value of 1.88 ± 0.09 sec for the propylene glycol intravenous group (t = 4.33, df = 7, $p < 0.01$ for the difference from the overall mean of 1.49 sec).

Figure 1 summarizes the difference between I and the vehicle in

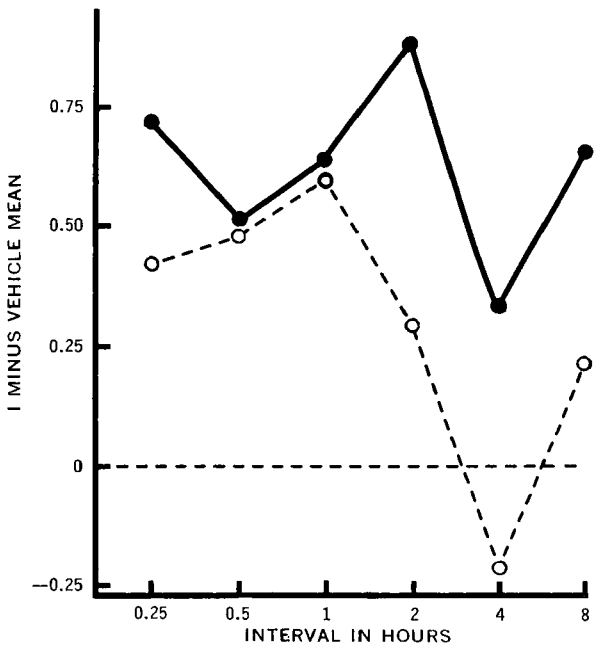

Figure 1—*Difference between the I and vehicle groups in mean latency in seconds to convulsion at intervals of 0.25–8 hr after oral administration of I (20 mg/kg) or its vehicle, showing a comparison between groups with propylene glycol* (●—●) *and polyvinylpyrrolidone* (○- - - -○) *as the vehicle.*

[3] Swiss-Webster, Hilltop Lab Animals, Inc., Scottdale, Pa.

Table II—Effect of I Measured by Mean Difference in Latency between I and Vehicle Groups Shown in Table I

Route of Administration	Vehicle[a]				Average
	Bovine Serum Albumin (5%)	Polysorbate 80 (1%)	Polyvinyl-pyrrolidone (3%)	Propylene Glycol (10%)	
Oral	0.06	0.18	0.47**	0.45**	0.29
Subcutaneous	0.27*	0.18	1.02***	1.64***	0.78
Intraperitoneal	0.13	0.13	1.26***	1.92***	0.86
Intravenous	0.49*	0.84***	1.90***	2.00***	1.31
Average	0.24	0.33	1.16	1.50	0.81

[a] * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

mean convulsive latency at six time intervals after oral administration, comparing two vehicles (polyvinylpyrrolidone and propylene glycol). An analysis of variance revealed a highly reliable increase in latency caused by I, averaging all six time intervals and both vehicles ($F = 50.26$, $df = 1/168$, $p < 0.001$), and a reliably greater increase in latency when I was in the propylene glycol vehicle, averaging all time intervals ($F = 6.46$, $df = 1/168$, $p < 0.05$). Without I, the mean latencies to convulsion were closely similar for the two vehicles (1.70 for propylene glycol and 1.67 for polyvinylpyrrolidone). An analysis of variance showed no statistically significant difference between vehicles ($F = 0.20$, $df = 1/84$). The difference among time intervals approached statistical significance ($F = 1.93$, $df = 5/84$, $p < 0.10$) because at the 4-hr interval, latency was elevated (1.88 sec for the propylene glycol and 1.91 sec for the polyvinylpyrrolidone vehicle). This may account for the diminished effect of I at that time interval (Fig. 1). A more prolonged duration of effect of I with the propylene glycol vehicle was indicated by t tests showing a statistically significant difference ($p < 0.05$) between the I and control groups at each time interval with propylene glycol but only at the first three time intervals with polyvinylpyrrolidone.

DISCUSSION

The present study demonstrates that I in the 5% bovine serum albumin and 1% polysorbate 80 vehicles had weak effects after administration by the intraperitoneal route, as reported previously (1), and also after administration by the oral, subcutaneous, and intravenous routes. Even the intravenous route, which produced a statistically significant effect of I in both vehicles, was much less effective than with the other two vehicles tested.

Borgen and Davis (6) tested the effects of I on operant behavior in rats, with four vehicles and the intraperitoneal and subcutaneous routes of administration. Contrary to the present results, 1% polysorbate 80 was a satisfactory vehicle, although weaker than 10% polyvinylpyrrolidone with the subcutaneous route. They added 100% polysorbate 80 to I, followed by gradual dilution of the fluid with saline[4], similar to the method that was used for preparing a suspension of I in polysorbate 65 (7). This procedure apparently minimized the disadvantage of the insolubility of I in saline; the present procedure, as described previously (1), was to add the 1% polysorbate 80 solution to I. It is evident that the effectiveness of a suspension depends not only on the vehicle but also on the method of preparation, which should be specified in detail by the experimenter.

Other vehicles are also possible. Rosenkrantz *et al.* (8), in a comparison of vehicles, recommended the use of sesame oil with 0.4–1% polysorbate 80 or 4–5% polyvinylpyrrolidone added to it. In the present experiment, 3% polyvinylpyrrolidone and 10% propylene glycol were the most effective vehicles for I. Propylene glycol appeared to be the superior vehicle, because it resulted in a stronger effect of I at 1 hr after administration by most of the four routes (Table I) and in a more prolonged effect on I by the oral route (Table II).

Concentrations of propylene glycol greater than 10% should be avoided, especially by the intravenous route. Table I shows an anticonvulsant effect of this vehicle when administered intravenously. This finding is consistent with a report (9) that 25 and 50% concentrations of propylene glycol, when administered intraperitoneally to mice, significantly elevated the maximal tonic extensor threshold to intravenous infusion of pentylenetetrazol and strychnine. Concentrations of propylene glycol ranging from 20 to 70% did not protect against electroconvulsive shock; however, these investigators did not report on latency to hindlimb tonic extension produced by electroshock, nor did they use concentrations of propylene glycol below 20%. In the present investigations, the vehicle containing 10% propylene glycol likewise did not protect against tonic extension produced by electroshock when administered *via* any route of administration. The results of several pilot experiments[5] showed that propylene glycol in concentrations as low as 30% increased the latency to electroshock when given intraperitoneally or subcutaneously, and a similar effect was observed after oral administration of a 50% propylene glycol solution.

[4] L. A. Borgen and W. M. Davis, University of Mississippi, University, Miss., personal communication.

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* Present address: Department of Pharmacology, Wallace Laboratories, Cranbury, NJ 08512

‡ Present address: Medical Research Department, ICI America Inc., Wilmington, DE 19899

ˣ To whom inquiries should be directed.

[5] Unpublished data.

Selected Pharmacological Studies of *Luffa operculata*

RONALD CHAMPNEY, NOEL M. FERGUSON*, and GARY G. FERGUSON[x]

Abstract □ An aqueous extract of *Luffa operculata* Cong. Cucurbitaceae (Cabacinha) was tested in mice for the following pharmacological effects: toxicity, analgesia ("grid shock" method), alteration of neuromuscular coordination (rotarod), and modification of barbiturate metabolism (hexobarbital sleeping time). The extract was evaluated in rats for analgesic effects (heat method); effects on electroshock seizure threshold; effects on blood pressure, pulse, electrocardiographic, and respiratory patterns; and anti-inflammatory activity. It was tested in guinea pigs for protective effect against histamine-induced bronchospasm. The extract was found to be inactive in all except analgesic studies in mice, in which a significant elevation of pain threshold was seen with 160 mg/kg ip. The 24-hr LD_{50} of the extract in mice was 160 mg/kg ip. A possible mechanism of the action is discussed.

Keyphrases □ *Luffa operculata*—pharmacological studies □ Pharmacological screening—extracts of *Luffa operculata* screened for activity

The saponins are found in some 500 plant species. Those of pharmacological importance are found in the families Rosaceae, Araliaceae, Liliaceae, Scrophulariaceae, and Polygalaceae (1).

Luffa operculata belongs to the family Cucurbitaceae (2). The active constituent was isolated and found to be isocucurbitacin B, which is believed to be responsible for its bitter taste (3). Other properties reported are foaming upon shaking, decongestant action on mucosa, hemolytic action on red blood cells, sternutatory effects (2), purgative, and abortifacient effects (4).

The extracts of *L. operculata* are highly toxic to mice and fish due to their hemolytic action on red blood cells (4). Because of this property, the saponin-containing plants are used as fish poisons in Africa and South America (1).

This study of *L. operculata* was begun because of interest in its decongestant property. An aqueous extract of the crude drug was studied, and a profile of pharmacological activity was obtained through the use of a screening program. Results of this study are reported here.

EXPERIMENTAL

Extraction—A quantity of 5.7 g of the ground, dried fruit of *L. operculata*[1] was boiled for 1.5 hr in 114 ml of distilled water. The remaining fluid was poured off, another 114 ml of distilled water was added to the marc, and the mixture was boiled for an additional 2.5 hr.

The resulting solution was filtered, and both filtrates were consolidated to form a dilution of 1:40. A volume of 28.5 ml of distilled water was added to the extract to make a final solution representing 1 g of crude drug in 25 ml (4%). This volume included water lost by evaporation.

Toxicity Studies—White, male, Swiss-Webster mice, weighing approximately 25 g, were injected intraperitoneally with the following arbitrarily chosen doses of 4% (40 mg/ml) *Luffa* extract: 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 ml. The mice were observed for effects such as sedation, excitation, salivation, defecation, and piloerection for 2 hr. Maximum drug effect appeared after 1 hr. A 1-hr period after injection was also allowed in most other studies, under the assumption that other responses would be at a high level after this period of drug incubation. The mice were again observed after 24 hr, and deaths were recorded. When an approximate LD_{50} dose was thus determined, 10 similar mice were injected with that dose and a more precise determination of the LD_{50} was made using similar techniques.

Rotarod Studies—Six white, male, Swiss-Webster mice, weighing approximately 25 g, were injected intraperitoneally (three controls and three experimentals). Each of three mice was treated intraperitoneally with 0.025, 0.050, and 0.075 ml, respectively, of a 4% (40 mg/ml) solution of *Luffa* extract; the three controls were injected intraperitoneally with equal volumes of distilled water.

All mice were placed on a rotarod having a speed of 6 rpm for 5 min, and the number of falls was recorded. The number of falls from the rotarod within a *short* time (to minimize the effect of fatigue) gives an estimate of the effect of the studied compound on neuromuscular coordination. Each mouse was given a 10-min rest between trials, and a total of four trials was performed.

Analgesia (Heat Method)—Twenty-eight white, male, Wistar rats were used (14 controls and 14 experimentals). The rats were weighed and injected intraperitoneally (14 with *Luffa* extract and 14 with distilled water). In each case, the dose was 80 mg/kg of the 4% extract.

The rats' tails were placed above the heating coil in a Dolorimeter[2] and the pain threshold was recorded (°C). The end-point was raising of the tail from the hot area ("tail flick" response).

Analgesia ("Grid Shock")[3]—Twenty white, male, Swiss-Webster mice were used in three separate trials. The mice were weighed and injected with 160 mg/kg ip of 4% *Luffa* extract or an equal volume of distilled water. Each mouse was placed in the grid shock apparatus for 1.5 hr. At 10-min intervals, the voltage was gradually increased from zero to the end-point (pain threshold), and the value thus obtained was recorded. The end-point was rapid and repeated lifting of a hindleg. Details of the testing procedure were previously reported (6).

Hexobarbital Sleeping Time—Twenty white, male, Swiss-Webster mice were used (10 experimentals and 10 controls) in each of three trials. A dose of 120 mg/kg ip of 4% *Luffa* extract or an equal volume of distilled water was used. After 1 hr of drug incubation, the mice were injected with 70 mg/kg ip of a 4-mg/ml solution of sodium hexobarbital. Sleeping time was considered to be the time interval from the point when the mouse lost the righting reflex to the point when the righting reflex was regained. This procedure was repeated two additional times.

Electroshock Seizure Threshold—Twenty white, male, Wistar rats were used. Each was shocked with corneal electrodes to determine the threshold for maximal tonic seizure. Normal saline solution was placed on each cornea to assure proper conductance. Thresholds were measured (milliamperes) with an electroshock apparatus[2], which provided a shock of 110 v with variable amperage and a duration of 0.2 sec.

After maximal tonic thresholds were determined, 10 rats were injected with 2 ml/kg ip of distilled water and 10 were injected with 80 mg/kg ip of 4% *Luffa* extract. After 1 hr, the rats were then shocked at their predetermined thresholds to observe any change.

Blood Pressure, Electrocardiographic (ECG), Pulse, and Respiration Studies—Ten white, male, Wistar rats were used.

[1] Collected in Northeast Brazil and identified by Dr. Lauro Xavier. A voucher specimen is deposited at the Universidade Federal Da Paraiba, Joao Pessoa, Paraiba, Brazil.

[2] Metro Industries, Farmingdale, N.Y.

[3] Consisting of a wooden box having a floor composed of brass rods wired in parallel to deliver a mild electric shock to the animal's feet.

Normal tracings for indirect blood pressure, ECG, pulse, and respiration were determined with the Physiograph[4], using the pneumatic pulse transducer[4], high gain preamplifier[4], and impedance pneumograph[4] in the measurement of the respective parameters. Respiration, pulse rate, and ECG were monitored using needle electrodes placed beneath the skin of conscious rats, which were restrained in Physiograph small animal housings[4]. Indirect blood pressure was obtained from the tail with the pulse transducer using an electrosphygmomanometer[4] which automatically inflated and deflated a pressure cuff around the tail and allowed for measurement of systolic blood pressure. Seven rats were then injected with 40 mg/kg iv of 4% *Luffa* extract, and three rats were injected with 160 mg/kg iv of 4% *Luffa* extract. Each animal was injected three times with its particular dose of extract. Tracings of blood pressure, ECG, pulse, and respiration were again obtained following each injection to observe any alterations in patterns.

Foot Volume Plethysmography—Fifteen white, male Wistar rats were used in each of two trials. In the first trial, following initial measurement of foot volumes, 15 rats were injected with 0.1 ml of 1% silver nitrate solution through the Achilles tendon of the left hindfoot to induce swelling of the foot pad. After 18 hr, the foot volume was again measured (in arbitrary units) with the plethysmograph[5] to confirm the presence of pedal edema. Five rats were injected with a solution of oxyphenbutazone (50 mg/kg ip solubilized in 5% polyethylene glycol), which served as a reference anti-inflammatory compound. Five rats were injected intraperitoneally with 5% polyethylene glycol solution in a volume equal to that of reference compound. Five rats were injected with 80 mg/kg ip of 4% *Luffa* extract. After a 1-hr incubation period, each rat's foot volume was measured at 1-hr intervals for 2 hr.

The second trial was conducted in the same manner except for the following changes: 0.2 ml of 1% silver nitrate solution, 1 ml/kg of distilled water as control, 1 mg/kg of dexamethasone to replace oxyphenbutazone, and 160 mg/kg of 4% *Luffa* extract.

Foot volume was measured hourly for 4 hr after the 1-hr incubation period. The procedure is similar to that previously reported (5).

Histamine Antagonism—Eight adult, male guinea pigs were weighed and then divided into two groups of four (controls and experimentals). The experimental animals received 120 mg/kg ip of 4% *Luffa* extract 30 min prior to testing, and controls received 3 ml/kg ip distilled water in an identical treatment. After incubation, each animal was placed in an airtight chamber into which was sprayed 0.5 ml of 0.5% histamine diphosphate solution. The animals were observed for signs of bronchoconstriction, hypoxia, or asphyxia.

RESULTS AND DISCUSSION

Toxicity Studies—The following observations were made during the test period. Regardless of the dose of *Luffa*, the effects were similar. At first, defecation increased followed in approximately 30 min by watery diarrhea. Spontaneous motor activity appeared to decrease. All *Luffa*-treated mice remained in a crouched position with eyes closed and head lowered. Piloerection was present in some animals. Some mice lost the righting reflex. Controls appeared normal at the times these observations were made. The 24-hr LD_{50} was approximately 160 mg/kg ip of 4% *Luffa* extract in mice.

Rotarod Performance; Analgesia (Heat Method); Hexobarbital Sleeping Times; Electroshock Seizure Threshold; Blood Pressure, ECG, Pulse, and Respiration Studies; Foot Volume Plethysmography; and Antihistamine Studies—No significant results were observed ($p > 0.05$).

Analgesia (Grid Shock)—The grid shock testing showed an elevated pain threshold for up to 1.5 hr after injection of *Luffa* extract. Statistical analysis showed a significant difference as compared to controls (Table I).

Table I—Pain Thresholds in Control and Treated Animals[a]

Time after Injection, min	Control	Experimental	T^b
10	13	17	3.7
30	14	20	2.7
50	16	24	2.0
70	15	26	3.2
90	17	28	3.7

[a] Each value, expressed in volts, represents the average of nine individual determinations. For details of treatment, see text. [b] $p < 0.05$.

The absence of activity of the extract in the cardiovascular, anti-inflammatory, and antihistaminic studies was surprising, considering the previously reported stimulant action of the drug on toad heart, guinea pig ileum, and rabbit duodenum; its pressor effects in cats; and its colloquial use in the treatment of nasal congestion (2, 4). Most drugs that are beneficial in the treatment of nasal congestion act by means of adrenergic stimulation, producing vasoconstriction of the capillaries in the nasal mucosa, or by antihistaminic or anti-inflammatory effects, resulting in reduced engorgement of the nasal mucosa (7). While the degree of engorgement of the nasal mucosa and resultant resistance to nasal air passage is difficult to evaluate in animals, the studies performed should have provided a parallel means of evaluating the effect of *Luffa* extract on these parameters in terms of effects on certain cardiovascular functions, changes in response to histamine, or modification of experimentally induced inflammatory edema. Thus, if the drug has marked vasoconstricting effects, some elevation in blood pressure or change in pulse rate would be expected in animal studies. Likewise, if the drug acts as an anti-inflammatory agent, modification of silver nitrate-induced pedal edema in rats would be expected. If the primary action of the drug is as an antihistaminic, partial or complete protection of guinea pigs from histamine-induced bronchospasm should be seen. If the action or a portion of the action of *L. operculata* is as an analgesic, it must be assumed to be of a mild degree since near lethal doses were required for the effect in mice and this effect was not seen in the heat method studies but required the more sensitive grid shock method for its detection.

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* Present address: College of Pharmacy, University of Houston, Houston, TX 77004

x To whom inquiries should be directed.

[4] Narco Industries, Houston, Tex.

[5] Plethysmographe V3, Ugo Basile, Milan, Italy. Volume is measured by mercury displacement, using a balanced bridge electronic system and meter-type dial.

Chemical Constituents of Gentianaceae IX: Natural Occurrence of Erythrocentaurin in *Enicostemma hyssopifolium* and *Swertia lawii*

S. GHOSAL[x], A. K. SINGH, P. V. SHARMA, and R. K. CHAUDHURI

Abstract □ Erythrocentaurin was isolated from two gentianaceous plants—*viz.*, *Enicostemma hyssopifolium* (Willd.) Verd. (synonymous with *E. littorale*) and *Swertia lawii* Burkill., as a native compound. Its natural occurrence was demonstrated for the first time by using a modified isolation procedure. The identity of the compound was established by chemical transformations and spectral (UV, IR, NMR, and mass spectra) evidence. Phylogenetic significance of the cooccurrence of erythrocentaurin and a number of structurally related monoterpene alkaloids in *E. hyssopifolium* is discussed.

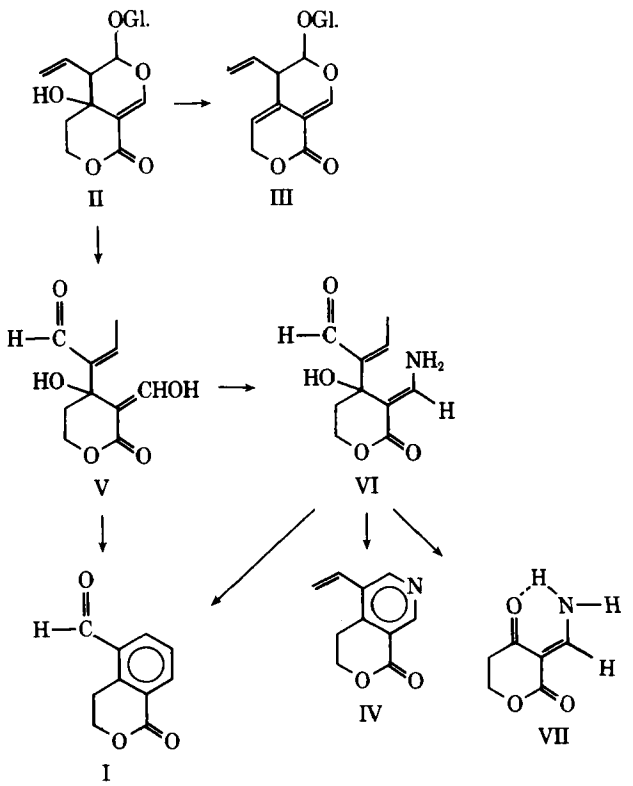
Keyphrases □ Erythrocentaurin—isolation and identification from *Enicostemma hyssopifolium* and *Swertia lawii* □ *Enicostemma hyssopifolium*—natural occurrence of erythrocentaurin and structurally related monoterpene alkaloids □ *Swertia lawii*—natural occurrence of erythrocentaurin and gentianine □ Enicoflavine—spontaneous transformation into erythrocentaurin, gentiocrucine, and gentianine

Erythrocentaurin (I) was first isolated by Méhu (1) from commercial samples of *Erythrea centaurium*. Subsequently, it was obtained (2) by the enzymatic hydrolysis of swertiamarin (a native glucoside of *Swertia japonica*) and of erytaurin (a native glycoside of *Er. centaurium*) (3). The structure of I was established later by Kubota and Tomita (4), who suggested it to be an artifact formed by the hydrolysis of swertiamarin (II). Compound I was also isolated (5) from *Gentiana lutea*. But this time, the process of isolation, involving alkalization of the root extracts of *G. lutea*, did not eliminate the possibility of its formation from swertiamarin present in the plant in appreciable quantities. Thus, the question of whether erythrocentaurin is an artifact or a native compound in these plants remained unsettled. Evidence is presented in this report establishing that erythrocentaurin is a native compound in two gentianaceous plants—*viz.*, *Enicostemma hyssopifolium* (Willd.) Verd. (synonymous with *E. littorale* Blume) and *Swertia lawii* Burkill. (synonymous with *S. corymbosa* Wight). In addition, the cooccurrence of erythrocentaurin with a number of structurally related monoterpene alkaloids in *E. hyssopifolium* (6) is appraised in the light of a common biogenetic sequence.

RESULTS AND DISCUSSION

E. hyssopifolium and *S. lawii* whole plants were separately extracted with petroleum ether. The petroleum ether extracts were processed for neutral, basic, and glycosidic constituents. Although it seemed unlikely that a weakly polar extract would contain appreciable quantity of any strongly polar constituent, *e.g.*, swertiamarin (II) and gentiopicroside (III), tests for these compounds were carried out to dispel any gap in the finding. The absence of swertiamarin and gentiopicroside in the petroleum ether extracts and isolation of erythrocentaurin from the neutral fraction established that erythrocentaurin occurs as such in these two species. The identity of erythrocentaurin was established by chemical transformations and spectral (UV, IR, NMR, and mass spectra) evidence. The NMR and mass spectral data of this compound were not reported previously. The basic fraction of the two plant extracts afforded gentianine (IV). Previous studies showed (7) that gentianine can exist as a native alkaloid in small quantities, but the relatively high yield of gentianine, reported in many plants, results from the action of ammonia on nonnitrogenous precursors, *e.g.*, swertiamarin (II) and gentiopicroside (III). The present report of isolation of gentianine from *E. hyssopifolium* and *S. lawii*, without involving treatment with ammonia, should be regarded as additional evidence in favor of labeling it as a natural alkaloid. The aglucone (V) of swertiamarin, which remains to be isolated, is an accepted intermediate to gentianine (5, 7, 8). Compound V could also act as an intermediate to erythrocentaurin in plants. The transformation of V to I would involve only minor molecular rearrangement followed by the elimination of water.

Recently, isolation of two new monoterpene alkaloids, enicoflavine (VI) and gentiocrucine (VII), from *E. hyssopifolium* was reported (9). Enicoflavine was shown to transform spontaneously into gentianine, gentiocrucine, and erythrocentaurin. Upon electron impact (in the mass spectrometer), it was converted into gentianine and gentiocrucine (6). If the premise is granted that



Scheme I—Hypothetical route to genesis of monoterpene alkaloids and erythrocentaurin in E. hyssopifolium

enicoflavine is formed from the same aglucone (V) in *E. hyssopifolium*, then a plausible common sequence of genesis of the congener compounds would be as shown in Scheme I.

The conversion of V into VI would involve simple amination (the source of nitrogen *in vivo* is, however, not known) at the hydroxymethylene group. The subsequent transformation of VI into VII would proceed *via* retroaldolization; the accompanying crotonaldehyde was also detected (6). Further details of these findings will be reported.

EXPERIMENTAL[1]

Extraction of *E. hyssopifolium*[2]—Air-dried and milled whole plants (1.6 kg), collected from the local area and properly identified[3], were extracted (16 hr) under reflux in a soxhlet apparatus with petroleum ether (bp 60–80°). The petroleum ether extract was concentrated to about 400 ml, and a portion of the concentrate (300 ml) was extracted with aqueous citric acid (12.5%, 200 ml) using a mechanical stirrer (8 hr). The acidic aqueous layer was kept for further processing for erythrocentaurin. The other portion (100 ml) of the petroleum ether extract was extracted with ethyl acetate (3 × 100 ml). The combined ethyl acetate extracts were washed with water, dried (anhydrous sodium sulfate), and concentrated (about 25 ml). The concentrate was passed through a column of alumina[4] (24 × 1.8 cm), and the column was eluted with methanol (500 ml). The methanol eluate was concentrated (20 ml) and examined for the monoterpene glucosides, swertiamarin (11) and gentiopicroside (11), by analytical TLC in the presence of authentic markers. The two glucosides were absent in this fraction. Only a weakly polar constituent, R_f 0.8 [*n*-butanol–acetic acid–water (4:1:2)], was detected in the methanol eluate.

Isolation of Erythrocentaurin (I)—The aqueous citric acid layer was extracted with ether (3 × 100 ml). The combined ether extracts were washed, dried, and concentrated (about 20 ml). The ether concentrate, after sitting, afforded brown crystals of erythrocentaurin (27 mg). It crystallized from acetone–petroleum ether (1:1) as colorless needles, mp 132–133° [lit. (11) mp 140–141°]; R_f 0.40 [benzene–chloroform (100:20)], 0.79 [*n*-butanol–acetic acid–water (4:1:2)]; UV: λ_{max} (ethanol) 224 (log ϵ 4.28) and 290 nm (3.09); IR: ν_{max} (mineral oil) 1718 (conjugated lactone C=O) and 1695 cm^{-1} (conjugated —CHO); mass spectroscopy *m/e* 176 (M^+, 100%) and significant fragment ion peaks at *m/e* 148 (14), 147 (17), 131 (8), 120 (40), 119 (43), 118 (46), 117 (11), and 105 (8); NMR (100 MHz): δ ($CDCl_3$) 3.6 and 4.6 [two 2H triplets, the large downfield shift due to $-CH_2-CH_2-$ moiety attached to a hetero atom, associated with the functionality —O—C(=O)—], 7.65 (1H, t, J = 8 Hz), 8.15 (1H, dd, J = 8 Hz, J' = 1.6 Hz), 8.42 (1H, dd, J = 8 Hz, J' = 1.6 Hz) (three aromatic protons), and 10.3 (1H, s, —CHO).

Anal.—Calc. for $C_{10}H_8O_3$: C, 68.18; H, 4.54. Found: C, 67.74; H, 4.73.

Direct comparison with the reported (11) erythrocentaurin was not possible since that material was not available. After sitting and being exposed to light, the compound gradually turned reddish brown and showed several spots with analytical TLC.

The semicarbazone, prepared in the usual way, crystallized from ethanol as colorless needles, mp 217–218° [lit. (11) mp 219–220°].

Anal.—Calc. for $C_{11}H_{11}N_3O_3$: C, 56.65; H, 4.72; N, 18.02. Found: C, 56.41; H, 4.98; N, 17.83.

Isolation of Gentianine (IV)—The clarified acidic aqueous layer, after separation of erythrocentaurin, was made basic with sodium bicarbonate. The liberated bases were extracted with ether (3 × 100 ml). The combined ether extracts were washed, dried, and evaporated. The residue crystallized from petroleum ether–benzene as colorless needles (12 mg), melting point and mixed melting point with authentic gentianine 80°; co-TLC with authentic gentianine showed a single spot, R_f 0.68 [*n*-butanol–acetic acid–water (4:1:2)]; λ_{max} (ethanol): 220 (log ϵ 4.39), 245 inflection (3.88), and 275–278 nm (3.20).

Anal.—Calc. for $C_{10}H_9NO_2$: C, 68.57; H, 5.14; N, 8.00. Found: C, 68.83; H, 4.90; N, 7.57.

Extraction of *S. lawii*[5]—Air-dried and milled whole plants (1.2 kg) were extracted with petroleum ether, and the extracts were processed in the same way as described for *E. hyssopifolium* when erythrocentaurin (24 mg) and gentianine (17 mg) were obtained from the neutral and basic fractions, respectively.

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Part VIII of this series: S. Ghosal, R. K. Chaudhuri, M. P. Tiwari, A. K. Singh, and F. W. Wehrli, *Tetrahedron Lett.*, **1974**, 403.

[x] To whom inquiries should be directed.

[1] The general directions are same as reported in a recent paper (10).

[2] The plant material was supplied by Mr. B. Singh, Varanasi, India. A herbarium specimen has been preserved at the Department of Pharmaceutics, Banaras Hindu University, Varanasi-5, India.

[3] Through the courtesy of Dr. C. S. P. Rao, Department of Botany, Faculty of Sciences, Banaras Hindu University.

[4] Brockmann activity grade about IV.

[5] The plant material was supplied by Soil Conservation, Research Training and Demonstration Centre, Ootacumund, the Nilgiris, India. A voucher specimen has been deposited at the Department of Pharmaceutics, Banaras Hindu University.

Synthesis of Substituted Benzthiaxanthones for Possible Schistosomicidal Activity

I. NABIH[x] and A. ZAYED

Abstract □ New substituted benzthiaxanthones were synthesized in an attempt to find new compounds that may have schistosomicidal activity.

Keyphrases □ Benzthiaxanthones, substituted—synthesized as possible schistosomicidal agents □ Thiaxanthones—synthesized containing methyl group *para* to diethylaminoethylamino side chain as possible schistosomicidal agents □ Schistosomicidal agents, potential—synthesis of substituted benzthiaxanthones

Some substituted thiaxanthones, represented by 1-(2-diethylaminoethylamino)-4-methylthiaxanthone[1], have shown schistosomicidal (1) and carcinostatic activity (2). The structural feature necessary for the biological activity of this agent is a methyl group *para* to a dialkylaminoalkylamino side chain on the aromatic ring (3). The reactivity of these groupings necessary for the biological activity was discussed previously (4–7).

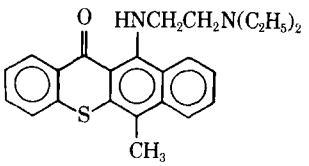
DISCUSSION

These considerations suggested the synthesis of a new compound of the thiaxanthone type containing a methyl group *para* to the diethylaminoethylamino side chain and incorporating the naphthalene system. This was suggested since, in mammalian systems, molecular oxygen is directly incorporated in the naphthalene metabolism through an oxygenation process (8, 9). Such oxygen carriers may affect glycolysis in the parasite, which seeks its main source of energy for living and reproduction through this metabolic process.

Condensation of thiosalicylic acid with 2-bromo-1-methyl-4-nitronaphthalene (10, 11) in the presence of anhydrous sodium carbonate gave 1-methyl-4-nitro-2-naphthyl-*o*-carboxyphenyl sulfide (II).

Reduction of the nitro group in II, using iron and acetic acid, gave 4-amino-1-methyl-2-naphthyl-*o*-carboxyphenyl sulfide (III), which was cyclized under the effect of polyphosphoric acid to give 11-amino-6-methylbenz[*b*]thiaxanthen-12-one (IV). Upon condensation of IV with 2-diethylaminoethyl chloride, the desired product, 11-(2-diethylaminoethylamino)-6-methylbenz[*b*]thiaxanthen-12-one (V), was obtained.

Furthermore, it was observed that 1,4-naphthalenediamine killed adult *Schistosoma mansoni in vitro* at a drug concentration as low as 25 μg/ml (12). The potent chemotherapeutic effect of various *N*-(dialkylaminoalkyl)-1,4-naphthalenediamines (13–15) against infections of *S. mansoni* and *S. japonicum* in experimental animals was attributed to the affinity of these compounds to exist in the oxidation state as quinoid structures of the naphthoquinone types within these series (16). It was already known that naphthoquinone can reduce the rate of glycolysis in schistosomes substantially (17).



V

Condensation of thiosalicylic acid with 2-iodo-4-nitro-1-naphthylamine (18) in the presence of anhydrous sodium carbonate afforded 1-amino-4-nitro-2-naphthyl-*o*-carboxyphenyl sulfide (VI).

Reduction of VI with iron and acetic acid gave 1,4-diamino-2-naphthyl-*o*-carboxyphenyl sulfide (VII), which underwent ring closure with polyphosphoric acid to give 6,11-diaminobenz[*b*]thiaxanthen-12-one (VIII). This reacted with 4-diethylaminoethyl chloride to yield the product 6,11-bis(2-diethylaminoethylamino)benz[*b*]thiaxanthen-12-one (IX).

Upon acetylation of VI with acetic anhydride, 1-acetamido-4-nitro-2-naphthyl-*o*-carboxyphenyl sulfide (X) was obtained. This compound was similarly reduced to 1-acetamido-4-amino-2-naphthyl-*o*-carboxyphenyl sulfide (XI).

Cyclization of XI with polyphosphoric acid gave 6-acetamido-11-aminobenz[*b*]thiaxanthen-12-one (XII). Upon reaction with 2-diethylaminoethyl chloride and hydrolysis, XII gave 6-amino-11-(2-diethylaminoethylamino)benz[*b*]thiaxanthen-12-one (XIII).

Furthermore, *p*-aminophenols were found to undergo biological transformations to benzoquinone-type structures (19, 20). For this purpose, the present synthesis emphasized a benzthiaxanthone structure bearing a hydroxyl group *para* to the diamine side chain.

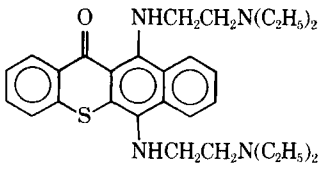
Condensation of 2-iodo-4-nitro-1-naphthol (18) with thiosalicylic acid gave 1-hydroxy-4-nitro-2-naphthyl-*o*-carboxyphenyl sulfide (XIV). However, this product, XIV, could also be obtained by boiling VI with alkali.

Reduction of the nitro group in XIV, using iron and acetic acid, afforded 4-amino-1-hydroxy-2-naphthyl-*o*-carboxyphenyl sulfide (XV). This was cyclized using polyphosphoric acid to give 11-amino-6-hydroxybenz[*b*]thiaxanthen-12-one (XVI). Finally, XVI was reacted with 2-diethylaminoethyl chloride to give 11-(2-diethylaminoethylamino)-6-hydroxybenz[*b*]thiaxanthen-12-one (XVII).

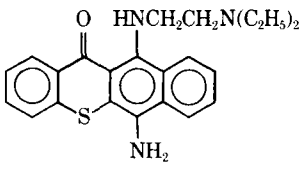
Biological studies on V, IX, XIII, and XVII will be presented in a separate report.

EXPERIMENTAL[2]

Compound II—A mixture of thiosalicylic acid (5.8 g, 0.037



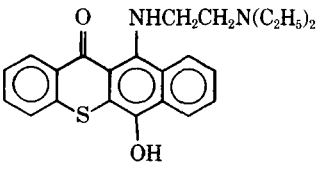
IX



XIII

[1] Lucanthone or Miracil D, Farbenfabriken Bayer AG, Leverkusen, West Germany.

[2] Melting points were taken in open capillary tubes by use of a sulfuric acid bath and a Gallenkamp melting-point apparatus and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, National Research Centre, Cairo, U.A.R. IR spectra were determined on a UR.10 Zeiss-Jean IR spectrophotometer using KBr pellets.



XVII

mole), 2-bromo-1-methyl-4-nitronaphthalene (10 g, 0.038 mole), anhydrous sodium carbonate (4.3 g, 0.041 mole), and amyl alcohol (60 ml) was refluxed at 140° for 16 hr. The alcohol was removed by steam distillation, and the reaction mixture was boiled with excess water and then filtered. The cold filtrate was acidified with acetic acid; the precipitated product was collected, washed with water, and dried to give 9 g (71%) of II, mp 153° (from ethanol).

Anal.—Calc. for $C_{18}H_{13}NO_4S$: C, 63.70; H, 3.86; N, 4.13. Found: C, 63.39; H, 3.74; N, 4.51.

Compound III—A suspension of II (7 g) in acetic acid (100 ml, 70%) was stirred on a steam bath, and then iron powder (7 g) was added portionwise. Stirring and heating were continued for 3 hr. The mixture was poured into ice-cold water. The solid product was collected, dissolved in boiling ammonium hydroxide solution, and filtered. The filtrate was acidified with acetic acid. The precipitated product was isolated to give 4.5 g (72%) of III, mp 190° (aqueous ethanol); IR: 700 (—S—), 1655 (—COOH), and 3330 (—NH_2) cm^{-1}.

Anal.—Calc. for $C_{18}H_{15}NO_2S$: C, 69.88; H, 4.89; N, 4.53. Found: C, 69.67; H, 4.81; N, 4.70.

Compound IV—Compound III (2 g) was heated with polyphosphoric acid (20 g) at 140° for 2 hr. The reaction mixture was poured into ice-cold water, and the solid product was collected and washed with boiled ammonium hydroxide solution. After drying, it gave 1.7 g (90%) of IV, mp 151° (from ethanol).

Anal.—Calc. for $C_{18}H_{13}NOS$: C, 74.20; H, 4.50; S, 11.00. Found: C, 73.85; H, 4.21; S, 10.79.

Compound V—Compound IV (1 g) was treated with 2-diethylaminoethyl chloride (0.5 ml) at 160° for 4 hr. The reaction mixture was boiled with dilute hydrochloric acid and then filtered. The cooled filtrate was neutralized with dilute sodium hydroxide solution, and the solid precipitate was collected and dried to give 1 g (75%) of V, mp 113° (from aqueous ethanol).

Anal.—Calc. for $C_{24}H_{26}N_2OS$: C, 73.81; H, 6.71; N, 7.17. Found: C, 73.49; H, 6.53; N, 7.56.

Compound VI—A mixture of thiosalicylic acid (3.66 g, 0.0238 mole), 2-iodo-4-nitro-1-naphthylamine (7.6 g, 0.024 mole), anhydrous sodium carbonate (2.6 g, 0.0255 mole), copper powder (0.1 g), and amyl alcohol (60 ml) was refluxed at 150° for 15 hr. The alcohol was steam distilled, and the mixture was boiled with excess water and then filtered. The cold filtrate was neutralized with acetic acid and the formed precipitate was collected. After drying, it gave 6 g (75%) of VI, mp 230° (from ethanol); IR: 690 (—S—), 1505 (—NO_2), 1680 (—COOH), and 3330 (—NH_2) cm^{-1}.

Anal.—Calc. for $C_{17}H_{12}N_2O_4S$: C, 59.99; H, 3.56; S, 9.42. Found: C, 60.27; H, 3.88; S, 9.33.

Compound VII—A suspension of II (4.5 g) in acetic acid (60 ml, 70%) was stirred on a steam bath, and iron powder (4 g) was added portionwise. After 4 hr, the reaction mixture was poured into ice-cold water. The precipitated product was isolated, dissolved in boiling ammonium hydroxide solution, and filtered. The cold filtrate was acidified with acetic acid, and the formed precipitate was collected, washed with water, and dried to give 3 g (73%) of VII, mp 180° (from ethanol).

Anal.—Calc. for $C_{17}H_{14}N_2O_2S$: C, 65.78; H, 4.55; N, 9.04. Found: C, 65.54; H, 4.32; N, 9.28.

Compound VIII—Compound III (2 g) was treated with polyphosphoric acid (20 g) at 140° for 3 hr, and the reaction was poured into ice water. The solid product was isolated, washed with boiling ammonium hydroxide solution, washed with water, and dried to give VIII in 82% yield, mp 165° (from ethanol).

Anal.—Calc. for $C_{17}H_{12}N_2OS$: C, 69.84; H, 4.14; N, 9.58. Found: C, 69.68; H, 4.31; N, 9.36.

Compound IX—A mixture of IV (1.5 g, 0.005 mole) and 2-diethylaminoethyl chloride (1.4 g, 0.11 mole) was heated at 160° for 4 hr. The reaction mixture was boiled with excess water and a few milliliters of dilute hydrochloric acid and then filtered while hot. The cold filtrate was made basic with sodium hydroxide solution. The formed precipitate was collected, washed with water, and dried to give 1.7 g (61%) of IX, mp 144° (from aqueous ethanol).

Anal.—Calc. for $C_{29}H_{38}N_4OS$: C, 72.41; H, 7.81; S, 6.53. Found: C, 72.15; H, 7.63; S, 6.78.

Compound X—A solution of VI (5 g) in acetic acid (30 ml) and acetic anhydride (3 ml) was heated under reflux for 5 hr. The cooled reaction mixture was poured into ice water, and the formed precipitate was collected and recrystallized from aqueous ethanol to give 5 g (89%) of X, mp 246°.

Anal.—Calc. for $C_{19}H_{14}N_2O_5S$: C, 59.69; H, 3.69; N, 7.33. Found: C, 59.38; H, 3.61; N, 7.59.

Compound XI—Reduction of VI (4 g) with acetic acid (50 ml, 60%) and iron powder (4 g), as described for III, gave 3 g (82%) of XI, mp 216° (from aqueous ethanol).

Anal.—Calc. for $C_{19}H_{16}N_2O_3S$: C, 64.77; H, 4.58; N, 7.98. Found: C, 64.45; H, 4.39; N, 8.26.

Compound XII—This compound was prepared by the cyclization of XI with polyphosphoric acid, as described for VIII, in 83% yield, mp 158° (from ethanol).

Anal.—Calc. for $C_{19}H_{14}NO_2S$: C, 71.23; H, 4.40; N, 4.37. Found: C, 70.86; H, 4.13; N, 4.69.

Compound XIII—A mixture of XII (2 g) and 2-diethylaminoethyl chloride (1 ml) was heated at 170° for 4 hr. The reaction mixture was refluxed with dilute hydrochloric acid (20 ml) for 10 hr and then filtered after the addition of water. The cold filtrate was neutralized with aqueous sodium hydroxide. The precipitated product was collected, washed with water, and dried to give 1.8 g (75%) of XIII, mp 106° (from aqueous ethanol).

Anal.—Calc. for $C_{23}H_{25}N_3OS$: C, 70.55; H, 6.44; N, 10.73. Found: C, 70.36; H, 6.47; N, 10.58.

2-Iodo-4-nitro-1-naphthol (17)—To a solution of 4-nitro-1-naphthol (6 g) in acetic acid (50 ml) was added iodine monochloride (5.5 g) in acetic acid (12 ml). The mixture was kept at room temperature for 3 days and then poured into ice water. The precipitated product was collected, washed with water, and dried to give 7 g (70%) of practically pure 2-iodo-4-nitro-1-naphthol, mp 115° (17).

Compound XIV—*Method A* —A mixture of 2-iodo-4-nitro-1-naphthol (4.5 g, 0.0143 mole), thiosalicylic acid (2.2 g, 0.0142 mole), anhydrous sodium carbonate (16 g, 0.015 mole), and amyl alcohol (45 ml) was heated under reflux for 15 hr. The product was isolated as described for VI to give 3.5 g (72%), mp 200° (from ethanol); IR: 700 (—S—), 1505 (—NO_2), 1690 (—COOH), and 3400 (—OH) cm^{-1}.

Anal.—Calc. for $C_{17}H_{11}NO_5S$: C, 59.73; H, 3.25; N, 4.11. Found: C, 60.03; H, 3.44; N, 4.36.

Method B—A solution of VI (2 g) in sodium hydroxide solution (40 ml, 10%) was refluxed for 8 hr. The hot mixture was filtered, cooled, and acidified with dilute hydrochloric acid. The precipitated product was collected and dried to give 1.6 g (80%) of XIV identified by melting point and mixed melting point.

Compound XV—Reduction of XI with iron and acetic acid, as described for VII, gave XV in 74% yield, mp 211° (from aqueous ethanol).

Anal.—Calc. for $C_{17}H_{13}NO_3S$: C, 65.64; H, 4.21; S, 10.31. Found: C, 65.43; H, 4.26; S, 10.53.

Compound XVI—Cyclization of XV with polyphosphoric acid, as described for VIII, and washing with sodium carbonate solution gave XVI in 68% yield, mp 186° (from ethanol).

Anal.—Calc. for $C_{17}H_{11}NO_2S$: C, 69.76; H, 3.78; S, 10.93. Found: C, 69.53; H, 3.61; S, 10.79.

Compound XVII—Compound XVI (2 g) was treated with 2-diethylaminoethyl chloride (1 ml) at 160° for 5 hr. The reaction mixture was boiled with dilute hydrochloric acid and then filtered. The cold filtrate was neutralized with sodium carbonate solution, and the formed precipitate was isolated and dried to give 1.9 g (74%) of XVII, mp 122° (from aqueous ethanol).

Anal.—Calc. for $C_{23}H_{24}N_2OS$: C, 70.38; H, 6.16; N, 7.14. Found: C, 70.16; H, 6.23; N, 7.42.

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* To whom inquiries should be directed. Present address: Medicinska Nobelinstitutet, Biokemiska Avdelningen, Karolinska Institutet, Solnavägen 1, 104 01 Stockholm 60, Sweden.

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B. J. SANDMANN*, R. U. NESBITT, Jr.*, and R. A. SANDMANN

Abstract □ Silver sulfonamides can be successfully recrystallized from strong ammonia solution. The spectral characterization of silver sulfadiazine, silver sulfamethazine, silver sulfanilamide, and silver sulfapyridine indicates that the silver sulfonamide formed on reaction of silver nitrate with the free sulfonamide or its sodium salt is correctly represented by a classical salt structure. Complexations of silver sulfonamides with morpholine yield 1:1 crystalline complexes, which can be analyzed by NMR spectroscopy directly. Spectral studies of the silver sulfonamides were carried out using an indirect NMR technique.

Keyphrases □ Silver sulfadiazine and related compounds—recrystallization and spectral characterization, structure determination □ Silver sulfonamides—recrystallization and spectral characterization, structure determination □ Sulfonamides, silver—recrystallization and spectral characterization, structure determination □ NMR spectroscopy—structure determination, silver sulfonamides

Since 1968, interest in silver sulfadiazine has increased steadily due to its effectiveness in the topical treatment of *Pseudomonas* infections associated with thermal burns. The compound contains both silver with its oligodynamic effect and sulfonamide antibiotic activity to give a broad range of effectiveness against microorganisms. Silver ion in solution is toxic, but salts of silver have been shown to be safe and effective against bacteria in concentrations as small as 1 ppb. When used in combination with, or when reacted with, a sulfonamide, silver offers additional advantages in the treatment of burns. These include the lack of sensitization characteristics of antibiotics usually employed for these conditions, the broader range of antibacterial activity as well as activity against mycotic and viral infections, and the failure of organisms to develop resistant strains to silver-ion activity.

Silver sulfadiazine offers many therapeutic advantages in topical use over other silver salts. Fox (1) reported that, unlike silver nitrate, silver sulfadiazine is odorless, stainless, easy to apply, and painless and often reduces the need for skin grafts. In addition, the compound does not deplete the applied area of essential body salts and, therefore, does not require simultaneous administration of supplementary fluids as is necessary with silver nitrate. Sulfonamides used as sodium salts are also inferior to their silver salts, because the sodium derivatives have been shown to cause extensive tissue damage because of their high alkalinity.

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Table I—Elemental Analyses and Melting Points of Silver Aminosulfonamides

Complex	Melting Point	Analysis, %	Calc.	Found
Morpholine silver sulfadiazine	263–265°	C	37.83	37.59
		H	4.08	4.24
		N	15.75	15.65
Morpholine silver sulfapyridine	220–223°	C	40.65	40.91
		H	4.32	4.46
		N	12.64	12.44

silver sulfonamides. No conclusive evidence about the structures has been reported.

The preparation of crystalline silver sulfonamides has not been indicated since all previous reports refer to silver sulfadiazine and the other silver sulfonamides as colloids or precipitates. The intents of this study were to purify and crystallize the silver sulfonamides prepared and to establish the structures for these compounds using spectral analytical methods. During this investigation, additional reactions of the silver sulfonamides with amines were studied and the results indicate complex formation with different stoichiometry than that previously reported (8). Additional studies on the physical properties of the silver sulfonamides are underway, and the results will be reported subsequently.

EXPERIMENTAL

Equipment—A grating IR spectrophotometer[1] and an NMR spectrometer[2] were used.

Materials and Reagents—All chemicals used were of analytical or reagent grade. Silver nitrate and sodium sulfadiazine were supplied commercially[3].

Sulfadiazine, sulfamethazine, and sulfapyridine were recrystallized from 95% ethanol. The sulfanilamide was recrystallized from distilled water.

Synthesis of Sodium Salts of Sulfonamides—The sodium salts of sulfadiazine, sulfamethazine, sulfapyridine, and sulfanilamide were prepared by dissolving 1.0 g of the sulfonamide base in 7% excess of 5 *N* NaOH and adding distilled water to form a clear solution, which was evaporated to dryness. The sodium salts were recrystallized from 95% ethanol and vacuum dried overnight. IR spectra were obtained using potassium bromide disks.

Synthesis of Silver Salts of Sulfonamides—Silver sulfadiazine was prepared by the method of Braun and Towle (9) and vacuum dried overnight at 40°. The silver salts of sulfamethazine, sulfapyridine, and sulfanilamide were prepared by the method of Rosenzweig and Fuchs (8) and vacuum dried overnight at 40°.

Purification of Silver Sulfonamides—The dried silver sulfonamides were dissolved in ammonium hydroxide with gentle heating, if needed. The solution was filtered and allowed to evaporate partially at room temperature. The colorless crystals remaining after partial evaporation were filtered and washed with distilled water and acetone. The silver sulfadiazine was vacuum dried overnight at 40°. Silver sulfamethazine, silver sulfapyridine, and silver sulfanilamide were dried at 110° for 72 hr. The melting decomposition points of the silver sulfonamides are as follows: silver sulfadiazine, 286–288°; silver sulfamethazine, 298–299°; silver sulfapyridine, 248–250°; and silver sulfanilamide, 212–215°. IR spectra were obtained using potassium bromide disks.

NMR—A procedure for the determination of active hydrogen by NMR was described previously (10), and a similar method was used to study the silver sulfonamides. The NMR spectra of the silver sulfonamides were obtained by preparing a sample tube containing 40 mg of the silver sulfonamide, 0.25 ml deuterated trifluoroacetic acid[4] (CF_3COOD), and 4 mg of sodium 2,2-dimethyl-2-silapentane-5-sulfonate[4] (I) as the reference standard. Another sample tube was prepared containing 0.25 ml deuterated trifluoroacetic acid and 4 mg of I as the reference standard.

The NMR spectrum of both samples was scanned up to 980 Hz. The number of exchangeable hydrogens was obtained by integrating the residual OH peak at 11–12 ppm of deuterated trifluoroacetic acid in both samples at a constant instrument setting. These integrations were carried out by successively removing and replacing the sample tubes for each integration. By subtracting the average height of the residual OH peak in deuterated trifluoroacetic acid from the average height of the residual OH peak in the silver sulfonamide spectrum and dividing by the height contributed by one hydrogen as determined from the average integral heights of the other peaks on the spectrum, the number of active hydrogens was determined.

Synthesis of Silver Aminosulfonamides—One gram of the silver sulfonamide was dissolved in 40 ml of warm morpholine[5]. The solution was filtered and the complex was forced out of solution with approximately 100 ml of anhydrous ether[5] and recrystallized from a morpholine–ether solvent system. The elemental analyses and melting points of the silver aminosulfonamides are given in Table I. NMR spectra of the complexes were obtained in deuterated dimethyl sulfoxide[4] with scanning up to 980 Hz.

Table II—Asymmetric SO_2 Stretching Vibrational Frequencies (Centimeters^{-1})

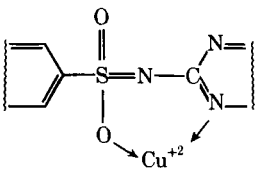
Compound	Free Base	Sodium Salt	Silver Salt
Sulfanilamide	1320	1190	1250
Sulfadiazine	1330	1430	1425
Sulfamethazine	1315	1430	1425
Sulfapyridine	1270	1330	1310

RESULTS AND DISCUSSION

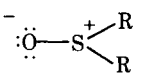
IR—Spectral identification of the structures of the silver sulfonamides requires a pure and preferably crystalline material and solution of the compound in the analytical solvent. Previously prepared silver sulfonamides have been colloidal in nature and insoluble in the commonly used spectroscopic solvents.

The IR spectra of sulfonamides reacted with copper ion were investigated (11) and indicated a difference in the SO_2 absorption of the free sulfonamides when compared to the copper reaction product. The proposed chelate structure, II, for sulfadimethoxine, sulfamerazine, and sulfadimethoxypyridine was based on SO absorption band shifts from 1316 to 1282 cm^{-1}, indicating coordination of the metal with oxygen. This shift to a lower SO stretching frequency is seen with metal complexes with sulfoxides where Structure III dominates where there is oxygen coordination (12). It seemed reasonable to investigate the IR spectra of the silver sulfonamides to determine if a similar SO shift is seen with the silver as was reported with copper (Table II).

The shifts in the SO bands of the silver and sodium salts are similar. The SO shifts for sulfanilamide are toward lower frequency



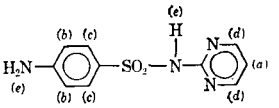
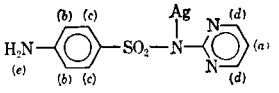
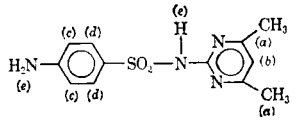
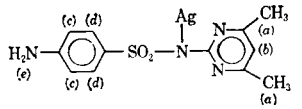
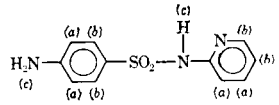
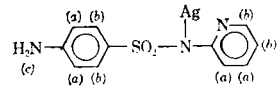
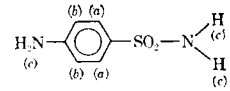
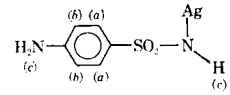
II



III

[1] Perkin-Elmer 457.
[2] Varian T60.
[3] Marion Laboratories, Kansas City, Mo.
[4] Merck Sharp and Dohme of Canada Limited.
[5] Fisher Scientific Co., Fair Lawn, N.J.

Table III—NMR Spectra of Silver Sulfonamide Analogs (Parts per Million) (in Deuterated Trifluoroacetic Acid Using I as the Reference)[a]

Compound	*a*	*b*	*c*	*d*	*e*	Number of Active Hydrogens per Mole	
						Theoretical	Found
	7.60 t	7.90 d	8.46 d	9.03 d	11.75 s	3	2.67
	7.30 t	7.80 d	8.36 d	8.93 d	11.75 s	2	1.67
	2.72 s	7.13 s	7.83 d	8.35 d	11.56 s	3	3.00
	2.66 s	7.0 s	7.70 d	8.21 d	11.9 s	2	2.13
	7.83 m	8.36 m	11.53 s	—	—	3	3.01
	7.83 m	8.35 m	11.68 s	—	—	2	1.89
	7.75 d	8.13 d	10.86 s	—	—	4	3.97
	7.71 d	8.15 d	11.35 s	—	—	3	2.64

[a] s = singlet, d = doublet, m = multiplet, and t = triplet.

for the salts, whereas the shifts for the other sulfonamides are toward higher frequency. The shift to lower frequency for silver and sodium sulfanilamide could indicate possible oxygen coordination with the silver and sodium ions in sulfanilamide. The SO shifts to higher stretching frequency for salts of sulfadiazine, sulfamethazine, and sulfapyridine indicate little if any oxygen coordination with the sodium or silver ions. Therefore, these data suggest that the silver salts of substituted sulfonamides do not involve coordination with oxygen but have a classical salt structure similar to the sodium salts.

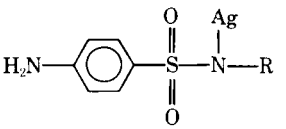
The amide–amine region of the IR spectra was investigated to determine the presence or absence of the amide hydrogen in silver sulfadiazine. This region for the silver and sodium sulfonamides was very similar to that of the free sulfonamide base. Thus, it was impossible to determine the presence or absence of the amide hydrogen on the basis of IR data alone.

NMR—Because the results obtained from the IR data were inconclusive relative to the presence of an amide hydrogen in the silver sulfonamides, NMR studies were undertaken. NMR studies of the free sulfonamide bases in deuterated dimethyl sulfoxide are well documented (13).

Deuterated dimethyl sulfoxide did not prove to be a suitable solvent for the silver sulfonamide salts. Various amines were tried as solvents, but none provided the solubility needed to obtain a NMR spectrum. Deuterated trifluoroacetic acid was found to be the solvent of choice. The exchange of the deuterium of the acid with the active hydrogens of the sulfonamides was utilized as a method for counting the number of active hydrogens in the compounds. The acid also has the advantage of forming stable solutions with the silver sulfonamides studied; in addition, the residual OH peak at 11.34 ppm of the acid is easily integrated because of its position downfield from the rest of the spectrum.

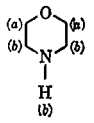
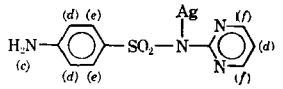
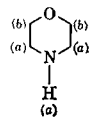
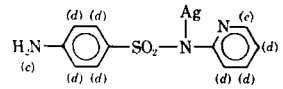
The exchange reactions occur rapidly enough to give one peak for the exchanged protons. In this case the integrated peak height at 11–12 ppm minus the integrated peak height of the residual OH in the deuterated trifluoroacetic acid at 11.34 ppm represents the total active hydrogen content of the silver sulfonamide. The results (Table III) indicate the complete displacement of the amido hydrogen by the silver in the reaction of a solution of silver nitrate with a solution of the free sulfonamide base or a solution of the sodium salt of the sulfonamide.

Additional NMR data of the silver sulfonamides complexed with morpholine were studied. The morpholine complexes of the prepared silver sulfonamides were synthesized to make them soluble in deuterated dimethyl sulfoxide. Only the morpholine complexes of silver sulfadiazine and silver sulfapyridine were soluble



IV

Table IV—NMR Spectra of Silver Sulfonamide Morpholine Complex (Parts per Million) (in Deuterated Dimethyl Sulfoxide Using I as the Reference)[a]

Compound	*a*	*b*	*c*	*d*	*e*	*f*
	2.9 m	3.7 m	5.6 s	6.75 m	7.78 d	8.51 d
						
	2.9 m	3.7 m	5.8 m	7.1 s	—	—
						

[a] s = singlet, d = doublet, m = multiplet, and t = triplet.

enough in deuterated dimethyl sulfoxide to obtain a NMR spectrum. Rosenzweig and Fuchs (8) indicated that the silver sulfonamides form a dimorpholinamino complex. The data (Tables III and IV) indicate that only a monomorpholinamino complex is formed under the experimental conditions used. These data also suggest that the amido hydrogen is absent in the silver sulfadiazine–morpholine complex.

It was concluded from the studies using deuterated trifluoroacetic acid and complexation data that the amide hydrogen is not present in silver sulfonamides and that Structure IV represents the compound.

SUMMARY

The first successful crystallization of silver sulfonamides has been achieved. The spectral characterization of these compounds, including silver sulfadiazine, silver sulfamethazine, silver sulfanilamide, and silver sulfapyridine, indicates that silver ion reacts with the sulfonamide displacing the amide hydrogen to give a classical silver sulfonamide salt.

Complexation studies of silver sulfonamides with morpholine indicate that one morpholine reacts with one silver sulfonamide to give a stable complex which is soluble in deuterated dimethyl sulfoxide. Although the morpholine complexes could be analyzed directly with NMR solvents, the silver sulfonamides could only be analyzed by an indirect method. IR data were inconclusive in establishing the structures of the silver sulfonamides.

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* Fellow, American Foundation for Pharmaceutical Education.

x To whom inquiries should be directed.

Effect of Cadmium *In Vivo* on Glucose Catabolism in Normal and Alloxan Diabetic Rats

DIONYSSIS S. ITHAKISSIOS*§, WAYNE V. KESSLER*x, JAMES N. ARVESEN‡, and GORDON S. BORN*

Abstract □ A radiorespirometry experiment was conducted to determine the effect of single and multiple doses of cadmium on the catabolism of D-glucose-^{14}C (uniformly labeled) in normal and alloxan diabetic rats by the collection of $^{14}CO_2$. Cumulative amounts of $^{14}CO_2$ were determined at 1, 3, 6, and 12 hr. The effect of cadmium on glucose catabolism was related to the administered dose as well as to the manner of dosing, single or repeated.

Keyphrases □ Cadmium—effect *in vivo* on glucose catabolism, normal and alloxan diabetic rats, radiorespirometry, single and multiple doses □ Glucose catabolism, *in vivo*—effect of single and multiple doses of cadmium, normal and alloxan diabetic rats, radiorespirometry □ Radiorespirometry—determination of effect of single and multiple doses of cadmium on glucose catabolism, normal and alloxan diabetic rats

An increasing amount of cadmium in the environment is thought to be a risk to the general population. The human hazard usually results after long-term accumulation of relatively small daily increments of cadmium in the microgram range. It has been reported that cadmium alters *in vitro* the activity of certain enzymes (1–5), decreases insulin secretion (6, 7), and produces changes in blood glucose levels (2).

In previous work (8), multiple doses of cadmium did not produce differences in blood glucose or insulin levels, although cadmium did increase the evolution of respiratory $^{14}CO_2$ in rats catabolizing D-glucose-^{14}C (uniformly labeled). It was of interest to investigate further the influence of single and multiple doses of this metal on the rate and extent of evolution of respiratory $^{14}CO_2$ in normal rats. Alloxan diabetic rats were also used to determine if cadmium can disturb the amount of respiratory CO_2 when the amount of insulin present is decreased.

EXPERIMENTAL

Design and Procedures—Forty-two female rats[1] from the same shipment and weighing 195–205 g were used. Four normal and three alloxan diabetic groups, each containing six animals, were established by random assignment. One animal from each group was tested on one of six alternating days. Within each group, the day for testing was established by random assignment. Prior to experimentation, the animals were housed individually in metal cages for 10 days. They were allowed free access to food and tap water but were fasted 1 night before testing to decrease the endogenous glucose level.

Treatments for two normal and two alloxan diabetic groups consisted of either a single dose of 1.0 mg cadmium/kg or a multiple dose of 0.75 mg/kg given for 4 consecutive days, including the day for testing. In addition, a 3.0-mg/kg dose was chosen for one normal group to examine the effect of a single large dose. This dose was not used in the alloxan diabetic group because only seven metabolism chambers were available. The remaining groups served as controls and received saline solution. During the period of multiple dosing, animals receiving single doses were given saline solution.

Table I—Effect of Cadmium on $^{14}CO_2$ Expiration by Rats Metabolizing D-Glucose-^{14}C

Time Interval, hr	Dose, mg Cadmium/kg				
	Normal			Alloxan Diabetic	
	1.0	3.0	4 × 0.75	1.0	4 × 0.75
0–1	NS[a]	NS	NS	NS	NS
0–3	*[b]	NS	**[c]	**	**
0–6	*	NS	**	**	**
0–12	*	NS	**	**	**

[a] Nonsignificant. [b] Significant at the $p = 0.05$ level. [c] Significant at the $p = 0.01$ level.

The cadmium was given intraperitoneally as cadmium acetate dissolved in water. The dose was based on cadmium ion and not on cadmium acetate. The single dose or the last of the multiple doses was followed at once by 3.0 ml *per os* of a 50% (w/v) glucose-water solution containing 5 μCi/3.0 ml of D-glucose-^{14}C (uniformly labeled). Immediately after dosing, the animals were placed in metabolism chambers where neither water nor food was provided. The $^{14}CO_2$ was collected for 12 hr, with the trapping solutions being changed and sampled at 1, 3, 6, and 12 hr. At the end of the collection, the animals were sacrificed and discarded.

Alloxan Diabetic Rats—The diabetic condition was produced by intravenous injection of a 10% aqueous solution of alloxan[2], injected within 10 min after its preparation. The dose administered was 55 mg/kg. Blood samples for glucose determination were obtained by orbital sinus puncture 36 hr after alloxan dosing. Plasma glucose levels were determined enzymatically by a glucose oxidase method[3]. The animals were considered to be diabetic when their plasma glucose level was 350 mg % or greater. All diabetic animals received alloxan 4 days before administration of labeled glucose.

Metabolism Chambers and Radioactivity—Seven chambers were used in the manner previously described (8). Determination of the radiochemical purity of the labeled glucose, which was greater than 99%, and counting of the $^{14}CO_2$ samples were also identical.

RESULTS AND DISCUSSION

The results (Figs. 1 and 2) were analyzed by a two-factor analysis of variance (treatment and time) to assess the differences between the control and treatment groups. The analysis was run separately for the normal and the alloxan diabetic animals and was performed using the square roots of the cumulative amounts, in disintegrations per minute, of $^{14}CO_2$ collected. The square roots yielded a response with a variance approximately independent of the mean. Since the F values for the treatment by time interaction were significant, a Newman-Keuls comparison was done on the cell means (9). At the end of each time interval, the results for the control and the treatments were tested for significant dif-

[1] Sprague-Dawley descendants, Laboratory Supply Co., Indianapolis, Ind.

[2] Alloxan monohydrate (Lot 31C-0220), Sigma Chemical Co., St. Louis, Mo.

[3] ERA model 2001, Beckman glucose analyzer, Fullerton, Calif.

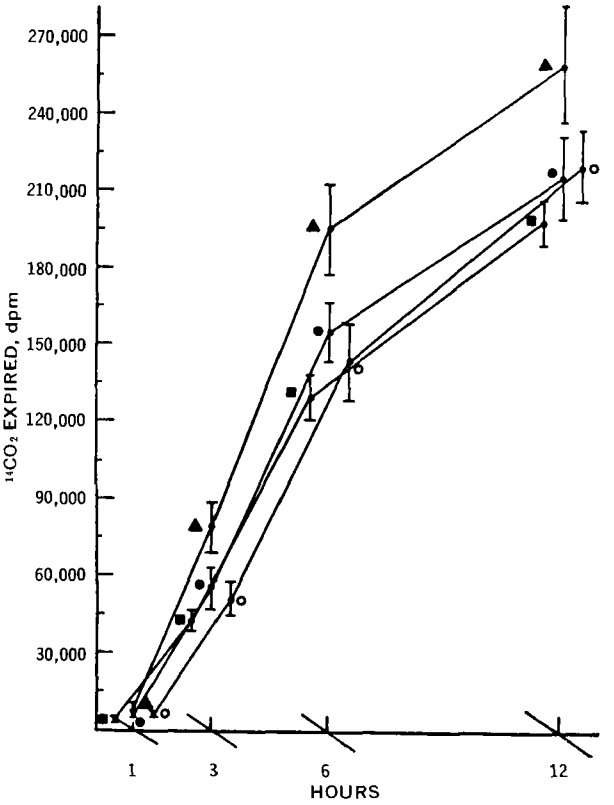

Figure 1—*Cumulative amount of $^{14}CO_2$ expired by normal rats receiving* D-*glucose-^{14}C. Data points are staggered to include error bars which indicate ±1* SD. *Key:* ●, *control;* ■, *1.0 mg/kg;* ○, *3.0 mg/kg; and* ▲, *0.75 mg/kg daily for 4 days.*

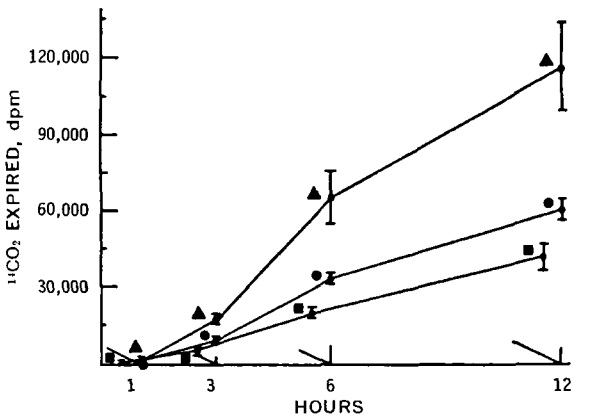

Figure 2—*Cumulative amount of $^{14}CO_2$ expired by alloxan diabetic rats receiving* D-*glucose-^{14}C. Data points are staggered to include error bars which indicate ±1* SD. *Key:* ●, *control;* ■, *1.0 mg/kg; and* ▲, *0.75 mg/kg daily for 4 days.*

ferences at the $p = 0.01$ and $p = 0.05$ levels (Table I). In the first time interval, there were no significant differences in the amounts of $^{14}CO_2$ produced. This was probably related to the availability of labeled glucose at the site of catabolism.

The data for the single doses of cadmium in the normal animals showed that the cadmium effect on glucose catabolism was related to the administered dose. Rats receiving the 1.0-mg/kg dose produced significantly ($p = 0.05$) smaller amounts of $^{14}CO_2$ at the 3-, 6-, and 12-hr intervals, due perhaps to an increase in endogenous glucose levels after administration of cadmium. Mechanisms by which this increase can be explained are: *(a)* cadmium directly stimulates glycogenolysis (2), *(b)* cadmium stimulates release of epinephrine from the adrenals which induces hepatic glycogenolysis and inhibits insulin secretion from pancreatic β-cells (10), and *(c)* cadmium decreases somewhat the hypoglycemic effect of insulin (11). An increase in blood glucose level would decrease the specific activity of the $^{14}CO_2$ because of a decrease in labeled glucose utilization.

With the 3.0-mg/kg dose, there was no significant difference in the amount of $^{14}CO_2$ expired when compared to control at any time interval. Increasing the dosage of cadmium may affect, in addition, a number of enzymes which introduce further changes in glucose catabolism mechanisms and increase the $^{14}CO_2$ evolution rate. Affected mechanisms may involve glycolysis and the tricarboxylic acid cycle.

Increased $^{14}CO_2$ evolution ($p = 0.01$) was found at the 3-, 6-, and 12-hr intervals when 3.0 mg/kg was given in four multiple doses of 0.75 mg/kg. This same effect was seen (8) when 20 doses of 0.25 mg/kg were given over 40 days. The increase in $^{14}CO_2$ evolution with the multiple dose may be due to an uncoupling effect of cadmium on oxidative phosphorylation (12), which increases the rates of glycolysis and the tricarboxylic acid cycle.

From the data in Figs. 1 and 2, it can be seen that the diabetic animals produced decreased amounts of $^{14}CO_2$ in comparison to the normal animals. This was expected because of the higher concentration of glucose in the blood of diabetic animals due to a lack of insulin, with a resulting decrease in labeled glucose utilization. However, except for this overall decrease in labeled glucose utilization in the diabetic animals, cadmium affected evolution of $^{14}CO_2$ in a similar way in both normal and diabetic animals. That is, a single dose of 1.0 mg/kg decreased ($p = 0.01$) $^{14}CO_2$ evolution and the multiple dose increased ($p = 0.01$) $^{14}CO_2$ evolution in both cases. These results indicate that cadmium affects glucose catabolism by a mechanism independent of the presence of insulin.

The results of this study indicate the *in vivo* effect of cadmium on carbohydrate catabolism in relation to the amount of the administered dose and the manner in which the dose is given. Further investigations on the specific pathways and the enzymes involved are needed.

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§ Fellow, Purdue Research Foundation. Present address: Nuclear Products Division, 3M Center, St. Paul, MN 55101

ˣ To whom inquiries should be directed.

Determination of Methyldopa in Pharmaceutical Dosage Forms and Biological Fluids Based on Oxidation at the Tubular Carbon Electrode

JAMES T. STEWART[x], HOO C. LO*, and WILLIAM D. MASON‡

Abstract □ A method for the determination of methyldopa in pharmaceutical dosage forms based on electrochemical oxidation at the tubular carbon electrode is presented. The method is highly specific and may be used to analyze methyldopa in the presence of other types of pharmaceuticals. A comparison with two other colorimetric procedures of analysis shows the new method to have fewer manipulative steps with comparable precision and accuracy. In addition, the utility of the method in determining methyldopa in urine, plasma, and whole blood is demonstrated.

Keyphrases □ Methyldopa—method for determination in pharmaceutical dosage forms, urine, plasma, and whole blood, electrochemical oxidation, tubular carbon electrode, compared to compendial and modified colorimetric procedures □ Oxidation, electrochemical—use of tubular carbon electrode for determination of methyldopa in pharmaceutical dosage forms, urine, plasma, and whole blood □ Tubular carbon electrode—determination of methyldopa in pharmaceutical dosage forms and biological fluids

Various chemical methods for the determination of methyldopa are available. Paper chromatography, potentiometric titrimetry in a nonaqueous medium, UV spectrophotometry, colorimetry, and fluorescence have been applied to the determination of methyldopa in dosage forms and biological fluids (1–7). Although highly sensitive and specific, these methods involve separation and other manipulative steps including controlled reactions for color or fluorescence formations (2–4, 6, 7). A possible alternative method for methyldopa may be an electrochemical method similar to the ones presented for ascorbic acid and levodopa (8, 9).

The methods for ascorbic acid and levodopa are based on continuous analysis in flowing streams by oxidation of the drugs at the tubular carbon electrode (TCE) (10). This electrochemical method has the advantages over other commonly employed methods of being extremely fast and simple without any significant loss in accuracy and precision. Studies (9, 11) of the electrochemical oxidation of levodopa and catecholamines suggested that methyldopa may be assayed in this manner. Thus, an investigation of the oxidation of methyldopa at the TCE was undertaken to develop an analytical method. In this paper a method for the determination of methyldopa in pharmaceutical dosage forms is presented and compared to the official USP method and a colorimetric procedure. In addition, the utility of the new electrochemical method in determining methyldopa in urine, plasma, and whole blood is demonstrated.

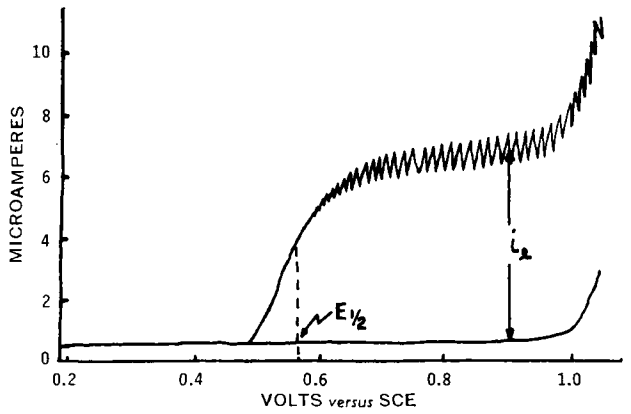

Figure 1—*Methyldopa current–voltage curve using a 10^{-4} M solution in 1 N hydrochloric acid, a flow rate of 7 ml/min, and a scan rate of 0.2 v/min.*

EXPERIMENTAL

Apparatus—The electrode assembly, flow system, pump, and polarography system were the same as reported previously (8).

Chemicals—All chemicals were commercially available and were utilized as received. Standard solutions of methyldopa[1] were prepared by dissolving weighed amounts of the powder in aqueous 1 *N* hydrochloric acid.

Procedures for Determining Methyldopa—Current–voltage curves were determined using a TCE which had previously been cleaned with ethyl acetate. A volume flow rate of 7 ml/min was commonly employed and was controlled to ±1%. The voltage was scanned anodically from zero volt *versus* the saturated calomel electrode (SCE) at 0.20 v/min. Calibration plots of limiting current *versus* methyldopa concentrations were prepared and used directly to calculate drug concentration in unknown samples.

Method A: Analysis of Solid Dosage Form—Tablets were weighed and powdered, and a weighed aliquot was dissolved in 1 *N* hydrochloric acid to give approximately a 10^{-4} *M* solution. This solution was filtered, if necessary, and then pumped directly to the TCE for analysis.

Method B: Analysis in Urine—A 10-ml sample of urine containing 10^{-4} *M* methyldopa was diluted to 100 ml with distilled water. The pH of the solution was adjusted to 2.5 with concentrated hydrochloric acid and passed through a column containing 5 ml of cation-exchange resin[2] at a flow rate of 1.5 ml/min. The resin was previously prepared for use by washing with 100 ml of 4 *N* hydrochloric acid, followed by washing with distilled water until neutral to litmus. Methyldopa was then eluted from the column by the addition of 250 ml of 1 *N* hydrochloric acid solution. The acid solution was pumped directly to the TCE for analysis. A blank urine sample was also prepared by this method.

Method C: Analysis in Plasma—A 1-ml sample of plasma containing 10^{-4} *M* methyldopa was diluted to 100 ml with 1 *N* hydrochloric acid solution. The solution was pumped directly to the TCE for assay. An appropriate blank solution was also prepared.

Method D: Analysis in Whole Blood—A 2-ml sample of rabbit whole blood containing 10^{-4} *M* methyldopa was centrifuged for 30 min. One milliliter of the plasma was diluted to 100 ml with 1 *N* hydrochloric acid and pumped directly to the TCE for analysis. An appropriate blank sample was also prepared.

[1] Merck Sharp and Dohme Laboratories, Rahway, N.J.

[2] Amberlite IR-120, 20–50 mesh, hydrogen form, Mallinckrodt Chemical Works, St. Louis, Mo.

Table I—Methyldopa Analysis

Product	Amount Declared per Sample, mg	Percent Recovery ± SD[a]		
		TCE Method[b]	Colorimetric Method[c]	Colorimetric Method[d]
A[e]	25.0	100.26 ± 0.60	98.77 ± 1.07	98.00 ± 2.90
B[f]	25.0	98.73 ± 1.55	98.15 ± 3.20	98.53 ± 1.38
C[g]	25.0	98.73 ± 1.55	99.08 ± 1.30	98.70 ± 1.83

[a] Calculated on basis of three assays of a single aliquot. [b] TCE method with 1 *N* HCl as medium. [c] Ref. 7. [d] Ref. 12. [e] Commercial tablet containing methyldopa. [f] Commercial tablet containing methyldopa plus hydrochlorothiazide. [g] Commercial tablet containing methyldopa plus chlorothiazide.

To determine if other antihypertensive drugs or compounds commonly found in dosage forms with methyldopa alter its current–voltage curve or are oxidized at the TCE, the following studies were performed. Solutions of guanethidine monosulfate, hydrochlorothiazide, chlorothiazide, triamterene, chlorthalidone, hydralazine hydrochloride, reserpine, and phenobarbital (10^{-4} *M*) were prepared with and without methyldopa. Each solution was pumped through the TCE, and the voltage was scanned from 0 to 1.0 v *versus* the SCE.

RESULTS AND DISCUSSION

A typical current–voltage curve for the oxidation of methyldopa at the TCE is shown in Fig. 1. The limiting current was linear with respect to concentration over the 10^{-3}–10^{-5} *M* range. With the volume flow rate at 7 ml/min, a typical calibration plot has a slope of 6.2 μamp/10^{-4} *M* with a zero intercept. A plot of the logarithm limiting current *versus* logarithm flow rate has a slope of 0.30, which agrees with theoretical considerations of the tubular electrode geometry (11).

Current–voltage scans on 10^{-4} *M* solutions of the compounds listed under *Experimental* showed that only hydralazine hydrochloride and reserpine interfere with the analysis of methyldopa. The other compounds listed did not show an oxidation wave between 0 and 1 v *versus* the SCE.

The accuracy of the TCE method in determining methyldopa content of solid dosage forms is shown in Table I. The new method gives data in agreement with existing colorimetric procedures (7, 12) with comparable precision. The sensitivity of the TCE method as well as the colorimetric procedures is in the range of 10^{-4}–10^{-5} *M*. In comparison with the colorimetric methods, the TCE procedure was faster and simpler. Once the sample solutions were prepared, 25–30 determinations could be run each hour. The reproducibility of the TCE, as determined by repeatedly assaying a single sample dilution, is indicated by a deviation of the mean of 0.95% for five determinations.

A study of the analysis of methyldopa in urine, plasma, and whole blood was performed utilizing the new TCE method. In the determination of urine samples, a cation-exchange resin procedure (13) was employed to separate methyldopa from oxidizable components present in urine and gave polarographic waves between 0 and 1.0 v that interfered with the analysis of the drug. The separation was achieved by adjusting the pH of the urine sample containing methyldopa to 2.5 and passing the solution through the resin. Subsequent elution with 1 *N* hydrochloric acid gave an analytical solution that was free of the interfering species. A blank urine sample prepared by the same method gave identical results. The use of 1 *N* hydrochloric acid solution was ideal in many ways; not only was it satisfactory for the elution of methyldopa from the resin, but it also provided the necessary electrolyte and pH for the successful analysis of the drug as predetermined in the development of the TCE method. Percent recovery of methyldopa from urine samples was 89.86 ± 0.62%.

A resin cleanup procedure was not required for plasma and whole blood determinations since background current for blank samples did not reveal any interferences from components present in the biological fluids. Quantities of methyldopa (10^{-4} *M*) were added to plasma and whole blood samples. In whole blood determinations, samples were centrifuged and analyzed. Percent recoveries of drug from plasma and whole blood samples were 99.67 ± 0.40 and 79.57 ± 0.40%, respectively. The differences in recovery percentages between plasma and whole blood suggest that there may be some binding of methyldopa to components present in whole blood. Pretreatment of whole blood samples to cause hemolysis of blood cells and/or the use of a protein denaturant should increase the percent recovery of the drug.

The preparation of samples is rapid for plasma and whole blood and somewhat slower for urine due to passage of the sample through the cation-exchange column. Nevertheless, the analysis of methyldopa in these biological samples by the TCE method is convenient, fast, and simple with acceptable precision and accuracy.

Although automation was not employed in this study, the method may readily be incorporated into automated or semiautomated systems because it employs continuous analysis in a flowing stream of sample.

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* National Science Foundation Undergraduate Research Participant.

‡ Present address: School of Pharmacy, University of Missouri–Kansas City, Kansas City, MO 64110

ˣ To whom inquiries should be directed.

Effect of pH and Citrate Ions on Adsorption of Benzoic Acid by Sulfamethazine

R. N. NASIPURI and SALEH A. H. KHALIL ˣ

Abstract □ The adsorption of benzoic acid on sulfamethazine particles was found to be pH dependent. At pH values above 4.2 (the pKa of benzoic acid), a gradual suppression of the adsorption occurred; at pH 4.9 and above, no adsorption was noted. The effect of sodium citrate on adsorption is attributable to pH and ionic strength effects. Citric acid had an insignificant effect on the adsorption. The role of pH and citrate ions on the dissolution of sulfamethazine in this system is discussed.

Keyphrases □ Benzoic acid—adsorption on sulfamethazine, pH and citrate-ion effects □ Sulfamethazine—benzoic acid adsorption, pH and citrate-ion effects □ Sulfonamides—adsorption of benzoic acid on sulfamethazine, pH and citrate-ion effects

The spoilage of the BPC mixture of sulfamethazine (sulfadimidine) for infants was attributable to possible adsorption of the preservative, benzoic acid, by the suspended sulfamethazine particles (1). The adsorption isotherm for benzoic acid on sulfamethazine and the suppressive effect of three hydrophilic polymers on the adsorption were studied (2). Both the equilibrium solubility and dissolution rate of sulfamethazine were remarkably inhibited due to saturation of the sulfonamide surface by adsorbed benzoic acid (3). The present article reports the effect of pH and citrate ions on the adsorption–dissolution relationship in the sulfamethazine–benzoic acid system.

EXPERIMENTAL

The materials used and the methods adopted were as previously reported (3). Adjustment of pH was made by the addition of a predetermined volume of either 0.05 *N* NaOH or 0.05 *N* HCl to the benzoic acid solution, and the pH value of the final system was checked[1]. In studying the effect of citrate ions, either sodium citrate[2] or citric acid[2] (both AR grade) was dissolved in the benzoic acid solution before the latter was added to sulfamethazine powder. All experiments were carried out at 24 ± 0.2°.

RESULTS AND DISCUSSION

Figure 1 shows the effect of citric acid and sodium citrate on the adsorption of 0.1 g % benzoic acid by sulfamethazine. In the concentration range studied, citric acid had no apparent effect on benzoic acid adsorption in systems containing varying concentrations of sulfamethazine. The incorporation of sodium citrate resulted in a gradual suppression of the adsorption, the effect being dependent on sodium citrate concentration. Complete suppression of adsorption was noted in the presence of about 1.7 mmoles/liter. The difference between the effect of citric acid and sodium citrate was attributable to changes in the pH of the system. The effect of pH on adsorption was, therefore, investigated. The results (Fig. 2) show that benzoic acid adsorption by sulfamethazine is pH dependent. An increase in pH value produced a suppression of adsorption, and almost complete suppression occurred at and above pH 4.9. No change in adsorption was observed at pH values below 3.1.

[1] Pye pH meter, model 291.
[2] BDH Chemicals Ltd., Poole, England.

Table I—Percentages of Benzoic Acid Adsorbed at Various pH Values with and without Sodium Citrate[a]

	Sulfamethazine Concentration, g %					
	0.1		0.2		0.4	
pH	I[b]	II[c]	I[b]	II[c]	I[b]	II[c]
3.5	35.8	30.6	63.1	48.2	76.4	73.8
3.8	36.0	28.1	60.8	40.3	56.5	54.0
4.0	34.6	26.8	47.9	35.1	48.3	45.3
4.2	34.2	24.3	36.9	29.0	39.1	36.1

[a] Initial concentration of benzoic acid = 0.1 g %. [b] I = with sodium citrate. [c] II = without sodium citrate.

The data plotted in Fig. 1 are similar to those of Fig. 2 in that adsorption was unchanged in systems containing citric acid (pH <3.1) while adsorption was suppressed in the presence of sodium citrate due to an increase in pH. That the role of sodium citrate on the adsorption is not solely due to pH effect is shown in Table I, where the percentages of benzoic acid adsorbed are shown for systems with and without citrate ions. At an identical pH value, the presence of citrate ions produced an increase in adsorption, possibly due to ionic strength effects. Changes in pH values affect both the degree of ionization of benzoic acid and the net charge on the sulfamethazine surface. Within the pH range of adsorption studied (pH 2.1–3.1, Fig. 2), sulfamethazine with an acidic pKa of 7.4 and a basic pKa of 2.2 (determined electrometrically at 24°) will be positively charged; the extent of ionization of the amino group is 55.7 and 11.2% at pH 2.1 and 3.1, respectively. Benzoic acid (pKa = 4.2), on the other hand, exists predominantly in the unionized form in the pH range of adsorption.

It is suggested, therefore, that the adsorption process involves the unionized form of benzoic acid since no adsorption was noted at pH values above 4.9. Clarke and Armstrong (4) found that, in the adsorption of benzoic acid on kaolin, the negatively charged anion was adsorbed on the clay. As shown in Figs. 1 and 2, benzoic acid adsorption was not altered in the 2.1–3.1 pH range in spite of the change in the ionization of the amino group from 55.7 to 11.2%. Sulfadiazine, sulfathiazole, and sulfapyridine also did not adsorb benzoic acid to any significant extent.

From the foregoing, it is assumed that the adsorption of benzoic acid by sulfamethazine is of a physical type and probably in-

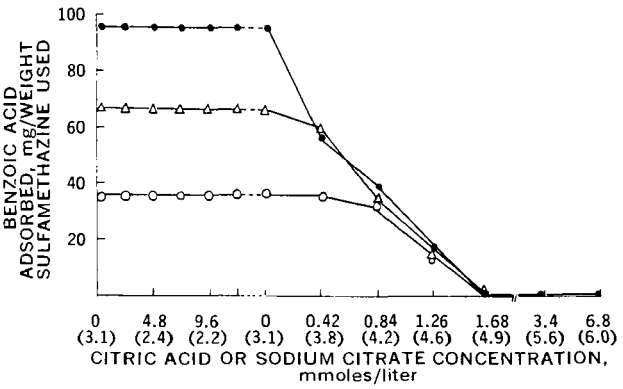

Figure 1—*Effect of citric acid and sodium citrate on the adsorption of 0.1 g % benzoic acid by sulfamethazine. The concentrations of sulfamethazine in the system (g %) were: ○, 0.1; △, 0.2; and ●, 0.3, 0.4, 4, and 10. Figures in parentheses are the pH values of the systems.*

Table II—Effect of Sodium Citrate on Benzoic Acid Adsorption and Sulfamethazine Solubility[a]

Sodium Citrate Concentration, mmoles/liter	Sulfamethazine Concentration, g %							
	Benzoic Acid Adsorbed, mg %				Sulfamethazine Solubility, mg %			
	0.1	0.2	4	10	0.1	0.2	4	10
0	36.4	67.6	95.6	95.8	4.4	4.8	39.8	40.4
0.42	36.0	60.8	54.5	60.7	7.2	30.8	40.3	40.3
0.84	34.2	36.9	39.3	38.8	21.9	36.2	40.6	39.4
1.68	1.3	1.4	1.1	3.1	39.6	39.8	39.6	40.1
3.36	1.1	0	0	0	40.3	40.4	40.5	40.6
5.04	0	0	0	0	40.1	39.7	39.9	39.4
6.72	0	0	0	0	39.8	40.3	39.7	39.8

[a] Initial concentration of benzoic acid = 0.1 g %.

Table III—Effect of Citric Acid on Benzoic Acid Adsorption and Sulfamethazine Solubility[a]

Citric Acid Concentration, mmoles/liter	Sulfamethazine Concentration, g %							
	Benzoic Acid Adsorbed, mg %				Sulfamethazine Solubility, mg %			
	0.1	0.2	4	10	0.1	0.2	4	10
0	36.8	68.1	95.8	96.1	4.6	4.9	40.3	39.6
0.6	36.4	67.7	95.6	94.8	4.1	5.2	46.1	45.8
1.2	36.2	67.2	96.1	95.8	4.3	6.4	50.3	52.1
2.4	35.9	66.9	95.5	96.2	6.6	6.8	56.9	55.9
4.8	36.2	68.1	95.8	94.8	7.0	7.0	65.1	66.4
7.9	36.1	67.9	93.9	95.1	7.3	6.9	73.1	74.0
9.6	36.1	66.8	94.7	95.8	7.8	7.6	80.7	81.3
12.0	35.8	67.4	95.1	96.3	8.1	8.0	84.1	85.5

[a] Initial concentration of benzoic acid = 0.1 g %.

volves the unionized form of the acid and the dimethylpyrimidine moiety of the sulfonamide.

In the concentration range of 0.1–0.3 g % sulfamethazine, benzoic acid adsorption was proportional to the amount of sulfamethazine in the system. At pH 3.1, benzoic acid (0.1 g % initial concentration) was adsorbed to the extent of about 34 mg/0.1 g sulfonamide (or 0.76 mmole/mmole). This constant value represents the "saturation" of the sulfonamide surface by the adsorbed benzoic acid. At and above 0.3 g % sulfamethazine, the amount of benzoic acid adsorbed was constant, approaching about 96 mg.

The effect of pH on the dissolution of sulfamethazine in the presence of 0.1 g % benzoic acid is plotted in Fig. 3. The dissolution plot of the sulfonamide in the absence of benzoic acid is shown for comparison. Depending on the sulfamethazine content in the system, the pH produced either an increase or a decrease in the equilibrium solubility of sulfamethazine. As previously reported (3), when the sulfonamide surface becomes saturated with the adsorbed benzoic acid, inhibition of sulfamethazine dissolution occurs. This is shown in Fig. 3 in systems containing 0.1 and 0.2 g % sulfamethazine. In the pH range where no change in adsorption occurred (pH 2.1–3.1), the equilibrium solubility ranged between 4.2 and 8.1 mg %. These values are to be compared with 39.8 and 84.6 mg % in systems having pH 3.1 and 2.1, respectively, but in these systems the adsorbed benzoic acid did not saturate the surface. At pH values higher than 4.2, the equilibrium solubility of all systems studied reached almost a constant value of about 40 mg %, since no adsorption occurred (Figs. 2 and 3).

The effect of sodium citrate and citric acid on sulfamethazine solubility in the presence of benzoic acid can be attributed to pH effect and adsorption of benzoic acid. Table II correlates, as a function of sodium citrate concentration, the amount of benzoic acid adsorbed and the equilibrium solubility of sulfamethazine. The suppression of benzoic acid adsorption restored the inhibited sulfamethazine solubility to the equilibrium value of about 40 mg %. In systems containing citric acid, the effect of pH on sulfa-

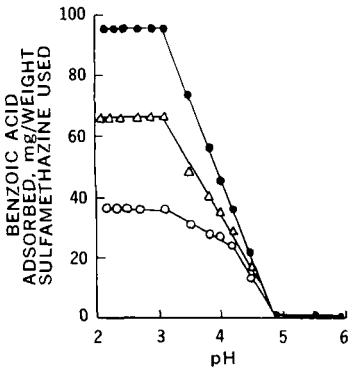

Figure 2—*Effect of pH on the adsorption of 0.1 g % benzoic acid by sulfamethazine. The concentrations of sulfamethazine in the system (g %) were: ○, 0.1; △, 0.2; and ●, 0.3, 0.4, 4, and 10.*

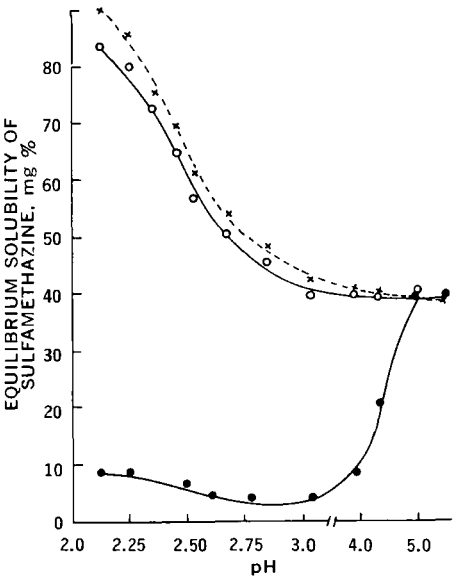

Figure 3—*Effect of pH on the equilibrium solubility of sulfamethazine in the presence of 0.1 g % benzoic acid. The concentrations of sulfamethazine in the system (g %) were: ●, 0.1 and 0.2; and ○, 4 and 10 (×, solubilities in the absence of benzoic acid).*

methazine solubility was dependent on the sulfonamide content in the system (Table III). At low sulfamethazine concentrations (0.1 and 0.2 g %), citric acid did not significantly increase the lowered sulfamethazine solubility because of the saturation of the surface by the adsorbed benzoic acid. At higher sulfamethazine concentrations (4 and 10 g %), citric acid increased the equilibrium solubility by virtue of its effect on lowering the pH.

In conclusion, the effect of electrolytes such as sodium citrate on adsorption should be interpreted not only on the basis of changing the dielectric constant of the system (5) but also on the possible effect of the electrolyte on the system pH. This may alter the ionization of electrolytic adsorbates and the charge characteristics of the adsorbent.

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Isolation of Myricadiol, Myricitrin, Taraxerol, and Taraxerone from *Myrica cerifera* L. Root Bark

B. D. PAUL, G. SUBBA RAO*, and GOVIND J. KAPADIA[x]

Abstract □ Isolation and identification of three triterpenes, myricadiol, taraxerol, and taraxerone, and a flavonoid glycoside, myricitrin, from the root bark of *Myrica cerifera* L. are reported.

Keyphrases □ Myricadiol—isolation and identification from *Myrica cerifera* root bark □ Myricitrin—isolation and identification from *Myrica cerifera* root bark □ Taraxerol—isolation and identification from *Myrica cerifera* root bark □ Taraxerone—isolation and identification from *Myrica cerifera* root bark □ *Myrica cerifera* L.—isolation and identification of three triterpenes and one flavonoid glycoside

"Bayberry bark" (myrtle wax or southern bayberry), the dried root bark of *Myrica cerifera* L. (Myricaceae), is known to exhibit astringent, emetic, and antipyretic activities when administered orally (1, 2). In medical practice, it has been used externally as a stimulant for indolent ulcers (1). Recently, the aqueous extracts and the tannin fractions of *Acacia villosa, Krameria ixina,* and *K. triandra* were prepared, bioassayed, and found to be tumorigenic in rats (3, 4). In a continuation of these studies, the authors isolated tannins of *M. cerifera* L. root bark and leaves and are currently examining them for carcinogenicity. The present paper describes the identification of four crystalline nontannin compounds isolated during the fractionation of tannins from the root bark of *M. cerifera.*

EXPERIMENTAL

Isolation of Triterpenes—The powdered root bark[1] of *M. cerifera* (1 kg) was extracted with 3 liters of petroleum ether (bp 38–47°) in a continuous extractor for 24 hr. The defatted bark powder was then similarly extracted with benzene (3 liters) for 24 hr. The benzene extract was concentrated (~25 ml) and chromatographed on a column (2.5 cm i.d. × 30 cm) of neutral alumina[2] (40 g). The following sequence of solvents with increasing polarity was used for elution: petroleum ether (1 liter), petroleum ether–benzene (2:1 mixture, 1 liter; 1:1 mixture, 1 liter), benzene (1 liter), and benzene–chloroform (2:1 mixture, 1.5 liters).

Taraxerone (I)—The petroleum ether–benzene (2:1) eluant of the alumina column was evaporated to dryness, and the residue was crystallized from petroleum ether–benzene to yield 280 mg of taraxerone, mp 238–239° [lit. (5) mp 242–244°]; mass spectrum: m/e 424 (M^+), 409 ($M - CH_3$), and 300 ($M - C_9H_{16}$). The IR and mass spectra of the isolated compound were identical to those of reference taraxerone. The melting point of the reference compound remained unchanged when mixed with the plant isolate.

Taraxerol (II)—Removal of the solvents from the petroleum ether–benzene (1:1) eluant of the alumina column and crystallization of the resulting residue from methanol yielded 141 mg of taraxerol, mp 282–283° [lit. (5) mp 282–283°]; mass spectrum: m/e 426 (M^+), 411 ($M - CH_3$), and 302 ($M - C_9H_{16}$). It formed a monoacetate derivative (IV), mp 302° [lit. (5) mp 304–305°]; mass spectrum: m/e 468 (M^+), 453 ($M - CH_3$), 408 ($M - HOCOCH_3$), and 344 ($M - C_9H_{16}$). The IR and mass spectra of both the isolated alcohol and its acetate derivative were identical to those of reference taraxerol and its acetate, respectively. The melting points of both reference compounds were unaltered when admixed with the respective plant products from *M. cerifera.*

Myricadiol (III)—The benzene–chloroform (2:1) eluant of the alumina column deposited a semicrystalline precipitate when left overnight at room temperature. The precipitate was crystallized from hot alcohol (95%) to afford 450 mg of myricadiol, mp 267–268° [lit. (6) mp 273–274°]; mass spectrum: m/e 442 (M^+), 427 ($M - CH_3$), and 302 ($M - C_9H_{16}O$). Acetylation of the isolated compound with acetic anhydride–pyridine gave a diacetate (V), mp 254° [lit. (6) mp 256.5°]; mass spectrum: m/e 526 (M^+), 511 ($M - CH_3$), 466 ($M - HOCOCH_3$), and 344 ($M - C_{11}H_{18}O_2$). The IR

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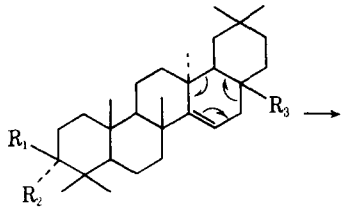
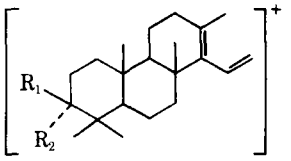
[1] *The plant material* used was collected in South Carolina and provided by Dr. J. F. Morton, Morton Collectanea, University of Miami, Coral Gables, Fla.

[2] Brockmann activity I, 80–200 mesh, Fisher Scientific Co.



I: $R_1, R_2 = O, R_3 = CH_3$
II: $R_1 = OH, R_2 = H, R_3 = CH_3$
III: $R_1 = OH, R_2 = H, R_3 = CH_2OH$
IV: $R_1 = OCOCH_3, R_2 = H, R_3 = CH_3$
V: $R_1 = OCOCH_3, R_2 = H, R_3 = CH_2OCOCH_3$



a

m/e 300, $R_1 = R_2 = O$
m/e 302, $R_1 = OH, R_2 = H$
m/e 344, $R_1 = OCOCH_3, R_2 = H$

Scheme I

and mass spectra of both the isolated diol and its diacetate derivative were identical to those of reference myricadiol and its diacetate, respectively. The melting points of both reference compounds remained unchanged when mixed with the respective plant isolates.

Isolation of Myricitrin—Following benzene extraction of the defatted bark powder, the marc was extracted with 95% alcohol (3 liters). The alcohol extract was concentrated (~150 ml) and, upon cooling, deposited a crystalline precipitate. The precipitate was recrystallized from alcohol to furnish 2.5 g of myricitrin, mp 197–199° [lit. (7) mp 197–199°]; mass spectrum: *m/e* 464 (M^+). Hydrolysis of the isolated compound by refluxing with 4% aqueous sulfuric acid for 15 min gave a sugar, rhamnose, and myricetin, mp 350° dec. [lit. (7) mp 350° dec.]; mass spectrum: *m/e* 318 (M^+). Rhamnose was identified by paper chromatography [solvent system of *n*-butanol–acetic acid–water (4:1:5)] and by preparation of the phenylosazone derivative, mp 183° [lit. (8) mp 185°], which did not depress the melting point of reference rhamnose phenylosazone when admixed. The IR, NMR, and mass spectra of isolated myricitrin and its aglycone, myricetin, were identical to those of respective reference compounds. The melting points of both reference compounds were unchanged when mixed with respective isolates from bayberry bark.

RESULTS AND DISCUSSION

The three triterpenes (I–III) isolated from the root bark of *M. cerifera* were readily identified as derivatives of Δ^{14}-taraxerene by their mass spectra, which showed the characteristic fragment *a* (Scheme I) resulting from retro-Diels–Alder reaction involving the 14,15-double bond (9, 10). The characterization of the isolated terpenoids as taraxerone (I), taraxerol (II), and myricadiol (III) was carried out by comparison with the reference samples, with identical IR and mass spectra and undepressed mixed melting points being the criteria for identity. Similarly, the acetate derivatives of II and III were identical to those of the reference compounds.

Identification of the isolated flavonoid glycoside as myricitrin was established by the NMR spectra of the glycoside and its acid hydrolysis product, myricetin, which were identical to the published reference spectra (11, 12). Similarly, the IR and mass spectra of the isolated myricitrin and its aglycone, myricetin, were identical to those of the reference compounds. Further characterization of the glycoside was carried out by the identification of rhamnose as a product of its mineral acid hydrolysis. Finally, the melting points of both reference myricitrin and myricetin were unchanged when mixed with respective compounds obtained from *M. cerifera* root bark.

These four compounds have been encountered in other *Myrica* species (13, 14). Two of these compounds have been found to be biologically active. Myricitrin is reported to exhibit choleretic (15), bactericidal, paramecicidal, and spermatocidal (16) activities, while myricadiol has mineralcorticoid activity (17).

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* Present address: Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institutes of Health, Bethesda, MD 20014

x To whom inquiries should be directed.

Anticonvulsant Activity of Substituted 4-Thiazolidones

S. P. SINGH*, THEODORE K. AUYONG‡, and SURENDRA S. PARMAR*‡ˣ

Abstract □ Several 3-(3,4-dimethoxyphenylethyl)-4-oxothiazolin-2-yl substituted hydrazones were synthesized and evaluated for their anticonvulsant activity. All thiazolidones were found to afford protection against pentylenetetrazol-induced seizures.

Keyphrases □ Hydrazones, 3-(3,4-dimethoxyphenylethyl)-4-oxothiazolin-2-yl substituted—synthesized and screened for anticonvulsant activity □ Thiazolidones—synthesized and screened for anticonvulsant activity □ Anticonvulsants, potential—synthesis and screening of 3-(3,4-dimethoxyphenylethyl)-4-oxothiazolin-2-yl substituted hydrazones

Diverse biological properties have been shown to be associated with substituted thiazolidones. These include anticonvulsant (1, 2), hypnotic (3), and local anesthetic (4) activities. Recent observations of the depression of the central nervous system (CNS) by thiazolidones, as evidenced by reduced locomotor activity, ataxia, hindlimb weakness, and loss of the righting reflex (5), led to the present synthesis of 3-(3,4-dimethoxyphenylethyl)-4-oxothiazolin-2-yl substituted hydrazones and evaluation of their anticonvulsant activity.

EXPERIMENTAL

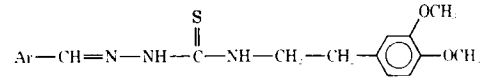
4-(3,4-Dimethoxyphenylethyl)-3-thiosemicarbazide—To an ethanolic solution of 3,4-dimethoxyphenylethylamine (0.1 mole) was slowly added 16.5 ml of concentrated ammonia solution (sp. gr. 0.91). After the mixture was cooled below 30°, carbon disulfide (7.5 ml) was added dropwise over 20 min and the mixture was allowed to stand at room temperature for 1 hr. To this mixture was added an aqueous solution of the sodium salt of monochloroacetic acid (0.1 mole). This resulted in an exothermic reaction, and hydrazine hydrate (0.1 mole, 80%) was then added to the warm solution. The mixture was cooled overnight and the crude thiosemicarbazide which separated out was filtered and recrystallized from ethanol, mp 140°, yielding 80%.

Anal.—Calc. for $C_{11}H_{17}N_3O_2S$: C, 51.76; H, 6.67; N, 16.86. Found: C, 51.24; H, 6.38; N, 16.66.

4-(3,4-Dimethoxyphenylethyl) 1-Substituted 3-Thiosemicarbazones—An ethanolic solution of 4-(3,4-dimethoxyphenylethyl)-3-thiosemicarbazide (0.01 mole) was added to a boiling solution of the suitable aldehyde (0.01 mole) in ethanol. The mixture was refluxed for 2 hr on a steam bath, and the reaction mixture was concentrated under reduced pressure. The solid mass which separated out on cooling was recrystallized from ethanol. All compounds were characterized by their sharp melting points and elemental analyses (Table I).

3-(3,4-Dimethoxyphenylethyl)-4-oxothiazolin-2-yl Substituted Hydrazones—4-(3,4-Dimethoxyphenylethyl) 1-substituted 3-thiosemicarbazone (0.005 mole), monochloroacetic acid (0.005

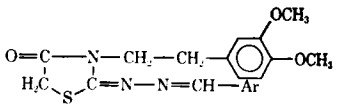
Table I—Physical Constants of 4-(3,4-Dimethoxyphenylethyl) 1-Substituted 3-Thiosemicarbazones



Compound Number	Ar	Melting Point[a]	Yield, %	Formula	Analysis, %	
					Calc.	Found
I	C_6H_5	125°	80	$C_{18}H_{21}N_3O_2S$	C 62.97 H 6.12 N 12.24	62.88 6.08 12.14
II	2-Cl—C_6H_4	167°	55	$C_{18}H_{20}ClN_3O_2S$	C 57.22 H 5.29 N 11.13	57.00 5.24 11.24
III	4-Cl—C_6H_4	135°	90	$C_{18}H_{20}ClN_3O_2S$	C 57.22 H 5.29 N 11.13	57.22 5.26 11.00
IV	3-Cl, 4-Cl—C_6H_3	198°	84	$C_{18}H_{19}Cl_2N_3O_2S$	C 52.43 H 4.61 N 10.14	52.28 4.58 10.17
V	2-CH_3—C_6H_4	115°	60	$C_{19}H_{23}N_3O_2S$	C 63.86 H 6.44 N 11.76	63.72 6.32 11.56
VI	4-OCH_3—C_6H_4	110°	88	$C_{19}H_{23}N_3O_3S$	C 61.13 H 6.17 N 11.26	61.12 6.15 11.24
VII	3-OCH_3, 4-OCH_3—C_6H_3	115°	78	$C_{20}H_{25}N_3O_4S$	C 59.55 H 6.23 N 10.42	59.34 6.00 10.34
VIII	3-OCH_3, 4-OC_2H_5—C_6H_3	122°	75	$C_{21}H_{27}N_3O_4S$	C 60.43 H 6.47 N 10.07	60.13 6.31 10.00
IX	4-$(CH_3)_2N$—C_6H_4	124°	82	$C_{20}H_{26}N_4O_2S$	C 62.18 H 6.74 N 14.50	61.88 6.55 14.37
X	C_6H_5—CH=CH	140°	68	$C_{20}H_{23}N_3O_2S$	C 65.04 H 6.23 N 11.38	64.98 6.18 11.29

[a] Melting points were taken in open capillary tubes and are uncorrected.

Table II—Physical Constants of 3-(3,4-Dimethoxyphenylethyl)-4-oxothiazoline-2-yl Substituted Hydrazones and Their Anticonvulsant Activity



Compound Number	Ar	Melting Point[a]	Yield, %	Formula	Analysis, %		Anticonvulsant Activity[b], % Protection	Pentylenetetrazol Mortality[c], %
					Calc.	Found		
XI	C_6H_5	130°	60	$C_{20}H_{21}N_3O_3S$	C 62.66 H 5.48 N 10.97	62.32 5.50 10.86	60	30
XII	2-Cl—C_6H_4	143°	45	$C_{20}H_{20}ClN_3O_3S$	C 57.48 H 4.79 N 10.60	57.21 4.63 10.78	40	50
XIII	4-Cl—C_6H_4	165°	75	$C_{20}H_{20}ClN_3O_3S$	C 57.48 H 4.79 N 10.60	57.42 4.70 10.58	60	30
XIV	3-Cl, 4-Cl—C_6H_3	185°	66	$C_{20}H_{19}Cl_2N_3O_3S$	C 53.09 H 4.20 N 9.29	52.92 4.00 9.24	30	40
XV	2-CH_3—C_6H_4	132°	48	$C_{21}H_{23}N_3O_3S$	C 63.48 H 5.79 N 10.58	63.42 5.58 10.36	60	40
XVI	4-OCH_3—C_6H_4	145°	70	$C_{21}H_{23}N_3O_4S$	C 61.02 H 5.57 N 10.17	61.00 5.44 10.22	50	30
XVII	3-OCH_3, 4-OCH_3—C_6H_3	150°	68	$C_{22}H_{25}N_3O_5S$	C 59.59 H 5.64 N 9.48	59.49 5.66 9.38	60	40
XVIII	3-OCH_3, 4-OC_2H_5—C_6H_3	138°	66	$C_{23}H_{27}N_3O_5S$	C 60.39 H 5.90 N 9.19	60.11 5.82 9.12	20	50
XIX	4-$(CH_3)_2$—N—C_6H_4	166°	72	$C_{22}H_{26}N_4O_3S$	C 61.97 H 6.10 N 13.15	61.87 6.00 13.12	10	80
XX	CH=CH—C_6H_5	144°	62	$C_{22}H_{23}N_3O_3S$	C 64.55 H 5.62 N 10.27	64.33 5.48 10.25	70	20

[a] Melting points were taken in open capillary tubes and are uncorrected. [b] Anticonvulsant activity was determined at a dose of 100 mg/kg as described under *Experimental*. [c] Represents mortality during 24 hr in each group of animals administered pentylenetetrazol.

mole), and fused sodium acetate (0.0075 mole) were mixed in 15 ml of acetic acid, and the mixture was refluxed for 6 hr. The reaction mixture was poured over ice-cold water and refrigerated overnight. The solid mass which separated out was filtered, washed with water, and recrystallized from acetic acid. All compounds (Table II) were characterized by their sharp melting points and elemental analyses.

Determination of Anticonvulsant Activity—Anticonvulsant activity against pentylenetetrazol-induced seizures was determined in mice of either sex weighing 25–30 g. The mice were divided into groups of 10, with weights being kept nearly the same as possible. Various thiazolidones were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compounds were administered to a group of 10 animals at a dose of 100 mg/kg ip. Four hours after the administration of these compounds, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol had been shown not only to produce convulsions in almost all untreated mice but also to exhibit 100% mortality during the 24-hr period. On the other hand, no mortality was observed during 24 hr in animals treated with 100 mg/kg of the test compounds alone. The mice were then observed for seizures for 60 min. An episode of clonic spasm that persisted for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks or tremulousness was not counted. Animals not exhibiting threshold convulsions during 60 min were considered protected. The number of animals protected in each group was noted, and the anticonvulsant activity of these thiazolidones was represented as percent protection. The animals were then observed for 24 hr and their mortality was recorded.

RESULTS AND DISCUSSION

The anticonvulsant activity exhibited by these thiazolidones at a dose of 100 mg/kg against pentylenetetrazol-induced seizures and the mortality due to the toxic effects of pentylenetrazol during 24 hr are shown in Table II. All 3-(3,4-dimethoxyphenylethyl)-4-oxothiazolin-2-yl substituted hydrazones afforded protection ranging from 10 to 70%. Maximum protection was observed with Compound XX; Compound XIX exhibited the lowest anticonvulsant activity. There appears to be some connection in these data but no uniform trend toward a correlation between the percent protection against pentylenetetrazol-induced seizures provided by these thiazolidones and their percent protection against pentylenetetrazol mortality (*e.g.*, Table II: 60% protection = 30 or 40% mortality, 50% protection = 30% mortality, and 30% protection = 40% mortality). These results have failed to supply evidence for a specific structural requirement for anticonvulsant activity in the molecular makeup of these thiazolidones.

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Forks, ND 58201

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[x] To whom inquiries should be directed.

Cannabis sativa L. (Marijuana) V: Pharmacological Evaluation of Marijuana Aqueous Extract and Volatile Oil

ALVIN B. SEGELMAN*[x], R. DUANE SOFIA‡, FLORENCE P. SEGELMAN*, JOHN J. HARAKAL‡, and LINDA C. KNOBLOCH‡

Abstract □ The aqueous extract (marijuana tea) and volatile oil prepared from marijuana were compared with (−)-*trans*-Δ^9-tetrahydrocannabinol for their effect on hexobarbital sleeping time and analgesic action in mice. All three substances prolonged hexobarbital sleeping time with an order of potency of (−)-*trans*-Δ^9-tetrahydrocannabinol > aqueous extract > volatile oil. Each agent produced significant analgesic activity. However, the potencies of the aqueous extract and the volatile oil were similar to each other but only 1/200 that of (−)-*trans*-Δ^9-tetrahydrocannabinol.

Keyphrases □ *Cannabis sativa* L.—pharmacological evaluation of marijuana aqueous extract and volatile oil, hexobarbital sleeping time and analgesia, mice □ Marijuana—pharmacological evaluation of aqueous extract and volatile oil, hexobarbital sleeping time and analgesia, mice

A recent report (1) described the preparation of marijuana[1] tea, a simple aqueous extract that was obtained by continuously heating *Cannabis* plant material in water under reflux for 6 hr. In addition, a volatile oil was removed and retained. Since the use of marijuana tea has been stated to prolong and intensify significantly the effects resulting from smoking marijuana (1), the effects of marijuana tea were compared to (−)-*trans*-Δ^9-tetrahydrocannabinol (I), the major psychoactive constituent of marijuana (2, 3). Two pharmacological parameters known to be affected by I (4, 5) were used, *i.e.*, hexobarbital sleeping time and alteration of painful stimuli. Moreover, since no pharmacological data have been reported for the volatile oil, it was also included.

EXPERIMENTAL

Test Animals—Male, albino mice[2] (CD-1 strain) weighing 20–28 g at the time of testing were used. The animals, housed in groups of eight each, were allowed at least 5 days of acclimation to laboratory housing conditions of 12 hr of light and 12 hr of dark at a temperature of 21–23°, with food and water continuously available up until the time of testing.

[1] The term marijuana as used here refers generally to the crushed and broken tops, including leaves and small stems, derived from flowering or nonflowering *Cannabis sativa* L. plants of either sex.

[2] Charles River Breeding Laboratories, Wilmington, Mass.

Table I—Effects of I, III, and IV on Hexobarbital Sleeping Time in Mice

Test Drug	Dose, mg/kg ip	Mice	Sleeping Time, Minutes (Mean ± *SE*)	Increase, %
Propylene glycol vehicle	—	16	49 ± 4	—
I	5.0	8	49 ± 2	0
	10.0	8	100 ± 7[a]	104
	20.0	8	105 ± 12[a]	114
IV	40.0	8	53 ± 5	8
	80.0	8	75 ± 6[a]	53
	160.0	8	87 ± 6[a]	78
	320.0	8	140 ± 11[a]	186
Distilled water vehicle	—	16	41 ± 2	—
III	12.5	8	44 ± 4	7
	25.0	8	58 ± 5[a]	41
	50.0	8	59 ± 3[a]	44
	100.0	8	81 ± 8[a]	98

[a] When compared with its respective vehicle-treated control group, $p \leq 0.05$.

Preparation of Aqueous Extract from Marijuana Tea—Marijuana tea was prepared from 100 g of marijuana[3] and lyophilized as described previously (1) to give 30 g of aqueous extract (II). Then II was purified by stirring with petroleum ether (bp 30–60°) to remove trace amounts of I and other petroleum ether-soluble fast blue B reactive substances, which were detected by TLC (6). The petroleum ether-extracted II was dried *in vacuo* at 40° to give a cannabinoid-free aqueous extract (III)[4].

Preparation of Volatile Oil—Marijuana plant material[3] was subjected to direct steam distillation. The water-insoluble volatile constituents were recovered from the aqueous distillate using an oil separator and dried (anhydrous sodium sulfate) to give a pale yellow aromatic oil, yielding 0.18% (w/w) (n_D^{20} 1.494 and d_{20}^{20} 0.8788) (IV). This IV was found to contain no detectable amounts of cannabinoids[5] as indicated by TLC (6).

Dosage Forms—All doses of I[6], III, and IV were prepared in a concentration to permit a constant volume of 0.1 ml/10 g body

[3] Female plant material representing a Mexican strain of *C. sativa* (1).

[4] Extract III was used for all pharmacological studies, although preliminary pharmacological experiments showed no significant differences in results when II and III were compared.

[5] The volatile oil obtained by water distillation during the preparation of marijuana tea (1) was shown by TLC (6) to contain significant amounts of I and thus was not used in the present pharmacological study.

[6] Obtained from the National Institute of Mental Health, Rockville, Md.

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[2] Charles River Breeding Laboratories, Wilmington, Mass.

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Test Drug	Dose, mg/kg ip	Mice	Sleeping Time, Minutes (Mean ± *SE*)	Increase, %
Propylene glycol vehicle	—	16	49 ± 4	—
I	5.0	8	49 ± 2	0
	10.0	8	100 ± 7[a]	104
	20.0	8	105 ± 12[a]	114
IV	40.0	8	53 ± 5	8
	80.0	8	75 ± 6[a]	53
	160.0	8	87 ± 6[a]	78
	320.0	8	140 ± 11[a]	186
Distilled water vehicle	—	16	41 ± 2	—
III	12.5	8	44 ± 4	7
	25.0	8	58 ± 5[a]	41
	50.0	8	59 ± 3[a]	44
	100.0	8	81 ± 8[a]	98

[a] When compared with its respective vehicle-treated control group, $p \leq 0.05$.

Preparation of Aqueous Extract from Marijuana Tea—Marijuana tea was prepared from 100 g of marijuana[3] and lyophilized as described previously (1) to give 30 g of aqueous extract (II). Then II was purified by stirring with petroleum ether (bp 30–60°) to remove trace amounts of I and other petroleum ether-soluble fast blue B reactive substances, which were detected by TLC (6). The petroleum ether-extracted II was dried *in vacuo* at 40° to give a cannabinoid-free aqueous extract (III)[4].

Preparation of Volatile Oil—Marijuana plant material[3] was subjected to direct steam distillation. The water-insoluble volatile constituents were recovered from the aqueous distillate using an oil separator and dried (anhydrous sodium sulfate) to give a pale yellow aromatic oil, yielding 0.18% (w/w) (n_D^{20} 1.494 and d_{20}^{20} 0.8788) (IV). This IV was found to contain no detectable amounts of cannabinoids[5] as indicated by TLC (6).

Dosage Forms—All doses of I[6], III, and IV were prepared in a concentration to permit a constant volume of 0.1 ml/10 g body

[3] Female plant material representing a Mexican strain of *C. sativa* (1).

[4] Extract III was used for all pharmacological studies, although preliminary pharmacological experiments showed no significant differences in results when II and III were compared.

[5] The volatile oil obtained by water distillation during the preparation of marijuana tea (1) was shown by TLC (6) to contain significant amounts of I and thus was not used in the present pharmacological study.

[6] Obtained from the National Institute of Mental Health, Rockville, Md.

Table II—Effect of I, III, and IV in the Hot-Plate Analgesia Test in Mice

Test Drug	Dose, mg/kg ip	Control[a] Reaction Time, sec	Drug[a] Reaction Time, sec	Increase in Mean Reaction Time, %	Number Analgesic[b] / Number Tested	ED_{50}, mg/kg[b] (95% Confidence Limits)
Propylene glycol vehicle	—	7.8 ± 0.7	7.7 ± 0.6	0.0	0/8	—
I	0.3	8.3 ± 0.6	10.0 ± 1.5	20.7	3/8	1.3
	0.625	7.4 ± 0.5	11.6 ± 1.5[c]	57.3	4/8	(0.5–3.1)
	1.25	7.6 ± 0.4	11.0 ± 1.0[c]	45.2	5/8	
	2.5	7.5 ± 0.6	11.7 ± 1.1[c]	56.2	5/8	
	5.0	7.6 ± 0.7	12.7 ± 2.1[c]	68.7	5/8	
	10.0	7.4 ± 0.6	16.8 ± 1.1[c]	126.2	7/8	
IV	100.0	8.0 ± 0.7	9.1 ± 1.0	14.1	2/8	260.0
	200.0	7.4 ± 0.7	11.6 ± 1.0[c]	56.2	4/8	(138.0–489.0)
	400.0	8.6 ± 0.6	13.3 ± 1.6[c]	55.0	4/8	
	800.0	7.0 ± 0.6	19.5 ± 0.5[c]	178.4	8/8	
Distilled water vehicle	—	7.9 ± 0.2	7.9 ± 0.5	0.0	0/8	—
III	100.0	8.1 ± 0.7	8.9 ± 0.9	11.0	2/8	216.0
	200.0	7.0 ± 0.5	9.6 ± 0.5[c]	37.0	4/8	(135.0–346.0)
	300.0	8.0 ± 0.6	12.4 ± 1.7[c]	54.7	6/8	
	400.0	7.5 ± 0.6	12.0 ± 1.0[c]	60.6	7/8	

[a] These data represent the mean ± *SE* for eight mice in a group. [b] The number of mice per group showing a 40% or greater increase in their predrug reaction time. These data are used to calculate the ED_{50} value. [c] $p \leq 0.05$ when compared with its respective predrug control reaction time.

weight to be given each animal. The vehicle used for I and IV was 10% propylene glycol–1% polysorbate 80[7]–0.9% saline (7), while III was dissolved in glass-distilled water. All injections were by the intraperitoneal route.

Hexobarbital Sleeping Time—Thirty minutes following administration of the test drugs or their vehicles, each mouse was given an injection of sodium hexobarbital (125 mg/kg ip). Sleeping time for each animal was measured by the time in minutes from the loss to the regaining of the righting reflex observed for at least 10 sec after the animal was placed on its back. The mean sleeping time for each group was calculated, and a statistical comparison between groups was evaluated by the Student *t* test.

Hot-Plate Test—This method for assessing analgesic activity was based on the reaction time of mice to lick their forepaws and/or jump after exposure to a copper surface hot plate heated and maintained at 54–56° (8). A control reaction time (measured to the nearest 0.1 sec) was obtained 24 hr prior to any test for drug effect. Only mice with a control reaction time of 10 sec or less were used. On the test day, mice were administered the test drugs or their vehicles; 30 min later, each mouse in a group was re-exposed to the hot-plate surface and reaction time once again was recorded. A cutoff time of 30 sec was used. The percent change in mean reaction time was obtained and statistically evaluated by comparing postdrug and predrug values. In addition, the number of mice in each group that showed a 40% or greater increase in reaction time from their respective predrug control value was noted and ED_{50} values were determined (9).

RESULTS

Hexobarbital Sleeping Time—Table I shows the results of each treatment on hexobarbital-induced anesthesia in mice. A significant prolongation of sleeping time was observed in mice treated with either 10.0 or 20.0 mg/kg of I. However, this effect with the data of this study appeared not to be dose related since a similar degree of activity was obtained with both doses, 104 or 114%, respectively.

Varying the pretreatment dose of IV produced a significant prolongation of hexobarbital sleeping time after the three highest doses. The 80.0- and 160.0-mg/kg doses produced similar degrees of enhancement (53 and 78%, respectively), while 320.0 mg/kg drastically increased hexobarbital sleeping time by 186%. Finally, a pattern of activity similar to that obtained with IV was noted when the pretreatment regimen was III. The low dose of 12.5 mg/kg was ineffective, while doses of 25.0 and 50.0 mg/kg produced 41 and 44% increases in hexobarbital anesthesia, respectively. When the dose of III was increased to 100.0 mg/kg, sleeping time was increased by 98%. Based on the data presented in Table I, the order of potency (on a milligram per kilogram basis) to prolong hexobarbital sleeping time was I > III > IV.

Hot-Plate Test—Table II summarizes the data obtained in this test system with each treatment group. All doses of I, except 0.3 mg/kg, produced a significant increase in mean reaction time to the noxious stimulus (heat). The intermediate doses of 0.625–5.0 mg/kg produced similar elevations in pain threshold (45.2–68.7%), while the highest dose of I (10.0 mg/kg) caused a 126.2% increase in mean reaction time. Both III and IV showed equal analgesic effectiveness when compared with each other but were much less potent than I (ED_{50} values of each were approximately 1/200 that of I). Neither the 10% propylene glycol–1% polysorbate 80–0.9% saline nor the distilled water vehicles produced analgesic activity.

DISCUSSION

These results demonstrate that III and IV prolong hexobarbital sleeping time and possess analgesic activity in mice similar to, but less potent than, I. Since no measurable amounts of I or any other cannabinoids could be detected in either III or IV, their pharmacological activity must be due to one or more chemical entities as yet unidentified. Further work is underway in these laboratories to isolate and identify the constituents in III and IV responsible for the observed activities.

Moreover, since it has been claimed that the effects of smoked marijuana in humans can be augmented by the ingestion of marijuana teas (1) and because it is reasonable to expect that some *Cannabis* volatile oil constituents cooccur with I in the mainstream smoke resulting from smoking marijuana, animal experiments have been initiated to determine the extent of any interaction of either III or IV or both with I.

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Effect of γ-Radiation on Yield of Insulin from Beef Pancreas Glands

PAUL SKIERKOWSKI*x and MARTIN BEASLEY‡§

Abstract □ Beef pancreas glands were subjected to varying doses of γ-radiation to inactivate proteolytic enzymes and thus allow greater insulin yields *via* normal extraction procedures. An increase in yield was seen with doses greater than 10,000 rads, with the maximum yield obtained from a 20,000-rad dose.

Keyphrases □ Radiation, gamma—effect on yield of insulin from beef pancreas glands □ Insulin—increased yields by γ-irradiation of beef pancreas glands

Recent beef shortages as well as recommended restrictions by the Food and Drug Administration on the use of oral hypoglycemics (1) have placed a serious strain on the supply of insulin from cattle and hogs. Although an increase in animal slaughter will somewhat alleviate this shortage, an increasing population carries with it an increasing demand for insulin. Thus, there is a need for methods of increasing the yield of insulin extracted from the pancreas glands of these animals.

The commercial insulin procedure is a combination of acid-alcohol extractions and isoelectric precipitations to achieve a purified insulin (2). Frozen glands in 45.9–68.1-kg (100–150-lb) lots are ground and placed in large extraction tanks filled with cold acidified alcohol. The acid–alcohol extracts the insulin from the glands and inactivates enzymes that could destroy the insulin in the extract. This step is most important, and it is felt that a loss of insulin in the grinding–extraction process could still be due to some action of the proteolytic enzymes on the insulin. Thus, if enzymes could be further inactivated, less degradation of insulin would occur and the yield would be improved.

One such means of inactivating the enzymes would be through the use of ionizing radiation (3). Irradiation of frozen glands prior to the grinding step could inactivate the enzymes that may be degrading the insulin during the subsequent steps.

Table I—Results of Insulin Assays of Irradiated Pancreas Glands

Sample Dose, rads	Yield, Units/ 0.454 kg	Change from Control, %	*p*
0 (Control)	1456 ± 54	—	—
4,000	1469 ± 45	+0.9	—
6,000	1393 ± 48	−4.3	<0.02
8,000	1278 ± 55	−12.2	<0.001
10,000	1457 ± 102	0	—
12,000	1742 ± 104	+19.6	<0.001
14,000	1702 ± 123	+16.9	<0.001
16,000	1657 ± 126	+13.8	<0.001
18,000	1831 ± 155	+25.8	<0.001
20,000	1954 ± 160	+34.2	<0.001
50,000	1820 ± 80	+25	<0.001
100,000	1490 ± 174	+2.03	—

EXPERIMENTAL

Approximately 11.6 kg (25 lb) of beef pancreas glands was obtained from a commercial slaughterhouse. The glands had been collected during the previous day's slaughter and were stored at 5°. These glands were transported in a refrigerated container to the laboratory and then placed in a −10° freezer for 24 hr. The frozen glands were chopped into pieces approximately 2.54–5.08 cm (1–2 in.) square, and all pieces were uniformly mixed. This procedure was done to approximate a uniform sample and avoid individual gland variation. Lots (0.2 kg, 0.5 lb) of the mixed frozen glands were then placed in polyethylene bags for irradiation.

Samples (0.9 kg, 2 lb) of the glands were then irradiated in 0.2-kg (0.5-lb) lots in a cobalt-60 (^{60}Co) pool-type irradiator[1] (7 cm i.d.; 1.85×10^2 rads/sec) for varying doses. All samples remained frozen during the irradiation. There was no induced radioactivity in the sample as determined by using a NaI (Tl) detector. Following irradiation, the glands were ground, weighed, and extracted with cold (1–4°) 95% ethanol acidified to pH 2.5 with concentrated phosphoric acid. The extraction time was 30 min and the temperature of the final extraction mixture was measured; then this mixture was filtered to collect the crude alcoholic extract. Ten aliquots of the alcoholic filtrate were collected for assay.

The radioimmunoassay technique of Herbert *et al.* (4) was uti-

[1] Lockhead-Georgia Co., Marietta, Ga.

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RESULTS AND DISCUSSION

Irradiation of the beef pancreas glands at doses between 10,000 and 20,000 rads produced significant increases in insulin yield when compared to the control sample (Table I). Beginning at 12,000 rads, an increase in yield occurs. The maximum yield obtained was an increase of 34.2% over the control with a dose of 20,000 rads.

From the data in Table I, it appears that 20,000 rads is the most effective dose of ^{60}Co γ-radiation for increasing insulin yields. Although it remains to be proven that it is a specific enzyme inactivation that results in the increased yield, this effect of γ-irradiation can be hypothesized. It is also possible that the irradiation could affect the insulin molecule. However, experiments conducted in this laboratory using reference standards in simulated gland concentrations have shown no significant loss in insulin as a result of γ-irradiation in these dose ranges. The insulin molecule, a small protein of molecular weight of approximately 6000, is much smaller than most enzymes. In addition, enzymes usually have unique subgroups attached to them which make them more susceptible to the effects of ionizing radiation (4).

The radioimmunoassay method used in this experiment does not assure the safety of these insulin extracts in humans. Further biological testing is necessary to allow clinical application. However, considering the present factors of increased population and increased utilization of insulin, in lieu of oral hypoglycemic agents with reported side effects, utilization of γ-radiation to increase the insulin yield of pancreas glands may be a means of increasing this insulin supply.

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§ National Science Foundation Undergraduate Research Program Participant, 1973, University of Mississippi.

ˣ To whom inquiries should be directed.

New Compounds: Monoesters of α-Aminobenzylphosphonic Acid

W. FRANKLIN GILMOREˣ and HILMER A. McBRIDE

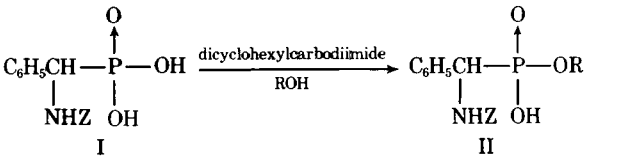
Abstract □ The reaction of α-(*N*-carbobenzoxyamino)benzylphosphonic acid with *n*-propanol or ethanol in the presence of triethylamine and dicyclohexylcarbodiimide gave the title compounds in good yields.

Keyphrases □ α-Aminobenzylphosphonic acid monoesters—synthesis □ Phosphonic acid monoesters, α-aminobenzyl—synthesis

While investigating methods of incorporating α-aminophosphonic acids into peptides, the conversion of aminophosphonic acids to monoesters became of interest. It is well known that phosphonic acids can be converted to monochlorides by use of 1 equivalent of phosphorus pentachloride. Addition of the monochlorides to alcohols furnishes the monoesters (1). While this method is satisfactory, it is laborious. Monoalkyl esters of *N*-substituted α-aminophosphonic acids may be prepared by treating a Schiff base with an alkaline salt of an alkyl dihydrogen phosphonate (2). This method is not satisfactory for preparing the monoesters of α-(*N*-carbobenzoxyamino)phosphonic acids. Partial hydrolysis of the diesters of aminophosphonic acids has been used as a source of the monoesters (3).

Dicyclohexylcarbodiimide has been used for the synthesis of esters of phosphorus acids (4–8). The preparation of monoesters from α-(*N*-carbobenzoxyamino)benzylphosphonic acid (I) by treatment with alcohols and dicyclohexylcarbodiimide is the subject of this report. Scheme I illustrates this synthesis.

The α-(*N*-carbobenzoxyamino)benzylphosphonates (II) can be converted to the deblocked amine hydrobromides by treatment with hydrogen bromide in acetic acid. The water-soluble hydrobromide salts can be converted to the amino esters by use of silver oxide.



I

Z = carbobenzoxy

II

R = ethyl or propyl

Scheme I

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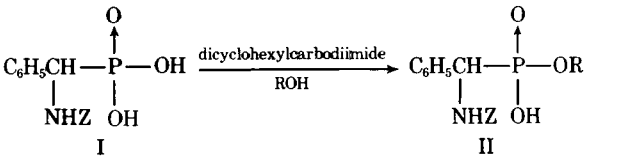

I

Z = carbobenzoxy

II

R = ethyl or propyl

Scheme I

EXPERIMENTAL[1]

***n*-Propyl-α-aminobenzylphosphonate**—A solution of 6.4 g (20 mmoles) of I (9), 4.21 g (20 mmoles) of dicyclohexylcarbodiimide, and 2.5 g (25 mmoles) of triethylamine in 100 ml of *n*-propanol was stirred for 16 hr at 25° and the dicyclohexylurea was removed by filtration. Evaporation of the *n*-propanol, partitioning of the residue between methylene chloride and 1 *N* HCl, and evaporation of the dried methylene chloride gave 7.9 g of a solid. Recrystallization of this solid from acetone afforded 5.5 g (76%) of the propyl ester, mp 181–182°; IR (KBr): 3300 (N—H), 1725 (C=O), and 1250 (P—O) cm^{-1}; NMR (d_6-dimethyl sulfoxide): δ 0.89 (t, 3, CH_3), 1.50 (m, 2, CH_3CH_2), 3.82 (m, 2, $CH_3CH_2CH_2O$), 5.12 (s, 2, $CH_2C_6H_5$), and 7.65 (s, 10, aromatic).

Anal.—Calc. for $C_{18}H_{22}NO_5P$: C, 59.50; H, 6.10; N, 3.86; P, 8.52. Found: C, 59.46; H, 6.17; N, 3.83; P, 8.12.

The ethyl ester, mp 188–190° [lit. (3) mp 183–189°], was obtained in a similar manner.

Anal.—Calc. for $C_{17}H_{20}NO_5P$: C, 58.45; H, 5.77; N, 4.01; P, 8.86. Found: C, 58.70; H, 5.81; N, 3.93; P, 8.69.

Propyl-α-aminobenzylphosphonate Hydrobromide—To a suspension of 3.6 g (10 mmoles) of the propyl ester II in 30 ml of acetic acid was added 10 g (5.5 mmoles/g) of 45% HBr in acetic acid. The suspension was stirred for 40 min, after which all of the ester had dissolved. The solution was diluted with dry ether to precipitate the hydrobromide, which was collected by filtration and recrystallized three times from methanol–ether to yield 2.5 g (81%) of the hydrobromide, mp 156–158°; IR (KBr): 1210 (P—O) cm^{-1}, no carbonyl absorption; NMR (D_2O): δ 0.85 (t, 3, CH_3), 1.60 (m, 2, CH_3CH_2), 3.83 (m, 2, $CH_3CH_2CH_2O$), 4.65 (d, 1, J = 16 Hz, CH—P), and 7.62 (s, 5, phenyl); mass spectrum (70 ev): *m/e* (relative intensity) 124(6), 122(10), 81(9), 79(10), 43(100), and 41(30).

Anal.—Calc. for $C_{10}H_{17}BrNO_3P$: C, 38.73; H, 5.53; N, 4.52; P, 9.99. Found: C, 39.20; H, 4.94; N, 4.55; P, 9.96.

[1] Melting points were determined with a Thomas-Hoover Unimelt melting-point apparatus and are corrected. NMR spectra were taken on a Jeolco model C-60-HL spectrometer using tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard. IR spectra were taken on either a Perkin-Elmer model 137 or 256 spectrophotometer. Mass spectra were taken by Dr. John K. Baker using a DuPont CEC model 21-492 spectrometer. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn.

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COMMUNICATIONS

Interaction of Salicylic Acid with Adenosine and Adenosine Triphosphate: Potential Mechanism of Intensifying Aspirin-Induced GI Blood Loss

Keyphrases □ Salicylic acid—interaction with adenosine and adenosine triphosphate, related to mechanism of intensifying aspirin-induced GI blood loss □ Adenosine and adenosine triphosphate—interaction with salicylic acid, mechanism of aspirin-induced GI blood loss □ Aspirin—salicylic acid-adenosine interaction, possible mechanism of intensifying aspirin-induced GI blood loss

To the Editor:

Several studies (1–4) indicated that aspirin-induced gastric or GI bleeding is usually a local effect resulting from contact of aspirin particles, or saturated solutions of aspirin surrounding these particles, with the mucosa. Maudlin (5) also stated that bleeding and ulceration are due to a local focal necrosis. However, Woznicki and Mrtek (6) expressed the opinion that the mechanism of aspirin-induced GI blood loss is due to a combination of both local and systemic effects. They explained the systemic effect by the role of aspirin in reducing platelet aggregation through elimination of the second wave of platelet aggregation induced by adenosine diphosphate. These authors defended their opinion by the fact that aspirin is being used experimentally to prevent formation of thromboemboli. However, no work has been published to support this theory.

Preliminary investigations in our laboratories have indicated interaction between salicylic acid and adenosine or adenosine triphosphate. The presence of the phosphate moiety does not appear to be critical for the complexation. The complexes formed also

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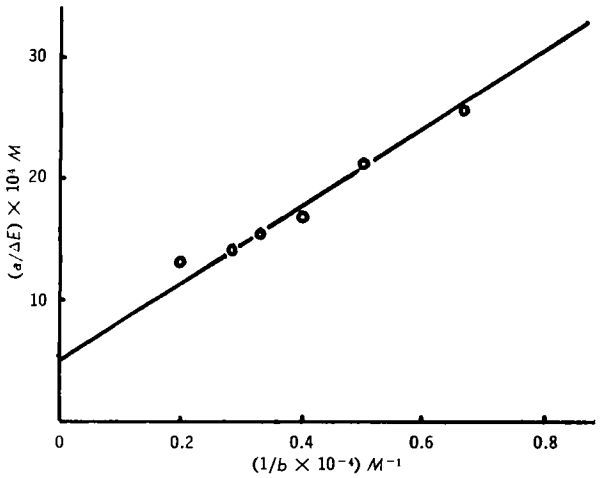
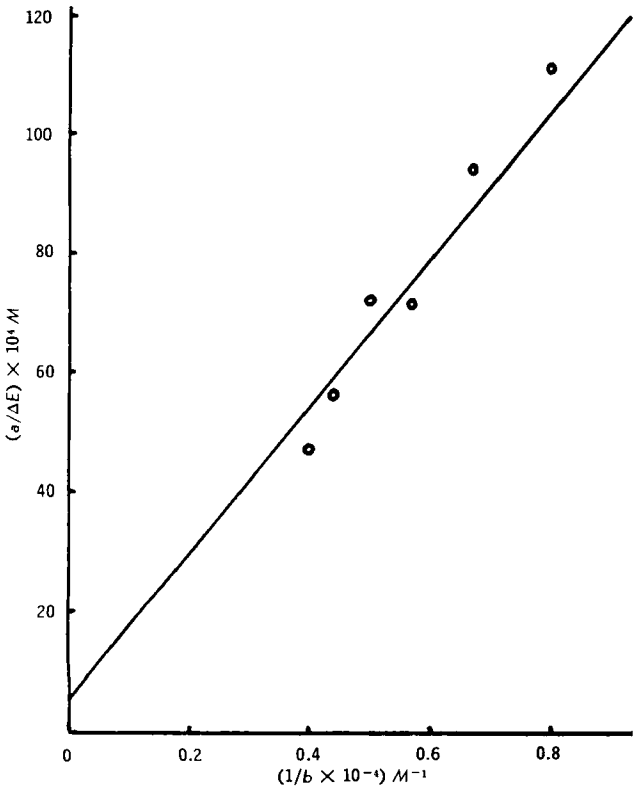


Figure 1—*Benesi–Hildebrand plot for salicylic acid–adenosine complexation in 0.05* M *phosphate buffer at pH 7 after storage for 19 hr at 27°.*

possessed greater surface activity. These factors are believed to contribute to the reduction of platelet aggregation, thus aggravating and prolonging the local effect.

Spectrophotometric techniques were used to evaluate complex formation between salicylic acid and adenosine or adenosine triphosphate. The absorbance decrease at 296 nm was measured in the presence of various concentrations of adenosine or adenosine triphosphate in 0.05 *M* phosphate buffer at pH 7. The Benesi–Hildebrand equation (7) was applied to the data obtained to form the plots shown in Figs. 1



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A. F. Asker
C. W. Whitworth[x]
School of Pharmacy
University of Georgia
Athens, GA 30601

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[x] To whom inquiries should be directed.

O-Alkyloxime Derivatives: A New Group of Compounds with Antibacterial Activity

Keyphrases □ *O*-Alkyloxime derivatives—synthesized and screened as potential antibacterial agents □ Antibacterial agents, potential—synthesis and screening of *O*-alkyloxime derivatives

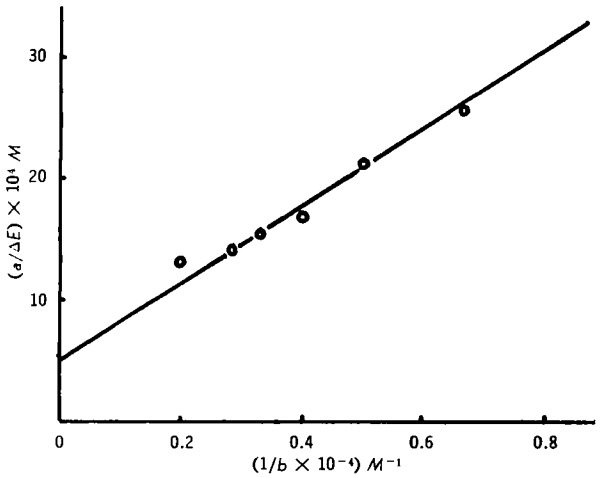
To the Editor:

Medicinal agents with various types of activity incorporate the pharmacophoric grouping Am-C-C-X, where Am is amino, alkylamino, or dialkylamino and X is C, N, O, or S. Interest in the alkoxyimino grouping as a potential pharmacophore led to the preparation of some new polyfunctional compounds in which Am is alkoxyimino rather than amino or substituted amino.

The new compounds that were prepared and tested as antimicrobials are listed in Table I. The one aldehyde, 2-methoxyiminophenylacetaldehyde (VIII), was inactive, but its bisulfite (XI), dioxolane (X), and semicarbazone (IX) derivatives showed some activity. Of the two sulfides, one was active and one was not. The one sulfonic acid derivative was active, as were all of the *S*-acetyl-2-alkoxyiminoalkylmercaptans.

Preparation of the aldehyde was through the selenium dioxide oxidation of acetophenone oxime *O*-methyl ether (1). The aldehyde was converted to its biologically active derivatives by standard methods (2, 3).

Acylmercaptans, sulfones, sulfonates, and sulfides were prepared by established procedures from the corresponding 2-alkoxyiminoalkyl bromides by displacing bromide with thiolacetate anion (4), sulfi-



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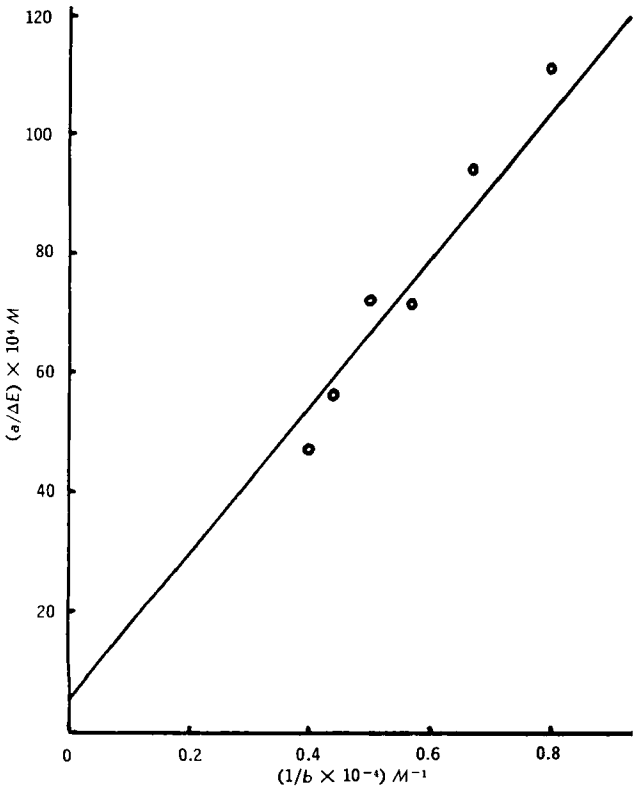

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Table I—Antimicrobial Activity of Oxime Derivatives

$R_1—C(=NOR_2)—X$

Compound	R_1	R_2	X	Melting Point	Boiling Point/torr ($\eta/T°$)	Yield, %	*S. aureus* Smith Strain	*K. pneumoniae* AD Strain
I	CH_3	CH_3	$CH_3CH_2CH_2SCH_2$	—	97–98°/34 (1.4735/23)	67.2	+	−
II	C_6H_5	CH_3	$CH_3CH_2CH_2SCH_2$	—	118–120°/0.10 (1.5539/23)	42.4	−	−
III	C_6H_5	CH_3CH_2	$C_6H_5SO_2CH_2$	108–109°	—	80.0	+	−
IV	CH_3	CH_3	CH_2SO_3Na	—[a]	—	34.2	+	−
V	CH_3	CH_3	CH_3COSCH_2	—	88–90°/28 (1.4879/23)	32.5	+	+
VI	CH_3	CH_3CH_2	CH_3COSCH_2	—	97–98°/9 (1.4851/24)	61.7	+	+
VII	C_6H_5	CH_3CH_2	CH_3COSCH_2	—	104°/0.02 (1.5598/24)	63.4	+	+
VIII	C_6H_5	CH_3	CHO	—	63–65°/0.035 (1.5455/20)	49	−	−
IX	C_6H_5	CH_3	$CH{=}NNHC(=O)NH_2$	195°	—	41	+	+
X	C_6H_5	CH_3		81–82°	—	52	+	+
XI	C_6H_5	CH_3	$CH(OH)SO_3Na$	—[a]	—	30	+	−

[a] Decomposes.

Table II—Elemental Analysis of Oxime Derivatives

Compound	Molecular Formula	Analysis, % Calculated C	H	N	S	Found C	H	N	S
I	$C_7H_{15}NOS$	52.17	9.32	8.70	19.88	52.24	9.26	8.57	20.20
II	$C_{12}H_{17}NOS$	64.58	7.62	6.28	14.35	64.78	7.77	6.40	14.17
III	$C_{16}H_{17}NO_3S$	63.37	5.61	4.62	10.56	63.36	5.89	4.73	10.50
IV	$C_4H_8NNaO_4S$	25.40	4.23	7.41	16.90	25.64	4.20	7.51	—[a]
V	$C_6H_{11}NO_2S$	44.72	6.83	8.70	19.88	43.27	6.29	8.83	19.80
VI	$C_7H_{13}NO_2S$	48.00	7.43	8.00	18.29	48.07	7.38	7.60	18.30
VII	$C_{12}H_{15}NO_2S$	60.76	6.33	5.91	13.50	59.84[b]	6.09	5.93	13.55
VIII	$C_9H_9NO_2$	—[c]	—	—	—	—	—	—	—
IX	$C_{10}H_{12}N_4O_2$	54.09	5.41	24.54	—	54.32	5.33	24.92	—
X	$C_{11}H_{13}NO_3$	63.77	6.28	6.76	—	63.63	6.45	6.56	—

[a] Sulfur analysis unsatisfactory even after repeated crystallizations. [b] Could not be improved on recrystallization. [c] Analyzed as semicarbazone (Compound IX) after liberating the aldehyde from the bisulfite addition product with sodium carbonate.

nate anion (5), sulfate (6), and mercaptide (7), respectively. Preparation of the 2-alkoxyiminoalkyl bromides has been reported (8). Analytical data for new compounds reported are collected in Table II.

In the biological evaluation, with the exception of II and VIII (Table I), all compounds were active against *Staphylococcus aureus* and *Klebsiella pneumoniae* in an *in vivo* test. In this test, the compounds are added to agar medium and the test organisms are inoculated (about 3×10^5 cells) on the surface. The end-point (Table I) is the concentration of compound that prevents emergence of visible growth (200 ppm in all cases).

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*Larry A. Sternson**
Dominick A. Coviello[x]

Department of Medicinal Chemistry
College of Pharmacy
University of Illinois at the Medical Center
Chicago, IL 60612

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* Present address: School of Pharmacy, University of Georgia, Athens, Ga.

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Urinary Excretion of Acetaminophen by the Rat

Keyphrases □ Acetaminophen—urinary excretion by the rat, suitability of the rat as an experimental animal □ Metabolism—urinary excretion of acetaminophen by the rat, suitability of the rat as an experimental animal □ Excretion, urinary—acetaminophen by the rat, rat-human metabolic patterns compared

To the Editor:

Acetaminophen, after a therapeutic dose, is excreted in the urine of humans primarily as the glucuronic acid conjugate (1, 2). Büch *et al.* (3) reported that acetaminophen glucuronide was also the major metabolite excreted in the urine by rats 4 hr after administration of large doses of acetaminophen (300 and 600 mg/kg). Recently, the rat was used to investigate a potential interaction between relatively low doses of acetaminophen (10 mg/kg) and salicylic acid (4). We are not aware of reports concerning the metabolism and excretion of acetaminophen in rats after lower doses, which are more comparable to those used therapeutically in humans. It was of interest, therefore, to determine whether the rat excreted acetaminophen in a manner similar to that reported for humans.

We determined the amounts of acetaminophen and its glucuronide and sulfate conjugates excreted in the urine of two strains of rats given a 25-mg/kg dose of acetaminophen. An aqueous solution of acetaminophen, 2.5 mg/ml, was administered orally or intraperitoneally to male Wistar rats (290–340 g) and intraperitoneally to male Sprague–Dawley rats (290–370 g). Urine for analysis was collected for 24 hr. Preliminary experiments showed that excretion of the drugs was essentially completed within this period. Acetaminophen and its conjugates in urine were determined using selective enzyme hydrolysis followed by GLC analysis. The conjugates were hydrolyzed enzymatically, and acetaminophen was extracted with ether using a modification of the method of Levy and Yamada (2). This procedure was followed by back-extraction into 0.1 *N* NaOH, with subsequent removal of the organic phase and neutralization of the aqueous phase to pH 7.0. Acetaminophen was extracted from the aqueous solution with ethyl acetate. The organic layer was evaporated to dryness, and the acetaminophen was derivatized with bis(trimethylsilyl)trifluoroacetamide[1] and was analyzed by GLC using a modification of the method of Thomas and Coldwell (5).

The results (Table I) indicate that, after a dose of 25 mg/kg, both strains of rats excreted acetaminophen principally as the sulfate conjugate. A very small percentage of the dose was excreted as the glucuronide conjugate or as unchanged drug. The GI tract is an important site for formation of glucuronic acid conjugates (6). Acetaminophen was administered orally to rats to determine if the low amount of acetaminophen glucuronide excreted in the urine following an intraperitoneal dose of acetaminophen was due to the unavailability of this site of glucuronidation. Acetaminophen was probably not conjugated with glucuronic acid during absorption because oral administration resulted in no change in the urinary excretion of the drug when compared to the results of intraperitoneal administration.

These data from rats differ considerably from urinary excretion data following administration of acetaminophen to humans. Cummings *et al.* (1) reported that 49% of a 12-mg/kg dose was excreted as acetaminophen glucuronide, with smaller amounts excreted as acetaminophen sulfate (25%) and unconjugated drug (4%). Levy and Yamada (2) reported similar results for humans following 1- or 2-g doses (63% as acetaminophen glucuronide, 26% as acetaminophen sulfate, and 3% as unconjugated drug). We observed in three humans that 47.8 ± 3.8% (±*SEM*) of a 10-mg/kg dose of acetaminophen was excreted as the glucuronide conjugate while 32.3 ± 5.9% of the dose was excreted as acetaminophen sulfate.

Table I—Urinary Excretion of Acetaminophen in the Rat following a 25-mg/kg Dose

Strain	Route	Percent of Dose[a] Excreted in 24 hr as		
		Acetaminophen	Acetaminophen Glucuronide	Acetaminophen Sulfate
Sprague–Dawley (n = 4)	Intraperitoneal	1.9 ± 0.3	2.7 ± 0.7	68.9 ± 6.2
Wistar (n = 4)	Intraperitoneal	0.8 ± 0.1	3.8 ± 0.7	68.6 ± 6.5
Wistar (n = 4)	Oral	1.2 ± 0.2	4.8 ± 0.7	77.0 ± 7.4

[a] Expressed as the mean ± standard error.

Büch *et al.* (3) reported a saturation of the sulfate-conjugating pathway in the rat following 300- and 600-mg/kg doses of acetaminophen. Levy and Yamada (2) suggested that the pathway for sulfate conjugation in humans was saturated at doses of 12–32 mg/kg. The differences between the urinary metabolite pattern in rats given high doses of acetaminophen and that in rats treated with a 25-mg/kg dose may be due to saturation of the sulfate-conjugating pathways at high dosages, with a resultant increase in acetaminophen glucuronide formation.

We conclude from these studies that the rat is not an appropriate animal model for acetaminophen me-

[1] Regisil, Regis Chemical Co., Chicago, Ill.

tabolism in humans. Acetaminophen appears to be excreted in the urine primarily as acetaminophen sulfate when administered to rats in doses comparable to human doses. In contrast, the glucuronide conjugate constitutes the largest fraction of the dose of acetaminophen excreted in the urine by humans. Therefore, results from studies of the interference by other drugs upon acetaminophen conjugation in the rat cannot be readily extrapolated to similar situations in humans. This possibly explains why Ramachander *et al.* (4) observed an interaction between acetaminophen and salicylate in rats while Levy and Regårdh (7) could not demonstrate this in humans.

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R. P. Miller
L. J. Fischer[x]
Toxicology Center
Department of Pharmacology
University of Iowa
Iowa City, IA 52242

Received January 31, 1974.
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[x] To whom inquiries should be directed.

Decomposition of Cannabidiol in Chloroform Solution

Keyphrases □ Cannabidiol—stability in chloroform and ethanol, GLC, TLC □ Stability—cannabidiol in chloroform and ethanol, GLC, TLC

To the Editor:

Concurrent with our investigation of the stability of the cannabinoids, we observed what appeared to be an unusually large loss of cannabidiol when stored in a chloroform solution. Since chloroform is frequently employed in cannabinoid extraction and analysis (1–10), we felt it imperative to investigate the stability of cannabidiol in this solvent. Previous reports on the stability of cannabinoids in solution were concerned primarily with crude marijuana extracts. Kubena *et al.* (11) reported that cannabidiol was one of the cannabinoids present in a 43-year-old alcoholic solution of marijuana extract, and Turner *et al.* (12) recently indicated that the extractable cannabinoids, including cannabidiol, are stable in chloroform for at least 6 days. However, we wish to report a marked decomposition of cannabidiol in chloroform as compared to its relative stability in ethanol.

Table I—GLC Analysis[a] of Cannabidiol in Ethanol and in Chloroform

Day	Cannabidiol Peak Area[b]		Cannabidiol–Steroid Ratio[c]	
	A(Ethanol)	B(Chloroform)	C(Ethanol)	D(Chloroform)
0	100	100	1.00	1.00
1	105	91	1.02	0.88
2	85	61	0.97	0.68
3	80	54	0.96	0.56
4	85	34	0.89	0.31
5	82	23	0.97	0.19
6	86	14	0.96	0.09
7	87	9	0.97	0.04
8	84	5	0.96	0.01

[a] Average of two or three determinations where integrated peak area differences were not greater than 8%. [b] Percent relative to the peak area on Day 0. [c] Normalized to 1.00 on Day 0; actual values on Day 0 were 1.10 for C and 1.05 for D.

Four 1-ml solutions, each containing 1.0 mg of cannabidiol[1], were prepared: Solution A, in absolute ethanol; Solution B, in spectrograde chloroform[2]; Solution C, in absolute ethanol containing 1.0 mg 4-androstene-3,17-dione[3] (steroid) as an internal standard (7–9); and Solution D, in spectrograde chloroform containing 1.0 mg of steroid as an internal standard. Except during analysis, the solutions were stored at room temperature in the dark in tightly stoppered 1-ml volumetric tubes.

The GLC analysis was performed using a gas chromatograph[4] equipped with a digital integrator[5] and dual 0.6-cm (0.25-in.) by 1.8-m (6-ft) glass columns packed with 3% OV-17 on 100–120-mesh Gas Chrom Q. The experimental conditions were: injection port temperature, 232°; detector temperature, 240°; column temperature, 218°; and nitrogen flow rate, 50 ml/min[6]. Dual flame-ionization detectors were employed, and the attenuation was adjusted so that a 1.0-μl sample of each solution produced a cannabidiol peak of approximately 70% full-scale deflection on Day 0.

The data (Table I) show that there was a significant decrease in the cannabidiol content in chloroform, even in the first 24 hr, while the cannabidiol content in ethanol remained essentially unchanged. The GLC data of Solutions A and B represent absolute values of peak areas and are subject to the usual experimental variations[7]. The consistency of the cannabidiol to steroid ratio in Solution C indicates that the cannabidiol content in ethanol is relatively stable and does not decrease as might be concluded from the data for Solution A alone.

The TLC technique employed was that of Korte *et al.* (10). After impregnation with dimethylformam-

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[2] Fisher Scientific, Fair Lawn, N.J.
[3] E. M. Laboratories Inc., Elmsford, N.Y.
[4] Perkin-Elmer model 990.
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R. P. Miller
L. J. Fischer[x]
Toxicology Center
Department of Pharmacology
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ide–carbon tetrachloride (3:2) and air drying, the plates[8] were spotted with 1.0-μl aliquots of the four solutions and standards of Δ^1- and $\Delta^{1(6)}$-tetrahydrocannabinol[1]. Visualization was accomplished by spraying with 0.1% aqueous di-*o*-anisidine tetrazolium chloride[9]. The TLC data paralleled that of the GLC in the disappearance of cannabidiol from the chloroform solutions. Initially, a single orange component (R_f 0.13) was present in all solutions. For 15 successive days, the solutions of cannabidiol in ethanol (A and C) remained unchanged. However, in the case of the chloroform solutions (B and D), the size and color intensity of the spot decreased in the first 24 hr; by the 8th day, the presence of cannabidiol could not be detected in these solutions. Concomitant with the disappearance of cannabidiol was the appearance of a deep-orange spot at the origin.

On the 9th day, a "micro"[10] Duquenois–Levine test (13) was performed on 5.0-μl aliquots of the four solutions. The ethanol solutions (A and C) showed strongly positive results while the two chloroform solutions (B and D) were negative, indicating the absence of cannabidiol or of Δ^1- or $\Delta^{1(6)}$-tetrahydrocannabinol (14). Formation of cannabinol or of Δ^1- or $\Delta^{1(6)}$-tetrahydrocannabinol in the chloroform solutions was not observed in either the GLC or TLC analyses, as determined from the retention and R_f values of authentic samples[1]. However, a new peak (representing less than 1% of the original cannabidiol content) was present near the Δ^1-tetrahydrocannabinol region in the 8th-day gas chromatograms of the chloroform solutions. This peak may correspond to the major pyrolytic product of cannabidiol reported by Kuppers *et al.* (15). In addition, the chloroform solutions developed a deep-yellow color while the ethanol solutions remained colorless throughout the analysis.

Thus, the decomposition of cannabidiol in chloroform solutions, but not in ethanolic solutions, was demonstrated by GLC, TLC, and colorimetric analysis. Caution should be exercised in the use of chloroform as a solvent for prolonged extraction and storage of cannabidiol. Further study on the stability of cannabidiol in other solvents and the elucidation of the nature of the decomposition products is in progress.

[8] Silica gel, 250 μm, on glass, Brinkmann Inc., Westbury, N.Y.

[9] Fast Blue Salt B, E. M. Laboratories.

[10] One hundred microliters of Duquenois reagent, followed by 5 drops of concentrated hydrochloric acid, is added to the residue from the 5-μl aliquots in a 2-ml glass vial. Within 3 min, a pinkish-violet color is produced. Then 200 μl of chloroform is added with stirring; a violet color in the organic layer indicates a positive test for cannabinoids.

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James M. Parker[x]
Mitchell L. Borke
Lawrence H. Block
Todd G. Cochran

Department of Pharmaceutical Sciences
School of Pharmacy
Duquesne University
Pittsburgh, PA 15219

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[x] To whom inquiries should be directed.

Micellar Properties of Drugs: Micellar and Nonmicellar Patterns of Self-Association of Hydrophobic Solutes of Different Molecular Structures — Monomer Fraction, Availability, and Misuses of Micellar Hypothesis

PASUPATI MUKERJEE

Abstract □ The nature of multiple, sequential, self-association equilibria and the significance of the average degrees of association are discussed. Characteristic patterns of association are examined on the basis of available experimental evidence and suitable theoretical models of self-association and their relationship to molecular structure is indicated. The meaning and significance of the simple two-phase approximation of the micellar hypothesis to the CMC are examined and its application is discussed.

Keyphrases □ Micellar properties of drugs—symposium □ Micelle and nonmicelle patterns of self-association, hydrophobic solutes of different molecular structures—consideration of monomer fraction, availability, and correct application of micellar hypothesis □ Self-association of hydrophobic solutes—micellar and nonmicellar patterns, discussion and equations □ Solutes, hydrophobic—micellar and nonmicellar patterns of self-association, discussion and equations

Self-association of hydrophobic molecules in aqueous solution is not confined to micelle formation of soaps and detergents. Many hydrophobic drugs, dyes, physiological surfactants such as bile salts, and hydrophobic proteins show self-association. However, the *patterns* of self-association may be very different for solutes with different molecular structures and geometry. Hydrophobic solutes are classified on the basis of their structures. The nature of multiple, sequential, self-association equilibria and the significance of the average degrees of association are discussed. Some characteristic patterns of association are examined on the basis of available experimental evidence and suitable theoretical models of self-association. These include formation of unique oligomers, *e.g.*, a dimer, open-ended continuous self-association, and cooperative association to form micelles of limited and wide size distributions. The relationship of these patterns to molecular structures is indicated.

Since self-association in various situations is often described using the simple two-phase approximation of the micellar hypothesis after the isolation of a critical micelle concentration (CMC), the meaning and significance of this hypothesis with respect to the CMC in practical situations are examined in detail. It is shown by some suitably constructed examples that different experimental approaches may give rise to apparent CMC values for many systems exhibiting nonmicellar patterns of association. However, the application of the micellar hypothesis to such systems may be seriously misleading. The importance of the monomer fraction as a measure of the activity or "availability" of the self-associating system is underscored. The micellar hypothesis is especially inappropriate for estimating the monomer fraction in systems showing nonmicellar patterns of association and may seriously underestimate the extent of self-association in dilute solutions, *i.e.*, below the apparent CMC. These conclusions should be particularly relevant for many physiological surfactants and aggregating drug systems.

HYDROPHOBIC INTERACTIONS AND HYDROPHOBIC ASSOCIATION

In aqueous media, essentially all molecules containing exposed organic groups, *i.e.*, ones that are not protected by polar groups on more than one side, show some degree of hydrophobicity. Methanol (CH_3OH) and phenol (C_6H_5OH), for example, containing the smallest alkyl and aromatic groups substituting for a proton in the water molecule itself, reduce the surface tension of water and are, therefore, surface active. In a qualitative sense, this surface activity is a result of the tendency of the organic groups of the molecules constituting the hydrophobic moiety to leave the contact of water and seek out relatively nonpolar substrates. The hydrophobicity also expresses itself in many other ways. For example, lipophilicity, as reflected in the ability of the molecules to partition into nonpolar phases, is closely related to hydrophobicity. Similarly, adsorption and binding to nonpolar substrates, which may be as diverse as charcoal, proteins, enzymes, membranes, or receptor sites, and solubilization in micelles or other lipoidal systems are often caused by the hydrophobicity of the molecules.

The intermolecular forces and water structure effects responsible for this hydrophobicity have been the focus of much attention but are still not well understood (1–4). The magnitude of the problems involved has been discussed (3). For correlation with molecular structure, one fruitful qualitative idea that has had considerable application in many guises and forms is the additivity of the hydrophobic interactions: as the hydrophobic moiety gets larger, its interfacial activity, lipophilicity, adsorbability, *etc.*, tend to increase and often do so in a parallel fashion (3, 5). This generalization seems to hold particularly well for a homologous series of compounds (3).

Another expression of the hydrophobicity of organic molecules in aqueous solution is their tendency to exhibit aggregation in solution, *i.e.*, self-association and mutual association. Here again, qualitatively, the larger the hydrophobic moiety the stronger is the tendency toward association. Thus, the association of small molecules may be appreciable only at high concentrations, whereas molecules containing large hydrophobic moieties can show self-association at extremely low concentrations. For example, many

long-chain nonionic surfactants have CMC's of the order of 10^{-5}–10^{-6} mole/liter (6). Detailed applications of the theories of micelle formation (7) to such systems suggest that the monomer concentration may not exceed this value by very much even if the total concentration is 10^{-1} mole/liter, *i.e.*, 10^{4}–10^{5} times as much as the CMC. Thus, at these high concentrations, the monomer fraction can be as low as 10^{-4}–10^{-5}. This can be an important factor if the concentration of free monomers is the important variable, as it often is.

The self-association and mutual association of hydrophobic solutes are usually ascribed to "hydrophobic bonding." This catch-all expression summarizes various interactions, most of which are difficult to quantify or even to examine separately (1–4). Van der Waals' forces, water-structure effects, and/or interfacial free energy changes are some of the attractive interactions involved. These are opposed by short-range electron cloud repulsions and repulsions between any charges or higher multipoles that may be present. As in all reactions between solute molecules in a liquid medium, the "net" interactions are the result of the balance between solute–solute and solvent–solvent interactions on the one hand and solute–solvent interactions on the other. The extent of association is determined by the interplay of these interactions with the ever present thermal energy of the molecules and the loss in the entropy of mixing on association (1, 4).

SELF-ASSOCIATION

Equilibria—Stepwise self-association of monomers can lead to an immense variety of distributions of aggregates of different sizes. Various techniques are available and are being developed for the analysis of such multiple equilibria (7–11). The equilibria for ideal, nonionic systems can be written as a set of stepwise equilibria involving addition of a monomer to a q-mer containing q monomers or as a set of overall equilibria in which the q-mer is depicted as a result of the association of q monomers. The equilibria (Scheme I) will often be applicable to charged systems if the ionic strength and the counterion concentration and composition are kept constant (7, 9) and the aggregates can be assumed to behave ideally, *i.e.*, the interaction between aggregates is negligible.

The simplest self-association equilibrium is dimerization, as represented by Scheme I and Eq. 1:

$$2b_1 \overset{K_2}{\rightleftharpoons} b_2$$

Scheme I

$$K_2 = \frac{[b_2]}{[b_1]^2} \qquad \text{(Eq. 1)}$$

where b_1 is the monomer, b_2 is the dimer, and K_2 is the association constant for the dimer.

All concentration units used here will be moles or equivalents per liter, which are practical units for dilute solutions. For thermodynamic analysis, the mole fraction concentration scales are more appropriate (1, 4). The equilibria described here can easily be transformed into the mole fraction or molality scale.

Dimerization is the first step in any self-association scheme. As stepwise association continues, larger multimers form (Scheme II).

$$b_2 + b_1 \overset{K_3}{\rightleftharpoons} b_3$$

$$b_3 + b_1 \overset{K_4}{\rightleftharpoons} b_4$$

$$b_{q-1} + b_1 \overset{K_q}{\rightleftharpoons} b_q$$

Scheme II

The overall association constant for the formation of b_q from b_1 in Scheme III:

$$qb_1 \rightleftharpoons b_q$$

Scheme III

is represented by:

$$\beta_q = \frac{[b_q]}{[b_1]^q} \qquad \text{(Eq. 2)}$$

where:

$$\beta_q = \prod_{2}^{q} K_q \qquad \text{(Eq. 3)}$$

β_q is the product of all stepwise association constants K_2, K_3, . . . , up to K_q.

The total molar concentration of all species, M, is given by:

$$M = \sum [b_q] \qquad \text{(Eq. 4)}$$

The total equivalent concentration of all species, C, the concentration that is usually known experimentally from the composition of a solution, is given by:

$$C = \sum q[b_q] \qquad \text{(Eq. 5)}$$

If the quantity G is defined as:

$$G = \sum q^2[b_q] \qquad \text{(Eq. 6)}$$

then the average degrees of association of all species, including the monomer, can be defined as:

$$N_n' = \frac{C}{M} \qquad \text{(Eq. 7}a\text{)}$$

$$N_w' = \frac{G}{C} \qquad \text{(Eq. 7}b\text{)}$$

where N_n' is the number average degree of association, and N_w' is the corresponding weight average value. N_n' is usually determined from colligative measurements such as freezing-point depression or vapor pressure lowering, and N_w' is obtained from light scattering and equilibrium ultracentrifugation. Various other methods also are available.

For many systems, particularly micelle-forming systems, the average degree of association of interest is that pertaining to the aggregates only, *i.e.*, *excluding the monomer.* Such degrees of association are defined as:

$$N_n = \frac{C - [b_1]}{M - [b_1]} \qquad \text{(Eq. 8}a\text{)}$$

$$N_w = \frac{G - [b_1]}{C - [b_1]} \qquad \text{(Eq. 8}b\text{)}$$

In experimental situations, the assumption is usually made that the monomer concentration above the CMC in micellar systems is represented by the value of the CMC itself. This assumption leads to the approximation:

$$N_w \approx \frac{G - \text{CMC}}{C - \text{CMC}} \qquad \text{(Eq. 9)}$$

These equilibria lead to some general conclusions about the degree of association from the fact that $[b_q]$ is proportional to $[b_1]^q$. Therefore, as $[b_1]$ increases, the concentrations of all q-mers increase. Thus, irrespective of which or how many q-mers form, N_n' and N_w', from their definitions (Eqs. 7*a* and 7*b*), must increase as the total concentration, C, increases. On the other hand, as infinite dilution is approached, *i.e.*, as $C \to 0$, then $N_n' \to 1$, $N_w' \to 1$, and $[b_1] \to C$.

If one considers the N_n and N_w values, *i.e.*, the degrees of association of the aggregates only, then if a *unique*, single oligomer (containing few monomers) or multimer (containing many monomers) forms, *i.e.*, the aggregate is *monodisperse*, clearly $N_w = N_n$ and both are independent of concentration. However, if there is a distribution in aggregate sizes, *i.e.*, the aggregates are polydisperse, since $[b_q]$ is proportional to $[b_1]^q$ and therefore the fractional increase in $[b_q]$ with increasing $[b_1]$ is larger as q increases, the definitions of N_n and N_w (Eqs. 8*a* and 8*b*) and Eqs. 4–6 lead to the important result that N_n and N_w must increase with concentration (7). For polydisperse aggregates, $N_w > N_n$ and the ratio N_w/N_n is a measure of the polydispersity. For a monodisperse system, this ratio is unity.

Structural and Geometrical Factors—In the self-association

equilibria discussed previously, the absolute values of K_q and β_q are the quantitative measure of the tendency toward self-association. Thus, the higher these values are, the more extensive is the association and the lower is the concentration at which the association becomes important. The pattern of the association, however, *i.e.*, whether the products of the self-association are unique oligomers (containing a few monomers) or multimers (containing many monomers), a narrow distribution of oligomers or multimers, or a broad distribution of oligomers or multimers, depends upon the *relative* variation of K_q or β_q with q. The geometrical and structural characteristics of the hydrophobic solutes are reflected both in the absolute and the relative values of K_q and β_q.

Hydrophobic solutes can be separated into four broad classes depending upon their structures.

Class I—The first class consists of flexible chain compounds containing polar head groups. Soaps, many synthetic detergents, cationic and nonionic surfactants, and phospholipids fall into this class. The association of these molecules is mainly due to the flexible chains, although these sometimes include unsaturated groups or aromatic moieties. These compounds are characterized by having nonpolar and polar ends joined together.

Class II—The second class consists mainly of aromatic or heteroaromatic ring or fused ring structures which are rigid. Many of these molecules may be quite flat or planar. Many dyes, purines, pyrimidines, *etc.*, fall into this class, which also contains a large number of drugs. Some of these molecules may possess one or more short flexible chains, with or without a polar group attached to the ring structure. If these chains are not large in comparison to the rings, the self-association is expected to be controlled by the planar portion of the molecule. Because of the presence of π-electron systems and slightly polar groups such as ring nitrogens, which may be capable of hydrogen bonding, these molecules tend to be less hydrophobic than the hydrocarbon chains of the same molecular weight.

An important characteristic that distinguishes this class of compounds from the Class I compounds is that the molecules do not possess a particularly polar or nonpolar end; they are roughly symmetrical with respect to their hydrophobicity on both sides of their flat or nearly flat structures. These molecules are thus capable of a "stacking"-type association in which each associating monomer can lie flat on top of a stack containing one or more monomers and there are no geometrical restrictions to open-ended continuous self-association. When polar or nonpolar groups are attached to the planar ring systems, these are radially disposed and, unless they are very bulky, they do not prevent face-to-face association.

Phenothiazines and similar molecules containing ring structures that are somewhat bent or kinked in the middle form a subclass of this class of solutes. The self-association of a monomer with another monomer probably involves the association of the convex side of one to the concave side of the other. The dimer in this configuration still has a concave and convex side open for further association, so stepwise self-association can continue indefinitely somewhat in the manner of a stacking of tiles.

Class III—This class consists mainly of alicyclic fused ring systems which may be inflexible but are not planar, *e.g.*, bile acids, cholesterol, and similar compounds. When polar functions are present, because of the tetrahedral geometry of most of the carbon atoms, the polar groups are directed away from the backbone of the molecule more to one side than the other. This tends to make one side more hydrophobic than the other (12).

Class IV—This class consists of macromolecular solutes, particularly proteins and enzymes. The self-association of proteins is of considerable importance in biochemistry and physiology. Hydrophobic interactions between exposed side chains probably play an important role in these association reactions, but complex conformational changes may be involved. The association equilibria can formally be treated in terms of stepwise self-association (10, 11). These solutes are not discussed further in this article.

DATA TREATMENT, METHODS, AND OTHER CONSIDERATIONS

The complete description of a self-associating system requires a knowledge of the values of K_q or β_q for all relevant values of q. Even when only a few oligomers or multimers are involved, this task is extremely difficult because experimental uncertainties of one K_q value affect those of all the others (8, 9).

Of the various possible experimental approaches available for studying self-associating systems, the nonequilibrium methods (*e.g.*, those based on spectroscopy of any kind, polarography, conductance, *etc.*) are usually unsuitable (13, 14). Such methods require the determination of a characteristic parameter for every oligomer or multimer other than the formation constant. For example, methods based on spectral changes require the estimate of the absorptivity for every oligomer or multimer (15), NMR methods require the estimate of a chemical shift, and conductance methods require the estimate of an equivalent conductance (16). This additional set of unknown parameters makes the determination of K_q or β_q values extremely difficult. The equilibrium methods generally depend upon the estimate of the free monomer concentration $[b_1]$ as a function of the total equivalent concentration C (8, 13, 14), the osmolarity M from colligative measurement, the number average degree of association $N_n' = M/C$, or the weight average degree of association $N_w' = G/C$. Different kinds of data usually require different kinds of analysis (7-11). Even when equilibrium data are available, if they are of ordinary precision, it is in general extremely difficult to extract more than two or three association constants precisely, although mathematical methods of data analysis are available in principle (8, 9).

It is, therefore, frequently necessary to devise suitable models of association in which the stepwise association constants are allowed to vary in some predetermined sequence and no more than two or three independent parameters are used to describe the models. For systems exhibiting unique oligomers or multimers (*e.g.*, dimers) or monodisperse micelles, two suitable parameters are β_q and q, for example. For systems showing continuous self-association, there may not be any restriction on allowed q values, and the association model may involve the use of one or two parameters which describe the variation of K_q with q, with q ranging from 2 to ∞, according to some predetermined sequence. Various models of this kind have been devised (8, 9).

Different Patterns of Self-Association: Structural and Geometrical Factors—Considering the complexities of interactions and geometrical factors involved, it is not surprising that self-association reactions are not simple. However, some limiting simple patterns of self-association are *approached* by some systems. Quantitative treatments based on these simple patterns can then be used to *approximate* the behavior of real systems.

The forces of interactions between monomers and multimers in hydrophobic association, as discussed earlier, are usually of short range unless coulombic repulsions are involved between similarly charged particles. The coulombic repulsions themselves are strongly dependent on the ionic strength of the medium. Higher ionic strengths, therefore, stabilize aggregates of ionic solutes. At high ionic strengths, salting-out and salting-in of the nonionic moiety may be appreciable also (3).

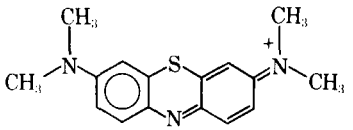
The different patterns of self-association are generated by the different variations in K_q or β_q with q. The patterns of association are seldom known completely. Even when they are known reasonably well from an experimental point of view, it is not often clear what factors control the patterns of association. In general, large multimers, *i.e.*, q values above 10 or 20, form as a result of stepwise association of monomers and not because of the concerted aggregation of 10 or more monomers. Collisional processes involving 10 or more monomers are extremely improbable in dilute solutions. Furthermore, it is unlikely that when a 20-mer, for example, is a major species, 19-mers and 21-mers are completely absent. Polydispersity should thus be the rule rather than the exception. On the other hand, the distribution in sizes can be quite narrow in many cases. A detailed understanding of the system requires not only the understanding of how stepwise self-association proceeds but how it is stopped.

Formation of Unique Oligomers—The formation of monodisperse aggregates, *i.e.*, q-mers where $(q - 1)$-mers or $(q + 1)$-mers are negligible, is more of a possibility for small oligomers than for large multimers. In hydrophobic association, only a few cases are known where the evidence suggests that a unique oligomer may exist over a range of concentrations. Long-chain fatty acid anions have been shown to be dimerized in dilute solutions (13). From equilibrium partition data, the average number of monomers in the average aggregate could be derived without assuming any

model of association. This value was found to be 2 over a wide range of concentrations varying by a factor of about 1000 (13). This means that K_3 and higher K_q values are negligible with respect to K_2. It has been argued that the association is limited to dimers for the ionic surfactants in dilute solution because stepwise trimerization entails greater charge repulsion (16) and less hydrophobic interaction when compared to the dimerization reaction (3).

For sodium cholate in aqueous solutions, light-scattering studies suggested a degree of association of 2, *i.e.*, dimer formation (17). Higher degrees of association are found in the presence of added salt (17–19). This fact, coupled with the uncertainty of the interpretation of light-scattering data in the absence of added salt and other lines of evidence, makes it difficult to assert that only dimers form. The structure of the cholate ion, however, suggests that it is not physically unreasonable to assume that dimerization may be somewhat favored in this system, *i.e.*, K_2 may be significantly higher than K_3 or K_4. Cholic acid, with three hydroxyl groups directed to one side of the molecule, has a hydrophobic back side which should allow dimerization in a back-to-back configuration. This configuration does not allow further association in the same manner because that would entail front-to-front or front-to-back association, involving the side containing the polar groups. Thus, the formation of trimers and higher multimers is likely to be reduced because of the special geometry of the molecule. Multimer formation may require different arrangements of the molecules (12).

Continuous Stepwise Self-Association—In contrast to sodium cholate, rigid planar molecules, aromatic or heteroaromatic, should allow stacking interactions, as described earlier. A model system, the cationic dye methylene blue, was investigated in detail recently (4, 9, 14, 20, 21); Structure I is one of four resonance forms of this dye ion.



I

It was possible, by using two new experimental techniques, to estimate the monomer concentration as a function of the total concentration of methylene blue directly. This information is valuable for deriving association constants and patterns of self-association (9). Many different models of association were examined. Some evidence was found for cooperative interactions in the early stages of association. Thus, in spite of the greater charge repulsion involved in stepwise trimerization, K_3 was found to be greater than K_2. The best fitting model indicated that higher K_q values decrease in a mild sequence, in agreement with the expected build-up of the charge repulsion. However, these variations were not very large.

This study (9) suggested that with stacking-type interactions for many charged systems undergoing self-association, if the charge repulsions in self-association are of minor importance compared to the hydrophobic interactions, and for uncharged systems generally, a particularly simple model of continuous self-association often provides a good description of the associating system. This model uses only one parameter, namely a stepwise association constant which is assumed to be the same for all oligomers and multimers (8). The model thus states that:

$$K_2 = K_3 = K_4 = K_q = K_\infty = K \qquad \text{(Eq. 10)}$$

This one-parameter model, because of its simplicity and widespread applicability, is examined in some detail.

Relationships between various observable quantities can be easily derived. The product of the equilibrium constant K and the equilibrium monomer constant $[b_1]$ is defined as a pure number X:

$$X = K[b_1] \qquad \text{(Eq. 11)}$$

The total molar concentration M is obtained as follows:

$$M = [b_1] + [b_2] + [b_3] + \ldots [b_q] \ldots \qquad \text{(Eq. 12a)}$$

$$M = [b_1]\{1 + K[b_1] + K^2[b_1]^2 + K^3[b_1]^3 \ldots + K^{q-1}[b_1]^{q-1} \ldots\} \qquad \text{(Eq. 12b)}$$

$$M = [b_1](1 + X + X^2 \ldots X^q \ldots) \qquad \text{(Eq. 12c)}$$

$$M = \frac{[b_1]}{1 - X} \text{ for } X < 1 \qquad \text{(Eq. 12d)}$$

In any real system obeying Eqs. 12a–12d, the value of X is less than 1. In a similar manner, the equivalent concentration C is given by:

$$C = \frac{[b_1]}{(1 - X)^2} \qquad \text{(Eq. 13)}$$

so that:

$$\left(\frac{[b_1]}{C}\right)^{1/2} = 1 - K[b_1] \qquad \text{(Eq. 14)}$$

Equation 14 permits the determination of K if $[b_1]$ is known as a function of the total concentration C (8, 9).

The molar osmotic coefficient ϕ can be defined as the ratio of the osmolarity M to the equivalent concentration C; ϕ is related to $[b_1]$ through the equation:

$$\frac{M}{C} = \phi = 1 - K[b_1] \qquad \text{(Eq. 15)}$$

K can be determined from osmotic coefficient data obtained from colligative measurements (20) by using:

$$K = \frac{1 - \phi}{C\phi^2} \qquad \text{(Eq. 16)}$$

The number average degree of association N of all species, including the monomer, namely N_n', is given by:

$$N_n' = \frac{C}{M} = \frac{1}{1 - X} \qquad \text{(Eq. 17)}$$

Similarly:

$$N_w' = \frac{G}{C} = \frac{1 + X}{1 - X} \qquad \text{(Eq. 18)}$$

The number average degree of association of all aggregate species, *i.e.*, excluding the monomer, can be derived from Eqs. 19–21 derived from the model:

$$\frac{M - [b_1]}{[b_1]} = \frac{X}{1 - X} \qquad \text{(Eq. 19)}$$

$$\frac{C - [b_1]}{[b_1]} = \frac{X(2 - X)}{(1 - X)^2} \qquad \text{(Eq. 20)}$$

$$\frac{G - [b_1]}{[b_1]} = \frac{2X}{(1 - X)^3} + \frac{X(2 - X)}{(1 - X)^2} \qquad \text{(Eq. 21)}$$

It follows from these equations that:

$$N_n = \frac{C - [b_1]}{M - [b_1]} = \frac{2 - X}{1 - X} \qquad \text{(Eq. 22)}$$

Similarly:

$$N_w = \frac{G - [b_1]}{C - [b_1]} = \frac{2}{(1 - X)(2 - X)} + 1 \qquad \text{(Eq. 23)}$$

This model of association fits or approximates the self-association behavior of many purine and pyrimidine bases of nucleosides that show stacking interactions (22, 23).

The characteristic features of the association pattern described by the one-parameter model, which leads to the well-known most probable distribution (7), are that N_n and N_w are slowly varying functions of the total concentration (compare, for example, Eqs. 13 and 22), that the aggregates are polydisperse so that N_w/N_n is greater than unity (compare Eqs. 22 and 23), and that the polydispersity index N_w/N_n approaches the value of 2.00 as X and, therefore, C increase, so the molecular weight distribution is

wide. The average degree of association of the aggregate species can be quite low, even when a major fraction of the molecules is associated. Thus, when the value of X is 0.30, $C/[b_1]$ from Eq. 13 is 2.04, so about half of the monomers are aggregated. At this concentration, however, $N_n = 2.43$ (Eq. 22) and $N_w = 2.68$ (Eq. 23). The value of $N_n = 11$ when $X = 0.9$. The corresponding $C/[b_1]$ ratio, however, is 100, indicating only 1% free monomers.

Several other models of continuous stepwise self-association involving changes in K_q values with q were devised and their predictions were examined (8, 9). In these models, a predetermined explicit relationship between the successive equilibrium constants K_q as a function of q above and including K_3 is invoked whereas K_2 is considered to be an independent parameter. The variation in K_q in the model might indicate that successive association constants become more favorable or less favorable, *i.e.*, the model might include an element of positive or negative cooperativity. These models are likely to be more realistic than the simple one-parameter model in many cases (9).

Formation of Large Multimers (Micelles) of Narrow Size Distributions—In contrast to the rigid monomers discussed, flexible chain surfactants with polar head groups produce aggregates of different geometry. The chains can coil around each other and fill up space by themselves. Thus, spheroidal aggregates can form, with the cores consisting of hydrocarbon chains, whereas the polar groups remain exposed to water at the surface. This arrangement partially satisfies both the hydrophobic and hydrophilic tendencies of the amphipathic monomers. The aggregates described are called micelles. Various physical properties of long-chain surfactants can be understood in terms of the formation of micelles. Micelle formation is one of the best known and most spectacular examples of self-association.

A characteristic feature of a typical micelle-forming system is that above the CMC, the first detectable aggregate (micelle) may be already quite large. Thus, only a small fraction of the solute may be aggregated and yet the value of N_w may be in the 30–120 range (24). The pattern of the association is thus quite different from those described earlier. The micelle sizes are *approximately* consistent with those expected for spherical micelles whose radii are equal to the lengths of the fully stretched monomers. The degree of aggregation, N_w, depends upon various factors including the chain length, the ionic strength, and the nature of the counterions for ionic surfactants, the nature of the head groups, and the temperature (24). The degree of aggregation, N_w, is often nearly constant and independent of the concentration above the CMC in many systems. When this is the case, the molecular weight (size) distribution is narrow, *i.e.*, $N_w \approx N_n$ (7), as shown recently by an analysis of the multiple equilibria involved.

The formation of large multimers of narrow molecular weight distributions requires some special types of variations in K_q with q. As discussed previously, when K_q is independent of q, polydisperse aggregates result and high N_n or N_w values can be obtained only when monomer fractions are extremely low. If K_q increases monotonically with q, a narrow molecular weight distribution independent of concentration cannot be obtained. If K_q decreases monotonically with q, the formation of large aggregates in preference to smaller ones is not possible. Therefore, typical micellar systems showing high N_w values close to the CMC require that K_q *increases* with q at the early stages of the growth of micelles, so that larger micelles are more stable than smaller ones. Thus, there must be a positive cooperativity of association in the early stages of growth. On the other hand, the narrow range of q values indicates that the growth must be limited beyond certain values of q, *i.e.*, K_q must eventually decrease with q at higher values of q. Thus, K_q values must show a maximum at some value of q.

When considering the factors responsible for micelle formation, the rationale for this pattern of the variation of K_q with q derives from the opposing effects of hydrophobic interactions and head-group interactions. Hydrophobic interactions are mainly responsible for the formation of micelles. The head groups usually oppose micelle formation. When the head groups are charged, the addition of a monomer to an aggregate involves a charge repulsion (25, 26). When the head groups are nonionic, the repulsive interactions arise from the crowding of the head groups at the micelle surface (3, 7). As small spherical micelles grow, the hydrophobic interactions involved in the addition of successive monomers increase because the total amount of hydrocarbon interface lost on the addition of a monomer is greater as the size of the sphere increases, as simple geometrical considerations will show. This factor, therefore, leads to an increase in K_q with q. As the sphere increases in size, however, the volume of the micelle and, therefore, q increase proportionately to r^3, where r is the radius of the sphere, whereas the surface increases as r^2 increases. Therefore, the density of the head groups also increases with q, as do the charge repulsion factor and the self-interaction of the head groups on crowding. These factors should, therefore, reduce the value of K_q as q increases.

It is generally accepted that when the micelles are very large, *i.e.*, for q values above several hundred, the micelles are rod-like (27, 28), presumably with spherical ends. When small micelles grow to be too large for spheres, there must be a transition in shape from a sphere to a sphero-cylinder-type structure (28). During this transition also, both the hydrophobic interactions and head-group and charge interactions increase. It is possible that the sphere–rod transition is ultimately responsible for limiting the growth of the micelles. Qualitatively, however, it seems that the interplay of geometrical factors, the hydrophobic interactions, and the charge and head-group effects is responsible for an increase in K_q with q at low values of q and a subsequent decrease at some higher values.

When q values are large, only small variations in the average free energy of micelle formation per monomer can produce narrow distributions in molecular weights. To demonstrate this, as is customary (3), the formation constants of the micelles from the monomers, the β_q values, are used. These provide a direct measure of the standard free energy changes associated with micelle formation for nonionic systems corresponding to the process of Scheme III:

$$q\,\Delta G_q = \Delta G_q' = -RT\,\ln \beta_q \qquad \text{(Eq. 24)}$$

Here ΔG_q is the average free energy change per monomer when the q-mer forms, $\Delta G_q'$ is the total change, R is the molar gas constant, and T is the absolute temperature. Since β_q is a product of the K_q values (Eq. 3), $\ln \beta_q/q$, which equals $-\Delta G_q/RT$, will not show a maximum at the same value of q as K_q itself.

Figure 1 illustrates a model calculation in which the following *arbitrary* relationship between β_q and q is assumed:

$$\ln \beta_q = 2(q-1)\ln(q-1) - 0.02(q-1)^2 + 2.7896(q-1) \qquad \text{(Eq. 25)}$$

An equation of this form (25) is chosen because it shows a slight minimum in ΔG_q (Fig. 1). The equivalent concentrations of the q-mers, $q[b_q]$, are then calculated using the appropriate β_q values and Eq. 25 for an assumed monomer concentration corresponding to $\ln[b_1] = -10.000$, ($[b_1] = 4.11 \times 10^{-5}$ mole/liter). A narrow size distribution with a maximum at about $q = 97$ and a half-width of less than 10 is obtained, even though ΔG_q changes by less than 2% over the whole range of 70–120 for q. Thus, only mild minima in the free energy profile (ΔG_q *versus* q curves) are needed to produce narrow size distributions of micelles. Sharper minima produce narrower distributions. This point also illustrates the difficulty of predicting such size distributions exactly, because extremely precise calculations of the ΔG_q values are required which involve the incompletely understood hydrophobic interactions and head-group effects.

Formation of Polydisperse Giant Micelles—In many micellar systems, particularly when the monomer chains are very long, very large rod-like micelles form in dilute solutions (7, 27–30). For these giant micelles, a relatively simple model of self-association can be derived which seems to account for many of their properties (7).

The basic assumption of the model is that for a cylindrical micelle, if the end effects can be neglected and if the growth of the micelle does not change the minor axes, the introduction of a monomer involves the same amount of hydrophobic and head-group interactions irrespective of the length of the cylinder (7). When q is very large, above 200–300 for most surfactants, K_q becomes independent of q. The real system, of course, must also have aggregates for which q values are low. For the giant micelles ($q > \sim 200$) to appear at low solute concentrations, K_q must increase with q over certain ranges of low q values as with ordinary small

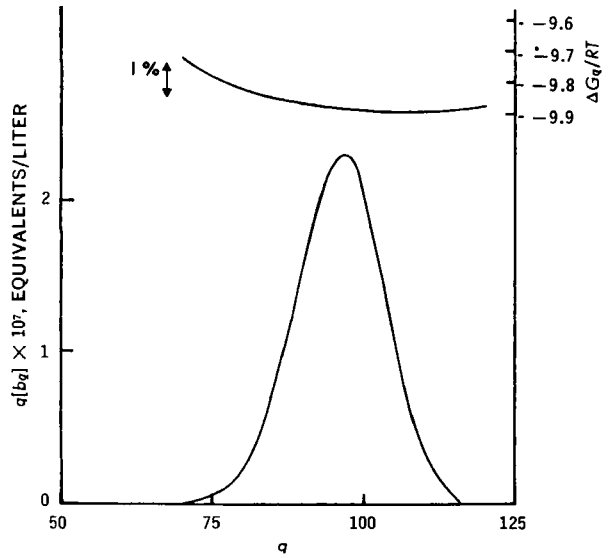


Figure 1—*Variation in the concentration of monomers existing as micelles,* $q[b_q]$, *as a function of the number of monomers in the micelle,* q, *for an assumed free energy profile,* ΔG_q versus q, *upper curve (see text).*

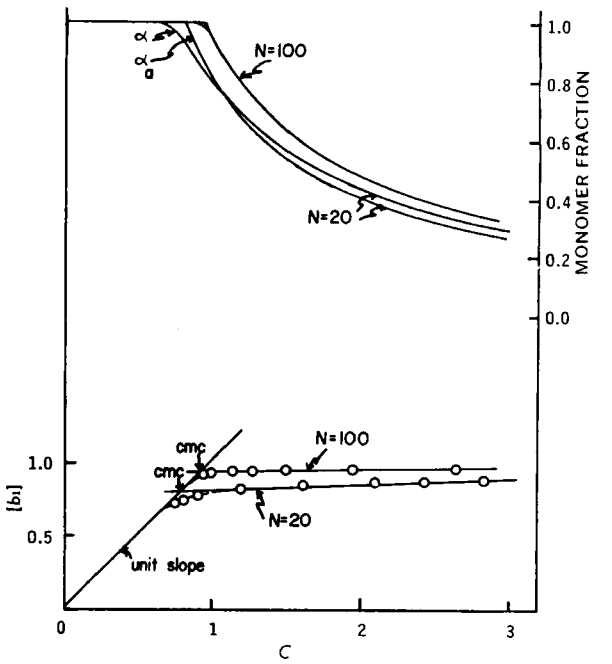


Figure 2—*Variation in the monomer concentration* $[b_1]$ *as a function of the total solute concentration* C *for monodisperse micelles containing 20 and 100 monomers/micelle.* α *is the true monomer fraction, and* α_n *is the fraction calculated using the micellar hypothesis (see text).*

micelles. This gradual increase is replaced by a step function in an approximate manner by invoking the following model of association:

$$K_2' < K \qquad K_3 = K_4 = K_q = K_\infty = K \qquad \text{(Eq. 26)}$$

Here, instead of a gradually increasing function of K_q with q at low q values, followed by an uncertain transition region around $q \approx 50$–200 corresponding to the sphere to rod transition which, in turn, is followed by a constant value of K_q at very high q values ($q > \sim 200$), a *hypothetical* K_2' is chosen which is probably a good deal lower than the real K_2, followed by all subsequent K_q values equal to each other. The replacement of the gradually increasing function by a step function has some important consequences. One is that the model is appropriate only when moderate q values do not make a major contribution to the micelle size distribution, *i.e.*, average N_w values that are large ($N_w > \sim 300$).

Given the limitations of the model, the predictions are easy to examine. The model differs from the previous one-parameter model for continuous self-association by having an extra parameter K_2'. On summing the series for M, C, and G in terms of $X = K[b_1]$, the following relationships are obtained (7):

$$\frac{M - [b_1]}{[b_1]} = \frac{K_2'}{K}\left(\frac{X}{1 - X}\right) \qquad \text{(Eq. 27)}$$

$$\frac{C - [b_1]}{[b_1]} = \frac{K_2'}{K}\left(\frac{X(2 - X)}{(1 - X)^2}\right) \qquad \text{(Eq. 28)}$$

$$\frac{G - [b_1]}{[b_1]} = \frac{K_2'}{K}\left(\frac{2X}{(1 - X)^3} + \frac{X(2 - X)}{(1 - X)^2}\right) \qquad \text{(Eq. 29)}$$

These relationships are identical with Eqs. 19–21 when $K_2' = K$.

The predictions of the model are in some ways similar to the previous one-parameter model. In particular, N_w and N_n values are given by Eqs. 22 and 23. When N_w is large, $N_w \approx 2N_n$ and the size distribution is wide. When X is close to unity, $C >> [b_1]$ and N_w is proportional to $\sqrt{C}$, a prediction that has been confirmed (7).

The difference between the two-parameter model leading to the formation of a series of giant aggregates and the one-parameter model leading to the formation of a continuous series of aggregates is a matter of degree and derives from the ratio K_2'/K. To make this comparison, let us take a numerical example in which $K = 10^5$ (liters/mole) and $X = 0.999$, so that $[b_1] = 9.99 \times 10^{-6}$ mole/liter and N_w is then about 2000 (Eq. 23), a value large enough so that the two-parameter model applies. From Eq. 20, the value of C needed to produce this degree of aggregation for the one-parameter model is 10 equivalents/liter, a very high concentration indeed. From Eq. 28, however, if $K_2'/K = 10^{-4}$, a value of 2000 for N_w is obtained at a concentration of only about 10^{-3} equivalent/liter. The values of K, K_2', and $[b_1]$ used for illustrative purposes are similar to some values pertaining to a real nonionic micelle-forming surfactant (7). Some other consequences of this model have been examined, including the prediction of a sharp CMC when K_2'/K is 10^{-3} or lower. The initial cooperativity of micellar association can thus be represented in these cases by the choice of a low ratio of K_2'/K (7).

Micellar Hypothesis and Significance of CMC—In contrast to the systems showing small oligomers only, either unique ones or those having a distribution over a narrow range, and systems showing continuous self-association in which all stepwise association constants are roughly of the same magnitude, typical micelle-forming systems exhibiting high degrees of association ($N_w > 20$) show no evidence of micelle formation until the CMC characterizing the system is reached. This is the result of the cooperativity of association in the initial growth of micelles. The significance of the CMC is of central importance in the discussion of micelle formation and the application of the micellar hypothesis to self-associating systems.

It has been shown that typical micellar systems involving small micelles (*i.e.*, the ones that are roughly consistent with spherical micelles of radius r where r is the length of the stretched-out monomer) in many cases have narrow distributions in molecular weight (7). Such a micellar system, showing an N_w value of 50, has negligible amounts of 10-mers, 20-mers, 30-mers, 70-mers, and 80-mers. Narrow distributions of this sort can be *approximated* by monodispersity, so that the monomer–micelle equilibrium for nonionic systems can be represented to a good approximation by Scheme IV:

$$Nb_1 \rightleftharpoons M$$

Scheme IV

where the micelle M contains N monomers. It is well known that when N is large ($N > 20$), Scheme IV predicts the existence of a critical concentration range somewhat below which micelles are absent and somewhat above which essentially all additional solute forms micelles (31).

Figure 2 illustrates this fact and the effect of N on the sharpness of the transition at the CMC. The equilibrium constant, K_m,

governing Scheme IV is given by:

$$K_m = \frac{[M]}{[b_1]^N} \qquad \text{(Eq. 30)}$$

for ideal uncharged systems. By assuming that $K_m = 1$, the monomer and micellar concentrations have been calculated for $N = 20$ and $N = 100$. Any other choice of the K_m value merely shifts the concentration scales and not the shapes of the curves. For $N = 100$, as C increases from zero, the value of $[b_1]$ increases with unit slope, *i.e.*, there is no detectable self-association, until C approaches 0.9. When $C = 0.929$, $[b_1]$ equals 0.915, *i.e.*, only about 1.5% of the solute is micellized. On increasing C further, however, the value of $[b_1]$ increases very slowly and the equivalent concentration of micelles, $C - [b_1]$, increases roughly as C itself. Thus, over a narrow range of concentrations of the order of a few percent only, as C changes from about 0.92 to 0.97, the distribution of the solute species changes abruptly. Any measured physical property of the system that reflects the concentration (activity) of the monomer, the equivalent concentration of the micelle, or any unequally weighted combination of the two will register or display an abrupt change of slope over this concentration range, *i.e.*, show a "kink" in the curve, when the property is plotted as a function of the concentration. Even when the value of N is as low as 20, corresponding to very small micelles, the change in composition of the solution around the critical region is still fairly sharp. Thus, when $C = 0.75$, the fraction of the solute micellized is only 4%, and the value of $[b_1]$ is 0.72. Beyond the transition region, as C increases, $[b_1]$ increases faster than in the case of $N = 100$ but is still only 0.88 when $C = 2.43$. If now N is allowed to increase continuously from a minimum value of 20, the rate of increase of $[b_1]$ with C will be reduced further. Thus, polydispersity of the giant micelles does not invalidate the argument about the sharpness of the changes but only strengthens it. For the giant micelles, N_w is large and increases with C.

The key requirement for the existence of a narrow concentration range across which the fraction of added solute that forms micelles changes from nearly zero to nearly unity is the initial cooperativity of association that makes large or intermediate sized micelles so much more stable with respect to smaller ones that the latter do not appear to any significant extent. However, no careful study has demonstrated a "break" in any curve, *i.e.*, a discontinuity, at the CMC. All properties of a solution in the CMC region vary in a continuous manner.

Although the change in the nature of the solute species distribution occurs over a narrow range of concentrations rather than at one concentration, only very precise techniques allow the transition range itself to be studied (3, 16). The usual practice is to extend observed segments of the curves of a chosen property plotted against a suitable concentration scale below the transition region and above the transition region linearly to meet at a point which is then defined as the CMC. Figure 2 gives examples of how CMC values may be determined from $[b_1]$ *versus* C curves. The CMC thus represents some value of the concentration within the transition range. Many methods are available for determining the CMC (6). Different experimental methods may give somewhat different estimates of the CMC, depending upon how monomers and micelles are reflected in the measured property (6). For typical micellar systems, however, they agree within a few percent. The CMC is an approximate measure of the concentration at which micelles become detectable.

The micellar hypothesis, therefore, represented by Scheme IV and its proper modification for charged systems (3), leads to the following approximations which are usually valid to within small degrees of error. These approximations are frequently used when micellar systems are encountered in many practical situations and are closely related to the micellar hypothesis itself.

1. In the dilute concentration range, up to very near the CMC (*i.e.*, below roughly 0.9 × CMC), micelle formation can be completely neglected. Unless there is some premicellar formation of small aggregates, the evidence for which is uncertain (3), one can assume that the monomer concentration equals the total concentration and the monomer fraction is unity.

2. Above the CMC, the monomer concentration for the ideal uncharged system and the monomer activity in general increase very slowly with total concentration so that the CMC is a reasonable measure of the activity of the monomers above the CMC up to fairly high concentrations. The monomer fraction above the CMC is given by CMC/C.

3. The micellar concentration is negligible until shortly before the CMC. Above the CMC, the equivalent concentration of micelles can be approximately equated to C − CMC.

4. The CMC can be used to calculate thermodynamic quantities related to micelle formation, such as the free energy of micelle formation, and the degree of dissociation for charged systems using various approaches (3).

5. Light-scattering and various data dealing with properties of micelles can be interpreted assuming monodisperse micelles. This approximation has been used for very large (giant) micelles also. In view of the polydispersity inferred for such systems (7), this particular approximation appears to be unsuitable for such systems.

These approximations are clearly of importance in all situations where monomer concentrations (activity) or micellar concentrations are involved. These include equilibrium or transport processes of the monomer–micelle system itself; adsorption, binding, and interfacial activity of the monomer; and processes involving micelles such as solubilization. In quantitative treatments of physiological activities of associating systems such as bile salts or hydrophobic drugs for which CMC values are reported, the ancillary approximations are often made. This need arises because the detailed information that would make the approximations unnecessary is not usually available. Indeed, properties of the micelles themselves above the CMC, such as molecular weights (24), charge (32), electrophoretic mobility and electrokinetic potentials (33), hydration (34), and solubilizing powers (35), are usually investigated by making the approximation that C − CMC represents the equivalent concentration of micelles.

These approximations are really not approximations but are exact consequences of the two-phase model of micelle formation (3) according to which the CMC corresponds to a phase separation point. Evidence from various sources (3) indicates that the two-phase model is only approximately valid for micellar systems. A theoretical framework has recently become available for examining the approximations for ideal systems, *i.e.*, systems in which intermicellar interactions can be neglected (7). For example, Eq. 31, representing the change in the concentration of the monomers above the CMC, is applicable to all such systems of any degree of polydispersity:

$$d \ln [b_1] = \frac{C - [b_1]}{G - [b_1]} d \ln (C - [b_1]) = \frac{d \ln [b_1]\left(\frac{C}{[b_1]} - 1\right)}{N_w(C)} \qquad \text{(Eq. 31)}$$

where $N_w(C)$ represents the fact that N_w can be a function of (C) if the system is polydisperse. Equation 31 indicates that the change in $[b_1]$ with C is reduced as N_w becomes higher, and as $C/[b_1]$ increases.

The approximations discussed are closely connected to the estimated value of the CMC. The meaning and significance of the CMC in many other connections were examined in detail as a part of a critical discussion of the methods of determining CMC values and of a critical compilation of some 4600 CMC values of 720 different compounds (6). The important point to be made here is that when a concentration value for a system is reported as a CMC, these approximately valid conclusions about the system usually become part of the description of the system. It is, therefore, of some general interest to examine how the micellar hypothesis can be misused for many systems and what some consequences of this misuse are for self-associating systems to which the micellar hypothesis does not apply to a suitable degree of approximation.

Apparent CMC Values that May Be Misleading—The genesis of a narrow critical concentration range in micellar systems from the initial cooperativity of association of monomers to micelles has been discussed. Whereas the CMC is usually determined from a change in the concentration dependence of some solution property, not all real or apparent changes in such dependence correspond to CMC's. Indeed, many solution properties of systems showing self-association, micellar or nonmicellar, show curvatures in graphical plots against concentration; many of these curved plots may appear to show kinks resembling critical con-

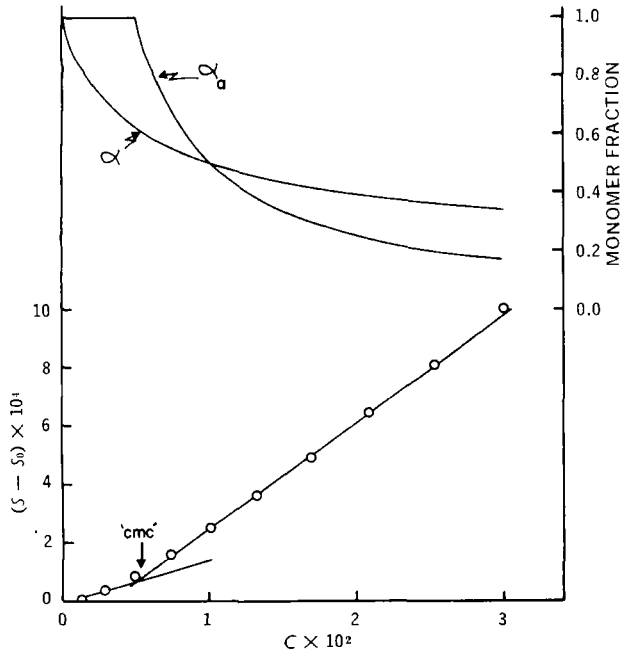


Figure 3—*Simulated solubilization curve for a system showing only dimerization. α is the true monomer fraction, and α_a is the fraction calculated using the micellar hypothesis (see text).*

centration ranges, particularly if a limited amount of experimental data is available. If all of these kinks are interpreted as CMC values and the micellar hypothesis is applied along with the approximations associated with the pre-CMC region, the post-CMC region, and the CMC itself for micellar systems, rather substantially erroneous conclusions about the self-associating system may be drawn. Several examples of how such apparent CMC values may arise in nonmicellar self-associating systems in practical situations are given here. These do not include such relatively trivial examples as the reaching of a solubility limit or the leveling of the degree of hydrolysis of a hydrolyzing system.

Figure 3 shows an example of a model system in which it is assumed that a nonionic hydrophobic solute undergoes only dimerization ($2b_1 \rightleftharpoons b_2$) with $K_2 = 10^2$ liters/mole. It is now assumed that a substance P, with a low solubility in water, is solubilized according to the association reaction shown in Scheme V:

$$b_1 + P \rightleftharpoons b_1P$$

Scheme V

At saturation, if the concentration of P in solution is S and the solubility in water is S_0, then $S - S_0$ is the amount solubilized, which equals $K_sS_0[b_2]$ where K_s is a constant. The ordinate of Fig. 3 plots this excess solubility, $S - S_0$, assuming $K_sS_0 = 10^{-2}$. The diagram thus simulates a solubilization experiment. The concentration of b_2P was neglected in calculating C. The data can now be represented by two lines, showing an apparent CMC value at about 5×10^{-3} equivalent/liter. Any other choice of K_sS_0 produces the same curve with a change in the ordinate scale. A different K_2 value does not change the shape of the curve and, therefore, the existence of the apparent CMC. Clearly, however, this apparent CMC cannot have the same significance as the CMC of a micelle-forming substance and the generalizations that hold *at least approximately* for micelle-forming systems are completely inapplicable here.

A second example is shown in Fig. 4. Here the model system is assumed to show continuous self-association, with all stepwise association constants having the identical value (Eq. 10). By assuming this value of K to be 10^2 liters/mole (any other value merely changes the scales of the ordinate and the abscissa), the calculated molar concentration M (Eq. 12) is plotted against the equivalent concentration C (Eq. 13). This is equivalent to plotting osmotic pressure data or osmolarity data from vapor pressure lowering (36). The system again shows an apparent CMC, which depends somewhat upon which segments of the curve are used to derive it. This apparent CMC again cannot have the same kind of significance as the CMC of a typical micellar system because of the assumption of the model chosen.

A third example is shown in Fig. 5. Here the function G (from Eq. 21) is plotted against C for the continuous self-association model in which all the stepwise association constants are the same, these values being 10^2 liters/mole. Any other value is represented by the same curve by a change in scale of the ordinate and the abscissa. The definition of G shows that this plot is equivalent to plotting turbidity data from light scattering, assuming that the optical and refractive characteristics of all aggregates and the monomer are the same. The curve again can be represented approximately by two straight lines to show an apparent CMC value. From the slope of the plot above the apparent CMC, a value of 7 is derived for N_w.

Figures 4 and 5 illustrate that systems showing typical stacking-type interactions lend themselves fairly easily to interpretation in terms of micelle formation. This interpretation and the use of the attendant generalizations about micellar systems can be substantially erroneous. Many drug systems with rigid structures are likely to show patterns of self-association of the kind used for Figs. 4 and 5 or some mild variants of it such as the ones examined for methylene blue self-association (9, 14). Considerable care should be exercised in the application of the micellar hypothesis to such systems. Close examinations of all such systems in terms of various models of multiple self-association equilibria are desirable before the micellar hypothesis is forced on the systems. In general, if the degree of association close to the CMC is low, less than 20, and if there is evidence of association well below the apparent CMC, care should be taken.

Monomer Fraction and Problems of Availability—An obvious result of self-association is to decrease the value of the monomer fraction, α, from unity. This is also one of the most important effects of self-association. Hydrophobic self-association equilibria are usually rapidly attained, so that for most processes any change in the total concentration C results in a rapid adjustment of the value of α to the equilibrium value. For many processes, the effective concentration is the monomer concentration, αC. Indeed, for ideal self-associating systems that are uncharged, α is the activity coefficient of the system (23) and $\alpha C = [b_1]$ measures the activity of the system. In any process where this activity is important, such as binding to a substrate or adsorption to an interface, α and its dependence on C are thus of crucial importance in all quantitative calculations. For self-associating drug systems in particular, one can expect that α will be an important parameter in the control and understanding of binding to proteins or re-

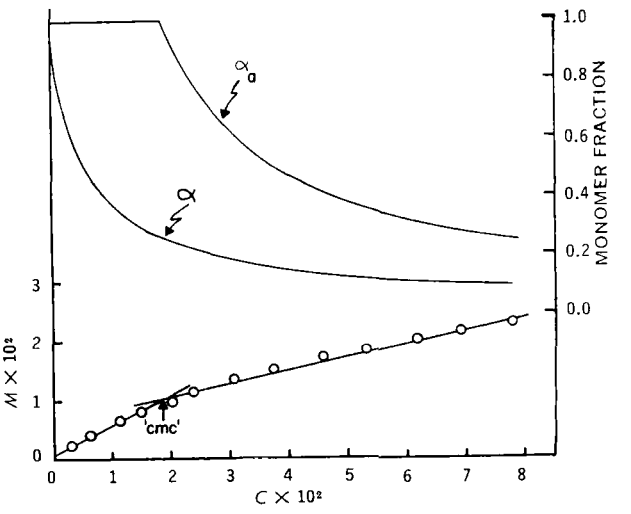


Figure 4—*Calculated osmolarity, M, for a system showing continuous self-association (Eq. 10) with all stepwise association constants having the same value, 10^2 liters/mole, plotted against the total solute concentration C. α is the true monomer fraction, and α_a is the fraction calculated using the micellar hypothesis (see text).*

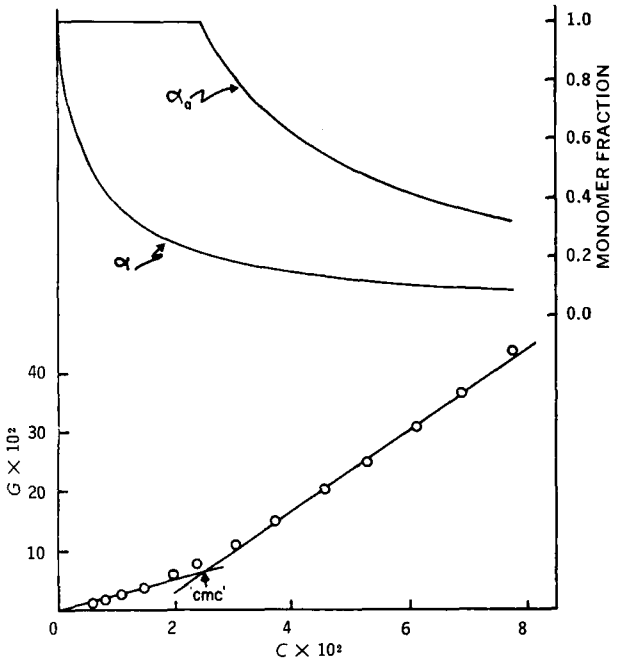


Figure 5—*Simulated turbidity–concentration plot for a system showing continuous self-association (Eq. 10) with all stepwise association constants having the same value, 10^2 liters/mole. Ordinate* G *(Eq. 6) is proportional to the solute turbidity obtained in light-scattering experiments under idealized conditions (see text), α is the true monomer fraction, and α_a is the fraction calculated using the micellar hypothesis (see text).*

ceptor sites, binding to membranes prior to transport, adsorption to interfaces, and rates of solution and diffusional transport; α is a measure of the "availability" of the drug or any other hydrophobic solute in many situations.

In view of the importance of this parameter and its close interrelationship with the micellar model or any other model of self-association, it is of interest to examine to what extent the approximations of the micellar hypothesis apply to other systems. These approximations are that α is unity up to the CMC and has the value of CMC/C above the CMC. The true values calculated for various model systems are compared here to this approximate value, designated as α_a.

Figure 2 shows how α compares with α_a for monodisperse micellar systems. Above the CMC, α decreases rapidly. As mentioned previously, for micellar systems α or CMC/C in some experimental situations may be as low as 10^{-4}–10^{-5}. In the limited range covered in Fig. 2, the agreement between α_a and α is better when N = 100 than when N = 20, as is to be expected. The important point to note is that even when N = 20, *i.e.*, when the micelle is rather small, α and α_a do not differ by more than 0.07 in the region covered. In dilute solutions in particular, somewhat below the CMC, α_a, with a value of unity, provides a very good approximation.

In contrast to these micellar systems, systems for which other patterns of self-association hold show considerable differences between the true α and the α_a calculated using the micellar hypothesis. For the dimerization case (Fig. 3), for example, α has a value of 0.62 at the apparent CMC and the monomer fraction is appreciably less than below the CMC unity. The deviations below the apparent CMC are even more marked in Figs. 4 and 5. Considerably below the CMC, in dilute solutions, when the micellar hypothesis would indicate negligible association, the value of α is well below unity. In the dilute solutions, therefore, which are often the most important in physiological systems, the true α may be considerably lower than that predicted by the micellar hypothesis, even though the latter hypothesis assumes large aggregates. Thus, in any situation where the availability or activity of the monomer is important, assuming α to be unity leads to errors. The misuse of the micellar hypothesis for such systems also

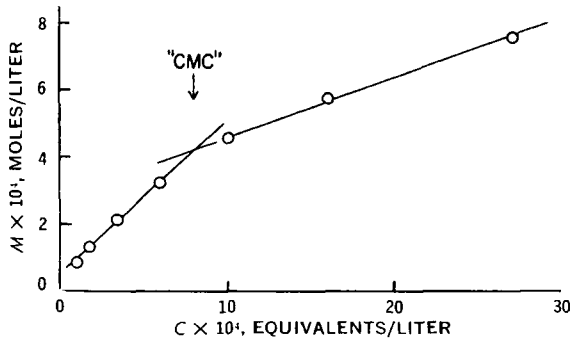


Figure 6—*Apparent CMC for methylene blue in aqueous solutions at 25°. (The data for* M *and* C *are from Ref. 14.)*

leads to serious errors in any quantitative calculation involving the CMC, such as the free energy of micelle formation, because the monomer fraction at the apparent CMC may be as low as 0.25 instead of being close to unity.

Lack of Significance of a CMC Value for Methylene Blue—Figure 6 shows an example of a real system for which an apparent CMC value can be obtained but for which the application of the micellar hypothesis leads to erroneous results. The self-association of methylene blue has been studied in detail and analyzed in terms of multiple stepwise association equilibria (4, 9, 14). From the experimentally determined variation in the equilibrium monomer concentration [b_1] with the total concentration C, the values of M, the molarity sums of all the methylene blue species (Eq. 4), were calculated as a function of C by graphical integration to examine the variation of N_n with C in the original work (14). The data were obtained at an approximately constant ionic strength so that Schemes I–III and Eqs. 1–8 apply because the charge effects can be assumed to be constant (9, 14). Changes in M for this system, therefore, represent changes in the osmolarity contribution of the aggregating species of methylene blue.

Figure 6 shows a plot of M *versus* C. An apparent CMC value can be obtained at a concentration of 8×10^{-4} mole/liter of methylene blue. This value, if interpreted as a true CMC, gives a very misleading description of the system. The aggregation of methylene blue is extensive at much lower concentrations, and the experimental value of the monomer fraction at this apparent CMC is only about 0.24 (14).

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Micellar Properties of Drugs: Determination of Molecular Weight, Size, and Molecular Interactions of Drug Micelles Using the Analytical Ultracentrifuge

JOEL KIRSCHBAUM

Abstract □ This article illustrates the usefulness of the analytical ultracentrifuge in investigating micellar properties of drugs. Also discussed are some examples of micelle forms of drugs and the working hypothesis that the same binding forces that hold together the aggregate found at high concentrations may be responsible for the binding of monomer (at physiological concentrations) to biological structures.

Keyphrases □ Micellar properties of drugs—symposium □ Ultracentrifugation—determination of molecular weight, size, and molecular interactions of drug micelles □ Sedimentation velocity and coefficient—determination of micellar properties of drugs □ Molecular weight of drug micelles—determination from sedimentation data □ Density gradient centrifugation—determination of micellar properties of drugs

The analytical ultracentrifuge is frequently used by physical, biological, and polymer chemists to investigate molecular weights, subunit structure, and purity of molecules and small particles from diverse sources. The investigation of drug micelles is facilitated by the described techniques since ultracentrifugation affords a direct view of some properties of molecules and small particles. For example, a recent controversy (1, 2) concerning whether or not theophylline aggregated in aqueous solutions was resolved when molecular weight studies performed with an analytical ultracentrifuge (3) showed that the self-association of theophylline is concentration dependent; that is, monomers are found at low concentration and multimers (dimers, trimers, and tetramers) are found at higher concentrations. This paper discusses some ultracentrifugal techniques, some physical properties of several drugs that form micelles, and the question of whether aggregation of these agents is a manifestation of a physical property required for activity *in vivo*.

All analytical ultracentrifuges operate on the same principle, *i.e.*, they cause molecules to move toward the bottom (outer edge) of the ultracentrifuge cell under the influence of the artificial gravitational field created by a rotor spinning from 800 to 60,000 rpm.

Results obtained with different instruments are similar (4), as expected from calculations based on thermodynamics and mass transfer phenomena.

Separations of the sample within the ultracentrifuge cell may be achieved by: *(a)* differences in size and shape, as in the sedimentation velocity methods; *(b)* differences in mass, as in approach-to-sedimentation equilibrium and equilibrium procedures; or *(c)* differences in density, as in the density gradient (isopycnic or isodensity method).

SEDIMENTATION VELOCITY

Figure 1*a* shows a cross section of the filled ultracentrifuge cell. Before the ultracentrifuge run begins, solute is distributed equally throughout the solution. As the cell is accelerated, solute molecules move toward the bottom of the cell. Complete redistribution by diffusion is prevented by maintaining a sufficiently high speed to cause net sedimentation of solute. This speed depends on the size, shape, and partial specific volume (which is related to recip-

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Micellar Properties of Drugs: Determination of Molecular Weight, Size, and Molecular Interactions of Drug Micelles Using the Analytical Ultracentrifuge

JOEL KIRSCHBAUM

Abstract □ This article illustrates the usefulness of the analytical ultracentrifuge in investigating micellar properties of drugs. Also discussed are some examples of micelle forms of drugs and the working hypothesis that the same binding forces that hold together the aggregate found at high concentrations may be responsible for the binding of monomer (at physiological concentrations) to biological structures.

Keyphrases □ Micellar properties of drugs—symposium □ Ultracentrifugation—determination of molecular weight, size, and molecular interactions of drug micelles □ Sedimentation velocity and coefficient—determination of micellar properties of drugs □ Molecular weight of drug micelles—determination from sedimentation data □ Density gradient centrifugation—determination of micellar properties of drugs

The analytical ultracentrifuge is frequently used by physical, biological, and polymer chemists to investigate molecular weights, subunit structure, and purity of molecules and small particles from diverse sources. The investigation of drug micelles is facilitated by the described techniques since ultracentrifugation affords a direct view of some properties of molecules and small particles. For example, a recent controversy (1, 2) concerning whether or not theophylline aggregated in aqueous solutions was resolved when molecular weight studies performed with an analytical ultracentrifuge (3) showed that the self-association of theophylline is concentration dependent; that is, monomers are found at low concentration and multimers (dimers, trimers, and tetramers) are found at higher concentrations. This paper discusses some ultracentrifugal techniques, some physical properties of several drugs that form micelles, and the question of whether aggregation of these agents is a manifestation of a physical property required for activity *in vivo*.

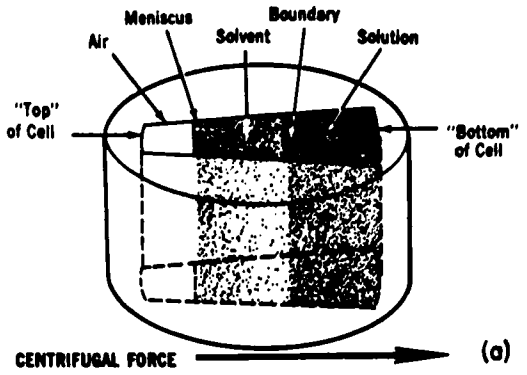
All analytical ultracentrifuges operate on the same principle, *i.e.*, they cause molecules to move toward the bottom (outer edge) of the ultracentrifuge cell under the influence of the artificial gravitational field created by a rotor spinning from 800 to 60,000 rpm.

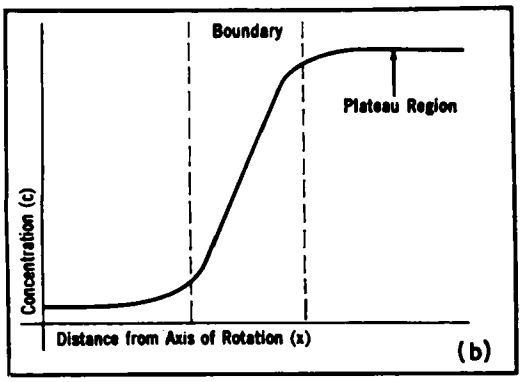
Results obtained with different instruments are similar (4), as expected from calculations based on thermodynamics and mass transfer phenomena.

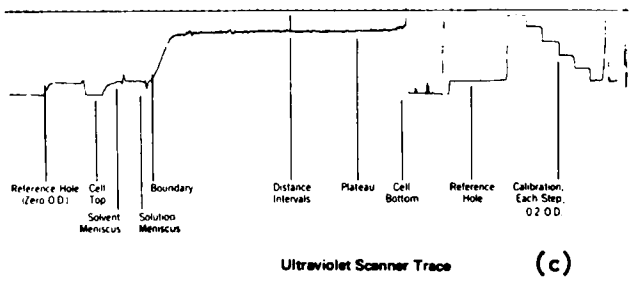
Separations of the sample within the ultracentrifuge cell may be achieved by: *(a)* differences in size and shape, as in the sedimentation velocity methods; *(b)* differences in mass, as in approach-to-sedimentation equilibrium and equilibrium procedures; or *(c)* differences in density, as in the density gradient (isopycnic or isodensity method).

SEDIMENTATION VELOCITY

Figure 1*a* shows a cross section of the filled ultracentrifuge cell. Before the ultracentrifuge run begins, solute is distributed equally throughout the solution. As the cell is accelerated, solute molecules move toward the bottom of the cell. Complete redistribution by diffusion is prevented by maintaining a sufficiently high speed to cause net sedimentation of solute. This speed depends on the size, shape, and partial specific volume (which is related to recip-







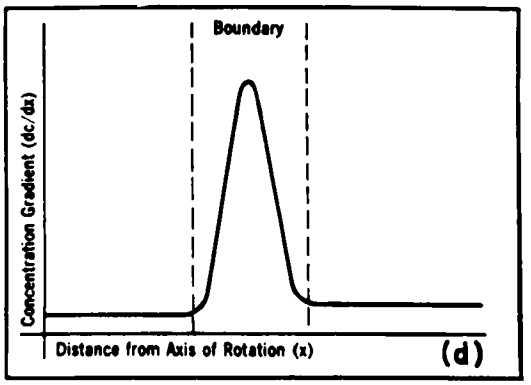


Figure 1—(a) *The centerpiece filled with solution in a sedimentation velocity experiment.* (b) *The variation in solute concentration in the centerpiece after a sufficiently high speed has been reached to sediment solute from the meniscus and these changes in concentration with distance as visualized by the* (c) *UV scanner and* (d) *schlieren optical system. (Portions of this figure are courtesy of Beckman Instruments, Inc.)*

rocal density) of the solute and the density of the solvent. When there is zero solute concentration at the meniscus (Fig. 1*b*), the UV optical system, which is based on absorptivity, visualizes this as shown in Fig. 1c. The schlieren optical system, which produces images based on differences in indexes of refraction caused by

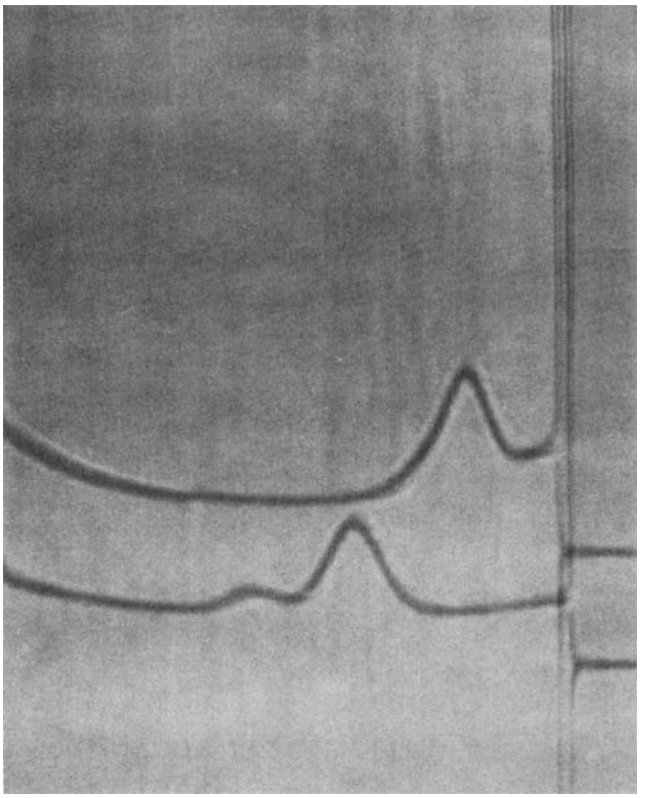


Figure 2—*Upper portion: aged γ-globulin in 5* M *NaCl solution. Lower portion: aged γ-globulin in tromethamine buffer. Sedimentation is from right to left.*

local differences in concentration, visualizes the region of increasing solute concentration as a peak (Fig. 1*d*). As sedimentation continues, the region devoid of solute increases and the plateau region (Fig. 1*b*) decreases. The peak is seen to travel toward the bottom of the cell.

Such an ultracentrifuge run can aid in determining the purity of such sedimenting species, as illustrated in Fig. 2. This figure is from a photograph of aged γ-globulin in dilute tromethamine buffer. The lower tracing shows two peaks; the larger one is the slower moving monomer, and the smaller peak is a faster moving dimer. The upper tracing is an aliquot of the same γ-globulin in 5 *M* NaCl. No dimer is visible, because it has been cleaved to the monomer by this concentrated salt solution. If the logarithm of the distance of the peak from the center of rotation is plotted *versus* time and the material is approximately 95% pure (depending on the molecular weight or size differences between compound and impurity), a straight line usually results. The slope of this line is used to calculate the sedimentation coefficient *(s)*:

$$s = \frac{\log_e x_t - \log_e x_0}{\omega^2(t_t - t_0)} \qquad \text{(Eq. 1)}$$

in which $(t_t - t_0)$ = time interval during which the boundary moves from x_0 to x_t, and ω is the angular speed of rotation, which is dependent on revolutions per minute. Such a determination requires 10–180 min of centrifugation, depending on the size of the macromolecule or particle. In Eq. 1, s is usually given in units of 10^{-13} cm sec^{-1} (S) or Svedbergs. The value of s is usually corrected for the density, ρ, and viscosity, η, of water at 20° (6) and is then written as $s_{20,w}$.

Particles as large as viruses can be investigated by this method. For example, a virus preparation with s values of 400 and 1400 S was interpreted[1] as containing both individual viruses and clumps of viruses, and the interpretation was subsequently verified by electron microscopy.

It is mandatory to calculate sedimentation coefficients at different concentrations of solute when one is examining micelles,

[1] Unpublished studies from this laboratory.

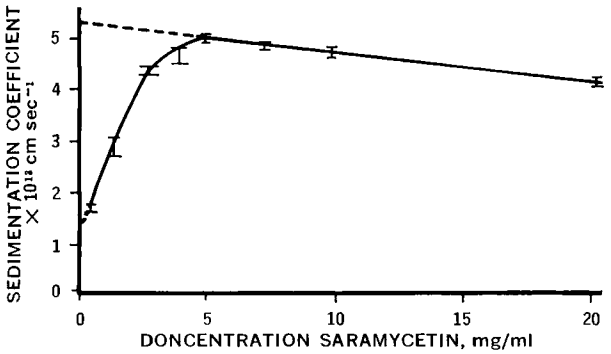


Figure 3—*Plot of the dependence of the sedimentation coefficient of saramycetin at different concentrations of 0.2* M *sodium chloride–0.02* M *sodium phosphate buffer, pH 6.85, with both regions of the curve extrapolated to zero concentration.*

because dilution will eventually disrupt the aggregate. Figure 3 illustrates the different sedimentation coefficients found for the antifungal antibiotic saramycetin at various concentrations of solute. At concentrations greater than 5 mg saramycetin/ml 0.2 *M* sodium chloride–0.02 *M* sodium phosphate buffer, pH 6.85, the micelle has an $s^0_{20,w}$ value of 5.3 S. The sedimentation coefficient increases initially, due to diminishing solute–solute interactions. At lower concentrations of solute, the micelle is disrupted to yield an $s^0_{20,w}$ value of 1.2 S. These data are supported by diffusion coefficient measurements; with disruption, the diffusion coefficient (*D*, Ref. 8) increases from 6×10^{-7} to 34×10^{-7} cm^2 sec^{-1}.

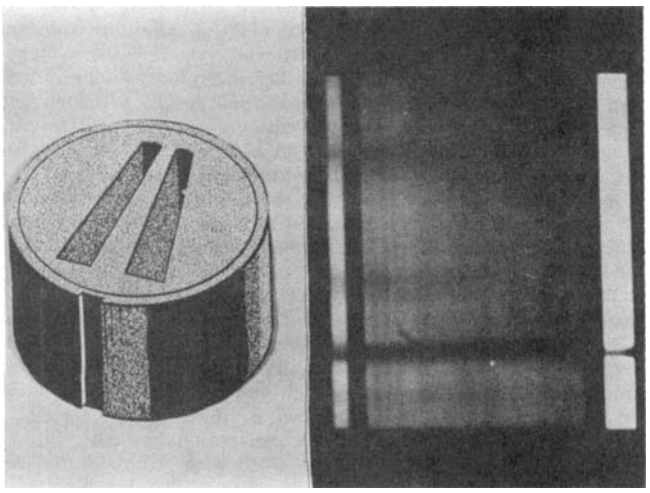
MOLECULAR WEIGHT DETERMINATIONS

The values of M_{app} from various methods used in determining the apparent molecular weight from sedimentation data are similar, as expected from the common thermodynamic basis of the calculations. The commonly used methods, their advantages, and disadvantages are summarized as follows.

Estimation from Sedimentation Coefficient—The approximate M_{app} of spherical micelles (8) can be determined as follows:

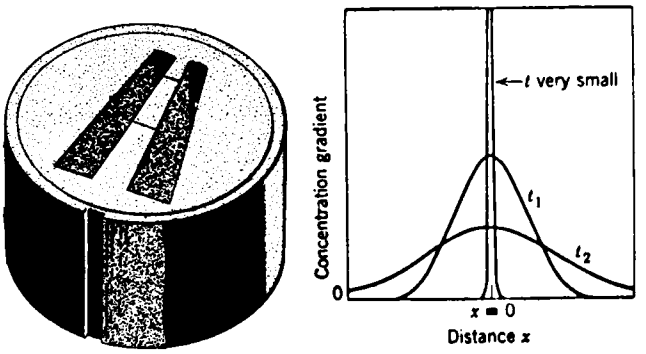
$$\frac{s^0_{20,w}\,(\text{unknown})}{s^0_{20,w}\,(\text{standard})} = \frac{M_{app}\,(\text{unknown})}{M_{app}\,(\text{standard})} \qquad \text{(Eq. 2)}$$

where $s^0_{20,w}$ is the sedimentation coefficient corrected for the viscosity and density of the solvent at 20°. This method is fast but relatively inaccurate.



(a) (b)

Figure 4—(a) *Double-compartment centerpiece used to measure the concentration gradient,* dc/dr, *for determining the apparent molecular weight by the approach-to-sedimentation equilibrium technique.* (b) *Concentration gradients at the meniscus and the cell bottom as visualized by the schlieren optical system.*



(a) (b)

Figure 5—(a) *Double-compartment capillary centerpiece used to determine total concentration.* (b) *Representation of peak produced by schlieren optical system. Note that the peak broadens and decreases with time due to diffusion.*

Sedimentation–Diffusion Method—The value of M_{app} can be determined using Eq. 3, where:

$$M_{app} = \frac{RTs}{D(1 - \bar{v}\rho)} \qquad \text{(Eq. 3)}$$

in which R is the gas constant, T is the absolute temperature, D is the diffusion coefficient, $\bar{v}$ is the partial specific volume of the solute, and ρ is the density of the solvent. The partial specific volume can be determined by pycnometry (9), Archimedes principle using sinkers or floats (9), and microdrop density in organic fluids (10) or from two ultracentrifuge runs, one in water and the other in a heavy isotope of water, followed by simultaneous solution of the two equations (11).

This method is good for mixtures and large molecules or micelles. The disadvantage is that both s and D must be determined at several different concentrations. The concentration of solute needed depends on methods of visualization available.

Archibald Approach-to-Sedimentation Equilibrium—In this procedure (12), M_{app} is determined using Eq. 4:

$$M_{app} = \frac{RT}{\omega^2(1 - \bar{v}\rho)} \frac{dc}{dr} \frac{}{Cr} \qquad \text{(Eq. 4)}$$

where ω is the angular speed of rotation, which depends on revolutions per minute, C is the concentration, dc/dr is the concentration gradient, and r is the distance from the center of rotation; C, dc/dr, and r are determined separately at the meniscus and cell bottom. Here, dc/dr is determined in a two-compartment centerpiece (Fig. 4*a*); one side is filled with solvent and the other with solution. Figure 4*b* shows the schlieren visualization of dc/dr—the heights of the schlieren curves at the meniscus and cell bottom.

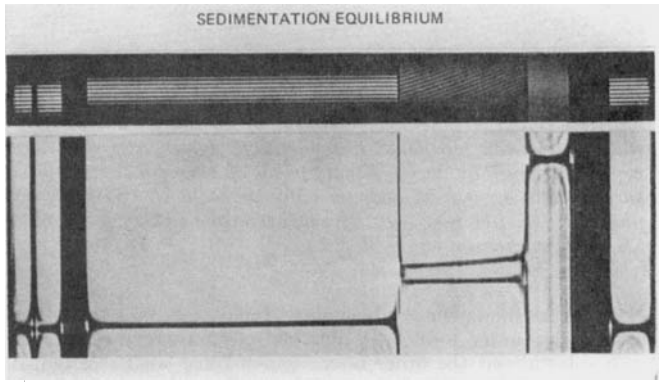


Figure 6—*Visualizations by Rayleigh interference (upper portion) and schlieren optical (lower portion) systems of low-speed equilibrium method for determining molecular weight. (After Schachman, Ref. 15.)*

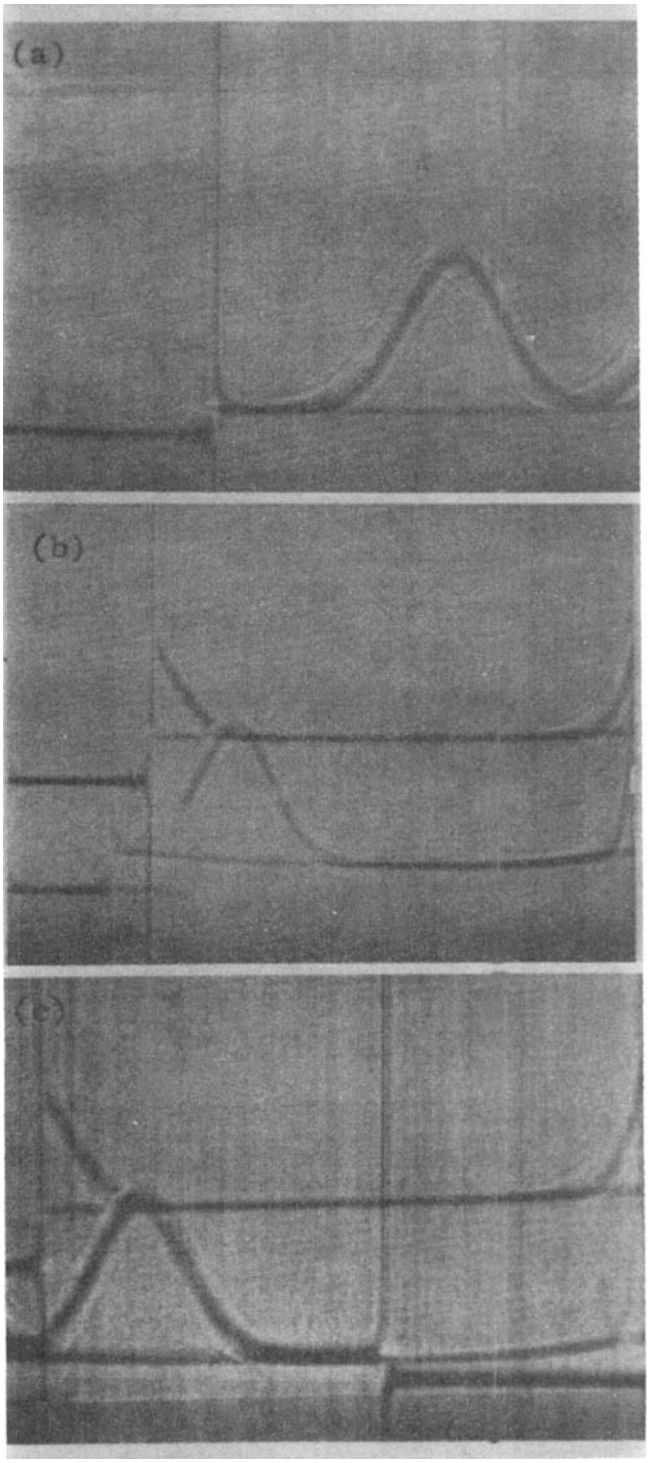


Figure 7—(a) *Total concentration of 10 mg saramycetin/ml ethanol, as seen by the schlieren optical system.* (b) *Schlieren peak produced by the sedimentation of this concentration of saramycetin in 0.2* M *sodium chloride–0.02* M *sodium phosphate buffer, pH 6.85.* (c) *The superimposition of* (a) *on* (b). (*After Kirschbaum* et al., *Ref. 22.*)

Figure 5*a* shows the double-compartment capillary centerpiece used to determine total concentration; one compartment is filled with solvent and the other is one-fourth filled with solution. As the ultracentrifuge cell is accelerated, solvent is forced into the solution compartment by its increasing apparent weight. Figure 5*b* shows the schlieren visualization of the different concentrations in the cell (originating from differences in indexes of refraction). With time, diffusion causes the peak height to diminish

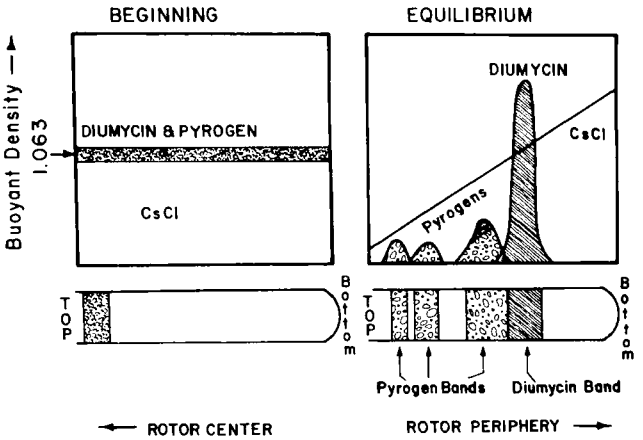


Figure 8—*Diagram of preparative centrifuge tube and distribution of diumycin, contaminating pyrogen, and cesium chloride in the tube prior to the start of centrifugation and after equilibrium had been achieved in the final density gradient.*

and the peak to broaden. Total concentration minus area under the schlieren curve at the meniscus (Fig. 4*b*) equals C at the meniscus. Total concentration plus area under the schlieren curve at the cell bottom equals C at the cell bottom. Note that the schlieren optical system visualizes concentrations at the meniscus and the cell bottom as positive, or upward, deflections. The advantages of this method are that as little as 1 hr of operating time is required, D can be calculated from the total concentration data, and M_{app} values as low as 100 can be calculated. Similar M_{app} values at both the meniscus and the cell bottom usually indicate homogeneity. The major disadvantage is that the values of the concentration gradients (height of schlieren curves at the meniscus and the cell bottom) are often difficult to measure, occasionally introducing large errors.

Sedimentation Equilibrium—The value of M_{app} can also be measured (13, 14) using Eq. 5:

$$M_{app} = \frac{2RT}{(1 - v\rho)\omega^2} \frac{d \ln c}{dr^2} \qquad \text{(Eq. 5)}$$

where $d \ln C/dr^2$ is the slope of the Rayleigh fringe (Fig. 6, upper part) or schlieren (Fig. 6, lower part) displacement *versus* radius squared. This method is accurate, but up to 3 days may be needed to achieve equilibrium, which may lead to new intermolecular interactions (16).

Several modifications exist to shorten the amount of time required for an accurate determination of M_{app}. One procedure involves accelerating the rotor, or overspeeding (17), to a higher speed than has been calculated as being needed for equilibrium and then decelerating to the desired final speed. Although less time is needed than with the conventional method, more distortion of cell windows is found and the method is limited to higher M_{app} values for solutes than is the conventional method.

In the short column method (18) the height of solution is limited to 1–3 mm. Less time is needed to achieve equilibrium than with the conventional method, but only one to three good values for M_{app} can usually be obtained.

The meniscus depletion method (19) involves running the centrifuge faster than is needed for the conventional equilibrium procedure to deplete the meniscus of solute. The advantages are that less time is needed, the total concentration can be calculated at any point in the ultracentrifuge cell, and the method is sensitive to polydispersity (20). This method is limited to higher M_{app} values than can be determined by the conventional method.

Data for M_{app} can often be combined with sedimentation velocity data to yield other useful information. The deviation from sphericity of a micelle can be approximated (21) from M_{app}, $s^0_{20,w}$, solvent viscosity (η), $\bar{v}$, and ρ. First, the diffusion coefficient is calculated (if it has not been determined directly) by a rearrangement of Eq. 3: $D = RTs/M_{app}(1 - \bar{v}\rho)$. Then, f/f_{min} is calculated, where $f = kT/D$ (k is Boltzmann's constant), and $f_{min} = 6\pi\eta\,(3M_{app}\,\bar{v}/4\pi N)^{1/3}$ (N is Avogadro's number). Numbers much above 1 indicate asymmetry.

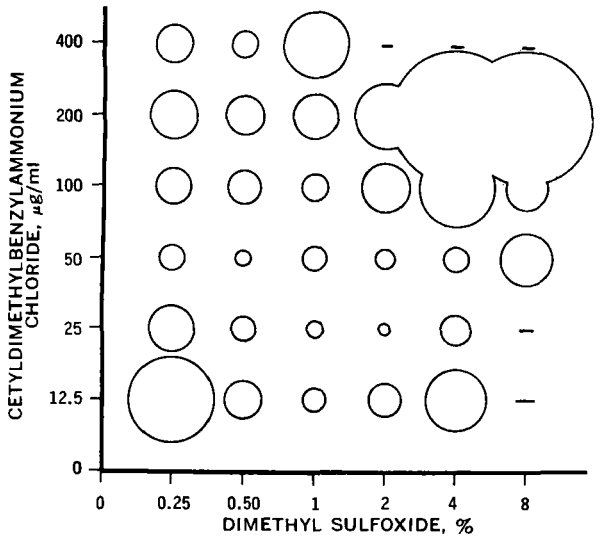


Figure 9—*Relative sedimentation coefficients of 100 μg amphotericin B/ml of various concentrations of cetyldimethylbenzylammonium chloride and dimethyl sulfoxide.*

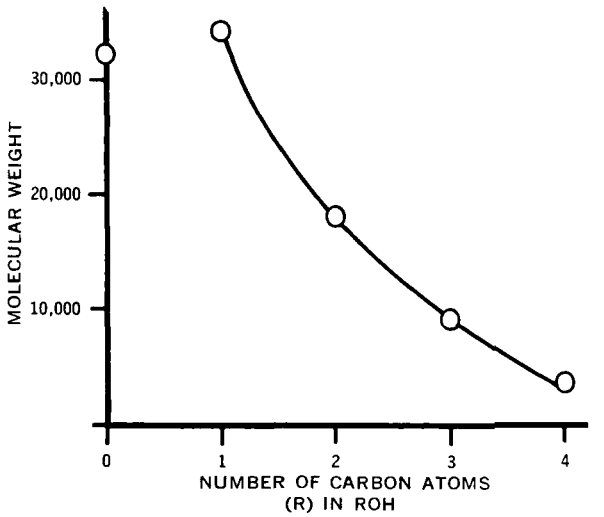


Figure 10—*The M_{app} values of diumycin in 95% 0.2 M sodium chloride–0.02 M sodium phosphate buffer, pH 6.85, containing 5% ROH, where R = CH_3—, CH_3CH_2—, $CH_3CH_2CH_2$—, or $CH_3CH_2CH_2CH_2$—.*

Sedimentation velocity and total concentration data can also be used to determine how much of the solute is in the form of the aggregate. Figure 7*a* is a reproduction of the photographic plate (22) recording the total concentration of 10 mg saramycetin/ml ethanol (M_{app} = 2100 daltons or molecular weight units). Figure 7*b* is a reproduction of the photograph of the schlieren peak produced during the sedimentation-velocity study of the same concentration of saramycetin in 0.2 *M* sodium chloride–0.02 *M* sodium phosphate buffer, pH 6.85; the solute is in the form of an aggregate (M_{app} = 55,000 daltons). Figure 7*c* is the superimposition of part *a* on *b*. The exact superimposition demonstrates that essentially all saramycetin in the aqueous buffer is in the form of the aggregate.

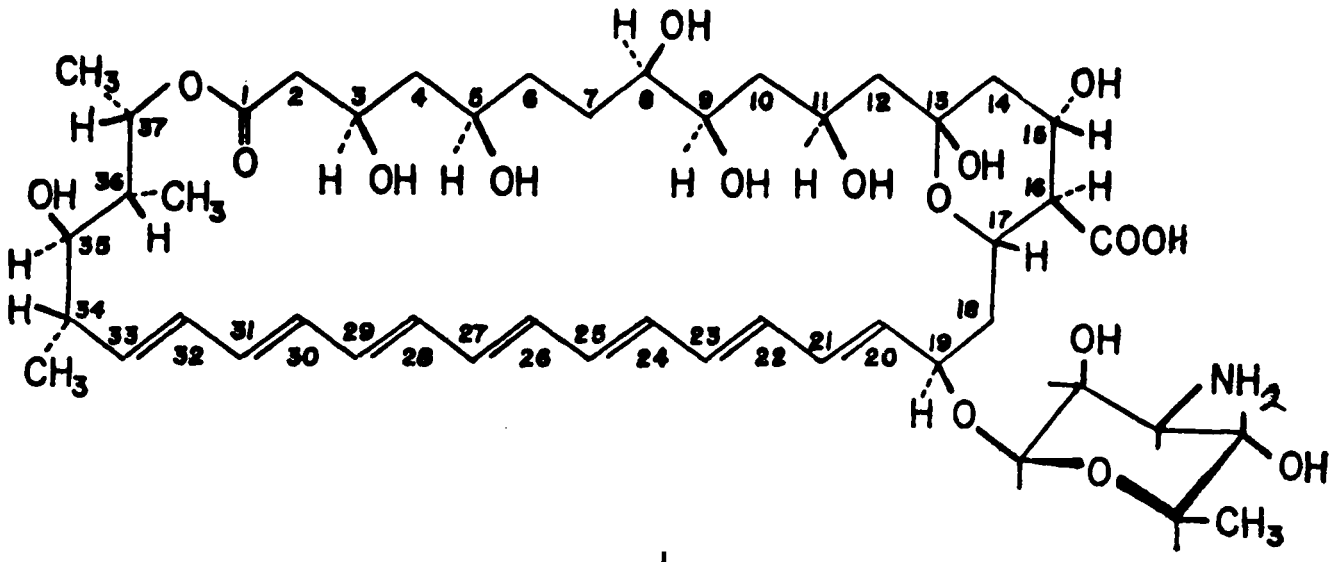
DENSITY GRADIENT CENTRIFUGATION

This method for separating and characterizing macromolecules is also known as isodensity or isopycnic centrifugation. It is based on the tendency of molecules to sink or float in a density gradient until they find the region corresponding to their own density. If a solution of cesium chloride is centrifuged at moderate to high speed (20,000 rpm or more) for 16–48 hr, the salt redistributes to form a density gradient. If a macromolecule is in the cesium chloride solution, it migrates to the region corresponding to its density and forms a band. The width of the solute band is inversely proportional to the molecular weight of the macromolecule; macromolecules or micelles with M_{app} values in the millions form bands 1 mm or less in width. Multiple bands would indicate heterogeneity, and the variety of their widths could be caused by either a wide spread in molecular weights or concentration (23).

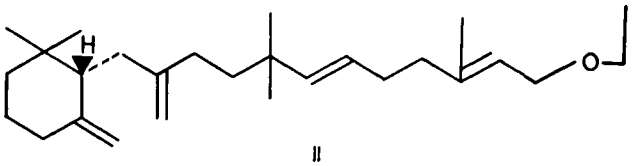
This isopycnic method was performed in a preparative ultracentrifuge to confirm the contamination of a sample of diumycin (26) with pyrogen. Diumycin micelles are essentially intact in cesium chloride. Figure 8 shows the distribution of diumycin, pyrogen, and cesium chloride, both prior to the start of centrifugation and after the attainment of equilibrium. The low molecular weight micelle (M_{app} ~ 30,000 daltons) formed a band about 1 cm wide. Fractions were collected, and the cesium chloride was removed by dialysis. Because most diumycin was in the form of the micelle, it did not diffuse through the cellophane membrane. The pyrogen was found to be heterogeneous, as shown by several fractions or bands that, upon injection, increased the temperature of rabbits.

FURTHER APPLICATIONS OF ULTRACENTRIFUGAL TECHNIQUES TO MICELLAR SYSTEMS

The site of action of polyene antibiotics is the cell membrane (24), at which compounds such as amphotericin B induce alterations in permeability. Amphotericin B (I) contains hydrocarbon

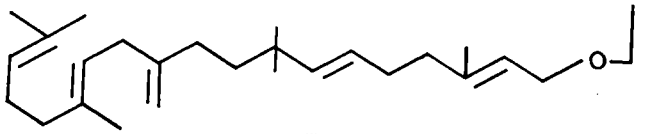


I



II

lipid side chain of diumycin (29)



III

lipid side chain of prasinomycin (30)

regions, hydroxyl groups, and amino and carboxyl groups capable of forming a zwitterion (25). Because of these structural features, it is not unexpected that this molecule forms micelles and that these micelles are sensitive to additions of the detergent cetyldimethylbenzylammonium chloride or of dimethyl sulfoxide, which breaks intermolecular hydrogen bonds. Figure 9 illustrates these changes as reflected in values for the sedimentation coefficient of the micelle in different solutions of cetyldimethylbenzylammonium chloride and dimethyl sulfoxide; small changes in the concentration of either agent greatly affect the sedimentation coefficient.

Diumycin (26) and prasinomycin (27) are closely related mixtures of antibiotics that aggregate in solution. Diumycin and prasinomycin contain 2-amino-1,3-cyclopentanedione, phosphoglyceric acid, phosphate, sugars such as glucose and glucosamine, and a 340-dalton hydrocarbon side chain (II and III). The M_{app} values of the micelles are approximately 30,000 daltons.

Since the molecular weight of each monomer is approximately 1800–1900 daltons, the micelle must be composed of 16 subunits. Figure 10 illustrates the M_{app} values of diumycin as alcohols of increasing side-chain length are used in the solvent system. As the length of the hydrocarbon side chain increases, with the concentration of each alcohol kept constant at 5%, M_{app} values decrease, indicating hydrophobic rather than hydrogen bonding (31). Chemical modifications of the water-soluble portions of the antibiotic, such as esterification or acetylation, or changes in pH or ionic strength of the solvent do not disrupt the micelle, indicating that ionic bonding is not involved. Without the hydrophobic side chains, there is neither aggregation nor microbiological activity. Figure 11 illustrates the most likely structure of the micelle. The core is a tangle of 16 interacting lipid side chains bound by hydrophobic forces. Surrounding this hydrocarbon core is a mantle of water-soluble (or hydrophilic) moieties of the antibiotic. Calculations show that the diumycin micelle can be spherical, since the minimum volume of the hydrophobic core, as calculated (32) from the length of the side chain as the radius (volume = $\frac{4}{3}\pi R^3$), is less than the calculated volume of the 16 lipid side chains.

The forces binding the micelle appear to be a manifestation of the same physical property of the lipid that is required for drug activity. This working hypothesis is based on evidence[2] that these antibiotics act prior to the site of penicillin action (33) to inhibit cell wall biosynthesis by interfering with the enzyme, peptidoglycan synthetase, that synthesizes phospholipid intermediates of cell wall glycopeptides (34). (The cell wall glycopeptide is synthesized from pentapeptide-phospholipid and acetylglucosamine-phospholipid intermediates.) These antibiotics may resemble the substrate and bind to the enzyme through phospholipid and the hydrophilic moiety, since both portions of the antibiotic are required for microbiological activity.

The lipid side chain may also be responsible for the long half-life *in vivo* of these antibiotics, because both diumycin and prasinomycin bind avidly to human serum albumin and red blood cell membranes, as demonstrated by molecular weight, NMR, and

[2] F. L. Weisenborn (Squibb Institute for Medical Research, Princeton, NJ 08540) and J. L. Strominger, personal communication.

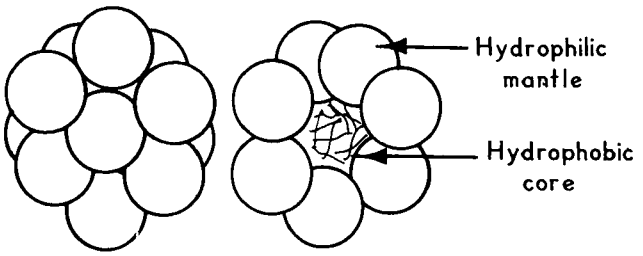


Figure 11—*Representation of the 16-subunit micelle of diumycin, consisting of hydrophobic lipid side chains surrounded by hydrophilic sugars.*

equilibrium-binding studies in this laboratory. The same agents that disrupt the aggregate, such as urea, also displace the antibiotics from human serum albumin.

The enzyme L-asparaginase hydrolyzes the amino acid asparagine to aspartic acid and ammonia and is used in antileukemia therapy. This enzyme (35) is similar to many others (36) in consisting of subunits. Subunit structure is affected by the addition of such compounds as urea, sodium chloride, and sodium lauryl sulfate. The $s^0_{20,w}$ value diminishes from 8.7 S in dilute buffer to 4.0 S in sodium chloride or sodium lauryl sulfate solutions; the M_{app} diminishes from 133,000 to 60,000 daltons. These decreases in M_{app} and $s^0_{20,w}$ and in activity *in vitro*, caused by the addition of sodium lauryl sulfate, can be reversed by adding sodium sulfate, sodium citrate, or potassium dihydrogen phosphate (37) to this solution. Optical rotatory dispersion and UV difference studies show that the spatial relationship of at least some atoms in the molecule is first changed by the addition of detergent and then restored by the addition of the inorganic salt.

MICELLE BINDING FORCES AND INTERACTION OF MONOMER WITH BIOLOGICAL STRUCTURES

At such unphysiologically high concentrations as were used in these studies, many compounds, including drugs, form micelles. The bonding available to maintain the aggregate at these concentrations may be the same forces that bind monomer to biological structures at physiological concentrations. Since all micellar systems of drugs studied in this laboratory dissociate with decreasing concentration and, at physiological concentration, are calculated to be in the form of the monomer (based on uniform distribution of compound in body fluids), the drug substance could bind to the receptor *via* the bonds available to maintain the aggregate at the higher, nonphysiological, concentrations.

Amphotericin B, which forms micelles at the unphysiologically high concentrations used in the model systems *via* bonds affected by the addition of a detergent and/or a hydrogen bond-breaking agent, may, at physiological concentrations, interact with cell membranes *via* these bonds.

Prasinomycin (22) and diumycin (38) form micelles at concentrations higher than those found necessary for antibiotic activity *in vivo* through the lipid side chains that, at physiological concentration, are probably needed only singly to interact with the enzyme to inhibit cell wall biosynthesis.

The enzyme L-asparaginase represents a special group of molecules for which subunit-aggregate equilibrium *in vitro* was previously shown to affect the activity of many enzymes (39, 40), usually through molecular association (41). These macromolecules may have evolved in response to the survival requirements of organisms under various environmental conditions. They provide the final, although special, example of the biological significance of the binding forces that maintain the aggregate.

In summary, the ultracentrifuge has been shown to be useful in investigating some physical properties of drug micelles, including molecular weight, sedimentation coefficients, and mode of binding. The hypothesis is proposed that the intermolecular forces that maintain micellar structure may be a manifestation of the same forces that bind monomers to active sites.

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Micellar Properties of Drugs: Properties of Micellar Aggregates of Phenothiazines and Their Aqueous Solutions

D. ATTWOOD*, A. T. FLORENCE[x], and J. M. N. GILLAN‡

Abstract □ The solution properties of a series of phenothiazine hydrochlorides were studied using light-scattering, viscosity, conductivity, dialysis, and NMR techniques. The aggregates formed are considered to be true micelles, which in water are probably composed of about 10 monomers vertically stacked, with the alkyl side chain and nitrogen group alternating to form an approximately spherical unit in solution. In the presence of sodium chloride the micelles grow and it is thought that the conformation of the aggregate also changes. Calculations of the monomer concentration as a function of total drug concentration above the CMC's give good agreement with data obtained previously from NMR shift data. The free energy of inserting a monomer into a micelle was calculated, and the values obtained are discussed in the light of free energies of solution of the hydrophobic bases. The significance of aggregation, mixed micelle formation, and complexation in biological and pharmaceutical systems containing phenothiazines is discussed.

Keyphrases □ Micellar properties of drugs—symposium □ Micelle aggregates of phenothiazines—determination of properties by light scattering, viscosity, conductivity, dialysis, and NMR spectroscopy □ Phenothiazines—micellar aggregation properties determined by light scattering, viscosity, conductivity, dialysis, and NMR spectroscopy

The aggregation of four phenothiazines (chlorpromazine, promazine, promethazine, and thioridazine) was previously studied by NMR techniques (1, 2). Measurements of the changes in chemical shift produced in solutions of the four compounds by varying their concentration above and below their critical micelle concentration (CMC) allowed calculation of the proportion of monomer and micelles present in solution. Further work has been carried out to find out more about the nature of the aggregation process in solutions of phenothiazine derivatives and to compare the monomer–micelle ratios obtained using the mass action approach with those obtained from treatment of the NMR results. Aggregates composed of phenothiazine molecules stacked vertically with the *N*-alkyl side chain alternating were compatible with the magnitudes and directions of the chemical shifts.

The number of drugs observed to aggregate or to form micelles in aqueous solution continues to increase. This is not entirely a surprising observation because the structural requirements for surface activity or micelle formation are often similar to those for interaction of drug with receptor sites. Possession of a well-defined hydrophobic head provides opportunities for hydrophobic interactions, and the existence of a charged hydrophilic head group—often a nitrogen-containing group—provides the opportunities for hydrogen bonding interactions with receptor molecules. Discrete hydrophobic and hydrophilic regions are also prerequisites for surface activity and micelle-forming ability.

Although micellar concentrations of surface-active drugs are not frequently encountered *in vivo*, the actions of these drugs cannot be fully understood without a knowledge of the interactions that they can undergo. Micelle formation is the result of intimate interactions between the drug molecules. Micelle formation is primarily the result of hydrophobic bonding interactions. Hydrophobic bonding plays a part also in the binding of phenothiazines to bovine serum albumin (3) and in the interactions of phenothiazines and flavins (4), dyes (5), and erythrocytes (6). One can envisage instances, for example in pharmaceutical dosage forms, when the concentration of drug will exceed the CMC. In many experimental systems, high concentrations of drugs are used and anomalies caused by the aggregation of the solute may unwittingly be misinterpreted (7). There are instances where aggregation of drug molecules into micelles results in an alteration in biological activity. One can understand why this should be so: micelles have a larger radius and diffuse more slowly than the monomeric species, and the micellar solution has a lower thermodynamic activity than an ideal solution. A diminution in biological activity has been noted with surface anesthetics and bacteriostatic agents (8, 9); where the active species is the monomer, it is useful to know about the monomer–micelle equilibrium in detail.

MASS-ACTION TREATMENT

Scheme I describes the aggregation of j monomers, $[D^+]$, and $(j - Z)$ anions, $[X^-]$, to form positively charged micellar species $[M^{Z+}]$:

$$jD^+ + (j - Z)X^- \rightleftharpoons M^{Z+}$$

Scheme I

To utilize Scheme I, the values of j, from light-scattering or osmotic pressure measurements and of Z, the number of unit charges per micelle, are required.

If the micelle is considered to be monosized (10), the thermodynamic equilibrium constant for this reaction is:

$$K = \frac{M^{Z+}}{(D^+)^j(X^-)^{j-Z}} \qquad \text{(Eq. 1)}$$

or, in a more accessible form:

$$1 = \frac{(M^{Z+})^*}{(D^+)^{*j}(X^-)^{*j-Z}} \qquad \text{(Eq. 2)}$$

each concentration being divided here by $K_m^{1/(Z+1-2j)}$ and denoted by $(\,)^*$.

EXPERIMENTAL

Light Scattering—Measurements were made at a wavelength of 436 nm using an instrument fitted with an automatic scanning device and recording system, the cell being thermostatted to 34 ± 0.1°. The construction and calibration of the instrument were described previously (11). Solutions were clarified by ultrafiltration[1].

The Rayleigh ratios for the solutions were measured at 90° (R_{90}) to the incident beam, and the corresponding measurement for a solution at the CMC was subtracted to give the excess Rayleigh ratio, ΔR_{90}.

The specific refractive index increment, dn/dc, for the micellar species was determined with a Hilger-Rayleigh interference refractometer using Bauer's (12) technique for monochromatic light.

Micellar aggregation numbers, j, were evaluated by the method of Anacker and coworkers (13, 14). In a solution containing no added electrolyte:

$$p = \frac{M_0 S\,\mathrm{CMC} + (2M_0 S\,\mathrm{CMC})^{1/2}}{M_0 I(1 - M_0 I/2)} \qquad \text{(Eq. 3)}$$

$$j = \tfrac{1}{2}[p + 1/M_0 I] + \tfrac{1}{2}[(p + 1/M_0 I)^2 - p(p + 1)]^{1/2} \qquad \text{(Eq. 4)}$$

where p = effective thermodynamic charge[2]; M_0 = monomer molecular weight; I and S are the intercept and slope, respectively, of plots of $K(C - \mathrm{CMC})/\Delta S_{90}$ *versus* $(C - \mathrm{CMC})$, where $K = 2\pi^2 n_0^2 (dn/dc)^2 V_0/\lambda^4$; and the other symbols have their usual significance.

Electrophoretic Mobility—The electrophoretic mobilities of the micellar species were determined at 34 ± 0.5° by the dye-tracer method using an apparatus similar in design to that described by Hoyer *et al.* (15). The micelles were "tagged" by shaking the dispersions with the dye Orange O.T. for 3–4 days, and the dye concentrations were estimated from the E_{492}. The ζ-potential of the micelles was calculated from the experimentally obtained electrophoretic mobilities (Fig. 1) using the data computed by Wiersema *et al.* (16), which account for both electrophoretic retardation and relaxation effects. The micellar charge was estimated using the numerical solutions of the Poisson-Boltzmann equation obtained by Loeb *et al.* (17).

Conductivity—The conductivity of the solutions at 34 ± 0.01° was determined using a conductivity bridge[3]. CMC's were determined from plots of conductivity *versus* concentration (Fig. 2), the values agreeing well with those obtained from pH and NMR data (2). The effective micellar change, p, was estimated from the conductance data using an equation proposed by Evans (18):

$$\frac{p^2}{j^{1/3}}(1000\,S_1 - \Lambda_R) + \frac{p\Lambda_R}{j} = 1000\,S_2 \qquad \text{(Eq. 5)}$$

where Λ_R is the equivalent conductance of the counterion at infinite dilution, j is the aggregation number of the micelles, and S_1 and S_2 are the slopes of the specific conductance-concentration plots below and above the CMC, respectively.

Viscosity—Viscosity measurements were made at 25 and 34° (±0.01°) with a suspended-level dilution viscometer. The viscosities were obtained relative to water or 0.15 M NaCl as solvents, and corrections were made for the CMC. Measurements were made well above the CMC region, and there is little error in assuming that the monomer concentration is constant in the range studied. Intrinsic viscosities, $[\eta]$, were obtained by extrapolation of the reduced specific viscosities to zero micelle concentration, *i.e.*, where C_0 = CMC:

$$\lim_{(C - C_0) \to 0} \frac{\eta_{sp}}{C - C_0} = [\eta] \qquad \text{(Eq. 6)}$$

Dialysis[4]—The cells were of 10-ml capacity, and the solution and solvent sides were separated by a cellophane membrane[5]. The cells were rotated at 24 rpm along their vertical axis. Samples were taken from the solvent side at intervals and analyzed spectrophotometrically for phenothiazine. The rate of dialysis was constant up to 2 hr.

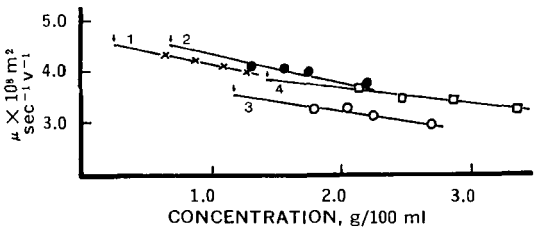

Figure 1—*Electrophoretic mobilities of the phenothiazine micelles as a function of concentration. Key: 1, thioridazine; 2, chlorpromazine; 3, promazine; and 4, promethazine. Arrows indicate CMC's. Temperature = 34°.*

Materials—The phenothiazines used were described previously (2). A commercial sample of triflupromazine was used as received. Sodium chloride was Analar grade material, and water was twice distilled from a glass apparatus.

RESULTS AND DISCUSSION

The light-scattering results (Table I) show that the aggregates formed by the phenothiazines are small. Those of chlorpromazine and promethazine have aggregation numbers of 11 ± 1, and the smallest aggregate, formed by thioridazine hydrochloride, consists of eight monomers. This is the order of magnitude expected from bulky molecules with relatively rigid hydrophobic ring systems in the absence of salt. A number of dyes with similar ring systems, *e.g.*, thionine and methylene blue, form only dimers in dilute solution (19). Sodium fusidate forms aggregates composed of only a few monomers (20), behaving like the structurally related cyclopentenophenanthrene derivative, sodium taurocholate, which is trimeric in 0.01 M NaCl (20). Similarly, the xanthone derivatives studied by Scholtan and Gonnert (21), with the exception of lucanthone hydrochloride which has an aggregation number of 445, form small micelles. While many drugs will be surface active, few will be capable of forming large micelles in water.

The relatively high CMC's (Ref. 2 and Figs. 2 and 3) are in the

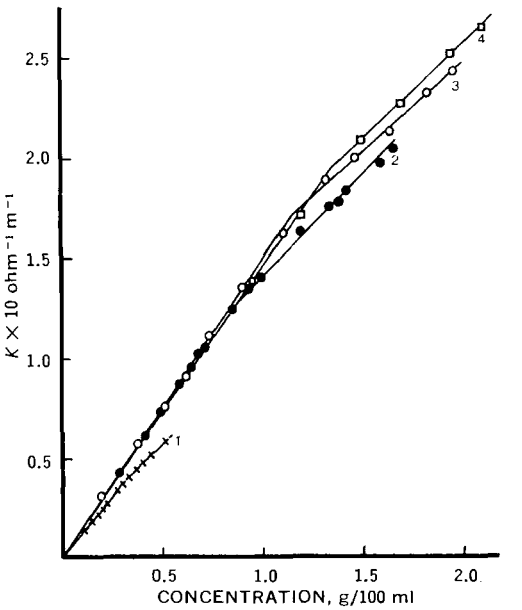

Figure 2—*Specific conductance of phenothiazine solutions as a function of concentration. Key: 1, thioridazine; 2, chlorpromazine; 3, promazine; and 4, promethazine. All show distinct breaks in the CMC region.*

[1] Millipore filters, 0.1 μm.

[2] Charge from light scattering is an effective thermodynamic charge, because ideality is assumed in its calculation. This charge is not the true charge at the shear plane of the micelle but its effective charge, taking into account all deviations from ideality [see K. J. Mysels, *J. Colloid Sci.*, **10**, 507(1955).] z is the micellar charge.

[3] L. K. B. Instruments Ltd., South Croydon, Surrey, United Kingdom.

[4] Dialysis cells were constructed from Gallenkamp filtration units.

[5] Visking.

Table I—Properties of Phenothiazine Micelles

Compound	Aggregation Number, j		Number of Unit Charges per Micelle, z			
			Experimental			
	Experimental: Light Scattering	Mass-Action Calculations	ζ-Potential Measurements	Conductivity Measurements	Mass-Action Calculations	ζ-Potential, mv
Thioridazine	8	8	6[a]	6	7	+88
Chlorpromazine	11	11	8	6	8	+83
Promazine	11	11	6[a]	6	11	+59
Promethazine	9	10	8	6	8	+70

[a] Value calculated using the simplified equation of Mysels and Stigter [K. J. Mysels and D. Stigter, *J. Phys. Chem.*, **57**, 104(1953)] was 7.

same region as found for sodium fusidate and sodium taurocholate (20). Therefore, one may assume that micellization of the phenothiazines and many other drug molecules is unimportant *in vivo*. What is important is the ability of the molecules to interact in the special way encountered in micelles. Interaction of the phenothiazines with various biological materials has been noted (4, 6). For example, Moriguchi *et al.* (22) recently reported the interaction of chlorpromazine with adenosine phosphate; the association of phenothiazines with bovine serum albumin (3, 23), caffeine, and riboflavin (27) was also reported.

The differences in the aggregation numbers j found for the four compounds cannot be regarded as significant, because the precision of the light-scattering technique allows measurement of j to ±1 monomer only. The authors are convinced, however, that the systems studied are in fact true micellar systems which are not behaving like the methylene blue systems investigated by Mukerjee and Ghosh (24). They interpreted the solution properties of methylene blue using a multiple equilibrium model for association from dimers to pentamers. Although the ring systems are similar, methylene blue differs from the phenothiazines in that the charge on the methylene blue monomer is delocalized due to resonance. Resonance cannot occur in the phenothiazines studied, and the compounds are more like conventional surfactants than dye molecules, having a fairly distinct separation of hydrophobic and hydrophilic groups. Methylene blue has hydrophilic groups on both sides of the ring.

Low concentrations of salt have a pronounced effect on the size of the aggregates formed. In 0.154 *M* NaCl, thioridazine micelles contain 57 monomers at 34°; pH measurements in water as a function of drug concentration at 20 and 34° show abrupt changes at the CMC and even more abrupt changes in the presence of sodium chloride (2). This behavior points to there being a true micellization phenomenon.

Mass-Action Calculations—Previously (2), the ratios of monomer to micelle in solutions of the phenothiazines were calculated from NMR data on the assumption that the observed chemical shift at any drug concentration, $\bar{\delta}$, was a numerical weight average of the resonance frequencies of the protons of the monomeric and micellar species (25). It was also assumed that the monomeric and micellar forms contributed shifts δ_{mon} and δ_{mic}, respectively, the latter value being obtained by an extrapolation procedure (2). The weight fractions, x, of the two species were obtained by substitution in the equation (25):

$$\delta_{mic} = \frac{\bar{\delta} - \delta_{mon}}{\delta_{mic} - \delta_{mon}} \qquad \text{(Eq. 7)}$$

To verify that this simple approach may be used, the authors sought to fit the results so obtained by calculating monomer and micelle concentrations using Eqs. 1 and 2 and Scheme I and solving graphically. The parameters j and Z were adjusted to give the best fit with experimental data points and compared with the values of j obtained by light-scattering measurements and Z values from ζ-potential and conductivity measurements (Table I). In Fig. 4 the continuous line is the calculated line (j, Z as in Table I), the points representing the NMR values. Very good fits were obtained when charge values and aggregation numbers close to those obtained experimentally were used in the calculations. Results for promazine do not match so well, but, in general, the agreement between the two sets of data lends weight to the use of NMR data and suggests that the extrapolations involved are valid. In addition, the view that these systems form true micelles with definite CMC's, which can be interpreted in the usual way, is strengthened.

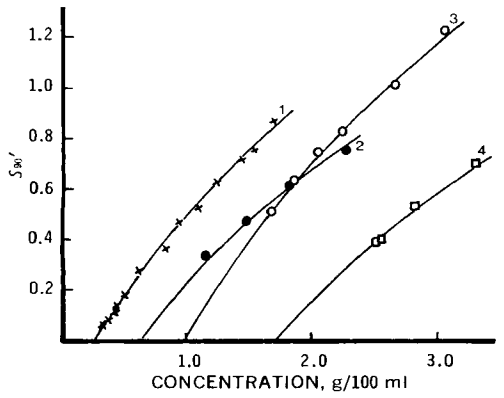

Figure 3—*Light-scattering results in water at 34°. Excess scattering at 90° to the incident beam, ΔS_{90}, plotted as a function of concentration. Key: 1, thioridazine; 2, chlorpromazine; 3, promazine; and 4, promethazine.*

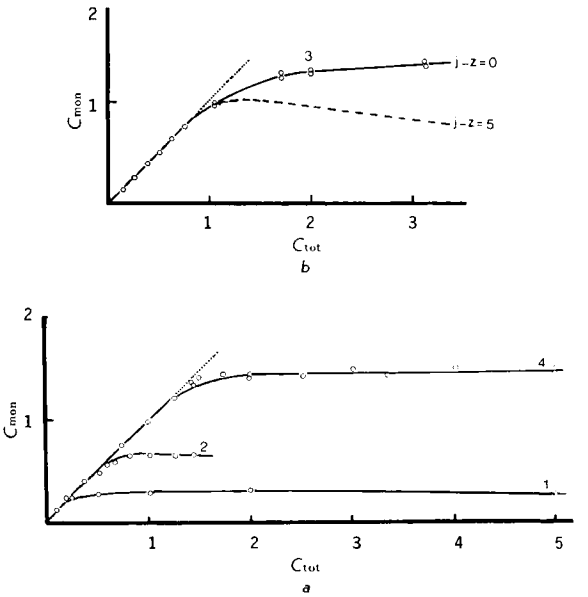

Figure 4—(a) *Monomer concentrations, C_{mon}, as a function of total concentration, C_{tot} (% w/v), for thioridazine (1), chlorpromazine (2), and promethazine (4). The continuous line is that calculated using Eq. 2 and the parameters recorded in Table I.* (b) *Results for promazine show best agreement between calculated line and NMR-derived results when* j − z = 0. *Conductivity and electrophoretic data indicate that* j − z *should be 5. The sensitivity of the calculated concentrations to* (j − z) *is shown in this diagram.*

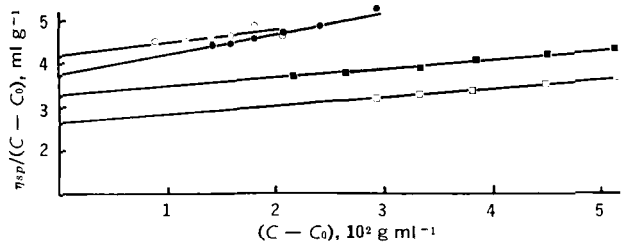

Figure 5—*Viscosity results for representative systems in 0.9% (0.154* M*) NaCl* $\eta sp/(C - CMC)$ *plotted as function of* $(C - CMC)$ *to obtain intrinsic viscosity,* $[\eta]$*, as intercept. Key:* ● *and* ○*, thioridazine at 25 and 34°, respectively; and* ■ *and* □*, chlorpromazine at 25 and 34°, respectively.*

Viscosity Results—Viscosity measurements (Fig. 5 and Table II) indicate that the phenothiazine micelles are spherical or nearly so, because the intrinsic viscosities, $[\eta]$, are close to the value expected for spherical units in solution, *i.e.*, 2.5/density. The intrinsic viscosities at 34° are lower than those at 20° in 0.154 *M* NaCl for chlorpromazine, promazine, and promethazine. With these compounds also, the intrinsic viscosities in the salt solutions are lower than those in distilled water, as would be expected from a reduction in the charge of nearly spherical units whose shape has not altered. The intercepts of 2.6 and 2.54 for chlorpromazine and promazine, respectively, must be an indication of their nearly spherical shape in solution. The micelles of thioridazine in a salt solution at 34° contain over 50 monomers, according to light-scattering results. The corresponding intrinsic viscosity is 4.35 ml g^{-1}, suggesting that some asymmetry is developing; 50 monomers cannot stack in a vertical mode without forming a very asymmetrical unit. The results of Scholtan and Gonnert (21) show that there is a marked decrease in micellar size with increasing temperature, and this probably explains the general decrease in intercept with rise in temperature. No such change should occur if the micelles are conventionally shaped spherical micelles. The difference in behavior between thioridazine and the other compounds may be related to the differences in the nature of the side-chain systems on these molecules. In a vertical stack, the side chains assume relatively little importance. Once larger, more intimate associations take place, the side chains should play a part in determining micelle size.

Transport across Membranes—Nonequilibrium dialysis measurements of the rate of transfer of thioridazine and chlorpromazine hydrochlorides indicate clearly the onset of aggregation. The results of transport rate—as a function of total concentration—can be explained using mass-action monomer–micelle ratio data and assuming simple partition of the monomeric species. The micelles are not expected to dialyze readily through the membrane, which has a pore size of 0.80-μm diameter; their rate of permeation is assumed to be sufficiently slow to allow one to account for the rates by considering the monomers alone. The results are shown in Fig. 6, where calculated points are superimposed on the experimental data.

Addition of salt (sodium chloride) to the system decreases the net rate of transport of the drug by inducing the formation of larger micelles and by depressing the CMC. Caffeine, sodium saccharin, and riboflavin decrease the rate of transport of chlorpromazine across dimethylpolysiloxane membranes (27), presumably as a result of complexation; micellization is one form of self-complexation, and both micelle formation and interactions with extraneous materials decrease the proportions of free drug available for passage through membranes. By addition of salt and by addition of surfactants to form mixed micelles, the rate of transport can be controlled. The pharmaceutical possibilities of this phenomenon have yet to be investigated.

Mixed Micelles in Phenothiazine Systems—A preliminary study was made of the aggregation of promethazine hydrochloride in the presence of the more hydrophobic compound chlorpromazine hydrochloride. It is well known that the addition of a more hydrophobic homolog to a lower surfactant homolog can result in the depression of the CMC of the latter (9). Measurement of the break in pH–concentration plots was used as a measure of the CMC in the mixed solutions. The results (Table III) suggest that the CMC of promethazine is depressed below the CMC of either component, a phenomenon not predicted by previous work (28) on straight alkyl chain ionic surfactants.

Table II—Intrinsic Viscosities of Phenothiazine Micelles (Milliliters Gram^{-1})

Phenothiazine	Solvent	25°	34°
Thioridazine	Water	—	3.8
	0.154 *M* NaCl	3.8	4.3_5
Chlorpromazine	Water	—	3.6_3
	0.154 *M* NaCl	3.2	2.6
Promazine	Water	—	3.8_8
	0.154 *M* NaCl	4.0_5	2.5_4
Promethazine	Water	—	3.5
	0.154 *M* NaCl	3.9	3.3_5

It is possible that there is a twofold effect here. The added chlorpromazine may be inducing easier micelle formation by the normal mechanism, but the free hydrogen chloride may be acting to reduce the charge on the protonated nitrogens of the alkyl side chains, with the result that the CMC is reduced. Further work with solutions of controlled ionic strength is required to confirm these results.

Thermodynamics of Aggregation Process—Since micelle formation is a relatively simple process, it should provide a clear indication of the energy, enthalpy, and entropy changes to be expected when micelle-forming drugs participate in interactions involving hydrophobic bonds. The free energy change on micellization per mole of monomer, ΔG_m, can be obtained from the CMC (mole fraction units). From the mass-action equilibrium (2):

$$\Delta G_m = (2 - z/j)RT \ln \text{CMC} \qquad \text{(Eq. 8)}$$

If j counterions are firmly bound to the micelle so that, when j is large, z is zero, Eq. 8 reduces to:

$$\Delta G_m = 2RT \ln \text{CMC} \qquad \text{(Eq. 9)}$$

If $z = j$, Eq. 9 becomes:

$$\Delta G_m = RT \ln \text{CMC} \qquad \text{(Eq. 10)}$$

which is the equation applicable to nonionic surfactants. More valuable from the biological point of view is gaining an understanding of the energy involved in adding a monomer to the "most probable" micelle. The incorporation of an additional surfactant ion into an aggregate containing j surfactant ions involves the equilibrium of $D^+ + M^{+j} = M^{+(j+1)}$, for which the free energy change can be written:

$$\Delta G_m = -RT \ln \frac{F(M^{+(j+1)})}{(D^+)(M^{+j})} \qquad \text{(Eq. 11)}$$

If equilibrium concentrations at the CMC are used and ln F is much smaller than ln CMC:

$$\Delta G_m = RT \ln \text{CMC} \qquad \text{(Eq. 12)}$$

This equation avoids complications arising from consideration of the formation of the double layer. There are conceptual difficulties with the definition of micellar standard state. In Eq. 12, the standard state has been taken to be the micelle at unit mole frac-

Table III—Apparent CMC of Promethazine in Presence of Chlorpromazine[a]

Concentration of Chlorpromazine $\times 10^2$ *M*	Apparent CMC of Promethazine $\times 10^2$ *M*	Promethazine–Chlorpromazine Molar Ratio
0	4.35	—
0.28	3.12	11.14
0.34	1.49	4.42
1.27	1.56	1.23

[a] CMC determined from break in pH *versus* concentration curve.

Table IV—Calculated Free Energies of Micelle Formation and Free Energy of Solution of Phenothiazines (kcal mole^{-1})

Compound	ΔG_m[a] (20°)	ΔG_m[a] (34°)	ΔG_m (0.154 *M* NaCl) (20°)	ΔG_m[b] (20°)	ΔG_{ma}[c] (20°)	ΔH_m[d]	ΔG_s (RT ln X_w)	ΔG_s[e] (RT ln X_w[corr])
Thioridazine	−5.25	−5.57	−6.0 (34°)	−10.5	−5.91	+1.5	+10.3	+7.4
Triflupromazine	−4.72			−9.44			+9.6	+7.2
Chlorpromazine	−4.66	−4.86	−5.46	−9.32	−5.93	−0.6	+9.3	+6.9
Promazine	−4.36	−4.45	−5.05	−8.72	−4.36[f]	−2.3	+8.2	+5.9
Promethazine	−4.15	−4.34	−4.98	−8.30	−4.98	−0.2	+8.15	+5.85

[a] RT ln CMC, with CMC in mole fraction units. [b] $2RT$ ln CMC. [c] $[2 - (j/z)]$ RT ln (CMC), using mass-action j and z values. [d] Calculated from limited data between 20 and 37°. [e] Solubility X_w in mole fraction units. Free energy of solution was corrected for side-chain contribution, using estimates based on data of G. Nemethy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773(1962). Standard states are such that mole fractions are unity and solution properties are characteristic of infinitely dilute solution. In the case: drug (solid) → drug (solution) and drug (solution) → drug (micelle), the solution standard states will be identical. But while the micellar standard state represents a hydrated species, the solid standard state will be pure solid. The differences in magnitude between ΔG_{soln} and ΔG_m would represent the differences in those standard state conditions. [f] −6.34 if alternative j and z values in Table I are used.

tion, devoid of double layer. The values of ΔG_m obtained by the three main equations are quoted in Table IV.

Micelle formation involves the removal of the hydrophobic portion of the molecule completely or partially from the water. Hence, one would expect to obtain some indication of the energy involved in the aggregation process from the free energy of solution of the phenothiazines in their basic form. Solubilities of the bases were taken from Green's (29) data. By neglecting activity corrections (because of the extreme dilution), the free energy of solution, ΔG_s, was calculated from:

$$\Delta G_s = -RT \ln X_w \qquad \text{(Eq. 13)}$$

where X_w is the mole fraction solubility of the base (Table IV). Green's method involves precipitation of the base from its solution as the hydrochloride. The bases separate out as oils (see Ref. 29) so the equilibrium studied is base (oil) ⇌ base (solution). When ΔG_m is plotted as a function of ΔG_s, there is a linear relationship for the promazine derivatives (Fig. 7), but the thioridazine and promethazine values deviate from the line. The solubility values can predict the rank order of the CMC but not its absolute value.

The unitary free energy of dimerization of methylene blue (24), which involves the loss of two faces of the ring system and is therefore equivalent to the insertion of one molecule into a micelle, is −6.95 kcal mole^{-1}. After correcting for the positive free energy contribution due to electrical interaction, the hydrophobic contribution has been estimated to be approximately −8 kcal mole^{-1}. The methylene blue molecule differs from the phenothiazines in that its inner ring is aromatic; the phenothiazine inner ring is quinoid. A rough estimate of the corresponding value for the phenothiazine is, therefore, −5.3 kcal mole^{-1}, close to the values obtained experimentally, which range from −4.2 to −5.3 kcal mole^{-1} at 20°. This gives weight to the picture of the micelle containing the monomers stacked in an alternating mode, with the *N*-alkyl side chain still partially in an aqueous environment. Nevertheless, changes in the side chain (*cf.*, promazine and promethazine) do result in differences in ΔG_m.

The free energy of binding of chlorpromazine to serum albumin was found to be −5.83 kcal mole^{-1} (31). Kwant and Seeman (6) quoted a value of −9.4 kcal mole^{-1} for the energy of binding of chlorpromazine to hemoglobin-free erythrocyte ghost membranes. The interaction of the phenothiazine with the albumin is regarded to involve one benzene ring and part of the aliphatic side chain. The value obtained by Kwant and Seeman is close to the free energy of solution of chlorpromazine (−9.30 kcal mole^{-1}), suggesting that the ring system and aliphatic side chain are removed from water. The discrepancy between the solution free energies (from −8.15 to −10.3 kcal mole^{-1}) and the free energy of micelle formation (from ∼−5 to −7 kcal mole^{-1}) may be due to two factors in the case of the small drug aggregates encountered in water:

1. When the alternately stacked micelles form, the alkyl side chain is not completely removed from the water. Attempts to correct the solubility data to exclude the *N*-alkyl side-chain contribution (Fig. 7 and Table IV) bring the solubility and micellization energies closer, but only to within 1-2 kcal of each other.
2. The rings do not perfectly coincide in the stack.

The larger micelles formed in salt solutions have a more substantial hydrophobic core; the hydrophobic portions of the molecules are more completely removed from the water on micellization and, hence, ΔG_m is larger.

CONCLUSIONS

The aggregation process in phenothiazine solutions is akin to that observed in conventional surface-active agents and can similarly be described by the law of mass action. A CMC does exist, as demonstrated by nonequilibrium dialysis measurements of the transfer of a monomer across a membrane. The rate of transfer increases negligibly above the CMC region. The extreme hydrophobic character of the compounds and the sensitivity of the aggregation number to salt make the analysis of the transfer from

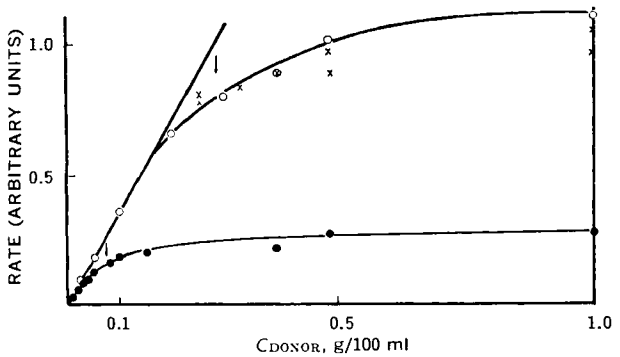

Figure 6—*Rate of dialysis of thioridazine across cellophane membranes as a function of drug concentration above and below the CMC in water* (○) *and 0.154* M *NaCl* (●) *at room temperature.* × = *calculated rates of transport assuming exclusive passage of monomer across the membrane, based on NMR data. Arrows denote CMC values.*

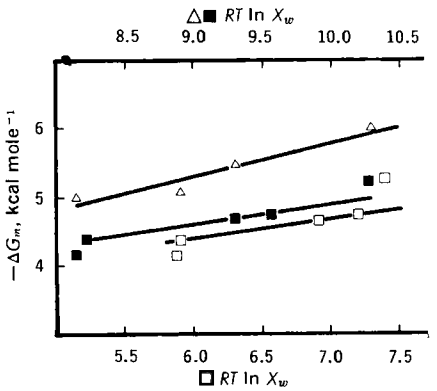

Figure 7—*Free energy relationships. The free energy of micelle formation as a function of the free energy of solution of the phenothiazine bases calculated from the solubility data of Green (29). Key:* ■, *from CMC values in water; and* △, *from CMC values in 0.9% NaCl. The corrected values for the free energy of solution represent data adjusted by subtracting an estimated contribution for the* N*-alkyl side chain* (□, *data points*).

water to a nonpolar environment difficult. Their surface-active properties and their ability to complex with other drugs having similar ring systems render doubtful the conclusions drawn by many workers as to the biological significance of enzyme inhibition studies and complexation with a variety of biological materials. While caution must be observed in interpreting such studies, it becomes clear that the colloidal properties of the drugs must be linked to their biological action. It should be no coincidence that a range of drugs with diverse structure, but with pharmacological properties similar to those of phenothiazines, was demonstrated to be surface active and to form micelles in solution.

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ˣ To whom inquiries should be directed.

BOOKS

REVIEWS

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Reviewed by Durward F. Dodgen
Food Chemicals Codex
National Academy of Sciences–
National Research Council
Washington, DC 20418

Aromatic and Heteroaromatic Chemistry, Vol. 1. C. W. BIRD, Senior Reporter. The Chemical Society, Burlington House, London W1V OBN, England, 1973. 445 pp. 14 × 22 cm. Price £11.

This volume is the first of a new series of Specialist Periodical Reports on Aromatic and Heteroaromatic Chemistry which is planned as an annual publication. The work reported in this initial volume has been compiled from the literature covered by *Chemical Abstracts* from July 1971–June 1972. It is a credit to the editors that so extensive an amount of work has been collected into one volume in such a well-organized and readable manner. The text meets the high standards set by other Chemical Society publications (Annual Reviews, Chemical Society Reviews, *etc.*) and of necessity is extensively referenced.

The subject material is divided into an extensive chapter on aromatic systems including discussions of exotic systems such as cyclophanes, circulenes, helicenes, and annulenes among other unusual aromatic and heteroaromatic systems, to provide the reader with a concept of the current understanding of aromaticity. The remaining chapters (14) deal with the synthesis of aromatic systems (4 chapters), aromatic substitution reactions (6 chapters), side-chain reactions (1 chapter), and finally a review (3 chapters) of naturally occurring compounds. A section on theoretical aspects is to be included in the second volume.

The authors of the various chapters have included, where appropriate, a brief, well-referenced survey of the less recent subject material, prior to the discussion of the 1971–1972 literature. This is particularly useful to the reader wishing to update knowledge of areas in which he or she has not kept abreast. For the medicinal chemist, both teacher and researcher, this volume would seem to be a necessary addition to a personal, or at the very least, departmental library. It is most important that our scientists keep abreast of recent advances for possible applications to the development of superior drugs. This volume satisfies the criteria for accomplishing these objectives and its attractive format adds to its desirability as a valuable text.

Reviewed by I. W. Mathison
College of Pharmacy
University of Tennessee
Memphis, TN 38103

Antihistamines; Barbiturates; Cytotoxic Agents; Drug Dependence; Ganglionic Blocking Drugs; Hypoglycemic Sulfonylureas and Biguanides; Oral Contraceptives; Parasympathomimetic and Anticholinergic Drugs; Pituitary Hormones and Factors; Psychotherapeutic Drugs; Renal Therapeutic Agents; and Thyroid Functions, Hormones, and Diseases. The articles vary in depth and breadth of coverage, the one on antibiotics, for example, being quite extensive, while those on antihistamines and psychotherapeutic drugs receive only token coverage (although the individual drug articles in the book make up the difference). In general, however, these articles (averaging about eight pages in length and ranging from less than two to about 30 pages) provide concise, useful outlines in pharmacology and therapeutics for the various classes of drugs covered.

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Food Chemicals Codex
National Academy of Sciences–
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Aromatic and Heteroaromatic Chemistry, Vol. 1. C. W. BIRD, Senior Reporter. The Chemical Society, Burlington House, London W1V OBN, England, 1973. 445 pp. 14 × 22 cm. Price £11.

This volume is the first of a new series of Specialist Periodical Reports on Aromatic and Heteroaromatic Chemistry which is planned as an annual publication. The work reported in this initial volume has been compiled from the literature covered by *Chemical Abstracts* from July 1971–June 1972. It is a credit to the editors that so extensive an amount of work has been collected into one volume in such a well-organized and readable manner. The text meets the high standards set by other Chemical Society publications (Annual Reviews, Chemical Society Reviews, *etc.*) and of necessity is extensively referenced.

The subject material is divided into an extensive chapter on aromatic systems including discussions of exotic systems such as cyclophanes, circulenes, helicenes, and annulenes among other unusual aromatic and heteroaromatic systems, to provide the reader with a concept of the current understanding of aromaticity. The remaining chapters (14) deal with the synthesis of aromatic systems (4 chapters), aromatic substitution reactions (6 chapters), side-chain reactions (1 chapter), and finally a review (3 chapters) of naturally occurring compounds. A section on theoretical aspects is to be included in the second volume.

The authors of the various chapters have included, where appropriate, a brief, well-referenced survey of the less recent subject material, prior to the discussion of the 1971–1972 literature. This is particularly useful to the reader wishing to update knowledge of areas in which he or she has not kept abreast. For the medicinal chemist, both teacher and researcher, this volume would seem to be a necessary addition to a personal, or at the very least, departmental library. It is most important that our scientists keep abreast of recent advances for possible applications to the development of superior drugs. This volume satisfies the criteria for accomplishing these objectives and its attractive format adds to its desirability as a valuable text.

Reviewed by I. W. Mathison
College of Pharmacy
University of Tennessee
Memphis, TN 38103

Antitumor versus *Cytotoxicity*

A recent paper by Miles *et al.*[1] bears the title "Tumor Inhibitors I . . . ." However, the authors state that extracts of the plant under investigation showed no activity in the L-1210 and Walker carcinosarcoma test systems (both are *in vivo* systems). They did show that certain extracts exhibited cytotoxicity in the 9 KB system, but none of the isolated compounds was shown to be cytotoxic. (Inference was made, however, that betulinic acid isolated in their investigation might be cytotoxic because of its high concentration in the 9 KB active fraction.)

Not too long ago[2], I addressed this forum on the subject of investigators alluding to "antitumor" activity when in essence they were speaking of "cytotoxic" activity. The two terms are not synonymous and not all cytotoxic compounds have *in vivo* antitumor activity, nor do all antitumor compounds exert cytotoxicity. Apparently, neither the authors of the paper in question nor the reviewers of that paper are aware of the differences that exist between cytotoxicity and antitumor activity, and they also do not read the *Open Forum* section of *J. Pharm. Sci.*

It would be well for those investigating natural products for the presence of antitumor or cytotoxic agents to have their manuscripts reviewed by the Drug Research and Development Branch of the National Cancer Institute[3] (if this is where the testing is done) prior to submission for publication.

Norman R. Farnsworth
Department of Pharmacognosy and Pharmacology
College of Pharmacy
University of Illinois at the Medical Center
Chicago, IL 60612

Received April 25, 1974.

[1] D. H. Miles, U. Kokpol, L. H. Zalkow, S. J. Steindel, and J. B. Nabors, *J. Pharm. Sci.*, **63**, 613(1974).
[2] N. R. Farnsworth, *ibid.*, **62** (5), iv(1973).
[3] J. L. Hartwell, Head, Natural Products Section, Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, 8300 Colesville Road, Silver Spring, MD 20010

Journal of Pharmaceutical Sciences

JULY 1974

VOLUME 63 NUMBER 7

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EXTREMISM IN THE PURSUIT OF DRUG ABUSE PREVENTION

In recent years, sociologists and others who have an interest in criminology, the court system, and the judicial process have increasingly been questioning whether society, in its effort to protect and safeguard the rights of the guilty, may at the same time be intruding upon the rights and privileges of the innocent. The purpose of this editorial is to note that a similar and parallel problem appears to have developed within the health-care complex relative to drugs and medication.

The situation can be described quite succinctly by quoting a portion of a letter which was published in the March 11, 1974 issue of the *Journal of the American Medical Association*. A frustrated physician from Owensboro, Kentucky, wrote:

> *"Hey, out there! Somebody listen to me. I'm getting sick and tired of these restrictions on the drugs I prescribe for my patients. Sure, I know we have a drug problem in this country, but I'm not a part of it, and my patients are not and the pharmacists are not.*
>
> *"Why can't I phone in a prescription for a few sleeping pills any more? Why must my patient be required to come to me for a new prescription for nerve pills each time he runs out? How much worse is it going to get? And finally, who is doing all this?"*

On a more personal basis, we had an experience ourselves during this past winter flu season in which attempts to obtain a bottle of an exempt narcotic cough syrup met with no success in a number of local pharmacies. The pharmacists on duty at these pharmacies refused to dispense the requested cough syrup despite offers of identification and despite the fact that the request was being made by a local resident.

When asked for an explanation, these pharmacists, without exception, indicated that their refusal to dispense the medication was a personal decision and it did not reflect policy dictated by some manager, owner, or corporate employer. These pharmacists also indicated that their actions were due primarily to their fear of entrapment by zealous government narcotic officers, and only partly to prior experience with drug abusers. No matter that a family, including small children, was suffering and was being kept awake much of the night due to incessant coughing.

In essence, therefore, it seems that the laws which have been developed and the manner in which these laws are being enforced with regard to drug addiction have become so radical and extreme as to inhibit the proper functioning of health-care practitioners and to preclude satisfying legitimate medication requirements of law-abiding citizens.

There is no question but that drug abuse constitutes a very serious problem for a portion of the population. As such, suitable efforts must be made to control and eliminate this problem. In our opinion, however, when such controls and practices extend to the point that they prevent suffering individuals from obtaining appropriate and necessary medication—not to mention interfering with duly qualified health personnel in the discharge of their professional obligations to the public—then the pendulum has been permitted to swing much too far to one side. What obviously is needed is a reassessment of the obligations of government to meet adequately the needs of all elements of society.

Edward G. Feldmann

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Edward G. Feldmann

1974

July 29–August 1
Annual Conference on Pharmaceutical Analysis, The Land O'-Lakes Conferences, Extension Services in Pharmacy, University of Wisconsin—Extension and the School of Pharmacy, University of Wisconsin, Madison, Wis.

August 3–8
American Pharmaceutical Association, Annual Meeting, Chicago, Ill.

August 5–9
Gordon Research Conference on Medicinal Chemistry, New London, N.H. (Contact A. M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881)

August 8–13
American Podiatry Association, Annual Meeting, Marriott Motor Hotel, Atlanta, Ga.

September 2–7
General Assembly of the International Pharmaceutical Federation, Rome, Italy

September 8–13
American Chemical Society, National Meeting, Atlantic City, N.J. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

September 8–13
International Conference on Alcohol, Drugs, and Traffic Safety, Toronto, Canada (Contact Dr. W. S. Schmidt, 33 Russell St., Toronto, M5S 2S1, Canada)

September 9–13
International Symposium on Medicinal Chemistry, Medicinal Chemistry Section, Royal Netherlands Chemical Society, Noordwijkerhout, The Netherlands (Contact Secretariat, Merck Sharp and Dohme B. V., External Relations, Waarder-weg 39, P.O. Box 581, Haarlem, The Netherlands)

September 19–21
Seminar on the Relevance of Animal Models to Human Dermatopharmacology and Dermatotoxicity, Medical Sciences Building, University of California, San Francisco, Calif. (Contact Continuing Education in Health Sciences, Room 570-U, University of California, San Francisco, Calif.)

September 24–27
National Congress of Pharmaceutical Sciences, Hungarian Pharmaceutical Association, Hungarian Academy of Sciences, Budapest, Hungary [Contact Office for Conference Organization (Motesz), H-1361 Budapest, POB 32, Hungary]

October 8–12
American Society of Hospital Pharmacists, Midyear Meeting, Hollywood, Fla.

October 13–15
St. Louis Society of Pharmacists in Industry, Annual Seminar, St. Louis Marriott, St. Louis, Mo. (Contact Cecil W. Propst, Lewis-Howe Co., 319 S. 4th St., St. Louis, MO 63102)

October 14–17
Association of Official Analytical Chemists, Annual Meeting, Marriott Hotel, Twin Bridges, Washington, D.C. (Contact L. G. Ensminger, AOAC, Box 540, Benjamin Franklin Station, Washington, DC 20037)

October 20–24
American Public Health Association, New Orleans, La.

October 25
APhA Academy of Pharmaceutical Sciences, Industrial Pharmaceutical Technology Section, Cherry Hill Inn, Cherry Hill, N.J. (Contact A. J. Scarpone, Publicity Committee, Lederle Laboratories, Pearl River, NY 10965)

October 27–29
Symposium on Unusual and Under-Utilized Drug Information Resources, Drug Information Association, Hilton Inn, Williamsburg, Va. (Contact Dr. Fred Salter, VADICS Center, Virginia Commonwealth University, Medical College of Virginia, Richmond, VA 23298)

October 31–November 3
American College of Apothecaries, Annual Convention, Alameda Plaza Hotel, Kansas City, Mo.

November 7–12
American Association of Colleges of Pharmacy, Fairmont Hotel, New Orleans, La.

November 10–14
APhA Academy of Pharmaceutical Sciences, National Meeting, Fairmont Hotel, New Orleans, La.

November 10–14
American Dental Association, Annual Meeting, Washington, D.C.

November 30–December 4
American Medical Association, Clinical Meeting, Portland, Ore. (Contact Dr. E. B. Howard, 535 N. Dearborn St., Chicago, IL 60610)

December 7–11
American Society of Hospital Pharmacists, Midyear Meeting, Bal Harbour, Fla.

1975

January 27–31
American Association for the Advancement of Science, Annual Meeting, Americana Hotel and City Squire, New York, N.Y.

March 9–13
Society of Toxicology, Williamsburg Lodge, Williamsburg, Va. (Contact R. E. Scala, Esso Research and Engineering Co., P. O. Box 45, Linden, NJ 07036)

April 6–11
American Chemical Society, National Meeting, Philadelphia, Pa. (Contact Meetings Department, American Chemical Society, 1155 16th St. N.W., Washington, DC 20036)

April 19–24
APhA Annual Meeting, San Francisco, Calif.

May 26–30
International Conference on Prostaglandins, Florence, Italy (Contact Dr. G. C. Folco, Scientific Secretary, Institute of Pharmacology and Pharmacognosy, University of Milan, via Andrea Del Sarto, 21-20129, Milan, Italy)

June 15–19
American Medical Association, Annual Meeting, Atlantic City, N.J. (Contact Dr. E. B. Howard, 535 N. Dearborn St., Chicago, IL 60610)

Items to be considered for inclusion or corrections should be addressed to

J. Pharm. Sci. Calendar
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Journal of Pharmaceutical Sciences

JULY 1974

VOLUME 63 NUMBER 7

REVIEW ARTICLE

Pharmaceutical Sciences — 1973: Literature Review of Pharmaceutics

ALLEN K. HERD*x and JOHN K. HALEBLIAN‡

Keyphrases □ Pharmaceutical sciences—1973 literature review of pharmaceutics □ Technology, pharmaceutical—sterility, dosage forms, cosmetics, packaging equipment □ Physical pharmacy—dissolution, solubility, permeability, complexation, surface phenomena □ Antibiotics—pharmaceutical aspects □ Radiopharmaceuticals—pharmaceutical aspects □ Biopharmaceutics—drug properties, formulation effects, absorption mechanisms and control □ Pharmacokinetics—literature review □ Literature review—pharmaceutical sciences 1973

CONTENTS

General Pharmacy 996
Preservatives 996
Flavor, Aroma, and Color 996
Stability 997
Stability Kinetics 998
Antibiotic Stability 999
Vitamin Stability 1000
Pharmaceutical Technology 1000
Parenterals 1001
Ophthalmics 1002
Sterility and Sterilization 1002
Tablets and Capsules 1004
Comminution, Mixing, Granulation, and Drying 1004
Powder Characteristics 1005
Compression 1005
Effects of Excipients 1006
Tablet Coating 1007
Suspensions 1007
Emulsions 1007
Ointments and Creams 1008
Suppositories 1009
Aerosols 1009
Timed Release 1010
Cosmetics 1011
Microbiological Contamination of Cosmetics 1011
Aspects of Formulation and Technology of Cosmetics 1011
Packaging 1012
Equipment 1013
Physical Pharmacy 1014
Dissolution 1016
Solubility-Solubilization Phenomena 1018
Membrane Permeation and Release 1019
Complexation 1020
Interactions of Drugs with Biological Substances 1020
Interactions of Drugs with Nonbiological Substances 1021
Surface Phenomena 1022
Interface Studies 1022
Adsorption Studies 1022
General Properties of Surfactants 1023
Micelle Studies 1023
Dispersion Stabilization 1024
Rheology 1025
Pharmaceutical Aspects 1026
Antibiotics 1026
Radiopharmaceuticals 1026
Biopharmaceutics 1026
Effects of Physicochemical Properties 1029
Effects of Formulation 1030
Absorption Control and Alteration 1030
Absorption Mechanisms 1032
Pharmacokinetics 1033
References 1037

This review of the literature represents a comprehensive cross section of the research and development effort in various selected disciplines of pharmaceutical sciences. As in past years, the scope of this endeavor has been limited to a review of the area of pharmaceutics because annual reviews of the literature related to other areas of pharmaceutical sciences are published elsewhere. This is the 12th annual review in the series (1–12). To compile it, numerous journals, periodicals, and selected sections of *Chemical Abstracts* were abstracted.

The review was prepared to provide a convenient method for pharmaceutical scientists to review the literature of the past year and to supply a source of references to articles of preferred interest. Except for minor changes, the well-accepted format of last year's review was retained.

GENERAL PHARMACY

A review of the chemistry, pharmacology, toxicity, metabolism, specific side effects, and antiallergic properties *in vitro* and *in vivo* of disodium cromoglycate (cromoglycic acid, disodium salt) was presented (13). Other reviews presented during the past year considered the use of dimethyl sulfoxide in dermatology (14); chemistry, biological activity, and therapeutic activity of the prostaglandins (15); and current aspects of pharmaceutics (16). The latter included dissolution, absorption of drugs, physical pharmacy, chemical stability, pharmaceutical microbiology, and tableting technology.

Preservatives—In an investigation of the inhibitory action of 3-phenylpropan-1-ol, 2-phenylethanol, and benzyl alcohol against *Pseudomonas aeruginosa*, 3-phenylpropan-1-ol was the most effective and benzyl alcohol was the least effective, as shown by growth rate, minimum inhibitory concentration (MIC), and determination of sterilization times (17). The three compounds enhanced the bactericidal action of benzalkonium chloride in the same rank order. The effect of polyvalent cations on growth inhibition of *Streptococcus faecalis* was investigated (18). With the exception of chromium, there was a rank-order correlation between the ability of the polyvalent metal salts to reduce the ζ-potential of the bacteria and to inhibit their growth. Suggestions were presented as to how the critical micelle concentration (CMC) of a nonionic surfactant could be increased and thereby lessen the possibility of reducing the activity, or even obtaining synergistic germicidal activity, of a cationic surfactant (19).

Multicomponent preservative systems containing up to six active components were used in cosmetics and pharmaceutical formulations to provide a wider spectrum of antimicrobial activity (20). Each preservative was screened alone and then with one other preservative before proceeding to more complex mixtures. The degradation of 14 commonly used preservatives in a 2% aqueous solution of polyvinylpyrrolidone was determined at 30° for up to 63 days (21). It was shown that the degradation of nonphenolic preservatives in the presence of polyvinylpyrrolidone was no greater than that in aqueous solutions. The effect of polyethylene and polyvinyl chloride granules on the stability of commonly used antimicrobial agents and antioxidants was studied. The potency losses of these agents were mainly attributed to their sorption affinities toward the plastic materials (22).

The effects of 11 different pharmaceutical materials on the bactericidal activities of benzyl alcohol, chlorobutanol, chlorhexidine diacetate, chlorocresol, methyl *p*-hydroxybenzoic acid ester, phenylmercuric nitrate, and benzalkonium chloride were investigated using viable count techniques (23, 24). The most antagonistic agents were colloidal magnesium aluminum silicate (Veegum), magnesium trisilicate, and polysorbate 80. The preservative efficiency of organomercuric compounds, *p*-hydroxybenzoic acid esters, and sorbic and benzoic acids was tested in emulsions containing sunflower oil, castor oil, and paraffin oil (25). Sorbic acid (1%) was the most efficient preservative. In another study of the preservation of oil and water emulsion ointment bases, sorbic acid (0.2%) or dimethyldodecylbenzylammonium chloride (0.01%) was recommended (26). The same type of study, but on anhydrous ointment bases, indicated that hexachlorophene and quaternary ammonium compounds possessed the highest antibacterial activity (27). Phenylethanol and sodium edetate were shown to be excellent preservatives for sulfacetamide eye drops against *P. aeruginosa* (28). The irritating action of some commonly used preservatives in preparation of collyria was studied (29). Of the preservatives tested, the organomercuric compounds, especially thimerosal, proved the best. Exceptional irritation and low bactericidal activity were demonstrated by a mixture of methyl- and propylparaben.

Additional references relative to preservatives can be found in Table I.

Table I—Additional References on Preservatives

Reference	Topic
30	Recommended procedures for conducting challenge tests for pharmaceutical preparations
31	Fundamental concepts for preservation of cosmetic formulations
32	Review of preservatives for cosmetics and some directions for manufacturing cosmetic preparations
33	Effectiveness of furoic acid and ethyl, propyl, and butyl esters against *P. aeruginosa*
34	Antiseptic properties of perfume oils and perfume chemicals
35	Bactericidal properties of aromatic substances and essential oils
36	Review of ophthalmic preservatives and vehicles
37	Review of adsorption, permeation, and chemical reactivity of 27 preservative agents for different types of plastics
38	Review of mode of action and parameters affecting activity of preservatives

Flavor, Aroma, and Color—A series of 2,7-dialkylcarbonate esters of lincomycin was synthesized (39) to enhance the pediatric acceptability of this antibiotic. Four diester derivatives exhibited oral bioactivity comparable to lincomycin. The physiology of bitterness was described (40). Bitter taste could be masked by local anesthesia of the tongue, modification of the chemical structure, or correction of the taste by special galenical preparations such as the addition of sweetness, increased viscosity, or modification of ionic concentration. Procedures to decrease or suppress the bitter taste of quinine hydrochloride as a solution or in a syrup were described (41). The linguistic difficulties in describing specific sensations of taste and smell were discussed (42). Experts in different countries were urged to communicate to work out a uniform terminology.

An encyclopedic evaluation of the literature in the

Table II—Additional References on Flavor, Aroma, and Color

Reference	Topic
55	Review of identification of flavors
56	Use of continuous process for preparing essential oils by steam distillation
57	Review of natural essential oils produced in Yugoslavia and their physicochemical characteristics
58	Essential oils of South America
59	Review of natural and synthetic aromatic elements and their use in the creation of perfumes and flavors
60	Description of method to improve the yield of rose oil
61	Listing of compounds with a jasmine odor
62	State of the art of perfuming aerosol products
63	Review of artificial and natural pineapple compounds
64	Use of ferric oxide as pigment dye for drug forms
65	Recent progress in jasmine research
66	Flower oils of lily-of-the-valley and lilac
67	Degradation of acid dyes by irradiation plus oxidation

perfumery materials field was continued (43). The composition affecting a perfume's fragrance is always distorted by the substrate due to inequality of association between each molecular species in the substrate (44). Suggestions for elimination of this distortion were discussed. A computer was used for routine blending and comparison of olfactory and gustatory components in essences and scents (45). Objective criteria for the actual smell had to be found so that the precise data could be fed into the computer. The smell masking of wax-containing products, foam rubber, textiles, synthetic leather products, and solvents in candles was described (46). A new class of perfumery ingredients, alkoxyalkylpyrazines, was developed, which is of particular interest since changes in the structure of the basic molecule lead to distinct and progressive changes in odor characteristics (47). Some general operative principles for the preparation of aerosol products were reviewed, and perfume dosages for various cosmetic products were given (48). It was pointed out that the perfume in cosmetics should give a distinct and elegant character and should mask the odor of the starting ingredients (49). Two examples of a popular perfume and the necessary modifications for its use in a cosmetic were given. The process of microencapsulation of perfumes and aromas was explained, and the application of this new technique in the cosmetic industry was reviewed (50). Anonis (51) described an experiment attempting to relate four major colors to corresponding odors.

The liquid and solid solution interactions of eight FD&C dyes with pharmaceutical gelatins and the effects of the dyes on disintegration behavior of gelatins were studied (52). All dyes interacted with Type A gelatin, but none interacted with Type B gelatin. In simulated gastric fluid without pepsin, FD&C Red No. 3 dye greatly diminished the average disintegration rate of both gelatins. 4,5-Dinitrofluorescein was discussed as to toxicology and legal aspects as a new coloring substance for cosmetics (53). Data on the properties of 16 common dyes were listed (54). Those properties considered were solubility in different solvents, light fastness, stability to heat, pH, oxidizing and reducing agents, and compatibility with citric and ascorbic acids, glucose, lactose, saccharose, sodium bicarbonate, and gelatin.

Table II lists additional references on flavor, aroma, and color.

Stability—The effect of certain commonly used vehicles on the decomposition of 5 and 20% sodium aminosalicylate solutions was studied (68). In all cases, the 20% solutions degraded more than the 5% solutions, but solutions containing glycerol and propylene glycol were more stable than those containing water. The decomposition of aspirin in a polyethylene glycol base was inhibited by citric and tartaric acids (69). Even when water was added, citric acid tended to slow the decomposition process. Fats of vegetable origin were incorporated into a polyethylene glycol base in an attempt to inhibit the decomposition of aspirin in this type of mixture (70). Degradation was retarded at 26°, but little effect was noted at 4 and 45°. The stability of erythromycin base compounded in lanolin, petrolatum, and their admixtures was determined for a year at room temperature (71). The optimal ointment consisted of 40 parts lanolin and 60 parts petrolatum. Sodium salicylate solutions (10 and 15%) were stabilized after the addition of 0.1% sodium bisulfite and 0.05% sodium edetate (Trilon B); they then could be autoclaved at 120° for 5 min and then stored for 1 year (72).

The decomposition of physostigmine salicylate solution sterilized at 120° for 20 min in the presence of various quantities of ascorbic acid and air or nitrogen was investigated (73). The addition of 50 mg of ascorbic acid to 100 ml of solution prevented oxidation, even when the ampul filling was carried out in air. Air in the presence of 1×10^{-3}% copper (II) caused considerable decomposition of procaine (Novocaine) solutions (74). This decomposition was significantly retarded by 0.01% sodium metabisulfite and 0.05% sodium bisulfite. For solutions of dexamethasone sodium phosphate, an empirical method was developed for determining the proper package size for preserving sodium bisulfite based on the volume of air per volume of product; the method was independent of any loss due to interaction of the preservative with other components of the formulation (75). Degradation of isoproterenol, phenylephrine, and epinephrine was studied by adding these compounds to aqueous oxymix systems and assaying for the remaining parent compound (76). After 30 min, recoveries of isoproterenol and phenylephrine were 89–100 and 89–108%, respectively, while the recovery of epinephrine was 53–79%.

Due to reports of transacetylation of acetaminophen (paracetamol) and codeine by aspirin, Roller (77) investigated the possible acetylation of morphine by aspirin. His conclusion was that this reaction between these two compounds is insignificant. The oxidation of aqueous cholesterol dispersions was studied in the absence and presence of various lecithins at 85° (78). More than half of the cholesterol was oxidized within 8 hr in the absence of phospho-

Table III—Additional References on Stability

Reference	Topic
87	Effect of structure of chelating agents on stability of apomorphine chloride solutions
88	Thermal stability of tocopherol acetate solutions in oil
89	Description of method for testing and preserving dihydroxyacetone
90	Effect of light on stability of aqueous solution of metaproterenol (orciprenaline) sulfate
91	Stability of chlorhexidine during autoclaving
92	Preservation of shark liver with sodium bisulfite
93	Thermal and storage stability of desoxycorticosterone acetate solutions in oil
94	Effect of light on stability of solutions of cardenolides
95	Stability of physostigmine eye drops
96	Influence of reducing agents on oxidation of epinephrine (adrenaline) with potassium iodate
97	Stability testing of sterilized adenosine solutions
98	Determination of decomposition rate of nicotinoyltauraminothiazole
99	Stability of aqueous guanethidine sulfate solutions
100	Degradation of phenylbutazone in aqueous solutions
101	Stability of elixir of diacetylmorphine (diamorphine) and cocaine
102	Chemical stability of homatropine and survival of bacteria in frozen buffered homatropine eye drops
103	Effect of freezing on pH of buffered aqueous solutions
104	Incompatibility of nonionic surfactants with oxidizable drugs
105	Properties of physiological solution of sodium chloride during different storage times
106	Stability and shelflife of drugs in liquid dispersion form
107	Physical and chemical interactions of injectable drugs, particularly antibiotics
108	Influence of various factors on stability of amino acid infusion solutions
109	Stability of casein hydrolyzate preserved at different temperature regimes
110	Chemistry of degradation of drugs in pharmaceutical systems
111	Method for evaluating stability of pharmaceutical products
112	Review of nonisothermal stability studies
113	Stability testing of pharmaceutical preparations
114	Review of methods available for accelerated aging studies
115	Review of cybernetic principles and use of charts as means of controlling drug quality

lipids, but less than 10% oxidation occurred in the presence of the lecithins. Factors involved in the browning of antacid tablets containing glycine were investigated (79). Initial pH was found to be the most important factor, with the greatest discoloration being at high pH. The content uniformity of nitroglycerin tablets was shown to be affected by volatilization and intertablet migration of the active ingredients (80), and the factors influencing these parameters were discussed. The conversion of lanatoside C to digoxin was shown to take place at 37° *in vitro* below pH 3 (81). Thus, moderate hydrolysis of the drug by gastric juices might be expected if taken between meals and substantial hydrolysis might be expected if taken with meals.

The stability of drug substances in solid pharmaceutical systems was discussed and theoretical models for various situations were proposed (82). As indicated by accelerated aging tests of compressed tablets containing eucalyptol, adsorbents with large surface area were the most effective in preserving the volatile agent (83). High humidity and compression force were unfavorable for good preservation. A simplified method for determining the chemical stability of drug substances in pharmaceutical suspensions was described (84), and the value of the method was confirmed experimentally using aspirin. A comparative study of the influence of ultrasound and heat on the hydrolysis of some organic esters was presented (85). The hydrolysis rate of the organic esters was nine times higher when subjected to ultrasound with heat as opposed to heat alone. In a study aimed at developing a stable solution of essential amino acids for parenteral nutrition, tryptophan was the only amino acid that degraded in considerable amounts (86); the use of amber vials and a 6-month expiration date were recommended.

Other papers of interest relating to stability are listed in Table III.

Stability Kinetics—The degradation of prostaglandins E_1 and E_2 was studied at 60° at pH 1–10 (116). A consecutive first-order reaction appeared to be operative above pH 4 for the dehydration and rearrangement reactions, and the data suggested that the *cis*-Δ^5-double bond participated actively in the rearrangement reaction of PGE_2 and was responsible for the greater reactivity of this molecule compared to PGE_1. The kinetics of hydrolysis of the carbamoyl group of 4-benzoylphenyl *N*-methylcarbamate in 50% aqueous ethanol showed that the reaction was first order with respect to both hydroxide ion and carbamate (117). The calculated half-life at pH 7.3 and 37° was 73 min. By studying the kinetics of the hydronium-ion-catalyzed hydrolysis of the ketal group in dexoxadrol hydrochloride, Brown and Forist (118) predicted that 91% of orally ingested dexoxadrol remained intact for a resident time of 60 min at pH 2 in the stomach. Previous investigations suggested that various 1-acyl-3,5-dimethylpyrazoles might owe their hypoglycemic activity to a nonenzymatic hydrolysis *in vivo* to the potent compound 3,5-dimethylpyrazole (119). To test this hypothesis, relative rates of hydrolysis at pH 2 and 6.7 were determined for a representative series of compounds covering a wide range of hypoglycemic potencies but no correlation between hydrolysis rate and activity was observed.

By selective formulation of aqueous solutions of methylprednisolone, it was observed that the stability of this compound could be affected significantly (120). A mechanism of solubilization and stabilization, based on hydrogen bonding and inclusion of the methylprednisolone into the polyoxyethylene exterior of polysorbate 80 micelles, was proposed. The acid degradation of sulfisoxazole in hydrochloric acid solutions in concentrations of 10–24% at 108° was studied (121). Two parallel pathways of molecular degradation, which led simultaneously to the formation of sulfanilic acid and sulfanilamide as final products, were determined and both were shown to be pseudo-

Table IV—Additional References on Stability Kinetics

Reference	Topic
128	Stability of aqueous solutions of aminopyrine (amidopyrine)
129	Autoxidation of drotaverine in aqueous solutions
130	Chemical stability of cyclophosphamide in aromatic elixir
131	Use of viscosimetry in the study of chemical kinetics in aqueous carbomer- (Carbopol) based gels
132	Stability of aspirin in substituted and nonsubstituted polyethylene glycol bases
133	Autoxidation of paverine in aqueous solutions
134	Kinetic behavior of phenylbutazone in four solvent systems
135	Chemical change of pentazocine in aqueous acidic media
136	Stability of 5-allyl-5-(2-hydroxypropyl)barbituric acid in solution
137	Stability of curcuma pigments in solution
138	Incompatibilities with thimerosal

first order. The kinetics of hydrolysis of benoxinate (oxybuprocaine) in aqueous citrate buffer solutions were studied over pH 1–6.5 (122). The pH–rate profile indicated that this molecule is subject to both acid and base catalysis, and the most stable pH was 3.0. An investigation of the decomposition of phenylephrine in buffered solution at pH 6.8 indicated that the major decomposition products, as identified by GLC–mass spectrometry, were 1,2,3,4-tetrahydro-4,6-dihydroxy-2-methylisoquinoline and the 4,8-dihydroxy analog (123). A possible mechanism for the degradation reaction was discussed. A kinetic study was made on norphenylephrine in aqueous solution (124). The decomposition rate depended on the rate constants of three dissociated ionic species. Hydronium ion and hydroxyl ion had no catalytic activity.

Hou and Poole (125) demonstrated that the β-lactamase-catalyzed hydrolysis of several penicillins followed Michaelis–Menton kinetics. Penicillins with a lipophilic side chain had the highest affinity for the enzyme. The introduction of a polar group into the side chain and/or a bulky diester group on the penicillin nucleus drastically lowered the binding. Exposure of dilute solutions of *N*-(2,6-dichloro-*m*-tolyl)anthranilic acid to visible or UV light resulted in fairly rapid decomposition with concurrent formation of approximately equimolar amounts of 8-chloro-7-methylcarbazole-1-carboxylic and 8-chloro-5-methylcarbazole-1-carboxylic acids (126). The kinetics of the hydrolysis of polysorbate 80 in aqueous buffers were studied over pH 1–10 (127). The hydrolysis appeared to be specific acid catalyzed at pH values below 3 and specific base catalyzed at pH values greater than 7.6. Both the acid- and base-catalyzed hydrolyses exhibited an unusual initial micellar surfactant concentration–rate dependence, opposite to that previously reported for the hydrolysis of anionic surfactants of the *n*-alkyl sulfate type.

Other papers related to stability kinetics are listed in Table IV.

Antibiotic Stability—The kinetics of the inactivation of some broad-spectrum antibiotics (tetracycline, chlortetracycline, and demeclocycline) were studied (139). The rate constants, activation energies, reaction half-times, and frequency factors were calculated. The toxic impurities, anhydrotetracycline and 4-epianhydrotetracycline, in commercial tetracycline products were determined spectrophotometrically (140). The storage temperature of tetracycline had a profound effect on the accumulation of these impurities, but temperature had practically no adverse effect on the biological activity or color of the preparations. Schlecht and Frank (141) used NMR to monitor the epimerization of tetracycline, since the dimethylamino resonance of tetracycline and that of its C-4 epimer differ by 0.1 ppm. This method eliminated the need for acidification in the previously reported spectrophotometric method, thereby lessening the possibility of anhydro formation which occurs in the presence of mineral acids. The radiochemical purity of three commercially available tritium-labeled tetracycline products was reported (142). All three products contained, in addition to tetracycline, substantial amounts of radioactively labeled 4-epitetracycline.

Decomposition of benzylpenicillin was measured over 24 hr at 25° in normal saline and in a solution containing one part of a tyloxapol-containing product (Alevaire) with four parts normal saline (143). In normal saline, a satisfactory degree of penicillin activity was retained for 24 hr, but penicillin decomposition was approximately 28% in solutions containing the tyloxapol product and saline. The presence of 10% sodium sulfacetamide had a stabilizing effect on sodium penicillin G eye drop preparations (144). In the formulation of suppositories with penicillin, degradation was enhanced greatly when the antibiotic was associated with antiseptic ingredients (145). Incompatibilities occurred, which led to as much as 95% inactivation of penicillin in the presence of creosote and guaiacol. Nystatin powder, which is known to be relatively unstable in its powder form and particularly sensitive to temperature, was shown to be more temperature stable in suppository bases (146). The stability of solutions containing amphotericin B was studied under the following conditions: exposure to 100 ft-candles of light at 23°, protection from light at 23°, exposure to 35 ft-candles at 4°, and protection from light at 4° (147). There was no appreciable loss of active material during an 8-hr exposure under these conditions. In the presence of phosphate–citrate buffers, nystatin and amphotericin B were optimally stable between pH 5 and 7 (148). Loss of biological activity followed first-order kinetics except under acid conditions. The decomposition rates of cephalosporin C in aqueous buffers and in fermentation broth at 25° were determined and found to obey the rate law $-dC/dt = [0.27(H^+) + 0.005 + 500(OH^-)]C$ mole/liter/hr (149). In a typical fermentation, nonenzymatic hydrolysis destroys about 15% of the product.

The stability of oleandomycin phosphate was determined at pH 2–8 at various storage times and temperatures (150). The decomposition rate was shown to be first order and the compound was most stable at pH 4. The stability of solid α-cycloserine was extremely dependent on moisture content (151).

Table V—Additional References on Antibiotic Stability

Reference	Topic
153	Effect of heat on biological activity of tetracycline and oxytetracycline hydrochloride
154	Effect of insoluble penicillins on stability of sodium penicillin G in aqueous solutions
155	Vacuum evaporation of water–butanol solutions of penicillin in a thin film
156	Stability of penicillin and streptomycin in tissue culture media
157	Influence of various concentrations of polysorbate 80 on stability of chloramphenicol
158	Effect of sodium benzoate and sodium salicylate on stability of chloramphenicol
159	Effect of sodium benzoate and sodium salicylate on solubility of chloramphenicol
160	Hydrolysis of chloramphenicol (levomycetin) esters
161	Alkaline degradation products of cephradine
162	Effect of honey, sugar syrup, and pasteurization methods on activity of kanamycin and benzathine penicillin G (Bicillin)
163	Thermodynamic and structural aspects of antibiotic–surfactant mixtures

At 0.15% moisture, little decomposition occurred; but at 1.3%, a sharp drop in activity was observed at 40°. The stability of antibiotics in infusion solutions was examined, and possible causes of inactivation were discussed (152). There were no general rules for the stability of antibiotics in these systems and specific stability studies are required.

Other references relating to antibiotic stability can be found in Table V.

Vitamin Stability—The effect of addition of 0.015% *p*-butoxytoluene antioxidant on the quality of soybean oil and the stability of vitamin A acetate solutions in soybean oil was studied (164). Although vitamin A was slowly oxidized in systems with and without the antioxidant, the rate was three times faster than that in the soybean oil containing the antioxidant. The stability of vitamin A alcohol, an intermediate product of vitamin A acetate, was determined in aqueous ethanolic solutions (165). The stability of this vitamin in 60–100% alcoholic solutions decreased gradually as water content increased. A photometric and spectrometric study related to the stability of vitamin A in dermatological preparations was presented (166). Vitamin A losses in anionic and nonionic hydrophilic bases and in polyethylene glycol gels were 3–17 and 100%, respectively, after 18 months of storage. In an effort to relate the stability of vitamin A in cod liver oil to filtration at 0–5°, it was determined that the filtration of the oil was not beneficial (167).

McAlpine *et al.* (168) studied the photoxidation of ascorbic acid in water at pH 4.5–11.6 using electron spin resonance spectroscopy. Results were consistent with a primary photochemical step involving the ejection of an electron from the monoanion of ascorbic acid to form the ascorbate. Sixteen commercial liquid multivitamin preparations were stored at 5, 25, and 40° for 1 year, and the contents were assayed for vitamins B_1 and C (169). As might be expected, degradation at 40° was more rapid than at 25°, and considerable difference was found among the commercial preparations. The results of an investigation of microbial contamination in oral vitamin preparations was reported (170). The occurrence of microorganisms in all preparations was higher than the values suggested in the literature, and pathogenic bacteria were found in 11 of 17 investigated preparations.

PHARMACEUTICAL TECHNOLOGY

In vitro test methods were described for the evaluation of antacid preparations (171). By using these procedures, a mixture of 400 mg dihydroxyaluminum L-histadinate and 400 mg magnesium hydroxide was compared with 15 experimental antacid mixtures. Phares and Hramchenko (172) described the advantages of colloidal alumina (Sol-Al; Baymal) and its chemical and physical properties in pharmaceutical applications. The test methods used by several investigators, or recommended by official compendia, for the microbial control of pharmaceutical and cosmetic products were reviewed and discussed (173). Attention was drawn to important factors that could influence test results such as product composition, sample preparation, time and temperature of incubation, and culture media. Fifty pharmaceutical grade samples of lactose were examined for microbial content (174). None showed greater than 6×10^4/g total aerobic colony-forming units. A basic description of the physical principles of accelerated freeze drying and the best methods for practical application were given (175). Methods for determining optimum conditions for freeze drying and for monitoring the progress of the cycle were described. Addition of carboxymethylcellulose, sodium alginate, gum tragacanth, pectin, and gum arabic increased the rate of solution of the lyophilized product (176). Viscosity of the reconstituted solutions was slightly decreased with respect to that of the unlyophilized gums, but viscosity did not decrease after storage.

Application of cation- and anion-exchange resins for the purification of ascorbic acid solutions from heavy metal and anion contamination was described (177). Finished material satisfied all compendial requirements. A paper reviewed the surface forces controlling the properties of dispersions of particles smaller than 1 μm in diameter (178). It was pointed out that distinction must be made between the process of deposition of such particles onto solid surfaces and their subsequent removal. The latter is a more complicated problem because of deformation of the materials at points of contact, the extreme closeness of the surfaces, and the possible formation of chemical bonds. For measurement of the angular intensities of scattered light of an aerosol, a method was devised without the aid of a cell with glass windows (179). This method was compared with the method using the glass cell and the Raleigh ratio. A technique was developed whereby a formulation with optimum properties, according to predetermined criteria, could be selected by computer analysis (180). The results of a statistically designed set of experiments, based on five independent variables, were used as the data input. An example was presented of the selection of an optimum formulation by this procedure and of its preparation in the laboratory.

Table VI—Additional References on Pharmaceutical Technology

Reference	Topic
181	Progress in pharmaceutical technology in 1970 and 1971
182	Use of nonaqueous solvents for preparing solutions of *N*-acetylsarcolycine peptides
183	Use of separate granulations in production of sodium barbital tablets with diphenhydramine hydrochloride (Dimedrol)
184	Fat-soluble vitamins and related compounds
185	Formation of gelatinous aluminum hydroxide and some properties of the derived alumina
186	Production and properties of highly disperse precipitated calcium carbonate for use in the pharmaceutical industry
187	Effect of technology on effervescent strength of piperazinium effervescent preparations
188	Structure and properties of natural hydrocolloids
189	Sulfurous acid salts as pharmaceutically useful antioxidants
190	Review of antioxidants used in pharmacy
191	Experiment planning methods on pilot plant apparatus for crystallization of trichloroacetic acid
192	Review of percutaneous absorption of chemical agents with particular emphasis on toxicity and side effects

Additional references on pharmaceutical technology are listed in Table VI.

Parenterals—Groves presented reviews dealing with the nature, origin, and hazard of particulate material in intravenous fluids (193), the detection of particulate contamination (194), and the existing standards and proposed new standards for particulate contamination in intravenous fluids (195). Efforts to produce solutions to specifications are defeated when the solution becomes contaminated with particulate matter from administration sets and cannulas (196). Therefore, it was suggested that standards be applied to all solution administration equipment. A comparative determination of particulate matter indicated that stable antibiotic solutions and lyophilized antibiotics have the lowest particulate contamination levels (197). In contrast, sterile powder-filled antibiotics have contamination levels two to three times higher.

The current trend toward quantitating trace particulates in parenteral preparations has resulted in an increased emphasis on the membrane filtration technique (198). A simplified method for carrying out this procedure was described. In a comparative study of particulate contamination in ampuls, standards based only on Coulter counter readings were not the whole answer (199). Visual examination was also found to be necessary. Results of a membrane filtration study of particulate matter contamination of intravenous fluids manufactured by four firms were reported (200). Three-year comparative data showed that considerable improvement in particulate matter contamination had taken place and that solutions in flexible plastic containers were the least contaminated. A manual counting method and an electronic scanning method were compared for determining particulate contamination in several small-volume commercial products (201). It was determined that, for large numbers of particles in the 5–25-μm range, the electronic method resulted in higher counts; in general, this method was more precise and more rapid than the manual counting method.

Ryan *et al.* (202) concluded that both the design and utilization of presently available equipment necessary for administering intravenous solutions are apparently deficient. In a study of 100 postoperative patients, the effectiveness of a 0.45-μm membrane filter dramatically minimized acute phlebitis and thrombophlebitis. The influence on flow rates of infusion fluids, when they are used with commercially available final filtration devices, was studied along with their ability to remove particulate matter (203). Commonly used infusion fluids flowed satisfactorily with these devices, and the filters did not significantly influence the pH and were effective in removing particulate matter. Intravenous administration of latex particles in doses of 8×10^6/kg with 0.4–10-μm diameter particles and of 4×10^5/kg with 40-μm diameter particles was well tolerated by rats (204). No histopathological effects, including inflammation, were seen except for infrequent focal myocardial degeneration with 10- and 40-μm particles. Dosage of 8×10^6/kg with 40-μm particles killed acutely. The factors causing drug additive compatibility problems were discussed, with the conclusion that general methods for determining compatibility are limited (205). Until more complete information is available, it was suggested that the health-care professions attempt to limit the number of drugs added to any single large-volume injectable solution. A review of the physical incompatibility of parenteral drugs was presented (206). It included the effects of concentration and temperature, stability of parenteral solutions, pH patterns for intravenous additives, and the presence of two or more drugs in the same parenteral vehicle including carbenicillin and gentamicin, carbenicillin and other drugs, antibiotics and synthetic sweetening agents, cardiovascular and psychotherapeutic agents with sodium ethacrynate, admixtures of two or more preoperative drugs, and penicillin and tetracycline.

The ability of large-volume vacuum-packed parenteral containers to maintain vacuum under adverse conditions of temperature and agitation was studied (207). In all cases, the vacuum seal was maintained under the test conditions; it was concluded that such factors as decomposition of ingredients and faulty glassware should be suspected if vacuum loss is found with these types of containers. The hypothesis that contamination of large-volume parenteral containers may be due to improper handling techniques was examined (208). Statistical evaluation of the results indicated that certain procedures may, indeed, result in contamination in many cases, whereas there was no significant contamination in any system tested when recommended procedures were followed. *In* a similar test, a series of different drug additives was added to intravenous glass bottles of Ringer's solution in a hospital ward (209). None of the bottles became contaminated during this procedure, but the bottles collected from bedside after administration to patients were found to be contaminated at a 3% level with fungi or bacteria. The effectiveness and safety

Table VII—Additional References on Parenterals

Reference	Topic
215	Suspended substances in commercial infusion solutions
216	Review of particulate matter in intravenous infusions
217	Parenteral dosage forms with prolonged action
218	Effect of oil solutions and suspensions for prolongation of vitamin B_{12} action
219	Methods for preparation of prolonged-action injectable preparations
220	Differential thermal analysis as screening technique for adjuvants in parenteral formulation
221	Parenteral incompatibilities in hospital pharmacy
222	Incompatibilities and other problems involved in adding drugs to intravenous solutions
223	Incompatibilities in multicomponent liquids used for injections
224	Interactions between penicillin and aminoglycoside antibiotics in parenteral solutions
225	Growth of microorganisms in parenteral nutritional fluids
226	Use of neutral olive oil for injections
227	Plastic containers for liquid parenteral drugs
228	Method for preparing ethyl oleate, a solvent for parenteral administration
229	Solubilization, emulsification, and dispersion with surfactants in parenteral preparations
230	Preparation of sodium bicarbonate solutions for injection
231	Stable parenteral dosage form of orotic acid
232	Preparation of parenteral dosage forms of trypsin and α-chymotrypsin
233	Preparation of stable injectable solution of *N*-benzyl-*N*-(β-benzoylethyl)piperazine dihydrochloride
234	Development of spasmolytic and analgesic preparation in injectable form
235	Problems in preparation and control of injectables in hospital pharmacy
236	Review of oil emulsions for parenteral administration
237	Problems in preparation of Ringer's injection, lactated Ringer's injection, sodium lactate, Trometanol, and urea solutions for parenteral infusion
238	Influence of siliconization on hydrolytic glass resistance
239	Evaluation of methods for testing of ampuls for undissolved impurities
240	Microbiology training for pharmaceutical production personnel
241	Design, construction, and operation of sterile filling facility

of a plastic two-way transfer needle for aseptically reconstituting parenteral drug powders, or transferring parenteral liquids, were studied, and it was found suitable for these purposes over a broad pH range (210).

In studies to evaluate methods for detecting leaks in glass ampuls, it was concluded that the best dye test is a pressure test (211). However, for routine testing, the pressure used should be the highest possible consistent with no ampul damage. A suggested approach to the selection of an elastomer compound for use with various parenteral solutions was presented (212). Many factors, including type of solvents, solvent pH, and the effect of the sterilization process, must be considered. Since many freeze-dried pharmaceutical preparations contain very small amounts of active medicaments, it is often necessary to add other ingredients to provide a supporting matrix of solids for the dried product (213). A method that measured fracturability of the plug was developed and was shown to give reproducible and meaningful results in evaluating these additive materials. In an effort to produce a long-acting intramuscular injection, trifluoperazine pamoate (trifluoperazine embonate) was microencapsulated by interfacial polymerization techniques (214). The time of duration of suspensions of microencapsulated drug was shown to be approximately double that of the same drug in polyethylene glycol 400 solution.

Other papers of interest in the area of parenterals are listed in Table VII.

Ophthalmics—Drainage of an instilled drug solution away from the eye is responsible for a considerable loss of drug and, hence, affects the biological activity of drugs in the eye (242). The rate of this drainage is related to the volume of drug solution instilled and increases with increasing volume. In a study conducted on anesthetized and unanesthetized rabbit eyes, it was concluded that the drop size of ophthalmic delivery systems should be reduced from its present 50–75-μl size to, at most, 5–10-μl drops. In a review article on ophthalmic preservatives and vehicles, Mullen *et al.* (243) indicated that the most widely used ophthalmic preservatives were benzalkonium chloride, chlorobutanol, and phenylmercuric nitrate or acetate. The most commonly used vehicles were hydroxypropyl methylcellulose and polyvinyl alcohol. Rabbit corneas were denuded of epithelium and the rate of reepithelization was measured to determine the toxicity of various commercially available wetting solutions used for contact lenses (244). Of six commercial solutions, three were inhibitory, but no consistent pattern was revealed as to which components were toxic.

Eighty-four semisolid, water-soluble, anhydrous bases for possible ophthalmic use were formulated and evaluated on the basis of pH and desirable physical spreading characteristics over 0–50° (245). One was selected on the basis of several attributes as the best formulation for ophthalmic use. Pilot plant facilities were designed and built for scale-up activity based on accumulated data from investigations of conventional sterilizing procedures and aseptic techniques for manufacturing sterile anhydrous ophthalmic ointments (246). Because of batch sizes normally made by production, practical means for sterilizing and operating on a large scale under aseptic conditions were evaluated. The detection of metal particles as an impurity in ophthalmic ointments dispensed in collapsible metal tubes was studied (247). A modification of the USP method was given in which the disturbing solid was extracted by a suitable solvent before the final inspection. The dilution effect of drugs introduced as solutions into the conjunctival sac could be minimized by use of a hydrophilic contact lens made of glycol methacrylate polymer (248). Lenses composed of this polymer were immersed in various antibiotic solutions and placed in the eye with no deleterious effects and with prolonged duration of action.

Sterility and Sterilization—A review of the improvements made over recent years in sterilization

procedures and sterility testing methods for the aseptic manufacturing of parenteral products was presented (249). Methods were investigated for isolating and enumerating *Escherichia coli*, inoculated into white, soft petrolatum, by a membrane filtration technique (250). Superior recovery levels were obtained by homogenizing the ointment base with a polysorbate 80–peptone–water mixture, which permitted the estimation of contamination in up to 250 mg of material. Factors influencing the reisolation of inoculated organisms in ointments and creams were also examined employing a membrane filtration technique (251). Ointments were less sensitive to experimental design and had more consistent recovery rates, while creams required more rigid conditions and low levels of contamination could remain undetected because of the difficulty of increasing sample size. Bruch (252) reported that the best proof that a lot of sterilized material has a high probability of being sterile is the destruction of calibrated doses of microorganisms of defined resistance carried by a few samples from the lot. In a study comparing thioglycolate medium with four other medium broths, it was determined that thioglycolate was superior in terms of accuracy and time required (253).

Studies indicated the doses of ethylene oxide injected subcutaneously that would cause tissue reaction (254). Factors that affect the elution of ethylene oxide from medical materials were discussed. To determine the necessary aeration time of materials before they can be determined safe for use, the desorption characteristics of ethylene oxide from three types of silicone polymer were studied (255). Very rapid desorption of gas to low residual levels was observed in two nonreinforced silicone rubbers. The third material, Dacron-reinforced silicone sheeting, lost the gas at a considerably slower rate due to retention by the Dacron reinforcement. An acute ocular toxicity test for ethylene oxide, ethylene glycol, and ethylene chlorohydrin was described (256). In general, the ocular damage was most intense with ethylene oxide and least intense with ethylene glycol. It was reported that the establishment of effective economic ethylene oxide sterilization cycles for medical devices involved three major considerations (257). These were the determination of total microbial load on the product prior to sterilization, the use of the total load information to determine the number of samples to be tested, and a comparison of the resistance to sterilization of the indicator with that of the microorganisms on the product.

Schulman (258) presented a review on the fundamentals of interaction of ionizing radiation with chemical, biochemical, and pharmaceutical systems. Thirteen antibiotics belonging to different groups were exposed to 5, 10, and 15 Mrads of neutron and γ-radiation (259). With γ-radiation, oxytetracycline at 15 Mrads and penicillin G at 5 and 10 Mrads showed a considerable decrease in activity. Other antibiotics did not show significant change in activity by this method. When subjected to γ-radiation at 2.5 Mrads, procaine hydrochloride crystals showed no chemical changes from the nonirradiated material (260). A slight yellow color was attributed to minute traces of chemically nondetectable compounds. Injectable solutions and powders of ephedrine hydrochloride, atropine sulfate, scopolamine hydrobromide, strychnine nitrate, morphine hydrochloride, codeine phosphate, and neostigmine (proserine) were radiation sterilized at low temperature (261). All preparations underwent some decomposition accompanied by changes of color and pH, appearances of foreign impurities, decreases in biological activity, and increases in toxicity. Disposable tube stoppers and adaptors, made of both high and low density polyethylene, were successfully sterilized by γ-radiation (262). No changes between irradiated and nonirradiated material were detected.

A discussion of deep and surface filtration, the design, production, and efficiency of membrane filters, process filtration with membrane filters, cold sterilization with membrane filters, techniques of filtration, and filtration carried out just prior to filling containers was presented (263). Ultrasound of 800 kHz frequency was successfully used in sterilizing a *Bacillus subtilis* spore suspension in 0.9% sodium

Table VIII—Additional References on Sterility and Sterilization

Reference	Topic
269	Laminar flow in the pharmaceutical industry
270	Use of *Bacillus stearothermophilus* as biological indicator
271	Sterility testing of large-volume aqueous pharmaceutical products by the membrane filtration technique
272	Further developments in *Limulus* amebocyte lysate pyrogen testing
273	Membrane filtration tests for sterility
274	Bacteriological control of pharmaceutical emulsions through laser radiation scattering
275	Applicability of Attest indicators for testing sterile solutions
276	Ethylene oxide sterilization of nylon-wrapped materials
277	Sterilization by ethylene oxide and its application to respirators
278	Decontamination of artificial respirators by ethylene oxide
279	Antimicrobial treatment with ethylene oxide and formaldehyde
280	Effect of neutron and γ-radiation on drugs
281	Sterilization of biochemical products by γ-irradiation
282	Effects of γ-radiation on physical and chemical properties of disposable infusion assemblies
283	Radiosterilization of chamomile
284	Effect of ionizing radiation on essential oils
285	Description of sterilization procedure for eye drops
286	Continuous, automatic sterilization system for parenteral solutions
287	Review of sterilizing filtration of liquids
288	Filtration of liquids and gases by calibrated porous membrane
289	Effect of sterilization process on pH of solutions of 6% dextran (Hemodex) and 10% dextran (Reoisodex)
290	Germicidal effect of thimerosal on biological products
291	Review of actions, mechanisms, and uses of formaldehyde as sterilizing agent
292	Review of antimicrobial activity and chemical properties of glutaraldehyde as cold sterilizing agent
293	Peracetic acid as suitable disinfectant for disinfecting rooms
294	Review of sterilization by antiseptic liquids

chloride solution (264). Increased spore sensitivity to ultrasound, in the presence of very small amounts of preservatives, was attributed to changed permeability of the spore membrane. A review discussed the possible applications of β-propiolactone as a sterilizing agent (265). The bactericidal activities of isomeric trifluoromethylphenols against *E. coli* were found to be in the order: *meta* > *para* > *ortho* (266). These activities were related to the partition coefficients of the compounds between polar organic phases and aqueous buffers. The advantages and disadvantages of the application of sodium hypochlorite, potassium hypochlorite, peracetic acid, β-propiolactone, propylene glycol, phenolic agents, and formaldehyde solutions by aerosol spray were discussed (267). The importance of the limiting speed of sprayed droplet collapse, the exposed surface being covered by 1 ml of sprayed liquid, and the vapor pressure of a monodispersed droplet as a function of its diameter was presented. Ceramic and plastic tiles, as well as joint sealing materials, were sprayed with five disinfectants of specific action against Gram-positive and Gram-negative test organisms and fungi (268). Polished and glossy surfaces were more resistant to decontamination than dull or rough surfaces, and spraying left four times as many organisms as scrubbing.

Other papers relating to sterility and sterilization are listed in Table VIII.

Tablets and Capsules—A computer optimization technique was used to indicate the directions necessary to improve various characteristics of a production tablet (295). Data from experimental and production size batches were compared with computer predictions. The relationships of temperature, relative humidity, size of inoculant, and duration of storage on survival of *Staphylococcus aureus* inoculated onto surfaces of 17 commercial tablets and one gelatin capsule were determined (296). Within limits of the experiment, decreased survival was associated with an increase in each variable. Several other articles dealing with the general topic of tablets and capsules concerned identification coding (297), a discussion of hard capsule dosage forms (298), advances in tablet production (299), and quality control of tablet production (300–302).

The other numerous articles dealing with pharmaceutical technology of tablets and capsules have been subdivided into the following classifications to facilitate a search of the literature: comminution, mixing, granulation, and drying; powder characteristics; compression; effects of excipients; and tablet coating. For a thorough review, consideration of the entire section is advised due to the obvious overlap in the subject matter.

Comminution, Mixing, Granulation, and Drying—Jet grinding was evaluated as a method for reducing particle size of several forms of pharmaceuticals (303). Although different pharmaceuticals behaved differently, the powders did not lose their medicinal properties after grinding by this method. Improved measurement and digital control techniques resulted in in-line blending schemes that offer significant time and money savings (304). Three industrial blending situations were described which demonstrated the advantages of in-line blending. Because direct measurement of individual granule strength by crushing techniques is tedious and invariably gives a wide scatter of results for irregular granules, Hunter (305) described a method based on a friability test which correlated well with the direct crushing method. The effects of intragranule porosity and granule strength on some tablet properties were investigated (306). It appeared that compaction pressures eliminated any of these effects except at low pressures. The influences of five spheronization process variables—water content, extruder speed, extruder screen size, spheronizer speed, and residence time in the spheronizer—were evaluated on several granulation parameters (307). By using a complete factorial design, it was possible to define statistically significant effects of these process variables as well as any linear interactions. A mixture of starch and sulfanilamide, processed in a rotary granulator, was compared with one processed in an oscillating granulator with respect to porosity, specific surface, grain fluidity, and dissolution time (308). The effect of differences in density and compactness on the tablet was discussed.

In a study of the influence on pharmaceutical granulations of the type and capacity of mixers, it was determined that the initial densification of the wet mass was directly related to the capacity, but the rate of densification and binder distribution were similar for the three mixers studied (309). The influences of batch size, droplet distribution, adhesive strength and concentration, and drying times were factors that determined the size distribution of granules formed during granulation in a fluidized bed (310). Various methods for drying pharmaceuticals were discussed and evaluated with respect to the time required to achieve satisfactory dryness (311). In the study, the fluidized-bed dryer–granulator approach was found to be the best. The effect of temperature on the drying process was analyzed (312). The kinetics of constant rate and decreasing rate were described. The mathematical equations fitting the drying process using a fluidized bed were deter-

Table IX—Additional References on Comminution, Mixing, Granulation, and Drying

Reference	Topic
316	Influence of addition of organic and inorganic powders on degradation of polyvinylpyrrolidone by ball-milling in air
317	High frequency drying of pharmaceutical tablets and their physical properties
318	Review of vacuum drying in chemical and pharmaceutical industries
319	Optimization of processing of pharmaceuticals including fluidization
320	Discussion of granulation theories and mechanism of granule formation and growth
321	Mathematical evaluation of granule growth rate
322	Theories of granulation and different types of granulators
323	Production of granules by compaction
324	Effect of humidity on disintegration rates and hardness of compressed tablets

mined (313). From the corresponding nomograms, the rates of the drying time in relation to a given initial moisture content could be determined. Pitkin and Carstensen (314) determined that granules dry by a diffusional process and that, therefore, moisture content is a function of particle size. Where granules are not case hardened, the moisture levels equilibrate and the granulation is uniform when it reaches the tablet press. The following new techniques for tablet processing were discussed: drying by microwave, granulation and drying in one operation, and dry granulation without subsequent drying (315).

Additional references on comminution, mixing, granulation, and drying are listed in Table IX.

Powder Characteristics—Fell (325) investigated the influence of fines on the flow and compaction properties of conventionally processed and spray-dried lactose. He concluded that the fines of spray-dried lactose may well be formed in a different manner from the bulk of the material and, thus, behave differently under load. Flow or fracture may occur more readily, providing greater areas of bonding, leading to stronger tablets. The effect of crystal habit and particle shape upon the physical properties of tablets produced from cubic and dendritic crystals was reported (326). Tablets formed from the dendritic crystals, which had the larger shape index, were stronger than those formed from the cubic crystals. The effects of the particle shape and the ratio of interparticle cohesion–particle weight on the initial fractional voidage before tapping, the rate of tapping–compaction, and the final fractional voidage attained by tapping were studied (327). The initial fractional voidage of rod-shaped particles was much greater than that of the irregular or spherical ones and was explained in terms of the strong mechanical resistance caused by interlocking of particles due to the high elongation of the rod-like particles.

The specific volumes of three bulk solids at moisture contents up to 50% (w/w) under various consolidating stresses were shown to be dependent on the nature of the bulk solids (328). The influence of moisture on the packing properties and tensile strength of the three bulk solids was discussed in terms of the granulation process. An attempt to overcome the difficulties of the Jenike-type shear cell and to use a simpler procedure in a different shear cell, and thereby to reduce the quantity of powder required, was described (329). The yield loci obtained were very nearly linear. The process of sintering and its effect on the strength, pore structure, and rate of release of potassium chloride from a matrix tablet, prepared from a vinyl acetate–vinyl chloride copolymer, were investigated (330). Although the tensile strength of the tablets increased on sintering, the porosity and mean pore radius also increased, leading to a marked increase in release rate.

Additional articles relative to powder characteristics are listed in Table X.

Compression—It was shown that certain enzyme preparations lose activity when subjected to pressure (339). The concentration and distribution of stress by compression at several different pressures in convex-type tablets were studied (340), measuring specific enzyme activities of proteinase as a parameter. A new zone, which gave increased damage to the enzyme activity, was found that appeared about two-thirds from the center of the tablet in the longitudinal direction, and this zone was related to structural breaking during compression. Two strain gauges, diametrically opposed, were utilized on a tableting machine to study powder compression in tablet production (341). Granule size, crystal structure, humidity, and powder discharge were contributing factors to pressures required. A rotary tablet press was instrumented to permit production of tablets that would remain within preset weight tolerances (342). Tablets falling outside specifications were automatically rejected. The effect of compaction force on tablet dissolution using simple direct compression systems was investigated (343). For some systems, increase in pressure caused an increase in dissolution rate, while for other systems, increase in pressure caused a reduction in dissolution rate; the results were discussed in terms of the effects of the compaction process on interparticle bonding, fragmentation, and other tablet properties. A study was reported on the effect of the melting point of additives on the tensile strength of tablets measured by using a diametrical compression test (344). Tablets of equal density containing the same amount of additives showed a general decrease in strength as the melting point of the fatty acid additive increased, and this was attributed to different amounts of interparticle bonding resulting from melting of asperites on the particles at temperatures below their conventional melting points.

Compression runs on several powders of different particle size and hardness were made at a relatively low pressure range where fragmentation of particles did not take place (345). It was confirmed that the compaction parameters obtained were related to the pressure transmission ratio and the hardness of the material being compressed. For lactose tablets, it was shown that the effect of granule size on tablet hardness was less pronounced at high pressures (346). In another study, a linear relationship was found between compression pressure and disintegration time and hardness (347). This relationship oc-

Table X—Additional References on Powder Characteristics

Reference	Topic
331	Granulometric methods in testing pharmaceutical preparations
332	Manufacture and properties of highly dispersed calcium carbonate precipitate
333	Flow of granulation containing antibiotics and its effect on physicochemical behavior of tablets
334	Cohesion and flow of particulate solids
335	Studies on flowability of powder and interparticle cohesion
336	Pharmaceutical studies on physical properties of powders
337	Effect of sintering on pore structure and strength of plastic matrix tablets prepared from polyvinyl chloride
338	Moisture transfer effect in hard gelatin capsules of sodium cromoglycate (cromoglycic acid, sodium salt)

Table XI—Additional References on Compression

Reference	Topic
349	Compression cycles of crystalline substances for pharmaceutical use
350	Evaluation of creep curves from process of dynamic compression
351	Use of compressional force measurements in tableting
352	Use of compaction for obtaining tableting mixtures and limitations involved in this process
353	Elastic, plastic, and viscous properties, and characteristics of deformation processes in tablet masses
354	Effect of technological factors on release of active material
355	Review of dry compaction of pharmaceutical powders
356	Compression of medicinal preparations without pregranulation
357	Use of lubricants to avoid slip-stick during ejection of tablets
358	Production of tablets by direct compression

curred in all cases, regardless of the weight and granule size of the particles. The importance of punch length uniformity to the control of tablet weight variation was demonstrated on a rotary tablet press (348). Analysis of the relationship between punch length and compression force pointed to the uncertainty of the relationship between tablet weight and compression force when variations of upper and lower punch length are considered.

Other articles concerning compression are listed in Table XI.

Effects of Excipients—By using a scanning electron microscope, the location and structure of starch grains in experimental and commercial aspirin tablets and aspirin–phenacetin–caffeine tablets were studied (359). By observing tablet faces and cross sections before and after the addition of water, rupture of the surfaces was shown to occur where starch agglomerates were found; it was postulated that disintegration occurs by hydration of the hydroxy groups of the starch molecules, causing them to move apart. Disintegrant properties of sodium starch glycolate and a cation-exchange resin were examined in various tablet systems (360). Extreme efficiency, even at low concentrations, was demonstrated for both compounds. A study of the effects of six disintegrants on a water-soluble tablet showed that rice starch and bentonite caused a decrease in the disintegration time with little or no effect on the mechanical characteristics (361). An evaluation was made of the properties of five tablet disintegrants in two insoluble direct compression matrixes: dicalcium phosphate dihydrate and calcium–phosphato–carbonate complex (362). The effect of concentration of disintegrants on disintegration time was interpreted in terms of the difference in mechanisms of disintegrant action. Cross-linked polyvinylpyrrolidone was studied for its disintegration property in comparison with starch USP and alginic acid (363). Cross-linked polyvinylpyrrolidone demonstrated superiority over both these agents, and it was postulated that capillary activity of the cross-linked polyvinylpyrrolidone is responsible for its disintegration property. The dissolution and disintegration of a number of experimental formulations of chloramphenicol capsules were dependent on capsule size, diluents, lubricants, and filling methods (364). Measurements of the rate of liquid penetration through powder beds of similar compounds to those used in capsules did not adequately reflect the dissolution profiles of the various formulations.

Sodium alginate was evaluated as a tablet binder for lactose tablets in comparison with acacia and gelatin (365). Sodium alginate compared favorably with regard to mechanical properties of the tablet produced, but the disintegration time of tablets was not increased as sodium alginate concentration increased above 1%. In a study of binder dilution effects on granulation, it was concluded that this factor had insignificant effects on the average granule size and granule density of granulations manufactured in a fluid-bed spray granulator (366). However, considerable influence was observed on granule friability, bulk density, interparticulate porosity, and, thus, flow rate. In three of the four formulations tested, the surfactant magnesium lauryl sulfate was equivalent to the hydrophobic magnesium stearate as a lubricant (367). Magnesium lauryl sulfate was superior to sodium lauryl sulfate; thus the goal of finding a compound with excellent lubricating properties but without waterproofing liability was achieved. A limiting concentration of 1.5% was determined for sodi-

Table XII—Additional References on Effects of Excipients

Reference	Topic
373	Tableting properties of sulfameter (sulfamethoxydiazine) formulations
374	Factors affecting capsule shell dissolution rate
375	Pharmaceutical studies on cyclandelate fine granules
376	Direct compression of pure excipients and excipients in presence of phenobarbital on reciprocating machine
377	Comparative evaluation of excipients for direct compression
378	Pharmaceutical-technological problems in preparation of tablets with colored active ingredients
379	Effect of certain tablet formulation factors on antimicrobial activity of tetracycline hydrochloride and chloramphenicol
380	Release of easily soluble drug from hydrophobic tablet matrix
381	Mechanism of action of flow-regulating agents
382	Effect of stearic acid and calcium stearate on quality of tablets
383	Effect of moisture on crushing strength of sucrose tablets
384	Dependence of pill disintegration and active ingredient release on pill mass composition and other factors
385	Dragées with tocopherol acetate
386	Effect of dibasic calcium phosphate and polysorbate 80 on disintegration and rate of release of active substance from tablets
387	Use of carboxymethyl starch as binding and disintegration rate-improving additive in tablet production
388	Binding activity of some adjuvants and their influence on physical characteristics of granules and tablets
389	Influence of starch and lactose on release rate of drugs from hard gelatin capsules
390	Influence of excipient type and drug particle size upon small-scale mixing process
391	Use of adsorbents to prevent mottling in colored tablets

um and magnesium stearates as lubricants for assisting the flow of granules into tablet dyes (368). Aluminum stearate was unsuitable during compression due to its inadequate coating of the granules or to its intense adsorption to granules, making it unavailable for lubrication.

The shear strength of fatty acids decreased with increasing carbon chain length to an optimum of 18 carbon atoms, followed by an increase (369). Lubricating efficiency, however, first increased and then decreased under the same conditions. In an attempt to avoid intertablet dose variation in nitroglycerin sublingual tablets, a formulation containing polyethylene glycol was prepared (370). With concentrations of polyethylene glycol equivalent to 85% of the nitroglycerin, the tablets maintained their content uniformity for long periods even at 37 and 45°, thus assuring a more uniform and predictable dose to the patient. The effect of finely divided silica on the antifoaming properties of polydimethylsiloxane, when formulated in a typical antiflatulent tablet, was examined (371). A dynamic froth test was used to show that removable or extractable dimethicone by ether extraction of the tablet powder markedly reduces the antifoaming properties of the powder despite retention of a small percentage of the polydimethylsiloxane dispersed on the surface of the silica. A rather novel method to study the relation of porosity to other physical properties of tablets was reported (372). The method consisted of granulating tablet mixtures with varying amounts of ammonium carbonate as an additive and allowing the tablets to sit for certain periods as the ammonium carbonate decomposed, leaving pores in the tablets.

Additional references on effects of excipients are listed in Table XII.

Tablet Coating—Heyd (392) evaluated the operational characteristics of an automatic, airless, spray tablet-coating system, with particular emphasis on volume of fluid delivery and spray pattern characteristics. He determined that a linear relationship existed between the pressure employed and the volume of fluid delivered and that viscosity had very little effect on the volume of fluid delivered. The influence of the solvents, ethanol and isopropyl alcohol, on the tablet bed temperature during spray coating was investigated (393). The temperature at equilibrium of the rotating tablet bed depended on tablet composition, solvent, solvent temperature, and temperature and velocity of the forced drying air. The effect of surface coating on the dissolution rate of potassium chloride solid disks was reported (394). It appeared that the major factor controlling the effect of a hydrophobic substance on the dissolution rate of a solid was the attachment of the substance to the surface of the solid.

The effects of additives on a solvent-free formulation suitable for use as a film coating were described (395). Coatings were applied by spraying molten mixtures onto tablets in a conventional coating pan; of the additives evaluated, only castor oil, cocoa butter, and isopropyl myristate improved the basic formulations. In a study describing the measurement of physical and chemical characteristics of film coating, it was determined that cellulose films had higher tensile strength and elastic deformation than methacrylic acid polymers and that the addition of plasticizers increased, whereas pigments decreased, viscosity, tensile strength, and tear resistance (396). Dissolution rate–pH profiles of several films for enteric coatings were also determined. The various standard methods for tablet coating were compared economically as well as in light of modern pollution requirements (397). A review compared the traditional sugar coating and coloring methods to the modern film coating and pigment-suspension coloring methods (398).

Suspensions—A comprehensive review of suspensions, including formulation, reduction of particle size, augmentation of the viscosity of the liquid phase, influence of concentration, and addition of surfactants, was presented (399). The degree of flocculation of sulfamerazine suspensions, as represented by relative sedimentation volume, was determined as a function of both surfactant and electrolyte concentrations (400). The results emphasized the importance of knowing the location, as well as the concentration, of the surfactant and were consistent with the Derjaquin, Landau, Verwey, and Overbeek theory. The particle-size distribution of commercial samples of hydrocortisone and norgestrel suspensions was determined by use of the Coulter counter, model B, and the raw experimental data so obtained were digitalized by use of the Weiball equation (401). It was shown that the concept of controlled aggregation may be usefully applied to the formulation of these suspensions. The sedimentation volume of procaine penicillin G suspensions varied with the concentration and molecular weight of polyvinylpyrrolidone (402).

Kayes (403) studied the effect of surface-active agents on the ζ-potential of particles in a model suspension system containing polyoxyethylene monohexadecyl ethers and sodium lauryl sulfate. The results suggested some type of multilayer adsorption with sodium lauryl sulfate adsorbed in reverse orientation, possibly attached to the ethylene oxide chains after they had covered the particle surface. A review was presented on the measurement of the ζ-potentials in nonaqueous systems (404). A flow-through diffusion chamber was developed to determine the release rate of the active ingredient from salicylamide suspensions as a function of its concentration and the concentration of dispersing and stabilizing additives (405). Release rates decreased with increasing concentrations of additives, with sodium carboxymethylcellulose having a higher release rate-retarding effect than tragacanth gum. Although sulfathiazole (norsulfazole) suspensions could not be prepared using sodium bentonite alone, easily prepared suspensions were made possible by the addition of methylcellulose or sodium carboxymethylcellulose (406).

Emulsions—Criteria were discussed for the selection of emulsion stabilizers (407). Some surface-active agents used as stabilizers were listed and their characteristics were given. In evaluating the method of preparation on the critical hydrophilic-lipophilic

Table XIII—Additional References on Emulsions

Reference	Topic
417	Stability of emulsions of oxyethylated alcohols
418	Glycerides of pyroglutamic and malic acids as emulsifiers
419	Pharmacotechnical study of fatty esters of polyethylene glycol in emulsion systems
420	Testing of emulsion stability and emulsion uniformity
421	Review of water-in-oil emulsions
422	Review of methods for choosing emulsifying agents
423	Review of nonaqueous emulsion systems
424	Review of various concepts of emulsion stability
425	Review of emulsification and emulsion stability
426	Review of determination of potency of emulsifying agents

balance (HLB) of a liquid petrolatum emulsion, it was discovered that the method of manufacturing changed the critical HLB (408). In this particular study, emulsions made at lower temperatures required higher HLB values for satisfactory products. The rate of separation of oil from mineral oil–water emulsions stabilized with sodium lauryl sulfate and several other stabilizing agents was determined at 5–30° in an ultracentrifuge (409). The emulsions were all found to be less stable at higher temperatures, but the change in stability with temperature was not caused primarily by changes in viscosity. In studying the relationship between globular size and ζ-potential, an exponential relationship between the apparent ζ-potential and mean globule size was found (410). The emulsions showed apparent instability behavior when the ζ-potential was reduced to approximately 25.0 mv. The emulsifying properties of linseed mucilage were evaluated, and it demonstrated considerable advantages over those of polysorbate 80, gum arabic, and gum tragacanth (411). While stable emulsions of liquid paraffin or cod liver oil with 4% aqueous methylcellulose solution showed rather high stability, those made with gelatin were less stable and their stability was dependent upon the purity of gelatin and the amount of acid added (412). No stable emulsion could be prepared with carboxymethylcellulose.

Liquid petrolatum emulsions with hydrophilic and hydrophobic surfactants were used to compare two methods for determining the stability of emulsions (413). Both the HLB method and a method using phase diagrams were evaluated, with the latter being the more accurate. Studies of phase diagrams confirmed that the Schulman microemulsion was not an emulsion but a solubilized solution (414). The conditions necessary to produce microemulsions with 5–10% solubilizer were elucidated. Reichmann and Petersen (415) prepared emulsions of glycerin and mineral oil using anionic, cationic, and nonionic surfactants. The effects of temperature and aging on droplet size and viscosity were evaluated, and the decrease in viscosity correlated well with droplet size growth. *Physical* methods for determining types of emulsion were recapitulated briefly, and the theory of emulsion stability was considered (416). Tests employed in the laboratory were summarized, and the physical and chemical methods available for process control were discussed with illustrative measurement curves.

Other references relative to emulsions are listed in Table XIII.

Ointments and Creams—The use of aqueous borax in formulating cold creams with beeswax dissolved in mineral oil provided whiteness, stability, and ease of formulation (427). The best samples were obtained with half-neutralization of the beeswax acid, and the borax behaved as if supplying 1 molecule sodium hydroxide/molecule of borax under these conditions. The water-release capacity of creams was determined by the diffusion of salicylate added to the creams (428). The consistency of emulsions strongly affected water diffusion, with the water-release capacity being much greater from liquid emulsions than from thick creams. A lubricant gel of optimal consistency containing a local anesthetic was formulated which could be sterilized by γ-irradiation (429). The formulation consisted of 1% carbomer gel, neutralized by 2% lidocaine (lignocaine) base, and the biological availability of the local anesthetic was assessed using an *in vitro* method. The degree of dispersion in various types of ointment bases except petrolatum was in direct relation to the release of salicylic acid from the base (430). Increased hydrophilicity of the ointment base improved the release rate. Emulsions containing 7.5% *o*-phenylphenol and the emulsifiers polysorbates, sorbitan esters, and a polyoxyethylene derivative of ricinoleic acid (Cremophor) had only a slight residual disinfectant activity (431). On the other hand, emulsions with natural emulsifiers such as gum arabic retained their activity for 53 days. The penetration of 1% neomycin sulfate through the skin and into the blood of rabbits was enhanced fivefold from a water–oil ointment base containing 30% dimethyl sulfoxide (432).

Bone oil (and the fatty ointment bases prepared from it) had the chief disadvantage of low water absorbability, aggravated by the addition of water-soluble drugs and remedied by the addition of emulsifiers (433). Emulsion ointment bases with the highest water content and the lowest concentration of emulsifying agent were obtained with HLB values of 4.5–5.5. A continuous manufacturing method, including the necessary equipment (434), was discussed for cosmetic creams. Consistencies of petrolatums of different melting points and of liquid–solid paraffin mixtures were determined (435). Suitable consistencies could be obtained by replacement of a part of the petrolatum with mixtures of solid and liquid paraffin to the extent of 12.5–15%. In hydrocarbon ointments, the strength of plastic gels was proportional to the log of the molecular weight of the solid gel phase (436). No such regular relationship could be found for polyethylene glycol gels. The rheological conditions operative during spreading of topical preparations on the skin were determined for a series of aqueous gels and oil-in-water emulsions (437). Where relevant, all results were compared with previously reported work on lipophilic formulations.

Table XIV—Additional References on Ointments and Creams

Reference	Topic
439	Gel stabilization and emulsifying properties of cholesterol under conditions of heat sterilization
440	Evaluation of quality of petrolatum in relation to its composition
441	Emulsion ointment bases using oxyethylated aliphatic alcohols
442	Pharmaceutical ointments utilizing a 5% solution of methylcellulose as a base
443	Formulations for waterproof film-forming preparations
444	Use of bone fats as ingredient in ointment bases and stabilization of them with antioxidants
445	Interactions of anthralin, salicylic acid, and zinc oxide in pastes
446	Analysis of local anesthetic preparations of different basic materials
447	Comparison of different pharmacopeial prescriptions using wool alcohol ointments
448	Optimization experiments for the formulation of wool alcohol ointments
449	Comparative analysis of different polyethylene-type ointment bases
450	Choice of suitable base for ammoniated mercury ointment
451	Granulometric composition of boric acid suspension ointments
452	Preparation of suspension ointments with sodium sulfacetamide
453	Cooling effect of currently used, topical dermatological preparations
454	Physical, chemical, and microbiological methods for evaluating liberation of medicinal substances from ointments
455	Shea butter as base for ointments and creams
456	Mowrah butter as ointment base ingredient

Hersey and Cook (438) stressed the importance of dose, or sample size, and particle size for assuring the homogeneity of suspension-type pharmaceutical ointments.

Other references pertaining to ointments and creams are listed in Table XIV.

Suppositories—The course of melting and resolidification of suppository bases was followed by consequent variations in transparency (457). Extreme and intermediate values of light transmission provided the data for plotting a melting-point transparency curve, by means of which it was possible to control the composition and homogeneity of these and similar pharmaceutical bases. The effects on the uniformity of drug distribution in suppositories and of the addition of lanolin, higher alcohols and their esters, emulsifying wax, polysorbate 80, and polysorbate 60 to various suppository bases were studied (458). The addition of surface-active compound improved uniformity only when it was appropriately matched with the suppository base. The changes, under storage conditions, in breaking strength, disintegration time, and consistency of various suppository bases were studied (459). Quality grades for the characterization of consistency were suggested.

The release of halidor fumarate from suspension-type suppositories of different composition was investigated by *in vitro*, chemical, and biological methods (460). The release rate was increased in all cases by increasing the surfactant added to the base. Salicylate suppositories were prepared using polyethylene glycol 1540 and polyethylene glycol 6000 in various proportions (461). Salicylate release differed markedly for bases composed solely of polyethylene glycol 1540 or 6000; but when suppositories contained a mixture of the two polyethylene glycols, the rate of release approximated that observed for a base composed solely of polyethylene glycol 6000. By applying mathematical methods to the optimization of the processing of medicinals, sedimentation of sodium chloride in suppositories prepared by molding was studied (462). By using mathematical calculations, optimum conditions of preparation could readily be determined. A review of *in vitro* and *in vivo* testing methods for the release of drugs from suppositories was presented (463). Physicochemical properties of various suppository bases, such as wetting characteristics, viscosity, melting time, and melting range, were discussed.

Table XV—Additional References on Suppositories

Reference	Topic
464	Experimental justification of rectal route for administration of doxapram
465	New formulations for suppository bases for tropical use
466	Release and adsorption of amobarbital (barbamyl) from polyethylene glycol base rectal suppositories
467	Hydrogenated peanut oil as fatty base for suppositories
468	Student experiment in preparation and evaluation of suppository formulations

Additional references on suppositories can be found in Table XV.

Aerosols—Reviews in this field included the technology of production and sterilization of aerosols (469), propellants used for aerosols (470), different methods for studying particle size and concentration in aerosols (471), the growth of the aerosol market (472), and a general review of medicinal aerosols (473). In a study to determine the optimum methods for formulation of aerosol emulsion concentrates, the most suitable were obtained by adding aqueous triethanolamine at room temperature to a myristic acid–mineral oil solution at 54.4° (474). A theory to account for the efficiency of this procedure was proposed, which involved the formation of a triethanolamine myristate–myristic acid complex during the initial addition of the aqueous phase. Aerosol foam stability was determined by visual observation of time-dependent changes in foams of different propellant composition (475). Compositions containing both a liquid crystal phase and a liquid phase gave rise to foams with pronounced stability compared with those where only a liquid phase was present.

A multistage liquid impinger was used to determine the particle-size distribution of aerosols generated by several pressurized inhalers containing isoproterenol (isoprenaline) in solution and as a suspension (476). Although suspension-type inhalers delivered aerosols containing much less coarse materials than solution-type inhalers, the use of finely ground powders was not judged sufficient in itself to ensure the absence of coarse material. For evaluation of oral in-

halation aerosols, Karig (477) designed a model lung chamber which was a compartmentalized unit based on certain parameters of the human respiratory tract. A vacuum system was used to regulate the flow rate through the chamber, and it was found to be a suitable device for evaluating medicinal and pharmaceutical aerosol units.

Timed Release—A simple method for the production of drug-impregnated silicone rubber implants for sustained-release drug administration was described (478). The method involved incorporating the drug in unpolymerized silicone rubber, adding a catalyst, and casting the drug–silicone rubber mixture in a hemicylindrical shape in a methacrylate mold. Silicone membranes containing various concentrations of testosterone were prepared by mechanical mixing (479). The amount of testosterone ranged from 5 to 20% by weight, and a definite increase in permeability was observed with an increasing amount of the drug. Low density polyethylene, homogeneously loaded with 2% progesterone and 12% barium sulfate, was tested as a matrix from which to fabricate biochemically active intrauterine devices (480). Progesterone release rates were initially high but reached a constant value of 14 μg/day/cm^2 after the 6th day; the release patterns *in utero* were closely simulated by those in a continuous-flow system and in the rat peritoneal cavity.

The release of pyrimethamine from epoxy resin and silicone disks was compared (481). After 2 months, 32.5% of the totally incorporated drug was released from the silicone disk while the epoxy disk released 16.7%. An investigation of the diffusion rate of chloroquine diphosphate from silicone rubber disks was reported (482). All samples showed high release rates during the first few days, followed by much slower and more or less constant drug release over a period of months. Long-term release studies of antimalarials from polymers and copolymers of lactic and glycolic acids were carried out to evaluate their potential as sustained-release implants (483). In general, the release rate of the drugs was shown to be more strongly dependent on chemical loading of the polymer–chemical matrix than on the type of polymer used in these tests.

A solid dosage form with constant release was developed based on the concept of a constant driving force, a constant diffusion path, and a constant surface for mass transport during drug release (484). Constant release tablets were manufactured by compressing an insoluble, nondisintegrating, and noneroding porous coat onto a soluble substance like potassium chloride, sodium salicylate, or sodium pentobarbital. Boylan and Banker (485) described a physicochemical approach to the preparation of drug-containing matrix systems in which a soluble anionic drug was entrapped, on a molecular scale, in coagulated polymer emulsion systems. The resulting dry product was designed to provide controlled, prolonged release. An investigation was described for the development of a process utilizing suspension polymerization for the production of prolonged-release medicated dosage forms (486). The effect of production variables upon dissolution of acetaminophen from these dosage forms was reported. The relationship between the release rate of cyclazocine from composites of polylactic acid and the molecular weight of the polymer and the form of the composite, as a film sealed in an envelope or as discrete small particles, was investigated (487). Phenobarbital microcapsules and floccules showed that sustained release of the drug, as exhibited by different dissolution rates, ranged from 1.5 to 24 times that of the powder (488). The most pronounced sustained release was demonstrated by floccules where cellulose triacetate was the coating material.

Formulations and procedures were described for the coating of tablets with polymethacrylate films to achieve a time-controlled release of active substances in selected areas of the digestive tract (489). L-Asparaginase was immobilized in semipermeable microcapsules which retained the enzyme and allowed asparagine to diffuse in (490). After intraperitoneal injection in mice, the encapsulated enzyme lowered plasma asparagine levels to zero and maintained that level for 7 days. Due to its biodegradable and noninflammatory nature, collagen was suggested as the ideal material for prolonging drug activity in the eye (491). Estradiol, progesterone, and methyltestosterone were incorporated into 8–10-mm blocks of 10% polyacrylamide (cyanogum-41) gel and implanted in rats (492). The effect of introduced hormones was

Table XVI—Additional References on Timed Release

Reference	Topic
493	Development of sustained-release antispasmodic tablet
494	Development of slow release in potassium chloride tablets
495	Acrylic coatings in controlled-release tablet manufacture
496	Review of biopharmaceutical aspects of sustained-release drugs
497	Methods for producing prolonged effects of drugs
498	Review of oral sustained-release dosage forms
499	Higher fatty acids and their glycerol esters in orally administered medicines with prolonged action
500	Bioavailability of potassium from slow-release tablet
501	Production of long-acting tablets using sugarcane wax
502	Lithium absorption from ordinary and sustained-released tablets
503	Prolonged-release hydrocortisone therapy
504	Polymer coatings for retardation of drug release
505	Effects of formaldehyde vapor and solution on drug release from hardened medicated pellets
506	Sustained-release formulations of tolbutamide
507	Use of ethylcellulose as excipient in slow-release drug preparations
508	Comparison of bioavailability of aminophylline in conventional base and continuous-release base
509	*In vitro* and *in vivo* diffusion of antitumor agent from silicone rubber capsules
510	Coating of pharmaceuticals by phase separation of cellulose derivatives
511	Microencapsulation and flocculation techniques in pharmaceutical formulations
512	Effect of palmitic and lauric acids and their glycerol esters on delayed drug release

noted 3 days after implantation and lasted for at least 33 days.

Additional references pertaining to timed release may be found in Table XVI.

Cosmetics—Microbiological contamination of cosmetic products and its control continue to be subjects of interest to the cosmetic industry. To facilitate a literature search relevant to this critical problem, the subject has been divided into the following subclassifications: microbiological contamination of cosmetics and aspects of formulation and technology of cosmetics.

Microbiological Contamination of Cosmetics—The primary sources of contamination present in cosmetic preparations were found to be makeup water and raw materials (513). Microbial organisms from a city water supply can multiply rapidly in a deionizing column and seed an entire distribution system. Techniques for reducing microbial levels in deionized water systems such as formalin decontamination, recirculation, and point-of-use filtration and the principles of clean system design were discussed. In addition to good manufacturing practices, adequate preservation is necessary together with tests to ensure the adequacies of the preservative used (514). Testing required for quality control must be accurate, simple to perform, and rapid. Goldman (515) described how the aerobic plate count technique fulfills these criteria. The major variables associated with control of microbial growth and antimicrobial action in cosmetics were considered (516). The advantages and disadvantages of approximately a dozen preservatives generally accepted for use in cosmetic preparations were reviewed. Physicochemical techniques allowing quantitative assessment of interactions between preservatives and other formulation ingredients were described (517). A survey of microbiological methods for indicating the practical significance of such interactions was included.

The microbiological quality of a variety of used and unused cosmetics and cosmetic applicators was investigated (518). The incidence of contamination was significantly higher for used cosmetics than for unused cosmetics. Likewise, 100% of the used cosmetic applicators were heavily contaminated, whereas only 27.5% of the unused applicators contained bacteria. A preservative evaluation procedure was described which involved a 13-week incubation using staphylococci and Gram-negative bacteria, fungi, and several microorganisms specific to the manufacturing area (519). Systems difficult to preserve were water-in-oil emulsions containing pigments and high concentrations of nutrient oils and proteins. Stable emulsions containing polysorbates and sorbitan esters increased the effectiveness of water-insoluble antibacterial materials, including essential oils such as thyme (520). Six commercially available ointment products were studied for sterility before and after use in a hospital patient care area (521). Of the partially used tubes, 93% were contaminated whereas 11% of the tubes were contaminated prior to use. A list of preservatives was presented which included quaternary and phenolic compounds, salicylanilides, acids, esters, salts, alcohols, and several miscellaneous compounds; suggested analytical methods were also discussed (522).

Table XVII—Additional References on Microbiological Contamination of Cosmetics

Reference	Topic
523	Microbial content of cosmetics and nonsterile drugs
524	Microbial contamination of cosmetics and toiletries
525	Antimicrobial properties of bay oil and other phenolic essential oils
526	Microbial profile of used eye cosmetics by examination of both applicator and product
527	Microbiological profile of eye cosmetics by examination of product only
528	Triclosan (Irgasan DP 300) activity as preservative in cosmetics
529	Microbiological and physical factors relative to preservatives
530	Discussion of preservation of cosmetic products
531	Antimicrobial contact sensitization in humans

Table XVII lists additional references on microbiological contamination of cosmetics.

Aspects of Formulation and Technology of Cosmetics—The objective evaluation of cosmetic properties on skin can be difficult because of the sensitivity of skin to many variables (532). New techniques were devised to measure the physical components of smoothness and, by using these, a psychophysical equation for skin smoothness was established. Fatty products applied as lipstick base components showed different rheological properties depending on their gel structure (533). Addition of metallic salts increased the viscosity value and thixotropic index. A description of techniques, developed to show that protein treatment of hair fibers results both in deposition on outer surfaces and penetration of the cuticle, was presented (534). While water is recognized as an effective plasticizer of dry skin, cosmetic moisturizers help retain inherent moisture in the skin (535). Thus, the oil bath was shown to be an effective lubrication method for generalized skin dryness.

Toxicological evaluation of cosmetic preparations was discussed in terms of toxicity, irritancy, allergic sensitivity, photosensitivity, and carcinogenicity (536). In each case, techniques and methods of evaluation were presented. The effectiveness of suntan lotions and related skin protective cosmetics was tested by the Jadasson patch test (537). Since many of these lotions are used in athletics and it is possible that some lotion moves with the sweat into the eye, the effect of the lotion on the conjunctival sac had to be investigated. Hematoxylin–eosin-stained paraffin sections of the upper eyelids of rabbits were examined by light microscopy for residual pigmentation and cellular reaction in rabbits killed 1, 7, and 21 days following injection of cosmetic colors into the eyelids (538). Although none of the materials tested was an irritant, it was concluded that a more sensitive test is needed to identify the potential of a color to cause conjunctival pigmentation.

Surfactants present in cosmetic formulations can interact with preservatives, and these interactions

Table XVIII—Additional References on Aspects of Formulation and Technology of Cosmetics

Reference	Topic
548	Evaluation of chemical binders and their effect on pressed powders
549	Use of natural products in cosmetic and toiletry preparations
550	Effect of cosmetic oils in aerosol antiperspirant formulations
551	After-bath powders
552	Bath salts
553	Evaluation of foaming power of bubble bath preparations
554	Review of after-bath emollients
555	Types of bath oils
556	Liquid bubble bath preparations
557	Dry bubble bath formulations
558	Perfuming of bubble bath preparations
559	Monomolecular film bath oils
560	Preparation and stabilization of bath satins
561	Review of bath and body lotions
562	General principles of deodorant soaps
563	Properties of polyacrylamides in cosmetic preparations
564	Chemical aspects of jojoba oil and its application to cosmetic preparations
565	Use of egg oil in cosmetic products
566	Uses of sucrose esters in cosmetic formulations
567	Physical properties of hydrophilic cosmetic oils
568	Deodorant and antiperspirant cosmetic preparations
569	Skin irritancy testing for perfuming agents in cosmetics
570	Use of collagen and gelatin in cosmetic preparations
571	Cosmetic application of vitamins
572	Physical and chemical properties of cosmetics with isostearics
573	Review of cosmetic chemistry advances during 1972
574	Review of fatty materials in cosmetic formulations
575	New allantoin derivatives for cosmetic and dermatological applications
576	Review of gums in drugs, cosmetics, and foods (Part I)
577	Review of gums in drugs, cosmetics, and foods (Part II)
578	Review of composition of hypoallergenic cosmetics
579	Application of extract of *Lithospermi radix* in cosmetics
580	Review of cosmetic oils and fats and their derivatives
581	Galenical and cosmetic aspects of lipstick
582	Physical and chemical characteristics of synthetic triglycerides for pharmaceutical and cosmetic industries
583	Preparation and characteristics of biologically active principles for cosmetic use
584	Use of specialty starch products in treating oily hair
585	Industrial production of cosmetic emulsions in Germany
586	Defatting and fat regeneration of scalp and hair after shampooing with various surfactants
587	Review of hair-coloring agents
588	Review of basic requirements for shampoo preparations
589	Possibilities for improving quality of hair sprays
590	Preparation of cream shampoos from domestic raw materials
591	Review of effects of surfactants on hair (Part I)
592	Review of effects of surfactants on hair (Part II)
593	Review of commonly used perfuming agents for shampoos
594	Survey of raw materials and formulations of shampoos
595	Review of hair-setting lotions and hair sprays
596	New types of hair-setting sprays having semipermanent properties
597	Effects of shampoo on hair, skin, and hair follicles
598	Influence of hormones on hair growth

can reduce or, in some cases, enhance the preservative efficiency. Coates (539) presented guidelines to aid the formulator in choosing preservative and surfactant materials. In the same vein, interactions between preservatives and thickening agents must be considered, and a listing of known reactions was surveyed (540). Crystallization of cetyl alcohol from cosmetic emulsions removed it from the phase interface and caused the emulsion to break (541). This crystallization could be reduced by controlling the concentration of the emulsifier in the formulation. Current theories of autoxidation and antioxidants were reviewed, with special reference to cosmetics and raw materials used in the manufacture of cosmetics (542). The relationship between dispersion of inorganic pigments in water in the presence of surface-active agents and protection of the dispersed pigments from gel formation was discussed (543). A new color-control scale, giving good results in the routine control of high quality cosmetics, was discussed (544).

A major breakthrough in the formulation and processing of soap, which led to a new type of bar soap, was reported (545). Comparisons of its properties with those of opaque and transparent soaps were presented. The active ingredients of hair-setting formulations were discussed and their curl-retention properties were compared (546). The importance of spreading action in hair sprays was discussed from the points of view of the wettability of hair fibers by hair spray solutions and the rate of spreading of such solutions in bundles of hair fibers (547). The rate of spreading was shown to depend mainly on the viscosity of the solution, the rate of evaporation of the solvent, and the rate of increase in viscosity due to evaporation.

Other papers related to aspects of formulation and technology of cosmetics are listed in Table XVIII.

Packaging—A brief review of the historical, chemical, and practical aspects of pharmaceutically useful plastic materials was presented (599). Stability parameters such as temperature and vapor transmission, as related to drug packaging, were discussed (600). The predominant types of sorption found in reactions between dissolved medicinals and plastics were adsorption and absorption (601). General relationships and effects of such factors as plastic material, solvent base, active substance, and various external parameters were detailed. Sorption of leucinocaine, butacaine, tetracaine, oxybucaine, and tetracaine hydrochloride (Farmocaine) in aqueous solutions by polypropylene, polystyrene, polyvinyl chloride, and polycarbonate did not affect stability (602). On the other hand, polyamides and polyethylene

Table XIX—Additional References on Packaging

Reference	Topic
610	Stability of injection solutions in polyethylene containers
611	Normalization of plastics with regard to sorption of drugs from aqueous solutions
612	Protection of pharmaceuticals in solutions against light-induced decomposition by use of amber glass
613	Light transmission of pharmaceutical containers
614	Design of child-resistant closure for glass bottles
615	Review of available child-safe closures
616	Use of blister and strip packaging in child-safety packaging
617	Ethylene oxide gas sterilization of packaging materials
618	Review of progress in industrial pharmaceutical packaging technology
619	Review of modern trends in pharmaceutical packaging
620	Description of new bicompartmental pack
621	Description of legal standards and measuring technique directions for the pharmaceutical industry
622	Packaging and delivery of nitroglycerin tablets with regard to loss of strength

caused a decrease in concentration due to sorption. The influence of two preservatives, benzalkonium chloride and thimerosal, on the adsorption of phenylephrine hydrochloride by polyethylene was investigated (603). The data indicated apparent binding of phenylephrine hydrochloride to low density polyethylene by means of a preservative agent, and their interaction was most significant at room temperature, with elevated temperature disrupting the apparent weak binding. It was reported that solutions of sorbic acid stored in polypropylene, polyvinyl chloride, polyethylene, and glass containers showed significant loss except when refrigerated or when an antioxidant was present (604). A readily consultable table of preservative losses in widely used plastic containers was presented (605).

The effect of temperature, pressure, and velocity of heat sealing on the mechanical strength of a polyethylene seam was studied (606). The strength depended on pressure and was independent of temperature and velocity of sealing. A rationale used to predict which esters can safely be included in anhydrous cosmetic formulations placed in polystyrene containers was illustrated (607). Under consideration were anhydrous products existing as pastes, creams, and molded units at ambient temperatures. The advantages of the unit dose system suggested its application to the packaging of biological products (608). Selection of the particular unit dose system was dependent on the stability and compatibility of the biological with the packaging component. It was reported that roll-feed systems are reaching higher and higher speeds and can offer pharmaceutical manufacturers a high level of product security by instrumental means (609).

Other papers in the area of packaging can be found in Table XIX.

Equipment—Descriptions of the evaluation of various types of automatic ampul and vial inspection devices were presented. Included were the evaluation of the Autoskan equipment (623), the Rota machine (624), and the Strunck machine (625). Washing of ampuls was facilitated and improved by preliminary exposure of the ampuls to ultrasonic waves (626). A modified ampul-washing machine which incorporated an ultrasound generator was described. The determination of particle size in dispersed systems with the Coulter counter, using data processing, was described (627). The quantitative determination of large particles could also be calculated. A review of the theory, techniques, and equipment employed in the laboratory and in the industrial plant for lyophilization of pharmaceuticals was presented (628). Methods of freezing and sublimation, including ideal temperatures for this process, were discussed. A new apparatus, used on an industrial scale for freezing vials to be lyophilized, resulting in a dried mass in the shape of an internal hollow paraboloid with very thin layers, was described (629). Water purification by filtration, distillation, and ion exchange was compared with reverse osmosis (630). The ability of reverse osmosis to produce sterile, pyrogen-free dialysate, in addition to rejecting 95% of all salts and organic compounds with a molecular weight of 200 or more, presented advantages to manufacturers and hospitals.

A model lung chamber was designed for the evaluation of oral inhalation aerosols (631). The chamber was a compartmentalized unit based on certain parameters of the human respiratory tract and a vacuum system was used to regulate the flow rate through the chamber. Solutions of varying strengths of isoproterenol hydrochloride and phenylephrine hydrochloride, aerosolized using several common aerosol devices, demonstrated the model lung chamber to be a suitable device for evaluating medicinal and

Table XX—Additional References on Equipment

Reference	Topic
635	Description of equipment for eliminating introduction of air during mixing operations
636	Description of weighing control procedures in automatic cosmetic manufacturing systems
637	Description of in-line continuous mixing and processing operation for cosmetic products
638	Use of foam heat-exchange apparatus in ampul production
639	Evaluation of Marzocchi ampul-filling machine
640	Description of improved machine design for stopper washing
641	Use of compulsion gauges in automatic selection of tablets
642	Review of available methods for automatic control of tablet weight
643	Instrumentation for evaluation of dustiness of tableting materials
644	Review of various capsule-filling machines
645	Pilot size equipment available for pharmaceutical plants
646	Use of rotor-pulsation apparatus for preparing disperse drugs
647	Description of apparatus based on pulsating air currents for drying granulates of pharmaceutical preparations
648	Description of new powder proportioner for pharmacy use
649	Particle-size measurements using πMC computer for particle counting and measurement

pharmaceutical aerosol units. A Zanasi LZ/64 capsule-filling machine was instrumented with strain gauges and fitted to a modified dosator piston which had been calibrated with a loaded beam (632). Three main regions could be distinguished on the oscillograph: a force representing compression of powder into the dosator, a retention force carryover of the plug to the capsule body, and an ejection force as the plug is ejected into the capsule. Goodhart *et al.* (633) presented a study to determine the relative merits of various tablet hardness testers now being used. From the results obtained, it was apparent that there are distinct advantages to using a hardness tester with a mechanical drive rather than a pneumatic type because more uniform force application rates may be achieved and there is less maintenance work and less need for calibration checks. A study was conducted to design and build a laboratory extruder and to evaluate various factors relating to its use for the preparation of both wet and hot fusion granulations (634). Data were reported on torque, powder throughput, liquid required, and attainment of uniform consistency.

Additional references on equipment can be found in Table XX.

PHYSICAL PHARMACY

Two crystalline modifications of meprobamate were prepared (650) and each crystalline modification was identified by melting point, X-ray diffraction pattern, and IR spectra. In contact with water, the metastable phase underwent reversion to the stable phase. Different thermodynamic properties, *e.g.*, heats of fusion, heats of solution, enthalpy, entropy, and free energy difference and transition temperature, were also determined. Two polymorphic forms of tolbutamide were identified by IR spectroscopy, X-ray diffraction, and differential thermal analysis. Both crystalline modifications had similar dissolution rates in distilled water and simulated intestinal fluid; when given to fasting beagles, there was no difference in their absorption rates (651).

Of seven different sulfonamides, sulfamethoxypyridazine, sulfacetamide, sulfathiazole, and sulfaguanidine were identified as existing in different polymorphic forms (652). In work with labile triazinoindoles, two aqueous topical suspensions in fine particles were prepared (653). Upon aging, both suspensions produced crystal growth, even when protective colloid methylcellulose was added. These crystals were found to be monohydrates of the respective triazinoindoles and they readily lost water of hydration under mild heat. Both hydrates could be air milled with no water loss. One hydrate was formulated in the suspension form and found to be free of crystal growth for 2 years.

The dehydration kinetics of theophylline monohydrate transformation directly to a crystalline anhydrous form, with apparent zero-order kinetics, were studied as well as the dehydration kinetics of ampicillin trihydrate transformation to an amorphous state (654). Micronized ampicillin trihydrate, which is commercially available, contained small amounts of excipients and, as a result, exhibited a different kinetic order and a faster rate of transformation. The thermal dehydration of cortisone acetate was studied using IR spectroscopy (655); the unstable monohydrate phase changed to anhydrous Forms B, C, and D. The kinetics of interconversion of sulfameter (sulfamethoxydiazine) crystalline phases and the effect of various additives on the rate of transformation of the more energetic sulfameter phase to the water-stable phase in the aqueous suspensions were studied using representative structurally related compounds, viscosity-imparting agents, surfactants, and coloring agents to inhibit this transformation (656, 657). Significant transformation-retarding effects were observed in most cases. The effects varied from slight retardation to almost complete inhibition of the transformation for more than 1 year (*e.g.*, using 1% polyvinylpyrrolidone).

The anomalous behavior of oxyclozanide polymorphs was studied, and three polymorphs were identified using X-ray powder diffraction (658). The different polymorphic phases exhibited variable behavior both toward aqueous solubility and stability in suspension in terms of phase transformation and crystal growth. The polymorphism of aspirin was studied (659), and the two polymorphic phases previously recommended were prepared. X-ray diffraction analysis showed both forms to be monoclinic and could not identify any polymorphic differences. The dissolution rates of Forms I and II were within tolerance and depended upon the different crystal surface. This effect was also responsible for the different heats of solution. The electronic scan microscope clearly showed the different surfaces of Forms I and II. The saturation solubility and the dissolution rate of sulfathiourea were determined (660). Only two of the varieties on the market were suitable for solubility investigations. The two crystalline phases were enantiotropic and possessed identical solubilities at 24°, but the variety with the lower melting point was 14% more soluble at 50° than the variety with the higher melting point.

By using differential scanning colorimetry, the properties of two metastable phases of chloramphenicol palmitate, the δ-form and the sub-α-form, and the occurrence of the sub-$\dot{\alpha}$-form during the manufacture of chloramphenicol palmitate oral suspension were clarified (661). The pharmaceutical application of inclusion compounds was reviewed (662) in which the actions of urea, thiourea, deoxycholic acid, cyclodextrin, and perhydrotriphenylene were covered. In a study of the formation of a mesomorphic (liquid crystalline) system by highly concentrated solutions of dioctyl sodium sulfosuccinate in an homologous series of five even-numbered chain length *n*-aliphatic hydrocarbon solvents, dioctyl sodium sulfosuccinate concentration, solvent chain length, and temperature significantly affected the formation of the mesomorphic phase (663). At constant temperature, the concentration of dioctyl sodium sulfosuccinate required to produce a mesomorphic phase increased as the solvent chain length increased from octane to dodecane and decreased to hexadecane.

The mutual diffusion coefficient, D_v, for the binary

solution of sucrose in water at 23 points over the concentration range of 9–300 kg/m^3 at 298°K was measured (664). Experiments with the concentrated solutions showed that for a given concentration the areas of the fringe deviation graphs varied with the initial concentration difference, $\Delta\rho$; but since D_v did not vary with $(\Delta\rho)^2$, the diffusion coefficient was not "concentration dependent." The capillary method was used to determine the diffusive transport properties of radioactive salicylate ion in the presence of a commonly used polyelectrolyte, sodium carboxymethylcellulose (665). In a solution of this polymer, the self-diffusion coefficient of salicylate decreased only moderately, even though the bulk macroscopic viscosity increased to about 2–3 orders of magnitude. These data were evaluated with two theories from the literature. The tracer drug release rate out of the polymer solutions was also studied and was more rapid than if the polymer were not present. Increases of up to 40% were observed. The equilibrium dialysis of salicylic acid from solutions of polysorbate 20 and 80 was studied, simulating the system using an analog computer (666). Rate constants for the partition of salicylic acid out of polysorbate micelles into water were obtained by fitting computer-generated curves to experimentally determined data. The release of salicylic acid from polysorbate micelles was independent of surfactant concentration but dependent on the micelle–aqueous partition ratio of salicylic acid and its concentration in the donor and recipient cells.

In a series of experiments, the diffusion rate of the encapsulated drug was a function of microcapsule size (667). The influence of the coating upon diffusion and the determination of the thickness of the different coatings were also included. An equation was developed and was verified by microscopic measurements of microspheres previously sliced with a microtome. Four microencapsulation and three flocculation techniques were investigated using phenobarbital as the model drug; gelatin, sodium alginate, and sodium carboxymethylcellulose as water-soluble coating materials; and ethylcellulose and cellulose triacetate as water-insoluble coating materials (668). The amount of phenobarbital bound to the product was determined quantitatively, and the coating–core ratio of each product was calculated. Microcapsules of phenacetin were prepared by coacervation of aqueous cellulose acetate phthalate solutions (669). Appropriate solutions were made by dissolving cellulose acetate phthalate in an equivalent concentration of disodium hydrogen phosphate. A triangular phase diagram of a coacervation system was elaborated by using sodium sulfate as the coacervating agent, and the coacervation and encapsulation conditions were optimized. The amount of drug encapsulated had no significant effect on particle-size distribution of the capsules; however, it did influence the release rates of the drug, indicating that the drug diffusion through the shells was the controlling step.

By modifying gelatin, which is a proven packaging material because its protection to small particles permits controlled release, it was possible to relate the type of capsule wall material to the chemistry of the phase to be encapsulated (670). The consumption of glutaraldehyde by various acid- and alkali-precursor gelatin–gum arabic coacervate gels over a range of conditions was studied (671), and glutaraldehyde consumption by several gelatin gels was determined. All gels consumed 0.3–1.6 mmoles glutaraldehyde/g gelatin. Acid and alkali gelatin–gum arabic gels had similar glutaraldehyde uptakes at 4°. Glutaraldehyde consumption by acid-precursor gelatin–gum arabic gels increased significantly with increasing gelation temperature (4–28°) due to the temperature-dependent changes in the gel structure. Microencapsulation of various specific pharmaceuticals was reviewed by several authors, with special emphasis on coacervation (672–674).

In the microbiological determination of drug partitioning, the functional dependence of the partition coefficient on the drug concentration and also partition coefficient profiles of several antimicrobial drugs were developed (675, 676). The usefulness of this method was demonstrated by comparing the partition coefficients of the same antimicrobial drugs obtained with traditional chemical analysis. By using literature data, the contribution of hydroxyl and carboxyl groups to solute activity and partition coefficients was investigated (677). Both polar functions

Table XXI—Additional References on Physical Pharmacy

Reference	Topic
678	Physical constants and purity profiles of 115 drug substances
679	Physicochemical properties of prostaglandin $F_{2\alpha}$
680	Physicochemical properties of chloral hydrate
681	Physicochemical properties of clidinium bromide
682	Physicochemical properties of dexamethasone
683	Physicochemical properties of dioctyl sodium sulfosuccinate
684	Physicochemical properties of 5-fluorouracil
685	Physicochemical properties of isopropamide
686	Physicochemical properties of levallorphan tartrate
687	Physicochemical properties of sulfamethoxazole
688	Diffuse reflectance, its quantitative application to acetaminophen (*N*-acetyl-*p*-aminophenol) excipient-induced degradation
689	Current aspects of pharmaceutics
690	Review of lipophilic character and biological activity of drugs (the parabolic case)
691	Review of water-soluble cellulose derivatives
692	Surface diffusion in monomolecular films
693	Kinetics of degradation of crystalline networks of aspirin and aminopyrine (aminophenazone) associated in a solid phase
694	Differential thermal analysis and X-ray diffraction studies of griseofulvin–succinic acid solid dispersions
695	Physical properties of cholesterol and cholesteryl esters
696	Coacervate formation by sodium salicylate with benzalkonium chloride
697	Effect of hydroxy group on coacervate formation by sodium hydroxybenzoates with benzalkonium chloride
698	Distribution and selectivity coefficients at optimum pH values of buffers
699	Misconceptions and thermodynamic untenability of deviations from pH–partition hypothesis

gave rise to reduced activity coefficients for aromatic solutes in water, and differences existed between ring and α-substitution. Partition coefficients were similarly affected, but the magnitude of the group contribution to the free energy of transfer was dependent on the nature of the solute and the organic partition solvent.

Additional references relating to physical pharmacy are listed in Table XXI.

Dissolution—The dissolution kinetics of gallstones based on four different models were developed (700). One model was based upon simple diffusion-controlled dissolution and another upon a leaching process. The remaining two models related to interfacial coat-type barriers. Calculations were carried out employing models with reasonable input parameters. The theoretical dissolution rate based on a simple dissolution model would predict that a 2.5-mm stone should dissolve in several days into an undersaturated bile solution. Both clinical and *in vitro* experiments showed that much longer times were needed. Experiments were designed for investigating and comparing the *in vitro* dissolution kinetics of human cholesterol gallstones and cholesterol monohydrate compressed pellets (701), and the rates of gallstone dissolution compared well with the dissolution rate obtained with the cholesterol monohydrate pellets in the solvents investigated. The dissolution rates for both stones and pellets in organic aqueous solvents were extremely rapid and were diffusion-controlled processes. In a sodium cholate solution, the dissolution rates were two to three times slower than rates predicted by diffusion theory; the data generated suggested a modest interfacial resistance to dissolution. The rates obtained in 2% bile acid–1% lecithin solutions were about 17 times slower than diffusion-controlled processes. These results point to an interfacial barrier to dissolution which may be very important clinically.

By using dextrose, galactose, and sucrose, glass dispersions were developed with corticosteroids, and by using mannitol, partial solid solutions were prepared (702). The results, using the NF XIII dissolution method, revealed a marked increase in the rate of dissolution of the corticosteroids contained in a solid dispersion when compared to the dissolution rate of the plain corticosteroid powder. The increase in the dissolution rates was attributed to: *(a)* the presence of the corticosteroid in a very fine state of subdivision, *(b)* the increased wettability of the corticosteroid powder, and *(c)* the molecular dispersion of the drug in the partial solid solution. In work on the dissolution behavior of solid drugs, a method was developed for determining the transition temperature between two polymorphs and between the hydrate and anhydrous phases of drugs by measuring the initial dissolution rates (703, 704). An equation was derived describing the dissolution of monodisperse particles beyond the point where concentrations are small compared to solubility. If it was assumed that a stagnant layer model applied, the thickness of these layers was of the same order of magnitude as calculated *via* the Hixson–Crowell treatment but dissolution rate constants were 1.5–2 times as large. The application of the equation to the dissolution of hydrocortisone, levodopa, and *p*-hydroxybenzoic acid was shown (705). The equilibrium solubility and dissolution rate of sulfamethazine were remarkably inhibited in the presence of benzoic acid (706). The effect was dependent on the concentration of both benzoic acid and sulfamethazine in the system. The inhibition of sulfamethazine dissolution was synchronous with the adsorption of the benzoic acid on the sulfonamide particles. As low as 5 mg % polyvinylpyrrolidone prevented the adsorption of the benzoic acid on sulfamethazine and, consequently, the suppressive effect of benzoic acid on sulfamethazine dissolution was no longer shown.

A eutectic mixture of phenobarbital and urea was prepared and its dissolution and absorption were compared with those of pure phenobarbital (707). The drug in eutectic form had faster dissolution and absorption rates than in pure material. The factors involved in the growth and dissolution of crystals were reviewed, with special emphasis on the pharmaceutical and biological implications of these phenomena (708).

Correlation of the dissolution rate of digoxin tablets and their bioavailability was discussed by several investigators who found that there were wide dissolution rate differences between lots from different companies and between different lots from the same company (709–712). It was recommended that a minimal acceptable level of dissolution rate be established for commercial digoxin tablets. Fresh samples of 11 brands of prednisone tablets were tested for dissolution time, hardness, and disintegration time (713). Four samples failed to comply with the USP XVIII monograph requirement for prednisone tablets and a fifth sample was very doubtful. There was no correlation between tablet hardness, dissolution rate, and disintegration. The dissolution method gave consistent results with deaerated water only. Rank-order correlations were demonstrated among dissolution, disintegration, and several measures of bioavailability for tablets of aminosalicylic acid and its salts (714). The correlation between the disintegration time and the percent of the dose excreted in the urine was examined quantitatively using both linear and quadratic models. The results supported the use of either the disintegration or the dissolution test to control the availability of this drug and suggested control limits for these tests.

With the USP dissolution apparatus, the times required for 60% release of the active ingredient in two clinically different brands of chlorpromazine hydrochloride tablets were 5.7 and 35.4 min (715). The data obtained for these two products indicated that, if the apparatus was carefully standardized, the USP method yielded reproducible results. However, mesh size of basket, depth of the basket-stirrer assembly in the dissolution container, and stirrer speed alter the dissolution profiles. The effect of 5 years of storage on dissolution of two different sodium salicylate tablets was investigated (716). The first tablet was manufactured with gelatin and the second one with polyethylene glycol 4000 as binders. The effect of storage was most noticeable in the dissolution of tab-

Table XXII—Additional References on Dissolution

Reference	Topic	Reference	Topic
729	Classification of dissolution tests on basis of their hydrodynamics	742	Evaluation of effective surface areas of micronized powders from dissolution rate measurements
730	Disintegration and dissolution tests for dextran sulfate sodium tablets	743	Practical significance of dissolution rate determinations
731	Dissolution–dialysis method of assessing *in vitro* drug availability of prednisolone tablets	744	Effects of various hydrodynamic conditions on dissolution rate determinations
732	Dissolution and solubility of triamcinolone acetonide	745	Experimental verification of solid–liquid interfacial concentration developed during interfacially controlled dissolution of a solid
733	Dissolution rates of cholesterol monohydrate crystals and human cholesterol gallstones in bile acid–lecithin solutions, and the enhancing effect of added alkyl quaternary ammonium salts	746	Review of dissolution rates of slightly water-soluble drugs
		747	Review of physical and chemical factors affecting dissolution rate of drugs
734	Effect of formulation on dissolution of sodium warfarin tablets	748	Dissolution properties of poly(alkyl vinyl ether–maleic anhydrides) and partial ester derivatives
735	Dissolution studies on sulfameter (sulfamethoxydiazine) formulations	749	Influence of micelle–drug solubilization on dissolution rates
736	*In vitro* determination of dissolution rates of oxytetracycline tablets and interlaboratory comparison of USP XVIII method, beaker method with propeller, and beaker method with magnetic stirring	750	Dissolution rate as parameter in structure–activity studies
		751	Dissolution tests and interpretation of anomalies observed in dissolution process of sulfoquinoxaline based on salt formation
737	*In vitro* determination of dissolution rates of tablets, and interlaboratory comparison of USP XVIII method with two other methods using 15 batches of tablets of eight different brands	752	*In vitro* dissolution of solid pharmaceuticals for oral administration
		753	Dissolution profile of log-normal powders, exact expression
738	Compendial dissolution characteristics of commercial formulations	754	Importance of considering variables when using magnetic basket dissolution apparatus
739	Effect of solvent flow on dissolution rate of nondisintegrating potassium chloride disk	755	Prediction of dissolution rates of slightly water-soluble drugs questioned
740	Effect of dissolution medium and moisture content of powder on dissolution of chloramphenicol capsules	756	Comparative analysis of solution rate of active ingredients from tablets, using USP and Becherglas methods
741	Improvement and simplification of dissolution rate measurement and its application to solubility determinations	757	Preservative release from creams and emulsions, dissolution method

lets containing gelatin. This result may be due to changes in tablet structure during storage because the content of sodium salicylate in the tablet was not changed. The storage had the least effect on tablets containing polyethylene glycol 4000.

The *in vitro* release rates of potassium ions from slow-release potassium chloride tablets from 12 sources were investigated (717). Dissolution rates were determined using a modified nonsink method and a modified sink method. Medicament release in both methods was continuously monitored using a potassium-ion specific electrode. The release rate constants were derived and the applicability of the Noyes–Whitney equation, the Hixson–Crowell cube root law, and Higuchi's equation was studied. The most satisfactory products appeared to be those prepared from a fat-wax potassium chloride matrix, an insoluble wax coat on a nondisintegrating wax core, and a combination of potassium chloride, cellulose acetate phthalate, ethylcellulose, and polyethylene glycol.

Haringer *et al.* (718) compared the dissolution of tablets using the official USP–NF rotating basket, the bent basket, and the newly designed L-shaped Teflon holder–stirrer. In these studies, the latter two devices were superior to the official device. A rotating filter–stationary basket *in vitro* dissolution test apparatus was designed and evaluated (719). The major advantages of this apparatus were suggested to be: *(a)* precision-controlled variable intensity of mild laminar liquid agitation; *(b)* continuous or intermittent filtration of representative dissolution fluid samples through a nonclogging filter; *(c)* convenient means for introducing solid samples in a stationary basket and positioning at a set level in the fluid medium; *(d)* minimal mechanical impacts, abrasion, and wear of the solid samples; and *(e)* simultaneous determinations of the disintegration–dissolution rates of tablets and capsules. Dissolution rates of five different lots of an antidiabetic tablet were evaluated by the new apparatus and correlated with *in vivo* activity.

In experiments comparing the accuracy of four dissolution models, using aspirin and prednisone tablets, the modified Levy beaker method was the most accurate at relatively low agitation frequencies as encountered *in vivo* after oral administration (720). Several different formulations of pentobarbituric acid tablets were manufactured and were used to illustrate the ability of the magnetic basket dissolution apparatus to differentiate between the dissolution of tablets with differences in particle size of active ingredients, in formulation, and in hardness (721). An *in vitro* technique for testing the disintegration and dissolution of tablets and capsules was developed (722). The apparatus consisted of a beaker with a cylindrical well in the bottom into which is placed a platform containing the dosage form. Comparison between the official and the new method indicated that the official test does not differentiate

between capsule formulations containing hydrophobic lubricants. The effects of capsule formulation factors such as type and level of lubricant and disintegrant, as well as the presence of surfactants, were also determined. The disintegration and dissolution of tablets containing starch, alginic acid, sodium starch glycolate, and two types of calcium sodium alginate were studied (723). The dissolution times were determined with both the beaker and the basket methods. The results indicated that when tablets contain adhesive substances, such as the alginates, an adhesive gel is formed which tends to block the basket pores. It was recommended that great care should be taken when estimating the dissolution of tablets containing such substances; for these products, there are probably advantages in the use of the beaker-type apparatus.

The rate of solution of potassium chloride and sodium chloride from compressed disks was determined under controlled conditions in water at 25° in solutions of varying concentrations of polyvinylpyrrolidone, polyethylene glycol 6000, and cetomacrogol 1000 (724). As expected, dissolution rate constants decreased with increasing bulk solution viscosity, but no one equation relating the rate constant and bulk viscosity fitted the results for the systems studied. In studies on dissolution rates, the effect of flow rates using a continuous-flow, column-type apparatus was determined (725, 726). The flow method of agitation was compared to three other types used with the static beaker procedure. The advantages of the column-type apparatus in obtaining low intensity of agitation without sacrificing accuracy and homogeneity were discussed. The solvent flow patterns in the column-type apparatus also were determined by using a flow visualization technique. A supporting bed of glass spheres in the dissolution chamber ensured laminar flow, and this method was preferred over the complex, poorly defined flow found with static beaker methods.

The data available on the influence of fluid motion in dissolution rate determinations and the knowledge regarding GI motion were reviewed (727). The use of a continuous flowing-stream apparatus to follow tablet dissolution was studied (728). A dissolution chamber with a commercially available filter unit was designed to follow tablet dissolution through use of either a sodium-ion electrode or a spectrophotometric analytical module. The effect of variation of flow rate on the dissolution profile and the ability of the apparatus to differentiate between the common tablet parameters of hardness and drug potency were also shown.

Table XXII lists additional references on dissolution.

Solubility-Solubilization Phenomena—The solubility of chloramphenicol palmitate in propylene glycol-water systems was determined (758). The solubility was 0.004 mg/ml in pure water and 4.95 mg/ml in pure propylene glycol. Additions of propylene glycol to water did not significantly alter the water solubility of the drug until about 60% (w/w) was added. Then the solubility increased logarithmically in a manner approximately proportional to the propylene glycol concentration of the system. By using available data on the dielectric constants of these solvents, the solubility parameter for chloramphenicol palmitate was calculated to be 11.8. The aqueous solubility of cholesterol, testosterone, progesterone, and diethylstilbestrol at 30° was reported using a radioactive assay procedure (759). The solubility of platyphylline, seneciphylline, and their hydrogen tartrates was determined for 19 solvents at 20° and at the solvent boiling temperatures (760). Mayer and Kata (761) developed a method for determining the solubility of substances in ointment and suppository bases; the drug is added to the base and stirred for 1 hr at 37°, the undissolved portion is separated with a membrane filter, and solubility is then determined.

The solubilization of several pairs of preservatives by the nonionic surfactant cetomacrogol was reported (762). In all cases the addition of a second preservative altered the equilibrium of the first to an extent that depended on a particular cosolute and the concentration added. The tetramethyl-substituted amides of pimelamide, suberamide, and sebacamide markedly enhanced the solubility of glutethimide in a solution (763). Partition studies, surface tension measurements, and light-scattering measurements strongly suggested that the amides are associated at infinitely dilute concentrations and that further aggregation of these associated molecules occurred with the possible formation of micelles at concentrations slightly higher than those observed for surfactants. Solubility of glutethimide was increased significantly above the CMC and, from the nature of the solubility curve, a micellar type of solubilization appeared to be dominant.

Solubilization of khellin by caffeine was reported, where the solubilizing effect was attributed to complex formation (764). The degree of solubilization of heptane, toluene, and heptanol by nonionic-ionic surfactant mixtures (sodium oleate and alfapol) depended on the degree of ethoxylation and the hydrophilic properties of the alkylphenol (765). Investigations of the solubilization behavior of mixed micelles of the nonionic surfactant polyethylene glycol, monododecyl ether, and anionic surfactants, *e.g.*, phenylalkanesulfonates, or alkylbenzenesulfonates toward a water-insoluble dye showed that the position of the benzene ring in the hydrocarbon chain of the anionic surfactant significantly affected the dye solubility (766).

Table XXIII—Additional References on Solubility-Solubilization Phenomena

Reference	Topic
767	HLB and molecular size as factors in increasing mutual solubility of oil and water by solubilizers
768	Solubilization efficiency of surfactants
769	Solubilization properties of nonionic surfactants
770	Content of free surfactant in aqueous phase of the aqueous emulsifying ointment DAB 7 and its solubilizing effect on pharmaceuticals

Other papers in the area of solubility–solubilization phenomena are listed in Table XXIII.

Membrane Permeation and Release—A transport cell was designed for examining *in vitro* solute transfer across biological membranes (771). When using the intestine as a model membrane, a primary advantage of the method was that there was no need to evert the intestine. This eliminated the influence of eversion on the structural and functional integrity of the intestine and thus its possible influence on solute transfer. Preliminary studies using salicylamide were performed to quantitate various parameters of the apparatus. A structure–activity model based on diffusional theories was developed, where the ability of each member of the biologically active homologous series to reach the receptor site was equated with the relative ability to permeate the biological barriers (772). The equations are generally applicable for transport across membranes and can be of use in describing a variety of passive absorption or permeation phenomena.

In the 1–5 pH range, a linear relationship was found between the rate constant for salicylic acid transfer across a cellophane membrane and the fraction of ionized drug present at different pH's (773). The rate constant for ionized molecules was about 60% of that for unionized molecules. The presence of polysorbates 20 and 80 in a drug solution markedly decreased the apparent transfer rate constant of salicylic acid at low pH. The effect of various substances on amobarbital permeation through polydimethylsiloxane membranes was studied (774). The rate of drug transfer across the membrane depended on the pH because only the unionized species were eligible for transfer.

The simultaneous dissolution and permeation of a drug from solid oral dosage forms were analyzed by a treatment based on Fick's second law of diffusion (775). The dissolution rate constants were obtained from the permeation lag time, while the coefficients of permeability were estimated from the rate of drug transfer across the membrane under steady-state conditions. Drug–excipient interactions, if any, would be expected to affect the rate of transfer across the membrane. Therefore, from a single experiment, the dissolution rate and the extent of drug and excipient interaction could be obtained. The permeability of cellophane membranes to the nonionic surfactant cetomacrogol 1000 was investigated using equilibrium dialysis, dynamic dialysis, and an ultrafiltration technique; cellophane and silicone rubber membranes also were compared in an equilibrium dialysis study of the interaction of chlorocresol with cetomacrogol (776). The permeability and interaction studies showed that the cellophane membranes were permeable to the surfactant.

The transport of solute into and through a heterogeneous system involving interfacial barriers was theoretically investigated (777). The system consisted of a "donor" bulk aqueous phase, the aqueous diffusion layer, and a matrix in which oil droplets of uniform size were dispersed. The theoretical method employed accounted quantitatively for the barriers existing at the oil droplet–water interface, and the technique had general applicability to all situations involving a heterogeneous medium in which the local interface equilibrium was achieved slowly.

The effect of six commercial nonionic surfactants on thioridazine absorption in goldfish was compared to the effect of polysorbate 80 (778). The index of absorption rate used was the reciprocal death time when the fish were immersed in the solution under study. Three of the surfactants tested had no effect below their CMC; the remaining three surfactants increased the reciprocal death time with a biphasic response. The factor determining whether the surfactant increases the absorption rate appeared to be the configuration of the surfactant molecule rather than the HLB or surface activity.

The transport of 3-*O*-methyl-D-glucose in liposome dispersions prepared from lecithin–dicetyl phosphate (10:1) and lecithin–dicetyl phosphate–cholesterol (10:1:1) was studied (779). The transport results with 3-*O*-methyl-D-glucose yielded a permeability coefficient 50 times larger than that for D-glucose. The dispersions prepared from lecithin–dicetyl phosphate containing 10% cholesterol yielded a permeability coefficient that was 2.4 times smaller than the dispersions prepared without cholesterol. The analysis of the results indicated that for relatively large permeability coefficients, as obtained in these studies, the dilution–release experiments showed greater sensitivity in the determination of this parameter compared to the direct release experiments. The transport of taurocholic acid-[cholic-^{3}H(G)] was studied in liposome dispersions prepared from lecithin–dicetyl phosphate in buffered glucose solutions at different pH's (780). The pH dependence of the permeability coefficient from the study indicated that the unionized form of taurocholic acid was preferentially transported at low pH and that the taurocholate ion was the main species involved at high pH.

The release of sulfathiazole from different ointment bases was studied (781, 782), and the effect of polysorbate 80 and sorbitan monostearate on the release of sulfathiazole from bases containing either lard or petrolatum was determined. The gelatin plate, chromatography, and dialysis test were adopted and used under identical conditions for evaluations of release of drugs from ointment bases. The release rate of drugs was found to be dependent on: *(a)* the composition of the ointment base, *(b)* the concentration of the drug, and *(c)* the composition of the diffusion medium (783). The influence of five types of ointment bases on the release of tetracycline hydrochloride, chlortetracycline (biomycin) hydrochloride, chlornitromycin, and erythromycin base was studied (784). There was a difference in the degree of the *in vitro* release of the active substances from the various ointment bases studied. Under the same experimental conditions, various concentrations of hyaluronidase did not particularly influence the degree of release of the various antibiotics.

The effect of surfactant concentration on drug release from ointment bases was studied, and the concentration of nonionic surfactants between 0.05 and 0.10% gave the optimum release (785). The suitabili-

Table XXIV—Additional References on Membrane Permeation and Release

Reference	Topic
788	Systems approach to study of drug transport across membranes using suspension cultures of mammalian cells
789	Effects of membrane materials and viscosity of aqueous phase on permeability of polyamide microcapsules toward electrolytes
790	Permeation behavior of surfactant solutions through cellulose acetate membranes
791	Prediction of permeation rates and potential usefulness of polyethylene as *in vitro* membrane for drug availability prediction
792	Release of water-soluble and water-insoluble active drugs from anhydrous lipid ointment bases
793	Review of drug release from ointments and drug penetration through skin
794	Review of release and absorption of medicinal substances from ointment bases, and factors affecting therapeutic effectiveness of ointment
795	Dynamics of drug release from ointment bases, and influence of dimethyl sulfoxide on release of salicylic acid
796	*In vitro* release of an amine salified by a water-soluble polyelectrolyte, and factors influencing the release rate
797	*In vitro* release of an amine salified by a hydrosoluble polyelectrolyte, and comparison with other types of gels and consideration of the mechanism of release
798	Release of nystatin from ointment bases

ty of several water-soluble and oleaginous ointment bases for use with sodium sulfacetamide and sulfanilamide was studied (786). A formulation containing 5% sodium sulfacetamide in a base comprised of 60% polyethylene glycol 400 and 40% polyethylene glycol 4000 gave the best release of the drug and the highest antibacterial effect. The addition of polysorbate 80 had no significant effect. The water-soluble sulfonamide was released more efficiently than the water-insoluble sulfonamide. Oleaginous bases were less satisfactory.

New polymers in drug delivery were studied (787). Pilocarpine in 0.3, 1.0, and 3.0% solutions was absorbed by disks of a hydrophilic plastic of the sort used in soft contact lenses. The *in vitro* release of the active drug was compared with *in vivo* contact lenses which were fitted to rabbits whose pupil dilation responses were measured as a response to the amount of drug released. As control experiments, rabbits were given drugs topically in the usual manner. The results showed that pilocarpine can be administered in a hydrophilic plastic which takes it up and that the prolongation of release gives a mode of treatment that is as effective as a series of topical installations.

Additional references on membrane permeation and release are listed in Table XXIV.

Complexation—Studies related to complexation phenomena are categorized into: *(a)* interactions of drugs with biological substances, and *(b)* interactions of drugs with nonbiological substances.

Interactions of Drugs with Biological Substances—The binding of antibiotics to bovine plasma protein was studied by an ultrafiltration technique at 4° (799). The binding rate decreased in the order: tetracyclines > macrolides > chloramphenicols > penicillins > glycosides. Gentamicin did not bind to bovine plasma proteins. Tetracycline had the highest recovery rate, whereas aminodeoxykanamycin had the highest binding activity rate. The optimum pH for binding was 5–8. The binding of demeclocycline and oxytetracycline to bovine serum albumin was studied using fluorescent methods (800) and was shown to be hydrophobic. Two strong binding sites, at or near the tryptophan residues of bovine serum albumin, were found for both tetracyclines. The equilibrium constants for tetracyclines increased with increasing protein concentration, and the number of binding sites on the protein decreased with increasing protein concentration. This finding suggested the possibility of the sharing of one tetracycline molecule by more than one protein molecule at relatively high protein concentrations. The same investigators also calculated the equilibrium constants and the number of binding sites for the binding of 1-anilinonaphthalene-8-sulfonate to human and bovine serum albumins, using the fluorescent method and an equation derived from the Scatchard multiple-equilibrium treatment (801). They compared the results and the equations with other equations used in protein binding studies. Treatment of data by this modified method showed a clear determination of the number of binding sites.

By using *in vitro* experiments with eye fluids and tissues as well as *in vivo* studies in rabbits, the concept of competitive inhibition as a means of significantly improving drug bioavailability was supported (802). Pilocarpine nitrate, a miotic drug with low binding affinity for albumin, had a 10-fold increase in biological activity in the presence of the competitive inhibitor cetylpyridinium chloride. An ultrafiltration method was devised for determining binding constants for some common antibiotics to plasma proteins (803). For penicillin derivatives, the binding decreased in the order: dicloxacillin > cloxacillin > oxacillin > penicillin G > carbenicillin > meticillin > ampicillin. The degree of binding of 37 antibiotics to bovine and ovine serums, after treatment with therapeutic doses, was determined by equilibrium dialysis and ultrafiltration (804). The extent of binding varied from 3% for cephalosporin and kanamycin to greater than 95% for novobiocin and fusidic acid. The capacity of bovine and ovine serums to bind antibiotics was similar to the reported capacity of human serum.

The interaction of L-(1-^{3}H)-methadone with solution of purified human γ-globulin and human plasma was studied by equilibrium dialysis (805). The percent of methadone bound to γ-globulin was dependent on drug and protein concentrations. Serum protein binding determinations for erythromycin, lincomycin, and clindamycin utilizing ultrafiltration of serum containing antibiotic in a concentration of 5 μg/ml and tube dilution techniques revealed a high degree of binding: erythromycin base, 73%; erythromycin propionate, 93%; lincomycin, 72%; and clindamycin, 94% (806). In studies on complex formation between macromolecules and drugs, sodium sac-

charin was bound much more than *N*-methylsaccharin to human serum albumin, demonstrating that the type of binding was ionic (807). Hydroflumethiazide and bendroflumethiazide were more bound when ionized. The bonding of acetaminophen (paracetamol) to human and porcine plasma at both toxic and therapeutic concentrations was investigated by ultrafiltration and equilibrium dialysis over the range of 50–300 μg/ml (808). Plasma protein binding occurred at acetaminophen concentrations greater than 60 μg/ml. The extent of protein binding at the plasma concentration of 280 μg/ml of the drug was between 15 and 21% for both pigs and humans.

Following the intravenous injection of sulfamethylphenazole, sulfamerazine (sulfamethyldiazine), sulfadimethoxine, sulfamethazine, and sulfaphenazole to horses, their biological half-lives were calculated to be 8–12 hr (809). Plasma protein binding was greatest with sulfamethylphenazole and least with sulfamerazine. Based on the plasma protein binding of the sulfonamides, large amounts of sulfamethylphenazole and small amounts of sulfamerazine have to be administered to be therapeutically effective. When binding of amphetamine and related compounds to plasma proteins was investigated, 3,4-methylenedioxyamphetamine was bound to plasma protein to a greater extent than amphetamine, which may explain the differences in the concentration of drug transported into the central nervous system (CNS) (810). The protein binding of three tritiated muscle relaxants was studied and, after electrophoretic separation, ^{3}H-dimethyltubocurarine was bound most by γ-globulin whereas ^{3}H-gallamine and ^{3}H-decamethonium were bound mainly to the β-globulin fraction (811).

Table XXV—Additional References on Interactions of Drugs with Biological Substances

Reference	Topic
814	Competitive binding of 2-(4′-hydroxybenzeneazo)benzoic acid and α-(4-chlorophenoxy)-α-methylpropionic acid to serum albumins
815	Binding of propranolol to human plasma
816	Influence of protein binding on pharmacokinetics of sulfanilamides in rats
817	Excretion, plasma protein binding, and dosage of sulfonamides in cattle
818	Excretion, plasma protein binding, and dosage of sulfonamides in swine
819	Binding of fenoprofen to human plasma albumin
820	Competitive binding of two drugs for single binding site on albumin
821	Effect of protein binding on degradation of hexobendine in plasma
822	Interaction of penicillins with phospholipids
823	Binding of calcium 2,5-dihydroxybenzosulfonate-^{35}S to human serum
824	Binding of drugs to plasma proteins of swine during perinatal period
825	Interaction of mechlorethamine and isophosphamide with bovine serum albumin
826	Review of physical methods for studying drug-protein binding
827	Review of effects of binding to plasma proteins on distribution, activity, and elimination of drugs
828	Review of factors affecting drug binding to plasma proteins

Trichloroacetic acid precipitation caused the binding of phenylthiourea to the erythrocytes of a number of vertebrates (812). In rats, mice, and dogs, Scatchard plots of the binding data indicated that two sets of receptors may be involved in the binding. The total binding capabilities of the sets of receptors were of the same order of magnitude, but one set was of much greater affinity than the other. The induced binding of phenylthiourea to erythrocytes was probably related to acidity, because a neutral precipitating agent did not cause binding. Judis (813), utilizing equilibrium dialysis for the estimation of protein binding, studied several coumarin derivatives for their abilities to displace sulfonylureas from human serum albumin. All coumarin derivatives caused reduction in the binding of the sulfonylureas, although there was no clearcut pattern with a variation in the pH.

Additional references pertaining to interactions of drugs with biological substances are given in Table XXV.

Interactions of Drugs with Nonbiological Substances—The interactions between dextroamphetamine sulfate and dextrates in buffered solutions at three different temperatures and dextroamphetamine sulfate concentrations were studied (829). On heating, solutions containing dextroamphetamine sulfate and dextrates became progressively darker than solutions containing dextroamphetamine sulfate or dextrates alone. The rate of browning in these solutions increased with increasing temperature and pH and decreased with increasing dextroamphetamine sulfate concentration. From the relationship shown in the plots of absorbance against time, the browning reaction was assumed to follow an apparent zero-order rate law. In work on the influence of auxiliary material on pharmaceuticals, the interactions between surface-active poly(oxyethylene)ethers and esters of nicotinic acid (niacin) took place in the hydrophobic interior and in the hydrophilic exterior of the micelles (830). The degree of binding of the esters to the micelles depended particularly on the physicochemical properties of the esters. A strict relationship for the surface-active poly(oxyethylene)ethers in their ability to bind and to stabilize esters of nicotinic acid was also observed (831). The higher the ability to bind, the better was the stabilizing effect. A crystalline oxytetracycline–magnesium nicotinate (1:1) complex was prepared which was more soluble and stable than plain oxytetracycline base (832). The decomposition of the oxytetracycline–magnesium nicotinate complex was first order.

The heat of bonding of sodium lauryl sulfate with polyvinylpyrrolidone and the amount of sodium lauryl sulfate bound to polyvinylpyrrolidone in an aqueous medium were measured at 25° by a twin-type conduction calorimeter and by an equilibrium dialysis method, respectively; both the heat and the amount of bonding increased with an increasing concentration of sodium lauryl sulfate up to the CMC and then reached a plateau (833). The heat of bonding increased linearly with the increase in the amount of bonding below the CMC and became con-

Table XXVI—Additional References on Interactions of Drugs with Nonbiological Substances

Reference	Topic
835	Interaction between chlorpromazine and polysorbate 80
836	Caffeine-gentisic acid complexes with low water solubility and their potential use
837	Review of interactions between alcohol and drugs
838	Effect of titanium dioxide surface reactions with sodium lauryl sulfate on efficacy of medicinal preparations
839	Influence of temperature and nature of hydrophobic groups on thermodynamic parameters of hydrophobic bonding in model polymeric system and their implications in drug–biopolymer interactions
840	Complexing capacity of barbituric acids and its relation to solubility
841	Review of drug interactions with particular reference to their metabolism
842	Interaction of tetracyclines with metal ions
843	Interaction between chlorpromazine base and sorbitan monooleate

stant, indicating that the heat of micelle formation was almost the same as the heat of bonding. Hem *et al.* (834) studied the formation of 1:1 molar complexes between sucrose and the following penicillins: potassium penicillin G USP, potassium phenoxymethyl penicillin USP, sodium dicloxacillin monohydrate, and anhydrous ampicillin. The degree of complexation was greatest with sodium dicloxacillin and least with ampicillin. The rate of degradation of complexed penicillin was five to six times the rate for the uncomplexed penicillin.

Additional references related to interactions of drugs with nonbiological substances are listed in Table XXVI.

Surface Phenomena—The publications dealing with surface phenomena are divided into four major categories: *(a)* interface studies, *(b)* adsorption studies, *(c)* general properties of surfactants, and *(d)* micelle studies. However, because of the obvious overlap among these categories, the reader with special interest in this field should consider the entire section.

Interface Studies—Bond *et al.* (844) devised and improved an apparatus for measuring both the interfacial tension by the DuNouy ring method and the film elongation, which uses a stepmotor to raise or lower the interface past the ring. An apparatus for studying monomolecular films, by which the subphase can be changed without disturbing the film, was developed and the effectiveness of the apparatus was studied (845). Almost 100% of the subphase could be replaced when 1500 ml of the subphase was exchanged over 20 min. The effect of subphase exchange on human serum albumin films of different concentrations was studied. The films became condensed when a water subphase was exchanged with water, apparently because of loss of some protein from the surface. The effect was more pronounced when films were spread using larger amounts of human serum albumin.

Three recent methods for investigating the physical state existing in boundary layers formed between contacting solid surfaces, with and without a lubricant, were reviewed (846). The error in the calculation of surface tension was discussed (847). The calculation of surface tension from air-bubble data led to erroneous results when the dimension of the vessel in which the surface tension was determined was disregarded. An equation was developed taking into account the size effect of the experimental vessel.

Table XXVII—Additional References on Interface Studies

Reference	Topic
852	Orientation of high molecular weight surfactants at interface and in stable emulsions
853	Membrane potentials of sodium lauryl sulfate solutions at 25°
854	Cohesive force of 2-methyl-5-vinylpyridinium sulfate–2-vinylpyridinium sulfate at oil–water interface
855	Modified Flachsbart–Anliker model for study of interfaces

A study was conducted on the oil–water partitioning and interfacial adsorption of ^{3}H-2-pyrrolidylmethyl *N*-methylcyclopentylphenylglycolate (848). Among the physical parameters examined were partition coefficient, permeation constant, stability constant, and rate constant of the parent drug in a two-phase system of water and a lipid, didodecyl phosphate. Didodecyl phosphate greatly accelerated the rate of oil–water partitioning of the parent compound and exhibited interfacial adsorption with it. The presence of polyanions, such as hyaluronic acid, in the aqueous phase promoted the transfer of drug from the oil to water phase. The interaction of sorbitan esters in liquid paraffin with sodium lauryl sulfate in water to form spontaneous interfacial emulsions was studied by photographing the interfacial phenomenon at different stages (849). Sorbitan trioleate did not demonstrate spontaneous emulsification as readily as the monolaurate or monooleate compounds. A film balance approach using continuous compression was used to study the interaction of the carcinogen 1,2:5,6-dibenzanthracene and the noncarcinogen 1,2:3,4-dibenzanthracene with insoluble monolayers of cholesterol (850). Surface pressure and surface potential of mixed dibenzanthracene–cholesterol films spread on water and saline were studied. The results showed an association of 1,2:5,6-dibenzanthracene and no association of 1,2:3,4-dibenzanthracene with cholesterol. Surface potential data supported the concept of carcinogen–cholesterol association. Microemulsions, which were spontaneously produced upon mixing hexadecane, hexanol, potassium oleate, and water in specific proportions were studied (851). The drop-volume measurements of the hexadecane–water interface in the presence of hexanol or potassium oleate revealed that these surfactants decreased the interfacial tension of the hexadecane–water interface.

Additional references on interface studies are listed in Table XXVII.

Adsorption Studies—The uptake of benzoic acid on sulfamethazine (sulfadimidine) particles was studied (856). Depending on the concentration of sulfamethazine in the system, benzoic acid was adsorbed to the extent of 94%. Data from the adsorp-

Table XXVIII—Additional References on Adsorption Studies

Reference	Topic
862	Use of thermal gravimetric analysis, GLC, and mass spectrometry in sorption studies, and evaluation of clustering functions of ethanol–water–polyurethane system
863	Adsorption and structure formation of polyvinyl alcohols on interfaces in emulsions
864	Selection of sorption conditions for purified diphtheria toxoid prepared on different culture media
865	Thermodynamic consistency test for adsorption of liquids and vapors on solids

tion experiments were shown to fit a Langmuir plot for systems containing up to 0.2 g/100 ml of sulfamethazine. The suppressive effect of three hydrophilic polymers on the benzoic acid adsorption was also studied; the results followed the sequence polyvinylpyrrolidone > methylcellulose > sodium carboxymethylcellulose. The effect of pH on sorption of sulfonamides on nylon 6 was reported (857). The bonding of the sulfonamides resulted in an uncharged condition through H bridges to acid amide groups (cross-links) and hydrophobic reactions, with the sorption maximum lying at the isoelectric point.

In vitro adsorption of oxyphenonium bromide, oxyphencyclimine hydrochloride, clidinium bromide, chlordiazepoxide hydrochloride, and hydroxyzine hydrochloride was determined (858). The adsorbents used were aqueous suspensions of magnesium oxide, magnesium trisilicate, calcium carbonate, aluminum hydroxide, kaolin, and bismuth subcarbonate. In most cases, magnesium oxide and magnesium trisilicate showed the highest adsorptive capacity, calcium carbonate and aluminum hydroxide were intermediate, and kaolin and bismuth subcarbonate showed the least adsorptive capacity. The adsorption of benzoic acid and crystal violet on kaolin was investigated to elucidate the influence of the system dielectric constant and the electrolyte content on this drug–adjuvant interaction (859). The differing results obtained with the two adsorbates reflected the dissimilar adsorption sites on the kaolin and the different mechanisms involved in the uptake of acids and bases on clays. The experimental data were satisfactorily explained by a consideration of the effect of both the dielectric constant and the electrolyte concentration and valency on adsorbent and adsorbate characteristics.

The interaction between colloidal titanium dioxide and tetradecylpyridinium chloride, in aqueous suspensions containing various inorganic chloride salts at pH 8, was evaluated using a two-phase titration to determine the cation content and conductometric measurements to determine chloride ion (860). Surfactant adsorption was dependent on the inorganic salt content and varied with type of anion and cation. Surfactant adsorption appeared to affect the exchange equilibrium at the titanium dioxide interface and the aggregation properties of the amphiphilic cations. The relationship between adsorption from liquids and adsorption from unsaturated vapors was derived (861). The surface excess for adsorption from liquids could be calculated from equilibrium data on adsorption of unsaturated gases. The equations for the surface excess were derived from simple type I and type II models of gas adsorption.

Table XXIX—Additional References on General Properties of Surfactants

Reference	Topic
872	Review of balanced amphoteric surfactants
873	Review of determination and uses of HLB of surfactants
874	Review of determination of HLB of surfactants
875	Review of physicochemical studies of surfactants in nonaqueous medium
876	Review of solubilization properties of oil-soluble surfactants
877	Review of applications of surfactants
878	Review of action of surfactants in nonaqueous media
879	Review of chemical structure, classification, and properties of surfactants
880	Effect of molecular weight on surfactant properties of sulfonmethane (sulfonol) chloride
881	Review of properties and uses of surfactants

Additional references on adsorption studies are listed in Table XXVIII.

General Properties of Surfactants—The determination of the HLB of surfactants using GLC was investigated (866, 867). The use of partial molal volume to correlate with the HLB of nonionic surfactants was recommended (868). Measurements showing the wetting power of solutions prepared with nonionic nonylphenol polyglycol ether surfactants containing different numbers of ethylene oxide units increased with surfactant concentration up to the CMC (869). Above the CMC, the surfactants with HLB values of 10.9–17.6 gave poorer wetting with an increase of the HLB value. The oil concentration of stable emulsions was characteristic of the emulsion stability and was explained in terms of lowering of the interfacial tension by the emulsifier. The cloud point of aqueous solutions of nonionic surfactants prepared by ethoxylation of *o*-cresol, isooctylphenol, C_9–C_{18} alcohols, and coconut fatty acid monoethanolamide on addition of sodium chloride, potassium chloride, ammonium chloride, calcium chloride, magnesium chloride, and barium chloride was independent of the hydrophobic part of the surfactant, decreased linearly with electrolyte concentration, and was dependent on the lyotropic number of the cation, increasing in the order given above (870). The method of reversed GC was used to determine the polarity index on nonionic surface-active agents (871). The correlation of the real HLB value with the polarity index did not indicate any linear character.

Additional studies on the general properties of surfactants are listed in Table XXIX.

Micelle Studies—Solutions of optically active amphetamine isomers, in various concentrations, were used for the preparation of nonionic surfactant solutions, and the CMC was determined using interference refractometry (882). No significant effect of the amine antipodes on the CMC of the surfactant was observed. Parrott and Braun (883), using micellar diffusion coefficients calculated by means of dissolution rate and solubility measurements, employed the

Table XXX—Additional References on Micelle Studies

Reference	Topic
889	Model describing kinetics of micellization at concentrations exceeding the second CMC
890	Review of recent advances in chemistry of micelles
891	Critical concentrations of micelle formation of polyethylene glycol alkyl ethers
892	Review of kinetics of micelle formation by various relaxation methods
893	Determination of CMC of surfactants in organic solvents
894	Review of methods used for determination of critical concentration for micelle formation of surface-active agents in solution
895	Review of micelle formation in surfactant solutions
896	Review of current theories and recent results on thermodynamics of micelle formation and structure of micelles of nonionic surfactants in water
897	Electrostatic and electrokinetic potentials of surfactant micelles in aqueous solutions
898	Comparative study of properties of solutions of surfactants using surface tension measurements
899	Methods for determining second CMC of aqueous solution of sodium lauryl sulfate using fluorescence depolarization
900	Formation of micelles from ionic surfactants, and interpretation of surface tension curve minimum
901	Micellar behavior in solutions of the three-component system of bile acid salt–*n*-decanol–water
902	Calculation of thermodynamic parameters controlling micellization, micellar binding, and solubilization
903	Spectrophotometric determination of CMC of nonionic surfactants
904	Methods for determining second CMC of aqueous solution of sodium lauryl sulfate using light scattering

Stokes-Einstein equation to estimate the relative micelle size and micellar weight of benzoic acid–micelle species in solutions of polysorbate 80. The necessity of considering the relative viscosity of the micellar solutions and the variance of diffusion coefficients with concentration was demonstrated. Under the experimental conditions described, it did not seem justifiable to use the Stokes–Einstein equation in the determination of micellar weight and/or size. The CMC's were determined in mixed aqueous solutions of two homologous nonionic–anionic or nonionic–ionic surface-active agents from surface tension–concentration curves (884). The results were compared with the previous equation for CMC as a function of the mixing ratio, indicating that the mixed micelles behaved thermodynamically like ideal mixtures. The CMC values measured in homologous systems agreed with the equation. In nonionic–ionic systems, the CMC values of the mixed solutions were smaller than calculated by the equation.

The CMC of different polysorbates was determined by measuring the density of their aqueous solutions at 24.88° with a new instrument which can measure accurately the change in natural frequency of a hollow oscillator of constant volume when filled with a solution of an unknown density (885). The frequency change is transformed into density by calibrating the instrument with liquids of known densities. By applying the intercept method, partial specific volumes of water and the surfactant were obtained from the density of each surfactant solution. These partial quantities indicated that the formation of micelles was associated with an increase in the partial specific volume of the surfactant and a concomitant decrease in that of water.

The CMC of three nonionic surfactants was studied by interferometric measurements of refractive index, UV spectroscopy, surface tension, and dye solubilization determination (886). Good agreement was shown to exist between the results obtained by the various methods. The effects of various concentrations of testosterone and both *l*- and *d*-amphetamine isomers on the CMC values of the three surfactants were also determined, and the changes in the free energies of micellization were calculated. It was shown that the effect of the solubilized species on the thermodynamic parameters controlling the micellization process could be complex. Temperature dependence of the rate of micellization of sodium octyl sulfate was determined at 288–333°K, where the experimentally determined CMC passed through a minimum (887). The Beer's law curve for aqueous solutions of typical surface-active azo dyes and two typical colorless surfactants (hexadecyltrimethylammonium bromide and hexadecylpyridinium bromide), when plotted to relatively high concentrations, reveals a normal type of deviation toward the concentration axis over the initial concentration range, followed by a linear branch with positive slope (888). The intersection of the two branches occurred at a concentration corresponding to the CMC determined by other physical means.

Additional references on micelle studies are given in Table XXX.

Dispersion Stabilization—Hallworth and Carless (905) investigated the effect of a long-chain alcohol on the stability of paraffin-in-water emulsions containing alkyl sulfates. *n*-Octadecanol increased the stability of emulsions of light petroleum with sodium lauryl sulfate and caused a greater increase in light petroleum emulsions with sodium cetyl sulfate. The most likely cause of increased stabilization brought about by octadecanol was thought to be increased coherence of the interfacial film, which has considerably greater viscoelasticity than films without the alcohol and is thus able to stabilize the oil droplets against coalescence.

Two aqueous triethanolamine myristate–halogenated hydrocarbon (Freon) propellant emulsions with different degrees of stability were studied in conjunction with their corresponding foams to determine if any relationship existed between the properties of the emulsions and those of the foams (906). Microscopic and visual observations showed that the surfactant system producing emulsified propellant droplets with the smaller diameters also produced foams with an initially smaller bubble size and a slower increase in bubble size after discharge. The systems with the small emulsified droplets were the most stable and produced the most stable foams. An experimental model was developed in which the stability of water-in-oil emulsions, used in effecting prolonged or enhanced responses to vaccines, could be

Table XXXI—Additional References on Dispersion Stabilization

Reference	Topic
912	Dispersion state of protein-stabilized emulsions, and dependence of globule size and size distribution upon pH in concentrated oil-in-water systems
913	Comparison of theoretical equations for potential energy of electrostatic repulsion of colloidal particles at constant surface charge
914	Relationship of stability of emulsions with energy of adsorption, average volume of a single drop, and concentration of surface-active stabilizers
915	Polymer-induced flocculation of pharmaceutical suspensions
916	Sedimentation in dilute emulsion
917	Photometric investigations regarding agglomeration and dispersion in suspensions
918	High viscosity and high stability emulsions for cutaneous use
919	Measurement of forces between particles in disperse systems
920	Review of colloidal state including classification of colloidal systems, factors affecting stability of colloidal state, and flocculation
921	Conditions for formation and stability of emulsions forming near critical mixing temperature of tricosane-8-hydroxyquinoline system
922	Efficiency of control methods in evaluation of emulsion quality
923	Adsorption of nonionic surfactants on sulfathiazole and naphthalene, and flocculation-deflocculation behavior of these suspensions
924	Role of molecular diffusion in bulk stability of oil-in-water hydrocarbon emulsions
925	Charge of emulsified oil particles, and case of anionic emulsifying agent
926	Industrial application of ζ-potential studies, and effect of electrolytes on dilute suspensions
927	Effect of surfactants on electrochemical activity of oil-in-water-type emulsions stabilized by solid emulsifiers
928	Electron microscopic study of mechanism of flocculation
929	Stability of emulsions stabilized by nonionic surfactants

studied in simple environments of known constitutions (907). A decrease in emulsion stability was noted with a decrease in pH and constant ionic strength, with an increase in ionic strength and constant pH, or with an increase in temperature at a constant pH and ionic strength. Protein concentration did not affect the stability.

A survey of 68 oil-in-water emulsions, containing 20–40% liquid paraffin oil and 5–10% emulsifier, showed that 40 were stable for less than 3 months; the best emulsions contained secondary and tertiary esters of phosphoric acid with lauryl tetraglycol ether (908). Most of the apparently stable emulsions were finely dispersed and homogeneous. Changes were found in the particle size of either salicylic acid or ethionamide suspended in petrolatum base, particularly when nonionic surfactants were present during storage (909). Depending on their concentration, surfactants reduced the degree of dispersion to a substantially great extent, whereby the larger particles grew at the expense of the smaller ones. Tsukiyama and Takamura (910) studied the effects of emulsifying agents on water-in-oil-type emulsions; in the systems studied, they found that sorbitan trioleate was the best emulsifying agent. Tagats surfactants, comparable to polysorbates and polyoxyethylene derivatives of fatty alcohols (Myrjs and Brijs), had the same stabilizing effect on a suspension of phenoxymethyl penicillin and an emulsion of castor oil (911). The optimal hydrophilic–lipophilic equilibrium value for a suspension of 10% phenoxymethyl penicillin was two, while the value for castor oil emulsion was 12.

Additional references on dispersion stabilization are given in Table XXXI.

Rheology—Liquid paraffin-in-water emulsions, stabilized by mixed emulsifiers of a surfactant (cetrimide or cetomacrogol) and a long-chain alcohol, were used as a model system to represent pharmaceutical semisolids (930). They were examined in their linear viscoelastic regions using creep and oscillatory techniques. The agreement between transformed functions and those obtained directly from oscillatory measurements was generally good. In work on oscillatory testing of oil-in-water emulsions containing mixed emulsifiers of the surfactant–long-chain alcohol type, the self-bodying action and the influence of surfactant chain length were studied (931, 932). The rheology of a new topical vehicle, fatty alcohol–propylene glycol (FAPG) cream base, was investigated over 25–37° (933). Continuous shear rheograms obtained with the Ferranti-Shirley cone and plate viscometer were hysteresis loops with a spur point; loop areas, yield values, and apparent viscosities decreased with an increase in temperature. In creep, the base was viscoelastic with a low limit of linearity with respect to strain. Patient acceptance of skin spreadability of the base was assessed by using a master curve concept; the spreading properties were close to the preferred values for maximum patient acceptance.

The influence of alcohol chain length and homolog composition on the rheological stability of cetrimide emulsions was also studied (934). Upon aging, the consistency of emulsions prepared with cetostearyl alcohol increased while that of emulsions prepared with cetyl and stearyl alcohols decreased. Measure-

Table XXXII—Additional References on Rheology

Reference	Topic
936	Rheological characteristics of a base containing petrolatum (Vaseline), white wax, cholesterol, and stearyl alcohol
937	Relationships among structure, properties, and stability of salves containing surface-active substances, and interaction between emulsifiers and liquid phase of gels
938	Rheology of oily suspension suppositories of mefenamic acid
939	Rheological characteristics of cold cream
940	Review of uses of rheology in pharmaceutical field
941	Viscosity and rheological changes in bentonite gel following addition of various salts
942	Effect of aging on rheological properties of gelatin gels
943	Rheological properties of some cellulose solutions
944	Structural and mechanical properties of bases containing methylcellulose solutions
945	Rheological study of fatty acid–soap systems
946	Rheological properties of lipophilic bases applied to cosmetics
947	Rheological study of salve bases

ment of viscosity of carbomer dispersions showed a reduced viscosity which increased very rapidly with an increasing concentration of carbomer (935). The increase in viscosity was further decreased by addition of sodium chloride. Measurements of ionic strength of these dispersions by a viscometric method gave results that were 10–20% of the theoretical strength as calculated from the normality of the ionized carboxylic acid group. These results were then discussed in relation to interactions between macromolecules.

Additional references on rheology are provided in Table XXXII.

PHARMACEUTICAL ASPECTS

Antibiotics—The influence of binding, disintegration, and lubricating filling substances on the flow of granules containing oxacillin or ampicillin, as well as on the hardness and disintegration of the prepared tablets, was discussed (948). Oxacillin was electrostatically charged, but no electrostatic charges were established in ampicillin. Therefore, ultraamylopectin, which was also electrostatically charged, was unsuitable as an additive for tablets containing oxacillin but was successfully used in ampicillin tablets. A silicone lubricant (Aerosil) neutralized the electric charges in the two antibiotics, improved the flow of the granules, and increased the hardness and disintegration of the tablets. Gelatin glue and 5% ethylcellulose were the most suitable binding agents.

The stabilization of aqueous solutions of sodium penicillin G was investigated using a mixture of 0.5% methenamine and 1% disodium edetate and compared to using methenamine sodium citrate (949). The rate of decomposition was less with disodium edetate. With this combination, the penicillin remained stable for 3 days at 30°. The influence of trichloroacetate anion on the *n*-octanol–aqueous phosphate buffer apparent partition coefficient of various tetracyclines was determined (950). At acidic pH values, the presence of trichloroacetate significantly increased the apparent partition coefficients of most tetracycline analogs studied, presumably through intermolecular ion-pairing in the positively charged tetracycline moiety and the trichloroacetate anion. From intermediate to mildly alkaline pH values, trichloroacetate had essentially no effect on partitioning. The alteration of the apparent partition coefficients of the tetracycline antibiotics by intermolecular ion-pair formation was discussed in terms of its relationship to the absorption of these compounds.

The compatibility of five antibiotics with seven polyethylene glycol gels was studied immediately after preparation as well as after 1, 3, 7, 12, and 36 weeks (951). Chloramphenicol and neomycin sulfate were stable in all of the gels studied. Erythromycin lactobionate was stable for 7 weeks. An antifungal preparation containing nystatin (stamycin) was stable in an emulsifier-free gel. Polyethylene glycol gels were not appropriate for tetracycline hydrochloride. An examination was made of the suspension characteristics of antibiotic granules or fine powders with various concentrations of sodium carboxymethylcellulose and distilled water (952). Antibiotic suspensions with sodium carboxymethylcellulose solution of 0.125% concentration and distilled water showed the same sedimentation profile, resulting in a hard cake formation of dispersed antibiotics. Due to its high viscosity, sodium carboxymethylcellulose solution in a concentration of 0.5% showed low resuspendability for the dispersed phase. Sodium carboxymethylcellulose at 0.25% was suitable as a suspending medium for syrupy antibiotic suspensions.

Table XXXIII—Additional References on Antibiotics

Reference	Topic
953	Penicillin G and ampicillin interactions with phospholipids
954	Measurement of wettability of antibiotic powders
955	Effect of processing on stability of troleandomycin and erythromycin propionate suspensions
956	Review of pharmaceutical aspects of parenteral penicillins
957	Review of interactions of antibiotics with other pharmaceuticals
958	Effect of oleandomycin and its combinations with tetracycline, chloramphenicol, erythromycin, lincomycin, and 1′-demethyl-4′-depropyl-4′-(*R* and *S*)-*n*-pentylclindamycin on microbial generation of *E. coli*
959	Formation of polyvinylpyrrolidinone complexes of amphotericin B during coprecipitation by an aprotic solvent
960	Production of thermostable chloramphenicol (synthomycin) and sulfamidochrysoidine (streptocid) emulsions using the new nonionic emulsifier oxysterone

Additional references on antibiotics are found in Table XXXIII.

Radiopharmaceuticals—The use of radionuclides for diagnostic purposes, with descriptions of the preparation of a ^{99m}Tc–iron–ascorbic acid complex and a ^{99m}Tc–sulfur colloid, using a Mo-Tc generator, was reviewed (961). Radiopharmaceuticals used for diagnosis of blood, liver, pancreas, and heart diseases were reviewed (962), as was the design of the synthesis of radioisotope-labeled drugs with their stability and storage (963). The development and production of various radioactive drugs were reviewed (964), and other radiopharmaceutical reviews were presented (965, 966).

BIOPHARMACEUTICS

The various publications dealing with biopharmaceutics were subdivided according to the area of special interest. However, because of the obvious overlap in subject matter, the reader seeking a thorough review should consider the entire section.

A model was proposed for determining the bioavailability of drugs whose elimination from a one-compartment body model occurs by one or more apparent first-order processes in parallel with one capacity-limited elimination process (967). Another model was based on estimates of renal clearance, plasma clearance, and urinary excretion of unchanged drug. The method was totally compatible with pharmacokinetic models and lent itself to a more flexible sampling schedule which made it independent (968). The average amount of drug in the body at steady state upon repetitive dosing in a two-compartment

open system was related to the average steady-state plasma level by the apparent volume of distribution (V_β) at steady state rather than by the apparent volume of distribution at pseudodistribution equilibrium, despite the fact that the average steady-state plasma level is directly proportional to $1/V_\beta$ (969).

A series of review articles dealt with: bioavailability, clinical effectiveness, and the public interest (970); design of *in vivo* studies of bioavailability, biometrical considerations (971); use of physical and animal models to assess bioavailability (972); overview of the analysis and interpretation of bioavailability studies in humans (973); and physiological and pharmacokinetic complexities in bioavailability testing (974).

As a single entity, the bioavailability of digoxin tablets attracted the most attention in the field of biopharmaceutics. The absorption of a commercial digoxin tablet preparation of low as well as high therapeutic potency and of digoxin dissolved in alcohol was studied in digitalized patients and compared with the dissolution rates of the tablets (975). The bioavailability of the low potency tablet was markedly inferior to that of the high potency tablet, which did not differ significantly from that of digoxin in alcoholic solution. A significant correlation between bioavailability and the dissolution rate was also found. The investigators suggested that measurement of the dissolution rate might be used as a guide to the bioavailability of various digoxin tablet preparations. The commercial digoxin radioimmunoassay procedure was modified so that the sensitivity was increased 20-fold (976). By using this assay, significant differences in plasma levels in different brands of digoxin tablets were found as determined by the analysis of variance at 0.5, 1, 72, and 240 hr, and the peak plasma levels differed significantly. Beveridge *et al.* (977) recommended following urinary excretion in addition to plasma levels to estimate the bioavailability of different digoxin formulations. In an evaluation of the bioavailability of digoxin preparations available to a Naval hospital, variations in the early absorption of four digoxin oral preparations were found; however, serum levels at 5 hr were essentially the same and the urinary recovery was similar (978).

The bioavailability of dicumarol from three different commercial tablets was determined by measuring plasma levels in dogs and by an *in vitro* dissolution rate test (979). The products showed significant difference in plasma levels, area under plasma level curves, peak prothrombin time responses, and rates of dissolution, even though they contained the labeled amount of dicumarol. A comparative study was done with methyltestosterone sublingual and oral tablets to find if there were any differences in their bioavailability (980). The sublingual methyltestosterone was absorbed faster. Its extent of bioavailability approached twice that resulting from an equal dose of orally administered drug. This may explain the clinical experience that milligram for milligram, methyltestosterone sublingual tablets have greater potency than orally administered tablets.

Various dosage forms of butaperazine were administered to patients, and the levels of butaperazine in the blood were determined at regular intervals (981). The oral concentrate formulation produced the highest blood level, the intramuscular form produced the lowest, and the tablet form was intermediate. When the blood and urine levels of tetracycline hydrochloride were monitored in normal subjects throughout a 96-hr treatment period, the bioavailability of film-coated tetracycline hydrochloride tablets was inferior to that of capsules (982). In general, the dissolution time of capsule preparations was shorter than that of tablet preparations. The bioavailability of some steroids administered orally was followed when they were put into oily and aqueous vehicles (983). The highest amount of GI absorption occurred from oily solutions.

Clindamycin availability in humans from clindamycin 2-palmitate and clindamycin 2-hexadecylcarbonate compared to clindamycin hydrochloride was determined (984); the palmitate ester was the superior ester and was bioequivalent to clindamycin hydrochloride in humans. The initial urinary excretion rate of chloramphenicol was decreased in humans when the drug was administered orally with milk instead of water (985). This difference was suggested to be due to the drug–milk protein binding. The bioavailability of potassium from orally administered 10% potassium chloride solution was compared to a slow-release potassium chloride tablet (986). The urinary potassium excretion increased sooner and reached greater peak levels after the solution was administered. In both cases, greater amounts of potassium appeared in the urine when it had been administered in the fasting state rather than in the postprandial state. Hydrochlorothiazide tablets from 10 of 39 Canadian companies failed to meet the USP XVIII dissolution requirements (987). However, the bioavailability in drug absorption parameters did not give correlations of predictive value with the formulation dissolution times. It was also noted that the dissolution time of some commercial hydrochlorothiazide tablets was decreased after storage.

The factors affecting the therapeutic activity of rectal preparations, such as chemical modifications of the drug, presence of adjuvants in vehicle, various dosage forms, and pharmaceutical processes used in formulation, were reviewed (988). The absorption rate of sulfanilamide and sulfisoxazole in rabbits decreased in the order: oral > subcutaneous > rectal administration (989). When comparing water-soluble, emulsified, or oleaginous substances such as suppository bases, it was found that the emulsified base suppositories gave similar values to oral and subcutaneous administration, while the drugs prepared with oleaginous bases had smaller absorption rate constants.

The *in vivo* bioavailability and the *in vitro* dissolution rates of several commercial tolbutamide tablets were compared (990), and no detectable statistically significant differences among the dosage forms in terms of percent drug recovered, maximum excretion rate, or time of maximal excretion rate could be found. However, there were marked differences in the *in vitro* dissolution rates among the formulations

Table XXXIV—Additional References on Biopharmaceutics

Reference	Topic
1005	Review of biological availability of drugs
1006	Review of statistical methods in evaluation of *in vivo* performance of dosage forms
1007	Review of factors influencing drug absorption, administration, and elimination
1008	Review of factors influencing drug availability at receptor site
1009	Review of bioavailability of peroral dosage forms and factors influencing it
1010	Review of materials for injection with immediate effect
1011	Review of problems encountered when using animals for bioavailability testing
1012	Review of basis of biopharmaceutical testing of drug bioavailability
1013	Review of sites of drug transport and disposition, with special emphasis on permeability of blood–brain barrier to drugs
1014	Review of chemical, biological, and clinical evaluation of drug equivalency
1015	Review of routes of drug administration, with special emphasis on diffusion and active transport of drugs through cell membranes
1016	Review of factors that modify drug activity and patient response
1017	Chemical equivalence or therapeutic equivalence
1018	Chemical equivalence and therapeutic nonequivalence of various drugs in pharmaceutical forms
1019	Bioavailability of aspirin tablets registered in Finland
1020	Urinary excretion of ampicillin administered in four dosage forms in humans
1021	Clindamycin dose–bioavailability relationships
1022	Bioavailability of codeine preparations in rats
1023	Comparison of oral and percutaneous routes in humans for systemic administration of ephedrines
1024	Review of bioavailability of griseofulvin, tetracycline, tolbutamide, chloramphenicol, nitrofurantoin, and sulfadiazine
1025	Bioavailability of nitrates in the dog
1026	Biological equivalence of oxytetracycline hydrochloride capsules from Colombian markets
1027	*In vitro* availability of phenobarbital from solubilized systems
1028	Comparative availability of Czechoslovakian and foreign phenoxymethyl penicillin-type preparations in blood serum of volunteers
1029	Bioavailability of salicylamide from tablets
1030	Review of bioavailability of sodium warfarin
1031	Vehicle and route of administration as parameters affecting operant behavioral effects of Δ^9-tetrahydrocannabinol
1032	Biopharmaceutical studies on macrolide antibiotic SF-837
1033	Absorption and topographic distribution of 4-bromophenylisothiocyanate-^{35}S on normal guinea pig skin in various bases
1034	Percutaneous absorption of heparin-^{35}S from specially prepared oil-in-water emulsion
1035	Modification of cutaneous permeability after application of dermal preparations, and action of hydrocortisone ointment
1036	Absorption of hydrocortisone acetate from hydrophilic ointments
1037	Absorption, excretion, and metabolism of subcutaneously and topically applied mafenide acetate
1038	Percutaneous absorption and distribution of 2-naphthol in humans
1039	Effect of excipients on percutaneous absorption of nicotinic acid esters
1040	Optimization of dermatological formulations of retinoic acid
1041	Bioavailability of different ointments containing sodium sulfacetamide (sodium sulfacyl) and potassium iodide
1042	Comparative bioavailability of proprietary hydrophilic topical steroid preparations
1043	Biopharmaceutical studies on percutaneous absorption of vitamin A from ointments
1044	Review of release and cutaneous absorption of drugs from dermatological preparations
1045	Barrier and reservoir function of human stratum corneum for topically applied drugs
1046	Percutaneous absorption of drugs, and time course of cutaneous reservoir of drugs
1047	Quantitative effect of topically applied anti-inflammatory agents on external ocular inflammation in rats

examined in pH 6.45 buffer. A crossover absorption study of seven oral oxytetracycline and two tetracycline formulations was made (991). *In vitro* analysis of disintegration and dissolution was also carried out on these preparations. When the rate of dissolution was correlated with absorption, it was found that with one preparation the dissolution rate was slow and incomplete and the absorption was inferior, whereas with another preparation the dissolution was quite rapid and the absorption was quite good. An *in vitro* method for studying the release of active ingredients from suppositories was devised (992). The suppository was surrounded by a pH 7.8 buffer solution in a dialysis chamber which was continuously perfused with artificial plasma; the latter was pumped through a flow cell for direct, continuous spectrophotometric reading. These results were compared with data obtained by *in vivo* urinary excretion of proxyphylline after it was administered as a suppository to human subjects. A good correlation between the percent absorbed *in vivo* by the rectal route and the percent released *in vitro* was observed when the amount of proxyphylline released *in vitro* was between 15 and 85%.

In work on the mechanism of percutaneous absorption, the permeation rates of a homologous series of primary alcohols (C_1–C_{10}) through skin were studied (993). The alcohols were applied from aqueous solutions and as pure liquids. The permeation behavior in the two cases was compared in terms of: *(a)* transport rates of the alcohols, *(b)* distribution equilibrium between the tissue and vehicle, and *(c)* damage to the tissue produced by the vehicle. Fick's law was found to hold as an approximation for both the aqueous and the liquid alcohol systems. An electrometric study was done to find the effects of ionic surfactants on the permeability of human epidermis (994). Three ionic surfactants of a homologous series were studied (R—COONa, R—OSO_3Na, and R—NH_3Cl). A similar pattern of relative surfactant activity was shown for each series of surfactants. The C_8 compounds showed no effect, the C_{10} compounds showed slow but distinct increases in conductance, and maximal effects occurred at C_{12} and C_{14} but less at C_{16}.

Percutaneous absorption through the damaged skin lacking stratum corneum greatly increased as compared with that of intact skin (995). The initial

absorption pattern, which was distinctly recognized in the intact skin, almost disappeared in the damaged skin; the absorption of the drugs occurred by simple diffusion of a first-order rate from the start of the experiment. The absorption of the drugs in the intact skin was almost negligible when the drugs were in an ionized state; however, in damaged skin, there was a distinct absorption of ionized drugs. Comparison of the rate of absorption through mouse skin of several water-soluble substances from emulsion-type ointments decreased with an increase in the molecular size of the compound studied (996). Using different water–glycerin ratios, different solutions of methyl nicotinate with identical thermodynamic activity were prepared and their percutaneous absorption was followed (997). The results showed that the time of onset of erythema was the same for all solutions of equal thermodynamic activity. Thus, the rate of penetration was mainly dependent on the thermodynamic activity of the penetrant.

Application of dimethyl sulfoxide to the human skin increased skin permeability for electrolytes, with the maximum effect after 20 min and a lessening effect afterward (998). Similarly, application of sodium lauryl sulfate to human skin increased electrolyte permeability, thus facilitating the entrance of potentially harmful detergent ingredients such as metals (999). The *in vitro* penetration of four different radiolabeled hydrocortisone creams was measured in human skin (1000) and the highest activity was found in the horny layer of the skin. When using a water-in-oil emulsion, a longer penetration period was observed with a large reservoir in the horny layer. The oil-in-water emulsions gave no distinct reservoir. The amount of hydrocortisone found in the epidermis and the cutis was low when petrolatum and water-in-oil emulsions were used. Absorption of salicylic acid and *p*-aminosalicylic acid in 5% ethanol through intact rat skin decreased as pH increased and followed the pH–partition hypothesis (1001).

The properties of the cutaneous barrier, methods for measuring the efficiency of the cutaneous barrier and *in vivo* and *in vitro* percutaneous absorption, and the importance of physicochemical factors were reviewed (1002). With the extrathermodynamic approach, for many series of drugs the percutaneous absorption through intact skin is highly dependent upon the lipophilic character, measured by the log partition coefficient from octanol–water, ether–water, and other suitable solvent systems (1003). A review of the absorption of drugs through the skin was presented (1004).

Additional references on biopharmaceutics are listed in Table XXXIV.

Effects of Physicochemical Properties—Different physicochemical factors affecting the absorption of sulfonamides from rat small intestine were studied (1048) including partition coefficient, adsorption to rat blood, adsorption to activated charcoal, molecular weight, and dissociation constants. The intestinal absorption of sulfonamides was satisfactorily predicted with regression analysis. Absorption of isomeric and N^1-heterocyclic sulfonamides from rat small intestine also was studied, and the physicochemical properties were correlated with absorption of unionized sulfonamides (1049). The rate of absorption of the undissociated form of the compounds could be correlated to partitioning to *n*-octanol and molecular weight.

Plasma half-lives, plasma metabolites, and anticoagulant efficacies of the enantiomers of warfarin were studied in humans (1050). *S*-(−)-Warfarin was a more potent anticoagulant than *R*-(+)-warfarin in humans. However, *S*-warfarin was cleared more rapidly from the plasma compared to *R*-warfarin. The different mean plasma half-lives for *R*- and *S*-warfarin were 45.4 and 33 hr, respectively. The influence of raw materials used in drugs on the bioavailability of the active components was studied (1051). The various effects of polymorphism, granulation, valence, pK, partition coefficient, and ester formation were considered. Beckett and Shenoy (1052) studied the effect of *N*-alkyl chain length and stereochemistry on the absorption, metabolism, and urinary excretion of *N*-alkylamphetamines in humans. The total metabolism of (+)-methyl-, ethyl-, and *n*-propylamphetamine was greater than that of the corresponding (−)-isomers, but there was no difference in the total metabolism of (+)- and (−)-*n*-butylamphetamine. Molecular weight and chemical structure as factors in the biliary excretion of sulfonamides in the rats were studied (1053). In the N^4-position, when substituted with various carboxyacyl groups, and the N^2-position, with acetyl or 2-thiazolyl, there appeared to be a threshold value for the molecular weight above which biliary excretion became appreciable.

The bioavailability of digoxin was also studied as affected by the particle size of the different formulations studied (1054). Tablets and capsules made of crude material of digoxin, which passed the British Pharmacopoeia requirements, and after it had been crushed to a finer particle size were given to seven volunteers. The serum digoxin levels were followed, and the results showed that formulations made with the finer particles gave higher area under concentration curves than did formulations made with coarse material. Human plasma levels of digoxin tablets prepared with 3.7-, 12-, and 22-μm particles were compared, and it was found that the smaller the particle size the higher the plasma levels (1055). Absorption of sulfanilamide and sulfisoxazole from the rabbit rectum and intestine was influenced by the particle size of these drugs, with the absorption rate constant being proportional to the log of the reciprocal of the particle size while the dissolution rate constant was proportional to the reciprocal of the particle size (1056).

A homologous series of straight-chain lincomycin 2-monoesters, comprised of both acyl and carbonate esters, was synthesized (1057). Good antibacterial activity was observed with esters of chain lengths from C_4 to C_{16}. Esters with chain lengths from C_{12} to C_{16} were tasteless and highly active *in vivo*. The long-chain lincomycin 2-esters gave the desired properties required for formulation as a tasteless pediatric preparation. Clindamycin was chemically modified to provide *in vivo* reversible derivatives that

Table XXXV—Additional References on Effects of Physicochemical Properties

Reference	Topic
1060	Statistical approach to evaluating effect of physical and chemical factors on fecal excretion of chlorophenothane
1061	Review of effect of chemical modification of drugs on biological availability
1062	Effect of stereochemistry of warfarin on its metabolism
1063	Review of newer physicochemical methods useful in bioavailability studies
1064	Chance correlations in structure-activity studies using multiple-regression analysis
1065	Structure-activity correlations of 1,3-benzodioxole synergists
1066	Structure-activity correlation of mitomycin derivatives
1067	Structure-activity correlation for substrates of phenylethanolamine *N*-methyltransferase
1068	Structure-activity correlation of antineoplastic drugs
1069	Structure-activity correlations of kinins
1070	Effectiveness of pharmaceuticals related to their physical form
1071	Effect of chain length in homologous series of anionic surfactants on irritant action and toxicity
1072	Substituent-effect analyses of rates of metabolism and excretion of sulfonamide drugs
1073	Utilization of operational schemes for analog synthesis in drug design
1074	Physicochemical studies on elucidating the distribution behavior of aminopyrine
1075	Correlations between physical and physicochemical properties (pH value, binding agents, coating, *etc.*) of medicinals and various forms of administration
1076	Review of effect of particle size of different dosage forms on pharmacological action
1077	Effect of particle size on bioavailability of trimethoprim-sulfamethoxazole preparations

might be utilized in special dosage forms (1058). A series of 2- and 3-monoesters improved the taste properties. Highly water-soluble salts were also prepared to provide intramuscular injectable preparations. A review of different ways of changing the pharmacokinetics of drugs by modifying the properties of the multicompartment system or by changing the physicochemical properties of the active drug was presented (1059).

Additional studies on the effects of physicochemical properties are listed in Table XXXV.

Effects of Formulation—The general applicability of the polyvinylpyrrolidone coprecipitation technique as a method for enhancing GI absorption of orally administered hydrophobic drugs was explored with digitoxin (1078). The relative absorption characteristics of digitoxin alone and as a 1:9 physical mixture and coprecipitate with polyvinylpyrrolidone were determined indirectly by measuring the oral LD_{50} values in rats. The *in vivo* data obtained provided evidence that digitoxin was absorbed from the coprecipitate at a significantly faster rate and was present in the body at a much higher level than when equivalent doses of either the drug alone or a physical mixture with polyvinylpyrrolidone was orally administered. A correlation was found between the *in vitro* dissolution rates of these systems at 37° and their *in vivo* toxicities. *In vitro* dissolution and *in vivo* absorption characteristics of various nitrofurantoin and nitrofurantoin–deoxycholic acid preparations were studied (1079). *In vitro* particulate dissolution studies showed that the dissolution rate of a 1:5 molar ratio nitrofurantoin–deoxycholic acid coprecipitate was approximately six times greater than the dissolution rate of the 1:5 physical mixtures. Similar results were also obtained with *in vivo* absorption where the 1:5 coprecipitate showed significant increases in both the initial urinary excretion and the total cumulative amount of unchanged nitrofurantoin. The GI absorption characteristics of micronized griseofulvin from an oil-in-water emulsion dosage form, an oil suspension, and an aqueous suspension were assessed in rats (1080). The emulsion form produced a mean peak plasma antibiotic level 1.5 and 2.3 times higher than the oil and aqueous suspensions, respectively, after a slight delay.

Bioavailability studies of acetaminophen rectal dosage forms with various polyethylene glycol bases and water in humans showed statistically significant differences both in the rate and the extent of absorption (1081). The dissolution rate studies showed that one factor controlling the initial rate of absorption was the speed with which polyethylene glycol bases released the drug into the rectal fluids. Another factor was the apparent partition coefficient which controlled the transfer of drugs across a lipoidal barrier. The appearance of radioactivity in the blood plasma of dogs was more delayed and of longer duration after administration of coated sustained-release tablets containing 300 mg of ^{14}C-*O*-(β-hydroxyethyl)rutoside than after administration of rapid-release capsules that contained the same amount of drug (1082).

Dissolution rate and absorption for one film-coated and four sugar-coated oxytetracycline tablet preparations were determined (1083). The dissolution in 0.1 *M* hydrochloric acid and in simulated intestinal fluid of film-coated tablets was faster than with sugar-coated tablets, although absorption studies in humans did not show these differences. The urinary excretion of a conventional and a slow-release tablet of 300 mg heptaminol was followed (1084). The average peak excretion occurred in the first 2 hr after ingestion and after 4–6 hr for the conventional tablet and the delayed-action tablet, respectively. Starting with the 6–8-hr period, excretion levels became higher for the slow-release tablets as compared with the conventional ones. In simple suspensions, particle size of sulfisoxazole influenced both the rates of absorption and dissolution (1085). However, when formulated in a vehicle containing a surface-active flocculating agent and methylcellulose, the particle-size differences were not as pronounced. Berlin *et al.* (1086) determined the bioavailability of diazepam in various formulations from their steady-state plasma concentration data. The suspension form showed lower values during steady state as a result of incomplete absorption.

Absorption Control and Alteration—Absorption of phenolsulfonphthalein by healthy adults was decreased significantly during the 1st hr when the drug

was administered in very viscous sodium alginate solution or when the subject had been premedicated with propantheline (1087). Sodium alginate had no effect on the total amount of phenolsulfonphthalein absorbed; pretreatment with an anticholinergic agent increased the total amount of phenolsulfonphthalein recovered in the urine from an average of 16 to 24% of the dose. The effect was attributed to a decrease of the GI transit rate. The influence of dosage form on the antipyretic activity of acetaminophen administered intraperitoneally in the rat was studied; the solution form produced an antipyretic response twice that of the same dose administered as a suspension (1088). This experiment indicated that ED_{50}, LD_{50}, and relative potency calculations may be inaccurately determined if dosage form influences on bioavailability of an insoluble drug are not considered in the design and evaluation of animal drug testing procedures.

Alterations in the absorption of dicumarol by various excipient materials were studied (1089). The drug was combined with the excipient by an equilibration process and was administered orally. Significant differences were noticed in the plasma levels of dicumarol with six of the 10 excipients studied. Both magnesium oxide and magnesium hydroxide significantly increased the plasma levels of dicumarol while talc, colloidal magnesium aluminum silicate, aluminum hydroxide, or starch resulted in significantly lower plasma levels of the drug. The investigators suggested that these types of interactions may be an explanation for differences in the bioavailability of dicumarol from different dosage formulations. The effect of adding polyethylene glycol 400 or polysorbate 80 on the absorption of virginiamycin was studied (1090, 1091). Blood concentrations of virginiamycin were 2.5 times higher when the antibiotic was administered to rats orally in conjunction with 5% polyethylene glycol 400. This result was attributed to the increase of solubility of the antibiotic. Above 5% concentration, the blood levels progressively decreased due to the partition coefficient, which favored the aqueous over the oily phase, causing a decrease in the amount of antibiotic available for absorption. When polysorbate 80 was administered with virginiamycin, it increased the solubility and the absorption of virginiamycin. Similarly, polysorbate 80 increased the availability of aspirin in humans as compared to dosing with aspirin alone (1092). The possible effect of complex formation between drugs and adjuvants on the dissolution rates of drugs from tablets was determined (1093). Generally, the higher the adjuvant–drug ratio and the greater the drug interaction tendency between drug and adjuvant, the more pronounced was the influence of excipient on the dissolution rate of drugs.

Liquid chlorpromazine, with or without gel antacids, was given orally to humans (1094). Plasma chlorpromazine levels were significantly lower with gel antacid–chlorpromazine combinations than with chlorpromazine alone. The effect of nonsteroidal anti-inflammatory drugs on the absorption and excretion of sulfadimethoxine in rabbits was studied (1095); the simultaneous administration of indomethacin or benzydamine altered the absorption of sulfadimethoxine by delaying gastric emptying and thus increasing the time for sulfadimethoxine to reach its primary absorption site.

A set of *in vivo* and *in vitro* experiments was designed to show the drug–protein interaction in tissues and fluids of the eye and the effect that this interaction had on drug bioavailability (1096). Equilibrium dialysis experiments, using pilocarpine nitrate, demonstrated that extensive binding to proteins in tears, cornea, and aqueous humor does occur. This was confirmed by using pilocarpine nitrate and following its effect on the pupillary diameter. Theophylline alone gave better *in vitro* dissolution and *in vivo* availability than a theophylline–phenobarbital complex, indicating that bioavailability may be influenced by complexation or interaction of the two drugs in the tablet formulation (1097).

Patients on digoxin therapy, when using propantheline, had an increase of serum digoxin levels; when using metoclopramide, they had reduced digoxin serum concentrations (1098). These results showed that any change in GI motility produced by the two drugs was responsible for the altered bioavailability of digoxin. By using premicellar concentrations of sodium taurocholate, sodium tauroglycholate, and sodium deoxycholate, the dissolution rate of aspirin and salicylamide was enhanced (1099). Similarly, an increase in the intestinal absorption of both drugs was observed in rats. This increased absorption may be due to alterations in permeability characteristics of intestinal mucosa cells. In a study designed to determine the rate of gastric emptying of phenol red in the rabbit, aminopyrine considerably delayed the gastric emptying of phenol red (1100). However, caffeine, which has an increasing effect on human gastric secretion, did not affect the rate of emptying of phenol red.

The effect of sodium glycocholate upon the partitioning behavior and GI absorption of isopropamide iodide was studied (1101). Sodium glycocholate progressively increased the partitioning of isopropamide from a physiological aqueous buffer into *n*-octanol below the CMC of the bile salt, but increased partitioning was inhibited above this value. The formation of a lipid-soluble ion-pair between the bile salt anion and the quaternary cation was suggested as the mechanism of enhanced partitioning. Absorption from the rat ileum *in situ* in the presence of sodium glycocholate below and above its CMC did not follow a similar pattern. It was suggested that the GI absorption of the isopropamide cation cannot be increased in the presence of bile salt molecules through ion-pair formation or mixed micelle formation. A study was conducted to evaluate the influence of dietary calcium carbonate and sodium sulfate in chick rations containing oxytetracycline (1102); the sodium sulfate improved the absorption of oxytetracycline, while calcium carbonate at concentrations greater than 1% inhibited the absorption of oxytetracycline.

Ramachander *et al.* (1103) studied the effect of concurrent administration of choline salicylate and acetaminophen on their mutual biotransformation in

Table XXXVI—Additional References on Absorption Control and Alteration

Reference	Topic
1111	Effect of surfactants on drug absorption, and changes in drug stability in both biosurfactant and synthetic surfactant solutions estimated by thiol-disulfide exchange reaction *in vitro*
1112	Effect of surfactants on drug absorption, and mechanism of action of sodium glycocholate on absorption of benzoylthiamine disulfide in presence of sodium lauryl sulfate and polysorbate 80
1113	Effect of sodium taurodeoxycholate on biological membranes, and release of phosphorus, phospholipid, and protein from everted rat small intestine
1114	Mechanisms of drug absorption and drug solution
1115	Review of relationships among drug plasma concentrations, elimination pathways, extent of renal impairment, and dosage regimen modifications in patients with renal impairment
1116	Review of drug-induced malabsorption
1117	Release of active substance after oral administration of a β-cyclodextrin inclusion compound to humans
1118	Distribution of phenothiazines between aqueous and organic phases and buccal absorption
1119	Quantitative approach to *in vitro* availability of drugs from nonionic surfactant solutions
1120	Review of drugs affecting gastric emptying rate and drug absorption
1121	Modifications of absorption and excretion of trimethoprim and sulfamethoxazole predicted from buccal mucosa
1122	Calculation of rate constants in forward drug transfer reactions
1123	Influence of polysorbate 20 and sodium cholate on uptake of *p*-hydroxybenzoates by frogs, *Rana pipiens*
1124	Programmed release of active drugs from preparations for oral use
1125	Excretion of *trans*-Δ^9-tetrahydrocannabinol and its metabolites in intact and bile duct-cannulated rats
1126	Influence of sodium taurocholate, cholestyramine, and simethicone-containing formulation (Mylanta) on intestinal absorption of glucocorticoids in the rat

the rat. Concurrent administration of the two drugs did not alter the plasma salicylic acid levels but resulted in a slight increase of the plasma acetaminophen values at 5 and 10 min. Differing degrees of glucuronidation of the two drugs may explain this interaction which, however, appeared too low to be of clinical significance. The absorption of a quaternary ammonium compound, *N,N*-bis(phenylcarbamoylmethyl)dimethylammonium chloride, was studied *in situ* in the small intestine of the rat in the presence of salicylate and trichloroacetate (1104), and both significantly enhanced the disappearance of the drug from the intestinal lumen. Both ionic agents exerted their greatest influence on the initial absorption rate of the drug and the effect tended to decline with time thereafter. The effect of fenfluramine on the intestinal absorption of triglycerides was studied; *dl*-fenfluramine hydrochloride decreased plasma triglycerides in fed rats but had no effect in fasted rats (1105). It was postulated that the lowering of the plasma triglycerides by *dl*-fenfluramine hydrochloride was partially due to inhibition of intestinal absorption of triglycerides.

Jaffe and coworkers (1106, 1107) studied the effect of altered urinary pH on tetracycline and doxycycline excretion in humans. Alkalinization of the urine significantly enhanced the cumulative renal excretion of both drugs as compared with acidic urine treatments. Altered tubular reabsorption was considered to have caused this effect, since tetracyclines have been shown to be more lipid soluble at their isoelectric pH (approximately the pH of the acidic urine treatment) than at more alkaline pH's. The effect of food on the GI absorption of amobarbital was studied in the rat (1108). The presence of food in the GI tract decreased significantly the serum and brain levels of amobarbital and also reduced the sleeping time induced by the drug. Since amobarbital is mostly absorbed from the small intestine, it was assumed that the decreased pharmacological activity of amobarbital was due to delayed gastric emptying. The urinary excretion of fenfluramine and ethylamphetamine and their main metabolites norfenfluramine and amphetamine was studied in humans with different urinary pH's as a result of their different diets (1109). More (+)-fenfluramine hydrochloride and (+)-ethylamphetamine hydrochloride were excreted when the urine was acidic. Milk and soluble milk proteins decreased the rate of dialysis of caffeine through semipermeable membranes, indicating complex formation (1110). This was also verified by determining the half-lives of absorption of black coffee and black coffee with milk, which were 15 and 30 min, respectively.

Additional references on absorption control and alteration are listed in Table XXXVI.

Absorption Mechanisms—Levodopa was found to be actively transported in rat intestine (1127). As a result of this active transport, it was suggested that there would be a greater difference in the bioavailability of this drug compared to drugs that are absorbed by passive diffusion alone. The transport and binding of methotrexate in rats were studied following intravenous doses of 0.05, 0.25, 2.5, and 25 mg/kg (1128). The transport was saturable and influenced by a strong intracellular binding. Following intragastric administration of thiazolidinecarboxylic acid (hepalidine) to rats, the drug passed rapidly from the stomach to the intestine, where it was rapidly absorbed in the blood (1129). Thiazolidinecarboxylic acid appeared to be absorbed intact by an active transport mechanism. Sodium warfarin was absorbed in the dissociated form by the human stomach and small intestine by a first-order reaction (1130). Curves obtained after intraduodenal and intravenous administrations gave a good simultaneous fit with a two-compartment model, which included an additional compartment accounting for the delay of sodium warfarin in the gastric mucosa. Sulfobromophthalein was excreted in bile of rats 85 and 81% after intravenous and retrograde biliary administrations, respectively (1131). After administration of sulfobromophthalein by either means, metabolites of the drug were observed in bile. No transformation of

the drug in the bile *in vitro* was detected. The data provided evidence that sulfobromophthalein was absorbed from the biliary system into hepatocytes. The transfer behavior of cephalexin across the everted rat intestine was studied using physiological phosphate buffers (1132), and it appeared that a passive transfer process was involved. The rate of transfer of cephalexin across the everted rat intestine increased as the mucosal solution was made more alkaline.

PHARMACOKINETICS

Not only has the number of papers dealing with pharmacokinetics increased yearly, but for the first time a journal dealing specifically with pharmacokinetics is available, *i.e.*, the *Journal of Pharmacokinetics and Biopharmaceutics*. According to the introductory statement by the editors, this journal will be devoted to illustrating the importance of pharmacokinetics and biopharmaceutics in understanding the mechanisms of drug action, design, therapy, and evaluation.

Pharmacokinetics is the study of the kinetics of absorption, distribution, metabolism, and excretion of drugs and their corresponding pharmacology and therapeutic and toxic responses in animals and humans. As a result of this definition, the section called drug absorption which was reviewed separately in previous years will be combined as one section with pharmacokinetics.

Wagner and Sedman (1133) derived equations that quantitatively describe the rate of GI and buccal absorption of acidic and basic drugs as a function of the pH of aqueous lumenal contents and time. They also derived an equation that describes the renal clearance of acidic or basic drugs as a function of urinary pH. In essence, these equations quantitate the pH–partition hypothesis and explain most related observed data in the literature. The results suggested that the aqueous diffusion layer may not rate-limit absorption of monomeric drug molecules but that absorption was rate limited by transfer of drug out of the membrane *in vivo*. Another model was presented for relating turnover time in goldfish to the concentration of drug in the bathing solution (1134). The model was based on passive transport and the existence of a critical concentration of drug within the fish which was necessary for turnover. The developed model was applied to the dose–response data of eight homologous esters of *p*-aminobenzoic acid.

The most commonly used methods for studying intestinal drug absorption, the *in vitro* everted rat gut and the *in situ* rat intestinal loop, were compared with GI absorption using various penicillins, cephalosporins, and tetracyclines (1135). Based on these observations, the *in situ* intestinal loop of the rat appeared to be the most suitable animal model for predicting human drug absorption.

The principle of area analysis was used in the development of a metabolic and pharmacokinetic model for an extensively biotransformed drug (1136). N_4-Ethoxyacetylsulfamethoxazole and its three biotransformation products were administered intravenously to a monkey on separate occasions. Excellent agreement was obtained between the simulated and the actual blood levels of the intact drug and its three biotransformation products. Pharmacokinetic studies were carried out to determine the possible effects of product inhibition on drug elimination (1137). Theoretical considerations and digital computer simulations showed that an apparent increase in the biological half-life of a drug with increasing dose may result from product inhibition if the dissociation constant for the drug metabolite–enzyme complex is appreciably lower than the Michaelis constant for drug–enzyme complexes, if drug metabolite levels remain relatively constant for some time due to slow elimination of the metabolite, and if the level of drug in the body does not appreciably exceed the apparent *in vivo* Michaelis constant.

Wagner (1138) reviewed some old equations and derived some new equations that indicate certain properties of the Michaelis–Menton equation and its integrated forms. He derived an equation that accurately estimates the slope of the linear decline of concentrations from the values of C_0, k_m, and V_m. It was also pointed out that if a metabolite is formed by Michaelis–Menton kinetics, then: *(a)* one would not expect linear plots of cumulative amount of metabolite excreted in the urine *versus* time, and *(b)* the plasma clearance of the drug would change with dose and would be expected to be different following administration of the same dose in a rapidly available and a slowly available dosage form. The kinetics of a drug eliminated by first-order processes in a perfusion-limited isolated perfused organ system were examined and compared with a compartmental model (1139). In the perfusion model, the mean clearance determined by dividing the dose by the area under the blood concentration profile and the steady-state clearance were shown to be equal. A computer program called COMPT was developed for optimizing the solution of integral compartmental models of drug distribution by nonlinear regression analysis (1140). This version of COMPT was designed to solve the two-compartment open model of intravenous drug administration.

A mathematical analysis was made of the type of results to be expected by the classic pharmacokinetic treatment of plasma level data when, in addition to absorption, the drug was simultaneously lost to an extravascular compartment *via* either a parallel first- or zero-order process (1141). A method was presented to develop individualized dosage regimens for cardiac antiarrhythmic drugs by applying steady-state kinetics to pharmacokinetic parameters obtained from single-dose studies (1142, 1143). Dosage regimen calculations were given for intravenous infusion of lidocaine and for oral administration of procainamide and propranolol. An iterative least-squares method for finding the best fit multiexponential equation to observed data was presented (1144). This computer program, written in ALGOL, permitted the routine fitting of most pharmacokinetic data. Theoretical pharmacokinetics, which included the simple one-

compartment model, the one-compartment model with parallel first-order elimination, the two-compartment model, the multiple-compartment model, and the nonlinear nonhomogeneous multiple-compartment model, were reviewed (1145). A method for calculating oral drug absorption constants with two-compartment disposition, based mainly on the maximum point of the concentration–time curve, was developed further and shown to give stable values when random noise was applied to concentration values (1146). Perrier and Gibaldi (1147) warned that calculation of absorption rate constants from plasma drug concentration–time data by commonly employed methods cannot be performed with any degree of confidence unless the drug is known to be completely absorbed as such.

A mathematical technique for estimating the kinetic parameters that control patient response to oral anticoagulant administration was presented (1148). The technique utilized routinely obtained and recorded data such as anticoagulant dose regimen and prothrombin times. Computer-simulated single and multicompartment analyses on the kinetics of placental drug metabolism indicated the need to characterize fully drug metabolism in the mother (1149). Only a measure of relative exposure provided a meaningful basis for determining the potential effects on the fetus of drugs given to the mother during gestation. A mathematical model incorporating features of current theories of pharmacokinetics was advanced using control system theory and was tested on the pharmacokinetics of digitalis glycosides (1150). An attempt was made to simplify two-compartment models into one-compartment models and to see if they could adequately approximate the two-compartment models and be useful for clinical purposes (1151). A review of the effect of temperature on pharmacokinetics including drug absorption, distribution, metabolism, and excretion was presented (1152), as was a review of pharmacokinetics and molecular modification and their implications in drug design and evaluation (1153).

Last year produced the first report of the American Pharmaceutical Association bioavailability pilot project, which outlined the various pharmacokinetic tests and parameters recommended by the task force (1154). The GI absorption of chlormadinone acetate and norethindrone was studied in a rat *in situ* preparation (1155). Both steroids were absorbed to a greater extent in the intestine. The effects of bile duct cannulation, ethanol, and exogenous bile salts were also investigated. Results of studies with flurazepam hydrochloride using rats demonstrated a rapid and complete absorption of the compound, which was followed by a rapid excretion with no apparent accumulation in any tissue (1156). The blood level profile of flurazepam was also studied in humans (1157). The major metabolite was found to be *N*-desalkylflurazepam, which reached steady-state levels after 7 days.

The absorption, distribution, and metabolic fate of seclazone was studied in the rat, dog, and rhesus monkey (1158). Seclazone was readily absorbed by all species. The half-life of blood radioactivity following oral administration of radioactive drug was 10, 8.5, and 6 hr in the rat, beagle hound, and rhesus monkey, respectively. Following oral administration of fenfluramine hydrochloride in human volunteers, fenfluramine was rapidly absorbed by the intestine, accumulating in the tissues (1159). Two hours after administration, norfenfluramine was detected in the plasma. Both fenfluramine and its metabolite were excreted slowly.

Several studies were done on the absorption, metabolism, and excretion of various drugs, including droperidol in humans (1160); macrolide antibiotic SF-837 in rats, dogs, and humans (1161); ^{14}C-niflumic acid [2-(3-trifluoromethylanilino)nicotinic acid] in dogs and humans (1162); propylthiouracil in albino rats (1163); methimazole in albino rats (1164); oxandrolone in humans (1165); and ^{35}S-2-mercaptopropionylglycine in rats (1166).

Clindamycin hydrochloride was administered both orally and parenterally to rats and dogs (1167). Based upon the identical areas under the plasma *versus* time curves, plus the fact that the distribution of the drug excreted in urine and feces was independent of the route of administration, it was concluded that, after oral administration, the absorption of this drug into the bloodstream was almost complete and little appeared to pass through the GI tract for direct elimination. Based on the metabolic patterns found in the urine of both the rat and the dog dosed with clindamycin hydrochloride, sulfoxidation and *N*-demethylation were the predominant metabolic routes in the rat, while conjugation of glucuronic acid and sulfoxidation were the major pathways in the dog (1168). The *in vivo* absorption patterns of four sulfonamides after administration to rabbits as suppositories, with or without buffer reagents, were compared by estimating the blood concentration of sulfonamide as a function of time (1169). These studies showed that both the rate and extent of absorption of sulfonamides were considerably enhanced by the rectal administration of the buffered suppository.

Doluisio *et al.* (1170) studied the pharmacokinetics of intramuscularly administered kanamycin in normal healthy adults and found them to be independent of dose. Plasma levels were described by a one-compartment body model. Kanamycin serum levels during repetitive dosing were accurately predicted from single-dose studies. The pharmacokinetics of oral and intravenous doses of ampicillin were studied when ampicillin was given directly and as its inactive precursor, hetacillin (1171). The major effect of intravenous hetacillin was that early plasma, urine, and peripheral compartment levels of ampicillin resemble those from a rapid absorption process. In oral use in fasting subjects, ampicillin capsules were absorbed to only 32% while hetacillin capsules were absorbed 38%.

The pharmacokinetics of iodochlorhydroxyquin was studied in humans (1172). A clearcut dose–plasma concentration relationship was obtained, and no evidence of accumulation was found. Clindamycin phosphate, when given intramuscularly or intravenously to humans, was rapidly hydrolyzed in the

serum to active clindamycin (1173). The active drug was then distributed rapidly to other fluids and tissues and was handled similarly after intravenous and intramuscular administrations. In a study of the pharmacokinetics of tetracycline hydrochloride, the absorption of the solid dosage form proceeded by a two-step mechanism: *(a)* dissolution in the stomach, which was a function of the dose, formulation variables of the drug, and the pH of the gastric fluid contents; and *(b)* gastric emptying into the intestine where the lumen fluids are favorable for absorption of drug in solution but contribute very little to the dissolution of the emptied particles unless well formulated (1174). In the presence of sulfaethidole, the serum levels of dicloxacillin and penicillin G were significantly increased while the serum levels of oxacillin, methicillin, and ampicillin were relatively unchanged (1175). The extent of protein binding influenced the renal clearance of the penicillins. As the percent of bound penicillin increased, the renal clearance decreased. The total clearance of penicillins from the body did not exhibit any relationship with the extent of protein binding.

The concentrations of diphenylhydantoin in the blood of male rats after intravenous injection were determined over a sufficiently wide range to permit comparison of rates of decline at the same absolute and relative concentrations (1176). This comparison led to the conclusion that the elimination of diphenylhydantoin in the rat cannot be described by first-order or simple Michaelis–Menten kinetics but was qualitatively consistent with product inhibition of diphenylhydantoin metabolism. A pharmacokinetic analysis of digoxin in humans was applied to digoxin dosage adjustment in severe renal failure (1177). The analysis revealed that digoxin distribution and elimination in humans could be described adequately by a two-compartment open kinetic model. Because of the finding of a pronounced decrease in the apparent volume of distribution of digoxin in severe renal failures, it was suggested that the loading dose be decreased in these patients.

The pharmacokinetics of sotalol in dogs was studied, and unchanged sotalol was excreted up to 90% in the urine (1178). There was no protein binding, and the partition coefficient between plasma and the red blood cells was unity. Pharmacokinetics of diazepam in humans following single intravenous and oral administration and chronic oral administration were studied (1179). The intravenous blood level data were fitted with a three-compartment open-model system containing both a "shallow" and a "deep" compartment. Orally administered diazepam was rapidly and completely absorbed. Following chronic administration, the minimum and the maximum steady-state levels of diazepam were successfully predicted utilizing pharmacokinetic parameters obtained following intravenous administration. The oral bioavailability and pharmacokinetics of soluble and resin-bound forms of amphetamine and phentermine were studied in humans (1180). The one-compartment open model with first-order drug absorption was fitted to the data from each subject by nonlinear regression methods which provided an excellent fit. In both cases, the rate constant for absorption was significantly lower and less variable for the resinated compound.

The pharmacokinetics of ^{3}H-methotrexate were studied in 22 patients with malignancies (1181). Following the intravenous administration, the plasma disappearance was triphasic. It was observed that methotrexate had a long terminal half-life, which may explain the high incidence of toxicity in patients receiving chronic low-dose methotrexate therapy.

Pharmacokinetics of pentobarbital were determined after intravenous administration (1182). Pentobarbital was distributed in at least two kinetically distinct body compartments: *(a)* a central or "serum" compartment, and *(b)* a peripheral or "tissue" compartment. In oral administration, it was found that the presence of food significantly reduced the apparent absorption rate but not the total amount absorbed. In addition, several other papers appeared on the pharmacokinetics of specific drugs: propylthiouracil in humans (1183), warfarin in humans (1184), intravenous theophylline in humans (1185), isosorbide in humans (1186), minocycline in humans (1187), adriamycin in humans (1188), tobramycin and gentamicin in humans (1189), tolbutamide in rabbits (1190), digoxin in humans (1191), (+)-, (−)-, and (±)-hexobarbital in humans (1192), and phentermine and chlorphentermine in rats (1193).

Additional references on pharmacokinetics are listed in Table XXXVII.

Table XXXVII—Additional References on Pharmacokinetics

Reference	Topic	Reference	Topic
1194	Routes of drug administration, and absorption of drugs from GI tract	1200	Review of kinetics of drug absorption, elimination, placental transfer, and biliary excretion
1195	Routes of drug administration, and buccal absorption of drugs	1201	Review of absorption and distribution of drugs in the organism
1196	Routes of drug administration, and subcutaneous and intramuscular injection of drugs	1202	Review of individual pharmacokinetic differences in humans
1197	Routes of drug administration, and absorption, distribution, and excretion of gaseous anesthetics	1203	Review of pharmacokinetics and cell population growth models in cancer chemotherapy
1198	Routes of drug administration, and aerosols	1204	Review and general discussion of pharmacokinetics
1199	Review of absorption, distribution, and excretion of drugs	1205	Biotransformation of iomeglamic acid

(continued)

Table XXXVII—*Continued*

Reference	Topic
1206	Review of absorption, distribution, and metabolism of various classes of drugs in animals and humans
1207	Review of compartmental modeling of pharmacokinetic action
1208	Review of theoretical aspect of pharmacokinetics
1209	Review of role of pharmacokinetics in pharmaceutical research
1210	Review of pharmacokinetics of intravenous injection
1211	Sartorius absorption model for investigating absorption of passively transported drugs *in vitro* with artificial lipid barriers
1212	Effect of route of administration on drug metabolism
1213	Solvent drag influence on intestinal absorption of aminopyrine and antipyrine
1214	Methods of obtaining preliminary estimates to fit two-term exponential model to blood concentration data
1215	Review of implication of biotransformation of a medicament on therapeutic potentiality and dangers of its use
1216	Significance of linear decrease in plasma drug levels for pharmacokinetics
1217	Review of theory and applications concerning intermittent dosing, continuous applications, nonaccumulative dose schedules, and calculation of initial doses
1218	Review of role of pharmacokinetics in biopharmaceutical research
1219	Graphical determination of rate constants of first-order reactions
1220	Definition and determination of important pharmacokinetic parameters for dosage regimen in one-compartment model
1221	Pharmacodynamic model for cell-cycle-specific chemotherapeutic agents
1222	Drug transfer across intact rat intestinal mucosa following surgical removal of serosa and muscularis externa
1223	Absorption, excretion, and urinary metabolic pattern of ^{3}H-albuterol aerosol in humans
1224	Synergistic effect of ampicillin and dicloxacillin, and absorption and excretion
1225	Distribution of aspirin in rumen and corpus tissues of rat stomach during first 4 min after administration
1226	Metabolism of benzydamine hydrochloride, and species differences and identification of unconjugated metabolites in rabbit urine
1227	GI absorption and anti-inflammatory effect of bromelain
1228	Factors influencing intestinal absorption of calcium
1229	Absorption of organic arsenical compounds from rat small intestine
1230	Absorption kinetics of different brands of chloramphenicol
1231	Metabolism of cloxazolam, and distribution, excretion, and biotransformation in rats and mice
1232	Cyclophosphamide metabolism in the rabbit
1233	Intestinal absorption of ^{3}H-digitalis glycosides in experimental animals and humans
1234	Absorption of orally given digoxin preparations
1235	Double peak in the plasma–drug curve after oral digoxin and lanatoside C
1236	GI absorption of iopanoic acid
1237	Physiological disposition of a new diuretic, ^{14}C-metolazone, in dogs
1238	Absorption and distribution of naloxone in rats after oral and intravenous administrations
1239	Absorption of nicotine 1′-*N*-oxide and its reduction in GI tract in humans
1240	Metabolic fate of nitrofuran derivatives, and degradation by small intestinal mucosa and absorption from GI tract
1241	Metabolism of oxaflumazine
1242	Kinetics of absorption of pralidoxime chloride in dogs
1243	Prazepam metabolism in rats
1244	Accumulation and steady-state concentrations during chronic oral administration of propranolol in humans
1245	Excretion and metabolism of reserpine in renal failure
1246	Plasma concentrations and urinary excretions of sulfamethoxazole and trimethoprim in humans
1247	Absorption of drugs from rat lung
1248	Dissolution and absorption of sulfathiazole
1249	Absorption and distribution of ^{14}C-sulfobenzylpenicillin
1250	Absorption of tetracyclines in sheep intestines
1251	Pharmacokinetics of antibacterial activity of a new semisynthetic penicillin, 6-(1-aminocyclohexylcarboxamido)penicillanic acid
1252	Elimination kinetics of amobarbital in mothers and their newborn infants
1253	Comparative study of pharmacokinetics of amphetamine
1254	Effects of diethyl dithiocarbamate and ethanol on *in vivo* metabolism and pharmacokinetics of amphetamine in the rat
1255	Ampicillin pharmacokinetics in mice and humans
1256	Kinetics of three structurally related bicyclic antidepressant compounds in the dog
1257	Routes and kinetics of excretion of arsenic in rats administered organoarsenical drugs
1258	Biopharmaceutical and pharmacokinetic aspects of aspirin intoxication in humans
1259	Pharmacokinetics of apazone-^{14}C (azapropazone-^{14}C) in rats
1260	Pharmacokinetic studies on apazone-^{14}C dihydrate in humans
1261	Absorption, distribution, biotransformation, and excretion of azidocillin in mice, rats, and dogs
1262	Intestinal absorption, intestinal distribution, and excretion of ^{14}C-butylscopolamine in rats
1263	Pharmacokinetics of carbenicillin in neonates of normal and low birth weight
1264	Pharmacokinetic studies of carbenicillin in newborns and prematures
1265	Serum and blood concentration of sodium cephalexin in humans given single intramuscular and intravenous injections
1266	Pharmacokinetics of a new cephalosporin, cephacetrile sodium (CIBA BA 36278A)
1267	Comparative billiary excretion of different cephalosporins
1268	Cephradine absorption and excretion in fasting and nonfasting volunteers
1269	Pharmacokinetics of (2-chlorophenyl)diphenyl-1-imidazolylmethane
1270	Biopharmaceutical evaluation of catechin (cyanidanol) tablets using pharmacokinetic techniques
1271	Metabolism and pharmacokinetics of a new nonsteroid, anti-inflammatory agent, clonixin, in rats, dogs, and monkeys
1272	Pharmacokinetics of creatinol *O*-phosphate
1273	Absorption, organ distribution, and metabolism of diethylaminoethanol after oral administration to rats
1274	Metabolism and excretion of diethylpropion in humans under acidic urine conditions
1275	Pharmacokinetics of digoxin and its 4‴-acetyl and methyl derivatives in rats
1276	Pharmacokinetics of cardiac glycosides, digoxin and digitoxin, in dogs
1277	Metabolism of antihypertensive agent, 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester
1278	Pharmacokinetics of diphenyl(2-chlorophenyl)-1-imidazolylmethane-^{14}C after topical applications

Table XXXVII—*Continued*

Reference	Topic
1279	Pharmacokinetics and distribution of diphenylhydantoin in kittens
1280	Pharmacokinetic study of patient with diphenylhydantoin toxicity
1281	Pharmacokinetics of dipropylacetamide in rats after oral administration
1282	Absorption, distribution, and excretion of doxapram hydrochloride in rats
1283	Behavior of doxycycline administered orally or intravenously
1284	Effect of route of administration on distribution of ellipticine in mice
1285	Kinetics of entry and distribution of fluorouracil in cerebrospinal fluid and brain following intravenous injection in a primate
1286	Pharmacokinetic studies on flupenthixol and flupenthixol decanoate in humans
1287	Pharmacokinetics of ^{14}C-fominoben in rats and gravid mice
1288	Pharmacokinetics of gentamicin after repeated administration
1289	Pharmacokinetics and metabolism of glyburide (glibenclamide) in presence of phenylbutazone
1290	Pharmacokinetics of glibornuride
1291	Pharmacokinetics of guanazole in humans
1292	Pharmacokinetics of heparin and heparinoids
1293	Pharmacokinetics of ^{3}H-hexoprenaline in rats
1294	Pharmacokinetics, toleration, and safety of indanylcarbenicillin in humans
1295	Pharmacokinetics of indomethacin in pregnant women and in parturient women and their newborns
1296	Pharmacokinetics of iron absorption with a new iron preparation
1297	Pharmacokinetics of isoniazid in humans
1298	Comparative pharmacokinetics of a semisynthetic aminoglycoside derived from kanamycin (BB-K8) and kanamycin in dogs and humans
1299	Lidocaine pharmacokinetics in advanced heart failure, liver disease, and renal failure in humans
1300	Half-life, metabolism, and excretion of tritiated luteinizing hormone-releasing hormone in humans
1301	Metronidazole pharmacokinetics in nonpregnant women
1302	Pharmacokinetics of metronidazole and tinidazole in humans
1303	Plasma levels and derived pharmacokinetic characteristics of unchanged nitrazepam in humans
1304	Pharmacokinetics of nortriptyline in humans after single and multiple oral doses, and predictability of steady-state plasma concentrations from single-dose plasma level data
1305	Genetic and pathological basis for individual differences in pharmacokinetics of nortriptyline and diphenylhydantoin in humans
1306	Review on interindividual differences in pharmacokinetics of nortriptyline and diphenylhydantoin
1307	Pharmacokinetics of ^{14}C-labeled penicillamine
1308	Determination and pharmacokinetics of oxacillin and ampicillin on simultaneous administration
1309	Comparative pharmacokinetics of oxazepam and nortriptyline after single oral doses in humans
1310	Pharmacokinetics of pirazocillin in dogs and rats
1311	Pharmacokinetics of polymyxin methanesulfonic acid in rats
1312	Pharmacokinetic studies of practolol in humans
1313	Pharmacokinetics of dextro-, levo-, and racemic propranolol in humans
1314	Pharmacokinetics of rifampin in patients with impaired renal function, before and during hemodialysis
1315	Sodium salicylate pharmacokinetics in dogs
1316	Biotransformation and pharmacokinetics of salicylate in newborn animals
1317	Renal contribution to overall metabolism of drugs, and biotransformation of salicylic acid to salicyluric acid in humans
1318	Pharmacokinetics of capobenate sodium [sodium 6-(3,4,5-trimethoxybenzamido)hexanoate; C-3] in humans
1319	Pharmacokinetics of succinic acid dinitrile in animals and humans
1320	Pharmacokinetics of sulfaclomide in early pregnancy
1321	Pharmacokinetics of sulfaguanole, and multiple-dose kinetics in humans
1322	Absorption, distribution, excretion, and metabolism of sulfamethoxazole in rats
1323	Pharmacokinetics of trimethoprim and its combination with sulfamethoxazole in humans after single and chronic oral administration
1324	Bacteriological and pharmacokinetic studies of sulfamethoxazole–trimethoprim
1325	Pharmacodynamics and pharmacokinetics of sulfamethoxazole and trimethoprim
1326	Pharmacokinetic data of combination of sulfamethoxazole plus trimethoprim in patients with renal impairment
1327	Pharmacokinetics of tetraethylammonium in cats
1328	Uptake and distribution of thiopental after oral, rectal, and intramuscular administration, and effect of hepatic metabolism and injection site blood flow
1329	Pharmacokinetic studies of tobramycin and gentamicin in humans
1330	Pharmacokinetics of trichloroethanol and metabolites, and interconversions among variously referenced pharmacokinetic parameters
1331	Pharmacokinetics of the antibiotic virginiamycin in rats
1332	Pharmacokinetic aspects of drug absorption, distribution, and elimination

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ˣTo whom inquiries should be directed.

Physicochemical Properties, Solubility, and Protein Binding of Δ^9-Tetrahydrocannabinol

EDWARD R. GARRETT[x] and C. ANTHONY HUNT

Abstract □ The rate and extent of glass binding of Δ^9-tetrahydrocannabinol in aqueous solution depend on the surface area and pretreatment of glass and the concentration of the drug. A total of 20 and 40% at 0.1 and 0.05 μg/ml, respectively, was bound to 50-ml volumetric flasks but could be minimized by silyl pretreatment of the glass. The drug rapidly diffused into plastics, and 70–97% was taken up by the rubber closures used for plasma vials. These bindings precluded classical methods of solubility determination, so spectral and particle-size counting determinations, which observed those concentrations at which true solution was terminated, were used. The aqueous solubility was a linear function of both the ethanol concentration (increasing) at constant ionic strength and the square root of the ionic strength (decreasing) at constant ethanol concentration. The salting-out coefficient was of high magnitude and typical solubilities were 2.8 mg/liter in water and 0.77 mg/liter in 0.15 *M* NaCl at 23°. The bindings also precluded the use of the classical methods of equilibrium dialysis and ultrafiltration to determine the protein binding of tetrahydrocannabinol. A method of variable plasma concentrations was devised, so protein binding was determined from the pseudoplasma concentrations of the drug after the separation of the pseudoplasma from the red blood cells added to form pseudoblood with known concentrations of Δ^9-tetrahydrocannabinol. This use of the competition between the high partitioning of drug between red blood cells with plasma water (D = 12.5) and the binding to plasma protein permitted an estimate of 97% binding which was not drug concentration dependent. The spectrophotometric pKa′ of Δ^9-tetrahydrocannabinol was 10.6. Δ^9-Tetrahydrocannabinol degraded readily in acid solutions. Subsequent to a rapid loss, the kinetics appeared to be first-order and specific hydrogen-ion catalyzed. Concomitantly, small amounts of Δ^8-tetrahydrocannabinol were produced, as were two GLC observable products, P_2 and P_3, and the rate of their appearance appeared to parallel the rate of Δ^9-tetrahydrocannabinol degradation. A peak, P_1, also appeared almost instantaneously but did not parallel drug degradation.

Keyphrases □ Tetrahydrocannabinol—physicochemical properties, solubility, and protein binding □ Marijuana—physicochemical properties, solubility, and protein binding of tetrahydrocannabinol □ Protein binding—tetrahydrocannabinol

Tetrahydrocannabinol is highly insoluble in water (1–3). This can be a critical factor in its bioavailability, pharmacokinetics, and pharmacological action. Large differences in the bioavailability of tetrahydrocannabinol from various solutions and administrative routes have been reported (2, 3). Metabolism is extremely rapid (4–6) and appears to be even faster than chloral hydrate (7) which has a half-life of 3 min. Nevertheless, tetrahydrocannabinol persists in the plasma for 72 hr after a single intravenous dose (4, 5). This is a possible consequence of a slowly releasing deep compartment (8). Evidence has been presented (1) that tetrahydrocannabinol's solubility may be exceeded in plasma, resulting in its possible precipitation and fortuitous localized accumulation in body organs which may act as such a deep compartment.

Stabilities have been reported for tetrahydrocannabinol on filter paper (9) and in various organic solvents (10), but no report is available on its aqueous stability or pKa′.

All relevant physicochemical properties of a drug should be obtained prior to initiating detailed pharmacokinetic investigations (8). This paper presents the results of studies on the solubility, pKa′, stability, partitioning, and protein binding of tetrahydrocannabinol and their biopharmaceutic and pharmacokinetic relevance. The fact that tetrahydrocannabinol is so highly bound to plasma proteins that its extent is difficult to determine by classical procedures has led to the development of a new method of variable plasma concentrations that permits estimation by extrapolation.

EXPERIMENTAL

Determination of Solubility—*Method A*—Twelve stock solutions of aqueous ethanol were prepared at varying ionic strengths. At each ionic strength the ethanol concentrations were 5.0, 7.5, 10.0, and 12.5% (v/v). The ionic strengths at each ethanol concentration were adjusted to 0.05, 0.1, and 0.15 with sodium chloride. Δ^9-Tetrahydrocannabinol[1] was dissolved in absolute ethanol to yield a stock solution containing 4 mg/ml.

Four milliliters of a stock solution was pipeted into matched 1-cm cells. A small volume (0.25–1.0 μl) of the tetrahydrocannabinol stock solution was added to one cell which was stoppered and shaken. The absorbance was then recorded[2] against the blank between 350 and 215 nm. This process was repeated until turbidity was clearly evident. The cell was washed and the process was repeated.

Beer's law plots were obtained by plotting the absorbance at one wavelength *versus* the amount of tetrahydrocannabinol added. The solubility of tetrahydrocannabinol in these solutions was assigned to that concentration at which deviation from Beer's law was observed.

Method B—The procedure of Saad and Higuchi (11) for determining solubility by using a particle-size counter[3] was modified. All solutions were filtered through a 0.45-μm pore size filter[4], and all samples were counted using a 30.0-μm aperture. The coincidence corrections for the counter were made on a calculator[5] programmed in accordance with the coincidence correction chart

[1] S.S.C. Lot 61591, furnished by the Department of Health, Education, and Welfare, U.S. Public Health Service, National Institute of Mental Health, Bethesda, MD 20014

[2] Cary model 15, Applied Physics Corp., Monrovia, Calif.

[3] Coulter counter, model ZBI, Coulter Electronics, Hialeah, Fla.

[4] Millipore Filter Corp., Bedford, Mass.

[5] Wang 700, Wang Laboratories Inc., Tewksbury, Mass.

supplied in the manufacturer's manual. The total counts were also corrected for background. The background for each sample averaged 5000/50 μl.

Twenty-four milliliters of filtered 0.9% NaCl was added to each of two 30-ml serum vials. Five microliters of a tetrahydrocannabinol solution (1.45 mg/ml in 95% ethanol) was added to each vial. The contents were stirred for 1 min with a 0.6-cm (0.25-in.) Teflon-coated magnetic stirring bar. Fifty microliters of the first vial was counted, and 50 μl from the second vial was then transferred to the first. Five microliters of the stock tetrahydrocannabinol solution was again added to each vial; the vials were counted and then the procedure was repeated until turbidity was clearly evident.

Spectrophotometric pKa′ Determination—The molar absorptivities of tetrahydrocannabinol and its anion were obtained between 210 and 350 nm using 0.5 *N* HCl and 0.5 *N* NaOH in 50% aqueous ethanol. A maximum difference in molar absorptivity of 8.5×10^3 occurred at 240 nm.

Buffer solutions with pH values between 7.1 and 13 (12) were adjusted to 0.1 ionic strength with sodium chloride. Buffer solution (29.5 ml) was added to each of two 10-cm cells. Tetrahydrocannabinol stock solution (5.00 μl) *(Determination of Solubility, Method A)* was added to one cell. The cell was stoppered and shaken, and the absorbance was recorded against the blank at 240 nm. This process was repeated until a valid Beer's law plot was obtained, and three such plots were obtained at each pH. The slopes of these plots (molar absorptivity) were used to calculate the pKa′.

Analytical Methods—The concentration of tetrahydrocannabinol was monitored by GLC. All quantitative analyses of Δ^9- and Δ^8-tetrahydrocannabinol, when at least 0.1 μg/sample was available for analysis, were performed with flame-ionization detection as described previously (14). When less than 0.1 μg was available, the pentafluorobenzoate ester of tetrahydrocannabinol was prepared and analyzed with electron-capture detection (13). This latter method does not differentiate between the Δ^8- and Δ^9-compounds.

Glass Binding—The binding of tetrahydrocannabinol to glass in various solutions was followed with time by analyzing the tetrahydrocannabinol concentration by GLC.

Method A—Clean volumetric flasks (50 ml) were filled with isotonic pH 7.4 phosphate buffer and binding was investigated. Two-tenths milliliter of ethanol, containing either 5 or 2.5 μg of tetrahydrocannabinol, was added to each flask to establish tetrahydrocannabinol concentrations of 0.1 and 0.05 μg/ml, respectively. The flasks were stoppered and shaken, and the contents were transferred to a 125-ml separator containing 15 ml of methylene chloride[6]. The extracting methylene chloride layer was transferred to a 15-ml glass-stoppered test tube and dried under nitrogen in a 55° water bath. The aqueous layer was extracted a second time with 10 ml of methylene chloride. The second extract was dried in the same tube, 100 μl of chloroform[7] with the internal standard was added, and aliquots were analyzed on the gas chromatograph. One hundred percent recovery was defined as the peak height ratio between tetrahydrocannabinol and the internal standard tetraphenylethylene obtained when 50 ml of an aqueous solution was extracted directly from a 100-ml volumetric flask.

In addition, similar studies were conducted using a water-soluble silicone concentrate[8] and trimethylsilyl-treated glassware. The former was used in accordance with the manufacturer's instructions (14) and the latter was effected by filling the glassware with a 1% solution of a silylating reagent[9] in chloroform and allowing this to stand for 30 min at room temperature. The silylating solution was then discarded and the glassware was rinsed once with chloroform and dried in an oven at 120° for 2 hr.

Method B—The extent of glass binding of tetrahydrocannabinol was also studied at low concentrations (1-100 ng/ml) of ^{14}C-Δ^9-tetrahydrocannabinol[10] in buffer, plasma, and whole blood. The ^{14}C-Δ^9-tetrahydrocannabinol and the unlabeled drug were combined in 50% aqueous ethanol to yield solutions with equivalent disintegrations per minute per milliliter and varying tetrahydrocannabinol concentrations. The details of the procedure are presented subsequently.

Method C—Serum vials[11] (5 and 10 ml) were washed, soaked in ethanol, and allowed to air dry. Four 10- and four 5-ml vials were filled with 9 and 4.5 ml, respectively, of pH 7.55 isotonic phosphate buffer. Three solutions of Δ^9-tetrahydrocannabinol and the ^{14}C-labeled compound were prepared to yield 0.0785, 0.785, and 24.8 ng/μl and 13.49 dpm/μl in each case. An aliquot (90 μl) of each solution was added to one each of the 5-ml vials, and 180 μl was added to one each of the 10-ml vials. The solutions were stirred for 5 min with a 0.6-cm (0.25-in.) Teflon stirring bar. The stirring bars were then removed (no significant radioactive residue remained on the stirring bar) and the solutions were allowed to stand for 30 min. Three milliliters was removed for analysis from the 10-ml vials and 2 ml from the 5-ml vials. These samples were extracted as described later for plasma samples. Each vial was then stoppered with its original rubber stopper and shaken slowly (60 rpm) on an automatic shaker for 1 hr. Duplicate volumes were again removed for analysis.

Protein Binding by Classical Methods—Equilibrium dialysis and ultrafiltration were used to determine the protein binding of tetrahydrocannabinol to plasma proteins. The details of these procedures were reported previously (15, 16). In both cases, isotonic phosphate buffer (pH 7.4) was used as a reference solution and drug concentrations of 0.05, 0.1, and 1.0 μg/ml were investigated.

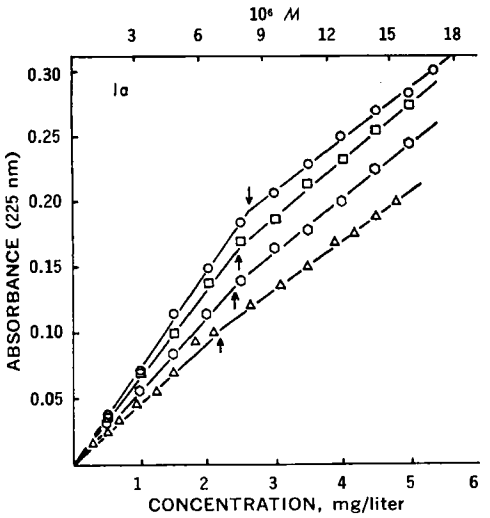
Protein Binding by Method of Variable Plasma Concentrations—Fresh, heparinized whole dog blood was centrifuged. The plasma was transferred to a glass-stoppered test tube; the red blood cells were washed with isotonic phosphate buffer and centrifuged, and the buffer phase was discarded. This procedure was repeated three times. The packed red blood cells were gently mixed and duplicate hematocrits (H_1) were taken.

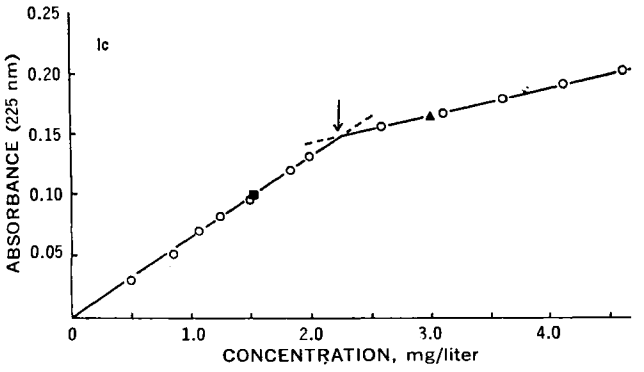
Appropriate amounts of plasma and isotonic phosphate buffer (pH 7.5) to give mixtures with 0, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, and 100% plasma were prepared. The desired mixtures of ^{14}C-labeled and unlabeled drug in absolute ethanol were added to 50-ml glass centrifuge tubes and dried under nitrogen. The drug was reconstituted in 0.2 ml of isotonic 50% ethanol-water, and 3.00 ml of a particular plasma-buffer mixture was added so that concentrations of 1, 5, 10, 25, 50, 75, 100, 250, 500, and 1000 ng tetrahydrocannabinol/ml pseudoplasma could be studied. Five milliliters of 95% ethanol was added to one of these tubes to serve as a blank. All tubes but the blank were stoppered and placed in a 37.5° water bath for 10 min, at which time 2 ml of the packed red blood cells at 37.5° was added to each tube. Duplicate hematocrits (H_2) were made from each tube, which were then centrifuged at 3000 rpm for 10 min. An aliquot (2.5 ml) of plasma was removed for subsequent tetrahydrocannabinol analysis. The packed red blood cells were resuspended in the plasma residue, and a third hematocrit (H_3) was made on two samples of this resuspension. One milliliter of this suspension was transferred to a 50-ml glass test tube containing 1 ml of distilled water and hemolyzed.

One milliliter of a saturated sodium chloride solution was added to the 2 ml of the 2.5 ml of plasma previously removed and to the 2.0-ml sample containing the hemolyzed red blood cells. Heptane (15 ml) with 1.5% isoamyl alcohol was added to each. The tubes were stoppered and shaken on an automatic shaker for 20 min. The tubes were then centrifuged to separate the layers, and the heptane layers (14 ml) were transferred to liquid scintillation counting vials and dried under nitrogen. Ten milliliters of a liquid scintillation cocktail[12] was added to each vial, and each sample was counted[13] three times for 10 min. Addition of a quantitative amount of ^{14}C-toluene (904 dpm) indicated no significant quench.

An aliquot (2.00 ml) of the blank was transferred directly to a counting vial and dried to represent 100% recovery. Since the

[6] Nanograde dichloromethane, Mallinckrodt Chemical Works, St. Louis, Mo.
[7] Nanograde chloroform, Mallinckrodt Chemical Works, St. Louis, Mo.
[8] Siliclad, water-soluble silicone concentrate, Clay Adams, Parsippany, N.J.
[9] Regisil, Regis Chemical Co., Chicago, Ill.
[10] Lot CP-1, R.T.I., Research Triangle Park, N.C.
[11] Vacutainer, nonheparinized, Becton-Dickinson, Rutherford, N.J.
[12] Spectrafluor, Amersham Searle, Arlington Heights, Ill.
[13] Packard Tri-Carb, series 3000, Packard Instrument Co., Downers Grove, Ill.





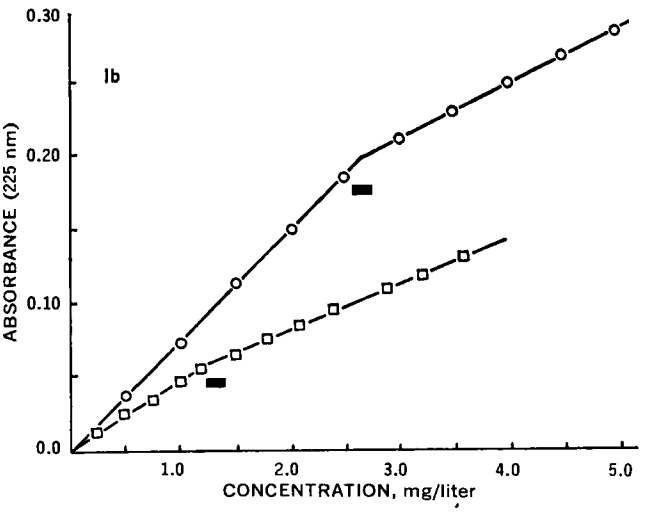


Figure 1—*Typical absorbances of tetrahydrocannabinol at 225 nm in various aqueous ethanol solutions as a function of concentration. (a) An ionic strength of 0.05 was used. Key: ○, 12.5% ethanol; □, 10% ethanol; ○, 7.5% ethanol; and △, 5% ethanol (v/v). The arrows indicate the concentrations where deviations from Beer's law occurred. Each point is the mean of two replicate studies. (b) The solid bars indicate the range of* ± **1 SD** *in the estimated concentration where deviations from linearity occurred. Key: ○, 12.5% ethanol (v/v) and 0.05 ionic strength; and □, 5% ethanol and 0.15 ionic strength. Each point is the mean of three replicate studies. (c) The absorbance (○) of tetrahydrocannabinol in 12.5% ethanol at 0.1 ionic strength where the arrow indicates the concentration at which the deviation from Beer's law occurred. The ▲ value was obtained by a 1:0.67 dilution of the final solution (4.6 μg/ml). The ■ value was obtained by a 1:0.33 dilution of the final solution.*

original packed and washed red blood cells obtained from whole blood retained a significant fraction of the washing buffer solution (12–22%), it was necessary to recalculate the percentage of plasma in the synthetic diluted plasma–buffer mixtures.

The extent of red blood cell-plasma distribution was monitored as a function of time.

Stability of Δ^9-Tetrahydrocannabinol in Acid Solutions—The stability of 1 liter of 1 μg/ml Δ^9-tetrahydrocannabinol was followed with time at 55° in 0.5, 0.1, 0.05, and 0.01 *N* HCl, with the ionic strength of 0.1 with sodium chloride in the last two cases. An aliquot of 1 ml of ethanol containing 1 mg of tetrahydrocannabinol was added to each liter of solution. Aliquots (48.0 ml) were sampled after vigorous shaking and transferred to a 125-ml separator containing 5 ml of enough sodium hydroxide to neutralize the hydrochloric acid. The solution was extracted twice with 15 ml of methylene chloride, and the pooled organic extracts were analyzed quantitatively by GLC.

RESULTS AND DISCUSSION

Determination of Solubility—Attempts to use classical methods of solubility determination were unsuccessful. When a saturated solution was filtered, the filtrate showed no detectable tetrahydrocannabinol due to the high affinity of the dissolved tetrahydrocannabinol for the filter paper. When a saturated solution was ultracentrifuged and the clear solution was decanted, this solution showed little and variable tetrahydrocannabinol due to the more rapid diffusion of solution tetrahydrocannabinol into the plastic (polycarbonate and nitrocellulose) of the centrifuge tube over the rate of reequilibration of the sedimented excess tetrahydrocannabinol with the solution. This hypothesis was validated further by the fact that the amount of tetrahydrocannabinol in the clear solution in the ultracentrifuged tube decreased with time of standing.

Typical data used to determine the solubility of tetrahydrocannabinol spectrophotometrically in various aqueous ethanol solutions at constant ionic strength are given in Fig. 1. The data initially followed Beer's law. Subsequent to the deviation from linearity, each addition of tetrahydrocannabinol resulted in increasing turbidity. The concentration where the deviation occurred was reproducible. The standard deviations (as percent of mean) of the concentrations at which such deviations occurred ranged from 6.32% in 5% ethanol at 0.15 ionic strength to 2.5% in 12.5% ethanol at 0.05 ionic strength. The dilution of a turbid solution below the apparent solubility limit resulted in the same absorbance obtained by the addition of increasing amounts of tetrahydrocannabinol. This reversibility was consistent with the assignment of the limit of the solubility of tetrahydrocannabinol to that concentration where the deviation from Beer's law occurred.

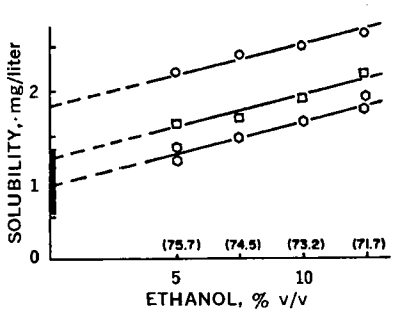


Figure 2—*Solubility as estimated from the observed deviations from Beer's law for tetrahydrocannabinol at various ionic strengths plotted as a function of percent ethanol in aqueous solutions. Key: ○, 0.05 ionic strength; □, 0.1 ionic strength; and ○, 0.15 ionic strength. The solid bar at the intercept includes the mean and the standard deviation for the determined solubility (Method B) in pure water at physiological ionic strength (0.9% NaCl). The numbers in parentheses are the dielectric constants for the four ethanol–water solutions used.*

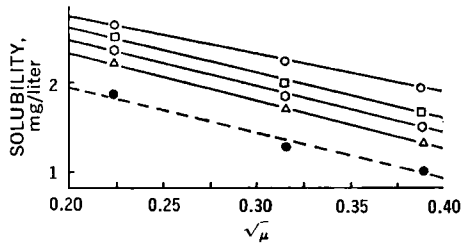


Figure 3—*Solubility of tetrahydrocannabinol in various aqueous ethanol solutions as a function of the square root of the ionic strength, $\sqrt{\mu}$. Key: ○, 12.5% ethanol; □, 10% ethanol; ○, 7.5% ethanol; △, 5% ethanol; and ●, solubility in pure water as obtained from extrapolation to 0% ethanol.*

The solubility was a linear function of both ethanol concentration (at constant ionic strength) (Fig. 2) and the square root of the ionic strength (at constant ethanol concentration) (Fig. 3). The solubility of tetrahydrocannabinol in water at various ionic strengths was estimated from the extrapolated intercepts of Fig. 2 and also are plotted in Fig. 3, where its extrapolated intercept estimates a solubility of 2.8 mg/liter for tetrahydrocannabinol in pure water at 23.0°. Although reasonable reproducibility in estimated solubilities was obtained with fixed ionic strengths, enormous variability in solubility estimates were obtained among replicate studies in pure water.

An often used empirical equation employed in the study of solubilities of unionized solutes in water (17) is:

$$\log S_0/S = k_s C + \log \alpha \qquad \text{(Eq. 1)}$$

where S_0 is the solubility of the solute in pure water, S is the solubility in a dilute salt solution of concentration C, k_s is the "salting-out coefficient," and the constant $\log \alpha$ is taken as zero.

The nonzero intercepts, $\log \alpha = 0.8$ and $\alpha = 6.3$, of such plots for tetrahydrocannabinol solubilities (Fig. 4) were reproducible on replication and when the solvent systems were carefully purified. A possible explanation may be that the observed solubility is not linearly related to ionic strength below the lowest ionic strength measured and it is invalid to accept the extrapolated intercept at zero salt concentration (Fig. 3) or that the solubility is overestimated since the solute does bind at the cell-solvent interface.

The apparent solubility in pure water may be corrected for this phenomenon so that $S_0' = S_0/\alpha$. The k_s values of Eq. 1 are plotted in the insert of Fig. 4 as a function of percent ethanol, and the

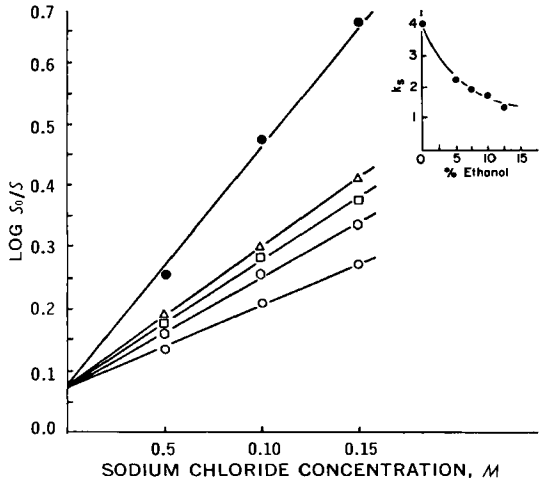


Figure 4—*Function log S_0/S plotted against the concentration of sodium chloride. S_0, the apparent solubility of tetrahydrocannabinol in pure water, was obtained from the extrapolation of the solubilities to 0% ethanol and zero ionic strength and S was the observed solubility. Key: ●, 0% ethanol; △, 5% ethanol; □, 7.5% ethanol; ○, 10% ethanol; and ○, 12.5% ethanol (v/v). The insert is a plot of the salting-out coefficients, k_s, as a function of percent ethanol (v/v).*

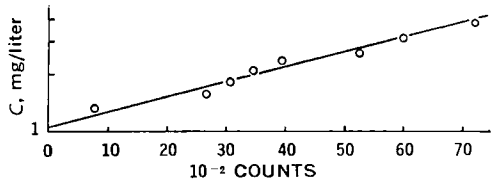


Figure 5—*Solubility of tetrahydrocannabinol in 0.9% NaCl estimated by the particle-size counting method on the postulate that the function log tetrahydrocannabinol concentration (C), against counts, corrected for background, is linear. The correlation coefficient for the data plotted in this manner is r = 0.986. The intercept, which estimates the solubility in 0.9% NaCl, is 1.05 mg/liter.*

log of the apparent solubility, S, at 23.0° may be expressed as:

$$\log S = \log S_0/\alpha - k_s C \qquad \text{(Eq. 2)}$$

for $\alpha = 6.3$ and for the salt concentration, C, in moles per liter.

These salting-out coefficients, k_s, calculated for tetrahydrocannabinol are larger than those reported for more polar neutral solutes such as phenol (0.22) and salicylic acid (0.196) (18).

The particle-size counting procedure of Saad and Higuchi (11) overestimated the true solubility since there is a limit below which particle size cannot be counted.

A plot of the corrected counts *versus* concentration of tetrahydrocannabinol after serial addition of the drug is given in Fig. 5. The intercept at zero net counts gives a mean solubility estimate of 1.05 mg/liter (σ = 0.38 mg/liter) for the solute in 0.9% NaCl (0.154 *M*). This is in good agreement with the water solubility of 0.77 mg/liter in 0.15 *M* NaCl calculated from Eq. 2.

As with cholesterol suspensions (11), size-distribution analyses by this method of the various concentrations of solutes gave no evidence of a preferred micellar size. The frequency of observable particles apparently increased semilogarithmically with size.

Spectrophotometric pKa'—A 1.19×10^{-5} *M* solution of tetrahydrocannabinol, more than three times the solubility of the drug in water at 0.1 ionic strength, is required for a 0.1 difference between the absorbances of completely ionized and unionized solutions at 240 nm.

The pKa' for tetrahydrocannabinol may be calculated by employing the modified form of the Henderson-Hasselbalch equation (19):

$$\log[(\epsilon_b - \epsilon)/(\epsilon - \epsilon_a)]_\lambda = \text{pH} - \text{pKa}' \qquad \text{(Eq. 3)}$$

where ϵ_a (5850) and ϵ_b (12,266) are the molar absorptivities of the unionized and completely ionized drug measured at a given wavelength, λ (240 nm), respectively, and ϵ is the apparent molar absorptivity of a solution measured at a particular pH.

The apparent molar absorptivity (ϵ) of the drug in various

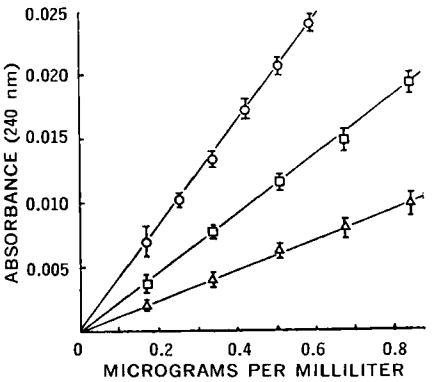


Figure 6—*Typical Beer's law plots for tetrahydrocannabinol in various aqueous buffer systems. A 10-cm cell was used (29.5 ml). The values shown are the mean and standard deviation of three determinations. Key: ○, pH 12.92; □, pH 10.35; and △, pH 7.15.*

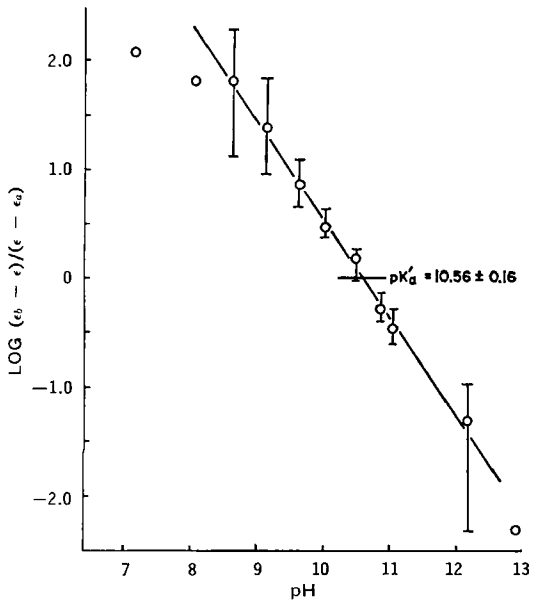


Figure 7—*Spectrophotometric determination of the pKa′ of Δ⁹-tetrahydrocannabinol in accordance with log* $[(\epsilon_b - \epsilon)/(\epsilon - \epsilon_a)] = pH - pKa'$, *where* ϵ_a *and* ϵ_b *are the molar absorptivities of the unionized and completely ionized drug at 240 nm, respectively, and* ϵ *is the apparent molar absorptivity at the stated pH value.*

buffer systems was calculated from the slopes of the plots of absorbance *versus* concentration at 240 nm; typical plots are given in Fig. 6. The absorptivities of the ionized and unionized species were estimated similarly using 0.5 *N* HCl and 0.5 *N* NaOH. The pKa′ was 10.56 ± 0.16 (σ), obtained from the plot of Fig. 7 in accordance with Eq. 3. This pKa′ is higher than would be anticipated for *ortho*- and *meta*-substituted phenols since *o*-methoxyphenol has a pKa′ of 9.98, *m*-methoxyphenol has a pKa′ of 9.65, and *m*-hydroxyphenol has a pKa′ of 9.8 (20).

A molecular model of the drug shows that the free rotation of the phenolic hydrogen is hindered by the Δ⁹-hydrogen and can explain Δ⁹-tetrahydrocannabinol's higher pKa′. Of course, if solvated dimers, trimers, *etc.*, exist with hydrophobic bonding, the observed pKa′ could be a hybrid pKa′ for a solution of such polymers.

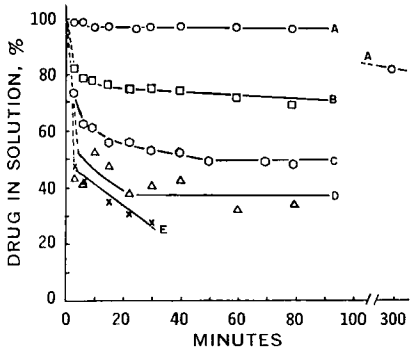


Figure 8—*Glass binding of Δ⁹-tetrahydrocannabinol showing the percent of drug added that remained in solution at various times for: (A) trimethylsilyl-treated 50-ml volumetric flasks from an aqueous drug concentration of 0.1 μg/ml, (B) an aqueous drug concentration of 0.1 μg/ml in untreated 50-ml volumetric flasks, (C) an aqueous drug concentration of 0.1 μg/ml in water-soluble silicone concentrate-treated 50-ml volumetric flasks, (D) an aqueous drug concentration of 0.05 ug/ml in untreated 50-ml volumetric flasks, and (E) an aqueous drug concentration of 0.1 ug/ml in a 20-ml stainless steel ultracentrifuge tube. Each point is the mean of four separate determinations.*

Table I—Percent Retention of Tetrahydrocannabinol from 0.1 mg/ml Solution after Various Seconds of Contact with Full Glass Pipets

Pipet Size, ml	Seconds of Contact		
	1	3	6
50	0.5	0	0.3
10	1	1	1
4	2.5	3	4
2	4	7	11
1	5	11	15

Table II—Binding of Δ⁹-Tetrahydrocannabinol to Serum Vials and Loss to Rubber Closures

Concentration, ng/ml	Vial Size, ml	Fraction Bound, *B*, to Glass[a]	Fraction Lost, *L*, to Closure[b]
1.57	5	0.48	0.71
1.57	10	0.26	0.98
15.7	5	0.30	0.80
15.7	10	0.26	0.92
494	5	0.24	0.85
494	10	0.22	0.75

[a] On the assumption that an equilibrium is established between drug in solution and drug bound to glass, a binding constant, K_B, may be defined for each vial: $K_B = B/V_0C = B'/V'C'$, where $B = (T - V_0C)/T$ is the amount bound prior to exposure to the closure, T is the total drug added, V_0 is the initial volume, and C is the observed concentration prior to exposure to the closure; B' is the amount bound after exposure, V' is the volume remaining after exposure, and C' is the concentration after exposure. [b] The total amount remaining, T', after the first sample is given by $T' = T - (V_0 - V')C$, and the amount bound to glass is given by $B' = K_BV'C'$. The fraction lost to the stopper, L, can be defined as $L = T' - B' - V'C'/T'$.

Glass Binding—The rate and extent of tetrahydrocannabinol in aqueous solution binding to glass depend on the surface area of the glass, the pretreatment of the glass, and the concentration of the drug. Curves B and D of Fig. 8 show the extent of binding, about 20 and 40%, respectively, of tetrahydrocannabinol as a function of time at concentration of 0.1 and 0.05 μg/ml in 50-ml volumetric flasks. At high drug concentrations (0.5–1.0 μg/ml) and after full equilibration, less than 7% of the amount in an aqueous solution was bound to glass from various flasks and test tubes.

Preconditioning glass with strong acid or alkali did not decrease the extent of binding. New, unused glassware bound the drug to a similar extent. Treatment of the flasks with a water-soluble silicone concentrate increased the extent of binding (Fig. 8, curve C). However, vigorous shaking immediately prior to sampling reduced the degree of binding, but no more than 50% in any case, although the fraction bound rapidly increased on further standing. All of the initial samples plotted in Fig. 8 were obtained immediately after vigorous shaking.

Trimethylsilyl treatment of glassware significantly reduced binding (Fig. 8, curve A) so that binding in 50-ml flasks and pipets was negligible for 80 min at 24° and for 24 hr at 0°. However, at room temperature the bound fraction of 0.1 μg/ml solution increased to the pretreatment level after 300 min. The equilibration binding of solutions to silyl-treated glassware with high concentrations of tetrahydrocannabinol (0.5–1.0 μg/ml) was negligible.

Polycarbonate, polypropylene, Teflon, and stainless steel (Fig. 8, curve E) containers showed more extensive binding than glass.

Aqueous solutions of tetrahydrocannabinol (0.1 and 0.05 μg/ml) were transferred by glass pipets. Significant retention of the pipeted tetrahydrocannabinol was observed, which increased with decreasing glass pipet size and with increased time of contact of the solution in a full pipet before delivery was permitted (Table I).

When aliquots of phosphate buffer solutions of tetrahydrocannabinol in serum vials were removed without prior shaking, significant amounts were bound to glass and the percent bound increased with decreasing concentration (Table II). However, of greater importance in light of the normal handling of samples of biological fluids is the fact that 70–97% of the drug in the vials

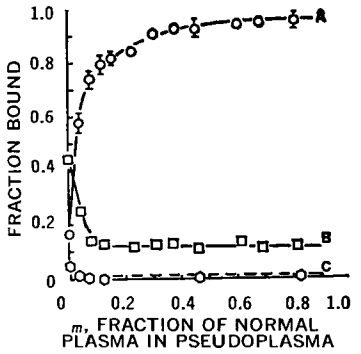


Figure 9—*Protein binding and glass binding of Δ⁹-tetrahydrocannabinol in pseudoblood for various fractions, m, of normal plasma in the pseudoplasma. (A) The fraction of drug in pseudoblood that is in pseudoplasma, A_P/A_T. The vertical bars give the mean ± 1 SD for 10 different drug concentrations. The open circles are for a concentration of 1.0 ng/ml in pseudoblood. (B) The fraction of drug added that is bound to glass, A_{BG}/A_0 for a concentration of 1 μg/ml in pseudoblood.*

was lost to the rubber stopper after 1 hr of shaking of the stoppered vials.

Tetrahydrocannabinol in whole blood or plasma also binds to glass (Fig. 9). However, since tetrahydrocannabinol protein binding is very large, glass binding is significantly reduced. In general, only one-fifth of the glass binding observed from saline solutions was observed from plasma solutions of tetrahydrocannabinol. The binding of drug from plasma to the silyl-treated glassware was negligible.

Protein Binding by Classical Methods—When tetrahydrocannabinol in isotonic phosphate buffer was dialyzed against isotonic phosphate buffer, 50–100% of the drug was bound to the tubing used as the membrane[14]. All of the drug was bound below 0.05 μg/ml. Ultrafiltration was equally unsuccessful, since only 0–5% of the drug in isotonic phosphate buffer was recovered in the ultrafiltrate. Attempts were made to saturate both dialysis membranes and ultrafiltration cones, but the membranes could not be saturated. The variability among repetitive samples with ultrafiltration was such that it could only be concluded that the fraction bound to plasma proteins was in the 0.85–1.0 range.

Protein Binding by Method of Variable Plasma Concentrations—The unbound, unionized fraction of a drug in whole blood may be presumed to partition into the red blood cells (7) in accordance with Scheme I. In Scheme I, the partition constant be-

$$A_{RBC} \overset{D}{\rightleftharpoons} A_P^u + P \overset{K}{\rightleftharpoons} AP$$

Scheme I

tween concentrations of drug in the red blood cells and unionized, unbound drug in plasma is:

$$D = [A_{RBC}]/[A_P^u] \quad \text{(Eq. 4)}$$

and the association constant for unionized, unbound drug, $[A_P^u]$, with the unoccupied protein binding sites, $[P]$, in plasma is:

$$K = [AP]/[P][A_P^u] \quad \text{(Eq. 5)}$$

where $[AP]$ is the apparent concentration of bound drug, on the presumption that the reactivity of binding sites is equivalent and that the occupancy of one does not modify the binding power of another.

If $[P_T]$ is the total concentration of binding sites, then:

$$[P_T] = m[P_1]_T = [P] + [AP] \quad \text{(Eq. 6)}$$

where the total concentration of binding sites may be expressed in terms of fractions, m, of the normal concentration of binding sites in plasma.

When the numerator and the denominator of the second term of Eq. 5 are divided by the total concentration of drug in the plasma, $[A_P]$:

$$K = ([AP]/[A_P])/([P][A_P^u]/[A_P]) = f_b/[P]f_u \quad \text{(Eq. 7)}$$

since the fraction of drug in the plasma that is bound is $f_b = [AP]/[A_P]$ and the fraction of drug in the plasma that is unbound is $f_u = [A_P^u]/[A_P]$. If there are excess numbers of binding sites, $[P_T]$ is not significantly depleted and:

$$[P] \sim [P_T] = m[P_1]_T \quad \text{(Eq. 8)}$$

Substitution of the approximation of Eq. 8 into Eq. 7 results in:

$$m[P_1]_T K = f_b/f_u \quad \text{(Eq. 9)}$$

A constant volume, V_{PC} = 2.0 ml, of washed, packed red blood cells of hematocrit H_1 (usually in the 0.68–0.88 range) was added to a volume V = 3.0 ml, of plasma-buffer mixtures to give pseudoblood of volume V_{WB} = 5.0 ml, with the hematocrit H_2 (usually in the 0.26–0.38 range). The volumes of red blood cells, V_{RBC}, and plasma, V_P, are given by:

$$V_{RBC} = H_1 V_{PC} = H_2 V_{WB} \quad \text{(Eq. 10)}$$

and:

$$V_P = V + (1 - H_1)V_{PC} = (1 - H_2)V_{WB} \quad \text{(Eq. 11)}$$

The V_{RBC} and V_P values had to be the same when calculated from either the observed H_1 or H_2 values or hemolysis was assumed and the preparation was discarded. The total concentration of drug in the plasma, $[A_P]$, after centrifugation of the pseudoblood was determined radiochemically in V_P' = 2.5 ml of plasma based on the fact that 96% of the drug was extracted from *all* plasma-buffer mixtures. Therefore:

$$[A_P] = [A_P^u] + [AP] \quad \text{(Eq. 12)}$$

and apparently the salting-out procedure used minimized any variability in the extraction efficiency with variations in plasma protein concentration.

Hematocrit H_3 (usually in the 0.50–0.61 range) was taken on the red blood cells resuspended in the residual plasma. The concentration of the total drug in the plasma containing the resuspended red blood cells was determined radiochemically as C. Thus, the total amount, A_T, of drug in the original pseudoblood could be calculated from:

$$A_T = V_P'[A_P] + (V_{WB} - V_P')C \quad \text{(Eq. 13)}$$

Since:

$$C = \{A_{RBC} + (1 - H_3)(V_{WB} - V_P')[A_P]\}/(V_{WB} - V_P') \quad \text{(Eq. 14)}$$

the amount of drug in the red blood cell fraction, A_{RBC}, may be calculated from a combination of Eqs. 13 and 14:

$$A_{RBC} = A_T - V_P'[A_P] - (1 - H_3)(V_{WB} - V_P')[A_P] \quad \text{(Eq. 15)}$$

The red blood cell distribution coefficient, Eq. 4, was determined directly for red blood cell dispersions in buffer where P_T = 0 and was D = 12.5 for the dog red blood cells.

The fractions of drug unbound and bound are:

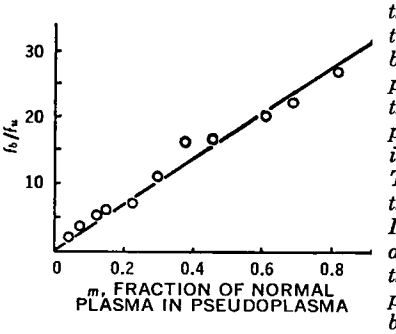


Figure 10—*The ratio of the fraction of drug bound to the fraction of drug unbound in the pseudoplasma as a function of the fraction, m, of normal plasma in pseudoplasma in accordance with Eq. 9. The intercept is zero and the slope, $[P_1]_T K$, is 34. In true whole blood with an equivalent hematocrit, the fraction bound to plasma proteins is given by 34 $(1 - f_b) = f_b$ and f_b = 0.97.*

[14] Visking.

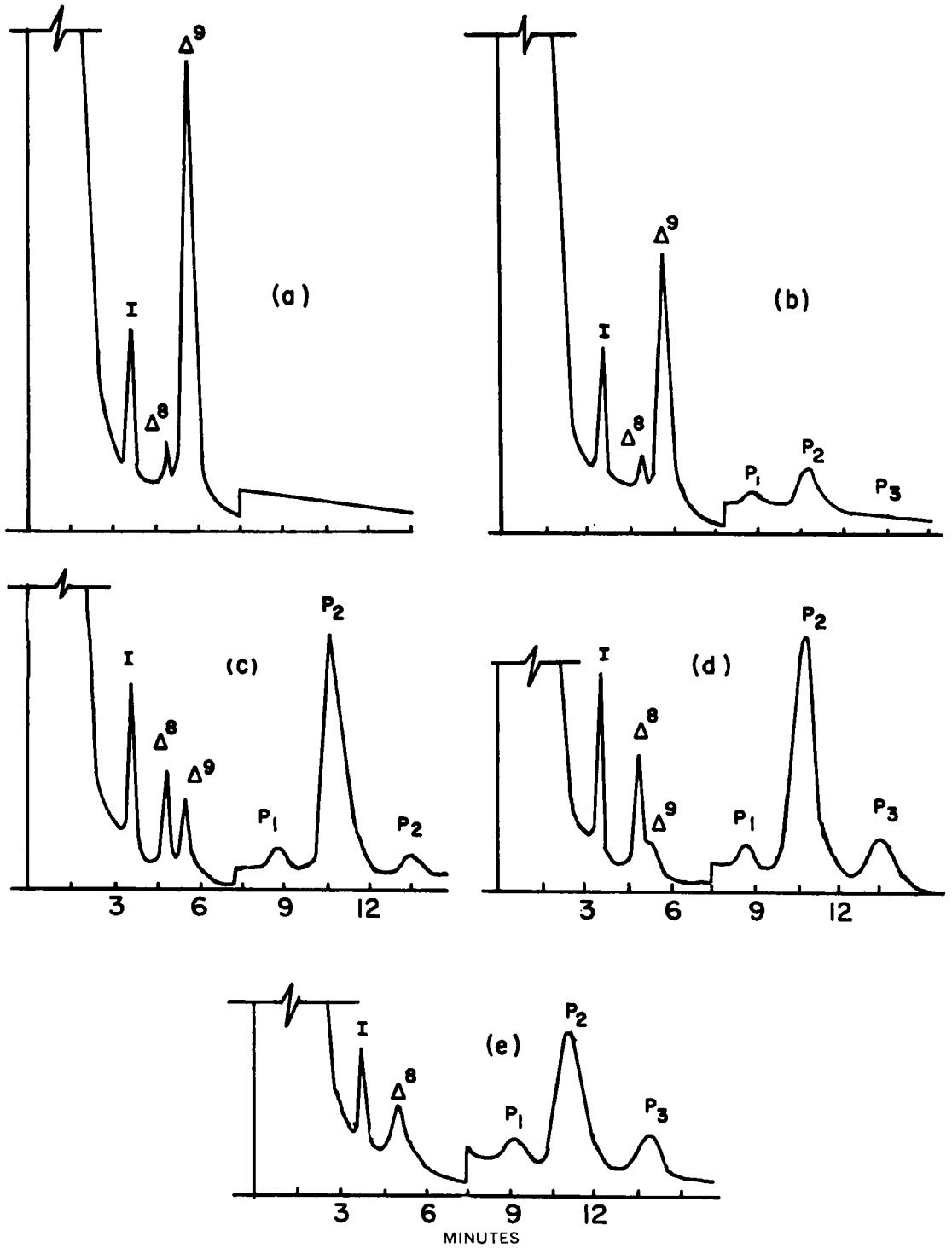


Figure 11—*GLC scan of organic extracts at various times of an aqueous 0.5 N HCl solution of Δ^9-tetrahydrocannabinol reacted at 55°. The first peak, I, following the solvent peak is the internal standard. The second and third peaks correspond to authentic Δ^8- and Δ^9-tetrahydrocannabinol, respectively. The last three peaks, P_1, P_2, and P_3, are for the three major degradation products. (a) Starting levels, t = 0, of Δ^8- and Δ^9-tetrahydrocannabinol. (b) A sample taken at 3 min shows loss of Δ^9-tetrahydrocannabinol and the appearance of P_1 and P_2. (c) A sample taken at 35 min shows an increase in Δ^8-tetrahydrocannabinol, $\Delta^8 > \Delta^9$, and shows P_2 to be the major product under these conditions. (d) A sample taken at 65 min shows that the loss of Δ^9-tetrahydrocannabinol is almost complete. (e) A sample taken at 780 min shows little change from the 65-min sample to suggest a final equilibrium among P_1, P_2, P_3, and Δ^8-tetrahydrocannabinol. The abscissas give the retention times.*

$$f_u = A_P^u/A_P = A_{RBC}V_P/DV_{RBC}A_P \quad \text{(Eq. 16)}$$

on consideration of Eq. 4, and:

$$f_b = AP/A_P = (V_P[A_P] - A_P^u)/A_P = 1 - f_u \quad \text{(Eq. 17)}$$

where the values of V_{RBC}, V_P, and A_{RBC} can be determined from Eqs. 10, 11, and 15, respectively, and where $A_P = V_P[A_P]$ was determined from the assayed total concentration of drug in plasma.

Due to the fact that the drug binds significantly to glass, the amount, A_0, of drug added and the total amount, A_T, calculated (Eq. 13) in the pseudoblood may not agree. The fraction of the total amount added that is bound to the glass container would be:

$$f_{BG} = (A_0 - A_T)/A_0 \qquad \text{(Eq. 18)}$$

The fraction of the amount of drug in the pseudoblood that is in the pseudoplasma, A_P/A_T, is plotted as curve *A* in Fig. 9 as a function of the fraction, *m*, of normal plasma in the plasma–buffer mixtures. The 10 concentrations of tetrahydrocannabinol studied were in the 1 to 1000-ng/ml range, and curve A showed no significant variation among such concentrations. The extent of the vertical lines through the mean values plotted represents the standard deviation of the values obtained for all such concentrations.

Curves B and C in Fig. 9 show the fractions of drug (Eq. 18) added that were bound to glass as a function of *m* for 1 ng and 1 μg, respectively, of tetrahydrocannabinol added to the various pseudoblood preparations. Since it is apparent that the fraction of tetrahydrocannabinol bound to plasma protein is independent of drug concentration (Fig. 9, curve A), the premise of Eq. 8 holds and the quotient f_b/f_u, with values calculated from Eqs. 16 and 17, is plotted against the fraction, *m*, of pseudoplasma that is normal plasma in accordance with Eq. 9. The resultant plot (Fig. 10) was completely linear with zero intercept in agreement with Eq. 9 and the slope estimated $K[P_1]_T = 34$. This permits extrapolation to true plasma conditions when $m = 1$ and the resultant $f_b/f_u = 34$, so that the fraction of tetrahydrocannabinol in plasma that is bound at normal protein concentrations is 0.972.

The only other known study on the binding of Δ^9-tetrahydrocannabinol to plasma proteins was performed with electrophoretic techniques on tritium-labeled material and human plasma (21). The compound was 80–95% associated with lipoproteins.

The apparent partition coefficient, *D* (Eq. 4), between buffer and red blood cells and of the fraction bound, f_b, to plasma proteins was determined by the described procedures at various times after the addition of drug to the preparation. The true equilibrium values were readily established within the 4.5 min used to add and suspend the red blood cells in plasma, centrifuge, and sample.

This procedure of estimating protein binding by the method of variable plasma concentrations permits estimates at high degrees of protein binding, which the normal errors in classical methods would not allow. The procedure can be used routinely to determine the protein binding of individual subjects.

Stability of Δ^9-Tetrahydrocannabinol in Acid Solutions—The degradation of Δ^9-tetrahydrocannabinol was followed by GLC with flame-ionization detection in aqueous hydrochloric acid solutions at 55°. Four major peaks could be detected in addition to that for Δ^9-tetrahydrocannabinol (Fig. 11).

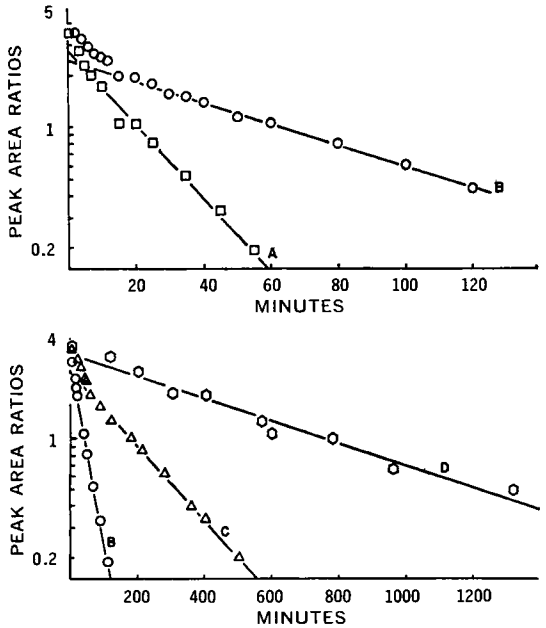


Figure 12—*Semilogarithmic plots of peak area ratios of Δ^9-tetrahydrocannabinol and internal standard as a function of time at 55°. Key: A, 0.5 N HCl; B, 0.1 N HCl; C, 0.05 N HCl (μ = 0.1 with sodium chloride); and D, 0.01 N HCl (μ = 0.1 with sodium chloride).*

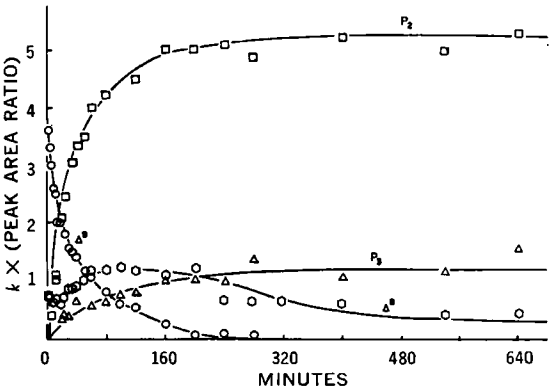


Figure 13—*Peak area ratios of Δ^8- and Δ^9-tetrahydrocannabinol and P_2 and P_3 plotted as a function of time for 0.1 N HCl at 55°. Key: ○, Δ^9-tetrahydrocannabinol, k = 1; ○, Δ^8-tetrahydrocannabinol, k = 3; △, P_3, k = 2; and □, P_2, k = 2.*

The semilogarithmic plots of peak area ratios of Δ^9-tetrahydrocannabinol to the internal standard, tetraphenylethylene, with time (Fig. 12) show an initial rapid drop with a subsequent linearity to indicate a possible rapid equilibration with one or more degradation products. The subsequent loss of Δ^9-tetrahydrocannabinol was apparent first order.

The retention time for one product corresponded to authentic Δ^8-tetrahydrocannabinol, and three others, P_1, P_2, and P_3, were observed with larger retention times (Fig. 11).

Plots of the peak area ratio of the products *versus* time (Fig. 13) showed that the peak corresponding to Δ^8-tetrahydrocannabinol increased rapidly and then decreased at a slower rate than that for the loss of Δ^9-tetrahydrocannabinol. Analysis of authentic Δ^8-tetrahydrocannabinol under similar aqueous conditions showed Δ^8-tetrahydrocannabinol to be more stable than Δ^9-tetrahydrocannabinol. Although this implicated the Δ^8-isomer as a product of Δ^9-tetrahydrocannabinol degradation, the facts that the retention time of the former was less and the peak area was also low indicated that it was not a major product.

Two of the other three products observed by GLC (Figs. 11 and 13), P_2 and P_3, increased slowly to a final equilibrium value. The rate of generation of these peaks appeared to parallel the apparent first-order loss of Δ^9-tetrahydrocannabinol within the experimental error, and the attainment of the final equilibrium value appeared to correspond to the complete loss of Δ^9-tetrahydrocannabinol. The other product, P_1, appeared almost instantaneously and gave a relatively small peak, which appeared to remain constant with time. The ratios of peak area values for the final equilibrium concentrations of P_2 and P_3 were independent of the hydrochloric acid concentrations studied, although the magnitudes varied.

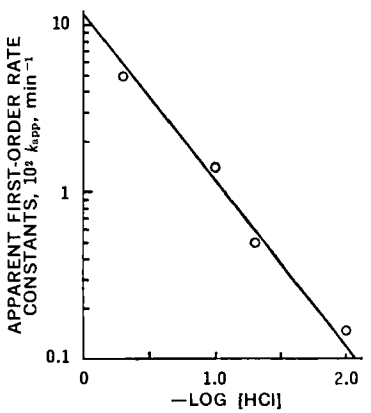


Figure 14—*Linear plot with slope of unity of the terminal apparent first-order rate constants, k_{app}, for loss of Δ^9-tetrahydrocannabinol (Fig. 12) as a function of −log [HCl].*

A plot of the apparent first-order rate constants obtained from the terminal slopes of Fig. 12 for the loss of Δ^9-tetrahydrocannabinol against $-\log[HCl]$ (Fig. 14) demonstrated a straight line consistent with a slope of unity to indicate hydrogen-ion-catalyzed degradation and an intercept $\log k_{H^+}$ that may characterize a bimolecular rate constant, k_{H^+}, of 0.002 liter/mole/sec. This observed instability of Δ^9-tetrahydrocannabinol in acid solution can account in part for the poor and variable availability of the drug when given orally. Studies are continuing to establish the complete log k *versus* pH profile for Δ^9-tetrahydrocannabinol and to identify the various degradation products.

Biopharmaceutical and Pharmacokinetic Implications of Physicochemical Properties of Δ^9-Tetrahydrocannabinol—The extremely low solubility of Δ^9-tetrahydrocannabinol (2.8 mg/liter in water and 0.77 mg/liter in 0.15 *M* NaCl at 23°) most certainly affects its bioavailability on oral dosing of amounts wherein the solubility is exceeded (2, 3). The premise that its solubility in the plasma is readily exceeded certainly is not inconsistent with the observation of its possible precipitation and localized accumulation in body organs (1). Tetrahydrocannabinol in excess of its solubility instantaneously forms a stable emulsion or micellar dispersion.

The rapid diffusion of Δ^9-tetrahydrocannabinol into the plastic of containers and into the rubber stoppers normally used as closures for plasma vials (70–97%) and the significant binding to glass at low tetrahydrocannabinol concentrations (20 and 40% at 0.1 and 0.05 μg/ml, respectively, in 50-ml volumetric flasks) definitely demand careful techniques in the handling, storage, and assay of this compound from aqueous and biological fluids. In fact, the results of any pertinent study where these conditions were not held in account should be quantitatively suspect.

The high degree of partition into lipid phases and of adsorption to all and any surfaces by tetrahydrocannabinol (lipoprotein binding may be included in these categories) implies that oral administration of the drug in lipid vehicles that are relatively immiscible with aqueous fluids would drastically reduce the bioavailability of the drug. A common practice of administering tetrahydrocannabinol admixed with lipid-containing feed in animal experiments would cast suspicion on the dose-response relationships proposed from the results of such studies.

The high sequestering and binding of Δ^9-tetrahydrocannabinol are consistent with the proposal of a pharmacokinetically deep compartment, especially with the known rapidity of its metabolism. The prolonged but lowered blood levels of administered drug (13) must be rationalized by its slow rate-determining release from such compartments. The lack of significant renal excretion of unchanged drug (13) is readily understandable since its high lipophilicity should result in complete tubular reabsorption.

The partitioning of tetrahydrocannabinol from plasma water into red blood cells is enormously high since $D = 12.5$. This may also be ascribed to the high surface affinity of the drug. However, the competition of a large degree of unsaturable binding (97%) to plasma proteins minimizes the amounts in the red blood cells of whole blood, although possibly large adherence of the drug to the walls of blood vessels and to the surfaces of tissues must be anticipated. The fact that the plasma binding is largely assigned to the lipoprotein fraction (21) may result in large individual variability in binding dependent upon individual and species variations in lipoprotein and fat content. Thus, the proposed method of variable plasma concentration may be useful in the routine determination of the protein binding of individual subjects.

The large instability of Δ^9-tetrahydrocannabinol in acid solution, which can be estimated minimally as $t_{1/2} = 1$ hr at pH 1.0 (55°) *after* an initial rapid drop in tetrahydrocannabinol content, implies that the drug may be significantly degraded in the normal stomach. Again, this intimates that oral administration may not be an optimum route on which to establish dose-response correlations.

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[x] To whom inquiries should be directed.

Improved Method for Estimating Chlorthalidone in Body Fluids

M. G. TWEEDDALE* and R. I. OGILVIE*

Abstract □ The reliable estimation of chlorthalidone in whole blood, plasma, urine, or bile depends upon solvent extraction followed by deamination in alkali at elevated temperature. The concentration of chlorthalidone is quantitatively determined from the difference in absorbance at 262 nm before and after deamination. The conditions required for quantitative extraction from the different body fluids are described. The assay permits detection of 1 mg/liter, which is 10 times better than the previously described assay of chlorthalidone. Mass spectroscopy showed the assay to be specific for unchanged chlorthalidone. The assay was applied to the measurement of chlorthalidone in human blood and urine, and results suggest that chlorthalidone has a long half-life and is extensively metabolized in humans. Red cell concentrations of chlorthalidone are 7–10 times greater than plasma concentrations.

Keyphrases □ Chlorthalidone—spectrophotometric determination in biological fluids □ Diuretics—improved assay of chlorthalidone in biological fluids □ Spectrophotometry—improved assay of chlorthalidone in biological fluids

While studying the antihypertensive actions of chlorthalidone[1] in humans, it became necessary to measure the concentrations of this drug in blood and urine. The only published assay for chlorthalidone in body fluids (1) was considered by its authors to be unsuitable for clinical purposes, because concentrations of chlorthalidone below 10 mg/liter could not be detected. The modifications described in this paper resulted in a 10-fold increase in sensitivity of the assay. The method described is used in this laboratory to study the pharmacokinetics of chlorthalidone in humans and to monitor diuretic and antihypertensive effects of this drug.

EXPERIMENTAL

Materials—All chemicals[2] used were of analytical purity. A concentrated stock solution (300 mg/liter) of chlorthalidone[3] in 0.1 *N* Na_2CO_3 is used to prepare the internal standards. Reference standards are prepared in bulk by adding known quantities of chlorthalidone to the appropriate biological fluid obtained from a "normal" subject. Both reference standards and biological samples are stored at −10° until required. Such storage for over 1 year does not result in deterioration of reference standards.

Procedure—All samples are analyzed for chlorthalidone in duplicate. Each 2-ml aliquot of a chlorthalidone-containing sample is added to 2 ml of buffer solution in a glass-stoppered bottle (125-ml capacity). (The buffer solution is described later.) Twenty-five milliliters of ether is added, and the mixture is shaken mechanically for 10 min. After settling, the ether layer is decanted into a graduated cylinder from which 20 ml is transferred to a glass separator (60-ml capacity, fitted with a Teflon stopcock) containing 2 ml 2 *N* NaOH. The mixture is agitated mechanically for 5 min and, after allowing time for separation, the aqueous layer is drawn off into a glass tube fitted with a plastic screw-cap.

Single aliquots of four reference standards (0, 5, 10, and 20 mg/liter chlorthalidone) and two reagent blanks (2 ml distilled water) are carried through with each run of the assay. One reagent blank serves as the spectrophotometric reference solution, while the other is converted to an internal standard by the addition of chlorthalidone to give a final concentration of 20 mg/liter. This internal standard is subjected to spectrophotometry and deamination along with the other extracts and is used to calculate the fractional recovery of chlorthalidone from the reference standards. In addition, this internal standard serves as a control for the deamination process.

The absorbance of the final alkaline extract from each sample is measured at 262 nm against the reagent blank, using silica cells of 5-mm width and 10-mm light path. Each extract is then returned to its tube and the cap is screwed down tightly. All tubes are immersed in a water bath at 97° for 2 hr, after which they are cooled to room temperature by standing in cold water. Finally, the absorbance of each incubated extract is measured at 262 nm against the incubated reagent blank. Two spectrophotometers[4] were used to measure absorbance, and identical results were obtained.

Determination of Chlorthalidone Concentration—The absorption spectra of chlorthalidone before and after incubation in alkali are shown in Fig. 1. The difference between the absorbance readings $(A_2 - A_1)$ is proportional to the concentration of chlorthalidone. When chlorthalidone is extracted from a biological fluid, this absorbance difference must be corrected for background activity by subtraction of the absorbance difference $(A_2^B - A_1^B)$ of a "blank" sample taken prior to the administration of chlorthalidone. (Experience has shown that this blank value ranges from zero to ±2 mg/liter and is usually negative when urine or bile samples are assayed.) The difference $(A_2 - A_1) - (A_2^B - A_1^B)$ is referred to as the final absorbance, A_F, of a sample. Values of A_F are linearly related to the concentration of chlorthalidone present in the original sample (Fig. 2).

The chlorthalidone content of a sample is determined either by reading off the A_F values from a standard curve or by use of Eq. 1:

$$\text{chlorthalidone concentration (mg/liter)} = \frac{A_F}{b \times f \times e} \quad \text{(Eq. 1)}$$

where b is the slope of the standard curve for chlorthalidone in 2 *N* NaOH, f is the fraction of the ether layer used for back-extraction of chlorthalidone into alkali, and e is the fractional recovery of chlorthalidone from the reference standards.

Assay Conditions with Differing Biological Fluids—Consistent extraction of chlorthalidone from whole blood or from packed red cells requires prior hemolysis of the cells, which was accomplished by slow freezing and thawing. The buffer system giving optimal recovery of chlorthalidone from whole blood or packed red cells is a citric acid–disodium hydrogen phosphate mixture at pH 5.0 and of approximately 1 *M* ionic strength (60 g citric acid plus 108.8 g disodium hydrogen phosphate dissolved in water and diluted to 1 liter). This buffer system also proved satisfactory for the recovery of chlorthalidone from plasma and serum; but when used with samples of urine or bile, it resulted in such high background activity (due to extraction of pigments) that chlorthalidone was undetectable. Milder, alkaline extraction conditions are necessary to recover chlorthalidone from these body fluids. A suitable buffer system (0.25 *M* at pH 8.5) contains 6.2 g boric

[1] Hygroton, Geigy Pharmaceutical Co.
[2] Obtained from either J. T. Baker Chemical Co. or British Drug Houses.
[3] A pure sample of chlorthalidone (lot 538) was obtained through Ciba-Geigy (Canada) Ltd.
[4] Hitachi Perkin-Elmer model 139 and Heath-Schlumberger model EU-701.

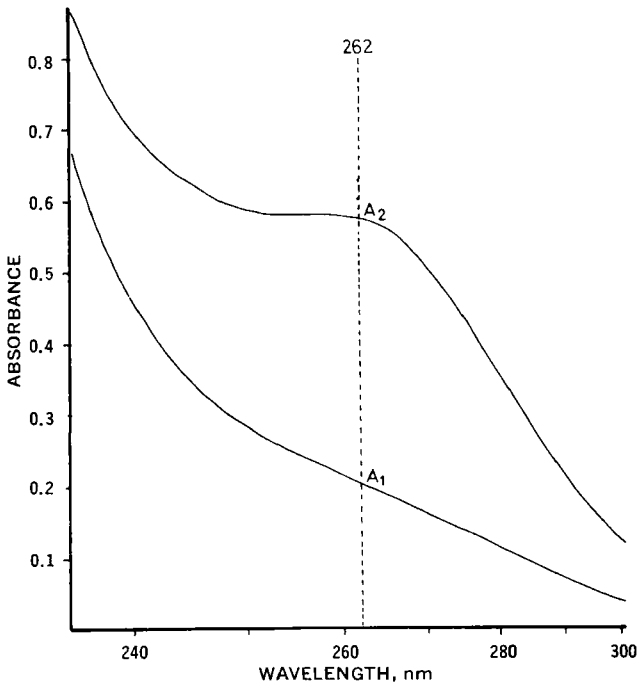

Figure 1—*Absorption spectra of chlorthalidone in 2 N NaOH before (lower curve) and after (upper curve) incubation at 98° for 2 hr. The difference between the readings at 262 nm* ($A_2 - A_1$) *is proportional to the concentration of chlorthalidone in the solution.*

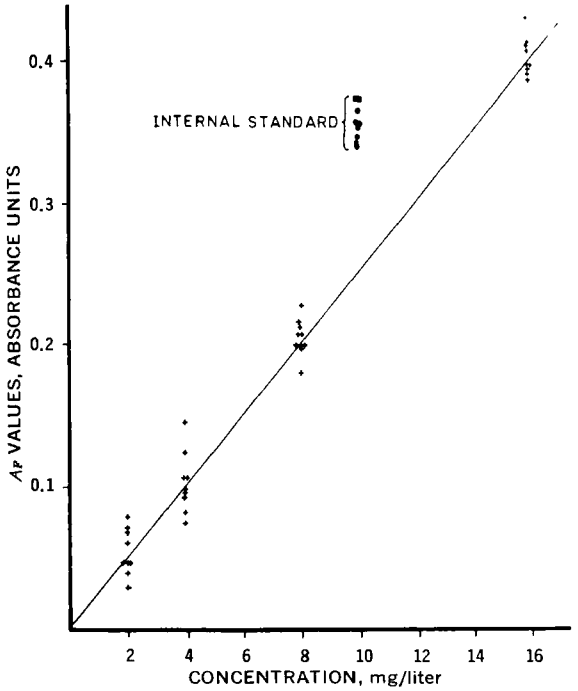

Figure 2—*Assay of reference standards of chlorthalidone in normal human plasma. The results of nine completed assays are shown (+) together with the simultaneous values for the internal standards (●). The least-squares line of best fit is shown. All samples were assayed singly; duplicate assays would reduce the scatter by about one-third.*

acid, 7.26 g KCl, and 1 g NaOH in each liter of solution. Because of the relatively low buffering capacity of this system, acid urines are made alkaline prior to extraction by the addition of a few drops of 5 *N* NaOH. Despite the use of this buffer system, pigment extraction from some very concentrated samples of bile is still great enough to impart a marked color to the ether layer. When this occurs the ether layer is "washed" by transferring it to another bottle containing 4 ml fresh buffer solution. Mechanical agitation for 10 min is followed by separation of the ether layer and back-extraction into 2 *N* NaOH in the usual manner. Although this additional extraction procedure makes the assay unreliable at concentrations of chlorthalidone below 3 mg/liter, concentrations greater than this can be estimated with an error comparable to that for chlorthalidone in urine or blood.

RESULTS

Standard Curves—The A_F values obtained from the first nine runs using reference standards of chlorthalidone in normal human plasma are shown in Fig. 2. This figure also shows the values obtained from the internal standard during the same runs. It can be seen that the best straight line obtained by least-squares linear regression analysis passes through zero and that the relationship between A_F and the concentration of chlorthalidone is linear over the experimental range. Other experiments showed that this relationship remains linear over the 1-40-mg/liter range. With concentrations of chlorthalidone greater than 40 mg/liter, the absorbance after incubation with alkali is so great that the readings are unreliable. Such samples can only be measured after dilution to bring the concentration into the usual range. Apart from minor differences in slope, reference standards in packed red cells, plasma, and bile provided standard curves similar to that shown (Fig. 2).

Recovery and Reproducibility—The mean recovery of chlorthalidone from reference standards in packed red cells was 86.3 ± 1.1% (mean ± *SE*, $n = 36$). The recovery from reference standards in urine was slightly less at 83.8 ± 0.70% (mean ± *SE*, $n = 47$). The reproducibility of the assay was assessed from the day-to-day variations in *(a)* the slope of the standard curve, and *(b)* the A_F value of the internal standard. For each of these parameters, the percent deviation of individual values from the grand mean was determined. Thirty-six complete runs were made using reference standards in packed red cells. Twenty-two (60%) of the values for the slope of the standard curve lay within ±5% of the mean, while only three values (12.5%) varied from the mean by more than ±10%. Similarly, 47 runs were completed with reference standards in urine. Of these, 36 (76%) lay within ±5% of the mean, and no value differed from the mean by more than ±10%. The results for the internal standard were more variable (45% of the values were within ±5% of the mean, and 15% differed from the mean by more than ±10%, $n = 83$), but this was not surprising since preparation of the internal standards involved the repeated accurate measurement of small quantities of fluid.

Accuracy—A series of 16 "unknown" samples containing 1–20 mg/liter chlorthalidone in plasma were assayed in duplicate. The mean error of estimation was 0.5 mg/liter with a range of from −1.1 to +0.8 mg/liter. Similar results were obtained with unknown samples dissolved in urine, but the determination of unknown samples in whole blood or packed red cells was not so accurate, the mean error being 0.8 mg/liter with a range of from −1.6 to +1.2 mg/liter ($n = 12$).

Specificity—A normal adult male volunteer received a single oral dose of 400 mg of chlorthalidone (as 100-mg tablets), and all urine was collected for 3 days. The urine was made alkaline and then extracted repeatedly with ether until assay of the remaining aqueous material revealed that all chlorthalidone had been removed. The ether extracts were pooled and evaporated to dryness under a stream of warm air. The residue was reconstituted in 2 ml 99% ethanol and diluted with distilled water to 50 ml. This reconstituted solution was again extracted with ether, and the process was repeated until material assayable as chlorthalidone accounted for 70% of the weight of the residue left after evaporation of the ether. This final residue was recrystallized from 99% ethanol and the crystals were submitted to mass spectrometry[5]. The mass spectra of the material extracted from urine were compared with that of chlorthalidone extracted from chlorthalidone tablets by solution in, and crystallization from, 99% ethanol. The mass spectra obtained were identical.

Assay Control—Statistical control of the assay was main-

[5] Mass spectra were obtained and interpreted by Morgan-Schaffer Enterprises, Montreal, Quebec, Canada.

tained by day-to-day plotting of six variables. These were the slope, the Y-intercept, and the standard error of Y on X (obtained by the method of least squares from A_F values for the reference standards), the value of the internal standard, the percent recovery from the reference standards, and the within-assay variation. The latter parameter was defined as the mean difference between the duplicate values of the unknown samples. On the basis of the results from the first 10 runs of the assay, means and 95% confidence intervals for each parameter were determined. The assay remained in acceptable control throughout these experiments.

Interfering Substances—Whole blood and urine samples from patients receiving a number of drugs in addition to chlorthalidone were assayed. These drugs included digitalis, chlorpropamide, benzodiazepines, phenobarbital, aspirin, allopurinol, methyldopa, and an unidentified sulfonamide preparation (for a urinary tract infection). Only the latter compound interfered with the assay of chlorthalidone.

Blood Concentrations and Urinary Excretion of Chlorthalidone in Humans—A normal, fasting, adult male subject received two 100-mg tablets of chlorthalidone. Blood samples were collected at intervals over the subsequent 5 days, and the cumulative urinary excretion of chlorthalidone was measured over the same period. Chlorthalidone became detectable in the whole blood in less than 1 hr, and levels reached a peak of 6.3 mg/liter at 6 hr. Thereafter, the concentrations declined steadily to reach 1.5 mg/liter at 5 days. Cumulative urinary excretion of chlorthalidone during the experimental period amounted to 52 mg.

The 24-hr urinary excretion of chlorthalidone was assessed in three hospitalized male patients who had received stable doses of chlorthalidone (100 mg daily) for at least 1 month. In these subjects, the daily excretion of chlorthalidone ranged from 36 to 64 mg. Heparinized blood samples were obtained from 15 subjects receiving chlorthalidone for the control of hypertension in doses ranging from 25 to 200 mg/day. The chlorthalidone content of these samples ranged from 4 to 34 mg/liter. When the serum from these blood samples was assayed separately, it was observed that the serum concentrations were markedly lower than those obtained from whole blood. In only three of the subjects was there any agreement between the whole blood and serum concentrations, and each of these serum samples showed evidence of hemolysis.

These results led to an investigation of the distribution of chlorthalidone in whole blood. Heparinized blood was obtained by venipuncture from a normal volunteer. Chlorthalidone was added to portions of the blood to give final concentrations of 0, 3, 6, 12, and 24 mg/liter. Aliquots were withdrawn from each lot and were assayed to confirm the accuracy of the initial dilution. After separation of the red cells and serum, aliquots of each layer were assayed separately. The results are shown in Fig. 3 where the nominal concentration in whole blood is plotted against the actual concentrations in the red cell and plasma layers. It can be seen that the concentration of chlorthalidone in the red cell layer is approximately 7 times that of the serum. In this experiment, 90% of the chlorthalidone originally added to the whole blood was recovered. Similar experiments were repeated on two other occasions using outdated blood from the blood bank. The results were again similar, the concentration in the red cells exceeding that of the plasma by 8 and 10 times, respectively. It would appear from these experiments that chlorthalidone is extensively bound in or on the red cell.

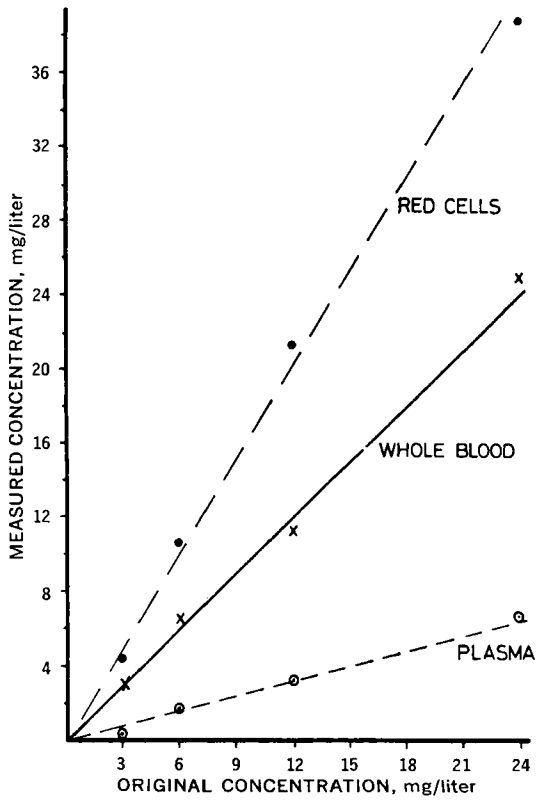

Figure 3—*Distribution of chlorthalidone in blood. Chlorthalidone was added to whole blood and aliquots were assayed for chlorthalidone content* (×). *Red cells* (●) *and plasma* (○) *from the chlorthalidone-containing blood were separated and assayed independently. High concentrations of chlorthalidone were found in the red cells and correspondingly low concentrations in the plasma. (In the experiment illustrated, the hematocrit was 45%.)*

DISCUSSION

The present paper describes modifications of the only published assay for chlorthalidone in biological materials (1). As a result of these modifications, the assay sensitivity was increased 10-fold, so that concentrations of chlorthalidone occurring with normal therapeutic regimens can now be readily measured. The modified assay for chlorthalidone appears to be specific for the unchanged compound and has an acceptable degree of reproducibility and accuracy. Although no formal search was made for interfering compounds, it appears that the assay is valid in the presence of various drugs commonly used with chlorthalidone.

There are two main reasons for the increased sensitivity of the current assay over that described by Pulver *et al.* (1). These are the initial extraction conditions and the volume of 2 N NaOH into which the ether layer is back-extracted. These experiments showed that the extraction conditions described by Pulver *et al.* will not quantitatively remove chlorthalidone from whole blood or packed red cells. Furthermore, the original extraction technique yielded high background activity when used with samples of urine, thereby obscuring the presence of small quantities of chlorthalidone. Although the modified extraction conditions failed to extract 14–18% of the chlorthalidone present, they nonetheless extracted chlorthalidone both quantitatively and reliably from different biological fluids and provided low background activity. The original assay used sample sizes from 2 to 5 ml which, after ether extraction, were back-extracted into 10 ml 2 N NaOH, thereby diluting considerably any chlorthalidone present in the original sample. In contrast, the use of small volume cells with a 1-cm light path allows the transfer of chlorthalidone from a 2-ml sample to an equal volume of alkali, thereby avoiding this diluting effect. Furthermore, Pulver *et al.* calculated the concentration of chlorthalidone by a standard formula which took no account of either differing recovery rates of chlorthalidone from various biological fluids or of the sample blank values. The methods described in the present paper allow for both of these variables.

Pulver *et al.* (1) reported that, after a single oral dose of chlorthalidone (600 mg) administered to a normal subject, only 31% was recovered in the urine during 24 hr. In the present experiment, only 26% was recovered in the urine during the 5 days following a single oral dose of 200 mg. Twenty-four-hour collections from three patients on a stable dose of chlorthalidone (in which daily input should equal daily output) showed that the daily excretion of chlorthalidone ranged from 30 to 60% of the daily dose. Since the mass spectrometry results show the present assay to be specific for unchanged chlorthalidone, it appears that another significant route of elimination of this drug exists in the human as compared with the dog (1) and rat (2). In the latter paper (2), the

authors showed that 6 times as much chlorthalidone was present in the red cell fraction of rat blood as in the plasma. The present study reports similar findings in humans where from 8 to 10 times as much chlorthalidone is found in the red cells as in the plasma. On the basis of the results described in this paper, approximately 90% of a given dose of chlorthalidone is bound in the tissues. It is this tissue binding that probably accounts for the long half-life (54 hr) noted in the normal human subject and for the prolonged diuretic action of chlorthalidone in humans (3).

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* Present address: Faculty of Medicine, Memorial University of Newfoundland, St. John's, Newfoundland, Canada.

ˣ To whom inquiries should be directed.

Dependence of Toxicity on Molecular Structure: Group Theory Analysis

J. L. COHEN*, W. LEE, and E. J. LIEN ˣ

Abstract □ Group theory was applied to a wide variety of toxic substances, drugs, and endogenous organic compounds to test the apparent empirical relationship between toxicity of a molecule and its degree of molecular symmetry. Many compounds in each classification were analyzed and separated into individual symmetry groups using "space-filling" molecular models. With few exceptions, an apparent relationship was noted between each symmetry group and the relative toxicities. A brief introduction to group theory is presented as well as the rationale and implications of these observations.

Keyphrases □ Structure-activity relationships—group theory analysis, toxicity-molecular symmetry □ Toxicity—dependence on molecular structure, group theory analysis of various substances □ Molecular structure—role in toxicity, group theory analysis of various substances

Numerous reports in the literature attempt to correlate the activities or toxicities of drugs and chemicals with various structural or physical-chemical parameters of the molecules (1-3). These approaches, in general, have shown the greatest applicability for structurally related series of compounds, but there has been no satisfactory general correlation which explains the activities of molecules with significantly different structures and physical-chemical properties. The reason for this may be that fundamental parameters, such as molecular symmetry, or other relevant properties of molecules have not been included among the physical-chemical parameters used in existing quantitative structure-activity correlations. Because the overall objective of all correlations of this type is to design drugs with very specific activities and limited toxicities, the approach along specific drug classification lines or specific disease lines is clearly appropriate. Therefore, the implication is that a fundamental explanation of the general type stated may direct future research to include additional specific parameters required to design drugs of specific pharmacological activity.

Table I—Mean LD_{50} Values for Oral Doses in Rats of Different Symmetry Groups

Group	Number of Compounds	LD_{50}[a]	*df*	*t*	Significance Level, %
C_1	29	17.60 ± 2.59	46	4.26	99.5
C_{2v}	19	3.57 ± 0.91	—	—	—
C_{1h}	5	5.21 ± 2.58	32	1.95	95
D_{2h}	5	3.21 ± 1.54	32	2.27	97.5

[a] LD_{50} values in rats, millimoles per kilogram, mean ± standard error ($S\bar{x}$, where $S\bar{x} = S/\sqrt{n}$).

In this paper the empirical relationship between molecular symmetry and toxicity is explored with various drugs, toxic chemicals, and endogenous organic compounds.

GROUP THEORY

Group theory invariably differentiates between all possible geometric isomers and places them into their respective symmetry groups. Once the compounds are classified into their respective symmetry groups and subgroups, the similarities and contrasts within the groups can be compared. An extensive mathematical discussion of group theory will not be presented here; however, several references (5, 6) discuss the theoretical details of group theory analysis. Most appropriate to this discussion is the actual application of some principles to the compounds analyzed in the present study.

Group symmetry operations are mathematical operators which perform linear transformations on molecular orbital wave func-

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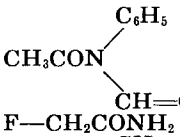
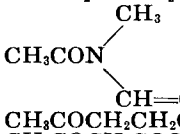
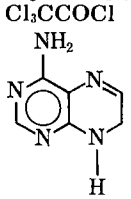
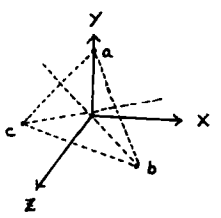
Group symmetry operations are mathematical operators which perform linear transformations on molecular orbital wave func-

Table II—Oral Toxicity in Rats: C_1 Group Symmetry

Number	Compound	LD_{50}, mg/kg	Molecular Weight	LD_{50}, mmoles/kg
1		2460	113	21.77
2	$F—CH_2CONH_2$	85	77	1.10
3		2830	99	28.59
4	$CH_3COCH_2CH_2CON(C_2H_5)_2$	4760	172	43.37
5	$CH_3COCH_2COOC_2H_5$	3980	130	30.62
6	$HOCH_2COCH_3$	2200	74	29.73
7	$FCl_2CCOCClF_2$	277	214	1.29
8	$4\text{-}NH_2—C_6H_4COCH_3$	1870	135	13.85
9	$3\text{-}CH_3,4\text{-}CH_3O—C_6H_3COCH_3$	1500	164	9.15
10	$3\text{-}NO_2—C_6H_4—COCH_3$	3250	165	19.70
11	$Cl_2HC—COCl$	2460	134	18.36
12	Cl_3CCOCl	600	180	3.33
13		745	133	5.60
14	$CH_3CHOH—CH_2CHO$	2180	88	24.77
15	$3\text{-}Cl—C_6H_4NH_2$	880	127	6.93
16	$2,4,5\text{-}(CH_3)_3—C_6H_2NH_2$	1585	135	11.74
17	$n\text{-}C_3H_7—C_6H_4—CHO$	1600	164	9.76
18	$C_6H_5CH_2OH$	1600	108	14.81
19	$CH_3—CHOH—C_6H_5$	400	122	3.28
20	$4\text{-}Cl—2\text{-}(C_6H_5CH_2)—C_6H_3OH$	1700	218	7.80
21	$(C_2H_5)_2CHCHO$	3900	100	39.00
22	$(C_2H_5)_2NH$	649	73	8.89
23	$(C_3H_7)_2NH$	930	101	9.21
24	$H_2N—CH_2CH_2OH$	3320	61	54.43
25	$CH_3NHCH_2CH_2OH$	2340	75	31.20
26	$n\text{-}C_6H_{13}OH$	2380	116	20.52
27	$C_2H_5OCH_2CH_2OH$	3000	90	33.33
28	$CH_2=CH—CH_2—OCHCH_2$	550	84	6.55
29	$C_2H_5O—(n\text{-}C_4H_9)$	1870	114	1.84

tions. The set of operators that sends a symmetrical figure into itself is said to form a "group." For example, given a triatomic molecule placed in three-dimensional (orthogonal) space, the operators that send the molecule into itself are:

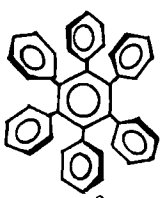
1. The identity operation $\hat{E}$, which leaves each point unchanged.
2. $\hat{\sigma}xy$, reflection in the plane passing through point b and perpendicular to the line joining a and c.
3. σyz, reflection in the yz plane passing through point a and perpendicular to bc.
4. $\hat{\sigma}d$, reflection in the plane passing through point b and perpendicular to ac.
5. $\hat{C}_3^1$, clockwise rotation through 120°.
6. $\hat{C}_3^{-1}$, counterclockwise rotation through 120°.



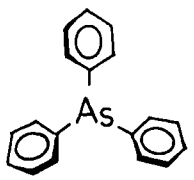
Other symmetry operations are possible; however, they turn out to be equivalent to one of these six operations. For example, a clockwise rotation through 240° is an operation identical to $\hat{C}_3^{-1}$, and a rotation through 180° about the y-axis is identical to $\hat{\sigma}_d$. The successive application of any two of the operations discussed here is equivalent to some other single operation.

C_1 Symmetry Group—This is the simplest point group possible, consisting of only the identity operation $\hat{E}$. Examples of this point group that have no symmetry at all are adenosine, glucose, cholesterol, and many other endogenous and exogenous compounds.

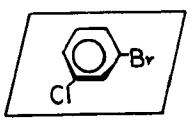
C_n Symmetry Group—This group is associated with an n-fold rotational axis and consists of the operations $\hat{E}$, $\hat{C}_n^1$, $\hat{C}_n^2$, . . ., $\hat{C}_n^{n-1}$; there is no limit to the possible value for n in these compounds. Examples of compounds in this symmetry group are:



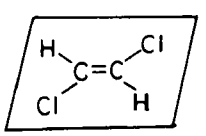
$n = 6$



C_{nh} Symmetry Group—This group is classified by two independent operations, C_n and σ_h, which add to a horizontal plane to the molecule. Examples of compounds in this group are:



C_{1h}



C_{2h}

Table III—Oral Toxicity in Rats: C_{2v} Group Symmetry

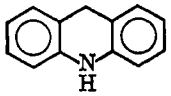
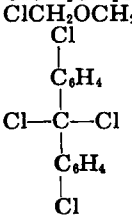
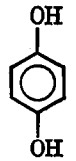
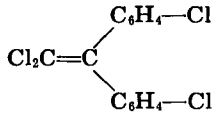
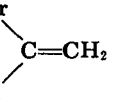
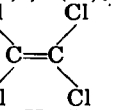
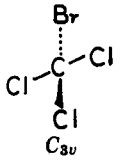
Number	Compound	LD_{50}, mg/kg	Molecular Weight	LD_{50}, mmoles/kg
1	$ClCF_2COCF_2Cl$	61	198	0.31
2		2140	179	11.96
3	$CH_2{=}CHCH_2{-}O{-}CH_2CH{=}CH_2$	320	98	3.27
4	$(ClCH_2CH_2)_2{-}N{-}C_6H_5$	123	213	0.58
5	$4\text{-}Cl{-}C_6H_4{-}NH_2$	300	127	2.36
6	$(CH_3)_2N{-}C_6H_5$	1410	121	11.65
7	$2,4,6\text{-}(CH_3)_3{-}C_6H_2{-}NH_2$	506	135	3.75
8	$n\text{-}C_4H_{10}$	658	58	11.34
9	$BrCH_2CH{=}CH{-}CH_2Br$	75	214	0.35
10	$ClCH_2CH{=}CH{-}CH_2Cl$	89	126	0.71
11	$(CH_2{=}CH{-}CH_2)_2NH$	650	97	6.70
12	$(CH_3)_3CNHC(CH_3)_3$	550	129	4.26
13	$Cl(CH_2)_2O(CH_2)_2{-}Cl$	105	142	0.74
14	$CH_3CHCl{-}O{-}CHCl{-}CH_3$	105	142	0.74
15	$ClCH_2CH_2OH$	89	80	1.11
16	$ClCH_2OCH_2Cl$	210	114	1.84
17		168	308	0.54
18		320	110	2.90
19		880	320	2.75

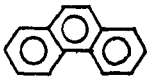
Table IV—Oral Toxicity in Rats: $C_{\infty v}$ Group Symmetry

Number	Compound	LD_{50}, mg/kg	Molecular Weight	LD_{50}, mmoles/kg
1	$Br{-}C{\equiv}C{-}Br$	117	184	0.64
2	$Cl{-}C{\equiv}C{-}Cl$	770	94	8.19
	C_{1h} Group Symmetry			
1	$CH_2{=}CHOH$	64	44	1.45
2	$4\text{-}Cl{-}1,3\text{-}(NO_2)_2{-}C_6H_3$	640	202	3.17
3	$Cl_2C{=}CCl{-}CCl{=}CCl_2$	300	258	1.16
4		500	106	4.71
5	$CH_2{=}CH{-}CH{=}CH{-}CH{=}CH_2$	1210	80	15.12
	D_{2h} Group Symmetry			
1	$1,2,4,5\text{-}(Cl)_4{-}C_6H_2$	1500	214	7.01
2		200	166	1.20
3	$ClCH_2CH_2Cl$	680	98	6.94
4	$BrCH_2CH_2Br$	117	188	0.62
5	$BrCH{=}CHBr$ (*trans*)	62	186	0.29

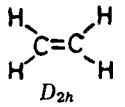
C_{nv} Symmetry Group—This group is derived from C_n and also has a vertical plane of symmetry σ_v through the axis. Examples of molecules of this group are:



C_{3v}

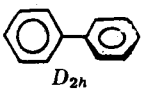

C_{2v}

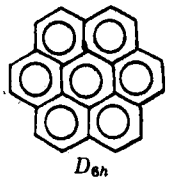
D_n, D_{nh}, and D_{nv} Symmetry Groups—Molecules that possess one or more twofold axes of rotation belong to the D_n group, while the addition of a σ_h (horizontal plane) produces the D_{nh} group and the addition of a σ_v (vertical plane) produces the D_{nv} group. Examples of these include:



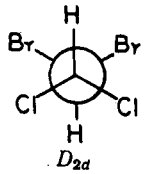
D_{2h}


D_{6h}


D_{2h}


D_{6h}

D_{nd} Symmetry Group—Another series of groups arises when a set of vertical planes, σ_v, is added to D_n through the axis C_n, bisecting the angles between the twofold axis. An example of D_{nd} is:



D_{2d}

$C_{v\infty}$ Symmetry Group—This group is obtained by adding a vertical plane, σ_v, to C_∞, where C_∞ implies an infinite-fold axis for which rotation by all multiples of an infinitesimal angle ($2\pi/n$, where $n \rightarrow \infty$) is a symmetry operation. A heteronuclear diatomic molecule has this symmetry, with the C_∞ axis along the bond axis, *e.g.*, carbon monoxide (CO).

EXPERIMENTAL

The molecular symmetry of the compounds under investigation was assigned based upon the observation of the spatial orientation of the "space-filling" molecular models[1]. These models depict the linearity and planarity of the molecules. For example, hydrocarbon side chains were found to be nonlinear due to sp^3 (tetrahedral) orbitals, whereas sp^2 bonded atoms such as in olefins were found to be trigonal and in the same plane. The possible alteration of spatial orientation by stretching, rotational, and translational energies was also considered. Although stretching energies of the molecules are the most significant of the three mentioned, there was no demonstrated alteration in the symmetry of the molecule. The rotational and translational energies were subjected to restrictions consistent with the most stable conformation. In all cases the most apparently stable conformation was selected. Compounds that were too large for model construction or too complex due to heterocyclic ring structures were assigned to symmetry groups after detailed inspection of their structural formulas or portions of the molecular models.

Compounds selected for study were assumed to be relatively stable in biological systems and do not readily undergo chemical or biological modification. For purposes of this discussion, only neutral and relatively inert compounds, which are presumed to act in an inhibitory or regulatory manner in biological systems, are included. Toxicity data for the compounds studied were obtained (7) and converted to LD_{50} values expressed in millimoles per kilogram.

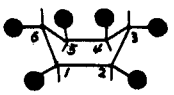
[1] CPK, Ealing Corp., Cambridge, Mass.

RESULTS AND DISCUSSION

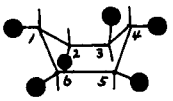
When using compounds from Ref. 7, an apparent relationship between the degree of molecular symmetry and acute lethal toxicity was found. For example, when the oral toxicity data in rats are arranged according to the different groups of symmetry of the compounds in Tables II–IV, the data can be summarized as in Table I.

Utilization of an unpaired *t* test indicated that the mean LD_{50} for the C_1 symmetry group is statistically different from the other three groups. This implies that the C_1 group, which is least symmetrical, has the lowest degree of toxicity. All constituents of living matter listed in "Geigy Documenta Scientific Tables" (8) also belong to the C_1 group. These include compounds from the following general classifications: monosaccharides, sugar phosphates, polyhydric alcohols, amino acids, purines and pyrimidines, porphyrins, fatty acids, steroids, *etc.* As expected, these endogenous compounds have low degrees of toxicity.

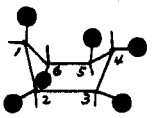
Although the sample sizes for the higher symmetry groups apparently account for the lack of statistical significance between the difference in mean LD_{50} values, the general trend indicates that the greater the degree of symmetry, the greater the degree of toxicity. Among the groups listed, D_{2h} has the greatest degree of symmetry and the highest acute lethal toxicity (lowest mean LD_{50}). The trend of this relationship and the usefulness of symmetry grouping are strengthened by the example of benzene hexachloride (lindane). Three different isomers (α, γ, and δ) are known, with the γ-isomer being the most potent (8) although their octanol-water partition coefficients (log *P*) differ only by 0.4 log unit[2]. Dreiding's models of these compounds show that the most stable conformation of the γ-isomer has all the chlorine atoms at the equatorial positions, which places it in the D_{6h} group while the other two isomers belong to the C_1 group:



benzene hexachloride γ-isomer
1,2,4,5/3,6
LD_{50} = 125 mg/kg (oral rat)
log *P* = 3.72
D_{6h} group symmetry



benzene hexachloride α-isomer
1,2,4/3,5,6
LD_{50} = 500 mg/kg
log *P* = 3.80
C_1 group symmetry



benzene hexachloride δ-isomer
1,2,3,5/4,6
LD_{50} = 1000 mg/kg
log *P* = 4.14
C_1 symmetry group

Another example of interest is the comparison of Compound 7 of the C_{2v} group in Table III with Compound 16 of the C_1 group in Table II. Compound 7, the more symmetrical of the two, is nearly four times as toxic yet the two molecules are isomers.

Considering the large number of chemical classes of compounds represented in Tables II–IV, the apparent relationship becomes even more interesting. Although a number of fundamental questions obviously remain to be answered, the symmetry parameter is apparently useful in predicting something about activity and toxicity of various chemical compounds. Why should all endogenous compounds used as natural building blocks belong to the C_1 symmetry group? Why do compounds of the same symmetry group have the same magnitude of toxicity regardless of their chemical nature and the mechanism of toxicity? Empirically, the answer to the first question may be governed by the building blocks of DNA which are all the C_1 purines and pyrimidines. This should not be confused with the gross symmetry of the two double-helical DNA molecules after replication occurs, which is probably responsible for the gross symmetry of almost all living organisms.

[2] T. Fujita, unpublished data through the courtesy of Dr. C. Hansch.

The answer to the second question may be that the acute lethal toxicity (LD_{50}) is a very nonspecific type of biological response which depends upon nonspecific interactions with biopolymers or macromolecules. It is well known that compounds of higher degrees of symmetry usually have higher melting points and/or boiling points, indicating a greater degree of binding force with the neighboring molecules.

CONCLUSIONS

An apparent relationship was found between the degree of molecular symmetry and the acute oral lethal toxicity in animals as represented by the LD_{50}. This approach has some obvious limitations including the uncertainty in conformation assignments for large molecules and the lack of comparable data on large numbers of compounds of interest of higher order symmetry groups. Furthermore, the approach may not be directly applicable to drugs or substances that exert very specific biological activity by interaction with enzymes, such as antimetabolites, or to drugs that are rapidly metabolized in the body to other species with altered biological activity.

In general, however, this approach may have uncovered a new parameter which should be considered in the design of potentially specific, nontoxic drugs. In a given chemical classification, for example, this parameter may be included along with other physical-chemical parameters such as lipophilicity and steric and electronic factors utilized in current approaches to structure-activity correlations. Group theory can also be taken one step further by using quantum mechanical calculations of molecular interaction in the case of a specific process where a significant amount of information is known about the interaction between the drug and the receptor. Additional studies on specific pharmacological groups or agents will further demonstrate the validity and usefulness of this working hypothesis.

REFERENCES

(1) "Drug Design," vol. I, E. J. Ariens, Ed., Academic, New York, N.Y., 1971, pp. 271–337.

(2) C. Hansch, "Biological Correlations—The Hansch Approach," Advances in Chemistry Series 114, American Chemical Society, Washington, D.C., 1972, pp. 20–40.

(3) J. G. Topliss and R. J. Costello, *J. Med. Chem.*, **15**, 1066(1972).

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(6) R. M. Hochstrasser, "Molecular Aspects of Symmetry," Benjamin, New York, N.Y., 1966.

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(8) "Documenta Geigy Scientific Tables," 6th ed., Geigy Pharmaceuticals, Ardsley, N.Y., 1968.

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GLC Determination of Plasma Levels of Enantiomers of α-[4-(1-Oxo-2-isoindolinyl)phenyl]propionic Acid

G. P. TOSOLINIˣ, E. MORO, A. FORGIONE, M. RANGHIERI, and V. MANDELLI

Abstract □ A quantitative GLC determination of each α-[4-(1-oxo-2-isoindolinyl)phenyl]propionic acid enantiomer in human plasma after oral administration of the racemate is described. After extraction and purification of the extract through partition steps, the substances were converted to the diastereoisomeric amides *via* the acid chlorides. These derivatives were separated and quantitated by GLC. The sensitivity limit is 0.3 μg of each enantiomer/ml plasma. In the concentration range of 0.62–5.00 μg/ml plasma, the percent recovery (± standard deviation) of the *d*- and *l*-enantiomer was 67.51 ± 3.11 and 67.44 ± 3.14, respectively, whereas the coefficients of variation of the ratios between these recoveries and those of the internal standard were 1.03 and 1.05%, respectively.

Keyphrases □ α-[4-(1-Oxo-2-isoindolinyl)phenyl]propionic acid—GLC determination of plasma levels of enantiomers □ α-Phenylpropionic acid derivatives—GLC determination of plasma levels of enantiomers □ GLC—determination, enantiomers of α-[4-(1-oxo-2-isoindolinyl)phenyl]propionic acid, plasma levels, humans

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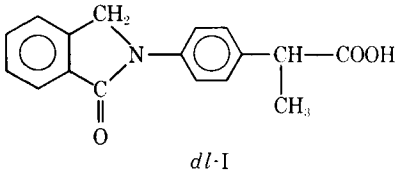
Pharmacokinetics studies (3) showed that the substance in its racemic modification was completely and rapidly absorbed from the GI tract when administered in capsules to healthy volunteers (peak plas-



dl-I

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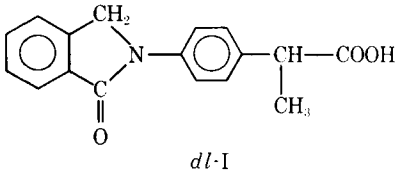


dl-I

ma levels between 30 min and 2 hr) and rapidly eliminated ($t_{1/2\beta}$ between 2.16 and 5.40 hr). These values were obtained from plasma level determinations by techniques (radioactivity and GLC) that do not distinguish between the enantiomers and, therefore, reflect the parameters for the *dl*-form.

Since the high degree of stereospecificity of the anti-inflammatory action of several pairs of α-methylarylacetic acid enantiomers is known (4), the determination of the pharmacokinetic parameters of each enantiomer of *dl*-I after administration of these substances and of their racemate was desirable. The results should indicate whether the two enantiomers are characterized by different pharmacokinetic parameters and whether any interaction exists regarding their absorption, elimination, and metabolism. Therefore, a method for the determination of the plasma levels of the two enantiomers was required. The method described here is based on the conversion of the two enantiomeric acids, extracted from plasma, into their chlorides and reaction of the latter with *l*-α-methylbenzylamine; the resulting two diastereoisomeric amides can be easily separated and determined by GLC.

EXPERIMENTAL

Reagents—*l*-α-Methylbenzylamine[1] and other solvents[2] or reagents[3] were used as purchased.

Reference Compounds—*dl*-I and *dl*-II were synthesized by the methods described in the literature (1). *d*-I, *l*-I, and *d*-II were obtained from their racemates by repeated fractional crystallizations of their salts with *l*-α-methylbenzylamine; the optical purity of these compounds, ascertained by the GLC and NMR spectra of their amides with *l*-α-methylbenzylamine, was greater than 98%. These amides and the amide of *dl*-II with cyclohexylamine were prepared by the following method. The acid (0.2 g) was suspended in anhydrous benzene (25 ml) and treated with thionyl chloride (25 ml), the suspension was refluxed until complete dissolution (about 1 hr), and the solvent and thionyl chloride were then evaporated. The evaporation was repeated twice after addition of anhydrous benzene (25 ml). To the residue, dissolved in anhydrous chloroform (25 ml), the amine (1 ml) in chloroform (10 ml) was added in small portions. After 30 min of agitation at room temperature, the solution was washed with 0.1 *N* HCl (10 ml) and then twice with water (10 ml). The organic phase was evaporated under vacuum and the residue was crystallized twice from the solvent indicated for each amide in Table I. Table I also reports the structural and empirical formulas and the physical characteristics of the reference compounds used in this work and gives the numbers by which these compounds are identified.

GLC—For the GLC determinations, a gas chromatograph[4] equipped with a flame-ionization detector (hydrogen = 50 ml/min, air = 300 ml/min) was utilized. A coiled glass column (length 2 m, i.d. 3 mm) packed with 1% OV-7[5] on acid-washed, silanized Chromosorb W (60–80 mesh) was used; the support was coated by the evaporation technique. Nitrogen was used as the carrier gas (flow rate = 90 ml/min). The temperatures were as follows: column, 280°; and injector port and detector, 300°. The retention times of the compounds are reported in Table I. Figure 1 shows a gas chromatogram of the reference compounds employed. In quantitative GLC determinations, the peak area obtained by multiplying the peak height by the width of the peak at half its height was used.

GLC Reference Compound Calibration Curves—The following reference compound solutions were prepared.

A. Quantitation standard solution: 50 μg *dl*-V/ml chloroform.

B. Stock solutions of reference compounds *d*-III and *l*-III: dissolve 25 mg *d*-III and *l*-III in 25 ml Solution A.

C. Stock solution of reference compound *d*-IV: dissolve 25 mg *d*-IV in 25 ml Solution A.

D. Working solutions: pipet 0.25, 0.5, 1.0, 1.5, and 2.0 ml of B and 1 ml of C in five 10-ml calibrated flasks and dilute to volume with A. Each solution contained 50 μg/ml of *dl*-V and 100 μg/ml of *d*-IV together with 25.0, 50.0, 100.0, 150.0, and 200.0 μg/ml of *d*-III and *l*-III.

GLC data for each solution of D gave the same linear relationship, passing through the origin, as was obtained by plotting the ratios of peak area *d*-III/peak area *dl*-V and peak area *l*-III/peak area *dl*-V as a function of the concentration of *d*-III and *l*-III, respectively. For each reference compound, a "specific peak area ratio" can, therefore, be calculated. For *d*-III, this is given by:

$$\text{specific peak area ratio} = \frac{\text{peak area } d\text{-III}}{\text{concentration } d\text{-III}} \times \frac{\text{concentration } dl\text{-V}}{\text{peak area } dl\text{-V}} \qquad \text{(Eq. 1)}$$

The same expression is used to calculate the specific peak area ratio for *l*-III and *d*-IV.

Quantitative Conversion of d-*I,* l-*I, and* d-*II into* d-*III,* l-*III, and* d-*IV*—The following standard solutions were prepared.

E. Stock solution of *dl*-I: dissolve 25 mg *dl*-I in anhydrous methanol (25 ml). The stock solution was refrigerated; fresh solutions were made monthly.

F. Stock solution of *d*-II (internal standard): dissolve 25 mg *d*-II in anhydrous methanol (25 ml). The stock solution was refrigerated; fresh solutions were made monthly.

G. Working solutions: pipet 0.25-, 0.5-, 1.0-, and 2.0-ml aliquots of E and 1-ml aliquots of F into four 10-ml calibrated flasks and dilute to volume with anhydrous methanol.

Conversion Procedure—Aliquots (0.1 ml) of each solution of G were transferred into four test tubes and evaporated at 40° under a stream of nitrogen; each sample thus contained 10.0 μg of *d*-II together with 1.25, 2.50, 5.0, and 10.0 μg of *d*-I and *l*-I, respectively. The residue was redissolved in anhydrous ethyl acetate (0.2 ml), treated with thionyl chloride (25 μl), and heated for 1 hr at 60° in stoppered test tubes. The solution was then evaporated at room temperature under a stream of nitrogen; anhydrous ethyl

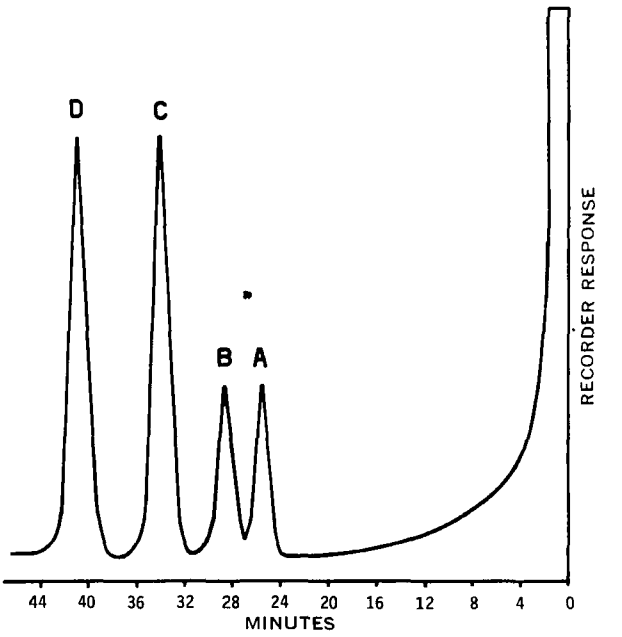
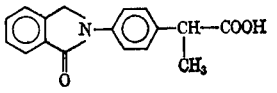
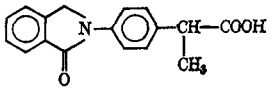
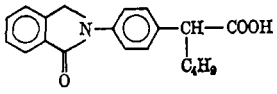
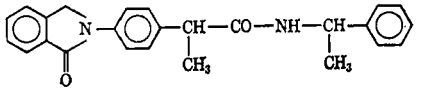
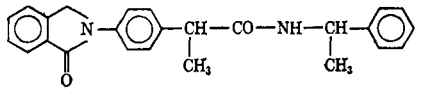
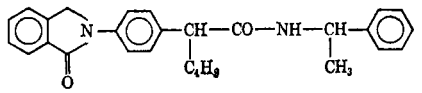
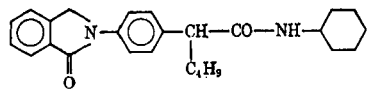


Figure 1—*Gas chromatogram of the reference compounds. Key: A,* d-*III; B,* l-*III; C,* dl-*V (quantitation standard); and D,* d-*IV (internal standard). Conditions were: 1% OV-7 on Chromosorb W (60–80); nitrogen flow rate, 90 ml/min; column temperature, 280°; and recorder chart speed, 30 cm/hr.*

[1] Aldrich Chemical Co.
[2] RS per pesticidi, Carlo Erba.
[3] RP, Carlo Erba.
[4] Carlo Erba, model GI.
[5] Applied Sciences Laboratories.

Table I—Physical Characteristics of Isoindolinylphenylpropionic Acids and Their Derivatives

Compound[a]	Structural Formula	Molecular Formula	Crystallization Solvent	Melting Point	Retention[b] Time, min
d-I		$C_{17}H_{15}NO_3$	Ethanol	209–212°	—
l-I		$C_{17}H_{15}NO_3$	Ethanol	209–212°	—
d-II		$C_{20}H_{21}NO_3$	Ethanol	163–164°	—
d-III		$C_{25}H_{24}N_2O_2$	Benzene	197–198°	24.9
l-III		$C_{25}H_{24}N_2O_2$	Methanol	235–236°	28.2
d-IV		$C_{25}H_{30}N_2O_2$	Ethanol–water	131–133°	40.2
dl-V		$C_{26}H_{32}N_2O_2$	Ethanol–water	188–189°	33.6

[a] The *d*- and *l*-symbols of amides refer to the rotatory power of the starting acids; the optically active amides were prepared from *l*-α-methylbenzylamine.
[b] For GLC conditions, see *Experimental*.

Table II—Conversion Yields of *d*-I, *l*-I, and *d*-II

Micrograms of Compound in Each Sample			Average Conversion[a] ± *SD*, %			Average Conversion Ratios[a] ± *SD*		
d-I	*l*-I	*d*-II	*d*-I	*l*-I	*d*-II	*d*-I/*d*-II	*l*-I/*d*-II	*d*-I/*l*-I
1.25	1.25	10.0	89.20 ± 1.04	87.40 ± 0.76	91.92 ± 0.71	0.970 ± 0.0045	0.950 ± 0.0071	1.020 ± 0.0039
2.50	2.50	10.0	89.65 ± 3.00	88.02 ± 3.31	92.05 ± 4.19	0.974 ± 0.0074	0.957 ± 0.0080	1.018 ± 0.0046
5.0	5.0	10.0	94.70 ± 1.51	93.02 ± 1.39	97.35 ± 0.97	0.973 ± 0.0091	0.955 ± 0.0100	1.018 ± 0.0045
10.0	10.0	10.0	91.10 ± 2.72	89.27 ± 2.73	93.10 ± 2.01	0.978 ± 0.0083	0.959 ± 0.0099	1.020 ± 0.0040
Overall average ± *SD*						0.974 ± 0.0074	0.955 ± 0.0080	1.019 ± 0.0040

[a] Mean of four determinations.

acetate (0.1 ml) was added and the evaporation was repeated. The residue was redissolved in anhydrous ethyl acetate (0.1 ml), and *l*-α-methylbenzylamine (10 μl) was added. After 15 min at room temperature, the solvent and amine were vacuum evaporated at 60° under nitrogen. Ethyl acetate (0.1 ml) was added and the evaporation was repeated. Then 0.1 ml of Solution A was added and a suitable amount of the solution (1–5 μl) was injected into the chromatograph. By using each peak, an experimental specific peak area ratio was calculated by Eq. 1; the ratio between this value (×100) and the theoretical one obtained as described in the preceding paragraph gives the conversion percentage of *d*-I, *l*-I, and *d*-II into *d*-III, *l*-III, and *d*-IV, respectively. The results obtained by this procedure (mean of four determinations for each sample) are reported in Table II; they permit the conclusion that, since the average conversion ratios *d*-I/*d*-II and *l*-I/*d*-II fall in a narrow range (0.974 ± 0.007 and 0.955 ± 0.008, respectively), *d*-II is a suitable internal standard to control the conversion of *d*-I and *l*-I. The same data also show that the mean conversion yield of *d*-I is higher than that of *l*-I, the ratio between them being 1.019 ± 0.004. TLC examination [on silica gel[6] in the solvent system of benzene–ethyl acetate–acetic acid (130:24:30)] of the products obtained by the conversion procedure shows that the conversion is quantitative and that no side-products are formed; losses may occur only during the evaporation steps.

Recoveries of *d*-I, *l*-I, and *d*-II from Human Plasma—*Partition Characteristics*—The determination of partition coefficients between aqueous buffers with different pH and organic solvents showed that *d*-I and *l*-I are quantitatively extracted with 4 volumes ether from aqueous solution at pH < 2 and with 0.1 volume 0.1 *N* NaOH from ether. In stability studies, these compounds were stable in 0.1 *N* HCl and 0.1 *N* NaOH; the same partition and stability characteristics were shown by *d*-II used as the internal standard to check the recovery of *d*-I and *l*-I.

Extraction Procedure—Each Solution G (0.1 ml) was taken to dryness at 40° under a stream of nitrogen and treated with 2 ml

[6] Stratocrom SIF RS, Carlo Erba.

Table III—Recoveries of *d*-I, *l*-I, and *d*-II from Plasma

Micrograms of Compound Added to Each Milliliter Plasma Sample			Average Recovery[a] ± *SD*, %			Average Recovery Ratios[a] ± *SD*		
d-I	*l*-I	*d*-II	*d*-I	*l*-I	*d*-II	*d*-I/*d*-II	*l*-I/*d*-II	*d*-I/*l*-I
0.62	0.62	5.0	69.35 ± 2.46	69.10 ± 2.48	72.12 ± 2.88	0.962 ± 0.0066	0.958 ± 0.0048	1.003 ± 0.0032
1.25	1.25	5.0	66.40 ± 2.01	66.35 ± 1.91	70.52 ± 2.38	0.942 ± 0.0099	0.941 ± 0.0080	1.001 ± 0.0065
2.50	2.50	5.0	67.75 ± 3.32	67.90 ± 3.43	71.25 ± 2.87	0.951 ± 0.012	0.953 ± 0.014	0.998 ± 0.0039
5.0	5.0	5.0	66.53 ± 4.06	66.40 ± 4.20	69.13 ± 3.59	0.962 ± 0.010	0.956 ± 0.013	1.002 ± 0.0032
Overall average ± *SD*						0.954 ± 0.0098	0.953 ± 0.010	1.001 ± 0.0046

[a] Mean of six determinations.

human plasma; each sample thus contained 5 μg of *d*-II and 0.62, 1.25, 2.5, and 5.0 μg of *d*-I and *l*-I/ml plasma, respectively. After equilibration at 37°, plasma samples were acidified by addition of 4 ml 0.1 *N* HCl and extracted for 15 min with ether (40 ml) by mechanical shaking. The organic phase was washed with 5 ml distilled water and reextracted with 0.1 *N* NaOH (5 ml). From the aqueous phase after washing with ether (40 ml) and acidification with 1 *N* HCl, *d*-I, *l*-I, and *d*-II were extracted by mechanical shaking for 15 min with 40 ml ether. After washing with distilled water (5 ml), the ether was taken to dryness at 30° under a stream of nitrogen. The residue obtained was then partitioned between methanol (1 ml) (containing 2 drops water) and hexane (3 ml) by shaking for 15 min. The hexane phase (upper) was removed by aspiration and discarded, the methanol phase was taken to dryness, and derivatization of the residue was accomplished as described under *Conversion Procedure*. The final derivatives were chromatographed under the experimental conditions described previously. The percent recoveries of *d*-I, *l*-I, and *d*-II from plasma were calculated by the ratios (×100) between the experimental specific peak area ratios obtained for each sample by this procedure and the theoretical value of the same parameter deduced from the calibration curves. The recoveries (mean of six determinations) of increasing amounts of *d*-I and *l*-I and a constant amount of *d*-II from plasma are reported in Table III. On the basis of the results obtained in recovery determinations, the following method was developed to determine *d*-I and *l*-I present in human plasma.

Determination of *d*-I and *l*-I in Unknown Samples of Human Plasma—Compound *d*-II (internal standard) (10 μg) was added to 2 ml human plasma; the sample was acidified with 4 ml 0.1 *N* HCl and submitted to the extraction and conversion procedures described. Plasma samples were stored frozen at −20° until assayed; during 3 weeks of storage under these conditions, no change was noted in the concentrations of *d*-I and *l*-I.

RESULTS AND DISCUSSION

Derivative Preparation—The derivative most suitable for GLC separation of *d*-I from *l*-I was found by the preparation of the amides of these compounds with *l*-α-methylbenzylamine. Esters of the same enantiomers with optically active alcohols (*e.g.*, 1,2-methyl-1-butanol), even if easier to prepare, were not separable by GLC. OV-7 (1%) proved the most efficient stationary phase to achieve the separation of the diastereoisomeric amides mentioned; OV-17 can produce separation of these compounds but with less satisfactory results.

Method Precision—The data of Table III show that the amounts of *d*-I and *l*-I recovered from plasma and calculated with reference to the recovery of *d*-II are significantly related (r = 0.999 for $p < 0.01$) to the amount added by the following linear relationship:

$$\text{amount recovered} = \text{amount added} \times b + a \qquad \text{(Eq. 2)}$$

For *d*-I, $b \pm SD = 0.964 \pm 0.0039$ and $a \pm SD = -0.037 \pm 0.022$ (a is not significantly different from zero for $p = 0.05$). For *l*-I, $b \pm SD = 0.962 \pm 0.0044$ and $a \pm SD = -0.031 \pm 0.025$ (a is not significantly different from zero for $p = 0.05$). These data and those of Table III indicate the improvement in precision with the internal standard technique in comparison to the procedure that

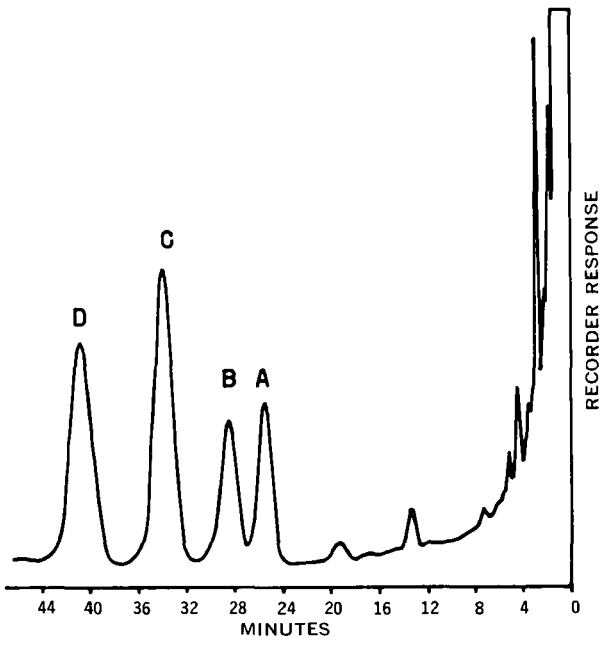


Figure 2—*Gas chromatogram of an extract of 2 ml human plasma of a subject treated with 200 mg of* dl-*I orally. Key and conditions: same as in Fig. 1.*

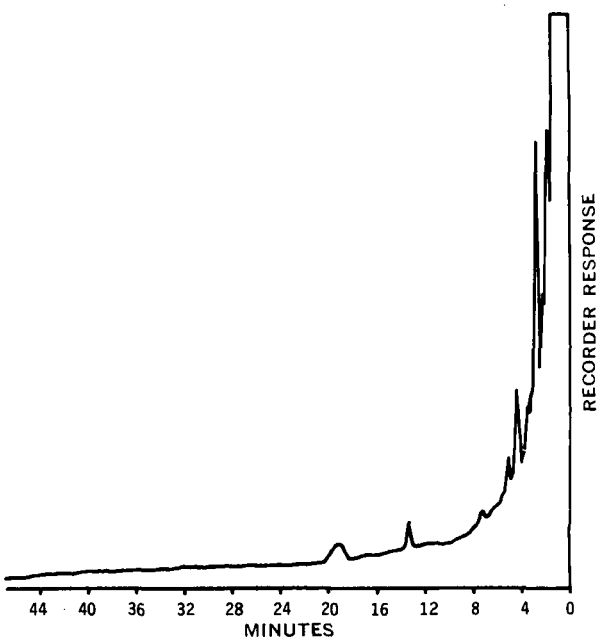


Figure 3—*Gas chromatogram of an extract of 2 ml blank human plasma. Conditions: same as in Fig. 1.*

Table IV—Plasma Levels (Micrograms per Milliliter) of *d*-I and *l*-I in a Subject Treated with 200 mg of *dl*-I Orally[a]

Hours	*d*-I	*l*-I	*A*	*B*	*A* − *B*
0.5	0.92	1.01	1.93	—	—
1.0	6.70	6.16	12.86	12.90	−0.04
1.5	6.94	5.46	12.40	12.60	−0.20
2.0	6.29	4.31	10.60	10.46	+0.14
3.0	4.84	3.03	7.87	8.03	−0.16
6.0	3.39	1.61	5.00	5.03	−0.03
8.0	2.55	1.10	3.65	3.68	−0.03
10.0	1.62	0.66	2.28	2.22	+0.06

[a] *A* = (*d*-I) + (*l*-I) plasma levels obtained by the present method. *B* = (*d*-I) + (*l*-I) plasma levels obtained by the method described in Ref. 3.

uses as reference recovery the mean recovery of each substance to be determined. The addition of the internal standard (*d*-II) to the initial plasma samples makes unnecessary the total recovery of extracts during the extraction procedure. Since (as already stated) the amounts of *d*-I and *l*-I in unknown plasma samples are deduced by reference to the recovery of the internal standard, the quantitation standard (*dl*-V) added at the end of the derivatization should not be necessary. However, it is used to check the efficiency of the extraction and conversion procedures.

Method Specificity—Figure 2 shows the gas chromatogram for the determination of *d*-I and *l*-I in the plasma extract of a subject treated with 200 mg of *dl*-I. Figure 3 represents a blank plasma extract of the same subject; in this chromatogram there are no peaks interfering with those of the substances under examination or with those of internal and quantitation standards. Such interferences are absent even if the equivalent of 4/100th of an extract of 4 ml plasma is injected.

Method Sensitivity—By using 4 ml of plasma in the extraction and derivatization and injecting 4/100th of the final solution, up to 0.3 μg of *d*-I and *l*-I/ml plasma can be determined.

Figure 4 shows the plasma levels of *d*-I and *l*-I in a subject treated with 200 mg of *dl*-I orally; these curves show that *d*-I and *l*-I are characterized by comparable absorption rates but different elimination half-lives[7]. Table IV reports the comparison between the sum (*d*-I) + (*l*-I) plasma levels deduced from the data of Fig. 4 and those obtained experimentally by using the same plasma samples in the extraction and derivatization to obtain this sum directly. This procedure (3) is based on the same extraction steps described in this paper but applies a conversion reaction (formation of 2,2,2-trifluoroethyl esters of *dl*-I) which does not permit a distinction between *d*-I and *l*-I plasma levels. Table IV shows the good accordance between the results obtained by these two methods.

[7] An extensive study on this difference will be published elsewhere.

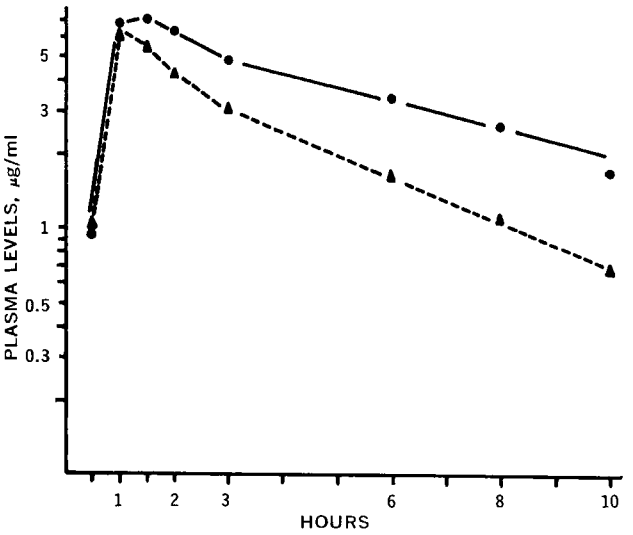


Figure 4—*Plasma levels (micrograms per milliliter) of* d-*I and* l-*I in a subject following administration of 200 mg of* dl-*I orally. Key:* ●—●, d-*I; and* ▲- - -▲, l-*I.*

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[x] To whom inquiries should be directed.

Microbial Kinetics of Drug Action against Gram-Positive and Gram-Negative Organisms I: Effect of Lincomycin on *Staphylococcus aureus* and *Escherichia coli*

SAMUEL M. HEMAN-ACKAH

Abstract □ Lincomycin-affected *Staphylococcus aureus* cultures show two phases of steady-state generation curves whose characteristics are different from those of lincomycin-affected *Escherichia coli*. In both cases, an initial (phase I) steady-state generation, expressed as $\ln N = \ln N_0 + k(\text{app}_\text{I})t$, is followed by an ultimate (phase II) steady-state generation, $\ln N = \ln N_0 + k(\text{app}_\text{II})t$, at the same dose level, where $k(\text{app}_\text{I})$ and $k(\text{app}_\text{II})$ are the apparent generation rate constants for phase I and phase II generations, respectively; N is the number of organisms per unit volume at time, t; and N_0 is the number of organisms per unit volume at some initial time, 0, during this generation phase. The $k(\text{app}_\text{I})$ was less than $k(\text{app}_\text{II})$ for lincomycin-affected *Staph. aureus*, which suggests development of organism adaptation or resistance to drug action, whereas $k(\text{app}_\text{I})$ was greater than $k(\text{app}_\text{II})$ for lincomycin-affected *E. coli*, which indicates enhanced organism inhibition during the later stages of drug-bacteria reaction at any concentration level. The generation rate constants for lincomycin-affected *Staph. aureus* in phase I and phase II generations show similar functional dependencies on drug concentration as the lincomycin-affected *E. coli* in phase I generation and suggest the same mechanism of action, which is different from that of the lincomycin-affected *E. coli* in phase II generation. The lincomycin (phase I) action on *Staph. aureus* is about 1000 times as potent (on a weight basis) as that on *E. coli* at 37.5° and pH 7.05. However, the extent of generation inhibition of *Staph. aureus* and *E. coli* by lincomycin increases as some function of pH, which indicates that the unprotonated fraction of drug concentration contributes to the activity.

Keyphrases □ Microbial kinetics—effect of lincomycin on *Staph. aureus* and *E. coli*, drug-bacteria reactions, total and viable count methods □ Lincomycin—comparison of action on *Staph. aureus* and *E. coli*, microbial kinetics, drug-bacteria reactions, generation curves □ *Staphylococcus aureus*—effect of lincomycin, compared to lincomycin effects on *E. coli*, drug-bacteria reactions, generation curves, microbial kinetics □ *Escherichia coli*—effect of lincomycin, compared to lincomycin effects on *Staph. aureus*, drug-bacteria reactions, generation curves, microbial kinetics

The quantification and prediction of antimicrobial action by microbial kinetics have been demonstrated for a number of antibiotics and chemotherapeutic agents (1). Kinetic parameters, which are derived from generation curves of drug-free and drug-affected *Escherichia coli* cultures, have been used to: *(a)* characterize drug-receptor interactions and elucidate possible mechanisms of action (2–5), *(b)* estimate minimal inhibitory concentration (MIC) (6) and relative potencies (7–9), *(c)* determine structure-activity relationships (5, 6, 9–12), and *(d)* evaluate combination effects of the drugs (5, 7–9, 13–15).

A particle-size counter is employed to monitor organism population of drug-free and drug-affected cultures (16). In all cases, coincident plots of total counts (obtained by the particle-size counter method) *versus* time with those of viable count (obtained by the pour plate method) were obtained, indicating the suitability of the particle-size counter and the total method for determining generation rates of the cultures. The technique is very rapid and permits evaluation of drug action on the cultures within 2–5 hr. It produces highly reproducible and precise kinetic parameters of drug-free and drug-affected cultures (7). Unlike conventional methods employed in the *in vitro* evaluation of antimicrobial activity, such as agar diffusion and serial dilution methods, it is not subject to restraints of drug diffusivity in agar medium or errors of definitive end-point inherent in experimental design.

E. coli has been the only test organism employed in microbial kinetic studies because it does not form clumps or chains and it produces uniform cell suspensions (17, 18) suitable for obtaining total counts. It is a classical organism employed in the evaluation of antimicrobial activity of many compounds. However, it is a typical Gram-negative organism and its use as a test organism for studies on the action of macrolide and lincosaminide antibiotics has been criticized[1]. It is claimed that these antibiotics are normally more active against Gram-positive than Gram-negative organisms (19, 20) and that the two types of organisms vary in gross morphology (17, 18). The data obtained with *E. coli* may not be readily extrapolated to other Gram-negative bacteria without experimental evidence to substantiate this implied claim. The spectrum of activity for macrolide and lincosaminide antibiotics is reason enough to select a Gram-positive coccus for the study.

It was of interest to use *Staphylococcus aureus* as a test organism representative of Gram-positive organisms in studies of drug action by microbial kinetics. This report presents the results of a study on lincomycin action on *Staph. aureus* and a comparison of the data with those obtained on *E. coli*.

EXPERIMENTAL

Test Organism—Replicate slants of *Staph. aureus* (ATCC 6538) and *E. coli* (ATCC 12407) were used in all experiments. The slants were prepared from single isolated colonies of the respective organisms and were stored in a refrigerator at 4°.

Culture Media—Antibiotic medium III[2] was prepared according to the specification of the manufacturer, filtered twice through 0.45-μm filters[3], and autoclaved at 121° for 15 min. The pH of the medium was 7.05 ± 0.05, with the exception of media used to study the antibacterial activity as a function of pH.

[1] Personal communications from Dr. J. B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich., and Dr. F. Kavanagh, Eli Lilly & Co., Indianapolis, Ind., to Dr. E. R. Garrett, University of Florida, Gainesville, Fla.

[2] Difco Laboratories, Detroit, Mich.

[3] Millipore HA.

Table I—Analysis of Variance on Generation Rate Constant (k_0 in seconds^{-1}) of Drug-Free *Staph. aureus* Cultures at 37.5° and pH 7.05 within and between Day Experiments

Experiments	Days 1	2	3	4	5
	$k_0 \times 10^{-5}$ sec^{-1}				
1	39.81	40.31	39.42	40.82	41.82
2	41.69	40.13	43.65	40.68	40.32
3	40.13	39.82	40.91	42.43	42.01

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Between days	4	4.1038×10^{10}	1.0260×10^{10}
Within days	10	14.9588×10^{10}	1.4959×10^{10}
Total	14	19.0626×10^{10}	

Variance ratio (F_4^{10}) = 1.4580; [a] $p > 0.10$
Mean generation rate constant (k_0) = 40.93×10^{-5}
Standard error of the mean = 0.55×10^{-5}

[a] From Table V of "Statistical Tables for Biological Agricultural and Medical Research," 6th ed., R. A. Fisher and Yates, Eds., Hafner Publishing, New York, N.Y., 1963.

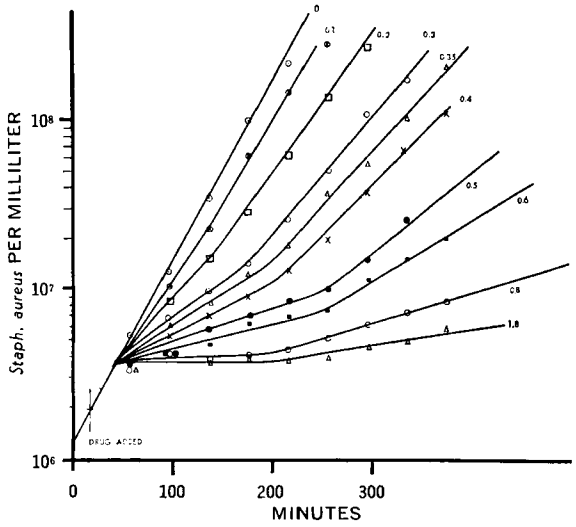


Figure 1—*Typical generation curves of* Staph. aureus *in antibiotic medium III at pH 7.05 and 37.5° in the absence and presence of various concentrations of lincomycin. The curves are labeled according to drug concentration in micrograms per milliliter.*

Antibiotic—An assayed sample of lincomycin hydrochloride[4] (860 μg base equivalent/mg) was used.

Bacterial Cultures—A 5-ml aliquot of culture medium in a screw-capped test tube was inoculated with the organism from a fresh slant and incubated at 37.5° for 12–15 hr. A sample of 0.5 ml *(E. coli)* or 1.0 ml *(Staph. aureus)* was then transferred into 50 ml fresh culture medium contained in a 125-ml loosely capped erlenmeyer flask and maintained at 37.5° in a 57-liter (15-gallon) constant-temperature shaker water bath[5]. The generation of the culture was followed up to 2.0×10^7 cells/ml. An aliquot of this culture (*i.e.*, 1.0 ml *Staph. aureus* culture/50 ml medium or 1.0 ml *E. coli* culture/100 ml medium) was added to a bulk amount of culture medium contained in a Pyrex flask fitted with a 50-ml dispenser. The "seeded" culture medium was kept in an incubator at 37.5° for 30 min with intermittent shaking. Aliquots (50 ml) of the seeded culture medium were then aseptically transferred with the aid of the dispenser into replicate erlenmeyer flasks, maintained at 37.5 ± 0.1° in the water bath.

Total Count Method—Culture samples (0.5 or 1.0 ml) were withdrawn at 20–30-min intervals and diluted to obtain counts within a range of 5000–20,000 counts/50 μl on the particle-size counter[6] equipped with a 30-μm orifice. The settings of the instrument were: 1/aperture current, 1.0; 1/amplification, 2.0; gain, 8.0; matching switch, 40K; lower threshold, 5; and upper threshold, at maximum. These operational conditions were found effective for the counting of *Staph. aureus* and *E. coli* cells without significant loss of counts or interference from the background noise. The counts were also corrected for background count on the batch of medium used when suitably diluted in the same manner as the culture samples. In general, the background count did not exceed 500 counts/50 μl. The diluent was an aqueous solution of 0.85% sodium chloride and 1.0% formaldehyde, filtered twice through a 0.45-μm filter[3].

Viable Count Method—Culture samples (0.5 or 1.0 ml) were withdrawn at 20–30-min intervals and diluted serially in sterile 0.9% NaCl solution and finally in a culture medium to obtain a concentration of 50–150 cells/ml. Aliquots of 1.0 ml from the final broth dilution were pipeted into each of three replicate sandwich agar plates. The plates were incubated at 37.5° for 48 hr, and the colonies were counted on a colony counter[7].

Effects of Antibiotic Concentration on Generation Rates—Fresh solutions of the antibiotic were aseptically prepared for each experiment. They were diluted suitably so that aliquots of 0.5 or 1.0 ml added to replicate 50 ml volumes of seeded culture medium yielded the desired concentrations of the antibiotic. The solutions were added to the cultures growing at 37.5° in the logarithmic phase at an organism concentration of 2.0×10^6 cells/ml. Samples were withdrawn every 20–30 min, and organism population was determined by the appropriate method. Generation curves for *Staph. aureus* cultures and for *E. coli* cultures affected with 0–1.0 and 0–600 μg/ml lincomycin, respectively, were obtained by the total count method. Generation curves for *Staph. aureus* cultures affected with selected concentrations of lincomycin (0, 0.4, 1.0, 1.2, and 1.4 μg/ml) were also obtained by both the total and viable count methods.

Effect of Nutrient Concentration on Drug-Affected Generation Rates—Culture media of half, normal, and twice the concentration of both ingredients were prepared and adjusted to pH 7.05 ± 0.05, where necessary. They were used for determining the generation curves at 37.5° of replicate *Staph. aureus* cultures affected with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml lincomycin as described in the previous section. The organism concentration at the time of drug addition was 2×10^6 cells/ml. Total counts were obtained on samples withdrawn every 20–30 min.

Effect of Organism Population on Drug-Affected Generation Rates—Three sets of normal strength culture media were prepared and used for determining the generation curves at 37.5° of replicate *Staph. aureus* cultures affected with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/ml lincomycin. The organism concentrations at the time of drug addition were 3.0×10^5, 2.0×10^6, and 3.0×10^7 cells/ml in the set of culture medium. Total counts were obtained on samples withdrawn every 20–30 min.

Effect of pH on Drug-Affected Generation Rates—Sufficient amounts of 2.0 *N* HCl and 2.0 *N* NaOH were added to culture media to obtain pH values of 6.2, 6.4, 6.8, 7.0, 7.4, and 7.7. They were used for determining generation curves at 37.5° of replicate *Staph. aureus* cultures affected with different predetermined concentrations of lincomycin at each pH level. The organism concentration at the time of drug addition was 2×10^6 cells/ml. Total counts were obtained on samples withdrawn every 20–30 min.

RESULTS

Generation Rate Constants of Drug-Free Cultures—Balanced cultures of *Staph. aureus* and *E. coli* showed an exponential phase of generation expressed as:

$$\ln N = \ln N_0 + k_0 t \qquad \text{(Eq. 1)}$$

where N is the number of organisms per unit volume at time, t: N_0 is the number of organisms per unit volume at some initial

[4] Supplied by Dr. J. B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich.
[5] Model RW 65, New Brunswick Scientific Co., New Brunswick, N.J.
[6] Coulter counter, model ZB1, Coulter Electronics Co., Hialeah, Fla.
[7] Lab-line Instruments, Inc., Melrose Park, Ill.

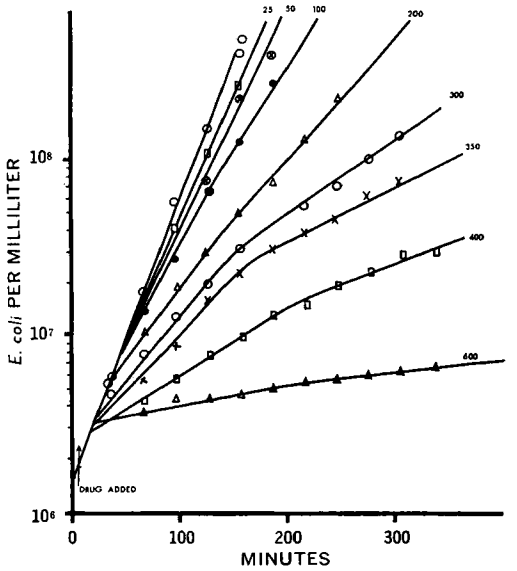


Figure 2—*Typical generation curves of* E. coli *in antibiotic medium III at pH 7.05 and 37.5° in the absence and presence of various concentrations of lincomycin. The curves are labeled according to drug concentration in micrograms per milliliter.*

time, 0, during this generation phase; and k_0 is the apparent generation rate constant. Apparent generation rate constants, k_0 in seconds^{-1}, obtained from the slopes of plots of ln N *versus* t from generation curves of *Staph. aureus* were highly reproducible in experiments performed within a day and between days on different batches of antibiotic medium III (Table I). The mean generation rate constant, k_0, obtained from the results of 15 experiments for *Staph. aureus* was 40.93×10^{-5}/sec. The generation rate constant obtained for *E. coli* in previous experiments (8, 9) and determined in the present study was 60.67×10^{-5}/sec. Thus, the mean generation time (t_{mgt}) can be calculated for each organism from the expression:

$$t_{mgt} = 0.693/k_0 \qquad \text{(Eq. 2)}$$

The values found were 28.21 and 19.04 min for *Staph. aureus* and *E. coli* cultures, respectively.

Shape of Generation Curves for Drug-Affected Cultures—The addition of graded concentrations of lincomycin to balanced cultures of *Staph. aureus* (Fig. 1) and *E. coli* (Fig. 2) decreased the generation rates after lag periods of approximately 30 and 20 min, respectively. Subsequent to the deviation from the curve of the drug-free culture, a new steady-state phase of generation (phase I) was established, followed after 1–2 generations of the drug-affected *Staph. aureus* or 3–5 generations of the drug-affected *E. coli* by an ultimate steady-state phase of generation (phase II) during the later stages of drug-bacteria reaction. Apparent generation rate constants (k_{app} in seconds^{-1}) of the drug-affected cultures were obtained from the slopes of the linear portions of the plot of ln N *versus* t in accordance with the expression:

$$\ln N = \ln N_0 + k_{app}t \qquad \text{(Eq. 3)}$$

The $k(\text{app}_\text{I})$ was less than $k(\text{app}_\text{II})$ for lincomycin-affected *Staph. aureus*, whereas the $k(\text{app}_\text{I})$ was greater than $k(\text{app}_\text{II})$ for lincomycin-affected *E. coli*, where $k(\text{app}_\text{I})$ and $k(\text{app}_\text{II})$ are the generation rate constants for the phase I and phase II generations, respectively.

Comparison of Generation Curves of Drug-Affected Cultures by Total and Viable Count Methods—Semilogarithmic plots of total and viable counts *versus* time for the action of selected concentrations of lincomycin (0, 0.4, 1.0, 1.2, and 1.4 μg/ml) against *Staph. aureus* are given in Fig. 3. Parallel generation curves were obtained from the plots of the total and viable counts for the drug-free culture and for drug-affected cultures in the subcompletely inhibitory range of lincomycin concentrations (curves A, A_1; B, B_1; and C, C_1). However, the total count was 60–80% of the viable count. At concentrations greater than 1.0 μg/ml lincomycin, complete bacteriostatic inhibition was demonstrated in 170 min for both the total and viable counts (curves D, D_1; and E, E_1). Thereafter, there was a rapid drop in the viable counts, which may indicate a bactericidal or lytic phenomenon.

Coincident generation curves were, however, obtained from plots of total and viable counts *versus* time for drug-free and drug-affected *E. coli* cultures in the subcompletely inhibitory concentration range of lincomycin (3).

Effect of Drug Concentration on Generation Rates—A plot of k_{app} *versus* concentration is given in Fig. 4. The extent of generation inhibition is directly proportional to lincomycin concentration, L, in the range 0–0.35 μg/ml for the phase I action (curve A) and 0–0.55 μg/ml for the phase II action (curve A) against *Staph. aureus* or in the range 0–100 μg/ml for the phase I action (curve C) against *E. coli*, in accordance with the expression:

$$k_{app} = k_0 - k_L L \qquad \text{(Eq. 4)}$$

where k_L is defined as the specific inhibitory rate constant. Above these concentration ranges, the k_{app} is not a linear function of the increasing drug concentration, but it asymptotically approaches zero.

On the other hand, a nonlinear decrease of the k_{app} with drug concentration is observed for the phase II lincomycin action (curve B) against *E. coli* throughout the concentration range studied. Coincidence of plots of k_{app} *versus* drug concentration is obtained (curve A) when the actual concentrations for the phase I and phase II lincomycin action against *Staph. aureus* from the data of Table I are multiplied by factors of 1.0 and 0.67, respectively. The plots for the phase I and phase II lincomycin action (curves C and B) against *E. coli* are not superimposable, which suggests different functional dependencies of the respective k_{app} values on drug concentrations.

Applicability of Saturable Receptor Site Model to Action of Lincomycin against *Staph. aureus* and *E. coli*—Figure 5 gives a plot of $L/(k_0 - k_{app})$ *versus* L in accordance with a previously derived (3) receptor site model:

$$L/(k_0 - k_{app}) = L(k_b/k_a) + 1/k_a \qquad \text{(Eq. 5)}$$

where k_a and k_b are constants of proportionality related to drug availability in the biophase and drug affinity for receptor or binding sites. Adherence to the model is observed from linear plots obtained for concentrations of lincomycin greater than 0.35 μg/ml

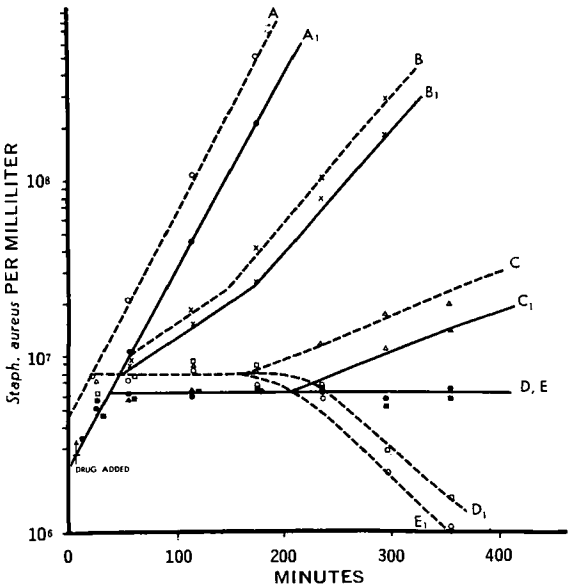


Figure 3—*Generation curves of* Staph. aureus *at pH 7.05 and 37.5° in the absence and presence of lincomycin, obtained by total* (—) *and viable* (---) *counts. The curves and respective drug concentrations (micrograms per milliliter) were: A, A_1, 0.0; B, B_1, 0.4; C, C_1, 1.0; D, D_1, 1.2; and E, E_1, 1.4.*

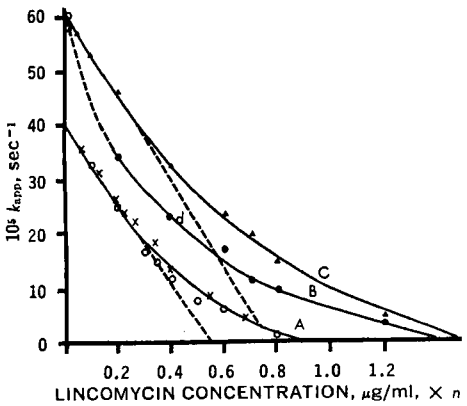


Figure 4—*Dependence of apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, for* Staph. aureus *and* E. coli *on concentrations of lincomycin (micrograms per milliliter) at pH 7.05 and 37.5°. The actual concentrations are multiplied by a factor,* n*, where* n = *1.0 in curve A for* Staph. aureus *in phase I generation* (○), n = *0.67 in a coincident curve for* Staph. aureus *in phase II generation* (×), *and* n = *0.002 in curves B and C for* E. coli *in phase I and phase II generations, respectively.*

in the phase I action (curve A) and greater than 0.55 μg/ml in the phase II action (curve B) against *Staph. aureus* or greater than 100 μg/ml in the phase I action (curve D) against *E. coli.* In all cases, deviations occur in the lower concentration ranges. Strict adherence to Eq. 5 is observed for all concentrations of lincomycin in the phase II action (curve C) against *E. coli.* The values of k_a and k_b calculated from slopes and intercepts of such plots are given in Table II. At the MIC, when there is complete bacteriostasis and $k_{app} = 0$, Eq. 5 reduces to:

$$L = k_0/(k_a - k_0 k_b) \qquad \text{(Eq. 6)}$$

where lincomycin is at the MIC. Calculated MIC values of lincomycin action against *Staph. aureus* and *E. coli* are given in Table II. The relative potency of lincomycin action (on a weight basis) calculated in accordance with Eq. 6 from the data of Table II is (*Staph. aureus* to *E. coli*) 852:1 and 611:1 for the phase I and phase II generations, respectively.

Effect of Culture Medium Variations—The plot of apparent

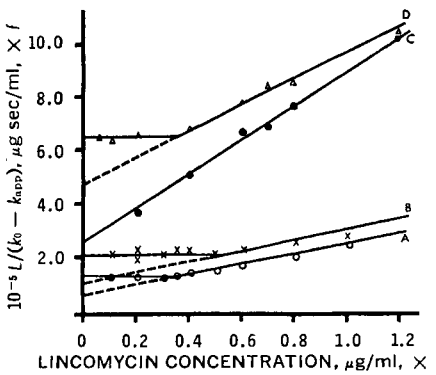


Figure 5—*Demonstration of saturation kinetics for the dependency of the apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, of* Staph. aureus *and* E. coli *at higher concentrations of lincomycin (micrograms per milliliter) at pH 7.05 and 37.5°. Key: A,* Staph. aureus*-affected culture in phase I generation; B,* Staph. aureus*-affected culture in phase II generation; C,* E. coli*-affected culture in phase II generation; and D,* E. coli*-affected culture in phase I generation. The curves were plotted in accordance with Eq. 5. The actual values of* $L/(k_0 - k_{app})$ *are multiplied by a factor,* f*, where* f = *0.01 for curves A and B and* f = *1.0 for curves C and D. The actual lincomycin concentrations,* L*, are also multiplied by a factor,* n*, where* n = *1.0 for curves A and B and* n = *0.002 for curves C and D.*

Table II—Derived[a] Kinetic Parameters from Generation Curves of Lincomycin-Affected *Staph. aureus* and *E. coli* Cultures

Kinetic Parameter	*Staph. aureus* Phase I	*Staph. aureus* Phase II	*E. coli* Phase I	*E. coli* Phase II
$10^5 k_L$ (ml/μg sec)[b]	72.73	48.73	0.16	—
$10^5 k_a$ (ml/μg sec)[c]	153.85	91.50	0.20	0.40
$10^5 k_a/k_b$ (sec^{-1})[c]	57.19	52.29	102.77	78.95
$(k_a/k_b)/k_0$[c]	1.42	1.30	1.68	1.29
$10^2 k_b$ (ml/μg)[c]	267.69	174.76	0.19	0.51
MIC (μg/ml)[d]	0.88	1.13	749.50	690.67

[a] Derived from data of Fig. 1. [b] Calculated from the slope of the plot of k_{app} *versus* concentration from 0 to 0.35 μg/ml lincomycin in phase I action on *Staph. aureus* and from 0 to 0.55 μg/ml lincomycin in phase II action of *Staph. aureus* or 0–100 μg/ml lincomycin in phase I action on *E. coli* according to the expression $k_{app} = k_0 - k_L L$, where L is the concentration of lincomycin. [c] k_a, k_a/k_b, $(k_a/k_b)/k_0$, and k_b are estimated from the slopes and intercepts of the plots of $L/(k_0 - k_{app})$ *versus* L from the expression, $L/(k_0 - k_{app}) = L(k_b/k_a) + 1/k_a$ for concentrations >0.35 μg/ml lincomycin in phase I action on *Staph. aureus* and >0.55 μg/ml lincomycin in phase II action on *Staph. aureus* or > 100 μg/ml lincomycin in phase I action on *E. coli* and all studied concentrations of lincomycin in phase II action on *E. coli.* [d] Calculated from the expression $L = k_0/(k_a - k_0 k_b)$, where lincomycin is at the MIC.

generation rate constants (k_{app}) of *Staph. aureus* affected by different concentrations of lincomycin (L) in three culture media of different compositions is given in Fig. 6. The variation of these generation rate constants among different culture media is not significantly different from that observed in the daily variation in organism generation rates. This indicates that there is no significant inactivation or binding of lincomycin by broth constituents that interferes with drug action on *Staph. aureus.* Similar evidence was established previously for lincomycin action on *E. coli* (3).

Effect of Organism Population on Drug-Affected Generation Rates—The plot of apparent generation rate constants (k_{app}) affected by various concentrations of lincomycin (L) for three different organism populations at the time of drug addition is given in Fig. 7. There are no significant differences among the generation rate constants of different organism populations at any drug concentration level studied, as was previously found for *E. coli* (3).

Effect of pH on Drug-Affected Generation Rates—The apparent generation rate constants obtained for *Staph. aureus* cultures in media of pH 6.2–7.7 in the absence and presence of graded concentrations of lincomycin are given in Fig. 8. The generation rate constant (k_0) of the drug-free culture is invariant with the pH of the culture medium in the range studied. The phase I generation rate constants [$k(\text{app}_I)$] of the drug-affected cultures are significantly decreased with increasing pH values for the same lincomycin concentration. Therefore, progressively larger amounts of lincomycin are required to produce the same generation inhibition as the pH is decreased. The calculated values of k_L obtained

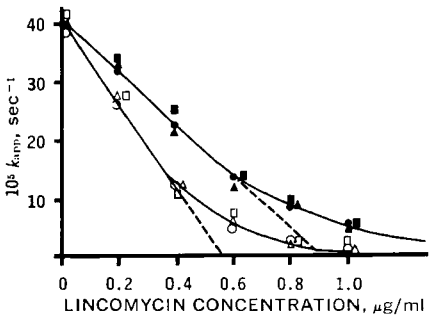


Figure 6—*Apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, of* Staph. aureus *as functions of concentration of lincomycin (micrograms per milliliter) in culture media of different compositions at pH 7.05 and 37.5°. Curve A (open symbols) is for phase I generations, and curve B (closed symbols) is for phase II generations. The culture media were: ●,○, half strength; ▲,△, single strength; and ■,□, double strength.*

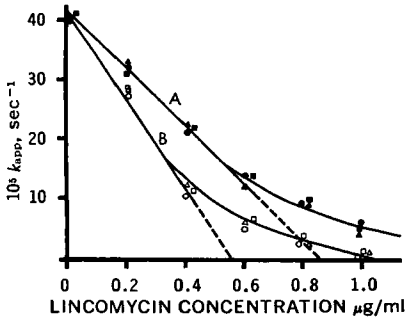


Figure 7—*Apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, of* Staph. aureus *as functions of concentration of lincomycin (micrograms per milliliter) at pH 7.05 and 37.5° for various organism populations at time of drug addition. Curve A (open symbols) is for phase I generations, and curve B (closed symbols) is for phase II generations. The organism concentrations were: ●,○, 3.0 × 10⁵ cells/ml; ▲,△, 2.0 × 10⁶ cells/ml; and ■,□, 3.0 × 10⁷ cells/ml.*

from the slopes of plots of $k(\text{app}_I)$ *versus* lincomycin concentration in accordance with Eq. 4 are plotted as a function of pH in Fig. 9. The values of k_L increase about 10-fold for a unit increase in pH over the 6.2–6.8 range, but the rate of change tends to lessen to a null slope above pH 6.8 (curve A). Similar results are obtained from plots of the data of Mielck and Garrett (3) for the action of lincomycin against *E. coli* in media with a pH range of 5.80–7.50 (curve B).

DISCUSSION

Staph. aureus is known to divide with no set pattern in the orientation of successive division planes to form irregular clumps (18). The total count method employed for monitoring organism population in broth does not differentiate between single discrete and clustered particles in counts on sample suspensions. Therefore, it was expected that the use of the particle-size counter for counting drug-free and drug-affected *Staph. aureus* would produce erratic counts and yield nonreproducible generation rates of cultures. If this were the case, it would necessitate the application of certain physicochemical concepts and basic microbiological techniques to break down clumps and disperse cells before counting or the coupling of the Channelyzer[8] and other devices to the particle-size counter to determine and characterize the distributive pattern of the cells and thereby permit an estimate of true and reproducible counts on the culture samples.

The work reported here shows that the total count method is applicable for determining the generation rates of drug-free and drug-affected *Staph. aureus* cultures with none of the anticipated problems. Although the concentrations of cells at any time, *t*, as determined by the total count method and the pour plate method were quite different (Fig. 3), the proportion of total cells to viable cells remained relatively constant in the drug-free and drug-affected cultures. The higher count obtained from viables may be

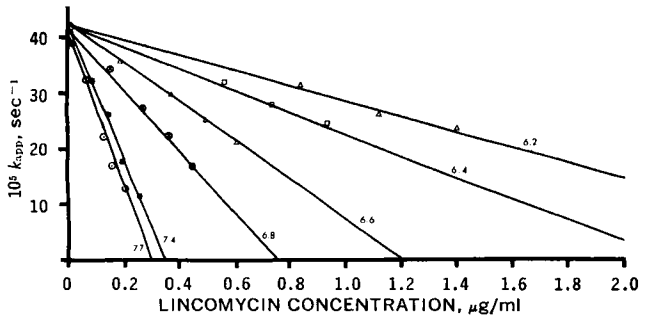


Figure 8—*Dependence of the apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, for* Staph. aureus *on concentrations of lincomycin (micrograms per milliliter) in culture media of various pH values at 37.5°. Each curve is labeled as to the pH values of the culture media.*

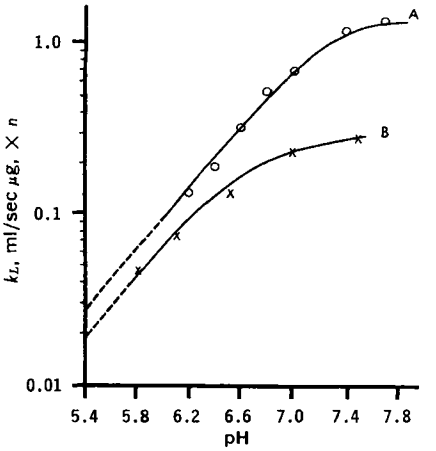


Figure 9—*Semilogarithmic plot of the apparent inhibitory rate constant,* k_L *in milliliter per microgram second, for the action of lincomycin on* Staph. aureus *(curve A) and* E. coli *(curve B) in culture media of various pH values at 37.5°. The drawn lines are consistent with Eq. 14. The actual values of* k_L *are multiplied by a factor,* n, *where* n = 10^3 *for* Staph. aureus *and* n = 10^5 *for* E. coli.

rationalized by subsequent generation of the culture samples, during the interval between withdrawal of the samples and the subsequent serial dilution procedures in saline and finally in broth before plating on agar. Alternatively, the high dilution employed to reduce the cell concentration to a countable size on the agar plate possibly caused a dispersion of randomly distributed clusters with a consequent increase in colony counts. Such effects were conceivably nonexistent in the total count procedure where the diluent contained formaldehyde, which promptly arrested generation of the culture samples which were also counted at low dilutions. It is evident that there is no kill or death of the organisms superimposed on generation inhibition by the action of lincomycin at the subcompletely inhibitory level. The generation rate constants obtained by the two methods were identical and within limits of experimental error. Size frequency analysis[8] indicates (Fig. 10) that the drug-free (curve A) and drug-affected (curve B) cultures have the same distributive pattern, except that the cell volume (μm^3) of the drug-affected culture is increased

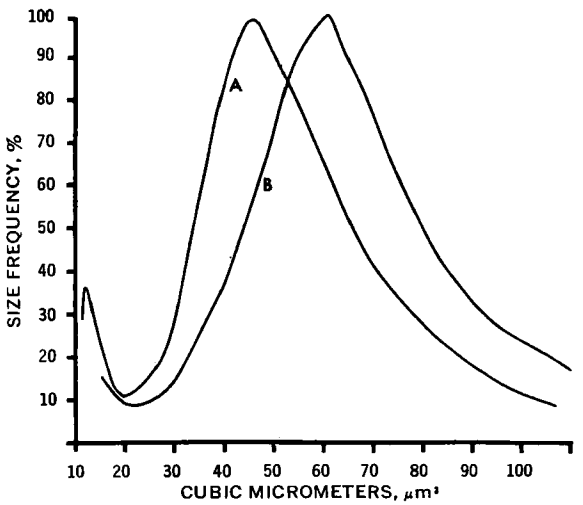


Figure 10—*Size frequency distribution curves of* Staph. aureus *cultures. Curve A is for the drug-free culture, and Curve B is for culture treated with 0.5 μg/ml lincomycin 30 min after drug addition. The curves were obtained from a Channelyzer calibrated with a threshold factor, 0.034, at base channel threshold 5 and window width 100.*

[8] Coulter Electronics Co., Hialeah, Fla.

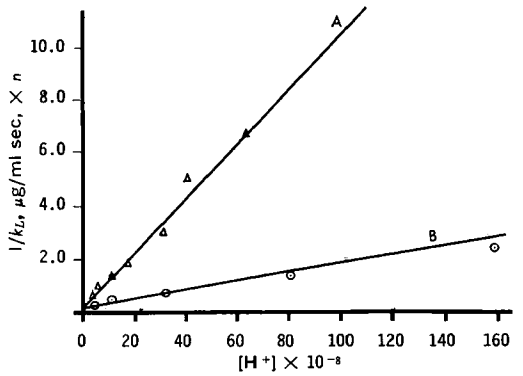


Figure 11—*Demonstration of linearity in plots of* $1/k_L$ *(micrograms per milliliter second)* versus $[H^+]$, *in accordance with Eq. 15, for the action of lincomycin on* Staph. aureus *(curve A) and* E. coli *(curve B). The curves were drawn in accordance with Eq. 15. The actual values of* $1/k_L$ *are multiplied by a factor,* n, *where* $n = 10^{-3}$ *for* Staph. aureus *and* $n = 10^{-6}$ for E. coli.

relative to that of drug-free culture, due undoubtedly to alterations in shape and form of the cells (18) by lincomycin action. There was, however, evidence of a rapid drop in the viable count (Fig. 3) for the action of lincomycin on *Staph. aureus* at concentrations above the bacteriostatic level, which may indicate a bactericidal or lytic phenomenon.

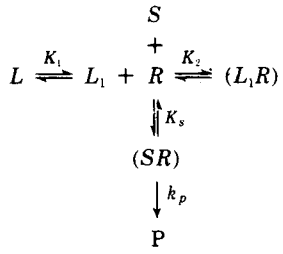
The generation rate constants of the drug-free and drug-affected *Staph. aureus* are not affected by variations in concentrations of constituents of the nutritive media (Fig. 6), indicating that no significant inactivation or binding of the lincomycin by broth constituents occurs to interfere with drug action on the cultures. Likewise, variations in inoculum size at the time of drug addition (Fig. 7) do not affect generation rate constants of cultures, which indicates that the drug is neither metabolized by the organisms, nor depleted in the medium as a result of adsorption to cellular components, nor inactivated by metabolic by-products and other interactions as functions of *Staph. aureus* numbers.

Balanced drug-free cultures of *E. coli* grow and divide at a rate 1.50 times faster than *Staph. aureus* in antibiotic medium III, which may be due to inherent variations in generation characteristics of the organisms or may suggest that the medium supports the growth of *E. coli* better than that of *Staph. aureus*. There are definite lag periods (Figs. 1 and 2) after drug addition before new steady-state generations of the cultures are established. The lag period is longer for *Staph. aureus* cultures (about 30 min) than for *E. coli* cultures (about 20 min). This may be attributed to differences in gross morphology of the organisms (17, 18) which possibly influence the rates of passage of the drug through cell membranes and formation of drug–receptor complexes that determine the onset of generation inhibition (14).

Lincomycin-affected *Staph. aureus* and *E. coli* cultures exhibit biphasic steady-state generation curves (Figs. 1 and 2) whose characteristics are, however, different. The generation rate constant $[k(\text{app}_\text{I})]$ of the phase I generation is less than the generation rate constant $[k(\text{app}_\text{II})]$ of the phase II generation of the drug-affected *Staph. aureus* and suggests development of organism resistance to drug action in the later stages of the drug–bacteria reaction. The reverse is the case with drug-affected *E. coli*, where $k(\text{app}_\text{I})$ is greater than $k(\text{app}_\text{II})$ and indicates an enhanced generation inhibition at the same concentration levels of the lincomycin.

The functional dependency of k_{app} on lincomycin concentration for the drug-affected *Staph. aureus* in phase I generation is the same as that in its phase II generation. This suggests the same mechanism (22) of lincomycin action in the phase I and phase II generations of the drug-affected cultures. The k_{app} *versus* concentration curves for the phase I and phase II generations are superimposable by a potency factor of 0.67 over the entire concentration range studied (Fig. 4). This indicates that only 67% of total drug concentration is effective against *Staph. aureus* in the phase II generation.

An operative kinetic model (Scheme I) similar to that which defined the action of many antibiotics and chemotherapeutic agents on *E. coli* (1) may be applied.



Scheme I

In this model, L is the lincomycin concentration in the medium, which is in equilibrium with L_1, the lincomycin concentration within the cell or biophase that reversibly binds with free receptor sites, R, to form a drug–receptor complex, L_1R; S is the normal substrate utilized by the receptor site, R, to synthesize the protein product, P, from the substrate–receptor complex, SR; K_1 is the drug partition constant through the cell membrane; K_2 is the drug affinity constant for the receptor site, R; K_s is the substrate affinity constant for the receptor site, R; K_s is the rate constant for protein synthesis.

The model implies that the receptor sites unreacted with lincomycin are engaged in protein synthesis. It may be assumed that a minimum fraction of protein synthesis is necessary for life-sustaining processes and that the remaining synthesis is utilized in cell growth and division. Therefore, the rate of increase in microbial numbers, dN/dt, must be proportional to a net rate of protein synthesis, dP'/dt, above a certain minimum rate, k_p', and the number of organisms, N, present:

$$dN/dt = k_m(dP'/dt)N = k_m[(dP/dt) - k_p']N = k_{app}N \quad \text{(Eq. 7)}$$

where dP/dt is the overall rate of protein synthesis, k_m is the constant of proportionality related to metabolic activity of the organism, and k_{app} is the generation rate constant of the drug-affected culture. Then:

$$dP/dt = q(1 - \theta)R_T \quad \text{(Eq. 8)}$$

where q is the constant of proportionality related to synthesis of protein from a constant supply of substrate; and θ is the fraction of total receptors, R_T, reversibly bound by lincomycin. Therefore, $(1 - \theta)\,R_T = R$ in Scheme I. Then:

$$\theta = L_1R/R_T = K_1K_2L/(1 + K_1K_2L) \quad \text{(Eq. 9)}$$

Combination of Eqs. 7–9 yields:

$$k_{app} = (qk_mR_T - k_mk_p') - qk_mR_TK_1K_2L/(1 + K_1K_2L) \quad \text{(Eq. 10)}$$

In the absence of any drug effect, Eq. 10 simplifies to:

$$k_{app} = qk_mR_T - k_mk_p' = k_0 \quad \text{(Eq. 11)}$$

where k_0 is the generation rate constant of the drug-free culture.

Substituting Eq. 11 in Eq. 10 and simplifying yield:

$$k_{app} = k_0 - k_aL/(1 + k_bL) \quad \text{(Eq. 12)}$$

where $k_a = qk_mR_TK_1K_2$, and $k_b = K_1K_2$. Therefore:

$$k_a/k_b = qk_mR_T > k_0 \quad \text{(Eq. 13)}$$

Equation 12 describes the functional dependencies of k_{app} on the drug concentration for drug-affected *Staph. aureus* in phase I and phase II generations (Fig. 4). At low drug concentrations of 0–0.35 μg/ml lincomycin in phase I action or of 0–0.55 μg/ml lincomycin in phase II action (where only 67% of the concentration is indicated to be effective), it is possible that generation inhibition is effected by reaction of the drug with only a small fraction of available receptor sites. Thus, $k_bL \ll 1$ and Eq. 12 simplifies to

Eq. 4, which is the expression for the observed linear dependence of k_{app} on drug concentrations in these concentration ranges (Fig. 4). At higher concentrations, it is possible that the already complexed receptor sites reduce the availability of the remaining sites by steric effects, protective colloid action, or other mechanisms. Therefore, it takes progressively greater concentrations of the drug to bind the remaining sites and the k_{app} does not remain a linear function of lincomycin concentration. The arithmetical transformation of Eq. 12 is Eq. 5, which is adhered to by the data of Fig. 4 from the linear plots of $L/(k_0 - k_{app})$ *versus* concentration of lincomycin greater than 0.35 μg/ml in phase I action (Fig. 5, curve A) and greater than 0.55 μg/ml in phase II action (Fig. 5, curve B) on *Staph. aureus* and may indicate a saturable mechanism similar to that describing the lincomycin phase I action on *E. coli* at concentrations greater than 100 μg/ml (Fig. 5, curve D) and that of phase II action on *E. coli* at all concentrations studied (curve C).

The phase II generation of lincomycin-affected *Staph. aureus* takes place after 1–2 successive generations of the culture in phase I generation and indicates a decreased inhibitory effect of lincomycin during the later stages of the drug–bacteria reaction at the same drug concentration level. Since the drug is neither consumed nor inactivated by the organisms during the phase I and phase II generations (Fig. 7), the organisms may have developed a resistance to drug action. Data[9] obtained from within day and between day experiments confirm that the organisms develop resistance to lincomycin action possibly by adaptation or mutation to resistant strains (23–28). Furthermore, the increased resistance to drug action in this generation phase is irreversible. The experimentally determined values of $(k_a/k_b)/k_0 > 1$ (Table II) agree with theoretical predictions in Eq. 13. However, the values remain the same for the phase I and phase II generations of drug-affected *Staph. aureus* cultures, which implies no alteration in metabolic activity of the cultures in both generation phases. Therefore, it can be argued that the development of adaptation or resistance to drug action (28) is not associated with an induction or a repression of some enzyme system resulting in overproduction of cellular RNA and nonribosomal proteins or the utilization of alternative metabolic pathways (26, 29).

On the other hand, the calculated values of k_a and k_b for the phase I generation are 1.50 times the corresponding values for the phase II generation (Table II). Since k_a and k_b are parameters derived from K_1 and K_2, as defined in Scheme I, it may be presumed that the affinity of ribosomal receptor sites for lincomycin is decreased due to structural modifications (9, 21). Alternatively, the rate of partitioning of the drug into the biophase may be decreased due to altered permeability of the cell membrane, although this is not recognized as being likely in terms of ribosomal mechanisms (29). Studies on the binding of lincomycin to ribosomes in cell-free extracts (26, 30) have shown that ribosomes of resistant strains of *E. coli*, *Staph. aureus*, and *Bacillus subtilis* have greater binding capacity than those of sensitive strains. Thus, it may be rationalized that treatment of *Staph. aureus* with subcompletely inhibitory concentrations of lincomycin results in a single mutational step to resistant phenotypes whose ribosomal components have less binding affinity for the drug than the initial strains. This may account for the observation that only 67% of the total drug concentration can be shown kinetically to be active in the phase II generation of the drug-affected cultures.

The functional dependency of k_{app} on lincomycin concentrations for drug-affected *E. coli* in phase I generation is similar to that of the drug-affected *Staph. aureus* in phase I and phase II generations but different from that of drug-affected *E. coli* in phase II generation. The k_{app} *versus* concentration curve for drug-affected *E. coli* in phase I generation is not coincident with that of its phase II generation when normalized by a potency factor (Fig. 4). This indicates that the phase I lincomycin action on *E. coli* has a different mechanism from that of the phase II action (Fig. 5). A possible kinetic model to rationalize the biphasic action of lincomycin on *E. coli* has been proposed (9). The explanation offered for the lincomycin phase II action is a blockade of additional receptor sites engaged in the synthesis of an intermediate product. The intermediate product is utilized by the receptor site, which is the binding site for lincomycin phase I action in a "sequential blocking" (31, 32) of protein synthesis. Therefore, there is an enhanced generation inhibition. The derived kinetic parameters for lincomycin action on *E. coli* (Table II) are quite consistent with the proposed model. The calculated k_b value for lincomycin-affected *Staph. aureus* in phase I generation is, however, 1400 times greater than that of the lincomycin-affected *E. coli* in phase I generation. This may imply that either the drug is more readily partitioned into the biophase of *Staph. aureus* than *E. coli* or the binding affinity of the receptors for lincomycin is greater in *Staph. aureus* than *E. coli*. Literature evidence (26, 30) supports the latter hypothesis, since it has been shown that ribosomal cell-free extracts of *Staph. aureus* bind relatively larger amounts of lincomycin than do those of *E. coli*. The observation that lincomycin is about 1000 times (on a weight basis) more active against *Staph. aureus* than *E. coli*, as indicated by the relative MIC values (Table II), may be attributed to greater binding capacity of the receptors in *Staph. aureus* for lincomycin. The decreased relative potency factor of 340 observed for the phase II generations may be due to perturbations in the kinetics of drug action on the respective organisms, as previously discussed.

The extent of generation inhibition by lincomycin action on *Staph. aureus* increases as some function of the pH value of the culture medium (Fig. 8). The constant, k_L (milliliters per microgram second), as defined in Eq. 4 for various pH values (Fig. 9, curve A) adhered to the expression:

$$k_L = k_L^* f = k_L^* K_a/(K_a + [H^+]) \qquad \text{(Eq. 14)}$$

where k_L^* is the intrinsic inhibitory rate constant of the unprotonated drug, f is the fraction of the drug unprotonated, K_a is the dissociation constant of the unprotonated base, and $[H^+]$ is the hydrogen-ion concentration. The plot of log k_L *versus* pH approaches a slope of unity when $[H^+] > K_a$ and it approaches a slope of 0 when $K_a > [H^+]$. A similar trend is observed for plots of the data (17) of lincomycin action on *E. coli* (Fig. 9, curve B). Arithmetical transformation of Eq. 4 yields:

$$1/k_L = [H^+]/(k_L^* K_a) + 1/k_L^* \qquad \text{(Eq. 15)}$$

The pKa of lincomycin, derived from the slopes and intercepts of the linear plots of $1/k_L$ *versus* $[H^+]$ (Fig. 11), is 7.70 for the data of lincomycin-affected *Staph. aureus* cultures and 7.21 for the data of lincomycin-affected *E. coli* cultures. These kinetically determined pKa values are in reasonably good agreement with the experimentally determined values of 7.6 obtained by potentiometric titration (33). The k_L^* values obtained from the intercepts of the plots are 10×10^{-3} (milliliters per microgram second) and 10×10^{-6} (milliliters per microgram second) for the lincomycin action on *Staph. aureus* and *E. coli*, respectively. Thus, it is concluded that the unprotonated fraction of lincomycin concentration contributes to antibacterial activity and that the uncharged drug has intrinsic activity against *Staph. aureus* that is about 1000 times its activity against *E. coli*.

[9] Unpublished data obtained in this laboratory.

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Improved Method for Microdetermination of Plasma Vitamin E in Laboratory Rats

MAHASEN M. MESHALI and CHARLES H. NIGHTINGALE ˣ

Abstract □ The method generally used to assay human serum for vitamin E (tocopherol) was found to be inapplicable to rat plasma. However, a modified fluorometric method was suitable for both human and rat plasma. This microassay is relatively rapid and reproducible and was utilized to monitor the onset of the tocopherol-deficiency state in rats fed a vitamin-free diet.

Keyphrases □ Vitamin E—improved microdetermination, rat plasma □ Tocopherol—improved microdetermination, rat plasma □ Fluorometry—microdetermination of vitamin E, rat plasma

Problems associated with the analysis of vitamin E (tocopherol) in biological fluids obtained from laboratory animals include the small sample size and the endogenous materials that interfere with tocopherol determinations. The present study describes a modified procedure that improves the fluorometric analysis (1) of tocopherol in humans. The modified microassay is relatively rapid and extremely reproducible, and it may be used for small laboratory animals as well as humans.

EXPERIMENTAL

Rats, Sprague-Dawley[1], weighing 156 ± 10 g, were divided into three groups of 60 each. The normal control animals were fed ordinary laboratory chow[2], while the others were fed either a tocopherol-deficient diet (deficient group) or the deficient diet supplemented with 20 μg/g of α-tocopheryl acetate (supplemented control group). Food and water were allowed *ad libitum*. The composition of the deficient diet was as suggested by Bieri and Privel (2). Both the deficient and supplemented diets[3] were prepared in powdered form in these laboratories. The rat food was assayed colorimetrically at 534 nm for tocopherol content by the method of Bieri (3).

To establish whether the decline in tocopherol plasma levels was a result of the diet and not to a lack of food intake, the rate of animal growth as well as food consumption was monitored.

The method of analysis for free and total tocopherol plasma levels in rats was a modification of the fluorometric procedure of Hansen and Warwick (1) which was suggested for humans. These authors used a 0.1-ml serum sample to which were added 1 ml of water, 1 ml of ethanol, and 2 ml of hexane. The mixture was shaken with a mechanical shaker and centrifuged; then a portion of the clear supernatant layer was assayed fluorometrically, against a reagent blank, for free tocopherol at 295 and 340 nm, the wavelengths of maximum excitation and emission, respectively. Total tocopherol (free plus acetate) was determined by redissolving 1 ml of the remaining supernate, after evaporation to dryness, in 2 ml of hexane and adding 0.5 ml of a solution containing 15.0 mg $LiAlH_4$ dissolved in 10 ml of anhydrous ether. Three milliliters of 0.1 N H_2SO_4 was added, the solution was mixed and centrifuged, and the fluorescence was measured.

In this study the assay procedure was modified by increasing the sample size to 0.2 ml of plasma and reducing the volume of

[1] Blue Spruce Farms, Altamont, New York.

[2] Ralston Purina Co., St. Louis, Mo.

[3] All dietary constituents were purchased from General Biochemicals, Chagrin Falls, Ohio, with the exception of the stripped lard which was obtained from Eastman Kodak Co., Rochester, N.Y. All materials were used as received.

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Rats, Sprague-Dawley[1], weighing 156 ± 10 g, were divided into three groups of 60 each. The normal control animals were fed ordinary laboratory chow[2], while the others were fed either a tocopherol-deficient diet (deficient group) or the deficient diet supplemented with 20 μg/g of α-tocopheryl acetate (supplemented control group). Food and water were allowed *ad libitum*. The composition of the deficient diet was as suggested by Bieri and Privel (2). Both the deficient and supplemented diets[3] were prepared in powdered form in these laboratories. The rat food was assayed colorimetrically at 534 nm for tocopherol content by the method of Bieri (3).

To establish whether the decline in tocopherol plasma levels was a result of the diet and not to a lack of food intake, the rate of animal growth as well as food consumption was monitored.

The method of analysis for free and total tocopherol plasma levels in rats was a modification of the fluorometric procedure of Hansen and Warwick (1) which was suggested for humans. These authors used a 0.1-ml serum sample to which were added 1 ml of water, 1 ml of ethanol, and 2 ml of hexane. The mixture was shaken with a mechanical shaker and centrifuged; then a portion of the clear supernatant layer was assayed fluorometrically, against a reagent blank, for free tocopherol at 295 and 340 nm, the wavelengths of maximum excitation and emission, respectively. Total tocopherol (free plus acetate) was determined by redissolving 1 ml of the remaining supernate, after evaporation to dryness, in 2 ml of hexane and adding 0.5 ml of a solution containing 15.0 mg $LiAlH_4$ dissolved in 10 ml of anhydrous ether. Three milliliters of 0.1 N H_2SO_4 was added, the solution was mixed and centrifuged, and the fluorescence was measured.

In this study the assay procedure was modified by increasing the sample size to 0.2 ml of plasma and reducing the volume of

[1] Blue Spruce Farms, Altamont, New York.

[2] Ralston Purina Co., St. Louis, Mo.

[3] All dietary constituents were purchased from General Biochemicals, Chagrin Falls, Ohio, with the exception of the stripped lard which was obtained from Eastman Kodak Co., Rochester, N.Y. All materials were used as received.

Table I—Free Tocopherol Content of Human Plasma[a]

Subject	Mean Plasma Level[b] ± *SD*	
	Method of Hansen and Warwick (1)	Modified Method
1[c]	23.0 ± 0.51	25.8 ± 0.63
2	13.0 ± 0.54	13.8 ± 0.31

[a] No dependence on agitation intensity could be detected using human plasma. [b] Mean of six determinations for each subject. No statistical difference (Student's *t* test) in tocopherol levels exists between the two methods of analysis. [c] Subject 1 supplements a normal diet with 100 mg/day of tocopherol.

water from 1.0 to 0.2 ml. It was found that 0.5 ml of a solution of 200–300 mg of finely powdered lithium aluminum hydride dissolved in 20 ml of cold freshly distilled ether was necessary for complete reduction of plasma tocopherol. A reciprocating shaker[4] and a high agitation mixer[5] of the type recommended by Hansen and Warwick (1) were used.

All fluorometric measurements were made with a spectrophosphormeter[6] at 295 and 330 nm, the wavelengths of maximum excitation and emission, respectively.

Tocopherol recovery determinations were carried out using the modified procedure by adding a quantity of the vitamin to rat plasma. The amount of tocopherol added to blank plasma samples ranged from 0.5 to 10 μg/ml and represented quantities of tocopherol in the plasma of rats and humans found during these experiments. A mean amount ± *SD*, representing 97.6 ± 5.3% of the vitamin added to the plasma, was recovered after accounting for endogenous tocopherol.

Plasma samples were usually assayed immediately; however, storage of the samples in the frozen state for as long as 3 weeks did not result in apparent tocopherol loss. All reagents were purified according to the method of Hansen and Warwick (1).

RESULTS AND DISCUSSION

Analysis of the normal laboratory chow and the supplemented diet yielded tocopherol values of 17.0 and 17.5 μg/g food, respectively. Analysis of the deficient diet yielded tocopherol levels below the limits of the assay procedure (0.33 μg/g diet). Food consumption for the control diet (supplemented group) and the test diet (deficient group) was found to be statistically[7] identical, *i.e.*, 17.3 ± 0.4 and 16.9 ± 0.5 g/rat/day, respectively, and within the range for this strain of rat fed ordinary laboratory chow. The gain in body weight (Fig. 1) was identical for the supplemented control and deficient animals; however, both groups showed a significant weight gain difference from the normal control animals. This difference in growth patterns for animals fed the diet of Bieri and Privel (2) has not been previously reported and illustrates that one should be cautious in the choice of a control diet when performing vitamin-deficiency studies. The results indicate that both the supplemented and deficient diets were equally well accepted by the animals. Visual observation of each experimental group over 8 months did not reveal any gross differences among the animals.

The results of the analysis of human plasma by either the method of Hansen and Warwick (1) or the modified method were found to be extremely reproducible and within the normal range reported by these authors (Table I).

Similar results were not obtained when rat plasma was analyzed for free tocopherol content. Table II shows that the method of Hansen and Warwick yielded low plasma values for free tocopherol when a reciprocating shaker was used and abnormally high values when a high agitation mixer was used. The modified method, however, resulted in reproducible values regardless of the shaking procedure. The values obtained with the modified method were within the normal range of values reported previously by Bieri (4) using an independent method of analysis.

With rat plasma the modified extraction procedure resulted in a heavy, flocculent precipitate, which is believed to consist mainly of plasma proteins; only a slight haziness occurred when extraction was accomplished by use of the Hansen and Warwick (1) technique. It appears, therefore, that the modified assay technique is relatively insensitive to agitation intensity because plasma proteins were efficiently precipitated and the vitamin was released from its binding sites. In the Hansen and Warwick procedure, protein precipitation did not appear to be complete. This resulted in either incomplete extraction when weak agitation was used for partitioning or extraction of highly fluorescent substances together with the free tocopherol when a high agitation mixer was used in the assay. Table II shows that an apparently low tocopherol content (1.7 μg/ml) was observed with the Hansen and Warwick procedure using the reciprocating shaker and an abnormally high value (19.5 μg/ml) was found when the high agitation mixer was employed. Abnormally high values were also obtained using blank plasma samples. Tryptophan is the only component nor-

Table II—Free Tocopherol Content of Normal Rat Plasma[a]

	Method of Hansen and Warwick (1)	Modified Method
Reciprocating shaker[b]	1.70 ± 0.28[c] (4)	4.65 ± 1.06 (4)
High agitation mixer[d]	19.52 ± 5.71[c] (6)	4.76 ± 1.3 (11)

[a] Micrograms per milliliter ± *SD*. Values in parentheses denote number of determinations. [b] Extraction time was found to be maximal after 20 min at the fastest setting. [c] Statistically different from all other values (Student's *t* test); $p < 0.05$. [d] Extraction time was found to be maximal after 3 min.

Table III—Total Tocopherol Content of Normal Rat Plasma[a]

	Method of Hansen and Warwick (1)	Modified Method
Reciprocating shaker[b]	2.29 ± 1.10[c] (7)	4.60 ± 0.11 (4)
High agitation mixer[d]	4.85 ± 0.78 (4)	5.05 ± 0.84 (4)

[a] Micrograms per milliliter ± *SD*. Values in parentheses denote number of determinations. [b] Extraction time was found to be maximal after 20 min at the fastest setting. [c] Statistically different from all other values (Student's *t* test); $p < 0.005$. [d] Extraction time was found to be maximal after 3 min.

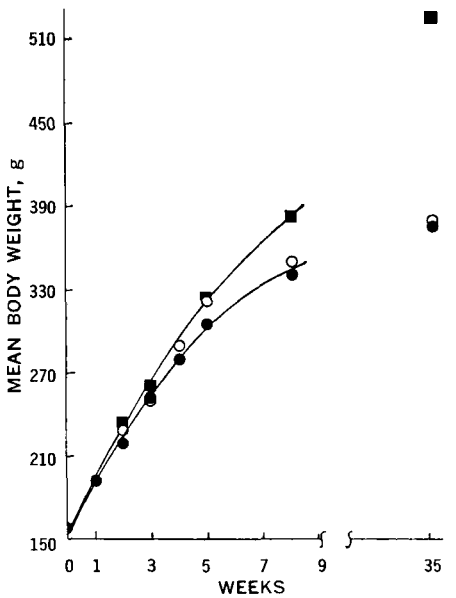

Figure 1—*Growth patterns of rats maintained on test diets. Key:* ●, *deficient;* ○, *supplemented; and* ■, *regular chow.*

[4] Variable-speed reciprocating shaker, Eberbach Corp.
[5] Vortex-Genie, Scientific Industries.
[6] Aminco-Keirs, with fluorescent attachment.
[7] Student's *t* test, $p > 0.05$.

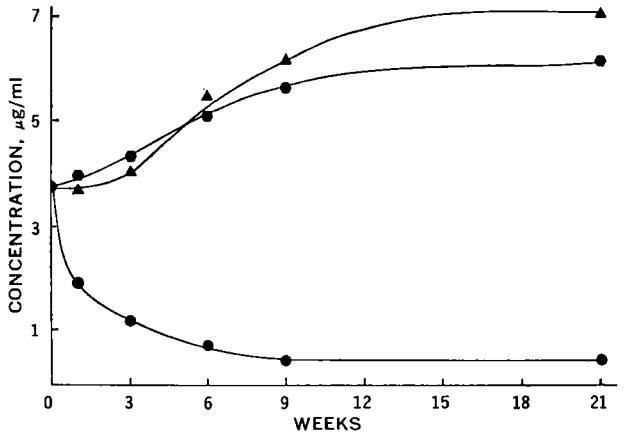

Figure 2—*Tocopherol plasma levels of rats maintained on test diets. Key: ●, deficient group; ▲, supplemented control; and ●, normal control.*

mally present in plasma extracts in concentrations high enough to show a measurable fluorescence (5). A twofold greater tryptophan concentration exists in rat blood as compared to human blood (6), and it is not unreasonable to assume that the high fluorescent values were caused by tryptophan. These abnormally high fluorescent values did not occur in the analysis of total tocopherol, probably because the fluorescent materials were not transferred to the organic phase after treatment with lithium aluminum hydride (Table III). This phenomenon also does not occur in the analysis of free tocopherol by use of the modified method where protein precipitation is more efficient nor with human plasma, probably due to differences in plasma constituents (6, 7). When using human plasma, both methods yielded the same degree of apparent protein precipitation.

Table III illustrates that the method of Hansen and Warwick resulted in incomplete extraction of tocopherol from rat plasma when a reciprocating shaker was used. The modified method, however, gave reproducible results regardless of agitation intensity.

The values for free and total plasma tocopherol in rats appear to be identical when determined by the modified method. Although it was not possible in this study to compare the ratio of free to total tocopherol using the Hansen and Warwick procedure, these authors could not demonstrate a statistical difference between concentrations of the two forms (free and acetate) of the vitamin in human serum. Since the concentration of free and total tocopherol in serum or plasma appears to be identical, one can accurately follow the time course of the vitamin in the body, using the modified method, by measuring only the free form and thus eliminating the longer procedure of the reduction of tocopheryl acetate.

The data shown in Table IV further demonstrate the utility of the modified assay procedure for the analysis of rat plasma tocopherol levels. Tocopherol-deficient rats can be differentiated from normal animals by use of this assay method; moreover, tocopherol depletion in the deficient group was similar to that observed by Bieri (4). Analysis of the same data, for the first 6 weeks, by the method of Hansen and Warwick resulted in an apparent tocopherol plasma content of approximately 2.5 μg/ml regardless of length of time on diet or deficiency state.

The results shown in Fig. 2 suggest that, with a reasonably deficient diet, one can expect that rats will deplete most of their body tocopherol stores in approximately 6 weeks. This finding is confirmed by the work of other authors using various physiological parameters such as kidney autolysis (8), muscular dystrophy (9), oxygen consumption in isolated perfused hearts (10), decrease in testicular weights (11), and composition of nonphosphatides from adipose tissue (12).

Table IV—Free Tocopherol Analysis as a Function of Time Using the Modified Method

Weeks on Diet	Normal[a] Control Group	Supplemented[a] Control Group	Deficient[a] Group
0	3.8 ± 0.7	3.8 ± 0.7	3.8 ± 0.7
1	4.1 ± 0.6[b]	3.7 ± 0.7[b]	1.9 ± 0.6
3	4.5 ± 0.5[b]	3.8 ± 0.7[b]	1.2 ± 0.3
6	5.1 ± 1.6[b]	5.5 ± 1.2[b]	0.7 ± 0.3
9	5.6 ± 0.5[b]	6.2 ± 0.8[b]	0.4 ± 0.2
21	6.2 ± 1.6[b]	7.0 ± 2.8[b]	0.4 ± 0.2

[a] Mean of five animals (μg/ml ± *SD*). [b] Statistically different from deficient groups (Student's *t* test); $p < 0.05$.

SUMMARY

It was observed that the method of Hansen and Warwick (1) for human serum tocopherol determinations could not be used without modification to assay the vitamin in rat plasma. Their procedure does not efficiently precipitate plasma proteins; it results in poor tocopherol extraction when using a low agitation shaker and in the transfer of fluorescent impurities when using a high agitation mixer. The modified procedure, by increasing the organic to aqueous volume ratio, produces efficient precipitation of plasma proteins without dependence on shaking intensity. The modified procedure was used to differentiate between normal and tocopherol-deficient rats, and the results were essentially identical to those found by other workers using an independent method of analysis (4).

No difference was observed between the Hansen and Warwick procedure and the modified method in the analysis of human plasma tocopherol levels.

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[x] To whom inquiries should be directed.

Synthesis of Peptides Containing Aminophosphonic Acids

W. FRANKLIN GILMORE[x] and HILMER A. McBRIDE

Abstract □ Dipeptides containing C-terminal α-aminobenzylphosphonic acid were prepared in both aqueous and nonaqueous media. The synthesis of intermediates necessary for the preparation of peptides containing α-aminophosphonic acids is also discussed. Similarities and differences in coupling aminophosphonic and aminocarboxylic acids by means of dicyclohexylcarbodiimide, acyl chlorides, and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline are described.

Keyphrases □ Aminophosphonic acids—as terminal portion of synthesized peptides □ Peptides—synthesized containing terminal α-aminobenzylphosphonic acid in aqueous and nonaqueous media □ Coupling agents—evaluation of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and dicyclohexylcarbodiimide for esters of aminophosphonic acids and carbobenzoxyamino acids

In a program designed to prepare peptide hormones with altered biological activity, methods of incorporating α-aminophosphonic acids at the C-terminal position of peptides were investigated. α-Aminophosphonic acids are dibasic, having one pKa larger and one pKa smaller than aminocarboxylic acids; thus, it is anticipated that peptides containing these phosphonic acids will have altered isoelectric points, altered binding properties for biological molecules, altered metabolism, and other altered biological properties when compared to the corresponding peptides containing only aminocarboxylic acids.

Initial results of endeavors to incorporate an α-aminophosphonic acid into a peptide are reported. Despite the long existence of α-aminophosphonic acids, no simple broadly applicable method of preparation has been described. Satisfactory methods have been described (1–10) for the synthesis of phosphonic acid analogs of the simple aminocarboxylic acids. A recent report (11) described the synthesis of the optically active α-aminobenzylphosphonic acid, but this method does not appear to be broadly applicable. The synthesis of peptides containing an α-aminophosphonic acid has been explored only recently (12–15). Now a number of modern peptide methods have been examined in the synthesis of peptides containing aminophosphonic acids.

RESULTS

Addition of a dioxane solution of carbobenzoxyglycyl chloride to an aqueous alkaline solution of α-aminobenzylphosphonic acid (I) gave carbobenzoxyglycine and unchanged I. Despite the use of a number of bases such as magnesium oxide, triethylamine, and sodium hydroxide, peptide formation could not be detected. In contrast, addition of a dioxane solution of phthalylglycyl chloride to I in a cold aqueous solution, adjusted to pH 8.5 with sodium hydroxide and maintained at pH 8.5 by periodic addition of sodium hydroxide solution, gave peptide formation. Treatment of the blocked dipeptide with hydrazine hydrate in ethanol gave the dipeptide hydrochloride (II). The dipeptide (II) as the free base was also prepared in one step by an adaptation of the *N*-carboxyanhydride method (16).

Addition of a solution of carbobenzoxyglycine and triethylamine in acetonitrile to a solution of the hydrobromide salt of diethyl α-aminobenzylphosphonate (III) and dicyclohexylcarbodiimide failed to give peptide formation. Conversion of III to the free amine (IV) was accomplished by use of silver oxide in methylene chloride. The free amine and carbobenzoxyglycine in the presence of dicyclohexylcarbodiimide gave peptide formation. Peptide formation from carbobenzoxyglycine and IV was also efficiently accomplished using 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (17) in tetrahydrofuran. Treatment of the blocked dipeptide with hydrogen bromide in acetic acid removed the carbobenzoxy group and hydrolyzed the phosphonate to give VI. Hydrogenolysis of V in ethanol–hydrochloric acid removed only the carbobenzoxy group and gave VII (Scheme I).

DISCUSSION

From these results, it is apparent that 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and dicyclohexylcarbodiimide are excellent coupling agents for esters of aminophosphonic acids and carbobenzoxyamino acids. Contrary to what has been found with salts of amino acid esters (18), the addition of a solution of a blocked amino acid and triethylamine to a solution of the hydrobromide salt of the aminophosphonic acid ester and dicyclohexylcarbodiimide was not satisfactory. From IR spectra, the formation of anhydride from the blocked amino acid was observed. However, peptide formation did not occur. Although without direct evidence, it is postulated that *N*-acylurea formation is faster than peptide formation. *N*-Acylurea formation in the dicyclohexylcarbodiimide coupling reaction is believed to be catalyzed by triethylamine (19, 20). In this case, it appears that *N*-acylurea or some other product is formed from the blocked amino acid anhydride. In retrospect, carbobenzoxyglycine was a poor choice since it is known that the corresponding anhydride rearranges in the presence of bases (20).

Perhaps one of the most surprising results and a major difference between the synthesis of peptides containing aminophosphonic acids and peptides containing only aminocarboxylic acids is that, at the dipeptide stage, treatment of the carbobenzoxyamino dipeptide ester with hydrogen bromide in acetic acid caused hydrolysis of both the carbobenzoxy group and the esters of the phosphonic acid. Preliminary evidence shows that the carbobenzoxy group is hydrolyzed faster than the esters. Thus, with a short reaction time, salts of the dipeptide esters should be obtained. The problem is best avoided by hydrogenolysis of the carbobenzoxy group. When the peptide acid is the desired product, treatment with hydrogen bromide in acetic acid is of considerable utility. This result is surprising since treatment of VIII with hydrogen bromide in acetic acid gave III. One explanation is that in the dipeptide, neighboring group participation through a six-membered transition state (IX and/or the intermediate X) is possible. This is not possible for VIII.

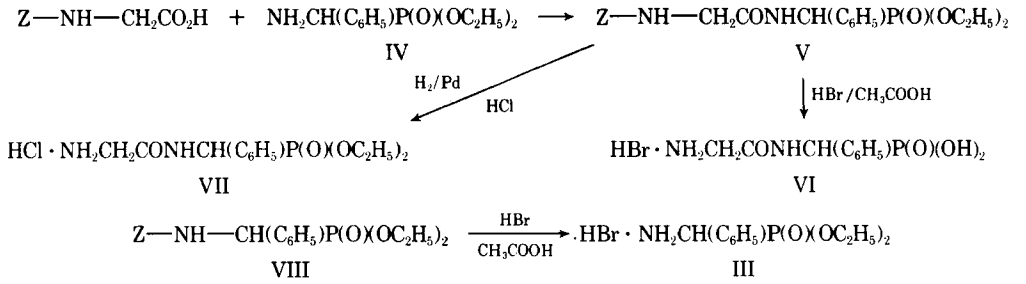
In IX and X, the phosphorus is pentacoordinate, thus having trigonal-bipyramidal geometry. In this case, at least one ethoxy group could occupy one of the longer apical bond positions. This would make hydrolysis easier than it would be in the case of the tetrahedral phosphorus of VIII.

The antimicrobial properties of II were investigated[1]. At a con-

$$HCl \cdot NH_2—CH_2—CONH—CH(C_6H_5)—P(O)(OH)_2$$

II

[1] Tests were performed by Dr. Larry Robertson and Mrs. Nanci Youngblood, Department of Pharmacognosy, University of Mississippi.



Z = carbobenzoxy

Scheme I

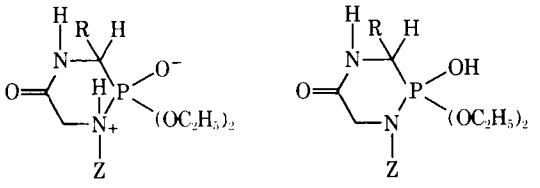
centration of 10 mg/ml, this compound was inactive against selected bacteria and fungi.

EXPERIMENTAL[2]

***N*-Phthalylglycyl-α-aminobenzylphosphonic Acid**—To a stirred suspension of 0.94 g (5 mmoles) of I in 30 ml of water was added 5.0 ml of 1 *N* NaOH, and the pH was adjusted to 8.0 with additional sodium hydroxide. The solution was cooled in an ice bath, and the pH was monitored by a pH electrode placed directly in the solution. To the cold alkaline solution, a solution of 1.18 g (5 mmoles) of phthalylglycyl chloride (21) in 20 ml of dry dioxane was added dropwise over 30 min. The pH was adjusted by periodic addition of sodium hydroxide during the reaction, and the pH stabilized in approximately 1 hr. The solution was stirred an additional 15 min, acidified to pH 1.0 with 6 *N* HCl to obtain a copious precipitate, collected by filtration, and dried (*in vacuo*, 80°). The crude solid was recrystallized from ethanol to yield 1.6 g (86%) of fine white needles, mp 253–254°; IR (KBr): 3275 (N—H), 1710 (glycine C=O), and 1725 and 1775 (phthalyl C=O) cm^{-1}; NMR (D_2O, NaOD): δ 4.32 (s, 2, CH_2CO), 5.01 (d, 1, J = 18 Hz, CH—P), 7.65 (s, 5, phenyl), and 7.81 (s, 4, phthalyl).

Anal.—Calc. for $C_{17}H_{15}N_2O_6P$: C, 54.55; H, 4.04; N, 7.48; P, 8.27. Found: C, 54.58; H, 4.14; N, 7.59; P, 8.13.

Carbobenzoxy-α-aminobenzylphosphonic Acid—A suspension of 8.4 g (0.047 mole) of I in 50 ml of water was treated dropwise with 4 *N* NaOH until solution was effected, and the pH was adjusted to 9.5. The solution was cooled to 0°, and 8.0 g (0.047 mole, 95% purity) of carbobenzoxy chloride was added dropwise over 45 min. The pH was maintained at 9.0–9.5 during the reaction by periodic addition of 4 *N* NaOH. The pH was monitored by a pH electrode in the solution and stabilized after 6 hr. The solution was warmed to 25°, stirred for 2 hr, and extracted with 50 ml of ether, which was discarded. The aqueous phase was added to a mixture of 30 ml of water, 20 ml of concentrated hydrochloric acid, and 100 g of crushed ice. The oil which separated was extracted into two 100-ml portions of ether and the two extracts were combined, dried (magnesium sulfate), and evaporated to yield the crude solid product. The crude solid was recrystallized from ethyl acetate–*n*-hexane to afford 12.7 g (84%) of long needles, mp 152–153°; IR (KBr): 1680 (C=O) and 1240 (P—O) cm^{-1}; NMR (CH_3OH-d_4): δ 5.20 (s, 2, CH_2), 5.22 (d, 1, J = 22 Hz, CH—P), and 7.50 (m, 10, aromatic).



IX X

[2] Melting points were determined with a Thomas–Hoover Unimelt melting-point apparatus and are corrected. NMR spectra were taken on a Jeol model C-60HL spectrometer using tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal standards. IR spectra were taken on either a Perkin-Elmer 137 or 257 spectrophotometer. Microanalyses were performed by either Chemalytics, Inc., Tempe, Ariz., or Galbraith Laboratories, Knoxville, Tenn. The aminobenzylphosphonic acid was racemic.

Anal.—Calc. for $C_{15}H_{16}NO_5P$: C, 56.08; H, 5.02; N, 4.36; P, 9.65. Found: C, 56.30; H, 4.86; N, 4.13; P, 9.57.

Diethyl Carbobenzoxy-α-aminobenzylphosphonate (VIII)—Using a modified procedure described by Nicholson *et al.* (22), a suspension of 6.42 g (0.02 mole) of carbobenzoxy-α-aminobenzylphosphonic acid in 25 g of triethyl orthoformate was slowly heated at 80°. The ethanol and ethyl formate were continually removed by a Dean–Stark apparatus. When all of the acid had dissolved, the temperature rose from 80 to 145°. The solution was refluxed for 20 min and cooled. After standing for 12 hr, the diester precipitated and was collected by filtration. Recrystallization of the crude ester from ethyl acetate–*n*-hexane yielded 7.0 g (93%) of fine white needles, mp 118–119°. An analytical sample was recrystallized from methylene chloride–*n*-hexane to give fine needles, mp 118–118.5° [lit. (13) mp 108–109°]; IR (KBr): 1160 (P—OCH_2CH_3) and 1735 (C=O) cm^{-1}; NMR ($CDCl_3$): δ 1.15 (m, 6, CH_3), 4.00 (m, 5, CH_2—O), 5.22 (s, 2, benzyl CH_2), 5.25 (m, 1, CH—P), and 7.55 (m, 10, aromatic).

Anal.—Calc. for $C_{19}H_{24}NO_5P$; C, 60.47; H, 6.41; N, 3.71; P, 8.21. Found: C, 60.26; H, 6.21; N, 3.81; P, 8.09.

Diethyl α-Aminobenzylphosphonate Hydrobromide (III)—A solution of 6.8 g (18.3 mmoles) of VIII in 15 ml of acetic acid was treated with 11.0 g (5.5 mmoles/g) of 45% HBr in acetic acid and stirred at 25° for 45 min. The solution was diluted with dry ether until the hydrobromide precipitated. The suspension was stirred for 30 min and the crystals were collected by filtration and recrystallized from methanol–ether to yield 5.3 g (95%) of a fine white crystalline powder, mp 268–270°; IR (KBr): 1160 (P—OCH_2CH_3) cm^{-1}; NMR (D_2O): δ 1.38 (m, 6, CH_3), 4.36 (m, 4, CH_2—CH_3), 5.10 (d, 1, J = 18 Hz, CH—P), and 7.72 (s, 5, phenyl).

Anal.—Calc. for $C_{11}H_{19}BrNO_3P$: C, 40.77; H, 5.91; N, 4.32; P, 9.57. Found: C, 40.58; H, 5.71; N, 4.57; P, 9.66.

Diethyl *N*-Carbobenzoxyglycyl-α-aminobenzylphosphonate (V)—*Method A*—A suspension of 1.5 g (4.8 mmoles) of III in 40 ml of methylene chloride was treated with 30 ml of 10% K_2CO_3 solution. The organic phase was separated and the aqueous layer was extracted with an additional 30 ml of methylene chloride. The organic extracts were combined, dried (magnesium sulfate), and evaporated to dryness to yield 0.97 g (4.22 mmoles, 88% recovery) of IV. The ester was dissolved in 20 ml of tetrahydrofuran and added at once to a solution of 1.07 g (4.22 mmoles) of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and 0.88 g (4.22 mmoles) of carbobenzoxyglycine in 20 ml of tetrahydrofuran. The solution was stirred for 24 hr at 25° and evaporated to yield an orange-brown oil, which was dissolved in 30 ml of methylene chloride and extracted with two 25-ml portions of 0.1 *N* HCl, 30 ml of 10% K_2CO_3, and finally 30 ml of water. The organic phase was dried (magnesium sulfate), evaporated, and dried by high vacuum (0.10–0.05 mm) to yield 1.60 g (91%) of the crude dipeptide, which was recrystallized from ethyl acetate–*n*-hexane to give white needles, mp 89–91° [lit. (13) mp 88–89°]; IR (KBr): 1175 (P—O—C), 1680 (glycine C=O), and 1735 (carbamate C=O) cm^{-1}; NMR ($CDCl_3$): δ 1.18 (m, 6, CH_3), 4.03 (m, 6, CH_3—CH_2 and glycine CH_2), 5.16 (s, 2, benzyl CH_2), 5.65 (m, 1, CH—P), and 7.40 (s, 10, aromatic).

Anal.—Calc. for $C_{21}H_{27}N_2O_6P$: C, 58.06; H, 6.26; N, 6.45; P, 7.13. Found: C, 58.04; H, 6.16; N, 6.28; P, 6.86.

Method B—To a solution of 0.66 g (3.20 mmoles) of dicyclohexylcarbodiimide and 0.77 g (3.20 mmoles) of IV in 25 ml of methy-

lene chloride was added 0.67 g (3.20 mmoles) of carbobenzoxyglycine. Urea formation was noticed in 15 min. The suspension was stirred for 14 hr and the urea was removed by filtration (627 mg, 82%). The solution was extracted with 50 ml of 1 *N* HCl and two 25-ml portions of 10% Na_2CO_3, dried (magnesium sulfate), and evaporated to yield 1.40 g of a light-green oil which solidified upon standing. The solid was recrystallized from ethyl acetate–*n*-hexane to afford 1.00 g (72%) of the peptide as white needles, mp 89–91°; IR (KBr): 1175 (P—O—C), 1680 (glycine C=O), and 1735 (carbamate C=O) cm^{-1}; NMR ($CDCl_3$): δ 1.20 (m, 6, CH_3), 4.09 (m, 6, CH_3—CH_2 and glycine CH_2), 5.18 (s, 2, benzyl CH_2), 5.70 (m, 1, CH—P), and 7.47 (s, 10, aromatic).

Diethyl *N*-Glycyl-α-aminobenzylphosphonate (VII Free Amine)—To a solution of 1.00 g (2.32 mmoles) of V in 30 ml of 95% ethanol and 1.00 ml of concentrated hydrochloric acid was added 400 mg of 5% palladium-on-charcoal, and hydrolysis was performed at 1.4 kg/cm^2 of hydrogen for 18 hr. Removal of the catalyst followed by evaporation of the solvent yielded 0.79 g (100%) of a yellow crystalline solid. The solid was dissolved in 7 ml of absolute ethanol and treated with dry ether until clouding occurred. Stirring overnight at 25° failed to give a precipitate. The solvent was evaporated and the solid was dissolved in 30 ml of water and extracted with 20 ml of methylene chloride which was discarded. The aqueous layer was basified with 15 ml of 10% K_2CO_3 and extracted with three 20-ml portions of methylene chloride, dried (magnesium sulfate), and evaporated to yield a light-green oil which solidified in platelets on standing. The platelets were recrystallized from methylene chloride–*n*-hexane to give 0.41 g (60%) of white needles, mp 66–70°; IR (KBr): 1240 (P—O), 1160 (P—O—C), and 1680 (C=O) cm^{-1}; NMR ($CDCl_3$): δ 1.25 (m, 6, CH_3—CH_2—), 1.88 (s, 2, NH_2), 3.43 (s, 2, CH_2—CO), 4.07 (m, 4, CH_2—O), 5.67 (m, 1, NH—CH—P, collapses to a doublet with D_2O exchange), 7.58 (m, 5, phenyl), and 8.75 (s, 1, NH—CO).

Anal.—Calc. for $C_{13}H_{21}N_2O_4P$: C, 51.99; H, 7.05; N, 9.33; P, 10.31. Found: C, 51.86; H, 7.13; N, 9.19; P, 10.24.

***N*-Glycyl-α-aminobenzylphosphonic Acid (VI Free Amine)**—*Method A: From Deblocking* N-*Phthalylglycyl-α-aminobenzylphosphonic Acid*—An adaption of the procedure of Sheehan and Frank (21) was followed. To an ethanolic solution of 1.87 g (5 mmoles) of the phthalyl-blocked dipeptide was added 5.0 ml of 2 *N* hydrazine hydrate in ethanol, and the solution was refluxed for 1 hr. During this time, a flocculent precipitate formed. The ethanol was removed by evaporation and the solid residue was treated with 30 ml of 2 *N* HCl and warmed to 50° for 15 min; the insoluble phthalylhydrazide was removed by filtration. Evaporation of the acidic solution yielded a mixture of the dipeptide hydrochloride and hydrazine hydrochloride. Recrystallization of the crude solid from *n*-propanol afforded 1.3 g (93%) of the dipeptide hydrochloride as small white crystals, mp 119–122° dec. Elemental analysis of the hydrochloride gave unacceptable values for carbon and phosphorus. A solution of 0.75 g (3 mmoles) of the dipeptide hydrochloride was dissolved in a small volume of water and treated with 0.35 g (3 mEq) of silver oxide, and the silver chloride was removed by filtration. The filtrate was passed through an ion-exchange column[3] (1 × 10 cm). Evaporation of the eluate yielded 0.60 g of slightly brown crystals, which were recrystallized from water–acetonitrile, mp 234–239° dec.; IR (KBr): 1700 (C=O) and 3450 (N—H) cm^{-1}; NMR (D_2O): δ 3.81 (s, 2, CH_2—CO), 5.28 (d, 1, J = 18 Hz, CH—P), and 7.55 (s, 5, phenyl).

Anal.—Calc. for $C_9H_{13}N_2O_4P$: C, 44.27; H, 5.36; N, 11.47; P, 12.28. Found: C, 44.16; H, 5.66; N, 11.50; P, 11.97.

Method B: From Glycine N-*Carboxyanhydride*—Since the *N*-carboxyanhydride procedure is subject to a number of variables, it is described in detail. To a metal-jacketed blender[4] fitted with an ice water supply was added 80 ml of 1 *M* H_3BO_3, previously cooled at 0°. A pH electrode calibrated at pH 10.0 and 0° was placed directly in the solution, and the pH was adjusted to 10.2 with 4 *N* NaOH. To the moderately stirred buffer was added 1.87 g (10 mmoles) of I, and the pH was adjusted to 10.2. The blender was turned to high speed, 1.20 g (12 mmoles) of glycine *N*-carboxyanhydride was added in four portions, and the pH was rapidly adjusted to 10.2 after each addition. After the last addition, the solution was stirred for 1 min. With rapid stirring the solution was acidified to pH 2.0 with concentrated hydrochloric acid. Cooling at 0° for 12 hr did not give crystals. Evaporation of the solution *in vacuo* at 40° followed by extraction with 100 ml of hot methanol yielded 2.0 g of a white crystalline solid. The NMR spectrum in D_2O showed that the solid contained the product but was contaminated. The solid was dissolved in 50 ml of water and treated with excess silver oxide. The suspension was stirred for a few minutes and the silver chloride and excess silver oxide were removed. The filtrate was passed through an ion-exchange column[3] (1 × 10 cm) and the eluate evaporated to yield a slightly purple solid. The solid was dissolved in 30 ml of water and treated with hydrogen sulfide, 0.5 g of activated charcoal was added, and the suspension was stirred and filtered. Evaporation of the solution yielded 0.8 g (33%) of a crystalline solid, mp 228–238° dec. The spectra were identical to those given under *Method A*. Recrystallization from water–acetonitrile failed to give the pure dipeptide.

Method C: From Diethyl N-*Carbobenzoxy-α-aminobenzylphosphonate (V)*—Diethyl *N*-carbobenzoxy-α-aminobenzylphosphonate (1 g, 2.3 mmoles) was dissolved in 10 ml of acetic acid, treated with 2.0 g of 45% (5.5 mmoles/g) HBr in acetic acid, and stirred for 1 hr. Addition of dry ether gave a waxy solid which, upon recrystallization attempts from methanol–ether, yielded an oil. The solvent was evaporated and the solid residue was dissolved in 30 ml of water and extracted with 50 ml of methylene chloride and 30 ml of ether. Evaporation of the aqueous layer yielded 0.63 g (84%) of *N*-glycyl-α-aminobenzylphosphonic acid hydrobromide (VII). The hydrobromide was dissolved in a little water and treated with 0.134 g (1.61 mEq) of silver oxide. The silver chloride was removed by filtration and the filtrate was passed through an ion-exchange column[3]. Evaporation of the eluate to dryness yielded 0.48 g (97%) of the free dipeptide, mp 234–239° dec. The spectra were identical to those given under *Method A*.

[3] Amberlite IRC-50.
[4] Waring.

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Keyphrases □ 1-Methyl-4,4-disubstituted 3,5-pyrazolidinediones —synthesis, evaluation of anticonvulsant activity □ 3,5-Pyrazolidinediones, 1-methyl-4,4-disubstituted—synthesis, evaluation of anticonvulsant activity □ Anticonvulsant activity—synthesis and evaluation of 1-methyl-4-substituted 3,5-pyrazolidinediones

It has been firmly established that an important pharmacophoric grouping among anticonvulsant agents is the imide group (1). Cyclic hydrazides, which may be represented by Structure I, have rarely been studied for their anticonvulsant properties. The two compounds (2, 3) of Structure I that were examined were found to be inactive; however, neither contains alkylated nitrogen atoms.

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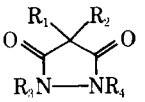
In a program designed to synthesize molecular modifications of the imide grouping, the 3,5-pyrazolidinediones (I) were investigated. This report describes the preparation of a series of compounds in which R_3 = alkyl, R_4 = H, and R_1 and R_2 = a combination of H, alkyl, and aryl groups.



I

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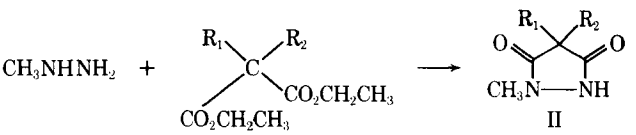

II

Scheme I

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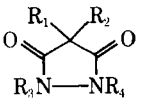

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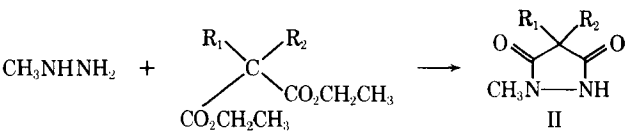
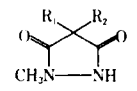


II

Scheme I

Table I—1-Alkyl-4,4-disubstituted 3,5-Pyrazolidinediones



Compound	R_1	R_2	Method	Melting Point	Yield, %	Crystallization Solvent[a]	Formula	Analysis, % Calc.	Found
II*a*	CH_3	CH_3	A	115–117°	74	X	$C_6H_{10}N_2O_2$	C 50.69	50.83
								H 7.09	6.94
								N 19.71	19.74
II*b*	—$CH_2CH_2CH_2$—		A	89–91°	55	X	$C_7H_{10}N_2O_2$	C 54.54	54.52
								H 6.54	6.75
								N 18.17	18.21
II*c*	CH_3	C_2H_5	A	98–100°	16	Y	$C_7H_{12}N_2O_2$	C 53.83	53.73
								H 7.74	7.66
								N 17.94	18.04
II*d*	—$CH_2CH_2CH_2CH_2$—		A	107–109°	39	Y	$C_8H_{12}N_2O_2$	C 57.13	57.30
								H 7.19	7.07
								N 16.66	16.82
II*e*	C_2H_5	C_2H_5	A	108–110°	23	Y	$C_8H_{14}N_2O_2$	C 56.45	56.64
								H 8.29	8.36
								N 16.46	16.46
II*f*	C_6H_5	H	C	185–187°	68	—[b]	$C_{10}H_{10}N_2O_2$	C 63.15	63.07
								H 5.30	5.49
								N 14.73	14.68
II*g*	C_6H_5	CH_3	A	177–178.5°	69	Y	$C_{11}H_{12}N_2O_2$	C 64.69	64.65
								H 5.92	5.86
								N 13.72	13.62
II*h*	C_6H_5	C_2H_5	A	171–172°	62	Y	$C_{12}H_{14}N_2O_2$	C 66.04	66.23
								H 6.47	6.54
								N 12.84	12.86
II*i*	n-C_4H_9	n-C_4H_9	B	158–159°	59	Y	$C_{12}H_{22}N_2O_2$	C 63.69	63.55
								H 9.80	9.51
								N 12.38	12.39
II*j*	C_6H_5[c]	C_2H_5	D	136–138°	78	Z	$C_{13}H_{16}N_2O_2$	C 67.22	67.23
								H 6.94	6.92
								N 12.06	11.99

[a] X = cyclohexane–ethyl acetate, Y = hexane–ethyl acetate, and Z = cyclohexane–benzene. [b] Could not be recrystallized. [c] This compound contains an *N*-ethyl group in place of *N*-methyl.

product obtained in this way were identical with those of the dialkylation product.

Compound II*f* also reacted with 2 equivalents of acetyl chloride in pyridine and furnished the di-*O*-acetate (VI). The pyrazole structure is proposed since the IR spectrum of the product shows a single carbonyl band at 1780 cm^{-1}, reminiscent of an enol acetate group (6).

Table I records relevant data for the series of 1-alkyl-4-substituted 3,5-pyrazolidinediones. The IR spectra (chloroform) exhibit two strong absorption bands in the ranges of 1668–1682 and 1732–1737 cm^{-1}, with the former band being the more intense. Table II contains the PMR data for these compounds.

PHARMACOLOGY

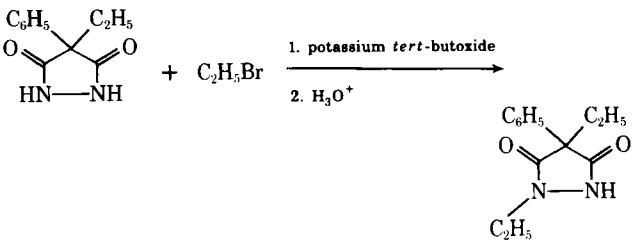
The anticonvulsant activity of each test compound was evaluated by maximal electroshock seizure and pentylenetetrazol seizure threshold tests (7) with modifications. Parallel procedures were performed using both the vehicle and drugs of proven anticonvulsant potency.

Diphenylhydantoin[1], phensuximide[2], or the experimental compounds were mixed with 0.5% methylcellulose, using five strokes of a Potter-Elvehjem homogenizer, and administered intraperitoneally. The concentration of each preparation was such that a volume of 0.1 ml was injected for every 10 g of body weight.

Fifteen male albino Swiss-Webster mice, weighing 20–27 g, were injected with each compound for each procedure[3]. A group of five animals was tested at 1, 2, or 3 hr following drug administration, each animal being used only once. In the maximal electroshock procedure, animals were stimulated with 60 v of 60-cycle alternating current for 0.3 sec, delivered through saline-wetted corneal electrodes. A variable transformer was used to regulate the applied voltage. The duration of the stimulus was kept constant by the inclusion of a relay in the circuit operated by a square wave stimulator[4]. Mice not demonstrating full tonic extension of both hindlimbs following stimulation were considered protected. In the pentylenetetrazol seizure threshold, test animals were injected with an aqueous solution of pentylenetetrazol[5] (85 mg/kg sc) and observed for 45 min. The absence of 3 continuous sec of clonic muscular activity was considered to indicate protection. For the duration of the test procedure, mice were kept individually in 1-liter glass beakers separated by opaque material.

An attempt was made to detect possible neurological toxicity of each compound prior to the pentylenetetrazol seizure test. Toxicity was considered present if the animal failed to climb to the top of a 1-liter beaker after being suspended from the rim by its forelimbs three times within 1 min.

Results of the anticonvulsant tests are shown in Tables III and IV. No compound displayed the marked protection obtained with diphenylhydantoin in the maximal electroshock procedure or with



Scheme II

[1] Dilantin, Parke, Davis & Co., Detroit, Mich.
[2] Prepared by the procedure described by C. A. Miller and L. M. Long, *J. Amer. Chem. Soc.*, 73, 4895(1951).
[3] Laboratory Supply, Inc., Indianapolis, IN 46241. Food and water were supplied *ad libitum*. All animals were allowed several days to recover from shipping stress before use.
[4] Grass S-4.
[5] Metrazol, Knoll Pharmaceutical, Whippany, NJ 07981

Table II—PMR Data of Substituted 3,5-Pyrazolidinediones

Compound	1-Methyl	2-NH	Other Substituents
II*a*[a]	3.28	9.40	1.36 (s) (4,4-dimethyl)
II*b*[a]	3.28	9.50	2.46 (m) (4,4-$CH_2CH_2CH_2$)
II*c*[a]	3.25	9.72	1.34 (s) (4-methyl), 0.89 (t), 1.80 (q) (4-ethyl)
II*d*[a]	3.21	9.42	1.96 (broad s) (4,4-$CH_2CH_2CH_2CH_2CH_2$—)
II*e*[a]	3.22	8.93	0.83 (t), 1.77 (q) (4,4-diethyl)
II*f*[b]	3.24	8.80	7.0–8.1 (m) (4-aromatic H), 8.80 (s) (3- or 5-OH)
II*g*[a]	3.26	7.68	1.75 (s) (4-methyl), 7.35–7.75 (m) (4-phenyl)
II*h*[a]	3.23	8.42	0.96 (t), 2.19 (q) (4-ethyl), 7.26–7.7 (m) (4-phenyl)
II*i*[a]	3.37	10.01	0.90 (t), 1.10–2.20 (m) (4,4-di-*n*-butyl)
II*j*[a]	—	9.0	0.98 (t), 2.25 (q) (4-ethyl), 7.5 (m) (4-phenyl), 1.25 (t), 3.37 (q) (1-ethyl)

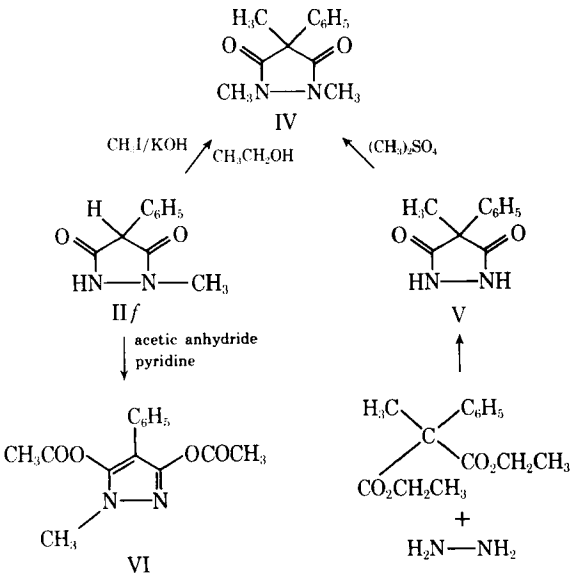
[a] Chemical shift given in δ units; multiplicity is reported as: s = singlet, t = triplet, and q = quartet. Spectra were determined in $CDCl_3$ with tetramethylsilane as internal reference. [b] Spectrum recorded in dimethyl sulfoxide-d_6 with tetramethylsilane as the internal reference.

phensuximide in the pentylenetetrazol test. Only sporadic protection against pentylenetetrazol-induced seizures was observed with several of the more lipid-soluble compounds. At the doses employed, no neurological toxicity was observable with any preparation tested.

EXPERIMENTAL[6]

3,5-Pyrazolidinediones—*Method A*—A solution of 0.04 mole of the substituted diethyl malonate, 15 ml of anhydrous methylhydrazine, and 5.5 g (0.102 mole) of sodium methoxide in 150 ml of anhydrous methanol was refluxed with stirring for approximately 70 hr. The solution was concentrated *in vacuo*, the gummy residue was treated cautiously with 30 ml of 18% hydrochloric acid, and the resulting solution was refluxed for 1 hr. The solution was neutralized to pH ~6 with 40% aqueous sodium hydroxide and concentrated *in vacuo* to one-half of its original volume. The resulting crystalline solid was filtered, dried, and recrystallized.

Method B—A solution of 0.05 mole of the substituted diethyl malonate and 0.07 mole of methylhydrazine in 200 ml of absolute ethanol containing 0.11 mole of sodium was heated in an autoclave at 80° for 20 hr. The temperature was raised to 135° and heating was continued for another 6 hr. The solution was cooled and evaporated to dryness, and the residue was dissolved in water and extracted twice with ether. Acidification of the aqueous solution with 20% aqueous hydrochloric acid afforded a solid which was filtered, dried, and recrystallized.



Scheme III

Method C—A solution of 9.7 g (0.041 mole) of diethyl phenylmalonate and 16 ml of anhydrous methylhydrazine in 150 ml of anhydrous methanol was refluxed for 24 hr. The mixture was evaporated *in vacuo* and the oily residue was treated with 40 ml of 18% aqueous hydrochloric acid. After refluxing for 2 hr, the mixture was cooled and neutralized with aqueous sodium hydroxide to pH ~3. The solid which precipitated was filtered and washed with hot ethyl acetate. The product amounted to 5.1 g.

Method D—A mixture of 1.0 g (4.9 mmoles) of 4-ethyl-4-phenyl-3,5-pyrazolidinedione and 554 mg (4.9 mmoles) of potassium *tert*-butoxide in 50 ml of dry 1,2-dimethoxyethane was stirred vigorously under nitrogen at 45° for 1 hr. After cooling, the mixture was treated with a solution of 535 mg (4.9 mmoles) of ethyl bromide in 2 ml of 1,2-dimethoxyethane. The resulting mixture was heated at 40–50° for 16 hr. An additional 100 mg of ethyl bromide was added and the heating was continued overnight. The solution was evaporated to dryness and the semisolid residue was treated with 20 ml of 10% hydrochloric acid and 95 ml of ethanol. The mixture was refluxed for 6 hr. Cooling afforded 900 mg of the product as white needles.

4-Methyl-4-phenyl-3,5-pyrazolidinedione (V)—A mixture of 10.0 g (0.40 mole) of diethyl methylphenylmalonate and 15 ml of anhydrous hydrazine was refluxed with stirring for 64 hr. The excess hydrazine was removed by distillation at atmospheric pressure and the oily residue was dissolved in water and acidified with 5% hydrochloric acid. Upon attempted extraction of the aqueous phase with chloroform, the product precipitated from solution. The solid was filtered and dried to give 3.7 g (48%) of material, mp 196.5–199°. Recrystallization from ethyl acetate afforded the pure product, mp 198.5–200°; IR (KBr): 1738 and 1660 cm^{-1}.

Anal.—Calc. for $C_{10}H_{10}N_2O_2$: C, 63.15; H, 5.30; N, 14.73. Found: C, 62.84; H, 5.27; N, 14.61.

Table III—Maximal Electroshock Seizure Test

Compound	Dose, mg/kg	Number of Animals Protected 1 hr	2 hr	3 hr
II*a*	500	0/5	0/5	0/5
II*b*	500	0/5	0/5	0/5
II*c*	500	0/5	0/5	0/5
II*d*	500	0/5	0/5	0/5
II*e*	500	0/5	0/5	0/5
II*f*	500	0/5	0/5	0/5
II*g*	500	0/5	0/5	0/5
II*h*	500	0/5	0/5	0/5
II*i*	500	0/5	0/5	0/5
II*j*	500	0/5	0/5	0/5
III	500	1/5	1/5	—
Diphenylhydantoin	25	5/5	5/5	5/5
Methylcellulose, 0.5%	—	0/5	0/5	0/5

[6] Melting points were determined with a Thomas-Hoover melting-point apparatus and are uncorrected. The structures of the compounds were confirmed by their IR and NMR spectra. IR spectra were obtained on a Beckman IR-8 spectrophotometer. NMR spectra were determined on a Varian A-60A spectrometer, using tetramethylsilane as the internal reference. Microanalyses were performed by Dr. Kurt Eder, Geneva, Switzerland, and PCR, Inc., Gainesville, Fla.

Table IV—Pentylenetetrazol Seizure Threshold Tests

Compound	Dose, mg/kg	Number of Animals Protected		
		1 hr	2 hr	3 hr
II*a*	500	0/5	0/5	0/5
II*b*	500	1/5	0/5	0/5
II*c*	500	0/5	0/5	0/5
II*d*	500	0/5	1/5	0/5
II*e*	500	2/5	2/5	1/5
II*f*	500	0/5	1/5	2/5
II*g*	500	3/5	0/5	1/5
II*h*	500	0/5	1/5	1/5
II*i*	500	1/5	1/5	1/5
II*j*	300[a]	2/5	0/5	0/5
III	500	0/5	0/5	0/5
Phensuximide	200	5/5	4/5	3/5
Methylcellulose, 0.5%	—	0/5	0/5	0/5

[a] Dosage reduced due to small quantity of compound available.

1,2,4-Trimethyl-4-phenyl-3,5-pyrazolidinedione (IV)—*Method A*—To a solution of 1.0 g (5.25 mmoles) of II*f* in 80 ml of 95% ethanol was added 30 ml of 0.5 *M* ethanolic potassium hydroxide followed by 4.0 g (28 mmoles) of methyl iodide. The mixture was stirred at room temperature for 6 hr and then refluxed gently overnight. The solution was neutral to pHydrion paper. Evaporation afforded a residue which was triturated with chloroform and filtered. The filtrate was evaporated to dryness and gave 880 mg of red oil whose TLC [silica gel, ethyl acetate–acetone (4:1)] indicated one intense spot (R_f 0.48) along with three other components. Column chromatography [60 g of silica gel, elution with ethyl acetate–hexane (1:1)] afforded 620 mg (53%) of an oil; IR ($CHCl_3$): 1730 and 1675 cm^{-1}; NMR ($CDCl_3$): δ 1.75 (s, 3, CH_3—C), 3.35 (s, 6, CH_3N), and 7.45–7.75 (m, 5, ArH). The compound could not be induced to crystallize.

Method B—A mixture of 1.0 g (5.3 mmoles) of V and 4.5 g (36 mmoles) of dimethyl sulfate was heated at 140° for 4 hr. The dark-brown liquid was cooled and cautiously hydrolyzed with saturated aqueous sodium carbonate solution overnight. After dilution with 10 ml of water, the mixture was extracted thoroughly with chloroform. The chloroform extract was dried and concentrated to afford 720 mg (62%) of an oil, which was subsequently evaporatively distilled twice *in vacuo* (0.2 mm) at a bath temperature of 80°. The colorless oil so obtained was identical in its spectral and TLC properties with the oil described in *Method A*.

Anal.—Calc. for $C_{12}H_{14}N_2O_2$: C, 66.04; H, 6.47; N, 12.84. Found: C, 65.79; H, 6.78; N, 12.68.

3,5-Diacetoxy-1-methyl-4-phenylpyrazole (VI)—To a solution of 1.0 g (5.25 mmoles) of II*f* in 15 ml of dry pyridine was added dropwise 840 mg (10.7 mmoles) of acetyl chloride at room temperature. The mixture was stirred for 10 min and filtered, and the filtrate was diluted with water and extracted with chloroform. The chloroform phase was washed with 5% hydrochloric acid several times, dried, and concentrated to afford 1.0 g (70%) of white solid. Recrystallization from hexane–ethyl acetate gave pure material, mp 122.5–124°; IR ($CHCl_3$): 1780 cm^{-1}; NMR ($CDCl_3$): δ 2.24 (s, 3, CH_3CO), 2.30 (s, 3, CH_3CO), 3.65 (s, 3, CH_3N), and 7.40 (s, 5, ArH); mass spectrum: *m/e* 274 (M^+); UV (ethanol): 237 nm (log ϵ 4.08).

Anal.—Calc. for $C_{14}H_{14}N_2O_4$: C, 61.31; H, 5.14; N, 10.21. Found: C, 61.45; H, 5.20; N, 10.29.

2-Acetyl-1,4-dimethyl-4-phenyl-3,5-pyrazolidinedione (III)—To a solution of 2.6 g (12.7 mmoles) of II*g* in 20 ml of dry pyridine was added 1.0 g of acetyl chloride at ice bath temperature. The solution was stirred with ice bath cooling for 20 min and then allowed to warm to room temperature over 2.5 hr. The mixture was poured onto 100 g of crushed ice and afforded 1.7 g (54%) of a white solid, mp 91–93.5°. Recrystallization from hexane–ethyl acetate gave pure material, mp 91–93°; IR ($CHCl_3$): 1770 and 1715 cm^{-1}; NMR ($CDCl_3$): δ 1.80 (s, 3, CH_3—C), 2.63 (s, 3, CH_3CON), 3.61 (s, 3, CH_3N), and 7.70 (s, 5, ArH).

Anal.—Calc. for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38. Found: C, 63.29; H, 5.83; N, 11.54.

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[x] To whom inquiries should be directed.

GLC Determination of Chlorphenesin Carbamate in Serum

D. G. KAISER[x] and S. R. SHAW

Abstract □ A method for the determination of chlorphenesin carbamate in human serum was developed to measure the concentrations of intact drug in circulation after therapeutic doses (≥400 mg). The method, based on a chloroform extraction of the buffered (pH 7.6) specimen and subsequent GLC analysis of the trimethylsilyl derivatives of the extract residue, is sensitive to 0.5 μg chlorphenesin carbamate/ml serum. Statistical analyses indicate an average recovery (±*SD*) of 98.3 ± 7.8%. Mass spectrometric analysis, in conjunction with GLC, confirmed the specificity of the method for intact drug. The procedure was successfully applied to drug absorption studies in humans.

Keyphrases □ Chlorphenesin carbamate—GLC analysis in human serum □ GLC—analysis, chlorphenesin carbamate in human serum

Chlorphenesin carbamate[1] [3-(*p*-chlorophenoxy)-1,2-propanediol 1-carbamate, I] is a well-tolerated, orally active agent effective in the treatment of skeletal muscle trauma and inflammation (1–5). The pharmacology (6), physical and analytical characterization (7–12), metabolism in humans and animals (13–16), and pharmacokinetics in humans (17) have been reported for this drug.

The only available data for serum levels of I in humans were obtained by Forist and Judy (17), following oral administration of large single doses (2–3 g); they utilized modifications of the procedure developed by Morgan *et al.* (18) for the determination of mephenesin carbamate and methocarbamol. The method involved: (*a*) alkaline hydrolysis of I in diluted serum to yield chlorphenesin (II), (*b*) chloroform extraction of II, (*c*) periodate oxidation of II (8, 9), and (*d*) spectrophotometric determination of the resulting formaldehyde by reaction with chromotropic acid.

Determination of serum I levels at therapeutic doses (≥400 mg) required a more sensitive and specific method. To meet this need, a GLC procedure was developed and is the subject of this report. Recently, GLC methods were described for the determination of II in plasma (20) and glyceryl guaiacolate in blood (21).

EXPERIMENTAL

Reagents and Materials—Compound I was synthesized[2]. Hydrocarbon-stabilized chloroform[3], *n*-docosane[4], and silylating reagent[5] (1-ml ampuls) were used as supplied. Stock solutions of *n*-docosane (35 μg/ml) and I (25, 50, 75, and 100 μg/ml) in hydrocarbon-stabilized chloroform were stored in glass containers. McIlvaine's buffer (0.5 *M*, pH 7.6) was prepared by dissolving 26.61 g dibasic sodium phosphate and 1.24 g citric acid in sufficient water to make 1 liter of solution. Silicone gum rubber (OV-17) on 60–80-mesh Gas Chrom Q (3% w/w) was used as supplied[6].

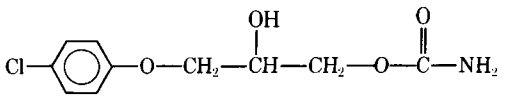
Instrumentation—A two-speed reciprocating shaker[7] was used for shaking the samples in the horizontal position. A mixer[8] was used to aid in preparing the silyl derivatives. Chromatographic measurements were made with a gas chromatograph[9] equipped with a hydrogen flame-ionization detector and a −0.2–1.0-mv recorder[10]. All cylinders of gases used for chromatography (helium, hydrogen, and oxygen) were fitted with filters containing molecular sieve 4Å.

Chromatographic Conditions—All chromatography was conducted on U-shaped glass columns [1.8 m (6 ft) × 3 mm i.d.] of 3% (w/w) OV-17 on 60–80-mesh Gas Chrom Q. All newly prepared columns were preconditioned at 250° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and detector block were maintained isothermally at 220, 220, and 250°, respectively. Helium, hydrogen, and oxygen flow rates were 60, 40, and 400 ml/min, respectively. Under these conditions, *n*-docosane and the disubstituted trimethylsilyl derivative of I have retention times of 3.0 and 6.2 min, respectively (Fig. 1). The monosubstituted trimethylsilyl derivative of I has a retention time of 7.2 min.

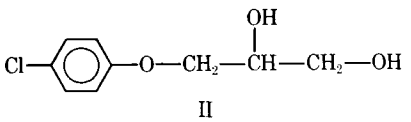
Assay Procedure—*Preparation of Standards*—Pipet aliquots of the I chloroform stock solution equivalent to 2.5, 5.0, 7.5, and 10.0 μg into glass-stoppered centrifuge tubes. Add 0.1-ml aliquots of the *n*-docosane in chloroform stock solution. Evaporate to dryness with a gentle stream of nitrogen gas. Add 1 ml control serum to each centrifuge tube and mix well with the mixer. Prepare an appropriate blank. Add 0.5 ml McIlvaine's buffer to all standards and extract in the same manner as described later for the serum specimens.

Preparation of Samples—Place 0.1-ml aliquots of the *n*-docosane stock solution in a series of glass-stoppered centrifuge tubes and evaporate to dryness with a gentle stream of nitrogen gas. Add 1 ml serum, 0.5 ml McIlvaine's buffer, and 4 ml hydrocarbon-stabilized chloroform. Shake in the horizontal position for 20 min, and centrifuge for 10 min at 2000 rpm. Transfer a 2-ml aliquot of the chloroform layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with a gentle stream of nitrogen gas. Wash down the walls of the centrifuge tube with 0.5 ml chloroform and evaporate to dryness with nitrogen gas. Add 0.1 ml silylating reagent to each chloroform extract residue. Rotate each tube to permit the reagent to contact the lower 1.2 cm (0.5 in.) of the centrifuge tube wall, and allow the reagent to react for at least 2 hr. Then inject a 2-μl aliquot of the reaction mixture for analysis into the chromatograph.

Calculations—The peak heights for *n*-docosane and the disubstituted trimethylsilyl derivative of I are measured. Peak height ratios are obtained by dividing the peak height of the I derivative by the peak height of *n*-docosane. Calibration curves for known



I



II

[1] Maolate, The Upjohn Co.
[2] Research Division, The Upjohn Co.
[3] Matheson, Coleman and Bell, Milwaukee, Wis.
[4] Analabs, Inc., Hamden, Conn.
[5] Sil-Prep, Applied Science Labs., State College, Pa.
[6] Applied Science Labs., State College, Pa.
[7] Eberbach and Sons, Ann Arbor, Mich.
[8] Vortex model K-500, Scientific Industries, Queen's Village, N.Y.
[9] F & M model 400, Hewlett-Packard Co., Avondale, Pa.
[10] Electronik 15, Honeywell, Inc., Philadelphia, Pa.

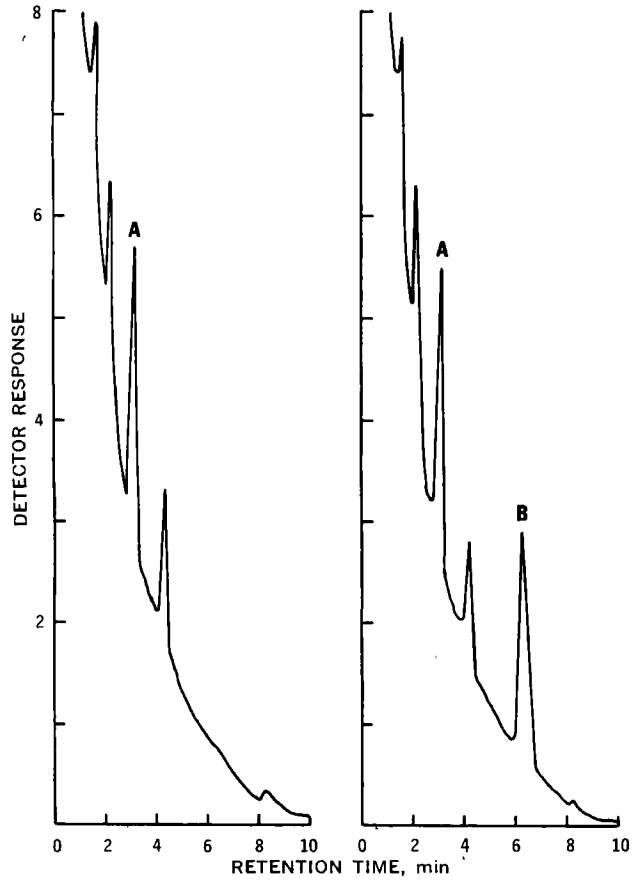


Figure 1—*GLC of human serum extracts. Left: normal serum specimen. Right: serum specimen from subject at 1 hr after dose 30 of a multiple-dose regimen (400 mg every 6 hr). Key: A, n-docosane internal standard; and B, disubstituted trimethylsilyl derivative of I.*

concentrations of I in serum are prepared by plotting peak height ratios *versus* I concentration expressed as micrograms per milliliter serum. Values for unknown concentrations of I in serum specimens, obtained in the same manner, are then read directly from the graph or calculated from the slope of the standard curve.

Drug Administration to Humans—Four normal adult male volunteers, ages 28–37 years and ranging in body weight from 66.4 to 87.3 kg, received 400 mg I in hard-filled gelatin capsules every 6 hr for a total of 30 doses. At zero time (just prior to the 30th dose) and at 0.5, 1, 2, 3, 4, 6, and 8 hr postadministration of the 30th dose, blood specimens (20 ml) were withdrawn. The serum was harvested and stored at −18°.

RESULTS AND DISCUSSION

Silylation of Chlorphenesin Carbamate—The thermal stabilities of carbamates show a marked dependence upon the degree and type of *N*-substitution (22). Structural transformations observed during GLC of intact carbamates show a similar dependence (23). Preparation of suitable derivatives, as well as careful selection of column supports and chromatographic temperatures, circumvents or minimizes thermal degradation of carbamates during GLC analysis (24). Trimethylsilyl derivatives retard the thermal breakdown of pesticidal carbamates and ureas, resulting in better symmetry of the GLC peaks (25). As shown in Fig. 2, four drug-related materials, with retention times of 1.25, 1.5, 6.2, and 7.2 min, were observed in the chromatogram of a I silylation mixture. GLC in conjunction with IR and mass spectrometry indicated that the materials were the: (*a*) disubstituted trimethylsilyl derivative of II (R_T = 1.25 min), (*b*) monosubstituted trimethylsilyl derivative of II (R_T = 1.5 min), (*c*) disubstituted trimethylsilyl derivative of I (R_T = 6.2 min), and (*d*) monosubstituted trimethylsilyl derivative of I (R_T = 7.2 min).

Table I—Recovery of Chlorphenesin Carbamate from Human Serum

Added, μg/ml	Found, μg/ml	Recovery, %
1.0	0.95	95.0
2.5	2.43	97.2
5.0	4.48	89.6
7.5	7.69	102.5
10.0	9.39	93.9
15.0	16.76	111.7
	Mean ± *SD* = 98.3 ± 7.8	

A series of samples containing known amounts of I and *n*-docosane was prepared to determine the optimal reaction time for formation of the disubstituted trimethylsilyl derivative. To minimize thermal degradation, the injection port flash heater was maintained at the same temperature as the column. The results indicated that formation of the disubstituted derivative was completed within 2 hr and that little, if any, monosubstituted derivative was present (Fig. 3). Stability studies showed that the I disubstituted derivative was stable for at least 72 hr in the silylating reagent. In the presence of known amounts of water, the I disubstituted derivative was rapidly hydrolyzed to the monosubstituted derivative. GLC also showed increased amounts of the II monosubstituted derivative, consistent with the greater thermal instability of *N*-unsubstituted carbamates. Small amounts of the II disubstituted derivative, found in the chromatograms, resulted from thermal cleavage of I mono- and disubstituted derivatives and subsequent silylation in the flash heater of the chromatograph. Ancillary studies showed that increasing the temperature of the injection port flash heater markedly increased the amount

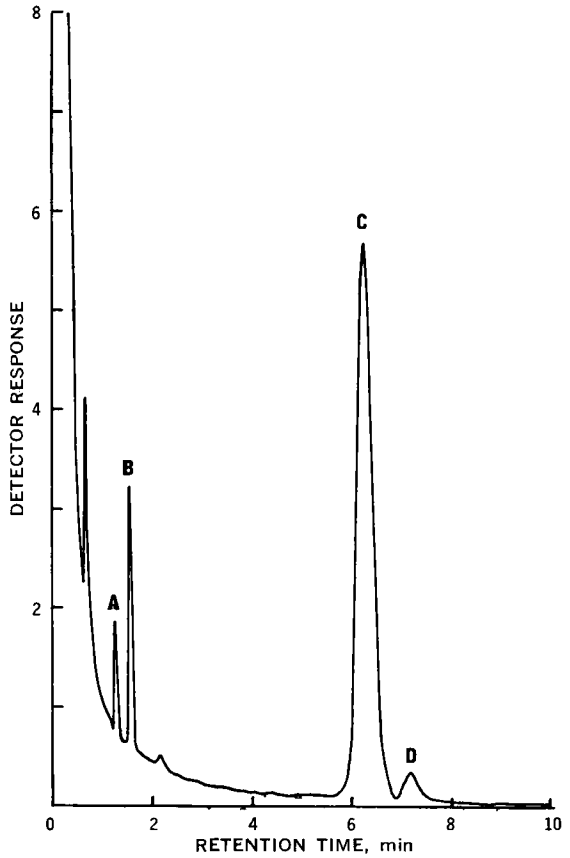


Figure 2—*GLC of chlorphenesin carbamate silylation mixture. Reaction time = 30 min. Key; A, disubstituted trimethylsilyl derivative of II; B, monosubstituted trimethylsilyl derivative of II; C, disubstituted trimethylsilyl derivative of I; and D, monosubstituted trimethylsilyl derivative of I.*

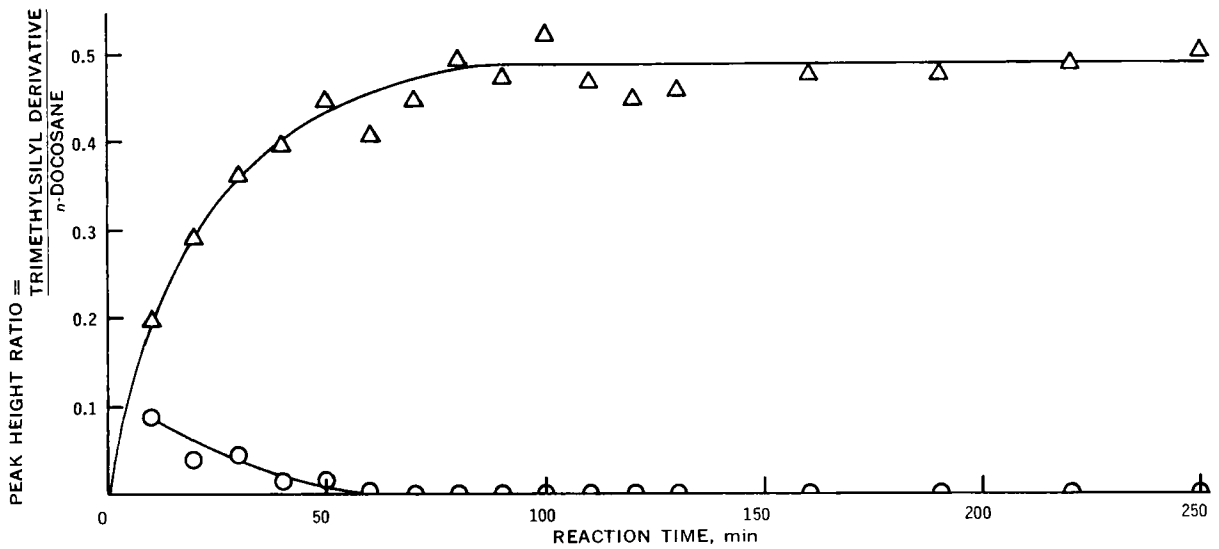


Figure 3—*Effect of reaction time on formation of I trimethylsilyl derivatives. Key: △, disubstituted derivative; and ○, monosubstituted derivative.*

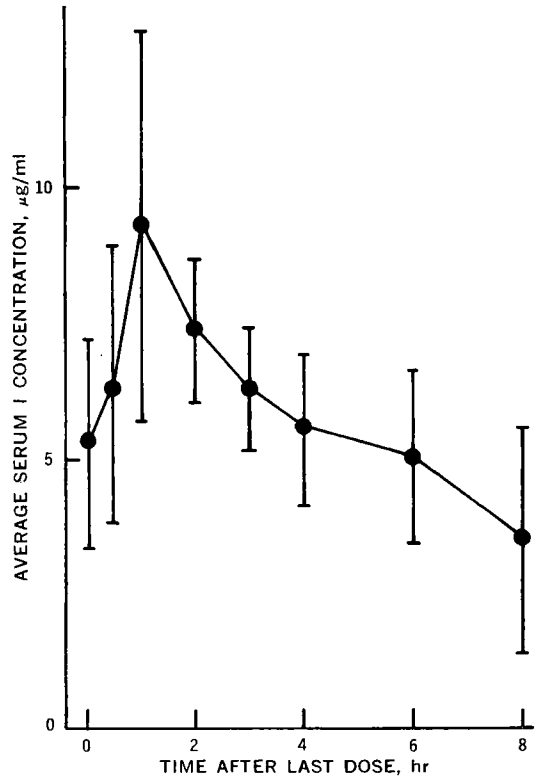


Figure 4—*Average* (±SD) *serum I concentrations* versus *time in humans* (n = 4) *after dose 30 of a multiple-dose regimen (400 mg every 6 hr).*

of the II monosubstituted derivative found in the chromatogram of the I disubstituted derivative.

Selection of Internal Standard—Pilot studies, using 3-(*p*-chlorophenoxy)-1,2-propanediol cyclic carbonate as an internal standard, showed that it had a retention time of about 6.7 min as compared to 6.2 min for the I disubstituted trimethylsilyl derivative. As shown in Fig. 2, the monosubstituted derivative of I had a retention time of 7.2 min. To ensure that no hydrolysis of the I disubstituted derivative had occurred, it was highly desirable to maintain this region of the chromatogram free of other materials. *n*-Docosane was subsequently selected to replace 3-(*p*-chlorophenoxy)-1,2-propanediol cyclic carbonate as the internal standard. Although *n*-docosane eluted from the chromatograph in a region where there was no interference, it only served as a measure of sample injection reproducibility.

Assay Sensitivity and Specificity—At a sensitivity of 3.2×10^{-10} amp/mv, 0.98 μg of I as its disubstituted trimethylsilyl derivative produced a full-scale response. However, under the assay conditions described, the lower limit of assay detection sensitivity for I in extracts of human serum is 0.5 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under the assay conditions described, a linear relationship between detector response and concentration is obtained for I over the 0–20-μg/ml range. Quantification from a standard curve is adequate. Analysis of serum specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay is the disubstituted trimethylsilyl derivative of I. Known metabolites of I—*viz.*, *p*-chlorophenoxylactic acid, *p*-chlorophenoxyacetic acid, and *p*-chlorophenol (13), do not interfere in the assay.

Recovery Experiments—Known amounts of I and *n*-docosane in chloroform were evaporated to dryness in centrifuge tubes and water or serum was added. The samples were thoroughly mixed, 0.5 ml McIlvaine's buffer was added, and the solution was extracted with chloroform. All extract residues were silylated and analyzed chromatographically. The results (Table I) indicate that recovery of I from serum is essentially quantitative (98.3 ± 7.8%) as compared to simple aqueous samples.

Serum Levels of Chlorphenesin Carbamate in Humans—Results from the measurement of serum I concentrations in normal human subjects, after multiple-dose oral drug administration, demonstrate the utility of the analytical methodology (Fig. 4). A peak mean (±*SD*) level of I (9.29 ± 3.62 μg/ml) was observed at 1 hr postadministration, indicating rapid drug absorption from hard-filled gelatin capsules. The serum drug disappearance half-life, as estimated graphically from the average serum drug concentrations, was 4.6 hr, showing good agreement with the calculated half-lives (2.32–4.30 hr) following single-dose oral administration of 2–3 g drug (17). The combined results from these investigations show that the GLC method can be used for: (*a*) determining serum I concentrations after therapeutic doses (≥400 mg) of drug, (*b*) evaluating the pharmacokinetics, and (*c*) selecting an optimum dosage regimen for I administration to humans.

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Colestipol hydrochloride[1] is a high molecular weight anion-exchange polyethylenepolyamine polymer with 1-chloro-2,3-epoxypropane which may have approximately one of five amine nitrogens protonated as the chloride salt. Investigators have shown that orally administered polymer binds bile salt anions in the small intestine and reduces serum cholesterol levels in experimental animals (1–3) and in humans (4–8). Since colestipol may also bind other substances, including drugs that might be used as concurrent therapy in hypercholesterolemic subjects, the effects of the polymer on the absorption of a series of drugs in the rat were studied. Results with radiolabeled aspirin, L-thyroxine, phenobarbital, cortisone, and sul-

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Table I—Kinetic Parameters for Aspirin with Concurrent Administration of Colestipol or Cellulose Control

Parameters[a]	Dose, 71.5 mg/kg		Dose, 214.5 mg/kg		Standard Deviation, %[d]
	Control[b]	Colestipol	Control	Colestipol[c]	
Model:					
K_A[e]	6.55	6.44	8.36	4.68*	49.7
$A_{1/2}$	0.111	0.116	0.102	0.165*	51.3
K_E	0.161	0.179	0.177	0.156	24.9
$E_{1/2}$	4.65	4.08	4.37	4.67	33.0
Peak radioactivity	4,900	4,702	5,052	3,759**	13.1
Area	34,960	30,313	34,129	28,210	25.2
Observed:					
Peak radioactivity	4,860	4,628	5,012	3,641**	12.7
Area	29,005	25,882	27,804	23,671*	15.7

[a] K_A(hr^{-1}) = rate of absorption; $A_{1/2}$(hr) = absorption half-life; K_E(hr^{-1}) = rate of elimination; $E_{1/2}$(hr) = elimination half-life; peak radioactivity is in dpm/0.1 ml serum, and area under the time–concentration curve is in dpm/0.1 ml × hr. The abbreviations are the same in all tables. [b] Represents mean of nine animals; all other values represent mean of 10 animals. [c] Significant difference between colestipol means and mean of corresponding control at (*) $p < 0.05$ and (**) $p < 0.01$. [d] Obtained from analysis of variance over all four groups. [e] Data converted to logarithms to achieve more homogeneous within-group variances; values expressed as antilog of logarithm means.

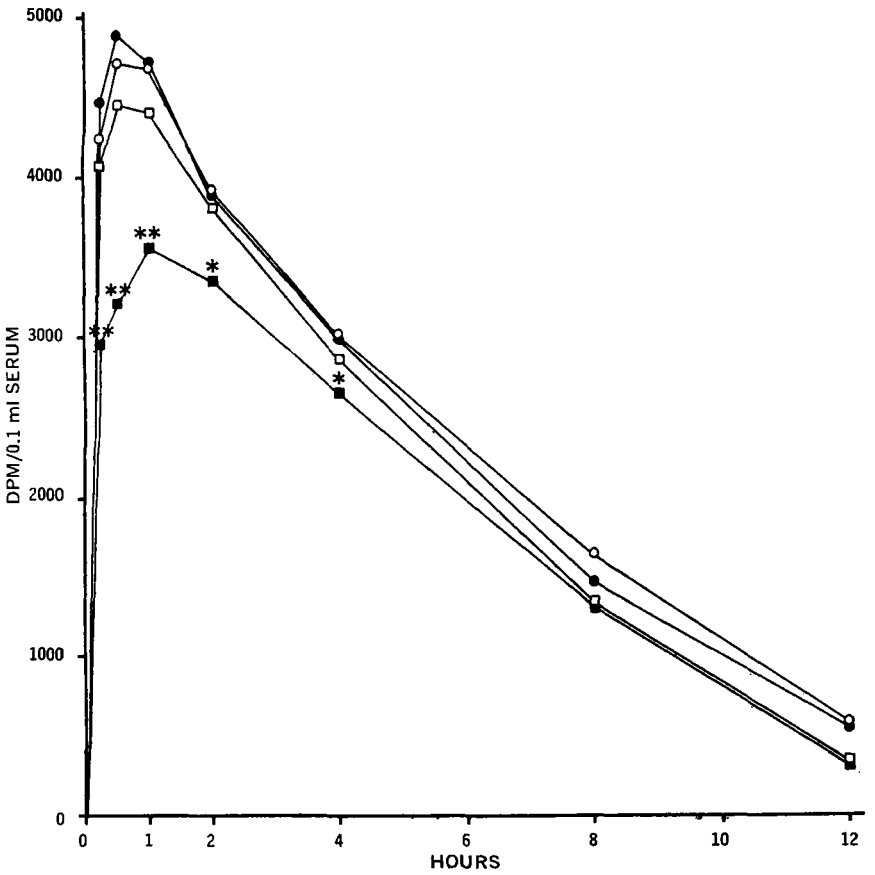


Figure 1—*Effect of colestipol on absorption of aspirin-carboxyl-^{14}C. Key: cellulose control at 71.5 (○) and 214.5 (●) mg/kg; colestipol at 71.5 (□) and 214.5 (■) mg/kg; and significant difference between colestipol mean and mean of corresponding control at* $p < 0.05$ (*) *and* $p < 0.01$ (**).

fadiazine, whose pharmacokinetics can be described by a one-compartment open model, are reported here.

EXPERIMENTAL

Ten male rats[2] with an average weight of approximately 240 g were used in each group. The rats were fasted for 18 hr prior to and during each experiment; water was allowed *ad libitum*. Colestipol was suspended in 0.25% aqueous methylcellulose and administered by stomach tube at 71.5 and 214.5 mg/kg. Controls received equal amounts of a bulk material, microcrystalline cellulose[3], suspended and dosed similarly to colestipol. These levels correspond on a weight basis to 5 and 15 g, respectively, of material in a 70-kg human. After treatment, each rat was immediately, within 10 sec, administered the radioactive drug dissolved or suspended in 0.25% methylcellulose vehicle by stomach tube. The total doses of the drugs, after addition of nonradioactive compound[4], ranged generally within the amount given to humans on a weight basis. In the sulfadiazine experiment, each rat received about 1.9 μCi/100 g body weight; in all other experiments each received about 0.45 μCi. The rats were then bled from the jugular vein (0.25 ml) at various time periods (9). Radioassay of 0.1 ml serum was made in a liquid scintillation spectrometer[5]; quenching was determined using the automatic external standard and a prepared efficiency correlation curve. Correction for ^{35}S decay was also made in the sulfadiazine experiment. The scintillation fluid

[2] Upj:TUC(SD)spf.
[3] Avicel, PH-102, FMC Corp., American Viscose Division, Marcus Hook, Pa.
[4] Aspirin, phenobarbital, cortisone acetate, and sulfadiazine were USP grade; supplementation with L-thyroxine was not necessary.
[5] Model 3375, Packard Instrument Co., Downers Grove, Ill.

Table II—Kinetic Parameters for L-Thyroxine with Concurrent Administration of Colestipol or Cellulose Control

Parameters	Dose[a], 71.5 mg/kg		Dose[a], 214.5 mg/kg		Standard Deviation, %
	Control	Colestipol[b]	Control	Colestipol[b]	
Model:					
K_A	0.176	0.221	0.169	0.270**	27.4
$A_{1/2}$	4.08	3.21	4.24	2.68**	27.9
K_E	0.028	0.028	0.023	0.027	29.7
$E_{1/2}$	26.5	27.1	32.5	28.0	29.8
Peak radioactivity	1,352	1,050**	1,476	1,081**	12.3
Area	72,650	54,150*	93,046	55,385**	25.6
Observed:					
Peak radioactivity	1,420	1,112**	1,564	1,153**	12.9
Area	58,222	43,375**	67,905	43,639**	15.6

[a] Mean values represent 10 animals per group. [b] Significant difference between colestipol mean and mean of corresponding control at (*) $p < 0.05$ and (**) $p < 0.01$.

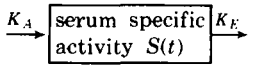
Table III—Kinetic Parameters for Phenobarbital with Concurrent Administration of Colestipol or Cellulose Control

Parameters	Dose, 71.5 mg/kg		Dose, 214.5 mg/kg		Standard Deviation, %
	Control[a]	Colestipol	Control	Colestipol	
Model:					
K_A[b]	7.63	6.84	5.69	5.22	52.3
$A_{1/2}$	0.106	0.116	0.135	0.138	47.5
K_E	0.088	0.072	0.082	0.085	20.5
$E_{1/2}$	8.45	9.92	8.62	8.32	20.3
Peak radioactivity	1,196	1,164	1,197	1,198	8.5
Area	15,219	17,427	15,801	15,364	17.1
Observed:					
Peak radioactivity	1,214	1,168	1,197	1,187	9.2
Area	13,869	15,032	14,316	14,050	12.0

[a] Represents mean of nine animals; all other values represent mean of 10 animals. [b] Data converted to logarithms; values expressed as antilog of logarithm means.

contained 8.8% serum solubilizer[6], 0.4% 2,5-diphenyloxazole, and 0.005% *p*-bis[2-(5-phenyloxazolyl)]benzene in toluene. The radioactive drugs [aspirin-carboxyl-^{14}C[7], L-thyroxine-^{14}C (uniformly labeled)[7], cortisone-4-^{14}C acetate[7], sulfadiazine-^{35}S[7], and 5-ethyl-5-phenylbarbituric-2-^{14}C acid[8]] were found to be radiochemically pure by TLC on silica gel and scanning with a radiochromatogram scanner[9].

The statistical analysis of the data for each drug was made in the following manner. The observed specific activity curves (dpm/0.1 ml serum) for each rat were analyzed by the one-compartment open model (Scheme I):



Scheme I

where the serum activity-time function is described by:

$$S(t) = D\left(\frac{K_A}{K_E - K_A}\right)(e^{-K_A t} - e^{-K_E t}) \qquad \text{(Eq. 1)}$$

Nonlinear techniques were used to estimate the parameters of this model (10). The absorption half-life, $A_{1/2}$ (hours), was obtained as ln $2/K_A$, and the elimination half-life $E_{1/2}$ (hours) was obtained as ln $2/K_E$, where K_A (hr^{-1}) and K_E (hr^{-1}) are the rates of absorption and elimination, respectively. The curve generated for each rat with the estimated parameter values in the serum activity-time function was used to obtain the area under the curve [(dpm/0.1 ml) × hr] from time zero to infinity and peak radioactivity. Peak radioactivity was also obtained from the observed curve, and the area from time zero to the end of the test was calculated with the trapezoidal rule.

For each parameter described, a one-way analysis of variance (11) was performed on the values from the four groups of animals; comparisons were made between each colestipol mean and the mean of the corresponding control. This procedure was also carried out on the specific activity values observed at each sampling period. In some analyses the data were transformed to logarithms to achieve more homogeneous within-group variances; values were then reported as the antilog of logarithm means. When more than two samples were lost during centrifugation or the rat died from the bleeding procedure, the data from that animal were not included in the analyses.

[6] Bio-Solv BBS-3, Beckman Instruments, Inc., Fullerton, Calif.
[7] Amersham/Searle.
[8] New England Nuclear.
[9] Model 885, Vanguard Instrument Corp., La Grange, Ill.

RESULTS AND DISCUSSION

Aspirin-carboxyl-^{14}C—The results obtained with aspirin administered at 4.4 mg/kg are presented in Table I and Fig. 1. The differences in serum drug levels with 71.5 mg/kg colestipol and the corresponding control were not significant at any sampling period. Estimated model parameters for absorption, elimination, peak radioactivity, and area under the time-concentration curve (zero to infinity) did not differ significantly from control. Values for observed peak radioactivity and the area under the curve (0–12 hr) were unaltered significantly. However, 214.5 mg/kg colestipol caused a significant reduction in serum drug levels at 0.25, 0.5, 1, 2, and 4 hr. The rate of absorption of aspirin decreased (from 8.36 to 4.68 hr^{-1}) and absorption half-life increased (from 0.102 to 0.165 hr^{-1}); the rate of elimination and the elimination half-life did not vary significantly from the control values. Drug availability as measured by the area under the time-concentration curve from the model and the observed area were reduced by 17 and 15%, respectively; only the latter was significantly different from control. Model and observed peak radioactivities were both reduced significantly by 27%.

Cholestyramine in rats did not reduce absorption of aspirin at 71.5 mg/kg but did at 357.5 mg/kg, as measured by ^{14}C appearance in the plasma during a 2-hr study (12). Although the level of resin was greater than used in the present studies, the results indicate that both anion-exchange drugs can bind aspirin when administered concurrently to the rat.

L-Thyroxine-^{14}C (Uniformly Labeled)—Serum levels of L-thyroxine after oral administration of 0.029 mg/kg were measured at various time intervals over 3 days. Serum drug levels significantly

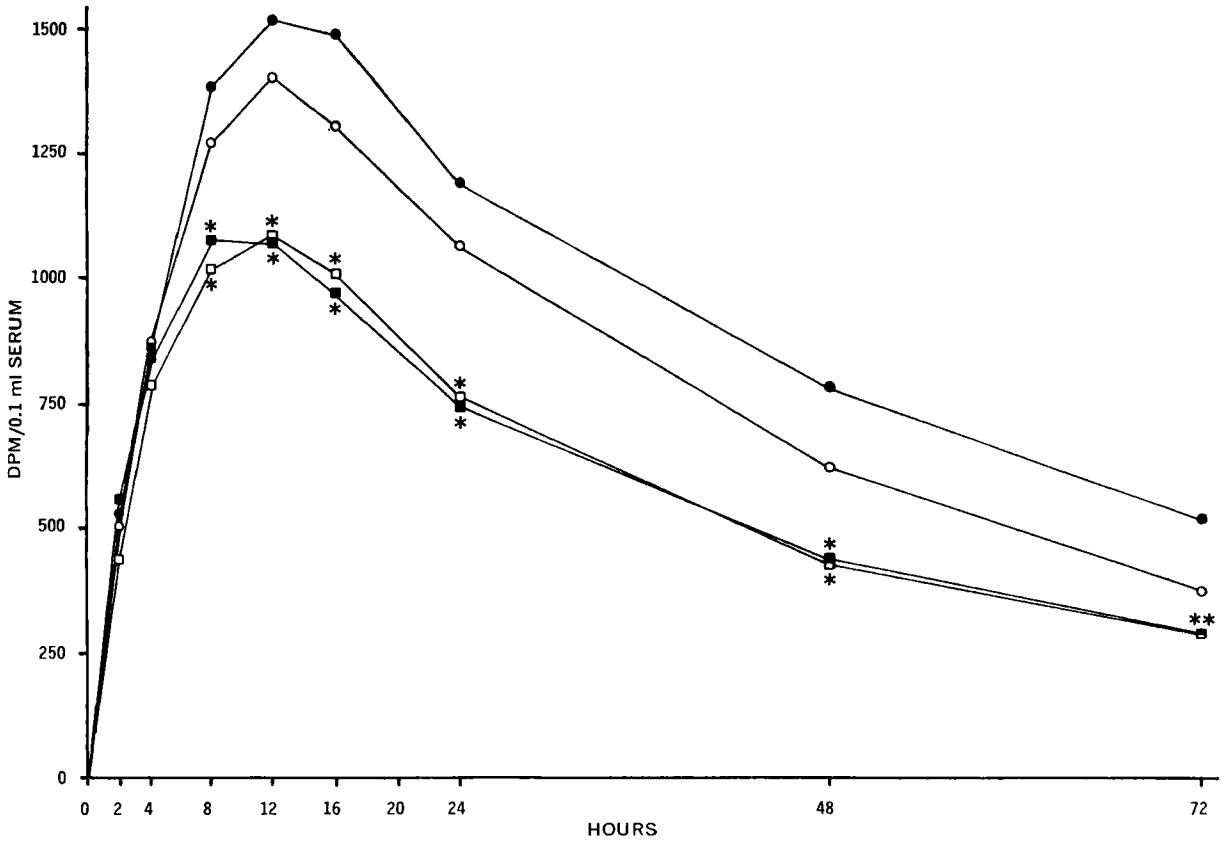


Figure 2—*Effect of colestipol on absorption of* L-*thyroxine-*^{14}C*(uniformly labeled). Key: cellulose control at 71.5* (○) *and 214.5* (●) *mg/kg; colestipol at 71.5* (□) *and 214.5* (■) *mg/kg; and significant difference between colestipol mean and mean of corresponding control at* $p < 0.01$ (*) *and* $p < 0.01$ *for high level of colestipol only* (**).

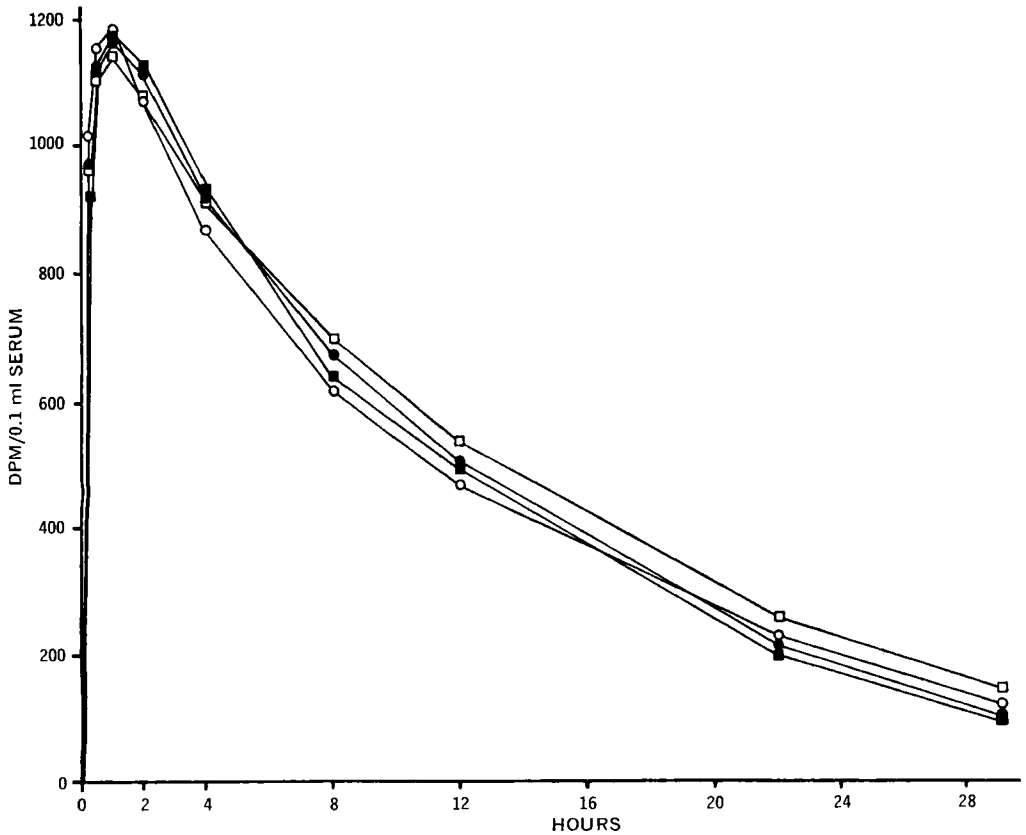


Figure 3—*Effect of colestipol on absorption of phenobarbital-2-*^{14}C*. Key: cellulose control at 71.5* (○) *and 214.5* (●) *mg/kg; and colestipol at 71.5* (□) *and 214.5* (■) *mg/kg.*

Table IV—Kinetic Parameters for Cortisone with Concurrent Administration of Colestipol or Cellulose Control

Parameters	Dose, 71.5 mg/kg		Dose, 214.5 mg/kg		Standard Deviation, %
	Control	Colestipol[a]	Control[a]	Colestipol	
Model:					
K_A[b]	9.87	12.48	7.82	9.66	46.0
$A_{1/2}$	0.079	0.059	0.092	0.082	44.0
K_E	0.939	1.037	1.122	1.124	22.0
$E_{1/2}$	0.797	0.692	0.642	0.646	25.7
Peak radioactivity	583	590	531	573	21.6
Area[b]	800	720	659	683	22.7
Observed:					
Peak radioactivity	561	581	526	571	22.0
Area	785	704	672	689	22.1

[a] Represents mean values of nine animals; all other values represent mean of 10 animals. [b] Data converted to logarithms; values expressed as antilog of logarithm means.

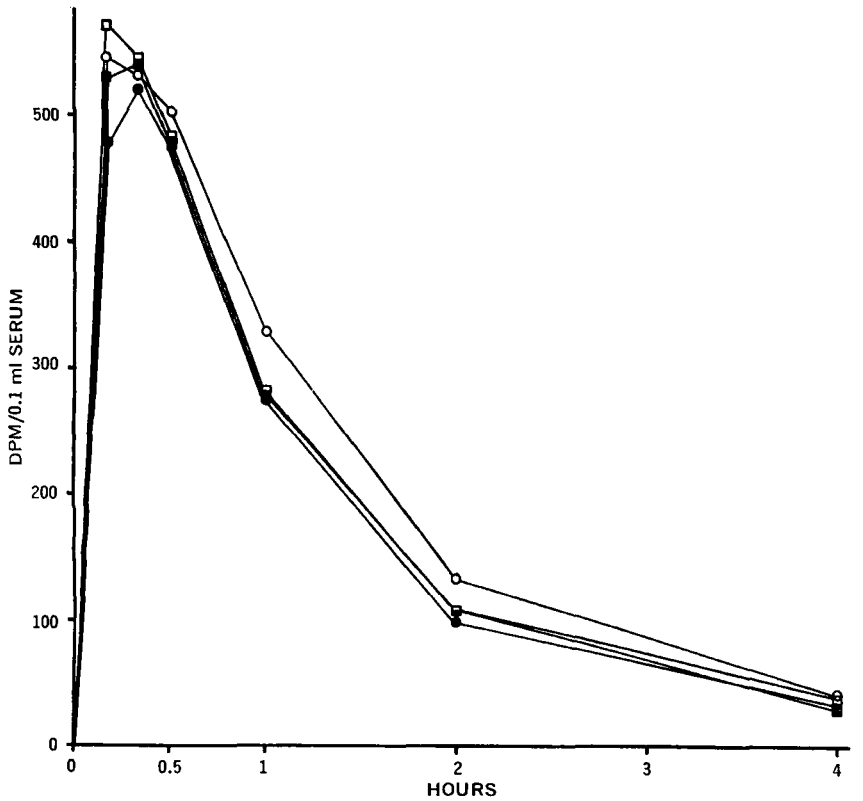


Figure 4—*Effect of colestipol on absorption of cortisone-4-^{14}C acetate. Key: cellulose control at 71.5 (○) and 214.5 (●) mg/kg; and colestipol at 71.5 (□) and 214.5 (■) mg/kg.*

decreased with the low level of colestipol at 8, 12, 16, 24, and 48 hr (Table II and Fig. 2). Model and observed peak radioactivities and areas under the curves were reduced significantly and to the same extent, 22 and 25%, respectively; none of the other changes differed significantly from control values. At the high level of colestipol, the amount of radioactivity in the serum decreased significantly at all time intervals except at the 2- and 4-hr sampling periods. There was a significant increase in the rate of absorption (from 0.169 to 0.270 hr^{-1}) and a decrease in the absorption half-life of the drug (from 4.24 to 2.68 hr). This apparent increase in the rate of L-thyroxine absorption may be due to reduced availability of the drug at the absorption site since it has been shown that overestimation of the absorption rate constant increases as bioavailability decreases (14). The rate of elimination and the elimination half-life did not vary significantly from the control values. Observed peak radioactivity and drug availability decreased significantly by 27 and 36%, respectively. Significant decreases of model values for these parameters were essentially the same as observed values.

Phenobarbital-2-^{14}C—The results obtained with 1.34 mg/kg phenobarbital-2-^{14}C appear in Table III and Fig. 3. The changes in dpm/0.1 ml serum and kinetic and observed parameters with colestipol did not differ significantly from corresponding controls.

Cholestyramine retarded the absorption of phenobarbital in the rat (12). At the lower dose of resin (71.5 mg/kg), drug plasma levels were reduced at the 15- and 30-min sampling periods; at the higher dose (357.5 mg/kg), absorption was significantly reduced at all time intervals during the 2-hr test. There appears to be a difference between the binding capacities of cholestyramine and colestipol for phenobarbital; however, comparisons should be made under identical experimental conditions.

Cortisone-4-^{14}C Acetate—Cortisone-4-^{14}C acetate was administered at 1.13 mg/kg to each rat. None of the serum radioactivities at various sampling periods or kinetic parameters was influenced at either level of colestipol (Table IV and Fig. 4).

Sulfadiazine-^{35}S—The results obtained with 14 mg/kg sulfadiazine-^{35}S appear in Table V and Fig. 5. The differences in serum radioactivities for the 71.5-mg/kg dose and the control were not significant at any sampling period. Colestipol at 214.5 mg/kg decreased radioactivity at 1 hr; other differences did not vary significantly. The changes in kinetic parameters were not significant at either level with the polymer.

Colestipol is an anion-exchange polymer that would be expected to bind with ionized acidic drugs by electrostatic forces (*e.g.*, between the negatively charged carboxyl groups of aspirin and the positively charged nitrogen atoms of colestipol). Since colestipol interfered with absorption of aspirin (pKa 3.5) but not phenobarbital (pKa 7.2), one could speculate that the stability of the

Table V—Kinetic Parameters for Sulfadiazine with Concurrent Administration of Colestipol or Cellulose Control

Parameters	Dose, 71.5 mg/kg[a]		Dose, 214.5 mg/kg[a]		Standard Deviation, %
	Control	Colestipol	Control	Colestipol	
Model:					
K_A[b]	0.30	0.32	0.32	0.31	49.8
$A_{1/2}$	2.57	2.30	2.43	2.63	51.5
K_E[b]	0.10	0.10	0.10	0.09	33.0
$E_{1/2}$	7.07	6.74	7.49	8.88	48.6
Peak radioactivity	21,657	19,977	20,620	18,411	16.6
Area[b]	386,872	337,995	408,478	408,478	27.1
Observed:					
Peak radioactivity	22,491	20,008	21,326	19,467	18.2
Area	348,726	308,770	334,263	317,547	16.2

[a] Mean values represent 10 animals per group. [b] Data converted to logarithms; values expressed as antilog of logarithm means.

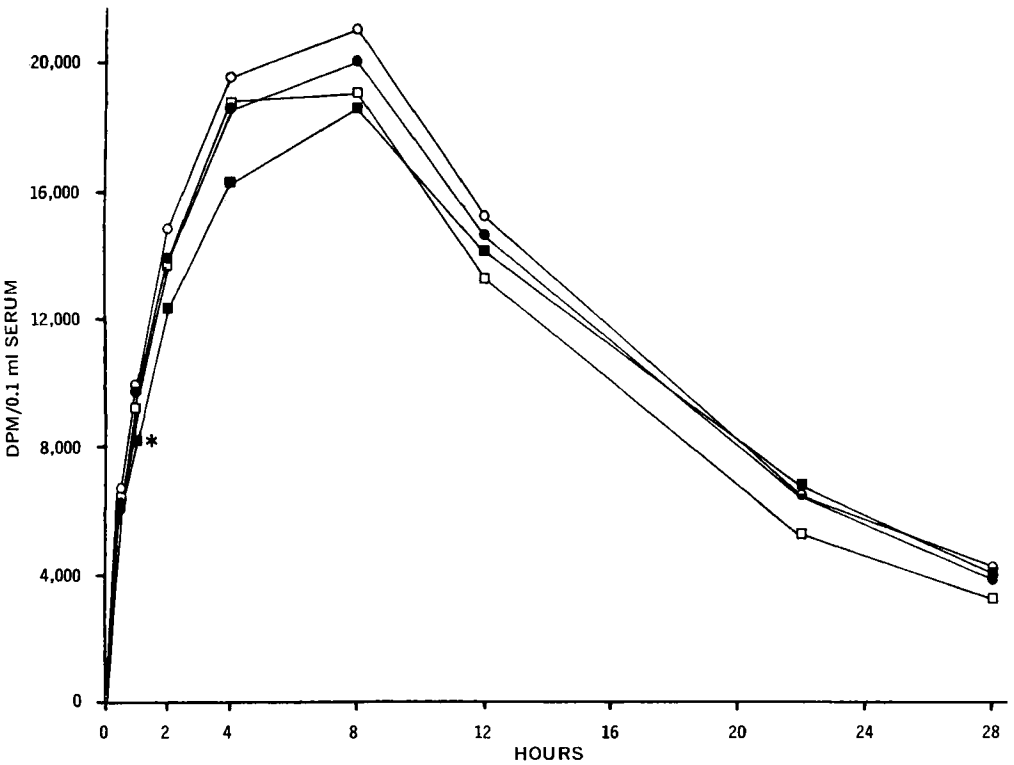


Figure 5—*Effect of colestipol on absorption of sulfadiazine-^{35}S. Key: cellulose control at 71.5 (○) and 214.5 (●) mg/kg; colestipol at 71.5 (□) and 214.5 (■) mg/kg; and significant difference between high colestipol mean and mean of corresponding control at* p *<0.05* (*).

drug–polymer interaction was related to the pKa of these drugs. This pKa relationship to drug and polymer interaction may not always determine the extent of binding, since other forces such as hydrogen bonding, dipole–dipole interactions, van der Waals forces, nonelectrostatic interactions, and intermolecular attraction of like molecules also can play an important part in the process.

The absorption of L-thyroxine was most markedly reduced by colestipol in comparison to the other drugs tested. In addition to the primary ionic interaction between the anionic carboxyl group of L-thyroxine and the basic insoluble polymer, there can be a reinforcement by a nonelectrostatic force existing between the hydrophobic portion of L-thyroxine and the polymer molecules. Evidence for the importance of the hydrophobicity of the adsorbate molecule was reported in studies with cholestyramine (14). Since the phenolic hydroxyl of L-thyroxine has a pKa of 6.73 and is over 50% ionized at physiological pH (15), it is possible that the phenolic hydroxyl may have influenced binding to the polymer by hydrogen bonding or electrostatic attraction. Although thyroxine is known to undergo marked enterohepatic circulation in the rat (16), the polymer had no significant effect on rates of excretion of drug from the serum at either level.

Colestipol had little or no effect on the absorption of the weakly acidic drugs phenobarbital (pKa 7.2) and sulfadiazine (pKa 6.5). Competition for binding sites on the polymer of these drugs with bile acid anions and inorganic physiological anions (*e.g.*, phosphate, chloride, and bicarbonate) present in the GI tract could possibly explain these results. Since the rat does not possess a gallbladder, there could be a continuous flow of bile salts and electrolytes competing for binding sites on the polymer.

The present study indicates that colestipol, like cholestyramine, can inhibit the absorption of other therapeutic agents from the GI tract of the rat. Whether results obtained in rats are predictive of results in humans remains to be determined. However, potential drug interaction could be minimized by separating administration of colestipol and other drugs as much as is practical.

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Keyphrases □ Succinylsulfathiazole—preparation, characterization, and interconversion of different crystal forms, kinetics, Arrhenius plots □ Polymorphism—preparation, characterization, and interconversion of succinylsulfathiazole crystal forms, kinetics, Arrhenius plots □ Crystal forms, succinylsulfathiazole—preparation, characterization, and interconversion

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Succinylsulfathiazole was reported to be dimorphic, and methods of preparation of the two crystal forms were described previously (7). Shefter and Higuchi (4) also described methods of preparation of an anhydrous form, three hydrates, and a pentanol solvate of succinylsulfathiazole. Later, Mesley and Houghton (8) described the preparation of four crystal forms and an amorphous form of the same compound. Uncertainty concerning the number of crystal forms of this compound and the lack of reproducibility in preparing and characterizing them were observed. A more thorough investigation of this polymorphic system seemed necessary.

The present work is concerned with the preparation, characterization, interconversion, and kinetics of transformation of the various crystal forms of succinylsulfathiazole.

EXPERIMENTAL[1] AND RESULTS

Materials—Three commercial samples of succinylsulfathiazole[2] were used during this investigation. The purity of the starting materials and the products of crystallization was checked by paper chromatography using the solvent system described by Steel (9). Solvents used for crystallization were of USP or BP quality.

Preparation of Crystal Forms—The general procedure for the preparation of the different crystal forms involved crystallization from specific solvents. For this purpose, 0.2 g of the drug was dissolved in a suitable volume of an appropriate solvent to form a saturated solution at the boiling point of that solvent. The solution was allowed to cool slowly and stand at room temperature until most of the solid crystallized out. The crystals were then separated by filtration through a sintered-glass disk[3], dried in a current of air at room temperature (25°), and stored in a desiccator. Crystals prepared from aqueous solvents had to be dried between two filter papers. Optimum conditions for the preparation of the different crystal forms are summarized as follows.

[1] IR spectra were measured with a Perkin-Elmer double-beam grating IR spectrophotometer, model 237 B. X-ray diffraction measurements were made with a General Electric XRD-6 diffractometer under the following conditions: 3° beam slit and 0.2° detector slit, nickel-filtered CuKα radiation (30 kv, 11 mamp). A Unicam SP500 UV spectrophotometer was used to determine the molar absorptivity of the solution of the various crystal forms.

[2] Boots, England, Katwuk, Holland, and a Chinese brand, all supplied by Chemical Industries Development, Guiza, Egypt.

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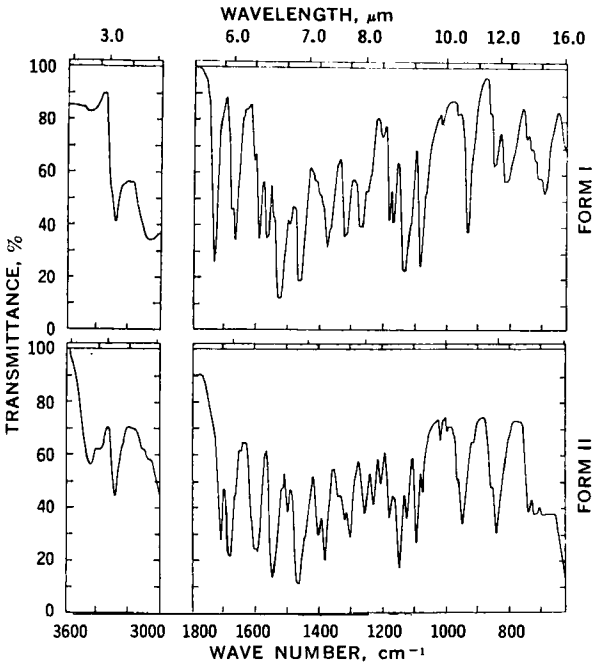

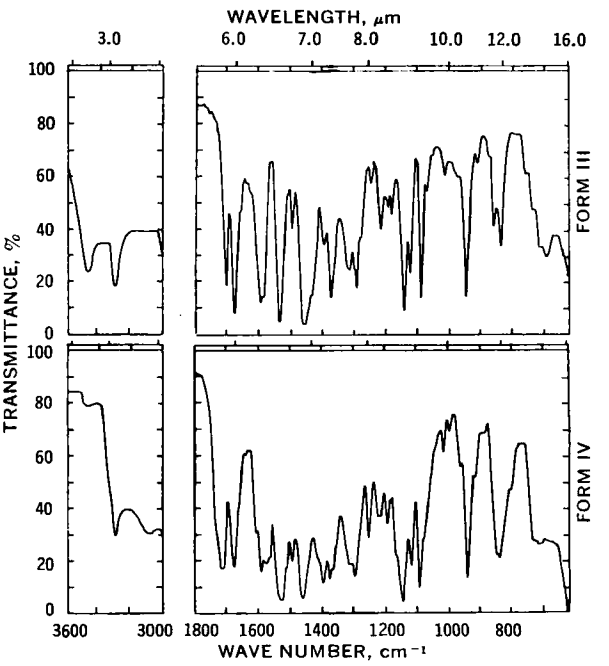

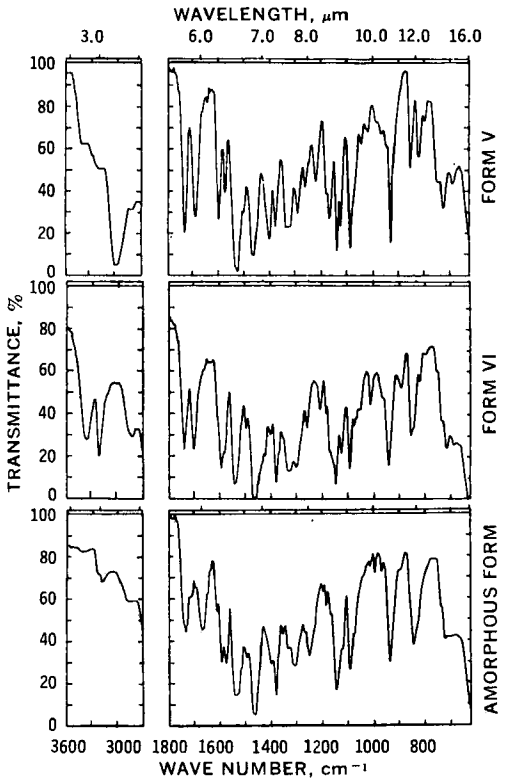

Figure 1—*IR spectra of succinylsulfathiazole crystal forms in mineral oil mulls.*

Form I was prepared by evaporation to dryness of a solution in acetone on a water bath. The same form was also encountered as one of the available commercial samples.

Form II was not recovered from any solvents used for crystallization. However, suspension of any of the other crystal forms in water effected transformation to Form II in a time varying from a few hours to several weeks. Two of the available commercial samples of succinylsulfathiazole existed as Form II.

Form III was prepared by crystallization from water, methanol, or ethanol. Precipitation from a solution in sodium hydroxide by the addition of hydrochloric acid also gave Form III.

Form IV was prepared by evaporation on a water bath of a solution in acetone until the first crystals separated. The solution was then left to stand at room temperature.

Form V was prepared by crystallization from *n*-butanol, and Form VI was prepared by crystallization from *n*-pentanol. An amorphous form was also prepared by melting any of the crystal forms (≃200°) followed by slow cooling of the melt.

Characterization of Crystal Forms—The IR spectra of Forms I–VI and the amorphous form in mineral oil mulls are shown in Fig. 1, and characteristic bands and absorbance ratios are presented in Table I. X-ray diffraction patterns of Forms I and II (being the most frequently encountered in commercial preparations) are shown in Fig. 2. The IR spectra and X-ray diffraction patterns show distinct differences which can be used for the characterization of the crystal forms.

Interconversion of Crystal Forms—*Crystallization*—Any crystal form can be converted to another by crystallization from the appropriate solvent as described under *Preparation of Crystal Forms.*

Heating—Heating at a temperature above the melting point (≃200°) followed by slow cooling of the melt resulted in a transformation of any of the crystal forms to the amorphous form. In the transformation of Forms V and VI, solvent was given off at 120–140° and Form I was produced. Form I subsequently transformed to the amorphous form at its melting point.

Suspension in Water—Suspension of all forms in water resulted in a transformation to Form II. Form III was detected as an intermediate in all cases except for the transformation of Form I, which changed directly to Form II. The time required for complete transformation varied according to the particle size of the crystals and the temperature of the aqueous suspension. Heating, shaking, and grinding under water accelerated the transformation to Form II.

Crystal growth accompanied the transformation of commercially available micronized succinylsulfathiazole Form I to Form II. This resulted in the formation of long needles which, by interlocking together, formed a cement-like cake. Photomicrographs of some stages in the transformation of Form I to Form II are shown in Fig. 3.

A summary of the course of interconversions is illustrated in Fig. 4.

Determination of Solvent—Solutions of the various crystal forms (4.23×10^{-5} *M*, assuming the molecular weight of succinylsulfathiazole is 355.4) in 0.1 *N* sulfuric acid were prepared. Absorbance values of these solutions were measured at 258 nm. Calculation was made as to the number of molecules of solvent associated with each molecule of succinylsulfathiazole in those cases where the presence of the solvent of crystallization was suspected (Table II); similar treatment was previously reported (4).

Table I—Characteristic Absorbance Bands of Succinylsulfathiazole Crystal Forms by IR Spectroscopy[a]

Form	800–900 cm^{-1}	1100–1200 cm^{-1}	1200–1300 cm^{-1}	1500–1600 cm^{-1}	3200–3500 cm^{-1}	Other Characteristic Bands and Absorbance Ratios (r)
I	2b (825, 852)	doublet (1172, 1185)	b (1270), s	b (1530), doublet (1568, 1595)	b (3300)	A_{1730}/A_{1670} = 1.333
II	b (845), s	b (1150), 2s*	3b (1210, 1235, 1260)	2b (1548, 1595)	2b (3295, 3450)	A_{1700}/A_{1670} = 0.825, no b between 1700 and 1750
III	2b (845, 865)	3b (1130, 1150, 1185)	3b (1225, 1250, 1295)	2b (1540, 1600)	2b (3320, 3480)	No b between 1700 and 1750
IV	b (842), s	3b (1125, 1150, 1198), s	2b (1225, 1260)	2b (1535, 1595), s	b (3300)	—
V	2b (825, 855)	3b (1130, 1142, 1170), s	3b (1225, 1265, 1295)	2b (1530, 1575)	b (3220)	—
VI	2b (855–895), s	b (1150), 2s*	2b (1210, 1260)	2b (1540, 1595)	2b (3350, 3450)	—
Amorphous	b (848)	b (1148), 2s*	b (1252), s	b (1540), doublet (1575, 1595)	b (3310)	—

[a] Figures in parentheses are the specific absorption maxima; b = band, s = shoulder, and * denotes shoulders on either side of the band.

Table II—Solvent Determination in Succinylsulfathiazole Crystal Forms by Measuring Their Molar Absorptivity at 258 nm

Form	Expected Solvent Molecule per Molecule Drug	Absorbance of 4.23×10^{-5} M Solution (Experimental)	Calculated Absorbance for Equimolar Solution of Crystals Containing Solvent	Solvent Found, Molecule Solvent/ Molecule Drug
Amorphous	—	0.797	0.797	
I	—	0.794	0.797	
IV	—	0.800	0.797	
III	1 molecule water	0.750	0.758	1
II	2 molecules water	0.716	0.723	2
V	1–2 molecules butanol	0.597	0.609	3/2
VI	0.5–1 molecule pentanol	0.688	0.684	2/3

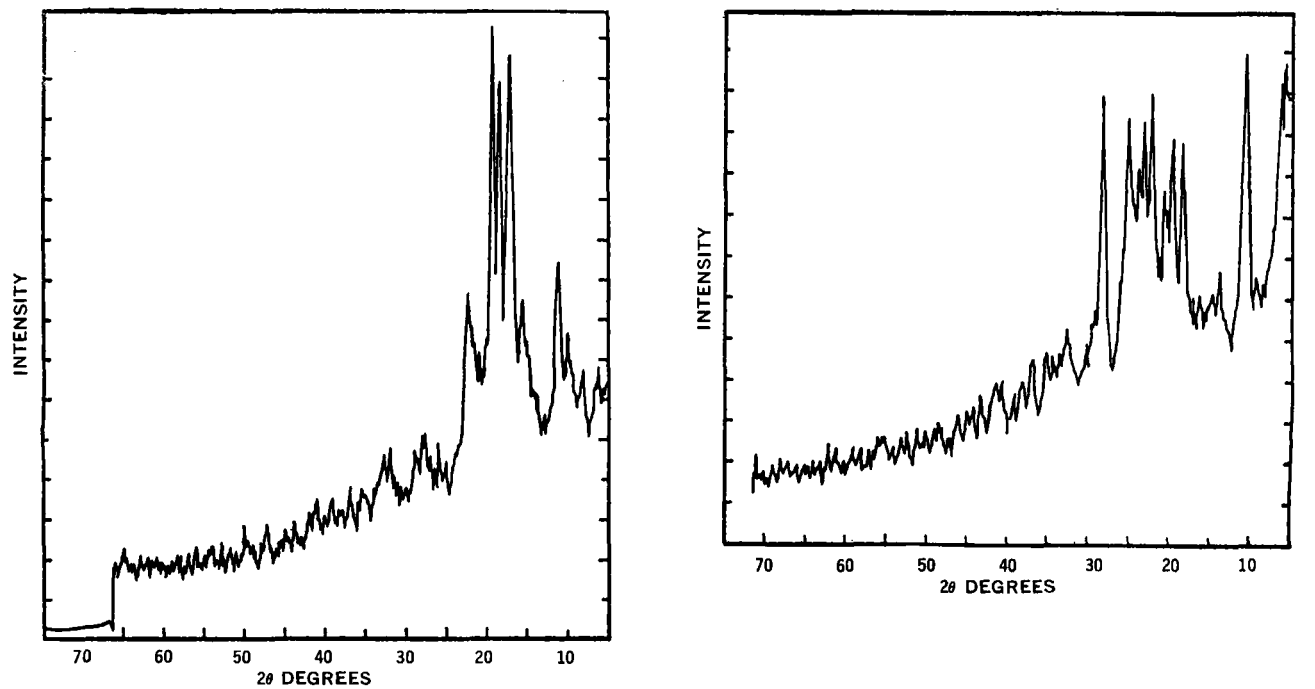

Figure 2—*X-ray diffraction data of succinylsulfathiazole crystal Form I (left) and Form II (right).*

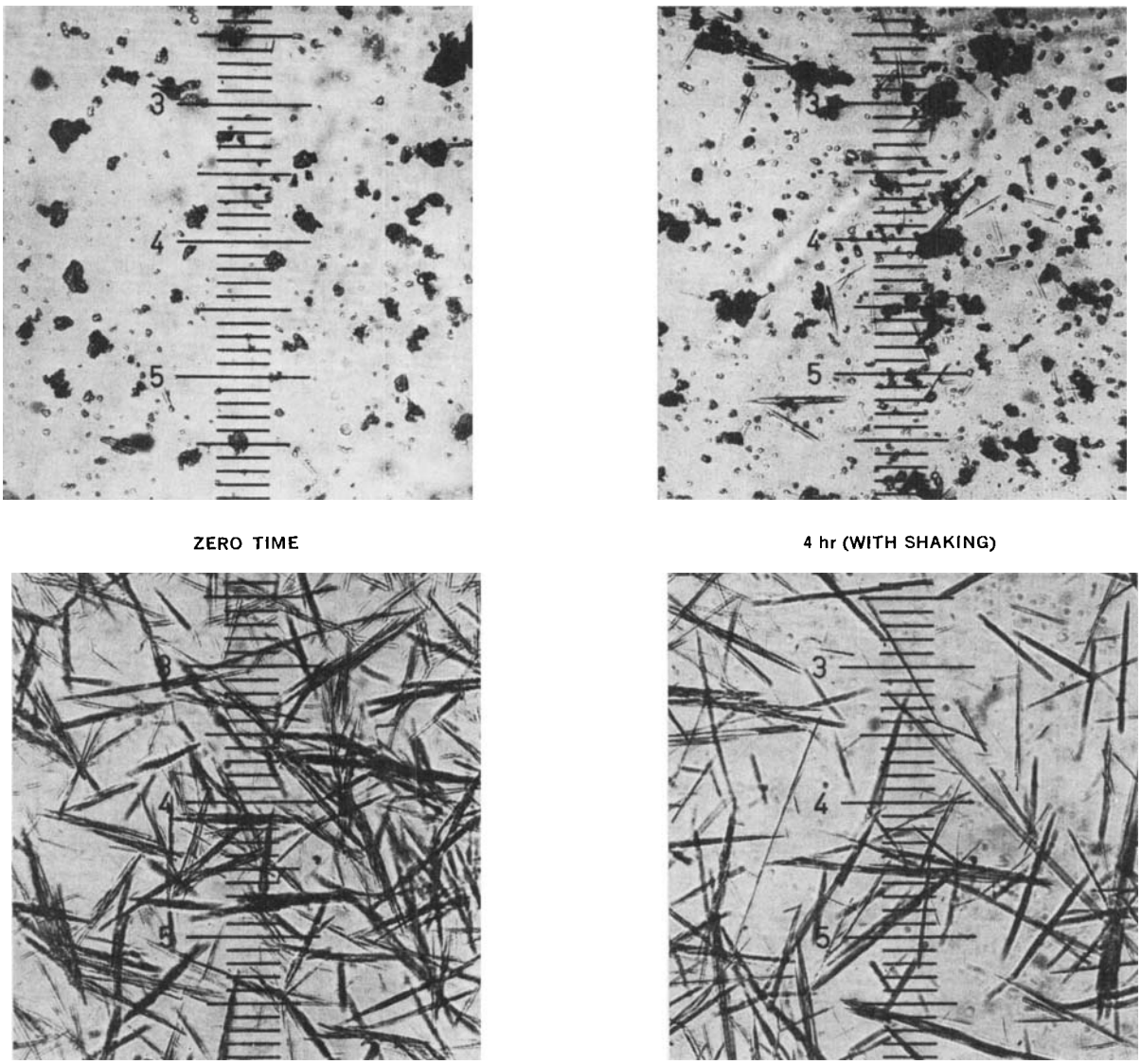

Figure 3—*Photomicrographs representing various stages in the crystal growth accompanying the transformation of succinylsulfathiazole Form I to Form II in aqueous suspension (1 small micrometer division = 100 μm).*

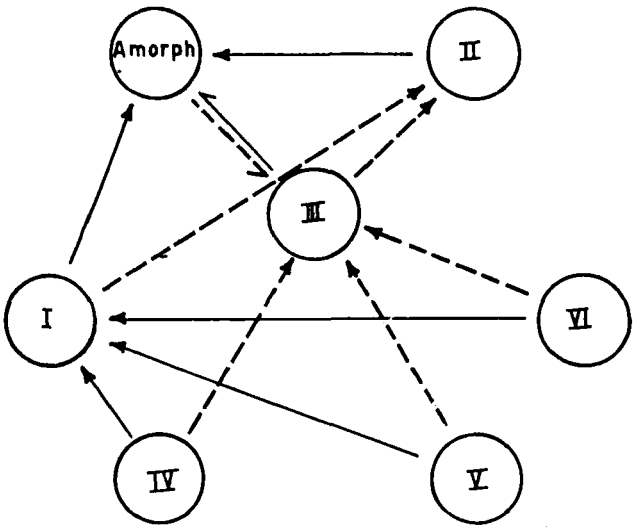

Figure 4—*Interconversion of succinylsulfathiazole crystal forms. Key: ——, heating; and ---, suspension in water.*

Kinetics of Transformation of Form I to Form II—The transformation of succinylsulfathiazole Form I to Form II in aqueous suspensions is of importance because of its effect on the stability of pharmaceutical preparations containing this compound. Therefore, the rate of transformation under normal conditions was studied. For this purpose, the quantitative IR method previously used to study the kinetics of interconversion of various sulfamethoxydiazine crystal forms (10) was employed. This method utilizes an application of the absorbance ratio procedure (11) in which specific bands characteristic of each crystal form are selected from the IR spectrum. The bands 1730 cm^{-1} for Form I and 1700 cm^{-1} for Form II were used, and the band at 1670 cm^{-1} common to both forms of succinylsulfathiazole was selected as an internal marker for absorbance ratio measurements.

The absorbance ratios are calculated as follows.

From the IR spectrum of Form I, $A(1670\ cm^{-1}) = \log I_0/I = \log 66.5/9.5 = 0.845$, and $A(1730\ cm^{-1}) = \log I_0/I = \log 83/6 = 1.141$. The absorbance ratio is $A(1730\ cm^{-1})/A(1670\ cm^{-1}) = 1.141/0.845 = 1.350$. The means of four runs (IR spectrum was recorded for four samples taken from the same mull) (10) = 1.333 (Table I).

Following the same procedure, the following values were calculated: Form I, $A(1730\ cm^{-1})/A(1670\ cm^{-1}) = 1.350$; Form II, $A(1700\ cm^{-1})/A(1670\ cm^{-1}) = 0.846$; the mixture of Form I and

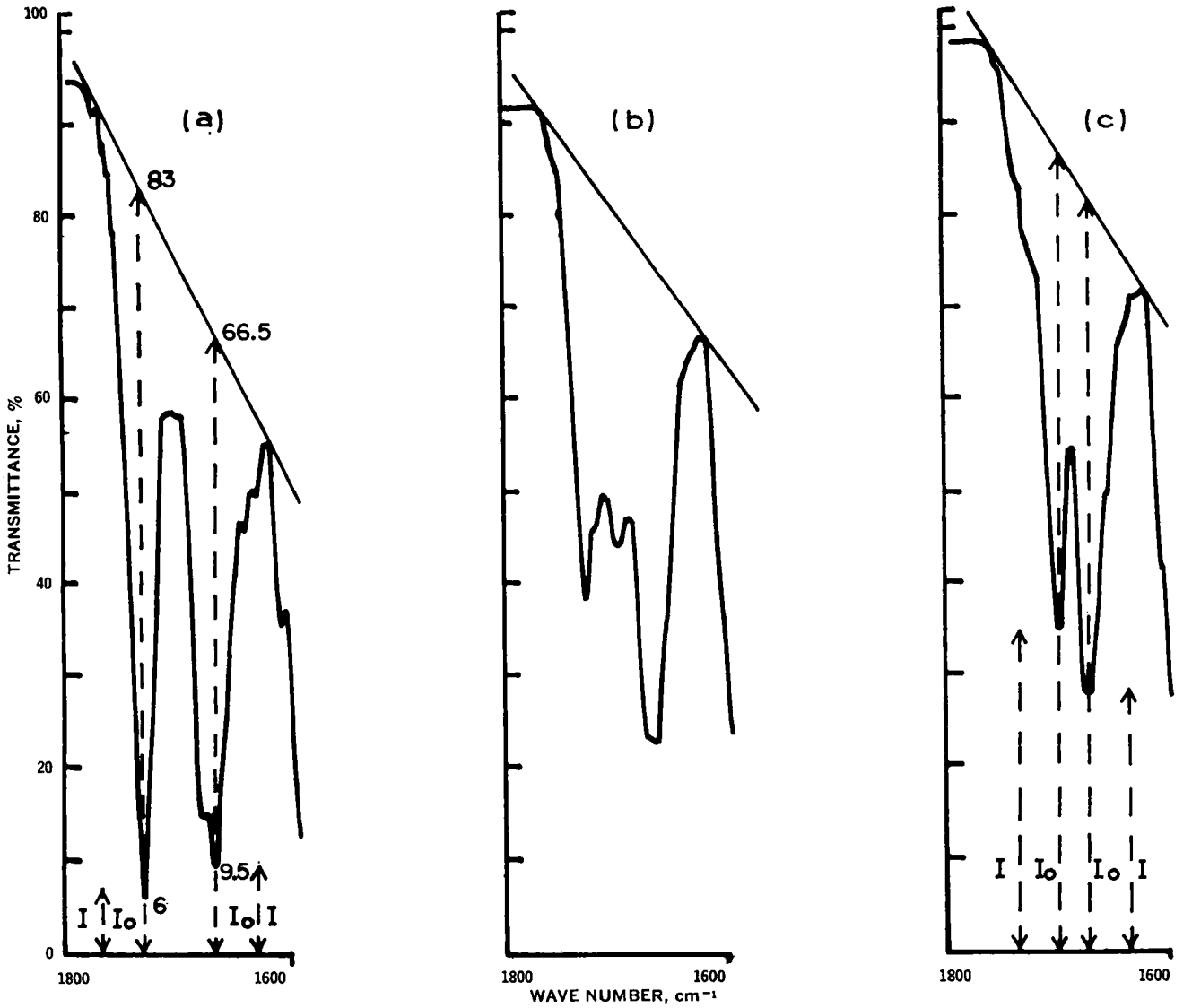

Figure 5—*Measurement of absorbance ratio of succinylsulfathiazole. Key: a, Form I; b, 40:60 mixture of Form I and Form II; and c, Form II.*

Form II (40:60), $A(1730\ cm^{-1})/A(1670\ cm^{-1})$ = 0.680; and the mixture of Form II and Form I (60:40), $A(1700\ cm^{-1})/A(1670\ cm^{-1})$ = 0.505. In these measurements, the baseline minimums for each form and mixture were measured from 1770 to 1630 cm^{-1}, respectively (Fig. 5). Additional absorbance ratios for mixtures of Form I and Form II and of Form II and Form I were recorded and plotted against the concentration of the respective crystal form (Fig. 6).

The rate of transformation of Form I to Form II was studied by preparing 1% (w/v) suspensions of Form I in distilled water and placing them in thermostated water baths at 22, 37, 40, 55, 60, and 70 ± 0.1°. Samples of each suspension were taken at various time intervals and filtered, and the residue was dried by pressing between two filter papers. The concentration of Form II in the residue was determined from the calibration curve (Fig. 6), and the concentration of Form I was calculated by the difference. Results of the transformation of Form I at various temperatures are shown in Fig. 7.

DISCUSSION

Succinylsulfathiazole exists in the solid state in one of six or more crystal forms or in an amorphous state. Forms I and IV are anhydrous, Forms II and III are hydrates, and Forms V and VI are solvates of butanol and pentanol, respectively.

In the present study, the methods of preparation of succinylsulfathiazole crystal forms (4, 7, 8) were often difficult to reproduce. No evidence could be found in favor of the claim that one crystal form was stable below 35° and the other was stable above that temperature (7). The suggestion (7) that Form II (mp 180°) changed through solid phase transformation to Form I (described as a pseudopolymorph of Form II, mp 125°) is against expectations based on thermodynamic considerations, which would favor a transformation in the opposite direction—*viz.*, from a lower melting-point crystal form to a higher melting-point form. Under the conditions of the present study, crystallization from 25% eth-

Table III—Kinetic Parameters of Transformation of Succinylsulfathiazole Form I to Form II in Aqueous Suspension[a]

Rate Constant ($K \times 10^4$), min^{-1}					
22°	37°	40°	55°	60°	70°
6.33	33.6	40.30	203.82	361.80	959.42

[a] K at 25° = 8.5 × 10^{-4} min^{-1}, $t_{1/2}$ at 25° = 13.6 hr (by extrapolation), and Ea = 21.05 kcal/mole.

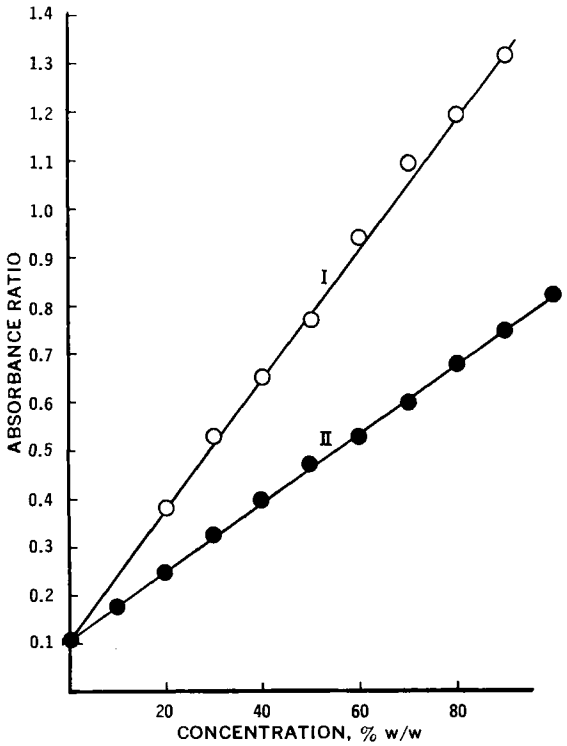

Figure 6—*Calibration curve of succinylsulfathiazole. Key:* ○, *Form I in presence of Form II using the ratio* A(*1730 cm*$^{-1}$)/A(*1670 cm*$^{-1}$); *and* ●, *Form II in presence of Form I using the ratio* A(*1700 cm*$^{-1}$)/A(*1670 cm*$^{-1}$).

anol (7) gave Form III, which did not undergo any transition below its melting point.

Shefter and Higuchi (4) used 25% ethanol to prepare a monohydrate (Form II), which probably matches Form III of the present study. The same form was also recovered from various solvents in addition to an alkali-acid treatment. The water-stable hydrate (Form I) of Shefter and Higuchi (4) matched Form II of the present work. The preparation of a pentanol solvate (4) and its conversion through partial melting at 127–136°, which probably accompanied loss of solvent to a form that melted at 191° (probably Form I of the present study), was in agreement with the present findings.

Mesley and Houghton (8) recommended dissolution of succinylsulfathiazole in sodium hydroxide solution and precipitation with dilute hydrochloric acid to prepare Form B. The present results

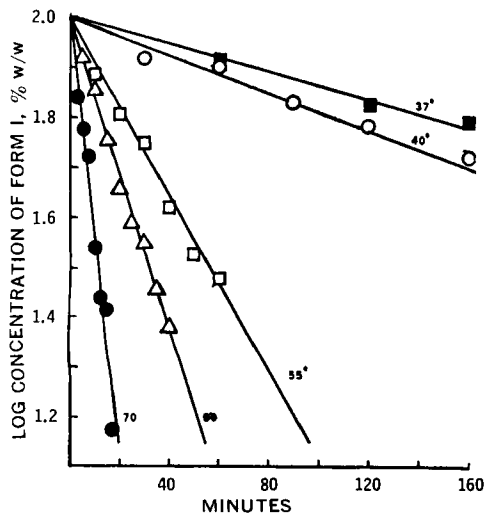

Figure 7—*Transformation of succinylsulfathiazole Form I to Form II in aqueous suspension at various temperatures.*

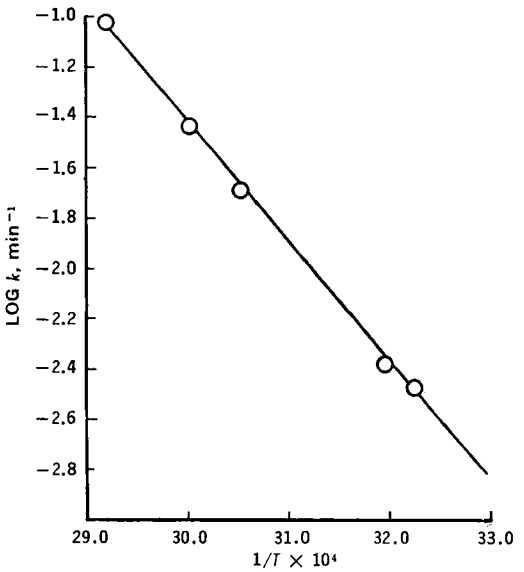

Figure 8—*Arrhenius plot of the transformation of succinylsulfathiazole Form I.*

would agree with this recommendation for identification purposes, the crystal form produced being Form III. Although Mesley and Houghton (8) suggested other treatments such as evaporation at room temperature of solutions of Forms A and D in acetone for the preparation of their Form B, similar treatments in the present study produced Form I. Heating Form A of Mesley and Houghton's (8) study resulted in its transformation to their Form D, a transformation that could not be reproduced in the present investigation. However, some crystal forms, namely Forms IV, V, and VI, changed to Form I by heating at about 150°; Forms II and III transformed through partial melting and resolidification to the amorphous form. In all cases, transformation to the amorphous form occurred when any crystal form was melted (melting point of all forms 191–193°) and allowed to resolidify slowly from the melt. The preparation of the amorphous form by crystallization from alcoholic solvents (8) is also questionable, since most alcoholic solvents used in the present study gave other crystal forms.

Molar absorptivity studies of the various crystal forms in 0.1 *N* sulfuric acid (Table II) suggested that Form II existed as a dihydrate, that Form III existed as a monohydrate, and that butanol and pentanol in a ratio of approximately 1 molecule solvent/molecule succinylsulfathiazole accompanied Forms V and VI, respectively, probably as adducts or clathrate forms. The relative ease with which solvent is given off by heating above its boiling point and below the melting point of the crystalline drug, as shown by the evolution of bubbles from an oil mount on a microscope hot stage, is further evidence for the existence of butanol and pentanol solvates in the clathrate form. Shefter and Higuchi (4) also reported the existence of a pentanol solvate containing 0.9 molecule solvent/molecule succinylsulfathiazole.

IR spectroscopy proved to be a valuable technique for both the identification and quantitative estimation of succinylsulfathiazole crystal forms. Use of the calibration curve of Form II (Fig. 6) gave more accurate results than those obtained when the calibration curve of Form I was used. This is probably due to the fact that both absorption bands used in measuring the absorbance ratio characteristic of Form II merge together from a common background, represented by the tangent to both bands between the points at 1630 and 1770 cm^{-1}. The band at 1730 cm^{-1}, characteristic of Form I, merges separately. Therefore, a greater error is anticipated when the latter band is used for the quantitative determination of Form I in mixtures. Accordingly, Form II was measured in all samples and Form I was found out by difference.

Log concentration–time plots for the decrease in concentration of Form I in aqueous suspensions of succinylsulfathiazole are shown in Fig. 7. An Arrhenius plot of the log of the first-order rate constants determined at various temperatures *versus* the reciprocal of the absolute temperature is shown in Fig. 8. Kinetic data including rate constants, activation energy, and half-life for the

transformation of Form I to Form II in aqueous suspension, as well as extrapolated values at 25°, are shown in Table III. The half-life value obtained by extrapolation (≃14 hr) was in fair agreement with the experimental value (≃16 hr) determined for suspensions stored at room temperature.

According to the results of the present study, it might be concluded that the preparation of physically stable aqueous suspensions of succinylsulfathiazole could best be obtained by using the water-stable Form II. If Form I is available commercially, as is frequently the case, adequate measures have to be taken to inhibit the transformation of the crystal form and its accompanying crystal growth, caking, and difficult resuspendability. A study of the effect of potential transformation retardants will be the subject of future reports.

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Inhibitory Effects of Central Hypertensive Activity of Angiotensin I and II by 1-Sar-8-ala-angiotensin II (Saralasin Acetate)

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Abstract □ The cerebroventricular administration of 0.25–2.00 μg/kg of angiotensin I or II into α-chloralose-anesthetized cats produced significant increases in mean systemic pressure. These central hypertensive effects were inhibited by the intraventricular injection of 0.5 or 1.0 μg/kg of 1-sar-8-ala-angiotensin II (saralasin acetate). The response to angiotensin I was attenuated to a lesser extent than that to angiotensin II with this preparation. However, when both the agonists and antagonists were administered intravenously, there was equal inhibition of the effects of both angiotensin I and II. Saralasin acetate alone had little or no effect on mean blood pressure or heart rate when administered by either route; when injected intravenously, it did not significantly alter the bradycardia induced by vagal stimulation, the pressor responses to bilateral carotid occlusion or intravenously administered epinephrine, and the depressor effect of intravenous acetylcholine. The difference in the levels of antagonism at central and peripheral sites suggests that the receptors for the angiotensins are not identical in these two areas.

Keyphrases □ Angiotensin I and II—inhibition of central hypertensive activity by saralasin acetate, intravenous and intraventricular administration, cats □ Saralasin acetate (1-sar-8-ala-angiotensin II)—inhibitor of central hypertensive activity of angiotensin I and II, intravenous and intraventricular administration, cats □ 1-Sar-8-ala-angiotensin II (saralasin acetate)—inhibitor of central hypertensive activity of angiotensin I and II □ Hypertensive activity, central—inhibition of effects of angiotensin I and II by saralasin acetate

Bickerton and Buckley (1) presented evidence that angiotensin II (an octapeptide) could exert an effect on the central nervous system (CNS). Utilizing the dog cross-circulation technique, they showed that administration of the peptide into the vascularly isolated head of the recipient produced a pressor effect due to peripheral vascular constriction. Since these initial observations, several investigators (2–11), utilizing similar as well as other preparations, have confirmed this effect of angiotensin. This action appears to be mediated *via* an increase in sympathetic outflow from the CNS, since it may be blocked by the intravenous administration of α-adrenergic blockers (1) into the periphery and consists mainly of an increase in peripheral resistance, with cardiac activity only slightly altered (12).

This effect of angiotensin II has been postulated to play an important role in the central control of the cardiovascular system (13), and the possibility exists that the interaction of angiotensin II with central receptor sites contributes to the development of cardiovascular hypertensive disease (14). A strong cor-

transformation of Form I to Form II in aqueous suspension, as well as extrapolated values at 25°, are shown in Table III. The half-life value obtained by extrapolation (≃14 hr) was in fair agreement with the experimental value (≃16 hr) determined for suspensions stored at room temperature.

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Table I—Effects of Saralasin Acetate on Mean Blood Pressure, Heart Rate, and Blood Pressure Responses Induced by Bilateral Carotid Occlusion and Intravenously Administered Epinephrine and Acetylcholine in α-Chloralose-Anesthetized Cats[a]

	Route of Saralasin Administration	Dose of Saralasin, μg/kg/min		
		Control (n = 10)	1.0 (n = 6)	5.0 (n = 4)
Mean blood pressure, mm Hg	Intravenous	92.3 ± 9.9	60.4 ± 3.4[b]	87.5 ± 20.9
	Intraventricular	103.8 ± 13.4	95.6 ± 8.5	85.8 ± 14.3
Heart rate, beats/min	Intravenous	181.9 ± 15.5	178.5 ± 12.1	197.7 ± 16.2
	Intraventricular	174.5 ± 13.6	165.3 ± 11.8	159.4 ± 15.1
		Changes in Mean Blood Pressure, mm Hg		
Epinephrine, 1.0 μg/kg iv	Intravenous	55.6 ± 7.9	56.5 ± 8.3	54.2 ± 13.9
Acetylcholine, 0.5 μg/kg iv	Intravenous	−40.9 ± 4.5	−28.3 ± 9.7	−38.3 ± 7.4
Bilateral carotid occlusion	Intravenous	30.9 ± 5.6	23.2 ± 5.4	36.4 ± 4.8

[a] α-Chloralose, 70 mg/kg iv. [b] $p < 0.05$.

relation has been shown between the blood pressure of patients with essential hypertension and the concentration of a pressor peptide, possibly angiotensin I (a decapeptide), in the cerebral spinal fluid (15).

The centrally mediated pressor effects of angiotensin may be inhibited by blockade of the central sympathetic receptors, as demonstrated by the intraventricular (ivt) administration of phentolamine, an α-adrenergic blocking drug (16). It was suggested (16) that phentolamine could be a useful tool for studying the central effects of angiotensin II. However, this does not enable the investigator to elucidate further the direct interaction between angiotensin and specific receptor sites in the CNS. For this purpose, a compound that competes with the peptide for these sites would be helpful.

Recently, an angiotensin II analog, which exhibits properties characteristic of specific competitive antagonism of the vascular action of angiotensin II, was synthesized (17). The compound, 1-sar-8-ala-angiotensin II (saralasin acetate), was utilized in an attempt to counteract different models of hypertension (17). It had no effect on the blood pressure of normotensive, spontaneously hypertensive, metacorticoid hypertensive, and unilateral renal hypertensive rats. However, in the acute phase of experimental renal hypertension, there was a dose-related reduction in blood pressure with doses of the compound ranging from 0.62 to 10 μg/kg/min iv. The investigators concluded that angiotensin II played a role in the development of this type of hypertension.

A comparison of the two types of Goldblatt hypertension with reference to this inhibitor was made by Brunner *et al.* (18). In the one-kidney Goldblatt preparation (left renal artery clipped and contralateral kidney removed), saralasin acetate did not lower the blood pressure; in the two-kidney Goldblatt preparation (left renal artery clipped and the other kidney left intact), arterial blood pressure was significantly reduced. Therefore, these authors surmised that the renin–angiotensin system plays a causal role in the two-kidney type of hypertension but that a different mechanism was responsible for maintenance of one-kidney renal hypertension.

If the central action of angiotensin contributes to the development of sustained cardiovascular hypertensive disease, it would be of interest to determine whether saralasin acetate competes with angiotensin II for receptors in the CNS. The purpose of the present study was to investigate the effects of saralasin acetate on the centrally induced pressor effects of angiotensin I and II in cats.

EXPERIMENTAL

Drugs and Chemicals—1-Sar-8-ala-angiotensin[1] II (saralasin acetate), angiotensin[2] I (94% pure), and angiotensin[3] II were dissolved in artificial cerebral spinal fluid for intraventricular administration and in saline for intravenous administration.

Perfusion of Cerebroventricular System—Ten adult cats of either sex, weighing 2–3 kg, were anesthetized with α-chloralose (70 mg/kg iv). The right femoral artery was catheterized and connected to a pressure transducer[4], and blood pressure was recorded *via* a polygraph[5]. A tracheotomy was performed and a tracheal catheter was inserted for connection to a respiration pump[6]. The animal was then affixed into a stereotaxic instrument[7], and the calvarium was surgically exposed along the sagittal suture line. Then a small hole was made[8] in the skull above the right lateral ventricle. An unbeveled 22-gauge stainless steel needle, approximately 35 mm in length, was stereotaxically lowered into the ventricle according to the coordinates described by Snyder and Niemer (19). The coordinates used were: frontal, 15 mm; horizontal, 6.75 mm; and lateral, 2.5 mm.

The cannula was affixed to the skull with dental acrylic cement, and the cerebral ventricles were perfused with artificial cerebral spinal fluid (20) after the method of Bhattacharya and Feldberg (21). The cerebral spinal fluid entered the lateral ventricle; passed through the third ventricle, the aqueduct of Sylvius, and the fourth ventricle; and drained through a polyethylene catheter inserted into the cisterna magna. The perfusion rate was kept constant at 0.1 ml/min by means of a pump[9] connected by polyethylene tubing to a three-way stopcock attached to the stainless steel cannula. The artificial cerebral spinal fluid was maintained at 37° using a heated water jacket. Injections of angiotensin I and II were made through the stopcock without interruption of the perfusion. Angiotensin I and II (0.25–2.00 μg/kg) were injected into the ventricular catheter prior to and during intraventricular infusion of saralasin acetate (1.0 or 5.0 μg/kg/min). Saralasin acetate was infused for 15 min prior to administering the peptides and infusion was continued for approximately 90 min.

General Pharmacological Tests—Ten adult cats of either sex, weighing 2.2–3.5 kg, were anesthetized and catheterized in the manner previously described. A second catheter was inserted into the ipsilateral femoral vein for administration of angiotensin I

[1] Norwich Pharmacal Co.
[2] Schwarz-Mann, Orangeburg, N.Y.
[3] Hypertensin, Ciba-Geigy.
[4] Statham P23AC.
[5] Grass model 7.
[6] Model 606 Harvard.
[7] Trent H. Wells, Jr.
[8] With the aid of a dental burr and a model 2 Moto-tool (Dremel).
[9] Harvard Peti pump.

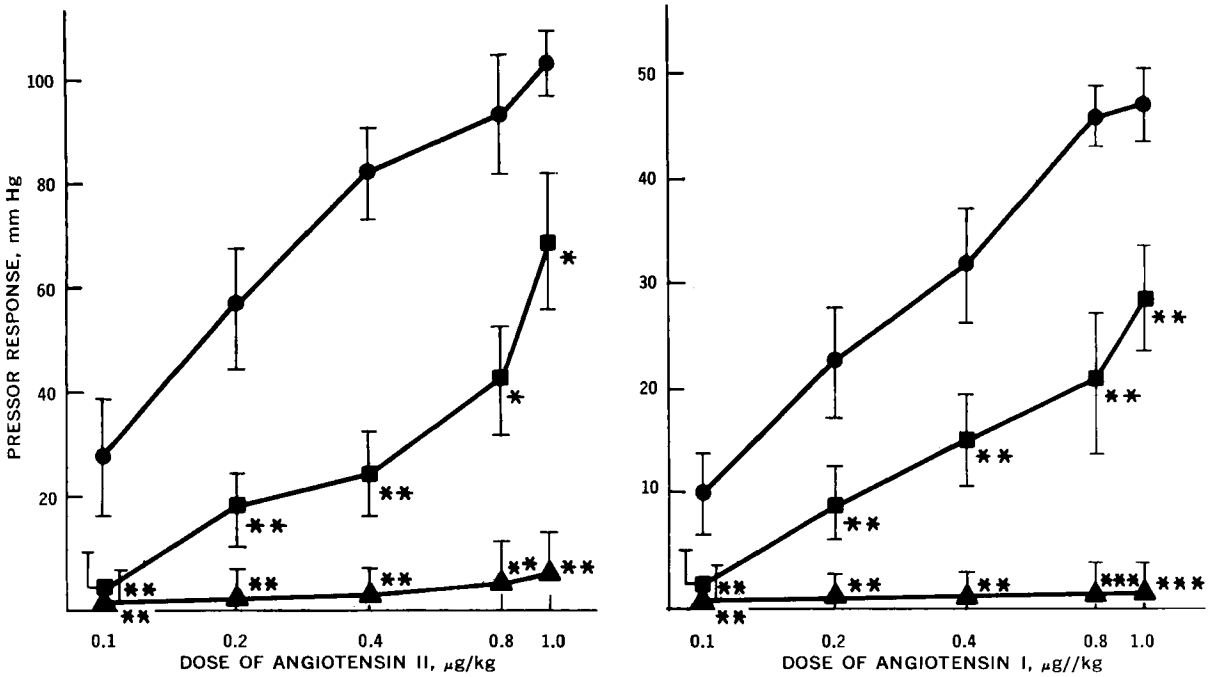

Figure 1—*Effects of intravenous saralasin acetate on the pressor response to intravenous angiotensin I and II in α-chloralose-anesthetized cats* (n = *10*). *Key:* ●, *control;* ■, *during infusion of 1.0 μg/kg/min saralasin acetate;* ▲, *during infusion of 5.0 μg/kg/min saralasin acetate;* *, $p < 0.05$; **, $p < 0.01$; *and* ***, $p < 0.005$.

and II and saralasin acetate. The left and right carotid arteries were isolated so that effects on the bilateral carotid occlusion pressor reflex could be studied. The left vagus was isolated for supramaximal stimulation (v = 3–4.5, duration = 1 msec, frequency = 1.5–12 Hz, 15 sec) by means of bipolar electrodes using a stimulator[10]. The preganglionic sympathetic trunk to the nictitating membrane was also isolated for supramaximal stimulation (v = 3–4.5, duration = 1 msec, frequency = 1.5–12 Hz, 15 sec) in the same manner. The nictitating membrane tension was measured with a force-displacement transducer[11].

Test doses of angiotensin I and II (0.1–1.0 μg/kg), epinephrine (1.0 μg/kg), and acetylcholine (0.5 μg/kg) were administered intravenously prior to and during intravenous infusion of saralasin acetate (1.0 and 5.0 μg/kg/min). Saralasin acetate was infused for 15 min prior to administering other compounds or stimulating the neurons, and perfusion was continued for approximately 110 min. The Student paired *t* test was used to determine statistical significance unless otherwise indicated.

RESULTS

Table I summarizes the effects of saralasin acetate on blood pressure and heart rate and the blood pressure changes induced by epinephrine, acetylcholine, and bilateral carotid occlusion in normotensive cats. Saralasin acetate (1.0 or 5.0 μg/kg/min) did not alter heart rate when administered *via* the intravenous or intraventricular route. Intravenous perfusion of saralasin acetate produced a nondose-related effect on blood pressure; 1.0 μg/kg/min significantly lowered blood pressure ($p < 0.05$) but 5.0 μg/kg/min did not. The intravenous perfusion of saralasin acetate in doses of 1.0 and 5.0 μg/kg/min did not significantly alter the vascular responses to bilateral carotid occlusion, 0.5 μg/kg acetylcholine, or 1.0 μg/kg epinephrine and caused no consistent alterations in the frequency–response curve to vagal stimulation (decrease in heart rate) or stimulation of the sympathetic innervation to the nictitating membrane (contraction of the membrane). There was a nonsignificant decrease in mean blood pressure when saralasin acetate was perfused *via* the lateral ventricle of the cat.

Figure 1 summarizes the inhibitory activity of saralasin acetate on the pressor responses to intravenously injected angiotensin I and II. Saralasin acetate, 1.0 and 5.0 μg/kg/min, significantly attenuated the pressor effects of both peptides at all dose levels. The percent decrease in the pressor activity of angiotensin I and II during intravenous infusion of saralasin acetate (1.0 μg/kg/min) is plotted in Fig. 2 to compare the effects on the peptides. As can be seen, the percent inhibition of the pressor responses of angiotensin I and II was similar. Figure 3 summarizes the inhibitory effects of intraventricularly perfused saralasin acetate on the pressor effects induced by the intraventricular administration of the decapeptide and the octapeptide. The responses of angiotensin I and II were markedly attenuated by both doses of saralasin acetate. Saralasin acetate, 1.0 μg/kg/min, produced an average of 48.5% inhibition of the pressor effects of intraventricularly administered angiotensin I, and 5.0 μg/kg/min produced an average of 83.5% inhibition; the pressor effects of angiotensin II were decreased by 84.9 and 98.1%, respectively. When the data were

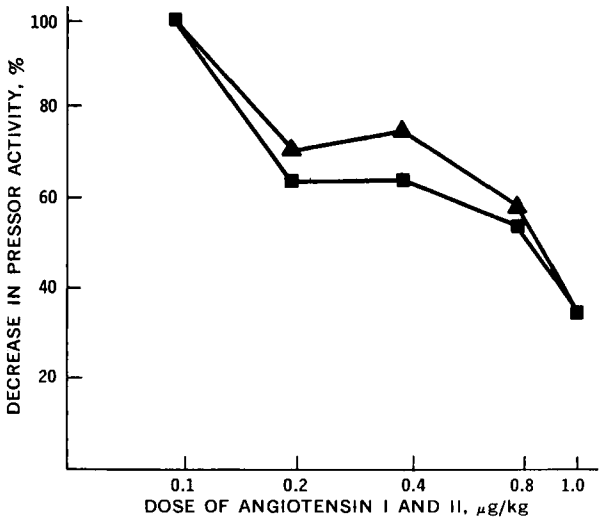

Figure 2—*Percent decrease in pressor activity of intravenous angiotensin I and II, induced by concomitant infusion of saralasin acetate, 1.0 μg/kg/min iv. Key:* ■, *angiotensin I; and* ▲, *angiotensin II.*

[10] Grass model SD5.
[11] FT03 Grass.

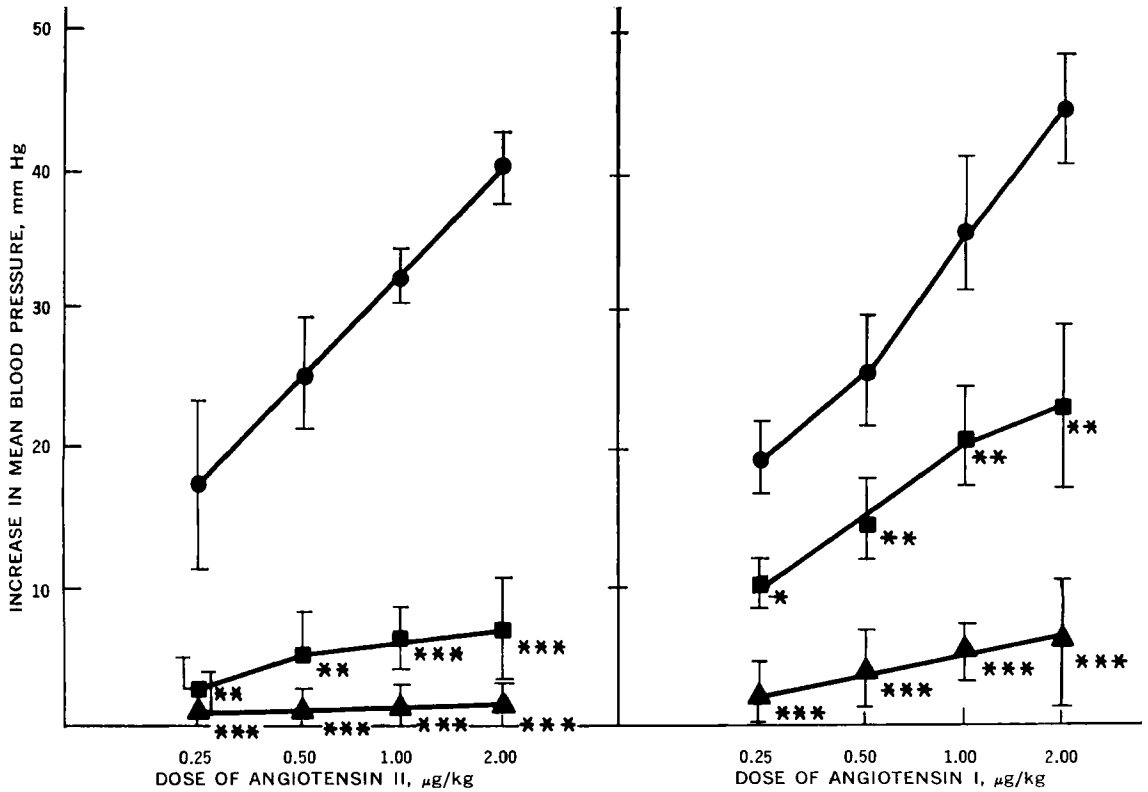

Figure 3—*Effects of intraventricular saralasin acetate on the pressor response to intraventricular angiotensin I and II in α-chloralose-anesthetized cats* (n = *10*). *Key:* ●, *control;* ■, *during infusion of 1.0 μg/kg/min saralasin acetate;* ▲, *during infusion of 5.0 μg/kg/min saralasin acetate;* *, $p < 0.05$; **, $p < 0.01$; *and* ***, $p < 0.001$.

subjected to an analysis of variance, the antagonism of angiotensin II by the low dose of saralasin acetate was significantly greater ($p < 0.01$) than that of angiotensin I. However, there was no significant difference with the higher dose of the inhibitor.

DISCUSSION

Elucidation of the involvement of the renin–angiotensin system in cardiovascular hypertensive disease is of great clinical significance. Recently, Ganten *et al.* (22) demonstrated the presence of renin in brain tissue and Finkielman *et al.* (15) showed a direct correlation between blood pressure in hypertensive patients and the concentration of an angiotensin I-like substance in the cerebral spinal fluid. In line with these reports, Ferrario *et al.* (13) stated: "The findings that angiotensin and renin are present in brain tissue suggest the possibility that angiotensin may play an important, but as yet unidentified, function in the central regulation of cardiovascular control." If the central component of activity of the renin–angiotensin system is also of importance in the development and maintenance of cardiovascular hypertensive disease, then clarification of the events occurring centrally is of even greater importance. Saralasin acetate, a specific angiotensin II antagonist, was utilized in this study to derive information that could be helpful in this clarification.

Evidence indicating that angiotensin does play a role in the development of this pathological condition is accumulating but is by no means conclusive. Whether the central or peripheral effect of these pressor substances is of more importance in this role is argumentative. Nevertheless, if alterations in the activity of angiotensin are indeed responsible for certain types of this cardiovascular disease, then one obvious pharmacological solution to this problem could be to inhibit the renin–angiotensin system at one step in this system's normal pattern of events. Formation of angiotensin II may be affected by inhibition of the synthesis and/or release of renin or by the conversion of angiotensin I to angiotensin II. Also, direct blockade of the receptor sites for angiotensin II (and possibly angiotensin I) could result in abolishing, or at least inhibiting, the pressor effects.

Since the possibility that angiotensin I may directly stimulate receptors was recently suggested (23–26), this compound was utilized along with angiotensin II in this study. The physiological events resulting from the administration of the decapeptide into the CNS were of specific importance. A comparison of how saralasin acetate affected angiotensin I and II centrally and peripherally was made to characterize the activity of angiotensin I.

Saralasin acetate failed to produce significant effects on mean blood pressure or heart rate when administered intraventricularly to normotensive cats. When infused intravenously, it had no effect on the blood pressure responses to bilateral carotid occlusion or intravenously administered epinephrine and acetylcholine. There was, however, an irregular pattern of effects on mean blood pressure upon peripheral administration. The depressor effects produced by 1.0 μg/kg/min were not in accord with the findings of other investigators (17, 18) nor with the effects seen with the higher dose (5.0 μg/kg/min) of saralasin acetate infused by this route. Saralasin acetate is reported to be a competitive antagonist of angiotensin II in the periphery (17, 18) of rats, and results obtained in cats in this study are similar to these previous findings. This can be seen by the decrease in the inhibitory effects of saralasin acetate as the dose of angiotensin I or II was increased (Fig. 2). There was no difference in activity between the peptides in this respect. Increasing doses of angiotensin I or II also override the effect of saralasin acetate in the cat lateral ventricle preparation, suggesting the possibility of competitive inhibition. However, angiotensin II is inhibited to a greater degree than angiotensin I by this route. The fact that angiotensin I retains some of its activity while that of angiotensin II is virtually abolished supports the hypothesis that angiotensin I possesses activity that is not reliant on its conversion to angiotensin II. Also, the difference in the levels of antagonism produced by central and peripheral routes of administration indicates that perhaps the receptors for the polypeptides are not identical in these two areas.

Although the activity of angiotensin I injected intravenously was significantly lower than that of angiotensin II, no significant difference between the pressor activity of the two peptides was observed upon administration into the perfused ventricular sys-

tem of cats (27). Dog cross-circulation experiments verified these results and also demonstrated that both peptides have the ability to cross the blood–brain barrier[12]. Certain observations on the central effects of angiotensin I suggest that the peptide may have activity exclusive of that mediated through conversion to angiotensin II. However, each of these points is debatable. There is a relatively small amount of converting enzyme in the brain (28), but it may be adequate for full conversion to occur. Also, angiotensin I produced a greater response than angiotensin II in some preparations, but this means little since there is a great variation in the central response to the peptides. There was no difference in the time for onset of the responses to angiotensin I and II, while a delay might be expected if conversion of angiotensin I to angiotensin II is necessary for activity. On the other hand, the rate of conversion may be such that the time required would be undetectable in this preparation.

These results with saralasin acetate are significant in that they give additional evidence to the possibility that angiotensin I is an active compound *per se*. Also, when comparing the activity of saralasin acetate centrally and peripherally, it appears that the receptive areas for angiotensin in the CNS are dissimilar in certain respects from those seen in areas exclusive of the brain.

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* Present address: Laboratory of Cardiodynamics, School of Medicine, Johns Hopkins University, Baltimore, MD 21205

x To whom inquiries should be directed. Present address: Department of Pharmacology, College of Pharmacy, University of Houston, Houston, TX 77004

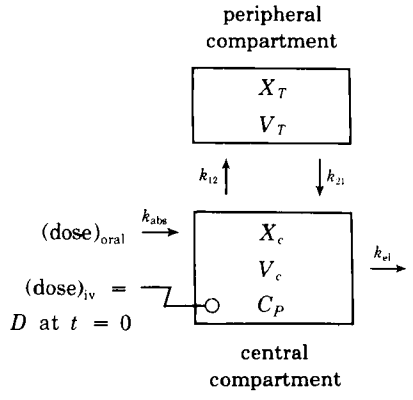
Pharmacokinetics and Distribution Properties of Pentobarbital in Humans following Oral and Intravenous Administration

M. EHRNEBO

Abstract □ The pharmacokinetics of intravenously and orally administered pentobarbital (100 mg) were studied in humans by GC analysis of plasma, and the data were simulated with the aid of digital computation. The drug had an apparent disposition half-life of 22.3 ± 4.0 *(SD)* hr when administered intravenously to seven healthy subjects and after oral dosage the half-life was about the same. An examination of the individual pharmacokinetic constants showed that the volume of the peripheral compartment in the two-compartment open model appeared to increase with increased body weight in the subjects. Furthermore, this influenced the tissue to central compartment distribution of the drug. Formulas are presented which allow calculation of fractions of amount in the central compartment distributed to plasma water, plasma proteins, blood cells, and associated fluid. The application of these equations showed that of the total amount in the central compartment, about 87% was in the associated fluid; of the 13% in the systemic circulation, 4% was in plasma water, 5% was bound to plasma proteins, and the other 4% was distributed to blood cells. Analysis of the distribution volumes referenced to plasma water concentration gave volumes that considerably exceeded the total body water, so it is concluded that pentobarbital exhibits extensive tissue binding.

Keyphrases □ Pentobarbital—pharmacokinetics after intravenous and oral administration, volume of distribution □ Absorption kinetics—pentobarbital □ Bioavailability—pentobarbital, application of intravenous clearance □ Pharmacokinetics—pentobarbital after intravenous and oral administration, volume of distribution □ Drug distribution—pentobarbital, influence of body weight

Pentobarbital [5-ethyl-5-(1-methylbutyl)barbituric acid] has widespread use as a hypnotic and as a sedative and is commonly used in clinical practice. Until 1973, little information was available on the pharmacokinetics of the drug in humans following therapeutic dosage. A half-life of 35.1 ± 6.1 hr for ^{14}C-pentobarbital was reported (1) after oral administration of 6 mg/kg to four healthy subjects. The plasma levels of the drug after the intravenous injection of 750–1000 mg to six volunteers were described, but no rate constants were evaluated (2). Based on these data, Riegelman *et al.* (3) determined the half-life of the drug to be 41 hr. In the present study, 100 mg pentobarbital was given by intravenous and oral routes to seven young healthy subjects and the plasma levels were followed using a sensitive GC technique (4). Recently, Smith *et al.* (5) examined the pharmacokinetics of pentobarbital following intravenous and oral administration of 50 mg. The results of these investigators deviate to some extent from the data reported here, and the reasons behind the discrepancy will be discussed.



Scheme I

EXPERIMENTAL

Intravenous Study—Seven healthy male and female volunteers received a bolus dose of 2.2 ml of pentobarbital[1] solution equivalent to 100 mg of the acid. None of the volunteers had taken drugs regularly during the month prior to the study.

Venous blood was sampled through an indwelling catheter during the first 3 hr with the subjects lying down and thereafter by venous puncture at appropriate times. The sampling times were 6, 12, 18, 24, 30, and 45 min and 1.5, 2.0, 3.0, 6.0, 11.0, 23.0, and 47.0 hr. Each blood sample (3 ml) was centrifuged within 3 hr and the plasma was frozen (−20°).

The experiments were carried out by a surgical department with equipment for resuscitation available. No adverse effects were seen, and only three subjects fell into a light sleep for 20–30 min. The blood pressure was measured repeatedly during the first 3 hr; only a slight lowering (10–20 mm Hg) in the systolic pressure was recorded.

Oral Study—In a second experiment, the same volunteers who had participated in the intravenous test also received an oral dose of pentobarbital[2]. The subject ingested one 100-mg pentobarbital tablet on an empty stomach with approximately 100 ml water; no food was taken for at least 3 hr. Blood samples (3 ml) were drawn by venous puncture at 40 and 80 min and 2.0, 3.0, 6.0, 12.0, 24.0, and 48.0 hr after drug administration.

GC Assay of Pentobarbital—The plasma concentration of pentobarbital was determined by the method of Ehrnebo *et al.* (4) with a minor modification as follows: 500 μl of plasma was buffered to pH 5.5 with 50 μl 4 *M* NaH_2PO_4, a standard of 40 μg/ml sodium secobarbital in methanol (25 μl) was introduced, and 1.0 ml of ether was added. After mechanical agitation, the ether was separated by centrifugation, and the whole volume was transferred to another tube and shaken with an aqueous solution (25 μl) of trimethylanilinium hydroxide. To decrease the alkaline decomposition of the barbiturates in the injector, the basic extract was withdrawn and neutralized to pH 7 with 4 *M* hydrochloric acid (~1 μl). An aliquot (1.5–3.5 μl) was then injected into the chromatograph. Duplicate extractions and determinations were made on each plasma sample. This method allows the accurate determination of pentobarbital to as low as 0.25 μg/ml (4).

Calculations—The obtained plasma data indicate that the time course of the drug in the body might be properly described by assuming that the body acts as a two-compartment open model (Scheme I). Thus, in the intravenous study, the plasma concentrations (C_P) declined biexponentially with time according

[1] Nembutal, Abbott S.A., Belgium.
[2] Mebumal, Lot No. S2101, ACO Läkemedel AB, Solna, Sweden.

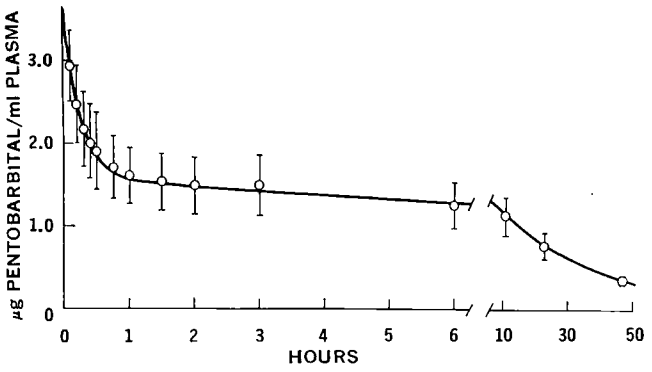


Figure 1—*Mean* (±SD) *plasma levels of pentobarbital following rapid intravenous injection of 100 mg to seven subjects. The line is drawn by an analog computer programmed with the mean constants from Table II and the mean plasma concentration at zero time,* $(C_P)_0$.

to Eq. 1:

$$C_P = Ae^{-\alpha t} + Be^{-\beta t} \quad \text{(Eq. 1)}$$

where A, B, α, and β were used to calculate the rate constants k_{12}, k_{21}, and k_{el} (Scheme I) as shown previously (6). The absorption rate constant (k_{abs}, Scheme I) and apparent absorption lag time (t_0) were evaluated using the equation for first-order absorption (7), where t_0 was included in the exponents. The various rate constants of this model and the lag time of absorption were evaluated with the aid of a digital computer[3] by a method for nonlinear least-squares fit. The plasma concentrations were given equal weights of unity.

The volumes of the central compartment (V_C) and the tissue compartment (V_T) are obtained with the expressions (8):

$$V_C = D/(C_P)_0 \quad \text{(Eq. 2)}$$

$$V_T = V_C k_{12}/k_{21} \quad \text{(Eq. 3)}$$

where D is the dose of pentobarbital (free acid) administered by rapid intravenous injection, $(C_P)_0$ is the concentration in plasma at zero time, and $V_C + V_T$ is the total distribution volume at steady state. In addition to these two volume entities, a third volume, $(Vd)_\beta$, was calculated (9):

$$(Vd)_\beta = D/\beta \int_0^\infty C_P\, dt \quad \text{(Eq. 4)}$$

where $\int_0^\infty C_P\, dt$ is the area under the plasma concentration–time curve following intravenous administration.

The ratio between the amount in the peripheral compartment (X_T) to the amount in the central compartment (X_C) during the β-phase is obtained as shown previously (10) with the expression:

$$(X_T/X_C)_\beta = k_{12}/(k_{21} - \beta) \quad \text{(Eq. 5)}$$

The bioavailability of the oral preparation ($F\%$) was determined by comparing the areas under the time–concentration curves:

$$F\% = 100(AUC_{oral}/AUC_{iv}) \quad \text{(Eq. 6)}$$

where the areas after oral and intravenous administration, AUC_{oral} and AUC_{iv}, respectively, were estimated by the trapezoidal rule including the residual area up to infinite time.

Fraction of Amount in Central Compartment Distributed to Plasma Water, Plasma Proteins, Blood Cells, and Associated Fluid—Recently, Garrett and Lambert (11) made a detailed pharmacokinetic analysis of trichloroethanol and metabolites in the dog. They presented complex formulas for the different distribution volumes of the central compartment in the three-compartment body model with reference to plasma water concentrations. The following is a simplified and condensed approach to the distribution properties of a drug in the two-compartment body model using common pharmacokinetic expressions.

[3] IBM 360; revised BMDX85 program.

Table I—Data Obtained following a Single Intravenous Injection of Pentobarbital (100 mg) to Humans; $C_P = Ae^{-\alpha t} + Be^{-\beta t}$

Subject	A, μg/ml	α, hr^{-1}	B, μg/ml	β, hr^{-1}	Area[a], (μg × hr)/ml
H.K.	2.16 (0.15)[b]	5.20 (0.50)	1.22 (0.03)	0.0293 (0.0030)	41.9
N.K.	2.07 (0.19)	4.44 (0.59)	1.39 (0.04)	0.0283 (0.0035)	49.4
L.L.	2.57 (0.16)	4.73 (0.42)	1.75 (0.03)	0.0250 (0.0020)	70.5
M.E.	1.76 (0.11)	3.60 (0.36)	1.79 (0.03)	0.0340 (0.0022)	53.0
G.P.	1.56 (0.20)	2.65 (0.61)	1.88 (0.08)	0.0309 (0.0042)	61.3
K.K.	1.95 (0.10)	4.64 (0.35)	1.16 (0.02)	0.0309 (0.0023)	37.9
U.H.	2.07 (0.14)	3.72 (0.41)	2.18 (0.04)	0.0468 (0.0031)	47.3

[a] Calculated as $A/\alpha + B/\beta$. [b] The standard deviations of the parameter (estimated by the BMDX85 program) are shown in parentheses beneath the value of the parameters.

The assumptions made are: *(a)* the drug instantaneously equilibrates between the different components in the central compartment; and *(b)* the binding capacity of both blood cells and plasma proteins is independent of concentration, *i.e.*, the dose is sufficiently low to give a linear relationship between bound and free drug with reference to plasma and whole blood. The slight difference between the corrected venous hematocrit value (blood cell volume per volume venous whole blood) and the total hematocrit value (total blood cell volume per total blood volume) is not taken into account.

As seen in the *Appendix*, the following expressions for the fraction of amount in the central compartment distributed to plasma water (F_{PW}), to plasma proteins (F_{PP}), to blood cells (F_{BC}), and to associated fluid (F_{AF}) are readily obtainable:

$$F_{PW} = \lambda_{PW}(1 - H)V_{Btrue}/(\lambda_{PW} + \lambda_{PP})V_C \quad \text{(Eq. 7)}$$

$$F_{PP} = \lambda_{PP}(1 - H)V_{Btrue}/(\lambda_{PW} + \lambda_{PP})V_C \quad \text{(Eq. 8)}$$

$$F_{BC} = \lambda_{BC}(1 - H)V_{Btrue}/(\lambda_{PW} + \lambda_{PP})V_C \quad \text{(Eq. 9)}$$

$$F_{AF} = 1 - (1 - H)V_{Btrue}/(\lambda_{PW} + \lambda_{PP})V_C \quad \text{(Eq. 10)}$$

where λ_{PW}, λ_{PP}, and λ_{BC} are the fractions of amount in whole blood distributed to plasma water, plasma proteins, and blood cells, respectively; H is the hematocrit as fraction volume blood cells per whole blood volume; and V_C is the apparent distribution volume of the central compartment (Eq. 2).

Distribution Volume of Central and Peripheral Compartment with Reference to Plasma Water Concentrations—Garrett and Lambert (11) defined the distribution volume, $V_P u$, in a formula equal to the following expression:

$$V_P u = (a_{AF} + a_{PW})/C_{PW} = [(a_{AF})_0 + (a_{PW})_0]/(C_{PW})_0 \quad \text{(Eq. 11)}$$

where $V_P u$ is the volume of distribution of associated fluid and plasma water with reference to the concentration of drug in plasma water (C_{PW}), a_{AF} is the amount of drug in associated fluid, and a_{PW} is the amount in plasma water. The 0-indexed parentheses indicate the situation at zero time in the two-compartment open model.

It is evident that:

$$a_{AF} + a_{PW} = X_C(F_{AF} + F_{PW}) = V_C C_P(F_{AF} + F_{PW}) \quad \text{(Eq. 12)}$$

which makes it possible to express $V_P u$ as:

Table II—Rates Constants and $t_{1/2}$ ($0.693/\beta$) to Fit the Plasma Pentobarbital Levels following a Single Intravenous Injection (100 mg)

Subject	Sex	Body Weight, kg	$t_{1/2}$ hr	k_{12}, hr^{-1}	k_{21}, hr^{-1}	k_{el}, hr^{-1}
H.K.	Male	82	23.6	3.26	1.89	0.0807
N.K.	Male	71	24.5	2.60	1.80	0.0700
L.L.	Female	57	27.7	2.76	1.93	0.0613
M.E.	Male	60	20.4	1.74	1.84	0.0668
G.P.	Female	50.5	22.4	1.16	1.46	0.0560
K.K.	Male	79.5	22.4	2.84	1.75	0.0820
U.H.	Female	47	14.8	1.75	1.93	0.0901
Average			22.3	2.30	1.80	0.0724
SD of averages			4.0	0.76	0.16	0.0123
Coefficient of variation, %			18	33	8.9	17

$$V_{Pu} = V_C(F_{AF} + F_{PW})/(1 - f_P) \quad \text{(Eq. 13)}$$

where f_P is the fraction of drug bound in plasma.

The distribution volume of the peripheral compartment during the β-phase referenced to plasma water concentration, $(V_Tu)_\beta$, can be defined as:

$$(V_Tu)_\beta = (X_T)_\beta/(C_{PW})_\beta \quad \text{(Eq. 14)}$$

where $(X_T)_\beta$ is the amount in the peripheral compartment of the plasma water concentration, $(C_{PW})_\beta$, during the β-phase. Since $C_{PW} = C_P(1 - f_P)$ and $(X_T)_\beta = C_P(Vd)_\beta - C_PV_C$, Eq. 14 can also be written as:

$$(V_Tu)_\beta = [(Vd)_\beta - V_C]/(1 - f_P) \quad \text{(Eq. 15)}$$

where $(Vd)_\beta$ and V_C are defined in Eqs. 4 and 2, respectively.

By relating V_Pu and $(V_Tu)_\beta$ to the volume of the body water, information is obtained concerning to what degree a drug is bound to body tissues.

RESULTS AND DISCUSSION

Intravenous Study—Figure 1 shows the mean plasma concentrations following the intravenous injection of 100 mg pentobarbital, with the standard deviation at each level indicated with bars. There was a rapid fall in the plasma levels during the 1st hr, from about 3 μg/ml at 6 min to about 1.6 μg/ml at 1 hr (mean values). The line in Fig. 1 was calculated by an analog computer using the mean values of k_{12}, k_{21}, and k_{el} in Table II. Hence, as seen in the figure, there was good agreement between predicted and observed plasma concentrations.

The plasma concentrations following the intravenous administration were found to fit very well to the biexponential function (Eq. 1) in all seven subjects; the computer solution of the parameters A, α, B, and β (Table I) gave low standard deviations of the parameters (values in parentheses). The individual and average rate constants of elimination and distribution are shown in Table II. The results indicate that both k_{12} and k_{21} are greater than k_{el} and that the ratio k_{12}/k_{21} ranged from 0.79 to 1.72 in the subjects. As indicated by the coefficient of variation, the least individual variation of the parameters in Table II was found in k_{21}, showing a coefficient of variation of only 8.9%.

The distribution volumes referenced to plasma concentrations following the 100-mg dose of pentobarbital are given in Table III. The average volume of distribution per kilogram body weight (0.990 liter/kg) showed a coefficient of variation of only 4.9%. The volume of the central compartment varied very little between the individuals (average 27.8 liters), and no significant increase in V_C with body weight was observed (Fig. 2). On the other hand, the volume of the peripheral compartment (V_T) at steady state (Eq. 3) showed an increase in volume with an increase in body weight (Fig. 2). The regression line for V_T $(= y)$ *versus* body weight $(= x)$ was $y = 0.922x - 23.1$ ($r = 0.972$). Consequently, an increase in body weight primarily seems to influence the peripheral volume of distribution, probably due to additional adipose tissue and muscles, whereas the central volume appears to be on the whole unaffected by the body weight. These findings are consonant with the proposal (6) that the main constituents of the peripheral compartment are adipose tissue, muscles, and skin tissue.

The body weight also influences the $(X_T/X_C)_\beta$ distribution ratio (Eq. 5) of pentobarbital (Fig. 3). Since $k_{21} > \beta$, this depends mainly on the ratio k_{12}/k_{21}, which increases with the body weight. This relationship is in accordance with the comparatively consistent volume of the central compartment and the increase in peripheral volume with body weight which, according to Eq. 3, should influence the ratio k_{12}/k_{21} in this way. The regression line for $(X_T/X_C)_\beta$ $(= y)$ *versus* body weight $(= x)$ was $y = 0.0243x - 0.261$ ($r = 0.885$).

As seen in Table IV, most pentobarbital in the central compartment was distributed to the associated fluid and only about 13% was present in the blood compartment where about 4% was in the plasma water, 5% was bound to plasma proteins, and 4% was distributed to blood cells. Furthermore, as seen in Table V, the value of V_Pu (distribution volume of plasma water and associated fluid referenced to plasma water concentration) far exceeded the total body water. The value of the distribution volume of peripheral compartment referenced to plasma water concentrations, $(V_Tu)_\beta$, also far exceeded total body water. Therefore, pentobar-

Table III—Distribution Volumes of Pentobarbital following Intravenous Injection of 100 mg with Reference to Plasma Concentrations

Subject	V_C, liters	V_T, liters	$V_C + V_T$, liters	$(V_C + V_T)$/body weight, liters/kg	$(Vd)\beta$, liters
H.K.	29.6	50.9	80.5	0.982	81.3
N.K.	28.9	41.9	70.8	0.997	71.5
L.L.	23.1	33.1	56.3	0.987	56.7
M.E.	28.2	26.7	54.9	0.915	55.4
G.P.	29.1	23.2	52.3	1.036	52.8
K.K.	32.1	53.3	84.4	1.061	85.4
U.H.	23.5	21.2	44.7	0.952	45.2
Average	27.8	35.6	63.4	0.990	64.0
SD of averages	3.3	12.9	15.2	0.049	15.4
Coefficient of variation, %	12	36	24	4.9	24

Table IV—Fraction of Amount in Central Compartment Distributed to Plasma Water (F_{PW}), Plasma Proteins (F_{PP}), Blood Cells (F_{BC}), and Associated Fluid (F_{AF}) following 100 mg Pentobarbital Intravenously

Subject	V_{Btrue}[a], liters	Hematocrit[b]	C_B/C_P[b]	λ_{PW}[b]	λ_{PP}[b]	λ_{BC}[b]	F_{PW} ×100	F_{PP} ×100	F_{BC} ×100	F_{AF} ×100
M.E.	3.94	0.47	0.714	0.257	0.407	0.336	2.9	4.5	3.7	88.9
H.K.	5.38	0.44	0.854	0.262	0.394	0.344	4.1	6.1	5.3	84.5
K.K.	5.22	0.42	0.833	0.279	0.417	0.304	3.8	5.7	4.1	86.4

[a] Calculated as 65.6 ml/kg body weight. [b] Unpublished data of M. Ehrnebo and I. Odar-Cederlöf.

bital shows extensive tissue binding in both the central and peripheral compartments.

Oral Study—Table VI shows that the tablet with 100 mg of pentobarbital was rapidly and almost completely absorbed from the GI tract. The half-life of the absorption process was 13 min (k_{abs} = 3.1 hr^{-1}). The lag time (t_0) until absorption began was about 30 min from the time of tablet intake (Table VI).

The average apparent biological half-life after oral administration was 25.1 ± 5.6 *(SD)* hr (n = 7) and apparently about the same as after intravenous administration (22.3 ± 4.0 hr).

The maximum availability of the 100-mg tablet was determined by the following equation (12):

$$\theta = 1 - \frac{f_m(\text{dose})_{iv}}{\dot{V}_{BL}\lambda\left(\int_0^\infty C_{\text{plasma}}\,dt\right)_{iv}} \qquad \text{(Eq. 16)}$$

where θ is the fraction of the administered dose appearing in the rest of the body, f_m is the fraction of dose metabolized in the liver, $\dot{V}_{BL}$ is the liver blood flow, λ is the ratio of the concentration of drug in blood to the concentration of the drug in plasma (C_B/C_P), and $(\int_0^\infty C_{\text{plasma}}\,dt)_{iv}$ is the area under the plasma concentration–time curve from time zero to infinity following intravenous administration of the $(\text{dose})_{iv}$. For pentobarbital, f_m approaches 1 as shown by Brodie *et al.* (2). Using 91,800 ml/hr for liver blood flow (12), values for C_B/C_P (Table IV), and areas (Table I) following intravenous administration, it can be shown that the maximum bioavailability of orally administered pentobarbital is about 97%. The average bioavailability in Table VI calculated from the ratios between areas under plasma concentration curves is in agreement with this value.

Comparison between Reported Pharmacokinetic Constants after Different Dosage Levels of Pentobarbital—Following the intravenous administration of 1000 mg of pentobarbital (2), a β-phase half-life of 41 hr (n = 6) was observed (3). With a 6-mg/kg body weight dose of oral ^{14}C-pentobarbital, half-lives of 35 hr (n = 4) were reported (1). In a recent publication (5), a mean β-phase half-life of about 50 hr was reported when 50 mg of pentobarbital was administered intravenously. A more precise value of this half-life can be obtained using the formula for the relationship between β and k_{12}, k_{21}, and k_{el} (8). This gives the mean value 60.3 hr (n = 5) for the half-life of pentobarbital in the latter study. In the present paper, the average β-phase half-life following 100 mg pentobarbital intravenously was 22.3 hr (n = 7). Accordingly, the half-life following 50 mg pentobarbital in the report of Smith *et al.* (5) gave almost three times the half-life found in the present investigation. Both Smith *et al.* (5) and the present investigator, in contrast to the other authors, used GC techniques selective for the parent drug.

The results of Smith *et al.* (5) showed deviations from the data reported here. In summary, they obtained the following values for the pharmacokinetic constants of distribution and elimination: k_{12} = 1.162 hr^{-1}, k_{21} = 0.515 hr^{-1}, k_{el} = 0.038 hr^{-1}, and V_C = 41 liters. A comparison with the data in Table II shows that the rate constants in the present report attained much higher values and that the volume of the central compartment referenced to plasma concentrations reported here is a smaller value. A minor difference in the experimental technique, disregarding the dose size, is that Smith *et al.* (5) administered the 50-mg dose by 5 min zero-order infusion compared with 30–45 sec in the present study. However, the deviations cannot solely be due to the differences in infusion time *per se* (13) and must be due to model-dependent pharmacokinetics of a more complex nature (14). This may include, among other things, the increased or decreased binding to tissues at different dose sizes, dose-induced changes in hepatic lipid–water ratios (11), or some physiological action (lowered blood pressure or decreased peripheral blood flow) that may be caused by the central effect of pentobarbital. Accordingly, a more detailed knowledge of pentobarbital pharmacokinetics will require further examinations.

The data of Smith *et al.* (5) for the oral administration of 50 mg sodium pentobarbital are, according to these investigators, as follows (mean values in five subjects): availability = 98.5%, k_{abs} = 2.00 hr^{-1}, and lag time = 0.30 hr. A comparison with the values in Table VI (100 mg pentobarbital as acid) shows that the availability and k_{abs} are about the same, whereas the lag time for the 50-mg sodium pentobarbital in capsule form appears to be shorter than that of 100 mg acid in tablet.

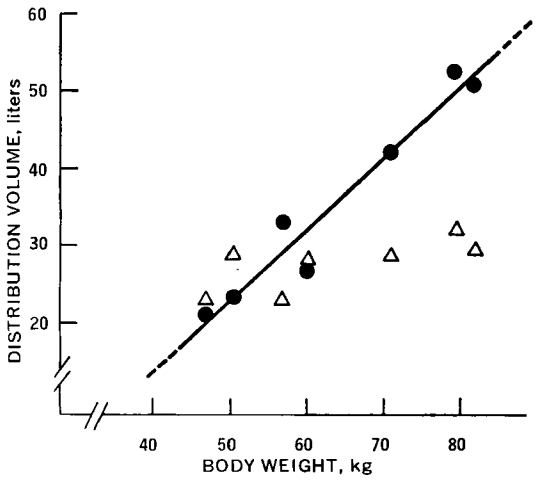


Figure 2—*Distribution volumes in liters referenced to plasma concentrations* versus *body weight in seven healthy volunteers following the administration of 100 mg pentobarbital by rapid intravenous injection. Key:* △, *central compartment; and* ●, *peripheral compartment (at steady state). The regression line* (y = 0.922x − 23.1; r = 0.972) *for peripheral volume* (= y) versus *body weight* (= x) *is drawn.*

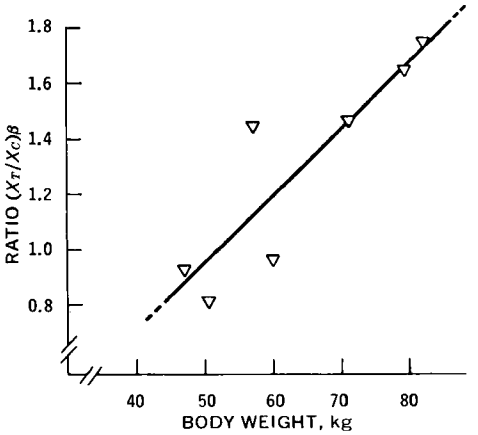


Figure 3—*Ratio of amount in peripheral compartment/amount in central compartment during the β-phase* versus *body weight in seven healthy volunteers following administration of 100 mg pentobarbital by rapid intravenous injection. The regression line* (y = 0.0243x − 0.261; r = 0.885) *is drawn.*

Table V—Distribution Volumes of Pentobarbital (100 mg) with Reference to Plasma Water Concentrations

Subject	V_C, liters	f_P[a]	V_{Pu}, liters	$(V_T u)\beta$, liters	Total Body Water, liters[b]
M.E.	28.2	0.613	66.8	70.3	36
H.K.	29.6	0.601	65.7	129.7	49
K.K.	32.1	0.600	72.4	133.3	48

[a] Fraction drug bound in plasma, unpublished data of M. Ehrnebo and I. Odar-Cederlöf. [b] Calculated as 60% of body weight (14).

CONCLUSIONS

It is concluded that pentobarbital pharmacokinetics following 100 mg administered intravenously is adequately described by the two-compartment open model. The β-phase half-life was 22 hr, and the half-life was about the same after oral dosage of 100 mg. The body weight appears to influence the peripheral to central compartment distribution ratio of the drug so that the drug is further distributed to the peripheral compartment with increased body weight. In the central compartment the drug was mainly located in the associated fluid, and only 13% of the total amount in the central compartment is present in the systemic circulation where 4% was in plasma water, 5% was bound to plasma proteins, and 4% was distributed to blood cells. Consequently, since only about 5% of the amount in the central compartment is bound to plasma proteins, the interaction of other drugs for plasma protein binding sites will have little influence on pentobarbital distribution in the body. As shown by the distribution volumes referenced to plasma water concentration, it is concluded that pentobarbital exhibits extensive tissue binding in both the central and peripheral compartments. The biological availability of the drug following oral administration was high (94%), and this value is in agreement with the calculated bioavailability of completely absorbed oral pentobarbital by application of intravenous clearance concepts.

APPENDIX

By defining λ_{PW}, λ_{PP}, and λ_{BC} as the fraction of amount in whole blood distributed to plasma water, plasma proteins, and blood cells, respectively, it is evident that $a_{PW} = \lambda_{PW}a_B$, $a_{PP} = \lambda_{PP}a_B$, and $a_{BC} = \lambda_{BC}a_B$, where a_{PW}, a_{PP}, and a_{BC} are the amounts of drug distributed to plasma water (and in the plasma), plasma proteins, and blood cells, respectively, and a_B is the amount of drug in blood.

The amount in plasma (a_P) is:

$$a_P = C_P V_{P\text{true}} = C_P(1 - H)V_{B\text{true}} \qquad \text{(Eq. A1)}$$

where $V_{P\text{true}}$ and $V_{B\text{true}}$ are the true plasma and whole blood volumes in the body, respectively. Accordingly, $a_B = C_B V_{B\text{true}}$.

It is evident that the following relationship exists between plasma and whole blood concentrations:

$$C_P = C_B(\lambda_{PW} + \lambda_{PP})/(1 - H) \qquad \text{(Eq. A2)}$$

which makes it possible to calculate the amount in blood as:

$$a_B = C_P V_{B\text{true}} = (1 - H)/(\lambda_{PW} + \lambda_{PP}) \qquad \text{(Eq. A3)}$$

Since $a_{PW} = \lambda_{PW}a_B$ and $X_C = V_C C_P$, the following expression for the fraction of amount in the central compartment distributed to plasma water (Eq. 7) is readily obtainable:

$$F_{PW} = a_{PW}/X_C = \lambda_{PW}V_{B\text{true}}(1 - H)/(\lambda_{PW} + \lambda_{PP})V_C \qquad \text{(Eq. A4)}$$

In the same way, Eqs. 8 and 9 are derived. Furthermore, $F_{AF} = 1 - (F_{PW} + F_{PP} + F_{BC})$ and $\lambda_{PW} + \lambda_{PP} + \lambda_{BC} = 1$, which allows the calculation of Eq. 10. The fraction of total drug amount in the body distributed to plasma water, *etc.*, during the β-phase can be obtained if V_C is replaced by $(Vd)_\beta$.

Table VI—Biopharmaceutical Data Obtained following an Oral Dose of Pentobarbital (100 mg in Tablet) to Humans

Subject	Availabilities, %	k_{abs}, hr^{-1}	Lag Time, hr
H.K.	95.9	4.32	0.64
		(12.01)[a]	(0.07)
N.K.	101.9	—[b]	—
L.L.	74.0	—	—
M.E.	88.7	2.72	0.56
		(0.78)	(0.04)
G.P.	93.5	5.71	0.74
		(8.36)	(0.32)
K.K.	94.0	1.82	0.64
		(0.50)	(0.04)
U.H.	107.5	0.79	0.61
		(0.08)	(0.04)
Average	93.6	3.07	0.64
SD of averages	10.6	1.96	0.07
Coefficient of variation, %	11	64	11

[a] The standard deviations of the parameter (estimated by the BMDX85 program) are shown in parentheses beneath the values of the parameters. [b] Not evaluated.

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Estimation of Pharmacological, Biophasic, and Biological Half-Lives of Quinidine in Rabbits

V. E. ISAACS* and R. D. SCHOENWALD[x]

Abstract □ Pharmacological response intensities were measured from a lengthening of QT intervals and corresponding RR intervals following slow and rapid intravenous infusion of quinidine gluconate to rabbits for the purpose of determining the kinetics of pharmacological response. Measurements of the lengthening of the QT interval were corrected for heart rate, not according to Bazett's formula as has been done in the past but using a bisegmented linear relationship which more accurately relates the two variables. The temporal variation in response intensities was additionally used to quantify biophasic drug levels. This was accomplished by the use of a dose-effect curve, which served as a calibration curve to provide graphically the relationship between response and the amount of drug directly responsible for eliciting the response. Plasma concentrations of quinidine were also determined following intravenous dosing. Semilogarithmic time plots of response intensities, derived biophasic drug levels, and plasma concentrations were best fitted with similar biexponential relationships from which half-lives of 24.1–33.6 min were calculated. The results indicated that once the drug reaches the biophase, binding as well as debinding is instantaneous, and that the rate-determining step to decline in response is removal of drug from the biophase. It was further reasoned that the biophase is located within tissues kinetically indistinguishable from plasma. Distribution to a pharmacologically inert depot compartment was also apparent from the results.

Keyphrases □ Quinidine—estimation of pharmacological, biophasic, and biological half-lives, rabbits, pharmacological response intensities, ECG monitoring □ Half-lives, pharmacological, biophasic, and biological—estimation, quinidine, rabbits □ Pharmacological response intensities—monitored by ECG, estimation of pharmacological, biophasic, and biological half-lives of quinidine, rabbits □ Pharmacokinetics—estimation of pharmacological, biophasic, and biological half-lives of quinidine, rabbits, pharmacological response intensities, ECG monitoring □ Biological half-life—quinidine, rabbits □ Biophase distribution—quinidine, rabbits

The usefulness of quinidine in maintaining normal sinus rhythm following conversion from refractory cardiac arrhythmias is often limited by the difficulty of determining an adequate dosage and/or a suitable dosing interval. To provide some means of estimating dosages and dosing regimens without risking the unexpected development of toxicity, clinicians have sought a relationship between plasma drug levels and the therapeutic effect. Qualitatively, the therapeutic effect as measured by changes in QRS duration or heart rate-corrected QT intervals has been shown to correlate with plasma quinidine concentrations (1). However, a more quantitative correlation has not been possible because of the large degree of scatter generally displayed by ECG measurements. This is due in part to the difficulty in making precise measurements (1) and in part to a poorly defined baseline or reference response from which subsequent measurements influenced by drug must be compared.

This report shows that once a stable baseline is established for pharmacological response, it is possible to quantify the response intensities associated with quinidine for the purpose of estimating the kinetics of pharmacological response, calculating biophasic drug levels, and comparing these processes to plasma levels of drug as a function of time in rabbits following intravenous dosing.

METHODS

A commercially obtained quinidine gluconate solution[1] was injected slowly over 1 min in the marginal ear vein of each of two rabbits; this procedure will be referred to as rapid intravenous dosing. The doses in terms of quinidine base were 0.31, 0.62, 1.24, 2.5, 3.75, 5.0, and 6.23 mg/kg of body weight. Quinidine prolongs the QT interval of the ECG at the time the antiarrhythmic effect of the drug is observed (2); it follows then that QT is the most relevant parameter to quantitate. In addition to QT intervals, the cycle length RR was measured to correct for changes in QT dependent on RR but independent of drug concentrations. This was accomplished through the use of Eq. 1:

$$QT = (bRR + a)Z + (1 - Z)QT_B \qquad \text{(Eq. 1)}$$

When determined for individual subjects before dosing, the QT interval is related to RR according to the bisegmented linear relationship expressed as Eq. 1 and shown in Fig. 1, where a and b represent the intercept and slope of the diagonal linear segment, respectively, and QT_B represents the baseline value of QT independent of RR. The parameter Z is referred to as a "dummy variable" and is defined as:

$$Z = \begin{cases} 1, & \text{if } RR \leqslant E \\ 0, & \text{if } RR > E \end{cases} \qquad \text{(Eq. 2)}$$

Point E represents the intersection or join point of the two linear segments; QT and RR were measured directly from the stored sweep displayed by an oscilloscope[2] equipped with a dual-time base[3] consisting of two sweep modes and a high gain differential amplifier[4].

To measure the ECG intervals, a precordial-type lead was placed over the anterior aspect of the rabbit's chest with the indifferent lead positioned over the caudal region of the lower back. The rabbits were placed in a suitable restrainer to minimize postural effects on the measurements. Disposable silver electrodes[5] coated with a small quantity of electrolyte paste were affixed to the exposed skin. Although various formulas (3–6) have been used to correct QT for the cycle length, including the commonly used square-root relationship (7), it was found that Eq. 1 more closely described the relationship between QT and RR for individual data in a number of species including humans[6]. The procedure consisted of first recording QT–RR data at relatively slow heart rates by permitting the animal to remain inactive in the restrainer. Measurements corresponding to faster heart rates were obtained by removing the animal from the restrainer and with electrodes left in place inducing exercise in a treadmill fashion. Measurements were taken as soon as the rabbit was returned to the

[1] Eli Lilly and Co.
[2] Model 5103N, Tektronix, Inc., Beaverton, Ore.
[3] Model 5B12N, Tektronix, Inc., Beaverton, Ore.
[4] Model 5A22N, Tektronix, Inc., Beaverton, Ore.
[5] Electronics for Medicine, Inc., White Plains, N.Y.
[6] R. D. Schoenwald and V. E. Isaacs, to be published.

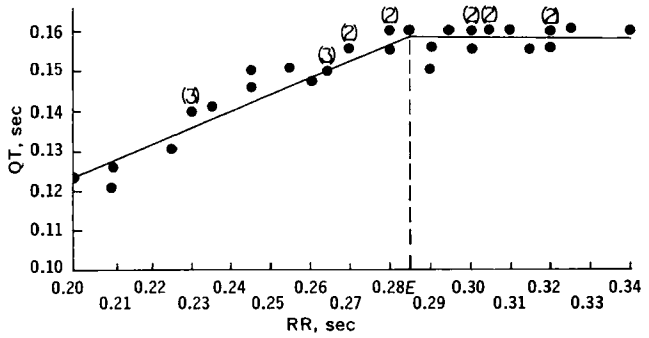

Figure 1—*Bisegmented linear relationship between the experimentally obtained QT and RR for a single rabbit. The numbers in parentheses represent the number of replications recorded at that point. The line denotes the computer least-squares line of best fit, which is represented by* $QT = (0.44 + 0.0355)Z + (1 - Z)0.158$. *The join point is symbolized by* E.

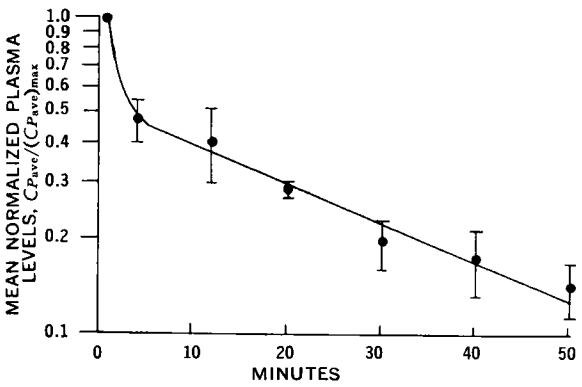

Figure 2—*Time course of mean normalized plasma levels of quinidine. The computer least-squares line of best fit is* $C_{Pave}/(C_{Pave})_{max} = 3.57e^{-0.535t} + 2.01e^{-0.0288t}$. *Each point is an average of one determination made on each of two rabbits; the vertical bars represent 1* SD.

restrainer. A few repetitions of this exercise produced a full range of QT–RR measurements so that predosing parameter values could be reliably obtained. Curve-fitting programs were written in FORTRAN IV for the digital computer[7] to calculate the parameter values of Eq. 1.

To ascertain the join point E, calculations were made by computer in which values of E were chosen in a successive manner, beginning with the third lowest RR value and proceeding to the maximum RR value. A sum of squares solution was written for Eq. 1 as outlined by Hudson (8) for fitting segmented curves whose join points are estimated from the data. By this approach, a least sum of squares is calculated for every possible join point. The line of best fit is determined by the lowest sum of squares computed, and the parameter values calculated for a, b, and QT_B were taken to describe the data best. Each set of data points was given equal weight; however, when duplicate points were recorded, they were included in the calculations, thereby indirectly weighting that particular determination by the number of replications recorded for that point. The primary advantage in using Eq. 1 to correct QT for heart rate over other formulas is that QT_B represents a reference point independent of RR, which permits greater precision to be obtained in the individual case because of the closer fitting relationship. The QT–RR relationship was well defined just before dosing and was assumed to apply for the duration of the experiment[8]. A new QT–RR relationship was determined just before the administration of each dose.

Following dosing, the response measurements, QT and RR, were recorded in triplicate at 1, 2, 3, 6, 9, 11, 13, and 15 min and every 5 min thereafter until measurements returned to the baseline value, QT_B. For most determinations, gain settings were chosen that permitted one or two RR intervals to be enlarged in sweep A whereas the QT interval corresponding to the first cycle in sweep A was enlarged in sweep B. The position control was adjusted so previous sweeps could be stored; with this procedure, two or three sets of sweeps could be displayed in a matter of seconds. This method of recording the ECG allowed for the simultaneous display of two or three tracings, each representing essentially the same point in time; in addition, it minimized the chances of mistaking a T wave for a partially merged T plus U complex and permitted measurements of the QT interval to within 0.005 sec. When an irregular baseline or artifact occurred or if U wave interference was suspected, the measurement was discarded.

Additional measurements of response were performed on two rabbits (4.30 and 4.25 kg) during the time course of a slow intravenous infusion of a 2.5-mg/ml solution of quinidine prepared from the commercial quinidine gluconate solution and distilled water. The solution was infused using a syringe pump[9] at a zero-order rate of 0.0247 ml/min (0.0145 mg/kg/min).

Nonweighted fits to all experimental data used in this report were obtained[10]. Beginning with an initial set of parameter values, the program obtains a least-squares fit to experimental data by means of stepwise Gauss–Newton iterations (9). Exponential relationships were fit to a summation of exponential terms. To obtain initial estimates, data were plotted as a function of time on semilogarithmic paper. By using back-extrapolation procedures (10), the curves were resolved into the appropriate number of exponential components to yield values for the equation parameters A_i's and m_i's. For the determination of plasma levels of quinidine, each of the two rabbits received a 3.75-mg/kg dose in separate experiments. Following dosing to the marginal vein of the left ear, venous blood samples were collected from the marginal vein of the right ear at 1, 5, 12, 20, 30, 40, and 50 min. Response was monitored only to ascertain the point in time when drug effects had dissipated. Quinidine concentrations were determined by the method of Cramer and Isaksson (11), in which quinidine is first extracted into benzene and finally transferred to sulfuric acid before fluorometric[11] determinations are made.

THEORETICAL

A method was developed by Smolen and Schoenwald (12) and applied to the mydriatic response intensity of tropicamide to quantitate the amount of pharmacologically active drug in the biophase following ophthalmic administration (13, 14). The results indicate that the drug was passively absorbed across the cornea as well as surrounding peripheral tissues, *i.e.*, nasolacrimal duct and scleral tissue, following dosing to rabbits. The bioavailability of tropicamide from the ophthalmic, oral, and intramuscular routes of administration was calculated entirely from pharmacological data and determined to be a function of pH and vehicle adjuvants. This method offers an *in vivo* approach to quantifying drug levels from pharmacological response intensities by the use of a dose–effect curve, which serves as a calibration curve to provide graphically the relationship between pharmacological response and the amount of drug directly responsible for eliciting the response. This method obviates the necessity for deriving the-

[7] CDC digital computer, Computer Science Center, University of Washington, Seattle, Wash.

[8] An attempt was made to determine QT changes solely as a function of RR during steady-state biophasic drug levels (slow intravenous infusion of quinidine), but interpretation was difficult because of fluctuating response values, presumably due to changing biophasic drug levels induced by exercise. This was attempted on two occasions, but a great deal of scatter resulted. The E value as well as QT_B was estimated to be the same as that determined before dosing, but values of a and b could not be reliably calculated. The effect of exercise on k_{ij} values is not well known; therefore, changes in parameter values could reflect changing biophasic drug levels and not necessarily a changing QT–RR relationship. Therefore, it was assumed that whatever stimuli were responsible for QT changing in response to RR, *e.g.*, simultaneous or separate positive and negative chronotropic effects of quinidine, the QT–RR relationship and the parameters a, b, QT_B, and E remained constant during the experiment.

[9] Harvard Apparatus Co., Millis, Mass.

[10] By use of the BMDX 85 FORTRAN IV computer program written for the CDC 6400.

[11] Flurospec, SF-100, Baird-Atomic Inc., Bedford, Mass.

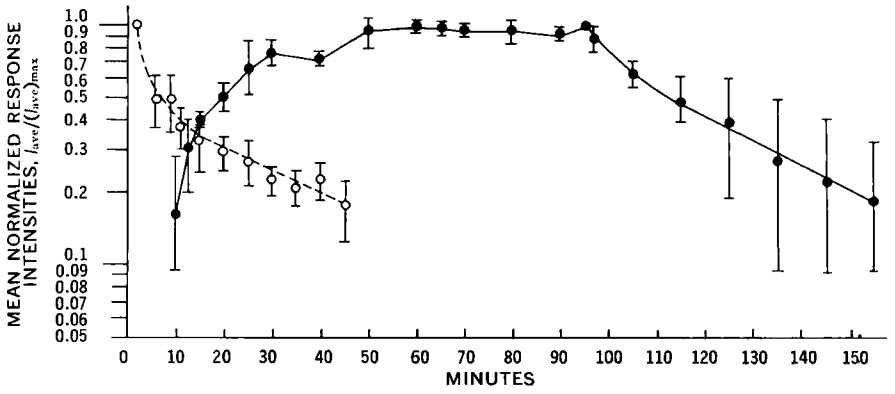

Figure 3—*Time course variation in response intensities following rapid (○) and slowly infused (●) intravenous doses of quinidine. Each point represented by open circles is an average of eight determinations for two rabbits given four doses of 2.5, 3.75, 5.0, and 6.25 mg/kg. The dashed line is a computer least-squares line of best fit represented by* $I_{ave}/(I_{ave})_{max} = 1.32e^{-0.461t} + 0.495e^{-0.0241t}$. *The solid line is a computer least-squares line of best fit for a slowly infused dose of quinidine of 0.0145 mg/kg/min to each of two rabbits for the postinfusion data beginning at* $t = 95$ *min and represented by* $I_{ave}/(I_{ave})_{max} = 0.224e^{-0.247(t-95)} + 0.777e^{-0.0240(t-95)}$. *The vertical bars represent 1* SD.

oretical relationships between pharmacological response and body drug concentration and concerns itself with the compartment of most interest, the biophase.

Expressions were derived previously (12, 15) that describe the relationship between response intensities and the quantity of drug in the biophase, Q_B (Eq. 3), as well as the time course variation of proportional biophasic drug levels, $f(I)$ (Eq. 4):

$$Q_B = f(I)\beta_{t_{max}} \qquad \text{(Eq. 3)}$$

$$f(I) = \frac{f(I)_{max}}{\beta_{t_{max}}} \sum_{i=1}^{n} A_i e^{-m_i t} \qquad \text{(Eq. 4)}$$

The values of $f(I)$ are obtained from the dose–effect curve by finding the experimentally observed response intensity value (I) on the ordinate and directly reading the corresponding abscissa value in milligrams per kilogram. In this way, I values as a function of time can be converted into the time course of proportional biophasic drug levels. The parameter $f(I)_{max}$ is a constant equal to the dose when the drug is given intravenously. The A_i's and m_i's are equation parameters evaluated from the data; n denotes the number of compartments included in the model. The constant $\beta(t_{max})$ equals the summation, $A_i e(-m_i t_{max})$; its value is unity for any model in which the biophase is kinetically indistinguishable from the central systemic compartment. Equation 4 assumes that the kinetics of drug disposition are first order and that the response is instantaneous once drug enters the biophase.

RESULTS AND DISCUSSION

Time Course of Plasma Levels of Quinidine following Rapid Intravenous Dosing—Figure 2 depicts the time course of logarithmic quinidine concentrations in plasma as a function of a 3.75-mg/kg rapid intravenous dose to two rabbits. Because the data points could be best represented by a well-fit biexponential relationship, the kinetics of quinidine distribution and elimination may be characterized by a two-compartment linear model with elimination most likely occurring from the central compartment. The latter log linear segment of the computer least-squares line of best fit yielded a biological half-life of 24.1 min.

Although the double-extraction fluorometric assay of Cramer and Isaksson (11) was reported by Resnekov *et al.* (16) to be more specific in terms of measuring unchanged quinidine than other often used assays (17–19), it is not presently known to what degree metabolic derivatives, probably possessing different pharmacokinetic properties from the parent compound, contribute to the assumed serum concentrations of quinidine. Consequently, with the specificity of the assay in question, a two-compartment linear model can only be assumed. In addition, the model is only assumed to be correct for the length of time the data were collected.

Time Course of Pharmacological Response Intensities—To evaluate the response characteristics of quinidine in individual rabbits as a function of time following intravenous dosing, it was necessary to correct measured QT intervals, in which the corresponding RR values were less than or equal to the join point E. This was achieved by the following relationship:

$$QT_t = \frac{QT_B}{(b\,RR + a)} QT_t' \qquad \text{(Eq. 5)}$$

where QT_t represents the ECG QT calculated as a function of time; and a, RR, and b are as previously defined. The symbol QT_t' represents the uncorrected ECG QT measured as a function of time following dosing. The ratio (≥ 1) of $QT_B/(b\ RR + a)$, when multiplied by QT_t', compares all QT_t measurements from a common reference point, QT_B, independent of RR; therefore, QT_t in effect takes on theoretically corrected values that would have been generated if QT_B and any RR greater than E had been the actual baseline values.

When Eq. 5 was applied to ECG measurements made before dosing or when the effect of quinidine on QT was no longer apparent at large values of t, QT_t was within the 95% confidence limits of QT_B. During the time course of effect, QT_t was significantly greater than QT_B; therefore, the fractional increase in response in

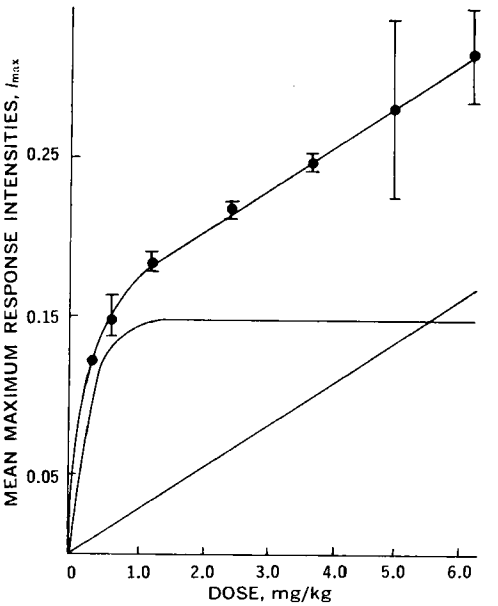

Figure 4—*Relationship between response intensities of quinidine calculated from QT lengthening at maximum intensities and the corresponding rapid intravenous dose of quinidine. The experimental points are averages of two determinations and computer fitted to a sum of two components classical receptor theory and intrabiophasic partitioning. The vertical bars represent 1* SD. *The resolution of the dose–effect curve into each component is shown separately.*

Table I—Equation Parameters[a] Characterizing the Kinetics of Pharmacological Response, Derived Biophasic Drug Levels and Plasma Concentrations of Quinidine following Slow and Rapid Intravenous Infusions to Two Rabbits

Type of Data[b]	Mode of Administration	A_1	m_1[c]	A_2	m_2[c]	A_3	$t_{1/2}$[d]
Pharmacological	Rapid intravenous[e]	1.32	0.461	0.495	0.0241	—	28.8
	Slow intravenous, postinfusion[f]	0.224	0.247	0.777	0.0240	—	28.9
Plasma	Rapid intravenous[f]	3.57	0.535	2.01	0.0288	—	24..1
Biophasic	Rapid intravenous[e]	3.48	0.687	0.127	0.0265	—	26.2
	Slow intravenous, postinfusion[f]	0.809	0.177	0.170	0.0206	—	33.6
	Slow intravenous infusion[f]	0.0016	0.648	1.39	0.0239	1.16	29.0

[a] Determined from nonlinear, least-squares, unconstrained computer fits to the experimentally obtained data. [b] All data were normalized by dividing each value by the maximum value recorded; therefore, A_i's are dimensionless. [c] Expressed as minutes^{-1}. [d] Calculated from the equation $t_{1/2} = 0.693/m_2$; expressed in minutes. [e] Parameters were computed from an average of eight determinations, four doses given to each of two rabbits. [f] Parameters were determined from an average of two determinations, one dose given to each of two rabbits.

reference to QT_B was calculated according to Eq 6:

$$I_t = \frac{QT_t - QT_B}{QT_B} \qquad \text{(Eq. 6)}$$

where I_t represents the intensity of response attributed to quinidine as a function of time for the rapid intravenous doses (Fig. 3). The response intensities obtained from each rabbit and at each dose were normalized with respect to maximum values at each time following dosing. The normalized response intensities were then averaged for each rabbit and dose. The computerized least-squares line of best fit was best represented by a biexponential relationship from which the latter log linear region of the curve yielded a pharmacological half-life of 28.8 min. The response intensities averaged for each rabbit but expressed separately for each dose could be fit by biexponential relationships producing pharmacological half-lives of 29.9, 38.4, 37.9, and 43.9 min for doses of 2.5, 3.75[12], 5.0, and 6.25 mg/kg, respectively. Response intensities significantly greater than baseline values were observed for 50–60 min following injection with the exception of the lower doses, 0.31, 0.62, and 1.24 mg/kg, which rapidly dissipated to predosing values within 15–30 min and were, therefore, not included in the averaging of data. Maximum response intensities for these lower doses, however, were used in establishing the dose-effect relationship.

The purpose of averaging the individual data was to obtain a more reliable fit to the data than might be obtained from treating the data from each dose separately. Justification for averaging response intensities over all doses comes from the fact that not only could the normalized response intensities for each dose be fit by a biexponential relationship but that pharmacological half-lives showed no apparent trend with dose. The rapid development of the maximal response, 2.17 ± 0.98 min, following dosing indicates the rapidity with which quinidine can enter the biophase and bind to receptors to elicit a response; consequently, the biophase can be kinetically assigned to the same compartment as the plasma. Under these conditions, $\beta(t_{max})$ for quinidine can be assigned a value of unity. The initial decline of plasma concentration for the rapid intravenous dosing was calculated to be approximately the same as the corresponding decline of response intensities as judged by the ratio of m_1 for each determination, 1.16 (Table I). Once equilibrium is reached, the continued monoexponential declines in response intensities and plasma levels nearly parallel one another. This would suggest that removal of drug from the biophase and not debinding of drug from receptor sites is the rate-determining step to decline in response. Garrett *et al.* (20) showed, by analog computer simulations, that the apparent half-life calculated from the decline in response may approach the half-life determined from plasma level measurements provided the number of receptor sites is relatively large, the affinity of drug for sites is low, and the rates of binding as well as debinding are large. These postulates, as far as can be ascertained from the data, are in agreement with the observed results.

[12] Calculated for one rabbit only; the other rabbit died of unknown causes before the dose could be administered.

The pharmacological response intensities attributed to quinidine in this study are a consequence of quinidine as well as any pharmacologically active metabolites; however, Conn (21) reported that antiarrhythmic activity in the rabbit is mainly the result of the cellular effects of unchanged quinidine. He found that dihydroquinidine, a contaminant in levels of 5–9% in commercial quinidine preparations (22) and also detected in rabbit urine[13] (21), was the only metabolite to produce significant antiarrhythmic activity. For the purpose of presenting new information and due to the lack of specific knowledge to the contrary, the percentage of contaminants was considered to be negligible in this report.

Figure 3 graphically demonstrates the logarithmic change in response intensities during the time course of the slow intravenous infusion of quinidine as well as the decline in response intensities following termination of the infusion after 95 min. The latter log linear decline in response intensities yielded a pharmacological half-life of 28.9 min, decidedly similar to the rapid intravenous results.

Intravenous Dose-Effect Curve—Figure 4 represents the dose-effect curve constructed from I values recorded at t_{max} for the corresponding rapid intravenous dose. The curve represents a summation of two components, hyperbolic and linear. The hyperbolic component is suggestive of a dose-effect relationship in which the "occupancy assumption" applies; that is, one drug molecule combines with one receptor site, response is proportional to the concentration of bound sites, and a negligible fraction of total drug is combined so that the response intensities can be related to total biophasic drug levels in preference to unknown free drug levels (23). The linear component of the dose-effect curve suggests a second set of receptor sites exhibiting a different affinity for drug in which partitioning of quinidine occurs under apparent nonsaturable conditions over the dose range studied; additionally, the possibility of one class of sites exhibiting mutual interaction should not be excluded. According to these assumptions, the response intensities can be related to Q_B using Eq. 7:

$$I = \frac{I_{max} Q_B^n}{K_D + Q_B^n} + P Q_B^n \qquad \text{(Eq. 7)}$$

where K_D represents the quantity of drug in the biophase at one-half of I_{max} for the hyperbolic component of the dose-effect curve, P represents the proportionality constant for the linear component, and n is a constant value which is 1 for a hyperbolic relationship. As n becomes greater than 1, the curve becomes more sigmoidal, implying multiple and cooperative binding sites for drug (24). Substituting dose [$\equiv f(I)_{max}$] for Q_B and assuming $\beta(t_{max})$ to be equal to 1.0, the values of I_{max} = 0.151 mg/kg, K_D = 0.116 mg/kg, n = 0.981, and P = 0.0281 mg/kg were determined from computer calculations and used in generating the theoretically based line in Fig. 4. The closeness of n to unity suggests that occupation theory is correct in describing the hyperbolic component of the dose-effect curve.

[13] Percentage not specified.

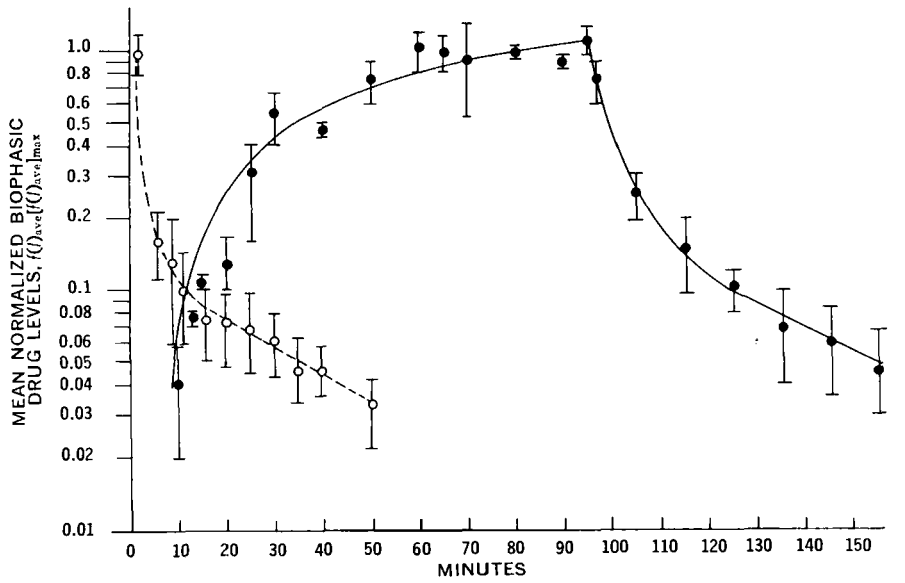

Figure 5—*Time course variation in biophasic drug levels following rapid* (○) *and slowly infused* (●) *doses of quinidine. Each point represented by open circles is an average of eight determinations calculated from response intensities measured in two rabbits given four doses of 2.5, 3.75, 5.0, and 6.25 mg/kg. The dashed line is a computer least-squares line of best fit represented by* $f(I)_{ave}/[f(I)_{ave}]_{max} = 3.48e^{-0.687t} + 0.127e^{-0.0265t}$. *The solid line is a computer least-squares line of best fit for a slowly infused dose of quinidine of 0.0145 mg/kg/min to each of two rabbits. The postinfusion data beginning at* $t = 95$ *min are represented by* $f(I)_{ave}/[f(I)_{ave}]_{max} = 0.809e^{-0.177(t-95)} + 0.170e^{-0.0206(t-95)}$. *The computer line of best fit for the infusion data up to and including* $t = 95$ *min was represented by* $f(I)_{ave}/[f(I)_{ave}]_{max} = 1.16 - 0.0016e^{-0.468t} - 1.39e^{-0.0239t}$. *The vertical bars represent 1* SD.

Time Course of Biophasic Drug Levels—Figure 5 depicts the time course of fractional amounts of quinidine in the biophasic compartment of the body. For the rapid intravenous dosing, the averages of eight determinations obtained for two rabbits and four doses are shown; the average of a single-infusion run administered to each of two rabbits is also shown. Biophasic half-lives were calculated from the slopes of the latter linear segments of the semilogarithmic plots for the rapid and postinfusion data, yielding values of 26.2 and 33.6 min, respectively. The independence of these values on dose is indicated by their similarity. Additionally, a lack of trend in half-lives was observed for the rapid intravenous doses of 2.5, 3.75, 5.0, and 6.25 mg/kg, corresponding to 22.6, 10.3, 42.3, and 37.5 min, respectively, which justified the averaging of the normalized data. A confirmation in biophasic half-life was further demonstrated from the calculation of the half-life from the infusion data up to 95 min using Eq. 8:

$$Q_B = f(I) = A_3 - A_1e^{-m_1t} - A_2e^{-m_2t} \qquad \text{(Eq. 8)}$$

Equation 8 corresponds to a two-compartment model where the A_i's and m_i's are equation parameters and the m_i's have the same meaning as in the rapid intravenous case (25). As a result of the kinetic characteristics of quinidine, $f(I)$ is considered to be directly equal to Q_B. From a least-squares computerized fit to the infusion data shown in Fig. 5, an m_2 value of 0.0239 was calculated to yield a biophasic half-life of 29.0 min.

In terms of ideal compartment theory, the drug is instantaneously dispersed within a body compartment so that the drug within these tissues can be considered as kinetically homogeneous. Therefore, once pseudoequilibrium is reached, as in the case of a multicompartment model, the monoexponential decline of drug sampled from any region of the compartment will be identical. In the case of quinidine, the rapid development of a maximum response indicates for all practical purposes an instantaneous dispersion within the central compartment of which the biophase is a part. In addition, the similar biological and biophasic half-lives calculated directly from plasma concentrations and indirectly from response intensities, respectively, suggest a kinetically homogeneous relationship between plasma and biophase. A rapid distribution phase, kinetically similar to that of the plasma data, was apparent with biophasic drug levels and represents equilibration into a pharmacologically inert depot compartment.

SUMMARY

In summary, pharmacological, biological, and derived biophasic half-lives were found to be similar following rapid and slow intravenous infusions. From direct measurements of response intensities, a maximum response was observed to be immediate following the rapid intravenous injection of quinidine. Although an insufficient number of replications was performed to draw firm conclusions, certain postulates were considered to be consistent with the observed results. The results indicate that the rate-determining step to decline in response is removal of drug from the biophase and that binding as well as debinding is instantaneous once drug reaches the biophase. Moreover, the biophase is located within tissues from which drug is in rapid instantaneous equilibration with plasma concentrations. Distribution to a pharmacologically inert depot was also apparent from the results. A comparison of the kinetics of pharmacological response to derived biophasic drug levels and plasma concentrations as determined by computerized fits of the data is summarized in Table I.

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ˣ To whom inquiries should be directed. Present address: Alcon Laboratories, Fort Worth, TX 76101

Synthesis of Substituted Anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes: Correlation between Anticonvulsant Activity and Monoamine Oxidase Inhibitory and Antihemolytic Properties

C. DWIVEDI*, RAYMOND D. HARBISON*, B. ALI‡, and SURENDRA S. PARMAR‡ˣ

Abstract □ Several substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes were synthesized and characterized by their sharp melting points, elemental analyses, and IR spectra. Their ability to inhibit monoamine oxidase and to afford protection against hypoosmotic hemolysis in dog erythrocytes was found to bear no relationship with their anticonvulsant activity against pentylenetetrazol-induced seizures in mice.

Keyphrases □ Anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes, substituted—synthesis, characterization, monoamine oxidase, antihemolytic, and anticonvulsant activity □ Anticonvulsant activity—substituted benzylidenes, relationship to monoamine oxidase inhibitory and antihemolytic activity □ Antihemolytic activity—substituted benzylidenes against hypoosmotic hemolysis, relationship between monoamine oxidase inhibitory activity—substituted benzylidenes, relationship between antihemolytic and anticonvulsant activity

Hydrazine derivatives (1) and semicarbazides and thiosemicarbazides (2–4) have been shown to inhibit monoamine oxidase [EC 1.4.3.4 monoamine: O_2 oxidoreductase (deaminating)]. Monoamine oxidase inhibitors have also been shown to possess pronounced anticonvulsant properties (5). Evidence has been put forward to demonstrate that various drug responses are presumably mediated due to alterations in the physical properties of cell membrane and drug receptor interactions (6, 7). Studies with various central nervous system depressants, tranquilizers, and biogenic amines (8–11) revealed the ability of these compounds to stabilize the red blood cell membrane. Furthermore, substituted benzylidenes have been reported to possess psychotropic properties (12). These observations led to the synthesis of substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes. The ability of these benzylidenes to inhibit monoamine oxidase and to exhibit membrane-stabilizing properties was investigated. The anticonvulsant activity of these compounds against pentylenetetrazol-induced seizures was also determined in an attempt to correlate their anticonvulsant activity with their monoamine oxidase inhibitory property and their ability to afford protection against hypoosmotic hemolysis using dog red blood cells.

The various substituted benzylidenes were synthesized by following the methods outlined in Scheme I.

EXPERIMENTAL[1]

Chemistry—Substituted anilines (I) on condensation with vanillin (II) gave substituted anilino-(3-methoxy-4-hydroxy)benzylidenes (III and IV). Treatment of these compounds with ethyl chloroacetate resulted in the formation of substituted anilino-[3-methoxy-4-(ethoxycarbonylmethyleneoxy)]benzylidenes (V and VI) which, on treatment with hydrazine hydrate, gave substituted anilino-[3-methoxy-4-(hydrazinocarbonylmethyleneoxy)]benzylidenes (VII and VIII). These benzylidenes, on further treatment with aryl isothiocyanates, yielded substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes (IX–XXIV).

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Substituted Anilino-[3-methoxy-4-(ethoxycarbonylmethyleneoxy)]benzylidenes (V and VI)—To a solution of 0.08 mole of III and IV in dry acetone were added 0.08 mole of ethyl chloroacetate and 0.08 mole of anhydrous potassium carbonate, and the reaction mixture was refluxed on a steam bath for 15 hr. The reaction mixture was filtered and excess acetone was removed by distillation. The crude product, which separated on cooling, was filtered, dried, and recrystallized from ethanol (Table I).

[1] Analyses for carbon, hydrogen, and nitrogen were performed by the Central Drug Research Institute, Lucknow, India. Melting points were taken in open capillary tubes and are uncorrected.

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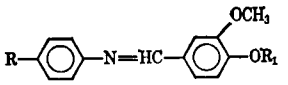
Table I—Physical Constants of Substituted Anilino-3-methoxy-4-substituted Benzylidenes

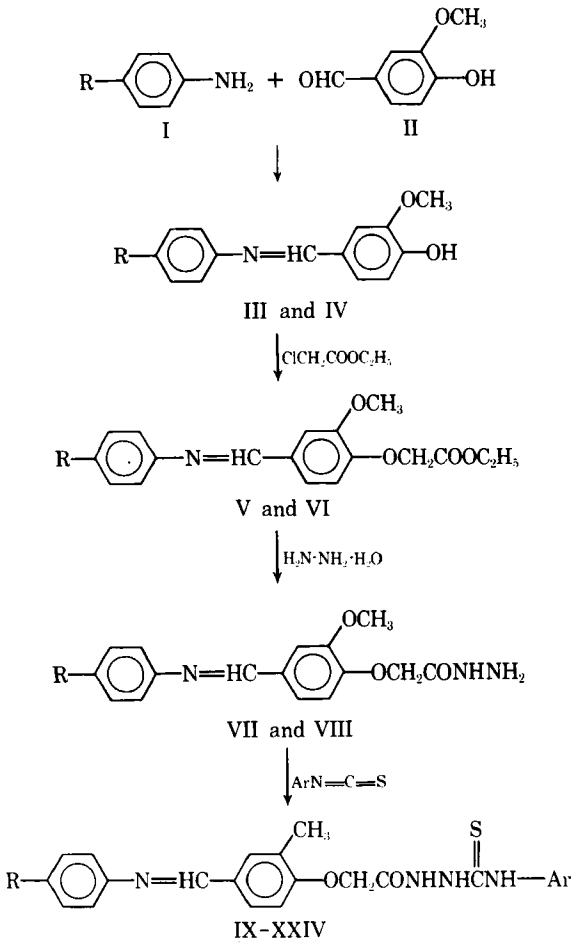


Compound	R	R_1	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Found
III	CH_3	H	120°	85	$C_{15}H_{15}NO_2$	C 74.68	74.92
						H 6.22	6.01
						N 5.80	5.65
IV	OCH_3	H	122°	84	$C_{15}H_{15}NO_3$	C 70.03	70.02
						H 5.83	5.76
						N 5.44	5.16
V	CH_3	$CH_2COOC_2H_5$	110°	80	$C_{19}H_{21}NO_4$	C 69.72	69.48
						H 6.42	6.51
						N 4.28	4.56
VI	OCH_3	$CH_2COOC_2H_5$	128°	80	$C_{19}H_{21}NO_5$	C 66.47	66.52
						H 6.12	5.06
						N 4.08	3.92
VII	CH_3	$CH_2CONHNH_2$	168°	65	$C_{17}H_{19}N_3O_3$	C 65.17	65.36
						H 6.07	5.92
						N 13.41	13.28
VIII	OCH_3	$CH_2CONHNH_2$	124°	62	$C_{17}H_{19}N_3O_4$	C 62.00	61.92
						H 5.77	6.02
						N 12.76	12.56

[a] All melting points were taken in open capillary tubes and are uncorrected.

Substituted Anilino-[3-methoxy-4-(hydrazinocarbonylmethyleneoxy)]benzylidenes (VII and VIII)—A mixture of 0.06 mole of V and VI and 99–100% hydrazine hydrate (0.09 mole) in absolute ethanol was refluxed on a steam bath for 12–15 hr. Excess ethanol was removed by distillation. The crude product, which separated on cooling, was filtered, dried, and recrystallized from benzene (Table I).

Substituted Anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes (IX–XXIV)—The substituted anilino-[3-methoxy-4-(hydrazinocarbonylmethyleneoxy)]-benzylidenes (VII and VIII) (0.004 mole) and suitable aryl isothiocyanates (0.004 mole) were mixed in dry benzene, and the mixture was refluxed on a steam bath for 3–5 hr. Excess benzene from the reaction mixture was removed by distillation under reduced pressure. The solid mass, which separated on cooling, was filtered, dried, and recrystallized from ethanol. Compounds IX–XXIV, thus synthesized, are recorded in Table II. The presence of the characteristic bands of the N—C(=S)—N (1490 cm^{-1}) and C=N (1640 cm^{-1}) groups in the IR spectra of these compounds provided further support in confirmation of their molecular structure.



Scheme I

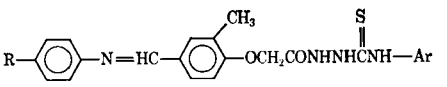
Determination of Monoamine Oxidase Activity—*Spectrophotofluorometric Method*—A spectrophotofluorometric method was used for the determination of monoamine oxidase activity of rat brain homogenate, using kynuramine as the substrate (13). The 4-hydroxyquinoline formed during oxidative deamination of kynuramine was measured fluorometrically in a spectrophotofluorometer[2] using activating light of 315 nm and measuring fluorescence at the maximum of 380 nm.

Male adult rats weighing approximately 150–200 g were killed by decapitation. Brains were quickly removed and homogenized[3] in ice-cold 0.25 *M* sucrose. The reaction mixture in a final concentration consisted of 0.5 ml phosphate buffer (0.2 *M*, pH 7.5), 1×10^{-4} *M* kynuramine, and 0.5 ml brain homogenate (equivalent to 10 mg of wet weight of the tissue). The monoamine oxidase activity of the brain homogenate was determined by incubation at 37° in air for 30 min. The various substituted benzylidenes were added to the brain homogenate to produce a final concentration of 5×10^{-5} *M* and incubated for 10 min before adding kynuramine. The mixture was then incubated for an additional 30 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (w/v), and the precipitated proteins were removed by centrifugation. Suitable 1-ml aliquots of the supernate were taken in 2 ml of 1 *N* NaOH solution and were assayed for 4-hydroxyquinoline. An increase in absorbance provided a direct measurement of the 4-hydroxyquinoline formation, which was taken as an index of the enzyme activity. The percent inhibition was calculated from the decrease observed in the absorbance and this provided an index of the inhibitory property of these substituted benzylidenes.

Warburg Manometric Method—The monoamine oxidase activity of rat brain homogenate was determined by the conventional Warburg manometric technique, using tyramine as the substrate (14). The decrease in oxygen uptake for 1 hr during oxidative deamination of tyramine in the presence of the substituted benzylidenes was used as an index of enzyme inhibition. The reaction mixture in a final concentration consisted of 50 m*M* phosphate

[2] Aminco-Bowman.
[3] Potter-Elvehjem homogenizer.

Table II—Physical Constants of 4-Substituted Anilino-[3-methoxy-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes



Compound	R	Ar	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Found
IX	CH_3	C_6H_5	120°	80	$C_{24}H_{24}N_4O_3S$	C 64.28	64.46
						H 5.35	5.29
						N 12.50	12.35
X	CH_3	2-CH_3—C_6H_4	175°	72	$C_{25}H_{26}N_4O_3S$	C 64.93	65.21
						H 5.62	5.54
						N 12.12	11.96
XI	CH_3	3-CH_3—C_6H_4	138°	74	$C_{25}H_{26}N_4O_3S$	C 64.93	64.74
						H 5.62	5.70
						N 12.12	12.02
XII	CH_3	4-CH_3—C_6H_4	160°	82	$C_{25}H_{26}N_4O_3S$	C 64.93	64.86
						H 5.62	5.58
						N 12.12	12.23
XIII	CH_3	4-OCH_3—C_6H_4	155°	81	$C_{25}H_{26}N_4O_4S$	C 62.67	62.82
						H 5.43	5.38
						N 11.71	11.63
XIV	CH_3	4-Cl—C_6H_4	156°	82	$C_{24}H_{23}ClN_4O_3S$	C 59.68	59.82
						H 4.76	4.62
						N 11.60	11.48
XV	CH_3	4-Br—C_6H_4	162°	84	$C_{24}H_{23}BrN_4O_3S$	C 54.64	54.38
						H 4.36	4.45
						N 10.62	10.56
XVI	CH_3	4-I—C_6H_4	166°	82	$C_{24}H_{23}IN_4O_3S$	C 50.17	49.98
						H 4.00	4.12
						N 9.77	9.58
XVII	OCH_3	C_6H_5	123°	79	$C_{24}H_{24}N_4O_4S$	C 62.06	61.82
						H 5.17	5.46
						N 12.06	11.98
XVIII	OCH_3	2-CH_3—C_6H_4	118°	73	$C_{25}H_{26}N_4O_4S$	C 62.76	63.05
						H 5.43	5.28
						N 11.71	11.46
XIX	OCH_3	3-CH_3—C_6H_4	135°	75	$C_{25}H_{26}N_4O_4S$	C 62.76	62.68
						H 5.43	5.52
						N 11.71	11.66
XX	OCH_3	4-CH_3—C_6H_4	130°	80	$C_{25}H_{26}N_4O_4S$	C 62.76	62.61
						H 5.43	5.38
						N 11.71	11.62
XXI	OCH_3	4-OCH_3—C_6H_4	156°	80	$C_{25}H_{26}N_4O_5S$	C 60.72	61.01
						H 5.26	5.12
						N 11.33	11.24
XXII	OCH_3	4-Cl—C_6H_4	158°	82	$C_{24}H_{23}ClN_4O_4S$	C 57.77	58.08
						H 4.61	4.48
						N 11.23	10.96
XXIII	OCH_3	4-Br—C_6H_4	166°	84	$C_{24}H_{23}BrN_4O_4S$	C 53.03	53.38
						H 4.23	4.16
						N 10.31	10.19
XXIV	OCH_3	4-I—C_6H_4	170°	83	$C_{24}H_{23}IN_4O_4S$	C 48.81	49.12
						H 3.89	3.66
						N 9.49	9.28

[a] All melting points were taken in open capillary tubes and are uncorrected.

buffer (pH 7.4), 5 m*M* tyramine, and the homogenate (equivalent to 250 mg of fresh tissue) in a total volume of 3.0 ml. The inhibitors, used at a final concentration of 1×10^{-3} *M*, were incubated with the enzyme preparation for 20 min before the substrate was added. The enzyme system was then incubated for an additional hour at 37°, using oxygen as the gaseous phase. Readings of oxygen uptake were made every 10 min.

Determination of Hypoosmotic Hemolysis—The procedure of Despopoulos (10), slightly modified, was used for the assay of hypoosmotic hemolysis; fresh blood of healthy dogs was used without oxygenation. An aliquot of 0.1 ml of dog blood was added to 3.0 ml of a buffer solution (0.425% Na_2HPO_4-NaH_2PO_4 buffer, 5 m*M*, pH 7.4; total osmolality 135 mosmoles/liter). The tubes, with and without the test compounds, were shaken gently and allowed to stand at room temperature for 10 min; then these tubes were centrifuged for 5 min at 1000×*g* to separate the cells. Under these conditions, hemolysis of the blood cells occurred but in no case exceeded 50%. Absorbance of the supernate was read at 540 nm in a colorimeter. Percent protection was calculated by comparing the absorbance values observed in the presence of the test compounds with that observed in the control tubes without the test compounds. The control value was represented as 100% hemolysis. Absorbance values for the blank containing 3 ml of 0.85% NaCl (normal saline) were subtracted from the values for both the control and experimental tubes. All test compounds were dissolved in the pH 7.4 phosphate buffer (osmolality 135 mosmoles) and were used at a final concentration of 1×10^{-4} *M*.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined (15) in mice of either sex weighing 25–30 g. The mice were divided in groups of 10, keeping the group weights as near the same as possible. Each benzylidene was suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compound was injected in a group of 10 animals at a dose of 100 mg/kg ip. Four hours after administration of the substituted benzylidenes, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol not only produced convulsions in almost all untreated mice but also exhibited 100% mortality during 24 hr. On the other hand, no mortality was observed during 24 hr in animals treated with 100 mg/kg alone of the test compounds. The mice were then observed 60 min for seizures. An episode of clonic spasm that persisted for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks or tremulousness was not counted. Animals devoid of threshold convulsions during 60 min were considered protected. The number of animals protected in each group was recorded and the anticonvulsant activity of these substituted

Table III—Monoamine Oxidase Inhibitory, Antihemolytic, and Anticonvulsant Properties of Substituted Anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes

Compound	Monoamine Oxidase Inhibition, %		Protection of Hypoosmotic Hemolysis[b], %	Anticonvulsant Activity[c], % Protection	Pentylenetetrazol Mortality, %
	Kynuramine[a]	Tyramine[a]			
IX	34.85 ± 0.56	30.61 ± 0.48	49.46 ± 0.75	50	10
X	46.91 ± 0.64	43.82 ± 0.56	43.17 ± 0.69	40	20
XI	38.87 ± 0.58	30.56 ± 0.47	47.96 ± 0.72	40	20
XII	32.17 ± 0.49	26.82 ± 0.43	52.66 ± 0.81	20	30
XIII	33.74 ± 0.52	21.90 ± 0.28	50.42 ± 0.80	60	10
XIV	68.25 ± 0.72	30.83 ± 0.36	41.04 ± 0.68	40	40
XV	64.34 ± 0.68	29.32 ± 0.18	48.18 ± 0.76	50	30
XVI	44.23 ± 0.59	27.35 ± 0.27	49.46 ± 0.88	60	20
XVII	33.51 ± 0.63	31.32 ± 0.42	31.37 ± 0.56	60	0
XVIII	36.19 ± 0.48	25.77 ± 0.38	63.83 ± 1.06	40	0
XIX	33.51 ± 0.42	37.20 ± 0.41	33.36 ± 0.58	40	10
XX	34.46 ± 0.46	31.38 ± 0.36	21.76 ± 0.54	70	0
XXI	32.17 ± 0.48	30.66 ± 0.44	34.75 ± 0.62	60	10
XXII	32.46 ± 0.50	40.68 ± 0.52	58.84 ± 0.78	80	0
XXIII	30.28 ± 0.40	35.29 ± 0.47	53.73 ± 0.69	60	0
XXIV	29.49 ± 0.32	25.68 ± 0.32	56.92 ± 0.76	60	0

[a] Compound numbers are those recorded in Table II. Each experiment was done in duplicate, and figures indicate mean values of three separate experiments with ± standard error of the mean. Vessel contents and assay procedures are as indicated in the text. The final concentrations of the inhibitors used were 5×10^{-5} and 1×10^{-3} *M* during oxidative deamination of kynuramine and tyramine, respectively. [b] Each experiment was done in duplicate and figures indicate mean values of three separate experiments with ± standard error of the mean. Assay procedure and contents of the reaction mixture are as indicated in the text. All compounds were used at a final concentration of 1×10^{-4} *M*. [c] Compounds were administered at a dose of 100 mg/kg ip. The screening procedure is as indicated in the text.

benzylidenes was represented as percent protection. The animals were then observed for 24 hr, and their mortality was recorded.

RESULTS AND DISCUSSION

In the present study, substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes were tested for their monoamine oxidase inhibitory property, using kynuramine and tyramine as the substrates, and for their ability to inhibit the membrane-stabilizing properties of dog erythrocytes. The monoamine oxidase inhibitory property of these benzylidenes, at final concentrations of 1×10^{-3} *M* and 5×10^{-5} *M*, was determined during oxidative deamination of kynuramine and tyramine, respectively (Table III). Inhibition of the enzyme activity was significantly higher with kynuramine as compared to that with tyramine. Similar changes in the degree of monoamine oxidase have also been observed with other inhibitors during oxidative deamination of different substrates (4, 16, 17). The presence of different substituents on the phenyl group of the thiosemicarbazide moiety of both 4-methyl- and 4-methoxyanilinobenzylidenes influenced their ability to inhibit monoamine oxidase. The effect of a methyl group on the degree of monoamine oxidase inhibition was found to be in the order of 2-methyl > 3-methyl > 4-methyl substituent (X–XII and XVIII–XX). The 4-methoxy substituent produced a similar degree of enzyme inhibition, as was observed with compounds possessing an unsubstituted phenyl nucleus (IX, XIII, XVII, and XXI). Among compounds possessing a 4-halogen-substituted phenyl nucleus, 4-chlorophenyl substituted compounds (XIV and XXII) produced a greater degree of monoamine oxidase inhibition than the corresponding 4-bromophenyl- or 4-iodophenyl-substituted compounds (XV, XVI, XXIII, and XXIV).

All substituted benzylidenes were found to protect red blood cells against hypoosmotic hemolysis (Table III). Introduction of various substituents at position 4 of the thiosemicarbazide moiety of 4-methylanilinobenzylidenes produced no significant effect on their ability to protect against hemolysis (IX–XVI). Maximum protection was observed with Compound XII. On the other hand, attachment of the various substituents at position 4 of the thiosemicarbazide moiety of 4-methoxyanilinobenzylidenes was found to play a definite role in their antihemolytic activity. The presence of the 2-methylphenyl substituent at position 4 was found to be most effective in exhibiting the antihemolytic property (XVIII). Variations in the position of the methyl group of the phenyl substituent attached to position 4 of the thiosemicarbazide moiety decreased the antihemolytic activity (XIX and XX). A similar decrease was also observed on the introduction of the 4-methoxyphenyl group at position 4 of the thiosemicarbazide moiety (XXI). On the other hand, the presence of a 4-halosubstituted group at position 4 of the thiosemicarbazide moiety produced increased protection against hypoosmotic hemolysis (XXII–XXIV).

The anticonvulsant activity observed with these benzylidenes at a dose of 100 mg/kg against pentylenetetrazol-induced seizures ranged from 20 to 80% (Table III). Maximum protection was observed with Compound XXII. As is evident from Table III, all 4-methoxyanilinobenzylidenes (XVII–XXIV) not only elicited a relatively higher degree of protection but also protected the experimental animals against pentylenetetrazol-induced mortality during 24 hr. These observations do not prove that the membrane stabilization and monoamine oxidase inhibitory properties of these substituted benzylidenes are the basis for their anticonvulsant activity. Further studies dealing with pharmacological and toxicological properties and their effects upon the activity of other purified enzyme preparations may possibly reflect a biochemical basis for the anticonvulsant activity of these substituted benzylidenes.

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x To whom inquiries should be directed. Present address: Department of Physiology and Pharmacology, School of Medicine, University of North Dakota, Grand Forks, ND 58201

PHARMACEUTICAL ANALYSIS

Determination of Silicon in Streptomycin by Atomic Absorption

R. J. HURTUBISE

Abstract □ An atomic absorption method was developed for determining silicon in streptomycin without prior separation. The method has a reproducibility of ±2.2 ppm and a detection limit of 5 ppm silicon. Ash-fused and untreated samples were compared, and a deuterium arc background corrector indicated a moderately high bias in the results.

Keyphrases □ Silicon—atomic absorption determination in streptomycin □ Streptomycin—atomic absorption determination of silicon content □ Atomic absorption spectroscopy—determination of silicon in streptomycin

Because the manufacturing of pharmaceuticals involves many complex chemical and physical processes, contaminants can be introduced in many places before the final product is obtained. Thus, there is a need for precise, accurate, and sensitive analytical methods to determine contaminants. Silicon can be introduced by raw materials, water, and other sources into many in-process manufacturing samples. In the pharmaceutical industry, the control of the amount of silicon is important because it can directly or indirectly cause turbidity in solutions of product samples.

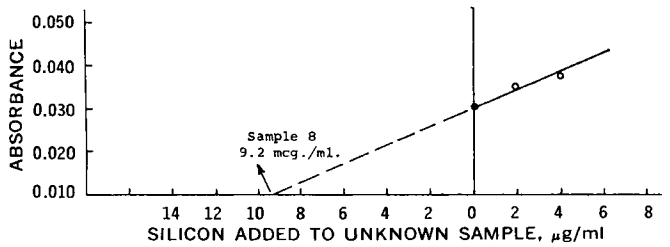

Figure 1—*Typical standard addition curve for streptomycin.*

Few atomic absorption methods have been published for the determination of silicon in pharmaceuticals (1, 2). This paper describes a rapid, sensitive, and accurate atomic absorption method for the determination of silicon in the antibiotic, streptomycin. Ashing and alkaline fusion steps are not needed, and a deuterium arc background corrector may be employed, depending on the accuracy desired.

EXPERIMENTAL

Apparatus—An atomic absorption spectrophotometer[1] equipped with a nitrous oxide burner and a deuterium arc background corrector[2] were used according to the manufacturer's instructions. A single-element silicon hollow-cathode lamp[3] was used for all silicon determinations. The following instrument settings were used for all measurements: resonance line, 251.6 nm; slit 4; lamp current, 40 mamp; nitrous oxide flow, 13 liters/min; and acetylene flow, 12 liters/min. Experimental results indicated that Pyrex volumetric glassware was suitable for this work.

Reagents—Fisher certified sodium silicate atomic absorption standard was used to prepare all silicon standards.

Procedures—*Determination of Silicon*—Between 24.0 and 26.0 g of streptomycin sulfate was weighed accurately into a 50-ml volumetric flask and diluted to volume with distilled water. Ten-milliliter aliquots of this solution were transferred quantitatively to three 25-ml volumetric flasks. Aliquots (1 and 2 ml) of 50 μg/ml standard sodium silicate solution were added to two of the volumetric flasks, respectively, and the contents of all three flasks were diluted to volume with distilled water. The atomic absorption unit was adjusted so there was a fuel-rich flame, the absorbance was set at zero with distilled water, and the absorbance of the three solutions was determined. A standard addition curve was prepared by plotting absorbance against micrograms of silicon per milliliter added to streptomycin solutions. The line inter-

[1] Perkin-Elmer model 403.
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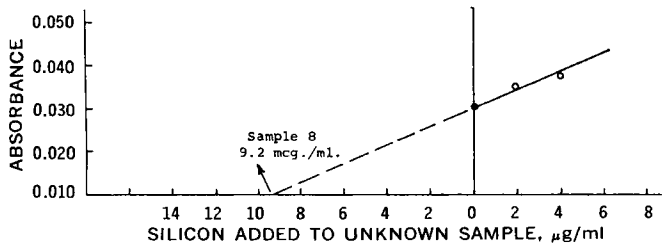

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Table I—Recovery of Silicon

Sample	Silicon[a] Present, ppm	Silicon Added, ppm	Silicon[b] Found, ppm	Silicon Added, ppm	Silicon[b] Found, ppm	Silicon Added, ppm	Silicon[b] Found, ppm
1	47	9.5	9.0	19	19	47	47
2	70	10.0	7.8	20	17	52	42
3	65	9.8	7.4	20	15	49	37
4	66	9.8	7.4	20	15	49	37
5	70	10.0	8.0	20	17	50	42
6	65	9.9	9.9	20	20	50	48
7	54	10.0	10.0	20	20	50	47
8	46	9.9	8.5	20	18	50	47
9	55	10.0	8.4	20	17	50	47
10	56	9.9	8.4	20	17	49	46
11	74	10.0	8.0	20	15	50	40
12	54	9.6	9.6	19	17	48	45
Average		9.9	8.5	20	17	50	44
Average difference		1.4 ppm		3.0 ppm		6.0 ppm	
Average recovery, %		86		85		88	

[a] Results obtained from standard addition method. [b] Results corrected for original silicon content present in the sample.

secting the absorbance axis was extrapolated to zero absorbance to obtain the concentration of silicon in the 25-ml unknown solution and then the parts per million of silicon in the streptomycin was calculated (Fig. 1).

Determination of Silicon with Fusing—A 5.0-g sample of streptomycin sulfate was weighed accurately into a platinum crucible and then carefully ashed over a burner until all carbon was removed. One gram of sodium bicarbonate was added to the carbon-free sample and heated on a hot plate for approximately 10 min; then the sample was heated over a Meker burner until a clear or slightly turbid melt was obtained. The sample was cooled and 11.5 ml of 1 *N* H_2SO_4 was added slowly with stirring. The sample was then heated for approximately 10 min on a hot plate. The sulfuric acid solution was transferred quantitatively from the crucible to a 25-ml volumetric flask and diluted to volume with distilled water. Five-milliliter aliquots of streptomycin solution were added to three 10-ml volumetric flasks. To two of the volumetric flasks were added 1 and 2 ml of a 20 μg/ml standard sodium silicate solution, respectively. The contents of all three flasks were then diluted to volume with distilled water. The absorbance readings and the concentrations of silicon were obtained as described under *Determination of Silicon*, except the atomic absorption spectrophotometer was set at zero absorbance with a water solution that contained the same amount of sodium bicarbonate and sulfuric acid as the unknown solution.

RESULTS AND DISCUSSION

Table I indicates the accuracy of the method and gives the average percentage recovery and average difference for silicon added as sodium silicate to 12 different streptomycin samples which originally contained different amounts of silicon. The values of "silicon found, ppm" were obtained as follows. The unknown absorbance was subtracted from the absorbance of the corresponding spiked samples; these absorbance difference values were used to obtain the corresponding concentrations of silicon from a calibration curve prepared from water solutions of sodium silicate; finally the parts per million of silicon found was calculated. The average percentage recovery was low in all cases, and the average difference between the "added" and "found" silicon showed a proportional increase with increasing amounts of silicon. These effects are most likely due to the complex composition of streptomycin resulting in both chemical and physical interferences in the nitrous oxide–acetylene flame. To minimize these interferences, the standard addition method was used. Since the unknown and standards were submitted to the same experimental conditions, the same proportional decrease in absorbance was manifested in both, so proportional errors were eliminated.

Table II gives the average absorbance and standard deviation corresponding to the samples in Table I. Slight composition variation of the different samples was not considered important. The results indicate that as the concentration of silicon increases, the absorbance values become less reliable. Because of the poorer reliability at high silicon concentrations, it was decided to use only two standard additions per determination.

The precision of the method (Table III) was obtained from replicate determinations on five different samples of streptomycin. If the results from Sample 14 are omitted, an average reproducibility of ±2.2 ppm silicon can be expected. The poorest precision for Sample 14, which contained about twice as much silicon as the other samples, was probably due to incomplete dissociation of silicon compounds in the nitrous oxide–acetylene flame. The results in Table I support this because, as the amount of silicon increases, the difference between the added silicon and the experimental silicon increases.

Since the standard addition method only minimizes proportional errors, sample size was varied to detect constant errors. The results in Table IV show that variation in sample size does not indicate any large constant errors.

Table II—Precision of Absorbance Values[a]

	2 μg Silicon/ml Added	4 μg Silicon/ml Added	10 μg Silicon/ml Added
Average absorbance	0.004	0.008	0.020
Standard deviation	0.000	0.001	0.002

[a] Absorbance values corrected for original silicon content present in samples.

Table III—Precision of Standard Addition Method

Sample	Silicon[a], Average ± ppm	Number of Determinations
8	47 ± 4.3	6
12	56 ± 2.8	6
13	46 ± 0.97	24
14	105 ± 12	6
15	52 ± 0.56	6

[a] Determined at the 95% confidence level.

Table IV—Variation of Sample Size[a]

Sample Weight, g	Silicon, ppm
11.025	52
16.797	50
22.260	52
26.043	49
31.520	48
	$\overline{50}$ ± 2.2 ppm[b]

[a] Sample 13. [b] Precision determined at 95% confidence level.

Table V—Comparison of Ashed-Fused and Untreated Samples

Sample	Silicon (Ashed-Fused), ppm	Silicon (Untreated), ppm	Difference, ppm Silicon
12	64	56	+8
16	51	46	+5
17	60	53	+7
18	41	51	−10
19	58	53	+5
20	27	36	−9
21	30	41	−11
22	36	45	−9
23	41	44	−3
24	51	61	−10
		Average	−2.7

Because silicon can exist in many forms such as silicates, soluble silica, and colloidal silica (3), the silicon content was determined in both ashed-alkaline fused samples and untreated samples to decide if total silicon was determined by the proposed standard addition method. The results (Table V) show a random distribution of difference values, indicating that indeterminate errors are present. Because the proposed standard addition method has an average reproducibility of ±2.2 ppm silicon, the indeterminate errors most likely result from the ashing and fusing steps. Application of the statistical t test at the 95% confidence level indicated that there was no significant difference between the results from ashed-fused samples and untreated samples. Thus, total silicon was determined in streptomycin by the proposed standard addition method.

Because the standard addition method does not eliminate background absorption such as molecular absorption and absorption due to the solvents or salts aspirated into the flame (4), a deuterium arc background corrector was used to see if effects of this type were present. Aqueous silicon standards gave identical readings with and without background absorption. The results in Table VI indicate that without using the deuterium arc background corrector the results for streptomycin samples were biased high by approximately 6 ppm silicon. Consequently, to obtain more accurate results, a deuterium arc corrector should be used. However, the error introduced was not large and, depending on the accuracy needed, the use of the deuterium arc corrector could be omitted. No attempt was made to identify the source of this error, but ions such as calcium, iron, and sodium can enhance the response of silicon (5). These ions are present at trace levels in streptomycin.

Table VI—Comparison of Results with and without Deuterium Arc Background Corrector

Sample	Silicon[a], ppm		Difference	Number of Determinations
	Arc	No Arc		
25	58 ± 3.7	63 ± 3.0	−5	6
26	80 ± 2.4	86 ± 2.4	−6	6

[a] Precision determined at the 95% confidence level.

To determine the limit of detection, it was necessary to have silicon-free samples of streptomycin. Because such samples were not readily available, the limit of detection was calculated as the concentration of silicon that would give an absorbance of 0.002. Since a sample with 50 ppm silicon would have an absorbance of 0.020, a sample with 5 ppm would give an absorbance of 0.002. Thus, the limit of detection is near 5 ppm silicon.

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New Method for Quantitative Determination of D-Dopa in Levodopa Samples[x]

A. VIDI, C. FRANCO, and G. BONARDI

Abstract □ A simple method for the determination of D-dopa present in levodopa samples is reported. The method is based on the property of the L-amino acid oxidase to convert quantitatively levodopa to 3,4-dihydroxyphenylpyruvic acid while D-dopa remains unchanged. The latter is assayed fluorometrically, and contaminant amounts of D-dopa present in levodopa samples can be detected.

Keyphrases □ Levodopa—quantitative determination of D-dopa contaminant □ D-Dopa—quantitative determination in levodopa samples □ Fluorometry—analysis, D-dopa in levodopa samples

The analytical assay of the purity of levodopa preparations, used in the treatment of Parkinson's disease, allows the detection of the D-enantiomer which may cause undesirable side effects (1, 2).

Previous work (3) suggested a method for determining minimal amounts of D-dopa present in levo-

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Previous work (3) suggested a method for determining minimal amounts of D-dopa present in levo-

Table I—Determination of D-Dopa in Levodopa Standard Preparations

Standard Preparations	D-Dopa Found[a] mg	%
Levodopa, 10.000 mg, + D-dopa, 0.500 mg	0.492	98.32
Levodopa, 10.000 mg, + D-dopa, 0.200 mg	0.195	97.42
Levodopa, 10.000 mg, + D-dopa, 0.100 mg	0.095	95.60
Levodopa, 10.000 mg, + D-dopa, 0.050 mg	0.048	96.66
Levodopa, 10.000 mg, + D-dopa, 0.025 mg	0.027	103.02
	Mean ± *SD*	98.20 ± 1.28
Levodopa, 10.000 mg	0.000	0.000
D-Dopa, 10.000 mg	9.998	99.980

[a] Values represent average of three determinations.

dopa samples. This method is based on the property of the L-amino acid oxidase (EC 1.4.3.2) enzyme to convert selectively and quantitatively levodopa into 3,4-dihydroxyphenylpyruvic acid. According to Anton and Sayre (4), the latter does not interfere in the subsequent fluorometric assay of the unchanged D-dopa.

EXPERIMENTAL

In a test tube (26 × 110 mm) fitted with a cap and equipped with tubes for the bubbling of oxygen (1 bubble/sec), 10.0 mg of standard levodopa[1], various amounts of standard D-dopa[1], 10.0 mg of pure L-amino acid oxidase[1], 30.0 μg of flavine-adenine dinucleotide[1] in a total volume of 10.0 ml of 0.1 *M* phosphate buffer (pH 6.5) were mixed. The mixture was incubated at 37° for 3 hr in the dark and subsequently centrifuged at 40,000×*g* for 20 min at 4°. The supernate was diluted 1:200 with 0.1 *M* phosphate buffer (pH 6.5), and D-dopa was determined fluorometrically[2] according to Anton and Sayre (4). The excitation and emission wavelengths were 330 and 380 nm (uncorrected data), respectively.

[1] Sigma.

[2] A model MPF-2A spectrophotofluorometer (Hitachi, Ltd.) was used.

RESULTS AND DISCUSSION

From the results reported in Table I, the validity of the method is confirmed by incubating separately pure samples of levodopa and D-dopa with amino acid oxidase enzyme. Levodopa is converted to 3,4-dihydroxyphenylpyruvic acid quantitatively while D-dopa is recovered unchanged. The dilution of the supernate is performed to obtain minimal interferences and D-dopa concentrations lower than 2 μg/ml where the response to the fluorometric assay is linear. The sensitivity limit for the determination of D-dopa in the presence of levodopa is 0.25%.

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[x] Inquiries should be directed to Professor Marcello Gaetani, De Angeli Institute, Via Serio 15—P.O.B. 3197, Milan, Italy.

Determination of Disodium Edetate Dihydrate in Streptomycin by Atomic Absorption Spectrophotometry

R. J. HURTUBISE

Abstract □ An atomic absorption method was developed for determining disodium edetate dihydrate (I) in the antibiotic streptomycin. It is free of phosphate interference and has a reproducibility of ±1.3 μg/g and a limit of detection near 4 μg/g. Quantitation involves the formation of a nickel–I complex, release of the complexed nickel by pH adjustment, and the determination of nickel by atomic absorption. The amount of nickel determined is directly proportional to the amount of I.

Keyphrases □ Streptomycin—determination of disodium edetate dihydrate, atomic absorption spectroscopy □ Disodium edetate dihydrate—determination in streptomycin, atomic absorption spectroscopy □ Edetate disodium dihydrate—determination in streptomycin, atomic absorption spectroscopy

In the production of pharmaceuticals, small amounts of disodium edetate dihydrate (I) may be used to remove alkaline earth impurities and to improve the efficiency of production processes. To control low levels of I in pharmaceuticals, rapid, accurate, and precise analytical methods are important. A search of the chemical literature yielded one method for I in pharmaceuticals (1). Other methods have been developed for I in waste water, urine, and protein solutions (2–6). No methodology was reported

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Levodopa, 10.000 mg, + D-dopa, 0.050 mg	0.048	96.66
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Table I—Percentage Recovery of Disodium Edetate Dihydrate in Streptomycin Sulfate[a]

Sample	Present, μg/g	Added, μg/g	Found, μg/g	Recovery, %
1	60	14	74	100
1	60	35	91	89
2	25	36	60	97
3	17	49	63	94
3	17	49	64	96
5	9.8	50	58	96
6	27	50	75	96
4	17	71	82	92
4	17	71	82	92
1	60	71	127	94
1	60	106	161	95
7	7.2	144	144	95
3	17	196	193	90
3	17	196	192	89
			Average	94

[a] Replicate samples of 14.0 ± 0.2 g were used.

for determining I in the antibiotic streptomycin in the presence of triphosphate. One method[1] for I in boiler water can tolerate triphosphate up to 12 μg/ml; however, higher levels of triphosphate result from initial steps in the purification of streptomycin.

The method developed, which proved free of phosphate interference, involves the formation of a nickel–I complex. The amount of nickel reacting is directly proportional to the amount of I present in streptomycin. Excess nickel is removed by precipitation with dimethylglyoxime; the nickel complexed by I is released by pH adjustment and is then determined by atomic absorption. Darbey (7) used a similar approach for colorimetric determination of I.

EXPERIMENTAL

Apparatus[2]—A single element nickel hollow-cathode lamp[3] was used for the determinations. The following instrument settings were used for all measurements: resonance line, 2324 Å; slit 2; lamp current, 40 mamp; air flow, 21.2 liters/min; and acetylene flow, 6.22 liters/min.

Reagents—For the nickel solution, dissolve 13.3 g of reagent grade nickel sulfate hexahydrate in distilled water and dilute to 1 liter with distilled water. For the dimethylglyoxime solution, dissolve 3.75 g of reagent grade dimethylglyoxime in 3-A alcohol and dilute to 250 ml with 3-A alcohol.

The following reagents were also used: 3-A alcohol–5% (v/v) methanol in ethanol and analytical grade I, ammonium hydroxide, hydrochloric acid, and sodium hydroxide.

Procedure for Standards—Standards of 8.0, 4.0, 2.0, and 1.0 μg/ml of I were prepared in distilled water with a few drops of 1 *N* NaOH added so the final volume was 100 ml and the pH was 6.0–6.5. A 100-ml distilled water blank was prepared by adding a few drops of 1 *N* HCl so the pH was 6.0–6.5. Fifteen milliliters of nickel solution was added to the standards and blank and after 10 min, 5 ml of concentrated ammonium hydroxide was added. After 5 min and occasional swirling, 15 ml of dimethylglyoxime reagent was added to all solutions. After 5 min, nickel dimethylglyoxime was filtered off and about 60 ml of each filtrate was collected. Fifty milliliters of each filtrate was pipeted into 125-ml erlenmeyer flasks and 2.5 ml of concentrated hydrochloric acid was added to each flask. The absorbance of the standards was determined, and then absorbance *versus* total micrograms of I was plotted on linear graph paper.

[1] ASTM Method D 3113-72T.

[2] A Perkin-Elmer 403 atomic absorption spectrophotometer was used as described in the Perkin-Elmer instruction manual.

[3] P-E Intensitron Lamp.

Table II—Reproducibility of Disodium Edetate Dihydrate in Streptomycin Sulfate[a]

Sample		Micrograms per Gram
2		24
		25
		24
		27
		25
		27
	Average	25
Standard deviation = 1.4		
95% confidence limit = 25 ± 1.5 μg/g		
5		9.5
		9.5
		8.5
		11
		9.5
		11
	Average	9.8
Standard deviation = 0.98		
95% confidence limit = 9.8 ± 1.0 μg/g		
6		27
		28
		25
		27
		25
		27
	Average	27
Standard deviation = 1.3		
95% confidence limit = 27 ± 1.4 μg/g		

[a] Replicate samples of 14.0 ± 0.2 g were used.

Procedure for Sample—Solutions of accurately weighed 14-g samples of streptomycin were prepared in distilled water with a few drops of 1 *N* NaOH added so the final volume was 100 ml and the pH was 6.0–6.5. The streptomycin solution was treated exactly as the standards beginning with: "Fifteen milliliters of nickel solution was added. . . ." The micrograms of I were obtained from the calibration curve and the micrograms per gram of I was calculated.

RESULTS AND DISCUSSION

The results in Table I show that the average percent recovery, 94, is somewhat low over a concentration range of 14–196 μg/g I added to streptomycin. Darbey (7) showed that ferric, calcium, and magnesium ions in water solution do not interfere in the complex formation of nickelous ion with I at 40 μg/ml and subsequent precipitation of excess nickelous ion with dimethylglyoxime. Ferric, calcium, and magnesium ions are present at micrograms per gram levels in streptomycin; with the method developed, I is about 3.0 μg/ml in the test solution. At such low concentrations of I, a negative bias could be introduced into the results if ferric ion partially complexed I in favor of nickelous ion.

In water solution at pH 6, ferric ion has a conditional formation

Table III—Effect of Sodium Triphosphate on Determination of Disodium Edetate Dihydrate in Streptomycin Sulfate[a]

Sample	Calculated Phosphate Added, μg/g	I, μg/g
8	0	7.4
	500	7.4
	1000	7.4
	5000	7.4
9	0	16
	500	15
	1000	14
	5000	15

[a] Replicate samples of 15.0 ± 0.1 g were used.

Table IV—Effect of Sodium Dihydrogen Phosphate Monohydrate on Determination of Disodium Edetate in Streptomycin Sulfate[a]

Sample	Calculated Phosphate Added, μg/g	I, μg/g
8	0	7.4
	500	7.4
	1000	8.4
	5000	7.4
9	0	16
	500	15
	1000	15
	5000	14

[a] Replicate samples of 15.0 ± 0.1 g were used.

constant of $10^{14.6}$ while nickelous ion has a conditional formation constant of $10^{13.8}$ (8). Ferric ion, with the greater conditional formation constant, probably would compete effectively with nickelous ion for I in a streptomycin solution. However, because the ferric-ion concentration is so low and nickelous ion is present in excess in the test solution, the reaction between nickelous ion and I should be favored over the reaction of ferric ion and I. Thus, the magnitude of error introduced by ferric ion should be small. Calcium and magnesium ions would not be expected to compete strongly for I, having conditional formation constants of $10^{5.9}$ and $10^{3.9}$, respectively, at pH 6.0 (8). Other factors that might contribute to the low percentage recovery are adsorption of I-complexed nickel on the precipitated nickel dimethylglyoximate and the complex composition of streptomycin solutions causing matrix effects. A systematic investigation of interferences in solution and interferences in the flame was not undertaken.

Table II indicates that within a concentration range of 10–24 μg/g I, an average reproducibility of ±1.3 μg/g I can be obtained at the 95% confidence level. The results also show that good precision is obtainable at the relatively low concentrations of I, *e.g.*, 9.5 μg/g. The limit of detection is near 4 μg/g I.

Tables III and IV show the effect of sodium triphosphate and sodium dihydrogen phosphate monohydrate on the determination of I. The results indicate that there is no interference from phosphate species. This conclusion is contrary to that of Darbey (7), who suggested that phosphates would give high results with his colorimetric method because these anions complex nickel.

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Abstract □ The procedure described here is applicable for determining edetic acid (ethylenediaminetetraacetic acid) at the 0.05–0.2% level in ophthalmic solutions. This method employs a spectrophotometric titration, using a magnesium-ion solution as titrant and arsenazo I as the indicator. The chemistry involved is an adaptation of a procedure to determine water hardness using edetic acid as titrant. The apparatus employed is an automated system utilizing a probe colorimeter to monitor the titration. Good precision was demonstrated with relative standard deviations of less than 1%. The method was also shown to be applicable to commercially available ophthalmic solutions, either decongestants or hard contact lens cleaning solutions.

Keyphrases □ Edetic acid—spectrophotometric titration in ophthalmic solutions □ Preservatives—spectrophotometric titration of edetic acid in ophthalmic solutions □ Titration, spectrophotometric—determination of edetic acid in ophthalmic solutions

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The classical colorimetric determination of the ferric iron–edetic acid complex (6) suffered from fading color and poor sensitivity with these ophthalmic solutions. Fritz *et al.* (7) reported a procedure for determining water hardness by an edetic acid titration using arsenazo I as an indicator; they showed spectrophotometric titration curves for magnesium and calcium. They also determined formation constants for these metals with arsenazo I. Based on these data, it seemed feasible that edetic acid could be titrated with magnesium ion using arsenazo I as the indicator.

To automate the method, a spectrophotometric ti-

Table IV—Effect of Sodium Dihydrogen Phosphate Monohydrate on Determination of Disodium Edetate in Streptomycin Sulfate[a]

Sample	Calculated Phosphate Added, μg/g	I, μg/g
8	0	7.4
	500	7.4
	1000	8.4
	5000	7.4
9	0	16
	500	15
	1000	15
	5000	14

[a] Replicate samples of 15.0 ± 0.1 g were used.

constant of $10^{14.6}$ while nickelous ion has a conditional formation constant of $10^{13.8}$ (8). Ferric ion, with the greater conditional formation constant, probably would compete effectively with nickelous ion for I in a streptomycin solution. However, because the ferric-ion concentration is so low and nickelous ion is present in excess in the test solution, the reaction between nickelous ion and I should be favored over the reaction of ferric ion and I. Thus, the magnitude of error introduced by ferric ion should be small. Calcium and magnesium ions would not be expected to compete strongly for I, having conditional formation constants of $10^{5.9}$ and $10^{3.9}$, respectively, at pH 6.0 (8). Other factors that might contribute to the low percentage recovery are adsorption of I-complexed nickel on the precipitated nickel dimethylglyoximate and the complex composition of streptomycin solutions causing matrix effects. A systematic investigation of interferences in solution and interferences in the flame was not undertaken.

Table II indicates that within a concentration range of 10–24 μg/g I, an average reproducibility of ±1.3 μg/g I can be obtained at the 95% confidence level. The results also show that good precision is obtainable at the relatively low concentrations of I, *e.g.*, 9.5 μg/g. The limit of detection is near 4 μg/g I.

Tables III and IV show the effect of sodium triphosphate and sodium dihydrogen phosphate monohydrate on the determination of I. The results indicate that there is no interference from phosphate species. This conclusion is contrary to that of Darbey (7), who suggested that phosphates would give high results with his colorimetric method because these anions complex nickel.

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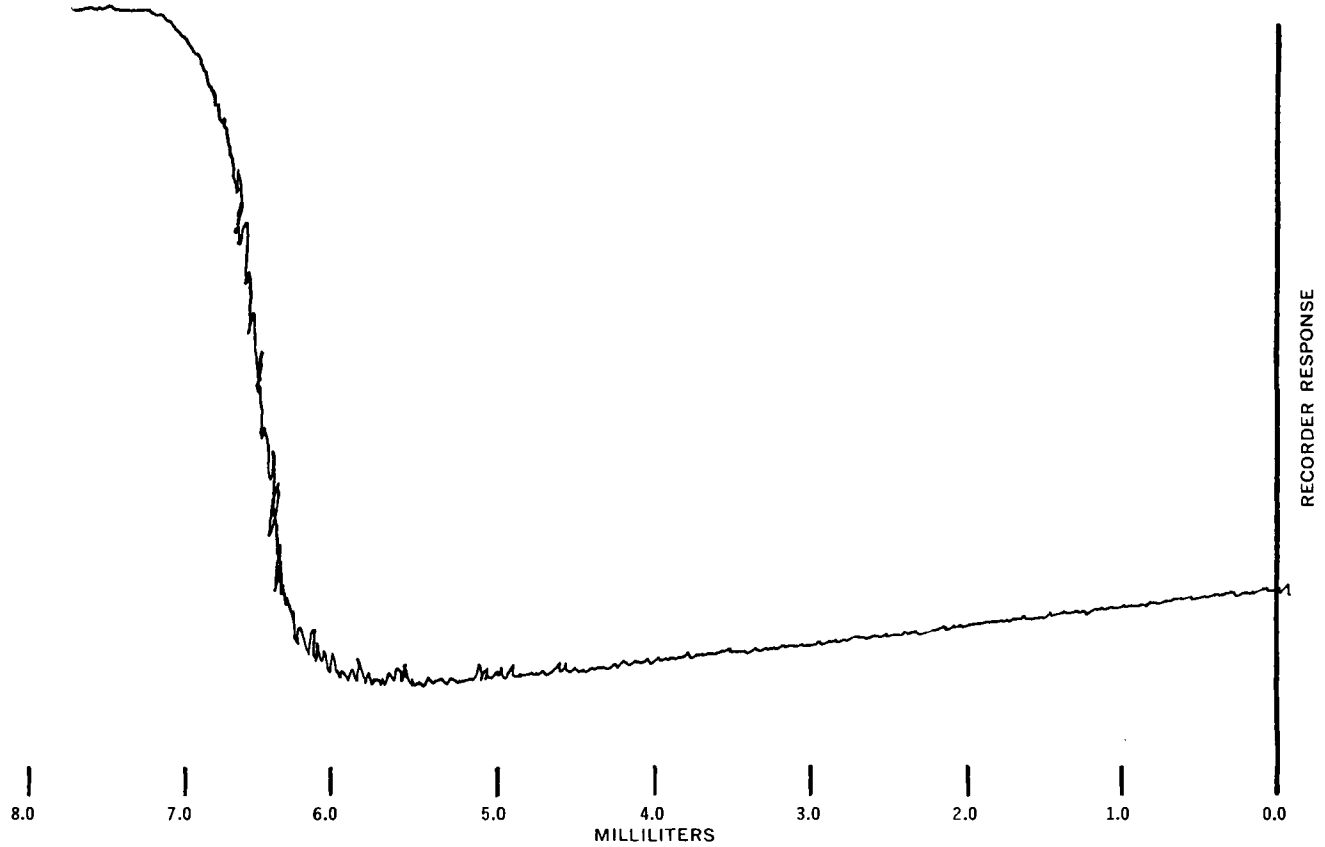

Figure 1—*Spectrophotometric titration curve of Solution A.*

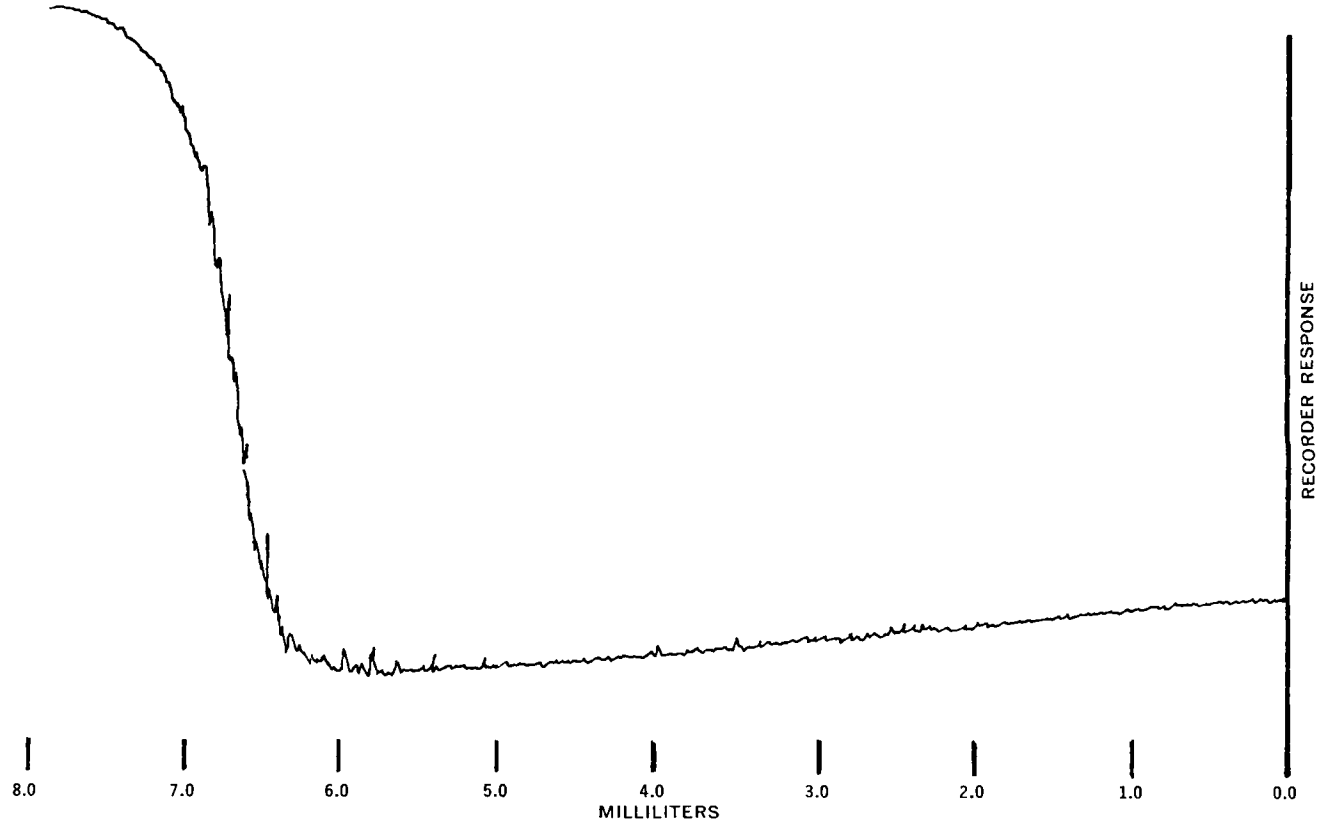

Figure 2—*Spectrophotometric titration curve of Solution B.*

Table I—Precision Data

	Solution A, Edetic Acid Found[a], mg/ml	Solution B, Edetic Acid Found[a], mg/ml
First day	0.968	0.959
	0.963	0.933
	0.975	0.943
Second day	0.964	0.938
	0.972	0.939
	0.968	0.946
Third day	0.961	0.939
	0.961	0.936
	0.975	0.943
Mean (mg/ml) ($\bar{x}$)	0.967	0.941
Standard deviation[b] (mg/ml)	±0.006	±0.007
Relative standard deviation	0.59%	0.76%

[a] $\text{mg/ml found} = \dfrac{\text{ml titrant} \times M \text{ titrant} \times 372.24 \text{ mEq/ml}}{\text{sample volume}}$

[b] $\text{Standard deviation} = \left[\dfrac{1}{n-1}\sum(x_i - \bar{x})^2\right]^{1/2}$

where $n = 9$, $\bar{x}$ = the mean, and x_i = each individual result.

Table II—Standard Addition–Recovery Experiments

	Edetic Acid Added, mg	Edetic Acid Found, mg	Recovery[a], %
Solution A	0.916	0.878	95.9
	1.373	1.365	99.4
	1.831	1.788	97.7
Solution B	0.916	0.986	107.6
	1.373	1.422	103.6
	1.831	1.874	102.3

[a] Percent recovered = [(mg found − mg initial)/mg added] × 100.

tration procedure was desired. A probe colorimeter filled the requirements for a sensitive, accurate, and rapid flowthrough measurement system. The system is further automated by using an automatic delivery buret and a recorder to trace the titration curve.

EXPERIMENTAL

Equipment—A colorimeter[1] with a stainless steel probe tip is used with an automatic constant-rate 10-ml buret[2]. The titration curve is plotted using a variable range recorder[3].

Reagents—A 1-mg/ml solution of arsenazo I trihydrate indicator[4] is prepared by dissolving 100 mg of indicator and 1.0 g of tromethamine (the buffer) in 10 ml of 2-propanol and diluting with water in a 100-ml volumetric flask.

Prepare pH 10 buffer by dissolving 13.1 g of tromethamine in about 40 ml of water, adding 4.0 ml of concentrated hydrochloric acid, and then adding 30 ml of concentrated ammonium hydroxide. Dilute the resulting solution to 100 ml with water, mix well, and then further dilute 1 to 10 with water.

The standard magnesium-ion solution (0.002 *M*) is prepared by transferring about 122 mg of magnesium metal ribbon to a 1000-ml volumetric flask and adding 75 ml of water before completely dissolving the magnesium with the slow addition of 3 *N* hydrochloric acid. Dilute the solution to volume with deionized water and then further dilute 200 to 500 ml with deionized water. Calculate the molarity based on the exact weight of magnesium ribbon.

Procedure—Pipet 5.0 ml of sample (containing approximately 0.1% edetic acid) into a 50-ml Griffin low-form beaker containing a magnetic stirring bar. Then add 10.0 ml of pH 10 buffer, 10.0 ml of deionized water, and 0.5 ml of indicator. The pH of the solution should now be 10.

Set the filter on the colorimeter at 570 nm and the range on the recorder at 125 mv. Insert the probe tip port and delivery tube into the beaker and, while stirring, set the pen on the recorder paper between 70 and 90 by adjusting the "absorbance zero" knob. It is important to ensure that the probe tip port is below the solution level and free of air bubbles or the noise level of the recorder tracing will become excessive.

Place the standard magnesium-ion solution in the automatic buret, open the delivery tube stopcock, and titrate to the end-point. In an identical manner, prepare and titrate a reagent blank solution.

[1] Brinkman PC/1000.
[2] Sargent-Welch.
[3] Sargent model SR.
[4] Aldrich Chemical Co.

Table III—Ingredients of Ophthalmic Solutions

Solution	Edetic Acid Concentration	Other Preservatives	Vasoconstrictor
A	0.1%	Benzalkonium chloride, 0.01%	Naphazoline hydrochloride, 0.012%
B	0.1%	Benzalkonium chloride, 0.1%	—
C	0.05%	Benzalkonium chloride, 0.01%	Phenylephrine hydrochloride, 0.2%
D	0.1%	Benzalkonium chloride, 0.01%	Tetrahydrozoline hydrochloride, 0.05%
E	0.05%	Thimerosal, 0.002%	—
F	0.2%	Benzalkonium chloride, 0.01% Chlorobutanol, 0.4%	—

RESULTS AND DISCUSSION

The titration curves for the titration of two ophthalmic solutions (A and B) are shown in Figs. 1 and 2. The end-point breaks are at least 30 scale units in each case, thus facilitating the location of the inflection point. The noise level was not a detrimental factor.

One variable investigated was the effect of indicator concentration on the titration curve. Accordingly, various amounts of indicator were used in titrating solutions *via* the recommended procedure. The shapes of the curves and the end-point determination were not affected when the volume of indicator was varied from 0.20 to 0.75 ml (40–150% of the recommended concentration).

The precision of this method was determined by performing three separate assays per day for 3 days on each ophthalmic solution (Table I).

Standard addition–recovery experiments were performed to insure the lack of interference in the procedure (Table II). The additional edetic acid was added by pipeting 1.0, 1.5, and 2.0 ml of a standard solution to respective sample solutions and titrating as before. Since a graduated 5.0-ml pipet was used for the trans-

Table IV—Titration Results Using Edetic Acid

Solution	Label Claim, mg/ml	Found, mg/ml	Label Claim, %
A	1.0	0.967[a]	96.7
B	1.0	0.941[a]	94.1
C	0.50	0.505[b]	101.0
D	1.0	1.000[b]	100.0
E	0.5	0.429[c]	85.7
F	2.0	2.042[b]	102.1

[a] Average of nine determinations. [b] Average of five determinations. [c] Average of three determinations.

fer, these recoveries are considered adequate to indicate the lack of any significant interferences.

An additional way to indicate lack of interference and also to demonstrate the applicability of the method is to analyze other ophthalmic solutions[5]. Decongestant ophthalmic drugs contain preservatives, vasoconstrictors, and other ingredients such as buffers and cleaning agents. Hard contact lens cleaning solutions do not contain the vasoconstrictors (8).

The common vasoconstrictor drugs available for over-the-counter use in ophthalmic preparations include phenylephrine, naphazoline, and tetrahydrozoline. The common preservatives are chlorobutanol, benzalkonium chloride, thimerosal, and edetic acid. Table III lists the combinations present in Solutions A–F, and Table IV lists the results for titration of these solutions by the proposed method. There were no problems with the titrations, and curves with end-point breaks similar to Figs. 1 and 2 were obtained. The low result for Solution E might be due to the more viscous nature of this solution, but sampling in triplicate did produce identical results.

CONCLUSION

This spectrophotometric titration procedure for determining edetic acid in ophthalmic solutions is simple and accurate. A complete analysis can be accomplished in 15 min using the automated equipment described herein. The precision and accuracy of the method have been quantitatively demonstrated.

[5] The commerical solutions used in this work were: A, Clear Eyes; B, Lensine; C, Degest; D, Visine; E, Adapt; and F, Soquette.

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Abstract □ 3-Methyl-2-oxazolidone was evaluated as a solvent for the titration of selected barbiturates and sulfa drugs. Its high dielectric constant and wide liquid range contribute to its outstanding solvent properties. Tetrabutylammonium hydroxide was used as the titrant. End-points were determined potentiometrically using a glass–calomel electrode system. Data evaluation was performed by suitable computer programs, and relative acid strengths were determined.

Keyphrases □ 3-Methyl-2-oxazolidone—evaluated as solvent for titration of barbiturates and sulfa drugs, determination of relative acid strengths □ Barbiturates—potentiometric titration using 3-methyl-2-oxazolidone as solvent, relative acid strengths determined □ Sulfa drugs—potentiometric titration using 3-methyl-2-oxazolidone as solvent, relative acid strengths determined

Information is available concerning the physical properties, preparation, and pharmacological usefulness of highly substituted 2-oxazolidones. Since most of these compounds are solids at room temperature, they have little usefulness as analytical solvents. However, a few *N*-alkyl-substituted derivatives of the parent compound are liquids and have a wide liquid range. Little is known regarding 2-oxazolidones as possible solvents for acid–base titrations (1). Some liquid *N*-substituted 2-oxazolidones were suggested as promising solvents (2).

In this study, 3-methyl-2-oxazolidone was chosen due to its high dielectric constant and relatively low melting point. When pure, the compound is odorless and colorless. Some of its important physical properties at 25° (2) are: mp, 15.9°; bp at 1 mm, 74–75°; dielectric constant at 1 MHz, 77.5; viscosity, 2.450 cps; density, 1.1702 g/ml; and refractive index, 1.4522. A series of important weak acids of pharmaceutical interest was used to determine the suitability of 3-methyl-2-oxazolidone as a solvent for such analyses.

EXPERIMENTAL

Apparatus—A digital pH/mv meter[1] equipped with an electrode switch[2] was used for potential measurements. Glass indicator electrodes[3] were used in conjunction with porous ceramic junction calomel reference electrodes[4]. The saturated aqueous potassium chloride solutions of the calomel electrodes were replaced with a saturated solution of potassium chloride in ethanol. The electrodes were soaked for several days in 3-methyl-2-oxazolidone prior to use. To stabilize potential readings, any one pair of electrodes was resoaked in 3-methyl-2-oxazolidone before reuse.

The titrant was dispensed from a 5-ml microburet[5] graduated in 0.01-ml divisions, which meets National Bureau of Standards Specification NNN-B-789. The storage vessel was fitted with a

[1] Orion model 601.
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Table I—Acid–Base Titration Data in 3-Methyl-2-oxazolidone

Acid[a]	Milliequivalents Taken	Milliequivalents Found	Recovery[b], %
Sulfamerazine	0.1307	0.1320	99.34 ± 1.59
	0.1128	0.1103	
	0.1122	0.1112	
Sulfadiazine	0.1231	0.1260	101.92 ± 1.38
	0.2261	0.2267	
	0.1086	0.1117	
Sulfapyridine	0.2017	0.2052	100.92 ± 0.83
	0.1438	0.1452	
	0.1647	0.1649	
Barbituric acid	0.4121	0.4152	100.94 ± 0.29
	0.2589	0.2616	
	0.1363	0.1377	
Phenobarbital	0.1717	0.1712	100.50 ± 0.89
	0.1246	0.1264	
	0.1490	0.1495	
Secobarbital	0.1226	0.1239	100.08 ± 1.77
	0.1581	0.1601	
	0.1812	0.1777	
Amobarbital	0.1487	0.1479	100.75 ± 1.17
	0.1653	0.1682	
	0.2234	0.2256	
Barbital	0.1662	0.1665	99.99 ± 0.46
	0.2449	0.2437	
	0.2595	0.2605	

[a] Barbituric acid was Eastman reagent grade; barbiturates and sulfa drugs were Eli Lilly USP grade. [b] Standard deviation based on three reported values.

rubber stopper and nitrogen purge line. The titrations were performed in a 200-ml beaker[6] fitted with a rubber stopper with provisions for the electrodes, nitrogen purge line, and buret tip. To prevent titrant loss or evaporation, the buret height was adjusted so that the delivery tip just penetrated the titration solution.

Reagents—3-Methyl-2-oxazolidone was prepared by the Homeyer (3) method and purified by fractional freezing as described earlier (4). Tetrabutylammonium hydroxide[7] was diluted with methanol and standardized against benzoic acid, which was previously purified by sublimation. All samples were dried *in vacuo* prior to use and stored over anhydrous calcium sulfate. Further purification was not attempted due to limited quantities.

Procedure—Each sample weight was determined to four significant figures using an electrobalance[8]. To provide quantitative transfer of samples, all weights were determined by difference. Once the solid was transferred to the titration vessel, it was dissolved in approximately 50 ml of 3-methyl-2-oxazolidone. To avoid atmospheric contamination, the head space was flushed with nitrogen which was passed through asbestos coated with sodium hydroxide[9] to remove carbon dioxide and through anhydrous magnesium perchlorate to remove any residual traces of water.

The electrodes were allowed to stabilize for 10 min before the addition of titrant. Readings were taken after the potential had stabilized (which generally occurred in 2 min or less) to ±0.2 mv. The average time per titration was 20 min. The determination of the equivalence point and inflection point was accomplished with the use of suitable FORTRAN programs[10] by a first and second derivative *versus* volume technique.

RESULTS AND DISCUSSION

Table I lists the recovery data for all drugs studied, and Figs. 1 and 2 show the titration curves. At the present time there is no established indicator for acid–base titrations in 3-methyl-2-oxazolidone.

In addition to the good analytical results, it is possible to determine relative acid strengths from the size of the break in the titration curve in the vicinity of the equivalence point. The relative acid strengths of the barbiturates studied are barbituric acid > phenobarbital > secobarbital > amobarbital > barbital. Similarly, the relative strengths of the sulfa drugs are sulfamerazine > sulfadiazine > sulfapyridine. The samples were readily soluble in 3-methyl-2-oxazolidone. The recoveries were as good or better than those reported previously (5–8). With respect to barbital and phenobarbital, the recoveries and precision are especially encouraging. The recoveries and precision of the sulfa drugs deviate

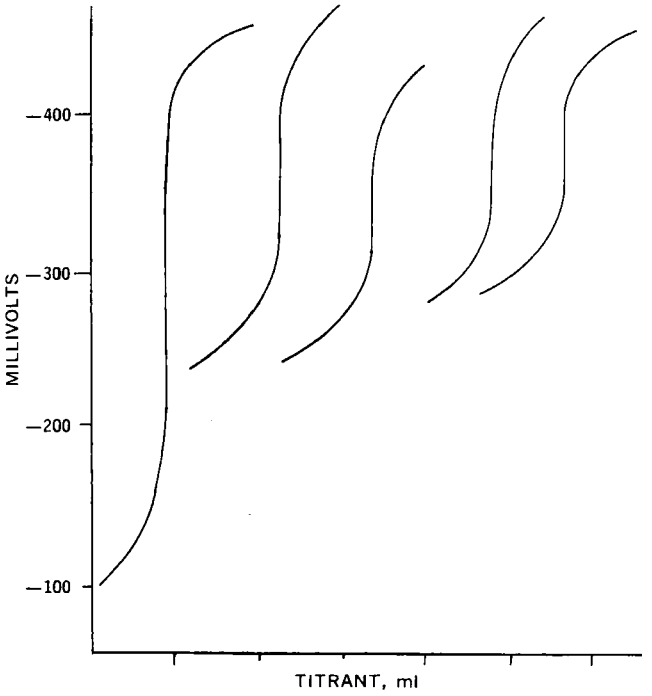

Figure 1—*Typical titration curves for (left to right) barbituric acid, phenobarbital, secobarbital, amobarbital, and barbital.*

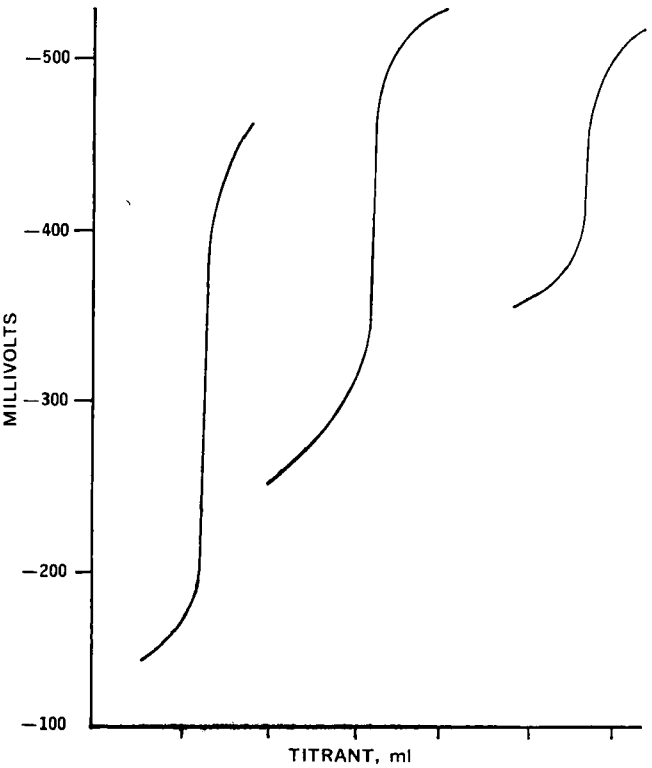

Figure 2—*Typical titration curves for (left to right) sulfamerazine, sulfadiazine, and sulfapyridine.*

[6] Berzelius.
[7] Eastman No. 7774, 25% solution in methanol.
[8] Cahn model G-2.
[9] Ascarite.
[10] University of Cincinnati IBM 370/165 Computer System.

more than expected. To explain such deviations, several blank determinations were performed to ascertain if any solvent-titrant reaction occurred. No discernible reaction was observed, which suggests that the precision might be improved by further purification of these materials.

It has been found in these laboratories that the hydroxyl and phenolic protons of salicylic acid can be titrated separately in this solvent. Work on the differentiating ability of the solvent is currently in progress. This preliminary study shows 3-methyl-2-oxazolidone to be a promising solvent in pharmaceutical assays by titration.

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PHARMACEUTICAL TECHNOLOGY

Automated Analysis of Riboflavin in Multivitamin Preparations

OMER PELLETIER [x] and RENÉ BRASSARD

Abstract □ An automated procedure was developed for the determination of riboflavin by following the steps of the USP manual procedure. The automated procedure is applicable to different types of multivitamin products and yielded results equivalent to the manual procedure.

Keyphrases □ Riboflavin—automated analysis, multivitamin preparations, compared to official method □ Multivitamin preparations—automated analysis of riboflavin, compared to official method □ Automated analysis—riboflavin in multivitamin preparations

Methods recognized officially by the Association of Official Analytical Chemists (1) or USP (2) for the determination of riboflavin in pharmaceutical preparations are described exclusively as manual techniques. These determinations consist of measuring the fluorescence of riboflavin in an acetic acid medium after destroying interfering materials by permanganate oxidation and decolorizing excess permanganate with hydrogen peroxide. Other interfering materials are measured separately after reducing riboflavin with sodium hydrosulfite. Automated procedures (3–7) previously proposed differed from the official methods (1, 2) either by omitting some steps or by using different chemical principles. An automated system was developed that maintains the specificity and validity of the manual procedures by performing exactly the same steps.

EXPERIMENTAL

Equipment—The analytical system[1] consisted of the following modules: liquid sampler II, a proportioning pump (model II), a fluorometer II, and a recorder.

Reagents—*Hydrochloric Acid, 0.1* N—Dilute 8.5 ml of concentrated hydrochloric acid (analytical reagent) to 1 liter with distilled water.

Acetic Acid and Sodium Acetate Solution (pH 2.4)—Dissolve 10.25 g sodium acetate (analytical reagent) in 300 ml distilled water, add 600 ml acetic acid, and dilute to 1 liter with distilled water.

Potassium Permanganate, 0.2% (w/v)—Dissolve 0.4 g of potassium permanganate (analytical reagent) in 200 ml distilled water. Prepare fresh daily.

Hydrogen Peroxide, 0.15% (w/v)—Dilute 1 ml of 30% H_2O_2 (analytical reagent) to 200 ml with distilled water. Prepare fresh daily.

Sodium Hydrosulfite, 5% (w/v)—Dissolve 5 g of anhydrous sodium hydrosulfite (analytical reagent) in distilled water and dilute to 100 ml. Prepare daily just before use and keep in an amber bottle under toluene.

Ascorbic Acid, 4% (w/v)—Dissolve 4 g ascorbic acid[2] in 100 ml distilled water.

Standards—*Standard Riboflavin Stock Solution (10 μg/ml)*—

[1] Technicon.

[2] Nutritional Biochemicals Corp.

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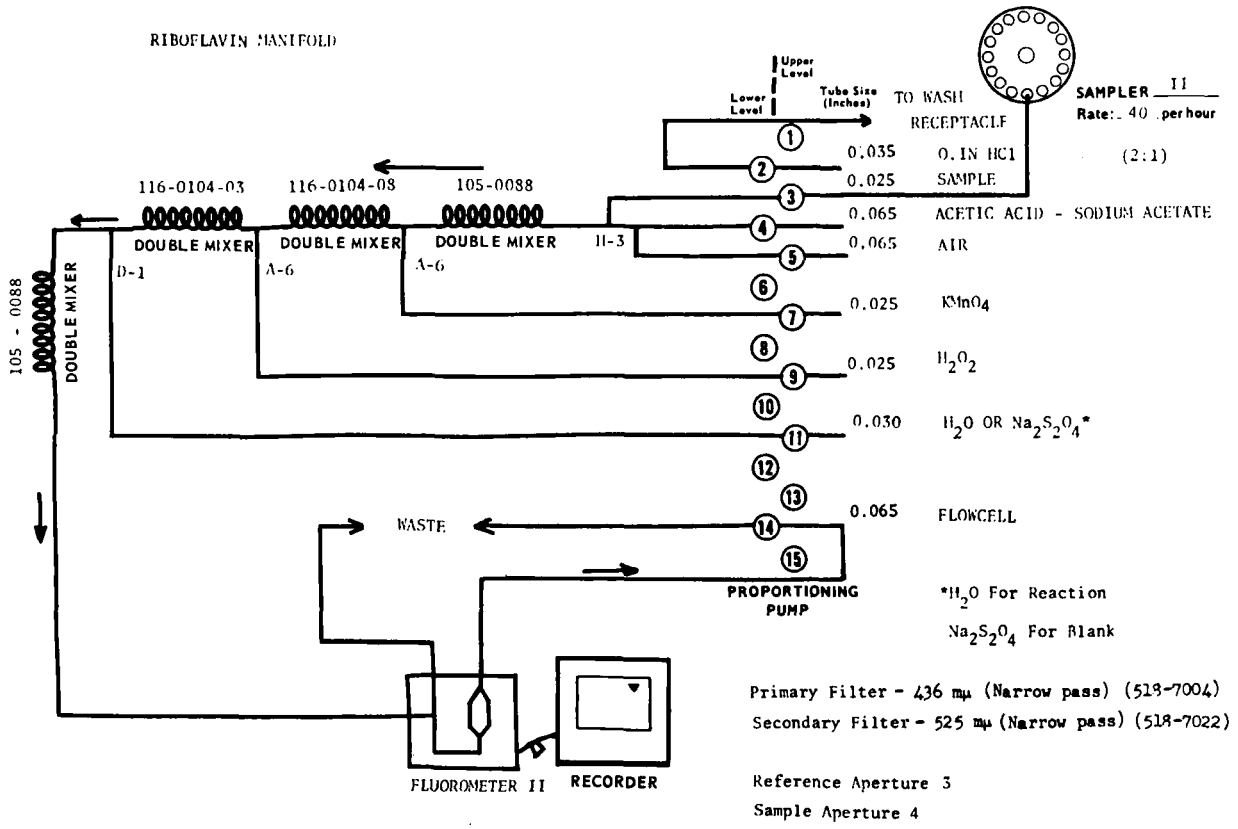

Figure 1—*Diagram of flowthrough system for determining riboflavin.*

Prepare as described for the USP procedure (2).

Intermediate Riboflavin Solution (1 μg/ml)—Dilute 10 ml standard riboflavin stock solution to 100 ml with 0.1 *N* HCl.

Working Riboflavin Solutions (0.05, 0.10, 0.15, 0.20, and 0.25 μg/ml)—Dilute 5, 10, 15, 20, and 25 ml of intermediate riboflavin solution (1 μg/ml) to 100 ml with 0.1 *N* HCl.

Controls—*Stock Riboflavin Control Solution (9 μg/ml)*—Prepare in the same way as described for the standard riboflavin stock solution (2) by weighing 45 mg and diluting to 9 μg/ml.

Working Control Solution (0.18 μg/ml)—Dilute 20 ml stock riboflavin control solution to 1 liter with 0.1 *N* HCl. Transfer volumes of approximately 40 ml into 50-ml brown[3] bottles. Store in a freezer and prepare monthly. Thaw one control bottle daily and mix vigorously before use.

Sample Preparation—Sample preparation and all the other steps of the analytical procedure must be performed in subdued light to prevent destruction of riboflavin by light.

Tablets and Capsules[4]—Grind a sample of at least five tablets to a fine powder and transfer quantitatively to a 1-liter flask with approximately 900 ml 0.1 *N* HCl. For soft core tablets or capsules, replace the grinding process by blending for about 2 min with 0.1 *N* HCl in the glass container of a blender[5]. Similarly, blend hard core capsules with 0.1 *N* HCl but use 2 ml of ethylene dichloride and 0.2 ml of acetic acid per capsule. Transfer the blended material quantitatively with 0.1 *N* HCl to a 1-liter volumetric flask and make to a volume of approximately 900 ml. Heat flask in a hot (nearly boiling) water bath with frequent shaking until a good dispersion is obtained and continue heating for an additional 15 min or autoclave at 15 lb of pressure for 15 min. Let the solution cool and dilute to 1 liter with 0.1 *N* HCl. Filter and dilute an aliquot with 0.1 *N* HCl to an expected concentration of about 0.2 μg riboflavin/ml.

Liquid Materials—Dilute an aliquot of the sample to an expected concentration of about 0.2 μg riboflavin/ml with 0.1 *N* HCl.

[3] Nalgene.

[4] Automated Technicon equipment (solid sampler) is also available to solubilize riboflavin from tablets and capsules.

[5] Waring.

Procedure—Start the automated system (Fig. 1) about 30 min before initiating the analysis. Set the reference and sample aperture of the fluorometer at the No. 3 and No. 4 positions, respectively. To avoid the formation of manganese dioxide precipitate, introduce the acetic acid and sodium acetate solution before potassium permanganate. Pump all reagents until an equilibrium is obtained and set the baseline between 2 and 5%.

Place the standards, samples, and controls into polystyrene cups of 2 ml after rinsing each cup with the solution to be analyzed to avoid contamination. Run the sequence as described in Table I.

Before determining blanks, run three cups of the 0.20-μg/ml standard. To obtain blanks, pump 5% sodium hydrosulfite in place of distilled water through tube No. 11 (Fig. 1) and run the sequence described in Table I. Rinse tube No. 11 by pumping dis-

Table I—Disposition of Standards[a] and Samples[b]

Baseline, 0.1 *N* HCl
Standard curve 1
0.05 μg/ml
0.10 μg/ml
0.15 μg/ml
0.20 μg/ml
0.25 μg/ml
Baseline, two cups 0.1 *N* HCl
Standard curve 2 (as curve 1)
Baseline, two cups 0.1 *N* HCl
Standard, 0.20 μg/ml
Control, 0.18 μg/ml
Ten samples
Baseline
Standard, 0.20 μg/ml
Control, 0.18 μg/ml
Ten samples
Baseline
Standard, 0.20 μg/ml[c]

[a] For blanks, run the standard of 0.20 μg/ml in triplicate before and after the sodium hydrosulfite treatment. [b] Every hour, run a cup of 4% ascorbic acid to remove traces of coating by potassium permanganate. [c] For additional samples, continue the same sequence starting with one standard and one control before every 10 samples.

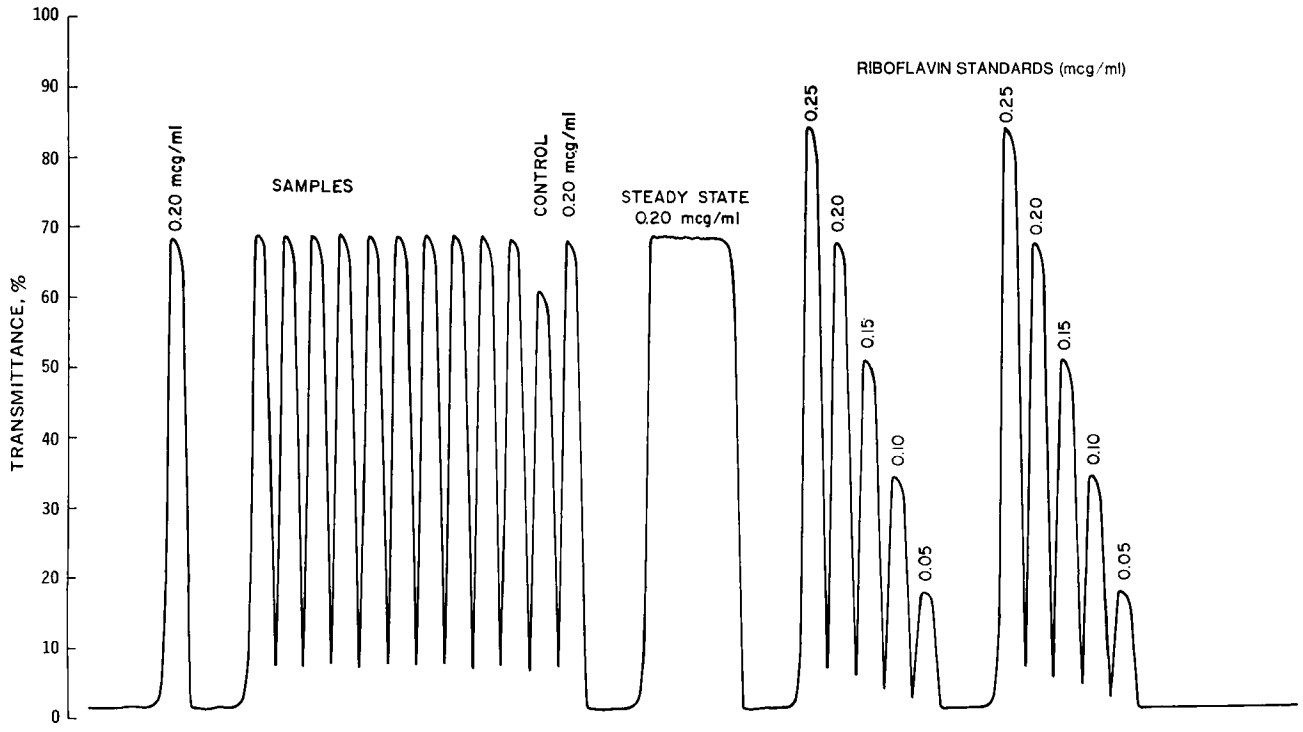

Figure 2—*Typical recordings from the automated analysis of riboflavin.*

tilled water for about 5 min before running three additional cups of the 0.20-μg/ml standard.

After completing the analysis, pump distilled water through all of the tubing except No. 14, which must be placed into water only after the potassium permanganate solution is pumped out.

Calculations—*Standard Curves*—Calculate the net fluorescence (% F) of each level of standard by subtracting from the peak the baseline averaged from before and after the standards. Calculate the slope, b, by:

$$b = \sum Y / \sum X \qquad \text{(Eq. 1)}$$

where X is the concentration, and Y is the net % F of the standard. Using the net % F of the 0.20-μg/ml standard, calculate its concentration and calculate its efficiency, E, by:

$$\text{concentration} = \frac{Y}{b} \qquad \text{(Eq. 2)}$$

and:

$$E = \frac{\text{concentration} \times 100}{0.2} \qquad \text{(Eq. 3)}$$

Follow the same procedure for the second standard curve and obtain the average slope, $\bar{b}$, and efficiency, $\bar{E}$, for the two standard curves.

Samples—Before and after every group of 10 samples, average the % F of baselines and subtract from the mean % F of the two peaks of 0.20 μg/ml standard to obtain the net % $\bar{F}_{1,2}$. Also subtract the average baseline value from the control and sample peaks to obtain the net % $F(Y)$.

Calculate the corrected slope, b^1, the percentage change of b^1 relative to b, and the concentration, X, by the following:

$$b^1 = \frac{\%\ \bar{F}_{1,2}}{0.01\bar{E} \times 0.2} \qquad \text{(Eq. 4)}$$

$$\%\ \text{change of}\ b^1 = \frac{b^1 \times 100}{\bar{b}} \qquad \text{(Eq. 5)}$$

$$X = \frac{Y}{b^1} \qquad \text{(Eq. 6)}$$

Blanks—Average the % F of the baseline before and % F of the baseline after the blanks of all of the samples, and subtract from the mean % F of three peaks of the 0.20-μg/ml standard which precede and follow these blanks. Subtract the average baseline (before and after 10 samples) from the blanks of the standards, control, and samples treated with sodium hydrosulfite. A value of about zero should be obtained with the standards, control, and most samples. Make calculations of the corrected slope, the percent change of the slope, and concentrations with Eqs. 4–6. Subtract the blank value from the corresponding sample value and multiply by the dilution factors to obtain the true riboflavin concentration.

Controls—Calculate the mean value of the controls and their standard deviations to evaluate the reproducibility on the day of analysis and the variation from day to day.

Table II—Sample Interactions[a] at Four Levels

Sample Levels, μg/ml	Range[b] of Interaction	Range[b] of Coefficient of Variation	
		Random	Continuous
0.05	1.00–1.02	0.5–0.7	0.4–0.7
0.10	1.00–1.01	0.5–1.0	0.4–0.5
0.15	1.00–1.01	0.4–0.9	0.3–0.4
0.20	1.00–1.00	0.4–0.8	0.2–0.8

[a] Interaction = ratio of 20 samples at one level randomly mixed with 20 samples from each of the other three levels over 20 samples of the same level in one continuous sequence. [b] Range obtained from assays on 3 different days.

RESULTS AND DISCUSSION

The system (Fig. 1) performs the manual procedure (2) automatically. The sampler regulates the sampling and wash times to 1 and 0.5 min, respectively. Reproducible volumes of standards or samples are introduced by the constant flow rate induced by the action of the proportioning pump on the flexible manifold tubing. Air bubbles divide each sample or wash in several segments to prevent interaction due to diffusion and to ensure proper mixing of the reagents as they are added to the continuous flow in the same sequence as in the manual procedure. The wash that separates each sample further eliminates residual interactions by removing traces left from the previous sample. The air bubbles are removed immediately before the reaction product is pumped

Table III—Comparison of Results by USP Manual Method and Automated Procedure with Various Vitamin Preparations

Sample	Composition	Riboflavin[a], mg/Tablet, Capsule, or ml		Coefficient of Variation	
		USP	Automated	USP	Automated
Tablets	Multivitamin	3.01	3.09	1.68	0.78
Capsules	Multivitamin	2.20	2.19	4.87	1.44
Tonic	Water-soluble vitamins plus minerals	0.12	0.12	5.69	1.57
Syrup	Water-soluble vitamins	0.16	0.16	4.17	1.30
Capsules	Multivitamin plus minerals	5.20	5.15	4.64	1.24
Tablets	Multivitamin plus minerals	5.62	5.63	3.00	1.37
Tablets	Multivitamin plus minerals	3.03	3.04	2.40	0.86
Tablets	Multivitamin	0.83	0.83	3.12	0.77

[a] Values represent the mean of eight assays on 8 different days.

through the flow cell, where the fluorescence is continuously measured and recorded on a chart. An example of such recording is shown in Fig. 2. Sample interactions were found to be effectively negligible (Table II) by analyzing levels of 0.05, 0.10, 0.15, and 0.20 μg riboflavin/ml arranged in groups of 20 containing one single concentration and four groups of 20 containing random mixtures of the four levels.

As shown in Table III, results obtained with the automated procedure compared favorably with those of the USP method in the analysis of eight different types of vitamin products. The mean values from eight assays on 8 different days were essentially the same by both procedures. The coefficient of variation with each sample was significantly smaller by the automated procedure.

In summary, results obtained in these laboratories indicate better reproducibility by the proposed automated procedure in comparison to the official USP manual procedure.

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NOTES

TLC Separation and Identification of Diastereomers of D-Ergonovine Maleate

DAVID L. SONDACK

Abstract □ The resolution of D-lysergic acid D-2-propanolamide from D-lysergic acid L-2-propanolamide (ergonovine) by TLC and column chromatography is described. The compound was identified by comparison with authentic samples using TLC, circular dichroism, and other physical characteristics.

Keyphrases □ Ergonovine maleate—TLC separation and identification of diastereomers □ Lysergic acid derivatives—TLC separation and identification of diastereomers of D-ergonovine maleate □ TLC—separation and identification of diastereomers of D-ergonovine maleate

During TLC examination of some samples of D-lysergic acid L-2-propanolamide maleate (ergonovine maleate), in preparation for gas–liquid partition chromatography (1), a hitherto unobserved minor spot became evident. Since it had not been observed before in this laboratory, even in aged samples, it

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Capsules	Multivitamin	2.20	2.19	4.87	1.44
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Table I—Solvent Systems

Components	Solvent System[a]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Chloroform	114	9	—	15	5	—	5	4	10	10	—	—	—
Acetone	—	—	25	10	4	15	10	4	10	—	—	—	—
Methanol	—	—	4	5	—	—	—	2	10	5	—	3	—
Triethylamine	—	—	1	1	1	—	—	—	—	—	—	—	—
Concentrated ammonia	—	—	—	—	—	0.1	0.1	0.1	0.3	0.1	1	—	—
Ethanol	29	1	—	—	—	—	—	—	—	—	—	—	5
n-Propanol	—	—	—	—	—	—	—	—	—	—	88	—	—
n-Butanol	—	—	—	—	—	—	—	—	—	—	—	2	—
Benzene	37	—	—	—	—	—	—	—	—	—	—	—	—
Acetic acid	—	—	—	—	—	—	—	—	—	—	—	—	3
Water	—	—	—	—	—	—	—	—	—	—	12	—	2

[a] The numbers in each column refer to the parts by volume of the individual solvent making up each solvent system.

was reasoned that its presence was not due to degradation. The unknown was subsequently identified with the aid of preparative column chromatography and UV, IR, optical rotatory dispersion (ORD), circular dichroism (CD), and mass spectral analysis as D-lysergic acid D-2-propanolamide.

EXPERIMENTAL

TLC—Generally, 25 μg of each substance was applied to a 20 × 20-cm plate[1], 250 μm thick, as a solution in methanol. The plates were developed in preequilibrated paper-lined tanks containing approximately 100 ml of solvent. Each chromatogram was developed until the solvent reached 1 cm from the top of the plate. Visualization of the spots was by examination under 254 nm illumination.

Thirteen solvent systems (Table I) were tested.

Liquid Chromatography ("Dry Column")—Glass columns, 190 × 23 mm, were packed firmly with silica gel[2]. A 1-g sample of ergonovine maleate was applied to the dry column as a solution in 2 ml of methanol plus 1 ml of triethylamine. Elution was performed with chloroform–acetone–methanol–triethylamine (15:10:5:1) with a pressure head of 3 lb/in.[2] of nitrogen to produce a flow rate of approximately 20 ml/hr. Five-milliliter fractions were collected[3]. Each fraction was examined by TLC, and the appropriate tubes were pooled, concentrated, and rechromatographed on a fresh column until essentially single-peak material was obtained. The purified material was concentrated in methanol and precipitated as the maleate salt by the addition of ether (10 volumes) (2).

Spectral Studies—UV spectra were obtained[4] using methanol solutions of the various alkaloids being tested. IR spectra were obtained[5] as KBr pellets.

ORD and CD spectra were obtained from methanol solutions on a spectropolarimeter[6] equipped with a circular dichroism attachment[7]. Mass spectra were also obtained[8].

RESULTS AND DISCUSSION

Since it was reasoned that the impurity observed in the sample of ergonovine (I) was not due to degradation, because it had not been seen in aged samples, attempts to identify the impurity proceeded along two lines: isolation and comparative TLC. Preparative TLC on plates 250 μm thick provided sufficient material for UV, IR, and mass spectral analysis. The UV and IR spectra closely matched literature data (3) for ergonovine maleate, and the mass spectrum was analogous to that reported for lysergic acid 2-aminobutan-1-olamide (4). This was considered to be evidence for a stereoisomer arising from an impurity in either of the two precursors, *i.e.*, D-lysergic acid or L-2-aminopropanol.

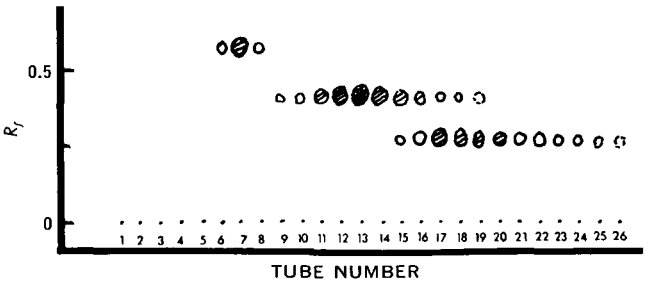
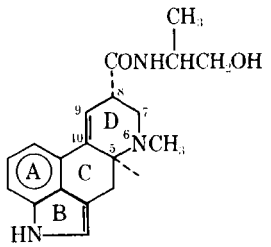


Figure 1—*TLC examination of fractions obtained from dry column chromatography. Both TLC and column chromatography were run in System 4. Key: ergometrinine, peak tube 7; ergonovine, peak tube 13; and unknown, peak tube 17.*

Authentic samples of L-lysergic acid L-2-propanolamide maleate (II), D-lysergic acid D-2-propanolamide maleate (III), and D-isolysergic acid L-2-propanolamide nitrate (IV) were obtained and subjected to comparative TLC in 13 solvent systems (Table I) along with the sample of I containing the impurity. The R_f values of II and III were identical to that of the impurity in all systems. The R_f values of the compounds tested are listed in Table II for the eight systems that gave the largest mobility. Compound IV is the result of epimerization of I at C-8 occurring readily under basic conditions.



I

System 4, which provided the best separation of the unknown from I, was used as the eluting solvent in dry column preparative chromatography. Figure 1 shows the elution pattern of the column as determined by TLC of each fraction obtained. The order of elution and relative resolution was similar to that obtained by TLC. True dry column chromatography, where the column is extruded and cut into sections, was attempted and found to be unsatisfactory. The closeness of mobility of I and the unknown required the extra running length and, indeed, rechromatography.

[1] Commercially available coated glass plates (Merck silica gel F254, Mallinckrodt Chromar 7GF and Analtech Uniplate silica gel GF) were used. The so-called preparative plates (500–2000 μm thick) afforded no resolution of the investigated minor spot.

[2] Silica gel GF254, according to Stahl, Merck.

[3] Golden Retriever Pup, model 1100, Instrumentation Specialties Co.

[4] Cary recording spectrophotometer, model 14.

[5] Beckman IR 12.

[6] Cary model 160.

[7] Cary 6001.

[8] Consolidated Electronic Corp. 21-110.

Table II—$R_f \times 100$ for Some Stereoisomers of Ergonovine

Compound	Solvent System							
	3	4	8	9	10	11	12	13
D-Lysergic acid D-2-propanolamide	37	33	44	57	62	51	40	52
L-Lysergic acid L-2-propanolamide	37	33	44	57	62	51	40	52
D-Lysergic acid L-2-propanolamide (ergonovine)	45	45	50	61	66	52	46	52
D-Isolysergic acid L-2-propanolamide(ergometrinine)	61	69	66	70	74	62	52	47

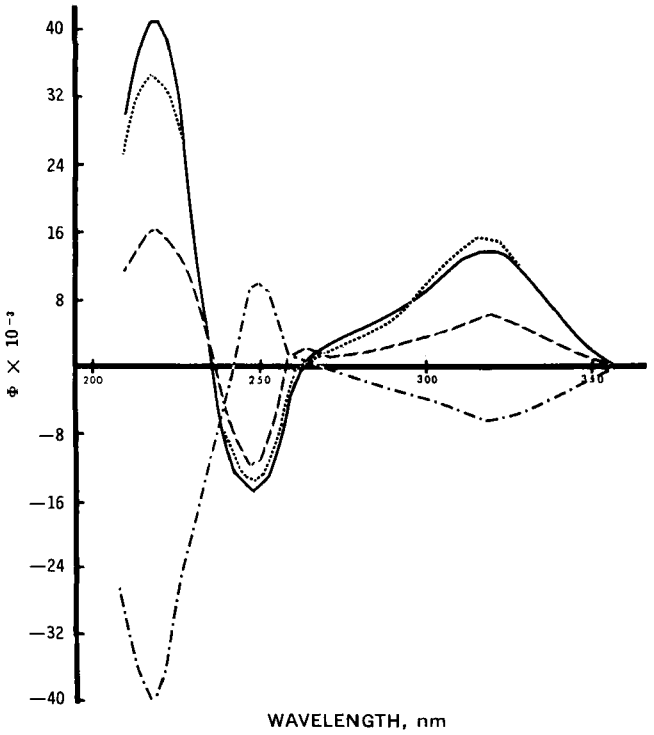


Figure 2—*CD spectra. The sample contained a drop of potassium hydroxide to aid in dissolution. Since this causes equilibration of the isolysergic and lysergic forms, the absolute values of Φ are not those of the pure compound. Key:* —, *ergonovine;* ·–· -, *II;* . . . ., *III and unknown; and* – –, *IV.*

The purified material (~5 mg), I, II, III, and IV were submitted for ORD–CD measurements and also for UV, IR, and mass spectral analyses. The latter three tests gave results unchanged from those previously obtained. CD measurements distinguished between II and III as expected (Fig. 2). The CD spectrum of the unknown resembled that of III and its identity was thereby assigned.

Unlike TLC, gas–liquid partition chromatography (1) is only capable of distinguishing derivatives of the *cis*-lysergic series from those of the diastereomeric *trans*-isolysergic series but not diastereomers within each series, *i.e.*, where a third chiral site is involved. Apparently the molecular interaction with the stationary phase in gas–liquid partition chromatography is governed by the C5–C8 relationship to the exclusion of the third chiral site in the side chain. This emphasizes the necessity for a judicious choice of derivatizing agent for gas–liquid partition chromatography resolution of enantiomers by derivatization to diastereomers, as was recently pointed out (5). Obviously, lysergic acid would not be a reagent of choice.

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NMR Spectra of Tetracyclines: Assignment of Additional Protons

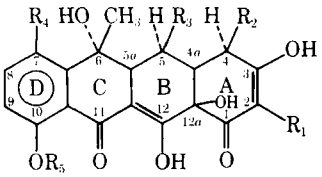
G. L. ASLESON, L. J. STOEL*, E. C. NEWMAN, and C. W. FRANK[x]

Abstract □ The assignments of four additional signals observed in the NMR spectrum of tetracycline hydrochloride were made with the help of modified tetracyclines and model compounds. The signals, all occurring downfield from 8 ppm, were caused by exchangeable protons. The signals were observed at 9.10, 9.59, 11.80, and 15.11 ppm and were assigned to the two amide protons, the 10-position hydroxyl proton, and the 12-position hydroxyl proton, respectively. The signals observed in the NMR spectra of other tetracyclines were also assigned.

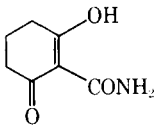
Keyphrases □ Tetracyclines—NMR spectra, assignment of additional protons □ NMR spectroscopy—assignment of additional protons in spectra of tetracyclines

NMR spectroscopy has been applied in the study of tetracyclines to elucidate stereochemical and structural features (1–6), to determine the microscopic dissociation constants (7, 8), and to monitor the kinetics of epimerization at the 4-position (9). The assignments of some protons in 16 tetracyclines were reported (10). The application of NMR to problems in tetracycline chemistry has not been extensive because the spectra obtained are fairly complex and the solubility properties of tetracyclines limit the choice of solvents. Observations of signals between 4 and 16 ppm for the exchangeable protons of tetracycline have been reported, but no assignments have been made (10).

Four exchangeable protons, all downfield from 8 ppm *versus* tetramethylsilane, were observed in the NMR spectrum of tetracycline hydrochloride (I) in deuterated dimethyl sulfoxide at 9.10, 9.59, 11.80, and 15.11 ppm (Fig. 1). Similar NMR spectra were obtained for oxytetracycline hydrochloride (II) and chlortetracycline hydrochloride (III) with signals at 9.15, 9.65, 11.75, and 15.19 ppm for the former and at 9.18, 9.67, and 12.28 ppm for the latter. The structure of the tetracycline molecule contains seven easily exchangeable protons, of which five are hydroxyl protons and two are associated with the amide functional group.



	R_1	R_2	R_3	R_4	R_5
I:	$CONH_2$	$N(CH_3)_2$	H	H	H
II:	$CONH_2$	$N(CH_3)_2$	OH	H	H
III:	$CONH_2$	$N(CH_3)_2$	H	Cl	H
IV:	CN	$N(CH_3)_2$	H	H	H
V:	CN	$N(CH_3)_2$	H	H	$SO_2C_6H_5$
VI:	$CONH_2$	H	H	H	H



VII

The variability of hydroxyl proton chemical shifts is caused principally by hydrogen bonding which, in turn, depends upon concentration, temperature, and solvent. Intramolecular hydrogen bonds are much less susceptible to change by these factors than intermolecular bonds. The reasons for a downfield shift resulting from hydrogen bonding are not completely understood, but it is generally found that the magnitude of the downfield shift increases with the strength of the hydrogen bond (11). The 6- and 12*a*-position hydroxyl protons would not be expected to have chemical shifts downfield from 8 ppm.

EXPERIMENTAL

NMR Spectra—The NMR spectra were obtained using high-resolution NMR spectrometers[1] at probe temperatures of 33 ± 1°. The chemical shift data were measured relative to tetramethylsilane[2] as an internal standard, and all values are presented as parts per million. All NMR spectra were recorded using d_6-dimethyl sulfoxide as a solvent unless otherwise noted. Deuterated solvents were obtained from commercial sources[3].

Compounds—Samples of tetracycline hydrochloride[4], oxytetracycline hydrochloride[4], dedimethylaminotetracycline[4], and chlortetracycline[5], as well as the model compounds and starting materials[6] for the syntheses of model compounds, were purchased from commercial sources.

2-Carbamoylcyclohexane-1,3-dione was synthesized by the procedure of Tomino (12). 8,9,10-Trihydroxy-1-keto-1,2,3,4-tetrahydroanthracene was synthesized by the procedure of Hochstein *et al.* (13). 2-Cyanotetracycline was prepared according to Söder and Siedel (14). The synthesis of 10-benzenesulfonyl-2-cyanotetracycline followed the procedure of McCormick *et al.* (15).

RESULTS AND DISCUSSION

A number of model compounds have structures similar to portions of the tetracycline molecule. These compounds and modified tetracyclines were investigated to aid in the assignments of the observed exchangeable protons of tetracycline. The data shown in Table I for some model compounds give the range of the chemical shift of a hydroxyl proton in phenols beta to a carbonyl, in β-diketones, and in β-triketones.

Signals of identical shape were observed in the NMR spectrum of tetracycline hydrochloride at 9.10 and 9.59 ppm. The NMR spectrum of 2-carbamoylcyclohexane-1,3-dione (VII), which is similar to the A-ring in tetracycline, produced signals at 8.7 and 9.5 ppm which were assigned to the amide protons. The assignments of

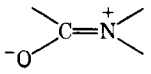
[1] Varian A-60 and HA-100.
[2] Matheson, Coleman and Bell.
[3] Stohler Isotopes and Bio-Rad Laboratories.
[4] Pfizer, Inc.
[5] American Cyanamid Co.
[6] Aldrich Chemical Co.

Table I—Chemical Shifts of Hydroxyl Protons

Compound	Parts per Million
Phenols	
1,8-Dihydroxyanthraquinone	12.0
1-Amino-4-hydroxyanthraquinone	13.9
5-Hydroxy-1,4-naphthoquinone	12.0
1,8-Dihydroxynaphthalene	11.0
1-Amino-4-hydroxy-2-methoxyanthraquinone	14.22[a] (16)
Chrysin, galangin, quercetin, kaempferol, myricetin, morin	12.40 to 12.86[b] (17)
Pinocembrin, naringenin, hesperetin, dihydroquercetin, dihydrokaempferol	11.95 to 12.20[b] (17)
β-Diketones	
2,4-Pentanedione	15.1
1-Phenyl-1,3-butanedione	16.3
1,3-Diphenyl-1,3-propanedione	17.2
1,3-(4,4′-Dibromo)diphenyl-1,3-propanedione	16.61[a] (16)
Ethyl anisoylacetate	15.3[a] (16)
β-Triketones	
2-Carbamoyl-5,5-dimethylcyclohexane-1,3-dione	18.7[c] (18)
2-Carbamoyldecalin-1,3-dione	18.98[c] (18)
3-Acetylpentane-2,4-dione	17.4[d] (19)
2-Acetyl-5,5-dimethylcyclohexane-1,3-dione	18.1[d] (19)
2-Hydrocinnamoyl-5,5-dimethylcyclohexane-1,3-dione	18.1[d] (19)
2-Cinnamoyl-5,5-dimethylcyclohexane-1,3-dione	18.6[d] (19)
Ceroptene	18.9[d] (19)

[a] *d*-Chloroform. [b] d_6-Dimethyl sulfoxide. [c] d_5-Pyridine. [d] Carbon tetrachloride.

these protons in 2-carbamoyl-5,5-dimethylcyclohexane-1,3-dione and 2-carbamoyldecaline-1,3-dione to signals observed between 9.6 and 10.3 ppm in d_5-pyridine solution were previously made (18). No signals were observed in the spectra of either 2-cyanotetracycline (IV) or 10-benzenesulfonyl-2-cyanotetracycline (V) in the 9.0–10.5-ppm range. Thus, the signals at 9.10 and 9.59 ppm in the spectrum of tetracycline hydrochloride were assigned to the amide protons. The nonequivalence of these protons can be explained by the hindered rotation about the carbon–nitrogen bond due to the contribution of the canonical form (20):



The third downfield signal in the spectrum of tetracycline hydrochloride was observed at 11.80 ppm. As shown in Table I, most phenolic protons, favorably situated for chelation with a carbonyl, have chemical shifts in the range of 12 ppm. The only tetracyclines shown in Table II that have no observed signals near 12 ppm are 10-benzenesulfonyl-2-cyanotetracycline and dedimethylaminoanhydrotetracycline (IX). The absence of this signal for 10-benzenesulfonyl-2-cyanotetracycline is easily explained because the phenolic proton has been replaced by the benzenesulfonyl ester. Thus, the signal at 11.80 ppm in the spectrum of tetracycline hydrochloride was assigned to the phenol in the 10-position. The case of dedimethylaminoanhydrotetracycline is discussed later.

The final peak was observed in the spectrum of tetracycline hydrochloride at 15.11 ppm. All tetracyclines in Table II have a signal in the 15–17-ppm range with the exception of chlortetracycline hydrochloride. The β-diketones in Table I also have their enolic proton chemical shifts in the 15–17-ppm range. Thus, the signal at 15.11 in tetracycline hydrochloride was assigned to the hydroxyl proton in the 12-position.

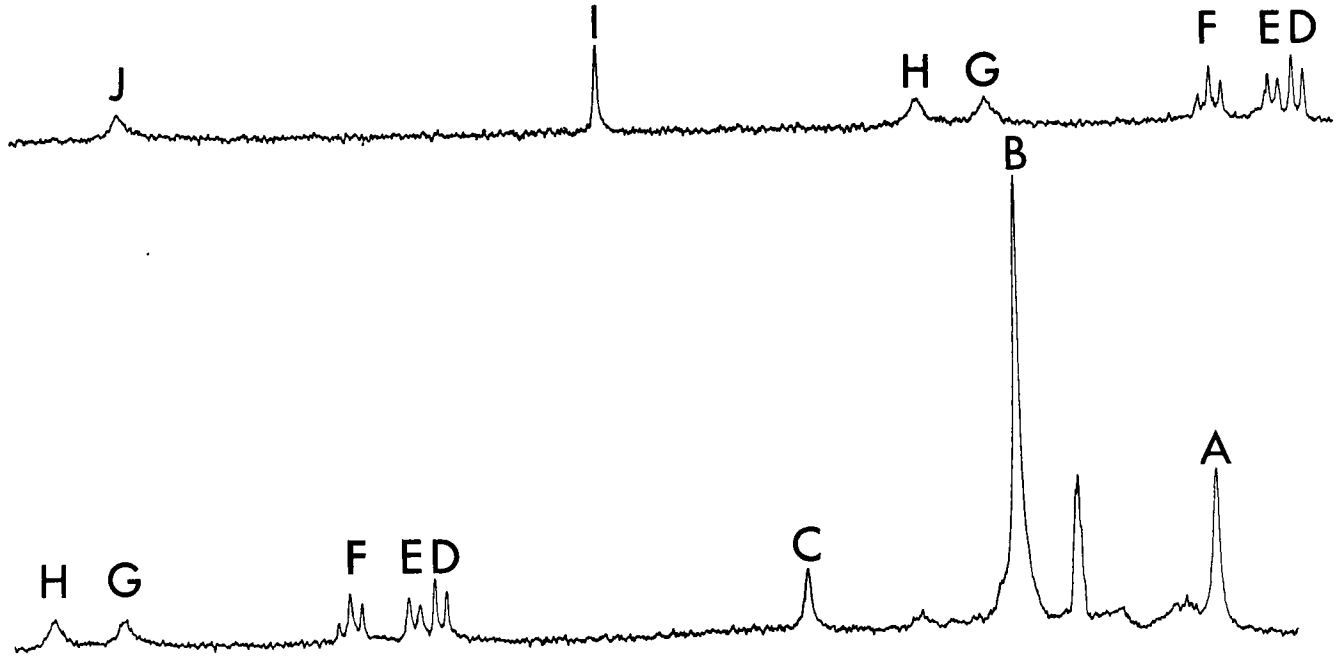


Figure 1—*NMR spectrum of tetracycline hydrochloride in* d_6*-dimethyl sulfoxide (0.20* M*). Key: A, 1.54 ppm, C-6,* CH_3*; B, 2.92 ppm, C-4,* $N(CH_3)_2$*; C, 4.38 ppm, C-4, H; D, 6.92 ppm, C-9, H; E, 7.10 ppm, C-7, H; F, 7.55 ppm, C-8, H; G, 9.10 ppm; H, 9.59 ppm; I, 11.80 ppm; and J, 15.11 ppm. See Table II for additional assignments.*

Table II—Proton Resonance Data for Tetracyclines

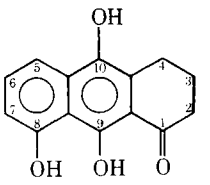
Compound	NH_1	NH_2	10-OH	12-OH	3-OH
I	9.10	9.59	11.80	15.11	—[b]
II	9.15	9.65	11.75	15.19	—[b]
III	9.18	9.67	12.28	—[b]	—[b]
IV	—[a]	—[a]	11.90	15.28	—[b]
V	—[a]	—[a]	—[a]	16.26	—[b]
VI	8.70	8.94	11.83	15.24	18.34
IX	8.85	8.98	9.90	15.29[c]	18.16

[a] Proton not present in compound. [b] Not observed in NMR spectrum. [c] 11-Position hydroxyl proton.

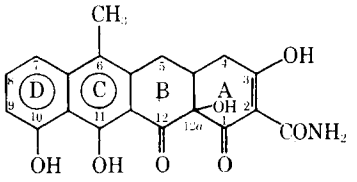
The assignments of the 10- and 11-position hydroxyl protons for dedimethylaminoanhydrotetracycline (Table II) were aided by investigation of the model compound 8,9,10-trihydroxy-1-keto-1,2,3,4-tetrahydroanthracene (VIII). Three signals for the exchangeable protons for this compound were observed at 8.60, 9.84, and 15.65 ppm. The 8.60-ppm signal was assigned to the 10-hydroxyl proton, because it is not capable of intramolecular hydrogen bonding. The 8-hydroxyl proton would not be expected to hydrogen bond intramolecularly as strongly as the 9-hydroxyl proton. Therefore, the signal at 9.84 ppm was assigned to the 8-hydroxyl proton and the signal at 15.65 ppm was assigned to the 9-hydroxyl proton. The 15.65-ppm signal fell into the range of chemical shifts for enolic protons of β-diketones, providing additional evidence for this assignment. The signals observed at 9.90 and 15.29 ppm in the spectrum of dedimethylaminoanhydrotetracycline were assigned to the 10-position and 11-position hydroxyl protons, respectively.

Finally, it must be explained why only four signals were detected in the downfield portion of the NMR spectrum of tetracycline hydrochloride instead of the predicted five. As shown in Table II, signals were observed for dedimethylaminotetracycline (VI) and dedimethylaminoanhydrotetracycline at greater than 18 ppm and were assigned to the 3-position hydroxyl proton on the basis of the data for β-triketones in Table I.

It has been observed that the presence of the dimethylamino group increases the acidity of the 3-position hydroxyl (21, 22).



VIII



IX

Therefore, the signal of the 3-position enolic proton in tetracycline hydrochloride was not observed because of rapid exchange.

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* Present address: Department of Chemistry, Augustana College, Rock Island, Ill.

x To whom inquiries should be directed.

Biotransformation of Isosorbide Dinitrate in Humans

W. H. DOWN ˣ, L. F. CHASSEAUD, and R. K. GRUNDY

Abstract □ An oral dose of ^{14}C-isosorbide dinitrate was rapidly absorbed and eliminated by human subjects. Up to 99% was excreted in the urine. The drug was completely biotransformed, nearly 50% to the principal metabolite, an isosorbide conjugate, presumably with glucuronic acid. Up to 13% was excreted as free and conjugated 5-isosorbide mononitrate, and 1% was excreted as the free 2-isosorbide mononitrate only. D-Sorbitol possibly accounted for a further 18% of the radioactivity; an unknown metabolite, less polar than isosorbide, accounted for 6%.

Keyphrases □ Isosorbide dinitrate, radiolabeled—biotransformation and excretion after oral administration, humans □ Biotransformation—isosorbide dinitrate, excretion after oral administration, humans □ Metabolism—isosorbide dinitrate, humans

Despite the widespread use of isosorbide dinitrate as a coronary dilator (1–4), there is little reported regarding its metabolism in humans. Dietz (5) detected isosorbide mononitrates, but not unchanged drug, in the urine of patients. Isosorbide dinitrate was rapidly biotransformed by isolated perfused rat liver (6) through the glutathione-dependent organic nitrate reductase system (7), which is also present in human liver (8).

Metabolism studies are greatly facilitated by the use of radioisotopes, and this paper describes the fate of ^{14}C-isosorbide dinitrate in humans.

EXPERIMENTAL

Materials—Uniformly labeled ^{14}C-isosorbide dinitrate of specific activity 2.36 mCi/mmole[1] and radiochemical purity >97% was used. Isosorbide dinitrate, isosorbide 2-mononitrate, isosorbide 5-mononitrate, and isosorbide were used as reference compounds[2].

Drug Administration—The subjects[3] did not take other drugs during the study and were under suitable medical supervision. Adverse effects attributable to the drug were not reported by the subjects.

After a 12-hr fast, each subject swallowed a gelatin capsule containing 5 mg of isosorbide dinitrate dissolved in acetone, together with 150 ml of water. The subjects fasted for a further 4 hr postadministration but took 150 ml of water hourly to maintain urine volume. All the urine and feces excreted during 5 days were collected and stored at −20°.

Extraction of Radioactivity—Samples of urine were freeze dried for 16 hr and each residue was extracted with methanol. After filtration of the residue, the methanol solution was evaporated under reduced pressure for examination by TLC, with a recovery of radioactivity of 99.6%. Feces were macerated with methanol and, after centrifugation, the radioactivity was determined in samples of the supernate and the residue.

Measurement of Radioactivity—Radioactivity was measured in a liquid scintillation analyzer[4] with automatic quench correction by external standard channels ratio (9). Urine samples and solvent extracts of urine and feces were mixed with a toluene–polyethylene glycol alkyl aryl ether[5]-based scintillator (10). Samples of the residues from solvent extractions of feces were combusted using a modified plastic bag technique (11), and the combustion products were absorbed into a β-phenylethylamine-based scintillator system (12).

TLC and Detection of Metabolites—TLC was carried out on prelayered kieselgel F_{254} plates[6] of 0.25-mm thickness. The solvent systems used were: *(a)* benzene–ethyl acetate (1:1 v/v) and *(b)* isopropanol–concentrated ammonium hydroxide (4:1 v/v).

^{14}C-Labeled metabolites were detected by autoradiography using X-ray film[7]. The radioactive areas of the silica gel were removed and measured for radioactivity in a toluene–polyethylene glycol alkyl aryl ether[5]-based scintillator gel (13). This technique gave recoveries of radioactivity exceeding 95%. Isosorbide mononitrates were located by spraying the plates with 1% (w/v) diphenylamine in ethanol and exposing to UV light for 5 min (5). They appeared as brown spots. Isosorbide was located by spraying with a metaperiodate–permanganate reagent (14), and it appeared as a yellow-brown spot on a violet background.

RESULTS

Excretion of Radioactivity by Human Subjects—Following a single oral dose of 5 mg of ^{14}C-isosorbide dinitrate, the radioactivity was readily absorbed and rapidly excreted by human subjects (Fig. 1). Up to 25% was excreted in the urine in 6 hr, 49% in 12 hr, and 78% in 24 hr. After 5 days, up to 99% of the radioactive dose had been excreted in the urine and 0.8% in the feces. The urinary excretion data show that an oral dose of ^{14}C-isosorbide dinitrate was rapidly and almost completely absorbed from the GI tract of humans.

The urinary excretion half-life for about 95% of the radioactivity was 10 hr (9.8–10.2 hr). The remainder was excreted more slowly[8].

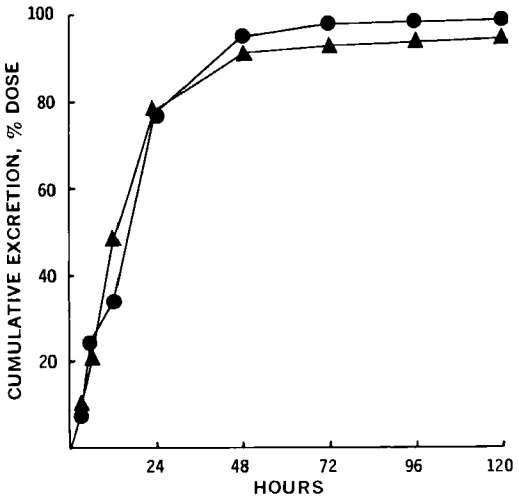


Figure 1—*Cumulative excretion of radioactivity in the urine of Subject 1 (●) and Subject 2 (▲) after an oral dose of 5 mg of ^{14}C-isosorbide dinitrate.*

[1] Synthesized by New England Nuclear Chemicals GmbH, Frankfurt, Germany.

[2] Provided by Sanol-Arzneimittel Dr. Schwarz GmbH, Monheim, Germany.

[3] With the approval of the Isotope Advisory Panel of the Medical Research Council, two male human subjects, both with normal renal and hepatic function, volunteered to take part in the studies. After the aim of the studies and the nature of the drug had been explained, the subjects gave their consent.

[4] Philips N. V., Holland.

[5] Octoxynol-9, Fisons Ltd., Loughborough, England.

[6] Merck A.-G., Darmstadt, Germany.

[7] Kodirex, Kodak Ltd., Hemel Hempstead, England.

[8] Blood also was withdrawn in this study. The results obtained will be reported elsewhere.

Table I—Urinary Metabolites of Isosorbide Dinitrate

Metabolite	R_f[a]	Administered Dose, % 0–12 hr	12–24 hr	24–48 hr	48–120 hr	Total Excreted, %
Subject 1:						
1	0–0.05	27.02	41.80	15.86	1.91	86.59
2	0.23 (5-mononitrate)	2.86	0.91	0.51	0.08	4.36
3	0.31 (2-mononitrate)	1.09	0.04	—	—	1.13
Subject 2:						
1	0–0.05	42.23	28.27	11.79	1.70	83.99
2	0.23 (5-mononitrate)	2.51	0.69	0.26	0.09	3.55
3	0.31 (2-mononitrate)	0.31	0.10	0.03	—	0.44

[a] Solvent system *a*.

Table II—Urinary Metabolites of Isosorbide Dinitrate after Deconjugation with β-Glucuronidase–Aryl Sulfatase

Metabolite	R_f[a]	Administered Dose, % 0–12 hr	12–24 hr	24–48 hr	48–120 hr	Total Excreted, %
Subject 1:						
1	0–0.05	22.29	40.71	15.93	2.24	81.17
2	0.23 (5-mononitrate)	10.03	1.30	1.45	0.06	12.84
3	0.31 (2-mononitrate)	0.78	0.06	0.08	—	0.92
4	0.57 (dinitrate)	0.38[b]	—	—	—	0.38
Subject 2:						
1	0–0.05	39.94	28.03	12.13	1.84	81.94
2	0.23 (5-mononitrate)	6.71	1.10	0.32	0.04	8.17
3	0.31 (2-mononitrate)	0.57	0.07	0.07	—	0.71
4	0.57 (dinitrate)	0.15[b]	—	—	—	0.15

[a] Solvent system *a*. [b] Excreted during 0–3 hr only.

Table III—Urinary Metabolites of Isosorbide Dinitrate after Deconjugation with β-Glucuronidase–Aryl Sulfatase

Metabolite	R_f[a]	Administered Dose, % 0–12 hr	12–24 hr	24–48 hr	48–120 hr	Total Excreted, %
Subject 1:						
1	0.10 (D-sorbitol)	4.09	6.33	3.38	0.43	14.23
2	0.55 (isosorbide)	10.86	23.18	9.86	0.63	44.53
3	0.64 (unknown)	1.90	2.78	1.11	0.26	6.05
4	0.75[b]	8.83	2.69	1.45	0.24	13.21
Subject 2:						
1	0.10 (D-sorbitol)	11.92	4.59	1.62	0.30	18.43
2	0.55 (isosorbide)	22.60	18.73	7.81	0.83	49.97
3	0.64 (unknown)	2.60	1.55	0.71	0.19	5.05
4	0.75[b]	6.20	1.67	0.83	0.21	8.91

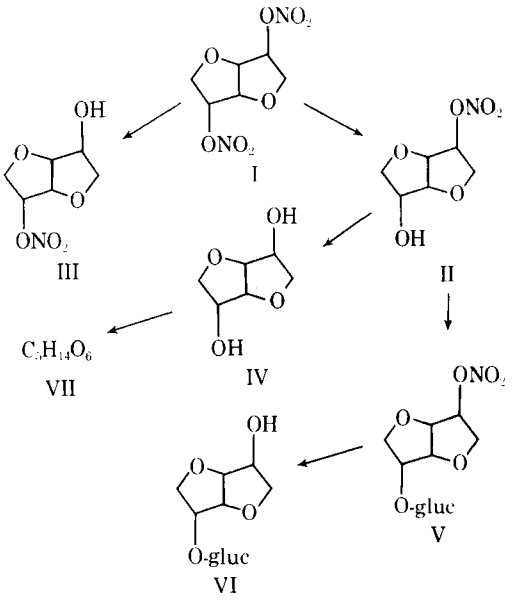
[a] Solvent system *b*. [b] R_f values: dinitrate, 0.81; 5-mononitrate, 0.76; and 2-mononitrate, 0.74.

Detection and Identification of Metabolites—The proportions of metabolites excreted in the urine of human subjects following oral administration of ^{14}C-isosorbide dinitrate are shown in Tables I–III. Isosorbide 5-mononitrate, isosorbide 2-mononitrate, and isosorbide were identified by cochromatography with authentic compounds.

More than 80% of the radioactivity was excreted in the urine as polar material and only about 4% corresponded to the 5-mononitrate (II) and 1% to the 2-mononitrate (III) (Table I). Unchanged isosorbide dinitrate (I) was only detected in trace amounts in the 0–3-hr urine (Table II). Incubation of the methanolic urinary extracts with a β-glucuronidase/aryl sulfatase preparation[9] or acid hydrolysis suggested that isosorbide 5-mononitrate was excreted partly conjugated, whereas isosorbide 2-mononitrate was excreted only as the free compound. The results (Table II) indicated that about 8% of the dose was excreted as conjugated isosorbide 5-mononitrate (V).

Most polar material was excreted as conjugated isosorbide (VI) produced by complete denitration of isosorbide dinitrate (Table

[9] Type H-2, Sigma Chemical Co. Ltd., Kingston, Surrey, England.



Scheme I—Possible biotransformation of isosorbide dinitrate in humans (gluc represents glucuronic acid)

III). Since sulfates of isosorbide (IV) or isosorbide 5-mononitrate would not be expected to be hydrolyzed by the enzyme preparation used, which contained an aryl sulfatase, it may be concluded that the conjugates are glucuronides (Tables II and III).

An unidentified metabolite, which was less polar than isosorbide (IV), accounted for about 6% of the dose and a more polar metabolite for about 16% (Table III). This latter polar metabolite was chromatographically similar to D-sorbitol (VII), which would be produced by ring opening of IV. Further characterization of the metabolites by mass spectrometry was precluded by the low dose administered resulting in only small quantities of metabolites.

DISCUSSION

Denitration of organic nitrates has been extensively studied (7, 15–21). In animals, denitration is the major biotransformation route of isosorbide dinitrate (5–8, 22–24) and this is so in humans (Tables I–III).

Identification of the radioactive components excreted in the urine showed that only traces of isosorbide dinitrate, the parent drug, were excreted (Table II). Both isomeric mononitrate metabolites were excreted, the 2-mononitrate more rapidly and in smaller amounts than the 5-mononitrate (Tables I and II). Greater production of the 5-mononitrate *(endo)* than of the 2-mononitrate *(exo)* cannot be explained on the basis of sterically assisted enzymic denitration since the chemical displacement of the 5-*endo* group is more facile (25).

Isosorbide 5-mononitrate was excreted partly conjugated and isosorbide (IV) was excreted completely conjugated, probably with glucuronic acid. In dogs, isosorbide glucuronide (VI) was apparently derived from isosorbide 5-mononitrate glucuronide (V) and not from isosorbide (IV), which was excreted unchanged when administered to the dog (22, 23). Since IV is also excreted unchanged by humans (22, 26), VI was probably derived from V in humans (Tables II and III) and not from isosorbide (IV) itself. A possible metabolic pathway for isosorbide dinitrate in humans is shown in Scheme I.

These data are qualitatively similar to those obtained from rats (24) and dogs (22, 23), except that Sisenwine and Ruelius (23) did not detect isosorbide 2-mononitrate (III) in dog urine whereas Reed *et al.* (22) did. However, larger doses were administered to the animals than the clinical dosage used in the present studies.

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[x] To whom inquiries should be directed.

Comparison of Fluorometric Procedures for Assay of Amphetamine

ANIL C. MEHTA and STEPHEN G. SCHULMAN ˣ

Abstract □ Five methods for the fluorometric determination of amphetamine were examined. The acetylacetone–formaldehyde procedure for colorimetric analysis of amphetamine was modified, and its accuracy and precision compared favorably with those obtained with a fluorescamine procedure. Only these two methods were suitable for the trace analysis of amphetamine. The formaldehyde–sulfuric acid method is less sensitive and slightly inferior in precision to these two procedures. The native fluorescence method is simple but far less sensitive, and the 2,5-hexanedione method was unsuitable for the quantitative analysis of amphetamine.

Keyphrases □ Amphetamine—comparison of five fluorometric methods □ Fluorometry—determination, amphetamine, comparison of five methods

The continued use of amphetamine and its derivatives as drugs of abuse stimulates continued interest in the evaluation of selective and sensitive methods for the detection and determination of these compounds. In particular, there is interest in the fluorometric determination of amphetamines because of its great sensitivity.

Chemically, amphetamine is a primary alkyl amine having weak native fluorescence. Therefore, most fluorometric procedures for its determination depend upon reactions involving the primary aliphatic amino group. Since many methods of this type have recently appeared, it was decided to undertake the present comparison and, where appropriate, modification of the available methods. This information should be useful in selecting the fluorometric procedure most suited for a particular situation.

EXPERIMENTAL

Fluorescence spectra (uncorrected) were taken on a spectrophotofluorometer[1] equipped with a 150-w xenon source, R-106 photomultiplier, strip chart recorder[2], and 10-mm quartz cells. The instrument was operated in the ratio recording mode, and the slit settings allowed for 6- (excitation) and 10- (emission) nm bandpass.

Dextroamphetamine sulfate[3], sodium amobarbital[4], reagent grade sulfuric acid[5], boric acid[5], hydrochloric acid[5], acetic acid[5], sodium acetate[5], sodium chloride[5], sodium hydroxide[5], reagent grade 2,5-hexanedione[6], acetylacetone[6], *n*-pentanol[6], 40% formaldehyde[6], spectroquality acetone[6], spectroquality ethanol[6], spectroquality chloroform[6], spectroquality dioxane[6], spectroquality acetonitrile[6], and fluorescamine[7] were used without further purification.

The pH 9 borate buffer was prepared by titrating 0.2 *M* boric acid with 0.1 *M* sodium hydroxide. Solutions of required concentration were prepared by dilution with distilled deionized water.

Procedure I—This method is based on the native fluorescence of amphetamine. Amphetamine shows native fluorescence in 0.1 *N* sulfuric acid at the wavelength combination of 260 (excitation) and 282 (emission) nm (1). This observation was confirmed in the present experiments, although the intensity was found to be very weak. Amphetamine did not show fluorescence in other solvents such as distilled water, 0.1 *M* sodium hydroxide, and ethanol.

Procedure II—A procedure of Wachsmuth and Koeckhoven (2) for the colorimetric analysis of amphetamine was used. Amphetamine reacts with 2,5-hexanedione in acetic acid to form 1-(1′-phenylisopropyl)-2,5-dimethylpyrrole, which gives a green fluorescence under UV radiation. The aim was to make this method more sensitive by using the fluorescence rather than absorption. To a 0.5-ml aliquot of amphetamine sulfate in 1 *N* acetic acid, 0.2 ml of 2,5-hexanedione and 5.0 ml of pentanol were added. The mixture was heated in a stoppered tube on a boiling water bath for 50 min. After cooling to room temperature, its fluorescence was observed. The wavelength combination was found to be 400/490 nm (excitation/fluorescence).

Procedure III—A colorimetric procedure of Wachsmuth and Koeckhoven (3) was modified. In this procedure, amphetamine reacts with acetylacetone and formaldehyde to give a colored 1,4-dihydrolutidine derivative. This compound was also found to be fluorescent. A mixture of 2.0 ml of amine solution in 0.1 *N* sulfuric acid, 4.0 ml 50% sodium acetate, 0.25 ml acetic acid, 0.15 ml acetylacetone, and 0.25 ml 35% formaldehyde was heated at 60° for 20 min with occasional stirring. After cooling, it was quantitatively transferred to a 10-ml volumetric flask and diluted to volume with 50% ethanol and its fluorescence was observed. A linear relationship between relative fluorescence intensity (395/475 nm) and concentration was obtained for amphetamine sulfate concentrations below 10 ppm (3.7×10^{-5} *M*). The lower limit of detection (that concentration of amphetamine sulfate giving a signal equal to twice that of the solvent blank) of amphetamine sulfate by this method was found to be 2.3×10^{-9} *M*.

Procedure IV—A general fluorometric procedure for assaying primary amines using fluorescamine reagent was reported (4, 5). This procedure was applied to amphetamine determination without modification. The excitation and emission maxima of the fluorophore lie at 395 and 475 nm, respectively. A 0.2-ml sample aliquot was buffered to pH 9 with 3.0 ml of sodium borate buffer. To this, 1.0 ml of a solution of fluorescamine in acetone (24 mg/100 ml) was added. Rapid addition and constant stirring of the solution are essential for optimal results. The mixture was diluted to 10.0 ml with borate buffer. The calibration curve drawn at 395/475 nm was linear below 10 ppm amphetamine sulfate (3.7×10^{-5} *M*). The lower limit of detection of amphetamine sulfate was 5.1×10^{-11} *M*, employing derivatization with fluorescamine.

Procedure V—A fluorogenic reaction (6) was used without modification. A 1.0-ml sample aliquot in 0.1 *N* sulfuric acid mixed with 1.0 ml formaldehyde–concentrated sulfuric acid reagent (1:20) and 5.0 ml concentrated sulfuric acid was heated for 15 min at 60°. It was cooled and diluted to 50 ml; then, after 15 min, the intensity of fluorescence was measured at 385/445 nm. The calibration curve constructed at the same wavelength combination was linear below 150 ppm amphetamine sulfate (5.5×10^{-4} *M*). A lower limit of detection of 7.3×10^{-6} *M* amphetamine sulfate was obtained.

Determination of Amphetamine Mixtures—Three synthetic mixtures of dextroamphetamine sulfate and sodium amobarbital were prepared. Each mixture was dissolved in 0.1 *N* sulfuric acid. Amobarbital was removed by extraction with chloroform. Stock

[1] Perkin-Elmer model MPF-2A, Perkin-Elmer Corp., Norwalk, Conn.
[2] Hitachi model QPD-33.
[3] Smith Kline and French, Philadelphia, Pa.
[4] Eli Lilly and Co., Indianapolis, Ind.
[5] Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Matheson, Coleman and Bell Inc., East Rutherford, N.J.
[7] American Instrument Co., Silver Spring, Md.

Table I—Analysis of Amphetamine in Mixtures

Procedure	Mixture[a]	Amphetamine Found[b], mg	Error, %	Standard Deviation[c], mg
III	1	4.85	−3.0	
	2	5.10	+2.0	
	3	5.25	+5.0	±0.11
IV	1	4.80	−4.0	
	2	4.80	−4.0	
	3	4.95	−1.0	±0.13
V	1	4.85	−3.0	±0.18

[a] Each mixture contained 5 mg amphetamine sulfate and 5, 10, and 25 mg amobarbital in Mixtures 1, 2, and 3, respectively. [b] Analysis done in duplicate. [c] Calculated from 10 measurements.

solution (5.0 ml) was pipeted into a 100-ml separator and extracted three times with 20-ml portions of chloroform. The organic phase was discarded and the aqueous phase was diluted with 0.1 *N* sulfuric acid to a region of linear response for the method under consideration. A blank solution was prepared by treating 5.0 ml of 0.1 *N* sulfuric acid in a manner similar to that for the sample. The sample reading was corrected for the blank. Analysis of amphetamine was done using Procedures I–V (Table I).

Determination of Amphetamine in Urine—Known amounts of dextroamphetamine sulfate were added to the normal urine *in vitro* and the samples were kept in a refrigerator. Amphetamine was isolated from urine by extraction of alkaline urine with chloroform and back-extraction with 0.1 *N* sulfuric acid. To the urine sample, 1 *N* sodium hydroxide was added until the pH was about 10. Then 5.0 ml of this was saturated with sodium chloride and extracted with 20 ml and three quantities each of 15 ml of chloroform. Amphetamine was reextracted from the combined chloroform layers into 0.1 *N* sulfuric acid (3 × 10 ml). The acid extract was diluted with 0.1 *N* sulfuric acid to a volume suitable for adequate fluorescence and analyzed by one of the procedures (Table II). A blank was determined in a similar way by treating 5.0 ml of drug-free urine, and the sample reading was corrected for the blank. The use of large quantities of chloroform for extraction minimized emulsion formation.

RESULTS AND DISCUSSION

Procedure I was studied recently in great detail (1) and was not investigated further. This procedure, although simple, is not sensitive. Procedure II gives weak and unstable fluorescence, and the concentration–fluorescence relationship is not linear even at low concentrations. It is also time consuming and was not investigated further. Procedures III and IV are quite sensitive and may be recommended for the trace analysis of amphetamine. Procedure IV is more sensitive, simpler, and less time consuming but the reagent fluorescamine is considerably more expensive than the acetylacetone and formaldehyde used in Procedure III. In Procedure III, it is advisable to take readings after a fixed period of time since the fluorescence intensity slowly increases with time. The intensity is stable for several hours in Procedure IV.

Procedure V is quite satisfactory from the standpoint of stability and reproducibility, but its sensitivity is poor and the precision is slightly inferior to that of the Procedures III and IV. However, it gives higher fluorescence intensity than that obtained from the native fluorescence method. The blank is negligible and the calibration range is linear between 25 and 150 ppm. The fluorescence intensity is stable for a few hours. The fluorophore has two excitation peaks (at 272 and 385 nm), which offer a large wavelength range for excitation. This could be useful in avoiding certain interferences (selective excitation).

Table II—Analysis of Amphetamine in Urine

Procedure	Sample[a]	Amphetamine Sulfate Found[b], ppm	Error, %
III	1	46.5	−7.0
	2	92.0	−8.0
IV	1	46.5	−7.0
	2	90.0	−10.0
V	2	92.5	−7.5

[a] Samples 1 and 2 contained 50 and 100 ppm of amphetamine sulfate, respectively. [b] Analysis done in duplicate.

The presence of twofold excesses of amobarbital does not interfere in these methods. Most amphetamine–amobarbital drug combinations contain more than fivefold amounts of amobarbital, requiring its removal by extraction with chloroform.

About 90% of amphetamine was recovered from urine by the extraction procedure employed. In the present work, amphetamine was added to the urine *in vitro* because of legal complications; however, the situation may be little different when doing analysis of urine from a subject who has received the drug. A few points should be borne in mind. For example, excretion of amphetamine is markedly dependent upon urinary pH, being greatly increased in acid urine (7). After large doses, amphetamine may be detected in urine for several days (7). The presence of *p*-hydroxyamphetamine (a metabolite) will interfere in the methods; however, this metabolite could be separated by TLC (7) prior to the amphetamine determination.

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[x] To whom inquiries should be directed.

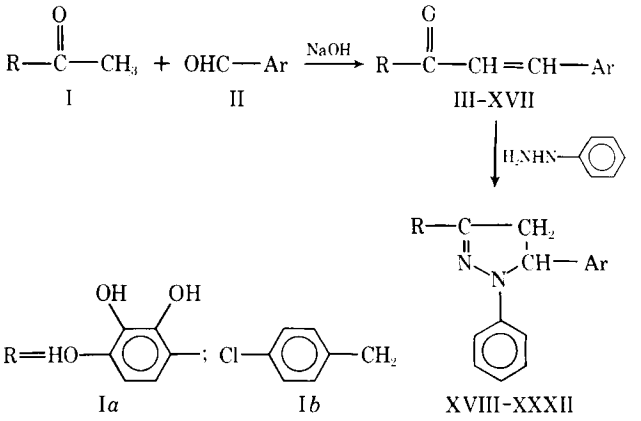
Anticonvulsant Activity and Monoamine Oxidase Inhibitory Properties of 1,3,5-Trisubstituted Pyrazolines

SURENDRA S. PARMAR*x, B. R. PANDEY*, C. DWIVEDI‡, and RAYMOND D. HARBISON‡

Abstract □ Several substituted pyrazolines were synthesized and characterized, and all of these pyrazolines inhibited rat brain monoamine oxidase. Evaluation of these compounds revealed appreciable anticonvulsant activity, reflected by their ability to afford protection against pentylenetetrazol-induced seizures in mice. The anticonvulsant activity possessed by these pyrazolines was unrelated to their monoamine oxidase inhibitory effectiveness.

Keyphrases □ Pyrazolines, 1,3,5-trisubstituted—synthesis, relationship between anticonvulsant and monoamine oxidase inhibitory properties □ Monoamine oxidase inhibitors—substituted pyrazolines, synthesis, anticonvulsant activity □ Anticonvulsant activity—substituted pyrazolines, relationship to monoamine oxidase inhibitory properties □ Structure-activity relationships—monoamine oxidase inhibitory property-anticonvulsant activity

The ability of pyrazole derivatives to exhibit tranquilizing, muscle relaxant, psychoanaleptic (1), hypnotic (2), and anticonvulsant (3, 4) activities prompted the synthesis of some 1,3,5-trisubstituted pyrazolines. The anticonvulsant activity of these pyrazolines was investigated against pentylenetetrazol-induced seizure in albino mice. The monoamine oxidase [EC 1.4.3.4-monoamine: O_2 oxidoreductase (deaminating)] inhibitory property possessed by these pyrazolines was also investigated to study the biochemical mechanism of action for the anticonvulsant activity of pyrazolines. The various pyrazolines were synthesized following the methods outlined in Scheme I.



Scheme I

EXPERIMENTAL

Chemistry—Appropriate ketones (I) were condensed with arylaldehydes (II) to yield chalcones (III-XVII). These chalcones (III-XVII), on treatment with phenylhydrazine hydrochloride in acetic acid, were cyclized to the corresponding pyrazolines (XVIII-XXXII).

Analyses for carbon, hydrogen, and nitrogen were performed[1]. Melting points were taken in open capillary tubes and are uncorrected. These results are presented in Tables I and II.

Gallacetophenone (Ia)—This compound was prepared by the Friedel-Crafts reaction with acetic anhydride on pyrogallol using anhydrous zinc chloride as the catalyst, mp 171° (5).

Chalcones III-VIII—A mixture of gallacetophenone (0.043 mole), an appropriate aromatic aldehyde (0.041 mole), sodium hydroxide (0.21 mole), ethanol (13 ml), and water (50 ml) was stirred vigorously until a thick paste formed and stirring was no longer effective. The reaction mixture was kept in a refrigerator overnight, and the solid mass was dissolved in water and neutralized with hydrochloric acid. The separated chalcones were collected by filtration, recrystallized from ethanol, and then characterized by their sharp melting points and elemental analyses (Table I).

Chalcones IX-XVII—4-Chlorobenzyl methyl ketone (0.043 mole), a suitable aromatic aldehyde (0.041 mole), sodium hydroxide (0.054 mole), ethanol (13 ml), and water (50 ml) were mixed and stirred vigorously. The reaction mixture, after cooling overnight in a refrigerator, was neutralized with hydrochloric acid. The resulting yellow oil, which could not be crystallized, was used for subsequent reactions without further purification.

1,3,5-Trisubstituted Pyrazolines (XVIII-XXXII)—A mixture of chalcone (III-XVII) (0.01 mole) and phenylhydrazine hydrochloride (0.02 mole) was refluxed in acetic acid (20 ml) for 4–5 hr. The reaction mixture was cooled and poured in ice water. The solid mass which separated was collected by filtration, washed with water, and recrystallized from benzene. These trisubstituted chalcones were characterized by their sharp melting points and elemental analyses (Table II).

Determination of Monoamine Oxidase Activity—A spectrophotofluorometric method was used for the determination of monoamine oxidase activity of rat brain homogenate, using kynuramine as the substrate (6). The 4-hydroxyquinoline formed during oxidative deamination of kynuramine was measured fluorometrically[2] using activating light of 315 nm and measuring fluorescence at the maximum of 380 nm.

Male adult rats, weighing approximately 150–200 g, were killed by decapitation. Brains were quickly removed and homogenized[3] in ice-cold 0.25 *M* sucrose. The reaction mixture, in a final concentration, consisted of 0.5 ml of phosphate buffer (0.2 *M*, pH 7.5), 1×10^{-4} *M* kynuramine, and 0.5 ml of brain homogenate (equivalent to 10 mg of wet weight of the tissue). The monoamine oxidase activity of the brain homogenate was determined by incubation at 37° in air for 30 min. The various substituted pyrazolines were added to the brain homogenate to produce a final concentration of 5×10^{-5} *M* and incubated for 10 min before adding kynuramine. The mixture was then incubated for an additional 30 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (w/v), and the precipitated proteins were removed by centrifugation. Suitable 1-ml aliquots of the supernate were taken in 2 ml of 1 *N* NaOH solution and were assayed for 4-hydroxyquinoline. An increase in absorbance provided a direct measurement of the 4-hydroxyquinoline formation, which was taken as an index of enzyme activity. The percent inhibition was calculated from the decrease observed in the absorbance, and this

[1] Central Drug Research Institute, Lucknow, India.

[2] Aminco Bowman spectrophotofluorometer.

[3] Potter-Elvehiem homogenizer.

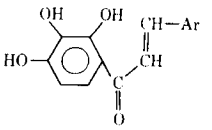
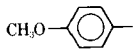
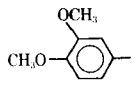
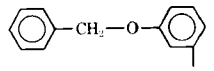
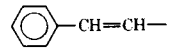


Table I—Physical Constants of 2,3,4-Trihydroxychalcones

Compound	Ar	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found
III[b]		112°	64	—	—	—
IV		225°	62	$C_{15}H_{11}ClO_4$	C 61.85 H 3.72	61.72 3.49
V		140°	58	$C_{16}H_{14}O_5$	C 67.13 H 4.89	67.38 4.72
VI		105°	59	$C_{17}H_{16}O_6$	C 64.55 H 5.06	64.31 4.99
VII		120°	67	$C_{22}H_{18}O_5$	C 72.92 H 4.97	73.10 5.23
VIII		125°	63	$C_{23}H_{18}O_4$	C 77.09 H 5.02	77.18 5.18

[a] All melting points were taken in open capillary tubes and are uncorrected. [b] *Curr. Sci.*, **34**, 18(1965).

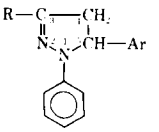
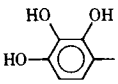
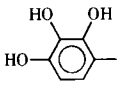
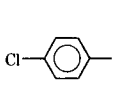
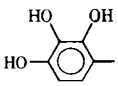
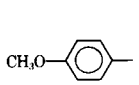
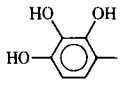
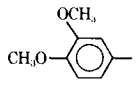
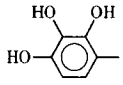
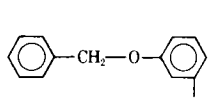
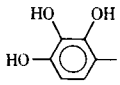
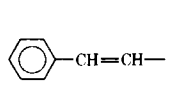
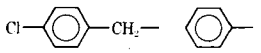
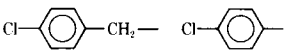
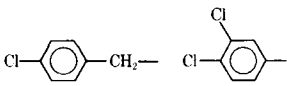
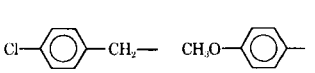
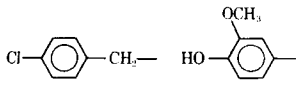
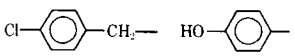
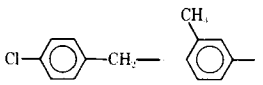
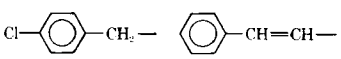
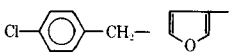


Table II—Physical Constants of 1,3,5-Trisubstituted Pyrazolines

Compound	R	Ar	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found
XVIII			150°	61	$C_{21}H_{18}N_2O_3$	C 72.83 H 5.20 N 8.09	72.62 5.00 8.31
XIX			215°	59	$C_{21}H_{17}ClN_2O_3$	C 66.14 H 4.46 N 7.34	66.44 4.58 7.28
XX			108°	62	$C_{22}H_{20}N_2O_4$	C 70.21 H 5.31 N 7.44	70.10 5.52 7.31
XXI			142°	65	$C_{23}H_{22}N_2O_5$	C 67.98 H 5.42 N 6.89	68.28 5.22 6.58
XXII			137°	62	$C_{28}H_{24}N_2O_4$	C 74.33 H 5.31 N 6.19	74.01 5.58 6.01
XXIII			130°	59	$C_{23}H_{20}N_2O_3$	C 74.19 H 5.37 N 7.52	74.31 5.30 7.22
XXIV			128°	60	$C_{22}H_{19}ClN_2$	C 76.08 H 5.46 N 8.06	76.31 5.63 8.35
XXV			115°	68	$C_{22}H_{18}Cl_2N_2$	C 69.29 H 4.72 N 7.34	69.00 4.51 7.64
XXVI			133°	64	$C_{22}H_{17}Cl_3N_2$	C 63.53 H 4.09 N 6.73	63.33 4.39 6.60
XXVII			122°	65	$C_{23}H_{21}ClN_2O$	C 73.30 H 5.57 N 7.43	73.01 5.50 7.62

(continued)

Table II—(*Continued*)

Compound	R	Ar	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found
XXVIII			110°	69	$C_{23}H_{21}ClN_2O_2$	C 70.31 H 5.35 N 7.13	70.55 5.40 7.38
XXIX			165°	68	$C_{22}H_{19}ClN_2O$	C 72.82 H 5.24 N 7.72	73.00 5.13 7.46
XXX			126°	64	$C_{23}H_{21}ClN_2$	C 76.56 H 5.82 N 7.76	76.31 5.90 7.99
XXXI			135°	63	$C_{24}H_{21}ClN_2$	C 77.31 H 5.63 N 7.54	77.51 5.70 7.44
XXXII			103°	67	$C_{20}H_{17}ClN_2O$	C 71.32 H 5.05 N 8.32	71.62 5.09 8.21

[a] All melting points were taken in open capillary tubes and are uncorrected.

provided an index of the inhibitory property of these substituted pyrazolines.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined (7) in mice of either sex weighing 25–30 g. The mice were divided in groups of 10, keeping the group weights as near the same as possible. Each pyrazoline was suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compound was injected intraperitoneally in a group of 10 animals at a dose of 100 mg/kg. Four hours after the administration of the substituted pyrazolines, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol has been shown not only to produce convulsions in almost all untreated mice but also to exhibit 100% mortality during 24 hr. On the other hand, no mortality was observed during 24 hr in animals treated with 100 mg/kg alone of the test compounds. The mice were then observed for 60 min for seizures. An episode of clonic spasm that persisted for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks or tremulousness was not counted. Animals devoid of threshold convulsions during 60 min were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of these substituted pyrazolines was represented as percent protection. The animals were then observed for 24 hr and their mortality was recorded.

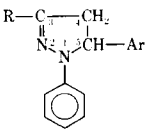
RESULTS AND DISCUSSION

The ability of these substituted pyrazolines to inhibit rat brain monoamine oxidase, using kynuramine as the substrate, and their anticonvulsant activity are recorded in Table III. All 3-(2,3,4-trihydroxyphenyl)-substituted pyrazolines produced inhibition of rat brain monoamine oxidase at a final concentration of 5×10^{-4} *M*, while the presence of a 3,4-dimethoxyphenyl substituent at position 5 of the pyrazoline ring (XXI) produced maximum inhibition of monoamine oxidase of 74.28%. However, the presence of a 4-chlorophenyl substituent at position 5 of the pyrazoline ring (XIX) produced minimum inhibition of 17.14% of monoamine oxidase. The presence of a styryl group at position 5 (XXIII) also produced a greater degree of monoamine oxidase inhibition as compared to a pyrazoline possessing an unsubstituted phenyl ring (XVIII).

These results indicated that the presence of an electron-donating substituent on the phenyl group present at position 5 of the pyrazoline ring (XX–XXII) produced a relatively higher degree of enzyme inhibition while an electron-withdrawing atom (XIX) produced a lesser degree of monoamine oxidase inhibition. Among the 3-(4-chlorobenzyl)-substituted pyrazolines, the compound possessing a 3-methoxy-4-hydroxyphenyl substituent at position 5 (XXVIII) produced the maximum inhibition of 90.00% of monoamine oxidase. The pyrazolines possessing an electron-donating substituted phenyl group at position 5 (XXVII–XXX) showed an increased degree of enzyme inhibition, while an electron-withdrawing substituted phenyl group at position 5 (XXV and XXVI) decreased the degree of enzyme inhibition in a similar manner as was observed with 3-(2,3,4-trihydroxy)phenyl-substituted pyrazolines. The presence of two electron-withdrawing atoms at the 5-phenyl group (XXVI) decreased further their ability to inhibit monoamine oxidase.

Results of anticonvulsant activity studies (Table III) indicate significant protection of 50–90% by 3-(2,3,4-trihydroxyphenyl)-substituted pyrazolines against pentylenetetrazol-induced seizures at a dose of 100 mg/kg. The presence of a 4-chlorophenyl substituent at position 5 of the pyrazoline nucleus (XIX) produced maximum anticonvulsant activity. The protection afforded by 3-(4-chlorobenzyl)-5-substituted pyrazolines ranged from 30 to 70%, and 3-(4-chlorobenzyl)-5-phenylpyrazoline (XXIV) was the

Table III—Monoamine Oxidase Inhibition and Anticonvulsant Properties of 1,3,5-Trisubstituted Pyrazolines



Compound	Monoamine Oxidase Inhibition[a], %	Anticonvulsant Activity[b], Protection, %	Pentylenetetrazol Mortality, %
XVIII	32.86 ± 0.56	50	50
XIX	17.14 ± 0.44	90	Nil
XX	32.86 ± 0.52	80	20
XXI	74.28 ± 0.98	60	30
XXII	47.71 ± 0.86	80	20
XXIII	44.28 ± 0.75	80	10
XXIV	46.66 ± 0.78	70	30
XXV	22.22 ± 0.42	60	30
XXVI	18.59 ± 0.38	50	40
XXVII	41.11 ± 0.46	60	40
XXVIII	90.00 ± 1.25	40	50
XXIX	48.57 ± 0.98	30	70
XXX	32.22 ± 0.62	40	60
XXXI	50.00 ± 0.46	40	50
XXXII	56.66 ± 0.98	50	50

[a] Assay procedure and contents of the reaction mixture are as indicated in the text. Inhibitors were used at a final concentration of 5×10^{-4} *M*. Each experiment was done in duplicate, and figures indicate mean values of three separate experiments with ± standard error of the mean. [b] Screening procedure was as indicated in the text. Each compound was used at the dose of 100 mg/kg ip, and pentylenetetrazol mortality was observed during 24 hr. In the present study, administration of an equivalent amount of 5% gum acacia solution was found to possess no anticonvulsant activity.

most active compound. As is evident from Table III, all pyrazolines exhibiting a greater degree of protection elicited a lower pentylenetetrazol-induced mortality during 24 hr in the experimental animals.

These studies, exhibiting pronounced anticonvulsant activity of substituted pyrazolines, were unable to provide a correlation between their anticonvulsant activity and their ability to inhibit monoamine oxidase activity as a biochemical basis of their anticonvulsant activity. Further studies dealing with the synthesis of other related structures carrying different substituents and the determination of their ability to inhibit purified enzyme preparations may possibly reflect the biochemical basis of the anticonvulsant activity of substituted pyrazolines.

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ˣ To whom inquiries should be directed. Present address: Department of Physiology and Pharmacology, School of Medicine, University of North Dakota, Grand Forks, ND 58201

Novel Synthesis of Bifunctional Acid-Esters

SUDHAKAR KASINA, KIM WAH NG, and JAY NEMATOLLAHIˣ

Abstract □ A novel method was developed for the synthesis of acid-esters *via* monodemethylation of a dimethyl ester by 1,1-dimethylhydrazine and subsequent conversion of the resulting trimethylhydrazonium ester to the desired acid-ester. The method is facile and seems to have a wide range of applicability.

Keyphrases □ Acid-esters, bifunctional—novel synthesis using 1,1-dimethylhydrazine and a dimethyl ester and conversion of the resulting trimethylhydrazonium ester □ Trimethylhydrazonium ester—intermediate in synthesis of bifunctional acid-esters □ 1,1-Dimethylhydrazine—utilized in novel synthesis of bifunctional acid-esters

Quite often the synthesis of compounds containing both an acid and an ester moiety (acid-ester) requires tedious experimental design and manipulation. This is particularly true with cyclic systems from which no anhydride (a widely used precursor for the acid-ester) can be synthesized, due either to nonproximity of the functional groups or to development of severe strain if the anhydride is formed.

This paper describes a novel and efficient solution to the problem of preparing bifunctional compounds containing both a carboxy and a carbomethoxy functional group.

DISCUSSION

The developed method utilizes 1,1-dimethylhydrazine to effect the monodemethylation of an aliphatic or aromatic dimethyl ester and subsequent conversion of the resulting trimethylhydrazonium ester to the desired acid-ester (Scheme I). Either the addition of dilute hydrochloric acid to the trimethylhydrazonium salt followed by ether extraction or the use of silica gel column chromatography provided the free acid-ester. The latter method, although laborious, is an attractive feature, particularly for compounds containing acid-sensitive moieties or whose acid-esters exist in zwitterion forms and are, therefore, highly water soluble and ether insoluble.

That this method of synthesis of acid-esters is facile and has broad application in both aliphatic and aromatic systems is shown by the examples in Table I. A possible exception seems to be five-membered heterocyclic rings with vicinal methyl ester substituents. Under the generally applied reaction conditions, no bifunctional acid-ester compounds were afforded from the dimethyl esters of either 4,5-imidazoledicarboxylic acid or 3,4-pyrazoledicarboxylic acid[1]. However, more five-membered rings with vicinal diesters must be investigated before generalizations can be made concerning their reactions with 1,1-dimethylhydrazine.

The reaction of dimethyl 3,5-pyridinedicarboxylate with 1,1-dimethylhydrazine (Scheme I) exemplifies the general procedure. The structural formulas of all synthesized compounds as well as their melting points, spectral data, and elemental analyses are shown in Table I.

The molecular structures of the reaction intermediates, the bifunctional trimethylhydrazonium esters, and the final products, the acid-esters, were elucidated by using IR, NMR, and mass spectrometry. Elemental analyses were carried out for only a few crystalline hydrazonium salts[2], in an effort to establish an unequivocal correlation between molecular structures and spectral

[1] The reaction product is somewhat complex and is currently being investigated.

[2] Some hydrazonium salts were observed to be semisolid and extremely hygroscopic, and no elemental analyses were attempted for them. Spectral data of the given trimethylhydrazonium and its corresponding acid-ester were used for determining molecular structure.

most active compound. As is evident from Table III, all pyrazolines exhibiting a greater degree of protection elicited a lower pentylenetetrazol-induced mortality during 24 hr in the experimental animals.

These studies, exhibiting pronounced anticonvulsant activity of substituted pyrazolines, were unable to provide a correlation between their anticonvulsant activity and their ability to inhibit monoamine oxidase activity as a biochemical basis of their anticonvulsant activity. Further studies dealing with the synthesis of other related structures carrying different substituents and the determination of their ability to inhibit purified enzyme preparations may possibly reflect the biochemical basis of the anticonvulsant activity of substituted pyrazolines.

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ˣ To whom inquiries should be directed. Present address: Department of Physiology and Pharmacology, School of Medicine, University of North Dakota, Grand Forks, ND 58201

Novel Synthesis of Bifunctional Acid-Esters

SUDHAKAR KASINA, KIM WAH NG, and JAY NEMATOLLAHIˣ

Abstract □ A novel method was developed for the synthesis of acid-esters *via* monodemethylation of a dimethyl ester by 1,1-dimethylhydrazine and subsequent conversion of the resulting trimethylhydrazonium ester to the desired acid-ester. The method is facile and seems to have a wide range of applicability.

Keyphrases □ Acid-esters, bifunctional—novel synthesis using 1,1-dimethylhydrazine and a dimethyl ester and conversion of the resulting trimethylhydrazonium ester □ Trimethylhydrazonium ester—intermediate in synthesis of bifunctional acid-esters □ 1,1-Dimethylhydrazine—utilized in novel synthesis of bifunctional acid-esters

Quite often the synthesis of compounds containing both an acid and an ester moiety (acid-ester) requires tedious experimental design and manipulation. This is particularly true with cyclic systems from which no anhydride (a widely used precursor for the acid-ester) can be synthesized, due either to nonproximity of the functional groups or to development of severe strain if the anhydride is formed.

This paper describes a novel and efficient solution to the problem of preparing bifunctional compounds containing both a carboxy and a carbomethoxy functional group.

DISCUSSION

The developed method utilizes 1,1-dimethylhydrazine to effect the monodemethylation of an aliphatic or aromatic dimethyl ester and subsequent conversion of the resulting trimethylhydrazonium ester to the desired acid-ester (Scheme I). Either the addition of dilute hydrochloric acid to the trimethylhydrazonium salt followed by ether extraction or the use of silica gel column chromatography provided the free acid-ester. The latter method, although laborious, is an attractive feature, particularly for compounds containing acid-sensitive moieties or whose acid-esters exist in zwitterion forms and are, therefore, highly water soluble and ether insoluble.

That this method of synthesis of acid-esters is facile and has broad application in both aliphatic and aromatic systems is shown by the examples in Table I. A possible exception seems to be five-membered heterocyclic rings with vicinal methyl ester substituents. Under the generally applied reaction conditions, no bifunctional acid-ester compounds were afforded from the dimethyl esters of either 4,5-imidazoledicarboxylic acid or 3,4-pyrazoledicarboxylic acid[1]. However, more five-membered rings with vicinal diesters must be investigated before generalizations can be made concerning their reactions with 1,1-dimethylhydrazine.

The reaction of dimethyl 3,5-pyridinedicarboxylate with 1,1-dimethylhydrazine (Scheme I) exemplifies the general procedure. The structural formulas of all synthesized compounds as well as their melting points, spectral data, and elemental analyses are shown in Table I.

The molecular structures of the reaction intermediates, the bifunctional trimethylhydrazonium esters, and the final products, the acid-esters, were elucidated by using IR, NMR, and mass spectrometry. Elemental analyses were carried out for only a few crystalline hydrazonium salts[2], in an effort to establish an unequivocal correlation between molecular structures and spectral

[1] The reaction product is somewhat complex and is currently being investigated.

[2] Some hydrazonium salts were observed to be semisolid and extremely hygroscopic, and no elemental analyses were attempted for them. Spectral data of the given trimethylhydrazonium and its corresponding acid-ester were used for determining molecular structure.

Table I—Data for Trimethylhydrazonium Esters and Acid-Esters

Compound	Yield, %	Analysis, %[a] Calc.	Analysis, %[a] Found	Mass Spectroscopy Results	IR Bands, cm^{-1}	NMR Peaks[b,c], δ	Melting Point	Reference
I		C 56.69 H 7.08 N 11.02	56.46 7.34 11.22	163[d]	3220(NH); 1730, 1580(C=O)[e]	3.45(s, NCH_3); 4.00(s, OCH_3); 7.52–7.85(m, C_6H_5)	144°	1
II	90			180	1730, 1698(C=O)	4.00(s, OCH_3); 7.52–8.15 (m, C_6H_5)	75–78° (lit. 84°)	2
III	43			180[f]	3210(NH); 1725, 1625, 1580(C=O)	3.53(s, NCH_3); 4.15(s, OCH_3); 7.58–8.90(m, C_6H_5)	134–138°	
IV	90			180	1730, 1680(C=O)	4.05(s, OCH_3)	189–192° (lit. 193°)	3
V	51	C 56.69 H 7.08 N 11.02	56.43 6.99 11.05	180[f]	3210(NH); 1730, 1592, 1550 (C=O)	3.50(s, NCH_3); 4.12(s, OCH_3); 8.33(s, C_6H_5)	176–178°	
VI	90			180	1728, 1690(C=O)	4.12(s, OCH_3); 8.27(s, C_6H_5)	228–229° (lit. 230°)	4
Mixture of VII and VIII	23			164[d]	1740, 1605(C=O)	3.47(s, NCH_3); 4.18 and 4.20(s, OCH_3); 8.40–9.27(m, pyridine)		
IX					1735, 1705(C=O)	4.20(s, OCH_3); 8.35–9.10(m, pyridine)	105°	5
X					1735, 1695(C=O)	4.17(s, OCH_3); 8.33–9.18(m, pyridine)	123°	5
XI	61			181[f]	3400(NH); 1725, 1610(C=O)	3.45(s, NCH_3); 4.20(s, OCH_3); 9.73–9.86(m, pyridine)	133–138°[g]	
XII	91	C 53.04 H 3.87 N 7.74	53.43 4.23 8.00	181	1735, 1600(C=O)	4.18(s, OCH_3); 9.68–9.76(m, pyridine)	216–218°	
XIII	20			129[d]	1750, 1640(C=O)	3.44(s, NCH_3); 3.78(s, OCH_3); 1.70–2.80(m, CH_2)		
XIV	80			129[h]	1735, 1710(C=O)	3.82(s, OCH_3); 1.83–2.83(m, CH_2)		6
XV	31			169[d]	1730, 1575(C=O)	3.45(s, NCH_3); 3.78(s, OCH_3); 1.10–2.90(m, cyclohexane)	78–80°	
XVI	85			169[h]	1739, 1705(C=O)	3.77(s, OCH_3); 1.20–2.90(m, cyclohexane)	94–96° (lit. 96°)	7

[a] Schwarzkopf Microanalytical Laboratories, Woodside, N.Y. [b] The solvent was trifluoroacetic acid. [c] The reference was sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionate. [d] The number represents the OH-cleaved molecular ion M^+ (*m/e*) of the corresponding acid-ester which, in turn, was formed by the cleavage of the trimethylhydrazonium cation and abstraction of a hydrogen. [e] The frequency values listed here are for the recrystallized sample and differ somewhat from those reported previously (1). [f] Same as *d* but no OH cleavage. [g] The melting range is for the solvent-washed product whose recrystallization from various solvents was unsuccessful; it became oily. [h] Molecular ion minus 17 (OH).

data, and for those acid-esters that had not been reported previously.

Because of high reactivity inherent to ester groups, the acid-esters offer a wide applicability in the synthesis of biologically active substances. For example, hydrazides analogous to currently used drugs, isoniazid or isocarboxazid, can easily be prepared from these acid-esters. Such hydrazides, however, due to their amphoteric character, would be considered unique.

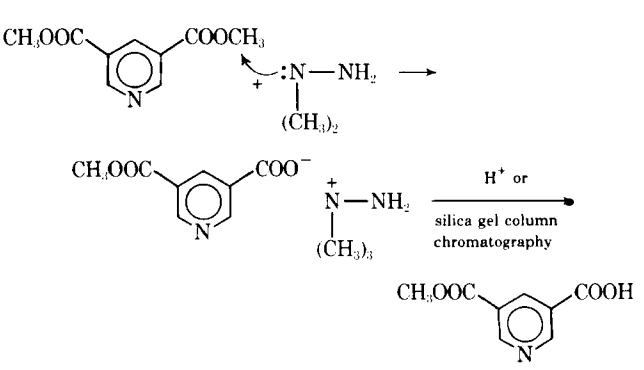
EXPERIMENTAL[3]

A general procedure for the synthesis of the acid-esters and their precursor trimethylhydrazonium esters is exemplified by the experimental procedures described for XI and XII. An additional description is given for the reaction procedure of dimethyl 2,3-pyridinedicarboxylate, since it was somewhat different from that of the general procedure.

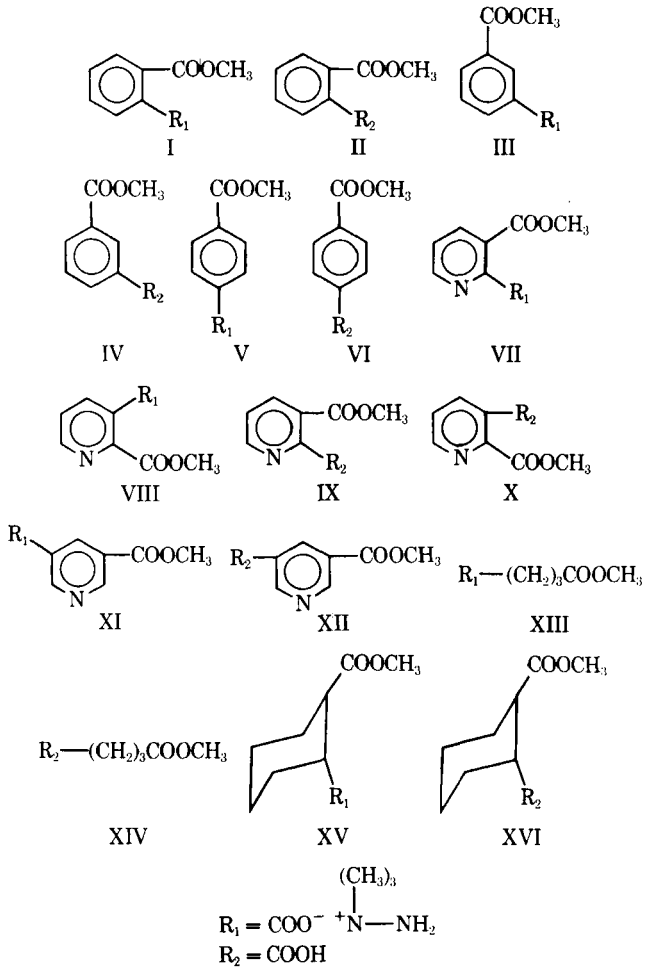
[3] The melting points were determined in a Thomas-Hoover Uni-Melt apparatus, using open capillaries, and are uncorrected. The IR spectra were determined on a Beckman IR-8, using KBr disks for the solid compounds *and a sodium chloride* window sandwich for the semisolid and liquid compounds. Only the bands for N—H and C=O stretching frequencies (ν_{max}) in reciprocal centimeters were reported. The NMR spectra were determined on a Jeol C-60 HL, using trifluoroacetic acid as a solvent and sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionate as a reference. The peak positions were reported in parts per million (δ); s and m designate singlets and multiplets, respectively. The mass spectra were determined on a DuPont 21-491, and only the parent ions (*m/e*) are reported. The TLC was done on microscope slides coated with silica gel (HF 254+366, Brinkmann Instruments, Inc.).

Trimethylhydrazonium 5-Carbomethoxy-3-pyridinecarboxylate (XI)—To 0.5 g (0.002 mole) of dimethyl 3,5-pyridinedicarboxylate was added 3.0 g (0.05 mole) of 1,1-dimethylhydrazine. The mixture was heated under reflux for 6 hr. After cooling to room temperature, the reaction product, whose formation was observed by TLC, was washed successively with ether and carbon tetrachloride to eliminate the unreacted ester and the excess 1,1-dimethylhydrazine. Removal of solvent *in vacuo* afforded 0.4 g of XI (60%).

5-Carbomethoxy-3-pyridinecarboxylic Acid (XII)—To 0.40 g (0.0016 mole) of XI was added, dropwise, sufficient 5% HCl to effect its dissolution. After 10 min at room temperature, the solu-



Scheme I



tion gave a precipitate[4] of XII which, on filtration and drying *in vacuo*, weighed 0.26 g (91%). It was characterized by IR, NMR, and mass spectrometry. The sample for elemental analysis was crystallized once from a mixture of about 1% methanol in benzene.

Trimethylhydrazonium 3-Carbomethoxy-2-pyridinecarboxylate (VII) and Trimethylhydrazonium 2-Carbomethoxy-3-pyridinecarboxylate (VIII)—To 5.0 g (0.026 mole) of dimethyl 2,3-pyridinedicarboxylate was added 12.0 g (0.2 mole) of 1,1-dimethylhydrazine. The mixture was left at room temperature for 24 hr, at which time a gummy substance was formed. Washing successively with ether and carbon tetrachloride eliminated the unreacted ester and 1,1-dimethylhydrazine. The residue was dried under vacuum to give 1.5 g (23%) of a mixture of VII and VIII, as determined by IR, NMR[5], and mass spectrometry. Attempts to separate the two isomers by differential solubility and chromatography were unsuccessful.

3-Carbomethoxy-2-pyridinecarboxylic Acid (IX) and 2-Carbomethoxy-3-pyridinecarboxylic Acid (X)—A crude mixture (1.5 g, 0.006 mole) of VII and VIII in 5 ml methanol was chromatographed on a column containing 25 g silica gel, and was developed with 15% methanol in methylene chloride. The fourth 10-ml fraction of the eluate gave a small quantity of the unreacted diester. The combined fifth to 12th fractions contained 0.2 g (20%) of a mixture of IX and X. The individual acid-esters were separated from the mixture by differential solubility in ethyl acetate and identified by comparing their spectral data with those of the authentic samples prepared by a method described by Kenyon and Thaker (5).

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[x] To whom inquiries should be directed.

[4] For acid-esters that were liquid or that would not precipitate upon addition of hydrochloric acid, ether extraction and/or silica gel column chromatography (described for IX and X) were used.

[5] Two peaks of about 3:1 ratio (δ 4.20 and 4.18, respectively) for OCH_3 protons were indicative of the presence of a mixture of VII and VIII.

Quinazolines as Inhibitors of Xanthine Oxidase

D. G. PRIEST [x], J. B. HYNES, C. W. JONES, and W. T. ASHTON*

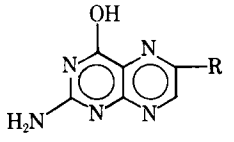
Abstract □ A series of 23 quinazolines and 5,6,7,8-tetrahydroquinazolines was evaluated for inhibition of bovine milk xanthine oxidase. The synthesis of four of these compounds has not been reported previously. Several 2-amino-4-hydroxyquinazolines, as well as 2-amino-4-mercaptoquinazoline, were effective inhibitors of this enzyme. 2-Amino-4-hydroxy-6-hydroxymethylquinazoline was a threefold better inhibitor than its pteridine counterpart, while 2-amino-4-hydroxyquinazoline-6-carboxaldehyde was the most potent compound studied.

Keyphrases □ Quinazolines—synthesis, evaluated as xanthine oxidase inhibitors □ Tetrahydroquinazolines—synthesis, evaluated as xanthine oxidase inhibitors □ Xanthine oxidase—synthesis of four and evaluation of 23 quinazolines and 5,6,7,8-tetrahydroquinazolines as inhibitors

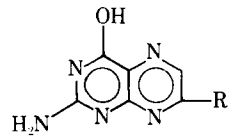
Suitably substituted members of various heterocyclic ring systems have been shown to be inhibitors of and/or substrates for xanthine oxidase. In addition to numerous purine derivatives (1), several pteridines were of interest in this regard. For example, 2-amino-4-hydroxy-6-hydroxymethylpteridine (I*a*) and 2-amino-4-hydroxypteridine-6-carboxaldehyde (I*b*) were shown to be good inhibitors, having approximately equal potency against this enzyme (2). On the other hand, 2-amino-4-hydroxypteridine (II*a*) was oxidized at essentially the same rate as xanthine, yielding 2-amino-4,7-dihydroxypteridine (II*b*), an effective inhibitor (3). In addition to other pteridines (4), certain 1*H*-pyrazolo[4,3-*c*]pyridines were recently reported to display modest inhibitory action against xanthine oxidase (5).

From a clinical standpoint, however, the most important agent acting on this enzyme is allopurinol (III*a*), which is currently employed in various conditions characterized by hyperuricemia (6). The compound is not only an inhibitor of xanthine oxidase but is also a substrate that is converted to oxypurinol (III*b*), another effective inhibitor (7, 8).

In general, the known heterocyclic inhibitors of xanthine oxidase consist of two fused unsaturated rings, each containing at least one hetero nitrogen atom. An exception is that one pyrazine derivative, 2-amino-3-pyrazinecarboxylic acid, is also a moderately effective inhibitor (4). However, since the removal of either the amino group or the carboxyl function results in total loss of activity (4), it seems more logical to consider this compound as a quasicyclic analog of II*a*. It was of interest, therefore, to determine the effects of quinazolines upon xanthine oxidase. These compounds may be regarded as analogs of either purines or pteridines which contain only one heterocyclic ring.



I*a*: R = CH_2OH
I*b*: R = CHO



II*a*: R = H
II*b*: R = OH

EXPERIMENTAL

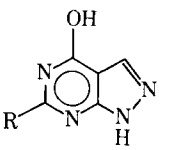
Melting points were taken with a capillary apparatus[1] and are uncorrected. Analytical samples were vacuum dried at 100° prior to analysis. TLC was performed using silica media[2]. UV absorption measurements were made using a recording spectrophotometer[3].

Enzymology—Ammonium sulfate suspensions of highly purified bovine milk xanthine oxidase[4] were used without further purification. The specific activities of the two preparations employed were 0.1 and 0.56 unit/mg protein (no signifcant kinetic differences were observed with these two preparations). Velocities were determined from absorbance changes at 293 nm in a cell chamber maintained at 28° by circulating water. Initial velocities were taken as the tangent to time plots at zero time. Reactions were usually initiated by the addition of 25 μl of enzyme solution containing 1.0 mg/ml protein to 3.0-ml reaction cells.

The substrate, xanthine, as well as each inhibitor was prepared at a final concentration of 10 μM in 0.05 *M* potassium phosphate, pH 8.0. Each inhibitor was tested separately at 10-μM concentration for possible substrate activity which could interfere with the spectrophotometric assay. In no case was there a significant absorbance change in the absence of xanthine. To test more rigorously for substrate activity, 5 ml of a 100 μM solution was allowed to react overnight in the presence of 25 μl of the xanthine oxidase solution. A compound was assumed to be a substrate if a significant spectral change was observed in the 230–350-nm region.

Chemistry—Compounds I*a*[5], II*b*[4], III*a*[4], IV[6], V, XXVII[4], and XXVIII[4] were obtained commercially. The synthetic procedures for the preparation of the remaining compounds, with the exception of those presented here, were described previously (9–11).

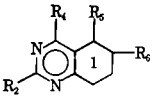
2-Amino-4-hydroxy-6-chloroquinazoline (XVI) and 2,6-Diamino-4-hydroxyquinazoline (XX)—Compounds XVI and XX were prepared by refluxing the corresponding 2,4-diaminoquinazolines (10, 11) in 2 *N* HCl for 2 and 3.5 hr, respectively. After



III*a*: R = H
III*b*: R = OH

[1] Mel-Temp, Laboratory Devices.
[2] SAF, Gelman Instrument Co.
[3] Acta C III, Beckman Instruments.
[4] Sigma Chemical Co.
[5] Raylo Chemicals Ltd.
[6] Aldrich Chemical Co.

Table I—Inhibition of Xanthine Oxidase by Selected Quinazolines and Related Compounds



Compound	R_2	R_4	R_5	R_6	Ring 1[a]	Velocity[b]	I_{50}, μM[c]	Substrate[d]
IV	H	H	H	H	Ar	4.95 ± 0.76	—	(+)
V	H	OH	H	H	Ar	6.77 ± 0.48	—	(+)
VI	H	SH	H	H	Ar	2.37 ± 0.43	26.0	(−)
VII	H	NH_2	H	H	Ar	4.69 ± 0.09	—	(+)
VIII	OH	OH	H	H	Ar	6.45 ± 0.80	—	(−)
IX	NH_2	NH_2	H	H	Ar	5.40 ± 0.36	—	(−)
X	OH	NH_2	H	H	Ar	4.68 ± 0.48	—	(−)
XI	NH_2	OH	H	H	Ar	0.50 ± 0.00	0.9	(−)
XII	CH_3CONH	OH	H	H	Ar	1.15 ± 0.63	1.0	(−)
XIII	NH_2	SH	H	H	Ar	0	0.1	(−)
XIV	SH	NH_2	H	H	Ar	3.65 ± 0.44	—	(−)
XV	NH_2	OH	Cl	H	Ar	0.53 ± 0.05	1.0	(−)
XVI	NH_2	OH	H	Cl	Ar	0.45 ± 0.14	0.7	(−)
XVII	NH_2	OH	CH_3	H	Ar	3.72 ± 0.36	—	(−)
XVIII	NH_2	OH	H	CH_3	Ar	0.47 ± 0.05	1.2	(−)
XIX	NH_2	OH	H	(7)CF_3	Ar	0.40 ± 0.08	0.6	(−)
XX	NH_2	OH	H	NH_2	Ar	1.38 ± 0.05	6.5	(−)
XXI	NH_2	OH	H	CN	Ar	0.37 ± 0.09	1.0	(−)
XXII	NH_2	OH	H	CH_2OH	Ar	0	0.3	(−)
XXIII	NH_2	OH	H	CHO	Ar	0	0.07	(+)
XXIV	NH_2	OH	H	COOH	Ar	2.52 ± 0.19[e]	9.7	(−)
XXV	NH_2	OH	H	H	H_4	4.80 ± 0.17	—	(−)
XXVI	NH_2	OH	H	CH_3	H_4	5.06 ± 0.58	—	(−)
XXVII	Isocytosine					4.37 ± 0.81	—	(−)
XXVIII	Purine					3.03 ± 0.73	—	(+)
I*a*	2-Amino-4-hydroxy-6-hydroxymethylpteridine					0.16 ± 0.04	0.9	(−)
II*b*	2-Amino-4,7-dihydroxypteridine					1.25 ± 0.68	2.5	(−)
III*a*	Allopurinol					0	1.7[f]	(+)

[a] Ar = aromatic; H_4 = 5,6,7,8-tetrahydro. [b] Control velocity = 5.07 ± 0.28; this represents the absorbance change (×200) over 1.7 min using 10 μM inhibitor and 10 μM xanthine in 0.05 M potassium phosphate buffer (pH 8.0). [c] Compounds that showed significant inhibition at 10 μM were also assayed at other concentrations to determine the level producing 50% of the control velocity (I_{50}). [d] Concentration = 100 μM; incubation time = 12 hr. [e] A final 10% dimethyl sulfoxide concentration was necessary to maintain the inhibitor in solution. (Control velocity = 3.74 ± 0.19.) [f] This value may be compared with the 0.87 μM value obtained using 8.1 μM hypoxanthine in tromethamine buffer (pH 7.4) containing 10% dimethyl sulfoxide (Ref. 1).

neutralization with concentrated ammonium hydroxide, each compound was recrystallized from dimethylformamide–water containing excess ammonium hydroxide. Compound XVI was obtained in 31% yield, mp 370–375° dec. [TLC, homogeneous in dimethylformamide–acetonitrile (1:1)].

Anal.—Calc. for $C_8H_6N_3O$: C, 49.12; H, 3.09; N, 21.48. Found: C, 49.10; H, 3.16; N, 21.26.

Compound XX was obtained in 42% yield, mp > 300° dec. [TLC, homogeneous in dimethylformamide–acetonitrile (1:1)].

Anal.—Calc. for $C_8H_8N_4O$: C, 54.53; H, 4.57; N, 31.80. Found: C, 54.46; H, 4.45; N, 31.69.

2-Amino-4-hydroxy-6-hydroxymethylquinazoline (XXII)—A 0.47-g (2.5-mmole) sample of 2,4-diaminoquinazoline-6-carboxaldehyde (10) in 100 ml of 5% aqueous acetic acid was shaken in the presence of 100 mg of 10% palladium-on-charcoal under 3.5 atm of hydrogen for 3 hr at ambient temperature. At this point, the hydrogen uptake had ceased and TLC (dimethylformamide) showed no aldehyde present. The solution was filtered through diatomaceous earth[7] and then evaporated to dryness *in vacuo*. After stirring with 5% NH_4OH for 1 hr, the solid product was separated by filtration, washed with water and acetone, and dried *in vacuo*. A cream-colored solid, 0.31 g (65% yield), was obtained, mp 248.5–250.5° dec. (TLC showed a trace impurity at the origin which was not removed by recrystallization from a variety of solvent combinations).

A 0.20-g sample of this material was refluxed in 3 ml of 2 N HCl for 2.5 hr. After neutralization with concentrated ammonium hydroxide, the product was isolated by filtration, washed successively with water and acetone, and dried *in vacuo*. A white powder, 0.144 g (72% yield), was obtained, mp > 360° dec. (TLC showed a trace of impurity at the origin in dimethylformamide). This material was extracted with ethanol made basic with concentrated ammonium hydroxide and filtered, and then the filtrate was concentrated until precipitation began. The resulting solid was separated by filtration, washed with absolute ethanol, and dried *in vacuo* (TLC, virtually homogeneous in dimethylformamide).

Anal.—Calc. for $C_9H_9N_3O_2 \cdot 1.5H_2O$: C, 49.54; H, 5.50; N, 19.26. Found: C, 49.29; H, 5.63; N, 19.25.

2-Amino-4-hydroxyquinazoline-6-carboxylic Acid Hydrochloride (XXIV)—To a heated suspension of 2.78 g (15 mmoles) of 6-cyano-2,4-diaminoquinazoline (10) in 37.5 ml of ethylene glycol was added dropwise 15 ml of 40% NaOH. After refluxing for 4 hr, the solution was treated with activated charcoal and then filtered through diatomaceous earth[7], the filter cake being washed with additional ethylene glycol. The filtrate was reheated, made strongly acidic with 2 N HCl, diluted further with water at the boiling point, and then cooled. The precipitate was collected on a filter and washed with water, methanol, and acetone. This solid was dissolved in boiling water made basic with concentrated ammonium hydroxide. The solution, which contained a small amount of insoluble material, was treated with charcoal, filtered through diatomaceous earth, and then reheated and made distinctly acidic as before. Crystallization occurred upon cooling; the product was collected on a filter, washed with water, methanol, and acetone; and finally dried *in vacuo*. White crystals, 2.2 g (61% yield), were obtained, mp > 450° gradual dec. (TLC, homogeneous in dimethylformamide).

Anal.—Calc. for $C_9H_7N_3O_3 \cdot HCl$: C, 44.73; H, 3.34; N, 17.39. Found: C, 44.66; H, 3.39; N, 17.21.

RESULTS AND DISCUSSION

The enzyme data obtained in this study are presented in Table I. Quinazoline (IV) and its 4-OH (V) and 4-NH_2 (VII) derivatives serve as substrates for this enzyme and are ineffective as inhibitors of xanthine oxidation. However, 4-mercaptoquinazoline (VI) is a weak inhibitor with apparently no substrate activity. As expected, purine (XXVIII) also serves as a substrate. Of the 2,4-disubstituted quinazolines, VIII–XIV, only those possessing a 2-NH_2 and either OH or SH in position 4 show significant inhibitory action. 2-Amino-4-mercaptoquinazoline is an exceptionally

[7] Celite.

potent inhibitor and acetylation of the 2-NH_2 group of XI to afford XII does not alter activity significantly.

A series of derivatives of XI was selected to test the effect of substitution in the benzenoid nucleus on inhibitory potency. The 4-mercapto analogs were not prepared for this study in view of the extreme difficulty encountered in the synthesis of the unsubstituted compound XIII (9). The addition of a chlorine in the 5-position (XV) did not alter activity, while the inclusion of a 5-CH_3 (XVII) was highly deleterious. Conversely, the 6-Cl (XVI) and 6-CH_3 (XVIII) derivatives had similar potencies to XI. The presence of a 6-CN (XXI) or 7-CF_3 (XIX) caused little change in the I_{50}'s, while the 6-NH_2 derivative (XX) was some sixfold less inhibitory than the parent compound, XI. Compounds XXII–XXIV were synthesized as analogs of the pteridines, I*a* and *b*. Both the 6-CH_2OH (XXII) and 6-CHO (XXIII) derivatives were excellent inhibitors, with XXII being three times more potent than its pteridine counterpart, I*a*. In addition, XXIII was fourfold more inhibitory than XXII (which was apparently not the case with respect to I*a* and I*b*) and also served as a slow substrate, apparently being converted into the 6-COOH derivative (XXIV) based upon spectrophotometric observations. As was the case in the pteridine series (2), the introduction of a 6-COOH group (XXIV) yielded a substantially less inhibitory compound.

The 5,6,7,8-tetrahydroquinazolines (XXV and XXVI) were examined together with isocytosine (XXVII) to determine whether a second ring fused to the 2-amino-4-hydroxypyrimidine moiety was required for good activity and, if so, whether this ring needed to be unsaturated. None of these compounds showed inhibitory effects at 10 μM, so this structural feature appears to be required for good inhibition.

CONCLUSION

From the results discussed, it is apparent that suitably substituted quinazolines, but not related 5,6,7,8-tetrahydroquinazolines, can act as potent inhibitors of xanthine oxidase, rivaling or even surpassing their highly potent pteridine analogs. Furthermore, structure–activity considerations indicate that the quinazolines more closely resemble the pteridines with regard to inhibition of this enzyme than the analogous purines or pyrazolo[3,4-*d*]pyrimidines such as III*a*. For example, 2,4-dihydroxyquinazoline (VIII) is far less inhibitory than the reported (1) activity for III*b*, while XIII is substantially more inhibitory than its purine counterpart 2-amino-6-mercaptopurine (1).

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* Present address: Merck Sharp and Dohme Research Laboratories, Rahway, NJ 07065

ˣ To whom inquiries should be directed.

Chemical Constituents of *Piper sylvaticum* (Roxb) and *Piper boehmerifolium* (Wall)

P. K. MAHANTAˣ, A. GHANIM*, and K. W. GOPINATH‡

Abstract □ A phytochemical investigation of the roots and stems of *Piper sylvaticum* (Roxb) resulted in the isolation and identification of piperine, piperlonguminine, *N*-isobutyldeca-*trans*-2-*trans*-4-dienamide, and β-sitosterol. In addition, two unidentified compounds were detected. The stems of *P. boehmerifolium* (Wall) included piperine, but further investigation was not attempted.

Keyphrases □ *Piper sylvaticum* (Roxb)—phytochemical investigation of roots and stems, isolation and identification of piperine, piperlonguminine, *N*-isobutyldeca-*trans*-2-*trans*-4-dienamide, and β-sitosterol □ *Piper boehmerifolium* (Wall)—phytochemical investigation of stems, isolation and identification of piperine

Piper sylvaticum and *P. boehmerifolium* are members of the Piperaceae and are distinguished by perennial roots and branches creeping on the ground or rooting like ivy on trees. These plants are abundantly found in the upper and lower parts of Assam in India (1). In the Ayurvedic system of medicine in India, these roots have been used for their laxative, anthelmintic, and carminative properties and in bronchitis, diseases of the spleen, and the treatment of tumors (2). Although no phytochemical studies on

potent inhibitor and acetylation of the 2-NH_2 group of XI to afford XII does not alter activity significantly.

A series of derivatives of XI was selected to test the effect of substitution in the benzenoid nucleus on inhibitory potency. The 4-mercapto analogs were not prepared for this study in view of the extreme difficulty encountered in the synthesis of the unsubstituted compound XIII (9). The addition of a chlorine in the 5-position (XV) did not alter activity, while the inclusion of a 5-CH_3 (XVII) was highly deleterious. Conversely, the 6-Cl (XVI) and 6-CH_3 (XVIII) derivatives had similar potencies to XI. The presence of a 6-CN (XXI) or 7-CF_3 (XIX) caused little change in the I_{50}'s, while the 6-NH_2 derivative (XX) was some sixfold less inhibitory than the parent compound, XI. Compounds XXII–XXIV were synthesized as analogs of the pteridines, I*a* and *b*. Both the 6-CH_2OH (XXII) and 6-CHO (XXIII) derivatives were excellent inhibitors, with XXII being three times more potent than its pteridine counterpart, I*a*. In addition, XXIII was fourfold more inhibitory than XXII (which was apparently not the case with respect to I*a* and I*b*) and also served as a slow substrate, apparently being converted into the 6-COOH derivative (XXIV) based upon spectrophotometric observations. As was the case in the pteridine series (2), the introduction of a 6-COOH group (XXIV) yielded a substantially less inhibitory compound.

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From the results discussed, it is apparent that suitably substituted quinazolines, but not related 5,6,7,8-tetrahydroquinazolines, can act as potent inhibitors of xanthine oxidase, rivaling or even surpassing their highly potent pteridine analogs. Furthermore, structure–activity considerations indicate that the quinazolines more closely resemble the pteridines with regard to inhibition of this enzyme than the analogous purines or pyrazolo[3,4-*d*]pyrimidines such as III*a*. For example, 2,4-dihydroxyquinazoline (VIII) is far less inhibitory than the reported (1) activity for III*b*, while XIII is substantially more inhibitory than its purine counterpart 2-amino-6-mercaptopurine (1).

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P. K. MAHANTAˣ, A. GHANIM*, and K. W. GOPINATH‡

Abstract □ A phytochemical investigation of the roots and stems of *Piper sylvaticum* (Roxb) resulted in the isolation and identification of piperine, piperlonguminine, *N*-isobutyldeca-*trans*-2-*trans*-4-dienamide, and β-sitosterol. In addition, two unidentified compounds were detected. The stems of *P. boehmerifolium* (Wall) included piperine, but further investigation was not attempted.

Keyphrases □ *Piper sylvaticum* (Roxb)—phytochemical investigation of roots and stems, isolation and identification of piperine, piperlonguminine, *N*-isobutyldeca-*trans*-2-*trans*-4-dienamide, and β-sitosterol □ *Piper boehmerifolium* (Wall)—phytochemical investigation of stems, isolation and identification of piperine

Piper sylvaticum and *P. boehmerifolium* are members of the Piperaceae and are distinguished by perennial roots and branches creeping on the ground or rooting like ivy on trees. These plants are abundantly found in the upper and lower parts of Assam in India (1). In the Ayurvedic system of medicine in India, these roots have been used for their laxative, anthelmintic, and carminative properties and in bronchitis, diseases of the spleen, and the treatment of tumors (2). Although no phytochemical studies on

these species have appeared in the literature, the genus *Piper* has received considerable attention in recent years (3–6).

EXPERIMENTAL[1]

Extraction of Plant Material[2] and Isolation of Piperine—The air-dried, crushed roots and stems of *P. sylvaticum* (3 kg) were extracted with benzene in a soxhlet apparatus for 36 hr. The extract was concentrated to 50 ml of an oily residue (A) which, after refrigeration, gave a light-yellow product, mp 125°. After recrystallization from benzene, the product yielded needles, mp 128–129° [lit. (7) mp 130°]. The IR and NMR spectra of this compound were identical with that of an authentic sample of piperine isolated from *P. nigrum* (7). The identity of the compound as piperine was further established by a mixed melting-point determination with an authentic sample of piperine; the melting point was undepressed. In a similar procedure, piperine was isolated and identified from the air-dried stems of *P. boehmerifolium*.

Isolation of β-Sitosterol—After the isolation of piperine, the crude alkaloid mixture was chromatographed over neutral alumina[3] packed in a column. On elution with petroleum ether–ethyl acetate (95:5), a neutral crystalline compound was obtained. On recrystallization from methanol, the material gave a white solid, mp 136–138° [lit. (8) mp 136–137°]. While the product did not show any absorption in the UV region of 220–400 nm, the IR spectrum indicated characteristic signals at 3450 (OH), 2950 (C—H stretching of C—CH_3), 2880, 1460, 1050, and 950 cm^{-1}. The compound gave a positive Libermann-Burchard test for steroids. An acetate derivative was prepared, mp 130–132° [lit. (8) mp 134°]. The identity of the compound as β-sitosterol was further established by an IR spectrum which was superimposable with that of an authentic sample.

Isolation of Piperlonguminine—The material remaining on the column was further eluted with petroleum ether–ethyl acetate (9:1). The eluent was concentrated to yield a residue which, when spotted on silica gel G plates, developed with a solvent system composed of petroleum ether–ethyl acetate (1:1), and sprayed with Dragendorff's reagent, showed a single spot. On recrystallization from benzene–petroleum ether (2:1), the crude alkaloid gave a colorless crystalline material, mp 167–168°; UV: λ_{max} 245, 256, 307, and 340 nm (log ε 3.90, 4.02, 4.33, and 4.52); IR: 3275, 1645 (monosubstituted unsaturated carboxy amide), 1650 (extended conjugation), 1375, 1325, and 995 (isopropyl, methylenedioxy, and *trans*-configuration) cm^{-1}; NMR: τ 4.10 (2H, methylenedioxy), 3.20–3.04 (3H, aromatic), 3.99, 3.68, 3.25, 2.50 (4H, olefinic), 8.95 (6H, methyl, d, J = 6 Hz), 7.90 (isopropyl), and 6.55 (2H, methylene adjacent to NH). The product had identical UV, IR, and NMR spectra and an identical melting point with those of an authentic sample of piperlonguminine, which had previously been synthesized by the condensation of piperic acid chloride with isobutylamine (4).

Anal.—Calc. for $C_{16}H_{19}NO_3$: C, 70.6; H, 6.98. Found: C, 70.83; H, 7.55.

Isolation of *N*-Isobutyldeca-*trans*-2-*trans*-4-dienamide—Six spots were found by TLC of the crude alkaloid extract (A) on silica gel G plates, developed with a solvent system composed of petroleum ether–ethyl acetate (1:1). The sample was chromatographed over a column packed with neutral alumina[3], and chlorophyll was removed by elution with petroleum ether. The remaining extract was separated into different fractions on preparative TLC [145 g silica gel G, with petroleum ether–ethyl acetate (1:1) as the developing solvent]. Fraction 2, on washing with petroleum ether, afforded a crude alkaloid. On recrystallization from petroleum ether, the crude alkaloid yielded colorless needles, mp 66–68°; UV: λ_{max} 258 nm (log 4.81, conjugated system); IR: ν_{max} 997 (*trans*-double bond), 947 (2,4-diene system with *trans*-configuration), 3300 (NH), 1625 (C=O), and 1650 (>C=C<) cm^{-1}. The IR spectrum was superimposable with that of the authentic sample of *N*-isobutyldeca-*trans*-2-*trans*-4-dienamide (5).

Anal.—Calc. for $C_{14}H_{25}NO$: C, 75.28; H, 11.28. Found: C, 74.89; H, 11.54.

A low melting, unstable alkaloid and a terpene, mp 195°, were also isolated. Their structural elucidation is in progress.

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* Present address: Central Arid Zone Research Institute, Jodhpur, Rajasthan, India.

‡ Present address: Ranbaxy Laboratories, OKHLA, New Delhi-20, India.

ˣ To whom inquiries should be directed.

[1] All melting points are uncorrected. The maximim wavelength of absorption in the UV spectrum was determined on a Carl Zeiss Jena spectrophotometer in 95% ethanol. The IR spectra were recorded on a Perkin-Elmer model 237B in mineral oil. The NMR spectra were recorded with a Varian T-60 instrument in $CDCl_3$ with tetramethylsilane as the internal reference.

[2] *P. sylvaticum* was collected in Nowgong, Assam, during April 1969, and *P. boehmerifolium* was collected in the Kamrup district during February 1970. The identity of the plant material was confirmed by Mr. L. C. Rabha, Division of Medicinal and Economic Plants, Regional Research Laboratory, Jorhat-6, Assam. The voucher specimens of *P. sylvaticum* and *P. boehmerifolium* have been preserved at the Herbarium, Division of Medicinal and Economic Plants, Regional Research Laboratory, Jorhat-6, Assam, India.

[3] Brockmann activity I.

Antitumor Agents VIII: Synthesis and Cytotoxic Activity of *O,O'*-Bis(acrylyl)-α,ω-alkanediols

KUO-HSIUNG LEE*[x], SUN-HYUK KIM*, CLAUDE PIANTADOSI*, ENG-SHANG HUANG‡, and T. A. GEISSMAN§

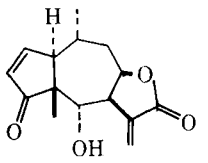
Abstract □ A short series of *O,O'*-bis(acrylyl)-α,ω-alkanediols was prepared to evaluate the potential significance of the O=C—C=CH_2 system for cytotoxic activity against the growth of tissue culture cells originating from human epidermoid carcinoma of larynx (HEp-2). These compounds showed significant cytotoxic activity; however, the chain length bridging the two O=C—C=CH_2 systems appeared to have no effect on cytotoxicity. Several *O,O'*-bis(β-bromopropionyl)-α,ω-alkanediols were prepared, tested, and found to have marginal cytotoxicity.

Keyphrases □ *O,O'*-Bis(acrylyl)-α,ω-alkanediols—synthesized as possible antitumor agents, cytotoxicity evaluated □ Helenalin analogs—synthesized as possible antitumor agents, cytotoxicity evaluated □ Antitumor agents, potential—synthesis and cytotoxic activity of *O,O'*-bis(acrylyl)-α,ω-alkanediols

In previous studies (1–3), the most immediate and direct factor responsible for cytotoxicity among the sesquiterpene lactones was the introduction of the O=C—C=CH_2 system, whether it be in the lactone or ketone. Additional alkylating groups appear to enhance cytotoxicity significantly (4, 5). In an effort to evaluate these hypotheses, several bisacrylyl esters (II*a*–II*e*) were prepared as bifunctional alkylating open-chain analogs related to helenalin (I).

DISCUSSION

The desired new bifunctional alkylating agents (II*a*–II*e*) (Table I) were best synthesized by dehydrobromination of *O,O'*-bis(β-bromopropionyl)-α,ω-alkanediols (III*a*–III*e*) (Table II), which were obtained by reaction of diols with β-bromopropionyl chloride (IV). Compound IV was prepared by refluxing β-bromopropionic acid (V) with thionyl chloride (Scheme I). Since all of these bisacrylyl derivatives (II*a*–II*e*) were unstable at room temperature, traces of *p*-hydroquinone were added to prevent polymerization.



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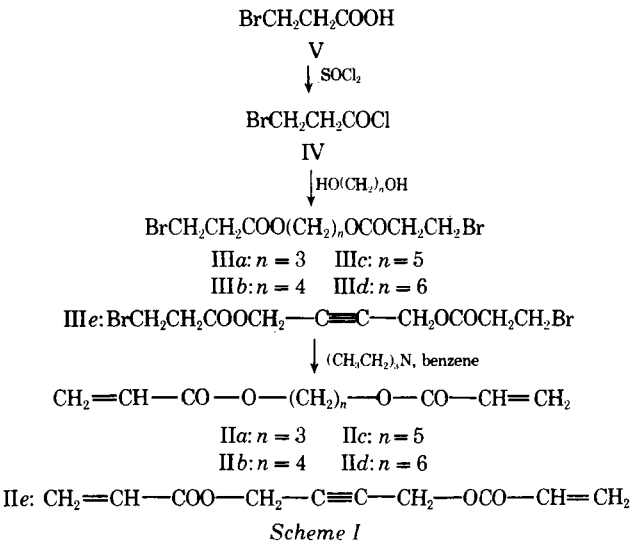

Scheme I

Compounds II*a*–II*e* and III*a*–III*e* were assayed for inhibitory activity *in vitro* against cells originating from human epidermoid carcinoma of larynx (HEp-2), according to a rapid microtiter method previously described (6). A comparison of the ED_{50} values for the cytotoxicity of the compounds listed in Table I indicated that the monofunctional alkylating acrolein and ethyl acrylate were inactive, whereas the bifunctional alkylating derivatives (II*a*–II*e*) were about equally active although they were about 7–10 times less active in comparison with helenalin (I). These findings are in agreement with the previous hypotheses indicating that the structural requirement for significant cytotoxicity is to

Table I—*O,O'*-Bis(acrylyl)-α,ω-alkanediols

Compound	Melting Point	Analysis[a], % Calc.	Found	IR[b]	NMR[c]	ED_{50}, μg/ml (HEp-2)
II*a*	—[d]	—[e]		—[f]	2.08 (2H, q, J = 6.0, OCH_2CH_2) 4.30 (4H, t, J = 6.0, OCH_2)[g]	0.84
II*b*	—[d]	C 60.59 H 7.12	60.54 7.21	—[f]	1.80 (4H, m, OCH_2CH_2) 4.22 (4H, m, OCH_2)[g]	0.89
II*c*	—[d]	C 62.25 H 7.60	61.77 7.70	—[f]	1.62 (6H, m, OCH_2CH_2) 4.28 (4H, m, OCH_2)[g]	0.77
II*d*[h]	—[d]	C 63.70 H 8.20	63.65 8.08	—[f]	1.55 (8H, m, OCH_2CH_2) 4.20 (4H, m, OCH_2)[g]	0.95
II*e*[i]	—[d]	C 61.85 H 5.19	61.77 5.20	1735, 1745, 1640 cm^{-1}	4.81 (4H, s, OCH_2) 5.75–6.72 (6H, m, olefinic protons)	0.76
Acrolein[j], CH_2=CH—CHO						>20
Ethyl acrylate[j], CH_2=CH—$COOCH_2CH_3$						>20
Mechlorethamine hydrochloride[k]						0.33
Helenalin 170–172° (Ref. 5)						0.10

[a] Performed by Atlantic Microlab, Inc. [b] Determined in liquid film with a Perkin-Elmer 257 grating IR spectrophotometer. [c] Measured in $CDCl_3$ with a Jeolco C 60 HL NMR spectrometer, using tetramethylsilane as an internal standard. Chemical shifts are reported in δ units (parts per million); s refers to singlet, t to triplet, q to quintet, and m to multiplet, and the J values are in hertz. [d] Oil. [e] The attempted analysis of this compound was unsuccessful because of rapid polymerization at room temperature. [f] Showed IR absorption maxima at 1725 (C=O) and 1635 and 1618 (C=C) cm^{-1}. [g] 5.72–6.78 (6H, m, olefinic protons). [h] Reference 7 reported the synthesis of this compound in 27% yield. [i] Reference 8 reported the synthesis of this compound in 18% yield. [j] Aldrich Chemical Co., Milwaukee, Wis. [k] Sigma Chemical Co., St. Louis, Mo.

Table II—*O,O'*-Bis(β-bromopropionyl)-α,ω-alkanediols

Compound	Melting Point	Yield, %	Analysis[a], % Calc.	Found	IR[b]	NMR[c]	ED_{50}, μg/ml (HEp-2)
III*a*	—[d]	75	C 31.23 H 4.07	31.48 4.19	—[e]	2.02 (2H, q, J = 6.0, OCH_2CH_2) 4.25 (4H, t, J = 6.0, OCH_2)[f,g]	7.17
III*b*	—[d]	80	C 33.35 H 4.47	33.56 4.57	—[e]	1.75 (4H, m, OCH_2CH_2)[f,g,h]	5.20
III*c*	—[d]	90	C 35.31 H 4.85	35.49 4.93	—[e]	1.58 (6H, m, OCH_2CH_2)[f,g,h]	4.76
III*d*	—[d]	90	C 37.13 H 5.19	37.21 5.28	—[e]	1.50 (8H, m, OCH_2CH_2)[f,g,h]	5.80
III*e*	—[d]	90	C 33.73 H 3.39	33.61 3.37	—[e]	3.55 (4H, t, J = 6.0, $BrCH_2$) 4.75 (4H, s, OCH_2)[f]	2.36
III*g*[i], $BrCH_2CH_2COOCH_2CH_3$							>20

[a] Performed by Atlantic Microlab, Inc. [b] Determined in liquid film with a Perkin-Elmer 257 grating IR spectrophotometer. [c] Measured in $CDCl_3$ with a Jeolco C 60 HL NMR spectrometer, using tetramethylsilane as an internal standard. Chemical shifts are reported in δ units (parts per million); s refers to singlet, t to triplet, q to quintet, and m to multiplet, and the J values are in hertz. [d] Oil. [e] Showed IR absorption maximum at 1740 cm^{-1} (C=O). [f] 2.95 (4H, t, J = 6.0, CH_2CO). [g] 3.60 (4H, t, J = 6.0, $BrCH_2$). [h] 4.18 (4H, m, OCH_2). [i] Aldrich Chemical Co., Milwaukee, Wis.

have an O=C—C=CH_2 system as the active alkylating center, especially when two of these systems are linked together. The chain length bridging these two systems does not appear to affect cytotoxicity among the compounds studied (II*a*–II*e*). The marginal cytotoxicity exhibited by III*a*–III*e* (Table II) suggests that there might be a dehydrogenation or dehydrobromination reaction for these compounds to take place in tissue culture cells. The dehydrogenated or dehydrobrominated products might act in a manner analogous to II*a*–II*e*. The monofunctional bromoester (III*g*) was completely inactive. Pipobroman [1,4-bis(3-bromopropionyl)-piperazine, NSC-25154], which has two N—$COCH_2CH_2Br$ moieties, is listed in the National Cancer Institute compilation[1] of "Drugs Active in Clinical Cancer."

EXPERIMENTAL

General Synthetic Method for *O,O'*-Bis(β-bromopropionyl)-α,ω-alkanediols (III*a*–III*e*)—Diols (1 mmole) were added dropwise to β-bromopropionyl chloride (2 mmoles) with stirring in an ice bath, and the resulting mixture was heated to 70° for 5 hr. Ether was then added, and the mixture was washed with 1% sodium bicarbonate and water, dried over anhydrous sodium sulfate, and distilled *in vacuo*, leaving a viscous oily product which was purified by column chromatography on silica gel[2] by elution with benzene.

General Synthetic Method for *O,O'*-Bis(acrylyl)-α,ω-alkanediols (II*a*–II*e*)—To III*a*–III*e* (1 mmole) in dry benzene (5 ml) was added triethylamine (3 mmoles) and a small amount of *p*-hydroquinone (~1% of the reactants). The mixture was refluxed for 18–20 hr. The solid triethylamine salt, which separated upon cooling, was filtered and the filtrate was evaporated *in vacuo*. The product was purified in benzene by passing through a column (1.3 × 3 cm) of silica gel[2] to furnish the quantitative yield of II*a*–II*e*, which was rapidly stabilized by addition of traces of *p*-hydroquinone. TLC [silica gel[3] G developed with chloroform–acetone (3:1)], IR, and NMR data indicated that these esters (II*a*–II*e*) were homogeneous.

[1] *Chemotherapy*, Sept. 1971.
[2] Baker A.R. No. 3405.
[3] Merck.

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[x] To whom inquiries should be directed.

Effect of Temperature and Sodium Chloride on Drag Reduction by Sodium Carboxymethylcellulose and Guar Gum

DEVENDRA SHAH[x] and BERNARD ECANOW

Abstract □ The effect of variations in temperature and sodium chloride concentrations on drag reduction by sodium carboxymethylcellulose and guar gum was studied with water in turbulent flow, using a simple flow tube. Two different drag-reducing mechanisms apparently exist for sodium carboxymethylcellulose and guar gum. Sodium chloride significantly reduced the drag-reducing property of sodium carboxymethylcellulose but did not appear to affect drag reduction by guar gum. The effect of variations in temperature (10–45°) on drag reduction was different for each drag-reducing agent.

Keyphrases □ Sodium carboxymethylcellulose—effects of temperature and sodium chloride on drag reduction □ Guar gum—effects of temperature and sodium chloride on drag reduction □ Drag-reducing agents—effects of temperature and sodium chloride on sodium carboxymethylcellulose and guar gum

Drag reduction may be defined as the effect that occurs when the rate of flow of fluid at a constant pressure gradient is increased upon addition of a colloidal additive (generally in amounts insufficient to cause large changes in the viscosity of the fluid). Drag reduction occurs in turbulent flow and is, therefore, of great potential value to the processing industry where most flows are turbulent. Most such drag-reducing agents are polymers of high molecular weight (1–3).

Recently, Rodriguez (4) observed the drag reduction by polymeric aluminum soap using a simple flow tube. The flow tube is L-shaped Pyrex glass tubing with a 500-ml capacity reservoir connected to the upper end of the vertical portion. This apparatus can be used by measuring "jet action" as the fluid leaves the horizontal tube. Previously, sodium carboxymethylcellulose, guar gum, and many other polymers were reported to be drag-reducing agents (2).

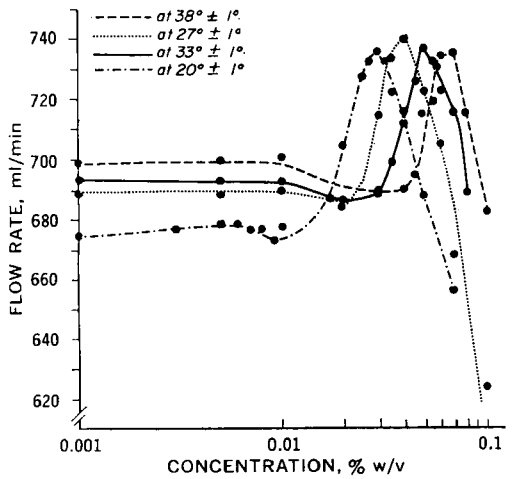

Figure 1—*Effect of concentration of sodium carboxymethylcellulose on flow rate of water at different temperatures.*

Table I—Effect of Sodium Chloride on Some Aqueous Systems at 27 ± 1°

System	Rate[a], ml/min
Water (distilled)	686.5 ± 1.11
Water plus:	
0.05% guar gum	814.78 ± 2.58
0.05% guar gum + 0.9% NaCl	812.57 ± 1.98
0.05% sodium carboxymethylcellulose	723.59 ± 1.96
0.05% sodium carboxymethylcellulose + 0.9% NaCl	670.55 ± 2.51
0.9% NaCl	688.4 ± 1.73

[a] Rate ± standard deviation for five readings.

This paper reports the effect of temperature and sodium chloride concentrations on the drag reduction by sodium carboxymethylcellulose and guar gum, in turbulent flow of water, using a flow tube as previously reported (4).

EXPERIMENTAL

Experiments were carried out in three different categories.

Effect of Temperature on Drag Reduction at Various Concentrations of Sodium Carboxymethylcellulose—The different concentrations of sodium carboxymethylcellulose[1] from 0.001 to 0.1% were run through the flow tube, the time required for 500 ml of solution to flowthrough was noted, and the rate (milliliters per minute) of flow was calculated and plotted against log concentration at 20 ± 1, 27 ± 1, 33 ± 1, and 38 ± 1° (Fig. 1).

Effect of Temperature on Drag Reduction by Guar Gum—The different concentrations of guar gum[2] from 0.001 to 0.15% were run through the tube. The time required for 500 ml of suspension to pass through the tube was noted, and the rate was plotted against log concentrations at 27 ± 1° (Fig. 2). The 0.05% guar gum suspension, which gave a maximum drag reduction at 27°, was tested at 9 ± 1, 15 ± 1, 19 ± 1, 27 ± 1, and 45 ± 1°. The rate was calculated and plotted against the log temperature (Fig. 3).

Effect of Sodium Chloride on Drag Reduction by Sodium Carboxymethylcellulose and Guar Gum—To study the effect of

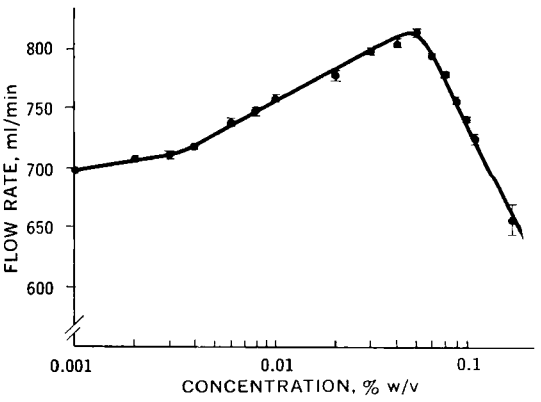

Figure 2—*Effect of concentration of guar gum on flow rate of water at 27°.*

[1] Mallinckrodt Chemical Works, St. Louis, Mo.
[2] S. B. Penick & Co., Chicago, Ill.

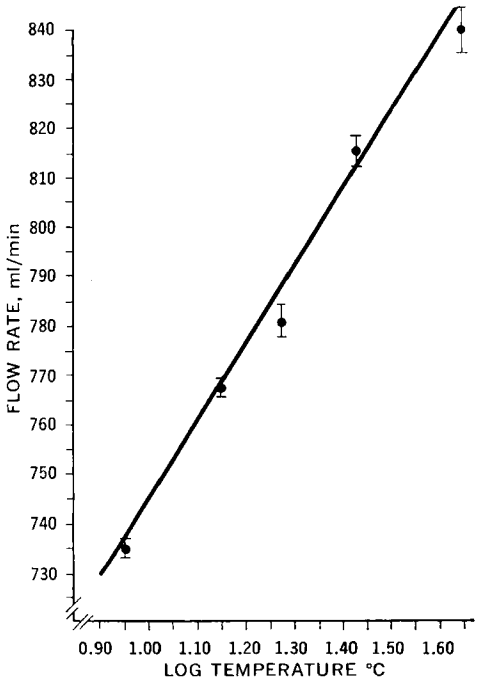

Figure 3—*Effect of temperature on flow rate of 0.05% guar gum suspension.*

sodium chloride[1] on the drag-reducing property of sodium carboxymethylcellulose, 0.05% sodium carboxymethylcellulose was run through the tube with different concentrations of sodium chloride from 0.0001 to 0.05% and the rate of flow was calculated. The rate of flow was plotted against log concentration of sodium chloride (Fig. 4).

No significant change in rate was observed when 0.05% guar gum suspension was run through with 0.9% NaCl (Table I).

RESULTS AND DISCUSSION

In the study of effect of temperature on the flow rate of sodium carboxymethylcellulose, it was observed that an increase in temperature increased the flow rate at initial concentrations. This was probably due to a decrease in viscosity of the solution. An increase in temperature also increased the concentration required to obtain maximum drag reduction. The maximum amount of drag reduction that can be achieved by sodium carboxymethylcellulose remained practically the same at all temperatures. Any increase or decrease of temperature in a system at maximum drag reduction concentration resulted in a decreased flow rate. The effect of temperature on the flow rate of guar gum differed from that of sodium carboxymethylcellulose; there was an increase in the flow rate of a 0.05% guar gum suspension with an increase in temperature.

In the study of the effect of sodium chloride on the drag-reducing property of sodium carboxymethylcellulose, it was observed that up to about 0.0008% NaCl concentration, there was no significant change in flow rate of 0.05% sodium carboxymethylcellulose solution. At around 0.001% NaCl concentration, there was a slight increase in the flow rate followed by a sharp decrease.

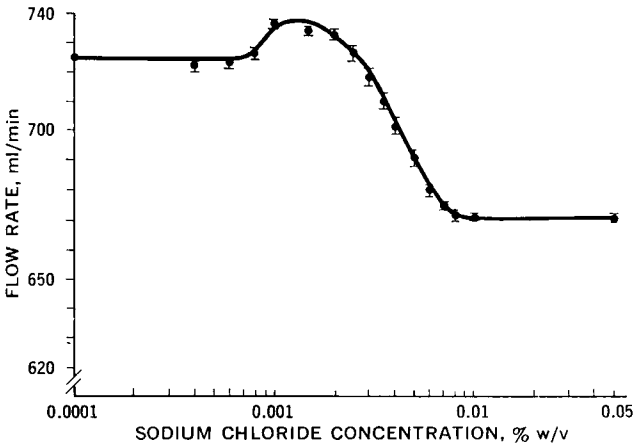

Figure 4—*Effect of sodium chloride concentration on flow rate of 0.05% sodium carboxymethylcellulose at 27°.*

The increase in the rate of flow in 0.05% sodium carboxymethylcellulose with increasing sodium chloride concentrations around 0.001% can be explained as follows. The 0.05% sodium carboxymethylcellulose solution at 27 ± 1° is a drag-reducing concentration, but it is above the concentration required for maximum drag reduction, which is 0.04% sodium carboxymethylcellulose. Apparently, the concentration of the sodium carboxymethylcellulose at the interface is reduced when sodium chloride is added to 0.05% sodium carboxymethylcellulose solution, since there is a significant increase in flow rate at the initial concentrations of sodium chloride, which corresponds to the maximum drag reduction concentration. Further addition of sodium chloride reduces the concentration of sodium carboxymethylcellulose at the interface below the concentration required for maximum drag reduction and, therefore, produces a reduction in the flow rate.

From the data, it would appear that two different mechanisms of drag reduction are operative.

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[x] To whom inquiries should be directed. Present address: School of Pharmacy, Florida A & M University, Tallahassee, FL 32307

Isolation and Characterization of a Hypoglycemic Agent from *Xanthium strumarium*

FLOYD P. KUPIECKI*[x], C. DWAYNE OGZEWALLA‡, and FRED M. SCHELL‡§

Abstract □ A compound isolated from seeds of *Xanthium strumarium* exhibited potent hypoglycemic activity in the rat. Partial characterization of the crystalline compound showed that it contains only carbon, hydrogen, oxygen, and sulfur and that the sulfur appears to be present as the sulfonate group. The agent differs from all compounds previously isolated from *X. strumarium*.

Keyphrases □ *Xanthium strumarium* L. (Compositae)—isolation and characterization of a hypoglycemic agent □ Hypoglycemic activity—isolation and characterization of crystalline compound from *Xanthium strumarium*

Biological studies have been reported on extracts or compounds isolated from *Xanthium strumarium* L. (Compositae), the common cocklebur. An attempt to verify arthralgic activity in *Xanthium* resulted in isolation of a depressor substance which proved to be identical with choline (1). A tincture of seed stimulated respiration in the frog at low doses and depressed respiration at high doses (2). Leaves of the plant yielded xanthinin (3), a plant growth-regulating compound isolated earlier from *X. pennsylvanicum* (4). An investigation into the cause of the often reported toxicity of cocklebur led to the conclusion that hydroquinone is responsible for the toxic effects produced by ingestion of various species of *Xanthium* (5). This paper describes the isolation and characterization, from seed of *X. strumarium*[1], of a new crystalline compound that lowers blood sugar in the rat.

EXPERIMENTAL

Partial Purification—An extract was prepared by boiling 300 g of coarsely ground seed in 1300 ml of water in an open beaker for 30 min. After cooling and centrifuging, the volume was adjusted to 1000 ml. Extracts prepared in this way contained 23–40 mg solids/ml and were fractionated through the following four steps before chromatography[2].

1. The solution was concentrated to 185 mg/ml and 2 volumes of acetone were added with stirring at room temperature. After 0.5–1 hr, the solids were removed by centrifugation and discarded. A 3rd volume of acetone was added and the precipitate was removed as described and discarded. The solution was concentrated to remove acetone and brought back to a solids concentration of 185 mg/ml. Absolute ethanol was added to a final concentration of 95% during 10–15 min while the solution was stirred and maintained between 65 and 75°. After it cooled to room temperature, the flask was placed in ice and held at 0° for 24–36 hr. The loose precipitate was harvested on a sintered-glass funnel and dissolved in water to a concentration of 100 mg/ml for Step 2.

2. Absolute ethanol was added to a final concentration of 90%, the flask was cooled, and the precipitate was harvested as in Step 1.

[1] Identified by Dr. C. D. Ogzewalla, University of Cincinnati, Cincinnati, OH 45221. Specimens are deposited at the Herbarium, University of Cincinnati, Cincinnati, OH 45221

[2] Amberlite XAD-2 resin.

Table I—Comparative Hypoglycemic Activity of Crude Extract and Material Obtained in Step 1 of Purification

Fraction Injected	Dose, mg/kg	Blood Glucose, mg%	Percent Lowering	*p* (*versus* Control)
Saline (control)	—	63[a]	—	---
Crude extract	15	49	22	<0.05
Crude extract	30	41	35	<0.01
Purified, Step 1	12	29	54	<0.01

[a] Each number is the mean of five rats dosed by intraperitoneal injection and bled 3 hr later. The standard error of a treatment mean is 3.25.

3. The solids from Step 2 were dissolved in water to a concentration of 50 mg/ml. Acetone (4 volumes) was added in 5–10 min while the solution was stirred in a 55° bath. The solution was removed from the bath and, after 1 hr, the precipitate was removed by centrifugation and discarded. The solution was stored at from −55 to −70° for 24–48 hr and the precipitate was harvested on a sintered-glass funnel at that temperature and dried.

4. The solids from Step 3 were dissolved in water to a concentration of 85 mg/ml. Absolute ethanol (6 volumes) was added in 10–15 min while the solution was stirred in a bath held at 75–80°. The solution was removed from the bath, allowed to reach room temperature, and placed in a freezer at from −15 to −20° for 24–48 hr. The precipitate was harvested as in Step 3.

Chromatography on a Resin[2] Column—In a typical procedure, 100 mg of material from Step 4 was fractionated on a 10-ml column in a 10-ml pipet at an elution rate of 1% column volume/min. Water was passed through the column until 2.4 column volumes were collected. This was followed by 1.2 column volumes of 50% methanol.

Crystallization—*Method 1*—The material that came off the resin with 50% methanol was dried and dissolved in 50% ethanol at a concentration of 50 mg/ml. The solution was stored in ice for 2–4 days and the fine colorless needles were harvested by filtration. Recrystallization was accomplished from 50% ethanol in the same way.

Method 2—The solids from Step 4 were dissolved in acetic acid at a concentration of 50 mg/ml. One volume of *n*-butanol and 2 volumes of ethyl acetate were added and the solution was stored in ice overnight. The precipitate was removed by centrifugation at 0° and discarded. The remaining solids were recovered by evaporation under vacuum and crystallized from 50% ethanol as in Method 1.

Progress in purification was followed by assaying for hypoglycemic activity and by TLC on precoated silica gel plates in three solvent systems: System 1, *n*-butanol–acetic acid–ether–water (45:30:15:5); System 2, *n*-butanol–methanol–water–acetic acid (80:10:10:1); and System 3, *n*-butanol–methanol–water (80:10:10). Routinely plates were developed once in System 1 and twice in Systems 2 and 3. The hypoglycemic component appeared as a red-violet spot at R_f 0.60, 0.45, and 0.14 in Systems 1, 2, and 3, respectively, when plates were sprayed with vanillin in phosphoric acid and heated 5 min at 140°.

Chemical Methods—Since the crystalline product yielded a residue of 17–20% when analyzed for carbon, hydrogen, and nitrogen, it appeared to be a salt. Therefore, it was passed through a resin column[3] in the H^+ form at 4° (100–200 mg/5 ml column)

[3] Amberlite IR-120.

Table II—Comparative Activity of Crude, Partly Purified, and Crystalline Hypoglycemic Agent

Fraction Injected	Dose, mg/kg	Blood Glucose, mg%	Percent Lowering	*p* (*versus* Control)
Saline (control)	—	70[a]	—	—
Crude extract	30	42	40	<0.01
Purified, Step 3	8	23	67	<0.01
Purified, Step 3	12	19	73	<0.01
Purified, Step 4	6	17	76	<0.01
Purified, Step 4	12	9	87	<0.01
Purified on resin[b]	5	21	70	<0.01
Crystalline	1.25	33	53	<0.01
Crystalline	2.50	14	80	<0.01

[a] Each number is the mean of five rats dosed by intraperitoneal injection and bled 3 hr later. The standard error of a treatment mean is 2.08. [b] Amberlite XAD-2.

and titrated with potassium hydroxide. Titration showed two strong and two weak acid groups per molecular weight of approximately 885. Other aliquots were passed through the column, collected in dilute ammonium hydroxide, and dried for analyses. The results for the ammonium salt showed: C, 42.82; H, 6.8; N, 4.74; O, 37.29; and S, 7.15; therefore, a formula of $C_{32}H_{52}O_{21}S_2$ for the free acid was indicated. The two sulfur atoms and the two strong acid groups appear to be sulfonates, since elemental analyses showed that the sulfur was not lost when the compound was warmed with 0.1 *N* $Ba(OH)_2$ at 37° for 4 days. The IR spectrum (mineral oil) of the crystalline compound showed hydroxyl at 3440, carbonyl at 1720, and SO_3H at 1240 cm^{-1}. Peaks at 1040 and 1000 cm^{-1} could be attributed to SO_3H and/or alcohol C—O. Other peaks occurred at 1625, 1560, 1375, 1165, 1120, 925, 900, and 810 cm^{-1}. The mass spectrum, after trimethylsilylation of the compound, showed a fragment at 1042 which, by use of the deuterated silyl reagent, was found to contain six trimethylsilyl groups. This indicates a fragment with a mass of 610. Further structure studies were complicated by the fact that the crystalline compound, when passed through the resin[3], decomposed spontaneously to yield six red-violet spots on TLC in System 3 unless it was collected in alkaline solution.

Assay for Hypoglycemic Activity—Male rats[4], weighing 130–150 g, were injected intraperitoneally or subcutaneously with the agent in saline after an overnight fast. Immediately after treatment, they were injected subcutaneously with 1 g/kg of glucose in 1 ml of saline. They were bled from the abdominal aorta after cyclopentobarbital[5] anesthesia. Blood glucose was measured using an automated analyzer by a modification of the ferricyanide procedure of Hoffman (6).

RESULTS AND DISCUSSION

Tables I and II show the comparative hypoglycemic activity of the crude extract and the products of the several steps of purification to crystallinity. The results show a good dose–response relationship at each step of purification. The time course of hypoglycemia from 1 to 7 hr is shown in Table III. There is no lowering of blood glucose after 1 hr. After 2 hr the lowering of glucose is significant, but maximal lowering occurs at 3 hr and persists at this level through Hours 5 and 7. To eliminate the possibility that the drug acts directly on the liver when injected into the peritoneal cavity, the activity of subcutaneously and intraperitoneally injected doses was compared. The two routes of injection produced equal effects on blood glucose (Table IV). The crude material, partly purified fractions, and crystalline compound had only weak hypoglycemic activity when given orally at 3–5 times the injected doses.

The demonstrated presence of only carbon, hydrogen, sulfur, and oxygen in the molecule eliminates the various classes of compound containing nitrogen. The possibilities are narrowed further by absence of peaks in the UV spectrum (2 mg/10 ml water). The presence of sulfate ester or sulfonate is shown by titration, and the results of alkaline hydrolysis suggest the latter. The following reagents for specific classes of compounds or functional groups (7) repeatedly failed to react with the purified compound on TLC under conditions that worked with standards: antimony trichloride for steroids and glycosides, basic lead acetate for uronic acids and flavonoids, *p*-toluenesulfonic acid for steroids and flavonoids, benzidine–sodium metaperiodate and silver nitrate–sodium hydroxide for sugars and sugar alcohols, and 2,4-dinitrophenylhydrazine for aldehydes and ketones.

Partial characterization of the compound demonstrates that the hypoglycemic activity is not due to compounds isolated previously from cocklebur (1–5). Further attempts at identification of the agent are underway.

[4] Upj:TUC(SD) spf.
[5] Cyclopal.

Table III—Time Course of Activity of Partly Purified Hypoglycemic Agent

Hours	Blood Glucose		Percent Lowering	*p* (*versus* Control)
	Control	Treated		
1	61[a]	63[a]	0	NS
2	60	38	37	<0.01
3	63	21	67	<0.01
5	65	21	68	
3	77	24	69	<0.01
5	72	29	60	<0.01
7	76	31	59	<0.01

[a] Each number is the mean of five rats dosed by intraperitoneal injection. The standard error for 1-, 2-, 3-, and 5-hr groups is 2.48, 1.19, 3.23, and 2.88, respectively; for 3-, 5-, and 7-hr groups, it is 1.91, 2.25, and 1.73, respectively. Each treated animal was dosed with 6 mg/kg of material purified through Step 4.

Table IV—Comparison of Routes of Injection on Activity of Partly Purified Hypoglycemic Agent

Experiment Number	Route	Blood Glucose		Percent Lowering	*p* (*versus* Control)
		Control	Treated		
1	Intraperitoneal	70[a]	21[a]	70	<0.01
	Subcutaneous	61	22	64	<0.01
2	Subcutaneous	57	15	74	<0.01

[a] Each number is the mean of five rats. The standard error for lines 1, 2, and 3 is 2.66, 4.07, and 1.98, respectively. Each treated animal was dosed with 5 mg/kg of material purified through the resin step.

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§ Present address: Department of Chemistry, University of Tennessee, Knoxville, TN 37916

ˣ To whom inquiries should be directed.

Circular Dichroism Study on Inclusion Complexes of β-Cyclodextrin with Anti-Inflammatory Fanamates

Keyphrases □ Cyclodextrin-fanamate inclusion complexes—circular dichroism studies □ Anti-inflammatory fanamate-cyclodextrin inclusion complexes—circular dichroism studies □ Circular dichroism—analysis of cyclodextrin-fanamate inclusion complexes □ Complexes, inclusion (cyclodextrin-fanamates)—stoichiometric ratio, stability constants, circular dichroism

To the Editor:

Cyclodextrins are known to form inclusion complexes with various drug molecules (1–7). When optically inactive compounds interact with optically active compounds, new circular dichroism (CD) bands are induced in the absorption bands of optically inactive compounds (8). Thakkar *et al.* (9) recently reported that barbiturates generated the induced CD upon binding to β-cyclodextrin (cycloheptaamylose) and estimated the stability constants of the inclusion complexes from the CD bands. Azo dye, iodine, and benzoic acid also have been shown to generate extrinsic Cotton effects upon binding to cyclodextrins (10–13).

Since the induced Cotton effects can give useful information on the nature of inclusion complexes (9–13), we studied, by means of a CD technique, the interactions of β-cyclodextrin with anti-inflammatory fanamates such as flufenamic, mefenamic, and meclofenamic acids in aqueous solution. In this communication, we report that the analysis of these induced CD bands could afford the conformational elucidation of the guest molecules of fanamates within the cavity of β-cyclodextrin.

The CD[1] and UV[2] spectra were taken in 0.1 *M* phosphate buffer (pH 7.0) at 25°. The optical anisotropy factor ($g = \Delta\epsilon/\epsilon$), which is proportional to the magnitude of the induced Cotton effects, was calculated from the following expression:

$$g = \frac{[\theta]}{3300 \times \epsilon} \qquad \text{(Eq. 1)}$$

where $[\theta]$ is the molar ellipticity, and ϵ is the molar absorption coefficient of the drug in the presence of β-cyclodextrin (1.0×10^{-2} *M*) at the maximum wavelength of the CD spectrum.

Figure 1A shows the effect of β-cyclodextrin on the UV absorption spectrum of flufenamic acid. The absorption maximum of the drug shifted to a longer wavelength in the presence of β-cyclodextrin, with concomitant change in molar absorption coefficients. Figure 1B shows the CD spectrum for flufenamic acid in the presence of β-cyclodextrin, with positive peaks at 287 and 328 nm. Since intrinsic Cotton effects of β-cyclodextrin itself exhibit below 220 nm, the observed optical activity above 220 nm can be attributed to the induced Cotton effect of flufenamic acid, generated by the formation of an inclusion complex with β-cyclodextrin. The binding of β-cyclodextrin with other fanamates, mefenamic acid and meclofenamic acid, also showed similar changes in UV spectra and generated the induced CD bands.

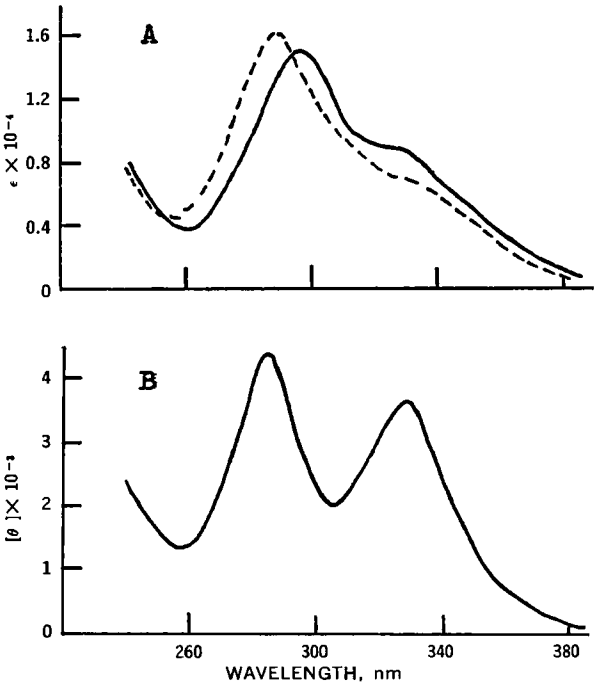
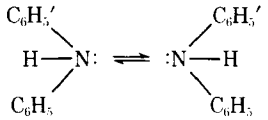


Figure 1—(*A*) *Effect of β-cyclodextrin* (1.0×10^{-2} M) *on UV spectrum of flufenamic acid* (5.0×10^{-5} M). *Key: - - -, flufenamic acid alone; and ——; flufenamic acid plus β-cyclodextrin.* (*B*) *CD spectrum of flufenamic acid* (5.0×10^{-5} M) *in the presence of β-cyclodextrin* (1.0×10^{-2} M).

To determine what factors are important for the generation of the induced Cotton effects, interactions of other *N*-phenylanthranilates and their related compounds with β-cyclodextrin were studied. Table I summarizes the *g* values obtained for these compounds bound to β-cyclodextrin. With the exception of anthranilic acid, all compounds studied generated the induced Cotton effects. This may indicate that the diphenylamine moiety in drugs is essential to generate the optical activity. It is assumed from these findings that the uncomplexed diphenylamine, which resonates between two pyramidal structures (Scheme I), is fixed in one conformation by the formation of the inclusion complex.

By application of the continuous variation method to the induced Cotton effects, the stoichiometric ratio of the complexes was found to be 1:1 for β-cy-



Scheme I

[1] Jasco model 20A recording spectropolarimeter.
[2] Hitachi EPS-3T spectrophotometer.

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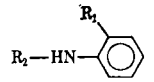

Compound		UV Maximum		CD Maximum		
R_1	R_2	Wavelength, nm	ε, × 10^{-4}	Wavelength, nm	$\Delta\epsilon^a$	g^b, × 10^4
COOH	H	240	0.74			
		310	0.30		(Not observed)	
H	C_6H_5	283	1.40	273	1.21	1.03
COOH	C_6H_5	291	1.26	285	2.36	1.90
		332	0.53	332	0.61	1.15
$COOCH_3$	C_6H_5	287	1.40	293	2.06	1.62
		343	0.64	335	0.78	1.28
COOH	2-COOH—C_6H_4	—	—	233	1.00	0.81
		—	—	242	−0.58	−0.73
		289	1.17	292	−1.45	−1.27
		338	0.73	345	−0.23	−0.32
COOH	3-CF_3—C_6H_4 (Flufenamic acid)	292	1.46	287	1.30	0.93
		320	0.86	328	1.09	1.36
COOH	2,3-Xylyl (Mefenamic acid)	283	0.70	280	−0.33	−0.29
				315	0.61	1.13
		336	0.46	335	0.73	1.16
COOH	2,6-Dichloro-*m*-tolyl (Meclofenamic acid)	285	0.48	275	0.43	0.91
		320	0.43	320	0.03	0.07

[a] Molar ellipticity (deg cm^2 $dmole^{-1}$). [b] Optical anisotropy factor (see text).

clodextrin and fanamates. Stability constants ($K_{1:1}$) determined by the CD method for β-cyclodextrin with flufenamic, mefenamic, and meclofenamic acids were 1300, 630, and 450 M^{-1}, respectively. In contrast to β-cyclodextrin, α-cyclodextrin (cyclohexaamylose) showed no appreciable complex formation. This suggests that the cavity size of α-cyclodextrin is not large enough to include the bulky guest molecules of drugs.

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Ken Ikeda[x]
Kaneto Uekama
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Faculty of Pharmaceutical Sciences
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Tanabe-dori, Mizuho-ku
Nagoya, Japan

Masahiro Hatano

Chemical Research Institute of Non-aqueous Solutions
Tohoku University
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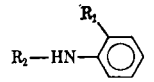

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Dried, ground root material was percolated consecutively with hexane, chloroform, 95% ethanol, water, and 5% hydrochloric acid. The potassium content of the first four extracts prepared for infusion[2] was determined by atomic absorption spectroscopy and found to be 0.109, 0.181, 16.9, and 36.0 mmoles, respectively[3]. Ten Ham *et al.* (2) reported the potassium content of their infusion-prepared ethanol–water extract by flame photometery to be 140 mmoles/liter. Cardiac activity, although different from that observed by Ten Ham *et al.* (2), was found in the hexane, chloroform, and ethanol extracts; however, no activity of a detrimental nature was observed in the aqueous or 5% hydrochloric acid extracts.

Cardiac screening was done using anesthetized male guinea pigs. The right jugular veins were cannulated with polyethylene tubing to a distance of approximately 1 cm from the heart. Infusion of the dried extracts (emulsified in 2% sorbitan ester[4], 3% polysorbate 60, and 4% ethanol in water)[2] was then carried out at the rate of 0.2 ml/min and was continued until the heart stopped or for 90 min. The ECG was monitored through bipolar leads attached on either side of the thorax at the base of the front limbs[5].

For each extract, the ECG of a vehicle-only control animal was recorded which showed slight bradycardia, an increase in the PR interval, and an increase in the QRS complex amplitude. The hexane extract was fractionated into polar and nonpolar portions[6]. The cardiac activity was mainly associated with the less polar fraction which contained insignificant traces of potassium. Infusion of the hexane extract had a rapid and pronounced effect on the heart. Bradycardia, prolonged PR intervals, and A–V conduction blockade developed quickly. After 18 min, atrial activity had disappeared, with death occurring following 23 min of continuous infusion of the extract. The chloroform extract[2] produced effects similar to those caused by infusion of a 140-mmole solution of potassium chloride, as described previously (2); however, there was no inversion of the T wave. The A–V conduction was severely impaired and the heart irreversibly stopped. The ethanol extract[2], in addition to causing irreversible heart stoppage, produced a more pronounced arrhythmia than any other extract; during later stages of infusion, a prominent conduction blockade was observed within 10 min after the appearance of multiple P waves. The heart then entered a short period of ventricular fibrillation and collapsed. Once definite arrhythmias were observed, termination of infusion did not stop the progressive deterioration of the heart.

The aqueous and 5% hydrochloric acid extract infusions[2] were apparently not toxic and, indeed, when compared with the control animals receiving the suspension vehicle alone, fewer changes in the ECG were observed. Bradycardia was not seen, but three of four subjects showed an increase in heart rate of 25–40%.

These preliminary data strongly suggest that *Cannabis* roots of Mexican origin do contain organic component(s) that produce cardiac activities quite different than those classically observed with potassium ions. It is quite possible that Mexican *Cannabis* roots contain chemicals not found in other "variants." This is the case for the cannabinoids (3, 4). Thus, this could explain the difference in findings by Ten Ham *et al.* (2) and our group. Further fractionations of these extracts are underway in an effort to isolate the principle(s) responsible for the observed cardiac activities.

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M. Leonard Mole
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Lidocaine: A Case of Intraamide Hydrogen Bonding

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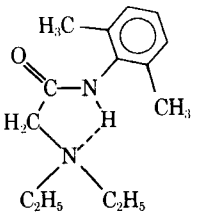

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ration for the molecule and dimerization by intermolecular hydrogen bonding.

Related data were obtained in this Institute following some IR studies on the effect of systematically changing the steric and polar properties of R and R′ on the configurational and hydrogen bonding properties of the amide group in *N*-alkylamides and benzanilides of the general type R—CONH—R′. In some of these molecules, *cis*- and *trans*-forms of the amide group exist in equilibrium with one another in dilute solution (2, 3). But if an attractive polar interaction is introduced between an electronegative atom in the R group and the amido hydrogen, the *trans*-planar configuration of the —CONH— group is stabilized to the exclusion of the *cis*-configuration (3).

In the lidocaine molecule, there is a possibility of polar interaction between the nitrogen atom of the 2-diethylamino group and the amido hydrogen. Three 2′,6′-xylidides [$RCONHC_6H_4(CH_3)_2$, in which R = $CH_3CH_2CH_2$, CH_3OCH_2, and $(C_2H_5)_2NCH_2$] were prepared, and the IR spectra of 0.001 *M* solutions of these compounds in carbon tetrachloride were recorded.

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R. Lumley Jones

Chester Beatty Research Institute
Royal Cancer Hospital
London SW3 6JB, England

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Effects of Hydraulic Pressure and Nozzle Orifice Size on Delivery Rates of Sprayed Materials

Keyphrases □ Spray processes—effects of hydraulic pressure and nozzle orifice size on delivery rates □ Delivery rates, sprayed materials—effects of hydraulic pressure and nozzle orifice size □ Coating processes—effects of hydraulic pressure and nozzle orifice size on delivery rates of sprayed materials

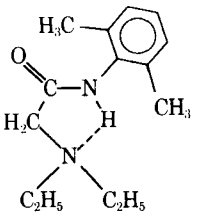
To the Editor:

Spraying processes are important in many phases of the development and production of certain pharmaceutical dosage forms. The manner in which these processes are carried out can affect the physical appearance, stability, and bioavailability of the product. Therefore, the judicious choice of nozzle size and hydraulic pressure for a particular process should improve product uniformity as well as batch-to-batch reproducibility.

A practical equation derived from fundamental flow theories (1–3) applicable to an airless spray system is:

$$R = KD^2\sqrt{\frac{P}{\rho}} \qquad \text{(Eq. 1)}$$

where R = volumetric rate of fluid delivery, D = diameter of nozzle orifice, P = hydraulic pressure, ρ = density of material, and K = proportionality constant. This equation predicts that the flow rate is directly proportional to the cross-sectional area of the nozzle orifice and to the square root of pressure. Also, flow is inversely proportional to the square root of the density of the liquid. Therefore, graphs of flow rate *versus* square root of pressure, diameter



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ration for the molecule and dimerization by intermolecular hydrogen bonding.

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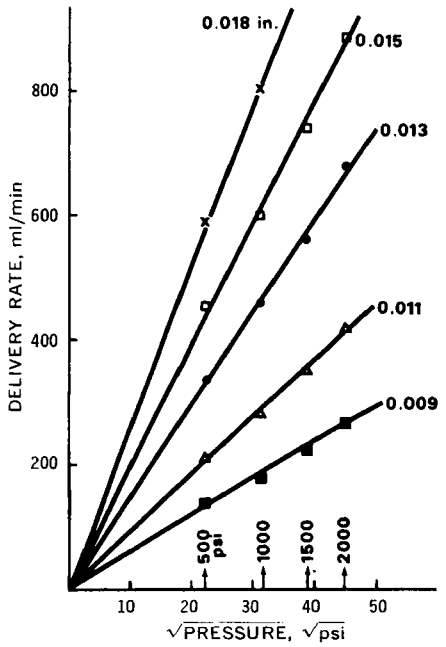

Figure 1—*Characteristic profile showing the effects of pressure on the delivery rates of water.*

squared, or density$^{-1/2}$ will all result in straight lines passing through the origin.

A recent article (4) reported the effects of hydraulic pressure and nozzle size on the spray rate of various liquid systems. For the cases studied, it was concluded that: *(a)* a linear relationship existed between hydraulic pressure and spray rate, and *(b)* a linear relationship existed between nozzle orifice diameter and spray rate, although direct dependence on orifice area had been expected. While the author indicated that the second finding was unexpected, the first finding may also be considered outside the generally

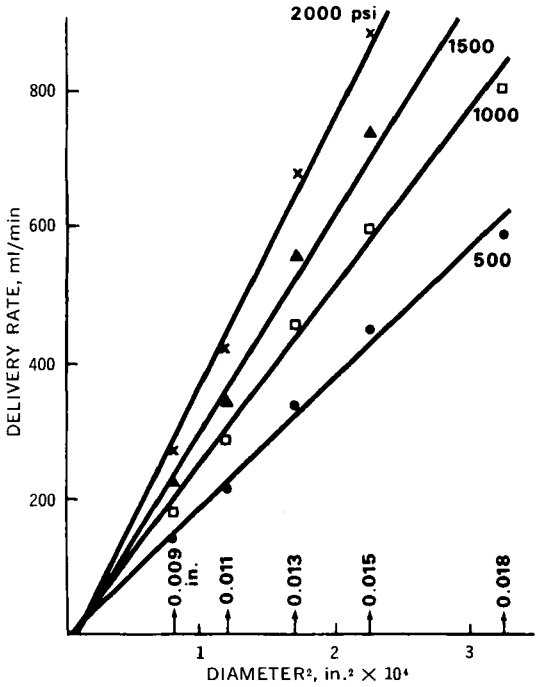

Figure 2—*Dependency of the delivery rates of water on nozzle orifice size.*

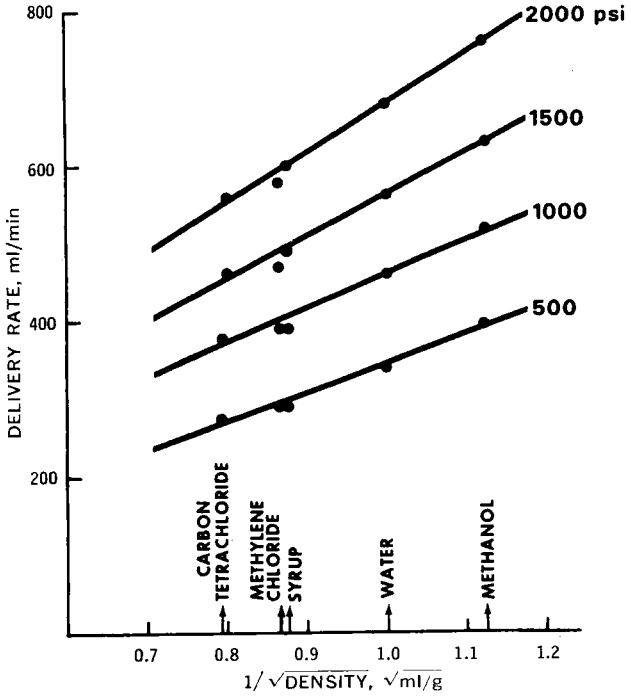

Figure 3—*Effects of density on the delivery rates of liquids.*

accepted theory of fluid mechanics as described by Eq. 1. Cognizance is taken of the fact that if the published data relating to the nozzle orifice diameter had been evaluated on the basis of Eq. 1, some agreement with theory would have been observed.

To test the validity of the accepted theory on pharmaceutical systems, studies were designed to determine the influence of pressure, orifice size, and density on spray rate. The volume of liquid delivered from airless spray equipment[1] in 1 min was measured for pressures from 500 to 2000 psi and spray tips from 0.02 to 0.05 cm (0.009 to 0.023 in.) in diameter. Water, methylene chloride, carbon tetrachloride, methanol, and syrup (65% w/w sucrose) were evaluated.

Figure 1 shows the effect of pressure on the delivery rate of water for five nozzle orifice sizes. The linear relationship is evident when the delivery rates are plotted as a function of the square root of pressure. Furthermore, the data extrapolate to zero delivery at zero pressure for all nozzle sizes as predicted by Eq. 1. These data points can be replotted as a function of the nozzle diameter squared. When presented in this manner, the expected linearity is obtained (Fig. 2). Here again, extrapolation of the least-squares line through the origin is in agreement with theory.

The effects of pressure on the delivery rates of methanol, methylene chloride, carbon tetrachloride, and syrup were compared with water. Each of these four liquids showed the same dependencies on pressure and orifice size as did water but varied in the amounts delivered under the same conditions. Since these liquids differed in both viscosity and density,

[1] Alemite 7860 pump, Alemite Division, Stewart-Warner Corp., Chicago, Ill., and flat spray tungsten carbide nozzles, Spraying Systems Co., Wheaton, Ill.

independent measurements were made on methylcellulose solutions having essentially constant densities but varying viscosities up to 200 cps. The delivery rates of all of the methylcellulose solutions were nearly identical, indicating that viscosity is not a determining factor under the study conditions. Based on these observations, the differences in delivery rates can be attributed to the effect of density. The data in Fig. 3 illustrate this dependency on density. Here the inverse square-root relationship predicted by Eq. 1 seems to hold well for all test conditions.

The experimental data appear to be in good agreement with theoretical predictions. Using airless spray equipment, the delivery rate was found to be: *(a)* directly proportional to the square root of pressure, *(b)* directly proportional to the area of the nozzle orifice, and *(c)* inversely proportional to the square root of density.

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Philip W. Stern

Pharmaceutical Chemistry
Smith Kline & French Laboratories
Division of SmithKline Corporation
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Metabolism of Δ^1-Tetrahydrocannabinol by Mouse Hepatic Microsomes: Identification of 6α-Hydroxytetrahydrocannabinol

Keyphrases □ Tetrahydrocannabinol—metabolism by mouse hepatic microsomes, identification of 6α-hydroxytetrahydrocannabinol □ 6α-Hydroxytetrahydrocannabinol—identification, metabolism of tetrahydrocannabinol by mouse hepatic microsomes □ Marijuana—metabolism of tetrahydrocannabinol by mouse hepatic microsomes, identification of 6α-hydroxytetrahydrocannabinol

To the Editor:

The body of knowledge concerning the biotransformations of Δ^1-tetrahydrocannabinol has grown considerably in the past few years (1). However, the nature of the transformations in the mouse has not been firmly established, even though it has been used in studying the pharmacology of tetrahydrocannabinol. Several reports on distribution (2–5) suggested that the principal metabolites in this species are hydroxytetrahydrocannabinols, in analogy with those isolated from rats, rabbits, monkeys, and humans. The purpose of this study was to identify the major metabolites of ^{14}C-Δ^1-tetrahydrocannabinol produced by the microsomal fraction of mouse liver.

Table I—Metabolism of Δ^1-Tetrahydrocannabinol by Liver Microsomes

TLC Zone	R_f	Assignment[b]	Percent Recovered Products[a] Mouse[c]	Rat[d]
1	0.67	Δ^1-Tetrahydrocannabinol acetate	9.42	19.2
2	0.40	6α-Hydroxy-Δ^1-tetrahydrocannabinol diacetate	26.3	10.0
3	0.30	7-Hydroxy-Δ^1-tetrahydrocannabinol diacetate	34.1	53.3
4	0.13	6α,7-Dihydroxy-Δ^1-tetrahydrocannabinol triacetate (?)	30.2	17.6

[a] Recoveries of added radioactivity were approximately 50%. [b] All materials were acetylated prior to TLC with a mixture of acetic anhydride and pyridine. [c] Adult male CD-1 mice (30–33 g). [d] Adult male Sprague–Dawley rats (190–230 g).

The preparation of the microsomes, the incubation conditions, and the extraction procedure were identical to those previously reported for the rat (6). The residue obtained after extraction of the incubation mixture and evaporation of the solvent was acetylated to minimize decomposition of the products during isolation. The mixtures of acetates were separated by silica gel TLC (30% ether in hexane, developed twice), and the locations of the radioactive zones were determined by autoradiography. The four major zones were eluted and chromatographed a second time in the same manner to obtain materials pure enough for GLC–mass spectrometry. Comparison of

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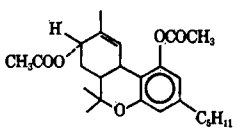
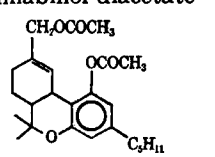
 6α-Hydroxy-Δ^1-tetrahydrocannabinol diacetate	7.0	372 (3.4), 354 (100), 339 (21), 312 (82), 297 (65), 295 (18)
 7-Hydroxy-Δ^1-tetrahydrocannabinol diacetate	7.5	372 (3.8), 354 (43), 312 (100), 297 (28), 259 (31)

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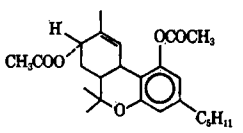
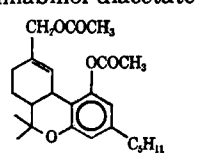
 6α-Hydroxy-Δ^1-tetrahydrocannabinol diacetate	7.0	372 (3.4), 354 (100), 339 (21), 312 (82), 297 (65), 295 (18)
 7-Hydroxy-Δ^1-tetrahydrocannabinol diacetate	7.5	372 (3.8), 354 (43), 312 (100), 297 (28), 259 (31)

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the products with authentic standards[1] by TLC, GLC, and GLC–mass spectrometry led to the identification of three of the four zones. A parallel study was done with rat liver microsomes for comparison purposes (Tables I and II). The identification of zone four rests mainly on its TLC mobility, because it was not possible to isolate sufficient pure material for GLC–mass spectrometry analysis. A possible metabolite, 6β-Δ^1-tetrahydrocannabinol, can be excluded on the basis of its markedly different TLC mobility from that reported (8).

It can be seen from the results that mouse liver microsomes metabolize Δ^1-tetrahydrocannabinol even more extensively than the analogous rat system. The most notable difference is the greater proportion of 6α-hydroxy-Δ^1-tetrahydrocannabinol *versus* 7-hydroxy-Δ^1-tetrahydrocannabinol. Concurrent results from another laboratory have also shown that 6α- and 7-hydroxy-Δ^1-tetrahydrocannabinols are major products when Δ^1-tetrahydrocannabinol is incubated with mouse liver microsomes[2]. Since both metabolites are psychoactive (7), evidence for their presence in a particular species is of importance in interpreting pharmacological studies with Δ^1-tetrahydrocannabinol. The production of 6α-hydroxy-Δ^1-tetrahydrocannabinol becomes of equal interest to that of 7-hydroxy-Δ^1-tetrahydrocannabinol since the former has been tentatively identified in human plasma (8).

Our results may also help to establish the nature of one unknown metabolite from rat lung and liver reported by Nakazawa and Costa (9). While we have not been able to make a direct comparison, it seems likely that their R_f 0.48 metabolite is 6α-hydroxy-Δ^1-tetrahydrocannabinol, since both our and their rat liver experiments produced only one substance intermediate in mobility between Δ^1-tetrahydrocannabinol and 7-hydroxy-Δ^1-tetrahydrocannabinol.

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Zvi Ben-Zvi
Sumner Burstein[x]
John Zikopoulos

Worcester Foundation for Experimental Biology
Shrewsbury, MA 01545

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[x] To whom inquiries should be directed.

[1] Labeled and unlabeled Δ^1-tetrahydrocannabinol as well as samples of the metabolites were obtained from the National Institute of Mental Health.

[2] Professor S. Agurell, Faculty of Pharmacy, Stockholm S-113 86, Sweden, personal communication.

BOOKS

REVIEWS

Selective Toxicity, Fifth Edition. By ADRIEN ALBERT. Chapman and Hall, London, England, 1973. 597 pp. 15 × 23 cm. Price £7.

"Selective toxicity" is defined by Adrien Albert as "the injury of living matter without harming another kind with which the first is in intimate contact." Those organisms (including humans) that are not injured are referred to as "economic species," while those which are harmed are classified as "uneconomical species." Examples can be given where chemical agents that are toxic to one type of plant have little effect upon other plants growing on the same plot of land. In the animal world, insecticides are so chosen to bring death to insects and pest without harming useful animals and plant life. Selective toxicity as interpreted by Albert is applicable to all drugs since they will alter one or more physiological or biochemical processess to help bring about a state of normalcy. For a large number of drugs the toxic effect is reversible, for example, the general anesthetics, sedatives, and drugs used to relieve pain. Other drugs such as the antibiotics bring about a nonreversible toxic effect, in this case to the noneconomic species, the bacteria, without harm to the host (economic species).

In an oversimplification, selective toxicity may be considered a general approach of helping rid the world of undesirable organisms through chemical agents while permitting useful ones to flourish. Even though the mechanisms by which one chemical agent can harm one species while having relatively little effect upon another are complex and often unknown, Albert approaches the subject in a very methodical and deliberate manner and bases his thesis, as in the past, upon the chemical structure and physical properties of the agents.

The present edition is the fifth and when compared to the first (1951) and second editions (1960) monumental growth of the book is apparent. For example, the 1960 issue was a mere 233 pages *versus* the present 597 pages. While the 1960 edition was a relatively breezy approach to the subject which could be read with

the products with authentic standards[1] by TLC, GLC, and GLC–mass spectrometry led to the identification of three of the four zones. A parallel study was done with rat liver microsomes for comparison purposes (Tables I and II). The identification of zone four rests mainly on its TLC mobility, because it was not possible to isolate sufficient pure material for GLC–mass spectrometry analysis. A possible metabolite, 6β-Δ^1-tetrahydrocannabinol, can be excluded on the basis of its markedly different TLC mobility from that reported (8).

It can be seen from the results that mouse liver microsomes metabolize Δ^1-tetrahydrocannabinol even more extensively than the analogous rat system. The most notable difference is the greater proportion of 6α-hydroxy-Δ^1-tetrahydrocannabinol *versus* 7-hydroxy-Δ^1-tetrahydrocannabinol. Concurrent results from another laboratory have also shown that 6α- and 7-hydroxy-Δ^1-tetrahydrocannabinols are major products when Δ^1-tetrahydrocannabinol is incubated with mouse liver microsomes[2]. Since both metabolites are psychoactive (7), evidence for their presence in a particular species is of importance in interpreting pharmacological studies with Δ^1-tetrahydrocannabinol. The production of 6α-hydroxy-Δ^1-tetrahydrocannabinol becomes of equal interest to that of 7-hydroxy-Δ^1-tetrahydrocannabinol since the former has been tentatively identified in human plasma (8).

Our results may also help to establish the nature of one unknown metabolite from rat lung and liver reported by Nakazawa and Costa (9). While we have not been able to make a direct comparison, it seems likely that their R_f 0.48 metabolite is 6α-hydroxy-Δ^1-tetrahydrocannabinol, since both our and their rat liver experiments produced only one substance intermediate in mobility between Δ^1-tetrahydrocannabinol and 7-hydroxy-Δ^1-tetrahydrocannabinol.

(1) S. H. Burstein, in "Marihuana: Chemistry, Pharmacology, Metabolism and Clinical Effects," R. Mechoulam, Ed., Academic, New York, N.Y., 1973, chap 4. (See chap. 1 for a discussion of nomenclature.)

(2) A. Ryrfeldt, C. H. Ramsay, I. M. Nilsson, M. Widman, and S. Agurell, *Acta Pharm. Suecica*, **10**, 13(1973).

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(4) R. Q. Freudenthal, J. Martin, and M. E. Wall, *Brit. J. Pharmacol.*, **44**, 244(1972).

(5) H. D. Christensen, R. I. Freudenthal, J. Gidley, R. Rosenfeld, G. Boegli, L. Testino, D. R. Brine, C. G. Pitt, and M. E. Wall, *Science*, **172**, 165(1971).

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(7) Z. Ben-Zvi, R. Mechoulam, H. Edery, and G. Porath, *Science*, **174**, 951(1971).

(8) M. E. Wall, D. R. Brine, C. G. Pitt, and M. Perez-Reyes, *J. Amer. Chem. Soc.*, **94**, 8579(1972).

(9) K. Nakazawa and E. Costa, *Nature*, **234**, 48(1971).

Zvi Ben-Zvi
Sumner Burstein[x]
John Zikopoulos

Worcester Foundation for Experimental Biology
Shrewsbury, MA 01545

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[x] To whom inquiries should be directed.

[1] Labeled and unlabeled Δ^1-tetrahydrocannabinol as well as samples of the metabolites were obtained from the National Institute of Mental Health.

[2] Professor S. Agurell, Faculty of Pharmacy, Stockholm S-113 86, Sweden, personal communication.

BOOKS

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This publication has been used routinely by the Journal staff to verify and confirm drug names and has been found to be both thorough and accurate, particularly in the area of international trade names. Since it is only revised every 2 years, it lacks the currentness of the AMA Drug Nomenclature Printout; it is however to be recommended for persons needing a readily available, accurate, and complete drug nomenclature reference.

Staff Review

British Pharmacopoeia 1973. British Pharmacopoeia Commission, London, England. Available from Pendragon House, Inc., 220 University Ave., Palo Alto, CA 94301, 1973. 506 pp. plus Appendixes. 22 × 30 cm. Price $40.00.

The British Pharmacopoeia 1973 includes 1277 monographs establishing standards for the quality of a wide range of articles used in medicine including simple inorganic salts, complex synthetic compounds, vegetable drugs, radioactive preparations, antibiotics, hormones, vitamins, blood products, immunobiologicals, and biological products.

Specifications set forth in the monographs include the definition and description and tests for identification, purity, and potency as well as information concerning solubility, labeling, action, use, and storage, where appropriate.

The Appendixes include specifications for reagent chemicals, descriptions of physical, chemical, and biological methods of analysis, tests for microbial contamination, blood grouping procedures, and statements on radioactive preparations and plastic containers for medicinal preparations.

Monographs for items also included in the first two volumes of the "European Pharmacopoeia" have been revised to conform to "European Pharmacopoeia" specifications.

Staff Review

NOTICES

Corneal Graft Failure, Ciba Foundation Symposium 15. Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands, 1973. 17 × 24.5 cm.

Medical Care of Prisoners and Detainees, Ciba Foundation Symposium 16. Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands, 1973. 17 × 24.5 cm.

Drug Effects on Neuroendocrine Regulation, Progress in Brain Research, Vol. 39. Edited by E. ZIMMERMANN, W. H. GISPEN, B. MARKS, and D. DE WIED. Elsevier, 52 Vanderbilt Ave., New York, NY 10017, 1973. 502 pp. 19.5 × 27 cm. Price $69.00.

Encyclopedia of Industrial Chemical Analysis, Vol. 18. Edited by F. D. SNELL and L. S. ETTRE. Wiley, 605 Third Ave., New York, NY 10016, 1973. 545 pp. 18.5 × 26 cm. Price $40.00, single copy; $35.00, subscription.

Lithium: Its Role in Psychiatric Research and Treatment. Edited by S. GERSHON and B. SHOPSIN. Plenum, 227 West 17th St., New York, NY 10011, 1973. 358 pp. 15.5 × 23.5 cm. Price $19.50.

Hagers Handbuch der Pharmazeutischen Praxis: Vollstandige (Vierte) Neuausgabe. By P. H. LIST and L. HORHAMMER. Springer-Verlag, Berlin, Germany, 1973. 1226 pp. 17 × 25 cm. *(German).*

Contact Dermatitis, 2nd ed. By ALEXANDER A. FISCHER. Lea & Febiger, 600 Washington Square, Philadelphia, PA 19106, 1973. 448 pp. 18 × 26 cm. Price $19.50.

Handbook of Experimental Pharmacology. Herausgeber, O. EICHLER. Springer-Verlag, Heidelberger Platz 3, Berlin, Germany, 1973. 316 pp. 17 × 25.5 cm *(German).*

Trypanosomiasis and Leishmaniasis with special reference to Chagas' disease. Ciba Foundation Symposium 20 (new series). Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands, 353 pp. 18 × 25 cm.

Medical Research Systems in Europe. Ciba Foundation Symposium 21 (new series). Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands, 335 pp. 18 × 28 cm.

La Pharmacopée Sénégalaise Traditionelle, Plantes, Médicinales, et Toxiques. J. KERHARO and J. G. ADAM. Vigot Fréres, 23, Rue de l'Ecole de Medecine, 75006 Paris, France, 1974. 1011 pp. 18 × 27 cm. Price 370 fr. *(French).*

The Human Lens—in Relation to Cataract. Ciba Foundation Symposium 19 (new series). Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands. 324 pp. 18 × 25 cm.

Protides of the Biological Fluids, 21st Colloquium 1973. Edited by H. PEETERS. Pergamon Press, Inc., Maxwell House, Fairview Park, Elmsford, NY 10523, 1974. 677 pp. 18 × 26 cm. Price $51.00.

Carbohydrate Chemistry, Volume 6. Senior Reporter, J. S. BRIMACOMBE. The Chemical Society, Burlington House, London, W1V, OBN, England, 1973. 620 pp. 14 × 22 cm. Price £ 8.00.

The Biochemical Basis of Neuropharmacology. By J. R. COOPER, F. E. BLOOM, and R. H. ROTH. Oxford University Press, 200 Madison Ave., New York, NY 10016, 1974. 272 pp. 14 × 21 cm. Price $4.95.

Index Nominum 1973/74, 7th ed. Edited and published by the Swiss Pharmaceutical Society, Berne, Switzerland, 1973. xxvi + 1295 pp. 15 × 33 cm. Price 130 Sw. fr.

"Index Nominum" is an alphabetical list of nonproprietary ("generic") and trade names of internationally available products containing one active ingredient. Information is given on International Nonproprietary Names (INN) and the nonproprietary names, trade names and manufacturers, chemical names and structures, a listing of pharmacopeial monographs on each drug, and main therapeutic uses. This edition includes about 3300 substances, more than 18,000 entries, and introductions in French, German, and English. The entry terms are also given in French, but this presents only minimal problems in use.

This publication has been used routinely by the Journal staff to verify and confirm drug names and has been found to be both thorough and accurate, particularly in the area of international trade names. Since it is only revised every 2 years, it lacks the currentness of the AMA Drug Nomenclature Printout; it is however to be recommended for persons needing a readily available, accurate, and complete drug nomenclature reference.

Staff Review

British Pharmacopoeia 1973. British Pharmacopoeia Commission, London, England. Available from Pendragon House, Inc., 220 University Ave., Palo Alto, CA 94301, 1973. 506 pp. plus Appendixes. 22 × 30 cm. Price $40.00.

The British Pharmacopoeia 1973 includes 1277 monographs establishing standards for the quality of a wide range of articles used in medicine including simple inorganic salts, complex synthetic compounds, vegetable drugs, radioactive preparations, antibiotics, hormones, vitamins, blood products, immunobiologicals, and biological products.

Specifications set forth in the monographs include the definition and description and tests for identification, purity, and potency as well as information concerning solubility, labeling, action, use, and storage, where appropriate.

The Appendixes include specifications for reagent chemicals, descriptions of physical, chemical, and biological methods of analysis, tests for microbial contamination, blood grouping procedures, and statements on radioactive preparations and plastic containers for medicinal preparations.

Monographs for items also included in the first two volumes of the "European Pharmacopoeia" have been revised to conform to "European Pharmacopoeia" specifications.

Staff Review

NOTICES

Corneal Graft Failure, Ciba Foundation Symposium 15. Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands, 1973. 17 × 24.5 cm.

Medical Care of Prisoners and Detainees, Ciba Foundation Symposium 16. Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands, 1973. 17 × 24.5 cm.

Drug Effects on Neuroendocrine Regulation, Progress in Brain Research, Vol. 39. Edited by E. ZIMMERMANN, W. H. GISPEN, B. MARKS, and D. DE WIED. Elsevier, 52 Vanderbilt Ave., New York, NY 10017, 1973. 502 pp. 19.5 × 27 cm. Price $69.00.

Encyclopedia of Industrial Chemical Analysis, Vol. 18. Edited by F. D. SNELL and L. S. ETTRE. Wiley, 605 Third Ave., New York, NY 10016, 1973. 545 pp. 18.5 × 26 cm. Price $40.00, single copy; $35.00, subscription.

Lithium: Its Role in Psychiatric Research and Treatment. Edited by S. GERSHON and B. SHOPSIN. Plenum, 227 West 17th St., New York, NY 10011, 1973. 358 pp. 15.5 × 23.5 cm. Price $19.50.

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Contact Dermatitis, 2nd ed. By ALEXANDER A. FISCHER. Lea & Febiger, 600 Washington Square, Philadelphia, PA 19106, 1973. 448 pp. 18 × 26 cm. Price $19.50.

Handbook of Experimental Pharmacology. Herausgeber, O. EICHLER. Springer-Verlag, Heidelberger Platz 3, Berlin, Germany, 1973. 316 pp. 17 × 25.5 cm *(German).*

Trypanosomiasis and Leishmaniasis with special reference to Chagas' disease. Ciba Foundation Symposium 20 (new series). Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands, 353 pp. 18 × 25 cm.

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The Biochemical Basis of Neuropharmacology. By J. R. COOPER, F. E. BLOOM, and R. H. ROTH. Oxford University Press, 200 Madison Ave., New York, NY 10016, 1974. 272 pp. 14 × 21 cm. Price $4.95.

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COMPENDIA UNIFICATION BECOMES A REALITY

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For many years, there have been repeated suggestions from various quarters, including government, industry, and the health professions, that unification of the NF and USP would be desirable. Following a period of preliminary discussions, this possibility became the subject of intense serious discussion in 1970 when each of the two organizations responsible for publication of the official compendia separately took actions directing their respective officers and staffs to determine if and how merger of the two compendia could be satisfactorily achieved.

An immense amount of time and effort was devoted to such explorations during the ensuing four years. Unfortunately, however, despite total dedication of the negotiating parties to the goal of unification, basic problems or objections continually surfaced which apparently made the original goal of a pure merger impossible to achieve. Meanwhile, the patience of interested onlookers—including pharmacists, physicians, Food and Drug Administration personnel, industry scientists, and the many participants in the respective compendium programs—began to diminish as a result of their desire to see some meaningful progress made toward compendia consolidation. Therefore, increasing pressures mounted that some solution be found and action be taken.

Having been frustrated in all their efforts to this point, the negotiating parties decided to explore somewhat different approaches to achieve the desired end result. Out of this exploration, an agreement was concluded early in July whereby the USP and the NF would be unified through the sale of all rights and responsibilities relating to the NF to the publisher of the USP, the United States Pharmacopeial Convention, Inc.

This dramatic development required substantial concessions on the part of both organizations. Undoubtedly, such concessions were felt to be not only justified but, in fact, necessary by the respective organizations in order to accomplish the desired goal, which in everyone's view was paramount.

A major consideration which swayed the APhA in reaching its decision on this matter was its conclusion that survival of the drug standard setting function within the private sector—and divorced from direct control from either industry or government—is even more important than having APhA continue direct authority over one phase within such a program (*i.e.*, the National Formulary).

Now that this dream is to become reality, it is our opinion that unification of the official compendia will be of enormous benefit in strengthening the so-called independent "third force"—as distinguished from government and industry—in our three-sided system of responsibility for drug quality.

Over the years, the compendia have contributed very substantially to assuring a quality drug supply. However, due to the advent of many potent new drugs—as well as new techniques of analysis and new knowledge relative to drug properties requiring standardization—the compendia need to muster all possible resources and strength in order to meet present day challenges.

When the agreement to acquire the NF was announced, USP Executive Director William M. Heller cautioned that unification of the compendia is not an end in itself, but that this should serve as an efficient conduit whereby the emerging new challenges involving the nation's drug supply can be successfully met in a way that will result in the most objective scientific decisions possible in order to ensure the safest and most effective drug supply. We echo Dr. Heller's comments, and we add our full support toward this end. —EGF

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REVIEW ARTICLE

Metabolically Oxygenated Compounds: Formation, Conjugation, and Possible Biological Implications

ERIC C. SCHREIBER

Keyphrases □ Metabolism—introduction of oxygen in biotransformations, major conjugation reactions, aspects in mutagenicity and carcinogenicity, review □ Oxygenation in drug metabolism—role in biotransformations, major conjugation reactions, aspects in mutagenicity and carcinogenicity, review □ Mutagenicity and carcinogenicity—implications of metabolically oxygenated compounds, review

The development of drug metabolism into an essential aspect of new drug discovery has gone through a number of interesting phases. Originally, it was considered to be the domain of a few individuals who were more or less happily puttering about. The data being gathered were treated as interesting, but their implications made little or no impact on the larger scientific community concerned with the discovery, evaluation, and development of therapeutic entities. It borders on the incredible that, for many years, an almost complete lack of appreciation of the value of drug metabolism studies existed among medicinal chemists, pharmaceutical scientists, and the medical profession in general.

Today, it is inconceivable that any serious development of a new therapeutic agent would not include extensive drug metabolism studies which influence every step of a drug's history from its synthesis and early animal pharmacology through toxicology, formulation development, and human clinical trials. Perhaps the pendulum has swung too far and now drug metabolism is being regarded as the means of answering every therapeutic or toxicological puzzle, which, in fact, it cannot hope to do.

Drug metabolism is a term that is sorely in need of definition. We are all aware of the old story of the four blindfolded men standing about an elephant. One has his hand placed on the end of the elephant's trunk, another holds a tusk, one man's hands are placed around a leg, and the fourth holds the tail. Each man is then asked to describe an elephant. The reader can use his or her own imagination as to the variety of descriptions of an elephant that would be forthcoming. Unfortunately, all too often, the answer to the question of what is drug metabolism reflects the interest of the person to whom the query is posed. A pharmaceutical scientist couches an answer in terms of bioavailability and biopharmaceutics, the medicinal chemist thinks of biotransformation and active metabolites, toxicologists and pharmacologists refer to selective toxicity and species differences, biochemists mention events at the enzyme level, and more mathematically inclined individuals respond in terms of rate constants, half-lives, and compartmental models. It is all of these and more. Drug metabolism is that branch of science devoted to studying all aspects of the fate of foreign molecules in biological systems under *in vivo* or *in vitro* conditions.

Since the investigations described as "drug metab-

olism studies" frequently do not include either drug substances *per se* (*e.g.*, pesticides and the influences of adjuvants) or biotransformation of the parent compound, perhaps a new term should be considered. "Xenobionics," which loosely translated from the Greek means "the fate of strangers in a biological system," is perhaps more generally appropriate than drug metabolism.

It is not possible to present all aspects of such a multifaceted area in one review article. A number of recent texts, one of which is particularly outstanding (1), include discussions of particular matters such as absorption, protein binding, transfer of drugs across membranes, and routes of excretion. The present article will be confined to the introduction of oxygen in selected cases of biotransformations and the major conjugation reactions of these compounds that have been published recently. Some aspects of the possible implication of highly reactive metabolites in mutagenicity and carcinogenicity will be discussed.

DETOXICATION

The relationship of enzymic or hydrolytic biotransformations and the resulting physiological consequences of the exposure of biological systems to foreign chemical agents are no longer open to discussion; they have been demonstrated in numerous studies. Unfortunately, there is a tendency to extrapolate findings inappropriately and to make generalizations that are not quite justified.

Biotransformation of drugs into their metabolites is often referred to as "detoxication" (2) and is considered by some to be a relatively nonspecific mechanism developed by animals for eliminating foreign substances. The continued habit of calling biotransformation detoxication is unfortunate in view of the fact that it requires involving an almost supernatural power to suggest that any structural modification taking place *in vivo* will automatically reduce the toxic potential of a substance. Indeed, when one considers the concept of an inactive prodrug being converted into a therapeutically active molecule, the term detoxication seems even more inappropriate today.

An example illustrative of the effect of metabolism on the toxicity of two closely related compounds is the case of sulfadiazine and sulfamethazine. Both compounds are conjugated to the corresponding N^4-acetyl derivatives. In the mouse, the LD_{50} of sulfadiazine is 1.6 g/kg; N^4-acetylsulfadiazine is more toxic, 0.6 g/kg. With sulfamethazine (LD_{50} of 0.9 g/kg), the opposite is true; the LD_{50} of N^4-acetylsulfamethazine is greater, 1.3 g/kg (2).

Another frequently stated generalization is that biotransformation increases the aqueous solubility of compounds. This statement also must be accepted cautiously. When *o*-chlorophenylacetic acid is metabolized to *o*-chlorophenylaceturic acid, the metabolite has 1.5 times the aqueous solubility of the parent compound. On the other hand, when *p*-chlorophenylacetic acid is converted to its aceturic metabolite, the solubility of the conjugate is only one-half that of the parent compound. The aqueous solubility of metabolites can have important therapeutic consequences. Sulfamethazine and sulfathiazole have roughly the same aqueous solubility. Both compounds are metabolized to acetyl conjugates, which are excreted as such. The acetyl metabolite of sulfamethazine is more water soluble than the parent compound. In the sulfathiazole case, however, the acetyl metabolite is only one-tenth as soluble as the parent compound. The occurrence of kidney blockage caused by crystalline deposits within the kidney is rare with sulfamethazine but very frequent with sulfathiazole (2).

These cases are cited to point out that adherence to generalizations about the probable physicochemical, therapeutic, or toxicological consequences of biotransformations must be undertaken with considerable reservation. This does not mean that they cannot be used as the basis of "educated guesses." It is *usually* true that metabolic alterations *do* lead to more polar substances which are less lipophilic, more water soluble, and more acidic, leading to an accelerated termination of pharmacological activity and more rapid excretion by the renal tubule. The importance of these factors in the elimination of drugs from the body is exemplified by pentobarbital. According to one researcher (3), if biotransformations were not present, pentobarbital would exert its pharmacological effects in the body for nearly a century because of its high lipid solubility.

The hydroxylation of compounds and the subsequent conjugation of these products probably can be considered as being among the most important events that occur among the myriad of biotransformation reactions.

Aside from the implications of these transformations, insofar as they affect the excretion of foreign compounds from the animal because of altered hydrophilicity and/or acidity, the possibility of serious physiological consequences of some reactions of oxygen with foreign molecules are alarming. Although imperfectly understood, there can be little doubt that the carcinogenicity of many compounds is due to highly reactive transitory molecular forms which may occur during the process of biotransformation (4–6).

ALIPHATIC HYDROXYLATION

The hydroxylation of many aliphatic compounds has been reported, including fatty acids (7), alkanes (8, 9), substituted barbituric acids (10), and alkylamines (11). The aliphatic chains are most commonly oxidized at a terminal methyl group to form the corresponding primary alcohol. Usually, this primary alcohol undergoes further biotransformation to the aldehyde and then the acid. However, hydroxylation is not confined exclusively to terminal methyl groups. In addition to the oxidation at this position, hydroxylation at the penultimate carbon atom ($\omega - 1$) is also frequently encountered (7, 12–14). Metabolism of the fatty acids seems to involve hydroxylation at both the ultimate and penultimate carbon atoms.

Stearic acid has been reported (7) as being such a case; in this instance, terminal methyl oxidation is favored 3:1 over methylene group oxidation.

Hydroxylation of benzylic carbon atoms is also well known and the initial product, as in the case of purely aliphatic systems, is a primary alcohol (15–18). As with aliphatic compounds, the final product is often a carboxylic acid. Hydroxylation is not confined to methyl substituents but also is known to occur with ethyl, propyl, or butyl substituents. The stereospecific formation of *S*-(+)-1-indanol from indane has been reported (18).

Systematic studies (19, 20) of *o*-, *m*-, and *p*-methylbenzenes suggest that steric factors play an important role in determining whether the metabolic pathway will proceed by benzylic oxidation or an alternative pathway. *para*-Substituted methylbenzenes undergo benzylic oxidation, as with *trans*-2-(*p*-tolyl)cyclohexanol (20), whereas the corresponding *ortho*-component does not undergo benzylic oxidation but is hydroxylated in the cyclohexyl ring instead. In studies with isomeric methylanisoles (19), *p*-methylanisole underwent benzylic oxidation; *o*-methylanisole afforded the *p*-hydroxy analog.

Under *in vitro* conditions, cyclohexanol is formed (21) from cyclohexane. The position at which oxygenation of the cyclohexane ring occurs seems to be under the influence of stereochemical factors, wherein the bulk of the substituent groups plays a major role in the selection of the sites of attachment. Thus, when methylcyclohexane was studied (22), approximately equal amounts of 3- and 4-hydroxylated products were formed. On the other hand, when *trans*-2-(*o*-tolyl)cyclohexanol was studied, axial hydroxylation took place at the ring position opposite the bulky tolyl substituent as the principal reaction (20).

Particularly interesting studies were reported where the disposition of decalin and tetralin was investigated. *trans*-Decalin was metabolized to (±)-*trans*-*cis*-2-decalol containing an equatorial hydroxyl group, whereas *cis*-decalin yielded (±)-*cis*-*cis*-2-decalol with an axial hydroxyl (23). Tetralin was converted largely to α-tetralol, with β-tetralol being a minor product (24, 25). When ethinamate (1-ethynylcyclohexanol carbamate) (26) or 1-cyclohexanol (27) undergoes hydroxylation, it is at the 3- or 4-position of the alicyclic ring. The major metabolites found in the urine contain the hydroxyl moiety in the 4-equatorial or 3-axial orientation. Relative amounts of 3- and 4-hydroxylated products and the stereochemical nature of the metabolite in studies with acetohexamide {1-[(*p*-acetylphenyl)sulfonyl]-3-cyclohexylurea} were shown to be a function of species differences.

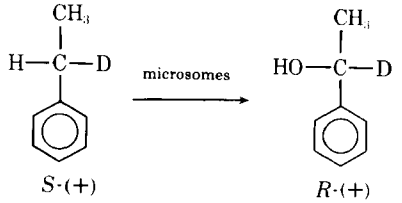
MECHANISMS OF HYDROXYLATION

The hydroxylation of carbon atoms has been exhaustively investigated. The oxygen entering the molecule is derived from molecular oxygen and not water, as one might intuitively expect. That the oxygen source is O_2 rather than —OH has been confirmed by a number of researchers. These studies include the hydroxylation of phenethylamine by dopamine-β-hydroxylase (28). These workers also employed phenylalanine hydroxylase, enzyme hydroxylation of steroids (29), and the benzylic hydroxylation of ethylbenzene in microsomal preparations (30). McMahon *et al.* (30) also demonstrated an isotope effect in the reaction, which indicates the rate-limiting role of the insertion step; the benzylic hydroxylation to yield methylphenylcarbinol had a K_H/K_D ratio of 1.8, which the corresponding benzylic hydroxylation of the oral antidiabetic agent, tolbutamide, did not have (15).

Early work with microbial systems (31, 32) showed that aliphatic hydroxylation of steroids in microbiological systems proceeded without inversion at the 11-position. In the rat, hydroxylation of cholesterol-7α-^{3}H resulted in the formation of the 7α-metabolite without loss of tritium (33). Additional examples were reported (34, 35), and it appears that monooxygenase catalyzes the hydroxylation of aliphatic hydrocarbons stereospecifically with retention of configuration. An interesting case is the fate of ethylbenzene *in vitro* when the system is composed of normal rat liver microsomes or those derived from rats treated with phenobarbital. In the normal case, ethylbenzene is oxidized *in vitro* to a mixture which is four parts *R*-(+)- to one part *S*-(−)-methylphenylcarbinol (36). The stereospecificity of this reaction was greatly reduced when the microsomes of rats previously dosed with phenobarbital were used (30, 36). Unequivocal evidence, demonstrating a case of hydroxylation in the presence of microsomes proceeding with retention of configuration, was also obtained (30, 36). Incubation of *S*-(+)-α-^{2}H-ethylbenzene with normal rat microsomes resulted in a product that was 92% *R*-(+)-α-^{2}H-methylphenylcarbinol, with 14% of the deuterium having been lost (Scheme I).

AROMATIC OXIDATION

The susceptibility of various aromatic rings to attack by oxygen during biotransformation is reminiscent of the reactivity of such compounds in the synthetic reactions of aromatic systems undergoing electrophilic substitution. Thus, aromatic rings that contain electron-donating groups are described as being "*ortho*–*para* directing" by chemists engaged in organic synthesis, whereas groups that are electron withdrawing are called "*meta* directing." The presence of groups (*e.g.*, —NH_2, —CH_3, and —OCH_3) (37, 38) that increase the electron density of the aromatic ring cause it to undergo metabolism more readily to



Scheme I

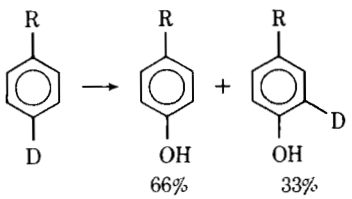
para-phenols. On the other hand, substituents that tend to decrease the electron density of the ring (*e.g.*, $-NO_2$, $-CONH_2$, and $-COOH$) make the compound more resistant to hydroxylation (37).

As with almost all other biotransformations, steric factors play an important role in aromatic hydroxylations. There is a pronounced tendency for such substitutions to take place in the *para*-position if it is available (37, 39–42). The ratio of *ortho*- to *para*-hydroxylation should be 2:1 if only electron density is a factor in determining the point of entry of oxygen into the molecule. In any particular instance, this ratio should be a constant if only one enzyme system is involved. The ratio of *ortho*- to *para*-hydroxylation products is species dependent and can be altered by enzyme induction. This latter observation strongly implies that several P-450 cytochromes can be present within a species. Even in the presence of a strong *meta*-directing group such as is found in nitrobenzene (37, 38), significant *para*-hydroxylation was noted. The entry of substituents into the *para*-position, even when it is a less favorable position in terms of electron density, is most probably ascribable to steric factors.

When more than one substituent is present in the aromatic substrate as in di- or trisubstituted compounds, the position where hydroxylation will most probably take place appears to be reasonably predictable on the basis of the electron-donating characteristics of the individual substituents, keeping in mind that *para*- rather than *ortho*-substitution is preferred (43–49). When the aromatic system contains more than one ring, whether it be aromatic, heterocyclic, or alicyclic, projection of the possible or probable site of hydroxylation is not simple because both electronic and steric factors interact. Some of the many papers dealing with these systems include skatole (50), zoxazolamine (51), and tricyclic compounds acting on the central nervous system (CNS) (52–54). The metabolism of β-blocking agents was recently reviewed (55), and an older review (2) is especially thorough.

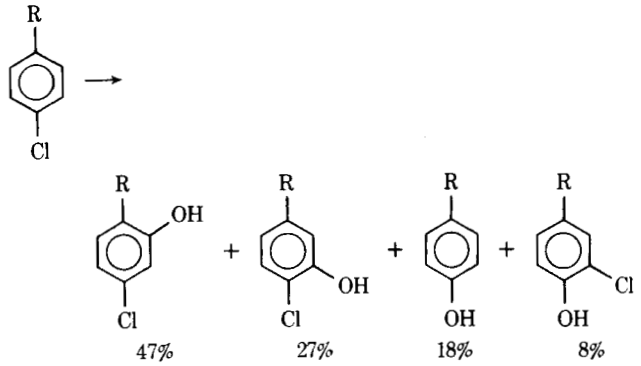
As was the case with aliphatic compounds undergoing hydroxylation, molecular oxygen is also involved when aromatic compounds are metabolized (28, 56). A number of studies have been conducted with substrates containing deuterium labels; in no instance could any isotope effect on the rate of hydroxylation be demonstrated when ^2H was inserted in place of ^1H (57–59).

An important feature of many carbon–oxygen bond formation steps is the "NIH shift," and an understanding of this phenomenon is necessary for the explanation of some seemingly strange metabolic changes. Briefly stated, the NIH shift is the phenomenon whereby the intramolecular migration of a ring substituent takes place during aromatic hydroxylation (Scheme II) (60–64).



R = $NHCOCH_3$

Scheme II



R = $NHCOCH_3$

Scheme III

The fate of deuterium or tritium labels has been studied in a number of cases. The label is largely retained if it is originally introduced in a position adjacent to the carbon atom undergoing hydroxylation.

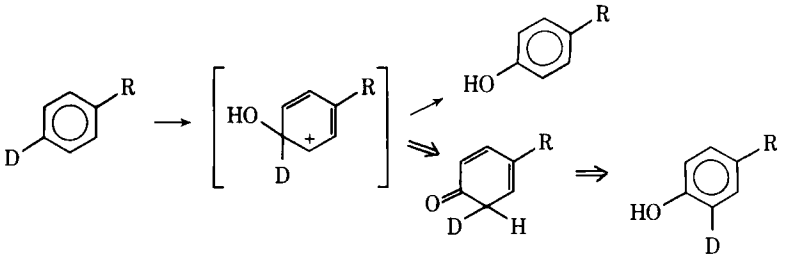
A series of investigations established that the NIH shift is a general reaction associated with the hydroxylation of aromatic compounds by animal, bacterial, fungal, and plant systems. The only reported exception is the aerobic hydroxylation of acetanilide-4-^2H and anisole-4-^2H with peroxidase in the presence of dihydroxyfumaric acid, which serves as a reducing agent. Free radical hydroxylation of aromatic compounds does not lead to migration of substituents (65). In the case of the peroxidase system, it may be presumed that the lack of migration is a reflection of the free radical nature of this hydroxylase system.

The migration of a number of substituents from positions undergoing hydroxylation has been demonstrated. Halogen or methyl groups migrate from the *para*- to the *meta*-position when *para*-substituted phenylalanines are subjected to hydroxylation with phenylalanine hydroxylase (61, 66, 67). When *p*-chloroacetanilide was exposed to liver microsomes from rats treated with the enzyme-inducing agent benzpyrene (68), a variety of products was obtained (Scheme III). In studies of the oxidative hydroxylation of *p*-methylacetanilide or *p*-methylanisole in liver microsomal systems, it was not possible to demonstrate migration of the methyl group (69); instead, hydroxylation of the methyl group ($-CH_2OH$) was found.

Although the examples cited have involved *in vitro* systems, the NIH shift has been demonstrated under *in vivo* conditions with 2-[(*p*-chlorophenyl)thiazol-4-yl]acetic acid (70).

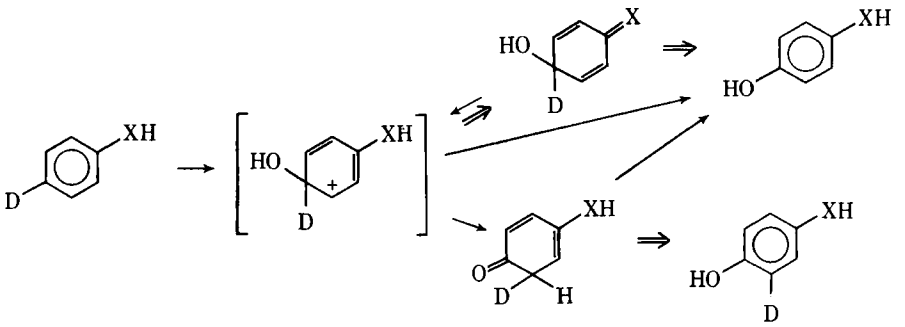
A very incisive and reasonable explanation of the mechanism of the NIH shift and of factors that influence it has been suggested (71); the observed data have been interpreted in terms of cationoid intermediates (Scheme IV). The investigators classified the

Group I



R =	OCH_3	CH_3	C_6H_5	$C{\equiv}N$	$CONH_2$	NO_2	Cl	Br	F	$NCH_3SO_2C_6H_5$
% D retained	60	54	64	41	42	40	54	40	47	53

Group II



X =	O	$NSO_2C_6H_5$	NH	NCOH	$NCOC_6H_5$	$NCOCH_3$
% D retained	0	1	6	19	21	30

Scheme IV

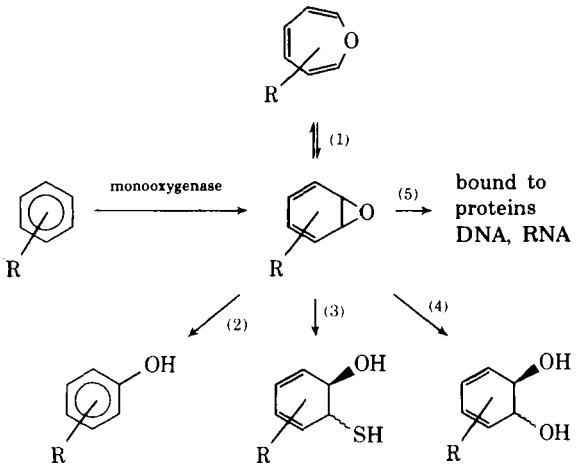
substrates as Group I or II. Group I is comprised of those substrates with substituents that do not have a labile proton adjacent to the aromatic system and have high retention values. The Group II substrates can form a stable cationoid by losing a proton from a heteroatom adjacent to the aromatic ring; these substrates show low retentions. The degree of retention is influenced by the acidity of the substituent and by pH. Retention in Group II substrates decreases as the ability of the substituent to donate electrons is increased. A recent publication (72) amplified the suggested mechanisms to include the formation of *ortho*-isomers in the metabolism of chlorobenzene.

The highly unstable nature of some oxygenated metabolites make their detection very difficult. As early as 1950, the formation of arene oxides, which are compounds arising from the epoxidation of a double bond in an aromatic ring system, was suggested and speculations were made as to their possible role in chemical carcinogenesis. The question as to whether arene oxides could be formed under biological conditions was not answered until much later (73, 74). Shortly thereafter, evidence showed that the carcinogenicity of polycyclic hydrocarbons having planar configurations was probably due to these reactive intermediates (75). The arene oxide intermediates rearrange nonenzymatically to phenols, which subsequently are converted to either vicinal diols by epoxide hydrases or to glutathione conjugates by glutathione *S*-epoxide conjugases. In addition, the glutathione *S*-epoxide conjugation seems to proceed without enzymic participation (76). The glutathione *S*-epoxide transferases are found in the cytoplasm (76), whereas the epoxide hydrases have been shown to be present in endoplasmic reticulum (77). The hydrases are inducible (77), whereas the glutathione *S*-transferases are not (78). Surprisingly, a number of investigators have been unable to demonstrate that hydration of arene oxides can take place without the participation of enzymes (79, 80).

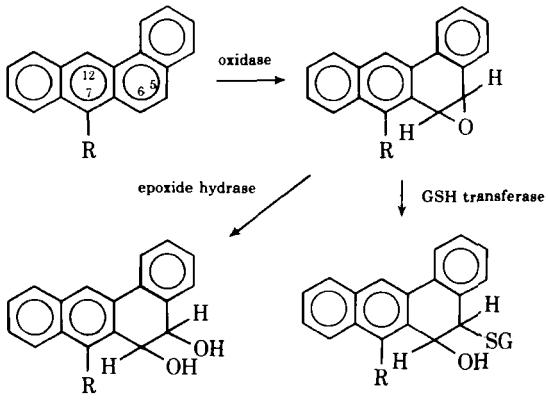
Arene oxides have been demonstrated as being microsomal metabolites of several benzanthracenes (81–83), pyrene, and benzo[*a*]pyrene (84). Also, naphthalene oxide was prepared in a reconstituted P-450 system (85).

The action of monooxygenases is not confined to aromatic double bonds. A related situation also has been found with alkene oxides when epoxidation of olefinic bonds has been reported. These epoxides have been found among a number of insecticides (86–89), allyl-substituted barbituric acids (90, 91), and the 10,11-epoxide of carbamazepine isolated from human urine (92), as well as a variety of other compounds. The alkene epoxides are far more stable than the arene oxides and are not as subject to enzymatic attack or spontaneous alteration. Frequently, vicinal glycols, but not epoxides, are found as products of oxidative metabolism of olefins (93–99). Theoretically, glycols could be formed by the direct dihydroxylation of an olefinic bond or by means of an intermediate epoxide. Cyclohexene, indene, and styrene form intermediate epoxides (100), although the glycol is the isolated product. The presence of intermediate epoxides was demonstrated in the case of octenes (101).

A discussion of some mechanistic considerations of



Scheme V



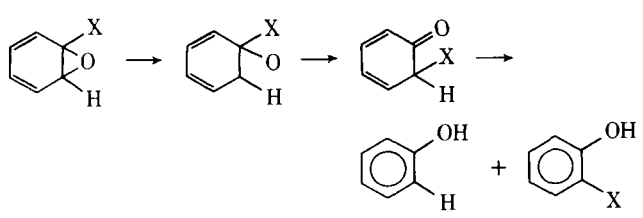
R = CH_2OH
GSH = glutathione

Scheme VII

oxirane formation was published (78). A review of those proposals, shown in Scheme V, offers an appreciation of the unusual nature of the oxirane system and why it may have great importance in the toxicity of chemical agents.

The aromatic substrate is acted upon by a monooxygenase to yield a corresponding arene oxide. The arene oxide, once formed, has a multiple number of paths available to follow. Pathway 1 shows that it may undergo carbon–carbon bond scission to form the seven-membered oxepin ring system. The equilibrium between the oxepin and arene oxide forms is a valence bond tautomerism and the equilibrium may be greatly displaced in either direction. Naphthalene 2,3-oxide appears to exist only in the oxepin form (102), whereas naphthalene 1,2-oxide has been shown to exist only as the oxide (103, 104). The failure of naphthalene 1,2-oxide to enter into an equilibrium with its tautomeric oxepin form is thought to be due to the fact that such a step would cause the aromatic ring to lose its aromaticity. Oxepins that are not converted to the arene oxide do not isomerize to the phenol. To date, only those arene oxides that at least partly exist in the oxide form have been demonstrated to be metabolic intermediates. The isomerization of the oxide (Pathway 2) is an internal spontaneous reaction (105–107) which occurs under basic or neutral conditions and pH < 6. This isomerization of the arene oxide to the phenol is frequently accompanied by the NIH shift previously discussed (108–111). The sequence shown in Scheme VI has been proposed (110) to take account of the role of the NIH shift in the arene–phenol conversion.

The third pathway (Pathway 3) open to the arene



NIH shift

Scheme VI

oxide is conjugation with glutathione by direct coupling without the involvement of enzymes or through catalysis by glutathione *S*-epoxide transferase (74, 112–114). The glutathione conjugates undergo conversion to premercapturic acids, which in the presence of acid are dehydrated to mercapturic acids (115).

The arene oxides can undergo hydration (Pathway 4) under the influence of epoxide hydrases to form *trans*-1,2-diols. These diols may then conjugate with glucuronic acid or undergo enzymatic dehydrogenation to form catechols (116, 117).

The remaining pathway of Scheme V (Pathway 5) is the covalent binding of an arene oxide structure to the macromolecules of biological systems. A number of investigators (107, 114, 118) described the formation of covalent bonds between the arene oxides and nucleophilic substances such as methanol, azide, and sulfhydryl compounds. Aromatic hydrocarbons do not bind with the nucleophilic centers present in DNA or RNA under *in vitro* conditions if NADPH is excluded from a liver microsome system, but they do bind readily if all cofactors of the system are present (119, 120). Pretreatment of the rats with an enzyme-inducing agent (120) known to stimulate the liver microsomal system results in considerable enhancement of the binding to DNA by the hydrocarbon when exposed to these microsomes. These findings strongly suggest, but do not prove, that the moiety that binds to the DNA of the system or tissue may be the arene oxide.

Other work tends to reinforce the likelihood that this is the case. In the absence of a microsomal system, firm binding of arene oxides to DNA, RNA, and histone was found under *in vitro* conditions (121). The same study demonstrated that the parent aromatic hydrocarbons and diols did not bind to these macromolecules. Cell culture studies in systems having the capability of causing biotransformations by oxidative pathways demonstrated that the parent hydrocarbons, their arene oxides, and the phenols and diols derived from them all firmly bound to DNA, RNA, and other proteins present in the medium; however, the arene oxides underwent covalent bonding to a much greater extent than did the other components (122, 123). It has been demonstrated

glucose-1-phosphate + uridine triphosphate $\xrightarrow{\text{pyrophosphorylase}}$ uridine diphosphate glucose + pyrophosphate

uridine diphosphate glucose + $2NAD^+ + H_2O \xrightarrow{\text{uridine diphosphate glucose dehydrogenase}}$ uridine diphosphate glucuronic acid + $2NADH + 2H^+$

Scheme VIII

that both alkene and arene oxides are active as alkylating agents (124, 125). The latter study is particularly interesting in that "K-region" (126) activity is involved (Scheme VII).

CONJUGATION REACTIONS

Conjugation with glucuronic acid is a common phenomenon in the metabolism of foreign compounds in mammalian species. Although this review is primarily concerned with the introduction of oxygen as a biotransformation step, glucuronide conjugation will be discussed on a somewhat broader basis.

The mechanism of glucuronide formation involves the reaction of the compound with D-glucuronic acid (127). For the condensation to proceed, glucuronic acid is activated by biosynthesis of uridine diphosphate glucuronic acid. The synthetic sequence is initiated by the formation of glucose-1-phosphate, as shown in Scheme VIII. The formation of uridine diphosphate glucuronic acid from uridine diphosphate glucose is under the mediation of a dehydrogenase present in the supernatant fraction of liver preparations.

The condensation of uridine diphosphate glucuronic acid with the compound undergoing conjugation (RZH) takes place upon catalysis of the reaction by a solubilized microsomal enzyme (128), glucuronyl transferase, which is found primarily in the liver. Glucuronyl transferase is also present in other body tissues. The reaction proceeds according to Scheme IX. The C^1 atom of glucuronic acid is present in the α-configuration in uridine diphosphate glucuronic acid but appears in the β-configuration in the conjugates formed.

Glucuronic acid forms conjugates with the hydroxyl groups of primary, secondary, or tertiary aliphatic compounds, phenols, enolic compounds, and hydroxylamines. The carboxyl group of straight-chain aliphatic, heterocyclic, carbocyclic, and arylalkyl acids form such conjugates. Aromatic amines, carboxyamides, and sulfonamides have also been reported as reacting with glucuronic acid (129).

Alcohols and phenols tend to form "ether-type" glucuronides. Many aromatic and aliphatic carboxylic compounds form "ester-type" glucuronides. Aromatic amines form *N*-glucuronides enzymatically through glucuronyl transferase, but some compounds apparently form these conjugates by nonenzymatic reactions (130).

MERCAPTURIC ACID SYNTHESIS

Conjugation with glutathione to form mercapturates is probably much more widespread in animals than in humans (131, 132). Hydrocarbons, aliphatic or acylhalogen compounds, nitro compounds, aromatic amines, sulfonate esters, carbamic esters, *etc.*, have all been reported to undergo this type of conjugation *in vivo*. Thus, the acetylcystyl residue of glutathione has been shown to replace H, halogen, $-NO_2$, $-SO_3CH_3$, $-NH_2$, and $-SO_2NH_2$ groups from a wide variety of structures.

uridine diphosphate glucuronic acid + RZH $\xrightarrow{\text{glucuronyl transferase}}$ RZ-glucuronic acid + uridine diphosphate

$$Z = O, \overset{O}{\overset{\|}{C}}O, NH, \text{or } S$$

Scheme IX

The reactive position of the drug molecule reacts with glutathione under mediation of glutathione *S*-transferase and glutathionkinase, which are present in the soluble fraction of liver, kidneys, and heart. Some compounds seem to react with glutathione in the absence of enzyme involvement (133). The initial complex undergoes transpeptidization to lose glutamate followed by peptidase cleavage of glycine. The final step of mercapturic acid synthesis requires *N*-acetylation.

Normally stable cyclic hydrocarbons have been shown to be excreted as mercapturates. In contrast to the displacement reactions already mentioned, the reaction of glutathione with aromatic hydrocarbons does not result from the displacement of an atom, in this case, hydrogen. In this instance, it is thought that an addition reaction involving an intermediate epoxide structure takes place (Scheme VII).

SULFATE CONJUGATION

This form of conjugation is quantitatively less important than those involving glucuronic acid, probably because the body pool of sulfate is relatively small. Primarily aliphatic alcohols and phenols react to form "ethereal sulfates" (129, 131). During the reaction, the phenol reacts with 3′-phosphoadenosine-5′-phosphosulfate in the presence of a sulfokinase to yield the sulfate and 3′-phosphoadenosine-5′-phosphate. "*N*-Sulfates," or sulfamates, are formed from aromatic amines in the rat, rabbit, and guinea pig. Conjugation takes place in soluble fractions of the liver (132, 133).

Morphine is treated similarly by the cat (134, 135) as are codeine, norcodeine, and normorphine (136, 137). Although the cat acetylates sulfadimethoxine reasonably well (6%), it forms no glucuronide with this compound (138).

Since biotransformations other than conjugation reactions frequently take place, a situation often exists where a series of competing reactions occurs simultaneously. It is not rare for excretion of unchanged drug, conjugation of the parent substance, molecular alteration of the parent, conjugation of the daughter compound, and excretion of these various entities to take place at the same time. This phenomenon is demonstrated by an experimental anti-inflammatory agent, I (139).

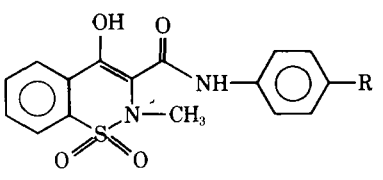
Table I—Conjugation of Phenylacetic Acid

Species (Number, Sex)	Route of Administration and Dose, mg/kg	Metabolite, % in 24-hr Urine[a]			
		1	2	3	4
Human (2M)	po 1	—	<0.05	91	7
Rhesus monkey (2F)	ip 80	41	0.9	24	9
Cynomolgus monkey (1F, 1M)	ip 80	9	0.6	46	2
Green monkey (1M)	im 25	10	0.4	69	3.5
Red-bellied monkey (1M)	im 8	3.4	0.5	39	1.2
Mona monkey (1F)	im 8	6.8	0.2	4.8	3.2
Mangabey (1M)	im 8	43.5	0.4	24.5	5.9
Drill (1F)	im 8	34	0.2	14.6	3.6
Baboon (1M)	im 2	5	0.1	85	10
Squirrel monkey (1F, 2M)	im 50	2.9	1.1	52	12
Capuchin (2F)	ip 80	1.2	5.3	14	10
Marmoset (2M)	im 80	3.5	0.6	56	0.3
Bushbaby (1M)	ip 80	—	60	—	9
Slow loris (1M)	ip 80	16.2	56.7	—	8.1
Dog (3F)	ip 80	0	76	—	3
Cat (3F)	ip 80	0.5	74	—	0.5
Ferret (3F)	ip 80	3	41	—	30
Rabbit (3F)	ip 80	2	82	—	0.4
Rat (3F)	ip 80	0	94	—	1
Mouse (8F)	ip 80	17	30	—	3.5
Hamster (3F)	ip 80	44	40	—	0.9
Guinea pig	ip 80	3.4	59	—	0.3
Vampire bat (3F)	ip 80	—	75	—	—
Pigeon (3F)	po 80	2	43	—	28
Chicken (3F)	po 80	4	4	(Metabolite 5, 51%)	

[a] Metabolites: 1, phenylacetic acid; 2, phenylacetylglycine; 3, L-(—)-phenylacetylglutamine; 4, phenylacetyltaurine; and 5, L-(+)-diphenylacetylornithine.

Several phenomena are to be noted. Of special interest is that the parent compound (I), 4-hydroxy-2-methyl-2*H*-1,2-benzothiazin-3-carboxanilide 1,1-dioxide, has a shorter half-life (21 hr) in humans than does its hydroxy metabolite (37 hr), which is in contradiction to the generalization that increased hydrophilicity speeds the rate of elimination. In the dog, a more normal pattern is seen, with the half-life of the administered compound being 30 hr and that of the hydroxy metabolite being only 14 hr. The rat, however, presents an entirely different case; here the half-lives of the parent and metabolite are essentially the same. After a 300-mg daily dose, a human eliminated 30% of the administered drug in the form of the conjugate of the parent compound, no free drug was found, and 70% was eliminated as the hydroxy metabolite and its conjugate. The dog, after receiving 10 mg/kg daily, excreted 10% of the dose as free drug and 70% as the hydroxy metabolite and its conjugate. Rats and monkeys afforded values between those observed for humans and dogs.

In addition to the unusual half-life observations alluded to earlier, this paper forcefully demonstrates how species differences can markedly affect the validity of animal toxicity and pharmacological efficacy data when extrapolation to humans is undertaken. The hydroxy metabolite (II) was tested for anti-inflammatory activity (140) and was classified as non-



I: R = H
II: R = OH

active. Obviously, testing the parent compound in a species that excretes a substantial portion of the drug unchanged would make the compound appear much more potent than with a species that conjugates it rapidly or converts it to its primary biotransformation product.

It is of interest to see how phenylacetic acid is excreted and conjugated by animals (Table I). In one study (141), 25 species were given phenylacetic acid and urine was then collected for 24 hr. The urine samples were investigated as to the percent of unchanged drug excreted; the glycine, glutamine, taurine, and ornithine conjugates were individually determined. Table I shows that humans excrete the glutamine conjugate, a small amount of the taurine metabolite, and no unchanged compound. Except for significant amounts of unchanged material being eliminated by the kidney, old world monkeys follow the same pattern. New world monkeys, however, excrete little unchanged phenylacetic acid but more of the taurine conjugate. The nonprimates do not form glutamine conjugates nor, with the exception of the ferret and pigeon, do they form large amounts of the taurine conjugate. Only the chicken is reported as excreting the ornithine conjugate (51%).

Table II shows some data from this study (141) and data from another experiment conducted by the same laboratory (142). *p*-Chlorophenylacetic and *p*-nitrophenylacetic acids were studied in some of the same species. The *p*-chloro substituent apparently had little effect on altering the metabolic pattern as was seen with phenylacetic acid in humans, rhesus monkey, and rats. With the capuchin monkey, which had eliminated virtually no unchanged phenylacetic acid, 24% of the administered dose appeared in urine following dosing with *p*-chlorophenylacetic acid. The *p*-nitrophenylacetic acid was largely eliminated un-

Table II—Species Differences in Conjugation of Phenylacetic Acids[a]

	Species	Unchanged	Glycine Conjugate	Glutamine Conjugate
Phenylacetic acid	Human	<2	~0	91
	Rhesus monkey	41	1	24
	Capuchin	1	5	14
	Rat	0	94	1
p-Chlorophenylacetic acid	Human	5	0	90
	Rhesus monkey	27	<1	38
	Capuchin	24	<1	14
	Rat	3	92	0
p-Nitrophenylacetic acid	Human	87	0	0
	Rhesus monkey	80	0	0
	Rat	28	61	0

[a] Values are percent of administered material excreted in urine.

changed by humans (87%) and rhesus monkey (80%), with neither the glycine nor glutamine conjugates having been formed. The rat excreted 28% unchanged and 61% as the glycine conjugate. Both humans and rhesus monkey excreted approximately 7% as a conjugate which, upon acid hydrolysis, afforded *p*-nitrophenylacetic acid. The investigators had available to them *p*-acetamidophenylacetic acid, but it did not appear to be a metabolite formed by any of these species. It would seem that the strong electron-withdrawal properties of a nitro group in the *para*-position reduces electron density sufficiently to impart a considerable degree of chemical inertness to the molecule.

Several other major studies designed to investigate species differences involving conjugation reactions have appeared recently. One (143) reports on the sulfate and glucuronide formation of phenol or its metabolite, hydroquinone, in 19 species (Table III). Another (138) reports on N^4-acetylation and N^1-glucuronide formation after exposing 18 species to sulfadimethoxine [N^1-(2,6-dimethoxy-4-pyrimidinyl)sulfanilamide]. The N^1-glucuronide was the primary urinary metabolite in humans, rhesus monkey, squirrel monkey, baboon, capuchin, bushbaby, slow loris, and tree shrew. It was a minor metabolite in dog, rat, mouse, guinea pig, Indian fruit bat, and hen. However, the N^4-acetyl compound, which was excreted to some extent by all species except the dog, was the major urinary metabolite in the green monkey, guinea pig, and rabbit.

The ferret, which has been suggested by some individuals to be a good biotransformation model for humans, certainly does not resemble humans, especially insofar as conjugation reactions are concerned. In the case of phenylacetic acid, humans excrete 91% of the dose as the glutamine conjugate, whereas the ferret does not excrete this metabolite at all but does eliminate roughly equal amounts of the glycine and taurine forms. Similar differences appear with phenol and sulfadimethazine.

NITROGEN

The hydroxylation reactions previously described have been confined to events involving the formation

Table III—Conjugation of Phenol[a]

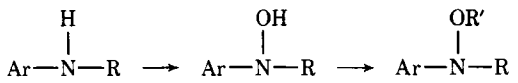
Species	Sulfate	Glucuronide	*p*-Hydroxyphenyl Sulfate	*p*-Hydroxyphenyl Glucuronide
Human	77	16	1	Trace
Rhesus monkey	60, 70	40, 30	—	—
Squirrel monkey	7	68	—	25
Capuchin	14	65	—	21
Ferret	28	40	30	—
Cat	87	—	13	—
Dog	33, 68	24, 12	43, 20	—
Pig	—	100	—	—
Hedgehog	63, 86	20, 10	17, 4	—
Fruitbat	9, 11	91, 89	—	—
Rabbit	45	46	9	—
Chicken	78	22	—	—
Rat	54	42	1	2
Mouse	46	35	5	1
Jerboa	61	26	12	1
Gerbil	42	35	19, tr	1
Hamster	27, 24	44	1	28, 27
Lemming	35	39	10	15
Guinea pig	13, 22	82	tr, tr	5, 5

[a] Data are expressed as percent of orally administered dose excreted in urine in 24 hr. All animals were given 25 mg/kg, except humans (0.01), rhesus monkey (50), pigs (21), and hedgehogs (20).

of a C—O bond. The introduction of oxygen into arylamines at the nitrogen atom has been studied extensively because of the association of these products with the development of malignancies in laboratory animals. The usual cause of the reaction is that a hydrogen atom of a primary or secondary arylamine is replaced by a hydroxy group; this metabolite then is further metabolized by conjugation with glucuronic or sulfuric acid (Scheme X). *N*-Hydroxylation reactions lead to the formation of highly reactive intermediates, which are frequently more toxic than the parent compounds, particularly in the area of chemical carcinogenicity. Only in recent years has an understanding of this reaction come about; this long delay is probably due to the fact that, as in the case of arene oxides, many of these *N*-hydroxy metabolites are quite unstable and difficult to isolate.

The conversion of arylamines to *N*-hydroxy metabolites under *in vivo* conditions was unambiguously demonstrated in 1959. The development of a new assay procedure (144) made it possible to assay arylhydroxylamines and arylnitroso compounds in tissues, blood, and urine. The formation of methemoglobin was studied in animals treated with aromatic amines, and a number of *in vitro* studies were undertaken in a series of experiments (145–150) wherein a variety of substituted aniline compounds was investigated. In compounds where groups that decreased ring hydroxylation were introduced into aniline, it was demonstrated that a corresponding increase in *N*-hydroxylation frequently occurred. Importantly, it was demonstrated that biochemical oxidation of *N*-alkylanilino compounds resulted in the formation of nitrosobenzene analogs.

The carcinogenic properties of *N*-2-fluorenylacetamide were intensively investigated. When rats were given single doses of *N*-2-fluorenylacetamide, no metabolites indicating *N*-hydroxylation were recovered but several ring-hydroxylated metabolites



Scheme X

were identified (151). Multiple exposures of rats to *N*-2-fluorenylacetamide administered in the diet led to the isolation of a previously unreported glucuronide (152). Enzymic hydrolysis, followed by exhaustive characterization of the metabolite, showed it to be an *N*-hydroxy derivative. This important breakthrough was vigorously pursued, and it was soon established that this metabolite was capable of producing all the effects, both physiological and pathological, of the parent compound when given to rats (153). The carcinogenicity study with the *N*-hydroxy metabolite of *N*-2-fluorenylacetamide produced the same tumors as did *N*-2-fluorenylacetamide and showed the metabolite to be more potent than the parent compound. Tumors were also formed at the site of injection of the *N*-hydroxy metabolite. Metabolites of *N*-2-fluorenylacetamide that had undergone ring hydroxylation reactions were shown to be either very weakly carcinogenic or inactive.

The *in vitro* *N*-hydroxylation of a series of *para*-substituted anilines was studied (154). These compounds all underwent *N*-hydroxylation more rapidly than did aniline itself. Reactivity was as follows: *p*-aminopropiophenone > *p*-phenetidine > *p*-chloroaniline > *p*-toluidine > aniline. When using the cat, it was shown that methemoglobin formation varied directly with reactivity. In another series of experiments by the same laboratory (155), aromatic amines known to be chemical carcinogens were investigated by measuring the generation of nitroso derivatives by rat liver microsomes. The formation of nitroso derivatives was in the order: 4-aminodiphenyl > 2-aminofluorene > 2-naphthylamine ≈ 4-aminostilbene ≈ aniline. As in the previous study, methemoglobin formation in the cat followed the pattern of reactivity in the microsomal system.

The presence of *N*-hydroxylating enzymes has been reported in many organs of several species. These tissues include the liver, lung, kidneys, brain, small intestine, and bladder, and the species include the rat, dog, guinea pig, rabbit, pig, cat, and hamster.

When the lung or liver of cats was perfused with a series of alkylanilines, the level of the amines was lowered and an increased level of nitroso compounds was noted. *N*-Alkylanilines yielded both aniline and nitrosobenzene (149). 2,4-Dichlorophenol reduced aniline formation but increased nitrosobenzene synthesis. The ability to synthesize nitrosobenzene was *N*-methylaniline > *N*-ethylaniline > *N*-butylaniline > aniline > *N,N*-dimethylaniline when rat liver microsomes were used. In a similar series of experiments (153, 156), no inhibition of nitrosobenzene formation could be demonstrated with proadifen hydrochloride[1], α,α-dipyridyl, cyanide, hydrogen peroxide, ascorbic acid, or catalase.

[1] SK&F 525-A.

Dogs pretreated with phenobarbital excrete much more 2-naphthylhydroxylamine after exposure to 2-naphthylamine than do normal controls (157). Phenobarbital treatment also strongly enhanced the microsomal activity in rabbits (158). Investigations (159–161) of the effect of enzyme-inducing agents on metabolic parameters such as the *N*-hydroxylation of *p*-chloroaniline, aniline, and *N*-alkylaniline; the C-hydroxylation of the *N*-alkylanilines; and effects on *N*- and *O*-dealkylation were all studied. *N*-Hydroxylation of *p*-chloroaniline was greatly increased with liver microsomes whose host had undergone stimulation with methylcholanthrene, some increase followed phenobarbital induction, but no discernible change followed exposure to chlorophenothane (DDT). A contrasting pattern was noted for ring hydroxylation, *N*-dealkylation of *p*-chloroaniline, and the formation of nitrosobenzene from *N*-alkylaniline. In the latter instance, all three inducers led to a reduced yield.

Although the question of N—O bonds and some of their physiological implications have been approached from the viewpoint of a biochemical synthetic reaction, it must be borne in mind that *N*-hydroxylamines can also be generated by reductive reactions. The hematopoietic consequences of nitrobenzene exposure have been documented in humans and animals, and fatalities have been reported after treatment with chloramphenicol. Nitrofurans have been implicated as chemical carcinogens. The experimental evidence gathered to date suggests that aromatic organic nitro compounds undergo conversion to arylamines by being first reduced to nitroso compounds and then to arylhydroxylamines. Such a reductive pathway need not be confined to the enzymatic characteristics of the host but may be produced by the microbiological components of the GI system (162).

Administration of toxic levels of nitrobenzene to cats resulted in the detection of nitrosobenzene in the blood (163, 164). The urine of rabbits treated with *m*-dinitrobenzene yielded *m*-nitrosonitrobenzene (165). Kinetic analysis of the reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid by rat liver microsomes clearly demonstrated that a hydroxylamino derivative was an intermediate step in the reductive sequence (166).

The known carcinogen, 4-nitroquinoline *N*-oxide, also undergoes biotransformation to the corresponding hydroxylamino compound, with the amine being the final product (167–171). In addition to the liver, this reductive sequence was also reported to occur in the subcutaneous tissue of the rat (172). A number of microorganisms have shown ability to reduce this compound to a hydroxylamino intermediate. *Pseudomonas aeruginosa* and *Escherichia coli* produce further reduction. Reduction in bacterial systems has been shown to proceed through sequences that include the intermediate hydroxylamine compound, which is reduced to the 4-aminoquinoline *N*-oxide and, in some cases, ultimately to 4-aminoquinoline. A considerable literature exists on the carcinogenic and mutagenic aspects of this compound (172–175).

The ability of these intermediates to react with DNA and other macromolecules has been extensively documented (176–181).

A number of investigations have been undertaken to elaborate those aspects of chemical structures or functional groups that are thought to be implicated in chemical carcinogenesis; mutagenic aspects, other than carcinogenicity, also have received attention. Thus, nitro compounds (182), epoxides (183), aromatic amines (184), and alkylating agents (185) have been studied in considerable detail.

Although many early studies designed to demonstrate the carcinogenicity of arene oxides and similar compounds under *in vivo* conditions did not permit any firm conclusions to be drawn (186–189), the most generally advanced explanation is that these highly energetic intermediates are too reactive to undergo any physical migration and must be generated *in situ*. A very comprehensive and systematic investigation (75) lends support to this hypothesis. In this study, K-region (126) epoxides of benz[*a*]anthracene, dibenz[*a,h*]anthracene, and 3-methylcholanthrene were studied in cell culture employing mouse prostate cells (G-23 line) and were evaluated in terms of toxicity and ability to produce malignant transformations (190). Comparisons were made using these epoxides, the parent hydrocarbons, and the corresponding K-region dihydrols. In this transformation assay, 3-methylcholanthrene was found to be weakly active, but benz[*a*]anthracene, dibenz[*a,h*]anthracene, and the *cis*- and *trans*-dihydrates of the three hydrocarbons were completely devoid of carcinogenic activity. The K-region phenol of 3-methylcholanthrene was moderately active. The K-region epoxides of the hydrocarbons were very active. One epoxide, the 8,9-epoxide of benz[*a*]anthracene, which is a non-K-region epoxide, was only weakly active if active at all. However, the situation is much more complex because the K-region epoxides of phenanthrene and chrysene, noncarcinogenic hydrocarbons, did not exhibit any transformations of this cell line. To complicate the question further, the K-region epoxide of 7-methylbenzanthracene was not as active a carcinogen as its parent hydrocarbon (although both compounds are only very weakly active).

Studies of the binding of epoxides to RNA, DNA, and the protein constituents of normal or transformed cells have not demonstrated a definitive relationship between the binding observed and cell transformation or toxicity (123). The mechanism of these malignant transformations still awaits elucidation (75). It seems that epoxide formation is essential in some cases, but recent papers (191, 192) suggested that certain hydrocarbons may be activated by metabolism of methyl groups through the formation of carbonium ions. Bromomethyl derivatives of benzanthracene and dimethylbenzanthracene were prepared as carbonium-ion-generating model compounds. The latter compound was found to be carcinogenic (192), but it was reported (75) that neither 7-bromomethylbenzanthracene nor 7-bromomethyl-12-methylbenzanthracene was active in the G-23 mouse prostate cell line. It is possible that metabolic activation is not a prerequisite for certain methylated hydrocarbons to transform cells.

An alternative possibility is that the intermediate metabolites forming these compounds are so reactive that they never survive long enough to reach this biochemical target within the cell. However, tissue culture studies (192) using Chinese hamster cells have shown that 7-methylbenz[*a*]anthracene and the bromomethyl compounds discussed earlier are mutagenic in that system. This makes the hypothesis less tenable. Also, the epoxides of chrysene and phenanthrene, which are highly toxic but do not transform mouse prostate cells, do transform hamster embryo cells.

A study of the metabolic disposition of 7-hydroxymethylbenz[*a*]anthracene 5,6-oxide in rat liver preparations was published (125). 7-Hydroxymethylbenz[*a*]anthracene 5,6-oxide was converted by rat liver microsomal preparations or rat liver homogenates into *trans*-5,6-dihydro-5,6-dihydroxy-7-hydroxymethylbenz[*a*]anthracene and into a glutathione conjugate by rat liver homogenates or by rat liver soluble fraction in the presence of glutathione. Sometimes trace amounts of 5-hydroxy-7-hydroxymethylbenz[*a*]anthracene was formed in these reactions, but it is felt that such a substance was probably the result of a nonenzymatic reaction. The 7-hydroxymethylbenz[*a*]anthracene was metabolized to a number of products, including the corresponding *trans*-5,6-dihydrol, when rat liver microsomes were used, but this was not seen when rat liver homogenate was employed in the incubation. No *cis*-dihydrol could be detected. When the glutathione conjugates of K-region epoxides are treated with acid, the unoxidized parent hydrocarbon is regenerated. However, when a non-K-region glutathione conjugate is treated similarly, the corresponding phenol is formed. In assessing the possible carcinogenicity of the 7-hydroxymethylbenz[*a*]anthracene 5,6-oxide in the 4-(*p*-nitrobenzyl)pyridine procedure, it was shown to be a less efficient alkylating agent than 7-methylbenz[*a*]anthracene 5,6-oxide which, in turn, is less efficient than the unoxidized parent hydrocarbon.

BIOTRANSFORMATION AND CARCINOGENICITY

Much of the preceding discussion has centered on the concepts of "drug" and "prodrug" or "carcinogens" and "procarcinogens" and "direct"- or "indirect"-acting carcinogens without making any special attempt to allude to them as such.

The distribution of the drug-metabolizing enzymes has not been vigorously pursued by most investigators of drug metabolism; efforts have been largely focused on the liver as the primary site of biotransformation. Recent studies (193–197) demonstrated that polycyclic hydrocarbon hydroxylase activity, at least, is widely distributed throughout the animal. The hydroxylase activity in the proximal position of the small intestine of the rat is about equivalent to that of the liver. Hydroxylase activity has also been demonstrated in the forestomach, the glandular stomach, the large intestine, the lung, the sebaceous glands of

the skin, the distal and proximal sections of the convoluted tubules of the kidney, and the adrenal of the rat. Hydroxylase activity increases in all tissues, except the adrenal, upon exposure of the rat to various enzyme inducers. Benzpyrene hydroxylase activity has been shown in the proximal small intestine of the baboon, bull, dog, guinea pig, hamster, human, monkey, mouse, and rabbit.

In early studies of the role of drug-metabolizing enzymes in the biochemical aspects of oncology, it was shown that administration of known microsomal enzyme inducers would protect animals from the carcinogenic effects of aminoazo compounds and *N*-2-fluorenylacetamide (197–199). Later studies also demonstrated protection against polycyclic hydrocarbons (200–203).

A provocative consideration of these findings and their implications was presented (204). This analysis suggested that the portals of entry into the animal, *i.e.*, the GI tract, the lungs, and the skin, act as a first line of defense to prevent the exposure of the internal organs to contact with the unaltered form of the foreign compounds and that "detoxification" here would result in a protective effect. The liver is considered a very potent second line of defense. Presumably, the microsomal enzymes of the kidney would be a third line of defense in their role of facilitating renal excretion. This hypothesis, however, does not appear to take into account that certain polycyclic hydrocarbons require metabolic activation to K-region epoxides before their carcinogenic effects can be demonstrated.

There is little doubt that there has been a continually increasing exposure of the population to airborne polycyclic hydrocarbons from fuel combustion and cigarette smoke and that these agents are probably the most widely distributed carcinogens present in our environment. Protection against pulmonary adenoma formation was achieved when β-naphthoflavone, an enzyme inducer, was given with orally administered 7,12-dimethylbenz[*a*]anthracene (203). Flavones are widely distributed in plants, and this finding implies that diet may have a hithertofore unappreciated role in carcinogenicity studies.

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Determination of Isoniazid and Metabolites in Biological Fluids

HAROLD G. BOXENBAUM* x and SIDNEY RIEGELMAN‡

Abstract □ Methods were developed for the determination of isoniazid and some of its metabolites in biological fluids. Isoniazid is determined in whole blood by a fluorometric procedure, and isoniazid and hydrazones, acetylisoniazid, isonicotinic acid, and isonicotinuric acid are determined in urine by colorimetry; each method is specific and accurate. Data are presented on these compounds following intravenous doses of isoniazid to both rapid and slow inactivator subjects of isoniazid.

Keyphrases □ Isoniazid and metabolites—fluorometric and colorimetric procedures for determination in biological samples □ Antituberculosis drugs—determination of isoniazid and metabolites in biological fluids □ Fluorometry—determination, isoniazid and metabolites in biological fluids □ Colorimetry—determination, isoniazid and metabolites in biological fluids

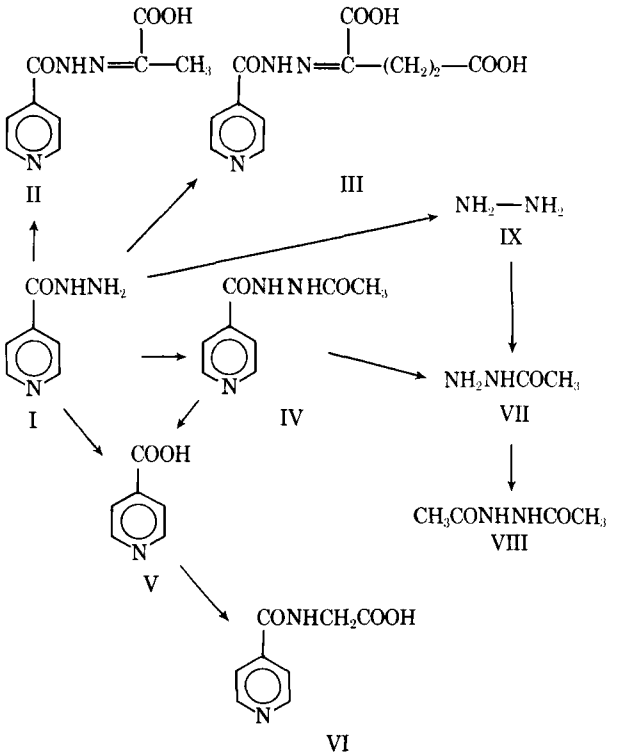
The synthesis of isoniazid (isonicotinic acid hydrazide) was first reported in 1912 (1), but it was not until the early 1950's that three independent research groups simultaneously discovered the marked antituberculosis activity of this compound (2). Presently, isoniazid is considered to be the most effective of the commonly employed antituberculosis drugs (3). Numerous methods have been reported for determining isoniazid and its metabolites in biological fluids (4, 5).

The major urinary excretion products resulting from isoniazid administration to humans are intact isoniazid (I), pyruvic acid isonicotinoylhydrazone (II), α-ketoglutaric acid isonicotinoylhydrazone (III), acetylisoniazid (IV), isonicotinic acid (V), isonicotinuric acid (VI), acetylhydrazine[1] (VII), and 1,2-diacetylhydrazine[1] (VIII) (4, 6). The pathways proposed[1] for the metabolism of isoniazid in humans are shown in Scheme I. Hydrazine (IX) was presumed to be formed from the hydrolysis of isoniazid to isonicotinic acid, but this compound was not detected. Examination of the literature indicates other metabolic routes may exist. Ziporin *et al.* (7, 8) reported an elevation of blood ammonia following isoniazid administration. Additionally, *in vitro* studies in which isoniazid was converted by animal tissue preparations into isonicotinic acid and hydrazine indicated that the hydrazine was subsequently converted to ammonia (9–13).

Schmidt (14) reported that pyruvic acid isonicotinoylhydrazone and α-ketoglutaric acid isonicotinoylhydrazone were cleaved promptly in pH 6–6.5 buffer to free isoniazid and the corresponding keto acids. Taking cognizance of the lability of the hydrazones, Schmidt suggested that their cleavage back to isoniazid in the body is possible. The direct conversion of isoniazid to isonicotinuric acid is suggested by the experiments of Wenzel (15–17). Incubation of isoniazid with glycine, with or without human serum, resulted in the production of isonicotinuric acid. Incubation of isonicotinic acid with glycine under the same conditions did not produce isonicotinuric acid.

Humans have been classified as either rapid or slow inactivators of isoniazid, and this capacity to inactivate (metabolize) isoniazid is a permanent hereditary characteristic (18). Approximately half of the Caucasian and American Negro population are rapid isoniazid inactivators and the other half are slow inactivators. Plasma half-lives in rapid inactivators range from 45 to 80 min; in slow inactivators, the half-lives range from 140 to 200 min. Isoniazid inactivator status results from the capacity to acetylate isoniazid (6).

The purpose of this investigation was to develop specific and accurate methods for the determination



Scheme I—Metabolism of isoniazid in humans

[1] G. A. Ellard and P. T. Gammon, to be published.

of isoniazid and some of its metabolites in biological fluids. In most instances, modifications of previously published methods were used. However, critical details of the methodology were found to be lacking, as was verification of the accuracy of methods in the presence of potentially interfering substances. Methods are reported here for the determination of isoniazid in blood as well as isoniazid plus hydrazones, acetylisoniazid, isonicotinic acid, and isonicotinuric acid in urine. Total isonicotinoyl compounds present in urine were determined by measurement of isonicotinic acid after strong acid hydrolysis. Verification of the utility of these methods is presented.

EXPERIMENTAL

Materials—Isonicotinic acid hydrazide[2], isonicotinic acid[2], barbituric acid[3], and vanillin[2] were purchased. Pyruvic acid isonicotinoylhydrazone and α-ketoglutaric acid isonicotinoylhydrazone were prepared by the method of Wenner (19), acetylisoniazid was prepared by the method of Fox and Gibas (20), isonicotinuric acid was prepared by a modification of the method used by Rohrlich (21) for the preparation of nicotinuric acid, acetylhydrazine was prepared by the method of Kost and Sagitullin (22), and 1,2-diacetylhydrazine was prepared by the method of Turner (23). All compounds were recrystallized or otherwise purified to constant melting point, and elemental analyses were obtained. Verification was obtained that the compounds were greater than 99% pure (24).

The following were used without further purification: chloramine-T[2], potassium cyanide (purified grade)[4], salicylaldehyde (from bisulfite addition compound)[2], 2-mercaptoethanol[2], *p*-dimethylaminobenzaldehyde[2], and hydrazine dihydrochloride[2].

The isobutanol[2] was extracted with equal volumes of 0.10 *N* HCl, 0.10 *N* NaOH, and water.

Apparatus—A fluorescence spectrophotometer[5] equipped with a xenon lamp[6] was used. A spectrophotometer[7] using 1.3-cm (0.5-in.) o.d. × 10.5-cm (4-in.) length colorimeter tubes was used for all colorimetric determinations. When filled with a colored solution absorbing at 600 nm, the absorbance from any two tubes differed by no more than 2.0%. A pH meter[8] was also utilized.

Methods—In each urine assay, standard solutions were prepared in water and carried through the experimental procedure. Water was also carried through the procedure and provided a blank. Unless otherwise stated, for each urine assay the standards and blanks prepared in water and urine resulted in identical calibration curves. For each assay, freshly prepared standards were run concomitantly with the unknowns. Metabolite interferences are reported on a molar basis. Representative calibration curves are described in each section; in every case, the intercept of the regression lines did not differ significantly from zero at the 5% level of significance (30).

Determination of Sum of Isoniazid and Its Hydrazones in Urine—The sum of isoniazid and its hydrazones in urine was determined, since it was demonstrated that the amount of free isoniazid in urine most probably does not reflect the amount of free isoniazid excreted by the kidney. Pyruvic acid, α-ketoglutaric acid, and many other keto-containing molecules are very reactive with isoniazid, forming hydrazones virtually spontaneously in aqueous solutions. Although these hydrazones may be preformed within the body, they are also very labile to hydrolysis at various urinary pH's. When using a concentration of pyruvic acid isonicotinoylhydrazone that might normally occur in the urine, approximately 30% of the pyruvic acid isonicotinoylhydrazone in 0.067 *M* pH 8.00 phosphate buffer was hydrolyzed to isoniazid in 30 min at room temperature (initial concentration of pyruvic acid isonicotinoylhydrazone was 49.7 μmoles/ml). When a freshly collected urine (pH 5.9) was spiked with an amount of pyruvic acid isonicotinoylhydrazone that might normally be present after an isoniazid dose, 35 min at room temperature resulted in approximately 25% hydrolysis to isoniazid. On the basis of these experiments, it seems likely that pyruvic acid isonicotinoylhydrazone in the urinary bladder would undergo hydrolysis. Hence, a measurement of intact urinary isoniazid would not reflect the actual amount excreted from the renal tubules. Therefore, it was decided to measure the sum of isoniazid and its hydrazones in urine.

The method involves the reaction between isoniazid and vanillin to form a colored hydrazone and is modified from the procedures of Peters (25) and Deeb and Vitagliano (26). In this method, a weak acid hydrolysis, modified from the procedures of Hughes *et al.* (27) and Short (28), is used to quantitatively split pyruvic acid isonicotinoylhydrazone and α-ketoglutaric acid isonicotinoylhydrazone to isoniazid.

Urine is diluted so as to contain approximately 4 μg/ml of isoniazid plus hydrazones (expressed in terms of isoniazid), and 5.00 ml is mixed with 1.00 ml of 0.200 *N* HCl in a culture tube. The capped tube is placed in a 52° water bath for 60 min and then cooled to room temperature. One milliliter of 1.00 *N* HCl and 1 ml of 10.0% (w/v) solution of vanillin in 95% ethanol are added. After 10 min the absorbance is determined at 365–370 nm against a blank. The yellow color of urine does add slightly to the absorbance, and a correction for each sample is necessary. For example, a 2 in 10 dilution of blank urine would have an absorbance of approximately 0.3. For many determinations, a common dilution is 3–5 in 100. Consequently, a second sample of diluted urine is also carried through the procedure, but 1.00 ml of 95% ethanol is used in place of the 1.00 ml of vanillin reagent. The absorbance of this solution is measured against its blank, and this absorbance is subtracted from the original absorbance value. Beer's law is obeyed ($r \geq 0.999$) for initial concentrations of isoniazid up to 8 μg/ml ($A \approx 0.35$). Isonicotinic acid, isonicotinuric acid, and 1,2-diacetylhydrazine do not interfere with this method, whereas 1% of the acetylisoniazid present is detected as isoniazid; presumably this results from its hydrolysis. Acetylhydrazine interferes to the extent of 1.8%, while hydrazine (not a urinary metabolite) interferes to the extent of 242%. Substances were present in blank urines that did react as apparent isoniazid, and these substances were equivalent, in a 24-hr period, to approximately 6 mg isoniazid. For a rapid isoniazid inactivator receiving a 700-mg dose of isoniazid, this interference would introduce an error of approximately 3.7%.

Determination of Acetylisoniazid in Urine—A modification of the Peters (29) extraction method is used; acetylisoniazid is extracted from diluted urine into an organic solvent mixture and is subsequently reextracted into dilute hydrochloric acid. Acetylisoniazid is separated from other hydrazino compounds by a modification of the ion-exchange chromatography procedure of Belles and Littleman (30). Unfortunately, the complete metabolic pathways of isoniazid were not known at that time, and the original ion-exchange method was developed solely to separate isoniazid and acetylisoniazid. The possibility that other isoniazid metabolites eluted with the acetylisoniazid was not determined. This is of critical importance because of the potential interference of such metabolites in the subsequent quantitation of acetylisoniazid.

In the present method, urine is diluted so as to contain approximately 50 μg acetylisoniazid/ml, and 3.00 ml is pipeted into a 90-ml prescription bottle containing 3.2 g ammonium sulfate. A 1.00-ml quantity of 0.100 *N* NaOH and 40.0 ml of organic extraction solvent (3:7 isopentyl alcohol–1,2-dichloroethane, water saturated) are added, and this mixture is vigorously shaken for 30 min followed by centrifugation. Thirty-five milliliters of the organic phase (top) is reextracted for 30 min with 6.00 ml of 0.100 *N* HCl and then separated by centrifugation. A 4.00-ml aliquot of the aqueous layer (top) is gently pipeted onto the ion-exchange column.

Prior to use, the resin[9] is soaked in 0.100 *N* HCl for at least 12 hr and then placed into columns. Disposable columns[10], glass barrel, 0.70 cm i.d., cut from 30 to a length of 15.0 cm, were used.

[2] Eastman.
[3] Aldrich.
[4] Mallinckrodt.
[5] Perkin-Elmer model 203.
[6] Perkin-Elmer model 150.
[7] Bausch and Lomb Spectronic 20.
[8] Corning model 10.

[9] AG 50W-X4, 100–200 mesh, hydrogen form, analytical grade cation-exchange resin, Bio-Rad Laboratories, Richmond, Calif.
[10] Bio-Rad.

The resin slurry is added to the column to a height of 3.0 cm, and 25 ml of 0.100 *N* HCl is passed through; if necessary, the resin bed height is then readjusted to 3.0 cm. Immediately prior to use, the 0.100 *N* HCl above the resin bed is permitted to fall to the level of the bed. Columns are prepared no sooner than 3 days prior to the assays, and 0.100 *N* HCl is permitted to remain on top of the resin bed during storage.

Just as the 4 ml of hydrochloric acid extract runs to the level of the resin bed, 4.00 ml of 0.100 *N* HCl is gently pipeted onto the column, and this is also permitted to run through. This hydrochloric acid wash does not elute acetylisoniazid but does remove substances that interfere with the assay. As soon as the 0.100 *N* HCl level runs to the level of the resin bed, a 25-ml volumetric flask is placed under the column to collect effluent. Immediately thereafter, elution solution is run through the column until the 25-ml flask is full. The elution solution consists of an aqueous solution of 0.100 *N* HCl, 0.200 *M* LiCl, 0.1 *M* KCl, and 10.0% (v/v) 95% ethanol. Reservoir caps for the columns, provided by the manufacturer, may be placed on top of the glass barrels to provide for a greater volume and pressure head for the elution solution (these caps hold approximately 7 ml). No attempt is made to control flow rate; however, a rate of approximately 1.5 ml/min is achieved with a maximum pressure head. Four milliliters of the 25.0 ml effluent is added to a calibrated test tube containing 1.00 ml of a freshly prepared solution of Ehrlich's reagent. Ehrlich's reagent is prepared fresh by adding 0.60 g *p*-dimethylaminobenzaldehyde to 20.0 ml of 10.0 *N* HCl. The uncapped tube is placed in a boiling water bath for 2.0 hr, thereby quantitatively converting acetylisoniazid to hydrazine. The solution is cooled to room temperature and made up to a volume of 5.0 ml with 0.100 *N* HCl, and the absorbance is measured at 450 nm. Beer's law is obeyed ($r \geq 0.999$) up to initial acetylisoniazid concentrations of 100 μg/ml ($A \approx 0.92$).

Acetylisoniazid is quantitatively eluted from the column, and the amount recovered in the 25 ml effluent is approximately 54% of that present in the initial 3.00 ml diluted urine. This recovery is a consequence of nonquantitative extraction and the use of aliquots (Fig. 1).

Isoniazid, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, isonicotinic acid, isonicotinuric acid, and 1,2-diacetylhydrazine do not interfere with this determination. Although isoniazid and the hydrazones are extracted, they are not eluted from the column with this procedure. Isonicotinic acid and isonicotinuric acid cannot interfere, since they have no hydrazine moiety. 1,2-Diacetylhydrazine is extracted but elutes prior to the addition of the elution solution. When urines were spiked with hydrazine and acetylhydrazine, these compounds interfered to the extent of approximately 7.8 and 9.0%, respectively. Whereas hydrazine is not found in urine after isoniazid administration, acetylhydrazine is definitely present (5). However, one can estimate the magnitude of this possible error; it will be shown that it results in a maximum error of 5%.

Determination of Isonicotinic and Isonicotinuric Acids in Urine—This two-component colorimetric analysis, modified from the method of Nielsch (31, 32) and Nielsch and Giefer (33), is based upon polymethine dye formation (33). Both isonicotinic acid and isonicotinuric acid form colored dyes: by measuring absorbance at two wavelengths, isonicotinic acid as well as isonicotinuric acid may be simultaneously determined. The original method is modified so as to achieve a greater degree of accuracy and to accommodate the rapid processing of large numbers of samples.

Acetate buffer is prepared according to Nielsch and Giefer (33), which on dilution with 4 parts water yields a solution of pH 5.0. Barbiturate buffer contains approximately 2% barbituric acid adjusted to pH 5.0 with sodium hydroxide. On standing, this solution develops a precipitate which is removed by filtration or decantation before use.

Urine is diluted so that the concentration of isonicotinic acid plus ½ isonicotinuric acid is approximately 10–15 μg/ml; this concentration may not exceed 20 μg/ml. To a 25-ml volumetric flask under a hood, the following are added with shaking in the following sequence: (*a*) 1.25 ml acetate buffer (pH 5.0); (*b*) 0.50 ml of 2% aqueous KCN, prepared fresh under a hood; (*c*) 5.0 ml of 1.0% chloramine-T trihydrate, prepared fresh; (*d*) 0.50 ml of 8.6% (v/v) acetic acid; (*e*) 6.50 ml barbiturate (pH 5.0) buffer; (*f*) 2.50 ml acetone; and (*g*) 1.00 ml unknown solution. By using automatic pipeting devices, 15–20 samples may be processed simultaneously; each reagent is added to all flasks before proceeding to the next reagent. The volume of each flask is brought to 25.0 ml with water, and the flasks are placed in the dark for 1.0 hr. Absorbances are determined at 590 and 625 nm.

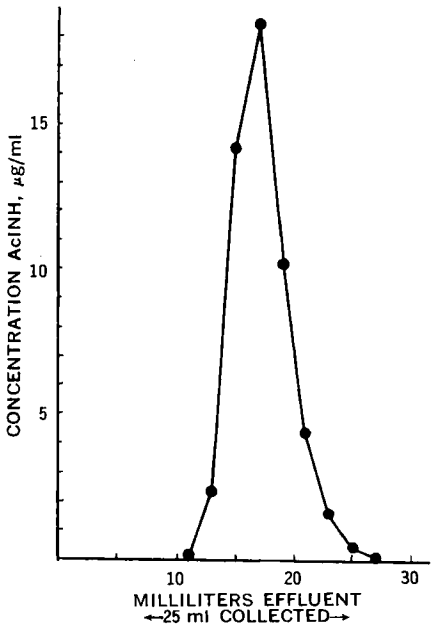


Figure 1—*Elution chromatogram for acetylisoniazid (AcINH).*

An important presumption in the method presented here is that the absorbances from the two components be additive; this presumption was shown to be valid. The absorbance maxima for the polymethine dyes of isonicotinic acid and isonicotinuric acid are 600 and 615 nm, respectively. The wavelengths of 590 and 625 nm were selected because they provided greater accuracy than 600 and 615 nm. To minimize errors, it is important to make all absorbance measurements at one wavelength setting before proceeding to the second setting, particularly since the wavelength settings on the spectrophotometer used are not precisely reproducible. Wavelength settings were selected to provide absorptivities similar to those calculated from Table I; these nominal settings would not be expected, however, to differ by more than about 5 nm from those reported here. Additionally, greater accuracy is achieved when the microgram per milliliter concentration of isonicotinic acid plus ½ isonicotinuric acid is 10 or above. The ratio of isonicotinic acid to isonicotinuric acid in a sample should be less than 4 and greater than 0.25 or accuracy in the determination of the lesser component is significantly reduced. Standard solutions of isonicotinic acid (10, 15, and 20 μg/ml) and isonicotinuric acid (20, 30, and 40 μg/ml) are carried through the procedure. Isonicotinic acid and isonicotinuric acid concentrations are determined by solving two simultaneous equations with two unknowns (33). Table I shows typical data from calibration curves.

Isoniazid, acetylisoniazid, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, hydrazine, acetylhydrazine, and 1,2-diacetylhydrazine do not interfere with this determination.

Determination of Total Isonicotinoyl Compounds in Urine (Total Isonicotinic Acid)—Total isonicotinic acid includes isonia-

Table I—Absorbances from Isonicotinic Acid and Isonicotinuric Acid from Typical Calibration Curves[a]

Absorbance[b]			
Isonicotinic Acid (590 nm)	Isonicotinic Acid (625 nm)	Isonicotinuric Acid (590 nm)	Isonicotinuric Acid (625 nm)
0.410 (0.9999)	0.103 (0.9991)	0.120 (0.9997)	0.135 (0.9994)

[a] Concentration of each component was 10 μg/25 ml final solution. [b] Values in parentheses indicate correlation coefficient of calibration curve.

zid, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, acetylisoniazid, isonicotinic acid, and isonicotinuric acid and is determined by a modification of the method of Peters (25). The principle of the method is to convert quantitatively all the aforementioned compounds to isonicotinic acid by strong acid hydrolysis. The isonicotinic acid is subsequently measured colorimetrically. The original Peters procedure utilizes hydrochloric acid as an acid catalyst. As will be discussed subsequently, this method consistently underestimates total isonicotinoyl compounds in urine.

In the modified procedure, urine is diluted so as to contain approximately 30–120 μg isonicotinoyl compounds/ml (expressed in terms of isonicotinic acid). Two milliliters of this solution is added to a 5-ml ampul containing 2.00 ml of 10.0 *N* H_2SO_4, and the ampul is sealed. The ampul is placed in an autoclave at 125–130° (27 psig) for 3.0 hr, and 1.00 ml of hydrolysate is neutralized with 5.00 ml of 1.00 *N* NaOH. Alternatively, 2.00 ml of hydrolysate may be neutralized with 5.00 ml of 2.00 *N* NaOH. An appropriate aliquot of the neutralized solution is subsequently used for isonicotinic acid determination. The colorimetric method is the same as that described for isonicotinic acid and isonicotinuric acid, except that the wavelength is 600 nm. One milliliter each of isonicotinic acid solutions (5, 10, 15, and 20 μg/ml) is used to prepare standards. Beer's law is obeyed ($r \geq 0.999$) up to 20 μg/25 ml final volume ($A \approx 0.88$).

With this procedure, all isonicotinoyl compounds are quantitatively converted to isonicotinic acid. While hydrochloric acid is entirely suitable for hydrolysis of aqueous isonicotinoyl solutions, it does not result in accurate quantitation of total isonicotinoyl compounds when urinary constituents are present; in such situations, recoveries of approximately 90–95% are obtained. The reason for this anomaly is unclear, since urines spiked with isonicotinic acid are not affected. One possibility is that a product other than isonicotinic acid is formed during the hydrolysis period.

The use of an autoclave is essential; placing these ampuls in a 130° oven frequently resulted in violent explosions.

Determination of Isoniazid in Whole Blood—The method used was modified from that of Scott and Wright (34) and is based upon formation of the isoniazid–salicylaldehyde hydrazone. The reduced form of the hydrazone, extracted into isobutanol, is highly fluorescent. Bisulfite is used to react with excess salicylaldehyde, thus making it nonfluorescent. Unfortunately, the method presented here requires precise handling of samples and completion of the total assay within 24 hr.

The salicylaldehyde–acetic acid reagent, zinc sulfate solution, and barium hydroxide solution are prepared fresh according to Scott and Wright (34). The acetate buffer consists of 0.120 *N* NaOH and 0.385 *M* sodium acetate; sufficient acetic acid or sodium hydroxide is added to this buffer so that 1.30 ml of buffer, 1.50 ml of water, and 0.40 ml of salicylaldehyde–acetic acid reagent result in a solution of pH 5.60 ± 0.05.

A 0.5-ml quantity of freshly drawn, heparinized blood is immediately placed in an acid-washed tube (Teflon cap) containing 15.0 ml of distilled water. The blood is permitted to hemolyze for about 1 min, after which time 1.00 ml of the zinc sulfate solution is added. The tube is vigorously shaken by hand for 5 sec; then 1.00 ml of barium hydroxide solution is added. After shaking vigorously for 5 sec, the mixture is immediately filtered through Whatman No. 1 filter paper into a thick-walled Pyrex culture tube (acid washed, Teflon-lined cap) resting in an ice water bath. The clear, colorless filtrate is immediately frozen by immersing in a dry ice–isopropanol mixture for 2 min and stored at −18°. Frozen filtrates gradually deteriorate; therefore, within 24 hr from the time of freezing, the filtrate is thawed and brought to room temperature in a 50° water bath; 1.50 ml of filtrate is mixed with 0.40 ml of salicylaldehyde–acetic acid reagent in an acid-washed tube with a Teflon-lined cap and is kept at room temperature for 20 min. After that reaction period, 1.35 ml of 2-mercaptoethanol–bisulfite buffer is added and the capped tube is placed in a 50° water bath for 10 min. The 2-mercaptoethanol–bisulfite buffer is prepared fresh by adding 1.92 ml of 2-mercaptoethanol (stench!) to 50.0 ml of acetate buffer containing 77.0 mg of sodium bisulfite (total volume approximately 51.92 ml).

The tube is cooled to room temperature by immersing in an ice water bath for 15 sec, and 4.00 ml isobutanol (water saturated) is added. The tube is vigorously shaken by hand for 5 sec to extract the reduced form of the hydrazone into the isobutanol. The tube is subsequently placed in an ice water bath for 5 min and is centrifuged for 5 min at 2400 rpm (without prior chilling, the heat generated by centrifugation causes turbidity). The top isobutanol layer is removed and fluorescence is determined (activation wavelength 392 nm, emission wavelength 478 nm). With the instrument used, the blank is set to 0 units and the standard of maximum concentration is set to 100 units. With a wide concentration range of unknowns, several calibration curves of limited range must be prepared (*e.g.*, 0.05–1 μg isoniazid/ml blood and 1–30 μg isoniazid/ml blood). In the examples just cited, linear curves were obtained with correlation coefficients of greater than 0.999. The lower limit of sensitivity is approximately 0.01 μg isoniazid/ml blood, whereas the upper limit of measurability is greater than 30 μg isoniazid/ml blood. Standards are prepared by having the requisite amount of isoniazid in the 15.0 ml of distilled water and adding 0.50 ml of blood.

Blood must be obtained from the subject receiving the isoniazid, and this should be drawn just prior to injection. In this way, standards are prepared immediately before the start of the experiment and carried through the procedure with the samples.

Isonicotinic acid, isonicotinuric acid, hydrazine, acetylhydrazine, 1,2-diacetylhydrazine, and acetylisoniazid do not interfere with this determination. The hydrazones α-ketoglutaric acid isonicotinoylhydrazone and pyruvic acid isonicotinoylhydrazone interfere to the extent of 1.1 and 7.8%, respectively. The potential for interference from hydrazones will be discussed.

RESULTS AND DISCUSSION

Potentially Interfering Substances in Assay Procedures—The assay of acetylisoniazid in urine requires one to hydrolyze the compound to hydrazine. The presence of acetylhydrazine in the sample can lead to an error since it also results in the formation of hydrazine. An estimation of the error resulting from the acetylhydrazine interference was calculated from the data of Ellard and Gammon[1]. These investigators administered 190 mg monoacetylhydrazinium fumarate orally to a slow isoniazid inactivator subject and recovered 44.5% of the dose as 1,2-diacetylhydrazine in the urine; although urinary acetylhydrazine could not be determined accurately, the maximum amount that could be excreted was 55.5% of the oral dose. This information may be applied to the data reported elsewhere (24).

After intravenous infusion of 670 mg of isoniazid to Subject B (rapid acetylator), equimolar cumulative amounts of acetylisoniazid and isonicotinic acid derivatives (isonicotinic acid and isonicotinuric acid) were excreted in the urine. Since isonicotinic acid and isonicotinuric acid do not contain a hydrazine moiety, their former hydrazine content would necessarily have to be accounted for elsewhere. For this calculation, it was assumed that these hydrazine moieties were solely converted to acetylhydrazine and 1,2-diacetylhydrazine. If the 55.5% figure of Ellard and Gammon is used, the ratio of cumulatively excreted acetylisoniazid–acetylhydrazine is 1:0.555. Since only 9.0% of acetylhydrazine is detected as acetylisoniazid, 0.05 mole (0.555 × 0.09) acetylhydrazine is detected as acetylisoniazid for each mole of acetylisoniazid actually excreted. This indicates that the contribution of acetylhydrazine to the acetylisoniazid assay results in a maximum 5% error. In rapid acetylator subjects, more acetylhydrazine is converted to 1,2-diacetylhydrazine, and the error would be even smaller. Conversion of the hydrazine moieties to ammonia would reduce the error to a greater extent.

Another measure of the error in the acetylisoniazid determination is provided by the cumulative urinary excretion data after intravenous administration of acetylisoniazid to a slow acetylator volunteer (Subject A) who received 587 mg by constant infusion over 60 min; urine pH was maintained at 8.0 ± 2.0 by administration of sodium bicarbonate (35), and urine was collected for 34 hr. The cumulative excretion of acetylisoniazid, isonicotinic acid, and isonicotinuric acid was 586 mg. Total isonicotinoyl compounds accounted for 585 mg. If there were a significant interference from acetylhydrazine in the acetylisoniazid determination, the sum of the urinary components would exceed total isonicotinoyl compounds; this did not occur.

In the assay of isoniazid in whole blood, there is a potential for interference from hydrazones that are converted in the processing into isoniazid. To quantitate the interference from hydrazones in these experiments, a study was undertaken with Subject A (slow

Table II—Experimental Trial of Urinary Assay Methods

Compound	Percent of Theoretical Amount Found	
	Rapid Inactivator Urine	Slow Inactivator Urine
Isoniazid plus hydrazones	99.6	98.0
Acetylisoniazid	96.9	97.0
Isonicotinic acid	95.6	97.9
Isonicotinuric acid	101	95.4
Total isonicotinoyl compounds	98.8	98.8

acetylator) with urine pH maintained at 8 by sodium bicarbonate administration (35). A dose of 670 mg isoniazid was infused at a constant rate over 5.48 min. Blood was drawn at approximately 50-min intervals for 550 min; assays were performed on each sample for isoniazid by the fluorometric method and for isoniazid plus hydrazones by a modification (24) of the vanillin colorimetric method of Deeb and Vitagliano (26). Isoniazid hydrazones were first hydrolyzed to isoniazid in the latter method. The concentrations at each time point were analyzed by the t test for paired observations (36); at a 5% level of significance, the concentrations were not identical, indicating that hydrazones were present in blood. By assuming that pyruvic acid isonicotinoylhydrazone was the only hydrazone in blood, the concentration of isoniazid and pyruvic acid isonicotinoylhydrazone in each sample was calculated by solving two equations with two unknowns:

V = 100% isoniazid concentration + 100% pyruvic acid isonicotinoylhydrazone concentration (Eq. 1)

F = 100% isoniazid concentration + 7.8% pyruvic acid isonicotinoylhydrazone concentration (Eq. 2)

where V = concentration determined by vanillin colorimetric method, and F = concentration determined by fluorometric method.

The two coefficients of 100% in Eq. 1 indicate that 100% of the isoniazid and 100% of the pyruvic acid isonicotinoylhydrazone are detected by the vanillin colorimetric method. The coefficients of 100 and 7.8% in Eq. 2 indicate that the fluorometric method measures 100% of the isoniazid present but only 7.8% of the pyruvic acid isonicotinoylhydrazone. Since these are two simultaneous equations with two unknowns, isoniazid and pyruvic acid isonicotinoylhydrazone concentrations are solvable.

The maximum concentration of pyruvic acid isonicotinoylhydrazone in any sample was 19.9% of the concentration of isoniazid present. The interference in the fluorometric method would be 7.8% of that value, for an approximate overall 1.6% error in the isoniazid blood level. The average blood level of pyruvic acid isonicotinoylhydrazone was calculated at 4.65% of the isoniazid blood level (average error of isoniazid by fluorometry approximately 0.36%). Russell (37) reported a similar finding in subjects receiving isoniazid; in five subjects, hydrazone blood levels averaged 15.5% of the isoniazid blood levels.

The original Scott and Wright (34) method was modified only slightly, in hope of eliminating the slight interference from the hydrazones. The hydrazones, however, interfere slightly in both methods, and this interference has been shown to be virtually insignificant due to the low levels of hydrazones in blood. The Scott and Wright (34) procedure or possibly the modification of Ellard *et al.* (5) are consequently preferred over the present method due to the instability problem inherent in the present method.

Experimental Trial of Urine Assay Methods—The data of Peters *et al.* (6) were utilized to prepare spiked urines containing isoniazid, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, acetylisoniazid, isonicotinic acid, isonicotinuric acid, and 1,2-diacetylhydrazine (at the time of these studies, acetylhydrazine had not been established as a urinary metabolite). 1,2-Diacetylhydrazine was added in equal molar concentration to the sum of isonicotinic acid and isonicotinuric acid. The urines were prepared in such a manner as to mimic what might be expected in the 24-hr urines of rapid and slow isoniazid inactivators who were administered a 1.00-g iv isoniazid dose; a urine flow rate of 1.00 ml/min was assumed.

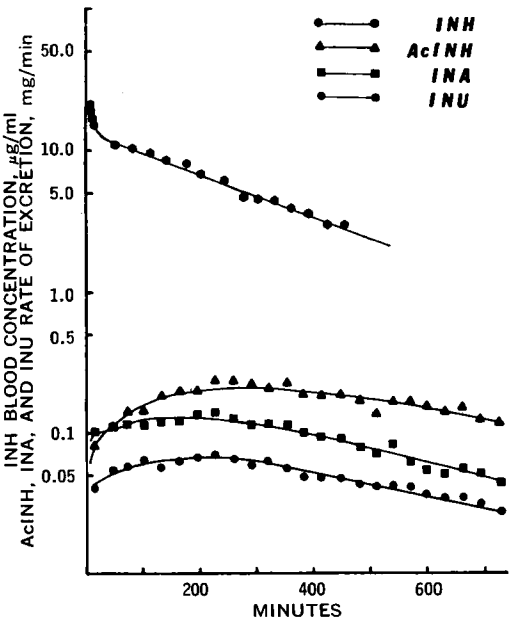


Figure 2—*Isoniazid blood levels and rates of urinary excretion of acetylisoniazid (AcINH), isonicotinic acid (INA), and isonicotinuric acid (INU) following intravenous administration of isoniazid (INH) to slow inactivator Subject A (all compound amounts corrected to correspond to mole equivalents of INH).*

The rapid inactivator urine contained the following molar percentages: isoniazid, 3.6; pyruvic acid isonicotinoylhydrazone, 2.9; α-ketoglutaric acid isonicotinoylhydrazone, 1.4; acetylisoniazid, 43.8; isonicotinic acid, 27.1; isonicotinuric acid, 21.3; and 1,2-diacetylhydrazine, 48.4. The slow inactivator urine contained the following molar percentages: isoniazid, 12.7; pyruvic acid isonicotinoylhydrazone, 13.9; α-ketoglutaric acid isonicotinoylhydrazone,

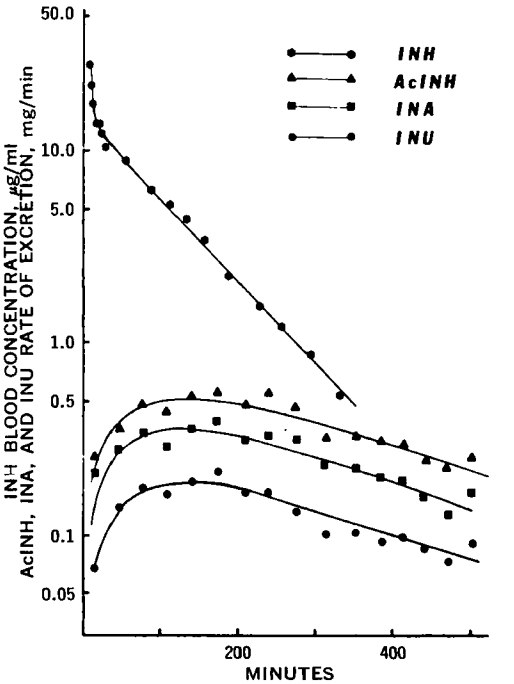


Figure 3—*Isoniazid blood levels and rates of urinary excretion of acetylisoniazid (AcINH), isonicotinic acid (INA), and isonicotinuric acid (INU) following intravenous administration of isoniazid (INH) to rapid inactivator Subject B (all compound amounts corrected to correspond to mole equivalents of INH).*

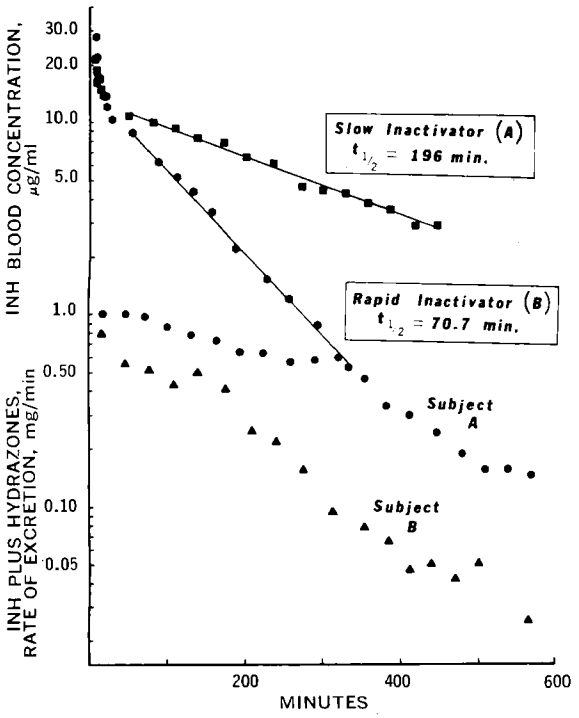


Figure 4—*Isoniazid blood levels and rates of urinary excretion of isoniazid (INH) plus hydrazones in rapid and slow inactivator subjects following intravenous isoniazid administration (all compound amounts corrected to mole equivalents of INH).*

6.9; acetylisoniazid, 34.3; isonicotinic acid, 18.1; isonicotinuric acid, 14.2; and 1,2-diacetylhydrazine, 32.3. Each urine assay was performed on these samples. On the basis of these results (Table II), it was concluded that the asays are valid. Acetylhydrazine interferes somewhat in the acetylisoniazid determination, as previously discussed.

Additionally, it was demonstrated from urine collected after isoniazid administration that these urine assays gave identical results when the urine was assayed shortly after collection and after 6.0 months of storage at −18°.

Isoniazid Administration Studies—Isoniazid was administered intraveneously to two human subjects in apparent good health. Subject A (Caucasian male, age 29 years, weight 69.0 kg, height 165 cm) was a slow inactivator who received 681 mg infused at a constant rate over 5.40 min. Subject B (Caucasian male, age 50 years, weight 80.0 kg, height 181 cm) was a rapid inactivator who received 670 mg infused at a constant rate over 5.20 min. Urine pH was maintained at pH 8.0 ± 0.2 in each study by oral administration of sodium bicarbonate (35) to eliminate the possibility of fluctuations in urinary excretion rates due to changes in urinary pH.

Figures 2 and 3 show isoniazid blood curves as well as rates of excretion curves for acetylisoniazid, isonicotinic acid, and isonicotinuric acid for Subjects A and B, respectively. Figure 4 compares the isoniazid blood curves to the rates of excretion curves for the sum of isoniazid and hydrazones. The slow inactivator subject had an isoniazid half-life of 196 min compared to 70.7 min for the rapid inactivator. The rapid decline of isoniazid blood concentrations during the first 50 min occurred during the so-called distributive phase. In separate experiments, without urine pH control, Subject A received 1.00 g isoniazid orally and Subject B received 350 mg isoniazid orally. The method of Scott and Wright (34) was used to determine isoniazid serum levels, and the half-lives were 148 and 69.9 min for Subjects A and B, respectively.

The validity of the urinary assay methods was verified in the two intravenous isoniazid administration studies by comparing the sum of isoniazid plus hydrazones, acetylisoniazid, isonicotinic acid, and isonicotinuric acid as determined by each individual method to total isonicotinoyl compounds, determined subsequent to strong acid hydrolysis. Theoretically, these results should be equal (Table III), and good agreement is observed.

Table III—Comparison of Sum of Urinary Isoniazid plus Hydrazones, Acetylisoniazid, Isonicotinic Acid, and Isonicotinuric Acid as Determined by Each Method to Total Isonicotinoyl Compounds Determined Subsequent to Strong Acid Hydrolysis

Minutes after Start of Injection	Sum of Isoniazid plus Hydrazones, Acetylisoniazid, Isonicotinic Acid, and Isonicotinuric Acid, (mg)	Total Isonicotinoyl Compounds, mg	Difference[a], %
Subject A (Slow Inactivator)			
35.5	44.45	50.1	+11.3
59.1	30.44	33.7	+9.67
88.5	37.75	39.9	+5.39
116.0	32.27	32.0	−0.844
150.0	38.71	38.1	−1.60
181.3	34.76	33.6	−3.45
208.7	28.41	27.1	−4.83
243.5	37.21	36.2	−2.79
276.3	32.59	33.2	+1.84
305.0	27.92	27.3	−2.27
339.3	33.69	32.0	−5.28
370.3	26.76	26.6	−0.602
396.0	17.18	16.5	−4.12
430.2	21.24	20.0	−6.20
466.0	20.23	20.0	−1.15
496.0	14.26	13.5	−5.63
524.0	11.32	10.4	−8.85
554.0	13.38	13.1	−2.14
586.0	13.18	12.7	−3.78
613.0	11.202	12.2	+8.18
648.0	12.82	11.6	−10.52
677.0	11.396	10.6	−7.51
710.0	10.68	9.25	−15.46
747.0	10.074	9.44	−6.72
Subject B (Rapid Inactivator)			
32.0	42.1	45.9	+8.28
62.0	39.9	43.3	+7.85
94.0	48.1	51.8	+7.14
126.0	42.3	43.4	+2.53
158.0	49.9	51.7	+3.48
195.0	57.7	59.9	+3.67
227.0	38.5	38.8	+0.773
259.0	40.5	39.7	−2.02
296.0	39.4	42.1	+6.41
335.0	29.0	29.6	+2.03
373.0	28.0	28.7	+2.44
400.0	17.9	18.5	+3.24
426.0	16.5	17.1	+3.51
458.0	17.3	17.2	−0.581
486.0	13.0	12.5	−4.00
516.0	16.8	17.0	+1.18

[a] Calculated from:

$$\frac{\left(\begin{array}{c}\text{total isonicotinoyl}\\ \text{compounds}\end{array}\right) - \left(\begin{array}{c}\text{sum of isoniazid plus}\\ \text{hydrazones, acetylisoniazid,}\\ \text{isonicotinic acid, and}\\ \text{isonicotinuric acid}\end{array}\right)}{\text{(total isonicotinoyl compounds)}} \times 100$$

Pharmacokinetic analyses will be reported at a later date.

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[x] To whom inquiries should be directed. American Foundation for Pharmaceutical Education Fellow, September 1967–September 1970. Present address: Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche, Inc., Nutley, NJ 07110

Interconversion of Ampicillin and Hetacillin *In Vitro*

GERALD N. LEVY, JOHN V. IOIA, and EDWARD J. KUCHINSKAS[x]

Abstract □ The conversion of hetacillin to ampicillin in aqueous solution was studied by hydroxylamine assay, IR analysis, and ^{14}C-labeling methods. Each technique indicates a half-life of at least 4 hr for hetacillin at physiological pH. The interconversion of ampicillin and hetacillin in aqueous solutions can be controlled by the concentration of added acetone. Excess acetone leads to the formation of hetacillin in aqueous solutions of ampicillin. A scheme for this interconversion is proposed, which accounts for the production of a Schiff base intermediate and penicillenic acid.

Keyphrases □ Hetacillin and ampicillin interconversion in aqueous solution—mechanism, effect of acetone concentration, determination by hydroxylamine assay, IR analysis, and ^{14}C-labeling □ Ampicillin and hetacillin interconversion in aqueous solution—mechanism, effect of acetone concentration, determination by hydroxylamine assay, IR analysis, and ^{14}C-labeling

Ampicillin and hetacillin degrade to different products in aqueous solution. Concentrated solutions of ampicillin form polymers while similar solutions of hetacillin do not produce polymers of the ampicillin type but lead instead to a penicillenic acid with a characteristic UV absorbance peak at 317 nm (1). In a previous report (2), the finding of different degradative pathways for ampicillin and hetacillin in solution indicated that hetacillin could not be rapidly and completely converted to ampicillin and acetone

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[x] To whom inquiries should be directed. American Foundation for Pharmaceutical Education Fellow, September 1967–September 1970. Present address: Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche, Inc., Nutley, NJ 07110

Interconversion of Ampicillin and Hetacillin *In Vitro*

GERALD N. LEVY, JOHN V. IOIA, and EDWARD J. KUCHINSKAS[x]

Abstract □ The conversion of hetacillin to ampicillin in aqueous solution was studied by hydroxylamine assay, IR analysis, and ^{14}C-labeling methods. Each technique indicates a half-life of at least 4 hr for hetacillin at physiological pH. The interconversion of ampicillin and hetacillin in aqueous solutions can be controlled by the concentration of added acetone. Excess acetone leads to the formation of hetacillin in aqueous solutions of ampicillin. A scheme for this interconversion is proposed, which accounts for the production of a Schiff base intermediate and penicillenic acid.

Keyphrases □ Hetacillin and ampicillin interconversion in aqueous solution—mechanism, effect of acetone concentration, determination by hydroxylamine assay, IR analysis, and ^{14}C-labeling □ Ampicillin and hetacillin interconversion in aqueous solution—mechanism, effect of acetone concentration, determination by hydroxylamine assay, IR analysis, and ^{14}C-labeling

Ampicillin and hetacillin degrade to different products in aqueous solution. Concentrated solutions of ampicillin form polymers while similar solutions of hetacillin do not produce polymers of the ampicillin type but lead instead to a penicillenic acid with a characteristic UV absorbance peak at 317 nm (1). In a previous report (2), the finding of different degradative pathways for ampicillin and hetacillin in solution indicated that hetacillin could not be rapidly and completely converted to ampicillin and acetone

Table I—Hydroxylamine Color of Penicillins

Compound	A_{515nm}/mg/ml
Potassium penicillin G	0.350
6-Aminopenicillanic acid	0.385
Potassium ampicillin	0.350
Potassium hetacillin	0.170

as had been claimed (3). In the present study, the stability of hetacillin solutions and the rate and manner of appearance of their degradation products were examined. Preliminary studies were reported earlier (2).

EXPERIMENTAL[1]

Ampicillin trihydrate, potassium ampicillin, hetacillin, and potassium hetacillin were obtained from a commercial source[2]. Acetone-2-^{14}C was also obtained commercially[3].

Hydroxylamine assays were performed using the reagents described previously (4). The method was modified by extending the reaction time of the penicillin with neutral hydroxylamine to 20 min before adding ferric ammonium sulfate. This modification produced higher and more consistent color values with the penicillins tested than did the shorter 3-min incubation originally described.

IR determination of intact hetacillin was based on the ratio of absorbances at 1720 (imidazolidinone carbonyl) and 1790 (β-lactam carbonyl) cm^{-1} (5). In this way the amount of intact β-lactam served as an internal standard for the determination of the γ-lactam (imidazolidinone). For this part of the study, 0.1% solutions of hetacillin were prepared in 0.05 *M* K-PO$_4$ buffer (pH 7.4) and subjected to aeration by nitrogen for various times. The solutions were then frozen, lyophilized, and examined as KBr pellets.

Hetacillin labeled with ^{14}C at the 2-position of the imidazolidinone ring was prepared by reacting ampicillin with acetone-2-^{14}C by a modification of the procedure of Hardcastle *et al.* (5). Excess ^{14}C-acetone was removed by washing with nonradioactive acetone and by vacuum drying of the product. The structure of the labeled hetacillin was verified by comparison of the IR spectrum with that obtained from a commercial source[2]. The hydrolysis of ^{14}C-hetacillin was followed by bubbling nitrogen through a 0.1% solution of ^{14}C-hetacillin in 0.05 *M* K-PO$_4$ (pH 7.4). At intervals, a 50-μl aliquot was withdrawn and counted by liquid scintillation spectrometry. Control experiments showed that the amounts of acetone included in the samples had no observable quench. All aging and gas-flushing experiments were conducted at 25°.

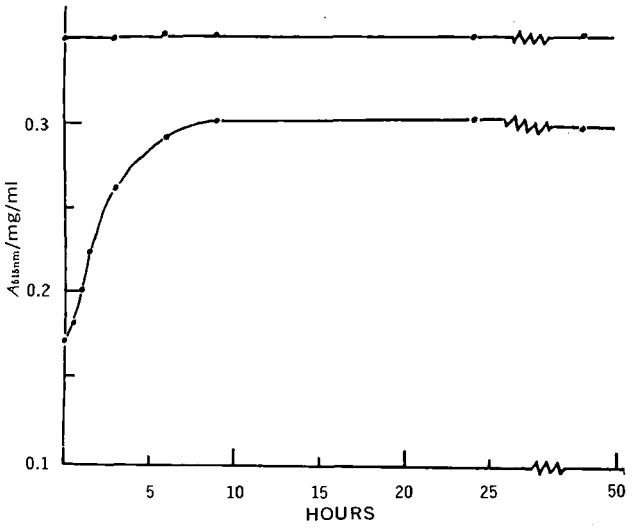


Figure 1—*Development of hydroxylamine color with time. Aqueous solutions of potassium ampicillin (upper curve) and potassium hetacillin (lower curve) in concentrations of 1 mg/ml. Each experimental point is the average of at least three determinations.*

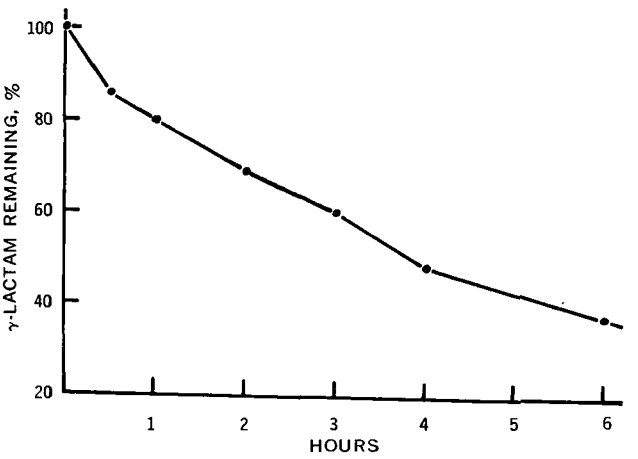


Figure 2—*IR analysis of samples from a hetacillin solution bubbled with nitrogen gas (see* Experimental*). Each data point is a separate flushing experiment for the specified time.*

RESULTS

Stability of Hetacillin—Solutions of hetacillin showed a lower reactivity with hydroxylamine than did solutions of ampicillin, penicillin G, or 6-aminopenicillanic acid. Table I shows that freshly dissolved hetacillin produced less that half the absorbance of other penicillins in the hydroxylamine assay.

When the 0.1% hetacillin solution was allowed to remain at room temperature and aliquots were removed at various times for assay, the color produced increased and approached the ampicillin color value. It was also found that 0.1% ampicillin solutions showed no significant change in their hydroxylamine color value. Figure 1 shows the relationship of the hydroxylamine color value of hetacillin and ampicillin with time.

IR determination of the γ-lactam showed a slow disappearance of 1720-cm^{-1} absorbance. The values for 1720-cm^{-1} absorbance were normalized by taking the ratio of 1720- to 1790-cm^{-1} absorbances. This provides an internal correction for any slight loss of the β-lactam. The decrease in this ratio is shown in Fig. 2.

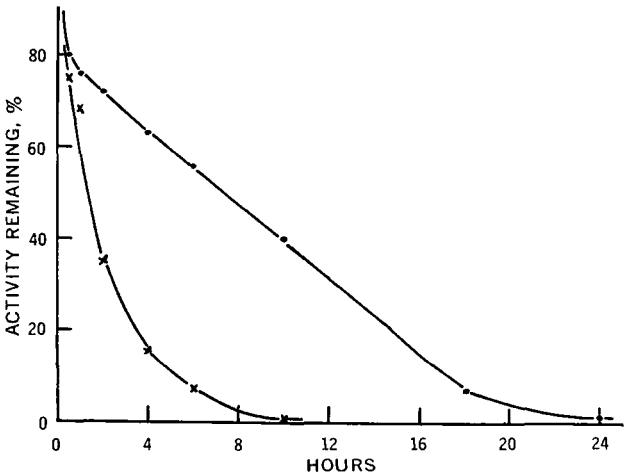


Figure 3—*Comparison of the loss of radioactivity from solutions of ^{14}C-hetacillin (●) and control solutions of ^{14}C-acetone (×) in a stream of nitrogen gas (see* Experimental*). The data shown are typical individual experimental runs performed at least three times.*

[1] IR spectral analyses were performed on KBr pellets using a Perkin-Elmer 21. UV spectra were obtained with a Cary 14, and additional UV measurements were made on a Beckman DU. Radioactivity was determined with a Packard Tri-Carb liquid scintillation counter. NMR spectra were obtained using a Varian A-60 spectrophotometer.

[2] Bristol-Myers Co., International Division.

[3] New England Nuclear.

The loss of radioactivity of ^{14}C-hetacillin is shown in Fig. 3. The volatilization and loss of ^{14}C-acetone from this buffer are also shown as a control. Acetone is removed from the solution far more quickly when free acetone is present than when acetone is chemically combined as hetacillin. The large difference in the loss of radioactivity between solutions of ^{14}C-acetone and ^{14}C-hetacillin indicates that hydrolysis of hetacillin to acetone and ampicillin is not an immediate result of solution.

The results of the three methods used to study the breakdown of hetacillin are compared in Fig. 4. The hydroxylamine method and IR measurements are in close agreement, giving a half-life for hetacillin of 3.5–4 hr. The loss of radioactive acetone was slower than the loss of the γ-lactam. The loss of one-half of the initial acetone required more than 7 hr.

Formation of Penicillenic Acid—A significant difference between solutions of ampicillin and hetacillin is the formation of 317-nm-absorbing material from hetacillin (1). In an attempt to produce UV-absorbing material from ampicillin, various amounts of acetone were added to 10% potassium ampicillin in water. The rate of formation and the final amount of penicillenic acid produced varied with the molar ratio of acetone to ampicillin. The form of this relationship is shown in Fig. 5. In the absence of acetone, ampicillin solutions did not produce any 317-nm absorbance in 24 hr. As the acetone concentration was increased, 317-nm absorption at 24 hr increased. The production of penicillenic acid from ampicillin reached a maximum at a molar ratio of about 4 acetone to 1 ampicillin. At higher concentrations of acetone, this production decreased in both rate and final amount. Similar experiments with hetacillin and acetone showed that adding acetone to 10% aqueous potassium hetacillin resulted in increased production of 317-nm material at 24 hr. Higher concentrations of acetone resulted in a decrease in 317-nm absorbance (Fig. 5).

The time course of the reaction leading to penicillenic acid is illustrated in Fig. 6. The early rate (up to about 2 hr) of production of A_{317nm} material by ampicillin and acetone is less than both the later rate and the rate of production by hetacillin and acetone. In the latter situation, a quick burst of 317-nm-absorbing material appeared within the 1st hr, after which the 317-nm absorbance increased at a somewhat slower rate.

Formation of Hetacillin from Ampicillin in Aqueous Solution—The material formed by incubating ampicillin with acetone in aqueous solution for 48 hr was able to react with neutral hydroxylamine. The amount of 515-nm absorbance produced after 48 hr of incubation was a function of the acetone to ampicillin ratio. Figure 7 shows that as the ratio of acetone to ampicillin was increased, the color value of the solution in the hydroxylamine assay decreased from the value for pure ampicillin to a value (at 20 acetone to 1 ampicillin) that equaled the color value of fresh hetacillin.

A decrease in hydroxylamine assay value could indicate either a loss of the β-lactam or a conversion to a less reactive β-lactam compound. To determine the identity of the material formed from ampicillin and acetone, an aqueous solution of 10% potassium ampicillin with 20 equivalents of acetone was incubated for 48 hr. At the end of the incubation time, the solution was frozen and lyophilized. Samples of the dry powder were examined by IR and NMR and found to give spectra identical to potassium hetacillin. A sample of this material was assayed with neutral hydroxylamine and gave the low extinction of hetacillin. On aging, the color produced by this solution increased to approach the ampicillin value in about 24 hr. Figure 7 shows the increase in color for the hetacillin formed in this way. The rate of conversion of this hetacillin back to ampicillin is very near that shown for hetacillin in Fig. 1.

DISCUSSION

Solutions of hetacillin in water or dilute buffer do not undergo a rapid and complete hydrolysis to ampicillin and acetone. As shown in Fig. 4, the hydrolysis of the γ-lactam of hetacillin has a half-life of nearly 4 hr at an initial concentration of 1 mg/ml and pH 7.4. The loss of IR absorbance characteristic of the γ-lactam carbonyl closely matches an increase in the reactivity of the β-lactam toward neutral hydroxylamine. These results do not distinguish between the conversion of hetacillin to ampicillin or to an intermediate compound. However, the formation of an intermediate with a significant lifespan is indicated by the slow loss of

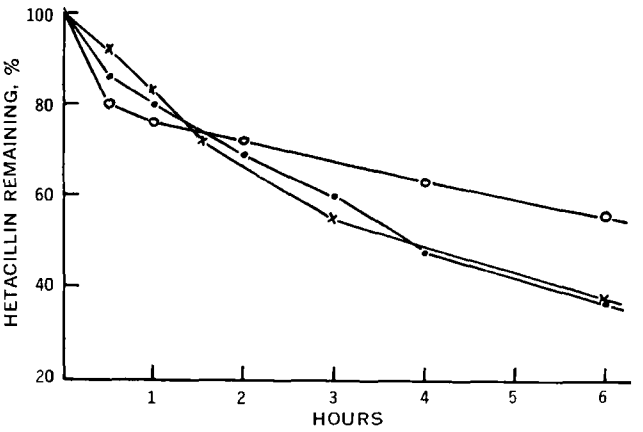


Figure 4—*Correlation of hydroxylamine assay* (×), *IR analysis* (●), *and loss of* ^{14}*C activity* (○) *in terms of remaining hetacillin. (Data derived from experiments of Figs. 1–3.)*

acetone by ^{14}C-hetacillin. The half-life for the loss of the labeled acetone group from ^{14}C-hetacillin is nearly 8 hr.

The intermediate compound formed in the degradation of hetacillin is probably the Schiff base postulated previously (1). This assumption is based on the known participation of the Schiff base in the conversion of free amino compounds to dimethylimidazolidinones (6) and on the direct evidence for the formation of Schiff base in the ampicillin–hetacillin reaction in NMR studies (7).

Since ampicillin does not undergo a penicillenic acid rearrangement to produce 317-nm-absorbing material, the appearance of 317-nm absorbance in solutions of ampicillin with added acetone indicates the presence of either hetacillin or the blocking of ampicillin's free amino group by reaction with acetone as the Schiff base. At high ratios of acetone to either penicillin, the formation of penicillenic acid can be completely blocked. This indicates that it is the Schiff base and not hetacillin that actually undergoes the rearrangement to 317-nm material. If hetacillin degraded directly to the oxazolone compound (penicillenic acid), the presence of excess acetone would have little effect on 317-nm absorbance. However, Figs. 5 and 6 show that excess acetone reduces the formation of penicillenic acid from hetacillin and the formation of penicillenic acid from ampicillin. Figure 6 demonstrates that the rate profiles of penicillenic acid formation differ, depending on whether the starting material is hetacillin or ampicillin.

Beginning with ampicillin and acetone, there is a lag period before the maximum rate of penicillenic acid production is reached. This lag may be accounted for by the formation of the Schiff base intermediate, which can then undergo rearrangement to the 317-nm-absorbing material. In contrast, when the reaction begins with hetacillin or with hetacillin and acetone, the required intermediate is formed by molecular rearrangement rather than by a bimolecular reaction. The more rapid formation of the intermedi-

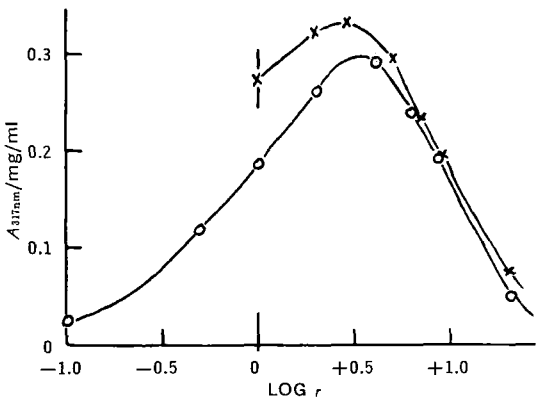


Figure 5—*Effect of the molar ratio* (r) *of* (○) *acetone to 10% potassium ampicillin or of* (×) *acetone to 10% potassium hetacillin on the production of 317-nm absorbance in 24 hr. Each experimental point is the average of at least three determinations.*

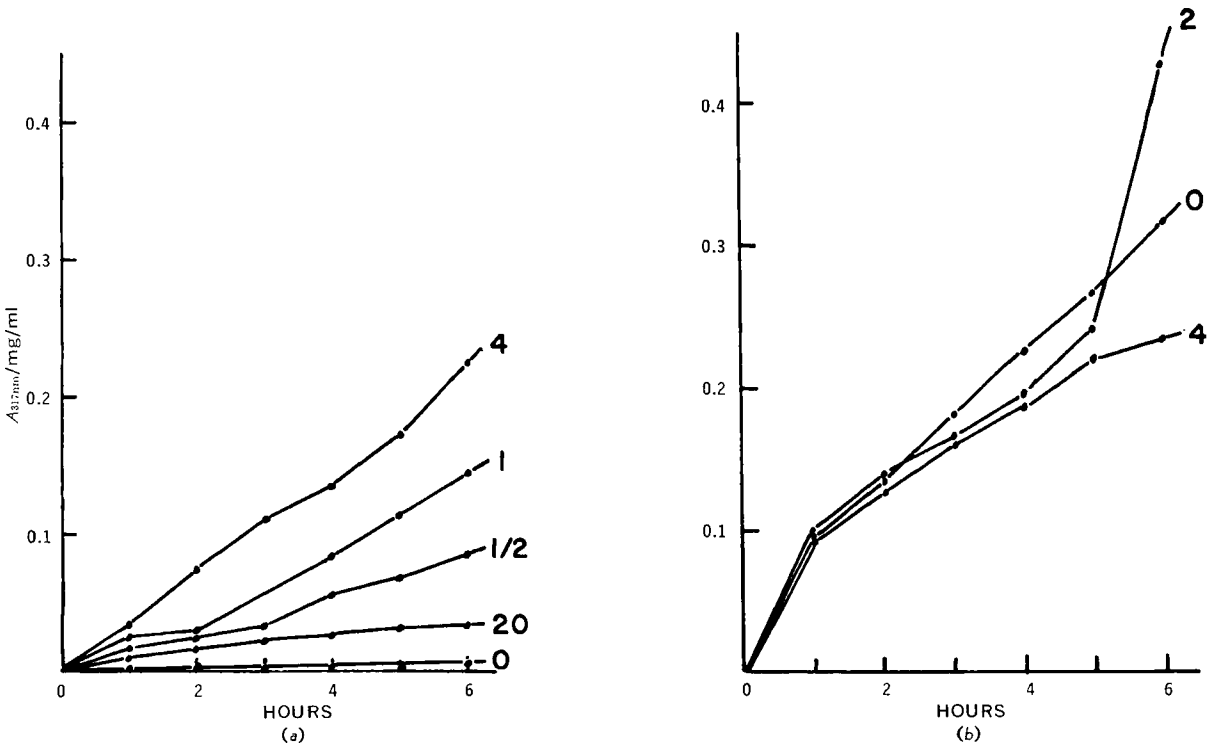


Figure 6—*Production of 317-nm absorbance with time. Key:* (a), *10% aqueous potassium ampicillin plus acetone to give specified molar ratios of acetone to ampicillin; and* (b), *10% aqueous potassium hetacillin plus acetone to give specified molar ratios of acetone to hetacillin.*

ate is reflected in the absence of any lag in penicillenic acid production.

The effect of large excesses of acetone on either hetacillin or ampicillin is to keep the penicillin in a form incapable of forming penicillenic acid. Although low amounts of acetone increase penicillenic acid production, higher amounts drive the reaction beyond the Schiff base intermediate to form hetacillin. The finding that hetacillin forms spontaneously from ampicillin and excess acetone in aqueous solution (Fig. 7) completely supports this proposal.

From the findings presented here, the system shown in Scheme I is proposed. In aqueous solutions of hetacillin, hetacillin and ampicillin are present in an equilibrium as is the Schiff base. A solution of ampicillin alone does not contain any free or potential acetone and cannot form either the Schiff base or penicillenic acid. If the ampicillin solution is concentrated enough, polymers of the type described previously (1) are formed.

The equilibrium between ampicillin, acetone, hetacillin, Schiff base, and water is complicated by the slow removal of material by the irreversible rearrangement of the Schiff base to the penicillenic acid (Step 3). The removal of material by this route would cause the rate of formation of penicillenic acid to be greater when starting with hetacillin than when starting with ampicillin and acetone. The reasoning for this is that the conversion of hetacillin to the Schiff base (Step 2) seems to be more rapid than the conversion of ampicillin plus acetone to the Schiff base (Step

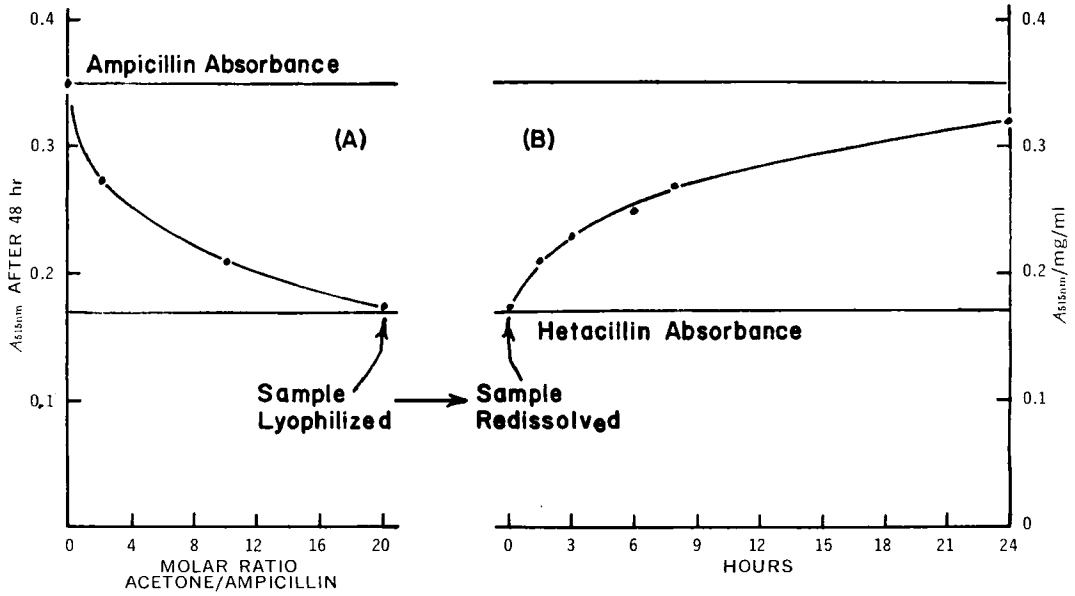
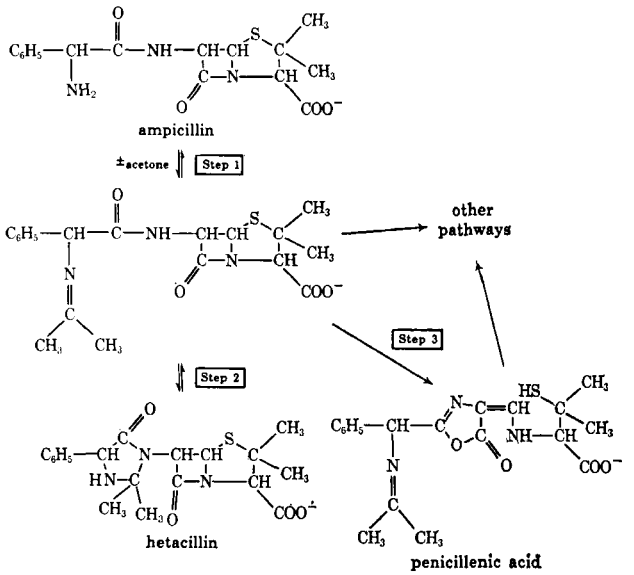


Figure 7—*Hetacillin production and degradation. Key:* (*A*), *production of hetacillin at different molar ratios of acetone and ampicillin in 48 hr reported as absorbances of milligrams per milliliter solution; and* (*B*), *degradation of hetacillin sample formed at a molar ratio of acetone to ampicillin of 20 in 48 hr. Each data point represents the average of at least three separate determinations.*



Scheme I

1). Thus, with hetacillin as the starting material, the Schiff base forms quickly and begins to be removed as penicillenic acid. The slower step of the equilibrium would then be the formation of ampicillin from the Schiff base. With such a mechanism, a definite quantity of penicillin would be lost as penicillenic acid before equilibrium is attained. The preequilibrium buildup of penicillenic acid would be much smaller starting with ampicillin and acetone than with hetacillin.

The effect of high acetone concentrations on ampicillin solutions is readily explained by Scheme I. High acetone drives the reaction to the Schiff base and prevents the back-reaction to ampicillin; moreover, since the Schiff base to hetacillin step is faster than the conversion of the Schiff base to penicillenic acid, hetacillin is the major product. The possible effect of acetone on inhibiting penicillenic acid rearrangement itself needs further study. Additional investigations are also required for an understanding of the factors controlling the conversion of Schiff base to penicillenic acid as well as the possible isolation of this unstable intermediate.

SUMMARY

Solutions of hetacillin in water hydrolyzed to ampicillin and acetone much more slowly than was reported by others. The half-life for the opening of the γ-lactam ring in hetacillin is about 4 hr, while the half-life for the removal of the acetone moiety from hetacillin is nearly 8 hr.

A penicillenic acid with a UV absorbance maximum at 317 nm is produced by solutions of hetacillin, hetacillin with acetone, and ampicillin with acetone. Ampicillin with no acetone present does not produce a 317-nm absorbance. The penicillenic acid is postulated to be derived from a Schiff base intermediate in the reversible interconversion of ampicillin and hetacillin.

These observations and the formation of hetacillin from ampicillin and acetone are accounted for in the proposed scheme. In the scheme neither ampicillin nor hetacillin can go directly to a penicillenic acid but must first form the Schiff base which rearranges to 317-nm-absorbing penicillenic acid. Step 2, the interconversion of the Schiff base and hetacillin, is thought to be faster than Step 1, the interconversion of ampicillin plus acetone and Schiff base. Step 3, the removal of material *via* penicillenic acid, is probably of an intermediate rate.

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[x] To whom inquiries should be directed.

Specific TLC Determination of Trimethoprim and Sulfamethoxazole in Plasma

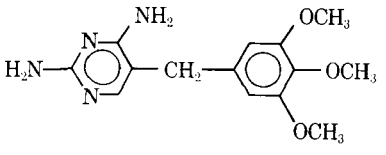
CARL W. SIGEL[x], MICHAEL E. GRACE, CHARLES A. NICHOL, and GEORGE H. HITCHINGS

Abstract □ A simple spectrodensitometric method for the direct determination of trimethoprim and sulfamethoxazole is based upon measurement of the absorbance of the two compounds on silica gel plates irradiated at 280 and 265 nm, respectively. Unlike the procedure for trimethoprim based on its fluorescence, which requires time for the development of the intensity of the spots, absorbance can be measured immediately. For both compounds, quantities as low as 0.005 μg can be detected and a linear relationship is observed between the integrated peak area and the amount on the plate between 0.02 and 0.5 μg. The percent recovery over the normal range of plasma concentrations for both compounds was 96–105%. This procedure for sulfamethoxazole is compared directly with a method based upon the Bratton–Marshall reaction.

Keyphrases □ Trimethoprim and sulfamethoxazole—analysis in plasma, TLC and spectrodensitometric method, compared to Bratton–Marshall reaction □ Sulfamethoxazole and trimethoprim—analysis in plasma, TLC and spectrodensitometric method, compared to Bratton–Marshall reaction □ TLC and spectrodensitometry—analysis, trimethoprim and sulfamethoxazole in plasma

Trimethoprim (I) and sulfamethoxazole (II) both inhibit the biosynthesis of folic acid in bacteria (1); the sulfonamide inhibits a step in the formation of dihydrofolic acid from *p*-aminobenzoic acid, and trimethoprim inhibits the conversion of dihydrofolic acid to tetrahydrofolic acid (1, 2). This relationship results in potentiation of the two activities and is the basis for a potent antibacterial combination[1] (3, 4). This report describes a new method that was applied to the specific determination of both drugs in plasma.

Previously reported analytical methods for the determination of trimethoprim in body fluids are based upon microbiological activity (5, 6), spectrophotometry (5), spectrofluorometry (7), scintillation spectrometry of ^{14}C-labeled trimethoprim (8), differential pulse polarography (9), a combination of TLC and the microbiological assay (10), and fluorescence TLC (11). Of all of these methods, the only specific ones are those including a TLC separation. Isolation from the TLC adsorbent or the wait for the development of trimethoprim fluorescence, as used in previously reported methods, can be circumvented by the direct measurement of the absorbance of I on silica gel plates. Trimethoprim "quenching" can be visualized on fluorescing silica gel plates irradiated with UV light (254 nm); however, this determination is based upon measurement of the absorbance and can be done using either fluorescing or nonfluorescing TLC plates.



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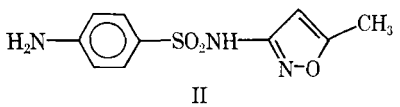

II

The most widely used method for the determination of sulfamethoxazole and other sulfonamides is a colorimetric procedure based upon the Bratton–Marshall (12) reaction. Falk and Kelley (13) and Kraml and Boudreau (14) reported adaptations of the method to automated systems. Various other improvements in the basic procedure have been introduced to improve specificity. Rieder (15) used the Bratton–Marshall reaction directly in an ethyl acetate extract of the nonhydrolyzed body fluid, thereby measuring what closely approximates the "bacteriostatically active fraction." His modification is generally applicable to the routine determination of sulfonamides in body fluids and potentially could be automated. Others have utilized various chromatographic methods including TLC (16–21), GLC (22), and partition chromatography (23, 24) to improve specificity for determinations of sulfonamides in both pharmaceutical preparations and biological samples. The present method is based upon the same principles as for the trimethoprim assay: separation on a silica gel thin-layer plate followed by direct determination by measuring the absorbance of sulfamethoxazole. A direct comparison between this TLC method and a modification of the Bratton–Marshall procedure was made and the results are discussed.

EXPERIMENTAL

Thin-Layer Plates—Silica gel 60 F-254 plates (20 × 20 cm, 0.25 mm)[2] were used. Nonfluorescing silica gel 60 plates gave identical results.

Solvents and Solutions—All solvents were reagent grade[3] and all, except ammonia, were distilled before use. The buffer for the sulfamethoxazole determination was 0.3 *M* citric acid–0.6 *M* sodium phosphate, dibasic (pH 4.1).

Standard Solutions—The following were prepared: trimethoprim (2.0 mg) in 100 ml chloroform–methanol (9:1), and sulfamethoxazole (1.0 mg) in 100 ml chloroform.

Instrumentation—Plates were spotted with an automatic spotter[4] or by hand. Absorbance was determined by scanning TLC

[1] Trimethoprim–sulfamethoxazole (1:5 combination); Septra, Burroughs Wellcome Co.

[2] E. M. Laboratories.

[3] Mallinckrodt.

[4] Analytical Instrument Specialties Multi-spotter.

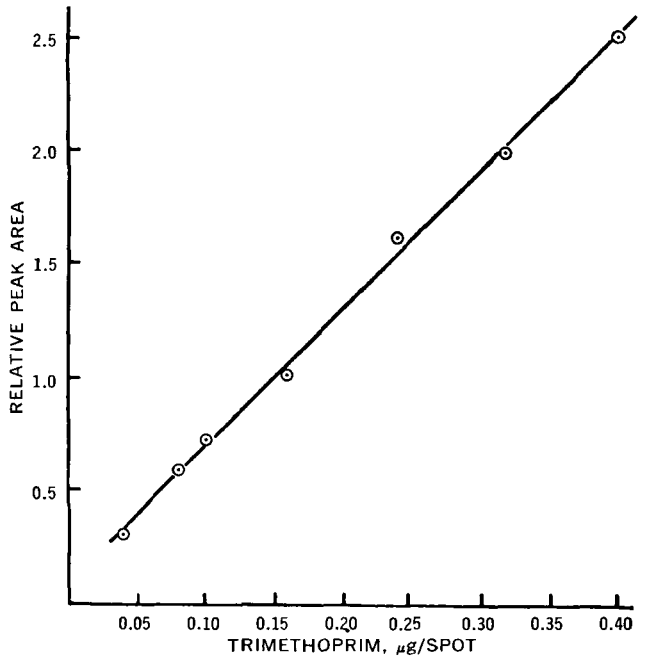

Figure 1—*Standard curve for trimethoprim.*

plates with a spectrodensitometer[5], using the reflectance mode and only the sample beam without a secondary barrier filter. Irradiation was at 280 nm for trimethoprim and at 265 nm for sulfamethoxazole. Absorbance was determined using a density computer[6] and the response was recorded[7]. Peak areas were electronically integrated[8] and standard curves were calculated using the method of least squares.

Trimethoprim Determination—Into a 50-ml glass-stoppered centrifuge tube, pipet 1.0 ml plasma and add 4 ml water and 0.1 ml 1 *N* NaOH. Add 20 ml isopropanol–dichloromethane (1:4), invert the tube gently 30 times, and centrifuge. Transfer 18 ml of the organic layer to a separate tube and decrease this volume to about 0.1 ml by heating the tube in a water bath at 40° under a stream of nitrogen. Transfer the residue to a 1-dram screw-capped vial using methanol–chloroform (1:9) and evaporate the solvent with a stream of nitrogen. Seal the vial with a septum-type lined screw-cap. With a syringe, add 100 μl of methanol–chloroform (1:9) to the vial, rotate it to dissolve the residue, and cool in an ice bath for a few minutes. Scan the TLC plate prior to spotting to check for uniformity of background. Apply 12 10-μl sample aliquots (up to about 50 μl can be spotted to each scored plate, 20 channels/plate) and a series of six concentrations of I (0.4–0.04 μg) by spotting appropriate volumes of the standard solution. Develop the plate in chloroform–*n*-propanol–28% aqueous ammonium hydroxide (80:20:1), allowing the solvent to migrate about 15 cm. Air dry the plate for 10 min and scan at 280 nm using only the sample beam. The trimethoprim peak ($R_f \simeq 0.42$) is detected by absorbance and appears on the recorder chart as a symmetrical peak well resolved from the trimethoprim metabolites, sulfamethoxazole and its metabolites, and common endogenous compounds.

Sulfamethoxazole Determination—Into a 15-ml glass-stoppered centrifuge tube, pipet 0.2 ml plasma and add 0.8 ml citrate–phosphate buffer and 4 ml chloroform–acetone (2:1). Invert the tube gently 30 times and centrifuge (1500×*g* for 3 min). Apply 80 μl of the organic phase (12 samples/plate) and a series of six sulfamethoxazole standards (0.50–0.05 μg) to each scored plate using an automatic spotter. Develop the plate in isopropanol–benzene (1:4), allowing the solvent to migrate about 15 cm. Air dry the plate for 10 min and scan at 265 nm in the same manner as for trimethoprim. The sulfamethoxazole spot appears at $R_f \simeq$ 0.5. The Bratton–Marshall procedure followed was based on the adaptation of Falk and Kelley (13).

[5] Schoeffel model SD 3000 using a reflectance mode assembly, SDA325.
[6] Schoeffel SDC30.
[7] Honeywell Electronik 194.
[8] Hewlett-Packard calculator, model 9100B.

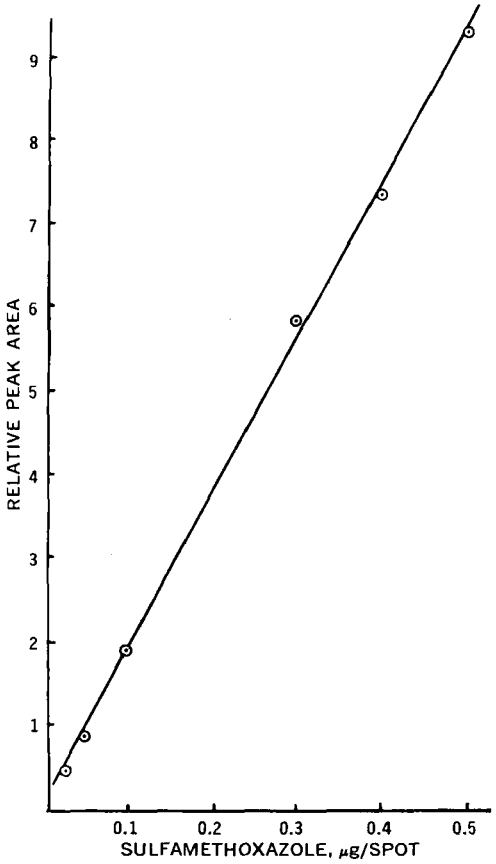

Figure 2—*Standard curve for sulfamethoxazole.*

RESULTS

Calibration curves for both compounds using the described procedures are shown in Figs. 1 and 2. A linear relationship between the integrated area under the peak and the amount on the plate

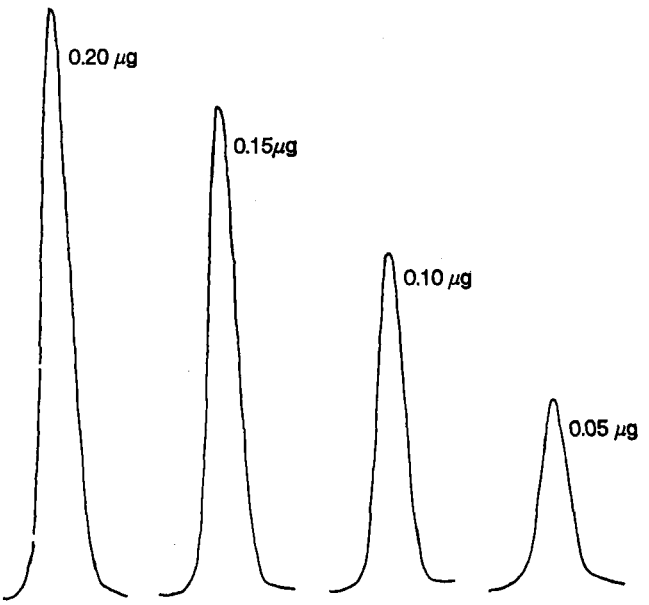

Figure 3—*Typical peaks observed when scanning the absorbance of trimethoprim standards on silica gel 60 F-254 plates.*

Table I—Reproducibility of Recovery of Trimethoprim from Spiked Human Plasma (1 ml)

Quantity Added, μg	Number of Samples	Mean Recovery	Percent	Standard Deviation
2.00	5	2.08	104	0.07
1.20	5	1.23	103	0.04
0.80	5	0.77	96	0.035
0.40	5	0.40	100	0.008

Table II—Reproducibility of Recovery of Sulfamethoxazole from Spiked Human Plasma (0.2 ml)

Quantity Added, μg/ml	Number of Samples	Mean Recovery	Percent	Standard Deviation
40.0	5	40.42	105	0.44
20.0	5	19.28	96	0.12
8.0	5	8.15	102	0.16

was observed between 0.02 and 0.5 μg. Typical peaks observed on the recorder chart after scanning four trimethoprim standards are shown in Fig. 3. Quantities as low as 0.005 μg can be detected for both I and II.

Both I and II appeared as well-resolved symmetrical peaks. Occasionally, an endogenous compound with R_f 0.5 was observed and overlapped slightly with trimethoprim. Development of the plate a second time in the same solvent separated the two peaks to recorder baseline.

Reproducibility and recovery data for a normal range of plasma concentrations for I and II are shown in Tables I and II, respectively. The percent recovery from plasma varied from 96 to 105%. An application of the method is summarized in Table III, which shows trimethoprim and sulfamethoxazole plasma concentrations for a human subject during the 25-hr period following an oral dose of 160 mg trimethoprim and 800 mg sulfamethoxazole[9]. In this case, 1 ml plasma was used for trimethoprim; however, volumes as low as 0.1 ml can be employed by spotting larger aliquots of the redissolved sample. Sample application to the plate can be done manually but is accomplished more readily with an automatic multispotter. For sulfamethoxazole, 0.2 ml plasma was used routinely.

DISCUSSION

For trimethoprim, the outlined method has several advantages over previously reported assays. It is specific, accurate, and rapid; 20 samples can be determined in 1 day by one person. The microbiological techniques are reproducible and sensitive but not specific. During therapy, when there is a change from another antibacterial to trimethoprim-sulfamethoxazole, the microbiological assay for I can be complicated by the presence of other agents. Spectrofluorometry is also sensitive but includes measurement of the metabolites or endogenous materials to some extent. The fluorescence TLC method reported earlier from these laboratories is specific and direct and can be used for the determination of I in plasma, urine, and tissues or the metabolites in urine (25). The fluorescence procedure, however, requires time for development of sufficient intensity of the spots for detection whereas absorbance can be measured immediately. Sensitivities of the two modes of operation are comparable. Application of the absorption procedure to the determination of urinary metabolites was not attempted because of the presence of endogenous compounds that also absorb.

Perhaps the only feature of the described method that might be considered as a limitation is the requirement for a good scanning spectrodensitometer. Considering the potential applications of this technique to assays in the clinical laboratory, however, such instrumentation will undoubtedly become standard in the near future. This technique can be applied to drugs of diverse structure (as in this instance, I and II). Also, in recent experience the alternative of using the fluorescence or absorbance mode has made possible the analysis of different types of drugs in body fluids.

For the routine assay of sulfamethoxazole levels in the clinical laboratory, the automated procedures based upon the Bratton-Marshall reaction are fast and provide a reliable measure of the bacteriostatically active fraction. A comparison between sulfonamide levels for both spiked and actual blood samples as determined by the TLC method and the Bratton-Marshall reaction as described by Falk and Kelley (13) was conducted and close agreement between the results of the two methods was observed (Table IV). The length of time required for both studies was similar; however, for a large number of samples, use of an automated Bratton-Marshall procedure would save considerable time. The agreement between two such different methods provides evidence that both procedures give reasonably accurate measures of the "active," nonconjugated sulfonamide fraction.

The major advantage of the TLC method is that one can be more confident that only the sulfonamide is being determined and not another drug or endogenous material that furnishes a chromophore with the Bratton-Marshall reagent. This problem of specificity, particularly for tissue level studies and for drug assay of preparations composed of sulfonamide combinations, has caused

[9] Two tablets of Septra.

Table III—Plasma Concentrations of Trimethoprim and Sulfamethoxazole following Oral Dose of 160 mg Trimethoprim and 800 mg Sulfamethoxazole

Hours	Trimethoprim Concentration, μg/ml	Sulfamethoxazole Concentration, μg/ml
0	—	—
0.5	1.95	26.33
1	1.77	47.53
2	1.67	60.33
3	1.43	46.33
5	1.19	47.66
13	0.73	23.00
22	0.41	15.50
25	0.29	14.4

Table IV—Comparison of the Determination of Plasma Concentrations of Sulfamethoxazole using Quantitative TLC and the Bratton-Marshall Reaction[a]

Spiked Plasma Samples

Sample	Quantity Added, μg/ml	TLC Assay	Colorimetric Assay[b]
Blank	—	—	0.3
1	10.0	10.8	10.4
2	10.0	11.3	10.8
3	10.0	10.8	11.1
4	10.0	10.0	10.7
5	40.0	42.9	39.2
6	40.0	39.9	39.1
7	40.0	39.6	39.7
8	40.0	39.2	40.3

Plasma Samples from Patients Receiving Trimethoprim/Sulfamethoxazole

	TLC Assay, μg/ml	Colorimetric, μg/ml
Pretreatment sample	—	0.6
1	33.2	28.9
2	52.5	51.4
3	33.8	31.5
4	28.8	28.9
5	31.0	31.4
6	26.0	26.9

[a] All samples were 0.2 ml. [b] Bratton-Marshall assay results corrected by subtraction of value for plasma blank or pretreatment sample.

others to devise various chromatographic procedures for sample preparation prior to spectrophotometric determination. The current method is an advancement over those procedures since the sulfonamide is determined directly on the separation medium. Presumably, the TLC method could be adapted for measurement of specific sulfonamide drug levels when sulfonamide mixtures are used. TLC conditions for separation of up to 15 different sulfonamides on one plate have been reported (19).

Finally, the lower limit of sensitivity of the TLC procedure is at least 20 times lower than is required for normal sulfonamide plasma concentration levels. This factor enabled the development of the very simple extraction procedure. In most laboratories, TLC has been utilized mainly as a qualitative tool. These new assay procedures for trimethoprim and sulfamethoxazole in plasma demonstrate that TLC can also provide a rapid, sensitive, accurate, and specific means for quantitative determinations.

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H. R. HELD[x] and S. LANDI

Abstract □ The interaction between the preservative 8-hydroxyquinoline sulfate and macromolecules present in tuberculin purified protein derivative solutions such as tuberculoprotein, nucleic acid, and polysaccharide was studied. In buffered solution (pH 7.38), 8-hydroxyquinoline sulfate is dissociated to 8-hydroxyquinoline and sulfuric acid, and it is the base 8-hydroxyquinoline that forms a reversible association with these macromolecules. The degree of binding of 8-hydroxyquinoline to tuberculoprotein, nucleic acid, and polysaccharide was shown to be a function of the concentration of these macromolecules. In commercial solutions of tuberculin purified protein derivative, the low concentrations of purified protein derivative used have little effect on the concentration of 8-hydroxyquinoline sulfate. Thus, for all practical purposes the antimicrobial activity of 8-hydroxyquinoline sulfate was not affected.

Keyphrases □ 8-Hydroxyquinoline sulfate—interaction with components of tuberculin purified protein derivative □ Tuberculin purified protein derivative—interaction of 8-hydroxyquinoline sulfate with solution components, binding of 8-hydroxyquinoline □ Binding—8-hydroxyquinoline to tuberculoprotein, nucleic acid, and polysaccharide (components of tuberculin purified protein derivative) □ Antimicrobial agents—interaction of 8-hydroxyquinoline sulfate with components of tuberculin purified protein derivative

8-Hydroxyquinoline sulfate[1] is added to tuberculin purified protein derivative solutions as an antimicrobial agent (2). In previous reports, the authors described how 8-hydroxyquinoline sulfate disappeared from these solutions when dispensed in glass vials stoppered with rubber closures. They showed that, in

[1] Chinosol (1), Eastman Organic Chemicals, catalog number 1776.

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a buffered solution (pH 7.38), 8-hydroxyquinoline sulfate is dissociated into 8-hydroxyquinoline and sulfuric acid and that most of the loss of 8-hydroxyquinoline sulfate from the solution was caused by sorption of 8-hydroxyquinoline by the rubber closures (3). Furthermore, it was shown that 8-hydroxyquinoline interacted with polysorbate 80, used as an antiadsorption agent in tuberculin purified protein derivative solutions (4), and that 8-hydroxyquinoline reacted with traces of metals present in tuberculin purified protein derivative solutions to form dark precipitates. These metals also affected the antimicrobial properties of 8-hydroxyquinoline (5, 6).

In a previous report (4), it was mentioned that the interaction of 8-hydroxyquinoline sulfate with some macromolecules other than polysorbate 80 such as tuberculoprotein, nucleic acid, and polysaccharide, which are present in tuberculin purified protein derivative solutions, would be reported subsequently. Since it is known that preservatives and other organic substances of small molecular weight interact with proteins (7–19), polysaccharides (20–23), and nucleic acids (19, 24–26) and that the bound preservative has no antimicrobial activity (15, 27), it was important to measure the degree of interaction that may take place between 8-hydroxyquinoline sulfate and these macromolecules.

The solubility method (28), the equilibrium dialysis method (28), and the spectrophotometric method were used to measure the degree of interaction between the components of tuberculin purified protein derivative and 8-hydroxyquinoline sulfate.

EXPERIMENTAL

Reagents—8-Hydroxyquinoline sulfate[1], 8-hydroxyquinoline[2], and polysorbate 80 (polyoxyethylene 20 sorbitan monooleate)[3] were used.

Buffer Solution (pH 7.38)—Isotonic phosphate-buffered solution (2), pH 7.38 (0.145% KH_2PO_4, 0.76% Na_2HPO_4, and 0.48% NaCl) was used. This buffer is also used to prepare the diluent (4, 5) for the preparation of tuberculin purified protein derivative solutions[4].

Tuberculin Purified Protein Derivative—Tuberculin purified protein derivative consists of tuberculoprotein, with its main impurities being mycobacterial nucleic acid and polysaccharide. It was of interest to determine the interaction of 8-hydroxyquinoline with each of these substances separately. The tuberculoprotein as well as the nucleic acid and the polysaccharide used was prepared from the culture filtrate of *Mycobacterium tuberculosis* grown on Long's synthetic medium (29). The first step of the preparation consisted of precipitation of tuberculin purified protein derivative with trichloroacetic acid (4% final concentration). Besides tuberculoprotein, the dry tuberculin purified protein derivative also contained 0.4% polysaccharide, 5% nucleic acid, and traces of metals (5). The tuberculin purified protein derivative was redissolved in a phosphate-buffered saline to a volume of approximately 4% of the original volume of the crude filtrate. This provided a stock solution of tuberculin purified protein derivative designated "tuberculin purified protein derivative stock solution" (29).

Tuberculoprotein—To decrease the nucleic acid content of the purified protein derivative, the tuberculin purified protein derivative stock solution was precipitated with an equal volume of saturated ammonium sulfate solution (pH 7.1). The precipitate was washed free of ammonium sulfate by repeatedly treating it with 1% trichloroacetic acid solution. Finally, to remove most of the metals, the precipitate was redissolved in buffer solution (pH 7.38) to form a 2% solution of purified protein derivative, and an excess of 8-hydroxyquinoline sulfate was added. This darkened the color of the purified protein derivative solution immediately, and a black precipitate formed within a few days and was removed by filtration[5] (pore size 0.45 μm). To the clear filtrate, trichloroacetic acid (1% final concentration) was added and the precipitate was dried with acetone and ether. The tuberculoprotein powder so obtained contained less than 0.1% nucleic acid and, when dissolved in buffer (pH 7.38), it did not form precipitates with 8-hydroxyquinoline sulfate.

Polysaccharide—The supernate from the trichloroacetic acid precipitation of the culture filtrate was neutralized with sodium hydroxide, concentrated under vacuum, and dialyzed against running tap water. After dialysis, the contents of the dialysis bags were concentrated under vacuum until they became viscous. To this viscous concentrate an equal volume of acetone was added, and the precipitate obtained was dried in a vacuum desiccator until it became brittle enough to be ground in a mortar. The yellowish powder so obtained was a crude tuberculopolysaccharide of relatively high molecular weight and was designated Crude Polysaccharide A. This polysaccharide was purified by passing it through a gel filtration column[6] using water as an eluant (30). The fractions collected from the column were scanned by UV spectrophotometry and were tested by the anthrone reaction (31). The fractions showing the presence of polysaccharide were pooled and precipitated with ethanol. After centrifuging and vacuum drying, the polysaccharide, a snow-white powder, was designated Purified Polysaccharide A.

The supernate of the acetone precipitation was evaporated under vacuum to dryness. The evaporation residue consisted of crude polysaccharides of medium and low molecular weights and was designated as Crude Polysaccharide B.

Nucleic Acid—The supernate from the ammonium sulfate precipitation of tuberculin purified protein derivative was dialyzed against running tap water. Then the contents of the dialysis bags were concentrated under vacuum and precipitated with acidified ethanol, washed with ethanol and ether, and vacuum dried. The white powder so obtained consisted of nucleic acid (29). A sufficient amount of this nucleic acid was further purified by paper electrophoresis (29).

Molecular Weight and Light Absorption Spectrum of Tuberculin Purified Protein Derivative—The molecular weight ranges of the tuberculoprotein, of the polysaccharides, and of the nucleic acid used were estimated from the elution patterns obtained when these substances were subjected to gel filtration on a column[6] (Fig. 1). The elution patterns of Blue Dextran, human serum albumin, egg albumin, ribonuclease, and phenol with approximate molecular weights of 2,000,000, 67,000, 45,000, 14,700, and 96, respectively, are shown for comparison (Fig. 1). The absorbance, measured at various wavelengths and concentrations, is recorded only to show the relative locations of the peaks. Therefore, no values are given for the absorbance (Fig. 1).

The UV light absorption spectra of the preparations used are shown in Fig. 2.

Preparation of Solutions—A 5% solution of the test substance in buffer (pH 7.38) was prepared first. Solutions of lower concentration were obtained by diluting the 5% solution with buffer (pH 7.38) to the required concentration. When the 5% solution of tuberculoprotein was prepared, the required amount of 1 *N* NaOH was added to maintain the pH of the buffer at 7.38. In the case of nucleic acid, the following procedure was used. Nucleic acid and an equivalent amount of sodium bicarbonate were dissolved in as little buffer as possible while stirring until the development of carbon dioxide stopped and the pH of the solution was pH 7.38. Then more buffer was added to obtain concentrations of nucleic acid of 5% or lower.

Determination of 8-Hydroxyquinoline Sulfate—The 8-hydroxyquinoline sulfate concentration of a solution was determined by measuring its absorbance[7] at 239 nm after dilution with buffer (pH 7.38) and comparing it with the absorbance at the same wavelength of a standard solution.

Since tuberculoprotein, polysaccharide, and nucleic acid also absorb UV light (Fig. 2), their own absorption had to be taken

[2] Fisher Scientific Co., catalog number 0-261.
[3] Tween 80, Atlas Chemical Industries, Brantford, Canada.
[4] Mantoux.
[5] Millipore.
[6] Sephadex G200, Pharmacia Fine Chemicals, Uppsala, Sweden.
[7] Beckman model DU spectrophotometer.

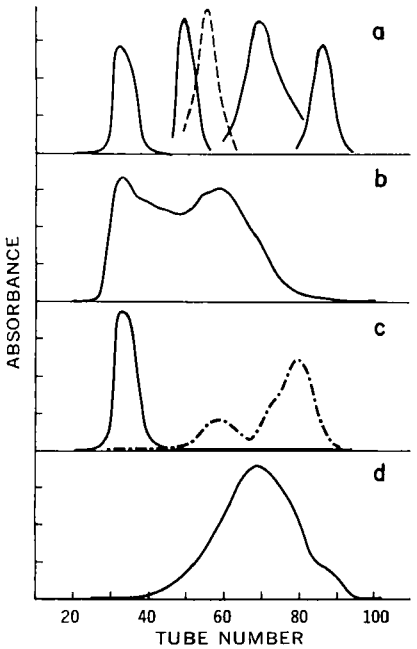

Figure 1—*Elution pattern of various substances after passing through one column. Key: a (from left to right), Blue Dextran, human serum albumin, egg albumin, ribonuclease, and phenol of molecular weights 2,000,000, 67,000, 45,000, 14,700, and 96, respectively; b, tuberculoprotein; c, Purified Polysaccharide A of high molecular weight (the solid line) and Crude Polysaccharide B (the broken line representing a mixture of crude polysaccharides of medium and lower molecular weight); and d, mycobacterial nucleic acid.*

into consideration when 8-hydroxyquinoline was measured in their presence. The absorption at 239 nm due to the known amount of tuberculoprotein or polysaccharide present was subtracted from the absorption of the mixture to obtain the absorption due to 8-hydroxyquinoline sulfate.

In the case of nucleic acid, this correction was considered too large to obtain precise results. Therefore, the following procedure was used to remove most of the nucleic acid from the samples. To 1 ml of the solution containing nucleic acid and 8-hydroxyquinoline sulfate was added 3 ml of acidified ethanol. The mixture was centrifuged[8] in closed Pyrex glass tubes at 5000 rpm for 10 min. A 0.1-ml aliquot of the clear supernate was added to 9.9 ml buffer solution (pH 7.38), and the UV absorption at 239 nm was measured. An identical solution containing the same amount of nucleic acid but no 8-hydroxyquinoline sulfate was submitted to the same procedure; to obtain the absorption due to 8-hydroxyquinoline sulfate, the absorption of this solution was subtracted from the absorption of the solution containing nucleic acid and 8-hydroxyquinoline sulfate.

Solubility Method—The solubility of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38), containing various amounts of the tested macromolecular substance, was evaluated by placing into glass ampuls 1.5 ml of this solution and an excess of 8-hydroxyquinoline sulfate (40 mg of a mixture consisting of equivalent amounts of 8-hydroxyquinoline sulfate and sodium bicarbonate). The ampuls were flame sealed and agitated for 48 hr at 23°. The contents of the ampuls were then clarified either by centrifugation in glass-stoppered tubes or by filtration[9] (pore size 0.45 μm), and the 8-hydroxyquinoline sulfate in the clear solution was determined spectrophotometrically. To reduce evaporation losses, pressure rather than vacuum was applied to the filter.

Dialysis Method—The dialysis membranes consisted of small, thin nylon bags[10] which had been preequilibrated against 8-hydroxyquinoline sulfate by keeping them for 10 days in 1 liter of buffered solution (pH 7.38) containing 0.08% 8-hydroxyquinoline sulfate. Into each bag was placed 2 ml of a buffered solution (pH 7.38) containing 0.08% 8-hydroxyquinoline sulfate and various amounts of the tested macromolecular substance. Each nylon bag was then placed in a glass-stoppered weighing bottle containing 2 ml of a 0.08% solution of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38). Each bottle was then stoppered tightly with the end of the nylon bag protruding to the outside of the bottle, thus providing also a tight closure for the nylon bag. To minimize evaporation losses, the weighing bottles were then placed in a larger closed glass container which also contained some buffer (0.08% 8-hydroxyquinoline sulfate), and the whole system was agitated for 4 days at 23°. After this time, an equilibrium between the inner and outer solutions of the dialysis membranes was reached, and samples were taken from the inside and outside of the nylon bags to determine the concentration of 8-hydroxyquinoline.

Spectrophotometric Method—If a solution contains two substances that do not interact, the light absorption of the solution at each wavelength will be the sum of the light absorption of each substance. If an interaction occurs, then the light absorption of the mixture will not be the same at all wavelengths and a shift in the spectrum will occur. Obviously, the shift will increase with an increasing percentage of the bound substance present. Therefore, these spectrophotometric observations had to be done at fairly high concentrations of 8-hydroxyquinoline sulfate (0.05%) and of the interacting substances, namely, 1 and 5% polysorbate 80, 0.3% tuberculoprotein, 1% polysaccharide, and 0.2% nucleic acid. At these concentrations, some absorbance measurements[7] had to be done in quartz cells, allowing light paths of 1, 0.1, and 0.01 cm.

Different light paths (0.1–10 cm) were also employed when measuring the spectrum of 8-hydroxyquinoline sulfate at various concentrations. The values for the 2- and 10-cm light paths were obtained by calculation from the absorbance of the 1-cm light path.

On the other hand, the quantitative determination of 8-hydroxyquinoline sulfate (1-cm cells) was not affected by the shift in the spectrum due to the presence of tuberculin purified protein derivative, since these measurements were done after a high dilution of the sample.

RESULTS

Interaction between Components of Tuberculin Purified Protein Derivative and 8-Hydroxyquinoline at pH 7.38—When 8-hydroxyquinoline sulfate is dissolved in a buffered solution (pH 7.38), it is dissociated into 8-hydroxyquinoline and sulfuric acid; it is the base 8-hydroxyquinoline that interacts with polysorbate 80 (4), with metals (5), or with other macromolecules such as tu-

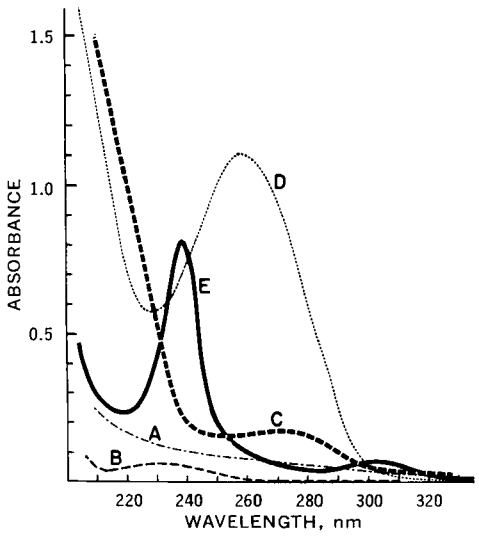

Figure 2—*UV absorption spectra in buffered solution (pH 7.38). Key: A, Purified Polysaccharide A (1000 μg/ml); B, polysorbate 80 (100 μg/ml); C, tuberculoprotein (100 μg/ml); D, nucleic acid (50 μg/ml); and E, 8-hydroxyquinoline sulfate (5 μg/ml).*

[8] Servall SS-3 centrifuge.
[9] Millipore HAWP1300.
[10] Supplied by Holland Rantos Co., New York, N.Y.

Table I—Degree of Binding of 8-Hydroxyquinoline by Tuberculoprotein Calculated[a] from the Solubility Increase of 8-Hydroxyquinoline Sulfate due to the Presence of Tuberculoprotein

Tuberculoprotein		Binding of 8-Hydroxyquinoline, Expressed as	
% w/v	Tu[b]/ml	Ratio of Total/Free	% of Total
5		1.94	48.45
3.75		1.709	41.49
2.50		1.470	31.97
1.25		1.235	19.03
1.0		1.188	15.82
0.625		1.1175	10.51
0.2	100,000	1.0376	3.62
0.1	50,000	1.0188	1.85
0.01	5,000	1.00188	0.188
0.001	500	1.00019	0.019
0.0001	50	1.00002	0.002
0	0	1.00000	0.000

[a] Calculated from the slope (0.188) of curve a (Fig. 3), using the following equation: % bound = 100% − (100/R)%. R = ratio of total/free. [b] The biological potency of tuberculin is expressed in tuberculin units (Tu). One Tu is biologically equivalent to 0.00002 mg of PPD-S (36), which is the international standard for tuberculin purified protein derivative.

berculoprotein, polysaccharide, and nucleic acid. Nevertheless, since in the literature dealing with tuberculin the concentration of this preservative is given as percentage of 8-hydroxyquinoline sulfate, the authors have expressed all measurements in 8-hydroxyquinoline sulfate equivalents.

Interaction Evidenced by Solubility and Dialysis Methods—*Tuberculoprotein*—Figure 3 (curve a) shows the degree of interaction of tuberculoprotein with 8-hydroxyquinoline sulfate, as determined by the solubility method. The degree of interaction, expressed as the ratio R = total/free preservative, was small when compared to the interaction of 8-hydroxyquinoline sulfate with nonionic surfactants, such as polysorbate 80 (Fig. 3, curve e) (4).

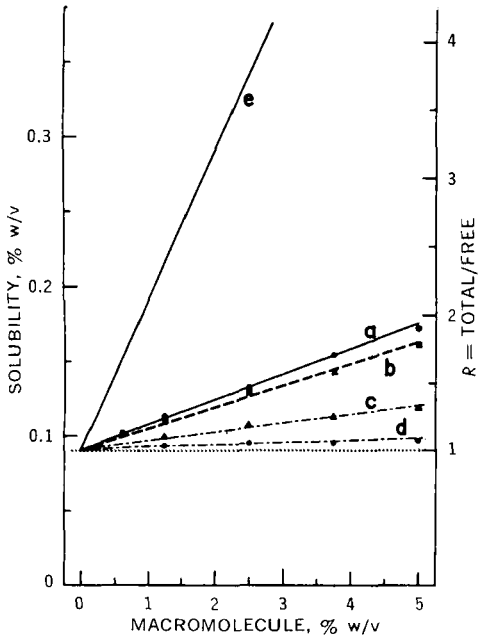

Figure 3—*Solubility of 8-hydroxyquinoline sulfate in a buffered solution (pH 7.38, 23°) containing various amounts of macromolecular substances present in tuberculin purified protein derivative. The increase in solubility represents the bound 8-hydroxyquinoline. The binding, calculated as the ratio R = total/free, is shown at the right scale. Key: a, tuberculoprotein; b, nucleic acid; c, Crude Polysaccharide A; d, Purified Polysaccharide A; and e, polysorbate 80. The slopes for the interaction curves (solubility method) were: a, 0.188; b, 0.160; c, 0.068; d, 0.018; and e, 1.06.*

Table II—Degree of Binding of 8-Hydroxyquinoline by Tuberculoprotein Calculated[a] from the Ratio C_i/C_o (Dialysis Method at pH 7.38 using a Nylon Dialysis Bag)

Tuberculoprotein, % w/v	Concentration of 8-Hydroxyquinoline Inside (C_i) and Outside (C_o) of Nylon Bag			Binding of 8-Hydroxyquinoline, Expressed as	
	C_i, %	C_o, %	C_i/C_o	Ratio of Total/Free	% of Total
5	0.105	0.057	1.84	1.84	45.6
3.75	0.102	0.062	1.65	1.65	39.4
2.5	0.093	0.065	1.43	1.43	30.0
1.25	0.086	0.071	1.21	1.21	17.4
0.625	0.082	0.074	1.11	1.11	9.9
0	0.08	0.08	1.0	1.0	0

[a] The ratio C_i/C_o is numerically equal to the ratio R = total/free preservative. For calculation of the degree of binding, the equation given as a footnote in Table I was used. The slope of the interaction curve (dialysis method) was 0.171.

Based on the slope of the interaction curve (Fig. 3, curve a), the amount of 8-hydroxyquinoline sulfate bound to tuberculoprotein could be calculated. Table I shows that in a concentrated stock solution (1% tuberculoprotein), approximately 15.8% of the total 8-hydroxyquinoline sulfate present was bound to the tuberculoprotein, while in a dilute solution [0.0001% tuberculoprotein, 50 tuberculin units (Tu)/ml)], a negligible amount of approximately 0.002% of the total 8-hydroxyquinoline sulfate present was bound to tuberculoprotein.

When the dialysis method was used for the determination of the interaction of 8-hydroxyquinoline sulfate with tuberculoprotein (Table II), the findings obtained by the solubility method were confirmed. In fact, Tables I and II show that the degree of binding of 8-hydroxyquinoline sulfate to tuberculoprotein as determined by these two methods was of the same order.

The interaction between tuberculoprotein and 8-hydroxyquinoline sulfate was reversible. This was evidenced by the fact that the nylon bags, from the dialysis experiment containing tuberculoprotein and 8-hydroxyquinoline sulfate, lost all of their 8-hydroxyquinoline sulfate when dialyzed against a large volume of buffer at pH 7.38.

Nucleic Acid—If mycobacterial nucleic acid and 8-hydroxyquinoline sulfate were simultaneously present in buffered solution (pH 7.38), the nucleic acid interacted reversibly with 8-hydroxyquinoline. Figure 3 shows that the degrees of interaction between 8-hydroxyquinoline sulfate and nucleic acid (curve b) and tuberculoprotein (curve a) as determined by the solubility method are of the same order.

Polysaccharide—Figure 3 (curves c and d) represents the interaction between 8-hydroxyquinoline sulfate and Crude Polysaccharide A and Purified Polysaccharide A, as indicated by the solubility method. The degree of interaction of 8-hydroxyquinoline sulfate with Crude Polysaccharide A and B was practically the same (curve c) but much less than the interaction between 8-hydroxyquinoline sulfate and tuberculoprotein (curve a) or nucleic acid (curve b). However, the degree of interaction with Purified Polysaccharide A was extremely small (curve d). Presumably, part of the interaction shown by the crude polysaccharides was due to some impurities.

Interaction Evidenced by Spectrophotometric Methods—By using the solubility and dialysis methods, it was found that the degree of interaction between 8-hydroxyquinoline and substances of relatively high molecular weight, which are present in tuberculin purified protein derivative, is small. Therefore, additional evidence of this interaction was sought by measuring the spectrum of 8-hydroxyquinoline in buffered solution alone and in buffered solution containing a fairly high concentration of these substances. The evidence that interaction had occurred was based on the change in the spectrum of 8-hydroxyquinoline in the presence of these substances. Polysorbate 80, which is known to interact strongly with 8-hydroxyquinoline (4), was included for comparison. To see the effect of the interaction, Figs. 4–7 each shows three curves obtained by measuring the absorption of UV light, namely of 8-hydroxyquinoline, of the tested macromolecular substance, and of the mixture of 8-hydroxyquinoline with the macromolecular sub-

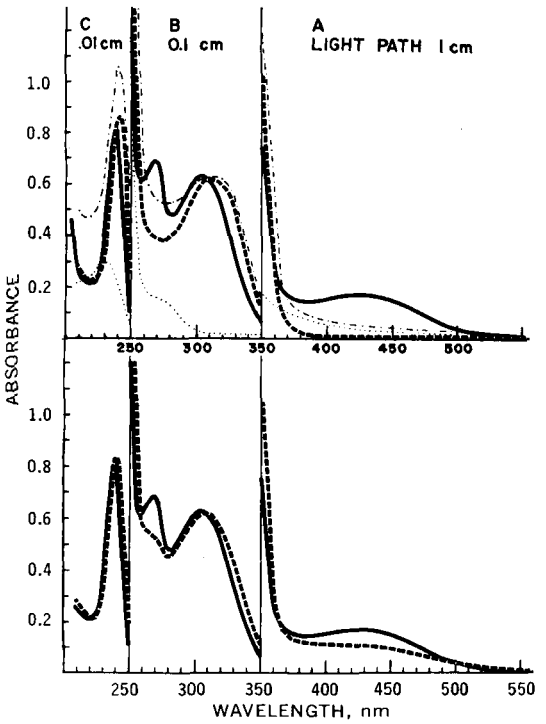

Figure 4—*Effect of polysorbate 80 on the absorption spectrum of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38). Key: . . ., 5% polysorbate 80; –·–·, 5% polysorbate 80 and 0.05% 8-hydroxyquinoline sulfate; ——, 0.05% 8-hydroxyquinoline sulfate; and - - -, 0.05% 8-hydroxyquinoline sulfate in the presence of, and corrected for, absorption due to 5% polysorbate 80 (upper section) and 1% polysorbate 80 (lower section).*

stance. In addition, to see more easily the effect of the macromolecule on the spectrum of 8-hydroxyquinoline, Figs. 4–7 show a fourth curve which was obtained by subtracting the absorption of the macromolecule from that of the mixture of 8-hydroxyquinoline with the macromolecule. This calculated curve represents the light absorbed by 8-hydroxyquinoline when in the presence of the macromolecule.

Polysorbate 80—Figure 4 shows the spectrum of 8-hydroxyquinoline (0.05%) alone and in presence of 5% polysorbate 80 (Fig. 4, upper section) and 1% polysorbate 80 (Fig. 4, lower section). Polysorbate 80 caused the disappearance of the absorption peak at 270 nm, and there was a spectral shift over the whole spectrum from 220 to 500 nm which was especially pronounced between 370 and 500 nm.

In the case of polysorbate, the spectral change was also visually recognizable by the disappearance of the yellow color of the 8-hydroxyquinoline solution when polysorbate 80 (5% final concentration) was added to it.

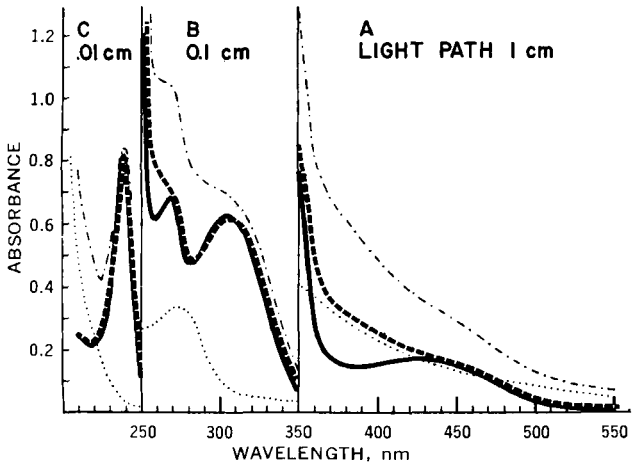

Figure 5—*Effect of tuberculoprotein on the absorption spectrum of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38). Key: . . ., 0.3% tuberculoprotein; –·–·, 0.3% tuberculoprotein and 0.05% 8-hydroxyquinoline sulfate; ——, 0.05% 8-hydroxyquinoline sulfate; and - - -, 0.05% 8-hydroxyquinoline sulfate in the presence of 0.3% tuberculoprotein and corrected for absorption due to tuberculoprotein.*

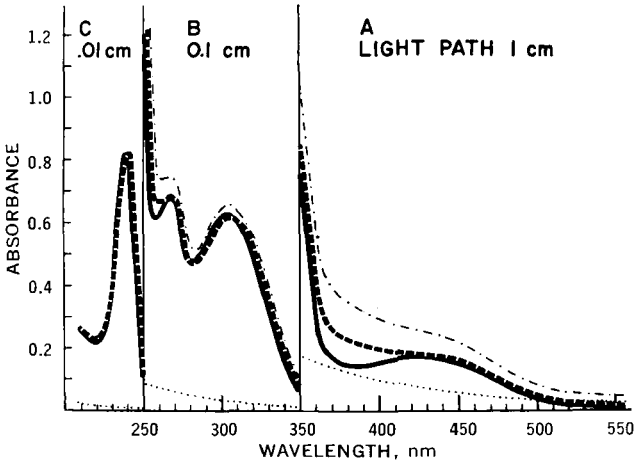

Figure 6—*Effect of polysaccharide on the absorption spectrum of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38). Key: . . ., 1% polysaccharide; –·–·, 1% polysaccharide and 0.05% 8-hydroxyquinoline sulfate; ——, 0.05% 8-hydroxyquinoline sulfate; and - - -, 0.05% 8-hydroxyquinoline sulfate in the presence of 1% polysaccharide and corrected for absorption due to polysaccharide.*

Tuberculin Purified Protein Derivative—Tuberculoprotein as well as Purified Polysaccharide A or nucleic acid caused an increase in absorption from approximately 350 to 420 nm (Figs. 5–7). In addition, tuberculoprotein and Purified Polysaccharide A caused the disappearance of the light absorption peak at 270 nm (Figs. 5 and 6). No attempt was made to measure the effect of nucleic acid on the spectral peak of 8-hydroxyquinoline at 270 nm, since the absorption of nucleic acid was too strong in this region (Fig. 7).

DISCUSSION

The fact that tuberculoprotein, mycobacterial nucleic acid, and polysaccharide present in tuberculin purified protein derivative interacted with the preservative 8-hydroxyquinoline sulfate was not surprising, since it was well known that other representatives of these groups of macromolecules interact with numerous substances. For instance, proteins such as plasma proteins (7–13), lactoglobulins (14), gelatin (15), bacterial proteins (16), and others (17–19) interact with numerous drugs, preservatives, detergents, dyes, and other substances. Polysaccharides such as starches (20), dextrins (21, 22), sucrose esters (23), and others (19) interact to a low degree with drugs, dyes, and other substances. Nucleic acids (19, 24–26) interact with peptides, dyes, antibiotics, and other substances. However, Table I shows that the loss of preservative due to its binding to tuberculoprotein was negligible because of the relatively low concentration in which tuberculin purified protein derivative is used[11]. Furthermore, the degree of interaction with some impurities contained in tuberculin purified protein derivative solutions, such as nucleic acid and polysaccharide, was still smaller and, therefore, the loss of preservative due to their presence was also negligible.

The small degree of interaction of 8-hydroxyquinoline with tuberculin purified protein derivative as measured by the solubility method and the dialysis method was confirmed by observing changes in the light absorption spectrum of 8-hydroxyquinoline

[11] Mantoux testing (0.0001%) and for Heaf testing (0.2%).

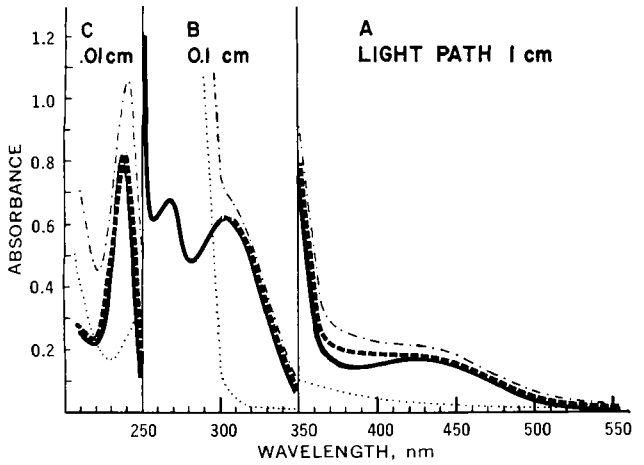

Figure 7—*Effect of nucleic acid on the absorption spectrum of 8-hydroxyquinoline sulfate in a buffered solution (pH 7.38). Key: . . ., 0.2% nucleic acid; —·—·, 0.2% nucleic acid and 0.05% 8-hydroxyquinoline sulfate; ——, 0.05% 8-hydroxyquinoline sulfate; and - - -, 0.05% 8-hydroxyquinoline sulfate in the presence of 0.2% nucleic acid and corrected for absorption due to nucleic acid.*

when tuberculoprotein, polysaccharide, or nucleic acid was present. No attempt will be made here to draw any conclusions from the spectral changes as to which groups of 8-hydroxyquinoline and of the macromolecules do interact except to say that the absorption peak of 8-hydroxyquinoline at 270 nm may be caused by association between several molecules of 8-hydroxyquinoline, since this peak disappeared at higher dilution but became more pronounced with increasing concentration of 8-hydroxyquinoline (Fig. 8). Thus, if the presence of polysorbate 80, tuberculoprotein, or polysaccharide reduced the size of the peak at 270 nm, it would indicate that the association of 8-hydroxyquinoline was retarded by interacting with these macromolecules, and this would explain the increase in solubility of 8-hydroxyquinoline in the presence of these macromolecules. Furthermore, the changes in the light absorption spectrum due to interaction are of interest, since they can be determined in a few minutes, while the solubility and dialysis studies require days to be completed. The spectral shifts shown in Figs. 4–7 were determined 5–10 min after the mixing of 8-hydroxyquinoline sulfate with the test substance, and there was no further change afterward. This would indicate that the interaction between 8-hydroxyquinoline and purified protein derivative may take place very fast after the mixing of these two substances.

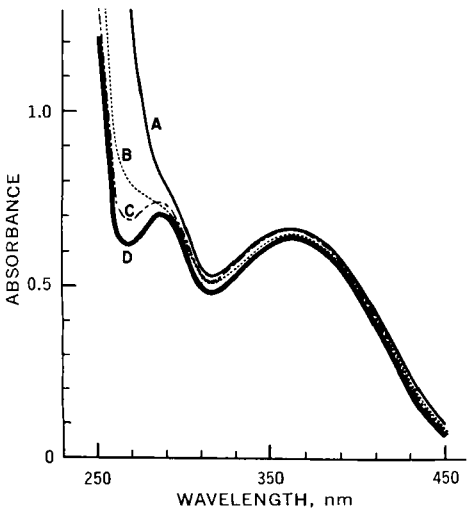

Figure 8—*Effect of various concentrations of 8-hydroxyquinoline sulfate on its absorption spectrum at pH 7.38. Concentration of 8-hydroxyquinoline sulfate was: A, 5 μg/ml; B, 25 μg/ml; C, 100 μg/ml; and D, 500 μg/ml. The light path was 10, 2, 0.5, and 0.1 cm for A, B, C, and D, respectively.*

One difficulty in obtaining valid measurements of the interaction between the components present in tuberculin purified protein derivative solutions and 8-hydroxyquinoline sulfate was due to the fact that tuberculoprotein is a very complex mixture of proteins which differ in their physical properties and which cover a wide range of molecular weights (32, 33); hence, the percentage distribution pattern of these proteins will vary from lot to lot of purified protein derivative. Therefore, the values determined for the interaction can only be expected to be strictly valid for the particular lot of tuberculoprotein, polysaccharide, or nucleic acid used. However, the degree of interaction of 8-hydroxyquinoline and, for instance, tuberculoprotein prepared from different lots of culture filtrate can be expected to be of the same order.

Some difficulties encountered when 8-hydroxyquinoline sulfate is used as a preservative (34) can be overcome if phenol, which is the preservative used in our tuberculin purified protein derivative solutions, is employed. In fact, work done in these laboratories (35) showed that the degree of interaction between phenol and the components present in tuberculin purified protein derivative solutions is much smaller for phenol than for 8-hydroxyquinoline sulfate. This is particularly true in the case of tuberculin solution for Heaf testing where the presence of glycerol binds 61.5% of 8-hydroxyquinoline sulfate as compared to 28.5% of phenol (35).

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[x] To whom inquiries should be directed.

Influence of Sodium Salicylate on Metabolism of Lathyrogen-Treated 3T6 Fibroblasts in Culture

JOSEPH J. ALEO

Abstract □ The effect of sodium salicylate on the metabolism of 3T6 fibroblasts treated with the lathyrogen β-aminopropionitrile was studied. A 5 m*M* concentration of the lathyrogen and/or 0.5 m*M* sodium salicylate was used to study proliferation, collagen synthesis, and mucopolysaccharide synthesis as measured by $^{35}SO_4$ incorporation. Sodium salicylate was able to reverse all lathyrogen-mediated changes to control or near control values. The diversity of these biosynthetic activities suggests that the influence of sodium salicylate on lathyrogen-treated cells is a result of a general metabolic effect rather than a specific one; the exact mechanism of action remains unknown.

Keyphrases □ Sodium salicylate—effect on metabolism of fibroblasts treated with β-aminopropionitrile, proliferation, collagen synthesis, and mucopolysaccharide synthesis □ Lathyrogens, β-aminopropionitrile—used to treat fibroblasts, effect of sodium salicylate on fibroblast metabolism □ β-Aminopropionitrile—used to study effects of sodium salicylate on metabolism of lathyrogen-treated fibroblasts □ Fibroblasts (3T6), β-aminopropionitrile treated—effects of sodium salicylate on metabolism, proliferation, collagen synthesis, and mucopolysaccharide synthesis □ Metabolism—β-aminopropionitrile-treated fibroblasts, effects of sodium salicylate

The lathyrogen β-aminopropionitrile fumarate in a concentration of 5 m*M* has been shown to depress cell proliferation while enhancing the synthesis of collagen, noncollagenous protein, glycogen, and mucopolysaccharides (1–4). Lesser concentrations of the lathyrogen do not exhibit these changes. Both the higher and lower concentrations, however, do affect collagen maturation, which is thought to be due to an inactivation of lysyl oxidase resulting in a deficiency of lysine-derived aldehydes (5). The discrepancies in data among studies are no doubt due to differences in cell lines used, experimental conditions, and, most important, concentrations of the lathyrogen employed.

Among the antirheumatic drugs, salicylates have been shown to have a beneficial influence on changes in experimental lathyrism by decreasing the elevated proportion of extractable collagen in tissues and the disappearance of the typical histological changes seen in the disease (6–8). Salicylates have also been shown to modify collagen and mucopolysaccharide synthesis to a greater extent than noncollagenous protein (9). Other researchers reported that they stimulate the turnover of collagen in general and its maturation in particular (10) while inhibiting the growth of human embryonic cells (11).

In the present study, an attempt was made to ascertain whether the influence of sodium salicylate was limited primarily to correcting the collagen maturation defect brought about by low concentrations of the lathyrogen (1 or 2 m*M*) or whether sodium salicylate affects other metabolic alterations such as those resulting from a 5 m*M* lathyrogen concentration.

EXPERIMENTAL

Materials and Methods—The 3T6 fibroblasts (12) were grown in the presence of 10% CO_2 in 60 × 15-mm plastic petri dishes in the Dulbecco–Vogt modification of Eagle's medium containing 10% calf serum. Five-day-old cultures were trypsinized and resuspended into a common culture having approximately 100,000 cells/ml. The common culture was then divided into four equal portions: β-aminopropionitrile was added to one at a concentration of 5 m*M*; sodium salicylate was added to the second at a concentration of 0.5 m*M*; β-aminopropionitrile (5 m*M*) and sodium salicylate (0.5 m*M*) were added to the third; and the fourth served as a control. The dosages of the lathyrogen and sodium salicylate used were established by assessing the toxicity of various concentrations. It was found that 5 m*M* β-aminopropionitrile and 0.5 m*M* sodium salicylate produced the maximum response without affecting cell viability.

Replicate cultures were prepared by dispensing 5 ml of cell suspension into each culture plate and incubating them at 37°. The medium was changed three times weekly for the duration of the experiment. Cells were harvested at 2, 4, 7, 9, 11, and 14 days. All media were pooled and saved for analysis, so the data represent total synthesis of material to the day of harvest. For analysis the medium was decanted and centrifuged at 200×*g* for 10 min to remove any dislodged cells or debris, and the cell layer was detached by using a razor blade or by incubation with 0.02% ethylenediaminetetraacetic acid in a phosphate buffer. Quantitation was based on both the cell count and DNA content (13), taking the average of three identically treated plates separately analyzed.

Chemical Analysis—*Collagen Synthesis*—Hydroxyproline in the medium and cell layer was determined by the method of Prockop and Udenfriend (14). Recoveries by this method averaged 86.8 ± 4.2%. All readings[1] were made at 560 nm. The medium was dialyzed exhaustively and then hydrolyzed in 6 *N* HCl for 16 hr at 104° before analysis. Unused medium served as a blank.

The solubility of the cell layer was determined as follows. Salt-

[1] Hitachi-Perkin-Elmer spectrophotometer.

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[x] To whom inquiries should be directed.

Influence of Sodium Salicylate on Metabolism of Lathyrogen-Treated 3T6 Fibroblasts in Culture

JOSEPH J. ALEO

Abstract □ The effect of sodium salicylate on the metabolism of 3T6 fibroblasts treated with the lathyrogen β-aminopropionitrile was studied. A 5 m*M* concentration of the lathyrogen and/or 0.5 m*M* sodium salicylate was used to study proliferation, collagen synthesis, and mucopolysaccharide synthesis as measured by $^{35}SO_4$ incorporation. Sodium salicylate was able to reverse all lathyrogen-mediated changes to control or near control values. The diversity of these biosynthetic activities suggests that the influence of sodium salicylate on lathyrogen-treated cells is a result of a general metabolic effect rather than a specific one; the exact mechanism of action remains unknown.

Keyphrases □ Sodium salicylate—effect on metabolism of fibroblasts treated with β-aminopropionitrile, proliferation, collagen synthesis, and mucopolysaccharide synthesis □ Lathyrogens, β-aminopropionitrile—used to treat fibroblasts, effect of sodium salicylate on fibroblast metabolism □ β-Aminopropionitrile—used to study effects of sodium salicylate on metabolism of lathyrogen-treated fibroblasts □ Fibroblasts (3T6), β-aminopropionitrile treated—effects of sodium salicylate on metabolism, proliferation, collagen synthesis, and mucopolysaccharide synthesis □ Metabolism—β-aminopropionitrile-treated fibroblasts, effects of sodium salicylate

The lathyrogen β-aminopropionitrile fumarate in a concentration of 5 m*M* has been shown to depress cell proliferation while enhancing the synthesis of collagen, noncollagenous protein, glycogen, and mucopolysaccharides (1–4). Lesser concentrations of the lathyrogen do not exhibit these changes. Both the higher and lower concentrations, however, do affect collagen maturation, which is thought to be due to an inactivation of lysyl oxidase resulting in a deficiency of lysine-derived aldehydes (5). The discrepancies in data among studies are no doubt due to differences in cell lines used, experimental conditions, and, most important, concentrations of the lathyrogen employed.

Among the antirheumatic drugs, salicylates have been shown to have a beneficial influence on changes in experimental lathyrism by decreasing the elevated proportion of extractable collagen in tissues and the disappearance of the typical histological changes seen in the disease (6–8). Salicylates have also been shown to modify collagen and mucopolysaccharide synthesis to a greater extent than noncollagenous protein (9). Other researchers reported that they stimulate the turnover of collagen in general and its maturation in particular (10) while inhibiting the growth of human embryonic cells (11).

In the present study, an attempt was made to ascertain whether the influence of sodium salicylate was limited primarily to correcting the collagen maturation defect brought about by low concentrations of the lathyrogen (1 or 2 m*M*) or whether sodium salicylate affects other metabolic alterations such as those resulting from a 5 m*M* lathyrogen concentration.

EXPERIMENTAL

Materials and Methods—The 3T6 fibroblasts (12) were grown in the presence of 10% CO_2 in 60 × 15-mm plastic petri dishes in the Dulbecco–Vogt modification of Eagle's medium containing 10% calf serum. Five-day-old cultures were trypsinized and resuspended into a common culture having approximately 100,000 cells/ml. The common culture was then divided into four equal portions: β-aminopropionitrile was added to one at a concentration of 5 m*M*; sodium salicylate was added to the second at a concentration of 0.5 m*M*; β-aminopropionitrile (5 m*M*) and sodium salicylate (0.5 m*M*) were added to the third; and the fourth served as a control. The dosages of the lathyrogen and sodium salicylate used were established by assessing the toxicity of various concentrations. It was found that 5 m*M* β-aminopropionitrile and 0.5 m*M* sodium salicylate produced the maximum response without affecting cell viability.

Replicate cultures were prepared by dispensing 5 ml of cell suspension into each culture plate and incubating them at 37°. The medium was changed three times weekly for the duration of the experiment. Cells were harvested at 2, 4, 7, 9, 11, and 14 days. All media were pooled and saved for analysis, so the data represent total synthesis of material to the day of harvest. For analysis the medium was decanted and centrifuged at 200×*g* for 10 min to remove any dislodged cells or debris, and the cell layer was detached by using a razor blade or by incubation with 0.02% ethylenediaminetetraacetic acid in a phosphate buffer. Quantitation was based on both the cell count and DNA content (13), taking the average of three identically treated plates separately analyzed.

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The solubility of the cell layer was determined as follows. Salt-

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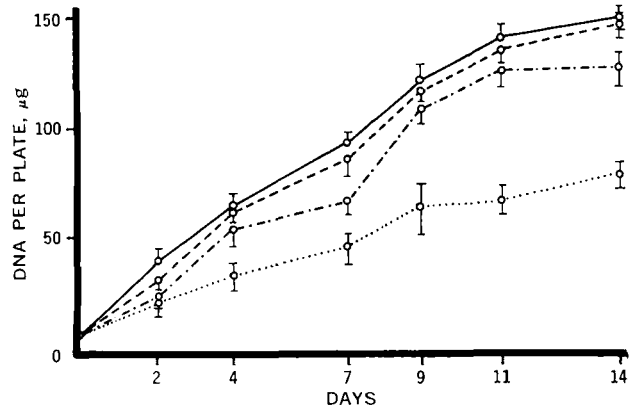

Figure 1—*Influence of 5 mM β-aminopropionitrile and/or 0.5 mM sodium salicylate on cellular proliferation for a 14-day period. Each point represents the average of three identically treated plates separately analyzed. Key: O—O, control; O- - -O, sodium salicylate; O···O, β-aminopropionitrile; and O-·-·O, β-aminopropionitrile and sodium salicylate.*

soluble collagen was extracted by pooling five plates of cells and shaking at 4° in 5 volumes of 1 *M* NaCl (pH 7.6) for 24 hr. Acid-soluble collagen was then extracted by shaking the residue in 5 volumes of 0.1 *N* acetic acid for 24 hr, and the remaining material was designated as insoluble collagen. Each fraction was hydrolyzed and analyzed for hydroxyproline.

$^{35}SO_4$ Uptake—Carrier-free Na_2-$^{35}SO_4$, 1 μCi/ml culture medium, was added to sulfate-free medium. Cells were harvested over a 7-day culture period, and both cells and media were analyzed for $^{35}SO_4$ incorporation. The medium was dialyzed exhaustively while cells were extracted four times with boiling 80% ethanol and then dissolved in 10% sodium hydroxide before counting[2].

RESULTS

Growth—All culture plates were seeded at 1×10^5 cells/ml medium used. Lathyrogen-treated cells showed a depression in cell growth as previously reported (1, 3), while sodium salicylate-treated cells showed very little, if any, change in growth. When both β-aminopropionitrile and sodium salicylate were used together, growth increased to within 85–90% of control values (Fig. 1).

To determine whether the sodium salicylate effect was indeed a true reversal, cells were grown in the presence of β-aminopropionitrile alone for 4 days, at which time the lathyrogen effect is clearly established. At this point, sodium salicylate was added

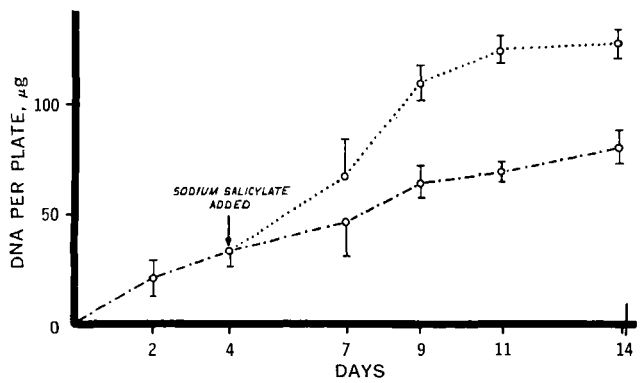

Figure 2—*Modification of cellular proliferation of β-aminopropionitrile-treated cells by 0.5 mM sodium salicylate added on the 4th day of culture. Each point represents the average of three identically treated plates separately analyzed. Key: O-·-O, β-aminopropionitrile; and O···O, β-aminopropionitrile and sodium salicylate.*

[2] Beckman LS-150 scintillation counter.

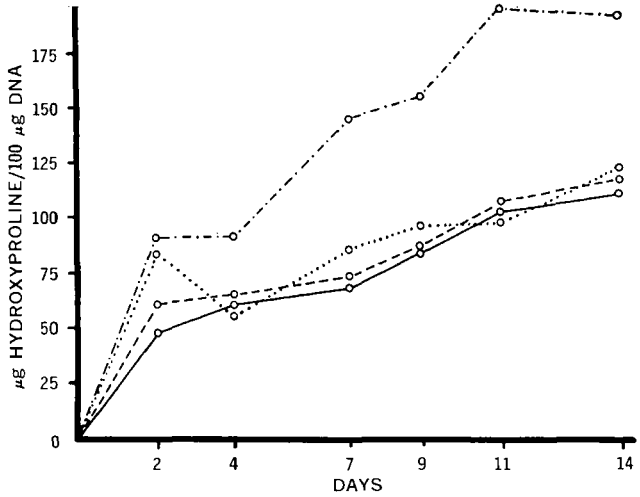

Figure 3—*Medium hydroxyproline measured over a 14-day period. All media were pooled and saved for analysis; therefore, the data represent total synthesis of material to the day of harvest. Each point represents the average of three plates separately analyzed. DNA was measured as previously described. Key: O—O, control; O- - -O, sodium salicylate; O-·-O, β-aminopropionitrile; and O···O, β-aminopropionitrile and sodium salicylate.*

and growth was monitored for an additional 10 days. At the end of 14 days, growth was the same as for those cultures in which both lathyrogen and sodium salicylate were added at the beginning of the experiment (Fig. 2).

Collagen Synthesis—*Medium*—At the end of a 14-day culture period, the lathyrogen produced the previously reported (1) increase in a hydroxyproline-containing macromolecule in the medium. Sodium salicylate alone had no effect on hydroxyproline synthesis, while the addition of sodium salicylate to the lathyrogen-treated cultures reduced hydroxyproline synthesis to control values (Fig. 3).

Cell Layer—There was virtually no significant difference in the total collagen associated with the cell layer. Salt-soluble fractions were similar in both control and sodium salicylate-treated cells, while the lathyrogen produced almost twice the amount of salt-soluble material. The addition of sodium salicylate to lathyrogen-treated cultures lowered the salt-soluble collagen and increased the insoluble fraction to close to control values. The acid-soluble fractions remained relatively unchanged throughout (Fig. 4).

$^{35}SO_4$ Uptake—Lathyrogen-treated cultures showed an increased uptake into a sulfated macromolecule in both the cell

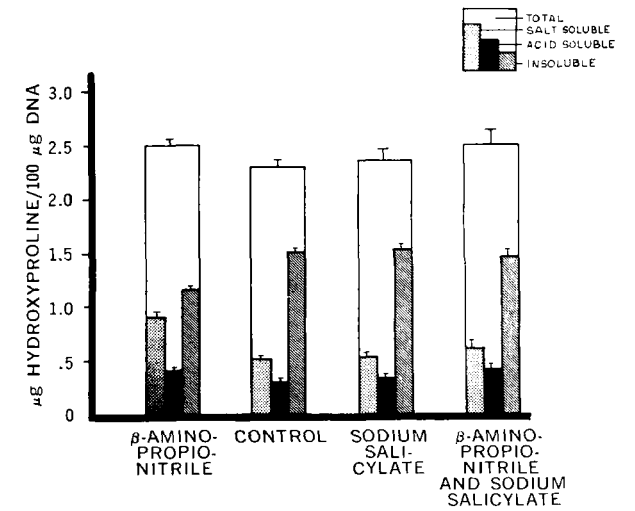

Figure 4—*Cell-associated hydroxyproline at the end of a 7-day culture period showing total, salt-soluble, acid-soluble, and insoluble fractions. Results are the average of three experiments; five plates of cells were pooled for each analysis.*

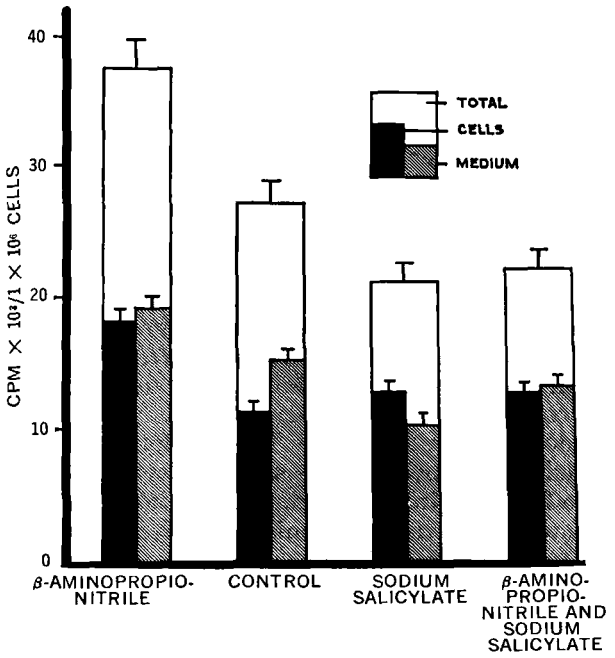

Figure 5—$^{35}SO_4$ *uptake (three plates per point, as previously described) at the end of a 7-day culture period showing total, cellular, and medium uptake.*

layer and the medium, while sodium salicylate lowered the uptake when compared to controls. The addition of sodium salicylate to lathyrogen-treated cultures lowered uptake below control values more closely to those obtained with sodium salicylate alone (Fig. 5).

DISCUSSION AND CONCLUSIONS

There have been conflicting reports in the literature on the *in vivo* effect of lathyrogens on macromolecule synthesis. Polysaccharides, for example, have been shown to exhibit no change, an increase, and a decrease in total synthesis (15–17). Collagen synthesis has also been reported to be normal (18, 19), retarded (20, 21), or accelerated (22). The *in vitro* model system used in this study (5 m*M* β-aminopropionitrile) consistently showed an increase in collagen synthesized into the medium (1), an increase in $^{35}SO_4$ uptake (3), and a decrease in proliferation (1, 3).

At a concentration of 0.5 m*M*, sodium salicylate had little or no effect on cell proliferation. This is a dose-response effect because higher concentrations have a variety of effects, depending on concentration and tissue involved (11, 23). The reversal of growth inhibition produced by the lathyrogen was pronounced whether the sodium salicylate was administered at the beginning of the culture period or on the 4th day when the lathyritic effect was clearly established.

Collagen in the medium was clearly increased by 5 m*M* β-aminopropionitrile, while the cell-associated material showed virtually little change in total amount. The only definitive change in the cell-associated material was an increase in the salt-soluble fractions and a slight decrease in the insoluble fractions of the lathyrogen-treated cells. Sodium salicylate reversed the lathyrogen-produced increase of a hydroxyproline-containing macromolecule in the medium and reduced the changes in the cell layer to near control values. While sodium salicylate by itself did not influence either medium- or cell-associated hydroxyproline, other studies using different model systems and dosages showed that salicylate can and does influence collagen metabolism (6).

Salicylates have been known to inhibit $^{35}SO_4$ incorporation and mucopolysaccharide synthesis in general (23, 24). However, at the dosage used in this study, sodium salicylate alone caused a slight reduction in total $^{35}SO_4$ uptake, while the combination of β-aminopropionitrile and sodium salicylate reduced the total to below control values but did not affect the ratio of cell-bound material to $^{35}SO_4$-containing macromolecule found in the medium.

Aside from the cross-linking defect, lower concentrations of β-aminopropionitrile do not seem to affect cell proliferation or other metabolic processes (25, 26). Higher concentrations, however, limit proliferation while stimulating the general synthetic capabilities of cells in culture (1–4). Experiments in this laboratory designed to test the possibility that limitation of proliferation is related to the phenomena observed demonstrated that these are separate and apparently unrelated events.

The fact that sodium salicylate tends to reverse seemingly diverse metabolic pathways is not surprising. Salicylates seem to act at several possible sites. They have been shown, among other things, to accelerate the metabolic turnover of collagen (6), inhibit the biosynthesis of noncollagenous protein (27) and mucopolysaccharide (24), increase oxygen uptake, and inhibit acetyl-CoA synthetase (28). The diversity of these biosynthetic activities suggests that the influence of the antirheumatic drug, sodium salicylate, on experimental lathyrism is a result of a general metabolic effect rather than a specific one; the exact mechanism of action remains unknown.

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Use of 8-Anilino-1-naphthalenesulfonic Acid in Radioimmunoassay of Triiodothyronine

MARY LOU BROWN[x] and JON METHEANY

Abstract □ The rationale for the use and effects of 8-anilino-1-naphthalenesulfonic acid in the radioimmunoassay of triiodothyronine is discussed. Historical background for the use of fluorescent probes, such as 8-anilino-1-naphthalenesulfonic acid, to study protein sites is outlined. By using 8-anilino-1-naphthalenesulfonic acid to block the binding of triiodothyronine to thyroxine binding globulin, it is possible to design a sensitive and precise radioimmunoassay for measurement of triiodothyronine in unextracted serum samples. Recovery of triiodothyronine added to serum was excellent, and hyperthyroid serum assayed at various dilutions produced a curve that was virtually identical with the standard curve. Standard curves are prepared either in triiodo-/tetraiodothyronine-free serum or human serum albumin to maintain the same milieu between standards and serum samples. Triiodothyronine values by radioimmunoassay ranged from 80 to 250 ng/100 ml in 243 euthyroid subjects. In patients with thyroid disease, the serum triiodothyronine concentrations were as follows: hypothyroid (n = 27), <36–145 ng/100 ml; and hyperthyroid (n = 42), 273– >1000 ng/100 ml. Twenty-two normal pregnant women had triiodothyronine serum concentrations ranging from 174 to 410 ng/100 ml. To assess a patient's thyroid status properly, serum triiodothyronine measurements are required. The triiodothyronine assay appears to be a sensitive index, particularly in the evaluation of thyroid hyperfunction.

Keyphrases □ 8-Anilino-1-naphthalenesulfonic acid—used as probe for radioimmunoassay of triiodothyronine, relevance to evaluation of thyroid hyperfunction □ Triiodothyronine—use and effects of 8-anilino-1-naphthalenesulfonic acid, radioimmunoassay □ Thyroid hyperfunction—triiodothyronine radioimmunoassay using 8-anilino-1-napththalenesulfonic acid as fluorescent probe □ Radioimmunoassay—8-anilino-1-naphthalenesulfonic acid as fluorescent probe in analysis of triiodothyronine □ Fluorescent probes—8-anilino-1-naphthalenesulfonic acid, radioimmunoassay of triiodothyronine

In a previous paper (1), it was demonstrated that highly specific antibodies to triiodothyronine can be produced by coupling it to human serum albumin and that the antiserum was suitable for the detection of picogram quantities of triiodothyronine from unextracted serum. Additional clinical experience with the radioimmunoassay of triiodothyronine has demonstrated quite clearly that serum proteins, particularly thyroxine binding globulin, compete with the antibody for binding of both labeled and unlabeled triiodothyronine. This interference has significant effects on the assay and, of course, on the triiodothyronine value reported for the patient.

Thyroxine, tetrachlorothyronine, diphenylhydantoin, sodium salicylate, and 8-anilino-1-naphthalenesulfonic acid are some of the compounds used by various investigators to block the binding of triiodothyronine to thyroxine binding globulin (2–6).

Additional problems may be caused by displacement of the radioactive label from the antibody in the presence of serum, even though it is free of the antigen in question. Therefore, most radioimmunoassays include antigen-free serum or plasma in the preparation of the standard curve. Mitsuma *et al.* (3) and Larsen (5) observed displacement of labeled triiodothyronine by triiodothyronine-free human serum. It has been suggested that the dextran-coated charcoal method of separating antibody bound ^{125}I-triiodothyronine from free ^{125}I-triiodothyronine has the advantage over double-antibody systems in that the effect of thyroxine binding globulin on the distribution of ^{125}I-triiodothyronine in the serum sample can be recognized and a correction applied (5). The comparative merits of these two systems in the radioimmunoassay of triiodothyronine are presently being evaluated in this laboratory.

This paper examines: (*a*) the rationale for the use of 8-anilino-1-naphthalenesulfonic acid in the triiodothyronine assay; (*b*) effects of adding 8-anilino-1-naphthalenesulfonic acid to the assay; and (*c*) any possible differences that may result from preparation of the standard curve in triiodo-/tetraiodothyronine-free serum, plain phosphate-buffered saline buffer, or human serum albumin.

EXPERIMENTAL

Materials—The following were used: L-triiodothyronine[1], charcoal[2], ^{125}I-triiodothyronine[3] with specific activity of 350–544 mCi/mg, and the probe, 8-anilino-1-naphthalenesulfonic acid[4]. Antiserum to triiodothyronine was produced in rabbits as described previously (1).

Preparation of Triiodo-/tetraiodothyronine-Free Serum—To maintain the same milieu between standards and serum sample and to eliminate any differences that may occur between standard curves prepared in buffer and in serum, a serum with a very low triiodo-/tetraiodothyronine content was prepared as follows. Ten grams of charcoal[2] was added to 100 ml of normal human serum and incubated at 4° for 24 hr. The serum was then centrifuged for three 1-hr periods at 15,000×g. The procedure removed over 95% of the triiodo- and tetraiodothyronine (comparing values before and after charcoal addition by radioimmunoassay and competitive protein binding assay) and did not affect thyroxine binding globulin binding capacity as shown by the triiodothyronine resin uptake test.

Determination of Quantity of 8-Anilino-1-naphthalenesulfonic Acid Required to Prevent Thyroxine Binding Globulin Interference—Various concentrations of 8-anilino-1-naphthalenesulfonic acid were incubated with ^{125}I-triiodothyronine, triiodothyronine antiserum, and serum samples from euthyroid, hyperthyroid, hypothyroid, and pregnant patients. In other experiments, an excess of the probe (700 μg/0.1 ml of serum) and no probe were added to the assay system to determine effects on the triiodothyronine values of a group of euthyroid patients. The object of using 8-anilino-1-naphthalenesulfonic acid in the assay of triiodothyronine is twofold: (*a*) to displace triiodothyronine bound to thyroxine binding globulin in the patient's serum so that the triiodothyronine can be recognized by the triiodothyronine antibody, and (*b*) to prevent binding of ^{125}I-triiodothyronine

[1] Sigma Chemical Co.
[2] Norit A, Sigma Chemical Co.
[3] Abbott Laboratories.
[4] Eastman Kodak Co.

to thyroxine binding globulin so that all of the ^{125}I-triiodothyronine is available for binding to the triiodothyronine antibody.

Patient Dilution Curve—Hyperthyroid serum was assayed at various dilutions from 0.010 to 0.100 ml. Triiodo-/tetraiodothyronine-free serum or human serum albumin (4.5%) was used to maintain a consistent serum sample size of 0.100 ml.

Triiodothyronine Radioimmunoassay Procedure—Reagents were added to disposable 10 × 75-mm polystyrene tubes as follows:

Preincubation—

1. Delivery of the triiodothyronine standard solution was such that the concentration ranged from 10 to 1000 pg for a 10-point standard curve or 0.1 ml of the serum to be assayed. Each point on the standard curve was assayed in triplicate.
2. Triiodo-/tetraiodothyronine-free serum (0.1 ml) or 4.5% human serum albumin was added to the 0% control tubes, the 100% control tubes, and tubes containing the standard curve.
3. A 1:5000 dilution (0.1 ml) of triiodothyronine antibody containing 1% normal rabbit serum and 0.05 *M* disodium edetate in phosphate-buffered saline buffer (pH 7.4) was added to all tubes except the 0% control tubes.
4. Phosphate-buffered saline buffer (pH 7.4) was added sufficient to adjust the final total volume to 1.0 ml. The tubes were vortexed and incubated at 4° for 24 hr.

Incubation 1—

5. Phosphate-buffered saline buffer (0.1 ml) containing 50 pg of ^{125}I-triiodothyronine (350–544 mCi/mg), 60 μg of 8-anilino-1-naphthalenesulfonic acid, and 0.25% human serum albumin was added to all tubes. The tubes were vortexed again and incubated at 4° for an additional 48 hr.

Incubation 2—

6. Precipitation of ^{125}I-triiodothyronine bound to antibody was accomplished by adding 0.1 ml of a previously titered goat anti-rabbit γ-globulin to all tubes and incubating at 4° for 24 hr.

Separation and Calculations—The details of separation of antibody-bound ^{125}I-triiodothyronine and free ^{125}I-triiodothyronine by centrifugation, correction for nonspecific binding, and calculation of results were described previously (1).

Serum Specimens—Serum was obtained from euthyroid healthy volunteers, normal pregnant women, and hyper- and hypothyroid patients[5]. The blood was allowed to clot, and the serum was separated by centrifugation and frozen at −20° unless assayed within 2 days.

RESULTS

Probe Concentration—Concentrations ranging from 70 to 1400 μg of the probe were added to serums containing different quantities of thyroxine binding globulin. Maximum percent binding of ^{125}I-triiodothyronine to triiodothyronine antiserum occurred when the probe concentration was approximately 70 μg/0.1 ml of serum in all samples (Fig. 1). Further dilution of the probe in a range of 3.75–150 μg/0.1 ml of serum showed maximum percent binding occurring between 37.5 and 75 μg. As a result, 60 μg/0.1 ml of serum was chosen for routine use in all triiodothyronine assays. The effects of no probe and an excess concentration of probe on the triiodothyronine assay were studied by assaying 18 euthyroid serum samples. Triiodothyronine serum concentrations in euthyroid serum samples containing no probe ranged from 330 to 540 ng % (437 ± 13.1, mean ± *SE*). In samples containing 60 μg of probe, the triiodothyronine concentration was 150–245 ng % (189 ± 5.9, mean ± *SE*). The samples containing 700 μg of probe contained 153–267 ng % triiodothyronine (196 ± 7.1, mean ± *SE*).

Standard Curves—Standard curves prepared in 4.5% human serum albumin or in plain phosphate-buffered saline buffer were virtually superimposable, while the curve prepared in triiodo-/tetraiodothyronine-free serum was shifted slightly to the right of that in plain phosphate-buffered saline buffer in the area of concentration of 10–200 pg triiodothyronine. Figure 2 shows a comparison in the displacement of ^{125}I-triiodothyronine by increasing quantities of triiodothyronine and a hyperthyroid serum. Results of diluting the serum with either triiodo-/tetraiodothyronine-free serum or 4.5% human serum albumin to maintain a consistent serum sample of 0.1 ml are shown in Table I. Displacement of 50% of ^{125}I-triiodothyronine from the antibody was produced by a mean triiodothyronine concentration of 118.6 ± 2.2 (*SE*) pg in 10 assays.

[5] Followed or treated at the Wilford Hall USAF Medical Center.

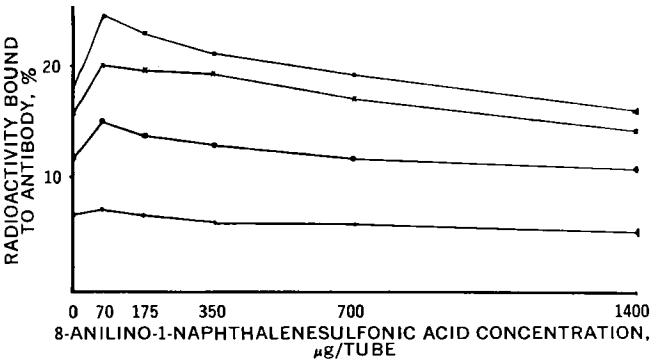

Figure 1—*Effect of addition of increasing quantities of 8-anilino-1-naphthalenesulfonic acid to different serum samples on the binding of ^{125}I-triiodothyronine to triiodothyronine antiserum. Key: ■, serum from hypothyroid subject; ×, serum from euthyroid subject; ○, serum from pregnant subject; and ●, serum from hyperthyroid subject.*

Recovery of Triiodothyronine Added to Serum—Serum from euthyroid, hypothyroid, and pregnant subjects was enriched with triiodothyronine in concentrations ranging from 50 to 500 ng/100 ml. The recovery of added triiodothyronine (Table II) averaged 105.3 ± 2.7% (mean ± *SE*).

Reproducibility of Triiodothyronine Assay—Within-assay variability was evaluated by duplicate determinations of serum from a euthyroid subject assayed as four pairs and was found to be 210 ± 1.3 (*SE*) ng %. The percent standard deviation within the assay was 1.9%. Between-assay variability was evaluated by duplicate measurements of euthyroid and hyperthyroid serums in the same and in 10 different assays by four technicians. The euthyroid serum triiodothyronine concentration was 160 ± 3.5 (*SE*) ng %, and the percent standard deviation was 6.9. The hyperthyroid serum was assayed as 283 ± 5.0 (*SE*) ng %, and the percent standard deviation was 7.3%.

Clinical Results—Triiodothyronine levels were determined in euthyroid subjects, hyper- and hypothyroid patients, and a group of normal pregnant patients (Table III). The quantity of serum assayed was 0.1 ml, and 27–52 pg of unlabeled triiodothyronine [36 ± 2.2 (*SE*) pg for 10 assays] produced meaningful inhibition (20%) of ^{125}I-triiodothyronine binding to antibody; therefore, the minimum detectable triiodothyronine concentration was generally 36 ng/100 ml. The 243 euthyroid subjects had tetraiodothyronine concentrations of 4.5–11.5 μg/100 ml [7.7 ± 1.5 (*SD*) μg %, uncorrected for extraction, extraction efficiency = 83.2%] as determined by the Murphy (7) procedure. The triiodothyronine resin

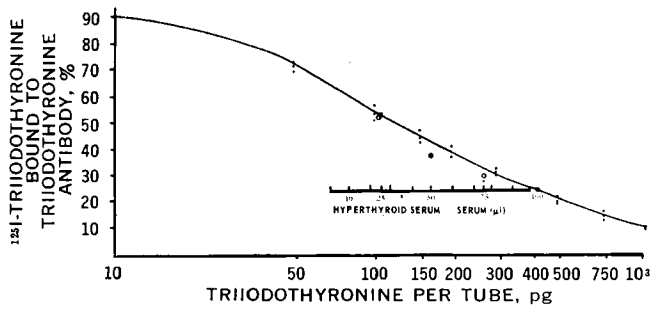

Figure 2—*Dose-response curve prepared in 0.1 ml of 4.5% human serum albumin, showing inhibition of binding of ^{125}I-triiodothyronine by unlabeled triiodothyronine and a hyperthyroid serum. Serum is matched to curve at the 0.1-ml dilution and shows reasonable fit at various dilutions along the standard curve. Key: ×, serum diluted to 0.1 ml with 4.5% human serum albumin; and ○, serum diluted to 0.1 ml with triiodo-/tetraiodothyronine-free serum.*

Table I—Triiodothyronine Concentrations in Dilutions of Serum from a Hyperthyroid Patient

Amount of Serum Assayed, ml	Triiodothyronine[a]	Triiodothyronine
0.1	410[b]	410[c]
0.075	335	305
0.050	205	205
0.025	103	105
0.010	44	54

[a] Triiodothyronine in ng/100 ml for all dilutions. [b] Serum diluted to 0.1 ml with 4.5% human serum albumin. [c] Serum diluted to 0.1 ml with triiodo-/tetraiodothyronine-free serum.

uptake values for this group ranged from 24.1 to 36.4% [30.4 ± 2.7 (*SD*) %]. In hyperthyroid patients, serum triiodothyronine concentrations ranged from 273 to >1000 ng/100 ml, all values being greater than the highest of the euthyroid group. There was some overlap between the hypothyroid and euthyroid subjects. Six patients in the hypothyroid group had triiodothyronine concentrations in the euthyroid range of 80–250 ng/100 ml. Normal pregnant women from 19.5 to 40 weeks of gestation had serum triiodothyronine concentrations of 170–410 ng/100 ml.

DISCUSSION

A major improvement in the radioimmunoassay of serum triiodothyronine has been accomplished by the use of 8-anilino-1-naphthalenesulfonic acid, which is a fluorescent dye with the general formula $C_6H_5NHC_{10}H_6SO_3H$. The use of dyestuffs to characterize human serum was introduced over 50 years ago when the ability of serum and its fractions to bind dyes was demonstrated (8, 9). Bennhold (10) proposed the transporting function of human serum albumin through the similar use of a variety of dyes.

Many studies from 1935 to 1955 elaborated on the use of dyestuffs for serum protein characterization. The practical importance of the phenomenon of protein absorption of dyes was demonstrated, thus establishing a foundation for research in this area (11, 12). In the early 1950's, Weber (13) and Lawrence (14) developed and utilized the method of measurement of the polarization of light emitted by excited fluorescent molecules in characterizing the binding properties of human serum albumin. They reported later on the significance of particular fluorescing dyes of the naphthalene and acridine series which possessed anilino or certain substituted anilino groups. They demonstrated that only in the presence of organic molecules such as those in serum would these dyes fluoresce in aqueous solvents (15).

By 1968, the development of the use of fluorescent probes had significantly advanced. Stryer (16) showed 8-anilino-1-naphthalenesulfonic acid to be an excellent probe in characterizing the binding of heme to apomyoglobin and apohemoglobin and utilized its ability to displace heme from the binding sites on each.

Table II—Recovery of Triiodothyronine Added to Serum

Patient Serum	Initial Serum Triiodothyronine[a]	Triiodothyronine Added	Triiodothyronine		Recovery, %
			Expected	Recovered	
Pregnant subject	255	50	305	300[b]	98.4
		100	355	345	97.2
		200	455	490	107.7
		500	755	920	121.9
Hypothyroid subject	62	50	112	112	100
		100	162	158	97.5
		200	262	260	99.2
		500	562	610	108.5
Euthyroid subject	159	50	209	205	98.1
		100	259	265	102.3
		200	359	390	108.6
		500	659	820	124.4
	Average (mean ± *SE*):				105.3 ± 2.7

[a] All triiodothyronine serum concentrations in ng/100 ml. [b] Mean of duplicate determinations.

Table III—Serum Triiodothyronine Concentrations

Clinical Status	Number	Range, ng/100 ml	Mean Range, ng/100 ml
Euthyroid	243	80–250	183 ± 41 (mean ± *SD*)
Hyperthyroidism	42	273–>1000	—
Hypothyroidism	27	<36–145	—
Normal pregnancy	22	174–410	275 ± 12.4 (mean ± *SE*)

McClure and Edelman (17–19) elaborated on this by using different fluorescent probes with several serum proteins and studying the binding characteristics of each.

More recently, Green *et al.* (20) used 8-anilino-1-naphthalenesulfonic acid to characterize the binding site of thyroxine binding globulin, demonstrating that 8-anilino-1-naphthalenesulfonic acid competes with thyroxine for the same binding site. In the field of radioimmunoassay, Chopra utilized this property of 8-anilino-1-naphthalenesulfonic acid to prevent protein interference in the radioimmunoassay of triiodothyronine (6) and thyroxine (21).

It has been established that thyroxine binding globulin is a glycoprotein consisting of a linear series of peptide bonds with amino acid and carbohydrate side chains (19, 22, 23). Due to environmental interaction with solvents, with other solutes, and within itself, thyroxine binding globulin takes on a folded and twisted configuration (19). With the use of fluorescent probes, thyroxine binding globulin has demonstrated only one binding site for triiodothyronine and tetraiodothyronine. This has been characterized as being in a severe fold of a hydrophobic nature interspersed with amino acid residues that possibly can ionize in the presence of strong electronegative groups to become positively charged (14–17, 19, 20, 22–27).

The bond also exhibits properties of hydrogen bonding with triiodothyronine and tetraiodothyronine, due perhaps to the presence of the imidazole ring of histidine, the nitrogens of which have a certain affinity for hydrogen (25, 27, 28). Thus, thyroxine binding globulin apparently binds to small molecules exhibiting a hydrophobic nature and capable of hydrogen bonding and/or possessing high electronegativity. Additional studies using analogs of tetraiodothyronine have shown that molecules possessing hydroxy groups, amino side chains, a preponderance of iodine on a phenyl ring structure (distal to the amino acid group), and large, bulky naphthoxy or naphthalene groups have a definite and greater affinity for thyroxine binding globulin (26, 27). An examination of the chemical structure of triiodothyronine and 8-anilino-1-naphthalenesulfonic acid shows that both of these small molecules meet these criteria and may, therefore, be expected to exhibit a certain affinity for thyroxine binding globulin.

The hydrophobic nature of the diphenyl ether structure of triiodothyronine or the naphthalene group of the probe satisfies one basic requirement of binding to thyroxine binding globulin (14–17, 26, 27). (The bulky naphthalene structure of the probe satisfies another requirement of strong affinity for thyroxine binding globulin. This suggests that the binding site of thyroxine binding globulin is better occupied by a molecule of tighter fit such as that exhibited by tetraiodothyronine analogs with naphthoxy or naphthalene groups.) The triiodothyronine molecule possesses iodine on the outer phenolic ring, and the iodine's electronegative character is particularly important in the distal conformation. This conformation shows not only tighter binding but more hormonal activity (29, 30). This further suggests that an induced positive ionic residue is in close proximity to the electronegative iodine of the distal conformation rather than the proximal triiodothyronine conformation due to the relative bond strengths (27).

Similarly, the sulfonate group of the probe possesses high electronegativity and would seem to induce electrostatic attractions with amino acid residues that easily assume a positive ionic behavior (14, 25). The hydrogen bonding proposed between the hydroxyl group of triiodothyronine and the thyroxine binding globulin binding site is also important. The presence of this bond would attest to a definite affinity of thyroxine binding globulin for triiodothyronine as proposed (25–28). The relative impor-

tance of each characteristic in the affinity of thyroxine binding globulin for triiodothyronine and 8-anilino-1-naphthalenesulfonic acid infers a difference in binding ability.

Comparisons of the relative affinity of tetraiodothyronine and triiodothyronine for thyroxine binding globulin have been made. When assuming that levothyroxine has a relative affinity of 100, distal triiodothyronine has an affinity of 3.3 and proximal triiodothyronine has an affinity of 0.8 (27). The association constants of tetraiodothyronine and the probe for thyroxine binding globulin have been reported as follows: K_a tetraiodothyronine = 2.35 ± 0.43 × 10^{10} m^{-1} at 23°, and K_{assoc} 8-anilino-1-naphthalenesulfonic acid = 2.09 ± 0.16 × 10^6 m^{-1} at 37° (20). Scatchard (31) reported the K_{assoc} 8-anilino-1-naphthalenesulfonic acid for thyroxine binding globulin as 4.19 ± 0.16 × 10^6 m^{-1} at 23°. Thus, when triiodothyronine and the probe compete for thyroxine binding globulin binding sites, there is indeed a competitive equilibrium reaction.

It can be stated, therefore, that thyroxine binding globulin has a decreasing affinity for tetraiodothyronine, triiodothyronine, and the probe, respectively, and that when the probe is present in sufficient concentrations, it displaces almost all of the tetraiodothyronine and triiodothyronine from thyroxine binding globulin. The quantity remaining bound to thyroxine binding globulin would be inconsequential.

If endogenous triiodothyronine was not displaced from thyroxine binding globulin, unmeasurable serum triiodothyronine concentrations would be expected. This is not the case. Recovery of triiodothyronine added to serum is also excellent, thus suggesting strongly the validity of the radioimmunoassay method. When 500 ng % of triiodothyronine was added to the serums from pregnant and euthyroid subjects, the percent recovery was much greater than expected. This was primarily due to the flattening out of the curve in the assay system at levels >500 ng/100 ml. When hypothyroid serum is used, percent recovery is excellent all along the standard curve. Excess concentrations of the probe appear to have no significant effects on the antigen–antibody reaction, as shown by the lack of significant differences (paired t-test, p >0.10) in mean serum triiodothyronine concentrations of 18 euthyroid subjects in assays containing 60 and 700 μg 8-anilino-1-naphthalenesulfonic acid/assay tube. This would also suggest that even with probe concentrations in excess of those required to displace triiodothyronine and tetraiodothyronine from thyroxine binding globulin since tetraiodothyronine has a greater affinity for thyroxine binding globulin than either triiodothyronine or the probe (thus requiring more 8-anilino-1-naphthalenesulfonic acid for displacement from thyroxine binding globulin), tetraiodothyronine does not alter the measured triiodothyronine value because there is little difference between the means of the 60 and 700 μg 8-anilino-1-naphthalenesulfonic acid–triiodothyronine values. This would be expected since the triiodothyronine antibody was previously shown to lack significant cross reaction with tetraiodothyronine (1).

Significant differences of triiodothyronine values did not occur between standard curves prepared in 4.5% human serum albumin or triiodo-/tetraiodothyronine-free serum. Since the standard curve containing the triiodo-/tetraiodothyronine-free serum was not displaced to the left of that prepared in buffer, it may be assumed that the triiodo-/tetraiodothyronine-free serum is, in fact, essentially free of triiodothyronine. The slight displacement of the serum standard curve to the right of the buffer curve may be due to nonspecific interactions of serum proteins on the equilibrium reaction. Tubes containing triiodothyronine antibody, ^{125}I-triiodothyronine, and either triiodo-/tetraiodothyronine-free serum or human serum albumin (100% tubes) have a lower percent binding than those containing only buffer. This suggests that triiodothyronine may be binding to certain serum proteins or, again, that there is a nonspecific inhibition by serum protein on the antigen–antibody equilibrium. Serum triiodothyronine concentrations are significantly higher when the standard curve is prepared in plain buffer, due to the higher percent binding of ^{125}I-triiodothyronine to the antibody when serum proteins are not present.

With the use of 8-anilino-1-naphthalenesulfonic acid as a thyroxine binding globulin blocking agent and either 4.5% human serum albumin or triiodo-/tetraiodothyronine-free serum in the preparation of the standard curve, the radioimmunoassay procedure is an accurate measure of the concentration of triiodothyronine in serum. The excellent recovery of exogenous triiodothyronine added to various serum samples, the virtually identical curve of different dilutions of serum from hyperthyroid subjects with that of the standard curve, and the satisfactory within- and between-assay variability all validate the radioimmunoassay procedure.

The triiodothyronine serum concentration in euthyroid subjects of 183 ± 41 (*SD*) ng % reported here is in reasonable agreement with the mean range of values of 102–218 ng/100 ml determined by other radioimmunoassay procedures (32, 33). Differences in triiodothyronine serum concentrations determined by different radioimmunoassay systems may be due to: (*a*) specific factors within the assay such as sensitivity, specificity, and nonspecific serum effects; (*b*) geographic location and dietary iodine; and (*c*) sample size.

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[x] To whom inquiries should be directed.

Ocular Evaluation of Methylcellulose Vehicle in Albino Rabbits

SUKHBIR S. CHRAI* and JOSEPH R. ROBINSON ˣ

Abstract □ Viscous solution drainage from the eyes of unanesthetized albino rabbits was determined and related to drug bioavailability. When using methylcellulose as the viscosity-inducing polymer, it was shown that the rate of solution drainage is related to viscosity and decreases with increasing viscosity. Over a range of 1–15 cps viscosity of the instilled solution, there is a threefold change in the drainage rate constant and a further threefold change over the viscosity range of 15–100 cps. The decline in precorneal drug concentration was determined to be first order in concentration, and the rate of decline was proportional to the viscosity of the instilled solution. When using pilocarpine nitrate as the test drug in both miosis and aqueous humor drug concentration studies, it was shown that, over a range of 1–15 cps solution viscosity, there is a linear relationship between the first-order drainage rate constant and both the miotic activity and aqueous humor drug levels attained. Comparison of the present study in rabbits to reported behavior in humans suggests that the vehicle viscosity influence is qualitatively similar but quantitatively different in both species, with the quantitative difference being due, in part, to the difference in rate of blinking and precorneal solution movement in rabbits as compared to humans.

Keyphrases □ Ophthalmic vehicle viscosity—effect on ocular drug bioavailability, pilocarpine nitrate in methylcellulose, rabbits, relationship to effect in humans □ Methylcellulose—effect as vehicle on ocular drug bioavailability of pilocarpine nitrate, rabbits, relationship to effect in humans □ Viscosity effect of ophthalmic vehicle (methylcellulose)—bioavailability of pilocarpine nitrate, rabbits

It has long been assumed that a substantial increase in ocular drug bioavailability can be achieved by placement of the drug in a high viscosity vehicle prior to instillation into the eye. Presumably, the highly viscous solution would prolong contact of the drug with eye tissues, resulting in a greater drug absorption into the appropriate eye area. Over the years, this belief has been strengthened by qualitative and semiquantitative studies on vehicle viscosity effects in both humans and experimental animals. A recent report (1) challenged this belief and concluded that increasing vehicle viscosity does not prolong ocular contact time nor markedly improve ocular drug bioavailability in humans. Specifically, these researchers showed that aqueous humor levels of fluorescein increased only slightly with relatively large increases in solution viscosity. This small increase was attributed to an elevated initial concentration of fluorescein in the tear film resulting from less initial loss of drug solution from the precorneal area following topical instillation.

This earlier work (1), while shedding considerable light on the influence of vehicle viscosity in humans, has not provided a complete quantitative picture of the influence of solution viscosity on ocular drug bioavailability, nor has it explained contradictory results in animals. The present study attempts to provide a quantitative picture of ophthalmic vehicle viscosity effects in albino rabbits and its relationship to behavior in humans.

EXPERIMENTAL

Materials—USP grade pilocarpine nitrate[1] was used as received, but the tritiated pilocarpine nitrate[2] was subjected to further purification (2) by repeated vacuum evaporation. Usually, three such evaporations were required to remove tritiated solvent, which was present as a result of tritium exchange. In each evaporation the solution was reduced to a very small volume and then brought back to volume by addition of water. The purification end-point was reached when the measured radioactivity reached a constant value and purity was established by TLC. The final isotopic activity in the drug solution was approximately 50,000 counts min^{-1} μl^{-1}. All other chemicals were either USP or reagent grade.

Adult, male, albino rabbits[3], weighing 1.8–2.4 kg, were used without any special pretreatment diets.

Solution Preparation—The preparation and composition of technetium sulfur colloid solutions, pilocarpine nitrate solutions, and anesthetic solutions were described earlier (2–5). Methylcellulose–technetium solutions were prepared by mixing equal volumes of an appropriate concentration of methylcellulose solution and technetium colloid solution. The methylcellulose solutions were prepared by addition of hot water to methylcellulose 100, followed by cooling of the resultant mixture until solution was effected. The final volume of the solution was then adjusted and the flask was equilibrated to the appropriate temperature prior to use. Methylcellulose–pilocarpine nitrate solutions were prepared in the same manner as the methylcellulose–technetium solutions, with the modification that Sørensen's pH 7.38 phosphate buffer solution was used instead of water in preparation of the methylcellulose solution. All solutions were prepared fresh.

Procedures—*Viscosity Measurements*—Solution viscosity was determined with a viscometer[4] at 25 ± 0.1°.

Drainage Studies—A complete description of the nonsampling isotopic technique for measuring solution drainage was reported previously (3). Unanesthetized, preconditioned rabbits were used for all experiments.

Miosis–Time Studies—Unanesthetized, preconditioned rabbits were used as described previously (6). No animal was used more than once, and at least five separate determinations were made for each experiment. Twenty-five microliters of 1×10^{-2} *M* pilocarpine nitrate solution, containing various concentrations of methylcellulose, was instilled into one eye of an experimental animal; the other eye served as a control. Pupillary diameter measurements commenced immediately postinstillation of drug and continued until the pupil size returned to its normal value (4).

Aqueous Humor Drug Studies—Experimental details of aqueous humor–drug concentration studies were reported previously (2). Unanesthetized rabbits were used in all experiments.

RESULTS

Effect of Vehicle Viscosity on Drainage Rate—Several methylcellulose–technetium solutions of varying viscosity were prepared and instilled into the eyes of unanesthetized rabbits, fol-

[1] Merck and Co., Rahway, N.J.
[2] New England Nuclear, Boston, Mass.
[3] Klubertanz, Edgerton, Wis.
[4] Brookfield Engineering Labs., Stoughton, MA 02072

Table I—Fractional Amount of Technetium Tracer Remaining in the Precorneal Pocket as a Function of Time for 25 μl of Solutions of Varying Viscosity

Seconds	Fractional Amount of Drug Remaining					
	100[a] (6)[b]	40 (4)	12.5 (6)	4.2 (6)	1.8 (8)	1.3 (7)
50	0.97 (0.03)[c]	0.86 (0.05)	0.78 (0.05)	0.72 (0.04)	0.59 (0.06)	0.51 (0.05)
100	0.91 (0.04)	0.74 (0.05)	0.71 (0.05)	0.55 (0.04)	0.47 (0.05)	0.42 (0.05)
150	0.87 (0.02)	0.64 (0.04)	0.63 (0.03)	0.48 (0.04)	0.42 (0.06)	0.36 (0.06)
200	0.85 (0.04)	0.57 (0.04)	0.55 (0.03)	0.43 (0.02)	0.36 (0.04)	0.32 (0.06)
250	0.81 (0.06)	0.54 (0.04)	0.48 (0.03)	0.39 (0.04)	0.35 (0.05)	0.29 (0.06)
300	0.79 (0.08)	0.52 (0.05)	0.45 (0.04)	0.37 (0.03)	0.34 (0.05)	0.28 (0.04)
350	0.76 (0.12)	0.51 (0.06)	0.43 (0.05)	0.36 (0.04)	0.34 (0.03)	0.26 (0.04)
400	0.74 (0.15)	0.49 (0.06)	0.41 (0.03)	0.35 (0.05)	0.32 (0.03)	0.25 (0.04)
450	0.73 (0.14)	0.48 (0.06)	0.40 (0.06)	0.35 (0.03)	0.30 (0.02)	0.24 (0.03)
500	0.71 (0.13)	0.47 (0.06)	0.40 (0.02)	0.34 (0.03)	0.30 (0.02)	0.23 (0.03)
550	0.72 (0.14)	0.46 (0.06)	0.40 (0.03)	0.34 (0.04)	0.30 (0.03)	0.23 (0.03)
600	0.71 (0.14)	0.45 (0.05)	0.37 (0.03)	0.34 (0.04)	0.30 (0.03)	0.23 (0.03)

[a] Viscosity of the instilled solution in centipoises. [b] Number of determinations. [c] Standard deviations are shown in parentheses.

lowed by measurement of technetium radioactivity in the tear film at different time intervals. In all cases, the volume of solution instilled was kept constant at 25 μl.

The fractional amounts of technetium tracer remaining in the tear film as a function of time are shown in Table I. Except for the 100-, 150-, and 200-sec points of the 40- and 12.5-cps runs, there is a statistically significant difference for all other values with 90% confidence limits and, in most cases, with 95% confidence limits. The data in Table I are presented graphically as smooth curves in Fig. 1.

Cessation in the decline of radioactivity occurred at about 600–700 sec for all viscosities (Table I and Fig. 1). In the case of the 100-cps viscosity solution, a large standard deviation is associated with the values obtained after 300 sec. This is due to the fact that in a few runs there was no decline in radioactivity after 250 sec and in others the decline was continuous, thus widening the gap between experimental values.

A possible explanation for the plateau region is based on the rate of blinking of the experimental animal. Since the rabbit blinks infrequently, the decrease or cessation in the decline of radioactivity is interpreted as due to formation of a relatively stable film. The fact that the curves for fraction of radioactivity remaining against time for 1.3- and 1.0-cps solutions are superimposable for the first 500 sec and then bifurcate—1.3 cps being almost a plateau and 1.0 cps continuing to decline—lends support to the postulate of a relatively stable film. When the animal blinked, this film appeared to lose its integrity and there was a further decrease in radioactivity. Thus, the plateau region in Fig. 1, from 600 to 1000 sec, is probably due to a lack of blinking by the animal together with perhaps temporary blockage of the puncta and/or formation of a film.

The thickness of the layer as physically observed at about 600 sec appeared to be proportional to the viscosity of the instilled solution. Since the film did not appear to expand over 5–15 min, it may be assumed that tear production is inhibited during this period. Mueller and Deardorff (7) felt that polymer solutions might decrease reflex lacrimation, so normal tear turnover might be inhibited. This possibility of tear inhibition appears unlikely in view of the results of Adler *et al.* (1) as well as the calculation of precorneal drug concentrations at various times postinstillation, as will be discussed. Thus, the plateau in the fractional amount remaining against time plot is ascribed to a lack of blinking by the rabbit. Destruction of the film by blinking or forcing the pool solution out by blinking results in a further decline of radioactivity. The rate of decline of radioactivity after rupture of the film at long time periods postinstillation is not close to the rate of turnover of tears. It was not possible to carry out the experiments long enough to determine when the rate of decline in isotopic activity could be ascribed to normal tear turnover.

The initial decline in radioactivity (Fig. 1) appears to be proportional to the viscosity of the instilled solution. Nonproductive drug loss by adsorption or absorption into eye tissues is expected to be minimal, since both methylcellulose and technetium sulfur colloid are not absorbed from the conjunctival sac. Adler *et al.* (1), in their vehicle studies in humans, reported that polymer solutions such as methylcellulose did not increase corneal contact time. This is clearly not the case in rabbits, as judged by the results shown in Fig. 1 as well as by the work of others (8–11). This inconsistency can perhaps be ascribed to the difference in blinking rate of humans *versus* rabbits as well as to the difference in tear turnover rate between these species (3).

First-order drainage rate constants were determined from the slope of the line of plots of logarithm of the volume remaining against time for the first few minutes postinstillation as shown in Eq. 1 (4):

$$V_r = V_0 e^{-k_{dv} t} \qquad \text{(Eq. 1)}$$

where k_{dv} is the drainage rate constant for viscous instilled solutions.

Table II shows the drainage rate constants for various viscosity solutions and clearly indicates that the drainage rate is significantly retarded as the viscosity of the solution is increased. For a 100-fold change in viscosity, there is an overall 10-fold decrease in the drainage rate constant. From the standard deviations pre-

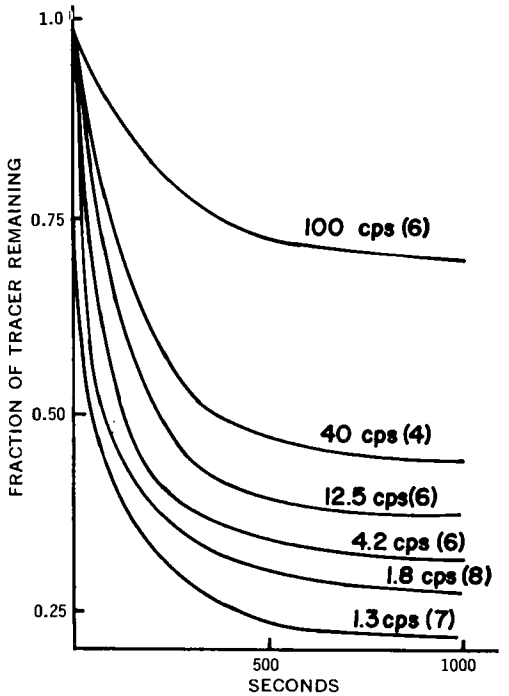

Figure 1—*Fractional amount of drug remaining as a function of time for 25 μl of solutions of varying viscosity. The number in parentheses indicates the number of runs conducted at each instilled volume, and the lines represent the mean values for these runs. Each line was generated from a minimum of 20 points, many of which are shown in Table I.*

Table II—First-Order Rate Constants for Drainage of Instilled Solution of Varying Viscosities[a]

Viscosity of Instilled Solution, cps	Drainage Rate Constant, k_{dv}, $sec^{-1} \times 10^3$
1.0	9.1
1.3	9.1
1.8	7.7
4.2	6.0
12.5	3.0
40.0	2.4
100.0	0.88

[a] Volume of the solution instilled was 25 μl.

sented in Table I, the calculated rate constants are probably accurate to ±5–10%.

To determine if there is a direct relationship between the drainage rate constant and vehicle viscosity, the calculated first-order drainage rate constants were plotted against the viscosity of the instilled solution (Fig. 2). Although a thorough investigation was not performed, it appears that the distribution of the experimental points is biphasic. The first phase is a relatively rapid decrease in the drainage rate constant with a small increase in viscosity, and the second phase is a small decrease in the drainage rate constant with a large increase in viscosity. The two lines appear to intersect each other at about 15–20 cps, which may be said to be the optimum viscosity of an instilled solution of methylcellulose for rabbits. A slight increase in viscosity above this optimum viscosity does not lead to a proportional improvement in drainage property.

The range of viscosity employed in this study was restricted at the lower end to a simple aqueous solution and at the upper end by the maximum viscosity the eye can tolerate without rapid blockage of the drainage duct. With the highest viscosity used, 100 cps, great care was needed in instilling the drug solution onto the cornea. In cases where the drug solution was inadvertently instilled into the cul-de-sac instead of onto the cornea, the animal would close its eye very tightly and keep the viscous solution from

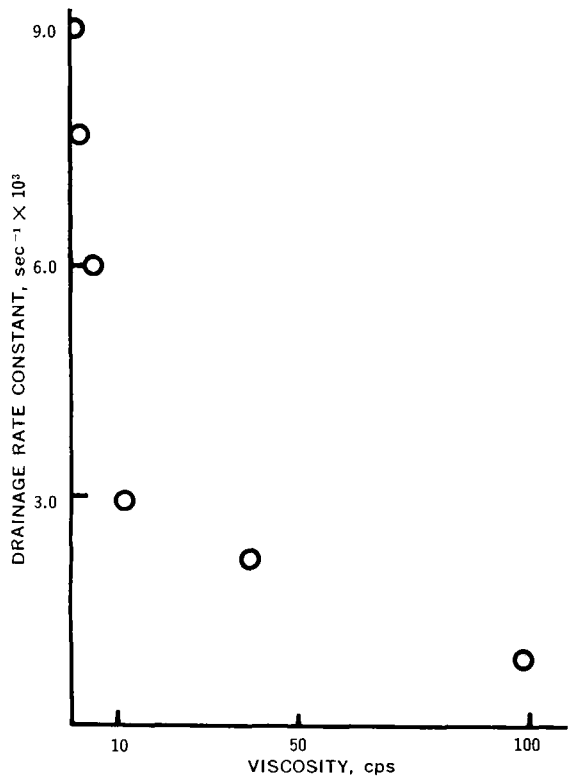

Figure 2—*Drainage rate constants,* k_{dv}, *as a function of the viscosity of the instilled methylcellulose–technetium solution. The volume instilled in all cases was 25 μl.*

Table III—Average Values for the Change in Pupillary Diameter at Random Times for Different Viscosity Solutions

Minutes	Average Change in Pupillary Diameter, mm		
	1[a] (6)[b]	1.8 (5)	4.2 (5)
20	1.87 (0.13)[c]	2.20 (0.16)	2.28 (0.15)
30	2.12 (0.11)	2.37 (0.06)	2.58 (0.14)
40	2.05 (0.12)	2.20 (0.16)	2.50 (0.17)
90	1.10 (0.20)	1.35 (0.21)	1.60 (0.20)

[a] Viscosity of the solution instilled in centipoises. [b] Number of experimental runs. [c] Standard deviations are shown in parentheses.

mixing and draining. Such holding of the solution in the pocket was observed in a few runs and these runs were discarded. There was no decline in radioactivity in such cases for the first few minutes postinstillation.

Miosis–Time Study to Demonstrate Effect of Vehicle Viscosity on Drug Activity—To determine the influence of viscosity of the instilled solution on drug activity, several methylcellulose solutions containing 1×10^{-2} *M* pilocarpine nitrate were prepared and instilled into the eyes of unanesthetized rabbits. The volume of solution instilled was maintained at 25 μl. If there is a change in the drainage rate for these solutions, it would be expected that the extent of drug absorption and hence the peak height, area under the miosis–time curves, and duration of the miotic effect would be different and proportional to solution viscosity.

The change in pupillary diameter due to the miotic effect of pilocarpine nitrate was determined at approximately 25–35 time points, representing the full range of drug activity (4) for several viscosity solutions. Table III illustrates the magnitude of the miotic effect and experimental reproducibility for a few random time points. There is a statistically significant difference with a 90% confidence limit between the results of the three viscosities used at all time points; for some time points, such as the values at the peak height, there is a difference with a 95% confidence limit.

Table IV provides the area under the miosis–time curves, duration of the miotic effect, and first-order rate constants for elimination and absorption. The first-order rate constants for elimination were determined from the terminal slope of the lines of logarithm change in pupillary diameter against time plots. The first-order rate constants for absorption were determined by graphical analysis using the feathering technique. From the standard deviations presented in Table III, the experimental or calculated values for the different parameters presented in Table IV are probably accurate to ±5–10%.

The areas under the miosis–time curves, the peak heights, and the duration of the miotic effect of pilocarpine nitrate are presumably proportional to the concentrations of drug reaching the target area which, in turn, is proportional to the concentrations of drug present in the tear fluid. Since all of these solutions initially had the same concentration, the increased miotic effect observed as the viscosity of the instilled solution is increased is due to the fact that the fraction of the instilled solution remaining in the tear film for different viscosity solutions is different. The time to reach a maximum in the miosis–time profile remained constant for all solutions tested, thus tending to rule out a vehicle-controlled availability. Figure 3 shows plots of the first-order drainage rate constants, k_{dv}, against the peak height of miotic effect, the area under the miosis–time curves, and the duration of miotic effect. Excellent linearity is observed in all three cases, supporting the idea that increased ocular drug bioavailability is due to a decreased rate of drainage.

Determination of Effect of Viscosity of Instilled Solution on Aqueous Humor Drug Concentration—Several solutions of 1×10^{-2} *M* pilocarpine nitrate of varying viscosity were prepared and instilled into the eyes of unanesthetized rabbits to determine the influence of drug solution viscosity on the aqueous humor drug concentration attained. Aqueous humor samples were withdrawn at 20 and 30 min postinstillation of drug, and the pilocarpine nitrate concentrations were determined. These particular time periods were chosen because they represent the time periods for maximum drug concentration in aqueous humor (2). The average concentrations of pilocarpine nitrate reaching the anterior

Table IV—Effect of Viscosity of Instilled Solution on Miotic Effect of Pilocarpine Nitrate in Rabbits

Viscosity of Instilled Solution, cps	Peak Height[a], mm	Area under Curve[b], cm^2	Duration of Miotic Effect[c], min	First-Order Rate Constant for Absorption, min^{-1}	First-Order Rate Constant for Elimination, min^{-1}
1	2.12	92	172	0.077	0.012
1.8	2.37	109	202	0.077	0.016
4.2	2.58	132	235	0.076	0.011

[a] The peak height is the average of maximum change in pupillary diameter and is taken as the change in pupillary diameter at 30 min. [b] Units are millimeters per minute. On the vertical axis, 1 cm equals 0.20 mm; on the horizontal axis, 1 cm equals 10 min. [c] Time for pupil to return back to normal, *i.e.*, the time when change in pupillary diameter is zero.

chamber, along with the number of runs and standard deviations, are presented in Table V. Since some of the standard deviations shown in Table V appear high, a rejection test was performed on the individual values. At the 90% level, no value could be rejected. In addition, the values at both 20 and 30 min were significantly different with a 90% confidence limit, except for the 1.8- and 4.2-cps values in the 30-min runs.

From Table V, it is clear that higher aqueous humor drug levels are achieved with increasing solution viscosity, indicating that the extent of drainage loss is not the same for each solution. There is approximately a twofold increase in aqueous humor drug levels for a 100-fold increase in solution viscosity.

The increase in aqueous humor drug concentrations might be ascribed to a slow release of drug from the vehicle at higher vehicle viscosities, *i.e.*, vehicle control. If this rate of release of drug from the vehicle is the controlling factor, it would be expected that the peak concentration of the aqueous humor drug concentration would shift toward the right and that the degree of shift would be proportional to the viscosity of the instilled solution. Careful examination of the results indicates that, at 30 min postinstillation, there is a twofold increase in aqueous humor drug concentration with a 100-fold increase in viscosity, the same as was observed for the 20-min study. Furthermore, the ratio of aqueous humor drug concentration at 20 min postinstillation to aqueous humor drug concentration at 30 min postinstillation for different viscosity solutions is similar within experimental error. This finding, when combined with the results from the miosis study, appears to rule out the possibility that the rate of release from the vehicle is the controlling factor.

These aqueous humor drug concentration data agree with earlier speculation concerning the biphasic nature of drainage as a function of viscosity (Fig. 2). There was a 1.6-fold increase in aqueous humor drug concentration at 20 and 30 min postinstillation for a 12.5-fold increase in the viscosity of the instilled solution below the "optimum" viscosity, and there was only a 1.2-fold increase in aqueous humor drug concentration for an 87.5-fold increase in the viscosity of the instilled solution above the optimum viscosity.

The increase in aqueous humor drug concentration is presumably due to higher drug concentrations in the conjunctival sac as a result of slow drainage. Figure 4 shows the relationship between drainage rate constants and aqueous humor drug concentrations for drug solutions of varying viscosities. A linear relationship is observed for both the 20- and 30-min postinstillation aqueous humor drug concentrations and the slope of both lines appears similar. The parallel linearity supports the idea that the aqueous humor drug concentration is a function of higher concentration of drug in the conjunctival sac and is *not* controlled by the rate of release of drug from the vehicle.

The area under the response–time profile for any biological response usually reflects the tissue concentration of the drug. By the same token, the area under the miosis–time curve due to pilocarpine nitrate solution should be a reflection of the aqueous humor concentration of the drug. Figure 5 shows that a linear re-

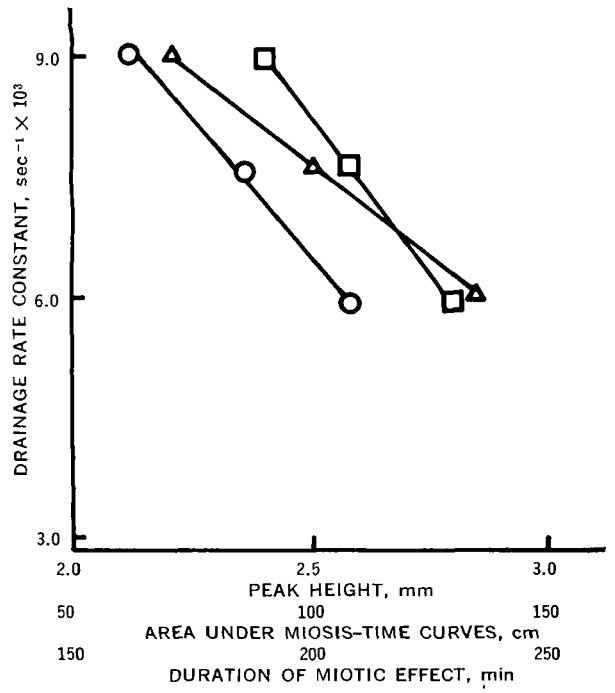

Figure 3—*Effect of the drainage rate constants on the peak height* (○), *area under the miosis–time curves* (□), *and duration of miotic effect* (△) *for pilocarpine nitrate.*

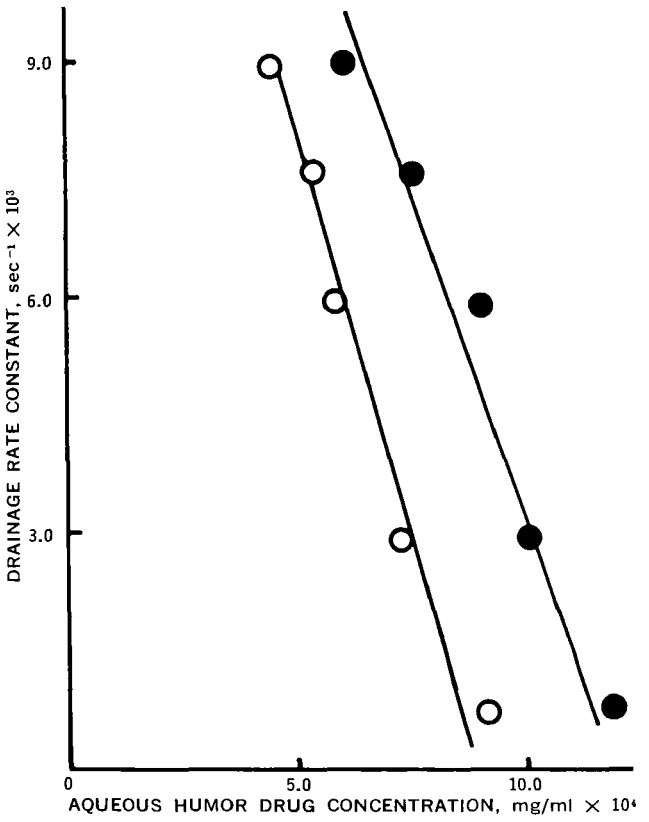

Figure 4—*Relationship between the drainage rate constants and the aqueous humor drug concentrations of pilocarpine nitrate attained at 30 min* (○) *and 20 min* (●) *postinstillation.*

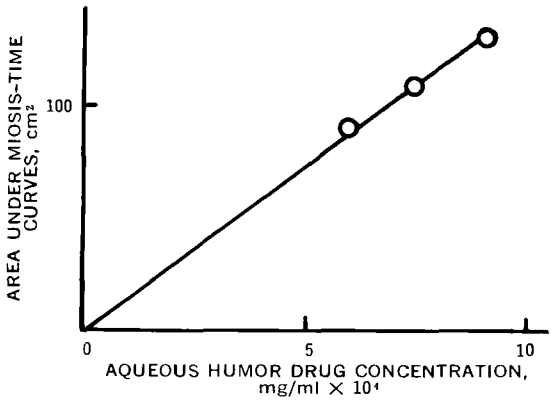

Figure 5—*Relationship between area under the miosis–time curves and aqueous humor drug concentration at 20 min postinstillation of pilocarpine nitrate.*

lationship exists between the area under the miosis-time curve and the aqueous humor drug concentration at 20 min postinstillation of 1×10^{-2} *M* pilocarpine nitrate solution of different viscosities. Naturally, there is also a linear relationship between aqueous humor drug concentration and duration of miotic effect as well as the maximum change in pupillary diameter, *i.e.*, peak height.

DISCUSSION

The quantitative studies on ocular vehicle effects in rabbits reported are in agreement with previously reported observations (8–11). For example, Blaug and Canada (8) observed in rabbits that an increase in contact time of methylcellulose ophthalmic vehicle was approximately proportional to the viscosity of the solution in the lower viscosity region, *i.e.*, 5–25 cps, and levels off at 55 cps or above.

The results presented in this report on the influence of vehicle viscosity on ocular drug bioavailability in rabbits are in some respects similar and in some respects different from those found in humans. Clearly, the fact that there is only a relatively small increase in aqueous humor drug level for a large increase in solution viscosity is common to both humans and rabbits. Higher levels are achieved in rabbits than humans, presumably due to the slower drainage rate of instilled solutions in rabbits as compared to humans (2).

Adler *et al.* (1) found no prolongation of the drug solution in the tear film with viscous drops, and they attributed the increase in aqueous humor drug level to a higher zero time concentration of the drug in the conjunctival sac due to less loss of unmixed drug solution on instillation. To the contrary, studies in rabbits show a substantial prolongation of contact time with viscous drops. Moreover, the increased aqueous humor drug levels in rabbits cannot be completely attributed to higher zero time concentration of the drug in the tear film. If drug concentration at zero time is the driving force, then the zero time concentration in the present study should be double for the 100-cps solution compared to the 1-cps solution. Earlier studies (3) with nonviscous solutions showed that the loss of unmixed drug solution for 1-cps solutions is about 3.5 μl when the instilled volume is 25 μl. When assuming that there is no loss of unmixed solution for a 100-cps solution and a loss of 3.5 μl for a 1-cps solution, the zero time concentration for instillation of 25 μl of 1% drug solution will give zero time concentrations of 0.77 and 0.66%, respectively, *i.e.*, $C_{100} = (25 \times 1)/32.5 = 0.77$ and $C_1 = (21.5 \times 1)/32.5 = 0.66$. These calculations are based upon a lacrimal fluid volume of 7.5 μl. The only way the zero time concentration for a 1-cps solution can be half of the zero time concentration for the 100-cps solution is if the loss of unmixed 1-cps solution is about 13 μl or the lacrimal fluid volume is about 40 μl, both of which are intuitively unacceptable values.

Table V—Aqueous Humor Drug Concentration after Instillation of 25 μl of 1×10^{-2} *M* Pilocarpine Nitrate Solutions of Varying Viscosities

Viscosity of Instilled Solution, cps	Aqueous Humor Drug Concentration at 20 min Postinstillation[a], mg/ml × 10^4	Aqueous Humor Drug Concentration at 30 min Postinstillation[a], mg/ml × 10^4
1	6.0 ± 0.5 (8)	4.5 ± 0.4 (12)
1.8	7.5 ± 1.6 (6)	5.3 ± 1.2 (6)
4.2	9.0 ± 1.2 (8)	5.7 ± 1.7 (6)
12.5	10.0 ± 1.5 (8)	7.2 ± 1.5 (6)
100	11.8 ± 1.7 (7)	9.3 ± 1.6 (6)

[a] Numbers in parentheses represent the number of samples of aqueous humor, *i.e.*, number of eyes.

Table VI—Calculated Concentration of Drug in the Conjunctival Sac after Instillation of Drug Solution[a] of Various Viscosities

Minutes	Concentration[b] in Conjunctival Sac[c], % w/v					
	100[d]	40[d]	12.5[d]	4.2[d]	1.8[d]	1.0[d]
0[e]	0.77	0.77	0.77	0.77	0.77	0.77
1	0.745	0.743	0.739	0.737	0.729	0.722
2	0.719	0.712	0.705	0.695	0.681	0.671
3	0.693	0.677	0.667	0.647	0.630	0.617
4	0.667	0.642	0.626	0.599	0.581	0.560
5	0.641	0.605	0.586	0.551	0.531	0.506

[a] Concentration of solution before instillation 1% w/v. [b] Concentration of the drug solution, assuming the turnover rate of tear fluid to be 0.5 μl/min (3). [c] Values to calculate volume remaining, V_r, were taken from Fig. 1. [d] Viscosity of instilled solution, centipoises. [e] Zero time concentration is the concentration after dilution with lacrimal tear fluid. The lacrimal tear fluid is assumed to be 7.5 μl, and it is further assumed that no loss of unmixed drug solution occurs (3).

An alternative explanation for higher aqueous humor drug levels is a slow decrease in precorneal drug concentration due to a decrease in the drainage rates. Table VI shows a hypothetical case, listing the concentration of drug in the precorneal pocket, calculated with the use of Eqs. 2 and 3, for the first 5 min postinstillation of drug for various viscosity solutions. For a tear turnover rate of 0.5 μl/min, dilution of the drug solution in the conjunctival sac will be different for different viscosity solutions because of the different drainage rates. The difference in concentration for different solutions will widen with time. At 1 min, the difference in concentration for solutions of 1 and 100 cps is 2%, whereas the difference widens to 14% at 5 min postinstillation for the same two solutions.

After instillation of a known concentration of drug solution into the eye, there will be a dilution effect due to the presence of the lacrimal fluid volume. The new concentration, which is also the concentration of the drug solution at time zero, $C_t = 0$, can be calculated by the use of Eq. 2 (4):

$$C_{t=0} = \frac{(C_0)(V_i)}{V_i + V_l} \qquad \text{(Eq. 2)}$$

The concentration of drug solution in the conjunctival sac at any time can be calculated by use of Eq. 3 (4):

$$C_t = \frac{(V_r - nF)}{V_r + nF} C_{t-n} \qquad \text{(Eq. 3)}$$

where C_t is the concentration to be calculated at time t, F is the turnover rate, and C_{t-n} is the known concentration at time $t - n$. This equation is valid only if $n < t > 0$. The slope of a plot of logarithm concentration against time for the first few minutes postinstillation provides the first-order rate constant, k_{con} min^{-1}, for the decline in concentration. Plots of the determined aqueous humor drug concentration against the calculated first-order rate constant for the decline in the concentration shows a linear relationship for both 20 and 30 min postinstillation (Fig. 6). Inspection of the values shows that there is approximately a twofold increase in concentration with a twofold decrease in the rate constant. This suggests that the increase in aqueous humor drug concentration with viscous solution may be partly due to the increased zero time concentration of drug in the conjunctival sac but is principally due to a decrease in the rate of decline of concentration for the first few minutes postinstillation.

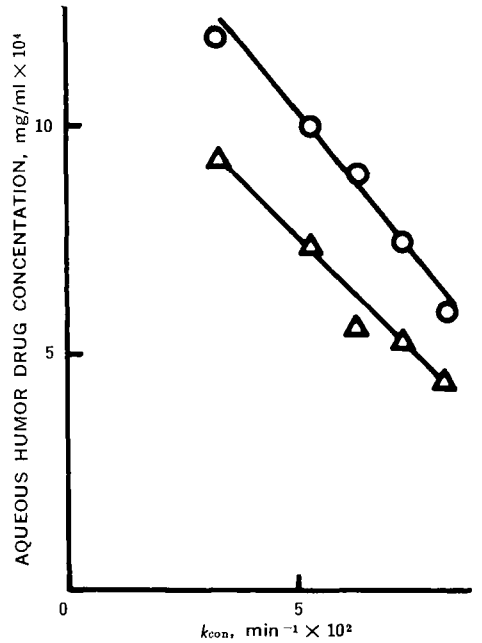

Figure 6—*Relationship between aqueous humor drug concentration and first-order rate constant for the decline of drug concentration in the cul-de-sac. Circles and triangles represent the aqueous humor drug concentrations at 20 and 30 min post-instillation, respectively.*

Considerable reservations may be felt about comparing results from rabbits with those from humans because of the differences between the physiology of tear flow and mixing and general anatomy. Nevertheless, the rabbit is the principal experimental animal in ophthalmology, so comparisons are needed.

Mueller and Deardorff (7), in human studies, found an increased cycloplegia and mydriasis from homatropine in 1% methylcellulose (about 60-cps solution) at all doses due to increased contact time. Their data suggest that double the quantity of drug would penetrate the cornea with increased viscosity, which is in agreement with the results of this study in rabbits. Furthermore, a 0.1% methylcellulose (about 1.4-cps solution) solution of drug gave a pharmacological response that was comparable to that of the nonviscous aqueous vehicle. This is also in agreement with results of the present study. Since both 1 and 0.1% methylcellulose in aqueous solution lowers surface tension to about 50 dynes/cm, the difference in effect of two solutions rules out an increased effect due to surface tension. The response is probably due to an increase in contact time, as suggested by the authors (7).

There has been a reported study (12) in humans of an enhanced pharmacological response to pilocarpine when applied in a methylcellulose vehicle. However, a comparison of that study to the present study could not be made because the viscosity of the solutions was not reported.

Linn and Jones (13) studied the rate of lacrimal excretion of various ophthalmic vehicles by determining the length of time necessary for various vehicles to pass through the drainage apparatus. Solutions of dye were instilled into the eyes of test subjects, and the dye loss was followed by placing a moistened cotton-tipped applicator in the inferior meatus of the nose and measuring the length of time for the dye to appear. This test determined the excretion time, which was defined as the length of time from instillation into the eye until the solution stained the cotton-tipped applicator. The reported result was 60 sec for an aqueous solution; for a viscous solution the results were as follows: 0.25%, 90 sec; 0.5%, 140 sec; 1.0%, 210 sec; and 2.5%, 255 sec. The corresponding viscosity of these solutions will be approximately 1, 3, 8, 65, and 33,000 cps. Qualitatively, these results agree with results in the present study; namely, that there is a large decrease in excretion rate with a small change in viscosity in the lower viscosity region and there is a small change in drainage rate with large changes in viscosity in the higher viscosity region. The fact that the results of the present study in rabbits correspond well to those in humans suggests that the mechanism of vehicle effect may be the same in both humans and rabbits. However, we are unable to offer an explanation of the finding by Adler *et al.* (1) of no increased contact time with viscous vehicles in humans.

The present study on viscosity vehicle effect in rabbits can be summarized as follows. Instillation of the drug solution of varying viscosities decreases the rate of drainage as the viscosity of the instilled solution increases. This increases the concentration, both at zero time and subsequent time periods, of the drug in the precorneal film, which results in a higher aqueous humor drug concentration. However, the magnitude of the increase in aqueous humor drug concentration is small over a 100-fold change in solution viscosity, in agreement with the observation in humans by Adler *et al.* (1).

Since there is more loss of unmixed nonviscous drug solution from the eye and a faster rate of drainage for larger volumes, it is postulated that the effect of vehicle viscosity will be greater in larger instilled volumes than in smaller instilled volumes. This is an important consideration since smaller drops will probably be used in the future and viscosity influence is expected to be less.

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* Present address: Schering Corp., Bloomfield, NJ 07003

ˣ To whom inquiries should be directed.

Drug Permeation through Membranes IV: Effect of Excipients and Various Additives on Permeation of Chlordiazepoxide through Polydimethylsiloxane Membranes

EDWARD G. LOVERING[x], DONALD B. BLACK, and MARY L. ROWE

Abstract □ Permeation experiments were used to look for interactions between chlordiazepoxide and some common excipients, nutrients, and surfactants. No effect on the permeability coefficient was observed in the presence of starch, calcium hydrogen phosphate, gelatin, lactose, silica, sucrose, cholesterol, or porcine mucin. The coefficient increased in the presence of magnesium stearate and decreased with talc, sodium saccharin, skim milk, egg lecithin, beef albumin, egg albumin, polysorbate 80, cetrimonium bromide, and sodium lauryl sulfate, indicating an interaction of these substances with the drug.

Keyphrases □ Chlordiazepoxide—permeation through polydimethylsiloxane membranes, effect of excipients and various additives □ Permeation—chlordiazepoxide through polydimethylsiloxane membranes, effect of excipients and various additives □ Membranes, polydimethylsiloxane—permeation of chlordiazepoxide, effect of excipients and various additives □ Polydimethylsiloxane membranes—permeation of chlordiazepoxide, effect of excipients and various additives □ Drug permeation—chlordiazepoxide through polydimethylsiloxane membranes, effect of excipients and various additives

A broadly based, continuing investigation of drug availability and absorption is being carried out in these laboratories. Part of the work involves dissolution and permeation studies aimed at developing relationships between physical properties measured *in vitro* and absorption parameters measured *in vivo*. Absorption of a drug is hindered by chemical or physical processes that decrease its effective concentration in the gut. Complex formation, partitioning into surfactant micelles, adsorption on surfaces, and precipitation are processes that interfere with absorption. Interactions that decrease the effective drug concentration can be detected by permeation experiments carried out *in vitro*, since the permeation rate of a drug in solution is directly proportional to its concentration. Factors affecting the permeation of chlorpromazine (1), amobarbital (2), and phenylbutazone (3) were reported previously.

This paper reports the effect of various excipients, nutrients, and surfactants on the permeation of chlordiazepoxide through polydimethylsiloxane membranes.

EXPERIMENTAL

Permeation Measurements—Cells of the type described by Garrett and Chemburkar (4) were used, except that the polydimethylsiloxane membranes were attached with silicone adhesive and the cells were stirred internally. The cells were placed in stirred beakers containing chlordiazepoxide solution, and the entire assembly was thermostated at 37 ± 1°. Drug solutions were buffered over a pH range of 3–8 using citrate or phosphate buffers (5). The desorbing solution within the cell was buffered at pH 1.2 to ensure zero concentration of unionized drug, thus preventing back-permeation. Due to an excess of drug outside the cell, concentrations were constant during the experiment and steady-state permeation rates were obtained about 1 hr after the experiment began. The desorbing solution was circulated through a UV spectrometer, and the absorption was measured at appropriate time intervals. The Beer's law molar extinction coefficient of chlordiazepoxide at 244 nm was 34,200 at pH 1.2.

Materials—Polydimethylsiloxane membranes (0.0025 cm), thought to contain silica filler, were cut from two squares as obtained from the manufacturer[1]. The squares were labeled A and B for identification purposes. The permeability coefficient of the drug measured in Membrane B was about 1.4 times greater than that measured in Membrane A under the same experimental conditions. The reason for the difference is not known. *cis*-1,4-Polybutadiene membranes (0.029 cm) containing no filler were lightly cross-linked using 2,5-bis(*tert*-butylperoxy)-2,5-dimethylhexane[2]. Chlordiazepoxide hydrochloride[3] was used as received.

Other materials used were: skim milk powder; sucrose; cholesterol[4], certified; sodium lauryl sulfate[4] USP; polysorbate 80[4]; magnesium stearate[4] USP; starch[4] USP; calcium hydrogen phosphate[4], certified; bovine albumin[5], fraction V; egg lecithin[5], 95–100%; magnesium sulfate[5], anhydrous reagent; cetrimonium bromide[5], reagent; sodium saccharin[5], reagent; sodium deoxycholate[5], reagent; gelatin[5] BP; talc[5], fine powdered, acid purified; porcine mucin[6], crude Type II; egg albumin[7], IX cryst, chicken; silica[8]; lactose[9] USP; and stearic acid[10], high purity.

RESULTS AND DISCUSSION

Permeation results were plotted as the moles of drug traversing the membrane against time. Slopes, m, were measured from the steady-state portion of the curves. Permeability coefficients, P, were calculated from:

$$P = \frac{ml}{AC_0}\left[1 + e^{2.303(\mathrm{pKa}-\mathrm{pH})} + \frac{1}{T}\right] \qquad \text{(Eq. 1)}$$

where l is the membrane thickness, A is the surface area, C_0 is the total drug concentration outside the cell, pKa is the dissociation constant, and $T = D_a l/K_p h D_m$; D_a and D_m are the diffusion coefficients of the drug in water and the membrane, respectively; h is the thickness of the aqueous diffusion layer; and K_p is the partition coefficient of unionized drug between the membrane and the aqueous solution. The term T^{-1} is negligible except when the permeation rate is limited by the aqueous diffusion layer at the membrane surface.

Effect of pH—The permeation rates of chlordiazepoxide were measured over the pH range of 3.02–8.01. The permeability coefficients were calculated from Eq. 1 by taking the pKa as 4.8 and neglecting the T^{-1} term. Based on the results of 26 experiments, P was found to be independent of pH and have a value of $(6.65 \pm 0.41) \times 10^{-8}$ cm² sec^{-1} (Membrane A). If T^{-1} were not negligible, P calculated in this manner would be pH dependent. The fact that T^{-1} could be neglected indicates that the rate-limiting step in the permeation is the membrane and not the aqueous diffusion layers in contact with it (6). Phosphate buffer (pH 7.0–7.2) was used in all experiments where an excipient or other

[1] General Electric Co., Schenectady, N.Y.
[2] Cis-4 1203, Phillips Petroleum Co., Bartlesville, Okla.
[3] Hoffmann-La Roche Inc., Montreal, Canada.
[4] Fisher Scientific Co.
[5] British Drug Houses.
[6] Sigma Chemical Co.
[7] Miles Laboratories Inc.
[8] Cab-O-Sil M-5, Cabot Corp.
[9] Merck.
[10] Fine Organics Inc.

Table I—Effect of Excipients on Permeability Coefficients

Excipient	Excipient Concentration, g liter^{-1}	pH of Solution	Permeability Coefficient, (cm^2 sec^{-1}) × 10^8
Starch	1.22	7.17	6.31
	2.04	7.18	5.64
	12.9	7.15	5.53
	33.6	7.16	5.42
			Mean 5.72 ± 0.40
Calcium hydrogen phosphate	1.26	7.16	5.86
	9.9	7.16	6.01
	29.7	7.16	5.71
			Mean 5.86 ± 0.15
Gelatin	9.7	7.01	6.49
	10.1	7.02	5.92
	31.9	6.65	5.53
			Mean 5.98 ± 0.48
Lactose	1.18	7.16	6.42
	2.49	7.15	5.41
	47.1	7.14	6.24
			Mean 6.02 ± 0.54
Silica	0.05	7.17	6.39
	0.50	7.17	5.89
	4.88	7.17	5.40
			Mean 5.89 ± 0.50
Talc	2.02	7.18	5.56
	9.9	7.20	3.94
	40.0	7.22	1.95
	40.0	7.16	1.75
	40.2	7.20	1.33
Magnesium stearate	0.053	7.16	7.46
	0.061	7.20	7.27
	0.40	7.20	7.39
	0.46	7.22	8.38
	0.55	7.18	8.38
	4.87	7.18	8.64
			Mean 7.92 ± 0.61

Table II—Effect of Nutrients on Permeability Coefficients

Nutrient	Nutrient Concentration, g liter^{-1}	pH of Solution	Permeability Coefficient, (cm^2 sec^{-1}) × 10^8
Sucrose	40.0	7.10	6.12
	60.0	7.08	5.98
	100.0	7.04	5.28
			Mean 5.79 ± 0.45
Cholesterol	1.01	7.13	6.20
	1.47	7.12	5.79
	2.00	7.13	5.58
			Mean 5.86 ± 0.32
Porcine mucin	2.00	7.10	6.74
	7.00	6.98	6.93
	15.0	6.82	5.78
			Mean 6.48 ± 0.62
Sodium saccharin	5.00	7.10	5.69
	5.03	5.95	5.44
	10.0	7.08	5.27
	10.0	6.89	5.43
	16.0	7.04	4.75
	16.0	6.78	4.33
Skim milk	40.0	7.10	4.11
	60.0	7.08	3.76
	80.0	7.06	2.76
Egg lecithin	0.96	7.12	4.52
	2.07	7.11	3.39
	3.28	7.10	2.69
Beef albumin	2.02	7.13	4.72
	5.00	7.13	4.00
	8.00	7.10	3.81
Egg albumin	0.20	7.10	5.50
	0.50	7.09	5.24
	0.79	7.09	5.20

Table III—Regression Coefficients of Nutrient: Permeability Coefficient Plots

Nutrient	Slope, cm^2 liters sec^{-1} g^{-1}	Intercept, cm^2 sec^{-1}	Correlation Coefficient
Sodium saccharin	-9.4×10^{-10} ($p < 0.05$)	6.12	−0.91
Skim milk	-3.4×10^{-10} ($p < 0.05$)	5.56	−0.96
Egg lecithin	-7.8×10^{-9} ($p < 0.05$)	5.19	−0.99
Beef albumin	-1.5×10^{-9} ($p < 0.10$)	4.94	−0.95

substance was added to the drug solution. Over this narrow pH range, the mean permeability coefficient was $(6.02 \pm 0.28) \times 10^{-8}$ cm^2 sec^{-1} (15 experiments with Membrane A). Permeability coefficients determined in the presence of nutrients, surfactants, and excipients are compared to this value.

Excipients—The effect on the permeability coefficient of excipients common in chlordiazepoxide formulations available in Canada is given in Table I. Relatively large quantities of excipients were used to increase the probability of detecting an interaction. A significant change in the permeability coefficient was deemed to have occurred if the observed coefficient differed by more than 2 *SD* ($p < 0.05$) from the coefficient for the drug itself. No significant change in the permeability was found in the presence of starch, calcium hydrogen phosphate, gelatin, lactose, or silica. The permeability decreased in the presence of large quantities of talc. A linear regression of the permeability coefficient against the concentration of talc in the slurry yielded a linear correlation ($p < 0.05$) with a regression coefficient of 0.98. The drug may be adsorbed on the surface of the talc or react with a minor impurity in it. Even though the quantities of talc used are larger then would be encountered in practice, the result suggests that talc should be used with caution in formulating chlordiazepoxide. It was recently shown that talc interferes with the *in vivo* absorption of dicumarol in dogs (7).

When magnesium stearate, a widely used excipient, was slurried in a chlordiazepoxide solution, the permeability coefficient increased from $(6.02 \pm 0.28) \times 10^{-8}$ to $(7.92 \pm 0.61) \times 10^{-8}$ cm^2 sec^{-1} (Membrane A). In subsequent experiments on the same membranes carried out without magnesium stearate in the drug solution, the permeability coefficient was $(8.09 \pm 0.59) \times 10^{-8}$ cm^2 sec^{-1}. Thus, magnesium stearate appears to have altered the properties of the membrane toward the permeation of chlordiazepoxide.

To investigate the cause of the increase in the permeability, new cells were prepared with fresh membranes. The mean coefficient for chlordiazepoxide, based on nine experiments, was $(8.72 \pm 0.50) \times 10^{-8}$ cm^2 sec^{-1} (Membrane B). Addition of magnesium sulfate to the drug solution led to some precipitation, probably of magnesium phosphate from the buffer, but to no significant change in the permeability coefficient [$(8.03 \pm 0.28) \times 10^{-8}$ cm^2 sec^{-1}; three experiments]. Therefore, the enhanced permeability does not appear to be due to the reaction of the drug with magnesium ions to form a chelate compound of greater solubility or diffusivity in the membrane than chlordiazepoxide. After the cells were allowed to stand overnight in a slurry of stearic acid and rinsed clean, the permeability coefficient of the drug increased to $(11.8 \pm 0.7) \times 10^{-8}$ cm^2 sec^{-1} (three experiments). The possibility that the enhanced permeability coefficient was the result of stearic acid adsorption on the membrane surface was eliminated by an experiment in which the drug traversed the membrane from the inside of the cell outward. The permeability was unchanged [$(11.7 \pm 0.9) \times 10^{-8}$ cm^2 sec^{-1}; three experiments]. Unequivocal confirmation that the increased permeability involved the polydimethylsiloxane membranes was obtained by replacing them with *cis*-1,4-polybutadiene membranes. The permeability coefficient of the drug through these membranes was $(8.26 \pm 0.88) \times 10^{-9}$ cm^2 sec^{-1} based on eight experiments. In the presence of stearic acid, the coefficient was unchanged [$(8.45 \pm 0.84) \times 10^{-9}$ cm^2 sec^{-1}].

It is unlikely that the observed increase in the permeation rate is due to plasticization of the membrane by stearic acid because no increase was observed in previous experiments with amobarbital (2) or phenylbutazone (3). The increased permeability may arise from the competitive interaction of the drug and stearic acid with the surface of the silica filler contained in the membrane, but the nature of the interaction is not known.

Nutrients—The permeability coefficient (Membrane A) of chlordiazepoxide was measured in the presence of various food constituents, either dissolved or slurried in the drug solution (Table II). Sucrose, cholesterol, and porcine mucin had no effect on the permeability coefficient. Sodium saccharin at concentrations much higher than would be encountered *in vivo* led to a small but definite decrease in the permeability, perhaps the result of ion-pair formation between chlordiazepoxide cations and saccharin anions. The protein-containing materials, skim milk, egg lecithin, and beef albumin, produced substantial decreases in the coefficient while a slight decrease was noted in the presence of small amounts of egg albumin. Plots of the permeability coefficient *versus* the concentration of protein yield linear correlations, the regression coefficients of which are given in Table III. The correlations suggest that adsorption or protein binding is taking place. Chemical reaction seems unlikely because the large excess of nutrient would probably drive any reaction far toward completion and, consequently, the permeability coefficient virtually to zero.

Surfactants—The decrease in the observed permeability coefficient with increasing concentrations of surfactant was used to calculate the partition coefficient, K_p, of drug partitioning between the surfactant micellar phase and the aqueous solution. Data were analyzed according to the equation:

$$\frac{Q}{C_u W_w} = \frac{K_p W_m}{\rho_m W_w} + \frac{1}{\rho_w} \qquad \text{(Eq. 2)}$$

where Q is the total quantity of drug in the system; C_w is the quantity of drug in the aqueous phase; W_m and W_w are the weights of the micellar and water phases, respectively; and ρ_m

Table IV—Effect of Surfactants on Permeation of Chlordiazepoxide

Total Drug, Q, (moles $liter^{-1}$) $\times 10^4$	pH	Weight of Surfactant, (g $liter^{-1}$)	Apparent Permeability Coefficient, P_a, (cm^2 sec^{-1}) $\times 10^8$
Sodium Lauryl Sulfate			
1.725	7.14	0.104	6.19
1.868	7.14	0.276	6.05
1.856	7.10	0.461	5.81
1.808	7.11	1.208	2.58
1.808	7.11	3.019	0.96
Cetrimonium Bromide			
1.880	7.15	0.116	4.60
1.904	7.14	0.512	3.40
2.153	7.03	2.00	1.51
1.915	7.12	5.00	0.85
Polysorbate 80			
1.999	7.15	0.552	4.42
1.832	7.14	1.236	5.00
1.796	7.14	4.26	3.21
2.011	7.14	10.88	1.52

Table V—Surfactant Regression Coefficients and Partition Coefficients

Surfactant	$K_p \rho_m^{-1}$, liter g^{-1}	ρ_w^{-1}, (liter g^{-1}) $\times 10^3$	r	K_p
Sodium lauryl sulfate	1.87	0.44	0.99	1880
Cetrimonium bromide	1.19	1.28	0.99	1190
Polysorbate 80	0.27	0.97	0.99	270

and ρ_w are the respective densities (2, 3); C_w was estimated from:

$$C_w = \frac{QP_a}{P} \qquad \text{(Eq. 3)}$$

where P_a is the apparent permeability coefficient measured in the presence of surfactant. The decrease in the permeability coefficient from 6.02×10^{-8} cm^2 sec^{-1} (Membrane A) upon addition of surfactant is assumed to be directly attributable to the disappearance of free drug. Plots of Q/C_wW_w against W_m/W_w were constructed from the experimental data (Table IV). The density of the surfactant was taken as unity. When assuming no volume change upon mixing, W_w and W_m were obtained directly. The regression coefficients of these plots are given in Table V, along with the partition coefficient estimated from Eq. 2.

The large partition coefficients found for cetrimonium bromide and sodium lauryl sulfate solutions are due to their ionic nature. At the pH of these experiments, chlordiazepoxide exists in the unionized and cationic forms. In addition to dissolution of unionized drug in the hydrocarbon interior of the micelles, reaction with the charged exterior occurs. Chlordiazepoxide cations react with lauryl sulfate anions to form ion-pairs while unionized drug forms dipole complexes with cetrimonium ions. The strong interaction of chlordiazepoxide with ionic surfactants suggests that the latter should be avoided in preparing formulations of this drug.

CONCLUSIONS

The interaction of chlordiazepoxide with talc and ionic surfactants suggests that care should be exercised in preparing formulations with these substances to avoid conditions under which absorption may be restricted by drug–excipient interaction.

The interaction of magnesium stearate with the polydimethylsiloxane membrane highlights the need for careful membrane selection. The ideal material would be a nonporous, homogeneous, polymeric membrane that contains no filler, crystallites, or other internal microphases which influence the diffusion rate or solubility of the drug in the membrane. Most commercially available, thin elastomeric membranes contain filler to impart physical strength.

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[x] To whom inquiries should be directed.

Synthesis of Substituted Piperidino Carbamides: Correlation between CNS Effects and Selective Inhibition of NAD-Dependent Oxidations

A. K. GUPTA*, C. DWIVEDI*, T. K. GUPTA*, SURENDRA S. PARMAR*‡[x], and WILLIAM E. CORNATZER‡

Abstract □ Several 1-(*N*-acetyl-substituted piperidino)-3-aryl carbamides were synthesized. All carbamides possessed anticonvulsant activity, as exhibited by protection against pentylenetetrazol-induced seizures, and potentiated pentobarbital-induced hypnosis. Inhibitory effects of these carbamides on the respiratory activity revealed selective inhibition of NAD-dependent oxidation of the various substrates by the rat brain homogenate. Such a selective inhibition of respiratory activity was in no way related with the pharmacological properties of the compounds that were tested.

Keyphrases □ Carbamides of substituted piperidines—synthesis, relationship between anticonvulsant activity and inhibition of respiratory activity □ Piperidine carbamides—synthesis, relationship between anticonvulsant activity and inhibition of respiratory activity □ Anticonvulsant activity—synthesis and evaluation of 1-(*N*-acetyl-substituted piperidino)-3-aryl carbamides □ Respiratory activity—synthesis and evaluation of carbamides as inhibitors □ CNS activity—synthesis and evaluation of 1-(*N*-acetyl-substituted piperidino)-3-aryl carbamides □ Structure–activity relationships—substituted piperidino carbamides, CNS effects

Continuing interest in the synthesis of substituted carbamides (1–3) and the study of their central nervous system (CNS) effects led to the synthesis of some 1-(*N*-acetyl-substituted piperidino)-3-aryl carbamides. In the present study, the anticonvulsant activity of these compounds against pentylenetetrazol-induced seizures and their ability to potentiate pentobarbital hypnosis were determined. The *in vitro* inhibitory effects of these carbamides on the respiratory activity of the rat brain homogenate were also investigated to elucidate their biochemical mechanism of action. These carbamides were synthesized by following the methods outlined in Scheme I.

and ρ_w are the respective densities (2, 3); C_w was estimated from:

$$C_w = \frac{QP_a}{P} \qquad \text{(Eq. 3)}$$

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Table I—Physical Constants of 1-(*N*-Acetyl-substituted piperidino)-3-aryl Carbamides



Compound	Ar	R	Melting Point[a]	Yield, %	Molecular Formula	Analysis, %	
						Calc	Found
I	2-$CH_3C_6H_4$	2-CH_3	65°	70	$C_{16}H_{23}N_3O_2$	C 66.43	66.18
						H 7.95	8.10
						N 14.53	14.62
II	3-$CH_3C_6H_4$	2-CH_3	67°	76	$C_{16}H_{23}N_3O_2$	C 66.43	66.23
						H 7.95	7.92
						N 14.53	14.68
III	4-$CH_3C_6H_4$	2-CH_3	70°	75	$C_{16}H_{23}N_3O_2$	C 66.43	66.38
						H 7.95	7.88
						N 14.53	14.50
IV	3,4-$(CH_3)_2C_6H_3$	2-CH_3	76°	60	$C_{17}H_{25}N_3O_2$	C 67.32	67.21
						H 8.25	8.13
						N 13.86	13.69
V	2-$OCH_3C_6H_4$	2-CH_3	95°	58	$C_{16}H_{23}N_3O_3$	C 62.95	63.02
						H 7.54	7.50
						N 13.77	13.82
VI	4-$OCH_3C_6H_4$	2-CH_3	68°	70	$C_{16}H_{23}N_3O_3$	C 62.95	62.92
						H 7.54	7.33
						N 13.77	13.69
VII	2-ClC_6H_4	2-CH_3	100°	62	$C_{15}H_{20}ClN_3O_2$	C 58.15	58.38
						H 6.46	6.48
						N 13.57	13.48
VIII	2-$CH_3C_6H_4$	3-CH_3	85°	45	$C_{16}H_{23}N_3O_2$	C 66.43	66.50
						H 7.95	8.00
						N 14.53	14.43
IX	4-$CH_3C_6H_4$	3-CH_3	80°	40	$C_{16}H_{23}N_3O_2$	C 66.43	66.46
						H 7.95	7.92
						N 14.53	14.44
X	3,4-$(CH_3)_2C_6H_3$	3-CH_3	92°	50	$C_{17}H_{25}N_3O_2$	C 67.32	67.52
						H 8.25	8.30
						N 13.86	13.77
XI	2-$OCH_3C_6H_4$	3-CH_3	105°	35	$C_{16}H_{23}N_3O_3$	C 62.95	62.88
						H 7.54	7.48
						N 13.77	13.82
XII	2-ClC_6H_4	3-CH_3	94°	32	$C_{15}H_{20}ClN_3O_2$	C 58.15	58.27
						H 6.46	6.43
						N 13.57	13.44
XIII	2-$CH_3C_6H_4$	4-CH_3	82°	70	$C_{16}H_{23}N_3O_2$	C 66.43	66.48
						H 7.95	7.93
						N 14.53	14.50
XIV	3-$CH_3C_6H_4$	4-CH_3	65°	70	$C_{16}H_{23}N_3O_2$	C 66.43	66.42
						H 7.95	7.93
						N 14.53	14.39
XV	4-$CH_3C_6H_4$	4-CH_3	80°	65	$C_{16}H_{23}N_3O_2$	C 66.43	66.50
						H 7.95	7.87
						N 14.53	14.46
XVI	3,4-$(CH_3)_2C_6H_3$	4-CH_3	73°	75	$C_{17}H_{25}N_3O_2$	C 67.32	67.33
						H 8.25	8.23
						N 13.86	13.88
XVII	2-$OCH_3C_6H_4$	4-CH_3	133°	90	$C_{16}H_{23}N_3O_3$	C 62.95	62.80
						H 7.54	7.50
						N 13.77	13.86
XVIII	4-$OCH_3C_6H_4$	4-CH_3	74°	78	$C_{16}H_{23}N_3O_3$	C 62.95	62.94
						H 7.54	7.52
						N 13.77	13.76
XIX	2-ClC_6H_4	4-CH_3	90°	80	$C_{15}H_{20}ClN_3O_2$	C 58.15	58.42
						H 6.46	6.42
						N 13.57	13.48

[a] Melting points were taken in open capillary tubes and are uncorrected.

EXPERIMENTAL

Synthesis—*Aryl Carbamides*—These compounds were synthesized by treating a solution of potassium cyanate with suitable anilines (4).

1-Chloroacetyl-3-aryl Carbamides—A mixture of aryl carbamide (0.1 mole) and chloroacetyl chloride (0.11 mole) in dry benzene was refluxed for 2–4 hr. On cooling, the solid mass which separated out was filtered, washed with water, dried, and recrystallized from suitable solvents (3,5,6).

1-(*N-Acetylmethyl-substituted piperidino)-3-aryl Carbamides*—To a solution of 1-chloroacetyl-3-aryl carbamide (0.01 mole) in dry benzene was added an appropriate methylpiperidine (0.02 mole), and the reaction mixture was refluxed for 6–8 hr. On cooling, the separated hydrochlorides were removed by filtration and the filtrate was concentrated under reduced pressure. The solid mass which separated out on cooling was filtered, washed with water, dried, and recrystallized from suitable solvents. These carbamides were characterized by their sharp melting points and elemental analyses (Table I).

Pharmacological—*Toxicity*—The approximate 50% lethal dose (LD_{50}) was determined in albino mice by the method of Smith (7). All substituted carbamides were suspended in 5% aqueous gum acacia and were administered intraperitoneally to each group of 10 mice.

Anticonvulsant Activity—Anticonvulsant activity was determined in mice of either sex weighing 25–30 g. The mice were divided in groups of 10, keeping the group weights as near the same

Table II—Pharmacological Properties of 1-(*N*-Acetyl-substituted piperidino)-3-aryl Carbamides

Compound[a]	Approximate LD_{50}, mg/kg	Anticonvulsant Activity[b], % Protection	Pentylenetetrazol Mortality[c], %	Potentiation of Pentobarbital Sleeping Time[d], min	Increase Times Control
I	600	50	20	58.3 ± 5.2	1.62
II	800	30	10	44.5 ± 3.5	1.23
III	1000	50	40	48.5 ± 3.8	1.35
IV	>1000	60	10	62.8 ± 6.5	1.74
V	750	30	10	55.8 ± 5.4	1.55
VI	250	20	80	60.5 ± 2.8	1.67
VII	800	30	20	56.5 ± 6.9	1.57
VIII	>1000	60	10	49.3 ± 5.9	1.37
IX	1000	60	0	39.5 ± 2.8	1.09
X	1000	40	20	39.0 ± 2.3	1.08
XI	>1000	60	10	52.0 ± 5.4	1.44
XII	>1000	10	50	55.5 ± 6.7	1.52
XIII	1000	20	50	59.0 ± 5.3	1.64
XIV	>1000	20	50	64.5 ± 7.0	1.79
XV	>1000	20	50	72.5 ± 8.9	2.01
XVI	>1000	40	50	41.0 ± 3.0	1.14
XVII	>1000	30	30	55.7 ± 7.2	1.54
XVIII	850	50	50	48.5 ± 4.5	1.34
XIX	>1000	20	60	57.0 ± 6.4	1.58

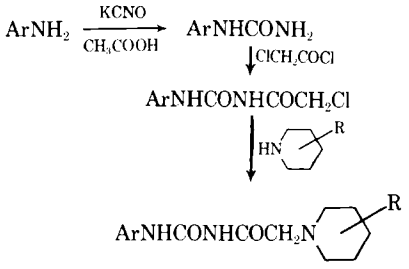
[a] Compound numbers are as reported in Table I. [b] Anticonvulsant activity was determined at a dose of 100 mg/kg ip, as described under *Experimental*. [c] Represents mortality during 24 hr in each group of animals administered pentylenetetrazol. [d] Control values for pentobarbital (40 mg/kg) sleeping time was 36 min, and test compounds were administered at a dose of 100 mg/kg ip, which was taken as 1 for comparison.

as possible. All substituted carbamides were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compound was injected in a group of 10 animals at a dose of 100 mg/kg ip. Four hours after the administration of the substituted carbamides, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol has been shown to produce convulsions in almost all untreated mice and also to exhibit 100% mortality over 24 hr. However, no mortality was observed during 24 hr in animals treated with 100 mg/kg alone of the test compounds.

The mice were observed for 60 min for the occurrence of seizures. An episode of clonic spasm that persisted for a minimum of 5 sec was considered a threshold convulsion (8). Transient intermittent jerks and tremulousness were not counted. Animals devoid of threshold convulsions during 60 min were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of these substituted carbamides was represented as percent protection. The animals were then observed for 24 hr and their mortality was recorded.

Potentiation of Sodium Pentobarbital Sleeping Time—The method of Winter (8) was followed to investigate the ability of substituted carbamides to potentiate pentobarbital-induced hypnosis. Mice weighing 20–25 g were taken in groups of six animals. One group of six was used for each compound while the other group of six served as the control. Pentobarbital, administered in a dose of 40 mg/kg ip to the control group, produced sleep. All substituted carbamides were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compounds were injected in a group of six animals at a dose of 100 mg/kg ip 30 min prior to the administration of pentobarbital. The administration time of pentobarbital in both the control and the experimental mice was recorded. The animals were observed regularly for sleep as evidenced by the observance of the loss of the righting reflex until the animal had awakened. The mean average sleeping time in each group was calculated. The degree of potentiation caused by these substituted carbamides was calculated by the total average time of the sleep observed in the experimental animals divided by the total average time of the sleep observed in the control animals.

Biochemical[1]—Male albino rats, kept on an *ad libitum* diet, were used for the assay of respiratory activity of rat brain homogenate. Rats weighing 150–200 g were sacrificed by decapitation. The brains were taken out immediately and homogenized in the



Scheme I

Table III—Effect of 1-(*N*-Acetyl-substituted piperidino)-3-aryl Carbamides on the Oxidation of Pyruvate, α-Ketoglutarate, NADH, and Succinate by Rat Brain Homogenates

Compound[a]	Inhibition of Substrate Oxidation[b], %			
	Pyruvate	α-Ketoglutarate	NADH	Succinate
I	75.9 ± 2.9	65.5 ± 2.0	43.0 ± 1.6	Nil
II	93.6 ± 2.9	83.2 ± 2.4	86.7 ± 2.1	Nil
III	81.7 ± 2.7	68.7 ± 2.8	63.4 ± 1.8	Nil
IV	79.3 ± 2.6	73.4 ± 2.7	78.4 ± 1.9	Nil
V	34.9 ± 1.9	39.9 ± 1.9	30.6 ± 2.0	Nil
VI	53.3 ± 1.4	49.0 ± 2.0	40.3 ± 2.2	Nil
VII	56.5 ± 1.4	40.5 ± 2.0	40.0 ± 1.8	Nil
VIII	46.8 ± 2.7	88.6 ± 2.9	82.3 ± 3.0	Nil
IX	87.4 ± 2.0	85.5 ± 2.6	89.3 ± 2.3	Nil
X	94.3 ± 3.0	95.4 ± 2.9	91.3 ± 3.0	Nil
XI	43.4 ± 1.7	44.1 ± 1.4	63.9 ± 1.9	Nil
XII	62.3 ± 1.9	49.0 ± 1.5	86.8 ± 2.4	Nil
XIII	57.3 ± 1.8	46.7 ± 1.5	55.0 ± 2.1	Nil
XIV	93.1 ± 3.0	94.1 ± 2.9	93.9 ± 2.8	Nil
XV	82.5 ± 2.8	84.4 ± 2.6	81.4 ± 2.4	Nil
XVI	90.0 ± 2.6	88.8 ± 2.4	85.5 ± 2.4	Nil
XVII	32.5 ± 1.9	30.7 ± 1.7	32.5 ± 1.2	Nil
XVIII	78.5 ± 2.1	53.1 ± 2.2	88.4 ± 2.9	Nil
XIX	75.2 ± 2.6	54.9 ± 2.1	80.1 ± 2.5	Nil

[a] Compound numbers are as recorded in Table I. [b] Each experiment was done in duplicate. All values represent mean values of percent inhibition with ± standard error of the mean calculated from three separate experiments. Inhibition was determined by the decrease in the oxygen uptake/125 mg wet tissue weight/hr. Assay conditions are as described in the text. All substituted carbamides were used at a final concentration of 1 m*M*. Different substrates and NADH were used at a final concentration of 10 and 0.5 m*M*, respectively.

[1] Commercial chemicals were used. Adenosine monophosphate, cytochrome c, NADH, α-ketoglutarate, sodium pyruvate, and sodium succinate were purchased from Sigma Chemical Co., St. Louis, Mo.

ratio of 1:9 (w/v) with a Potter–Elvehjem homogenizer in 0.25 *M* cold sucrose. Respiratory activity was determined by measuring the oxygen consumption by the conventional Warburg manometric method at 37° with air as the gas phase. Fresh brain homogenate of healthy albino rats, equivalent to 125 mg wet tissue weight, was used in each flask. The reaction mixture in a final volume of 3.0 ml consisted of 20 m*M* Na_2HPO_4 buffer solution (pH 7.4), 6.7 m*M* $MgSO_4$, 1 m*M* AMP (sodium salt), 33 m*M* KCl, and 500 μg of cytochrome c. The central well contained 0.2 ml of 20% KOH solution. All substituted carbamides were used in the final concentration of 1 m*M* to study their effects on the oxidation of the various substrates and NADH, which were used in a final concentration of 10 and 0.5 m*M*, respectively. The compounds were dissolved in propylene glycol (100%) and an equivalent amount of the solvent was added to the control vessels.

RESULTS AND DISCUSSION

Pharmacological properties exhibited by substituted carbamides are recorded in Table II. The approximate LD_{50} values, ranging from 250 mg/kg, reflect low toxicity for these compounds. Compound VI was the most toxic, with an approximate LD_{50} value of 250 mg/kg. As is evident from Table II, all carbamides possessed anticonvulsant activity and the protection afforded by these compounds against pentylenetetrazol-induced seizures ranged from 10 to 60%. Maximum protection was observed with carbamides having a 2-methyl, 4-methyl, or 2-methoxy substituent at the phenyl moiety at position 3 of 1-(*N*-acetyl-3-methyl)-piperidino carbamide and a 3,4-$(CH_3)_2$ substituent at the phenyl moiety at position 3 of the 1-(*N*-acetyl-2-methyl)piperidino carbamide. In the present study, compounds exhibiting 60% protection afforded greater protection from pentylenetetrazol mortality (0–10%). These results have, however, failed to indicate that greater anticonvulsant activity of these compounds is associated with a greater degree of protection against pentylenetetrazol mortality. All carbamides were found to potentiate sodium pentobarbital hypnosis in mice, where maximum potentiation was observed with the carbamide having a 4-methyl substituent at the phenyl moiety at position 3 of 1-(*N*-acetyl-4-methyl)piperidino carbamide (Compound XV). Their ability to potentiate pentobarbital-induced hypnosis ranged from 1.09 to 2.01 times that observed in normal control rats.

All carbamides were found to inhibit selectively *in vitro* NAD-dependent oxidation of pyruvate and α-ketoglutarate by rat brain homogenate whereas FAD-dependent or NAD-independent oxidation of succinate remained unaltered (Table III). These carbamides, like nitrobenzamides (9), were also found to inhibit the oxidation of NADH, indicating possible inactivation of the electron transfer process in the respiratory chain by acting presumably at the site of electron transfer from NADH to FAD. The variations in the aryl group at position 3 of these carbamides have been found to alter the ability of these carbamides to produce inhibition in the respiratory activity. Maximum inhibition of the respiratory activity was observed with Compound X while V and XVII, possessing a 2-methoxy substituent at the phenyl moiety of these carbamides, exhibited low inhibitory effects.

These results have not only failed to provide any structure–activity relationships with respect to the inhibitory effects or pharmacological properties but have also failed to show any correlation between the inhibition of respiratory activity with the ability to provide protection against pentylenetetrazol-induced seizures and to potentiate pentobarbital-induced hypnosis. Detailed study of the effects of these substituted carbamides on the activity of purified enzyme systems may possibly reflect a biochemical basis for their CNS effects.

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ˣ To whom inquiries should be directed.

Bioavailability and Dissolution Properties of Two Commercial Digoxin Tablets

PAUL R. KLINK*§, ROLLAND I. POUST*x, JOHN L. COLAIZZI*, and ROBERT H. McDONALD, Jr.‡

Abstract □ Six healthy male subjects were given 0.5 mg of digoxin on three separate occasions in the form of an elixir and two different commercially available brands of compressed tablets. Blood levels were measured by a radioimmunoassay technique at various time intervals up to 48 hr following drug administration. A previously unreported variable in the radioimmunoassay method was found. Varying amounts of digoxin-free serum added to aqueous samples such as urine or hydroalcoholic solutions led to significant changes in the apparent digoxin concentration measured in the sample. The elixir form gave much higher blood levels than either tablet during the first two sampling times, but the two tablets showed nearly identical blood levels and relative bioavailabilities. A previously reported dissolution rate test method showed wide differences between the two tablets and thus failed to correlate with the similar bioavailabilities observed *in vivo*.

Keyphrases □ Digoxin tablets and elixir—comparative bioavailability, variable in radioimmunoassay technique reported, tablet bioavailability compared to dissolution rate □ Bioavailability of digoxin tablets and elixir—compared using radioimmunoassay technique, variable in technique reported, tablet bioavailability compared to dissolution rate □ Radioimmunoassay—analysis, digoxin blood levels, variable in technique reported

Marked differences in the bioavailability of digoxin from commercial tablet dosage forms have been reported (1–6). Other reports (7, 8) indicated that digoxin is less than 100% absorbed from tablet dosage forms as compared to an oral solution or an intravenous injection. As a result of these reports, it has become increasingly apparent that the bioavailability of digoxin from a particular manufacturer's dosage form may be significantly reduced. Such incomplete or variable bioavailability of digoxin preparations represents a potential hazard to the patient because a change in the source of manufacture of digoxin or lot-to-lot variation from the same manufacturer may result in toxicity or underdigitalization.

Various methods have been used to estimate or predict the bioavailability of digoxin from commercially available products. With the advent of the radioimmunoassay technique for digoxin, a rapid, precise, and sensitive assay became available for repetitive assays of digoxin in biological fluids. However, as illustrated in this and other reports (9), certain limitations are inherent in the radioimmunoassay for digoxin. Similarly, other reported methods of predicting therapeutic effectiveness of the cardiac glycosides, including *in vitro* dissolution rate testing (5), are subject to limitations. Thus, the objectives of this article are to: *(a)* present a previously unreported variable in the radioimmunoassay technique for the determination of serum digoxin concentrations, *(b)* report the relative bioavailability of two commercially available digoxin tablets in comparison to an elixir, and *(c)* report the applicability of dissolution rate testing in predicting the relative bioavailability of digoxin from tablet dosage forms.

EXPERIMENTAL

Six healthy volunteer male subjects, ranging in age from 20 to 22 years and in weight from 54.6 to 74.2 kg, were selected. The subjects had no history of GI, liver, or kidney disease, and none admitted to taking any medication regularly. Each subject received a thorough physical examination. Values for the following clinical pathology laboratory tests were within normal ranges: hemoglobin, hematocrit, complete blood count, platelet count, differential count, serum creatinine, blood urea nitrogen, sodium, potassium, serum alkaline phosphatase, serum total bilirubin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, chest X-ray, and ECG. Urine was also collected and analyzed for creatinine to enable the determination of creatinine clearance. Digoxin elixir[1] USP was administered first to each subject to determine whether any malabsorption problems existed, a phenomenon previously reported for digoxin (10). Either digoxin tablets[2] USP (hereafter referred to as Treatment I) or another group of digoxin tablets[3] (Treatment II) were then administered to the same six subjects in a crossover manner.

All volunteer subjects were asked to abstain from alcoholic beverages for 48 hr preceding each experiment. They were also asked not to ingest any drug of any kind for at least 1 week preceding each experiment. All subjects were informed as to the nature of the experiments and signed a form prior to each experiment consenting to special studies that are ordinarily not a part of normal diagnosis and treatment of disease states.

Beginning about 8 am on the day of the experiment, two tablets, each containing 0.25 mg of digoxin, were administered orally with 100 ml of water, followed by an additional 100 ml of water 2 hr later. Representative samples from each lot of tablets and elixir were previously determined to meet USP XVIII specifications as to potency, disintegration time, and content uniformity.

Food was not permitted for 8 hr before and 4 hr after digoxin administration. Blood samples of 5–7 ml each were obtained by venipuncture or *via* an intravenous administration set at 0, 0.5, 1, 1.5, 2, 3, 5, 7, 12, 24, and 48 hr following drug administration. The samples were analyzed for digoxin utilizing commercially available radioimmunoassay kits[4] and solid crystal scintillation counting. The bound radioactivity separated from the free ^{125}I-digoxin derivative during the assay procedure was counted by an automatic control gamma scintillation spectrometer[5] for 5 min/sample.

Subjects were ambulatory during the experiment. All subjects were requested not to be in a horizontal position for at least 4 hr after the test dose of digoxin was administered, but they were permitted to sit in a semireclining position.

The dissolution method described by Wagner *et al.* (5) was employed to investigate the applicability of an *in vitro* dissolution rate method in predicting relative bioavailabilities of digoxin from tablet dosage forms. The dissolution medium in the current study was 900 ml of distilled water. One-milliliter samples were

[1] Lanoxin Elixir, Burroughs Wellcome & Co., Research Triangle Park, NC 27709

[2] Lanoxin Tablets, lot 059G, Burroughs Wellcome & Co., Research Triangle Park, NC 27709

[3] Lot 047105, Towne Paulsen and Co., Monrovia, CA 91016

[4] ^{125}I-Digoxin radioimmunoassay kit, Schwarz/Mann, Division of Becton, Dickinson and Co., Orangeburg, NY 10962

[5] Model 578, Packard Instrument Co., Downers Grove, IL 60515

Table I—Mean Digoxin Serum Levels (ng/ml ± Standard Error) and Areas under the Serum Level–Time Curves (ng/ml × hr) for Six Subjects following Oral Administration of 0.5 mg Digoxin in Three Different Forms along with the *F* Ratio and Level of Significance (*p*) Resulting from an Analysis of Variance of the Data

Hours	Digoxin Elixir		Treatment I		Treatment II			
	Mean	*SE*	Mean	*SE*	Mean	*SE*	F^a	*p*
0.0	0.00	0.00	0.00	0.00	0.00	0.00	—	—
0.5	2.76	0.51	0.83	0.30	0.19	0.12	14.92	<0.01
1.0	2.38	0.33	1.38	0.43	0.67	0.20	6.66	<0.01
1.5	1.59	0.16	1.12	0.31	1.56	0.37	0.82	n.s.[b]
2.0	1.09	0.14	0.81	0.14	1.27	0.21	1.61	n.s.
3.0	0.66	0.05	0.64	0.08	0.67	0.10	0.05	n.s.
5.0	0.33	0.06	0.47	0.06	0.37	0.09	0.96	n.s.
7.0	0.22	0.06	0.26	0.03	0.22	0.06	0.20	n.s.
12.0	0.15	0.06	0.22	0.04	0.17	0.07	0.39	n.s.
24.0	0.16	0.03	0.24	0.06	0.19	0.07	0.16	n.s.
48.0	0.04	0.03	0.09	0.02	0.12	0.04	1.91	n.s.
AUC_5	5.51	0.58	3.68	0.66	3.54	0.52	3.42	n.s.
AUC_{12}	6.97	0.56	5.59	0.77	5.11	0.82	1.71	n.s.
AUC_{24}	8.81	0.48	8.04	0.87	7.30	1.44	1.07	n.s.
AUC_{48}	11.17	0.33	11.32	1.08	11.10	2.58	0.01	n.s.

[a] Degrees of freedom (2, 15). [b] Not significant ($p > 0.05$).

obtained at 30, 60, and 120 min; they were filtered through a 0.22-μm membrane filter[6], diluted to 25 ml with 25% ethanol, and then assayed by the radioimmunoassay previously described for digoxin. The only variation in this procedure was that 50 μl of digoxin-free serum was added to each tube immediately after the addition of a 50-μl dissolution sample dilution.

RESULTS AND DISCUSSION

Radioimmunoassay—Linear standard curves were obtained by least-squares linear regression analysis of the reciprocal counts per minute of the bound ^{125}I-digoxin derivative *versus* digoxin concentration. Excellent correlation coefficients were obtained for each standard curve prepared during the assay of each group of samples. The mean correlation coefficient (±standard error) for 25 consecutive standard curves plotted in this way was 0.9988 ± 0.0001. These findings are consistent with reported results (12). Thus, it was concluded that the precision of the method of plotting corrected reciprocal counts per minute *versus* known digoxin concentrations is satisfactory for constructing a standard curve for the determination of unknown digoxin concentrations in samples of similar digoxin concentration.

The radioimmunoassay for digoxin was also investigated for its accuracy and reproducibility in the quantitative determination of digoxin in serum, urine, and hydroalcoholic samples to which a known quantity of digoxin had been added. Within experimental error, complete recovery was demonstrated for each. However, an interesting observation was made as to the necessity of maintaining a uniform quantity of serum in the samples used to prepare the standard curve and in the samples to be assayed. For example, two standard curves prepared simultaneously with different quantities of the same blank serum resulted in nonsuperimposable curves (Fig. 1). One standard curve prepared from 50 μl of blank serum/tube resulted in a *Y*-intercept of 0.00073999 and a slope of 0.00034044. The other standard curve prepared from 200 μl of blank serum resulted in a *Y*-intercept of 0.0016223 and a slope of 0.0012828. The correlation coefficients obtained by linear regression analysis were 0.99949 and 0.99977, respectively, indicating that both standard curves were linear. As illustrated in Fig. 1, a reciprocal count read as 1.0 ng/ml from the standard curve prepared from 200 μl of blank serum would be read as 6.4 ng/ml from the standard curve prepared from 50 μl of blank serum—an approximate sixfold difference in concentration resulting from the same reciprocal count. As can be seen from these plots, the error would become even greater at higher reciprocal counts. The total absence of blank serum from the standard curve and samples yielded erratic results and thus was not investigated further.

In light of this discussion, the results of the urinary excretion studies by Huffman and Azarnoff (7) might be subject to question. To determine the digoxin concentration in urine, they added 0.2 ml of blank plasma to each 0.1 ml urine sample except when the concentration exceeded the highest standard, in which case they simply diluted the sample with phosphate buffer (pH 7.4) containing bovine serum albumin (1 g/liter) to bring the concentration within the range of the standard curve. Unless the serum, plasma, or protein content was adjusted beyond that reported, different quantities of plasma and/or serum albumin were apparently included in the final dilution of some samples. As suggested by the current study, differences in the serum, plasma, or protein

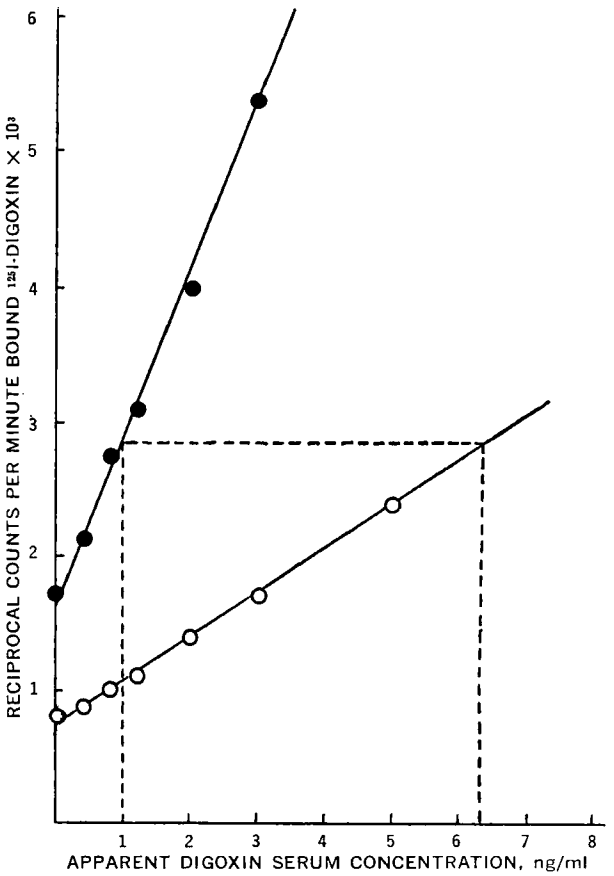

Figure 1—*Reciprocal plots of two standard curves prepared simultaneously utilizing 200 (●) and 50 (○) μl blank serum/tube, illustrating the markedly different apparent digoxin concentrations (indicated by the dashed lines) obtained from reciprocal counts read from the two plots.*

[6] Millipore Corp., Bedford, MA 01730

Table II—Mean Percent[a] Bioavailabilities (±Standard Error) of Commercial Digoxin Tablets Estimated from Area under the Serum Level–Time Curve during Selected Finite Time Periods following the Oral Administration of Two 0.25-mg Digoxin Tablets to Six Male Subjects

Hours	Bioavailability, %		t^b	p
	Treatment I	Treatment II		
0–5	70.98 ± 8.14	64.88 ± 8.92	0.616	n.s.[c]
0–12	83.28 ± 5.62	73.53 ± 13.13	0.931	n.s.
0–24	95.64 ± 7.17	85.17 ± 19.23	0.676	n.s.
0–48	106.38 ± 10.27	100.75 ± 24.09	0.238	n.s.

[a] Calculated as a percentage of the corresponding area under the serum level–time curve obtained from the elixir data. [b] Degrees of freedom = 5. [c] Not significant ($p > 0.05$).

content of either the samples used to prepare the standard curve or the unknown samples could result in markedly different apparent digoxin concentrations.

Relative Bioavailability Studies—Table I summarizes the mean serum levels at all times for the three treatments, along with the area under the serum level–time curves up to 5, 12, 24, and 48 hr as calculated by the trapezoidal rule. The serum levels at each sampling time, the individual serum levels at each sampling time, and the individual areas were analyzed by a one-way analysis of variance. Thus, the *F*-ratios and the significance levels of the treatment mean square are also shown in Table I. As can be seen, significant differences resulting from the treatments occurred only at the 0.5- and 1.0-hr periods, probably due to the much higher levels obtained following administration of the elixir. Thus, it would appear that there was very little difference in the rate and extent of digoxin absorption from the two brands of compressed tablet dosage forms investigated.

One criticism of the study by Lindenbaum *et al.* (1) was that serum concentrations of digoxin were determined for less than one-third of one half-life, a period generally thought to be too short to reflect accurately the total absorption and, thus, bioavailability. However, in a recent report (5), the area under the curve (AUC) for the 0–5-hr interval correlated "extremely well" with the area measured from 0 to 96 hr. Similar results were obtained in the current study, as can be seen from the various areas (Table I). However, relative bioavailabilities of the two tablet dosage forms appeared to be somewhat lower when calculated from data over shorter periods, as can be seen by comparing the relative bioavailabilities and taking the elixir data as 100% (Table II). The increase in the apparent relative bioavailabilities of both commercial digoxin tablets when estimated from data recorded over a longer period may be accounted for on the basis of a larger proportion of the total area under the curve occurring during the latter part of the time interval for the tablets in comparison to the elixir, which showed much earlier and higher peak levels. However, the relative bioavailabilities of the two tablets remained quite similar regardless of the time used to calculate the respective areas. When the individual values were statistically evaluated by a *t* test for paired observations, there were no significant differences between the two treatments at any time interval (Table II). Consequently, further treatment of the serum level data tends to provide additional evidence that there is indeed very little difference in the rate and extent of digoxin absorption from these two commercially available tablet dosage forms.

Blood levels of various drugs are frequently used by clinicians to determine the extent of an expected pharmacological or toxic response. However, such data have little meaning in the absence of supportive information. Concentrations obtained at the time of peak blood levels may lead to a different conclusion from concentrations obtained during the equilibrium plateau of the blood level curve. In the case of digoxin, as demonstrated in this study, individual peak serum levels may exceed 4 ng/ml[7] following a single oral dose of 0.5 mg of digoxin in the elixir dosage form (upper limit of 0.5-hr level). Such levels have been reported to be in the toxic range (11, 12).

Dissolution Studies—A commonly employed method of estimating bioavailability is testing the rate of dissolution of the drug from a compressed tablet. Recently, Wagner *et al.* (5) correlated rate of dissolution of digoxin from commercially available tablets to values for areas under the curve, and they concluded that their dissolution test was capable of distinguishing between "good" and "bad" lots of digoxin tablets. From the results of the current study, it appears that this dissolution method (5) may not be applicable to the comparative evaluation of the two brands of tablets employed in the current study. The mean percent (±standard error) of the labeled amount of digoxin in solution at 120 min for five Treatment I tablets (Table III) was 59.1 ± 3.9% whereas the mean percent in solution at 120 min for five Treatment II tablets was 19.4 ± 3.5%, a more than threefold difference. Although the Treatment I tablets were only about 59% dissolved after 2 hr as contrasted to a reported 80.3% dissolved in the study of Wagner *et al.* (5) for the same brand, this should not be surprising in light of a recent report by Lindenbaum *et al.* (6). These investigators showed a fairly wide range in dissolution rates for three different lots of tablets prepared by the manufacturer of the Treatment I tablets. The percent dissolved after 2 hr, estimated from Fig. 1 of the Lindenbaum *et al.* (6) report, ranges from slightly less than 60% to slightly more than 80% for the various lots tested. Although the methodology employed by these investigators was somewhat different than was utilized in the present study, the lower rate obtained compared to that reported by Wagner *et al.* (5) can be explained by an apparent, large batch-to-batch variation in the dissolution rate properties of digoxin tablets made by this manufacturer.

Table III—Mean Percent (±Standard Error) of Labeled Digoxin in Solution at Various Times following *In Vitro* Dissolution Testing on Five Tablets of Each Formulation

Minutes	Dissolved, %	
	Treatment I	Treatment II
30	34.9 ± 4.3	6.0 ± 1.2
60	46.9 ± 6.1	8.0 ± 0.8
120	59.1 ± 3.9	19.4 ± 3.5

However, as shown by the data in Tables I and II, no such difference was observed in the bioavailability of the tablets. This would suggest that the drug is readily available for absorption from both tablets and does, indeed, yield relatively high areas under the curve. The slower dissolution observed with Treatment II tablets did not have a significantly adverse effect on the bioavailability as shown in this study. It is possible that when certain differences in formulation or manufacturing specifications are involved between two brands, it may not be possible to correlate dissolution results and bioavailability for digoxin tablets. Thus, it is apparent that *in vivo* testing in the target species (humans) is necessary to prove bioavailability of a particular dosage form and that additional work is needed before a dissolution rate method can be used to predict digoxin bioavailability consistently.

[7] Although individual data have not been reported, Subject 2 showed a serum level of 4.70 ng/ml 30 min after drug administration.

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§ Present address: Eli Lilly and Co., Indianapolis, IN 46206

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K. K. MIDHAˣ, I. J. McGILVERAY, and C. CHARETTE

Abstract □ Sensitive specific methods are described for the determination of phenylbutazone and its metabolite oxyphenbutazone from the same plasma sample. The sample, to which an internal standard 5-(4-hydroxyphenyl)-5-phenylhydantoin (Standard II) is added, is first extracted with ether to remove interfering substances and then with *n*-heptane under acidic conditions to separate phenylbutazone, which is determined on a gas chromatograph by flash methylation (310°) with trimethylanilinium hydroxide using 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane as the external standard (Standard I). The aqueous acidic residue from which the phenylbutazone has been selectively removed is shaken again with ether to extract the oxyphenbutazone, which is analyzed with a different GLC system with flash methylation (310°) against Standard II. The methods are of sufficient sensitivity to determine plasma levels in humans after a 200-mg dose of phenylbutazone (phenylbutazone, 1 μg/ml; oxyphenbutazone, 0.5 μg/ml).

Keyphrases □ Phenylbutazone and its metabolite oxyphenbutazone—GLC analysis in plasma □ Oxyphenbutazone metabolite and phenylbutazone—GLC analysis in plasma □ GLC—analysis, phenylbutazone and oxyphenbutazone metabolite in plasma

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The classical method of Burns *et al.* (1) is not of sufficient sensitivity to estimate phenylbutazone and oxyphenbutazone in biological fluids following single doses of phenylbutazone. Although the need for a sensitive specific method for estimating phenylbutazone in plasma has been met by the recently reported high-speed liquid chromatography (HSLC) and GLC procedures (8, 9), no suitable methods are available for estimating oxyphenbutazone and hydroxyphenylbutazone following single doses of phenylbutazone in humans.

For the current pharmacokinetic study in these laboratories (10), a GLC method that is specific and sensitive for measuring oxyphenbutazone in plasma following single doses of phenylbutazone has been developed. The liquid chromatographic method reported earlier (9) for phenylbutazone is adequate, but the present method measures the levels of oxyphenbutazone as well as phenylbutazone in the same plasma sample.

EXPERIMENTAL

Reagents—Ether[1] and *n*-heptane[2] were distilled in glass prior to use. Stock solutions of phenylbutazone[3] containing 100 μg/ml were prepared (9) and diluted with phosphate buffer (pH 7.4) to the concentrations required (2–64 μg/ml) before use. Stock solutions of oxyphenbutazone[3] containing 16 μg/ml in ether were freshly prepared daily and diluted to the required range (1–16 μg/ml). Stock solutions of 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane[4] (Standard I), containing 50 μg/ml in *n*-heptane, and 5-(4-hydroxyphenyl)-5-phenylhydantoin[5] (Standard II), containing 22.2 μg/ml in 0.01 *N* NaOH, were prepared. A solution of Standard I was prepared daily and used as such. A solution of Standard II was prepared every week and diluted to 1.11 μg/ml with 0.01 *N* NaOH daily before use. A 0.2 *M* phosphate buffer (pH 11.2 ± 0.2) and 1 *N* and 2 *N* HCl were employed. A 0.2 *M* solution of trimethylanilinium hydroxide in methanol was synthesized according to the method of Barret (11).

Glassware—All glassware was silanized before use by soaking for 1 hr in 1% hexamethyldisilazane in ether and rinsing with ether, methanol, and finally water (distilled in glass) before oven drying. Evaporation tubes with narrow bore bases (12) were custom made from Teflon-lined screw-capped test tubes[5].

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[1] Diethyl ether (anhydrous), Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[2] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[3] Ciba-Geigy, Canada.
[4] Aldrich Chemical Co., Milwaukee, WI 53233.
[5] Canadian Laboratory Supplies Ltd., Montreal, Quebec, Canada.
[6] Butazolidin, Geigy Pharmaceuticals, Montreal, Quebec, Canada.
[7] Vacutainers, Becton Dickinson & Co., Mississauga, Ontario, Canada.

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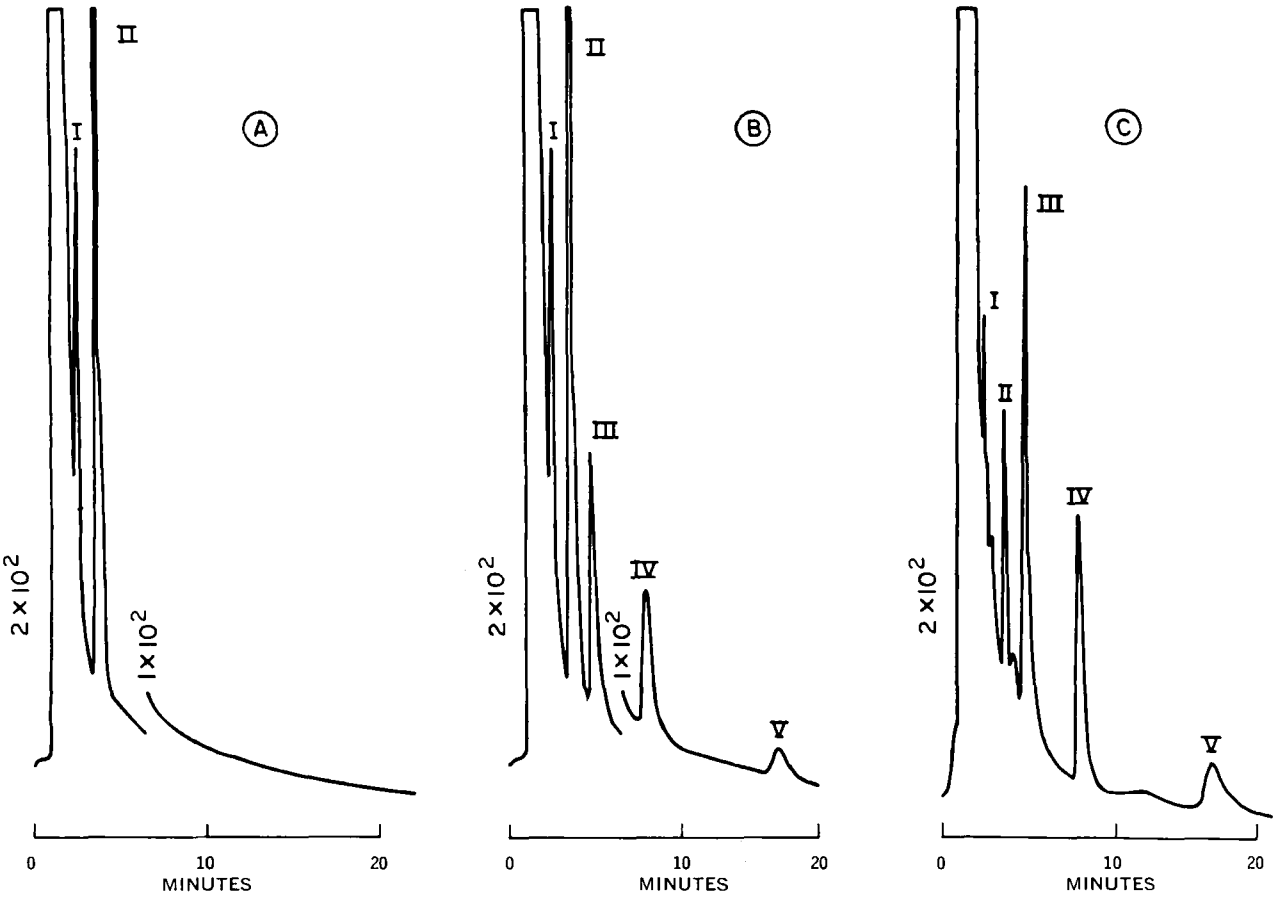


Figure 1—*GLC of human plasma. Key: A, control plasma; B, plasma containing 16 μg/ml of phenylbutazone and 50 μg/ml of Standard I; C, plasma from a human volunteer who had been given 400 mg of phenylbutazone; peaks I and II, endogenous material from plasma; peak III, Standard I; and peaks IV and V, major and minor peaks of flash-methylated phenylbutazone.*

II (1.11 μg/ml), 2 ml of 1 *N* HCl, and 5 ml of ether. The samples are extracted by mixing[8] for 10 min at 19 rpm followed by centrifugation at 2500 rpm for 10 min. Four milliliters of the ethereal layer is transferred into another Teflon-lined screw-capped centrifuge tube (20 ml) containing 5 ml phosphate buffer (pH 11.2). After repeating the extraction with 7 ml of ether, 7 ml of the organic layer is added to the previous extract. The combined extracts are mixed[8] with the phosphate buffer (10 min) and centrifuged (10 min) and the ethereal layer is discarded. The aqueous phosphate solution is acidified with 2 ml of 2 *N* HCl and extracted with 2 × 5-ml portions of *n*-heptane (mix[8] 10 min and centrifuge 10 min). Four milliliters of the *n*-heptane layer is transferred from the first extract and 5 ml from the second into an evaporating tube containing 1 ml of Standard I (50 μg/ml in *n*-heptane). The *n*-heptane extract is evaporated to dryness at 60° under a stream of dry nitrogen, and 50 μl of a methanolic solution of trimethylanilinium hydroxide (0.2 *M*) is added. The contents are dissolved by swirling on a mixer[9] before injecting 1–2 μl into gas chromatograph I.

Extraction of Oxyphenbutazone—The tubes containing the acidified phosphate buffer and remaining *n*-heptane are carried over for the determination of oxyphenbutazone. The remaining *n*-heptane is removed with a Pasteur pipet, a further 2-ml portion of *n*-heptane is added, the tube is swirled gently by hand, and *n*-heptane is removed with a Pasteur pipet. The aqueous acid is then extracted with 2 × 5-ml portions of freshly distilled ether by mixing[8] (15 min each time) followed by centrifugation (10 min each time) and removal of 4 and 5 ml of the ether extract successively into an evaporating tube. The ether extract is then evaporated in a dry bath at 40° under a stream of dry nitrogen; 20 μl of trimethylanilinium hydroxide (0.2 *M*) in methanol is added with gentle rotation followed by mixing[8]. One to two microliters is injected into gas chromatograph II.

GLC—GLC was effected on two gas chromatographs[10], both equipped with flame-ionization detectors. Stainless steel columns [2.4 m × 0.3 cm (8 ft × 0.125 in.) o.d. for phenylbutazone, column I; 1.2 m × 0.3 cm (4 ft × 0.125 in.) o.d. for oxyphenbutazone, column II] packed, respectively, with 3.0 and 5.0% OV-7 coated on acid-washed, DMCS treated, 80–100-mesh, high performance Chromosorb W[11] were used throughout the study. Both columns were conditioned by injecting a silylating mixture[12] and maintaining the columns at 300° for 18 hr with low nitrogen flow. For both columns the injection port and detector temperatures were 310 and 300°, respectively, and the column oven temperature was 260°. Nitrogen, as a carrier gas, was maintained at 40 ml/min for column I and 27 ml/min for column II. Hydrogen and compressed air flow rates were adjusted to give maximum response with each gas chromatograph.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to phenylbutazone or oxyphenbutazone by the height of the respective standard. Calibration curves were constructed from the results of spiked control plasma samples by plotting the concentration of phenylbutazone or oxyphenbutazone (micrograms per milliliter plasma) against the respective peak height ratios.

RESULTS AND DISCUSSION

Flash-heater methylation of phenylbutazone with trimethylanilinium hydroxide gave two peaks with retention times of 7.1 and 14.9 min (IV and V, Fig. 1B). The heights of the respective peaks were in the ratio 5:1. This ratio was independent of oven temper-

[8] Roto-Rack, Fisher Scientific Co.
[9] Vortex Genie, Fisher Scientific Co.
[10] Model F/11, Perkin-Elmer, Canada.
[11] Chromatographic Specialties, Brockville, Ontario, Canada.
[12] Silyl 8, Pierce Chemical Co., Rockford, Ill.

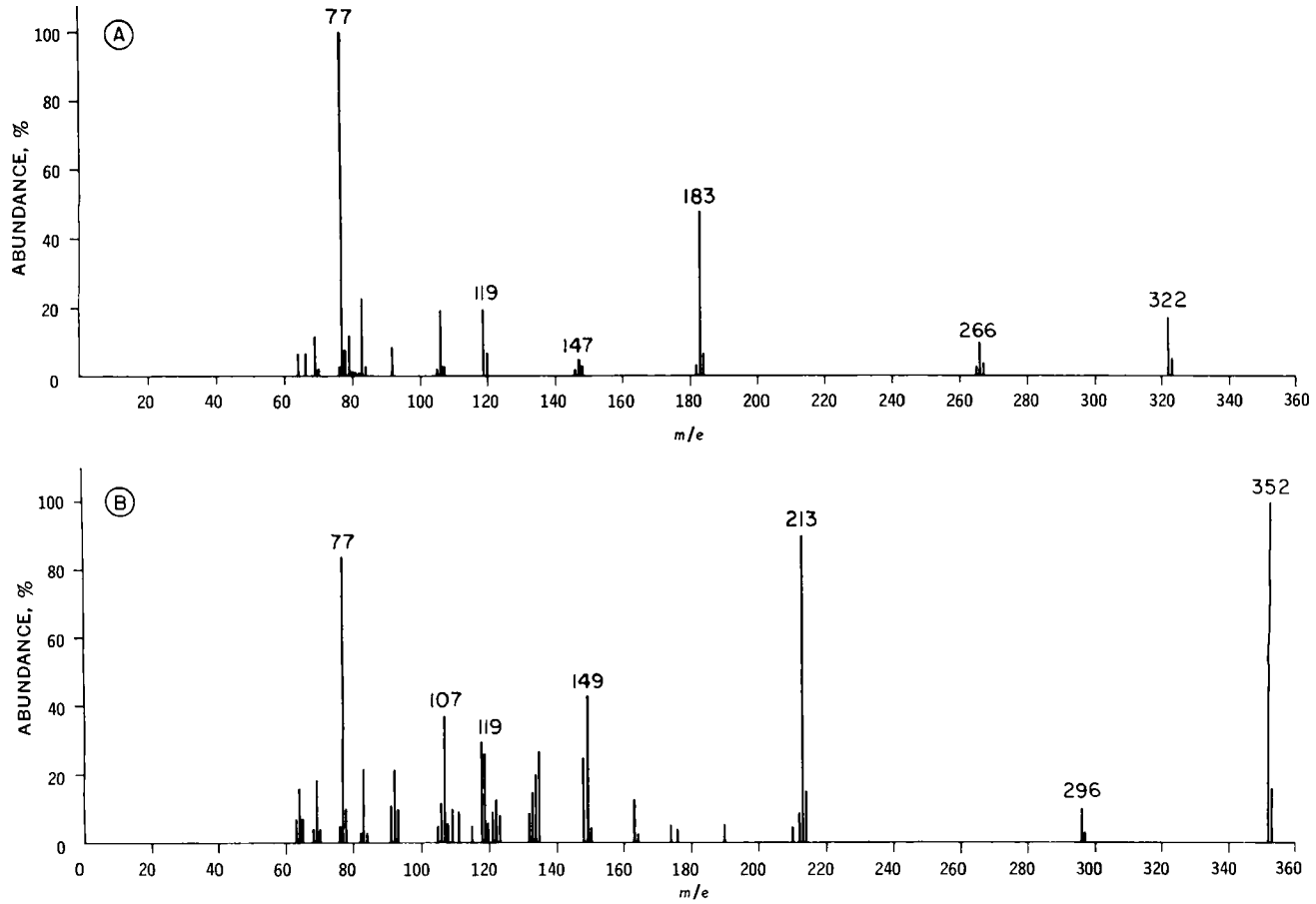


Figure 2—*GLC–mass spectra of major peaks of flash-methylated phenylbutazone (A) and oxyphenbutazone (B).*

ature, instrument, and sample size; therefore, the major peak that elutes first was used for quantitation. Standard I gave a single peak at 4.0 min upon flash methylation (III, Fig. 1B). To establish the structures of the compounds giving rise to these two peaks from trimethylanilinium hydroxide treatment of phenylbutazone, eluates from the GLC column were fed directly into the mass spectrometer[13] and the mass spectra of IV and V were recorded. The mass spectrum of each eluate showed a molecular ion peak at *m/e* 322. Abundant ions of the major component retention time (7.1 min, IV, Fig. 1B) were located at *m/e* 77, 119, 147, 183, and 266 (Fig. 2A). Structures I–V have been postulated for these ions (Scheme IA). These fragmentations suggest that the major derivative of flash-methylated phenylbutazone has the structure 1,2-diphenyl-3-methoxy-4-*n*-butyl-5-oxopyrazoline.

Flash-heater methylation of oxyphenbutazone with trimethylanilinium hydroxide gave two peaks with retention times of 9.3 min for the major peak and 18.4 min for the minor peak (VI and IX, Fig. 3C). As with phenylbutazone, both were formed in the same height ratio and this ratio was also independent of oven temperature, instrument, and sample size. GLC–mass spectroscopy of these two derivatives of oxyphenbutazone was employed. The mass spectrum of the derivative giving rise to the major peak (retention time 9.3 min) showed the molecular ion at *m/e* 352 (VII, Scheme IB) and that giving rise to the minor peak retention time (18.4 min) showed the molecular ion at *m/e* 338. The mass spectrum of the major product (Fig. 2B) had abundant ions at *m/e* 77, 107, 119, 149, 213, and 296, for which Structures I–VI (Scheme IB) have been postulated. The major product of flash-methylated oxyphenbutazone was tentatively assigned the structure 1-phenyl-2-(*p*-methoxyphenyl)-3-methoxy-4-*n*-butyl-5-oxopyrazoline and was used for quantitation.

Flash-heater methylation of Standard I with trimethylanilinium hydroxide consistently gave a sharp peak on column I under the described experimental conditions. Flash methylation of 5-(4-hydroxyphenyl)-5-phenylhydantoin (Standard II) was described previously (15). The combined GLC–mass spectral evidence obtained in the present study was consistent with a trimethylated structure for Standard II (the molecular ion being at *m/e* 310). The use of trimethylanilinium hydroxide as a methylating reagent offers the advantage that purified plasma extracts at high temperatures do not need to be completely moisture free—a factor that saves time. The reagent is stable over a long period and is easily prepared. The methylation reaction is instantaneous in the injection port at a temperature of 300° (14). One important precaution is that care must be taken to avoid transfer of trace amounts of acid during the extraction carried out at acidic pH since methylation with trimethylanilinium hydroxide does not occur under acidic conditions.

Figure 1A shows a typical chromatogram obtained by processing fresh blank plasma as described in the *Experimental* section for phenylbutazone but omitting the external standard (Standard I). The extraneous peaks (I and II) at retention times 1.6 and 2.8 min were observed in chromatograms of all human plasma samples. A chromatogram obtained when the method was applied to spiked plasma containing 16 μg/ml of phenylbutazone is shown in Fig. 1B, where it is clear that the extraneous peaks I and II do not interfere with the peaks from Standard I (peak III, retention time 4.0 min) or phenylbutazone (peak IV, retention time 7.1 min). Figure 1C shows a chromatogram from a 1-ml plasma sample from a volunteer who received 400 mg of phenylbutazone. The time required for one analysis is 18 min. Oxyphenbutazone and hydroxyphenylbutazone do not interfere with the assay because they are extracted with *n*-heptane only to the extent of 7% and have different retention times. The major and minor peaks of oxyphenbutazone on column I have retention times of 15.2 and 31.0 min, respectively. Hydroxyphenylbutazone[14], under similar condi-

[13] Hitachi Perkin-Elmer model RMS4 mass spectrometer with Perkin-Elmer gas chromatograph model 990.

[14] Reference specimen was provided by Dr. A. Sallman, Ciba-Geigy, Switzerland.

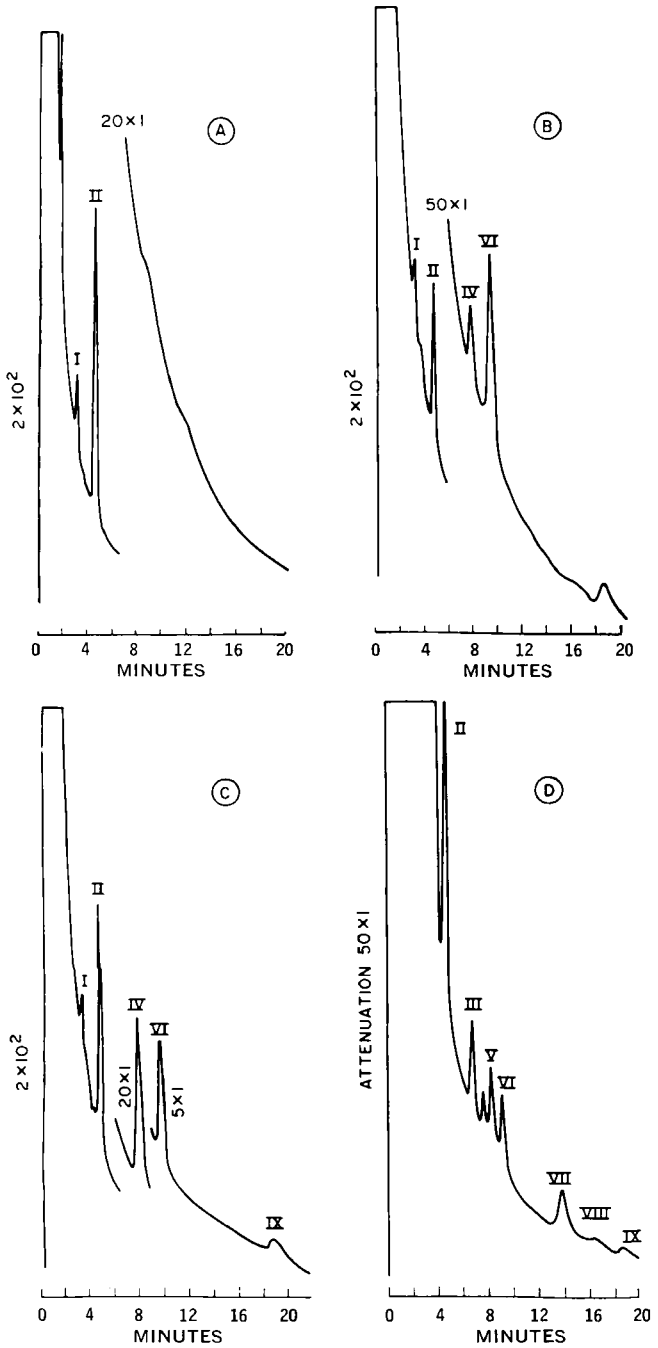
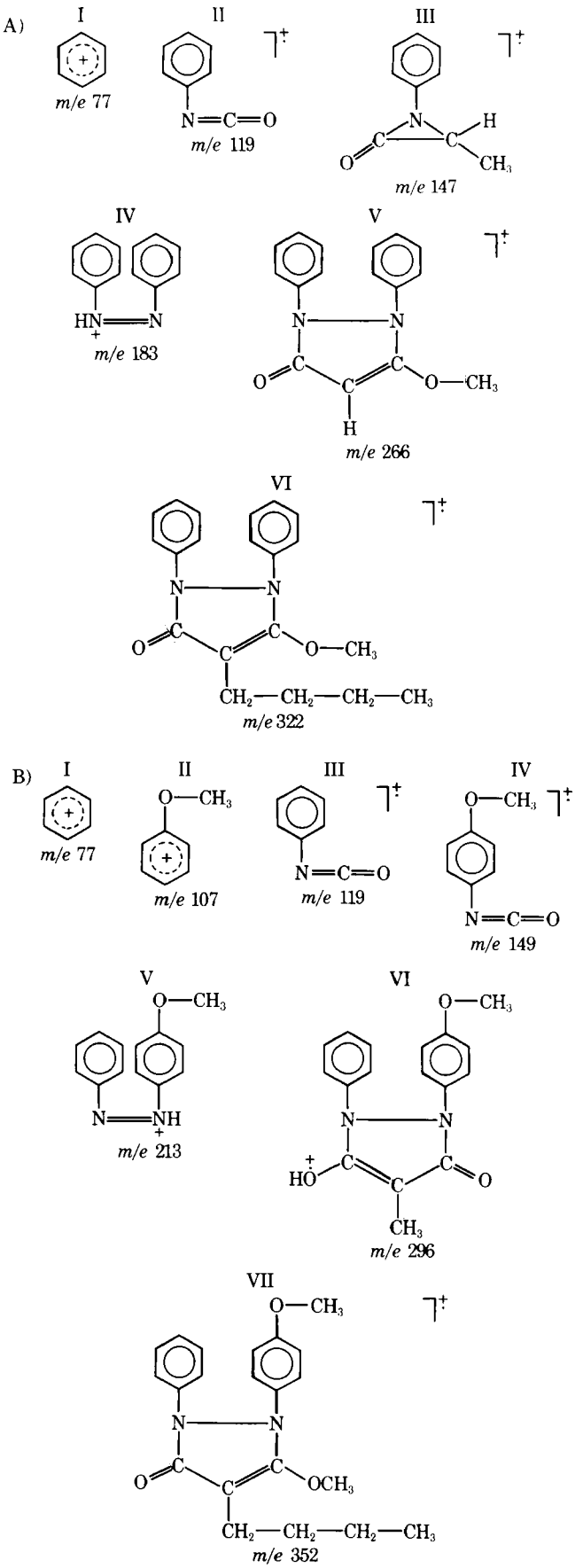


Figure 3—*GLC of human plasma. Key: A, control plasma; B, plasma containing 14.75 μg/ml of oxyphenbutazone and 1.11 μg/ml of Standard II; C, plasma from a human volunteer who had been given 200 mg (2 × 100-mg tablets) of phenylbutazone; D, plasma containing 9.44 μg/ml of oxyphenbutazone, 15.56 μg/ml of hydroxyphenylbutazone, and 1.11 μg/ml of Standard II; peaks I and II, endogenous material; peaks III, V, VII, and VIII, peaks due to flash-methylated hydroxyphenylbutazone; peak IV, Standard II; and peaks VI and IX, peaks due to flash-methylated oxyphenbutazone.*

tions, gives peaks with retention times of 10.5 and 14.2 min and thus does not interfere.

The response of the flame-ionization detector to phenylbutazone was linear with concentration in the 1.0–64.0-μg/ml range. The peak height ratio of the drug and external standard was used as the index of detector performance and overall efficiency of the analytical procedure. The overall recovery of phenylbutazone from plasma was more than 98%, a figure comparable to that reported earlier (8, 9). Figure 4 represents the calibration curve ob-



Scheme I—Postulated fragmentation of phenylbutazone (A) and oxyphenbutazone (B)

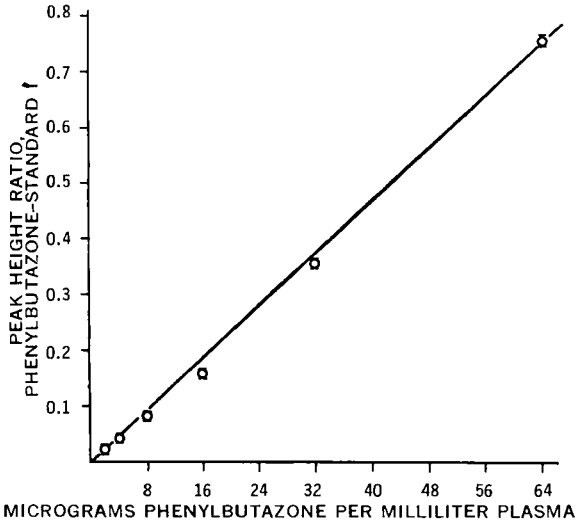


Figure 4—*Calibration curve of phenylbutazone extracted from human plasma. Each point is a mean of at least four determinations, except for 2 μg where a mean of eight determinations was taken.*

tained by plotting the peak height ratio of phenylbutazone/Standard I *versus* the concentration of phenylbutazone. The plot is a straight line ($y = mx$) over the range of 2.0–64.0 μg phenylbutazone/ml plasma. A mean slope value of 0.0117 ± 0.0003 ($r^2 = 1$) was obtained. The overall coefficient of variation was 4.65%.

A typical chromatogram obtained by processing fresh blank plasma as described in the *Experimental* section for oxyphenbutazone but omitting the internal standard (Standard II) is shown in Fig. 3A. The extraneous peaks (I and II) at retention times 3.2 and 4.6 min were observed in chromatograms of plasma from all human subjects (total of eight) including the pooled plasma from the Red Cross blood bank. The extraneous peaks (I and II) do not interfere with peaks due to Standard II (IV, retention time 7.7 min) and oxyphenbutazone (VI, retention time 9.3 min), as shown in the chromatogram for spiked plasma (Fig. 3B) containing 14.75 μg/ml of oxyphenbutazone. The time required for one analysis is 20 min. Phenylbutazone does not interfere because it is removed by partitioning into *n*-heptane. This partition of phenylbutazone into *n*-heptane was checked by preparing plasma samples containing various concentrations of phenylbutazone (up to 60 μg/ml) and treating each sample as described for the oxyphenbutazone extraction. No phenylbutazone was detected in the final ether extract.

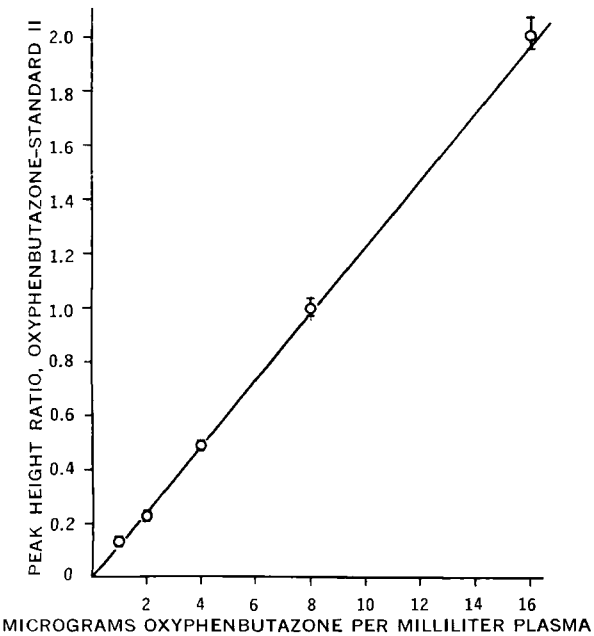


Figure 5—*Calibration curve of oxyphenbutazone extracted from human plasma. Each point is a mean of at least five determinations.*

Table I—Recovery of Oxyphenbutazone and Standard II from Plasma Determined by GLC Assay

Micrograms Added to 1 ml Plasma	*n*	Mean Micrograms Recovered	Mean Recovery, %	*SD* of Recovery, %
Recovery of Standard II				
1.11	5	0.77	69.72	2.37
Recovery of Oxyphenbutazone				
4.00	6	0.98	24.43	0.74
8.00	6	1.98	24.75	0.23
Mean 24.59 ± 0.55%				

There was no interference from hydroxyphenylbutazone because flash methylation of this metabolite gives peaks at 6.8, 8.4, 13.7, and 16.0 min—well separated from the methylated oxyphenbutazone (Fig. 3D). Figure 3C shows the chromatogram of a sample (72 hr) from a human subject who received 200 mg of phenylbutazone. The response of the flame-ionization detector to oxyphenbutazone was linear with concentration in the 1.0–16.0-μg/ml range. The peak height ratio of oxyphenbutazone and the internal standard (Standard II) was used as the index of detector performance for the second gas chromatograph and overall efficiency of the analytical procedure. The overall recovery of 4.0 and 8.0 μg of oxyphenbutazone and 1.11 μg of Standard II from plasma was of the order of 24.59 ± 0.55 and 69.72 ± 2.37%, respectively (Table I). The recovery of oxyphenbutazone in this procedure was low but was consistent.

Several attempts were made to improve the recovery of oxyphenbutazone by employing different solvents, such as dichloromethane and dichloroethane. There were large variations in the recovery, which were attributed to the breakdown of oxyphenbutazone and/or carryover of trace amounts of acid resulting in the inhibition of flash methylation. Ethylene dichloride, employed by Burns *et al.* (1), was also tested. No improvement in recovery of oxyphenbutazone was obtained, while the procedure became more difficult due to transference of ethylene dichloride (specific gravity 1.25). Figure 5 represents the calibration curve obtained by plotting the peak height ratio of oxyphenbutazone/Standard II *versus* the concentration of oxyphenbutazone. The plot is a straight line ($y = mx$) over the concentration range of 1.0–16.0 μg oxyphenbutazone/ml plasma. A mean slope value of 0.126 ± 0.002 ($r^2 = 1$) was obtained. Following single doses of up to 400 mg phenylbutazone, hydroxyphenylbutazone could not be detected. Calibration curves for oxyphenbutazone were also tested in the presence of various amounts of phenylbutazone and no change in slope was detected.

Refrigerated plasma samples containing phenylbutazone have been reported to be stable for several weeks (8, 9). It was found that refrigerated plasma samples containing oxyphenbutazone were also stable for several weeks. However, evaporated extracts

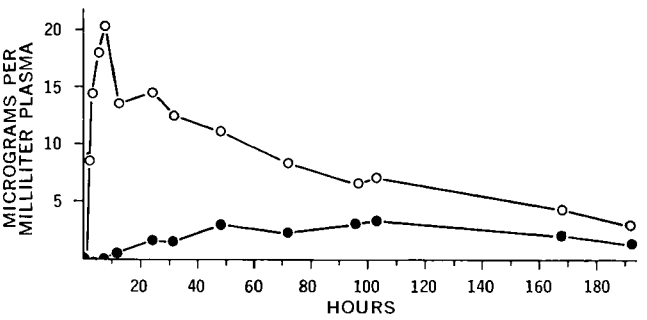


Figure 6—*Phenylbutazone and oxyphenbutazone concentrations in a human volunteer following single oral dose of 200 mg (2 × 100-mg tablets) phenylbutazone. Key: ○, phenylbutazone; and ●, oxyphenbutazone.*

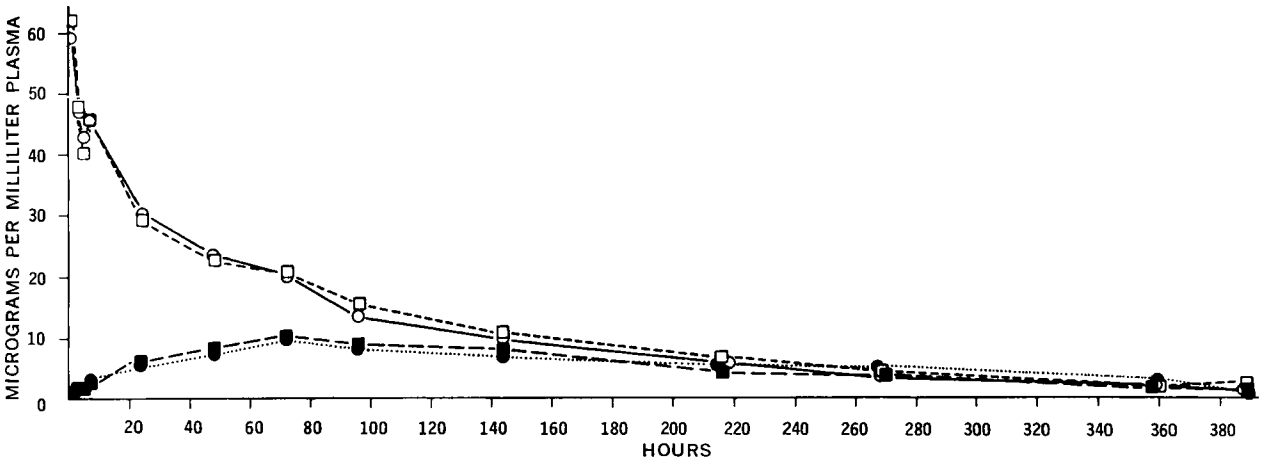


Figure 7—*Comparison of plasma levels determined by GLC method with those of a HSLC method following a single oral dose of 400-mg solution of phenylbutazone to a volunteer. Key:* ○, *phenylbutazone, GLC method;* ●, *oxyphenbutazone, GLC method;* □, *phenylbutazone, HSLC method; and* ■, *oxyphenbutazone, HSLC method.*

to which trimethylanilinium hydroxide had been added were unstable over 24 hr. The tubes containing evaporated extracts, when stored overnight in a freezer or in dry ice–acetone, showed lower amounts (4.7–5%) of oxyphenbutazone. Thus, extraction and GLC analysis of oxyphenbutazone should be completed on the same day.

Application of the method of plasma level determination of phenylbutazone and oxyphenbutazone by GLC is shown in Figs. 6 and 7, where it is compared with a recent HSLC procedure[15]. A 200-mg dose of phenylbutazone (2 × 100-mg tablets) was given to a healthy male volunteer [95.3 kg (210 lb)], and plasma was withdrawn at intervals over 192 hr and assayed for phenylbutazone and oxyphenbutazone using the GLC method (Fig. 6). A second male volunteer [90.8 kg (200 lb)] was given a 400-mg dose of phenylbutazone in solution, and plasma levels of phenylbutazone and oxyphenbutazone over 388 hr were compared by the GLC method and a recent HSLC procedure (Fig. 7). The methods compare favorably, with an overall 8.06% difference for phenylbutazone and 7.68% difference for oxyphenbutazone between the two methods.

The described GLC method is sensitive for the determination of phenylbutazone as well as oxyphenbutazone. It offers the advantage that determinations of the drug as well as its metabolite are made by extraction of the same plasma sample. It is a specific method, because the second metabolite hydroxyphenylbutazone is distinguished clearly; therefore, it can be employed for single- as well as multiple-dose pharmacokinetic studies.

[15] N. J. Pound, Drug Research Labs., Health Protection Branch, Ottawa, Canada, personal communication.

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[x] To whom inquiries should be directed.

Kinetics of Aerobic Oxidation of Ascorbic Acid in the Presence of Nonionic Surfactants

S. M. BLAUG[x] and B. HAJRATWALA*

Abstract □ The effect of two nonionic surfactants, poloxalkol and polysorbate 80, on the aerobic oxidation of ascorbic acid was studied at 67° and pH 4.55 and 6.60. Results show a relationship between surfactant concentration and oxidation rate, the rate increasing at surfactant concentrations well below the surfactant's CMC and decreasing as the surfactant concentration approaches its CMC. The thermodynamic parameters were obtained for ascorbic acid solutions containing 1% surfactant.

Keyphrases □ Ascorbic acid—aerobic oxidation, effects of nonionic surfactants poloxalkol and polysorbate 80, kinetic parameters □ Surfactants—effects of poloxalkol and polysorbate 80 on ascorbic acid aerobic oxidation kinetics □ Poloxalkol—effects on aerobic oxidation of ascorbic acid, kinetic parameters □ Polysorbate 80—effects on aerobic oxidation of ascorbic acid, kinetic parameters □ Oxidation—ascorbic acid in presence of nonionic surfactants, above and below CMC

As early as 1918, McBain and Bolam (1) found that soap solutions protected materials against hydrolysis. Nogami *et al.* (2) reported that the hydrolysis of aspirin was suppressed by cationic, anionic, and nonionic surfactants, and they also reported the stabilization of various other drugs through the use of surfactants (3, 4). Other investigators (5, 6) showed that vitamin A solubilized in aqueous solutions of nonionic surfactants was more resistant to oxidation than vitamin A in an oil solution. However, Patel *et al.* (7) reported that solubilized vitamin A decomposed faster than nonsolubilized vitamin A.

Carless and coworkers (8–13), using benzaldehyde oxidation as a model system, studied the role of surfactants in retarding oxidation. They found that solubilized systems were oxidized at lower rates and were less susceptible to the catalytic action of metallic ions than emulsified systems (10).

The stability of ascorbic acid in various pharmaceutical vehicles has been reported. Giral (14) attributed the stability of ascorbic acid in simple syrup to the viscosity of the syrup. Aqueous solutions of sucrose, sorbitol, glycerin, and propylene glycol were found to stabilize ascorbic acid (15), and similar findings were reported by other workers (16). Viscous solutions containing natural hydrophilic colloids appeared to accelerate the rate of oxidation of ascorbic acid. This was attributed to the presence of metal ion or impurities in the colloids (15).

Two recent studies (17, 18) of the copper-catalyzed oxidation of ascorbic acid in the presence of polysorbates 20 and 80 showed interesting results. When using saturated solutions of ascorbic acid containing varying concentrations of polysorbate 20 (17), the oxidation rate of ascorbic acid declined rapidly between 40 and 75% surfactant. The decrease in the rate at high concentrations of surfactant was attributed to a decrease in the diffusion of oxygen to the site of oxidation due to the high viscosity of the solution.

In the presence of polysorbate 80 (19), a rapid decline in the rate of oxidation of ascorbic acid was reported in solutions containing 3% ascorbic acid and varying concentrations of polysorbate 80. The rate declined in solutions containing up to 10% surfactant and remained constant at surfactant concentrations between 10 and 30%. The decrease in the oxidation rate of ascorbic acid was attributed to an increase in both micelle concentration and micelle aggregation number in solutions containing up to 10% polysorbate 80. The increase in viscosity produced at high surfactant concentration had no significant effect on the rate of oxidation of ascorbic acid.

A previous investigation (19) concerned the aerobic degradation of ascorbic acid in aqueous solutions over the 3.52–7.22 pH range. The purpose of the present investigation was to study the aerobic degradation of ascorbic acid in the presence of two nonionic surfactants at pH 4.55 and 6.60. Surfactants used in internal preparations must be nontoxic and effective as stabilizers in relatively low concentrations. The agents used in this study were polysorbate 80[1] and poloxalkol (polyoxyethylene–polyoxypropylene polymer)[2].

EXPERIMENTAL[3]

Materials—All reagents used were of analytically pure grade as previously reported (19). Surfactants were used as received without further purification.

Method of Analysis—Kinetic runs were carried out as previously described (19) using the 2,6-dichloroindophenol method.

Determination of Critical Micelle Concentration—The critical micelle concentrations (CMC) of polysorbate 80 and poloxalkol were determined at 25° in acetate buffers (pH 3.52, 4.55 and 5.45) and phosphate buffer (pH 6.60). The buffers were adjusted to ionic strength 0.4 by the addition of potassium chloride. The CMC's were determined from plots of surface tension *versus* concentration, using the point of intersection obtained by extrapolation of the straight-line portions of the curve as the CMC value.

Oxidation in Presence of Surfactants—To determine the effect of the surfactants on the analytical procedure previously described, ascorbic acid solutions containing 0.01 mole liter^{-1} of ascorbic acid and 5% polysorbate 80 or poloxalkol were prepared in acetate buffers (pH 3.52, 4.55, and 5.45) and phosphate buffer (pH 6.60). The solutions were analyzed for ascorbic acid using the 2,6-dichloroindophenol method.

The effect of polysorbate 80 and poloxalkol on the rate of oxidation of ascorbic acid was studied in acetate buffer (pH 4.55) and

[1] Tween 80, Atlas Chemical Co., Wilmington, Del.

[2] Pluronic F-68, Wyandotte Chemicals Corp., Wyandotte, Mich.

[3] Surface tension measurements were obtained using a Fisher surface tensiometer model 21 equipped with a 6-cm platinum–iridium ring. Viscosities were determined with an Ostwald-Fenske viscometer (size 100). A constant-temperature bath with a Haake thermoregulator series "ED" and a thermometer calibrated to 0.1° was used for all studies.

Table I—Analysis of Ascorbic Acid at Various pH's and Ionic Strength 0.4 in the Presence of Polysorbate 80 and Poloxalkol Using the 2,6-Dichloroindophenol Volumetric Method

Buffer	pH	Ascorbic Acid Added, moles liter^{-1} × 10^3	Ascorbic Acid Found, moles liter^{-1} × 10^3
	Polysorbate 80, 5% (w/v)		
Acetate	3.52	10.00	10.10
	4.55	10.00	10.02
	5.45	10.00	10.06
Phosphate	6.60	10.00	10.15
	Poloxalkol, 5% (w/v)		
Acetate	3.52	10.00	10.02
	4.55	10.00	10.15
	5.45	10.00	10.10
Phosphate	6.60	10.00	10.06

phosphate buffer (pH 6.60) at 67° and ionic strength 0.4. The concentrations of polysorbate 80 and of poloxalkol used were 0.0001, 0.001, 0.01, 0.05, 0.10, 0.50, 1.00, and 2.00% and 0.001, 0.01, 0.10, 0.50, 1.00, and 2.00%, respectively. Surfactant solutions containing 0.50% or more surfactant were prepared by dissolving accurately weighed quantities of surfactant in appropriate buffers. Solutions containing less than 0.50% surfactant were prepared by a dilution technique. The contents were analyzed for ascorbic acid using the previously described method (19).

Effect of Surfactant Concentration on Viscosity—Since high viscosity may contribute to the stability of a drug solution, the viscosities of aqueous solutions containing various concentrations of polysorbate 80 and poloxalkol were determined at 30 and 67°. Densities of the solutions were determined at the temperatures of the study using a pycnometer.

Effect of pH and Temperature on Stability of Ascorbic Acid Solutions Containing 1% Surfactant—The reactions were carried out in acetate buffer (pH 4.55) and phosphate buffer (pH 6.60) in the presence of 1% polysorbate 80 or poloxalkol at 50, 55, 60, and 67°. All buffers are adjusted to ionic strength 0.4 using potassium chloride. The concentration of ascorbic acid used was 0.01 mole liter^{-1}. Reactions were carried out for a maximum of 50 hr, and all aliquots were analyzed using the 2,6-dichloroindophenol method. Activation energies and $\Delta H\ddagger$, $\Delta S\ddagger$, and $\Delta F\ddagger$ values were determined from Arrhenius plots obtained by plotting log k *versus* $1/T$.

DISCUSSION

All rate constants were calculated from the first-order rate equation. The slopes of the lines were calculated by regression analysis (20, 21) on an electronic calculator[4]. All rate constants were calculated with n (number of points on the line) equal to 5–10. The correlation coefficient of all of the calculations was between −0.950 and −1.000. The thermodynamic parameters were calculated from Arrhenius plots using specially programmed cards.

The CMC of the two surfactants used in the study was determined at 25° using the surface tension lowering method. The CMC's were not significantly different at pH 3.52, 4.55, 5.45, and 6.60. The CMC's were approximately 10 mg/100 ml for polysorbate 80 and 100 mg/100 ml for poloxalkol at 25°. A typical plot of the effect of various surfactant concentrations on the surface tension of ascorbic acid solutions at pH 3.5 is shown in Fig. 1. The data shown in Table I indicate that the surfactants used did not interfere with the 2,6-dichloroindophenol method of analysis for ascorbic acid.

The effect of surfactant concentration on the rate of oxidation of ascorbic acid is shown in Fig. 2 and summarized in Tables II and III. The oxidation of ascorbic acid increased in dilute surfactant solutions containing a quantity of surfactant equal to 0.01 of the respective CMC values determined at 25°. Since this is an oxidation process, the magnitude of increase was approximately the same at pH 4.55 and 6.60 for both surfactants; *i.e.*, for polysorbate 80 at pH 4.55 and 6.60, the rate increased 1.53- and 1.60-fold over the rate obtained in surfactant-free buffer. In a dilute solution of poloxalkol, the increase in rate was 1.13- (pH 4.55) and 1.25- (pH 6.60) fold over the rate obtained in surfactant-free buffer. At surfactant concentrations equal to 0.1 of the respective CMC values, the rate was approximately the same as that obtained in surfactant-free buffer.

The increase observed in the oxidation rate of ascorbic acid in very dilute surfactant solutions may be due to the adsorption of

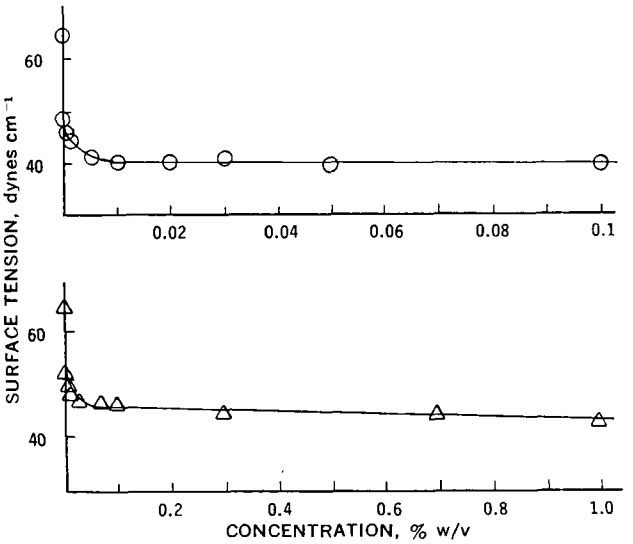

Figure 1—*Effect of concentration of surfactant on the surface tension of ascorbic acid (0.01 mole liter^{-1}) solution at 25° in acetate buffer pH 3.52 and ionic strength 0.4. Key: ○, polysorbate 80; and △, poloxalkol.*

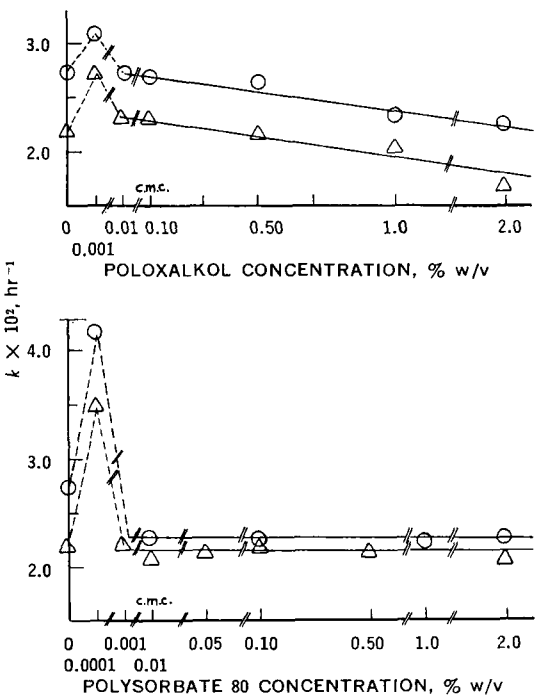

Figure 2—*Effect of various concentrations of surfactants on the rate of oxidation of ascorbic acid at 67° and ionic strength 0.4. Key: △, pH 4.55 (acetate buffer); and ○, pH 6.60 (phosphate buffer).*

[4] Wang model 360K/362K card programmer CP-1 with specially programmed cards.

Table II—Specific Rate Constants for the Oxidation of Ascorbic Acid at 67°, pH 4.55 and 6.60, and Ionic Strength 0.4 in the Presence of Polysorbate 80 and Poloxalkol

Buffer	pH	Polysorbate 80, % w/v	$k \times 10^2$, hr^{-1}	Poloxalkol, % w/v	$k \times 10^2$, hr^{-1}
Acetate	4.55	0.0	2.74	0.0	2.74
		0.0001	4.18	0.001	3.10
		0.01	2.26	0.01	2.74
		0.10	2.26	0.10	2.68
		1.00	2.23	0.50	2.64
		2.00	2.26	1.00	2.35
				2.00	2.25
Phosphate	6.60	0.0	2.20	0.0	2.20
		0.0001	3.52	0.001	2.74
		0.001	2.21	0.01	2.31
		0.01	2.12	0.10	2.31
		0.05	2.15	0.50	2.16
		0.10	2.20	1.00	2.07
		0.50	2.16	2.00	1.69
		2.00	2.08	—	—

Table III—Specific Rate Constants for the Oxidation of Ascorbic Acid at Various Temperatures and pH's in the Presence of 1% Surfactant

Surfactant	Buffer[a]	pH	Temperature	$k \times 10^2$, hr^{-1}
Polysorbate 80	Acetate	4.55	50.0°	1.03
			55.0°	1.42
			60.0°	1.96
			67.0°	2.23
	Phosphate	6.60	50.0°	2.36
			55.0°	2.59
			60.0°	2.30
Poloxalkol	Acetate	4.55	50.0°	1.07
			55.0°	1.34
			60.0°	1.92
			67.0°	2.35
	Phosphate	6.60	50.0°	2.49
			55.0°	2.21
			60.0°	1.70
			67.0°	2.07

[a] Ionic strength 0.4.

ascorbic acid molecules on the surface of the surfactant molecules, making them more susceptible to oxidative attack through surface catalysis. The increase in the oxidation rate might also be due to the formation of an association complex between ascorbic acid and surfactant molecules in which ascorbic acid is made more susceptible to oxidative degradation. These findings are in agreement with reported observations (17, 18).

Since the CMC's of nonionic surfactants decrease with increasing temperature, the CMC's determined at 25° were higher than those at the temperature of this study (67°). At surfactant concentrations equal to 0.1 of the CMC values determined at 25°, the oxidation rates of ascorbic acid in polysorbate 80 and poloxalkol solutions at 67° were approximately the same as the rates determined in surfactant-free buffer at the same temperature. Therefore, the CMC's of the two surfactants at 67° are probably approximately equal to 0.1 of their CMC's at 25°.

For polysorbate 80, no effect on the oxidation rate was observed at surfactant concentrations ranging from 0.1 of its CMC value (0.001%) up to 2% (w/v) surfactant; for poloxalkol, the oxidation rate of ascorbic acid decreased over the same surfactant concentration range. The effect can be shown by the following equations. For polysorbate 80 at pH 4.55:

$$k = 0.0225 \qquad \text{(Eq. 1)}$$

and at pH 6.60:

$$k = 0.0217 \qquad \text{(Eq. 2)}$$

For poloxalkol at pH 4.55:

$$k = 0.0272 - 0.0025(C) \qquad \text{(Eq. 3)}$$

and at pH 6.60:

$$k = 0.0233 - 0.0031(C) \qquad \text{(Eq. 4)}$$

where k is in $hour^{-1}$ and (C) is surfactant concentration from 0.01 to 2.00%. Equations 1–4 were obtained by regression analysis.

It is difficult to correlate the results (17, 18) of previous studies with those obtained in this study. Nixon and Chawla (17) studied saturated solutions of ascorbic acid containing high concentrations of polysorbate 20 and reported a sharp decrease in the copper-catalyzed oxidation rate in solutions containing about 40% surfactant. The decrease in the rate was attributed to a decrease in oxygen diffusion due to the high viscosity of the surfactant solutions. The decrease was also explained on the basis that, at very high polysorbate 20 concentrations, water was enclosed as a discontinuous pseudophase inside the micelles; the ascorbic acid dissolved in the water was protected from oxygen by the surfactant micelles.

In one study (18), solutions containing 3% ascorbic acid and varying amounts of polysorbate 80 were used. The solutions were not buffered and their pH varied from 2.5 to 4.0. Polysorbate 80 concentrations varied from 0.015 to 70%. The oxidation rate of ascorbic acid decreased in solutions containing 0.05–10% surfactant. The decrease in rate was attributed to an increase in micelle size.

An increase in the viscosity of a surfactant solution with increasing surfactant concentration could cause a decrease in the diffusion rate of oxygen, thereby decreasing the oxidative degradation rate of ascorbic acid. The viscosities of dispersions containing polysorbate 80 and poloxalkol were determined over the range of surfactant concentrations used (Fig. 3). The maximum increases in viscosity were 0.04 cps for polysorbate 80 and 0.09 cps for poloxalkol. These increases are not significant enough to affect markedly the diffusion rate of oxygen.

The differences in the behavior of polysorbate 80 (mol. wt. approximately 1270) and poloxalkol (mol. wt. approximately 8350) in stabilizing solutions containing ascorbic acid may be related to differences in the aggregation number and size of the micelles formed in each surfactant solution or to a difference in the number of available sites in the surfactant molecules at which ascorbic acid may bind. It is difficult to picture a geometrical arrangement for poloxalkol molecules that would produce large micelles with high aggregation numbers. This surfactant has very small lipophilic groups interrupted by a large number of polar ether oxygens. The ether oxygens do represent a large number of potential binding sites for ascorbic acid, so the decrease in the oxida-

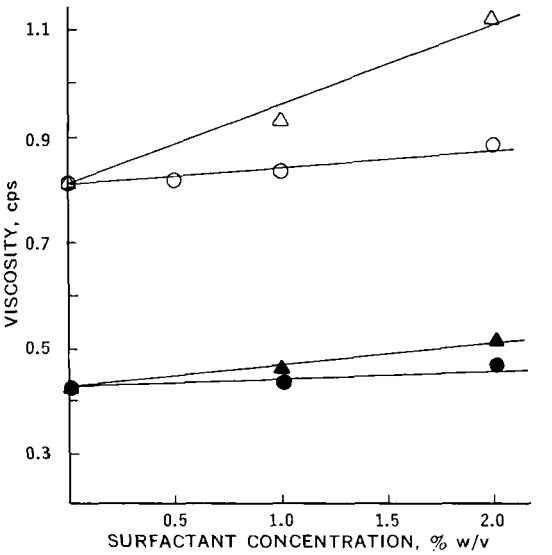

Figure 3—*Effect of various concentrations of surfactant on the viscosity. Key:* ○, *polysorbate 80 at 30°;* ●, *polysorbate 80 at 67°;* △, *poloxalkol at 30°; and* ▲, *poloxalkol at 67°.*

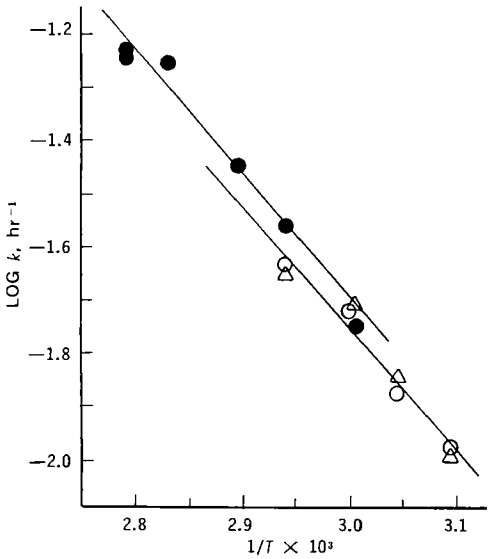

Figure 4—*Arrhenius plots showing the temperature dependence of ascorbic acid oxidation at pH 4.55 (acetate buffer) and ionic strength 0.4 in the presence of 1.0% surfactant. Key: ●, without surfactant; △, polysorbate 80; and ○, poloxalkol.*

tion rate of ascorbic acid may be due to interaction with these sites. Polysorbate 80 has a more favorable hydrophilic–lipophilic balance for the formation of spherical micelles. However, the polysorbate 80 molecules contain fewer potential binding sites than do the poloxalkol molecules. Also, ascorbic acid may be more sterically hindered from interacting with the binding sites in polysorbate 80 than with those in the straight chain poloxalkol.

The effects of pH and temperature on the aerobic oxidation of ascorbic acid are summarized in Table III. Figure 4 shows the plot of log k *versus* $1/T$ at pH 4.55. The thermodynamic parameters together with statistical parameters are shown in Table IV for ascorbic acid oxidation at 67° in pH 4.55 buffer and in buffer containing 1% surfactant. The large negative values of entropy of activation, $\Delta S\ddagger$, indicate that the degree of disorder in the activated complex and in the reaction is great.

At pH 6.60 the plot of log k *versus* $1/T$ obtained in the presence of either surfactant was not linear, so it was not possible to calculate the thermodynamic parameters at pH 6.60. The nonlinearity at pH 6.60 in solutions containing 1% surfactant was probably due to a complex mechanism. Either the mechanism proposed earlier (19) was not followed or additional reactions involving the surfactant molecules play a role in the rate-determining steps in the oxidative degradation of ascorbic acid.

CONCLUSIONS

Two nonionic surfactants, polysorbate 80 and poloxalkol, affected the oxidation rate of ascorbic acid. The rate increased sharply at surfactant concentrations equal to 0.01 of the CMC values. At surfactant concentrations equal to 0.1 of the CMC values, the rate was approximately the same as that obtained in surfactant-free water. For polysorbate 80, no effect on the oxidation rate was observed at surfactant concentrations ranging from 0.1 of the CMC value up to 2% surfactant, whereas for poloxalkol the oxidation rate decreased over the same surfactant concentration range.

The increase observed in the oxidation rate of ascorbic acid in very dilute surfactant solutions may be due to adsorption of ascorbic acid molecules on the surface of the surfactant molecules, making them more susceptible to oxidative attack through surface catalysis. The increase could also be due to the formation of an association complex between ascorbic acid and surfactant molecules.

The difference in the behavior of polysorbate 80 and poloxalkol in stabilizing solutions containing ascorbic acid may be related to differences in aggregation number and size of the micelles formed in surfactant solution or to a difference in the number of available sites in the surfactant molecules at which ascorbic acid may bind.

Table IV—Thermodynamic and Statistical Parameters Obtained from Arrhenius Plots for Ascorbic Acid Oxidation at 67°, pH 4.55, and Ionic Strength 0.4 in the Presence of 1% Surfactant

Parameter	No Surfactant[a]	Polysorbate 80	Poloxalkol
Thermodynamic			
Ea, kcal mole^{-1}	10.9	10.2	10.5
$H^{\ddagger}$, kcal mole^{-1}	10.3	9.5	9.8
$S^{\ddagger}$, cal mole^{-1} deg^{-1}	−52.1	−54.5	−53.6
$F^{\ddagger}$, kcal mole^{-1}	28.0	28.0	28.0
Statistical			
r (correlation coefficient)	−0.989	−0.968	−0.987
S_E (standard error of estimate)	0.035	0.046	0.030

[a] From Ref. 19, Table III.

Plots of log k *versus* $1/T$ yielded a straight-line relationship with a negative slope at pH 4.55 in buffer and in buffer containing 1% surfactant. The specific rate constants and half-life periods at 25° can be obtained from the plots by extrapolation. The nonlinearity of the Arrhenius plots at pH 6.60 in surfactant-containing solutions prevented the calculation of activation energies or other thermodynamic parameters.

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* Present address: Department of Pharmacy, University of Otago, Dunedin, New Zealand.

ˣ To whom inquiries should be directed.

GLC Determination of Quinidine from Plasma and Whole Blood

K. K. MIDHA[x] and C. CHARETTE

Abstract □ A method for the quantitative estimation of quinidine in biological fluids is described. Plasma containing quinidine, to which a known amount of cinchonidine is added as the internal standard, is extracted at pH 12.0 with benzene. The residue from the extract is mixed with 25 μl of trimethylanilinium hydroxide in methanol, and aliquots (1–2 μl) are injected into a gas chromatograph in which the injection port is held at 350°. The methyl derivatives of quinidine and the internal standard give well-separated symmetrical peaks. Detection by flame ionization gives a linear response over the range of 0.2–12.0 μg quinidine/ml plasma. The limit of detectability is 0.05 μg/ml and the method is adequate for following blood profiles of 200-mg quinidine sulfate doses in humans.

Keyphrases □ Quinidine—GLC determination in plasma and whole blood □ GLC—determination, quinidine in plasma and whole blood

The need for quantitative studies of blood concentrations in relation to dose and therapeutic effect of quinidine has been emphasized in recent years (1–4). Fluorometric (5–9), TLC (10–12), and GLC (5, 13) methods have been reported for the estimation of quinidine in pharmaceutical preparations and biological fluids. These methods are either cumbersome and time consuming or insensitive and nonspecific. The objective of this study was to develop a sensitive, specific, GLC method suitable for pharmacodynamic studies following single doses of quinidine sulfate (100–400 mg).

EXPERIMENTAL

Reagents—Quinidine sulfate[1], cinchonidine sulfate[2], and benzene[3] were obtained commercially. Methanolic trimethylanilinium hydroxide was synthesized according to the method of Barret (14). Plasma and blood were obtained from the Red Cross Blood Bank. All other chemicals employed were analytical grade.

GLC—A gas chromatograph[4] equipped with a flame-ionization detector was employed. The column was coiled stainless steel tubing[5], 1.2 m (4 ft) long by 0.3 cm (0.125 in.) o.d., packed with phenyl methyl dimethyl silicone[5] (OV-7) on acid-washed, dimethylchlorosilane-treated, high-performance Chromosorb W[5] support, 80–100 mesh. The column was conditioned by injecting 25–50 μl of column conditioner[6] [a mixture of *N,O*-bis(trimethylsilyl)acetamide, trimethylsilyldiethylamine, and hexamethyldisilazane] and maintaining the column at 300° for 18 hr with low nitrogen flow. Operating conditions were: injection port, 350°; column, 270°; and detector, 350°. The flow rate of nitrogen was 20 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

Procedure—To 1 ml plasma or whole blood samples in Teflon-lined, screw-capped centrifuge tubes (20 ml) are added 1 ml of distilled water, 1 ml of internal standard (≃2 μg cinchonidine/ml), 1 ml 1 *N* NaOH, and 10 ml benzene. The samples are extracted by shaking[7] for 10 min at 19 rpm followed by centrifugation at 2500 rpm for 10 min. Nine milliliters of the benzene layer is transferred into a custom-made evaporating tube[8] (15). The benzene extract is evaporated to dryness at 75° under a stream of dry nitrogen, and 25 μl of a methanolic solution of trimethylanilinium hydroxide (0.2 *M*) is added. The contents are dissolved by vibrating with a vortex mixer[9] before injecting 1–2 μl into the gas chromatograph. The retention times of flash-methylated quinidine and cinchonidine are 6.9 and 4.4 min, respectively.

Standards and Calibration Curve—Quinidine and cinchonidine standards were prepared by dissolving appropriate amounts of quinidine sulfate and cinchonidine sulfate in distilled water to obtain stock solutions of approximately 20 μg base/ml in each case. Appropriate dilutions of these stock solutions were made to obtain concentrations over the 0.2–12.0-μg/ml range. Peak height ratios were calculated by dividing the peak height of the quinidine methyl derivative by that of cinchonidine methyl derivative. Calibration curves were constructed from the results of spiked control plasma samples by plotting the peak height ratios against the concentration of quinidine.

Table I—Recovery of Quinidine and Cinchonidine from Plasma Determined by GLC Assay

Micrograms Added to 1 ml Plasma	*n*	Mean Micrograms Recovered	Mean Percent Recovery	Standard Deviation of Percent Recovery
Quinidine				
1.96	5	1.98	100.71	3.48
7.86	5	7.53	95.84	1.55
Mean 98.27 ± 3.61%				
Cinchonidine				
2.11	7	1.72	81.63	2.84

Table II—Estimation of Quinidine Added to Plasma by GLC

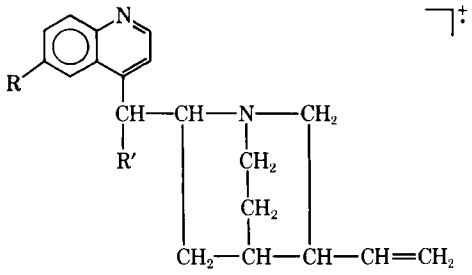
Quinidine Added, μg	*n*	Mean Peak Height Ratio	Standard Deviation	*CV*, %[a]
0.20	7	0.092	0.009	9.68
0.39	4	0.157	0.002	1.38
0.79	4	0.238	0.007	2.83
1.96	4	0.609	0.031	5.12
3.93	4	1.087	0.044	4.09
7.86	4	2.522	0.086	3.43
11.78	4	3.430	0.198	5.76

[a] Mean $CV = 4.61\%, y = mx$ where $m = 0.295 \pm 0.008$; $r = 1$.

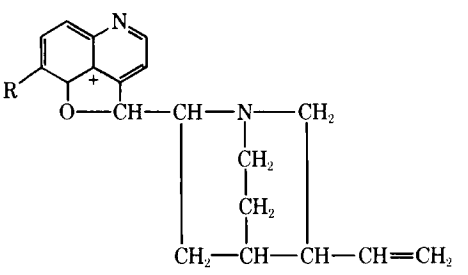
RESULTS AND DISCUSSION

The recently reported method of Smith *et al.* (5) for the measurement of impurities in quinine and quinidine preparations employs a silylation technique before GLC analysis. To adapt this

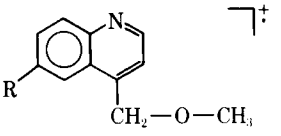
[1] J. T. Baker Laboratory chemicals.
[2] Merck Chemical Co.
[3] Caledon Laboratories, Georgetown, Ontario, Canada.
[4] Perkin-Elmer model F-11.
[5] Chromatographic Specialties, Brockville, Ontario, Canada.
[6] Silyl-8, Pierce Chemical Co.
[7] On a Roto-Rack, Fisher Scientific Co.
[8] Custom made by Canadian Laboratories Supplies Ltd., Montreal, Canada.
[9] Fisher Scientific Co.



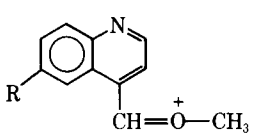
Ia: *m/e* 338, R = OCH_3, R′ = OCH_3
Ib: *m/e* 308, R = H, R′ = OCH_3
IIa: *m/e* 308, R = OCH_3, R′ = H
IIb: *m/e* 278, R = H, R′ = H



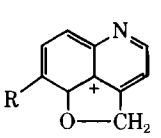
IIIa: *m/e* 323, R = OCH_3
IIIb: *m/e* 293, R = H



IVa: *m/e* 203, R = OCH_3
IVb: *m/e* 173, R = H



Va: *m/e* 202, R = OCH_3
Vb: *m/e* 172, R = H



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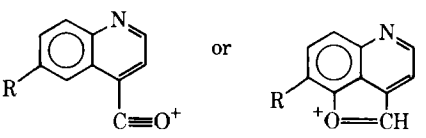
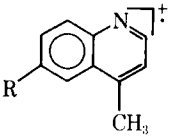
VIb: *m/e* 158, R = H



VIIa: *m/e* 186, R = OCH_3
VIIb: *m/e* 156, R = H



VIIIa: *m/e* 173, R = OCH_3
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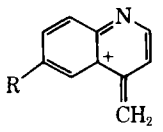

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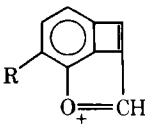
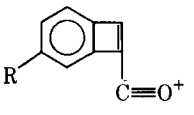
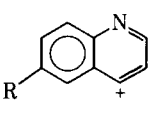


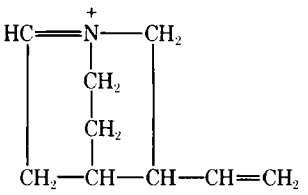
Xa: *m/e* 159, R = OCH_3
Xb: *m/e* 129, R = H



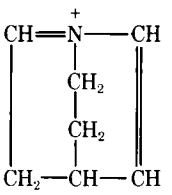
XIa: *m/e* 159, R = OCH_3
XIb: *m/e* 129, R = H



XIIa: *m/e* 158, R = OCH_3
XIIb: *m/e* 128, R = H



XIII: *m/e* 136



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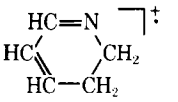

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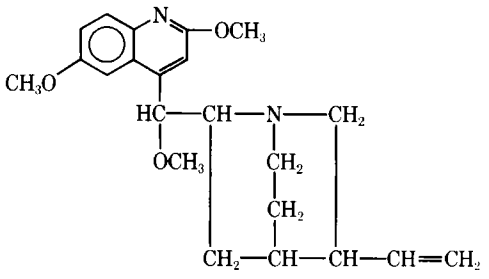
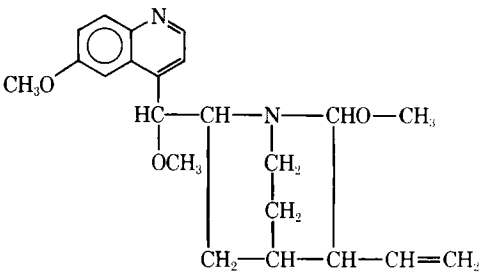


XVI



XVII

Postulated mass spectral fragmentation of flash-methylated quinidine and cinchonidine

method to biological fluids, the alkaloids must be extracted and freed from any trace of moisture before silylation. This drying step can be avoided by the use of trimethylanilinium hydroxide as a methylating reagent for GLC analysis. Plasma samples do not need to be dried completely and the methylation reaction in the injection port is instantaneous at 300° (16, 17).

Flash-heater methylation of quinidine and cinchonidine with trimethylanilinium hydroxide gave sharp peaks with retention times of 6.9 and 4.4 min, respectively, under the conditions described under *General Procedure*. The structures of flash-methylated quinidine and cinchonidine were established by combined GLC-mass spectrometry[10]. The mass spectrum of methylated quinidine (Fig. 1A) showed a molecular ion at *m/e* 338. Characteristic ions at *m/e* 323, 308, 203, 202, 188, 186, 173, 172, 159, 158, 136, 129, 108, and 81 were tentatively assigned the structures illustrated. These fragmentations suggest that flash-methylated quinidine has Structure Ia. The mass spectrum (Fig. 1B) of flash-methylated cinchonidine showed a molecular ion at *m/e* 308 and other major ions at *m/e* 293, 278, 173, 172, 156, 143, 142, 136, 129, 128, 108, and 81. Structures for these ions were postulated, indicating that flash-methylated cinchonidine has Structure Ib.

Figure 2A shows a typical chromatogram obtained by processing control blank plasma as described in the procedure, in which the internal standard, cinchonidine, was omitted. The extraneous peaks I, II, V, and VI at retention times 2.8, 3.1, 14.2, and 17.2 min, respectively, were observed in the chromatograms of all human as well as whole blood samples and remain unidentified.

[10] Perkin-Elmer model 900 gas chromatograph attached to a Hitachi-Perkin-Elmer model RMSU mass spectrometer through a jet separator.

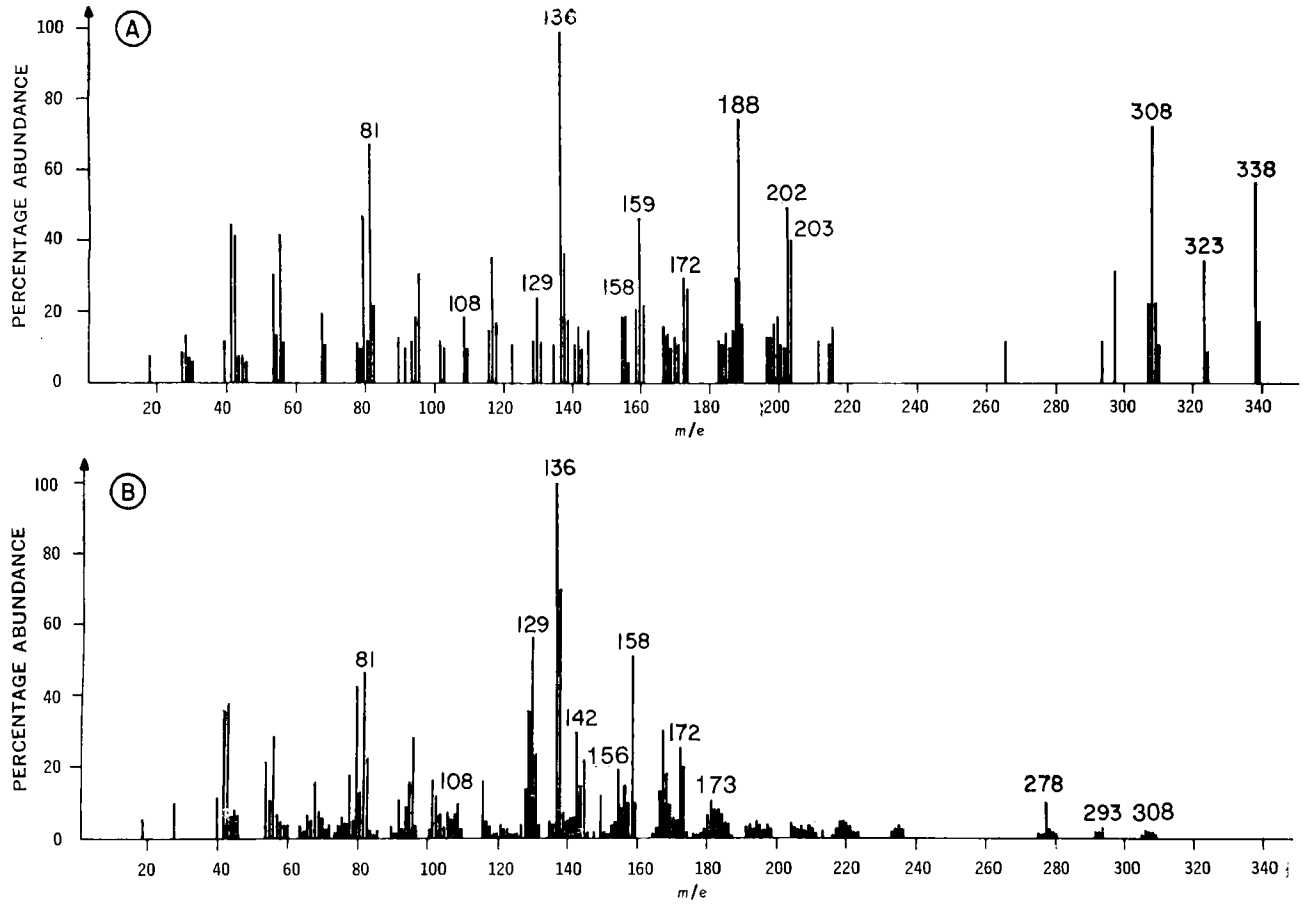


Figure 1—*GLC–mass spectrometry of flash-methylated quinidine (A) and cinchonidine (B).*

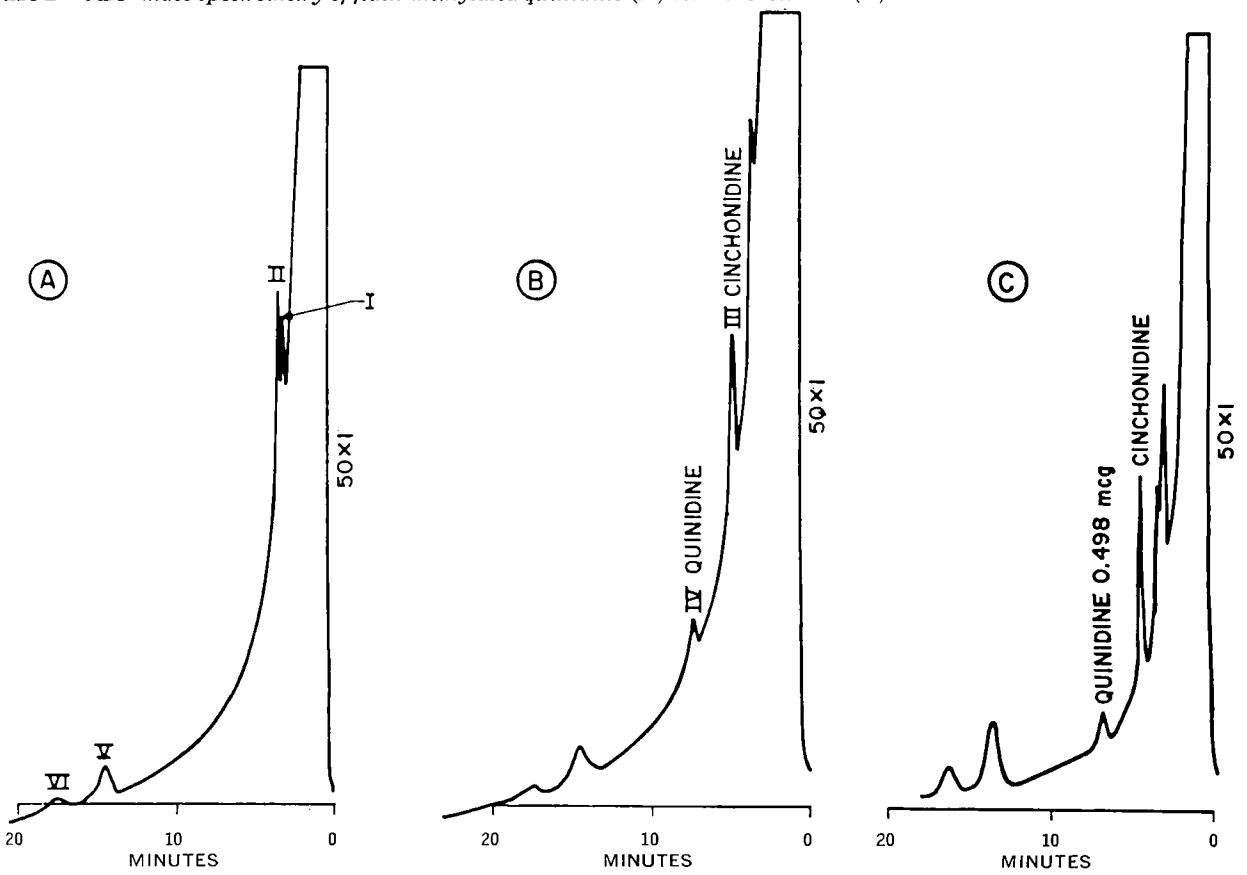


Figure 2—*Chromatograms obtained from flash methylation of plasma blank (A), plasma spiked with 0.79 μg quinidine and 2.11 μg cinchonidine (B), and plasma from volunteer receiving 200 mg quinidine sulfate (C).*

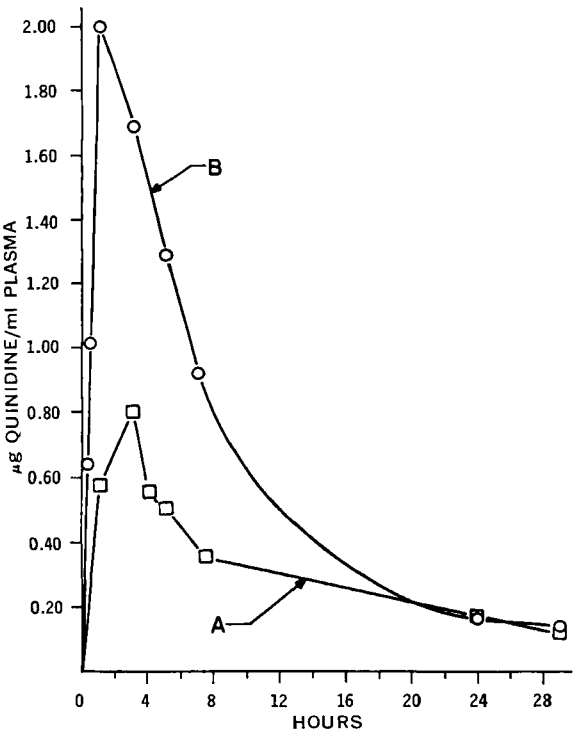


Figure 3—*Plasma profile of quinidine. Key: (A), healthy male volunteer (73 kg) receiving a 200-mg tablet of quinidine sulfate; and (B), dog receiving 10 mg quinidine sulfate/kg.*

A chromatogram obtained when the method was applied to spiked plasma containing 0.79 μg of quinidine and 2.11 μg of cinchonidine as the internal standard is shown in Fig. 2B, where it is clear that the extraneous peaks I, II, V, and VI do not interfere with the peak of quinidine IV (6.9 min) and the internal standard peak III (4.4 min). Figure 2C shows a chromatogram from a 1-ml plasma sample of a male volunteer who received a 200-mg tablet of quinidine sulfate[11]. An analysis time of 20 min was achieved. The metabolites of quinidine, *i.e.*, 2-hydroxyquinidine and monohydroxyquinidine (18), do not interfere with the assay, because on flash methylation (XVI and XVII) they gave retention times of 12.1 and 10.6 min, respectively.

The response of the flame-ionization detector to quinidine was linear with concentrations in the 0.2–12-μg/ml range (Table II). The overall recoveries of quinidine and cinchonidine from plasma at pH 12.0 by extraction with benzene are 98.27 ± 3.61 and 81.63 ± 2.84%, respectively (Table I). The ratio of the peak heights of quinidine to the internal standard plotted against concentration in the 0.2–12.0-μg/ml range gave a straight line passing through the origin (r = 1). A mean slope value of 0.295 ± 0.008 was obtained. The overall coefficient of variation was 4.61% (Table II).

The GLC procedure was compared with the spectrofluorometric method of Cramer and Isaksson (6). Routine analysis by both methods of duplicate plasma samples from a dog administered 10 mg/kg of quinidine sulfate[1] gave good agreement with an overall difference of 2% (Table III).

Application of the method to plasma level determinations was demonstrated (Fig. 3). A 200-mg dose of quinidine sulfate[11] was given to a healthy male volunteer (73 kg), and plasma was withdrawn at intervals over 29 hr and analyzed (Fig. 3A). A plasma profile of a dog (29.6 kg) receiving 10 mg quinidine sulfate[1]/kg is shown in Fig. 3B.

[11] Rougier Inc., Montreal, Canada.

Table III—Comparison of Plasma Levels by GLC and Spectrofluorometric Methods following Administration of Quinidine Sulfate (10 mg/kg) to a Dog (29.6 kg) (n = 2)

Hours after Administration	Concentration Determined by: GLC, μg/ml	Concentration Determined by: Spectrofluorometry, μg/ml
1	2.00	2.04
2	1.80	1.84
3	1.64	1.67

The described GLC method is simple, sensitive, and specific in that it distinguishes quinidine from its metabolites and can be employed for single- as well as multiple-dose pharmacodynamic studies.

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[x] To whom inquiries should be directed.

Comparison of Spironolactone Tablet Dosage Forms in Healthy Humans

L. M. HOFMANN[x], J. E. DUTT, L. G. DEYSACH, H. LONCIN, and L. TAO

Abstract □ A single-dose, crossover, comparative study of 25- and 100-mg spironolactone tablets was undertaken in 39 healthy males. Following a total dose of 200 mg, blood samples were drawn at 0, 1, 2, 3, 4, 6, and 24 hr and a single collection of urine was taken after the initial 6 hr. The dethioacetylated active metabolite was measured in these specimens and was employed as the indicator of drug absorption and elimination. A comprehensive statistical analysis, including: *(a)* multivariate hour-by-hour comparisons with plasma aldadiene levels, *(b)* comparisons with total plasma area and total urinary metabolite level, *(c)* bivariate comparisons with peak plasma aldadiene levels, and *(d)* urinary sodium–potassium ratio responses, examines the question of the biologieal equivalence of these two spironolactone preparations. The primary discriminators for comparing the two dosage forms were found to be plasma metabolite levels at Hours 3 and 4, peak plasma metabolite level, and total 24-hr area under the plasma level curve. Moreover, a single plasma level by itself does not appear to be sufficient to test for biological equivalence, so correlations among levels should not be ignored. The mean peak plasma metabolite levels for the 25- and 100-mg formulations reached 67.8 and 61.6 μg/100 ml, respectively, each at the same mean time of 160 min.

Keyphrases □ Spironolactone—statistical comparison of two tablet dosage forms (25 and 100 mg), analysis, humans □ Statistical analysis—comparison of spironolactone tablet dosage forms (25 and 100 mg), multivariate analysis, determination of discriminators □ Dosage forms—comparison of 25- and 100-mg spironolactone tablets, humans, multivariate statistical analysis (hour-by-hour comparisons) □ Discriminators—determined for comparison of spironolactone tablet dosage forms

Spironolactone is a specific antagonist of aldosterone, modifying electrolyte metabolism only in the presence of aldosterone-like compounds (1–5) and reversing all electrolyte-regulating effects of aldosterone regardless of the tissue studied (2, 3). Spironolactone is employed in the diagnosis (6) and treatment (7) of primary hyperaldosteronism and is often combined with other diuretics (thiazides) in the treatment of hypertension. The effects from such combinations in reducing blood pressure tend to be greater than the individual responses from either drug alone (8).

Spironolactone is presently marketed in the form[1] of 25-mg tablets. Since the drug is often given in daily dosages of between 200 and 400 mg (9, 10), it was thought that a 100-mg tablet dosage form could offer a greater convenience to patients who would otherwise be taking as many as four 25-mg tablets in a single dose. The primary intent of this study was to determine whether spironolactone absorption with 100-mg tablets was equivalent to absorption of the same dose in the form of 25-mg tablets. Also, based upon elevation in the urinary sodium–potassium ratio, it was of interest to determine whether a difference in pharmacological response between these two dosage formulations could be observed in healthy subjects.

[1] Aldactone, 25-mg spironolactone tablets, 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl) propanoic acid lactone, G. D. Searle & Co.

EXPERIMENTAL

A group of 39 males with no symptoms of chronic diseases participated in this single-dose crossover study, which consisted of one 24-hr test period and then a 2-week interval followed by a second 24-hr test period. The ranges of age and body weight were 21–39 years and 56.7–106.6 kg (125–235 lb), respectively. All subjects abstained from the use of all drugs for at least 3 days and from alcohol for 24 hr prior to a test day. Each test period was initiated approximately 2 hr after a light breakfast. About 4–5 hr into the test, a light lunch was consumed in which fried or fat meats were avoided. After the subjects emptied their bladders, each subject at zero time ingested 200 mg of spironolactone in a randomized order, either 8 × 25-mg or 2 × 100-mg tablets, along with two 250-ml glasses of water. In the second test period, the randomized order of drug ingestion was interchanged in crossover fashion. An additional two glasses of water were taken midway into the first 6 hr.

During each test period at 0, 1, 2, 3, 4, and 6 hr, 10-ml blood samples were collected using heparinized, sterile, disposable needles and syringes. An additional blood sample was drawn from all subjects 24 hr after the beginning in each test period. Levels of the dethioacetylated active metabolite of spironolactone were measured in plasma and urine by a spectrofluorometric method (11), and these levels served as indicators of drug absorption and elimination.

To compare the urinary sodium–potassium ratio, each subject collected 6-hr urine samples on the day prior to the first test period and in each test period. To minimize effect of diurnal variations, all samples were collected during the same 6-hr period (between 9 am and 3 pm). Side effects were not expected and were not observed. A 2-week interval was maintained between test periods.

STATISTICAL

The statistical analysis for comparing the 25- and 100-mg tablet formulations is based on the multivariate Hotelling T^2 method (12–14). The Hotelling T^2 method, a special case of the general multivariate linear model, permits comparisons between two groups at several time points and/or with several different variables simultaneously. Rahlfs and Bedall (15) discussed the appropriateness of the general multivariate linear model and other methods for analysis of time-dependent biological data, which, of course, includes the case discussed here.

The X and Y notation is hereafter used to distinguish the subject data corresponding to the two dosage forms: I, 25-mg tablets; and II, 100-mg tablets. A bold character distinguishes a vector from a scalar component. If N denotes the total number of subjects, then let the column vectors:

$$\mathbf{X}_i = \begin{bmatrix} X_{i1} \\ \cdot \\ \cdot \\ \cdot \\ X_{ik} \end{bmatrix} \qquad \mathbf{Y}_i = \begin{bmatrix} Y_{i1} \\ \cdot \\ \cdot \\ \cdot \\ Y_{ik} \end{bmatrix}_{i=1,\ldots,N} \qquad \text{(Eq. 1)}$$

denote K measurement variables relating to the ith subject with

respect to the two dosage forms, Groups I and II, respectively.

If d_{ik} denotes the difference between the kth measurement variables for Groups I and II relating to the ith subject:

$$d_{ik} = X_{ik} - Y_{ik} \qquad \text{(Eq. 2)}$$

the mean and variance of the kth difference variable are:

$$\bar{d}_k = (1/N)\sum_{i=1}^{N} d_{ik} \qquad \text{(Eq. 3)}$$

and:

$$s_k^2 = (1/N - 1)\sum_{i=1}^{N}(d_{ik} - \bar{d}_k)^2 \qquad \text{(Eq. 4)}$$

respectively. The correlation matrix of the K difference variables is defined as:

$$R = \begin{bmatrix} 1 & r_{12} & \cdots & r_{1K} \\ . & 1 & & . \\ . & & . & . \\ . & & & . \\ r_{K1} & \cdots & & 1 \end{bmatrix} \qquad \text{(Eq. 5)}$$

where $r_{km} = (1/N - 1)\,\Sigma^{N}_{i=1}(d_{ik} - \bar{d}_k)(d_{im} - \bar{d}_m)/s_k s_m$.

Since a correlation matrix is symmetrical, ($r_{ij} = r_{ji}$), the same correlations that appear above the main diagonal also appear below the main diagonal. Hereafter, the below-diagonal correlations will not be listed.

The Hotelling T^2 statistic is defined as:

$$T^2 = \mathbf{t}^* R^{-1}\mathbf{t} \qquad \text{(Eq. 6)}$$

where $\mathbf{t}$ is the column vector whose components $t_1, \ldots, t_K$ are the individual Student t statistics corresponding to the K measurement variables; R^{-1} is the inverse of the correlation matrix R; and the asterisk denotes vector transposition. The Student t statistic, t_k, is defined by:

$$t_k = \sqrt{N}(\bar{d}_k/s_k) \qquad \text{(Eq. 7)}$$

for $k = 1, \ldots, K$.

If the inverse of the correlation matrix is given by:

$$R^{-1} = \{\alpha_{ij}; 1 \le i,j \le K\} \qquad \text{(Eq. 8)}$$

then Eq. 6 becomes:

$$T^2 = \sum_{i=1}^{K}\sum_{j=1}^{K}\alpha_{ij}t_i t_j \qquad \text{(Eq. 9)}$$

In the particular case when all correlations are zero, Eq. 9 reduces to:

$$T^2 = t_1^2 + \ldots + t_K^2 \qquad \text{(Eq. 10)}$$

The Hotelling T^2 statistic is a positive real number which relates directly to a calculated F statistic value by:

$$F_{\text{calc}(K,m-K)}(\alpha) = [(m - K)/(m - 1)K]T^2 \qquad \text{(Eq. 11)}$$

where α is the level of significance and K, $m - K$ are the two degrees of freedom. The value of $m = N$ relates to pairwise comparisons or the so-called one-sample case, whereas $m = N_1 + N_2 - 1$ is used for unpaired comparisons or the case of two samples with sample sizes N_1 and N_2. Primary attention here is focused on the pairwise case, since each subject had both dosage forms in crossover fashion.

For a specific comparison using the Hotelling T^2, if the calculated value of F is less than the corresponding tabled value of F (16), then the difference between I and II is said to be *not* significant at the appropriate level of probability α. If the calculated F is greater than the tabled F, the comparison *is* considered significantly different at the level α.

For two variables ($K = 2$), Hicks (17) gave a useful comparison between the correlated and uncorrelated cases. It is possible, for example, at a fixed significance level, for values of t_1 and t_2 not to be significant while the bivariate (t_1, t_2) *is* significant. On the other hand, either t_1 or t_2 or both, taken separately, can be significant while the bivariate (t_1, t_2) is *not* significant.

In past pharmacokinetic studies, the area under the plasma level curve has been probably the single most important variable.

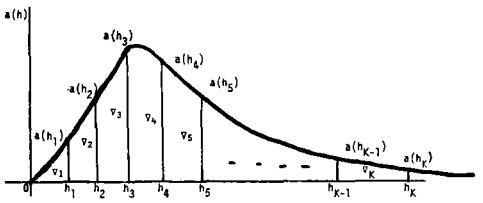

Figure 1—*Typical plasma level curve in which area is subdivided into the* **K** *subareas* $\nabla_1, \ldots, \nabla_K$.

By considering the plasma levels themselves, total plasma area, and certain other variables collectively in a multivariate framework, an assessment of the relative importance of area is obtained, as discussed in the *Results and Discussion* section. Here the role of subareas is considered.

In the multivariate approach, the area under the plasma level curve for each subject is broken up into K subareas (trapezoids), $\nabla_1, \ldots, \nabla_K$. Each subarea is determined by the difference between two successive time points, the corresponding plasma levels $a(h)$, and a straight line connecting two levels as in Fig. 1.

The K trapezoids, $\nabla_1, \ldots, \nabla_K$, are defined to be the K components of a vector which can relate to either X or Y, corresponding to the two dosage forms I and II, respectively. A Hotelling T^2 analysis can then be applied to subareas as the basic variates.

However, the obtaining of areas by straight lines implies geometrically that a linear transformation is applied to each data set, $a(h_1), \ldots, a(h_K)$. In other words, the kth trapezoid, ∇_k, is simply:

$$\nabla_k = [a(h_{k+1}) + a(h_k)]/2; \qquad k = 1, \ldots, K \qquad \text{(Eq. 12)}$$

But since the value of Hotelling T^2 remains unchanged with respect to nonsingular linear transformations (12, 13), calculations can be based on the plasma levels, $a(h_k)$, rather than the subareas, ∇_k. Moreover, for such biological comparisons, an explicit model for the plasma concentration curve is unnecessary.

In either case, using the levels, $a(h_k)$, or the areas, ∇_k, it is necessary that the time points, $h_1, \ldots, h_K$, be the same for both groups in the comparison. The choice of the number of variates, on the other hand, is often a delicate one and is decided on grounds that are only partly pharmacological and partly statistical. This question is not discussed here. However, later selection of variables or discriminators is obtained.

RESULTS AND DISCUSSION

In addition to the significance testing for differences, all 11 measurement variables, as well as subsets in the comparisons discussed here, are considered collectively, and stepwise selection of relative "best" discriminators are obtained. A set of m relative best discriminators in a set of K variables is here defined as that set of m variables, among all subsets of m variables, that accounts for the largest difference between the two groups under study. There are several accounts relating to the concepts of best discriminators and stepwise selection of discriminators (13, 14, 18, 19).

Multivariate (Hour-by-Hour) Comparisons with Plasma Metabolite Levels—As already mentioned, blood samples were obtained from each subject on each of the two dosage forms (I, 25 mg; and II, 100 mg) at the times, in hours, of $h_1 = 1$, $h_2 = 2$, $h_3 = 3$, $h_4 = 4$, $h_5 = 6$, and $h_6 = 24$. The mean values ± standard

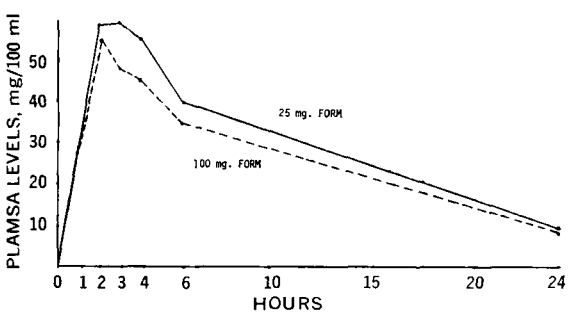

Figure 2—*Mean plasma levels of the 25- and 100-mg tablet forms.*

Table I—Mean Values of Plasma Levels at 1, 2, 3, 4, 6, and 24 hr for Two Tablet Forms

Dosage Form	1 hr	2 hr	3 hr	4 hr	6 hr	24 hr
I	27.3 ± 2.6	57.8 ± 3.7	58.4 ± 3.4	54.6 ± 2.2	39.5 ± 1.8	9.05 ± 0.53
II	27.6 ± 3.2	54.9 ± 5.6	47.7 ± 3.7	45.1 ± 2.9	34.3 ± 2.0	8.60 ± 0.58

errors of the six plasma metabolite levels in units of μg/100 ml are listed in Table I and displayed graphically in Fig. 2. (Hereafter, all mean values are listed with their corresponding standard errors in this fashion.)

The matrix R_1 of correlation coefficients of the plasma levels $X_1, \ldots, X_6$ for Form I is available in Table II, and the matrix R_2 of correlation coefficients of the levels for Form II is listed in Table III. The matrix R of correlation coefficients of differences between the six plasma levels for the two forms, I and II, and its inverse R^{-1} are found in Tables IV and V, respectively. The determinants of R_1, R_2, and R are in Table VI.

Correlated Case—For the computation of the Hotelling T^2, in addition to R^{-1}, it is necessary to have the individual pairwise Student t variables for the six levels. Their squares were found to be: $t_1^2 = 0.013$, $t_2^2 = 0.268$, $t_3^2 = 6.142$, $t_4^2 = 14.992$, $t_5^2 = 10.097$, and $t_6^2 = 0.757$.

When using the values of α_{ij} from R^{-1} in Table V, Eq. 9 becomes:

$$T^2 = (\alpha_{11}t_1^2 + \ldots + \alpha_{66}t_6^2) + 2(\alpha_{12}t_1t_2 + \ldots + \alpha_{56}t_5t_6) =$$
$$1.45t_1^2 + 2t_2^2 + 1.74t_3^2 + 3.94t_4^2 + 3.73t_5^2 + 1.13t_6^2 -$$
$$2[(0.61t_1t_2 + 0.31t_1t_3 + 0.62t_2t_3 + 0.17t_1t_4 + 0.86t_2t_4 +$$
$$0.71t_3t_5 + 2.99t_4t_5 + 0.25t_2t_6 + 0.15t_5t_6 + 0.08t_4t_6) -$$
$$(0.18t_3t_4 + 0.41t_1t_5 + 0.24t_2t_5 + 0.25t_1t_6 + 0.27t_3t_6)] \quad \text{(Eq. 13a)}$$

and now with the values of $t_1, \ldots, t_6$:

$$T^2 = 108.83 - 2[45.9 - 2.8] \quad \text{(Eq. 13b)}$$
$$= 22.63$$

For $N = 39$ and $K = 6$, F calculated by Eq. 11 is:

$$F_{calc} = 3.28 \text{ for } df_1 = 6, df_2 = 33 \quad \text{(Eq. 14)}$$

which, compared to a tabled F value (16) of about 3.4, is *not* significant at the 1% level (actually, 0.012).

Uncorrelated Case—The assumption that all correlations are zero implies, for the element of R^{-1}, that $\alpha_{ij} = 1$ for $i = j$ and $\alpha_{ij} = 0$ for $i \neq j$. In this case, calculation by Eq. 10 yields:

$$T^2 = t_1^2 + \ldots + t_6^2 = 32.27 \quad \text{(Eq. 15)}$$

or correspondingly, $F_{calc} = 4.68$, which for the same $df_1 = 6$, $df_2 = 33$, is *clearly significant* at the 1% level (actually 0.002).

Therefore, the *relative percent variation in* T^2 of the correlated result with respect to the uncorrelated one is:

$$\frac{32.27 - 22.63}{32.27} \sim 30\% \quad \text{(Eq. 16)}$$

Significance of Correlation—The significance of correlation can be seen in many ways.

From the point of view of hypothesis testing, Morrison (23, p. 113) obtains the likelihood ratio test for the complete independence of the components of a normally distributed K component vector as:

$$\chi^2 = -\left[N - 1 - \frac{(2K + 5)}{6}\right] \ln|R| \quad \text{(Eq. 17)}$$

The hypothesis of independence (*i.e.*, no correlation) is accepted if:

$$\chi^2 \leq \chi^2_{\alpha;K(K-1)/2} \quad \text{(Eq. 18)}$$

and rejected if $\chi^2 > \chi^2_{0.05;K(K-1)/2}$, where $\chi^2_{\alpha;K(K-1)/2}$ is the upper 100 α percentage point of the central χ-squared distribution with $K(K-1)/2$ degrees of freedom. If the criteria (Eq. 18) are overwhelmingly rejected, there is not only strong support for a multivariate analysis but a univariate approach may be quite misleading.

Application of Eq. 17 to R, the correlation matrix of $K = 6$ plasma level differences, yields from Table VI and for $N = 39$:

$$\chi^2 \cong -[38 - 3] \ln 0.0846$$
$$= 86.44 \quad \text{(Eq. 19)}$$

at 15 degrees of freedom. A $\chi^2 = 86.44$ compared to critical values of 25, 30.58, and 37.70 at significance levels of 5, 1, and 0.1%, respectively, is highly significant. This, therefore, gives strong motivation for a multivariate approach. Moreover, it is seen from Table VI that:

$$|R_1| \cong |R_2| \quad \text{(Eq. 20)}$$

This approximate equality not only gives support to the use of the Hotelling T^2 but pinpoints the major source of variation to be in the mean level differences.

Pursuing this latter point, the mean level differences at Hours 3, 4, and 6 are about 18, 17, and 13%, respectively; at other time points, they are essentially negligible. It is somewhat difficult to deal with these differences since the peak of a "volunteer" can occur at one of several times during 2, 3, 4, and 6 hr. However, it is later indicated that the mean peak levels for I and II only differ by about 9% and the mean time of peak occurrence was found to be 160 min for both I and II.

The significance of correlation can also be seen from the point of view of total area under the plasma level curve. As was inferred previously, the Hotelling T^2 is, in effect, a multivariate comparison of total area. Total area is broken up into six correlated subareas, which are then compared vectorily. It was previously noted (Eq. 14) that this comparison is not significant at the 1% level (actually 0.012). On the other hand, it will be seen later that the univariate comparison of total area yields a pairwise t_T =

Table II—Correlation Coefficients of Six Plasma Levels for 25-mg Tablet Form I

$$R_1 = \begin{bmatrix} 1 & 0.79 & 0.57 & 0.04 & 0.05 & 0.31 \\ & 1 & 0.72 & 0.19 & 0.24 & 0.40 \\ & & 1 & 0.40 & 0.41 & 0.42 \\ & & & 1 & 0.93 & 0.45 \\ & & & & 1 & 0.55 \\ & & & & & 1 \end{bmatrix}$$

Table III—Correlation Coefficients of Six Plasma Levels for 100-mg Tablet Form II

$$R_2 = \begin{bmatrix} 1 & 0.54 & 0.53 & 0.25 & 0.29 & 0.05 \\ & 1 & 0.70 & 0.64 & 0.53 & 0.26 \\ & & 1 & 0.56 & 0.57 & 0.21 \\ & & & 1 & 0.92 & 0.53 \\ & & & & 1 & 0.55 \\ & & & & & 1 \end{bmatrix}$$

Table IV—Correlation Coefficients of Six Plasma Level Differences between Forms I and II

$$R = \begin{bmatrix} 1 & 0.46 & 0.38 & 0.17 & 0.07 & -0.18 \\ & 1 & 0.55 & 0.54 & 0.43 & 0.08 \\ & & 1 & 0.44 & 0.46 & -0.11 \\ & & & 1 & 0.84 & 0.16 \\ & & & & 1 & 0.16 \\ & & & & & 1 \end{bmatrix}$$

Table V—Inverse Matrix of R

$$R^{-1} = \begin{bmatrix} 1.45 & -0.61 & -0.31 & -0.17 & 0.41 & 0.25 \\ & 2.0 & -0.62 & -0.86 & 0.24 & -0.25 \\ & & 1.74 & 0.18 & -0.71 & 0.27 \\ & & & 3.94 & -2.99 & -0.08 \\ & & & & 3.73 & -0.15 \\ & & & & & 1.13 \end{bmatrix}$$

Table VI—Determinants of R_1, R_2, and R

$\|R_1\|$	$\|R_2\|$	$\|R\|$
0.0106	0.0161	0.0846

Table VII—Statistics for the Pair (∇_T, U_T)

Dosage Form	$\bar{\nabla}_T$	$\bar{U}_T$	Correlation (∇_T, U_T)
I	703.4 ± 28.7	296.6 ± 19.7	0.01
II	619.8 ± 34.8	285.8 ± 21.5	0.11

2.94, which *is* significant at the 1% level (actually 0.006). One explanation for these two different results must relate to correlation.

Another view of significance of correlation, of course, relates to the *relative percent variation* in T^2 already indicated in Eq. 16. Equation 16 was computed with the pairwise t's of the six plasma levels. The t's for the levels at Hours 3, 4, and 6 are significant at α = 5% (0.017), 1% (0.001), and 1% (0.003), respectively, while the other t's at Hours 1, 2, and 24 are quite small. There is then a tendency to conclude that the effect of the set (1, 2, 24) on the overall process is not only small but also tends to dilute the role of the triplet (3, 4, 6). That this is not the case can be seen from the following.

First of all, the triplet (3, 4, 6) by itself yields a value of T^2 = 15.8. When the vector (1, 2, 24) is combined with (3, 4, 6), T^2 increases by about 43% to 22.63 (Eq. 13). This indicates that in a truly multivariate situation, t values are not necessarily indicators of the importance of variables.

Now, by making use of nonsingular linear transformations of the six plasma levels, three new variables can be found which have significant univariate t's (either at 5 or 1%). One such variable, of course, is total area which, in effect, is a sum of the six plasma levels. It was noted above that total area has a t_∇ = 2.94, which is significant at 1% (0.006). If total area replaces, say, plasma level 1, T^2 for the resulting six-variate comparison remains at 22.63 (Eq. 13) since a nonsingular linear transformation leaves T^2 fixed. However, T^2 for the uncorrelated case is T^2_{uncorr} = 40.94 and *the relative percent variation* in T^2 is now:

$$\frac{40.94 - 22.63}{40.94} = 44\% \qquad \text{(Eq. 21)}$$

This process can be continued to replace plasma levels at Hours 2 and 24 by, say, two new variables, Y and Z: Y = (level 2 + level 3 + level 4), and Z = (level 3 + level 6 + level 24). The t's of Y and Z are 2.19 and 3.11, respectively. The new six-variate vector (Y, Z, total area, level 3, level 4, and level 6) still gives T^2_{corr} = 22.63 but now T^2_{uncorr} = 54.34. The percent variation in correlation is:

$$\frac{54.34 - 22.63}{54.34} = 58\% \qquad \text{(Eq. 22)}$$

It seems clear that the correlations play a dominant role in these comparisons.

Comparisons with Total Plasma Areas and Total 6-hr Urines—Comparisons were made here using the pair (∇_T, U_T) where ∇_T in units of (micrograms per milliliter) × 24 hr denotes the total area (0–24 hr) under the plasma level curve, and U_T in milliliters relates to the total 6-hr urinary aldadiene level. The mean total plasma area, $\bar{\nabla}_T$, the mean total 6-hr urinary aldadiene level, $\bar{U}_T$, and the correlation between ∇_T and U_T for the two dosage forms are listed in Table VII.

When using a pairwise bivariate Hotelling T^2, the difference between the 25- and 100-mg dosage forms was found not to be significant at the 1% level (actually 0.021) and, moreover, ∇_T was found to be the better discriminator of the two. When the variables ∇_T and U_T were considered separately and when neglecting correlation, comparison with ∇_T showed a significant difference at the 1% level (actually 0.006), whereas with U_T no significant differences were observed. There is virtually no correlation between ∇_T and U_T for either dosage form.

Bivariate Comparison with Peak Metabolite Levels—The 2nd, 3rd, and 4th hr are generally the times of occurrence of the peak levels of individual curves. Therefore, it is of interest to make comparisons with the pair [h_m, max $a(h)$], where h_m is the time at the peak and max $a(h)$ is the peak plasma level.

Table VIII—Statistics for the Pair [h_m, max $a(h)$]

Dosage Form	Mean Time h_m	Mean max $a(h)$	Correlation h_m and max $a(h)$
I	160 ± 7.7 min	67.8 ± 3.2 μg/100 ml	−0.10
II	160 ± 7.5 min	61.6 ± 4.9 μg/100 ml	−0.35

Table IX—Statistics for Sodium and Potassium[a]

	$\bar{Na}$	$\bar{K}$	Correlation	$\overline{\text{Log (10 Na/K)}}$
I	67.9 ± 4.3	22.2 ± 1.2	0.50	1.5 ± 0.04
II	59.8 ± 4.1	18.7 ± 1.4	0.54	1.7 ± 0.2

[a] The units of sodium and potassium are mEq/6 hr.

The mean times, the mean peak levels, and the correlation between h_m and max $a(h)$ for the two dosage forms are listed in Table VIII. In general, mean peak levels are greater than the largest mean levels at specific measurement times. Consequently, the mean peak levels of 67.8 and 61.6 in Table VII are greater than the mean levels of 58.4 and 54.9 in Table I, respectively.

The mean peak level of the 25-mg dosage form is about 9% higher than that of the 100-mg form and the significant differences for the peak levels separately were found at the 5% level (actually 0.041). Since the mean times in each case are the same, a bivariate Hotelling T^2 showed the difference between the 25- and 100-mg dosage forms not to be significant at the 5% level (actually 0.124). The larger correlation, 35%, in Table VII for the 100-mg form probably relates to the slower passage of the larger tablet through the GI tract and/or to a smaller surface area relative to 4 × 25-mg tablets.

Comparisons with Urinary Sodium and Potassium—Comparisons with urinary sodium (Na) and potassium (K) data were made using a bivariate Hotelling T^2 on the pair (Na, K) and a univariate Student's t on the often used statistic log (10 Na/K). By using each statistic separately, the pair (Na, K) and the log ratio of Na/K, comparisons of the 6-hr test data were made for each dosage form with the corresponding data obtained the day prior to the testing, and no significant differences were found.

By using either the pair (Na, K) or the logarithm of the ratio Na/K, no significant differences at the 5% level were found between the two dosage forms during the test periods. The means of Na and K, the correlation (Na, K), and the mean of log (10 Na/K) for the two dosage forms are given in Table IX.

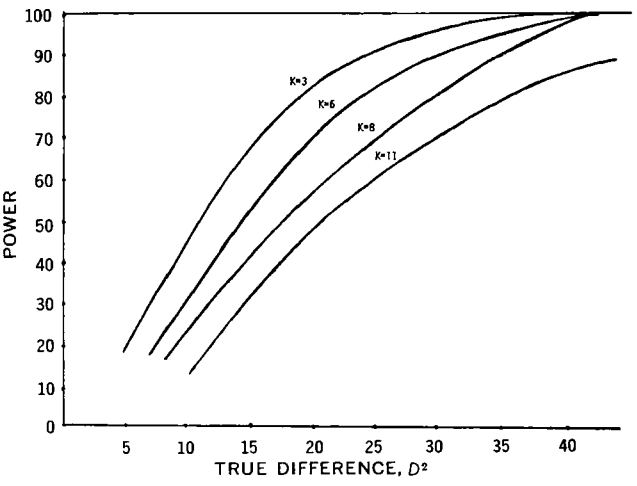

Figure 3—*Power (probability of detecting* D^2*) for Hotelling* T^2 *for* α = 1% *and* N = 39.

Table X—Correlation Coefficients of All 11 Variable Differences between Forms I and II

$\hat{R}$ =

1 hr	2 hr	3 hr	4 hr	6 hr	24 hr	Mean Time	Peak	Urine Volume	Log	Plasma Area	
1	0.46	0.38	0.17	0.07	−0.18	−0.16	0.44	0.28	0.07	0.31	1 hr
	1	0.55	0.54	0.43	0.08	−0.39	0.87	0.25	−0.09	0.71	2 hr
		1	0.44	0.46	−0.11	0.16	0.67	0.19	0.12	0.66	3 hr
			1	0.84	0.16	0.06	0.71	0.23	0.09	0.82	4 hr
				1	0.16	0.11	0.62	0.10	0.20	0.89	6 hr
					1	−0.23	−0.01	−0.12	−0.28	0.28	24 hr
						1	−0.14	0.02	−0.02	−0.01	Mean Time
							1	0.18	−0.02	0.78	Peak
								1	0.01	0.20	Urine Volume
									1	0.08	Log
										1	Plasma Area

All 11 Measurement Variables—In the previous sections, different subsets of a total of 11 measurement variables were used in the comparison of two dosage forms of spironolactone. It is now of interest to make comparisons in both the correlated (Eq. 9) and uncorrelated (Eq. 10) cases with all 11 measurement variables and to obtain some assessment of the relative importance of each variable with respect to its discrimination ability. Several accounts on the concept of best discriminator and stepwise selection of discriminators are available (13, 14, 18, 19).

The means for all 11 variables are listed in Tables I, VII, VIII, and IX, and the correlation matrix $\hat{R}$ of the 11 variable differences is given in Table X. When using the fact that $\hat{R}$ is not singular, despite redundancies in the measurement variables, the pairwise Hotelling T^2 for all 11 variables yields:

$$T^2_{corr} = 38.56 \qquad \text{(Eq. 23)}$$

while the corresponding uncorrelated Hotelling T^2 (Eq. 10) is found to be:

$$T^2_{uncorr} = 43.81 \qquad \text{(Eq. 24)}$$

The percent variation of correlated T^2 with respect to uncorrelated T^2 is about 12%.

The selection of variables were here obtained using a stepwise regression in which variables are entered by forward selection using the multiple correlation coefficient as the criterion for ordering. Selections can also be obtained based on Hotelling T^2 by selecting those variables that contribute most to T^2. Both procedures give generally consistent results. However, as is expected with different selection procedures, there are differences.

With the stepwise 11-variate regression procedure, the discriminators, in order of decreasing importance, were found to be: plasma level at Hour 4, plasma level at Hour 3, peak plasma level, total 24-hr plasma area, time of peak plasma level, urinary electrolyte ratio (log 10 Na/K), and plasma level at Hour 2. The plasma levels at Hours 1, 6, and 24 and the total 6-hr urine have about equally low discrimination power.

With the 11-variate Hotelling T^2, the corresponding order of discriminators was found to be: plasma level at Hour 4, total 24-hr plasma, plasma level at Hour 6, plasma level at Hour 3, peak plasma level, and plasma level at Hour 2. The plasma level at Hour 24 and the urinary electrolyte ratio have lesser power, while the plasma level at Hour 1, the time of peak, and the total 6-hr urine have equally low discrimination power.

There appears to be general agreement that the four key variables are plasma level at Hour 4, plasma level at Hour 3, peak plasma level, and total 24-hr plasma area. The plasma level at Hour 6 and the time of peak seem to have inversely important roles, depending upon the selection technique; the urinary electrolyte ratio, the plasma levels at Hours 1, 2, and 24, and total 6-hr urine have lesser roles of decreasing importance.

Finally, the selection procedure based on T^2 takes into account both mean and variance–covariance information, whereas it appears that the stepwise regression procedure puts less weight on the means of the variables. This accounts for the low position of time of peak, whose mean difference is zero, in the T^2 procedure. However, more study of stepwise selection procedures is needed.

Power of Hotelling T^2—Because of the presence of nonnegligible correlations, the statistical approach for comparing dosage forms is here based on a multivariate test of significance. Another approach might be based on confidence intervals. However, the use of univariate confidence intervals, as indicated by Westlake (20), appears not to be useful here since correlations are not taken into account. Using a new technique (22), tables are being prepared for obtaining multivariate (simultaneous) confidence intervals and prediction intervals that are more to the point.

The criterion often used to judge the desirability of a significance test is the power function. The method of Hotelling T^2 has not only a number of optimum properties (13), but curves of its power function can be obtained from Tang's tables (24).

For a sample of size 39 and significance level 1%, T^2 power curves for vectors of K = 3, 6, 8, and 11 components as a function of true differences, D^2 (*i.e.*, true values of Hotelling T^2), are given in Fig. 3. For each K, one can readily obtain the smallest D^2, which can be detected with a power of, say, 90%.

Other Studies—With three daily dosage regimens at 25, 100, and 200 mg of spironolactone and a placebo, Wolf *et al.* (25) investigated antihypertensive responses in 24 cases of benign, essential hypertension. Each dosage regimen was administered continuously in a randomized double-blind fashion for 6 weeks including two crossover periods. Systolic and diastolic blood pressures were significantly lower for all patients on spironolactone compared to placebo. The decreases in blood pressure were not significant at the low dosage level (25 mg), while the most efficacious response occurred with the 100-mg regimen. Treatment at the 200-mg dosage level appeared to be no more effective than at the 100-mg level.

In the present study, it is believed that absolute differences in plasma metabolite levels, particularly peak levels of 67.8 and 61.6 μg/100 ml observed for the 25- and 100-mg tablet formulations, respectively, would be clinically unimportant. This conjecture is consistent with the above-mentioned observations (25, 26) that, following a therapeutic dosage regimen with a relatively narrow dosage range, an optimum circulating level of active metabolites can be achieved and an optimal antihypertensive response of spironolactone can be realized. It is anticipated that a clinical requirement of 100 mg of spironolactone can be achieved with either four 25-mg tablets or one 100-mg tablet.

SUMMARY

A comparative study of two dosage forms of an important marketed drug was carried out using the techniques of multivariate statistical analysis. In studying the biological equivalence of dosage forms as a time-dependent process, two significant facts emerged. Correlations among measurement types should not be ignored and measurement variables should be selected according to their collective discrimination ability.

Specifically, comparisons of the 25- and 100-mg spironolactone tablet forms were examined by multivariate comparisons with: *(a)* hour-by-hour plasma metabolite levels, *(b)* total plasma area and total urinary metabolite level, *(c)* peak plasma levels, and *(d)* urinary electrolytes, sodium and potassium.

The key discriminators for comparing the two dosage forms were found to be plasma metabolite levels at Hours 4 and 3, peak plasma metabolite level, and total 24-hr area under the plasma level curve. The total 6-hr urinary metabolite level and the plasma levels at Hours 1 and 24 were relatively poor discriminators.

The mean peak plasma metabolite levels for the 25- and 100-mg formulations reached 67.8 and 61.6 μg/100 ml, respectively, at a mean time of 160 min.

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[x] To whom inquiries should be directed.

β-Hydroxyhomomethionine and β-Hydroxymethoxinine: Preparation and Separation of Diastereomers

THEODORE T. OTANI[x] and MARY R. BRILEY

Abstract □ β-Hydroxyhomomethionine and β-hydroxymethoxinine were prepared by a one-step condensation of the corresponding aldehydes with cupric glycinate in an alkaline medium. The diastereoisomers of each compound were separated by means of partition column chromatography with cellulose as the immobile phase and methyl ethyl ketone–*n*-butanol–ammonia–water as the eluant. The compounds were characterized by paper chromatography in five solvent systems, chemical ionization mass spectrometry, elemental analysis, Van Slyke ninhydrin determination of α-carbonyl carbon, Van Slyke nitrous acid determination of primary amino nitrogen, IR absorption, and NMR spectra.

Keyphrases □ β-Hydroxyhomomethionine—preparation and separation of diastereomers □ β-Hydroxymethoxinine—preparation and separation of diastereomers □ Methionine analogs—preparation and separation of diastereomers of β-hydroxyhomomethionine and β-hydroxymethoxinine

In studies on β-hydroxy-α-amino acids, it was noted that certain of these amino acids in the *N*-chloroacetylated form, even as racemic mixtures of the diastereomers, inhibited the growth of certain microorganisms in systems selected for antitumor screening (1). Of the 11 *N*-acyl-β-hydroxy-α-amino acids tested, *N*-chloroacetyl-β-hydroxynorleucine showed the most promise (1).

β-Hydroxynorleucine may be considered to be a structural analog of threonine and methionine, and it is possible that its growth-inhibitory activity is attributable to its structural resemblance to those amino acids. Since *N*-acylmethionine is a better substrate of hog renal acylase than is *N*-acylnorleucine (2), it appeared that, if the growth-inhibitory action was related to this hydrolysis, a methionine analog would be a better inhibitor.

With this in mind the preparation of two methionine analogs was undertaken. The present article reports the synthesis, the isolation of the diastereomers, and identification of β-hydroxyhomomethionine[1] and β-hydroxymethoxinine[1].

EXPERIMENTAL

Preparation of β-Hydroxyhomomethionine A, B, and AB[2]—Ten grams (47.2 mmoles) of cupric glycinate (3) was dissolved in 300 ml of 0.2 *M* sodium carbonate solution. The solution was cooled to 5° and 20 ml (about 186 mmoles) of 3-methylthiopropionaldehyde[3] was added in four equal portions over 8 hr. The alkalinity of the reaction mixture was tested before and after each addition of the aldehyde, and more 0.2 *M* sodium carbonate solution was added as required to keep the system basic. The reaction mixture was then set in the coldroom at 5° with constant stirring (magnetic stirrer) overnight (18–22 hr), after which time the reaction was terminated by acidification with acetic acid. A small aliquot was removed and spotted on Whatman No. 1 chromato-

[1] β-Hydroxyhomomethionine = 2-amino-3-hydroxy-5-methylthio-*n*-valeric acid; β-hydroxymethoxinine = 2-amino-3-hydroxy-4-methoxy-*n*-butyric acid.

[2] The letters A and B are arbitrarily assigned to the diastereomers moving faster and slower, respectively, when chromatographed on paper and developed in a methyl ethyl ketone–*n*-butanol–concentrated ammonia–water (3:5:1:1) solvent system.

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Table I—Elemental Analysis Data of the Diastereomers of β-Hydroxyhomomethionine and of β-Hydroxymethoxinine[a]

		β-Hydroxyhomomethionine		β-Hydroxymethoxinine	
		Diastereomer A	Diastereomer B	Diastereomer A	Diastereomer B
C	Calc.[b]	40.21	40.21	40.26	40.26
	Found	39.95	39.98	40.34	40.13
H	Calc.	7.31	7.31	7.44	7.44
	Found	7.44	7.50	7.51	7.39
N	Calc.	7.81	7.80	9.39	9.39
	Found	7.56	7.62	9.41	9.00
S	Calc.	17.89	17.89	—	—
	Found	17.31	17.54	—	—

[a] The analysis was performed by the staff of the Microanalytical Laboratory, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md. [b] Calculated for β-hydroxyhomomethionine, $C_5H_{11}NO_3S$, and for β-hydroxymethoxinine, $C_5H_{11}NO_4$.

Table II—Chromatographic Data of the Diastereomers of β-Hydroxyhomomethionine and of β-Hydroxymethoxinine[a]

Solvent System[b]	β-Hydroxyhomomethionine		β-Hydroxymethoxinine	
	Diastereomer A	Diastereomer B	Diastereomer A	Diastereomer B
Methyl ethyl ketone system	R_f 0.35–0.43 (0.39)	R_f 0.26–0.35 (0.31)	R_f 0.14–0.24 (0.19)	R_f 0.08–0.15 (0.12)
77% ethanol	0.40–0.49 (0.44)	Same as A	0.34–0.39 (0.36)	Same as A
80% pyridine	0.51–0.61 (0.56)	Same as A	0.39–0.45 (0.42)[c]	0.33–0.39 (0.36)[c]
Formic acid system	0.45–0.55 (0.50)	Same as A	0.32–0.42 (0.37)	Same as A
Phenol system	0.61–0.71 (0.67)	Same as A	0.61–0.71 (0.66)	Same as A

[a] Chromatographed on Whatman No. 1 chromatographic paper for 18–22 hr at 22–25°, ascending. Compounds were visualized by dipping the paper into a 0.25% acetone solution of ninhydrin and then warming the paper. Figures indicate range of values, and figures in parenthesis indicate the R_f value at the center of ninhydrin-positive spots. [b] Solvent systems: for the methyl ethyl ketone system, see text; formic acid system = formic acid–water–*tert*-butanol(15:15:70); phenol system = phenol saturated with 10% sodium citrate. [c] Sometimes closely situated double spots were noted with this system.

graphic paper and chromatographed in methyl ethyl ketone–*n*-butanol–concentrated ammonium hydroxide–water (3:5:1:1) (4) to determine the extent of the reaction. The main bulk of the reaction mixture was treated with hydrogen sulfide, and the precipitated cupric sulfide was removed by filtration (sintered-glass funnel, ultrafine porosity).

The clear, dark-colored filtrate was concentrated to a syrup on a flash evaporator at about 8 mm Hg and at a bath temperature of 42–50° with the condenser bulb immersed in a dry ice–alcohol bath. The residue was evaporated several times after addition of about 5–10 ml water to remove as much hydrogen sulfide as possible. To remove salts, the sticky mass was then redissolved in a small volume of water, adsorbed on an ion-exchange resin[4] column (H^+, 50–100 mesh, 4.5 × 90.0 cm) washed with water, and eluted with 2 *N* ammonia. The ammonia effluent was concentrated in the flash evaporator, the residue was dissolved in a small volume of the methyl ethyl ketone–*n*-butanol–ammonia–water system, and the solution was applied to a column (3.8 × 140 cm) packed with dry cellulose powder[5]. The column was eluted with the methyl ethyl ketone solvent system at a rate of about 2.7 ml/min, and the effluent was collected in 16-ml fractions at room temperature.

The effluent fractions were monitored for ninhydrin-positive material by spotting a drop from each tube onto a filter paper strip and treating it with an acetone solution of ninhydrin. The positive fractions were further identified by paper chromatography in the methyl ethyl ketone solvent system. By this means, it was noted that diastereomer A appeared between 248 and 418 ml of the effluent, a mixture of A and B appeared between 419 and 530 ml, and diastereomer B appeared between 531 and 1021 ml. Ninhydrin-positive material appeared beyond this volume (1022–1501 ml) and showed two spots [A, R_f 0.10–0.13 (0.12); and B, 0.08–0.10 (0.09)], probably the sulfoxides of isomers A and B, respectively. The fraction of 1502–1921 ml contained a mixture of "sulfoxides" and glycine.

The fractions corresponding to isomers A and B and to the diastereomeric mixture were concentrated at 42–50° on the flash evaporator, and the residue was further dried by passage of nitrogen over its surface. A small volume (about 5 ml) of water was added, and a large excess (about 200 ml) of absolute ethanol was added to the suspension. The turbid mixture was cooled at 5° in the refrigerator overnight, whereupon white crystals formed. The product was collected by filtration and was washed with cold absolute ethanol. The yield was 622.4 mg of diastereomer A, 940 mg of diastereomer B, and 1.1661 g of a mixture of the diastereomers. The total yield was 2.7015 g or 16% based upon the following reaction: 1 mole cupric glycinate yields 2 moles β-hydroxyamino acid. The diastereomeric mixture could be further separated by rechromatography in the same system. However, complete separation of the diastereomers by a single passage through the column could not be accomplished despite varying the geometry of the column, the flow rate of the eluant, and the size and mode of packing of the cellulose particles. Elemental analysis data for these compounds are shown in Table I.

DL-*β-Hydroxyhomomethionine A*—Molecular weight: calc. 179.2, obs. 179 (chemical ionization mass spectrometry)[6]. IR (KBr)[6]: 2.92 (—OH), 3.27 ($—NH_3^+$), 3.42 (—NH and —CH), 6.14 ($—COO^-$), 6.32 shoulder, and 6.71 ($—NH_3^+$) μm; unassigned bands as follows: 7.19 (medium intensity), 7.51 (medium), 7.92 (weak), 8.61 (weak), 9.57 (weak), 10.4 (weak), and 11.2 (weak) μm. NMR (D_2O, NaOD): δ 1.82 (m, 2H, γ-CH_2), 2.17 (s, 3H, —SCH_3), 2.70 (m, 2H, δ-CH_2), 3.20 (d, J = 4 Hz, 1H, α-CH), and 3.93 (m, 1H, β-CH).

DL-*β-Hydroxyhomomethionine B*—Molecular weight: calc. 179.2, obs. 179 (chemical ionization mass spectrometry). IR (KBr): 2.92 (—OH), 3.27 ($—NH_3^+$), 3.43 (—NH and —CH), 6.14 ($—COO^-$), 6.32 shoulder, and 6.71 ($—NH_3^+$) μm; unassigned bands as follows: 7.08 (medium), 7.42 (medium), 7.61 (medium), 7.88 (weak), 8.78 (weak), 9.52 (medium), 10.5 (weak) μm. NMR (D_2O, NaOD): δ 1.80 (m, 2H, γ-CH_2), 2.17 (s, 3H, —SCH_3), 2.70 (m, 2H, δ-CH_2), 3.20 (d, J = 4 Hz, 1H, α-CH), and 3.95 (m, 1H, β-CH).

[4] Dowex 50, × 8.

[5] Whatman cellulose CF-11.

[6] The authors are indebted to Mr. Noel F. Whittaker, Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md., for the IR absorption and NMR spectra and for the mass spectrometric analysis. The instruments used were: mass spectrometer, Finnigan 1015D; IR spectrophotometer, Perkin-Elmer 421; and NMR spectrometer, Varian A60.

Table III—Van Slyke Ninhydrin Determination of Carbonyl Carbon of α-Amino Acids[a]

	β-Hydroxyhomomethionine		β-Hydroxymethoxinine	
	Diastereomer A	Diastereomer B	Diastereomer A	Diastereomer B
α-Carbon taken, mg	0.407	0.407	0.407	0.407
α-Carbon found, mg	0.402	0.398	0.396	0.388
Percent theory	98.8	97.8	97.3	95.8
Percent theory, alanine control	97.8	97.8	99.0	99.0

[a] One milliliter of amino acid solution containing an equivalent of 0.407 mg α-carbon treated with 1.0 ml saturated picric acid in a reaction tube and about 100 mg of ninhydrin added.

Table IV—Van Slyke Nitrous Acid Determination of Primary Amino Nitrogen[a]

	β-Hydroxyhomomethionine		β-Hydroxymethoxinine	
	Diastereomer A	Diastereomer B	Diastereomer A	Diastereomer B
α-Amino nitrogen taken, mg	0.475	0.475	0.475	0.475
α-Amino nitrogen found, mg	0.481	0.507	0.490	0.485
Percent theory	101.2	106.7	103.1	102.2
Percent theory, leucine control	102.8	102.8	102.7	102.7

[a] One milliliter of amino acid solution contained an equivalent of 0.475 mg amino nitrogen after being diluted with 4.0 ml water and treated with 1 ml acetic acid and 2 ml of sodium nitrite solution (80 g/100 ml).

Preparation of β-Hydroxymethoxinine A, B, and AB—The method of preparation of these compounds is similar to that of β-hydroxyhomomethionine. Methoxyacetaldehyde was prepared by acid hydrolysis of its diethylacetal[7] in the conventional manner of preparation of aldehyde from its acetal. The reaction was carried out at room temperature (25°) overnight (about 16 hr) with constant stirring. The products were isolated by removal of the salt with the ion-exchange resin[4], passage of the salt-free solution through a dry cellulose column, and precipitation from aqueous solution with ethanol, as described for the preparation of β-hydroxyhomomethionine.

For 10.0 g (47.2 mmoles) of cupric glycinate in 300 ml of 0.2 *M* sodium carbonate solution, 14.2 ml (approximately 191 mmoles) of methoxyacetaldehyde was added.

For the separation of the diastereomers on the cellulose column, the column was eluted with the methyl ethyl ketone system at 0.75 ml/min at room temperature (22–25°) and 6-ml cuts were collected. The fraction emerging from 846 to 1115 ml contained an unidentified ninhydrin-positive material, that emerging from 1116 to 1600 ml contained diastereomer A, that emerging from 1601 to 1806 ml contained a mixture of the two diastereomers, and that emerging from 1807 to 2746 ml contained isomer B. The volume emerging beyond this fraction was still ninhydrin-positive, containing a mixture of isomer B and glycine (2747–3306 ml); from 3307 to 4806 ml, the fraction contained glycine only. Isomer A separated as a gelatinous precipitate upon addition of alcohol to the aqueous solution. The yield was 694.1 mg of isomer A, 920.7 mg of isomer B, and 49.1 mg of the mixture. The total yield was 1.6639 g (11.2 mmoles) or 23.7%. Isomer B could be separated from glycine by removal of the solvent by evaporation *in vacuo*, dissolution of the residue thus obtained in 80% pyridine, and passage of the solution through a second dry cellulose column and elution with 80% pyridine (β-hydroxymethoxinine emerges first in the system). Elemental analysis data for these compounds are shown in Table I.

DL-*β-Hydroxymethoxinine A*—Molecular weight: calc. 149.2, obs. 149 (chemical ionization mass spectrometry). IR (KBr): 2.92 (—OH), 3.27 ($—NH_3^+$), 3.41 (—NH and —CH), 6.16 ($—COO^-$), 6.32 (shoulder), and 6.69 ($—NH_3^+$) μm; unassigned bands as follows: 7.20 (medium intensity), 7.42 (medium), 8.00 (weak), 8.40 (weak), 9.00 (medium), 9.70 (medium), 10.5 (weak), 10.9 (weak), and 11.3 (weak) μm. NMR (D_2O, NaOD): δ 3.43 (s, 3H, $—OCH_3$), 3.67 (d, J = 4 Hz, 2H, γ-CH_2), 3.81 (d, J = 4 Hz, 1H, α-CH), and 4.33 (m, 1H, β-CH).

DL-*β-Hydroxymethoxinine B*—Molecular weight: calc. 149.2, obs. 149 (chemical ionization mass spectrometry). IR (KBr): 2.92 (—OH), 3.29 ($—NH_3^+$), 3.42 (—NH and —CH), 6.17 ($—COO^-$), 6.31 (shoulder), and 6.72 ($—NH_3^+$) μm; unassigned bands as follows: 7.14 (medium), 7.58 (medium), 8.40 (weak), 8.99 (medium), 9.54 (medium), 10.6 (weak), and 11.9 (weak) μm. NMR (D_2O, NaOD): δ 3.41 (s, 3H, $—OCH_3$), 3.67 (d, J = 4 Hz, 2H, γ-CH_2), 3.95 (d, J = 4 Hz, 1H, α-CH), and 4.33 (m, 1H, β-CH).

RESULTS AND DISCUSSION

Each diastereomer gave a single ninhydrin-positive spot when chromatographed in the methyl ethyl ketone system (which separates the diastereomers of these compounds) and in four other solvent systems, *i.e.*, 77% ethanol, 80% pyridine, formic acid–water–*tert*-butanol (15:15:70), and phenol saturated with 10% sodium citrate (Table II). The elemental analysis values are in agreement with values calculated for β-hydroxyhomomethionine and β-hydroxymethoxinine (Table I). Van Slyke ninhydrin determination of α-carbonyl carbon (5) and Van Slyke nitrous acid determination of primary amino nitrogen (6) yielded essentially quantitative amounts of carbon dioxide and nitrogen, respectively (Tables III and IV), confirming the α-amino carboxylic acid and free amino configuration of the compounds.

Although assignment of absolute configuration of the diastereomers could not be made at this time, comparison of the relative mobilities of the diastereomers of these compounds with those of the diastereomers of threonine (threonine and allothreonine) and of β-hydroxynorleucine[8] in the methyl ethyl ketone system shows that isomer A, or the faster moving compound, is probably related to the *threo*-configuration while isomer B, or the slower moving diastereomer, is related to the *erythro*-configuration.

In the synthesis of these compounds, varying the molar ratio of the reactants (from 1:0.01:1 to 1:1:3 glycine–cupric ion–aldehyde) over a range of temperature (5–100°) for from 15 min to 3 days showed that the products formed over a wide range of conditions.

[7] Aldrich Chemical Co., Milwaukee, Wis.

[8] The R_f values of the diastereomers in the methyl ethyl ketone system are as follows: diastereomer A, 0.43; and diastereomer B, 0.35. Diastereomer A moves with *threo*-β-hydroxynorleucine and diastereomer B moves with *erythro*-β-hydroxynorleucine in the Hardy–Holland (7) solvent system (*cf.*, Ref. 8).

The conditions were selected that gave the best yield for the shortest duration of reaction. Although these compounds could be obtained from glycine and cupric sulfate and the aldehyde as well as from cupric glycinate and aldehyde, the latter gave superior yields for the conditions studied. Lengthening the duration of reaction did not appear to improve the yield. Increasing the aldehyde concentration seems to result in larger formation of sticky material with no appreciable improvement of yield.

Removal of the cupric ion at the termination of the reaction by rapid heating gave inferior yields (8%). Use of the resin column for desalting can be eliminated when a smaller amount of sodium carbonate is used and the aqueous solution from which the sulfides have been largely removed is passed through a single cellulose column, but a smaller yield (8%) results.

The slow moving compounds appearing in trace amounts when β-hydroxyhomomethionine is chromatographed in the methyl ethyl ketone system are very likely the corresponding sulfoxides of the diastereomers. This is supported by the observation that the slower moving ninhydrin-positive material of each diastereomer is associated with the respective parent diastereomer during the separation procedure, being present when its parent compound is present and disappearing when its parent compounds disappear. When a small amount of diastereomer A and B was treated with hydrogen peroxide for a few minutes at room temperature and the resulting solution was chromatographed on paper in the methyl ethyl ketone system, a pronounced increase in the slower moving compound and a corresponding decrease in the parent compounds were noted. The ninhydrin-positive material that emerged ahead of β-hydroxyhomomethionine has not been identified.

Attempts have been made to prepare β-hydroxymethionine from methylthioacetaldehyde and cupric glycinate, starting with the diethylacetal of the aldehyde, but with no success thus far.

Enzymatic resolution of the racemates of each diastereomer of β-hydroxyhomomethionine and β-hydroxymethoxinine is now being studied in preparation for testing the pure enantiomers for activities in microbial antitumor screening systems and mammalian tumor systems.

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[x] To whom inquiries should be directed.

Influence of Repetitive Dosing and Altered Urinary pH on Doxycycline Excretion in Humans

JAMES M. JAFFE[x], ROLLAND I. POUST, STEPHEN L. FELD, and JOHN L. COLAIZZI

Abstract □ The effect of altered urinary pH on the renal excretion of doxycycline, with both single- and multiple-dose regimens, was studied in humans. Alkalinization of the urine resulted in higher cumulative amounts of doxycycline excreted during both single- and multiple-dose regimens. The increased excretion was reflected in larger renal clearances and shorter half-lives for both regimens when the alkaline condition was compared to the control condition. Half-life values increased from the single- to the multiple-dose regimen under both alkaline and control conditions. However, the increase in half-life values from single- to multiple-dose administration under the alkaline condition was shorter than the half-life change under control conditions.

Keyphrases □ Doxycycline—comparison of single-dose and multiple-dose regimens, effects of urine alkalinization on excretion, half-lives, humans □ Urinary pH—effects on excretion of doxycycline, single-dose and multiple-dose regimens, half-lives, humans □ Dosing regimens—comparison of single-dose and multiple-dose administration of doxycycline, effects of urinary pH on excretion, half-lives, humans □ Alkalinization, urine—effects on doxycycline excretion, comparison of single-dose and multiple-dose regimens, half-lives, humans □ Excretion, urinary—comparison of single-dose and multiple-dose doxycycline regimens, effects of alkalinization of urine, half-lives, humans

It was recently shown that altered urinary pH can significantly influence the urinary excretion of tetracycline and doxycycline (1). A treatment producing an alkaline urinary pH was shown to result in a 24% increase in cumulative tetracycline excretion when compared to a treatment producing an acidic urinary pH for the same time interval (48 hr). An even greater increase in cumulative excretion was shown for doxycycline under similar conditions. For doxycycline the alkaline urine condition resulted in a 54% increase in cumulative excretion as compared to an acidic urine condition.

This effect has been attributed to differences in the lipid solubilities of these antibiotics over the physiological pH range of the urine resulting in their increased or decreased reabsorption in the tubules of the kidney. The greater differences between alkalinization and acidification of the urine for the excretion of doxycycline than for tetracycline appear to confirm the theory that this antibiotic shows greater

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lipid solubility at its isoelectric pH (5.6) than does tetracycline (2).

Although there is no evidence in the literature indicating the effect of altered urinary pH on the excretion of tetracyclines administered in multiple-dose regimens, it has been shown that the apparent biological half-lives of tetracyclines increase when a multiple-dose regimen is compared to a single dose (3). For example, tetracycline and doxycycline biological half-lives increased from 6.3 to 10.0 hr and from 8.3 to 14.5 hr, respectively, when a single-dose was compared to a multiple-dose regimen.

Since the tetracycline antibiotics are generally administered on a multiple-dose regimen, a more clinically relevant finding would be to determine the effect of altered urinary pH on the excretion of tetracycline antibiotics administered on a multiple-dose regimen. Also of importance would be the determination of changes in biological half-lives under controlled urinary pH conditions to find if these effects were similar to those observed with uncontrolled urinary pH conditions. Since altered urinary pH has been shown to influence doxycycline to a greater extent than tetracycline (1), changes in excretion during multiple dosing, if any, should be more apparent with this antibiotic.

Thus, the objectives of this study were to: *(a)* investigate the effects of urinary pH changes on serum levels, urinary excretion, and related pharmacokinetic parameters of doxycycline in humans during a multiple-dose regimen; and *(b)* determine whether the lengthening of half-life that has been observed for tetracyclines administered on a multiple-dose regimen occurs for doxycycline when the urinary pH is significantly altered.

EXPERIMENTAL

Subjects—Six adult, male volunteers with no known diseases, between 65.9 and 79.5 kg in weight, between 172.7 and 185.4 cm in height, and between 19 and 29 years of age, served as subjects. Clinical laboratory tests for creatinine clearance, hemoglobin, hematocrit, and serum albumin determined that all subjects had normal blood and renal function.

Procedure for Subjects on Single-Dose Regimen—To provide a control to determine if subjects had relatively normal urinary pH, determinations of urine pH were made every 6 hr beginning 14 hr prior to drug administration. Subjects on the alkaline protocol took 6.0 g of sodium lactate[1] every 6 hr for the entire test period beginning 14 hr before drug administration. The sodium lactate was given in a vehicle of syrup USP (15 ml) and licorice flavor compound[2] (0.1 ml) to reduce the saline taste of the alkalinizer.

Following the 14-hr control period, 200 mg of doxycycline (as two 100-mg capsules[3]) was administered orally with 240 ml of water. The subjects were requested not to eat food of any kind or drink any beverage, except water, for 3 hr before and 3 hr after doxycycline administration. Drug administration always occurred at 8:00 am.

Total urine voids were collected immediately prior to drug administration and 2, 4, 9, 12, and 16 hr following drug administration and then every 6 hr until drug could no longer be detected in the urine.

[1] Fisher Scientific Co., Fair Lawn, N.J.
[2] Fritzsche Dodge and Olcott, Inc., New York, N.Y.
[3] Vibramycin, Pfizer Laboratories Division, Chas. Pfizer and Co., New York, N.Y.

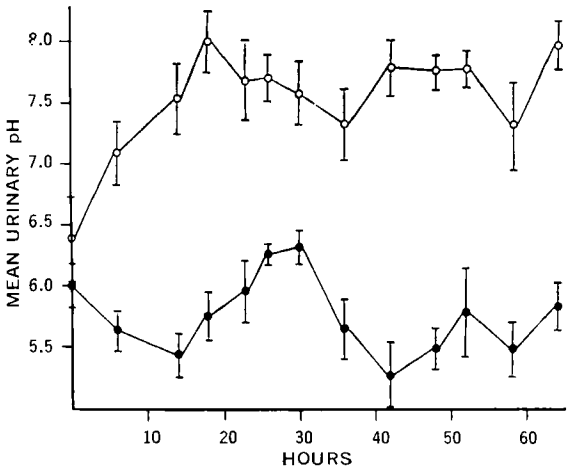

Figure 1—*Mean urinary pH* (±SE) *for six subjects on single-dose regimen. Key:* ●, *control condition; and* ○, *alkaline condition.*

Venous blood samples were taken immediately prior to the administration of the drug and 1, 2, 3, 4, 6, 9, 12, 22, 28, 34, 40, 46, and 52 hr following drug administration.

Procedure for Subjects on Multiple-Dose Regimen—Subjects followed the identical protocol as previously described for the single-dose regimen up to the time of administration of the test drug. At this time, an initial 200-mg loading dose of doxycycline was administered followed by 100 mg every 12 hr for a total of 12 doses. Drug administration always occurred at 8:00 am and 8:00 pm.

Urine voids were collected immediately prior to doxycycline administration and then every 6 hr for 72 hr. Urine voids were then collected at 73.5, 76, 77, 80, 82, and 84 hr after drug administration and then every 12 hr until 132 hr after administration of the initial drug dose (48 hr after the last dose).

Blood samples were taken immediately prior to the administration of the initial doxycycline dose and at 1.5, 3, 6, 9, 12, 24, 25.5, 27, 30, 33, 36, 48, 49.5, 51, 54, 57, 60, 72, 73.5, 75, 76, 77, 80, 82, 84, 96, 100, 104, 108, 120, 124, and 132 hr following the first dose of drug. Blood samples were always taken immediately prior to the administration of all doses (10, 12, 24, 36, 48, 60, 72, and 84 hr after the first dose).

Analytical—The analytical technique utilized in the determination of doxycycline in biological fluids was based on the method of Kohn (4), which involves the formation of an extractable complex of doxycycline with calcium ion and water-soluble barbiturate. The concentration of the resulting complex was then determined fluorometrically at an excitation wavelength of 400 nm and an emission wavelength of 520 nm[4]. A 0.5-ml serum sample and a 0.3-ml urine sample were employed for each determination. The assay is specific for unchanged doxycycline in blood or urine (5, 6).

Statistical Analysis of Data—Since each subject was tested under two different conditions (control and alkaline treatments), the statistical tests employed were by the method of paired observations or by a two-way analysis of variance (7). The t-test was employed for determining statistical significance of urinary pH values, cumulative excretion differences, and differences in various pharmacokinetic parameters. A two-way analysis of variance test was utilized for determining statistical significance for the blood level *versus* time curves in the excretory phases of the diagrams. The sources of variation in this analysis included time, the individual subjects, treatments to modify urinary pH, and residual.

RESULTS

The mean urinary pH for all subjects on the single-dose regimen under the two conditions is shown in Fig. 1. Throughout the entire excretion period, the sodium lactate treatment resulted in significantly higher mean pH values ($t = 11.68$, $df = 12$, $p < 0.01$) at all

[4] Model 110 Turner fluorometer, Turner Associates, Palo Alto, Calif.

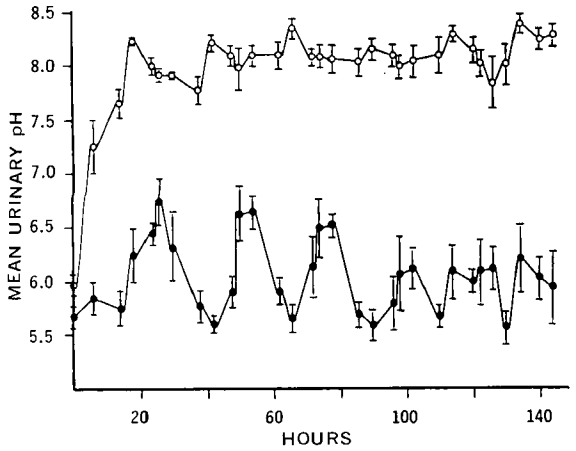

Figure 2—*Mean urinary pH* (±SE) *for six subjects on multiple-dose regimen. Key:* ●, *control condition; and* ○, *alkaline condition.*

time intervals when compared to the control treatment. A comparison of individual urine pH values under both conditions immediately prior to the alkalinizer treatment resulted in no significant difference.

Figure 2 shows mean urinary pH values for all subjects under the two conditions on the multiple-dose regimen. Over the entire excretion period, the sodium lactate treatment resulted in significantly higher mean pH values ($t = 22.26$, $df = 30$, $p < 0.01$) beginning with the first 12-hr period when compared with the control treatment.

The mean cumulative urinary excretion for all subjects under both conditions during the single-dose regimen is shown in Fig. 3. The alkaline treatment resulted in significantly higher mean cumulative excretion ($t = 15.61$, $df = 5$, $p < 0.01$) at the end of the 52 hr, the latest time at which all subjects excreted measurable amounts of drug. The control condition resulted in a mean of 37.1% of the dose excreted in the urine as compared to a mean of 60.8% excreted during the same time interval for the alkaline treatment. The values are in close agreement with similar work previously reported for doxycycline excretion (1).

Figure 4 shows the mean cumulative urinary excretion of doxycycline under both conditions during the multiple-dose regimen. The alkaline treatment resulted in significantly higher cumulative excretion ($t = 3.65$, $df = 5$, $p < 0.05$) at the end of the 132-hr test period. The control condition resulted in a mean excretion of

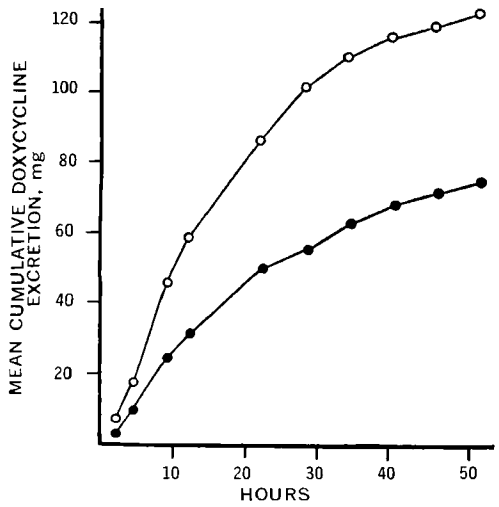

Figure 3—*Mean cumulative amounts of doxycycline excreted in the urine as a function of time for six subjects on single-dose regimen. Key:* ●, *control condition; and* ○, *alkaline condition.*

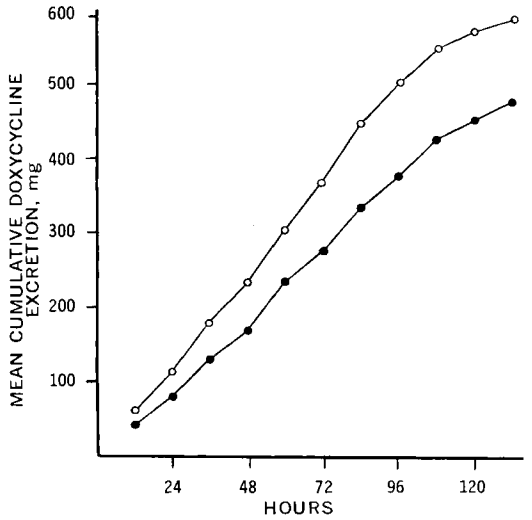

Figure 4—*Mean cumulative amounts of doxycycline excreted in the urine as a function of time for six subjects on multiple-dose regimen. Key:* ●, *control condition; and* ○, *alkaline condition.*

52.8% of the total administered doses over the entire collection period as compared to 65.0% for the alkaline condition.

Mean serum levels for the single-dose regimen under both conditions are shown in Fig. 5. Urinary alkalinzation resulted in significantly lower mean serum levels ($F = 13.20$; $df = 1,50$; $p < 0.01$) from 12 to 40 hr, the excretory phase for doxycycline, as compared to the control condition. A similar trend in doxycycline serum levels under controlled urinary pH conditions was shown in a previous study (1). Peak serum levels occurred at 3 hr during each treatment.

Figure 6 shows the mean serum levels for all subjects under both conditions during the multiple-dose regimen. As with the single-dose regimen, urinary alkalinization resulted in significantly lower mean serum levels ($F = 53.42$; $df = 1,140$; $p < 0.01$) as compared to the control condition. The values subjected to statistical analysis were those during the excretory phase for the drug after each dose of the multiple-dose regimen, which was always at least 10 hr after drug administration.

Both the single-dose (Fig. 5) and the multiple-dose (Fig. 6) semilogarithmic serum level *versus* time plots show appreciably different slopes during the postabsorptive phase (22–46 hr on the single-dose regimen and 100–132 hr on the multiple-dose regimen) when the two conditions are compared. These slopes (as determined by least-squares regression analysis) represent the elimination rate constants (K_E) of both regimens under both conditions (8). The single-dose regimen resulted in a mean elimination rate constant

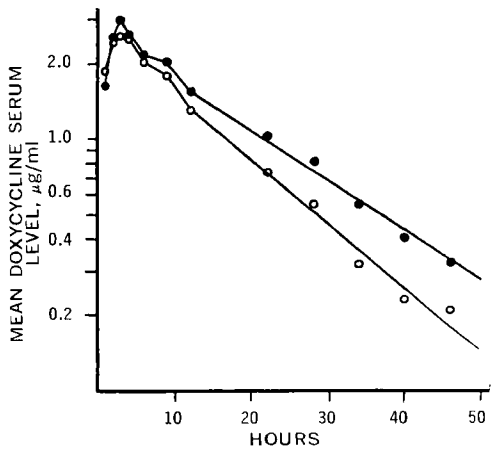

Figure 5—*Semilogarithmic plot of mean serum levels as a function of time for six subjects on single-dose regimen. Key:* ●, *control condition; and* ○, *alkaline condition.*

Table I—Pharmacokinetic Parameters for Doxycycline for Six Subjects during Single-Dose Regimen

Subject	Volume of Distribution Ratio, ($V_d^c/V_d^{OH^-}$)	Control Condition: Renal Clearance (R_c), ml/min	Control Condition: K_E, hr^{-1}	Control Condition: $t_{1/2}$, hr	Alkaline Condition: Renal Clearance (R_c), ml/min	Alkaline Condition: K_E, hr^{-1}	Alkaline Condition: $t_{1/2}$, hr
A	1.25	25.3	0.0568	12.2	61.8	0.0103	6.7
B	1.19	22.5	0.0337	12.4	51.5	0.1123	6.2
C	1.38	41.5	0.0583	11.9	60.0	0.1147	6.0
D	0.70	30.0	0.0701	9.9	42.5	0.0467	14.8
E	1.79	20.4	0.0400	17.3	24.8	0.0628	11.0
F	1.06	18.1	0.0446	15.5	43.6	0.0736	9.4
Mean	1.23	26.0	0.0542	13.2	47.4	0.0885	9.0
± *SE*	±0.15	±3.6	±0.0043	±1.1	±5.6	±0.0116	±1.4

Table II—Pharmacokinetic Parameters for Doxycycline for Six Subjects during Multiple-Dose Regimen

Subject	Volume of Distribution Ratio, ($V_d^c/V_d^{OH^-}$)	Control Condition: Renal Clearance (R_c), ml/min	Control Condition: K_E, hr^{-1}	Control Condition: $t_{1/2}$, hr	Alkaline Condition: Renal Clearance (R_c), ml/min	Alkaline Condition: K_E, hr^{-1}	Alkaline Condition: $t_{1/2}$, hr
A	1.61	26.2	0.0491	14.1	54.4	0.0988	7.0
B	0.88	10.7	0.0411	16.9	39.0	0.0689	11.3
C	0.55	20.0	0.0558	12.4	46.5	0.0502	13.8
D	0.91	18.2	0.0382	18.1	43.8	0.0555	12.5
E	1.21	21.8	0.0279	24.8	52.2	0.0535	13.0
F	0.98	29.4	0.0439	15.8	47.2	0.0543	12.8
Mean	1.03	21.1	0.0426	17.0	47.2	0.0618	11.8
± *SE*	±0.15	±2.7	±0.0038	±1.8	±2.3	±0.0074	±1.0

of 0.0542 hr^{-1} for the control condition as compared to 0.0885 hr^{-1} for the alkaline treatment, while the multiple-dose regimen resulted in mean elimination rate constants of 0.0426 and 0.0618 hr^{-1} for the control and alkaline conditions, respectively. Although a comparison of individual K_E values under the two different conditions on the single-dose regimen was just short of statistical significance, the multiple-dose regimen showed significantly different K_E values ($t = 2.57$, $df = 5$, $p < 0.05$) when the two treatments were compared.

Based on the K_E values, the alkaline treatment on the single-dose regimen resulted in a mean apparent biological half-life of 9.0 hr as compared to 13.2 hr for the control condition. The half-life for the control condition is shorter than most of those reported in the literature [15.0 hr (9, 10) and 15.1 hr (11)] but is much longer than that reported by Doluisio and Dittert [8.1 hr (3)]. The mean half-life for the alkaline and control conditions for the multiple-dose regimen were 11.8 and 17.0 hr, respectively. A comparison of individual half-lives for this regimen resulted in a significantly shorter half-life ($t = 2.91$, $df = 5$, $p < 0.05$) for the alkaline treatment as compared to the control condition. A comparison of the individual half-life values for the control conditions (single *versus* multiple dose) yielded a significantly longer half-life ($t = 2.69$, $df = 5$, $p < 0.05$) for the multiple-dose regimen. A comparison of the alkaline treatments (single *versus* multiple dose) showed a trend of longer half-life values for the multiple-dose regimen but this trend was short of statistical significance.

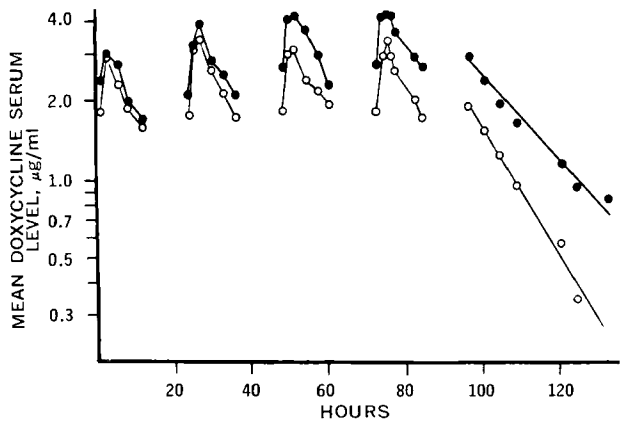

Figure 6—*Semilogarithmic plot of mean serum levels for six subjects as a function of time on multiple-dose regimen. Key: ●, control condition; and ○, alkaline condition.*

Renal clearance (R_c) values for the single-dose regimen were calculated based on Eq. 1 (8):

$$R_c = \frac{(Au)^{\infty}}{(AUC)_0^{\infty}} \qquad \text{(Eq. 1)}$$

where $(Au)^{\infty}$ is the total amount of drug excreted in the urine, and $(AUC)_0^{\infty}$ is the total area under the serum level–time curve.

Renal clearance values for the multiple-dose regimen were calculated based on Eq. 2 (8):

$$R_c = \frac{Au_{t(2)-t(1)}}{(AUC)_{t(1)}^{t(2)}} \qquad \text{(Eq. 2)}$$

where $(Au)_{t(2)-t(1)}$ is the amount of drug excreted in the urine during the steady-state interval between 72 and 84 hr, and $(AUC)_{t(1)}^{t(2)}$ is the area under the serum level–time curve during the same interval. Renal clearance, K_E, and biological half-life ($t_{1/2}$) for all subjects under both conditions for the single- and multiple-dose regimens are shown in Tables I and II, respectively.

The renal clearance values under both conditions on the single-dose regimen are significantly higher ($t = 4.43$, $df = 5$, $p < 0.01$) for the alkaline as compared to the control condition. Mean renal clearances were 26.0 and 47.4 ml/min for the control and alkaline treatments, respectively. A significant increase in renal clearance ($t = 14.6$, $df = 5$, $p < 0.01$) was also found for the multiple-dose regimen where the control and alkaline conditions showed mean renal clearances of 21.1 and 47.2 ml/min, respectively. No significant difference was observed when the renal clearance values from

the single- and multiple-dose studies under control conditions were compared. Likewise, no significant difference occurred when the alkaline conditions of the single- and multiple-dose regimens were compared.

In a previous paper (1), an equation for the volume of distribution ratios ($V_d^c/V_d^{OH^-}$) was derived to determine if the effects shown in this study were due to distribution changes. Ratio values of approximately unity indicate that the volume of distribution differences under each condition are negligible. As can be seen from Tables I and II, these changes were minimal for both the single- and multiple-dose regimens.

DISCUSSION

The findings of this study indicate that altered urinary pH can significantly change the urinary excretion of doxycycline administered in multiple doses to humans. It also confirms results of previous single-dose (1) and multiple-dose (3) studies.

As was expected on the basis of previous work (1), alkalinization of the urine (mean pH range 7.4–8.0) in the single-dose study resulted in 60.8% of the dose excreted in the urine as compared to the more acidic control condition (mean pH range 5.3–6.3) where only 37.1% of the dose was excreted. This effect is thought to be due to doxycycline having its maximum lipid solubility at its isoelectric pH of 5.6 (2) where enhanced tubular reabsorption would be favored. The shift to the alkaline pH condition resulted in a smaller fraction of doxycycline being present in the lipid-soluble form and hence less reabsorption and greater excretion.

This trend was also shown for the multiple-dose regimen where the alkaline condition (mean pH range 7.7–8.4) resulted in 65.0% excretion of the administered dose as compared to 52.8% of the dose excreted on the control condition (mean pH range 5.6–6.7). The trend of greater excretion of doxycycline due to alkalinization of the urine was also reflected in significantly lower serum levels with both regimens.

Although renal clearances of doxycycline are significantly greater for the alkaline condition as compared to the control condition under either regimen, renal clearance values are essentially identical when comparing conditions on the single dose with the same conditions on the multiple dose. This would indicate that the renal clearance of doxycycline is not influenced by repetitive dosing, at least not with the regimens employed in this study.

Mean elimination rate constants for the single-dose as compared to the multiple-dose regimen are lower with both conditions. This decrease is reflected in increased biological half-lives during the multiple-dose regimen as compared to the single-dose study. A mean increase of 3.8 hr occurred when control conditions for single- and multiple-dose regimens were compared in this study. A greater increase in biological half-lives of doxycycline (6.2 hr) for the uncontrolled urinary pH condition was shown in a previous study (3), although these apparently greater differences could possibly be attributed to the relatively short sampling times employed in the single-dose portion of the study. This could lead to an underestimation of the biological half-life and cause the change from single to multiple dosing to appear greater than actually was the case.

Alkaline urinary pH conditions also resulted in an increase in mean biological half-lives (2.8 hr) when the single-dose regimen was compared to the multiple-dose regimen in this study. Thus, altered urinary pH will also show a trend of increased biological half-lives between single and multiple dosing, but this difference is not as great as that observed under normal (uncontrolled) urinary pH conditions.

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ˣ To whom inquiries should be directed.

Effects of Aromatic Aldehydes on *O*-Methylation of Dopamine and Norepinephrine *In Vitro*

BENG T. HO[x], PATRICIA M. GARDNER, LEO F. ENGLERT, and K. E. WALKER

Abstract □ An increase in extracted radioactivity was found when the aromatic aldehydes 2-nitrobenzaldehyde or pyridine-4-carboxaldehyde were incubated with catechol-*O*-methyltransferase in the presence of ^{14}C-methyl-*S*-adenosylmethionine and dopamine or norepinephrine. The condensation products of dopamine with the two aldehydes were synthesized. The Schiff bases and tetrahydroisoquinoline derivatives were better substrates for catechol-*O*-methyltransferase than was dopamine or norepinephrine in terms of either K_m or V_{max}.

Keyphrases □ Aldehydes, aromatic—effects on catechol-*O*-methyltransferase and *O*-methylation of dopamine and norepinephrine *in vitro*, syntheses of Schiff bases and tetrahydroisoquinoline derivatives as substrates □ Catechol-*O*-methyltransferase—effects of aromatic aldehydes, *O*-methylation of dopamine and norepinephrine *in vitro* □ Dopamine—effects of 2-nitrobenzaldehyde and pyridine-4-carboxaldehyde on *O*-methylation *in vitro* □ Norepinephrine—effects of 2-nitrobenzaldehyde and pyridine-4-carboxaldehyde on *O*-methylation *in vitro*

The biological effects of aldehydes, derived endogenously or from the ingestion of foodstuffs, drugs, or other foreign compounds, are gaining widespread attention. The effects of aldehydes on the disposition of monoamines have been reported (1–3), as has evidence (4) that aldehydes derived from brain amines induced behavioral effects in laboratory animals. Recently, salsolinal and tetrahydropapaveroline, the condensation products of dopamine with acetaldehyde or 3,4-dihydroxyphenylacetaldehyde, respectively, were reported (5) to be better substrates for the enzyme catechol-*O*-methyltransferase than either dopamine or norepinephrine.

This report describes the effects of some aromatic aldehydes on the *O*-methylation of norepinephrine and dopamine *in vitro*.

EXPERIMENTAL[1]

4-(*N*-3,4-Dihydroxyphenethyliminomethyl)pyridine (II)—A mixture of 1.89 g (10 mmoles) of dopamine hydrochloride, 1.07 g (10 mmoles) of pyridine-4-carboxaldehyde, 3 g of sodium bicarbonate, and 100 ml of water was stirred under nitrogen for 2 hr. The resulting precipitate was filtered and recrystallized from dimethyl sulfoxide–water to give 2.25 g (93%) of a cream-colored solid, mp 239–240° dec. *N*-(2-Nitrobenzylidene)-3,4-dihydroxyphenethylamine (I) and *N*-(2-nitrobenzylidene)-3-methoxy-4-hydroxyphenethylamine (III) were prepared by this procedure. All compounds traveled as one spot on silica gel plates developed in *n*-butanol–acetic acid–water (4:1:1) and chloroform–methanol–ammonia (12:12:1.25). Table I lists their physical constants and analytical data.

1-(2-Nitrophenyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline Hydrochloride (IV)—A mixture of 0.31 g (1.1 mmoles) of I, 0.1 ml of concentrated hydrochloric acid, and 10 ml of *n*-propanol was refluxed for 6 hr and the resulting mixture was kept in a refrigerator for 18 hr. The solid was collected by filtration, yielding 0.27 g (75%), mp 226–232° dec. The compound traveled as one spot on silica gel plates developed in *n*-butanol–acetic acid–water (4:1:1).

1-Pyridyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline Hydrochloride (V)—To a solution of 1 g (5 mmoles) of dopamine hydrochloride in 16 ml of ethanol and 2 ml of methanol was added 0.5 g (5 mmoles) of pyridine-4-carboxaldehyde. The mixture was refluxed with stirring for 8 hr. After standing at room temperature for 24 hr, the solid was filtered and washed twice with a small amount of ethanol, yielding 71%, mp 235–240° dec. The compound traveled as one spot on silica gel plates developed in *n*-butanol–acetic acid–water (4:1:1). Table I lists the analytical data.

Enzyme Purification and Assay—Catechol-*O*-methyltransferase from rat liver was prepared according to Axelrod and Tomchick (6). Assay of the enzyme was performed according to Axelrod and Vesell (7) using 78.3 nmoles of either dopamine or L-norepinephrine and 50 nmoles (40 nCi) of ^{14}C-methyl-*S*-adenosylmethionine[2] in a total volume of 1 ml. Compounds I, II, III, 2-nitrobenzaldehyde, and pyridine-4-carboxaldehyde[3] were dissolved in dimethyl sulfoxide unless otherwise stated. Compounds IV and V were dissolved in water. When dimethyl sulfoxide was used as a solvent, a corresponding volume (0.1 ml) was added to the controls. The K_m and V_{max} values were determined from a Lineweaver–Burke plot constructed using the method of least squares.

Recovery Studies—3-Methoxytyramine hydrochloride[4] was dissolved in 0.01 *N* HCl and scanned on a spectrophotometer[5] between 190 and 340 nm; absorbance at 282 and 225 nm was found to be linear in a range of 7.5–150 nmoles in a volume of 3 ml. Twenty nanomoles of 3-methoxytyramine in 0.01 *N* HCl was added to a solution containing 0.3 ml of 0.05 *M* sodium phosphate buffer (pH 7.9), 0.1 ml of 0.02 *M* $MgCl_2$, 0.1 ml of dimethyl sulfoxide, and 0.42 ml of water for a final volume of 1 ml. A blank contained all but the 3-methoxytyramine. Following the addition of 0.5 ml of 0.5 *M* sodium borate buffer (pH 10), the mixture was extracted with 6 ml of toluene–isoamyl alcohol (3:2 by volume). Five-milliliter aliquots from three samples were pooled and lyophilized, and the residue was dissolved in 3 ml of 0.01 *N* HCl and transferred to cells. The absorbance at 282 and 225 nm was averaged from triplicate samples, and nanomoles of 3-methoxytyramine was determined from the standard curve. Eighty-four percent of the added 3-methoxytyramine was recovered.

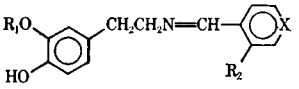
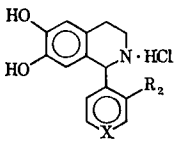
The recovery of III was performed similarly to that of 3-methoxytyramine, with the following exceptions: the compound was dissolved in methanol rather than 0.01 *N* HCl and it was quantitated by its absorbance at 280 nm, which was linear between 7.5 and 150 nmoles. For extraction, the compound was dissolved in dimethyl sulfoxide rather than 0.01 *N* HCl, extracted, and lyophilized, and the residue was dissolved in methanol. The amount of compound extracted was 74%.

Since the *O*-methylated derivatives of II, IV, and V were not available, their recoveries were determined using the corresponding ^{14}C-labeled forms synthesized enzymatically. Compound IV (0.4 μmole in water) was used as the substrate in the reaction mixture containing 0.1 ml dimethyl sulfoxide as described under *Enzyme Purification and Assay*. Following extraction of the product, 4-ml aliquots from two samples were pooled to obtain a reference sample and 4-ml aliquots from another eight samples were pooled

[1] Melting points were taken on a Mel-Temp apparatus and are uncorrected. Analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

[2] New England Nuclear Corp.
[3] Aldrich Chemical Co.
[4] Calbiochem.
[5] Beckman DB-G.

Table I—Physical Constants of Schiff Bases and Tetrahydroisoquinolines

Compound				Melting Point	Yield, %	Empirical Formula	Analysis, %	
Number	R_1	R_2	X				Calc.	Found
								
I	H	NO_2	C	117–119° dec.[a]	93	$C_{15}H_{14}N_2O_4$	C 62.93 H 4.93 N 9.79	63.03 5.04 9.68
II	H	H	N	239–240° dec.[b]	93	$C_{14}H_{14}N_2O_2$	C 69.40 H 5.82 N 11.56	69.36 5.70 11.50
III	CH_3	NO_2	C	67.5–69°[c]	61	$C_{16}H_{16}N_2O_4$	C 63.99 H 5.37 N 9.33	63.97 5.44 9.43
								
IV	—	NO_2	C	226–232° dec.	75	$C_{15}H_{15}ClN_2O_4$	C 55.82 H 4.68 N 8.68	56.08 4.65 8.61
V	—	H	N	235–240° dec.	71	$C_{14}H_{15}ClN_2O_2$	C 60.32 H 5.42 N 10.05	60.60 5.37 10.05

[a] Recrystallized from absolute ethanol. [b] Recrystallized from dimethyl sulfoxide–water. [c] Recrystallized from ethanol–water.

Table II—Effect of 2-Nitrobenzaldehyde and Pyridine-4-carboxaldehyde on *O*-Methylation of Dopamine *In Vitro*

Experiment Number	Product Formed[a,b], nmoles			
	Dopamine in Water	Dopamine in Dimethyl Sulfoxide	Dopamine plus 2-Nitrobenzaldehyde[c]	Dopamine plus Pyridine-4-carboxaldehyde[c]
1	3.34	4.96	6.19 (25%)	—
2	5.29	8.52	8.66 (2%)	—
3	5.30	8.19	10.65 (30%)	—
4	4.48	7.08	10.52 (49%)	—
5	4.56	7.72	7.80 (1%)	9.78 (27%)
6	9.62	10.70	10.90 (2%)	11.94 (12%)
7	3.12	5.12	—	8.28 (62%)
8	5.50	9.57	—	13.43 (40%)
9	5.16	10.51	—	12.28 (17%)
10	5.16	8.26	—	10.11 (22%)
11	10.96	—	—	12.03 (10%)[d]
12	7.46	—	—	8.14 (9%)[d]
13	9.20	—	—	10.23 (11%)[d]
14	9.61	—	—	10.84 (13%)[d]

[a] Each value represents the average of two samples. [b] Values in parentheses represent percent increase from controls. [c] Concentration of aldehydes was 1 m*M* in dimethyl sulfoxide unless otherwise noted. [d] Pyridine-4-carboxaldehyde was dissolved in water.

for preparative isolation of the product. The reference sample was lyophilized and the residue, dissolved in methanol, was then chromatographed on a silica gel F-243-coated plastic sheet[6] (20 × 20 cm) with a solvent system of *n*-butanol–triethylamine–ethyl acetate (3:2:2). The R_f value of the product was determined by cutting the sheet in 0.5-cm sections for measurement of radioactivity by liquid scintillation spectrometry.

After establishing the R_f value of the radioactive product (0.79), the corresponding area on a chromatogram of the preparative sample was cut out, minced, and eluted three times with 5 ml of methanol. The eluates were pooled and evaporated under nitrogen, and the residue was dissolved in 20 ml of methanol. After determining the specific activity of this solution, triplicate samples of 0.5 ml were transferred to 10-ml centrifuge tubes and evaporated to dryness; the residue was then dissolved in 0.1 ml of dimethyl sulfoxide before the addition of magnesium chloride, sodium phosphate buffer (pH 7.9), and water to a final volume of 1 ml. The samples were extracted with 6 ml of toluene–isoamyl alcohol (3:2) after being made basic with 0.5 ml of 0.5 *M* sodium borate buffer (pH 10). A 4-ml aliquot of the extract was transferred to a counting vial and the ^{14}C was assayed as already described with the addition of 3 ml of methanol and 15 ml of liquifluor. Recovery of the *O*-methylated derivative of IV determined by the radioactivity of the extracts was 92.5%.

The method used to determine the recovery of *O*-methylated derivatives of II and V was similar to that used for IV with the following exceptions: 78.3 nmoles of II (dissolved in dimethyl sulfoxide) and 47.0 nmoles of V (dissolved in water) were substituted as substrates, and the product was chromatographed on cellulose thin-layer plates[6] (20 × 20 cm) with *n*-butanol–water–triethylamine (25:4:1). The R_f values of the products formed from II and V were 0.89 and 0.91, respectively; the recovery of the products was then established at 87.8 and 87.5%, respectively.

DL-Normetanephrine-$^3H^2$ (7 nmoles, 20 nCi) was dissolved in water and added to quadruplicate samples containing 0.1 ml of di-

[6] Merck, Darmstadt, Germany.

Table III—Kinetic Constants of Substrates for Catechol-*O*-methyltransferase

Substrate	K_m, μM[a]	V_{max}, μmoles/hr
I	23.6	27.39
II	15.1	21.62
IV	28.5	38.66
V	25.9	33.44
Norepinephrine	51.1	25.55
Dopamine	32.7	18.51

[a] All assays were carried out in the presence of 0.1 ml of dimethyl sulfoxide.

methyl sulfoxide, sodium phosphate buffer (pH 7.9), magnesium chloride, and water to a final volume of 1 ml. The solution was made basic with 0.5 ml of 0.5 *M* sodium borate buffer (pH 10) and extracted with 6 ml of toluene–isoamyl alcohol (3:2). The radioactivity in 4-ml aliquots was assayed as already described, and only 26% of the added normetanephrine was found to have been extracted.

RESULTS AND DISCUSSION

The effects of 2-nitrobenzaldehyde and pyridine-4-carboxaldehyde on dopamine *O*-methylation are shown in Table II. In all experiments, an increase in extracted radioactivity (in terms of nanomoles of product) was found when these aldehydes were added to the reaction. Although dimethyl sulfoxide exerted activation in itself, this effect did not contribute to the action of pyridine-4-carboxaldehyde which, when dissolved in water instead of dimethyl sulfoxide, still produced an increase in ^{14}C (see last four experiments in Table II). One explanation for the increased product is an interaction of the aldehyde with the substrate dopamine. To examine the possibility that a Schiff base was being formed between these aldehydes and dopamine, providing an additional substrate for catechol-*O*-methyltransferase, or that the Schiff base cyclized *via* the Pictet–Spengler reaction, producing a tetrahydroisoquinoline, these derivatives were synthesized and examined as possible substrates for catechol-*O*-methyltransferase.

As seen in Table III, the Schiff bases and tetrahydroisoquinoline derivatives were better substrates for catechol-*O*-methyltransferase than either dopamine or norepinephrine; II was most active in terms of K_m (Fig. 1) while the tetrahydroisoquinolines had greater V_{max} values. All values were corrected for 100% recovery (see *Recovery Studies*). No attempt was made to distinguish between the *meta*- and *para*-*O*-methylated isomers. Although it is possible that one optical isomer of either IV or V might be a better substrate for catechol-*O*-methyltransferase than its counterpart, no attempt was made to resolve the stereoisomers.

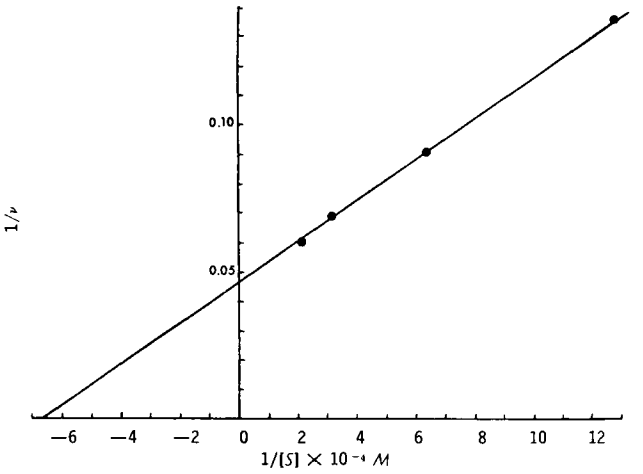


Figure 1—*Determination of kinetic constants of II for catechol-O-methyltransferase* (K_m = *15.1* μM, V_{max} = *21.62 nmoles/hr*).

Due to the wide variability in activation found when dopamine was used as substrate, it was thought that norepinephrine would yield more consistent results in the presence of the aldehydes. As seen in Table IV, activation with norepinephrine was of a greater magnitude and less variable than when dopamine was used. In addition, dimethyl sulfoxide had little or no effect on the methylation of norepinephrine; this was evident also in runs where pyridine-4-carboxaldehyde was dissolved in water. Concentration variation of the aldehydes (see Experiments 7, 13, and 16, Table IV) gave a linear response.

Preliminary findings using epinephrine or 3,4-dihydroxymandelic acid as a substrate indicate that the aldehydes were ineffec-

Table IV—Effects of 2-Nitrobenzaldehyde and Pyridine-4-carboxaldehyde on *O*-Methylation of Norepinephrine *In Vitro*

Experiment Number	Product Formed[a,b], nmoles: Norepinephrine in Water	Norepinephrine in Dimethyl Sulfoxide	Norepinephrine plus Pyridine-4-carboxaldehyde in Water[c]	Norepinephrine plus Pyridine-4-carboxaldehyde in Dimethyl Sulfoxide[c]	Norepinephrine plus 2-Nitrobenzaldehyde in Dimethyl Sulfoxide[c]
1	1.32	1.97	—	4.07 (107%)	—
2	1.76	2.00	—	4.10 (105%)	—
3	2.05	2.02	—	3.59 (78%)	—
4	1.58	1.99	—	3.81 (91%)	—
5	1.34	1.97	—	3.60 (83%)	—
6	2.33	3.26	—	4.62 (42%)	—
7	3.85	3.76	—	6.45 (72%) 5.20 (38%)[d] 3.99 (6%)[e]	—
8	2.05	—	3.55 (73%)	—	—
9	1.58	—	2.84 (80%)	—	—
10	2.03	—	3.45 (70%)	—	—
11	3.43	—	4.98 (45%)	—	—
12	3.74	—	6.31 (69%)	—	—
13	3.82	—	5.94 (56%) 5.34 (40%)[d] 4.09 (7%)[e]	—	—
14	2.31	2.47	—	—	4.65 (88%)
15	2.33	3.26	—	—	4.60 (41%)
16	3.97	3.70	—	—	6.37 (72%) 5.35 (45%)[d] 4.15 (12%)[e]

[a] Each value represents the average of two samples. [b] Values in parentheses represent percent increase from controls. [c] Concentration of aldehydes was 1 m*M* unless otherwise stated. [d] Aldehyde concentration was 0.5 m*M*. [e] Aldehyde concentration was 0.1 m*M*.

tive in producing activation, further supporting the hypothesis of Schiff-base formation for which a terminal primary amine group is required. It is hoped that results of these studies may provide further information concerning the metabolism of dopamine and norepinephrine in terms of endogenous aldehyde levels and agents that alter these levels.

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Synthesis and Screening of Potential Antimalarial Agent α-(2-Piperidyl)-2-(1-adamantyl)-6,8-dichloro-4-quinolinemethanol Hydrochloride

JAROSLAV NOVOTNY, CAROL H. COLLINS, and FRED W. STARKS[x]

Abstract □ The synthesis and biological testing of α-(2-piperidyl)-2-(1-adamantyl)-6,8-dichloro-4-quinolinemethanol hydrochloride and its *O*-acetyl and *N*-acetyl derivatives are reported. Direct hydrogenation of the α-pyridyl ketone gave very low yields. A novel procedure was developed which permits formation of piperidyl quinolinemethanols in satisfactory yields.

Keyphrases □ α-(2-Piperidyl)-2-(1-adamantyl)-6,8-dichloro-4-quinolinemethanol hydrochloride (and *N*-acetyl and *O*-acetyl derivatives)—synthesized and screened as potential antimalarial agents □ Quinolinemethanols, α-(2-piperidyl) analog and *N*-acetyl and *O*-acetyl derivatives—synthesized and screened as potential antimalarial agents □ Antimalarial agents, potential—synthesis and screening of α-(2-piperidyl)-2-(1-adamantyl)-6,8-dichloro-4-quinolinemethanol hydrochloride and *N*-acetyl and *O*-acetyl derivatives

Antimalarial activity has been reported for many derivatives of the quinine analog 4-quinolinemethanol. Mono- or disubstitutions at the 6-, 7-, or 8-position of the quinoline nucleus enhance activity relative to the unsubstituted compound, while substitution at the 2-position appears necessary to retard metabolic oxidation of the quinoline skeleton (1–4).

Unfortunately, the more active 2-aryl-4-quinolinemethanols also have undesirably high phototoxicity, which might detract from their use as antimalarial agents in humans (3, 5–8). Recently, a number of 4-quinolinemethanols were prepared with nonaryl substitution at the 2-position (9–11). Mixed results indicated further search for appropriate substitution at this position. Use of an adamantyl substituent at position 2 was suggested by the enhanced biological activity of this substituent in comparison to more conventional saturated moieties in other medicinal agents (12–14). Synthesis of several 2-adamantylquinolinemethanols has been reported (15). The promising antimalarial activity of α-(di-*n*-butylaminomethyl)-2-(1-adamantyl)-6,8-dichloro-4-quinolinemethanol (I*a*) (15) suggested that the α-(2-piperidyl) analog (I*b*) should be synthesized and tested.

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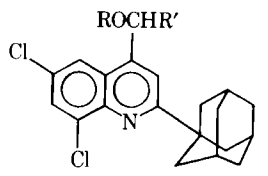

I*a*: R = H, R′ = $CH_2N(C_4H_9)_2$
I*b*: R = H, R′ = 2-piperidyl
I*c*: R = H, R′ = *N*-acetyl-2-piperidyl
I*d*: R = CH_3CO, R′ = 2-piperidyl

CHEMISTRY

Compound I*b* was prepared as illustrated in Scheme I. Acetyladamantane (II) (17, 18) was obtained in 59% yield, by the method of Tegner (19), from the addition of methyllithium to adamantane-1-carboxylic acid followed by hydrolysis. A by-product of this reaction, 2-(1-adamantyl)propan-2-ol (20), was also isolated in low yield (about 4%). The formation of this product was somewhat surprising, because Tegner (19) suggested that methyllithium should not react further with the dilithium salt of the ketone, even though phenyllithium has been shown to give tertiary alcohols in its reaction with carboxylic acids and their derivatives (21).

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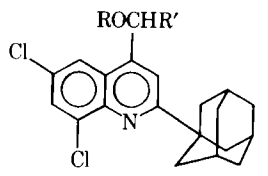

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In this study, the Pfitzinger (22) condensation of 5,7-dichloroisatin and II produced the cinchoninic acid (III) (15) in 48% yield. Slow addition of III to α-pyridyllithium (23, 24) at −74° followed by hydrolysis gave pyridyl ketone (IV) in 84% yield. When this reaction was carried out at −60°, according to literature directions (4), the yield was only 46%.

When the one-step selective hydrogenation (4, 16) of IV to *Ib* was attempted, the desired compound was obtained in only 4% yield. This occurred in conjunction with a theoretical absorption of hydrogen. Similar low product yields and the formation of side-products have been reported in the selective hydrogenation of several other 2-pyridyl quinolyl ketones that lacked a 2-aryl substituent (10, 11).

A satisfactory yield of *Ib* ensued by first reducing the carbonyl with sodium borohydride (99% yield), acetylating the carbinol (60%), hydrogenating this (59%), and, finally, hydrolyzing the reduced *O*-acetyl compound (*Id*) in 52% yield. Compound *Id* hydrolyzed smoothly to *Ib* in either sodium methoxide or concentrated hydrochloric acid. However, in ammonium hydroxide at room temperature, the acetyl group migrated to the piperidyl nitrogen. The molecular arrangement of the piperidyl nitrogen and methanol oxygen made probable the facile formation of a six-membered ring as an intermediate in this intramolecular migration under the mild conditions employed. Use of stronger reagents hydrolyzes either the *N*-acetyl or the *O*-acetyl compound.

BIOLOGICAL ACTIVITY

Compound *Ib* and the several intermediates were tested[1] for antimalarial activity against *Plasmodium berghei* in mice and against *P. gallinaceum* in chicks. Assessment of activity was based on the influence of various doses of the compounds upon the survival times of groups of mice in comparison with untreated controls (mean survival time 6.2 days). Those surviving more than 60 days were adjudged "cured." Compound *Ib* was active at the 320-mg/kg level against *P. berghei*, effecting two (out of five) cures at this level. None of the other compounds (*Ic*, *Id*, IV, V, and VI) showed antimalarial activity (Table I).

Compounds *Id*, *Ic*, and *Ib* were also evaluated against blood-induced *P. gallinaceum* infections in chicks (25)[1]. They showed no significant activity. A similar response to *P. berghei* and *P. gallinaceum* has been reported for the di-*n*-butylaminomethyl analog (*Ia*) (15).

Table I—Antimalarial Activity in Blood-Induced Infections

Dose, mg/kg	Increase in Survival Time, Days: Evaluation against *P. berghei* KBG Malaria in Young Noninbred ICR/HA Swiss Mice[a]		Evaluation against *P. gallinaceum* Brumpt 8A in 9–12-day-old White Leghorn Chicks[a]
	Ib	*Id*	*Ib*
10	0.3		
20	0.3 0.3	0.1	0.0
40	0.5 0.3	0.3	0.0
80	0.5 0.5	0.3	0.0
160	2.1 1.9	0.7	0.0
320	2/5C[b]	1.9	0.0
640	2/5C[b]	5.9	3.4

[a] The test results were provided by the Walter Reed Army Institute of Research through the courtesy of Dr. T. R. Sweeney and Dr. B. T. Poon. [b] C = cures, the number of mice surviving 60 days after infection.

EXPERIMENTAL[2]

1-Acetyladamantane (II)—In a modification of the method of Tegner (19), an excess of methyllithium (2 *M* in ether) was added dropwise to a stirred solution of 721 g (4.0 moles) of adamantane-1-carboxylic acid[3] in 6 liters of ether, maintained under nitrogen at from −10 to 0° by partial immersion in a dry ice–acetone bath. The mixture was stirred at room temperature for 1 hr and then hydrolyzed by dropwise addition of wet ether followed by water. The organic layer was separated, washed with water, and dried (magnesium sulfate). The solvent was evaporated *in vacuo* and the crude product was recrystallized from methanol to give 420 g (59%) of analytically pure material, mp 51–53° [lit. (17, 18) mp 53–54°]. The product was homogeneous to VPC with a retention time of 14.2 min.

Anal.—Calc. for $C_{12}H_{18}O$: C, 80.85; H, 10.18; O, 8.97. Found: C, 80.62; H, 10.18; O, 9.16.

2-(1-Adamantyl)propan-2-ol—The mother liquor from recrystallization of crude 1-acetyladamantane was spin evaporated to an oil which was distilled (bp 65–67°/10 mm). An analytically pure product was obtained by crystallization from aqueous ethanol, mp 65–67° [lit. (20) mp 77–80°]. The product was homogeneous to VPC with a retention time of 14.8 min and was identical to a sample synthesized independently from methylmagnesium iodide and acetyladamantane.

Anal.—Calc. for $C_{13}H_{22}O$: C, 80.36; H, 11.41. Found: C, 80.45; H, 11.50.

6,8-Dichloro-2-(1-adamantyl)cinchoninic Acid (III)—A mixture of 178 g (1.00 mole) of II, 197 g (0.910 mole) of 5,7-dichloroisatin[3], 1 liter of ethanol, 340 ml of water, and 161 g (2.90 moles) of potassium hydroxide was refluxed for 17 hr, cooled, and then evaporated *in vacuo* to a residue (22). The residue was partitioned between water and ether. The aqueous layer was separated, washed with ether, and then acidified by addition of concentrated hydrochloric acid to precipitate the product. The solid was collected, washed by resuspension in hot ethanol, dried, and recrystallized from acetone to yield 166 g (48%), mp 260–263° dec. [lit. (15) mp 258–261°]; λ_{max} ($\epsilon \times 10^{-3}$): 241 (29.0), 298 (4.6), and 332 (4.6) nm.

Anal.—Calc. for $C_{20}H_{19}Cl_2NO_2$: C, 63.84; H, 5.09; N, 3.72. Found: C, 64.07; H, 5.05; N, 3.68.

2-Pyridyl-2-(1-adamantyl)-6,8-dichloro-4-quinolyl Ketone (IV)—An ethereal solution of 2-pyridyllithium was prepared, according to the method of Wibaut *et al.* (23), from 166 g (1.05 moles) of 2-bromopyridine, 1000 ml of ether, and 425 g (1.00 mole) of a 15% solution of *n*-butyllithium in hexane. While maintaining the nitrogen atmosphere, the ethereal solution was cooled to −74° and 125 g (0.333 mole) of III was added portionwise over 1.25 hr. After the addition, the mixture was stirred for 1 hr at −73°, for 1 hr at between −70 and −68°, for 1 hr at between −68 and −63°, and for 1 hr between −63 and −59°. After warming slowly to 10°, 500 ml of water was cautiously added. After separating the layers, the aqueous portion was extracted several times with ether. The combined ether solution was dried (magnesium sulfate) and the solvent was removed *in vacuo*. The residue was triturated with ethanol to yield crude product, which was recrystallized from ethanol, yielding 122 g (84%), mp 138–140°, homogeneous to TLC; λ_{max} ($\epsilon \times 10^{-3}$): 232 (38.6) and 332 (4.0) nm.

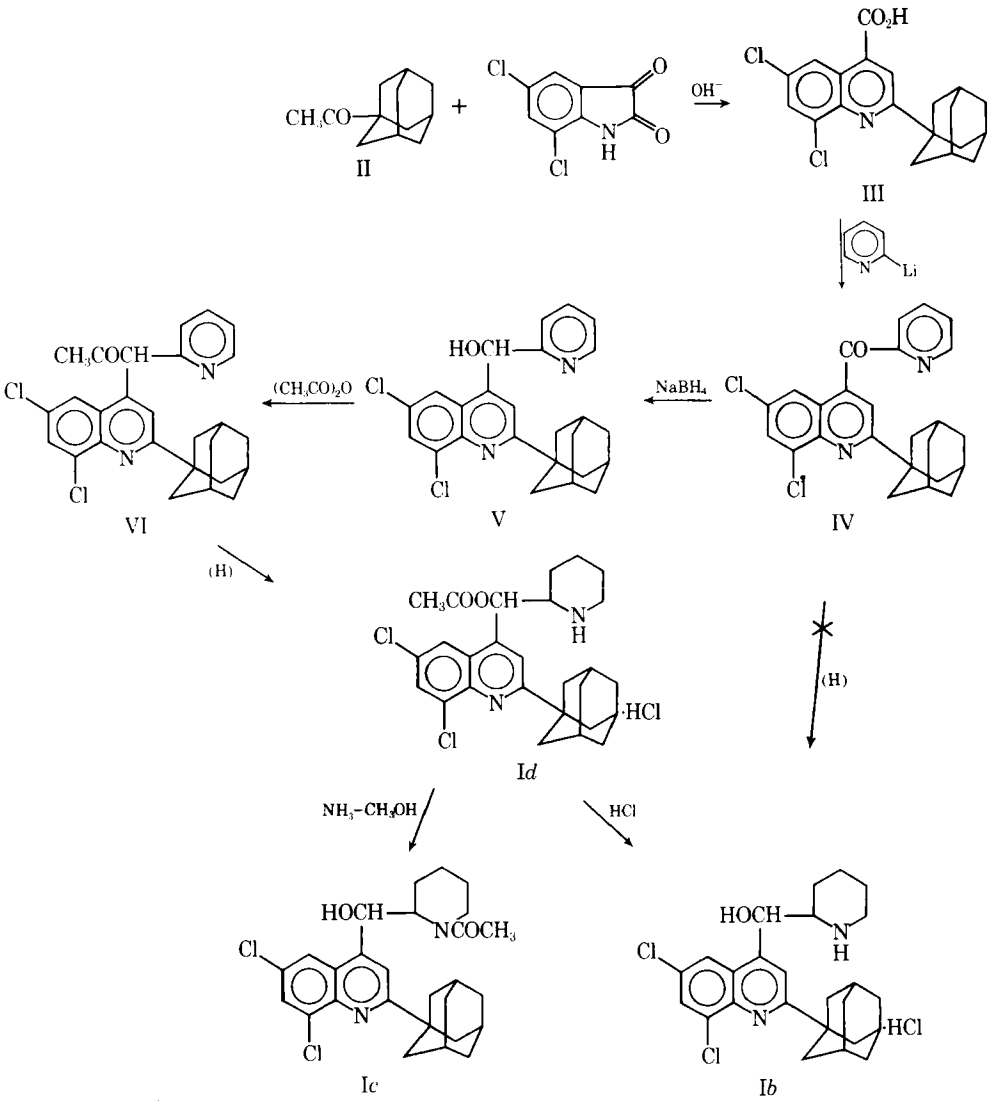
Anal.—Calc. for $C_{25}H_{22}Cl_2N_2O$: C, 68.66; H, 5.07; Cl, 16.21; N, 6.40. Found: C, 68.60; H, 5.24; Cl, 16.06; N, 6.60.

α-(2-Pyridyl)-2-(1-adamantyl)-6,8-dichloro-4-quinolinemethanol (V)—Sodium borohydride (51.3 g, 1.35 moles) was added portionwise to a stirred solution of 593 g (1.35 moles) of IV in 9 liters of ethanol. The mixture was stirred overnight at room temperature. The solvent was removed *in vacuo* and the residue was dissolved in chloroform, which was then washed with water, dried (magnesium sulfate), and removed by spin evaporation *in vacuo*. The residue was recrystallized from methanol to give 587 g (99%) of product, pure to TLC (four systems), mp 177–177.5°; λ_{max} ($\epsilon \times 10^{-3}$): 238 (31.2), 262 (4.3), 268 (4.3), 284 (4.2), 316 (2.2), and 330 (2.3) nm.

[1] By Dr. L. Rane of the Malaria Screening Laboratory, University of Miami (25). The test results were provided by the Walter Reed Army Institute of Research through the courtesy of Dr. T. R. Sweeney and Dr. B. T. Poon.

[2] Melting points were determined on a Fisher-Jones apparatus. Those below 230° are corrected. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. UV spectra were determined in ethanol with a Bausch and Lomb Spectronic 505 spectrophotometer. TLC was run on Eastman silica gel chromagram sheets 6060, using at least three different solvent systems for each compound. Vapor phase chromatography (VPC) was carried out using a Hewlett-Packard model 5750B, temperature programmed at 10°/min from 40 to 250°, using a 2-m × 3-mm column packed with 10% UC-W982 on 80–100-mesh Diatoport S.

[3] Aldrich Chemical Co.



Scheme I

Anal.—Calc. for $C_{25}H_{24}Cl_2N_2O$: C, 68.34; H, 5.51; Cl, 16.14; N, 6.38. Found: C, 68.38; H, 5.36; Cl, 16.10; N, 6.23.

α-(*O*-Acetyl)-α-(2-pyridyl)-2-(1-adamantyl)-6,8-dichloro-4-quinolinemethanol (VI)—A solution of 461 g (1.05 moles) of V in 1690 ml of acetic anhydride was heated at approximately 100° for 45 min. While still hot, the excess acetic anhydride was hydrolyzed with water. After cooling, the reaction mixture was poured into additional water. The white solid that separated was collected, washed with water, and recrystallized from ethanol to give 303 g (60% yield) of pure product (TLC), mp 174–175°; λ_{max} ($\epsilon \times 10^{-3}$): 240 (48.2), 268 (6.3), 286 (6.4), 319 (3.7), and 333 (3.8) nm.

Anal.—Calc. for $C_{27}H_{26}Cl_2N_2O_2$: C, 67.36; H, 5.44; Cl, 14.72; N, 5.81; O, 6.64. Found: C, 67.44; H, 5.47; Cl, 14.83; N, 5.83; O, 6.69.

α-(*O*-Acetyl)-α-(2-piperidyl)-2-(1-adamantyl)-6,8-dichloro-4-quinolinemethanol Hydrochloride (I*d*-HCl)—A mixture of 71.2 g (0.148 mole) of VI, 900 ml of absolute ethanol, 25 ml of concentrated hydrochloric acid, and 0.8 g of platinum oxide was hydrogenated at 50 psi until the theoretical amount of hydrogen (3 moles) had been consumed (4 hr). The resulting suspension was dissolved in 1700 ml of absolute ethanol and filtered through a diatomaceous earth[4] pad. The filtrate and washings were concentrated *in vacuo* to give a crude product, which was recrystallized from absolute ethanol to give 42 g (59%) of pure product (TLC), mp 205.5–207.5° dec.; λ_{max} ($\epsilon \times 10^{-3}$): 239 (55.1), 285 (5.8), 320 (3.6), and 334 (3.7) nm.

Anal.—Calc. for $C_{27}H_{33}Cl_3N_2O_2$: C, 61.90; H, 6.35; Cl, 20.32; N, 5.35; O, 6.11. Found: C, 61.83; H, 6.43; Cl, 20.13; N, 5.26; O, 6.07.

α-[2-(1-Acetylpiperidyl)]- 2-(1-adamantyl)- 6,8-dichloro- 4-quinolinemethanol (I*c*)—Dry methanol (1000 ml), saturated with ammonia at 0°, was added to 10.0 g (0.020 mole) of solid I*d*-HCl at 0°. The mixture was kept at 0° overnight. Volatiles were removed by spin evaporation *in vacuo*, and the resulting solid was dissolved in 500 ml of chloroform. This solution was washed with water, dried (magnesium sulfate), and concentrated *in vacuo* to give a crude product, which was recrystallized from methanol to yield 4.4 g (44%), mp 246–249°, pure to TLC; λ_{max} ($\epsilon \times 10^{-3}$): 238 (46.7), 285 (6.0), 317 (3.1), and 331 (3.1) nm.

Anal.—Calc. for $C_{27}H_{32}Cl_2N_2O_3$: C, 64.41; H, 6.41; Cl, 14.08; N, 5.56; O, 9.53. Found: C, 64.35; H, 6.51; Cl, 14.19; N, 5.51; O, 9.22.

α-(2-Piperidyl)- 2-(1-adamantyl)- 6,8-dichloro-4-quinolinemethanol Hydrochloride (I*b*-HCl)—A solution of 237 g (0.487 mole) of I*d*-HCl in 2370 ml of concentrated hydrochloric acid was refluxed overnight, cooled to 15°, and neutralized to pH 12 with 33% aqueous sodium hydroxide. The white precipitate was collected, washed with water, and then dissolved in 20 liters of chloroform. The solution was dried (magnesium sulfate) and then saturated with 230 g (16.3 moles) of anhydrous hydrogen chloride. The white solid was filtered, washed with ether, and dried before being dissolved in 38 liters of ethanol containing 45 ml of concentrated hydrochloric acid. The solution was filtered hot, and the filtrate was reduced in volume to about 5.5 liters. The product crystallized on cooling to yield 122 g (52%) of material pure to TLC, mp 253–255° dec.; λ_{max} ($\epsilon \times 10^{-3}$): 237 (46.5), 283 (5.8), 317 (3.1), and 330 (3.4) nm.

Anal.—Calc. for $C_{25}H_{31}Cl_3N_2O$: C, 62.31; H, 6.48; Cl, 22.07; N,

[4] Celite.

5.81. Found: C, 62.15; H, 6.70; Cl, 21.80; N, 5.67.

An identical product was obtained by treating 10 g of Id-HCl with 40 ml of a 1 *M* solution of sodium methoxide in 1000 ml of dry methanol.

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The authors thank Mr. Bruce Mayer, Mr. Walter Schreiner, and Mrs. Anita White for their valuable contributions to the synthetic work.

[x] To whom inquiries should be directed.

Binding of Quinidine to a Red Blood Cell Hemolysate Preparation

V. E. ISAACS[*] and R. D. SCHOENWALD[x]

Abstract □ A constant degree of binding of quinidine to a red blood cell hemolysate preparation was found for a clinically significant range of concentrations at 37° using the methods of equilibrium dialysis and ultracentrifugation. By repeating the binding experiments at 37° but over a much wider range of quinidine concentrations, it was possible to calculate an association constant (252 liters/mole) and the apparent number of binding sites (1.54). Quinidine appeared to be bound to a single binding site. In another series of experiments performed at therapeutic levels and 37°, the competitive binding of quinidine was studied in the presence of red blood cell hemolysate and serum, each placed in individual compartments of a dialysis cell but separated by a semipermeable membrane. Following attainment of equilibrium, free drug was separated from bound drug by ultracentrifugation. Calculations indicated that slightly more than half of the drug was contained within the serum compartment.

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Quinidine has been shown to interact with various proteins contained within the body's vascular pool. For example, elucidation of a quantitative quinidine–albumin relationship at pH 7.4 established the existence of one receptor location on an albumin molecule and an association constant of 7.7×10^3 (1). There is also extensive binding of quinidine to human platelets as evidenced by its large accumulation within the platelet, much greater than would be expected on the basis of pH partitioning (2). Moreover, quinidine has been found to penetrate red blood cells (3), which might be expected to occur for an organic base with a pKa of 8.6 (4) whose unionized form is lipid soluble (5). Because of its potential for binding, the purposes of this study were to determine the binding affinity of quinidine to an erythrocytic preparation obtained from freshly drawn human blood samples and to determine its competitive influence on the protein binding of quinidine in human serum.

5.81. Found: C, 62.15; H, 6.70; Cl, 21.80; N, 5.67.

An identical product was obtained by treating 10 g of Id-HCl with 40 ml of a 1 *M* solution of sodium methoxide in 1000 ml of dry methanol.

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EXPERIMENTAL

Blood Collection—Venous blood samples used in all experiments were drawn from the cephalic veins of two male Caucasians. Samples of 50–100 ml were collected from each donor and placed in a 500-ml erlenmeyer flask containing enough glass beads to cover about two-thirds of the bottom of the flask. The flask was gently swirled for about 10 min to remove the clotting fraction. The suspension of cells was then decanted into an appropriate container and centrifuged. The plasma and buffy layer were discarded and the packed red cells were washed two times with an isotonic pH 7.2 phosphate buffer preparation. The washed cells were resuspended with the isotonic buffer preparation to about 80% of their original volume and pooled.

Preparation of Red Blood Cell Hemolysate—The pooled resuspended cells were hemolyzed with an ultrasonic vibrator[1] and centrifuged at 5000 rpm to remove ghosts. The resulting solution was assayed for hemoglobin content. The volume of the hemolysate preparation was determined and enough additional buffer was added so that the final dilution contained the desired concentration of hemoglobin. The buffer preparation used to adjust the hemoglobin concentration as well as to resuspend the washed red blood cells was formulated to approximate the intracellular pH and ionic strength of the red blood cell. All chemicals used were reagent grade quality, and distilled water was used to prepare all solutions. The pH[2] of the buffer measured 7.2 and the ionic strength was calculated to be 0.18 *M*; the quantities of chemicals used per liter were: $Na_2HPO_4 \cdot 7H_2O$, 1.940 g; KH_2PO_4, 0.312 g; $MgCl_2 \cdot 6H_2O$, 1.017 g; NaCl, 0.512 g; and KCl, 9.76 g. This particular chemical composition yielded 16, 131, 10, and 167 mEq/liter of Na^+, K^+, Mg^{+2}, and Cl^-, respectively, which compared to 16, 150, 10, and 80 or more mEq/liter, respectively, for the same ionic species present in a red blood cell (6). The purpose of studying binding to a red blood cell hemolysate preparation instead of a relatively pure solution of crystallized hemoglobin was to mimic more closely the *in vivo* conditions and, thus, come closer to estimating drug affinity for protein under clinical conditions.

Equilibrium Dialysis—Protein binding to hemolysate components for two different hemoglobin concentrations was determined using a dialyzing system which consisted of a cylindrical Plexiglas cell separated into two compartments by a dialyzing membrane[3]. The cells were similar to those described by Patel and Foss (7), with the exception that a different compartment capacity (depth 5.6 mm, diameter 20.05 mm) was used. Each cell compartment had a capacity of 5.0 ml; however, solutions were added in volumes of 4.7 ml, thus leaving a small volume of air after the plastic screws were inserted into the sampling ports and producing a closed system. The membrane was cut to the proper size, soaked in 70% ethanol for approximately 3 hr to remove impurities, and stored in isotonic buffer under refrigeration until needed; the membranes were thoroughly rinsed with fresh buffer before use. Varying drug concentrations were dissolved in isotonic buffer and pipeted into one compartment of the cell designated the external compartment. The internal compartment contained red blood cell hemolysate. Equilibrium dialysis was carried out at 37° for 20–22 hr in a water bath equipped with a shaker[4]. Additional cells, containing isotonic buffer and drug in one compartment and buffer in the other, were incubated with shaking at 37° and used to determine the time of equilibrium. The extent of protein binding was calculated from the concentration of free quinidine on the external side.

The dialysis cells were also used to determine the binding of quinidine to the red blood cell hemolysate in competition with serum by placing each preparation in compartments separated by a dialysis membrane. The study was performed in a manner simulating *in vivo* conditions in that the initial serum and red blood cell hemolysate were adjusted to pH 7.35 by bubbling a 5% carbon dioxide–95% oxygen mixture through the solutions and were incubated in a 37° shaker water bath. The red blood cell hemolysate was prepared as previously described. The serum was obtained from separate samples of whole blood from the same donors and allowed to stand for approximately 10 min before centrifuging to facilitate removal of the clotting fraction as well as the red blood cells. The serum was decanted into an appropriate container and stored overnight under refrigeration before use. The study was performed at two concentrations of hemoglobin to simulate *in vivo* conditions of binding at normal and anemic hemoglobin concentrations, 0.002218 and 0.001478 *M*, respectively. The volume in each compartment was 4.7 ml. Aliquots of 0.5 ml were removed from each side following attainment of equilibrium and measured for total quinidine concentration, whereas free drug was determined by removing samples of 4.0 ml from each side and separating the free and bound forms by ultracentrifugal separation. In addition, 0.1 ml was removed from each side for sodium ion determination, which was used to correct for the Gibbs–Donnan effect.

Ultracentrifugation—Buffered isotonic solutions of the hemolysate preparation containing both hemoglobin and quinidine in known concentrations and a volume of 4 ml were placed into 8-ml plastic centrifuge tubes. The tubes[5] were then capped and spun at 50,000 rpm in the preheated preparative rotor of the ultracentrifuge[6]. Centrifugation was carried out for 2.5–3 hr at 37°, and the rotor was permitted to stop without use of the brake. Samples of 0.25–1.0 ml containing only free drug were removed from the clear supernatant upper layer and used to determine the extent of binding.

Analytical Methods—Although quinidine sulfate[7] USP was used throughout the study, all concentrations are expressed in molarity of the base, quinidine. Quinidine concentrations were determined by the method of Cramer and Isaksson (5) in which quinidine is first extracted into benzene and finally transferred to sulfuric acid before fluorometric determinations[8] are made. A calibration curve was prepared from solutions that were treated identically to the unknown drug–protein samples but contained only known amounts of quinidine; blank samples were similarly prepared but from solutions devoid of quinidine and protein.

The concentration of hemoglobin present in the hemolysate preparation was determined from the iron content according to the relationship (8):

$$\frac{\text{g hemoglobin/}}{\text{100 ml solution}} = \frac{\text{mg iron/100 ml}}{3.40} \qquad \text{(Eq. 1)}$$

The total iron content was determined in triplicate by a spectrophotometric method (9) and averaged. The hemoglobin concentrations were expressed in molarity by using a molecular weight of 67,000 (10). The pooled plasma samples were assayed for total protein to confirm the presence of a normal protein concentration[9].

Since quinidine exists predominately as the cation at pH values of 7.2–7.4, it will be distributed across the dialysis membrane in accordance with the Gibbs–Donnan equilibrium (11). To assess the Gibbs–Donnan effect, sodium ion was measured on the external and internal sides of the membrane by flame photometry[10].

All necessary calculations were performed by a digital computer[11]. A nonweighted least-squares fit of the experimental data (Fig. 1) was obtained by the use of the BMDX 85 FORTRAN IV computer program, which improves upon initial estimates by means of stepwise Gauss–Newton iterations (12).

RESULTS AND DISCUSSION

Corrections for Free Quinidine Concentration—For the equilibrium dialysis studies, which included a concentration range of 2.2–78 μM, it was necessary to correct for the space occupied by protein, of which 97.5% (w/v) was hemoglobin (6), for binding of

[1] Model LS75 sonifier equipped with a model S75 probe, Branson Instruments, Inc., Stanford, Conn.
[2] Model 28B Metrion III pH meter, Coleman Instruments, Maywood, Ill.
[3] Dialyzing cellophane, Union Carbide Corp., New York, N.Y.
[4] Model G-76 water bath and gyrotory shaker, New Brunswick Scientific Co., New Brunswick, N.J.
[5] High-speed polycarbonate tubes with Noryl caps, Beckman Instruments, Palo Alto, Calif.
[6] Model L2-65B preparative ultracentrifuge equipped with type Ti50 rotor, Beckman Instruments, Palo Alto, Calif.
[7] Merck and Co., Rahway, N.J.
[8] Fluorospec, SF-100, Baird Atomic Inc., Bedford, Mass.
[9] Kindly performed by the Clinical Laboratory, Harborview Hospital, Seattle, Wash.; serum protein content, 8.0 g %, and albumin content, 5.2 g %, were within normal limits.
[10] Model 403, atomic absorption spectrophotometer, Perkin-Elmer Corp., Norwalk, Conn.
[11] CDC 6400, Computer Science Center, University of Washington, Seattle, Wash.

Table I—Binding of Quinidine to Human Red Blood Cells Hemolysate[a] Studied over a Clinically Significant Range of Molar Concentrations at 37° Using Ultracentrifugation and Equilibrium Dialysis

Method Used	H_b[b], $\times 10^3$	D_t[c], $\times 10^5$	D_f[d], $\times 10^5$	D_b[e], $\times 10^5$	β[f]	nK_a[g], liters/mole
Ultracentrifugation	0.505	0.311	0.238	0.0726	0.234	—
		0.932	0.768	0.163	0.175	—
		1.55	1.31	0.239	0.154	—
		4.66	4.07	0.593	0.127	—
		7.76	6.45	1.32	0.170	—
Average (*SD*)					0.172 (0.0392)	411.3
Ultracentrifugation	1.011	0.311	0.191	0.0120	0.386	—
		1.55	1.03	0.0519	0.334	—
		3.11	2.04	0.107	0.343	—
		4.66	4.19	0.0472	0.101	—
		7.76	6.04	0.172	0.222	—
Average (*SD*)					0.277 (0.116)	379.0
Equilibrium dialysis[h]	1.33	0.216	0.120	0.0957	0.444	—
		0.647	0.360	0.287	0.444	—
		1.05	0.640	0.407	0.389	—
		2.13	1.24	0.889	0.418	—
		3.14	1.92	1.21	0.386	—
		5.42	2.97	2.44	0.451	—
Average (*SD*)					0.422 (0.0288)	549.0
Equilibrium dialysis[h]	1.33	0.223	0.112	0.109	0.493	—
		0.647	0.360	0.287	0.444	—
		1.05	0.640	0.407	0.389	—
		2.08	1.30	0.787	0.378	—
		3.12	1.94	1.18	0.378	—
		5.34	3.06	2.29	0.428	—
Average (*SD*)					0.418 (0.0458)	540.1
Equilibrium dialysis[h]	1.33	0.220	0.114	0.106	0.481	—
		0.655	0.351	0.304	0.465	—
		1.038	0.652	0.387	0.372	—
		2.11	1.26	0.855	0.450	—
		3.45	1.66	1.69	0.505	—
		5.28	3.14	2.14	0.405	—
Average (*SD*)					0.439 (0.0521)	588.5
Ultracentrifugation	1.77	1.08	0.591	0.493	0.455	—
		5.71	3.31	2.41	0.421	—
Average (*SD*)					0.438 (0.0239)	440.8
Equilibrium dialysis[i]	1.96	2.20	1.26	0.939	0.427	—
		3.38	1.77	1.61	0.476	—
		5.44	3.23	2.21	0.406	—
Average (*SD*)					0.436 (0.0362)	226.9
Equilibrium dialysis[i]	1.96	0.569	0.504	0.0652	0.115	—
		0.949	0.840	0.109	0.115	—
		2.05	1.47	0.575	0.281	—
		3.74	1.28	2.46	0.659	—
		5.21	3.56	1.65	0.317	—
Average (*SD*)					0.297 (0.223)	215.3
Equilibrium dialysis[i]	1.96	0.575	0.496	0.0790	0.137	—
		0.960	0.824	0.136	0.142	—
		2.04	1.48	0.554	0.272	—
		3.19	2.04	1.15	0.361	—
		5.15	3.63	1.52	0.295	—
Average (*SD*)					0.242 (0.0986)	162.7

[a] Represents erythrocytic contents with ghosts removed and diluted to appropriate hemoglobin concentrations using a pH 7.2 phosphate buffer containing approximate cell concentrations of K^+, Na^+, Mg^{+2}, and Cl^-. [b] Molar hemoglobin concentration, experimentally determined. [c] Total molar quinidine concentration. [d] Free molar quinidine concentration. [e] Bound molar quinidine concentration. [f] Fraction bound. [g] n = number of binding sites on hemoglobin; K_a = association constant. [h] Gibbs–Donan correction, Na^+ (internal)/Na^+ (external) = 1.032 ± 0.0362. [i] Gibbs–Donnan correction, Na^+ (internal)/Na^+ (external) = 1.080 ± 0.0389.

drug to the dialysis cell and/or membrane, and for the Gibbs-Donnan effect. Because of the space occupied by protein, a correction was made based upon the fractional water volume of a normal red blood cell, 0.7142 (13). Since the concentration of free quinidine in erythrocyte water exceeds the experimentally determined concentration based upon the total volume of the red blood cell hemolysate, the measured free molar drug concentration in the external compartment, D_f (external), is divided by 0.7142 to relate this quantity to the molar free drug concentration in the internal compartment. However, for hemoglobin concentrations [Hb] other than average for adult males, *i.e.*, 0.002388 *M* (12), the correction for D_f (internal) was achieved by use of Eq. 2:

$$D_f \text{ (internal) corrected for hemoglobin space} = 1 - (1 - 0.7142)\frac{[\text{Hb}]}{0.002388} \quad \text{(Eq. 2)}$$

Preliminary experiments showed that binding of quinidine to the plastic dialysis cells and/or membrane was on the order of 6.08 ± 1.10% (n = 10) over the concentration range studied and, therefore, necessitated a correction.

Because the ionic strength was not inordinately large and the pH of the red blood cell hemolysate, 7.2, was not too distant from the isoelectric point of hemoglobin, 7.07 (14), a small but numerically significant Gibbs-Donnan effect was measured (Table I). The correction for the Gibbs-Donnan effect was based upon the assumption that sodium is a freely diffusible ion whose asymmetric distribution is a direct consequence of the concentration of negatively charged hemoglobin present in the internal compartment of the dialysis cell. Thus, the requirements of the Gibbs-Donnan effect states that the ratio of the sodium-ion concentration in the internal chamber to the concentration of sodium ion in

Table II—Competitive Binding of Quinidine to Red Blood Cell Hemolysate[a] and Serum Proteins Studied over a Clinically Significant Range of Molar Concentrations at 37° Using Equilibrium Dialysis

Cell Number	Red Blood Cell Hemolysate Compartment					Serum Compartment				
	H_b[b], $\times 10^3$	D_t[c], $\times 10^5$	D_f[d], $\times 10^5$	D_b[e], $\times 10^5$	β[f]	P_t[g], $\times 10^3$	D_t[c], $\times 10^5$	D_f[d], $\times 10^5$	D_b[e], $\times 10^5$	β[f]
1	1.48	0.128	0.050	0.080	0.609	1.16	0.106	0.0408	0.0652	0.615
2	1.48	0.324	0.109	0.215	0.664	1.16	0.381	0.0766	0.304	0.800
3	1.48	0.505	0.238	0.267	0.529	1.16	0.858	0.160	0.698	0.813
4	1.48	1.00	0.415	0.588	0.586	1.16	1.66	0.300	1.36	0.819
5	1.48	1.62	0.632	0.988	0.610	1.16	2.32	0.709	1.61	0.694
6	1.48	3.24	2.15	1.09	0.335	1.16	3.94	1.30	2.64	0.670
Average (SD)[h]					0.551 (0.106)					0.735 (0.0868)
7	2.22	0.154	0.0781	0.0761	0.493	1.16	0.168	0.0351	0.133	0.791
8	2.22	0.241	0.0594	0.182	0.754	1.16	0.307	0.140	0.166	0.542
9	2.22	0.490	0.364	0.126	0.257	1.16	0.876	0.193	0.683	0.779
10	2.22	1.28	0.499	0.778	0.609	1.16	1.27	0.282	0.988	0.778
11	2.22	1.97	0.618	1.35	0.686	1.16	1.75	0.427	1.32	0.756
12	2.22	3.68	2.31	1.37	0.372	1.16	3.25	0.422	2.82	0.870
Average (SD)[i]					0.583 (0.152)					0.753 (0.110)

[a] Represents erythrocytic contents with ghosts removed and diluted to appropriate hemoglobin concentrations using a pH 7.35 phosphate buffer containing approximate cell concentrations of Na^+, K^+, Mg^{+2}, and Cl^-. [b] Molar hemoglobin concentration, experimentally determined. [c] Total molar quinidine concentration, experimentally determined. [d] Free molar quinidine concentration, experimentally determined by ultracentrifugation. [e] Bound molar quinidine concentration, calculated. [f] Fraction bound. [g] Total molar protein concentration; albumin concentration was 0.000754 M. [h] Gibbs–Donnan correction, Na^+ (serum)/Na^+ (red blood cell hemolysate) = 1.077 ± 0.0636. [i] Gibbs–Donnan correction, Na^+ (serum)/Na^+ (red blood cell hemolysate) = 1.121 ± 0.0603.

the external chamber is equal to a similar ratio of any membrane-permeable cations present in solution including the free quinidine cation. As a result, the following relationship is valid:

$$D_f \text{ (internal) corrected for Gibbs–Donnan effect} = \frac{[D_f \text{ (external)}][Na^+ \text{ (internal)}]}{Na^+ \text{ (external)}} \quad \text{(Eq. 3)}$$

The terms on the right-hand side of Eq. 3 are experimentally determined and permit the correction to be made.

The remaining parameters, D_b and D_t, the molar concentrations of bound drug and total drug present in the internal compartment, respectively, were calculated according to Eqs. 4 and 5:

$$D_t = D_i - D_f \text{ (external)} \quad \text{(Eq. 4)}$$

$$D_b = D_t - D_f \text{ (internal)} \quad \text{(Eq. 5)}$$

where D_i refers to the initial molar concentration placed into the external compartment of the dialysis cell.

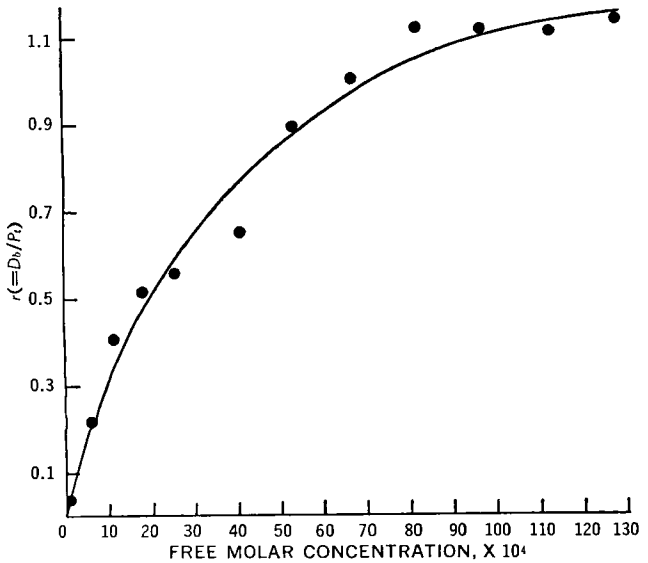

Figure 1—*Binding of quinidine to red blood cell hemolysate, of which 97.5% of the total proteins is hemoglobin* (P_t *is taken as hemoglobin concentration, 9.93* × 10^{-4} M). *Binding was studied at 37° and evaluated by ultracentrifugation. The computer-generated line of best fit yielded an association constant of 251.6 liters/mole.*

Analysis of binding utilizing the technique of ultracentrifugation obviated the need for correcting for the Gibbs–Donnan effect. In addition, no binding of quinidine to the centrifuge tubes could be detected. A correction for the space occupied by hemoglobin in the red blood cell hemolysate was made by use of Eq. 2.

Characterization of Binding—The relative binding of quinidine to red blood cell hemolysate was studied over a range of D_t values of approximately 30-fold (0.0466–1.40 μM) including therapeutic levels. It can be seen from Table I that a constant fraction of quinidine was bound to the red blood cell hemolysate when the concentration of hemoglobin was kept constant; however, as the concentration of hemoglobin was reduced, the fraction bound likewise was reduced. It was assumed that hemoglobin was primarily responsible for the protein binding of quinidine in human red blood cell hemolysate since it represents 97.5% of the total protein content within a red blood cell.

The interaction of quinidine, presumably with hemoglobin, was studied by ultracentrifugation at 37° over a concentration range of quinidine sufficient to characterize the binding parameters, n and K_a, which are the number of binding sites on the hemoglobin molecule and the association constant for the interaction, respectively. The binding parameters are related by Eq. 6:

$$r = \frac{D_b}{P_t} = \frac{nK_aD_f}{1 + K_aD_f} \quad \text{(Eq. 6)}$$

where r describes the ratio of D_b to total moles of protein P_t, and n, K_a, and D_f are as previously defined. Equation 6 makes the assumptions that binding involves only one protein in which each binding site is uninfluenced by the state of occupancy of other sites and that the affinity of each site for drug is identical (15). A linear transformation of Eq. 6 can be expressed as Eq. 7, which is the basis for the well-known Scatchard plot (16):

$$\frac{r}{D_f} = nK_a - rK_a \quad \text{(Eq. 7)}$$

the linearity of which indicates that only one class of sites needs to be considered mathematically. The experimental data conformed to Eq. 7, yielding n and K_a values from the slope and intercept of a linear (Pearson r = 0.914) plot. These parameter values were used as initial estimates in the nonlinear curve-fitting routine. When assuming quinidine to interact exclusively with hemoglobin, the data could be fitted according to Eq. 6 as shown by Fig. 1. The n and K_a values obtained from the fit are 1.54 and 252 liters/mole, indicating relatively weak binding.

The results of the binding experiments performed over a relatively small range of quinidine concentrations and at five different hemoglobin concentrations (Table I) can be described by Eq. 8 (17):

$$\beta = \frac{D_b}{D_t} = \frac{1}{1 + D_f/nP_t + 1/(nK_aP_t)} \quad \text{(Eq. 8)}$$

which is derived from mass balance considerations and Eq. 6 where β represents the fraction of total drug bound to hemoglobin. When D_f is small in comparison to nP_t, the second term in the denominator of the right-hand side of Eq. 8 becomes negligible and Eq. 8 can be expressed in Eq. 9 for the purposes of calculating nK_a:

$$nK_a = \frac{\beta}{(1 - \beta)P_t} \quad \text{(Eq. 9)}$$

where P_t is taken as the molar concentration of hemoglobin. Values of nK_a were calculated from Eq. 9 and are listed in Table I. The average of these values, 390.4 liters/mole, very closely agrees with the value of 389.4 liters/mole obtained from Fig. 1 using Eq. 6. When using Eq. 9 and an nK_a of 390 liters/mole, it was found that β varies from 0.449 to 0.511 for the normal range of hemoglobin for males and from 0.411 to 0.482 for females.

Competitive Binding—The competitive binding of quinidine in the presence of red blood cell hemolysate and serum was studied at two hemoglobin concentrations to determine the significance of hemoglobin variation to the fraction of quinidine bound in normal human serum (Table II). From the D_f values determined experimentally for each cell compartment, ratios of drug concentrations in serum to that in red blood cell hemolysate were calculated for each compartment and averaged, yielding values of 1.34 ± 0.329 and 1.15 ± 0.344 for the compartments containing normal and anemic hemoglobin concentrations, respectively. Expressed as a percentage, the same systems contained 57.3 and 53.5% of quinidine in the serum compartments.

Although the nK_a value for the quinidine–albumin system (1) is about 10 times larger than the nK_a determined for the quinidine–hemoglobin system, an almost equal percentage of quinidine equilibrates between each compartment because of the much larger concentration of hemoglobin than serum proteins. When both compartments were considered as a single system composed of serum separated from erythrocytic components by a semipermeable membrane, then the average percentage of total quinidine bound to proteins was calculated as 66.1 ± 0.0842 for the system containing normal serum and normal hemoglobin concentrations and 64.9 ± 0.0494 for the system containing normal serum and anemic hemoglobin concentrations.

It has been suggested (15, 17, 18) that elimination is a first-order process depending on free drug concentration. The differences in D_f in normal serum as a consequence of varying hemoglobin concentration is near 1% and, therefore, of doubtful significance to the elimination kinetics of quinidine. However, the binding of quinidine to hemoglobin as well as serum proteins would be important to the pharmacokinetics of quinidine, depending upon how rapidly an equilibrium is reestablished (protein binding as well as partitioning across red blood cells[12]) as drug is eliminated from the body. Under the conditions of a rapid readjustment of equilibrium, the instantaneous rate of drug loss would be directly proportional to the concentration of total drug in the circulating fluids and not to the concentration of free drug. As a consequence, assays currently in use (5, 19) that measure quinidine concentrations in plasma and not blood would overestimate the volume of distribution and, hence, total body clearance. However, if the process of elimination can be demonstrated as first order, either plasma or whole blood concentrations of quinidine measured as a function of time could be utilized in calculating the first-order rate constants characterizing drug transfer between the compartments. This is a result of the concentration of drug bound to hemoglobin as well as of plasma proteins remaining constant over a therapeutic range and, therefore, directly proportional to either D_f or D_t. Moreover, if elimination were a more rapid process than the binding and debinding of quinidine to hemoglobin and albumin, the elimination rate would become dependent upon the rate of binding and debinding. That is, the rates associated with the protein binding process would become rate determining to elimination, but the overall process of elimination would remain first order as long as the fraction bound was essentially constant over therapeutic drug levels (18, 20).

[12] It was estimated from a preliminary experiment that the partitioning of quinidine into red blood cells reaches an equilibrium between 2 and 6 min. Defibrinated whole blood (10 ml) was spiked with 100 μg of quinidine, and aliquots were placed into Wintrobe tubes and centrifuged for approximately 1 min. A volume of 50 μl of serum was removed and assayed. Timing was begun with the spike and ended when the serum was removed from the Wintrobe tube. Equilibration was two-thirds complete after 2 min. The experiment was performed at room temperature.

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x To whom inquiries should be directed. Present address: Alcon Laboratories, Inc., Fort Worth, TX 76101

Valence Tautomerism of Singly Protonated 9-Aminoacridine and Its Implications for Intercalative Interactions with Nucleic Acids

ANTHONY C. CAPOMACCHIA, JAY CASPER *, and STEPHEN G. SCHULMAN ˣ

Abstract □ Electronic absorption and fluorescence spectroscopy were used to show that the singly protonated 9-aminoacridine cation exists as the vinylogous cyclic amidine valence tautomer, the 9-iminoacridan monocation, in ground and excited states when in aqueous solution at physiological pH. The evidence indicates that the 9-aminoacridine monocation may intercalate into double-helical DNA in an orientation different from that employed by other acridine derivatives.

Keyphrases □ 9-Aminoacridine—valence tautomerism of singly protonated form, implications for intercalative interactions with nucleic acids, electronic absorption and fluorescence spectroscopy □ Acridine derivatives—valence tautomerism of singly protonated 9-aminoacridine, implications for intercalative interactions with nucleic acids, electronic absorption and fluorescence spectroscopy □ Nucleic acids—intercalative interactions, valence tautomerism of singly protonated 9-aminoacridine □ 9-Iminoacridan monocation—tautomer form of singly protonated 9-aminoacridine cation, intercalation with double-helical DNA

Aminoacridines are used extensively in the medical sciences as research tools and to a lesser extent clinically. 9-Aminoacridine has been used primarily as a probe to study the binding of small molecules to biologically important macromolecules. The binding of aminoacridines by intercalation into double-helical and denatured DNA and single-strand RNA has been extensively studied (1–5). Albert (6) demonstrated that the aminoacridine cation is necessary for bacteriostasis. The implication is that the singly protonated form is necessary for intercalation to occur. To date, the position and orientation of the 9-aminoacridine monocation during intercalation are undetermined. Studies have suggested that it may intercalate with the protonated heterocyclic nitrogen atom oriented toward the negatively charged phosphate ester linkages of double-helical DNA. However, it has recently been shown that 2- and 4-aminoquinolines exist as cyclic amidines rather than heterocyclic amines, having most of their positive charge at the exocyclic nitrogen atom when singly protonated (7). This implies that 9-aminoacridine, a benzolog of 4-aminoquinoline, may also exist as the cyclic amidine (I) rather than as the amino form (II) when singly protonated. Zanker and Wittwer (8) suggested the possibility of this structure as a resonance form of the monocation.

If the 9-aminoacridine monocation does exist as the cyclic amidine, its position when intercalated may differ from that of other aminoacridines due to residence of the positive charge of protonation on the exocyclic nitrogen.

The present study was undertaken to determine whether the singly protonated 9-aminoacridine cation exists as the amine or the cyclic amidine in aqueous solutions at physiological pH. Comparisons of electronic spectral shifts induced through changes in the state of protonation or solvent environment as well as dissociation constants of 9-aminoacridine and 2-aminoacridine (the latter was assumed to represent "ordinary" aminoacridines) were used to show that 9-aminoacridine behaves differently from arylamines and heterocyclic arylamines in particular.

EXPERIMENTAL

The pH measurements were made on a pH meter[1] with a combination silver–silver chloride glass electrode[2]. Electronic absorption spectra were taken in 1-cm silica cells on a grating-type spectrophotometer[2]. Fluorescence measurements were taken on a fluorescence spectrophotometer[3] whose monochromators were calibrated against the line emission spectrum of xenon. Emission spectra were corrected for the wavelength response of monochromators and the phototube by means of a corrected spectra accessory[3] employing a rhodamine-B quantum counter.

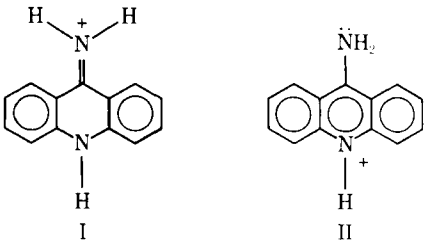
2-Aminoacridine[4], 9-aminoacridine[5], and 9-aminoacridine[6] monohydrochloride were purified by multiple recrystallizations from ethanol.

Reagent grade sulfuric acid[7] and spectroquality chloroform[8], *n*-heptane[8], and dioxane[8] were used as solvents. Benzene-free ethanol[8] and distilled, deionized water were used as necessary. Sodium hydroxide[7] USP was used to make an 18 *M* solution.

Ethanolic or aqueous solutions (about 1×10^{-3}) of the compounds to be studied were delivered from a 100-μl micropipet[9] into 10-ml volumetric flasks containing aqueous solutions at the desired pH. The added 0.1 ml caused negligible volume change. The ethanolic solutions employed were too dilute to affect pH. All solutions were prepared immediately before use to eliminate decomposition errors and errors due to pH changes of poorly buffered solutions in the mid-pH range.

RESULTS

Table I contains the long wavelength absorption and fluorescence band maxima for the prototropic species derived from 9-aminoacridine and 2-aminoacridine. The longest wavelength vibrational features of the absorption bands and the shortest wave-



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[1] Orion Research Inc., Cambridge, Mass.
[2] Beckman Instruments, Inc., Fullerton, Calif.
[3] Perkin-Elmer Corp., Norwalk, Conn.
[4] Dr. D. Jackman, Texas Technical University, Lubbock, Tex.
[5] Pfaltz and Bauer, Inc., Flushing, N.Y.
[6] Dr. F. C. Nachod, Sterling-Winthrop Research Institute, Rensselaer, N.Y.
[7] Mallinckrodt Chemical Works, St. Louis, Mo.
[8] Matheson, Coleman and Bell, Inc., East Rutherford, N.J.
[9] Cole-Parmer Instrument Co., Chicago, Ill.

Table I—Electronic Absorption (λ_a) and Fluorescence (λ_f) Maxima of the Doubly Protonated Cations (*D*), Singly Protonated Cations (M), Neutral Molecules (N), and Anions (A) Derived from 2-Aminoacridine and 9-Aminoacridine in Aqueous and Sulfuric Acid Media[a]

	D		M		N		A	
	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm
2-Aminoacridine:								
O–O band	425	—	—	—	—	—	—	—
Maximum	402	475	460	592	405	530	—	—
9-Aminoacridine:								
O–O band	440	475	423	430	426	445	443	470
Maximum	418	496	402	455	406	470	422	497

[a] The spectra of D were taken in solutions of 18 *M* H_2SO_4(H_0-10), the spectra of M were taken in water at pH 4.0, the spectra of N were taken in dilute sodium hydroxide in water (pH 11.5), and the spectra of A were taken in 14.2 *M* aqueous sodium hydroxide (H- 17.5).

length vibrational features of the fluorescence bands are taken as the O–O bands of absorption and fluorescence, respectively, for all compounds studied which demonstrated vibrational structure in their electronic spectra. The electronic absorption and fluorescence spectra of all species (anion, neutral, monocation, and dication) obtained from 9-aminoacridine (Figs. 1 and 2) exhibit well-defined vibrational features. The electronic absorption and fluorescence spectra of all species obtained from 2-aminoacridine (Figs. 3 and 4) exhibit diffuse spectral bands with the position of the O–O bands being uncertain, except the absorption spectrum of the dication which is structured.

The ground-state dissociation constants of 9-aminoacridine and 2-aminoacridine were determined by absorptiometric pH titrimetry and are presented in Table II. Also listed in Table II are the excited-state dissociation constants (pKa*) of 9-aminoacridine and 2-aminoacridine determined by fluorometric pH titrimetry. Excited-state dissociation constants were not obtained for the first protonation of 2-aminoacridine and 9-aminoacridine because the variations of the fluorescence quantum yields of the neutral species and monocations with pH followed ground-state titration characteristics. Apparently, the rates of proton exchange between these excited species are much slower than their rates of radiative deactivation.

A study of the spectra of 2-aminoacridine and 9-aminoacridine and their monocations in solvents of varying hydrogen-bonding capability and polarity produced the data in Table III. The electronic absorption and fluorescence spectra of the 9-aminoacridine monocation were barely affected by a change of solvent, showing a slight trend toward shorter wavelengths as solvent polarity was increased. In contrast, 2-aminoacridine and its monocation demonstrated trends toward substantially longer wavelengths in both their electronic absorption and fluorescence spectra as solvent polarity was increased.

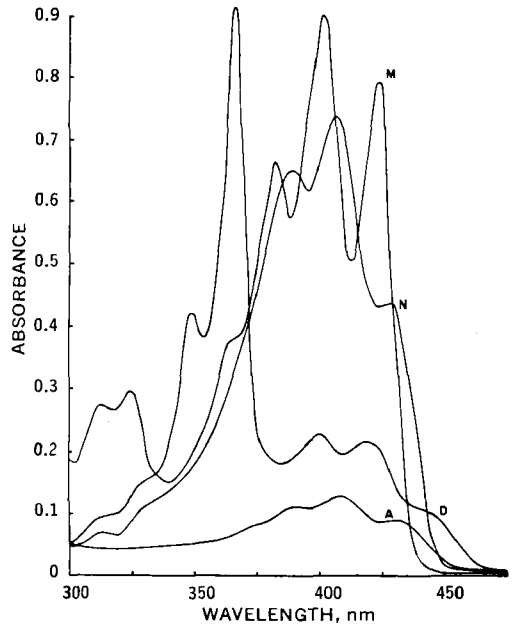


Figure 1—*Electronic absorption spectra of the dication (D), monocation (M), neutral species (N), and anion (A) derived from 9-aminoacridine at room temperature.*

DISCUSSION

2-Aminoacridine exemplifies the spectroscopic and prototropic behavior of a "well-behaved" *N*-heterocyclic arylamine (9–12). The neutral molecule has absorption and fluorescence maxima at longer wavelengths than those of acridine (λ_a = 362 nm, λ_f = 425 nm), the parent heterocyclic nucleus, while the singly protonated cation absorbs and fluoresces at wavelengths considerably longer than those of its parent heterocyclic nucleus, the acridinium cation (λ_a = 405 nm, λ_f = 481 nm). The doubly charged cation derived from 2-aminoacridine absorbs and fluoresces at wavelengths close to the corresponding spectral maxima of the acridinium cation. The pKa for the ground-state equilibrium between the neutral molecule and the singly charged cation of 2-aminoacridine is only slightly less acidic than that for the equilibrium between acridine and the acridinium cation [pKa = 5.6 (6)] while that for the equi-

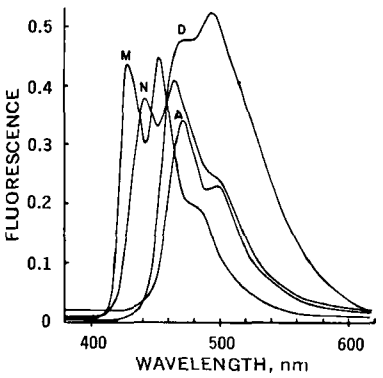


Figure 2—*Fluorescence spectra of the dication (D), monocation (M), neutral species (N), and anion (A) derived from 9-aminoacridine at room temperature.*

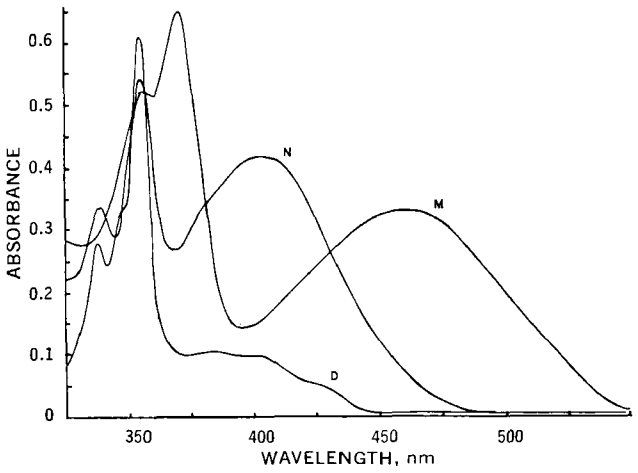


Figure 3—*Electronic absorption spectra of the dication (D), monocation (M), and neutral species (N) derived from 2-aminoacridine at room temperature.*

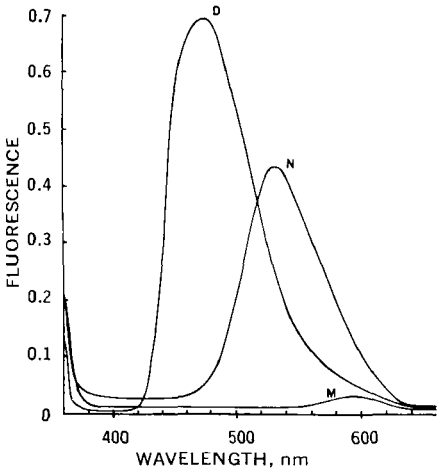


Figure 4—*Fluorescence spectra of the dication (D), monocation (M), and neutral species (N) derived from 2-aminoacridine at room temperature.*

librium between the singly and doubly charged cations is some three units more acidic than that for, say, 2-anthrylamine (12), a result of the protonated heterocyclic nitrogen atom in the acridine derivative.

The excited-state dissociation constant for the equilibrium between the singly and doubly charged cations derived from 2-aminoacridine is considerably more acidic than the corresponding ground-state dissociation constant. This, along with the previously described fluorescence behavior of 2-aminoacridine, indicates that this compound behaves as a typical *N*-heterocyclic amine in the lowest excited singlet state. The excited-state dissociation constant for the equilibrium between the singly charged cation and the neutral molecule could not be obtained by fluorometric titrimetry because the variations of intensities of the monocation and neutral molecule fluorescences with pH followed ground-state titration behavior. Apparently, the rates of fluorescence of the excited monocation and neutral molecule are too great for proton exchange with water to occur during the lifetimes of the lowest excited singlet state. However, in the pH 12–15 region, the fluorescence of the neutral molecule was quenched with increasing pH, although no change in the absorption spectrum of the neutral molecule was observed from pH 3 to 17.5. This behavior is attributed to ionization of the neutral amine in the lowest excited singlet state to form the excited iminate anion (Scheme I).

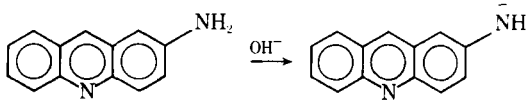
The failure of the absorption spectrum to change with increasing pH reflects that the ground-state iminate anion is too strong a base to be generated in aqueous solutions. This behavior, as well as the shift of the absorption and fluorescence spectra to longer wavelengths with increasing solvent polarity, is indicative of a much greater dipole moment of 2-aminoacridine in the lowest excited singlet state, the latter having a more charge-deficient amino group than in the ground state. This state of affairs is normal for arylamines.

The limited spectroscopic and pKa data available for the 1-, 3-, and 4-aminoacridines (13) suggest that these isomers also are "well-behaved" *N*-heterocyclic arylamines.

Table II—Ground (pKa) and Lowest Excited Singlet State (pKa*) Dissociation Constants for the Various Prototropic Equilibria of 2-Aminoacridine and 9-Aminoacridine[a]

	$D \rightleftarrows M + H^+$	$M \rightleftarrows N + H^+$	$N \rightleftarrows A + H^+$
2-Aminoacridine:			
pKa	1.1	5.9	—
pKa*	−5.8	—	12.4
9-Aminoacridine:			
pKa	−8.5	10.0	15.9
pKa*	−6.8	—	13.2

[a] The pKa values were determined by absorptiometric pH titrimetry. The pKa* values were determined by fluorometric pH titrimetry.



Scheme I

The absorption and fluorescence spectra of 9-aminoacridine and the acidity dependences of these spectra are quite different from those of 2-aminoacridine and are, in fact, similar to those of 4-aminoquinoline (7), the linear benzolog of 9-aminoacridine.

The absorption and fluorescence maxima of the monocation derived from 9-aminoacridine lie at wavelengths shorter than those of the acridinium cation as well as those of the monocation of 2-aminoacridine. This behavior is anomalous with regard to the expected spectral behavior of an amine-substituted aromatic ring(s) and suggests that the 9-aminoacridine is not an arylamine but rather the vinylog of a protonated amidine (protonated 9-iminoacridan). Similar spectroscopic behavior has been observed for protonated 9-aminoanthracene (12) relative to anthracene and for neutral acridone (8) (the oxygen analog of singly protonated 9-aminoacridine) relative to the acridinium cation and has been suggested to imply that protonated 9-aminoanthracene is a protonated imine rather than an arylammonium ion. The lactam structure of acridone is well known (6). The protonated heterocyclic nitrogen atom in 9-aminoacridine, which is more electronegative than the 10-carbon atom in 9-aminoanthracene, might be expected to encourage the "iminization" of the acridine derivative much as it does the lactonization of the 9-hydroxyacridine zwitterion to acridone.

The positive charge of the monocation thus appears to reside predominately at the exocyclic amino group. This conclusion is supported by the shifts of the absorption and fluorescence maxima of the monocation of 9-aminoacridine to shorter wavelengths with increasing solvent polarity. In well-behaved protonated heterocyclic amines, the spectra shift to longer wavelengths with increasing solvent polarity because the electronic dipole moment, which is directed from the amino group toward the highly electronegative, protonated, heterocyclic nitrogen atom, is increased in the lowest excited singlet state; the more polar solvent stabilizes the excited-state dipole relative to the ground-state dipole (10). The solvent shifts observed in the 9-aminoacridine monocation indicate that the ground state is relatively more stabilized than the excited state by increasing solvent polarity because the excited species has a lower dipole moment than the ground-state species. This is brought about by the flow of electronic charge away from the heterocyclic nitrogen atom and toward the exocyclic nitrogen atom in the excitation process and indicates that the exocyclic nitrogen atom is more electron withdrawing than the protonated heterocyclic nitrogen atom in singly protonated 9-aminoacridine.

Further evidence of the electron-withdrawing nature of the

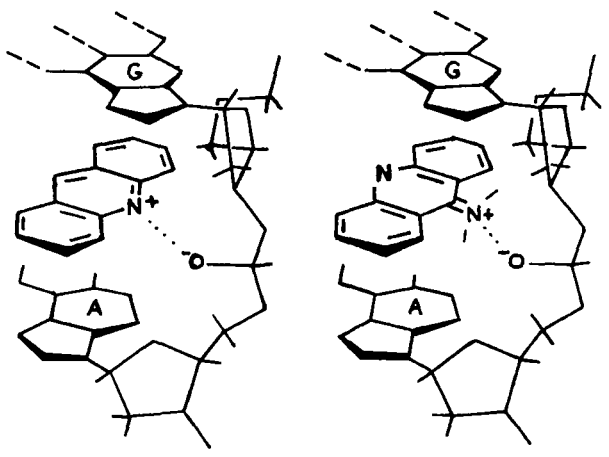


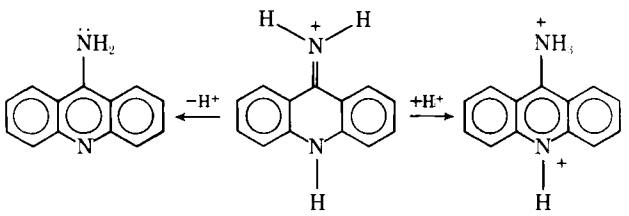
Figure 5—*Schematic illustration of:* (a) *the modified intercalation model with an unsubstituted acridinium cation intercalated between adjacent bases on a polynucleotide chain, and* (b) *the 9-aminoacridine monocation as the cyclic amidine in the same intercalation position.*

Table III—Electronic Absorption (λ_a) and Fluorescence (λ_f) Maxima of the Singly Charged Cations (M) and Neutral Molecules (N) Derived from 2-Aminoacridine, 9-Aminoacridine, and 10-Methyl-9-aminoacridinium Iodide in Solvents of Differing Polarity and Hydrogen-Bonding Capability

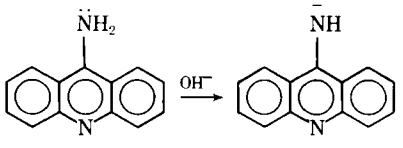
	Water		Ethanol		Dioxane		Chloroform		*n*-Heptane	
	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm	λ_a, nm	λ_f, nm
2-Aminoacridine:										
M (maximum)	460	592	470	550	475	558	460	540	480	500
N (maximum)	405	531	422	521	415	497	405	481	396	439
9-Aminoacridine:										
M (O–O band)	423	430	425	430	425	435	425	434	—	—
(maximum)	402	455	405	456	405	459	405	458	405	460
N (O–O band)	426	445	425	430	425	435	424	434	418	440
(maximum)	406	470	403	455	404	459	403	458	400	468

amino group and the electron-donating nature of the heterocyclic nitrogen atom of the 9-aminoacridine monocation is seen in the changes of the absorption and fluorescence spectra of the monocation accompanying dissociation from the heterocyclic nitrogen atom and protonation of the exocyclic nitrogen atom (Scheme II). Dissociation of the monocation, which entails loss of a proton from the heterocyclic nitrogen atom, shifts the electronic spectra to longer wavelengths, a phenomenon that is anomalous for dissociation from nitrogen atoms in six-membered heterocyclic rings (9–11). This type of spectral shift indicates that the excited state is stabilized by a loss of positive charge from the heterocyclic nitrogen atom and is commonly observed in protolytic dissociation from electron donor groups such as —OH and —NH_2 (14). This suggests that the heterocyclic nitrogen atom of the 9-aminoacridine monocation is amine-like. Moreover, the pKa for the dissociation of the monocation is about four units higher than those for the dissociation of other aminoacridines (6), an observation that is difficult to reconcile with simple substituent perturbations. Protonation of the monocation to yield the dication results in spectral shifts to longer wavelengths, an observation that is impossible to reconcile with the withdrawal of two electrons from the aromatic system by protonation of the amino group. Furthermore, ground-state protonation of the monocation occurs in media about nine orders of magnitude more acidic than for any other aminoacridine monocation while excited-state protonation of the 9-aminoacridine monocation occurs in solutions less acidic than those in which ground-state protonation occurs. These observations are reminiscent of the prototropic and spectral behavior of 2- and 4-aminoquinolines (7) and strongly support the contention that the amino group is the site of positive charge in the 9-aminoacridine monocation.

The exact nature of the neutral species derived from 9-aminoacridine is somewhat more difficult to establish than that of the monocation. Nonaqueous IR studies (15, 16) have yielded conflicting conclusions as to whether the neutral amine has imine-like character. However, at least one study in aqueous media suggests that an imino tautomer may comprise about 10% of the total neutral 9-aminoacridine population (17). In very concentrated alkali solutions, the absorption and fluorescence spectra of the neutral species shift to longer wavelengths due to, in our opinion, the dissociation of the amino group (Scheme III).



Scheme II



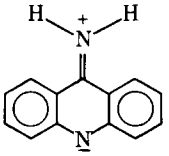
Scheme III

The shifts of the spectra to longer wavelengths upon dissociation and the fact that the fluorescence spectra shift at lower pH than the absorption spectra (*i.e.*, pKa* < pKa) are typical of arylamines. However, in ordinary arylamines the ground-state dissociation normally occurs at a pH too basic to be accessible and the shifts are normally much greater than observed here. To our knowledge, this is the first time that the dissociation of a neutral amine in the ground state has been observed in aqueous media.

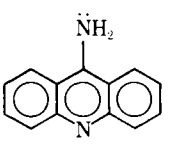
The spectra of the neutral 9-aminoacridine are amine-like in that they show a trend toward longer wavelengths with increasing solvent polarity. However, the shifts are much smaller than in other heterocyclic amines. This and the ease of ionization of the neutral amine in the ground electronic state suggest that even in the neutral molecule the amino group is somewhat charge deficient and that its actual electronic structure is intermediate between the zwitterionic structure (III) and the true amino form (IV).

The intercalation model proposed by Lerman (1, 2) depicted the ligand aminoacridine molecule, proflavin, as binding to double-helical DNA by insertion between, and centered over, adjacent base pairs. The amino groups are in the 3,6-position, each being directed toward a negatively charged phosphate ester linkage in the sugar backbone of each polynucleotide chain (3), and the long axis of the aminoacridine cation is nearly parallel to the hydrogen bonds of the nearest base pairs. The model did not explain how acridinium ions and singly protonated molecules with the 9-aminoacridine nucleus that have a bulky group attached at the 9-amino function, such as quinacrine (18), were able to intercalate with double-helical DNA. This consideration and others led to the proposal of a modified intercalation model (Fig. 5*a*), which called for the insertion of the acridine or 9-aminoacridine cation between adjacent bases on one of the polynucleotide chains comprising the double helix (4). The positive charge on the cation is closely associated with the negatively charged polynucleotide phosphate group on the same chain, such that the long axis of the cation is nearly perpendicular to the hydrogen bonds of the nearest base pairs. The position of the 9-carbon atom on acridine to which an amino group may be attached is no longer in the center of the helix, as dictated by the Lerman model, but is positioned to allow the amino group and bulky groups thereon access to the exterior of the helix. These two models apparently illustrate the two extremes that ligand molecules can assume upon intercalation into double-helical DNA.

Localization of the positive charge of protonation on exocyclic nitrogen should cause the 9-aminoacridine monocation, described by the modified intercalation model, to be "flipped over" so that positively charged exocyclic nitrogen is closely associated with the negatively charged polynucleotide phosphate group (Fig. 5*b*). When the monocation is in this position, bulky substituents on the exocyclic nitrogen, as found in quinacrine, would not seriously hamper intercalation by steric hindrance because they would have access to the exterior of the helix. However, some rotation or repositioning of the monocation about the point of ionic interaction



III



IV

could be expected due to the steric effect of bulky substituents attached at different sites on the aromatic nucleus or on the exocyclic nitrogen. The concentration of positive charge in 9-aminoacridinium cations at the exocyclic nitrogen atom also appears to be in agreement with the observation that 2,7-di-*tert*-butyl-9-aminoacridinium monocation intercalates with DNA (5), while 2,7-di-*tert*-butylproflavin monocation does not (19). In the former compound the bulky *tert*-butyl groups are directed toward the exterior of the helix where steric hindrance is minimal. In the proflavin derivative, however, the *tert*-butyl groups would be directed toward the interior of the helix if intercalation occurred, resulting in a strongly destabilizing steric interaction.

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[x] To whom inquiries should be directed.

Correlation and Prediction of Mass Transport across Membranes II: Influence of Vehicle Polarity on Flux from Solutions and Suspensions

SAMUEL H. YALKOWSKY [x] and GORDON L. FLYNN*

Abstract □ The effects of systematic alteration of vehicle composition on the release rate of drugs from their vehicle were studied. Equations were developed which quantitatively predict the rate of transport of a drug across a membrane separating two identical binary aqueous solvents. Separate equations were derived for solutions and for suspensions, which account for both the resistance of the membrane and the resistance of the solvent to drug diffusion.

Keyphrases □ Membrane diffusion—effect of vehicle polarity on flux from solutions and suspensions, systematic alteration of vehicle composition, equations □ Vehicle polarity—effect of systematic alteration of vehicle composition on release rates of drugs, equations □ Drug release rates—effects of systematic alteration of vehicle composition, solutions and suspensions, equations

One significant factor governing drug activity is the release rate of the drug from its vehicle. For nonsolid dosage forms (fluids, ointments, *etc.*), the ability of the vehicle to retain the drug can be conveniently altered by the addition of a second fluid, which may be a solvent or a nonsolvent for the drug. Of the many recent papers dealing with drug release from fluid vehicles, few have attempted to study, generalize, and quantitate the effects of incremental changes in vehicle composition systematically. The work of Poulsen (1) is a notable exception. In this regard, the authors have attempted to show the effects of vehicle composition upon solubility, the membrane vehicle partition coefficient, and, ultimately, the drug release rate.

THEORETICAL

Dependence of Flux upon Solubility and Partition Coefficient—The resistance, R_m, of a membrane to transport of a substance is proportional to the membrane's thickness, h_m, and inversely proportional to the diffusivity, D_m, of the substance in the membrane:

$$R_m = \frac{h_m}{D_m} \qquad \text{(Eq. 1)}$$

If the membrane separates two similar solvent phases, the regions of unstirred solvent (diffusion layers) adjacent to the membrane also offer resistance to the transport of a solute from the donor to the receptor phase. This solvent resistance is given by:

$$R_s = \frac{h_s}{D_s} \qquad \text{(Eq. 2)}$$

could be expected due to the steric effect of bulky substituents attached at different sites on the aromatic nucleus or on the exocyclic nitrogen. The concentration of positive charge in 9-aminoacridinium cations at the exocyclic nitrogen atom also appears to be in agreement with the observation that 2,7-di-*tert*-butyl-9-aminoacridinium monocation intercalates with DNA (5), while 2,7-di-*tert*-butylproflavin monocation does not (19). In the former compound the bulky *tert*-butyl groups are directed toward the exterior of the helix where steric hindrance is minimal. In the proflavin derivative, however, the *tert*-butyl groups would be directed toward the interior of the helix if intercalation occurred, resulting in a strongly destabilizing steric interaction.

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THEORETICAL

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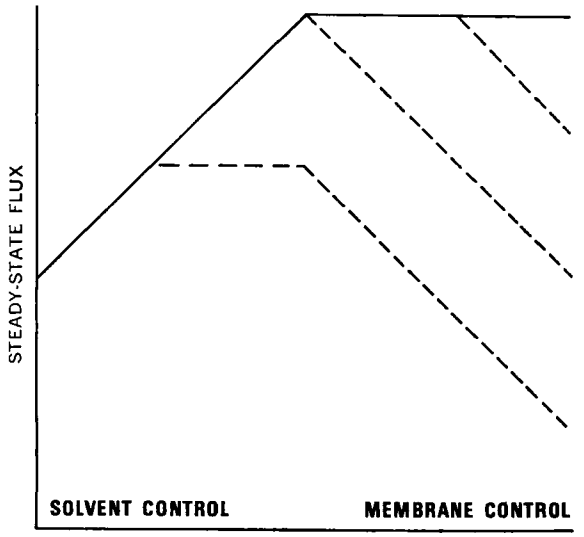

Figure 1—*Idealized relationships between solvent composition and membrane transport. Saturated solutions are indicated by a solid line; equimolar solutions of arbitrary concentrations are represented by the dashed lines. These curves are based on the assumption of a linear dependency of log (solubility) on the volume fraction of cosolvent.*

where h_s is the sum of the donor and receptor diffusion layer thicknesses, and D_s is the solute diffusivity in the solvent.

If C is the concentration gradient across the diffusion layer membrane system and PC is the membrane–solvent partition coefficient, the rate of solute transport or flux, F, across the membrane is (2–5):

$$F = \frac{C}{R_s + \frac{R_m}{PC}} \qquad \text{(Eq. 3)}$$

or, in logarithmic form:

$$\log F = \log C + \log PC - \log [R_s(PC) + R_m] \qquad \text{(Eq. 4)}$$

Generally, for a given membrane system, R_m and R_s are constants. Changes in R_s values resulting from alteration of solvent viscosity or solvation and changes in R_m resulting from alteration of the membrane by the solvent are ignored in this report. The flux is determined only by the values of C and PC. If the maximum possible flux of a particular compound across a given membrane is desired, Eqs. 3 and 4 can be modified by replacing C by S, the solubility of the compound. (This is physically accomplished by saturating the donor phase and maintaining the receptor phase at zero concentration, the perfect sink condition.) Under this condition, Eq. 3 becomes:

$$F^s = \frac{S}{R_s + (R_m/PC)} \qquad \text{(Eq. 5)}$$

and Eq. 4 becomes:

$$\log F^s = \log S + \log PC - \log [R_s(PC) + R_m] \qquad \text{(Eq. 6)}$$

Some ramifications of the chain-length dependencies of Eqs. 4 and 6, in terms of solute structure, were extensively discussed previously (5–7) and will not be repeated here. However, it can be seen that any alteration in either solubility or the partition coefficient will have a parallel effect on flux.

Effect of Binary Solvent Composition on Solubility—The solubility of a substance that is insoluble in a particular solvent can usually be increased significantly by the addition of a cosolvent in which the solute is more soluble. It has been shown (8) that for a large number of binary aqueous solvent systems, the logarithm of the solubility of various semipolar compounds is directly proportional to the volume fraction of cosolvent, f_c. That is:

$$\log S_f = \log S_w + \gamma f_c \qquad \text{(Eq. 7)}$$

where S_f and S_w are the solubilities of the solute in a system of a given cosolvent volume fraction and in water, and γ is a constant that is dependent on the polarity of the drug and the cosolvent. A review of the literature revealed that this relationship is sufficiently general so as to be useful in estimating or predicting solubilities in binary systems, provided that the polarity of the drug is significantly less than that of either solvent.

Table I—Solubility and Partition Coefficient Data for *p*-Aminoacetophenone

Percent Propylene Glycol in Water	Solubility in Binary Aqueous Solvent, mg/ml	Membrane–Solvent Partition Coefficient (P/D), mg/ml	Calculated Membrane Solubility, mg/ml
0	10.0	16.5	165
10	15.6	11.1	173
20	23.5	6.94	163
30	35.6	5.03	180
40	56.1	3.16	177
50	85.6	1.90	163
60	94.0	1.55	145
70	105	1.41	148
80	125	1.25	156
90	147	0.98	145
100	181	0.75	136
		Average	159

Effect of Binary Solvent Composition on Partitioning—The partition coefficient of a compound between a series of binary aqueous solvents and another totally immiscible nonpolar phase, such as a membrane, can be estimated by the ratio of the solubilities of the drug in the two phases. If the drug's solubility in the membrane is assumed to be independent of the composition of the binary solvent, the partition coefficient for a drug is equal to its solubility in the membrane, S_m, divided by its solubility in the binary solvent, which is described by Eq. 7; that is:

$$PC = \frac{S_m}{S_w 10^{\gamma f_c}} \qquad \text{(Eq. 8)}$$

or:

$$\log PC_f = \log PC_w - \gamma f_c \qquad \text{(Eq. 9)}$$

where PC_w is the ratio of the solubilities in the membrane and in water, S_m/S_w, or the membrane–water partition coefficient. The situation in which the solvent has an effect on membrane solubility was discussed by Poulsen (1).

Dependence of Flux on Binary Solvent Composition—From the previous sections, it can be seen that both log S and log PC are linearly dependent on f_c by Eqs. 7 and 9, respectively. If these equations are substituted into Eqs. 4 and 6, the following equations are obtained:

$$\log F^c = \log C + \log PC_w - \gamma f_c - \log [R_s PC_w 10^{-\gamma f_c} + R_m] \qquad \text{(Eq. 10)}$$

$$\log F^s = \log S_w + \log PC_w - \log [R_s PC_w 10^{-\gamma f_c} + R_m] \qquad \text{(Eq. 11)}$$

where the superscripts c and s represent equimolar concentration and saturation, respectively. Equations 10 and 11 each can be simplified under certain conditions, depending on whether the flux-determining step of the diffusion process is passage through the diffusion layers adjacent to the membrane.

Membrane Control of Flux—If the passage of the solute through the actual membrane is the rate-determining step in the diffusion process, the system is said to be under membrane control of flux (2–6). This condition is characterized mathematically by $R_m/PC \gg R_s$. The significance of this inequality can be seen when it is used to modify Eqs. 10 and 11. Under this condition, the last term of these equations becomes simply $-\log R_m$, so Eqs. 10 and 11 reduce to:

$$\log F^c = \log C + \log PC_w - \gamma f_c - \log R_m \qquad \text{(Eq. 12)}$$

and:

$$\log F^s = \log S_w + \log PC_w - \log R_m \qquad \text{(Eq. 13)}$$

respectively. Equation 12 predicts that at constant concentration

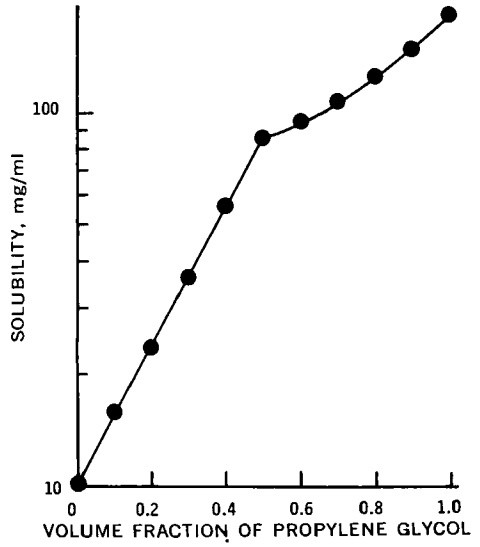

Figure 2—*Solubility of* p-*aminoacetophenone in propylene glycol–water mixtures.*

of diffusant, the flux is inversely proportional to the fraction of nonaqueous cosolvent present. On the other hand, according to Eq. 13, the flux from saturated solutions under membrane control is independent of solvent composition; that is, the effect of decreasing partition coefficient with increasing f_c is exactly offset by the resulting increase in drug solubility. These two relationships are graphically illustrated in the left-hand portion of Fig. 1.

Diffusion Layer Control of Flux—If, instead of lying within the membrane, the major barrier to transport is the stagnant solvent adjacent to the membranes, the system is said to be under diffusion layer control of flux. For this condition to exist, R_s must exceed R_m/PC and the last term of Eqs. 10 and 11 becomes $-\log[R_s PC_w\, 10^{-\gamma f_c}]$ or $-\log R_s - \log PC_w + \gamma f_c$. Using this approximation, Eq. 10 reduces to:

$$\log F^{\infty} = \log C - \log R_s \qquad \text{(Eq. 14)}$$

and Eq. 11 becomes:

$$\log F^{\infty} = \log S - \log R_s + \gamma f_c \qquad \text{(Eq. 15)}$$

Unlike the situation under membrane control, in diffusion layer control the flux at normalized concentration is independent of f_c while the flux from saturated solutions increases with increasing concentration of nonaqueous cosolvent. These relationships are illustrated in the right-hand portion of Fig. 1.

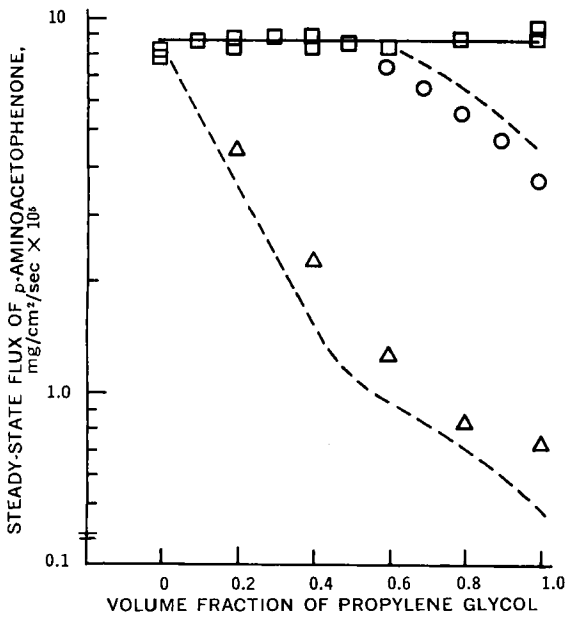

Figure 3—*Steady-state flux data for* p-*aminoacetophenone. Key:* □, *saturated solution;* ○, *equimolar solutions, 85 mg/ml;* △, *equimolar solutions, 10 mg/ml;* ——, *predicted by Eq. 6; and* - - -, *predicted by Eq. 4.*

In Fig. 1, it is possible to go from membrane to diffusion layer control or from constant concentration to saturation by simply increasing the volume fraction of water. This figure is only an attempt to illustrate the conditions described in this report. In general, a set of data ranging from 0 to 100% cosolvent will resemble either the right or left half of the figure. Whether or not the central portion, including the transition region, is observed depends upon the drug, the solvent system, and the experimental conditions.

EXPERIMENTAL

Materials—*p*-Aminoacetophenone[1] was used as received, and hexyl *p*-aminobenzoate was synthesized as described previously (4, 5). Deionized water, propylene glycol USP, and alcohol USP were used to prepare all solutions. Silicone rubber membranes were prepared as described previously (4, 5).

Procedure—All flux data were obtained on the same apparatus and by the same method described previously (4, 5). Solubilities in the pure and mixed solvents at 37° were determined as described previously (6).

RESULTS AND DISCUSSION

Solubility of *p*-Aminoacetophenone in Binary Aqueous Solvents—The solubility of *p*-aminoacetophenone in the binary solvent system of propylene glycol and water is shown in Fig. 2 as a function of the volume fraction of propylene glycol, f_{PG}. For f_{PG} up to 0.5, the solubility of *p*-aminoacetophenone (I) can be described by a specific form of Eq. 7:

$$\log S_f^{\,I} = \log (10.0) + 0.185\, f_{PG} \qquad \text{(Eq. 16)}$$

where $S_f^{\,I}$ is the solubility of the phenone in the binary solvent of volume fraction f_{PG} of propylene glycol, and 10.0 mg/ml is the drug's aqueous solubility. The complete range of solubilities described in Fig. 2 cannot be represented by such a simple equation but can be described by:

$$\log S_f^{\,I} = 1.0065 + 1.3386 \times 10^{-2} f_{PG} + 4.2419 \times 10^{-4} f_{PG}^{\,2} - 9.5312 \times 10^{-6} f_{PG}^{\,3} + 5.7986 \times 10^{-8} f_{PG}^{\,4} = 5.9172 \times 10^{-9} f_{PG}^{\,5} \qquad \text{(Eq. 17)}$$

with good correlation. The solubility data of a number of workers for many drugs in various binary aqueous solvents can be shown to fit equations such as Eq. 16 or 17. The qualification for a linear dependence of log (solubility) on the volume fraction of nonaqueous solvent component appears to be that the solubility parameters of the solvents are much higher than the solubility parameter of the drug. This point will be covered in a forthcoming publication.

Partition Coefficients and Membrane Solubility of *p*-Aminoacetophenone—If it is assumed that the membrane and solvent do not alter each other's properties, the partition coefficient of a drug between a membrane and a completely immiscible solvent can be approximated by the ratio of membrane to solvent solubilities of the drug. Conversely, if the partition coefficient is known along with the solvent solubility, it is possible to estimate the drug's solubility in the membrane. Table I shows the solubilities and membrane partition coefficients of *p*-aminoacetophenone for various propylene glycol–water mixtures. The partition coefficients were determined by dividing the previously determined permeabilities by the diffusivity of the phenone in the membrane (4). The membrane solubilities were determined by simply multiplying the partition coefficients by the binary solvent solubility for each mixture listed.

The consistency of the membrane solubility with solvent composition is noteworthy. The slight decrease in the calculated value with increasing propylene glycol composition results because the effects of viscosity on diffusivity and thus on solvent resistance are ignored. Some of these factors were discussed by Poulsen (1) for membrane control. This value is in good agreement with the experimentally determined solubility of *p*-aminoacetophenone in sili-

[1] Eastman.

cone fluid of 15 mg/ml. The utility of silicone fluid in studying silicone rubber permeability was discussed previously (5).

Calculation of Steady-State Flux under Membrane Control—At any volume fraction of propylene glycol, the steady-state flux of *p*-aminoacetophenone can be calculated from the solubility data by Eq. 10 provided that R_m and R_s are known and are nearly constant. Since these experiments were conducted with 47.6-nm membranes, in which Flynn and Smith (4) determined the diffusivity of *p*-aminoacetophenone to be 4.10×10^{-7} cm²/sec, the membrane resistance is 1.16×10^4 sec/cm.

The fluxes calculated, using these values for the resistances, the experimental donor phase concentration of drug, the silicone oil solubility, and the binary solvent solubilities given by Eq. 17 for various volume fractions of propylene glycol in water are shown in Fig. 3 along with the experimentally measured values. It can be seen that the agreement between the theoretical and experimental data is excellent. The decreasing permeability that accompanies an increase in the proportion of nonaqueous solvent parallels the reciprocal of the change in drug solubility with vehicle composition (Fig. 2).

This type of dependence of flux on solvent composition has been observed for other drugs and membranes (9–13) but has not been quantitatively correlated with physical-chemical parameters. Poulsen *et al.*'s (13) observation that the maximum release rate from propylene glycol–water mixtures containing a fixed concentration of drug occurs when a minimum amount of the glycol is used is also in agreement with this discussion.

The steady-state fluxes from saturated solutions of *p*-aminoacetophenone are indicated in Fig. 3. These values are again in excellent agreement with the theory. The compensating effects of increased solubility and decreased partition coefficient are obvious from the data and from the theory. (Note that the nonlinearity of the solubility data in Fig. 2 does not restrict the use of the model.) From a practical pharmaceutical point of view, the rate of release of a drug from a topical preparation can be expected to be proportional to the polarity of the vehicle for solutions of a given concentration. However, for suspensions of drug in the vehicle, the polarity has no effect on the release rate under membrane control.

Calculation of Steady-State Flux under Diffusion Layer Control—While the condition of diffusion layer control of flux is relatively uncommon in laboratory diffusion cells, it is frequently encountered in biological systems (3–7). To illustrate the effects of altering solvent composition when the system is under solvent control of transport, the value of R_m was minimized by reducing the membrane thickness from 0.047 mm (as was used in the previous experiments) to 0.005 mm. Further reduction in membrane thickness would produce a very fragile membrane. The value of PC was increased by choosing a solute having a low polarity. Previous studies (5) indicated that the diffusion of hexyl *p*-aminobenzoate from water across a 0.047-mm dimethylpolysiloxane membrane is under aqueous diffusion layer control. This ester was chosen because of this fact and the linear dependence of the logarithm of its solubility on propylene glycol or alcohol concentration, which was already determined as part of another study (8). Thus, it would be possible to illustrate the shift from membrane control to diffusion layer control simply by altering solvent composition.

The molar solubility expression for hexyl *p*-aminobenzoate (II) in alcohol–water solutions is[2]:

$$\log S^{II} = -4 + 4.3 f_{\text{ethanol}} \qquad \text{(Eq. 18)}$$

The theoretical fluxes were calculated by using this equation and assuming R_m and R_{aq} to be the same as the *p*-aminoacetophenone values. The relative experimental and theoretical fluxes for saturated solutions of hexyl *p*-aminobenzoate are shown in Fig. 4, with the flux from saturated purely aqueous solution being taken as unity. Here, as predicted by Eqs. 11 and 15, the flux from saturated solutions increases exponentially with increasing percentages of cosolvent present, with the slight deviation from theory being due to changes in viscosity and hydration with increasing alcohol composition. These solvent diffusion layer control data can be contrasted with the membrane control data from saturated solutions (Fig. 3). If the hexyl *p*-aminobenzoate data are normalized, the resulting data will of necessity show independence of equimolar flux on solvent composition as predicted by Eq. 14.

[2] The 37° molar solubilities of hexyl *p*-aminobenzoate in water and 10, 20, 30, and 40% alcohol are 1.07×10^{-4}, 2.7×10^{-4}, 7.3×10^{-4}, 1.9×10^{-3}, and 5.1×10^{-3}, respectively.

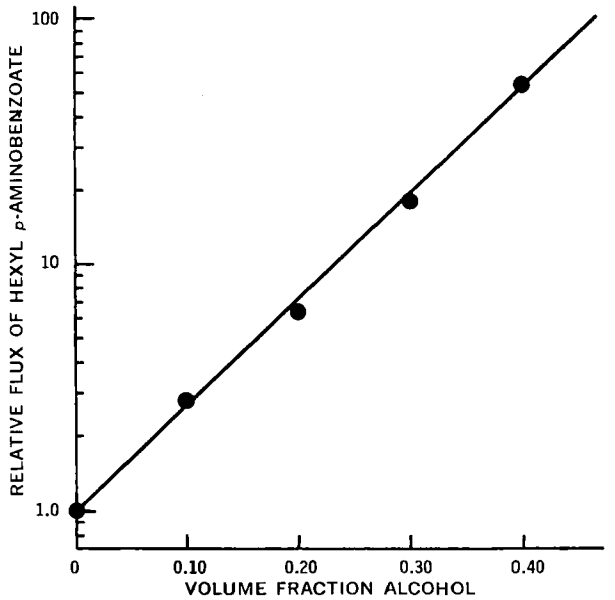

Figure 4—*Effect of cosolvent on steady-state flux from saturated solutions of hexyl* p-*aminobenzoate under solvent control. Key:* ——, *theoretical; and* ●, *experimental.*

CONCLUSIONS

The equations developed on the basis of diffusion theory and the relationship between solubility partitioning and solvent composition can describe and predict the rate of transport of a drug across a membrane separating two identical solvents as a function of solvent composition. Two distinct types of transport are described, one in which the membrane is the major transport barrier and the other in which diffusion through the unstirred solvent is rate limiting. In the former case, all saturated solutions produce equal fluxes and the flux from equimolar solutions is proportional to the polarity of the phase. Under the latter condition, the flux from equimolar solutions is independent of solvent polarity and the flux from saturated solutions is proportional to solvent polarity. These relationships have direct applicability to drug absorption from topical dosage forms where there is a great deal of flexibility in vehicle composition. The commonly observed relationships between solvent composition and permeability are those described by membrane control. These relationships, however, are not generally applicable to very nonpolar solutes whose transport can be limited by the solvent diffusion layers as described here.

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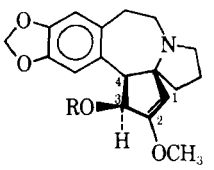
Keyphrases □ Harringtonine alkaloid analogs—partial synthesis, screened for antitumor activity □ *Cephalotaxus harringtonia* alkaloid analogs—partial synthesis, screened for antitumor activity □ Cephalotaxine esters—partial synthesis □ Antitumor agents, potential—partial synthesis of harringtonine analogs

Cephalotaxus harringtonia plant materials contain cephalotaxine (I) as well as esters of this alkaloid incorporating various dicarboxylic acids. Harringtonine (II) and certain other naturally occurring esters of cephalotaxine exhibit significant antitumor activity (1, 2). Because of its novel chemical structure and the pharmacological activity of its esters, the total synthesis of I was undertaken by at least three groups (3–5), two of which reported successful syntheses in preliminary form (3, 4). Cephalotaxine, although inactive, is by far the most abundant of the *Cephalotaxus* alkaloids; the active ester alkaloids needed for clinical testing remain in short supply.

The active alkaloids are not readily prepared by direct esterification of cephalotaxine because of marked steric hindrance at the reaction site in both the acid and alkaloid moieties. An α-hydroxy group in the acid moiety further complicates synthetic efforts. A sequence was developed for the preparation of a harringtonine analog (III) whose acid moiety (2-hydroxy-2-methylbutyric acid, V*a*) incorporates some structural features found in the active compounds. The purpose of the present study was to develop synthetic procedures by which active *Cephalotaxus* alkaloids could be prepared from cephalotaxine as well as to ascertain which structural features are essential for the antitumor activity of this group of alkaloids.



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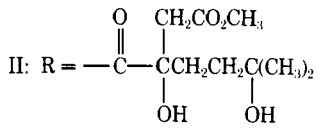

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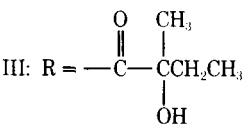

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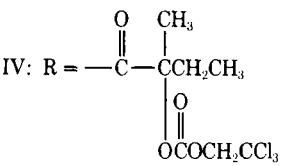

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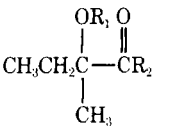
The first attempt to prepare 2-hydroxy-2-methylbutyrylcephalotaxine (III) was made with the α-hydroxy group blocked by a benzyl ether linkage. Treatment of cephalotaxine with the blocked acid chloride provided the desired ester, but the benzyl group could not be removed by hydrogenolysis. Therefore, the sequence described here was employed to solve the problem of protecting the hydroxy group. It is anticipated that this route will be useful in the preparation of other α-hydroxy esters of cephalotaxine.



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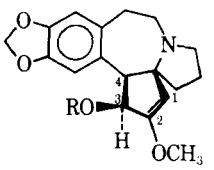

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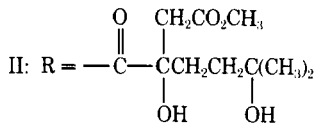

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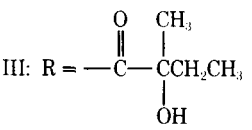

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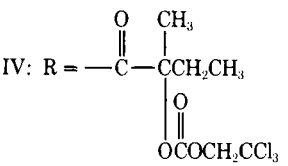

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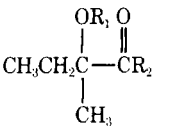

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V*d*: R_1 = —C(=O)—CH$_2$CCl$_3$, R_2 = OH

V*e*: R_1 = —C(=O)—CH$_2$CCl$_3$, R_2 = Cl

Although the benzyl ester (V*b*) and its derivative (V*c*) with the hydroxyl blocked by a trichloroethoxycarbonyl group are prepared readily, the hydrogenolysis and subsequent formation of the acid chloride (V*e*) occurred at a slower rate than expected. The trichloroethoxycarbonyl blocking group was readily removed from IV with zinc–acetic acid (6) after acylation of cephalotaxine.

The racemic form of the deoxyharringtonine dicarboxylic acid moiety (VI*a*), synthesized previously (2), was dehydrated (as its dimethyl ester) with thionyl chloride to provide two isomeric dehydration products, VII*a* and VIII*a*. These compounds were readily separated chromatographically and were distinguished by NMR spectra; the olefinic proton appeared in the spectrum of VII*a* as a singlet at δ 6.70, whereas the corresponding signal for VIII*b* was a triplet at δ 7.13. Hydrogenation of a mixture of VII*a* and VIII*a* afforded IX*a*. Diesters VII*a* and IX*a* were converted to the requisite half-esters by the partial esterification sequence developed previously (2) and were used to acylate cephalotaxine *via* the corresponding acid chlorides.

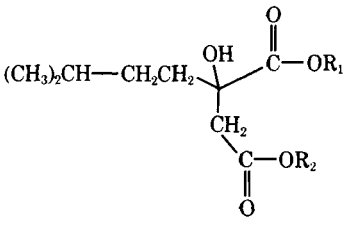
The alkaloid esters prepared were tested[1] for antitumor activity in the P-388 lymphocytic leukemia system. As shown in Table I, no activity was detected. Alkaloid ester X was prepared by partial synthesis previously[2], but test data have just become available. One test, at 20 mg/kg, indicated marginal activity, but the figure may not be reliable since tests at both higher and lower dose levels showed no activity. Alkaloid derivative III is an allylic ester. It has a hindered tertiary carboxyl group and an α-hydroxy group. Even though III incorporates these three features of the active natural cephalotaxine esters, its activity, if any, is of a much lower order of magnitude than that of the natural esters. This result exemplifies the high degree of structural specificity often required of antitumor compounds. However, some structural variation in the acyl moiety is allowed since homoharringtonine, isoharringtonine, and deoxyharringtonine, as well as harringtonine itself, all exhibit considerable antitumor activity (1, 2).

EXPERIMENTAL[3]

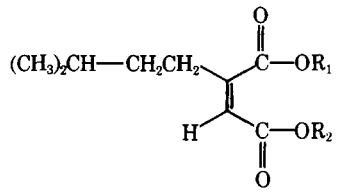
Hydroxy-Blocked Acid Chloride (V*e*)—Racemic V*a* (5.90 g), 5.00 ml of benzyl alcohol, and 0.080 g of *p*-toluenesulfonic acid catalyst were dissolved in 50 ml of benzene. Water formed during the 6-hr reflux period and was removed by azeotropic distillation with a Dean–Starke tube. The benzene solution was washed (2 × 10 ml) with 5% aqueous sodium bicarbonate and with water (3 × 10 ml) and was dried over anhydrous magnesium sulfate. Evaporation of benzene to constant weight gave V*b*. This benzyl ester (9.10 g) in 10 ml of dry chloroform was treated with 20 ml of pyridine and cooled to 0°. Then 1.10 equivalents of β,β,β-trichloroethoxycarbonyl chloride (6) in 20 ml of chloroform was added and the solution was stirred at 0° for 1 hr and then at 25° overnight. After most of the chloroform was evaporated, 100 ml of ether was added and the mixture was acidified with cold dilute hydrochloric acid. Product V*c* was recovered by ether extraction; NMR: δ 0.91 (t, J = 7.5 Hz, 3H, terminal —CH_3), 1.64 (s, 3H, branched —CH_3), 1.96 (m, at least eight lines visible, 2H, —CH_2CH_3), 4.66 (s, 2H, —CH_2CCl_3), 5.16 (s, 2H, —$CH_2C_6H_5$), and 7.33 (s, 5H, aromatic). The mass spectrum of V*c* showed no molecular ion but exhibited prominent ions at *m/e* 190 (10), 131 (15), 91 (100), 84 (78), and 63 (71).

Anal.—Calc. for $C_{15}H_{17}Cl_3O_5$: C, 47.12; H, 4.46; Cl, 27.58. Found: C, 47.52; H, 4.57; Cl, 28.11.

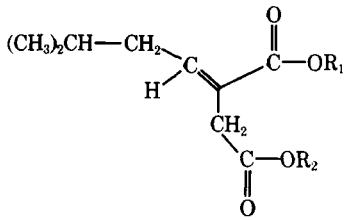
Hydrogenolysis of 5.70 g of V*c* dissolved in 15 ml of ethanol containing 0.30 g of 10% palladium-on-carbon for 4 hr at ambient conditions yielded V*d* as shown by the absence of the methylene singlet, δ 5.16, and the aromatic proton singlet, δ 7.33, from its NMR spectrum. Acid V*d* (3.85 g) was converted to its acid chloride V*e* by stirring for 65 hr at room temperature with 10 ml neat oxalyl chloride and then removing excess reagent under vacuum at room temperature. Products V*b*, V*c*, V*d*, and V*e* were recovered in essentially quantitative yields.



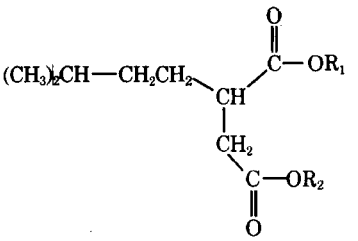
VI*a*: $R_1 = R_2 = H$
VI*b*: $R_1 = R_2 = CH_3$



VII*a*: $R_1 = R_2 = CH_3$
VII*b*: $R_1 = R_2 = H$
VII*c*: $R_1 = H$, $R_2 = CH_3$
VII*d*: R_1 = cephalotaxine moiety, $R_2 = CH_3$



VIII*a*: $R_1 = R_2 = CH_3$
VIII*b*: $R_1 = H$, $R_2 = CH_3$



IX*a*: $R_1 = R_2 = CH_3$
IX*b*: $R_1 = H$, $R_2 = CH_3$
IX*c*: R_1 = cephalotaxine moiety, $R_2 = CH_3$

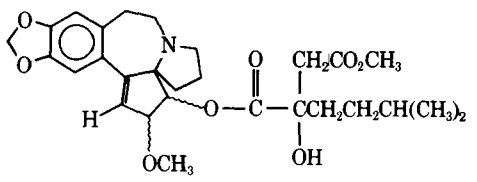
2-Hydroxy-2-methylbutyrylcephalotaxine (III)—Cephalotaxine (I), 2.60 g in 10 ml of pyridine was treated with small portions of a cold solution of 4.00 g of V*e*, also in pyridine (5 ml), and stirred at room temperature for 6 days [acid chloride–cephalotaxine (1.5:1)]. The solution was basified with 5 ml of 5% aqueous sodium carbonate solution and extracted with chloroform. Analytical TLC with methanol–chloroform (15:85) indicated the major product (IV) had R_f 0.58. Purification of IV by preparative TLC gave a yield of 40%; NMR: δ 0.62 and 0.66 (2 overlapping t[4], J = 7.5 Hz, 3H, terminal —CH_3), 1.24 and 1.27 (2s, 3H, branched —CH_3), 1.3–3.4 (overlapping m, methylene protons), 3.66 and 3.67 (2s, 3H, —OCH_3), 3.78 (d, J = 9 Hz, 1H, C-4 proton), 4.68 (s, 2H, —CH_2CCl_3), 5.04 (s, fine splitting, J < 1 Hz, 1H, vinyl), 5.84 (s, 2H,

[1] Assays were performed under Drug Research and Development auspices. Procedures are described in *Cancer Chemother. Rep.*, **25**, 1(1962).

[2] K. L. Mikolajczak, C. R. Smith, and R. G. Powell, unpublished results.

[3] NMR spectra were determined on $CDCl_3$ solutions with a Varian HA-100 instrument, and IR spectral analyses were done on 1% chloroform solutions with a Perkin-Elmer model 137 spectrometer. Low-resolution mass spectral data were determined with a Du Pont CEC 21-492-1 spectrometer, and high-resolution spectra were determined with a Nuclide 12-90 G spectrometer. Analytical TLC was done on Brinkmann silica gel F-254, 0.25-mm, precoated plates. Spots were visualized by observing the plates under UV light and by staining with iodide vapor. Preparative separations were achieved with 1-mm silica gel G plates developed with 7% methanol in chloroform. Spots were visualized with 0.5% bromthymol blue in ethanol solution.

[4] Since the α-hydroxy acid V*a* is racemic, the derived esters III and IV are mixtures of diastereomers; therefore, the chemical shifts of NMR signals associated with certain protons may be different for each isomer.



X

—OCH_2O—), 5.87 and 5.91 (2d, J = 9 Hz, fine splitting, J < 1 Hz, 1H, C-3 proton), and 6.59 (broadened s, 2H, aromatic). The mass spectrum of IV gave ions at m/e 589 (M^+, 6%), 314 (10), 298 (100), 271 (19), and 150 (17).

Anal.—Calc. for $C_{26}H_{30}Cl_3NO_8$: C, 52.98; H, 5.09; Cl, 17.82; N, 2.37. Found: C, 52.44; H, 5.21; Cl, 18.18; N, 2.24.

Ester IV (4.68 g) was stirred with an equal weight of activated zinc dust[5] in 11 ml of acetic acid for 48 hr at room temperature. This solution was diluted with 75 ml of ether and was washed with 5% aqueous sodium bicarbonate until the wash remained basic. The yield of III by preparative TLC was 60% of theoretical; IR: 3600 (—OH) and 1725 (—C=O) cm^{-1}; NMR: δ 0.40 and 0.75 ($2t^4$, J = 7.5 Hz, 3H, terminal —CH_3), 0.72 and 1.15 ($2s^4$, 3H, branched —CH_3), 1.3–3.3 (overlapping m, methylene protons), 3.68 (s, 3H, —OCH_3), 3.78 (d, J = 9 Hz, 1H, C-4 proton), 5.06 (s, 1H, vinyl), 5.85 (s, 2H, —OCH_2O—), 5.90 and 5.93 (2d, J = 9 Hz, 1H, C-3 proton), and 6.58, 6.59, and 6.62 [3s, 2H, aromatic (2s from one diastereomer and 1s from the other)]. The mass spectrum of III exhibited prominent ions at m/e 415 (M^+, 43%), 389 (13), 314 (21), 298 (100), 266 (17), and 150 (22).

Anal.—Calc. for $C_{23}H_{29}NO_6$: C, 66.50; H, 6.99; N, 3.37. Found: C, 66.01; H, 7.28; N, 3.44.

Methyl 2-Carbomethoxymethylene-5-methylhexanoate (VII*a*)—Compound VI*b* (2.40 g), prepared as described previously (2), was dissolved in 2.0 ml of pyridine at 0°; 1.90 ml of thionyl chloride was added and the mixture was heated under reflux 1 hr. Ice was added and the resulting mixture was extracted repeatedly with ether. The combined ether extracts were washed with aqueous sodium carbonate and then with water and were dried over anhydrous magnesium sulfate. Ether was evaporated to provide 2.09 g of the crude dehydration product. Analytical TLC of this mixture with hexane–ether–acetic acid (50:50:2) as the developing solvent revealed two spots; its IR spectrum showed no hydroxy absorption. The two components were separated by column chromatography; 1-g portions of the mixture were applied to a 2.4 × 30-cm column[6] and eluted successively with 100 ml of hexane, 200 ml of 5% ether in hexane, 200 ml of 10% ether in hexane, and finally 200 ml of 30% ether in hexane. The more mobile component (VII*a*) was a liquid representing 55% of the total product recovered; NMR: δ 0.91 (d, 6H, J = 6 Hz, isopropyl methyls), 1.2–1.5 (m, 1H at C-5), 1.38 (distorted t[7], 2H, J = 8 Hz, C-3 methylene protons), 2.78 (distorted t[7], 2H, J = 8 Hz, C-4 methylene protons), 3.73 and 3.77 (2s, 3H each, carbomethoxy protons), and 6.70 (s, 1H, olefinic proton). Mass spectroscopy showed: $M^+ - 15$, m/e 199.094; $C_{11}H_{18}O_4 - CH_3$ requires 199.097, and $M^+ - 31$, m/e 183.100; $C_{11}H_{18}O_4 - CH_3O$ requires 183.102.

The less mobile diester (VIII*a*), 45% of the dehydrated material resolved by column chromatography, was obtained as a liquid. Mass spectroscopy showed: $M^+ - 15$, m/e 199.094; $C_{11}H_{18}O_4 - CH_3$ requires 199.097, and $M^+ - 31$, m/e 183.100; $C_{11}H_{18}O_4 - CH_3O$ requires 183.102. Spectroscopic characterization of the corresponding half-ester (VIII*b*) is given in the next section.

2-Carbomethoxymethylene-5-methylhexanoic Acid (VII*c*)—Compound VII*a* (1.39 g) was stirred with 1 *N* sodium hydroxide in methanol–water (40:60) for 3 days at ambient temperature. The mixture was then acidified with dilute hydrochloric acid and extracted repeatedly with ether; the combined extracts were dried with anhydrous magnesium sulfate and evaporated to provide as an amorphous solid, 0.94 g of dicarboxylic acid VII*b*.

Anal.—Calc. for $C_9H_{14}O_4$: C, 58.16; H, 7.53. Found: C, 57.97; H, 7.41.

[5] Activated zinc was prepared by washing zinc dust for 10 min with 2 *N* HCl, then five times with water, and then three times with methanol before drying at 110°.

[6] Adsorbosil, Applied Science Laboratories.

[7] These two triplets, distorted by second-order splitting, are mirror images that represent a typical A_2X_2 system (7).

Table I—Test Data for Synthetic Analogs of Harringtonine in P-388 Lymphocytic Leukemia System[a]

Alkaloid	Dose, mg/kg	Survivors	Animal Weight Difference (T − C)	Survival Time, Days (T/C)	T/C, %
III	135	4/4	−1.7	13.5/10.0	135
	40	6/6	1.6	10.5/10.0	105
	10	6/6	0.5	9.0/10.0	90
	2.5	6/6	1.8	9.5/10.0	95
VII*d*	65	4/4	−1.7	12.5/11.0	113
	45	4/4	−0.3	13.0/11.0	118
	20	4/4	−0.7	10.5/11.0	95
	5	4/4	−2.2	11.5/11.0	104
IX*c*	40	6/6	0.1	11.5/11.0	104
	20	6/6	−1.8	13.0/11.0	118
	5	6/6	0.2	11.5/11.0	104
X	29	4/4	−0.3	11.5/10.0	115
	20	4/4	−1.5	14.0/11.0	127
	10	4/4	−0.2	11.0/11.0	100
	5	4/4	−0.3	11.0/11.0	100

[a] Materials were considered active if the survival time of animals treated (T) with them was ≥ 125% of that of the controls (C) (*i.e.*, T/C ≥ 125%).

This diacid was converted to the requisite half-ester (VII*c*), a viscous liquid, by treatment with dioxane–methanol–sulfuric acid, essentially as described previously for 3-carboxy-3-hydroxy-6-methylheptanoic acid (2).

Ester VIII*a* was similarly converted to the corresponding half-ester, 2-carbomethoxymethyl-5-methyl-2-hexenoic acid (VIII*b*), a viscous liquid; NMR: δ 0.92 (d, 6H, J = 6 Hz, isopropyl methyls), 1.2–2.0 (m, 1H, methine proton at C-5), 2.10 (overlapping dd, 2H, J = 7 Hz for each, C-4 methylene protons), 3.34 (s, 2H, methylene in side chain), 3.66 (s, 3H, carbomethoxy protons), and 7.13 (t, 1H, J = 7 Hz, olefinic proton at C-3).

2-Carbomethoxymethyl-5-methylhexanoic Acid (IX*b*)—The dehydration product from VI*a* was converted to a mixture of half-esters *via* the hydrolysis–esterification sequence described for VII*a*. A 1.20-g portion of the resulting product (VII*c* + VIII*b*) was hydrogenated in methanol with 10% palladium-on-carbon catalyst (ambient temperature, pressure). After the catalyst was removed by filtration, solvent was evaporated to afford the saturated half-ester IX*b*, a liquid; NMR: δ 0.86 (d, 6H, J = 6 Hz, isopropyl methyls), 1.0–1.8 (m, 1H, methine proton at C-5), 1.28 (td, 2H, $J_{3,4}$ = 6 Hz, $J_{4,5}$ = 0.5 Hz, C-4 methylene protons), 1.53 (dt, 2H, $J_{2,3}$ = 12 Hz, $J_{3,4}$ = 6 Hz, C-3 methylene protons), 2.2–3.0 (m, 1H, methine proton at C-2), 2.63 (dd, 2H, J = 13 Hz, side-chain methylene), and 3.63 (s, 3H, carbomethoxy protons). The mass spectrum of IX*a* exhibited ions at m/e 185 (29), 146 (60), 143 (42), and 114 (100). Found: $M^+ - 31$, m/e 185.116; $C_{11}H_{20}O_4 - OCH_3$ requires 185.118.

2 - Carbomethoxymethylene - 5 - methylhexanoylcephalotaxine (VII*d*)—Half-ester VII*c* (0.37 g) was treated for 2 hr with 2 ml of oxalyl chloride containing 1 drop of pyridine. After evaporation of solvents *in vacuo*, the acid chloride was stirred at room temperature with 0.30 g of cephalotaxine in 2 ml of pyridine for 72 hr. The resulting ester (VII*d*) was purified by preparative TLC yielding 57%; NMR: δ 0.84 and 0.87 (2d, 6H total, J = 6 Hz, isopropyl methyls), 1.1–3.2 (overlapping m), 3.67 (s, 6H, vinyl methoxy and carbomethoxy), 3.78 (d, 1H, J = 10 Hz, proton on C-4), 5.05 (s, 1H, vinyl of cephalotaxine moiety), 5.80 (s, 2H, methylenedioxy), 5.88 (d, 1H, J = 10 Hz, proton on C-3), 6.22 (s, 1H, vinyl of acyl moiety), and 6.51 and 6.53 (2s, 1H each, aromatic). Mass spectral analysis of VII*d* gave ions at m/e 497 (M^+, 34), 466 (11), 314 (48), 298 (100), 282 (8), 266 (18), 228 (4), 214 (6), 150 (16), and 137 (10).

Anal—Calc. for $C_{28}H_{35}NO_7$: C, 67.61; H, 7.04; N, 2.81. Found: C, 67.37; H, 7.11; N, 2.73.

2-Carbomethoxymethyl - 5 - methylhexanoylcephalotaxine (IX*c*)—Half-ester IX*b* (1.00 g) was treated with oxalyl chloride for 1 hr at room temperature, and excess reagent was evaporated *in vacuo*. The resulting acid chloride (1.27 g) in 3 ml of pyridine was added to 0.875 g of I in 10 ml of pyridine. The solution was heated at 50° for 3 hr and then stirred at room temperature for 3 days. The yield of IX*c* from preparative TLC was 81%; NMR: δ 0.78 and 0.83 (2d, 6H total, J = 6 Hz, isopropyl methyls), 1.1–3.2 (overlap-

ping m, methylenes and methines), 3.59 (s, 3H, vinyl methoxy), 3.64 and 3.66 (2s, 3H total, carbomethoxy), 3.72 (d, 1H, J = about 10 Hz, benzylic proton on C-4), 5.00 (s, 1H, vinyl), 5.82 (s and an indistinct overlapping signal, 2H plus 1H, methylenedioxy and proton on C-3), and 6.64 and 6.67 (2s, 1H each, aromatic). Its mass spectrum showed significant ions at m/e 499 (M^+, 40), 469 (12), 314 (19), 298 (100), 266 (17), 185 (36), 173 (29), 146 (71), and 114 (78); high-resolution M^+: calc. for $C_{28}H_{37}NO_7$, 499.257; found, 499.257.

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GLC Determination of Theophylline in Biological Fluids

VINOD P. SHAH and S. RIEGELMAN[x]

Abstract □ A specific GLC procedure for the determination of theophylline in plasma and saliva is described. The method selectively measures theophylline in the presence of its metabolites and other xanthines. The GLC method is sufficiently sensitive to detect 1 μg theophylline/ml plasma or saliva. Analysis for theophylline in plasma and saliva samples obtained from patients showed a saliva–plasma ratio of approximately 0.5.

Keyphrases □ Theophylline—GLC determination in biological fluids in presence of metabolites and other xanthines □ Xanthine derivatives—GLC determination of theophylline in biological fluids in presence of metabolites and other xanthines □ GLC—determination, theophylline in biological fluids

A specific and sensitive analytical method for the quantitative determination of a drug in blood and urine is needed to follow the kinetics of the substance in the body and to control the method of administration to secure optimal effects. Theophylline (1,3-dimethylxanthine) is a widely used xanthine for its antiasthmatic and cardiovascular actions. The method of Truitt *et al.* (1) for the determination of theophylline was based upon spectrophotometric measurement of the azo dye developed by coupling it with Fast Blue 2B. The method of Plummer (2) involved converting theophylline into a copper complex, mixing with potassium iodide solution, and titrating the liberated iodine with thiosulfate. Both these methods are tedious and require large quantities of blood. A UV spectrophotometric method (3–5) is more frequently used for the determination of theophylline in plasma. The sensitivity of this method is good but is subject to interference from other xanthines present in plasma, namely caffeine, theobromine, and theophylline metabolites. In addition, endogenous compounds such as xanthine, hypoxanthine, and uric acid contribute to the absorbance blank. Barbiturates, if present, also interfere in the absorption measurements of theophylline. The presence of these compounds results in an overestimation of the theophylline concentration in the sample. Gupta and Lundberg (6) described a differential spectrophotometric method for determination of theophylline in the presence of barbiturates.

A fast, sensitive, and selective GLC method for the determination of theophylline in plasma and saliva is reported here. It can be used in the presence of theophylline metabolites, caffeine, theobromine, or any barbiturates. If needed, the modified plasma extraction procedure can be used for spectrophotometric analysis.

EXPERIMENTAL

Materials—Analytical grade solvents and chemicals were used without further purification.

Apparatus—A chromatograph[1] equipped with a flame-ionization detector and a 1.83-m × 0.31-cm (6-ft × 0.125-in.) glass column packed with 3% OV-17 on Gas Chrom Q was used. The following conditions of the chromatograph were satisfactory for the separation of theophylline and its metabolites and the internal standard from the extracted components of plasma: injector port temperature, 265°; column temperature, 190°; detector block temperature, 280°; and nitrogen flow, 35 ml/min. Hydrogen and air flow were optimized for maximum response. The retention times

[1] Varian Aerograph model 1200.

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EXPERIMENTAL

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[1] Varian Aerograph model 1200.

Table I—Spectral Properties of Xanthines and Uric Acids[a] and Their Concentration after Theophylline Administration

	UV Absorption, 10 μg/ml			Normal Serum Concentration, μg/ml	Serum Level with Theophylline Therapy, μg/ml
	λ_{max}	A_{280}	ϵ_{280}		
Theophylline	274	0.42	7,600	—	5–20
3-Methylxanthine	271	0.48	8,000	—	3–4
1-Methyluric acid	284	0.55	10,100	—	—
1,3-Dimethyluric acid	285	0.48	9,400	—	—
Xanthine	266	0.37	5,600	1	6
Hypoxanthine	249	0.10	1,400	1.4	7–8.7
Uric acid	284	0.66	11,100	38–70	70–81

[a] Reference 8.

for theophylline and thiobarbital (internal standard) under these conditions were 3.6 and 3.0 min, respectively (Fig. 1).

A recording spectrophotometer[2] was used for UV absorption measurements.

Procedure—A 1-ml sample of plasma or saliva was pipeted into a nitric acid-washed test tube and extracted with 10 ml of organic solvent mixture [ether–dichloromethane–isopropanol (6:4:1)]. The tube was centrifuged for 10 min at 2500 rpm and cooled for 30 sec in a dry ice–acetone bath. The upper organic layer was decanted into another tube containing 2 ml of 1 *N* sodium hydroxide. The contents were mixed well, centrifuged, and cooled, and the upper organic layer was discarded. For UV determination of theophylline, the alkaline solution was used to scan the absorption from 310 to 260 nm. The alkaline solution was acidified with 0.4 ml of 8 *M* phosphoric acid (pH ~5), mixed with 1 ml of saturated solution of sodium sulfate, and extracted twice with 10 ml of the organic solvent mixture (by repeating mixing, centrifuging, and cooling). The internal standard, 30 μg of thiobarbital (or 5 μg of fluoranthene; see *Results and Discussion*), was added and the extract was evaporated to dryness under nitrogen at 40°. The residue was dissolved in 25 μl of tetrapropylammonium hydroxide. Duplicate 3-μl aliquots of the resulting solution were injected in the chromatograph, and the average peak height ratio of theophylline to the internal standard was calculated. The concentration of theophylline in the sample was then calculated from the standard curve.

Standard Calibration Curves—Normal plasma was spiked with known amounts of theophylline (0, 1, 2, 5, 10, 20, and 30 μg/ml) and extracted as described in the procedure for UV determination. To define the standard curve for UV measurements, the difference in absorbance at 275 and 310 nm was plotted against the respective concentration of theophylline. The spiked plasma samples were processed for GLC determination. To define the standard curve for GLC measurements, the average ratio of the height of the theophylline peak to the height of the internal standard peak was plotted against the theophylline concentration (Fig. 2). These standard curves represent an average of at least six samples at each of six data points. The average extraction efficiency in the organic solvent system was 85 ± 2% at each concentration.

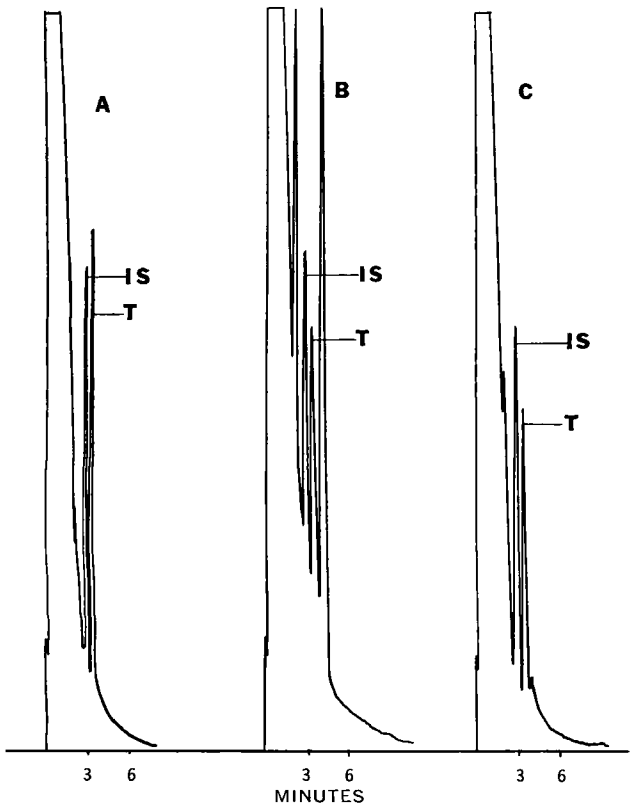

Figure 1—*GLC curves for theophylline (T) and thiobarbital (IS) in unextracted (A), extracted plasma (B), and extracted saliva (C) samples.*

RESULTS AND DISCUSSION

Theophylline is metabolized *in vivo* to 3-methylxanthine (13%), 1,3-dimethyluric acid (35%), and 1-methyluric acid (19%) (7). Administration of theophylline apparently results in increased plasma levels of xanthine, hypoxanthine, and uric acid (Table I)[3]. The UV absorption curves for theophylline and other xanthines are almost superimposable. Uric acids and hypoxanthine have significant absorption at 280 nm (Table I). Therefore, extraction of any of the xanthines, uric acids, or barbiturates with theophylline will

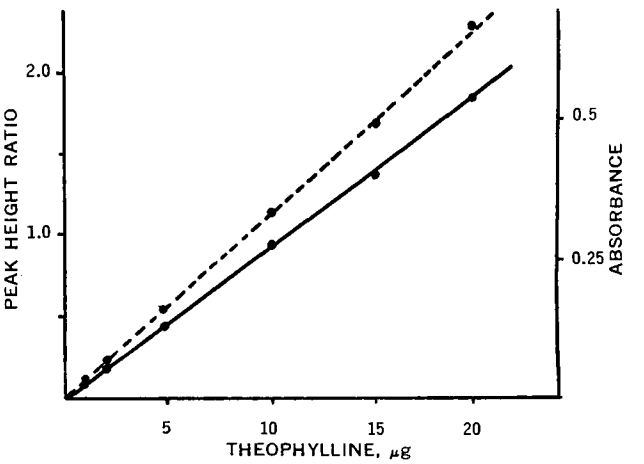

Figure 2—*GLC and UV standard curves for theophylline in unextracted (- - -) and extracted (—) plasma samples.*

[2] Cary model 15.

[3] Dr. Miles Weinberger, National Jewish Hospital and Research Center, Denver, Colo., personal communication.

Table II—Extraction Efficiency for Theophylline and Its Metabolites

Compound	Percentage Extracted in 10 ml of: Ether–Dichloromethane–Isopropanol (6:4:1)	Percentage Extracted in 10 ml of: Chloroform–Isopropanol (19:1)
Theophylline (1–20 μg/ml)	85[a]	85[a]
3-Methylxanthine (2.5–5 μg/ml)	40	40
1,3-Dimethyluric acid (2.5–5 μg/ml)	6	10
1-Methyluric acid (2.5–5 μg/ml)	10	33

[a] Range: 83–87%.

result in overestimation of the theophylline concentration by UV spectrophotometric determination.

The most commonly used assay procedure involves extraction of plasma with a chloroform–isopropanol mixture (19:1), reextraction of theophylline in base, and UV analysis of the alkaline solution (3). When the plasma is extracted with this solvent system, a thick gel forms and makes quantitative transfer of the underlying organic layer difficult. However, a solvent system consisting of ether–dichloromethane–isopropanol (6:4:1) is less dense than plasma and also does not form a gel. So, after extraction and centrifugation, the lower aqueous plasma layer can be frozen in a dry ice–acetone bath and the upper organic layer can be easily decanted. Both solvent systems are equally efficient in extracting theophylline (85 ± 2%) (Table II). However, other interfering components (as already discussed) will also be extracted from the plasma and will result in overestimation of theophylline. In the UV spectrophotometric method, one generally subtracts a constant blank value. In reality, this blank may vary, depending on the endogenous substances present in the plasma and on the length of time theophylline has been administered to the patient. Therefore, a selective and sensitive GLC method was developed for the determination of theophylline.

Theophylline, when converted by tetrapropylammonium hydroxide within the injector port to its propyl derivative, results in a symmetrical peak with a retention time of 3.6 min, distinct from any other xanthines, theophylline metabolites, and barbiturates (Fig. 1). The peak height is proportional to the amount of theophylline. The standard curve is linear in the range studied. The standard curves for extracted and unextracted plasma were determined to define the extraction efficiency (Fig. 2). The GLC method is sufficiently sensitive to detect 1 μg theophylline/ml plasma or saliva[4]. The metabolites of theophylline, namely 3-methylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid, when injected in tetrapropylammonium hydroxide are converted to their propyl derivatives, with retention times of 4.5, 5.5, and 6.1 min, respectively.

Theobromine, when injected in tetrapropylammonium hydroxide solution, is converted into its propyl derivative, with a retention time of 3.3 min. However, when caffeine is injected in aqueous tetrapropylammonium hydroxide solution, it is completely destroyed under the temperature and aqueous alkaline condition in the injection port. If injected in nonaqueous solvent, it is stable and has a retention time of 3.2 min. Barbiturates, if present, have a different retention time from theophylline. Thus, the selective and sensitive GLC method developed eliminates the potential sources of error in the detection of theophylline.

Thiobarbital used as an internal standard has one disadvantage. It is unstable to base and cannot be stored for more than 30 min at ambient temperature. Fluoranthene (5 μg) proved to be a satisfactory alternative internal standard[5]. When injected in the GLC with theophylline, fluoranthene has a retention time of 4.8 min.

[4] Use of an alkali flame-ionization detector using the same column conditions as described for the flame-ionization detector with hydrogen and air flow of 36 and 225 ml/min, respectively, allows one to detect theophylline levels as low as 0.1 μg/ml of plasma or saliva.

[5] Thiobarbital remains the best internal standard for the alkali flame-ionization detector.

Table III—Comparison of Plasma and Saliva Concentrations of Theophylline by UV and GLC Methods

Subject	Sample Source	Micrograms per Milliliter: UV	Micrograms per Milliliter: GLC	Saliva–Plasma Ratio, GLC Method
GC	Plasma	18.2	16.5	
PA	Plasma	5	5	
TA	Plasma	1.5	1.5	
AS	Plasma	17.0	8.2	
MK	Saliva	6.6	6.0	0.500
	Plasma	15.0	12.0	
NA	Saliva	12.0	6.25	0.521
	Plasma	27.0	12.0	
NC	Saliva	5.2	2.10	0.442
	Plasma	6.6	4.75	
JE	Saliva	11.0	10.0	0.513
	Plasma	27.0	19.5	

Plasma and saliva samples obtained from patients being treated with theophylline were analyzed by both UV spectrophotometric and GLC methods (Table III). In some instances, the UV method indicated a significantly higher theophylline concentration than did the GLC method. This may be due to interference from xanthines other than theophylline or from theophylline metabolites. All kinetic studies of theophylline reported in the literature apparently involve the drug determination by the UV method. Because of the potential error involved in the determination of theophylline concentration by the UV method, the kinetic parameters determined may be incorrect.

The saliva–plasma ratio of theophylline found in the four patients studied was about 0.5, thus suggesting that salivary measurements may be used to estimate plasma concentrations of theophylline. The salivary levels reflect the plasma water concentration and are, therefore, directly proportional to blood (or plasma) levels. Routine monitoring of theophylline levels in saliva rather than in plasma would preclude the inconvenience of venipuncture and be particularly suitable for use in children. A detailed kinetic study using the GLC method for plasma and saliva measurements is being conducted. Attempts will also be made to quantitate theophylline metabolites.

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[x] To whom inquiries should be directed.

Chemical Constituents of Gentianaceae X: Xanthone-*O*-glucosides of *Swertia purpurascens* Wall.

S. GHOSAL ˣ, P. V. SHARMA, and R. K. CHAUDHURI

Abstract □ Three tetraoxygenated xanthone-*O*-glucosides—*viz.*, 3,5,8-trihydroxyxanthone-1-*O*-glucoside (norswertianolin, I), 5,8-dihydroxy-3-methoxyxanthone-1-*O*-glucoside (swertianolin, II), and 1,5-dihydroxy-3-methoxyxanthone-8-*O*-glucoside (III), were isolated from *Swertia purpurascens* Wall. (Gentianaceae). The identity of the compounds was established by chemical transformation and by spectral (UV, IR, NMR, and mass) evidence. Among these compounds, III, named isoswertianolin, was not encountered before in nature or prepared synthetically. Swertianolin (II) was previously isolated from *S. japonica* and *S. tosaensis*, but its structure was only partially defined until this investigation. Selected pharmacological screening of the total xanthone-*O*-glucosides of *S. purpurascens* showed noteworthy CNS depressant, cardiovascular stimulant, and anticonvulsant activities. The pharmacological profile of activities of these compounds indicated that the curative properties ascribed to the plant extracts, in the Indian system of medicine, are essentially due to the xanthone-*O*-glucosides. Biochemical significance of the natural occurrence of xanthone-*O*-glycosides and their therapeutic potentiality were also appraised.

Keyphrases □ *Swertia purpurascens* Wall. (Gentianaceae)—isolation, chemical characterization of three tetraoxygenated xanthone-*O*-glucosides □ Xanthone-*O*-glucosides—isolation from *S. purpurascens* □ Norswertianolin—isolation, identification from *S. purpurascens* □ Swertianolin—isolation, characterization from *S. purpurascens* □ Isoswertianolin—isolation, characterization from *S. purpurascens* □ Gentianaceae—occurrence of xanthone-*O*-glucosides, phylogenetic significance

As part of a search for xanthonic constituents of the various genera of Gentianaceae, *Swertia purpurascens* Wall., collected in flowers from Jogindernagar, Himachal Pradesh, India, was examined. *S. purpurascens* is widely distributed in India in the temperate North Western Himalayas, 1524–3657 m (5000—12,000 ft) from Kashmir to Kumaon, and is used in the Indian system of medicine as a substitute for *S. chirata* Buch.-Ham. (1). Previous phytochemical investigations of *S. purpurascens* were limited to the isolation of oleanolic acid, swertiamarin, and swertisin (2). Several sister species are well known for liberally producing tetraoxygenated xanthones (3, 4). Naturally occurring xanthones were recently shown to produce a number of significant biological activities (4, 5). Mangiferin (1,3,6,7-tetrahydroxyxanthone-C_2-β-D-glucoside), the major xanthone of *Canscora decussata* (Gentianaceae) (6–8) and *S. chirata* (4), showed noteworthy monoamine oxidase inhibitor, cardiovascular stimulant, anticonvulsant, and choleretic activities (4, 5, 9) consistent with the uses of these plant extracts in the Indian system of medicine.

Norswertianolin, isolated from *S. randaiensis*, was reported to produce significant tuberculostatic activity (10). This activity was first reported for 1,3,8-trihydroxyxanthone, a degradation product of sterigmatocystin (11). Interestingly, some medicinal plants used in India as antitubercular vegetable drugs (1, 12) were subsequently found to contain polyoxygenated xanthones as their major chemical constituents (4, 6–8). Synthetic 1,3- and 1,6-dihydroxyxanthones, closely related to the xanthones of *Mammea americana* (Guttifereae), exhibited some degree of growth-inhibiting activity against Sarcoma 180 tumor cells (13). It was, therefore, thought worthwhile to investigate the xanthonic constituents of the title species and to determine the pharmacological profile of their activities. The present paper describes the details of isolation, characterization, and selected pharmacological screening of the xanthone-*O*-glucosides of *S. purpurascens*. Additionally, the biochemical significance and potential importance of the natural xanthone-*O*-glycosides as antipsychotic agents are appraised.

EXPERIMENTAL[1]

Extraction of *S. purpurascens*.—The general procedure followed for the isolation of the xanthone-*O*-glucosides of *S. purpurascens*[2] is shown in Scheme I.

Norswertianolin (I).—The solid, marked Fraction B, crystallized from about 200 ml of methanol–dioxane (1:1) as cream-colored microcrystals, mp 263–266° [lit. (10) mp 263–265°]; $[\alpha]_D^{25}$ −111° (c 0.28, 60% aqueous ethanol); UV: λ_{max} 250 (log ϵ 4.38), 275–280 (4.15), and 325 (3.84) nm; λ_{max} (C_2H_5OH–0.2% NaO-$COCH_3$): 250, 275, and 360 nm; NMR (dimethyl sulfoxide-d_6): δ 7.28 (1H, d, J = 9 Hz, C_6-H), 6.65 (1H, d, J = 9 Hz, C_7-H), 6.58 (1H, d, J = 3 Hz, C_4-H), 6.44 (1H, d, J = 3 Hz, C_2-H), 4.92 (1H, m, C_1-H of β-glucoside), and 3.50 (six glucosyl protons plus water).

Anal.—Calc. for $C_{19}H_{18}O_{11}$·H_2O: C, 51.81; H, 4.09. Found: C, 51.47; H, 4.44.

Norswertianolin Heptaacetate—Norswertianolin (24 mg), on treatment with acetic anhydride (1 ml) and pyridine (2 drops), gave the heptaacetate which crystallized from hexane–methylene chloride as colorless needles, mp 222–224°.

Hydrolysis of Norswertianolin—Norswertianolin (48 mg) was hydrolyzed with sulfuric acid (3%, 10 ml) for 2 hr on a steam bath. Extraction with ethyl acetate, evaporation of the solvent, and crystallization of the residue from methanol gave 1,3,5,8-tetrahydroxyxanthone, which had a melting point and mixed melting point of 295–298° (4). The presence of glucose in the aqueous hydrolysate

[1] Melting points were determined on a Toshniwal apparatus in open capillaries and are uncorrected. UV spectra were determined with 95% aldehyde-free ethanol, unless stated otherwise, on a Cary 14 or Spektromom 203 spectrophotometer. IR spectra were determined on a Perkin-Elmer 237 instrument in KBr pellets. NMR spectra were run on a Varian A-60 D or XL-100 spectrometer in $CDCl_3$ or dimethyl sulfoxide-d_6 using tetramethylsilane as an internal standard. Mass spectra were recorded with an AEI MS9 double-focusing spectrometer with an ionizing potential of 70 ev. Silica gel G (E. Merck) was used for analytical and preparative TLC (plate thickness 0.25 and 2 mm); iodine vapor was used for staining.

[2] The plant material was collected by Mr. M. R. Uniyal, Survey of Indian Medicinal Plants Unit, Central Council of Research in Indian Medicine and Homeopathy, Jogindernagar, Himachal Pradesh. A voucher specimen is available in the Department of Pharmaceutics, Banaras Hindu University, Varanasi-5, India.

was detected by partition paper chromatography[3] using *n*-butanol–acetic acid–water (4:1:5) as the developer, R_f 0.20.

Permethylation of Norswertianolin—Norswertianolin (56 mg) was methylated with dimethyl sulfate (2 ml) and potassium carbonate (1 g) in anhydrous acetone (50 ml) for 46 hr on a steam bath. The heptamethyl ether crystallized from methanol as yellow needles, mp 188–190°.

Hydrolysis of Norswertianolin Heptamethyl Ether—The heptamethyl ether (42 mg) was hydrolyzed with dilute sulfuric acid (3%, 10 ml) in the usual way. Extraction of the aglucone with ether, evaporation of the solvent, and crystallization of the residue from benzene gave 1-hydroxy-3,5,8-trimethoxyxanthone, which had a melting point and mixed melting point of 204–205°.

Swertianolin (II)—The first crop from Fraction C was obtained as pale-yellow needles (marked Fraction D). It showed one major spot and one minor spot on TLC. Repeated crystallization from methanol–dioxane gave the major product as a homogeneous entity, mp 204–205° [lit. (14, 15) mp 208 and 226°, respectively]; $[\alpha]_D^{25}$ −115° (c 0.31, 60% aqueous ethanol) [lit. (15) $[\alpha]_D^{18}$ −112.27°]; UV: λ_{max} 252 (log ϵ 4.40), 275 (4.22), and 325 (3.95) nm; NMR (dimethyl sulfoxide-d_6): δ 12.95 (1H, br s, C_8-OH), 7.30 (1H, d, J = 9 Hz, C_6-H), 6.68 (1H, d, J = 9 Hz, C_7-H), 6.60 (1H, d, J = 3 Hz, C_4-H), 6.52 (1H, d, J = 3 Hz, C_2-H), 5.0 (1H, m, C_1-H of β-glucoside), and 3.50 (six glucosyl protons plus water).

Anal.—Calc. for $C_{20}H_{20}O_{11}\cdot H_2O$: C, 52.86; H, 4.40. Found: C, 52.42; H, 4.76.

Swertianolin Hexaacetate—Swertianolin (28 mg) was acetylated with acetic anhydride (1 ml) and pyridine (2 drops) on a steam bath for 2 hr. The hexaacetate crystallized from hexane–methylene chloride as colorless needles, mp 226–228° [lit. (14) mp 226°].

Hydrolysis of Swertianolin—Swertianolin (42 mg) was hydrolyzed with dilute sulfuric acid (3%, 10 ml) in the usual way. Extraction with ethyl acetate, evaporation of the solvent, and crystallization of the residue from methanol afforded 1,5,8-trihydroxy-3-methoxyxanthone (bellidifolin), as shown by the melting point and mixed melting point of 270–271° and co-TLC (4). Presence of glucose in the hydrolysate was established by partition paper chromatography. The hydrolysate was evaporated *in vacuo* to a syrup, which was subjected to partition paper chromatography by a double-ascending method using *n*-butanol–pyridine–water (3:2:1 v/v) as the developer and *p*-anisidine hydrochloride as the staining reagent. The chromatogram showed a single spot (yellowish brown) identical with D-glucose run in parallel.

Swertianolin Dimethyl Ether—Swertianolin (98 mg) was methylated according to the method of Nakaoki and Hida (15). The product showed one major and two minor spots on TLC. The major component was separated by preparative TLC [chloroform–acetic acid (100:5) was used as the developer] as a yellow amorphous solid. It was subjected to hydrolysis without further purification.

Hydrolysis of Swertianolin Dimethyl Ether—The above compound (78 mg) was hydrolyzed with sulfuric acid (3%, 20 ml). Extraction of the aglucone with ether, evaporation of the solvent, and crystallization of the residue from ethanol afforded 1-hydroxy-3,5,8-trimethoxyxanthone, which had a melting point and mixed melting point of 204–205°.

Isoswertianolin (III)—The solid, marked Fraction E, crystallized from methanol as pale-yellow needles, mp 220–222°; $[\alpha]_D^{25}$ −110 ± 2° (c 0.25, 60% aqueous ethanol); UV: λ_{max} 252 (log ϵ 4.43), 275 (4.18), and 325 (3.94) nm; NMR (dimethyl sulfoxide-d_6): δ 13.18 (1H, br s, C_1-OH), 7.15 (1H, d, J = 9 Hz, C_7-H), 7.33 (1H, d, J = 9 Hz, C_6-H), 6.55 (1H, d, J = 3 Hz, C_4-H), 6.38 (1H, d, J = 3 Hz, C_2-H), 4.98 (1H, m, C_1-H of β-glucoside), and 3.50 (six glucosyl protons plus water).

Anal.—Calc. for $C_{20}H_{20}O_{11}\cdot H_2O$: C, 52.86; H, 4.40. Found: C, 52.49; H, 4.66.

Isoswertianolin Dimethyl Ether—To a solution of isoswertianolin (198 mg) in dimethylformamide (5 ml) and methanol (50 ml) was added an ethereal solution of diazomethane, prepared from nitrosomethylurea (2 g). The reaction was kept at room temperature (10–24°) overnight. Then the solvent was removed and the residue was triturated with methanol (2 ml) when a pale-yellow crystalline product (148 mg) was obtained. It showed one major and two minor spots on TLC [chloroform–methanol–water (7:3:1) lower layer]. The major component, being most polar, was separated by preparative TLC. The minor components could be due to partial methylation of the sugar moiety in III. The major component crystallized from ethanol as yellow needles, mp 178–180°; UV: λ_{max} 235 (log ϵ 4.30), 275–278 (3.94), 300 (3.90), 315 (3.82), and 360–365 (3.40) nm.

Hydrolysis of Isoswertianolin Dimethyl Ether—To a solution of emulsin (108 mg) in acetate buffer (pH 4.9, 20 ml), isoswertianolin dimethyl ether (98 mg) was added. The mixture was kept at 37° for 24 hr. The solution was then extracted with ether and the ether extract was processed in the usual way to give the aglucone as fine yellow needles (42 mg). Presence of glucose in the hydrolysate was established by partition paper chromatography.

1,3,5-Trimethoxy-8-hydroxyxanthone—The aglucone was crystallized from benzene as fine needles, mp 210–212°. It showed a depression in the melting point when admixed with authentic 1-hydroxy-3,5,8-trimethoxyxanthone, mp 204–205°. Co-TLC [chloroform–benzene (1:1)] also indicated that they were different. This new xanthone showed UV: λ_{max} 225 (log ϵ 4.02), 230–238 (4.17), 252 (4.38), 275–278 (3.90), and 330 (3.86) nm; NMR ($CDCl_3$): δ 13.30 (1H, s, C_8-OH), 7.38 (1H, d, J = 10 Hz, C_6-H), 6.80 (1H, d, J = 10 Hz, C_7-H), 6.64 (1H, d, J = 3 Hz, C_4-H), 6.42 (1H, d, J = 3 Hz, C_2-H), and 4.00–3.95 (9H, OCH_3); mass spectrometry: *m/e* 302 (M^+, 88%) and significant fragment ion peaks at *m/e* 287 (100), 272 (12), 259 (24), and 230 (8), consistent with the assigned structure.

Complete methylation of this compound with dimethyl sulfate and potassium carbonate in anhydrous acetone for 48 hr on a steam bath gave 1,3,5,8-tetramethoxyxanthone, which had a melting point and mixed melting point of 208–209° (4). Co-TLC [chloroform–benzene (1:1)] showed a single spot having the same R_f value and the IR spectra were superimposable.

RESULTS AND DISCUSSION

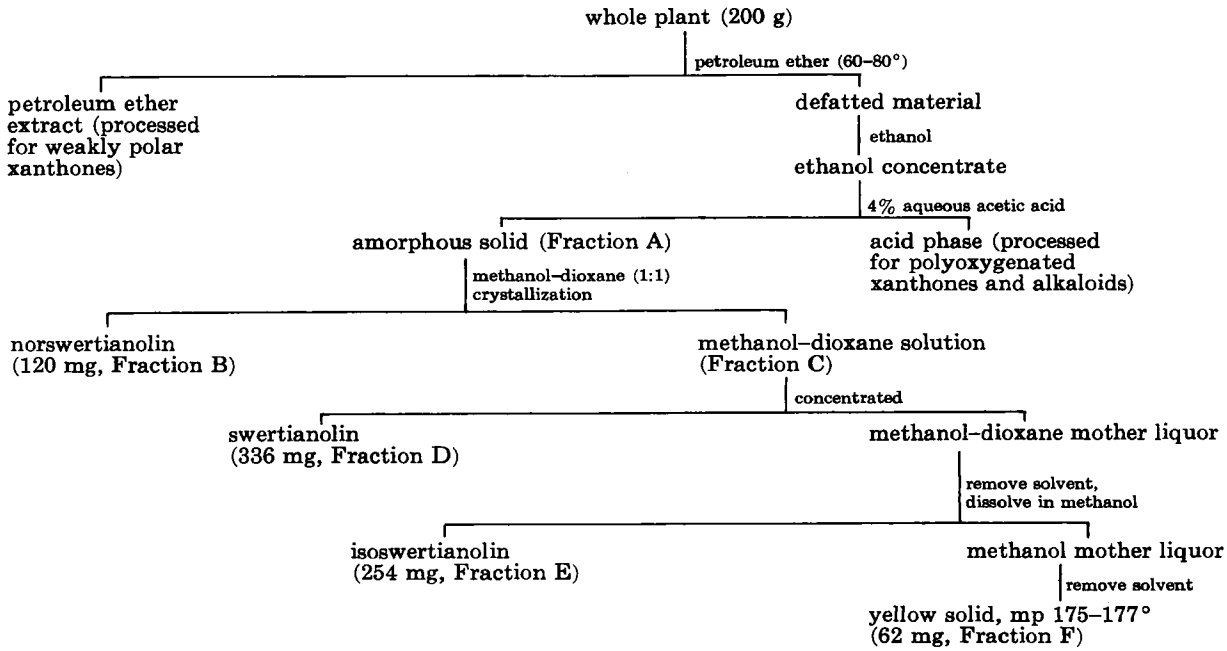
Three xanthone-*O*-glucosides were isolated from the alcoholic extract of *S. purpurascens*. The compounds were characterized by chemical transformation, such as derivatization, hydrolysis into xanthone and glucose, direct comparison of the two moieties so fragmented with reference materials where possible, and spectral (UV, IR, NMR, and mass) evidence. The characterization of these compounds will be described in order of isolation (Scheme I).

Xanthone-*O*-glucoside (I)—Compound I had the following characteristics: $C_{19}H_{18}O_{11}\cdot H_2O$, mp 263–266°, and $[\alpha]_D^{25}$ −111°; it formed a heptaacetate. The UV absorption spectrum showed close similarity with spectra of 1,3,5,8-tetraoxygenated xanthones (4, 16). In ethanolic sodium acetate, the UV spectrum showed a pronounced bathochromic shift of the longest wavelength maximum (λ 325 → 360 nm), indicating a free C_3-OH. The compound also responded to a quinol test (17), suggestive of the presence of free C_5- and C_8-OH groups. In the NMR spectrum, the compound showed signals due to four aromatic protons (δ 7.3–6.4), seven protons associated with glucosyl linkage (δ 4.9, 3.5) (18), and a strongly deshielded proton associated with a chelated OH. The aromatic protons appeared as *meta*- and *ortho*-split doublets associated with C_2-, C_4-, C_6-, and C_7-protons. The glucose anomeric proton showed a diaxial coupling with the $C_{2'}$-H, suggesting a β-glucosidic linkage (18) in the xanthone-*O*-glucoside. The strongly deshielded proton is ascribed to C_8-OH (6, 19). On hydrolysis with dilute sulfuric acid, it gave 1,3,5,8-tetrahydroxyxanthone (4) and glucose.

On the basis of these data, the xanthone-*O*-glucoside was identified as 3,5,8-trihydroxyxanthone-1-*O*-glucoside (I) (norswertianolin). Norswertianolin was previously isolated from *S. randaiensis* (10), but its detailed spectral data were not reported. It was reported to show noteworthy tuberculostatic activity (10). The tuberculostatic activity ascribed to *Swertia* plant extracts (1, 12) could be due to norswertianolin and the congener xanthone-*O*-glucosides.

Xanthone-*O*-glucoside (II)—Compound II had the following characteristics: $C_{20}H_{20}O_{11}\cdot H_2O$, mp 204–205°, and $[\alpha]_D^{25}$ −115°, it formed a hexaacetate and a dimethyl ether (with methyl iodide and silver oxide). The UV absorption spectrum was closely similar to that of norswertianolin. There was, however, no change in the UV maxima in ethanolic sodium acetate; the C_3-oxygen function is, therefore, substituted. In ethanolic sodium hydroxide, decomposition of the chromophoric system was observed. These observa-

[3] Whatman No. 50.



Scheme I—Isolation of xanthone-O-glucosides from S. purpurascens

tions, together with the positive quinol test shown by the compound, locate the *p*-hydroxy groups at C_5 and C_8. In the NMR spectrum of the compound, four aromatic protons appeared as *ortho*- and *meta*-doublets; these are associated with C_6-, C_7-, C_2-, and C_4-protons. In addition, there were seven protons due to β-glucoside linkage, three protons associated with an aromatic methoxyl (C_3-OCH_3), and a strongly deshielded proton associated with either C_1- or C_8-OH. The C_2-H in this compound appeared at a significantly lower field (δ 6.52) than the normal position of a 1,3,5,8-tetrahydroxy/methoxy xanthone (δ 6.36) (4). On this basis, the *O*-glucoside linkage appeared to be located at C_1. This conclusion was supported by the fact that the dimethyl ether, on hydrolysis, gave 1-hydroxy-3,5,8-trimethoxyxanthone. Therefore, the compound was identified as 5,8-dihydroxy-3-methoxyxanthone-1-*O*-glucoside (II) (swertianolin).

Swertianolin was previously reported to occur in *S. japonica* (14) and *S. tosaensis* (15), but its structure was only partially defined. The point of glucoside linkage (at C_1 or C_8) and the position of the methyl ether were not settled in earlier studies (14, 15). While the structure of the aglucone moiety was subsequently established (20), this study demonstrates the position of the glucoside linkage as in II.

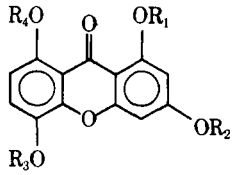
Xanthone-*O*-glucoside (III)—Compound III, $C_{20}H_{20}O_{11}\cdot H_2O$, mp 220–222°, $[\alpha]_D^{25}$ −110 ± 2°, is isomeric with swertianolin. The UV absorption spectrum was also closely similar to that of swertianolin. It formed a hexaacetate and a dimethyl ether (with ethereal diazomethane and formamide). On hydrolysis with dilute sulfuric acid, it furnished 1,5,8-trihydroxy-3-methoxyxanthone and glucose. In its mass spectrum, there was no discernible molecular ion peak but there was a fragment ion peak due to the aglucone moiety at *m/e* 274 (100%) and further fragmentation at *m/e* 245 (18) 244 (7), and 231 (14). The latter fragment ion peaks suggested the presence of C_3-OCH_3 and C_5-OH groups in the xanthone moiety (6, 7, 9). The NMR spectrum of the compound also showed a close similarity with that of swertianolin with only one exception: the C_7-H, instead of the C_2-H, showed a marked downfield shift (by about 0.4 ppm). The NMR spectrum of the acetyl derivative indicated six acetyl groups. Of these, two appeared at a significantly lower field (δ 2.40 and 2.48) than the remaining four (δ 1.85, 1.90, 2.02, and 2.08), suggesting their assignments as aromatic acetoxy. The upfield signals of the remaining acetoxy groups are in good agreement with the chemical shifts of the acetylated β-D-glucopyranoside (21, 22). The signals from the aromatic protons at C_2 and C_4 (δ 6.83 and 6.60, respectively) in the hexaacetate were practically the same as those from the corresponding protons of 1,5,8-triacetoxy-3-methoxyxanthone (bellidifolin triacetate) (δ 6.84 and 6.58) and swerchirin diacetate (δ 6.84 and 6.54) (23). However, the signals from C_6 and C_7 (δ 7.52 and 7.30) differed significantly from the corresponding positions of the last two compounds (δ 7.20 and 6.80 and δ 7.17 and 6.84, respectively).

On this basis, it appeared that the glucoside hexaacetate must bear an acetoxy at C_1 and that the glucoside linkage is at C_8. In consonance with this conclusion, the dimethyl ether, on hydrolysis, afforded 1,3,5-trimethoxy-8-hydroxyxanthone. This is a new tetraoxygenated xanthone, not encountered before in nature or prepared synthetically. Its identity was established from spectral data and from derivatization to 1,3,5,8-tetramethoxyxanthone (4). Therefore, the xanthone-*O*-glucoside was identified as 1,5-dihydroxy-3-methoxyxanthone-8-*O*-glucoside (III), which was named isoswertianolin. This study is the first demonstration of the occurrence of isoswertianolin in nature. This observation seems to be biogenetically significant since this is the first example of the natural occurrence of a xanthone-8-*O*-glucoside.

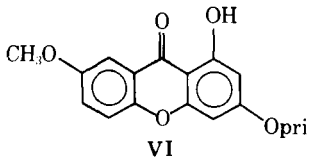
While the natural occurrence of xanthone-*O*-glycosides is restricted so far to the members of the family Gentianaceae (Table I), the related *C*-glucosides—*viz.*, mangiferin (IV) and isomangiferin (V), occur widely among angiosperms (3, 22, 24, 25). Mangiferin has also been reported to occur with its 3-methyl ether (homomangiferin) in Liliaceae (25). The available data in the literature indicate that there could be more xanthone-*O*-glycosides in

Table I—Distribution of Xanthone-*O*-glycosides in Gentianaceae

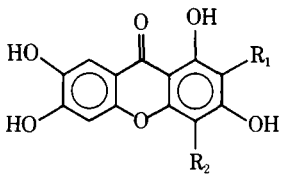
Xanthone	Species	Reference
Gentioside (VI)	*Gentiana lutea*	28
Gentiakochianoside (VII)	*G. kochiana, Swertia decussata*	29
Gentiacauloside (VIII)	*G. kochiana, G. acaulis*	28
Isogentiacauloside (IX)	*G. kochiana*	28
Norswertianolin (I)	*S. randaiensis, S. purpurascens*	10
Swertianolin (II)	*S. japonica, S. tosaensis, S. purpurascens*	14, 15
Isoswertianolin (III)	*S. purpurascens*	
Swerchirin glycoside (X)	*Frasera caroliensis*	30
Xanthone-*O*-glucoside (XI)	*S. bimaculata*	31
Xanthone-*O*-glucoside (XII)	*S. bimaculata*	31
Decussatin primeveroside (XIII)	*G. verna*	32
Corymbiferin-*O*-glycoside (XIV)	*G. corymbifera*	26



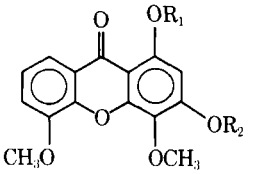
I: R_1 = glu, R_2 = R_3 = R_4 = H
II: R_1 = glu, R_2 = CH_3, R_3 = R_4 = H
III: R_1 = R_3 = H, R_2 = CH_3, R_4 = glu
X: R_1 = gly, R_2 = R_3 = CH_3, R_4 = H



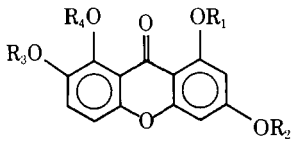
VI



IV: R_1 = glu, R_2 = H
V: R_1 = H, R_2 = glu



XI: R_1 = H, R_2 = glu
XII: R_1 = glu, R_2 = H



VII: R_1 = R_4 = H, R_2 = CH_3, R_3 = pri
VIII: R_1 = H, R_2 = R_4 = CH_3, R_3 = pri
IX: R_1 = R_3 = CH_3, R_2 = pri, R_4 = H
XIII: R_1 = pri, R_2 = R_3 = R_4 = CH_3

glu = β-D-glucosyl
gly = glycosyl (nature of sugar moieties unknown)
pri = primeverosyl

nature than are recorded to date. Polyoxygenated xanthones isolated from gentianaceous plants were often present in a bound (glycoside) form (16, 23, 26). The liberation of these compounds into polyoxygenated xanthones required treatment with dilute acids (16); hydrolysis also takes place during crystallization from protic solvents. When modified isolation procedures can be adopted, it will be interesting to see whether more xanthone-bearing taxa will be found to contain the corresponding *O*-glycosides. The results from such investigations would suggest biochemical significance of this blocking (glycosidation), which appears to be characteristic of the members of the Gentianaceae.

The study of xanthone-*O*-glycosides could also be therapeutically significant since a number of such compounds produced remarkable pharmacological and other biological activities (11, 27). The total xanthone-*O*-glucosides of *Canscora decussata* produced marked antipsychotic effects, while the corresponding aglucones and the *C*-glucoside, mangiferin, showed monoamine oxidase inhibitor activities (5, 9, 27). The therapeutic properties of *C. decussata* plant extracts (1, 12) could, therefore, be ascribed to the contained xanthone-*O*-glucosides. Mangiferin, constituting about 80% of the total xanthones of this plant, acts as a potentiator of the activities of the xanthone-*O*-glucosides.

Selected pharmacological screening of the total xanthone-*O*-glucosides (I–III), in doses of 25–50 mg/kg, produced definite signs of central nervous system (CNS) depression in albino mice and rats. The effect was manifested by an initial transient hyperactivity (with flag-tail in the case of albino mice) followed by moderate to deep depression, intact reflexes and response to external stimuli, potentiation (165%) of hexobarbital hypnosis in albino mice, and complete protection against amphetamine (20 mg/kg) and 5-methoxy-*N*,*N*-dimethyltryptamine (10 mg/kg) induced (33) toxicities. The total *O*-glucosides markedly potentiated (120%) the analgesic activity of subanalgesic doses (2 mg/kg ip) of morphine but had no analgesic activity *per se*. In higher doses (100 mg/kg ip) only, the total xanthone-*O*-glucosides offered protection to electroshock seizure (50%) and pentylenetetrazol- (70 mg/kg sc) induced convulsions (60%). Additionally, the total *O*-glucosides showed cardiovascular stimulant (in dose of 1 mg) and choleretic activities. These activities were determined according to methods reported earlier (5). The xanthone aglucones, on the other hand, produced dose-dependent weak CNS stimulant activities. The total xanthone-*O*-glucosides were nontoxic up to 200 mg/kg ip. These observations seem to indicate that the curative properties ascribed to *S. purpurascens* plant extracts (1) are primarily due to the contained xanthone-*O*-glucosides.

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PHARMACEUTICAL ANALYSIS

Quantitative TLC of Salicylazosulfapyridine

DAVID R. POWELL[x] and BRUCE A. BURTON

Abstract □ Nine lots of raw material salicylazosulfapyridine from six suppliers were analyzed by an early spectrophotometric method. The lower limit of purity set by this method is 88%. Using a highly purified reference standard, results of the spectrophotometric method were compared to a salicylazosulfapyridine-specific, quantitative TLC method. Results confirmed the nonspecificity of the spectrophotometric method. In an extreme case, material with a 94% spectrophotometric purity value was shown to be only 85% by quantitative TLC. As many as eight extraneous spots in addition to salicylazosulfapyridine were found in some lots of raw material when developed chromatographs were sprayed with an azo-indicating stain. The minimum visible detection level of the impurities was between 0.01 and 0.1% of the total salicylazosulfapyridine spotted. Isolated impurities showed spectrophotometric absorbance at the wavelength of the spectrophotometric method, resulting in erroneous purity values. Drug recovery by the quantitative TLC method was 98% with an assay standard deviation of less than ±0.5%. These results led to commercial upgrading of synthesis and purification, so that raw material salicylazosulfapyridine at the 96% level by quantitative TLC was subsequently available for drug production.

Keyphrases □ Salicylazosulfapyridine—quantitative TLC analysis, compared to literature spectrophotometric method □ TLC—analysis, salicylazosulfapyridine, compared to literature spectrophotometric method

Salicylazosulfapyridine (I), indicated for use in the treatment of ulcerative colitis, has been marketed in the United States for about 20 years. The first monograph for this drug appeared in 1953 (1). Chemical specifications for I found in that reference are as current as can be found and call for a purity of not less than 88%.

During the development of I dosage forms in this laboratory, it was necessary to explore analytical procedures for assurance of raw material purity as well as chemical stability. Three methods have been described: a titanium trichloride titration used in 1925 (2), a spectrophotometric procedure (2), and the more recent polarographic technique (3). Both the spectrophotometric and the azo titration procedures were included in Ref. 1. These methods were compared by Berggren and Hansen (2), who theorized that the similarity of potency values obtained for I raw material was due to the specificity of both assays for the azo linkage (—N=N—). More recent studies (3) showed that normal production lots of I contain azo impurities which have been proven to be spectrally active. Thus, neither method can be considered specific or meaningful for I potency unless azo impurities are first removed.

Consideration has been given to polarography (3) in an attempt to overcome the impurity disturbances of the spectral method; however, in the final analysis of this work, an average difference of only 0.6% between the two methods is found. This slight difference indicates that polarography is not a significantly more specific assay than spectrophotometry for production lots of I. It was further stated that the spectrophotometric method was unquestionably more sensitive and rapid. Therefore, it was evident from the literature reviewed that a specific, quantitative assay for I did not exist.

Kiger and Kiger (4) reported qualitative TLC techniques for I in their work on sulfonamide differentiation. Two distinct spots for this drug were reported, and one was designated as an impurity. After repeating the qualitative TLC procedure in these laboratories, it was confirmed that indeed two spots were visible with the naked eye at the reported R_f

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Table I—Physical–Chemical Properties of Salicylazosulfapyridine Reference Standard

	Found	Reported	Theory
Melting range	255–258°	240–245°[a]	—
Absorptivity	668[b]	658[c]	—
TLC	One spot	Two spots[d]	
Elemental analysis, %	C 54.83	55.19[c]	54.23
	H 3.52	3.75	3.54
	N 14.18	14.00	14.06
	S —	7.88	8.05

[a] Reference 6. [b] Calculated from Beer's law by least-squares method, n = 14. [c] Reference 2. [d] Reference 4.

values. However, further investigation using UV lamps and azo-indicating stains revealed as many as 10–12 additional spots in some raw material lots of I. By using TLC techniques, therefore, it was the objective to develop a quantitative isolating procedure for I and incorporate the highly sensitive spectrophotometric method for the purity determination.

EXPERIMENTAL

Chemicals and Equipment—Unless otherwise stated, all chemicals used in this study were analytical reagent grade quality. Precoated silica gel TLC plates (20 × 20 cm) were used. For the quantitative assay, silica gel with fluorescent indicator[1] was used; for the qualitative assay, additional types of TLC plates[2,3] were also employed. Other equipment included capillary pipets[4] (5 μl), a spectrophotometer[5], a mechanical shaker, a forced-air blower, a chromatographic sprayer, and short and long UV lamps.

Samples—Nine samples of raw material from salicylazosulfapyridine (I) production lots of six manufacturers were procured and dried at 105° for 90 min. Samples were then stored in a desiccator until required. The reference standard of I[6] was dried and stored in the same manner and was characterized by determining chromatographic purity, absorptivity, melting range, and carbon, hydrogen, and nitrogen content.

Spectrophotometric Assay—The spectrophotometric assay for I (1) was carried out on each sample in comparison to the reference standard. In this procedure, solutions of sample and standard were prepared by dissolving about 150 mg of I, accurately weighed, in enough 0.1 N sodium hydroxide solution to make 100 ml of solution. A 5-ml aliquot was transferred to a 1000-ml volumetric flask containing about 750 ml of water and 20 ml of 0.1 N acetic acid solution. Enough additional water was added to make 1000 ml. The absorbance of these solutions was read on a spectrophotometer at 360 nm against water, and the purity percentage of the sample was calculated. A Beer's plot of the reference standard was prepared according to the spectrophotometric procedure for the determination of the absorptivity value.

Quantitative TLC Assay—Standard and sample solutions were prepared by dissolving about 250 mg of I, accurately weighed, in 10 ml of dimethylformamide and diluting this solution to 25 ml with methanol. The chromatographic bath was prepared in a glass chamber (28 × 25 × 9 cm) using 60 ml chloroform and 15 ml each of acetone, n-butanol, and 88% formic acid. The chamber was lined on one side with filter paper, which was in contact with the mobile phase. The chamber was tightly sealed and allowed to equilibrate overnight for use on the next day only. Chromatographic plates were prerinsed with anhydrous methanol and thoroughly dried. Five microliters of sample and 5 μl of standard solutions were spotted alternately in triplicate on the TLC plates. To prevent thermal expansion, capillary pipets were held with a flag made of tape and the outer surface of the delivery end was coated with a thin film of silicone grease to prevent creep back of the solution. Pipet filling was done by capillary action using the nongreased end. Following each application, the pipet was rinsed with 5 μl of methanol, which was applied to its respective spot to ensure complete transfer. This step was then followed by rinsing the pipet with the next solution to be spotted. The same pipet was used for each six-spot series.

After drying the applied spots thoroughly, chromatographic development was started and continued until the solvent front was 10–12 cm above the origin. The plates were removed from the chamber and dried in a stream of forced warm air. The yellow drug spots visible at R_f 0.6–0.7 were outlined under long UV light to ensure total removal. These spots were individually cut from the plastic-backed plates. The three sample spots and the three standard spots were combined in separate 50-ml glass elution vessels. The drug was eluted from the silica gel with 18 ml of 0.016 N sodium hydroxide (one part 0.1 N sodium hydroxide plus five parts water) by mechanically agitating the tightly sealed vessels for 15 min. The mixtures were then transferred to a syringe-mounted, Teflon filter[7]. Six-milliliter aliquots of the filtrates were separately combined with 4 ml of 0.1 N acetic acid solution, and the absorbances were compared at the 360-nm maximum using water as the blank.

Qualitative TLC—For qualitative assessment of the raw material purity of I, developed chromatographs[8] were first viewed under short and long UV light followed by spraying with a solu-

Table II—Comparative Assays of Salicylazosulfapyridine Raw Material Samples

Sample Code	Purity[a], % Quantitative TLC Method	Purity[a], % Spectrophotometric Method (1)	Impurities[b], %	Melting Range[c]
A-1	97.7	98.7	2.4	256–258°
A-2	96.2	98.1	1.9	256–258°
A-3	92.1	94.4	3.8	249–251°
B-1	91.4	97.9	5.7	250–252°
C-1	90.2	93.7	5.2	248–250°
D-1	89.9	96.8	5.8	246–248°
B-2	85.6	94.1	10.2	247–249°
E-1	81.9	89.8	8.8	243–245°
F-1	77.4	88.9	12.5	237–239°

[a] All results based on reference standard. [b] Impurities isolated by TLC that show absorbance at 360 nm. [c] USP procedure 1a.

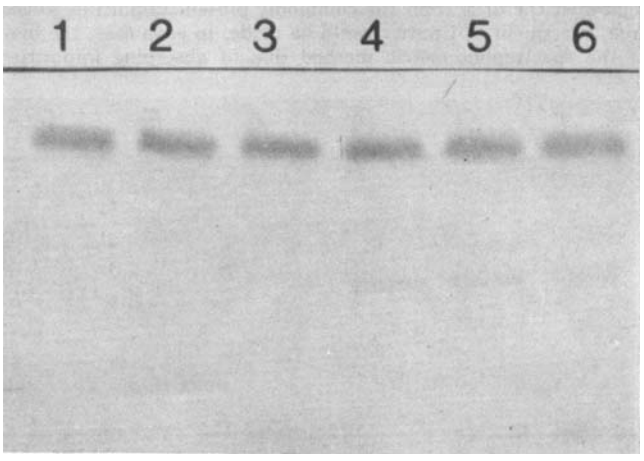

Figure 1—*Qualitative TLC of salicylazosulfapyridine (silica gel F254, fast running). Key: 1, reference standard; 2, A-2, 3, A-3; 4, D-1; 5, E-1; and 6, F-1. See Table II for purity values.*

[1] Eastman Chromagram (13181), Eastman Kodak Corp., Rochester, N.Y.
[2] Silica gel F254 (fast running), Brinkmann Instruments, Westbury, NY 11590
[3] MN silica gel N-HR/UV254, Brinkmann Instruments, Westbury, NY 11590
[4] Drummond Microcaps (55-mm length), Bolab, Inc., Derry, N.H.
[5] Beckman DB, Beckman Instrument Co., Fullerton, Calif.
[6] A gift of Orgamol Corp., Switzerland, through J. H. DeLamar, Inc., Chicago, Ill.
[7] Millipore Corp., Bedford, Mass.
[8] Spotted with a 10-μl applicator, Cordis Laboratories, Miami, Fla.

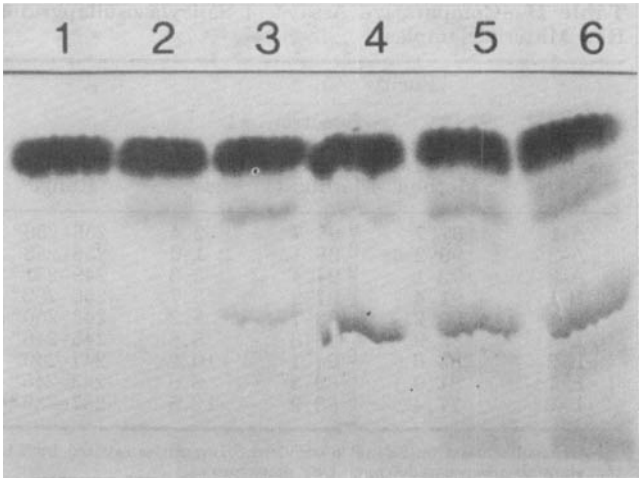

Figure 2—*Qualitative TLC of salicylazosulfapyridine (silica gel 13181). Key: same as Fig. 1. See Table II for purity values.*

tion prepared just prior to use by combining 20 parts of 4-dimethylaminocinnamaldehyde (II) solution (0.1% in a 50:50 mixture of glacial acetic acid and water) with one part of titanium trichloride solution (20% technical grade) (5).

RESULTS AND DISCUSSION

The reference standard of I used in this study was found to be higher in purity than any previously reported material, as shown by the comparative physical-chemical properties listed in Table I. The absorptivity reported by Berggren and Hansen (2) represents a calculated value based on their 98% standard. Our findings indicate that their standard was really 96.5% of I and that the use of the 658 absorptivity value in the spectrophotometric method results in a 1.5% positive bias. This indicates that the lower limit of purity by this currently accepted assay method is not 88% as stated but actually 86.5%.

The qualitative TLC system, capable of visualizing as little as 5 ng with respect to I, revealed that the reference standard was essentially homogeneous, with one trace band just above the drug band. The trace band was not consistently visualized and was considered to be insignificant.

Comparative raw material purity values for I samples from six sources are presented in Table II. The TLC system effectively separated the drug from the commonly present impurities so the true assessment of I purity could be made. In each case, the bias of the spectrophotometric method due to absorbing impurities

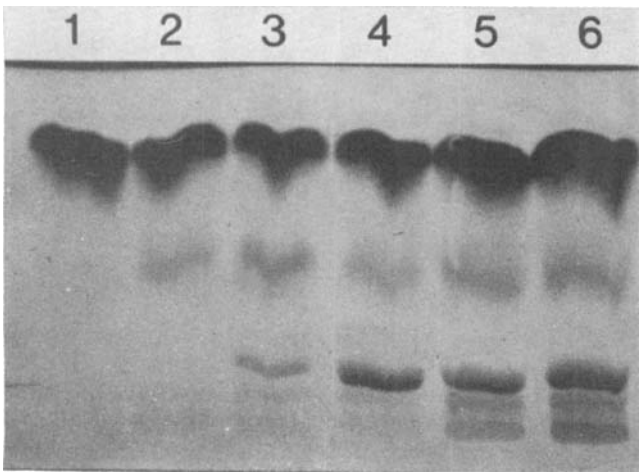

Figure 3—*Qualitative TLC of salicylazosulfapyridine (MN silica gel N-HR/UV254). Key: same as Fig. 1. See Table II for purity values.*

Table III—Statistical Evaluation of the Quantitative TLC Method

Sample Code	Purity[a], %	Mean Purity, %	Relative Standard Deviation, %
A-3	92.3[b]	92.1	±0.15
	92.0		
	92.0[c]		
	92.0		
D-1	89.9[b]	90.4	±0.39
	90.7[c]		
	90.5		
	90.6		
A-2	95.6[b]	95.8	±0.47
	96.2		
	95.3		
	96.2[c]		
			Average = ±0.34

[a] Individual assays based on reference standard. [b] Assayed on Day 1. [c] Assayed on Day 2.

was eliminated, resulting in significantly lower purity values by the quantitative TLC procedure. Impurity absorbance at 360 nm was confirmed by collective elution of all bands except I. Estimates for the total amount of impurities were calculated assuming the absorptivity to be similar to I. These values approximate the difference in percent purity found by the two methods.

All samples tested were within or above the currently accepted 220–240° melting range. As shown in Table II, melting ranges tended to indicate a rank-order correlation with I purity. The purity values obtained from the quantitative TLC method correlated better with melting temperature than did the purity values from the spectrophotometric analysis.

The methods of qualitative examination used for developed TLC plates revealed numerous impurities in most I samples studied. Figures 1–3 show six chromatographed samples of I ranked according to their quantitative TLC purity. A rank-order correlation was demonstrated between the quantitative TLC assay values and the number and intensity of impurity bands. Initially (Fig. 1), only one distinct impurity band could be seen at R_f 0.35 with the drug band at R_f 0.6–0.7. Spraying the chromatographs with the azo-reducing (titanium trichloride), amine-indicating solution of II allowed further visualization of this and other impurities (Figs. 2 and 3). The silica gel medium of the plate represented in Fig. 3 allowed for the greatest resolution of I from impurities. Although the R_f of the drug remained at about 0.7, the R_f 0.35 impurity was suppressed to about R_f 0.2.

As many as eight azo bands were located in the sample at position 6 (F-1). This material represented the lowest purity of I tested and, although it passed current specifications, it was found to contain only 77% of I by quantitative TLC. Chromatographs sprayed with the II solution alone showed only one free amine to be present in Sample F-1, which was subsequently confirmed as sulfapyridine.

Sample A-2 (position 2) and A-1 (not shown) represent production lots of I synthesized and purified by improved methods. This type of material is of the highest purity commercially available with essentially all azo impurities eliminated.

Some chromatographs viewed under short UV light contained a fluorescent band at a slightly greater R_f than the drug. This impurity was confirmed as being salicylic acid. No other short UV fluorescence was seen. Long UV fluorescence, common in all lots except A-1 and A-2, was observed at R_f 0.55, 0.2, and 0. These additional impurities were not the same as those responding to the azo spray and may account for the remaining impurities not seen by the spectrophotometric method.

The major impurity, found at R_f 0.35 in all I samples except A-1 and A-2, was isolated from TLC plates and shown to be similar to I in several regards. The UV spectrum and the IR spectrum were essentially the same for both the drug and this impurity. A qualitative examination of solubility showed that, like I, the major impurity was soluble at alkaline pH and could be precipitated by acidic conditions. Melting of this impurity occurred with decomposition over a broad range from about 240 to 260°. Further TLC studies of the isolate showed it to be homogeneous with an R_f of 0.35.

Table IV—Recovery of Salicylazosulfapyridine by the Quantitative TLC Method

Micrograms Recovered[a,b]	Percent Recovered	Micrograms Recovered	Percent Recovered
146.4	97.5	147.7	98.4
146.8	97.8	147.3	98.1
147.7	98.4	147.3	98.1
144.6	96.4	147.3	98.1
148.2	98.7	147.3	98.1
148.2	98.7	146.8	97.8
Mean percent recovered = 98.0 ± 0.65%			

[a] Reference standard, 150.1 μg, was spotted in each of the 12 assays. [b] A separate 5-μl pipet was used for each of the 12 assays.

A statistical evaluation of the quantitative TLC method was conducted on three lots of I. Assays were carried out in a randomized design so that six assays were run on each of 2 days. As shown in Table III, the quantitative TLC method has excellent reproducibility and can be carried out with a relative standard deviation of less than ±0.5%. The potency range of the three lots studied did not show a rank-order correlation with the standard deviation. Day-to-day assay variation did not appear to be a significant factor affecting the results.

Throughout the development of the quantitative TLC method, several techniques had been used. Prior to the final techniques described here, the basic eluate had been buffered to pH 4–5 in the presence of suspended silica gel from the TLC plates. It was subsequently learned that under these conditions erratic adsorption of drug by silica gel as well as various filtration systems resulted in only a 90% recovery with a relative standard deviation of ±1.4–2.5%. The final adjustment in technique, *i.e.*, filtration of the basic eluate for silica gel removal prior to buffering, reduced the assay variation to less than one-fourth that of the previous procedure while increasing the recovery to 98%.

The absorbance values for the reference standard from 12 individual assays were converted to micrograms of the drug recovered. This value was divided by the theoretical amount of standard spotted to obtain the percent recovery (Table IV). Blank assays showed no absorbance at 360 nm. The relative standard deviation of recovery corresponded to that of the delivery precision of the capillary micropipets as determined by Emanuel (7) (n = 12, ±0.64%) and tend to indicate that the TLC technique is highly reproducible.

Since the recovery of I by the quantitative TLC method was sufficiently high and had a low standard deviation, no attempt was made to determine the cause of the 2% drug loss.

SUMMARY AND CONCLUSIONS

A specific method with high precision was developed for the analytical control of salicylazosulfapyridine raw materials as well as dosage forms of this drug. Assay results showed that there are extremely wide purity variations among supplies of this drug. All nine samples tested would be considered acceptable by current spectrophotometric standards, although borderline cases contained as much as 23% of nonsalicylazosulfapyridine materials. The lack of specificity of the spectrophotometric assay for I was confirmed by showing that the isolated impurities possessed strong absorbance at 360 nm, causing assay interference that results in as much as 10–12% positive bias. Most impurities found in commercial grades of I were shown to contain an azo moiety; however, other impurities not responding to the azo test were revealed under UV light. The analytical findings based on the quantitative and qualitative TLC of I led to improvements of methods for synthesis and purification, so material at the 96% purity level was made available for drug production.

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[x] To whom inquiries should be directed.

Nonaqueous Titration of Methaqualone and Its Dosage Forms

L. G. CHATTEN[x], R. E. MOSKALYK, R. A. LOCOCK, and F. JANE SCHAEFER

Abstract □ The use of glacial acetic acid alone as a solvent for the nonaqueous titration of methaqualone dosage forms gives unsatisfactory overestimation due to interference of titratable excipients. A nonaqueous system of equal parts of chloroform and glacial acetic acid does provide a suitable medium for the visual and potentiometric titration of methaqualone and its hydrochloride. When applied to analysis of the dosage forms, the method gives recoveries that compare favorably with those of the manufacturer. The pKb of methaqualone, when determined potentiometrically, is 10.44.

Keyphrases □ Methaqualone and methaqualone hydrochloride tablets and capsules—nonaqueous titration □ Titrimetry, nonaqueous—analysis, methaqualone and methaqualone hydrochloride tablets and capsules

Since its advent on the market some 10 years ago, the hypnotic methaqualone has received considerable attention. Much work has focused on the detection and determination of the drug in various biological specimens. However, there are few reports concerned with the quantitative analysis of methaqualone and its hydrochloride in pharmaceutical dosage forms. Although the BP (1) offers a nonaqueous method with glacial acetic acid as the solvent system for the analysis of the raw material, no monograph is provided for the pharmaceutical dosage forms. This solvent does not give either a sharp visual or potentiometric end-point; however, it can be used for the titration of this weakly basic material provided no interfering excipients are present.

In 1969, King and Perry (2) published a nonaqueous titrimetic method for methaqualone tablets that employed glacial acetic acid as the solvent system and crystal violet as the indicator. Because of the solubility of a large number of interfering excipients, it has been demonstrated that this is not a suitable solvent to use when assaying tablets and capsules. On the other hand, very few excipients interfered with nonaqueous titrations when chloroform was first used to extract the active constituent from the tablet or capsule mass (3). Therefore, the use of this latter solvent system was investigated and its use as a means of eliminating the overestimation caused by interfering excipients when glacial acetic acid is employed as the extracting solvent is suggested.

EXPERIMENTAL

Apparatus—The following were used: a potentiometer[1] equipped with a glass–calomel electrode pair, a pH meter[2] fitted with a glass–calomel electrode system, a magnetic stirring apparatus, conventional laboratory glassware, and a platinum-tipped microburet graduated to 0.01 ml.

Reagents and Solutions—All of the following chemicals employed were ACS quality: acetone, chloroform, dioxane, glacial acetic acid, 0.1 *N* perchloric acid in dioxane (standardized against primary standard potassium acid phthalate), 3% mercuric acetate in glacial acetic acid, 1% crystal violet in glacial acetic acid, and 0.1 *N* HCl. The purity of the methaqualone free base and of the hydrochloride was determined by the BP procedure.

Procedures—*Pure Drugs*—Approximately 75 mg of the reference methaqualone or methaqualone hydrochloride was accurately weighed into a 150-ml beaker and dissolved in 25 ml of chloroform; then 25 ml of glacial acetic acid was added. Prior to commencing the titration for the hydrochloride salt, 2 ml of 3% mercuric acetate solution was added. The titrant was 0.1 *N* perchloric acid in dioxane. The end-point was determined potentiometrically using the pH meter for one set of samples and visually using crystal violet indicator for another set of samples.

Dosage Forms—The contents of 20 capsules were weighed or 20 tablets were weighed and then powdered to a very fine state. A uniform sample of the dosage form calculated to contain approximately 50 mg of methaqualone or its salt was accurately weighed into a 150-ml beaker. (For combinations with diphenhydramine hydrochloride, samples were calculated to contain 75 mg of methaqualone.) Twenty milliliters of chloroform was added and the mixture was stirred magnetically for 10 min to ensure dissolution of the active constituent. The solution was filtered under suction, the beaker was rinsed, and the filter was washed with an additional 5 ml of chloroform. The filtrate was transferred quantitatively to a clean 150-ml beaker by rinsing the flask with two 10-ml and one 5-ml aliquots of glacial acetic acid. When the hydrochloride salt was present, 2 ml of 3% mercuric acetate was added prior to the titration. The titrant was 0.1 *N* perchloric acid in dioxane and the end-point was detected from the first derivative plot of the potentiometer.

Comparative values were obtained by the method of King and Perry (2).

pKb Determination—The pKb of methaqualone was determined by the method according to Chatten and Harris (4) by using acetone–water solvent mixtures. Three concentrations of methaqualone were used (0.002, 0.0015, and 0.001 *M*). Each concentration was prepared in three different acetone–water

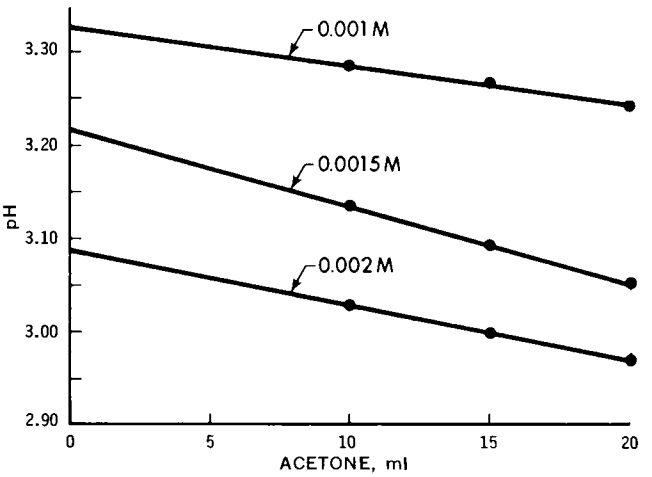

Figure 1—*Theoretical pH values obtained by extrapolation to 0 ml of acetone for three concentrations of methaqualone.*

[1] Model E336 Metrohm Potentiograph.
[2] Fisher model 230.

Table I—Comparison of Results Using Proposed Solvent System with Those of BP Method for Methaqualone and Its Hydrochloride Salt

	Chloroform–Glacial Acetic Acid (1:1), Recovery, %		BP 1973, Recovery by Visual Determination[a], %
	Potentiometric[a]	Visual[a]	
Free base	99.3 ± 0.3	98.6 ± 0.4	99.4 ± 0.9
Hydrochloride salt	99.9 ± 0.1	99.7 ± 0.3	98.2 ± 0.8

[a] Each figure is the average of five determinations.

mixtures, and the total volume of each mixture was constant at 50 ml. Thus, the acetone–water ratios were 10:40, 15:35, and 20:30 ml, respectively.

The solutions were titrated to the half-neutralization point with standardized 0.1 *N* HCl and the pH was measured. Because of the dilutions employed, no corrections were made for differences in activities.

RESULTS AND DISCUSSION

Since methaqualone and its hydrochloride are both readily soluble in chloroform, it was found to be a useful and convenient solvent for the extraction of the active constituent from pharmaceutical dosage forms. After filtration to remove the excipients, an equal quantity of glacial acetic acid was added to produce a stable, readily detectable potentiometric end-point. Simultaneous use of crystal violet indicator during the potentiometric titration of methaqualone or its hydrochloride revealed that the emerald-green color of the indicator corresponded to the maximum potentiometric inflection point. Although the indicator color change is not sharp, careful titration can produce good, reproducible results (Table I).

The following additional indicators were investigated in this and other solvent systems: malachite green, metanil yellow, methylene blue, methyl red, α-naphtholbenzein, neutral red, oracet blue B, phenol red, quinaldine red, and tropaeolin OO. None gave color changes that corresponded to the potentiometric end-point. Since the potentiometer plots a first-derivative curve, potentiometric end-point detection with this instrument is as rapid as a visual titration. Consequently, the indicator was not used with the dosage forms.

Comparative analyses between the results obtained with the proposed solvent system and those using the BP (1) method are presented in Table I for methaqualone and the hydrochloride. Other solvents investigated for the free base were various combinations of: *(a)* chloroform and acetonitrile, *(b)* chloroform and nitromethane, *(c)* acetone and hexane, and *(d)* benzene and nitromethane. In addition, both *(a)* and *(b)* were investigated for the hydrochloride. All of these combinations failed to yield quantitative results with the pure drugs.

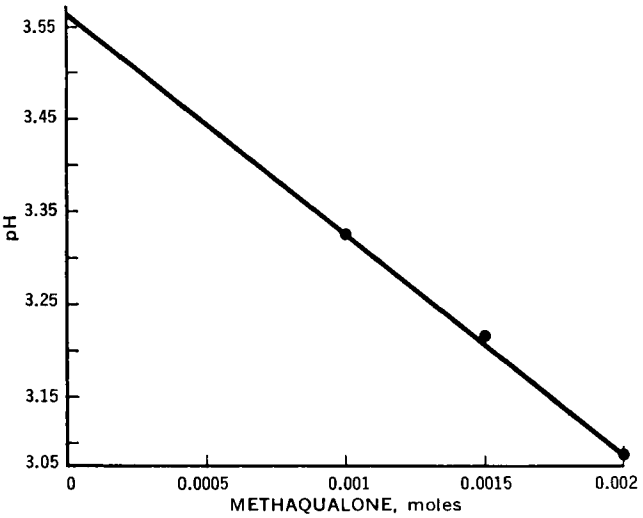

Figure 2—*Determination of pKa of methaqualone. The pH values from Fig. 1 are extrapolated to infinite dilution of methaqualone (pKa = 3.56).*

Table II lists the results obtained for the dosage forms, using the chloroform–glacial acetic acid (1:1) solvent system in which the end-point was determined from the first-derivative plot of the potentiometer. Comparative data were supplied by the manufacturer's quality control laboratory and are presented with the results obtained by the King and Perry (2) procedure. Although the values obtained with the proposed method generally agree well with those of the manufacturer, the use of glacial acetic acid alone (2) apparently resulted in serious overestimation in several instances. If the dosage form did not contain titratable excipients, the results of the glacial acetic acid procedure agreed with those of the proposed method. In addition, King and Perry (2) did not include a filtration step prior to the titration. This resulted in the presence of slowly soluble titratable excipients, which caused a fading or reversible indicator change in the vicinity of the end-point.

Synthetic mixtures of methaqualone and diphenhydramine hydrochloride (10:1) were analyzed for both constituents by the proposed method. When the end-point for methaqualone was passed, 1 ml of 3% mercuric acetate was added and the titration was continued. Based on the average of five determinations, the recovery for the methaqualone was 100.3 ± 0.7%. This compares favorably with the recovery of 99.3% reported in Table I for pure methaqualone. However, the recoveries for diphenhydramine hydrochloride were erratic. It is possible that the chloride ion furnished by the diphenhydramine hydrochloride, although small in amount, may

Table II—Comparison of Recoveries of Active Constituent from Commercial Dosage Forms Expressed as Percent Potency

Form	Product[a]	Labeled, mg	Proposed Method[b]	Manufacturer Data	King and Perry (2) Method
Capsule	A Methaqualone hydrochloride	150	99.7 ± 0.9	103.3	120.0
Capsule	B Methaqualone hydrochloride	250	98.9 ± 1.5	98.9	120.3
Capsule	C Methaqualone hydrochloride	300	99.9 ± 0.7	97.2	105.4
Tablet	D Methaqualone	300	99.5 ± 0.7	100.8	102.5[c]
Tablet	E Methaqualone hydrochloride	150	98.1 ± 1.2	Not available	107.7[c]
Tablet	F Methaqualone hydrochloride	300	99.5 ± 0.6	Not available	103.6[c]
Capsule	G Methaqualone hydrochloride	200	98.1 ± 1.4	98.6	101.7[c]
Capsule	H Methaqualone hydrochloride	400	95.4 ± 0.7	100.4	94.8
Capsule	I Methaqualone hydrochloride	300	100.0 ± 0.4	Not available	100.8
Capsule	J Methaqualone hydrochloride	150	101.1 ± 0.5	101.8	104.5[c]
Capsule	K Methaqualone hydrochloride	300	97.8 ± 1.9	100.5	96.7
Capsule	L Methaqualone	250	102.9 ± 0.3	103.3	98.8[c]
Tablet	M Methaqualone	250	102.9 ± 0.7	103.5	101.8[c]

[a] Trade names and suppliers of the products: A and B, Mequelon, Charles E. Frosst; C, Pexaqualone, Therapex; D, Quaalude, Rorer; E and F, Rougualone, Rougier; G and H, Somnafac, Cooper; I, Triador, Trianon; J and K, Tualone, ICN; and L and M, Mandrax, Roussel. [b] Each figure is the average of five determinations. [c] Indicator color reverses due to presence of excipients.

be responsible for the 1% discrepancy obtained for methaqualone in the mixtures. This would result in a significant error in calculating the diphenhydramine hydrochloride recoveries. Although samples equivalent to 75 mg of methaqualone were taken for the mixture, the procedure works equally well with mixtures containing 50 mg of methaqualone. The larger samples were used in the hope of being able to estimate accurately the antihistamine.

The visual and potentiometric end-points for methaqualone base are less distinct than are those for the CH_3COO^- species that is created from the hydrochloride by the addition of mercuric acetate. This indicates that methaqualone is a weaker base than the acetate anion. Furthermore, the presence of diphenhydramine hydrochloride further reduces the sharpness of the end-point with methaqualone base.

Because the pKb of methaqualone was not available from the literature for comparison, it was determined by a previously reported method (4). Figure 1 represents plots of pH *versus* milliliters of acetone for three concentrations of methaqualone. The pH values were measured at exactly half-neutralization. Figure 2 represents the three pH values obtained by extrapolation of the lines in Fig. 1 to 0 ml of acetone. Extrapolation to infinite dilution of the best straight line in Fig. 2 gives a resulting pH of 3.56. Since this is equal to pKa, the pKb of methaqualone was found to be 10.44.

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Improved Method for Salicylazosulfapyridine Analysis and Partial Characterization of Impurities in Commercial Salicylazosulfapyridine

J. C. STONE [x] and R. GORBY

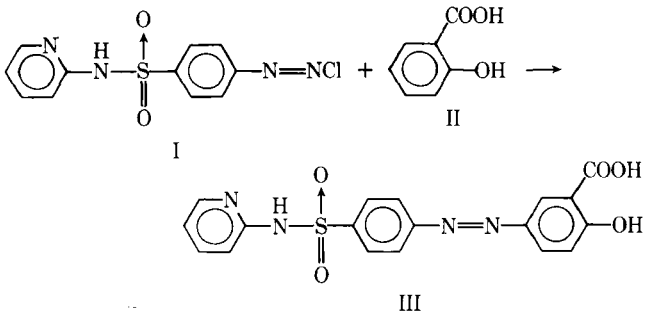
Abstract □ A column chromatographic system was developed that quantitatively separates salicylazosulfapyridine from impurities that contribute to the usual colorimetric method. The chromatographic–colorimetric method can be used to assay specifically for salicylazosulfapyridine as a raw material and in the final dosage form. Respective samples of two chromatographic mobile impurities were isolated, collected, and assayed by various means. Based on the data collected, partial characterizations of the chemical structure of these impurities were proposed. A third impurity, which was chromatographically immobile, was also studied and characterized as possibly being polymeric in nature.

Keyphrases □ Salicylazosulfapyridine bulk and tablets—chromatographic–colorimetric analysis, partial characterization of three impurities □ TLC—separation, salicylazosulfapyridine and three impurities □ Colorimetry—analysis, salicylazosulfapyridine and three impurities after TLC separation

The commercial synthesis (1) of salicylazosulfapyridine (III) involves the reaction between the diazonium salt of sulfapyridine (I) and *o*-hydroxybenzoic acid (II) (Scheme I).

The presence of the hydroxy *ortho–para*-director and the carboxy *meta*-director causes salicylazosulfapyridine to be the primary product. However, minor by-products which could interfere with the usual analytical method (2) are also expected to be present.

To determine if interfering impurities might be present, TLC analyses were performed on experimental salicylazosulfapyridine and commercial tablets, and it was possible to resolve three yellow-colored impurities in both types of samples. One impurity was essentially immobile (as indicated by the brown spot at the origin) and is speculated to be polymeric, resulting from the formation of a benzyne



Scheme I

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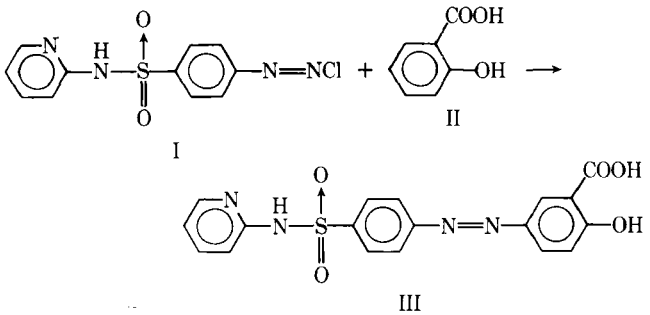


Scheme I



Figure 1—*Typical thin-layer chromatogram. Key: 1, salicylazosulfapyridine; 2, salicylazosulfapyridine tablets (recent lot); and 3, salicylazosulfapyridine (a lot several years older).*

intermediate (IV). The second impurity was positioned behind the salicylazosulfapyridine, and the data indicate that it is an isomer of the major component. The third impurity was positioned well ahead of salicylazosulfapyridine and was tentatively characterized by mass spectroscopy as an undiazotized sulfamide (V).

The knowledge acquired from the TLC work was eventually applied to the development of a liquid–solid chromatographic system to separate salicylazosulfapyridine from the impurities for subsequent quantitative analyses.

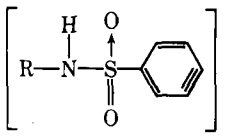
EXPERIMENTAL

Equipment—A spectrophotometer[1], glass columns (2.54 × 25.4 cm, 1 × 10 in.) with stopcocks, and filter paper[2] were used.

Materials—The following were used: acetone, analytical reagent; benzene, analytical reagent; methyl ethyl ketone, analytical reagent; pyridine, analytical reagent; Ottawa sand; glass wool; silicic acid[3], 100–200 mesh; 0.1 *N* sodium hydroxide; and 0.1 *N* acetic acid.

Column Preparation—Place a glass wool pledget in the bottom of the glass column and add 1.5–2 g of sand. Transfer a slurry containing 22 g of silicic acid and 50 ml of acetone into the column. Allow the acetone to drain from the column and then pass benzene

[1] Beckman DBG.
[2] Whatman No. 40.
[3] Mallinckrodt Chemical Co.



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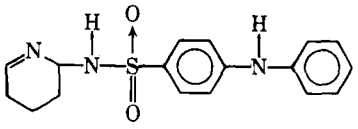

V

through the column until all of the acetone has eluted. Repeat the procedure using acetone to displace the benzene. Maintain a level of solvent (about 5 mm) above the surface of the partition column. Place a piece of appropriately cut filter paper on top of the adsorbent.

Sample Preparation—Weigh approximately 200 mg (±0.1 mg) of salicylazosulfapyridine into a 10.0-ml volumetric flask. Add about 6 ml of pyridine to dissolve the sample and then dilute to volume with pyridine.

Analysis—Place 1.0 ml of the sample solution onto the top of the column and develop using a solvent system of methyl ethyl ketone–acetone–water (16:16:1) at a flow rate of 1–2 ml/min. The first material to elute is yellow and is not collected. The second band is salicylazosulfapyridine and is characterized by an orange color. Collect this material in a 100-ml beaker, taking care not to include material from the yellow band following the major component. Approximately 50–70 ml of eluate is collected.

Remove the solvent by evaporation on a steam bath. Dissolve the sample in a minimum amount of 0.1 *N* sodium hydroxide, quantitatively transfer into a 10.0-ml volumetric flask, and dilute to volume with 0.1 *N* sodium hydroxide. Pipet 5.0 ml of the sample into a 1-liter flask and add about 750 ml of water. Pipet 20.0 ml 0.1 *N* acetic acid into the flask and dilute to volume with water. Determine the absorbance at 359 nm using water as a blank and calculate the percent purity using Eq. 1:

$$\% \text{ purity} = \frac{\text{absorbance} \times 2000}{a\ (\text{ml mg}^{-1}\ \text{cm}^{-1}) \times b\ (\text{cm}) \times \dfrac{\text{wt of sample (mg)}}{10\ \text{ml}}} \times 100 \quad \text{(Eq. 1)}$$

Absorptivity *(a)* was found to be 63.0 from a sample of salicylazosulfapyridine purified by repeated extractions with ether. The resulting material was free of impurities when analyzed by TLC. The literature value of 65.8 was used to allow direct comparison of the data to previously published data.

TLC Analyses—A TLC system was initially developed to monitor the relative purity of bench size and subsequent production size lots of salicylazosulfapyridine. The analysis was performed using silica gel-coated plates and a solvent system of methyl ethyl ketone–acetone–water (4:1:1). The sample was dissolved in pyridine, and about 2 μl of solution was spotted on the plate. The plate was air dried to allow most of the pyridine to evaporate, and the plate was then developed by ascending chromatography (Fig. 1).

Isolation of Impurities—Several attempts were made to use preparatory TLC to isolate the impurities for subsequent identification analyses; none was successful. Consequently, various approaches were investigated including liquid–solid chromatography. When using silicic acid as the adsorbent and methyl ethyl ketone–acetone–water (16:16:1) as the solvent system, resolution equivalent to that achieved by TLC was obtained. The column was prepared by gently tamping a total of 200 g of silicic acid into a glass chromatographic column. A filter paper disk was placed on top of the silicic acid and the column was washed with acetone, followed by benzene, and finally with acetone prior to use.

Approximately 250 mg of sample was dissolved in a minimum amount of pyridine and quantitatively placed on the column and chromatographed using the methyl ethyl ketone–acetone–water system. As in the TLC analysis, one brown immobile material and two yellow mobile impurities were resolved from salicylazosulfapyridine. All three mobile components were collected into individual containers as they eluted from the column.

Table I—Effectiveness of Chromatographic Separation

SW-1 Sample and Conditions	Salicylazosulfapyridine Found[a], %
As is (not chromatographed)	85.2
Salicylazosulfapyridine band from column	77.1
Two mobile impurities from column	5.7
All eluates collected and assayed as a unit	84.1

[a] Lit. (2) absorptivity value of 65.8 was used.

Table II—Evaluation of Salicylazosulfapyridine from Improved Process

SW-2 Sample and Conditions	Salicylazosulfapyridine Found[a], %
As is (not chromatographed)	95.5
Salicylazosulfapyridine band from column	92.0
Impurity preceding salicylazosulfapyridine	0.3
Impurity following salicylazosulfapyridine	4.8

[a] Lit. (2) absorptivity value of 65.8 was used.

To determine the purity and to confirm the relative identity of the collected eluates, each was analyzed by TLC. The first yellow eluate collected corresponded to the material that chromatographed ahead of salicylazosulfapyridine and contained a trace amount of the major component. The second eluate, salicylazosulfapyridine, was free of any detectable impurities. The third eluate corresponded to the material that traveled slower than salicylazosulfapyridine and appeared to be a single component.

RESULTS AND DISCUSSION

Characterization of Impurities—Because of the chromatographic nature of the immobile brown sludge, it was speculated to be structurally different than the major component and the two yellow mobile impurities. During discussions with the manufacturer[4], it was suggested that the brown sludge resulted from the alkaline decomposition of diazotized sulfapyridine. Consequently, diazotized sulfapyridine was decomposed with alkali and the resulting material was found to have the same chromatographic characteristics as the immobile sludge in commercial salicylazosulfapyridine.

The two yellow mobile impurities were assayed spectrophotometrically and found to have significantly different spectra. The later eluting material has a spectrum with maxima at 357, 323, 265, and 237 nm. Qualitatively, the spectrum is the same as salicylazosulfapyridine. Because of the similarity of the spectra and the orienting effect of both the hydroxy and carboxylic groups, it is proposed that this component is a position isomer of salicylazosulfapyridine.

The spectrum of the component that elutes first shows maxima at 323 and 237 nm. The isolated material was examined by mass spectrometry and major peaks were found at 261, 169, 94, 67, 64, and 48 amu. The dissimilarity between spectra of the unknown and salicylazosulfapyridine and sulfapyridine indicates that this unknown moiety is quite different than salicylazosulfapyridine. If it is assumed that SO_2 is present in the molecule, the mass spectra data lend themselves to a component having the proposed structure of an undiazotized sulfamide (V).

Effect of Impurities on Analyses—To determine the amount of contribution from the impurities using the colorimetric method (2), a sample of an experimental lot (SW-1) of salicylazosulfapyridine was assayed. One aliquot of the sample was assayed directly without a chromatographic separation, and a second aliquot was assayed using the proposed chromatographic separation. During the second analysis, the two mobile impurities were also collected as a sample as they eluted from the column and assayed in the same manner as salicylazosulfapyridine. A third sample was run to determine if loss of spectrophotometric absorbing material occurred from the chromatographic process in which all of the eluates were collected and assayed. The data are summarized in Table I.

A second sample (SW-2), synthesized using an improved process, was received from the manufacturer[4]. This sample was assayed in a similar manner to SW-1 except that each mobile impurity was collected and assayed individually; the experiment in which all of the eluates were collected and assayed as a composite was not repeated. During the analysis it was observed that the SW-2 sample contained significantly less of the immobile impurity than did the SW-1 sample (Table II).

Table III—Evaluation of Samples from Other Sources

Sample and Conditions	Salicylazosulfapyridine Found[a], %
Tablets as is	102% label amount
Tablets chromatographed	91% label amount
Powder A as is	94% purity
Powder A chromatographed	82% purity

[a] Lit. (2) absorptivity value of 65.8 was used.

Table IV—Precision of Assay

Sample	Purity Found, %
1	94.6
2	93.5
3	94.0
4	94.0
5	96.0

Analysis of Samples from Alternative Sources—Up to this point, the work was primarily associated with material from one source. In an effort to compare the composition of this material[4], samples of salicylazosulfapyridine tablets[5] and a 1-year-old laboratory sample of salicylazosulfapyridine powder (A) supplied by a second manufacturer[6] were assayed with and without a chromatographic separation (Table III).

Determination of Precision—Using the proposed chromatographic–spectrophotometric method, five aliquots of salicylazosulfapyridine were assayed (Table IV).

A brief statistical analysis shows an average purity of 94.4% and a standard deviation of ±1%. The confidence interval using the Student t test (0.05) was 94.4 ± 2.6% (91.8–97%).

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[x] To whom inquiries should be directed.

[4] Crompton-Knowles.

[5] Azulfidine.

[6] Salsbury Laboratories.

Spectrophotometric Determination of Pivampicillin Hydrochloride

E. MARCHI, G. MASCELLANI ˣ, and D. BOCCALI

Abstract □ A spectrophotometric method for the determination of pivampicillin hydrochloride is described based on the colorimetric reaction of chromotropic acid with formaldehyde, which is formed in a stoichiometric quantity on hydrolysis of pivampicillin hydrochloride. The analytical results are compared with those obtained by traditional methods.

Keyphrases □ Pivampicillin hydrochloride—spectrophotometric determination □ Colorimetry—spectrophotometric determination of pivampicillin hydrochloride □ Spectrophotometry—analysis, pivampicillin hydrochloride

Among the acyloxymethyl esters of ampicillin, interest has been aroused by pivampicillin (1), which can be administered orally with clinical and therapeutic advantages. With this antibiotic, because of its ready absorption and reasonable hydrolysis time, the concentrations of ampicillin reached in the blood in humans are about three times as high, and the quantities recovered from the urine at least twice as high, as those attainable by oral administration of a corresponding dose of ampicillin (1, 2).

It has been necessary to find a precise, accurate, and selective method of analysis for this series of products. Bricker and Johnson (3) adapted for spectrophotometry the well-known reaction between chromotropic acid and formaldehyde, which was proposed for quantitative use (4, 5). On the basis of these reports, a method was developed to obtain formaldehyde by acid hydrolysis of pivampicillin, as shown in Scheme I.

Subsequently, the quinonoid compound formed between chromotropic acid and formaldehyde (6) is determined.

EXPERIMENTAL[1]

Materials and Reagents—Pivampicillin hydrochloride was synthesized in these laboratories and purified to constant spectrophotometric values. The purity was also determined by TLC using 250-μm silica gel[2] GF_{254} plates and developed with methanol–isopropanol–water (50:50:10). Visualization was achieved by spraying with 0.3% ninhydrin in acetone and/or 1.5% sodium azide in 0.1 *N* iodine solution, giving R_f 0.65.

Concentrated sulfuric acid, 1.5 *N* hydrochloric acid, and a freshly prepared 4% aqueous solution of chromotropic acid[3] as the sodium salt were used.

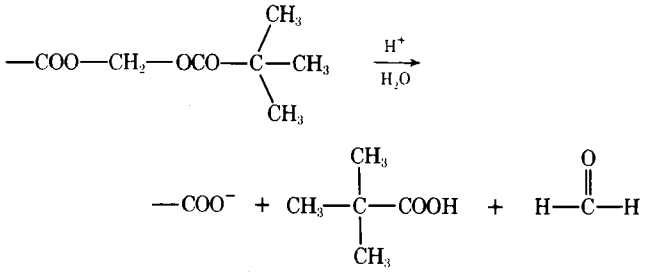
Absolutely aldehyde-free ethanol was prepared as follows. Five milliliters of 0.5% tetrazolium blue[4] in ethanol and 5 ml of 1% tetramethylammonium hydroxide[2] were added to 100 ml of alcohol. The solution was allowed to stand for 2 hr, and the ethanol was then distilled.

[1] A Perkin-Elmer twin-beam spectrophotometer (model 124) with a Perkin-Elmer recorder (model 165), a Beckman DU/2 single-beam spectrophotometer, a complete apparatus for TLC (Chemetron Milan), and a Beckman Expandomatic pH meter were used.

[2] Merck.

[3] Schilling.

[4] C. Erba.



Scheme I

The cupric chloride solution was prepared by dissolving 50 g of copper oxide in 500 ml of 3 *N* HCl in the cold and diluting to 1 liter. A Cu^{+2} concentration of 40 mg/ml was obtained.

Procedure—A sample of 40–80 mg of pivampicillin hydrochloride is accurately weighed and introduced into a 250-ml two-necked flask. A 100-ml aliquot of 1.5 *N* hydrochloric acid and 20 ml of cupric chloride solution are added. This is not necessary for pivampicillin hydrolysis, but cupric chloride keeps (according to experimental evidence) colored products otherwise easily distillable in the steam current. The flask is fitted with a condenser having an outlet adaptor dipping into a flask containing 6 ml of absolute ethanol and 15 ml of distilled water. A second flask containing water is connected in series with the first.

The solution in the distillation flask is heated to boiling (to effect the hydrolysis of the pivampicillin with consequent formation of formaldehyde) and slowly distilled in a gentle current of nitrogen. The distillation is continued until about 10 ml of liquid remains in the distillation flask. The distillate and the water used to wash the condenser, the outlet adaptor, and the receivers are combined and diluted to 200 ml in a graduated flask. The solution obtained in this way contains 0.3 μg/ml formaldehyde/mg pivampicillin hydrochloride in the weighed specimen.

The colorimetric reaction is then carried out in accordance with the details given by Mathers and Pro (7). One or more 0.2–1-ml samples are taken and transferred into an equal number of 25-ml graduated flasks. The volume of each sample is made to exactly 1 ml with 3% ethanol; a 0.5-ml aliquot of 4% chromotropic acid and 7.5 ml of concentrated sulfuric acid are then added to each flask

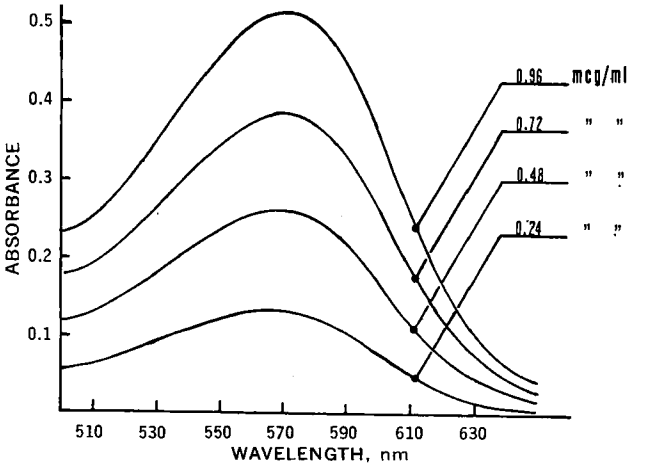


Figure 1—*Absorption spectra of the quinonoid compound of formaldehyde and chromotropic acid at various formaldehyde concentrations (temperature, 65°; heating time, 30 min; and chromotropic acid, 800 μg/ml).*

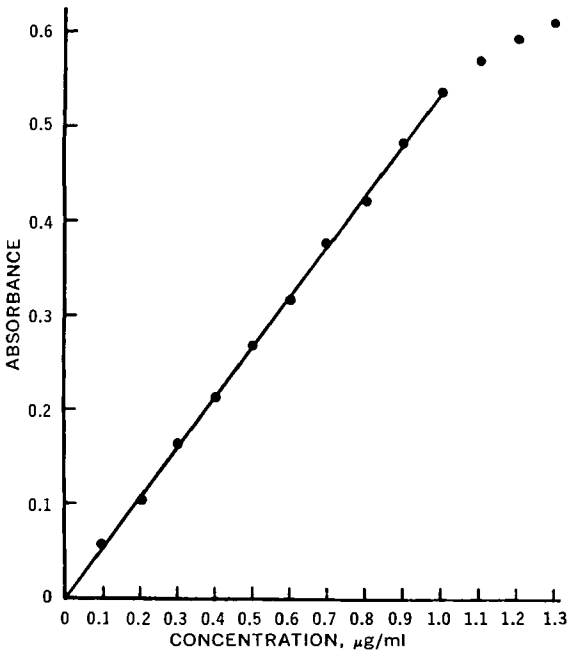


Figure 2—*Calibration curve of the formaldehyde at 570 nm (temperature, 65°; heating time, 30 min; and chromotropic acid final concentration, 800 μg/ml).*

with agitation in a bath of cold water. The solutions obtained are heated on a water bath at 65° for 30 min, immediately cooled, and then diluted to 25 ml with distilled water.

The absorbance of each solution is measured at 570 nm against a blank prepared in the same way but with an identical volume of 3% ethanol instead of the sample. The concentration of formaldehyde in the solution is determined from the absorbance with the aid of a suitable calibration curve, and the concentration of pivampicillin hydrochloride is then found by means of the factor 16.685.

The calibration curve can be constructed with substances that produce formaldehyde, such as methenamine or paraformaldehyde that has been previously standardized by the method described in the British Pharmacopoeia (8) or in the British Pharmaceutical Codex (9), respectively. The two substances, when treated as described, including the distillation, give identical and perfectly reproducible results.

Figure 1 shows some absorbance curves for the quinonoid compound. Figure 2 shows the calibration curve. The calibration curve, obtained by plotting at least 12 average values of the absorbance at 570 nm as functions of the concentrations, shows that the Beer-Lambert law is perfectly satisfied up to a concentration of 1 μg/ml.

According to the experimental results, the molar absorptivity of formaldehyde is $\epsilon = 1.61 \times 10^4$.

The experimental conditions described were chosen on the basis of preliminary investigations.

In agreement with other authors (7, 10), maximum absorbance values are obtained after heating for 30 min at 65°, with other conditions remaining constant. When the reaction is complete, the solution can be kept at ambient temperature for long periods with no appreciable decrease in the absorbance (decrease < 0.1%/min). According to the experiments, the concentration of chromotropic acid should not fall below 4%.

RESULTS AND DISCUSSION

The results obtained by the proposed method were compared with those obtained by two other procedures (Table I) and are the average ($\bar{X}$) and the standard deviation (SD) of six different determinations carried out on five samples of pivampicillin hydrochloride.

The results show that the proposed method is precise and accurate. It is a useful alternative for the determination of pivampicillin in pharmaceutical preparations together with excipients and/or other antibiotics that are not acyloxymethyl esters.

Table I—Comparison of Results Obtained with Different Methods

Pivampicillin Sample	Proposed Method		Ampicillin Method[a]		Microbiological Method[b],
	$\bar{X}$, %	SD, %	$\bar{X}$, %	SD, %	$\bar{X}$, %
1	97.1	1.09	96.2	1.25	101
2	98.6	0.87	98.4	0.83	100
3	99.6	0.92	99.0	0.91	102
4	96.8	1.15	95.3	1.12	99
5	97.7	1.17	99.2	1.05	104

[a] Adapted for pivampicillin hydrochloride (11), from the method proposed by Smith *et al.* (12) for ampicillin. This method has been approved for some time (13–15) and has been extended to other semisynthetic penicillins (16). [b] *Sarcina lutea* ATCC 9341 was used as the test microorganism, according to the general conditions described by Grove and Randall (17).

The formaldehyde–chromotropic acid colorimetric method can also be used for the analysis of all acyloxymethyl derivatives of well-known antibiotics, such as the cephalosporins including pivaloyloxymethyl cephaloglycin (18).

Chromotropic acid, which is mentioned in the British Pharmacopoeia (19) in the test for the identification of penicillins, may also be used under suitable conditions for the quantitative determination of compounds whose molecules contain groups of the type $-N{=}CH_2$ or $>N-CH_2-N<$ that yield formaldehyde on hydrolysis.

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[x] To whom inquiries should be directed.

Colorimetric Assay of Epinephrine

R. B. SALAMA[x] and S. K. WAHBA KHALIL*

Abstract □ A new colorimetric assay method for epinephrine, based on the reaction with thiosemicarbazide in alkaline medium, is presented. Beer's law is followed in the range of 25–300 μg. Data on precision, accuracy, and specificity are included. A special calculation is adopted in the presence of norepinephrine. The method, because of its simplicity, is particularly suited for routine analysis of official preparations of epinephrine.

Keyphrases □ Epinephrine—colorimetric assay, reaction with thiosemicarbazide □ Thiosemicarbazide—reagent in colorimetric assay of epinephrine □ Colorimetry—analysis, epinephrine, reaction with thiosemicarbazide

Several methods for the quantitative determination of epinephrine in pharmaceutical preparations are available. The official methods (1–3) have not proved completely satisfactory because they lack sensitivity to microquantities of the hormone and are time consuming.

In view of these disadvantages, several spectrophotofluorometric methods based on the oxidation of epinephrine to "adrenochrome" have been developed (4–7). These methods show high sensitivity, but the presence of certain antioxidants interferes with the development of the fluorescence (7). Other available methods either require previous separation from interfering substances, especially norepinephrine (8–10), or are based on color reactions given by the phenolic function of the molecule (11–17). Hence, the development of a simple and timesaving colorimetric method specific for the catecholic function is desirable.

EXPERIMENTAL[1]

Reagents—The following were used (all of analytical grade): epinephrine bitartrate, thiosemicarbazide solution (0.10%), and standard sodium hydroxide solution.

Assay Procedure—*Calibration Curve*—Pipet several aliquots of exactly 0.5–3 ml of epinephrine bitartrate solution (180 μg/ml is equivalent to 100 μg pure epinephrine/ml) into 25-ml volumetric flasks. Dilute each to 10 ml with water and then add, in order, 1.0 ml of thiosemicarbazide solution and 1.0 ml of 0.2 *N* NaOH. Mix well and let stand for 30 min. Dilute to volume with water and measure the absorption at 395 and 485 nm against a blank prepared under the same conditions using 1.0 ml of water in place of the thiosemicarbazide reagent.

Sample Preparation and Assay—Measure an aliquot of the epinephrine preparation containing 1.0 mg of epinephrine into a 10-ml volumetric flask and dilute to volume with water. Proceed as described for the calibration curve using 2.0 ml of this solution. Calculate the amount of epinephrine from the calibration curves.

Factors Affecting Color Formation—The absorption spectra were studied following the assay procedure for samples of 200 μg of epinephrine.

Interference—The effect of norepinephrine was determined by adding different amounts (by weight) to an epinephrine solution and performing the analysis according to the assay procedure.

The possible effect of the presence of adrenochrome as an oxidation product was studied. Samples of epinephrine solution were determined before and after oxidation with iodine (7).

The possible interference from other ingredients of epinephrine preparations was studied by adding the specific quantities found in official preparations.

Specificity to Catecholic Function—The procedure was also applied to solutions of other phenols and phenol-ethers with different numbers of hydroxy groups (carbolic acid, catechol, resorcinol, phloroglucinol, cresol, pyrogallol, and guaiacol).

Accuracy—Recovery trials were performed by adding known quantities of epinephrine bitartrate solution to different samples of epinephrine preparations. Analyses were made before and after addition.

[1] The absorption spectra were recorded on a Beckman DU-2 instrument.

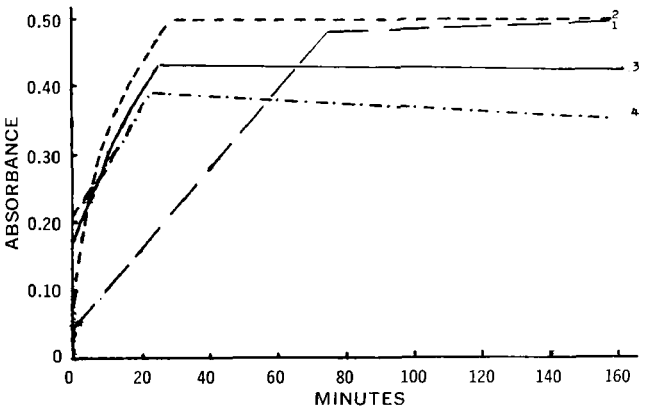

Figure 1—*Influence of pH on color formation. Key: 1, pH 11.0; 2, pH 11.3; 3, pH 11.5; and 4, pH 11.7. These values correspond in the assay to 1.0 ml of sodium hydroxide of 0.1, 0.2, 0.3, and 0.5* N, *respectively.*

RESULTS AND DISCUSSION

A characteristic orange-yellow color with an absorption maximum at 395 nm develops when epinephrine reacts with thiosemicarbazide in alkaline aqueous medium. A standard curve was plotted for various volumes of epinephrine solution. Beer's law was followed over a concentration range of 25–300 μg of pure epinephrine, and the color reached its maximum after 30 min at room temperature (25°) and remained stable for an additional 2 hr.

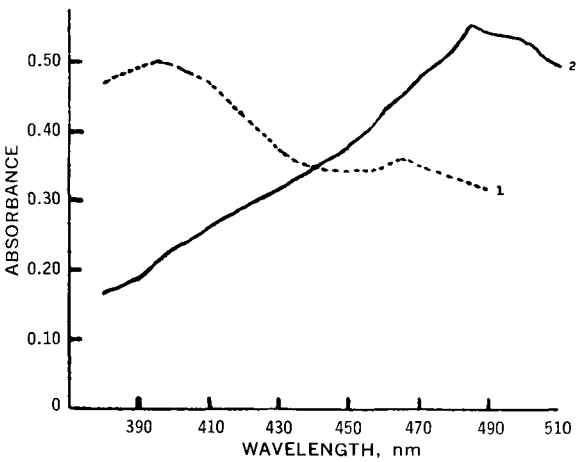

Figure 2—*Absorption spectra of the colors of (1) epinephrine and (2) norepinephrine.*

Table I—Determination of Epinephrine in Presence of Various Amounts of Norepinephrine

Epinephrine, μg	Norepinephrine, μg	Absorbance 395 nm	Absorbance 485 nm	Epinephrine, % Found	Deviation
200	0.00	0.500	0.330	100	0.0
180	20.0	0.480	0.350	90.5	+0.55
160	40.0	0.470	0.385	80.6	+0.75
120	60.0	0.425	0.465	60.3	+0.50

Table II—Colors Produced by Different Phenols and Phenol-Ethers with Thiosemicarbazide Reagent

Compound	Color Produced	Compound	Color Produced
Carbolic acid	None	Cresol	None
Catechol	Orange-yellow	Pyrogallol	None
Resorcinol	None	Guaiacol	None
Phloroglucinol	None		

The effects of temperature, the alkali concentration, and the presence of other chemicals on the color development were studied. Change of temperature had an insignificant effect on chromophore formation. Figure 1 shows that 0.2 *N* NaOH (pH of solution 11.3) is the optimum concentration for the color intensity and stability. The presence of sodium chloride, chlorobutanol, and sodium metabisulfite in the concentrations used in the official preparations produced no effect on color development, intensity, or stability. Adrenochrome produced no color under the experimental conditions. This result is to be expected, since adrenochrome lacks the catecholic function. But norepinephrine, when similarly treated, gave a red color with an absorption maximum at 485 nm and reached its maximum after 30 min. To determine the amount of intact epinephrine in the presence of its decomposition product norepinephrine, the absorption was measured at two wavelengths, 395 and 485 nm, the maxima for epinephrine and norepinephrine chromophores, respectively (Fig. 2). The amount of intact epinephrine was then deduced from the formula:

$$\% \text{ epinephrine} = \left(\frac{a_1 \times b_2}{a_2 \times b_1}\right) \times 100 \qquad \text{(Eq. 1)}$$

where a_1 = absorbance of sample at 395 nm, a_2 = absorbance of sample at 485 nm, b_1 = absorbance of standard epinephrine (200 μg) at 395 nm, and b_2 = absorbance of standard epinephrine (200 μg) at 485 nm.

The method was applied to various concentrations of epinephrine and norepinephrine mixtures (Table I).

It was found (Table II) that mono- and trihydroxybenzene derivatives do not produce any color under the experimental conditions. Moreover, dihydroxy compounds with the hydroxy substituent in the *meta*- or *para*-position fail to give the reaction.

Methylation of one or both hydroxy groups in the catechol molecule also prevents color development. Samples of aqueous epinephrine preparations were analyzed before and after the addition of known quantities of epinephrine solution (Table III).

Table III—Recovery of Epinephrine

Preparation	Epinephrine Added, μg	Absorbance Values Before Addition	Absorbance Values After Addition	Recovery, %	Deviation, %
Injection Solution	100	0.235	0.480	100	0.0
	50	0.230	0.350	100	0.0
Inhalation	100	0.245	0.490	100	0.0
Ophthalmic solution	50	0.245	0.362	97.5	2.5

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* Present address: College of Pharmacy, North Dakota State University, Fargo, ND 58102

x To whom inquiries should be directed.

Comparison of Analysis of Isoniazid and Its Dosage Forms by Several Methods

MARTIN I. BLAKE ˣ, DAVID BODE, and HAROLD J. RHODES

Abstract □ Four assay procedures for determining isoniazid as the powder and in its dosage forms are compared. These include iodometric titration, bromometric titration, nitrite titration, and nonaqueous titration with acetous perchloric acid. The presence of reducing sugars interfered with the iodometric titration. The four methods do not distinguish isoniazid and its hydrazone derivative. However, the presence of hydrazone may be a serious problem since it may interfere with the bioavailability of isoniazid. Ferrocyphen was found suitable as an internal indicator in the nitrite titration method.

Keyphrases □ Isoniazid powder, tablets, and syrup—comparative analysis by iodine, bromometric, nitrite, and nonaqueous titration techniques, effect of sugars in formulation □ Titrimetry, iodine, bromometric, nitrite, and nonaqueous—analysis, isoniazid powder, tablets, and syrup, comparative study □ Sugars—effect on analysis of isoniazid syrup □ Ferrocyphen—evaluation as internal indicator in nitrite titration for isoniazid analysis

Isoniazid and its dosage forms have been official since USP XV. The assay procedure has always involved the addition of an excess of standard iodine solution in the presence of sodium bicarbonate. After a definite standing period, the excess iodine is titrated with standard thiosulfate. The method was first proposed by Canback (1) who analyzed isoniazid powder and commercial tablets of isoniazid. At about the same time, a similar method was described (2) in which sodium hydroxide was used in place of sodium bicarbonate. Several workers (3–5) reported high results with the official procedure because of interference by excipients such as lactose and glucose, which are reducing sugars and susceptible to oxidation by iodine. Also, low results have been noted[1] with the drug entity (3, 6).

The BP (7) recognizes isoniazid and the tablet dosage form. The assay procedure involves residual bromometric titration. Excess bromine is determined by titration with thiosulfate solution. A direct visual bromometric titration procedure was reported (8), and a potentiometric bromometric titration method was described (9). The bromometric method was reported (6) to give results that were 0.5% high. However, the BP method for the drug entity and the tablet dosage form was reported (3) to be reliable, consistent, and superior to the iodine titration procedure.

A nitrous acid titration method was reported (10) using standard sodium nitrite solution as the titrant in the presence of dilute hydrochloric acid, with starch iodide as an external indicator. This is the method described in the USP (11) for the nitrite titration of sulfa drugs. Noxon (5) described a potentiometric titration procedure for analyzing isoniazid tablets based on the nitrite titration procedure for sulfonamides. This has been adapted as a semiautomated procedure by the National Center for Drug Analysis.

Nonaqueous titrimetric procedures have been suggested by several workers (2, 6, 10, 12). The titrant was either perchloric acid in acetic acid or perchloric acid in dioxane. Titrations were performed visually with crystal violet as the indicator or potentiometrically using a glass–calomel electrode system. The titration solvent was an acetonitrile–acetic anhydride mixture, acetic acid, acetic anhydride, or a mixture of acetic acid and acetic anhydride, and these methods have been reviewed (13).

Since 1952, various other methods, *e.g.*, colorimetry, spectrophotometry, titrimetry, and oxidation–reduction methods, have been proposed for the determination of isoniazid and its dosage forms, alone and combined with other therapeutic agents (4, 14). Wu *et al.* (15) investigated the solid-state interaction between isoniazid and the adjuvants magnesium oxide and lactose since it was felt that such solid–solid interactions may adversely affect drug bioavailability. Diffuse reflectance spectroscopic studies indicated that chemisorption and physical adsorption of isoniazid on magnesium oxide did take place. The browning reaction between isoniazid and lactose was also studied by this technique. Hydrazone formation between lactose and isoniazid was detected by TLC. These interactions between isoniazid and lactose apparently do not proceed to any great degree in the dry solid state at ambient temperatures; but in the presence of moisture or high humidity and at elevated temperatures over an extended period, hydrazone formation and the browning reaction could become significant factors.

In liquid dosage forms such as syrups, hydrazone formation is a distinct probability where a reducing sugar is used in the formulation. Rao *et al.* (16) found that in commercial syrups containing isoniazid, as much as 60–70% of the isoniazid became bound as the hydrazone within 24 hr. Studies involving human subjects indicated that absorption of isoniazid from such syrups was severely impaired as a result of this condensation. Furthermore, from a 6-hr absorption study in humans, it appeared that the bound isoniazid was not absorbed nor was there any indication that the bound form was converted to free isoniazid in the stomach or gut. The investigators recommended that sugars including glucose, fructose, and sucrose (though not a reducing sugar *per se*) not be employed in the formulation of isoniazid

[1] J. A. Mollica, Ciba-Geigy Corp., personal communication.

Table I—Analysis of Isoniazid Powder and Its Dosage Forms

Dosage Form	Method of Analysis			
	USP	Nitrite	BP	Nonaqueous
Powder	99.05 ± 0.22[a]	100.03 ± 0.45[b] 100.48 ± 0.28[c]	98.56 ± 0.66	100.46 ± 0.67
Tablets	95.95 ± 0.38	97.96 ± 0.41 95.01 ± 0.20	97.79 ± 0.84	95.78 ± 0.45
Syrup	98.09 ± 0.53	103.07 ± 1.22 102.36 ± 0.62	100.10 ± 0.22	97.53 ± 0.84

[a] Percent recovery based on at least four determinations ± *SD*. [b] Starch iodide indicator. [c] Ferrocyphen indicator.

Table II—Effect of Glucose, Sucrose, and Lactose on Analysis of Isoniazid

Sugar Content, % (w/v)	USP Method Recovery, %		BP Method[a] Recovery, % Isoniazid	Nitrite Method[a] Recovery, % Isoniazid
	No Isoniazid	Isoniazid		
Glucose				
10	57.61 ± 1.15	113.75 ± 1.38	99.99 ± 0.32	99.92 ± 0.84
20	67.89 ± 0.80	121.41 ± 2.39	99.57 ± 0.44	99.70 ± 1.26
30	75.16 ± 0.97	138.25 ± 0.73	98.63 ± 0.44	100.24 ± 0.54
40	78.50 ± 1.59	135.88 ± 2.52	97.23 ± 1.55	101.09 ± 0.12
50	82.07 ± 0.16	136.34 ± 0.32	98.19 ± 0.98	99.43 ± 0.54
Sucrose				
10	0.31 ± 0.23	98.40 ± 0.09	97.56 ± 0.62	99.47 ± 0.57
20	0.80 ± 0.31	98.42 ± 0.22	97.10 ± 0.33	99.69 ± 0.18
30	1.11 ± 0.17	98.56 ± 0.13	97.42 ± 0.07	99.38 ± 0.33
40	1.12 ± 0.06	98.14 ± 0.10	98.40 ± 0.44	98.84 ± 0.31
50	1.11 ± 0.07	98.44 ± 0.09	97.51 ± 0.20	100.10 ± 0.36
Lactose				
10	49.59 ± 1.01	105.37 ± 0.23	94.34 ± 0.31	98.80 ± 0.10
20	63.65 ± 2.67	110.33 ± 0.66	95.33 ± 0.38	97.89 ± 0.30

[a] The percent recovery was 0.00 in the absence of isoniazid.

syrup. Sorbitol, however, appears to be quite acceptable.

In the present study, a comparison is made of the four major methods of assay used for determining isoniazid as the powder and in its dosage forms. These include the iodine titration procedure (USP XVIII), bromometric titration (BP 1968), nitrite titration (proposed for adoption in USP XIX), and nonaqueous titration with perchloric acid. Also included in this study is the effect of the presence of certain sugars on the analytical procedure. Ferrocyphen is evaluated as an internal indicator in the nitrite titration method.

EXPERIMENTAL

Methods of Analysis—*Iodine Titration*—Isoniazid powder and the tablet and syrup dosage forms were analyzed by the procedure described in USP XVIII (11).

Bromometric Titration—Isoniazid powder and tablets were analyzed according to the method described in the BP (7). Isoniazid syrup USP was analyzed by the procedure described in the BP for the tablet dosage form. A 5.0-ml aliquot of the syrup was treated in a similar manner as the aliquot of the powder mass described in the BP assay.

Nitrite Titration—A combination of the procedure described in USP XVIII (11) and that of Noxon (5) was employed. About 100 mg of isoniazid, accurately weighed, was dissolved in 50 ml of a solvent containing 10% potassium bromide in 2 *N* HCl contained in a 100-ml beaker. For the syrup, an accurately measured volume equivalent to 100 mg of isoniazid was taken for analysis. For the tablet dosage form, 20 tablets were weighed and reduced to a fine powder and an aliquot of the powder mass equivalent to 100 mg of isoniazid, accurately weighed, was taken for analysis. The syrup aliquot or the aliquot of the powdered tablet mass was dissolved in 50 ml of 10% potassium bromide in 2 *N* HCl. The procedure then followed was that described in the USP (11) under nitrite titration beginning with "cool to 15°."

A series of nitrite titrations was performed on isoniazid and its dosage forms using ferrocyphen indicator solution. The indicator solution was prepared as directed by Banick and Valentine (17). Titrations were conducted as referred to previously, except 1 ml of indicator solution was added to the titration solution in place of using starch iodide as an external indicator. Solutions were titrated to a violet end-point which persisted for at least 3 min.

Nonaqueous Titration—Isoniazid powder was analyzed by transferring about 100 mg of powder, accurately weighed, to a 100-ml beaker. The powder was dissolved in 50 ml of glacial acetic acid or a mixture of acetonitrile–acetic anhydride (1:1). The solution was titrated potentiometrically with 0.1 *N* perchloric acid in glacial acetic acid or dioxane, prepared and standardized as directed in the USP (11). Titrations were performed with a titrimeter[2] equipped with a calomel-glass electrode system. The calomel electrode was modified by replacing the aqueous bridge in the calomel cell with a saturated solution of potassium chloride in methanol. Increments of 0.1 ml of titrant were added in the vicinity of the end-point. The exact end-point was determined from the inflection in the curve obtained by plotting millivolts *versus* milliliters of titrant added.

Isoniazid tablets were analyzed nonaqueously by weighing and finely powdering 20 tablets. An aliquot of the powder equivalent to about 500 mg of isoniazid, accurately weighed, was treated in

Table III—Analysis of 1-Isonicotinyl-2-D-glucosylhydrazine

Method	Recovery, %
Nitrite	99.71 ± 0.93
USP	103.92 ± 1.20
BP	99.32 ± 0.45
Nonaqueous	100.31 ± 0.17

[2] Fisher model 35.

the same manner as described under isoniazid tablets in the USP (11) up to the point where the directions state: "Pipet 20 ml. of the methanol solution into a beaker." This solution was evaporated to dryness, the residue was dissolved in 50 ml of glacial acetic acid or a mixture of acetonitrile–acetic anhydride (1:1), and this solution was titrated nonaqueously as described earlier.

Isoniazid syrup was analyzed by transferring a 5.0-ml aliquot to a beaker and diluting to about 50 ml with water. The solution was transferred quantitatively to a chromatographic column containing a strong cation-exchange resin[3]. The resin column was prepared and treated as described earlier (18). When the last of the diluted syrup solution just disappeared below the resin surface, water was added to the column until at least 500 ml of eluate was collected. The eluate contained sugar and other nonionic components of the syrup. The column was then eluted with 60 ml of 10% ammonium hydroxide in alcohol. The eluate was evaporated to dryness, the residue was dissolved in 50 ml of glacial acetic acid, and the solution was titrated potentiometrically with 0.1 *N* perchloric acid as described previously.

Interference by Hydrazone Formation—Since isoniazid reacts with reducing sugars to form an isonicotinyl hydrazone, the effect of hydrazone formation on the analysis for isoniazid was studied. 1-Isonicotinyl-2-D-glucosylhydrazine was synthesized according to the method of Fox (19). Its identity and purity agreed with those of the known compound.

The product, crystallized from absolute methanol, melted at 159–161° [lit. (19) 160° dec.]. It showed IR absorption maxima (mineral oil) at 3550–3450 (OH), 1668 (C=N), 1580 (CONH), and 1375 (CH_2OH, CHOH) cm^{-1}. Elemental analysis for carbon, hydrogen, and nitrogen content of a sample (98% purity) gave the following results:

Anal.—Calc. for $C_{12}H_{17}N_3O_6$: C, 48.16; H, 5.73; N, 14.04. Found: C, 48.18; H, 5.95; N, 13.89.

1-Isonicotinyl-2-D-glucosylhydrazine was analyzed by the four procedures described earlier.

Interference by Sugars—A series of solutions was prepared containing the following sugars: sucrose, 10, 20, 30, 40, and 50%; glucose, 10, 20, 30, 40, and 50%; and lactose, 10 and 20%. Each solution contained 10 mg isoniazid/ml. A similar series of the solutions contained no isoniazid and served as controls. All solutions were analyzed by the iodometric, bromometric, and nitrite titration procedures for isoniazid content as described under the specific procedure.

RESULTS AND DISCUSSION

The analysis of isoniazid as the drug entity, in dosage forms, and in combination with other drugs has been the subject of extensive study over the past 20 years. While many procedures have been proposed for the assay, the USP and BP have only recognized the iodometric method and the bromometric procedure, respectively. The present study was undertaken because of shortcomings reported for the iodometric method. As a result of this investigation, the nitrite titration procedure has been recommended for adoption by the USP for isoniazid and its dosage forms.

Four methods of assay for isoniazid and its dosage forms were subjected to a comparative study (Table I). No significant difference was noted in the analysis of the powder by the four procedures. The iodometric, nitrite, and bromometric methods were rapid and accurate, producing readily discernible end-points and quantitative recovery data with good precision. The nonaqueous procedure did not permit a suitable visual titration, although satisfactory results were obtained by potentiometric titration. However, this is time consuming since plotting of data is required for end-point determination. Consistent results were obtained for the analysis of the tablet dosage form by the four methods. Data are also reported in Table I for the analysis of a commercial syrup containing isoniazid.

Difficulties were encountered when the nonaqueous titration procedure was applied to the analysis of the syrup dosage form. In a preliminary study, an aliquot of the syrup was heated at 70–80° until all volatile material was driven off. The thick semisolid residue was dissolved in glacial acetic acid and titrated potentiometrically with acetous perchloric acid. No detectable end-point was obtained. The presence of sugars apparently interferes and prevents successful nonaqueous titration. This problem was resolved by passing the syrup aliquot through a cation-exchange resin which extracted the isoniazid. Washing the column thoroughly with water assured removal of the sugar and other nonionic components. Elution of the isoniazid with ammonium hydroxide in ethanol and subsequent evaporation of the solvent produced pure isoniazid which was then titrated nonaqueously. While quantitative recovery data were obtained, the method was overly time consuming and burdensome.

The nitrite titration procedure produces equally satisfactory results whether end-point detection is effected potentiometrically, using a saturated calomel–platinum electrode system, or visually, using starch iodide paper as an external indicator or ferrocyphen indicator solution as an internal indicator.

It was noted earlier (3–5), and confirmed by supplied data[4], that certain sugars interfere in the analysis of isoniazid by the USP XVIII method. A systematic study was undertaken to determine the extent of interference by glucose, sucrose, and lactose in the assay for isoniazid (Table II). The poor solubility of lactose permitted the study of only two concentration levels.

Wu *et al.* (15) and Rao *et al.* (16) showed that glucose and lactose may form a hydrazone with isoniazid in solid and liquid dosage forms. It was of interest to determine whether hydrazone formation does, in fact, interfere with the official assay for isoniazid. Also of concern was whether reducing sugars would interact with the iodine solution titrant, thus causing high results. It is apparent from the data in Table II that sugars in the absence of isoniazid do react positively to the USP assay for isoniazid. Even sucrose, which is not a reducing sugar, does titrate in high concentrations with iodine solution, although interference in the assay in the presence of isoniazid was not perceptible. No interference by sugars was noted with the bromometric or nitrite procedures. Titrant was not consumed in the absence of isoniazid, and quantitative recoveries are reported for syrups containing isoniazid.

The isonicotinyl hydrazone of glucose was synthesized and characterized as described under *Experimental*. The hydrazone was assayed by the four procedures studied (Table III). The official method produced results that were somewhat higher than theory. The iodine may have interacted with glucose formed by hydrolysis of the hydrazone during the assay procedure. This problem was not pursued. If hydrazone formation does occur in the tablet or syrup dosage form, interference with the nitrite, BP, and nonaqueous procedures would not be expected; *i.e.*, quantitative recovery for isoniazid would be obtained whether the isoniazid was free or combined as hydrazone. In other words, these methods do not distinguish isoniazid from its condensation product. It was shown earlier that the official method cannot be used in the presence of reducing sugars. Although hydrazone formation does not interfere with the three methods indicated, its presence may represent a more serious problem in terms of the interference with the bioavailability of isoniazid, as noted by Rao *et al.* (16). This aspect of the problem was beyond the scope of this study.

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[x] To whom inquiries should be directed.

PHARMACEUTICAL TECHNOLOGY

Automated Fluorescence Analysis of Perphenazine in Dissolution Rate Testing of Tablet Formulations

J. M. KONIECZNY and E. McGONIGLE [x]

Abstract □ An automated fluorescence method is described for determining perphenazine in large numbers of samples, as might be required in dissolution studies. Tablets containing perphenazine and amitriptyline were dissolved in 0.1 *N* HCl. Approximately 5 ml was transferred to the automated system where amitriptyline was removed as the ion-pair by extraction with chloroform and discarded. Perphenazine in the aqueous phase was reacted with permanganate solution to form a fluorophore which is quantitative. No sample blank was required. Analyses may be carried out at a rate of 30/hr. Sensitivity was approximately 0.1 μg/ml with a relative standard deviation of ±1.7%.

Keyphrases □ Perphenazine from perphenazine–amitriptyline tablets—automated fluorescence analysis in dissolution studies □ Dissolution rate testing of perphenazine–amitriptyline tablets—automated fluorescence analysis of perphenazine □ Fluorometry—analysis, automated, perphenazine from perphenazine–amitriptyline tablets during dissolution studies

A procedure based on the reaction between palladium (II) chloride and certain phenothiazines, including perphenazine, was reported previously (1, 2). This procedure was shown to be specific for perphenazine in the presence of certain oxidative degradates, specifically the sulfoxide. However, some interferences were encountered when applying this procedure to determinations of perphenazine. The interferences were due to excipients and could be avoided only by employing sample blanks. Furthermore, the palladium chloride assay was not sensitive enough to determine the low levels of perphenazine found in dissolution studies (0.5–5 μg/ml).

Mellinger and Keeler (3) described the identification of some phenothiazine drugs, including perphenazine, by monitoring the fluorescence obtained after reacting the drugs with potassium permanganate. This procedure has been automated and applied to the determination of perphenazine in dissolution studies of perphenazine–amitriptyline[1], a tranquilizer–antidepressant combination, in various proportions. Amitriptyline was removed by extraction prior to the permanganate reaction because it (or the oxidative products) interfered with the fluorescence of perphenazine. No sample blank was required.

EXPERIMENTAL

Materials—The following were used: chloroform, hydrochloric acid (0.1 *N*), and potassium permanganate solution (0.01% in 0.1 *N* HCl). Methanol and 0.1 *N* HCl served as purge solutions. All materials were reagent grade.

Apparatus—The analytical train consisted of the following: a liquid sampler II[2] and a proportioning pump[2], model I; a recording fluorescence spectrophotometer[3] equipped with a ratio mode and a flow cell (0.5-ml capacity and designed to measure fluorescence at an angle of 90° to the excitation beam); and a recorder[4].

[1] Triavil, Merck & Co.
[2] Technicon Corp., Tarrytown, N.Y.
[3] MPF-2A, Perkin-Elmer Corp., Norwalk, Conn.
[4] Sargent model SRL.

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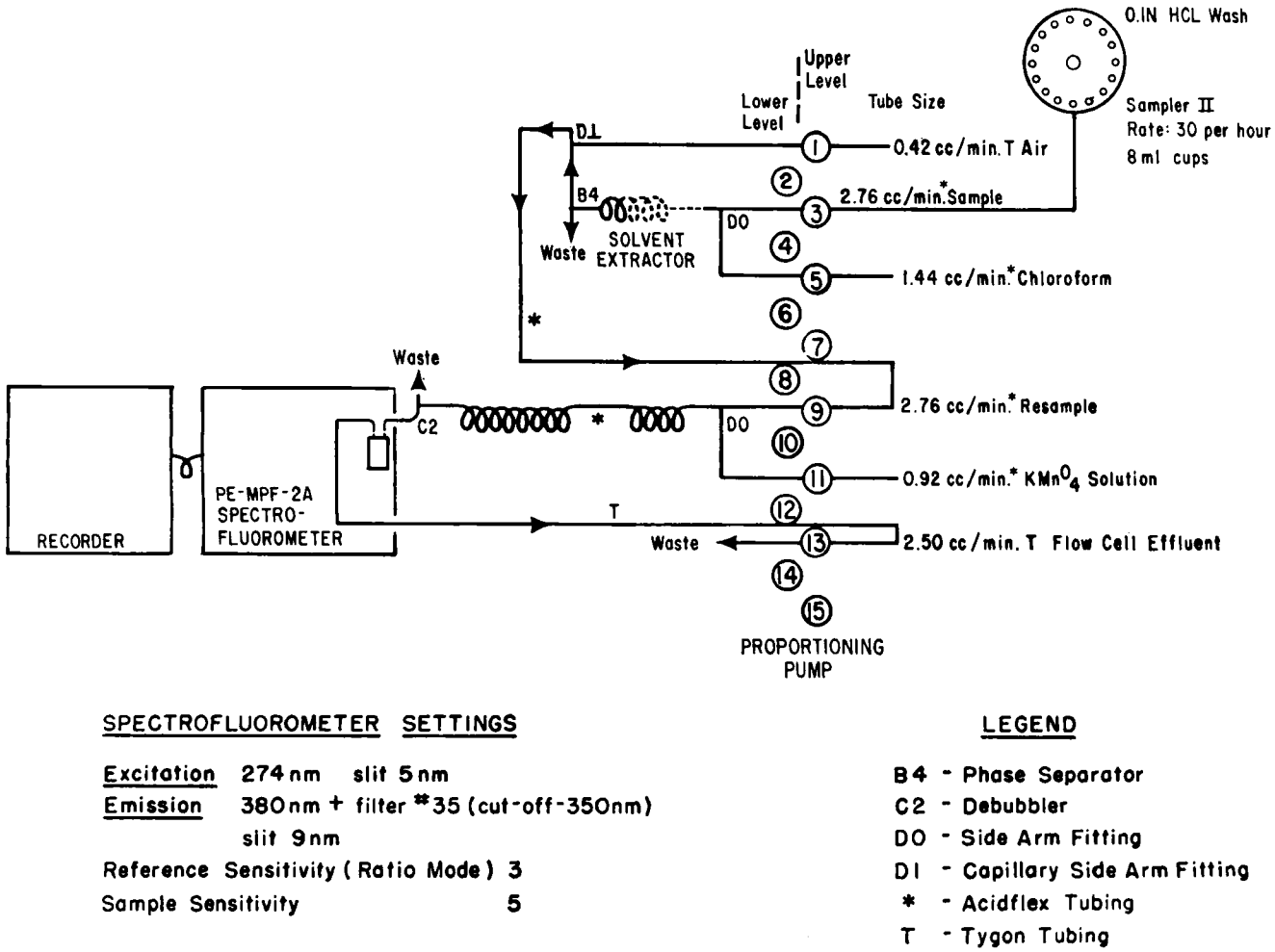

Figure 1—*Flow diagram for automated fluorescence perphenazine assay.*

Standard Preparation—Standards were prepared in 0.1 *N* HCl at concentrations of 1.2, 1.8, 2.4, and 3.0 μg/ml. For dissolution studies, this was equivalent to a sample concentration range of 45–110% of tablet claim. All standards were stored away from sunlight. Ordinary laboratory light was acceptable. Standards were prepared fresh daily.

Sample Preparation—In dissolution studies of tablets containing 2 mg of perphenazine, no dilution was required (sample concentration 0–2 mg/750 ml).

In dissolution studies involving tablets containing 4 mg of perphenazine, standard concentrations can be doubled to avoid sample dilution. (A lower instrument sensitivity can be employed.)

Approximately 5 ml of solution, sampled through a fritted gas dispersion tube, was transferred to the automatic sampler. Samples were stored away from sunlight.

Procedure—A schematic diagram of the automated system is shown in Fig. 1. All tubing was rinsed with methanol for 2 min and with 0.1 *N* HCl for 2 min prior to the introduction of reagents. The 0.1 *N* HCl was introduced first, followed by chloroform and potassium permanganate solution.

The most concentrated standard was introduced and the spectrofluorometer was adjusted as follows: excitation wavelength, a maximum near 274 nm; emission wavelength, a maximum near 380 nm; excitation slit, 5 nm; emission slit, 9 nm; emission cutoff filter, No. 35 (cuts out wavelengths below 350 nm); reference sensitivity, 2; and sample sensitivity, 5.

The sampling rate was 30/hr. The standard highest in concentration was included after every sixth sample to monitor possible

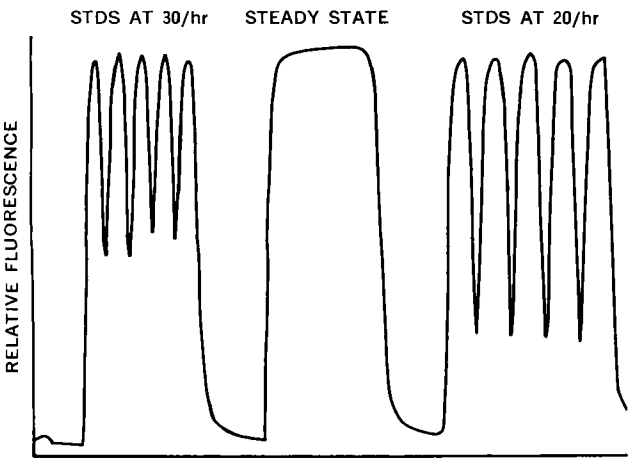

Figure 2—*Strip chart showing system response of standards at 3 μg/ml at rates of 20 and 30/hr compared to steady state.*

Table I—Correlation between Manual Palladium Chloride and Automated Fluorescence Methods

Lot	Palladium Chloride, % Claim	Automated Fluorescence, % Claim
1	104	102
2	101	101
3	108	107
4	99.5	98.0
5	94.5	94.5
6	95.5	94.5
7	96.0	98.0

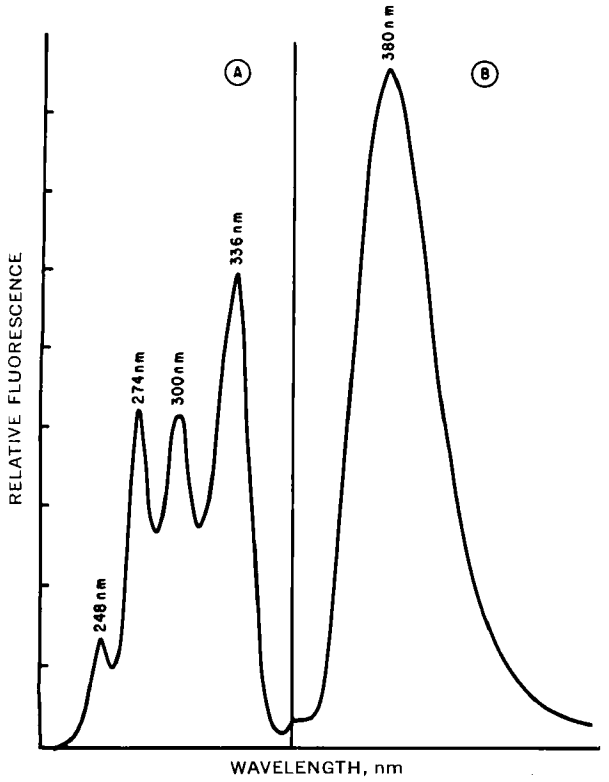

Figure 3—*Excitation (A) and emission (B) spectra for permanganate-oxidized perphenazine fluorophore.*

drift. Steady-state curves along with the flow response of standards sampled at rates of 20 and 30/hr are shown in Fig. 2.

DISCUSSION

The excitation spectrum of permanganate-oxidized perphenazine consists of four maxima of different intensities near 248, 274, 300, and 336 nm. The emission spectrum consists of one maximum near 380 nm. (See uncorrected spectra, Fig. 3.) Maximum sensitivity and reproducibility with minimum background interferences (<1%) from reagents and excipients were obtained when fluorescence was measured at an excitation wavelength near the maximum of 274 nm (emission at 380 nm). A filter fluorometer did not provide this resolution and lacked sufficient sensitivity. A fluorescence spectrophotometer[3] equipped with a xenon source was employed. The excitation slit was set at 5 nm for optimum resolution. The ratio mode incorporated in the amplifier of this equipment provided a constant output signal for several hours.

At an excitation wavelength of 274 nm and an emission wavelength of 380 nm, the fluorophore was stable for 3–10 min after addition of permanganate solution. The analytical train was arranged so that reaction time was 4 min. The fluorophore was not as stable at other excitation maxima.

Amitriptyline distorted the excitation spectrum, causing positive interference in some areas and quenching in others. This difficulty was avoided by extracting amitriptyline as an ion-pair from 0.1 *N* HCl using chloroform. In the analytical train, this operation was carried out before the addition of permanganate solution using an extraction coil and a phase separator, with the chloroform going to waste.

Ordinary laboratory light had no effect on fluorophore stability. However, solutions of perphenazine in 0.1 *N* HCl (20 μg/ml) exposed to sunlight for 2 hr on a windowsill exhibited a 15–20% loss when assayed by permanganate oxidation–fluorescence. Throughout the analysis, solutions of perphenazine and the analytical train were protected from sunlight.

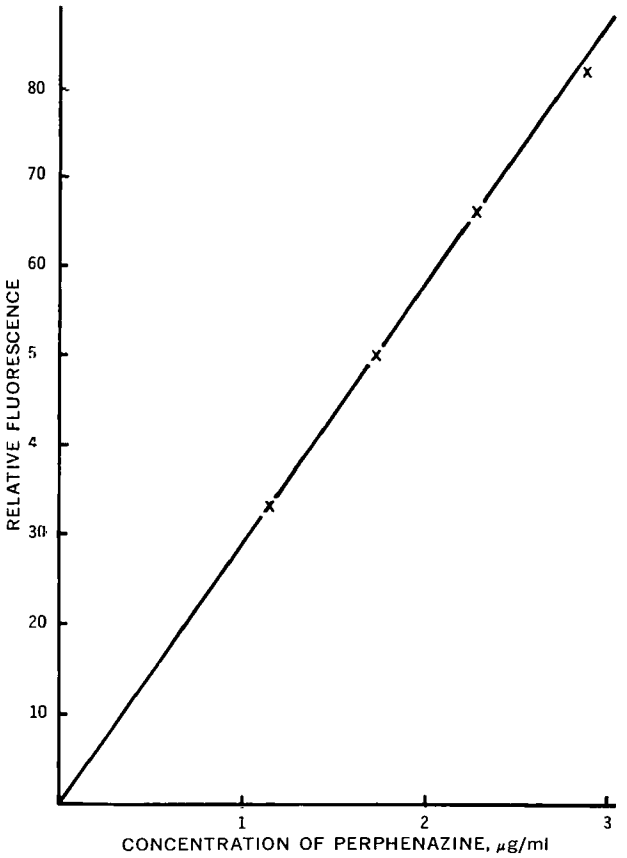

Figure 4—*Plot of fluorescence* versus *concentration of permanganate-oxidized perphenazine fluorophore.*

RESULTS

Following the described procedure, the relative fluorescence of perphenazine over a concentration range of 1.2–3.0 μg/ml was a straight line passing through the origin (Fig. 4). An identical tracing was obtained over the same concentration range for perphenazine plus a placebo containing all excipients including amitriptyline. Recovery from the placebo was 100% (at sampling rates of 20 and 30/hr), indicating that there was no positive interference and no quenching due to excipients.

Analyses of 32 standard solutions over a concentration range of 1.2–3.0 μg/ml plus all ingredients of a perphenazine–amitriptyline product[1] gave a relative standard deviation of ±1.7%. The samples were run at a 30/hr rate in staggered sequence. Sample peaks were 93–95% of steady state, depending upon the sampling rate (Fig. 2).

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[x] To whom inquiries should be directed.

Rapid Evaluation of Effect of Excipients on Color Fading II

P. TURI [x], R. A. TAUSENDFREUND, A. F. MICHAELIS, and C. STEREN

Abstract □ Model solutions of certified dyes (FD&C Blue No. 2, FD&C Yellow No. 5, and FD&C Yellow No. 6) were exposed, alone or with added selected excipients, in a fadeometer to accelerate light-induced color fading. At predetermined intervals, the solutions were tested spectrophotometrically. The method is a valuable rapid screening technique for predicting the comparative light stability of dyes used in pharmaceutical formulations.

Keyphrases □ Colorants, FD&C Blue No. 2, FD&C Yellow No. 5, and FD&C Yellow No. 6—rapid evaluation of effect of excipients on color fading □ Certified dyes—effect of excipients on color fading, method for evaluation □ Dyes, certified—effect of excipients on color fading, method for evaluation

Coloring agents have been used in medicine since ancient times to achieve a more attractive appearance. Recently, colors have become even more important in pharmaceuticals due to the necessity of rapid product identification. Natural dyes, usually obtained from the plant or animal kingdom, were gradually replaced in modern medicine by synthetic products with well-defined stability and uniformity. However, many artificial coloring agents previously permitted for foods and pharmaceuticals were recently eliminated or their use was severely restricted because of intensive research showing carcinogenesis or teratological damage in animals when massive doses of the coloring agents were used. "Martindale's Extra Pharmacopoeia" (1) lists 39 synthetic coloring agents used in food products. The size of this list, however, is sharply reduced if one selects only those approved in most industrialized countries, because the legislation regulating colorants varies widely. Only three synthetic coloring agents are almost universally accepted (2): FD&C Yellow No. 5, FD&C Yellow No. 6, and FD&C Blue No. 2. The objective of this study was to develop a rapid screening method for evaluating the compatibility of these three coloring agents with generally used excipients in pharmaceutical formulations.

Table I—Composition of Sample Solutions

Colorant		Additive	
Name	%	Name	%
FD&C Yellow No. 5	0.025	—	—
	0.025	Lactose	0.25
	0.025	Sucrose	0.25
FD&C Yellow No. 6	0.025	—	—
	0.025	Lactose	0.25
	0.025	Sucrose	0.25
FD&C Blue No. 2	0.030	—	—
	0.030	Lactose	0.75
	0.030	Sucrose	0.75
	0.030	Poloxalene	0.075
	0.030	Polyethylene glycol 6000	0.075
	0.030	Polyvinyl-pyrrolidone	0.075
	0.030	Citric acid	0.075
	0.030	Ascorbic acid	0.015

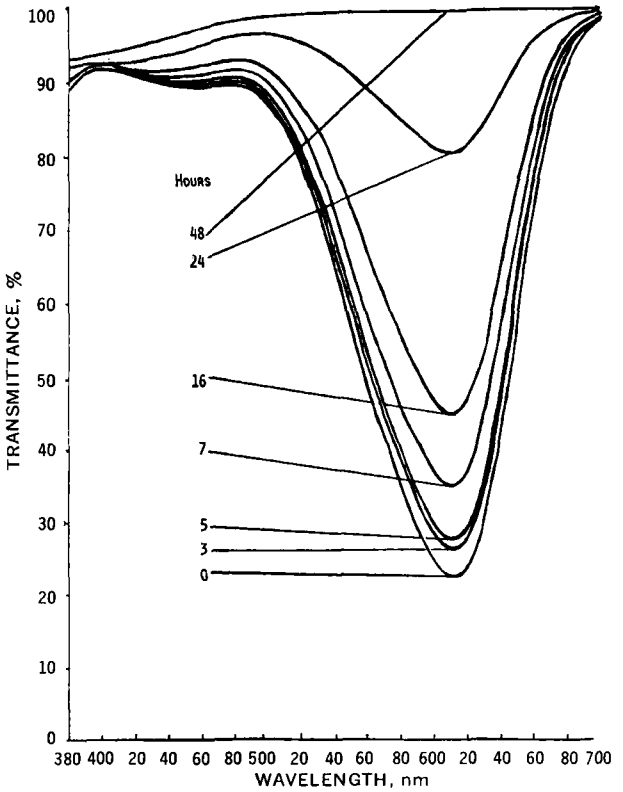

Figure 1—*Typical transmittance curves of dye solutions exposed in the fadeometer.*

It was reported earlier (3) that the fadeometer can be used successfully to predict the color stability of tablets. In the present study, the fadeometer was used also as the principal tool to obtain accelerated data on the color stability of model solutions prepared with the three selected colorants. Frequently used water-soluble excipients were added to the model solutions to study their effects on fading. These results were compared with those obtained using standard, more time-consuming techniques.

EXPERIMENTAL

Materials—The following were used: FD&C Yellow No. 5 (tartrazine), FD&C Yellow No. 6 (yellow orange S), FD&C Blue No. 2 (indigo carmine), lactose USP, sucrose USP, polyvinylpyrrolidone, poloxalene [poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene)], polyethylene glycol 6000, citric acid USP, and ascorbic acid USP.

The listed materials were obtained from commercial sources in reagent or pharmaceutical grade and were used without further purification.

Equipment—A light stability cabinet, equipped with 12 30-w

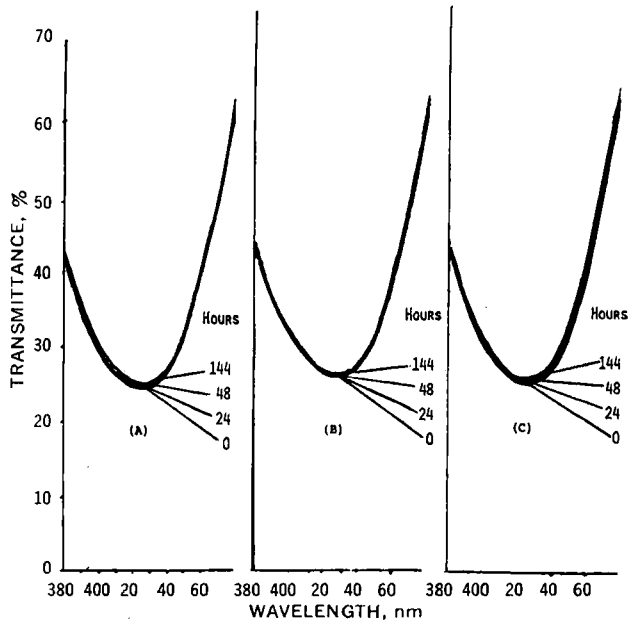

Figure 2—*Fading of FD&C Yellow No. 5 solutions. Key: (A), dye only; (B), dye plus lactose; and (C), dye plus sucrose.*

fluorescent tubes[1] and four 20-w fluorescent tubes[2], was used. The light was adjusted so that the illumination at the surface of the center of the shelf was 1100 foot candles as measured with a foot candle light meter[3].

The fadeometer[4] was equipped with a carbon arc, 167.4 w/ft^2,

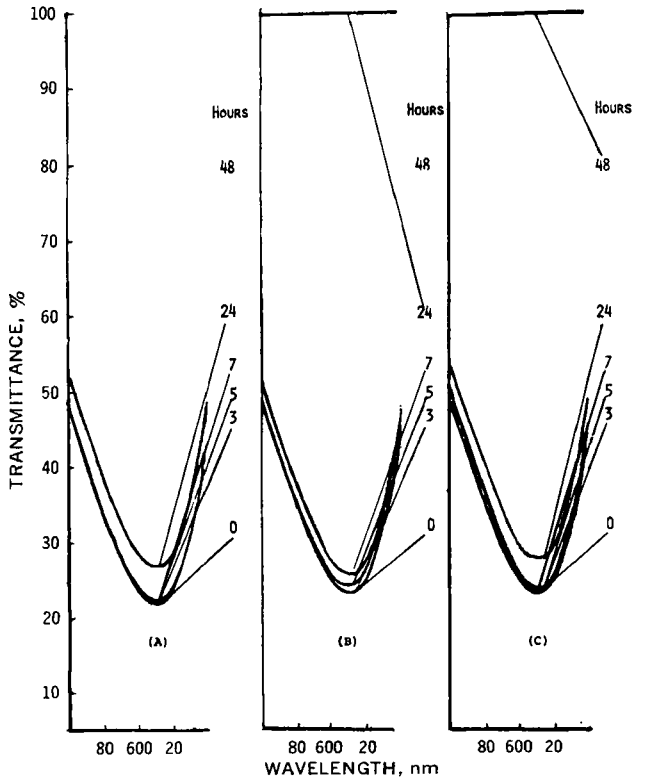

Figure 3—*Fading of FD&C Blue No. 2 solutions. Key: (A), dye only; (B), dye plus lactose; and (C), dye plus sucrose.*

[1] Cool White, Westinghouse, F30T8/CW.
[2] Cool White, General Electric F20T12/CW.
[3] Gossen Tri-Lux.
[4] Atlas Fade-Ometer, model FO-2428, Type FDA-R.

Table II—Discoloration of FD&C Blue No. 2 Solutions with Excipients Using the Fadeometer

Excipient	$t_{90\%}$, hr	Relative Order[a]
None	42.0	0
Poloxalene	30.0	1
Polyethylene glycol 6000	21.4	2
Polyvinylpyrrolidone	3.5	3
Citric acid	2.4	4
Ascorbic acid	1.5	5

[a] Relative order of initial fading velocity values.

sample distance 25.4 cm (3). Two recording spectrophotometers were utilized[5,6].

Preparation of Samples—The applied solutions are listed in Table I. Aliquots (12 ml) of the solutions were transferred into transparent glass containers and exposed in the fadeometer or the light cabinet. At predetermined intervals, the samples were withdrawn, 5.0-ml aliquots of the irradiated solutions were diluted to 100.0 ml, and transmittance (or absorbance) curves were obtained with the aid of a spectrophotometer. (A typical set of transmittance curves is shown in Fig. 1.) Total exposure time was 24–144 hr in the fadeometer and up to 12 days in the light cabinet.

RESULTS AND DISCUSSION

The earlier study using tablets showed that the intensive light source of the fadeometer caused a discoloration about 50–100 times greater than the light cabinet; therefore, a 24-hr exposure in the fadeometer was usually sufficient to judge the relative color stability of the system (3). In the first of the recent experimental series, the sample solutions were exposed in the fadeometer for 24 hr; however, the yellow solutions showed practically no fading during this time. Therefore, the experiment with the yellow solutions was continued in the fadeometer for up to 144 hr. Figure 2 shows the transmittance curves of the exposed FD&C Yellow No. 5 solutions.

Apparently, these solutions did not fade at all in 24 hr. FD&C Yellow No. 6 solutions yielded similar curves, indicating very good stability under the experimental conditions. On the other hand, the FD&C Blue No. 2 solutions faded quite signifi-

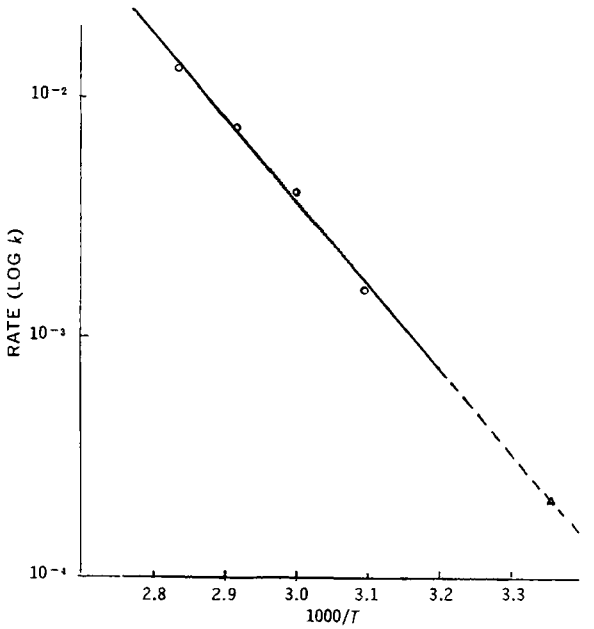

Figure 4—*Arrhenius plot for fading of 0.03% FD&C Blue No. 2 solution (pH 7).*

[5] Model "Hardy," General Electric.
[6] Cary model 14.

Table III—Discoloration of FD&C Blue No. 2 Solutions with Excipients Using the Light Cabinet

Excipient	$t_{90\%}$, days	Relative Order[a]
None	9.1	0
Poloxalene	4.3	1
Polyethylene glycol 6000	3.3	2
Polyvinylpyrrolidone	0.7	3
Citric acid	0.6	4
Ascorbic acid	0.2	5

[a] Relative order of initial fading velocity values.

cantly (Fig. 3). After 24 hr, the dye solution without additive was visibly faded and the excipients had accelerated the discoloration so rapidly as to show 100% transmittance at 610 nm in 24–48 hr. The log A values plotted *versus* time showed a rather slow initial rate (correlated to the induction period), followed later by a rapid discoloration. Since a 10% decrease in the color is observable, $t_{90\%}$ values were determined from the initial fading rates using the following equation:

$$t_{90\%} = \frac{2.303}{k} \log \frac{100}{90} \qquad \text{(Eq. 1)}$$

The data showed that the addition of the lactose accelerated the discoloration significantly, while the sucrose increased the fading at a much slower rate: $t_{90\%}$ (in hours), dye only, 42.0; dye plus lactose, 15.8; and dye plus sucrose, 36.5 (fadeometer exposure).

For the purposes of this study, the FD&C Blue No. 2 (indigo carmine) was selected as the model for further investigation. The buffered dye solution (pH 7) at room temperature fades comparatively slowly. Rate-temperature dependence curves for 0.03% FD&C Blue No. 2 solution were obtained in the usual manner by determining the absorbance maxima of the light-protected solutions kept at various temperatures. By extrapolating the log k *versus* $1/T$ plot, the half-life was found to be 234 days at 25° (Fig. 4).

The degradation of indigo carmine has been investigated (4–10). It was shown that, among others, organic acids, reducing agents, polyalcohols, and certain ions accelerate the color transition from dark blue to pale yellow. In the present study, selected pharmaceutical excipients belonging to one or more of these groups of chemicals were added to the dye solution and exposed in the fadeometer and the rates of discoloration were determined in the described manner (Table II).

For the purpose of comparison, the solutions listed in Table II were also kept in the light cabinet for up to 256 hr, exposed to 1100 foot candles illumination. Fading rates from this test series are listed in Table III.

As expected, the solutions in the light cabinet faded at a slower rate but in the same relative order of fading velocity as in the fadeometer.

It is evident that the fading of the indigo carmine solution is accelerated in the presence of the tested excipients. Other conditions being constant, the rates vary with the type of additive. In solid dosage forms, however, the interaction of these excipients and the rate of fading may be different. In a followup study, model tablets incorporating the listed excipients will be exposed to the fadeometer with the aid of a suitable holder and the rate of discoloration will be determined from the periodically obtained reflectance curves (3) to compare the actual fading of the tablets with the discoloration pattern of the dye solutions.

CONCLUSIONS

This study shows that the fadeometer method of exposing dye solutions with prospective excipients and the subsequent spectrophotometric testing for discoloration appears to be a rapid screening technique for aiding the pharmaceutical formulator in selecting the most suitable additives and eliminating those capable of accelerating the rate of fading.

In addition, the results of the experiments with FD&C Blue No. 2 corroborate earlier investigations and indicate the following:

1. Lactose accelerates the discoloration of the dye solution more than the nonreducing sucrose; however, due to its hydrolysis, even sucrose promotes the fading of the solution.

2. The poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene) wetting agent and polyethylene glycol have only a moderate effect on the fading rate.

3. Polyvinylpyrrolidone and particularly the strongly reducing ascorbic acid and polycarboxylic citric acid cause a rapid fading of the indigo carmine solution.

Under the experimental conditions, solutions of FD&C Yellow No. 5 and FD&C Yellow No. 6 (alone or with added lactose or sucrose) show practically no fading when exposed to the accelerated light effect of the fadeometer.

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[x] To whom inquiries should be directed.

Urinary Metabolites of D,L-1,2-Bis[5-(6)-methoxy-2-benzimidazolyl]-1,2-ethanediol in Mice

NELSON R. TRENNER, JOSEPHINE R. CARLIN, BYRON H. ARISON, and W. J. A. VANDENHEUVEL ˣ

Abstract □ The major urinary metabolite of D,L-1,2-bis[5-(6)-methoxy-2-benzimidazolyl]-1,2-ethanediol in the mouse was isolated and identified by various chromatographic and spectrometric techniques as the mono-*O*-demethylated phenol. Minor urinary components include the parent drug and a conjugate of the major metabolite.

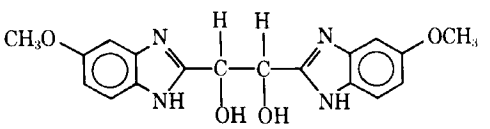
Keyphrases □ D,L-1,2-Bis[5-(6)-methoxy-2-benzimidazolyl]-1,2-ethanediol—urinary metabolites isolated and identified, mice □ Metabolism—urinary metabolites of D,L-1,2-bis[5-(6)-methoxy-2-benzimidazolyl]-1,2-ethanediol isolated and identified, mice □ Urinary metabolites—isolation and identification of D,L-1,2-bis[5-(6)-methoxy-2-benzimidazolyl]-1,2-ethanediol metabolites, mice

The inhibitory effect of D,L-1,2-bis[5-(6)-methoxy-2-benzimidazolyl]-1,2-ethanediol (I) on poliovirus was first demonstrated by Akihama *et al.* (1). More recently, data were presented indicating this compound to be a potent inhibitor of rhinoviruses and its absorption, distribution, and excretion in mice were reported (2). The isolation and identification of the urinary metabolites of I in the mouse are now reported.

EXPERIMENTAL

Urine from 30 mice (20 g, ICR/Ha strain) injected with I (68 mg/kg ip; 2-^{14}C-labeled) was collected for 18–20 hr; the pooled urine contained 45% of the dosed radioactivity. Extraction of an aliquot of the pooled mouse urine with 2 equal volumes of ethyl acetate at pH 6.5 removed 75% of the total urinary radioactivity.

Analytical TLC [silica gel G plates developed with isopropanol–ethyl acetate–ammonium hydroxide (7:9:5)] of this extract gave a zone of R_f 0.57, which accounted for >90% of the extracted radioactivity. A small amount of parent drug (R_f 0.68) was also observed. A reverse isotope dilution assay (3) indicated that it accounts for only 5% of the total radioactivity in the urine. Ion-exchange purification of the metabolite was effected by adsorption on a styrene-type cation-exchange resin[1] (sulfonic acid, ammonium cycle) and elution with 10% ammonium hydroxide in methanol. The effluent contained 21% and the eluate contained 63% of the charged radioactivity. This eluate was further purified by adsorption on a styrene-type anion-exchange resin[2] (quaternary ammonium, acetate cycle). The eluate obtained with 10% acetic acid in methanol contained 51% of the original urinary radioactivity. This material was subjected to preparative TLC with the previously described system. The major zone (R_f 0.57) was eluted from the plate with methanol and was found to contain 36% of the original urinary radioactivity. The TLC eluate was evaporated to dryness and partitioned between ethyl acetate and water; 92% of the radioactivity passed into the organic solvent. Removal of the ethyl acetate *in vacuo* yielded 2.2 mg of a solid, II, which possessed a specific activity 70% that of the parent drug.



I

NMR spectroscopy[3] was carried out in deuteromethanol. Mass spectra[4] were obtained *via* direct probe using a trap current of 60 μamp, an ionizing potential of 70 ev, an accelerating potential of 3.5 kv, and a source temperature of 270°. Derivatization with bis(trimethylsilyl)acetamide[5] (III) (4) directly in a probe tube (Procedure A) involved treatment of the sample (~5 μg) with 5 μl III; the tube was immediately (<30 sec) inserted into the vacuum lock, and the reagent was pumped off through the vacuum system of the spectrometer. The more vigorous derivatization (Procedure B) involved heating the sample (5–10 μg) in III (20 μl) in a tightly capped centrifuge tube for 20 min at 70° and then placing an aliquot of the solution in a probe tube for analysis.

RESULTS AND DISCUSSION

Comparison of the TLC mobilities of I (R_f 0.68) and II (R_f 0.57) suggested a metabolic transformation resulting in an increase in polarity. Furthermore, the metabolite zone (but not the zone of the parent drug) gave a positive color reaction when sprayed with diazotized sulfanilic acid and base, indicating the presence of a phenolic group. The UV spectrum (methanol) of the metabolite was similar to that of I, except that it exhibited a pH dependence not displayed by the drug itself, supporting the possibility that II is phenolic in nature.

NMR spectroscopy of the metabolite disclosed the presence of two nonequivalent 1,2,4-trisubstituted aromatic patterns. This clearly reflected a loss in symmetry compared with the parent molecule and suggested a structural change involving one site. This position was readily identified by the observation that the methoxyl signal (τ 6.19 relative to tetramethylsilane) was reduced to one-half of its original area. The fact that the protons in the modified ring showed only a small downfield displacement led to the proposal that the new substituent was a hydroxyl group.

Mass spectrometry (direct probe) of the metabolite yielded a rather uninformative spectrum; the radioactivity had not volatilized from the probe tube even at a temperature of >200°. This may have been caused by adsorption of the metabolite on residual silica gel from the TLC plate used for final purification (5). Mass spectrometry of the metabolite following treatment (Procedure A) with III proceeded smoothly, and a volatile compound IIa (probe temperature 200°) possessing a molecular ion of 556 amu was observed (Fig. 1). Similar treatment of the parent drug (mol. wt. 354) yielded a trimethylsilyl derivative with a molecular ion of 498 amu; this compound is the di-*O*-trimethylsilyl ether [354 + (2 × 72) =

[1] AG-50W-X2, BioRad Laboratories.
[2] AG-1-X8, BioRad Laboratories.
[3] Varian HA 100 instrument.
[4] LKB model 9000 instrument.
[5] Supelco.

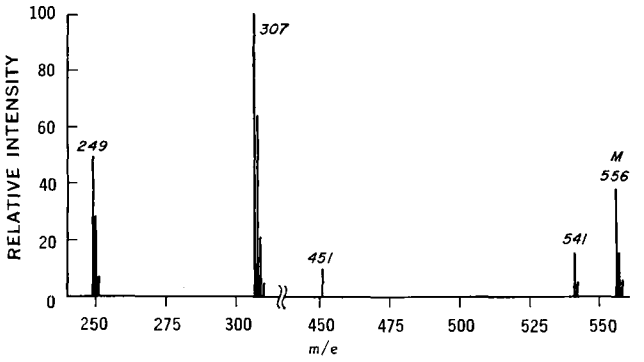
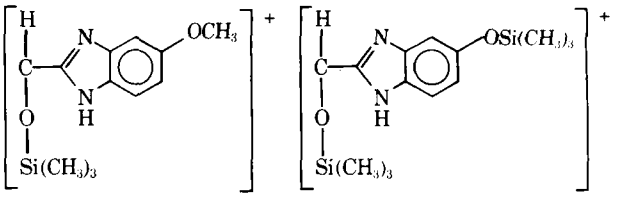


Figure 1—*Mass spectrum of the partially trimethylsilylated (Procedure A) major urinary metabolite.*

498] because imidazole NH groups are not readily derivatized under these mild reaction conditions. The base peak in the spectrum of the parent drug is at *m/e* 249 and results from symmetrical scission of the molecule to form ion IV. Although an intense signal is also observed at *m/e* 249 in the spectrum of II*a*, the base peak is found at *m/e* 307 and can be ascribed to the related ion V.

Derivatization of I with III using Procedure B forms the tetratrimethylsilyl drug with a molecular ion of 642 amu and a base peak at *m/e* 321. Under these more vigorous reaction conditions the metabolite is transformed to a compound (II*b*) with a molecular ion of 700 amu and intense signals at *m/e* 321 and 379 (base peak), which can be ascribed to ions possessing Structures IV and V, respectively, in which the imidazole NH has been transformed to N—$Si(CH_3)_3$. The mass spectral data from both derivatives clearly confirm the unsymmetrical nature of the metabolite and are compatible with the proposal that II is the mono-*O*-demethylated phenol (mol. wt. 340).

The metabolic transformation observed is perhaps not unexpected because the *O*-demethylation of codeine to form morphine is well known (6, 7) and a more recent report involves *O*-demethylation of aflatoxin B_1 (8). One might expect, however, that di-*O*-demethylation would occur with I. A very minor component (<1%) of the ethyl acetate urinary extract with an R_f of 0.44 was detected but not characterized. A considerable quantity of highly polar drug-related material is present in the mouse urine; 25% of the radioactivity remains in the aqueous phase following exhaustive extraction with ethyl acetate. The water-soluble material possesses an R_f of 0.1. Hydrolysis (1 *N* HCl at 100° for 1 hr) followed by neutralization and extraction with ethyl acetate resulted in nearly one-half of the radioactivity passing into the organic solvent. The extracted material possessed TLC behavior compatible with that of II and gave the same positive phenol test, suggesting that much of the water-soluble material is a conjugate of II. (In the case of monkeys dosed with I, all urinary radioactivity is extractable with ethyl acetate. TLC of the extract yields only one radioactive zone,



IV: *m/e* 249 V: *m/e* 307

with mobility and color test corresponding exactly to II.)

No evidence has been found to suggest that the glycol bridge of I undergoes metabolic cleavage. The metabolism of this drug in the mouse, on the basis of urinary metabolites, is essentially mono-*O*-demethylation followed, to a slight extent, by conjugation of the phenolic product.

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[x] To whom inquiries should be directed.

Synthesis of Benzoxazino- and Naphthoxazinoquinoline Derivatives as Possible Antitumor Agents

M. NASR[x], M. M. ABBASI, and I. NABIH

Abstract □ The synthesis of 2-methoxy-6-methyl-1-nitro-12*H*-quino[3,4-*b*][1,4]benzoxazine (III*a*), its 10-chloro derivative (III*b*), 2-methoxy-6-methyl-1-nitro-14*H*-naphth[1′,2′:5,6][1,4]oxazino[2,3-*c*]quinoline (IV*a*), and 2-methoxy-6-methyl-1-nitro[1,4]benzodioxino[2,3-*c*]quinoline (VI) is described. Compounds III*a*, III*b*, and IV*a* were condensed with benzaldehyde to give the corresponding styryl derivatives III*c*, III*d*, and IV*b* in an attempt to introduce new compounds that may have antitumor activity.

Keyphrases □ Benzoxazinoquinoline derivatives—synthesized as possible antitumor agents □ Naphthoxazinoquinoline derivatives—synthesized as possible antitumor agents □ Styrylquinoline derivatives—synthesis of benzoxazinoquinolines and naphthoxazinoquinolines □ Antitumor agents, potential—synthesis of benzoxazinoquinoline and naphthoxazinoquinoline derivatives

The preparation of benzoxazine derivatives *via* the Turpin (1) reaction of 2-aminophenol with picryl chloride has created much interest both from the synthetic and biological points of view (2–5). Crossley *et al.* (6) prepared a series of phenoxazine derivatives, and their differential tissue staining and tumor growth retardation activity were determined. Moreover, representatives of this class of compounds have exhibited antitubercular (7), sedative, antiepileptic, and tranquilizing (8) activities. Also, spasmolytic activity has been shown by phenoxazine carboxylic acid derivatives (9, 10).

In view of the fact that styrylquinolines have also been reported to exhibit antitumor activity (11), new agents that would structurally include systems of oxazine and benzoxazine fused with styrylquinolines might prove to be useful as antitumor agents. Therefore, the synthesis of some new benzoxazino- and naphthoxazinoquinoline derivatives *via* the Turpin reaction is reported here.

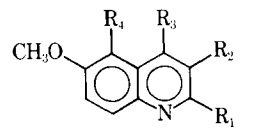
DISCUSSION

Synthesis was achieved by nitration of 4-hydroxy-6-methoxy-2-methylquinoline (I*a*) (12) to give the corresponding 3,5-dinitro derivative (I*b*) which, upon treatment with phosphorus oxychloride, yielded 4-chloro-3,5-dinitro-6-methoxy-2-methylquinoline (I*c*). Treatment of I*c* with benzaldehyde in the presence of fused zinc chloride gave 3,5-dinitro-4-hydroxy-6-methoxy-2-styrylquinoline (I*d*), which upon reaction with phosphorus oxychloride gave the 4-chloro derivative I*e*. When I*c* was allowed to condense with 2-aminophenol or 2-amino-4-chlorophenol, the product was either the 2-methoxy-6-methyl-1-nitro-12*H*-quino[3,4-*b*][1,4]benzoxazine (III*a*) or its 10-chloro derivative (III*b*).

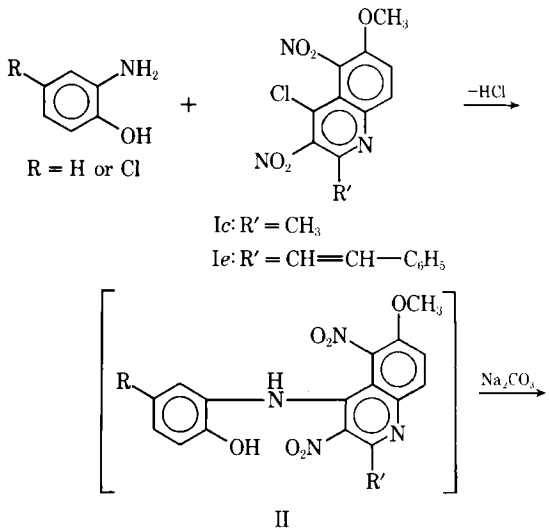
One can adequately explain the formation of III*a* and III*b* from the condensation of 2-aminophenols with I*c* and I*e* on the basis of initial formation of intermediate II *via* hydrochloric acid elimination (Scheme I). This, followed by intramolecular nucleophilic displacement of the nitro group by the phenoxide moiety under the influence of alkali, gave III*a* and III*b*.

Treatment of III*a* or III*b* with benzaldehyde in the presence of fused zinc chloride gave the styryl derivatives III*c* or III*d*. Compounds III*c* and III*d* were also obtained when the styryl derivative I*e* was allowed to condense with 2-aminophenol and 2-amino-4-chlorophenol, respectively. Similarly, reaction of I*c* with 2-amino-1-naphthol gave 2-methoxy-6-methyl-1-nitro-14*H*-naphth[1′,2′:5,6][1,4]oxazino[2,3-*c*]quinoline (IV*a*). The styryl derivative IV*b* was obtained when IV*a* was treated with benzaldehyde in the presence of fused zinc chloride.

Condensation of I*c* with phenol in dry pyridine gave the phenoxy derivative I*f*. This condensation suggested the use of catechol instead of 2-aminophenol in the Turpin reaction with I*c*. Thus, catechol condensed with I*c* in dry pyridine to give 3,5-dinitro-4-

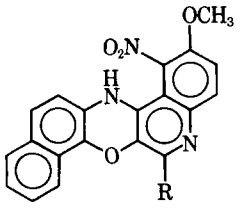


I*a*: $R_1 = CH_3, R_2 = R_4 = H, R_3 = OH$
I*b*: $R_1 = CH_3, R_2 = R_4 = NO_2, R_3 = OH$
I*c*: $R_1 = CH_3, R_2 = R_4 = NO_2, R_3 = Cl$
I*d*: $R_1 = CH{=}CH{-}C_6H_5, R_2 = R_4 = NO_2, R_3 = OH$
I*e*: $R_1 = CH{=}CH{-}C_6H_5, R_2 = R_4 = NO_2, R_3 = Cl$
I*f*: $R_1 = CH_3, R_2 = R_4 = NO_2, R_3 = OC_6H_5$



III*a*: R = H, R′ = CH_3
III*b*: R = Cl, R′ = CH_3
III*c*: R = H, R′ = $CH{=}CH{-}C_6H_5$
III*d*: R = Cl, R′ = $CH{=}CH{-}C_6H_5$

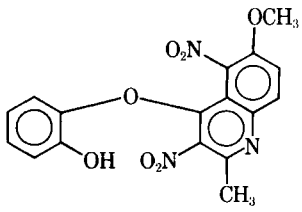
Scheme I



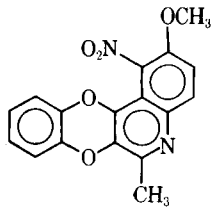
IV*a*: R = CH_3

IV*b*: R = CH=CH—C_6H_5

(2′-hydroxyphenoxy)-6-methoxy-2-methylquinoline (V), which gave 2-methoxy-6-methyl-1-nitro[1,4]benzodioxino[2,3-*c*]quinoline (VI) when gently warmed with alkali.



V



VI

EXPERIMENTAL[1]

3,5-Dinitro-4-hydroxy-6-methoxy-2-methylquinoline (I*b*)—Compound I*a* (12) (3 g, 0.0016 mole) was added portionwise with stirring to 30 g of cooled nitric acid (specific gravity 1.39), and the mixture was kept at room temperature for 24 hr and then poured onto 200 g of crushed ice. The precipitated material was collected, washed with water and then with ethanol, and recrystallized from dimethylformamide to give 2.8 g of I*b* (62%) as pale-yellow needles, mp 334–336° dec. The IR spectrum showed bands at 3300 (OH stretching), 1090 (—OCH_3), 1370 ($C_6H_5NO_2$), and 1550 (C=N) cm^{-1}. The NMR spectrum showed: *(a)* τ 7.3 (protons of the 2-CH_3 group, s), *(b)* τ 5.9 (protons of the 6-OCH_3 group, s), and *(c)* τ 2.3 and 1.7 (seven and eight aromatic protons, two doublets showing an *AB* system), indicating nitration at the 5-position.

Anal.—Calc. for $C_{11}H_9N_3O_6$: C, 47.32; H, 3.22; N, 15.05. Found: C, 47.54; H, 3.14; N, 14.85.

When a solution of I*b* in ethanol was treated with ammonia, ethylamine, diethylamine, or benzylamine, the corresponding salts were obtained.

With Ammonia—Anal.—Calc. for $C_{11}H_9N_3O_6 \cdot NH_3$: C, 44.59; H, 5.05; N, 18.92. Found: C, 44.55; H, 5.12; N, 18.88.

With Ethylamine—Anal.—Calc. for $C_{11}H_9N_3O_6 \cdot C_2H_5NH_2$: C, 48.15; H, 4.93; N, 17.28. Found: C, 48.15; H, 4.93; N, 16.99.

With Diethylamine—Anal.—Calc. for $C_{11}H_9N_3O_6 \cdot (C_2H_5)_2NH$: N, 15.90. Found: N, 15.90.

With Benzylamine—Anal.—Calc. for $C_{11}H_9N_3O_6 \cdot C_6H_5CH_2NH_2$: N, 14.51. Found: N, 14.51.

4-Chloro-3,5-dinitro-6-methoxy-2-methylquinoline (I*c*)—A mixture of 1 g (0.0036 mole) of I*b* and 5 ml of phosphorus oxychloride was refluxed for 30 hr. Excess phosphorus oxychloride was distilled off and the reaction mixture was cooled and then diluted with water. The precipitate formed after neutralization with ammonium hydroxide was filtered off, washed with water, and recrystallized from ethanol to give I*c*, 0.8 g (75%), as yellow needles, mp 210–212°.

Anal.—Calc. for $C_{11}H_8ClN_3O_5$: C, 44.37; H, 2.96; Cl, 11.93; N, 14.11. Found: C, 44.60; H, 3.30; Cl, 11.77; N, 14.23.

3,5-Dinitro-4-hydroxy-6-methoxy-2-styrylquinoline (I*d*)—A mixture of 1 g (0.0034 mole) of I*c*, 0.5 g (0.0047 mole) of benzaldehyde, and 0.01 g of fused zinc chloride was heated at 140–150° for 4 hr. The reaction mixture was cooled and treated with an alcoholic sodium hydroxide solution (20 ml, 2%). The solid product was collected, 0.5 g (41%), and recrystallized from dimethylformamide to give I*d* as colorless crystals, mp >350°.

Anal.—Calc. for $C_{18}H_{13}N_3O_6$: C, 58.86; H, 3.54; N, 11.44. Found: C, 58.32; H, 3.63; N, 11.07.

Attempted preparation of I*d* by treating I*b* with benzaldehyde in the presence of fused zinc chloride or acetic anhydride was unsuccessful.

4-Chloro-3,5-dinitro-6-methoxy-2-styrylquinoline (I*e*)—A mixture of 0.5 g (0.0014 mole) of I*d* and 5 g of phosphorus oxychloride was refluxed for 30 hr. Excess phosphorus oxychloride was distilled off and the reaction mixture was kept cold while being diluted with water and then basified with ammonium hydroxide. During continued cooling, a precipitate was formed. It was collected on a filter and washed several times with water and then with alcohol. Recrystallization from chloroform gave 0.3 g (57%) of I*e* as yellow crystals, mp 260–262°.

Anal.—Calc. for $C_{18}H_{12}ClN_3O_5$: C, 56.03; H, 3.11; Cl, 9.21; N, 10.89. Found: C, 56.26; H, 3.30; Cl, 9.21; N, 10.55.

3,5-Dinitro-4-phenoxy-6-methoxy-2-methylquinoline (I*f*)—A mixture of 0.6 g (0.002 mole) of I*c* and 0.28 g (0.003 mole) of phenol in 1 ml of dry pyridine was heated on a steam bath for 6 hr. After evolution of excess pyridine, the residue (0.5 g, 70%) was collected and recrystallized from ethanol to give I*f* as colorless needles, mp 140–141°.

Anal.—Calc. for $C_{17}H_{13}N_3O_6$: C, 57.46; H, 3.66; N, 11.83. Found: C, 57.46; H, 3.66; N, 11.47.

2-Methoxy-6-methyl-1-nitro-12*H*-quino[3,4-*b*][1,4]benzoxazine (III*a*)—A mixture of 2 g (0.0067 mole) of I*c*, 0.77 g (0.007 mole) of 2-aminophenol, and 1 g of sodium carbonate in 20 ml of ethanol was refluxed for 4 hr. Cooling and addition of water (100 ml) precipitated a material which was filtered off, washed with water and then with ethanol, and recrystallized from ethanol to give 1.2 g of III*a* (55%) as pale-yellow needles, mp 206–208°. The IR spectrum showed absorption bands at 3280 (NH-stretching) and 1590 (NH-bending) cm^{-1}. The NMR spectrum showed: *(a)* τ 7.2 (protons of the 2-CH_3 group, s), *(b)* τ 5.94 (protons of the 6-OCH_3 group, s), and *(c)* τ 2.4–3 (six aromatic protons, m).

Anal.—Calc. for $C_{17}H_{13}N_3O_4$: C, 63.16; H, 4.02; N, 13.00. Found: C, 62.76; H, 3.91; N, 12.92.

The hydrochloride salt was obtained by refluxing III*a* (0.32 g) with 15% HCl (10 ml) for 10 min. Cooling gave 0.25 g of the salt as bright-yellow needles, mp 254–256°.

Anal.—Calc. for $C_{17}H_{13}N_3O_4 \cdot HCl$: C, 56.74; H, 3.89; Cl, 9.87; N, 11.68. Found: C, 56.99; H, 3.89; Cl, 9.60; N, 11.68.

The salt regenerated the free base III*a* when gently warmed with water.

10-Chloro-2-methoxy-1-nitro-6-methyl-12*H*-quino[3,4-*b*][1,4]benzoxazine (III*b*)—This compound was prepared by the method used for III*a* from I*c* and 2-amino-4-chlorophenol to give III*b* (70%) as yellow needles from ethanol, mp 240–242°.

Anal.—Calc. for $C_{17}H_{12}ClN_3O_4$: C, 57.06; H, 3.36; Cl, 9.95; N, 11.75. Found: C, 57.20; H, 3.70; Cl, 10.00; N, 11.42.

Compounds III*a* and III*b* gave the styryl derivatives III*c* and III*d*, respectively, upon condensation with benzaldehyde in the presence of fused zinc chloride. The latter compounds were also obtained when I*e* was allowed to react with 2-aminophenol and 2-amino-4-chlorophenol, respectively.

When recrystallized from chloroform, III*c* had a melting point of 244–246°.

Anal.—Calc. for $C_{24}H_{17}N_3O_4$: C, 70.07; H, 4.13; N, 10.21. Found: C, 70.02; H, 4.13; N, 10.21.

When recrystallized from chloroform, III*d* had a melting point of 265–268°.

Anal.—Calc. for $C_{24}H_{17}ClN_3O_4$: C, 64.64; H, 3.59; N, 9.42. Found: C, 64.05; H, 3.91; N, 8.99.

In the same way, IV*a* was obtained by reacting I*c* with 2-amino-1-naphthol (60% yield). It was recrystallized from chloroform as violet crystals, mp 270–272°.

Anal.—Calc. for $C_{21}H_{15}N_3O_4$: C, 67.56; H, 4.02; N, 11.26. Found: C, 67.99; H, 3.98; N, 10.99.

Treatment of IV*a* with benzaldehyde in the presence of fused zinc chloride gave 40% yield of the styryl derivative IV*b*, mp 310–312°.

Anal.—Calc. for $C_{28}H_{19}N_3O_4$: C, 72.89; H, 4.12; N, 9.11. Found: C, 73.08; H, 4.02; N, 8.99.

3,5-Dinitro-4-(2′-hydroxyphenoxy)-6-methoxy-2-methylquinoline (V)—A mixture of 0.6 g (0.002 mole) of I*c*, 0.22 g (0.002 mole) of catechol, and 4 g (0.005 mole) of dry pyridine was heated on the steam bath for 10 hr. The residue left after evaporation of

[1] All melting points are uncorrected. The IR spectra were recorded with a Carl Zeiss Infracord spectrophotometer model UR 10 using KBr. NMR spectra were performed on a Varian A-60 spectrometer in $CDCl_3$ or CF_3COOH solution, using tetramethylsilane as an internal standard.

pyridine was extracted with ether and dried (sodium sulfate). After evaporation of the ether, the product was recrystallized from benzene to give 0.3 g (40%) of V as pale-yellow crystals, mp 128–130°.

Anal.—Calc. for $C_{17}H_{13}N_3O_7$: C, 54.98; H, 3.50; N, 11.32. Found: C, 54.80; H, 3.60; N, 11.40.

2-Methoxy-6-methyl-1-nitro[1,4]benzodioxino[2,3-*c*]quinoline (VI)—A stirred mixture of 0.37 g (0.001 mole) of V and 10 ml of an aqueous solution of sodium hydroxide (10%) was gently warmed on the steam bath for 2 hr. It was then cooled and neutralized with hydrochloric acid (pH 7). The precipitated product was recrystallized from ethanol to give 0.17 g (52%) of VI as yellowish-green needles, mp 208–210°. The IR spectrum showed the absence of an OH group. The NMR spectrum showed: *(a)* τ 7.41 (protons of the 6-CH_3 group, s), *(b)* τ 5.98 (protons of the 2-OCH_3 group, s), *(c)* τ 1.9 and 2.6 (three and four aromatic protons, two doublets showing an *AB* system), and *(d)* τ 3.02 (five aromatic protons, s).

Anal.—Calc. for $C_{17}H_{12}N_2O_5$: C, 62.96; H, 3.70; N, 8.64. Found: C, 63.06; H, 4.09; N, 8.46.

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[x] To whom inquiries should be directed. Present address: College of Pharmacy, University of Michigan, Ann Arbor, MI 48104

Separation of Anthraquinone Glycosides and Aglycones Using Electropaperography

TU-MING (CHENG) CHANG* and N. M. FERGUSON[x]

Abstract □ A method was developed using an electropaperography apparatus for the separation of hydroxyanthraquinone derivatives, both as glycosides and as aglycones. By using this technique, a mixture of emodin, alizarin, and chrysophanic acid was separated and identified by paper chromatography. Similarly, emodin maltoside and chrysophanic acid maltoside were separated and identified, and the glycosides of senna leaflets were separated and their corresponding aglycones were identified.

Keyphrases □ Anthraquinone aglycones and corresponding glycosides—extraction and separation from plant material by electropaperography □ Senna leaflets—extraction, separation, and identification of glycosides and aglycones by electropaperography □ Electropaperography—separation and identification of anthraquinone glycosides and aglycones

For a number of years, considerable work has been done on the identification of naturally occurring hydroxyanthraquinone derivatives. Numerous methods can be found for the separation of anthraquinone derivatives in several vegetable laxative drugs. Most methods involve the use of either TLC (1) or column (2) or paper (3) chromatography. Characterizations of the anthraquinone derivatives by paper electrophoresis were first described by Core and Kirch (4) and Siesto and Bartoli (5). These workers used single strip paper electrophoresis to accomplish the separation of some hydroxyanthraquinones. The R_f value of each individual compound was determined after each run.

The principle of applying, to a flowing liquid inside a porous medium, an electric field at right angles to the central stream of the mixture to be separated was initially proposed by several investigators (6–9). This method, usually referred to as electropaperography or continuous electrophoresis, is particularly useful for the preparative separations of multicomponent mixtures. The electrolyte solution flows in a direction normal to the lines of force of the electrical field, and the mixture to be separated is added continuously at a small spot in the flowing medium. Components of the mixture are deflected in different directions according to their electrophoretic mobilities and, after passage through the entire plane of the flowing medium, can be collected continuously at various positions. Electropaperography differs from other methods in one important and fundamental respect, *i.e.*, the more slowly migrating components are not required to move over a path that has been previously traversed by the more rapidly migrating components; as a result, absolute separations can be effected. The main purpose of this research was to use this method in the separation of certain plant anthraquinone derivatives, a method that has not been reported previously for these compounds.

pyridine was extracted with ether and dried (sodium sulfate). After evaporation of the ether, the product was recrystallized from benzene to give 0.3 g (40%) of V as pale-yellow crystals, mp 128–130°.

Anal.—Calc. for $C_{17}H_{13}N_3O_7$: C, 54.98; H, 3.50; N, 11.32. Found: C, 54.80; H, 3.60; N, 11.40.

2-Methoxy-6-methyl-1-nitro[1,4]benzodioxino[2,3-*c*]quinoline (VI)—A stirred mixture of 0.37 g (0.001 mole) of V and 10 ml of an aqueous solution of sodium hydroxide (10%) was gently warmed on the steam bath for 2 hr. It was then cooled and neutralized with hydrochloric acid (pH 7). The precipitated product was recrystallized from ethanol to give 0.17 g (52%) of VI as yellowish-green needles, mp 208–210°. The IR spectrum showed the absence of an OH group. The NMR spectrum showed: *(a)* τ 7.41 (protons of the 6-CH_3 group, s), *(b)* τ 5.98 (protons of the 2-OCH_3 group, s), *(c)* τ 1.9 and 2.6 (three and four aromatic protons, two doublets showing an *AB* system), and *(d)* τ 3.02 (five aromatic protons, s).

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[x] To whom inquiries should be directed. Present address: College of Pharmacy, University of Michigan, Ann Arbor, MI 48104

Separation of Anthraquinone Glycosides and Aglycones Using Electropaperography

TU-MING (CHENG) CHANG* and N. M. FERGUSON[x]

Abstract □ A method was developed using an electropaperography apparatus for the separation of hydroxyanthraquinone derivatives, both as glycosides and as aglycones. By using this technique, a mixture of emodin, alizarin, and chrysophanic acid was separated and identified by paper chromatography. Similarly, emodin maltoside and chrysophanic acid maltoside were separated and identified, and the glycosides of senna leaflets were separated and their corresponding aglycones were identified.

Keyphrases □ Anthraquinone aglycones and corresponding glycosides—extraction and separation from plant material by electropaperography □ Senna leaflets—extraction, separation, and identification of glycosides and aglycones by electropaperography □ Electropaperography—separation and identification of anthraquinone glycosides and aglycones

For a number of years, considerable work has been done on the identification of naturally occurring hydroxyanthraquinone derivatives. Numerous methods can be found for the separation of anthraquinone derivatives in several vegetable laxative drugs. Most methods involve the use of either TLC (1) or column (2) or paper (3) chromatography. Characterizations of the anthraquinone derivatives by paper electrophoresis were first described by Core and Kirch (4) and Siesto and Bartoli (5). These workers used single strip paper electrophoresis to accomplish the separation of some hydroxyanthraquinones. The R_f value of each individual compound was determined after each run.

The principle of applying, to a flowing liquid inside a porous medium, an electric field at right angles to the central stream of the mixture to be separated was initially proposed by several investigators (6–9). This method, usually referred to as electropaperography or continuous electrophoresis, is particularly useful for the preparative separations of multicomponent mixtures. The electrolyte solution flows in a direction normal to the lines of force of the electrical field, and the mixture to be separated is added continuously at a small spot in the flowing medium. Components of the mixture are deflected in different directions according to their electrophoretic mobilities and, after passage through the entire plane of the flowing medium, can be collected continuously at various positions. Electropaperography differs from other methods in one important and fundamental respect, *i.e.*, the more slowly migrating components are not required to move over a path that has been previously traversed by the more rapidly migrating components; as a result, absolute separations can be effected. The main purpose of this research was to use this method in the separation of certain plant anthraquinone derivatives, a method that has not been reported previously for these compounds.

EXPERIMENTAL

Separation of Emodin, Alizarin, and Chrysophanic Acid Aglycones—After the components of the electropaperography apparatus[1] (10) were thoroughly cleaned and the apparatus was assembled, the two electrode vessels were filled with 0.01 *M* sodium borate buffer, having an ionic strength of approximately 0.025 (pH 9.6), to approximately 2.5 cm above the horizontal portion of the platinum electrodes. The three liquid compartments or solvent vessels on top of the fractionation unit were then filled with the borate buffer to a given level, depending on the desired speed of fluid flow down the curtain. The buffer levels of the three liquid compartments were kept constant by adding fresh buffer solution. The fractionation surface was then placed on the position that makes an angle of 18° with the fractionation unit. The precut paper curtain[2] was positioned with the fractionation area centered over the supporting fractionation surface plate; the electrode flaps as well as the three solvent flaps were then inserted in their respective containers. The curtain was permitted to be wetted by capillarity for 2 hr, and the effluent was removed before applying the sample to paper.

A 1% solution of equal parts of pure samples of emodin[3], alizarin[4], and chrysophanic acid[3] (11) were prepared using 0.1 *M* aqueous potassium hydroxide as the solvent. The sample solution was then fed by a 1-mm wide filter paper wick to the prewetted paper curtain at point 1 marked on the paper curtain. A potential of 700 v was then applied across the platinum electrodes. After a steady state was established, a current of 14 mamp was observed. To avoid pH gradients that are formed during the experiment, the electrodes were washed with fresh buffer every 24 hr.

After the potential was applied for 96 hr, the sheet was removed and dried in a stream of warm air directed perpendicularly to the fractionation area of the paper. When the sheet was inspected under visible light, three distinct bands could be seen; the red band led to drip points 5–7, the purple band led to drip points 9–12, and the yellow band led to drip points 16 and 17. The various fractions in the collecting tubes were then acidified with hydrochloric acid and extracted with chloroform. The combined chloroform fractions of each tube were evaporated in air to about 0.5 ml.

The chloroform fractions were then spotted on paper[5] and chromatographed by the ascending method using toluene alone as the running solvent (3). The paper was allowed to remain in the sealed jar for about 2 hr. Following removal from the jar, the paper was dried and sprayed with a 0.5% solution of magnesium acetate in methanol and heated in the 90° oven for a few minutes. The developed chromatogram showed purple to pink coloration in daylight and in UV light. The fractions from tubes 5–7 resulted in a spot having an R_f value of 0.55, which was found to represent emodin when compared on the same chromatograph with a reference sample of pure emodin[3]. Similarly, fractions from tubes 9–12 were shown to contain alizarin (R_f 0.99) when chromatographed with pure alizarin[4].

The final proof of identity was established through a comparison of IR spectra of the separated aglycones with those of the pure compounds.

Separation of Emodin Maltoside and Chrysophanic Acid Maltoside—The sample was prepared by dissolving equal parts of emodin maltoside[3] and chrysophanic acid maltoside[3] (11) in approximately 0.1 *M* cold aqueous potassium hydroxide to result in a concentration of 1%. The background electrolyte used was 0.01 *M* sodium borate buffer solution. The precut paper curtain[2] was wetted for 2 hr, and the sample solution was then fed by a 1-mm wide filter paper wick to point 3 on the paper. A potential of 600 v was then applied; after a steady state was established, a current of 9 mamp was observed. At the end of 144 hr, the curtain was removed and dried in the oven. On inspection under visible light, two distinct bands were seen; the red band led to drip points 6–10 and the yellow band led to drip points 20–22. The contents in tubes 6–10 were then combined, as were also the contents in tubes 20–22. Each fraction from the resulting collecting tubes was acidified and allowed to evaporate in air to about 0.5 ml. Samples of each fraction as well as samples of the pure glycosides were chromatographed on paper[5] using a mixture of water–acetone–benzene (2:1:4). After 2 hr, the paper was dried and developed with magnesium acetate solution. Fractions from tubes 6–10 showed a single spot (R_f 0.10) corresponding to emodin maltoside when compared with the reference sample of emodin maltoside[3]. Fractions from tubes 20–22 showed a single spot (R_f 0.32) corresponding to chrysophanic acid maltoside when compared in like manner with pure chrysophanic acid maltoside[3].

As a further proof of the completeness of separation of the glycosides, samples from each set of tubes were hydrolyzed with 10% hydrochloric acid by refluxing in a boiling water bath followed by extraction with chloroform. The chloroform fractions were evaporated in air and chromatographed using toluene as the running solvent. Tubes 6–10 yielded a single spot (R_f 0.55) corresponding to emodin when chromatographed with the pure emodin[3]. Similarly, tubes 20–22 yielded chrysophanic acid (R_f 0.98) when chromatographed with pure chrysophanic acid[3].

Table I—Fractionation of Senna Leaflet Glycosides and Their Aglycones

	Fraction					
	14	15	16	17	18	19
	R_f Value					
Glycosides, sodium borate complexed	0.78	0.70	0.64	0.46	0.45	0.41
Glycosides, in acid form	0.64	0.68	0.75	0.10	0.51	0.72
Aglycones, potassium hydroxide hydrolyzed	0.98	0.96	0.94	0.92	0.93	0.91
Aglycones, hydrochloric acid–ferric chloride hydrolyzed	0.98	0.21	0.98 0.20	0.83	0.99 0.82	0.98

Separation and Identification of Senna Leaflet Hydroxyanthraquinone Glycosides and Their Aglycones by Electropaperography—The extraction procedure of the crude drug was based on the method described by Su and Ferguson (12) using powdered senna[6].

Separation of Aglycones—The chloroform layer was completely air dried, and a 1% solution of the residue was prepared using 0.1 *M* aqueous potassium hydroxide solution. The background electrolyte solution (0.01 *M* sodium borate buffer, pH 9.6) was fed continuously at point 3 by a 1-mm wide filter paper wick to the prewetted[2] paper curtain. A voltage of 400 and a current of 12 mamp were maintained for 144 hr after which fractions 1–22 were removed, air evaporated to about 5 ml, spotted on paper[5], and chromatographed using toluene as the running solvent. Pure samples of rhein[3], aloe-emodin[3], and chrysophanic acid[3] were spotted on the same paper for comparison. Following removal from the jar, the paper was developed using the magnesium acetate solution previously described.

Fractions 12 and 13 yielded single spots with R_f values of 0.02 and 0.03, respectively, corresponding to rhein. Similarly, fractions 15 and 16, with R_f values of 0.98 and 0.99, respectively, corresponded to chrysophanic acid. Fractions 17 and 18, with R_f values of 0.83 and 0.82, respectively, corresponded to aloe-emodin.

Separation of Glycosides from Senna—The alcoholic extract was air dried, and a 1% solution was prepared in 0.1 *M* aqueous potassium hydroxide. This solution was fed continuously by a 1-mm wide wick to the borate buffer prewetted paper curtain[2] at position 3. A voltage of 400 and a current of 10 mamp were maintained during 96 hr, after which the contents of fractions 1–20 were transferred to beakers and air evaporated to about 5 ml. These fractions containing the sodium borate-complexed glycosides were then spotted on paper[5] and chromatographed using water–acetone–benzene (2:1:4). The chromatogram was developed using magnesium acetate solution. The R_f values of those fractions showing spots are

[1] Misco electropaperography apparatus, Microchemical Specialties Co., Berkeley, Calif.
[2] Schleicher and Schuell 598.
[3] Synthesized by N. M. Ferguson.
[4] Fisher Scientific Co., New York, N.Y.
[5] Whatman No. 1.
[6] Senna leaves, *Cassia augustifolia*, Delile, Tinnevelly No. 1, NF X, 40 mesh, Lot No. WEB-414, E-10317, S. B. Penick & Co., New York, N.Y.

shown in Table I. The remaining solution from each fraction was then acidified to release the glycosides from the sodium borate complex, dissolved in alcohol, spotted, chromatographed, and developed as before. The R_f values obtained are shown in Table I.

To determine the exact composition of the glycosides of senna leaves as separated by electropaperography, samples of each fraction were treated with potassium hydroxide and refluxed for 2 hr in a boiling water bath. The solutions, after being cooled, were acidified with hydrochloric acid followed by extraction with chloroform. The latter solutions were in turn evaporated in air to 20 ml, and samples were chromatographed on paper[5] using toluene as the running solvent. Development of the spots with magnesium acetate solution resulted in the R_f values shown in Table I.

The remaining chloroform fractions were then evaporated in air, dissolved in dilute potassium hydroxide solution, and transferred to the reflux flasks. They were hydrolyzed with a combination of hydrochloric acid and ferric chloride by refluxing in a boiling water bath for 3 hr. The content of each flask was then evaporated in air to one-half the volume and extracted with chloroform. The chloroform solutions were evaporated in air to 3 ml and chromatographed on paper[5] using toluene as the running solvent. Development of the spots with magnesium acetate solution resulted in the R_f values shown in Table I.

RESULTS AND DISCUSSION

Although the technique of electropaperography was applied in the last decade to the separation of proteinaceous substances, it has not yet been developed to a state where it can be routinely applied to the separation and purification of many plant constituents.

In this study, this method was applied to the separation of some hydroxyanthraquinone glycosides and their aglycones, using 0.01 *M* sodium borate buffer solution as the background electrolyte. The borate ions complex with the free hydroxy groups and, since the position of the free hydroxy groups varies with each isomer, the degree of borate complexing also varies and, in turn, changes the molecular charge on the compound. The results were reproducible when constant conditions were maintained with respect to the concentration of the buffer and the sample, the pH, the applied potential, the rate of fluid flow, and the kind of paper curtain employed.

Electropaperography is not always the best method of separation even for the substances that it can separate. Older methods such as fractional precipitation by organic solvents, salting out, dialysis, ion exchange, paper chromatography, and TLC are often considered to be faster or more efficient. Electropaperography should be used only for those separations for which it is more efficient. It is an ideal tool for the separation of substances that have high electrophoretic potentials and large differences in mobility.

Good separations were obtained when using a mixture of the pure aglycones chrysophanic acid, alizarin, and emodin. A mixture of pure glycosides consisting of chrysophanic acid maltoside and emodin maltoside was satisfactorily separated, and the identity of the fractions was proved by determination of their R_f values as well as the R_f values of their aglycones. Further proof was obtained from IR spectral studies. In applying this method to a crude drug, senna leaflets were extracted and the free aglycones were separated and identified. The glycosides present were separated, hydrolyzed, and identified through the R_f values of their aglycones. Since some senna leaflet glycosides occur in the dimeric form, more than one aglycone may be produced through hydrolysis.

From Table I it is seen that the senna leaflet glycosides were separated into six fractions. Fraction 14, on hydrolysis with potassium hydroxide, produced chrysophanic acid (R_f 0.98). No further change in R_f value occurred on hydrolysis with hydrochloric acid–ferric chloride solution, showing fraction 14 to consist of the monomeric glycoside chrysophanic acid glycoside. Fraction 15 yielded a single spot (R_f 0.96) on hydrolysis with potassium hydroxide. On further hydrolysis in hydrochloric acid–ferric chloride solution, only one spot (R_f 0.21) was seen. Therefore, this fraction consisted principally of di-rhein glycoside. Through a similar line of reasoning, fraction 16 was found to contain the aglycones rhein and chrysophanic acid, resulting in the dimeric compound rhein–chrysophanic acid glycoside. Similarly, fraction 17 consisted of di-aloe-emodin glycoside, fraction 18 of aloe-emodin–chrysophanic acid glycoside, and fraction 19 of di-chrysophanic acid glycoside.

SUMMARY AND CONCLUSIONS

Methods were developed for the isolation and identification of some hydroxyanthraquinone compounds from plant materials and for the isolation of some hydroxyanthraquinone glycosides from plant material together with a means of identifying them through their corresponding aglycones. By using these techniques, senna leaflets were shown to contain rhein, chrysophanic acid, and aloe-emodin as free aglycones together with chrysophanic acid glycoside, di-rhein glycoside, rhein–chrysophanic acid glycoside, di-aloe-emodin glycoside, aloe-emodin–chrysophanic acid glycoside, and di-chrysophanic acid glycoside. The methods described here should find wide application in the isolation of other types of plant constituents from drug plants, because electropaperography provides larger amounts of individual constituents than the conventional paper chromatography or TLC systems.

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* Present address: 928 N. Highlands, Orange, CA 92667

ˣ To whom inquiries should be directed.

Complexation of Theophylline with Sodium Benzoate: An NMR Study

ARVIND L. THAKKAR[x] and LOWELL G. TENSMEYER

Abstract □ The interaction of theophylline with sodium benzoate was examined by NMR to understand the mode of interaction. Chemical shifts of theophylline protons were determined as a function of sodium benzoate concentration in D_2O at 30°. Several different concentrations of theophylline were used. Signals of both methyl groups undergo significant upfield shifts, indicating vertical or plane-to-plane stacking.

Keyphrases □ Theophylline–sodium benzoate complexation—studied by NMR □ Sodium benzoate–theophylline complexation—studied by NMR □ Interactions—theophylline–sodium benzoate complexation, studied by NMR □ NMR—analysis, theophylline–sodium benzoate complexation, mode of interaction

Although complexes of theophylline, caffeine, and other xanthines with drug molecules have been studied extensively, the mode of association for such complexes has remained largely a matter of speculation. For crystalline complexes, direct information has been provided regarding the geometry of the components in complexes of caffeine and theophylline with 5-chlorosalicylic acid (1, 2). On the basis of a linear correlation between the standard unitary free energy change and the planar area of neutral as well as ionic interactants for several xanthine complexes, Connors and coworkers (3, 4) suggested that the interacting species have a plane-to-plane arrangement. Similar views were advocated by other workers (5, 6) as well; however, direct experimental evidence that might aid in understanding the mode of interaction and the geometry of the interacting species *in aqueous solution* has been lacking.

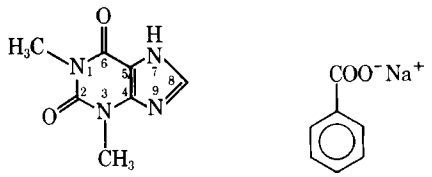
This paper concerns the interaction between theophylline (I) and sodium benzoate (II) in aqueous solution as examined by NMR spectroscopy. This system was chosen as a model because of its simplicity and because of conflicting reports on whether these two components interact (7, 8).

EXPERIMENTAL

Materials—Theophylline NF, sodium benzoate USP, and deuterium oxide (99.7 mole %)[1] were used as received.

Methods—Several sets of solutions were prepared; within each set the concentration of theophylline was maintained constant while that of sodium benzoate was varied from 0 to 1.0 *M*. The following concentrations of theophylline were used: 0.004, 0.010, 0.015, and 0.020 *M*. NMR spectra were recorded[2] in duplicate at a probe temperature of 30 ± 1°. External tetramethylsilane was used as the reference and as the source of a lock signal. Bulk susceptibility corrections were not made. Approximate calculations indicated that these corrections are too small in comparison with the large induced chemical shift changes due to complexation. Accuracy of the observed chemical shifts is estimated to be better than ±0.005 ppm.

[1] Merck Sharp and Dohme of Canada Ltd., Montreal, Canada.
[2] Varian HA-100.



I

II

RESULTS AND DISCUSSION

The NMR spectrum of a dilute (0.004 *M*) D_2O solution of theophylline consists of three signals of relative intensities, 1:3:3, at about 8.46, 4.01, and 3.80 ppm (from external tetramethylsilane) corresponding to the proton at C-8 and the 3- and 1-methyl protons, respectively. Signal assignments are based upon a comparison with the spectrum of caffeine (9). Theophylline, like caffeine and other xanthines, undergoes self-association in aqueous solution, and this behavior is reflected in the NMR spectrum by the upfield shift of all three signals as the concentration of theophylline is increased (10). In the present study, the concentration of theophylline was kept rather low to minimize the possibility of interactions of the self-associated species.

When sodium benzoate is added to a solution of theophylline, all three signals in the NMR spectrum move upfield. Stamm (11)

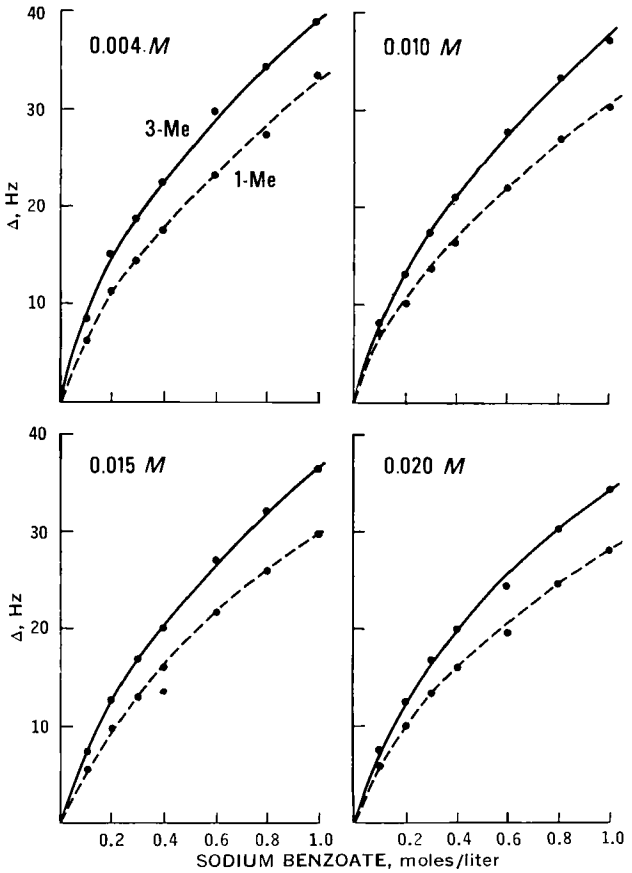


Figure 1—*Induced chemical shift changes for the methyl protons of theophylline as functions of sodium benzoate concentration.*

Table I—Formation Constants (K) and Complexation Shifts (Δ_0) for the Interaction of Theophylline with Sodium Benzoate at 30°

Theophylline, M	K, M^{-1}						Δ_0, Hz					
	Method I[a]		Method II[b]		Method III[c]		Method I[a]		Method II[b]		Method III[c]	
	1-CH_3	3-CH_3	1-CH_3	3-CH_3	1-CH_3	3-CH_3	1-CH_3	3-CH_3	1-CH_3	3-CH_3	1-CH_3	3-CH_3
0.004	1.00	1.26	1.00	1.38	1.05	1.44	64	69	63	66	65	92
0.010	1.00	1.07	1.20	1.20	1.34	1.25	60	72	54	68	68	82
0.015	1.00	1.11	1.01	1.18	1.05	1.22	58	68	58	66	59	78
0.020	1.14	1.24	1.35	1.42	1.42	1.47	52	61	48	57	65	81

[a] Method of Johnston *et al.* (12). [b] Method of Nakano *et al.* (13). [c] Method of Foster and Fyfe (14).

observed a similar effect upon the interaction of sodium benzoate with caffeine. Figure 1 shows the induced chemical shift change for the two methyl groups of theophylline as a function of sodium benzoate concentration. A similar plot could not be made for the C-8 proton because the signal appeared in the same spectral region as the signals due to the aromatic protons of benzoate and it could not always be distinguished. As the figure shows, both methyl signals move appreciably upfield.

The upfield changes in chemical shift suggest that the complex is formed in such a way that the two methyl groups of theophylline come under the shielding region associated with the anisotropic benzoate moiety. Since the conical shielding regions of the aromatic ring are perpendicular to and above and below the plane of the aromatic ring, it follows that the complex is formed by vertical stacking. If the opposite, horizontal (side-by-side or end-to-end) arrangement were the case, then chemical shift changes in the opposite direction would have been observed. The larger value of the upfield shift of the 3-methyl group (0.71 ppm) as compared with that of the 1-methyl group (0.60 ppm) provides information about the time-averaged, relative orientation of theophylline and benzoate; the 3-methyl group spends somewhat more of its time nearer the axis of the phenyl ring than does the 1-methyl group.

Apparently, three types of interactions are involved in the formation of the theophylline–benzoate complexes. Hydrophobic interactions tend to localize theophylline and benzoate molecules in the neighborhood of each other in such a manner as to minimize *effectively the surface exposed* to water. Maximum overlap of the two molecules would result in minimum exposure to water. A plane-to-plane arrangement, as in vertical stacking, would provide such a condition. Interactions between the π-electron systems would reinforce the plane-to-plane arrangement.

The hydrophobic and π interactions by themselves would permit free rotation of the two molecules about a common axis. If such were the case, one would not expect differences in the shielding experienced by the two methyl groups of theophylline. Specific interactions between the carboxyl ion of the benzoate and the imino group of theophylline would tend to hinder rotation of the molecules. The specific interaction would tend also to reduce the parallelism of the two molecules. The observed chemical shift difference indicates that the time-averaged position of the 3-methyl group is nearer the axis of the phenyl ring than is that of the 1-methyl group.

The chemical shift data were treated by three different methods to obtain the apparent formation constant K. All of the methods assume a 1:1 stoichiometry. Two of the methods, those of Johnston *et al.* (12) and Nakano *et al.* (13), apply over all concentration ranges. The third method, given in different but mathematically equivalent forms by Foster and Fyfe (14) and by Hanna and Ashbaugh (15), imposes the restriction that the molar concentration of one reactant be much larger than that of the reactant giving rise to the signals.

According to Johnston *et al.* (12):

$$\Delta = \frac{[1 + A_0K + B_0K] - [(1 + A_0K + B_0K)^2 - 4K^2A_0B_0]^{1/2}}{2KA_0}(\Delta_0) \qquad \text{(Eq. 1)}$$

where Δ is the difference between the observed chemical shift, δ_{obs}, and the chemical shift, δ_A, of a given signal of A in the absence of any B; Δ_0 is the chemical shift of that signal in the fully complexed A; A_0 and B_0 are the initial molar concentrations of theophylline and sodium benzoate (in this case), respectively; and K is the equilibrium constant in terms of M^{-1}.

A nonlinear least-squares computer program was used to fit the data (δ_{obs}, A_0, and B_0) to Eq. 1 and to compute the values of K and Δ_0. The computer[3] printout included a curve calculated from the computed values of K and Δ_0 along with the actual experimental values.

Nakano *et al.* (13) derived a general expression in which the equilibrium concentration of the complex, C, appears as a parameter:

$$\frac{B_0}{\Delta} = \left(\frac{1}{\Delta_0}\right)(A_0 + B_0 - C) + \frac{1}{K\Delta_0} \qquad \text{(Eq. 2)}$$

This expression can be solved graphically by successive approximations, plotting $B_0\Delta^{-1}$ *versus* $A_0 + B_0$, the first slope being approximately Δ_0^{-1}. This value for Δ_0 is utilized in:

$$C = \left(\frac{\Delta}{\Delta_0}\right)A_0 \qquad \text{(Eq. 3)}$$

to gain successive, convergent values for C. When the Nakano expression is solved with a computer, Eqs. 2 and 3 are solved simultaneously, and the method is essentially equivalent to the Johnston approach.

When $B_0 \gg A_0$, Eq. 2 reduces to Hanna and Ashbaugh's (15) equation:

$$\frac{B_0}{\Delta} = \frac{B_0}{\Delta_0} + \frac{1}{K\Delta_0} \qquad \text{(Eq. 4)}$$

or to Foster and Fyfe's (14) equivalent form:

$$\frac{\Delta}{B_0} = -\Delta K + \Delta_0 K \qquad \text{(Eq. 5)}$$

In concentration regions where $B_0 \gg A_0$, plots of ΔB_0^{-1} *versus* Δ should be linear with a slope of $-K$ and an intercept of Δ_0K. In each experiment reported here, $B_0 \gg A_0$.

The values of K and Δ_0, calculated by the three methods, are shown in Table I. The average value of K, 1.20 ± 0.13 M^{-1}, demonstrates that a significant interaction does take place between theophylline and sodium benzoate. The absolute value of K should be regarded with reservation, however, since the ionic strength was not maintained constant in each determination. The formation constant is only approximate. The reproducibility in the Δ_0 values indicates the validity and reliability of the NMR method.

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Tumor-Inhibitory Agent from *Zaluzania robinsonii* (Compositae)

S. D. JOLAD, R. M. WIEDHOPF, and J. R. COLE [x]

Abstract □ The chloroform extract of *Zaluzania robinsonii* Sharp has shown activity against the P-388 lymphocytic leukemia test system. The constituent responsible for this activity was a guaianolide, identified as zaluzanin C ($C_{15}H_{18}O_3$). The identity was proven by melting point, mixed melting point, elemental analysis, IR, PMR, mass spectroscopy, and preparation of derivatives.

Keyphrases □ *Zaluzania robinsonii* (Compositae)—isolation and identification of zaluzanin C as tumor-inhibiting agent □ Zaluzanin C—isolation and identification from *Z. robinsonii*, tested for tumor-inhibitory activity □ Antitumor agents, potential—isolation, identification, and screening of zaluzanin C from *Z. robinsonii*

As a result of the continuing search for plants having tumor-inhibiting constituents, the ethanol extract of the leaves, stems, flowers, and roots of *Zaluzania robinsonii* Sharp (Compositae)[1] was found to have inhibitory activity toward the P-388 lymphocytic leukemia test system (3PS)[2].

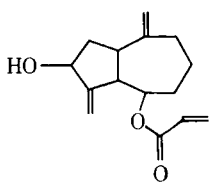
DISCUSSION

The chloroform extract, obtained from an ethanol extract by partition between chloroform and water, was subjected to systematic fractionation employing partition and solvent extraction followed by column chromatography on silica gel. The sequence of steps in the fractionation procedure leading to the isolation of the guaianolide is outlined in Scheme I. The guaianolide was identified as zaluzanin C (I) by means of its melting point, elemental analysis, mass spectroscopy, and comparison of IR and PMR spectra with authentic sample spectra. In addition, no depression in a mixed melting point and a superimposable IR spectrum of the acetyl derivative with an authentic sample and identical physical data of the dehydro derivative further proved the identification of the guaianolide as zaluzanin C. The compound had been previously isolated from two closely related species, *Z. augusta* (lag) Schultz, Bip. (1) and *Z. triloba* Pers. (2).

This compound demonstrated an activity of 161% test/control (T/C) at 150 mg/kg in the 3PS test system. Activity in the 3PS system is defined as an increase in the survival of treated animals over that of controls resulting in a T/C ≥ 125% (3).

EXPERIMENTAL[3]

Isolation Procedure—The roots, stems, leaves, and flowers (8.5 kg) of *Z. robinsonii* were ground and extracted exhaustively in a Lloyd-type extractor with ethanol. After removal of the solvent in air, the residue was subjected to three-funnel partition in three lots between benzene–methanol–water (8:5:1) using 1700 ml of each phase. All upper phases were combined, the solvent was removed in air, and the residue was stirred mechanically twice with ethyl acetate and filtered. The combined ethyl acetate-soluble fraction yielded a residue weighing 46 g. The lower phases (second and third) obtained from these partitions were similarly combined and the solvent was removed. The residue was stirred twice with *n*-hexane and filtered. The hexane-insoluble residue, weighing 8.5 g, was then mixed with the ethyl acetate-soluble residue (46 g). A portion of this mixture (39 g) was subjected to silica gel (1700 g) column (10 × 152 cm) chromatography. The column was eluted



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[2] Of the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md.

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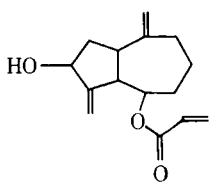

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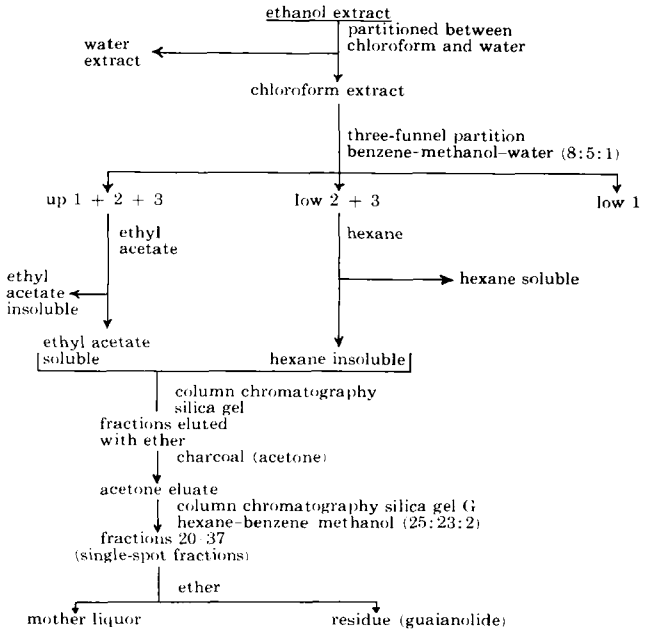

Scheme I—Isolation of zaluzanin C

with various solvents, starting with hexane, with increasing order of polarity. The fraction (20 g) from the ether eluate, which showed essentially one large spot on TLC, demonstrated antitumor activity.

Isolation of Zaluzanin C—A portion of this active fraction (6 g) was adsorbed on activated charcoal, and the resulting dry pulverized adsorbate was placed on the top of a column packed with a small amount of sand and eluted with acetone. The residue (5.07 g) obtained after removal of the solvent *in vacuo* was subjected to silica gel (60 g) column (3 × 68 cm) chromatography using hexane–benzene–methanol (25:23:2) as the solvent system for elution. Fractions (20–37; each fraction 15 ml) displaying a single spot on TLC were combined and the solvent was removed *in vacuo*. The resulting residue, on treatment with ether (15 ml), gave nearly colorless crystals of zaluzanin C. The mixture was filtered after standing overnight in the refrigerator and was washed with a small amount of cold ether to give 1.09 g, mp 93–94°. Crystallization from acetone–hexane afforded pure zaluzanin C in colorless prisms, mp 94–95° [lit. (2) mp 95–96°], $[\alpha]_D^{27}$ +38° (c 1% in $CHCl_3$). The IR and PMR spectra were identical with the authentic sample spectra[4]. Mass spectrometry indicated a parent peak of 246.

Anal.—Calc. for $C_{15}H_{18}O_3$: C, 73.15; H, 7.37; mol. wt. 246. Found: C, 73.06; H, 7.58; *m/e* 246.

Zaluzanin C Acetate—Acetylation of zaluzanin C with acetic anhydride in pyridine for 2 hr at room temperature yielded the acetate, which crystallized from hexane–isopropyl ether as colorless plates, mp 100°, $[\alpha]_D^{27}$ +24°(c 1% in $CHCl_3$). The mixed melting point with the authentic sample[4] showed no depression. The IR spectra were superimposable. Mass spectrometry indicated a parent peak of 288.

Dehydrozaluzanin C—Chromic acid oxidation of zaluzanin C in pyridine at ice-cold temperature yielded the dehydro product, which was purified by preparative TLC followed by crystallization from acetone–hexane, mp 132–133°; IR maxima (KBr): 1760, 1720, 1632, and 885 cm^{-1} (3).

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[x] To whom inquiries should be directed.

[4] The authors are grateful to Dr. J. Romo, Instituto de Quimica, Mexico 20, D.F., for providing the spectra and the sample.

Bioavailability of Organically Bound Iodine

B. O. NEMOITIN[x] and E. A. LAZO-WASEM

Abstract □ Total serum iodine levels were measured for 24 hr in human subjects following a single, oral, therapeutic dose of organically bound iodine administered as iodinated glycerol. In one volunteer, measurements were taken during continued dosing for 6 days and for 48 hr thereafter. Peak levels were attained 1–2 hr after administration of a single dose, with a gradual fall to almost basal levels by 24 hr. The experiment with continued dosing for 6 days indicated that once peak blood levels were reached, they continued at this concentration until the drug was discontinued; then they progressively dropped to pretreatment figures in about 48 hr. The bioavailability of the iodinated glycerol was demonstrated following aqueous solution, elixir, and tablet formulations. No significant effect on blood thyroxine iodine levels was noted.

Keyphrases □ Iodine, organically bound—bioavailability from iodinated glycerol as aqueous solution, elixir, and tablet formulations, humans □ Thyroxine—blood levels following administration of aqueous solution, elixir, and tablet formulations of iodinated glycerol, humans □ Bioavailability—iodine from iodinated glycerol formulated as aqueous solution, elixir, and tablet, humans

Any ingested drug whose pharmacological activity is dependent upon reaching the circulating blood should be detectable in the circulation as a basic step in proving potential therapeutic efficacy. The purpose of the present study was to establish the bioavailability of organically bound iodine as iodinated glycerol[1] when administered as a tablet or in liquid formulation, the latter as an elixir or aqueous solution.

EXPERIMENTAL

Study A—A group of three healthy, human subjects was used for each of the three dosage forms studied. Each subject had blood drawn for total blood iodine after a low-fat breakfast, and the test material was then administered orally at its usual recommended dose.

Group I received 5 ml of iodinated glycerol elixir containing 30 mg of organically bound iodine; blood iodine was determined at 0, 1, 2, 4, 6, 8, and 24 hr. Group II received two iodinated glycerol tablets containing a total of 30 mg organically bound iodine; blood iodine was measured at 0, 1, 2, 4, 8, and 24 hr. Subjects in Group III received 20 drops of iodinated glycerol in aqueous solution containing 25 mg organically bound iodine; blood iodine was estimated at 0, 4, 8, and 24 hr (Fig. 1).

In the case of iodinated glycerol in aqueous solution, both total iodine and thyroxine iodine were determined in three individuals at 0, 4, 8, and 24 hr after a single dose. Mean results are shown in Fig. 2.

Study B—In another subject, therapeutic doses of iodinated glycerol in aqueous solution were administered for 7 days (20 drops, four times daily at 8 am, 12 noon, 4 pm, and 8 pm). Total blood iodine levels were determined at the onset of the study and at 4-hr intervals to 192 hr (Fig. 3). Blood was drawn for iodine determination prior to each hour stated in Fig. 3; *i.e.*, the 24-hr iodine determination was made just prior to the first dose of the 2nd day (8 am), the 32-hr determination was made just prior to the 3rd dose (4 pm) of the 2nd day, *etc.* No iodine determinations were made on the 6th and 7th days. Blood was drawn at 8 am on the 8th and 9th days of the study, 12 and 36 hr, respectively, after the last dose of the drug.

Blood thyroxine iodine levels in this subject were measured in the pretreatment sample and at the terminal blood drawing, 48 hr after the last dose of medication (8th day after the study started).

The method used for total iodine determination consisted of wet ashing followed by the ceric ammonium sulfate reaction[2] (1). The thyroxine iodine test (2) was determined by a radioisotope dilution method.

RESULTS

The results of Study A are indicated in Fig. 1, which shows the total blood iodine levels attained in the serum after the oral administration of a single dose of the various iodinated glycerol formulations. Within the measurements made, all three formulations tested showed peak serum concentration between 1 and 4 hr after administration and demonstrated comparable levels. A progressive decline over 24 hr followed, when an appreciable amount of iodine was still detectable.

In the case of the iodinated glycerol elixir, a mean maximum

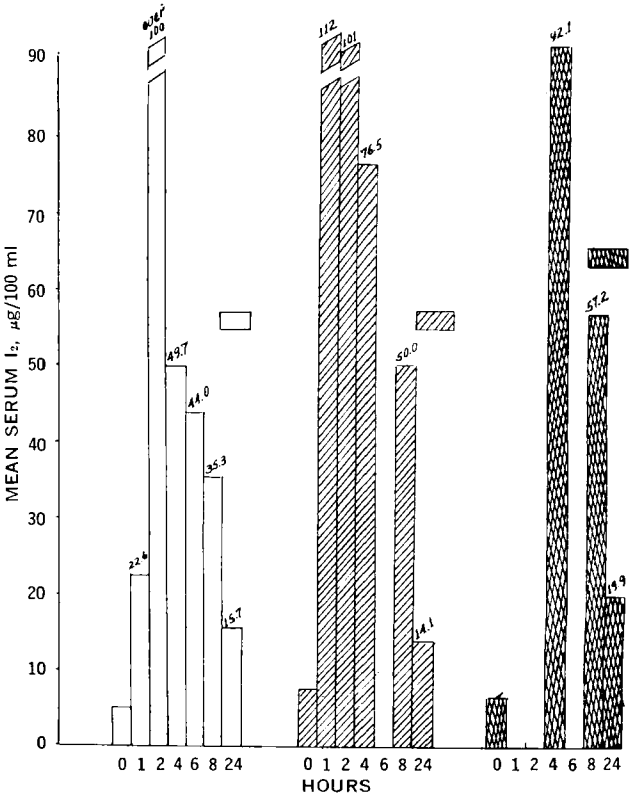

Figure 1—*Effect of single doses of iodine-containing products on iodine serum levels in adult subjects over 24 hr. Key:* □, *iodinated glycerol elixir, 30 mg organically bound iodine;* ▨, *iodinated glycerol tablets, 30 mg organically bound iodine; and* ▩, *iodinated glycerol solution, 25 mg organically bound iodine.*

[1] Organidin elixir: 60 mg iodinated glycerol (30 mg organically bound iodine)/5 ml with alcohol 24%; Organidin aqueous solution: 50 mg iodinated glycerol (25 mg organically bound iodine)/ml; Organidin tablets: 30 mg iodinated glycerol (15 mg organically bound iodine)/tablet; Wampole Labs., Stamford, Conn.

[2] Using a Technicon AutoAnalyzer.

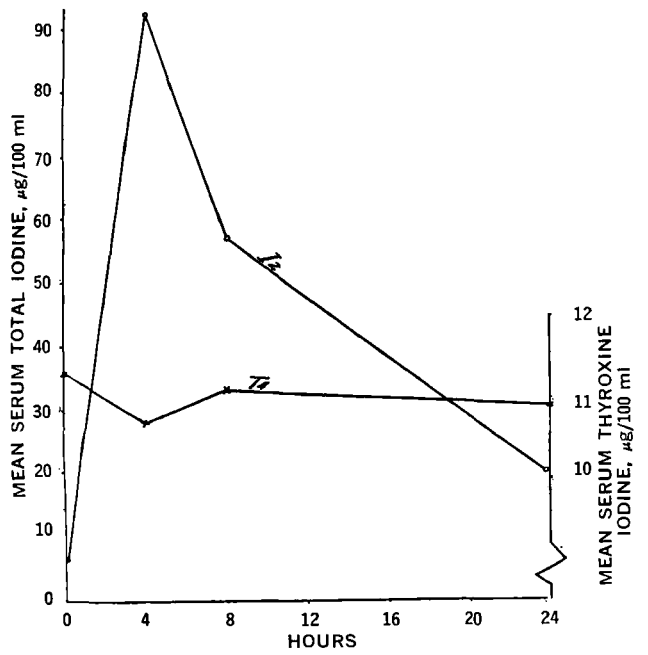

Figure 2—*Serum levels of total iodine (I_2) and thyroxine iodine (T_4) following a single dose of iodinated glycerol solution, 25 mg organically bound iodine.*

level of 100 μg/100 ml was reached in 2 hr, which dropped in progressive steps to 15.7 μg at 24 hr (2.5 × the mean initial level of 5.95 μg). A mean peak level of 112 μg/100 ml was attained after 1 hr when the tablets were used, and within 24 hr the level fell to 14.1 μg (2.4 × the initial level). Following the administration of the aqueous solution, a mean peak level of 92.1 μg was recorded at 4 hr. No blood was drawn in this instance after 1 and 2 hr where the peak undoubtedly occurred, based on experience with the other groups. At 24 hr after medication, levels of iodine in this instance dropped to 19.9 μg (3.3 × the basal level).

There has been some concern that thyroid function tests might be affected by the administration of iodine-containing drugs. Friend (6) found that iodinated glycerol solution administered to a group of healthy subjects in a dose exceeding that recommended as a mucolytic agent did not cause any striking elevation in the protein-bound iodine. The thyroxine iodine test was done to confirm this opinion that iodinated glycerol in the recommended therapeutic dose does not affect thyroid function testing. Figure 2 shows the data along with the corresponding total iodine levels and confirms that there is no effect on thyroxine iodine following administration of a single therapeutic dose of iodinated glycerol solution.

For Study B, the total serum iodine levels following repeated daily doses of iodinated glycerol solution at recommended therapeutic levels is shown in Fig. 3. There was a rapid rise in blood iodine; 4 hr after treatment it reached 65.2 μg from an initial level of 4.9 μg. The iodinated glycerol solution produced a maximum concentration of 100–133 μg, and this level was maintained through 5 days of medication. After administration of iodinated glycerol solution was discontinued on the 7th day, serum iodine levels dropped progressively to pretreatment levels in 36 hr.

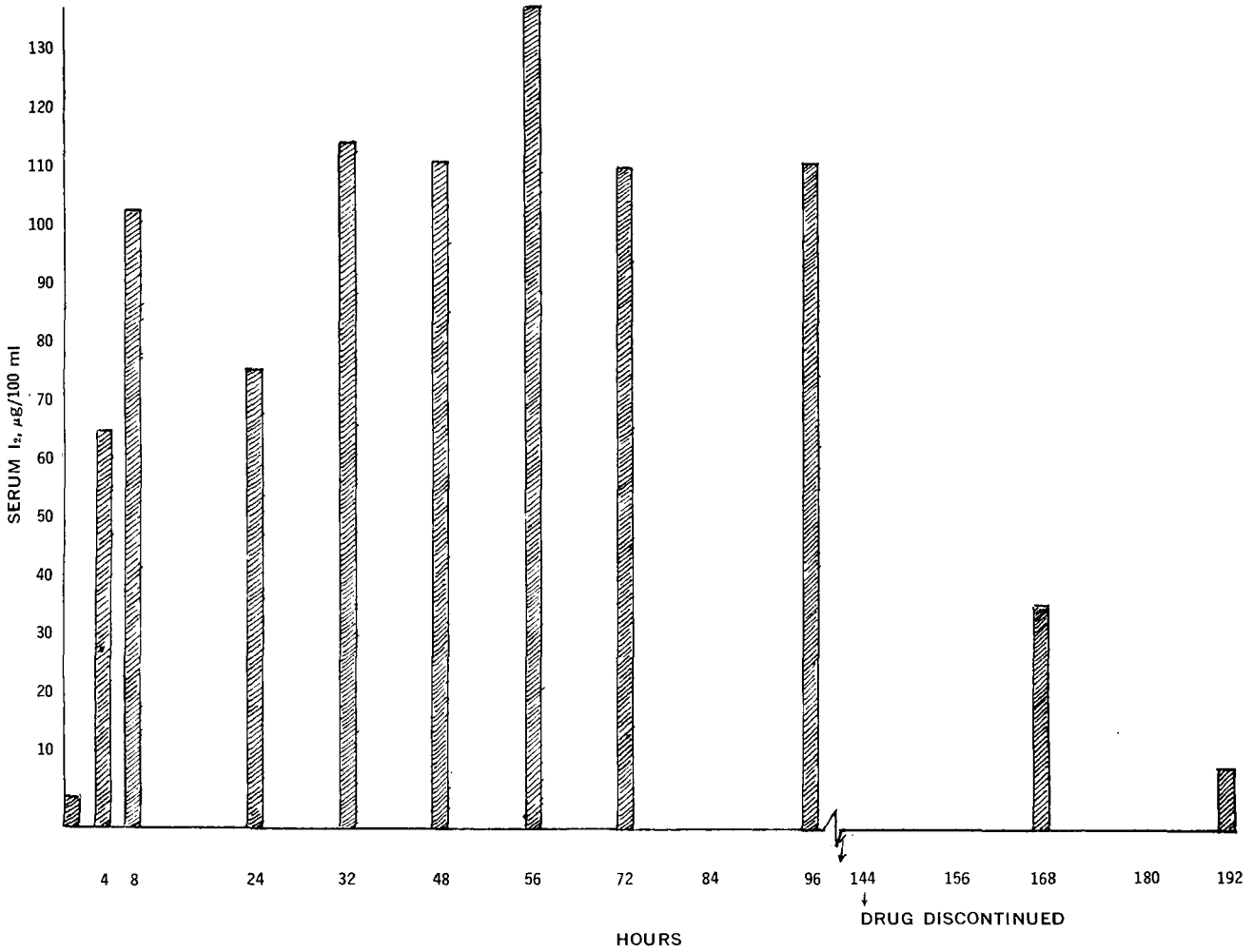

Figure 3—*Serum levels of total iodine following repeated therapeutic doses of iodinated glycerol solution, 25 mg organically bound iodine four times daily.*

The thyroxine iodine level was determined at the onset of the 6-day experiment and again 36 hr after discontinuance of the drug, when only a 4% difference in thyroxine iodine level was noted (7.9 μg/100 ml initially *versus* 8.2 μg/100 ml after drug discontinuation); this difference is considered within the normal range.

DISCUSSION

Organically bound iodine as iodinated glycerol has been widely used as a mucolytic expectorant for over 50 years. It has been reported to be as effective an expectorant as a saturated solution of potassium iodide and to have fewer side effects (3–5), the latter being attributed to the smaller amounts of iodine needed.

Gastric distress is frequently encountered with the use of a saturated solution of potassium iodide and, less commonly, iodism as evidenced by skin rash. Thyroid enlargement is not uncommon with longrange therapy with this drug. On the other hand, Seltzer (3) reported that in 100 consecutive cases treated with iodinated glycerol, there was no instance of thyroid enlargement, iodism, or complaints of gastric irritation. In 100 cases treated with a saturated solution of potassium iodide, he found four cases of iodism and three of thyroid enlargement.

It is self-evident that one must seek that expectorant activity of iodine that can be accomplished with the lowest possible dose and the least side effects, such as can be brought about with organically bound iodine formulations, as long as the formulation is bioavailable. This study has demonstrated that three such formulations containing organically bound iodine are not only bioavailable but are also approximately equiavailable in relation to time and dose.

CONCLUSIONS

The bioavailability of orally administered organically bound iodine as iodinated glycerol was demonstrated in adult, human, healthy subjects.

Total blood iodine levels following single recommended therapeutic doses of iodinated glycerol showed a rapid rise and a progressive fall over the experimental 24-hr period, at which point there was still an elevated level above initial concentrations. The thyroxine iodine level was not significantly affected after such single doses.

When daily doses of iodinated glycerol were continued for 6 days, it was demonstrated that the total blood iodine rose rapidly, maintained a constant level, and, after discontinuation, returned progressively within 48 hr to preadministration levels. The thyroxine iodine level remained within the normal range after continued medication over this period.

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Enhancing Effect of Calcium Ions on Transport of Cholesterol from Aqueous Sodium Taurocholate–Lecithin Micellar Phase to Oil Phase

VIJAY SURPURIYA and W. I. HIGUCHI[x]

Abstract □ Experiments on the influence of calcium ions upon the transport of cholesterol from bile salt–lecithin micelles into an oil phase were carried out using previously developed methods. As the calcium-ion concentration is increased, the sterol transport rate increases until a limiting maximum rate is reached at around a 0.03 *M* concentration of calcium ions. This limiting rate corresponds to an interfacial barrier permeability coefficient, *P*, of about 1×10^{-6} cm/sec, which is 35–40 times larger than that obtained in the absence of calcium. These results are consistent with the interfacial barrier-controlled mechanism in which the bile salt–lecithin micelle is involved in the rate-determining step at the aqueous–lipid interface.

Keyphrases □ Calcium ions—enhancing effect on cholesterol transport from sodium taurocholate–lecithin micelles to oil phase □ Cholesterol transport—from bile salt–lecithin micelles into oil phase, enhancing effect of calcium ions □ Bile salt–lecithin micelles—transport of cholesterol, influence of calcium ions

Recent investigations (1–6) in these laboratories led to the conclusion that the transport of cholesterol and other sterols from aqueous to oil phases may be interfacial barrier controlled rather than bulk phase diffusion controlled. The implications of these findings in biology should be broad, because the understanding of sterol transport is vital to the understanding of sterol absorption in the intestine, of the pathogenesis of atherosclerosis and of cholesterol gallstones, and of the metabolism of sterols generally. That interfacial kinetics rather than bulk diffusion and convection are more important relegates thermodynamics into somewhat of a secondary role as far as transport is concerned. Previous studies (5, 6) showed not only that the kinetics in this situation are interfacial barrier controlled but that the bile salt–lecithin micelle is a key participant in the rate-determining step.

The present report represents a portion of the effort made to understand the mechanistic role of the bile salt–lecithin–cholesterol micelle in the transport of cholesterol at the lipid–aqueous interface. The present data demonstrate a dramatic effect of calci-

The thyroxine iodine level was determined at the onset of the 6-day experiment and again 36 hr after discontinuance of the drug, when only a 4% difference in thyroxine iodine level was noted (7.9 μg/100 ml initially *versus* 8.2 μg/100 ml after drug discontinuation); this difference is considered within the normal range.

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VIJAY SURPURIYA and W. I. HIGUCHI[x]

Abstract □ Experiments on the influence of calcium ions upon the transport of cholesterol from bile salt–lecithin micelles into an oil phase were carried out using previously developed methods. As the calcium-ion concentration is increased, the sterol transport rate increases until a limiting maximum rate is reached at around a 0.03 *M* concentration of calcium ions. This limiting rate corresponds to an interfacial barrier permeability coefficient, *P*, of about 1×10^{-6} cm/sec, which is 35–40 times larger than that obtained in the absence of calcium. These results are consistent with the interfacial barrier-controlled mechanism in which the bile salt–lecithin micelle is involved in the rate-determining step at the aqueous–lipid interface.

Keyphrases □ Calcium ions—enhancing effect on cholesterol transport from sodium taurocholate–lecithin micelles to oil phase □ Cholesterol transport—from bile salt–lecithin micelles into oil phase, enhancing effect of calcium ions □ Bile salt–lecithin micelles—transport of cholesterol, influence of calcium ions

Recent investigations (1–6) in these laboratories led to the conclusion that the transport of cholesterol and other sterols from aqueous to oil phases may be interfacial barrier controlled rather than bulk phase diffusion controlled. The implications of these findings in biology should be broad, because the understanding of sterol transport is vital to the understanding of sterol absorption in the intestine, of the pathogenesis of atherosclerosis and of cholesterol gallstones, and of the metabolism of sterols generally. That interfacial kinetics rather than bulk diffusion and convection are more important relegates thermodynamics into somewhat of a secondary role as far as transport is concerned. Previous studies (5, 6) showed not only that the kinetics in this situation are interfacial barrier controlled but that the bile salt–lecithin micelle is a key participant in the rate-determining step.

The present report represents a portion of the effort made to understand the mechanistic role of the bile salt–lecithin–cholesterol micelle in the transport of cholesterol at the lipid–aqueous interface. The present data demonstrate a dramatic effect of calci-

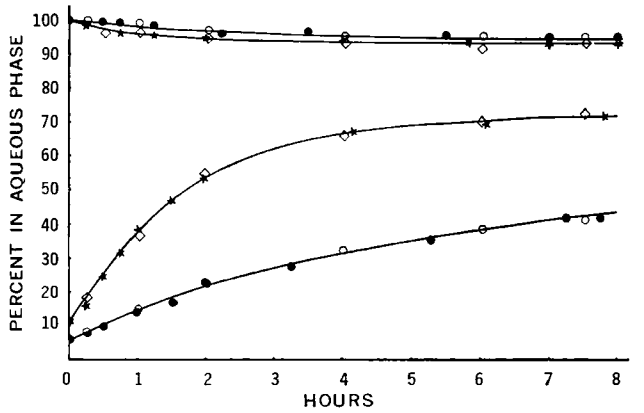

Figure 1—*Comparison of experimental data with theory for the uptake and release of cholesterol in the sodium taurocholate–lecithin system with 0.8% hexadecane. Key: ●, experimental, 2% sodium taurocholate + 1% lecithin; ★, experimental, 2% sodium taurocholate + 1% lecithin + 0.01 M calcium chloride; and ○ and ◊, duplicate experiments. Smooth curves represent theoretical predictions.*

um ions upon the cholesterol transport rate in the context of an interfacial barrier-controlled situation and, therefore, provide a significant clue toward the determination of the nature of the "activated complex" and of the various factors contributing to the interfacial transfer process.

EXPERIMENTAL

The same general procedures utilized previously (5, 6) were followed. Basically, the experiments involved the preparation of master emulsions with hexadecane[1] and appropriate sodium taurocholate[2]–lecithin[2] solutions by homogenizing in a blender[3], shaking for about 15–20 min, and then diluting in the sodium taurocholate–lecithin solutions in which appropriate amounts of calcium chloride[4] and/or sodium chloride[4] were added beforehand. Radioactive cholesterol[5] was either added to the oil (for release experiments) or to the sodium taurocholate–lecithin solution (for uptake experiments). The mixture was then shaken gently at 30° in a wrist-action shaker[6]. Five-milliliter samples were pipeted out at different time intervals, and the aqueous supernatant phases were separated from the oil by high speed centrifugation[7] for 1–1.5 min. Out of the clear aqueous solution collected, 1 ml was pipeted into a liquid scintillation vial and 10 ml of a liquid scintillation cocktail[8] was then added to the vial. The samples were quantitatively analyzed in a liquid scintillation counter[9].

Individual emulsion particle-size distribution data[10] were obtained for each emulsion, as before. Generally, no significant changes in the particle-size distribution occurred in the reaction systems during the run, even when the maximum levels (0.1 *M*) of calcium ions were present.

RESULTS

The plots of a typical transport experiment (Fig. 1) give the percent of the radioactive compound in the aqueous phase as a function of time. As can be seen, the reproducibility of the experiments was quite satisfactory.

The experimental results were treated by the following equations, developed earlier (5), based upon the physical model shown in Fig. 2:

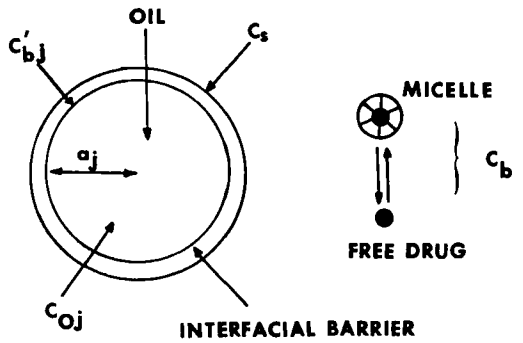

Figure 2—*Physical model describing the uptake and/or release of solutes by the oil droplet;* a_j = *radius of oil droplet,* C_{oj} = *solute concentration in oil phase,* C_s = *aqueous solute concentration just outside adsorbed film,* C_b = *solute concentration in aqueous phase, and* C'_{bj} = *solute concentration at oil–water interface.*

$$\frac{dC_{oj}}{dt} = \frac{3DP(C_b - C_{oj}/K)}{a_j(D + a_jP)} \qquad \text{(Eq. 1)}$$

$$\frac{dC_b}{dt} = -\frac{4\pi}{3V_w}\sum_{j=1}^{L} a_j^3 \Delta N_j \frac{dC_{oj}}{dt} \qquad \text{(Eq. 2)}$$

where:

a_j = radius of oil droplet
P = apparent permeability coefficient for interfacial barrier
D = relevant diffusion coefficient for cholesterol in bile salt–lecithin solution
C_b = total bulk aqueous solute concentration
C_{oj} = concentration of solute in oil droplet
K = effective hexadecane–(bile salt–lecithin) partition coefficient for solute
V_w = volume of aqueous phase
ΔN_j = number of droplets of sizes between A_j and A_{j+1}
L = largest oil droplets in system

Equation 1 expresses the rate of change in the concentration of the solute in the oil droplet, and Eq. 2 describes the material balance in the system. These two equations were solved[11] for C_b when V_w, D, P, K, and the particle-size distribution were known. Fitting of the experimental data by the theory was accomplished by using P as the only adjustable parameter. The K values were

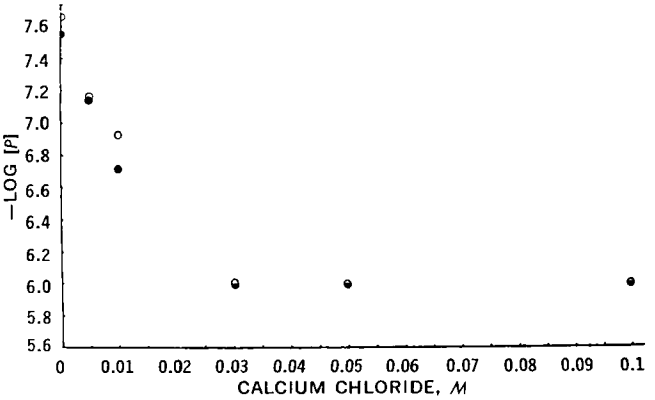

Figure 3—*Effect of increasing concentrations of calcium chloride on the permeability coefficient* (P). *Plot shows* −*log* [P] *versus molar concentration of calcium chloride. Key: ○, 1% sodium taurocholate + 0.05% lecithin; and ●, 2% sodium taurocholate + 1% lecithin.*

[1] Matheson, Coleman and Bell, Norwood, Ohio.
[2] Schwarz/Mann, Orangeburg, NY 10962
[3] Waring, Sargent Welch Scientific Co., Chicago, Ill.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] Cholesterol-4-C^{14}, New England Nuclear, Boston, Mass.
[6] Burrell, Burrell Corp., Pittsburgh, Pa.
[7] Lourdes Instrument Corp., Brooklyn, N.Y.
[8] The cocktail consisted of 2,5-diphenyloxazole (7 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (50 mg), naphthalene (50 g), and dioxane *q.s.* (1000 ml).
[9] Beckman Instruments, Inc., Fullerton, Calif.
[10] Using the Coulter counter, model A, Coulter Electronics, Hialeah, Fla.
[11] Using an IBM-360 digital computer.

Table I—Effect of Increasing Amounts of Calcium Chloride and/or Sodium Chloride on the Partition Coefficient (K) and the Effective Permeability Coefficient (P) for Cholesterol in the Sodium Taurocholate–Lecithin System

2% Sodium Taurocholate + 1% Lecithin					1% Sodium Taurocholate + 0.5% Lecithin				
Electrolyte, M			$P \times 10^7$, cm/sec		Electrolyte, M			$P \times 10^7$, cm/sec	
Calcium Chloride	Sodium Chloride	K	Uptake	Release	Calcium Chloride	Sodium Chloride	K	Uptake	Release
0.00	0.00	10.0	0.30	0.28	0.00	0.00	19.4	0.22	0.21
0.005	0.00	10.0	0.71	0.70	0.005	0.00	19.0	0.69	0.68
0.01	0.00	9.0	1.9	1.8	0.01	0.00	18.0	1.20	1.20
0.03	0.00	10.0	10.0	10.0	0.03	0.00	18.0	9.80	9.70
0.05	0.00	10.0	10.0	10.0	0.05	0.00	18.0	10.0	10.0
0.1	0.00	10.0	10.0	10.0	0.1	0.00	17.0	9.8	10.0
0.00	0.1	8.0	0.40	0.40	0.00	0.1	20.0	0.32	0.32
0.005	0.1	9.0	20.0	20.0					
0.01	0.1	9.0	20.0	20.0					

calculated from the plateau portions of the uptake and release plots of the experimental data. The computer simulation calculations are shown as the smooth curves in Fig. 1.

Table I gives the K values and the best P values for cholesterol in the sodium taurocholate–lecithin system with added calcium chloride and/or sodium chloride. As can be seen, the P values obtained from the uptake experiments and those obtained from the release experiments are in close agreement.

DISCUSSION

Table I shows the dramatic effect of added calcium chloride upon the P values of cholesterol in the sodium taurocholate–lecithin system. As the concentration of calcium chloride is increased, the P values increase until a maximum of 1×10^{-6} cm/sec is reached (Fig. 3). At 0.01 M calcium chloride, a P value of about $1.2\text{–}1.8 \times 10^{-7}$ cm/sec is obtained, which is about six to seven times larger than the value obtained without calcium chloride; at 0.1 M calcium chloride, a P value of about 1×10^{-6} cm/sec is obtained, which is about 35–40 times larger than the control. Table I also shows the effect of added sodium chloride upon the P values, and that effect is nothing like that exhibited by calcium. Even at 0.1 M sodium chloride concentration, a P value is increased by only a factor of about 1.5.

Table I shows that varying the concentration of sodium taurocholate and lecithin but keeping their ratio constant does not affect P; *i.e.*, P is independent of the taurocholate–lecithin concentration and, therefore, of K. This supports the previously proposed mechanism (5, 6) implicating the micelle in the rate-determining step. The presence of lecithin in the micelle would be expected to increase the affinity of the micelle for the sterols greatly. Consequently, it is reasonable to expect that the release tendency for the sterol from the micelle should be reduced when lecithin is present in the micelle.

The aqueous to oil transport of cholesterol from sodium lauryl sulfate micelles probably follows an entirely different mechanism (5). The effect[12] of sodium chloride on the transport rates of cholesterol in the sodium lauryl sulfate was that the electrolyte significantly reduced the P values. In this case, the free solute transport mechanism (5) rather than the micelle transport mechanism is probably the rate-determining one.

The present finding that calcium ion greatly increases the permeability coefficient for cholesterol in the sodium taurocholate–lecithin–hexadecane system is very provocative. The calcium-ion effect is much larger than might be expected on the basis of simple ionic strength effects, *e.g.*, the influence of sodium chloride. Also when calcium ions and sodium ions are added together in this sodium taurocholate–lecithin system, the P value for cholesterol is increased to 2×10^{-6} cm/sec (Table I), which indicates that the effect of calcium ions and sodium ions is synergistic rather than additive and strongly supports the proposal that the effect of calcium ions may be different and more specific than sodium ions and that it can play a key role in lowering the free energy of the activated complex involved in the transport process.

[12] V. Surpuriya and W. I. Higuchi, unpublished data.

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[x] To whom inquiries should be directed.

GLC Determination of Fluorouracil

KOPPAKA V. RAO[x], KARON KILLION, and YASAR TANRIKUT

Abstract □ A GLC assay method for fluorouracil, based on the flash-methylation technique using trimethylanilinium hydroxide, was developed. It is a convenient, rapid, and sensitive method for the determination in urine and other biological fluids. An ion-exchange recovery method was also developed, using a combination of strong acid and weak base resins and elution from the latter with aqueous ethanolic ammonia.

Keyphrases □ Fluorouracil—GLC analysis, flash methylation using trimethylanilinium hydroxide □ Ion-exchange chromatography—separation of fluorouracil from urine □ GLC—analysis, fluorouracil, flash methylation using trimethylanilinium hydroxide

The importance of fluorouracil (I) in the treatment of certain forms of cancer was reviewed (1). However, biopharmaceutical studies of this drug have been hampered by the lack of a suitable analytical procedure. Part of the problem is due to the lack of distinctive features which can facilitate the development of a convenient method of assay. The spectrophotometric method (2) lacks sensitivity as well as specificity, and the microbiological assay, although selective, can become unsuitable when the patient is on simultaneous antibiotic therapy. The combination of dialysis and silylation techniques (3) requires overnight operation, so it is not convenient for a routine assay.

Recently, Cohen and Brennan (4) developed a GLC procedure for fluorouracil coupled with extraction using 16% *n*-propanol in ether. The fluorouracil in the extract was silylated with 50% *N,O*-bis(trimethylsilyl)trifluoroacetamide to give a reasonable retention time on the gas chromatograph. In their method, the temperature of the column has to be raised after fluorouracil comes out of the column to permit the emergence of the internal standard, anthracene, in a reasonable time (12 min). Also, use of a silylating agent requires reconditioning of the column every 2–3 days and injection of a mixture of trimethylsilyl donors. The detector also has to be cleaned every 2–3 days.

Brochmann-Hanssen and Oke (5) developed a GLC assay method for barbiturates, phenolic alkaloids, and xanthine bases using flash methylation with 0.1 *M* trimethylanilinium hydroxide. The possibility of the application of this method for the determination of fluorouracil prompted the present study. Also, since the microbiological assay has certain advantages such as simplicity and sensitivity, an improved method for the recovery of fluorouracil from biological fluids would extend its utility. This paper describes an ion-exchange method for recovery and a GLC method for the determination of fluorouracil.

EXPERIMENTAL

Microbiological Assays—These assays were performed according to the method of Hunt and Pitillo (6) using *Escherichia coli* (ATCC 9637) as the test organism. Depending on the amount of inoculum, the range of sensitivity was 0.3–3 μg/ml. Although somewhat less sensitive (1–10 μg/ml), the *Bacillus subtilis* (ATCC 6633) plate assay has some advantages; *e.g.*, the culture can be stored as a relatively heat-stable spore suspension and the amount of inoculum can be more easily duplicated. The assay medium for both organisms had the following composition (in grams per liter): K_2HPO_4, 7; KH_2PO_4, 3; sodium citrate, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; glucose, 2; and agar, 15.

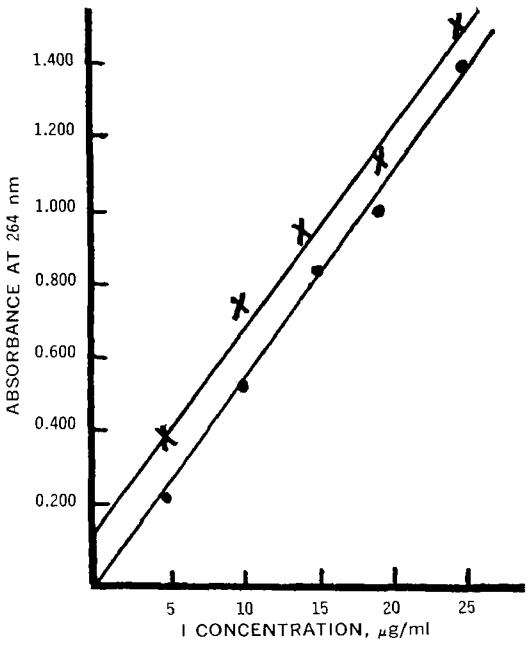

Figure 1—*Calibration curve for ion-exchange method of recovering fluorouracil. Key:* ●, *before the sulfonic acid-type column; and* ×, *after the primary amine-type column.*

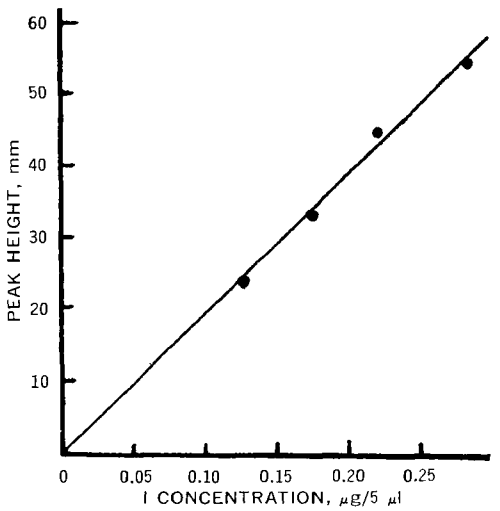

Figure 2—*Calibration curve for fluorouracil extracted from aqueous solution.*

Table I—Recovery of Fluorouracil (I) Added to Urine

I Added, μg/ml	I Recovered, μg/ml (Microbiological)	I Recovered, μg/ml (GLC)
1.24	1.06	1.14
2.48	2.10	2.23
3.72	3.12	4.01
4.96	4.16	5.10

Filter paper disks[1] were dipped in standard solutions of fluorouracil in the appropriate concentration range, placed on the seeded agar plates, and incubated at 37° overnight. A plot of the diameter of the zone of inhibition against the log of the concentration of fluorouracil gave the standard curve.

Ion-Exchange Methods—Analytical grade ion-exchange resins of the sulfonic acid[2] type (100–200 mesh) with 4% cross-linking agent and the primary amine[3] type (50–100 mesh) were used in this study. The acid resin was washed with 2 *N* HCl followed by 50% aqueous ethanol and water. The basic resin was washed with 10% aqueous potassium carbonate followed by 80% aqueous ethanol and water. Both resins must be freshly washed before use.

For the recovery of fluorouracil from urine, a filtered sample (100 ml) was passed through a column (18 × 150 mm) of the acid resin, previously washed as described, at a rate of 5–8 ml/min. The column was then washed with two bed volumes of distilled water. The effluent and wash, as they emerged, were passed through a second column containing the basic resin (18 × 150 mm). In this sequence, fluorouracil passed unabsorbed through the acid resin but was completely retained on the basic resin. After the latter column was washed with water, fluorouracil was eluted by two bed volumes of aqueous ethanolic ammonia [ethanol–water–concentrated ammonium hydroxide (3:1:0.2)].

A gas chromatograph[4] equipped with a flame-ionization detector was used for the GLC analyses. A glass U-shaped column, 0.63 cm o.d. and 183 cm long, was packed with 2.8% OV-210 + 3.2% OV-1 on 80–100-mesh Chromosorb W (MP)[5] and conditioned at 250° for 18 hr. Helium, the carrier gas, was maintained at 55 ml/min, the flow rate for hydrogen was 45 ml/min, and that for air was 300 ml/min. The injector temperature was 250°, the detector temperature was 300°, and the column oven temperature was maintained at 125°. Under these conditions, fluorouracil emerged as a sharp peak with a retention time of 7.7 ± 0.2 min.

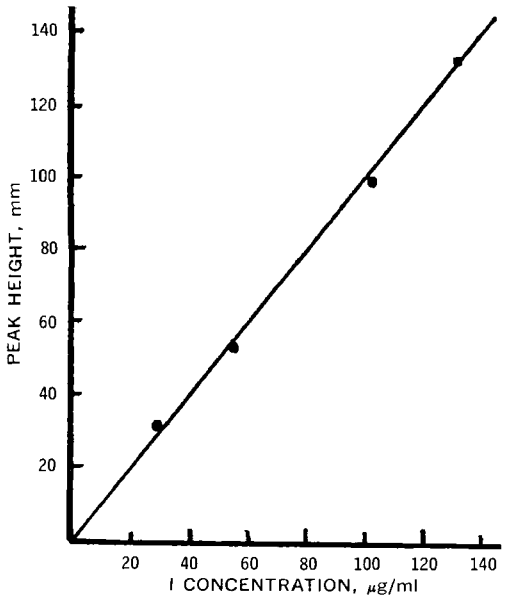

Figure 3—*Calibration curve of fluorouracil extracted from urine.*

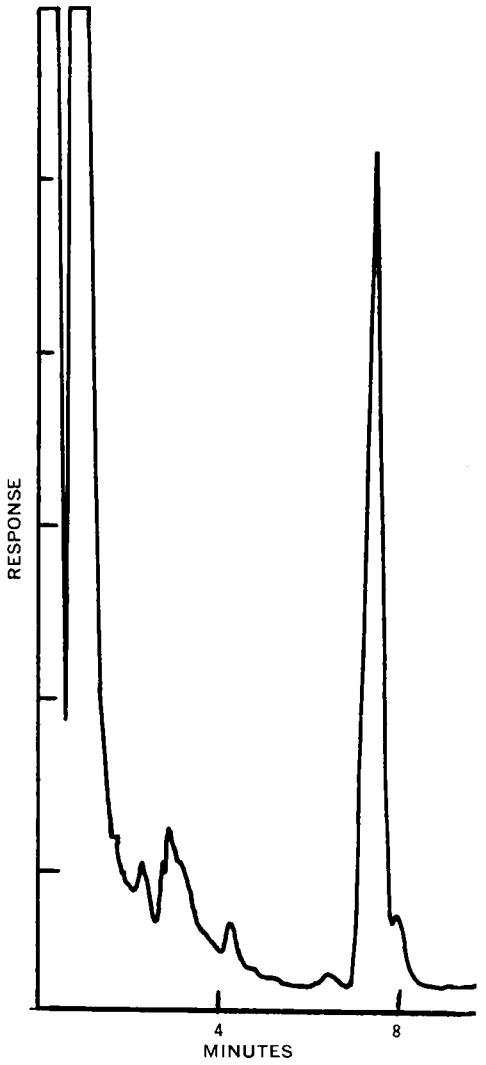

Figure 4—*Gas chromatogram of a urine sample of fluorouracil with the addition of trimethylanilinium hydroxide (fluorouracil concentration = 1.0 mg/ml).*

The extraction method of Cohen and Brennan (4) was followed. If necessary, the pH of the urine sample was adjusted to 6.0 using 1 *M* acetate buffer. After the extraction, a 10-ml aliquot of the ether–*n*-propanol solution was pipeted into a centrifuge tube and evaporated to dryness in a hot water bath (50°) under a gentle stream of air filtered through glass wool.

The evaporated sample was dissolved in 0.2 ml of 0.1 *M* trimethylanilinium hydroxide (5). The reaction mixture was kept cold until injected. A 5-μl sample of the solution was injected into the gas chromatograph. Each analysis was carried out in duplicate.

RESULTS AND DISCUSSION

The recovery of fluorouracil from the ion-exchange procedure based on absorbance at 264 nm is shown in Fig. 1. Because of some leaching from the resin of impurities that absorb in the 260–265-nm region, a blank must be run using the same samples of washed resins. Nevertheless, a method based on absorbance at 264 nm was found to be suitable for biological fluids only for concentrations of 100 μg or higher. For lower concentrations (up to 1 μg/ml), a microbiological assay was used and it gave the expected recovery within the limits of accuracy characteristic of this meth-

[1] Schleicher and Schuell No. 740-E.
[2] Dowex-50, Biorad Laboratories, Richmond, Calif.
[3] Amberlite IR-45, Biorad Laboratories, Richmond, Calif.
[4] Varian model 2100 (Varian Aerograph, Walnut Creek, Calif.) equipped with a Varian model A-25 strip-chart recorder.
[5] Column packing prepared by M. R. J. Perchalski, Medical Research, VA Hospital, Gainesville, Fla.

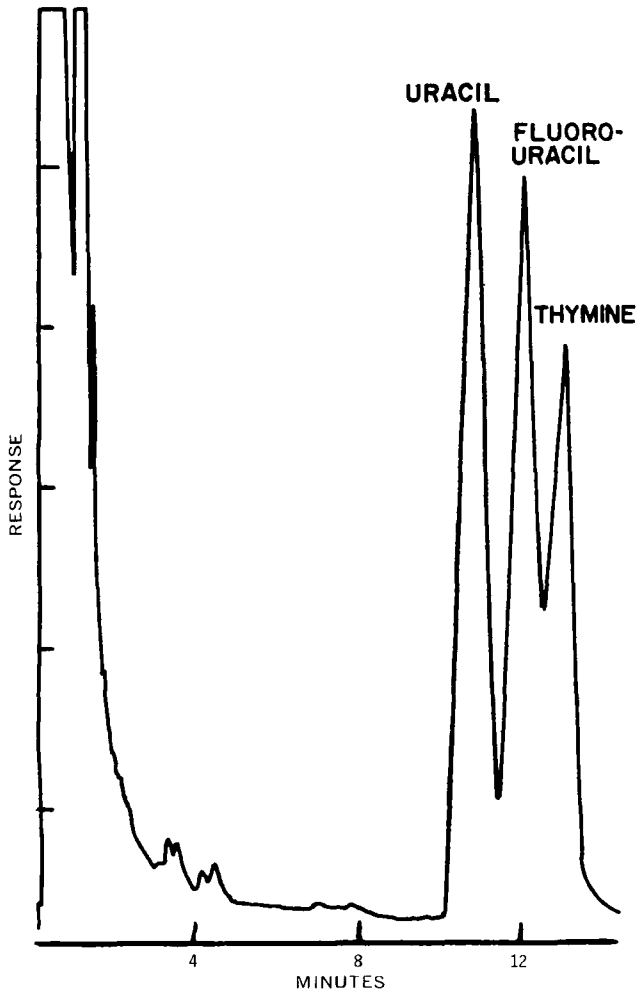

Figure 5—*Gas chromatogram of uracil, fluorouracil, and thymine with the addition of trimethylanilinium hydroxide. Flow rate for helium in this experiment was 27 ml/min.*

od (±20%) (Table I). The applicability of the ion-exchange procedure in the lower concentration range was also tested using GLC (Table I).

Figure 2 shows the standard curve for the GLC determination of fluorouracil after extraction from aqueous solutions. Recovery of added fluorouracil to a urine sample is shown in Fig. 3. The efficiency of extraction was approximately 85%, as indicated by Cohen and Brennan (4).

To establish the identity of the peak corresponding to flash-methylated fluorouracil, it was compared with the peak obtained from fluorouracil methylated with diazomethane. The two showed the same retention time of 7.5 min at the column oven temperature of 125°. Figure 4 shows a typical chromatogram of fluorouracil after extraction from urine sample and flash methylation.

Since biological fluids are likely to contain related pyrimidines such as uracil or thymine, their separation from fluorouracil was studied using these procedures. The three could be separated when the column temperature was maintained at 120°. A typical chromatogram is shown in Fig. 5. The separation of these three components could be effected even more readily when the mixture was subjected to flash ethylation with triethylanilinium hydroxide followed by GLC at a column oven temperature of 100°.

Although the extraction method is more convenient for the GLC assay, the ion-exchange method of recovery has certain advantages. It is capable of providing purer samples of fluorouracil as, for example, when recovery of labeled samples is desired. It is also capable of greater selectivity, especially when separation from certain basic or neutral constituents (such as antibiotics) is necessary.

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[x] To whom inquiries should be directed.

Papaver bracteatum Lindl: Population Arya II

I. LALEZARI[x], P. NASSERI*, and R. ASGHARIAN*

Abstract □ A new population of *Papaver bracteatum* named Arya II was found in Mahabad (western Iran) and produced 3.5% of thebaine in dried capsules. No other alkaloid was detected in this new population. The method of extraction is described.

Keyphrases □ *Papaver bracteatum* Lindl—thebaine content of Arya II population, extraction method □ Thebaine—analysis of content in new Arya II population of *P. bracteatum* □ Arya II, *P. bracteatum*—analysis of thebaine content, extraction method

Previously it was reported that the dried latex obtained by incision of the immature capsules of *Papaver bracteatum*, growing wild in the Albourz mountains in northern Iran, contained 26% thebaine (1). It was also demonstrated that the dried latex of *P. bracteatum* obtained from wild plants growing in the Polour region (northern Tehran) contained 26% thebaine along with 2.3% alpinigenine (2).

Recently, a new population[1], Arya II, of *P. bracteatum* was found in Mahabad (western Iran)[2] and produced thebaine almost exclusively in very high yields. The thebaine content of this new population is 3.5% in dried capsules, more than three times higher the content of previously reported thebaine-producing plants.

The content of the pigments and resinous material in Arya II is very low. Thus, almost pure thebaine could be obtained after one crystallization of extracted material. This finding was supported by IR, NMR, and mass spectroscopy. TLC of the crude extract of capsules revealed that no other alkaloid was present in the dry capsules. The thebaine content of the dried aerial parts of the plant is summarized in Table I.

Table I—Thebaine Assay Values of *P. bracteatum* Lindl, Population Arya II

Aerial Part	Number of Analyses	Percent Thebaine[a], Mean $\bar{X}$ (Range)	Standard Deviation, %
Petals	5	1.02 (0.85–1.15)	0.11
Stigmas	5	0.63 (0.55–0.75)	0.08
Bracts	5	1.81 (1.61–2.12)	0.19
Young leaves	2	0.02 (0.02–0.02)	—
Stems[b]	5	1.03 (0.91–1.21)	0.13
Capsules[c]	50[d]	3.58 (3.35–3.75)	0.16

[a] Figures are based on dried material. [b] Collected prior to flowering. [c] Mature capsules. [d] Ten separate collections were made during June. Each collection consisted of 200 capsules, and five analysis were carried out on each collection.

EXPERIMENTAL[3]

Plant Material—The yellow-green capsules collected just prior to maturation contained the maximum of thebaine. Capsules were dried in the open air in the shade, seeds were separated, and the residue was dried at 60° to constant weight and finely ground.

UV Spectrophotometry—A mixture of 1.0 g of powdered capsules and 20 ml of benzene containing 2 ml of concentrated ammonia was stirred for 1 hr at room temperature. The mixture was filtered with suction, and the residue was stirred with 20 ml of benzene for 1 hr and filtered. Then the residue was washed with 10 ml of benzene. The remaining plant material was subjected to extensive analysis for alkaloid content, but no alkaloid was detected. The combined extracts and benzene washings were evaporated under reduced pressure, and the residue was divided into two equal parts. One part was subjected to TLC (silica gel), using acetone–chloroform–triethylamine–methanol (4:3:2:1) as an eluting solvent. A single spot (R_f 0.83) was obtained. An authentic sample of thebaine was used as a reference. A preparative TLC technique followed by recrystallization gave a product, mp 198°. The IR (KBr) and NMR (deuterochloroform) spectra of this thebaine were identical with those of an authentic sample. The remainder of the extract was dissolved in 0.1 *N* HCl and diluted with the same solvent to 500 ml (a small amount of charcoal could be used in the colored solutions). The extinction at 285 nm (maximum) was measured using an $E_{1\%}$ (1 cm) value of 250.

Gravimetric Method—The residue from evaporation of benzene extracts of 10 g of powdered capsules (using a total of 100 ml of benzene and 5 ml of concentrated ammonia) was dissolved in 50 ml of 0.1 *N* HCl and extracted with three 15-ml portions of ether to remove the coloring matter. To the aqueous layer, concentrated ammonia was added until alkaline reaction and the mixture was extracted with three 10-ml portions of chloroform. After evaporation of the solvent, the crystalline residue was recrystallized from 96% alcohol, mp 198° [lit. (1) mp 198°]; molecular weight by mass spectroscopy: *m/e* 311. It had IR and NMR spectra superimposable with those of an authentic sample of thebaine. Use of either chloroform or methanol instead of benzene gave identical results. Also, a 50% aqueous solution of sodium carbonate could be used instead of ammonia.

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* Research assistant on a grant supported by the U.S. Department of Agriculture.

[x] To whom inquiries should be directed.

[1] The name Arya II was adopted by the Second International Working Group on *P. bracteatum*, Sept. 13–17, 1973, Tehran, Iran; United Nations Secretariat Publication ST/SOA/SER, J/2 12, Sept. 1973.

[2] A herbarium sample of the plant material has been deposited in the Missouri Botanical Garden.

[3] Melting points were taken on a Kofler hot-stage microscope. The IR spectra were determined on a Leitz model III spectrograph. NMR spectra were taken on a Varian A-60A instrument. UV spectra were recorded on a Varian-Techtron 635 instrument. Mass spectra were obtained using a Varian Mat 111 spectrograph.

Cardiac Stimulant Action of Extracts of Coelenterates on Rat Atria

S. SHIBATA, D. F. DUNN, M. KUCHII, M. KASHIWAGI, and T. R. NORTON[x]

Abstract □ Crude aqueous ethanol extracts of nine out of 12 species of anthozoans and the one scyphozoan examined showed varying degrees of positive inotropic and chronotropic effects on isolated rat atria. This is the first report of what may be rather widely occurring heart stimulants of possible practical utility among the Coelenterata.

Keyphrases □ Coelenterates—cardiac stimulant action of extracts, rat atria □ Anthozoans—ethanol extracts, cardiac stimulant activity, rat atria □ Scyphozoan—ethanol extract, cardiac stimulant activity, rat atria □ Cardiac stimulant activity—ethanol extracts of coelenterates, rat atria

While examining extracts of coelenterates for antitumor activity (1, 2) and central stimulant action (3), the toxicity of the extracts aroused interest in examining their effect on cardiac muscle. Extracts of nine of 12 species of anthozoans and the one scyphozoan examined showed varying degrees of positive inotropic effect.

About 70 of some 9000 known species of coelenterates have been reported capable of causing intoxication in humans, either from contact with the nematocysts (stinging organelles) or by ingestion of uncooked tissues (4). Many workers have examined the chemical nature and pharmacology of these toxins, and several have examined their cardiac action (4). Huang and Mir (5) noted that an extract of the tentacles of *Calliactis polypus* (a sea anemone) caused a brief lowering of coronary outflow, heart rate, and amplitude of cardiac contractions in isolated rabbit heart, while higher doses produced irregular cardiac contractions, usually resulting in cardiac arrest.

This is the first report of what may be rather widely occurring heart stimulants of possible practical utility among the Coelenterata.

EXPERIMENTAL

The sea animals were preserved in ethanol immediately after collection. They were homogenized with 50 ml 30% ethanol/100 g animal wet weight and allowed to stand for 24 hr. The solid residue was removed by centrifugation, and the combined supernate and preserving alcohol were flash evaporated at $\leq 40°$ to a volume equal in milliliters to the wet weight of the animal in grams. The aqueous solution was filtered or centrifuged and the clear solution was used for testing. This crude extract was lyophilized for dry weight determination.

Bioassay of the solutions was performed on isolated atria of rat hearts. The atrium was separated from the rest of the heart and suspended in an isolated organ bath (20, 25, or 50 ml) containing Krebs–Ringer bicarbonate medium (pH 7.4) of the following composition in distilled deionized water (in mmoles): Na^+, 145; K^+, 6.02; Ca^{+2}, 1.22; Mg^{+2}, 1.33; Cl^-, 126; HCO_3^-, 25.3; PO_4^{-3}, 1.2; SO_4^{-2}, 1.33; and glucose, 5.5. The temperature of the organ bath was maintained at 30°, and the Krebs–Ringer medium was continuously aerated with 95% O_2–5% CO_2.

The spontaneously beating atrial preparation was connected by a thin silk thread to a force-displacement transducer[1], and the contractile movements were recorded on a six-channel polygraph[2]. The preparation was allowed to equilibrate under 750 mg tension for 60 min prior to beginning an experiment. After this equilibration, during which the preparations were washed out every 30 min, the spontaneous beat rate of the atria remained constant, the change during a 10-min observation being less than 5 beats/min. The changes in contractile force and rate produced by the test agents are expressed as a percentage increase or decrease in tension and rhythm, with the period immediately preceding addition of test solution to the tissue bath as the baseline for comparison. These data are preliminary, one solution being tested on preparations from three different animals at most.

RESULTS AND DISCUSSION

Table I summarizes the cardiac stimulant action (inotropic and chronotropic) of crude extracts from 13 species of coelenterates. Figure 1 shows the tracings for the crude extracts of *Anthopleura xanthogrammica* and *A. elegantissima*, where the effects were observed for 70 and 30 min, respectively.

Pretreatment of the preparations with propranolol and metalol[3] (both 10^{-5} *M*, both β-adrenergic receptor blocking agents) and with phentolamine (10^{-6} *M*, an α-adrenergic receptor blocking agent) had no effect on the inotropic response of the atria to the stimulatory test agents. Atria from either 6-hydroxydopamine-treated rats (50 mg/kg iv, 24 hr prior to sacrifice) or reserpine-treated rats (4 mg/kg im, 24 hr prior to sacrifice) showed responses identical to those of normal animals. Therefore, it appears that the action of the coelenterate stimulant substance(s) is

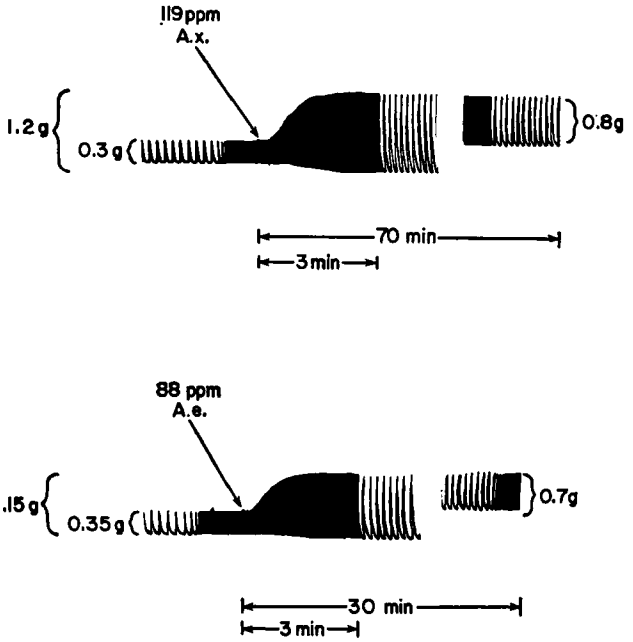

Figure 1—*Tracings for the crude extracts of* A. xanthogrammica *(top) and* A. elegantissima *(bottom) (rat atria, 1 g rest tension, chart speed of 10 mm/min and 5 mm/sec).*

[1] Grass model FT.03.
[2] Grass model 7.
[3] MJ-1998, Mead Johnson.

Table I—Effect of Extracts of Various Species of Coelenterates on Rat Atria

Species	Source	Concentration, ppm	Percent Increase, Inotropic[a]	Percent Increase, Chronotropic[a]
Boloceroides mcmurrichi (Kwietniewski)	Oahu, Hawaii	—[b]	20–51	0
Palythoa psammophilia Walsh and Bowers	Oahu, Hawaii	—[b]	0	0
Zoanthus pacificus Walsh and Bowers	Oahu, Hawaii	—[b]	0	0
Macranthea cookei Verrill	Oahu, Hawaii	—[b]	0	0
Tealia coriacea (Cuvier)	Bodega Bay, Calif.	500	>100	0
Tealia lofotensis (Danielssen)	Bodega Bay, Calif.	350	10–20	0
Metridium senile (L.)	Bodega Bay, Calif.	50	125–150	67
Anthopleura xanthogrammica (Brandt)	Bodega Bay, Calif.	119	300[c]	30
Anthopleura elegantissima (Brandt)	Bodega Bay, Calif.	88	230[d]	14
Tealiopsis nigrescens Verrill	Oahu, Hawaii	100	230	85
Stoichactis kenti Haddon and Shackleton[e]	Tahiti	10	50–85	0–30
Tubastrea aurea Quoy and Gaimard	Oahu, Hawaii	100	210	43
Cassiopeia mertensi Brandt	Oahu, Hawaii	100	0	0

[a] Increases were noted for at least 5 min. [b] A 0.1-ml crude extract diluted 200:1. [c] Increase observed for 70 min, dropping to 170% at 70 min. See Fig. 1. [d] Increase observed for 30 min, dropping to 100% at 30 min. See Fig. 1. [e] Purified fraction. Procedure described in Turlapaty *et al.* (6).

not related to any adrenergic mechanism and that the compound(s) probably acts directly on the heart muscle.

The isolation, characterization, mode of action, dose–response, and other pharmacological studies of the active principle(s) are being pursued.

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(3) P. Turlapaty, S. Shibata, M. Kashiwagi, and T. R. Norton, *Pharmacologist*, **13**, 255(1971).

(4) M. H. Baslow, "Marine Pharmacology," Williams & Wilkins, Baltimore, Md., 1969, pp. 100–115.

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(6) P. Turlapaty, S. Shibata, T. R. Norton, and M. Kashiwagi, *Eur. J. Pharmacol.*, **24**, 311(1973).

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New Compounds: Convenient Selective Esterification of Aromatic Carboxylic Acids Bearing Other Reactive Groups Using a Boron Trifluoride Etherate–Alcohol Reagent

PANKAJA K. KADABA

Abstract □ A mixture of commercial boron trifluoride etherate and an alcohol functions as an effective reagent in the direct esterification of a number of aromatic carboxylic acids bearing additional functional groups such as —OH, —NH_2, >C=O, —O—, and —S—, either alone or in conjunction with another group. Unlike the conventional Fischer esterification procedure where strongly acidic conditions prevail, the boron trifluoride etherate–alcohol reagent is unique in that it is both mild and effective. It esterifies the carboxyl group without affecting the other functionality in the molecule or the stability of the acid itself. The boron trifluoride procedure does not suffer from a lack of generality; it satisfactorily meets the esterification requirements of a greater number of different classes of carboxylic acids than does any other single reagent known. The method offers a simple and convenient esterification route for organic acids in general, in a direct, single-step reaction, using the alcohols themselves.

Keyphrases □ Carboxylic acids (aromatic) with reactive groups—selective esterification using boron trifluoride etherate–alcohol □ Esterification—aromatic carboxylic acids bearing other reactive groups, boron trifluoride etherate–alcohol reagent □ Boron trifluoride etherate–alcohol—used as reagent for convenient selective esterification of aromatic carboxylic acids with reactive groups

Although boron trifluoride complexed with methanol, $BF_3 \cdot CH_3OH$, is a common esterification reagent for stable carboxylic acids prior to GLC analysis (1), it is not generally considered useful for preparative scale esterifications. Recently, however, a mixture of an alcohol and commercial boron trifluoride etherate

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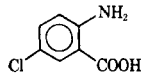
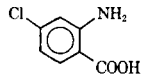
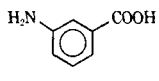
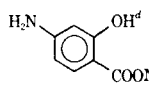
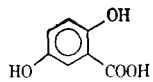
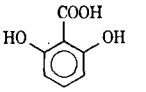
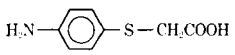
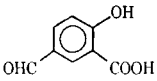
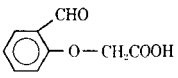
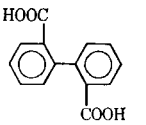
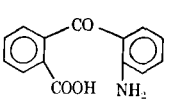
Table I—Esterification of Carboxylic Acids Bearing Other Functional Groups Using a Boron Trifluoride Etherate–Alcohol Reagent

$$R_1COOH + R_2OH \xrightarrow{BF_3\cdot(C_2H_5)_2O} R_1COOR_2 + H_2O$$

R_1COOH	Mole	R_2	$BF_3\cdot(C_2H_5)_2O$, mole	Time of Reflux, hr	Yield of Ester[a], %	Melting Point[b]
	0.05	CH_3	0.1	30	29	68–69°[c] (76°)
	0.025	CH_3	0.05	30	30	66–68° (68–69°)
	0.025	CH_3	0.075	24	22	66–68°
	0.0125	CH_3	0.025	96	58	64–66°
	0.05	CH_3	0.1	24	26	37–38° (39°)
	0.1	CH_3	0.4	17	84[e]	120–122° (120–121°)
	0.1	CH_3	0.3	19	84	120–122°
	0.1	CH_3	0.1	19	15	118–122°
	0.05	C_2H_5	0.15	48	52	114–115° (114–115°)
	0.05	n-C_3H_7[f1]	0.15	24	41	104–105°
Hydrochloride salt of acid	0.05	CH_3	0.1	24	48	118–122°
	0.05	CH_3	0.15	24	81	85–87° (88°)
	0.05	C_2H_5	0.15	18	79	77–78° (77°)
	0.05	n-C_3H_7[f2]	0.15	21	80	62–64°
	0.05	$CH_2CH_2OCH_3$[f3]	0.15	24	76	88–90°
	0.05	n-C_4H_9[f4]	0.15	24	69	65–66.5°
	0.05	CH_3	0.15	52	55[g]	70–72° (69–71°)
	0.05	C_2H_5[f5]	0.15	66	34	51°
	0.05	n-C_3H_7[f6]	0.15	51	32	37–39°
	0.05	CH_3[f7]	0.1	23	73[h]	192–195°[j]
	0.1	CH_3	0.2	24	78[i]	192–195°[j]
	0.05	C_2H_5	0.1	23	60[i]	152–154°[j,k]
	0.02	CH_3	0.06	24	78[l]	81–82° (82–83°)
	0.02	C_2H_5	0.06	23	68[l]	69–69.5°[m] (75°)
	0.02	CH_3	0.04	20	72	55° (55°)
	0.02	CH_3	0.04	24	78	72–74° (74°)
	0.02	CH_3	0.06	18	—[n]	—

[a] Refers to crystallized products. [b] The literature melting points are given in parentheses. [c] The product gave the same melting point after repeated crystallizations. [d] Reference 8 reports the preparation of the methyl, ethyl, and isopropyl esters of this acid using 4.5 moles of $BF_3\cdot(C_2H_5)_2O$ for each mole of acid. By allowing the reaction mixture to stand at room temperature for 10–30 days, approximately 70% yields of esters have been obtained. [e] Reference 8. The preparation of the methyl ester with sulfuric acid in methyl alcohol gave less than 10% yield. The primary product was *m*-aminophenol. [f1–f7] The melting points for these esters could not be located in the literature. Characterization was made by elemental and IR analysis. *f*1 calc.: C, 61.52; H, 6.71; N, 7.18. Found: C, 61.51; H, 6.73; N, 7.08. *f*2 calc.: C, 61.21; H, 6.17. Found: C, 61.21; H, 6.18. *f*3 calc.: C, 56.60; H, 5.70. Found: C, 56.68; H, 5.65. *f*4 calc.: C, 62.84; H, 6.71. Found: C, 62.78; H, 6.69. *f*5 calc.: C, 59.33; H, 5.53. Found: C, 59.25; H, 5.50. *f*6 calc.: C, 61.21; H, 6.17. Found: C, 61.35; H, 6.20. *f*7 calc.: C, 46.26; H, 5.19. Found: C, 46.21; H, 5.14. [g] Reference 9 reports the same yield after 4 days of reflux using a boron trifluoride–methanol reagent (Matheson, Coleman and Bell). Fischer esterification of the acid gave only 36% yield. [h] The ester was isolated as the hydrochloride salt. The reaction mixture was treated with water (50 ml) and made basic to litmus by adding solid sodium carbonate. The ester, partially soluble in water, was recovered by repeated extraction with ether and converted to the hydrochloride salt by addition of concentrated hydrochloric acid to the dried ethereal solution. [i] In these runs, the reaction mixture was well cooled and treated directly with ether followed by concentrated hydrochloric acid, when the hydrochloride salt of the ester separated out as beautiful crystals. [j] Melting point of the hydrochloride salt. [k] *Anal.*—Calc.: C, 48.48; H, 5.66; S, 12.93. Found: C, 48.31; H, 5.57; S, 12.98. [l] The ester was not treated with sodium carbonate. The crude product obtained by dilution of the reaction mixture was recrystallized from ethanol to give the pure ester. The product gave the phenylhydrazone in quantitative yield, mp 120–121°. [m] A sample purified through the bisulfite addition product had the same melting point. [n] The acid yielded the lactam in 78% yield, mp 252–254° [lit. (11) mp 254–255°]. The boron trifluoride etherate appears to aid lactam formation, since the same reaction performed under identical conditions in the absence of $BF_3\cdot(C_2H_5)_2O$ gives only 26% of the lactam, the rest being unchanged acid.

was found to be an effective reagent in the direct esterification of 4-aminobenzoic acid (2), unsaturated organic acids (3, 4), and heterocyclic carboxylic acids (5). Unlike the conventional proton-catalyzed esterifications, where strongly acidic conditions prevail, the boron trifluoride–alcohol procedure is thorough and mild; the reaction is selective at the carboxyl group and the acids are converted cleanly to their esters.

DISCUSSION

Esterification is an important reaction frequently used in biochemical and pharmaceutical research and in medicinal chemistry. However, the usual procedures for the esterification of carboxylic acids are often attendant with unexpected difficulties (6). For instance, in the case of substituted acids, the other functionality in the molecule may be sensitive to the strongly acidic conditions of the standard methods (7) or the acid itself may undergo decomposition (8). Two-step esterification procedures involving reaction of the silver salts of the acids with alkyl halides may help to overcome some difficulties but are expensive (9).

The alkyl esters of a number of aromatic acids, bearing additional functional groups such as —OH, —NH_2, >C=O (as CHO), —O—, and —S— which are susceptible to attack in conventional procedures, are useful as intermediates in the synthesis of biologically interesting heterocycles[1] (10). These acids can be conveniently esterified, using the mild and inexpensive boron trifluoride etherate–alcohol reagent, in a single-step reaction. The results (Table I) indicate that the reagent is effective in the direct esterification of the carboxyl group without affecting other functional groups or the stability of the acids themselves. The present investigation, along with those conducted earlier (2, 3, 5) clearly indicates that the boron trifluoride etherate–alcohol reagent is unique in that it is both mild and effective and it does not suffer from a lack of generality as do other methods (6). It satisfactorily meets the esterification requirements of more classes of carboxylic acids than does any other single reagent known. The boron trifluoride etherate–alcohol procedure offers a simple and convenient esterification route for organic acids in general, in a direct, single-step reaction, using the alcohols themselves.

EXPERIMENTAL

A mixture of the acid, the appropriate alcohol, and boron trifluoride etherate was refluxed for a period of time determined by the reactivity of the acid.

The amount of boron trifluoride etherate to be used was determined by the number and nature of the functional groups present. Groups such as —OH, —NH_2, and >C=O each required an additional equivalent of boron trifluoride etherate for complex formation. Thus, for 1 mole of sodium *p*-aminosalicylate, 3 moles of boron trifluoride etherate should be employed for good results; reducing the reagent to 1 mole reduced the yield of ester from 84% to a mere 15% of low quality material (Table I). When the acid was used in the form of the hydrochloride salt, 2 moles of boron trifluoride etherate was sufficient in theory; however, due to the insoluble nature of the hydrochloride, a heterogeneous reaction mixture resulted followed by lower yields.

In the case of the anthranilic acids and the phenoxy- and phenylmercaptoacetic acids, 2 moles of boron trifluoride etherate seemed sufficient (Table I).

In all cases, the alcohols were used 10 times in excess of the boron trifluoride etherate.

The esters were precipitated by diluting the cooled reaction mixture with water or a 10% solution of sodium carbonate, occasionally after removal of the excess alcohol under reduced pressure, followed by filtration or extraction with ether (whichever was more convenient). To remove unreacted acid, esters obtained by dilution with water were triturated with a dilute solution of sodium carbonate. However, sodium carbonate should be used very sparingly in the case of the hydroxy acids, especially formylsalicylic acid where the hydroxyl group was so acidic that the esters showed a tendency to dissolve in sodium carbonate. Final purification of the esters was effected by crystallization from appropriate solvents.

In the case of *p*-aminophenylmercaptoacetic acid, it was found more convenient to isolate the esters as their hydrochloride salts.

o-Anthraniloylbenzoic acid yielded the lactam (11); there was no ester formation.

[1] Unpublished data.

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The author is a visiting associate professor of chemistry at the University of Ljubljana, Yugoslavia, 1973–1974.

New Compounds: 4-Substituted 5,6-Dihydro-2-*o*-hydroxyphenyl-4*H*-1,3,4-oxadiazine-5-ones, Potential Psychopharmacological Drugs

SUSANA M. SICARDI, SAMUEL LAMDAN, and CARLOS H. GAOZZA[x]

Abstract □ The synthesis of 16 derivatives of a 1,3,4-oxadiazine, each of which may be envisioned as forming a tricyclic structure, was carried out by 4-alkylation and further reaction with a secondary amine. The compounds are proposed as potential psychopharmacological drugs.

Keyphrases □ 5,6-Dihydro-2-*o*-hydroxyphenyl-4*H*-1,3,4-oxadiazine-5-ones, 4-substituted—synthesized as potential psychopharmacological drugs □ Psychopharmacological agents, potential—synthesis of 4-substituted 5,6-dihydro-2-*o*-hydroxyphenyl-4*H*-1,3,4-oxadiazine-5-ones □ Oxadiazines—synthesis of 4-substituted 5,6-dihydro-2-*o*-hydroxyphenyl-4*H*-1,3,4-oxadiazine-5-ones

As a part of a program of synthesis of potential new psychopharmacological drugs derived from little explored heterocycles[1], several derivatives were obtained from a previously described compound, 5,6-dihydro-2-*o*-hydroxyphenyl-4*H*-1,3,4-oxadiazine-5-one (4). An outstanding feature of these oxadiazines is their potential ability to form a tricyclic plane structure due to the ease with which a stable hydrogen bond may form between the *o*-phenolic group of the aromatic substitution on carbon 2 and the position 3 nitrogen, in effect producing a cryptophenol.

The *N*-4-alkylations involved two different approaches: direct alkylation, and a two-step process consisting of direct alkylation to yield an α- or β-chloroalkyl derivative followed by treatment of the 4-chloroalkyloxadiazine with a secondary amine.

EXPERIMENTAL[2]

5,6-Dihydro-2-*o*-hydroxyphenyl-4-(2-chloroethyl)-4*H*-1,3,4-oxadiazine-5-one (III)—A suspension of 0.01 mole (1.92 g) of 5,6-dihydro-2-*o*-hydroxyphenyl-4*H*-1,3,4-oxadiazine-5-one in 35 ml of acetone was dissolved by adding, with stirring, 3.5 ml of 10% sodium hydroxide. After a few minutes the sodium salt crystallized; 0.026 mole (2.2 ml) of 1-bromo-2-chloroethane was then added and the mixture was refluxed for 2 hr. On standing overnight, the product crystallized (Table I).

5,6-Dihydro-2-*o*-hydroxyphenyl-4-(3-chloropropyl)-4*H*-1,3,4-oxadiazine-5-one (IV)—This compound was obtained by using the procedure for III with 1-bromo-3-chloropropane (Table I).

4-Alkyl Oxadiazines: General Procedure—*Method A*—To a suspension of 0.55 g of sodium methoxide in 35 ml of acetone was added, with stirring, 0.01 mole of 5,6-dihydro-2-*o*-hydroxyphenyl-

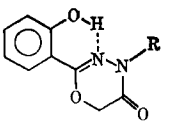


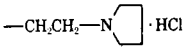
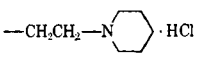
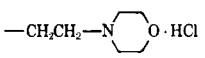
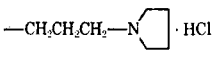
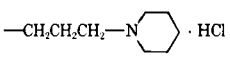
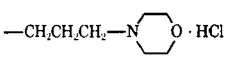
Table I—Physical Constants of Compounds I–XVI

Compound	R	Method	Melting Point	Yield, %	Crystallization Solvent	Analysis, % Calc.	Analysis, % Found
I	$-CH_2CH_3$	A	78°	75	Methanol	C 60.00 H 5.40 N 12.73	59.92 5.38 12.70
II	$-CH_2CH_2CH_3$	A	93°	67	Ethanol–water	C 61.53 H 5.98 N 11.96	61.50 5.93 11.95
III	$-CH_2CH_2Cl$	—	164–165°	63	Dioxane	C 51.86 H 4.32 N 11.00	52.00 4.40 10.85
IV	$-CH_2CH_2CH_2Cl$	—	168–169°	74.6	Dioxane	C 53.63 H 4.84 N 10.42	53.50 4.78 10.39
V	$-CH_2-C_6H_5$	A	115°	62	Ethanol–water	C 68.08 H 4.96 N 9.92	68.00 4.99 9.89
VI	$-CH_2CH_2O-C_6H_5$	A	110°	50	Cyclohexane–ethanol	C 65.38 H 5.13 N 8.97	65.35 5.15 8.96
VII	$-CH_2CH_2O-C_6H_4-Cl$	A	133–139°	59	Ethanol	C 58.87 H 4.32 N 8.08	58.85 4.40 7.98
VIII	$-CH_2CH_2O-C_6H_4-CH_3$	A	102–109°	60	Methanol	C 66.25 H 5.52 N 8.58	66.21 5.49 8.55

[1] Little information is available about 1,3,4-oxadiazines with pharmacological activity (1-3), and nothing is available concerning the corresponding 5-ones.

[2] Melting points (Buchi apparatus) are uncorrected; IR spectra recorded on Perkin-Elmer 137B Infracord; spectral data consistent with structure assigned; OH band, 3000 cm^{-1}; C=O, 1670 cm^{-1} (potassium bromide).

Table I—(*Continued*)

Compound	R	Method	Melting Point	Yield, %	Crystallization Solvent	Analysis, % Calc.	Found
IX	$-CH_2CH_2-N(CH_3)_2 \cdot HCl$	B	196°	54	Methanol–benzene	C 52.08 H 6.01 N 14.02	51.98 6.12 13.97
X	$-CH_2CH_2-N(C_2H_5)_2 \cdot HCl$	B	199°	58	Methanol	C 54.96 H 6.71 N 12.82	55.05 6.75 13.00
XI		B	205–206°	35	Methanol–benzene	C 55.29 H 6.14 N 12.90	55.18 6.16 12.82
XII		B	231–232°	79.4	Ethanol	C 56.55 H 6.48 N 12.37	56.47 6.55 12.26
XIII		B	256–258°	60	Methanol–benzene	C 52.70 H 5.85 N 12.30	52.72 6.01 12.42
XIV		B	177–178°	50.3	Methanol	C 56.55 H 6.48 N 12.37	56.65 6.50 12.28
XV		B	204–205°	35	Methanol	C 59.38 H 6.98 N 9.31	59.29 7.00 9.30
XVI		B	223–224°	70.6	Methanol	C 54.01 H 6.19 N 11.81	53.96 6.30 11.90

4*H*-1,3,4-oxadiazine-5-one. After a few minutes the sodium salt crystallized. Then 0.02 mole of the appropriate bromo derivative was added, and the mixture was refluxed for 5 hr and filtered. The solvent was evaporated *in vacuo* and the solid residue was crystallized from an appropriate solvent (Table I).

Method B—A mixture of 0.01 mole of III or IV and 0.06 mole of the corresponding secondary amine was heated at 80° in an oil bath for 6 hr and filtered, and the excess base was evaporated *in vacuo* at 80°. The residue was dissolved in benzene and the hydrochloride was precipitated by adding a solution of anhydrous hydrochloric acid in ether. It was recrystallized from an appropriate solvent (Table I).

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[x] To whom inquiries should be directed.

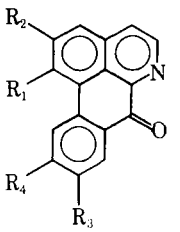
Nonbasic Aporphine Alkaloids from *Liriodendron tulipifera* L.

Keyphrases □ *Liriodendron tulipifera* L.—alkaloids identified in the heartwood □ Aporphine alkaloids (nonbasic)—isolated and identified from *L. tulipifera* □ Structure elucidation—*N*-acetylaporphine alkaloids by IR, UV, NMR, and circular dichroism

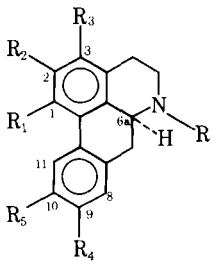
To the Editor:

The heartwood of *Liriodendron tulipifera* L. (Magnoliaceae) had previously yielded the alkaloids liriodenine (I*a*), 1,2,9,10-tetramethoxyoxoaporphine (I*b*), and *d*-glaucine (II*a*) (1–4). Investigation of the nonbasic fraction of the heartwood has led to the isolation of two alkaloids, (+)-*N*-acetylnornantenine (II*b*) and (+)-3-methoxy-*N*-acetylnornantenine (II*e*).

Percolation of the dried ground heartwood[1] with alcohol followed by evaporation of the solvent left a dark residue which was partitioned between ether and 2% aqueous citric acid. The ether solution was evaporated and then chromatographed over silicic acid using chloroform and increasing amounts of methanol in chloroform as the eluent. Elution with 2% methanol in chloroform yielded a fraction that was still a mixture as shown by TLC[2]. Rechromatography of this fraction over silica gel G using ether as the eluent yielded the two alkaloids in pure form.



I*a*: $R_1 + R_2 = OCH_2O$, $R_3 = R_4 = H$
I*b*: $R_1 = R_2 = R_3 = R_4 = OCH_3$



II*a*: $R = CH_3$, $R_1 = R_2 = R_4 = R_5 = OCH_3$, $R_3 = H$
II*b*: $R = COCH_3$, $R_1 = R_2 = OCH_3$, $R_3 = H$, $R_4 + R_5 = OCH_2O$
II*c*: $R = R_3 = H$, $R_1 = R_2 = OCH_3$, $R_4 + R_5 = OCH_2O$
II*d*: $R = CH_3$, $R_1 + R_2 = OCH_2O$, $R_3 = R_4 = R_5 = OCH_3$
II*e*: $R = COCH_3$, $R_1 = R_2 = R_3 = OCH_3$, $R_4 + R_5 = OCH_2O$

Alkaloid A had a melting point of 283–284° (alcohol)[3] and $[\alpha]_D^{26}$ + 340° (c 0.50 in $CHCl_3$). It exhibited a parent ion peak at *m/e* 367.1390 corresponding to the formula $C_{21}H_{21}NO_5$ (calculated *m/e* 367.1420), which was also supported by elemental analysis. The UV spectrum [$\lambda_{max}(CH_3OH)$ nm (log ϵ): 215 (4.61), 281 (4.04), and 307 (4.12)] was indicative of an aporphine substituted at C-1,2,9,10 (5); the IR spectrum [ν_{max}(KBr) 1635 cm^{-1}] suggested the presence of a tertiary amide carbonyl. The 60-MHz NMR spectrum ($CDCl_3$) showed three 3H singlets at δ 2.17 (—$NCOCH_3$), 3.60, and 3.82 (—OCH_3), a 2H singlet at δ 5.85 (—OCH_2O—), and three 1H singlets at δ 6.48, 6.62, and 7.82 (Ar—H). The presence of a 2H singlet for the methylenedioxy hydrogens required that the two methoxy groups be placed at C-1 and C-2 and the methylenedioxy group at C-9,10 since the hydrogens of a methylenedioxy group at C-1,2 will be split into two doublets (5). This assignment is also supported by the fact that one methoxy group appears at higher field (δ 3.60) than the other and thus must be located at C-1 (5). The circular dichroism spectrum[4] showed a large positive Cotton effect at 241 nm ([θ] = +198,000; 1.90 mg/50 ml), which has been correlated with the *S*-configuration at C-6*a* (6).

These data suggested that Alkaloid A be represented by Structure II*b*. Direct comparison of an authentic sample of (+)-*N*-acetylnornantenine (II*b*)[5] [lit. (7) mp 294°, $[\alpha]_D$ + 349° (c 0.44 in $CHCl_3$)], an alkaloid obtained by acetylation of nornantenine (II*c*), with Alkaloid A showed them to be identical in all respects (melting point, mixed melting point, TLC, IR, UV, and circular dichroism). A synthesis of (±)-*N*-acetylnornantenine has also been reported (8).

Alkaloid B [mp 216–217° (alcohol)[3], $[\alpha]_D^{26}$ + 271° (c 0.37 in $CHCl_3$)] gave a parent ion peak at *m/e* 397.1527 corresponding to the formula $C_{22}H_{23}NO_6$ (calculated *m/e* 397.1525), which was also supported by elemental analysis. A carbonyl absorption in the IR spectrum [ν_{max}(KBr) 1638 cm^{-1}] along with a 3H singlet at δ 2.20 in the NMR spectrum suggested the presence of an *N*-acetyl group. Other features of the NMR spectrum included three 3H singlets at δ 3.68, 3.83, and 3.90 (—OCH_3), a 2H singlet at δ 5.87 (—OCH_2O—), and two 1H singlets at δ 6.63 and 7.77 (Ar—H). Comparison of the NMR spectrum of (+)-*N*-acetylnornantenine (II*b*) with that of Alkaloid B indicated the two alkaloids to be closely related and that Alkaloid B was probably a pentaoxygenated aporphine with three methoxy groups and a methylenedioxy group. Since positions 1 and 2 are always substituted in naturally occurring aporphine alkaloids (5, 9) and one of the aromatic hydrogens ap-

[1] The plant material was obtained from a tree growing in southern Lafayette County, Miss. The tree was identified by Dr. Maynard W. Quimby; a voucher specimen has been deposited in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, University of Mississippi.

[2] TLC analyses were carried out on silica gel-coated plates using ether or acetone–methanol–benzene (1:1:8) as the solvent.

[3] The melting point was determined with a Thomas-Hoover Uni-Melt capillary melting-point apparatus and is not corrected.

[4] Circular dichroism measurements were performed on a Jasco model J-40 automatic recording spectropolarimeter using methanol as solvent.

[5] The authors thank Dr. S. R. Johns of C.S.I.R.O., Melbourne, Australia, for kindly providing an authentic sample for comparison.

pears downfield at δ 7.77 (*cf.*, 7.82 in II*b*) and one of the methoxy groups appears upfield at δ 3.68 (*cf.*, 3.60 in II*b*), a methoxy can be placed at C-1 and a hydrogen at C-11 (5, 10, 11)[6]. The UV spectrum [λ_{max} (CH_3OH) nm (log ϵ): 217 (4.65), 280 (4.08), 307 (4.16), and 313 sh (4.16)] is characteristic of a 1,2,3,9,10-pentaoxygenated aporphine (12, 13) and distinctly different from a 1,2,8,9,10-pentaoxygenated aporphine (10). Thus, the only substitution pattern for Alkaloid B that is consistent with both the UV and NMR spectra (singlets for the two aromatic hydrogens) is a C-1,2,3,9,10-pentaoxygenated aporphine with a C-1 methoxy group.

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Charles D. Hufford[x]
*Mary Jo Funderburk**

Department of Pharmacognosy
School of Pharmacy
University of Mississippi
University, MS 38677

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Synthesis and Psychotropic Activity of 2-Hydroxy-4,5-dimethoxyphenethanolamine, a Potential Endogenous Psychotogen, and Its Methylenedioxy Analog

Keyphrases □ 2-Hydroxy-4,5-dimethoxyphenethanolamine and methylenedioxy analog—synthesis and psychotropic activity, potential endogenous psychotogen □ Phenethylamine derivatives—synthesis and psychotropic activity of 2-hydroxy-4,5-dimethoxyphenethanolamine, role in schizophrenia □ Schizophrenia—synthesis and psychotropic activity of 2-hydroxy-4,5-dimethoxyphenethanolamine, a potential endogenous psychotogen

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In continuing investigations on peyote and related alkaloids (6, 7), the authors extended the work to the study of I and its methylenedioxy analog (II). The synthesis of racemates of I and II is reported here

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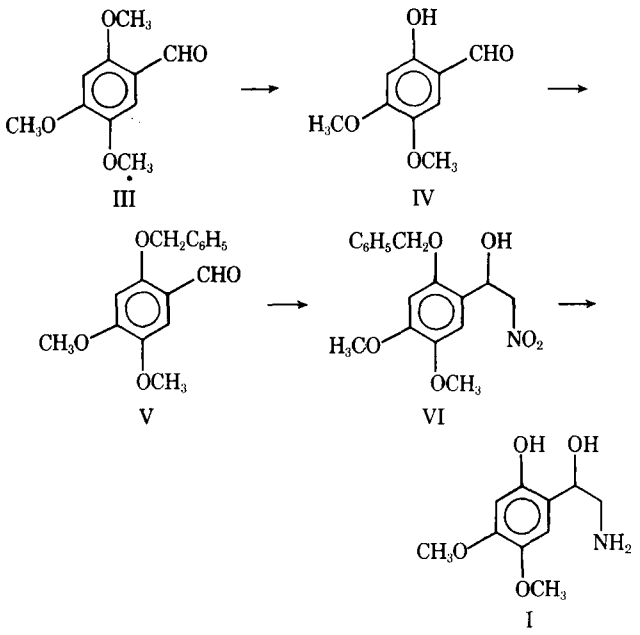


Scheme I

along with their behavioral response in mice, which could be correlated with hallucinogenic activity in humans (8).

Under base-catalyzed conditions, the reaction of nitromethane with aromatic aldehydes usually yields a mixture of nitrostyrene and the corresponding 1-phenyl-2-nitroethanol. The authors recently observed that the yield of nitro-alcohol could be increased by varying reaction conditions such as temperature and workup procedure (9, 10). The nitro-alcohol could then be reduced to the corresponding phenethanolamine as reported earlier for the synthesis of β-hydroxymescaline (11). The modified procedure for the preparation of 1-phenyl-2-nitroethanols has now been applied in the synthesis of the phenethanolamines I and II.

Scheme I outlines the synthesis of the proposed endogenous psychotogen (I). Demethylation of 2,4,5-trimethoxybenzaldehyde (III) by boron trichloride (12) gave the requisite benzaldehyde (IV), which was converted to its 2-benzyloxy derivative (V) and reacted with nitromethane at 0–5° for 30 min in the presence of potassium hydroxide to yield on acidification the key intermediate, 1-(2-benzyloxy-4,5-dimethoxyphenyl)-2-nitroethanol (VI), mp 75–77°. Hydrogenation of VI in the presence of 10% palladium-on-carbon catalyst furnished (±)-2-hydroxy-4,5-dimethoxyphenethanolamine (I), mp 151–152° dec., in 43% overall yield; mass spectrum: *m/e* 213 (M^+), 195 ($M-H_2O$), and 183 ($M-CH_2NH_2$); NMR (solvent, *CDCl*$_3$ containing a few drops of CD_3OD): δ 2.96–3.06 (m, 2, α-CH_2), 3.82 (s, 3), 3.86 (s, 3) (C_4 and C_5 di OCH_3), 4.68–4.78 (t, 1, β-CH), 6.46 (s, 1, aromatic C_6-H), and 6.65 (s, 1, aromatic C_3-H) ppm.

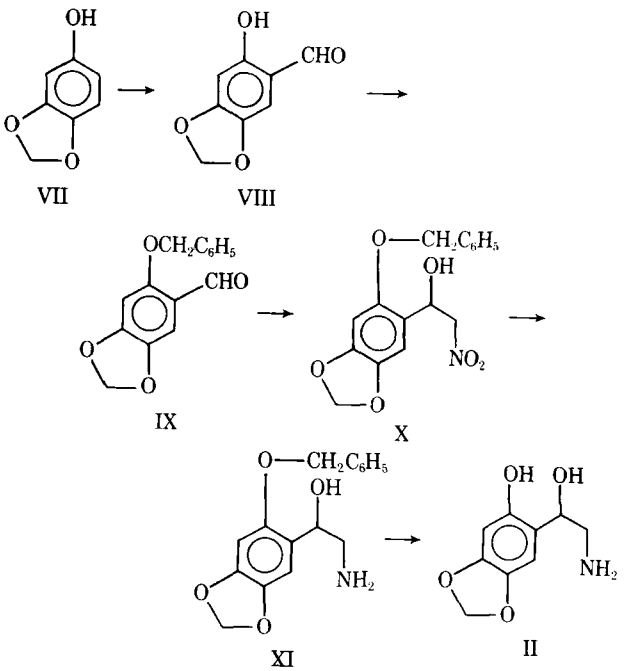
Anal.—Calc. for $C_{10}H_{15}NO_4$: C, 56.33; H, 7.09; N, 6.57. Found: C, 56.26; H, 7.04; N, 6.43.

The synthesis of the methylenedioxy analog II is described in Scheme II. Formylation of sesamol (VII) with *N*-methylformanilide and phosphorus oxychloride furnished the benzaldehyde (VIII), which was converted to its 2-benzyloxy derivative (IX). Compound IX was reacted with nitromethane at 0–5° in the presence of potassium hydroxide to afford on acidification the key nitroethanol intermediate (X), mp 120–122°. Compound X was first reduced to the corresponding amine (XI) (hydrochloride, mp 155–156° dec.) by hydrogen over platinum oxide. The benzyloxyamine (XI) was then hydrogenolyzed in the presence of 10% palladium-on-charcoal catalyst to give (±)-2-hydroxy-4,5-methylenedioxyphenethanolamine (II) (hydrochloride, dec. ~150°) in 36% overall yield; mass spectrum: *m/e* 197 (M^+), 179 ($M-H_2O$), and 167 ($M-CH_2NH_2$); NMR (D_2O solvent): δ 3.33–3.42 (m, 2, α-CH_2), 5.22–5.27 (m, 1, β-CH), 6.05 (s, 2, aromatic OCH_2O at C_4, C_5), 6.66 (s, 1, aromatic C_6-H), and 7.03 (s, 1, aromatic C_3-H) ppm.

Anal.—Calc. for $C_9H_{11}NO_4$·HCl: C, 46.25; H, 5.13; N, 5.99. Found: C, 46.45; H, 5.16; N, 5.93.

The hallucinogenic potencies of the synthetic compounds I and II were determined according to the method of Corne and Pickering (8), which has shown a good correlation between drug-induced hallucinations in humans and head twitches in mice. Male mice, weighing 20–40 g (5 g weight variation in each group), were injected with a single intraperitoneal dose of the test compounds in water and observed for head twitches over 30 min. Twelve mice were used at each dose level, and each compound was tested at four different doses ranging from 2.5 to 40 mg/kg. Mescaline was used as a positive control. The ED_{50} values (with 95% limits) were estimated according to the method of Litchfield and Wilcoxon (13).

The head twitch responses produced by the test compounds in mice are given in Table I. Onset of head twitch in individual animals following drug administration occurred within 18 min, and the effect lasted up to 28 min. The racemic phenethanolamine



Scheme II

Table I—Head Twitch Responses in Mice Produced by Phenethylamine Derivatives

Compound	ED_{50} (95% Limits), mg/kg ip[a]	Onset, min	Duration, min
(±)-2-Hydroxy-4,5-dimethoxyphenethanolamine (I)	17 (12.1–23.8)	2–18	2–24
(±)-2-Hydroxy-4,5-methylenedioxyphenethanolamine (II)	56 (25.4–123.2)	2–6	2–22
Mescaline	5.3 (3.6–7.3)	2–14	2–28

[a] The ED_{50} values reported are calculated for the free base concentrations of the test compounds.

derivatives I and II were about one-third and one-tenth as active as mescaline, respectively. From these results it appears that I could be an endogenous psychotogen responsible for schizophrenic manifestations as postulated by Shulgin *et al.* (4).

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Govind J. Kapadia[x]
Bejoy K. Chowdhury
*G. Subba Rao**
Department of Biomedicinal Chemistry
College of Pharmacy and Pharmacal Sciences
Howard University
Washington, DC 20001

S. N. Pradhan
Department of Pharmacology
College of Medicine
Howard University
Washington, DC 20001

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Chemical Constituents of Gentianaceae XI: Antipsychotic Activity of Gentianine

Keyphrases □ Gentianine—antipsychotic activity evaluated □ Gentianaceae—chemical constituents, antipsychotic activity of gentianine evaluated □ Antipsychotic activity—gentianine screened, chemical constituents of Gentianaceae

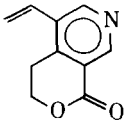
To the Editor:

In an attempt to locate the active principle(s) of *Swertia chirata* (1), we examined the psychopharmacological profile of its major alkaloid, gentianine (I). The first report (2) on the central nervous system (CNS) activity of this compound was limited to the observation that "in weak doses it appears to stimulate the central nervous system, but in larger amounts it shows a paralyzing action." Preliminary pharmacological screening of the alkaloid, however, reflected its CNS depressant activity even in small doses (3). The results obtained in the present investigation point to a persuasive explanation of this activity and prompt us to report the antipsychotic profile of action of this compound.

Pharmacological studies were conducted on albino mice[1] (18–25 g) and albino rats[1] (80–120 g). The animals were fed on standard pellet diet[2]. All experiments were conducted at ambient temperature of 28 ± 2°. Unless stated otherwise, gentianine was used in a dose of 20 mg/kg ip and pretreatment time was 1 hr. At least 10 animals were used for drug-treated and control groups, the latter receiving only the vehicle, distilled water.

In primary observational tests (4), gentianine, in small doses (10–20 mg/kg ip), markedly diminished spontaneous motility and produced sedation and ptosis in albino mice and rats, but reflexes were intact and the animals responded to external stimuli. On increasing the dose (50–100 mg/kg), hind-limb paralysis and catalepsy were produced. The cataleptic animals remained stationary when placed on a vertical wire netting and maintained the awkward posture when one hind limb was placed on a cork. However, they responded to painful stimuli and retained the righting reflex. Gentianine produced hypothermia in albino rats as recorded by a rectal thermister probe. Since these observations are indicative of antipsychotic activity, gentianine was subjected to further pharmacological screening as follows.

The effects of gentianine on hexobarbital hypnosis, amphetamine toxicity and stereotypy, and lysergide-induced symptoms were evaluated. Gentianine sig-



I

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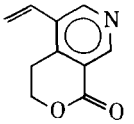
In primary observational tests (4), gentianine, in small doses (10–20 mg/kg ip), markedly diminished spontaneous motility and produced sedation and ptosis in albino mice and rats, but reflexes were intact and the animals responded to external stimuli. On increasing the dose (50–100 mg/kg), hind-limb paralysis and catalepsy were produced. The cataleptic animals remained stationary when placed on a vertical wire netting and maintained the awkward posture when one hind limb was placed on a cork. However, they responded to painful stimuli and retained the righting reflex. Gentianine produced hypothermia in albino rats as recorded by a rectal thermister probe. Since these observations are indicative of antipsychotic activity, gentianine was subjected to further pharmacological screening as follows.

The effects of gentianine on hexobarbital hypnosis, amphetamine toxicity and stereotypy, and lysergide-induced symptoms were evaluated. Gentianine sig-



I

[1] B. N. Ghosh & Co, Calcutta, India.
[2] Hindusthan Levers, Calcutta, India.

nificantly potentiated (80%) hexobarbital (100 mg/kg ip) sleeping time in albino mice (5); it markedly diminished (88%) amphetamine (20 mg/kg ip) toxicity in aggregated mice (6) and antagonized amphetamine- (10 mg/kg sc) induced stereotypy (continuous sniffing, biting, and compulsive gnawing) in albino rats (7). The alkaloid completely inhibited lysergide- (3 mg/kg sc) induced piloerection and tremors in mice (8).

Using rats and mice, the effects on the rotarod test, conditioned avoidance response, and induced aggressive behavior were examined. Gentianine markedly inhibited (80%) the ability of trained mice to remain on a rotating rod for a maximum time trial of 180 sec (9). The alkaloid selectively blocked the avoidance response to the conditioned stimulus (buzzer), without affecting the escape response to the unconditioned stimulus (electric shock), when tested on trained rats (10). However, in higher doses (50 mg/kg ip), there appeared to be an appreciable motor deficit as characterized by suppresssion of the escape response (40%) to the unconditioned stimulus in these animals. Gentianine inhibited (60%) foot-shock-induced fighting behavior in paired mice (11).

The effects of gentianine on morphine analgesia, anticonvulsant action of diphenylhydantoin, electroshock seizure, and pentylenetetrazol convulsion were also determined. Gentianine markedly potentiated (150%) the analgesic activity (12) of subanalgesic doses (2 mg/kg ip) of morphine (13) but had no analgesic activity *per se* at this dose (20 mg/kg). It significantly potentiated (60%) the anticonvulsant activity of a subanticonvulsant dose (2.5 mg/kg ip) of diphenylhydantoin but had no anticonvulsant activity *per se*. With higher doses (50 mg/kg ip), however, it showed noteworthy anticonvulsant activity (40%) as tested by the electroshock seizure method (14). In higher doses (50–100 mg/kg ip), gentianine offered significant protection (70%) against pentylenetetrazol- (70 mg/kg sc) induced convulsion (15).

The toxicity (16) and the LD_{50} of gentianine after intraperitoneal administration in albino rats were studied; the LD_{50} was calculated as 276 mg/kg. The drug appears to possess only a moderate to low order of toxicity as evidenced from the lack of any obvious toxicity on prolonged intraperitoneal administration, 20 mg/kg daily for 3 weeks.

Gentianine exhibited significant antipsychotic activity in the battery of tests accepted for arriving at such a conclusion (17). It has the added advantage of its minimal toxicity. The alkaloid, bearing a skeleton (lactonic monoterpene) different from those of known antipsychotic agents, is thus of potential importance as an antipsychotic drug.

(1) S. Ghosal, P. V. Sharma, R. K. Chaudhuri, and S. K. Bhattacharya, *J. Pharm. Sci.*, **62**, 926(1973).

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S. K. Bhattacharya

Department of Pharmacology
Institute of Medical Sciences
Banaras Hindu University
Varanasi-5, India

S. Ghosal[x]
R. K. Chaudhuri
A. K. Singh
P. V. Sharma

Pharmaceutical Chemistry Research Laboratory
Department of Pharmaceutics
Banaras Hindu University
Varanasi-5, India

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[x] To whom inquiries should be directed.

Renal Contribution to Drug Biotransformation

Keyphrases □ Renal metabolism—contribution to drug biotransformation, true renal clearance values □ Drug biotransformation—renal contribution, assessment of determination of true renal clearance values □ Metabolism, drug—renal contribution to drug biotransformation, assessment of determination of true renal clearance values

To the Editor:

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S. K. Bhattacharya

Department of Pharmacology
Institute of Medical Sciences
Banaras Hindu University
Varanasi-5, India

S. Ghosal[x]
R. K. Chaudhuri
A. K. Singh
P. V. Sharma

Pharmaceutical Chemistry Research Laboratory
Department of Pharmaceutics
Banaras Hindu University
Varanasi-5, India

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To the Editor:

Wan and coworkers (1–4) recently described an imaginative pharmacokinetic approach for assessing the contribution of the kidneys to the biotransforma-

tion of drugs in the body. Their method is based on the measurement of *(a)* the true renal clearance of a drug metabolite at the steady state during intravenous infusion of that metabolite and *(b)* the apparent renal clearance of this metabolite at the steady state during intravenous infusion of its precursor. An increase in apparent renal clearance over the true renal clearance is used as a measure of the rate of metabolite formation by the kidneys.

In discussing the theoretical basis of their method, Wan and coworkers emphasized the need to monitor the true renal clearance of the metabolite during the entire experiment since it may be affected by a number of factors, particularly by possible competitive effects of the precursor (1). They, therefore, infused radioactive metabolite during all phases of their apparent renal clearance measurements so that any changes in true renal clearance could be recognized readily. Unfortunately, they measured only the rate of excretion of the radioactive metabolite and not its concentration in the plasma during infusion of the precursor. This precluded determination of true renal clearance.

It is essential to determine not only the rate of excretion but also the concentration in the plasma of radioactive metabolite before and during precursor administration when using the method of Wan and Riegelman (1) for assessing the contribution of the kidneys to the biotransformation of drugs in the body. Failure to do so in the studies reported to date (1–4) makes it advisable to consider the results as tentative, pending further investigations according to a more appropriate protocol.

These researchers (4) stated that if the rate of excretion of radioactive metabolite stays constant during the experiment (*i.e.*, while precursor is infused at various rates), then the true renal clearance of the metabolite is in fact constant. This reasoning is incorrect. If a metabolite is eliminated entirely by renal excretion (for example, salicyluric acid in humans as in the study described in Ref. 4), then the rate of excretion during constant infusion of this metabolite is equal to the rate of infusion (*i.e.*, rate in = rate out) at the steady state by definition. This is so regardless of the true renal clearance value. What is affected by a change in the true renal clearance is the steady-state concentration of radioactive metabolite in the plasma since this concentration is inversely proportional to the true renal clearance. Failure to determine the plasma concentration of radioactive metabolite during all phases of the experiments makes it impossible to determine whether the true renal clearance did, in fact, remain constant.

Wan and coworkers stated that, under conditions when renal clearance is constant, the observed excretion ratio of cold to labeled compounds should be the same as the infusion ratio of cold and labeled compounds. If the two infused species are not metabolized, then their steady-state excretion rate ratio must be the same as the infusion ratio (rate in = rate out) irrespective of any changes in the true renal clearance of one, the other, or both compounds. If the cold compound is biotransformed partially while the labeled compound is eliminated only by excretion, then the ratio of excretion rates at the steady state equals the infusion rate of cold compound times f divided by the infusion rate of the labeled compound, where f is the fraction of cold compound excreted as such. A constancy in the steady-state excretion rate ratio of the compounds under these conditions indicates only constancy in f. This could (but need not) reflect constancy in the renal clearance of unchanged drug, since a change in the rate constant of one of several parallel elimination pathways changes the quantitative ratio of the products of elimination. It definitely does not reflect a possible change in the renal clearance of a metabolite.

If a precursor is eliminated solely by biotransformation (as is practically the case with benzoic acid in the study described in Ref. 1), then a change in true renal clearance would have no effect on the ratio of excretion rates of labeled and unlabeled metabolite at the steady state because the rate in equals the rate out at the steady state. In any event, a change in the rate of infusion of the precursor obviously causes a change in the ratio of excretion rates, so that it is impossible to detect a precursor concentration-dependent change in the true renal clearance of the metabolite. As Wan and Riegelman (1) pointed out, such a change, if unrecognized, can lead to erroneous values for the rate of metabolite formation by the kidneys.

We wish to emphasize that we consider the method of Wan and Riegelman (1) as a potentially valuable research tool and that our critical comments are limited entirely to an aspect of its execution in the studies reported until now (1–4).

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Burde L. Kamath
Gerhard Levy [x]

Department of Pharmaceutics
School of Pharmacy
State University of New York at Buffalo
Buffalo, NY 14214

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[x] To whom inquiries should be directed.

REVIEWS

Analytical Profiles of Drug Substances, Volumes 1, 2, and 3. Edited by KLAUS FLOREY. Academic, 111 Fifth Ave., New York, NY 10003. Volume 1, 480 pp., 16 × 24 cm, Price $14.00; Volume 2, 575 pp., 16 × 24 cm, Price $18.50; Volume 3, 581 pp., 16 × 24 cm, Price $19.50.

The series, *Analytical Profiles of Drug Substances*, Volumes 1, 2, and 3, was originated by the members of the Section on Pharmaceutical Analysis and Quality Control of the Academy of Pharmaceutical Sciences to provide a collection of available information for drugs recognized in the official compendia. Although some data about these drugs were readily available, much of the information was scattered through the literature or stored in files of pharmaceutical research laboratories. When scientists needed chemical or physical data about a drug substance, they could be fairly certain that the data existed somewhere, but they could also be reasonably certain that the particular information could not be found without an extensive literature search or through correspondence with other scientists. Only scientists who had experienced the frustration of looking for the elusive data could have recognized that a compilation of the available data about drug substances could be important enough to warrant the tremendous task of collecting and organizing the data into a series of books.

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When conflicting data for certain properties have appeared in the literature, all of the data are reported, but the authors of the monographs usually indicate which data have been accepted as most accurate. However, the reader need not simply accept the author's evaluation because references for the original sources are given. All of the monographs are liberally referenced, and these references can be as valuable to the research scientist as the collected data.

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Staff Review

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This case-bound edition combines Parts I and II of Volume 9, which were issued in limp covers during 1972. The book represents a timely offering of reviews of subjects that are of current interest including natural products having antitumor activity, the study of drug metabolism using isotope techniques, and the therapy of parkinsonism.

Too often naturally occurring substances are classified according to the taxonomy of plants, which may be confusing to the reader who is not trained in botany or pharmacognosy. After reviewing the methods used for detecting antitumor activity, the authors of the chapter on naturally occurring antitumor agents chose to classify the agents as tertiary amines, heterocyclic amines, amides and imides, nitro and nitroso compounds, epoxides, lactones, quinones, aminoquinones, quinone methides, oxidized terpenoids, glycosides, and proteins. This approach more closely represents a chemical classification and will provide less difficult reading to members of other disciplines.

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While not of any lesser importance, the other chapters will have a narrower range of readers. The remaining three chapters will be of interest primarily to chemists and medicinal chemists and include a review of the chemistry of chromone-2- and -3-carboxylic acids and their derivatives (4-oxopyranoazoles and 4-oxopyranoazines) and adrenochrome and related compounds. Brief discussions of the biological properties of some of the agents are included.

The few errors that were noticed in the book are obvious to the reader and should present no particular problems of interpretation. These include such things as a pyrazole ring is referred to as an imidazole and a hydrogen atom is joined to a carbon atom by a triple bond. Obviously the hydrogen should be a nitrogen atom.

The book should find an interested audience among medicinal and natural products chemists as well as pharmacologists.

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The book should find an interested audience among medicinal and natural products chemists as well as pharmacologists.

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DESTRUCTIVE FALLOUT

Our initial reaction, after reading the Report of the Drug Bioequivalence Study Panel to the Office of Technology Assessment (OTA), was one of shock and dismay.

True, the Panel had done a fine job of answering the one question embodied in the charge given it by Senator Kennedy and the OTA. However, the Panel then went off wildly in all directions criticizing: (*a*) how the drug industry conducts in-process quality controls and adheres to good manufacturing practices; (*b*) how the official compendia operate and perform; and (*c*) how the Food and Drug Administration monitors the nation's drug supply. And then the Panel proceeded to make sweeping recommendations for changes in all of these areas.

In leveling their criticism, the severest and harshest blast was directed at the official compendia. The Panel did not like anything about how the compendia operate, how they are organized and financed, what analytical methodology they employ, the design of the test procedures, the specifications and tolerances, the attributes tested or not tested, the frequency of issuing revisions, and even the fact that the participants contribute their services without monetary compensation.

Well, having gone so completely off the deep end—with neither the background, experience, nor information to make valid judgements in these areas—it is not surprising that the resulting report is filled with errors of fact and interpretation. This is especially the case concerning the comments, conclusions, and recommendations of the Panel relative to the *United States Pharmacopeia* and the *National Formulary*. And we told Senator Kennedy as much, in just so many words, when we testified before his Senate Subcommittee on Health at its hearing on July 22.

But now that the dust has begun to settle just a bit, we ask ourselves: Why? Why did the Panel attack the official compendia with such a frenzy? Why did the Panel feel the need to be so vicious with particular respect to the official compendia?

At best, we can only speculate as to the answer. We suspect, however, that the reason lies in the assessment made by various persons, organizations, and groups—most notably the HEW Task Force on Prescription Drugs—to the effect that "lack of clinical equivalency among drug products meeting all the official compendia standards has been grossly exaggerated as a major hazard to the public health."

By attacking the official compendia and attempting to discredit their standards, one can indirectly undermine this general assessment of drug equivalency. Although the strategy itself was clever, its execution was inept. The Panel's criticisms were obviously unfounded; their lack of understanding of the compendia content, purpose, and coverage was embarrassingly apparent; and their suggestions and recommendations ranged from the impractical to the unlawful. And so, hopefully, the Panel's effort to destroy the compendia has failed.

But before dismissing this incident, we feel compelled to make an observation.

However correct the Panel may have thought it was—did the end justify the means? Over many years, countless scientists and practitioners from pharmacy, medicine, and the allied health fields have given an enormous amount of unselfish, dedicated effort to create, develop, maintain, and improve the official compendia. They did this primarily for two reasons: altruistic dedication to their profession and belief in a voluntary system whereby the professions themselves would function as the guardians of drug quality.

In one massive blow, the Panel sought to destroy this entire system. If they had succeeded, and if they were justified, then so be it. But if they had succeeded, *and they were not justified* in wiping out the system, then their action warrants severe condemnation. In our opinion, destroying the official compendia system would not only have been a disservice to the public, but it would have had the effect of destroying a small but important part of the health professions themselves.

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REVIEW ARTICLE

Pharmacokinetics and Temperature

BERTON E. BALLARD

Keyphrases □ Pharmacokinetics—effects of body and environmental temperature on human physiological changes and physicochemical properties, absorption, distribution, and metabolism of drugs, review □ Temperature, human body and environmental —effects on physiology and physicochemical properties, absorption, distribution, and metabolism of drugs, review □ Drugs—effects of body and environmental temperature on physicochemical properties, absorption, distribution, and metabolism, review □ Environmental temperature—effect on drug pharmacokinetics, review □ Absorption and distribution, drug—effects of body and environmental temperature □ Elimination, drug and metabolite—effects of body and environmental temperature □ Metabolism, drug—effects of body and environmental temperature

The objective of this review is to discuss what effect body or environmental temperature variations might have on the physiological changes observed in humans, the physicochemical properties of a drug, drug absorption, drug distribution, drug metabolism, and drug or metabolite elimination. Information on this topic is widely scattered throughout the biological, medical, chemical, mathematical, pharmaceutical, pharmacokinetic, and related literature. As a consequence, it is highly probable that some key references may be inadvertently overlooked. It is beyond the scope of this review to consider in any great detail the possible effects that body or environmental temperature might have on a drug's pharmacological response.

The literature of pharmacokinetics has grown rapidly since the era of modern pharmacokinetics began in 1937 (1). The growth has been accelerated by the development of computers (2–7), which make the solution of pharmacokinetic and statistical equations a routine matter, and the development of sensitive analytical tools, which make the analysis of drug and metabolite concentrations in biological fluids less difficult than previously. The growth has also been accelerated because of an increased awareness among politicians and biomedical and pharmaceutical scientists that information derived from pharmacokinetic and biopharmaceutical studies may have an important bearing on patient care (8). Most pharmacokinetic studies have been conducted in animals and humans having "normal" temperatures. Several investigators have noted that pharmacokinetic and drug action studies in animals and particularly in humans under conditions of hypo- or hyperthermia are limited (9–11). Various review papers on pharmacokinetics have been presented (9, 12–23).

Humans have been known to survive when rectal temperatures have reached as low as 18° (24) and as high as 44.4° (25). Patients who are hypo- or hyperthermic may have received drugs prior to the onset of a body temperature change (26, 27) or after the body temperature change became evident (28–31).

Two groups in the general population are particularly sensitive to environmental temperature changes. They are the human newborn, whose body temperature under normal delivery room conditions may fall as much as 2–3° (32), and the elderly whose

home environments may put them "at risk" of developing hypothermia. The coldest elderly individuals tend to be the ones least aware of their problem. Fox *et al.* (33, 34) did not discuss drug utilization in the elderly population. Bender (35) and Hall (36) noted that the elderly tend to exhibit a decrease in both drug absorption and elimination, but they did not comment upon the possible effect of body temperature on these or other processes. The world energy crisis may make the home heating problem even more severe for the elderly and others in the near future. Weihe (10) stated that: "Once physicians as well as physiologists and pharmacologists become aware of the thermal environment, they will notice modifications of drug action in man and will be able to relate this data to ambient thermal conditions."

Buckley (37) indicated that a new facet in pharmacology, which could be called "environmental pharmacology," has emerged; it involves the possible alterations in drug action by environmental stimuli and the alteration of the response of the organism to its environment by drugs. A parallel field of "environmental (thermal) pharmacokinetics"[1] might also be emerging wherein conditions such as environmental or body temperature might affect the kinetics of drug absorption, distribution, metabolism, or elimination.

PHYSIOLOGICAL CHANGES

Temperature changes in the environment or within the animal may affect physiological functions and pharmacokinetic modeling. The literature in this field is extensive, and it is beyond the scope of this paper to review this topic. However, some major points should be mentioned. Many references cited in this section are review articles and should be consulted for references to the original work.

Some body reactions to high temperature have been summarized (38). The initial response to heat is peripheral vasodilatation followed by perspiration, which results in a large fraction of the total blood volume being circulated through the skin vessels for cooling. Venous return of blood to the heart is reduced, and a drop in blood pressure occurs. Compensatory mechanisms stimulate an increase in heart rate and cardiac output. External heat generally tends to lower blood pressure; however, the response is variable and depends upon the degree of vasodilatation, heart rate, cardiac output, pulse pressure, and body position. During artificial fever, the number and motility of leucocytes in blood are increased. High temperature increases respiratory rate, and a decrease in carbon dioxide alveolar tension results in a tendency for alkalosis to occur. In a hot environment, slight increases in rectal temperature are not accompanied by significant increases in renal blood flow.

Changing the environmental temperature influences the volume and composition of blood. Bass and Henschel (39) reviewed the effects of heat and cold on body fluid compartments and stated that one early response to abrupt heat exposure is the dilution of the plasma. This dilution is usually less than 5% and occurs during the first 30 min of exposure and before sweating occurs. The hemodilution is probably due to an influx of extravascular fluid as a result of peripheral vasodilation. There is also a decrease in urine flow and sodium- and chloride-ion excretion. After longer exposure to heat (49°) for up to 5 days, blood volume was shown to increase as a result of the addition of a fluid almost identical in composition to plasma. The increase in plasma volume was about 27% and the increase in interstitial fluid volume was about 14%. After 14 days at this environmental temperature, the total blood volume and circulating protein concentration returned nearly to control values.

One review (40) points out some physiological effects of environmental cold in humans. Initially, exposure to cold causes shivering, peripheral vasoconstriction, an increase in oxygen consumption, a fall in alveolar carbon dioxide, a fall in minute volume, an increase in respiratory rate, an increase in pulse rate, an elevation of blood pressure, an increase in cardiac output, and a progressive fall in blood pH. After the rectal temperature has fallen to about 35°, the metabolic rate begins to decline. Shivering usually ceases between 30 and 33°. After the initial increase in respiratory rate, a decrease in rate occurs, which is almost proportional to the fall in body temperature. Once rectal temperature begins to fall and cooling is continued, heart rate decreases. If the temperature falls below 28°, heart block may occur along with ventricular extrasystoles or nodal rhythm. Either ventricular fibrillation or cardiac asystole may occur if cooling is continued much below 28°.

After the initial rise in blood pressure and cardiac output, continued cooling causes a gradual decline in blood pressure due chiefly to a reduction of cardiac output. During deep hypothermia, blood viscosity increases. Cerebral blood flow and oxygen consumption both are reduced during hypothermia. Hypothermia causes a decrease in brain volume and an increase in extracerebral space volume. Splanchnic blood flow to the liver is decreased during hypothermia. The glomerular filtration rate, renal blood flow, and normal tubular reabsorptive capacity for glucose are decreased during hypothermia. Some acid–base and electrolyte disturbances observed in hypothermia have been reviewed (41).

The effects of cold on fluid compartments are not as clearcut as are the effects of heat. In an acute cold exposure experiment, nude reclining subjects exposed to cold (16°) for 2 hr showed a significant 7% decrease in plasma volume and an insignificant 1.6% decrease in circulating protein (39). In addition to hemoconcentration, acute cold frequently results in increased urine flow and increased sodium- and chloride-ion excretion. Prolonged exposure to cold, however, shows that blood volume is little affected. The composition of blood and plasma remains normal with respect to the concentration of plasma proteins, hematocrit, and plasma concentrations of sodium and chloride ions (39).

[1] The phrase "environmental pharmacokinetics" was defined more broadly by E. J. Ariëns, in "Drug Design," vol. I, E. J. Ariëns, Ed., Academic, New York, N.Y., 1971, p. 17.

Table I—Relationship between Gastric Emptying Time and Temperature

Species (Sex)	Number of Subjects	Age Range, Years	Experimental Conditions	Results	Reference
Human (♂)	9	—	Barium sulfate meal with 250 ml of ice water	Gastric emptying time for four subjects was increased by 15 min, for four subjects it was increased by 30 min, and for one subject there was no change	50
Dog	5	—	Barium sulfate meal; gastric emptying time measured in dogs exposed 20 days at −10°, room temperature (20–26°), and high temperature (32°) for 5 days	Gastric emptying time at −10° was shorter by 17%, and at 32° it increased about 10% compared to dogs at 20–26°	51
Dog	—	Puppies	Prolonged exposure to low environmental temperature	Increased rate of evacuation and decreased secretory activity of gastric glands	52
Human	24	—	Barium sulfate meal on fasting stomach; X-rays taken; first meal at about 37°, second at about 3°, and third at about 62°	Compared to 37° meal, the gastric emptying time for 3° meal was initially more rapid and the 62° meal was initially slower	53
Human (♂ and ♀)	—	—	Ingestion of 250 ml ice water (1.1–2.2°) on fasting stomach Ingestion of 90 g ice cream (−11.1–+6.7°)	Average gastric emptying time for water was delayed 30–45 min Gastric emptying time was delayed 30–45 min	54
Human (♂)	—	—	Irrigation of stomach with cold (9°) and hot (46°) water	Introduction of any fluid into stomach inhibited contractions temporarily; cold water had a longer inhibitory effect than hot water, and this effect did not occur when fluid was irrigated through in-lying balloon	55
Human (♂)	17	18–28	Barium sulfate meal on fasting stomach; X-rays taken; room temperatures of 25 and 49°	Gastric emptying time was faster in 16 subjects at 45° compared to that at 25°	56
Human (♂)	16	—	Barium sulfate and ice cream meal at −8, 26(32), or 65°; room temperature defined as 26 or 32°	Gastric emptying time was fastest with the 26° meal, slowest for the 65° meal, and intermediate for the −8° meal; no statistically significant relationship seen between gastric emptying time and meal temperature	57
Rat (♂)	54	Adult	*Acute studies:* rats ate a standard protein meal at 22° and then were transferred to a low (3°) or high (37°) environmental temperature *Chronic studies:* rats were maintained at 3, 22, or 37° for 6 days and then fed test meal	*Acute studies:* transfer of rats from 22 to 3 or 37° environment caused delays in gastric emptying time *Chronic studies:* gastric emptying time similar to that at 22°; sudden change of environmental temperature was responsible for delayed gastric emptying time	

In reviews on human circadian rhythms (42–44), studies were cited where a circadian rhythm has been observed in body temperature; circulatory function, such as pulse rate, blood and pulse pressures, stroke volume, and circulating blood volume; and renal function, such as urine volume, phosphate-, potassium-, sodium-, and chloride-ion excretion, and urine pH. At night, humans have a small flow of dark, acid urine, while in the morning, usually after awakening, both urine flow and urine pH increase. This phenomenon has been called the "alkaline tide" by some investigators.

The magnitude of human body temperature variations attributable to the normal circadian rhythm can be found in Blake's (45) work, which followed the sublingual body temperature in 74 humans. The lowest mean body temperature of about 36.2° occurred at 4 am and the highest mean body temperature of about 36.9° occurred near 8 pm. In this panel, approximately a 0.7° difference between maximum and minimum body temperatures was recorded.

Gastric Emptying Time—The duration of the gastric emptying time is a physiological variable that may influence the rate of drug absorption. This topic will be discussed later. The mechanics and regulation of gastric emptying have been reviewed (46, 47), as have factors affecting the regulation of gastric motility (48). Coates *et al.* (49) found that the gastric emptying time in human volunteers following the ingestion of a "standard egg sandwich" containing indium-113m chloride had a $t_{50\%}$ (time for half the radioactivity to leave the stomach) ranging from 42 to 95 min. Previous studies cited in the paper, using other radioactive elements, suggested that gastric emptying appears to follow a monoexponential curve. However, gastric emptying followed neither apparent first- nor zero-order kinetics. Table I summarizes chronologically some studies on the effects that meal temperature might have on gastric emptying.

Fordtran and Saltin (59) found that human gastric emptying of a solution containing 13.3% glucose, 1% polyethylene glycol (as a nonabsorbed marker), and 0.3% sodium chloride was not affected by severe ex-

ercise but the gastric emptying of a second solution of water with 1% polyethylene glycol was slightly inhibited by such exercise. Other factors affecting gastric emptying have been discussed (60). Elevation of body temperature above normal conditions resulted in an inhibition of gastric motility, but colonic motility was not altered (61).

Gastric pH and GI Transit Time—The pH of the gastric juice in the normal resting stomachs of dogs and of healthy male volunteers was measured (62). In 403 tests on 24 dogs, 77% had gastric pH values of 6.0 or above. In 1556 tests on 312 humans in four different geographical locations, the results from three locations showed a bimodal effect and one was unimodal. The gastric pH for 41% of all subjects was 3.5 or lower, 35% was 6.0 or greater, and 24% had pH values between 3.5 and 6.0. Among the normal humans studied, the gastric pH values ranged from 0.5 to 9.5.

For many years, it has been known that febrile patients may exhibit changes in gastric secretion patterns. Chang (63) studied 106 patients with various diseases having a temperature range of 37.4–41.3°. When using the histamine method, on an average there was a decrease in gastric acidity to about one-third that of normal. Chang noted that the decrease in gastric function was directly proportional to the magnitude of the fever temperature.

The reduction of gastric secretions in gastric pouch dogs given intravenous injections of a pyrogenic substance was measured (64). The gastric acid secretion was stimulated by subcutaneous injections of histamine solutions. The mean percent inhibition of gastric secretion was nearly proportional to the mean rise in body temperature or the log dose of the pyrogenic substance. These results were confirmed (65). Masuda *et al.* (66) found that the gastric free acidity in humans was significantly increased by immersing humans below the neck in a bath at 22–24°.

Beresford *et al.* (67) noted that it is a common clinical observation that febrile illness in humans generally resulted in reduced transit time through the GI tract. Reviews on the digestive system and GI tract motility have been presented (68–73).

Selected Organs and Organ Systems—Beltz and Reineke (74) showed that the heart rate of neonatal rats increased with an increase in intraperitoneal temperature in accordance with the Arrhenius equation over a 16–29° range. They cited evidence that the heart beat rate in other animal species is similarly affected by temperature change.

Merrill and Pelletier (75) studied the rheological properties of human blood over a range of shear rates from 0.1 to 100 sec^{-1} and over a temperature range from 10 to 37°. At a constant hematocrit level, the relative viscosity of whole blood (viscosity of blood/viscosity of water) remained nearly constant at shear rates between 1 and 100 sec^{-1} over the temperature range. At shear rates below 1 down to 0.1 sec^{-1}, the relative viscosity increased slightly with increasing temperature.

Reemtsma and Creech (76) measured the viscosity of dog blood at various hematocrit levels, dog plasma, and some plasma substitutes over a temperature range of 5–40°. As the temperature was lowered from 40 to 5°, *(a)* the blood viscosity increased more steeply at the higher hematocrit levels, *(b)* the viscosity of plasma more than doubled, and *(c)* the viscosity of dextran solutions of various molecular weights increased more sharply as molecular weight increased.

The apparent pK′ value or a "complex constant-of-convenience" (77, 78) for carbonic acid in serum was determined (79, 80) at various pH and temperature values. The pK′ value is a mixed constant involving the pKa for carbonic acid (81), the hydration of carbon dioxide in solution to form carbonic acid, and the activity and degree of dissociation of bicarbonate ion. While the thermodynamic pKa of carbonic acid is probably not affected by pH, the value for pK′ is. Both pKa and pK′ are influenced by temperature.

The rate of bile flow in isolated liver preparations and in intact animals has been shown to decrease with a reduction in liver temperature (82–84).

Individuals exposed to extremes of cold or heat or who are febrile are usually treated in part by varying periods of bed rest. Even among normal subjects, prolonged bed rest can affect certain physiological functions. Vogt *et al.* (85) found that blood volume changes during bed rest were variable, possibly because they used the radioiodinated serum albumin plasma volume method for their assay. However, with bed rest, diuresis was observed and there was an increase in both sodium- and potassium-ion excretion over control values. These and other physiological effects to cold and heat have been reviewed in detail (86–96).

PHYSICAL AND CHEMICAL PROPERTIES OF DRUGS

While changes in environmental and body temperature have important bearings on many physiological functions, one cannot ignore possible changes in the physical and chemical properties of the drug with temperature, which also may be important in the pharmacokinetic modeling process.

Solubility—As a general rule, the aqueous solubility of inorganic and organic solid drugs increases with increasing temperature (Table II). Different polymorphic forms of the same drug can exhibit this type of solubility behavior (105). Exceptions to this rule include calcium acetate and calcium hydroxide (106). In contrast, the aqueous solubility of gaseous drugs decreases with increasing water temperature (107). For nitrous oxide dissolved in water, the Ostwald solubility coefficient (the ratio of the volume of the absorbed gas to that of water) decreases from 0.693 at 20° to 0.435 at 40° (108). The Ostwald solubility coefficient for halothane in water, human blood, and plasma all decrease over the 25–37° temperature range (109). In plasma, the solubility, S, of carbon dioxide equals $[H_2CO_3$ (mmoles/liter)]/$[P(CO_2)$(mm Hg)], and increases from 0.0288 at 40° to 0.0554 at 15° (110).

pKa—Over the range of temperatures compatible with living organisms, the pKa of drugs in water generally decreases with increasing temperature

Table II—Solubility and pKa Values of Selected Weak Acids in Aqueous Solution at Different Temperatures

Drug	Temperature	Solubility, g/100 ml	Reference	pKa	Reference
Barbital	20°	0.629	97	8.06	98
	37°	0.949	97	7.82	98
Phenobarbital	20°	0.088	97	7.60	99
	37°	0.184	97	7.2	100
Sulfadiazine	20°	0.00616	101	6.35	102
	38°	0.0099	101	6.28	101
Tolbutamide	25°	—	—	5.42	103
	27°	0.0077	104	—	—
	37.5°	0.0142	103	5.32	103

(Table II). The pKa for water is also temperature dependent and follows the equation (81):

$$\mathrm{pKa}(H_2O) = \frac{4471.33}{T} - 6.0846 + 0.017053T \qquad \text{(Eq. 1)}$$

where T is the absolute temperature. The pKa for water is 14.17 at 20° and decreases to 13.63 at 37°.

Partition Coefficient—Renkin (111, 112) measured the olive oil–water partition coefficients of antipyrine and other 5-pyrazolone derivatives at various temperatures. Within the 2–31° range, the logarithm of the partition coefficient of a particular compound was a linear function of temperature. Munson and Eger (113) summarized the blood–gas and oil–gas partition coefficients of the anesthetic agents cyclopropane, fluroxene, halothane, and methoxyflurane over the 27–42° temperature range. The partition coefficients all decreased as the temperature of the *in vitro* system increased.

Diffusion Coefficient—For the majority of drugs, data on the aqueous diffusion coefficients are not known as a function of temperature, and the temperature dependency of the diffusion coefficient of drugs in biological fluids is virtually nonexistent. To circumvent the problem, the following equation was derived (114):

$$D_r = \frac{T}{T^0}\left(\frac{\eta r^0}{\eta r}\right)_{H_2O} \cong \frac{T}{T^0}\left(\frac{\eta r^0}{\eta r}\right)_f \qquad \text{(Eq. 2)}$$

where D_r is the relative diffusion coefficient, T and T^0 are two absolute temperatures (T^0 being the reference temperature), $(\eta r^0/\eta r)_{H_2O}$ is the ratio of the relative viscosities of water at temperatures T^0 and T, and $(\eta r^0/\eta r)_f$ is the ratio of the relative viscosities of body fluids such as blood serum or plasma at temperatures T^0 and T.

Over a 1–37° temperature range, the aqueous relative diffusion coefficient for dextrose calculated from Eq. 2 differed by less than 3% from the experimental relative diffusion coefficient calculated from Longsworth's data (114, 115).

Dissolution Rate and Tablet Disintegration—Wurster and Taylor (116) reviewed the many factors affecting solid dissolution rate. Generally, the rate of dissolution of solid drugs in water at a constant agitation rate increased with increasing temperature. The dissolution rate of 80–100-mesh crystals of benzoic acid from 25 to 40° was greater at the higher temperature (117). Gibaldi (118) showed that the dissolution rates of salicylic acid, benzoic acid, and phenobarbital all increased as the pH of the system increased from 1.5 to 9.0. In his review, Lowenthal (119) cited cases where the tablet disintegration rate in water was generally greater at 37° than at 18–20° (120–123); however, in one study, no difference in disintegration rate was noted between 20 and 40° (124).

Drug Stability—Garrett (125) pointed out that "... kinetic and predictive studies of drugs in the pharmaceutical literature are of relatively recent vintage. In fact, *ca.* 1950 can be considered the start of the modern era in the quantitative comprehension and prediction of drug stability." Some factors that may affect a drug's *in vitro* stability in aqueous systems include the concentrations or activities of hydrogen ion, hydroxide ion, and other charged species present, which may be components of buffers or excipients. The ionic strength and temperature of the system may also affect the observed velocity of the drug degradation reaction. For example, at pH 7.2, the apparent half-life of fluprednisolone acetate in aqueous solution is approximately 85.6 days at 25° and 27.7 days at 40° (126). From a manufacturer's standpoint, this drug was not considered sufficiently stable to be a component in ophthalmic solutions.

The stability of drugs in aqueous solutions that are introduced into body compartments having various temperatures within the physiological range could create pharmacokinetic modeling problems, particularly if the *in vitro* half-lives are short. Indeed, such drug degradation might be misinterpreted as being attributed to drug metabolism.

ABSORPTION

A few papers dealing with the effect of temperature of drug solutions and pellet implants from a number of anatomical sites have appeared, including intramuscular, subcutaneous, lymph sac, intraperitoneal, and GI sites.

A problem encountered in the early days of penicillin therapy occurred when the aqueous drug solution was administered intramuscularly. The drug tended to disappear rapidly from both the injection site and the bloodstream. If the drug was to be effective, it had to be administered at frequent intervals. One method of prolonging the action of a single dose of penicillin administered intramuscularly was to cool the injection site with an ice bag (127–129). Trumper and Hutter (128) suggested that the absorption rate of the penicillin solution was retarded by slowing the circulation around the injection site.

McInally *et al.* (130) studied the clearance of $^{24}NA^+$ following an injection of isotonic saline into the subcutaneous tissue of the legs of normal humans. The clearance of $^{24}Na^+$ from the injection site was increased in the subjects whose trunk and upper extremities were heated by an electric blanket compared to the clearance observed when the same subjects were tested at a room temperature of 18–20°. The investigators concluded that the clearance of $^{24}Na^+$ from the subcutaneous tissues is largely

governed by the blood flow surrounding the injection region, which is presumably greater in the heated subjects.

Franke *et al.* (131) injected $^{24}Na^+$ in physiological saline intramuscularly and subcutaneously into dogs and followed the rate of drug clearance. A decrease in the drug clearance rate over controls was observed both when an ice–salt pack was placed over the injection site and when radiant heat from an IR source was directed toward the injection site. In the latter experiment, the skin surface temperature was 55° and hyperemia was evident, which might explain the decreased clearance rate observed over controls.

The effect that body temperature has on pellet implant absorption was studied (114). Pellets of acetanilid and sulfanilamide were placed on separate occasions in the dorsal lymph sac of frogs, which had been paralyzed by an injection of tubocurarine chloride. Frogs were selected because it was possible to study drug absorption over a 0–29° range while the paralyzed animals were able to respire through their moist skin. The investigators showed that if the absorption rate per unit area of a pellet were known at one reference temperature, say 0°, it would be possible to predict the absorption rate of the same drug at any other temperature up to 29° by use of the equation:

$$(\bar{R}/\bar{A})_r = (K_r)(D_r)[(Ss)_r/\delta_r] \qquad \text{(Eq. 3)}$$

where $(\bar{R}/\bar{A})_r$ is the mean pellet absorption rate per mean area at a given temperature relative to that at 0°, K_r is a proportionality constant, D_r is the relative diffusion coefficient, $(Ss)_r$ is the relative solubility of drug, and δ_r is the relative diffusion layer thickness. A plot of $(\bar{R}/\bar{A})_r$ *versus* (D_r) $[(Ss)_r/\delta_r]$ yielded a straight line with a slope, K_r, of 1.09 for the two compounds. Theory would predict a value of 1.0 if the heart beat rate could be reduced to a hypothetical zero value. The advantages of using pellets for studying the effect of temperature on the drug absorption rate from this absorption site are that a quantitative estimate of the amount of drug remaining at the absorption site can be made at any time by removing and weighing the dried pellets and that the area of the tissue or membrane across which the drug molecules diffuse is maintained almost constant. In contrast, when drugs are administered orally, rectally, or parenterally (not intravenously), it is difficult and often impossible or impractical to maintain the area of the biological membrane in contact with the drug molecules constant or to recover the unabsorbed drug quantitatively from the absorption site.

Kalser *et al.* (132) found, by LD_{50} measurements, that rats exposed for 49 days to 5° were able to tolerate twice the amount of intraperitoneally administered atropine sulfate compared to rats exposed to 5° for only 1 day. This tolerance to atropine in the chronically cold-exposed rats was attributed to a slower absorption rate of drug from the peritoneal cavity compared to the absorption rates of acutely cold-exposed rats or controls maintained at 25°. The authors concluded that the cold-acclimated rat exhibited a decreased toxicity to atropine due to a limiting of atropine absorption *via* the portal circulation rather than due to an increase in drug excretion.

GI Absorption—There has been a paucity of experimental information regarding the effect of body or environmental temperature on the rate of drug absorption from the GI region. Several reviews included the many factors affecting the GI absorption of drugs and other substances (133–137). These factors generally have been classified under the broad headings of the physicochemical properties of the drug and the dosage form, the physiological functions of the GI tract, the metabolic activities of the GI epithelium, and the structure of the intestinal epithelium as an absorbing surface.

Levy (133) pointed out that the absorption of drugs administered as solid dosage forms (*e.g.*, tablets) can be described as a two-step process. The solid dosage form must disintegrate into its primary particles, followed by dissolution of the drug in the particles into the fluids present in the GI tract. Once the drug is in solution, it passes through the GI epithelium by one or more absorption mechanisms into the fluids of distribution in the body. In many cases, the dissolution or disintegration step, which depends upon the physicochemical properties of the drug and dosage form, is the rate-limiting factor in the absorption process. When this step is rate limiting, one might predict that the drug absorption rate would be reduced at lower temperatures compared to higher temperatures, because the rate of dissolution or disintegration of solid dosage forms is generally slower at the lower temperatures at constant pH and velocity of agitation. The *in vivo* testing of this hypothesis, however, has not been performed to date.

The physiological factors having a possible effect on the GI drug absorption rate include the gastric emptying rate, GI pH, intestinal motility, and GI blood flow.

The gastric emptying rate often affects the drug absorption rate in normal subjects, whether the drug is administered in solution or as a solid dosage form, because of the pH–partition effects (138) in the stomach and intestine and the relative differences in the areas of the two absorbing membranes. The effect of the gastric emptying rate on the drug absorption rate is usually more pronounced with solid drugs that are organic weak acids or bases. The dissolution rate of organic weak bases is enhanced in the low pH of the gastric fluids, while the dissolution rate of solid organic acids is not. Because of pH–partition considerations, the absorption rate of organic weak base molecules in solution per unit area of absorbing membrane is enhanced in the fluids of the intestinal tract that generally have a higher pH than the stomach fluids, while the absorption rate of organic weak acid molecules in solution is retarded in the intestine. A detailed treatment of this topic was presented (133).

The effects of hot and cold test liquids and meals on gastric emptying time are shown in Table I. Inspection of Table I shows that it is difficult to make

generalizations from animal and human data, which would be useful in pharmacokinetic modeling. Nonetheless, liquid and meal temperatures seem to have an effect on gastric emptying time even though the effect appears to be mixed, possibly depending upon the population studied and/or the experimental conditions used.

In the *Physiological Changes* section, it was noted that the gastric pH of "normal" humans had a range of 9 pH units in a large population. In febrile subjects, the pH tended to increase; subjects exposed to cold showed a decrease in gastric pH compared to the normal subjects in the studies cited. How important these gastric pH changes due to body or environmental temperature changes are seems to be unclear at present in terms of the pharmacokinetic modeling process.

As pointed out previously, during febrile illness the transit time through the GI tract is generally reduced. Beresford *et al.* (139) noted that ^{59}Fe-labeled ferrous ascorbate absorption was profoundly depressed in Jamaican children who had a febrile illness or a febrile response to diphtheria–pertussis–tetanus immunization. They proposed several mechanisms that might explain the decrease in iron absorption. One explanation was that pyrexia increases small bowel motility, so iron salts pass more rapidly through the duodenum and have less time for absorption. Cortell and Conrad (140) found that rats injected intraperitoneally with an endotoxin derived from *Escherichia coli* showed 12 hr later a decreased intestinal absorption of ^{59}Fe as ferrous citrate, an accelerated clearance of iron from the plasma, and a decreased serum iron concentration when compared to controls.

In a review on factors affecting drug absorption, the influence of blood flow on the intestinal absorption of drugs was noted (136). For example, the absorption of tritiated water is nearly blood flow limited, while that of adonitol (ribitol) is nearly independent of blood flow. Thus, any change in blood flow to the intestinal region due to hypo- or hyperthermic conditions may affect the absorption rate of drugs that are partially or totally affected by blood flow rate. Cordier *et al.* (141) found for the fish, *Tinca vulgaris*, that the percent intestinal absorption after 17 hr increased as temperature increased over the 2–20° range. For any given temperature, the rate of intestinal absorption for various sugars was: glucose $\geq$ galactose > fructose > xylose.

Block and Corbin (142) administered 300 mg of acetaminophen in aqueous solution orally to healthy subjects; this solution was followed by the ingestion of water at 4, 25, 37, or 50° in a complete crossover study. The urinary excretion of free acetaminophen and its metabolites was measured as a function of time. They concluded that changes in intragastric temperature resulting from the ingestion of water over a 4–50° range had no effect on acetaminophen absorption.

The everted intestinal closed sac technique has been widely used for studying *in vitro* drug absorption. The observations of Levine (134) indicate that at 37° such preparations may undergo serious morphological changes as a function of incubation time. For example, after 30 min of incubation, between 50 and 75% of the normal epithelial tissue had disappeared, and there was total destruction of the epithelial borders after 1 hr. The only intact structures present were at the deepest regions of the crypts where no drug absorption takes place. When the everted sac preparation was incubated at 23°, the tissue damage appeared more slowly than at 37°. According to Levine (134), the morphological changes were probably due to an excess accumulation of fluids in the intestinal tissue of the everted sac, which normally would have been removed *via* the lymphatic or blood drainage under *in vivo* conditions. The slower rate of appearance of damage at 23° compared to that at 37° was attributed to a slowing in the metabolic processes that transfer water and salts from the mucosal to the serosal intestinal surfaces.

DISTRIBUTION

Shortly after a drug is injected or absorbed into the general circulation, the drug molecules tend to distribute among many tissues, organs, and compartments. Changes in body or environmental temperature may influence the rate and extent of drug distribution.

Protein Binding—A great many drugs interact or bind to proteins. The interaction between drugs and serum albumin probably has been studied most extensively (143–146). Drug molecules are in a rapidly reversible dynamic equilibrium between the free molecules in solution and those occupying the several binding sites on the protein. The unbound drug molecules are the species that are diffusable through membranes and are believed responsible for eliciting the pharmacological effects for drugs in which a correlation between serum drug concentration and intensity of a given pharmacological response has been established. Thus, any factor, such as an increase in body temperature over normal, that tends to weaken the various bonds involved in the formation of the drug–protein species would result in a shift in equilibrium so that a greater fraction of free or unbound drug is present for any given total drug and protein concentration. With increasing temperature, the fraction of drug bound to proteins and the binding constants tend to decrease for a number of drugs including ampicillin (147), benzylpenicillin (148), diazepam (149), diphenylhydantoin (150), phenoxymethyl penicillin (penicillin V) (147), propicillin (147), sulfadiazine (151), sulfaethidole (152), sulfamethoxypyridazine (153), urate ion (154), and warfarin (155, 156).

Schoenemann *et al.* (157) considered the pharmacokinetic consequences of plasma protein binding of drugs and developed equations appropriate for both one- and two-compartment open models. In the theoretical examples, the number of binding sites on the protein was varied from one to 10 and the percent plasma or tissue binding was varied from 9 to 90%. One implicit assumption in their equations is

that the temperature of the system is constant or has no effect.

Plasma Clearance—After intravenous injections of sulfobromophthalein into anesthetized dogs, the disappearance of sulfobromophthalein from the plasma was followed as a function of time (158). Hypothermic dogs having a rectal temperature of 30° showed the slowest disappearance rate; those having a rectal temperature of 35° showed a slightly faster rate. It was postulated that the reduced sulfobromophthalein disappearance rate in the hypothermic dogs could be due to a decrease in metabolic activity at the removal site and/or reduced hepatic blood flow.

Brain and Cerebrospinal Fluid—The rate of penetration of drugs into the central nervous system (CNS) at a constant blood flow rate seems to depend mainly upon the magnitude of the lipid–water partition coefficient of the unionized form of the drug and upon the degree of ionization at a physiological pH (159, 160). Kurz (161) perfused the heads of dogs at a constant flow rate *via* the carotid arteries with an isotonic drug solution saturated with oxygen and at pH 7.4. The perfusion fluid was held at 0 or 37°. Some drugs studied were aniline, antipyrine, and barbital. Samples of the cerebrospinal fluid were collected from the cisterna magna after the perfusion began. Aniline had the greatest heptane–water (pH 7.4) partition coefficient at a given temperature and tended to penetrate the cerebrospinal fluid most quickly, while barbital had the lowest partition coefficient at a given temperature and tended to penetrate most slowly. Antipyrine occupied an intermediate position. At 37°, all three drugs had greater rates of penetration than they did at 0°. A decrease in temperature appeared to cause a greater decrease in the rate of drug diffusion through a lipid layer than was found for an aqueous phase (agar–gel). Renkin (112) noted that the temperature coefficients of lipids must be considered in discussions of the diffusion of lipid-soluble molecules. The temperature coefficients of viscosity for lipids tend to be greater than those for water. The results just described (161) for the *in vitro* dog's head preparation should be viewed with some caution, since in the intact dog it is unlikely that the perfusion rate of drug through the carotid arteries would remain constant at two widely different temperatures because of functional changes in the cardiovascular system.

One area of research largely ignored by those interested in drug distribution involves the possible role of the process of phagocytosis. Gomez-Luz (162) injected microcrystals of oxytetracycline subcutaneously into humans and shortly thereafter found crystals of the drug in peripheral blood, some intracellular and some free. Microcrystals, both inside and outside of leucocytes, were found in the exudates of patients suffering from osteomyelitis and urethritis due to gonococcus or staphylococcus and were also found in human bone marrow. The effect of temperature on the rate of phagocytosis by leucocytes of several animal species on a variety of inorganic particles and microorganisms has been reviewed (163, 164). McCutcheon (165) showed that the velocity of locomotion of human neutrophilic polymorphonuclear leucocytes appeared to double *in vitro* by a 10° rise in temperature up to about 40°, but at 42° there was a depression of locomotion.

Noordhoek (166) studied the pharmacokinetics and dose *versus* sleeping times of mice administered hexobarbital. He found that at low doses (80 mg/kg) the elimination of hexobarbital from the brain was monoexponential 10 min after intravenous administration of the drug. At 48 mg/kg, the sleeping time was 11.8 min at 23° and 9.6 min at 37°. At 75 mg/kg, the sleeping time was 28.9 min at 23° and 27.5 min at 37°. Statistically, the differences in the duration of sleeping time at a given dose were not significant at the two temperatures. The sleeping time is determined by the rate at which the drug is eliminated from the brain and the brain level of drug at the time of awakening. During the first 10 min after intravenous injection of hexobarbital in mice, the drug equilibrates among the plasma, brain, and other tissue compartments. Afterward, the decrease in drug concentration in the brain parallels the decrease in drug plasma concentration, which is affected by metabolism, renal excretion, and diffusion of drug from the plasma to the fat depots. No attempt was made to explain why temperature appeared to have little effect on sleeping times for a particular dose of hexobarbital.

Urinary Bladder—Borzelleca and Lowenthal (167) considered several factors that might influence the rate of movement of drugs across the isolated urinary bladder of the rabbit. A nicotine solution at pH 8.1 disappeared from the bladder in an apparent first-order manner. At 27, 32, 37, and 42°, the apparent first-order rate constants were 0.117, 0.177, 0.266, and 0.139 hr^{-1}, respectively. Thus, the rate of drug movement was greatest at 37° and decreased as the temperature of the bath either increased or decreased from 37°.

Heat and Exercise—Swartz and Sidell (168) injected pyridine-2-aldoxime methochloride and the sodium salt of *p*-aminohippuric acid intravenously into male volunteers and followed the plasma concentrations of both substances as a function of time. The disappearance of pyridine-2-aldoxime methochloride and *p*-aminohippuric acid from the plasma could be described by the biexponential equation:

$$C_1 = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 4)}$$

where C_1 is the plasma concentration of drug at any time t; β is -2.303 times the negative slope of the terminal linear segment of the plot of the logarithm of the plasma concentration *versus* time, with the half-life $(t_{1/2})_\beta$ for this segment equaling $\ln 2/\beta$; B is the extrapolated zero-time intercept of this line; α is -2.303 times the negative slope of a line plotted by the method of residuals from the initial plasma level values; A is the extrapolated zero-time intercept of this line; and α and β are hybrid rate constants for the distribution and metabolism or excretion in and out of the central (plasma) and peripheral (tissue) compartments, respectively. At time zero after the

Table III—Pharmacokinetic Constants following Intravenous Administration of Pyridine-2-aldoxime Methochloride (5 mg/kg) and Sodium *p*-Aminohippurate (900 mg Total Dose) into Human Males (168)

Condition[a]	V_1, ml/kg	V_2, ml/kg	$(t_{1/2})_\beta$, min	Percent Dose Recovered 0–3 hr	Percent Dose Recovered 0–24 hr
		Pyridine-2-aldoxime Methochloride			
R	369	393	71.2	84.3	85.3
E	425	481	87.7	74.6	84.5
H	444	475	71.1	73.2	82.6
HE	404	476	86.2	73.6	84.2
		Sodium *p*-Aminohippurate			
R	261	165	43.8	77.5	78.8
E	294	214	50.9	69.9	71.1
H	334	214	42.9	71.6	73.0
HE	298	193	41.1	66.5	67.3

[a] For pyridine-2-aldoxime methochloride and sodium *p*-aminohippurate *R* = resting for 3 hr at 21.1°, *E* = exercise consisting of walking on a treadmill at 4.83 km/hr for 20 min of each half-hour for 3 hr at 23.9°, *H* = resting for 3 hr at 40.6° dry bulb/23.9° wet-bulb, and *HE* = walking at 40.6°/23.9° for 3 hr.

intravenous injection, all of the dose is in plasma Compartment 1 at an initial concentration of $C_1^{(0)}$, and V_1 is the volume constant for Compartment 1. The concentration of drug in Compartment 2, or the tissue compartment, at any time is C_2. The term V_2 is the volume constant for Compartment 2.

Each of the six subjects in this study experienced nonstress or rest *(R)* conditions and three "stress" conditions including walking exercise *(E)*, environmental heat *(H)*, and simultaneous heat and exercise *(HE)* (Table III). For both drugs, the V_1 and V_2 terms all increased when going from *R* conditions to a stress condition, although the level of statistical significance varied. For both drugs, the plasma half-lives, $(t_{1/2})_\beta$, increased significantly when going from *R* to *E* conditions. For both drugs, the percent of dose recovered in the urine after the first 3 hr following administration was two-thirds or greater, and there was a significantly lower 3-hr recovery under all stress conditions compared to *R* conditions. As estimated by *p*-aminohippuric acid clearance, exercise decreased renal plasma flow, and this decrease may affect pyridine-2-aldoxime methochloride elimination, which is excreted 80% or more unchanged *via* the kidney. The increases in V_1 and V_2 during stress conditions compared to *R* conditions suggest that the two drugs are distributed to tissues not receiving as much drug at rest conditions. It is possible that drug metabolism may also occur in the newly perfused areas. The results of this study provide the best quantitative evidence so far to suggest that environmental or body temperature may affect the pharmacokinetics of drugs. It is of interest to know how the body temperature under conditions of exercise, heat, or both compares to that under conditions of rest.

METABOLISM

The conversion of drugs to one or more active or inactive metabolites is usually controlled by various enzymes found in many organs. A drug that is an ester may undergo both enzymatic and nonenzymatic hydrolysis in the body (169). Temperature changes may have major effects upon the rates of enzyme-catalyzed reactions and of inactivation of the enzyme (170). There has been a long-standing debate concerning the most appropriate method of plotting the rates of enzymatic reactions or biological processes *versus* temperature (171–173); however, the Arrhenius equation is most commonly employed:

$$k = Ae^{-E/RT} \qquad \text{(Eq. 5)}$$

where k is the reaction rate, A is a constant, E is the activation energy, and R and T are the gas constant and absolute temperature, respectively (174). Thus, a plot of $\log_{10} k$ *versus* $1/T$ usually yields a straight line over a limited temperature range with the slope equal to $-E/2.303\,R$.

During hyperthermia, organ damage may occur and enzymes may "leak" into the blood. Burger *et al.* (175) maintained groups of rats at a room temperature of 18° and in water baths at 37.5 and 42°. After hyperthermia, the serum of rats showed an increase over controls in the levels of alkaline phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, and lactate dehydrogenase. The serum levels of glutamate dehydrogenase and sorbitol dehydrogenase at first increased and then decreased.

The effect of temperature on drug metabolism in the intact animal is poorly understood for most drugs. A few studies should illustrate the present state of the art. Cottle and Veress (176) measured the total urinary glucuronide excretion from two groups of rats acclimated to 5 or 25° for 1 month. After a 1-day control urine collection, both groups were injected intraperitoneally with a solution containing 50 mg of sodium benzoate. Urine was collected for 2 days. Table IV shows that cold-acclimated rats excreted more total glucuronide in the urine compared to control values than did rats living under the 25° conditions. The capacity of the conjugation mechanism appears to be increased in the cold-acclimated animals compared to those at 25°.

Rink *et al.* (177) and Gray *et al.* (178) utilized a perfusion technique in the isolated rabbit liver to study morphine and thiopental metabolism at 24 and 37°. The biological half-life for the loss of free morphine from the plasma was 94 min at 24° and 3.7

Table IV—Excretion of Glucuronides in Urine of Wistar Rats (176)

	Rats Acclimated to 25°, mg/day	Rats Acclimated to 5°, mg/day
Control period	10.3	15.6
0–24 hr after sodium benzoate	13.2	21.1
24–48 hr after sodium benzoate	12.6	22.3

min at 37°. The biological half-life for the loss of free thiopental from the plasma was 185–530 min at 24° and 46 min at 37°.

A series of papers (179–182) was published on drug metabolism under hypothermic conditions using a perfused rat liver preparation. The acetylation of sulfanilamide by the liver was temperature sensitive. Most acetylated drug appeared in the blood, and the half-lives for the metabolite's appearance were 70 min at 37° and 750 min at 15° (179). In a similar study with pentobarbital, the half-lives for the disappearance of unchanged drug from the blood were 315 min at 37° and 1325 min at 20° (180). Hydrolysis of procaine in this perfusion system occurred only in the liver and not in rat plasma. Procaine disappearance from the blood at 37° had a half-life of 3 min; at 17°, it had a half-life of 35 min (181). In the perfused rat liver system, a ^{14}C-label on atropine disappeared from the plasma in an apparent polyexponential manner. The half-lives for the fastest component were 6 min at 37° and 20 min at 17° (182).

Creaven *et al.* (183) showed that the 10,000×*g* supernate of rainbow trout liver homogenate was able to *O*-dealkylate six 2- and 4-alkoxy-substituted biphenyls. The dealkylating activity in the trout preparations was more active at around 20° than at 37°. Frog liver preparations, *Rana temporaria,* also dealkylated the same substrates, but the optimum enzyme activity was at 37° instead of at 20°.

Following oral drug administration, extrahepatic drug metabolism may occur with normal intestinal microorganisms. As Scheline (184) pointed out, drugs may undergo reduction in molecular size and change their physicochemical properties. Goldman (185) recently reviewed the therapeutic implications of the metabolism of such drugs as salicylazosulfapyridine and levodopa by intestinal microflora. Neither investigator mentioned how changes in intestinal temperature might influence the rate of such drug metabolism.

Drug (or foreign chemical) metabolism has been shown to occur within various segments of the GI tract wall (186–188). Levy (189) found that hydrolysis of aspirin occurred in everted segments of the rat small intestine and that the percent hydrolysis decreased with increasing aspirin concentration. A linear relationship between the reciprocal of the aspirin hydrolysis rate and the reciprocal of the initial aspirin concentration was found on a Lineweaver–Burk-type plot. Possible temperature effects on metabolism were not studied by these investigators.

Extrahepatic metabolism occurred with some ester-type drugs in the serum or plasma of the rabbit (169) and the human (190–193). Both enzymatic and nonenzymatic hydrolysis of atropine occurred in rabbit serum and was affected by serum pH and temperature. At pH 8.4, the optimum activity of the tropine esterase occurred at 38° (169), and the hydrolysis was an apparent zero-order reaction.

Wislicki (194) studied the blocking action of succinylcholine on the gastrocnemius muscle in anesthetized cats during artificial hypo- and hyperthermia. Following a lowering of rectal temperature to 30°, the duration and magnitude of the neuromuscular blocking effect of succinylcholine were about double compared to animals at normal body temperature. The artificial warming of animals lessened the response of the gastrocnemius muscle to succinylcholine. The *in vitro* activity of succinylcholine incubated in cat plasma was measured using frog recti abdominis muscles to detect drug activity over a 30–45° temperature range. The rate of inactivation of succinylcholine increased from 30 to 40°, reached a maximum between 40 and 42.5°, and then decreased at 45°. The correlation between the *in vivo* and *in vitro* experiments suggests that the temperature dependence of enzymatic hydrolysis of succinylcholine may play an important role in determining the magnitude and duration of the neuromuscular block at various body temperatures. However, other factors might also be involved; for example, variations in blood circulation to muscle tissue at different temperatures could affect the results.

ELIMINATION

Although drug and metabolite elimination can occur *via* many routes, only the effect of temperature on renal and biliary elimination will be considered in this report. Several reviews discussed factors affecting drug excretion by the kidney (195–197) and the bile (195–200), but temperature effects were usually omitted.

Page (201) studied the effects of progressive hypothermia on the renal function of dogs maintained at a 20° room temperature for at least 5 days. After sodium pentobarbital anesthesia, intravenous infusions of *p*-aminohippurate and creatinine were initiated. At 20°, control values for the glomerular filtration rate were measured using exogenous creatinine clearance, the effective renal plasma flow was calculated from *p*-aminohippurate clearance, and the maximum tubular secretory capacity for *p*-aminohippurate, ^{Tm}p-aminohippurate, was determined. The dogs were then transferred to a cold room held at about −25° where urine and blood samples were collected and rectal temperatures were recorded. During progressive hypothermia, the glomerular filtration rate and effective renal plasma flow decreased in a linear manner with decreasing rectal temperature. An Arrhenius plot of the logarithm of ^{Tm}p-aminohippurate *versus* the reciprocal of the absolute rectal temperature yielded a linear plot with a negative slope. The average activation energy, *E,* for four normal dogs was 23.5 kcal/mole.

Previously, it was mentioned that there are many human circadian rhythms including those related to urine and plasma pH and body temperature. During sleep, urine and plasma pH values are lower than after awakening. Body temperature tends to be lowest 4 hr after midnight and highest at about 8 pm. An error in logic would occur if it were stated that just because two events occur simultaneously, or nearly so, one event was the cause of the other. However, the possibility of causal relationships between urine pH and body temperature cannot be ruled out pending the completion of suitable experiments.

When the urine pH is shifted from low to higher values, or the reverse, due to normal processes such as the circadian rhythm or due to the oral administration of acidifying agents (*e.g.*, ammonium chloride) or alkalinizing agents (*e.g.*, sodium bicarbonate), the rate of urine excretion and the biological half-life of some weak organic acids and bases may be altered. The physical properties of the drug, such as the pKa and the lipid–water partition coefficient, may determine whether the biological half-life of an organic weak acid or base will be affected by changes in urinary pH. For example, Dettli *et al.* (202) conducted a crossover study with human volunteers using two sulfonamides. The urine pH was not controlled in one test period ("spontaneous period"), and in the other test period ("alkali period") the urine pH was maintained at 8 with sodium bicarbonate. The mean serum biological half-lives of unchanged sulfalene (pKa′ = 6.1) were 7.3 hr for the alkali period and 14.0 hr for the spontaneous period, and the mean half-lives for sulfasymazine (pKa′ = 5.5) were 32.46 hr for the alkali period and 71.58 hr for the spontaneous period. Thus, for both drugs the biological half-life during the alkali period was approximately half that for the spontaneous period.

Dettli and Spring (203) found that the mean biological half-life of sulfasymazine (pKa′ = 5.5) in humans at night was about 2.6 times longer (35.0 hr) than that found during the day (13.5 hr). The mean biological half-life for sulfanilamide (pKa′ = 10.5) at night (12.7 hr) was nearly the same as that found during the day (11.1 hr). Spring *et al.* (204) noted that the biological half-life of sulfalene (pKa′ = 6.1) in human plasma was about three times longer at night (145.7 hr) than it was during the day (46.2 hr).

According to the pH–partition hypothesis (205), the unionized form of lipid-soluble drugs that are organic weak acids or bases can diffuse passively through cell membranes within the body. At any given physiological pH and temperature, the degree of ionization, α, of an organic weak acid, for example, is:

$$\alpha = \frac{1}{1 + 10^{pKa' - pH}} \qquad \text{(Eq. 6)}$$

Thus, sulfanilamide is nearly completely unionized at any physiological pH, while the sulfonamides having pKa′ values of 5.5 and 6.1 have a large fraction of the total sulfonamide present in the ionized form at pH 8 and less in the ionized form at lower pH values, the actual fraction being determined by pH.

Sulfonamides having pKa′ values of 5.5 and 6.1 would not tend to be as readily reabsorbed into the tubular cells from an alkaline urine as an acidic one. Dettli *et al.* (202) suggested that the decrease in biological half-life of an acidic drug in serum during an alkalization of urine is partly due to a diminished nonionic tubular reabsorption of drug and may be partly due to nonionic redistribution of drug within the organism. If the intracellular pH is less affected than the pH of the extracellular fluid due to the administration of alkali, then the fraction of unionized molecules of drug will decrease in the extracellular fluid. Unionized molecules will tend to diffuse from the cells into the extracellular fluid. Since the amount of sulfonamide eliminated per unit of time equals the product of the renal clearance and the plasma concentration, an increase in drug elimination rate would result as the plasma concentration increases. Thus, the observed increase in elimination rate following alkali administration could be due to diminished nonionic tubular reabsorption as well as to nonionic redistribution within the organism. A drug like sulfanilamide, which is nearly completely in the unionized form at all physiological pH values, would exhibit little increase in its elimination rate following administration of alkali.

The effects that body or environmental temperature may have on the biliary elimination or excretion of drugs has been investigated for such substances or drugs as bilirubin, sulfobromophthalein, glycerol, ethanol, liothyronine, and thyroxine. Roberts *et al.* (206) studied the influence of temperature on the maximum biliary excretion (T_m) of bilirubin and sulfobromophthalein in anesthetized rats and mice. A decrease in rectal temperature from 39 to 31° reduced both bilirubin and sulfobromophthalein T_m values in a linear manner. Bile flow in both species decreased linearly with a decrease in rectal temperature.

Larsen (207) measured the effect of body cooling on the liver function of anesthetized cats. Liver function was measured by following the elimination rate of glycerol and ethanol and the hepatic uptake of indocyanine green which were infused into the femoral vein. A 1–2° reduction of the body temperature reduced the elimination rate of glycerol and ethanol and the hepatic uptake of indocyanine green by about 40%. The changes in liver function following a slight decrease in liver temperature might be explained by a reduction in the interhepatic circulation and reduction in functional capacity of the liver in the anesthetized animal.

Cottle (208) found that the biliary clearance of liothyronine in rats acclimated at 5° was greater than in rats acclimated at 25°. Hillier (209) confirmed these results and also showed that the biological half-life of thyroxine in the blood following an intravenous injection was shorter for cold-adapted rats (4°) than for warm-adapted rats (22°).

CONCLUSIONS

The fragmentary evidence presented in this review

suggests that environmental or body temperature may influence the pharmacokinetic behavior of a drug. The magnitude or importance of this role for any one particular drug in intact animals or humans under hypo- or hyperthermic conditions cannot be defined precisely, because in nearly all instances the critical experiments needed to define pharmacokinetic constants have yet to be performed under controlled conditions. The potential for research in the area of temperature and pharmacokinetics is great and may prove to have valuable therapeutic consequences in determining dosages and dosing regimens in patients whose body temperature is not within the "normal" range throughout the day.

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Received from the *Department of Pharmacy, College of Pharmacy, University of Rhode Island, Kingston, RI 02881*

Drug Release from Hydroxypropyl Cellulose–Polyvinyl Acetate Films

SAUL BORODKIN ˣ and FLORENCE E. TUCKER

Abstract □ The release of methapyrilene, pentobarbital, and salicylic acid dispersed in films composed of different ratios of hydroxypropyl cellulose and polyvinyl acetate was investigated. The results indicate that drug release follows a diffusion-controlled matrix model, where the quantity released per unit area is proportional to the square root of time. The release rate was found to increase with increasing hydroxypropyl cellulose–polyvinyl acetate ratios, with the logarithm of the rate constant proportional to the fraction of hydroxypropyl cellulose in the matrix. The release rates were also shown to be proportional to drug concentration but independent of film thickness.

Keyphrases □ Drug release (methapyrilene, pentobarbital, salicylic acid) from hydroxypropyl cellulose–polyvinyl acetate films—diffusion-controlled matrix model, effects of polymer ratio and drug concentration on release kinetics □ Polymer films, hydroxypropyl cellulose–polyvinyl acetate—effects of polymer ratio and drug concentration on drug (methapyrilene, pentobarbital, salicylic acid) release kinetics

The use of drugs dispersed in inert polymer matrixes to achieve controlled release by diffusion has received considerable attention. This method has long been favored for the preparation of sustained-release tablets for oral ingestion (1–3). More recently, the concept has been suggested for other uses including catheters coated with antibiotic-impregnated polymers (4), prolongation of the release of pilocarpine in eye administration (5), long-term buccal absorption of drugs (6), dermatological applications (7, 8), and long-acting implants (9, 10).

Higuchi (11) suggested that release from a planar system having dispersed drug in a homogeneous matrix should follow the relationship:

$$Q = [D(2A - C_s)C_s t]^{1/2} \quad \text{(Eq. 1)}$$

where Q is the amount of drug released after time t per unit exposed area, D is the diffusivity of the drug in the matrix, A is the initial drug concentration, and C_s is the drug solubility in the matrix. He later derived a similar relationship for planar release from a granular matrix system in which diffusion occurs through channels (12):

$$Q = \left[\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t\right]^{1/2} \quad \text{(Eq. 2)}$$

where D and C_s refer to diffusivity and solubility in the permeating fluid, respectively; τ is the tortuosity of the matrix; and ϵ is the porosity of the matrix. Although the two equations are for different mechanisms, they both describe drug release as being linear with the square root of time:

$$Q = k_H t^{1/2} \quad \text{(Eq. 3)}$$

where for the homogeneous matrix system:

$$k_H = [D(2A - C_s)C_s]^{1/2} \quad \text{(Eq. 4)}$$

and for the granular matrix system:

$$k_H = \left[\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s\right]^{1/2} \quad \text{(Eq. 5)}$$

The validity of the relationships has since been confirmed experimentally by a number of workers using various systems (13–25).

The release rate of drugs from a polymer matrix may be controlled by the concentration, surface area, leaching solvent, and polymer system. However, where a specific dose, size, and solvent are defined by required use, the release rate in a specific polymer is limited by the physicochemical properties of the drug. It would, therefore, be desirable to achieve a wide range of release rates for a drug by simple modification of a polymer system. Shah and Sheth (26) demonstrated that modifying the hydroxypropyl methylcellulose–ethylcellulose ratio in barrier films could drastically alter the permeability of dye.

This paper describes studies of drug release from thin films containing drug dispersed in physical mixtures of hydroxypropyl cellulose and polyvinyl acetate. The polymers were selected based on film-forming characteristics, compatibility, demonstrated pharmaceutical applicability, and differing solubility and hydrophilicity characteristics. Pentobarbital, methapyrilene, and salicylic acid were selected as model drugs to study the effect of polymer ratio modifications on drug release kinetics.

EXPERIMENTAL

Chemicals—The hydroxypropyl cellulose[1] used in these studies was a food or pharmaceutical grade, with an average molecular weight of 100,000, which gives a viscosity of 75–150 cps as a 5% water solution. The polyvinyl acetate[2] used had an average molecular weight of 500,000 and a viscosity of 90–110 cps as an 8.6% solution in benzene. Sodium pentobarbital and salicylic acid were USP grade, while methapyrilene hydrochloride was NF grade. The pH 7.0 sodium phosphate buffer was made 0.5 N in phosphate (0.274 mEq/ml Na^+) by dissolving 28.9 g $Na_2HPO_4 \cdot 7H_2O$ and 8.05 g $NaH_2PO_4 \cdot H_2O$/liter of solution (27).

Film Preparation—Generally the films were cast from a solution containing 10% solids (drug plus polymer), using a methylene chloride–methanol mixture (9:1) as the solvent. The polymers were

[1] Klucel LF, Hercules, Inc., Wilmington, DE 19899
[2] Gelva V-100, Monsanto Co., St. Louis, MO 63166

Table I—Comparison between First-Order and Q *versus* $t^{1/2}$ Treatments of Pentobarbital Release Rate Data

Concentration[a], %	Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio	Number of Runs	First Order		Q *versus* $t^{1/2}$	
			t_{lag}, min[b]	Correlation Coefficient[b]	t_{lag}, min[b]	Correlation Coefficient[b]
36.4	10:0	4	−3.2	0.996	3.1	0.997
18.2	10:0	4	−14.0	0.957	2.6	0.993
18.2	9:1	2	−9.8	0.986	2.8	0.996
18.2	8:2	2	−6.5	0.986	2.3	0.993
18.2	6:4	2	−84.0	0.982	1.4	0.996
18.2	4:6	4	−142.0	0.983	0.3	0.998
18.2	2:8	4	−176.0	0.981	0.6	0.998
18.2	1:9	4	−176.0	0.982	0.7	0.998
18.2	0:10	3	−214.0	0.979	1.0	0.997
9.1	0:10	4	−199.0	0.981	1.3	0.990

[a] Weight of drug per weight of dry film. [b] All t_{lag} and correlation coefficient values expressed are mean values.

Table II—Effect of Film Thickness on Pentobarbital Release Rate Constant and Half-Life

Drug Concentration[a], %	Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio	Wet Film Thickness Setting, mm	Dry Film Thickness[b], μm	k_H, mg cm^{-2} min$^{-1/2}$	Correlation Coefficient	$t_{1/2}$, min
36.4	10:0	0.64	44.0 ± 1.1	0.33	0.996	14.8
		1.27	56.2 ± 2.1	0.29	0.996	25.2
		1.91	100.2 ± 4.6	0.35	0.998	49.8
		2.54	109.3 ± 3.3	0.32	0.998	60.2
18.2	4:6	0.64	61.4 ± 4.9	0.032	0.999	360
		1.27	113.2 ± 9.9	0.034	0.999	1,280
		1.91	145.0 ± 8.0	0.034	0.999	2,280
		2.54	204.2 ± 3.1	0.037	0.997	3,850
18.2	1:9	0.64	76.8 ± 1.2	0.0101	0.996	8,520
		1.27	118.4 ± 2.6	0.0102	0.999	19,200
		1.91	202.8 ± 2.2	0.0101	0.998	50,900
		2.54	268.0 ± 2.4	0.0102	0.998	83,600

[a] Weight of drug per weight of dry film. [b] Thickness = mean ± standard deviation of five measurements.

added as dry powders by slow addition to the vigorously stirring solution. Salicylic acid was added as the dry powder, and pentobarbital and methapyrilene were incorporated from stock methylene chloride solutions prepared from the drug salts. The pentobarbital solution containing 60.8 mg/ml was prepared by dissolving 12.0 g sodium pentobarbital in 50 ml water, adding 5 ml concentrated hydrochloric acid, extracting three times with 50-ml portions of methylene chloride, and adjusting the volume of the combined extract to 180 ml. The methapyrilene base solution containing 58.5 mg/ml was prepared by dissolving 12.0 g methapyrilene hydrochloride in 60 ml water, adding 10 ml 5 *N* NaOH, extracting three times with methylene chloride, and adjusting the volume of the combined extract to 180 ml.

Films were cast from the solutions at various wet thicknesses (0.64–2.54 mm) using a knife[3] on Teflon-coated plate glass. The films were allowed to air dry at least 48 hr before evaluation. The percent drug in the dry film was calculated from the ratio of drug and polymer weights used.

Release Rate Determination—Rectangular films measuring 2.2 × 4.0 cm (8.8 cm^2) were obtained by cutting a selected portion of the cast film with a razor blade, using a microscope cover glass as a template. The film was weighed on an analytical balance, and the thickness was measured at the four corners and center with a micrometer[4]. A thin coating of high vacuum silicone lubricant[5] was applied to a 2.54 × 7.62-cm microscope slide; the film was carefully pressed into the slide, making sure that all edges adhered and no lubricant touched the exposed surface. Silicone lubricant was found superior to solvent-based adhesives by virtue of its noninteracting compatibility with the film. In addition to its capacity to maintain adhesion of the film to the slide, its water repellency provided secondary assurance of only single surface release. When the exposed surface of film was coated with silicone lubricant, only insignificant drug release was obtained. The slide was placed at an angle into a 250-ml beaker in a 37° water bath containing 200 ml of pH 7.0 buffer preheated to 37°. A nonagitated system was selected to eliminate any effect of turbulence on the release rate as well as to assure that no disruption of the film occurred. Periodic assay samples (approximately 10/run) were obtained by removing the slide, stirring the solution, and pipeting a 5-ml sample. The slide was quickly reinserted, making sure that the film remained completely immersed throughout the release study. The beaker was kept covered[6] throughout the run to prevent evaporation. The run was continued for at least 7 hr or until the assays indicated complete release had occurred. With many extremely slow releasing systems, the runs were continued for 31 hr.

All samples were assayed by UV spectrophotometry[7]. Pentobarbital was assayed at 240 nm in 0.1 *N* NH_4OH, methapyrilene at 312 nm in 0.1 *N* HCl, and salicylic acid at 297 nm in 0.1 *N* NaOH.

RESULTS AND DISCUSSION

Kinetics of Drug Release—Adherence of drug release to the Q *versus* $t^{1/2}$ relationship discussed in Eqs. 1–5 requires that the drug concentration in the undepleted zone far exceeds the solution concentration at the interface ($A \gg C_s$ or ϵC_s). A first-order release relationship, with the release rate proportional to the drug concentration remaining within the film, might also be considered possible if this condition does not exist:

$$\ln (Q_\infty - Q) = -k_1 t + \ln Q_\infty \qquad \text{(Eq. 6)}$$

Sciarra and Gidwani (7, 8) reported that gentian violet, cetylpyridinium chloride, and benzalkonium chloride were released from

[3] Gardner Laboratories, Bethesda, MD 20014
[4] L. S. Starrett Co., Athol, Mass.
[5] Dow Corning Corp., Midland, MI 48640
[6] Parafilm M, American Can Co., Neenah, Wis.
[7] Coleman model 124, Perkin-Elmer Co., Norwalk, Conn.

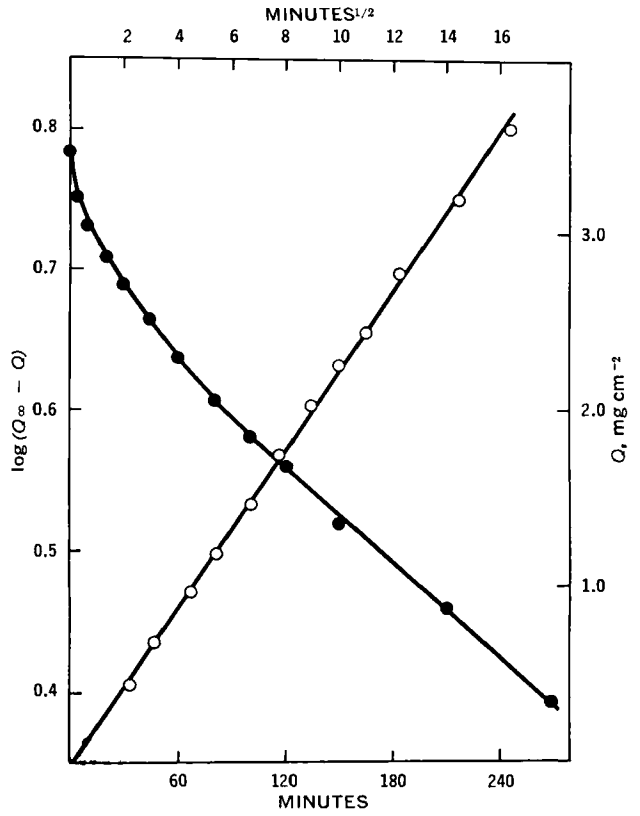

Figure 1—*Comparison between first-order release treatment and* Q versus $t^{1/2}$ *treatment of data from a film containing 26.3% methapyrilene at a 5:5 ratio of hydroxypropyl cellulose–polyvinyl acetate. Key:* ●, *log* (Q_∞ − Q) versus t; *and* ○, Q versus $t^{1/2}$.

various films by this first-order relationship. Schwartz *et al.* (22) rigorously examined the release data from wax matrixes by both first-order and the *Q versus* $t^{1/2}$ treatments before concluding that the latter was most applicable.

The release data obtained in this study were treated by both methods to ascertain which relationship gave the best fit. The correlation coefficients for the best statistical lines and the lag times (time intercept extrapolated to $Q = 0$) were used as the principal criteria for evaluation. The data comparing the two treatments for a number of pentobarbital systems where multiple runs were made are shown in Table I. Although the relatively high correlation coefficients obtained with first-order treatment indicate that this type of release can easily be misconstrued as following such a relationship after an initial surge of drug, the data continually showed the *Q versus* $t^{1/2}$ relationship to be clearly superior. The curvature with first-order treatment is shown in Fig. 1, a representative graph obtained when the data for a methapyrilene release experiment are plotted by both equations.

The linear relationships obtained by plotting *Q versus* $t^{1/2}$ generally gave correlation coefficients greater than 0.995. When plotted, deviation appeared to be random rather than due to any curvature. In experiments run through drug exhaustion, linearity generally held through greater than 75–80% of drug release, with negative deviation occurring in the last 10–20%. This latter deviation may be attributed to exhaustion of the drug suspension phase when the diffusion gradient distance in the film equals the film thickness. Unevenness in film thickness, where depletion of the suspension phase would be nonuniform, could also cause this negative deviation.

The lag times necessary to establish the release mechanism were generally no greater than several minutes. However, they appeared to become more significant with slower release rates, and some early assay points were observed to indicate deviation from linearity in the initial release stages.

The derivation of the *Q versus* $t^{1/2}$ relationship requires a constant concentration gradient throughout release. Therefore, the drug concentration in the buffer solution must remain insignificant compared to its solubility. This condition was easily met with salicylic acid and methapyrilene, where the maximum concentration that could be encountered with complete drug release was well below 1% of its solubility. In the case of pentobarbital, this value could rise to as high as 10% of solubility. However, the experimental results gave no indication of this causing deviation from the linear relationship.

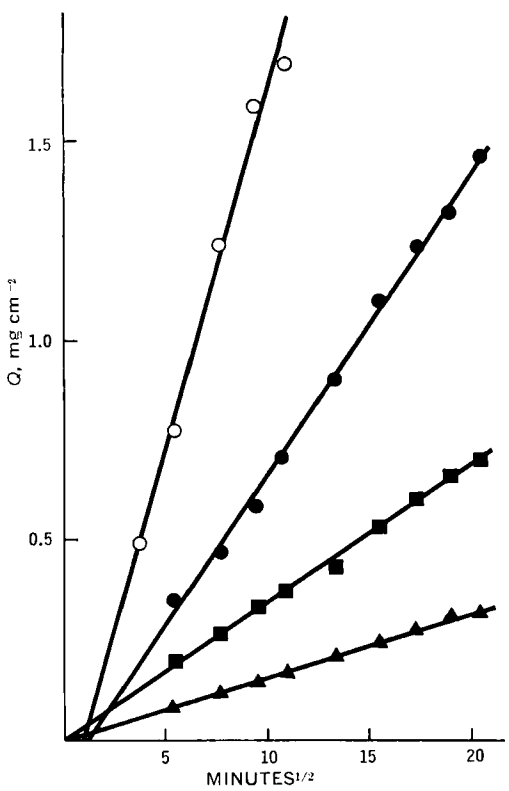

Figure 2—*Drug release from films containing 18.2% pentobarbital at different hydroxypropyl cellulose–polyvinyl acetate ratios. Key:* ○, *8:2;* ●, *6:4;* ■, *4:6; and* ▲, *2:8.*

Effect of Film Thickness—The release rate constant, k_H, has the dimensions of weight per area per square root of time and is, therefore, independent of film thickness. The constancy of k_H with varied film thickness is shown in Table II, where the results obtained with several pentobarbital systems are compiled.

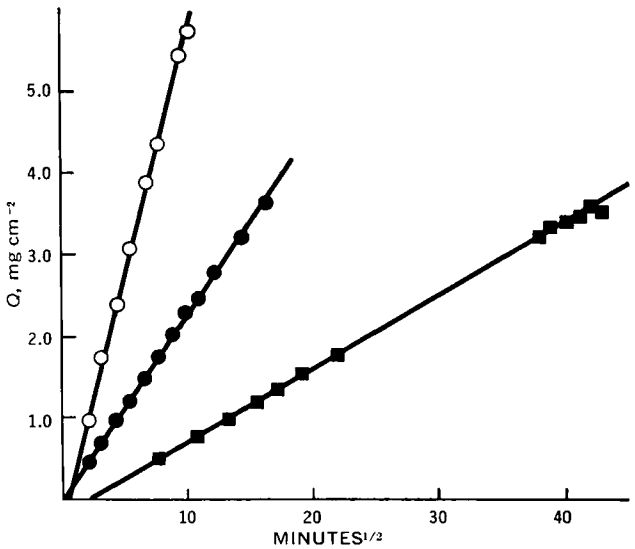

Figure 3—*Drug release from films containing 26.3% methapyrilene at different hydroxypropyl cellulose–polyvinyl acetate ratios. Key:* ○, *8:2;* ●, *5:5; and* ■, *2:8.*

Table III—Effect of Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio on the Release Rate from Films Containing 26.3% Methapyrilene

Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio	Dry Film Thickness[a,b], μm	k_H, mg cm^{-2} min$^{-1/2}$	Correlation Coefficient	$t_{1/2}$, min
10:0	210 ± 7.4	0.549	0.999	33
9:1	210 ± 6.5	0.593	0.997	28
8:2	216 ± 9.1	0.497	1.000	42
7:3	181 ± 7.8	0.272	0.995	99
6:4	218 ± 4.4	0.217	0.998	226
5:5	208 ± 4.6	0.225	0.999	190
4:6	142 ± 4.5	0.191	0.997	124
3:7	157 ± 3.3	0.094	0.998	630
2:8	136 ± 3.3	0.089	0.999	523
1:9	131 ± 6.0	0.075	0.998	681
0:10	185 ± 0.9	0.074	0.996	1404

[a] Thickness = mean ± standard deviation of five measurements. [b] All films cast using a wet thickness setting of 2.54 mm.

Although film thickness does not affect the rate constant, it determines the duration of drug release. By utilizing Eq. 3, the time, t_F, required for release of any fraction, F, of the drug in the film may be expressed as:

$$t_F = \left[\frac{Q_\infty F}{k_H}\right]^2 \quad \text{(Eq. 7)}$$

Additionally, the initial amount of drug in the film, $Q_\infty \times$ area, equals the concentration, A, times the volume. Dividing both sides by area, Q_∞ may be equated to Ah, where h is the film thickness. By substituting in Eq. 7, t_F is shown to be proportional to the square of thickness:

$$t_F = \left[\frac{AF}{k_H}\right]^2 h^2 \quad \text{(Eq. 8)}$$

The calculated values of 50% drug release ($t_{1/2}$) are included in Table II. Since the fractional time is proportional to the square of the fraction released, the calculated times for complete drug release would be four times the half-lives. However, since negative deviation from linearity occurs in the last stages of release, essentially complete depletion would be expected to take somewhat longer.

Effect of Polymer Ratio—Altering the hydroxypropyl cellulose–polyvinyl acetate ratio in the polymer composite mixture markedly affected the release rate (Figs. 2 and 3); the results obtained with the three drugs tested are compiled in Tables III–V. The results, with some exceptions, showed an acceleration of the release rate with an increasing proportion of hydroxypropyl cellulose in the polymer matrix. Pure hydroxypropyl cellulose gave k_H values seven to eight times those obtained with pure polyvinyl acetate. Based on Eq. 8, this would correspond to half-lives 50–75 times longer with polyvinyl acetate films than with hydroxypropyl cellulose films of identical thickness and concentration. The variability from this range indicated in Tables III–V, with half-lives being 42, 37, and 348 times greater for polyvinyl acetate films, is due to differences in the film thicknesses. Although the release rates differed substantially, there appeared to be little effect on the release mechanism as shown by the constancy of the *Q versus* $t^{1/2}$ relationship.

Table IV—Effect of Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio on the Release Rate from Films Containing 20.0% Salicylic Acid

Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio	Dry Film Thickness[a,b], μm	k_H, mg cm^{-2} min$^{-1/2}$	Correlation Coefficient	$t_{1/2}$, min
10:0	189 ± 2.8	0.403	0.997	28
9:1	223 ± 5.6	0.336	0.997	57
8:2	210 ± 0.9	0.328	0.993	53
7:3	229 ± 2.5	0.241	0.998	116
6:4	209 ± 6.6	0.216	0.984	120
5:5	204 ± 7.9	0.175	0.997	175
4:6	145 ± 0.5	0.115	0.997	206
3:7	156 ± 3.4	0.122	0.997	210
2:8	181 ± 7.9	0.112	0.998	338
1:9	222 ± 4.3	0.073	0.998	1190
0:10	162 ± 1.8	0.057	0.996	1040

[a] Thickness = mean ± standard deviation of five measurements. [b] All films cast using a wet thickness setting of 2.54 mm.

Table V—Effect of Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio on the Release Rate from Films Containing 18.2% Pentobarbital

Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio	Wet Thickness Setting, mm	Dry Film Thickness[a], μm	k_H, mg cm^{-2} min$^{-1/2}$	Correlation Coefficient	$t_{1/2}$, min
10:0	1.27	98 ± 5.4	0.225	0.995	20
9:1	2.54	163 ± 3.4	0.224	0.995	57
8:2	1.27	87 ± 6.4	0.178	0.987	25
6:4	2.54	236 ± 7.1	0.0767	0.997	1,010
4:6	1.91	145 ± 8.0	0.0342	0.999	2,280
2:8	1.27	94 ± 1.9	0.0161	0.999	3,630
1:9	1.27	118 ± 2.6	0.0102	0.999	19,200
0:10	1.91	210 ± 15.5	0.0260	0.998	6.960

[a] Thickness = mean ± standard deviation of five measurements.

Attempts to relate mathematically the release rate constant, k_H, to the polymer ratio yielded the best results when log k_H was plotted against the fraction of hydroxypropyl cellulose in the composite matrix. The relationship between the rate constant and polymer ratio may be empirically depicted by the following equation:

$$\log k_H = k_R F_H + \log k_p \quad \text{(Eq. 9)}$$

where k_R is a constant specific for each drug and concentration, F_H is the fraction of hydroxypropyl cellulose in the matrix, and k_p approximates k_H in pure polyvinyl acetate ($F_H = 0$). Table VI shows the results obtained when applying this relationship to the three drugs tested, and Fig. 4 graphically depicts the results with salicylic acid and pentobarbital.

The value for k_H obtained in the pentobarbital system with the pure polyvinyl acetate matrix deviated considerably from the relationship. Repeated determinations of k_H in pure polyvinyl acetate gave reproducible results, indicating the validity of this value. The most likely cause of this deviation would appear to be the plasticizing effect of hydroxypropyl cellulose on polyvinyl acetate. With total elimination of hydroxypropyl cellulose, mechanical changes in the film matrix alter the release rate from that otherwise expected. This deviation may not occur with methapyrilene and salicylic acid because the drugs themselves provide sufficient plasticity.

Although hydroxypropyl cellulose is water soluble, even with this pure polymer as the matrix no visual dissolution of the films was observed until all of the drug was released. Since the proposed diffusion mechanism requires maintenance of the matrix, adherence to the *Q versus* $t^{1/2}$ relationship confirms the observations. Lapidus and Lordi (14, 21) likewise observed adherence to the diffusion-controlled matrix model with drug release from water-soluble hydroxypropyl methylcellulose.

The best explanation to account for the large k_H changes with

Table VI—Linear Relationship of Log k_H to Hydroxypropyl Cellulose Fraction in the Polymer Matrix

Drug	Concentration, %	Number of Ratios Tested	k_R[a]	Log k_p[a]	Correlation Coefficient
Salicylic acid	20.0	11	0.83	−1.196	0.973
Methapyrilene	27.3	11	1.01	−1.209	0.950
Pentobarbital	18.2	7[b]	1.58	−2.110	0.985

[a] k_R and log k_p are defined by Eq. 9. [b] Covers hydroxypropyl cellulose–polyvinyl acetate ratio range of 10:0–1:9.

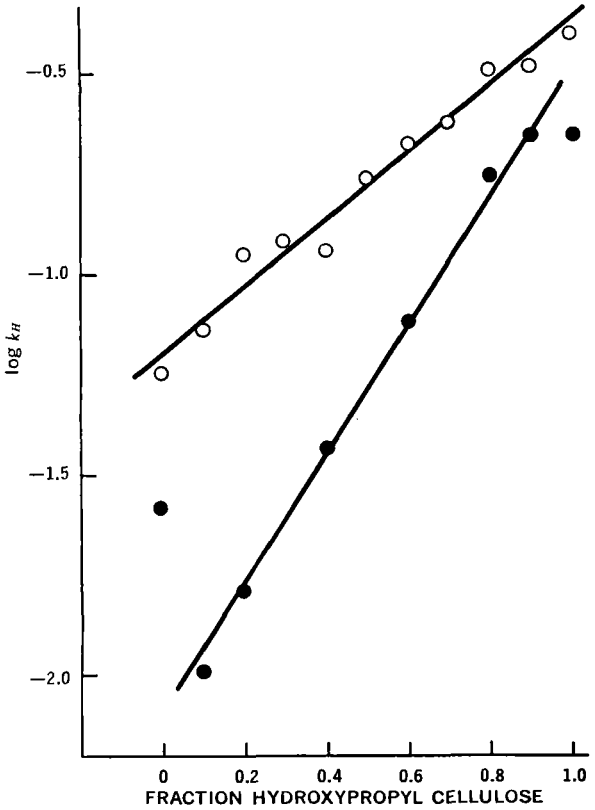

Figure 4—*Relationship of log* k_H *to fraction of hydroxypropyl cellulose in the polymer matrix. Key:* ○, *20.0% salicylic acid films; and* ●, *18.2% pentobarbital films.*

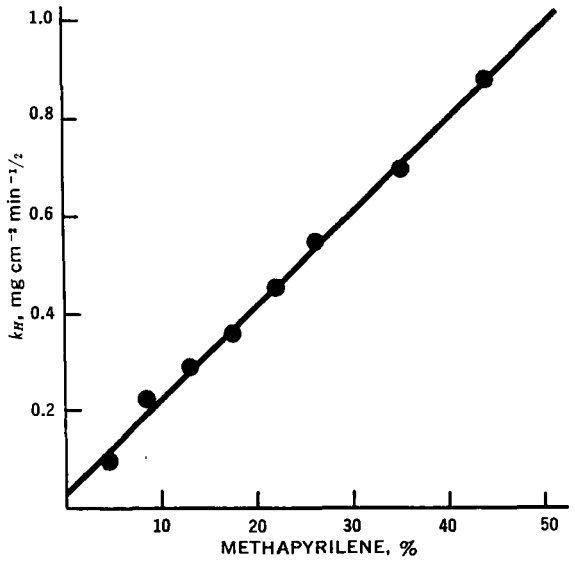

Figure 5—*Relationship of* k_H *to percent methapyrilene in 100% hydroxypropyl cellulose films.*

polymer ratio in terms of the parameters shown in Eqs. 4 and 5 would be through consideration of the hydrated film obtained upon immersion in the buffer as the actual diffusion matrix. Weight gain studies with films suspended in a 100% relative humidity atmosphere at 37° showed very rapid equilibration, with the percentage moisture absorbed increasing with hydroxypropyl cellulose content from about 4% with pure polyvinyl acetate to 32% with pure hydroxypropyl cellulose. In terms of the homogeneous matrix system (Eq. 4), the increasing k_H values obtained with increasing hydroxypropyl cellulose may occur primarily through higher solubility of the drug, C_s, in the more hydrophilic matrix. Alternatively, in terms of the granular matrix system (Eq. 5), increased porosity, ϵ, and decreased tortuosity, τ, would accompany the greater hydration of films containing increasing ratios of hydroxypropyl cellulose.

When the release rate constants of the different drugs were compared, after adjustment for drug concentration, methapyrilene and salicylic acid were found to give similar results. This may be attributed to their similar high solubilities of greater than 600 mg/ml in pH 7.0 buffer. Pentobarbital, with a solubility of only about 3 mg/ml at pH 7.0, gave much lower concentration-adjusted k_H values in comparative polymer systems. However, when release from several pentobarbital films was studied in 1 *N* ammonium hydroxide, where its solubility is comparable to methapyrilene and salicylic acid at pH 7.0, the relative rate constants were quite similar to those obtained with these latter two drugs in pH 7.0 buffer. Although solubility in the release medium appears to be an important parameter of the k_H value, it did not quantitatively fit a linear k_H *versus* $C_s^{1/2}$ relationship which might be expected from Eq. 5. The use of the undissociated drug forms in the films could cause the effective value of C_s at the dispersed drug–solvent interface to be substantially less than the drug solubility in the buffer.

Drug Concentration—The effect of drug concentration on the release rate constant was tested using eight different methapyrilene concentrations, from 4.39 to 43.9%, in a pure hydroxypropyl cellulose matrix. The results showed a linear relationship (correlation coefficient = 0.997) between k_H and methapyrilene concentration (Fig. 5). Similar relationships were observed in other systems where only two or three different concentrations were tested. This implies that the fraction of drug released from a given film composition is independent of drug concentration.

This relationship is difficult to explain in terms of Eq. 4, where k_H might be expected to be linear with the square root of drug concentration. However, it may be rationalized on the basis of drug concentration effect on D and C_s through film hydration. A more plausible explanation may be made on the basis of Eq. 5 for a granular matrix system. By assuming all matrix porosity necessary for the diffusion pathway is due to that vacated by the dispersed drug, ϵ may be equated to A. Assuming also that $2A \gg \epsilon C_s$, Eq. 5 may be simplified to:

$$k_H = \left(\frac{DC_s}{\tau}\right)^{1/2} A \qquad \text{(Eq. 10)}$$

Since D, C_s, and τ should be constants for this system, the release rate constant would be proportional to the drug concentration, the relationship obtained experimentally. Huguchi (12) hypothesized just such a concentration relationship in his development of Eq. 2.

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Microultrafiltration Technique for Drug–Protein Binding Determination in Plasma

VINOD P. SHAH, SYLVIA M. WALLACE [*], and SIDNEY RIEGELMAN [x]

Abstract □ A rapid microultrafiltration technique for determining plasma protein binding of drugs in small volumes of plasma was developed. The reliability of the method was tested by determining protein binding of acetazolamide, diphenylhydantoin, and salicylic acid in undiluted human plasma. The results are compared to literature values.

Keyphrases □ Drug–protein binding determination—microultrafiltration technique, plasma □ Plasma protein binding—microultrafiltration technique □ Protein binding, plasma—microultrafiltration technique □ Ultrafiltration, micro—technique for drug–protein binding determination in plasma

Plasma protein binding of drugs can affect their distribution, elimination, and thus the intensity and duration of their pharmacological action. The drug binding characteristics of plasma proteins may be altered in disease states by changes in the concentration of plasma proteins and by structural changes in the protein molecules themselves. Such structural perturbations may influence the affinity of drug–protein binding and/or the number of binding sites available on the protein molecule. Since the fraction of drug bound is frequently a function of drug concentration, determination of binding at a single concentration is not sufficient. Unfortunately, existing methods require relatively large volumes of plasma (up to 30 ml for measurements at six drug concentrations) and prolonged equilibration. Yet, one is limited by the volume of blood that may be obtained from a single patient. Therefore, a method is needed that allows characterization of an individual patient's drug–protein binding capacity using only a small volume of blood. This paper reports a simple, rapid, ultrafiltration method capable of defining the binding characteristics of a drug in sufficiently small volumes of plasma to make the method practicable for use with patients. The validity of the method was tested using salicylic acid, diphenylhydantoin, and acetazolamide in undiluted human plasma at 37°.

EXPERIMENTAL

Materials—Acetazolamide[1], diphenylhydantoin[2], and salicylic acid[3] were used as obtained from standard sources without further purification. ^{14}C-Diphenylhydantoin[4] (54 mg/mCi) and ^{14}C-salicylic acid[4] (28.7 mg/mCi) were appropriately diluted with nonradioactive drug to yield counts at least four to five times greater than background in the plasma water samples.

Microultrafiltration membranes were cut to size (13 and 25 mm diameter circles) from ultrafiltration membrane cones[5].

Apparatus—The binding of salicylic acid, diphenylhydantoin, and acetazolamide to plasma proteins was determined in small volumes (0.4 ml) of undiluted human plasma by a modified ultrafiltration technique. A circular membrane (13 mm in diameter) cut from ultrafiltration cones was secured inside an adapter[6]. A disposable needle was attached to the lower end of the adapter to deliver the filtrate to the glass tube below, and the whole assembly was inserted in a plastic tube (Fig. 1).

Procedure—Plasma (0.4 ml) containing known concentrations of drug (eight to 10 concentrations for each drug) was equilibrated for 2 hr at 37°. After a 20-μl aliquot was removed for analysis of total drug, the remaining plasma was introduced into the reservoir above the membrane using a Pasteur pipet. Centrifugation[7] of the apparatus for 10 min at 2200 rpm yielded 25–30 μl of plasma water in the lower glass tube (less than 10% of the total plasma volume). A 20-μl aliquot of plasma water was analyzed for free drug concentration. If the sensitivity of the analytical procedure requires a larger sample of plasma water, an adapter[8] may be used. This adapter accommodates approximately 1.5 ml of plasma and yields 125 μl of plasma water. The extent of drug–membrane binding was assessed using the same apparatus by substituting water for plasma. The protein concentration in plasma water determined by the method of Lowry *et al.* (1) was between 0.25 and 0.75% of the total plasma proteins.

Diphenylhydantoin and Salicylic Acid Radioisotope Measurements—Aliquots (20 μl) of whole plasma and plasma water obtained by microultrafiltration were added to 10.0 ml of scintillator solution[9], and the radioactivity was determined in a liquid scin-

[1] Sigma Chemical Co., St. Louis, Mo.
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[3] Aldrich Chemical Co., San Leandro, Calif.
[4] New England Nuclear, Boston, Mass.
[5] Centriflo, 2100 CF-50, Amicon Corp., Lexington, Mass.
[6] Millipore Swinnex 13, Millipore Filter Corp., Bedford, Mass.
[7] International centrifuge model UV, International Equipment Co., Needham, Mass.
[8] Swinnex-25.
[9] Aquasol, New England Nuclear, Boston, Mass.

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[5] Centriflo, 2100 CF-50, Amicon Corp., Lexington, Mass.
[6] Millipore Swinnex 13, Millipore Filter Corp., Bedford, Mass.
[7] International centrifuge model UV, International Equipment Co., Needham, Mass.
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[9] Aquasol, New England Nuclear, Boston, Mass.

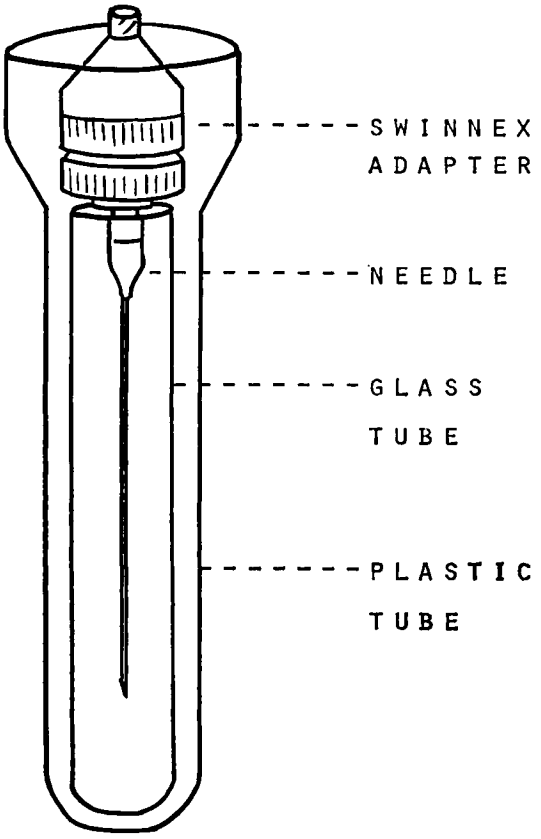

Figure 1—*Microultrafiltration assembly.*

tillation spectrometer[10] using an external standard ratio method for quench correction.

Salicylic Acid Fluorescence Measurements—Aliquots (20 μl) of plasma water were added to 5 ml of 0.1 *M* phosphate buffer (pH 7), and the concentration of salicylate was determined from the fluorescence measured[11] at excitation and emission wavelengths of 300 and 400 nm, respectively (2).

Acetazolamide GLC Measurements—Acetazolamide was determined in plasma and plasma water by GLC using a ^{63}Ni-electron capture detector[12]. Samples were processed and chromatographed on a glass column packed with 3% OV-17 on Gas Chrom Q[13], using fluoranthene as the internal standard. The details of the methods will be published subsequently.

RESULTS AND DISCUSSION

Protein binding of diphenylhydantoin, acetazolamide, and salicylic acid was studied over the therapeutic range of plasma concentrations. While a relatively constant percentage of diphenylhydantoin was bound to plasma protein, the percentage of both salicylic acid and acetazolamide bound decreased with increasing concentration (Fig. 2). A small, but constant, percent (6.8 ± 1.8%) of diphenylhydantoin was bound to the membrane. No corrections are made for this binding in the calculations. Salicylic acid and acetazolamide showed no significant membrane binding.

It is generally found that drugs that bind to plasma proteins interact primarily with albumin. Reynolds and Cluff (3) showed that salicylate binds solely to plasma albumin, and diphenylhydantoin is bound primarily to plasma albumin (4). Insufficient data are available to characterize the binding of acetazolamide to plasma proteins. In our studies, the binding parameters, k and n, were calculated assuming that the drug binds only to albumin.

[10] Packard Tri-Carb model 3375 liquid scintillation spectrometer, Packard Instrument Co., Downers Grove, Ill.

[11] Perkin-Elmer MPF-2A fluorescence spectrometer, Perkin-Elmer Corp., Norwalk, Conn.

[12] Varian series 1200 Aerograph, Walnut Creek, Calif.

[13] Applied Science Laboratories, Inc., State College, Pa.

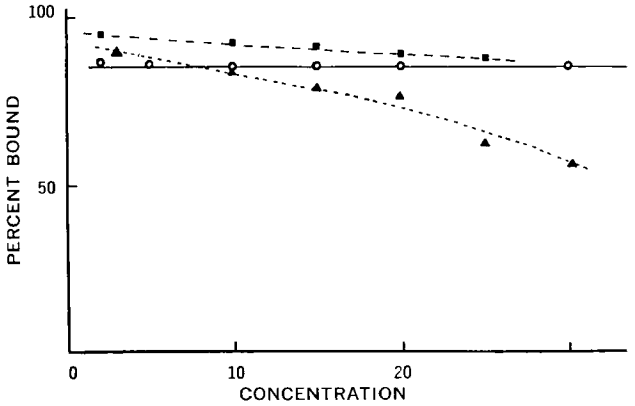

Figure 2—*Percentage of drug bound to plasma protein as a function of concentration for acetazolamide (μg/ml, ■), diphenylhydantoin (μg/ml, ●), and salicylic acid (μg/0.1 ml, ▲).*

Binding of acetazolamide and salicylic acid to plasma protein results in a curvilinear Scatchard plot (5) (Figs. 3–5), indicating that more than one class of binding sites exists. Binding constants were calculated using a nonlinear regression computer[14] program[15] on the assumption that two classes of binding sites are involved, *i.e.*:

$$r = \frac{n_1 k_1 D_f}{1 + k_1 D_f} + \frac{n_2 k_2 D_f}{1 + k_2 D_f} \qquad \text{(Eq. 1)}$$

where:

r = moles of drug bound per mole of protein (albumin concentration, 5×10^{-4} *M*)

n_1, n_2 = number of independent binding sites of Class I and Class II, respectively

k_1, k_2 = intrinsic association constant for binding to sites in Class I and Class II, respectively

D_f = concentration of free drug, *i.e.*, concentration of drug in plasma water

The binding constant (k_1) determined for salicylic acid in two different plasma samples using fluorometric and radioactive analysis is of the same order of magnitude as that reported in the litera-

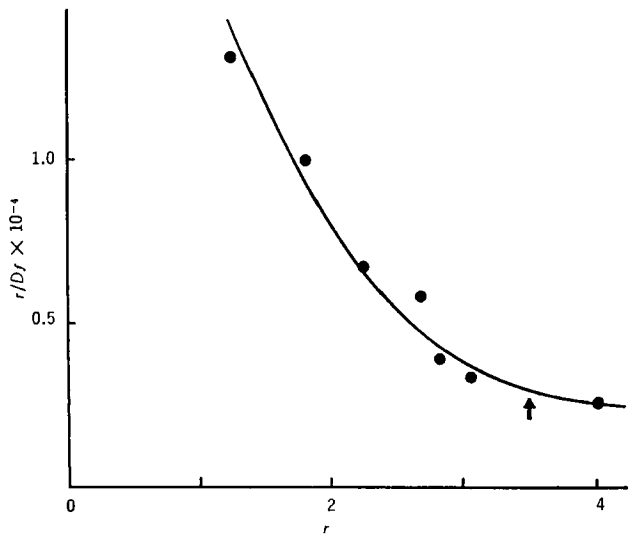

Figure 3—*Scatchard plot of the binding of ^{14}C-salicylic acid to plasma protein at 37°. (Arrow indicates a total drug concentration of 400 μg/ml.)*

[14] IBM 360-50 digital computer.

[15] Listing of Dr. M. C. Meyer's program obtained through Mr. S. J. A. Kazmi, University of British Columbia, Canada.

Table I—Binding Constants for the Interaction of Acetazolamide, Salicylic Acid, and Diphenylhydantoin with Undiluted Human Plasma

Drug	Concentration Range, μg/ml	Method[a]	Fraction Bound	Binding Constants[b]			
				k_1, liters/mole	n_1	k_2, liters/mole	n_2
Salicylic acid	25–500	Experimental: MUF, 37°, SPF, ^{14}C	0.9–0.5	1.19×10^4	1.99	1.38	6.82×10^2
			0.9–0.5	0.95×10^4	2.51	1.55	6.75×10^2
	130–370	Lit. (6): ED, 4°	0.80–0.45	0.54×10^4[c]	1.40	—	—
Diphenylhydantoin	1–40	Experimental: MUF, 37°	0.86–0.83	—	—	—	—
	2–11	Lit. (6): ED, 4°	0.86	—	—	—	—
	5–300	Lit. (7): UF, 23–26°	0.91–0.92	—	—	—	—
	7–80	Lit. (8): ED, 4°	0.95–0.92	—	—	—	—
Acetazolamide	2–30	Experimental: MUF, 37°	0.95–0.88	4.91×10^5	0.098	1.31×10^2	69
	11–66	Lit. (9): ED, room temperature	0.95–0.84	—	—	—	—

[a] MUF = microultrafiltration, SPF = spectrophotofluorometry, ED = equilibrium dialysis, and UF = ultrafiltration. [b] The computer program as presently available gives an estimate of the binding constant without providing detailed statistical analysis of the error space. [c] Reciprocal of the dissociation constant reported.

ture (Table I). The variation in binding constants calculated from fluorometric (spectrophotofluorometric)and radioactive (^{14}C) determinations (Table I) may be attributed to variations in plasma samples rather than assay techniques. Kudla *et al.* (6) reported a *single* binding constant of 0.54×10^4, while our data result in a k_1 value of 1.19×10^4 and 0.95×10^4 with k_2 values of 1.38 and 1.55. Although in the lower range of therapeutic concentrations the secondary binding sites may only contribute a small proportion to the total binding, they are, nonetheless, significant.

If binding is assessed only over a limited range of drug concentrations, such secondary binding sites may be overlooked and the affinity of binding at primary sites may be underestimated. Thus, to determine binding parameters accurately, it may be necessary to measure binding over a wider range of concentrations than is encountered therapeutically. For example, data obtained for salicylic acid at concentrations below 400 μg/ml (left of the arrow in Figs. 3 and 4) can be adequately described by a linear Scatchard plot, thus suggesting a single class of binding sites. However, the binding constant will differ from that determined over a wider concentration range where the curvature of the Scatchard plot becomes apparent.

Results were compared only to those studies using undiluted human plasma. Significantly different binding constants result from studies with albumin from different species and at different concentrations. For example, association constants (k_1 and k_2) for the interaction of salicylic acid with 0.4% bovine serum albumin were reported (10) as 20.0×10^4 and 1.75×10^3; with 0.3% crystalline human albumin, k_1 and k_2 were 7.07×10^4 and 3.3×10^3, respectively (11). On the other hand, the affinity constant determined by Kudla *et al.* (6) in undiluted human plasma, *i.e.*, at an albumin concentration of approximately 4%, was 0.54×10^4.

Literature data for plasma protein binding of acetazolamide are incomplete. Although Travis *et al.* (9) reported the fraction of acetazolamide bound to plasma protein, they did not calculate the binding constants. The fraction of the drug bound to plasma protein as determined by microultrafiltration compares favorably with the data reported in the literature. Affinity constants (k_1 and k_2) calculated from the curvilinear Scatchard plots (Fig. 5) were 4.91×10^5 and 1.31×10^2.

Since the fraction of diphenylhydantoin bound to plasma protein largely remains unchanged over the therapeutic concentration range (1–40 μg/ml), binding is characterized by the average fraction bound (0.85) rather than an association constant. Similarly, Kudla *et al.* (6) found the fraction bound to be 0.86 in the concentration range of 2–11 μg/ml. Lunde *et al.* (7), studying protein binding over a wider range (5–300 μg/ml), found that the fraction bound ranged from 0.91 to 0.82.

Plasma protein binding determined by microultrafiltration is

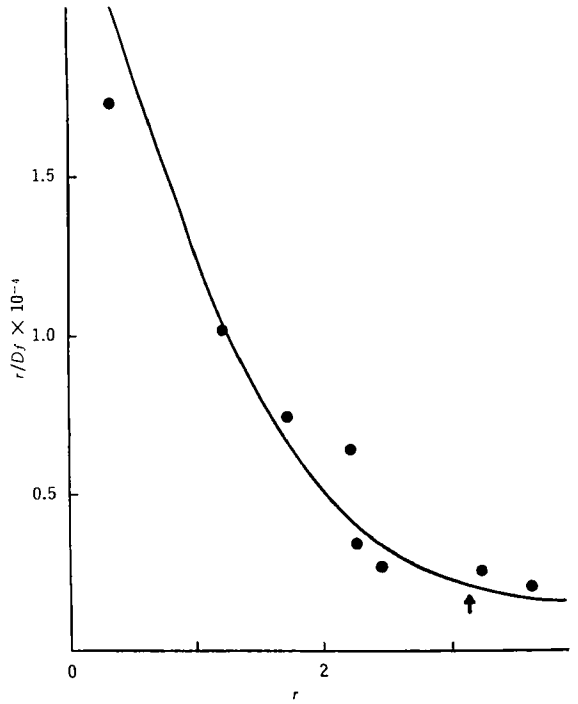

Figure 4—*Scatchard plot of the binding of salicylic acid (determined spectrophotometrically) to plasma protein at 37°.*

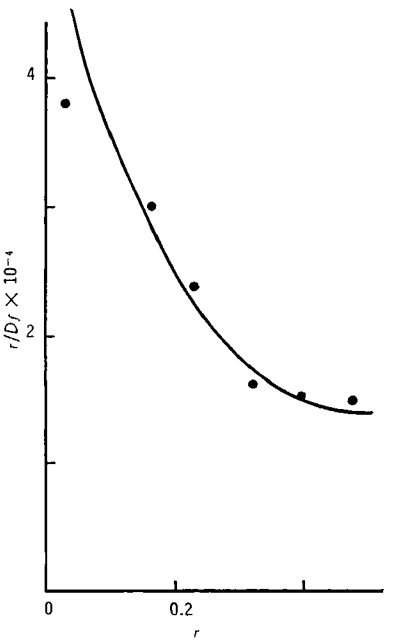

Figure 5—*Scatchard plot of the binding of acetazolamide to plasma protein at 37°.*

comparable to that determined by standard equilibrium dialysis or ultrafiltration techniques. The microultrafiltration technique is rapid and requires a total of only 4 ml of plasma for binding determinations at 10 drug concentrations. The technique, however, does require a sufficiently sensitive analytical method to detect the amount of free drug in 20–100 μl of plasma water.

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Effect of Aspirin on Biotransformation of ^{14}C-Acetaminophen in Rats

BARRY H. THOMASˣ, WALTER ZEITZ, and BLAKE B. COLDWELL

Abstract □ Aspirin (210 mg/kg po) administered to rats concomitantly with ^{14}C-acetaminophen (150 mg/kg po or 75 mg/kg iv) caused: *(a)* a reduction in the rate but not in the extent of acetaminophen absorption from the GI tract; *(b)* an enhanced blood level of radioactivity during the postabsorptive phase, irrespective of the route of administration of acetaminophen; *(c)* large changes in the proportions of acetaminophen metabolites and acetaminophen excreted in the urine; *(d)* a reduction in sulfate conjugation; and *(e)* an increase in glucuronide and mercapturate conjugation. These effects were not significantly altered by caffeine or codeine.

Keyphrases □ Aspirin—effect on acetaminophen absorption, rat □ Biotransformations, rat—acetaminophen, acetaminophen–aspirin interaction □ Acetaminophen—effect of aspirin on absorption and biotransformation, rat

Aspirin has been shown to alter the metabolism of phenacetin (1) and salicylamide (2) in humans, but two studies using conventional estimation procedures failed to detect any effect on the metabolism of acetaminophen (3, 4). The acetaminophen metabolites estimated by these authors were the glucuronide and sulfate conjugates.

Since acetaminophen is used in some analgesic formulations that include aspirin, it was considered desirable to investigate any possible effect on acetaminophen metabolism. The use of ^{14}C-ring-labeled acetaminophen in the rat permitted the estimation of a metabolite not included in the earlier studies in humans. The dose used was chosen to represent abusive use in humans. In an unpublished 200-day chronic toxicity study in rats, a mixture of aspirin, acetaminophen, caffeine, and codeine at the dose used in the present study produced some renal pathology but did not affect weight gain. The LD_{50} for acetaminophen in the rat is 3.7 g/kg (5) and for aspirin it is 1.48 g/kg (6). Based on data of Clark *et al.* (7), it would appear that a 25-g dose of acetaminophen in humans (approximately 0.36 g/kg) is lethal in about 50% of the cases. In view of the much lower toxicity of acetaminophen in rats compared to humans, it was considered reasonable to use a dose near the lethal dose in humans but well below the lethal dose in rats.

EXPERIMENTAL

^{14}C-Acetaminophen (*N*-acetyl-*p*-aminophenol-ring-UL-^{14}C), specific activity 17.24 μCi/mg, was custom synthesized[1]. Aspirin[2], caffeine citrate[3], codeine phosphate[4], and unlabeled acetaminophen[5] were obtained from commercial sources.

Male Wistar rats, 200–250 g, were deprived of food but not of water for 16 hr prior to dosing. In the oral dosing experiments, the rats were randomly divided into three treatment groups of six rats per group. Group 1 received a mixture of aspirin (210 mg/kg), acetaminophen (150 mg/kg), caffeine citrate (30 mg/kg), and codeine phosphate (7.5 mg/kg) in 0.25% gum tragacanth (Mixture II). Group 2 received a mixture of aspirin (210 mg/kg) and acetaminophen (150 mg/kg) in 0.25% gum tragacanth (Mixture I). Group 3 received acetaminophen (150 mg/kg) in 0.25% gum tragacanth. All three treatments were given orally as single doses in a volume of 10 ml/kg. The dose of ^{14}C-acetaminophen was 25 μCi/kg. The formu-

[1] Mallinckrodt, St. Louis, MO 63160. UL = uniformly labeled.
[2] J. T. Baker Chemical Co., Phillipsburg, NJ 08865
[3] K & K Laboratories Inc., Plainview, NY 11803
[4] B. D. H. Canada Ltd., Toronto, Canada.
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Table I—Blood Concentration of Radioactivity, Expressed as Micrograms of Acetaminophen per Milliliter of Blood, in Groups of Six Rats Dosed Orally with ^{14}C-Acetaminophen (150 mg/kg)[a]

Hours	Acetaminophen	Treatments[b] Mixture I	Mixture II
0.25	98.49 ± 6.03	77.78 ± 5.65 $p < 0.05$	45.30 ± 3.07 $p < 0.001$
0.5	133.18 ± 3.71	110.73 ± 3.52 $p < 0.005$	79.76 ± 2.66 $p < 0.001$
1.0	106.99 ± 4.10	95.14 ± 3.14 $p < 0.05$	82.30 ± 4.34 $p < 0.005$
2.0	51.44 ± 2.93	54.69 ± 2.83 N.S.	56.04 ± 4.45 N.S.
4.0	9.75 ± 1.43	21.94 ± 1.35 $p < 0.001$	25.42 ± 2.59 $p < 0.001$
6.0	6.19 ± 0.62	14.81 ± 2.19 $p < 0.005$	8.80 ± 1.65 N.S.
12.0	4.82 ± 0.28	8.82 ± 0.55 $p < 0.001$	5.68 ± 0.78 N.S.
24.0	2.11 ± 0.09	2.81 ± 0.29 $p < 0.05$	2.39 ± 0.39 N.S.

[a] Values are means ± *SE*, and the *p* values represent the probability when compared with the control group (acetaminophen). [b] Mixture I = aspirin and acetaminophen; and Mixture II = aspirin, acetaminophen, caffeine, and codeine.

lation of Mixture II corresponds to popular phenacetin-containing analgesic tablets sold over-the-counter in Canada, with acetaminophen being substituted for phenacetin.

Duplicate blood samples (10 μl) were collected from the tail at 0.25, 0.50, 1, 2, 4, 6, 12, and 24 hr after dosing. The blood was digested and counted in a liquid scintillation counter as previously described (8). Urine was also collected at 12 and 24 hr after dosing and estimated for radioactivity (8). Some urines were incubated overnight at 37° with an equal volume of β-glucuronidase[6] or with β-glucuronidase–sulfatase[7]. When the mixture was used, 0.5 ml of 0.2 *M* acetate buffer (pH 5.0) was added to 0.5 ml of urine followed by 50 μl of β-glucuronidase–sulfatase.

When acetaminophen was administered intravenously, two treatment groups were used. Group 1 received an oral dose of aspirin (210 mg/kg) in 0.25% gum tragacanth, and Group 2 received a similar dose of the vehicle alone. After 30 min, both groups were

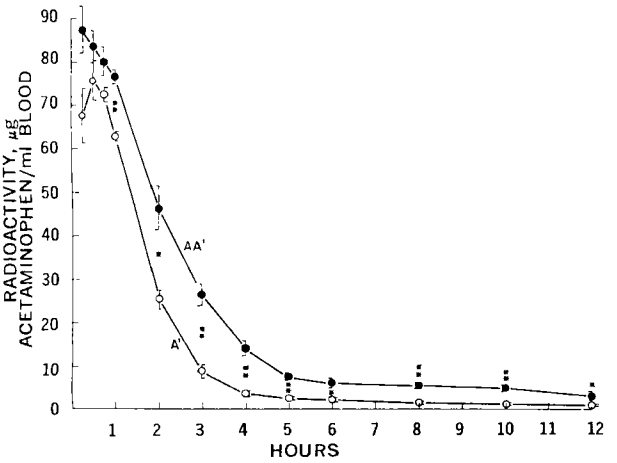

Figure 1—*Blood concentration of radioactivity expressed as micrograms of acetaminophen per milliliter of blood. Mixture I is aspirin and acetaminophen. Values are means* ± SE *from four rats per group dosed intravenously with* ^{14}*C-acetaminophen (75 mg/kg). Key:* ○, *acetaminophen alone;* ●, *Mixture I;* *, p < *0.05; and* *, p < *0.005.*

[6] Ketodase, Warner-Chilcott, Morris Plains, N.J.
[7] Glusulase, Endo Laboratories, Inc., Garden City, NY 11530

Table II—Effect of β-Glucuronidase–Sulfatase on the Paper Chromatography of 0–12-hr Urine from Single Rats Dosed Orally with Either Acetaminophen or Mixture I

Drug Treatment[a]	Enzyme Treatment	Chromatography Peaks[b], % I	II	III	IV
Acetaminophen	—	22.5	7.9	64.1	5.5
	β-Glucuronidase	1.7	8.4	64.0	26.0
	β-Glucuronidase–sulfatase	2.9	7.9	0	89.3
Mixture I	—	32.2	16.1	38.3	13.4
	β-Glucuronidase	3.2	16.4	37.4	42.9
	β-Glucuronidase–sulfatase	4.0	16.4	0	79.6

[a] Abbreviations as in Table I. [b] I = glucuronide, II = mercapturate?, III = sulfate, and IV = unchanged drug.

injected in the saphenous vein with ^{14}C-acetaminophen (75 mg/kg, 25 μCi/kg) in a volume of 1.0 ml/kg of 50% ethanol under light ether anesthesia. Duplicate blood samples (10 μl) were collected from the tail at 0.25, 0.50, 0.75, 1, 2, 3, 4, 5, 6, 8, 10, and 12 hr after the dose of acetaminophen and estimated for radioactivity. Urine was also collected up to 12 hr.

Metabolites of acetaminophen in urine were determined using the alkaline paper chromatography system described by Shahidi (9, System C). Urine (10–25 μl) was applied as a band to strips of chromatography paper[8]. Radioactivity on the developed chromatograms was detected by serial sectioning into 1-cm strips and counting in a liquid scintillation counter. A few of the urines were also chromatographed on a resin column[9] using a scaled down version of that described by Jagenburg *et al.* (10). The column dimensions were: diameter, 0.9 cm; height, 45 cm; and total bed volume, 30 ml.

RESULTS

The blood concentrations of radioactivity in the three treatment groups given ^{14}C-acetaminophen by the oral route are shown in Table I. The two groups given drug combinations had lower blood levels during the 1st hr than the group given acetaminophen alone. This effect was greater with Mixture II than with Mixture I. At 4 hr the effect was reversed with higher blood levels in the drug combination groups. The group that received Mixture I comtinued to have significantly higher blood levels than the acetaminophen

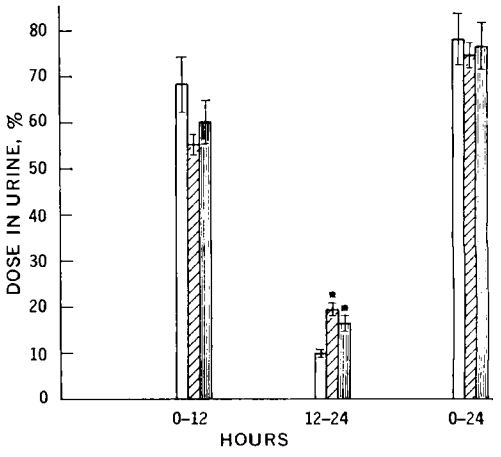

Figure 2—*Urinary excretion of radioactivity in rats dosed orally with* ^{14}*C-acetaminophen (150 mg/kg). Mixture II is aspirin, acetaminophen, caffeine, and codeine; and Mixture I is aspirin and acetaminophen. Values are means* ± SE *from six rats per group. Key:* □, *acetaminophen alone;* ▨, *Mixture I;* ▥, *Mixture II; and* *, p < *0.05 compared to acetaminophen alone.*

[8] Whatman No. 1.
[9] Sephadex gel G10.

Table III—Urinary Excretion of Acetaminophen and Its Metabolites in Rats Dosed Orally with ^{14}C-Acetaminophen (150 mg/kg)[a]

Hours	Metabolite	Acetaminophen	Treatments[b] Mixture I	$p<$	Mixture II	$p<$
0–12	Glucuronide	21.13 ± 0.97	34.60 ± 1.91	0.001	29.35 ± 1.61	0.005
	Mercapturate	7.46 ± 0.23	16.60 ± 0.64	0.001	17.22 ± 0.91	0.001
	Sulfate	64.27 ± 1.37	34.56 ± 1.94	0.001	40.02 ± 1.97	0.001
	Unchanged, acetaminophen	7.14 ± 0.77	14.24 ± 1.25	0.001	13.41 ± 1.75	0.01
12–24	Glucuronide	12.29 ± 0.72	16.92 ± 1.66	0.05	16.81 ± 1.24	0.02
	Mercapturate	5.07 ± 0.42	14.36 ± 1.25	0.001	9.77 ± 0.75	0.001
	Sulfate	80.04 ± 0.94	64.45 ± 1.84	0.001	67.03 ± 2.24	0.001
	Unchanged, acetaminophen	2.60 ± 0.59	4.27 ± 1.14	N.S.	6.38 ± 3.08	N.S.

[a] The proportion of each metabolite is expressed as a percentage. Values are means ± *SE*, and the *p* value represents the probability when compared with the control group (acetaminophen). [b] Abbreviations as in Table I.

alone group throughout the 4–12-hr period studied. To avoid complications due to interaction between the drugs during absorption from the GI tract, acetaminophen was given intravenously. The blood profiles shown in Fig. 1 indicate that aspirin does increase the blood level of radioactivity. When these data were plotted on semilogarithmic graph paper, it was found that elimination fell into two phases in both groups. There was rapid elimination up to 4 hr followed by a slower rate from 5 to 12 hr. The effect of aspirin was most apparent during the first phase (half-life: acetaminophen alone, 0.72 hr; Mixture I, 1.13 hr).

The total urinary excretion of radioactivity in the three treatment groups given oral acetaminophen is shown in Fig. 2. During the 0–12-hr period, there was no significant differences; but during the 12–24-hr period, there was a significant increase in excretion in the Mixture I and Mixture II groups compared to the controls. This effect could be due to a slower rate of absorption and/or a reduced elimination rate in the initial time period. The cumulative 24-hr excretion of radioactivity was high in all groups, indicating that absorption was essentially complete.

Paper chromatoraphy of the urines separated the radioactivity into four distinct peaks. Treatment of some urines with β-glucuronidase–sulfatase gave results such as those shown in Table II. β-Glucuronidase reduced only peak I and proportionately increased peak IV, which has the R_f of authentic acetaminophen. This indicates that peak I is largely acetaminophen glucuronide. When sulfatase was present, peak III was also eliminated; therefore, this peak is acetaminophen sulfate. Peak II was unaffected by either enzyme and its identity remains uncertain. In terms of R_f, it corresponds with that reported (10) for acetaminophen mercapturate. It also gives the color tests reported (10). The percentage of the dose excreted as unchanged drug and the three metabolites is shown in Fig. 3. The data in Table III show the proportions of each metabolite excreted in urine. There were some major shifts in metabolism due to the presence of the other drugs, but aspirin is apparently the major cause since there is little difference between the Mixture I and Mixture II groups.

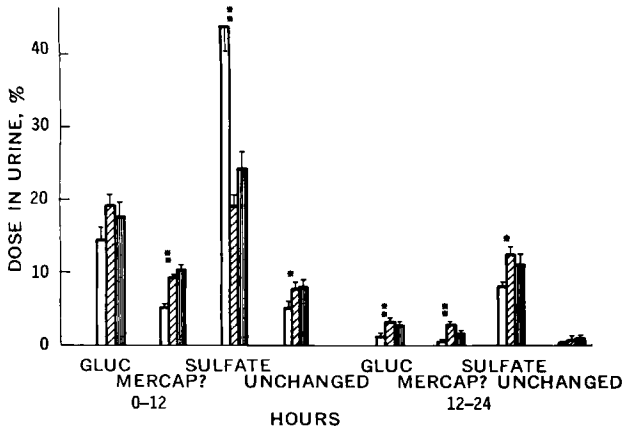

Figure 3—*Urinary excretion of acetaminophen and its metabolites in rats dosed orally with ^{14}C-acetaminophen (150 mg/kg). Mixture II is aspirin, acetaminophen, caffeine, and codeine; and Mixture I is aspirin and acetaminophen. Gluc represents the glucuronide conjugate and mercap is the mercapturate conjugate. Values are means ± SE from six rats per group. Key: □, acetaminophen alone; ▨, Mixture I; ■, Mixture II; *, p < 0.05 (compared to acetaminophen alone); and **, p < 0.005 (compared to acetaminophen alone).*

The paper chromatography data obtained from the rats dosed intravenously with acetaminophen are shown in Fig. 4. The results were similar to those obtained after oral administration except that acetaminophen glucuronide was significantly higher in the aspirin-treated rats. This experiment confirmed that the metabolic changes observed in urine were due to an effect of aspirin on metabolism and not to a slower rate of absorption.

Column chromatography confirmed the values for free acetaminophen obtained by paper chromatoraphy. A small amount of a metabolite described by Jagenburg and Toczko (11) as a cysteine conjugate was detected, but it constituted less than 0.2% of the dose.

DISCUSSION

The combination of acetaminophen with aspirin, caffeine, and codeine appeared to reduce the rate of acetaminophen absorption. Aspirin alone had a similar but less marked effect. The evidence for this was the slower rise in the blood concentration of radioactivity in the Mixture I and Mixture II groups compared to acetaminophen alone. Despite the slower rate, the total absorption was not diminished since there was a high recovery of radioactivity in the 24-hr urines from all three treatments.

During the postabsorptive phase, the blood level of radioactivity tended to be higher in the groups that received the drug combinations, particularly the Mixture I group. When acetaminophen was given intravenously, the same effect on the blood level was seen. It is deduced from these data that aspirin reduced the elimination rate of acetaminophen during the first few hours after dosing.

Over the urine collection periods studied, the other drugs had

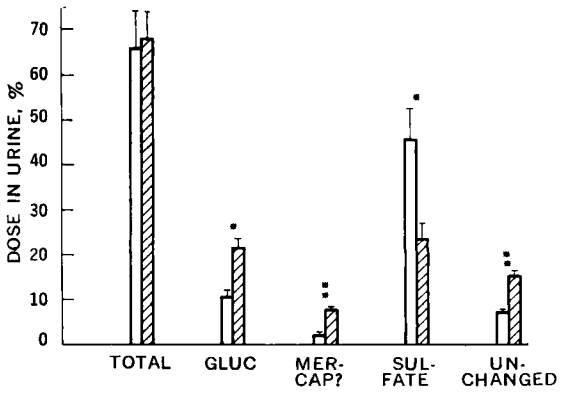

Figure 4—*Paper chromatography data obtained from rats dosed intravenously with acetaminophen. Key: □, acetaminophen alone; ▨, Mixture I; *, p < 0.05 (compared to acetaminophen alone); and **, p < 0.005 (compared to acetaminophen alone).*

only a slight effect on the total renal excretion of radioactivity. When given orally, there was an increase in the 12–24-hr period excretion in the Mixture I and Mixture II groups, but this could have been due to delayed absorption. It is possible that there was reduced excretion during the first few hours which was not apparent by 12 hr when the urine was collected.

The most significant effect of aspirin was on the proportions of the major metabolites of acetaminophen excreted in urine. This effect was seen whether acetaminophen was given orally or intravenously. The principal change was the reduction in both the amount and the proportion of acetaminophen sulfate. The increased proportions of free acetaminophen and the glucuronide and mercapturate metabolites could well be consequences of diminished sulfate conjugation. The reason for this effect of aspirin is not immediately apparent, since salicylate is not known to be conjugated with sulfate; therefore, competition for the available sulfate was not possible. However, Boström *et al.* (12) showed that salicylate inhibits the biosynthesis of mucopolysaccharide sulfates and the excretion of ester sulfates in urine. These investigators suggested that the known oxidative uncoupling action of salicylate reduced the supply of adenosine triphosphate and, hence, active sulfate (3′-phospho-5′-adenosinephosphosulfate), which is necessary for sulfate conjugation. It is possible, therefore, that the reduced amount of acetaminophen sulfate could be due to less active sulfate being available in the aspirin-treated rats.

Levy and Regardh (3) and Amsel and Davison (4) did not see any changes in acetaminophen sulfate in human urine when doses of aspirin in the high therapeutic range were given. This could be due to sulfate conjugation being quantitatively less important in humans than in rats or to the doses being less than abusive. The increased excretion by the aspirin-treated rats of a metabolite assumed to be acetaminophen mercapturate could be toxicologically important since this type of metabolite usually arises from a highly reactive precursor. In humans, both the mercapturate (10) and its immediate precursor, the cysteine conjugate (11), are found in urine after administration of normal doses of acetaminophen. In the rat, very little of the cysteine conjugate is found. If the greater excretion of unchanged acetaminophen in urine represents higher levels in the body, the duration of action of acetaminophen will be longer when aspirin is also administered.

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Abstract □ The field desorption mass spectra of azathioprine, I, and some of its metabolites, II–IV, were investigated and compared to their electron impact spectra. The field desorption spectra were shown to be especially well suited for the determination of molecular weights in drug metabolism studies of drugs conjugated with amino acids and peptides. This technique was applied to a metabolite of azathioprine isolated from rat urine and proved to be useful.

Keyphrases □ Azathioprine and metabolites—field desorption mass spectra, compared to electron impact spectra, application to molecular weight determinations □ Mass spectroscopy—comparison of field desorption and electron impact spectra, azathioprine and metabolites, application to molecular weight determinations □ Field desorption mass spectra—azathioprine and metabolites, compared to electron impact spectra, application to molecular weight determinations

Azathioprine[1] (I), [(1-methyl-4-nitroimidazol-5-yl)thio]purine, is the most widely used immunosuppressive agent in clinical organ transplantation. The metabolism of I has been the subject of extensive studies (1–7). When the metabolic fate of the methylnitroimidazole moiety of ^{14}C-azathioprine, labeled in the imidazole ring, was investigated (3, 5, 7), evidence was found that nucleophilic attack occurred on the nitromethylimidazole ring *in vivo*, leading to the formation of 5-substituted derivatives. In recent studies in the rat (3, 6), the following metabolites of azathioprine were characterized: 1-methyl-4-nitro-5-(*S*-glutathionyl)imidazole (II), 1-methyl-4-nitro-5-(*N*-acetyl-*S*-cysteinyl)imidazole (III), and 1-methyl-4-nitro-5-carboxymethylaminoimidazole (IV).

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[1] Imuran, Burroughs Wellcome Co.

only a slight effect on the total renal excretion of radioactivity. When given orally, there was an increase in the 12–24-hr period excretion in the Mixture I and Mixture II groups, but this could have been due to delayed absorption. It is possible that there was reduced excretion during the first few hours which was not apparent by 12 hr when the urine was collected.

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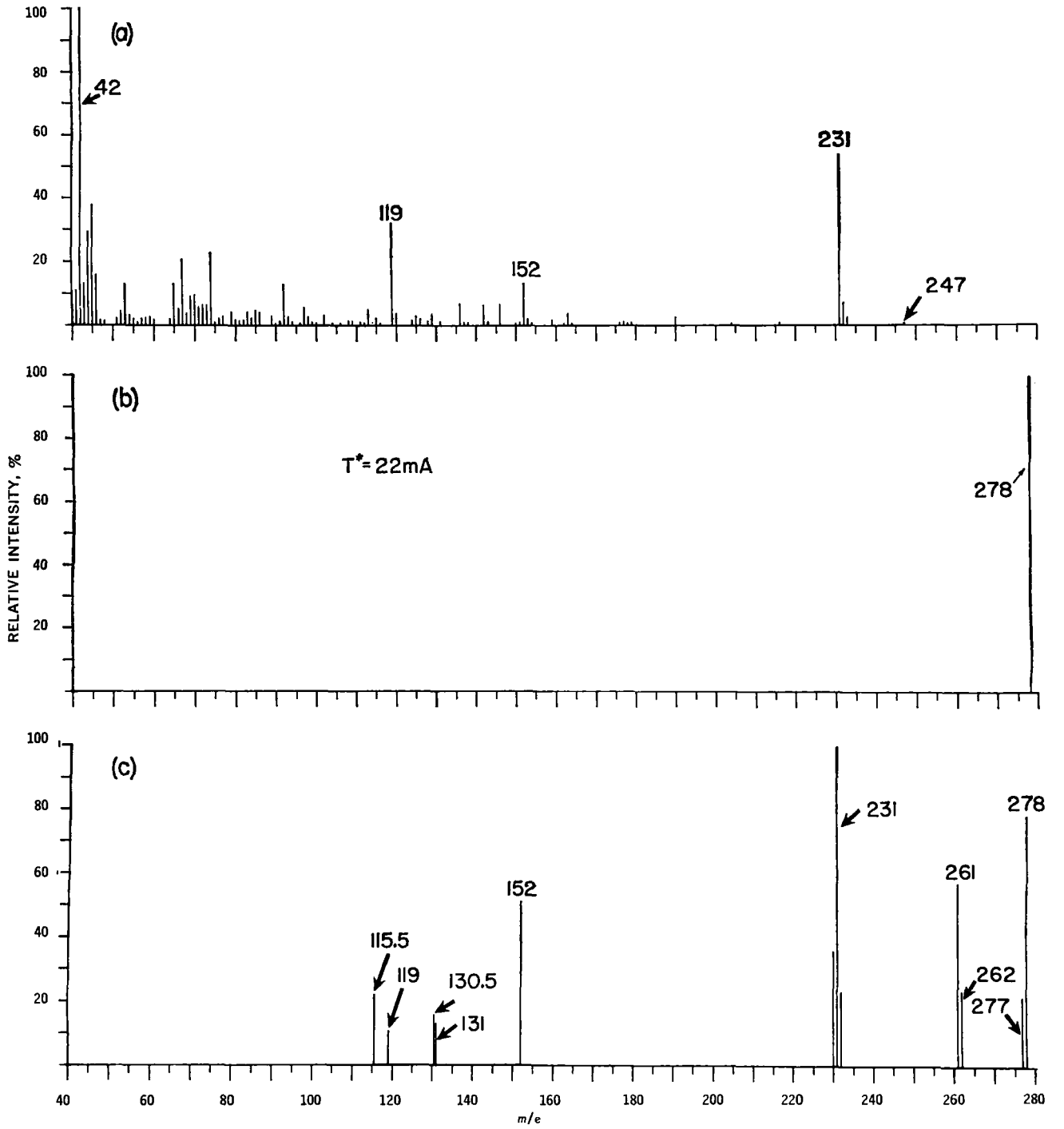


Figure 1—*Mass spectra of azathioprine (I). (a) Electron impact, using a direct probe at a temperature of 210°. (b) Field desorption, using 22 mamp emitter heating current. (c) Field desorption, using 25 mamp emitter heating current.*

tify II–IV were liquid chromatography and UV spectroscopy. Conventional electron impact mass spectrometry was of limited value in the study, because the resulting spectra had small to nonexistent molecular ions and weak high mass fragments. Therefore, molecular weights and/or elemental compositions of these metabolites were difficult or impossible to obtain by electron impact mass spectrometry.

Recent advances in the field of mass spectrometry have yielded techniques that allow the determination of the molecular weight of highly polar compounds whose volatility and/or thermal stability are limited. One such technique is field desorption mass spectrometry (8). Both low- (9–12) and high- (13, 14) resolution spectra can be obtained from polar compounds without derivatization. The observation that unprotected amino acids and oligopeptides exhibit high molecular ion intensities with field desorption mass spectrometry (9, 10) prompted the exploration of this method for the study of metabolites that are conjugates of drugs with amino acids and oligopeptides.

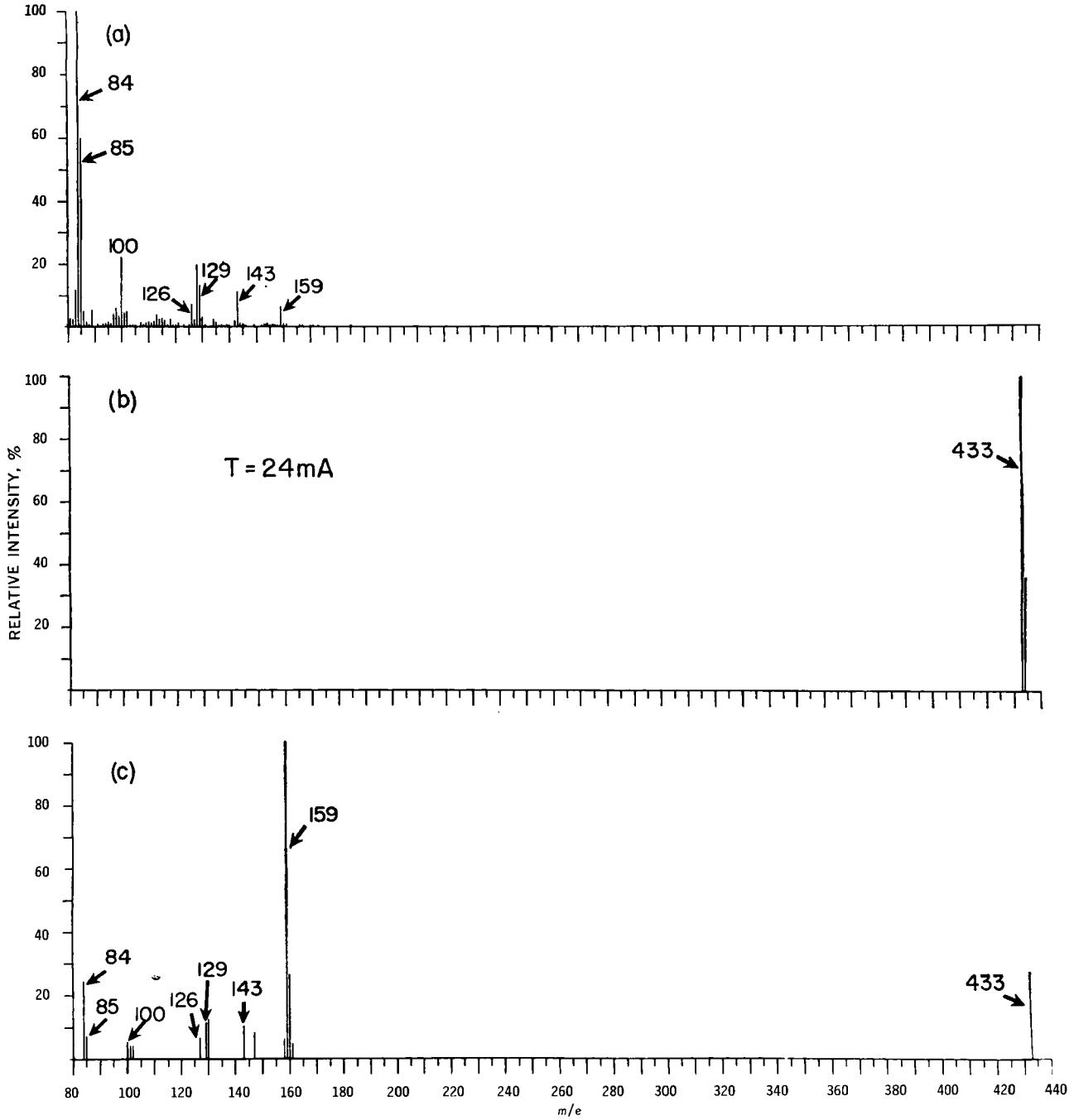


Figure 2—*Mass spectra of II. (a) Electron impact, using a direct probe at a temperature of 195°. (b) Field desorption, using 24 mamp emitter heating current. (c) Field desorption, using 27 mamp emitter heating current.*

EXPERIMENTAL

Compounds I–IV were synthesized[2] (3, 6).

The high-resolution field desorption mass spectra were recorded on a modified double-focusing mass spectrometer[3] equipped with an emitter micromanipulator (14), using vacuum evaporated silver bromide plates[4] for photographic detection. The emitters were 10-μm tungsten wires activated at high temperature (15). The average length of the carbon microneedles produced was about 25 μm. These emitters are of high chemical and mechanical stability; they offer a large adsorption surface and yield outstanding ionization efficiency. The accelerating voltages were +10 kv for the field anode and −2 kv for the slotted cathode plate. The observed total emission measured at the slotted cathode plate was in the range of 10^{-7} amp when optimal emitter heating current was reached. The samples were processed using the emitter dipping technique (8). All samples were adsorbed onto the emitter from aqueous solutions, except I which was applied as a suspension in acetone. The sample consumption was in the order of 5×10^{-8} g of adsorbed material on the emitter surface. For precise mass measurements the photoplates were evaluated by a comparator in connection with a computer[5]. The resolution exceeded 15,000 (10% valley definition) for all spectra. The error in the mass determination was 10 ppm on the average.

[2] At the Wellcome Research Laboratories.

[3] A CEC21-110B double-focusing mass spectrometer was used for accurate mass measurements.

[4] Ionomet, Burlington, Mass.

[5] PDP 8/E.

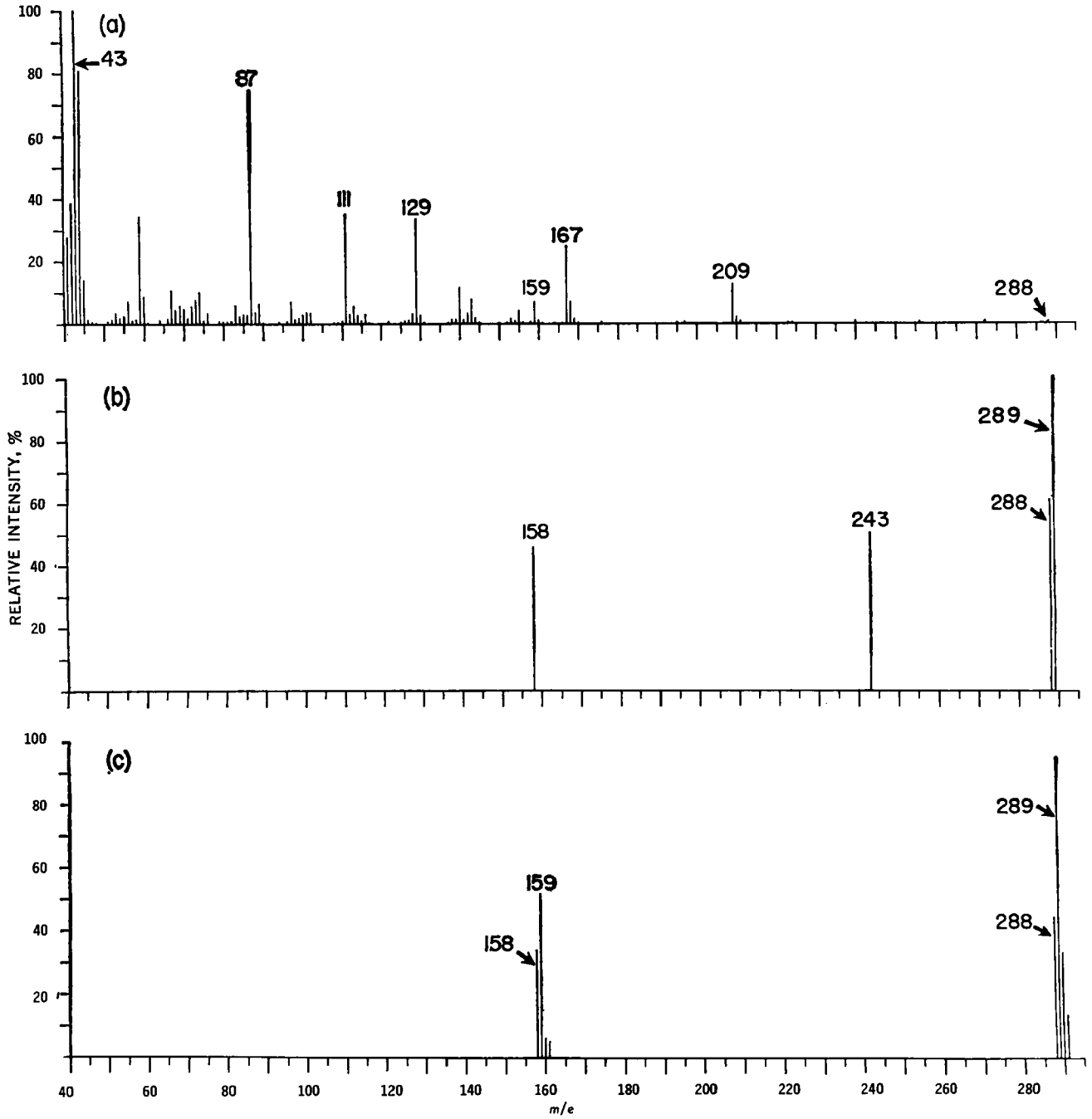


Figure 3—*Mass spectra of III. (a) Electron impact, using a direct probe at a temperature of 150°. (b) Field desorption, using 19 mamp emitter heating current. (c) Field desorption, using 24 mamp emitter heating current.*

The low- and high-resolution electron impact mass spectra were obtained on a second double-focusing mass spectrometer[6] equipped with a data system[6]. Accurate mass measurements were taken by peak matching at 10,000 resolution (10% valley definition). Low-resolution field desorption mass spectra were run on the same system using a source constructed as reported (16). The emitters were also used in the low-resolution work. Accelerating voltages of +3 kv for the field anode and −5 kv for the slotted cathode were applied. A distance of 2 mm was set between the anode and cathode. The secondary electron voltage of the electron multiplier was adjusted to 3 kv. The multiplier used had a gain of 10^6 at 1.7 kv. A scan speed of 2 sec/decade and a computer sampling rate of 6 kHz were selected.

The computer was calibrated by utilizing a previously obtained electron impact calibration and adjusting it to a field ionized spectrum of perfluorotributylamine (V) taken with the field desorption–field ionized source[7]. The field ionized spectrum of V was run at a source pressure of 5×10^{-5} torr and scanned immediately after the emitter was heated, using a current of 50 mamp, and cooled. Under these conditions, 11–22 peaks could be recorded and were used for mass calibration.

RESULTS AND DISCUSSION

Compound I exhibits no M‡ in the electron impact spectrum (Fig. 1a). A small peak at *m/e* 247 ($C_9H_7N_6OS$) represents the loss

[6] Varian Mat CH5DF mass spectrometer equipped with the spectrosystem 100 data system.

[7] The adjustment is accomplished by a program called XCAL contained in the Varian software.

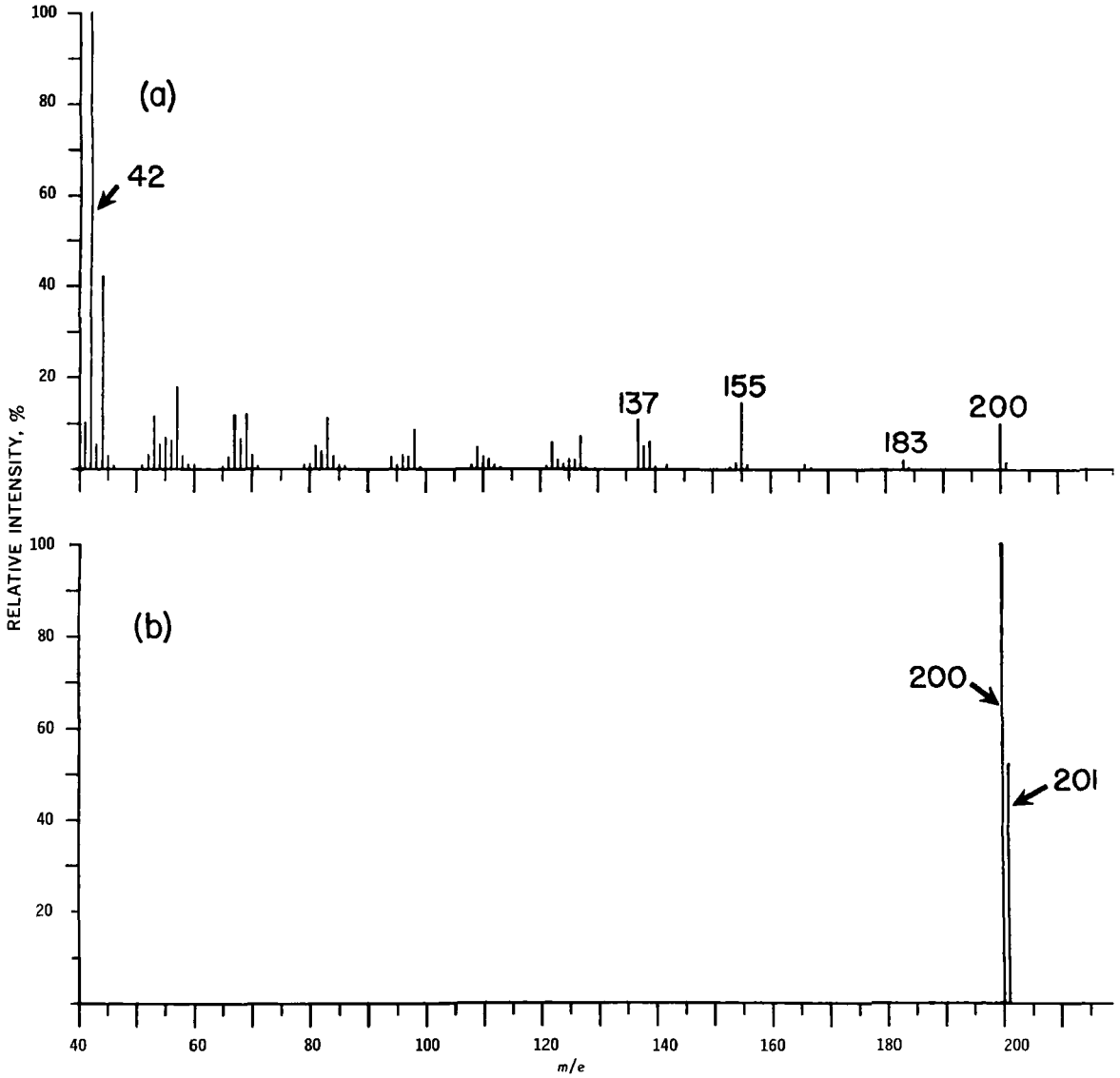


Figure 4—*Mass spectra of IV. (a) Electron impact, using a direct probe at a temperature of 240°. (b) Field desorption, using 26 mamp emitter heating current.*

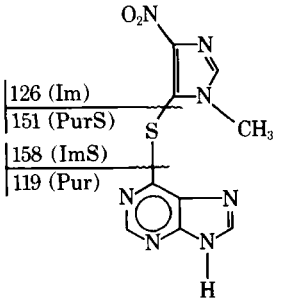
of NO from the molecular ion. The most intense high mass ion, *m/e* 231 ($C_9H_7N_6S$), is formed by the loss of NO_2. Fission of the sulfur–imidazole bond with rearrangement of a hydrogen to the purine moiety, $(PurS + H)^{\ddagger}$, results in an ion isomeric with 6-mercaptopurine at *m/e* 152 ($C_5H_4N_4S$). Cleavage between sulfur and the purine ring with retention of charge on the purine ring, $(Pur)^+$, accounts for the formation of the ion at *m/e* 119 ($C_5H_3N_4$). The base peak of the spectrum is *m/e* 42 (C_2H_4N).

In contrast, the field desorption mass spectrum gives an $(M + 1)^+$ ion [*m/e* 278 ($C_9H_8N_7O_2S$)] at T* (Fig. 1*b*). The T* is the optimal emitter heating current needed to obtain the most intense $M^{\ddagger}$ or $(M + 1)^+$ peaks. When the emitter current is raised (Fig. 1*c*), fragmentation is induced. The field desorption mass spectrum of I at 25 mamp emitter current is: *m/e* 278, 79%, $(M + 1)^+$; *m/e* 277, 22%, $M^{\ddagger}$; *m/e* 262, 24%, $M^{\ddagger} - CH_3$; *m/e* 261, 57%, $M^{\ddagger} - CH_4$; *m/e* 231, 100%, $M^{\ddagger} - NO_2$; *m/e* 152, 51%, $(PurS + H)^{\ddagger}$; *m/e* 130.5, 16%, doubly charged mass 261; *m/e* 131, 13%, doubly charged mass 262; *m/e* 119, 11%, $(Pur)^+$; and *m/e* 115.5, 22%, doubly charged mass 231.

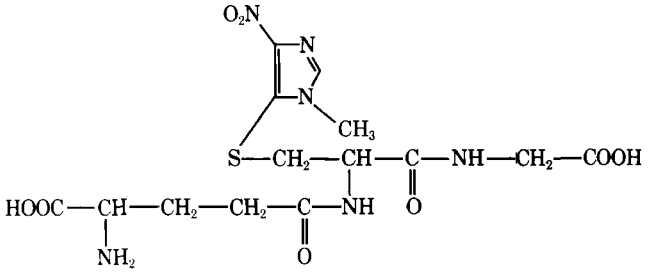
The fragments at *m/e* 231, 152, and 119 are common to both the electron impact and field desorption spectra of azathioprine. While both techniques offer fragments that can aid in characterizing I, the quasimolecular ion is only available in the field desorption spectra.

The electron impact spectrum of II (Fig. 2*a*) results primarily from pyrolytic decomposition. This spectrum is remarkably similar to the low-resolution field desorption spectrum taken at 27 mamp emitter heating current (Fig. 2*c*), with the exception of the molecular ion. The T* for II is 24 mamp (Fig. 2*b*). Under this mild thermal condition, only the $(M + 1)^+$ signal is seen. As the emitter temperature is increased, fragments are generated (17), yielding valuable structural information (Fig. 2*c*). The $(M + 1)^+$ peak is still large (26% relative intensity), and the base peak is now *m/e* 159 [$C_4H_5N_3O_2S$, $(ImS + H)^{\ddagger}$]. Also, $(ImS)^+$, *m/e* 158, and $(ImS + 2H)^+$, *m/e* 160, are present. The high-resolution field desorption mass spectrum has an ion at *m/e* 274 ($C_{10}H_{16}N_3O_6$), which is the loss of (ImS) from the molecule. Thus, the $(M + 1)^+$ and *m/e* 159 and 274 ions characterize this molecule.

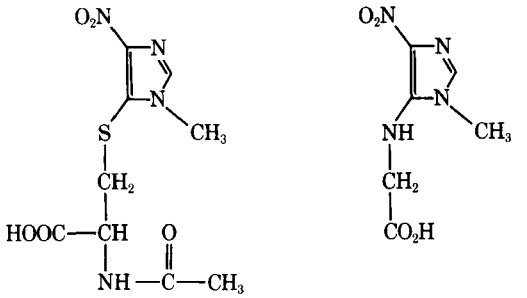
Compound III has a small molecular ion at *m/e* 288 (0.8% relative intensity) with electron impact mass spectrometry (Fig. 3*a*). The ion at *m/e* 209 has an elemental composition of $C_8H_9N_4OS$. Loss of ketene from *m/e* 209 yields an ion at *m/e* 167 ($C_6H_7N_4S$). The relative intensity of the $(ImS + H)^{\ddagger}$ ion is 7%. Loss of NO from the *m/e* 159 ion accounts for a small portion of the *m/e* 129 peak ($C_4H_5N_2OS$). The major portion of the *m/e* 129 peak is accounted for by the $(M^{\ddagger} - ImSH)$ ($C_5H_7NO_3$). The loss of H_2O from the *m/e* 129 ion gives *m/e* 111 ($C_5H_5NO_2$), and the loss of ketene yields an ion at *m/e* 87 ($C_3H_5NO_2$). The base peak of the electron impact mass spectrum is *m/e* 43 ($CH_3—C{\equiv}O^+$). Although the electron impact spectrum offers considerable structural information, the intensities of the high mass ions are small. The field desorption mass spectra of III have intense molecular ions (Figs. 3*b*



I ($C_9H_7N_7O_2S$, mol. wt. 277)



II ($C_{14}H_{20}N_6O_8S$, mol. wt. 432)



III ($C_9H_{12}N_4O_5S$, mol. wt. 288)

IV ($C_6H_8N_4O_4$, mol. wt. 200)

and 3*c*). At 24 mamp emitter current, $(M + 1)^+$, $(ImS)^+$, $(ImS + H)^+$, and $(ImS + 2H)^+$ appear. On the photoplate run, an ion was recorded at *m/e* 577 $(2M + 1)^+$, a common observation of cluster formation with field ionization.

Compound IV is relatively volatile, yielding a complete electron impact spectrum (Fig. 4*a*) with a substantial M‡ at *m/e* 200 (10%). The M‡ − CO_2H peak, *m/e* 155, is 15% as intense as the base peak, *m/e* 42 (C_2H_4N). An (M‡ − OH) ion at *m/e* 183 has a relative intensity of 2%. The ion at *m/e* 137 (11%) has the elemental composition $C_5H_5N_4O$. The field desorption spectrum of IV gave only M‡ and $(M + 1)^+$ ions (Fig. 4*b*). One explanation for the lack of induced fragmentation is that IV volatilizes from the emitter before enough thermal energy is transferred from the emitter to cause fragmentation.

To explore the potential of the field desorption method in its usefulness for the detection of drug metabolites, a sample of III isolated from the urine of rats given an oral dose of azathioprine was examined by field desorption mass spectrometry. Compound III was isolated by column chromatography[8] and purified by paper chromatography (3). Low-resolution field desorption mass spectrometry revealed the molecular weight of this metabolite. In an interval of the emitter heating current between 0 and 24 mamp, only two intense signals were obtained at *m/e* 288 (100% relative intensity) and 289 (40% relative intensity).

After this field desorption measurement was completed, observation of the emitter in a light microscope, 1:100 magnification, showed that the microneedles were still covered with a considerable amount of the sample. Consecutive high-resolution field desorption mass spectrometry with the double-focusing machine indicated that these residues are due to sodium and potassium salts, mainly phosphates, *e.g.*, *m/e* 80.974 $(HPO_3 + H)^+$, 96.969 $(HPO_4 + H)^+$, and 164.931 $(Na_3PO_4 + H)^+$. The photographically recorded spectrum showed Na^+ and $^{39,41}K^+$ peaks with saturated blackening.

In view of the importance of knowing the molecular weight of a metabolite, field desorption mass spectrometry is especially suited to studies of this type. The molecular weights of the compounds studied were easily accessible by field desorption mass spectrometry. In addition, valuable structural information can be obtained from fragments produced by higher emitter temperatures.

[8] The column used was DEAE Sephadex A-25 (acetate).

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ˣ To whom inquiries should be directed.

In Vivo Method for Determining Effectiveness of Spray-on Bandages Containing Anti-Infectives

M. LUONGO, J. J. SCIARRA ˣ, and C. O. WARD

Abstract □ Several topical spray-on bandages were developed and studied to determine a suitable method for evaluating the effectiveness of this group of products. Several formulations were prepared using neomycin sulfate and triclosan. Ethylcellulose and polyamide resins served as the film-forming agents, while tributyl citrate and hexadecyl alcohol were chosen as the plasticizers. Neomycin sulfate and triclosan preparations were evaluated using an *in vivo* method. Standardized, contaminated wounds in guinea pigs served as the testing system. The inflammatory response of the wounds was evaluated using several indexes including skin temperature, degree of induration, and presence of pus. The results indicate that neomycin sulfate and triclosan were released from their formulations. The spray-on bandages reduced the degree of infection about the wound. No difference was noted between the results obtained using either film former.

Keyphrases □ Spray-on bandages containing anti-infectives (neomycin sulfate and triclosan)—determination of *in vivo* effectiveness, guinea pigs □ Anti-infective spray-on bandages (neomycin sulfate and triclosan)—determination of *in vivo* effectiveness, guinea pigs □ Aerosol anti-infectives (neomycin sulfate and triclosan)—determination of *in vivo* effectiveness of spray-on bandages, guinea pigs

In recent years, great interest has developed regarding the use of medicated polymeric films in managing contaminated wounds. The value of using anti-infectives to control infections also has been firmly established over the years. On this basis, it seemed advantageous to formulate a preparation in which an anti-infective compound is released from a polymeric film following application onto the skin. A spray-on bandage formulated in this manner would have many advantages including ease of application, prevention of contamination of the product by the user, and, depending upon the nature of the polymeric film and therapeutic agent, a slow release of the active compound.

Spray-on bandages using polyvinyl alcohol in combination with polyvinyl acetate or acrylic resins and other water-soluble resins were investigated (1), and butyl cyanoacrylate was shown to be an effective chemical compound (2). In one study (3), optimal therapeutic responses were seen in the contaminated soft tissue wounds in rats when an antibiotic was administered to the affected area and covered with a spray dressing of bucrylate (isobutyl cyanoacrylate). Chloramphenicol and nitrofurazone were incorporated into solutions of ethylcellulose and sodium carboxymethylcellulose (4).

The possibility of infections developing after elective surgical procedures has been a recurring problem for both the physician and the patient. An antibiotic triad consisting of polymyxin, bacitracin, and neomycin was developed in the form of an aerosol dressing using fluorinated hydrocarbons as the propellant (5). The value of using aerosol sprays of oxytetracycline and a neomycin sulfate–polymyxin B sulfate product was demonstrated (6). Further work also was done in regard to using an antibiotic aerosol in minor surgery (7). Previous studies (8–10) indicated the use of certain polymeric films for formulating spray-on bandages and showed the release rate of different therapeutic agents from these films.

The purpose of this study was to select the proper combination of film former, plasticizer, and solvent to yield a film that permits drug release. Once the formulation is developed, an *in vivo* study will be conducted to determine the suitability of the method used as well as the relative effectiveness of the formulations.

Table I—Composition of Aerosols

Ingredient, % w/w	Formulation Number 5	5A	23	25
Polyamide resin[a]	—	2.5	—	25
Ethylcellulose	2.5	—	2.5	—
Neomycin sulfate	0.175	0.175	—	—
Triclosan	—	—	1.0	1.0
Tributyl citrate	0.25	0.25	0.25	0.25
Hexadecyl alcohol	0.25	0.25	0.25	0.25
Absolute ethanol	11.825	—	11.0	—
Absolute isopropyl alcohol	—	11.825	—	11.0
Propellant [trichloromonofluoromethane–dichlorodifluoromethane (50:50)]	85	85	85	85

[a] Emerez 1538.

EXPERIMENTAL

The polymers used included ethylcellulose[1] and two polyamide resins[2]. Hexadecyl alcohol and tributyl citrate[3] were used as the plasticizers. The therapeutic agents used included neomycin sulfate and triclosan[4].

Formulation—Formulations were prepared on a weight percent basis using varying combinations and amounts of the film-forming resins, plasticizers, solvents, and drugs. Absolute ethanol served as the solvent system for preparations containing ethylcellulose, while absolute isopropyl alcohol was used as the solvent for the polyamide resins. Heat was used to aid in the dissolution of the film formers.

The solution of film former, plasticizer, and solvent was added to a 202 × 214 internally lined, three-piece, tin-plated aerosol container and then fitted with an aerosol valve[5] and sealed. A propellant mixture of trichloromonofluoromethane and dichlorodifluoromethane (50:50) was added by the pressure method. The container was placed into a water bath at 56° to ensure that no leakage took place. A quick drying film was obtained when the concentrate was 15% (w/w) and the propellant was 85% (w/w) of the total

[1] Ethylcellulose N-10, Hercules Powder Co., Wilmington, Del.
[2] Emerez 1533, 1538, Emery Industries, Cincinnati, Ohio.
[3] Citroflex-4, Chas. Pfizer and Co., New York, N.Y.
[4] Irgasan DP-300, Ciba-Geigy Corp., Ardsley, N.Y.
[5] Precision Valve Corp.; 0.04 × 0.03-cm (0.018 × 0.013-in.) stem with a 0.2-cm (0.80-in.) housing; 0.016 mechanical breakup actuator.

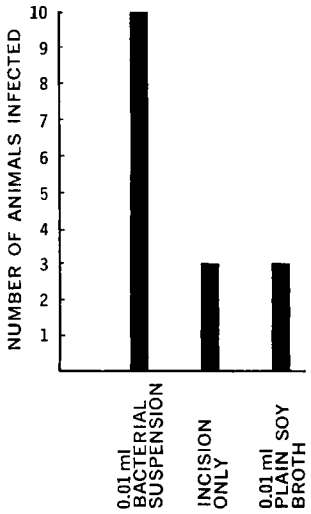

Figure 1—*Bar graph showing* in vivo *testing using controls.*

formulation. Various aerosol formulations were evaluated on the basis of film appearance and flexibility. These were then used for the *in vivo* test procedure. Table I shows the composition of the formulations selected for further study. These formulations gave satisfactory drying characteristics and films.

Delivery Rate—The delivery rate was determined using a suitable apparatus (11) which timed the product discharge. Each container was accurately weighed and then placed into a water bath at 25 ± 1°. After allowing the contents to come to equilibrium (about 1 hr), the container was removed from the water bath, dried, and placed into the discharge rate apparatus. The timer was set for 10 sec and the product was then allowed to be automatically discharged for this period of time. The can was removed from the apparatus and reweighed. Three determinations were made on each container. The discharge rate was determined by calculating the grams per second of product discharged. These results are shown in Table II and include the average of three determinations made on each of two cans of the same formulation.

***In Vivo* Study**—Adult male albino Hartley guinea pigs, weighing between 250 and 350 g, were used. Several hours before testing, the hair from the animal's back was clipped using an electric clipper and then a depilatory[6] was applied. The area was then washed with 70% ethanol. The skin temperature of the guinea pigs was taken at the star' of the test using a temperature probe. The animals were then anesthetized with sodium thiopental, 55 mg/kg body weight, administered intraperitoneally.

One group of animals received two incisions per animal, with each incision being treated with a different formulation; another group received only one incision per animal. Each incision was 3 cm long and parallel to and equidistant from the vertebral column. The incision was made through the skin down to the fascia using a scalpel blade fitted with a guide so that the skin would be cut to the proper depth.

Preparation of Bacterial Culture—*Staphylococcus aureus* (ATCC 12600) was obtained in the form of stable, standardized disks[7]. Each disk is standardized to contain 10^5–10^7 organisms when reconstituted in the proper culture medium. The disk was aseptically removed from the vial and placed into a capped sterile test tube containing 2 ml of sterile trypticase soy broth[8]. The tube was incubated at 35–37° for 30 min. It was then shaken and incubated at this temperature for an additional 4 hr.

Treatment of Animals—Once the bacterial suspension was prepared, the wounds were inoculated with 0.01 ml of the suspension delivered from a micropipet. This amount of suspension would contain approximately 5000–50,000 organisms.

The aerosol formulation was sprayed onto the wounded surface using a back-and-forth motion to cover the entire incision uniformly. The aerosols were sprayed onto the area for 5 sec at a distance of 15.2–20.3 cm (6–8 in.) from the wound surface. The aerosol film served as the only wound dressing. Where two incisions were made per animal, one incision was covered with gauze bandage while the therapeutic agent was being added to the other incision.

[6] Calcium thioglycolate, Nair-Carter Products, N.Y.

[7] Bact-Chek bacterial control cultures, Roche Diagnostics, Division of Hoffmann-La Roche, Inc., Nutley, N.J.

[8] BBL, Division of Becton, Dickinson and Co., Cockeysville, Md.

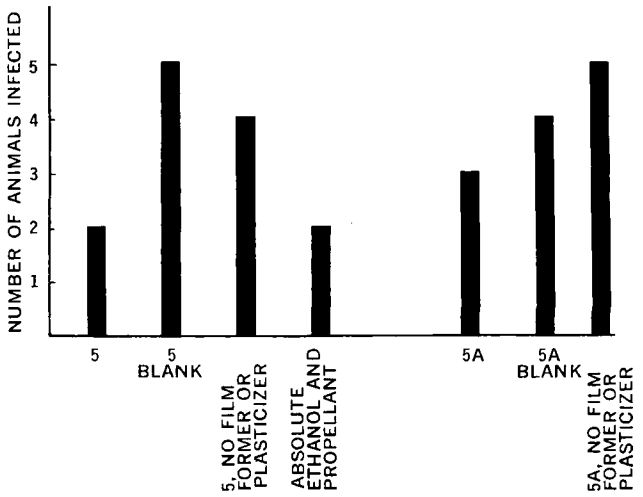

Figure 2—*Comparison of the results obtained from the* in vivo *testing of neomycin sulfate aerosol formulations. Key: 5, neomycin sulfate with ethylcellulose; and 5A, neomycin sulfate with polyamide resin.*

When neomycin sulfate ointment was used, approximately 1 g of the ointment was applied to the wound after it had been inoculated with the bacterial suspension. The wound was then covered with a sterile gauze pad and fastened with micropore tape. This ointment contained 3.5 mg of neomycin base. Neomycin sulfate ointment USP[9] was used in addition to the ointment prepared in the laboratory.

To determine the effect of the inactive ingredients contained in each aerosol preparation, blanks were also prepared containing no active ingredient, film former, or plasticizer. In addition, another blank consisted of alcohol and propellant alone.

Three control groups were included in this study. One group was inoculated with 0.01 ml of bacterial suspension only with no further treatment; another group received the 3-cm incision only, and the last group was treated by inoculating the wound with 0.01 ml of plain trypticase soy broth.

Evaluation of Wound Response—The inflammation of the infected wounds was evaluated in several ways including the deter-

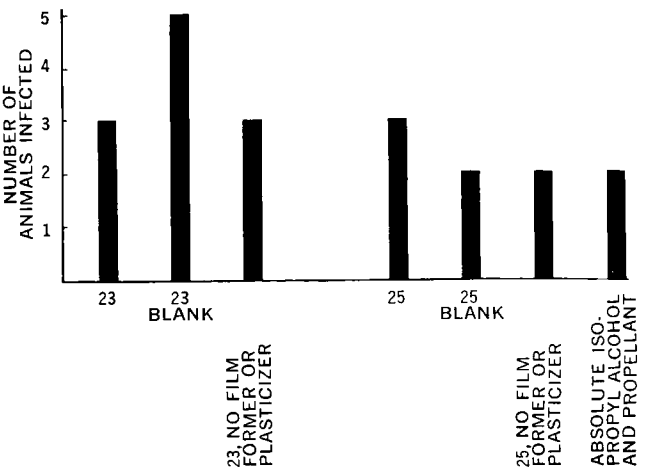

Figure 3—*Comparison of the results obtained from the* in vivo *testing of triclosan aerosol formulations. Key: 23, triclosan with ethylcellulose; and 25, triclosan with polyamide resin.*

[9] Myciguent Ointment, The Upjohn Co., Kalamazoo, Mich.

Table II—Delivery Rate and Active Ingredient Weight of Aerosols Selected for Animal Testing

Formula Number	Delivery Rate, g/sec	Active Ingredient, % in Film	Weight of Total Product/5 sec Spray	Weight of Non-volatile Film Remaining, g	Active Ingredient in Remaining Film, mg
5	0.551	5.512	2.755	0.08748	4.82
5A	0.673	5.512	3.365	0.10684	5.89
23	0.487	25.0	2.435	0.0974	24.35
25	0.474	25.0	2.370	0.0948	23.70

mination of skin temperature of the wounded area by observation of the color change of a thermographic liquid crystal tape[10] applied to the animal's back, induration of wound edges, and the presence or absence of pus. The animals were examined for these responses on the 4th postoperative day.

The skin temperature of the incision area was measured with a thermometer[11]. The liquid crystal tape was applied to the animal's back and allowed to remain there until the color stabilized. The area of induration was determined by palpating the margin of each wound and then measuring the indurated area in millimeters. After these responses were obtained, the wound was opened to detect any evidence of pus. The degree of infection that resulted was classified into one of four categories:

uninfected	no induration
minimal	1 mm induration or less
moderate	1.1–1.5 mm induration
massive	1.6 mm or more induration or presence of pus

The degree of infection caused by each control is shown in Fig. 1. Figure 2 illustrates the results using the formulation containing neomycin, and Fig. 3 shows these results using triclosan. A comparison of the results obtained using these formulations compared to an ointment preparation containing the same active ingredients is shown in Fig. 4.

DISCUSSION

Both ethylcellulose and polyamide films were found to be suitable for use with either neomycin or triclosan. The films were flexible and clear. In addition, when cast upon a substrate of mercury, a continuous film was formed.

Different results were obtained when one incision was made on each animal instead of two incisions. It is probable that there is some absorption of the active ingredient or test agent into the bloodstream. The agent may be transported *via* the circulatory system to the other incision and alter the results. For example, when the spray product containing neomycin sulfate was applied to the left incision on the back of the guinea pig and 0.01 ml of the bacterial suspension was applied to the right incision, only seven out of 10 incisions on the right side became infected while four of the 10 animals developed an infection on the left incision. These responses were different when the study was repeated with only one incision and one agent per animal. In these cases, all 10 animals inoculated with the bacterial suspension became infected and only three of the 10 animals developed an infection when the neomycin spray was used on the incision.

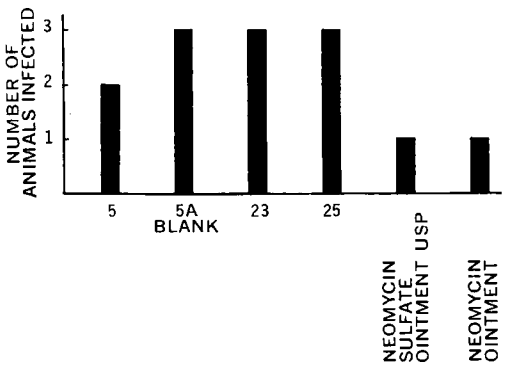

Figure 4—*Effectiveness of aerosol formulations* versus *ointments. Key: 5, neomycin sulfate with ethylcellulose; 5A, neomycin sulfate with polyamide resin; 23, triclosan with ethylcellulose; and 25, triclosan with polyamide resin.*

These results seem to indicate that the bacterial suspension applied to the right incision entered the bloodstream and contaminated the left and/or the aerosol spray applied to the left incision entered the bloodstream to reduce the total number of infected incisions on the animal's right side. Based on this possibility, the *in vivo* tests in this study were conducted using only one incision per animal in an effort to obtain more reliable results. In addition, only one agent was tested per animal. In previously cited studies (12–14), two incisions were made on each animal and then each incision was dealt with in a different way. The results obtained from those studies should be viewed with the possibility of cross-contamination between the two incisions.

As can be seen from Figs. 2–4, neomycin sulfate and triclosan were released from the ethylcellulose and polyamide films. While no spray was 100% effective in eliminating an infection, similar results were obtained with the controls and other substances studied such as ethanol, isopropyl alcohol, and an ointment prepared from neomycin or neomycin sulfate. However, when an infection did develop in the presence of the antibiotic or anti-infective, the degree of the infection was usually less intense, indicating that the drugs were released from the films and that the products did possess some degree of effectiveness. A higher concentration of therapeutic agent might be necessary to control the infection completely. While it was difficult to discern several borderline results, for the most part the method used was effective in differentiating the effects of different formulations.

An attempt was made to correlate the skin temperature on the 1st and 4th postoperative days with the degree of infection, but valid results were not obtained. Several factors interfered with obtaining an accurate skin temperature including ambient room temperature, the state of excitement of the animal, and the basal metabolic state of the animal. The thermographic tape used was obviously of too great a range for the detection of the fairly small temperature changes noted. Further studies are underway to modify this procedure by using a different range of thermographic tape.

Finally, no difference was noted in the results obtained using either the polyamide or ethylcellulose as the film former. The same held true for the plasticizers.

SUMMARY AND CONCLUSIONS

The formulation of a topical antibiotic spray-on bandage was attempted to develop an effective preparation for treating contaminated wounds. Many varied formulations were prepared using neomycin sulfate and triclosan as the anti-infective agents; ethylcellulose and polyamide resins served as the film-forming agents, and tributyl citrate and hexadecyl alcohol were chosen as the plasticizers.

Standardized, contaminated wounds in guinea pigs served as the easily reproducible laboratory *in vivo* testing system. The inflammatory response of the wounds was evaluated using several indexes including skin temperature, area of induration about the incision, presence or absence of pus, and color of the thermographic liquid crystal tape applied to the wounded area.

This method was effective in evaluating the *in vivo* release of therapeutic agents from a film. The results indicate that neomycin sulfate and triclosan were released from their formulations. The spray-on bandages did reduce the degree of infection about the wound. No difference was noted between the results obtained

[10] Hoffmann–La Roche, Inc., Nutley, N.J.
[11] Tele-Thermometer.

using either film former. The skin temperature of the animal and the use of a liquid crystal thermographic tape did not prove to be useful evaluative tools because additional factors may interfere with obtaining a true reading.

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Conformationally Constrained Analogs of Mescaline

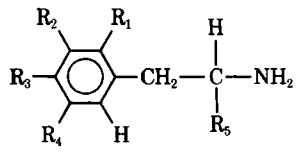
ROBERT J. WOLTERS [x], A. J. BEJ, and N. S. TANNER

Abstract □ The syntheses of 3-(3,4,5-trimethoxyphenyl)piperidine, 2-(3,4,5-trimethoxybenzyl)piperidine, and 2-(3,4,5-trimethoxyphenyl)morpholine are described. In addition, preliminary pharmacological data comparing these compounds with mescaline are given.

Keyphrases □ Mescaline—synthesis of conformationally constrained analogs, pharmacological data □ Hallucinogens—synthesis of conformationally constrained analogs of mescaline, pharmacological data

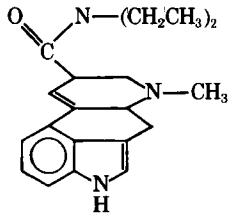
Recent years have seen extensive research in the synthesis of hallucinogens. Most of these compounds have been related to mescaline (I) and methoxyamphetamines (II) rather than to the more complex lysergic acid diethylamide (lysergide, LSD) (III) and tetrahydrocannabinol (IV) analogs (1). The latter two compounds are fairly rigid molecules; however, mescaline's side chain is conformationally mobile.

Most modifications of mescaline have been on the aromatic ring (1–3). However, Walters and Cooper (4) prepared *trans*-2-(3,4,5-trimethoxyphenyl)cyclopropylamine (V) in which the side chain was placed in a cyclopropyl ring. This analog produced mescaline-like activity. Later, Cooper (5) synthesized VI, the *cis*-isomer of V, and Trager and Huitric (6) prepared two related compounds, *cis*- and *trans*-2-(3,4,5-trimethoxyphenyl)cyclohexylamine (VII and VIII) by placing the side chain in a cyclohexyl ring.

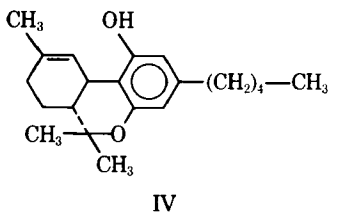


I: $R_1 = R_5 = H$, $R_2 = R_3 = R_4 = OCH_3$

II: R_1, R_2, R_3, R_4 = H or OCH_3, $R_5 = CH_3$



III



IV

The purpose of this investigation was to impose conformational restraint on the aliphatic side chain of mescaline by placing it in various heterocyclic ring systems, namely piperidine and morpholine. This report discusses the synthesis of 3-(3,4,5-trimethoxyphenyl)piperidine (IX), 2-(3,4,5-trimethoxybenzyl)-piperidine (X), and 2-(3,4,5-trimethoxyphenyl)morpholine (XI).

DISCUSSION

The procedure of Telang and Smith (7) was employed to synthesize 3,4,5-trimethoxyphenylacetonitrile (XII). Compound XII was

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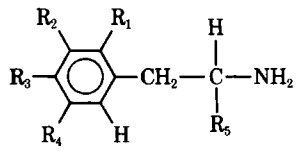

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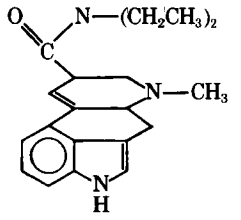
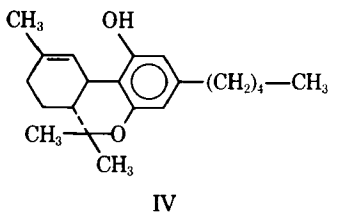


III

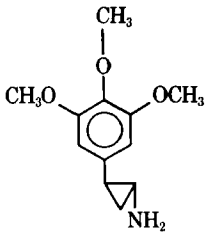


IV

The purpose of this investigation was to impose conformational restraint on the aliphatic side chain of mescaline by placing it in various heterocyclic ring systems, namely piperidine and morpholine. This report discusses the synthesis of 3-(3,4,5-trimethoxyphenyl)piperidine (IX), 2-(3,4,5-trimethoxybenzyl)-piperidine (X), and 2-(3,4,5-trimethoxyphenyl)morpholine (XI).

DISCUSSION

The procedure of Telang and Smith (7) was employed to synthesize 3,4,5-trimethoxyphenylacetonitrile (XII). Compound XII was



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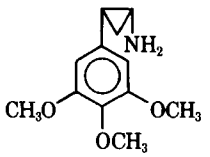
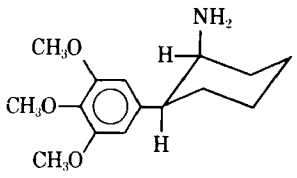
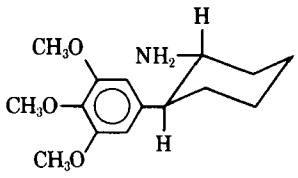


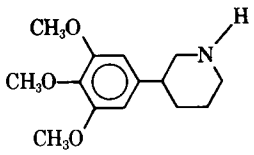
VI



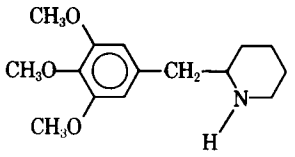
VII



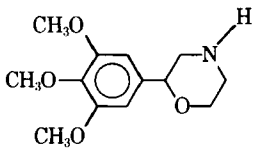
VIII



IX



X



XI

reacted with 3-bromopropionitrile and the resulting dinitrile (XIII) was hydrolyzed with sodium hydroxide solution to form the dicarboxylic acid (XIV). The glutarimide (XV) was synthesized by heating XIV with urea. Reduction of XV with lithium aluminum hydride afforded the desired compound, 3-(3,4,5-trimethoxyphenyl) piperidine (IX) (Scheme I).

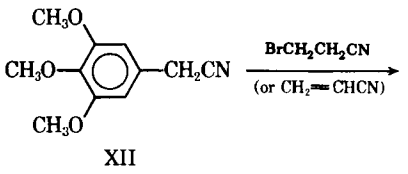
In an alternative procedure, XIII was synthesized *via* the Michael reaction by reacting XII with acrylonitrile in the presence of sodium. The yield by this method was low, 10–15% compared to a 50% yield obtained by the first method.

For the synthesis of 2-(3,4,5-trimethoxybenzyl)piperidine (X), 3,4,5-trimethoxybenzoic acid (XVI) was reacted with 2-pyridyl lithium to yield 3,4,5-trimethoxyphenyl 2-pyridyl ketone (XVII). Compound XVII was reacted with hydrazine to form the hydrazone (XVIII). Then XVIII was reduced by the Wolff–Kishner reaction to yield 2-(3,4,5-trimethoxybenzyl)pyridine (XIX). Catalytic hydrogenation of XIX afforded the desired Compound X (Scheme II).

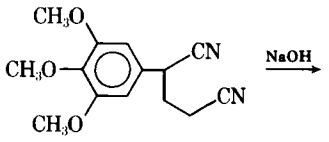
The synthetic route for the morpholine derivative is shown in Scheme III. The starting material, β-hydroxymescaline (XX), was synthesized according to the procedure of Friedman *et al.* (8). Compound XX was reacted with chloroacetyl chloride to form the *N*-acetyl chloride (XXI) followed by cyclization with base, yielding the morpholone (XXII). Reduction of XXII with lithium aluminum hydride or Red Al [sodium dihydro-bis(2-methoxyethoxy)-aluminate] afforded the desired Compound XI.

PHARMACOLOGY

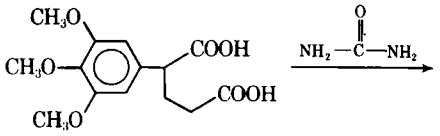
The spontaneous activity of Male Cox (SW) mice, 20–30 g, was determined and recorded by means of activity chambers[1]. Following 3 days of acclimation in a 12-hr light–12-hr dark cycle, the mice were randomly selected for pairing and assignment to an activity chamber (two mice per chamber). Pairing was done with animals from the same cage to avoid interaction in the activity chamber. Saline or the test compounds (50 mg/kg) were randomly assigned and administered intraperitoneally to each group. The animals were placed in the chambers and their activity was measured during 1-min periods at 5, 10, 20, 30, 40, 50, 60, 75, 90, 105, and 120 min following drug administration and was recorded as counts per minute. In addition, amphetamine (5 mg/kg), methylphenidate (10 mg/kg), and mescaline (50 mg/kg) were used as standards for comparison. The data for each time period (treatment *versus* control) were analyzed using the Student *t* test.



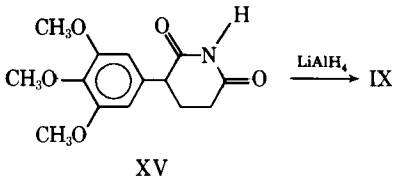
XII



XIII



XIV



XV

Scheme I

The saline-treated mice were recorded with an initial high activity, possibly due to exploratory behavior, after which the activity decreased markedly. The amphetamine and methylphenidate treatments significantly increased activity to a level greater than that of saline or mescaline. The activity of methylphenidate treatment decreased with time more rapidly than did amphetamine. Mescaline was associated with an activity level that was less than that of saline initially and decreased only slightly during the study period.

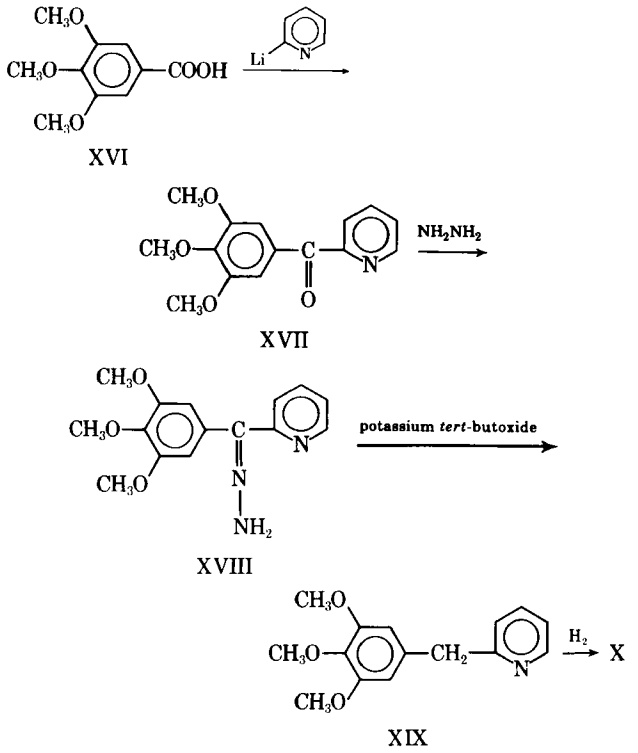
The compounds at the 50-mg/kg dosage induced locomotor activity corresponding to that of saline in the first 40 min. Beyond 40 min the rate of decrease in activity was more gradual than with saline. The tendency toward the mescaline characteristic was XI > X > IX. The stereotyped behavior of mescaline, scratching, was not observed with the test compounds at this dose.

EXPERIMENTAL[2]

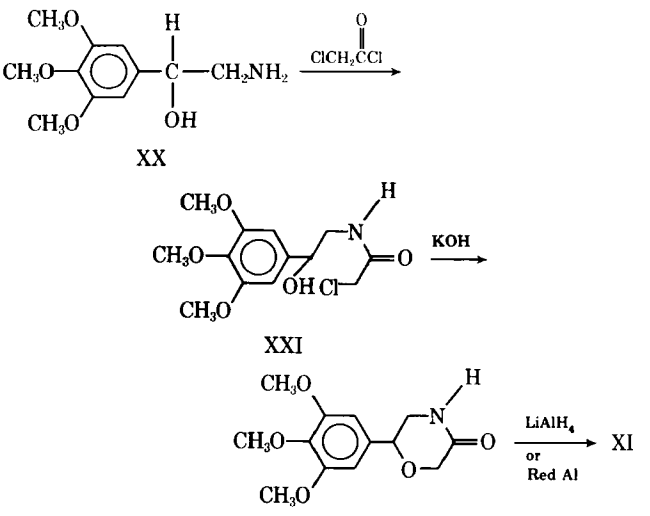
2-(3,4,5-Trimethoxyphenyl)glutaronitrile (XIII)—*Method A*—The procedure of Sugimoto and Kugita (9) was modified in the preparation of XIII. To a flask heated in an oil bath to 110° were added 8.00 g (0.0386 mole) of XII and 0.20 g (0.00087 g-atom) of sodium, and the temperature of the bath was increased to 120°. Subsequently, 1.20 g (0.0226 mole) of acrylonitrile was added dropwise. After the addition was completed, the mixture was allowed to cool, treated with 1 ml of acetic acid, extracted with ethyl acetate, and distilled. The third fraction gave 1.42 g (14%) of prod-

[1] Woodard Research Corp., Herndon, Va.

[2] IR spectra were determined on a Perkin-Elmer model 337 spectrophotometer using potassium bromide pellets. NMR spectra were determined on the Varian model A-60A using tetramethylsilane as the internal standard and deuterochloroform as the solvent in approximately 30% concentration. The letter abbreviations used follow: s = singlet, 2 s = two singlets, t = triplet, m = multiplet, and b = broad. Melting points were determined in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Microanalyses were performed by Alfred Bernhardt Mikroanalytishes Laboratorium, Fritz-Pregel-Strasse, West Germany.



Scheme II



Scheme III

uct, bp 190–200°/0.22 mm. After recrystallization the product had a melting point of 94.5–96°. The IR spectrum showed a peak at 2250 cm^{-1}, indicative of nitriles.

Anal.—Calc. for $C_{14}H_{16}N_2O_3$: C, 64.60; H, 6.20; N, 10.77. Found: C, 64.52; H, 6.34; N, 10.93.

Method B—A solution of 9.00 g (0.043 mole) of XII and 125 ml of anhydrous dimethylformamide was cooled in an ice bath treated with 2.5 g (0.052 mole) of sodium hydride in an oil dispersion. After cessation of hydrogen evolution, 5.85 g (0.0436 mole) of 3-bromopropionitrile was added dropwise. When the addition was completed, the ice bath was removed and the mixture was stirred at room temperature for 15 hr. About 2 liters of water was added to the reaction mixture, and the product was extracted with ether. The ether layer was concentrated and the precipitate was filtered and recrystallized from ether to yield 5.67 g (50%) of product, mp 94–95°. The melting point of a mixture of this product with a sample obtained by Method A was undepressed. The IR spectra were identical.

2-(3,4,5-Trimethoxyphenyl)glutaric Acid (XIV)—The procedure of Elvidge *et al.* (10) was adopted for this preparation. A mixture of 6.09 g (0.0234 mole) of XIII and 12 g of sodium hydroxide in 35 ml of water and 18 ml of ethanol was refluxed for 2.5 hr. After the solution attained room temperature, it was acidified to pH 2 with 10% hydrochloric acid and the precipitate was filtered and air dried to yield 5.4 g (77%) of crude product. On recrystallization from water, the melting point of the product varied between 80 and 90°, depending on the rate of heating. The IR spectrum showed a broad peak from 3600 to 3350 cm^{-1}, indicative of a hydroxyl group, and a peak at 1740 cm^{-1}, indicative of a carbonyl group.

Anal.—Calc. for $C_{14}H_{18}O_7$: C, 56.37; H, 6.08. Found: C, 56.22; H, 6.11.

3-(3,4,5-Trimethoxyphenyl)glutarimide (XV)—The procedure of Degutis and Sukeliene (11) was adopted for this preparation. A flask containing 6.24 g (0.0202 mole) of XIV and 3.0 g (0.64 mole) of urea was placed in an oil bath preheated to 135°. The temperature was raised to 165–170° and, after 1.5 hr, the reaction mixture was poured into 30 ml of 10% sodium bicarbonate solution and heated on a steam bath for 20 min. The solid which separated was filtered and recrystallized from methyl ethyl ketone, yielding 1.99 g (36.6%) of product, mp 185–187°. The IR spectrum showed a peak at 3200 cm^{-1}, indicative of NH, and a doublet at 1700 cm^{-1}, indicative of a carbonyl group. The NMR spectrum showed peaks at δ 2.6 (b, 4H, methylene), 3.6 (s, 1H, CH), 3.8 (s, 9H, methoxy), 6.4 (s, 2H, aromatic), and 8.3 (b, 1H, NH).

Anal.—Calc. for $C_{14}H_{17}NO_5$: C, 60.20; H, 6.14; N, 5.02. Found: C, 60.32; H, 6.29; N, 4.98.

3-(3,4,5-Trimethoxyphenyl)piperidine Hydrochloride (IX)—The procedure of Rice and Scott (12) was adopted for reduction of XV. To a mixture of 125 ml of anhydrous tetrahydrofuran and 3.5 g (0.075 mole) of lithium aluminum hydride was added 2.16 g (0.00774 mole) of XV, and the mixture was refluxed for 4 hr. The excess lithium aluminum hydride was decomposed by the dropwise addition of ice water. The solid which separated was removed by filtration and washed twice with ethyl acetate. The combined filtrate and washings were concentrated *in vacuo*. The oily residue was dissolved in benzene and extracted with 10% hydrochloric acid, followed by neutralization by the addition of sodium carbonate and extraction of the product with benzene. The benzene layer was dried over anhydrous magnesium sulfate and filtered. Dry hydrogen chloride was bubbled into the benzene solution to yield 1.45 g (59.5%) of product. Recrystallization from *n*-propanol gave the product, mp 215–216°. The NMR spectrum of the free base showed peaks at δ 2.1 (b, 8H, methylene), 3.1 (s, 1H, CH), 3.8 (2 s, 9H, methoxy), 4.6 (b, 1H, NH), and 6.3 (s, 2H, aromatic).

Anal.—Calc. for $C_{14}H_{21}NO_3$·HCl: C, 58.43; H, 7.71; N, 4.87. Found: C, 58.26; H, 7.52; N, 4.79.

3,4,5-Trimethoxyphenyl 2-Pyridyl Ketone (XVII)—The procedure of Boykin *et al.* (13) was adopted in the synthesis of XVII. The requisite 2-pyridyl lithium (from a solution of 31.6 g of 2-bromopyridine in 100 ml of anhydrous ether and 3.7 g of lithium) was prepared by the method of Wibaut *et al.* (14). Under nitrogen at −45°, 21.2 g (0.100 mole) of XVI was added to 2-pyridyl lithium along with 200 ml of anhydrous ether. The mixture was stirred for 1 hr and allowed to equilibrate to room temperature. The ketone (XVII) was extracted from the ether layer with 10% hydrochloric acid and neutralized with a 10% sodium hydroxide solution. The precipitate which separated was filtered and air dried to yield 14.6 g (53.5%) of product, mp 108.5–110°, after recrystallization from ether. The IR spectrum showed a peak at 1660 cm^{-1}, indicative of carbonyls.

Anal.—Calc. for $C_{15}H_{15}NO_4$: C, 65.92; H, 5.53; N, 5.13. Found: C, 65.91; H, 5.65; N, 5.31.

3,4,5-Trimethoxyphenyl 2-Pyridyl Ketone Hydrazone (XVIII)—The procedure of Boyer *et al.* (15) was adopted for the synthesis of XVIII. Compound XVIII was prepared in 66% yield with a melting point of 158–160° after recrystallization from ethanol. The IR spectrum showed a doublet at 3380 and 3280 cm^{-1}, attributed to the hydrazone.

Anal.—Calc. for $C_{15}H_{17}N_3O_3$: C, 62.72; H, 5.95; N, 14.62. Found: C, 62.68; H, 6.13; N, 14.79.

2-(3,4,5-Trimethoxybenzyl)pyridine Hydrochloride (XIX)—The Cram *et al.* (16) modification of the Wolff–Kishner reaction was utilized to reduce XVIII. To a solution of 8.0 g (0.073 mole) of

potassium *tert*-butoxide and 50 ml of dimethyl sulfoxide was slowly added, over 10 hr, 8.00 g (0.0278 mole) of XVIII. The solution was allowed to stand overnight. Then 200 ml of benzene was added and the product was extracted with 10% hydrochloric acid, neutralized with 10% sodium hydroxide, and reextracted with benzene. The solvent was removed and the residue was chromatographed on 400 g of a neutral alumina column, 3 cm in diameter. The column was first eluted with 50% petroleum ether–benzene solution until the first band was completely eluted and discarded. The column was then eluted with a 90% benzene–10% chloroform solution to elute XIX. The hydrochloride salt was made in the usual manner and recrystallized from methyl ethyl ketone, yielding 2.09 g (29.1%) of product, mp 250–252°. The NMR spectrum of the free base showed peaks at δ 3.9 (2 s, 9H, methoxy), 5.7 (b, 2H, CH_2), 6.8 (s, 2H, aromatic), 7.7 (b, 2H, pyridine ring), and 8.2 (b, 2H, pyridine ring).

Anal.—Calc. for $C_{15}H_{17}NO_3$·HCl: C, 60.91; H, 6.14; N, 4.74. Found: C, 60.82; H, 6.26; N, 4.35.

2-(3,4,5-Trimethoxybenzyl)piperidine (X)—A mixture of 1.80 g (0.00694 mole) of XIX, 10 ml of acetic acid, and 5 mg of platinum oxide was shaken with hydrogen (initial pressure of 55 psi) for 20 hr. The mixture was filtered, the filtrate was diluted with 50 ml of water, and the cloudy dispersion was extracted with ether. The aqueous phase was neutralized with sodium carbonate and the product was extracted with ether. The hydrochloride salt was made in the usual manner and recrystallized from methanol, yielding 1.81 g (74.3%) of product, mp 250–252°. The base melted at 59–60°. The NMR spectrum showed peaks at δ 2.6 (b, 6H, methylene), 2.9 (b, 4H, methylene), 3.5 (b, 1H, CH), 3.9 (2 s, 9H, methoxy), 4.2 (b, 1H, NH), and 6.4 (s, 2H, aromatic).

Anal.—Calc. for $C_{15}H_{23}NO_3$: C, 67.90; H, 8.73; N, 5.28. Found: C, 67.85; H, 8.59; N, 5.40.

1 - (3,4,5 - Trimethoxyphenyl) - 2 - chloracetamidoethanol) (XXI)—The procedure of Fodor *et al.* (17) was adopted to synthesize XXI. To a solution of 5.00 g (0.0220 mole) of XX [synthesized by the published method (8)] in 125 ml of 1,2-dichloroethane was added a solution of 1.3 g (0.032 mole) of sodium hydroxide in 25 ml of water. The mixture was placed in an ice bath and, after the mixture had equilibrated, 3.5 g (0.031 mole) of chloroacetyl chloride was added dropwise over 1 hr. The ice bath was removed and the mixture was stirred for 3 hr. The layers were separated and the organic phase was washed successively with 20 ml of water, 20 ml of 10% sodium hydroxide, two 20-ml portions of 10% hydrochloric acid, and 20 ml of water. The organic layer was dried over anhydrous sodium sulfate and filtered. The solvent was removed *in vacuo* and the solid residue was recrystallized from benzene, yielding 4.56 g (68.6%) of product, mp 109–110°. The IR spectrum showed peaks at 3450, 3250, and 1650 cm^{-1}, indicative of a hydroxyl proton, an amide proton, and a carbonyl group, respectively. The NMR spectrum showed peaks at δ 3.5 (b, 2H, N—CH_2), 3.9 (2 s, 9H, methoxy), 4.1 (s, 1H, OH), 4.1 (s, 2H, Cl—CH_2), 5.9 (s, 1H, CH), 6.6 (s, 2H, aromatic), and 7.2 (b, 1H, NH).

Anal.—Calc. for $C_{13}H_{18}ClNO_5$: C, 51.41; H, 5.97; N, 4.61. Found: C, 51.41; H, 6.02; N, 4.53.

2-(3,4,5-Trimethoxyphenyl)-5-morpholone (XXII)—To a solution of 3.57 g (0.0118 mole) of XXI in 125 ml of ethanol was added dropwise a solution of 0.90 g (0.017 mole) of potassium hydroxide in 4.5 ml of water. During the addition of potassium hydroxide, the pH of the ethanolic solution was not allowed to exceed 11. The precipitate that separated during the addition was removed by filtration. The solvent was removed *in vacuo* and the residue was recrystallized from methyl ethyl ketone to yield 2.29 g (72.5%) of product, mp 159–160°. The NMR spectrum showed peaks at δ 3.6 (b, 2H, N—CH_2), 3.9 (2 s, 9H, methoxy), 4.4 (s, 2H, O—CH_2), 4.8 (t, 1H, CH), 6.7 (s, 2H, aromatic), and 7.7 (b, 1H, NH).

Anal.—Calc. for $C_{13}H_{17}NO_5$: C, 58.42; H, 6.41; N, 5.24. Found: C, 58.49; H, 6.50; N, 5.29.

2-(3,4,5-Trimethoxyphenyl)morpholine Hydrochloride (XI)—*Method A*—The procedure of Rice and Scott (12), as previously described for the reduction of XV, was adopted for the reduction of XXII using 1.0 g (0.0037 mole) of XXII and 0.50 g (0.013 mole) of lithium aluminum hydride. The product, after recrystallization from methyl ethyl ketone, yielded 0.47 g (43%), mp 216–219°.

Method B—The procedure of Cerny *et al.* (18) was adopted to reduce XXII. A solution of 0.90 g (0.0034 mole) of XXII in 25 ml of anhydrous benzene was added dropwise to 2.5 g (0.012 mole) of Red Al in 25 ml of anhydrous benzene cooled to 0°. After the addition was completed, the flask was allowed to warm to room temperature and the solution was refluxed for 4 hr. The excess Red Al was decomposed with 6 ml of water and the resulting mixture was filtered. The filtrate was extracted with two 15-ml portions of 10% hydrochloric acid, neutralized with 10% sodium hydroxide, and reextracted with ether. The hydrochloride salt of the product was made in the usual manner from the dried ethereal solution, yielding 0.67 g (69%) of product, mp 214–218°. The analytical sample had a melting point of 222.5–224°. The IR spectra of the hydrochloride salt from Methods A and B were identical. The NMR spectrum of the free base showed peaks at δ 3.2 [b, 4H, N—$(CH_2)_2$], 3.9 (2 s, 9H, methoxy), 4.2 (b, 2H, O—CH_2), 4.5 (b, 1H, CH), 5.0 (b, 1H, NH), and 6.7 (s, 2H, aromatic).

Anal.—Calc. for $C_{13}H_{19}NO_4$·HCl: C, 53.89; H, 7.00; N, 4.83. Found: C, 53.92; H, 7.03; N, 4.76.

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[x] To whom inquiries should be directed. Present address: Food and Drug Administration, HFD-110, Rockville, MD 20852

Synthesis and Antiperoxidase Activity of Propylthiouracil Derivatives and Metabolites

RAYMOND H. LINDSAY ˣ, HASSAN Y. ABOUL-ENEIN *, DOMINIQUE MOREL, and STEVE BOWEN

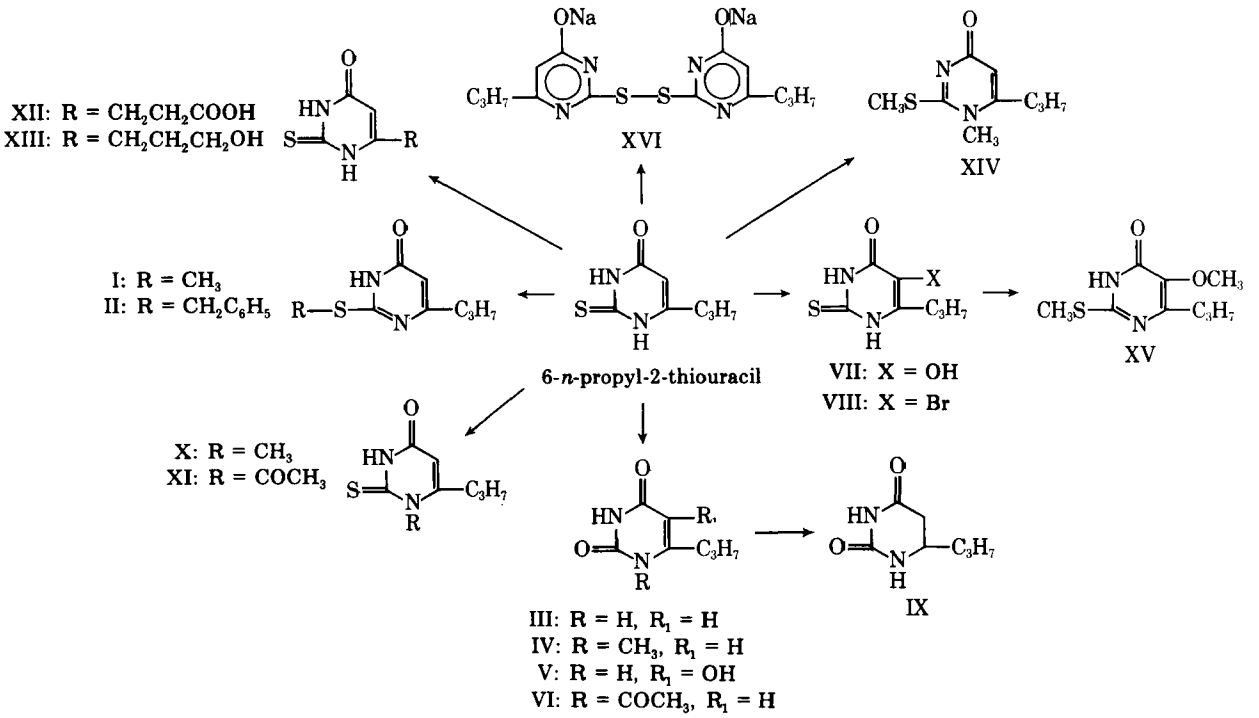
Abstract □ Some metabolites and potential metabolites of the antithyroid drug propylthiouracil (6-*n*-propyl-2-thiouracil) were synthesized and examined for antithyroid peroxidase activity. The compounds positively identified as metabolites of propylthiouracil or implicated in its metabolism were, at the concentrations examined, either completely inactive as thyroid peroxidase inhibitors or only weakly active. Propylthiouracil glucuronide was the most active metabolite, with a potency slightly more than 10% of that of the parent compound. All derivatives in which the sulfur was replaced by oxygen were completely inactive. Assays of propylthiouracil derivatives not implicated in metabolism demonstrated that 1-acetyl-6-*n*-propyl-2-thiouracil and 5-hydroxy-6-*n*-propyl-2-thiouracil were equally as potent as propylthiouracil and that 2-thiouracil-6-propanol and 2-thiouracil-6-propionic acid were weakly active. The data presented demonstrate that biotransformation of propylthiouracil, which occurs primarily at the S group, results in a substantial loss in antiperoxidase activity.

Keyphrases □ Propylthiouracil derivatives and metabolites—synthesis and antiperoxidase activity □ Antithyroid activity—synthesis and evaluation of propylthiouracil derivatives and metabolites □ Antiperoxidase activity—synthesis and evaluation of propylthiouracil derivatives and metabolites □ Biotransformation—propylthiouracil, activity of metabolites

Information relating to the pharmacology of antithyroid drugs was obtained primarily in the 1940's and 1950's with the compounds of interest at that time, thiourea and thiouracil. Because of toxicity, these drugs have been superseded by propylthiouracil (6-*n*-propyl-2-thiouracil) and methimazole, which are currently the drugs of choice in the treatment of hyperthyroidism. Little information is available concerning the pharmacology of these newer compounds and their metabolism is just now receiving attention (1–4).

The primary action of propylthiouracil is on the thyroid gland. It inhibits thyroid peroxidase, resulting in a blockade of iodide utilization for thyroid hormone synthesis (5). Propylthiouracil also inhibits the peripheral action of thyroxine (6) and apparently crosses the placenta, producing adverse effects in the developing fetus (7).

In this study, a number of metabolites and potential metabolites of propylthiouracil were synthesized (Scheme I) and investigated to determine if they inhibit thyroid peroxidase activity. The compounds found to be active in this initial screening test will be assayed *in vivo* for antithyroid activity and for activity as an inhibitor of peripheral thyroxine effects and to determine if the compounds cross the placenta. The ultimate goals are: *(a)* to identify the metabolites of propylthiouracil and to determine if they are biologically active, *(b)* to develop a propylthiouracil analog with potent antithyroid effects but with no inhibitory action on peripheral thyroxine effects, and *(c)* to develop a propylthiouracil analog that retains



Scheme I—6-n-Propyl-2-thiouracil Derivatives and Metabolites

Table I—Data for 6-*n*-Propyl-2-thiouracil and 6-*n*-Propyluracil Derivatives

Compound	Melting Point	Yield, %	UV Absorption Peaks, nm: 0.1 *N* HCl	pH 7.4[a]	0.1 *N* NaOH	Empirical Formula	Analysis, %:	Calc.	Found
I	155–157°	65	223[b] 250 270 (s)	228 248 (s)[c] 274 (s)	247 276	$C_8H_{12}N_2OS$	C H N	52.14 6.58 15.20	52.09 6.66 15.22
II	132–133°	60	253 270 (s)	253 270 (s)	248 277	$C_{14}H_{16}N_2OS$	C H N	64.58 6.20 10.76	64.63 6.22 10.81
III									
Method A	219–221°[d]	93	262	262	272	—		—	—
Method B	219–221°	56	262	262	272	—		—	—
IV	192–193°	70	262	262	282	$C_8H_{12}N_2O_2$	C H N	57.12 7.19 16.66	57.07 7.32 16.54
V	232–234°	86	278	278	288	$C_7H_{10}N_2O_3$	C H N	49.40 5.92 16.47	49.62 5.68 16.59
VI	230–232°	89	262	262	272 281 (s)	$C_9H_{12}N_2O_3$	C H N	55.10 6.17 14.28	54.94 6.27 14.37
VII	249–252°	58	277	269 315 (s)	265 295 (s)	$C_7H_{10}N_2O_2S$	C H N	45.14 5.41 15.06	44.93 5.34 15.11
VIII	226–227°	91	278	270 310 (s)	266 295 (s)	$C_7H_9BrN_2OS$	C H N S	33.75 9.07 11.25 12.87	33.64 3.57 11.31 12.49
IX	215–217°	88	No absorption						
X	170–172°	72	277	277	310 260	$C_8H_{12}N_2OS$	C H N	52.14 6.58 15.20	52.13 6.45 14.97
XI	225–227°	52	274	273	227 260 310 (s)	$C_9H_{12}N_2O_2S$	C H N	50.93 5.70 13.20	49.61 6.06 13.28
XII	282–284°	29	274	274	260 300 (s)	$C_7H_8N_2O_3S$	C H N	41.99 4.03 13.99	41.58 4.26 13.82
XIII	247–249°	36	274	274	260 300 (s)	$C_7H_{10}N_2O_2S$	C H N	45.14 5.41 15.04	45.44 5.22 14.98
XIV	40–42°	57	283 240	288	288 240	$C_9H_{14}N_2OS$	C H N	54.51 7.12 14.13	54.26 7.24 14.06
XV	90–92°	70	235 250 (s) 290	251 290	251 290	$C_9H_{14}N_2O_2S$	C H N	50.44 6.58 13.08	50.52 6.25 13.21
XVI	—	43	Decomposition to propylthiouracil		270 307 (s)	$C_{14}H_{16}N_4Na_2O_2S_2$	C H N	43.97 4.21 14.65	43.39 3.99 14.28
XVII	—	35	Decomposition to thiouracil		275			—	—

[a] Buffer was 0.05 *M* potassium phosphate, pH 7.4. [b] The first peak listed is the λ_{max}. [c] The (s) indicates a shoulder. [d] Lit. (21) mp 221–223°.

its potent antithyroid effects but does not cross the placenta.

EXPERIMENTAL[1]

***S*-Methyl-6-*n*-propyl-2-thiouracil (I)**—Compound I was prepared by the method described by Barrett *et al.* (8) for the synthesis of *S*-methyl-2-thiouracil. A mixture of 8.5 g (0.05 mole) of 6-*n*-propyl-2-thiouracil and 2.2 g of sodium hydroxide was dissolved in a minimum amount of water (about 15 ml) at 70°. The solution was cooled to about 30°, and 30 ml of ethanol and 3.7 ml of methyl iodide were added. The mixture was heated at 70° for 20 min and then cooled. The precipitate that formed was filtered and recrystallized from ethanol.

***S*-Benzyl-6-*n*-propyl-2-thiouracil (II)**—A modification of the procedure of Wheeler and Liddle (9) was adopted. Propylthiouracil (8.5 g, 0.05 mole) and 2.2 g of sodium hydroxide were dissolved in a minimum amount of water, and 5.0 g of benzyl chloride was added. Ethanol was then added until the oil went into solution. The reaction took place immediately and the colorless prisms that formed were filtered and recrystallized from ethanol.

6-*n*-Propyluracil (III)—*Method A*—An adaptation of the Wheeler and Liddle method (9) for the preparation of uracil from 2-thiouracil was used. An aqueous solution of 1.7 g of chloroacetic acid was mixed with 1.5 g (8.8 mmoles) of propylthiouracil. The solution was refluxed for 2 hr and then allowed to cool. The crystalline product was filtered and recrystallized from ethanol. 1-Methyl-6-*n*-propyluracil (IV), 5-hydroxy-6-*n*-propyluracil (V), and 1-acetyl-6-*n*-propyluracil (VI) were also prepared by this procedure using the appropriate propylthiouracil derivative. Table I lists their physical constants.

Method B—A mixture of 2.0 g (7.7 mmoles) of II in 25 ml of 1 *N* HCl was refluxed for 1 hr. The volatile benzyl mercaptan was removed by lyophilization under vacuum. The solid remaining was recrystallized from ethanol to yield III. When I was similarly treat-

[1] Melting points were determined on a Thomas-Hoover Unimelt capillary apparatus and are uncorrected. IR spectra were determined with a Beckman IR-20A spectrophotometer in a KBr phase. UV absorption spectra were measured with a Perkin-Elmer 124 double-beam spectrophotometer in 0.1 *N* NaOH, 0.1 *N* HCl, and 0.05 *M* potassium phosphate buffer, pH 7.4. NMR spectra were obtained with a Varian HA-60 IL using tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal standards. All IR and NMR spectra were in agreement with the assigned structure. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and by PCR, Inc., Gainesville, Fla.

Table II—Inhibition of Thyroid Peroxidase by 6-*n*-Propyl-2-thiouracil and Its Metabolites

Compound	Concentration, $\times 10^{-5}$ *M*	Specific Activity of Peroxidase	Inhibition, %
None	—	1.95 ± 0.11	—
6-*n*-Propyl-2-thiouracil	50	0.22 ± 0.01	88.7
	10	1.17 ± 0.08	40.0
	5	1.52 ± 0.08	22.1
6-*n*-Propyluracil	50	1.96 ± 0.14	0
	10	1.90 ± 0.08	2.5
	5	1.93 ± 0.14	1.0
S-Methylpropyl-thiouracil	100	2.01 ± 0.12	0
	50	1.98 ± 0.11	0
	10	1.99 ± 0.12	0
Propylthiouracil disulfide	50	1.72 ± 0.05	11.8
	10	1.83 ± 0.01	6.2
	5	1.88 ± 0.05	3.6
Propylthiouracil glucuronide	50	1.34 ± 0.08	30.8
	10	2.06 ± 0.08	0
	5	2.04 ± 0.08	0

ed with 1 *N* HCl, it was quantitatively converted to III with the evolution of methyl mercaptan.

5-Hydroxy-6-*n*-propyluracil (V)—Following the procedure described by Wang (10) for the conversion of 5-bromouracil to 5-hydroxyuracil, a mixture of 3.0 g (12.9 mmoles) of 5-bromo-6-*n*-propyluracil, prepared according to the procedure of Baker and Kawazu (11), and 100 ml 10% sodium bicarbonate solution was refluxed for 12 hr under nitrogen. The solution was then acidified to about pH 2 with hydrochloric acid and cooled. The white crystalline solid that formed was filtered and recrystallized from aqueous ethanol.

5-Hydroxy-6-*n*-propyl-2-thiouracil (VII)—The procedure followed was essentially the same as that used to prepare V, using 1.4 g (5.6 mmoles) of 5-bromo-6-*n*-propyl-2-thiouracil (VIII) and 50 ml of 10% sodium bicarbonate solution. The white crystalline solid obtained was recrystallized from aqueous ethanol.

5,6-Dihydro-6-*n*-propyluracil (IX)—A modification of the method of DiCarlo *et al.* (12) for the preparation of 5,6-dihydrouracil was used. A solution of 1.23 g (7.99 mmoles) of III in acetic acid was subjected to catalytic hydrogenation at room temperature for 72 hr under an initial pressure of 50 psi, using platinum oxide as a catalyst. After the reduction was complete (as indicated by the loss of UV absorption at 262 nm), the catalyst was filtered and the solvent was removed under vacuum. The product was recrystallized twice from water. Repeated attempts to synthesize 5,6-dihydro-6-*n*-propyl-2-thiouracil by catalytic reduction of *S*-benzyl- or *S*-methylpropylthiouracil using platinum oxide or 5% rhodium on powdered alumina were unsuccessful.

1-Methyl-6-*n*-propyl-2-thiouracil (X)—A modified procedure of Anderson *et al.* (13) for the preparation of 6-*n*-propyl-2-thiouracil was used. A solution of sodium ethoxide was prepared by dissolving 2.3 g (0.1 g-atom) of sodium metal in 50 ml of absolute ethanol; then 6.3 g (0.07 mole) of *N*-methylthiourea and 7.91 g (0.05 mole) of ethyl butyrylacetate were added simultaneously. The mixture was refluxed for 24 hr and the alcohol was removed by distillation under vacuum. The residue was dissolved in 50 ml of water and acidified to pH 3 with concentrated hydrochloric acid. The white precipitate was filtered, washed, and recrystallized from water.

A similar procedure was used to prepare 1-acetyl-6-*n*-propyl-2-thiouracil (XI), by condensing *N*-acetylthiourea and ethyl butyrylacetate, and 2-thiouracil-6-propionic acid (XII), by condensing thiourea and ethylmethyl-3-ketoadipate prepared by the method of Korman (14).

2-Thiouracil-6-propanol (XIII)—An ethanolic solution of 1.14 g (5 mmoles) of an ethyl ester of XII was reduced with 3.78 g (0.1 mole) of sodium borohydride. The solution was refluxed for 2 hr after the evolution of hydrogen ceased. The solvent was removed under vacuum and the residue was dissolved in 100 ml of water. The pH was adjusted to 3 with hydrochloric acid, and the precipitate obtained was filtered and recrystallized from aqueous ethanol.

Table III—Inhibition of Thyroid Peroxidase by Derivatives of 6-*n*-Propyl-2-thiouracil

Compound	Concentration, $\times 10^{-5}$ *M*	Specific Activity of Peroxidase	Inhibition, %
None	—	1.93 ± 0.11	—
6-*n*-Propyl-2-thiouracil	50	0.19 ± 0.02	90.2
	10	1.11 ± 0.07	42.5
	5	1.47 ± 0.07	24.0
X	100	1.62 ± 0.07	16.0
	50	1.79 ± 0.13	7.3
	10	1.92 ± 0.13	0
XI	50	0.14 ± 0.01	92.8
	10	1.00 ± 0.05	48.2
	5	1.31 ± 0.08	21.3
V	50	0.24 ± 0.05	87.5
	10	1.18 ± 0.02	39.0
	5	1.37 ± 0.07	29.0
XV	100	1.97 ± 0.08	0
	50	1.84 ± 0.09	4.7
	10	1.89 ± 0.05	2.1
XII	10	1.82 ± 0.05	5.7
	5	1.90 ± 0.08	1.6
	1	1.93 ± 0.10	0
XIII	10	1.75 ± 0.07	9.3
	5	1.86 ± 0.08	3.4
	1	1.94 ± 0.05	0

1,2-Dimethyl-6-*n*-propyl-2-thiouracil (XIV)—A solution of 0.85 g (4.0 mmoles) of X in 10 ml of 0.5 *N* NaOH was treated with a solution of 0.45 ml of methyl iodide in 20 ml of ethanol. The mixture was stirred for 2 hr at 70°, acidified with hydrochloric acid, and extracted with chloroform. The extract was dried over anhydrous magnesium sulfate, filtered, and condensed under vacuum to yield an oily substance which solidified after several hours.

5-Methoxy-2-methyl-6-*n*-propyl-2-thiouracil (XV)—Compound VII (500 mg, 2.7 mmoles) was dissolved in 1 ml of water containing 0.11 g of sodium hydroxide, 0.4 ml of dimethyl sulfate was added, and the mixture was stirred for 10 min at room temperature. The mixture was acidified with hydrochloric acid and extracted with chloroform. The chloroform extract was dried and filtered, and the solvent was removed under vacuum to yield a white solid.

6-*n*-Propyl-2-thiouracil Disulfide (XVI) and 2-Thiouracil Disulfide (XVII)—These compounds were synthesized by an adaptation of the procedure of Miller *et al.* (15) and were recrystallized from a water–acetone mixture. Several attempts to synthesize 6-*n*-propyl-2-thiouracil glucuronide were unsuccessful. However, this compound was isolated from rat bile after 6-*n*-propyl-2-thiouracil administration and subsequent column chromatography[2] and also preparative chromatography on cellulose TLC plates.

The purity of each compound was verified by TLC on precoated plastic sheets[3] run in 0.05 *M* ammonium carbonate, on cellulose sheets[4] run in 1 *M* ammonium acetate–ethanol (15:75), or on silica gel sheets[5] run in benzene–isopropanol (6:1).

Peroxidase Assay—Porcine thyroid peroxidase was prepared as previously outlined (16) and assayed by a modification of the guaiacol test (17). The assay medium contained 190 μmoles of guaiacol, 108 μmoles of tromethamine hydrochloride (pH 7.4), 1.0 mg of enzyme protein, and various concentrations of propylthiouracil derivative (in 0.3 ml of ethanol or water) in a final volume of 3.0 ml. The reaction was initiated by the addition of 2 μmoles of hydrogen peroxide. The increase in absorbance at 470 nm was measured for 15 sec during the initial linear reaction using a recording spectrophotometer[6]. Protein was determined by the method of Lowry *et al.* (18).

[2] Bio-Gel P-2.
[3] CEL 300 DEAE, Brinkmann Instruments, Inc.
[4] Eastman Kodak 6064.
[5] Eastman Kodak 13179.
[6] Perkin-Elmer 124.

RESULTS AND DISCUSSION

Some derivatives of propylthiouracil were synthesized and pertinent physical constants are shown in Table I. These compounds were used as authentic standards in studies of propylthiouracil metabolism. Several radioactive metabolites of propylthiouracil in bile and urine were identified by comparing the chromatographic properties of the compounds in Table I with those of radioactive metabolites isolated and purified by chromatographic methods (19).

In recent studies (1, 2) two metabolites of propylthiouracil were identified. Propylthiouracil glucuronide was reported in human urine and plasma and in rat bile and plasma. Sulfate was also found in human urine and thyroid and in rat bile, thyroid, and plasma. Subsequently, propylthiouracil glucuronide was reported (4) to be the major metabolite of propylthiouracil in both rat urine and bile. Results obtained in this laboratory (19) confirmed the presence of propylthiouracil glucuronide in rat bile and urine and identified three other urinary metabolites as sulfate, *S*-methylpropylthiouracil, and 6-*n*-propyluracil. In addition to these metabolites which have been positively identified, Desbarats-Schönbaum *et al.* (3) reported that highly alkalinized guinea pig urine contained a metabolite with the same R_f value as propylthiouracil disulfide in a TLC system. Several metabolites in bile and urine have chromatographic properties different from any compound shown in Table I and remain unidentified.

The metabolites of propylthiouracil were examined to determine their effects on thyroid peroxidase, the primary site of the antithyroidal action of propylthiouracil in the thyroid gland (Table II). The nature of the metabolites makes it immediately apparent that a major site of metabolism in the propylthiouracil molecule is the S group. In addition to the compounds obviously formed by alteration of the S, preliminary data (20) indicate that propylthiouracil glucuronide is a S conjugate. None of the metabolites was as active as the parent compound. Metabolic replacement of the sulfur with oxygen (6-*n*-propyluracil) and conjugation of the sulfur with a methyl group abolished the inhibitory effect on peroxidase. The disulfide was slightly active but was less than 10% as potent as propylthiouracil. Although the material used was chromatographically pure, this compound is very unstable, readily forming propylthiouracil. Part of the activity of the disulfide may be due to formation of the parent compound. The glucuronide of propylthiouracil was the most active metabolite and appears to be slightly more than 10% as active as propylthiouracil.

Although all of the metabolites in rat bile and urine have not been identified, the S group of propylthiouracil appears to be the major site of alteration; biotransformation at this site results in a total or major loss of antiperoxidase activity. All compounds shown in Table I in which the sulfur of propylthiouracil was replaced by oxygen to form the 6-*n*-propyluracil derivative were completely inactive as inhibitors of thyroid peroxidase. This demonstrates that sulfur is essential for the antiperoxidase activity to be expressed. However, the oxygen analogs are useful for identification of the sulfur analogs, which can be readily desulfurated.

The results of assays of the antiperoxidase activity of propylthiouracil derivatives which have not been implicated in metabolism are presented in Table III. Methylation of the 1-*N* group (Compound X) reduced antiperoxidase activity to less than 5% of that of propylthiouracil. However, substitution at the same position with an acetyl group (Compound XI) produced no alteration in antiperoxidase activity. 5-Hydroxy-6-*n*-propylthiouracil is also equally as active as the parent compound. The 6-*n*-propyl side chain was converted to a 6-propionic acid group (Compound XII) or a 6-*n*-propanol side chain (Compound XIII) with a marked reduction in antiperoxidase activity. Higher concentrations of these two substances could not be assayed due to their limited solubilities in aqueous solutions.

The results presented demonstrate that the antiperoxidase activities of all known metabolites of propylthiouracil are substantially lower than that of the parent compound, demonstrating that this biological effect is reduced or eliminated upon biotransformation. None of the 6-*n*-propyluracil derivatives was an inhibitor of peroxidase and only two propylthiouracil derivatives, XI and VII, were as potent as the parent compound.

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* Present address: The Toxicology Center, Department of Pharmacology, University of Iowa, Iowa City, IA 52242

[x] To whom inquiries should be directed.

Photoelectron Spectra, Hydrolytic Stability, and Antimicrobial Activity of *N*-Chlorinated Piperidines

NICOLAE BODOR*x, JAMES J. KAMINSKI*, S. D. WORLEY‡, R. J. COLTON§, T. H. LEE§, and J. W. RABALAIS§

Abstract □ The lone-pair ionization energies for N, O, and Cl heteroatoms in four derivatives of piperidine were measured from UV photoelectron spectra. The various lone-pair interactions in each piperidine were investigated. The hydrolytic stability and the antimicrobial activity for the *N*-chloropiperidines also were determined. The results suggest a correlation between the stability of the nitrogen lone pair, as determined from photoelectron spectroscopy, with the antibacterial activity of the corresponding *N*-chloramine.

Keyphrases □ Piperidines, *N*-chlorinated—photoelectron spectra, hydrolytic stability, and antimicrobial activity, correlations □ *N*-Chloramines—photoelectron spectra, hydrolytic stability, and antimicrobial activity, correlations □ UV spectrophotometry—determination of photoelectron spectra of four *N*-chlorinated piperidines, correlation of nitrogen lone-pair stability and antimicrobial activity □ Antimicrobial activity—correlated to hydrolytic stability of *N*-chlorinated piperidines, photoelectron spectra

Photoelectron spectroscopy provides a versatile method for determining ionization potentials and, thus, orbital energies for molecules. The energies of the molecular orbitals that describe localized or semilocalized electrons ("lone pairs")[1] depend on the chemical environment of the electronic group in question. Therefore, the corresponding ionization potentials exhibit characteristic "chemical shifts" in the photoelectron spectrum. The nature and magnitude of such shifts can thus provide information concerning the interactions that take place between the localized group and its environment. These interactions may be mediated "through space" (direct) or may be hyperconjugative in nature, occurring through intermediate sigma bonds (indirect) (1, 2).

Recently, the magnitude and mode of the interactions between a carbonyl group and one or more carbon–carbon double bonds were investigated (3). These investigators concluded that through-space interactions have no significant effect upon the ionization potentials corresponding to orbitals primarily confined to the carbon–carbon or carbon–oxygen double bonds. This observation suggests that any $\pi CC \rightarrow \pi CO$ interactions in the compounds investigated must occur through a hyperconjugative through-bond mechanism.

In this study, the photoelectron spectra of several piperidines and their *N*-chlorinated derivatives were investigated to define the extent of the interaction, if any, between the nitrogen lone pair and a carbonyl group or an *N*-chloro function. It is well known that *N*-chlorinated organic molecules exhibit antibacterial properties. The antibacterial activity of these *N*-chloramines is affected by a number of factors including concentration, solubility, molecular size, dipole moment, water–lipid partitioning coefficient, and solution pH. It was the hypothesis that the most important factor contributing to the antibacterial activity of these compounds would be the "chlorine potential" (4, 5) of the *N*-chloramine. The *N*-chlorine bond in an *N*-chloramine is covalent. However, the *N*-chlorine bond has properties different from those of the covalent carbon–chlorine bond. For many purposes, it is convenient to regard chlorine when bonded to a nitrogen atom as "positive." Therefore, the *N*-chloramine can be considered as a source of "positive" chlorine (Scheme I):

$$R_2\ddot{N}—Cl \longrightarrow R_2N:^- + Cl^+$$

Scheme I

or in an aqueous solution (Scheme II):

$$R_2N—Cl + H_2O \overset{Kcp}{\rightleftharpoons} R_2NH + HOCl$$

Scheme II

The reaction in aqueous solution has lead to the development of the chlorine-potential concept, pKcp (Eq. 1*b*) (4, 5):

$$Kcp = \frac{[R_2NH]\,[HOCl]}{[R_2NCl]\,[H_2O]} \qquad \text{(Eq. 1}a\text{)}$$

$$pKcp = -\log Kcp \qquad \text{(Eq. 1}b\text{)}$$

The chlorine potential has been defined in terms of the equilibrium constant for the hydrolysis of the *N*-chloramine to hypochlorous acid. When using this approach, the chlorinating ability of *N*-chloramines can be expressed quantitatively. The existence of a qualitative relationship between *N*-chloramine's antibacterial activity and its chlorinating ability as defined by its chlorine potential has also been considered[2].

Although the qualitative relationship between the acidity of an amine (pKa) and its stability relative to the corresponding *N*-chloramine (pKcp) has been demonstrated (4, 5), no general relationship could be elucidated that would correlate *N*-chloramines with substantially different chlorine potentials, *e.g.*, *N*-chloramines and *N*-chloramides. It is the hypothesis that the orbital energies (ionization potentials) of the

[1] In a rigorous description of ionization phenomena, one should use molecular orbital terminology and ionize nonbonding electrons from nonbonding orbitals rather than equivalent orbital terminology which considers "lone pairs." However, in a qualitative sense (particularly for use in reaction mechanisms), it is sometimes helpful to consider nonbonding orbitals as localized and to call them "lone pairs."

[2] J. Kaminski and N. Bodor, unpublished results.

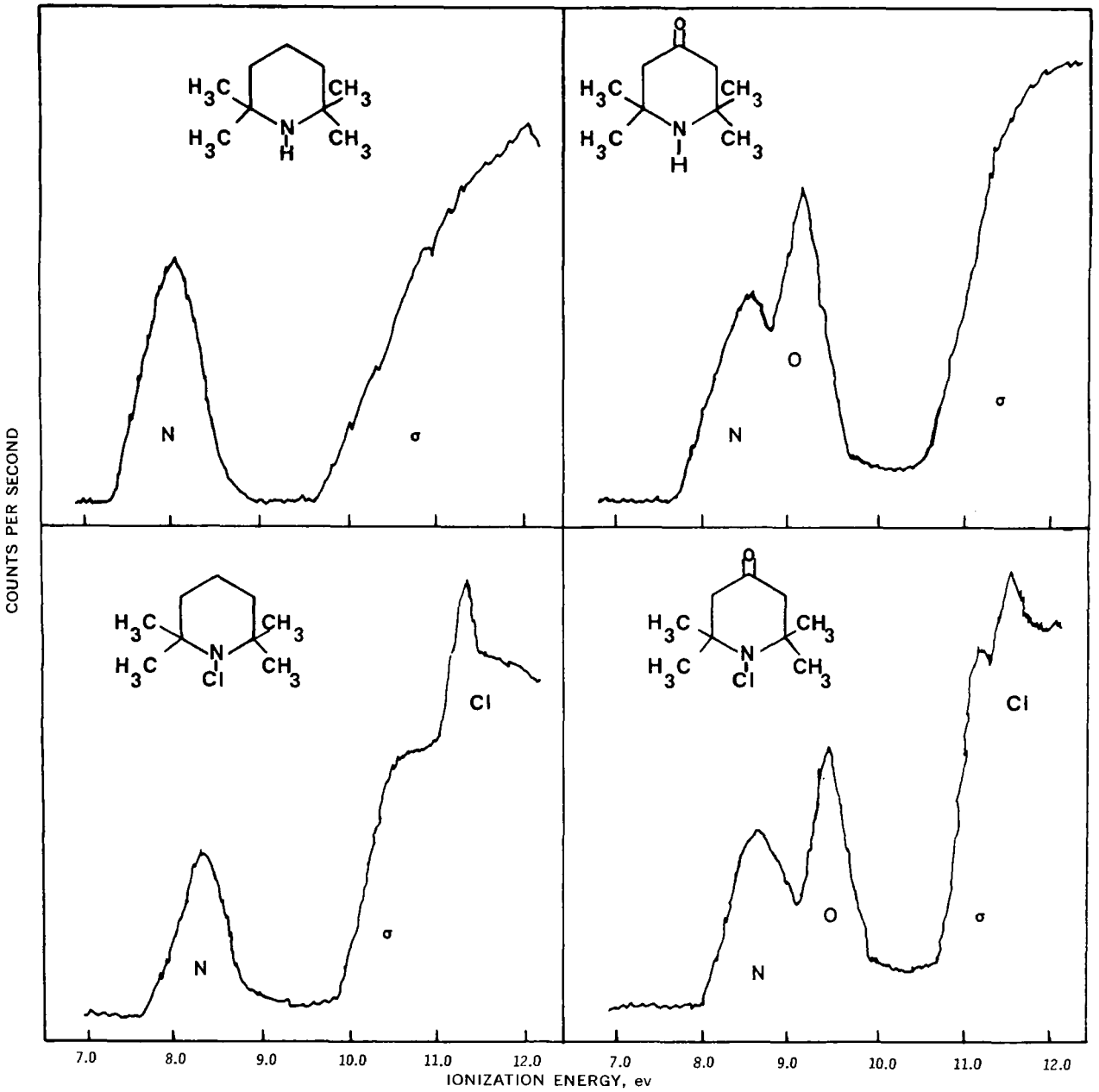


Figure 1—*Photoelectron spectra of some piperidine derivatives excited by the He I resonance line (21.22 ev). Key: top left, I; top right, II; bottom left, III; and bottom right, IV.*

nitrogen lone pair in *N*-chloramines may give a better and perhaps a unitary basis for comparing the chlorine potentials of structurally different *N*-chloramines than does comparison of the basicities of the parent amines.

In the present study, the effect of chlorine directly bonded to nitrogen in *N*-chloramines on the ionization potentials for the nitrogen lone pair was examined. The extension of this study to include novel *N*-chloramines, for which antibacterial activity has been determined, to establish a possible quantitative relationship between the ionization potentials of the nitrogen lone pair in *N*-chloramines and the stability of the *N*-chlorine bond toward hydrolytic displacement (chlorine potential) and/or antibacterial activity is intended.

RESULTS AND DISCUSSION

The relevant regions of the photoelectron spectra for the four piperidines considered are shown in Fig. 1. The ionization potentials derived from the photoelectron spectra are listed in Table I. The assignments of the various bands in the spectra to N, O, or Cl lone-pair ionization were based on comparisons with previously assigned spectra for other molecules having the same functional groups (6–9).

As the data in Table I indicate, relative to the first ionization potential for piperidine, 8.31 ev (6), the inductive effect of the four α-methyl groups in 2,2,6,6-tetramethylpiperidine (I) apparently decreases the nitrogen lone-pair ionization potential by 0.92 ev. The magnitude of this difference is in good agreement with that (1 ev) observed (10) between the first ionization potentials of hydrazine and 1,1,2,2-tetramethylhydrazine, although the marginal increase of the difference in the latter case is presumably due to the methyl groups being directly bonded to the nitrogen atom.

Comparison of the ionization potentials for the nitrogen lone pair in 2,2,6,6-tetramethyl-4-piperidone (II) relative to 2,2,6,6-

Table I—Ionization Potentials of Some Piperidines and Their *N*-Chlorinated Derivatives

Compound	N Lone Pair a[a]	N Lone Pair b[b]	O Lone Pair a	O Lone Pair b	σ-Onset	Cl Lone Pair a	Cl Lone Pair b
I	7.39	8.04	—	—	9.63	—	—
II	7.74	8.52	8.75	9.02	10.55	—	—
III	7.64	8.31	—	—	9.86	10.86	11.14
IV	8.01	8.68	9.09	9.27	10.60	11.10	11.43

[a] Adiabatic ionization potentials (ev); represents band onsets in Fig. 1. Vertical ionization potentials (ev); represents band maxima in Fig. 1.

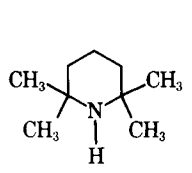
Table II—Ionization Potential Differences between Some Piperidines and Their *N*-Chlorinated Derivatives

ΔIP (ev)	Effect
II − I = 0.35	4-Carbonyl
IV − III = 0.37	4-Carbonyl
III − I = 0.25	*N*-Chlorine
IV − II = 3.27	*N*-Chlorine

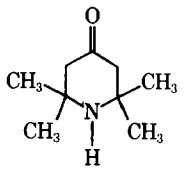
tetramethylpiperidine (I) suggests that the 4-carbonyl function in II exhibits a significant stabilizing–delocalizing effect on the nitrogen lone pair: *IP* (II) − *IP* (I) = 0.35 ev. A comparison of the activation energies for nitrogen inversion (11) between 1,2,2,6,6-pentamethylpiperidine, E_{act} = 10.8 kcal/mole, and 1,2,2,6,6-pentamethyl-4-piperidone, E_{act} = 10.5 kcal/mole, also demonstrates the significant stabilizing–delocalizing effect of the 4-carbonyl group on the nitrogen lone pair.

The delocalizing effect is further evidenced by comparison of the difference between the vertical and adiabatic ionization potentials observed in I ($I_v - I_a$ = 0.65 ev) and II ($I_v - I_a$ = 0.78 ev). (For completely localized lone-pair orbitals, the adiabatic and vertical ionization potentials should be the same.) The observed ionization potentials are in qualitative agreement and consistent with the trend observed in the basicities of the corresponding amines, pKa (I) = 11.18 (12) and 11.07 (13), and pKa (II) = 7.90 (14). However, the difference in the observed pKa's, ΔpKa = 3.17 or 3.28, is more pronounced than that observed in the nitrogen lone-pair ionization potentials, ΔIP = 0.35 or 0.48 ev. This must reflect the differences existing between the solvation properties of I and II. Undoubtedly, the differences in the solvation energies for these amines is an extremely important factor which affects the pKa value. As a consequence, the solvation phenomenon may substantially alter the electronic environment of the solvated nitrogen lone pair relative to that existing in the gaseous state. In support of this view, I and piperidine have essentially the same pKa of 11.12 (15) and 10.98 (12), respectively. However, the difference between the nitrogen lone-pair ionization potentials for the two amines is 0.92 ev.

Comparison of the ionization potential difference between I and II or 1-chloro-2,2,6,6-tetramethylpiperidine (III) and 1-chloro-2,2,6,6-tetramethyl-4-piperidone (IV) relative to the differences in



I



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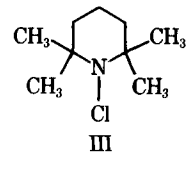
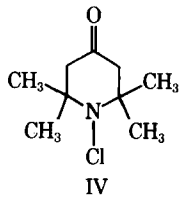


III



IV

Table III—Hydrolytic Stability of 1-Chloro-2,2,6,6-tetramethylpiperidine (III) and 1-Chloro-2,2,6,6-tetramethyl-4-piperidone (IV) at 40°

Compound	Solvent	pH	Half-Life, min	Rate Constant × 10^{-3} min^{-1}	Correlation Coefficient[a]
III	0.1 *M* $NaOCOCH_3$	4.6	168	4.12	0.993
	0.1 *M* NaH_2PO_4	7.0	221	3.13	0.990
	0.1 *M* $Na_2B_4O_7$	9.3	226	3.07	0.994
IV	0.1 *M* $NaOCOCH_3$	4.6	559	1.24	0.990
	0.1 *M* NaH_2PO_4	7.0	203	3.42	0.997
	0.1 *M* $Na_2B_4O_7$	9.3	16	42.6	0.999

[a] Correlation coefficients were determined from a linear regression analysis of the experimental data.

the ionization potentials between I and III or II and IV demonstrates that the 4-carbonyl group has a greater effect in stabilizing the nitrogen lone pair than does the *N*-chlorine group (Table II). In addition, it appears that the effects of the 4-carbonyl group and the *N*-chlorine function on the ionization potential of the nitrogen lone pair are nearly additive. Comparison between the oxygen lone-pair ionization potentials of IV and II indicates that the presence of the *N*-chlorine group stabilizes the oxygen lone-pair orbital by about 0.3 ev. Also, comparison between the chlorine lone-pair ionization potentials of IV and III indicates that the presence of the 4-carbonyl group stabilizes the chlorine lone-pair orbital by about 0.3 ev. Furthermore, it can be seen that the 4-carbonyl group in II and IV significantly increases the σ-onsets (0.92 and 0.74 ev, respectively), whereas the presence of the *N*-chloro function in III and IV has less effect on the onset of σ-ionization (0.23 and 0.05 ev, respectively).

The hydrolytic stability and the antibacterial activity of III and IV were investigated (Tables III and IV). For each *N*-chloramine, the rate of change in the concentration of positive chlorine in the solution was determined using the iodometric method of analysis. The data were interpreted as pseudo-first-order kinetic processes and the stability of the *N*-chloramines III and IV under the experimental conditions was characterized using the half-life as the reaction constant. Based on the ionization potential of the nitrogen lone pair determined from the photoelectron spectra, one would predict that the *N*-chloramine having the more stabilized nitrogen lone pair (higher *IP*) would also be more susceptible to base-catalyzed and less susceptible to proton-catalyzed hydrolytic cleavage of the N—Cl bond. Comparison of the stability data described in Table III for III and IV supports this hypothesis. For example, IV, which has a more stabilized nitrogen lone pair, *IP* = 8.01 ev, relative to III, *IP* = 7.64 ev, also exhibits a significant instability in base, $t_{1/2}$ = 16 min, relative to III, $t_{1/2}$ = 226 min, under the same experimental conditions (pH 9.3).

The antibacterial activity determined for III and IV is also consistent with the hydrolytic stability of the *N*-chloramines. As anticipated, the *N*-chloramine IV, which possesses a higher chlorine potential relative to III, also exhibits a higher antibacterial activity. Unfortunately, due to the limitations in the time scale for sampling in the antibacterial experiment, this observation is only evident in a comparison of the antibacterial activity times required to sterilize only the more resistant microorganisms *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027). Hopefully, it will be possible to correlate the stability of the nitrogen lone pair, as determined from photoelectron spectroscopy, with antibacterial activity.

Although no quantitative correlations can be established based on this limited investigation, it is encouraging to observe at least the existence of a qualitative relationship among the electronic, chemical, and microbiological properties of these compounds. This method of analysis will be extended to other *N*-chloramine derivatives to establish a quantitative correlation between the physical and microbiological properties investigated.

Table IV—Antibacterial Activity of 1-Chloro-2,2,6,6-tetramethylpiperidine (III) and 1-Chloro-2,2,6,6-tetramethyl-4-piperidone (IV)

Compound	Solvent	Concentration Data		Antibacterial Activity Time, min				
		Compound	Positive Chlorine	ATCC 12228[a]	ATCC 10536[b]	ATCC 10031[c]	ATCC 9027[d]	ATCC 6538[e]
III	0.1 *M* $NaOCOCH_3$ pH 4.6	0.91×10^{-3} *M*	30 ppm	0.5	0.5	0.5	2	2
IV	0.1 *M* $NaOCOCH_3$ pH 4.6	0.97×10^{-3} *M*	30 ppm	0.5	0.5	0.5	0.5	0.5

[a] *Staphylococcus epidermidis.* [b] *Escherichia coli.* [c] *Klebsiella pneumoniae.* [d] *Pseudomonas aeruginosa.* [e] *Staphylococcus aureus.*

EXPERIMENTAL

Synthesis—*2,2,6,6-Tetramethylpiperidine (I)*—A commercial sample[3] of 2,2,6,6-tetramethylpiperidine (I) was used after distillation from barium oxide, bp 150–151°.

2,2,6,6-Tetramethyl-4-piperidone (II)—2,2,6,6-Tetramethyl-4-piperidone (II) was obtained by basification of a commercial sample of 2,2,6,6-tetramethyl-4-piperidone hydrochloride[3]. Compound II was used after vacuum sublimation at room temperature (*0.1 mm*), mp 59–60°.

1-Chloro-2,2,6,6-tetramethylpiperidine (III)—To a solution of 7.05 g (0.05 mole) of I in 20 ml of water was added dropwise, with stirring, 63 ml (0.05 mole) of 0.8 *M* sodium hypochlorite solution. During the addition, the reaction solution was maintained between pH 4 and 6 using 1 *N* HCl. The *N*-chloramine separated from the reaction mixture as an oil and was extracted with ethyl acetate. The aqueous layer was separated and extracted with ethyl acetate. The extracts were combined and dried over anhydrous sodium sulfate. Following filtration, the ethyl acetate was removed at room temperature under reduced pressure to afford a pale-yellow oil. Vacuum distillation of this material gave 7.4 g (0.042 mole), 84%, of III, bp 28–34° (0.2 mm); UV (H_2O): λ_{max} 273 nm, ϵ = 152 M^{-1} cm^{-1}; PMR ($CDCl_3$): δ 1.38 (s, 12H) and 1.77 (s, 6H) ppm.

Anal.—Calc. for $C_9H_{18}ClN$: C, 61.52; H, 10.32; Cl, 20.2; N, 7.97. Found: C, 61.18; H, 9.95; Cl, 20.2; N, 8.11.

1-Chloro-2,2,6,6-tetramethyl-4-piperidone (IV)—To a solution of 4.80 g (0.025 mole) of II-HCl in 10 ml of water was added dropwise, with stirring, 31 ml (0.025 mole) of a 0.8 *M* sodium hypochlorite solution. During the addition, the reaction solution was maintained between pH 4 and 6 using 1 *N* HCl. The *N*-chloramine separated from the reaction mixture as an oil and was extracted with ethyl acetate. The aqueous layer was separated and extracted with ethyl acetate. The extracts were combined and dried over anhydrous sodium sulfate. Following filtration, the ethyl acetate was removed at room temperature under reduced pressure to afford a pale-yellow oil. Vacuum distillation of this material gave 3.63 g (0.019 mole), 75%, of IV, bp 44–48° (0.25 mm); UV (H_2O): λ_{max} 270 nm, ϵ = 299 M^{-1} cm^{-1}; PMR ($CDCl_3$): δ 2.73 (s, 4H) and 1.43 (s, 12H) ppm.

Anal.—Calc. for $C_9H_{16}ClNO$: C, 56.98; H, 8.50; Cl, 18.7; N, 7.39. Found: C, 56.59; H, 8.78; Cl, 18.5; N, 7.29.

Photoelectron Spectra—The spectra were recorded on a photoelectron spectrometer[4] which incorporates a 10-cm radius cylindrical electrostatic field-deflection analyzer. The apparatus was described previously (16). The He I resonance line at 584 Å (21.22 ev) served as the ionizing source. The samples studied had low vapor pressures and had to be heated to produce sufficient vapor for obtaining good electron fluxes. The temperature of the collision chamber was monitored by a thermocouple placed ~1 cm above the chamber slit. It was possible to maintain the temperature within ±1° for extended periods. The spectra were recorded at temperatures that gave the best balance between electron flux and sample stability. The spectra were calibrated by use of the $Ar^2P_{3/2}$ (15.759 ev) and $^2P_{1/2}$ (15.937 ev) lines with the $Kr^2P_{3/2}$ (13.999 ev) and $^2P_{1/2}$ (14.665 ev) lines as internal standards.

Hydrolytic Stability of III and IV—For each determination of the reaction constant for III at a given pH, a stock solution of ~0.80×10^{-3} *M* III was prepared by dissolving 28.00 mg (0.16 mmole) of III in the appropriate buffer solution to a final volume of 200 ml. Approximately 15-ml aliquots of the stock solution were dispensed into ampuls and sealed. The ampuls were equilibrated for 0.5 hr in a constant-temperature bath operating at 40°. Periodically, samples were removed from the bath, and the concentration of positive chlorine in the sample was determined iodometrically using 10^{-3} *N* sodium thiosulfate.

Analysis as a first-order kinetic process gave the half-lives listed in Table II.

For each determination of the reaction constant for IV at a given pH, a stock solution of 1.25×10^{-3} *M* IV was prepared by dissolving 236.25 mg (1.25 mmoles) of IV in the appropriate buffer solution to a final volume of 100 ml. Approximately 5-ml aliquots of the stock solution were dispensed into ampuls and sealed. The ampuls were equilibrated for 0.5 hr in a constant-temperature bath operating at 40°. Periodically, samples were removed from the bath, and the concentration of positive chlorine in the sample was determined iodometrically using 10^{-3} *N* sodium thiosulfate.

Analysis as a first-order kinetic process gave the half-lives listed in Table II.

Antibacterial Activity of III and IV—The procedure used to determine the antibacterial activity of III and IV was based primarily on a modification of the serial dilution method of analysis. Instead of determining the minimum inhibitory concentration (MIC) parameters for the *N*-chloramine, only the bactericidal endpoint for a given concentration of the *N*-chloramine was determined. Therefore, the screening procedure only determines the time required for complete sterilization of the microorganism when exposed to a given concentration of the *N*-chloramine.

For each *N*-chloramine, a stock solution of known concentration was prepared using the appropriate buffer solution. To 5 ml of the *N*-chloramine solution is added 0.2 ml of an overnight broth culture containing the particular microorganism being investigated. At time intervals of 0.5, 1, 2, 3, 4, 5, . . ., min, a loop of this suspension is subcultured into 5 ml of sterile nutrient broth. All samples are then incubated at 37° for 7 days, with daily observation for evidence of bacterial growth. The time intervals reported are for that sample in which no bacterial growth was observed after the incubation period.

In addition, several controls were also conducted.

Control 1—This control is designed basically to check the overnight broth culture.

To 5 ml of sterile 0.9% NaCl solution is added 0.2 ml of an overnight broth culture containing the particular microorganism being investigated. A loop of this suspension is subcultured into 5 ml of sterile nutrient broth and incubated at 37° for 7 days.

Control 2—This control is designed to ensure that the dilution factor of the nutrient broth is beyond any bacteriostatic activity of the *N*-chloramine solution tested.

To 5 ml of sterile nutrient broth is added a loop of the *N*-chloramine solution being evaluated and the solution is mixed immediately. To this solution is then added a loop of overnight broth culture which has been diluted 25 times with 0.9% NaCl solution. Incubate at 37° for 7 days.

Control 3—This control is used to ensure that the bacterial growth observed is due to the organism being tested rather than to contamination from a foreign organism.

At the same time intervals used for subculturing the test solution into nutrient broth during the screening procedure, a loop of the test solution is also subcultured onto sterile nutrient agar plates. This technique is useful for observing the morphology of the surface colony characteristic for each organism.

Control 4—This control was used initially to ensure that the pH of the solution and the concentration of the buffer species did not inhibit the bacterial growth during the time intervals used in the screening procedure.

[3] Aldrich Chemical Co.
[4] Perkin-Elmer model PS-16.

The entire screening procedure was conducted for each buffered solution rather than the *N*-chloramine solution in the procedure.

SUMMARY

The lone-pair ionization energies for the N, O, and Cl heteroatoms in four derivatives of piperidine were measured from UV photoelectron spectra. The interaction between the various lone pairs in each piperidine was studied. The hydrolytic stability and antimicrobial activity of the *N*-chloro piperidines also were determined. The current results suggest that it may be possible to correlate the stability of the nitrogen lone pair, as determined from photoelectron spectroscopy, with the antibacterial activity of the corresponding *N*-chloramines.

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ˣ To whom inquiries should be directed.

Cardiotonic Steroids I: Importance of 14β-Hydroxy Group in Digitoxigenin

BALA K. NAIDOO‡, THOMAS R. WITTY‡, WILLIAM A. REMERS‡ˣ, and H. R. BESCH, Jr.†

Abstract □ Analogs of digitoxigenin and its 3-acetate, in which the stereochemistry and nature of the substituent at the 14-position are varied, were synthesized and assayed for inhibition of myocardial Na^+,K^+-adenosine triphosphatase. Among the 3-acetates, the 14β-H and 14α-H analogs were less active than 3-acetoxydigitoxigenin by a factor of 1000, with the 14β-chloro analog slightly more active than the others. The corresponding 14β,15β-epoxide was 0.03 as active as 3-acetoxydigitoxigenin, but the isomeric 14α, 15α-epoxide was virtually inactive. In the 3-OH series, digitoxigenin was significantly more active than any analog. The 14β-H analog was 10-fold more active than the 14α-H analog. These results suggest the desirability of *cis*-C,D-ring fusion and a highly selective function for the 14β-OH group of digitoxigenin, possibly based upon its ability to receive a hydrogen in hydrogen bonding. Syntheses of the 14β-H, 14α-H, and 14β-Cl analogs were accomplished by converting the known 15-ketones to ethylene thioketals and desulfurizing with Raney nickel. Attempts to prepare a 14β-NH_2 analog were unsuccessful.

Keyphrases □ Digitoxigenin, 14β-H, 14α-H, 14β-Cl, and acetate analogs—synthesis and inhibition of Na^+,K^+-adenosine triphosphatase □ Cardiotonic steroids—synthesis of digitoxigenin analogs (14β-H, 14α-H, 14β-Cl, and acetates), inhibition of Na^+,K^+-adenosine triphosphatase □ Structure–activity relationships—digitoxigenin analogs (14 substituents) and inhibition of Na^+,K^+-adenosine triphosphatase

It has been accepted generally that the 14β-OH group of cardiotonic steroids and their glycosides is essential to their inotropic activity (1). Derivative structures such as the corresponding 14(15)-enes and epoxides are considered much less active in assays based upon lethality in cats (1), and 14-epidigitoxigenin showed no activity in a Na^+,K^+-adenosine triphosphatase assay (2). These observations tend to confirm the need for a *cis*-C,D-ring fusion as well as a hydroxy group at the 14-position. However, recent reports (3, 4) on uzarigenin derivatives showed that the 14β-H analog (I) was one-third as active as uzarigenin itself (II) in the isolated frog heart, indicating that the 14β-hydroxy group is not indispensible for positive inotropic activity. This report prompted a more extensive investigation of the requirements in stereochemistry and the nature of substituents at the 14-position of cardenolides for inotropic activity.

The digitoxigenin nucleus was chosen for investigation since it is more active than the uzariginen nucleus due to its *cis*-A,B-ring fusion. This factor appeared important for structure–activity relation-

The entire screening procedure was conducted for each buffered solution rather than the *N*-chloramine solution in the procedure.

SUMMARY

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Cardiotonic Steroids I: Importance of 14β-Hydroxy Group in Digitoxigenin

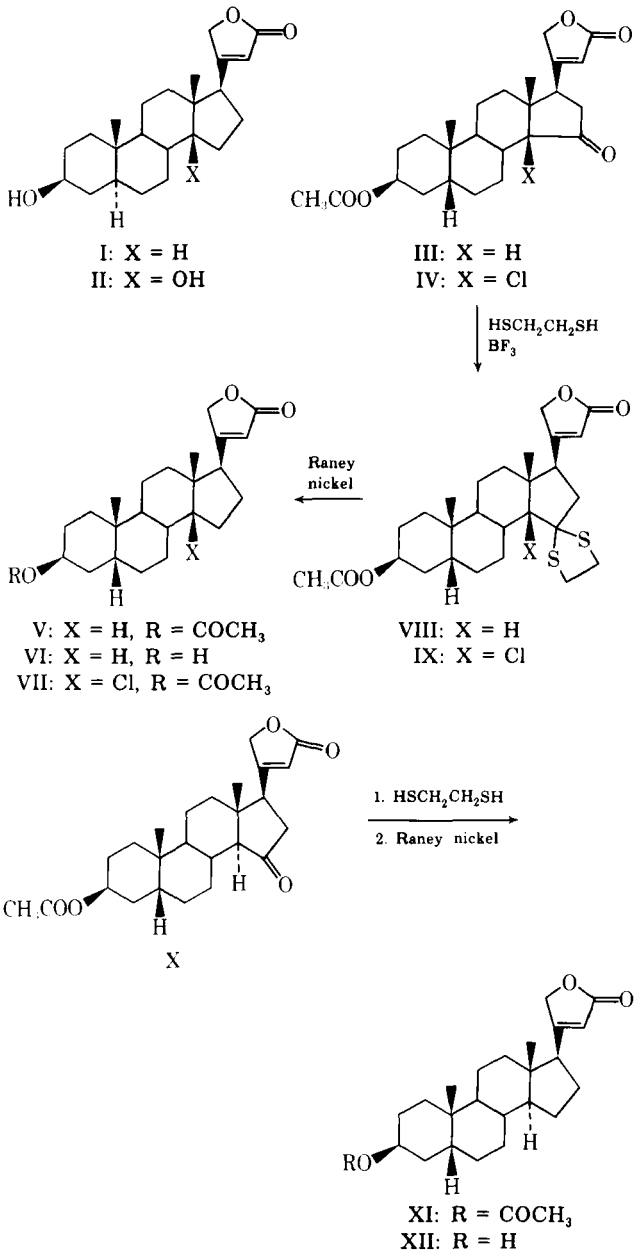
BALA K. NAIDOO‡, THOMAS R. WITTY‡, WILLIAM A. REMERS‡ˣ, and H. R. BESCH, Jr.†

Abstract □ Analogs of digitoxigenin and its 3-acetate, in which the stereochemistry and nature of the substituent at the 14-position are varied, were synthesized and assayed for inhibition of myocardial Na^+,K^+-adenosine triphosphatase. Among the 3-acetates, the 14β-H and 14α-H analogs were less active than 3-acetoxydigitoxigenin by a factor of 1000, with the 14β-chloro analog slightly more active than the others. The corresponding 14β,15β-epoxide was 0.03 as active as 3-acetoxydigitoxigenin, but the isomeric 14α, 15α-epoxide was virtually inactive. In the 3-OH series, digitoxigenin was significantly more active than any analog. The 14β-H analog was 10-fold more active than the 14α-H analog. These results suggest the desirability of *cis*-C,D-ring fusion and a highly selective function for the 14β-OH group of digitoxigenin, possibly based upon its ability to receive a hydrogen in hydrogen bonding. Syntheses of the 14β-H, 14α-H, and 14β-Cl analogs were accomplished by converting the known 15-ketones to ethylene thioketals and desulfurizing with Raney nickel. Attempts to prepare a 14β-NH_2 analog were unsuccessful.

Keyphrases □ Digitoxigenin, 14β-H, 14α-H, 14β-Cl, and acetate analogs—synthesis and inhibition of Na^+,K^+-adenosine triphosphatase □ Cardiotonic steroids—synthesis of digitoxigenin analogs (14β-H, 14α-H, 14β-Cl, and acetates), inhibition of Na^+,K^+-adenosine triphosphatase □ Structure–activity relationships—digitoxigenin analogs (14 substituents) and inhibition of Na^+,K^+-adenosine triphosphatase

It has been accepted generally that the 14β-OH group of cardiotonic steroids and their glycosides is essential to their inotropic activity (1). Derivative structures such as the corresponding 14(15)-enes and epoxides are considered much less active in assays based upon lethality in cats (1), and 14-epidigitoxigenin showed no activity in a Na^+,K^+-adenosine triphosphatase assay (2). These observations tend to confirm the need for a *cis*-C,D-ring fusion as well as a hydroxy group at the 14-position. However, recent reports (3, 4) on uzarigenin derivatives showed that the 14β-H analog (I) was one-third as active as uzarigenin itself (II) in the isolated frog heart, indicating that the 14β-hydroxy group is not indispensible for positive inotropic activity. This report prompted a more extensive investigation of the requirements in stereochemistry and the nature of substituents at the 14-position of cardenolides for inotropic activity.

The digitoxigenin nucleus was chosen for investigation since it is more active than the uzariginen nucleus due to its *cis*-A,B-ring fusion. This factor appeared important for structure–activity relation-



Scheme I

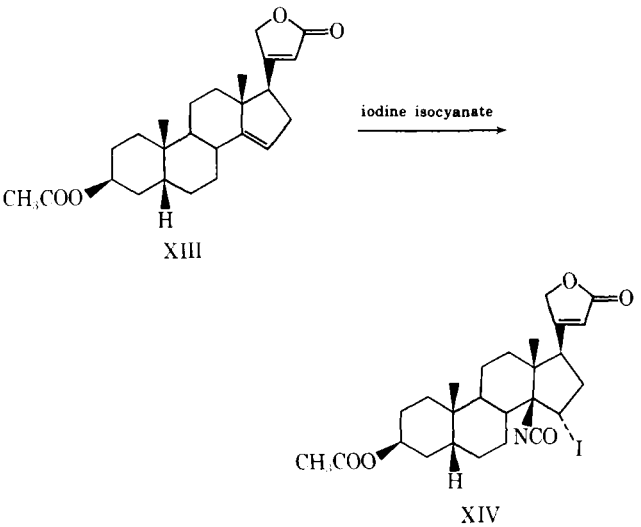
ships, and it suggested potential clinical utility if the digitoxigenin analogs retained good activity. The 14β-H analog (VI) of digitoxigenin was chosen as a target compound by analogy to 14β-H,14-dehydroxyuzarigenin (I), and the known (5) 14α-H epimer (XI) was chosen to check the C,D-ring fusion requirement. The 14β-chloro group (of analog VII) seemed important since it would have the same size and stereochemistry as the 14β-hydroxy group of digitoxigenin but not its ability to hydrogen bond to the receptor. The 14β-amino group would be able to participate in hydrogen bonding. Finally, the known 14β,15β-epoxide (XXI) is interesting as an analog because it has oxygen in nearly the same position as that of the 14β-OH group (although the steroid nucleus is distorted). It could receive a hydrogen in hydrogen bonding but it could not furnish one, in contrast to the hydroxy group. Since the 3-acetates of these target analogs were intermediates in their syntheses, it was reasonable to assay both the 3-acetates and the 3-hydroxy target compounds, using digitoxigenin and its 3-acetate as standards.

RESULTS AND DISCUSSION

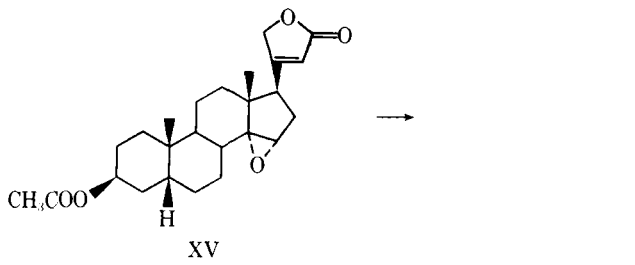
Synthesis—The 3-acetates (V and XI, respectively) of 14β-H- and 14α-H-dehydroxydigitoxigenins were prepared from the known 15-oxo derivatives (III and X, respectively) (6–8) by conversion to thioketals and desulfurization with deactivated Raney nickel (Scheme I). They were then hydrolyzed to the 3-hydroxy analogs VI and XII. Compound XII was also prepared by catalytic hydrogenation of 14(15)-anhydrodigitoxigenin according to the known (5) procedure. The 14β-chloro analog was prepared as its 3-acetate (VII) by a parallel route from the known (8) 14β-chloro-15-oxo compound (IV). However, it was not possible to hydrolyze VII to the corresponding 3-hydroxy compound due to an insufficient amount of VII. An alternative route to this 3-hydroxy compound involving hydrolysis of IV (9) prior to thioketal formation was attempted but was unsuccessful. Subsequent attempts to repeat the preparation of VII were only partially successful since the pure product was not obtained.

Two routes to the 14β-amino analog were investigated. In one route (Scheme II), the 14(15)-ene (XIII) was converted into the corresponding 15α-iodo-14β-isocyanato derivative XIV by treatment with iodine isocyanate. The stereochemistry of XIV is assigned on the basis of the mode of addition of iodine isocyanate to double bonds. Thus, an iodonium ion should form on the less hindered α-side and be opened by the attack at carbon-14 of isocyanate ion from the β-side of the molecule. This mechanism is analogous to the mechanisms that are presumably involved in the addition of hypobromous acid to XIII and and the acidic ring opening of 14α,15α-epoxide (XV); in each of these cases, the 14β,15α-product is formed (7, 8). The facile elimination of the elements of iodine isocyanate from XIV supports this structural assignment. Unfortunately, this elimination occurred during all attempts to convert XIV into the desired 14β-NH_2 analog and this route was abandoned. The second route (Scheme III) was based upon hydrazoic acid ring opening of 14α,15α-epoxide (XV). This reaction gave, in low yield, a product that appeared to be the azidohydrin XVI, according to its IR, NMR, and mass spectra, but it could not be purified because of its instability.

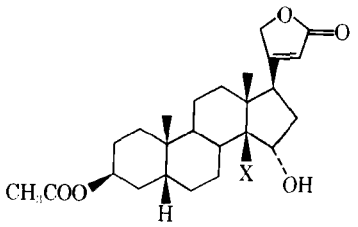
Biological—Several cardenolides and certain intermediates leading to them were tested for their ability to inhibit a Na^+,K^+-stimulated, Mg^{+2}-dependent adenosine triphosphatase isolated from calf or canine myocardium (10). The ability of a large number of cardenolides to inhibit specifically this adenosine triphosphatase has been shown to correlate well with their positive inotropic activity (11, 12). As recorded in Table I, digitoxigenin acetate has a K_I of 2×10^{-8}. The next most active compound was the 14β,15β-epoxide (XXI), which had about 0.03 of this activity. The 14β-H, 14α-H, 14β-Cl, and 14(15)-ene analogs were about 0.001 as active,



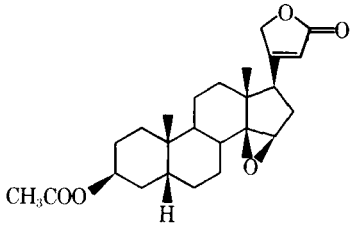
Scheme II



XV



XVI: R = $COCH_3$, X = N_3
XVII: R = $COCH_3$, X = OH
XVIII: R = $COCH_3$, X = Cl
XIX: R = H, X = OH
XX: R = H, X = Cl



XXI

Scheme III

with the 14β-Cl analog (VII) the most active of this group. Little difference was found between the acetates (V and XI) of the 14β-H and 14α-H compounds, due possibly to the low solubility of the former. However, the corresponding 3-hydroxy analogs (VI and XII) and the 14β-H compound were 10 times as active, which indicates the superiority of the *cis*-C,D-ring fusion. The 14α,15α-epoxide (XV) was so inactive that its K_I could not be determined conveniently.

The relatively high potency of the 14β,15β-epoxide (XXI) is surprising in view of Chen's (13) observation that the 3-hydroxy analog of this epoxide was not toxic to cats. However, that observation might be due to pharmacokinetic considerations. At the receptor enzyme, XXI appears to be more like digitoxigenin acetate than any of the other analogs tested in the present study, despite certain conformational distortions in XXI. Thus, its epoxide ring deforms the C-ring from a perfect chair formation, which causes small changes throughout the molecule in the positions of atoms relative to those in digitoxigenin acetate. Molecular models do show that the 14-oxygen in XXI is in almost the same position as the corresponding oxygen in digitoxigenin acetate.

Since the 14β-Cl analog (VII), which has correct C,D-ring fusion and substituent size at the 14-position, is poorly active whereas the 14β,15β-epoxide is relatively potent, it appears that the 14-oxygen fulfills a specific role. This role possibly is one of receiving a hydrogen in hydrogen bonding with some functional group on the adenosine triphosphatase. The 14β-OH group of digitoxigenin could furnish the hydrogen as well as receive it. The requirement of a 14β-oxygen group is supported by both the very poor activity of the 14α,15α-epoxide (XV) and the previously reported inactivity of 14-epidigitoxigenin relative to digitoxigenin (2).

In a previous report, Shigei *et al.* (14) were surprised to find that 15α-hydroxydigitoxigenin (XIX) was inactive in the frog heart whereas the corresponding 14β-Cl analog (XX) was 0.1–0.3 as active as digitoxigenin. Since the intermediate 3-acetates (XVII and XVIII) corresponding to these compounds had been prepared, they were tested for inhibition of Na^+,K^+-adenosine triphosphatase. As shown in Table I, the 14β-Cl,15α-OH derivative (XVIII) was approximately three times more potent than the corresponding compound without the 15α-OH substituent (VII) and about four times more active than 15α-hydroxydigitoxigenin 3-acetate (XVII).

Table I—K_I Values for the Inhibition of Na^+,K^+-Adenosine Triphosphatase

	3-OH,5β-Card-20(22)-enolide		K_I
	14β-OH	3-acetate	2×10^{-8}
V	14β-H	3-acetate	3.5×10^{-5a}
XI	14α-H	3-acetate	3.5×10^{-5}
VII	14β-Cl	3-acetate	1.1×10^{-5}
XIII	14(15)-ene	3-acetate	7.8×10^{-5}
XV	14α,15α-epoxide	3-acetate	$>3 \times 10^{-5}$
XXI	14β,15β-epoxide	3-acetate	6.2×10^{-7}
XVII	14β-OH,15α-OH	3-acetate	1.7×10^{-5}
XVIII	14β-Cl,15α-OH	3-acetate	4.2×10^{-6}
	14β-OH (digitoxigenin)		6×10^{-8}
VI	14β-H		1.6×10^{-6}
XII	14α-H		1.5×10^{-5}
	14(15)-ene		2.0×10^{-5}

[a] Not completely soluble.

During this investigation, it was reported that an impure sample of 14β-H analog (VI) was about 0.1 as active as digitoxigenin in a frog heart preparation (14).

EXPERIMENTAL[1]

3β-Acetoxy-15-oxo-5β,14β-card-20(22)-enolide, Cyclic Ethylene Mercaptole (VIII)—A mixture of 0.2 ml of 1,2-ethanedithiol, 0.26 ml of boron trifluoride etherate, and 100 mg of 3β-acetoxy-15-oxo-5β,14β-card-20(22)-enolide (III) (6–8) was kept at room temperature for 24 hr, treated with 0.5 g of solid sodium carbonate, and extracted with two portions (200 ml) of chloroform. The combined extracts were washed successively with 5% aqueous sodium hydroxide and water, dried (sodium sulfate), and concentrated under reduced pressure. The white solid residue (92 mg) was purified by chromatography on silica gel with chloroform–acetone (5:1) as solvent. This procedure gave 76 mg (65%) of VIII as white flakes, mp 262–263°; IR ($CHCl_3$): 5, 6, and 5.77 (butenolide, acetate) μm; the mass spectrum showed M^+ at *m/e* 490.

Anal.—Calc. for $C_{27}H_{38}O_4S_2$: C, 66.10; H, 7.75; S, 13.08. Found: C, 65.87; H, 7.79; S, 12.94.

3β-Acetoxy-15-oxo-5β,14α-card-20(22)-enolide, Cyclic Ethylene Mercaptole—This compound was prepared by the procedure described for VIII. From 80 mg of 3β-acetoxy-15-oxo-5β,14α-card-20(22)-enolide (X) (6–8) was obtained 63 mg (73%) of the thioketal as white powder, mp 269–272°; IR ($CHCl_3$): 5.59, 5.71, and 5.77 (butenolide, acetate) μm; the mass spectrum showed M^+ at *m/e* 490.

Anal.—Calc. for $C_{27}H_{38}O_4S_2$: C, 66.10; H, 7.75; S, 13.08. Found: C, 65.91; H, 7.88; S, 12.86.

3β-Acetoxy-5β,14β-card-20(22)-enolide (V)—A mixture of 68 mg of VIII, Raney nickel prepared from 3 g of alloy and deactivated by heating with acetone (15), and 40 ml of ethanol was heated at reflux temperature for 150 min, filtered, and concentrated. The residual solid (51 mg) was purified by chromatography on silica gel with chloroform–acetone (5:1) as the solvent. Recrystallization from ether gave a low yield of V as white plates, mp 180–181°; IR ($CHCl_3$): 5, 6, and 5.77 (butenolide, acetate) μm; the mass spectrum showed M^+ at *m/e* 400.

Anal.—Calc. for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06. Found: C, 75.36; H, 8.55.

3β-Acetoxy-5β,14α-card-20(22)-enolide (XI)—This compound was prepared by the procedure described for V. From 50 mg

[1] Melting points were determined on a Mel-Temp apparatus and are uncorrected. IR absorption spectra were recorded using $CHCl_3$ solutions or KBr pellets and a Perkin-Elmer 237B spectrophotometer. NMR spectra were determined in $CDCl_3$ using tetramethylsilane as the reference on a Jeol 60-MHz spectrometer. Mass spectra were determined on a Consolidated Electronics Corp. 21-110B mass spectrometer. Microanalyses were carried out by Midwest Microlab Ltd., Indianapolis, Ind.

of the thioketal (prepared from X) was obtained a low yield of XI, mp 171–173°; IR ($CHCl_3$): 5.61 and 5.77 (butenolide, acetate) μm; the mass spectrum showed M^+ at *m/e* 400.

Anal.—Calc. for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06. Found: C, 75.21; H, 8.85.

3β-Hydroxy-5β,14β-card-20(22)-enolide (VI)—A solution of 20 mg of V in 3 ml of 70% aqueous ethanol containing 5% HCl was heated at reflux temperature for 3 hr, cooled in an ice bath, neutralized with 5% $NaHCO_3$ solution, and concentrated under reduced pressure. The residue was extracted with chloroform and this extract was washed with water, dried (magnesium sulfate), and concentrated. The crude product was purified by chromatography on silica gel with chloroform–acetone (5:1) as the solvent, followed by recrystallization from ether–hexane. This procedure gave 11 mg (60%) of VI as white plates, mp 168–170°; IR: 2.85 (OH), 5.6, and 5.71 (butenolide) μm; the mass spectrum showed M^+ at *m/e* 358.

Anal.—Calc. for $C_{23}H_{34}O_3$: C, 77.05; H, 9.56. Found: C, 77.41; H, 9.35.

3β-Hydroxy-5β,14α-card-20(22)-enolide (XII)—This compound was prepared by the procedure described for VI. From 18 mg of XI was obtained 8 mg (50%) of XII as white needles, mp 223–224° [lit. (5) mp 224–225°]; IR: 2.86 (OH), 5.6, and 5.71 (butenolide) μm; the mass spectrum showed M^+ at *m/e* 358.

3β-Acetoxy-14-chloro-5β,14β-card-20(22)-enolide (VII)—A mixture of 48 mg of 3β-acetoxy-14-chloro-15-oxo-5β,14β-card-20(22)-enolide (IV) (8), 0.15 ml of 1,2-ethanedithiol, and 0.12 ml of boron trifluoride etherate was warmed until a solution was obtained and kept 2 days at room temperature. Solid sodium carbonate was added and the mixture was extracted with chloroform. Purification as described for the preparation of VIII gave 35 mg of the thioketal (IX) as a gum which could not be crystallized. It was treated directly with deactivated Raney nickel in refluxing ethanol as described in the preparation of V. After the silica gel chromatography, 8 mg (27% overall) of VII was obtained as white powder, mp 157–159°; IR ($CHCl_3$): 5.61, 5.72, and 5.8 (butenolide, acetate) μm; the mass spectrum showed $M^+ - HCl$ at *m/e* 398.

Anal.—Calc. for $C_{25}H_{35}ClO_4$: Cl, 8.07. Found: Cl, 7.73.

3β-Acetoxy-15α-iodo-14-isocyanato-5β,14β-card-20(22)-enolide (XIV)—A slurry of 0.25 g of silver cyanate (16) and 0.25 g of iodine in 5 ml of tetrahydrofuran was stirred 1 hr at −20° and then treated dropwise with 0.4 g of 3-acetoxy-5β-card-14(15),20(22)-dienolide (XIII) (17) in 5 ml of tetrahydrofuran. The resulting mixture was stirred 1 hr at −20°, stirred 4 hr at room temperature, filtered, and concentrated under reduced pressure. The residue was dissolved in methylene chloride, washed with 0.1 *N* sodium thiosulfate and water, dried (magnesium sulfate), and concentrated. Recrystallization of the residue (350 mg) from chloroform–ether gave a white solid, mp 167–168°; IR (KBr): 4.42 (isocyanate), 5.61, 5.73, and 5.78 (butenolide, acetate) μm; NMR ($CDCl_3$): 5.90 (m, 1, vinyl of butenolide), 5.06 (m, 1, C-3 methine), 4.72 (m, 2, methylene of butenolide), 2.02 (s, 3, acetate), and 0.97 and 0.90 (each s, each 3, angular methyls) ppm.

Anal.—Calc. for $C_{26}H_{34}INO_5$: C, 55.03; H, 6.04; N, 2.47. Found: C, 55.16; H, 6.28; N, 2.25.

Biological Assay—Na^+,K^+-adenosine triphosphatase activity was determined by continuously monitoring[2], at 340 nm, the oxidation of NADH linked *via* a series of enzymatic reactions to the hydrolysis of ATP (18). In this assay, the ADP produced by the adenosine triphosphatase is rephosphorylated to ATP by the action of pyruvate kinase, which converts phosphoenolpyruvate to pyruvate. This reaction is coupled to the oxidation of NADH *via* lactic dehydrogenase, which reduces the pyruvate to lactate. For total adenosine triphosphatase activity, each cell contained 0.1 *M* NaCl, 0.01 *M* KCl, 0.05 *M* $MgCl_2$, 0.025 *M* tromethamine–ATP, 0.025 *M* tromethamine hydrochloride (pH 7.4), 0.05 *M* NADH, 0.025 *M* phosphoenolpyruvate, about 15 units of pyruvate kinase/ml, and about 15 units of lactic dehydrogenase/ml. After temperature equilibration at 37°, the reactions were started by the addition of the Na^+,K^+-adenosine triphosphatase preparation (usually about 100 μg). The Na^+,K^+-stimulated portion of the total adenosine triphosphatase was taken as the difference in activity of the adenosine triphosphatase in the absence and the presence of 0.001 *M* ouabain. Inhibition of Na^+,K^+-adenosine triphosphatase activity by the cardenolides was measured over a 10^{-9}–10^{-3} *M* range of concentrations. With some cardenolides, the degree of inhibition did not reach its maximum until after about 15 min of incubation. The K_I values reported were extrapolated from curves describing this steady level of inhibition at each concentration of cardenolide.

[2] A Gilford recording spectrophotometer equipped with a Haake constant-temperature bath was used.

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[x] To whom inquiries should be directed.

Effects of Milling on Granulation Particle-Size Distribution

G. STEINER, MAHENDRA PATEL, and J. T. CARSTENSEN ˣ

Abstract □ A series of lactose granulations made with various amounts of starch paste and water was subjected to milling through different screens and the particle sizes were determined. The distributions were log-normal when the size of the coarse material was determined by sieve analysis and the size of the fine material was determined microscopically. An equation was developed to describe the increase in surface area, ΔO, when material is milled through a screen with n holes of diameter d; this equation takes the form: $\log[n(\Delta O)] = -q(\log d) + p$, where q and p are constants.

Keyphrases □ Milling—effects on granulation particle-size distribution, equations □ Particle-size distribution—effects of milling on granulations, equations □ Granulations, lactose, starch, and water—used to determine effects of milling on granulation particle-size distribution, equations

Although milling is an essential step in tablet and capsule manufacture, the basic concepts of pharmaceutical comminution have received only a small degree of attention in the literature. The treatise by Parrott (1) is the only theoretical approach reported on milling of *granulations*. Milled granulations are usually considered to be log-normally distributed (2), although frequent deviations seem to occur.

Pharmaceutical granulations are, of course, heterogeneous, porous substances. In contrast, milling of pure and compact substances has been treated extensively, *e.g.*, in the literature of the mining and engineering sciences. Log-normal size distributions have frequently been reported (3–12), as have other distributions (13–15). The usual distribution functions in these latter instances are the Rosin–Rammler (16) and Alyavdin (17) equations. Harris (18) employed these and the Weibull (15) distribution in construction of special grids with a wide range of utility in powder description.

THEORETICAL

The Weibull (15) distribution has gained wide use, primarily due to its universal character; it is strictly phenomenological and based on a fractional undersize function, $f(x)$, which may be described by:

$$f(x) = 1 - e^{-\psi(x)} \qquad \text{(Eq. 1)}$$

According to Weibull (15), the function $\psi(x)$ is subject only to the general condition that it be a positive, nondecreasing function that vanishes at a value x_u. The value of x_u is not necessarily zero, and the simplest general function meeting these requirements is:

$$\psi(x) = [x - x_u]^\lambda / k \qquad \text{(Eq. 2)}$$

Harris (18) applied this to particle-size distributions. In this case, x is a dimension of the particle, *e.g.*, diameter, largest dimension, or cube root of volume. It is assumed that x_u is zero, so Eq. 2 then takes the form:

$$\psi(x) = [x/k]^\lambda \qquad \text{(Eq. 3)}$$

Table I—Composition of Granulating Fluid in the Granulations and Loss on Drying

Designation	Starch, g	Water, g	Loss on Drying[a], %
B-2	50	450	0.83
B-3	50	495	1.10
B-4	50	540	1.18
C-2	60	540	0.79
C-3	60	594	1.34
C-4	60	648	2.20
D-2	55	495	0.79
D-3	55	545	0.90
D-4	55	594	1.40

[a] One hundred grams of dried granulation was dried further until constant weight (w g) was recorded. The difference ($100 - w$ g) is assumedly moisture, so the percent loss on drying is $100 - w$.

where $f(x) = 1 - e^{-\psi(x)}$ is the fraction oversize in the particle-size analysis. The fraction undersize, y, is then given by:

$$y = 1 - f(x) = e^{-\psi(x)} = \exp[-(x/k)^\lambda] \qquad \text{(Eq. 4)}$$

or:

$$1/y = \exp[(x/k)^\lambda] \qquad \text{(Eq. 5)}$$

The logarithmic transformation of this is:

$$\ln(1/y) = [x/k]^\lambda \qquad \text{(Eq. 6)}$$

$$\log[\ln(1/y)] = \lambda[\log(x/k)] \qquad \text{(Eq. 7)}$$

Normal and log-normal distributions, as will be shown, "almost" fit this type of presentation; indeed, Eq. 5 is a function of wide applicability.

As opposed to the statistical empirical approach leading to Eq. 5, some model approaches for pure substances lead to log-normal distributions. For instance, Irani and Callis (12) showed that crystal growth leads to a log-normal distribution truncated from above. Fines destruction in crystallizers accomplish truncation from below, so, indeed, many crystalline substances are log-normally distributed. One purpose of this study was to show that pharmaceutical comminution results in a log-normal distribution rather than a Weibull distribution. It was also the aim to advance a rational method for treating sieve analysis data. Finally, a study of the energetics of milling was a logical outcome of the investigation.

EXPERIMENTAL AND RESULTS

Granulations were made by mixing 2325 g of lactose USP[1] and 175 g of starch[2] for 8 min in a 10-liter mixer[3]. Starch pastes of the compositions shown in Table I were made in a steam-heated vessel by adding cold distilled water to the starch and heating it under stirring until it became translucent. After cooling to 30°, it was added to the powder mix and kneaded for 17 min at low speed (50 rpm). The wet mix was passed through a No. 4A perforated round hole screen [opening 7.92 mm (0.312 in.)] in a comminuting machine[4] at 957 rpm.

The granulations were dried on trays in an oven at 54° for 4 hr. One hundred-gram samples were left in the oven until constant weight had been reached and then the loss on drying was calculated (Table I). As expected, the loss on drying values were di-

[1] Lot 08915, Ruger Chemical Co., Irvington, NJ 07111
[2] Pure corn starch ARGO, Best Foods Division, CPC International Inc., Englewood Cliffs, NJ 07632
[3] Hobart mixer, Hobart Manufacturing Co., Troy, Ohio.
[4] Fitzpatrick model M55, Fitzpatrick Co., Elmhurst, IL 60126

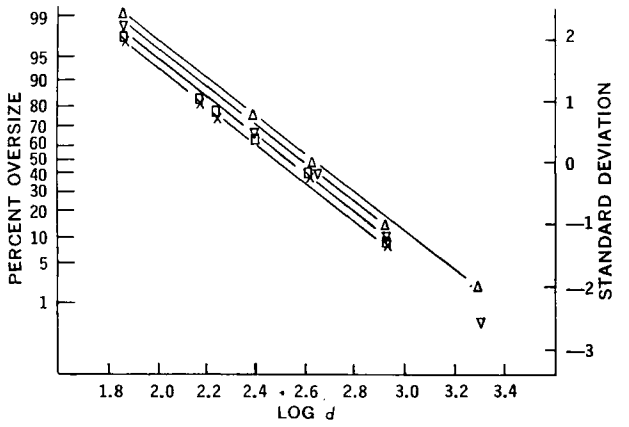

Figure 1—*Log-normal distribution of experimental Sample B-2. Key: △, screened through a 2B screen; ▽, screened through a 2A screen; □, screened through a 2AA screen; and ×, screened through a 0065 screen.*

rectly proportional to the amounts of water added in each group. The dried granulations were milled through a comminuting machine[4] at 1610 rpm using the following screens: 2A, 2AA, 2B, and 0065 with knives forward. The characteristics of these screens are listed in Table II.

Sieving tests were performed through series of screens of U.S. Standard sizes as follows: 10, 20, 40, 60, 80, 100, and pan or 20, 40, 60, 80, 100, 200, and pan. In general, a 5-min mimimum is required for good sieving operations; the shaker[5] used in these experiments was tested by the method of Fahrenwald and Stockdale (19) and it was found that 200 sec sufficed for 100% efficiency. Therefore, the general procedure for the sieving tests was to shake a sample of 100 g for 200 sec on the shaker[5] and to determine the weights of powder retained by the screens used. Specific gravity of the granulations was determined by a pycnometer, using toluene as the liquid. Particle-size distributions are shown graphically as percent oversize either on a log-probability grid when the distribution is log-normal or according to Eq. 7. The notation d is used to denote the largest dimension of the screen opening in the sieving tests; d is an estimate of x as described under *Theoretical*. Examples of data fitting a log-normal distribution are shown in Fig. 1, and examples of data presented according to Eq. 7 are shown in Fig. 2; least-squares fit values of λ and log k are shown in Table III.

Knowing the specific gravity and the mesh analysis allows calculation of the geometric surface areas (Table IV). The material

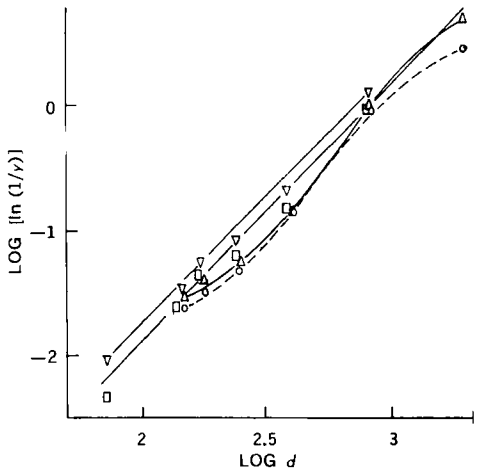

Figure 2—*Weibull plotting of data from experimental Sample C-2. Key: ○, screened through a 2B screen; △, screened through a 2A screen; □, screened through a 2AA screen; and ▽, screened through a 0065 screen.*

[5] Ro-Tap testing sieve shaker, W. S. Tyler Co., Cleveland, Ohio.

Table II—Screen Specifications

Screen Number	Number of Holes	Diameter[a] Inches	Diameter[a] Millimeters	Log of Diameter, mm
2B	2784	0.109	2.77	0.442
2A	2632	0.093	2.36	0.37
2AA	4200	0.079	2.01	0.303
0065	4165	0.065	1.65	0.21

[a] From: "Operating Instructions and Parts Manual, Model D Comminuting Machines," Fitzpatrick Co., Elmhurst, Ill.

passing the finest screen onto the pan was subjected to a microscopic count as described previously (20). A total of 10,000 particles was counted in each case. This is taken into account in Tables IV and V and Figs. 5 and 6 but not in Tables III and VI and Figs. 1–4; this will be subject to discussion.

DISCUSSION

The first point of discussion is the particle-size distributions. Only three samples (B-2, C-4, and D-2) appeared to be distributed log-normally on a weight basis. The remaining distributions, when plotted in log-normal fashion, yielded curves with downward curvature (Fig. 3). All of the curves, when plotted as Weibull functions (Eq. 7), give fairly good linearity (Fig. 2). One interesting aspect, as shown in Table III, is that the intercepts are fairly constant; that is, log k is, within an order of magnitude, the same for all of the granulations. More important, however, is the general tendency for slight S-shapes. On the surface, this might not seem significant; however, a normal or log-normal distribution will show exactly such an almost linear, but slightly S-shaped, relation when plotted according to Eq. 7 (Fig. 4). The original Weibull function was not purported to have a theoretical basis but was justified by the fact that the lack of theoretical basis is true for "other distribution functions applied to real populations from natural and biological fields" (15). The Harris grid

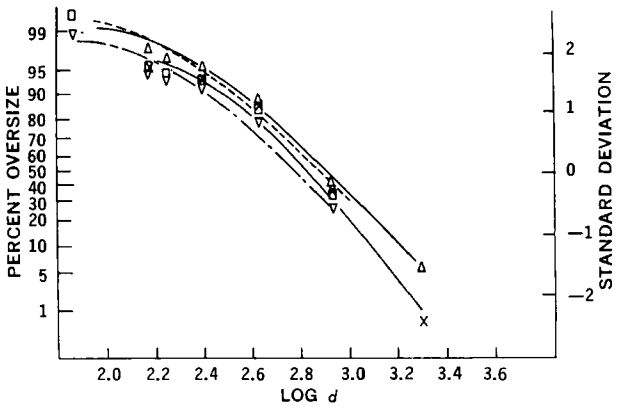

Figure 3—*Data from experimental Sample C-2 plotted in log-normal fashion. Key: △—, screened through a 2B screen; □- - -, screened through a 2A screen; × ——, screened through a 2AA screen; and ▽— - —, screened through a 0065 screen.*

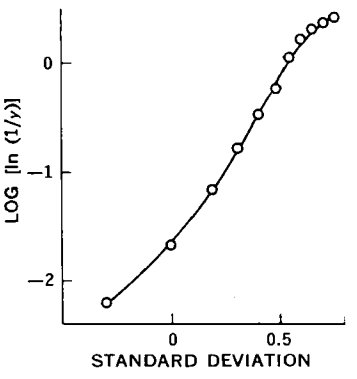

Figure 4—*Normal distribution plotted as a Weibull function.*

Table III—Weibull Parameters of Milled Granulations (Eq. 7)

Experiment Number	2B Screen		2A Screen		2AA Screen		0065 Screen	
	λ	log k	λ	log k	λ	log k	λ	log k
B-2	2.51	−3.1240	1.61	−2.7292	1.79	−2.6705	1.75	−2.6284
B-3	1.44	−2.9898	1.52	−2.8895	1.64	−2.8631	1.47	−2.8500
B-4	2.34	−3.0313	2.15	−3.0958	1.54	−3.3736	1.55	−3.1743
C-2	1.94	−3.0989	1.99	−2.9769	2.08	−2.9398	1.93	−2.9090
C-3	2.44	−2.848	2.08	−3.1477	1.04	−3.7728	1.13	−3.2810
C-4	1.34	−3.4113	1.35	−3.0350	0.90	−3.2127	0.88	−3.0937
D-2	1.73	−2.9367	1.82	−2.8744	2.03	−2.8014	1.89	−2.7893
D-3	2.51	−3.0411	2.28	−2.9994	1.75	−3.0901	1.80	−3.0544

Table IV—Geometric Surface Areas[a] of Test Granulations

Experiment Number	Unprocessed Granule	2.77-mm[b] Screen	2.36-mm[b] Screen	2.01-mm[b] Screen	1.65-mm[b] Screen
B-2	1.210	1.494	1.591	1.592	1.819
B-3	0.805	0.955	1.099	1.109	1.295
B-4	0.450	0.507	0.536	0.585	0.725
C-2	0.601	0.686	0.727	0.745	0.865
C-3	0.300	0.427	0.461	0.564	0.892
C-4	0.268	0.461	0.894	1.281	1.461
D-2	0.617	0.936	1.106	1.067	1.168
D-3	0.291	0.610	0.650	0.726	0.760
D-4	0.172	0.464	0.511	0.802	0.865

[a] Surface areas are in $m^2/100$ g. [b] Fitzpatrick round perforated plate size 2B, 2A, 2AA, and 0065 (see Table II).

was constructed to avoid "the kind of congestion frequently observed" (18) with other modes of plotting. An example is the conventional plotting of data in Figs. 1 and 3. Expanded scaling is necessary to show details in the graphs.

The distributions are not normal, so one is left with the choice of either an approximate fit to log-linear or a Weibull-type presentation. All curves are, however, only partially complete in the sense that the pan material from the sieve analysis is not broken down further. Therefore, samples were subjected to a microscopic count to estimate the particle-size distribution of the fine fraction (Table V and Fig. 5); the 2.36% by weight retained was broken down into subgroups (100–150, 50–100, 25–50, 10–25, and 5–10 μm, no particle being finer than 5 μm). It is noted from Fig. 5 that when the subdivision of the pan material is included, the distributions become log-normal. The distributions are shown as weight and number frequencies in Table V and as number distributions in Fig. 5; it is apparent from the Hatch-Choate (4) equation that there is no loss in generality in this conversion. In converting to the number distribution, more emphasis is placed on the finer fraction; the microscopic examination revealed that particles 50 μm and above in general were still agglomerates. Between 25 and 50 μm, some were prime particles (single crystals), and the 10–25-μm fraction appeared to be single crystals only. Therefore, it is logical to combine screen analysis data with a microscopic (or other suitable) count of the material retained on the pan and to treat the data as a number distribution in a log-normal fashion.

The milling experiments reported here give information relative to the energy actually imparted on the granulation during milling. To measure this directly is difficult; motor current can be used as an estimate of the amount of energy leaving the mill, but some (unknown) fraction of this is dissipated as heat, so that this (and other similar) measures are not indicative of the amount of energy transferred to the powder mass. There is evidence of long standing [as reported by Martin (21) and Gross and Zimmerley (22)] that the area of new surface (ΔO) produced in milling is proportional to the work input (E). This, essentially, is a direct

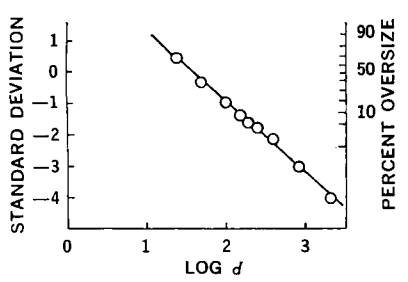

Figure 5—*Data from Table V plotted in log-normal fashion.*

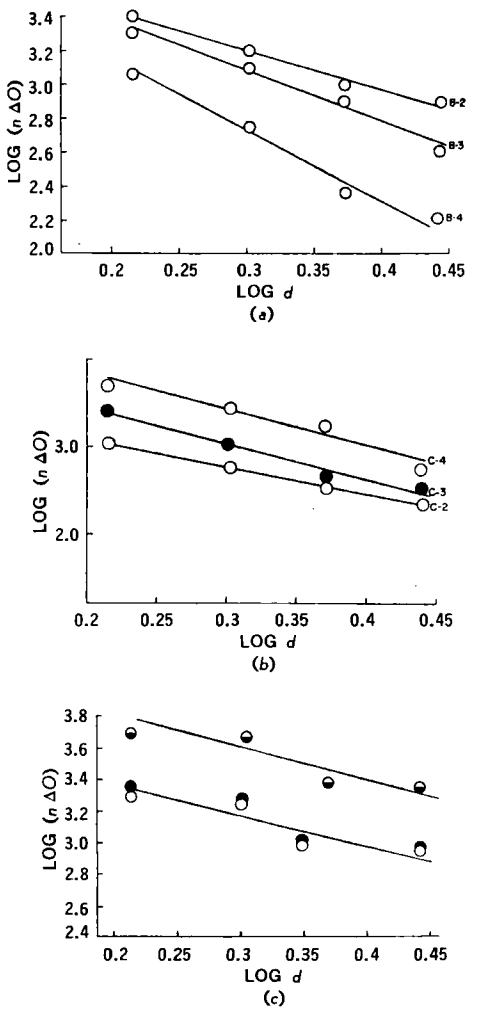

Figure 6—*Data from* (a) *B series,* (b) *C series, and* (c) *D series plotted according to Eq. 12. Key (for 6c):* ○, *Experiment D-2;* ●, *Experiment D-3; and* ◒, *Experiment D-4.*

Table V—Distribution of Sample C-2 Milled through a 2B Screen, with Inclusion of Distribution of Pan-Retained Material

Midsize Diameter, μm	Weight/100 g	10^{-3} × Number of Particles/100 g	Fraction by Number	Cumulative Fraction	Oversize Diameter, μm, d	Log d	Standard Deviation (t)
2680[a]	5.55	0.55	0.00003	0.00003	2000	3.301	−4.02
1420	35.15	23.45	0.001	0.001	840	2.924	−3.02
630	46.20	353	0.019	0.020	420	2.623	−2.05
335	8.55	435	0.023	0.043	250	2.398	−1.72
214	1.30	253	0.013	0.056	177	2.248	−1.58
163	0.80	353	0.019	0.075	149	2.173	−1.44
Pan:	2.45			0.075			
125	1.372	1342	0.071	0.146	100	2.00	−1.06
75	0.894	4049	0.213	0.359	50	1.699	−0.36
37.5	0.168	6087	0.321	0.679	25	1.398	+0.47
17.5	0.016	5705	0.300	0.979	10	1.00	+2.04
7.5	0.000086	390	0.021	1.000	5		

[a] Passes 6 mesh (3360 μm) and is retained by 10 mesh (2000 μm).

Table VI—Least-Squares Fit Statistics (Slope and Intercept) of Data in Table IV Fitted to Eq. 12

Experiment	Slope ($=-q$)	Intercept [$=\log(\beta/\gamma)$]	Moisture
B-2	−2.30	3.90	0.83
B-3	−3.04	4.00	1.10
B-4	−3.98	3.92	1.18
C-2	−3.03	3.69	0.79
C-3	−3.97	4.22	1.34
C-4	−4.15	4.66	2.20
D-2	−1.98	3.81	0.79
D-3	−1.74	3.70	0.90
D-4	−2.80	4.12	1.40

consequence of data originally published by Rittinger (23) and Dallavalle (24) and, when using these symbols, it takes the form:

$$E = \gamma(\Delta O) \qquad \text{(Eq. 8)}$$

where γ is a proportionality constant. It is noted in this study that there is a trend (Tables II and IV) that granule area increases as the screen plate hole area decreases.

In the screen, each hole offers a resistance associated with an energy expenditure, so that the energy is proportional to d^{-q}, where d is the diameter of the screen opening. The proportionality constant would have a dimension of erg cm^{+q}. The energy expenditure is inversely proportional to the number, n, of holes so that in all:

$$E = \beta(d^{-q}/n) \qquad \text{(Eq. 9)}$$

where β is an (unknown) proportionality constant. Combining this assumption with Eq. 8 then gives:

$$E = \beta(d^{-q}/n) = \gamma(\Delta O) \qquad \text{(Eq. 10)}$$

Rearranging gives:

$$\frac{\beta}{\gamma}(d^{-q}) = n(\Delta O) \qquad \text{(Eq. 11)}$$

The logarithmic form of Eq. 11 is:

$$-q(\log d) + \log(\beta/\gamma) = \log [n(\Delta O)] \qquad \text{(Eq. 12)}$$

Plotting of $\log[n(\Delta O)]$ *versus* $\log d$ is shown in Fig. 6. The least-squares fit parameters are shown in Table VI. It is noted that increasing water content increases q, *i.e.*, increases the resistance exponent. The term β/γ is (to the extent of order of magnitude comparisons) fairly constant as should be expected; *i.e.*, γ, the surface energy per square centimeter, is not a function of the size of the granulated particle or of the formulation. Thus, Eq. 12 appears to hold for the range of screens employed in the experiments.

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[x] To whom inquiries should be directed.

Diffusion Layer Effects on Permeation of Phenylbutazone through Polydimethylsiloxane

EDWARD G. LOVERING ˣ and DONALD B. BLACK

Abstract □ The permeation of phenylbutazone through polydimethylsiloxane was studied under conditions of varying membrane thickness, stirrer speed, and pH. The results are described in terms of the two-phase model of general transport theory and provide experimental evidence of its usefulness. It was found that permeation rates are controlled by the aqueous diffusion layer at the membrane surface, especially when permeation cells equipped with thin membranes are weakly stirred. Gentle agitation and thin membranes exist in the intestinal tract, so that drug absorption rates *in vivo* may be limited by a diffusion layer. Diffusion layer control may lead to a breakdown of the pH–partition theory because the ionic and nonionic forms of the drug diffuse through the aqueous diffusion layer. The diffusion of phenylbutazone in polydimethylsiloxane did not obey Fick's law of diffusion.

Keyphrases □ Phenylbutazone—diffusion layer effects on permeation through polydimethylsiloxane □ Permeation, drug—phenylbutazone through polydimethylsiloxane, diffusion layer effects □ Diffusion layer—effects on permeation of phenylbutazone through polydimethylsiloxane □ Polydimethylsiloxane—permeation of phenylbutazone, diffusion layer effects □ Membranes, polydimethylsiloxane—permeation of phenylbutazone, diffusion layer effects

As part of the drug absorption studies being carried out in these laboratories, the effects of dissolution rate, excipients, nutrients, pH, surfactants, and other materials on the permeation rate of drugs *in vitro* were investigated (1–3). During this work, it was found that the pH dependence of phenylbutazone permeation through polydimethylsiloxane membranes (3) could not be described by the pH–partition theory, as has usually been the case for drug permeation through synthetic membranes (1, 4–6). Breakdown of the pH–partition theory may arise from permeation of ions through the membrane or from aqueous diffusion layer effects at the membrane surface. Since permeation of ions through polydimethylsiloxane is unlikely, it appears that diffusion layer effects are responsible for the observed deviations from the pH–partition theory.

A two-phase model (7) of drug absorption was chosen to describe the experimental results. This model is suitable when the experimental conditions are chosen to maintain zero concentration of drug at the desorbing surface of the membrane. From the work of Suzuki *et al.* (7), it can be shown, for weak acids, that:

$$\frac{m}{C_0} = \frac{D_m K_p A}{l}\left\{1 + e^{2.303(\mathrm{pH}\,-\,\mathrm{pKa})} + \frac{1}{T}\right\}^{-1} \qquad \text{(Eq. 1)}$$

where:

$$T = \frac{D_a l}{K_p h D_m} \qquad \text{(Eq. 2)}$$

In these equations, m is the permeation rate of drug through the membrane; C_0 is the concentration of drug in the perfusing solution; D_m and D_a are the diffusion coefficients of the drug in the membrane and in the solution, respectively; K_p is the partition coefficient of the drug between the membrane and the solution; A and l are the area and thickness of the membrane, respectively; pKa is the ionization constant; and h is the thickness of the aqueous diffusion layer in contact with the membrane. Equation 1 reduces to the equation obtained from pH–partition theory if T^{-1} is small and can be neglected (8). The term T^{-1} is not negligible if the permeation rate is dependent upon or controlled by the aqueous diffusion layer in contact with the membrane. Conditions leading to diffusion layer control are low rates of agitation and very thin membranes.

Deviations from the behavior predicted by the pH–partition theory have been observed in experiments involving animal membranes (9–11), in cells constructed especially to demonstrate the diffusion layer problem (12), and in buccal absorption (13, 14). The effect of alkyl chain length on the diffusion layer has been reported (15). The phenylbutazone permeation results described in this paper, however, appear to be the first deviation involving a widely used drug and a synthetic rubber membrane. To ascertain whether the two-phase model is applicable to these results and to investigate some consequences of diffusion layer control, the effects of membrane thickness and stirrer speed on the permeation of phenylbutazone through polydimethylsiloxane were measured.

EXPERIMENTAL

Permeation Experiments—The effect of membrane thickness on permeation rate was measured using a cell similar to that described by Garrett and Chemburkar (4), except that the membranes were attached to the cells with silicone adhesive. Stirring rate experiments were done in the system depicted schematically in Fig. 1. The cells were placed in a beaker, at 37.0 ± 0.1°, containing drug solution buffered with a citric acid–disodium phosphate system having an ionic strength of 1.20. The desorbing solution within the cell was borate buffer at pH 10, ionic strength 0.78, ensuring a virtually zero concentration of unionized phenylbutazone within the cell. During the experiment, the change in concentration of the drug on both sides of the membrane was negligible and a steady rate of permeation was achieved. The drug concentration in the desorbing solution was measured by pumping the solution continuously through a UV spectrophotometer set at 265 nm. Phenylbutazone USP reference standard was used for the Beer's law calibration, giving a molar absorptivity of 21,050.

Agitation—During experiments designed to investigate the effect of membrane thickness on the permeation rate, the internal and external solutions were stirred gently to prevent the formation of concentration gradients within the solutions. In the stirring rate experiments, a three-bladed stainless steel impeller was mounted 3.2 cm above the membrane (Fig. 1). The impeller, 5 cm in diameter, had blades mounted at an angle of about 45° to the shaft, which was turned to drive the solution down onto the membrane.

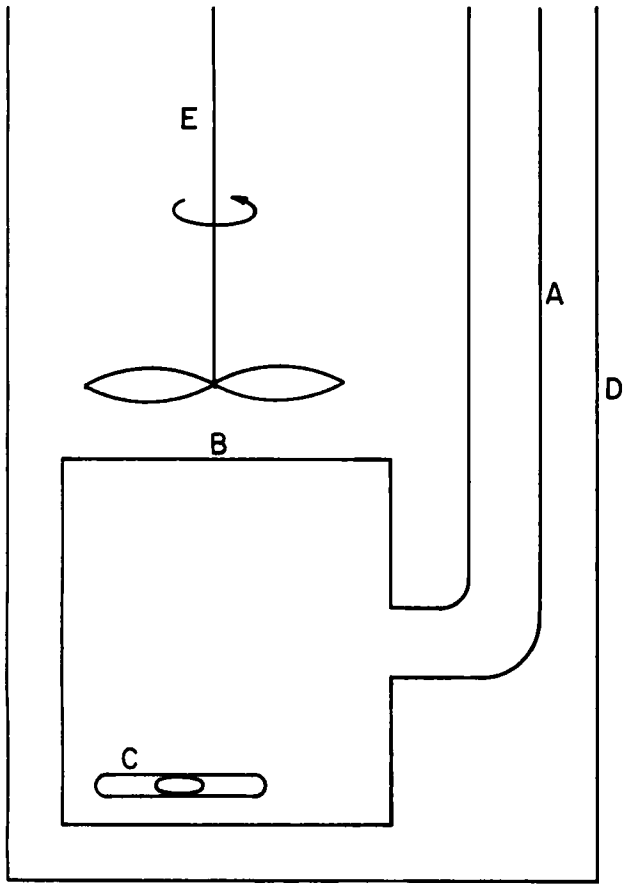

Figure 1—*Schematic diagram of the apparatus used to study stirring rate effects. Glass cell A with polydimethylsiloxane membrane B is stirred internally by magnetic stirring bar C. The cell is mounted in beaker D containing drug solution and stirred by impeller E.*

The shaft was driven by an adjustable stirrer[1] and the rate was measured with a tachometer. The inside of the cell was stirred to prevent the formation of concentration gradients.

Partition Coefficients—Weighed segments of polydimethylsiloxane membrane were immersed in buffered solutions of phenylbutazone, pH 4.0, for 48 hr at 37.0 ± 0.1°. Upon removal from the drug solution, the membrane was rinsed under distilled water, quickly patted dry, and placed in a borate buffer solution, pH 10, at 37°. The concentration of desorbed drug in the buffer was measured spectrophotometrically after allowing 48 hr for desorption. The density of the membrane was estimated from its weight and physical dimensions to be 1.13 g cm^{-3}.

Materials—Phenylbutazone[2] was used throughout. The thicknesses of the polydimethylsiloxane membranes were found by direct measurement to be 0.003[3], 0.015[4], and 0.025[4] cm. All are believed to contain about 30 parts of silica filler per 100 parts of polymer.

RESULTS AND DISCUSSION

The steady-state permeation rate of phenylbutazone through the polydimethylsiloxane membranes was measured under varying conditions of pH, agitation, and membrane thickness. The results were plotted as moles of drug permeated *versus* time. From these graphs the steady-state slope, m, and time lag, t^*, were estimated. Time lag is the point at which the extrapolated steady-state rate intersects the time axis.

[1] Fisher Stedi-Speed.
[2] Ciba Geigy Co., Montreal, Canada.
[3] General Electric Co., Schenectady, N.Y.
[4] Dow-Corning Co., Midland, Mich.

Table I—Parameters Used to Calculate Theoretical Curves for Varying Membrane Thickness

Membrane Thickness, cm	D_mK_p, (cm^2 sec^{-1}) × 10^5	T	pKa	Area, cm^2
0.003	8.16 ± 0.85	0.0093	4.5	8.62
0.015	6.41 ± 0.42	0.061	4.5	8.62
0.025	6.81 ± 0.98	0.082	4.5	8.62

Table II—Parameters Used to Calculate Theoretical Curves for Varying Agitation Rates

Agitation Rate, rpm	D_mK_p, (cm^2 sec^{-1}) × 10^5	T	pKa	Area, cm^2
47 ± 1	8.01 ± 0.28	0.119	4.5	4.46
372 ± 4	6.72 ± 0.56	0.54	4.5	4.46

Effect of Membrane Thickness—Permeation rates over a range of pH values were measured for membranes 0.003, 0.015, and 0.025 cm thick. The results are given in Fig. 2 as plots of m/C_0 *versus* pH. Figure 2 also includes theoretical curves of m/C_0 *versus* pH calculated from Eq. 1. Values of D_mK_p (by definition the permeability coefficient, $P = D_mK_p$) and T were adjusted by trial and error to give the best fit to the data for each membrane. The values used are given in Table I and, for D_mK_p, are in good agreement with those reported previously (3). Equation 2 predicts that the ratio of the membrane thicknesses will be the same as the ratio of T for each membrane. The actual ratios are 1:5:8.3 and 1:6.5:8.8, respectively. The difference between the actual and predicted values, which exceeds the experimental error, may arise from differences in filler content and cross-link density in the membranes studied. Variation in the latter would have a direct bearing on K_p and D_m. The displacement to the right of the m/C_0 curves (Fig. 2) as the membrane thickness decreases is a consequence of increasing diffusion layer control of the permeation process. Diffusion layer control may lead to a breakdown of the pH–partition theory because both the ionic and nonionic forms of the drug diffuse through the boundary layer. If diffusion is membrane controlled, only the nonionic form of the drug enters and diffuses through the membrane. The rate of partitioning into the membrane depends upon the concentration of nonionized drug in the solution, a function of the pH of the solution and the pKa of the drug.

The walls of the GI tract consist of a relatively thick diffusion layer (the mucus) and a relatively thin membrane (the cell wall). Under these conditions, diffusion layer control of the absorption rate might be expected. The *in vivo* absorption of acidic drugs may not, therefore, be adversely affected by the pH of the intestine to the extent predicted by the pH–partition theory. This postulate is corroborated to some extent by the observation that the peak blood level in human subjects given phenylbutazone in solution is observed within 30 min of ingesting the drug (16), although absorption from the stomach, while unlikely, cannot be precluded. Diffusion layer effects may account for the abnormalities observed by Hogben *et al.* (9) and Nogami and Matsuzawa (10, 11), making

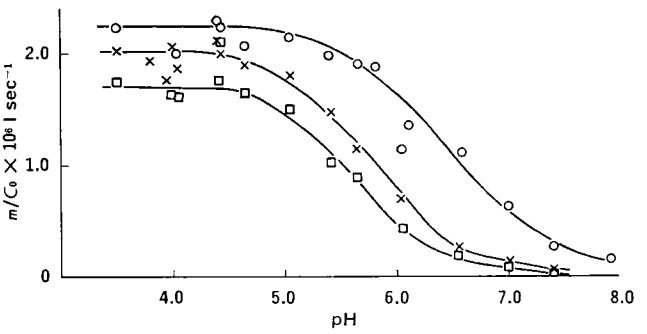

Figure 2—*Plot of* m/C_0 versus *pH. Membrane thicknesses were:* ○, *0.003 cm;* ×, *0.015 cm; and* □, *0.025 cm.*

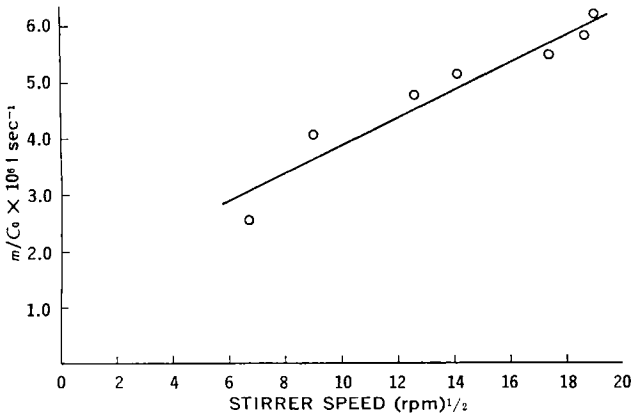

Figure 3—*Plot of* m/C_0 versus *the square root of the stirrer speed; pH = 5.03, l = 0.015, and* A = *4.46 cm².*

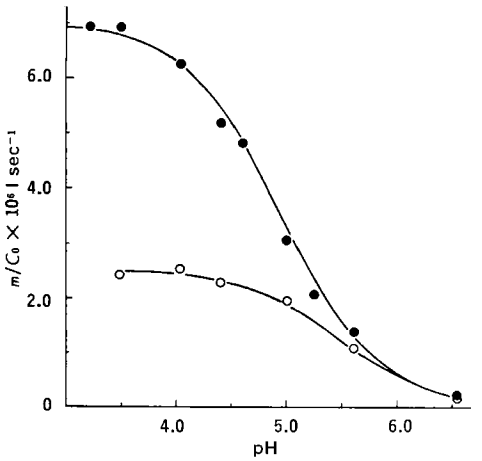

Figure 4—*Plot of* m/C_0 versus *pH; l = 0.015 cm, and* A = *4.46 cm². Stirring speeds were:* ○, *47 rpm; and* ●, *372 rpm.*

it unnecessary to invoke special mechanisms to account for their experimental results.

Effect of Agitation—According to Levich (17), the thickness of a diffusion layer is inversely proportional to the square root of the velocity at which liquid flows past the surface giving rise to the layer. In the present system, liquid is impelled onto the membrane surface and flows across the membrane at a velocity proportional to the stirrer speed, at least at low stirrer speeds. Under diffusion-controlled permeation, the term [exp 2.303(pH − pKa)] in Eq. 1 becomes small compared to T^{-1} and Eq. 1 reduces to:

$$\frac{m}{C_0} \sim \frac{AD_a}{h} \qquad \text{(Eq. 3)}$$

Equation 3 predicts that a plot of m/C_0 *versus* the square root of the stirrer speed will be linear, as was the case for data obtained at pH 5.03 (Fig. 3).

The effect of pH on the permeation rate through 0.015-cm membranes was measured at two levels of agitation, 47 ± 1 and 372 ± 4 rpm. The results are given in Fig. 4 along with the curves calculated from Eq. 1. The parameters used to obtain the best fit of Eq. 1 to the data are given in Table II. While D_mK_p remained essentially unaffected by the stirring rate, T increased by a factor of 4.5, thus showing its dependence on the diffusion layer h (Eq. 2). As the stirring rate increased, the thickness of the unstirred boundary diffusion layer decreased and the rate at which drug permeated the membrane increased. If stirring rates were increased sufficiently, the permeation rate would no longer be controlled by the aqueous diffusion layer.

Diffusion Coefficient—The diffusion coefficient of phenylbutazone in polydimethylsiloxane was determined by the time lag method and the permeation solubility method (18). Under steady-state conditions:

$$t^* = \frac{l^2}{6D_m} \qquad \text{(Eq. 4)}$$

where D_m is the diffusion coefficient of phenylbutazone in the studied membrane and l is the thickness of the membrane. The diffusion coefficients were (1.3 ± 0.7) × 10^{-7} and (1.3 ± 0.5) × 10^{-7} cm² sec^{-1} for the 0.015- and 0.025-cm membranes, respectively (Table III). The poor experimental reproducibility (coefficient of variation of 30–40%) is typical of the time lag method. Siegel and Coughlin (19) discussed the difficulties associated with this method.

The partition coefficient of phenylbutazone in equilibrium between the 0.025-cm polydimethylsiloxane membrane used in these experiments and aqueous buffer, pH 4.0, is 83 ± 9, based upon the mean of three experiments. The diffusion coefficient (0.025-cm membrane) calculated from the permeability coefficient (Table I) and the partition coefficient ($P = D_mK_p$) is 8.2 × 10^{-7} cm² sec^{-1}, significantly greater than the value obtained by time lag measurements (Table III).

The lack of agreement between the two methods indicates non-Fickian diffusion behavior and is not unexpected in view of the work of Most (20) and Flynn and Roseman (21), who studied the effect of silica filler on diffusion in polydimethylsiloxane. Diffusion in polyethylene membranes was recently shown to be non-Fickian (22–24). All polyethylenes are partially crystalline and do not meet the Fickian requirement of homogeneity, which was one boundary condition assumed in the derivation of the relationship $P = D_mK_p$. Since few polymeric membranes are actually homogeneous, it appears that the time lag and the permeation solubility methods are of limited practical applicability for the determination of diffusion coefficients.

Table III—Lag Times and Diffusion Coefficients

0.015-cm Membrane		0.025-cm Membrane	
t^*, sec	D_m, (cm² sec^{-1}) × 10^7	t^*, sec	D_m, (cm² sec^{-1}) × 10^7
420	0.9	360	2.8
420	0.9	1140	0.9
480	0.8	840	1.2
240	1.6	1080	0.9
300	1.3	660	1.5
180	2.1	900	1.1
360	1.0	720	1.4
120	3.1	1200	0.8
360	1.0	720	1.4
540	0.7	1260	0.8
540	0.7	960	1.1
180	2.1	780	1.3
420	0.9	540	1.9
350 ± 138	(1.3 ± 0.7) × 10^{-7}	860 ± 267	(1.3 ± 0.5) × 10^{-7}

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[x] To whom inquiries should be directed.

Diffusion of Benzocaine from Ointment Bases

JAMES W. AYRES[x] and PAUL A. LASKAR*

Abstract □ The release of benzocaine from oleaginous, absorption, emulsion (water-in-oil and oil-in-water), and water-soluble ointment bases *via* dialysis through a cellulose membrane to an aqueous sink was studied at 37.5°, and benzocaine release from some commercially available products was compared with experimental preparations. The rate of release was found to be greater from water-soluble bases than from other bases and was generally dependent on the concentration of drug in the base. An explanation is offered for the case in which the rate of release is not concentration dependent.

Keyphrases □ Benzocaine—release from various ointment bases, dialysis through cellulose membrane □ Ointment bases—comparison of rate of release of benzocaine, dialysis through cellulose membrane □ Vehicles—diffusion of benzocaine from various ointment bases

It is well recognized that one important function of an ointment base is the control it exerts over the release and, therefore, the therapeutic activity of the medication it carries. For topical preparations, there are two general approaches to the formulation problem of maximizing the absorption of the active ingredient from the vehicle. One approach is to include an agent that affects the barrier function of the epidermis, and the second is to alter the physical characteristics of the vehicle and, thus, the diffusion of the drug from the vehicle to the skin (1). When considering only the second approach, an experimental procedure that measures the release of drug from an ointment base should be of value in determining a base of choice for the formulation of an active ingredient. The methods available for measuring the release of drug from a semisolid were recently reviewed (2). Although some investigators have tried to develop models for the prediction of the relative release rates of a drug from various vehicles, it is generally felt to be impossible (2). Before it can be said that a drug is released best by a specific base, the release rates must be compared by some experimental means.

Local anesthetics have been widely used in therapeutic, diagnostic, and surgical situations because of their ability to inhibit pain and itch sensations by reversibly blocking both generation and conduction of impulses in nervous tissue. These agents have been administered by a variety of routes, but topical application for the relief of pain and pruritis is generally used by the lay public. A significantly large number and variety of these products are readily available for purchase as nonprescription items. Among the categories of products marketed that frequently include a local anesthetic in their formulation are burn and sunburn preparations, hemorrhoidal products, throat sprays and lozenges, eczema and psoriasis remedies, teething lotions, otic products, and topical first-aid preparations. Benzocaine (ethyl *p*-aminobenzoate) is an extensively used local anesthetic and is commercially available in concentrations varying from a declared amount of 0.5 to 20.0%.

An excellent method has been reported to measure the effectiveness of local anesthetics when applied to the intact skin of human subjects (3). Thirty commercially available products and some specially formulated solutions were evaluated with respect to their ability to block the sensation of itch and burning induced by an electrical current. Not all of the commercial products contained benzocaine; but of those that did, only one was found to be effective in obtunding the sensation of itch and burning associated with electrical stimulation of an area burned with UV light.

The purposes of this investigation were to: *(a)* utilize a simple dialysis cell method to compare the ef-

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Table I—Composition, Type, and Benzocaine Concentration of Various Ointment Bases

Ointment	Type of Base	Benzocaine, %
A	Oleaginous[a]	20.0
B	Oleaginous	10.0
C	Oleaginous	5.0
D	Absorption[b]	20.0
E	Absorption	10.0
F	Absorption	5.0
G	Emulsion (w/o)[c]	20.0
H	Emulsion (w/o)	10.0
I	Emulsion (w/o)	5.0
J	Emulsion (o/w)[d]	20.0
K	Emulsion (o/w)	10.0
L	Emulsion (o/w)	5.0
M	Water soluble[e]	20.0
N	Water soluble	10.0
O	Water soluble	5.0
P	Water soluble	2.5
Q	Water soluble	1.0
R	Water soluble[f]	20.0
S	Absorption[g]	—[i]
T	Emulsion (o/w)[h]	—[i]
U	Emulsion (w/o)[j]	1.25
V	Emulsion (o/w)[k]	2.0

[a] White petrolatum. [b] Aquaphor, Duke Laboratories, South Norwalk, Conn. [c] Aquaphor–water (1:1). [d] Neobase, Burroughs Wellcome and Co., Research Triangle Park, N.C. [e] Polyethylene glycol ointment USP. [f] Americaine Ointment, Arnar-Stone Laboratories, Mt. Prospect, Ill. [g] Rectal Medicone Ungent, Medicone Co., New York, N.Y. [h] Solarcaine Lotion, Plough, Inc., Memphis, Tenn. [i] Quantitative statement not provided. [j] Burn-A-Lay first aid cream, Kendall Co., Chicago, Ill. [k] Kip Sunburn Windburn Lotion, Rabin-Winters, El Segundo, Calif.

fect of various ointment bases on the rate and amount of drug (benzocaine) released; *(b)* prepare some experimental formulations and compare the release of benzocaine from these products with one previously found effective in humans (3), as well as some other commercially available products; and *(c)* determine the effect on dialysis rate of varying the concentration of benzocaine in an ointment base.

EXPERIMENTAL

Reagents—Ethyl *p*-aminobenzoate (mp 89–91°), *N*-1-naphthylethylenediamine dihydrochloride (mp 185–191°), sodium nitrite (reagent), ammonium sulfamate (practical), barium chloride (reagent), zinc sulfate (reagent), and polyethylene glycols were obtained from a single source[1]. The barium hydroxide and trichloroacetic acid were both reagent grade[2]. The dialysis membrane[3] used was from a single roll and was allowed to hydrate in distilled water for at least 24 hr prior to use.

Equipment and Methodology—Ointments were prepared by fusion or spatulation on a weight/weight basis. All commercial preparations were spatulated immediately prior to use to remove air bubbles and were tightly packed into glass drug reservoirs (3.1 cm deep and 2.4 cm in diameter). For the dialysis experiments, the hydrated membrane was placed over the opening of the drug reservoir in direct contact with the ointment. The surface was rolled gently with a wet stirring rod to smooth it and remove all air from between the ointment and the membrane, which was then secured in place with an O-ring. The dialysis cell was inverted and immersed slightly in 1000 ml of distilled water in a 1000-ml beaker maintained at 37.5 ± 0.5°, being careful to keep the water level below the O-ring. No attempt was made to control the temperature of the diffusion cell or to stir the receiving "sink." From time zero to 2 hr, at 20-min intervals, 5-ml samples were pipeted from the sink and replaced with 5 ml of distilled water at 37.5°. Care was taken to avoid stirring or agitation with the sampling pipet.

[1] J. T. Baker Chemical Co., Phillipsburg, N.J.
[2] Mallinckrodt, St. Louis, Mo.
[3] Cellophane 215 PD-62, E. I. duPont de Nemours and Co., Inc., Wilmington, Del.

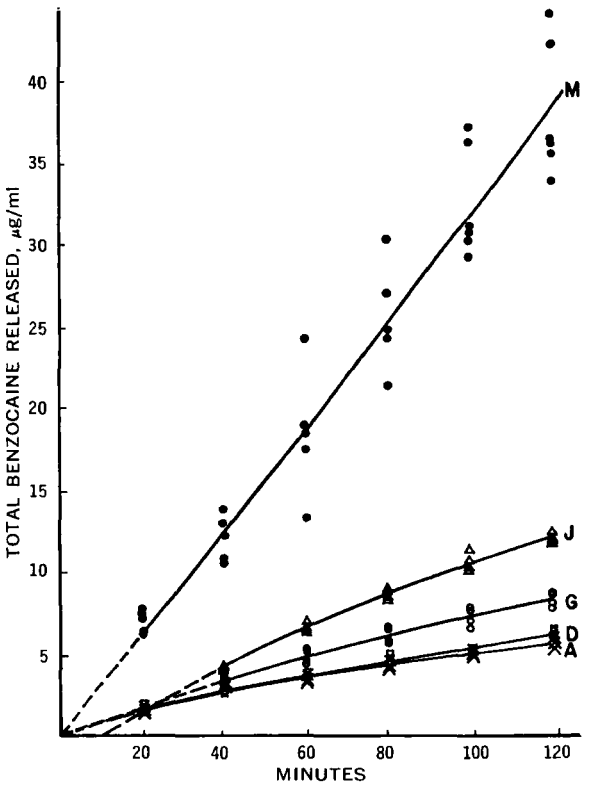

Figure 1—*Effect of ointment base composition on release of drug from preparations containing 20% (w/w) benzocaine. Key: see Table I.*

Analytical Method—The analysis of the benzocaine released during the *in vitro* test was carried out by the method of Matsumoto *et al.* (4). Aliquot portions of a sample solution were diluted appropriately, 5 ml of the dilution was pipeted into a test tube followed by 2 *N* HCl (2 ml) and 0.2% $NaNO_2$ (0.4 ml), and the

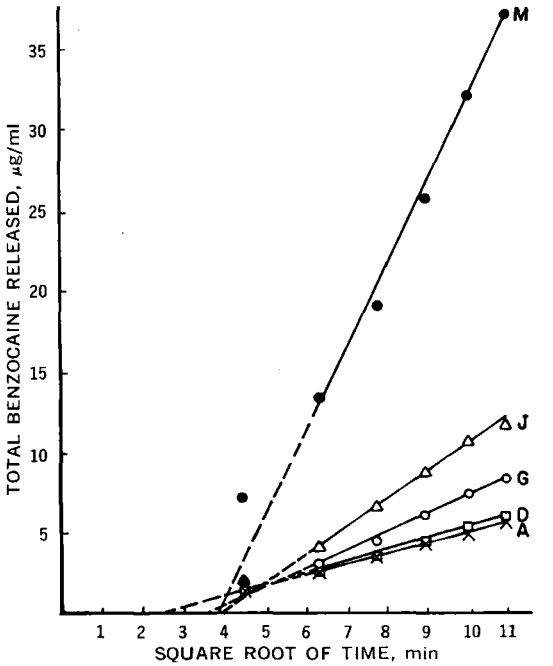

Figure 2—*Correlation between total benzocaine released and square root of time for preparations containing 20% (w/w) benzocaine. Key: see Table I.*

Table II—Summary of Calculated Statistical Parameters: Linear Model

Product	Intercept (b_0)	Standard Error of Intercept	Regression Coefficient (b_1)	Standard Error of Regression Coefficient	Sums of Squares: Regression	Degrees of Freedom: Regression	Sums of Squares: Residual	Degrees of Freedom: Residual
A	−1.368	0.121	0.633	0.015	46.386	1	0.534	22
B	−0.713	0.215	0.430	0.026	42.709	1	7.002	46
C	−0.713	0.103	0.281	0.012	9.127	1	0.383	22
D	−1.657	0.225	0.694	0.027	55.686	1	1.847	22
E	−0.992	0.109	0.472	0.013	5.767	1	0.433	22
F	−0.720	0.069	0.341	0.012	13.450	1	0.335	22
G	−2.786	0.347	1.009	0.042	117.770	1	4.385	22
H	−1.522	0.250	0.663	0.030	50.848	1	2.262	22
I	−1.337	0.156	0.604	0.019	42.175	1	0.886	22
J	−5.666	0.362	1.619	0.043	303.171	1	4.763	22
K	−3.742	0.270	1.207	0.032	168.337	1	2.639	22
L	−2.419	0.150	0.814	0.018	76.597	1	0.816	22
M	−16.881	1.481	5.022	0.177	4373.990	1	184.654	34
N	−38.588	2.779	9.037	0.332	14164.603	1	650.424	34
O	−40.600	3.375	8.882	0.403	20516.553	1	2152.743	51
P	−35.997	4.502	7.381	0.538	9449.051	1	1707.125	34
Q	−17.841	1.473	3.624	0.176	3036.200	1	329.837	46
R	−17.300	2.074	4.915	0.248	4188.757	1	362.245	34
S	−0.197	0.065	0.307	0.008	10.877	1	0.155	22
T	−0.011	0.144	0.292	0.017	9.832	1	0.752	22
U	−0.312	0.048	0.209	0.006	5.027	1	0.085	22
V	−1.077	0.132	0.369	0.016	27.586	1	1.999	40

mixture was shaken for 5 min. Then 0.5% $NH_4SO_3NH_2$ (0.4 ml) was added to this mixture and it was shaken for 3 min. Then 0.5% of *N*-1-naphthylethylenediamine dihydrochloride (1.0 ml) was added with shaking. After 30 min of intermittent shaking, the percent transmittance was measured[4] at 550 nm and the concentration of benzocaine was determined from a standard curve.

The dialysis of the polyethylene glycols was detected turbidimetrically (5). An appropriate volume of sample was diluted to 1.0 ml and added to 10 ml of distilled water. To this was added 1.0 ml of 10% (w/v) anhydrous barium chloride and 2.0 ml of 0.3 *N* barium hydroxide, with swirling after each addition. Next, 2.0 ml of 5% (w/v) zinc sulfate was added, the containers were covered with parafilm, and the mixture was shaken vigorously. After 10 min the contents were filtered[5] and 1.0 ml of the filtrate was mixed with 4 ml of 30% (w/v) trichloroacetic acid containing 5% (w/v) anhydrous barium chloride. All results were read 1 hr after adding the last reagent at 650 nm in a spectrophotometer[4], using the appropriate filter and light source. The amount of dialysis was determined using a standard curve constructed for either polyethylene glycol 400 or polyethylene glycol 4000.

RESULTS AND DISCUSSION

Table I shows the composition of the ointment bases selected to investigate variations in rate of release and dialysis of benzocaine. Since the active ingredient content of many commercial products is reported as a percentage rather than an amount, all products

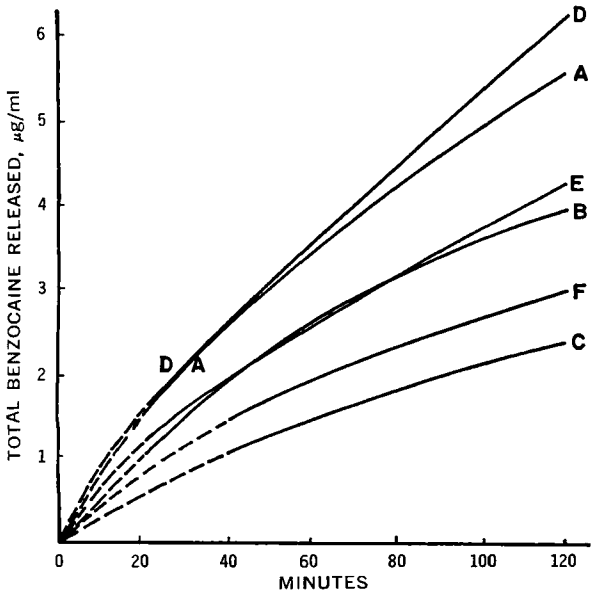

Figure 3—*Effect of varying drug concentration on release of benzocaine from oleaginous and absorption bases. Key: see Table I.*

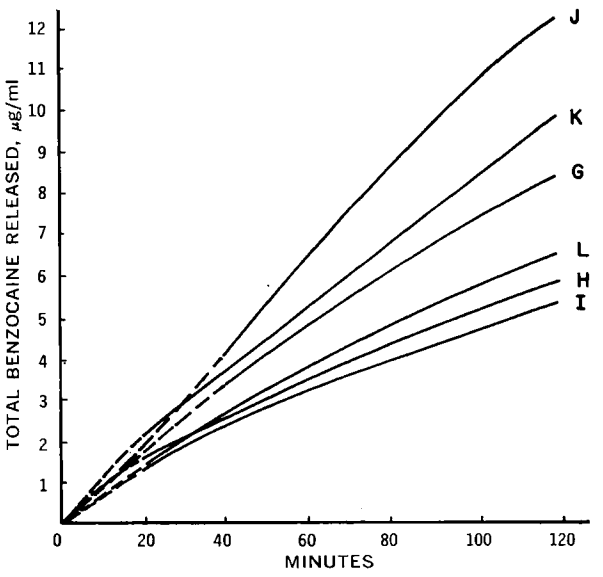

Figure 4—*Effect of varying drug concentration on release of benzocaine from emulsion (oil-in-water and water-in-oil) bases. Key: see Table I.*

[4] Spectronic 20, Bausch and Lomb, Rochester, NY 14602

[5] Whatman No. 42 filter paper, double thickness.

Table III—Individual Comparisons Made

Product	Product[a] V	U	T	S	R	Q	P	O	N	M	L	K	J	I	H	G	F	E	D	C	B	A
A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ns	*	*	–
B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ns	–	*	–	
C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	~*	–	–	–		
D	–	–	–	–	–	–	–	–	–	–	–	–	–	ns	ns	*	*	*	–			
E	–	–	–	*	–	–	–	–	–	–	–	–	–	*	*	–	*	–				
F	–	*	–	ns	–	–	–	–	–	–	–	–	–	*	–	–	–					
G	–	–	–	–	–	–	–	–	–	–	–	–	*	*	*	–						
H	–	–	–	–	–	–	–	–	–	–	*	*	–	ns	–							
I	–	*	–	*	–	–	–	–	–	–	*	–	–	–								
J	–	–	–	–	*	*	–	–	–	*	*	*	–									
K	–	–	–	–	–	–	–	–	–	–	*	–										
L	*	–	*	–	–	–	–	–	–	–	–											
M	–	–		–	–	ns	*	*	–	–												
N	–	–	–	–	*	*	ns	ns	–													
O	–	–	–	–	–	*	ns	–														
P	–	–	–	–	*	*	–															
Q	–	–	–	–	*	–																
R	*	*	*	*	–																	
S	–	*	–	–																		
T	~*	–	–																			
U	–	–																				
V	–																					

[a] ns = not significant, ~ * = barely significant, * = significant, and – = not tested for significance.

prepared were made on a percent weight/weight basis. The specific gravity of the bases used varied; therefore, the total benzocaine present also varied for products with different bases but the same concentration of benzocaine.

The penetration of a topically applied drug from an ointment can be modified simply by altering the composition of the vehicle (6). Figure 1 illustrates the variation in drug release from different ointment bases containing the same concentration (w/w) of benzocaine. Individual points are included to show the range of results obtained[6].

The diffusion of drug from semisolids can be described by equations derived for defined physical models. Most models require that the drug alone is allowed to diffuse out of the vehicle. In this experiment, both polyethylene glycol 400 and polyethylene glycol 4000 were found to diffuse through the membrane in the study of benzocaine diffusion from polyethylene glycol ointment USP. Regardless of which physical models are appropriate for the conditions and systems utilized in this study, it was determined that the experimental data were fairly well approximated by a straight line when the amount of drug released was plotted *versus* $t^{1/2}$ for the time period studied. The mean values of at least six determinations for the amount of drug released *versus* $t^{1/2}$ for the products in Fig. 1 are shown in Fig. 2. A least-squares line for the amount of drug released *versus* $t^{1/2}$ was generated for all products in Table I, and the parameters obtained were used to test the null hypothesis that the regression coefficients were identical. A summary of the calculated statistical parameters is given in Table II.

Table III shows the comparisons among rates of benzocaine release from the various semisolid bases as measured by the regression coefficient of the total benzocaine released on the square root of time. It is obvious that all comparisons made are not statistically independent. Therefore, when a comparison is noted as significant at the 95% confidence level, it is meant that the rates of release for the two products under comparison are likely to be different but at a confidence level slightly less than 95%. If the result is noted as "barely significant," then under more rigorous testing the result might not prove to be significantly different. It is true, however, that if regression coefficients were found not to be significantly different, it is highly unlikely that a Type II error was made.

Hydration of skin results from water diffusion from underlying epidermal layers or from perspiration that accumulates after the application of an occlusive vehicle or covering on the surface. Under occlusive conditions, the stratum corneum water content may change from the normal 5–15% to as much as 50% and permeability increases markedly (7). Oleaginous bases are occlusive and might, therefore, be considered desirable as vehicles for topically applied drugs. Figure 3 shows[7] the relative release of benzocaine from two oleaginous vehicles, each containing three different concentrations of drug.

Emulsion bases may exhibit either oleaginous or aqueous properties, depending on whether they are water-in-oil or oil-in-water emulsions, respectively. A survey of the literature concerned with the release of drugs from these vehicles leads to the conclusion that prediction of which base will best release the drug is not possible. The dialysis results utilizing varying concentrations of drug

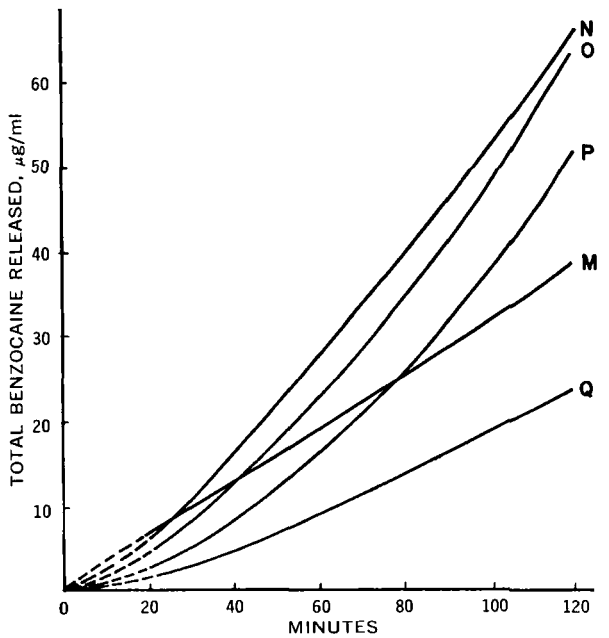

Figure 5—*Effect of varying drug concentration on release of benzocaine from a water-soluble base. Key: see Table I.*

[6] Similar ranges were obtained for all curves in Figs. 3–6 but were not included due to visual indistinguishability.

[7] In Figs. 3–6 the lines shown are those predicted using an iterative curve-fitting procedure and a digital computer. At least four determinations were made at each time, and the line was obtained from the following general equation: $Y = B_1 + B_2e^{B3X}$. Point spread about the lines was similar to that seen in Fig. 1.

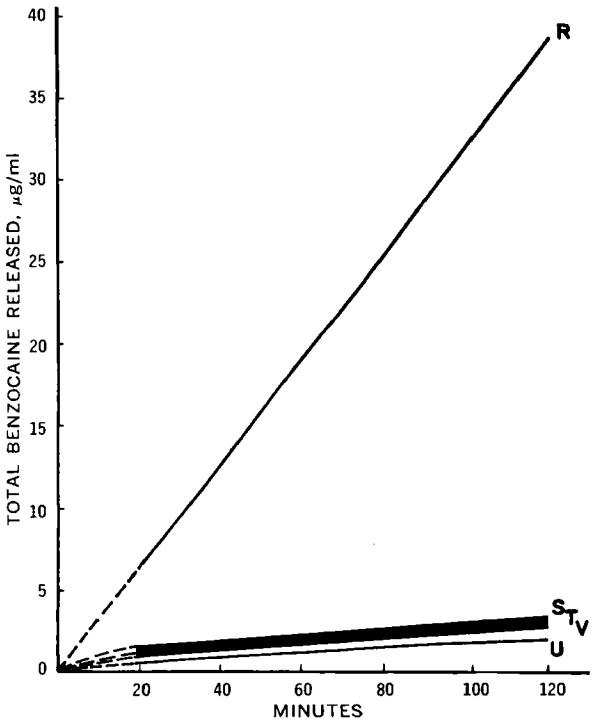

Figure 6—*Release of benzocaine from commercially available products. Key: see Table I.*

in water-in-oil and oil-in-water vehicles are shown in Fig. 4. It can be observed from Figs. 3 and 4 that benzocaine release from the vehicles used follows the same general trend as in Fig. 1 and is concentration dependent.

A general rule in ointment formulation is that if the drug is held firmly by the vehicle, such as when the drug dissolves in the vehicle, the rate of release of the drug will be slow. Conversely, if the drug has a low affinity for the vehicle, it will be readily released (7). Benzocaine is readily soluble in polyethylene glycols and might be predicted to be released quite slowly, at least compared to the other vehicles mentioned, in which benzocaine is not soluble. It can be seen in Fig. 1, however, that the polyethylene glycol vehicle released considerably more drug than any other vehicle. This ready release is further indicated by the curves in Fig. 5, which illustrate the dialysis of five different concentrations of benzocaine from the polyethylene glycol vehicle.

The decreased dialysis of 20% benzocaine in polyethylene glycol vehicle relative to the 2.5, 5.0, and 10% (w/w) preparations was not anticipated, but an interpretation may be offered. During dialysis, a dense, white precipitate formed between the ointment base and the dialysis membrane for the 20% preparation. A lesser amount of precipitate appeared for the 10% preparation and was not observed at all for the 5.0, 2.5, and 1.0% preparations. Water may diffuse through the membrane with formation of a water–polyethylene glycols solution. The precipitate is formed as a result of a marked decrease in solubility of benzocaine in the water–polyethylene glycols solution. A decreased rate of dialysis could be due to the need for an additional dissolution step for those preparations where a precipitate was observed or the precipitate may block the diffusion of the polyethylene glycols–benzocaine solution through the membrane.

Figure 6 shows the results of dialysis of benzocaine through the cellophane membrane from five different commercially available products. Different types of vehicles and varying concentrations of benzocaine are represented. Release studies have long been used as one criterion for judging the possible effect of vehicles on drug availability from topical formulations. There are obvious limitations to such methods, and conclusions must be drawn with great caution. However, the commercial product that gave the greatest release in this experiment gave a greater release than any of the experimental formulations and is the same as the one found effective *in vivo* (3). Furthermore, the relative release rates shown in Fig. 1 correlate with the *in vivo* rectal absorption in rats of radioactivity from benzocaine-^{3}H-containing ointments. These results will be the subject of a future report.

SUMMARY AND CONCLUSIONS

1. The rate of dialysis of benzocaine from various types of ointment bases was determined. Wide variations were found in the amount of benzocaine dialyzed.

2. The ability to release benzocaine for dialysis from ointment bases was found to be: polyethylene glycol > oil-in-water emulsion base > water-in-oil emulsion base > absorption base > white petrolatum.

3. One commercial product was found to release more benzocaine than any other commercial product or any experimental formulation tested.

4. Increasing the concentration of benzocaine in the vehicle caused a corresponding increase in the amount of benzocaine dialyzed through a cellophane membrane with the exception of the polyethylene glycol vehicle. The anomaly is explained in terms of the solubility of the drug and the vehicle.

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* Present address: Office of the Dean for Educational Development, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

ˣ To whom inquiries should be directed.

Metabolites of Mitragynine

JOHN E. ZAREMBO[x], BRYCE DOUGLAS, JOSEPH VALENTA, and JERRY A. WEISBACH

Abstract □ Microbial transformation of the alkaloid mitragynine by the fungus Helminthosporum sp. has elaborated two major metabolites. The compounds were isolated from the biological milieu and their structures were elucidated as mitragynine pseudoindoxyl and hydroxy mitragynine pseudoindoxyl. Tests to date have indicated quite high pharmacological activity by mitragynine pseudoindoxyl. Both metabolites were synthesized to verify structures.

Keyphrases □ Mitragynine—microbial transformation by Helminthosporum sp., isolation and identification of metabolites □ Microbial transformation—isolation and identification of two metabolites from Helminthosporum sp. transformation of mitragynine □ Indole alkaloids—microbial transformation of mitragynine by Helminthosporum sp., isolation and identification of metabolites

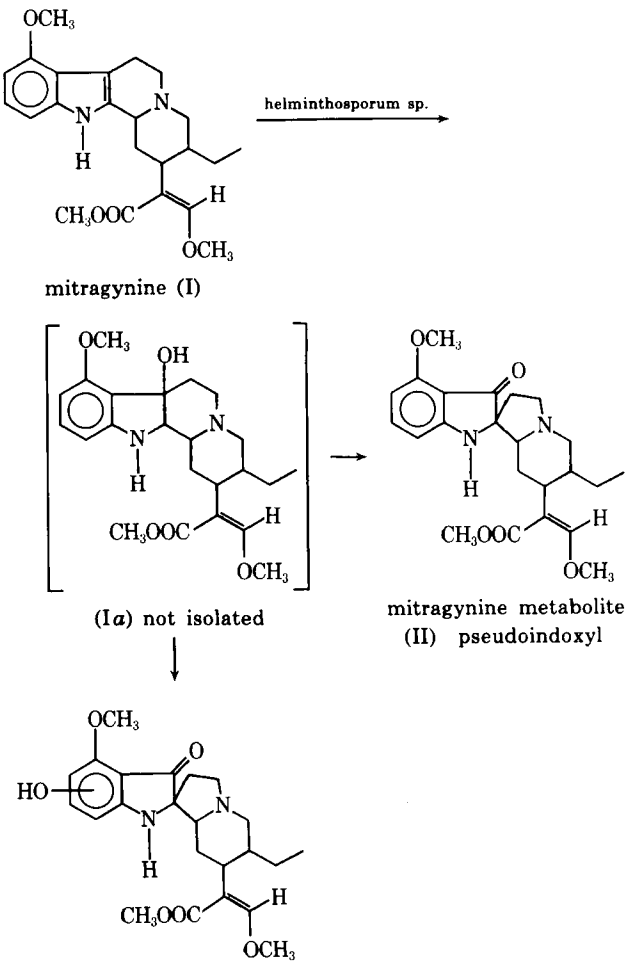
Mitragynine, an indole alkaloid isolated from the tree *Mitragynine speciosa*[1], indigenous to Borneo, Thailand, and New Guinea has been reported to exhibit analgesic and antitussive properties in experimental animals. The chemical structure of this material was defined in 1963 (1) and was confirmed by crystallographic studies (2). As part of continuing studies of the chemistry and biology of mitragynine, the pure material was subjected to biotransformation by the fungus Helminthosporum sp.[2]. A mixture including at least two major metabolites was found to be quite active pharmacologically; therefore, further work was undertaken to isolate each major metabolite and to test them biologically. The most active compound, II, $C_{23}H_{30}N_2O_5$, of this mixture was isolated in this laboratory and displayed analgesic activity in the D'Amour–Smith test (3) for analgesia almost 10-fold (9.4:1) over mitragynine, I, when administered by both oral and intraperitoneal routes to animals (4). A second active metabolite, III, $C_{23}H_{30}N_2O_6$, was also isolated in these laboratories in much smaller amounts but displayed lower activity (20–38%) compared to that of mitragynine. Considerable work has been reported (5, 6) describing many related alkaloids, but the two compounds isolated and identified in these studies have not been described.

The microbial transformation appears to proceed *via* oxidative and hydroxylative pathways (Scheme I). The primary metabolite, II, was isolated and identified as the pseudoindoxyl derivative of mitragynine [methyl ester of 6′-ethyl-2′,3′,6′,7′,8′,8′α-hexahydro-4-methoxy-α-(methoxymethylene)-3-oxospiro[indoline-2,1′(5*H*)indolizine]-7′-acetic acid]. The second metabolite, III, was identified as the ring A hydroxylated derivative of II.

Structure elucidation required extraction of the compounds from the fermentation mixture and chromatographic separation on a neutral alumina column followed by preparative TLC and spectral analysis. Selective chemical oxidation (7, 8) was carried out on mitragynine and resulted in the partial conversion of mitragynine into the major active metabolite, II. Spectral evidence obtained from both the synthesized and isolated compounds was identical. Because of the low level of biological activity associated with Compound III and the potential complexity of the synthetic problem, no attempt was made to prepare this compound chemically.

EXPERIMENTAL

Microbial Transformation—The pH of 9.5 liters of 0.5% glu-



Scheme I—Proposed microbial metabolic pathway for mitragynine

[1] The material used in this paper is *Mitragyna speciosa, KORTH*, a member of the Rubiacea. Specimens were collected by Dr. A. H. Beckett in November and December 1962 in the vicinity of Bangkok, Thailand. A voucher specimen of the plant has been deposited with Professor R. E. Schultes, Botanical Museum, Harvard University, Cambridge, Mass.

[2] ATCC 20154, available from the American Type Culture Collection, Rockville, Md.

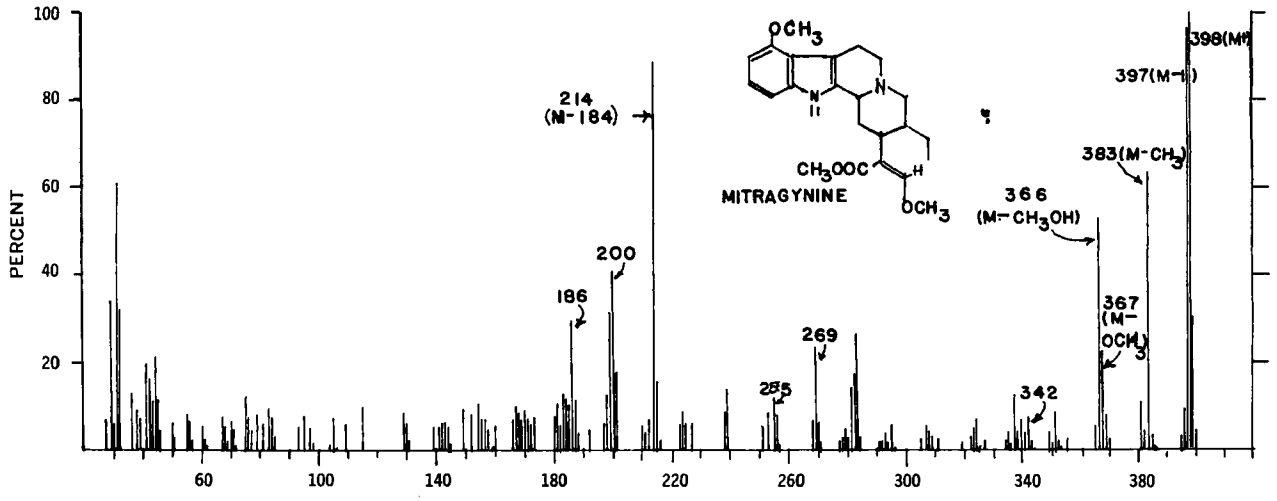


Figure 1—*Mass spectrum of mitragynine.*

cose in 2% corn steep liquor in a 10-liter fermentation flask was adjusted to pH 5.0 with dilute hydrochloric acid, and the solution was heated in an autoclave for 75 min at 17 psi and 121°. Helminthosporum sp. was grown in 500 ml of 0.5% glucose in 2% corn steep liquor for 48 hr. This culture was introduced into the prepared medium, and the system was incubated for 48 hr at 30° at 200 rpm with aeration at 0.5 v.v.m[3]. After this time the pH was adjusted to 8.0 with 1 *N* aqueous sodium hydroxide solution. Mitragynine hydrochloride (TLC pure, one spot) (10.88 g) in 100 ml of 95% ethanol and 100 ml of deionized water were added.

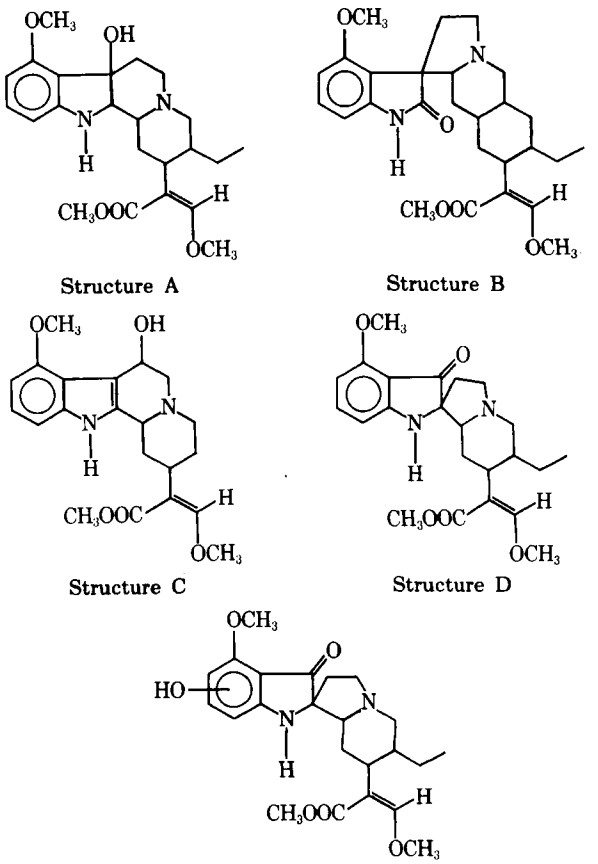
The fermentation was allowed to proceed at 30° with agitation at 250 rpm and aeration at 1 v.v.m. The pH was monitored with a pH meter and adjusted with dilute acid or alkali to maintain it at 8.0. Samples (4–5 ml) were taken at intervals and were adjusted to pH 9.2 and shaken with an equal volume of freshly opened peroxide-free ether. The ether phase was separated and concentrated *in vacuo* at 30°. The residue was taken up in chloroform–methanol (9:1) and spotted on neutral alumina chromatography plates (Fig. 1). The plates were developed for 10 cm in a paper-lined tank using chloroform–ethyl acetate (1:1), air dried, observed under UV light, and sprayed with modified Dragendorff reagent to determine the progress of the transformation.

After 50 hr the fermentation was terminated. The pH was adjusted to 9.2, the broth was centrifuged, and the supernate was decanted and extracted with half its volume of chloroform. The chloroform was backwashed with water and dried over anhydrous sodium sulfate. The chloroform extract was then concentrated to dryness *in vacuo*. Benzene was added to the residue, and the mixture was stirred and filtered. The benzene filtrate was evaporated to dryness *in vacuo*. Cyclohexane was added to the residue, and the mixture was filtered. The cyclohexane filtrate was then transferred onto a neutral alumina column and eluted with the following solvents: cyclohexane, toluene, ether, chloroform, and methanol.

The collected fractions were monitored by TLC. Fractions containing the active metabolites (R_f 0.13 and 0.23; Fig. 1) were combined and evaporated to dryness *in vacuo*. The residue was dissolved in cyclohexane with a small amount of toluene. This solution was placed on a second neutral alumina column and rechromatographed using the same eluting solvents. Examination of the fractions gave several cuts where single spots of each of the active metabolites were found. The overall yield was 73.5% of metabolized microbial product, 24.3% of metabolite II, and 1.4% of metabolite III. Two compounds were isolated and studied from both the structural and biological viewpoints. Biologically these were the most active metabolites when compared to mitragynine. Pharmacological work related to mitragynine was already reported (4).

Mass Spectral Analysis—Because many indole alkaloids are not readily distinguished from one another by their optical spectral properties and because mass spectral fermentation patterns offer convenient and very characteristic fingerprints for many alkaloid species (9–14), a mass spectral study was carried out to augment the UV, IR, and NMR spectral data. This consisted of: *(a)* 70-ev electron-impact spectra at medium resolution, *(b)* exact mass measurements of major peaks, and *(c)* mass spectral analysis of compounds after treatment with a silylating reagent, *N,O*-bis-(trimethylsilyl)trifluoroacetamide.

The mass spectra of mitragynine and its major metabolites are reproduced in Figs. 1 and 2. High-resolution mass measurements of the major fragmentation peaks were made and empirical formulas were established. Mitragynine was found to have a molecular weight of 398.2250 corresponding to an empirical formula of $C_{23}H_{30}N_2O_4$, while the major metabolite II had a molecular weight of 414.2160 corresponding to $C_{23}H_{30}N_2O_5$. Metabolite III, a hydroxylated mitragynine indoxyl, showed an increase of 16 amu corresponding to an empirical formula of $C_{23}H_{30}N_2O_6$. Exact mass



Structure A

Structure B

Structure C

Structure D

Structure E

Possible structures for metabolites

[3] v.v.m. = cubic liters of air per cubic liter of growth medium per minute.

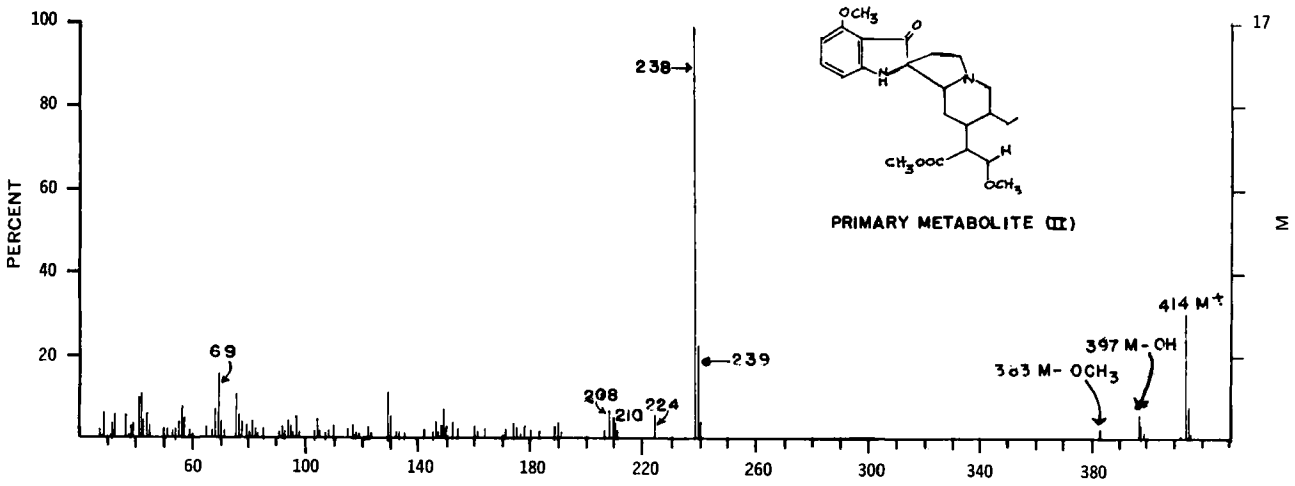


Figure 2—*Mass spectrum of mitragynine metabolite (II).*

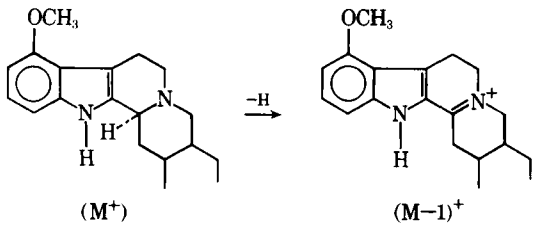
measurements were not made on III since the spectrum corresponded to that of the primary metabolite, the pseudoindoxyl II plus the additional 16 amu. Further evidence through derivatization as the silyl compound obviated the need for additional measurements. This increase of 16 amu for metabolite II and 32 amu for metabolite III over that of the parent mitragynine due to the addition of one and two atoms of oxygen, respectively, suggested four possible structures, A–D, for metabolite II and E (Fig. 1) for metabolite III.

Comparative examination of the mass spectral data of mitragynine and its major microbial metabolite II shows characteristic, unique fragmentation patterns (9). The $(M - 1)^+$ peak in mitragynine is a very large peak, whereas it is comparatively insignificant in the primary metabolite II. This $(M - 1)^+$ peak is large only in indole compounds having a tetrahydro-β-carboline moiety which can form an ammonium ion in conjugation with the indole system by losing one hydrogen. This is a very characteristic decomposition pattern which has been defined through extensive deuterium labeling (3) (Scheme II).

The mass spectra of indole alkaloids where an $(M - 1)^+$ peak is weak or absent provide an indication that the compound may be either an indole or pseudoindoxyl species (Scheme III) (9).

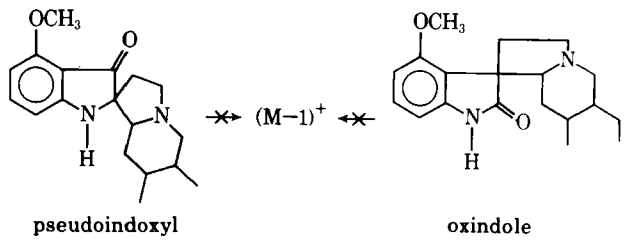
The base peak in the spectrum of mitragynine occurs at *m/e* 214, a loss of 184 amu, whereas the base peak in the major metabolite is at *m/e* 238. The difference of 24 amu between these two fragment ions does not correspond to a difference of 16 amu between the molecular weights of these two compounds. This suggests that fragmentation in both molecules occurs *via* different pathways (Schemes IV and V) and that the molecular ion shift technique (11) frequently used to establish similarities could not be applied because two basically different structures are present. The sequence of fragment formation is further substantiated by the presence of a metastable ion (*m** 115.4) for the transition and is verified by exact mass measurement of the peak at *m/e* 214 (214.111 = $C_{13}H_{14}N_2O$).

It is evident that since the mitragynine ion (214) has two nitrogens, the indole nucleus and the nitrogen in ring C contribute to its composition. In the major microbial metabolite, on the other hand, the most intense ion is at mass *m/e* 238 (238.1436 = $C_{13}H_{20}NO_3^+$), having only one nitrogen present. To obtain two such fragments from a compound, structural elements differing from mitragynine would have to be present. Such a moiety would occur from a pseudoindoxyl (*m/e* 238) or from an oxindole (*m/e* 239) (Scheme VI). Deuterium labeling (3) has shown that bonds 5–6 and 3–7 are broken by electron-impact mass spectrometry in oxindole compounds while bands 5–6 and 7–8 are broken in pseudoindoxyl compounds and that fragment ions (*m/e* 238 and 239) are derived from the alicyclic portion of the molecules. Since the most abundant peak in the mass spectrum of the mitragynine metabolite occurs at *m/e* 238 and not 239, the evidence is that the compound is a pseudoindoxyl and not an oxindole species. These conclusions are also in agreement with UV, NMR, and IR data provided. Other structural features are given in the fragmentation pathways shown in Schemes IV and V.



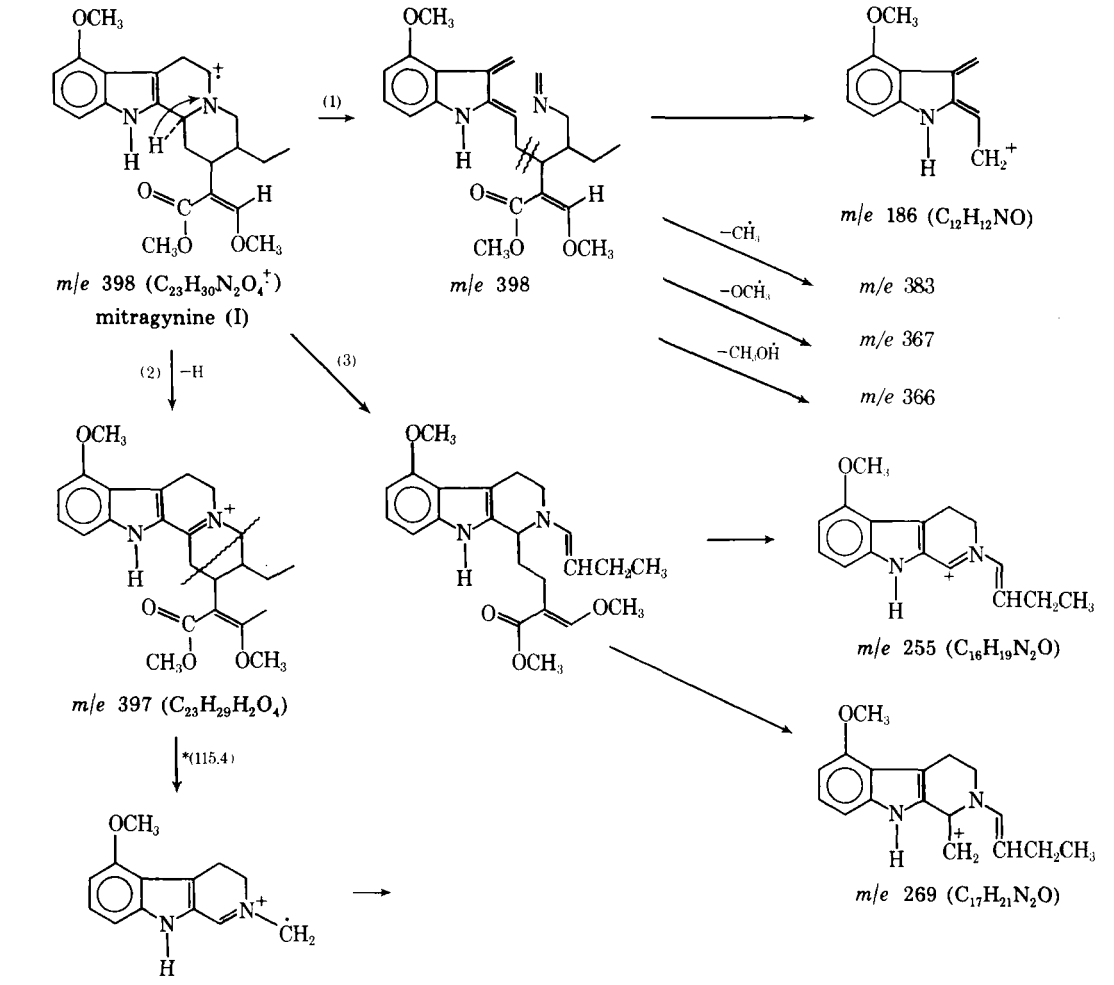
Scheme II

Additional diagnostic data were obtained from Compounds I, II, and III by treatment with diazomethane and a silylating reagent[4] prior to mass spectral fragmentation to establish the position and nature of the two added oxygens in metabolite III. The compounds (approximately 1–2 μg), dissolved in peroxide-free ether, were cooled in ice and treated with an excess of a freshly prepared diazomethane solution for approximately 30 min until the solution remained yellow. The reacted compounds were then isolated by evaporating the ether, and electron-impact mass spectra were obtained on them. The mass spectra of I and II were identical to the unreacted spectra, indicating that no reactive carboxylic acid, alkylatable phenolic, or reactive olefin groups were present. However, III showed an increase in mass of 14 amu (*m/e* 446) on the molecule, indicating alkylation had taken place on a phenolic hydroxyl in ring A. These same underivatized Compounds, I–III, were then dissolved in 5–6 μl of the silylating reagent[4] for 15 min and gently heated (~50°). The acetonitrile solvent was removed with a stream of nitrogen while heating at 50–60°. Upon rerunning the mass spectra of the compounds, the molecular ion of mitragynine had increased by 72 amu to *m/e* 470, equivalent to the addition of one trimethylsilyl group. This observation agrees with the structure of mitragynine, I, containing one replaceable or reactive hydrogen on the nitrogen of the indole nucleus. Metabolite II also increased by 72 amu to *m/e* 486. That only a single silyl derivative was produced agrees with earlier mass spectral assignments for II having no hydroxyl groups on the molecule. Metabolite III showed an increase in mass from *m/e* 430 to 574 (M^+ + two trimethylchlorosilane groups). The mass fragmentation pattern of each derivatized sample showed a loss of $M^+ - 15$, which was not diagnostic but sug-

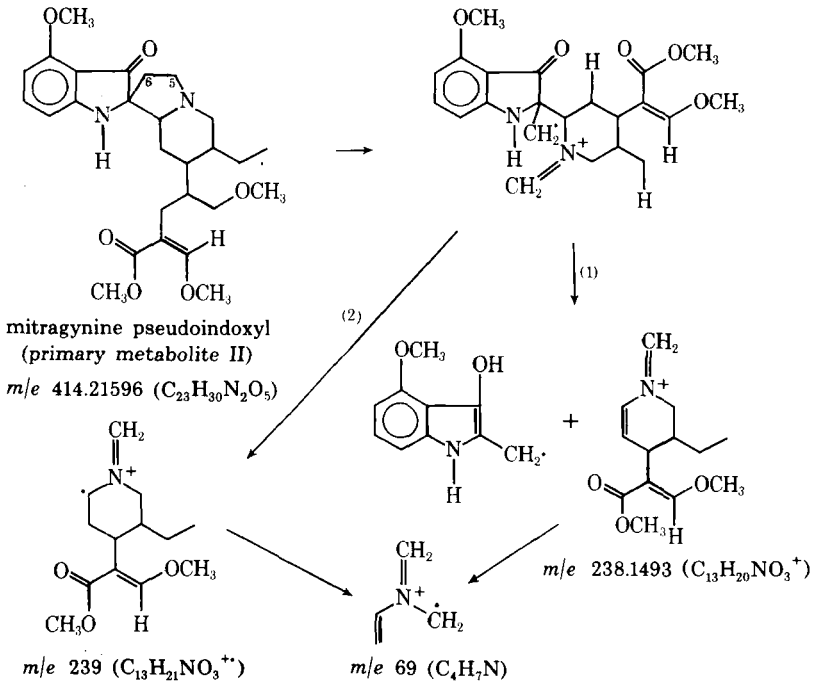


Scheme III

[4] *N,O*-Bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane, Pierce Chemical Co.



Scheme IV—Fragmentation of mitragynine



Scheme V—Fragmentation of mitragynine pseudoindoxyl

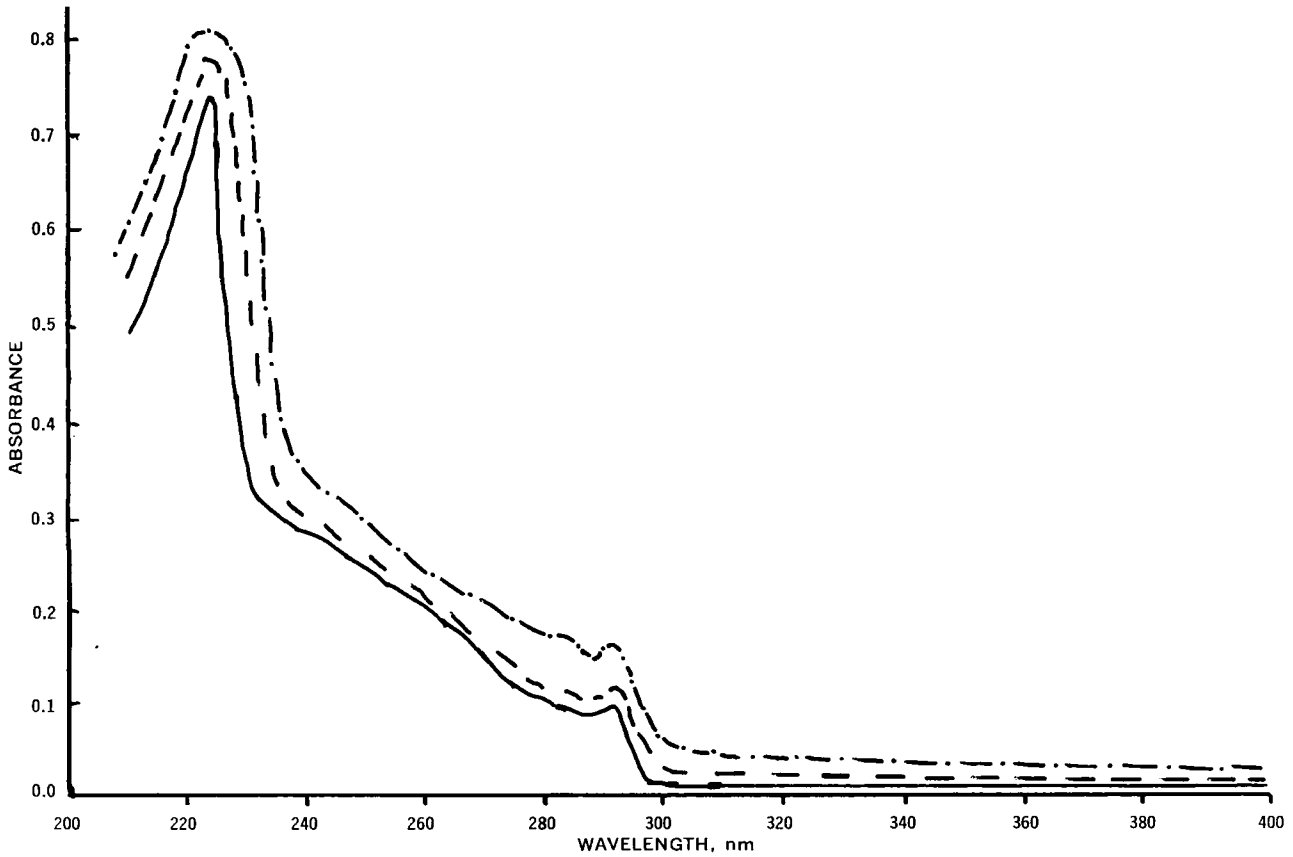


Figure 3—*UV absorption spectrum of mitragynine (I). Key: -·-, in neutral ethanol; ——, in acidified ethanol; and ---, in alkaline ethanol.*

gested the loss of a methyl from the trimethylsilyl compounds[5]. Metabolite III also showed the presence of an intense ion at *m/e* 238 plus a less intense one at *m/e* 239. Since this latter fragment ion had not increased in mass (mass shift), it was reasonable to assume that the hydroxyl is on the aromatic ring (A) of the indole and not on the alicyclic portion of the molecule. UV spectral data obtained in acid and alkaline solutions of these metabolites provided additional evidence that the hydroxyl is phenolic and not alicyclic, as discussed later.

These deductions and findings agree with those of Beckett *et al.* (15) and Lee *et al.* (16) who described the mass spectral data of several mitragynine-type indoles and oxindoles. These compounds have corresponding *m/e* 239 ions but no *m/e* 238 ions as do the pseudoindoxyl compounds isolated and identified here. To date, there is no evidence in the products obtained and isolated from the microbial transformation with Helminthosporum sp. that any oxindole compound was formed. This evidence shows that there has only been selective conversion to the pseudoindoxyl species.

On the basis of the mass spectral data, Structures A, B, and C were eliminated. Additional spectral evidence described here helps verify these deductions.

UV Data—Table I presents the UV spectral absorbance data of mitragynine (I), mitragynine metabolite II, and mitragynine metabolite III. Comparison of the UV absorption spectral curves (Figs. 3–5) of mitragynine metabolites II and III with that of mitragynine shows that the metabolites II and III have the characteristic indole absorption peaks at 285–290 nm and approximately 220–225 nm, which indicate the presence of an intact indole nucleus; however, metabolites II and III have two additional peaks: II at 239 and 296 nm and III at 242 and 320 nm. None of the absorption peaks in metabolite II is shifted with a change in pH of the solvent. When determined at pH 1.0 and 10.0, there were no detectable shifts of the absorption maxima or changes in their intensity. These data suggested that the additional oxygen present in metabolite II probably was located in a position other than ring A [*e.g.*, test for hindered phenol was negative (17)]. When this test was carried out on metabolite III, a positive (reddish-orange) color was produced.

Since the original sample of purified mitragynine showed the absence of an absorption peak above 290 nm, the presence of a peak above 360 nm indicated that the C ring—if metabolite II still had one intact—might have unsaturation present in conjugation with the indole portion of the molecule. Metabolite III did, however, show a shift in the position of the absorption maxima from 320 to 343 nm and from 242 to 262 nm, indicating that the oxygen

Table I—UV Absorption Spectral Data

Medium[a]	Mitragynine (I) λ_{max}	Mitragynine (I) Log ϵ	Metabolite II λ_{max}	Metabolite II Log ϵ	Metabolite III λ_{max}	Metabolite III Log ϵ
Ethanol (neutral)	225	4.67	216	4.40	222	4.45
			239	4.44	242	4.41
	290	3.88	288	3.89	285	4.22
			396	3.60	320	3.04
1 *N* HCl–ethanol[b]	225	4.73	216	4.32	222	4.46
			234	4.36	242	4.42
	290	3.77	290	3.83	285	4.21
			395	3.54	320	3.06
1 *N* NH_4OH–ethanol[c]	225	4.68	219	4.36	221	4.64
			237	4.43	285	4.24
	291	3.86	288	3.80	285	4.24
			395	3.53	343	3.43

[a] Alcohol USP. [b] Concentrated hydrochloric acid added to ethanol to produce a 1 *N* solution. [c] Concentrated ammonium hydroxide added to ethanol to produce a 1 *N* solution.

[5] See S. P. Markey, H. A. Thobhani, and K. B. Hammond, "Identification of Endogenous Urinary Metabolites by Gas Chromatography–Mass Spectrometry; A Collection of Mass Spectral Data," B. F. Stolinsky Research Laboratory, Department of Pediatrics, University of Colorado Medical Center, Denver, Colo., 1972.

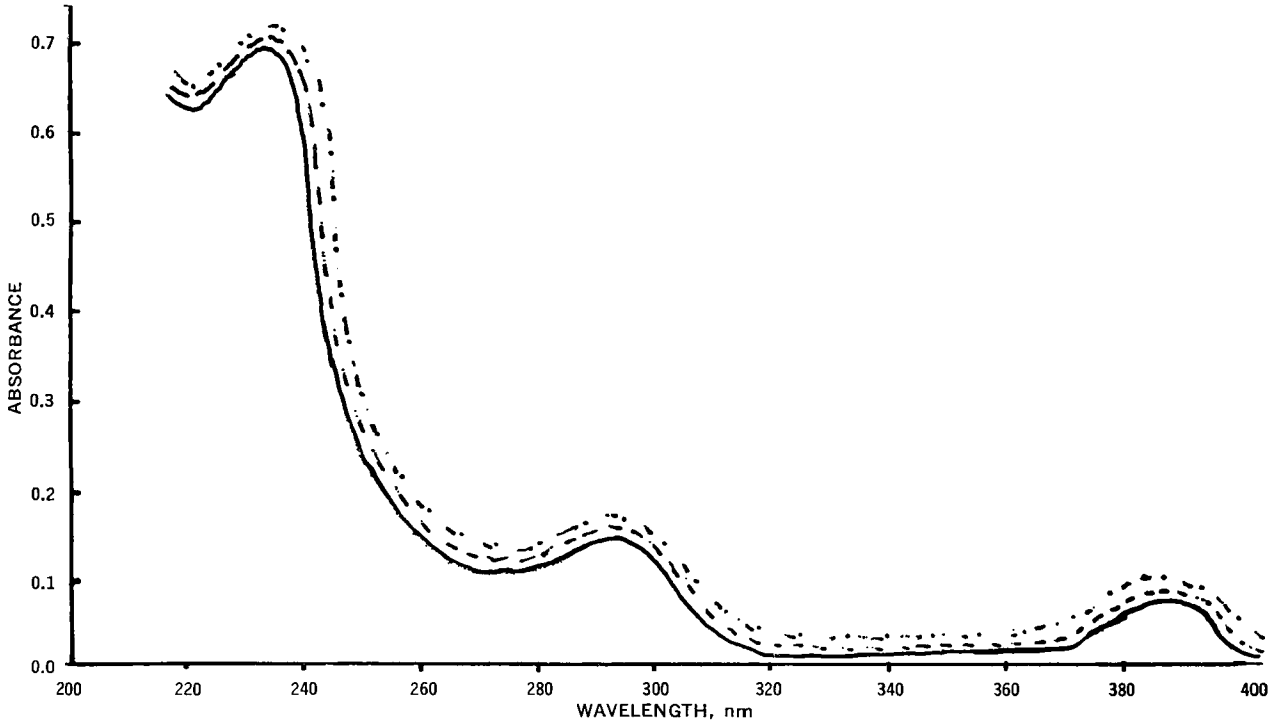


Figure 4—*UV absorption spectrum of mitragynine metabolite II. Key: –·–, in neutral ethanol; ——, in acidic ethanol; and ---, in alkaline ethanol.*

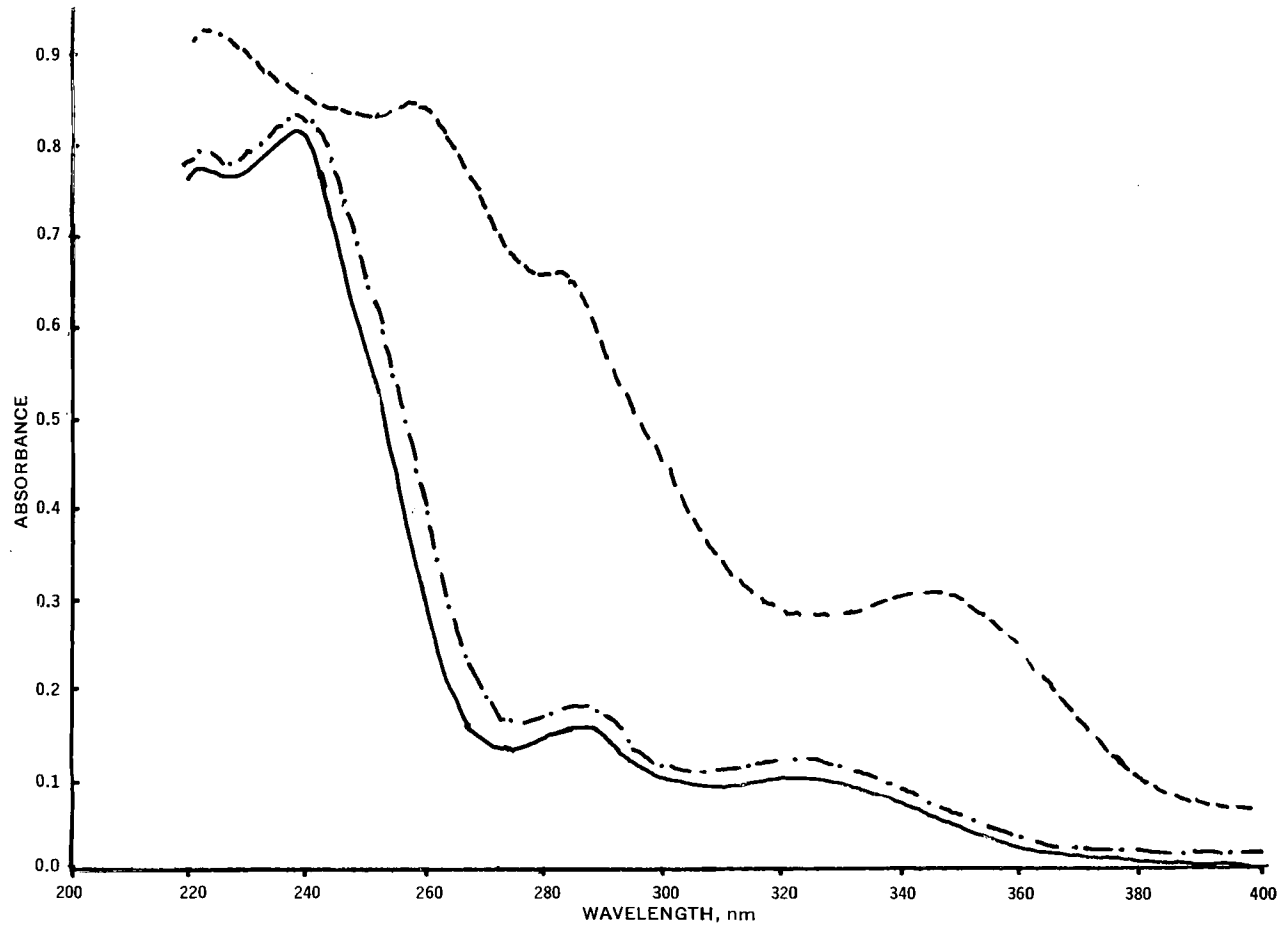


Figure 5—*UV absorption spectrum of mitragynine metabolite III. Key: –·–, in neutral ethanol; ——, in acidic ethanol; and ---, in alkaline ethanol.*

Table II—NMR Spectral Data[a]

Protons	Mitragynine (I)[b]			Metabolite II[b]		
	$CDCl_3$		D_2O	$CDCl_3$		D_2O
CH_3	0.88	Triplet	No change	0.88	Triplet	No change
CH_2 (ring)	2.0 –2.5	Multiplet	No change	2.0 –2.5	Multiplet	No change
OCH_3 (aromatic)	3.83	Singlet	No change	3.83	Singlet	No change
$OCOCH_3$	3.67	Singlet	No change	3.63	Singlet	No change
OCH_3 (vinyl)	3.64	Singlet	No change	3.58	Singlet	No change
CH_3 of ethanol	1.20	Triplet	No change	1.20	Triplet	No change
CH_2 of ethanol	3.45	Quartet	No change	3.45	Quartet	No change
One aromatic proton	6.42	Quartet	No change	6.25	Quartet	No change
Two aromatic protons	6.82–7.20	(Multiplet)	No change	7.10–7.40	(Multiplet)	No change
Olefinic protons	7.40	Singlet	No change	7.34	Singlet	No change
Proton on indole N—H	7.98	Singlet	Disappears	5.20	Singlet	Disappears

[a] Data values in parts per million from tetramethylsilane. [b] Compounds recrystallized and isolated as alcoholates.

of metabolite III was phenolic and was located on the aromatic A ring. The inference that metabolite II might have unsaturation in the C ring was not substantiated. A careful examination of the indole alkaloids listed in Ref. 18 failed to reveal a model structure with an absorption band above 360 nm. Reference to Sangster and Stuart's (19) review showed that pseudoindoxyl compounds and no other indole alkaloids reviewed had four absorption bands in their UV spectra in the approximate vicinity of metabolite II: 215–225, 225–235, 285–295, and 390–405 nm. Witkop and Patrick (20) reported these same absorption maxima for pseudoindoxyl compounds in their studies. Furthermore, they reported that these peaks were also insensitive to alkali and acid pH. A study of the UV spectral data in this review showed that oxindole alkaloid spectra were similar to indole alkaloids containing a tetrahydrocarboline moiety and that their UV spectra did not resemble metabolite II. These findings helped to substantiate the mass spectral findings and to exclude oxindoles from further consideration, thus adding support to the conclusion that Structure D fits all data for Compound II.

The limited investigation of metabolite III also suggests that Structure E is in accord with the spectral observations.

IR Spectral Data—The IR spectra of mitragynine and metabolites II and III show an N—H stretching vibration at approximately 3365 cm^{-1} (mineral oil mull). Carbonyl peaks attributable to the carboxymethoxy ether group for all three compounds are found at approximately 1690 and 1640 cm^{-1}. In addition, there is an OH stretching vibration at 3558 cm^{-1} in all three compounds due to the OH contributed by the ethanolate (see *NMR Data*). Structural changes attributed to changes in the indole ring are seen by the appearance of a moderately strong band at 1590 cm^{-1}. These findings are in agreement with the bands of the carboxyl region reported for other similar alkaloids having the pseudoindoxyl structure (7, 21–24) with similar functional groups on the indole nucleus. No evidence from the IR data could be found for oxindole formation.

NMR Data—The NMR spectra of mitragynine and the major metabolite II were run in deuterated chloroform (60 MHz) and were then shaken in deuterium oxide to exchange active hydrogens in these compounds (Table II). An insufficient amount of metabolite III was isolated to obtain a useful NMR spectrum. The observed chemical shifts for mitragynine very closely resembled those obtained by Trager *et al.* (25) and for indole pseudoindoxyls by Wenkert *et al.* (22) and Hendrickson and Sims (23). The NMR data agree with the structure assigned to Compound II. Examination of the NMR data shows that the indole nuclei, the proton on the indole nitrogen, and components of rings C and D are intact and that the methoxyl groups are present on mitragynine as well as the major metabolite II.

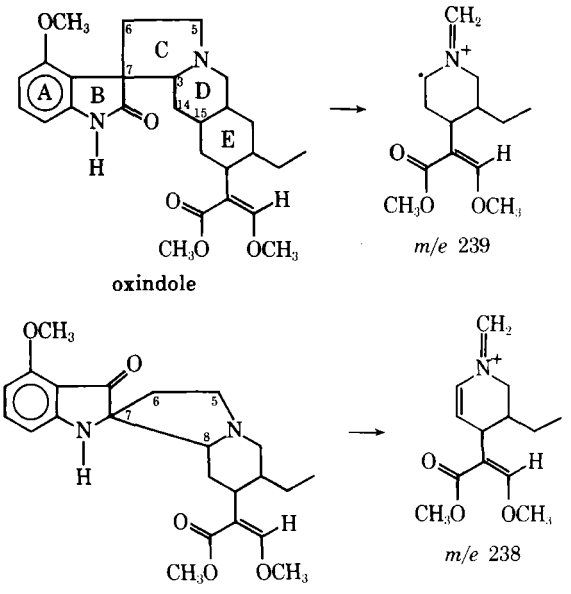
Table III—Microelemental Analysis Results

Element	Mitragynine I, $C_{23}H_{30}N_2O_4 \cdot C_2H_5OH$		Metabolite II, $C_{23}H_{30}N_2O_6 \cdot C_2H_5OH$		Metabolite III, $C_{23}H_{30}N_2O_6 \cdot C_2H_5OH$	
	Calc.	Found	Calc.	Found	Calc.	Found
C	67.54	67.34	65.20	65.48	63.01	63.21
H	8.16	7.92	7.88	7.66	7.61	7.49
N	6.30	6.18	6.08	6.12	5.88	5.88

Elemental Analysis Data—Both the major metabolites and mitragynine samples used in this study were isolated as alcoholates and run as such for elemental composition (Table III), NMR, and IR studies. Differential thermal analysis (DTA) examination of the samples showed that the alcohol was lost between 80 and 90° and that decomposition of the free base occurred near the melting points (mitragynine mp 102–104°, metabolite II mp 151–153°, and metabolite III mp dec.)[6].

TLC—TLC was employed throughout this study to check and verify the integrity of the samples and to prepare highly purified materials for analytical purposes. Trace impurities were removed from the compounds by preparative TLC; the separated spots were scraped from the plates and the highly purified samples were isolated and rechecked by additional TLC work. Only when a single TLC spot was found after this treatment was a sample used for structural characterizations. While this procedure might have been overly cautious, artifacts that frequently arise were avoided, thus making structural characterizations more definitive. Two TLC systems giving good separation were developed and employed throughout this work (Table IV).

Spot tests on TLC plates containing the metabolites as well as mitragynine gave positive reaction with Dragendorff reagent, modified Ehrlich reagent, iodoplatinate, and diazotized *p*-nitroaniline (weak red). When tested with Emerson reagent and 2,4-dinitrophenylhydrazine, the results were inconclusive because of the orig-



Scheme VI

[6] Melting-point determination on Mettler hot stage microscope.

Table IV—TLC Data for Mitragynine and Its Microbial Metabolites

Band	R_f	Color	Compound
		System 1[a]	
8	0.60	Yellow	?
7	0.49	Yellow	Mitragynine (I)
6	0.41	Yellow	?
5[b]	0.32	Yellow	?
4[c]	0.23	Yellow	Metabolite II
3[d]	0.13	Yellow	Metabolite III
2	0.05	Orange	?
1	0.00	Orange	?
		System 2[e]	
9	0.40	Yellow	Metabolite II
8	0.36	Yellow	Metabolite III
7	0.27	Yellow	?
6	0.25	Yellow	Mitragynine (I)
5	0.19	Yellow	?
4	0.13	Orange yellow	?
3	0.09	Orange yellow	?
2	0.05	Pink	?
1	0.00	Orange red	?

[a] Silica gel GF on 5.1 × 20.3-cm (2 × 8-in.) plates run for 10 cm; solvent of chloroform–ethyl acetate (1:1 v/v); equilibration time of 5 min. Compounds 1, 2, 6, and 8 were obtained in insufficient amounts for biological testing. [b] Slightly active. [c] Most active. [d] Active. [e] Silica gel GF on 5.1 × 20.3-cm (2 × 8-in.) plates run for 10 cm; solvent of benzene–methanol (95:5 v/v); equilibration time of 5 min.

inal color of the spots. All spots on the thin-layer plates were visible in ordinary white light as well as under short wavelength (254 nm) UV light. Test for hindered phenol employing ferric chloride in pyridine (17) was negative for Compounds I and II but showed a slight increase in color for Compound III. Since a positive test with 2,4-dinitrophenylhydrazine produces an orange-yellow reaction and the compounds had a yellow color, it was difficult to determine whether the test was weakly positive or negative. Since other spectral data (mass, IR, UV, and NMR) were more diagnostic and definitive, the spot test data were only used for semiqualitative purposes during the separations. The migration characteristics are described and the spots which indicate biological activity are listed in Table IV.

Synthesis of Metabolite II (Mitragynine Pseudoindoxyl)—Mitragynine and other indole alkaloids been reported to be converted into their corresponding indole and pseudooxindole analogs by reaction with oxidizing agents such as lead tetraacetate (7) and *tert*-butyl hypochlorite which convert the indoles to the β-hydroxyindolenines. Treatment of these compounds with mild alkali (sodium methoxide) converts the compounds into their corresponding pseudoindoxyl compounds. If the same reaction sequence is carried out and the β-hydroxyindolenine is treated with acid, the corresponding oxindole compounds are prepared (26).

Preparation of Mitragynine Pseudoindoxyl (II)—The procedure followed was essentially that of Finch *et al.* (7). In a 150-ml erlenmeyer flask, 0.25 mmole (111 mg) of mitragynine (as the alcoholate) was dissolved in 15 ml of methylene chloride and the solution was cooled in an ice–water bath. A freshly prepared 0.07 *M* solution of lead tetraacetate (50 ml), which was previously cooled in ice, was added dropwise over 10 min. Stirring and reaction were continued for an additional 60 min (cold), after which time the reaction mixture was poured over ice and stirred. The ice–water–organic mixture was then separated, washed several times with water, and dried (sodium sulfate). The volume was reduced to 10–15 ml in a stream of nitrogen. The resulting light-yellow reaction product was transferred to a neutral alumina[7] chromatographic column packed with 60 g of adsorbent.

Elution was carried out with pure methylene chloride followed by increasing amounts of methanol in methylene chloride. The fractions were collected and evaporated to dryness. Fractions containing the solvents from methylene chloride and 1% methanol in methylene chloride were combined. The dry fractions were then treated with 0.05 *N* sodium methoxide (prepared from sodium and methanol) and refluxed for 1 hr. The reaction product was treated with water, and methylene chloride solutions were dried with sodium sulfate, concentrated with a stream of nitrogen, and then prepared by TLC (System 1). The spots containing the desired compounds were scraped from the plates and eluted with chloroform–methanol (9:1). When a single spot was obtained on subsequent TLC, the compounds were recrystallized from a very small amount of ethanol and dried in a stream of nitrogen and spectral data were obtained. These data compared identically with spectral data obtained from Compound II isolated from the microbial transformation product and constituted final proof of structure.

One may speculate as to the action of the fungus at this time. It is possible that the products formed may simply have been the result of air oxidation of mitragynine during the fermentation. It is, however, unlikely that ring hydroxylation (Compound III) is the result of air oxidation since there was no evidence of this compound in the synthesis of Compound II from mitragynine. In the synthesis, however, it was possible to isolate some of Compound Ia. This aspect of the work requires further investigation and will be reported later.

[7] Woelm-Brockmann grade III activity.

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Browning of Dextrates in Solid–Solid Mixtures Containing Dextroamphetamine Sulfate

S. M. BLAUG[x] and WEN-TZY HUANG*

Abstract □ By using diffuse reflectance spectroscopy, the ethanol-mediated interaction between dextroamphetamine sulfate and dextrates in solid–solid mixtures was studied. Discoloration of the powder mixtures was accelerated by the presence of the amine and by storage at elevated temperatures. Heated samples showed two new absorption maxima at 330 and 300 nm in their reflectance spectra. The former was attributed to the chemisorption of the amine molecules on the surface of the dextrates, while the latter was attributed to the interaction between amine molecules and the dextrates. The rate of browning was determined by plotting remission function *versus* time at 300 nm for mixtures of dextroamphetamine sulfate and dextrates at three temperatures and two dextroamphetamine sulfate concentrations. Arrhenius-type plots were used to approximate the browning rate at 25°. The browning rate was considerably slower than that reported for solid–solid mixtures containing dextroamphetamine sulfate and spray-dried lactose USP.

Keyphrases □ Dextrates—browning in solid–solid mixtures containing dextroamphetamine sulfate, rate and variables □ Dextroamphetamine sulfate–dextrates—solid–solid mixtures, rate of browning, effects of temperature and concentration □ Discoloration—*dextrates–dextroamphetamine sulfate solid–solid mixtures*, rate of browning, effects of temperature and concentration □ Diffuse reflectance spectroscopy—monitoring rate of browning of dextrates–dextroamphetamine sulfate mixtures

Dextrates is dextrose prepared by the controlled hydrolysis of starch in combination with a special manufacturing process that yields spherical, porous beadlets[1]. It is composed of approximately 92% dextrose and 8% higher saccharides. The material is white, free flowing, and odorless and consists of aggregates of dextrose microcrystals intermixed and cohered with a small proportion of higher saccharides. The structure and dissolution characteristics of dextrates are unique when compared to those of dextrose USP and sucrose. Its absolute solubility is greater than that of dextrose USP but less than that of sucrose, yet its rate of solution is low when compared to dextrose USP and sucrose.

Henderson and Bruno (1) evaluated dextrates as an excipient in direct compression tablet production. It was concluded that dextrates and lactose[2] USP (beadlets) were superior to spray-dried lactose and lactose USP (anhydrous) for use as filler in direct compression. Both exhibited excellent flow characteristics, and dextrates showed improved compression and disintegration properties while lactose USP (beadlets) possessed greater physical stability.

A number of workers (2–4) reported that tablets prepared using lactose as a filler tend to discolor on storage. This phenomenon was accelerated by the presence of amines and/or certain lubricants and was dependent on the temperature, humidity, and light exposure at which the tablets were stored. With regard to color stability, tablets prepared with dextrates and stored at different temperatures and

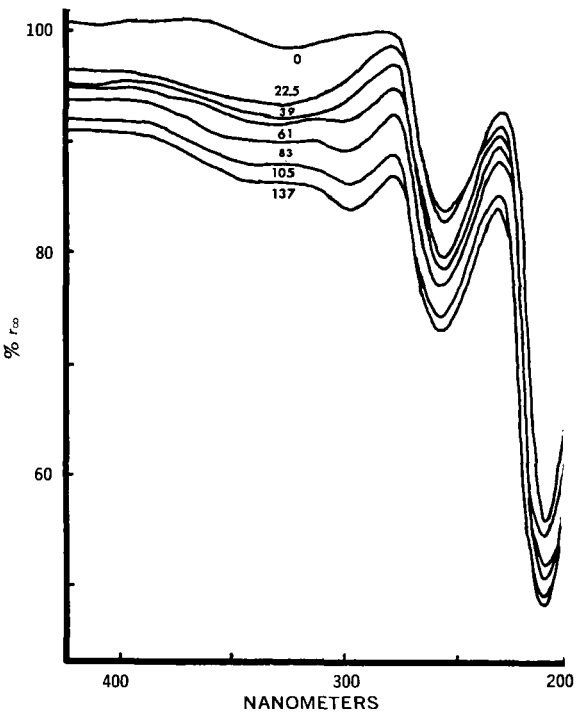

Figure 1—*Diffuse reflectance spectra of an equilibrated sample containing 10 mg of dextroamphetamine sulfate/g of dextrates after heating at 55° for various time periods (shown in hours).*

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[2] Foremost Dairies Inc., San Francisco, Calif.

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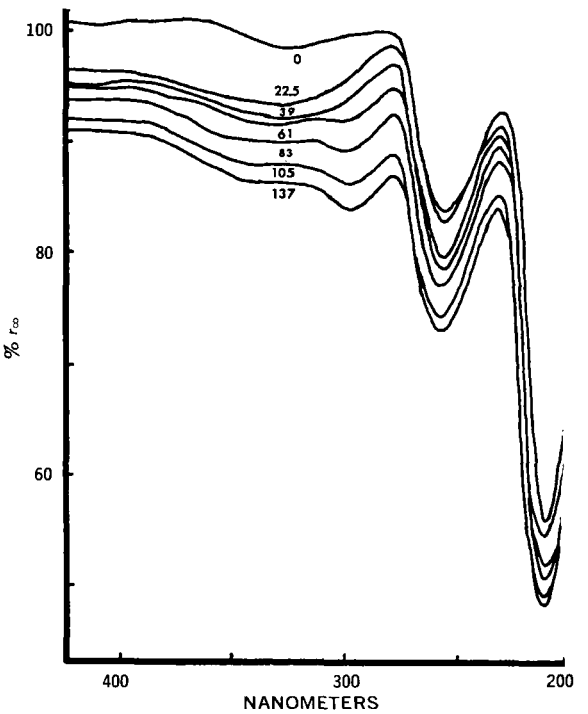

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Table I—Relationship between Reflectance Values and Time for an Equilibrated Mixture of Dextroamphetamine Sulfate and Dextrates after Heating at 60°

Hours at 60°	Reflectance, %/100			
	10 mg Dextroamphetamine Sulfate/g Dextrates		15 mg Dextroamphetamine Sulfate/g Dextrates	
	$r_\infty 300$	$f(r_\infty)300$	$r_\infty 300$	$f(r_\infty)300$
0	0.995	0	0.995	0
21.3	0.895	0.0062	0.860	0.0114
37.5	0.882	0.0079	0.840	0.0152
59.0	0.873	0.0092	0.825	0.0186
80.5	0.857	0.0120	0.795	0.0264
106.5	0.830	0.0174	0.780	0.0310
144.0	0.800	0.0250	0.745	0.0436

humidities showed no substantial improvement over those prepared with spray-dried lactose (1).

Blaug and Huang (5) studied the interaction of dextroamphetamine sulfate and spray-dried lactose in solid–solid mixtures using diffuse reflectance spectroscopy. Discoloration of the powder mixtures was accelerated by the presence of the amine and by storage at elevated temperatures. The brown material from the discolored samples was identified as dextroamphetamine–hydroxymethylfurfural. Blaug and Huang (6) also studied the interaction of dextroamphetamine sulfate with dextrates in solution. By using TLC, IR spectroscopy, and elemental analysis, the brown material isolated from the discolored solutions was also identified as amphetamine–hydroxymethylfurfural.

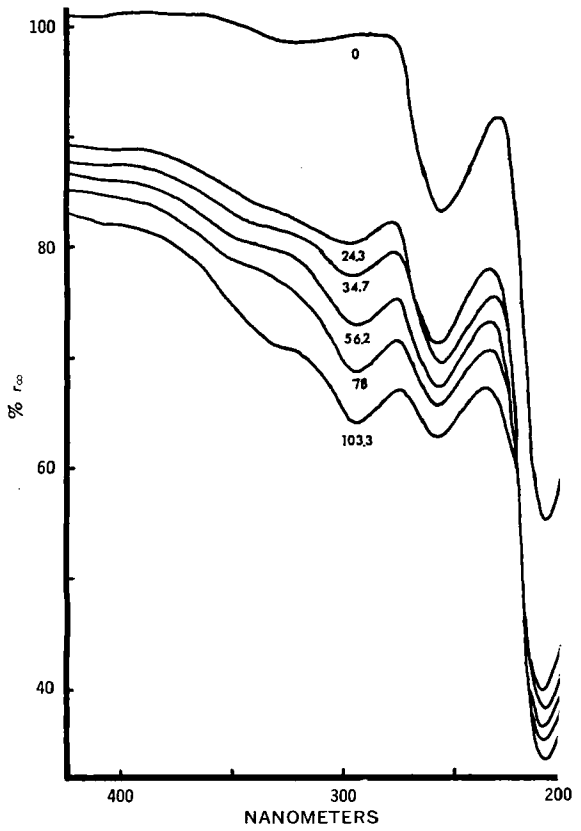

Figure 2—*Diffuse reflectance spectra of an equilibrated sample containing 10 mg of dextroamphetamine sulfate/g of dextrates after heating at 65° for various time periods (shown in hours).*

Table II—Relationship between Reflectance Values and Time for an Equilibrated Mixture of Dextroamphetamine Sulfate and Dextrates after Heating at 65°

Hours at 65°	Reflectance, %/100			
	10 mg Dextroamphetamine Sulfate/g Dextrates		15 mg Dextroamphetamine Sulfate/g Dextrates	
	$r_\infty 300$	$f(r_\infty)300$	$r_\infty 300$	$f(r_\infty)300$
0	0.0995	0	0.995	0
24.3	0.802	0.0244	0.700	0.0643
34.7	0.776	0.0323	0.695	0.0669
56.2	0.731	0.0495	0.670	0.0813
78.0	0.690	0.0696	0.646	0.0970
103.3	0.640	0.1013	0.613	0.1222

This study was undertaken to investigate and quantify the color reaction reported (1) to occur in tablets prepared using dextrates as a filler. A similar color reaction has been reported (4) in tablets prepared from dextrose. Since the discoloration is accelerated by the presence of amines, dextroamphetamine sulfate was used. Diffuse reflectance spectroscopy was used to study the interaction of dextrates with dextroamphetamine sulfate in the solid state.

EXPERIMENTAL[3]

Reagents—The following were used: dextroamphetamine sulfate[4] USP, mp >300°; and dextrates, mp 150–152°.

Procedure—*Preparation of Equilibrated Samples*—Forty- and sixty-milligram samples of dextroamphetamine sulfate were accurately weighed and transferred to 90-ml (3-oz) amber glass bottles. Sufficient absolute alcohol was added to dissolve the drug. Then 4 g of dextrates was accurately weighed and added to each solution. The bottles were sealed with screw caps lined with aluminum foil and attached to the submersion rotator. For reference use, a similar bottle was prepared containing 6 g of dextrates and the same amount of absolute alcohol used to prepare the sample bottle. The reference bottle was sealed and attached to the submersion rotator. The sample and reference bottles were tumbled for 24 hr at 25°. At the end of this time, the bottles were removed from the rotator and placed in the vacuum oven where the solvent was removed at room temperature using a vacuum pump to provide the vacuum. After drying, the contents of each bottle were triturated in a glass mortar to provide a uniform sample. All samples were stored in a calcium sulfate-charged vacuum desiccator for diffuse reflectance study.

Reflectance Measurement—The prepared samples and reference adjuvant (dextrates) were packed into the sample and reference cells previously described (7–10). Since it has been reported (11) that the quantity of material in the cell and the pressure used during the process of packing can cause fluctuations in reflectance values, care was taken to maintain the same conditions for every sample packed. After the initial reflectance spectra were recorded, each sample and reference adjuvant were placed in a constant-temperature oven maintained at 55, 60, and 65 ± 0.5°. At periodic time intervals, a sample and reference adjuvant were removed from the oven and allowed to cool to room temperature in a vacuum oven containing calcium sulfate. After cooling to room temper-

[3] All diffuse reflectance spectra were measured using a Beckman model DB-G spectrophotometer with reflectance attachment. The spectra were automatically recorded on a Beckman model 1005 25.4-cm (10-in.) linear potentiometric recorder. A Sargent analytical oven, low gradient, and a Precision Scientific model 524 vacuum oven were used for storing and drying samples. Drug–adjuvant samples were equilibrated using a submersion rotator, model SR-250-V (Scientific Industries, Inc., Queen Village, N.Y.), to tumble the powders.

[4] Hexagon Laboratories, Inc., New York, N.Y.

Table III—Relationship between Reflectance Values and Time for an Equilibrated Mixture Containing 15 mg of Dextroamphetamine Sulfate/g of Dextrates after Heating at 60°

Hours at 60°	Reflectance, %/100				
	$r_\infty 300$	$f(r_\infty)300$	$r_\infty 330$	$f(r_\infty)330$	$f(r_\infty)300/f(r_\infty)330$
0	0.995	0.0000	0.986	0.0001	0.100
21.3	0.860	0.0114	0.842	0.0148	0.769
37.3	0.840	0.0152	0.838	0.0157	0.973
59.0	0.825	0.0186	0.825	0.0186	1.000
80.5	0.795	0.0264	0.801	0.0247	1.069
106.5	0.780	0.0310	0.790	0.0279	1.112
144.0	0.745	0.0436	0.762	0.0372	1.174

ature, additional reflectance spectra were recorded by scanning from 700 down to 200 nm.

RESULTS AND DISCUSSION

Figures 1 and 2 indicate absorption maxima at 215 and 257 nm, which correspond to the absorption maxima obtained in the dextroamphetamine sulfate–spray-dried lactose interaction previously reported (5). The band at 257 nm is the characteristic band of dextroamphetamine sulfate in solution as well as in the solid state. The two bands were attributed to the physical adsorption of the dextroamphetamine sulfate molecules by the lactose or dextrates.

However, the equilibrated samples of dextroamphetamine sulfate and dextrates that had been heated showed two new absorption maxima, one at 330 nm and one at 300 nm. Browning of the samples occurred, particularly in the samples heated for long periods. As shown in Tables I and II, the intensity of the absorption maximum increased markedly at 300 nm with time, temperature, and dextroamphetamine sulfate concentration. This finding was analogous to the results previously reported (5, 6). The solid–solid interaction between dextroamphetamine sulfate and spray-dried lactose resulted in the appearance of a new absorption maximum at 295 nm and discoloration. In dextroamphetamine sulfate–dextrates solutions that had been heated for varying periods, a new absorption maximum appeared at 298 nm. The solutions became discolored, particularly those heated for long periods at elevated temperatures. In both studies the browning reaction was the result of a Schiff base-type reaction involving the amine and the carbonyl group of the sugars. The brown material from the discolored samples was identified as dextroamphetamine sulfate–hydroxymethylfurfural (5, 6).

Although the shoulder peak at 330 nm also increased in intensity with time and temperature, the rate of formation of the absorption maximum was faster at 300 than at 330 nm. Table III shows the reflectance values and remission functions at these two wavelengths for a mixture containing 15 mg of dextroamphetamine sulfate/g of dextrates that had been heated at 60° for varying periods. Table III also shows the ratio of remission function at 300 nm to remission function at 330 nm for each time period. A comparison of these values indicates that the rate of formation of the maximum at 300 nm was faster than it was at 330 nm. A plot of the remission function ratios *versus* time is shown in Fig. 3. From these data one can conclude that the rate of interaction between dextroamphetamine sulfate and dextrates was faster than the rate of chemisorption of dextroamphetamine sulfate. In other words, as soon as the drug was chemisorbed on the surface of the dextrates crystals, a Schiff base type of reaction occurred, leading to the formation of a new absorption maximum at 300 nm. Figure 3 shows that the curve gradually reached a plateau with time. Rideal and Trapnell (12) showed that there was no discontinuity between the end of chemisorption and the beginning of second layer formation. The end of first layer formation was continuous with the beginning of second layer formation. The formation of a second layer may be due either to a weak chemisorption or physical adsorption. The data in Table III and Fig. 3 indicate the formation of a weak second chemisorbed layer on the top of the first chemisorbed layer. This probably contributed to the increase in the intensity of the absorption at 330 nm with time, with a concomitant decrease in the rate of increase of the intensity of the band at 300 nm. Therefore, although the value of the ratio of $f(r_\infty)300/f(r_\infty)330$ increased with time, the increase became smaller, resulting in the plateau shown in Fig. 3. Browning did not occur until the appearance of the absorption band at 300 nm, and the intensity of the color increased with an increase in the intensity of absorption at 300 nm.

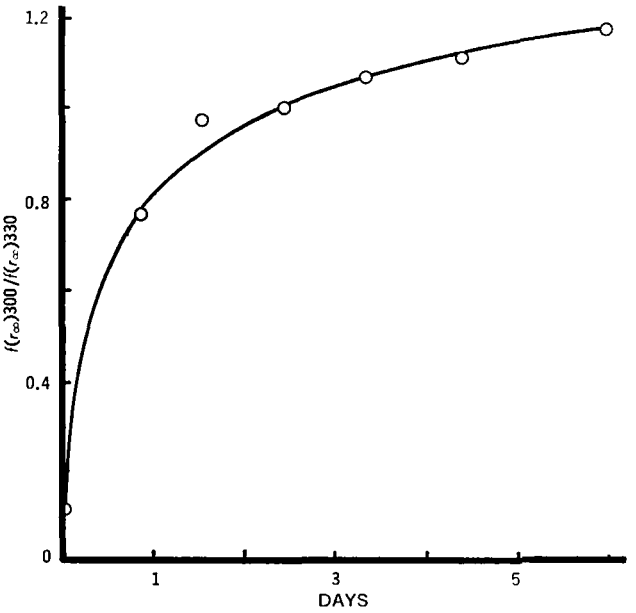

Figure 3—*Relationship between the remission function ratios at different wavelengths and time for an equilibrated sample containing 15 mg of dextroamphetamine sulfate/g of dextrates after heating at 60°.*

Since dextrates alone showed no browning, there was little doubt that the browning of the sample was due primarily to the interaction between the amine and the sugar on the chemisorbed surface, with the appearance of a new absorption peak at 300 nm. The latter wavelength corresponded to the new absorption peak at 298 nm in the heated solution containing dextroamphetamine sulfate and dextrates (6) and to the new peak at 295 nm in the equilibrated solid–solid mixtures of dextroamphetamine sulfate and spray-dried lactose (5).

The rate of the browning reaction was studied using the reflectance values obtained at 300 nm. The remission functions were calculated according to the Kubelka–Munk theory (13, 14). The results obtained at 60° are shown in Fig. 4. Critical examination of each curve showed that it consisted of two portions; in the first portion of the browning reaction, a hyperbolic curve was observed, whereas an almost linear relationship was obtained in the second

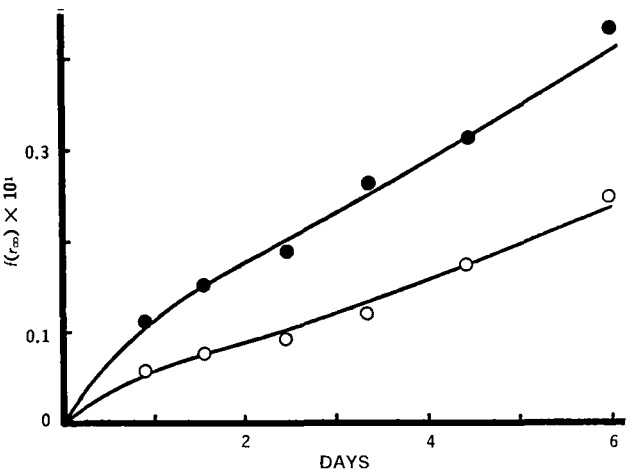

Figure 4—*Rate of browning of dextroamphetamine sulfate–dextrates equilibrated mixtures at 60°. Key: ●, 10 mg of dextroamphetamine sulfate/g of dextrates: and ○, 15 mg of dextroamphetamine sulfate/g of dextrates.*

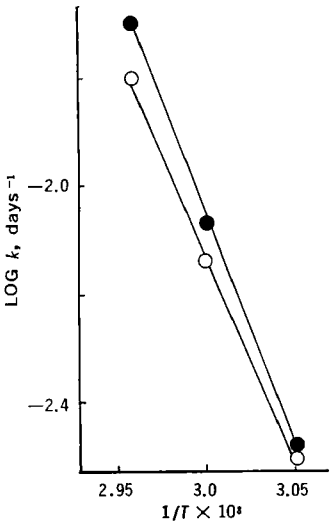

Figure 5—*Arrhenius-type plots showing the temperature dependence for the browning of dextroamphetamine sulfate–dextrates equilibrated mixtures. Key:* ●, *10 mg of dextroamphetamine sulfate/g of dextrates; and* ○, *15 mg of dextroamphetamine sulfate/g of dextrates.*

portion. The higher rate of browning in the initial stages may have been due to the liberation of small quantities of bound water from the dextrates, with the participation of the liberated water in the initial stages of the reaction due to the formation of a solution phase containing dextroamphetamine sulfate and dextrates molecules. Duvall *et al.* (3) reported that the rate of browning between dextroamphetamine sulfate and lactose was faster in solution than in the solid state, and Blaug and Huang (6) reported a high rate of browning in solutions containing dextrates and dextroamphetamine sulfate.

Figure 4 indicates that the rate of browning was faster in the samples that contained 15 mg of drug compared to the samples that contained 10 mg of drug. Similar results were obtained at 55 and 65°. Apparently, in the drug–dextrates mixtures the adjuvant surface was not saturated with dextroamphetamine sulfate molecules, so the surface coverage increased with increasing drug concentrations, resulting in a higher browning rate in the equilibrated mixtures containing 15 mg of drug as compared to those containing 10 mg.

Following the initial browning reaction (Fig. 4), a linear or almost linear relationship was obtained between remission function and time. Similar plots were obtained at 55 and 65°. From this relationship, the browning reaction may be assumed to follow an apparent zero-order reaction. Thus, the browning rate constants shown in Table IV were obtained from the slopes of the straight-line portions of the plots. Since the mechanism involved in the actual browning reaction was very complex, the apparent zero-order rate constants obtained do not have the usual kinetic significance. However, the rate constants do represent a total overall reaction rate that enables one to estimate the rate of browning at other temperatures.

Arrhenius-type plots of log k *versus* $1/T$ are shown in Fig. 5. The browning rate constants were determined at 25° by extrapolation, and the times required for browning to occur at 25° were calculated as approximately 845 and 793 days for the samples containing 10 and 15 mg of dextroamphetamine sulfate. These rates were considerably slower than the browning rate reported (5) for mixtures containing spray-dried lactose and dextroamphetamine sulfate. The differences observed in the browning rates could have been due to the unusual physical characteristics ascribed to the dextrates (1) due to their special manufacturing process that yields spherical, opaque, and highly aggregated crystals with a closed pore network structure of microcrystals. The microcrystals are reported[5] to be less than 5 μm in diameter. Each particle of dextrates has open connective pores between the microcrystals which are accessible to the incorporated drug.

Table IV—Apparent Zero-Order Rate Constants for Browning of Dextroamphetamine Sulfate–Dextrates Mixtures

Temperature	10 mg Dextroamphetamine Sulfate/g Dextrates		15 mg Dextroamphetamine Sulfate/g Dextrates	
	k, day^{-1}	Log k	k, day^{-1}	Log k
55°	0.310×10^{-2}	−2.5086	0.333×10^{-2}	−2.4776
60°	0.725×10^{-2}	−2.1397	0.859×10^{-2}	−2.0660
65°	1.550×10^{-2}	−1.8097	2.000×10^{-2}	−1.6990

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* Present address: State Hygienic Laboratory, Des Moines, IA 50309

ˣ To whom inquiries should be directed.

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Catharanthus Alkaloids XXXI: Isolation of Ajmalicine, Pericalline, Tetrahydroalstonine, Vindolinine, and Ursolic Acid from *Catharanthus trichophyllus* Roots

ALVIN B. SEGELMAN * and NORMAN R. FARNSWORTH ˣ

Abstract □ Certain active cytotoxic alkaloid fractions prepared from *Catharanthus trichophyllus* roots were investigated. This work resulted in the isolation of ajmalicine, pericalline, tetrahydroalstonine, vindolinine, and ursolic acid. These compounds were not responsible for the observed cytotoxic activity. Ajmalicine, tetrahydroalstonine, and ursolic acid were previously isolated from this plant, whereas vindolinine and pericalline are now reported for the first time from *C. trichophyllus*.

Keyphrases □ *Catharanthus* alkaloids—isolation of ajmalicine, pericalline, tetrahydroalstonine, vindolinine, and ursolic acid from *C. trichophyllus* roots, cytotoxic activity □ Vindolinine—isolation and identification from *C. trichophyllus* roots, cytotoxic activity □ Pericalline—isolation and identification from *C. trichophyllus* roots, cytotoxic activity

It has been reported that certain alkaloid fractions prepared from *Catharanthus trichophyllus* exhibit a significant inhibitory activity against the P-1534 leukemia in DBA/2 mice, as well as a cytotoxic activity against Eagle's 9KB carcinoma of the nasopharynx in cell culture (1). Prior to the present study, phytochemical investigations were limited to the aboveground parts of this plant and led to the isolation of ajmalicine (2), tetrahydroalstonine (1), vindorosine (1), and ursolic acid (1). The present phytochemical study dealt with the roots of *C. trichophyllus* and resulted in the isolation of ajmalicine, tetrahydroalstonine, vindolinine, pericalline, and ursolic acid.

EXPERIMENTAL[1]

Plant Material—The coarsely milled and air-dried roots[2] of *C. trichophyllus* (Bak.) Pich. (Apocynaceae) used in this study were collected in Madagascar during 1969.

Petroleum Ether Extraction and Fractionation (Scheme I)—A total of 35 kg of plant material was exhaustively extracted with petroleum ether (bp 35–60°) in a Lloyd extractor to furnish a total of 144 liters of combined concentrated extract (Extract A). On standing at room temperature, Extract A deposited 13 g of amorphous material (Extract A′) which was collected by filtration. The filtrate was concentrated *in vacuo* to a thick syrup (Extract B) and treated with 1 liter of methanol, and the solid material that separated was collected by filtration (Extract B′). The filtrate (Extract C) was evaporated *in vacuo* to give a residue which was redissolved in 500 ml of benzene. This benzene solution was mixed with 500 ml of warm 0.2 *M* citric acid solution, the benzene was removed *in vacuo* on a steam bath, and the aqueous mixture was cooled and filtered. The filtrate was reserved and the residue was redissolved in benzene and treated with citric acid, followed by removal of the benzene and filtration as before. This procedure was repeated three times to give 2.5 liters of acidic filtrate (Extract D) and 92 g of nonbasic, insoluble residue (Extract C′). Extract D was made alkaline with 58% NH_4OH and extracted with benzene (5 × 2.5 liters). The combined benzene extracts were washed with water, dried (anhydrous sodium sulfate), and taken to dryness *in vacuo* to give 2.42 g of crude, weakly basic alkaloids (Extract D′).

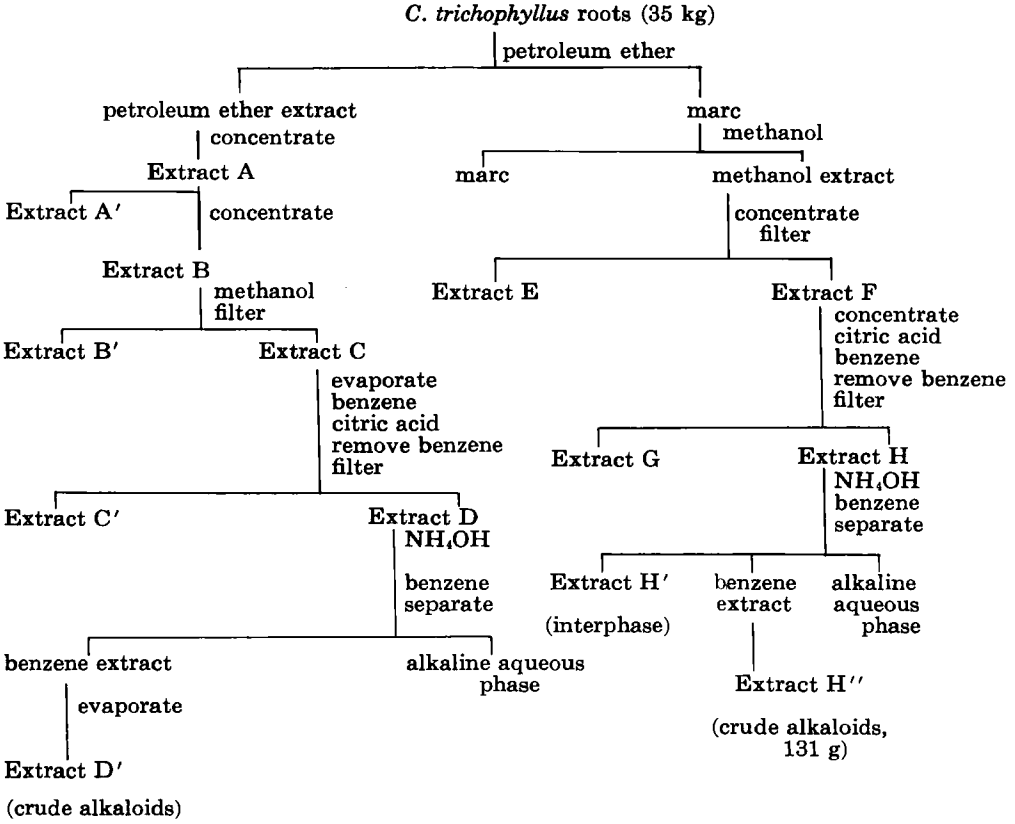
Isolation of Ursolic Acid—A sample (0.5 g) of Extract A′ was repeatedly crystallized from 95% ethanol to give colorless needles (0.018 g), mp 296–298°, $[\alpha]D^{28.5}$ + 55° (c 0.47 in pyridine). This isolate was homogeneous by TLC and gave R_f values of 0.70, 0.58, and 0.28 when analyzed in Solvent Systems A, D, and E, respectively. The UV spectrum was transparent over the 210–400-nm range. The mass spectrum showed the apparent molecular ion at *m/e* 456 ($C_{30}H_{48}O_3$), as well as additional fragments considered diagnostic for Δ^{12}-unsaturated oleananes and ursene-type triterpenes (5, 6). The IR, specific rotation, TLC, and mass spectroscopy data of the isolate were identical with those derived from authentic ursolic acid, and a mixed melting-point determination showed no depression. The isolate was further characterized by preparation of the acetate derivative, which proved to be identical (TLC and mass spectroscopy) with authentic acetylursolic acid.

Preparation of Total Crude Alkaloid Fraction (Scheme I)—The air-dried petroleum ether-extracted plant material was exhaustively extracted by repeated cold maceration and percolation, using a total of 1600 liters of anhydrous methanol. After the percolates were combined, they were concentrated *in vacuo* to 40 liters (Extract F), which resulted in the separation of 263 g of nonalkaloid material (Extract E) which was collected by filtration. The filtrate was further concentrated *in vacuo* to afford about 6 liters of a viscous syrup which was mixed with 6 liters of 0.2 *M* citric acid solution. This mixture was heated *in vacuo* on a steam bath to remove residual methanol and finally the mixture was cooled and filtered. This filtrate was set aside and the residue was redissolved in methanol and treated with citric acid solution, followed by removal of the methanol and filtration as before. Extract F was thus extracted a total of three times with citric acid solution to give 320 g of insoluble residue (Extract G) and 20 liters of combined acidic alkaloid filtrate (Extract H). Extract H (20 liters) was made alkaline (pH 9.0) with 58% NH_4OH and extracted with benzene (6 × 20 liters). Nonalkaloid interphase solids, which formed during the benzene extractions, were removed by filtration to give 320 g of Extract H′. The combined benzene extracts were washed with water, dried (anhydrous sodium sulfate), and taken to dryness *in vacuo* to give 131 g of crude alkaloid residue (Extract H″).

Dialysis Purification of Fraction H″ Alkaloids (Scheme II)—The following procedure is a modified version of the method described by Klasek (7). Fraction H″ (131 g) was worked up per batch as follows. Fraction H″ (24 g) was dissolved in 90 ml of citric

[1] Melting points were determined in open capillary tubes using a Thomas-Hoover apparatus and are uncorrected. Specific rotations were measured using a Rudolph model 70 precision polarimeter. UV spectra were taken in ethanol or methanol using a Perkin-Elmer model 202 spectrophotometer. IR spectra were measured in KBr pellets *versus* air with a Beckman model IR-8 spectrophotometer. Mass spectra were recorded at 70 ev using a LKB-9000 mass spectrometer (LKB Produkter, Stockholm, Sweden), operated in the direct probe mode. NMR spectra were determined in deuterochloroform, containing tetramethylsilane as the interior standard, using a Varian model A-60 spectrometer or HR-100 spectrometer. All concentrations and evaporations were carried out with water pump vacuum at less than 40°. Routine TLC was carried out using silica gel G (Merck) plates. The solvent systems used were: A, ethyl acetate–absolute ethanol (3:1); B, 1-butanol–acetic acid–distilled water (4:1:1); C, anhydrous methanol (100%); D, chloroform–acetone (5:4); E, benzene–ethyl acetate (7:2); and F, benzene–ethyl acetate–diethylamine (7:2:1). Nonalkaloid substances were detected on TLC plates by spraying with sulfuric acid followed by charring at 120°. Ceric ammonium sulfate reagent (3) and modified Dragendorff (4) reagent were used as spray reagents to visualize resolved alkaloids on TLC plates. Preparative thick-layer chromatography was carried out on silica gel PF_{254} (Merck) 1-mm layers, resolved components being detected by quenching under 254-nm UV light. Appropriate zones were scraped from the plates and pure components were recovered from the removed zones by elution with ethanol, followed by filtration and removal of the solvent.

[2] Obtained from the Curran Corp., South Hackensack, N.J. Voucher specimens from the collection are on deposit at the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, Ill. The identification was confirmed by N. R. Farnsworth.



Scheme I—Fractionation of C. trichophyllus *Roots*

acid solution (10 g of citric acid and 10 ml of distilled water), diluted to 180 ml with distilled water, and divided into six cellophane dialysis tubes[3]. These dialysis tubes, each containing 30 ml of acidic alkaloid solution, were suspended in a 2-liter beaker containing 1 liter of distilled water. The dialysate was stirred using a magnetic stirring bar, and the tubes were dialyzed against distilled water at 4°. At intervals of 8 hr, the dialysate was removed and reserved, fresh distilled water was added to the beaker, and the dialysis process was resumed. This protocol was repeated until each 24-g batch of alkaloids had been dialyzed for a total of four 8-hr periods. The combined acidic (pH 2.2) dialysates were made alkaline (pH 9.0) with 58% NH_4OH and exhaustively extracted with benzene to remove alkaloids. These combined benzene extracts were washed with water, dried (anhydrous sodium sulfate), and taken to dryness *in vacuo* to give the dialyzed alkaloid fraction (Extract I). In this manner, Extract H″ (131 g) yielded a total of 107.5 g of Extract I.

Chromatographic Separation of Extract I Alkaloids (Scheme II)—Extract I (107 g) was chromatographed batchwise as follows. Extract I (20 g) was dissolved in 30 ml of methanol–ethylene dichloride (7:3) and chromatographed on a 5 × 55-cm column[4] previously slurry-packed in methanol–ethylene dichloride (7:3). Elution was carried out using the same solvent at a flow rate of 75 ml/hr, and 5-ml fractions were collected. Individual fractions were monitored by TLC for the presence of alkaloids (Solvent System A), and consecutive fractions were combined on the basis of similar patterns of resolved alkaloids. A total of 570 fractions was collected from the column for each 20-g batch; these fractions were analyzed and combined into three groups. Removal of the solvent *in vacuo* for each group gave three fractions: J, K, and L.

Separation of Extract K Alkaloid Salts (Scheme II)—Treatment of a solution of Extract K (96 g) in 300 ml of cold absolute ethanol with dry hydrogen chloride gas resulted in the formation of a crystalline product, which was removed by filtration and dried over sodium hydroxide *in vacuo* to afford 22.9 g of Extract M. The filtrate was evaporated to dryness *in vacuo*, and the residue was dried over sodium hydroxide *in vacuo* to give 87.6 g of Extract N.

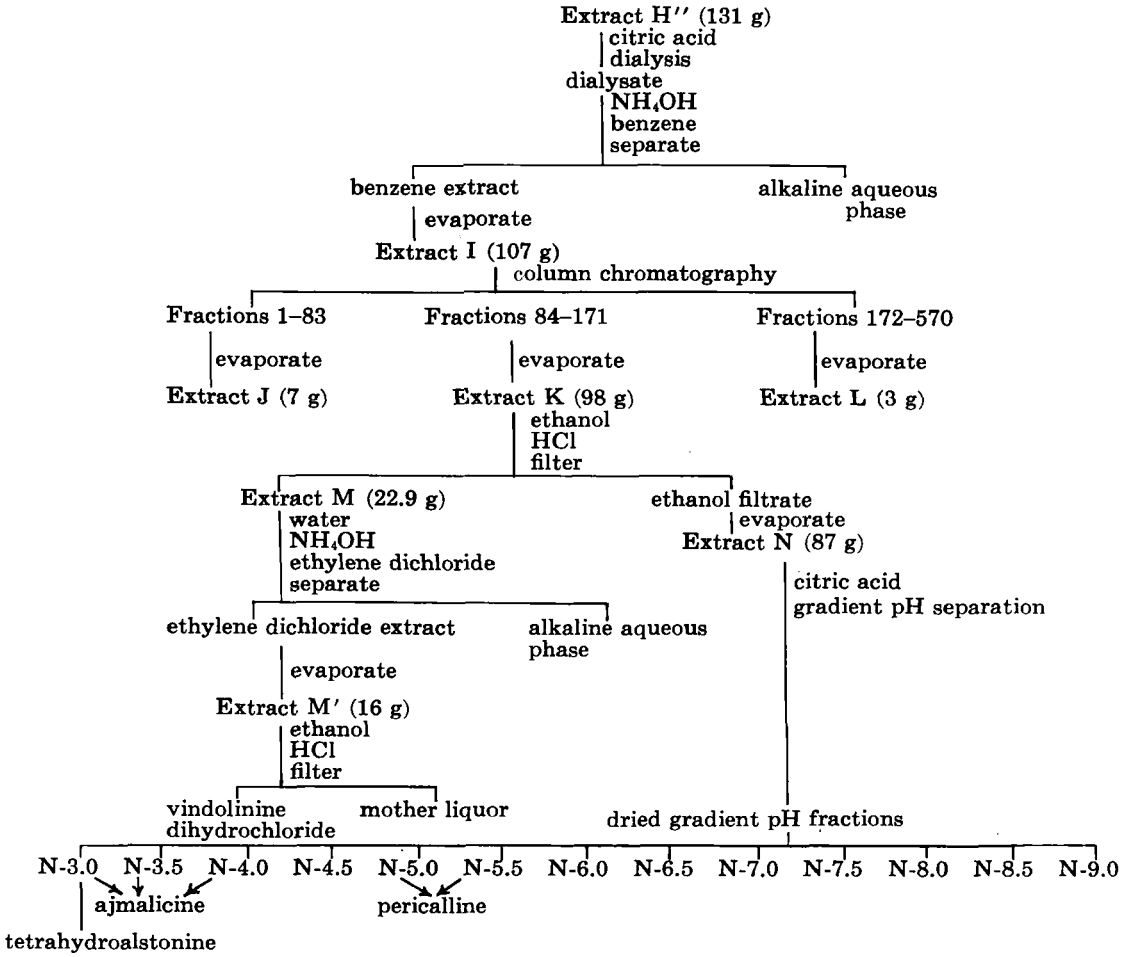
Isolation of Vindolinine Dihydrochloride—A solution of Extract M (22.9 g) in 1 liter of distilled water was decolorized with activated charcoal, filtered, alkalinized (pH 9.0) with 58% NH_4OH, and exhaustively extracted with ethylene dichloride. The combined ethylene dichloride extracts were washed with water, dried (anhydrous sodium sulfate), and evaporated *in vacuo* to give 16 g of Extract M′. A sample (0.25 g) of Extract M′ dissolved in 5 ml of absolute ethanol was treated with 1.5 ml of concentrated hydrochloric acid, and the solution was chilled. Crystals that formed were harvested and recrystallized from benzene–ethanol (1:1) to give 0.13 g of colorless needles, mp 218–220°, $[\alpha]D^{29.5}$ − 8.0° (c 0.5 in water). This isolate was homogeneous by TLC in Solvent System B (R_f 0.43) and displayed a red-orange chromogenic response with the ceric ammonium sulfate reagent. The UV absorption spectrum showed λ_{max} (C_2H_5OH) 206 (log ε 4.48), 246 (3.94), and 310 (3.44) nm, typical of dihydroindole alkaloids unsubstituted on both the indole nitrogen and the aromatic portion of the molecule (8). The IR, specific rotation, UV, NMR, TLC, and mass spectroscopy data were identical with those derived from authentic vindolinine dihydrochloride, and a mixed melting-point determination showed no depression.

Gradient pH Separation of Extract N Alkaloids (Scheme II)—A solution of Extract N (87 g) in 4.2 liters of 0.2 *M* citric acid was adjusted to pH 3.0 with 10% NH_4OH and extracted with benzene (2 × 4 liters). Following removal and pooling of the benzene extracts, the aqueous phase was adjusted to pH 3.5 with ammonium hydroxide and reextracted with benzene (2 × 4 liters) as before. This procedure was repeated, the pH of the aqueous phase being increased by 0.5 pH unit increments prior to each extraction with benzene, until a final pH of 9.0 was reached. The pooled benzene extracts from each pH level were washed with water, dried (anhydrous sodium sulfate), and taken to dryness *in vacuo* to give a total of 13 alkaloid fractions. Each of these 13 fractions was subjected to direct crystallization attempts using common solvents and solvent mixtures.

Isolation of Ajmalicine—Crystals harvested from the chilled acetone solutions of Extract N-3.5 and Extract N-4.0 were combined on the basis of similar TLC patterns. Further concentration

[3] Catalog number 8-667D, Fisher Scientific Co., Pittsburgh, Pa. Each tube was prepared from a 50-cm length of tubing.

[4] 250 g Sephadex LH-20, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.



Scheme II—Fractionation of Extract H'' Alkaloids

and chilling of the mother liquors gave additional crystals, which were combined with the first crop to afford a total of 0.71 g of crude pale-yellow crystals. Repeated crystallization of this material from hot acetone gave 0.33 g of pale-yellow stout rosettes, mp 257° dec., $[\alpha]D^{29.5}$ −85.4° (c 0.55 in chloroform). This isolate was homogeneous by TLC, displayed a yellow-green chromogenic response with the ceric ammonium sulfate reagent, and gave R_f values of 0.70, 0.53, 0.68, 0.67, 0.24, and 0.67 when analyzed in Solvent Systems A–F, respectively. The UV absorption spectrum showed λ_{max} (CH_3OH) 225 (log ϵ 4.60), 288 (3.85), and 290 (3.75) nm, typical of alkaloids containing a tetrahydro-β-carboline moiety (8). The IR, specific rotation, UV, NMR, TLC, and mass spectroscopy data were identical with those derived from authentic ajmalicine, and a mixed melting-point determination showed no depression.

Isolation of Pericalline—Crystals harvested from the chilled benzene solutions of Extract N-5.0 and Extract N-5.5 were combined on the basis of similar TLC patterns. Further concentration and chilling of the mother liquors gave additional crystals, which were combined with the first crop to afford a total of 0.6 g of crude yellow crystals. Recrystallization of this crude material from hot acetone gave 0.27 g of fine colorless needles, mp 195.5–196° dec., $[\alpha]D^{29.5}$ −182° (c 0.50 in chloroform). This isolate was homogeneous by TLC, displayed an initial purple chromogenic response with the ceric ammonium sulfate reagent, and gave R_f values of 0.18, 0.56, 0.25, 0.12, 0.03, and 0.54 when analyzed in Solvent Systems A–F, respectively. The UV absorption spectrum showed λ_{max} (C_2H_5OH) 208 (log ϵ 4.35) and 305 (4.18) nm with a shoulder at 230 nm. The IR, specific rotation, UV, NMR, TLC, and mass spectroscopy data were identical with those derived from authentic pericalline, and a mixed melting-point determination showed no depression.

Isolation of Tetrahydroalstonine—Crude brown crystals (0.1 g) harvested from a chilled benzene solution of Extract N-3.0 were shown to be a mixture of two alkaloids by TLC. A solution of these crude crystals (0.1 g) in 0.2 *M* citric acid was made alkaline with ammonium hydroxide solution, and the liberated alkaloids were extracted by repeated shaking with ethylene dichloride. The organic extracts were combined, washed with water, dried (anhydrous sodium sulfate), and evaporated *in vacuo* to give a pale-yellow oil, which was further separated into two components by preparative thick-layer chromatography (Solvent System E). The first component (R_f 0.24) crystallized from benzene to give a product identical in all respects with a reference sample of ajmalicine. The second component (R_f 0.51) crystallized from benzene–hexane (1:1) to give fine colorless needles (0.016 g), mp 227–228° dec., $[\alpha]D^{29.5}$ −103° (c 0.47 in chloroform). This isolate was homogeneous by TLC, displayed a yellow-green chromogenic response with the cerric ammonium sulfate reagent, and gave R_f values of 0.77, 0.55, 0.74, 0.76, 0.52, and 0.69 when analyzed in Solvent Systems A–F, respectively. The UV absorption spectrum showed λ_{max} (CH_3OH) 226 (log ϵ 4.71), 284 (3.99), and 291 (3.92) nm, typical of alkaloids containing a tetrahydro-β-carboline moiety (8). The IR, specific rotation, UV, TLC, and mass spectroscopy data were identical with those derived from authentic tetrahydroalstonine, and a mixed melting-point determination showed no depression.

Cytotoxicity and Antitumor Activities—Certain crude fractions and extracts were submitted to the Drug Research and Development Branch, National Cancer Institute, for bioassay using established protocols (9) in the P-388 leukemia (PS) and Eagle's carcinoma of the nasopharynx in cell culture (KB) systems. The results of these tests are presented in Table I. The alkaloids isolated in this study were previously shown to be devoid of antitumor and cytotoxic activity (10).

SUMMARY

The total crude alkaloid fraction derived from *C. trichophyllus* roots was found to exhibit a moderate degree of cytotoxicity

Table I—Cytotoxicity and Antitumor Test Results from Selected Fractions of *C. trichophyllus*

Extract	KB Results[a], ED_{50}, μg/ml	PS Results[b], mg/kg, T/C
A′	—[c]	—
B′	17.7	—
C′	100.0	—
C	—[c]	—
D′	27.7	50/90
E	100.0	50/95
G	100.0	50/95
H′	100.0	50/104
H″	2.66	25/104
I	2.77	25/113
J	0.56	12.5/104
K	5.99	12.5/140
L	Not tested	
M	100.0	25/95
N	3.33	12.5/131
N-3.0	25.5	50/90
N-3.5	2.33	50/118
N-4.0	3.99	25/118
N-4.5	2.22	50/131
N-5.0	3.22	12.5/125
N-5.5	2.77	12.5/127
N-6.0	4.77	25/90
N-6.5	24.4	25/90
N-7.0	22.2	25/90
N-7.5	25.5	100/104
N-8.0	100.0	—
N-8.5	200.0	—
N-9.0	27.7	—

[a] A fraction is considered active if it has an $ED_{50} \leq 10$ μg/ml. [b] Dose, mg/kg/prolongation of life (percent). A fraction is considered as active if it has a T/C of ≥125. [c] Not tested in KB; inactive in PS.

against the KB culture *in vitro*, and certain fractions were active to a moderate degree in the PS *in vivo* system. The major active fraction was separated and purified using a combination of dialysis, gel permeation chromatography, and gradient pH fractionation techniques. Further purification led to the isolation of ajmalicine, pericalline, vindolinine (dihydrochloride), and tetrahydroalstonine. The common triterpene ursolic acid was isolated from a nonalkaloid fraction. Since the alkaloids isolated in this study were previously shown to be devoid of cytotoxic and/or antitumor activity, work is being continued to elucidate the constituent(s) responsible for the observed activities. Ajmalicine and tetrahydroalstonine have been previously isolated from *C. trichophyllus* aerial parts, but this is the first record of their isolation from the roots of this plant. Vindolinine (dihydrochloride) and pericalline are being reported for the first time from this apocynaceous species.

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* Present address: Department of Pharmacognosy, College of Pharmacy, Rutgers–The State University, New Brunswick, NJ 08903

[x] To whom inquiries should be directed.

Interpretation of Protein–Drug Interaction through Fraction Bound and Relative Contribution of Secondary Sites

R. F. MAIS ˣ, S. KERESZTES-NAGY, J. F. ZAROSLINSKI, and Y. T. OESTER

Abstract □ Three mathematical procedures were devised which allowed for the interconversion and interpretation of protein–drug binding data in the literature. A minicomputer program enabled the calculation of the fraction of drug bound for a specified total drug and protein concentration, given the protein binding constants and the number of sites. The corollary problem of finding the total drug concentration for a particular fraction drug bound was included. In addition, the concept of partial fraction bound for binding systems with multiple classes of sites was introduced and a formula was derived which made possible the calculation of this value from the usual binding constants. Finally, an equation was derived which allowed the total drug concentration and the fraction bound to be obtained from the binding information for selected r values. These were obtained from the Scatchard plot and the associated binding constants. Examples of the utility of these procedures are supplied based on data for human serum albumin binding of four drugs of clinical interest. Evidence is given to show the prominence of the contribution by the secondary sites to the total binding at therapeutic levels of the drugs.

Keyphrases □ Protein–drug interaction—interpretation through fraction bound and relative contribution of secondary sites, interconversion of binding data, multiple classes of sites □ Drug–protein binding—multiple classes of sites, determination of partial fraction bound, secondary sites, interconversion of data □ Binding, drug–protein—fraction bound calculated from total drug, data interconversion and comparison, multiple classes of sites

Considerable information has been amassed in the last 2 decades on protein–drug interactions. The simplest way to describe such data is by the concept of fraction bound. This value is directly obtained from experimental free and total drug levels. Conversely, the calculation of the free and total drug levels for a specified fraction bound is also possible, but only for simple systems involving a single class of binding sites. For such a system, the Goldstein (1) equation relates the fraction bound to the binding parameters for a specified free drug level:

$$\beta = \frac{nP}{nP + K_d + c} \qquad \text{(Eq. 1)}$$

where n is the number of binding sites characterized by a common dissociation constant, K_d. The fraction bound, β, is described for each free drug concentration, c, for a specific protein level, P. However, this equation depends upon free drug concentration and cannot be used to calculate fraction bound for a designated total drug concentration, the most commonly known variable. This relationship does not provide for the interpretation of discrete multiple-site binding (*i.e.*, classes of binding sites differing significantly in their association constants).

Clearly, the calculation of fraction of drug bound from binding constants and concentration of protein and ligand will depend upon selection of a model that most closely approximates the true state of binding. Aside from the simplest case of a single class of sites, the model may include discrete multiple sites, which from a practical standpoint rarely exceed four classes. Alternative models that may be considered are one where there is heterogeneity of the binding sites on the protein molecules and one where prominent electrostatic effects of the ligand may exist. Combinations of these models are possible, and it is conceivable to have a system of multiple binding sites in which one or more classes display electrostatic interaction (2). Each of the models can be described mathematically. Selection of the proper model is based on the conformity of the values (such as fraction bound) predicted by the expression to the experimentally obtained data. Studies have shown that the simple two classes-of-sites model conforms most closely to our data for the binding of salicylate and warfarin to albumin (3, 4).

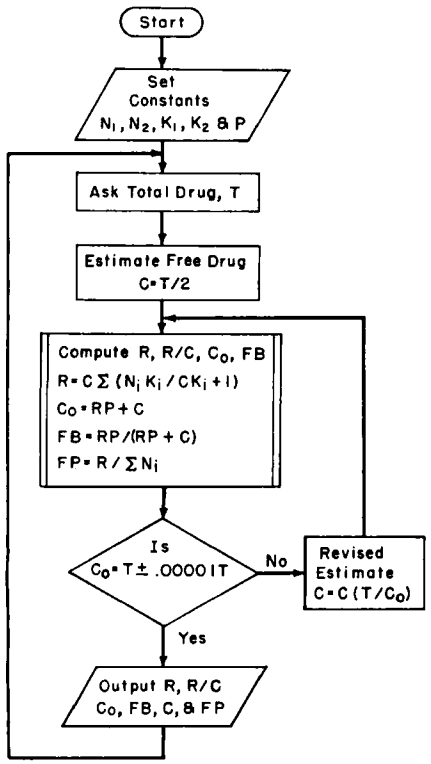
This paper expands upon the concept of fraction bound by extending the interpretation to the contribution of the individual classes of sites to total binding. This interpretation, called partial fraction bound, is analogous to partial pressures in gasometry. In addition, a computer program is presented which allows for the calculation of fraction bound and free drug concentration from protein and total drug concentrations when the binding parameters are known for a given binding system. Other equations are derived which are useful for the interconversion of binding parameters and readily allow comparison of literature values.

EXPERIMENTAL

Determination of binding parameters was made on the following drugs: salicylate, warfarin, chlorpromazine, and quinidine. All compounds except quinidine were studied with gel filtration chromatography using radioisotope dilution analysis. The quinidine data were obtained from equilibrium dialysis experiments in which quinidine was determined spectrophotometrically after extraction from the dialysis solutions. The frontal technique was used for the gel filtration, and the conditions employed were described previously (3).

Materials—All drug–human serum albumin solutions were made up to 0.3 g % albumin[1] in 0.067 M phosphate buffer, pH 7.4. The following ^{14}C-labeled drugs were used: salicylate-^{14}COOH, 31 mCi/mmole, with radiochemical purity of 98%[2]; warfarin-^{14}C, 23 mCi/mmole, with radiochemical purity of 99%[3]; and chlorpromazine-(ring-^{14}C), 31 mCi/mmole, with radiochemical purity of 99%[4]. Quinidine was employed as the hydrochloride[5]. For the quinidine study, the albumin concentration was approximately 2 g % to simulate literature data (5). The ^{14}C-labeled drugs were added to the

[1] Miles Laboratories, Inc., Kankakee, Ill.
[2] CFA. 185, Amersham/Searle, Arlington Heights, Ill.
[3] CFA. 449, Amersham/Searle, Arlington Heights, Ill.
[4] Applied Science Laboratories, State College, Pa.
[5] 14,592-0, Aldrich Chemical Co., Milwaukee, Wis.



Scheme I—Flowsheet for the PDP-8 computer program to derive the fraction bound from the total drug concentration and the binding constants

working drug solutions to provide at least 15,000 cpm/ml. The thixotropic phosphore[6] used as the scintillator for the β-ray spectrometry performed well with protein solutions.

Analytical Methods—The gel filtration frontal analysis technique of Cooper and Wood was used (6), and all samples were run in duplicate. A cross-linked dextran gel[7] was conditioned with the 0.067 *M* phosphate buffer, pH 7.4, at 25° for several hours prior to use. The polyacrylamide gel[8] used for the chlorpromazine study was similarly treated. Jacketed columns[9] and buffer and sample reservoirs were serviced by a thermoregulator system[10] which maintained the temperature at 25 ± 0.2°. Satisfactory elution profiles were obtained for the different drugs under the following conditions.

Salicylate—Forty-five-milliliter samples were applied at a rate of 4 ml/min to 1 × 25-cm columns[7]. Three aliquots for total drug were taken in plateau at 47–53 ml and four aliquots for free drug were taken at 60–64 ml.

Warfarin—Sixty-milliliter samples were applied at a rate of 4 ml/min to 1 × 25-cm columns[7]. Three aliquots for total drug were taken in plateau at 62–68 ml and four aliquots for free drug were taken at 77–81 ml.

Chlorpromazine—Sixty-milliliter samples were applied at a rate of 2.3 ml/min to 1 × 25-cm columns[11]. Three aliquots for total drug were taken in plateau at 62–69 ml and four aliquots for free drug were taken at 77–81 ml.

A discard sample, duplicating the highest concentration of drug, was run through the columns initially for each drug system to condition the column to offset any adsorption on the column that would upset the equilibrium of the system.

Equilibrium Dialysis—The dialysis was performed in 5-ml Karush cells[12], using regenerated cellulose[13] as the membrane. The dialysis, as previously described (3), required 16 hr of shaking at 60 strokes/min. The dialyzed solutions were extracted with ethylene dichloride under controlled pH conditions, and the extracts were measured for quinidine by UV spectrophotometry[14] (8).

Scintillation Spectrometry—One-milliliter aliquots of the samples were added to 10 ml of the scintillator solution. The samples were counted to less than 1% *SD* in a scintillation spectrometer[15]. Quenching of the solutions was evaluated by the channels ratios method of Baillie (9) and found to be insignificant at this protein level. The counts were converted to drug concentration by comparison with standards of similar composition and of known concentration. The concentrations were corrected for solute space and for the Donnan effect according to the procedure employed by Keen (10).

Calculations—Data from the scintillation (counts) and UV spectrometry (absorbance) were converted to the concentrations of bound and free drug. A minimum of eight points was used for derivation of the binding parameters for each system. The data were fit to the two classes-of-sites model described in Eq. 2 by means of a nonlinear least-squares technique using the computer program previously described (3).

ANALYSIS AND TREATMENT OF DATA

Derivation of Fraction Bound from Total Drug Concentration—The mass law expression for binding of a ligand to a macromolecule in which two classes of sites are involved is (11):

$$r/c = \frac{n_1K_1}{1 + K_1c} + \frac{n_2K_2}{1 + K_2c} \qquad \text{(Eq. 2)}$$

where n_1 and n_2 are the number of primary and secondary sites with corresponding association constants of K_1 and K_2. The free ligand concentration is c. By definition, r is the number of moles of ligand bound per mole of binding macromolecule, B/P, and the moles of ligand bound, B, is the difference between the total ligand and the free, $T - c$. From this follows:

$$r = \frac{T - c}{P} \qquad \text{(Eq. 3)}$$

Substitution of this expression for r in Eq. 2 and replacement of the association constants with their reciprocals, the dissociation constants ($1/K = k$), result in the following:

$$\frac{T - c}{Pc} = \frac{n_1}{c + k_1} + \frac{n_2}{c + k_2} \qquad \text{(Eq. 4)}$$

Clearing this expression of fractions yields:

$$c^3 + c^2[P(n_1 + n_2) + k_1 + k_2 - T] + c[P(n_1k_2 + n_2k_1) - T(k_1 + k_2) + k_1k_2] - Tk_1k_2 = 0 \qquad \text{(Eq. 5)}$$

The root of this complicated cubic equation defines c, the free ligand concentration, as a function of the total ligand, T. The fraction bound (FB) can then be written:

$$FB = \frac{T - c}{T} = \frac{T - f(T)}{T} \qquad \text{(Eq. 6)}$$

An extension of the above would show that a system of three classes of sites would result in a complicated fourth degree polynomial, *etc.*

For practical purposes, these equations are unmanageable. Therefore, an iterative procedure was devised for use with a digital computer[16]. This procedure not only solves the problem but gives additional information in a few seconds. The primary and secon-

[6] Insta-Gel, Packard Instrument Co., Downers Grove, Ill.
[7] Sephadex G25, coarse grade, Pharmacia Fine Chemicals, Piscataway, N.J.
[8] Bio-Gel P-4, 50–100 mesh, Bio-Rad Laboratories, Richmond, Calif.
[9] Adjusto-Chrom 5919, 1 × 60-cm set for 25-cm column, Ace Glass Inc., Vineland, N.J.
[10] Haake series FE constant-temperature circulator with KR refrigerated chiller, Polyscience Corp., Evanston, Ill.
[11] Bio-Gel P-4 was used in place of Sephadex G-25, which did not give a satisfactory free drug plateau for this size column with sample volumes less than 100 ml.
[12] No. 3221, Bellco Glass Inc., Vineland, N.J.
[13] Cuprophan, J. P. Bemberg Co., Wuppertal, West Germany.
[14] Beckman DB-G spectrometer.
[15] Model 3380, Packard Instrument Co., Downers Grove, Ill.
[16] PDP-8, Digital Equipment Corp., Maynard, Mass. The program was written in Focal 8 for the PDP-8 minicomputer; however, a similar approach should be possible with any of the more sophisticated programmable calculators that have decision-making capabilities.

Table I—Drug Binding Constants for Human Serum Albumin at 25° in 0.067 *M* Phosphate Buffer (pH 7.4)

Drug	n_1	K_1, M^{-1}	n_2	K_2, M^{-1}	Albumin, $\times 10^5$ *M*
Warfarin	1.46	241,000	2.42	5600	4.01
Salicylate	1.28	70,700	3.80	3300	4.21
	0.76	70,500	2.96	8600	42.8
Chlorpromazine	0.69	14,000	52.3	140	4.29
Quinidine	0.39	20,600	3.15	910	27.5

dary sites, n_1 and n_2, along with their corresponding association constants, K_1 and K_2, and the protein concentration are supplied to the computer. The program (Scheme I) calls for a total drug value, T. One-half of this value is taken as a first estimate of the free drug concentration, c. The total drug concentration for this estimate is calculated from $C_0 = rP + c$ and is then compared with the given value of T; a corrected estimate of c is then derived from $c(T/C_0)$. The iteration proceeds until the absolute difference is less than a small preset value based on the total drug. The program then prints out the usual Scatchard plot parameters r and r/c, along with the fraction bound, FB, and the fraction of protein sites occupied, FP, which is equal to $r/\Sigma n_i$. The final free drug concentration, c, which is printed out, is an exact value. This program is available (12) along with the corollary program for determining r, r/c, C_0, FP, and c from a selected fraction bound drug, FB. Unlike the computer program for the resolution of binding data (3), which is limited to two classes of sites, the present programs accommodate at least four classes. They also provide for changes in the level of protein, assuming that such changes do not influence the binding parameters. This was verified for the salicylate–albumin system in the range of 0.3–3.0 g % albumin (13).

Partial Fraction Bound—Many drug–protein systems are appropriately resolved into two or more classes of binding sites (10, 11, 14, 15). No expression relating the fraction bound to the observed variables is available for this situation. Analysis of the binding of salicylate (3) and warfarin (4) to human serum albumin at pH 7.4 requires such an expression. The experimental term used to describe the amount of bound ligand is fraction bound. This is commonly used in the literature and is related to the clinically important term fraction free by $FB = 1 - FF$. An expression will now be developed for fraction bound relevant to the individual classes of sites. Intuitively, fraction bound for a system of two classes of sites can be defined as:

$$FB = FB_1 + FB_2 \qquad \text{(Eq. 7)}$$

An equivalent expression, in terms of the binding constants n_1, n_2, K_1, and K_2, arises naturally from the relation of FB to r. By definition, the expression for fraction bound is:

$$FB = \frac{T - c}{T} = \frac{B}{T} \qquad \text{(Eq. 8)}$$

where T, B, and c are the total, bound, and free ligand concentrations, respectively. Since r is the moles of ligand bound per mole of binding macromolecule ($r = B/P$), substitution in Eq. 8 yields:

$$FB = \frac{rP}{T} \qquad \text{(Eq. 9)}$$

The mass action law expression for the binding system having i classes of sites is (16):

$$r = c\sum_{i=1}^{i}\frac{n_i k_i}{1 + K_i c} \qquad (i = 1, 2, \ldots, n) \qquad \text{(Eq. 10)}$$

Exchanging the value of r in Eq. 10 for that in Eq. 9 yields:

$$FB = \frac{Pc}{T}\sum_{i=1}^{i}\frac{n_i K_i}{1 + K_i c} \qquad \text{(Eq. 11)}$$

For two classes of sites, this is:

$$FB = \frac{Pc}{T}\left(\frac{n_1 K_1}{1 + K_1 c}\right) + \frac{Pc}{T}\left(\frac{n_2 K_2}{1 + K_2 c}\right) \qquad \text{(Eq. 12)}$$

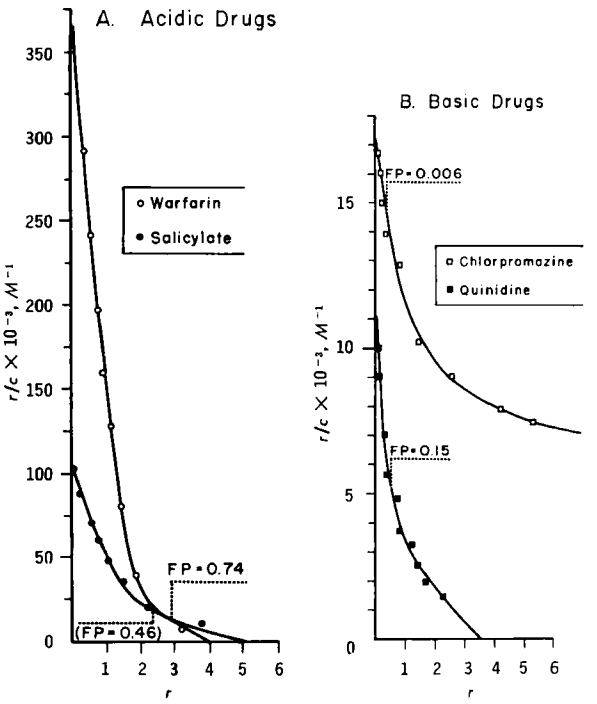


Figure 1—*Scatchard plots of four representative drugs illustrating the point (dotted line) at which the relative contributions of the primary and secondary sites to the binding are equal.* FP *is the calculated fraction of the protein sites occupied at the point of equal binding by primary and secondary sites.*

This operation confirms Eq. 7 and suggests the concept of partial fraction bound, which is the relative contribution of the independent classes of sites to the total bound fraction. Equation 11 and Goldstein's (1) Eq. 1 can be shown to be equivalent by process of identity when $i = 1$.

Determination and Interconversion of *r/c* Values from Specified Values of *r*—Frequently, it is necessary to determine equivalent total drug concentrations when comparing literature values or reproducing the work of others in the laboratory. The minimum data available in the literature usually consist of a Scatchard plot denoting a smoothed fit to experimental data (r values) and the binding parameters n and K for a constant protein level of P. The key here is to derive an expression relating the total drug to the desired r value. The free drug concentration, c, is very crucial for the comparison and, unfortunately, it cannot be obtained directly from the plot.

A solution to the problem of obtaining the total drug concentration for a given r value and the binding constants restricted to a two classes-of-sites system will now be given[17]. The following is obtained from the analytical geometry of the Scatchard plot for such a system:

$$r/c = \frac{y_1 + y_2 \pm \sqrt{(y_1 - y_2)^2 + 4a_1a_2}}{2} \qquad \text{(Eq. 13)}$$

where:

$$y_1 = a_1 - rK_1 \qquad (a_1 = n_1K_1) \qquad \text{(Eq. 14)}$$

$$y_2 = a_2 - rK_2 \qquad (a_2 = n_2K_2) \qquad \text{(Eq. 15)}$$

Equation 13 allows for the calculation of an exact r/c value from n_1, n_2, K_1, and K_2 for any given r value. The latter is reasonably specified from the plot. Consequently, the free drug concentration, c, can now be obtained:

$$c = \frac{r}{r/c} \qquad \text{(Eq. 16)}$$

[17]The derivation is involved and will not be given here. This approach is applicable to three or more classes of sites. The derivation is available from the authors on request.

Table II—Comparison of Fraction Bound Observed for Albumin Level of .3 g % with Fraction Bound Calculated from Constants Obtained for Binding of Salicylate to Albumin at 0.3-g % Level

(a) Total Drug $\times 10^5$ M	(b) FB Observed	(c) FB Calculated	(d) Percent Difference, $\frac{c-b}{b} \times 100$
2.39	0.970	0.977	0.78
10.22	0.966	0.974	0.88
25.57	0.956	0.976	1.17
40.00	0.953	0.958	0.50
59.70	0.931	0.940	0.97
102.56	0.891	0.893	0.22
184.39	0.728	0.785	7.77
		Average	1.75

Since $r = B/P$ or $B = rP$ and by definition $T = B + c$:

$$T = rP + c \qquad \text{(Eq. 17)}$$

Substituting this value for T in Eq. 9:

$$FB = \frac{rP}{rP + c} \qquad \text{(Eq. 18)}$$

The preceding relationships and their combinations permit a complete evaluation of most binding data from the literature and allow for satisfactory comparative use of such information as will be shown.

RESULTS AND DISCUSSION

Figure 1 shows the binding data in the form of Scatchard plots for the four drugs studied. The two-site computer resolution gives rise to the binding parameters for the acidic and basic drugs shown in Table I.

Quinidine has been reported (5) to bind to a single class of sites with $n = 1$ and $K = 7700$. Two classes-of-sites resolution of the data (Table I) results in considerably different values for fraction bound when calculated from parameters in this study using the concentrations employed by them. Comparison values are derived through the use of Eqs. 2 and 16–18. For their lowest concentration of free quinidine (3.0×10^{-6} M), these workers found the fraction bound to be 0.62 compared to the value of 0.74 in this study. The highest concentration they employed (2.2×10^{-3} M) resulted in 0.09 fraction bound compared to this study's value of 0.27. This disparity may reflect the variability of equilibrium dialysis as previously cited in the study of salicylate binding (3).

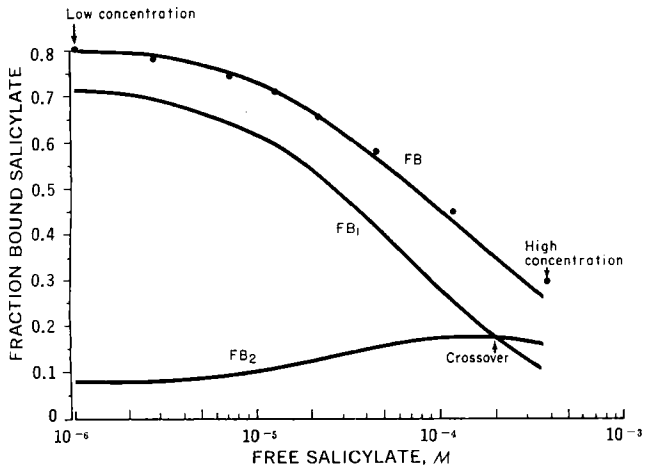


Figure 2—*Contribution of the partial fraction bound due to the primary and secondary sites,* FB_1 *and* FB_2, *to the total fraction of drug bound,* FB. *The curves were derived from the binding parameters obtained by computerized smoothing of the original binding data (3). Values for the curves were calculated with the aid of the computer program referred to in the text. The experimentally observed values for fraction bound are shown as points.*

Table III—Partial Fraction Bound Profiles for Selected Drugs

Drug Parameter	Salicylate	Warfarin	Chlorpromazine	Quinidine
Low concentration ($\times 10^5$ M)	0.2	1.6	0.4	1.6
FB[a]	0.79	0.92	0.42	0.74
Percent FR_1[b]	87	95	56	72
Crossover ($\times 10^5$ M)	27.7	37.5	3.7	24.0
FB	0.41	0.31	0.38	0.59
Percent FR_1	50	50	50	50
High concentration ($\times 10^5$ M)	50.0	60.0	100.0	235.0
FB	0.30	0.21	0.24	0.27
Percent FR_1	40	45	11	16

[a] FB = fraction bound. [b] Percent $FR_1 = (FB_1/FB) \times 100$, the percent contribution of the primary sites to the total binding.

The fraction bound–total drug interconversion program (Scheme I) was used to test the value of experimental binding constants in predicting binding information for protein levels other than those used to determine the constants (13). The binding constants from the 0.3 g % ($P = 4.21 \times 10^{-5}$ M) albumin study for salicylate binding (Table I) were employed in the computer to determine the total drug concentrations for a given range of fraction bound at the 3.0 g % level of albumin. Salicylate samples were then prepared at concentrations dictated by the computer, and the fraction bound was determined from these by gel filtration as described (Table II, columns a and b). The constants from this study are listed in Table I for $P = 42.8 \times 10^{-5}$ M. The same computer program was then used to verify the equivalence of the data from the two studies. This was done by computing the fraction bound for the total drug levels used in the 3.0 g % albumin study, but based on the constants determined in the 0.3 g % study (Table II, column c). The calculated values deviated by less than 2% from the observed fraction bound for the entire range of drug concentrations studied (Table II, column d). The large deviation reflects the poorer fit of the smoothed curve at very high concentrations.

The variation of the binding profiles of the four drugs is illustrated through the concept of partial fraction bound. Figure 2 illustrates the profile for the binding of salicylate in terms of fraction bound *versus* free drug concentration. The total binding is also resolved into the binding due to the primary and secondary sites using Eq. 12. The points of interest are the values for FB, FB_1, and FB_2 at the low, crossover, and high concentrations indicated in the figure. The crossover point is that concentration where the binding by the primary and secondary classes of sites is equal. It is calculated by a modification of the computer program (Scheme I) for which the conditional statement is changed to read: $FB - 2FB_1 = 0$. Results for the four drugs tested are shown in Table III. It can be seen that the point at which the binding is evenly accounted for by the primary and secondary sites varies considerably with the fraction bound for the various drugs.

Figure 1 shows that this point also occurs with considerable variation between drugs in respect to the molar ratio of bound drugs, r; the value is as small as 0.35 for chlorpromazine and as large as 3.0 for warfarin. This is also reflected in the variation of the fraction of protein sites occupied (FP) at the crossover point for the four drugs in Fig. 2. The basic drug chlorpromazine represents an extreme in this case; the crossover point occurs when only 0.6% of the albumin sites are occupied. Literature data on chlorpromazine are not available for comparison. It is also clear from Fig. 2 and Table III that the secondary sites are responsible for the majority of binding at higher therapeutic levels of the other drugs as well as for salicylate. The smoothed curve (Fig. 2) is calculated with the aid of Eq. 12. The extent to which this curve agrees with the actual data points attests to the suitability of the two-site model for salicylate.

An example of the use of the interconversion formula (Eq. 13) is

the comparison of this warfarin binding study with the study of O'Reilly (17). The data for warfarin in Table I were modified to make them conform to the conditions of temperature and protein concentration employed by O'Reilly. The association constants for warfarin at 27° were calculated from thermodynamic data using the van't Hoff equation; the revised values are K_1 = 238,100 and K_2 = 5200. Equations 13–18 were then used to calculate the value for r/c and FB from the binding parameters corresponding to the r values found by O'Reilly at the albumin concentration he used ($P = 2.32 \times 10^{-5}$ M). For the lowest concentration in his study on warfarin (r = 0.40 and r/c = 178,800), he obtained a value for fraction bound of 0.793. Corresponding values from the present study are r = 0.40, r/c = 259,800, and FB = 0.858. The highest concentration he used (r = 1.90 and r/c = 41,000) resulted in a fraction bound of 0.454. The values in this study are r = 1.90, r/c = 47,100, and FB = 0.522. The values for fraction bound averaged 8.0% higher than those he obtained for the entire concentration range studied. The thermodynamic data used to convert the warfarin binding constants of Table III to 27° resulted in a value for the free energy of binding of −7.29 kcal/mole. This compares favorably with the value O'Reilly obtained of −7.37 kcal/mole.

CONCLUSION

As binding data become more and more reliable due to improved techniques, it becomes apparent that multiple-class binding between serum albumin and various drugs is becoming the rule rather than the exception. This is well illustrated by the four examples presented in this paper. The two classes-of-sites resolution of the binding data provided the impetus to calculate the total fraction bound drug in terms of separate contributions by the two classes of sites. Such analysis revealed that the contributions of the secondary sites to total binding becomes significant at high drug concentrations in all four cases studied.

The computer program described here allows the calculation of protein bound drug at any total drug and protein concentration when the binding parameters are known. The usefulness of this program is illustrated in the case of salicylate, where the contributions of the two classes of sites are calculated at total therapeutic drug levels and physiological protein concentration.

Additional equations derived in this paper facilitate the interconversion of the various types of binding data for comparison and evaluation, as illustrated with warfarin and quinidine.

The concept of partial fraction bound, the computer programs, and the various equations reported here fulfill a need in the interpretation and communication of protein–drug binding information.

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[x] To whom inquiries should be directed.

Model Transport Studies Utilizing Lecithin Spherules IV: Transport of D-Glucose in Spherules Prepared from Lecithin, Dicetyl Phosphate, and Cholesterol

ZAKA-UD-DIN T. CHOWHAN[x] and WILLIAM I. HIGUCHI

Abstract □ The transport of D-glucose in liposome dispersions prepared from lecithin–dicetyl phosphate–cholesterol was studied. The transport experiments were carried out using previously developed techniques in which the release of the solute is studied from dispersions contained in dialysis bags. The experimental results of the direct-release and uptake-release experiments were analyzed by the monosize, multiconcentric layer Model 2, which was developed and used recently for the 3-*O*-methyl-D-glucose transport studies. Uniformly good fits of the experimental data with the model were observed, and the permeability coefficients and the aqueous–lipid partition coefficients were calculated for liposome dispersions containing different percentages of cholesterol. A direct relationship between the percent cholesterol in liposome dispersions and the effective bulk permeability coefficient was obtained. The incorporation of cholesterol into liposome dispersions does not seem to alter the effective aqueous–lipid partition coefficient.

Keyphrases □ Lecithin spherules—transport of D-glucose in lecithin–dicetyl phosphate–cholesterol spherules, permeability and partition coefficients □ D-Glucose—transport in lecithin–dicetyl phosphate–cholesterol spherules, permeability and partition coefficients □ Cholesterol—transport of D-glucose in lecithin–dicetyl phosphate–cholesterol spherules, permeability and partition coefficients □ Permeability—lecithin–dicetyl phosphate–cholesterol liposome dispersions, model transport studies □ Partition coefficients—lecithin–dicetyl phosphate–cholesterol liposome dispersions, model transport studies

In previous reports (1–3), quantitative methods were developed and used in the determination of effective bulk permeability coefficients for D-glucose, 3-*O*-methyl-D-glucose, and taurocholic acid-[cholic-^{3}H (G)] in complex aqueous liposome dispersions. The release of the solutes from dispersions contained in a dialysis bag was studied as a function of time using the direct-release, the dilution-release, and the uptake-release experimental procedures. The data were analyzed using the monosize, multiconcentric layer models, which assume that the spherules consist of multiconcentric bilayers of equal thickness separating aqueous compartments.

Since cholesterol is abundant in many biological membranes, several experimental model membrane systems have been used to understand its unique properties in the membranes. Several investigators used cholesterol in combination with lecithin for the preparation of liposome dispersions (4–14). The evidence derived from electron microscope studies (15) indicated that equimolar mixtures of lecithin and cholesterol arrange themselves in the form of concentric skins of bimolecular leaflet in aqueous solutions which are basically the same as lecithin alone. Reported observations (10–14, 16) indicated a reduction in permeability caused by the presence of cholesterol in liposome dispersions. The effective bulk permeability coefficient of 3-*O*-methyl-D-glucose was 2.4 times smaller for dispersions prepared from lecithin–dicetyl phosphate–cholesterol (10:1:1) as compared to dispersions prepared without cholesterol (2).

The purpose of this investigation was to use the liposome dispersion system prepared from lecithin–dicetyl phosphate–cholesterol in determining the effective bulk permeability coefficient of D-glucose. The effect of different percentages of cholesterol used in the preparation of liposome dispersions was expected to elucidate the role of cholesterol in biological membranes.

EXPERIMENTAL

Materials—Egg yolk lecithin was purified using the procedure reported previously (1), dicetyl phosphate[1] was used without further purification, and cholesterol[2] was recrystallized twice from ethanol. Anhydrous D-glucose[3] was analytical reagent grade. The dialysis bags[4] were used without pretreatment. The radioactive[5] D-glucose-^{14}C was obtained in crystalline solid form.

Procedure—The liposome dispersions were prepared from 5.0% egg yolk lecithin, 0.5% dicetyl phosphate, and varying percentages of cholesterol. The procedures used in the preparation of dispersions and for the transport of solute were the same as reported earlier (1). The dispersions were prepared in 5.1% anhydrous D-glucose solution, which also served as a sink solution. Five milliliters of the dispersion was added to the dialysis bag, and at zero time the dialysis bag holder–stirring assembly was lowered into 100 ml of sink solution maintained at 25°. The dispersion and the sink solution were stirred at 150 rpm by different stirring arrangements. At the end of each sampling time, the dialysis bag holder–stirring assembly was transferred to another water-jacketed beaker containing 100 ml of fresh sink solution. The overall rate of the solute transport represented both the movement of solute out of the spherules and the transfer of solute across the dialysis bag. The procedures for the determination of radioactivity and particle-size distribution[6] were reported earlier (1).

RESULTS AND DISCUSSION

The results of the direct-release experiment for the release of D-glucose in liposome dispersions containing different percentages of cholesterol are given in Fig. 1. The data are compared with the results of calculations utilizing the monosize, multiconcentric Model 2 (see *Appendix*). Since the results of the dilution-release experiments were very close to those of the direct-release experiments, and since the former were found to give the same good agreement between theory and experimental values, they are not shown here. The results of the uptake-release experiments are given in Fig. 2 and are compared to the results of computations using the same model and the same set of parameter values as were used for the direct-release case (Table I).

The generally good agreement obtained between the model-predicted results, the experimental results, and the results from different types of experimental procedures indicates the usefulness of

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Fisher Scientific Co., Fair Lawn, N.J.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Union Carbide Corp., Chicago, Ill.
[5] New England Nuclear, Boston, Mass.
[6] Using a Coulter counter.

Table I—Input Data Used in Calculations for D-Glucose Transport Studies in a Liposome Dispersion System Containing Cholesterol

Cholesterol, %	V_I, ml	Mean Surface Radius, μm	n	p_b ($S_b \times 10^3$), cm^3 sec^{-1}	p, cm sec^{-1}	k
0.2	0.97	0.655	45	1.919	1×10^{-8}	21
0.5	1.03	0.673	45	1.919	6×10^{-9}	21
0.8	0.94	0.653	45	1.919	2.5×10^{-9}	21

the method in determining the effective bulk permeability coefficients. The relatively low partition coefficient value for this solute and the range of permeability coefficients found in this study make the determination of this latter parameter very sensitive. Significantly different release curves are obtained (Fig. 1) only because of these differences in permeability coefficients. Furthermore, the incorporation of cholesterol into the liposome dispersions does not seem to alter the effective aqueous–lipid partition coefficients. An earlier report (14) on the decrease in permeability of glucose due to the presence of cholesterol may, therefore, be related to the reduction in the permeability coefficient. Although the liposome dispersion system prepared from lecithin–cholesterol in equal molar proportions has been described as basically the same as lecithin alone (15), the molecular arrangement in the two dispersion systems may not be exactly the same. While making comparisons between the two systems, one must recognize this limitation. Therefore, the results given in Fig. 3 showing the relationship between the percent cholesterol in liposome dispersions and the effective bulk permeability coefficient as defined in the *Appendix* (Eq. A5) were indeed interesting.

The linear relationship obtained in Fig. 3 indicates that the decrease in the permeability coefficient is directly related to the concentration of cholesterol used in the preparation of liposome dispersions. The investigations on mixed monolayers of cholesterol and phospholipids (17) demonstrated reductions in the average area per molecule compared with those in films of phospholipids alone. The direct relationship between the percent cholesterol in the liposomes and the permeability coefficient seen in this study could be explained on the basis of reductions in the average volume per molecule in the presence of cholesterol.

APPENDIX

Monosize, multiconcentric layer models assume that all spherules are of the same size and consist of $i = 1$ to $i = n$ number of segments. In Model 2, the outermost lipid layer is considered as a part of the spherule so that each segment consists of an aqueous phase and a lipid phase. The basic assumptions and equations were reported earlier (1, 2). A summary of the equations used in Model 2 is presented here.

Equation A1 relates the average solution concentration to the aqueous and lipid contributions for the $i = 1$ to $i = n$th segment:

$$\begin{aligned} C_{1,t} &= [v_a + (v_l)(k)]C_{1,a} \\ &\vdots \\ C_{n,t} &= [v_a + (v_l)(k)]C_{n,a} \end{aligned} \qquad \text{(Eq. A1)}$$

where $C_{1,t}$ and $C_{1,a}$ are the average and aqueous solution concentrations in segment 1, respectively; v_a and v_l are the aqueous and lipid volume fractions, respectively; and k is the effective lipid–aqueous partition coefficient.

The rates at which solute leaves segments $i = 1$ to $i = n$th are given by a set of equations:

$$-V_1[v_a + (v_l)(k)]\frac{dC_{1,a}}{dt} = S_1[p(C_{1,a} - C_{2,a})] \qquad \text{(Eq. A2}a\text{)}$$

$$V_2[v_a + (v_l)(k)]\frac{dC_{2,a}}{dt} = S_1[p(C_{1,a} - C_{2,a})] - S_2[p(C_{2,a} - C_{3,a})] \qquad \text{(Eq. A2}b\text{)}$$

$$\vdots$$

$$V_n[v_a + (v_l)(k)]\frac{dC_{n,a}}{dt} = S_{n-1}[p(C_{n-1,a} - C_{n,a})] - S_n[p(C_{n,a} - C_{II})] \qquad \text{(Eq. A2}c\text{)}$$

where V_1 is the volume of segment 1, S_1 is the surface area of segment 1, p is the permeability coefficient per segment, and C_{II} is the concentration of solute in Compartment II, which is the external phase of the dispersion. The rate expression of solute in Compartment II is given by Eq. A3:

$$V_{II}\left(\frac{dC_{II}}{dt}\right) = S_n[p(C_{n,a} - C_{II})] - (S_b)(p_b)(C_b) \qquad \text{(Eq. A3)}$$

where V_{II} is the volume of Compartment II; S_b and p_b are the surface area and effective permeability coefficient of the bag, respectively; and C_b is the concentration of solute in the bag.

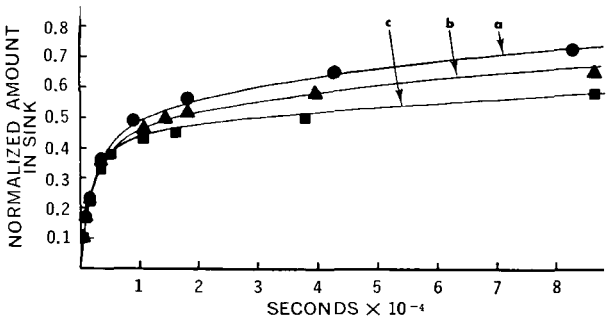

Figure 1—*Comparisons of the experimental data and results of computation using the monosize, multiconcentric layer Model 2 for the direct-release experiments using lecithin–dicetyl phosphate dispersion systems containing different percentages of cholesterol. Key (symbols represent experimental data): ●, 0.2% cholesterol; ▲, 0.5% cholesterol; and ■, 0.8% cholesterol. Curves a, b, and c represent calculations using parameter values taken from Table I for the 0.2% cholesterol, 0.5% cholesterol, and 0.8% cholesterol, respectively.*

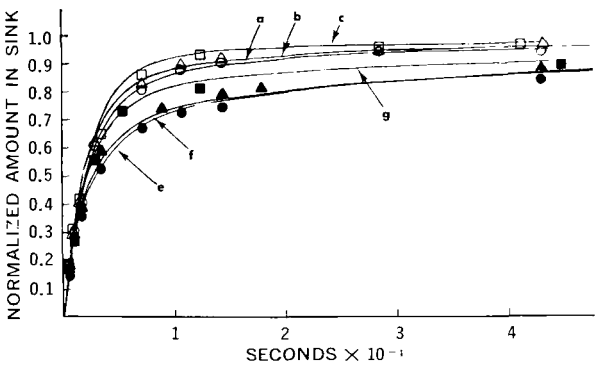

Figure 2—*Comparisons of the experimental data and results of computations using the monosize, multiconcentric layer Model 2 for the uptake-release experiments using lecithin–dicetyl phosphate dispersion systems containing different percentages of cholesterol. Key (symbols represent experimental data): ○, 0.2% cholesterol after 2 hr uptake; △, 0.5% cholesterol after 2 hr uptake; □, 0.8% cholesterol after 2 hr uptake; ●, 0.2% cholesterol after 10 hr uptake; ▲, 0.5% cholesterol after 10 hr uptake; and ■, 0.8% cholesterol after 10 hr uptake. Curve a (0.2% cholesterol), curve b (0.5% cholesterol), and curve c (0.8% cholesterol) represent 2-hr uptake calculations. Curve e (0.2% cholesterol), curve f (0.5% cholesterol), and curve g (0.8% cholesterol) represent 10-hr uptake calculations. The parameter values were taken from Table I.*

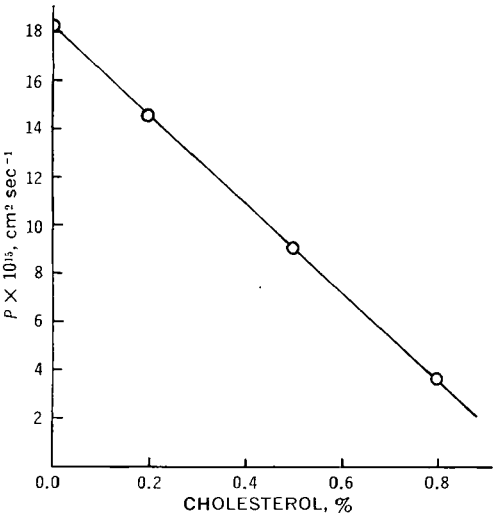

Figure 3—*Relationship between the percent cholesterol in liposome dispersions and the effective bulk permeability coefficient. The effective bulk permeability coefficient,* P, *was calculated using Eq. A5. The permeability coefficient per segment,* p, *value for the 0% cholesterol data point was taken from Ref. 1.*

The rate of appearance of solute in the sink is given by Eq. A4:

$$\frac{dA_s}{dt} = (p_h)(S_h)(C_h) \qquad \text{(Eq. A4)}$$

where A_s is the normalized amount of solute in the sink.

For multiconcentric layer models, it was shown earlier (1) that the choice for the permeability coefficient, p, depends upon both the radius, r, of the spherules and the number, n, of segments used in the calculations according to the relationship:

$$p = \frac{Pn}{r} \qquad \text{(Eq. A5)}$$

where P is regarded as the effective bulk permeability coefficient when the spherule is treated as a homogeneous sphere. At constant P, the convergence behavior of the theoretical curves was shown earlier (1) as n was increased. For $n = 45$, the release curve was approximately 2% higher than that for the $n \rightarrow \infty$ case. This was the basis for selecting the value of n used in the calculations.

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[x] To whom inquiries should be directed. Present address: Syntex Research, Palo Alto, CA 94304

Bioavailability of Aminosalicylic Acid and Its Various Salts in Humans IV: Comparison of Four Brands of the Sodium Salt

PERTTI J. PENTIKÄINEN, SUK HAN WAN ˣ, and DANIEL L. AZARNOFF

Abstract □ The relative bioavailability of four commercially available brands of the sodium salt of aminosalicylic acid was investigated in a crossover study with healthy volunteers. The estimation of bioavailability was based on comparison of areas under the plasma drug concentration–time curves and on cumulative excretion of aminosalicylate as well as its metabolites in urine, following a single dose of the drug. The total absorption of all products was similar, 86–90% of the dose being recovered in the urine during 24 hr. The fraction of dose available as the biologically active form of nonmetabolized aminosalicylate was significantly higher for one product. This difference, however, was probably due to the higher dose of this brand studied compared to the other brands (4.0 and 2.9 g as the free acid, respectively), since the main metabolic route, conjugation to *N*-acetyl-*p*-aminosalicylic acid, is known to be dose dependent and capacity limited. There were no significant differences in the bioavailability of the other three brands studied.

Keyphrases □ Sodium aminosalicylate tablets—bioavailability of four commercially available products compared □ Aminosalicylic acid, sodium salt, tablets—bioavailability of four commercially available products compared □ Bioavailability—comparison of four different commercially available sodium aminosalicylate tablets

Although its use is decreasing, aminosalicylic acid is still widely used in the treatment of tuberculosis as a supplement to other tuberculostatic agents. This drug is used in several chemical forms and pharmaceutical formulations developed to reduce its GI side effects. Blood levels of aminosalicylic acid obtained with different products vary considerably (1–4). The sodium salt is one of the most commonly used forms since it is relatively well tolerated and gives higher blood levels of aminosalicylate than other forms of the compound (5, 6). Although several brands of the sodium salt are commercially available, no reported studies have systematically compared the bioavailability of these products. The recent findings of inequivalencies between commercially available products of several drugs have emphasized the importance of evaluation of bioavailability of chemically equivalent products.

Several factors complicate the bioavailability studies of aminosalicylic acid. The interpatient variation in GI absorption of the compound is large (4), necessitating the use of relatively large groups of subjects. Crossover design and statistical methods help distinguish the differences in products from other variables. Since aminosalicylic acid is largely inactivated by metabolism, in part during the first pass, the measurement of unchanged drug only gives information about the fraction that is available in biologically active form but does not necessarily reflect total absorption. The latter can be better estimated by measuring the excretion of both unchanged drug and its metabolites in urine. In addition, the nonlinear pharmacokinetics of aminosalicylic acid (7) complicate the interpretation of results of previous absorption studies.

The bioavailability of four commonly used brands of the sodium salt was compared by measuring both unchanged drug and its main metabolite in plasma and urine in this crossover study in healthy individuals.

MATERIALS AND METHODS

Products—Four brands of sodium aminosalicylate from different manufacturers were tested: Product A[1], 0.5 g; Product B[2], 0.5 g; Product C[3], 0.5 g; and Product D[4], 0.57 g. All products were purchased by the Food and Drug Administration and met USP specifications for purity and content uniformity[5].

Subjects—Twelve healthy volunteers between 20 and 44 years of age, 11 men and one woman, weighing between 62.7 and 88.6 kg, participated. Before the study, in addition to written informed consent, a history, physical examination, and laboratory tests (complete blood count, serum alkaline phosphatase, serum glutamate pyruvate transaminase, serum glutamate oxalate transaminase, serum creatinine, and urinalysis) were obtained. The results of these examinations were within normal limits for all volunteers.

Drug Administration—Each subject received a single dose of 4 g of the sodium salt (equivalent to 2.9 g of free acid) of Products A, B, and C and a dose of 6.8 g (equivalent to 4 g of free acid) of Product D in a random order on four separate occasions. These doses were dictated by the potency of the different products. The drug was administered as eight tablets with 250 ml of water at 8 am after an overnight fast. Fasting was continued for the first 5 hr after drug administration. The use of other drugs was not allowed for at least 2 weeks and alcohol was not allowed for at least 3 days prior to as well as during the study. One week elapsed between the study of each product in each volunteer.

Sampling—Venous blood samples were drawn in tubes containing sodium heparin through a Teflon cannula inserted in an antecubital vein just before drug administration and at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, 8, and 10 hr thereafter. Urine was collected prior to ingestion of the drug and then continuously in 2-hr periods for 10 hr and finally from 10 to 24 hr. Plasma was separated by centrifugation, and the plasma and aliquots of each urine collection were kept frozen until analyzed.

Analysis of Aminosalicylic Acid—A modified Marshall method was used for the assay of aminosalicylic acid and *N*-acetyl-*p*-aminosalicylic acid as described earlier (7). With this modification, drug concentrations as low as 1 μg/ml could be measured accurately.

Analysis of Results—The plasma concentrations of aminosalicylic acid were corrected to a 70-kg body weight. In addition, a correction to a 2.9-g dose was made for Product D. Areas under the plasma drug concentration–time curve *(AUC)* were calculated by the trapezoidal rule. The half-lives of elimination of aminosalicylic acid and the acetyl conjugate were calculated[6] from a one-com-

[1] Pamisyl Sodium, Lot MB501, Parke, Davis & Co., Detroit, Mich.
[2] Parasal Sodium, Lot 73204, Panray Division, Englewood, N.Y.
[3] Pasdium, Lot 3727121, Kasar Laboratories, Niles, Ill.
[4] PAS Sodium, Lot X8646, Hellwig, Inc., Chicago, Ill.
[5] Analyses performed by FDA.
[6] Using the BMDX 85 program on an IBM 1130 computer.

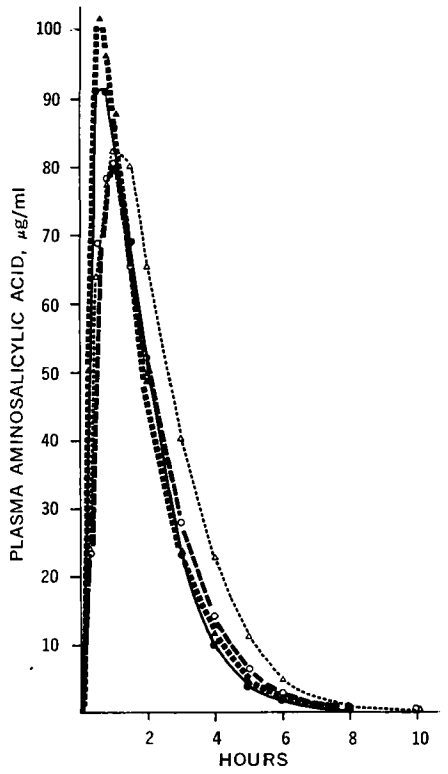

Figure 1—*Mean plasma concentration of aminosalicylic acid from 12 subjects following administration of four different brands of sodium aminosalicylate. Data corrected to 70 kg body weight and to a dose equivalent to 2.9 g aminosalicylic acid. Key: ●, Product A; ○, Product B; ▲, Product C; and △, Product D.*

partment open model. The cumulative excretion of total drug, non-acetylated drug, and acetylated drug in urine was calculated as a percent of the dose. The *AUC's*, the peak plasma concentrations of aminosalicylic acid independent of sampling time, the times to reach the peak level, and the urine data were analyzed by analysis of variance for crossover experiments. This method permitted the fractioning of the total variance into variance between the products, individuals, and periods or sequence of administration. Whenever significant differences ($p < 0.05$) between the products were found, Duncan's multiple-range test was applied to find the source of the difference (9).

RESULTS

Analysis of Plasma Data—The absorption of aminosalicylate from all products studied was rapid, as reflected by the relatively short time to attain the peak plasma drug level (Fig. 1 and Table I). The peak level after Product D was reached markedly later than that with the other products.

The peak plasma level attained following administration of Product C was significantly higher than that following Product B or D. Also, the peak level with Product A was significantly higher than with Product D, whereas no statistically significant difference was observed between Products B and D (Table I).

Table I—Peak Plasma Concentration of Aminosalicylic Acid and Time to Reach Peak Concentration

Product	Peak Concentration, μg/ml[a,b]	Peak Time, hr[a]
A	100.0 ± 6.0[c]	0.69 ± 0.06
B	89.0 ± 6.6	0.81 ± 0.09
C	106.6 ± 6.6[d]	0.65 ± 0.06
D	84.8 ± 7.3	1.19 ± 0.10

[a] Values are mean ± standard error ($n = 12$). [b] Results are corrected to 70 kg body weight and to a dose equivalent to 2.9 g free acid. [c] Significantly higher than Product D. [d] Significantly higher than Product B or D.

Table II—Areas under the Aminosalicylic Acid Plasma Concentration–Time Curves (*AUC*) and Their Statistical Analysis following Administration of Four Brands of Sodium Aminosalicylate

Product	*AUC*, μg hr ml^{-1}, mean ± *SE*
A	199.5 ± 11.2
B	199.9 ± 12.1
C	207.1 ± 9.5
D	245.4 ± 10.7

Analysis of Variance

Source of Variation	*df*	*ss*	*ms*	*F*
Between individuals	11	31707	2882	3.30
Between products	3	17113	57504	6.54
Between periods	3	5218	1739	1.99
Error	30	26181	872	
Total	47	80220		

SE of varietal mean = 8.528, $F_{0.05}$ (3.30) = 2.92, $F_{0.05}$ (11.30) = 2.12

Multiple-Range Analysis

	p	(2)	(3)	(4)
Shortest significant ranges		24.6	25.9	26.6
Product	A	B	C	D
AUC[a]	199.5	199.9	207.1	245.4

D − A = 45.9 > 26.6; significant
D − B = 45.5 > 25.9; significant
D − C = 38.0 > 24.6; significant
C − A = 7.6 < 25.9; not significant
C − B = 7.2 < 24.6; not significant
B − A = 0.4 < 24.6; not significant

[a] Any two or more means not underscored by the same line are significantly different.

The corrected *AUC* of aminosalicylic acid was significantly larger for Product D than for the other products (Table II). No statistically significant difference was found between the *AUC's* after Product A, B, or C. Significant interpatient variation was obvious, but there was no significant difference between periods.

The rate constant for elimination of aminosalicylic acid was the same for all products; the mean half-lives calculated for Products A, B, C, and D were 0.94, 1.03, 1.00, and 0.98 hr, respectively.

The peak plasma levels of the acetyl conjugate were obtained approximately 2 hr later than those of aminosalicylate (Fig. 2). The rate of elimination of the acetyl conjugate was slower than that of unchanged drug. The mean half-lives of elimination of the acetyl conjugate were 1.66, 1.40, 1.19, and 1.61 hr for Products A, B, C, and D, respectively.

Analysis of Urine Data—The cumulative excretion of total drug, nonacetylated drug, and acetylated drug in urine following administration of different products is illustrated in Figs. 3–5. The 24-hr values and their statistical analyses are presented in Table III.

The fraction of dose excreted in urine as aminosalicylate and its metabolites averaged 86–90%, and there were no significant differences between the products. The fraction excreted as nonacetylated compound during 24 hr was 47% of the dose of Product D and was significantly higher than the corresponding figures after Product A, B, or C (38–41%). There was a similar relationship between the products for the *AUC's* of aminosalicylic acid. A significant interpatient variation was found in the urinary excretion of nonacetylated drug, but the fraction of the dose excreted as the acetyl conjugate in urine during 24 hr was similar (43–49% of dose) for all products. However, the ratio of nonacetylated drug to acetylated drug was greater than 1 only in the case of Product D.

DISCUSSION

The importance of measuring both the unchanged drug and its metabolites in the bioavailability studies of aminosalicylic acid has been stressed (10). In humans, the drug is metabolized mainly to *N*-acetyl-*p*-aminosalicylic acid and to a lesser degree to its glycine conjugate, *p*-aminosalicyluric acid. These metabolites together ac-

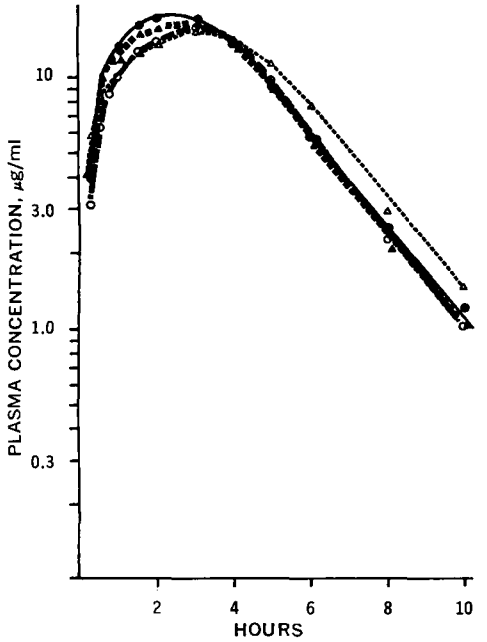

Figure 2—*Mean plasma levels of* N-*acetyl*-p-*aminosalicylic acid from 12 subjects following administration of four different brands of sodium aminosalicylate. Data corrected to 70 kg body weight and to a dose equivalent to 2.9 g free acid. Key:* ●, *Product A;* ○, *Product B;* ▲, *Product C; and* △, *Product D.*

count for more than 90% of the metabolites in urine (6, 11). Lauener *et al.* (7) showed that unchanged drug is the biologically active form, with the metabolites having virtually no bacteriostatic action. Thus, the biologically active fraction available from the dose can be estimated on the basis of the *AUC* and by measuring the excretion of unchanged drug in urine, whereas the cumulative excretion of total drug in urine better reflects the total absorption of the product.

The method used in the present study does not differentiate be-

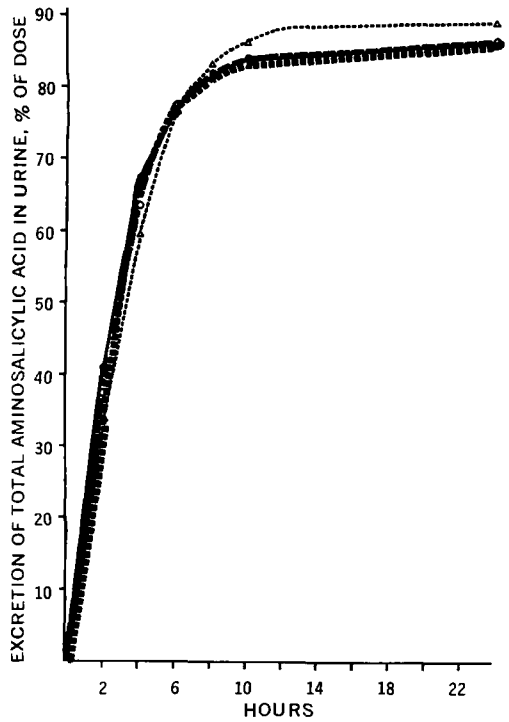

Figure 3—*Mean cumulative excretion of total drug in urine from 12 subjects following administration of four different brands of sodium aminosalicylate. Key:* ●, *Product A;* ○, *Product B;* ▲, *Product C; and* △, *Product D.*

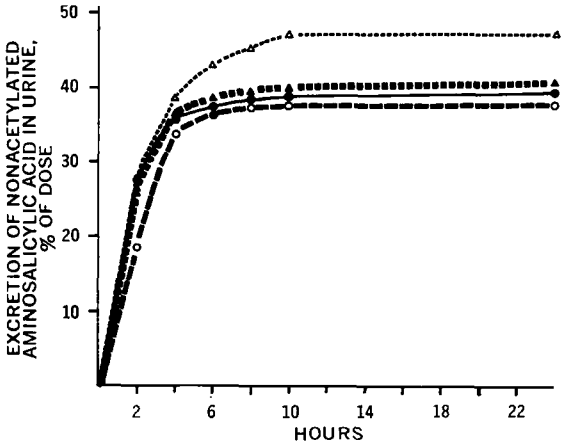

Figure 4—*Mean cumulative excretion of nonacetylated drug in urine from 12 subjects following administration of four different brands of sodium aminosalicylate. Key:* ●, *Product A;* ○, *Product B;* ▲, *Product C; and* △, *Product D.*

tween unchanged drug and the glycine conjugate; therefore, the term nonacetylated drug was used for the sum of these compounds in the urine. The overestimation of aminosalicylic acid in plasma as a result of the nonspecificity of the assay employed is small since the nonacetylated fraction in plasma consists of 95% unchanged drug and only 5% glycine conjugate (12). This ratio has been shown to be constant at different plasma levels of aminosalicylate (7). The ratio of these compounds is also constant in urine, although the fraction of glycine conjugate in urine is higher (20–25%) than in plasma (7). A specific method for aminosalicylate has been described but is impractical for a large-scale study (10). Considering the short half-lives of aminosalicylic acid (approximately 1 hr) and the acetyl conjugate (approximately 1.5 hr), the 24-hr collection period was long enough for virtually all excreted drug to be recovered and should satisfactorily reflect total absorption.

The sodium salt is rapidly and completely absorbed from a solution as shown earlier (8). The tablet formulation somewhat decreases the rate and the extent of absorption of the sodium salt, but the recovery of 86–90% of the dose in urine still indicates very good GI absorption of the sodium aminosalicylate tablets studied. As demonstrated earlier, the total absorption of sodium, potassium, and calcium salts in tablet form is essentially similar and markedly faster and more complete than the absorption of tablets of the free acid (13).

The four brands of the sodium salt compared in the present study did not differ in the total absorption of the compound. How-

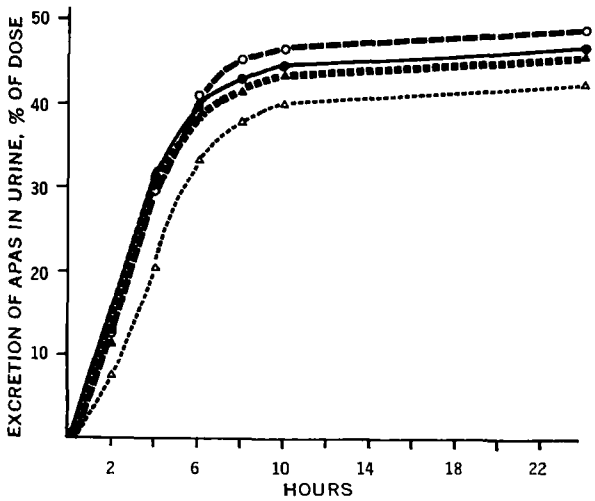

Figure 5—*Mean cumulative excretion of* N-*acetyl*-p-*aminosalicylic acid (APAS) in urine from 12 subjects following administration of four different brands of sodium aminosalicylate. Key:* ●, *Product A;* ○, *Product B;* ▲, *Product C; and* △, *Product D.*

Table III—Cumulative Excretion of Total Drug, Nonacetylated Drug, and Acetylated Drug in Urine during 24 hr following Administration of Four Brands of Sodium Aminosalicylate

Product	Total Excretion, % of Dose	Nonacetylated Drug, % of Dose	Acetylated Drug, % of Dose
A	86.0 (71.3–103.3)[a]	39.4 (27.6–48.9)	46.6 (35.2–60.9)
B	86.4 (69.1–121.9)	37.9 (19.3–55.4)	48.5 (33.5–70.9)
C	86.2 (57.2–96.2)	40.8 (26.1–54.5)	45.4 (31.2–62.9)
D	89.7 (65.9–101.4)	47.1 (31.7–63.7)	42.6 (23.8–55.4)

Analysis of Variance for Nonacetylated Drug

Source of Variation	*df*	*ss*	*ms*	*F*
Between individuals	11	2540	223	8.74
Between products	3	628	210	8.22
Between periods	3	193	65	2.54
Error	30	764	26	
Total	47	4037		

SE of varietal mean = 1.457, $F_{0.05}$ (3.30) = 2.92, $F_{0.05}$ (11.30) = 2.12

Multiple-Range Analysis

p	(2)	(3)	(4)
Shortest significant ranges	4.21	4.43	4.55
Product	B	A	C	D
Mean percent of dose excreted[b]	37.9	39.4	40.8	47.1

D − B = 9.20 > 4.55; significant
D − A = 7.70 > 4.43; significant
D − C = 6.30 > 4.21; significant
C − B = 2.90 < 4.43; not significant
C − A = 1.40 < 4.21; not significant
A − B = 1.50 < 4.21; not significant

[a] Means and ranges are given. [b] Any two or more means not underscored by the same line are significantly different.

ever, a significantly higher fraction of dose was available as nonmetabolized, therapeutically active product from Product D than from the other three brands, whether determined on the basis of the *AUC* of unchanged drug or the excretion of nonacetylated drug in urine. This finding can be at least partly explained on the basis of the higher dose of Product D (4 g) than A, B, or C (2.9 g), even though the results were normalized for dose and weight. The acetylation of aminosalicylic acid in liver and intestinal mucosa has been shown to be concentration dependent and capacity limited in humans (7). Near saturation of the enzyme is already achieved following a 1-g dose of drug (14). Thus, the larger the dose the larger will be the fraction of the dose that reaches the systemic circulation and is excreted in urine in the nonmetabolized form. The plasma elimination rate constant for aminosalicylic acid was the same following administration of all four products, suggesting that the dose-dependent effects on acetylation occur during the first pass through the intestinal wall and liver.

In our opinion, the nonacetylated fraction from the dose would be the same for all products studied if studied in equivalent doses. A different tablet size in the case of Product D complicated the dosing. The doses used in this study correspond to the recommendations of the manufacturers for single doses for tuberculosis treatment. The dose-dependent acetylation of aminosalicylic acid, with the consequent increase in the fraction of dose available as the biologically active form, supports the concept that single daily large doses may be preferred to the smaller multiple doses suggested (15). This would depend on the minimum inhibitory concentration *in vivo*, which is not known; however, the single dose would be preferred if intermittent high levels are required. The rapid and nearly complete absorption of sodium aminosalicylate tablets and the relatively good patient tolerance of this drug favor the choice of this form of aminosalicylic acid in the management of patients with tuberculosis. Moreover, a higher fraction of dose is available in a biologically active form from the sodium salt than from the free acid or as its potassium or calcium salts (13). According to the present results, the variation in total absorption of different brands of the sodium salt is small, further favoring the use of this form of aminosalicylic acid.

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[x] To whom inquiries should be directed.

Structure–Activity Studies on γ-Aminobutyric Acid-Like Agents

LEMONT B. KIER[x], JACK M. GEORGE*, and HANS-DIETER HÖLTJE[‡]

Abstract □ Molecular orbital calculations were conducted on the γ-aminobutyric acid-like agents hydrazinopropionic acid, taurine, homotaurine, and imidazoleacetic acid. The results predict that all but taurine prefer conformations in which the previously proposed γ-aminobutyric acid pharmacophore is contained. The evidence supports the hypothesis that the γ-aminobutyric acid pharmacophore is composed of an onium group separated from an oxygen atom by 5.8 ± 0.2 Å.

Keyphrases □ γ-Aminobutyric acid, related agents—structure–activity studies, molecular orbital calculations, relation to previously proposed pharmacophore □ Molecular orbital calculations—γ-aminobutyric acid-like agents (hydrazinopropionic acid, taurine, homotaurine, and imidazoleacetic acid), relation to proposed pharmacophore, structure–activity relationships □ Structure–activity relationships—γ-aminobutyric acid-like agents, evidence supporting possible pharmacophore, molecular orbital calculations

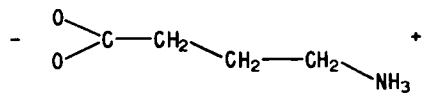
Accumulated evidence strongly supports the role of γ-aminobutyric acid (I) as an inhibitory transmitter at certain interneuronal synapses in the mammalian central nervous system (CNS) (1–5). γ-Aminobutyric acid appears to be associated with the excitability level of the brain as evidenced by relationships with epileptiform seizures (6), sodium barbital withdrawal convulsions (7), electroshock convulsions (8), and convulsions following high oxygen exposure (9). In view of its apparent role in maintaining a level of brain activity and its possible role in pathological conditions when deficient, structural characteristics influencing its biological effect are of considerable importance.

THEORETICAL

Some γ-aminobutyric acid-like agonists have been identified, including β-hydroxy-γ-aminobutyric acid (10) and muscimol (II) (11). More recently, the alkaloid bicuculline (III) was studied and concluded to be a competitive inhibitor of the central inhibitory effect of γ-aminobutyric acid (12), although some controversy has emerged on the specific nature of this antagonism (13–15).

In contrast to the central inhibitory transmission postulated for γ-aminobutyric acid, the amino acid glycine has been postulated as a distinctly different inhibitory transmitter functioning principally on motoneurons in the spinal cord (16–18). The effect of glycine was reported to be prevented by strychnine (18). The postsynaptic inhibition of strychnine has been questioned (19, 20) and defended (21).

The singularity of inhibitory effects produced by γ-aminobutyric acid and glycine is supported by the observations that strychnine, an inhibitor of glycine effects, does not depress the inhibitory effects of γ-aminobutyric acid (18). Likewise, bicuculline, an inhibitor of γ-aminobutyric acid effects, does not depress glycine-induced inhibition (12, 22). Taurine (2-aminoethanesulfonic acid) (23) and imidazoleacetic acid (14) have been suggested as inhibitory transmitters in addition to γ-aminobutyric acid and glycine. Further neurochemical evidence is required to support these possibilities.

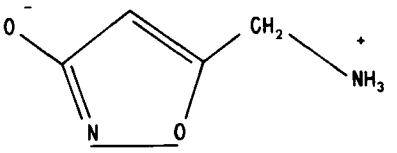


I

Some structural considerations have been studied among the γ-aminobutyric acid inhibitors in an effort to relate agonists and to deduce something about a γ-aminobutyric acid pharmacophore. In 1970, a molecular orbital study of the preferred conformation of γ-aminobutyric acid and γ-aminobutyric acid agonist muscimol (24) was reported. The calculations revealed a preference for an all-*trans* γ-aminobutyric acid zwitterion molecule, with free rotation predicted for the carboxylate. When assuming that the onium group and some part of the carboxylate are receptor active, it was predicted that the distance separating the nitrogen and one oxygen atom was 5–6 Å. The calculations on the muscimol betaine molecule predicted a conformation in which the O to N distance was 5.8 Å. From the calculated conformations, it was concluded that interaction of the two molecules with a common receptor was structurally allowed on the basis of similar charged regions separated by similar distances. It was also predicted that the γ-aminobutyric acid pharmacophore contained these two charged centers at least 5 Å, but more likely approximately 6 Å, apart.

Curtis *et al.* (12) commented on the similarity in distances separating these same structural features of γ-aminobutyric acid and muscimol on the basis of scale model examination. At the same time, they speculated that the γ-aminobutyric acid inhibitor bicuculline could conceivably present similarly charged structural features to a γ-aminobutyric acid receptor, thereby functioning as a competitive antagonist.

Another conclusion was reached by Van Gelder (25) who compared possible molecular arrangements of glutamic acid and γ-aminobutyric acid. He proposed that the steric dimension of the γ-hydrogens in γ-aminobutyric acid were important at a receptor and that cell wall transport was dependent on the diameter of the hydrated ions. He did presume a folded conformation for γ-aminobutyric acid. A preliminary report on the crystal structure of γ-aminobutyric acid zwitterion led to a provisional conclusion of some folding in the molecule, making the charged centers 4.5 Å or less apart (26). An X-ray study of γ-aminobutyric acid hydrochloride conformation revealed a *trans* or fully extended structure (27).



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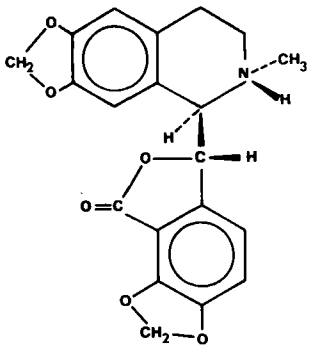

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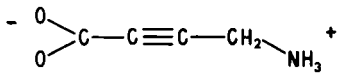

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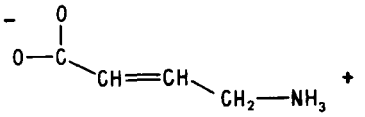

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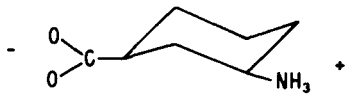


VI

More recent studies from this laboratory on β-hydroxy-γ-aminobutyric acid predicted the molecule to prefer both *trans-* (fully extended) and *gauche-* (Cα–Cβ bond) conformations (28). The *trans*-conformation is identical to the prediction of the γ-aminobutyric acid structure and is in agreement with the crystal structure (29), while the *gauche*-conformation is more consistent with the crystal study of Steward *et al.* (26) of γ-aminobutyric acid.

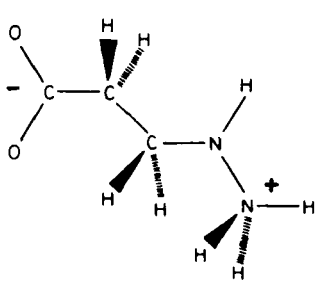
In the same study (28), the conformation of bicuculline was reported. It was found that the onium group distance from the carbonyl oxygen atom, in the preferred conformation, corresponds closely (5.6 Å) with the hypothesis of the γ-aminobutyric acid pharmacophore. Another study on bicuculline at the same time using molecular orbital calculations and NMR analysis appears to be in agreement (30).

An important consideration is the pharmacological mechanism whereby a compound may be exerting a γ-aminobutyric acid-like action. Actions mimicking the direct effect of γ-aminobutyric acid and requiring structural features comparable to γ-aminobutyric acid are: *(a)* a direct agonist action at the postsynaptic γ-aminobutyric acid receptor, *(b)* a competitive inhibition of γ-aminobutyric acid transaminase activity, and *(c)* a competitive inhibition of cellular uptake of γ-aminobutyric acid following postsynaptic action. γ-Aminobutyric acid transamination and cellular uptake constitute mechanisms whereby postsynaptic action is terminated. Inhibition of either process results in the preservation of γ-aminobutyric acid, presumably leading to the opportunity for continued interaction events at the postsynaptic receptor.

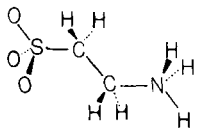
Some recent studies examined structural requirements promoting inhibition of γ-aminobutyric acid transaminase *in vitro* (31) and γ-aminobutyric acid uptake in cerebral cells *in vitro* (32, 33). The competitive inhibition of γ-aminobutyric acid transaminase was accomplished with 4-aminotetrolic acid (IV), a rigid molecule with N to O distances of 5.2 and 5.8 Å, quite similar to γ-aminobutyric acid in its fully extended conformation. A similar observation has been made that the rigid molecule *trans*-4-aminocrotonic acid (V) is an excellent substrate for this enzyme (34). Again the O to N distances are comparable to those found for the extended γ-aminobutyric acid conformation.

These studies led to the conclusion that the extended conformation of γ-aminobutyric acid is important in the interaction with γ-aminobutyric acid transaminase (31). It follows that substrates and competitive inhibitors should have this pattern of receptor-functional moieties.

Studies of competitive inhibitors of γ-aminobutyric acid uptake into cerebral gray matter cells *in vitro* have established that compounds with the potential for mimicking the extended γ-aminobutyric acid conformation are active (32). *trans*-4-Aminocrotonic acid was a competitive inhibitor of γ-aminobutyric acid uptake while the *cis*-isomer, with a much closer N to O distance, was inactive. Only the *cis*-3-aminocyclohexanedicarboxylic acid isomer (VI) was active—not the *trans*-isomer. Only in the diequatorial conformer of VI is the N to O distance identical to that found in the extended γ-aminobutyric acid.



VII



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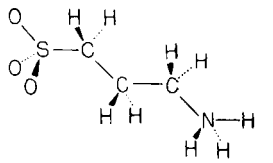
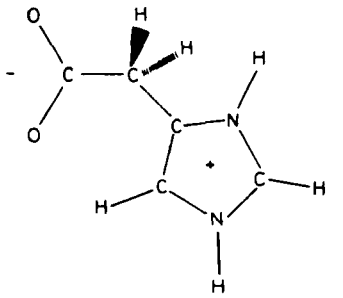


IX



X

This study consolidated previous results and tested the pharmacophore hypothesis by predicting the conformations of several agents acting at receptors responsive to γ-aminobutyric acid.

Hydrazinopropionic Acid—Hydrazinopropionic acid (VII) has been found to be an inhibitor of γ-aminobutyric acid transaminase and should, therefore, possess features comparable to γ-aminobutyric acid to be active at this enzyme (35). Van Gelder (35) commented on this possibility from an examination of scale molecular models. Accordingly, molecular orbital calculations have been performed on hydrazinopropionic acid to assess its preferred conformation and, therefore, its potential for mimicking γ-aminobutyric acid at the enzyme.

Taurine and Homotaurine—Taurine (VIII) and homotaurine (IX) have been observed to behave like γ-aminobutyric acid in their CNS effects (36). Taurine is a very prominent amino acid in the CNS and has been proposed as a possible neurotransmitter (23). Homotaurine has been reported to produce CNS effects more like γ-aminobutyric acid, while taurine appears to resemble the inhibitory effects of glycine. To determine whether either molecule possesses structural features resembling the hypothesized γ-aminobutyric acid pharmacophore, the conformational preferences of both molecules have been calculated using molecular orbital theory.

Imidazoleacetic Acid—Imidazoleacetic acid (X) has been found to produce an effect in the CNS mimicking γ-aminobutyric acid (37). Tests with inhibitors led to the conclusion that imidazoleacetic acid is acting on γ-aminobutyric acid-like receptors. To test the possibility that imidazoleacetic acid possesses a γ-aminobutyric acid pharmacophore, the conformational preferences of the protonated and neutral molecule in both tautomeric forms have been calculated using molecular orbital theory.

EXPERIMENTAL

Calculations of the total energies were made using extended Huckel theory (38) with parameters previously discussed (39) and

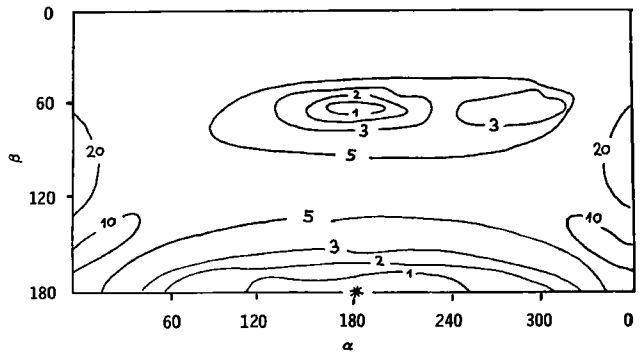


Figure 1—*Calculated energy contour map for hydrazinopropionic acid (VII). The 180° angle for bond α is a* trans*-conformation around bond CH_2—NH. The 180° angle for bond β is a* trans*-conformation around bond CH_2—CH_2. The carboxylate group is freely rotating.*

employed (24, 28). The minimum calculated energy was assumed to be associated with the preferred conformation.

Calculations on taurine and homotaurine were made using bond distances of 1.80 Å for C—S and 1.44 Å for S—O. The Coulomb integrals were used for S(3*s*) = −30.00 ev and S(3*p*) = −14.20 ev. The Slater exponent employed for sulfur was 1.817.

Imidazoleacetic acid at physiological pH certainly exists as the anion, and this form was used in the calculations. An additional problem is created by the high probability that the imidazole ring can tautomerize. The position of N—H is thus uncertain. In addition, the pKa for the ring may be very close to physiological pH. The pKa for imidazole is 6.7. It is probably more basic with the inductive effect of the methylene group. As a consequence, three forms must be considered as coexisting, the two tautomeric forms and the ring-protonated form. All three forms were considered in the calculations of preferred conformation.

RESULTS

Hydrazinopropionic Acid—Calculations of the minimum energy conformation of the hydrazinopropionic acid zwitterion revealed a preference for the fully extended molecule. The energy contours for the variation of the CH_2—CH_2 (β) and C—N (α) bonds are shown in Fig. 1. The carboxylate was found to rotate freely in the fully extended conformation while the N—N bond preferred a hydrogen eclipsing conformation. All possible hydrogen bonding conformations were calculated and found to have no preference. The β bond showed a modest flexibility, ±60°, from the fully extended conformation. The distances separating the onium nitrogen from the two oxygen atoms in the preferred conformation with the carboxylate group in the plane of the molecule were 5.1 and 6.1 Å.

Taurine—The calculations on the taurine zwitterion revealed a preference for a fully extended molecule. The contour diagram is shown in Fig. 2 for the rotations of the C—S and C—C bonds. The onium nitrogen to oxygen distances in the preferred conformation were 4.7, 4.7, and 5.3 Å.

Homotaurine—Calculations on the homotaurine zwitterion revealed a preference for a fully extended molecule. The contour diagram for the two C—C bonds is shown in Fig. 3. The calculated onium nitrogen to oxygen distances were 5.6, 5.6, and 6.6 Å.

Imidazoleacetic Acid—N_1—*H Tautomer*—The preferred conformation of this tautomer anion is shown in the contour diagram of Fig. 4. The plane of CO_2—CH_2—C_1 (ring) was found to be perpendicular to the ring plane. Some flexibility was revealed in this relationship. The carboxylate group was virtually free to rotate.

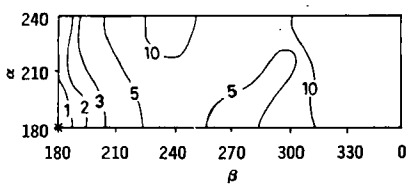


Figure 2—*Calculated energy contour map for taurine (VIII). The 180° angle for bond α is a staggered arrangement of oxygens and adjacent carbon atom substituents. The 180° angle for bond β is a* trans*-conformation around the CH_2—CH_2 bond. See Structure VIII.*

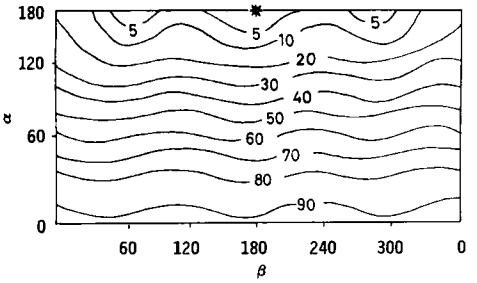


Figure 3—*Calculated energy contour map for homotaurine (IX). The 180° angle for bond α is a* trans*-conformation around the SO_3CH_2—CH_2CH_2 bond. The 180° angle for bond β is a* trans*-conformation around the CH_2CH_2—CH_2NH_3 bond. The SO_3 group is staggered to the adjacent methylene group.*

If the protonated nitrogen and the oxygens are identified as being significant structural features, interatom distances can be predicted in the preferred conformations, ranging from 3.8 to 4.2 Å.

N_3—*H Tautomer*—Calculations on the N_3—H tautomer anion revealed a conformational preference almost identical to that predicted for the N_1—H tautomer. The energy *versus* angle contour diagram is shown in Fig. 5. The range of N(H) to oxygen distances calculated was 5.5–5.9 Å.

The absolute energy values found for the N_3—H tautomer were consistently lower than those found for the N_1—H tautomer for comparable conformations. The energy differences (tautomer N_3—H minus tautomer N_1—H) were averaged at appropriate positions in the contour diagrams where there is a predicted energy <0.5 kcal above the global minimum. The average value of the increments is about 0.45 kcal. This corresponds to a predicted ratio of 2.1 in favor of the N_3—H tautomer.

Calculations on the conformational preference of the zwitterion

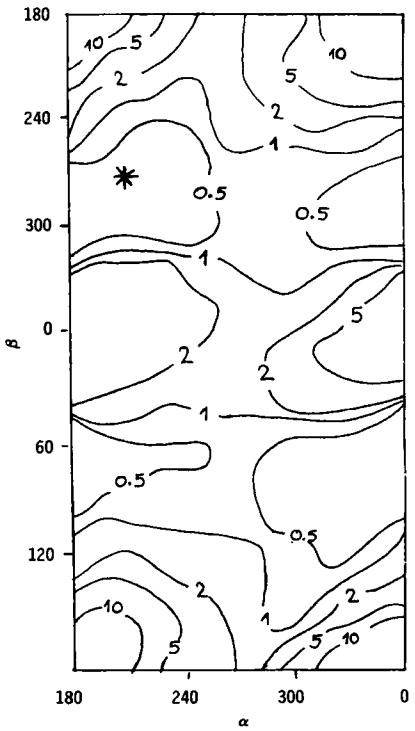


Figure 4—*Calculated energy contour map for imidazoleacetic acid anion (X) as the N_1(H) tautomer. The 0/180° angle for bond α is the conformation in which a C—O bond eclipses the ring—CH_2 bond. The 0° angle for bond β is the planer structure depicted by X.*

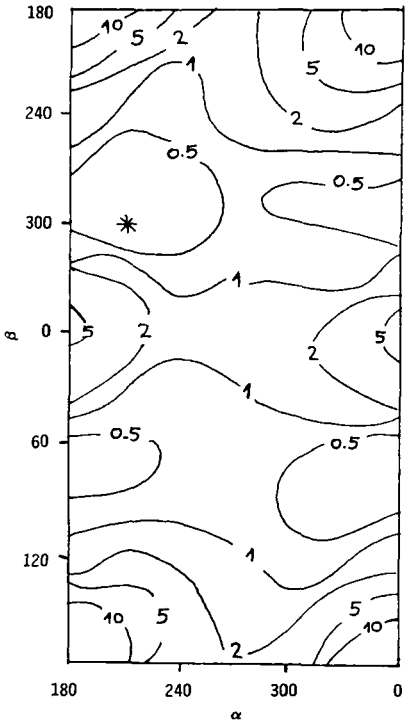


Figure 5—*Calculated energy contour map for imidazoleacetic acid anion as the $N_3(H)$ tautomer. The angles are the same as defined in Fig. 4.*

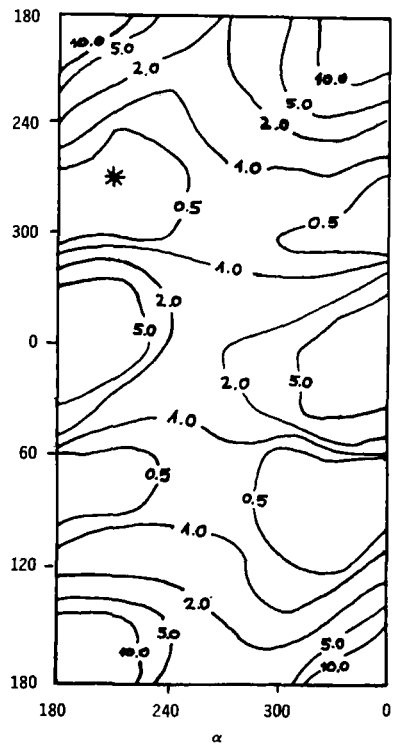


Figure 6—*Calculated energy contour map for imidazoleacetic acid zwitterion. The angles are the same as defined in Fig. 4.*

form of imidazoleacetic acid are shown in Fig. 6; the results are very similar to the two tautomeric anions. The distance range separating the N_3 and the most distal oxygen atom was identical, 5.5–5.9 Å, to that predicted for the N_3—H tautomer.

DISCUSSION

Calculations of the preferred conformation of hydrazinopropionic acid reveal a structure comparable to that of γ-aminobutyric acid. The oxygen to terminal nitrogen distance mirrors that of γ-aminobutyric acid. The competitive inhibition of γ-aminobutyric acid transaminase may be accounted for on the basis of this structural similarity. The comparison supports the hypothesis of the γ-aminobutyric acid pharmacophore (24). It further suggests that the same basic structural pattern may obtain in the enzyme reactions of the molecule as well as the postsynaptic receptor response.

Calculations on taurine predict a fully extended molecule, in contrast to the X-ray analysis revealing a *gauche* preference (40). The polar nature of the molecule would lead one to suspect that binding events in the crystal may be quite different from those found in solution. Nevertheless, an N to O distance in taurine is not predicted to be particularly close to that described by the hypothesized γ-aminobutyric acid pharmacophore. In taurine, this dimension is predicted to be 4.7, 4.7, and 5.3 Å, invoking each oxygen atom as being a candidate for receptor binding. Only the 5.3 Å value comes close to the range predicted for γ-aminobutyric acid. It is still short of the 5.6 or 5.8 Å that was predicted for comparable atoms in bicuculline (28) and muscimol (24).

If taurine is a neurotransmitter, as was discussed by Davison and Kaczmarek (23), the present structural studies argue against the possibility that it is acting at a γ-aminobutyric acid receptor. These results are in harmony with the findings of Curtis *et al.* (36) who observed that taurine is not γ-aminobutyric acid like based on the effect of competitive inhibitors.

Calculations on homotaurine predict a fully extended molecule to be preferred. This is in agreement with X-ray analysis (41), although it is not felt to be highly relevant to solution structure. The N to O distances in the predicted preferred conformation are 5.6, 5.6, and 6.6 Å. The 5.6-Å separation is closely related to the predicted γ-aminobutyric acid dimension. As a result, it is predicted that the γ-aminobutyric acid action observed (36) can be explained by the presence of the appropriate pharmacophore in homotaurine.

The calculations on the conformational preferences of the imidazoleacetic acid anions reveal a modest preference for the N_3—H tautomer. From a comparison of relative energies at highly preferred conformations, it can be concluded that this tautomer is in approximately a 2:1 ratio with the N_1—H tautomer. Recent experimental evidence on the tautomer ratio in histamine supports this prediction (42). The pKa of the acid is close to neutrality, so it can be concluded that approximately one-half of the molecules are zwitterionic. At physiological pH, over 80% of the population will be molecules with an N_3—H.

If it is presumed that the molecule is γ-aminobutyric acid like in its activity by virtue of possessing a pharmacophore, then the salient structural features of imidazoleacetic acid are the carboxylate oxygen atoms and an N—H group. This latter structural feature may be the receptor equivalent of an N—H moiety of γ-aminobutyric acid.

The distance interposing between at least one carboxyl oxygen atom and a protonated nitrogen atom should be comparable to that of γ-aminobutyric acid if this molecule is functioning at a γ-aminobutyric acid receptor in an agonistic or competitively antagonistic manner. The calculations reveal that this distance is indeed comparable in the preferred conformation of the imidazoleacetic acid N_3—H tautomer and zwitterion. Thus, it can be concluded that the molecule possesses the predicted pharmacophore in the preferred conformations. From a structural point of view, it is predicted that the molecule is capable of interacting with a γ-aminobutyric acid receptor.

In conclusion, the present predictions, coupled with previous studies, support the hypothesis that the γ-aminobutyric acid pharmacophore for the inhibitory receptor and very likely the transaminase and reuptake enzyme actions is the N—H feature separated from an oxygen atom by 5.8 ± 0.2 Å.

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* Present address: College of Medicine, Ohio State University, Columbus, Ohio.

‡ Present address: Fachgebiet 14.2–Pharmazeutische Chemie der Universitat des Saarlandes, Saarbrucken, Germany.

ˣ To whom inquiries should be directed.

Determination of Bromazepam in Blood by Electron-Capture GLC and of Its Major Urinary Metabolites by Differential Pulse Polarography

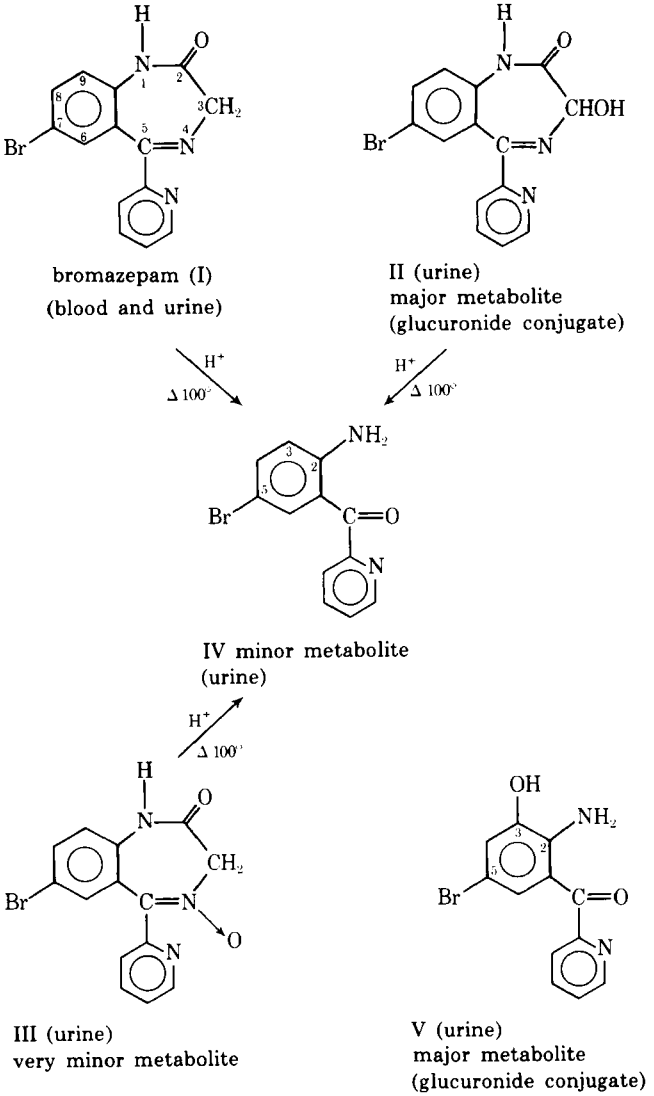
J. ARTHUR F. de SILVA[x], IHOR BEKERSKY, MARVIN A. BROOKS, ROBERT E. WEINFELD, WILLIAM GLOVER, and CARL V. PUGLISI

Abstract □ A sensitive and specific electron-capture GLC assay was developed for the determination of bromazepam (I), 7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one, in blood. The overall recovery of I from blood is 85 ± 5.0%, and the sensitivity limit of detection is 5–10 ng I/ml blood. In the determination of the urinary excretion, the major urinary metabolites, 3-hydroxy-bromazepam (II) and 2-amino-3-hydroxy-5-bromobenzoylpyridine (V), are selectively extracted into ether from urine buffered to pH 9.0 after incubation with glucuronidase–sulfatase, whereas I and 2-amino-5-bromobenzoylpyridine (IV) are directly extractable from urine into ether at pH 9.0. The residues of the respective ether extracts are dissolved in 1.0 *M* phosphate buffer (pH 5.5) and analyzed by differential pulse polarography, which yields two distinct peaks for the benzodiazepin-2-one and the benzoylpyridine component in each fraction. The overall recovery of I and IV is 80 ± 5.0% with sensitivity limits of 100 and 50 ng/5 ml urine, respectively; the recovery of II and V is about 45 ± 5.0% with sensitivity limits of 100 ng each/5 ml urine analyzed. Blood levels of bromazepam and the urinary excretion of the unchanged drug and its major metabolites were determined in humans following the administration of single 12-mg oral doses.

Keyphrases □ Bromazepam—electron-capture GLC analysis in blood, differential pulse polarographic analysis of major urinary metabolites □ GLC, electron capture—analysis, bromazepam in blood □ Differential pulse polarography—analysis, bromazepam urinary metabolites

Bromazepam, 7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one (I), is a member of the 1,4-benzodiazepine class of compounds and was synthesized by Fryer *et al.* (1). The pharmacology of the compound has been extensively described (2–4), and it is of clinical interest as an antianxiety agent (5–8).

The properties of I and the major known metabolites are given in Table I, and their chemical structures and reactions are given in Scheme I. Studies on the biotransformation of I in dogs, mice, and humans (9) and in dogs, rats, and rabbits (10, 11) showed that the compound was mainly metabolized by hydroxylation and hydrolysis, producing significant amounts of 3-hydroxy-bromazepam (II) and 2-amino-3-hydroxy-5-bromobenzoylpyridine (V). The parent drug (I) and 2-amino-5-bromobenzoylpyridine (IV) were excreted in lesser amounts in the urine, whereas the 4-*N*-oxide (III) was isolated as a urinary metabolite only in the dog (9). A sensitive electron-capture GLC assay for the determination of bromazepam in blood, employing acid hydrolysis to the benzoylpyridine derivative (IV) (12), was not specific for bromazepam since metabolites II and III, if present in blood, would also yield the same hydrolysis product (Scheme I) and would interfere with the specificity of the assay. An improved assay was developed for the simultaneous determination of bromazepam and its 3-hydroxy metabolite (II) as the intact 1,4-benzodiazepin-2-ones by a modification of published procedures (13, 14). When using this modified procedure, it was shown that II was not measurable in the blood of subjects chronically treated with bromazepam. Although this procedure was specific for I, it was time consuming; therefore, a simpler assay was developed which measures only bromazepam and excludes metabolites II, III, IV, and V. The method involves ether extraction of I at pH 9.0 from whole blood after protein precipitation. The metabolites are coprecipitated with the proteins and are not recovered. The



Scheme I—Chemical reactions of bromazepam and its known metabolites

Table I—Chemical Names and Physical Properties of the Compounds Referred to in Scheme I and in the Text

Compound	Chemical Name	Molecular Weight	Melting Point
I	7-Bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one (bromazepam)	316.16	237–238.5° dec.
II	7-Bromo-1,3-dihydro-3-hydroxy-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one	332.2	198–200°
III	7-Bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one 4-oxide	332.2	263° dec.
IV	2-Amino-5-bromobenzoylpyridine	277.12	97.5–99°
V	2-Amino-3-hydroxy-5-bromobenzoylpyridine	293.12	190–196°
VI	7-Bromo-1,3-dihydro-1-methyl-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one (methylbromazepam, reference standard for electron-capture GLC analysis)	330.19	135.5–137°

electron-capture GLC assay employs OV-17 as the liquid phase, which can resolve the intact drug (I) from its major metabolites (II, IV, and V), even if they are recovered in trace amounts. The high sensitivity of the ^{63}Ni-electron-capture detector is used in the pulsed dc mode for the determination of I with nanogram sensitivity. Methyl-bromazepam (VI) (Table I) is used as the reference standard in the assay.

The electron-capture GLC assay was used in the determination of blood levels of bromazepam in humans following the administration of a single oral 12-mg dose. The urinary excretion of I and its major metabolites was determined by differential pulse polarography.

EXPERIMENTAL

Electron-Capture GLC Analysis of Bromazepam in Blood

Conditions for GLC Analysis—*Column*—The column packing was a pretested preparation containing 3% OV-17 on 60–80-mesh Gas Chrom Q[1] packed in a U-shaped 1.2-m (4-ft), 4-mm i.d. borosilicate glass column. The column was conditioned at 325° for 4 hr with "no flow" of carrier gas, followed by 12 hr at 275° with carrier flowing at 40 ml/min. The useful lifespan of such a column was about 4–5 months of continuous use.

Instrumental Conditions—A gas chromatograph[2], equipped with a ^{63}Ni-electron-capture detector containing a 15.0-mCi ^{63}Ni β-ionization source was used. Argon–methane[3] (90:10), oil pumped and dry, was used as the carrier gas, with the column head pressure adjusted to 40 psig, the flow rate adjusted to 110 ml/min, and the detector purge gas adjusted to 30 ml/min. The temperature settings were as follows: oven, 245°; injection port, 270°; and detector, 325°. The conditions of column head pressure, flow rate, and oven temperature were adjusted so as to obtain retention times of 6.0 and 4.0 min for I and the reference standard (VI), respectively. A typical chromatogram is shown in Fig. 1. The solid-state electrometer[4] input was set at 10^2 and the output attenuation was 32, giving a response of 3.2×10^{-9} amp for full-scale deflection (fsd); the chart speed was 76.2 cm (30 in.)/hr, and the time constant on the 1.0-mv recorder[5] was 1 sec (fsd). The response of the ^{63}Ni-electron-capture detector (operated in the pulsed dc mode) to I showed maximum sensitivity at 60 v dc with a 150-μsec pulse rate and a 10-μsec pulse width. Under these conditions, 15 ng of I gives nearly full-scale deflection on the 1.0-mv recorder. The minimum detectable amount of I is 5.0–10.0 ng/ml of blood.

Preparation of Standard Solutions—The respective benzodiazepin-2-ones that are required as analytical standards are listed in Table I. Weigh out 10.0 mg of I and the reference standard (VI) into separate 10-ml volumetric flasks, dissolve, and dilute to volume with absolute ethanol to give stock solutions (A and A′) containing 1 mg/ml. Make suitable dilutions of A and A′ in benzene–ethanol–acetone (80:10:10) to prepare working standard solutions (B_1–B_4) in separate 10-ml volumetric flasks containing 25, 50, 75, or 100 ng of I and 50 ng of VI (added as the reference standard for electron-capture GLC analysis) per 0.1 ml of solution. Add 1 g of anhydrous sodium sulfate to these solutions to maintain their anhydrous state.

Aliquots (10 μl) of solutions B_1–B_4 are injected into the chromatograph to optimize the electron-capture detector response to I and VI and to establish the conditions for GLC analysis; 100-μl aliquots of these standard solutions are also added to control blood as the internal standards, which are used to determine the overall recovery of I and as the calibration curve for the quantitation of unknowns (Fig. 2).

After the electron-capture GLC conditions have been optimized

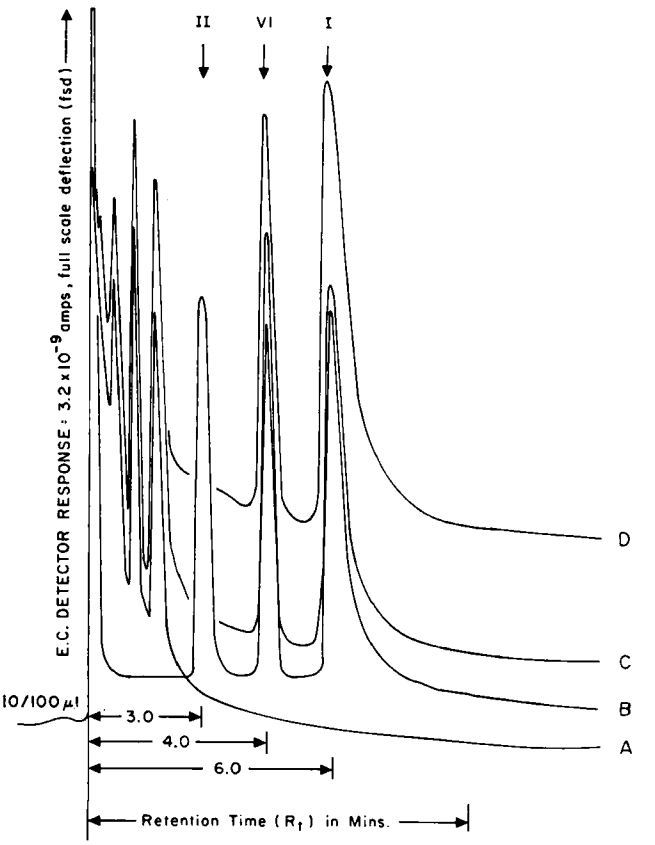


Figure 1—*Chromatograms of: (A) control blood extract; (B) authentic standards of I, II, and VI; (C) internal standard of 10 ng of I and 5 ng of methyl-bromazepam (reference standard) recovered from control blood; and (D) patient blood extract after 12-mg oral dose of I.*

[1] Applied Science Labs., Inc., State College, Pa.
[2] Micro-Tek model MT-220, Tracor Instruments, Austin, Tex.
[3] Matheson.
[4] Model 8169, Tracor Instruments, Austin, Tex.
[5] Model 194, Honeywell Instruments, Fort Washington, Pa.

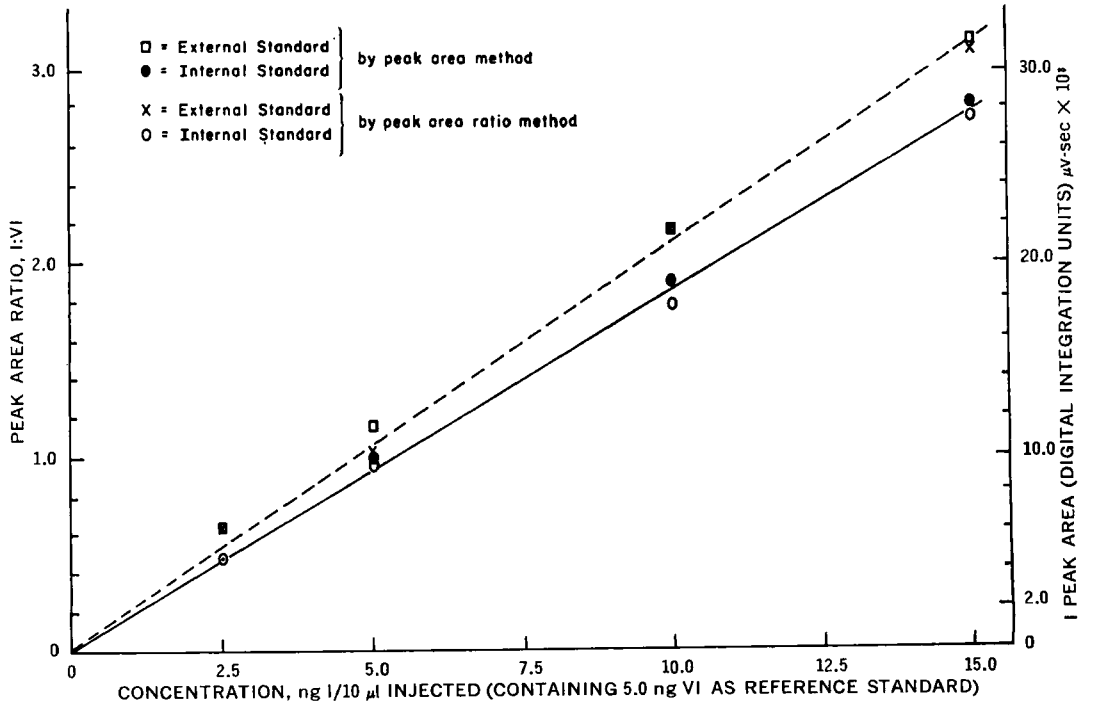


Figure 2—*Electron-capture detector calibration curves for I using either the direct calibration method [peak area (square centimeters)* versus *concentration (nanograms)] or the relative calibration method [peak area ratio of bromazepam–methyl-bromazepam (reference standard)* versus *concentration (nanograms)].*

using the pure authentic compound, the external calibration curve is prepared *after* the injection of a 2 × 10-μl aliquot of the control blood extract. *In practice,* 10-μl injections of the external standards are made *between every two or three consecutive biological samples.* The external standard curve is required only for the determination of percent recovery.

Reagents—All reagents were of analytical (ACS certified) reagent grade purity (>99%) and were used without further purification. All inorganic reagents were made up in double-distilled water. These included 1 *M* H_3BO_3–Na_2CO_3–KCl buffer (pH 9.0) prepared as previously described (12), 2 *N* H_2SO_4, and 2 *N* NaOH. Ether[6], benzene[6], acetone[7], methanol[7], ethanol[8], and a mixture of benzene–ethanol–acetone (80:10:10) (stored over anhydrous sodium sulfate) were the other reagents used.

Extraction of Blood for Determination of Bromazepam—Into a 50-ml centrifuge tube, add 2 ml of oxalated whole blood and 2 ml of distilled water, and mix well by inversion. Add 5 ml of 2 *N* H_2SO_4 dropwise and slurry vigorously on a vortex supermixer to precipitate the protein. Along with the samples, process four 2-ml specimens of control blood to which 25, 50, 75, or 100 ng of I (100 μl of solutions B_1–B_4 evaporated to dryness under nitrogen) has been added as the internal standard. The acidified specimen (including the precipitate) is washed with 2 × 15-ml portions of ether[9] by shaking for 5 min on a reciprocating shaker and centrifuged, and the supernatant ether layer is aspirated off and discarded. The ether-washed sample is then neutralized with 5.0 ml of 2 *N* NaOH, buffered with 2 ml of pH 9.0 borate buffer, mixed well on a supermixer, and adjusted to pH 9.0 with dilute alkali. The samples are then extracted with 2 × 10 ml of ether, centrifuged after each extraction at 2000 rpm (preferably at 0–4° in a refrigerated centrifuge), and the ether extracts are combined successively in a 15-ml conical centrifuge tube by evaporating them to dryness in the water bath of a rotary evaporator[10] at 35–40°. The residues are vacuum dried (over sodium hydroxide pellets) for 15 min and then dissolved in 100 μl of the benzene–ethanol–acetone (80:10:10) solvent mixture. A 10-μl aliquot of this solution is injected for electron-capture GLC analysis. The peaks due to bromazepam and the reference standard are identified by their respective retention times (Fig. 1), and their respective peak areas are determined either by measuring peak height (centimeters) × width at half-height (centimeters) using the slope baseline technique or by electronic digital integration.

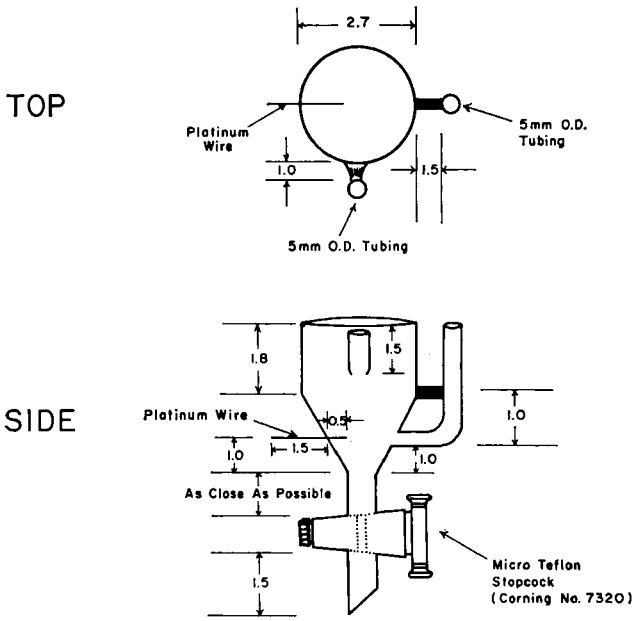


Figure 3—*Schematic diagram of the semimicropolarographic cell. (All dimensions are in centimeters except where noted, and the platinum wire used was B & S No. 22 gauge.)*

[6] Catalog No. 0848 and No. 1043, respectively; Mallinckrodt Chemical Works, St. Louis, Mo.

[7] Catalog No. A-18 and No. A-412, respectively; Fisher Scientific Co., Fairlawn, N.J.

[8] USP, 200 proof, Publicker Industries Inc., Philadelphia, Pa.

[9] The purity of ether for electron-capture GLC analysis is determined by evaporating 20 ml to dryness, dissolving the residue in 100 μl of benzene–ethanol–acetone (80:10:10), and injecting a 10-μl aliquot. The chromatogram must be free of interfering peaks in the retention area following 2 min after injection.

[10] Buchler Evapomix, Buchler Instruments, Fort Lee, N.J.

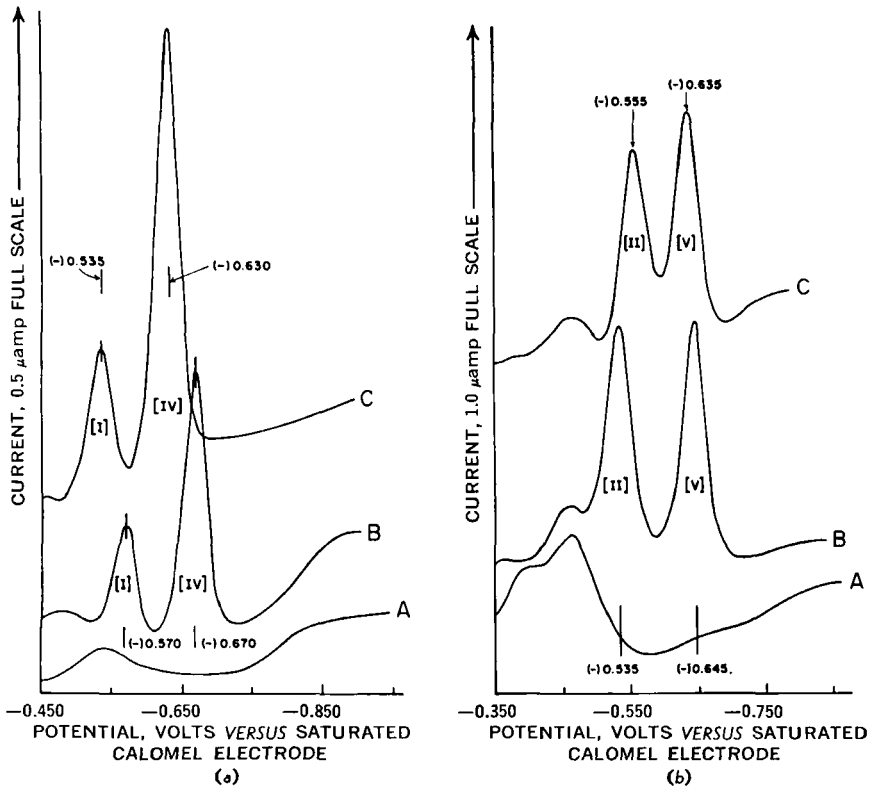


Figure 4—*Differential pulse polarograms of:* (a) *I and IV and* (b) *II and V obtained in 1.0* M *pH 5.5 phosphate buffer as the supporting electrolyte. Key: A, control urine blank; B, authentic standard mixture; and C, authentic compounds recovered from urine.*

Calculations—The concentration of bromazepam in the aliquots of the unknowns injected is calculated by interpolation from the internal standard curve (Fig. 2) by using either the direct calibration (peak area *versus* concentration) or the relative calibration (peak area ratio) techniques. The sensitivity of the electron-capture GLC assay is about 5–10 ng bromazepam/ml blood assayed.

Differential Pulse Polarographic Analysis of Major Urinary Metabolites of Bromazepam

The major urinary metabolites of I in humans are the 3-hydroxy metabolite (II) and the 2-amino-3-hydroxy-5-bromobenzoylpyridine metabolite (V), which are present mainly as glucuronide–sulfate conjugates (9). Small amounts of the intact drug (I) and metabolite IV are also excreted. The urine specimen is first extracted at pH 9.0 with ether, which quantitatively removes I and IV. The specimen is then titrated to pH 5.5 and incubated with glusulase enzyme to deconjugate metabolites II and V, which are then extracted into ether after adjusting the sample to exactly pH 7.75.

Reagents—*1.0* M *Phosphate Buffer (pH 5.5)*—Dissolve 138.0 g of $NaH_2PO_4 \cdot H_2O$ in distilled water and dilute to 1 liter. Titrate carefully with 1.0 *N* NaOH to pH 5.5 and shake well by inversion to effect equilibration.

Glusulase Enzyme[11]—Use the diagnostic reagent (100,000 units glucuronidase and 50,000 units sulfatase/ml).

Procedure—Into a 50-ml centrifuge tube, add 5 ml of urine, 5 ml of 1 *M* pH 9.0 borate buffer, and 10 ml of ether for the first extraction. Along with the unknowns, process a 5-ml specimen of control urine (taken preferably from the same subject prior to medication) and three 5-ml specimens of control urine to which 200, 400, or 600 ng each of I and IV is added as the internal standard. Extract all samples by shaking for 10 min on a reciprocating shaker, centrifuge at 2000 rpm, and transfer the supernate into a 15-ml conical centrifuge tube. Reextract the sample with another 10 ml of ether and combine the extracts by serial evaporation at 35–40° in the water bath of the rotary evaporator. This series of extracts (A) contain I and IV. The urine sample is then titrated potentiometrically to pH 5 by the dropwise addition of 6 *N* HCl and is buffered to pH 5.5 with 5 ml of pH 5.5 phosphate buffer after adding 0.2 ml of glusulase (2% of total volume). Then the entire contents are transferred into a 50-ml erlenmeyer flask.

The three extracted control urine specimens are treated in the same manner and are now spiked with 1, 2, or 3 μg each of II and V, the major conjugated metabolites. Stopper all flasks loosely with cotton and place in an incubation shaker[12] and incubate at 37° for 2.5 hr with mild shaking. After incubation, cool the samples to room temperature and adjust the solution to pH 7.75 (using a pH meter) by titrating the sample dropwise with 6 *N* NaOH. Transfer this solution quantitatively into a 50-ml stoppered centrifuge tube and extract twice with 15 ml of ether by shaking for 10 min and then centrifuging for 5 min. Combine the ether extracts in a 15-ml conical centrifuge tube by successive transfer and evaporation to dryness as before. This series (B) contains the conjugated metabolites II and V. The residues of both series A and B are solubilized with 100 μl of methanol, dissolved in 2 and 4 ml, respectively, of 1 *M* phosphate buffer (pH 5.5), and deaerated for 5 min with nitrogen bubbled through the sample with a sintered-glass (fritted) tube. Two milliliters of each solution is transferred into the semimicropolarographic cell[13] (Fig. 3), which contains the three operational electrodes, and analyzed by differential pulse polarography.

Conditions for Polarographic Analysis—A polarographic analyzer[14] equipped with a drop timer[15] was used in conjunction with a three-electrode polarographic cell comprised of a dropping mercury electrode, a saturated calomel electrode, and a platinum wire as the auxiliary electrode, as previously described (15). The drop time was 1.0 sec, and the drop rate was 2.74 mg/sec, where $(m^{2/3})(t^{1/6}) = 1.958$. The current range was set at 0.5 or 1 μamp for a peak response of full-scale deflection, the scan range was 1.5 v, and the scan rate was 2 mv/sec. The samples were scanned between −0.350 and −0.700 v *versus* the saturated calomel electrode, and the polarograms were obtained on an *X–Y* recorder[16]. The peak potential, E_p, due to the reduction of the azomethine ($>C_5{=}N_4{-}$) group of I and its 3-hydroxy metabolite (II) occurs at −0.535 and −0.555 v *versus* the saturated calomel electrode, respectively, whereas the peak due to the reduction of the carbonyl

[11] Endo Laboratories, Inc., Garden City, N.Y.

[12] Dubnoff, Precision Scientific, Inc., Chicago, Ill.

[13] Designed by M. R. Hackman and M. A. Brooks, Hoffmann-La Roche Inc., Nutley, NJ 07110 (1972).

[14] PAR model 174, Princeton Applied Research Corp., Princeton, N.J.

[15] PAR model 172A, Princeton Applied Research Corp., Princeton, N.J.

[16] Houston Omnigraph model 2200-3-3, Houston Instruments, a division of Bausch & Lomb, Inc., Bellair, Tex.

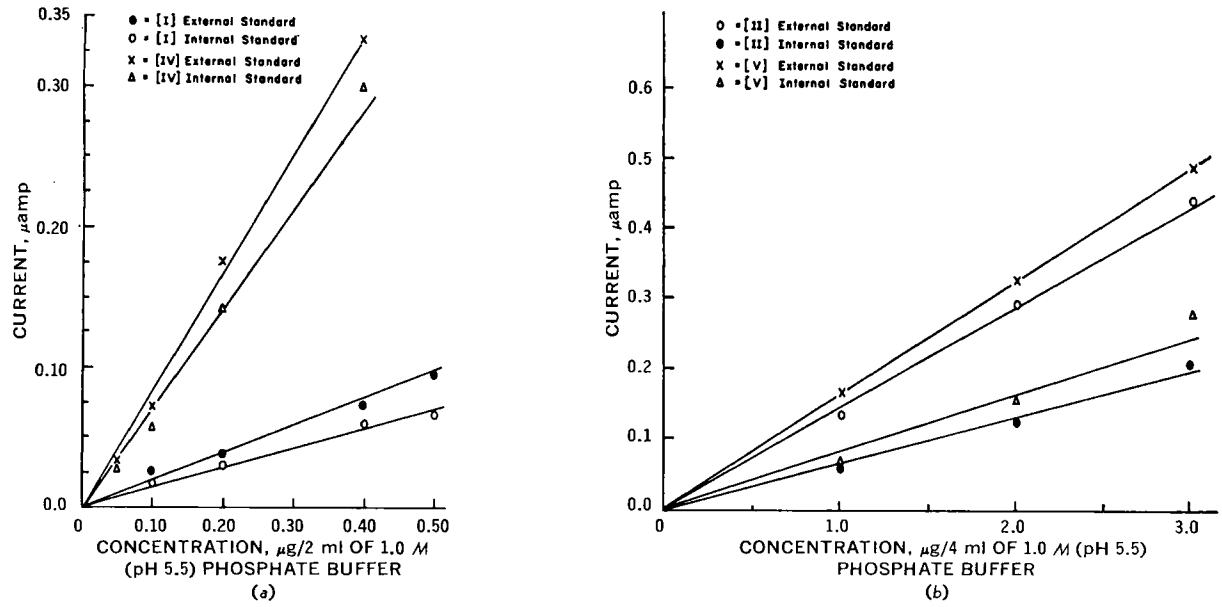


Figure 5—*Calibration curves for the differential pulse polarographic analysis of:* (a) *I and IV, and* (b) *II and V.*

(>C=O) group of metabolites, IV and V, occurs at −0.630 and −0.635 v *versus* the saturated calomel electrode, respectively (Fig. 4). The two analytical peaks are well resolved, with a peak-to-peak separation of nearly 100 mv. Therefore, series A, containing a mixture of I and IV, and series B, containing a mixture of II and V, can be analyzed and the two peaks readily distinguished.

Calculations—The current (microamperes) resulting from each peak and the overall recovery of each compound are determined as described previously (15, 16), while the concentration of each component in the unknowns is determined by interpolation from the respective internal standard curves (Fig. 5).

RESULTS AND DISCUSSION

The chromatographic analysis of bromazepam (I) and metabolite II manifested certain problems. Authentic standards of I and methyl-bromazepam (reference standard, VI), dissolved in benzene–ethanol–acetone (80:10:10) and analyzed by electron-capture GLC, indicated that although the two compounds were resolved, the peak of I showed excessive tailing. The chromatograms obtained from blood extracts containing both I and VI showed significantly improved, well-resolved Gaussian-shaped peaks for both compounds at the same retention times as before (Fig. 1), but with enhanced sensitivity to the electron-capture detector.

Table II—Blood Levels of Bromazepam in Three Human Subjects following Administration of a Single 12-mg Oral Dose

Subject	D.L.P. (m)	G.A.V. (m)	R.R.E. (m)
Weight, kg	68.10	104.42	87.62
Dose, mg/kg	0.176	0.115	0.137
Hours Postdosing	Blood Concentration, μg Bromazepam/ml		
0	—	—	—
0.5	0.12	0.05	0.13
1	0.12	0.08	0.13
2	0.12	0.11	0.13
4	0.10	0.10	0.13
6	0.10	0.10	0.12
8	0.10	0.10	0.13
12	0.09	0.07	0.13
24	0.05	0.05	0.08
30	0.03	0.03	0.06
36	0.02	0.02	0.05
48	0.01	0.01	0.02
72	N.M.[a]	N.M.	0.01

[a] N.M. = not measurable, <0.01 μg/ml.

These phenomena suggested that components in the blood extract probably form an adsorption complex with exposed active sites on the column, thereby reducing adsorption losses of I and VI and resulting in more symmetrical peaks with enhanced detector response. Consequently, pretreatment of the column by injecting control blood extracts is essential for obtaining reproducible analysis of I.

The electron-capture GLC behavior of metabolite II was not reproducible when injected as the pure solution or from a blood extract. Although authentic II was well resolved from I with a separation of about 3 min and was quite sensitive to the electron-capture detector, its overall recovery from blood (40–50%) and reproducibility (±10–15%) were unsatisfactory. Attempts at preparing the trimethylsilyl derivative of II, using mixtures of either hexamethyldisilazane and trimethylchlorosilane or bis(trimethylsilyl)acetamide in pyridine, did not yield reproducible derivatization and were not pursued further.

It has been reported that 3-hydroxy-*N*-desalkyl-1,4-benzodiazepin-2-ones such as oxazepam and lorazepam (17, 18) undergo thermolytic rearrangement during GC–mass spectrometric analysis, resulting in the formation of their respective quinazoline-carboxaldehydes. A similar thermolytic rearrangement may be responsible for the poor overall recovery and reproducibility of II during electron-capture GLC analysis. Although II is a major urinary metabolite, it is not present in blood (in the free or directly extractable form) even on chronic administration of I in any significant amount, so its quantitation can be omitted.

The N_4-oxide metabolite (III) was not isolated as a metabolite in humans or in mice, although it was excreted as a minor metabolite by the dog (9). Authentic III, when analyzed by electron-capture GLC, gave a peak with the same retention time as I. It is possible that it can undergo either a loss of oxygen to form I or a thermolytic rearrangement to give an epoxide (17) with the same retention time as I. Since this compound is not recovered in the final sample extract, it does not interfere with the determination of I. The benzoylpyridines IV and V, even if recovered in trace amounts, are eluted with the interfering peaks of the solvent front and cannot be quantitated under these conditions. The electron-capture GLC assay is, therefore, specific for the intact drug.

Differential pulse polarography has been successfully applied to the determination of several types of drugs in their intact form such as benzodiazepines (19, 20), the nitroimidazoles (21), and pyrimidine-containing compounds (16). Others such as glibornuride (a tolylsulfonylurea) (15), phenobarbital, and diphenylhydantoin (22) were determined as their nitro derivatives. The 1,4-benzodiazepin-2-ones and their benzophenones can be quantitated in the submicrogram range due to the ease of reduction of the (>C_5=N_4—) azomethine group of the former and the (>C=O) carbonyl group of the latter in dilute acids and to the relatively high sensi-

Table III—Excretion (0–72 hr) of Bromazepam and Its Major Urinary Metabolites in Humans Determined by Differential Pulse Polarography

Subject	Percent of Dose Excreted as				Dose Administered = 12 mg	
	I	II	IV	V	mg Recovered	% of Dose
D.L.P.	2.5	22.6	0.35	46.6	8.65	72.1
G.A.V.	1.9	27.8	0.75	29.9	6.91	57.6
R.R.E.	1.8	34.8	0.21	39.5	9.19	76.6

tivity (microamperes per microgram) achieved. Consequently, I, II, IV, and V can be selectively extracted as mixtures of I and IV and of II and V, successively, and quantitated with a minimum of cleanup because the coextracted impurities do not interfere in the potential region of interest. The use of the semimicropolarographic cell (20) (Fig. 3) with a minimum sample volume of 2 ml, a longer drop time of 1 sec, and a slower scan speed of 2 mv/sec contributed to increasing the reduction current generated and, consequently, the overall sensitivity of the assay.

The polarographic analysis of a mixture of authentic standards of I and IV (Fig. 4*a*) and of II and V (Fig. 4*b*) in 1.0 *M* pH 5.5 phosphate buffer shows two well-resolved analytical peaks for I at −0.570 v and IV at −0.670 v *versus* the saturated calomel electrode and for II at −0.535 v and V at −0.645 v *versus* the saturated calomel electrode (polarogram B). The same compounds recovered from urine again showed well-resolved peaks but with different E_p potentials. The peak potential E_p for I, IV, and V showed a shift toward a more positive potential, with the peak for I now at −0.535 v, for IV at −0.630 v, and for V at −0.635 v *versus* the saturated calomel electrode, whereas the peak for II shifted to a more negative potential at −0.555 v *versus* the saturated calomel electrode (polarogram C). These peak shifts are probably due to physicochemical interactions with impurities extracted from control urine (polarogram A). The calibration curves of authentic (external standards) of I, II, IV, and V and of the same compounds as internal standards recovered from urine (Fig. 5) show the linearity of the assay and its higher sensitivity for the benzoylpyridine metabolites IV and V, respectively.

The overall recovery of I and IV, which are extracted directly into ether from urine buffered to pH 9.0, was 80 ± 5.0%. No measurable amounts of II and V were present in this extract, as determined by electron-capture GLC analysis. Therefore, any interference is minimal and can be neglected.

Metabolites II and V are extracted after enzymatic deconjugation and analyzed as a mixture (Fig. 4*b*). Although the recovery of II and V into ether from urine buffered to pH 7.75 was only 45 ± 5.0%, the concentration of each present in the urine of treated subjects was sufficiently high for accurate quantitation. The overall recovery can be increased significantly (>75%) by using either ethyl acetate or methylene chloride (dichloromethane). It is achieved, however, at the expense of greatly increased impurities coextracted with the compounds of interest and precludes polarographic analysis without extensive cleanup of the sample.

Blood Levels of Bromazepam in Humans Determined by Electron-Capture GLC Analysis—Blood levels were determined in three subjects (in a pilot study) following the oral administration of a single 12-mg dose of the clinically used tablet formulation. The blood data (Table II) indicate the presence of measurable levels from 30 min to 72 hr postdosing, thereby demonstrating the clinical utility of the method. The assay was used to determine the blood levels in humans following single 12-mg oral doses and chronic administration of oral doses ranging from 3 to 9 mg/day over a 30-day dosing period in the evaluation of the pharmacokinetic profile of the drug in humans[17]. Blood specimens analyzed for II indicated that it was not present in any significant amounts following both single and chronic oral dosing.

Urinary Excretion of Bromazepam and Its Major Metabolites—The urinary excretion of bromazepam and its major metabolites following single 12-mg oral doses was determined by differential pulse polarography (Table III). The data indicate that II and V were the predominant metabolites excreted, accounting for a total of about 58–77% of the administered dose, along with lesser amounts of the intact drug (I) and its benzoylpyridine derivative (IV) over the 72-hr excretion period.

[17] S. A. Kaplan *et al.*, Hoffmann-La Roche Inc., Nutley, NJ 07110, unpublished data on file.

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Chloramine-T as Titrimetric Reagent in Potentiometric Determination of Isoniazid, Phenelzine, and Dihydralazine

SERGIO PINZAUTI ˣ, VITTORIO DAL PIAZ, and ENZO LA PORTA

Abstract □ A simple, rapid, and accurate direct potentiometric method was developed for the determination of isoniazid, phenelzine sulfate, and dihydralazine sulfate using chloramine-T as the titrimetric reagent. The oxidations were done at high temperatures in acidic medium. Quantitative recoveries are reported.

Keyphrases □ Chloramine-T—titrimetric reagent for potentiometric determination of isoniazid, phenelzine, and dihydralazine □ Potentiometric titration—isoniazid, phenelzine, and dihydralazine, chloramine-T as titrimetric reagent □ Hydrazine drugs—potentiometric determination of isoniazid, phenelzine, and dihydralazine, chloramine-T as titrimetric reagent

Hydrazine drugs have important therapeutic applications (1). Various oxidometric methods for their determination have been developed involving direct or indirect analysis. Iodometric and bromometric methods are used by the USP (2) and BP (3), respectively, for the assay of isoniazid. Hydralazine may be determined with the iodometric titration procedure (4) or with a standard iodate solution in hydrochloric acid medium (5). When an irreversible indicator (*p*-ethoxycrysoidine) is employed, the titration with iodate to the discharge of color can be used for isoniazid (6). The determination of isoniazid by the sodium nitrite method (7) and by cerate oxidometry using the residual titration procedure (8) was also discussed. Phenelzine can be determined iodometrically (2, 3) as well as with the bromine method (9). The bromometric assay of dihydralazine was reported (10). Potassium dichromate as the titrant in the presence of potassium bromide can also be used for determining dihydralazine (10).

Samek (11) noted that hydrazine can be titrated potentiometrically with chloramine-T in acid, as well as in neutral solution, in the presence of potassium bromide. Berka and Zyka (12) described the potentiometric titration of isoniazid using chloramine-T as titrant in the presence of added bromide. The behavior of chloramine-T as an oxidizing reagent and the methods of standardizing its solutions have been critically examined (13). Bishop and Jennings (14) studied the potentiometric titration of hydrazine with chloramine-T in the presence of added halides under varying conditions of acidity, but the results were unsatisfactory: the potentials at the end-point required more than 30 min to become steady (in chloride media), the end-points were not reproducible (in bromide media), the potentials were unsteady before the end-point, and the conditions for titration were rather critical (in iodide media). These investigators concluded that potentiometric titration with chloramine-T cannot be recommended for the accurate determination of hydrazine under any conditions. Nair and Nair (15) later described a back-titration procedure with chloramine-T as the oxidant for the determination of isoniazid. An excess of chloramine-T was employed and, after a suitable time, was determined by the well-known residual iodometric titration. When the reported (12) method for isoniazid was followed in this laboratory, low results and insufficient precision (93.39 ± 1.63% based on eight analyses) were obtained.

At room temperature the hydrazine–chloramine-T reaction is slow. In previous papers the effect of raising the temperature on the rate of reaction was not investigated. In the present work, it was established that hydrazine and some hydrazine derivatives react instantly and quantitatively with chloramine-T in acidic medium at about 60°, permitting simple, rapid, precise, and accurate direct potentiometric titrations. The proposed method is suitable for determining the antitubercular isoniazid USP and BP (isonicotinic acid hydrazide), the antidepressant phenelzine sulfate USP and BP (phenethylhydrazine sulfate), and the antihypertensive dihydralazine sulfate (1,4-dihydrazinophthalazine sulfate). However, with other hydrazine drugs, *e.g.*, nialamide BP and isocarboxazid BP, the proposed method was unsatisfactory due to secondary oxidative processes under the experimental conditions.

EXPERIMENTAL

Apparatus—Titrations were performed potentiometrically with an automatic titrator[1] equipped with a platinum electrode (a Pt flag) and calomel electrode[2]. The titration vessel is equipped with a hot plate magnetic stirrer.

Reagents and Solutions—The drug samples were the highest grades of commercially available materials and were used as received. Analysis by the USP and BP assays for isoniazid indicated a purity of >98%. Analysis by the USP and BP methods for phenelzine sulfate indicated a purity of >97 and 98%, respectively.

Chloramine-T (0.05 M*)*—Dissolve 7 g of chloramine-T (pure) in 500 ml of water and store in brown glass.

Hydrazine Sulfate—Weigh accurately about 350 mg of hydrazine sulfate (highest purity) and dissolve in 50 ml of water in a 100-ml volumetric flask and dilute to volume with 8 *N* sulfuric acid.

Standardization of 0.05 M *Chloramine-T Solution*—Pipet an

[1] Metrohm, Combi 3D.
[2] Metrohm, EA 404.

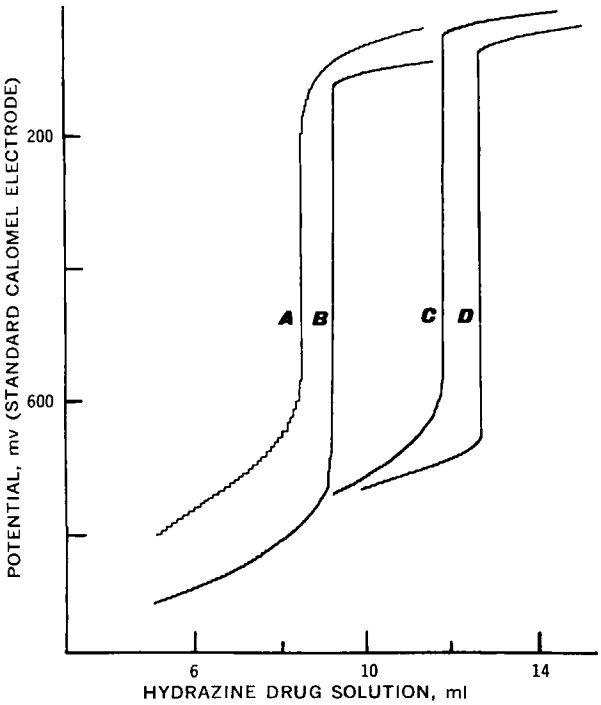


Figure 1—*Automatically recorded typical titration curves with chloramine-T as the oxidant (10 ml 0.05* M). *Key: A, standardization of chloramine-T with hydrazine sulfate (3.818 mg/ml); B, isoniazid (3.702 mg/ml); C, phenelzine sulfate (4.942 mg/ml); and D, dihydralazine sulfate (2.268 mg/ml).*

accurately measured aliquot (10–15 ml) of 0.05 *M* (0.1 *N*) chloramine-T into a 150-ml beaker and add about 30 ml of water. Adjust the temperature of the solution to 60 ± 3° with the hot plate magnetic stirrer and titrate potentiometrically against hydrazine sulfate solution using a platinum–calomel electrode system.

Procedure: Assay of Isoniazid, Phenelzine Sulfate, and Dihydralazine Sulfate—Weigh accurately about 300 mg of isoniazid or dihydralazine sulfate and about 500 mg of phenelzine sulfate. Place in a 100-ml volumetric flask and dissolve in, and dilute to volume with, 4 *N* sulfuric acid. Titrate an accurate volume (10–15 ml) of standardized chloramine-T with hydrazine drug solution, proceeding as for standardization of chloramine-T. From the volume of chloramine-T used, the normality of chloramine-T, and the factor for the hydrazine derivative, calculate the content of hydrazine derivative in the volume required for the titration. Each milliliter of 0.05 *M* chloramine-T is equivalent to 3.428 mg of isoniazid, 5.857 mg of phenelzine sulfate, and 2.883 mg of dihydralazine sulfate.

TLC Study—The solution from the chloramine-T–dihydralazine reaction, stopped at the end-point, was partially neutralized (pH 2.5) with 2 *N* NaOH and gently evaporated to dryness on a water bath. The residue was extracted with hot methanol, and the methanol-soluble extract was spotted on 10 × 20-cm silica gel plates[3] (0.25 mm) previously buffered by ascending development with a pH 7.6 McIlvaine buffer and removal of the solvent by heating for 2 hr at 70°. The mobile phase consisted of a mixture of ether and acetic acid (80:4). The location of migrating spots was found by UV light.

RESULTS AND DISCUSSION

Table I shows the results of the chloramine-T titration of three hydrazine drugs with potentiometric end-point detection. The recoveries by the proposed method were satisfactory, and a sharp fall in potential at the end-point was noted in the titration curves (about 500 mv) (Fig. 1).

[3] F_{254}, E. Merck, Darmstadt, Germany.

Table I—Results of Potentiometric Titrations with Chloramine-T

Drug	Theoretical Milligrams Consumed, Range	Found, % of Theoretical	Equivalents of Chloramine-T per Mole of Drug
Isoniazid	33.46–51.69	100.05 ± 0.44[a]	4
Phenelzine sulfate	57.30–88.22	99.53 ± 0.31	4
Dihydralazine sulfate	28.03–42.36	100.13 ± 0.29	10

[a] Standard deviation based on eight analyses.

In strong acid solution of chloramine-T, the free acid (RNHCl) and dichloramine ($RNCl_2$) predominate (11) and may react directly with hydrazine or the hydrazine part of isoniazid and phenelzine according to Scheme I:

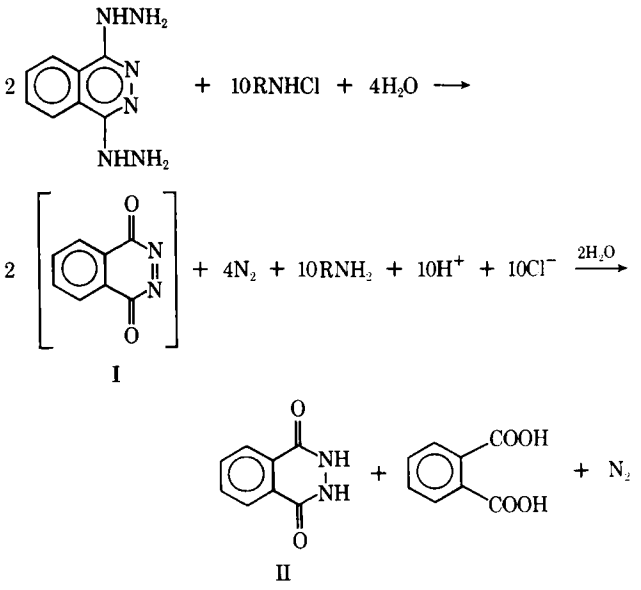
$$2RNHCl + N_2H_4 \longrightarrow 2RNH_2 + 2Cl^- + 2H^+ + N_2$$
$$RNCl_2 + N_2H_4 \longrightarrow RNH_2 + 2Cl^- + 2H^+ + N_2$$

Scheme I: R = p-Toluenesulfonyl

The hydrolytic products, isonicotinic acid or phenethyl alcohol, are not affected by chloramine-T under the present conditions. Four equivalents of the oxidant are consumed per mole of the reductant hydrazine derivative. However, in the case of dihydralazine, 10 equivalents of the oxidant are consumed per mole of drug, and this result is compatible with the proposed Scheme II. Following this route, 1 mole of 2,3-dihydrophthalazine-1,4-dione (II) and 1 mole of phthalic acid are produced.

Accordingly, the methanol-soluble extract of the chloramine-T–dihydralazine reaction yielded three spots in the TLC system described. The spots corresponding to *p*-toluenesulfonamide (R_f 0.92), II (R_f 0.57), and phthalic acid (R_f 0.065) were located by comparison with authentic samples treated similarly. On the other hand, studies of the oxidation of II with 1 mole of lead tetraacetate (14) provided evidence for the presence, in roughly equivalent amounts, of phthalic anhydride and unchanged II. The initial product of oxidation (I) is too unstable to be isolated (14). By taking advantage of its extraordinary reactivity as a dienophile, I was identified (14, 15) with butadiene as a Diels–Alder adduct.

Since the oxidation potential of chloramine-T is increased by lowering the pH, oxidations were carried out in a strong acid medium. Potentiometric analysis was performed with a reverse procedure. An accurately measured volume of standard chloramine-T solution, heated to about 60°, was titrated with hydrazine drug so-



Scheme II

lution in 4 *N* sulfuric acid. With increasing sulfuric acid concentration, from 4 to 16 *N*, no effect on the titration was observed; the rate of reaction decreased progressively toward the end-point as the sulfuric acid concentration was decreased from 4 to 0.5 *N*.

Hydrazine sulfate has been mentioned as a standard substance by several investigators (16); it is available in a highly purified form and its solutions are stable even if heated (17). The precision and accuracy of its potentiometric titration with chloramine-T under the proposed conditions suggested that it is suitable as a valuable primary standard substance in the standardization of chloramine-T solution. The possibility of adapting chloramine-T to the titrimetric analysis should not be overlooked. The stability of 0.05 *M* chloramine-T solution, stored in brown glass, was examined over 2 months and there was no detectable change in titer, in agreement with previous papers (11, 18).

The present method appears to offer a convenient alternative to the existing and more expensive oxidometric methods for the determination of the drugs listed in Table I.

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Spectrophotofluorometric Analysis of Procainamide and Sulfadiazine in Presence of Primary Aliphatic Amines Based on Reaction with Fluorescamine

JOHN M. STERLING and W. G. HANEY[x]

Abstract □ The potential of 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione (fluorescamine) for use as a reagent for determination of drugs containing the primary aromatic amino substituent in the presence of drugs containing primary aliphatic amino substituents was evaluated. Procainamide and sulfadiazine were used as test drugs in the evaluation. The selective determination is based on a difference in the extent of reaction of aromatic and aliphatic amines with fluorescamine at pH 5.5, as well as small differences in spectral characteristics of the two groups. Use of the reagent for microdetermination of aromatic primary amines was compared with that of the Bratton–Marshall and 9-chloroacridine techniques.

Keyphrases □ Procainamide—spectrophotofluorometric analysis with fluorescamine in presence of primary aliphatic amines □ Sulfadiazine—spectrophotofluorometric analysis with fluorescamine in presence of primary aliphatic amines □ Fluorescamine—reagent in spectrophotofluorometric analysis of procainamide and sulfadiazine in presence of primary aliphatic amines □ Spectrophotofluorometry—analysis, procainamide and sulfadiazine using fluorescamine in presence of primary aliphatic amines

Recently, Weigele *et al.* (1, 2) reported the synthesis and characterization of 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione (fluorescamine) as a reagent for the rapid preparation of highly fluorescent derivatives of primary amines. Udenfriend and coworkers (3, 4) have since shown the applicability of this reagent to the analysis of proteins, peptides, and amino acids in various biological systems. The reaction upon which the technique is based proceeds with a half-time in the range of milliseconds, giving fluorescent derivatives that can be determined in subnanomole per milliliter concentrations and that are stable for at least 24 hr.

Because of the rapid, quantitative nature of this reaction and the apparent stability of the reaction products, it was decided to evaluate fluorescamine as a reagent for use in drug microanalysis. This report presents its use as a reagent for the selective spectrophotofluorometric analysis of drugs containing the primary aromatic amino substituent (with procainamide and sulfadiazine as representative drugs) in the presence of primary aliphatic amines. In addition, the technique is compared with the Bratton–Marshall (5) and 9-chloroacridine (6) analyses for drugs with primary aromatic amino substituents.

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Table I—Analysis of Procainamide and Sulfadiazine Solutions

Sample	Component[a]	Amount Added, mole × 10^{-8}/ml	Percent of Theory Found (*SD* of Mean, %[b])		
			Fluorescamine	Bratton–Marshall	9-Chloroacridine
1	Procainamide	4.00	99.47 (0.52)	99.65 (0.46)	100.32 (0.64)
2	Sulfadiazine	4.00	100.43 (0.32)	99.94 (0.31)	99.41 (0.25)
3	Procainamide	1.00	99.25 (0.37)	100.86 (0.84)	99.12 (0.65)
4	Sulfadiazine	1.00	99.47 (0.26)	98.76 (0.94)	99.06 (0.76)
5	Procainamide	0.10	101.02 (0.98)	93.21 (5.24)	104.29 (4.65)
6	Sulfadiazine	0.10	99.08 (0.74)	92.61 (7.34)	105.71 (5.00)
7	Procainamide	0.08	98.76 (1.25)	— —	— —
8	Sulfadiazine	0.08	102.14 (1.98)	— —	— —

[a] Solutions analyzed by the fluorescamine procedure contained 4.0 × 10^{-8} mole/ml of dopa, dopamine, amphetamine, and histamine. [b] $n = 12$.

EXPERIMENTAL

Apparatus—The spectrophotofluorometer[1] was equipped with a 150-w, 7.5-amp, 17–23-v, dc xenon lamp[2] and a IP 21 photomultiplier tube. Meter multiplier settings varied from 0.1 to 0.005, and the photomultiplier photometer was operated at full sensitivity. The band passes were maintained at 2.0 mm. A spectrophotometer[3] was used for the Bratton–Marshall and 9-chloroacridine determinations.

Materials and Reagents—Histamine dihydrochloride[4], dopamine hydrochloride[4], fluorescamine[5], procainamide[6], sulfadiazine[7], dopa[7], and amphetamine[8] were used. Other materials and reagents were of the best grade commercially available and were used without further purification.

Solutions—Analyzed solutions of each drug were prepared by dilution of stock solutions of the individual drugs, the pH of which had been previously adjusted to 5.0 with 0.1 *N* HCl. Buffer solutions were prepared according to official procedures (7). Solutions necessary for the Bratton–Marshall and 9-chloroacridine analyses were prepared as previously outlined (6). In the initial studies, fluorescamine was used as a solution (5.0 mg/100 ml) in dioxane. Because of stability problems, the solvent was later changed to acetone.

Determination of pH Dependency—Solutions (1.0 ml, 4.0 × 10^{-8} *M*) of each drug were mixed with phosphate buffer (0.5 ml, pH 4.0–9.5). Fluorescamine solution (0.5 ml) was added immediately with shaking, and fluorescence determinations were made after 15 min. Excitation and emission wavelengths were 400 and 498 nm, respectively, for procainamide and sulfadiazine and 388 and 480 nm, respectively, for the remaining drugs.

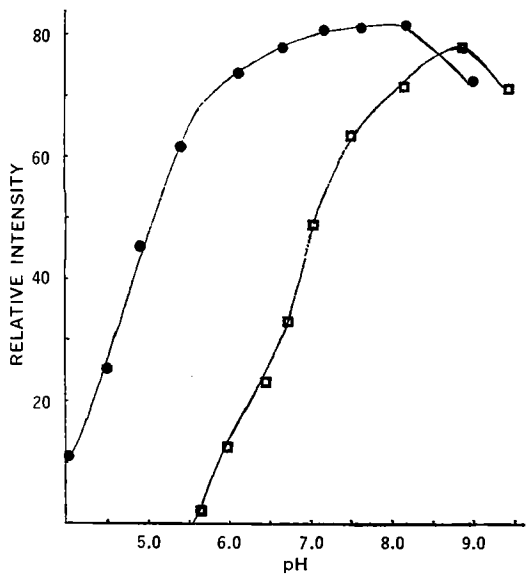


Figure 1—*pH dependency of the reaction of sulfadiazine* (●) *and amphetamine* (□) *with fluorescamine.*

Development Time and Fading Tendencies—Solutions (20.0 ml) of the drugs were prepared as already described in phosphate buffer (pH 5.5) and were mixed with fluorescamine solution (10.0 ml). Aliquots were taken for analysis at 1.5-min intervals for 6 min, at 5-min intervals for 1 hr, and at the end of 24 hr.

Spectrofluorometric Properties—Solutions (2.0 × 10^{-8} mole/ml) of procainamide and sulfadiazine were prepared in phosphate buffer (pH 5.5 and 8.5) for the remaining drugs. Fluorescamine (0.5 ml) was added, with shaking, to each solution. After 6 min, the pH of each solution was adjusted to 5.5 with 0.1 *N* HCl. Absorption and emission spectra were determined.

Analysis of Procainamide and Sulfadiazine—Solutions were prepared in the concentrations listed in Table I. Solutions analyzed by the fluorescamine-based procedure contained dopa, dopamine, amphetamine, and histamine (4.0 × 10^{-8} mole/ml) in addition to procainamide or sulfadiazine. An aliquot (2.0 ml) of each solution was mixed with fluorescamine solution (1.0 ml). After 6 min, the fluorescence of the solution was determined against a reagent blank, and the concentration was calculated by reference to previously determined calibration curves.

RESULTS AND DISCUSSION

Procainamide and sulfadiazine were chosen as representative drugs containing the primary aromatic amino substituent. Dopa, dopamine, amphetamine, and histamine were included to evaluate the method's potential for selectively determining drugs having a primary aromatic amino substituent in the presence of drugs with a primary aliphatic amino substituent.

Reaction of fluorescamine with primary amines has been shown to be strongly pH dependent (8), proceeding with increased rapidity as the pH is increased up to a maximum at 8.5–9.5. It has been suggested that protonation of the amine prior to reaction retards the reaction. Competing with the reaction that yields the fluorescent derivative is the reaction whereby the reagent is degraded to nonreactive products. Thus, a procedure for selective analysis of primary aromatic amines in the presence of primary aliphatic amines might utilize a pH at which fluorescamine reacts with aromatic amines but degrades before reacting with the more basic aliphatic amines.

On the basis of this rationale, the pH of the reaction medium was varied to observe the relative fluorescence of compounds in the study under these reaction conditions (Fig. 1). Since the fluorescence of fluorescamine derivatives was previously shown to be constant over the pH 4–9.5 range, fluorescent intensity at a particular pH is directly related to the extent of reaction at that pH. Sulfadiazine exhibited relatively high fluorescent intensity at pH 5.5, whereas amphetamine did not. Procainamide exhibited the same pH-dependency properties as sulfadiazine, and dopa and dopamine exhibited properties similar to those of amphetamine. Histamine gave a more intense fluorescence at pH 8.0 but did not fluoresce at pH 5.0. Thus, phosphate buffer of pH 5.5 was tentatively chosen as the reaction medium.

Figure 2 presents data regarding reaction time and stability of the reaction products for procainamide. Sulfadiazine studies gave similar results. On this basis, it appears that any time from 6 min

[1] Aminco-Bowman model 4-8202D.
[2] Hanovia 901 Cl.
[3] Cary model 118.
[4] ICN Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Roche Diagnostics, Nutley, N.J.
[6] E. R. Squibb, Princeton, N.J.
[7] USP reference standard.
[8] Analabs, North Haven, Conn.

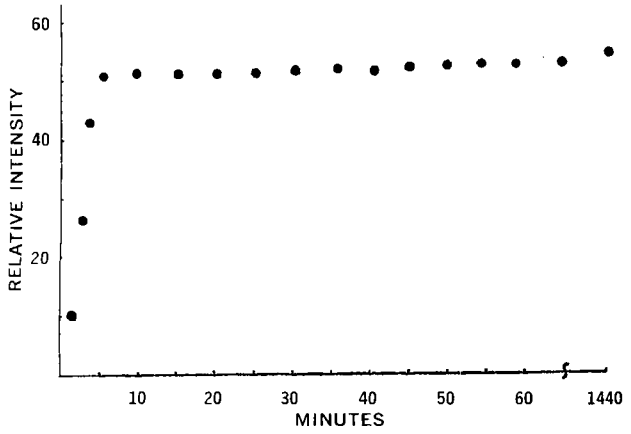


Figure 2—*Developing time and stability of products in the procainamide–fluorescamine reaction.*

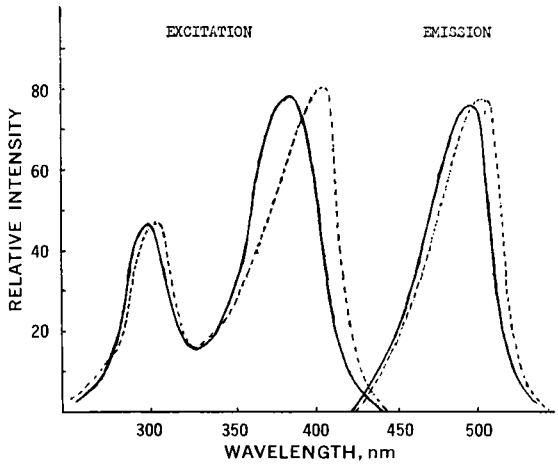


Figure 3—*Spectrofluorometric properties of sulfadiazine (– – –) and amphetamine (——) after fluorescamine reaction.*

after addition of fluorescamine up to 24 hr could be used for the analysis. The development time is somewhat longer than that previously reported for fluorescamine-based determinations, but this result is in accord with the lower pH of the reaction medium. The reaction time compared favorably with that of the 9-chloroacridine and Bratton–Marshall techniques, where the recommended development time is 15 min. In the latter procedure, product instability is severe, making a timed analysis essential.

Excitation and emission spectra of fluorescamine derivatives of sulfadiazine and amphetamine are presented in Fig. 3. The 10–15-nm increase in wavelengths of maximum absorption and emission is consistent with the data previously derived for aniline (10). This increase may be rationalized by reference to structures of the fluorescent derivatives for an aromatic amine (Structure A) and an aliphatic amine (Structure B).

In Structure A, the aromatic ring derived from the aromatic amine provides additional electron delocalization not present in the structure obtained from the aliphatic amine, shifting activation and emission maxima to longer wavelengths. Similar results were obtained when sulfadiazine was compared to dopa and dopamine. For histamine, both activation and emission maxima were shifted to shorter wavelengths with respect to the other aliphatic amines.

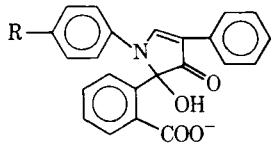
The difference in fluorescence spectra provides an additional procedure for ensuring selective analysis of primary aromatic amines in the presence of primary aliphatic amines. The fluorescent intensity resulting from the reaction of dopamine with fluorescamine in phosphate buffer of pH 8.5 was one-twelfth the value of that of an equimolar solution of procainamide when measured at the wavelength of maximum absorption and emission of procainamide. Comparable results were obtained with the other aliphatic amines. This difference in spectral properties, in combination with the more rapid reaction of aromatic amines at pH 5.5, provides the basis for selectivity of the analytical technique.

Standard curves for procainamide and sulfadiazine were prepared by plotting relative intensity against volumes taken of equimolar concentration of the two drugs. In the range of 0.5–125 nmoles, Beer's law holds for the system. Standard curves were prepared daily for 10 days. In initial studies, a large negative deviation in the slope of the curve was noted after 7 days, but a change of the fluorescamine solvent from dioxane to acetone removed this problem. The standard deviation of the slope of the curve for procainamide was 0.17%; it was 0.21% for sulfadiazine. The average correlation coefficient was 0.996 for procainamide and 0.991 for sulfadiazine. The average y-intercept value was −0.006 for procainamide and −0.009 for sulfadiazine.

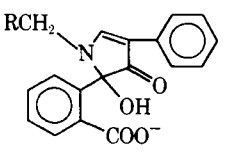
Results comparing the sensitivity and precision of the procedure to that of the Bratton–Marshall and 9-chloroacridine procedures are presented in Table I. With the drugs included in this study, fluorescamine reaction apparently allows analysis of solutions at least an order of magnitude more dilute than the other two procedures.

The potential for selective measurement of aromatic amines in the presence of aliphatic amines was evaluated by determining sulfadiazine and procainamide in solutions containing varying amounts of histamine, dopa, amphetamine, and dopamine. Concentration of the latter drugs 200 times higher than sulfadiazine or procainamide gave no observable effect on accuracy or precision.

Use of fluorescamine as a reagent for analysis of drugs containing primary aromatic amino substituents appears to have several advantages over the Bratton–Marshall or 9-chloroacridine techniques. Reaction proceeds more rapidly, and the fluorescence is stable for longer periods. Only one solution (fluorescamine in acetone) is required, and it has been shown to be stable at room temperature for up to 3 months. No tedious pH adjustments are necessary. Finally, the procedure gives data on solutions (0.08×10^{-8} mole/ml) with standard deviations comparable to those obtained on solutions (1.0×10^{-8} mole/ml) with the two other procedures, indicating that fluorescamine-based analysis gives reliable data on solutions at least 10 times more dilute than the Bratton–Marshall and 9-chloroacridine techniques.



Structure A



Structure B

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[x] To whom inquiries should be directed.

Complex Formation of Uranyl Acetate with Tetracycline and Its Utilization for Their Microdetermination

AFAF EL-SAID MAHGOUB[x], E. M. KHAIRY, and A. KASEM

Abstract □ Conductometric and spectrophotometric (covering the visible, UV, and IR ranges) studies as well as microanalyses of uranyl complexes with tetracycline revealed the existence of the 1:1 complex species. The mean stability constant of the 1:1 complex, as determined spectrophotometrically, amounted to 1.2×10^5. This finding permits the use of the procedure for the microdetermination of tetracycline using UO_2^{+2} ion or vice versa.

Keyphrases □ Uranyl acetate—complex formation with tetracycline, analysis of both antibiotic and uranyl ion □ Tetracycline—complex formation with uranyl acetate, analysis of both antibiotic and uranyl ion □ Complex formation, uranyl acetate and tetracycline—analysis of both antibiotic and uranyl ion □ Spectrophotometry—analysis, tetracycline and uranyl ion from antibiotic–uranyl acetate complex

Several tetracycline antibiotics are known including tetracycline, chlortetracycline, and oxytetracycline. The high affinity of chlortetracycline and oxytetracycline for cations of heavy metals was detected (1) and the parent substance, tetracycline, was found to chelate with heavy metal cations such as Fe^{+3}, Al^{+3}, Cu^{+2}, Ni^{+3}, Co^{+2}, Zn^{+2}, and Mn^{+2}, forming the 1:1 and 1:2 (metal ion–ligand) complexes (2). The stability constants of these chelates were shown to increase in the same order; values of log K ranging from about 9.9 to 4.4 were recorded, of the same order of magnitude as those corresponding to the chloro and oxy derivatives. Complexes of tetracyclines with the uranyl ion have not been studied frequently, but Ishidate and Sakaguchi (3) reported on complexes of the chloro derivative.

Several spectrophotometric methods, involving chelation with different ions, have been described for the determination of tetracycline. A ferric chloride colorimetric method was described (4). Tetracycline was determined using sodium hydroxide, where a yellow solution was produced, yielding an absorption maximum at 380 nm (5, 6). Colored materials have been also reported due to the interaction of tetracycline with several metal compounds including zinc chloride (7), zirconium oxychloride (8), thorium nitrate (9), and ammonium molybdate (10).

The present report concerns physicochemical studies, including conductometric, spectrophotometric, and microanalytical determinations, on uranyl–tetracycline complexes. Because of the pharmaceutical importance of tetracycline, a spectrophotometric method for its microdetermination using UO_2^{+2} was investigated. Application to microdetermination of the uranyl ion was also studied.

EXPERIMENTAL

Materials and Reagents—Tetracycline was prepared according to the procedure recommended by Duggar (11).

The uranyl–tetracycline (1:1) complex was prepared by mixing the organic reagent with uranyl acetate, both dissolved in a water–dioxane mixture [dielectric constant *(D)*, 30], and the complex separated out by evaporation under vacuum. The solid was filtered and washed thoroughly with redistilled water and then with pure dioxane to ensure complete removal of excess reagents. The uranyl–tetracycline complex was recrystallized from acetone, yielding brown crystals.

Procedures—For conductometric titrations, a solution of tetracycline in a water–dioxane mixture (3×10^{-3} *M*) and a uranyl acetate solution (3×10^{-4} *M*) in the same solvent were used. Both were previously thermostated at 25°. The reagent was added using a microburet to 20 ml of the uranyl acetate solution under a purified nitrogen atmosphere. After each addition the solution resistance was measured using an alternating current conductivity bridge[1] of >±0.02% accuracy. An audiofrequency electron tube oscillator was utilized as a source of alternating current (1000 Hz). A sensitive oscilloscope[2] served as a null indicator.

Spectrophotometric studies of the uranyl–tetracycline complexes in the visible, UV, and IR ranges were carried out at room temperature using a recording spectrophotometer[3]. The formation and composition of the uranyl complexes were detected by applying the slope ratio method as previously described (12).

Solutions of precipitated uranyl complex with tetracycline in the same solvent were also studied spectrophotometrically in the visible and UV ranges. The IR absorption spectra of the complexes studied were recorded[4] using the KBr disk technique.

The precipitated uranyl–tetracycline complex was also chemically analyzed[5] for uranium, carbon, and hydrogen.

RESULTS AND DISCUSSION

Conductometric Measurements—In Fig. 1, the specific conductance values, k, calculated from resistance measurements and

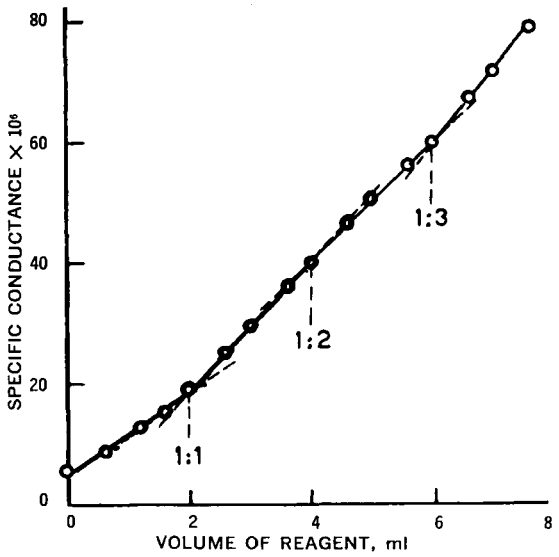

Figure 1—*Conductometric titration of* UO_2^{+2} *ions* (3×10^{-4} M) *against tetracycline* (3×10^{-3} M).

[1] Leeds and Northrup Co.
[2] Hartmann and Braun AG, Frankfurt/Main, West Germany.
[3] Beckman DK.
[4] Perkin-Elmer 337 spectrophotometer.
[5] In the Microanalytical Unit, University of Cairo, Giza, Egypt.

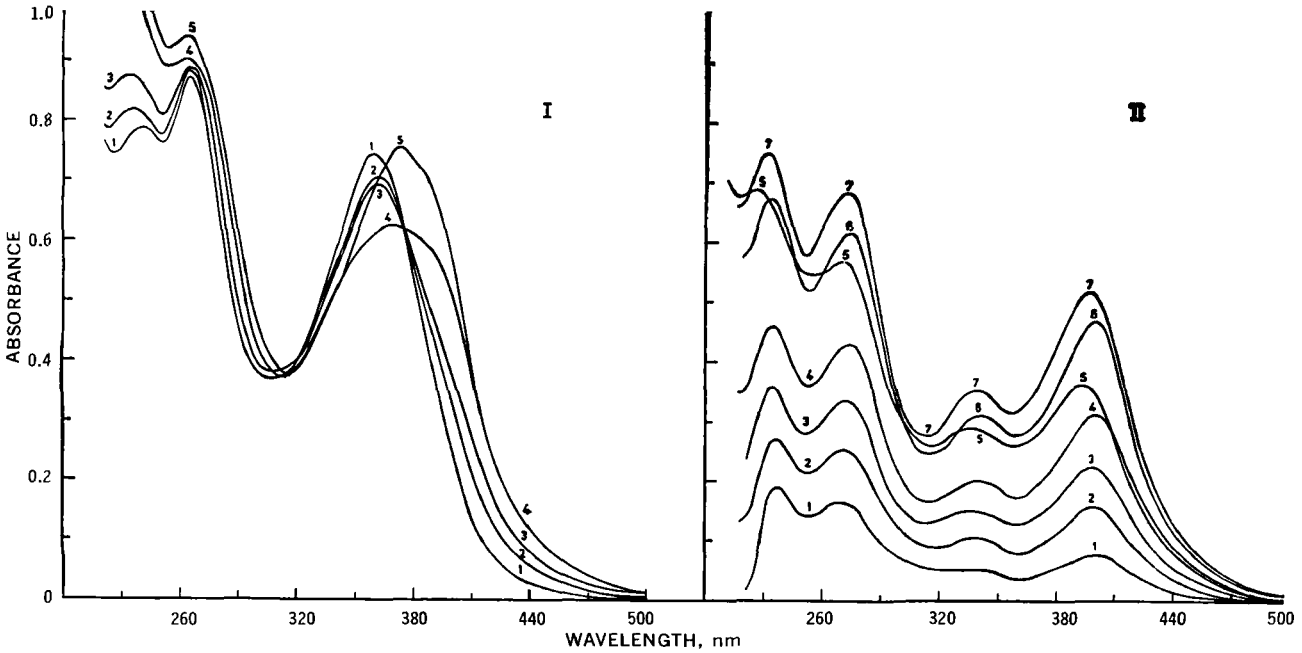

Figure 2—*Absorption spectra of uranyl acetate–tetracycline complex formed by the slope ratio method. Key: I, uranyl variable; and II, reagent variable.*

corrected for solvent conductance and volume changes are plotted against the volume of the ligand. The plot shows three breaks at the 1:1, 1:2, and 1:3 (uranyl–ligand) compositions. It reveals also a considerable increase in the conductance with an increase in the ligand concentration. This may be explained if it is assumed that the ligand behaves as a bidentate, using the carbonyl and the hydroxy groups in coordination with the uranyl cation. The H^+ ions released due to the participation of the OH group increase the conductance. Such an increase appears to be greater in the formation of the 1:2 complex. It is apparent that increased acidity is more effective in increasing conductance and overcomes the decrease of conductance due to partial balance of the charge of the central metal ion of the 1:2 species. In the same sense, the formation of the negatively charged 1:3 complex causes increased conductance at a greater rate than the 1:2 species. Further addition of excess reagent than required for the 1:3 species is accompanied by increased conductance, which may be plausibly attributed to partial acid dissociation of the free ligand. This effect is supposedly enhanced by masking the dissociated ligand in the 1:3 complex, thereby shifting the equilibrium toward further dissociation.

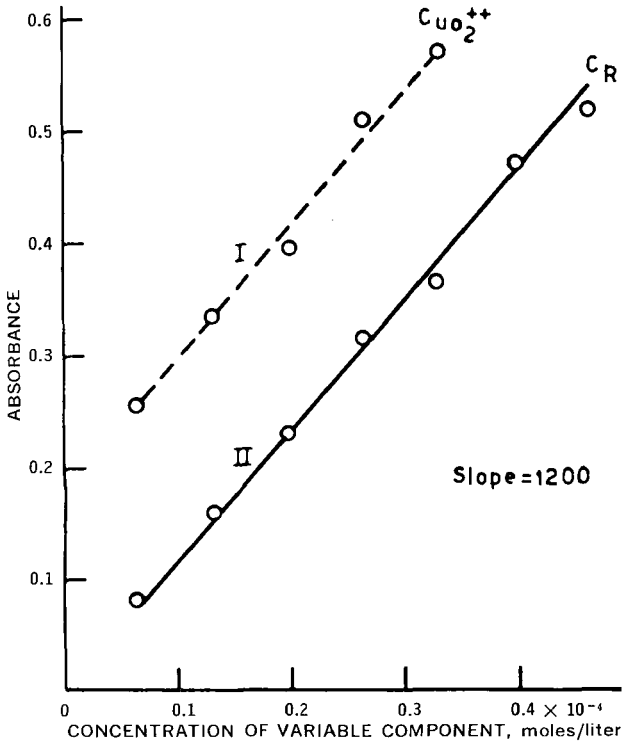

Figure 3—*Composition of uranyl–tetracycline complex by the slope ratio method (at* λ_{404}*).*

Spectrophotometric Studies—*Spectra of Uranyl–Tetracycline Complex Formed in Solutions*—The maximum absorption of uranyl acetate observed in pure dioxane occurs at 425 nm as was found previously (13). By applying the slope ratio method, plots of absorbance against wavelength are obtained (Fig. 2). Pure tetracycline yields λ_{max} 349 nm; the corresponding uranyl complex shows a characteristic band maximum at λ 404 nm. Absorption–concentration curves taken at that band maximum are given in Fig. 3. The slope ratio of uranyl to ligand is 1, revealing the major stability of the 1:1 species.

Absorption Spectra of Precipitated Complex—The complex prepared as described under *Experimental* separates as brown crystals. The spectra and chemical composition of the complex in the 1:1 mole ratio, as indicated by conductometric and spectrophotometric measurements, were studied to ascertain the behavior manifested in solutions of different reagents. For this purpose, the spectra of the complex, together with pure uranyl and pure tetracycline, were scanned in the UV, visible, and IR ranges.

The spectra in the 200–500-nm range are shown in Fig. 4. The spectra of the solutions of the complex in dioxane show three electronic transitions at λ_{max} 404, 350, and 270 nm. These transitions are shifted from those of pure uranyl acetate and have significantly greater intensities. The peaks at the maximum absorption of the complex are closely comparable with those obtained by admixing solutions of the pure reagents at the same mole ratio.

The absorption maxima, λ_{max}, together with the molar absorptivities of the complex are shown in Table I and are compared to those of pure reagents.

The IR spectra of tetracycline and its uranyl complex are shown in Fig. 5. The structure of tetracycline is complicated; it is difficult to assign all of the bands observed with certainty. However, the IR spectrum shows an intense broad band covering the 3700–2000-cm^{-1} region. In this region the ν_{OH}, ν_{N-H}, and ν_{C-H} are expectedly included. It also shows two carbonyl bands at 1630 and 1670 cm^{-1}, which can be assigned to the hydrogen-bonded ketone (14, 15) and amide-CO (16) groups, respectively.

The IR spectrum of the complex shows the absence of the high

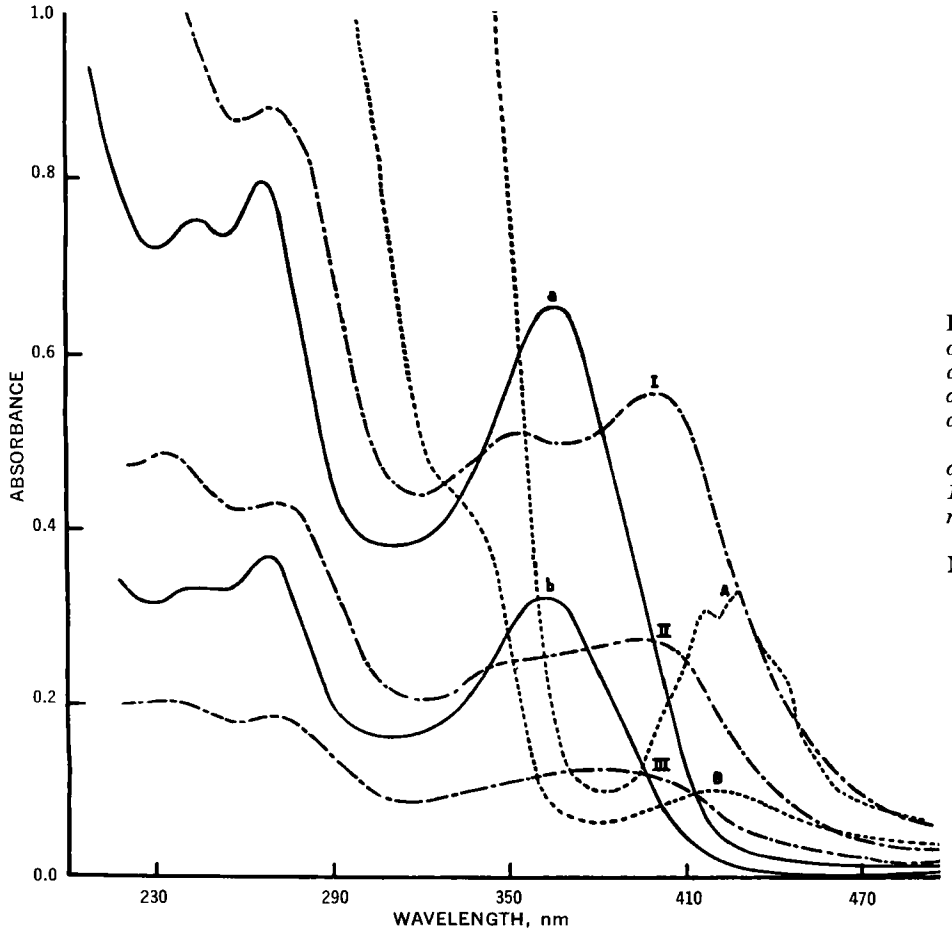

Figure 4—*Absorption spectra of uranyl acetate, tetracycline, and their complex in water–dioxane mixture. Key: ---, uranyl acetate, A = 6.25 × 10⁻³ M, B = 10⁻³ M; ——, tetracycline, a = 0.4 × 10⁻⁴ M, b = 2 × 10⁻⁵ M; and — - —, uranyl–tetracycline complex (1:1), I = 1 × 10⁻⁴ M, II = 0.5 × 10⁻⁴ M, and III = 0.2 × 10⁻⁴ M.*

frequency carbonyl stretching vibration at 1670 cm^{-1}. This suggests that the amide-CO group is involved in chelation with the UO_2^{+2} ion. The broad band within the 3000–3600-cm^{-1} range is considered to be due to a hydrogen-bonded hydroxy group (17).

Chemical Microanalyses of Precipitated Complex—The results of microanalyses of the precipitated recrystallized brown complex are: U, 29.6%; C, 33.6%; and H, 3.8%. These results are in good agreement with the calculated data for the complex composition $UO_2R{\cdot}3H_2O{\cdot}OH^-$, which are: U, 30.3%; C, 33.7%; and H, 3.8%.

Based on chemical analysis, it may be shown that the hydrogen content of the complex species and the difference in weight corresponding to the unanalyzed part of the product, principally oxygen, suggest the presence of 3 H_2O molecules in the precipitated complex. This suggests a 1:1 complex. The presumption of the presence of water molecules is confirmed by the IR data. The assigned structure (Structure I) is in agreement with the octahedral structure proposed by Szöke (18), who was able to build up a stereomodel which reflects the most suitable two-dimensional projection in the form of a regular hexagon. The uranyl axis stands perpendicular to this plane.

The stability constant *(K)* of the uranyl–tetracycline (1:1) complex is determined by the limiting logarithmic spectrophotometric method (19) using data obtained by the slope ratio method as described previously. A mean K value of 1.2×10^5 was found.

Spectrophotometric Microdeterminations—Complex formation between UO_2^{+2} ion and tetracycline was used for the microdetermination of both by a spectrophotometric method. For the determination of UO_2^{+2} ion, varying concentrations of the uranyl ion are added to a constant concentration of the ligand, as in the slope ratio method. The absorbances are scanned at different wavelengths, using the solvent (water–dioxane mixture, $D = 30$) as a blank. Plots of absorbances, corrected for excess tetracycline, against concentration of the metal ion (Fig. 6) indicate that the suitable wavelengths for such determination are λ_{446} and λ_{440} nm. The absorbance is a linear function of the metal-ion concentration in accordance with Beer's law at concentrations up to about 3.0×10^{-5} M, but at higher concentrations the absorbance deviates from Beer's law. The lowest concentration of UO_2^{+2} estimated with considerable accuracy, within the resolution of the instrument, corresponding to significant absorbance values, amounts to 6.6×10^{-6} M (about 2 ppm).

Because of the medical importance of tetracycline, the microdetermination was confirmed by carrying out another experiment in

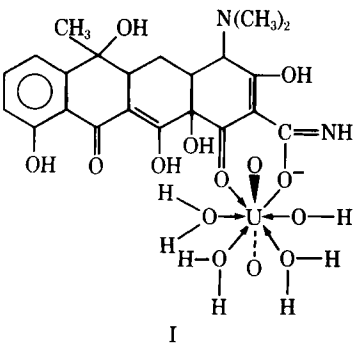

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Table I—Absorption Maxima and Molar Absorptivities

Compound	λ_{max}, nm	log ε, $mole^{-1}$ cm^{-1}
Uranyl acetate	428	1.72
	416	1.69
	335	2.64
Tetracycline	364	4.22
	270	4.28
Uranyl–tetracycline complex	404	3.74
	350	3.71
	270	3.94

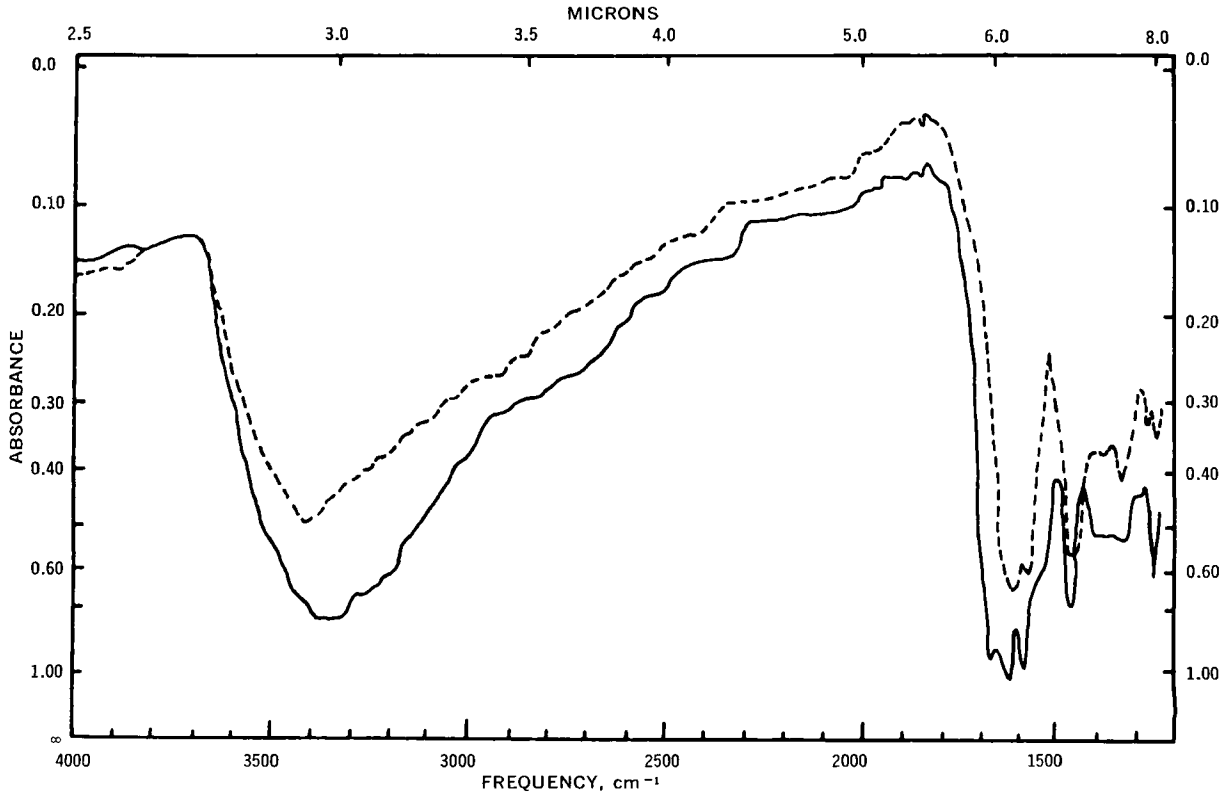

Figure 5—*IR absorption spectra of tetracycline and its complex with UO_2^{+2} ions. Key: ——, pure tetracycline; and ---, uranyl–tetracycline complex.*

which the ligand was added in different proportions to a fixed proportion of the uranyl ions. The plots of absorbance, corrected for excess UO_2^{+2} ion, against concentration of tetracycline at different wavelengths, corresponding to the peaks (Fig. 7) show that at λ_{404} and λ_{350} nm, a linear relationship exists to concentrations of about 5.0×10^{-5} and 3.0×10^{-5} *M*, respectively. The most suitable wavelength for such determination is λ_{404} nm. The highest concentration of tetracycline estimated within the range of applicability of Beer's law was about 22 ppm, while the lowest concentration determined with considerable precision was about 6.6×10^{-6} *M* (3 ppm).

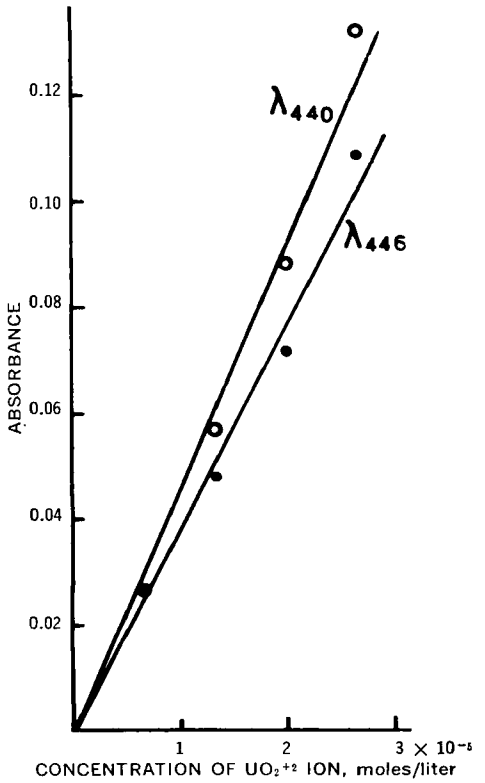

Figure 6—*Spectrophotometric microdetermination of UO_2^{+2} ion with tetracycline, corrected for excess tetracycline.*

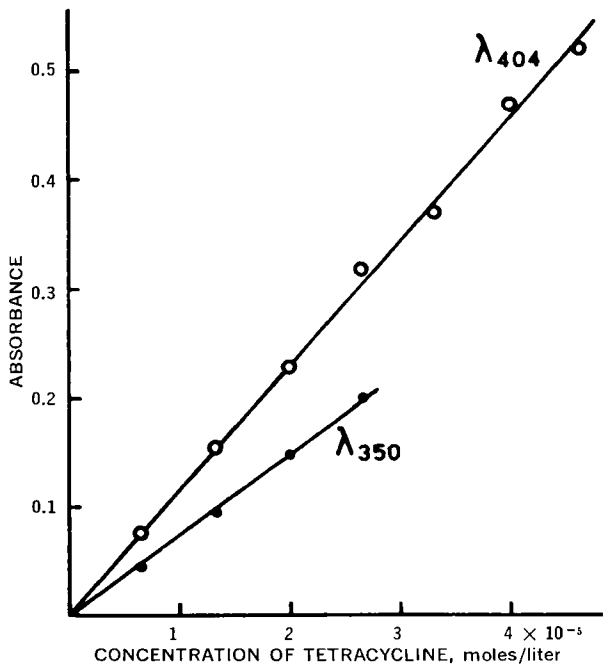

Figure 7—*Spectrophotometric microdetermination of tetracycline with UO_2^{+2} ion, corrected for excess uranyl.*

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[x] To whom inquiries should be directed.

Sodium Heparin Determination: Comparison of an Instrumental Method with the USP Method

M. REED, G. WILSON, W. DAVENPORT, T. SCHMITZ, and M. KLAMRZYNSKI [x]

Abstract □ An instrumental method for determining sodium heparin in aqueous solutions is described that has the advantages over the USP grading procedure of being simpler and quicker but just as reproducible. It is based on the principle that heparin concentration in solutions can be determined by measuring the recalcification clot time of heparinized sheep plasma by mechanical means. The concentration is read from a standard curve of clot time *versus* concentration of standard heparin.

Keyphrases □ Heparin—determination using instrumental method, compared to USP method □ Sodium heparin—determination using instrumental method, compared to USP method □ Blood clotting time—used in instrumental method for determination of sodium heparin in aqueous solutions, instrumental method compared to USP method

The USP (1) procedure for sodium heparin assay is rather cumbersome in both operation and calculation. This paper describes an instrumental method which offers ease of operation, calculation, and reliability.

The USP procedure is based on the increase in recalcification clotting time of sheep plasma with increasing concentration of heparin. The test is performed by comparing grades of clotting in assay samples with grades of clotting in a set of standards. Both sets of samples must be prepared within 20 min of each other, and the end-points are checked 1 hr after addition of the calcium chloride reagent.

With the instrumental method, standardized sheep plasma is used to develop a standard curve of known sodium heparin concentrations plotted against clotting time measured by the instrument. From this standard curve, sodium heparin concentrations can be read directly using clotting time obtained with assay samples.

The instrumental method differs from the USP method by measuring the time at which the clot forms rather than by grading the extent of clotting after 1 hr. The principle of the instrument is shown in Fig. 1.

EXPERIMENTAL

Each test vial contains a magnetic stainless steel ball. A drive motor moves the vial up and down in the reaction well. Before a clot forms, the stainless steel ball is held stationary by two calibrated permanent magnets as the vial moves. While in this stationary position, the ball interrupts a light beam directed through the vial at a photocell. As the vial moves up and down relative to the suspended ball, the test fluids flow back and forth around the ball, ensuring continuous and uniform mixing. When calcium chloride solution is added to the moving vial, an automatic timing device is activated. When clot formation occurs, the ball is pulled out of the magnetic field. Displacement of the ball more than 0.8 mm in either direction from its original suspended position permits the light beam to strike the photocell, stopping the timer.

The nominal bore diameter for the vial is 0.505 cm. The spherical nominal diameter of the stainless steel ball is 0.475 cm, having a weight of 0.4 g. The nominal clearance of the ball suspended in the vial is 0.015 cm on either side. Silicone oil standards of varying viscosities are used to factory calibrate each instrument. In brief, a master clot timing instrument is initially set up by using the most viscous plasma sample obtainable. A set point for the magnetic field is established with the viscous plasma. Silicone oil standards are prepared with the master instrument, which enable future adjustments to be made on subsequent production instruments.

One silicone oil standard has a viscosity that enables the photocell system to trip intermittently a timing motor relay circuit. This

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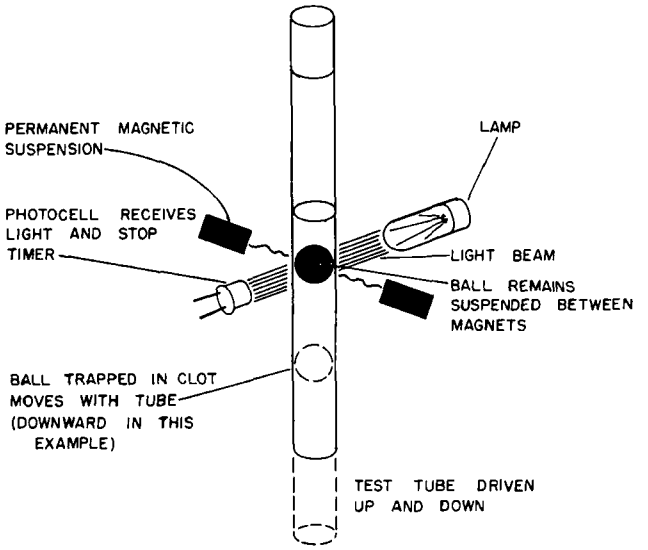

Figure 1—*Instrument for measuring the time at which blood clot forms.*

indicates that the magnetic field is weak enough. Another standard is made with a viscosity that does not enable the photocell system to trip the relay. This then indicates that the magnetic field is not too weak. The movement of the vial is two complete excursions per second with an absolute timing accuracy of ±0.2%

Apparatus—The following were used: a clot timing instrument[1] equipped with autoprecision pipeter, test vials of 5.0-ml capacity containing magnetic stainless steel balls, disposable plastic pipeter tips, graduated 2.0-ml pipets, and Whatman No. 1 filter paper. All glassware, stainless steel balls, and pipeter tips must be meticulously clean. If reused, they should be acid cleaned and thoroughly rinsed.

Reagents—The following were used: USP heparin sodium reference standard; normal saline, 9.00 g NaCl in distilled water, diluted to 1 liter; 0.02 *M* $CaCl_2 \cdot 2H_2O$[2]; and frozen sheep plasma[3], prepared for heparin assay in aliquots. All reagents must be equilibrated to 37° before use.

Standardization of Sheep Plasma—Each lot of sheep plasma exhibits a different clotting time and must be standardized against sodium heparin reference material. This was done by testing the sheep plasma against several concentrations of standard sodium heparin solution. The goal was to determine the concentration of sodium heparin that will give clotting times with the sheep plasma in the range of 100–150 sec. This clotting time range falls on the linear portion of the curve relating log of clotting time in seconds against sodium heparin concentration in units per milliliter.

The standardization procedure employed freshly thawed sheep plasma filtered through Whatman No. 1 filter paper and a sodium heparin standard solution prepared on the day of the assay. USP heparin sodium reference standard was made with normal saline to

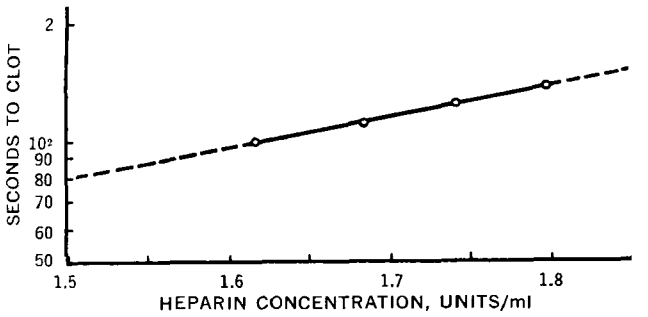

Figure 2—*Preparation of heparin standard curve.*

[1] Clotek system, Hyland Laboratories, Inc., Glendale, Calif.
[2] Chemical reagents are supplied with the Hyland Clotek system.
[3] Wilson Laboratories, Park Forest South, IL 60466

Table I—Comparison of Clot Timing Method with USP Method Using Range of Concentrations

Theoretical Concentration, Units/ml	Clot Timing Method, Mean[a] Values, Units/ml	USP Method, Mean[a] Values, Units/ml
68.38	66.15	66.22
70.13	70.36	68.72
72.07	73.55	69.56
74.20	75.51	71.08
76.15	77.04	73.38
78.03	78.74	76.07
80.14	81.50	80.90
82.16	83.70	82.64

[a] Mean of two determinations.

a concentration of 3 USP units/ml. Several dilutions of this sodium heparin solution were made to achieve the desired concentrations; a concentration of 1.80 units/ml gave a clotting time of 133.5 sec. This solution was further diluted as discussed under *Assay Procedure* to construct a standard curve.

Instrumental Operation—Empty test vials are placed in the reagent well containing one stainless steel ball each. Using the automatic pipeter, 0.1 ml of sheep plasma and 0.1 ml of a sodium heparin solution were added. The disposable tip was changed, and 0.1 ml of 0.02 *M* $CaCl_2$ was added while completely depressing the pipeter button to activate the timer. When the timer stopped, the time in seconds was automatically recorded.

Assay Procedure—Three dilutions were made from the heparin stock solution, described under *Standardization of Sheep Plasma,* which had a clotting time of 133.5 sec. Each standard dilution (1.80, 1.74, 1.68, and 1.62 units/ml) was tested twice with 0.1 ml sheep plasma. When plotted against the log of time in seconds (Fig. 2), a straight line was obtained for the clotting time range of 100–150 sec.

Test samples were diluted appropriately to bring their concentrations within the range of the standard curve. A 0.1-ml aliquot of the diluted sample was added to the test vial, and clotting time was determined with 0.1 ml sheep plasma. From the standard curve, the sodium heparin concentration of the test sample was calculated as follows: sodium heparin units per milliliter in sample equals sodium heparin units per milliliter in standard (from curve) times dilution of sample.

Equivalency—Eight samples of sodium heparin solution were prepared in duplicate spanning concentration ranges used in commercial sodium heparin blood collection units. These samples were assayed by both the USP and instrumental methods. The means and theoretical values for each sample are given in Table I.

In addition, 12 production batches of sodium heparin blood collection units were assayed by both methods. The results are given in Table II.

Reproducibility—Reproducibility of the clot timing instru-

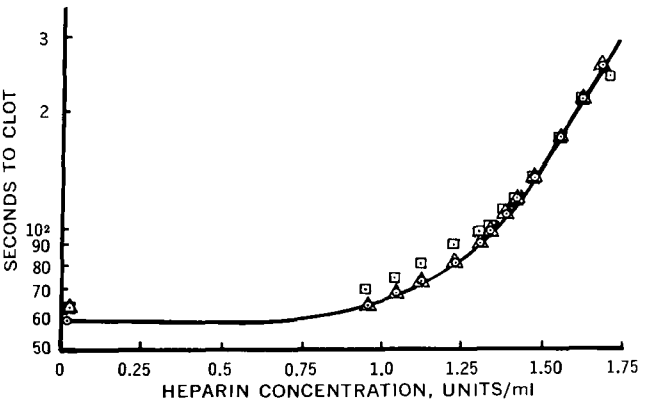

Figure 3—*Reproducibility of clotting times using the clot timing instrument. Key:* ⊡, *Stock Solution I tested on day stock solution was prepared;* ⊙, *Stock Solution II tested on day stock solution was prepared; and* ◬, *Stock Solution I tested 24 hr after stock solution was prepared.*

Table II—Comparison of Clot Timing Method with USP Method Using Production Batches at 75.0 Units/ml

Batch	Clot Timing Method			USP Method		
	Number of Determinations	Mean ± *SD*, Units/ml	Range	Number of Determinations	Mean ± *SD*, Units/ml	Range
1	7	78.66 ± 0.88	77.40–79.79	4	80.38 ± 5.36	75.80–83.06
2	5	79.38 ± 1.17	78.56–81.41	4	78.91 ± 3.05	75.80–81.53
3	5	79.68 ± 0.60	79.08–80.45	4	77.75 ± 3.86	73.90–82.80
4	7	77.09 ± 0.58	76.27–78.00	4	74.44 ± 2.68	71.07–76.60
5	6	76.71 ± 0.25	76.40–76.99	4	71.30 ± 4.36	66.90–75.14
6	5	71.89 ± 0.46	71.27–72.45	4	71.68 ± 2.64	69.06–75.19
7	5	70.80 ± 0.67	70.22–71.81	4	69.55 ± 2.36	66.80–71.83
8	8	73.48 ± 0.86	72.27–74.67	4	70.21 ± 1.87	67.52–71.53
9	5	74.57 ± 0.78	73.53–75.33	4	70.98 ± 1.02	69.87–72.13
10	8	73.55 ± 1.31	71.80–75.27	4	76.44 ± 1.35	74.73–77.67
11	7	74.05 ± 2.21	70.47–76.13	4	73.80 ± 1.02	72.37–74.57
12	8	75.23 ± 0.82	74.27–76.87	4	72.72 ± 0.91	71.85–73.87

Table III—Clot Timing Assays

ml Stock Solution/100 ml in Saline	Heparin Concentration[a], Units/ml	Stock Solution I[b] Dilutions, sec	Stock Solution I[c] Dilutions, sec	Stock Solution II[b] Dilutions, sec
Blank (saline)	0.0	61.0	61.0	58.8
1.30	0.921	67.9	64.4	64.6
1.50	1.063	74.5	70.8	70.5
1.60	1.134	80.5	75.1	75.8
1.70	1.205	—	—	—
1.75	1.240	88.8	85.0	86.8
1.80	1.276	—	—	—
1.85	1.311	97.2	95.5	96.0
1.90	1.347	103.4	103.2	102.6
1.95	1.382	110.4	111.5	112.0
2.00	1.418	120.8	120.4	119.6
2.10	1.488	140.0	141.8	141.9
2.20	1.559	166.9	167.7	168.9
2.30	1.630	206.7	207.7	—
2.40	1.701	243.2	265.5	—

[a] Stock Solutions I and II each contained 113.3 mg/250 ml in normal saline using USP heparin sodium reference standard (156.4 units/mg). [b] Tested on day when stock solution was prepared. [c] Tested 24 hr after stock solution was prepared.

ment method was established by preparing two stock solutions of sodium heparin (I and II) from which several dilutions of each were prepared with normal saline. These dilutions provided a final concentration range of 0.921–1.70 sodium heparin units/ml (Table III). On the day of preparation, both sets of diluted stock solutions were assayed by the clot timing instrument (Table III and Fig. 3). Twenty-four hours later, the instrumental assays were repeated on a second set of stock solutions made from Stock Solution I (Table III and Fig. 3).

RESULTS AND DISCUSSION

Table I shows that the estimates of reproducibility have 8 degrees of freedom each [(2 − 1) × 8]. The within-standard deviation for the USP method is 0.98, and it is 0.59 for the instrumental method. These values are not significantly different from one another, and the methods compare favorably in terms of reproducibility.

Table II shows that the clot timing instrument gave slightly higher values for sodium heparin concentration except for one instance. The differences between the two methods are significant for the eight samples in Table I and for the total of the eight in Table I plus the 12 in Table II in terms of accuracy. The mean difference between the two methods was 1.75 units or 2.3% at 75 units/ml. The instrumental method tended to overestimate the theoretical but not significantly (0.67 unit). The USP method underestimated the theoretical (1.59 units), giving a value significantly different from the zero bias.

The data in Table III show that the clotting time increases nonlinearly with increasing heparin concentration. Since it is desirable in a regression analysis that variability be equal at all concentrations, the log of time was analyzed as the dependent variable with heparin concentration as the independent variable. This log transformation equalized the variation, although it did not completely linearize the data for all heparin concentrations (Fig. 3). However, as seen in Fig. 2, the curve was linear with heparin concentrations that produced clotting times of 100–150 sec, and this portion was used as the "working curve."

Figure 3 indicates a small shift where stock dilutions were analyzed on succeeding days, but the average difference between the assays on the 2 days was not different from the zero bias.

The instrumental method required fewer and simpler dilutions and a shorter incubation period than the USP method. The instrumental assays were completed in about 25% of the time needed for the USP method.

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[x] To whom inquiries should be directed.

Determination of Sulfur Dioxide in Starch and Other Pharmaceutical Materials

C. M. HAMILTON and S. A. MOROS[x]

Abstract □ A sensitive and selective polarographic method for the measurement of sulfur dioxide in a wide variety of pharmaceutical materials is presented. It is based on the combination of its reducibility and volatility.

Keyphrases □ Starch—polarographic determination of sulfur dioxide content □ Sulfur dioxide—polarographic determination in starch and pharmaceutical materials □ Polarography—analysis, sulfur dioxide in starch and pharmaceutical materials

The polarographic reduction of sulfur dioxide in acid medium first was described by Gosman (1) and subsequently was elucidated by Kolthoff and Miller (2). The volatility of sulfur dioxide may be used to avoid a classical limitation of trace analysis: uncertainties in the blank and matrix corrections. This is accomplished by making the polarographic measurement, flushing sulfur dioxide from the system with an inert gas stream, and then remeasuring to obtain the residual current due to the sample, supporting electrolyte, impurities, *etc.*

EXPERIMENTAL

A suspension of starch (200 mg/10 ml of deoxygenated pH 8.3 Britton–Robinson buffer) was stirred while deoxygenating for 5 min in a polarographic cell thermostatted at 10°. After diverting the nitrogen flow over the surface of the solution, 1.0 ml of deoxygenated 3 *M* HCl was added, the suspension was stirred for 30 sec, and the current was measured. Nitrogen then was passed through the suspension to eliminate the sulfur dioxide and the residual current was measured. Finally, 50 μl of standard sulfite solution (5 mg $Na_2S_2O_5$/10 ml deoxygenated pH 8.3 buffer), corresponding to 80 ppm of sulfur dioxide in the sample, was added and the current was measured after stirring briefly. The current obtained after the standard addition of 80 ppm, the USP limit for sulfur dioxide in starch, was compared with that obtained for the sample originally. Alternatively, a calibration curve covering the concentration range of interest was prepared and the actual value was determined.

RESULTS AND DISCUSSION

In the case of capsule food starch samples, differential pulse polarography[1] gave a clearly defined and well-separated reduction peak at −0.35 v *versus* silver–silver chloride (saturated potassium chloride) electrode; the peak current was linear with sulfur dioxide concentration over the range studied: 5–200 ppm in the sample. Since none of the samples examined contained significant amounts of sulfur dioxide, both the precision and accuracy of the polarographic determination were evaluated on capsule food starch "spiked" with sulfur dioxide by equilibrating the sample with the gas in a closed vessel. The precision of the method (*ts*) was ±19% at the 95% confidence level for 6 degrees of freedom. In view of this precision, the polarographic average (223 ppm sulfur dioxide) is in good agreement with the value (244 ppm sulfur dioxide) obtained by the USP (3) iodometric titration method.

In the presence of starch, responses obtained upon standard additions of sulfur dioxide were 20% lower than in the absence of starch; calibration curves must be prepared using a sample suspension to account for such matrix effects. If no sulfur dioxide reduction wave is evident and if subsequent standard additions provide increases in current that are less than proportional to the amount added, the presence of oxidizing substances is indicated. With a few pregelatinized starch samples, a wave preceding that of sulfur dioxide also diminishes upon flushing with nitrogen, changing the residual current in the region of the wave; when this is compensated for, excellent recoveries are obtained upon addition of known quantities of sulfite.

In addition to starch, the method has been applied (using conventional dc polarographic equipment[2] or rapidly dropping mercury electrodes) to the determination of sulfite in the presence of menadiol sodium diphosphate, levodopa, canthaxanthin, iproniazid (4), trimethoprim, sulfisoxazole, sulfamethoxazole, influent and effluent water streams, and sulfuryl chloride. Only in the last case was it necessary to modify the procedure by using excess sodium hydroxide to neutralize acids generated as the sample dissolved. In all cases the sample should be acidified just prior to polarography and stirring should be kept to a minimum or the volatility of sulfur dioxide will lead to erratic results even at 10°.

Under the recommended conditions, the response is not significantly changed after 5 min; it decreases only 8% after 20 min with the starch samples, for example.

The recommended method does not require distillation, filtration, or other manipulation resulting in loss of sulfur dioxide. In addition to its relative simplicity, the method requires a minimal quantity of sample due to its high sensitivity, is virtually specific for sulfur dioxide, and is applicable to a wide variety of materials.

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[x] To whom inquiries should be directed.

[1] Princeton Applied Research model 174 polarographic analyzer.

[2] Metrohm Polarecord E261-R.

Microbiological Diffusion Assay I: Operations Studied with Cooper Equation

F. W. KAVANAGH

Abstract □ The Cooper equation relating zone diameter to certain parameters was used to study the influence of variations in technique upon the assay of antibiotics. Variations in prediffusion time, incubation temperature, density of inoculation, composition of medium, and thickness of agar affected zone size. From 80 to 90% of certain effects could be removed from the assay by applying the Food and Drug Administration's correction to the petri dish single-dose assay. The two-dose petri dish design was the least sensitive to variations in parameters. Large plate assay was sensitive to variations in prediffusion times but not to the other parameters. Since these variations can be minimized, large plate methods were less susceptible to operational details than the one-dose petri dish method. Suggestions are given for minimizing errors.

Keyphrases □ Antibiotics—microbiological diffusion assay, influence of variations in prediffusion time, incubation temperature, density of inoculation, composition of medium, and thickness of agar studied using Cooper equation □ Microbiology—diffusion assay of antibiotics, influence of variations in technique studied using Cooper equation □ Diffusion assay—microbiological assay of antibiotics, effects of prediffusion time, incubation temperature, density of inoculation, composition of medium, and thickness of agar on zone size

The theoretical basis of the diffusion assay was established primarily by Cooper and his students beginning with the paper by Cooper and Woodman (1). Other researchers (2–5) published equations more complex but no more useful than that of Cooper. For detailed discussion of the several equations and contributions by the other authors, see the review by Cooper (6) which is the general reference for this paper.

The Cooper equation will be used to show the size of error in an assay caused by variations in the several parameters of the equation. In addition to application to plate assays in the usual sense, the equation will also be applied to tests of sensitivity of bacterial isolates by the disk technique. The assumption will be made that the Cooper equation is applicable to disk, cylinder–plate, and cup (holes in the agar) methods as well as to linear diffusion in tubes (superposition method) for which it was derived. The lessons learned from this study of the Cooper equation as applied to the design of equipment and operating procedures of petri dish assaying will be published later.

Diffusion or plate assays have been the most important method for measuring concentrations of antibiotics for the last 35 years. Most official methods around the world are plate assays. It is the principal method used to assay millions of samples each year. Two petri dish designs, cup–plate methods employing large plates, and the disk plate methods had assumed essentially their present forms by 1945. Several parameters affecting the quality of these assays had been discovered by then. However, there was no unifying theory to guide development of operating procedures. That came later when Cooper and his students published their work which, however, was never applied.

EXPERIMENTAL

Several forms of the Cooper equations will be used. Zone sizes usually will be reported as X^2. The equations will be written using natural logarithms to permit easier computation using small scientific pocket calculators.

For the first part of this study, the simplifying assumption is made that isothermal conditions prevail; this is not true in an actual assay. The Cooper equation is:

$$X^2 = 4D(T_0 + h)(\ln m_0 - \ln m') \qquad \text{(Eq. 1)}$$

where X is the distance diffused from the solution–agar boundary, D is the diffusion coefficient of the antibiotic, T_0 is the critical time, m_0 is concentration of antibiotic in the cup, m' is the critical concentration of antibiotic, and h is the prediffusion time, *i.e.*, time of incubation of plates (after addition of antibiotic) at a temperature too low to permit growth of the test organism (D_h must be for the lower temperature for the time h). Critical time is the time required for a constant number of generations of the test organism to develop from a standard inoculum (7). Since the number of generations is the same for all growth conditions, anything that affects growth rate also affects the critical time period. Lag time may be thought of as a prediffusion time occurring at incubation temperature and is included in T_0. Therefore, the equation may be rewritten as:

$$X^2 = 4DT_0 \ln (m_0/m') + 4D_h \ln (m_0/m') \qquad \text{(Eq. 2)}$$

Prediffusion Time—Calculations will now be made to show the effect of prediffusion upon zone size of a petri dish assay. The situation in assays when done without special precaution may be to fill the standard cylinders 1 or even 2 hr before the last sample is filled into cylinders. The standard and first samples will have a prediffusion time of as much as 2 hr, but the last samples will have a prediffusion time of only a few minutes.

In this example, the constants in Eq. 2 obtained in the study of Cooper and Gillespie (8) were: D_{25} = 0.83 mm²/hr, D_{37} = 1.1 mm²/hr, T_0 = 8 hr, and m' = 1 μg/ml. Therefore, by substituting in Eq. 2, it becomes:

$$X^2 = 35.2 \ln (m_0) + 3.32h \ln (m_0) \qquad \text{(Eq. 3)}$$

In a Food and Drug Administration (FDA) one-dose design, the reference zone on the sample plate is used to correct the sample zone to a standard condition. For this example, the standard curve is taken as 32, 40, 50, 62.5, and 78.1 μg/ml as in an FDA design with the 50-μg/ml standard as the reference concentration. The standard curve is put on first in the assay, followed by the sample plates for the next hour. The examples are the last three sets of samples to show how a prediffusion time of the standard of 1 hr at room temperature affects the measured potency. The first sample is the reference of 50 μg/ml, the second sample is one at 70 μg/ml,

Table I—Influence of Prediffusion Time of 1 hr upon Zone Size in a Streptomycin Assay Using *Staphylococcus aureus*

Concentration, μg/ml	Zone Size, X^2, mm²		Increase in Zone Size, %
	Prediffusion Time		
	0	1 hr	
8	73.2	80.1	9.4
16	97.6	106.8	9.4
32	122.0	133.5	9.4
64	146.4	160.2	9.4
128	170.8	186.9	9.4

and the third one is at 40 μg/ml. Results are listed in Tables I and II.

Temperature—Temperature affects zone size through several mechanisms. The first to be considered is the effect upon the diffusion coefficient, D, and the critical time, T_0. The value of D increases with an increase in temperature whereas T_0 decreases with an increase in temperature. Since T_0 decreases more rapidly than D increases, the product DT_0 decreases with an increase in the temperature of incubation. For a streptomycin assay using *Staphylococcus aureus*, values of DT_0 were computed at 36 and 37° from the data of Cooper and Gillespie (8); DT_0 was 9.40 at 36° and 8.8 at 37°. These values were used to compute the influence of temperature upon zone size and, consequently, upon potency of the samples. Results are listed in Table III.

Thickness of Agar—Thickness of agar is not an element of the Cooper equation, which was derived for linear diffusion in tubes. Of the five equations listed by Cooper (6, Table II), only that of Humphrey and Lightbown (4) has a factor for agar thickness. They show that X^2 (where X is distance diffused) is related to logarithm of agar thickness as was found by the experimental work already mentioned. Since thickness occurs in the equation as the logarithm, zone size is not very sensitive to variations in thickness for layers thicker than 1 mm.

The data of Humphrey and Lightbown (4) for inhibition of *Sarcina lutea* by penicillin were used in their equation to investigate the effect of thickness of the agar layer upon zone size. Distance diffused and zone diameters were computed for agar thickness of 4 mm for the standard plates and of 3 mm for the sample plates. The samples were the two extreme standards. Results are given in Table IV for potencies before and after applying the FDA correction.

Biological Factors—To this point, the influences of three physical factors (prediffusion time, thickness of agar, and incubation temperature) upon zone size have been illustrated. Now the biological factors of lag time, L, generation time, G, inoculum size, N_0, and critical concentration, m', will be considered. To this end, a different form of the equation is used (7):

$$X^2 = [4D \ln (m_0/m')][L + 1.44269G \ln (N'/N_0)] \quad \text{(Eq. 4)}$$

where N' is the critical population that forms the zone edges only at the end of the lag period or after cell division. Prediffusion time will be considered to be zero. The lag, L, is a property of the organism, medium, and treatment of the culture. In the example here at 37°, L = 0.75 hr, D = 1.1, m' = 0.75 μg/ml, G = 0.466 hr, and $N' = 3 \times 10^7$. Rewriting Eq. 4 to incorporate the constants yields:

$$X^2 = [4.4 \ln (m_0/0.75)][0.75 + 0.672 \ln (3 \times 10^7/N_0)] \quad \text{(Eq. 5)}$$

To find the error caused by differences in the size of the inoculum, N_0, a standard curve was constructed using 10×10^3 as the inoculum.

Values of X^2 were computed for m_0 = 50 for different sizes of inoculum, and the apparent potencies were obtained by interpolating from the standard curve (Table V). The proportional change (error) in the apparent potency of a sample was less than the proportional change in the size of the inoculum because of the logarithmic relationship.

DISCUSSION

The Cooper equation has been used to investigate the influence of variations in prediffusion time, incubation temperature, concentration of test organism, growth rate, thickness of agar upon zone size, and, from these, potency of a sample. The most significant factors in influencing petri dish assays are prediffusion time and concentration of bacteria. Both are usually poorly controlled.

Prediffusion Time—Prediffusion of standards causes an increase in the size of the inhibition zone and, consequently, a negative bias in the uncorrected potencies of the sample. As shown in Table I, a 1-hr prediffusion time of standards and not of sample causes a substantial difference in zone size. This difference corresponds to overestimates of potency ranging from 22% at the 8-μg/ml level to about 60% at the 128-μg/ml level in uncorrected assays. A difference in prediffusion time of 4 min caused an error of about 2% in uncorrected potency at 50 μg/ml.

Two classes of errors are caused by differences in prediffusion times in petri dish assays. One is caused by differences of prediffusion times occurring among the containers within a plate; no correction for these errors is practical. The other is caused by differences in prediffusion times between plates; the FDA correction is applicable to these.

Differences in prediffusion times within the sets of standard plates can be a source of unrecognized and uncorrectable error. The usual practice in petri dish assays is to fill all cylinders holding a particular concentration of standard and then to fill the set containing the next concentration. Filling the cylinders may require considerable time and during this time the drug diffuses from the cylinders. Thus, prediffusion time is different for each concentration of standard on the set of standard plates. A time as short as 5 min between filling of successive concentrations of standards into the cylinders on a plate can cause a measurable error. The error is maximum if the order of filling sets of cylinders is such that the highest concentration is filled first and minimum if it is filled last. The FDA correction is not applicable to errors caused by within-plate differences in prediffusion times. The errors can be reduced to insignificance by working rapidly. The 18 cylinders in a three-dish set can be filled in about 1 min, which is a time too short to cause appreciable errors.

The FDA procedure (9) corrects the sample zones on a plate for the deviation of the reference zones on the sample plate from the reference zones of the standards. This correction can be expected to be only a first approximation because the basic assumption is made that any environmental or operational cause of change in zone size does not affect the slope of the dose–response line. Consideration of the Cooper equations reveals this assumption to be incorrect (10). Nonetheless, the FDA correction is a good first approximation as inspection of Table II reveals. Under the worst conditions (the highest potency), the FDA correction reduced by 90% the error caused by prediffusion.

Table II—Effect of Prediffusion and FDA Correction upon Accuracy of a Streptomycin Assay

Concentration of Standard, μg/ml	X^2, mm²	Not Corrected		FDA Correction			
		Potency	Error, %	Reference Zone	Corrected Sample	Potency	Error, %
32	133.5						
40	142.1						
50	150.7						
62.5	159.3						
78.1	167.8						
Sample 1	137.7	35.68	−28.6	137.7	150.7	50.0	0
Sample 2	149.5	48.47	−30.7	137.7	162.5	67.94	−2.9
Sample 3	129.8	29.1	−27.25	137.7	142.8	40.78	+1.95

Table III—Effect of Temperature upon Zone Size by Influencing Values of D and T_0 in the Cooper Equations

Concentration, μg/ml	X^2, mm²		37° Standard			
	Temperature				FDA Correction Applied	
	37°	36°	Potency at 36°	Error, %	Potency	Error, %
32	130.3	122.0	—	—	—	—
40	138.7	129.8	51.6	+29	39.38	−1.55
50	147.1	137.7	65.2	+30.4	50	0
62.5	155.5	145.6	83	+32.8	63.47	+1.55
78.1	163.8	153.4	—	—	80.34	+2.88

The FDA correction is effective only when the reference standard and sample are filled into cylinders of a plate at the same time. This means that the prediffusion times of the solutions in the six cylinders (or disks) on a plate must be identical which, of course, is not true in the usual cup or cylinder assay. It can be achieved in a disk method. The disks containing the samples are placed on glass plate and all are transferred to the agar at the same time. Both petri dish and large plate designs were described (11).

The principal factor affecting large plate assays is prediffusion time, which is different for each cup. However, if the filling of the cups proceeds at a constant rate, as Simpson (12) insisted it must to achieve the highest precision, the total prediffusion times are the same for all test solutions in a balanced Latin square or quasi-Latin square design and assure lack of bias from this source only if each row of cups is filled in sequence always starting from the same side of the array. Prediffusion causes error in completely random designs and no correction is possible. Such designs generally are considered to be of low precision. The other operations that can have such large effect in petri dish assays have minimal effect in large plate methods. This is one of their advantages.

Temperature—Results in Table III are given for both the uncorrected potencies and for potencies after FDA correction. Even a difference in incubation temperature of only 1° between standards and samples caused large error (~30%). The FDA correction reduced the error to less than 3%, depending upon the potency of the sample. This example shows the importance of having uniform incubation conditions for assays. Temperature differences of only 0.1° from one part of the plate to another would cause appreciable error in large plate assays unless the gradient was linear and the design was a balanced Latin square.

The usual practice was to place the large plate on a shelf in the incubator as soon as the last hole was filled or the last disk was put in place. The plates were not stacked in the incubator. Since Latin square designs usually were employed, temperature gradients (they should have been small) would not affect the assays significantly unless there was a strong nonlinear component from one part of the plate to another.

The situation with petri dish assays was quite different in an unfavorable manner. Usually the dishes were stacked in layers of three or five high. Quite often they were handled in wooden boxes. The numbers were so large that an assay could occupy a considerable volume in the incubator and, thus, be subjected to large temperature gradients. Each plate within a stack had a different temperature history and, consequently, different zone sizes for the same sample (13).

The general rule is that lowering the incubation temperature increases the zone size. In sensitivity testing by the Bauer–Kirby technique, substantial departure from the standard temperature of 35° could cause misclassification of a culture.

The Cooper equation represents the complete dose–response line. Usually only a small portion of the line is ever presented. To illustrate the effect of changing the parameters upon the line, computed dose–response lines for the streptomycin–*S. aureus* system are given at two incubation temperatures for a sensitive strain and at one temperature for a resistant strain. The data were taken from Cooper *et al.* (14). The assigned diameter of the cups was 8 mm. The results of the computations are given in Fig. 1 as zone diameter (not square of the distance diffused) *versus* concentration.

The dose–response lines show the influence of incubation temperature and sensitivity, m', of the test organism upon zone size. The line obtained at 30° has the steeper slope because of the larger DT_0. The difference between the two lines for the sensitive strain is caused solely by the difference in slope, DT_0, because m' is independent of temperature. The difference between the lines at 40° is caused by the different values of m'; DT_0 was the same for the two strains.

If the square of distance diffused, X^2, were plotted instead of zone diameter, the lines would be straight. The two lines for the sensitive strain would have different slopes because of the different values of DT_0. The two lines at 40° would be parallel because the slope is determined by physicochemical factors that are independent of sensitivity of the test organism. Decreasing susceptibility of the organism moves the line toward the right in Fig. 1. Note that the lines are not straight.

Thickness of Agar—As shown by the results in Table IV, the influence of variation in thickness upon potency is much smaller than the percentage change in thickness. Therefore, nonuniform thickness of the agar should be of minor significance because total thickness is easily controlled with the required precision.

A plot of zone diameter *versus* log penicillin concentration gave slightly curved lines that were not parallel. The FDA correction applied to such is not perfect (Table IV). The residual errors are caused by use of inappropriate, although customary, dose–response lines. The uncorrected answers had a large error (~33%) in agreement with the observations of Hayes (15). These results show the utility of the FDA correction for reducing the effect of this operational defect.

In large plate assays, the agar should be of uniform thickness because compensation and correction are not possible. However, a small gradient of thickness from one side to another probably would be compensated for in a balanced Latin square or quasi-Latin square design.

A source of error more serious than variations in total thickness of the agar is variations in thickness of the inoculated layer of two-layer plates. Usually the lower layer is 20 ml of uninoculated agar and the top layer is 4–6 ml of inoculated agar. Four milliliters of agar forms a layer 0.67 mm thick in a 90-mm dish. Fortunately for the analyst, the top layer unlike the lower layer does not assume a surface determined solely by gravity. The agar in the thin top layer begins to harden as soon as applied to the base layer and, consequently, thickness is determined by forces in addition to gravitational. A small variation in thickness of the inoculated layer can

Table IV—Influence of Agar Thickness upon Potency in a *Sarcina lutea* Penicillin Assay[a]

Concentration, Units/ml	Standard d = 4 mm	Sample d = 3 mm	Potency		Error, %	
			Uncorrected	FDA Correction	Uncorrected	FDA Correction
0.1	16.38	16.97	0.1330	0.1046	+33	+4.6
0.2	17.76	18.29	—	—	—	—
0.4	19.01	19.50	0.534	0.400	+33.5	0
0.8	20.17	20.63	—	—	—	—
1.6	21.24	21.68	2.135	1.537	+33.4	−6.2

[a] Reference concentration is 0.4 unit/ml.

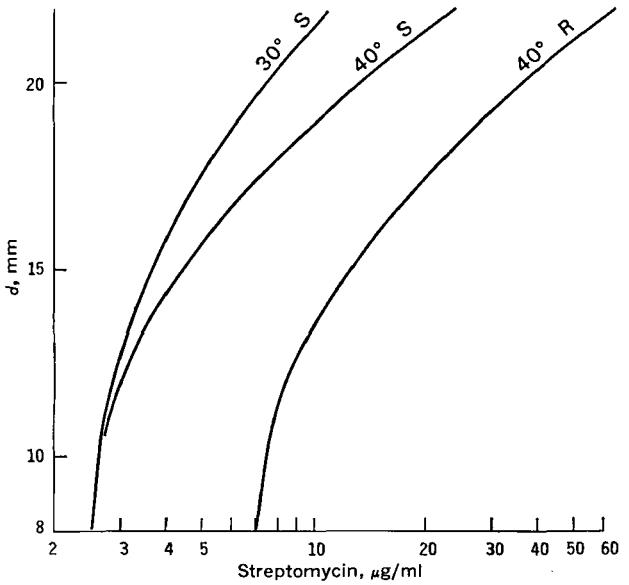

Figure 1—*Diameters of zones of inhibition by streptomycin of* Staphylococcus aureus *at 30 and 40°. The sensitive strain is S* (m′ = *2.5 μg/ml), and the more resistant strain is R* (m′ = *7 μg/ml). The diameters,* d, *were obtained from 2X + 8, where 8 was the inside diameter of the cup.*

cause a significant error because thinning of the layer, in effect, also reduces the concentration of the test organism. Errors caused by uneven top layer would not be corrected by application of the FDA procedure. Such error can only be avoided.

Biological Factors—The generation time, G, is a function of temperature, composition of medium, and inherent properties of the test organism. Therefore, composition of medium should be the same in all bottles of a multibottle assay. Changing the value of G changes the slope of the dose–response line. Therefore, the FDA correction would be incomplete. An increase in G of only 0.1 min would increase the uncorrected potency at the 50-μg/ml concentration of Table V by 1%. Vitamins, amino acids, minerals, and other growth substances present in a sample and not in the standards may increase the growth rate (decrease in value of G) of the test organism and, thereby, reduce the size of the zone. This kind of interference is well known to those who must assay low potency samples of natural origin such as those encountered in residue testing.

The critical concentration, m', of the antibiotic was independent of incubation temperature but not of medium composition for inhibition of *S. aureus* by streptomycin (6). Salts increase values of m' for inhibition by streptomycin, thus accounting for the well-known influence of salts upon streptomycin assays. The value of m' was greater for a resistant strain of *S. aureus*.

Changes in the value of m' will change the zone size but not the slope of the dose–response line. In the previous example, an increase of m' of 0.05–0.80 μg/ml caused a decrease in zone size equivalent to a decrease in potency of 6.25%.

A common practice is to use petri plates prepared from several bottles of agar. Each bottle is inoculated with *approximately* the same volume of inoculum. Results in Table V indicate that the inoculum must be identical if substantial error is to be avoided. However, if the assay is large plate or is one to which the FDA correction can be applied, the error caused by variation in inoculum can be reduced greatly. One advantage of the large plate method is immediately obvious; variation in inoculum cannot occur (if the inoculum is properly mixed with the agar) because the plate is poured from only one bottle.

Table V—Influence of Concentration, N_0 in the Cooper Equation, of Inoculum upon Computed Zone Size

Inoculum, $N_0 \times 10^{-3}$	X^2, mm²		Sample of 78.1 μg/ml			
	m_0		Uncorrected		FDA Correction	
	50	78.1	Potency	Error, %	Potency	Error, %
8	116.85	129.26	88	+12.5	78.96	+1.1
10	114.08	126.19	78.1	0	78.1	0
12	111.82	123.69	71.5	−8.45	77.41	−0.8

A 10-fold change in quantity of inoculum could cause a 3-mm change in zone diameter. The plates with the smaller inoculum would have the larger zones. This much change in zone size in a sensitivity test could move an organism from susceptible to resistant or vice versa as Linton (7) suggested. The example was for streptomycin inhibiting *S. aureus*. Other antibiotic–organism systems may be more sensitive to the size of the inoculum.

The first diffusion assays were done with agar inoculated on the surface. Surface inoculation was certain to produce large plate-to-plate and, perhaps, even within-plate variations in surface density of organisms. The importance of uniform inoculation (Table V) was not recognized at first. Surface inoculation was abandoned early by analysts (16). Ericsson (17), however, was still using surface inoculation in 1960 and it is used in sensitivity testing. All precise diffusion assays are now done with bulk-inoculated agar following the technique introduced by Foster and Woodruff (16).

In certain kinds of assays, of which testing for residual antibiotic in animal tissues is an example, the assays are operating at the limit of sensitivity. The day-to-day variation in responses may be large as in the examples of Kline and Rathmacher (18). Maximum sensitivity is sought in these assays; consequently, the assays are very sensitive to operating conditions. Every factor in Eq. 4 must be carefully controlled to produce consistent daily sensitivity. Here, the problem is not so much accuracy as it is the difficulty in producing a detectable zone reliably over several days.

Testing of clinical isolates of bacteria for susceptibility to antibiotics usually is done by procedures not suitable for quantitative work. The method of inoculating is certain to cause considerable plate-to-plate variation. However, this variation usually is not important because categorization of the organism usually is based upon the results from only one plate. Correlation of zone sizes with minimum inhibitory concentration (MIC) but, more importantly, with clinical efficacy is what is significant when using the test in hospitals. The results of such tests are only a guide to therapy and not an indication of the certainty of successful treatment. Nonetheless, attention to test details can increase reliability of the method.

CONCLUSIONS

The influence of operations upon four types of assays have been considered. They are the original single-dose petri dish assay, the single-dose petri dish assay incorporating the FDA correction, the two-dose petri dish assay of FDA, and the large plate assay. The single-dose assay is the most susceptible to variations in operations and the two-dose method is the least. Errors could be large in the single-dose method without the FDA correction. A two-dose assay is particularly attractive because each dish is a complete assay that can be quickly set up. The Latin square large plate design can be precise if filling is at a constant rate. A disk method can avoid the prediffusion problem. This study of the design of diffusion assays shows the advantages of the large plate method over the usual petri dish ones.

Nonuniform incubation temperature, agar thickness, and inoculum were potent sources of error. Uncorrectable errors were caused by differences in prediffusion times between reference standards and samples in all petri dish assays. Certain designs of tests and procedures will minimize errors caused by the inevitable variations in operations. These errors cannot be eliminated from petri dish assays but they can be reduced to insignificance. Procedures known to reduce errors are: *(a)* incubating the plates in one layer in an incubator free from temperature gradients, *(b)* preparing all plates used in a test from one bottle of inoculated agar, *(c)* pouring the agar into plates sitting on a truly level surface, and *(d)* filling all cups on a plate in 1 min or less. These conclusions concerning procedures needed to produce accurate assays were obtained from a quantitative study of operation using the Cooper equation.

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Automated System for Analytical Microbiology IV: Accuracy of Measurements

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Abstract □ The automated parts of a system designed for performing microbiological turbidimetric assays were tested for accuracy and precision of photometric and volumetric measurements. The system was tested with potassium ferricyanide solutions. The photometric measurements were accumulated and processed by an on-line computer. They had an average standard deviation of about 0.020. The diluter apparently could contribute an additional small variance to the measurements. The answers had a slight positive bias. Uncorrected multiple reflections in the cell can cause significant errors. The use of a multipoint standard curve makes it unnecessary to correct for multiple reflections in the cell in high precision measurements. Digitizing errors were very small.

Keyphrases □ Automated system—analytical microbiology, studies on precision and accuracy of photometric and volumetric measurements □ Microbiology, automated analytical—determination of accuracy of photometric and volumetric measurements □ Reproducibility—automated system for analytical microbiology, precision and accuracy of photometric and volumetric measurements evaluated

A system[1] for performing turbidimetric microbiological assays was described in detail previously (1, 2). Application of computer technology to the system was shown to improve precision and accuracy by reducing computational errors (3).

The system consists of a diluter, an incubation bath, and a reader. The diluter prepares dilutions of 66-fold and 100-fold in pairs and delivers the diluted sample to an array of test tubes in a carrier. The reader causes solution from the test tubes to flow through a fixed cell in a spectrophotometer and records percentage transmittance of the flowing solutions. The accuracy and precision of the measurements performed by the system are the results of the accuracy and precision of the measurements made by the two modules. The errors and variances were small because the philosophy governing the design was to reduce the electromechanical variations to such an extent that they would not contribute a significant variance to the microbiological assays. The goal was a maximum variation of 0.1% in dilutions or transmittances. Experience with routine measurements of dye solutions indicated probable achievement of the goals. It was also recognized that the accuracies and precisions reported were not necessarily the best values because of the limited resolution of the 3-digit voltmeter used with the spectrophotometer.

Improvements accompanying installation of an on-line computer made an evaluation of the system's accuracy and precision worthwhile. The system was tested by processing colored solutions. No assay, not even a high precision one, was suitable because of the introduction of variances external to the system being tested by the microbiological portion of an assay.

EXPERIMENTAL

The purpose was to test the accuracy and precision of the volumetric and photometric measurements of the system. Test solutions were prepared from solutions of potassium ferricyanide dissolved in pH 7 phosphate buffer. These solutions were used because they were shown (4) to follow Beer's law with requisite accuracy (1 part in 10,000). Measurements of transmittance were made at 415 nm by one of two spectrophotometers[2].

Two classes of test solutions were prepared. One was used to test photometric accuracy and the other to test the diluter as well as the photometer. The first class was prepared by diluting a careful-

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Table I—*Test of Photometric Module*[a]

Sample Number	Sample, % T		Concentration		Standard Deviation × 1000	Mean Responses
1	100	100	0			
	100	100				
2	25.64	25.64	60			
	25.64	25.64				
3	25.59	25.66	60.086	59.966	51	60.013
	25.64	25.64	60.000	60.000		
4	25.64	25.61	60.000	60.052	30	60.026
	25.61	25.64	60.052	60.000		
5	25.64	25.64	60.000	60.000	17	59.991
	25.66	25.64	59.966	60.000		
6	25.64	25.63	60.000	60.017	32	60.021
	25.64	25.60	60.000	60.069		
7	25.63	25.65	60.017	59.983	24	59.983
	25.66	25.66	59.966	59.966		
8	25.66	25.63	59.966	60.017	29	60.009
	25.63	25.62	60.017	60.034		
9	25.63	25.61	60.017	60.052	20	60.034
	25.61	25.63	60.052	60.017		
10	25.63	25.63	60.017	60.017	0	60.017
	25.63	25.63	60.017	60.017		

[a] The first two samples are standards. Samples 3 to 10 are the same solution as Sample 2. Each measurement is from a different tube in the carrier.

ly weighed portion of a stock solution and diluting in a 2-liter volumetric flask. Results of automated photometric measurements of the solution are given in Table I. These measurements tested stability of flow of liquid through the cell, the spectrophotometer, the signal conditioning amplifier and noise filter, the analog–digital converter, and the computer.

The second class of solutions was a series of samples of approximate concentrations ranging from 10 to 60 m*M* processed through the diluter. Relative concentrations of the samples were known to better than 1 part in 10,000. These measurements (Table II), simulating an antibiotic assay, test the entire measuring system.

The system has four channels, and results from each were treated separately, thereby avoiding all assumptions concerning identity of pairs of channels and ratios between the two sets of channels.

The solutions are listed in the tables in the order of measurement. The two values in the column headed 0.10 ml were measured before the two in the column headed 0.15 ml. The order of measurement was down the column. Precision of the set of four measurements on a sample was indicated by the standard deviations.

The dose–response lines (calibration lines) were point to point as absorbance *versus* concentration. The computer calculated four calibration lines for each test, one for each channel. Thus, four responses were obtained for each sample. Even when the colored solutions (Table I) were not processed through the diluter, they were kept in sets of four because the computer was programmed to handle data in such sets and in no other.

A detailed description of the system was given previously (2). Output of the spectrophotometer as % T was recorded to 3 digits on paper tape. A 1-digit uncertainty was placed in the least-significant (last) digit by the inherent uncertainty of the digital voltmeter. When acquisition and processing of the data by an on-line computer were instituted, resolution of voltage measurement was improved fourfold. As shown here, accuracy and precision were also improved. Output from the spectrophotometer (1 v = 100% T) was conditioned by an amplifier and amplified about 9.5-fold. The amplified and conditioned signal was applied to a 12-bit analog–digital converter and then to the computer for further processing. The computer[3] was programmed to sample the output of the spectrophotometer eight times in 200 μsec, to average the readings, and to store the average. When the data were converted to decimal form for printing, there was some rounding-off during conversion and rounding of potencies to 3 digits to the right of the decimal point of the output format. The analog-digital converter was calibrated at zero and 100% T before each day's measurements. The 100% T reading was equal to about 3400 bits.

The solutions were at the same temperature during measurement. This is important in high precision measurements because absorbance of dilute aqueous solutions will change by about 1 part in 4000 for each degree change in temperature (5) as a result of change in volume of the solution.

RESULTS AND DISCUSSION

The test reported in Table I was designed to measure photometric accuracy and precision. The tubes in the carrier were filled with portions of a 0.6 m*M* solution of potassium ferricyanide. To obtain a 5-digit answer, the standard was assigned a value of 60. One set of the measurements was selected to be the standard. The mean of all of the measurements was 60.019 with a standard deviation of 0.030. The range of individual measurements was from 59.966 to 60.052. These data also showed that any set of measurements (mean response of four individuals) was as good as any other. This is as it should be in a well-designed automated system. These measurements showed that a relatively inexpensive spectrophotometer and a fixed flow cell were capable of photometric accuracy and precision adequate for most analytical purposes.

In the real world of photometric analysis, much more than photometric accuracy is involved in determining the accuracy and precision of an analytical procedure. In this system, dilutions of samples and standards to assay concentration are performed mechanically by the diluter module. The module may be expected to contribute bias and variance to measurements of concentrations of samples. Experience indicated that only small errors were caused by leaking of valves or syringes of a properly maintained diluter. To find the extent of errors and variances of the system (reader and diluter) was the purpose of the experiment reported in Table II. Three samples, processed through the diluter and reader, were measured in terms of a 4-point standard curve. The samples were intermediate in concentration between the bracketing standards. Potency of a sample was obtained by interpolation from the straight line connecting the bracketing standards. The assumption of linearity applied only to the short segment of the calibration line used in obtaining the potency of the sample.

The precision of the four diluting channels was obtained from the data in Table II. The 0.10-ml channels were somewhat more variable than the 0.15-ml channels. The odd-numbered channels were slightly less variable than the even-numbered channels. One syringe of the dual-nozzle filler delivered diluent to the odd-numbered channels, and the other syringe delivered diluent to the even-numbered channels[4]. Since each syringe delivered diluent for both a 0.10- and a 0.15-ml measuring loop, the variance was caused

[3] Hewlett-Packard model 2100A.

[4] Diluent was delivered by a modified model DAB dual-nozzle filler, National Instrument Co., Inc.

Table II—Test of Diluter and Reader Modules as a System[a]

Sample Number	Sample Volume, ml		Concentration		Standard Deviation × 1000	Mean Responses
	0.10	0.15				
1	100.71	100.59	0			
	100.66	100.63				
2	63.36	49.78	20			
	63.05	49.62				
3	39.68	24.70	40			
	39.38	24.64				
4	25.00	12.36	60			
	24.78	12.39				
5	31.37	17.41	50.174	50.104	45	50.168
	31.10	17.35	50.192	50.205		
6	31.39	17.41	50.146	50.104	38	50.102
	31.20	17.41	50.053	50.104		
7	31.47	17.46	50.036	50.021	18	50.031
	31.23	17.44	50.012	50.054		
8	31.42	17.44	50.105	50.054	25	50.066
	31.20	17.44	50.053	50.054		
9	31.45	17.44	50.063	50.054	25	50.055
	31.18	17.46	50.081	50.021		
10	31.45	17.41	50.063	50.104	47	50.121
	31.12	17.38	50.164	50.154		
11	49.82	34.87	30.275	30.159	76	30.168
	49.66	34.85	30.144	30.095		
12	49.91	34.88	30.197	30.151	42	30.145
	49.67	34.85	30.136	30.095		
13	49.91	34.90	30.197	30.135	45	30.134
	49.70	34.85	30.110	30.095		
14	49.96	34.95	30.155	30.094	63	30.091
	49.70	34.96	30.110	30.005		
15	50.02	34.88	30.103	30.151	30	30.108
	49.73	34.85	30.084	30.095		
16	49.91	34.90	30.197	30.135	47	30.132
	49.70	34.86	30.110	30.087		
17	79.67	70.59	10.114	10.070	35	10.069
	79.55	70.59	10.062	10.029		
18	79.69	70.59	10.103	10.070	27	10.071
	79.53	70.57	10.073	10.037		
19	79.64	70.65	10.130	10.045	39	10.074
	79.53	70.54	10.073	10.049		

[a] The first four samples are standards. Percentage transmittances are given for sample volumes of 0.10 and 0.15 ml.

by the syringe and not by the loops and their attendant valves. Other data indicated that the 0.1-ml channels were slightly less variable than the 0.15-ml channels. When there are no mechanical difficulties with the measuring loops and their valves, variances should be the same. The sudden appearance of obviously erratic measurement in one channel is diagnostic of a mechanical problem associated with the measuring loop. Aberrant measurements associated with a pair of channels indicate difficulty with a syringe of the dual-nozzle filler.

A scan of the mean responses in Table II reveals any measurement of a sample to be as good as any other, the standard deviation to be independent of sample mean, and the bias to be essentially independent of sample. The reason for the biases is unknown.

A rough idea of the contribution of the dilution step to system variance may be obtained by comparing the pooled variances (0.024) of the "60" samples of Table I with the pooled variances of the "50" samples of Table II. The diluter probably is less variable than the reader. This conclusion is not unexpected because there are only four mechanical sources of variance in the diluter whereas there are at least seven electromechanical sources of variance in the reader.

Certain operational details peculiar to this automated system degrade accuracy slightly. One is the slight interaction between successive solutions differing in concentration. Such interaction is caused by the impossibility of completely removing all of the previous sample from the cell by a limited amount of succeeding solution. About 8 ml of solution is used to displace 0.25 ml of the previous sample from the cell by bubble-free, streamline flow. This error resides in the reader. An example of the interaction of successive different concentrations is the first measurement of Sample 11 in Table II. This sample also had the largest standard deviation of the six samples of the "30" concentration. This kind of interaction also occurred in standards. Such bias in the standard caused a corresponding bias in potency of samples for the first measurement of the 0.10-ml pair. Had carryover in the sampling system contributed to the interaction, both 0.10-ml samples would have given answers different from the 0.15-ml samples and from the 0.10-ml sample of succeeding identical solutions. Any cross-contamination from the sampling system caused errors too small to be detected.

The spectrophotometers that formed part of the reader modules achieved stability by being operated at constant voltage. This procedure was considered by Pardue and Rodriguez (6) to be inferior to optical feedback as a means of achieving stability. They reported repeatability of measurements made over 1 hr to be within 0.01% T for their manually operated instrument. The readings in Table I, obtained in about 6 min, had a mean of 25.633% T with a standard deviation of 0.018. Twelve readings differed from the mean by more than 0.01% T, indicating that this system was a little less precise than the spectrophotometer of Pardue and Rodriguez. The instruments developed by Pardue and coworkers (6, 7) probably are more accurate and precise than the system discussed here, but none of them was designed to measure samples at 10-sec intervals and to do it automatically.

A recent commercial instrument[5] and several manually operated ones designed 40 years ago were equal or slightly superior to the

[5] Cary 118 spectrophotometer, Varian Instrument Division.

Table III—Influence of Digitizing Errors upon Concentration of Samples when the Standard is 60

Concentration of Sample	Change in Absolute Value of Sample Caused by a +1-Bit Change in Measurement of		
	Blank	Sample	Standard
10		−0.016	
20	+0.012	−0.020	+0.017
30		−0.025	
50		−0.041	
60	+0.013	−0.052	+0.052

module used in this work. Both of the early instruments were null balancing types in which the photocells were used only to indicate a null condition and transmittance was read from a rotating sector. Follett (8) reported his measurements to four places. Kortüm (4) recorded transmittances to five places. Variations in measurements of his most favorable samples, gray glass and 2,4-dinitrophenol solutions, occurred mostly in the fifth place.

Mavrodineanu (9) gave details of an elaborate, high accuracy, single-beam spectrophotometer which spanned the spectrum from 275 to 800 nm. Provision was made for acquisition and processing of data by computer. Readings of the photomultiplier current were for 5 sec at the rate of 10/sec. Mean, standard deviation, and relative standard deviation of the 50 readings were printed as the data point for each determination. Samples were glass filters. Transmission of the air path was measured, then the filter, and then the air again. Transmittance of the filter was computed using the mean of the two air measurements. Two successive measurements of air differed by from 1 part in 4000 to 1 in 100,000. The precision of measurement of the glass filters was higher than the precision of measurement of solutions flowing through a cell as reported here.

Burke *et al.* (10) gave an excellent account of the many, and serious, problems associated with selection of substances to be used as light absorbance standards. They discussed chromates, cobalt and nickel salts, phthalate, certain dyes, and the liquid absorbance standard (SRM 931) to be issued by the National Bureau of Standards.

Östling (11) described the application of a discrete analyzer[6], designed for clinical analyses, to colorimetric water analysis. The photometer permitted "measurements with a precision of ±0.001 *A* up to 1.5 *A* with a drift of about 0.001 *A* per hour." The sample was sucked into the cell, measured, returned to the tube, and emptied by a puff of air. Carryover from one aqueous sample to the succeeding one varied between 0.1 and 2%, depending upon the size and construction of the cell.

Efforts to compare the photometric accuracy and precision of the system described here with those in the literature have been frustrating because of the inadequacy of published information. Such statements as "reproducibility of 0.001 *A* at 0.4 *A*" (12) are not exact enough. Usually the mean of some unknown number of measurements is given. A new instrument may be described without any indication of either accuracy or precision. Generally the indications of accuracy and precision of spectrophotometry are imprecisely defined and inadequate in quantity. The ambiguities and imprecision of statistical treatment would be removed by giving original measurements as is done here or by following the admonitions of Eisenhart (13).

The amount of stray light in the spectrophotometer at 415 nm was unknown. Stray light will cause a material not to follow Beer's law, with deviations increasing with the absorbance of the solutions (10). For example, stray light of 1% of that transmitted by a solution of 0.1 absorbance would decrease measured absorbance by 1.1%. That amount of stray light would cause an increase in the measured concentration of the 10 and 30 samples in Table II by about 0.1%. This result illustrates again the advantages of a point-to-point calibration line.

Theoretical treatment is needed of the sources and sizes of errors in a single-beam spectrophotometer containing a fixed cell (flow cell) when it is used as part of an automated data-gathering system. Drift over the extended measuring time and the relatively small number and infrequent measurements of the blank and other calibration points are significant differences from the manual procedures that have been so extensively studied during the last 20 years.

Multiple Reflection in Cell—Light passing through the cell is reflected at each interface. Some light passes through the solution more than once. The result of multiple reflections is an apparent increase in absorbance of the solution in the cell. Size of the increase is a function of absorbance and the relative indexes of refraction of solution and material of the cell faces. Both are functions of wavelength. Multiple reflections cause a high bias in the potency of samples lower in concentration than the standard. The bias decreases with an increase in concentration of the sample. If a single calibrating standard is used, as is the practice when absorbance of the solution follows Beer's law, all samples can have a bias significant in high precision assays. However, if multiple standards are employed as in Table II, the relative error in the sample is small because the sample is measured in terms of two standards bracketing the sample and not of a single standard far removed in concentration.

The table of Burnett (14) was used to calculate errors in samples for the two conditions. If a single standard of 60 is employed and no corrections are made for multiple reflections, the errors in samples of concentrations 10, 30, and 50 would be +0.135, +0.060, and +0.015%, respectively. When the calibration is multipoint, as in Table II, the errors caused by uncorrected multiple reflections become +0.044% for the 10 and +0.01% for the 50 concentrations. These results show that a multiple-point calibration line can greatly reduce the errors caused by uncorrected multiple reflections in the cell.

Digitizing Errors—The output voltage of the spectrophotometer had to be translated into a language understood by the computer. The analog signal from the spectrophotometer was transformed into binary numerical representation by an analog–digital converter. The numbers obtained from the analog–digital converter were linearly related to the voltage from the spectrophotometer and, therefore, to percent transmittance. The 12-bit analog–digital converter provided 4095 discrete values from its full-scale input. However, in this application, about 3400–3600 bits represented 100% *T*. Therefore, resolution of the spectrophotometer was 1 in 3400.

Two kinds of digitizing errors occur. One is the inherent 1-bit uncertainty of the digitizing process. The other is that of unknown size and frequency caused by noise and drift in the analog–digital converter. The random 1-bit uncertainty occurs in all digitized numbers and is independent of the size. Maximum deviation in a reading caused by inherent uncertainty of the digitizing process would be 2-bits: one from the sample and one from the standard of opposite sign. Digitizing error also could be zero; it is a chance event.

Influences of a 1-bit error on the computer concentration of samples are given in Table III using data from Table II. The influence of a fixed digitizing error depended upon whether it occurred in the blank, the standard, or the sample as well as upon the relative concentrations of the standard and the sample. The percentage error decreased somewhat with an increase in the concentration of the sample.

The 60 set of values in Table I changed by multiples of 0.017, a number one-third of that caused by a 1-bit change in the digitized value of transmittance. This small number showed that averaging the eight readings reduced the variation; otherwise, the concentrations would have changed by steps of 0.052. Only one of the 32 concentrations differed from 60 by more than 0.052.

The measuring system would be improved by using a more stable and less noisy signal conditioning amplifier and a 13-bit analog–digital converter. The increased stability and resolution would be of marginal value in most microbiological assays performed with the system.

[6] Autolab system, Linson Instrument AB, Stockholm, Sweden.

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NOTES

Amphetamine Derivatives: 10(e)- and 10(a)-Amino-1,2,3,4,4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrene

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Keyphrases □ 10(e)- and 10(a)-Amino-1,2,3,4,4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrene—synthesized and evaluated for amphetamine behavioral and hyperthermia effects □ Amphetamine derivatives—synthesis of 10(e)- and 10(a)-amino-1,2,3,4,4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrene, evaluated for amphetamine behavioral and hyperthermia effects

Conformationally rigid analogs have been used to study drug receptor systems, but few studies have been reported (1–5) with respect to amphetamines. In the cyclopropane series, the *trans*-isomer shows equivalent peripheral amphetamine-like effects, *e.g.*, pressor response, moderate central nervous system stimulation, and increased monoamine oxidase inhibition when compared to amphetamine. Some differences were noted in other rigid analogs, although only changes in motor activity and LD_{50} data have been reported (3, 4). The norephedrine analogs in the octahydrophenanthrene system were reported previously (6), in which a potentiation and blockade of the effects of norepinephrine were noted; a logical extension of this work was the preparation of these analogous amphetamine derivatives.

DISCUSSION

In a related study, the isomeric 9-hydroxy-10-amino-1,2,3,4,-4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrenes were prepared (6). The alcohols[1] III and IV were subjected to hydrogenolysis conditions. The conversion of III-HCl occurred smoothly using palladium-on-carbon in methanol containing hydrochloric acid. Similar results were obtained in a related tricyclic system (7). Attempted hydrogenolysis of the IV-HCl under these conditions failed. More rigorous conditions, acetic acid–hydrochloric acid, also failed. However, when using acetic acid–perchloric acid, hydrogenolysis did occur.

The mass spectra of 10(e)- and 10(a)-amino-1,2,3,4,4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrene, I and II, respectively, showed parent peaks at *m/e* 201.1494 and 201.1502, respectively (calculated 201.1518). The NMR spectra were not useful in assigning the relative stereochemistry because of similar chemical shifts of several protons in each spectrum. The small quantities of compound precluded preparation of suitable derivatives. When using a TLC developing system of ether–methanol–aqueous ammonia (90:8:2) and silica gel plates, R_f values of 0.46 and 0.28 were found for I and II, respectively, demonstrating that the potential hazard of dehydration to the intermediate enamine is not a likely process during the hydrogenolysis step.

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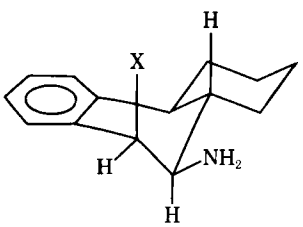

I: X = H
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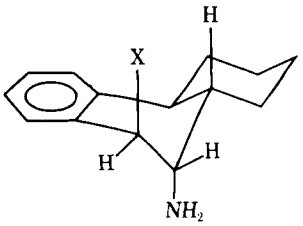

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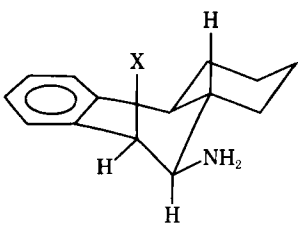

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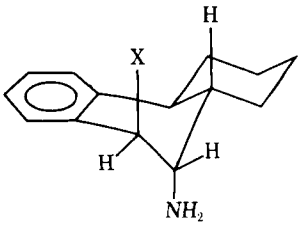

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[1] All materials are racemic, although only a single isomer is shown.

increased motor activity, piloerection, and salivation (3, 4, 8), were not observed when I and II were administered intraperitoneally to mice. Compound I produced decreased motor activity and sedation at doses of 50–100 mg/kg. Compound II produced slight tremors at 25–50 mg/kg and marked tremors and ataxia at 80–100 mg/kg. Significant hypothermia was also noted with the larger doses. No amphetamine-like hyperthermia (9) was noted in rabbits.

From these results, it was concluded that neither compound produces amphetamine-like effects. The pharmacology of II is under further investigation.

EXPERIMENTAL[2]

10(e)-Amino-1,2,3,4,4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrene Hydrochloride (I-HCl)—A mixture of 195 mg (0.9 mmole) 9(a)-hydroxy-10(e)-amino-1,2,3,4,4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrene (III) (6), 90 mg palladium-on-carbon (10%), and 0.7 ml of concentrated aqueous hydrochloric acid in 35 ml absolute methanol was reduced at an initial hydrogen pressure of 30 psi. After 24 hr the catalyst was removed by filtration, the solvent was evaporated, and the residue was crystallized from ether and recrystallized from isopropanol to give I-HCl, 185 mg (86% of theory), mp 290–300° dec.; IR (KBr): 3430 (NH), 2950 broad (CH), 1590, 1490, 1445, 770, and 735 cm^{-1}; NMR (dimethyl sulfoxide-d_6): δ 8.47 (s, broad, 3, $^+NH_3$), 7.37–7.07 (m, 4, ArH), 3.33–3.00 (m, 3, H_9, $H_{9'}$, H_{10}), 2.60–2.27 (m, 2, $H_{10a,4a}$), and 2.13–0.97 (m, 8, CH_2—CH envelope), addition of D_2O removed the absorption at 8.47, replaced by HOD at δ 3.65; mass spectrum (70 ev): *m/e* (relative intensity, fragment) 202 (14, M + 1), 201 (81, M), 200 (8, M − H), 184 (100, $C_{14}H_{16}$), 142 (51, $C_{11}H_{10}$), 141 (64, $C_{11}H_9$), 115 (33, C_9H_7), 91 (26, C_7H_7), and 77 (16, C_6H_5). Calc. *m/e* for $C_{14}H_{19}N$: 201.1518. Found: 201.1494.

Anal.—Calc. for $C_{14}H_{20}ClN$: C, 70.72; H, 8.48; N, 5.89. Found: C, 71.09; H, 8.58; N, 5.97.

10(a)-Amino-1,2,3,4,4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrene Hydrochloride (II-HCl)—A mixture of 63 mg (250 mmoles) 9(a)-hydroxy-10(a)-amino-1,2,3,4,4a,9,10,10a-(*trans*-4a,10a)-octahydrophenanthrene hydrochloride (IV) (6), 63 mg palladium-on-carbon (10%), and 0.5 ml of 70% aqueous perchloric acid in 30 ml acetic acid (99%) was reduced under an initial hydrogen pressure of 30 psi. After 24 hr the catalyst was removed by filtration and washed with water. The filtrate and washings were combined and made alkaline by slow addition of 10% aqueous sodium hydroxide. Ether was added, and the phases separated. The ether extract was washed with water, dried (magnesium sulfate), concentrated to 50 ml, and treated with hydrogen chloride gas. The precipitate was removed to give II-HCl, 37 mg (63%), mp 261–263° dec.; IR (KBr): 3450 (NH), 3050 (ArCH), 2900 (CH), 1600, 1500, 1445, 1380, 765, and 740 cm^{-1}; NMR (dimethyl sulfoxide-d_6): δ 8.03 (s, broad, 3, $^+NH_3$), 7.47–7.10 (m, 4, ArH), 3.47 (m, 1, H_{10}), 3.18 (m, 2, $H_{9,9'}$), 2.77–2.40 (m, 2, $H_{10a,4a}$), and 2.03–1.13 (m, 8, CH_2—CH envelope), addition of D_2O removed the signal at 8.03, replaced by HOD at 3.92; mass spectrum (70 ev): *m/e* (relative intensity, fragment) 202 (6, M + 1), 201 (82, M), 200 (4, M − H), 184 (100, $C_{14}H_{16}$), 142 (67, $C_{11}H_{10}$), 141 (86, $C_{11}H_9$), 115 (23, C_9H_7), 91 (15, C_7H_7), and 77 (11, C_6H_5). Calc. *m/e* for $C_{14}H_{19}N$: 201.1518. Found: 201.1502.

Anal.—Calc. for $C_{14}H_{20}ClN$: C, 70.72; H, 8.48; N, 5.89. Found: C, 70.90; H, 8.62; N, 5.71.

TLC Separation of I and II—About 2 mg of each amine hydrochloride salt was added to 10% aqueous sodium hydroxide and then extracted with ether. The ether solutions were evaporated and the amines were spotted on 20-cm silica gel TLC plates and developed with ether–methanol–aqueous ammonia (90:8:2). The R_f values (iodine visualization) were 0.46 for I and 0.28 for II. A mixture of the two amines produced similar results.

Pharmacological Testing—The compounds were screened for amphetamine-like effects by the method of Campbell and Richter (8) and for hypothermia by the method of Hill and Horita (9).

[2] IR data were recorded on a Beckman IR-5A spectrophotometer, and NMR data were determined from Varian A-60 and T-60 spectrometers. The solvents used are stated and tetramethylsilane was the internal standard. Mass spectra were determined using the AEI-MS9 mass spectrometer and a DEC PDP-12 computer equipped with suitable programs for data collection and reduction (Mass Spectrometry Laboratory, Department of Chemistry, University of Washington). Melting points were taken on a Thomas-Hoover capillary melting-point apparatus and are corrected. Microanalyses were performed by Dr. F. B. Strauss, Oxford, England.

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* Present address: Battelle Memorial Institute, Columbus, OH 43201

[x] To whom inquiries should be directed.

Synthesis and Physicochemical, Antimitogenic, and Antiviral Properties of a Novel Palladium (II) Coordination Compound

HASSAN A. TAYIM*, ARTIN H. MALAKIAN[‡], and ANWAR B. BIKHAZI[§x]

Abstract □ The synthesis, identification, and analytical characterization of a newly synthesized coordination complex, $PdCl_2$(2,6-diaminopyridine)·H_2O, are reported. The solubility of the compound in water and in different aqueous and basic solutions ranged between 7.5 and 14.2 × 10^{-4} *M*. Almost complete suppression (99.7%) of uptake of tritiated thymidine by phytohemagglutinin-induced transforming lymphocytes was produced with 50 μg of the drug. Furthermore, there was a good dose–response relationship between the amount of the drug used and its ability to suppress DNA synthesis. No lymphocytotoxic effect was produced with 50 μg of the drug after 1 and 72 hr of incubation at 37°. The antiviral activity of the complex was measured against vaccinia virus in Hela and chicken embryo fibroblast monolayer cultures. Complete inhibition of plaque-forming units was produced in both cell cultures.

Keyphrases □ Palladium (II) coordination compound—synthesis and physicochemical, antimitogenic, and antiviral properties of $PdCl_2$(2,6-diaminopyridine) □ Coordination complexes—synthesis and physicochemical, antimitogenic, and antiviral properties of $PdCl_2$(2,6-diaminopyridine), effect on DNA synthesis □ Antiviral agents, potential—synthesis and physicochemical properties of palladium (II) 2,6-diaminopyridine complex □ Antimitogenic agents, potential—synthesis and physicochemical properties of palladium (II) 2,6-diaminopyridine complex

Antineoplastic and immunosuppressive agents could be chemically classified as organic or organometallic compounds. The most recent compounds are the platinum coordination complexes (1–3). The mode of action of these complexes is still under investigation (2, 4).

One problem that has faced investigators in the field of the metal coordination complexes is formulating such complexes into pharmaceutically and biologically acceptable forms. The present article briefly outlines some preliminary experiments concerning the synthesis, physicochemical parameters, and antimitogenic and antiviral activities of a novel palladium (II) 2,6-diaminopyridine complex.

EXPERIMENTAL

Synthesis—To a solution of 0.072 g (0.66 × 10^{-3} mole) of 2,6-diaminopyridine in 10 ml methanol, 0.256 g (0.66 × 10^{-3} mole) of *cis*-$PdCl_2(C_6H_5CN)_2$ (5) in 15 ml methanol was added. A red precipitate formed immediately. The reaction mixture was continuously stirred for 3 hr and was then filtered, washed with methanol, and dried in a vacuum oven at room temperature. The product turned brown at 200° and melted at 245°. The IR spectrum (KBr) showed ν 3490 (OH), 3340 (NH), and 1620 and 1575 (C=N and C=C) cm^{-1}. The UV spectrum of the compound showed a peak at λ_{max} 280 nm with a molar absorptivity ($\epsilon^{M}{}_{1\,cm}$) of 4520, and another at λ_{max} 337 nm with a molar absorptivity ($\epsilon^{M}{}_{1\,cm}$) of 3860. The compound is formulated as $PdCl_2$(2,6-diaminopyridine)·H_2O.

Anal.—Calc. for $C_5H_9Cl_2N_3Pd$: C, 19.85; H, 3.01; Cl, 23.44; N, 13.90. Found: C, 20.23; H, 3.02; Cl, 23.15; N, 13.30.

Solubility Measurement—With a rotating-bottle disintegration apparatus[1], the following procedure was used to determine the intrinsic solubility at 30° of the metal complex in water at different pH's. Aliquots (5 ml) of the solvent were placed in six 10-ml screw-capped test tubes. Excess solid drug was placed into each test tube which was then very tightly capped. The test tubes were attached with rubberbands onto the shaft of the apparatus. The whole apparatus, including the attached test tubes, was then put into a stainless-steel tank which was filled with water thermostatically controlled at 30°. The test tubes should be well below the water level. As the motor rotated, mixing of the suspension in each test tube allowed single crystals of the suspended drug to expose maximum surface area for complete and fast equilibrium solubility measurements. Sampling was done at 2, 4, 8, 12, 24, and 48 hr. Each test tube was considered a sample and was randomly picked up from among the six. The contents of the test tube were immediately transferred into a preheated (around 35°) 10-ml disposable hypodermic syringe[2]. On the tip of the syringe, a preheated swinny adapter was attached[3] containing a white, plain, 13-mm, 0.45-μm filter[3]. The suspension was then forced through the filter and the clear filtrate was collected into a preheated clean test tube. The required dilutions were immediately made and the solutions were assayed spectrophotometrically at λ_{max} 280 and/or 337 nm.

Table I—Solubility of Palladium (II) 2,6-Diaminopyridine Complex in Different Aqueous Media

Stock Medium	pH of Stock Medium	pH of Saturated Solution of Stock Medium[a]	Solubility, moles
0.1 *M* HCl	1.00	1.15	Unstable[b]
0.001 *M* HCl	2.85	2.75	7.5 × 10^{-4}
0.0001 *M* HCl	4.00	3.00	8.25 × 10^{-4}
Boiled distilled water	7.00	3.10	12.5 × 10^{-4}
0.00001 *M* NaOH	8.80	4.50	14.2 × 10^{-4}
0.001 *M* NaOH	10.82	4.70	10.8 × 10^{-4}

[a] At time of sampling; the pH of solution was checked after filtration. [b] The λ_{max} 280 and 337 nm disappeared. New peaks appeared at λ_{max} 212, 233 (sh), and 330 nm.

Antimitogenic Activity—The antimitogenic effect was measured with phytohemagglutinin-induced blastogenic transformation of human lymphocytes. The cell cultures were prepared as described for lymphocytes (6, 7); the lymphocytes were isolated from heparinized blood[4,5]. Cell suspensions of 1 × 10^6 cells/ml were prepared in Medium 199[6], supplemented with 4% *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid[7] buffer solution (1.0 *N*, pH 8.1), 20% human AB serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml). The cell suspensions were distributed into screw-capped tissue culture glass tubes (15 × 150 mm), each receiving 1.0 ml. Blastogenic transformation was initiated with 0.02 ml of phytohemagglutinin-M[8]. To test the antimitogenic effect of the palladium compound, separate solutions containing 25–500 μg drug/1 ml tissue culture medium were prepared and filtered through membrane filters[9]. Next, 0.1 ml of each solution was

[1] Ernest D. Menold, Lesler, Pa.
[2] Jintan Terumo Co., Ltd., Japan.
[3] Millipore Corp., Bedford, Mass.
[4] Ficoll gradient, Pharmacia, Uppsala, Sweden.
[5] Isopaque gradient, Nyco, Oslo, Norway.
[6] Catalog No. 118EE, Gibco, Grand Island, N.Y.
[7] Calbiochem, Switzerland.
[8] Difco Labs., Detroit, Mich.
[9] Sartorius, Gottingen, West Germany.

Table II—Effect of Palladium (II) 2,6-Diaminopyridine on Tritiated Thymidine Incorporation by Phytohemagglutinin-Induced Lymphocytes

Lymphocyte Donors	Normal Cells	Cells with Phytohemagglutinin	Concentration of Drug in Culture, μg/ml				
			2.5	12.5	25.0	37.0	50.0
	Uptake of Tritiated Thymidine in Counts Per Minute (cpm)						
1	641[a]	130,463	119,163	90,321	45,127	13,659	369
2	400	135,866	120,096	63,447	45,440	14,799	640
3	325	150,678	139,285	134,053	75,766	10,479	374
4	436	105,000	95,000	82,053	30,278	2,416	121
Mean cpm	450	130,345	118,386	92,467	49,152	10,338	376
Mean percent suppression			9.2[b]	29.2	62.3	92.1	99.7

[a] Mean of duplicate values. [b] $100 - \frac{100 \times \text{cpm of cells containing phytohemagglutinin and drug}}{\text{cpm of control cells containing only phytohemagglutinin}}$.

added to each of the duplicate culture tubes subsequent to the addition of phytohemagglutinin and incubated at 37°. At 56 hr after the addition of phytohemagglutinin and the drug, tritiated thymidine, 2 μCi (specific activity[10] 2.0 Ci/mmole) was added to each culture tube and further incubated. Eighteen hours later the cells were washed with cold 0.9% NaCl solution and the nucleic acids were precipitated with cold 5% trichloroacetic acid. The precipitate was then washed with cold absolute methanol. The acid-insoluble fraction was then processed for liquid scintillation counting.

Antiviral Activity—The antiviral activity of the drug was measured against vaccinia virus in Hela and chicken embryo fibroblast monolayer cultures. The Hela cells have been maintained in this laboratory for the last 12 years[11]. The chicken embryo fibroblast monolayers were prepared as described in Ref. 8. The medium used for the growth of both cell cultures was the same as used for lymphocytes except that 10% fetal calf serum[12] was used instead of human serum. Plastic tissue culture flasks[13] of 25 cm^2 were used for preparation of the cell monolayers. Vaccinia virus[14] was subcultured in each cell monolayer until 50–100 plaque-forming units/flask were produced with 10^{-4} viral dilution. The plaque assays were prepared as described by Garabedian and Scott (9).

To measure the antiviral activity, 0.3-ml aliquots of the drug (230 μg/ml in medium) were mixed separately with 0.3 ml of 2×10^{-3} and 2×10^{-4} dilutions of the virus and mixed thoroughly. Immediately, 0.3 ml of each mixture was then inoculated into each of duplicate flasks of Hela and chicken embryo fibroblast cultures and incubated for adsorption at 37°. Two hours later the inocules were decanted and the monolayers were covered with 2.0 ml of chilled methylcellulose medium overlays and reincubated at 37°. Seventy-two hours later the overlays were washed out with a chilled solution of 0.9% NaCl and the monolayers were stained with 0.5% gentian violet solution.

RESULTS AND DISCUSSION

A plot for the amount of complex in solution per milliliter *versus* time was drawn. The plateau region was considered the equilibrium state and was attained after 4 hr in all cases. The asymptote to the plateau intercepting the concentration axis was considered to be the average intrinsic solubility of the metal complex in the respective solvent (Table I).

A pure sample of the ligand (2,6-diaminopyridine) when dissolved in water showed a UV spectrum with λ_{max} 212, 233 (sh), and 330 nm, which is identical to the spectrum of the complex when dissolved in 0.1 *M* HCl. This suggests that the complex decomposes at pH 1. The solubility of the complex over a 2–11 pH range is almost the same. This is because the pH of all of these solutions in the presence of the metal complex ranges between 2 and 5 (Table I).

Almost complete suppression (99.7%) of uptake of tritiated thymidine by the phytohemagglutinin-induced transforming lymphocytes was produced with 50 μg of the drug. Furthermore, there was a good dose–response relationship between the amount of the drug used and its ability to suppress DNA synthesis (Table II). Finally, no lymphocytotoxic effect was produced with 50 μg of the drug after 1 or 72 hr of incubation at 37°. The trypan blue exclusion test for viability was used for measuring the toxic effect (10).

Table III—Effect of Palladium (II) 2,6-Diaminopyridine on Vaccinia Virus Plaque Assay in Hela and Chicken Embryo Fibroblast Monolayers

Cell Culture	Plaque-Forming Units					
	Drug Control	Cell Control	Virus Control		Virus plus Drug[a]	
			1×10^{-3}[b]	1×10^{-4}	1×10^{-3}	1×10^{-4}
Hela (3)[c]	0	0	392[d]	39	0	0
Chicken embryo fibroblasts (3)	0	0	470	80	0	0

[a] See text for concentration. [b] The 10^{-3} and 10^{-4} indicate the virus dilutions used. [c] Figure in parentheses is the number of experiments. [d] Represents the average values of three experiments.

Complete inhibition of plaque-forming units was produced in both cell cultures (Table III). In the control flasks, the compound did not produce any visible harmful effect on fully grown or newly prepared cultures.

The $PdCl_2$(2,6-diaminopyridine)·H_2O is a novel palladium complex which has shown antimitogenic and antiviral activities. However, it is very poorly soluble in water and aqueous acidic or basic media, which makes it difficult to formulate into a dosage form. Derivatives of this complex with high water solubilities are currently being synthesized.

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[12] Microbiological Associates, Bethesda, Md.
[13] Flalcon Plastics, Oxnard, Calif.
[14] Institute Serotherapique et Vaccinal, Berne, Switzerland.

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Detection of Thioamides: Determination of Ethionamide with 2,3-Dichloro-1,4-naphthoquinone

M. B. DEVANI [x], C. J. SHISHOO, H. J. MODY, and P. K. RAJA

Abstract □ 2,3-Dichloro-1,4-naphthoquinone is reacted with thioamides in microquantities in the presence of ammonia in an alcoholic medium to give colored products showing absorbance maxima between 530 and 540 nm. The reaction is specific for thioamides and forms the basis of its spot test. The reaction is also used for the quantitative determination of ethionamide in tablets. The results compare favorably with those obtained by the official BP method. The method is simple, accurate, and precise.

Keyphrases □ Ethionamide—determination using 2,3-dichloro-1,4-naphthoquinone □ Thioamides—determination of ethionamide using 2,3-dichloro-1,4-naphthoquinone □ 2,3-Dichloro-1,4-naphthoquinone—colorimetric determination of ethionamide □ Colorimetry—determination of ethionamide using 2,3-dichloro-1,4-naphthoquinone

Thioamides are used widely as antitubercular agents (1). They are usually detected by fluorescein-1,3,6,8-tetramercuritetraacetate (2), Dragendorff reagent (3), sodium nitroferricyanide (4), sodium pentacyanoamine ferroate (5), and ammoniacal copper sulfate solution (6). Hydrogen sulfide, evolved on reacting thioamides with hydrazine hydrate, is detected with lead acetate paper (7).

Recently, 2,3-dichloro-1,4-naphthoquinone has been used in the detection and determination of sulfur-containing compounds such as thiosemicarbazones, thiosemicarbazides, and thioureas (8, 9). Therefore, it was of interest to use this reagent in the detection of thioamides and the determination of drugs containing the thioamide moiety. In the present work, reaction conditions were sought for a spot test for thioamides with 2,3-dichloro-1,4-naphthoquinone. The absorption maxima of colored products, obtained on reacting various thioamides with 2,3-dichloro-1,4-naphthoquinone, were determined.

Several methods for the estimation of ethionamide include iodometric (10, 11), acidimetric–alkalimetric (12–14), gravimetric (15), complexometric (16, 17), polarographic (18, 19), and spectrophotometric (20–29) procedures. However, most of them are not specific for the thioamide moiety of the molecule. In view of the specificity and sensitivity of this color reaction, 2,3-dichloro-1,4-naphthoquinone reagent is used for the quantitative estimation of ethionamide BP, a well-known antitubercular drug. Optimum conditions for the reaction have been studied.

EXPERIMENTAL

Apparatus—All spectral measurements were made with a spectrophotometer[1] having four matched 10-ml cells of 1-cm light path.

Reagents and Materials—Ethionamide BP, thioacetamide[2], thionicotinamide[3], and absolute alcohol (Ind.P.) were used. Thiobenzamide (30), thioisonicotinamide (31), and 2,3-dichloro-1,4-naphthoquinone (32) were synthesized by known methods. All other reagents were of analytical grade. Various brands of ethionamide tablets were obtained from the market.

Preparation of 2.5% Ethanolic Ammonia—Dry ammonia was passed into absolute alcohol at −5° until the weight had increased by about 20%. The solution was diluted with absolute alcohol to obtain 2.5% (w/v) of ammonia and was stored in a refrigerator.

2,3-Dichloro-1,4-naphthoquinone Reagent Solution—The concentration used was 0.026% (w/v) in absolute alcohol.

Standard Ethionamide Solution—A 0.03% (w/v) solution in absolute alcohol was prepared.

Detection of Thioamides—*Plate Method*—About 3 drops of ethanolic solution of thioamide (0.1 mg/ml) were spotted on a

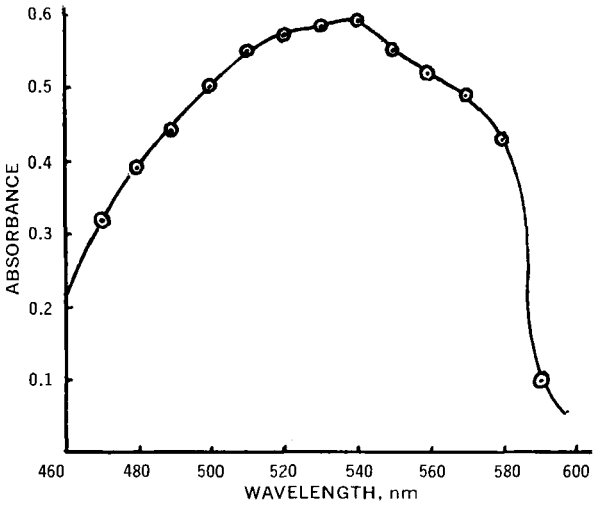

Figure 1—*Visible spectrum of the colored product obtained on reacting ethionamide with 2,3-dichloro-1,4-naphthoquinone reagent.*

[1] Spectronic 20, Bausch & Lomb.
[2] British Drug Houses.
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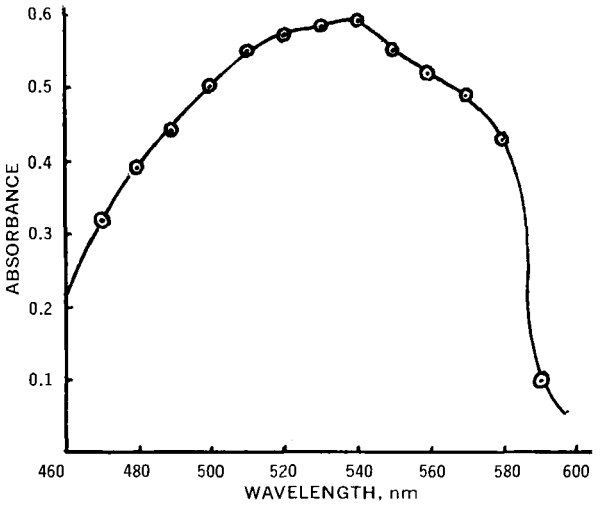

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Table I—Absorbance of Colored Products Obtained on Reacting Various Thioamides with 2,3-Dichloro-1,4-naphthoquinone Reagent

Thioamides	λ_{max}, nm	Absorbance at λ_{max}[a]
Thioacetamide[b]	530	0.085
Thiobenzamide[b]	540	0.255
Ethionamide[b]	540	0.590
Thionicotinamide[b]	530	0.450
Thioisonicotinamide[b]	530	0.615
Acetamide[c]	—	—
Benzamide[c]	—	—
Nicotinamide[c]	—	—

[a] Average value of three experiments. [b] Concentration of 5 μmoles/25 ml reaction mixture. [c] Concentration of 50 μmoles/25 ml reaction mixture.

white porcelain spot plate. Ethanolic ammonia (0.3 ml) and the 2,3-dichloro-1,4-naphthoquinone solution (0.5 ml) were added successively to it. After 5 min, the colored spot was compared with the yellowish reference spot of the reagents alone.

Spot Test on Paper—About 3 drops of the ethanolic test solution (0.1 mg/ml thioamide) were transferred onto a 2.54-cm (1-in.) wide filter paper[4] and dried. The 2,3-dichloro-1,4-naphthoquinone reagent (0.3 ml) was also spotted on it and dried. The yellowish spot thus obtained became purple when exposed to ammonia vapor for about 2 min. The colored spot was then compared with a yellowish reference spot of the reagents alone after 5 min.

Determination of Wavelength of Maximum Absorbance—An ethanolic solution of various thioamides (1 ml of a 5 μmole/ml solution) was mixed with ethanolic ammonia (4.5 ml) and the 2,3-dichloro-1,4-naphthoquinone solution (16.0 ml) in a 25-ml volumetric flask, diluted to volume with absolute alcohol, and allowed to stand for 20 min at room temperature. The absorbance was measured at 10-nm intervals from 400 to 600 nm against absolute alcohol. The blank contained the solution of 2,3-dichloro-1,4-naphthoquinone (16.0 ml) and ethanolic ammonia (4.5 ml) diluted to 25 ml with absolute alcohol. The blank readings were used to correct the absorbance of the sample (Table I).

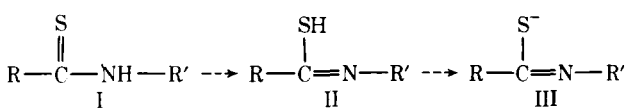
Analysis of Ethionamide Tablets—Twenty tablets were weighed and powdered. A portion of the powder, equivalent to about 30 mg of ethionamide, was accurately weighed. Four equal portions of 20 ml of absolute alcohol were used to extract ethionamide from the tablet powder, and each extract was filtered through filter paper[5]. The residue on the filter paper was then washed with 10 ml of alcohol. The filtrate and the washings were combined in a 100-ml volmetric flask and diluted to volume with ethanol. The solution was assayed as described.

Factors Affecting Reaction—*Concentration of 2,3-Dichloro-1,4-naphthoquinone*—The absorbance at 540 nm of the colored product formed by the reaction of the ethionamide solution (3.0 ml) with 2,3-dichloro-1,4-naphthoquinone increased with the increase in concentration of the reagent. The maximum absorbance was obtained in the presence of 16.0 ml of reagent in 25.0 ml of reaction mixture.

Concentration of Ammonia—A purple color was obtained on the addition of ammonia to the mixture of ethionamide solution (3.0 ml) and 2,3-dichloro-1,4-naphthoquinone reagent (16.0 ml). Maximum color intensity was obtained in the presence of 4.5 ml ammonia solution in 25.0 ml of reaction mixture.

Time of Reaction—The color intensity was found to be at a maximum when the reaction mixture (25.0 ml) containing ethionamide solution (3.0 ml), ethanolic ammonia (4.5 ml), and 2,3-dichloro-1,4-naphthoquinone reagent (16.0 ml) was kept at room temperature for 20 min. The color decreased slowly on standing.

Effect of Concentration of Ethionamide—The absorption at



Scheme I

[4] Whatman No. 1.
[5] Whatman No. 40.

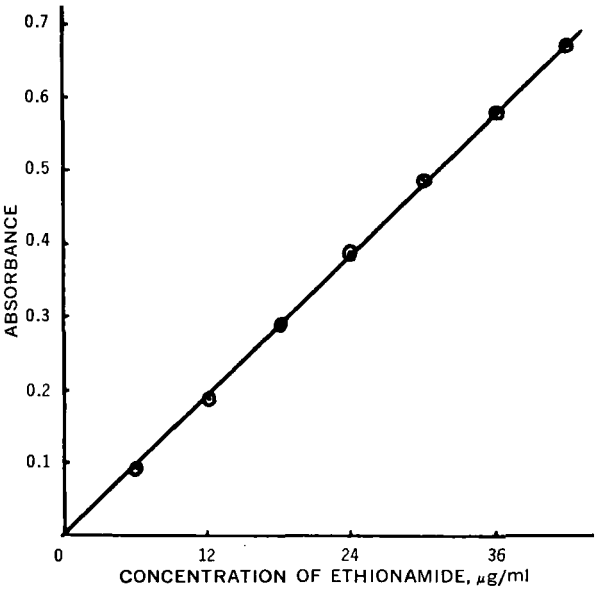


Figure 2—*Standard curve for ethionamide with 2,3-dichloro-1,4-naphthoquinone reagent.*

540 nm was proportional to the amount of ethionamide in the range of 6.0–42.0 μg/ml reaction mixture (Fig. 2).

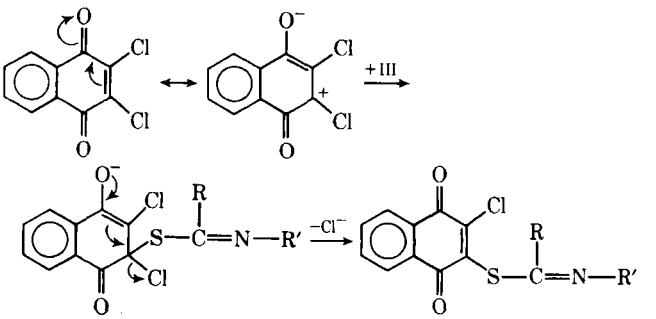
DISCUSSION

Thioamides are known to exist mainly in the thione form (I) (33). The reaction seems to be specific for compounds represented by a general formula R—C(=S)—NH—R′, provided the thioketo compound is able to enolize. In a nonpolar medium, the thio–thiol equilibrium shifts toward the thiol form (II) (34). In the presence of ammonia, thioenol (II) is converted into thioenolate ion (III) which, being a strong nucleophile, helps to remove one chlorine atom of 2,3-dichloro-1,4-naphthoquinone (Scheme I). The reaction shown in Scheme II is thereby initiated.

The color develops within 5 min after the reactants are mixed. Maximum color intensity is obtained after 20 min but decreases slowly on further standing. The absorption maxima of the colored products obtained on reacting various thioamides with the 2,3-dichloro-1,4-naphthoquinone reagent lie between 530 and 540 nm (Table I). The absorption curve for ethionamide is shown in Fig. 1. The corresponding amides such as acetamide, benzamide, and nicotinamide do not give any appreciable color even at a concentration 10-fold that used for thioamides.

Various substituents on the thioamide moiety seem to influence the color intensity of the reaction products: pyridyl > phenyl > methyl. This can be explained by the fact that an electron-withdrawing group like pyridyl or phenyl increases the acidity of the thiol group (II) and thus facilitates the formation of thioenolate ion (III). On the other hand, an electron-donating group like methyl decreases the acidity due to its positive inductive effect.

Ethionamide samples and tablets were assayed by the proposed procedure. The percent recovery and the standard deviation calculated from a series of experiments are given in Tables II and III.



Scheme II

Table II—Analysis of Ethionamide Samples

Sample	Recovery[a], %	
	Official BP Method (12)	Proposed Method
A	99.6 ± 0.852	99.7 ± 0.706
B	99.3 ± 0.856	99.8 ± 0.610

[a] Standard deviations were determined from the results of 10 experiments.

Table III—Analysis of Ethionamide Tablet

Sample	Labeled Amount, mg/Tablet	Recovery[a], mg/Tablet	
		Official BP Method (12)	Proposed Method
A	125.0	120.2	120.7
B	125.0	124.7	124.9

[a] Average result of three determinations.

The results compare favorably with those obtained by the official BP method (12). The usual tablet diluents and lubricants do not interfere in the proposed procedure. The method is simple, rapid, and accurate.

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[x] To whom inquiries should be directed.

Synthesis and Biological Evaluation of 2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate

RICHARD I. HO ˣ, LEONARD CORMAN, and WILLIAM O. FOYE

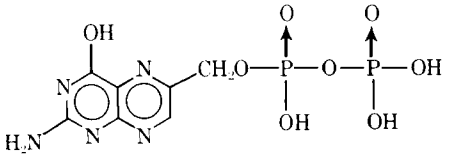
Abstract □ A method for synthesizing the title compound is described which represents a distinct improvement over previous procedures in regard to convenience, yield, and purity. IR and proton NMR spectral data are reported for this compound for the first time. The effectiveness of 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate as a substrate for the enzyme dihydropteroate synthetase, extracted from both *Neisseria gonorrhoeae* and *N. meningitidis*, was evaluated. Inhibition of this enzyme, using the pyrophosphate as substrate, was observed with sulfa drugs and related compounds.

Keyphrases □ 2-Amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate—synthesis and biological evaluation, effectiveness as substrate for dihydropteroate synthetase □ Dihydropteroate synthetase—effectiveness of 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate as substrate, effects of sulfa drugs and related compounds □ Enzyme inhibition—dihydropteroate synthetase by sulfa drugs and related compounds using 2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate as substrate

The enzymatic synthesis of dihydropteroate and dihydrofolate involves the coupling of hydroxymethylpteridine pyrophosphate with *p*-aminobenzoate or *p*-aminobenzoylglutamate (1–3). The first synthesis of hydroxymethylpteridine pyrophosphate (2-amino-4-hydroxy-6-hydroxymethylpteridine pyrophosphate) (I) was described by Shiota *et al.* (4, 5), and this method has been employed extensively for studies related to the biosynthesis of pteroate and analogs. This method of synthesis, however, is tedious and results in preparations that are invariably contaminated with simple pteridines, particularly pteridine monophosphate (5). Purification of these products is difficult, and yields are quite low (~5%). A similar synthesis of hydroxymethylpteridine pyrophosphate was reported (6), but it is also unsatisfactory since it yields a variety of phosphorylated pteridines, requiring extensive purification.

DISCUSSION

This article describes an improved synthesis of this compound, which is obtained free of contaminating pteridines; the monophosphate ester is completely absent. Furthermore, this procedure requires only one step, and no further purification is needed beyond recrystallization. In this procedure, an excess of crushed pyrophosphoric acid is melted slowly, and the hydroxymethylpteridine, prepared by the method of Forrest and Walker (7), is added slowly to avoid polymerization. The product is adsorbed on acid-washed active charcoal and eluted rapidly with a carefully controlled amount of ammonium hydroxide solution. If a solvent system of ammonium hydroxide and absolute ethanol is used, as with the Shiota *et al.* (5) procedure, other phosphorylated pteridines appear in the eluate. The eluate is checked for impurities by paper chromatography, using water as the solvent. The desired compound moves with the solvent front; if another spot appears, the extraction is stopped. This chromatographic technique has provided maximum yields and ensured purity of product. The compound is obtained as the diammonium salt, and its purity was substantiated by passage of the eluate through a column[1]; a single peak resulted.



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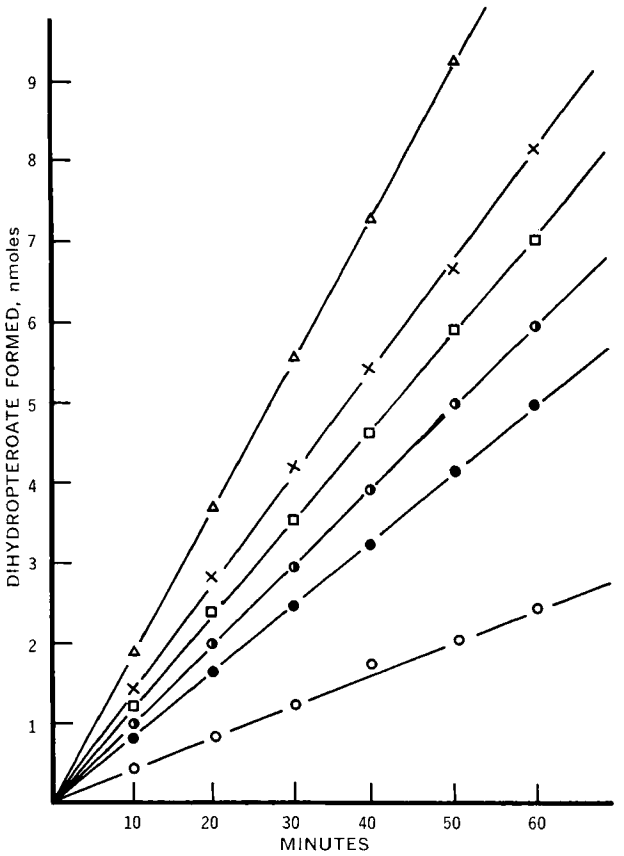

Figure 1—*Enzymic formation of dihydropteroate at 37° using different concentrations of substrate and incubation periods. The total volume of the incubation mixtures was 0.36 ml and included $MgCl_2$, 0.278 mM: mercaptoethanol, 8 mM; tromethamine–hydrochloric acid buffer (pH 8.0), 10 mM; dihydropteroate synthetase, 0.45 mg; and p-aminobenzoic acid-^{14}C, 4.4 × 10^{-2} mM. Key (hydroxymethyldihydropteridine pyrophosphate): ○, 0.034 mM; ●, 0.069 mM; ◑, 0.083 mM; □, 0.11 mM; ×, 0.138 mM; and △, 0.26 mM.*

An attempt was made to obtain the free acid form of the hydroxymethylpteridine pyrophosphate by passing the ammonium salt through a cation exchanger[2]. The free acid form was eluted rapidly and gave a single sharp peak. However, when the pooled fractions were lyophilized, the free acid was found to be less stable than the ammonium salt and it soon polymerized.

The hydroxymethylpteridine pyrophosphate obtained was iden-

[1] Sephadex G-10.
[2] AG 50W-X12.

Table I—Determination of Optimal Concentration of 2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate as Substrate for Dihydropteroate Synthetase

Concentration, m*M*	Activity[a], %
0.034	22
0.069	42
0.138	75
0.26	100
0.52	98
1.0	94
1.3	91
2.6	76

[a] Measured with enzyme extracted from *N. meningitidis* strain M-60 EUR, whose minimal inhibitory concentration by sulfanilamide on plates is 1 μg/plate. The extraction procedure was described previously (13).

tical to an authentic sample of this compound[3] in regard to both UV and IR absorption spectra and chromatographic behavior. The IR spectrum showed the presence of primary amino, phosphate hydroxyl, and pteridine ring stretching frequencies, and the proton NMR spectrum showed two methylene protons as a multiplet from splitting by phosphate. Neither IR nor proton NMR spectra nor a melting point have been previously reported for this compound.

Biological Evaluation—Reduction of the hydroxymethylpteridine pyrophosphate to its dihydro form using sodium dithionite (8) gave a product that showed full enzymatic activity with the dihydropteroate synthetase extracted from both *Neisseria meningitidis* and *N. gonorrhoeae*. By using the enzyme from one strain[4] of *N. meningitidis*, rates of formation of dihydropteroate for different concentrations of the hydroxymethyldihydropteridine pyrophosphate were measured.

The dihydropteroate, formed using *p*-aminobenzoate-^{14}C, was eluted from the origin and identified by its characteristic UV spectrum. Total dihydropteroate was radioassayed; this procedure was found to be highly reproducible. The rate of dihydropteroate synthesis was dependent on hydroxymethyldihydropteridine pyrophosphate concentration in the presence of a constant concentration of *p*-aminobenzoate (Fig. 1). A Lineweaver–Burke plot (of the reciprocal of rate of dihydropteroate synthesis *versus* the reciprocal of substrate concentration) gave a K_m value for the hydroxymethyldihydropteridine pyrophosphate of 2×10^{-4} *M* (Fig. 2), obtained from the average of three duplicate experiments. A K_m value was determined by Shiota *et al.* (5), using a cell-free extract from *Veillonella* strain V2 with the pyrophosphate as substrate; their value was 3.12×10^{-4} *M*.

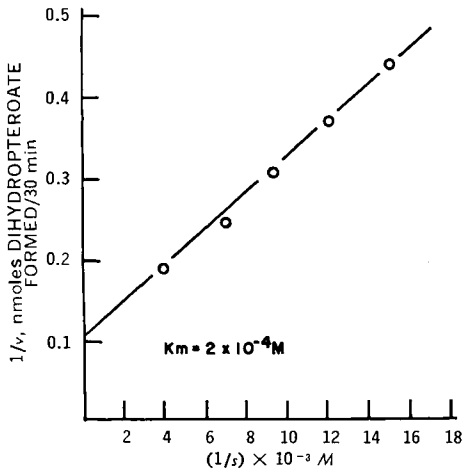

Figure 2—*Effect of hydroxymethyldihydropteridine pyrophosphate concentration on rate of enzymic synthesis of dihydropteroate. Velocity is given in nanomoles of product formed per milligram of protein per 30 min. Concentration of substrate was varied between 6.9 × 10⁻⁵ and 26 × 10⁻⁵ M. Experimental conditions were as described for Fig. 1.*

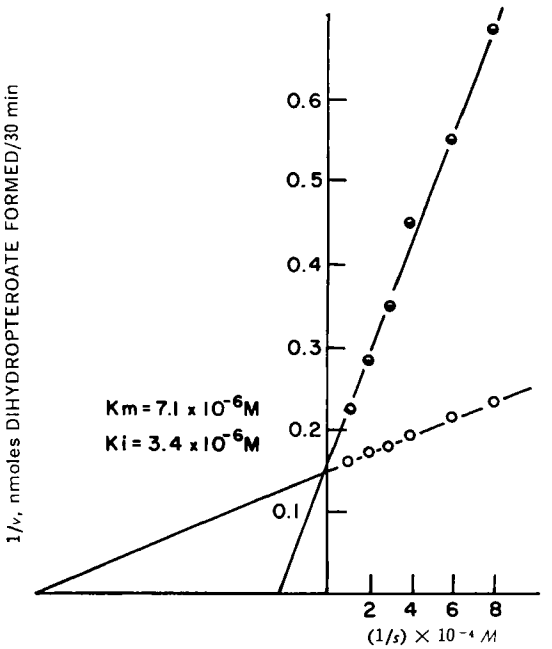

Figure 3—*Inhibition of dihydropteroate synthetase by dapsone (1.8 × 10⁻⁵ M) using varying amounts of p-aminobenzoic acid (0.012–0.098 mM) and a constant amount of hydroxymethylpteridine pyrophosphate (0.26 mM). Key: ○, control; and ◒, with dapsone.*

As shown in Table I, the optimal substrate concentration of the pyrophosphate for the dihydropteroate synthetase employed was 0.26 m*M*. The pyrophosphate becomes inhibitory beyond that concentration.

Inhibition Studies—Inhibition studies using the hydroxymethylpteridine pyrophosphate as substrate for the dihydropteroate synthetase of *N. meningitidis* were carried out using sulfanilamide, sulfadiazine, dapsone, and bis(4-aminophenyl) disulfide. Dapsone competitively inhibited the synthesis of dihydropteroate (Fig. 3), which is in accord with a previous finding that the antibacterial action of dapsone is reversed by *p*-aminobenzoate (9). This has been further substantiated by recent reports that dapsone (10) and the sulfonamides (11) were competitively inhibitory to the same enzyme from a cell-free extract of *Escherichia coli*. The other compounds observed also produced competitive inhibitions at concentrations of 1.7×10^{-5} *M*, in the following order of decreasing activity: sulfadiazine — dapsone > sulfanilamide > bis(4-aminophenyl) disulfide. The disulfide has been postulated to function analogously to dapsone in malaria chemotherapy (12).

EXPERIMENTAL[5]

2-Amino-4-hydroxy-6-hydroxymethylpteridine Pyrophosphate—Pyrophosphoric acid was crushed and dried under vacuum for 4 days, and 25 g was melted slowly in a glass-stoppered flask at 60–65°. 2-Amino-4-hydroxy-6-hydroxymethylpteridine (7) (300 mg, 0.0008 mole) was added slowly, and the flask was protected from light by aluminum foil. The mixture was stirred and heated at 60–65° for 2 hr. After addition of 80 ml of distilled water, the contents were transferred to a beaker, and an aqueous charcoal suspension[6] (3 g in 20 ml) was added. The mixture was stirred for

[3] Kindly supplied by Dr. T. Shiota.

[4] Strain M-60 EUR; obtained from the Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C., through the courtesy of Dr. M. Artenstein.

[5] The melting point was taken on a Mel-Temp apparatus and is uncorrected. Microanalyses were done by Galbraith Laboratories, Knoxville, Tenn. UV absorption spectra were determined with a Cary model 14 spectrophotometer, IR absorption spectra were obtained with a Perkin-Elmer model 137B spectrophotometer, and proton NMR spectra were obtained with a Varian T60 spectrometer using D_2O as the solvent.

[6] Darco 60.

30 min and was filtered through a 0.45-μm filter[7]. The charcoal pad was carefully removed from the filter and washed with 500 ml of distilled water to remove excess pyrophosphoric acid. The pteridine adsorbed on the charcoal pad was eluted by suspending the pad in 75 ml of 3 *N* ammonium hydroxide, and the mixture was stirred for 15 min. The resulting fine suspension was filtered through a fresh filter paper[7]. This procedure was repeated three times with the same quantity of 3 *N* ammonium hydroxide as eluant. The combined filtrate was evaporated under reduced pressure at room temperature until no odor of ammonia could be detected. The solution was lyophilized to yield about 300 mg (80%) of greenish-yellow amorphous powder. An analytical sample was obtained by recrystallization from aqueous ethanol, mp 166–175°; UV λ_{max} (0.1 *N* NaOH): 255 and 362 nm; IR ν_{max} (mineral oil): 3300, 1675 (NH_2), 1240 (P=O), 1040 (P—OH), and 820 (pteridine) cm^{-1}; NMR: δ 5.2 (m, 2H, CH_2) and 7.0 (d, 1H, aromatic H); R_f (water) 0.98.

Anal.—Calc. for $C_7H_{15}N_7O_8P_2$: C, 21.71; H, 3.90; N, 25.30; P, 15.99. Found: C, 21.86; H, 4.10; N, 24.50; P, 15.95.

[7] Millipore.

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Potential Anticancer Agents I: Confirming Evidence for the Structure of Fagaronine

M. TIN-WA, C. L. BELL, C. BEVELLE, H. H. S. FONG, and N. R. FARNSWORTH[x]

Abstract □ The structure of fagaronine was confirmed by spectral studies on its *N*-demethyl derivative.

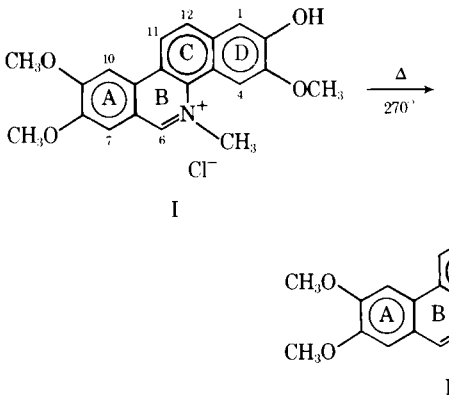
Keyphrases □ Fagaronine—structure confirmation □ Anticancer agents, potential—confirmation of fagaronine structure □ *Fagara zanthoxyloides* (Rutaceae) alkaloids—confirmation of fagaronine structure

Fagaronine (I), an alkaloid of *Fagara zanthoxyloides* (Rutaceae) (1), exhibits potent antileukemic properties and is currently being considered for preclinical evaluation by the National Cancer Institute. In an initial report, two possible structures for fagaronine were suggested, one of which was favored on the basis of mass spectral evidence. At that time, it was not possible to study the NMR spectrum in detail due to the poor solubility of I in all of the usual solvents. This problem has now been overcome by the preparation of *N*-demethylfagaronine (II) (Scheme I) which was found to be soluble in $CDCl_3$ and dimethyl sulfoxide-d_6 and from which good NMR data could be obtained.

DISCUSSION

The NMR spectrum (Table I) of *N*-demethylfagaronine in $CDCl_3$ and that of its hydrogen-bonded complex with triethylamine showed a significant increase in shielding of an upfield aromatic proton (δ7.47) by 0.17 ppm and little or no effect on the other aromatic protons. This increase in shielding would be expected for protons *ortho* and *para* to the phenolic OH position (2). The proton at C-4, which could be expected to produce a downfield signal due to its orientation with respect to the ring B nitrogen and the accompanying electron pair, was not affected, which suggested that the phenolic group was located at the C-2 position.

The use of dimethyl sulfoxide-d_6 as the NMR solvent offered the advantage of determining in the same solvent the chemical shifts of the free *N*-demethyl base as well as those of the phenolate anion of the base. From the chemical shift changes, it was possible



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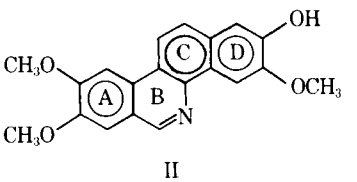

II

Scheme I—Preparation of N-Demethylfagaronine from Fagaronine

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Abstract □ The structure of fagaronine was confirmed by spectral studies on its *N*-demethyl derivative.

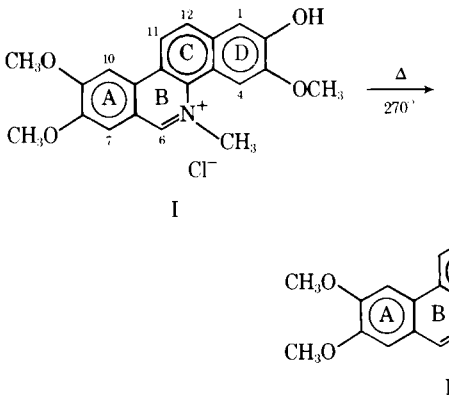
Keyphrases □ Fagaronine—structure confirmation □ Anticancer agents, potential—confirmation of fagaronine structure □ *Fagara zanthoxyloides* (Rutaceae) alkaloids—confirmation of fagaronine structure

Fagaronine (I), an alkaloid of *Fagara zanthoxyloides* (Rutaceae) (1), exhibits potent antileukemic properties and is currently being considered for preclinical evaluation by the National Cancer Institute. In an initial report, two possible structures for fagaronine were suggested, one of which was favored on the basis of mass spectral evidence. At that time, it was not possible to study the NMR spectrum in detail due to the poor solubility of I in all of the usual solvents. This problem has now been overcome by the preparation of *N*-demethylfagaronine (II) (Scheme I) which was found to be soluble in $CDCl_3$ and dimethyl sulfoxide-d_6 and from which good NMR data could be obtained.

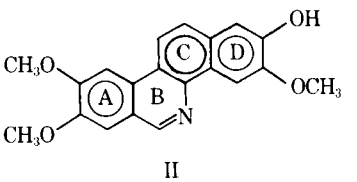
DISCUSSION

The NMR spectrum (Table I) of *N*-demethylfagaronine in $CDCl_3$ and that of its hydrogen-bonded complex with triethylamine showed a significant increase in shielding of an upfield aromatic proton (δ7.47) by 0.17 ppm and little or no effect on the other aromatic protons. This increase in shielding would be expected for protons *ortho* and *para* to the phenolic OH position (2). The proton at C-4, which could be expected to produce a downfield signal due to its orientation with respect to the ring B nitrogen and the accompanying electron pair, was not affected, which suggested that the phenolic group was located at the C-2 position.

The use of dimethyl sulfoxide-d_6 as the NMR solvent offered the advantage of determining in the same solvent the chemical shifts of the free *N*-demethyl base as well as those of the phenolate anion of the base. From the chemical shift changes, it was possible



I



II

Scheme I—Preparation of N-Demethylfagaronine from Fagaronine

Table I—NMR Data for *N*-Demethylfagaronine[a,b]

Protons	Chemical Shift (δ) in $CDCl_3$	Change (ppm) after Addition of Triethylamine[c]	Chemical Shift (δ) in Dimethyl Sulfoxide-d_6	Change (ppm) after Addition of NaOD
H_6	9.32	0	9.35	−0.08
H_4	8.82	0	8.68	−0.27
H_{11}	8.33 d, J = 9.0 Hz	−0.03	8.60 d, J = 9.0 Hz	−0.08
H_7 or H_{10}	7.93	0	8.17	−0.08
H_{12}	7.88 d, J = 9.0 Hz	−0.05	7.90 d, J = 9.0 Hz	−0.28
H_7 or H_{10}	7.43	0	7.72	−0.05
H_1	7.47	−0.17	7.37	−0.63
OCH_3	4.18	0	4.10 (A ring)	0
OCH_3	4.17	0	4.05 (C-3)	−0.10
OCH_3	4.07	0	3.98 (A ring)	0

[a] Chemical shifts measured downfield from internal tetramethylsilane. [b] Negative values indicate increased shielding. [c] Approximately 20% triethylamine.

not only to confirm the position of the phenolic group at C-2 but also to assign the remaining signals to the other aromatic protons. While the preparation of the phenolate by the addition of NaOD increased the shielding of all protons in the C and D rings due to the increased electron densities in these rings, it left the protons in the A and B rings largely unaffected.

The B ring proton singlet (H_6) was readily assigned by its most downfield position (δ9.35), which was due to its proximity to the ring nitrogen and by its insensitivity to anion formation. Two other singlets at δ7.72 and 8.17, unaffected by anion formation, were assigned to the remaining protons in the A ring, H_7 and H_{10}, and were not further differentiated from the data obtained.

In the study of Highet and Highet (2) of the chemical shift changes observed for 10 methyl- and methoxyphenols in dimethyl sulfoxide-d_6 upon anion formation, the magnitude of the shift changes offered a clear distinction between protons *ortho, meta,* and *para* to the OH site. They observed increased shielding for these protons at 0.50 ± 0.1, 0.28 ± 0.10, and 0.74 ± 0.05 ppm for the *ortho, meta,* and *para* protons, respectively.

The remaining aromatic singlets at δ7.37 and 8.68 in the spectrum of *N*-demethylfagaronine due to the D-ring protons gave shifts of 0.63 and 0.27 ppm, respectively, corresponding closely to those expected for *ortho* and *meta* protons. The only phenolic site possible consistent with these observations is at C-2 with the δ7.37 signal assigned to the H_1 proton *(ortho)* and the δ8.68 signal assigned to the H_4 proton *(meta)*. The downfield position predicted for the H_4 proton offered further verification of this assignment.

The two protons in the C ring constituted an *AB* system (J= 9.0 Hz) (Table I) and were both affected by the conversion of the phenolic group in the D ring to the phenolate, but by different magnitudes. The increased shielding shown by the upfield doublet (−0.28 *versus* −0.08 ppm) allows it to be assigned to the H_{12} proton by a consideration of the sites of increased electron density due to a phenolate group at C-2.

The methoxy group singlet at C-3 (δ4.05) could be distinguished readily from the signals due to the methoxy groups on the A ring since it alone shifted with anion formation.

From the data obtained on *N*-demethylfagaronine, the structure of fagaronine is thus confirmed as I.

EXPERIMENTAL[1]

Fagaronine was obtained as previously reported (1) from the roots of *Fagara zanthoxyloides* (Rutaceae). Fagaronine (100 mg) was heated at 270° for 5 min, during which time the original yellow color turned to a grayish white. The resulting product was mixed with 15 ml of chloroform, in which fagaronine is insoluble, and filtered. Following *in vacuo* removal of the chloroform, a 70-mg residue was obtained, which was purified by silica gel PF_{254} column chromatography, using an eluent of chloroform–methanol (9:1). The pure (TLC) *N*-demethylfagaronine (31 mg) exhibited a melting point of 250° and showed UV absorptions at λ_{max} 227 (log ϵ 4.30), 272 (4.67), and 280 (4.67) nm and a shoulder at 315 (4.04) nm. The IR spectrum was very similar to, but not identical with, that of fagaronine. The phenolic group was observed as a broad band at 3520 cm^{-1}. A molecular ion (M^+) was observed at *m/e* 335, followed by peaks at *m/e* 334, 320, 306, and 292 in the mass spectrum, the latter appearing very similar to the fragmentation pattern of fagaronine (1).

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[x] To whom inquiries should be directed.

[1] All NMR spectra were determined using a Varian T-60A instrument.

Isolation and Partial Purification of Hemolytic Toxin from Sea Anemone, *Stoichactis helianthus*

JOHN P. DEVLIN

Abstract □ The isolation of a toxic principle(s) from the sea anemone, *Stoichactis helianthus*, was effected by subjecting the anemones to a freeze–thaw cycle followed by immersion in deionized water. The crude toxic material was removed from the aqueous phase by precipitation with acetone and was partially purified by gel filtration. The partially purified toxin has a molecular weight greater than 5000 and an LD_{50} of 0.25 mg/kg in mice, and it causes complete hemolysis of a 1% suspension of sheep red blood cells at 0.5 μg/ml. The toxicity in mice is destroyed upon interaction of the partially purified toxin with concanavalin A.

Keyphrases □ *Stoichactis helianthus* (sea anemone)—isolation and partial purification of toxin with direct hemolytic activity □ Sea anemone—isolation and partial purification of toxin with direct hemolytic activity □ Hemolytic toxin, direct—isolation and partial purification from *Stoichactis helianthus* (sea anemone)

The pharmacological and chemical nature of coelenterate toxins has received much attention (1–5). While the properties of whole animal or whole tentacle homogenates employed in earlier studies are difficult to interpret, the more recent use of toxin preparations obtained from the rupture of isolated nematocysts (6–10) or the controlled discharge of attached nematocysts (11) has permitted a more definitive assessment of the mode of action of these substances and their chemical composition.

Various methods have been employed to effect rupture of the isolated nematocysts (6, 12). Of these methods the freeze–thaw cycle and immersion in distilled water, when effective, are perhaps the most gentle and the least destructive.

In this investigation, the isolation of a highly toxic and hemolytic principle from the sea anemone, *Stoichactis helianthus*, was most conveniently effected by applying the rupture techniques mentioned to the whole animals with neither homogenization nor nematocyst isolation. Partial purification was effected by the removal of low molecular weight constituents by gel filtration.

There is no direct proof that this substance is a constituent of the nematocyst apparatus; however, such a localization is implied by the high toxicity, the mode of preparation, and the fact that further extraction following homogenization failed to yield toxic material.

EXPERIMENTAL

The anemones (average 10 cm in diameter) were collected on the fringe reef off the St. James coast of Barbados, frozen (−20°), and shipped to these laboratories by air freight.

Crude Toxin Extraction—After storage (−20°) for 10 days the anemones (about 8 liters in volume) were defrosted, and the viscous liquid that separated was decanted (200 ml, Extract A). They were then gently mixed with deionized water (4 liters) and kept at room temperature for 4 hr with intermittent mixing. Care was taken in these operations to avoid mechanical tissue damage. The aqueous portion (5 liters) was decanted, filtered, and divided into two equal parts (Extracts B and C).

Table I—Isolation of Crude Toxin

Preparation	Weight, g	LD_{50}, mg/kg	Extract	Volume, ml	LD_{50}[a], ml/kg
A	3.6	4.5	A	200	0.25
B	25	3.3	B	2500	0.33
C	82	11.3	C	2500	0.34

[a] Extrapolated values (see text).

Table II—Gel Filtration of Water-Soluble Portion of Preparation B

Sample	Fraction	V_e/V_0[a] Range	Weight[b], mg	LD_{50}, mg/kg
1[c]	1–6	0.92–1.08	356	0.25
2	7–20	1.08–1.43	82	>10
3	21–35	1.43–1.80	205	>10
4	36–58	1.80–2.39	180	>10

[a] V_e = effluent volume, and V_0 = void volume. [b] Combined fractions were freeze dried. [c] Elemental analysis: C, 41.2%; H, 6.8%; N, 8.3%; P, nil; S, nil.

Crude Toxin Preparations—Extract A was diluted to 700 ml with acetone and kept at 0° for 4 hr. The precipitated material was collected, washed with acetone, and dried to constant weight under reduced pressure at room temperature. The resulting beige solid (9.6 g) was stirred with deionized water (100 ml) for 10 min, and the fine suspension that formed was centrifuged. The supernate was collected and freeze dried to provide Preparation A as a beige solid (3.6 g).

Extract B (2.5 liters) was diluted to 6 liters with acetone and kept at 0° for 24 hr. The precipitated material was collected, washed with acetone, and dried at room temperature under reduced pressure to provide Preparation B as a yellow-brown solid (25 g).

Extract C was freeze dried to provide Preparation C as a beige solid (82 g).

Gel Filtration of Water-Soluble Portion of Preparation B—The dextran gel[1] (500 g) was swollen in deionized water (saturated with chloroform) at 25° for 4 hr prior to use. The column (5 × 100 cm) had a void volume[2] (V_0) of 795 ml and was operated at a flow rate of 120 ml/hr (6 ml/cm²/hr). Fractions (20 ml each) were monitored by the biuret reaction according to the technique of Gornall *et al.* (13).

LD_{50} Determinations—The acute toxicity was determined in mice (female, ICR strain, approximately 2 months old) and based on a 6-day observation period. Intraperitoneal injections were given in a volume of 0.5 ml/20 g of mouse. Ten mice were used per dosage point, and the estimated LD_{50} was calculated by the method of Litchfield and Wilcoxon (14).

Hemolytic Studies—The hemolytic effect was determined by the addition of increasing toxin concentrations in isotonic saline (0.5 ml) to 4.5 ml of a 1.1% suspension of washed sheep red blood cells in isotonic saline. After 20 min, the suspensions were centri-

[1] Sephadex G-25 medium, Pharmacia, Montreal, Quebec, Canada.
[2] As determined with Dextran 2000, Pharmacia.

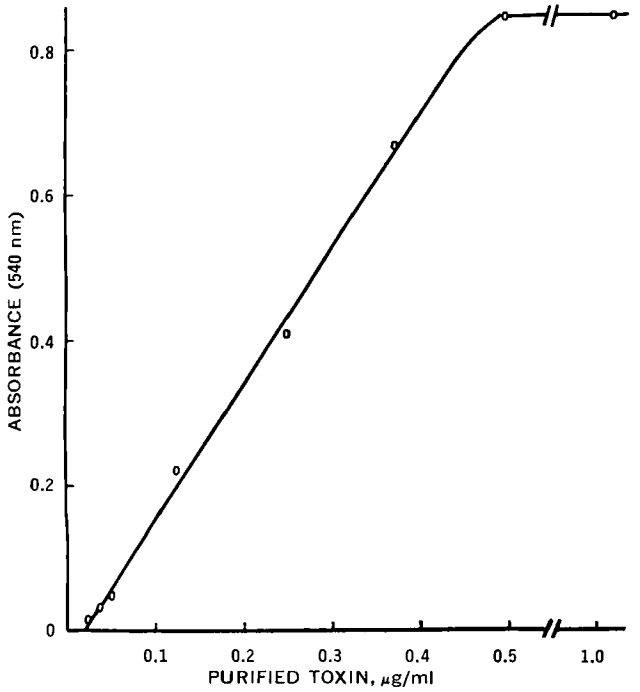

Figure 1—*Hemolysis of a 1% suspension of sheep red blood cells with the partially purified toxin in saline.*

fuged and the optical density of each supernate was measured[3] at 540 nm.

Interaction with Concanavalin A—The interaction of the purified toxin with concanavalin A[4] was determined by the development of turbidity upon the addition of a solution (3 ml) of concanavalin A to increasing toxin concentrations in a total volume of 4 ml. The medium employed was 15% aqueous sodium chloride, and the turbidity was measured[3] at 420 nm after 20 min at 25°. Corrections were made with respect to the absorption of the toxin solutions in the absence of concanavalin A.

RESULTS AND DISCUSSION

Isolation of Crude Toxin—The toxicities of Preparations A, B, and C are presented in Table I. Extrapolations of the toxicities of Preparations B and C back to the original extracts gave LD_{50} values of 0.33 and 0.34 ml/kg, respectively. Preparations B and C had a common origin and differed only in the mode of concentration, *i.e.*, acetone precipitation and lyophilization, respectively. Therefore, the toxic principle was insoluble in 50% aqueous acetone and apparently unaffected by this method of isolation.

Partial Purification—Preparation B (20 g) was stirred at room temperature with deionized water (300 ml) and then kept, without stirring, for 5 min. The milky aqueous portion was decanted. The insoluble material was treated twice more with deionized water (2 × 150 ml) in the same manner. The aqueous portions were combined and centrifuged (850×*g* for 1 hr at 5°). The supernate was decanted and the precipitate was mixed with water (200 ml) and again centrifuged (850×*g* for 1 hr at 5°). The latter supernate was combined with the former (total volume 800 ml), freeze dried to about 100 ml, and again centrifuged (850×*g* for 1 hr at 5°). The resulting clear pale-yellow solution was freeze dried. The beige solid (2.95 g, LD_{50} 0.6 mg/kg) obtained in this manner retained 90% of the toxic principle from Preparation B.

A portion (1 g) of this preparation (LD_{50} 0.6 mg/kg) was dissolved in deionized water (10 ml) and purified by gel filtration (see *Experimental*). The biuret-positive material was primarily in the first six fractions immediately following the void volume. The toxic effects were exclusively restricted to this narrow band (Table II) and represented an 86% recovery of the toxic principle. This behavior indicates that the molecular weight of the toxic principle(s) is greater than 5000.

Stability and Composition of Partially Purified Toxin—The toxin at this level of purity (Sample 1, Table II; hereafter referred to as toxin) was soluble in deionized water (>10%) and in 20% (w/v) sodium chloride solution (1%). At a concentration of 1 mg/ml, it was destroyed (LD_{50} >10 mg/kg) in acidic (0.05 *N* HCl at 25° for 1 day) and in basic (0.05 *N* NH_4OH at 25° for 1 day) media but was very stable (LD_{50} 0.25 mg/kg) in solution (25° for 2 days) in deionized water.

The toxin gave a positive biuret (protein) and a positive Molisch (carbohydrate) reaction.

Hemolytic Effects—The toxin caused complete hemolysis of washed red blood cells (sheep) at 0.5 μg/ml (Fig. 1). This effect was fully retained when a saline solution of the toxin at this concentration was kept at room temperature for 2 weeks. Since serum or lecithin addition was not required for this activity, the toxin can be classified as a direct hemolysin (15). The same level of activity was observed with whole blood (cat).

Direct hemolytic activity has been found in toxins of the sea wasp, *Chironex fleckeri* (16, 17), and the sea anemone, *Stoichactis kenti* (18), but not in that of the Portuguese man-of-war, *Physalia physalis* (7).

Interaction with Concanavalin A—Concanavalin A, a hemagglutinating protein from jack bean, *Canavalia ensiformis*, forms insoluble complexes with many polysaccharides (19) and glycoproteins (20). Therefore, the interaction of the toxin with concanavalin A was investigated (Fig. 2). Precipitation was complete at the ratio of 6 mg toxin to 1 mg concanavalin A. With this effect in mind, the toxin (60 mg) was treated with concanavalin A (15 mg) in 15% aqueous sodium chloride (15 ml). After 25 min (25°), the suspension was centrifuged and the supernate was decanted and diluted to 250 ml with deionized water. The resulting solution (equivalent of 0.25 mg toxin/ml saline) was found to be nontoxic at 50 ml/kg.

This result implies a close relationship of the polysaccharide moiety of the partially purified toxin with the toxic effect. The significance of the protein portion, however, remains unknown.

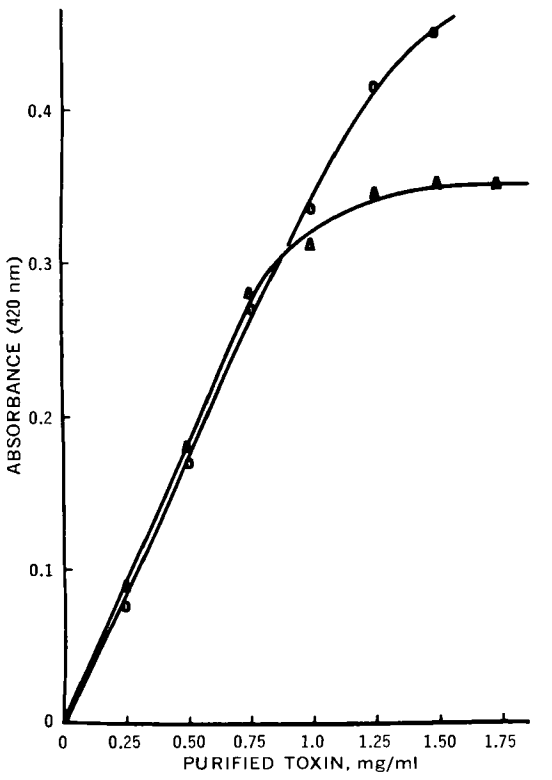

Figure 2—*Development of turbidity upon the interaction of the partially purified toxin with concanavalin A at concentrations of 0.250* (△) *and 0.375* (○) *mg/ml in 15% aqueous sodium chloride.*

[3] Spectronic 20, Bausch and Lomb Co.
[4] Pharmacia.

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Phosphorus–Nitrogen Compounds XVI: Phosphoramantadine Derivatives

LINDLEY A. CATES ˣ **and ROBERT L. GALLIO**

Abstract □ Nine derivatives containing adamantyl moieties were synthesized and screened for antileukemic properties. Five 1-*N*-adamantylphosphoramidate esters, phenyl *N*-1-adamantylphosphoramidochloridate, phenyl P-1-aziridinyl-*N*-1-adamantylphosphonamidate, and *N,N'*-diadamantyl-*N''*-bis(2-chloroethyl)phosphoric triamide were inactive. P,P-Bis(1-aziridinyl)-*N*-1-adamantylphosphinic amide, however, showed good activity when tested against L-1210 lymphoid and P-388 lymphocytic leukemia, giving %T/C values of 225 (20 mg/kg) and 244 (10 mg/kg), respectively.

Keyphrases □ Phosphoramantadine derivatives—synthesized and screened for antileukemic properties □ Phosphorus–nitrogen compounds—synthesis and screening of phosphoramantadine derivatives for antileukemic properties □ Amantadines, phosphorylated—synthesis and screening for antileukemic properties □ Anticancer agents, potential—synthesis and screening of phosphoramantadine derivatives □ Antileukemic agents, potential—synthesis and screening of phosphoramantadine derivatives

Amantadine (1-aminoadamantane, I) has been shown to inhibit the penetration of Rous Sarcoma virus in chick embryo cells (1) and to decrease mitosis and DNA synthesis in sea urchin ova (2), chick embryo fibroblasts, and Hela cell cultures (3). It also displayed activity *in vitro* against angiosarcoma and pancreatic sarcoma (4). The *N*-methyl, *N*-acetyl, and *N*-formyl derivatives of amantadine also gave an effect in the latter tumor system (4). Other compounds containing the adamantyl[1] moiety which have shown antineoplastic activity include those classified as thiosemicarbazones (5) and pyrimidines (6). Also, the insertion of an adamantyl grouping into cytarabine resulted in an immunosuppressant effect twice that of the parent molecule (7).

This paper reports the synthesis (Schemes I and II and Table I) and anticancer screening results (Table II) of nine phosphorylated amantadines. New ester derivatives (II–V) were synthesized by the phosphonate–carbon tetrachloride–amine method, while VII and XI were prepared by amidation of VI and IX, respectively. Compound VIII was synthesized by the reaction of I and bis(2-chloroethyl)phosphoramidic dichloride.

EXPERIMENTAL

Phosphoramantadine Diesters (II–V)—These compounds were synthesized by the reaction of I, carbon tetrachloride, and the appropriate phosphonate as previously reported for the diphenyl ester (8).

Di-*o*-chlorophenyl *N*-1-Adamantylphosphoramidate (X)—This derivative was prepared from IX, triethylamine, and *o*-chlorophenol according to a previously described procedure (8).

Phenyl *N*-1-Adamantylphosphoramidochloridate (VI)—To a stirred solution of 7.5 g (0.05 mole) of I and 5.0 g (0.05 mole) of triethylamine in 50 ml of anhydrous ether was added 10.5 g (0.05 mole) of phenyl phosphorodichloridate at 30° over 45 min. After remaining overnight, the reaction mixture was filtered and the residue was washed free of chloride with water. Recrystallization from ethanol gave the pure product.

Phenyl P-1-Aziridinyl-*N*-1-adamantylphosphonamidate (VII)—A solution of 3.3 g (0.01 mole) of VI and 1.3 g (0.03 mole) of aziridine in 50 ml of dioxane was refluxed for 4 hr and then left

[1] This name for the radical, rather than the Chemical Abstracts nomenclature of tricyclo(3,3,1,1^{3,7})dec-1-yl, is used in this paper.

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Phosphorus–Nitrogen Compounds XVI: Phosphoramantadine Derivatives

LINDLEY A. CATES ˣ **and ROBERT L. GALLIO**

Abstract □ Nine derivatives containing adamantyl moieties were synthesized and screened for antileukemic properties. Five 1-*N*-adamantylphosphoramidate esters, phenyl *N*-1-adamantylphosphoramidochloridate, phenyl P-1-aziridinyl-*N*-1-adamantylphosphonamidate, and *N,N'*-diadamantyl-*N''*-bis(2-chloroethyl)phosphoric triamide were inactive. P,P-Bis(1-aziridinyl)-*N*-1-adamantylphosphinic amide, however, showed good activity when tested against L-1210 lymphoid and P-388 lymphocytic leukemia, giving %T/C values of 225 (20 mg/kg) and 244 (10 mg/kg), respectively.

Keyphrases □ Phosphoramantadine derivatives—synthesized and screened for antileukemic properties □ Phosphorus–nitrogen compounds—synthesis and screening of phosphoramantadine derivatives for antileukemic properties □ Amantadines, phosphorylated—synthesis and screening for antileukemic properties □ Anticancer agents, potential—synthesis and screening of phosphoramantadine derivatives □ Antileukemic agents, potential—synthesis and screening of phosphoramantadine derivatives

Amantadine (1-aminoadamantane, I) has been shown to inhibit the penetration of Rous Sarcoma virus in chick embryo cells (1) and to decrease mitosis and DNA synthesis in sea urchin ova (2), chick embryo fibroblasts, and Hela cell cultures (3). It also displayed activity *in vitro* against angiosarcoma and pancreatic sarcoma (4). The *N*-methyl, *N*-acetyl, and *N*-formyl derivatives of amantadine also gave an effect in the latter tumor system (4). Other compounds containing the adamantyl[1] moiety which have shown antineoplastic activity include those classified as thiosemicarbazones (5) and pyrimidines (6). Also, the insertion of an adamantyl grouping into cytarabine resulted in an immunosuppressant effect twice that of the parent molecule (7).

This paper reports the synthesis (Schemes I and II and Table I) and anticancer screening results (Table II) of nine phosphorylated amantadines. New ester derivatives (II–V) were synthesized by the phosphonate–carbon tetrachloride–amine method, while VII and XI were prepared by amidation of VI and IX, respectively. Compound VIII was synthesized by the reaction of I and bis(2-chloroethyl)phosphoramidic dichloride.

EXPERIMENTAL

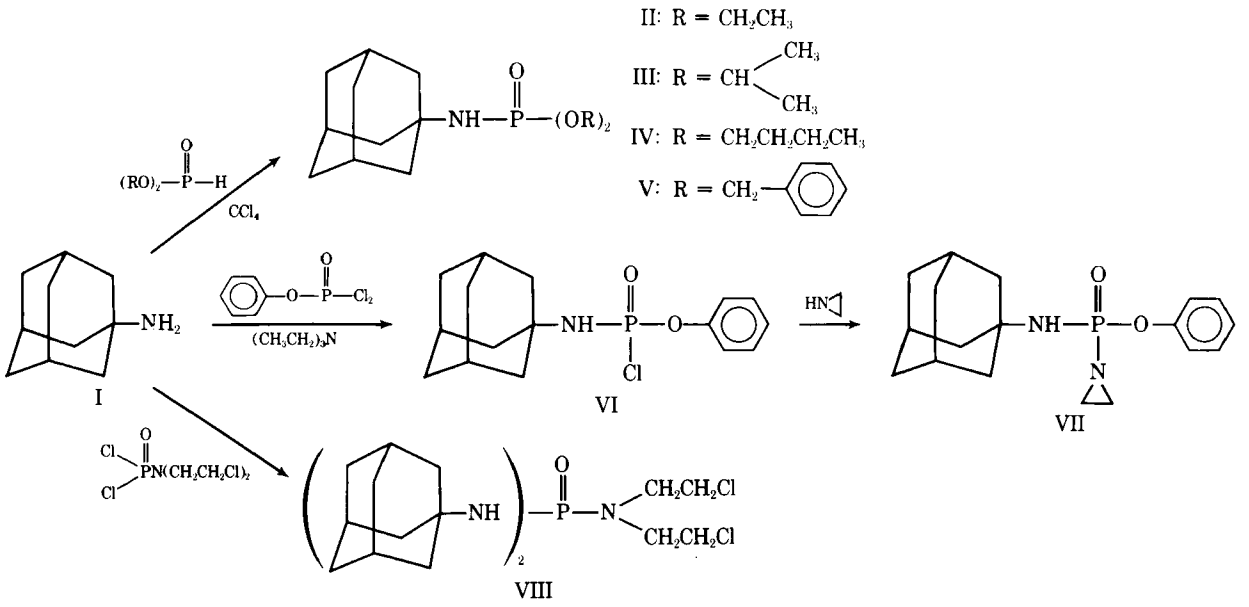
Phosphoramantadine Diesters (II–V)—These compounds were synthesized by the reaction of I, carbon tetrachloride, and the appropriate phosphonate as previously reported for the diphenyl ester (8).

Di-*o*-chlorophenyl *N*-1-Adamantylphosphoramidate (X)—This derivative was prepared from IX, triethylamine, and *o*-chlorophenol according to a previously described procedure (8).

Phenyl *N*-1-Adamantylphosphoramidochloridate (VI)—To a stirred solution of 7.5 g (0.05 mole) of I and 5.0 g (0.05 mole) of triethylamine in 50 ml of anhydrous ether was added 10.5 g (0.05 mole) of phenyl phosphorodichloridate at 30° over 45 min. After remaining overnight, the reaction mixture was filtered and the residue was washed free of chloride with water. Recrystallization from ethanol gave the pure product.

Phenyl P-1-Aziridinyl-*N*-1-adamantylphosphonamidate (VII)—A solution of 3.3 g (0.01 mole) of VI and 1.3 g (0.03 mole) of aziridine in 50 ml of dioxane was refluxed for 4 hr and then left

[1] This name for the radical, rather than the Chemical Abstracts nomenclature of tricyclo(3,3,1,1^{3,7})dec-1-yl, is used in this paper.



Scheme I

overnight at room temperature. The dioxane solution was decanted from the water-soluble oil and treated with water to yield the crystalline product.

P,P-Bis(1-aziridinyl)-*N*-1-adamantylphosphonic Amide (XI)—A solution of 3.9 g (0.09 mole) of aziridine in 50 ml of anhydrous ether was placed in a two-necked, round-bottom flask equipped with a dropping funnel and a condenser fitted with a drying tube. A solution of IX in 75 ml of ether was added dropwise with magnetic stirring at a rate sufficient to maintain reflux conditions. After remaining overnight, the precipitate was collected and washed free of chloride with water. Recrystallization of residue from acetone yielded the white crystalline product.

***N,N'*-Diadamantyl-*N''*-bis(2-chloroethyl)phosphoric Triamide (VIII)**—To a solution of 13 g (0.05 mole) of bis(2-chloroethyl)phosphoramidic dichloride (9) in 60 ml of chloroform was added dropwise a solution of 15.1 g (0.1 mole) of I in 300 ml of chloroform, with stirring and cooling to 0–5°. The reaction mixture was warmed to room temperature and refluxed for 2 hr. The chloroform was removed by spin evaporation, the residue was washed with water, and the remaining solid was dissolved in hot ethanol. The product precipitated in the form of white needles when the ethanol solution was cooled.

RESULTS AND DISCUSSION

The substitution of an adamantyl moiety for that of an aziridinyl results in a pronounced change in physical properties. Triethylenephosphoramide, for example, is extremely soluble in water, with a melting point of 41° (10), whereas XI has low water and high lipid solubility and melts at 177–178°. This increase in lipid solubility due to the introduction of an adamantyl fragment in any of the derivatives is expected to facilitate passage of the agents from the blood into the central nervous system (CNS). The penetration of the blood–brain barrier is achieved by only a few antineoplastic compounds such as 1,3-bis(2-chloroethyl)-1-nitrosourea. Leukemic cells in the CNS, which occur in about 80% of pa-

Table I—Phosphoramantadines

Compound	Melting Point[a]	Formula		Analysis[b], % Calc.	Found	NMR Spectra[c,d] δ, ppm	Signal	Protons
II	94.5–96.5°	$C_{14}H_{26}NO_3P$	C	58.52	58.67	3.95	m	4
			H	9.12	9.13	1.30	t	6
			N	4.87	4.94			
III	68–71°	$C_{16}H_{30}NO_3P$	C	60.93	60.86	4.54	m	2
			H	9.59	9.59	1.32	d	12
			N	4.44	4.50			
IV	36–39°[e]	$C_{18}H_{34}NO_3P$	C	62.95	62.50	3.98	m	4
			H	9.98	9.77	0.95	m	10[f]
			N	4.08	3.99			
V	96–97.5°	$C_{24}H_{30}NO_3P$	C	70.06	69.87	7.30	s	13
			H	7.35	7.41	5.08	d	4
			N	3.40	3.44			
VI	135–137°	$C_{16}H_{21}ClNO_2P$	Cl	10.88	10.78	7.30	s	5
			N	4.30	4.25			
VII	114–115°	$C_{18}H_{25}N_2O_2P$	C	65.25	65.33	7.25	s	5
			H	7.54	7.67	2.18	d	4
VIII	178–180°	$C_{24}H_{40}Cl_2N_3OP$	Cl	14.51	14.01	3.18 to	m	8
			N	8.60	8.50	3.82		
X	159–161.5°	$C_{22}H_{24}Cl_2NO_3P$	Cl	15.68	15.78	7.75	m	8
			N	3.10	3.13			
XI	177–178°	$C_{14}H_{24}N_3OP$	C	59.77	59.44	2.20	d	8
			H	8.60	8.58			
			N	14.94	14.87			

[a] Performed on a Fisher-Johns apparatus and are corrected. [b] Performed by Atlantic Microlab, Inc., Atlanta, Ga. [c] Taken on a Varian T-60 spectrometer using deuterated chloroform as the solvent and tetramethylsilane as the internal reference. Singlet, doublet, triplet, and multiplet splitting is abbreviated s, d, t, and m, respectively. [d] All products gave the characteristic three NMR signals for the adamantyl grouping which integrated for 3 (δ 2.00–2.04), 6 (δ 1.80–1.90), and 6 (δ 1.60–1.68) protons. [e] Bp 156–159°/0.2 mm. [f] The signal for the remaining four protons occurs at about δ 1.20–1.60 and overlaps with a portion of the signal from the adamantyl protons.

Table II—Antileukemic (L-1210 Lymphoid) Activity[a]

Compound	Dose[b], mg/kg	Toxicity Day[c] Survivors	T/C, %
II	400[d]	5/6	90
	200[d]		98
	100[d]		93
III	400[d]		100
	200[d]		96
	100[d]		95
IV	400[d]		96
	200[d]	5/6	92
	100[d]		100
V	300[e]		95
	150[e]		90
	75[e]		97
VI	75[e]	0/6	—
	50[e]	5/6	—
	25[e]		100
VII	260[f]		90
	130[f]		98
	65[f]		93
VIII	400[f]	5/6	98
	200[f]		94
	100[f]		105
X	400[e]		95
	200[e]		101
	100[e]		101
XI	80[d]	5/6	—
	40[d]		—
	20[d]		225
	20[e]	5/6	—
	13.3[d]		152
	10[e]		145
	9[d]		138
	6[d]		125
	5[e]		132
	4[d]		107
	10[e,g]		244
	6.6[e,g]		211
	4.4[e,g]		183

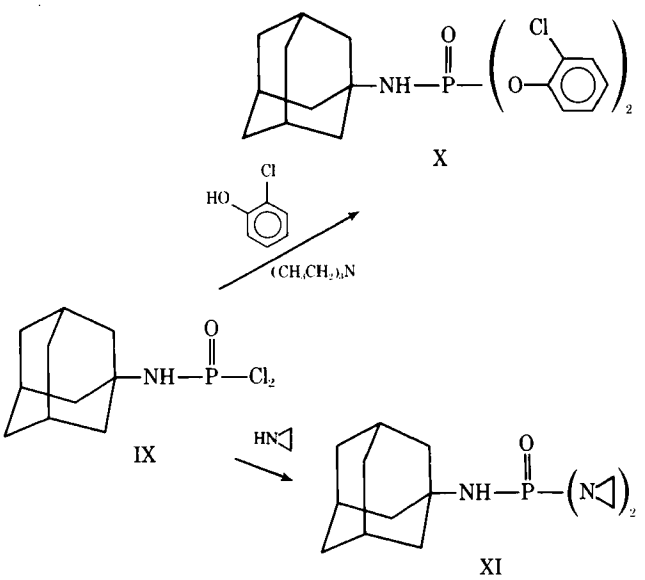
[a] Performed by the screening contractors of the National Cancer Institute, Bethesda, Md. [b] All initial injections were given at Day 1. [c] Number of survivors of total number of animals started on test (recorded on Day 5 in survival systems as a measure of drug toxicity); six out of six survivors unless otherwise indicated. [d] Three injections were administered at 4-day intervals. [e] Nine injections were administered on consecutive days. [f] One injection. [g] P-388 lymphocytic leukemia.

tients (11), can metastasize even though the disease is controlled peripherally, and the eradication of these cells is an important aspect in the chemotherapy of the disease (12).

Similar reasoning is applied to the series II–V[2] but with regard to the ester portion. As the carbon content of the ester grouping increases, so does lipid solubility and these changes in the hydrophilic–lipophilic balance are expected to produce differences in their *in vivo* distribution. The anticipated anticancer effect in these types of compounds is based on that shown by amantadine and/or adamantylphosphoramidic acids, both of which are possible hydrolytic products of the parent structures; the latter compounds are related to acylated amantadine with proven activity. Four derivatives possess moieties, in addition to the adamantyl, with demonstrated cytotoxic properties. Two of the groupings, the bis(2-chloroethyl)amino (in VIII) and the aziridinyl (in VII and XI), are well recognized for these activities.

Anticancer screening (Table II) indicates that a good antileukemic effect is produced by XI. The highest %T/C value was 225 (20 mg/kg), while acute toxicity was not noted until a fourfold dose was reached. Against lymphocytic P-388 in more limited testing, XI gave a %T/C value of 244 (10 mg). The other compounds have not, as yet, given indication of beneficial effect in this tumor system. As anticipated, the phosphoramidochloridate VI was the most toxic compound. The lack of activity by the aziridinyl derivative VII may be attributable to its operating as a monofunctional

[2] Compound II was independently synthesized and reported by V. D. Warner, D. B. Mirth, A. S. Dey, S. S. Turesky, and B. Soloway, *J. Med. Chem.*, **16**, 1185(1973).



Scheme II

alkylator. It has been established (14) that tumor systems vary in their sensitivity to these two chemical types. On this basis, however, it is difficult to rationalize the absence of activity in VIII, a nitrogen mustard derivative.

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[x] To whom inquiries should be directed.

Formulation and *In Vitro* Evaluation of Anti-Infective Dry Foams

P. N. CATANIA[x] and J. C. KING

Abstract □ To improve local therapeutic techniques for the massively burned patient, the concept of topical films was utilized to formulate a hydrophilic dry foam. An amphoteric surfactant, sorbitol solution, dextran, and water were selected as components for the dosage form. The release of therapeutic agents *in vitro* from medicated dry foam formulations was compared with that of corresponding ointment bases utilizing a modified agar diffusion method. When using *Pseudomonas aeruginosa* as the test bacteria, these tests showed that silver nitrate and silver sulfadiazine produced significantly larger zones of inhibition when used as the dry foam rather than as the ointment. Mafenide acetate and gentamicin sulfate produced equivalent release rates from both the dry foam and the ointment, while nitrofurazone was without effect.

Keyphrases □ Anti-infectives—formulation and *in vitro* evaluation of dry foams □ Topical films, dry hydrophilic—formulation and *in vitro* evaluation of anti-infective dry foams □ Dosage forms—formulation and *in vitro* evaluation of anti-infective dry foams

Local therapy for the massively burned patient usually includes the application of medicated ointments and dressings to minimize and eradicate *Pseudomonas* infection (1). As this approach to local therapy for denuded areas may present problems regarding application and removal techniques, the purpose of this study was to formulate a topical dosage form that may be applied to denuded areas of the skin without inunction. By using the concept of topical films and dressings, an aerated, hydrophilic dry film was formulated which is highly water soluble, flexible, nontoxic, and adherent to intact and abraded areas of the skin.

Because local drug action is dependent upon the rate of release of a drug from its vehicle (2), *in vitro* studies were performed to compare the release of medicaments from dry films with that of ointments. Therapeutic agents effective against *Pseudomonas* were selected for study (3).

EXPERIMENTAL

Formulation of Dry Films—Dextran[1], 4 g, was dissolved in 19 ml of water at a temperature of 70–75° on a water bath. To this solution were added 2 ml of sorbitol solution USP and 0.05 ml of an amphoteric surfactant[2]. The entire solution was whipped for 10 min with an electric beater, and the resultant foam was cast on a Teflon-coated surface to a depth of 2–3 mm and dried at 40–45° for 20–30 min.

After drying, the moisture content of the dry foam was determined using a moisture balance[3]. Average values for these measurements approximated 10%. In addition, the liquefaction time of the dry foam was ascertained; 2.5 cm^2 of the dry foam liquefied in 0.1 ml of water at ambient temperatures in 20 sec.

Batches of the dry foam formulation were prepared containing each of the following therapeutic agents in the concentration listed: 8.5% mafenide as the acetate[4], 0.2% nitrofurazone[5], 0.1% gentamicin as the sulfate[6], 0.5% silver nitrate, and 1, 2, and 3% silver sulfadiazine. According to the method of Fox (4, 5), silver sulfadiazine was prepared by dissolving 27 g of sodium sulfadiazine[7] and 17 g of silver nitrate, each in 50 ml of water. The solutions were mixed, and the precipitate was collected, washed, and dried to constant weight at 50–60°.

***In Vitro* Evaluation**—Because *Pseudomonas aeruginosa* has been frequently implicated as the causative bacteria in burn wound sepsis, a modified microbiological agar diffusion test (6) was utilized to compare the effectiveness of the dry foam with ointments as a vehicle for selected agents active against this bacterium. The *in vitro* drug release rates of the medicated dry foam preparations were compared with the corresponding commercially available ointment formulations, namely, mafenide cream, nitrofurazone cream, and gentamicin cream. Although not commercially available, 0.5% silver nitrate in an oil-in-water ointment base[8] and 1, 2, and 3% silver sulfadiazine in an oil-in-water ointment base[8] were prepared extemporaneously and utilized in this study.

After autoclaving sufficient quantities of Bacto Antibiotic Medium 2[9] at 121° and 15 psi for 25 min, approximately 20 ml was poured into 100 × 15-mm sterile, disposable petri dishes. Upon congealing, 0.1 ml of Bacto Antibiotic Medium 3[9] containing an overnight inoculum of *Ps. aeruginosa* (ATCC 9721) was spread onto the agar medium with the aid of a sterile, L-shaped glass rod. Each of 15 penicylinders was filled with approximately 0.3 g of the ointment formulation under investigation, placed upon the seeded agar, and incubated overnight at 37°. Penicylinders filled with the nonmedicated ointment served as the control. Zones of inhibition were measured after 24 hr with an antibiotic zone reader[10].

The corresponding medicated dry foam formulations were evaluated in a similar manner. However, rather than utilizing penicylinders, 15 disks of each medicated dry foam were prepared, each with a diameter of 7 mm, approximately equal to the diameter of the penicylinders. Nonmedicated dry foam disks were also prepared and evaluated as the dry foam control.

The results of the *in vitro* determinations are listed in Table I.

Stability—Preliminary studies were performed on replicate samples of medicated dry foams to determine the effect of varying levels of humidity and temperature upon stability. Mafenide and silver sulfadiazine dry foams were stored for 24 weeks in sealable, moistureproof, plastic bags[11]. Samples were stored at −5, 5, 25, 35, and 45° in a dark environment. Gross physical examination of the samples at weekly intervals indicated that the formulations will retain stability if packaging and storage conditions are designed to prevent moisture contact with the hydrophilic dry foams.

RESULTS AND DISCUSSION

To determine the effectiveness of the dry foam as a dosage form, a modified agar diffusion test was performed utilizing *Ps. aeruginosa* as the test bacteria. Mafenide, silver sulfadiazine, silver nitrate, gentamicin, and nitrofurazone were selected as typical topical antibacterials. The diameters of the zones of inhibition observed with the various medicated dry foam formulations were

[1] Sigma Dextran, A.M.W. 86,900, Sigma Chemical Co., St. Louis, Mo.
[2] Miranol 2MCA Modified, Miranol Chemical Co., Irvington, N.J.
[3] Cenco.
[4] Sulfamylon, Winthrop Laboratories, Division of Sterling Drug, Inc., New York, N.Y.
[5] Furacin, Eaton Laboratories, Division of The Norwich Pharmacal Co., Norwich, N.Y.
[6] Garamycin, Schering Corp., Bloomfield, N.J.
[7] Ruger Chemical Co., Irvington, N.J.
[8] Neobase Ointment, Burroughs Wellcome & Co., Research Triangle Park, N.C.
[9] Difco Laboratories, Detroit, Mich.
[10] Fisher-Lilly, Fisher Scientific Co., Pittsburgh, Pa.
[11] Zip-loc Plastic Bags, Dow Chemical Co., Midland, Mich.

Table I—*In Vitro* Microbiological Evaluation of Medicated Dry Foams

Formulation	Mean Diameter[a], Zone of Inhibition, mm	*SD*	Student *t* Test
0.5% silver nitrate dry foam	12.8	1.6	$p < 0.001$
0.5% silver nitrate ointment	9.3	1.2	
1% silver sulfadiazine dry foam	17.2	1.7	$p < 0.001$
1% silver sulfadiazine ointment	8.5	1.1	
2% silver sulfadiazine dry foam	16.6	2.1	$p < 0.001$
2% silver sulfadiazine ointment	9.7	0.9	
3% silver sulfadiazine dry foam	20.6	1.7	$p < 0.001$
3% silver sulfadiazine ointment	9.7	1.1	
8.5% mafenide dry foam	27.2	2.4	$p < 0.1 > 0.05$
8.5% mafenide ointment	25.9	1.0	
0.1% gentamicin dry foam	12.3	1.4	$p < 0.1 > 0.05$
0.1% gentamicin ointment[b]	13.6	1.6	
0.2% nitrofurazone dry foam[c]	—	—	—
0.2% nitrofurazone ointment[c]	—	—	—
Dry foam control[c]	—	—	—
Ointment control[c]	—	—	—

[a] Results listed are mean values for 15 determinations. [b] Gentamicin ointment liquefied at the incubation temperature. [c] No zones of inhibition were observed.

compared with those of the corresponding medicated ointments.

Mafenide was tested *in vitro* as the dry foam and also as the commercially available ointment. The mean diameter for 15 replicate determinations using mafenide dry foam measured 27.2 mm, while similar testing of the ointment formulation produced zones equal to 25.9 mm. Although the dry foam produced larger zones, a Student *t* test of these results suggested that a significant difference may not exist between the mean diameters of the zones of inhibition ($p < 0.1 > 0.05$). For this reason, it was assumed that the dry foam was as effective as the ointment when testing *in vitro* release of mafenide from these two dosage forms.

With respect to the silver sulfadiazine preparations, significance, as determined by the Student *t* test, could be assigned to the data obtained. That is, the mean diameter of the zones of inhibition was significantly larger for the dry foam formulation than for the corresponding ointment.

The mean diameters of the zones of inhibition measured 12.8 and 9.3 mm for the silver nitrate dry foam and the silver nitrate ointment, respectively. As was the case with silver sulfadiazine, the silver nitrate dry foam proved to be a better formulation *in vitro* than the corresponding ointment, as determined by the Student *t* test ($p < 0.001$).

In determining the *in vitro* effectiveness of the dry foam and ointment as a vehicle for 0.1% gentamicin, liquefaction of the ointment occurred during incubation. Due to the liquefaction and resultant flowing of the ointment onto the seeded agar plates, it is possible that exaggerated values were obtained. Nevertheless, zones were measured and evaluated. For the medicated dry foam, mean values of 12.3 mm were recorded, while values for the corresponding ointment equaled 13.6 mm. Since no statistically significant difference was noted between the means of the data obtained ($p < 0.1 > 0.05$), it was assumed that the dry foam and the ointment were equally effective as dosage forms for gentamicin.

The *in vitro* data indicated that drug release, as determined by the microbiological method, from the medicated dry foam formulations was at least as great as that from ointments. A medicated dry foam may be of value as a vehicle for topical use. The data obtained in this work indicated that the therapeutic concept of a medicated dry foam may be valid and should be subjected to clinical investigation at the earliest opportunity.

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[x] To whom inquiries should be directed.

Synthesis and Antibacterial Activity of 5-Nitro-2-furfurylidene *p*-Sulfonyl- and *p*-Sulfamoylbenzoylhydrazides VII

I. LALEZARI *x, V. GHARAVI ‡, and N. MAZHARI ‡

Abstract □ Thirteen 5-nitro-2-furfurylidene *p*-sulfonyl- and *p*-sulfamoylbenzoylhydrazides were synthesized. All compounds prepared were tested against 10 Gram-positive and Gram-negative bacteria and exhibited antibacterial activity.

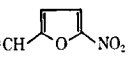
Keyphrases □ 5-Nitro-2-furfurylidene *p*-sulfonyl- and *p*-sulfamoylbenzoylhydrazides—synthesis, antibacterial activity □ Antibacterial activity—evaluation of 13 5-nitro-2-furfurylidene *p*-sulfonyl- and *p*-sulfamoylbenzoylhydrazides

In continuation of studies on the preparation of new 5-nitro-2-furfurylidene derivatives (1) and on the synthesis and pharmacological activity of compounds related to alkylsulfonyl-, arylsulfonyl-, and sulfamoylbenzoylhydrazides(2–5), a series of 5-nitro-2-furfurylidene benzoylhydrazides having sulfonyl or sulfamoyl groups in the *para*-position was synthesized. *p*-Sulfonyl- and *p*-sulfamoylbenzoylhydrazides were prepared by interaction of hydrazine hydrate and an appropriate ethyl ester (3, 4). The new benzoylhydrazides prepared are summarized in Table I.

Benzoylhydrazides were allowed to react with 5-nitro-2-furaldehyde to give the 5-nitro-2-furfurylidene derivatives (Scheme I). The 5-nitro-2-furfuryli-

Table I—*p*-Sulfamoylbenzoylhydrazides $p\text{-}RNHSO_2C_6H_4\text{—}CONHNH_2$

R	Melting Point	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found
CH_3	143°	65	$C_8H_{11}N_3O_3S$	C 41.92	41.88
				H 4.80	4.71
C_2H_5	150–152°	50	$C_9H_{13}N_3O_3S$	C 44.44	44.54
				H 5.34	5.28
$(CH_3)_2CH$	168–169°	80	$C_{10}H_{15}N_3O_3S$	C 46.69	46.73
				H 5.83	5.81
C_6H_5	220–222°	57	$C_{13}H_{13}N_3O_3S$	C 53.60	53.66
				H 4.46	4.52

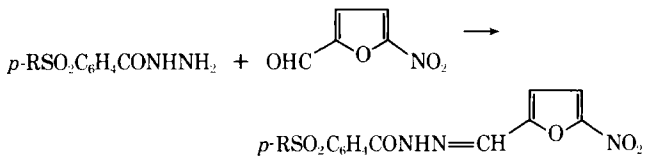
Table II—5-Nitro-2-furfurylidene *p*-Sulfonyl- and *p*-Sulfamoylbenzoylhydrazides $p\text{-}RSO_2C_6H_4\text{—}CONHN{=}CH$  NO_2

Compound	R	Melting Point	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found
1	CH_3	265°	82	$C_{13}H_{11}N_3O_6S$	C 46.29	46.25
					H 3.26	3.26
2	C_2H_5	240°	85	$C_{14}H_{13}N_3O_6S$	C 47.86	48.02
					H 3.70	3.75
3	C_6H_5	237°	92	$C_{18}H_{13}N_3O_6S$	C 54.13	54.14
					H 3.25	3.33
4	$p\text{-}FC_6H_4$	238–243°	90	$C_{18}H_{12}FN_3O_6S$	C 51.79	51.82
					H 2.87	2.77
5	NH_2	285°	83	$C_{12}H_{10}N_4O_6S$	C 42.60	42.61
					H 2.95	3.09
6	CH_3NH	297°	79	$C_{13}H_{12}N_4O_6S$	C 44.31	44.30
					H 3.40	3.44
7	C_2H_5NH	258°	81	$C_{14}H_{14}N_4O_6S$	C 45.90	45.99
					H 3.82	3.82
8	$(CH_3)_2CHNH$	254–259°	77	$C_{15}H_{16}N_4O_6S$	C 47.36	47.40
					H 4.21	4.40
9	C_6H_5NH	275°	75	$C_{18}H_{14}N_4O_6S$	C 52.17	52.17
					H 3.38	3.44
10	$(CH_3)_2N$	227°	89	$C_{14}H_{14}N_4O_6S$	C 45.90	45.80
					H 3.82	3.70
11	$(C_2H_5)_2N$	243°	93	$C_{16}H_{18}N_4O_6S$	C 48.73	48.69
					H 4.56	4.51
12	$(C_3H_7)_2N$	210°	89	$C_{18}H_{22}N_4O_6S$	C 51.18	51.18
					H 5.21	5.23
13	$(CH_2)_5N$	250°	79	$C_{17}H_{18}N_4O_6S$	C 48.98	49.11
					H 4.54	4.50

Table III—Antibacterial Activity

Compound	Zone of Inhibition, Average Size, mm					
	Staph. aureus	*Staph. albus*	*K. pneumoniae*	*S. fecalis*	*B. cereus*	*B. subtilis*
1	—	9.9	—	—	—	12.4
2	13	13.8	—	—	—	13
3	11	12	10.1	—	—	12
4	9.11	9.9	—	—	10.8	11.5
5	—	—	—	—	—	—
6	10.6	—	—	—	—	—
7	11.2	10.3	—	—	—	10.7
8	11.1	11.3	11.1[a]	—	—	10.7
9	10.8	11.8	—	—	11	11.4
10	9.5	11.3	—	—	—	11.3
11	13.3[a]	12.3	—	—	11.8[a]	14.3
12	—	11.9	—	—	—	10.4
13	11.2	13.9	—	—	11.8	13.8
Furazolidone	23.3	22.7	20.2	13.1	23.4	25.5

[a] Hazy zones.



1: R = CH_3 5: R = NH_2 9: R = NHC_6H_5
2: R = C_2H_5 6: R = $NHCH_3$ 10: R = $N(CH_3)_2$
3: R = C_6H_5 7: R = NHC_2H_5 11: R = $N(C_2H_5)_2$
4: R = p-FC_6H_4 8: R = $NHCH(CH_3)_2$ 12: R = $N(C_3H_7)_2$
13: R = $N(CH_2)_5$

Scheme I

dene *p*-sulfonyl and *p*-sulfamoyl derivatives are summarized in Table II.

PHARMACOLOGY

The compounds listed in Table II were tested against 10 Gram-positive and Gram-negative bacteria. Furazolidone was used as a control. The compounds were dissolved in acetone and diluted with water or phosphate buffer (pH 8) to a final concentration of 250 μg/ml. The solvent mixture was acetone–water (0.5:1.5) for Compounds 1, 11, and 17; acetone–buffer (1:1) for Compounds 5 and 6; and acetone–water (1:1) for all other compounds. Paper disks of 9-mm diameter were immersed in the solutions and put on the inoculated surface of penicillin assay seed agar.

All compounds and furazolidone were inactive against *Bordetella bronchiseptica* (ATCC 4617), *Sarcina lutea* (ATCC 341a), and *Proteus vulgaris*[1]. None of the compounds prepared showed significant activity against *Escherichia coli* (0128) at the test concentration. The antibacterial activities of other compounds against *Staphylococcus aureus* (ATCC 6538-P), *Staphylococcus albus* (ATCC 12228), *Klebsiella pneumoniae* (ATCC 10031), *Streptococcus faecalis* (ATCC 8043), *Bacillus cereus* (ATCC 1178), and *Bacillus subtilis* (NCTC 3610) are reported in Table III.

[1] Obtained from S. S. Pfizer Laboratories, Tehran, Iran.

EXPERIMENTAL[2,3]

***para*-Substituted Benzoylhydrazides**—A *para*-substituted benzoyl ethyl ester (0.02 mole) was dissolved in 25 ml of ethanol, and then 0.025 mole of 99% hydrazine hydrate was added to the hot solution. After refluxing the reaction mixture for 1 hr, the solvent was distilled off and the residue was crystallized from aqueous ethanol (Table I).

5-Nitro-2-furfurylidene *p*-Sulfonyl- and *p*-Sulfamoylbenzoylhydrazides—To a hot solution of 0.01 mole of the appropriate benzoylhydrazide in 25 ml of alcohol, a solution of 0.01 mole of 5-nitro-2-furaldehyde in 5 ml of alcohol was added. After standing for 3 hr at room temperature, the crystalline mass was filtered and recrystallized from alcohol (Table II).

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ˣ To whom inquiries should be directed.

[2] Melting points were taken on a Kofler hot stage microscope and are uncorrected. The IR spectra were recorded using a Leitz spectrograph. NMR spectra were obtained with a Varian A-60A instrument. The mass spectra were recorded on a Varian Mat 111 spectrograph.

[3] All compounds were subjected to IR, NMR, and mass spectroscopy and the results were as expected.

Preparation of 4-[*p*-[Bis(2-chloroethyl)amino]phenyl]butyramides as Potential Antitumor Agents I

JOHN H. BILLMAN and GERALD R. ROEHRIG ˣ

Abstract □ Six of the title compounds were synthesized and submitted for antitumor evaluation. Three of them exhibited significant activity in initial screening tests by the National Cancer Institute.

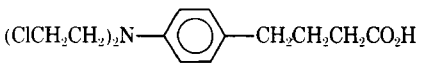
Keyphrases □ Chlorambucil—synthesis and screening of related 4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyramides as potential antitumor agents □ 4-[*p*-[Bis(2-chloroethyl)amino]phenyl]butyramides—synthesized and screened as potential antitumor agents □ Antitumor agents, potential—synthesis and screening of 4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyramides

Chlorambucil (I), 4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyric acid, has been shown to exhibit marked antitumor activity, primarily by virtue of its properties as an alkylating agent. It has found extensive clinical utilization in the treatment of chronic lymphocytic leukemia (1, 2) and ovarian carcinoma (3, 4) as well as in conjunction with other drugs (*e.g.*, 5–7). Chlorambucil exists predominately as the carboxylate ion at physiological pH; under these conditions the nitrogen mustard group has been shown to be significantly more reactive than when the molecule is in the undissociated form (8).

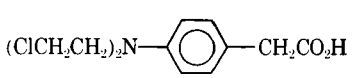
DISCUSSION

Linford (9, 10) postulated that the greater activity of chlorambucil in basic media involves interaction of the chloroethyl groups with the carboxylate groups of the chlorambucil molecule. This interaction would increase the reactivity of the nitrogen mustard group, thus explaining the effect of the length of the alkanoic acid chain and its point of attachment to the benzene ring. This is also thought to be a major factor in explaining the increased selectivity of chlorambucil for proteins. It was noted (11, 12) that with aralkyl carboxylic acids the diffusion through cellular membranes seems to be promoted by unionized carboxyl groups. Several instances noted in the recent literature suggest that various derivatives of cytotoxic aralkyl acids, notably esters and amides, significantly decrease the toxicity of these compounds and may considerably enhance therapeutic indexes. Niculescu-Duvaz *et al.* (13), in a study of nitrogen mustards of various methylbenzoic acids, noted that the methyl ester of one isomer (unavailable as the free acid) showed an LD_{50} value of 300 mg/kg, as opposed to a range of 15–62 for the other acids, with antineoplastic activity similar in degree to the other compounds.

Vollmer *et al.* (14, 15) and Schaeppi *et al.* (16) reported on phenesterine, estradiol mustard, and dehydroepiandrosterone mustard, which are esters of 4-[*p*-[bis(2-chloroethyl)amino]phenyl]acetic acid (II); these appear to be significantly less toxic and more suitable for use than II itself. Bardos *et al.* (17) and Masnyk (18), in comparing a number of nitrogen mustard derivatives of aryl amides, phenols, and aryl acids, showed that the amide and ester derivatives generally appear to be significantly less toxic and more selective. They suggested the possibility that the active component is the original acid, formed by enzymatic hydrolysis of the appropriate ester or amide linkage. Numerous studies have been



I



II

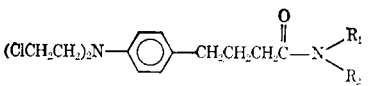


Table I—4-[*p*-[Bis(2-chloroethyl)amino]phenyl]butyramides

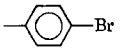
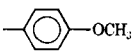
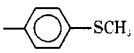
Compound	R_1	R_2	Formula	Pure Yield, %	Melting Point
1	H	C_6H_5	$C_{20}H_{24}Cl_2N_2O$	38.2	114–115°
2	CH_3	C_6H_5	$C_{21}H_{26}Cl_2N_2O$	49.5	Oil
3	H		$C_{20}H_{23}BrCl_2N_2O$	69.7	134.4–135.2°
4	H	$-CH_2CH(CH_3)_2$	$C_{18}H_{28}Cl_2N_2O$	72.7	86–87°
5	H		$C_{21}H_{26}Cl_2N_2O_2$	84.0	123.5–124.5°
6	H		$C_{21}H_{26}Cl_2N_2OS$	58.5	137.5–138.5°

Table II—Analytical Data for 4-[*p*-[Bis(2-chloroethyl)-amino]phenyl]butyramides

Compound	Nitrogen Analysis, %		IR Bands, cm^{-1}	
	Calc.	Found	N—H	C=O
1	7.39	7.56	3290	1655
2	7.12	7.17	—	1655
3	6.12	5.84	3260	1650
4	7.80	7.86	3260	1650
5	6.85	6.67	3270	1655
6	6.56	6.50	3270	1655

Table III—Antitumor Data of 4-[*p*-[Bis(2-chloroethyl)amino]phenyl]butyramides

Compound	NSC Number	L-1210[a] Dose Level[e]	L-1210[a] % T/C[f]	WA[b] Dose Level[e]	WA[b] % T/C[f]	KB[c] ED_{50}[g]	PS[d] Dose Level[e]	PS[d] % T/C[f]
1	136479	8	109	—	—	—	25	120
2	136480	8	110	—	—	—	—	—
3	145697	20	111	—	—	100	—	—
4	128885	8	112	5.3	606[h]	—	—	—
5	128827	8	110	6.1	571	—	—	—
6	128886	8	115	3.2	606[h]	—	—	—

[a] L-1210 is an ascites leukemia. [b] WA is Walker carcinosarcoma 256, intramuscular. [c] KB is a cell culture screen. [d] PS is P-388 lymphocytic leukemia. [e] Milligrams per kilogram body weight. [f] Measured as percentage increase in lifespan. [g] Micrograms per milliliter. [h] Maximum value allowed in test system prior to termination; comparable to chlorambucil.

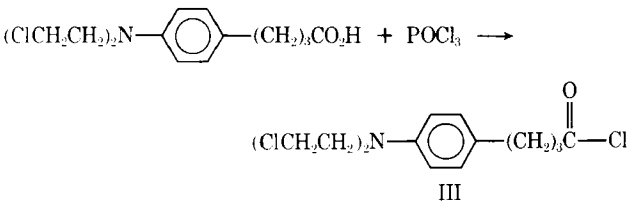
conducted on the premise that a lipophilic compound can be transported across cell walls considerably faster than the corresponding ionic forms, presumably by an appropriate carrier (19–23).

This information led the authors to prepare compounds similar in structure to chlorambucil but in which the carboxylic acid group was converted to a relatively nonpolar amide group. Hopefully, the amides would be transported to the neoplastic cells, perhaps more rapidly than the corresponding acid, after which they would be hydrolyzed to the acid form. Several of the prepared compounds exhibit encouraging antitumor activity in initial stages of testing.

The starting point for the preparation of the amides was the corresponding acid chloride (III), prepared according to the reaction shown in Scheme I. Formation of III was confirmed by appropriate IR spectral bands in the 1800-cm^{-1} region. Once the acid chloride was prepared, it was used immediately for the preparation of the appropriate amides (Scheme II). Tables I–III present analytical and antitumor screening data for the compounds reported.

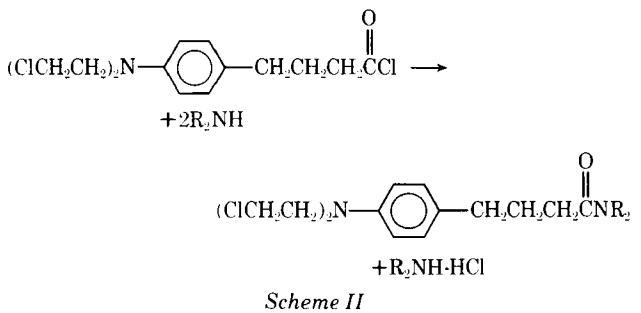
EXPERIMENTAL[1]

4-[*p*-[Bis(2-chloroethyl)amino]phenyl]butyryl Chloride (I) (Scheme I)—Phosphorus oxychloride (35.0 g, 0.229 mole) was added dropwise to a solution of chlorambucil (3.0 g, 9.86 mmoles) in 25 ml of dry chloroform in a 100-ml round-bottom flask fitted with a reflux condenser and drying tube. The reaction mixture was stirred with a magnetic stirrer and was heated with stirring to 60° for 4 hr. After cooling to room temperature, excess phosphorus oxychloride was removed *in vacuo*. The resulting oil was washed several times with petroleum ether (bp 30–60°) to remove any traces of phosphorus oxychloride, dissolved in 10 ml of dry chloroform or acetonitrile, and used immediately for preparation of the amide of choice. The formation of I was confirmed by the presence of the appropriate IR C=O band at 1800 cm^{-1}.



III

Scheme I



Scheme II

Synthesis of the Amides (Scheme II)—The appropriate amine (8.64 mmoles), dissolved in 25 ml of acetonitrile, was added dropwise to a stirring solution of the acid chloride previously obtained in 10 ml of acetonitrile at 0°. The reaction mixture was allowed to warm slowly to room temperature and was washed with water to remove the amine hydrochloride. The aqueous solution was extracted with chloroform, and the extract was dried and passed through a column of neutral and basic (3:1) alumina. The eluate containing the amide was evaporated to one-fourth of its original volume *in vacuo*, and petroleum ether was added. Crystals of the amide were obtained and recrystallized from chloroform–petroleum ether. In the case of the oils, the resulting oil was chromatographed a second time, using the same procedure as before, and the chloroform was removed *in vacuo*.

[1] All melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are corrected. IR spectra were determined on Perkin-Elmer 137 and 237B spectrophotometers. Elemental analyses were performed by Midwest Microlab, Indianapolis, Ind., and Micro-tech Labs, Skokie, Ill.

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[x] To whom inquiries should be directed.

Flame-Ionization and Electron-Capture GLC Determination of 1-(2,6-Dimethylphenoxy)-2-aminopropane in Plasma

ROBERT J. PERCHALSKI*[x], B. J. WILDER*[‡], and R. H. HAMMER[§]

Abstract □ A sensitive flame-ionization GLC method is described for the determination of the new anticonvulsant, 1-(2,6-dimethylphenoxy)-2-aminopropane, in plasma. The method is compared with a simultaneously developed electron-capture technique, which was found to give no advantage applicable to the analysis. Relative standard deviations of replicate samples extracted for analysis by the flame-ionization method are less than 7%. The lower limit of detection of this method is 7 ng/ml.

Keyphrases □ 1-(2,6-Dimethylphenoxy)-2-aminopropane—flame-ionization and electron-capture GLC analysis in human plasma □ GLC, flame ionization and electron capture—analysis, 1-(2,6-dimethylphenoxy)-2-aminopropane in human plasma

The new anticonvulsant drug, 1-(2,6-dimethylphenoxy)-2-aminopropane (I), was tested clinically and shown to be effective in the treatment of psychomotor seizures (1). Two urinary metabolites were characterized (2), and a method for the quantitation of I in urine was published (3). To determine the half-life of this drug and to establish an easily measurable parameter as a basis for dosage adjustment, a method for quantitation of I in plasma was developed[1].

EXPERIMENTAL

Reagents—1-(2,6-Dimethylphenoxy)-2-aminopropane[2] (I) and the internal standard, 1-(2,3-dimethylphenoxy)-2-aminopropane[2] (II), were supplied as the hydrochloride salts and were used without further purification. All extracting solvents were reagent grade for flame work and specially purified[3] for electron-capture work. Water for standards and buffers used in the electron-capture procedure was made by extracting 0.1 *N* HCl (distilled water) four times with 0.1 volume of chloroform, adjusting the pH to 12 with sodium hydroxide pellets, and repeating the extractions. The resulting aqueous phase was redistilled in an all-glass system and stored in a ground-glass stoppered bottle. Trifluoroacetic anhydride[4] and pentafluoropropionic anhydride[5] were used for derivatization.

Apparatus—A gas chromatograph[6] equipped with flame-ionization and scandium tritide (4) electron-capture detectors was used. The columns were 91.5-cm × 2-mm (i.d.) glass U-tubes packed with 2.8% OV-210–3.2% OV-1 on 80–100-mesh Chromosorb W-HP for flame-ionization analysis and 3% OV-17 on 100–120-mesh Gas Chrom Q for electron-capture analysis. Instrument temperatures were 270, 250, and 120° at the detector, injector, and column oven, respectively. The electron-capture detector was operated in the dc mode at a potential of 90 v. The flow of nitrogen through the OV-210–OV-1 column was set at 25.5 ml/min, giving retention times of 4.58 and 6.00 min for the trifluoroacetamide derivatives of I and II, respectively. Nitrogen was also used as carrier gas with the electron-capture detector at a flow rate of 12.0 ml/min, with a detector purge of 35.0 ml/min giving retention times of 3.00 and 4.37 min for the pentafluoropropionamide derivatives of I and II, respectively.

Special centrifuge tubes[7], tapered to a 1.0–1.5-mm diameter capillary at the bottom, were used to obtain almost total recovery of the final chloroform extract.

Procedure—In a 16 × 150-ml culture tube (Teflon-lined screw cap), 2.00 ml of plasma, 0.50 ml of the internal standard (800 ng II/ml of distilled water), 0.5 ml of 2.0 *N* NaOH, and 5 ml of ethyl acetate are combined and shaken for 10 min. After centrifugation, the ethyl acetate is transferred to a tapered centrifuge tube, 1.0 ml of 0.1 *N* HCl is added, and the mixture is shaken for 5 min. The ethyl acetate is aspirated completely after centrifugation, 0.5 ml of 2.0 *N* NaOH and 0.1 ml of chloroform are added to the aqueous phase, and the tube is shaken by hand for 1 min. The tube is centrifuged and the lower phase is transferred to a conical vial[8] with a microsyringe. Trifluoroacetic anhydride (50 μl) is added, and the vial is capped and allowed to stand at room temperature for 30 min. The chloroform and excess reagent are evaporated at room temperature under a stream of nitrogen, and the residue is dissolved in 25 μl of redistilled carbon disulfide. Approximately 1 μl is injected into the chromatograph. Quantitation is by peak height ratio (I/II).

For analysis by electron-capture detection, 1 ml of plasma is extracted and the internal standard concentration is lowered to 80 ng II/ml. All extractions prior to derivatization are performed as already described. Pentafluoropropionic anhydride is substituted for trifluoroacetic anhydride as the derivatizing reagent. After the 30-min reaction time, the chloroform and excess reagent are evaporated at room temperature under a stream of nitrogen and the residue is taken up in 100 μl of chloroform. The chloroform is transferred to a tapered centrifuge tube containing 0.5 ml of 2.0 *N* NaOH and the final extraction is repeated as already described. The organic phase is transferred and evaporated under nitrogen at

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[x] To whom inquiries should be directed.

Flame-Ionization and Electron-Capture GLC Determination of 1-(2,6-Dimethylphenoxy)-2-aminopropane in Plasma

ROBERT J. PERCHALSKI*[x], B. J. WILDER*[‡], and R. H. HAMMER[§]

Abstract □ A sensitive flame-ionization GLC method is described for the determination of the new anticonvulsant, 1-(2,6-dimethylphenoxy)-2-aminopropane, in plasma. The method is compared with a simultaneously developed electron-capture technique, which was found to give no advantage applicable to the analysis. Relative standard deviations of replicate samples extracted for analysis by the flame-ionization method are less than 7%. The lower limit of detection of this method is 7 ng/ml.

Keyphrases □ 1-(2,6-Dimethylphenoxy)-2-aminopropane—flame-ionization and electron-capture GLC analysis in human plasma □ GLC, flame ionization and electron capture—analysis, 1-(2,6-dimethylphenoxy)-2-aminopropane in human plasma

The new anticonvulsant drug, 1-(2,6-dimethylphenoxy)-2-aminopropane (I), was tested clinically and shown to be effective in the treatment of psychomotor seizures (1). Two urinary metabolites were characterized (2), and a method for the quantitation of I in urine was published (3). To determine the half-life of this drug and to establish an easily measurable parameter as a basis for dosage adjustment, a method for quantitation of I in plasma was developed[1].

EXPERIMENTAL

Reagents—1-(2,6-Dimethylphenoxy)-2-aminopropane[2] (I) and the internal standard, 1-(2,3-dimethylphenoxy)-2-aminopropane[2] (II), were supplied as the hydrochloride salts and were used without further purification. All extracting solvents were reagent grade for flame work and specially purified[3] for electron-capture work. Water for standards and buffers used in the electron-capture procedure was made by extracting 0.1 *N* HCl (distilled water) four times with 0.1 volume of chloroform, adjusting the pH to 12 with sodium hydroxide pellets, and repeating the extractions. The resulting aqueous phase was redistilled in an all-glass system and stored in a ground-glass stoppered bottle. Trifluoroacetic anhydride[4] and pentafluoropropionic anhydride[5] were used for derivatization.

Apparatus—A gas chromatograph[6] equipped with flame-ionization and scandium tritide (4) electron-capture detectors was used. The columns were 91.5-cm × 2-mm (i.d.) glass U-tubes packed with 2.8% OV-210–3.2% OV-1 on 80–100-mesh Chromosorb W-HP for flame-ionization analysis and 3% OV-17 on 100–120-mesh Gas Chrom Q for electron-capture analysis. Instrument temperatures were 270, 250, and 120° at the detector, injector, and column oven, respectively. The electron-capture detector was operated in the dc mode at a potential of 90 v. The flow of nitrogen through the OV-210–OV-1 column was set at 25.5 ml/min, giving retention times of 4.58 and 6.00 min for the trifluoroacetamide derivatives of I and II, respectively. Nitrogen was also used as carrier gas with the electron-capture detector at a flow rate of 12.0 ml/min, with a detector purge of 35.0 ml/min giving retention times of 3.00 and 4.37 min for the pentafluoropropionamide derivatives of I and II, respectively.

Special centrifuge tubes[7], tapered to a 1.0–1.5-mm diameter capillary at the bottom, were used to obtain almost total recovery of the final chloroform extract.

Procedure—In a 16 × 150-ml culture tube (Teflon-lined screw cap), 2.00 ml of plasma, 0.50 ml of the internal standard (800 ng II/ml of distilled water), 0.5 ml of 2.0 *N* NaOH, and 5 ml of ethyl acetate are combined and shaken for 10 min. After centrifugation, the ethyl acetate is transferred to a tapered centrifuge tube, 1.0 ml of 0.1 *N* HCl is added, and the mixture is shaken for 5 min. The ethyl acetate is aspirated completely after centrifugation, 0.5 ml of 2.0 *N* NaOH and 0.1 ml of chloroform are added to the aqueous phase, and the tube is shaken by hand for 1 min. The tube is centrifuged and the lower phase is transferred to a conical vial[8] with a microsyringe. Trifluoroacetic anhydride (50 μl) is added, and the vial is capped and allowed to stand at room temperature for 30 min. The chloroform and excess reagent are evaporated at room temperature under a stream of nitrogen, and the residue is dissolved in 25 μl of redistilled carbon disulfide. Approximately 1 μl is injected into the chromatograph. Quantitation is by peak height ratio (I/II).

For analysis by electron-capture detection, 1 ml of plasma is extracted and the internal standard concentration is lowered to 80 ng II/ml. All extractions prior to derivatization are performed as already described. Pentafluoropropionic anhydride is substituted for trifluoroacetic anhydride as the derivatizing reagent. After the 30-min reaction time, the chloroform and excess reagent are evaporated at room temperature under a stream of nitrogen and the residue is taken up in 100 μl of chloroform. The chloroform is transferred to a tapered centrifuge tube containing 0.5 ml of 2.0 *N* NaOH and the final extraction is repeated as already described. The organic phase is transferred and evaporated under nitrogen at

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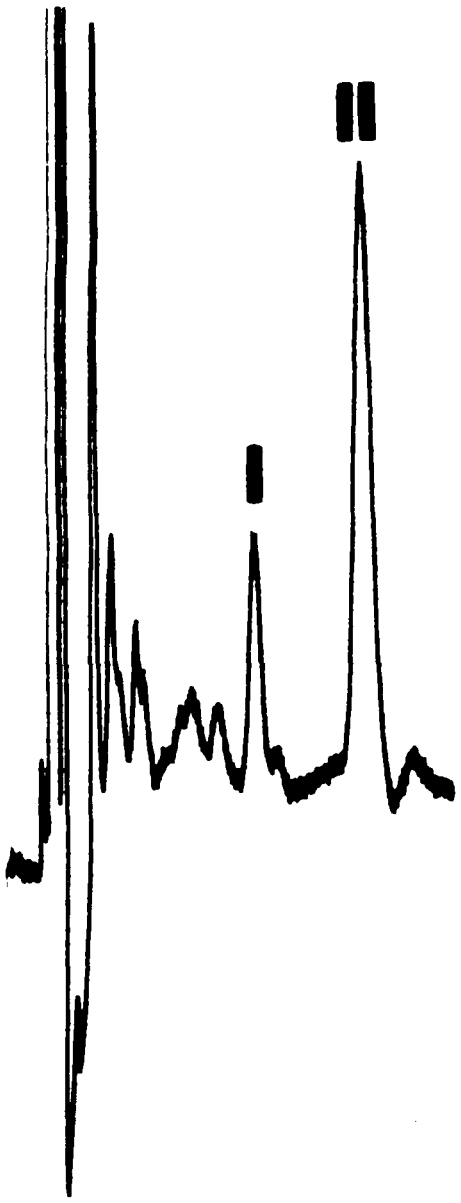

Figure 1—*Electron-capture chromatogram of extract of standard plasma (1 ml) with I and II added; I and II denote the pentafluoropropionamide derivatives of the drug (7.2 ng/ml) and internal standard (40.7 ng/ml), respectively.*

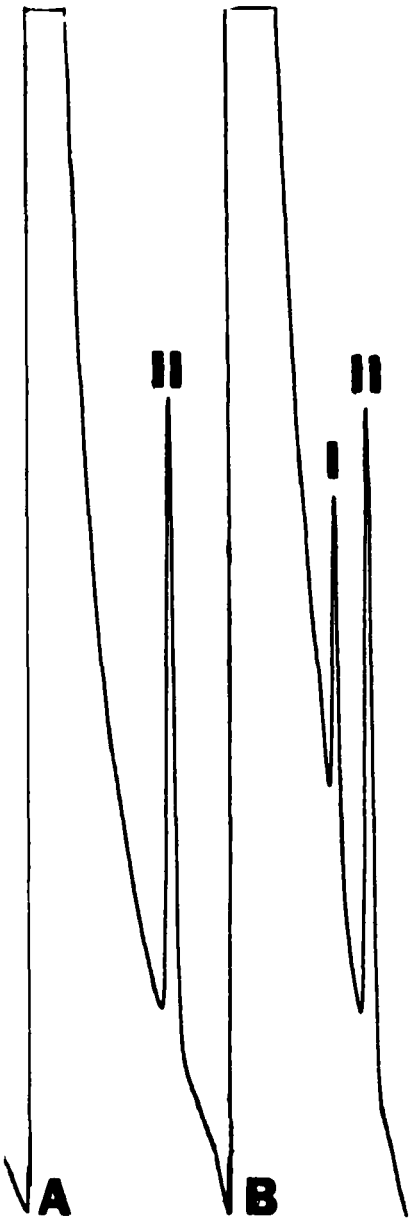

Figure 2—*Flame-ionization chromatograms of an extract of standard plasma (A) and an extract of plasma drawn from a patient taking 400 mg I/day in multiple doses (B) with the internal standard added to both; I and II denote the trifluoroacetamide derivatives of the drug and internal standard (202 ng/ml), respectively. The measured concentration of the drug in the patient sample was 99.3 ng/ml.*

room temperature, and the residue is dissolved in 25 μl of toluene. About 1 μl is injected into the chromatograph.

RESULTS AND DISCUSSION

Two primary factors prompted the consideration of both flame-ionization and electron-capture GLC during method development. Electron-capture GLC was indicated by the required lower limit of detection of 0–10 ng/ml. However, a scan of plasma samples from test patients on high doses of I (1200 mg/day) showed that the upper limit had to be at least 2000 ng/ml. Therefore, a wide linear dynamic range, characteristic of the flame-ionization detector, was also required.

The chromatogram of a plasma sample extracted according to the electron-capture procedure is shown in Fig. 1. Although the lower limit of detection of this technique was almost an order of magnitude lower than that of the flame-ionization technique, the linear range of the detector was such that multiple calibration curves were needed to cover the required concentration range. By using carbon disulfide as the final solvent, a lower limit of 7 ng I/ml was obtained with flame-ionization detection. Since this was well below the lowest recorded plasma concentration (16.8 ng/ml) of any patient taking the drug, this technique was chosen for routine analyses.

Figure 2 shows chromatograms of standard plasma and a patient's plasma extracted for flame-ionization detection. The standard plasma used was taken from a pool made up of samples from patients taking one or more of the common anticonvulsants, including ethosuximide, phenobarbital, primidone, diphenylhydantoin, and carbamazepine. No interference resulted from the presence of these drugs.

The stability of the trifluoroacetamide derivatives of I and II and the reproducibility of the flame-ionization method were checked simultaneously by chromatographing five replicate samples (102 ng/ml) six times over 90 hr. Table I shows the peak height ratios of the samples at various times after evaporation of the derivatizing reagent. The relative standard deviations of the ratios for each sample (RSD_{stab}) show that there is no apparent

Table I—Stability of Trifluoroacetamide Derivatives and Reproducibility of Flame-Ionization Procedure

Injection Time	Peak Height Ratio[a] Sample Number 1[c]	2[c]	3[c]	4[c]	5[c]	σ_{repr}	$\bar{X}$	RSD_{repr}, %
0.5 hr[b]	0.544	0.481	0.511	0.536	0.499	0.026	0.514	5
3.0 hr[b]	0.540	0.485	0.498	0.541	0.506	0.026	0.514	5
17.0 hr[b]	0.571	0.502	0.495	0.554	0.516	0.034	0.528	7
43.0 hr[b]	0.560	0.510	0.479	0.542	0.519	0.031	0.522	6
69.0 hr[b]	0.544	0.507	0.512	0.564	0.546	0.025	0.535	5
90.0 hr[b]	—	0.486	0.510	0.549	0.497	0.028	0.511	6
σ_{stab}	0.014	0.013	0.013	0.011	0.018			
$\bar{X}$	0.552	0.495	0.501	0.548	0.514			
RSD_{stab}, %	2.6	2.7	2.6	2.0	4			

[a] I-trifluoroacetamide/II-trifluoroacetamide. [b] Time after formation of trifluoroacetamide derivatives. [c] Replicate extracts of standard plasma with I and II added at 102 and 202 ng/ml, respectively.

degradation of the derivatives after 90 hr at room temperature in carbon disulfide. Sample 1 went to dryness between 69 and 90 hr, and the 90-hr result was rejected by application of the Q-test at a 99.5% confidence level. The relative standard deviations of the ratios of the five replicates at each time (RSD_{repr}) show satisfactory reproducibility of the technique. Standard calibration curves typically had linear correlation coefficients of 0.999, and the slopes of three separate curves run over a 2-month period had a relative standard deviation of 5%.

The particular sequence of extractions before the derivatization step served to eliminate any drying of a solution containing I in the free base form. Results indicated a large decrease in recovery and reproducibility if the amine was taken to dryness, even at room temperature. With this procedure, recovery was 70.3 ± 2.7% at a concentration of 65 ng I/ml and 73 ± 5% at 650 ng I/ml. Glassware was not silanized, but recovery may be increased by this technique.

The range of plasma concentrations found in patients taking normal amounts of I (200–400 mg/day in multiple doses) varied from 20 to 650 ng/ml. In single-dose studies, peak plasma levels were reached in 1–3 hr and the subsequent half-life varied from 4 to 7.5 hr. Chronic administration of I resulted in an increase in the low plasma levels over what would be expected from an equivalent dose given for 1 day; however, the peak plasma levels did not increase. For example, one patient was given 100 mg of I at 8:00 am, 2:00 pm, and 10:00 pm for 8 days. Blood was drawn at 1 and 4 hr after the initial morning dose. The plasma concentration at 1 hr increased from 45.9 to 193 ng/ml in 8 days, while the concentration at 4 hr remained at 243 ± 48 ng/ml over the entire period. The drug appears to be stable in refrigerated plasma for at least 1 month. Samples analyzed at the beginning and end of this period gave identical results within the limits of precision of the technique.

The metabolites of I (2) do not interfere with quantitation of the parent drug since the retention times of their trifluoroacetamide derivatives are much longer than those of the drug and internal standard under the chromatographic conditions employed. Preliminary investigation has indicated that the hydroxymethyl metabolite [1-(2-hydroxymethyl-6-methylphenoxy)-2-aminopropane] may be present in plasma in the low nanogram range; however, conclusive proof has not yet been obtained.

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ˣ To whom inquiries should be directed.

Effect of Cadmium on Palmitic Acid Metabolism by Rat Liver Homogenate

NAGY A. FARID *, GORDON S. BORN *x, WAYNE V. KESSLER *, and VIRGIL L. ANDERSON ‡

Abstract □ An *in vitro* study was conducted to determine the effect of cadmium on the metabolism of sodium palmitate-1-^{14}C by rat liver homogenate through the collection of $^{14}CO_2$. Concentrations of cadmium ion ranged from 8.9 to 115.7 μM. The results indicated a significant inhibitory effect of cadmium on palmitate metabolism. Increased cadmium concentrations caused a corresponding decrease in $^{14}CO_2$ production in a linear relationship.

Keyphrases □ Cadmium—effect on palmitic acid metabolism by rat liver homogenate, respirometry □ Palmitic acid—effect of cadmium on metabolism by rat liver homogenate, respirometry □ Metabolism, fatty acid—effect of cadmium on palmitic acid metabolism by rat liver homogenate

Many studies (1) on the toxicity and distribution of cadmium indicate that it accumulates mainly in the liver and kidneys regardless of the route of administration. It is not considered to be an essential element in humans or animals (2), but its influence on liver function has been demonstrated in both (3–8). In the liver, a large portion of the cadmium can be found in the homogenate supernate (9, 10) and it has been found in the mitochondria (10). The literature does not contain information about the effects of cadmium on fatty acid metabolism. Since the liver is one organ where cadmium accumulates to a large extent and the liver is known to oxidize about 50% of the free fatty acids in the body at rest (11), the effect of cadmium on the metabolism of sodium palmitate by rat liver homogenate was studied.

EXPERIMENTAL

The experimental design was a randomized complete block (12) with four replicates and eight cadmium concentrations plus a control in each. The liver of one rat was used in each replicate.

Compounds—Sodium palmitate-1-^{14}C[1] had a specific activity of 27.8 mCi/mmole. TLC of the free acid on silica gel G plates with two solvent systems showed one spot. The solvent systems were ethyl acetate–benzene–acetic acid (69:29:2) and petroleum ether (bp 60–80°)–ether–acetic acid (80:4:2), giving R_f values of 0.74 and 0.31, respectively. Of the total activity on the plate, 98% was detected in the palmitic acid spot. Unlabeled sodium palmitate was added to make the specific activity about 2 μCi/mg. Bovine serum albumin[2], fraction V, fatty acid poor, was used to solubilize the sodium palmitate. A fresh sodium palmitate–bovine serum albumin solution was prepared on the day of each replicate. The buffer used was calcium-free Krebs–Ringer phosphate buffer, pH 7.4. Calcium was omitted since it was found to decrease the solubility of the fatty acid (13).

Animals—Sprague–Dawley descendent male albino rats weighing 150–210 g[3] were used. Prior to use, each was fasted for 19 hr but had free access to water. All were sacrificed at about 10:45 am by decapitation on the day of use. The liver was removed and perfused with 15 ml of ice-cold buffer to remove blood. About 5 g was cut from different lobes and homogenized with 4 ml of buffer. The homogenate was centrifuged for 10 min at 2500 rpm to remove unground tissue.

System and Incubation—A Warburg respirometer with a shaking rate of 82 shakes/min was used. The temperature of the water bath was kept at 37°. The reaction flasks had two side arms, only one of which was used, and a center well. A 4 × 4.5-cm folded filter paper moistened with an agent[4] to trap the evolving $^{14}CO_2$ was placed in the center well. The total volume of fluid in each flask was 3 ml and consisted of 2.5 ml of buffer, 0.1 ml of cadmium acetate solution (or water), 0.2 ml of sodium palmitate–bovine serum albumin solution in the buffer in the side arm, and 0.2 ml of the liver homogenate supernate.

There were two flasks for each of the eight concentrations of cadmium and two that contained water for controls. The concentration of cadmium ion in the medium was 8.9, 17.8, 44.5, 53.4, 71.2, 89.0, 106.8, or 115.7 μM. The concentration of bovine serum albumin ranged from 40.6 to 43.5 μM based on a molecular weight of 69,000 (14) and that of sodium palmitate ranged from 0.100 to 0.110 mM (0.16–0.18 μCi/flask).

The flasks were attached to the manometers and placed in the water bath for 45 min, with the system opened to the air and shaking. After this incubation period, the systems were closed and the palmitate–bovine serum albumin solution was dropped into the main compartment from the side arm. Four hours after the addition of the palmitate–bovine serum albumin, the flasks were removed and placed in an ice bath.

Counting and Calculations—From each flask, a 0.2-ml sample was counted in a liquid scintillation spectrometer. The entire filter paper containing the $^{14}CO_2$ was also counted. Samples and background were counted three times for 10 min each. The counting data were converted to disintegrations per minute with correction for counter efficiency. The effect of cadmium on palmitate metabolism was determined using the following two equations:

$$\frac{A}{15B + A} \times 100 = N_f \text{ or } N_c \qquad \text{(Eq. 1)}$$

$$\frac{N_f}{N_c} \times 100 = Y \qquad \text{(Eq. 2)}$$

where A was the disintegrations per minute of $^{14}CO_2$ for a given flask, B was the disintegrations per minute of the 0.2-ml sample from a given flask, N_f was the percentage of total activity in the $^{14}CO_2$ for a given flask that contained cadmium, and N_c was the

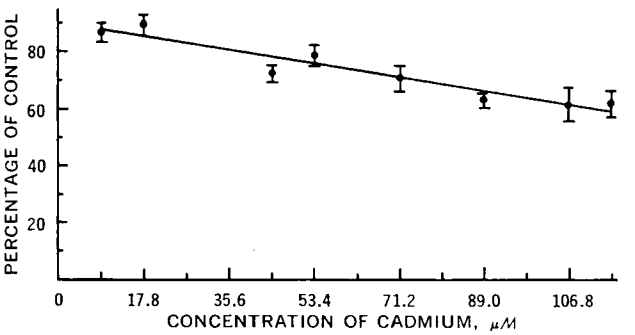

Figure 1—*Effect of cadmium on carbon dioxide production from palmitate by rat liver homogenate. Error bars represent the standard error of the mean.*

[1] International Chemical and Nuclear Corp., Irvine, Calif.
[2] Calbiochem, San Diego, Calif.
[3] Laboratory Supply Co., Indianapolis, Ind.

[4] Hydroxide of Hyamine 10-X, a 1 M solution in methanol, Packard Instrument Co., Downers Grove, Ill.

Table I—Effect of Cadmium on $^{14}CO_2$ Production

Cadmium Concentration in Flask, μM	Percentage of Control				
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average ± SE
8.9	95.24	91.25	72.81	95.83	86.41 ± 3.36
	88.57	86.25	71.05	90.28	
17.8	97.14	77.50	—[a]	97.22	89.18 ± 3.62
	91.43	78.75	—[a]	93.06	
44.5	76.19	80.00	69.30	83.33	72.39 ± 3.03
	—[a]	62.50	63.16	72.22	
53.4	87.62	71.25	62.28	83.33	78.52 ± 3.62
	86.67	73.75	—[a]	84.72	
71.2	77.14	63.75	67.54	95.83	70.65 ± 4.58
	78.10	68.75	54.39	59.72	
89.0	70.48	55.00	57.02	65.28	62.95 ± 2.62
	75.24	60.00	55.26	65.28	
106.8	80.95	62.50	47.37	77.78	61.56 ± 6.09
	—[a]	—[a]	53.51	47.22	
115.7	59.05	55.00	52.63	72.22	61.78 ± 4.59
	—[a]	—[a]	52.63	79.17	

[a] Rejected results. See text for explanation.

average percentage of total activity in the $^{14}CO_2$ for the two control flasks of each replicate; N_c was the 100% reference level for comparison, and Y was the result from a given flask relative to the control and was termed the percentage of control.

RESULTS AND DISCUSSION

The effect of cadmium on $^{14}CO_2$ production from labeled sodium palmitate is shown in Table I. For eight of the 64 flasks, the results were rejected because it was known that the filter papers were contaminated with the palmitate–bovine serum albumin solution and their activities did not represent only the collected $^{14}CO_2$. This problem was anticipated prior to the initiation of the experiments; the experimental design chosen allowed for the missing data.

An unequal subclass numbers least-squares analysis of variance (12) was run to determine differences between treatments and replicates. The difference between treatments was highly significant ($p = 0.005$), indicating that cadmium does affect carbon dioxide production from palmitate. A difference ($p = 0.005$) between replicates was probably due to day-to-day variability that was not entirely removed by expressing the results as the percentage of control. The treatment by replicate interaction was not significant even at the conservative probability level of $p = 0.25$ (15). Therefore, the values for the different doses could be pooled for calculation of the means shown in Table I.

A regression analysis was run on the means. The means rather than the individual values were used to average over the day-to-day variability and to allow for a better test on dose effects. The departures from a linear trend were not significant ($p = 0.25$), so the trend was considered linear. The equation for the straight line shown in Fig. 1 is:

$$\bar{Y} = 90.20 - 0.269X \qquad \text{(Eq. 3)}$$

where X is the concentration of cadmium ion in the flask. This equation is valid only for the range in cadmium-ion concentrations studied. It is not valid for concentrations less than 8.9 μM. The estimated linear correlation coefficient is 0.96, and the 95% confidence interval for the true correlation coefficient is $0.77 < \rho < 0.99$.

The number of concentrations studied was limited by the number of reaction flasks available on the Warburg respirometer. The highest concentration studied was dependent on the limited solubility of cadmium ion in the buffer, and the lowest concentration was chosen to give a substantial inhibitory effect. A preliminary study showed that the effect was slight and highly variable at concentrations lower than those used in this experiment. At low concentrations the inhibitory effect probably is not linear. Determining the relationship with certainty at this low range was not important enough to warrant running the large number of replicates required.

In this study, $^{14}CO_2$ was measured while the other oxidation products, the ketone bodies, were not. Ketone body formation from palmitic acid by rat liver homogenate has been reported to be strongly inhibited by arsenite (13), and the resemblance between cadmium and arsenite ions in their inhibitory actions has been cited (16). It was also reported that the production of the ketone bodies ceased 1 hr after the start of incubation (17). Their concentration in the medium then started to decrease gradually over the remaining 3 hr, while that of the $^{14}CO_2$ rose over this time. The net effect of not measuring the amounts of ketone bodies in this experiment was that the effect of cadmium on the metabolism of palmitate might have been slightly underestimated.

This *in vitro* study showed that increased cadmium concentration in the medium caused a corresponding decrease in the production of $^{14}CO_2$ resulting from the metabolic oxidation of sodium palmitate by rat liver homogenate. The inhibition was about 60% of the control at the highest cadmium concentration. This suggests that cadmium inhibits enzymes involved in the transformation of palmitic acid to acetyl coenzyme A and/or enzymes involved in the metabolism of acetyl coenzyme A to carbon dioxide.

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[x] To whom inquiries should be directed.

COMMUNICATIONS

Particle-Size Distributions of Milled Granulations and Powders

Keyphrases □ Particle size—distributions in milled granulations and powders, theory and equations □ Granulations, milled—particle-size distributions, theory and equations □ Powders—particle-size distributions, theory and equations

To the Editor:

Steiner *et al.* (1) recently demonstrated that milling of monodisperse granulations gives rise to log-normally distributed particle sizes. Log-normal distributions are not uncommon for solid particles; crystallization often leads to log-normal distributions that are truncated from below and above, a fact that has been explained (2) on theoretical grounds. However, no explanation has been put forth on why milling produces a log-normal distribution. Such an explanation may be sought in the following simplfied theoretical model.

Let it be assumed that a sample of N particles of size x_0 is milled and that a fraction, α, of the particles is "halved" on each impact. It follows that there will be $(N)(1 - \alpha)$ particles left of the original size after one impact and $(N)(1 - \alpha)^2$ left after two impacts. After the second impact there will be $(N)(4)(\alpha)(1 - \alpha)$ of size $x_0/2$ and $(N)(4)(\alpha^2)$ of size $x_0/(2^2)$. The total number of particles after the second impact is $(N)(1 + \alpha)^2$. The number of particles of size x_0, $x_0/2$, $x_0/(2^2)$, and $x_0/(2^3)$ after zero, one, two, three, and m impacts is listed in Table I. It is seen from the bottom line of the table that the total number of particles after m impacts is $(N)[(1 + \alpha)^m]$. After m impacts the possible particle sizes are $x_0/(2^p)$, where p is an integer between zero and m. The number of particles of size $x = x_0/(2^p)$ is seen from the last column of Table I to be:

$$N\begin{bmatrix} m \\ p \end{bmatrix}[(2\alpha)^p][(1 - \alpha)^{m-p}] \qquad \text{(Eq. 1)}$$

i.e., since the number of particles is $(N)[(1 + \alpha)^m]$, the fraction having a particle size of $x = x_0/(2^p)$ is given by:

$$Pr[(x_0/2^p)] = [(1 + \alpha)^{-m}]\begin{bmatrix} m \\ p \end{bmatrix}[(2\alpha)^p][(1 - \alpha)^{m-p}] \qquad \text{(Eq. 2)}$$

The right-hand side of Eq. 2 is a normalized binomial expression which, for large values of m, approaches a normal distribution. The *sizes* of the particles, however, are log-linear; for instance, $\log(2^{p+1}) - \log(2^p) = \log 2$, regardless of the value of p. Hence, for large m, the distribution described by Eq. 2 approaches a log-normal equation.

The same type of argument holds for any situation where the particle breaks into a number of equally sized fractions. The model is, of course, greatly simplified since it assumes that an impact will always produce the same (whether two, three, or more) fractional particles, and it does not account for removal of fines. However, the model describes the main feature of milling which accounts for the log-normally distributed end-product.

(1) G. Steiner, M. Patel, and J. T. Carstensen, *J. Pharm. Sci.*, 63, 1395(1974).

(2) R. R. Irani and C. F. Callis, "Particle Size: Measurement,

Table I—Number of Particles Remaining after Number of Indicated Impacts[a]

Size	Impacts				
	0	1	2	3	m
x_0	N	$(N)(1 - \alpha)$	$(N)[(1 - \alpha)^2]$	$(N)[(1 - \alpha)^3]$	$(N)[(1 - \alpha)^m]$
$x_0/2$		$(N)(2\alpha)$	$(N)(2)(2\alpha)(1 - \alpha)^b$	$(N)(2)(2\alpha)[(1 - \alpha)^2]$	$(N)\begin{bmatrix} m \\ 1 \end{bmatrix}(2\alpha)[(1 - \alpha)^{m-1}]$
$x_0/4$			$(N)(4)(\alpha^2)$	$(N)(3)[(2\alpha)^2](1 - \alpha)$	$(N)\begin{bmatrix} m \\ 2 \end{bmatrix}[(2\alpha)^2][(1 - \alpha)^{m-2}]$
$x_0/8$				$(N)[(2\alpha)^3]$	$(N)\begin{bmatrix} m \\ 3 \end{bmatrix}[(2\alpha)^3][(1 - \alpha)^{m-3}]$
Total	N	$(N)[(1 + \alpha)]$	$(N)[(1 + \alpha)^2]$	$(N)[(1 + \alpha)^3]$	$(N)[(1 + \alpha)^m]$

[a] Initial number is N. [b] The general procedure is to note that the amount of size $x_0/2$ disappearing is $N\alpha 2\alpha$; $N2\alpha(1 - \alpha)$ remains. The amount of $x_0/2$ produced from size x_0 is twice $N\alpha(1 - \alpha)$, so the amount of size $x_0/2$ produced is $(N)(2\alpha)(1 - \alpha)$. The total is $(2)(N)[\alpha(1 - \alpha)]$.

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Interpretation and Application," Wiley, New York, N.Y., 1963, p. 39.

J. T. Carstensen[x]
Mahendra Patel
School of Pharmacy
University of Wisconsin
Madison, WI 53706

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[x] To whom inquiries should be directed.

Alkaloids of *Lindera benzoin* (L.) Blume (Lauraceae) I: Isolation and Identification of Laurotetanine

Keyphrases □ Laurotetanine—isolation and identification from *L. benzoin* □ *Lindera benzoin* (L.) Blume (Lauraceae)—isolation and identification of laurotetanine

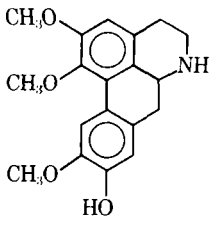
To the Editor:

The isolation of aporphine alkaloids from other *Lindera* species (1, 2) and the reported cytotoxic activity of certain aporphines (3, 4) led to this investigation of the native *Lindera benzoin* (L.) Blume (Lauraceae), known commonly as spicebush.

L. benzoin plants were collected locally[1]. The stems were separated from the leaves and roots, air dried, and ground for extraction. Preliminary TLC examination indicated one major and at least five minor nonquaternary alkaloids.

The stem material (36.3 kg) was defatted with *n*-hexane[2], and this alkaloid-free extract was reserved for further study. The defatted material was macerated and exhaustively extracted with methanol by percolation. The viscous residue (1.6 kg) remaining after removal of the methanol *in vacuo* at 40° was stirred with 0.1 *M* citric acid, vacuum distilled to remove the remaining traces of methanol, and filtered. The acid solution was alkalinized with ammonium hydroxide solution and extracted with chloroform. A phenolic fraction (38.5 g) and a nonphenolic fraction (4.0 g) were prepared from the chloroform solution by the method of Johns and Lamberton (5).

The dark-brown phenolic fraction was extracted with benzene to yield an orange benzene-soluble residue (23.5 g) which was found to contain the major alkaloid (I) by TLC [silica gel G, chloroform–methanol (9:1)]. A total of 0.4 g of I was separated from 0.6 g of the benzene-soluble phenolic residue by preparative



I

TLC [silica gel[3], chloroform–methanol (9:1)] and was further purified by precipitation from hot cyclohexane to yield a cream-colored amorphous precipitate (II) which failed to crystallize from the usual solvents. Compound II gave a single spot with several TLC solvent systems and $[\alpha]D^{25}$ + 94.6° (c 0.38 in ethanol). The mass spectrum showed the apparent molecular ion at *m/e* 327 (72%) followed by other prominent peaks at *m/e* 326 (100%), 312 (23), 310 (10), and 296 (16). The UV absorption spectrum showed λ_{max} (C_2H_5OH) 219 (log ε 4.51), 282 (4.2), and 304 (4.19) nm; λ_{max} (C_2H_5OH–NaOH) 232 and 329 nm; and λ_{min} (C_2H_5OH–NaOH) 270 nm.

The NMR spectrum run in deuterated chloroform, with tetramethylsilane as the internal standard, showed one aromatic proton at δ8.08, 6.61, and 6.08, three methoxyl protons at δ3.70, six methoxyl protons at δ3.91, and no *N*-methyl protons as shown by the absence of a signal at the δ2.5 region. The specific rotation (6), mass spectroscopy (7, 8), UV (9), and NMR (10) data suggested that II could be identified as the noraporphine alkaloid laurotetanine. The identification of II as laurotetanine was confirmed by co-TLC and by comparison of the IR, UV, and mass spectroscopy data with those derived from authentic laurotetanine. Furthermore, preparation of the *N*-methyl hydrobromide derivative of II furnished a crystalline product that gave melting points and IR, UV, and mass spectroscopy data in good agreement with those derived from authentic *N*-methyllaurotetanine hydrobromide.

This is the first report in the literature of the occurrence of laurotetanine in *L. benzoin*. The isolation and identification of other alkaloids from *L. benzoin* will be reported later.

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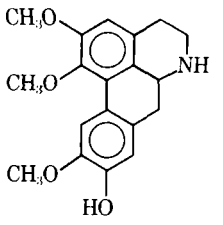

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Philip A. Babcock ˣ
Alvin B. Segelman

Department of Pharmacognosy
College of Pharmacy
Rutgers—The State University
New Brunswick, NJ 08903

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BOOKS

REVIEWS

A Guide to Molecular Pharmacology-Toxicology, Parts I and II. Edited by R. M. FEATHERSTONE. Dekker, 95 Madison Ave., New York, NY 10016, 1973. Part I, pp. 1-425; Part II, pp. 427-811. 16 × 24 cm. Price: Part I, $29.50; Part II, $27.75.

These two volumes consist of twenty-one chapters concerning a number of areas of what may be defined as molecular pharmacology-toxicology. Certainly, the emphasis in every chapter but one is quite heavily molecular. There is no apparent theme or continuity among the chapters which cover a number of topics including cell membranes, sugar transport, receptor isolation, analgesic and curare receptors, general anesthetics, conformational change, spectroscopic tools, quantum pharmacology, *etc.* Indeed the editor states that the book is intended as a guide, rather than a comprehensive map, to some areas of molecular pharmacology where the authors are actively working and to demonstrate to the reader how the systems discussed can generate information of general significance about big molecule-little molecule interactions. It would be generally agreed that it is a capital mistake for any scientist to confine his or her attention and reading to too narrow a span and there is a necessary role for essays and reviews that indicate the approaches and problems in near and distant fields.

The volumes in question contain a rather heterogeneous selection of essays with considerable variations in length, depth of treatment, and quality. Some chapters take the broad view and others are narrowly confining. Among the best chapters are those by Miller and Smith which present an admirably clear discussion of intermolecular forces and general anesthesia and by Shirachi, Chan, and Trevor on the isolation of pharmacological receptors both of which are very clear expositions taking care to outline the basic problems and the necessary background. More specialized chapters include that by Casy which is a most interesting review of analgesic structure-activity relationships and by Taylor and Kitz on the curare receptor and acetylcholinesterase, respectively, and by Martin, Rousseau, and Baxter on steroid hormones. Other chapters present fairly cursory surveys of NMR, ESR, ORD, and CD spectra and it is not apparent that they are likely to give much insight into the physical and intellectual power of these approaches. There is a certain amount of unnecessary overlap among the chapters—general anesthetic action is covered twice, curare receptors in three places, *etc.*

On the whole I think that the book is only partially successful in meeting its objectives. However, it is certainly worthy of general perusal and close reading of some chapters, but it is doubtful that many individuals will feel driven to purchase these volumes, the combination of uneven quality and high price likely proving an effective deterrent.

Reviewed by D. J. Triggle
Department of Biochemical Pharmacology
State University of New York at Buffalo
Buffalo, NY 14214

Reference Values in Human Chemistry. Edited by N. G. SIEST. S. Karger AG, Medical and Scientific Publishers, Arnol-Bocklin-Strasse 25, CH-4011 Basel, Switzerland. 347 pp. 17 × 24.5 cm. Price $46.90. (Distributed in United States by Albert J. Phiebig, Inc., P. O. Box 352, White Plains, NY 10602).

This publication contains material presented at the Second International Conference on Automatization and Prospective Biology in October 1972. Information on usual values in clinical biochemistry and reference values in human physiological and pathological biochemistry is presented.

The general theme of two of the seven sessions was a detailed study of analytical, physiological intraindividual and interindividual variations of reference values, followed by a study of variations due to age, sex, tobacco, and climatic factors. Chemical parameters, *e.g.*, urea, glucose, and electrolytes, steroids, and plasmatic enzymes are discussed.

The effects of food intake and standard and particular diets are described in both healthy and hyperlipemic subjects.

An examination of drug interferences both from an analytical point of view as well as from physiological and pharmacological points of view is of particular interest.

Staff Review

Alcohol Intoxication and Withdrawal: Advances in Experimental Medicine and Biology, vol. 35. Edited by M. M. GROSS, Plenum, 227 West 17th St., New York, NY 10011, 1973. 422 pp. 17 × 25.5 cm. Price $24.00.

This volume is based upon the symposium entitled "Experimental Studies of Acute Alcohol Intoxication and Withdrawal," a part of the Proceedings of the 30th International Congress on Alcoholism and Addiction, held in Amsterdam, The Netherlands, in September 1972.

Alcoholism is a worldwide social and medical problem. Experimental research on alcoholic intoxication and withdrawal is presented in this publication, including investigations on the metabolic, psychological, and neurological impact of alcohol and alcoholism. Human and animal research into such areas as mechanisms of alcohol, tolerance and physical dependence, biochemical changes in response to alcohol, and disruption of sleep, memory, and psychological equilibrium caused by alcohol intake and withdrawal is covered.

Staff Review

Philip A. Babcock ˣ
Alvin B. Segelman

Department of Pharmacognosy
College of Pharmacy
Rutgers—The State University
New Brunswick, NJ 08903

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Reviewed by D. J. Triggle
Department of Biochemical Pharmacology
State University of New York at Buffalo
Buffalo, NY 14214

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Philip A. Babcock ˣ
Alvin B. Segelman

Department of Pharmacognosy
College of Pharmacy
Rutgers—The State University
New Brunswick, NJ 08903

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The authors are grateful to Dr. M. Tomita of Kyoto College of Pharmacy, Kyoto, Japan, and to Dr. A. Rüegger of Sandoz Ltd., Basle, Switzerland, for generously supplying reference samples of laurotetanine and *N*-methyllaurotetanine hydrobromide, respectively.
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ADVICE, NOT ADVOCACY

Give us "advice, not advocacy." That's what Paul O'Neill, Director of the Human Resources Division of the Office of Management and Budget (OMB), told a meeting of the Advisory Council to the National Institutes of Health this past summer. In essence, he was a spokesman for the Federal government, and his message was directed at those in the scientific community who seek to have input into the process of government and of government decision making. His audience included scientists who were irritated because they felt their viewpoints were often ignored, and they wondered why this was so.

At least to a degree, this same distinction between *advice* and *advocacy* seems to have been involved over the past few years in the stresses and strains of the relationship between the American Pharmaceutical Association and its Academy of Pharmaceutical Sciences. In essence, the Academy has complained that the Association wasn't listening, and the Association has complained that the Academy was speaking the wrong language and shouting besides.

As generally follows in such situations, misunderstandings and ill-feelings grew on both sides.

However, it is not our purpose here to review this past history, but rather to take note of a very recent development that appears to signal that the communications gap has now been bridged.

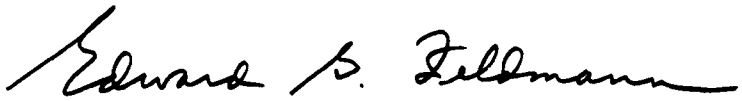
On rather short notice, the need arose for APhA to develop a list of drugs, based upon certain criteria, in order to be able to make recommendations to the Department of Health, Education, and Welfare in connection with the drug reimbursement proposal as first announced by HEW Secretary Weinberger last December.

One criterion which APhA wished to embody in developing its list was "the least potential or history of bioavailability differences or other significant quality related problems." Pure and simple, this is a technical question for which it appeared that a direct, science-based response could be given.

Recognizing this, APhA requested the Academy to undertake an assessment of those drugs fulfilling the various other criteria, and from among them, to identify the drugs meeting this specific criterion pertaining to drug quality.

The Academy responded to this challenge in a most gratifying manner. APS President Busse convened a special committee, and a well-organized, yet concise report was promptly submitted to the Association. Within hours after its receipt, the report was being used in discussions with HEW officials. The net result was that APS had effective input into an APhA activity in a constructive, mutually beneficial manner for the public interest.

We feel that, were he to be asked, OMB's Mr. O'Neill would attribute this successful experience to the fact that the APS report avoided advocacy in favor of sound, factual advice. In the final analysis, this is chiefly what science is all about.



Content Uniformity

The large amount of potentially useful work on computer simulation of the performance of various sampling tests by Flann[1] is to be applauded. The nonconventional, complex "operating characteristic" curves and the admittedly arbitrary measures used for reliability and robustness are to be lamented because they do not adequately summarize the data. Although the author refers to the two standard texts on variables sampling and several drug sampling papers, he does not subscribe to their use of "percentage defective" because he somehow feels the concept is not appropriate for drugs. This feeling produces the features mentioned above, so it deserves close scrutiny.

The author says (pp. 191, 192) that, for practical reasons, in the absence of pharmacological dose–response data ". . . pharmaceutical chemists have, for years, temporized . . . [by using] percentage of defectives . . . or outsiders, this classification implies that all units within the range are pharmacologically equally satisfactory and those outside the range are equally unsatisfactory." The word "equally" is the hinge upon which the matter turns. The author interprets it strictly, while most chemists (and others) interpret it loosely, in a practical sense.

Admittedly, the units within the specified range do differ in potency but, if the range is small enough, the resultant effect on patients would be swamped by other variations. The question then becomes: "How small must the range be for each drug?" The answer depends on, first, the dose–response curve and, second, the proportion that the drug content variation contributes to the *total* response variation composed of intrapatient variation in absorption, distribution, response, metabolism, and elimination as well as drug content variation.

The only study touching on this topic that comes to mind was one[2] in which aspirin at nine dose levels, at 2.5% intervals, was given to 20 fasted, young males *per os*. The authors stated that ". . . it is obvious that the inter-dose variation is quite trivial compared to that related to inter-individual divergencies and time-dependent individual differences."

However, this lack of definite information for many drugs is no reason to abandon the use of percentage of outsiders; the use of mean and standard deviation suffers from the same lack. What multiplier of the standard deviation is appropriate and what limit is best?

An advantage of the use of percentage of outsiders is that its interpretation is the same for any reasonable shape of a lot's distribution of unit drug content. Since, as Flann states, "There is ample evidence that an appreciable proportion of the many distributions of drug content are significantly nonnormal," the application of the mean and standard deviation (so easy for normal distributions) becomes more complicated and uncertain. For instance, with a mean of 100% label claim and a standard deviation of 5.8%, the theoretical number of single-dose units (per 100,000 units) beyond the ±15% limits is 1000 for a normal distribution (Flann, Table I), 60 for the platykurtic distribution defined in his Table IV, and 2000 for the leptokurtic distribution—the patients would think these are hardly lots of equal quality.

Another viewpoint of the nonnormality feature shows that three product lots with identical means of 100% label claim and the same percentage of outsiders, 1%, but with differently shaped distributions have standard deviations of 5.8% for normal, 7.0% for platykurtic, and 5.0% for leptokurtic distributions. The producers could say: "Here are three lots of equal quality. Why should the platykurtic one be more likely to be rejected, as would happen by the standard deviation test?" The use of percentage outsiders as the measure of uniformity seems fairer to both producer and consumer.

Finally, the reduction from two parameters, mean and standard deviation, to characterize content uniformity to one parameter, percentage outsiders, will simplify the analysis of the data and lead to a comprehensible presentation of results. The use of percentage outsiders has been vindicated so the author can, in good conscience, use his valuable simulation data to evaluate the various sampling tests.

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Tablet Strength Testing

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In any event, we feel that both objectives are essential for the growth of pharmaceutical sciences. On one hand, the testing and comparing of several tablet testers presently available on the market would lead to the knowledge of their performance in one's own laboratory, so that one becomes aware of the advantages and disadvantages associated with the testers. This knowledge should be disseminated and shared by others in the field. On the other hand, a study of the physical laws governing the mechanisms would lead to the development of new instruments to be marketed in the future. In either case, statistical inference should be used to arrive at valid conclusions.

Dr. Newton appears to have misunderstood the variation reported in our paper. He stated that the variation was limited and contradicted what one might expect. Table I of our paper consists of *geometric* means and geometric standard errors, as indicated in the footnote to the table. The use and presentation of the geometric means and geometric standard errors are appropriate because initially the data were transformed to logarithms for the purpose of *stabilizing* the variances.

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N. R. Bohidar
J. I. Karr
F. A. Restaino
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REVIEW ARTICLE

Effects of Prolonged Administration of Certain Antihypertensive Agents

BHAGAVAN S. JANDHYALA ˣ, DAVID E. CLARKE, and JOSEPH P. BUCKLEY

Keyphrases □ Antihypertensive agents—effects of prolonged administration, review □ Reserpine—effects of prolonged antihypertensive therapy, literature review of acute and chronic studies □ Hydrochlorothiazide—effects of prolonged antihypertensive therapy, literature review of acute and chronic studies □ Thiazide diuretics—effects of prolonged antihypertensive therapy, literature review of acute and chronic studies □ Guanethidine—effects of prolonged antihypertensive therapy, literature review of acute and chronic studies

Cardiovascular diseases are, for the most part, chronic conditions and the therapeutic agents used to treat these diseases must be administered over prolonged periods. One prevalent cardiovascular disease is hypertension, and it has been estimated that there are approximately 23,000,000 hypertensive patients[1] in the United States today and that hypertension is responsible for approximately 60,000 deaths per year. Hypertension has been identified as the major contributing factor to the development of atherosclerosis (1) and also appears to be of major importance in the development of congestive heart failure, coronary thrombosis, and uremia.

Most information concerning the pharmacology, toxicology, and mechanisms of action of the antihypertensive compounds has been obtained from acute experiments which, in many instances, utilized anesthetized animals and/or isolated tissues. Since one or more of these compounds might be administered throughout the lifespan of the hypertensive patient, it is possible that the effects of prolonged administration may vary greatly from those observed upon acute administration. The purposes of this paper are: *(a)* to review the available preclinical and clinical data on three of the more widely used antihypertensive compounds (reserpine, hydrochlorothiazide, and guanethidine); *(b)* to identify, wherever possible, disagreements that may have been found between the chronic effects of the compounds and data obtained from acute experiments; and *(c)* to point out the implications of these differences in relation to patient care.

It is feasible that prolonged administration of one or more of these compounds could influence markedly the absorption or metabolism of the compound administered, receptor sensitivity, the biochemical effects or even the known pharmacological actions of the drug, the control of the cardiovascular system by the central nervous system (CNS), the inherent characteristics of the autonomic innervation to the myocardium or the vasculature, and possibly other organ systems. Investigations concerning the prolonged effects of antihypertensive compounds in experimental animals or human patients could provide data that would lead to a better understanding of how the therapeutic agent produces its efficacious and/or toxic effects. This information should aid the practitioner in better utilizing these compounds in the treatment of

[1] See *NIH Record*, 25(3), 1(1973).

hypertensive cardiovascular disease and possibly other cardiovascular diseases, as well as in limiting toxic effects produced by the compounds themselves upon chronic administration. A review of the literature indicates that comparatively little is currently being done to investigate the pharmacological effects of antihypertensive compounds administered to experimental animals for prolonged periods.

RESERPINE

Since Wilkins (1) first introduced the use of rauwolfia serpentina in the United States for the treatment of hypertension, many studies have confirmed the efficacy of this drug and its active constituents in lowering blood pressure in hypertensive patients. Oral administration of large doses of reserpine (3–9 mg) or parenteral administration in a dosage as low as 1 mg can produce a marked reduction in arterial blood pressure in humans. Unfortunately, large doses of reserpine produce a high incidence of undesirable side effects, the more prominent being drowsiness and depression (2).

The hypotensive effect of reserpine is due to a selective interference with transmission of nerve impulses from postganglionic sympathetic nerve endings to smooth muscle cells in the arteriolar and venous beds. Norepinephrine, the chemical transmitter, is depleted by reserpine from its storage sites at postganglionic sympathetic nerve endings (3, 4); as a result, less transmitter is released by nerve impulses and little or no response of smooth muscle cell develops following reserpine administration. Sympathetic nerve blockade develops only after almost complete depletion of tissue norepinephrine has occurred. Lee (5) observed that no significant alterations occurred in the responses to sympathetic nerve stimulation in the right atrium, adrenal glands, and nictitating membrane unless the catecholamine content had fallen below 50% of normal values. On the other hand, measurable responses were still observed when catecholamine content was well below 10%. These studies further indicated that the sensitivity of the right atrium to the catecholamine-depleting action of reserpine was highest and that of the adrenal medulla was lowest. Similar findings concerning differential sensitivity of various organs were reported (6), suggesting that very small amounts of endogenous norepinephrine are capable of maintaining a residual function of the adrenergic nerves (7). These observations are consistent with the view (8) that the degree of depletion of catecholamine stores by reserpine depends not only on the dose of reserpine but also on the rate of turnover of the stores. The adrenal medulla is known to have a low rate of turnover and is very resistant to the depleting action of reserpine; the heart, on the other hand, has a high rate of turnover and is very sensitive to the depleting action of reserpine (9).

Since reserpine produces widespread depletion of peripheral sympathetic transmitters, it is widely believed that the action of reserpine on the sympathetic system is exclusively confined to the postganglionic neuronal sites. During the 1st to 3rd hr after intravenous injection of low and medium doses of reserpine, Bein (10–12) found no impairment of function in the peripheral sympathetic pathways, even though relaxation of the nictitating membrane, a decrease in the arterial pressure, bradycardia, and inhibition of centrally mediated pressor reflexes were fully apparent. Significant impairment of peripheral sympathetic transmission is usually found after a much longer latency period. For example, Bianchi and Fargier (13) noted that depletion of adrenal catecholamines in dogs and a lack of responsiveness to nerve stimulation could not be demonstrated until 8–24 hr after administration of reserpine. Reports concerning the effects of reserpine on preganglionic sympathetic activity are conflicting. Bein (11) observed a decrease in the electrical activity of preganglionic sympathetic cardioaccelerator nerve in the cat together with bradycardia, whereas Iggo and Vogt (14) found no change in the electrical activity of the cervical sympathetic fibers. A slow, progressive, and marked diminution in efferent splanchnic nerve activity was observed in cats and dogs (15), as was a moderate depression of efferent splanchnic nerve activity (16). Plummer (17) demonstrated that when cats are pretreated with reserpine, the nictitating membrane displays a greater hypersensitivity to epinephrine than to norepinephrine, the condition thus resembling preganglionic rather than postganglionic denervation.

The two main features of action of reserpine on which its widespread use in hypertension is based are the lowering of arterial blood pressure associated with bradycardia (which in animals appears to be species dependent) and a sedative effect (18). In general, depletion of the neurohumoral transmitter substances in the adrenergic system results in inhibition or block of adrenergic neurotransmission. Thus, one expects a fall in blood pressure, a reduction in peripheral vascular resistance, and bradycardia accompanied by a reduction in cardiac output. Reserpine, however, has been shown to increase renal plasma flow and the glomerular filtration rate in conscious dogs (19). In humans, the dilation of peripheral vessels is mainly confined to skin (20, 21). The ability of reserpine to diminish concentrations of norepinephrine in atrial appendages of humans has been demonstrated (22).

Whelan and Skinner (20) also found that reserpine exerted a prolonged dilator action on peripheral blood vessels but did not interfere with sympathetic reflex activity or with vasoconstrictor activity of ephedrine and amphetamine. Reserpine elicited its normal response in the chronically sympathectomized limb, whereas the constrictor response of the limb vessels to ephedrine and amphetamine has been shown to be dependent on the integrity of the sympathetic nerves. Thus, if reserpine exerts its effects by depleting tissue norepinephrine, the tissue norepinephrine stores may not play a part in reflex activity but would be involved in mediating tonic influences on the vessels (20). Other studies indicated that, following prolonged reserpine administration, reflex venoconstriction and reflex arteriolar constriction in

the forearm of normal subjects, elicited by leg exercises and by cold stimulation, are inhibited (23).

After prolonged treatment with reserpine, its pattern of activity against various vasoconstrictor agents changes. Therefore, results obtained in acute experiments do not necessarily apply to the more complex pharmacological situations arising after chronic treatment (24).

The usefulness of reserpine used alone or in combination with other hypotensive agents in controlled studies in reducing morbidity and the mortality rate due to hypertension has been demonstrated (25, 26). Moyer *et al.* (27) compared the effects of chronic reserpine therapy in humans to those observed in dogs following acute intravenous administration. Following intravenous administration of the drug to dogs, cardiac output was quite variable and was sometimes reduced during the reduction of blood pressure; however, these alterations were of short duration and usually of little consequence. Renal hemodynamics and electrolyte excretion rates were not altered significantly after intravenous administration of reserpine to dogs. When reserpine was administered either intravenously or orally for 3 months to patients with hypertension, renal blood flow, glomerular filtration rates, and renal excretion of water and electrolytes were not altered, and there was no evidence of renal toxicity or depression of renal function (27). Thus, at least in these studies, no visible differences were noted between acute and chronic studies.

Adams *et al.* (28) investigated certain physiological effects of prolonged oral administration of reserpine to mongrel dogs for a period of 12 months. The doses of reserpine (0.137–0.274 mg/dog/day)($\bar{X}$ = 26 μg/kg/day) selected were comparable to clinical doses used in veterinary medicine. In these studies, while hematologic values of untreated dogs were within normal limits (of canines), the hematocrit and hemoglobin content were consistently and significantly lower in reserpine-treated dogs; in addition, the leucocyte count was consistently lower in the treated population (28). These findings are in contrast with the reports by Earl (29), who failed to observe any effects of chronic reserpine upon hematologic components in dogs, and by West *et al.* (31), who found no effect of reserpine on leucocytosis in leukemic mice. However, in the studies of Earl, reserpine was administered for only 5 days a week in contrast to daily administration in the studies reported by Adams *et al.* (28). Marley and Pare (30) also observed a reduced hemoglobin value and an increased plasma portion of the hematocrit following reserpine treatment in humans, and they suggested that reserpine produced a hemodilution *via* fluid retention; they rejected the possibility of altered hematopoiesis. However, impaired hematopoietic processes could also induce a microcytic condition, and the peripheral edema observed by these investigators may have been a sequela to reserpine-induced cardiotoxicity (or failure), as noted by many investigators, rather than an indication of fluid retention leading to cardiac decompensation, as suggested by these workers. The most significant finding in the studies of Adams *et al.* (28) was the presence of right ventricular dilations in 50% of the reserpine-treated animals but in less than 10% of the placebo group.

Adverse effects of reserpine on myocardium have been the concern of many investigators. Cohen *et al.* (32) examined the cardiac effects of chronic administration of 0.25 and 0.5 mg reserpine/day for 20–48 days in patients with essential hypertension. Their studies indicated that therapeutically administered reserpine may decrease cardiac output, increase atrioventricular (A–V) conduction, and augment second degree heart block during induced tachycardia in a hypertensive population. Similar antihypertensive therapeutic doses of reserpine induced signs of cardiac failure in humans (30, 33). Disruption of normal ECG patterns and premature ventricular contractions likewise were associated with therapeutic dose levels of reserpine (34, 35). Perera (33) reported five cases that, within a week after daily administration of 0.4 mg of reserpine, developed edema accompanied by moderate exertional dyspnea; all symptoms were reversed within a week after discontinuation of the drug. In one patient, when reserpine therapy was reinstituted, the same symptoms reappeared within 1 week.

Histological examinations of the heart and other organs removed from animals pretreated for 1 or several days with 1–1.25-mg/kg total doses of reserpine revealed severe degenerative changes in cardiac muscle, characteristic signs of severe stress in the adrenal cortex, and centrilobular changes in liver indicative of heart failure and increased venous pressure (36). Similar histopathological alterations in myocardial structure of cats treated with large doses of reserpine were also noted (37). Large doses (1 mg/kg) or small doses administered over prolonged periods produced marked changes similar to those conditions in which oxygen supply or oxygen utilization of the myocardium is substantially decreased (38). Other investigators (39) conducted histochemical and electron microscopic studies on the effects of reserpine on the heart muscle of mice. In these studies, a disarrangement in lipid and glycogen metabolism and depression of mitochondrial oxidative enzymes were demonstrated. Ultrastructural studies showed significant changes in mitochondrial architecture in reserpine-treated mice. These findings supported the hypothesis that reserpine could cause damage to the myocardium and provided anatomical evidence of previously reported uncoupled oxidative phosphorylation in reserpine-treated animals. The doses used in these studies were 0.125 mg/mouse (or approximately 6 mg/kg), with two additional doses at the end of the 5th and 10th days. Wilcken *et al.* (40) also demonstrated mitochondrial swelling and fragmentation in dogs following daily administration of 25 μg/kg reserpine.

Withrington and Zaimis (41) studied the cardiovascular effects of prolonged administration of small doses of reserpine (10 μg/day for 5–26 weeks). In these studies, mean arterial blood pressure and heart rate were lowered significantly in reserpine-treated animals, and atropine treatment or bilateral vagoto-

my made little difference in these resting lower levels of heart rates. The administration of epinephrine and norepinephrine produced larger positive chronotropic and inotropic responses in reserpine-treated groups. Pressor responses induced by both epinephrine and norepinephrine in reserpine-treated cats were markedly greater than those noted in control groups, while the sensitivity of the hindlimb vasculature to epinephrine, norepinephrine, and isoproterenol was unchanged. Furthermore, epinephrine administration also resulted in various forms of cardiac irregularities, especially ectopic ventricular contractions in reserpinized cats, the hearts of these animals were unable to sustain the prolonged increase in heart rate brought about either by the administration of isoproterenol or by electrical stimulation. The investigators concluded that chronic administration of reserpine resulted in alterations in myocardial function; the greater pressor responses to epinephrine and norepinephrine in reserpine-treated cats were due to a greater increase in cardiac output rather than due to an increase in sensitivity of the vasculature (41). In these studies, no direct measurements of cardiac output were made. The investigators assumed that greater inotropic effects (contractile force) must have resulted in greater output from the heart, despite the occurrence of marked cardiac irregularities following the administration of catecholamines. If increased contractility did result in increased cardiac output to a greater degree than in untreated groups, one could assume that hearts of the treated animals were ejecting blood effectively, which would not be the case if cardiac function was indeed depressed by reserpine treatment. This is not to say that there were no alterations in cardiac function following reserpine treatment, evidence of which has been documented by these and other investigators. Normal relationships between contractility and cardiac output may not exist in a depressed heart, as shown in other studies (42). Administration of epinephrine and norepinephrine to dogs treated chronically with small doses of reserpine resulted in a greater increase in the blood pressure than in control groups (42). Following catecholamine administration, a greater elevation of contractile force did not result in a greater increase in cardiac output. These studies indicated that greater pressor responses to catecholamines in reserpine-treated animals were due to the marked elevation of total peripheral resistance rather than to cardiac output. Also, one cannot assume that the alterations in the responsiveness of the peripheral vasculature to catecholamines is essentially the same in all vascular beds. Investigators (43, 44) have clearly demonstrated that, following chronic reserpine treatment to dogs, there was a twofold increase in sensitivity of α-adrenergic receptor activity in femoral arteries, while no such alteration in the sensitivity was noted in the mesenteric vasculature. Thus, the assumptions made by Withrington and Zaimis concerning peripheral vascular sensitivity to reserpine may not be valid, and in these studies greater pressor responses to catecholamines could be due to greater increases in peripheral resistance in the reserpine-treated cats.

Several other workers (45, 46) disagreed with the opinion that reserpine may induce adverse myocardial changes. These studies demonstrated: *(a)* that reserpine does not change contractile force of the cat heart (46), *(b)* that it affects neither contractility in the papillary muscles of the cat (47) and in the isolated rabbit atria (48) nor the interval strength relationship of the isolated kitten heart (49), and *(c)* that it exercises beneficial influences on the recovery of isolated myocardium from anoxia (50). The oxygen consumption of rabbit atria has been found to undergo no changes after reserpine (48). Several investigators (34, 35, 51, 52) observed various alterations in human and animal ECG's following reserpine treatment. Hensler (51) reported the occurrence of extrasystoles and bigeminy which disappeared when the reserpine therapy was discontinued. Wilson and Wimberley (34) recorded premature ventricular contractions in hypertensive patients receiving reserpine. Schreader and Etzl (35) observed a consistent presence of premature ventricular contractions in patients receiving reserpine and digitalis. The investigators concluded that rauwolfia alkaloids produce premature ventricular contractions in all patients, but more readily in patients receiving digitalis (35). Lown *et al.* (52) demonstrated that reserpine enhanced the toxic effects of acetylstrophanthidin upon the atrium, A–V conduction system, and ventricles. Dick *et al.* (53) observed that simultaneous administration of reserpine and digitalis may induce arrhythmias and that this was not due to low serum potassium. The capacity of reserpine to enhance the toxicity of digitalis has been demonstrated by other investigators both *in vivo* and *in vitro* (54–56). Large doses of reserpine depressed digitalis-induced arrhythmias and this effect of reserpine was due to a nonspecific generalized depression of myocardium by reserpine (57, 58).

Cohen and colleagues (59) studied the effects of reserpine therapy on cardiac output and A–V conduction in patients with essential hypertension. In patients receiving reserpine (0.25–0.5 mg) daily for 20–48 days, there was a prolongation of A–V conduction time. This effect of reserpine may be due to an increased refractory period resulting from catecholamine depletion, as suggested by Gaffney *et al.* (60). These changes in A–V conduction (second degree A–V block) indicate a potential source of clinical difficulty when reserpine is administered to a patient with a preexisting A–V conduction disturbance (52, 60, 61).

Further observations on the adverse effects of chronic reserpine administration on myocardium were made (62). These studies in guinea pigs indicated that reserpine induced ECG changes characteristic of progressive myocardial damage. Similarly, Roberts and Modell (63) reported a high fatality rate from reserpine in dogs in which heart block was induced experimentally. In a series of four animals, two died after two doses of reserpine (0.1 mg/kg) given on successive days. The other two animals survived three such daily doses. Even in the case of a single dose of 0.1 mg, two of the 14 dogs so treated died

within a day of injection. Changes in QRS time and QRS configuration were often seen, and occasionally as many as four distinctly different QRS complexes were observed. Postmortem examination revealed an enlarged heart, ascites, and pulmonary edema, and the investigators concluded that the cause of death was probably heart failure. It was also found that *N*-methylatropine given at the time of the peak effect of reserpine did not alter the auricular and ventricular rates. The authors concluded that vagal mechanism seems to play no role in the bradycardia following reserpine administration (63).

Studies of Withrington and Zaimis (41) also supported this conclusion in that administration of atropine or sectioning of the vagi did not alter the resting heart rate levels in the reserpine-treated animals. These studies supported the view that bradycardia to reserpine involves a direct cardiac depressant component. Withrington and Zaimis (41) also reported that reserpine-treated cats were unable to sustain adequately a marked and prolonged increase in heart rate induced either by electrical stimulation or by the administration of isoproterenol. In the drug-treated animals, isoproterenol produced a marked and prolonged tachycardia, but the positive inotropic responses were less pronounced and were usually followed by a secondary depression of the heart contraction. The cardiac muscles in these animals apparently fatigued more easily under the influence of the abrupt and marked increase in the heart rate. Similar results were obtained when heart rates were increased by electrical stimuli. Possible adverse effects of reserpine on the myocardium received further support from the findings of Taylor (64), who noted that reserpine given to hypertensive patients with myocardial embarrassment may precipitate acute insufficiency if the heart is further subjected to additional stress such as heavy exercise, hypoxia, or anesthesia.

Withrington and Zaimis (41) postulated that catecholamine depletion is only one effect of reserpine and that the progressive deterioration of the myocardium is the result of a biochemical lesion which develops due to continued treatment or an increase in the daily dose. In support of this hypothesis, they indicated certain histochemical and biochemical changes accompanying reserpine-induced myocardial abnormalities. These included accumulation of stainable lipid and of glycogen and an increase in phosphorylase. Furthermore, it was possible to correlate these effects with disturbances in hydrogen transport associated with the Krebs cycle as demonstrated by the mitochondrial enzyme succinic dehydrogenase.

Effects of prolonged administration of reserpine (18–39 μg/kg po) to mongrel dogs for 12–13 months were reported (42). There was a moderate decrease in both systolic and diastolic blood pressures after 2 weeks of treatment; however, these alterations were restored to pretreatment levels during the subsequent 2–3 weeks. There was a gradual and consistent decrease in the heart rate of the treated animal; at the end of 12–13 months of treatment, heart rates remained significantly below control levels. A variety of autonomic tests conducted in these dogs revealed that prolonged reserpine treatment did not affect blood pressure responses to hypoxia, cold pressor test, exercise, and intravenous administration of sodium nitrite and phenylephrine. However, postural hypotension was evident when the animals were subjected to tilt (60° head-up position).

Hexamethonium produced a marked initial increase in heart rate and blood pressure in untreated dogs, followed by a prolonged hypotensive response. However, in the treated dogs, the hypotensive response was absent while the pressor and tachycardia responses of equal magnitude persisted. Since it has been established that tachycardia to hexamethonium in conscious animals is predominantly due to vagal blockade (65, 66), bradycardia to reserpine may not be due to enhanced vagal activity. While pressor responses to phenylephrine were essentially identical in both treated and untreated groups, the accompanying reflex bradycardia was markedly inhibited by reserpine treatment. Based on the pressor responses of phenylephrine, one may conclude that intrinsic α-adrenergic receptor activity was not altered by chronic reserpine treatment. Lack of a hypotensive response to hexamethonium, inhibition of reflex bradycardia to phenylephrine, and inhibition of tyramine pressor responses suggested marked inhibition in sympathetic tone to the cardiovascular system. These conclusions were further confirmed in the studies conducted in the same animals under pentobarbital anesthesia, in which blood pressure, responses to bilateral carotid occlusion, and hexamethonium were markedly inhibited. Furthermore, in the reserpine-treated animals, there was a reduction in cardiac output and left ventricular work in comparison with the untreated dogs. While there was a marked increase in contractile force and rate of tension development in the ventricular myocardium of the reserpine-treated dogs, there was no corresponding increase in stroke volumes. Systemic function curves obtained in these studies indicated that reserpine treatment did not alter the ability of the peripheral vasculature to return blood to the heart (42). Gross pathological examination revealed marked dilation of the right ventricles in these and other reserpine-treated dogs (28). The authors suggested that reserpine-induced ventricular dilation resulted in a stretch of initial ventricular fiber length. As a consequence, there were a greater contractile force and rate of tension development which were associated with increased energy consumption (67). However, this increase in energy consumption was not followed by an increase in work output, since stroke volumes were not altered. The authors suggested that these data indicated diminished efficiency of the right ventricle of the dogs treated with reserpine (42).

In a similar group of dogs that received reserpine for 12 months, there was an attenuation of sympathetic neuronal function in the isolated perfused mesenteric vessels (44). The decrease in sympathetic nerve activity could be restored by infusion of norepinephrine, indicating that chronic reserpine failed to alter neuronal uptake mechanisms. There was no alteration in α-adrenergic receptor activity in the per-

fused mesenteric vessels (44). *In vitro* studies also suggested that, while there was a twofold increase in α-adrenergic receptor activity in the femoral artery, no such alterations were noted in the superior mesenteric artery (43). In conscious reserpine-treated animals, pressor responses induced by phenylephrine were essentially the same as in control animals while pressor responses to norepinephrine were potentiated in the treated dogs under pentobarbital anesthesia. Thus, it is difficult to explain the differences in the responsiveness of reserpine-treated dogs to the pressor effects of phenylephrine and norepinephrine. Pentobarbital anesthesia itself may have some influence on the α-adrenergic receptor activity.

GUANETHIDINE

Guanethidine is an adrenergic neuronal blocking agent (68), which finds considerable use in the management of moderate to severe hypertension. Adrenal medullary release is unaffected (69) and the drug is contraindicated in the treatment of pheochromocytoma.

Guanethidine exerts multiple actions at the level of the adrenergic neuron which are of both pharmacological and clinical significance. Thus, the compound inhibits the neuronal uptake of sympathomimetic amines which are injected or endogenously released (70). Pharmacological doses of guanethidine enhance the peripheral sympathomimetic effects of direct-acting amines, whereas the effects of indirect-acting amines are depressed or abolished (69, 71, 72). Sneddon and Turner (73) showed that the local instillation of guanethidine (5% solution) into the human eye produces identical effects. However, following oral therapeutic doses of guanethidine (4–6 weeks), such interactions are less dramatic, indicating only a partial inhibition of the neuronal uptake process (74). Nevertheless, this inhibition is sufficient to potentiate the responses to injected norepinephrine and released adrenal medullary amines and adequately explains the contraindication for guanethidine in the patient with pheochromocytoma.

Guanethidine-induced impairment of adrenergic neuronal uptake arises because of the relative nonspecificity of this active transport process. Guanethidine itself is a good substrate for transport, thereby gaining access to intraneuronal sites (75–77). This fact partially explains why certain inhibitors of the uptake process (tricyclic antidepressants, some monoamine oxidase inhibitors, the amphetamines, *etc.*) attenuate the adrenergic neuronal blocking activity of guanethidine and reduce or prevent its clinical efficacy as an antihypertensive agent (78, 79). Amphetamine and its analogs are particularly potent antagonists of guanethidine-induced neuronal blockade and have been shown to restore readily the neurogenic function even after full inhibition (69, 80). These agents deplete guanethidine from certain intraneuronal storage sites (81), which are believed to provide a constant but slow release of the drug onto an active "receptor" intimately involved with neuronal blockade (82).

The initial entry of guanethidine into the adrenergic neuron induces a transient sympathomimetic effect which includes hypertension and cardiac stimulation. This effect is particularly evident in both humans and animals after intravenous injection and is dose related. This action may be qualitatively described as a tyramine-like effect of guanethidine and, as with tyramine (83), the effectiveness of the released norepinephrine is enhanced by the concomitant inhibition of neuronal uptake. Thus, an intravenous injection of guanethidine is of potential danger in hypertensive patients exhibiting crisis states or in those portraying cardiac arrhythmias. In contrast, the well-known depletion of endogenous norepinephrine by guanethidine occurs more slowly and may be attributed to a "reserpine-like" effect at the level of the intraneuronal vesicles (84, 85). This latter action is interesting from a pharmacological standpoint, since other neuronal blocking agents possessing almost identical guanethidine-like properties (bretylium, bethanidine, chlorobethanidine, and debrisoquin) fail to deplete endogenous norepinephrine (69, 79). All of these latter agents selectively inhibit intraneuronal monoamine oxidase (86–88); guanethidine is the lone exception (89–91). Thus, like guanethidine, other adrenergic neuronal blocking agents may interfere with amine storage mechanisms, but the liberated norepinephrine would be protected from deamination, whereas norepinephrine released by guanethidine is metabolized and lost from the nerve. In this respect, it should be recalled that monoamine oxidase inhibitors, including bretylium, can prevent guanethidine-induced depletion (92, 93). The lack of a monoamine oxidase inhibitory action of guanethidine is of some definite advantage clinically, since in contrast to other adrenergic neuronal blocking agents the responses to injected indirect-acting amines are not vastly exaggerated (94).

The actual mechanism whereby guanethidine blocks adrenergic neuronal function is still debated. Of the theories advanced, membrane stabilization (95), interference with calcium-induced release (96), and local anesthetic action (69, 95) appear the most attractive, although alternative views have been expressed (97–101). The high intraneuronal accumulation of guanethidine (75, 95) could well explain its marked potency and selectivity as a local anesthetic at postganglionic adrenergic neurons as compared with other neuronal elements. In addition, local anesthesia would readily explain membrane stabilization and its inhibitory effect upon calcium-induced release. In fact, the adrenergic neuronal blocking action of guanethidine is reversed by raising extracellular calcium (96, 102, 103). This antagonism should be borne in mind when guanethidine and hydrochlorothiazide diuretics are used concomitantly since overt hypercalcemia has been reported in both dogs (104) and humans (105) with this latter agent. The gross norepinephrine-depleting action of guanethidine plays little, if any, role in its neuronal blocking action. Depletion occurs secondary to inhibition of nerve function (69), even when it is well established. Spriggs (106) showed that the neurogenic function

could be quickly restored with dextroamphetamine. Finally, although it is quite clear that the peripheral adrenergic blocking action of guanethidine is responsible for its potent antihypertensive effect, the possibility of direct additional influences on vascular smooth muscle tone is suggested by experiments in which guanethidine elicited vasodilation in the perfused foreleg of reserpine-pretreated dogs (107).

The properties of guanethidine summarized so far were elucidated primarily from acute or subacute experimentation. By comparison, little detailed information is available concerning the chronic pharmacological and clinical effects of this compound. Recently, however, several publications have dealt with the chronic toxicity of guanethidine upon certain adrenergic neuronal elements in rats.

Gannon *et al.* (108) and Burnstock *et al.* (109) reported that the administration of guanethidine (5 mg/kg/day ip for 18 weeks) resulted in a greater depletion of norepinephrine in the genital organs than in the heart, cerebral arteries, or superior cervical ganglion. Within 2 weeks of discontinuing treatment, the fluorescence intensity of catecholamines in the ganglion, cerebral arteries, and heart appeared normal, whereas the genital organs failed to recover even after 6 months. Likewise, Evans *et al.* (110) demonstrated that chronic guanethidine treatment (10 mg/kg/day ip for 13 weeks) resulted in a marked depletion of norepinephrine from the short adrenergic neurons innervating the vas deferens. The nerves lost their ability to take up norepinephrine and showed markedly diminished responses to electrical stimulation even in the presence of a 15-fold increase in the effectiveness of added norepinephrine. Norepinephrine depletion existed for at least 6 months after the cessation of treatment and, although the sensitivity to this amine declined, an eightfold increase above normal still persisted. The sensitivity to acetylcholine was unaffected, strongly indicating the lack of any postjunctional, nonspecific supersensitivity.

These observations have a decided clinical relevance since there have been frequent reports of impotence and failure of ejaculation in males receiving chronic guanethidine therapy (111–114). In addition, the findings suggest that this inconvenient side effect may persist for long periods following the cessation of treatment due to functional damage (108) of the intramural neurons.

Although the genital organs are particularly susceptible to guanethidine (109), neurotoxicity has been found in certain sympathetic ganglia following prolonged treatment with the same or higher daily doses (usually 10 mg/kg and more). Using histochemical fluorescence techniques and electron microscopic methods, Burnstock *et al.* (109) found a severe reduction in amine fluorescence in the superior cervical ganglion and rat irides (25 or 30 mg/kg/day ip for 6 weeks). Less than 2% of the nerve cell bodies in the superior cervical ganglion remained at this time, and the mitochondria were badly damaged in these cells. This situation persisted for up to 4 months after the cessation of treatment (longest time period examined). Similar observations were made previously (115–119). These studies disclosed clear evidence of ganglionic cellular lysis, loss of specific and nonspecific cholinesterase activity (up to 70%), and marked catecholamine depletion (20 mg/kg/day ip for 2 weeks). A subsequent publication by these investigators (120) confirmed the presence of marked ultrastructural changes in rat sympathetic ganglia treated with guanethidine (4–40 mg/kg/day ip for 7–14 days) which were partially reversible after 26–56 days of discontinued treatment. Maximal alterations were observed with 20 mg/kg, whereas 4 mg/kg failed to produce any definite overt toxicity. The specificity of guanethidine for sympathetic ganglia was established since no changes were observed in the ganglion nodosum or dorsal root ganglia; furthermore, the liver and salivary glands were unaffected.

These observations might suggest that the neurotoxicity of guanethidine results from its ability to accumulate in adrenergic neurons (discussed previously), although this hypothesis has not been tested. On the other hand, the claimed local selectivity for the ganglion, as opposed to the postganglionic adrenergic terminals (120), would not be predicted, since both the neuronal uptake mechanism and the blood supply are relatively poor in the former region. In fact, the relative selectivity of 6-hydroxydopamine for terminal adrenergic fibers has been explained using this same reasoning (121). In view of the marked biochemical and structural changes reported in the rat superior cervical ganglion (122), it is surprising to find that Downing and Juul (122) reported only very slight or no qualitative impairment of ganglionic potentials in the isolated superior cervical ganglion preparation taken from guanethidine-treated rats (20–40 mg/kg ip for 5–21 days). Recorded action potentials were of normal form but did exhibit a diminished amplitude. The authors left their readers with indefinite conclusions but suggested that the majority of ganglion cells may be more susceptible than others to the damaging effects of guanethidine, thus accounting for the reduction in the amplitude of the recorded potentials. Finally, these workers acknowledged surprise at finding the same sensitivity to acetylcholine in guanethidine-pretreated ganglia as in control preparations in view of their previous reports concerning the marked reduction in cholinesterase activity (115–117).

Several other investigators studied the effects of guanethidine upon developing sympathetic ganglia utilizing newborn rats or mice. Eranko and Eranko (123, 124) described changes in the catecholamine fluorescence properties of the superior cervical and coeliac ganglia taken from newborn rats. Three weeks after the cessation of treatment with guanethidine (20 mg/kg/day ip for 8 days), the total number of ganglionic cells was reduced but the population of small, intensely fluorescent cells was increased markedly (300–500%). This latter interesting effect was not evident in adult rats, although an increased number of small, nonfluorescent, infiltrating cells was found. Whether these cells are related to small intensely fluorescent cells is still undecided, but Eranko and Eranko (123) felt that such a relationship is

unlikely. Presumably such cells correspond to proliferating connective tissue cells also seen in ganglia taken from guanethidine-treated newborn rats and mice (125). In cultured sympathetic ganglia derived from newborn rats, guanethidine failed to induce cytotoxic effects, but the increase in small intensely fluorescent cells was retained with a guanethidine concentration of 1 mg/ml (126). The relevance of this observation has been questioned (119) on the grounds that cultured tissues may not be fully representative of *in vivo* states. Although this objection must gain sympathetic support, considerable evidence to the contrary has been presented (127) in regard to many adrenergic aspects of functional ganglionic mechanisms. On the basis of their culture studies, Eranko *et al.* (126) speculated that guanethidine may evoke indirect cytotoxic effects *in vivo,* possibly by initiating an autoimmune response, by being converted to a toxic metabolite, or by initiating the endogenous formation of a toxic substance. Any or all of these suggestions might explain why guanethidine appears particularly toxic to ganglionic nerve cells as compared with the axons or nerve terminals. Experiments on cultured ganglia with tissue extracts or serum obtained from guanethidine-treated rats should aid in clarifying the possibilities. However, the guanethidine-induced increase in the population of small intensely fluorescent cells would appear to involve a direct component; the lack of such an increase in intact adult rats remains to be explained.

Guanethidine is not the only adrenergic neuronal blocking agent shown to exert ganglionic damage. Bretylium (128), 6-hydroxydopamine (129), debrisoquin, and, particularly, guanacline (130, 131) have also been implicated. Whether the cytotoxic actions of guanethidine and the other compounds occur therapeutically has not been established, but the persistence of postural hypotension after the withdrawal of treatment is a known complication of guanacline therapy (132). Many investigators are reluctant to translate effects seen with high daily doses of guanethidine in rodents to the clinical situation. While this caution is understandable, it should be recalled that very high therapeutic doses of guanethidine are used clinically and that rats are generally far more resistant to pharmacological and toxicological manipulations than are humans. However, even if ganglionic damage does occur in humans, the work of Downing and Juul (122) might suggest little resultant impairment of functional ganglionic transmission.

From the foregoing account, it is clear that the effects resulting from chronic guaethidine treatment can be expected to vary from those seen following single acute doses. However, with the exception of neural toxicity, little is known about such time-dependent variations. One early significant study concerning the chronic pharmacology of guanethidine was conducted by Boura and Green (133). They found that chronic guanethidine treatment could produce highly cumulative effects. For instance, subcutaneous doses of 2.5 mg/kg/day failed to cause nictitating membrane relaxation in cats after 1 or 2 days, but relaxation became progressively more pronounced between 3 and 12 days. Similarly, the responses to nerve stimulation to the same organ were much less after 2 weeks than after a single injection (2.5 mg/kg/day). The same pattern of events is seen clinically after oral administration. The blood pressure response often requires 3–4 days to become apparent and is cumulative in effect, requiring several additional days to attain maximal hypotensive effects (134). Conversely, Boura and Green (133) also found that when guanethidine was administered for longer periods (*e.g.,* 5 mg/kg/day sc for 5 weeks), pronounced tolerance developed, such that the mean frequency–response curve to nerve stimulation in the nictitating membrane was shifted markedly to the left of that found with the same dose given for only 3 days and approached control values. In the main, tolerance was attributed to the accompanying marked hypersensitivity to transmitter responses, and subsequent studies (135) reaffirmed this conclusion. However, in the femoral vascular bed of both cats and guinea pigs, the extent of the hypersensitivity to injected norepinephrine and epinephrine was considerably less than in the nictitating membrane. Stocks and Robertson (136) reported that tolerance to guanethidine was a major drawback to the long-term clinical management of severe hypertension (35 patients studied for 5 years); but in other investigations (137, 138), tolerance has been considered to occur only rarely or not at all. This lack of tolerance might be related to the use of concurrent therapy with other antihypertensive agents and/or to shorter periods of study. However, Stocks and Robertson (136) found that the addition of chlorothiazide medication to five tolerant guanethidine patients failed to produce beneficial effects on blood pressure control and a further increase in guanethidine dosage was required in three of the five subjects.

The successful use of guanethidine in the control of hypertension depends upon careful dosage adjustment (134). The effective dosage regimen can vary considerably from one individual to another, but the factors that give rise to this difference are not fully understood. Clinical observations on the ratio of the effective intramuscular and oral doses suggest that the variation in absorption is not the only factor responsible. In fact, although guanethidine absorption exhibits considerable biological variation, it has been reported to remain constant within a particular individual even during chronic therapy (139). However, other workers (140) refuted the claimed variability of patient absorption. McMartin *et al.* (141) pointed out that renal clearance, metabolism, and tissue uptake of guanethidine also play very important roles in determining the body's content of guanethidine. In addition, other factors that might account for variations in patient response are the severity and etiology of the hypertension and the development of hypersensitivity.

Clinical evidence suggests that the antihypertensive effect of chronic guanethidine therapy results primarily from a reduction in peripheral resistance rather than cardiac output (142–144), although this latter factor seems to be of importance during the

first few days of treatment. Thus, after 4–10 days of oral guanethidine therapy, the cardiac output in hypertensive patients was lower, even in the recumbent position; the decrease became more marked after tilting, but at this time the systemic resistance was only moderately decreased. Virtually opposite effects were seen after longer periods of treatment (10–32 days), since neither supine nor standing cardiac output was significantly lower, despite the presence of bradycardia, but systemic vascular resistance was decreased (143). Mason *et al.* (145) reported that reflexogenically induced arterial and venous constriction in the forearm of humans was completely inhibited in 10 of 17 subjects treated with guanethidine (30–50 mg/day for 26–43 days). No persistent impairment resulted, however, since normal reflexogenic regulation returned following the withdrawal of treatment. The authors suggested that reflexogenic impairment would be expected to reduce both venous return and cardiac output and thereby contribute to the antihypertensive properties of the compound. In fact, many workers have noted a pronounced postural and exercise hypotension in guanethidine-treated patients, which is of sufficient severity to be classified as a major complication inherent in the therapeutic management of hypertension with this agent (136, 146). Because of this effect, Stocks and Robertson (136) strongly recommended that all patients taking guanethidine should be adequately exercised while monitoring blood pressure to determine an effective and safe dose level. Exercise hypotension with guanethidine seems to be due to a greater than normal decrease in peripheral resistance and can be seen frequently even in the presence of a raised cardiac output (142). In this latter respect, it should be pointed out that although bradycardia may be marked with guanethidine, stroke volume is increased (142).

The effect of guanethidine on electrolyte excretion has received attention but has yielded conflicting data. Oral guanethidine in human subjects produced an enhanced natriuresis to saline loads and less salt retention in response to sodium-retaining steroids (147) and angiotensin (148). In another study (149), these investigators administered guanethidine to normal human subjects deprived of dietary sodium and obtained a marked sodium loss accompanied by a decreased creatinine clearance. The investigators suggested that these results were due to adrenergic neuronal blockade which gave rise to an inhibition of the tubular reabsorption of sodium. This conclusion is compatible with the previous observation of Wagner (150), who reported that patients with autonomic insufficiency excreted enhanced amounts of sodium during saline infusions. In a carefully thought-out acute study, Williams *et al.* (151) infused guanethidine directly into a renal artery of anesthetized dogs to divorce the systemic effects of the drug from direct renal actions. They found a unilateral increase in sodium, chloride, calcium, potassium, and water loss with no significant change in glomerular filtration rate or renal plasma flow. They were able to attribute these effects to local adrenergic neuronal blockade. However, the diuretic action of guanethidine, at least in dogs, may be short lived (152). In contrast with these studies, it has been shown that clinical treatment of hypertension with guanethidine may cause fluid retention (137, 153), reduced renal plasma flow, reduced glomerular filtration rate (154), and decreased excretion of sodium, potassium, and water. Such effects may account for guanethidine-induced weight gain (155). In a study on 10 patients treated with guanethidine for 7–21 days, Villarreal *et al.* (143) noted that the altered electrolyte excretion pattern was similar to that resulting from aldosterone release and suggested this or the lowering of renal perfusion pressure as a possible explanation. Thus, guanethidine would seem to exert both direct and indirect actions on the kidney and, although the precise nature of these actions requires clarification, it would seem wise to be cautious in treating hypertensive patients with accompanying renal insufficiency.

Two important studies sum up the side effects most likely to occur during the long-term use of guanethidine. Moser (134) reported on 9 years of clinical experience with guanethidine in treating more than 250 patients. Dizziness and muscular weakness were the most frequently noted side effects followed by diarrhea, bloating, "gas pains," and "indigestion." Occasionally, these latter symptoms persisted and were severe enough to warrant drug withdrawal. They are believed to be due to excessive parasympathetic activity in the face of sympathetic impairment. Muscular weakness is prominent on awakening and tends to disappear later in the day, suggesting alterations in fluid distribution overnight with early morning venous pooling (134). In addition, guanethidine exhibits neuromuscular blocking properties (69, 156) which could be extensive enough to account for this troublesome side effect. Tolerance, impotence, and failure of ejaculation were discussed earlier. Stocks and Robertson (136) encountered frequent depression, especially in female patients, but this symptom responded well to imipramine. Using this antidepressant, one patient experienced loss of blood pressure control (discussed previously). Out of 35 patients studied for up to 5 years, approximately 50% failed to complete the trial due in the main to severe side effects. Others suffered strokes (four patients), myocardial infarctions (four patients), and tolerance so that only 10 patients were still attending at the conclusion of the study, two of which experienced significant hypotensive symptoms. Stocks and Robertson (136) concluded that "despite its potency, guanethidine used alone is unlikely to be a satisfactory agent in the long-term therapy of more than one-third of young patients [average age was 46 years] with severe hypertension."

Recently, a time course study concerning the effects of guanethidine on the cardiovascular and peripheral autonomic nervous systems using purebred beagle dogs was completed (157, 158). The oral administration of 2.5 mg/kg of guanethidine failed to inhibit the chronotropic responses to cardiac sympathetic stimulation after 2 days, but continued administration of the same dose for 7 days significantly depressed cardiac sympathetic nerve activity. However,

following chronic administration of the same dose for 6–8 months, cardiac sympathetic nerve function returned completely to predrug levels, demonstrating the presence of a pronounced tolerance to sympathetic nerve activity with guanethidine (157, 158). A similar phenomenon was also noted in the hindlimb vasculature since the neurogenic tone and the vasoconstrictor responses to lumbar sympathetic stimulation (which were depressed after 7 days of treatment) were restored to placebo levels in dogs treated with guanethidine for 6–8 months (158). In addition, a progressive potentiation of the sympathetic cholinergic vasodilator activity was found as the duration of treatment increased. In this study, increased tolerance to guanethidine was not accompanied by any alteration in adrenergic receptor activity either in the heart or in the hindlimb vasculature, although a minor prejunctional increase in sensitivity to norepinephrine was found in the heart after both 7 days and 6 months of treatment. Thus, the mechanism responsible for tolerance differs from that reported in other studies (69, 133, 135), since it seems to be largely independent of transmitter sensitivity changes (157).

In contrast, no evidence for tolerance was found in the isolated perfused mesenteric arteries (157). The vasoconstrictor response to periarterial nerve stimulation was abolished after 7 days and 2, 6, and 8 months of treatment. Treatment for 1 day failed to alter significantly the frequency–response curve to periarterial nerve stimulation, but a twofold shift to the left in the dose–effect curve to injected norepinephrine was obtained. This twofold shift was evident throughout the study. However, the responses to nerve stimulation, as well as to norepinephrine, approached control values when guanethidine treatment was suspended for 2 months. At this time the nerves were fully susceptible to acute blockade by guanethidine when injected into the perfusing fluid. Other data revealed that, whereas dextroamphetamine could reverse the acute effects of guanethidine on neuronal transmission, it exerted little or no effect after 8 months of treatment[2]. There appeared to be a direct correlation between the duration of treatment and the inability of dextroamphetamine to reverse the neuronal blocking action of guanethidine. Although the mechanism of this effect has not been identified, the result reveals alterations in the normal pharmacology of the neuron upon chronic treatment, which, in turn, must reflect an increasingly deepening impairment of an important physiological process. Thus, these studies showed a much more persistent neuronal blocking action on the sympathetic innervation to the mesenteric arteries than on the myocardium and hindlimb vasculature. Functional transmission in the stellate ganglion and lumbar sympathetic chain was unaffected, as were both the monoamine oxidase activity and the catecholamine content as judged by the histochemical fluorescence technique. In accordance with previous observations, no alteration was observed in the population of small, intensely fluorescent cells. Heart norepinephrine was depleted by about 80% after 7 days of treatment and failed to recover even at 6 and 8 months. Therefore, tolerance to the inhibitory effect of guanethidine in the heart is not accompanied by a return in myocardial norepinephrine levels.

Despite these marked time-dependent changes in the functional responsiveness of sympathetic neurons, no detectable alterations in the neural regulation to the cardiovascular system was detected in conscious animals. Chronic administration of guanethidine (2.5 mg/day orally) over 8 months did not produce any significant alteration in body weight, blood volume, or blood pressure. There was, however, a slight reduction in heart rate, which was more prominent and significant in males than in females. The cardiovascular responses to tilt (head-up), mild exercise (on a treadmill, 12° inclination at 5 miles/hr for 4–5 min), ganglionic blockade, sodium nitrite, tyramine, angiotensin, phenylephrine, and norepinephrine did not differ significantly in treated animals compared with control dogs[2].

THIAZIDES

It is generally agreed that thiazide diuretics are moderately effective in the treatment of hypertension and are reported to have a relatively low incidence of side effects (159, 160). Frequently recognized toxic effects of these compounds are hypokalemia, infrequent hyperuricemia, possible exacerbation of previously controlled diabetes, prolongation of hypoglycemia even in normal patients, jaundice, skin eruptions, photosensitivity, thrombocytopenia, neutropenia, and pancreatitis. Although the compounds do initially decrease plasma volume and electrolytes, the mechanisms by which diuretics produce their antihypertensive effects (161–164) are still not understood.

As a rule, thiazides do not produce hypotension on acute administration and do not lower the arterial pressure of normotensive patients or experimental animals. Enhanced natriuresis, decreased blood volume, reduced extracellular fluid volume, and alteration in electrolyte balance are reported to play a role in the antihypertensive effects of these compounds (165–169). Winer (170) suggested that chlorothiazide has no effects on the CNS or on peripheral blood vessels. Based on their studies with normotensive as well as renal hypertensive rats receiving 0.5 mg/kg methyclothiazide for 14 days, Aoki and Brody (171) recorded electrical activity in the lumbar sympathetic nerves and measured the vascular responses in the perfused hindlimbs of the same animal. Treated renal hypertensive rats exhibited a higher basal nerve activity and greater changes in activity in response to asphyxia, carotid occlusion, and intravenous norepinephrine than did treated normotensive and untreated hypertensive and normotensive rats. The reflex increases in blood pressure produced by asphyxia and carotid occlusion were similar in all groups. Hypertensive rats showed greater vasoconstrictor re-

[2] Unpublished observations.

sponses to intraarterial angiotensin and tyramine, while responses to norepinephrine and epinephrine were similar in all rats. Thiazide treatment reduced the increased responsiveness to angiotensin in hypertensive rats to normal levels, and tyramine responses were unchanged. These data suggest that chronic renal hypertension is associated with increased vascular responsiveness to lumbar sympathetic nerve stimulation and intraarterial angiotensin and tyramine. Methyclothiazide treatment produced increased basal sympathetic nerve activity and greater reflex changes in nerve activity unaccompanied by blood pressure increases of similar magnitude and decreased vascular responsiveness to nerve stimulation and intraarterial angiotensin. The authors concluded that methyclothiazide may exert its hypotensive effect by interfering with norepinephrine liberation from sympathetic nerve terminals (171). Other studies indicated that renal hypertension is associated with larger than normal amounts of tissue sodium (172, 173), and thiazide treatment was ineffective in lowering blood pressure of rats with renovascular occlusive hypertension (172, 173). Willard (174) considered whether high tissue sodium levels interfered with the antihypertensive properties of thiazide. In these studies, hypertension was induced by deoxycortone acetate and sodium chloride in normal rats as well as in rats concomitantly treated with cyclothiazide (10 mg/kg/day for 16 weeks). Hypertension developed after 3–6 weeks of treatment with steroid and salt. The concomitant treatment with cyclothiazide did not alter the time for product or degree of hypertension. When the animals were returned to a normal diet, the blood pressure fell in both groups, but the reduction was significantly greater in animals treated with cyclothiazide. In another group, when thiazide treatment started 13 weeks after induction of the hypertensive state, there was a gradual reduction in blood pressure over 14 weeks. When the treatment stopped, the blood pressure returned to the original hypertensive level. The contrast between the slow hypotensive response and relatively fast recovery of hypertension corresponds to the results obtained clinically. These results seem to indicate that a high sodium diet is responsible for the slow onset of action. Therefore, it appears that in several other studies the failure of thiazide to lower blood pressure in renal hypertensive rats could be due to too short a duration of treatment employed (172, 175, 176). In these studies, since a high sodium diet appeared to decrease the effectiveness of thiazide therapy, sodium depletion did play a role in the antihypertensive properties of thiazides.

Decreased responsiveness of the vasculature to catecholamines was suggested as the antihypertensive mechanism for chlorothiazide (177–182). Eckstein *et al.* (177) reported that pressor responses to norepinephrine were due to increased cardiac output in dogs chronically treated with chlorothiazide. In untreated dogs in the same study, similar pressor responses to norepinephrine were due to increased peripheral vascular resistance since no essential changes occurred in cardiac output. These differences in the norepinephrine responses appeared to be due to the ability of chlorothiazide to reduce the effectiveness of cardio-inhibitory reflexes by reducing the responses of peripheral vasculature to the vasoconstrictor effect of norepinephrine. This effect was apparent both before and after ganglionic blockade, suggesting that modification of resting neurogenic vasomotor tone did not play a major role in the reduced responsiveness (177).

Feisal *et al.* (183) investigated the effects of graded intravenous infusions of norepinephrine on heart rate, mean blood pressure, forearm blood flow, and forearm vascular resistance in normal human subjects before and at the end of 7 days of chlorothiazide treatment. In these studies, chlorothiazide reduced the vasoconstrictor response to norepinephrine. The bradycardia accompanying the rise in blood pressure during norepinephrine infusions also appeared to be reduced. Forearm blood flow decreased with increasing doses of norepinephrine before chlorothiazide and increased with increasing doses after chlorothiazide. These results could also be explained on the basis of decreased responsiveness of the vasculature and of cardio-inhibitory reflexes of human subjects after chlorothiazide (183).

Zsoter *et al.* (184) studied the effects of prolonged administration of hydrochlorothiazide in rabbits and dogs for 6–8 weeks. In these studies, hydrochlorothiazide administration depressed mesenteric venoconstrictor responses to norepinephrine in rabbits but failed to alter the responses of acetylcholine, barium chloride, angiotensin, papaverine, and ATP. Hydrochlorothiazide also markedly reduced vasoconstrictor responses in the dog hindlimb to lumbar sympathetic stimulation and to intraarterial norepinephrine. These effects were not accompanied by any significant alteration in water, sodium, potassium, and calcium content of iliac artery or veins. The investigators concluded that diminished responses of the vessels to norepinephrine may be responsible for the antihypertensive effects after prolonged administration of hydrochlorothiazide.

Preziosi *et al.* (182) studied the pharmacological properties of chlorothiazide in rats, rabbits, and dogs. In these studies, chlorothiazide reduced pressor responses to carotid occlusion, epinephrine, norepinephrine, and angiotensin and slightly enhanced the responses to acetylcholine on the vasculature and on the isolated intestines, suggesting that chlorothiazide exerted a depressant action on smooth muscle fibers of the vasculature. The authors concluded that this depressant action might contribute to the chlorothiazide's antihypertensive effects as well as to its ability to potentiate other antihypertensive agents. These data do not support the view that chlorothiazide sensitizes carotid sinus buffer mechanisms (180). Preziosi *et al.* (188) also investigated acute and chronic effects of hydrochlorothiazide in mice and dogs. These studies demonstrated that hydrochlorothiazide had no influence upon carotid sinus baroreceptors, chemoreceptors, or vasomotor centers. Smaller doses inhibited the vasopressor responses to stimulation of adrenergic vasomotor fibers without influenc-

ing the vascular responses to catecholamines or angiotensin. Higher doses of hydrochlorothiazide were found to have adrenolytic, sympatholytic, and direct myolytic properties. Administration of hydrochlorothiazide (10 mg/kg acutely or for 7 days) also decreased catecholamine content of various tissues including the heart.

According to most investigators, the alterations in *fluid* volume or electrolyte balance do not play a major role in the chronic antihypertensive effects of thiazides. Daniel (185) found that hydrochlorothiazide did not significantly diminish inulin space, decrease concentrations of intracellular sodium, or increase the ratio of extracellular to intracellular sodium. Weller and Haight (186) administered 50 mg/kg chlorothiazide to rats for 6 weeks and found no consistent alterations in the electrolyte or water content of plasma or tissue of normotensive or hypertensive rats. On the contrary, administration of hydrochlorothiazide together with potassium chloride to hypertensive patients for 3 months resulted in a significant fall in total blood volume, exchangeable sodium, and blood pressure within 1–2 weeks and after 3 months of treatment (187). However, a fall in serum sodium and body weight was only significant after 1–2 weeks of treatment. Thus, in these studies, hypovolemia may be the explanation for the antipressor effect of thiazide even after 3 months of treatment (187).

Kusumoto *et al.* (189) administered 3.75 mg/kg/day hydrochlorothiazide chronically for 45 days to dogs, and the hydrochlorothiazide had no significant effect on blood pressure, blood and plasma volumes, and hematocrit. A significant decrease in plasma magnesium and potassium persisted throughout treatment, while plasma sodium and calcium remained unchanged. The potassium content of arterial tissue was significantly depressed and the calcium content was significantly enhanced in the left ventricular myocardium. Norepinephrine content was markedly reduced in all of these tissues. The juxtaglomerular index in four dogs examined was significantly elevated. Menard *et al.* (190) also reported an increase in plasma renin activity and juxtaglomerular index in normotensive rats treated with hydrochlorothiazide and lower sodium diet. The clinical and pharmacological significance of these findings has yet to be clarified, although it can be postulated that any compound decreasing plasma sodium content and/or renal blood flow would be expected to increase plasma renin levels.

Bourgoignie *et al.* (191) found elevated plasma renin levels during early phases of thiazide treatment only if the sodium intake was concurrently reduced and renin levels returned to normal levels with continued thiazide treatment. However, Tarazi *et al.* (192) noted that the blood pressure reduction during long-term thiazide therapy was associated with persistent plasma volume reduction, which occurred despite increased peripheral renin activity. There was no evidence for chronic intercellular dehydration, and variations in peripheral renin activity were related to changes in plasma volume and not to serum sodium concentration (192). The finding by Tobian *et al.* (175) of higher juxtaglomerular counts in normal rats given chlorothiazide for 5 weeks also seemed to indicate continued renin stimulation by these diuretics. The higher plasma renin activity and chronic weight reduction with continued thiazide therapy, followed by rapid extracellular water expansion with discontinuance as well as the continued potentiation of other antihypertensive agents by thiazides (193) and the reversal of their effects by higher sodium chloride intake (194), all correlate naturally with chronic reduction in plasma volume, extracellular fluid, and total exchangeable sodium as demonstrated in many studies (187, 195–197). It is by no means conclusive that this chronic hypovolemia is solely responsible for long-term hypotensive effect of thiazides in essential hypertension, but this volume-reducing effect may play a role in potentiating other antihypertensive agents. Despite numerous reports indicating a persistent decrease in fluid volume, the reduction in cardiac output appeared to play only a minor role in the antihypertensive effects of thiazides (169, 192, 198, 204). Conway and Lauwers (198) demonstrated the value of long-term therapy with chlorothiazide as the sole antihypertensive drug. In these studies, a significant fall in blood pressure occurred in 66% of the patients during the initial 2 weeks. There was a decrease in cardiac output accompanied by an increase in total peripheral resistance. However, after about 4 weeks, cardiac output was restored to predrug levels while the fall in blood pressure persisted due to reduction in total peripheral resistance. Although this initial decrease in cardiac output was usually related to a reduction in plasma volume, Frohlich *et al.* (199) noted a reduction in cardiac output unaccompanied by an alteration in fluid volume during acute infusion of chlorothiazide to anesthetized mongrel dogs. This reduction in cardiac output was associated to decreased venous return resulting from venodilation (200, 201). Few investigators, if any, considered the possibility that the reduction in cardiac output may be due to an alteration in contractility of myocardium induced by thiazides.

Studies on the prolonged administration of hydrochlorothiazide (10 mg/kg po for 12 months) to beagle dogs failed to produce any significant alterations in plasma volume, hematocrit, extracellular fluid, electrolyte balance (sodium, potassium, calcium, and magnesium), arterial blood pressure, and heart rate at any time during or at the end of the treatment period (202, 203). In conscious animals, hydrochlorothiazide treatment also did not affect cardiovascular alterations to tilt (60° head-up position) or exercise and blood pressure responses to sodium nitrite, angiotensin, tyramine, phenylephrine, and ganglionic blockade with mecamylamine[2]. Reflex tachycardia to sodium nitrite or reflex bradycardia to angiotensin, tyramine, and phenylephrine was not affected by chronic hydrochlorothiazide administration; however, while the depressor responses to mecamylamine were not influenced, tachycardia to ganglionic blockade was significantly inhibited by hydrochlorothiazide administration. Since it has been demonstrated that ganglionic blocking drugs induce tachycardia in

conscious dogs by essentially interfering with vagal tone (65), it appears from these studies that chronic hydrochlorothiazide may modify the inhibitory effects of ganglionic blocking drugs on vagal tone. These results may also explain the ability of thiazides to potentiate antihypertensive effects of several ganglionic blocking drugs. Prolonged hydrochlorothiazide administration for 10–12 months resulted in a shift of the pressure flow curves to the right, indicating a significant reduction in the neurogenic tone to the hindlimb vasculature (202). This inhibition was not due to any alteration in peripheral adrenergic mechanisms since vasoconstrictor responses to lumbar sympathetic stimulation and to intraarterial norepinephrine were essentially similar in treated animals to those obtained in placebo groups. Based on these findings, the authors postulated that hydrochlorothiazide inhibited neurogenic tone by an action mediated *via* the CNS and that these central effects may contribute to the antihypertensive properties of thiazide diuretics (202).

In another aspect of the same study, Clarke *et al.* (205) reported that chronic oral treatment of dogs with hydrochlorothiazide (10 mg/kg/day for 6 months) enhanced the frequency–response curve to periarterial nerve stimulation of mesenteric blood vessels *in vitro* without affecting vasoconstrictor responses to norepinephrine. However, this effect was no longer observable after an additional 6 months of treatment. Hydrochlorothiazide failed to affect the pharmacological interaction of certain agents (angiotensin, bethanidine, atropine, phentolamine, hexamethonium, and cocaine) with sympathetic neuronal receptor mechanisms. Furthermore, the *in vitro* accumulation of tritium, after incubation with ^{3}H-norepinephrine in atria, ventricle, and femoral arterial slices was unaltered by hydrochlorothiazide. These workers also demonstrated that acute infusion of hydrochlorothiazide (2.7 mg/min for 90 min) directly in mesenteric arteries *in vitro* failed to alter adrenergic mechanisms. These studies were in complete disagreement with previous reports that thiazide diuretics possess antiadrenergic properties (177, 184, 188). It may be that the adrenolytic effects of thiazide can only be demonstrated after a limited treatment period, as observed by Zsoter *et al.* (184) with hydrochlorothiazide. On prolonged treatment, peripheral sympathetic neural function may be restored to predrug levels.

The cardiovascular effects of prolonged hydrochlorothiazide administration (10 mg/kg/day for 12 months) in beagle dogs have been reported (203). Hydrochlorothiazide treatment for 12 months resulted in a significant reduction in cardiac output, stroke volume, and left ventricular stroke work without any accompanying changes in mean blood pressure and heart rate. Arterial pressures were maintained by an elevated total peripheral resistance. The reduction in cardiac output was not due to any alteration in plasma volume. Ventricular function curves of the treated animals were significantly depressed to the right, indicating marked diminution in the contractility and capacity of the myocardium to handle external loads. Thus, the decrease in cardiac output can be explained on the basis of reduced contractility. These effects cannot be related to alterations in the electrolyte pattern, either in serum or in the heart, since no such changes occurred. These cardiac effects of chronic hydrochlorothiazide can be reproduced by administering large doses (100 mg/kg/day po for 2 days).

Despite the widespread use of these compounds, little attention has been paid to their cardiac effects. As early as 1959, Barrett *et al.* (206) reported that small doses of hydrochlorothiazide, then a newly discovered diuretic, possessed negative inotropic properties in isolated heart preparations. Later, Preziosi *et al.* (182) observed similar effects with higher doses of chlorothiazide. Herbert and Buxton (208) warned against the danger of sensitization of the myocardium to the action of digitalis in obstetrical patients receiving concurrent treatment with hydrochlorothiazide. Such a toxicity was related to hypokalemia induced by thiazides, to the presence of cardiac decompensation, and to postpartum diuresis. Daniel (185) reported that the administration of hydrochlorothiazide to deoxycortone acetate-hypertensive rats resulted in marked alterations in the left ventricular function and suggested that more attention should be paid to the action of hydrochlorothiazide on the myocardium. More recently, Naylor *et al.* (207) demonstrated that diazoxide, a nondiuretic thiazide, displaced left ventricular function curves to the right in dogs, suggesting a diminution in the contractility of the left ventricle. Many of these reports clearly warrant a careful reevaluation of extensive use of thiazides in cardiovascular therapy.

SUMMARY AND CONCLUSIONS

The obvious conclusion that one can reach in reviewing the chronic pharmacological properties of antihypertensive agents is that the available literature is extremely limited. The dimension of time as an important variable in drug-induced effects has not been considered by most investigators, even in clinical studies; consequently, knowledge of these agents is largely restricted to their acute pharmacological properties. However, of the drugs discussed in this review, all have recorded chronic effects which were not readily observable from acute or even subacute studies. Thus, it cannot be assumed that the acute pharmacological spectrum of a drug will necessarily reflect or even predict its chronic pharmacological effects. Instead, investigators would be well advised to consider the opposite thesis, namely that drug-induced effects may, and probably will, vary with the duration of treatment. This contention is biologically sound, since it may be argued that drug challenges to physiological and pathological states will be resisted by prevailing homeostatic mechanisms. Such autoregulation requires time to develop and may be generalized or restricted to specific organ systems, depending upon the deposition of the drug and the biological limits of the system for adaptive change. The studies conducted by Jandhyala *et al.* (158) and

Clarke *et al.* (157) on guanethidine may well illustrate this point. The lack of persistence of adrenergic neuronal blockade in the heart and hindlimb vasculature compared with the mesentric blood vessels might be due to regional differences in the neurons to undergo adaptive change.

In addition to tolerance, toxicity may be subtle in onset and may involve indirect mechanisms. Alterations in metabolism, absorption, and cumulative effects of a drug may require time to become manifest. Indeed, certain findings in these respects have been discussed. The toxicities of the thiazide diuretics and reserpine on myocardial ventricular function are important findings which are now documented to occur following the repeated administration of relatively low daily doses of these drugs to otherwise healthy animals. However, as indicated earlier, the neuronal toxicity of guanethidine has yet to be discerned under similar circumstances.

Another significant factor, which appears to be almost entirely overlooked by pharmacologists, is the importance of studying the mechanisms of drug action in conscious animals. Perhaps it is considered that this area of study is adequately fulfilled by clinical studies; however, by necessity, these investigations are limited in scope and are mostly descriptive rather than conceptual in their conclusions. However, it should have become apparent to the reader that findings obtained in conscious animals often differed markedly from those obtained following general anesthesia. In general, the observed changes were far less dramatic and, in many instances, gave little or no indication of altered cardiovascular mechanisms. These discrepancies are unsettling and necessitate that due caution be observed when translating observations made in anesthetized animals or isolated tissue preparations to the clinical condition. However, it should not be construed that observations made in conscious animals are necessarily more valid. Under these conditions, the available technology for precise measurement is less well developed and it is extremely difficult to construct dose–effect relationships and elicit precise responses in specific vascular areas. In addition, studies using conscious normotensive animals under sheltered laboratory conditions may not reveal marked alterations in hemodynamic and/or autonomic parameters. Detailed studies involving conscious hypertensive animals subjected to rigorous exercise tests are urgently required.

Beside the general points raised, the literature revealed certain specific information that requires additional research for full comprehension. For instance, the time-dependent increase in cholinergic vasodilator activity in skeletal muscle blood vessels following chronic guanethidine treatment would not be predicted on the grounds of homeostatic readjustment. Neither can it be explained on the basis of an overactive cholinergic response in the face of adrenergic impairment, since the function of the latter neurons were restored to control levels. Thus, exertional hypotension in patients receiving guanethidine may be cholinergic in origin, and clinical studies using atropine or an atropine-like agent to seek further evidence for this contention would seem highly justified. Similarly, the bradycardia noted in clinical and experimental studies with guanethidine cannot be explained as an indirect result of adrenergic neuronal blockade. Although a central action of guanethidine has been generally ruled out with regard to sympathetic tone (209), it cannot be overlooked with respect to cholinergic activity. In this respect, chronic treatment with this agent has been shown to depress brain monoamine levels (210), indicating either direct or indirect effects on the CNS.

Certain other findings promote the possibility that physiological differences may exist with regard to the peripheral adrenergic innervation to various organs and vascular beds. In fixed-dose chronic studies (43, 44), the sensitivity of the femoral arterial smooth muscle to norepinephrine was increased by reserpine, whereas no sensitivity change occurred in the mesenteric vasculature. The opposite situation prevailed with guanethidine (157, 158). Neural toxicity with low doses of guanethidine was largely restricted to the genital organs and only became more diverse with increasing dosage (108, 109). It is apparent that these observations cannot be adequately explained on the basis of available information, and the need for more detailed chronic studies is further emphasized.

The mechanism of the antihypertensive property of the thiazide diuretics remains debatable. Certain findings suggest that at least part of their action may be mediated through an inhibition of peripheral adrenergic mechanisms, particularly at the level of the vasculature. However, chronic studies with hydrochlorothiazide have failed to reveal such effects with regard to the vascular responsiveness of injected norepinephrine. Instead, evidence from these studies indicates a possible central hypotensive effect of hydrochlorothiazide which appears to be mediated through a reduction in neurogenic tone to the vasculature. This latter effect was not noted in any reported acute or subacute studies.

In conclusion, it is apparent that chronic pharmacological studies are essential and exceedingly important, especially for compounds used for the control of chronic disorders. Based upon the available literature, one must conclude that chronic pharmacological investigations, employing relatively low dose levels of drugs, are likely to reveal important differences in regional physiological mechanisms and organ susceptibility to toxic effects and a deeper insight into the mechanism of action of the compounds themselves.

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[x] To whom inquiries should be directed.

Comparison of Theoretical, Absolute Interaction Energies with Heats of Complexation in Carbon Tetrachloride

GORDON L. AMIDON

Abstract □ The absolute interaction energies for the methylbenzene complexes with chloranil, fluoranil, and tetrachlorophthalic *anhydride* are calculated by using net atomic charges and bond polarizabilities. The theoretical results are then compared to the experimentally determined heats of complexation in carbon tetrachloride. The trend of increasing complex strength with methyl substitution within each series is qualitatively predicted by this method, but the theoretical increase per methyl group is less than the experimental solution value by a factor of two to three. The overall correlation between the complex strength and the absolute interaction energy is poor. These results indicate that the contribution of the nonpolar solvent carbon tetrachloride to the overall complex strength is significant and perhaps greater than the absolute interaction energy of the substrate and ligand.

Keyphrases □ Interaction energies—methylbenzene complexes with chloranil, fluoranil, and tetrachlorophthalic anhydride, net atomic charges and bond polarizabilities, compared to experimental heats of complexation in carbon tetrachloride □ Complexation, heats—absolute interaction energies for methylbenzene complexes with chloranil, fluoranil, and tetrachlorophthalic anhydride (net atomic charges and bond polarizabilities) compared to experimental heats of complexation in carbon tetrachloride □ Carbon tetrachloride—contribution to complex strength, methylbenzene complexes with chloranil, fluoranil, and tetrachlorophthalic anhydride, net atomic charge and bond polarizability theoretical results compared with experimental heats of complexation □ Solvents—contribution of carbon tetrachloride to the overall complex strength of methylbenzene complexes with chloranil, fluoranil, and tetrachlorophthalic anhydride

Complexation has been extensively discussed in the pharmaceutical literature. It is known, for example, that complexation can affect the solubility (1), dissolution (2), chemical reactivity (3–6), and membrane transport (7) properties of drugs. While extensive experimental studies have led to empirical correlations between the structure and the equilibrium constant of interacting molecules (8, 9), detailed molecular calculations of the forces (and energies) involved have not been reported. It is difficult to perform accurate calculations on molecules as large as those of pharmaceutical interest (10, 11). However, recent theoretical developments show considerable promise in this direction (12).

In this report the method of monopoles-bond polarizabilities (12), in which a molecule is considered as a collection of monopoles (net atomic charges) and a collection of bonds (bond polarizabilities), is discussed and used to calculate the structures and absolute interaction energies for the methylbenzene complexes with fluoranil, chloranil, and tetrachlorophthalic anhydride. These results are then compared with the experimentally determined heats of complexation in solution. In subsequent reports the effect of solvent on the energetics of complexation and the correlation between predicted structure and chemical reactivity will be discussed.

THEORETICAL

In the usual development of intermolecular force theory, two approximations are introduced (10, 11). The first is a truncation of the perturbation expansion to second order, leading to the electrostatic (first order) and polarization and dispersion (second order) terms. The second approximation is the development of the interaction terms in a multipole expansion, usually truncated after the first nonvanishing term (the dipole–dipole term for neutral molecules). Of these two approximations, the second seems to be the least reliable when the intermolecular distance is of the same order of magnitude as the dimensions of the interacting molecules (12). The method of monopoles-bond polarizabilities makes the following more reasonable approximations (12, 13):

1. For the first-order (electrostatic) term, the ground-state charge distribution is divided up into a set of monopoles located at the nuclei of the respective atoms. The electrostatic energy is then calculated by Eq. 1:

$$E_{el} = \sum_{1}\sum_{2} \frac{q\nu_1 q\nu_2}{r(\nu_1\nu_2)} \qquad \text{(Eq. 1)}$$

where $q\nu_1$ and $q\nu_2$ are the net atomic charges on nuclei ν_1 and ν_2 of molecules 1 and 2, respectively; $r(\nu_1\nu_2)$ is the ν_1, ν_2 distance; and the summations are over all nuclei in molecules 1 and 2.

2. For the second-order dispersion energy term, the transition charge density (14) is represented by a set of transition dipoles located at the center of the bonds. The dispersion energy is then a summation over all bonds and all excited states of each molecule. This latter summation, requiring a knowledge of the excited states of each molecule, is difficult to perform so a further approximation is to replace the bond transition dipole summations with the experimentally known bond polarizability tensors (15). To introduce the bond polarizability into the theoretical expression, two additional approximations are required: *(a)* each excitation must be localized over a single bond, and *(b)* a mean excitation energy must be used.

The assumption of localized excitations is reasonable for σ-electrons or π-electrons in a localized double bond but is questionable for delocalized π-electrons. This necessitates the use of an incremental π-electron polarizability (12, 13). The mean excitation energy is usually taken to be equal to the experimental ionization potential of the molecule. With these approximations, the dispersion energy is calculated using Eq. 2:

$$E_{disp} = -(I_1 \cdot I_2)/4(I_1 + I_2)\sum_{\mu_1}\sum_{\mu_2}$$
$$1/(r(\mu_1\mu_2))^6 \cdot \{6\alpha(\mu_1)^T\alpha(\mu_2)^T + \alpha(\mu_1)^T\delta(\mu_2)[3(\hat{\alpha}(\mu_2)^I \cdot \hat{r}(\mu_1\mu_2))^2 + 1] +$$
$$\alpha(\mu_2)^T\delta(\mu_1)[3(\hat{\alpha}(\mu_1)^I \cdot \hat{r}(\mu_1\mu_2))^2 + 1] + \delta(\mu_1)\delta(\mu_2)[3(\hat{\alpha}(\mu_1)^I \cdot \hat{r}(\mu_1\mu_2)) \cdot$$
$$(\hat{\alpha}(\mu_2)^I \cdot \hat{r}(\mu_1\mu_2)) - (\hat{\alpha}(\mu_1)^I \cdot \hat{\alpha}(\mu_2)^I)]^2\} \qquad \text{(Eq. 2)}$$

where $\delta_\mu = \alpha_\mu{}^l - \alpha_\mu{}^T$; the subscripts 1 and 2 refer to molecules 1

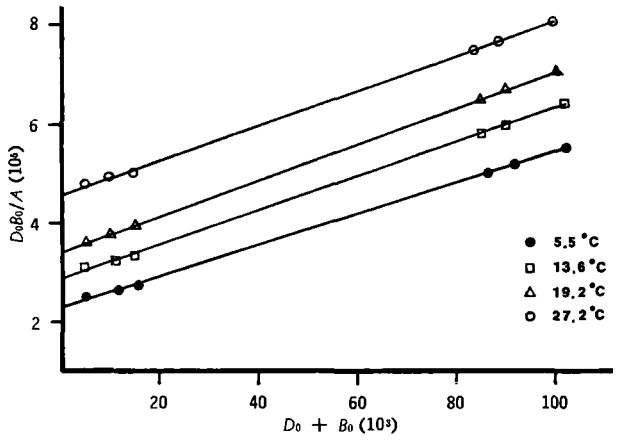


Figure 1—*Plots of* D_0B_0/A versus $D_0 + B_0$ *for the chloranil–hexamethylbenzene complexes in carbon tetrachloride.*

and 2, respectively; μ refers to a particular bond; α^l and α^r are the parallel and tangential polarizabilities, respectively; $\hat{\alpha}^l$ and $\hat{r}(\mu_1\mu_2)$ refer to the unit vectors in the direction of the bond and the intermolecular bond–bond distance, respectively; and the summation is over all bonds in molecules 1 and 2. If the bonds are considered isotropic, *i.e.*, $\alpha_\mu{}^l = \alpha_\mu{}^r = \alpha_\mu{}^m$, then $\delta_\mu = 0$ and Eq. 2 reduces to Eq. 3:

$$E_{\text{disp}} = -3/2\frac{I_1 \cdot I_2}{I_1 + I_2}\sum_{\mu_1}\sum_{\mu_2}\frac{\alpha(\mu_1)^m\alpha(\mu_2)^m}{[(r(\mu_1\mu_2)]^6} \qquad \text{(Eq. 3)}$$

which is the London equation summed over bonds (16).

The polarization contribution to the second-order interaction energy is calculated using a set of monopoles to represent the ground-state charge distribution of molecule 2 (the same set as used for the electrostatic calculation, Eq. 1) and bond polarizabilities for the other (polarized) molecule, as in the dispersion energy. The resulting equation is:

$$E_{\text{pol}} = -(1/2)\sum_{\mu_1}[\alpha(\mu_1)^r\cdot(\mathbf{E}(\mu_1)\cdot\mathbf{E}(\mu_1)) + \delta(\mu_1)\cdot(\mathbf{E}(\mu_1)\cdot\hat{\alpha}(\mu_1)^l)^2] \qquad \text{(Eq. 4)}$$

(1 by 2)

where:

$$\bar{\mathbf{E}}(\mu_1) = \sum_{\nu_2}[q\nu_2/(r(\nu_2\mu_1))^3]\hat{r}(\nu_2\mu_1)$$

$\mathbf{E}(\mu_1)$ represents the electric field at bond μ_1 due to the changes on molecule 2, and $r(\nu_2\mu_1)$ is the distance vector from nucleus ν_2 to the middle of bond μ_1. The remaining terms are as defined for Eq. 2. Again in the case of isotopic bonds, $\delta_\mu = 0$ and Eq. 4 reduces to the classical expression (10). Interchanging the subscripts 1 and 2 in Eq. 4 gives the analogous equation for the polarization of molecule 2 by 1.

In addition to the preceding "attractive" terms, varying as R^{-n}, $n = 1, \ldots, 6$, it is experimentally known that as the distance between two molecules decreases a repulsive force predominates. The physical basis for this repulsion is the electron exchange that takes place when the overlap of the electron clouds of the two molecules becomes significant. A theoretical method applicable at these intermolecular distances (3–4 Å) was recently developed (17–19). In this double-perturbation method, the ground-state product wave function is antisymmetrized, with the result that the longrange terms previously discussed appear along with additional terms involving the overlap that decrease exponentially. While this theory justifies the calculation of the longrange terms by the usual methods in the region of interest, the accurate calculation of the shortrange terms is extremely difficult (20). For this reason, most methods utilize an empirical repulsive potential, varying exponentially or as approximately r^{-12}, to calculate the repulsive term. In this study the exponential repulsive potential developed by Kitaygorodsky (21) and modified by Huron and Claverie (22) is employed:

$$E_{\text{rep}} = 30{,}000\mathbf{E}(\nu_1)\mathbf{E}(\nu_2)\ \exp[-11\cdot r(\nu_1\nu_2)/\sqrt{4R(\nu_1)\cdot R(\nu_2)}] \qquad \text{(Eq. 5)}$$

where $R(\nu)$ is the van der Waal radii of nucleus ν.

Detailed comparison of the method of monopoles-bond polarizabilities with other methods and with experimental results (12, 22–25) indicates that it is the most promising method available. For example, the molar volumes and vaporization energies of pyridine and benzonitrile and the solution energies of 11 small organic molecules were calculated and were in good agreement with experimental values (22, 26). Closely related methods were used to calculate the lattice energies and cell dimensions of benzene and triazine crystals and also were in good agreement with experimental values (27).

The theoretical method, as outlined here, gives no explicit recognition to the charge-transfer forces (28). While this force, arising from the resonance between the ground- and excited-state wave functions, may be important in strong complexes, for weak complexes such as those considered in this study, its contribution to the interaction energy appears to be significantly less than that due to the electrostatic, polarization, and dispersion forces (29–33).

EXPERIMENTAL

Materials—Carbon tetrachloride[1] was purified following the procedure of de Maine (34). Since no difference was observed in the spectrum of the donor, acceptor, or complex, it was used without further purification. Benzene[2] was distilled before use. Mesitylene[3], hexamethylbenzene[4], chloranil[5], and tetrachlorophthalic anhydride[4] were recrystallized to a constant melting point from a 7:3 mixture of ethanol and acetone before use.

Determination of Equilibrium Constant—Considering the simple complexation reaction $B + D \rightleftharpoons C$, where D = donor, B = acceptor, and C = complex, the following relationship is easily derived:

$$\frac{lD_0B_0}{A} = \frac{1}{\epsilon\cdot K} + \frac{B_0D_0}{\epsilon} - \frac{A}{\epsilon^2 l} \qquad \text{(Eq. 6)}$$

where A = absorbance; ϵ = molar absorptivity; l = path length; B_0 and D_0 = the initial amounts of acceptor and donor, respectively; and $K = [C]/([B][D])$. This relationship involves no assumptions other than Beer's law. If K is not very large, the last term in Eq. 6 can be neglected and Eq. 7 results:

$$\frac{lD_0B_0}{A} = \frac{1}{\epsilon\cdot K} + \frac{D_0 + B_0}{\epsilon} \qquad \text{(Eq. 7)}$$

This modified type of Scott equation becomes the Scott equation if $D_0 \gg B_0$ (35). Thus, a plot of D_0B_0/A *versus* $(D_0 + B_0)$ gives a straight line with slope $1/\epsilon$ and intercept $1/(\epsilon\cdot K)$. For the conditions of these experiments, $0.1 < K < 20$, $10 < [D]/[B] < 1000$, $[B] \simeq 10^{-3}$ M, and $\epsilon \simeq 10^3$, it has been shown that the corrections to the linear approximation (Eq. 7) are negligible (36) and ensure a good separation of K and ϵ (37). If the spectra of the acceptor (B) and complex (C) overlap, Eq. 8 is appropriate:

$$\frac{lD_0B_0}{A - \epsilon_BB_0} = \frac{1}{K(\epsilon_C - \epsilon_B)} + \frac{D_0 + B_0}{\epsilon_C - \epsilon_A} \qquad \text{(Eq. 8)}$$

where ϵ_B and ϵ_C refer to the molar absorptivity of the acceptor and complex, respectively.

While higher order complexes (2:1, *etc.*) may exist, they are not expected to be important in the concentration ranges used in this study. Trotter and Yphantis (38) studied the hexamethylbenzene–tetracyanoethylene complex in methylene bromide by ultracentrifugation and determined that, at a 1 M concentration, approximately equivalent amounts of 1:1 and 2:1 complexes are present. Since K = 21 liters/mole and $\Delta H°$ = 7.75 kcal/mole (39) for the 1:1 complex whereas $K \simeq 1.0$ liter/mole and $\Delta H° \simeq 2.0$ kcal/mole in this study (for the weaker complexes where higher concentrations are required for a good separation of K and ϵ), 2:1 complexation should be less significant in these systems. In addition, higher order complexes should be observed as a significant curvature in the plots according to Eq. 7 or 8 (40).

[1] Spectrophotometric grade, Aldrich Chemical Co.
[2] Spectrophotometric grade, Matheson, Coleman and Bell.
[3] Gold label, Aldrich Chemical Co.
[4] Purity of 99+%, Aldrich Chemical Co.
[5] Superior grade, Matheson, Coleman and Bell.

Table I—Summary of Experimental Results for the Hexamethylbenzene–Chloranil Complex

T	K, liters/mole	ϵ' (10^{-3}), liters/(mole·cm)	$K\epsilon'$ (10^{-4}), liters²/(mole²·cm)	ρ
27.20°	7.86 ± 0.22	2.82 ± 0.06	2.21 ± 0.02	1.000
19.24°	10.86 ± 0.40	2.72 ± 0.06	2.95 ± 0.05	1.000
13.60°	12.39 ± 0.64	2.84 ± 0.09	3.52 ± 0.10	1.000
5.52°	14.20 ± 0.29	3.11 ± 0.04	4.41 ± 0.05	1.000

Determination of Heats of Complexation—The heats of complexation were determined using the van't Hoff equation (Eq. 9):

$$\ln K = -\frac{\Delta H^\circ}{R}\cdot\frac{1}{T} + \frac{\Delta S^\circ}{R} \qquad \text{(Eq. 9)}$$

assuming that ΔH° is constant over the temperature range of interest (0–40° in this study). Because it is often difficult to separate K and ϵ, ϵ being nearly temperature independent (41), a plot of log $K\cdot\epsilon$ *versus* $1/T$ usually provides a more precise estimate of ΔH°. The experimental procedure can be further facilitated by noting that incorporation of Beer's law into the equilibrium constant expression and taking the logarithm of both sides give the following

$$\log K\cdot\epsilon = \log A - \log(D_0 - C)(B_0 - C) \qquad \text{(Eq. 10)}$$

If K and ΔH° for the complex are small, the last term on the left-hand side of Eq. 10 does not change significantly with temperature and a plot of log A *versus* $1/T$ is equivalent to a plot of log $K\cdot\epsilon$ *versus* $1/T$. This considerably increases the precision of the determination of ΔH°, since the absorbance data can be used directly and are independent of an accurate knowledge of concentrations[6]. Since ϵ usually decreases slightly over a temperature range of 0–40°, plots of log $K\cdot\epsilon$ or log A *versus* $1/T$ provide upper estimates of the heat of complexation.

Procedure—Spectrophotometric readings were taken on a spectrophotometer[7] equipped with thermal-jacketed cell holders. Samples were placed in glass-stoppered quartz cells and allowed to equilibrate in the cell compartment. The temperature in the compartment was monitored with two iron–constantan thermocouples. The blank in all cases was the initial stock solution of the acceptor.

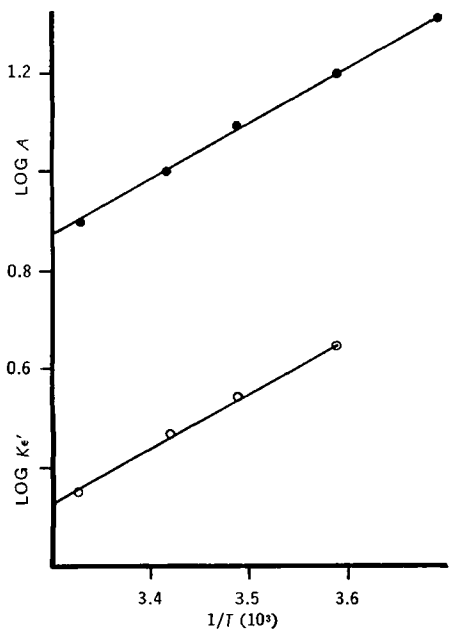


Figure 2—*Plots of log (absorbance) and log* K *versus* $1/T$ *for the chloranil–hexamethylbenzene complex.*

[6] The author thanks Professor Milton Tames for this suggestion.
[7] Both Beckman DU and Cary 14 spectrophotometers were used.

Table II—Experimental Heats of Complexation

Complex	Method	ΔH°, kcal/mole	ρ
Chloranil–hexamethylbenzene	log (Abs)	4.81 ± 0.26	0.999
	log ($K\epsilon$)	5.07 ± 1.29	0.997
Chloranil–mesitylene	log (Abs)	3.76 ± 0.11	0.999
	log (Abs)	3.84 ± 0.14	0.999
	log ($K\epsilon$)	3.97 ± 0.52	0.999
Chloranil–benzene	log (Abs)	2.36 ± 0.07	0.998
	log (Abs)	2.24 ± 0.12	0.996
	log ($K\epsilon$)	2.18 ± 0.62	0.996
Tetrachlorophthalic anhydride–benzene	log ($K\epsilon$)	4.19 ± 0.12	1.000

Table III—Thermodynamic Parameters at 20° for the Various Complexes

Complex	K_c, liters/mole	ΔH°, kcal/mole	$T\,\Delta S^\circ_c$, kcal/mole
Chloranil–hexamethylbenzene	10.04	−4.94	−3.57
Chloranil–mesitylene	2.12	−3.86	−3.42
Chloranil–benzene	0.39	−2.26	−2.81
Tetrachlorophthalic anhydride–benzene	6.87	−4.19	−3.07

The results were plotted according to Eq. 7 or 8 to determine the equilibrium constant and molar absorptivity and, according to Eq. 9 or 10, with appropriate density corrections, to determine the heat of complexation.

RESULTS

The systems studied experimentally are the chloranil complexes with benzene, mesitylene, and hexamethylbenzene and the tetrachlorophthalic anhydride complex with benzene in carbon tetrachloride. Figure 1 shows a typical plot of D_0B_0/A *versus* $D_0 + B_0$ (Eq. 8) for the chloranil–hexamethylbenzene complex (each point in Fig. 1 represents the determination from a single sample). The plots are linear and Table I summarizes the results. In this and the following tables, $\epsilon' = \epsilon_C - \epsilon_B$ and ρ represents the correlation coefficient and the uncertainty to the 95% confidence limits. The equilibrium constants (K) and $K\cdot\epsilon'$ products are all significantly different, with the latter being more precise. Figure 2 shows a plot of log A (density corrected) and log $K\cdot\epsilon'$ as $1/T$. Both plots are linear, giving values of 4.81 ± 0.26 and 5.07 ± 1.29 kcal/mole, respectively, for the heat of complexation. Table II presents the heats of complexation for all complexes studied. As expected, the log A method provides the more precise measurement of ΔH°.

Table III summarizes the thermodynamic parameters at 20° for the various complexes. The equilibrium constants (K) were determined from the $K\cdot\epsilon'$ product using an average ϵ' over the temperature range studied.

DISCUSSION

The experimental bond polarizabilities, ionization potentials, and van der Waals radii were taken from Refs. 15, 42, and 43, respectively. The net atomic charges were calculated[8] by the CNDO/2[9] method (44) for the independent molecules of fluoranil (I), chloranil (II), tetrachlorophthalic anhydride (III), benzene (IV), toluene (V), *p*-xylene (VI), mesitylene (VII), durene (VIII), pentamethylbenzene (IX), and hexamethylbenzene (X). The interaction energy is then calculated by summing the electrostatic, polarization, dispersion, and repulsive contributions (Eqs. 1, 4, 2, and 5, respectively).

[8] For these calculations, the orientation of each methyl group is such that one proton is in the plane of the ring and the other two are above and below the ring. With this orientation, the two planar surfaces are identical while there is a slight asymmetry in the plane containing the C_6 axis (of the benzene ring) and the methyl group of, for example, toluene. This asymmetry has virtually no effect on the reported results.
[9] Complete neglect of differential overlap, second parameterization.

Table IV—Contributions to Interaction Energy[a] for the Fluoranil Complexes

Fluoranil Complex	R^b	El[b]	Pol[b]	Disp[b]	Rep[b]	$E_{tot}{}^b$
Benzene	3.60	0.136	−0.364	−7.709	3.452	−4.394
Toluene	3.60	0.135	−0.376	−8.222	3.850	−4.613
p-Xylene	3.65	0.152	−0.361	−8.147	3.555	−4.802
Mesitylene	3.65	0.104	−0.406	−8.747	3.803	−5.241
Durene	3.65	0.189	−0.403	−9.142	4.036	−5.320
Pentamethylbenzene	3.65	0.196	−0.426	−9.593	4.310	−5.513
Hexamethylbenzene	3.70	0.218	−0.422	−9.473	3.947	−5.730

[a] In kilocalories per mole. [b] R = interplanar distance in Å, El = electrostatic energy, Pol = polarization energy, Disp = dispersion energy, Rep = repulsive energy, and E_{tot} = total interaction energy.

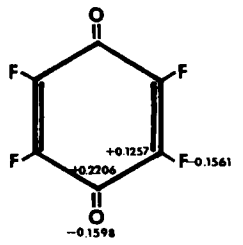
Table V—Contributions to the Interaction Energy[a] for the Chloranil Complexes

Chloranil Complex	R^b	El[b]	Pol[b]	Disp[b]	Rep[b]	$E_{tot}{}^b$
Benzene	3.40	0.135	−0.356	−15.863	7.521	−8.563
Toluene	3.45	0.127	−0.345	−15.810	7.231	−8.796
p-Xylene	3.45	0.149	−0.357	−16.832	8.003	−9.037
Mesitylene	3.55	0.095	−0.345	−16.184	7.248	−9.186
Durene	3.60	0.145	−0.324	−15.904	6.769	−9.314
Pentamethylbenzene	3.60	0.163	−0.342	−16.855	7.546	−9.488
Hexamethylbenzene	3.65	0.193	−0.338	−16.777	7.175	−9.747

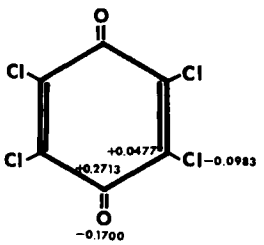
[a] In kilocalories per mole. [b] See Footnote *b* of Table IV.

When considering the methyl groups as fixed, the independent molecules have no internal degrees of freedom. The interaction energy is then a function of six variables, $E = E(\bar{R},\bar{\theta})$, three translational ($\bar{R}$) and three rotational ($\bar{\theta}$). Since no general methods are available for locating a global minimum of a nonlinear function of six variables (45), a systematic search was employed. The procedure consisted of calculating the total energy at approximately 50 different combinations of $\bar{R}$ and $\bar{\theta}$ to obtain a preliminary potential energy map, and then the energy values were further refined at these minima[10]. For the methylbenzene complexes with chloranil and fluoranil, the structure with the lowest minima, highest binding energy is with the two six-membered rings directly above one another, as shown for the toluene–fluoranil complex (XI). For the benzene, mesitylene, and hexamethylbenzene complexes, a single potential energy minimum exists by symmetry. For the toluene, *p*-xylene, durene, and pentamethylbenzene, two separate potential energy minima are observed. In the case of the toluene–fluoranil complex, the structures would correspond to the methyl group, on toluene, lying between the oxygen and fluorine atoms (XI) or between two of the fluorine atoms of the fluoranil. The energy difference between the two minima is calculated to be less than 0.2 kcal/mole for the fluoranil complexes and 0.5 kcal/mole for the chloranil complexes. This implies that with unsymmetrical donors, *e.g.*, toluene, complex structural isomers may exist. The skewing of the two rings in these complexes results from a reduced repulsive interaction in this position. While there is a falloff in the dispersion energy, the dispersion interaction being at a maximum when the bonds are parallel, the falloff in the repulsive energy is somewhat larger. In the case of the benzene–fluoranil and chloranil complexes, the calculated total interaction energy of the parallel (*i.e.*, all bonds being parallel) and skewed structures is less than 0.1 kcal/mole. An increasing preference for the skewed structure is observed with increasing methyl substitution on the donor due to the increased repulsion between the methyl groups and the oxygen or fluorines (chlorines) of the fluoranil (chloranil) molecule.

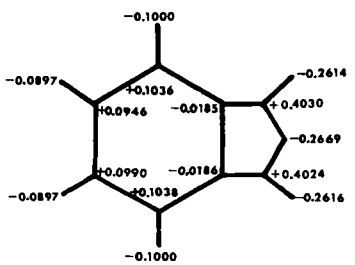
The contributions to the total interaction energy of the fluoranil–methylbenzene complexes at the primary potential energy minimum are presented in Table IV. The interplanar distance (R, in Å) increases with increasing methyl substitution on the donor due to the repulsive interaction. The electrostatic interaction energy is positive (repulsive), since the net atomic charges tend to be positive for the ring carbons and negative for the substituents in both donor and acceptor molecules (I–X). The polarization energy, due mainly to the polarization of the donor by the acceptor, in-



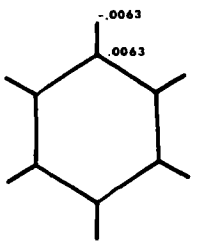
I: fluoranil



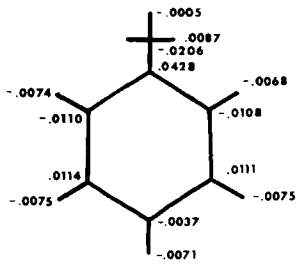
II: chloranil



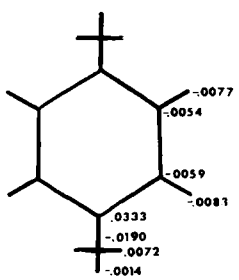
III: tetrachlorophthalic anhydride



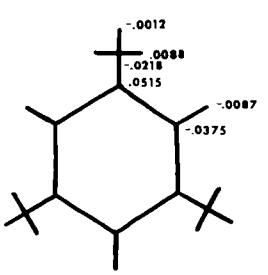
IV: benzene



V: toluene



VI: *p*-xylene



VII: mesitylene

[10] The reported results refer only to the lowest potential energy minimum. More complete information on the potential energy map can be obtained from the author on request.

Table VI—Contributions to the Interaction Energy[a] for the Tetrachlorophthalic Anhydride Complexes

Structure	Donor	R^b	El[b]	Pol[b]	Disp[b]	Rep[b]	E_{tot}[b]
XII	Hexamethylbenzene	3.80	0.21	−0.45	−12.76	5.25	−7.75
XII	Benzene	3.50	0.14	−0.45	−12.49	5.79	−7.01
XIII	Hexamethylbenzene	3.80	0.20	−0.44	−12.79	6.59	−6.44
XIII	Benzene	3.60	0.13	−0.37	−10.72	4.65	−6.31
XIV	Hexamethylbenzene	3.80	0.16	−0.43	−11.30	4.16	−7.41
XIV	Benzene	3.40	0.10	−0.46	−12.24	6.07	−6.53
XV	Hexamethylbenzene	3.80	0.21	−0.45	−12.78	5.40	−7.62
XV	Benzene	3.50	0.14	−0.45	−12.57	5.82	−7.06

[a] In kilocalories per mole. [b] See Footnote *b* of Table IV.

creases with each additional methyl substitution and is a result of the accompanying increase in polarizability of the donor.

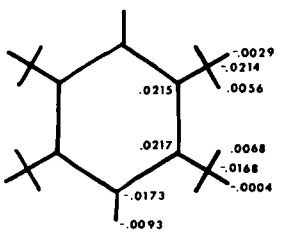
The most significant contributions to the total interaction energy are made by the dispersion and repulsive forces. Both forces significantly increase with each additional methyl substitution on the donor. The total (net) interaction energy, as well as the structure of these complexes, is thus essentially determined by the dispersion–repulsion balance. For example, in the case of the hexamethylbenzene complex, the dispersion and repulsive terms are actually lower than those of the pentamethylbenzene complex (Table IV). This is due to the larger equilibrium interatomic separation (3.70 *versus* 3.65 Å). At 3.65 Å, both the dispersion and repulsive contributions are larger in the hexamethylbenzene–fluoroanil complex.

Table V shows similar results for the chloranil–methylbenzene complexes. The trends observed are similar to those with fluoranil as the acceptor but with a large increase in the dispersion and repulsive forces. This is due to the larger polarizabilities and van der Waals radii of the chlorine atoms compared to the fluorine atoms.

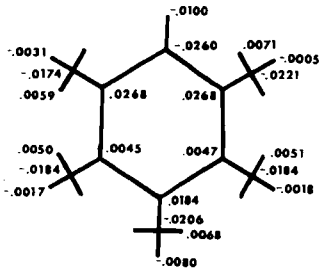
Several structures (XII–XV) were considered for the benzene and hexamethylbenzene complexes with tetrachlorophthalic anhydride. As can be seen from the interaction energies in Table VI, the maximum interaction occurs in both cases with the donor approximately over the center of the acceptor. Displacement to either side of the acceptor significantly reduces the interaction energy due to a reduction in dispersion and/or increase in repulsive energies in those positions.

A comparison of the theoretical and experimental results for the benzene and hexamethylbenzene complexes with the various acceptors is presented in Table VII. It is apparent that while the trend of increasing complex strength with increasing methyl substitution on the donor is predicted for a given acceptor, the theoretical increase underestimates the experimental value by a factor of two to three. Furthermore, the absolute interaction energy provides a considerable overestimate of the experimental heat of complexation in solution, and the overall correlation between the heat of complexation and absolute interaction energy is poor.

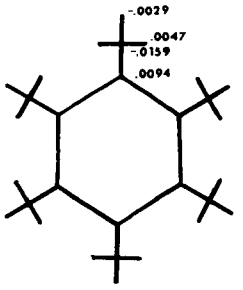
These results suggest an important role for the nonpolar solvent carbon tetrachloride. Solute–solvent interaction would be expected to reduce the interaction energies while the solvent–solvent term would tend to increase the complex strength. The net effects of adding these terms to the absolute interaction energy must be to reduce the net interaction and to magnify the increase in complex strength per methyl group.



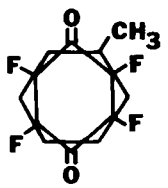
VIII: durene



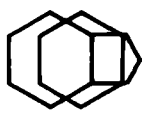
IX: pentamethylbenzene



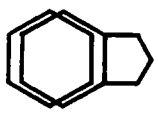
X: hexamethylbenzene



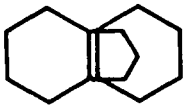
XI



XII



XIII



XIV



XV

CONCLUSIONS

The results of absolute interaction energy calculations for the methylbenzene complexes with chloranil, fluoranil, and tetrachlorophthalic anhydride indicate that the dispersion force is the major attractive contributor to the total interaction energy and that the balancing of the dispersion and repulsive forces predomi-

Table VII—Experimental and Theoretical (Gas Phase) Interaction Energies

Acceptor	Donor	Experimental $\Delta H°$, kcal/mole	Absolute Interaction Energy, kcal/mole
Fluoranil	Benzene	−2.0[a]	−4.39
Fluoranil	Hexamethylbenzene	−5.4[a]	−5.73
Difference		3.4	1.34
Chloranil	Benzene	−2.26[b]	−8.56
Chloranil	Hexamethylbenzene	−4.94[b]	−9.75
Difference		2.84	1.19
TCPA[c]	Benzene	−4.19[b]	−7.06
TCPA	Hexamethylbenzene	−5.75[d]	−7.72
Difference		1.56	0.66

[a] R. Foster, C. Fife, and M. Foreman, *Chem. Commun.*, **1967**, 913. [b] This work. [c] TCPA = tetrachlorophthalic anhydride. [d] J. Czekalla and K. Meyer, *Z. Phys. Chem.*, **27**, 185(1961).

nantly determines the structures and net interaction energies.

While the correlation between the absolute interaction energy and experimental heat of complexation in carbon tetrachloride is poor, the trends within each complexing series are predicted. However, the theoretical increase in the interaction energy per methylene group underestimates the experimental increase by a factor of two to three.

Since the comparison is being made between absolute (gas phase) and solution interaction energies, the results suggest that the solvent effects must account for the additional increase in complex strength per methylene group as well as a lowering of the net interaction energies in solution.

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A listing of the computer programs written by the author (in FORTRAN IV) to perform the absolute energy calculations reported in this paper may be obtained from the author on request.

Present address: School of Pharmacy, University of Wisconsin, Madison, WI 53706

Theoretical Calculation of Heats of Complexation in Carbon Tetrachloride

GORDON L. AMIDON

Abstract □ The theoretical absolute (gas phase) interaction energies of complexation are combined with a cavity model for the solvent carbon tetrachloride to calculate the theoretical heats of complexation for the complexes of chloranil, fluoranil, and tetrachlorophthalic anhydride in solution. The agreement between theoretical calculations and experimental results is excellent. The absolute interaction energy and the donor–solvent term increase in a parallel manner within each series, thus approximately canceling, and the overall complexation process may be considered as solvent driven. These results suggest that for planar complexing systems in solution, both the solvent term and the absolute interaction energy term will usually be maximized when maximum overlapping of area occurs.

Keyphrases □ Complexation, heats—theoretical calculation, chloranil, fluoranil, and tetrachlorophthalic anhydride complexes, gas phase interaction energies and cavity model □ Interaction energies (of complexation)—theoretical values combined with cavity model for carbon tetrachloride, used to calculate theoretical heats of complexation for chloranil, fluoranil, and tetrachlorophthalic anhydride complexes in solution □ Carbon tetrachloride—interaction with and contribution to chloranil, fluoranil, and tetrachlorophthalic anhydride complexes, theoretical interaction energies combined with cavity model, used to calculate theoretical heats of complexation □ Solvents—carbon tetrachloride, interaction energies of complexation and cavity model, used to calculate the theoretical heats of complexation for chloranil, fluoranil, and tetrachlorophthalic anhydride complexes in solution

In a preceding article (1), the theoretical, absolute interaction energies and structures of the complexes of fluoranil, chloranil, and tetrachlorophthalic anhydride with various methylbenzenes were reported. A comparison of the absolute interaction energies with the experimental values in carbon tetrachloride solution indicated that, while the general trends within each series were correctly predicted, the increase in the absolute interaction energy per methyl group was less than the experimental solution value. Furthermore, the predicted increase in the absolute interaction of chloranil over the fluoranil complexes with the various methylbenzenes is not present in the solution heats of complexation. These factors indicate the importance of the solvent in determining the overall heat of complexation. In this report, a cavity model for the solvent (2, 3) is used to calculate the

Table I—Molecular Surface Areas

Compound	$\bar{r}$, Å	Surface Area, Å²
Benzene	3.68	42.57
Hexamethylbenzene	4.90	75.39
Fluoranil	4.04	51.28
Chloranil	4.46	62.46
Tetrachlorophthalic anhydride[a]	—	78.19

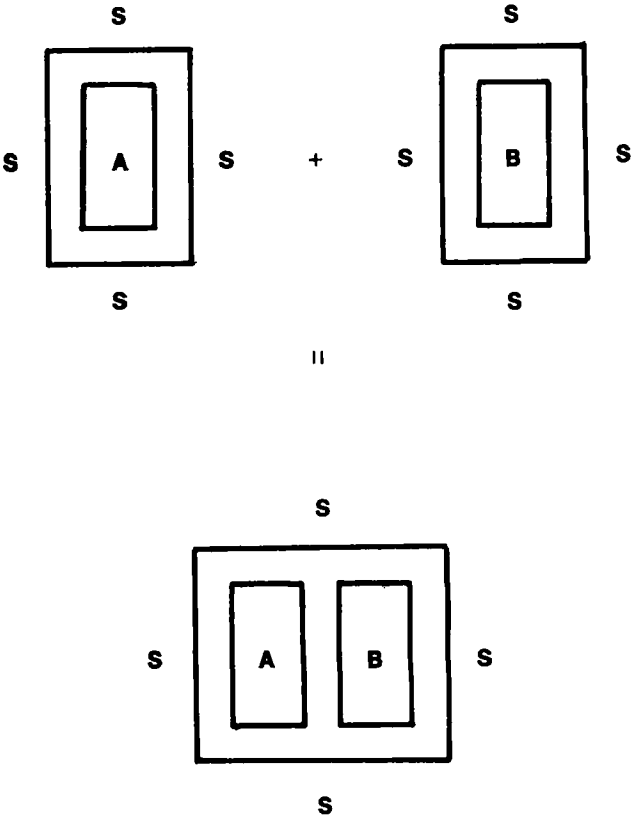
[a] A rectangular surface with length of 9.22 Å and width of 8.48 Å was used.

Table II—Solvent Contribution to the Heat of Complexation in Carbon Tetrachloride for the Fluoranil Complexes

Fluoranil Complex	Surface Area Loss, Å²	E_{SS}, kcal/mole	γ/γ_0	E_{SS}', kcal/mole
Benzene	93.85	−7.27	0.50	−5.94
Toluene	99.32	−7.70	0.51	−6.33
p-Xylene	104.79	−8.12	0.52	−6.72
Mesitylene	110.26	−8.55	0.53	−7.11
Durene	115.73	−8.97	0.54	−7.51
Pentamethylbenzene	121.20	−9.40	0.55	−7.91
Hexamethylbenzene	126.67	−9.82	0.56	−8.32

Table III—Solvent Contribution to the Heat of Complexation in Carbon Tetrachloride for the Chloranil Complexes

Chloranil Complex	Surface Area Loss, Å²	E_{SS}, kcal/mole	γ/γ_0	E_{SS}', kcal/mole
Benzene	105.02	−8.14	0.52	−6.73
Toluene	110.50	−8.56	0.53	−7.13
p-Xylene	115.96	−8.99	0.54	−7.52
Mesitylene	121.43	−9.41	0.55	−7.92
Durene	126.90	−9.84	0.56	−8.33
Pentamethylbenzene	132.37	−10.26	0.57	−8.74
Hexamethylbenzene	137.84	−10.68	0.58	−9.15



Scheme I—Cavity model of complexation

Table IV—Solvent Contribution to the Heat of Complexation in Carbon Tetrachloride for the Tetrachlorophthalic Anhydride Complexes

Tetrachlorophthalic Anhydride Complex	Surface Area Loss, Å²	E_{SS}, kcal/mole	γ/γ_0	E_{SS}', kcal/mole
Hexamethylbenzene	153.58	−12.67	0.63	−10.49
Benzene	120.75	−9.97	0.55	−7.87

solvent contribution to the overall heat of complexation. These results, combined with the absolute energies for the donor–acceptor (1), donor–solvent, and acceptor–solvent interactions can then be compared with the experimental solution values.

The goal of this work is to determine which, if any, terms dominate the complexation process in solution and if overall agreement between the theoretical and experimental complexation energies in solution can be achieved.

THEORETICAL

The model considered here is shown schematically in Scheme I for the complexation reaction $A + B = A{\cdot}B$. The energy of complexation is given by Eq. 1:

$$\Delta E_{comp} = E_{AB} + E_{SS} - E_{AS} - E_{BS} \quad \text{(Eq. 1)}$$

where E_{AB} is the absolute interaction energy of the complex, E_{AS} and E_{BS} are the solute–solvent interaction terms, and E_{SS} is the solvent term. Since the surface area of the $A{\cdot}B$ complex will usually be less than the surface area of A plus the surface area of B, the solvent term will tend to drive the reaction to the right—the "solvophobic force" (2). The magnitude of this contribution to the heat of complexation is given by the surface energy of the solvent per unit surface area, E^S, times the area lost on complexation, ΔSA (Eq. 2):

$$E_{SS} = E^S \cdot \Delta SA \quad \text{(Eq. 2)}$$

The surface energy, E^S, is calculated using the surface tension, γ, Eotvos constant, k_E, and molar volume, V, of the liquid (4):

$$E^S = \gamma - T\,d\gamma/dT \quad \text{(Eq. 3)}$$

$$\frac{d\gamma}{dT} = -k_E \cdot V^{-2/3} \quad \text{(Eq. 4)}$$

Since the surface energy, E_S, is relatively temperature independent, at points significantly below the critical temperature of the liquid, small changes in the choice of T have no effect on E^S.

Since surfaces of molecular dimensions are involved, it is necessary to consider a curvature-corrected surface energy. The surface energy, E^S, represents the energy required to move a molecule from a bulk situation to a planar surface, where it has a lower coordination number. Since the coordination number of a molecule is somewhat higher at a curved surface, E^S will be somewhat smaller, corresponding to a more bulk-like surface situation. For this purpose, the method of Wakeshima is employed (5).

Another method for considering the solvent effects is to construct the thermodynamic cycle (6) shown in Scheme II:

$$\begin{array}{ccccc} D(g) & + & A(g) & \xrightarrow{\Delta E(g)} & DA(g) \\ \downarrow \Delta E_D(\text{solv}) & & \downarrow \Delta E_A(\text{solv}) & & \downarrow \Delta E_{DA}(\text{solv}) \\ D(\text{soln}) & + & A(\text{soln}) & \xrightarrow{\Delta E(\text{soln})} & DA(\text{soln}) \end{array}$$

Scheme II

The energy of complexation in solution (soln) is then related to that in the vapor phase (*g*) by the following equation:

$$\Delta E(\text{soln}) = \Delta E(g) + [\Delta E_{DA}(\text{solv}) - \Delta E_A(\text{solv}) - \Delta E_D(\text{solv})] = \Delta E(g) + \Delta[\Delta E(\text{solv})] \quad \text{(Eq. 5)}$$

Table V—Acceptor–Solvent Interaction Energies[a]

Carbon Tetrachloride with	R[b]	El[b]	Pol[b]	Disp[b]	Rep[b]	E_{tot}[b]
Fluoranil	3.9	0.001	−0.507	−7.800	3.891	−4.414
Chloranil	3.6	−0.004	−0.568	−18.092	9.749	−8.914
Tetrachlorophthalic anhydride	3.8	0.012	−0.530	−12.555	6.232	−6.841

[a] In kilocalories per mole. [b] R = interplanar distance in Å, El = electrostatic energy, Pol = polarization energy, Disp = dispersion energy, Rep = repulsive energy, and E_{tot} = total interaction energy.

Table VI—Donor–Solvent Interaction Energies[a]

Carbon Tetrachloride with	R[b]	El[b]	Pol[b]	Disp[b]	Rep[b]	E_{tot}[b]
Benzene	4.0	0.000	−0.001	−7.152	3.095	−4.057
Toluene	4.0	0.001	−0.001	−7.328	3.123	−4.205
p-Xylene	4.0	0.002	−0.001	−7.571	3.152	−4.418
Mesitylene	3.9	0.001	−0.001	−9.326	4.371	−4.956
Durene	4.0	0.002	−0.001	−8.175	3.276	−4.899
Pentamethylbenzene	4.0	0.001	−0.001	−8.509	3.347	−5.162
Hexamethylbenzene	3.9	0.001	−0.002	−10.154	4.588	−5.567

[a] In kilocalories per mole. [b] See Footnote *b* of Table V.

Table VII—Summary of Results[a] for the Fluoranil Complexes in Carbon Tetrachloride

Fluoranil Complex	E_{AB}	E_{SS}	E_{SS}'	E (donor–CCl_4)	E_{th}	E_{th}'	E_{exp}[b]
Benzene	−4.39	−7.27	−5.94	−4.06	−3.19	−1.86	−2.0
Toluene	−4.61	−7.70	−6.33	−4.21	−3.69	−2.32	−2.3
p-Xylene	−4.80	−8.12	−6.72	−4.42	−4.09	−2.69	−2.7
Mesitylene	−5.24	−8.55	−7.11	−4.96	−4.42	−2.98	−3.0
Durene	−5.32	−8.97	−7.51	−4.90	−4.98	−3.52	−3.9
Pentamethylbenzene	−5.51	−9.40	−7.91	−5.16	−5.34	−3.85	−4.4
Hexamethylbenzene	−5.73	−9.82	−8.32	−5.57	−5.57	−4.07	−5.4

[a] In kilocalories per mole. [b] From Ref. 10.

Table VIII—Energy Term Differences between the Fluoranil–Hexamethylbenzene and Fluoranil–Benzene Complexes

Experimental	−3.40
Theoretical	−2.38
Theoretical (curvature corrected)	−2.21
Absolute (ΔE_{AB})	−1.34
Donor–solvent	−1.51
Solvent (ΔE_{SS})	−2.55
Solvent (curvature corrected) ($\Delta E_{SS}'$)	−2.37

within the context of the cavity model:

$$\Delta[\Delta E(\text{solv})] = [E_{DA}(\text{cavity}) + E(DA - \text{cavity}) - (E_A(\text{cavity})) + E(A - \text{cavity})] - [E_D(\text{cavity}) + E(D - \text{cavity})] \quad \text{(Eq. 6)}$$

where $E_X(\text{cavity})$, $X = A, D, DA$, is the energy required to create a cavity for X in the solvent, and $E(X - \text{cavity})$ is the interaction energy between X and the cavity walls (solvent). Rearranging Eq. 6, one has:

$$\Delta[\Delta E(\text{solv})] = [\Delta E(\text{cavity}) - \Delta E(\text{interaction})] \quad \text{(Eq. 7)}$$

where:

$$\Delta E(\text{cavity}) = E_{DA}(\text{cavity}) - E_D(\text{cavity}) - E_A(\text{cavity}) \quad \text{(Eq. 8)}$$

and:

$$\Delta E(\text{interaction}) = E(DA - \text{cavity}) - E(A - \text{cavity}) - E(D - \text{cavity}) \quad \text{(Eq. 9)}$$

By referring to Scheme I, the ΔE(cavity) term can be calculated by estimating the surface area lost on complexation, and the ΔE(interaction) is equal to the net solute–solvent interaction lost on complexation.

RESULTS

Cavity Term—The surface areas of the interacting planes of the various molecules were calculated using the standard van der Waal radii (7) and the values shown in Table I. The $\bar{r}$ represents the mean radius, from the center of the molecule, in two different directions, corresponding to the maximum and minimum values for r. The surface areas of the various methylbenzenes were calculated using a value of 5.47 Å^2 per methyl group. The surface tension and Eotvos constant of carbon tetrachloride at 20° were taken to be 26.75 dynes/cm (8) and 2.21 (9), respectively. The surface area lost on complexation was taken to be the sum of the interacting planar surface areas of the two complexing molecules.

Tables II–IV show the solvent contribution to the energy of complexation for the complexes of fluoranil, chloranil, and tetrachlorophthalic anhydride. In these tables, γ/γ_0 represents the ratio of the curvature-corrected surface tension to the surface tension of a planar surface. The radius of the cavity for the complex was taken to be the mean of the radii of the two complexing molecules. The terms E_{SS}' and E_{SS} are the solvent terms with and without curvature correction. These results indicate a significant contribution of the solvent to the increase in complex strength with increasing methyl substitution. In all cases the solvent term alone contributes 2–3 kcal/mole to the increase in the complex strength of hexamethylbenzene over the corresponding benzene complex.

Table X—Energy Term Differences between the Chloranil–Hexamethylbenzene and Chloranil–Benzene Complexes

Energy Term	ΔE, kcal/mole
Experimental	−2.84
Theoretical	−2.20
Theoretical (curvature corrected)	−2.10
Absolute (ΔE_{AB})	−1.19
Donor–solvent	−1.51
Solvent (ΔE_{SS})	−2.54
Solvent (curvature corrected) ($\Delta E_{SS}'$)	−2.42

The effect of the curvature correction is to lower the absolute value of the solvent contribution to the overall complexation reaction. The increase in the hexamethylbenzene complex strength over the benzene complex remains nearly the same as that without the curvature correction. It should be noted, however, that the curvature correction was applied to the surface free energy term, γ, in Eq. 3, but not the entropy term, $S^s = -d\gamma/dT$, in Eq. 3. This probably leads to an underestimate of the curvature correction to the surface energy since the entropy of surface formation is positive for carbon tetrachloride and would probably be less positive at a curved (more bulk-like) surface. For this reason, as well as the inherent uncertainty in the curvature correction to the surface tension, the curvature-corrected solvent contribution provides only an indication as to the effects of such considerations. Since the curvature correction depends on the radius of the cavity, which is nearly constant in these series, it affects mainly the absolute energy values and has little effect on energy differences.

Solute–Solvent Term—The two solute–solvent terms, E_{AS} and E_{BS} in Eq. 1, were determined by calculating the absolute interaction energies (1) of carbon tetrachloride with the planar faces of the donor and acceptor molecules from which they are displaced on complexation (Eq. 9). These values represent the difference in the solute–solvent interaction when the solute is free and complexed, *i.e.*, E_{AS} (complex) − E_{AS} (free), assuming one molecule of carbon tetrachloride is displaced from the planar surface of the solute. The tabulated values refer to the absolute interaction energy at the potential energy minima, and the results are shown in Tables V and VI[1]. The interactions are mainly dispersive and, of the acceptor molecules, chloranil shows the strongest interaction with the solvent. The donor–solvent interactions increase with each additional methyl substitution, with the planar face of hexamethylbenzene showing a 1.5 kcal/mole stronger interaction with the solvent than benzene.

Table IX—Summary of Results[a] for the Chloranil Complexes in Carbon Tetrachloride

Chloranil Complex	E_{AB}	E_{SS}	E_{SS}'	E (donor–CCl_4)	E_{th}	E_{th}'	E_{exp}[b]
Benzene	−8.56	−8.14	−6.73	−4.06	−3.73	−2.32	−2.26 (−1.67)
Toluene	−8.80	−8.56	−7.13	−4.21	−4.24	−2.81	
p-Xylene	−9.04	−8.99	−7.52	−4.42	−4.70	−3.23	
Mesitylene	−9.19	−9.41	−7.92	−4.96	−4.73	−3.24	−3.86
Durene	−9.31	−9.84	−8.33	−4.90	−5.34	−3.83	(−4.41)
Pentamethylbenzene	−9.49	−10.26	−8.74	−5.16	−5.68	−4.16	
Hexamethylbenzene	−9.75	−10.68	−9.15	−5.57	−5.95	−4.42	−4.94 (−5.15)

[a] In kilocalories per mole. [b] Values in parentheses are those of Briegleb *et al.* (14, 15).

DISCUSSION

These results can now be combined with those of the preceding paper (1) to provide an overall quantitative picture of complexation in the solvent carbon tetrachloride.

Table VII compares the experimental (10) and theoretical results for the complexes of fluoranil with the methylbenzenes. The experimental value in all cases lies between the two theoretical estimates. In Table VIII the differences in the various energy terms for the benzene *versus* hexamethylbenzene complexes with fluoranil are presented. The absolute interaction energy term, E_{AB},

[1] More complete potential energy maps are available from the author upon request.

Table XI—Summary of Results[a] for the Tetrachlorophthalic Anhydride Complexes in Carbon Tetrachloride

Tetrachlorophthalic Anhydride Complex	E_{AB}	E_{SS}	$E_{SS'}$	E (Donor–CCl_4)	E_{th}	$E_{th'}$	E_{exp}
Hexamethylbenzene	−7.75	−12.67	−10.49	−5.57	−8.01	−5.83	−5.75[b]
Benzene	−7.06	−9.97	−7.87	−4.06	−6.13	−4.03	−4.19

[a] In kilocalories per mole. [b] From Ref. 16.

the solvent terms, E_{SS} and $E_{SS'}$, and the donor–solvent term, E (donor–CCl_4), all increase with increasing methyl substitution. The increase in the donor–solvent term nearly cancels the increase in the absolute interaction energy term (Eq. 1). While this grouping of the terms is somewhat arbitrary (note also the paralleling of the two terms in Table VII), it is suggestive of the important role of even the nonpolar solvent carbon tetrachloride. The overall theoretical estimate still underestimates the experimental increase in the complex strength per methyl group. However, since the experimental values were determined by a plot of log K *versus* $1/T$, this difference may not be significant due to experimental uncertainties (1).

The results for the chloranil–methylbenzene complexes are shown in Tables IX and X. The agreement between theory and experiment is again good. The large increase in the absolute interaction term for a given methylbenzene complex with chloranil over fluoranil is essentially canceled by the larger acceptor–solvent term for chloranil. Thus, the theoretical estimate of the solution heat of complexation is nearly the same in the two series and is in line with the experimental values. Again, the solvent term is significant, with the donor–solvent and absolute interaction terms approximately canceling.

The tetrachlorophthalic anhydride complexes with benzene and hexamethylbenzene provide examples where the molecular sizes of the complexing systems are significantly different. The theoretical and experimental values agree quite well and, again, the solvent term is a predominant factor (Table XI).

Within the context of the cavity model, both the solvent contribution to the heat of complexation, E_{SS}, and the solute–solvent terms, E_{AS} and E_{BS}, depend on the surface area change on complexation. This would suggest that for complexes with little surface area change on complexation, the gas phase and solution heats of complexation would be nearly the same. This prediction is supported by data on the diethyl sulfide–iodine and benzene–iodine complexes (11) in which the solution and gas phase $\Delta H°$'s are approximately equal.

The solvent contribution, E_{SS}, also depends on the surface energy of the solvent, E^S; for solvents with a high E^S, the solvent term may well be the largest energy term. For example, since the E^S of water is about two to three times larger than that of carbon tetrachloride, the contribution of the solvent term to the energy of complexation would be correspondingly larger. Therefore, this may be the basis for the good correlations of $\Delta G°$ of complexation with surface area (12) and for the decreasing complex strength in mixed solvents of lower surface energy. The principle of maximum overlap of areas can also be justified by considering the absolute energy term, E_{AB}, since this term is also likely to be at a maximum when the areas of overlap are maximized. Thus, for planar-type complexes in solution, both terms that lower the energy of the complex relative to the independent molecules are likely to be maximized when the area of overlap is maximized. Since, for a given complex, many structures may be consistent with the maximum overlapping of areas while having different absolute energies, the actual structure of the complex will be determined by the relative absolute energy terms. This is probably the reason for some of the deviations observed in plots of complex strength *versus* maximum overlap area (13).

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Present address: School of Pharmacy, University of Wisconsin, Madison, WI 53706

Structure and Reactivity of Theobrominate and Theophyllinate Complexes with Methyl *trans*-Cinnamate

GORDON L. AMIDON

Abstract □ The structure of the methyl *trans*-cinnamate complexes with the theophylline and theobromine anions is calculated theoretically by maximizing the binding energy of the two interacting molecules. The binding energy is calculated using the monopoles-bond polarizabilities method. The predicted structures correlate well with observed reactivity of the ester in the two complexes.

Keyphrases □ Theobromine—structure and reactivity of complexes with methyl *trans*-cinnamate, theoretical calculation by maximizing binding energy □ Theophyllinate—structure and reactivity of complexes with methyl *trans*-cinnamate, theoretical calculation by maximizing binding energy □ Methyl *trans*-cinnamate—structure and reactivity of theobrominate and theophyllinate complexes

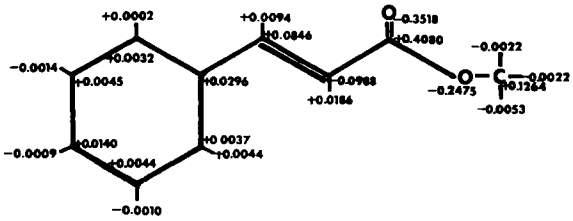
In a previous report (1), the method of monopoles-bond polarizability was discussed and applied to the calculation of the absolute interaction energy and structures of some electron donor–acceptor complexes. These results were then combined with a cavity model for the solvent to calculate the heat of complexation in solution (2). The agreement between theoretical calculations and experimental results was quite good. In this publication, attention is focused on the structure of complexes in solution.

The inhibition of a chemical reaction by a complexing ligand has frequently been observed. It was reported (3, 4) that caffeine inhibits the hydrolysis of benzocaine and procaine in aqueous solution, and this was attributed to complexation. The rate of alkaline hydrolysis of riboflavin and the ester 3-carbomethoxy-1-methylpyridinium cation was shown to be inhibited by xanthines (5, 6). The inhibition of the photodecomposition of menadione by electron donors has also been attributed to complexation (7).

Methods have been presented for separating out the reaction rate of the complexed drug (8, 9). The fractional decrease in reactivity of the complexed substrate was defined (8, 9) relative to the free substrate to be q_{11}:

$$q_{11} = 1 - \frac{k_{cs}}{k_s} \qquad \text{(Eq. 1)}$$

where k_s is the substrate reactivity, and k_{cs} is the reactivity of the substrate bound to the ligand. For the alkaline hydrolysis of the methyl *trans*-cinnamate ester–theobrominate complex, $q_{11} = 1.0$; for the corresponding theophyllinate complex, $q_{11} = 0.9$. Since the stability constants for the two ligands are about the same, a possible reversal of the xanthine–cinnamate orientation was suggested, with the theobrominate ligand occupying a position closer to the ester carbonyl (9). This orientation would be expected to lower the reaction rate on the basis of steric interference of the attacking nucleophile as well as a possible raising of the activation energy for tetrahedral intermediate formation.



I: methyl *trans*-cinnamate [(*S-cis*)]

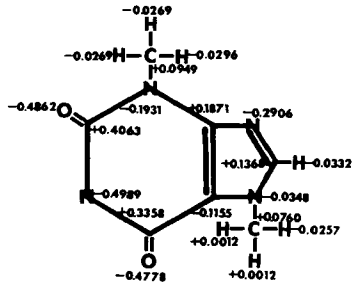
In this report this suggestion is tested by determining the structures of the two complexes theoretically.

THEORETICAL

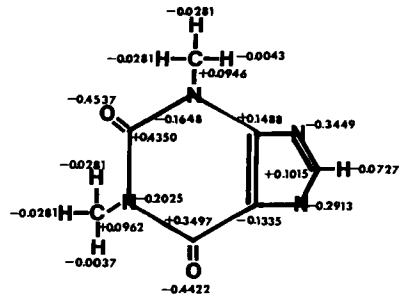
In a previous publication (2), it was concluded that the solvent term provides a general driving force toward the maximum overlapping of areas in carbon tetrachloride. This seems to be also true in aqueous solutions (2, 9, 10).

If several structures have nearly the same maximal overlap areas, then the preferred structure will be that which maximizes the absolute interaction energy. It is on this basis that a calculation of the complex structure is approached by maximizing the absolute interaction energy.

The absolute interaction energies are calculated using the monopoles-bond polarizabilities method (1, 11). The necessary data are taken from the references cited in a previous paper (1). The charge distributions were calculated by the CNDO/2[1] method (1, 2) for methyl *trans*-cinnamate (I) and the theobrominate (II) and theophyllinate (III) anions. As can be observed, the negative charge on the anions (II and III) does not reside solely at the nitrogen from which the proton was removed but is considerably delocalized to



II: theobromine anion



III: theophylline anion

[1] Complete neglect of differential overlap/second parameterization.

Table I—Interaction Energies[a] for the Methyl *trans*-Cinnamate–Theobrominate Complex

Structure	R	El[b]	Pol[b]	Disp[b]	Rep[b]	E_{tot}[b]
IV	3.4	−2.879	−3.720	−17.203	8.641	−15.162
V	3.3	−2.812	−3.792	−18.540	10.167	−14.978
	3.4	−2.712	−3.544	−16.157	7.444	−14.971
VI	3.4	−2.808	−3.089	−16.635	8.267	−14.264
	3.3	−2.903	−3.294	−19.129	11.327	−14.000
VII	3.4	1.402	−3.678	−15.849	7.338	−10.787

[a] In kilocalories per mole. [b] El = electrostatic, Pol = polarization, Disp = dispersion, Rep = repulsive, and E_{tot} = total energies.

Table II—Interaction Energies[a] for the Methyl *trans*-Cinnamate–Theophyllinate Complex

Structure	R	El[b]	Pol[b]	Disp[b]	Rep[b]	E_{tot}[b]
VIII	3.5	−1.121	−2.890	−15.268	7.173	−12.107
	3.4	−1.173	−3.069	−17.521	9.913	−11.850
IX	3.5	−1.340	−2.793	−15.653	7.231	−12.556
	3.4	−1.391	−2.965	−17.968	9.996	−12.328
X	3.4	−2.603	−2.953	−16.270	8.121	−13.707
XI	3.4	−1.452	−3.351	−17.163	8.273	−13.594

[a] In kilocalories per mole. [b] See Footnote *b* of Table I.

the remaining electronegative atoms (these calculations include both σ and π electronic charges). In general, the carbon atoms became slightly less positive and the electronegative atoms became considerably more negative on ionization. The theoretically calulated ionization energy, ΔE(ionization) = E(anion) − E(neutral xanthine), is larger for theobromine than theophylline, in line with its higher experimental pKa (10.1 *versus* 8.7). The charge distribution for methyl *trans*-cinnamate (I) is that of the *(S)-cis* form, referring to the arrangement of the two double bonds (12). The CNDO/2 method predicts the *(S)-cis* form to be about 1 kcal/mole more stable than the *(S)-trans* form, with the charge distributions being nearly identical. The results reported in this paper refer to the *(S)-cis* form.

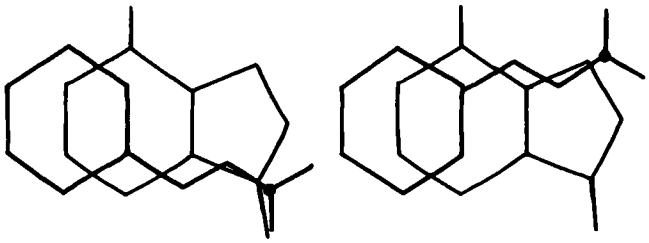
In determining the preferred structures of these complexes, an initial potential energy map was made to determine the location of the various potential energy minima and then the location and energy of each minimum were further refined. The reported results refer to the energetically preferred positions. Approximately 100 different structures for each complexing pair were considered to locate the region(s) of maximum binding.

RESULTS AND DISCUSSION

Several structures (IV–VII) were considered for the methyl *trans*-cinnamate–theobrominate complex, and the absolute interaction energies for these structures are presented in Table I. The interatomic distance, R, is the distance in Å between the planar rings of the two interacting molecules. The position of maximum binding for this complexing pair is Structure IV. Structure V shows a slightly lower interaction energy. Structure VI, with the six-membered ring of the cinnamate directly above the six-membered ring of the xanthine, is energetically less favorable due to a reduced dispersion energy. Structure VII is considerably less favorable than the other three structures due to a repulsive electrostatic energy. This results from the interaction of the negatively charged oxygens on methyl *trans*-cinnamate with the negatively charged nitrogen and oxygen of the theobrominate ion and illustrates the significant effect that complementary charge distributions in polar molecules can have on the absolute interaction energies.

These results for the methyl *trans*-cinnamate–theobrominate complex indicate that the substrate is positioned over the ligand in a broad potential energy well. The position of maximum binding, *i.e.*, the lowest point on the potential energy surface, places the reactive carbonyl carbon over the five-membered ring of the xanthine. This positioning is consistent with the proposed mechanism of reaction inhibition by the ligand.

Several structures (VIII–XI) were considered for the theophylli-



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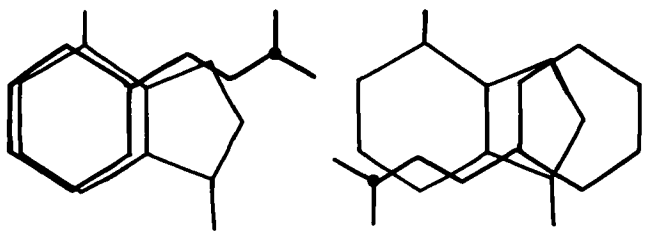

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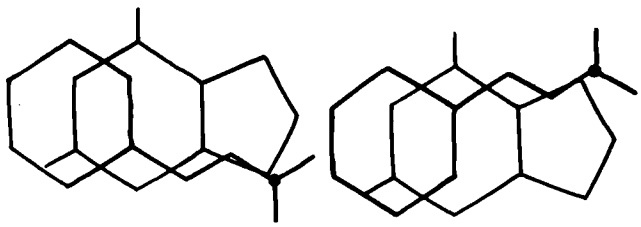

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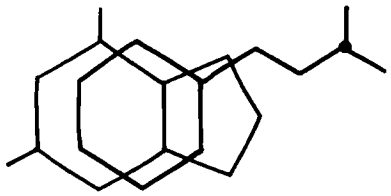

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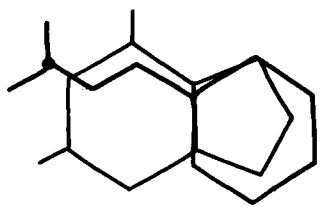

XI

nate–methyl *trans*-cinnamate complex, and the corresponding absolute energies are given in Table II. Structures VIII and IX are similar to the preferred structures for the theobrominate–methyl *trans*-cinnamate complex but are energetically less favorable compared to Structure X. In comparing Structures VIII and IX for the two ligands (Tables I and II) at an interplanar distance of 3.4 Å, it is seen that the reduced interaction energy for theophyllinate as compared to theobrominate results from an increased repulsion. This is due to the presence of the methyl group in the 1-position on the xanthine and a reduced electrostatic attraction, which results from the repulsive electrostatic interaction of the ester oxygens with the xanthine nitrogens. Structure XI, with the carbonyl carbon of methyl *trans*-cinnamate positioned approximately over the 2-position of the xanthine, is also energetically allowed.

This shift in position of the substrate over the ligand is even more evident in comparing the potential energy curves in Fig. 1. The solid curve and energy scale on the left of the figure correspond to the theobrominate complex, and the dashed curve and the scale on the right correspond to the theophyllinate complex. These curves were calculated, holding the interplanar distance constant and displacing the substrate either to the right (positive) or to the left (negative). For example, the zero position (displacement) corresponds to Structure VI while a displacement of −1.2 Å corresponds to Structure V. From Fig. 1, it is clear that the position of the potential energy minimum moves considerably to the right for the theophyllinate complex, corresponding to Structure X. These results on the theophyllinate–methyl *trans*-cinnamate complex indicate that Structure X, with the aromatic ring of the methyl *trans*-cinnamate positioned over the center of the xanthine, is energetically the most favorable. In this position, the reactive carbonyl carbon is considerably extended into the solvent. With this being the most energetically favorable position for the theophyllinate complex, the reactivity of the ester in this complex would be expected to be greater than the theobrominate complex. However, in both complexes the double bond of the cinnamate remains over the ligand and is consistent with a $q_{11} = 1$ for sulfite attack at this position (13).

From Table II it is also evident that Structure XI is energetically almost as favorable as Structure X. In this position it might be expected that the carbonyl carbon would be hindered and less reactive. Hence, there are at least two and probably more complex structural isomers that may have differing reactivities as well as equilibrium constants. The experimentally determined fractional decrease in reactivity, q_{11}, is obtained from the intercept of a plot of $k_s/(k_s - k_{s'})$ *versus* $1/L$, where k_s is the reactivity of the uncomplexed substrate, $k_{s'}$ is the observed reactivity in the presence of the ligand, and L is the ligand concentration (8, 13). If more than a single 1:1 complex exists (*e.g.*, isomeric 1:1 complexes or 2:1 complexes), the interpretation of the intercept is more complex (13). In the general case, if K_i is the equilibrium constant for the ith type of complex, either a 1:1 (of which there may be several) or 2:1, *etc.*, the equation becomes:

$$\frac{k_s}{k_s - k_{s'}} = \frac{1}{\Sigma q_i K_i(L)} + \frac{K}{\Sigma q_i K_i} \qquad \text{(Eq. 2)}$$

where q_i is the fractional decrease in reactivity of complex i:

$$K = \Sigma K_i \qquad \text{(Eq. 3)}$$

and the sum, Σ, is over all complexes[2]. Hence, in the general case:

$$\frac{1}{\text{intercept}} = \frac{\Sigma q_i K_i}{\Sigma K_i} \qquad \text{(Eq. 4)}$$

and is a weighted (according to the K_i) average of the q_i. Thus, the observed $q = 0.9$ for the theophyllinate–methyl *trans*-cinnamate complex could be ascribed to a $q_1 = 1$ for Structure XI, a $q_i = 0$ for all other complexes, and a ratio of $K_1/\sum_i K_i = 0.9$.

SUMMARY

A comparison of the theoretical results for the complexes of the theobrominate and theophyllinate anions with methyl *trans*-cinnamate indicates that the side chain of the methyl *trans*-cinnamate does not interact as strongly with the theophyllinate ligand as with the theobrominate ligand. This places the reactive carbonyl carbon of the substrate in a position more accessible to the solvent (and the nucleophile) when the ligand is the theophyllinate ion and, therefore, a more reactive complex. On the other hand, with the strong interaction of the substrate side chain with the theobrominate-ion ligand, the expected reactivity of the complex would be relatively low. These results are thus consistent with the proposed mechanism of reaction inhibition through complexation and indicate that the theoretical method may be a useful tool in studying such complexes.

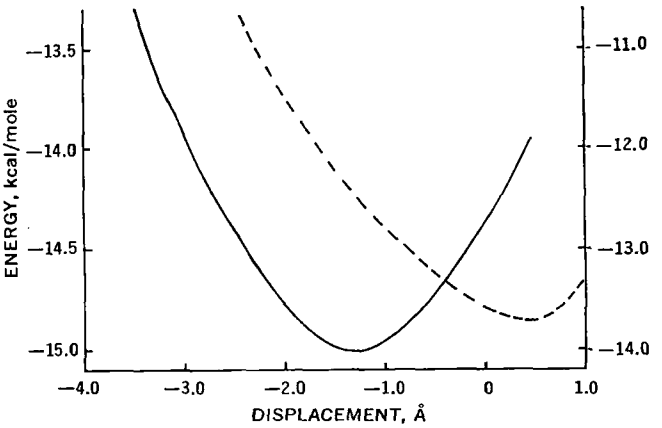

Figure 1—*Potential energy curves for the theobrominate (——) and theophyllinate (- - -) complexes with methyl* trans*-cinnamate.*

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Present address: School of Pharmacy, University of Wisconsin, Madison, WI 53706

[2] This is a straightforward extension of Eq. 49 in Ref. 8, in which there is a typographical error.

Comparative Studies on Distribution, Excretion, and Metabolism of Hydroxyzine-^{3}H and Its Methiodide-^{14}C in Rats

S. F. PONG ˣ and C. L. HUANG *

Abstract □ The comparative excretion, distribution, and metabolism of hydroxyzine-^{3}H hydrochloride and hydroxyzine methiodide-^{14}C were studied in rats after intraperitoneal administration. Both compounds were excreted mainly within the 1st day and mainly in feces *via* the bile. Hydroxyzine was rapidly distributed to the organs, with the highest specific activity found in the lungs, followed by fat, liver, spleen, and kidneys. It was completely metabolized in the bile and urine mainly to the glucuronides of the diphenylmethane derivatives. Based on differential solvent extraction and TLC, five metabolites, *p*-chloro-*p'*-hydroxybenzophenone, *p*-chlorobenzhydrol, *p*-chlorobenzophenone, piperazine, and 2-[2-(1-piperazinyl)ethoxy]ethanol, were identified. The distribution pattern of hydroxyzine methiodide was basically similar to that of hydroxyzine, except that it was restricted to fewer organs with lower specific activity. It was excreted mainly unchanged as studied by paper chromatography.

Keyphrases □ Hydroxyzine and methiodide, radiolabeled—comparative studies on distribution, excretion, and metabolism, rats □ Biotransformation—radiolabeled hydroxyzine hydrochloride and methiodide, comparative studies on excretion, distribution, and metabolism, rats □ Metabolism—radiolabeled hydroxyzine hydrochloride and methiodide, comparative study, rats

The central nervous system-depressant effect of hydroxyzine (I) provides the basis for its wide clinical application as a minor tranquilizer in recent years. Many reports have appeared attesting to its efficacy in the treatment of minor emotional disturbances such as anxiety, tension, fear, and agitation. The drug has been evaluated particularly for its ability to make disturbed patients more approachable and amenable to long-term psychotherapy (1–4). The efficacy of I as an adjunct to preoperative and postoperative sedative and analgesic medication has been well established (5–8). Its antihistaminic property, for which it was originally designed, has been recently reappraised (9, 10). However, little is known concerning the metabolic behavior and fate of this drug *in vivo*. Therefore, it is necessary to study the distribution, excretion, and metabolism of this drug. Since quaternization of the side-chain nitrogen often modifies the pharmacological properties of a drug, hydroxyzine methiodide (II) was included for comparison.

EXPERIMENTAL[1]

Preparation of Hydroxyzine Methiodide (II) and Hydroxyzine Methiodide-^{14}C (II-^{14}C)—Compound II was prepared by adding methyl iodide in acetone to the free base of I (11). The product was recrystallized from acetone–ether or ethanol to a white crystalline powder, mp 181–182°, UV_{max}: 214 and 228 nm.

Anal.—Calc. for $C_{22}N_{30}ClIN_2O_2$: C, 51.13; H, 5.85; N, 5.42. Found: C, 51.33; H, 5.91; N, 5.27.

Compound II-^{14}C was prepared according to the procedure described for II with a slight modification. The free base (0.9 mmole) of I was dissolved in acetone (1.5 ml) and pipetted into the upper part of the breakseal tube containing 1 mCi (0.6 mmole) of methyl iodide-^{14}C. The capillary seal was broken to allow 1 ml of the solution to enter the container under negative pressure. The tube was stoppered immediately and allowed to stand overnight at room temperature. A suitable amount of ether was added to the reaction mixture and left standing for 15 min for complete crystallization. Then the crystals were collected and washed with ether. The radiochemical purity of the product was checked by employing paper chromatography or TLC coupled with autoradiography and radiochromatographic scanning[2]. Only one spot with the same R_f value as authentic II in various solvent systems was obtained. Therefore, the product was radiochemically pure. The specific activity of the product was 3.36 μCi/mg.

Table I—R_f Values (TLC) of Hydroxyzine and Related Compounds

Compound	Solvent System[a] A	B	C	D	E	F
Hydroxyzine (I)	0.41	0.80	0.68	0.65	0.30	0
p-Chlorobenzophenone (IV)	0.69	0.90	0.81	0.76	0.83	0.80
p-Chloro-*p'*-hydroxybenzophenone (V)	0.04	0.85	0.80	0.74	0.80	0.03
p-Chlorobenzhydrol (VI)	0.52	0.86	0.81	0.78	0.84	0.41
Piperazine (VII)	0.05	0.20	0.31	0.06	0.04	0.06
2-[2-(1-Piperazinyl)-ethoxy]ethanol (VIII)	0.08	0.23	0.37	0.18	0.11	0.04

[a] A, cyclohexane–benzene–diethylamine (75:15:10); B, methanol–acetone (1:1); C, 95% ethanol; D, *n*-butanol–ethanol–water (5:2:2); E, water-saturated isoamyl alcohol containing 15% acetic acid; and F, chloroform–cyclohexane (1:1).

Tritiation and Purification of Hydroxyzine-^{3}H Hydrochloride (I-^{3}H)—Compound I-^{3}H was prepared commercially[3] according to the Wilzbach (12) tritium gas-exposure method, in which 1 g of I was exposed to 3 Ci of tritium gas at 250 mm Hg at 26° for 2 weeks. The product was purified by dissolving in methanol–chloroform (10:1) and evaporating to dryness *in vacuo* to remove labile tritium. The same procedure was repeated once and the residue was taken up in hot methanol. The I-^{3}H thus obtained gave only one spot with an R_f value corresponding to that of authentic I in Solvent Systems A–D (Table I) and was found to be 98% pure as determined by the TLC–zonal scraping–liquid scintillation counting to be described later.

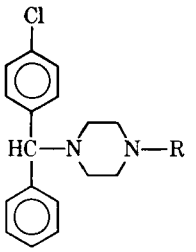
Preparation of Hydroxyzine *N*-Oxide (III)—Since it was speculated that III might play a role in the metabolism of I, synthesis of III was carried out. Compound I (3 g) was dissolved in water (50 ml), adjusted to pH 10, and extracted three times with ether. The combined ether extracts were evaporated to dryness *in vacuo* and the residue was dissolved in ethanol (10 ml). To this solution, 5 ml of 10% hydrogen peroxide was added and the mixture was stirred at room temperature for 2 days. After the ethanol was removed *in vacuo*, the residue was dissolved in water and extracted with chloroform. The chloroform extract was evaporated to dryness and the residue was recrystallized from acetone to white crystals, mp 189-190°, UV_{max}: 211 and 231 nm.

Anal.—Calc. for $C_{21}H_{27}ClN_2O_3$: C, 64.52; H, 6.96; N, 7.17.

[1] Melting point was measured on a Fisher-Johns melting-point apparatus. Elemental analysis was carried out by Galbraith Laboratory, Knoxville, Tenn. The UV spectrum was recorded in a Perkin-Elmer spectrophotometer model 202, using ethanol as a solvent.

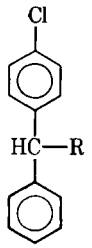
[2] Actigraph III, Nuclear-Chicago.

[3] New England Nuclear Corp.

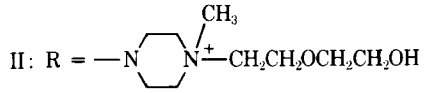


hydroxyzine I: R = $CH_2CH_2OCH_2CH_2OH$

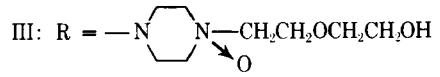
norchlorcyclizine IX: R = H



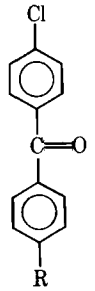
hydroxyzine methiodide



hydroxyzine *N*-oxide

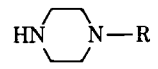


p-chlorobenzhydrol VI: R = OH



p-chlorobenzophenone IV: R = H

p-chloro-*p'*-hydroxybenzophenone V: R = OH



piperazine VII: R = H

2-[2-(1-piperazinyl)ethoxy]ethanol

VIII: R = $CH_2CH_2OCH_2CH_2OH$

Chemical structure of hydroxyzine and related compounds

Found: C, 64.41; H, 7.09; N, 7.24.

Lipid–Water Partition of Labeled Compounds—To find an optimal condition of extraction for metabolic study, a lipid–water partition study was carried out using the labeled compounds. Compounds I-^{3}H and II-^{14}C were dissolved in water and diluted with a series of buffers (pH 2.0–10.0). Each buffered solution was equilibrated with an equal volume of organic solvent. The radioactivity of the aqueous phase was compared with that of the original buffered solution. The result indicated that I-^{3}H was extractable in organic solvent only at alkaline pH, while II-^{14}C was only slightly soluble in organic solvents at all pH levels.

Animals and Method of Administration—Male albino rats of a Sprague–Dawley strain, weighing 170–250 g, were used. The animals were conditioned to the new environment and provided with food and water for 5 days. Compound I-^{3}H was dissolved in saline and Compound II-^{14}C was dissolved in 30% dimethyl sulfoxide in a concentration of 1.5–2.0 mg/ml. Rats were lightly anesthetized with ether and injected with 5–10 mg/kg ip of I-^{3}H, which is equivalent to a human dosage.

Table II—Urinary, Fecal, and Biliary Excretion of Hydroxyzine-^{3}H and Hydroxyzine Methiodide-^{14}C in Rats

Excretion	Time	Cumulative Percent of Administered Radioactivity (dpm ± *SE*)	
		Hydroxyzine-^{3}H Hydrochloride	Hydroxyzine Methiodide-^{14}C
Urinary	1 day	12.0 ± 0.8[a]	6.8 ± 0.4[b]
	2 day	14.3 ± 0.9	7.4 ± 0.4
	3 day	14.8 ± 0.9	7.7 ± 0.4
	4 day	15.2 ± 0.9	—
	5 day	15.5 ± 1.0	7.8 ± 0.1
Fecal	1 day	27.6 ± 4.6[a]	34.9 ± 3.5[b]
	2 day	32.2 ± 4.2	39.6 ± 2.5
	3 day	33.4 ± 4.1	40.0 ± 2.5
	4 day	34.3 ± 4.1	—
	5 day	34.9 ± 4.0	40.4 ± 2.3
Biliary	0.5 hr	19.8 ± 0.9[c]	1.8 ± 0.2[c]
	1 hr	34.9 ± 0.6	5.5 ± 0.7
	2 hr	44.3 ± 1.2	13.0 ± 0.9
	4 hr	49.9 ± 1.0	26.6 ± 1.7
	8 hr	53.7 ± 0.9	34.0 ± 4.2

[a] $n = 6$. [b] $n = 5$. [c] $n = 3$.

Urinary and Fecal Excretion—After the administration of the solutions, the animals were housed individually in metabolic cages and allowed food and water *ad libitum*. Urine and feces specimens were collected separately at 24-hr intervals for 5 days. The collected specimens were stored in a deep freeze until analyzed.

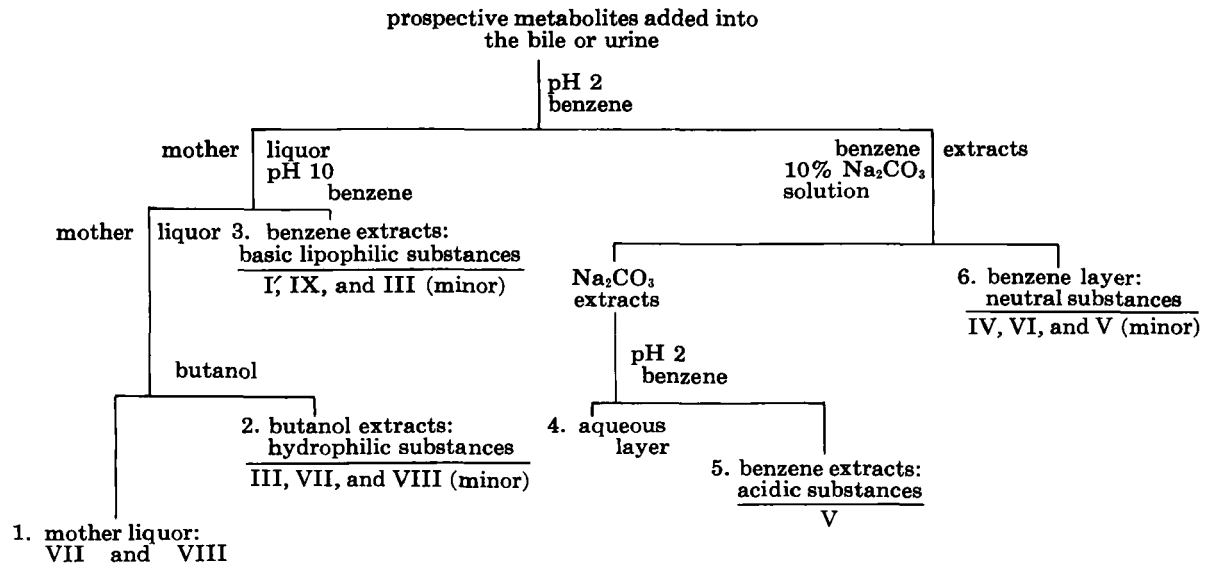
Pulmonary Excretion—This study was conducted to test the possible pulmonary excretion of $^{14}CO_2$ derived from *N*-demethylation of II-^{14}C. After intraperitoneal administration, the animals were maintained in a modified metabolic jar (13) for 24 hr with a constant flow of air current which was passed through two gas-washing bottles, each containing 150 ml of 30% NaOH. The carbon dioxide trapped in the gas-washing bottles was precipitated with 12% $BaCl_2$ solution containing a small amount of ammonium chloride (14). The barium carbonate thus formed was centrifuged, washed several times, and dried. The dried barium carbonate was ground to a fine powder, an aliquot of 50 mg was placed in a planchet, and the activity was recorded[4].

Biliary Excretion—Three rats were anesthetized with sodium pentobarbital (25 mg/kg sc). An abdominal incision was made and the bile duct was cannulated with a polyethylene tube (PE-50). Then 0.5 ml of the test solution was immediately dropped into the abdominal cavity, and the incision was closed by suture. Bile specimens were collected at the intervals of 0.5, 1, 2, 4, and 8 hr.

Tissue Distribution—The animals were administered intraperitoneally with the compound under study and were sacrificed at the following intervals: 0.5, 1, 2, 4, and 8 hr. Blood specimen was collected in a test tube containing 1 ml of 5% edetate (EDTA) and centrifuged at 2000 rpm for 10 min to separate the plasma. Tissues and organs were removed, rinsed thoroughly with water, and blotted on a filter paper. The fresh specimens were weighed and prepared for assay. The total blood volume was taken as 8% (v/w; hematocrit, 0.45); the weights of the muscle, bone, fat, and skin were calculated as 45, 22, 3, and 3%, respectively, of the body weight of the animal.

Radioactivity Counting—A liquid scintillation spectrometer[5] was used to record the activity. An aliquot of 100 μl of the urine and 20 μl of the diluted bile specimens were counted directly in 10 ml of Bray's scintillator (15). The bone, skin, and feces were prepared according to the method of Mahin and Lofberg (16). Specimens of bone, skin (100–200 mg), and feces (100 mg) were placed in a counting vial containing 0.2 ml of 60% perchloric acid and 0.4 ml of 30% hydrogen peroxide. The vial was tightly screw capped and the content was heated to 70–80° for 1–2 hr with occasional shaking. After the vial was cooled to room temperature, 5 ml of 2-ethoxyethanol[6] and 10 ml of scintillating solution containing 0.6%

[4] Geiger-Muller counter, Tracer-Lab, TGC-2.
[5] Corumatic 100, Tracerlab.
[6] Cellosolve.



Scheme I—Differential solvent fractionation of prospective metabolites added into the bile or urine according to the method of Soudijn and Van Wijngaarden (18)

2,5-diphenyloxazole in toluene were added and the activity was determined. The specimens (100–200 mg) of kidneys, lungs, spleen, heart, muscle, and fat were digested with 1.5–2.0 ml of hyamine hydroxide at 50–60° for from 12 hr to 2 days with constant shaking. Aqueous hydrogen peroxide was employed, if necessary, to bleach the specimens and 0.2 ml of concentrated hydrochloric acid was added to eliminate the phosphorescence (17) after the hyamine hydroxide digestion. Ten milliliters of Bray's scintillating solution was added and the activity was recorded. Control runs with the starting materials were conducted to correct for the loss of radioactivity due to the mechanical adsorption to the upper part of the vial and to the cap lining. An internal standard was used to estimate the counting efficiency and to correct for the quenching of the samples.

Metabolic Study of II-^{14}C—An aliquot of urine and bile from rats treated with II-^{14}C was placed linearly on a strip of Whatman No. 3 chromatographic paper (4 × 30 cm) and was developed ascendingly in Solvent Systems D and G. The dried chromatogram was scanned in a radiochromatogram scanner[2]. The R_f value of the radioactive peaks was compared with that of reference materials, and the percent of the radioactivity of each peak was estimated.

***Metabolic Study of* I-^{3}H**—A differential solvent extraction procedure (18) was carried out on authentic specimens of prospective metabolites of hydroxyzine added individually to control bile and urine. The procedure (Scheme I) was as follows. An aliquot of bile or urine containing a reference compound was acidified with concentrated hydrochloric acid to pH 2 and extracted with benzene. The combined benzene extracts were extracted with 10% Na_2CO_3 solution, leaving behind the neutral substance in the organic layer (Fraction 6). The sodium carbonate extract was acidified with concentrated hydrochloric acid to pH 2, and the acidic compound was extracted with benzene (Fraction 5). The initial aqueous solution was alkalized with concentrated ammonium hydroxide to pH 10 and extracted with benzene (basic lipophilic substance, Fraction 3) followed by *n*-butanol (hydrophilic substance, Fraction 2), leaving behind the mother liquor (Fraction 1). Each fraction was chromatographed by TLC and scanned under a UV lamp or sprayed with an appropriate color reagent to establish the identity of each compound. The same procedure was applied to the bile and urine specimens of rats receiving II-^{3}H. Each fraction obtained by solvent extraction was further fractionated by TLC, and the R_f was compared with that of the reference compounds. The percent ratio of the subfractions was quantified by zonal scraping–liquid scintillation counting.

TLC—Silica gel[7] and silica gel[8] IB-F were used. An aliquot of 5–10 μl of the sample solution was spotted 1.5 cm from the bottom of a chromatographic sheet and developed in a solvent system to a height of 10–15 cm. The developed chromatograms were dried and the spots were detected under a UV lamp and subsequently sprayed with Dragendorff reagent or exposed to iodine vapor. The R_f values of hydroxyzine and the related compounds are shown in Table I.

Autoradiography and fluorography (19) were carried out to check the purity of the substrate and to detect the metabolites on the chromatogram. The TLC plates containing ^{14}C samples were exposed to medical X-ray film[9] for 1–3 days. Those TLC plates containing a ^{3}H sample were first sprayed[10] and then exposed to medical X-ray film[11] over dry ice for 1–7 days. After the exposure, the films were processed according to the manufacturer's recommendation.

Zonal Scraping–Liquid Scintillation Counting (20)—This method was adapted to quantitate the radioactive metabolites on the chromatograms. A chromatogram (5 cm in width) was divided into two equal parts of 2.5 cm each by drawing a perpendicular line in the center of the adsorbent layer. One-half of the plate was used for reference compound and the other for the sample solution. After development, the sheet was cut into two pieces and the plate containing the reference compound was scanned to detect the spot. Silica gel zones (0.5–1 cm) of the specimen on the other half of the sheet were scraped into counting vials containing 12 ml of a scintillation solution[12]. The vials were shaken thoroughly and placed in a liquid scintillation spectrometer for activity recording. The profile for the distribution of the radioactivity was constructed by taking the net count accumulated in all zones and the percent of the activity calculated.

RESULTS AND DISCUSSION

Urinary, Fecal, and Biliary Excretion—Results of the urinary, fecal, and biliary excretion of I-^{3}H and II-^{14}C are summarized in Table II. Apparently, fecal excretion was the major route for these two compounds. The radioactivity of II-^{14}C in the feces appeared to be higher than that of I-^{3}H; however, the difference was insignificant ($p < 0.05$). Compound I-^{3}H (16%) exhibited a higher urinary excretion than II-^{14}C (8%) in 5 days. The majority (40–42%) of the administered radioactivity (urine and feces combined) was excreted in the 1st day for both compounds, and a slow excretion ensued thereafter. The higher biliary excretion of I-^{3}H (54%) than of II-^{14}C (34%) in 8 hr ($p < 0.01$) was consistent with the fecal excretion.

Pulmonary Excretion—No radioactivity was detected in the

[7] Eastman chromogram 6060.
[8] Baker-flex.
[9] Kodak no-screen.
[10] Omnispray, New England Nuclear Corp.
[11] Kodak Royal Blue.
[12] Containing 4% Cab-O-Sil.

Table III—Comparative Distribution of Radioactivity in the Rat after Intraperitoneal Injection of Hydroxyzine-^{3}H Hydrochloride and Hydroxyzine Methiodide-^{14}C Expressed in Terms of Relative Specific Organ Activity[a] (Disintegrations per Minute per Gram Wet Organ) (That in the Blood of Hydroxyzine-^{3}H Hydrochloride-Injected Rat Served as 1.00)

	Hours after Intraperitoneal Administration									
	Hydroxyzine-^{3}H Hydrochloride					Hydroxyzine Methiodide-^{14}C				
Organ	0.5	1	2	4	8	0.5	1	2	4	8
Blood	1.00	1.12	1.21	0.93	0.72	0.18	0.33	0.15	0.11	0.04
Brain	2.47	1.47	1.47	1.27	0.56	0	0	0	0	0
Liver	10.8	9.76	6.81	5.37	3.58	19.8	16.8	12.3	10.2	2.40
Intestines	9.75	13.0	20.9	22.4	31.0	9.90	23.7	29.0	32.0	35.4
Kidneys	6.91	6.52	4.63	2.62	1.18	9.20	5.48	3.32	2.33	0.91
Lungs	11.4	11.5	9.20	5.22	2.25	0.67	0.32	0.43	0.59	0.57
Spleen	8.65	6.96	4.14	4.09	2.50	3.72	2.72	3.30	1.32	0.81
Heart	2.98	2.25	1.57	0.87	0.72	0	0	0	0	0
Stomach	5.08	3.07	2.20	1.69	1.53	2.47	2.77	3.42	1.49	0.87
Testis	1.01	1.50	1.77	1.87	0.69	1.06	1.06	2.92	1.50	0.31
Muscle	1.11	1.02	0.90	0.56	0.32	0	0	0	0	0
Fat	11.2	5.27	3.53	2.65	1.06	12.0	3.86	4.04	5.28	1.79
Bone	3.14	2.43	1.70	1.52	1.15	—[b]	—	—	—	—
Skin	2.03	1.79	1.82	1.29	1.01	—	—	—	—	—

[a] Represents the average of three experiments. [b] Data not available.

trapped carbon dioxide in the expired air by the animal injected with II-^{14}C. This indicated that no *N*-demethylation of the quaternary ammonium salt occurred *in vivo* in the rat, which is in contrast to the documented *N*-demethylation of certain secondary and tertiary amine derivatives (21, 22).

Distribution Study—The radioactivity in the organs was expressed in terms of relative specific organ activity (disintegrations per minute per gram wet organ) for easy comparison, using activity in blood as 1.00 (Table III). The radioactivity of I-^{3}H was absorbed rapidly, reaching a peak level within 0.5 hr in most organs excepting blood, intestines, and testis; this finding verified the previous claim for hydroxyzine as a fast-acting tranquilizer (1). The specific activity was highest in the lungs, followed by the fat, liver, spleen, and kidneys. The sharp increase in the intestinal activity and urinary level coincided with the rapid fall of activity in the liver and kidneys. About 50% of the radioactivity was found in the intestines at 8 hr.

Compound II-^{14}C was also rapidly absorbed, with a peak level in the liver, fat, kidneys, and spleen within 0.5 hr, in the blood at 1 hr, and in the stomach and testis at 2 hr. The distribution pattern was similar to, but quantitatively less than, that of I-^{3}H, and the activity in most organs declined rapidly. At the end of 8 hr, about 57% of the radioactivity was recovered in the intestines.

From Table III, it is obvious that hydroxyzine, which is mostly nonionized at physiological pH, was more readily absorbed from the peritoneal cavity, while hydroxyzine methiodide, which is highly ionized at all pH's, was less readily absorbed but more rapidly released from organs after being absorbed.

Metabolic Study of II-^{14}C—As shown in Table IV, II-^{14}C gave three metabolites in the urine and bile as studied by paper chromatography. The R_f values of the major metabolite (about 77% in the urine and 75% in the bile) matched that of the parent compound. Therefore, it is apparent that the quaternary salt of hydroxyzine was excreted mainly unchanged. The nature of the two minor metabolites was not investigated.

Metabolic Study of I-^{3}H—As indicated in Table II, biliary excretion was the major route of elimination of I-^{3}H, with the majority of the radioactivity excreted within the 1st hr. Therefore, the combined bile collected during the 1st hr was used in the metabolic study using differential solvent extraction and TLC.

Table IV—Metabolites of Hydroxyzine Methiodide-^{14}C in Urine and Bile after Intraperitoneal Injection

	R_f Values (Paper Chromatography)		
Solvent System	Urine	Bile	Hydroxyzine Methiodide
D, butanol–ethanol –water (5:2:2)	0.90 (22)[a]	0.07 (6)	
	0.34 (3)	0.33 (19)	
	0.81 (75)	0.83 (75)	0.83
G, butanol–acetic acid–water (12:3:5)	0.06 (19)	0.06 (9)	
	0.52 (2)	0.54 (16)	
	0.89 (79)	0.91 (75)	0.91

[a] Figures in parentheses represent the percent of the total radioactivity.

The metabolites were fractionated according to the differential solvent extraction procedure (18) in which authentic specimens of prospective metabolites added in the bile served as a control to give constructive information as to the nature of the metabolites in each fraction (Scheme I). The percent radioactivity of the fractionated bile obtained from I-^{3}H-injected rats is shown in *Table V*. Only about 2% of the radioactivity was found in Fraction 3 (basic lipophilic substances, I and IX). This indicated that more than 98% of I-^{3}H was metabolized and only a small amount of IX, if any, was formed. The major metabolites were hydrophilic in nature, as evidenced by the findings that about 85% of the radioactivity was located in Fractions 1 and 2. The rest of the radioactivity (about 13%) was distributed into Fractions 4, 5, and 6, which comprised the neutral (IV and VI) and acidic (V) substances.

Studies with fluorograms indicated four metabolites in the bile on the thin-layer chromatogram developed in Solvent System D. Solvent System D (Table V) appeared to be the most efficient solvent system for the separation of the metabolites of I, so it was used preferably to separate the metabolites in the bile on TLC[13]. The developed chromatograms were then subjected to zonal scraping–liquid scintillation counting to obtain the TLC radioactivity profile (Fig. 1). It is obvious that the more hydrophilic the metabolites, the lower the R_f values in this solvent system. All fractions except Fraction 4 were chromatographed in other solvent systems, and the R_f values of the radioactive peaks and the percent radioactivity were recorded (Table V).

By comparing the R_f values of the radioactive peaks in the profile of Fraction 6 (Table V) with that of the authentic specimens (Table I), 2.5, 5.8, and 1.2% of the radioactivity were found to be attributable to IV, V, and VI, respectively. The R_f values of Fraction 5 (Table V) resembled that of V. Since the activity in Fraction 4 was insignificant, it was not further investigated. The R_f value of Fraction 3 in Solvent System B (Table V) indicated that the radioactive peak (R_f 0.82) did not match either IX (R_f 0.55) or III (R_f 0.45, Table I). The profile of radioactive peaks in Solvent System A (R_f 0.12 and 0.67) also excluded the presence of unchanged hydroxyzine (R_f 0.41). The nature of these two metabolites was unknown.

Fraction 2 was chromatographed on silica gel sheets in Solvent System D. The silica gel zone with radioactivity corresponding to the main metabolite 2d was scraped off and extracted with methanol. Its profile in Solvent System B (Fig. 2A) indicated the presence of two metabolites, 2d-1 and 2d-2. No radioactivity was found at R_f values higher than 0.60.

A study indicated that about 12% of the radioactivity in the bile was extractable in chloroform, and an additional 27% was extract-

[13] Baker-flex silica gel sheets.

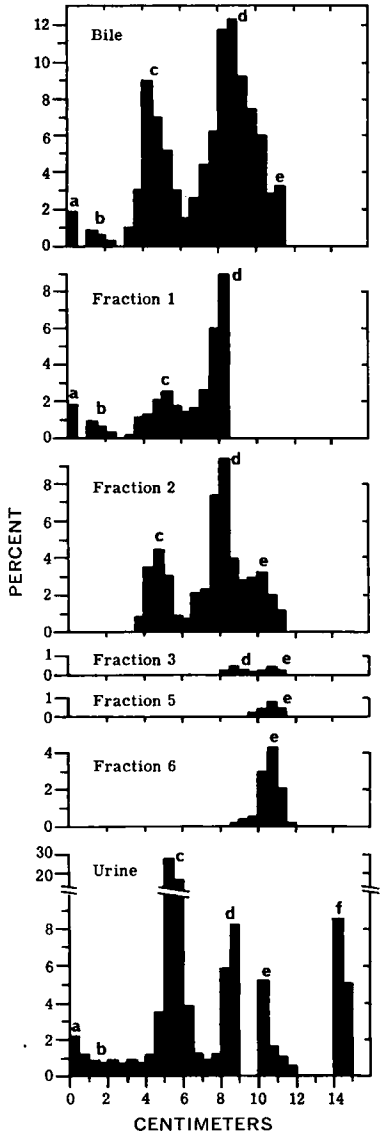


Figure 1—*TLC radioactivity profile in Solvent System D of fresh bile, fractionated bile (Fractions 1, 2, 3, 5, and 6), and the urine of the bile duct-cannulated rats injected intraperitoneally with hydroxyzine-^3H. Expressed in terms of percent radioactivity in the bile and urine, respectively.*

ed after the chloroform-extracted bile was incubated with β-glucuronidase[14] for 2 days. This finding was substantiated by the shift in the radioactivity profile of Fraction 2 in which the nonpolar subfraction, 2d-3, increased with a concurrent decrease in the polar subfractions, 2d-1 and 2d-2, after β-glucuronidase incubation. This finding indicated that the large fraction of the radioactivity was due to the glucuronide(s). The purified subfraction, 2d-3, gave a minor spot with R_f 0.56 and 0.45 in Solvent Systems A (Fig. 2C) and F (Table I), respectively, which corresponded to that of Compound VI in the same solvent systems. The majority of the radioactivity remained at the origin where Compound V was located (R_f 0.03–0.04). Therefore, the major metabolites of Fraction 2 were found to be the glucuronides of V (93%) and VI (7%). These findings coincided with the reported metabolic pathway of structurally related meclizine (23) and chlorcyclizine (24) in which formation of IV, V, VI, and VII was suggested. Metabolite 1d in Fraction 1 (Fig. 1) was similarly purified and incubated with or without β-glucuronidase according to the procedure described for 2d. Essentially the same result was obtained. Therefore, metabolite 1d was identified to be the same as Metabolite 2d.

[14] Sigma Chemical.

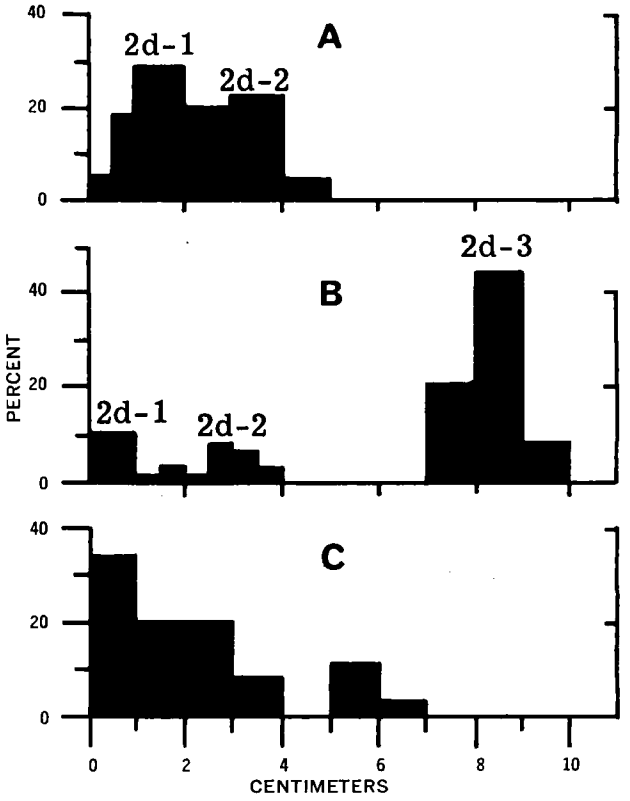


Figure 2—*TLC radioactivity profile in Solvent System B of Metabolite d in Fraction 2 (Fig. 1) after purification (A) and following incubation with β-glucuronidase (B); TLC radioactivity profile of purified 2d-3 in Solvent System A (C).*

When the profile of the 2-week-old bile in Solvent System D was compared with that of fresh specimens, it was apparent that the radioactive peak d decreased from about 60 to 40% with concurrent increase of peak e from 5 to 19%, while peak c did not change. This indicated that Metabolite c was stable in contrast to the unstable Metabolite d. Subfractions 1c and 2c were isolated from the silica gel sheets, combined together, and rechromatographed in Solvent Systems A, B, and F. More than 97% of the combined radioactivity was located at the origin. The chromatographic behavior of Metabolite c resembled that of VIII in Solvent Systems A and F but differed in D (R_f 0.34); therefore, the possibility that Metabolite c may be VIII was ruled out. The nature of Metabolite c remained unknown. Fractions 1a and 1b (Fraction 1, Fig. 1) were similarly isolated and rechromatographed in other solvent systems (Table V). By comparing the R_f values of the radioactive peaks with those of the reference compounds (Table I), 1a and 1b were identified to be VII and VIII, respectively. Thus, I, 5–10 mg/kg ip, was completely metabolized within 1 hr (Table V). Neither IX nor III was found to be the metabolites. The metabolites identified were V (7.2%) and its glucuronide (53.3%), VI (1.2%) and its glucuronide (4.1%), IV (2.5%), VII (2.2%), and VIII (3.3%), accounting for about 74% of the total radioactivity in the bile.

The urine specimens collected from the bladders of rats injected with I-^3H for the biliary metabolic study were chromatographed on silica gel sheets in Solvent System D to give the profile shown in Fig. 1. In comparison to the profile of bile, Metabolite c increased remarkably while d decreased appreciably, with about 14% of the metabolites appearing at R_f 0.95. As a result of the differential solvent extraction, Fractions 1, 2, and 3 contained about 72, 5, and 13%, respectively, of the radioactivity in the urine, while 13% was located in Fractions 4, 5, and 6. The profile in Solvent System A showed that only about 2% of the radioactivity was located at R_f 0.40 (I, 0.41; IX, 0.43). This finding indicated that more than 98% of I-^3H was metabolized and that the parent compound recovered or the amount of IX formed was not more than 2%. By comparing these data and the profile of the urine with that of the bile (Fig. 1 and Table V), inferentially about 7% of the radioactivity was at-

Table V—R_f Values (TLC) and Percent Radioactivity of Metabolites of Hydroxyzine-^{3}H in the Bile of Rats after Intraperitoneal Injection

Fraction Number	Radioactivity[a], %	R_f Value in Solvent System[b] A	B	D[c]	F	Metabolites[d]
1	34.6 ± 14.7[e]	Origin (34.6)[f]	Origin (34.6)	Origin (2.2) 0.12 (3.3) 0.35 (9.2) 0.55 (19.8)	Origin (34.6)	VII (2.2) VIII (3.3) VI–Gl. (1.4)[g] V–Gl. (18.4) Unknown (9.3)
2	50.2 ± 16.6	Origin (50.2)	0.15 (30.2) 0.34 (20.0)	0.32 (12.5) 0.55 (28.6) 0.68 (9.0)	Origin (50.2)	VI–Gl. (2.7) V–Gl. (34.9) Unknown (12.6)
3	1.9 ± 1.7	0.12 (1.6) 0.67 (0.3)	0.82 (1.9)	0.58 (1.2) 0.72 (0.7)	0.09 (1.6) 0.73 (0.3)	Probably spilled from Fraction 2
4	2.6 ± 0.4	—	—	—	—	Not investigated
5	1.3 ± 0.3	0.05 (1.3)	0.86 (1.3)	0.75 (1.3)	0.06 (1.3)	V (1.3)
6	9.4 ± 3.1	0.08 (5.5) 0.48 (1.0) 0.62 (2.9)	0.82 (9.4)	0.73 (9.4)	0.04 (6.0) 0.43 (1.3) 0.74 (2.1)	IV (2.5) VI (1.2) V (5.8)

[a] Expressed as the percent of total radioactivity in the bile. [b] See Table I. [c] Baker-flex silica gel IB-F sheets were used; solvent front 15 cm. For other solvent systems, Eastman chromagram sheets were used; solvent front 10 cm. [d] R_f values of prospective metabolites; see Table I. [e] n = 3. [f] Figures in the parentheses represent the percent radioactivity of the metabolite expressed in terms of the total radioactivity in the bile. [g] Gl. = glucuronide.

tributed to IV, V, and VI, about 15% to the glucuronides of the latter two compounds, and about 5% to VII and VIII.

SUMMARY

The excretion, distribution, and metabolism of hydroxyzine-^{3}H hydrochloride in the rat after intraperitoneal administration were compared with those of hydroxyzine methiodide-^{14}C. Both compounds were excreted largely within the 1st day and mainly in feces *via* the bile. Hydroxyzine-^{3}H was excreted more rapidly into the bile within 8 hr and exhibited a higher rate of urinary excretion within 5 days than the methiodide. Hydroxyzine-^{3}H was absorbed rapidly, with a peak level in most organs within 30 min, and decreased rapidly within 8 hr, confirming the previous claim for hydroxyzine as a fast-acting tranquilizer. The specific activity was the highest in the lungs, followed by the fat, liver, spleen, and kidneys; that in the blood, testis, and muscle was relatively low. The distribution pattern of hydroxyzine methiodide-^{14}C was basically similar to that of hydroxyzine-^{3}H. However, the distribution was more restricted, with insignificant radioactivity in the brain, heart, and muscle, and the specific activity in most organs was lower and declined more rapidly than hydroxyzine-^{3}H.

The metabolism of hydroxyzine methiodide-^{14}C, as studied by paper chromatography coupled with radiochromatographic scanning, indicated that it was excreted mainly unchanged in the urine (77%) and bile (75%) with two minor metabolites. The metabolism of hydroxyzine-^{3}H hydrochloride was studied by differential solvent fractionation and TLC, and this was found to be completely metabolized. Among the metabolites identified were *p*-chloro-*p'*-hydroxybenzophenone (7.2% in free form and 53.3% as glucuronide), *p*-chlorobenzhydrol (1.2% in free form and 4.1% as glucuronide), *p*-chlorobenzophenone (2.5%), piperazine (2.2%), and 2-[2-(1-piperazinyl)ethoxy]ethanol (3.3%), accounting for about 74% of the total radioactivity in the bile. About 7% of the radioactivity in the urine could be attributed to *p*-chlorobenzophenone, *p*-chlorobenzhydrol, *p*-chloro-*p'*-hydroxybenzophenone, about 15% to the glucuronides of the latter two compounds, and about 5% to piperazine and 2-[2-(1-piperazinyl)ethoxy]ethanol.

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* Present address: Department of Pharmacology, College of Pharmacy, Wayne State University, Detroit, MI 48202

ˣ To whom inquiries should be directed. Present address: Research and Development Department, Norwich Pharmacal Co., Norwich, NY 13815

Interfacial Properties of Polar Liquids against Nonpolar Phases

GEORGE ZOGRAFI*x and SAMUEL H. YALKOWSKY‡

Abstract □ The interfacial tension of 10 polar liquids against hexane and tetradecane and the contact angle against paraffin, polyethylene, and polytetrafluoroethylene were measured. Work-of-adhesion values against any one nonpolar phase, calculated from these data, are independent of the polar liquid used. Values for the same polar liquid, however, differ with the nonpolar phase used. These differences are attributed to differing surface group densities and, hence, a different number of dispersion force interactions per unit area. These results explain the variations occurring in the Fowkes dispersion component of surface tension, γ_L^d, for any liquid when it is determined using different nonpolar phases.

Keyphrases □ Polar liquids—interfacial properties against nonpolar phases, interfacial tension and contact angles measured, work-of-adhesion values calculated □ Nonpolar phases—interfacial properties of 10 polar liquids against hexane, tetradecane, paraffin, polyethylene, and polytetrafluoroethylene, interfacial tension or contact angle measured, work-of-adhesion values calculated □ Interfacial tension—10 polar liquids against hexane and tetradecane □ Contact angles—10 polar liquids against paraffin, polyethylene, and polytetrafluoroethylene, interfacial properties of polar liquids

In a study dealing with the free energy of solution per methylene group for a series of alkyl *p*-aminobenzoates in various polar solvents, it was found that a correlation exists between the free energy term and the interfacial tension of these solvents against the liquid alkane, tetradecane (1). Since the choice of tetradecane as a representative nonpolar surface was made arbitrarily for convenience, and since some solvents of interest were miscible with tetradecane, the use of other liquid and solid hydrocarbons for such correlations was considered. This led to the present study where the interfacial behavior of various polar liquids was measured against the liquids hexane and tetradecane and the solids paraffin, polyethylene, and polytetrafluoroethylene. By determining the interfacial behavior of any polar liquid against these systems, it was hoped that one could use such information more generally for the correlations mentioned.

THEORETICAL

Another reason for the interest in pursuing this study was the fact that polar liquids, such as glycerin or formamide, are often used to determine the surface free energy or some similar term for solids (2–5). The principle behind this approach centers on the work of Fowkes (2) who sought to define the interfacial free energy of systems in terms of measurable parameters such as surface tension, interfacial tension, and contact angle. The term of particular interest in this study is the work of adhesion, Wa^{12}, the free energy per unit area at the interface of phases 1 and 2. It is defined in terms of surface tension and interfacial tension as (6):

$$Wa^{12} = \gamma_1 + \gamma_2 - \gamma_{12} \qquad \text{(Eq. 1)}$$

When one phase is nonpolar and only contributes London dispersion forces, it can be shown (2) that:

$$Wa^{12} = 2(\gamma_1^d \gamma_2^d)^{1/2} \qquad \text{(Eq. 2)}$$

where γ_1^d and γ_2^d represent the part of the total surface tension of a substance that arises because of interactions due to dispersion forces. For the nonpolar phase, the surface tension is considered equal to γ^d, while for a semipolar substance:

$$\gamma = \gamma^d + \gamma^p \qquad \text{(Eq. 3)}$$

where γ^p is that part of the observed surface tension, γ, due to other forces of interaction.

For a solid–liquid system, one can utilize the Young equation to obtain the work of adhesion in terms of measurable parameters (6). Here:

$$\gamma_S - \gamma_{SL} = \gamma_L \cos\theta + \pi_{sv} \qquad \text{(Eq. 4)}$$

where S and L represent solid and liquid, respectively; θ is the contact angle; and π_{sv} is the surface energy change due to any adsorption of vapor from the liquid onto the solid. For systems where wetting is poor, this term is generally ignored (2). Thus, in general, the work of adhesion between a solid and liquid, Wa^{SL}, may be determined by combining Eqs. 1 and 4:

$$Wa^{SL} = \gamma_L + \gamma_L \cos\theta \qquad \text{(Eq. 5)}$$

and when one phase is nonpolar:

$$Wa^{SL} = 2(\gamma_S^d \gamma_L^d)^{1/2} \qquad \text{(Eq. 6)}$$

These expressions are useful because once the value of γ^d is known for a substance, it is then possible to determine γ^d for any substance that forms an interface with the first substance. This procedure has been utilized to determine γ_S^d for various solids, including polymers (2, 3), metals (7), skin (8), and different pharmaceuticals (9–11). The term γ_S^d provides a measure of the surface tension a completely nonpolar liquid would require to wet the solid completely (3, 4). In these cases the contact angle of various polar liquids of known γ_L^d is measured against the solid; the γ_L^d values are determined previously from contact angles measured on a nonpolar surface such as paraffin. The value of γ_S^d for paraffin, in turn, is determined from water–paraffin wetting data, while the value of γ_L^d for water originates from liquid hydrocarbon–water interfacial tension data and application of Eqs. 1 and 2.

In all of the calculations, it is necessary to assume that the value of γ_L^d for any liquid is independent of the system used as the second phase. However, in the system providing the first γ_L^d value, water–liquid alkane, the value of γ_L^d for water is somewhat dependent on the hydrocarbon chosen (12, 13). This has been attributed to changes in water structure due to different alkane chain lengths (13) and to an alteration of alkane surface density due to the dispersion force field of water molecules (14). No further work in this regard has been reported, particularly with respect to the possible changes occurring when other polar solvents are used. Hoernschemeyer (15) attempted to explain differences in the wettability of nonpolar surfaces by alkanes in terms of differences in the packing density of solid surface groups. Thus, he attributed differences to the number of dispersion interactions that can take place per unit area; the more interactions per unit area, the greater is the wetting. This difference in surface group density could account for the differences in interfacial tension between water and different liquid alkanes as well.

Since the large number of polar liquids considered in this report

Table I—Interfacial Tension of Various Polar Liquids against Hexane and Tetradecane at 25°

Liquid	Surface Tension, dynes/cm	Interfacial Tension, dynes/cm	
		Hexane	Tetradecane
Water	72.0	50.4	52.9
Glycerin	63.7	34.3	35.9
Formamide	58.3	29.1	31.8
1,3-Propanediol	49.2	17.9	20.6
Ethylene glycol	48.9	16.3	19.7
1,4-Butanediol	47.4	16.4	18.8
N-Methylformamide	40.2	9.3	12.3
1,3-Butanediol	39.1	11.0	13.0
1,2-Butanediol	38.0	9.6	12.7
N,N-Dimethylformamide	37.0	Miscible	4.6

are immiscible with liquid alkanes, results can be compared with liquid–liquid and liquid–solid systems over a wide range of conditions. Therefore, in the present study the interfacial tension of various polar liquids was measured against hexane and tetradecane, while the contact angle for these liquids was measured on paraffin, polyethylene, and polytetrafluoroethylene.

EXPERIMENTAL

Materials—The various polar solvents were of reagent grade quality and gave good agreement with available literature values of surface tension (2, 3). Hexane and tetradecane were of 99+% purity and gave surface tensions of 18.5 and 26.3 dynes/cm, respectively. These values agree well with reported values (12, 13). Water was doubly distilled from alkaline permanganate solution.

The polytetrafluoroethylene[1] had a bulk density of 2.13 g/cm^3, in good agreement with previously reported values (15). Contact angles with water, glycerin, and formamide were in good agreement with the literature (2–5). Paraffin likewise gave contact angles in good agreement with the literature (2–5). Smooth surfaces were prepared by melting and wiping with a clean glass slide just before solidification. Parafilm "M" [2], which is a paraffin-coated sheet, gave identical results to the block paraffin. A sample of "high-density" polyethylene[3] had a bulk density of 0.932 g/cm^3.

Interfacial Tension Measurement—Interfacial tension was measured using the Wilhelmy technique (13, 16). The platinum plate was attached to a microtorsion balance[4]. Values of interfacial tension of water against hexane and tetradecane at 25° were in good agreement with earlier results (12, 13). Values for most polar liquids were reproducible to 0.3 dyne/cm; however, with the more viscous liquids like glycerin, reproducibility amounted to about 0.5 dyne/cm.

Contact Angle Measurement—Contact angles were measured using a contact angle analyzer[5], which allows one to project a magnified image of a liquid drop so that direct measurement of the contact angle may be made. The drop was introduced at constant volume from a microsyringe at a volume of 0.02 ml. The angle measured was an advancing angle, independent of time. The results recorded are the averages of at least 10 measurements, and the range of angles was no greater than 3°. As indicated previously, results that could be compared with the literature agreed quite well (2–5).

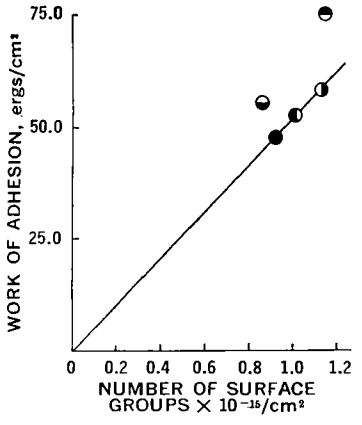

Figure 1—*Average work of adhesion for each polar liquid against a nonpolar phase* versus *surface group density of the nonpolar phase. Key:* ●, *hexane;* ◐, *tetradecane;* ◑, *paraffin;* ◒, *polytetrafluoroethylene; and* ◓, *polyethylene.*

[1] Dupont Co.
[2] American Can Co.
[3] University of Wisconsin Plant Service.
[4] Federal Pacific Co.
[5] Imass, Inc.

Table II—Contact Angle for Various Polar Liquids on Paraffin, Polyethylene, and Polytetrafluoroethylene

Liquid	Contact Angle		
	Paraffin	Polytetrafluoroethylene	Polyethylene
Water	106°	106°	85°
Glycerin	96°	97°	75°
Formamide	91°	91°	69°
1,3-Propanediol	78°	89°	54°
Ethylene glycol	79°	82°	56°
1,4-Propanediol	76°	81°	54°
N-Methylformamide	64°	68°	39°
1,3-Butanediol	62°	69°	41°
1,2-Propanediol	62°	67°	36°
N,N-Dimethylformamide	50°	59°	29°

RESULTS

Table I lists the various liquids utilized in the order of their surface tensions at 25° as well as their interfacial tensions against hexane and tetradecane. Only in one case, *N,N*-dimethylformamide with hexane, was miscibility complete, so that an interfacial tension could not be measured. Table II lists the contact angles for the various liquids. Comparison of the two tables reveals the same general order of effect at liquid or solid interfaces.

Table III contains values for the work of adhesion calculated from Eq. 1 for the liquid–liquid systems and from Eq. 5 for the solid–liquid systems. Comparison of values for any one nonpolar phase indicates very small differences, if any, in the work of adhesion for the various polar solvents[6]. In view of this finding, and for convenience of later computation and discussion, an average value for work of adhesion is presented in Table III for all liquids against each nonpolar phase. Comparison of values for each polar liquid on different nonpolar phases indicated differences in the work of adhesion. This is reflected also in Table IV, where values of γ_L^d are given. The γ_L^d terms for the polar solvents were obtained using Eqs. 2 and 6. The γ_L^d values for hexane and tetradecane were taken as their surface tensions, while the γ_S^d values for paraffin and polytetrafluoroethylene were taken as 25.5 and 19.5 dynes/cm, respectively (2). No calculation of γ_L^d was made from the polyethylene data because of the unknown character of polyethylene surfaces and the difficulty of choosing a correct γ_S^d (17). It is clear from Tables III and IV that significant changes do occur in the work of adhesion and, hence, γ_L^d as one changes the nature of the nonpolar phase used. Thus, this behavior is not limited to water–liquid alkane systems, as previously observed (13, 14). Moreover, except for water, the γ_L^d terms are not too different for the various polar liquids on any one nonpolar phase.

DISCUSSION

The results of this experiment clearly indicate that the value of γ_L^d obtained from interfacial tension or contact angle data for polar liquids against nonpolar phases depends on the nonpolar phase used. Thus, the observations made with water–liquid alkane systems (12–14) can be generalized to a wider range of other liquids. Previous discussion on this question has centered on specific effects of the nonpolar phase on water structure at the interface (13) or an effect of water on the surface density of the liquid hydrocarbons (14). The general effect on liquids ranging from water to *N,N*-dimethylformamide observed here and the fact that the effects are about the same for all liquids would suggest that a property of the polar liquid is not involved, but rather that an intrinsic

[6] The values for water tend to be slightly lower than the others in the case of the two liquid hydrocarbons, and this finding is commented on in the *Discussion* section.

Table III—Work of Adhesion[a] for Various Polar Liquids against Various Nonpolar Phases

Liquid	Work of Adhesion, ergs/cm²				
	Hexane	Tetradecane	Paraffin	Polytetrafluoroethylene	Polyethylene
Water	40.1	45.4	52.6	53.0	78.3
Glycerin	47.9	54.1	56.4	56.1	79.9
Formamide	47.7	52.8	57.6	59.7	79.4
1,3-Propanediol	49.8	54.9	59.5	58.4	78.0
Ethylene glycol	51.1	55.5	58.0	56.0	76.1
1,4-Butanediol	49.5	54.9	59.0	54.7	75.0
N-Methylformamide	49.4	54.0	58.0	55.8	71.4
1,3-Butanediol	46.6	52.4	57.5	53.1	68.5
1,2-Propanediol	46.9	51.3	55.9	53.2	68.8
N,*N*-Dimethylformamide	—	51.3	62.1	58.1	70.7
Average of all liquids	47.2	52.6	57.7	55.6	74.6

[a] Calculated from Eqs. 1 and 5. Based upon a maximum error of ±0.5 dyne/cm for interfacial tension and ±3° for contact angle, the maximum error associated with each work of adhesion is about ±0.5 erg/cm² and 3.0 ergs/cm² for liquid–liquid and liquid–solid systems, respectively.

property of the nonpolar phase such as the surface group density is responsible. Actually, these ideas were suggested earlier to explain differences in wettability of nonpolar solids by alkanes (15), but the concept has not been extended to polar liquid–nonpolar liquid or polar liquid–nonpolar solid systems to explain differences in γ_L^d values.

To see if this is a common factor for all systems studied, the number of methylene (CH_2) or difluoromethylene (CF_2) groups per square centimeter was calculated for each nonpolar phase using the two-thirds power of bulk group density (15). Figure 1 contains a plot of average work of adhesion (from Table III) *versus* surface group density. A line can be drawn through the three alkane data points such that it passes through the origin, while the two polymeric substances yield values of work of adhesion well above the straight line. A zero work of adhesion at zero surface group density (*i.e.*, a vacuum) makes physical sense. This linearity suggests that differences in surface group density and, hence, the number of interactions per square centimeter indeed can account for differences in γ_L^d when using different alkanes, liquid or solid. Thus, it should be possible to relate data obtained with one alkane to those obtained with another.

The work of adhesion for polyethylene falls considerably above the value expected from an extrapolation of the straight line, too far above to be explained by experimental error. Since the methylene or methyl (CH_3) groups involved in the interactions of polyethylene with the various liquids are also responsible for alkane interactions, it is concluded that the deviation is associated with the use of a bulk density to calculate surface group density. Indeed, it is well established that polyethylene can exhibit a different surface structure depending on the method of crystallization (17). Fowkes (14), for example, estimated that, in the extremes, polyethylene chains at the surface can align normally so that only methyl groups are exposed or they can align parallel to the surface so that mainly methylene groups are exposed. The number of methyl groups exposed per square centimeter in the former case should be about $0.4–0.5 \times 10^{15}$, whereas in the latter case the number of methylene groups per square centimeter should be about $1.75–2.0 \times 10^{15}$. In a real situation, any value in between could be the correct one. The sample of polyethylene used here is rated as a high density polyethylene and, indeed, from Fig. 1 an expected value for surface group density is about 1.5×10^{15} groups/cm². The liquid alkanes and paraffin have no crystallinity and, to a first approximation, can be regarded as being homogeneous up to and including their surface.

The correlation of work of adhesion with surface group density did not work with polar liquids wetting polytetrafluoroethylene, whereas it did work for alkanes on polytetrafluoroethylene (15). This could be attributed to uncertainties in the surface group density, but the same variation in crystallinity is not expected with this polymer as is seen with polyethylene. For example, the sample of polytetrafluoroethylene used here was identical in bulk density to that used by Hoernschemeyer (15) in his correlations of wetting with surface group densities. Thus, although one cannot discount an error in the surface group density, it would seem less likely to produce the effect noted in Fig. 1. Another contributing factor might be the tendency of polar group interactions to occur when difluoromethylene groups are present, thus giving rise to larger work-of-adhesion values than predicted. Indeed, fluorocarbon groups, such as in methyl fluoride or methylene fluoride, have a much higher dipole moment than the corresponding hydrocarbon groups (18).

From this discussion, therefore, it can be concluded that values of γ_L^d, obtained from interfacial tension or contact angle data, depend on the intrinsic properties of the nonpolar phase. In the case of the liquid alkanes and paraffin, differences are simply related to

Table IV—Values[a] of γ_L^d for Various Polar Liquids Using Different Nonpolar Phases

Liquid	γ_L^d, dynes/cm			
	Hexane	Tetradecane	Paraffin	Polytetrafluoroethylene
Water	21.7	19.6	27.1	35.9
Glycerin	30.0	27.8	31.2	40.3
Formamide	31.3	27.0	32.5	45.6
1,3-Propanediol	33.5	28.7	34.6	43.7
Ethylene glycol	35.1	29.2	33.0	40.2
1,4-Butanediol	33.1	28.7	34.1	38.2
N-Methylformamide	32.8	27.6	32.9	39.8
1,3-Butanediol	29.3	26.1	32.4	36.0
1,2-Propanediol	28.6	24.4	30.6	36.1
N,*N*-Dimethylformamide	—	31.9	37.2	41.3

[a] Calculated using Eqs. 2 and 6.

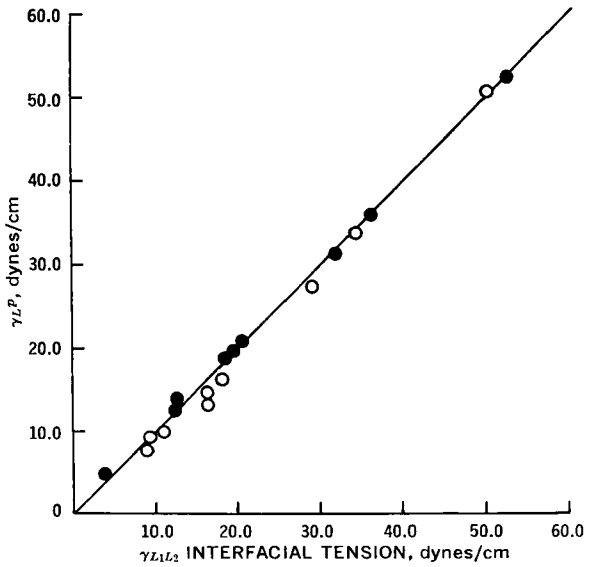

Figure 2—*Polar component, γ_L^p, for each polar liquid* versus *the interfacial tension of that liquid against:* (○) *hexane and* (●) *tetradecane. The solid line has a slope of one.*

the surface group density as calculated from bulk density, whereas the use of polymeric nonpolar phases introduces other uncertainties. From a practical point of view in evaluating the dispersion force component, γ_S^d, of polar solids using polar liquids, one can use a γ_L^d value obtained with one alkane and relate it to another; however, the use of polymers for this purpose is not appropriate. If one has to determine a γ_L^d for later use with solids, it is probably best, in a practical sense, to use data obtained with paraffin as the standard γ_L^d. The theoretical uncertainties of the entire concept behind γ^d, however, should still be kept in mind.

An earlier study (1) showed that the free energy of solution per methylene group for an homologous series of drugs correlates well with the interfacial tension of the various polar solvents used against tetradecane. Later unpublished results indicate that similar correlations exist for interfacial data obtained with hexane and paraffin. Explanations for these correlations are based upon the concept that this is a measure of the work required to create a cavity of given area in the polar solvent so as to allow a methylene group to enter and interact. From the results of this experiment, additional insight is gained into the major energetic factor contributing to this process. It is seen from the work-of-adhesion data that, regardless of the polar liquid used, the contribution of dispersion forces remains fairly constant. Water is a little lower in each case, possibly because of its smaller molecular size and polarizability relative to the organic solvents or because of some specific structure-forming ability. This suggests that the main difference between the various solvents is the work required to overcome polar–polar interactions.

By using the γ_L^d values obtained from hexane and tetradecane data and Eq. 2, it is possible to calculate γ_L^p for each liquid. This term is directly related to polar group interactions in the solvent. If γ_L^p is plotted *versus* the interfacial tension of that liquid against the corresponding liquid alkane, one can see that the points all fall on a line with a slope of unity or that in all cases γ_L^p is equal to interfacial tension. Thus, it is concluded that, when one sees a correlation with interfacial tension, as observed in the solubility work (1), one is dealing with energetic differences solely associated with the polar portion of the molecule.

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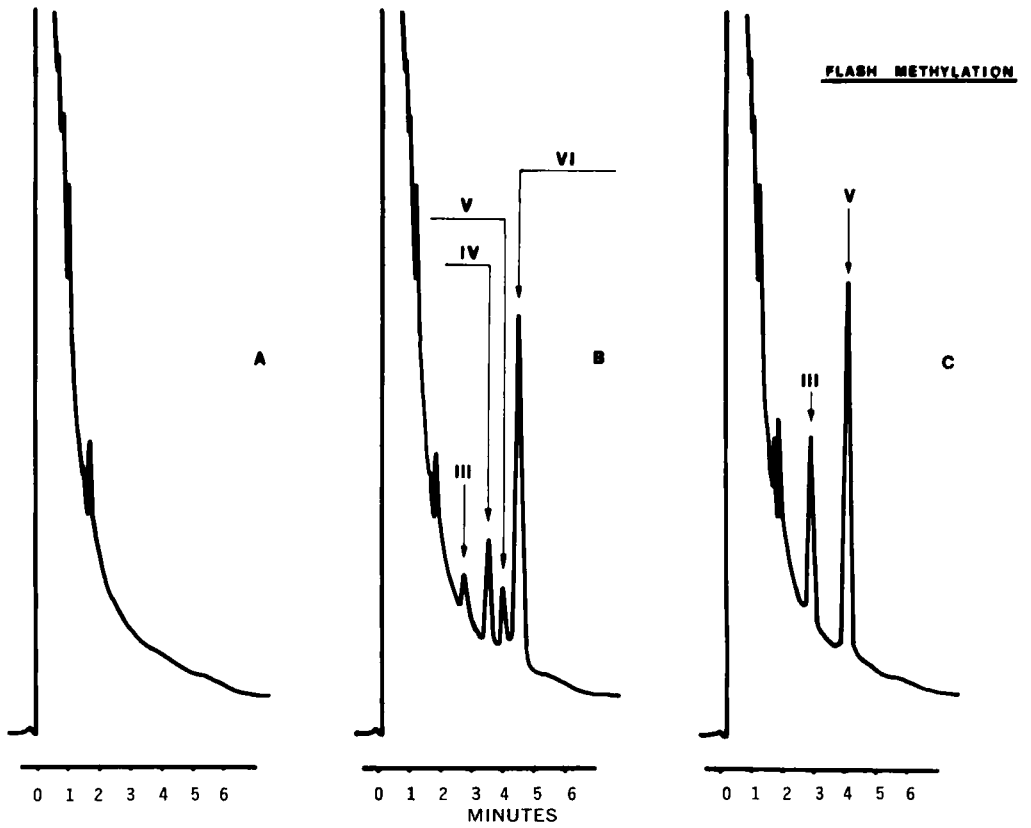


Figure 1—*GLC chromatograms. Key: A, urine blank; B, urine of rats treated with cyproheptadine (III), 40 mg/kg ip; and C, urine of treated rats after flash methylation using trimethylanilinium hydroxide. Retention times were: III, 2.8 min; IV, 3.5 min; V, 4.0 min; and VI, 4.5 min.*

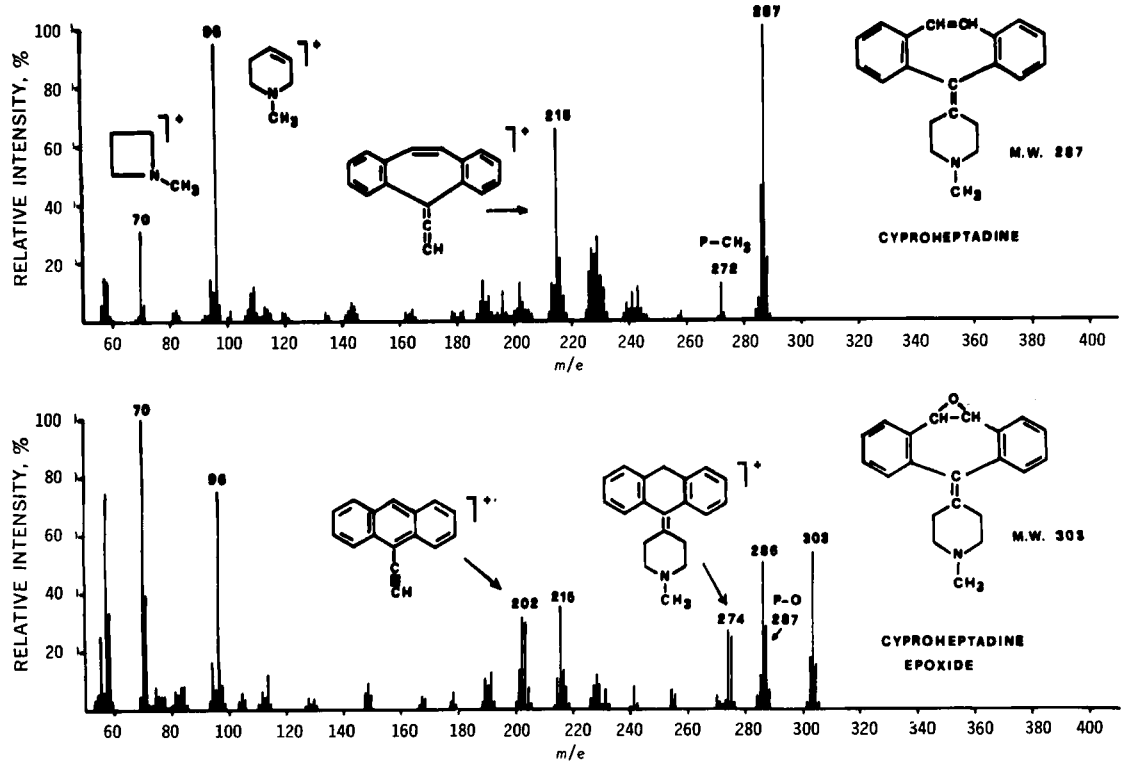
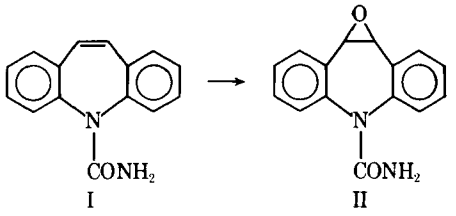


Figure 2—*Mass spectrum of authentic III and V. Similar mass spectra were obtained by introducing the corresponding eluted TLC spots by a direct inlet system or by a GLC procedure.*



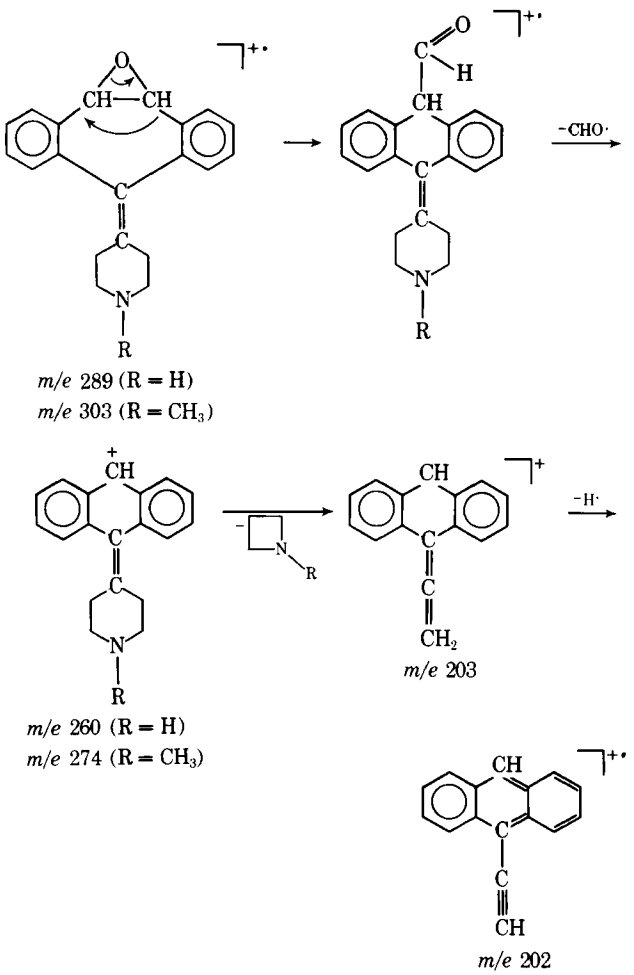
Scheme I—Epoxide formation from carbamazepine

cyproheptadine (III), 4-(5*H*-dibenzo[*a*,*d*]cyclohepten-5-ylidene)-1-methylpiperidine including the product of desmethylation and the epoxides of both cyproheptadine and desmethylcyproheptadine (IV).

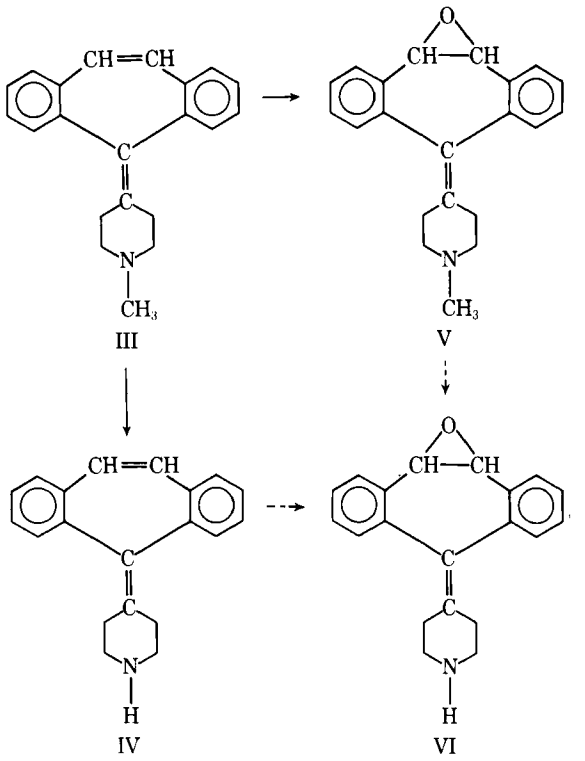
Cyproheptadine is a drug with antihistaminic and antiserotonin activities, and it is utilized for the treatment of allergic disorders (2) as well as for increasing the appetite of children and the elderly (3).

EXPERIMENTAL

Biological Samples—Two male Sprague–Dawley rats (200 g) were injected with 40 mg/kg ip of cyproheptadine[1] suspended in a 0.3% solution of hydroxypropyl cellulose (0.5 ml). Urine was collected at various times for 48 hr following drug administration and was kept frozen until analyzed. The drug, added to untreated rat urine and analyzed after 48 hr, did not show formation of any degradation products.



Scheme II—Suggested fragmentation pathway of cyproheptadine-10,11-epoxide and desmethylcyproheptadine-10,11-epoxide



Scheme III—Metabolic pathway of cyproheptadine: cyproheptadine (III), desmethylcyproheptadine (IV), cyproheptadine-10,11-epoxide (V), and desmethylcyproheptadine-10,11-epoxide (VI)

Urine samples (10–20 ml) were adjusted to pH 9 with 1 *N* NaOH and extracted with methylene chloride (20–40 ml). The organic phase was concentrated to dryness in a water bath at 65° under a gentle stream of nitrogen. This material was then used for TLC, GLC, and mass spectroscopic analyses.

General Procedures—Thin-layer chromatograms were prepared on 5 × 20-cm glass plates precoated with silica gel F-254[2] and developed at room temperature in carbon tetrachloride–methanol–diethylamine (85:10:5 v/v). Cyproheptadine and the possible metabolites were visualized under UV light at 254 nm.

GLC determinations were performed on a gas chromatograph[3] equipped with a flame-ionization detector. The chromatographic column was glass tubing (1 m long and 4 mm i.d.) packed with 100–120-mesh Gas Chrom Q and coated with 3% OV-17[4]. The operating conditions were: injection port temperature, 285°; oven temperature, 250°; detector temperature, 290°; nitrogen (carrier gas) flow rate, 50 ml/min; hydrogen flow rate, 20 ml/min; and air flow rate, 300 ml/min.

A gas chromatograph–mass spectrometer–computer system[5] was used at the following conditions: energy of the ionization beam, 70 ev; ion source temperature, 100°; and ionization current, 200 μamp. Sample introduction was carried out either by a direct inlet system at a probe temperature of 120° or by a GLC procedure utilizing a glass column (1 m long and 4 mm i.d.) packed with 3% OV-17 on 100–120-mesh Gas Chrom Q at the following conditions: injector temperature, 270°; oven temperature, 240°; and helium (carrier gas) flow rate, 30 ml/min.

Synthesis of Cyproheptadine-10,11-epoxide (V)—Cyproheptadine, 287 mg, was dissolved in 5 ml of acetone, and a mixture of 1 ml of acetonitrile and 2 ml of a 30% solution of hydrogen peroxide was added five times, keeping the pH constant at 9–10 with 1 *N* NaOH until oxygen was developed from the solution. After 48 hr at room temperature, the reaction mixture (20 ml) was extracted

[1] Donated by Merck Sharp & Dohme, Pavia, Italy.

[2] Merck.
[3] Carlo Erba Fractovap G1.
[4] Applied Science Laboratories.
[5] Finnigan quadrupole model 3100 and computer model 6000.

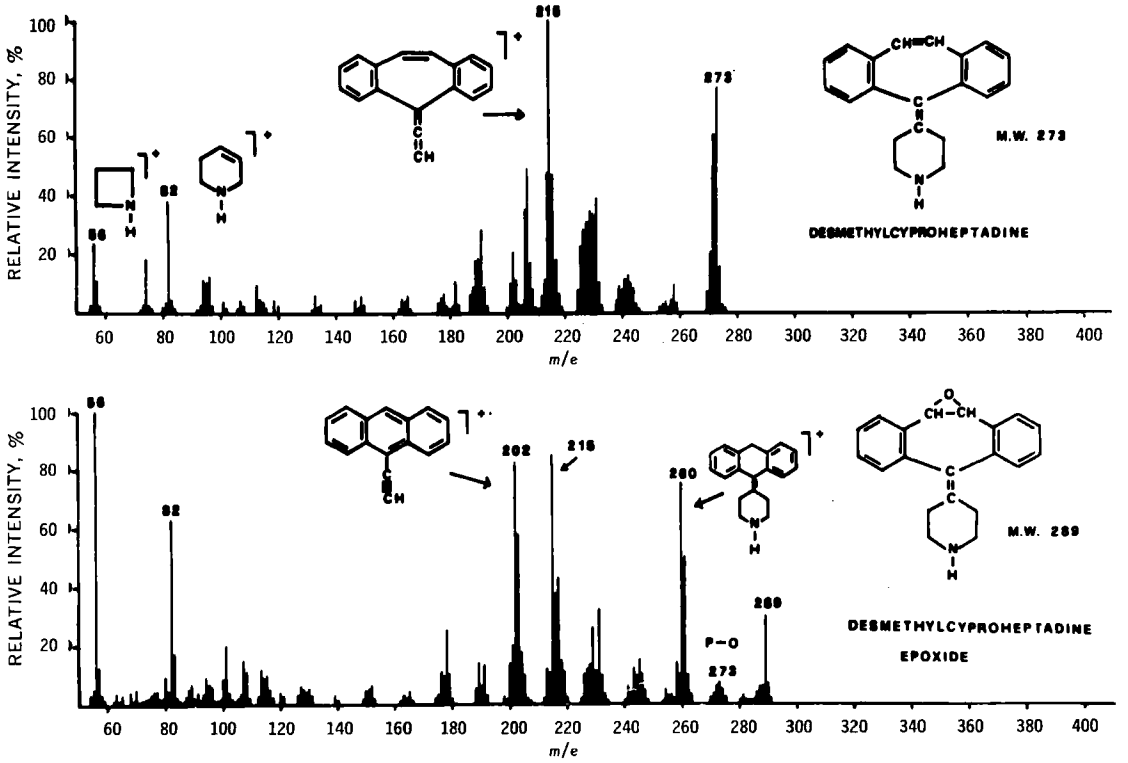


Figure 3—*Mass spectrum of IV and VI obtained by introducing the eluted TLC spots by a direct inlet system or by a GLC procedure.*

twice with an equal volume of methylene chloride, dried over sodium sulfate, and evaporated.

To reduce the *N*-oxide obtained from V, the solid was dissolved with 10 ml of 0.3 *N* $Na_2S_2O_4 \cdot 7H_2O$ and 5 ml of acetone. This solution was stored at room temperature for 15 hr. Then the solution was extracted twice with 15 ml of methylene chloride, and the organic layer was washed with 15 ml of 5% NH_4Cl. The organic phase was purified on a silica gel column (60 × 2 cm) eluted with cyclohexane–chloroform–diethylamine (70:20:10 v/v). The fractions collected (2 ml) were examined by GLC, and those containing the epoxide (retention time of 4.0 min) were pooled and evaporated.

The yield of epoxide, crystallized from methyl ethyl ketone, was 32%, mp 158–160°. The compound showed R_f 0.54 in the TLC system; NMR[6] ($CDCl_3$) showed: δ 4.22 (s, 2H,—HCO—epoxide), 2.21 (narrow s, 3H, N—CH_3), and 7.1–7.5 (m, 8H, ArH).

RESULTS AND DISCUSSION

The methylene chloride extract obtained from 20 ml of rat urine was concentrated to dryness, redissolved in 100 μl of methanol, and spotted on a TLC plate; carbon tetrachloride–methanol–diethylamine (85:10:5 v/v) was used as a solvent. The extracts of urine, obtained from rats treated with III (40 mg/kg ip), contained, in contrast to the extract of normal urine, four spots with R_f 0.60, 0.54, 0.47, and 0.39 when examined under UV light. The spot at R_f 0.60 corresponded to unchanged III, while the spot at R_f 0.47 corresponded to IV, a recently identified metabolite (4).

The GLC analyses showed that the methylene chloride extract of urines of cyproheptadine-treated rats, in contrast to controls, gave rise to four peaks (Fig. 1). The first two peaks corresponded to III and IV, respectively.

Further analyses were carried out by utilizing a GLC–mass spectroscopic procedure. The spot at R_f 0.60 eluted with methanol and introduced in the mass spectrometer with the direct inlet system and the substance yielding the first GLC peak gave a mass spectrum indistinguishable from the one obtained with authentic III (Fig. 2). The mass spectrum showed the molecular ion at *m/e* 287, corresponding to the base peak, and three main peaks at *m/e* 215, 96 (*N*-methyltetrahydropyridinil ion), and 70 (*N*-methylazetidinium ion), these two latter arising from the piperidine ring.

The mass spectrum of the eluate from the spot at R_f 0.54 and from the third peak obtained in GLC exhibited a molecular ion at *m/e* 303 (Fig. 2), suggesting, in respect to III, the gain of an oxygen atom as a result of the biotransformation. The fragmentation pathway suggests that the oxygen atom might be located at carbons 10 and 11 to form the epoxide derivative of III, as indicated in Scheme II. This scheme shows the possible fragmentation pathway of this molecule. The hypothesized structure was then confirmed by comparison with the mass spectrum of the synthetic compound.

The spot at R_f 0.47 and the second peak in GLC gave a mass spectrum corresponding to IV (Fig. 3). The mass spectrum of this metabolite showed a molecular ion at *m/e* 273 and other prominent peaks at *m/e* 215, 82, and 56; these latter two are due to a tetrahydropyridinil ion and an azetidinium ion, respectively.

The spot at R_f 0.39, as well as the fourth peak in GLC, was a substance with a molecular weight of 289 (Fig. 3). The fragmentation pathway is similar to V (Scheme II), the only differences being the peak at *m/e* 260 and the ions arising from the fragmentation of the piperidine ring (*m/e* 82 and 56). These findings suggest the introduction of an oxygen atom at carbons 10 and 11 in the structure of IV.

An indirect confirmation of this hypothesis was obtained by utilizing a flash methylation procedure (5). One microliter of the urine extract obtained from rats treated with III was injected into the gas chromatograph together with 1 μl of trimethylanilinium hydroxide. As shown in Fig. 1C, the peaks considered to be IV and desmethylcyproheptadine epoxide (VI) shifted to the peaks corresponding to the respective methylated compounds, III and V. The interpretation of the observed shift was then also confirmed by GLC–mass spectroscopic analysis.

The results obtained in rats suggest, but do not prove, that Scheme III is a possible metabolic pathway for III. The compound may undergo *N*-demethylation or epoxidation at carbons 10 and 11. The two metabolites may then be respectively epoxidated or *N*-demethylated to form the main metabolite VI.

Previous studies (6, 7) put forward the hypothesis that oxygenated metabolites of III could be responsible for the pancreatic toxicity induced in rats by high doses of the drug.

[6] Varian A60.

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[x] To whom inquiries should be directed.

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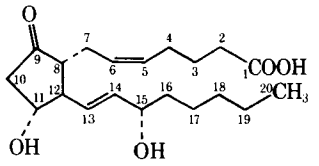
BERNARD SIMS [x] and SHELLY L. HOLDER [*]

Abstract □ Surface activities of four naturally occurring prostaglandins, PGE_2, $PGF_{2\alpha}$, PGA_1, and PGB_1, were investigated by examining the π–A compression isotherms obtained for insoluble monomolecular films of stearic acid spread on subphases containing the prostaglandins. All four compounds were observed to bring about increased instability of stearic acid monolayers, as evidenced by reductions in both transition and collapse surface pressures. Prostaglandin E_2 did not readily penetrate the monolayer but appeared capable of associating with the polar groups of the film, bringing about monolayer instability. PGB_1 exhibited a disruptive effect upon monolayer structure, causing some expansion of the π–A isotherm and increased instability. Prostaglandin $F_{2\alpha}$ penetrated into the stearic acid film, giving rise to increased surface pressure development in the initial regions of the π–A curve, resulting in a significantly expanded isotherm. This same effect, in the case of $PGF_{2\alpha}$, was observed using cholesterol and distearoyl phosphatidic acid monolayers. PGA_1 also appeared to penetrate the stearic acid monolayer, forming a mixed monolayer system and causing considerable rearrangement in monolayer structure as evidenced by an extended plateau region occurring in the π–A plot. The order of surface activities observed for the prostaglandins was $PGA_1 > PGB_1 > PGF_{2\alpha} > PGE_2$. The relative importance of polar functions, hydrophobic interactions, and hydrogen bonding are discussed with respect to the observed effects on monolayer stability. Some biological implications of the data are presented.

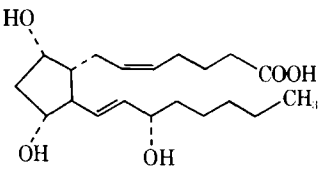
Keyphrases □ Prostaglandins E_2, $F_{2\alpha}$, A_1, and B_1—surface activity in presence of insoluble monomolecular films, π–A isotherms, biological implications □ Surface activity—four prostaglandins in presence of insoluble monomolecular films, π–A isotherms □ Films, insoluble monomolecular—surface activity of prostaglandins □ Monomolecular films, insoluble—surface activity of four prostaglandins

In recent years, great research interest has been directed toward the elucidation of the physical–chemical properties and pharmacological and physiological actions of a new class of hormones, the prostaglandins. Numerous review articles and publications have described their occurrence, synthesis, metabolism, and possible roles in biological processes (1–3). More recently, a journal has appeared devoted exclusively to prostaglandin research.

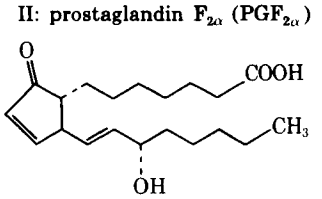
Surprisingly few investigations have concerned their surface activity (4, 5), especially since they have been implicated in various membrane-related functions, *e.g.*, vascular permeability changes (6), vascular resistance (7), intestinal transport of ions (8), binding of calcium to mitochondrial membrane (9), platelet aggregation (10), the inflammatory process (11), and smooth muscle stimulation (12). This study was undertaken to provide insight into the surface activity of four naturally occurring prostaglandins in the presence of simple model membrane systems.



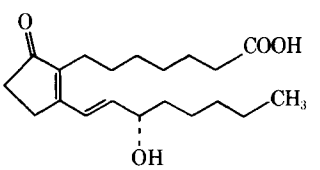
I: prostaglandin E_2 (PGE_2)



II: prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)



III: prostaglandin A_1 (PGA_1)



IV: prostaglandin B_1 (PGB_1)

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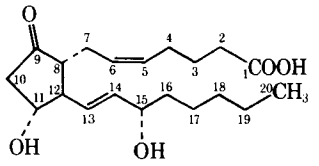
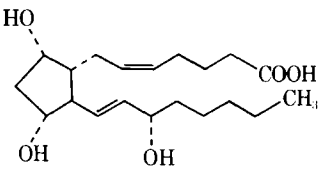
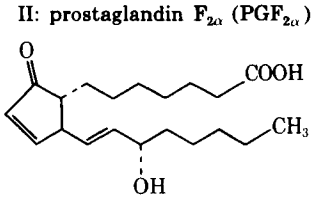


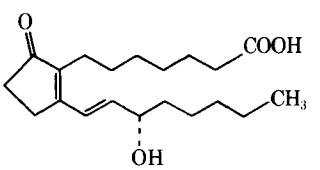
I: prostaglandin E_2 (PGE_2)



II: prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)



III: prostaglandin A_1 (PGA_1)



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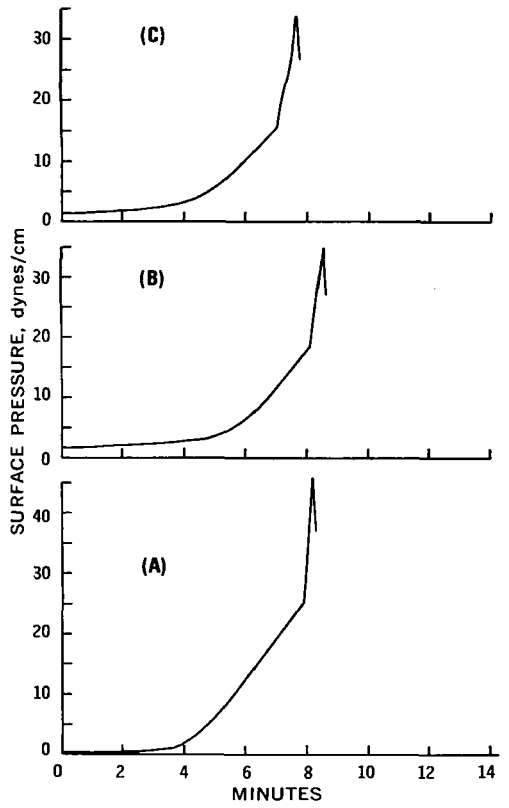


Figure 1—*The π–A compression curves for stearic acid monolayers spread on (A) 0.01 M HCl, (B) 0.01 M HCl with 2.0 × 10⁻⁵ M PGE_2, and (C) 0.01 M HCl with 4.0 × 10⁻⁵ M PGE_2. Area per molecule at zero time is 30 Å²; V_0 = 1.2 Å²/molecule/min.*

Stearic acid, distearoyl phosphatidic acid, and a bulky hydrophobic compound with a small polar moiety, cholesterol, were selected for monolayer formation. The prostaglandins investigated were PGE_2 (I), $PGF_{2\alpha}$ (II), PGA_1 (III), and PGB_1 (IV).

The prostaglandins are essentially long-chain unsaturated fatty acids containing a substituted cyclopentane ring system. $PGF_{2\alpha}$ differs from PGE_2 in that the carbonyl group at C_9 is replaced by a hydroxyl function. PGA_1 and PGB_1 are isomeric compounds which differ only in the position of the double bond in the cyclopentanone ring. The *π–A* compression behavior of the three monolayer compounds was examined on pure subphases (without substrate) and compared to the surface behavior exhibited by spreading the same films on subphases containing different concentrations of the prostaglandins. No surface activity was detectable for the prostaglandins alone at the concentrations employed in the subphase.

EXPERIMENTAL

Materials—GC of $PGF_{2\alpha}$[1], PGA_1[1], and PGB_1[1] and TLC–UV analysis of PGE_2[1] indicated a purity of 99.9% for all four compounds. Distearoyl phosphatidic acid[2], stearic acid[2], and cholesterol[2] had a stated purity of 99+%. Spectroscopic grade hexane–chloroform–methanol (20:2:1) was used as the spreading solvent, and water twice distilled with alkaline permanganate from an all-glass apparatus was used to prepare the subphase solutions.

Equipment—The monolayer balance consisted of a Teflon-covered trough having an area of 315 cm² and a width of 10.5 cm. The trough was hollowed out so that water at controlled temperatures could be circulated. The entire unit was enclosed in a large metallic box for protection against dust contamination. The barrier was driven at controlled rates of movement by means of constant revolutions per minute motors equipped with reversible drive and a positive clutch for instant stop.

Surface pressure was measured using the Wilhelmy plate method, utilizing a 5-cm roughened platinum plate. Mass changes were measured with an electromagnetic balance[3] and recorded[4]. The balance was housed in a large metallic box above the trough unit and was situated on an elevating stand to facilitate placement of the plate into the aqueous surface. Since vertical movement of the plate is negligible when this self-balancing unit is used (13, 14), it was possible to minimize error introduced by possible contact angle change at very high surface pressures.

General Procedure—Subphase solutions containing the prostaglandins were prepared in 0.01 *M* HCl at 2.0 × 10⁻⁵ and 4.0 × 10⁻⁵ *M* concentrations. Since it was difficult to assure complete solubility of these compounds above 4.0 × 10⁻⁵ *M*, preparation of more concentrated solutions was not attempted. Preliminary TLC of $PGF_{2\alpha}$, PGE_2, and PGB_1 solutions indicated the compounds to be chemically stable in 0.01 *M* HCl during the experiment. PGA_1 had previously been shown to be stable in acidic media (15). Monolayers of stearic acid, distearoyl phosphatidic acid, and cholesterol were prepared from 1-mg/ml solutions of each component in hexane–chloroform–methanol (20:2:1). These solutions were added to the surface of subphases containing the prostaglandins, utilizing

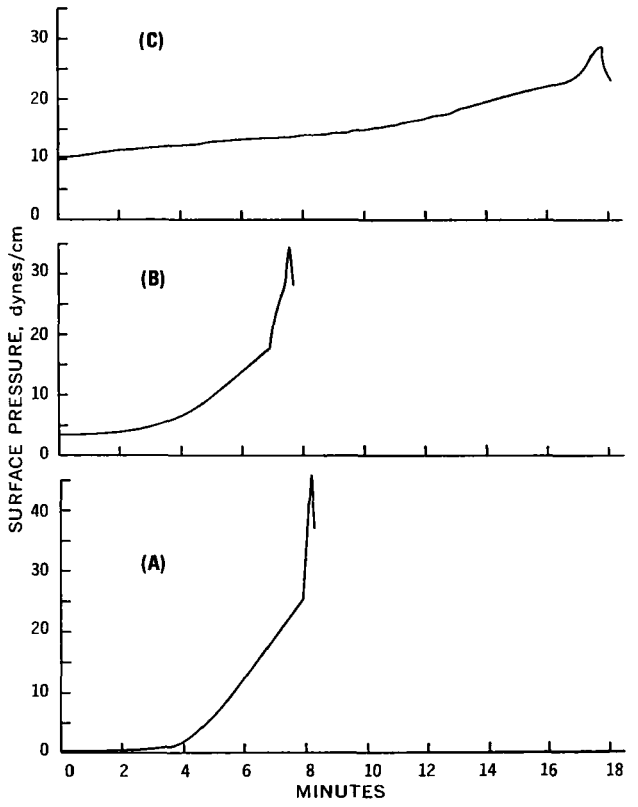


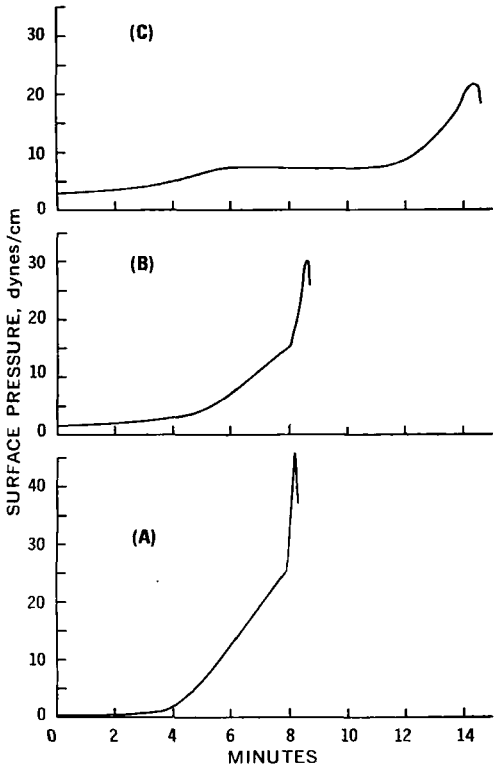
Figure 2—*The π–A compression curves for stearic acid monolayers spread on (A) 0.01 M HCl, (B) 0.01 M HCl with 2.0 × 10⁻⁵ M PGF_2, and (C) 0.01 M HCl with 4.0 × 10⁻⁵ M $PGF_{2\alpha}$. Area per molecule at zero time is 30 Å²; V_0 = 1.2 Å²/molecule/min.*

[1] $PGF_{2\alpha}$, PGE_2, PGA_1, and PGB_1 were prepared and made available by The Upjohn Co., Kalamazoo, MI 49001

[2] Applied Science, State College, Pa.

[3] Cahn R.G.

[4] Sargent Welch model SRLG.



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a micrometer syringe unit[5] which delivered 20–30 μg of material to the surface at an initially high area per molecule. After allowing 20 min for complete spreading and equilibration, the monolayer was compressed to its collapse point with constant velocity. Each subphase solution was used for only one compression experiment, which lasted for no longer than 20 min. The values reported for surface pressure represent the average of at least three to five independent measurements that differed by no more than ±0.4 dyne/cm. All studies were carried out at 25 ± 0.3°. Spreading of the mixed solvent system alone on 0.01 *M* HCl produced no detectable surface pressure after the 20-min waiting period.

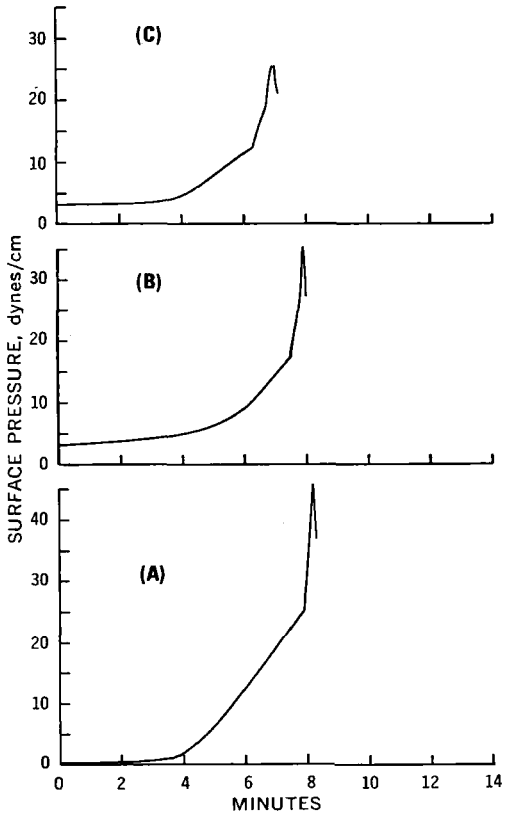
RESULTS AND DISCUSSION

The π–*A* compression plots obtained for stearic acid alone and on 2 × 10^{-5} and 4 × 10^{-5} *M* prostaglandin subphases are presented in Figs. 1–4. Values of specific surface parameters obtained from the isotherms are shown in Table I. The general π–*A* character and surface parameters obtained for stearic acid on 0.01 *M* HCl are in good agreement with results published previously (16). Each plot shown is an actual recorder tracing. Since compression in each case was carried out at constant velocity, the area per molecule at any time point on the graph can be easily calculated.

A great deal of information can be ascertained concerning surface activity of the various prostaglandins by evaluating their effect upon specific monolayer parameters such as $Å_L$, $Å_S$, π_{tr}, and π_c; $Å_L$ is defined to be the limiting area obtained by extrapolation of the liquid-condensed portion of the π–*A* isotherm. This area is generally considered to represent the point at which intermolecular interactions begin to occur among polar head groups of the film-forming molecules (17). It may be used to reflect the presence of molecules that have penetrated the film and that occupy area-determining positions. The term $Å_S$ is the limiting area obtained by extrapolation of the solid-condensed region of the curve and reflects whether molecules that penetrate the film in the liquid or expanded regions are expelled before transition to the solid phase occurs. The transition surface pressure, π_{tr}, and the collapse surface pressure, π_c, may be used to reflect changes that occur in the packing or organization of molecules within the film (17, 18) as well as film stability (19, 20). Transition from a liquid to a solid film represents a point of major reorganization among molecules in the film. Molecules capable of penetration into the surface region could presumably disrupt or alter molecular packing and bring about changes in the monolayer pressure for transition. The collapse surface pressure, π_c, represents the point of maximum monolayer (21) stability, since any effort to compress the film above this pressure results in film collapse. Therefore, a decrease observed in the collapse surface pressure indicates a trend toward increased film instability.

The π–*A* compression behavior observed for stearic acid monolayers on PGE_2 subphases is depicted in Figs. 1B and 1C. The values $Å_L$ and $Å_S$ are comparable to values observed for stearic acid alone on 0.01 *M* HCl (Table I). A pronounced effect of PGE_2 is observed upon the stability of the monolayer, as evidenced by a significant decrease in both the transition surface pressure and the collapse pressure. The transition pressure, at 2 × 10^{-5} *M* PGE_2, is decreased from 25.1 to 18.6 dynes/cm, while π_c is reduced from 46 to 35.2 dynes/cm. This same behavior is accentuated at the higher prostaglandin concentration. These data suggest that PGE_2 molecules do not readily penetrate into the surface region but can apparently associate beneath the surface with film molecules and bring about instability. This may result from dipolar interactions of the prostaglandin molecule with the carboxyl groups of stearic acid and a concomitant disruption in monolayer structure.

Stearic acid monolayers spread on $PGF_{2\alpha}$ subphases (Fig. 2) are noticeably expanded at both concentrations investigated, with $Å_L$ occurring at larger areas per molecule. The expanded character of the π–*A* isotherm suggests that penetration of the monolayer occurs and that prostaglandin molecules assume area-determining positions within the surface. Furthermore, $PGF_{2\alpha}$ alone will not form a stable monolayer when spread on 0.01 *M* HCl and stearic



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[5] Burroughs-Wellcome Agla.

Table I—Values of Specific Surface Parameters Obtained from π–A Isotherms for Stearic Acid Films Spread on Prostaglandin Subphases at 25°

Parameter[a]	0.01 M HCl	PGE_2		$PGF_{2\alpha}$		PGA_1		PGB_1	
		2×10^{-5} M	4×10^{-5} M	2×10^{-5} M	4×10^{-5} M	2×10^{-5} M	4×10^{-5} M	2×10^{-5} M	4×10^{-5} M
A_L, Å²	25.1	23.9	25.2	27.0	—	25.1	—	24.8	26.7
A_S, Å²	20.9	20.8	21.5	21.9	—	21.2	—	21.2	22.7
A_{tr}, Å²	20.5	20.2	21.5	21.8	—	20.3	—	21.0	22.5
A_C, Å²	20.1	19.7	20.7	21.0	8.7	19.6	12.8	20.5	21.7
π_{tr}, dynes/cm	25.1	18.6	15.4	17.5	—	15.2	—	17.1	12.0
π_C, dynes/cm	46.0	35.2	33.9	34.6	29.0	30.0	22.2	35.3	25.7
$\Delta\pi_C$, dynes/cm		10.8	12.1	11.4	17.0	16.0	23.8	10.7	20.3

[a] A_L = limiting area from liquid-condensed region of π–A isotherm; ±0.6 Å². A_S = limiting area from solid-condensed region of π–A isotherm; ±0.6 Å². A_{tr} = area of second-order transition; ±0.6 Å². A_C = area of collapse; ± 0.6 Å². π_{tr} = transition surface pressure, ±0.4 dyne/cm. π_C = collapse surface pressure; ±0.4 dyne/cm. $\Delta\pi_C$ = difference in monolayer collapse surface pressure on pure subphase and subphase containing prostaglandin substrate.

acid alone exhibits only minimal surface pressure in the initial portion of the π–A curve—*viz.*, 30–25 Å²/molecule at 0–4 min (Fig. 2A). The very pronounced surface pressure development in this region on the $PGF_{2\alpha}$ subphase indicates that considerable interaction occurs between the penetrant and film molecules. The presence of hydroxyl groups at both C_9 and C_{11} in $PGF_{2\alpha}$ might account for the increased surface pressure development if intermolecular hydrogen bonding with carboxyl groups of stearic acid occurred. Support for this interaction is obtained by noting in Fig. 1 that PGE_2, which has the C_9 hydroxyl replaced by a keto function (otherwise identical in structure to $PGF_{2\alpha}$), exhibits only minimal surface pressure development in the initial regions of the monolayer π–A curve.

One other activity of this compound on stearic acid monolayers is to decrease the film stability. As can be noted from Table I, at 4×10^{-5} M $PGF_{2\alpha}$, the collapse pressure is reduced from 46 to 29 dynes/cm. Unlike PGE_2, which brings about film instability apparently by association with film molecules, this same effect is achieved with $PGF_{2\alpha}$ by direct penetration of the monolayer and intermolecular association, such that a mixed film system having decreased stability is formed.

Stearic acid monolayers spread on PGA_1 subphases exhibit unusual π–A compression behavior (Fig. 3). At 2×10^{-5} M PGA_1, minimal effect is observed on the value of the limiting areas, $Å_L$ and $Å_S$. This indicates that PGA_1 molecules do not readily penetrate into the monolayer. However, the collapse pressure is substantially reduced from 46 to 30 dynes/cm, so some association apparently can occur among the prostaglandin molecules and stearic acid molecules in the film. In this respect, PGA_1 behaves in similar fashion to PGE_2 in bringing about instability of the monolayer. At the higher PGA_1 concentration (Fig. 3C), a plateau region occurs in the π–A plot at about 7 dynes/cm surface pressure. To account for the plateau, an appreciable number of PGA_1 molecules apparently do penetrate the monolayer at the higher concentration and

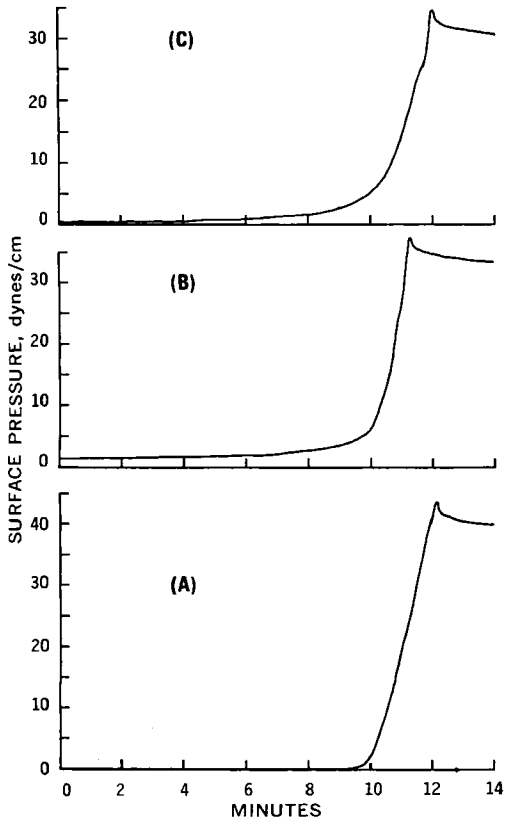

Figure 5—*The* π–A *compression curves for cholesterol monolayers spread on (A) 0.01* M *HCl, (B) 0.01* M *HCl with* 2.0×10^{-5} M PGE_2, *and (C) 0.01* M *HCl with* 4.0×10^{-5} M PGE_2. *Area per molecule at zero time is 75.9* Å²; V_0 = *3.45* Å²/*molecule/min.*

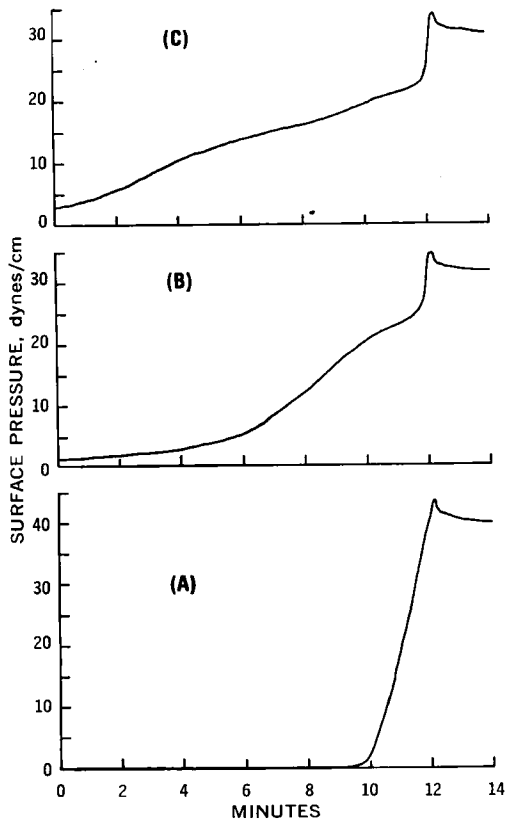

Figure 6—*The* π–A *compression curves for cholesterol monolayers spread on (A) 0.01* M *HCl, (B) 0.01* M *HCl with* 2.0×10^{-5} M $PGF_{2\alpha}$, *and (C) 0.01* M *HCl with* 4.0×10^{-5} M $PGF_{2\alpha}$. *Area per molecule at zero time is 75.9* Å²; V_0 = *3.45* Å²/*molecule/min.*

Table II—Values of Specific Surface Parameters[a] Obtained from π–A Isotherms for Cholesterol Films Spread on PGE_2 and $PGF_{2\alpha}$ Subphases at 25°

	0.01 M HCl	PGE_2		$PGF_{2\alpha}$	
		2×10^{-5} M	4×10^{-5} M	2×10^{-5} M	4×10^{-5} M
A_S, Å²	39.0	39.8	41.2	37.5	36.0
A_C, Å²	34.0	36.9	34.5	34.1	33.5
π_c, dynes/cm	43.8	38.0	34.9	34.4	33.1

[a] Surface parameters have the same definitions given in Table I.

drastically alter monolayer structure. There is considerable rearrangement of molecules within the film. This is evident by the large area over which the plateau extends (23.4–16.5 Å²/molecule) while the film pressure remains constant. The whole π–A curve, in essence, is displaced toward smaller areas per molecule. Following the plateau, monolayer stability is substantially reduced, as seen in the reduction of the collapse surface pressure from 46 to 22.2 dynes/cm. Hence, a dual role can be observed for PGA_1 molecules on the stearic acid system. At low prostaglandin concentrations, they decrease the stability of the monolayer, possibly by association with the polar groups of the film molecules; at higher concentrations, they appear capable of direct penetration into the surface in significant numbers, disrupting monolayer structure and causing significant expansion and rearrangement of film molecules which results in a mixed film system exhibiting decreased stability.

PGB_1, a structural isomer of PGA_1, behaves in a qualitatively similar fashion (Figs. 4B and 4C). At 2×10^{-5} M PGB_1, minimal effect is observed upon the limiting areas, $Å_L$ and $Å_S$. However, the collapse pressure, as in the case of PGA_1, is reduced from 46 to

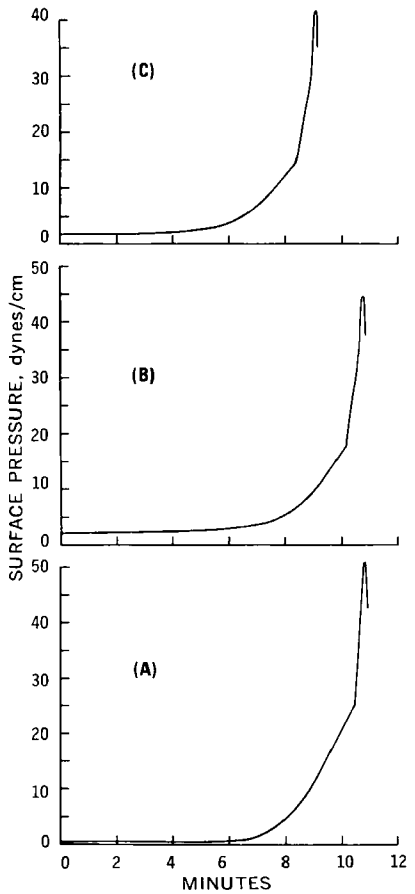

Figure 7—*The π–A compression curves for distearoyl phosphatidic acid monolayers spread on (A) 0.01 M HCl, (B) 0.01 M HCl with 2.0×10^{-5} M PGE_2, and (C) 0.01 M HCl with 4.0×10^{-5} M PGE_2. Area per molecule at zero time is 75.9 Å²; V_0 = 3.45 Å²/molecule/min.*

Table III—Value of Specific Surface Parameters[a] Obtained from π–A Isotherms for Distearoyl Phosphatidic Acid Films Spread on PGE_2 and $PGF_{2\alpha}$ Subphases at 25°

	0.01 M HCl	PGE_2		$PGF_{2\alpha}$	
		2×10^{-5} M	4×10^{-5} M	2×10^{-5} M	4×10^{-5} M
A_L, Å²	49.1	49.1	55.6	55.6	—
A_S, Å²	41.0	40.6	46.2	37.7	40.2
A_{tr}, Å²	40.0	40.9	47.0	41.0	—
A_C, Å²	38.6	38.8	44.5	35.6	38.4
π_{tr}, dynes/cm	24.5	17.7	14.3	19.2	—
π_C, dynes/cm	51.3	45.0	42.2	46.6	40.2

[a] Surface parameters have the same definitions given in Table I.

35.3 dynes/cm and the transition surface pressure is decreased from 25.1 to 17.1 dynes/cm. Hence, some association with the monolayer undoubtedly occurs. At 4×10^{-5} M PGB_1, expansion of the monolayer is evident. The value of $Å_L$ is increased from 25.1 to 26.7 Å²/molecule, and the value of $Å_S$ is increased from 20.9 to 22.7 Å²/molecule. The collapse pressure is further reduced to 25.7 dynes/cm. It is particularly interesting that no plateau region is observed in the π–A plot. The data support some penetration of the monolayer by PGB_1 at the higher concentration. The absence of a plateau might result from additional configurational constraints placed on PGB_1 molecules as a result of the more rigid, conjugated dienone system formed by the C_{8-12} double bond with the C_{12} side-chain double bond at C_{13} and the keto function at C_9. This could conceivably impose configurational requirements on the molecule such that the C_{12} side chain and cyclopentanone ring are constrained to limited positions within the surface. The observed instability following penetration of the monolayer at the higher PGB_1 concentration would then be attributed more to a disruptive effect upon monolayer structure and packing rather than to any specific interaction with the film species. The relative order of surface activities observed for the four prostaglandins in terms of increasing monolayer instability, as reflected by reductions in collapse surface pressure, $\Delta\pi_c$ (Table I), is $PGA_1 > PGF_{2\alpha} \approx PGE_2 \approx PGB_1$ at 2×10^{-5} M. These activities are delineated more clearly at the higher concentration, with $PGA_1 > PGB_1 > PGF_{2\alpha} > PGE_2$.

Cholesterol monolayers can provide a useful system for acquiring insight into the possible types of interactions that may be involved with the prostaglandin compounds. Intermolecular hydrogen bonding was suggested as one primary type of interaction involved in expansion of stearic acid monolayers and, if such is the case, the effect should be accentuated with cholesterol monolayers. Figures 5 and 6 depict π–A plots obtained for cholesterol monolayers spread on PGE_2 and $PGF_{2\alpha}$ subphases, respectively. The surface parameters are summarized in Table II. In each case, increased film instability occurs with an increasing concentration of prostaglandin, as evidenced by the decreases in collapse surface pressure (Table II). This surface behavior is in general agreement with similar effects observed for the two prostaglandins on stearic acid monolayers (Figs. 1 and 2 and Table I). A comparison of Figs. 5 and 6 illustrates the importance of the polar functions of the ring system, especially the hydroxyl group at C_9 in $PGF_{2\alpha}$. The cholesterol monolayer spread on 4×10^{-5} M $PGF_{2\alpha}$ is significantly expanded in the initial region of the π–A isotherm. This can be contrasted to the minimal expansion observed for cholesterol with PGE_2 at 4×10^{-5} M. $PGF_{2\alpha}$ molecules appear capable of film penetration, as was observed for stearic acid monolayers; but, in addition to penetration, $PGF_{2\alpha}$ apparently can effectively hydrogen bond with the hydroxyl group of cholesterol. The interaction results in an appreciably more expanded film, with increased surface pressure development occurring in the initial regions of the isotherm.

Another comparison is illustrated in Figs. 7 and 8 for the surface behavior for these two prostaglandins, using distearoyl phosphatidic acid monolayers. This system has a larger polar moiety than stearic acid or cholesterol and, at the same time, presents a rigid hydrophobic character to the prostaglandin molecules. It can be observed from the figures that PGE_2 and $PGF_{2\alpha}$ exhibit surface behavior similar to that observed in the case of the stearic acid mo-

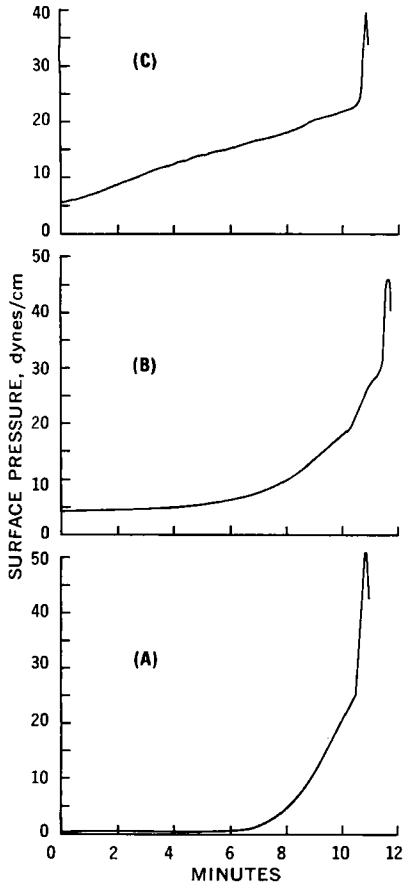

Figure 8—*The π–A compression curves for distearoyl phosphatidic acid monolayers spread on (A) 0.01 M HCl, (B) 0.01 M HCl with 2.0 × 10⁻⁵ M PGF₂α, and (C) 0.01 M HCl with 4.0 × 10⁻⁵ M PGF₂α. Area per molecule at zero time is 75.9 Å²; V₀ = 3.45 Å²/molecule/min.*

nolayers. PGE_2 causes minimal expansion of the monolayer but, nevertheless, increased instability, as evidenced by reductions in the transition and collapse surface pressures (Table III). $PGF_{2\alpha}$ brings about significant expansion of the distearoyl phosphatidic acid monolayers, with increased instability, and this is again similar to its effect on both stearic acid and cholesterol monolayers. Hence, no major differences in surface behavior of the two prostaglandins can be observed when stearic acid is replaced by a molecule having two C_{18} alkyl chains. One can conclude from this important observation that hydrophobic interactions within the film are not sufficient to counteract instability created in the monolayer by polar group interactions with the prostaglandin molecules.

In conclusion, all four of the naturally occurring prostaglandins investigated bring about increased instability in monomolecular films of stearic acid when dissolved in the aqueous subphase at very low concentrations. The relative order of surface activities is $PGA_1 > PGB_1 > PGF_{2\alpha} > PGE_2$. PGE_2 does not appear to penetrate the monolayer readily but apparently can associate with the polar groups of the film, bringing about instability. PGB_1 forms a mixed monolayer system with stearic acid and exhibits a disruptive effect upon monolayer structure that leads to increased film instability. Both $PGF_{2\alpha}$ and PGA_1 cause significant expansion in the π–A isotherms for stearic acid and appear capable of occupying area-determining positions within the surface. In addition, $PGF_{2\alpha}$, which has hydroxyl groups at C_9 and C_{11}, can effectively hydrogen bond with stearic acid molecules. This effect is accentuated when stearic acid is replaced by a cholesterol monolayer. Comparison of the π–A isotherms obtained for stearic acid and distearoyl phosphatidic acid monolayers did not indicate any significant differences in surface effects for the film penetrant $PGF_{2\alpha}$ or the PGE_2 molecule. Both prostaglandins showed similar behavior to that observed in the case of the stearic acid monolayer. From these findings, it is concluded that the primary instability created in the film resides within the polar groups of the film-forming molecules.

Surface activities for $PGF_{2\alpha}$ and PGE_2 follow the same order as that observed for F- and E-type prostaglandins in inhibition of sodium flux across the intestinal mucosa (8). However, this order is just the reverse of their uterine-stimulating activity observed for the pregnant human uterus (22). Such differences emphasize the multiplicity of actions which may be attributable to these compounds biologically and the inherent pitfalls involved in making any rash extrapolations to biological systems. However, the surface activities for PGA_1 and PGB_1 do follow closely the *trend* of biological depressor activity reported by Jones (23) for lowering cat and dog blood pressure after intraaortic injection, *i.e.*, $PGA_1 \gg PGB_1$. In addition, PGA_1 was observed to exhibit biphasic activity. Such activity parallels the dual role observed for PGA_1 in destabilization of stearic acid monolayers at the low and high concentration levels. However, more evidence is required to substantiate any extrapolations of the surface data toward mechanistic considerations for biological action of the prostaglandins.

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* Undergraduate research assistant from Xavier University with the Pharmacy Research Unit of The Upjohn Co., summer 1973.

ˣ To whom inquiries should be directed.

Reduced Acid-Neutralizing Velocity of Spray-Dried Agglomerated Magnesium Carbonate

Y. KAWASHIMA ˣ and H. TAKENAKA

Abstract □ The rate of neutralization of acid by spray-dried products bound with gum arabic, gelatin, polyvinyl alcohol, carboxymethylcellulose, methylcellulose, and polyvinylpyrrolidone was investigated to evaluate antacid and timed-release properties. A double-layer model was assumed to analyze the neutralization velocity. Diffusion and the reaction layers at the interface between solid and liquid were considered, and an acid neutralization rate equation was developed. The mass transfer coefficient and reaction rate constant for acid neutralization were determined to be 9.3 $\times 10^{-3}$–7.0 $\times 10^{-2}$ cm sec^{-1} and 400–500 cm^3 sec^{-1}, respectively. The percentage of spray-dried particles coated with binder was 80% for carboxymethylcellulose and 48–65% for gelatin, methylcellulose, and polyvinyl alcohol products. Gum arabic and polyvinylpyrrolidone products proved to be only agglomerated. Tableting the powdered spray-dried products increased the proportion of encapsulated material from 48–83 to 70–98%, and their timed-release capacity was greater than that prior to tableting.

Keyphrases □ Magnesium carbonate (spray-dried agglomerated)—reduced acid-neutralizing velocity, model, equations □ Acid-neutralizing velocity—reduced rate of spray-dried agglomerated magnesium carbonate, model, equations □ Neutralization rate of antacids—spray-dried products bound with gum arabic, gelatin, polyvinyl alcohol, carboxymethylcellulose, methylcellulose, and polyvinylpyrrolidone, model, equations □ Antacids—spray drying, effect on acid-neutralizing velocity □ Spray drying—magnesium carbonate, effect on acid-neutralizing velocity, model, equations

Spray drying has been widely applied in the pharmaceutical industry (1–3). One interesting application is as a microagglomeration and encapsulation technique for the preparation of timed-release dosage forms. Kornblum (4) prepared a sustained-action tablet using spray-dried powders, while Kawashima *et al.* (5) produced microencapsulated agglomerates by spray drying.

Magnesium carbonate is frequently used as an antacid and laxative, and many studies have evaluated its antacid capacity (6–8) and neutralizing velocity (9, 10). However, little is known about the neutralizing velocity of spray-dried agglomerated magnesium carbonate. The present work was undertaken to evaluate the antacid properties of the spray-dried material as a component of timed-release medicaments. Neutralizing velocity was analyzed by a double-layer model, which yielded separately the rate constants for diffusion and for reaction. The proportion of encapsulated product was also determined to allow various binders to be evaluated for effectiveness in producing timed-release behavior.

EXPERIMENTAL

Test Samples—Test antacids were unagglomerated magnesium carbonate, having a diameter of 2–8 μm and specific surface area by the nitrogen adsorption method of about 20 m^2 g^{-1}, and spray-dried agglomerated products bound with gum arabic, gelatin, polyvinyl alcohol, carboxymethylcellulose, methylcellulose, or polyvinylpyrrolidone. The binders were used at a level of 0.5–3% by weight of solids, and the aqueous slurries were atomized centrifugally and dried at 150 ± 10°. Details of the technique may be found in Ref. 11. Representative particle properties of the spray-dried products were as follows: volume surface mean diameter, 26–78 μm; true particle density, 2.1–2.3 g cm^{-3}; and specific surface area by adsorption, 13–20 m^2 g^{-1}.

Five samples of salicylic acid, having diameters of 4, 30, 58, 89, or 150 μm and a true density of 1.4 g cm^{-3}, were also used for dissolution rate studies.

Apparatus and Procedure—*Dispersed Powder in Beaker Method*—Powdered antacids (0.5 g) were added to 0.01 *N* HCl (450 ml) in the standard vessel with baffles (Fig. 1A) and maintained at 27, 37, or 47° in a water bath. A dispersed system was produced by constant agitation at 630, 1100, or 1640 rpm using a four-blade stirrer connected with a synchronous motor. At lower speeds, a considerable proportion of particles would float on the surface of the liquid. Acid neutralization was followed by measuring the pH of the solution at adequate intervals using a pH meter with calomel and glass electrodes.

The dissolution of salicylic acid particles into distilled water (450 ml) was investigated under the same experimental conditions. At suitable intervals, 1 ml of solution was withdrawn by pipet. After passage through a glass wool filter, the sample was diluted appropriately with distilled water and the salicylic acid content was determined spectrophotometrically at 298 nm. When powders smaller than 20 μm were used, a small amount of polyoxyethylene sorbitan monooleate was added to the acid solution to prevent agglomeration.

Rotating-Disk Method—Disks of 2.0 g and 2.0-cm diameter were prepared by compression of powdered antacids or salicylic acid at 50 kg cm^{-2} in a compression punch and die. Each disk was mounted on a six-blade agitator of 4.7-cm diameter with a suitable water-insoluble adhesive so that only one face remained exposed (Fig. 1B). The agitator was then immersed in bulk liquid at 27, 37, or 47° and rotated at 150, 241, 382, or 620 rpm. Analytical procedures were the same as for the beaker method.

Measurement of Diffusion Coefficients—Diffusion coefficients were measured by a diaphragm method (12, 13) using four cells with two chambers separated by a diaphragm. Cell constants were determined to be 6.37–9.70 using 0.1 *N* KCl, which is known to have diffusion coefficients of 1.68, 1.78, and 2.28 $\times 10^{-5}$ cm^2 sec^{-1} at 20, 25, and 37°, respectively. Diffusion coefficients of 2 ×

Table I—Diffusion Coefficients (centimeters^2 second^{-1})

	Temperature			
	27°	37°	47°	Literature Data
Salicylic acid[a]	4.10×10^{-6}	4.85×10^{-6}	5.75×10^{-6}	4.2×10^{-6} [b]
Hydrochloric acid[a]	2.31×10^{-5}	2.52×10^{-5}	2.79×10^{-5}	$2.38 \pm 0.04 \times 10^{-5}$ [c]

[a] Concentration of salicylic acid and hydrochloric acid is 2×10^{-3} mole liter^{-1} and 0.01 *N*, respectively. [b] Diffusion coefficient at pH 3 and 37°, Ref. 18. [c] Diffusion coefficient at 12°, Ref. 19.

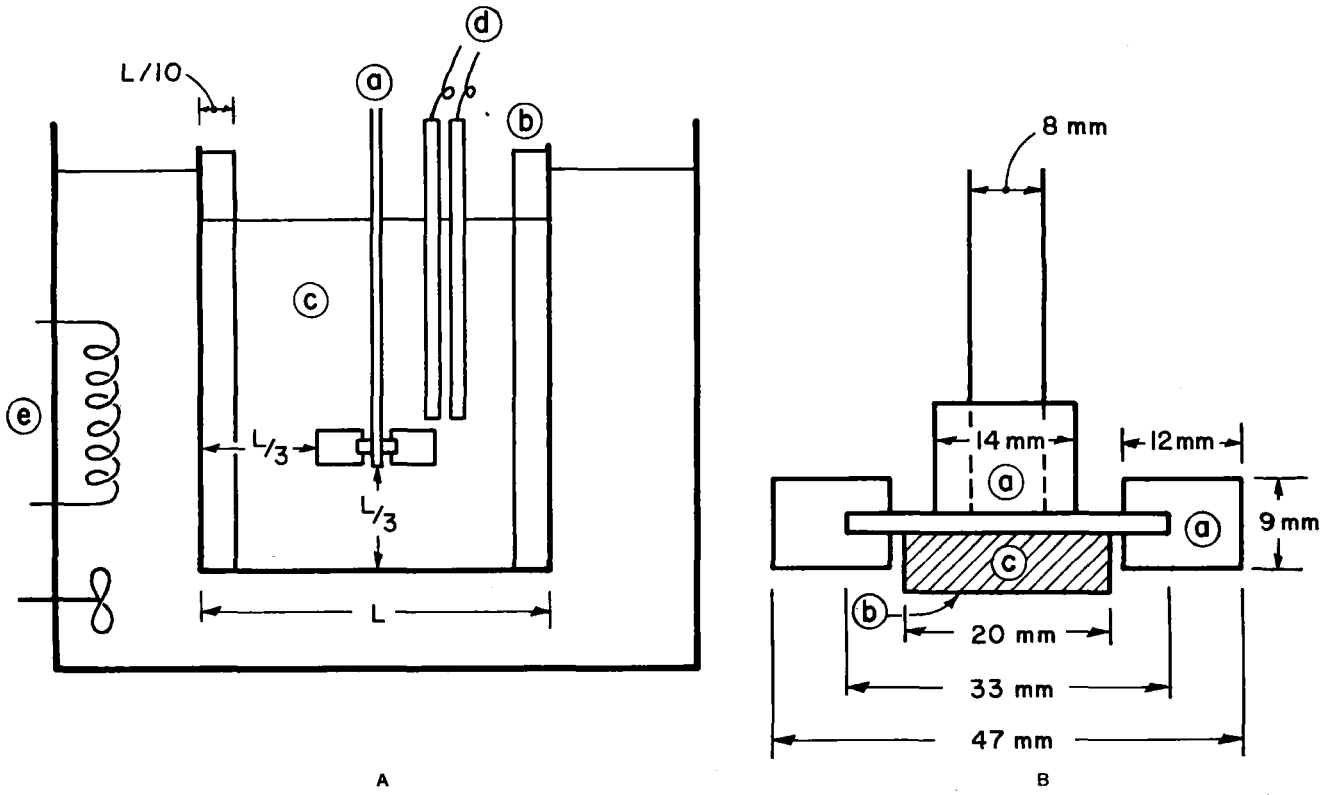

Figure 1—*Apparatus and procedure for measuring pH changes in dispersed system and rotating-disk method. (A) Dispersed system consisting of the standard vessel of 87 mm diameter. Key: a, stirrer with four blades; b, baffle; c, acid solution of 450 ml; d, pH meter; and e, water bath with thermal unit. (B) Rotating disk. Key: a, impeller with six blades; b, tablet surface exposed to acid solution; and c, adhesives.*

10^{-3} mole liter^{-1} salicylic acid and 0.01 *N* HCl were determined at 27, 37, and 47° (Table I).

Mechanism and Rate Equation of Acid Neutralization—In this work, the rate equation for acid neutralization was developed by considering both hydrogen-ion transfer and the neutralizing reaction at the interface between solid and bulk liquid. By assuming the presence of a reaction layer and a diffusion layer, a model for acid neutralization was introduced (Fig. 2).

Hydrogen ions diffuse through the diffusion layer into the reaction layer deposited on the solid surface and the neutralization reaction occurs in the reaction layer. Two assumptions were made:

1. The hydroxyl ions produced by dissolution of the magnesium carbonate reach the reaction layer at a much higher rate than the hydrogen ions coming through the diffusion layer.
2. A steady concentration of hydrogen ions in the reaction layer is established, and the net rate of change of hydrogen-ion concentration there is set equal to zero.

When using these assumptions, Eqs. 1 and 2 may be derived:

$$-\frac{d}{dt}(H_1) = \frac{k_1 A}{V}(H_1 - H_2) \qquad \text{(Eq. 1)}$$

$$-\frac{d}{dt}(H_2) = \frac{k_1 A}{\Delta v}(H_1 - H_2) - \frac{k_2}{\Delta v} H_2^{\alpha} = 0 \qquad \text{(Eq. 2)}$$

where:

A = surface area of antacid (centimeters^2)
k_1 = mass transfer coefficient (centimeters second^{-1})
k_2 = reaction rate constant (centimeters^3 second^{-1})
H_1 = hydrogen-ion concentration in bulk liquid (moles liter^{-1})
H_2 = hydrogen-ion concentration in reaction layer (moles liter^{-1})
t = time
V = volume of bulk liquid (centimeters^3)
Δv = volume of reaction layer (centimeters^3)
α = reaction order

The rate of reduction of hydrogen ions in the bulk liquid is given by:

$$-\frac{d}{dt}(H_1) = \frac{k_2}{V} H_2^{\alpha} \qquad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

Determination of Mass Transfer Coefficients and Reaction Rate Constants—If the neutralization reaction is first order with respect to hydrogen-ion concentration in the reaction layer (*i.e.*, $\alpha = 1$), Eq. 1 becomes:

$$-\frac{d}{dt}(H_1) = \frac{k_1 k_2 A}{V(k_1 A + k_2)} H_1 \qquad \text{(Eq. 4)}$$

Equation 4 may be integrated to yield Eq. 5, considering the initial condition $H = H_0$ or pH = pH_0 = 2.0 at $t = 0$:

$$-\ln H_1 = \frac{k_1 k_2 A}{V(k_1 A + k_2)} t - \ln H_0 \qquad \text{(Eq. 5)}$$

Equation 5 is transformed into a more convenient form:

$$pH - pH_0 = \frac{k_1 k_2 A}{2.303 V(k_1 A + k_2)} t \qquad \text{(Eq. 6)}$$

Acid neutralization of powdered unagglomerated magnesium carbonate was carried out in the dispersed system. Plots of the acidity change, pH − pH_0, are shown in Fig. 3. For clarity, only the data points taken at 10-sec intervals are shown, although readings were made every 5 sec in the early stages where pH was changing rapidly. After short induction periods of about 3–7 sec, linear relationships are obtained as required in Eq. 6. Their slopes, tan α, are represented as:

$$\tan\alpha = \frac{k_1 k_2 A}{2.303 V(k_1 A + k_2)} \qquad \text{(Eq. 7)}$$

The measured values of the slope are given in Table II where it

Table II—Mass Transfer Coefficients and Reaction Rate Constants for Acid Neutralization

Experimental Condition	tan α[a]	Average	Average Deviation, %	$X \times 10^{3}$[a]	$D \times 10^{5}$[a]	$k_1 \times 10^{2}$[a]	$k_2 \times 10^{-2}$[a,b]
37°, 630 rpm[a]	0.317 0.302 0.296	0.305 ± 0.009	2.6	2.30	2.52	1.10	
37°, 1100 rpm	0.320 0.326 0.314	0.320 ± 0.006	1.3	0.830	2.52	3.04	4.14
37°, 1640 rpm	0.390 0.378 0.387	0.385 ± 0.006	1.2	0.400	2.52	6.30	
27°							4.05
47°							4.52

[a] tan α = slope of pH *versus* time straight line (pH units second^{-1}); X = thickness of diffusion layer (centimeters); D = diffusion coefficient of hydrochloric acid (centimeters^2 second^{-1}); k_1 = mass transfer coefficient (centimeters second^{-1}); k_2 = reaction rate constant (centimeters^3 second^{-1}); rpm = agitation speed. [b] The reaction rate constant is the average of three experimental results at 630, 1100, and 1640 rpm.

is seen that these values were reproducible to within an average deviation of ±3% or less. As the reaction proceeds, the surface area of antacid decreases and the lines become curved. It is impossible to determine simultaneously both the mass transfer coefficient and the reaction rate constant from Eq. 7, so one of these constants must be estimated by another method (14).

Double-film theory (15) represents the mass transfer coefficient as:

$$K = \frac{D}{X} \qquad \text{(Eq. 8)}$$

where D = diffusion coefficient (centimeters^2 second^{-1}), K = mass transfer coefficient (centimeters second^{-1}), and X = thickness of diffusion layer (centimeters). The mass transfer coefficient for the hydrogen ion can be determined from Eq. 8 when the diffusion coefficient of hydrochloric acid and the thickness of the diffusion layer at the interface between antacid and bulk liquid are measured. Jost (16) expressed the diffusion layer thickness in the case of a solvent flowing linearly over a dissolving solid by:

$$X = \sqrt{\frac{\eta\xi}{d\gamma}} \qquad \text{(Eq. 9)}$$

where η = viscosity of the fluid (poises), ξ = linear dimension of the surface of the solid across which the dissolution medium flows (centimeters), γ = velocity of flow (centimeters second^{-1}), and d = density of the medium.

It is difficult to measure ξ in Eq. 9. In this study the thickness of the diffusion layer was estimated by analyzing the rate of powdered salicylic acid dissolution under the same experimental conditions (fluid properties, particle size, agitation, *etc.*) as those used in the acid neutralization study. The Hixson and Crowell (17) cube-root law for dissolution rate was rearranged to give:

$$\frac{d}{dt}\left(\frac{C_L}{C_e}\right) = \frac{DS_wC_e}{X}\left(1 - \frac{C_L}{C_e}\right)^{5/3} \qquad \text{(Eq. 10)}$$

where:

C_L = concentration of solute (moles liter^{-1})
C_e = equilibrium concentration of solute at $t = \infty$ (moles liter^{-1})
S_w = specific surface area given by $6/(\rho D_0)$ (centimeters^2 gram^{-1})
D_0 = mean particle diameter (centimeters)
ρ = true density of particles (grams centimeter^{-3})

The results of the dissolution experiments satisfied Eq. 10, yielding straight lines with slopes of 1.66 for log-log plots of $(d/dt)\ (C_L/C_e)$ against $[1 - (C_L/C_e)]$. The thickness of the diffusion layer was obtained from:

$$X = \frac{DS_wC_e}{(d/dt)(C_L/C_e)_{t=0}} \qquad \text{(Eq. 11)}$$

The relationships between diffusion layer thickness and the experimental conditions are shown in Fig. 4 for both the dispersed and the rotating-disk systems. As might be expected from Eq. 9, the thickness of the diffusion layer decreased with increasing temperature and agitator speed and increased with increasing particle size. Mass transfer coefficients of hydrogen ion were then determined by substituting the diffusion layer thickness, estimated from Fig. 4, and the diffusion coefficient for hydrochloric acid into Eq. 8. Subsequently, the reaction rate constants for acid neutralization were calculated from Eq. 7, using the initial slopes of the plots in Fig. 3. The mass transfer coefficients and the reaction rate constants for acid neutralization are given in Table II.

Determination of Encapsulation Ratio of Spray-Dried Products—A previous study (5), using a scanning electron microscope, showed that spray-dried products are not only agglomerated but that the surfaces of the individual particles in the agglomerates are coated with binder in varying degrees. The proportion of coated surface affects the rate of acid neutralization, and the effective surface area for reaction may be interpreted in terms of an "encapsulation ratio." The effects of spray drying are shown in Fig. 5, where it is seen that the neutralization rates are reduced

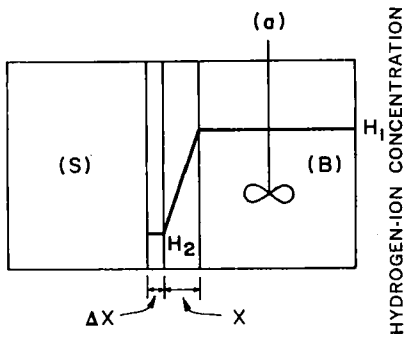

Figure 2—*Double-layer model for acid neutralization. Key: a, stirrer; B, bulk liquid; S, solid; and X and ΔX, diffusion and reaction layer.*

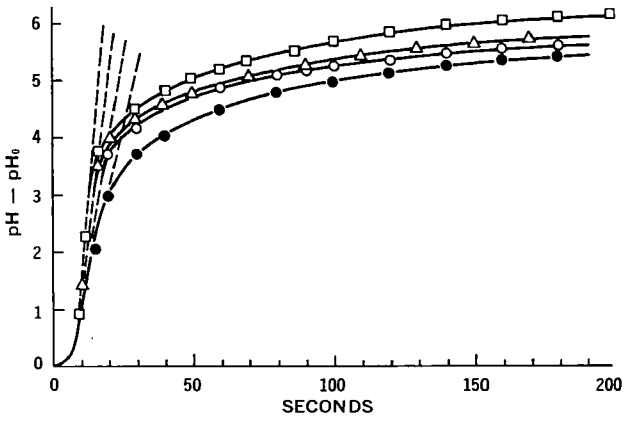

Figure 3—*The pH change* versus *time curves for unagglomerated magnesium carbonate powder at 37°. Key: □, 1640 rpm; △, 1100 rpm; ○, 620 rpm; and ●, 391 rpm.*

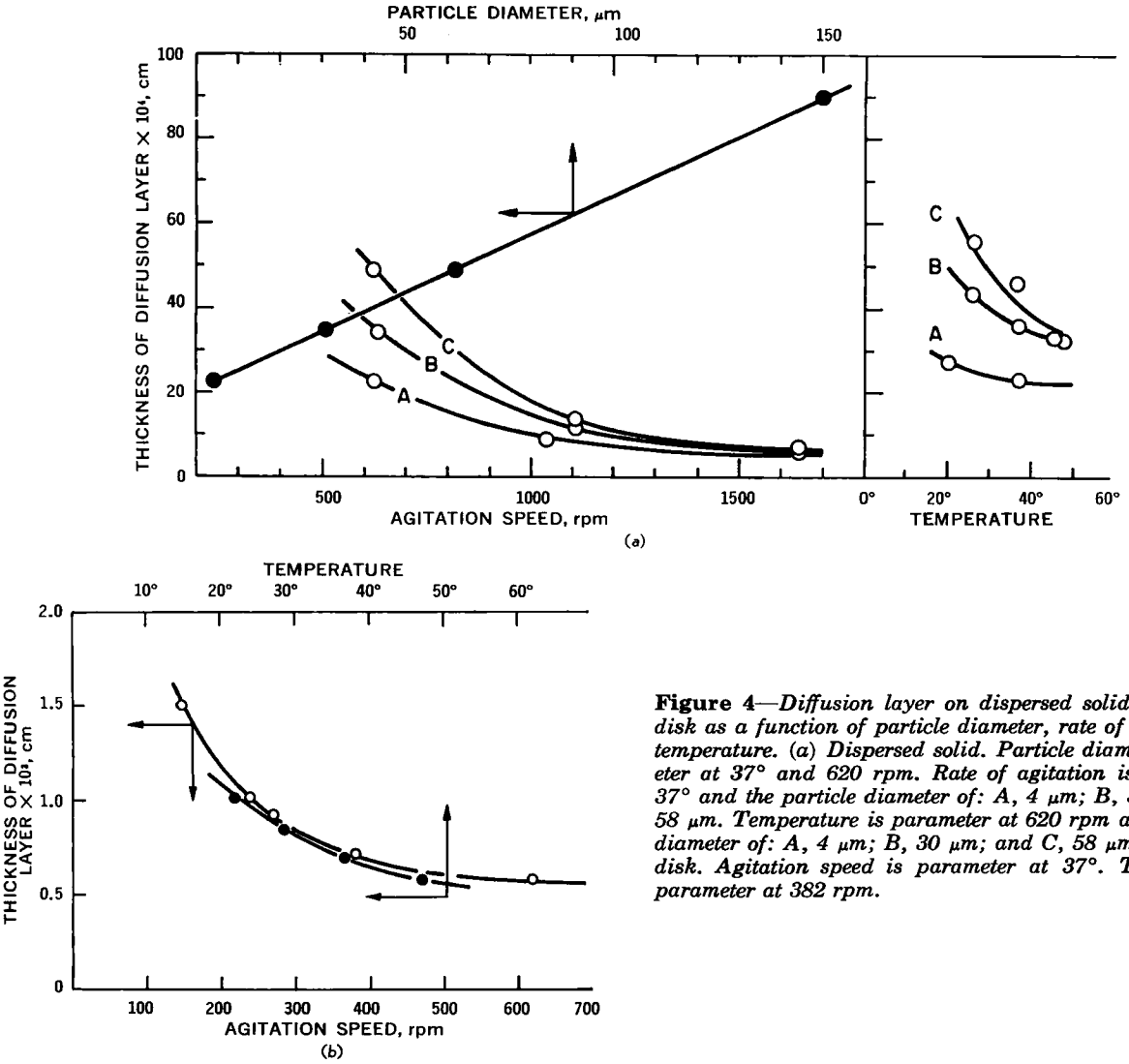

Figure 4—*Diffusion layer on dispersed solid and rotating-disk as a function of particle diameter, rate of agitation, and temperature. (a) Dispersed solid. Particle diameter is parameter at 37° and 620 rpm. Rate of agitation is parameter at 37° and the particle diameter of: A, 4 μm; B, 30 μm; and C, 58 μm. Temperature is parameter at 620 rpm and the particle diameter of: A, 4 μm; B, 30 μm; and C, 58 μm. (b) Rotating disk. Agitation speed is parameter at 37°. Temperature is parameter at 382 rpm.*

compared to those from the untreated magnesium carbonate. In Table III, the encapsulation ratios for the various products are given. The encapsulation ratio is defined by:

$$C_r = \left(1 - \frac{A_c}{A_0}\right) \times 100 \qquad \text{(Eq. 12)}$$

where A_c = effective surface area of particles for acid neutralization (centimeters^2), A_0 = surface area of particles measured by adsorption method (centimeters^2), and C_r = proportion of coated particles (percent).

The effective surface area was calculated by substituting the mass transfer coefficient estimated using Fig. 4 and Table I, the reaction rate constant listed in Table II, and the slope of pH change *versus* time straight line in Fig. 5 into:

$$A_c = \frac{2.303V \tan \alpha k_2}{k_1(k_2 - 2.303V \tan \alpha)} \qquad \text{(Eq. 13)}$$

Table III*a* shows that encapsulation was most effective with carboxymethylcellulose, gelatin, methylcellulose, and polyvinyl alcohol while gum arabic and polyvinylpyrrolidone products appeared to be uncoated; that is, they were only agglomerated.

The acid neutralization of tableted spray-dried products was also investigated using the rotating-disk method. The pH change *versus* time curves by this method at 37° and 382 rpm are shown in Fig. 6. As expected, the rate of pH change for the tablet was much slower than for the powder. Tablets of unagglomerated magnesium carbonate and polyvinylpyrrolidone products showed a similar pH profile. Disintegration of both tablets occurred after 1.5–~2.0 min of residence time. After the disintegration, pH change *versus* time curves were similar to those for the dispersed powder system. Acid neutralization velocities for gelatin and gum arabic products were slower than for unagglomerated and polyvinylpyrrolidone products. Methylcellulose and carboxymethylcellulose products showed a characteristic timed-release action and did not neutralize the acid completely after a day. These tablets did not disintegrate, and

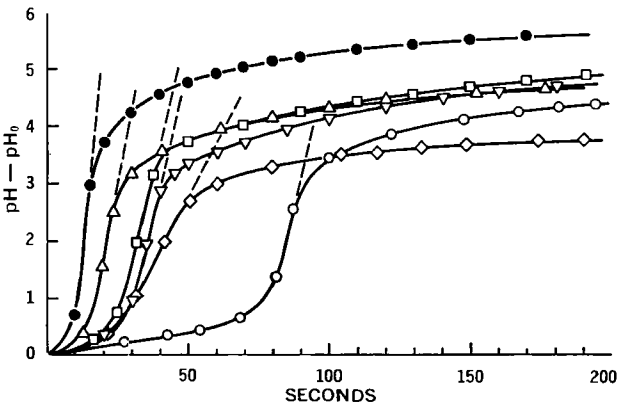

Figure 5—*The pH change versus time curves for powdered spray-dried magnesium carbonate. Key: ●, unagglomerated; △, gelatin; □, gum arabic; ◇, carboxymethylcellulose; ▽, polyvinyl alcohol; and ○, methylcellulose.*

Table III—Encapsulation Ratio of Spray-Dried Products of Magnesium Carbonate

Sample	Experimental Condition	$S_w \times 10^{-4}$ [a]	$A_0 \times 10^{-4}$ [a]	$A_c \times 10^{-4}$ [a]	C_{rd} [a]
(a) Encapsulation Ratios for Powdered Products					
Carboxymethylcellulose, 1% solution	27°, 620 rpm	21.1	10.70	1.75	
Carboxymethylcellulose, 1% solution	37°, 630 rpm	21.1	10.55	2.07	av. 82.8
Carboxymethylcellulose, 1% solution	47°, 620 rpm	21.1	10.62	1.66	
Gelatin, 1% solution		13.7	6.82	2.75	64.8
Gelatin, 3% solution		15.2	7.61	1.69	77.7
Gum arabic, 1% solution		18.4	9.23	9.25	0
Gum arabic, 3% solution		17.9	8.90	3.52	60.5 [b]
Methylcellulose, 1% solution		18.1	9.09	4.49	50.6
Polyvinylpyrrolidone, 0.5% solution		16.5	8.27	8.26	0
Polyvinyl alcohol, 0.5% solution		17.2	8.63	4.49	47.9

Sample	Experimental Condition	A_0	A_c	C_{rt} [a]
(b) Encapsulation Ratios for Tableted Products				
Unagglomerated	37°, 150 rpm		28.6	0.0
Unagglomerated	37°, 241 rpm	26.5 av.	29.7	0.0
Unagglomerated	37°, 382 rpm		22.2	0.0
Unagglomerated	27°, 328 rpm		25.5	0.0
Carboxymethylcellulose, 1% solution		26.5	0.604	97.7
Gelatin, 1% solution		26.5	7.10	73.2
Gelatin, 3% solution		26.5	1.48	94.4
Gum arabic, 1% solution		26.5	26.3	0 [c]
Gum arabic, 3% solution		26.5	1.77	93.3
Methylcellulose, 1% solution		26.5	1.50	94.3
Polyvinylpyrrolidone, 0.5% solution		26.5	27.7	0
Polyvinyl alcohol, 0.5% solution		26.5	7.79	70.6

[a] S_w = specific surface area (centimeters² gram^{-1}); A_0 = actual surface area (centimeters²); A_c = effective surface area for acid-neutralizing reaction (centimeters²); C_{rd} and C_{rt} = encapsulation ratio for powdered and tableted magnesium carbonate, respectively (percent). [b] Value is the average of three experiments at 27, 37, and 47° and 620 rpm. [c] Value is the average of three experiments at 150, 241, and 382 rpm and 37°.

their surfaces dissolved uniformly. The actual surface area of the tablet exposed to the acid solution was found to be 26.5 cm² by substituting experimental results of acid neutralization of unagglomerated magnesium tablet at various temperatures and agitations into Eq. 13. Encapsulation ratios for various kinds of tableted spray-dried products were determined in the same way as for powdered products and resulted in 70–98% (Table III*b*). Comparison of the encapsulation percent of tablets with that of powders (Table III) showed a distinct increase in the encapsulation percent after tableting, especially for the methylcellulose product.

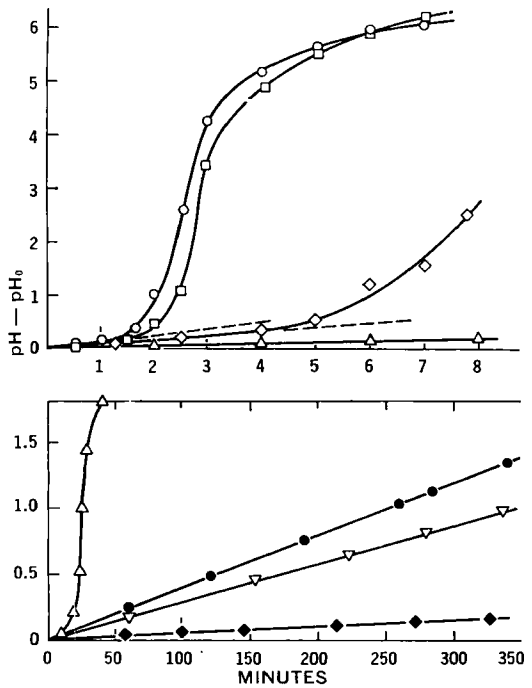

Figure 6—*The pH change versus time curves for tableted spray-dried magnesium carbonate. Key:* □, *unagglomerated;* △, *gelatin;* ▽, *methylcellulose;* ○, *polyvinylpyrrolidone;* ●, *polyvinyl alcohol;* ◇, *gum arabic; and* ◆, *carboxymethylcellulose.*

CONCLUSIONS

Timed-release behavior may be imparted to fine powders by the use of spray-drying agglomeration. In this study, using magnesium carbonate as a model system together with various binders, carboxymethylcellulose, methylcellulose, gelatin, and polyvinyl alcohol were the most effective in reducing the acid-neutralizing rate of the carbonate. Suitable binders apparently not only cause particle agglomeration but also encapsulate the individual particles.

Tableting of spray-dried products increased timed-release properties even further. Applied compression results in closer particle packing and filling of the pores with crushed particles and binder, leading to slower release of the reactive ingredient. Medicaments with release rates arbitrarily controlled over a wide range may thus be prepared using suitable combinations of spray-dried products.

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[x] To whom inquiries should be directed. Present address: Division of Chemistry, National Research Council of Canada, Ottawa K1A OR9, Canada.

Excretion of Probenecid and Its Metabolites in Bile and Urine of Rats

WALTER D. CONWAY [x] and SRIKUMARAN MELETHIL *

Abstract □ The metabolites of probenecid excreted in the bile of rats with and without ligation of the renal pedicles were investigated using a new GLC assay procedure. Within 8 hr after administration, 63.8% of a 40-mg/kg iv dose of probenecid was accounted for in the bile of normal, anesthetized rats. The metabolites found (as percent of dose) include probenecid (10.0%), probenecid glucuronide (15.7%), glucuronide of the *N*-2-hydroxypropyl derivative (20.3%), glucuronide of the *N*-3-hydroxypropyl derivative (14.2%), and the unconjugated *N*-2-carboxyethyl derivative (3.6%). Ligation of the renal pedicles increased the excretion of each metabolite, raising the total recovery to 86.6%. These oxidized metabolites, but not probenecid or its glucuronide, were excreted in urine in unconjugated form (3–5% each). The unconjugated mono-*N*-depropylated metabolite accounted for 11.2% of the dose in the urine of normal, unanesthetized rats but was not found in the bile of the anesthetized animals.

Keyphrases □ Probenecid—metabolites, excretion in bile and urine of rats with and without renal pedicle ligation, GLC analysis □ Metabolism—probenecid, metabolites excreted in bile and urine of rats with and without renal pedicle ligation, GLC analysis □ GLC—analysis, probenecid metabolites, rats

The metabolic fate of probenecid, which was introduced as a uricosuric agent in 1950, has only recently been elucidated in humans and rats (1, 2). One major metabolite in rat bile was suggested to be the ether glucuronide of *p*-(*N*-propyl-*N*-2-hydroxypropylsulfamoyl)benzoic acid (2). In contrast, a more recent study (3) claimed the major metabolite to be the acyl glucuronide of probenecid. The later conclusion was based on the observation that the peak for probenecid was the only one readily detected by GC of the aglycones released by enzymatic hydrolysis of bile samples from rats given probenecid, 40 mg/kg iv. In the first study (2), the renal pedicles of the animals were ligated; in the later study (3), such ligation was not indicated.

The lack of agreement on the relative amounts of the various metabolites excreted probably arises from the need for a reliable quantitative assay for probenecid and its metabolites in biological fluids. A quantitative procedure based on GLC of the methyl esters for the determination of probenecid and two of its oxidation products is presented. An assay based on the propyl esters, which can be used for all known metabolites of probenecid, will be more completely described in a subsequent publication. These assays were used to measure the excretion of probenecid and its metabolites in the urine and bile of rats and to study the effect of renal ligation on the disposition of the drug and its metabolites in bile.

EXPERIMENTAL

Instrumentation—A gas chromatograph[1], equipped with a flame-ionization detector and a 2.8-mm × 2-m (0.125-in. × 6-ft) stainless steel column packed with 10% OV-1 on 80–100-mesh Chromosorb W-HP, was used. Operating parameters were: column temperature, 225°; injection port, 280°; nitrogen carrier gas flow rate, 23 ml/min; and sensitivity, 2.5×10^{-11} amp full scale.

Procedure—To determine free metabolites, biological samples (1 ml of urine or 0.2 g of bile plus 0.8 ml of water) in 50-ml centrifuge tubes were acidified with 1 ml of 5 *N* HCl and extracted by shaking for 30 min with 20 ml of methylene dichloride. A 10-ml aliquot of the methylene dichloride extract was evaporated to dryness on a water bath (50°) under a stream of air. All subsequent evaporations were conducted in the same way. The residue was dissolved in 0.5 ml of methanol and treated with 2 ml of an ether solution containing approximately 0.3 mmole of diazomethane. The samples were kept at room temperature for 1 hr, after which excess diazomethane was removed by evaporation. The residue was taken up in approximately 2 ml of ether, and a 25–50-μl aliquot containing 75–150 μg of *N,N*-dibenzylbenzenesulfonamide in methanol was added as an internal standard. The ether was evaporated and the residue was taken up in 0.25–0.50 ml of methylene chloride. Approximately a 1-μl aliquot was injected into the gas chromatograph. The quantity of internal standard and the final volume of methylene chloride were chosen so as to keep the GC response within the established range of linearity. Reproducibility was significantly enhanced by making the injection with a 10-μl syringe first loaded with 1 μl of ether adjacent to the plunger and separated from the sample by about 2 μl of air.

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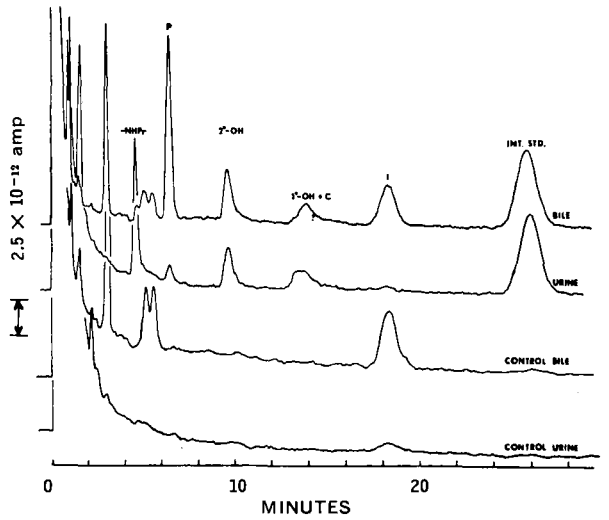

Figure 1—*GLC separation of probenecid metabolites in samples of 0–8-hr urine (1 ml) and bile (0.2 ml) from rats given 40 mg/kg iv of probenecid. See text for GLC operating conditions. Key: mono-N-propyl metabolite, —NHPr; probenecid, P; secondary alcohol, 2°-OH; primary alcohol, 1°-OH; terminal carboxy metabolite, C; impurity, I; and internal standard, int. std., N,N-dibenzylbenzenesulfonamide.*

Total metabolites were determined by heating the acidified biological sample prepared initially for 2 hr at 100° before proceeding with the methylene dichloride extraction. Conjugated metabolites were obtained by difference.

The efficiency of the extraction procedure was determined by assaying 1-ml aliquots of urine, to which were added known quantities of a methanolic solution of probenecid, the secondary alcohol, and the mono-*N*-propyl metabolite.

Standard Curves—Solutions containing approximately 2 mg/ml of authentic samples of probenecid, *p*-(*N*-propyl-*N*-2-hydroxypropylsulfamoyl)benzoic acid (the secondary alcohol metabolite), and *p*-(*N*-propylsulfamoyl)benzoic acid (the mono-*N*-propyl metabolite) were prepared in methanol. Following esterification of aliquots of these solutions (100–500 μl) as described, the dry residue was taken up in about 2 ml of ether and an aliquot of a methanol solution containing 750 μg of *N,N*-dibenzylbenzenesulfonamide was added as an internal standard. The ether solution was evaporated and the residue was taken up in 2.5 ml of methylene dichloride. Approximately 1-μl aliquots were injected into the gas chromatograph. The retention times (minutes) observed were: probenecid, 6.7; the secondary alcohol, 9.8; mono-*N*-propyl metabolite, 4.7; and internal standard, 26.0. A standard with this retention time was chosen to avoid interference from other metabolites and impurities that emerge over the 10–20-min interval (Fig. 1). The impurity emerging at about 18 min resulted from a contaminant in some batches of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide used to prepare the diazomethane.

Peak areas were determined by multiplying the peak height by the width at half height. The latter was measured using a comparator[2] (No. 30,325) fitted with a reticle (No. 30,323) having a 20-mm scale subdivided to 0.1 mm. A plot of the amount ratio (amount of sample per amount of internal standard) *versus* the area ratio (area of sample peak per area of internal standard peak) was linear over an amount ratio range of at least 0–1. Aliquots of a standard solution containing all three compounds were run daily—before, during, and after the series of biological samples being assayed. Each day the average area ratios for this series of standards was then used to calculate the concentrations in biological samples in the series.

Biological Studies—Male, Sprague–Dawley rats, weighing 420–530 g, were anesthetized with urethan (1.3 mg/g body weight ip). The animals were opened with a midline incision and the bile duct was cannulated with PE-10 polyethylene tubing. In some rats the renal pedicles were also ligated. The incision was covered with a gauze sponge which was kept moistened with 0.9% saline solution throughout the experiment. Probenecid was dissolved in a few drops of sodium bicarbonate solution and diluted with distilled water to a concentration of 20 mg/ml. A dose of 40 mg/kg was administered *via* the femoral vein. Bile samples were collected in tared vials at hourly intervals for 6 hr, and a single sample was collected for 6–8 hr. The quantity of bile was determined by weight. In animals in which the renal pedicles were not ligated, the penis was ligated to prevent loss of urine. After 8 hr the urine was withdrawn through the bladder wall with a syringe. The bladder was then rinsed with 1 ml of isotonic saline solution, which was combined with the urine.

To study urinary excretion in unanesthetized animals, male, Sprague-Dawley rats, weighing 300–350 g, were given 40 mg/kg probenecid *via* the femoral vein. The animals were housed individually in metabolism cages, permitting separate collection of urine and feces. Solid food was withheld for the first 8 hr postmedication. Water was given *ad libitum*. A few drops of acetic acid was added to the urine collection vessel, and both urine and feces containers were immersed in ice during collection to minimize hydrolysis of ester glucuronides. Urine was collected at intervals of 0–8, 8–24, 24–48, and 48–72 hr.

Synthetic Procedures—Metabolites of probenecid were prepared from *p*-carboxybenzenesulfonyl chloride (I), which was prepared as described previously (1), and the appropriate secondary amine.

p-(*N-Propyl-N-3-hydroxypropylsulfamoyl)benzoic Acid (II)*—A solution of 2.2 g (19 mmoles) of 3-propylaminopropanol (5) and the quantity of I obtained from 5 g (22 mmoles) of sodium *p*-carboxybenzenesulfonate in 30 ml of acetonitrile was allowed to stand for 18 hr at room temperature. Water (50 ml) was added, the acetonitrile was removed by distillation *in vacuo*, and the product was precipitated by acidification with hydrochloric acid. White crystals (II), 1.72 g, 34% yield, were obtained, mp 140–150°. Three recrystallizations from isopropanol–water (1:2, v/v) raised the melting point to 167–168° [lit. (4) mp 168–169°].

Anal.—Calc. for $C_{13}H_{19}NO_5S$: C, 51.81; H, 6.35; N, 4.65. Found: C, 51.70; H, 6.19; N, 4.51.

p-(*N-Propyl-N-3-propionitrilosulfamoyl)benzoic Acid (III)*—A solution of 2.8 g (24 mmoles) of propylaminopropionitrile (6), the quantity of I obtained from 6 g (27 mmoles) of sodium *p*-carboxybenzenesulfonate, and 15 ml of triethylamine in 35 ml of acetone plus 5 ml of methanol was allowed to stand for 18 hr at room temperature. The reaction mixture was diluted with 50 ml of water and acidified with hydrochloric acid. White crystals (III), 2.58 g, 41% yield, were obtained, mp 208–214°. Recrystallization from isopropanol raised the melting point to 217–217.5° [lit. (4) mp 213–215°]. The product was obtained in similar yield and purity by reacting 27 mmoles of I with 50 mmoles of propylaminopropionitrile in 10 ml of acetone, followed by the same isolation procedure.

p-(*N-Propyl-N-2-carboxyethylsulfamoyl)benzoic Acid (IV)*—Refluxing 1 g (39 mmoles) of the nitrile (III) for 1 hr in a mixture of 25 ml of acetic acid and 50 ml of concentrated hydrochloric acid followed by cooling, dilution with 50 ml of water, filtration, and drying gave 0.95 g (89% yield) of IV, mp 208–210°. Two recrystallizations from isopropanol–water (1:1, v/v) raised the melting point to 209–210.5° [lit. (4) mp 203–204°].

Anal.—Calc. for $C_{13}H_{17}NO_6S$: C, 49.51; H, 5.44; N, 4.44. Found: C, 49.34; H, 5.37; N, 4.33.

RESULTS AND DISCUSSION

Recoveries of probenecid and the mono-*N*-propyl and secondary alcohol metabolites from urine varied from about 85 to 94% for concentrations of 112–390 μg/ml (Table I). Recoveries from bile samples were not significantly different. The reason for lower recoveries from solutions containing 13 μg/ml was not apparent. Virtually none of the biological samples assayed contained less than 112 μg/ml; therefore, the mean recovery values for the solutions containing 112–390 μg/ml were used to correct the experimental results reported later.

A peak with a retention time of about 14 min, which was observed for extracts of bile from medicated but not from control animals (Fig. 1), was initially presumed to represent the methyl ester

[2] Edmund Scientific Co., Barrington, N.J.

Table I—Recoveries of Probenecid and Metabolites from 1 ml of Urine

Amount Added, μg	Percent Recovered[a]		
	Probenecid	Mono-*N*-propyl	Secondary Alcohol
336–390	87.1 ± 2.7	90.7 ± 2.9	94.0 ± 3.7
112–130	84.8 ± 3.3	87.3 ± 4.1	87.6 ± 8.4
11–13	47.3 ± 8.2	54.7 ± 6.5	75.7 ± 14.5

[a] Means of three trials ± standard deviation.

of either the primary alcohol or terminal carboxy metabolite which were reported to be resolved by GLC (2). When authentic samples of these metabolites were later prepared, using the procedures outlined under *Experimental*, their methyl esters were not resolved on the equipment using 10% OV-1 as the stationary phase. On the other hand, resolution of the mono-*N*-propyl metabolite from probenecid was better on 10% OV-1 than on 3% OV-1, 2.5% SE-30, or 3% OV-17. Therefore, composite samples were prepared of the biles from each group of rats and were assayed after conversion of the metabolites to the propyl esters, which were fully resolved by GLC on 10% OV-1. These results are presented in the last two columns of Table II. Details of this procedure using the propyl esters will be given in a later publication.

The excretion of probenecid and four of its metabolites in bile and urine is shown in Table II. Comparison of the results for Groups 1 and 2 illustrates that renal ligation has no significant effect on the qualitative composition of the metabolites excreted in bile. The total amount of each compound excreted in bile, particularly the primary alcohol, is moderately increased by ligation of the renal pedicles. The total of 86.6% of the dose recovered in the bile of rats with the renal pedicles ligated is in reasonable agreement with the 96% recovery obtained, using ^{14}C-labeled probenecid (2). Only probenecid is found in large amount in the unconjugated state in bile. The free terminal carboxy metabolite is also excreted but in much smaller amounts. It could not be ascertained whether the free probenecid was secreted into the bile by the liver in the free state or whether it arose by hydrolysis of some glucuronide conjugate during passage through the biliary network. However, the composition of the collected bile was stable over several weeks when stored at −15°.

The results for biliary excretion of probenecid in the free and conjugated forms are somewhat higher than the amounts of 10.6 and 7.2%, respectively, calculated from the data of Sabih *et al.* (3). Their failure to detect the remaining metabolites may have resulted from the insensitivity of their assay, which was not validated with authentic samples of these compounds.

The present results support the conclusion that, at a dose of 40 mg/kg, the major metabolites excreted in rat bile are the glucuronic acid conjugates of the alcohols formed by hydroxylation of an *N*-propyl group along with somewhat lesser amounts of both free and conjugated probenecid.

Biliary excretion appears to be the major pathway for excretion of probenecid and its metabolites in rats. Small amounts of the unconjugated alcohols, but very little conjugated material, are excreted in urine. Some metabolites excreted in urine may arise as a result of an enterohepatic circulation. This is in agreement with the finding of less than 5% of the administered dose excreted in the urine of the animals of Group 1, although the low excretion in this case might also be due to impairment of renal function as a result of anesthesia.

The unconjugated mono-*N*-propyl metabolite is excreted in significant amounts in urine, while only traces in either free or conjugated form are found in bile. Therefore, while it is advantageous to use renal-ligated rats to enhance biliary excretion and to provide a concentrated sample for isolation and identification of metabolites (2), it is important that urine also be collected to determine the complete disposition of the drug, since there may be large quantitative differences in the composition of the two fluids resulting from limited excretion of certain metabolites by one of the pathways.

This study was done with the relatively high dose of 40 mg/kg of probenecid to permit direct comparison with previously published studies (2, 3). However, the sensitive GLC assay presented should

Table II—Excretion of Probenecid and Its Metabolites in Urine and Bile of Rats after Intravenous Administration of a 40-mg/kg Dose

Animal Preparation				Amount Excreted in 8 hr, % of Dose[a]										
				Probenecid			Secondary Alcohol			Mono-*N*-propyl[b]	Primary Alcohol[c]		Terminal Carboxy[b,c]	All Metabolites
Group	Anesthesia	Renal Ligation	Excretion in	F[d]	G[d]	T[d]	F	G	T	F	F	G	T	F	T
1	Yes	No	Bile	10.0 ± 0.5	15.7 ± 2.2	25.7	—	20.3 ± 0.6	20.3	—[e]	—	14.2	14.2	3.6	63.8
2	Yes	Yes	Bile	12.8 ± 2.8	17.0 ± 3.5	29.8	—	23.1 ± 1.8	23.1	—[e]	—	27.9	27.9	5.8	86.6
3	No	No	Urine	Trace	Trace	Trace	5.4 ± 0.7	—[e]	5.4	11.2 ± 1.3	4.5	—	4.5	3.5	24.6

[a] Mean ± standard error ($s/\sqrt{n}$) for three animals based on assay of the methyl esters except as noted in c. [b] None excreted as the glucuronide conjugate. [c] Based on a single assay of a composite sample of all three animals as the propyl esters. [d] F = free compound; G = conjugate with glucuronic acid; T = F + G. [e] Not detectable.

also be applicable to urine and plasma samples obtained after administration of conventional doses of the drug.

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* Present address: College of Pharmacy and Allied Health Professions, Boston, MA 02115

ˣ To whom inquiries should be directed.

Quantitative GLC Determination of Cyclophosphamide and Isophosphamide in Biological Specimens

C. PANTAROTTO, A. BOSSI, G. BELVEDERE, A. MARTINI, M. G. DONELLI, and A. FRIGERIOˣ

Abstract □ A method for measuring the antitumor agent cyclophosphamide was developed and applied to the determination of the drug in biological specimens. After extraction with ether, cyclophosphamide and the internal standard isophosphamide are converted to their trifluoroacetyl derivatives and assayed by GLC, using either an electron-capture or a flame-ionization detector. The minimum detectable amount is 25 pg/injection using the electron-capture detector. Linearity was found up to microgram amounts of substance, without any interference of endogenous substrates. Values from serum, urine, and liver in mice treated with a single dose of cyclophosphamide (85 mg/kg) are also reported.

Keyphrases □ Cyclophosphamide—quantitative GLC determination in biological specimens □ Isophosphamide—quantitative GLC determination in biological specimens □ GLC—cyclophosphamide and isophosphamide, quantitative determination in biological specimens

Cyclophosphamide {2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide} (I) is a well-known anticancer drug utilized both in animal studies and in clinical practice (1–3).

Colorimetric, titrimetric, and IR methods have been used for estimating I (4–8), although they suffer from a lack of sensitivity and specificity, particularly when applied to biological specimens. Labeled I has been also utilized, but the results require validation for specificity because I undergoes a complicated metabolic pathway (9–12).

More recently, a GLC method for measuring I was described (13), but its usefulness in quantitative determination was limited by the presence of decomposition products. Compound I was also measured by direct injection into the ion source of a mass spectrometer, but the specificity of the determination was not accompanied by sufficient sensitivity (14).

The method described here does not suffer from some of these disadvantages. It is based on the formation of a stable trifluoroacetyl derivative of I suitable for GLC analysis of biological specimens (Scheme I). Isophosphamide [3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazaphosphorine-2-oxide] (II), an analog of I with similar antitumor activity (15–17), also forms a stable trifluoroacetyl derivative (Scheme I) and was chosen as the internal standard for quantitation of I.

EXPERIMENTAL

Standards and Reagents—The following reagents were used: trifluoroacetic anhydride[1], sodium hydroxide, ether, and ethyl acetate[2].

Cyclophosphamide[3] and isophosphamide[3] were used as the hydrate salts, with all concentrations expressed in terms of the free base. Drugs were dissolved in double-distilled water; methylaminochlorobenzophenone[4] (internal marker) was dissolved in ethyl acetate.

GLC—GLC was carried out on a gas chromatograph[5] equipped with a flame-ionization detector[6] or a ^{63}Ni electron-capture detec-

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[2] Carlo Erba, Milan, Italy.
[3] Supplied by Asta Werke AG, Brackwede, West Germany.
[4] Supplied by Ravizza, Milan, Italy.
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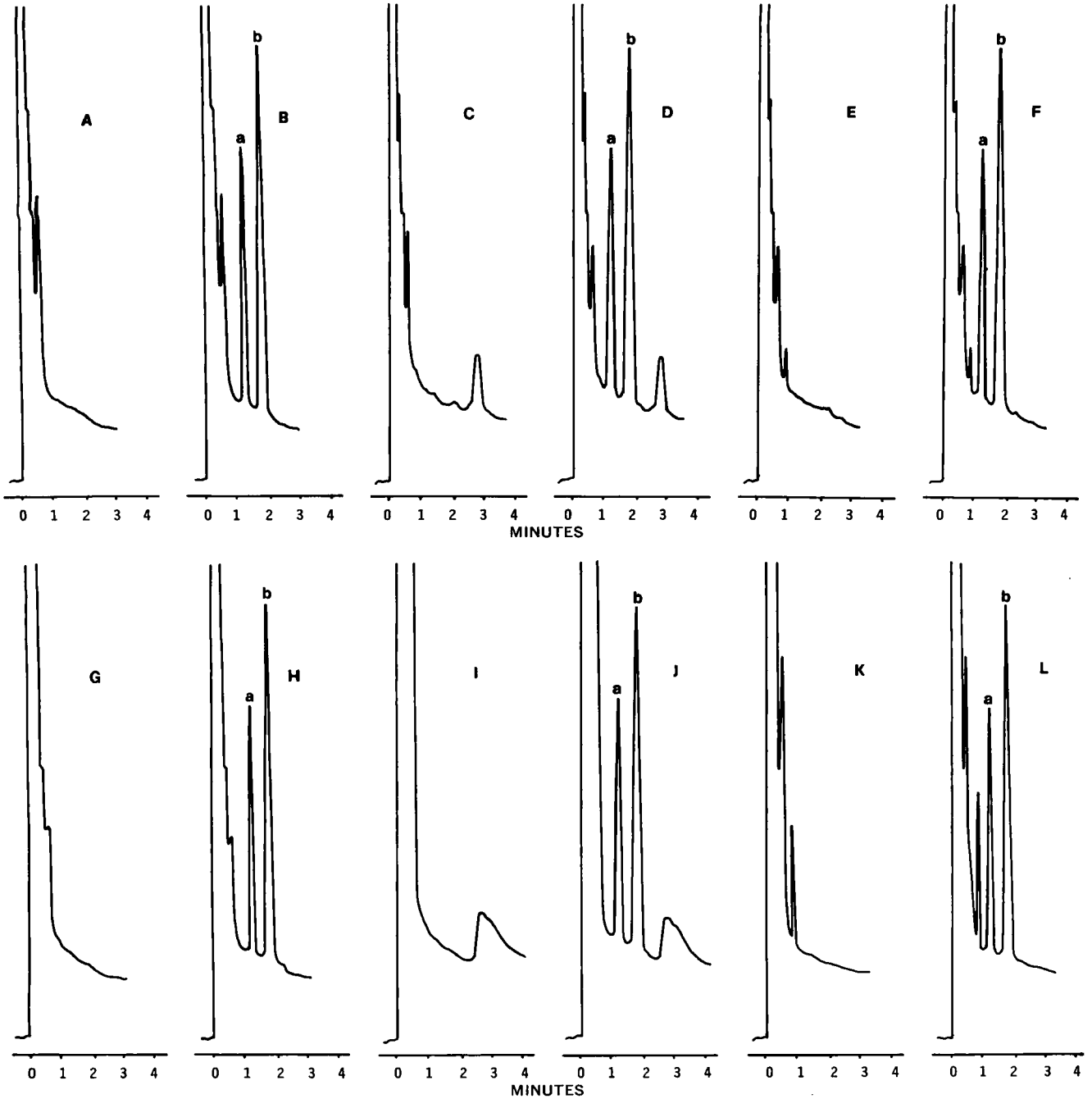


Figure 1—*Gas chromatograms of extracts by using an electron-capture detector: A, serum blank; B, I (peak a) and II (peak b) extracted from serum; C, liver blank; D, I (peak a) and II (peak b) extracted from liver; E, urine blank; and F, I (peak a) and II (peak b) extracted from urine. Gas chromatograms of extracts by using a flame-ionization detector: G, serum blank; H, I (peak a) and II (peak b) extracted from serum; I, liver blank; J, I (peak a) and II (peak b) extracted from liver; K, urine blank; and L, I (peak a) and II (peak b) extracted from urine. All other peaks present in the chromatograms are due to endogenous substances present in the final residue.*

tor[7]. In both cases the column was a glass tube, 1.5 m long and 4 mm i.d., packed with 100–120-mesh Chromosorb Q coated with 3% SE-30[8].

All newly prepared columns were conditioned at 280° for 1 hr without carrier gas flow and then for 12 hr with a carrier gas flow rate of 15 ml/min. During analysis, nitrogen was used as the carrier gas at a flow rate of 35 ml/min for both electron-capture and flame-ionization detection. Air and hydrogen flow rates were adjusted to give maximum response when operating with the flame-ionization detector. The operating conditions were: column oven temperature, 200°; injection port heater temperature, 250°; flame-ionization detector temperature, 250°; and electron-capture detector temperature, 280°. The electron-capture detector was used under the following conditions: pulse current; excitation voltage, 10 v; pulse width, 3 μsec; period, 30 μsec; and scavenger gas (nitrogen), 35 ml/min.

Mass Spectrometry—A mass spectrometer[9] coupled with a gas chromatograph was used under the following conditions: energy of the ionization beam, 70 ev; ion source temperature, 290°; acceler-

[7] Carlo Erba.
[8] Applied Science Laboratories, State College, Pa.

[9] LKB model 9000.

Table I—Extraction Recoveries of Cyclophosphamide and Isophosphamide from Water, Serum, Liver, and Urine

Drug	Percent Extraction Recovery from Water	Serum	Liver	Urine
Cyclophosphamide	77.4 ± 0.8	77.0 ± 1.5	78.3 ± 1.2	77.1 ± 1.3
Isophosphamide	76.2 ± 1.3	77.8 ± 1.9	80.7 ± 2.1	80.3 ± 1.8

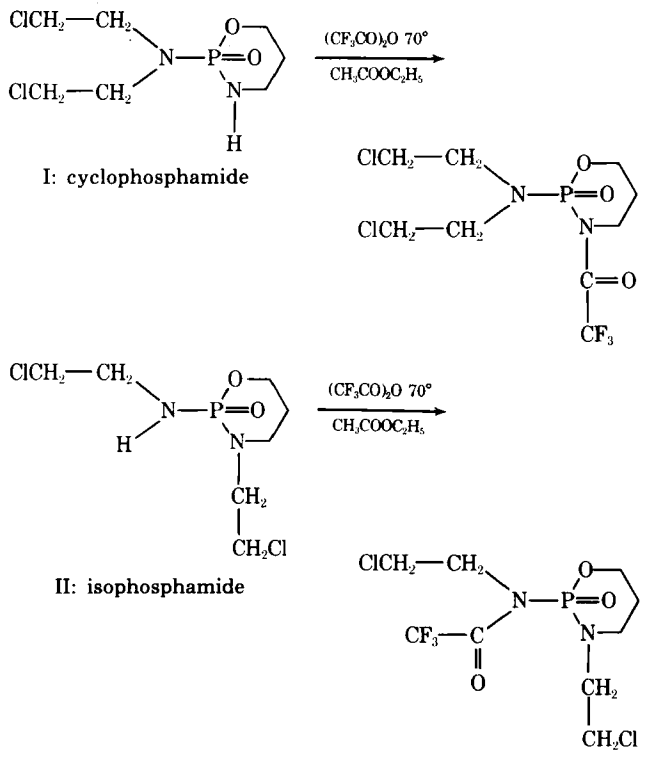
ating voltage, 3.5 kv; and trap current, 60 μmp. The introduction of the sample was carried out by a GLC procedure on a glass column, 1.5 m long and 4 mm i.d., packed with 3% SE-30 on Chromosorb Q (100–120 mesh) under the following conditions: injector temperature, 240°; oven temperature, 200°; helium (carrier gas) flow rate, 35 ml/min; and detector, total ion monitor.

Extraction Procedure from Water, Serum, Liver, and Urine—The procedure was designed to be suitable for the measurement of I in the range of picograms up to micrograms. To 0.1 ml of water, serum, or urine or to 100 mg of mouse liver (after homogenization) were added (depending on the anticipated concentration range for quantitation and the detector utilized) either 100 ng or 5 μg of II (internal standard), 0.9 ml of 0.1 *N* NaOH, and 5 ml of ether. The appropriate amount of internal standard to be added to 0.1 ml of specimen was determined from a preliminary experiment to find a suitable ratio of peak areas with respect to I (Fig. 1).

The test tubes were shaken mechanically for 10 min and, after centrifugation for 5 min at 4°, 4.5 ml of the organic phase was transferred to a second test tube. Then it was dried under a gentle stream of nitrogen at 35° in a water bath. The alkaline aqueous layer was extracted again with another 5 ml of ether and, after shaking and successive centrifugation, 5 ml of the organic phase was transferred into the corresponding test tubes and then dried.

The extraction recoveries of I and II from water, serum, liver, and urine are reported in Table I.

Trifluoroacetylation Reaction—The dry residue was redissolved in 150 μl of a mixture of trifluoroacetic anhydride and ethyl acetate (1:2 v/v), and the stoppered tubes were heated at 70° in a sand bath for 20 min. The samples were dried under nitrogen, 100 μl of ethyl acetate was added, and a 1-μl aliquot was injected into the gas chromatograph. Reaction kinetics of the trifluoroacetyl derivative formation of I and II at room temperature (25°) and at 70° and the stability of the trifluoroacetyl derivatives in relation to time are reported in Fig. 2. Those measurements were performed using methylaminochlorobenzophenone as an internal marker added after the reaction.



Scheme I

Table II—Urinary Excretion of Cyclophosphamide Expressed in Micrograms and Percentage of the Administered Drug

Time[a]	Micrograms ± *SE*	Percent Administered Dose ± *SE*
0–1 hr	114.91 ± 8.54	5.46 ± 0.31
1–4 hr	17.15 ± 9.53	0.81 ± 0.45
4–24 hr	1.98 ± 0.84	0.09 ± 0.04
Total	124.23 ± 7.20	5.91 ± 0.34

[a] Determinations at 1 and 4 hr were performed by using a flame-ionization detector; determinations at 24 hr were performed by using an electron-capture detector.

Biological Measurements of I in Serum, Liver, and Urine—The validity of this procedure for the *in vivo* determination of I was demonstrated by studying the elimination of the drug in mice. Male C57 Bl/6J inbred mice, 25 ± 2 g, were injected with a single dose of 85 mg/kg iv of I, prepared immediately prior to administration by dissolving the compound in double-distilled water. Groups of five animals were sacrificed, and biological samples (serum and liver) were collected at 1, 5, 15, 30, 60, and 120 min after drug injection.

The method of "feathering" was applied for calculating pharmacokinetic parameters (18, 19). Urines were collected from mice housed in glass metabolic cages, and collections were made 1, 4, and 24 hr after I administration.

RESULTS AND DISCUSSION

Figure 1 shows some typical chromatograms of extracts from urine, serum, and liver. No interfering peaks from endogenous substrates were found. The calibration curves for I in serum are illustrated in Fig. 3; the linearity of the method ranges from concentra-

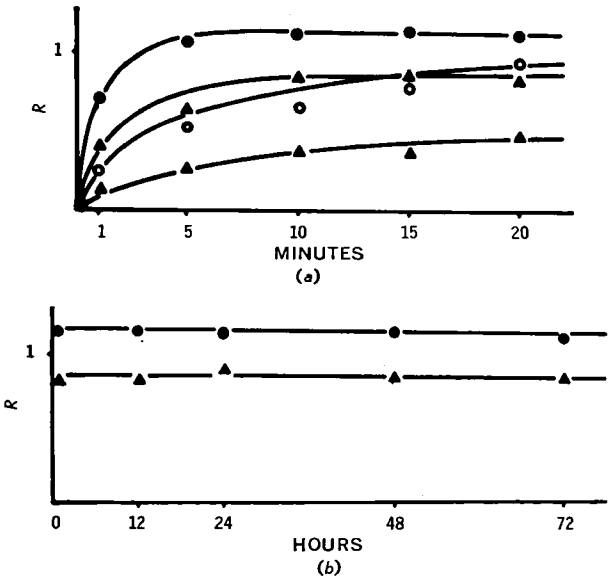


Figure 2—(a) *Reaction kinetics of I and II at room temperature and at 70°.* (b) *Stability of the trifluoroacetyl derivatives.* R = *the peak area ratio of I or II to the internal marker (methylaminochlorobenzophenone). Key:* △, *cyclophosphamide (25°);* ○, *isophosphamide (25°);* ▲, *cyclophosphamide (70°); and* ●, *isophosphamide (70°). These data were obtained from a concentration of 100 ng of I or II. Quantitation was performed using 0.5 μg of methylaminochlorobenzophenone as an internal marker added after the reaction.*

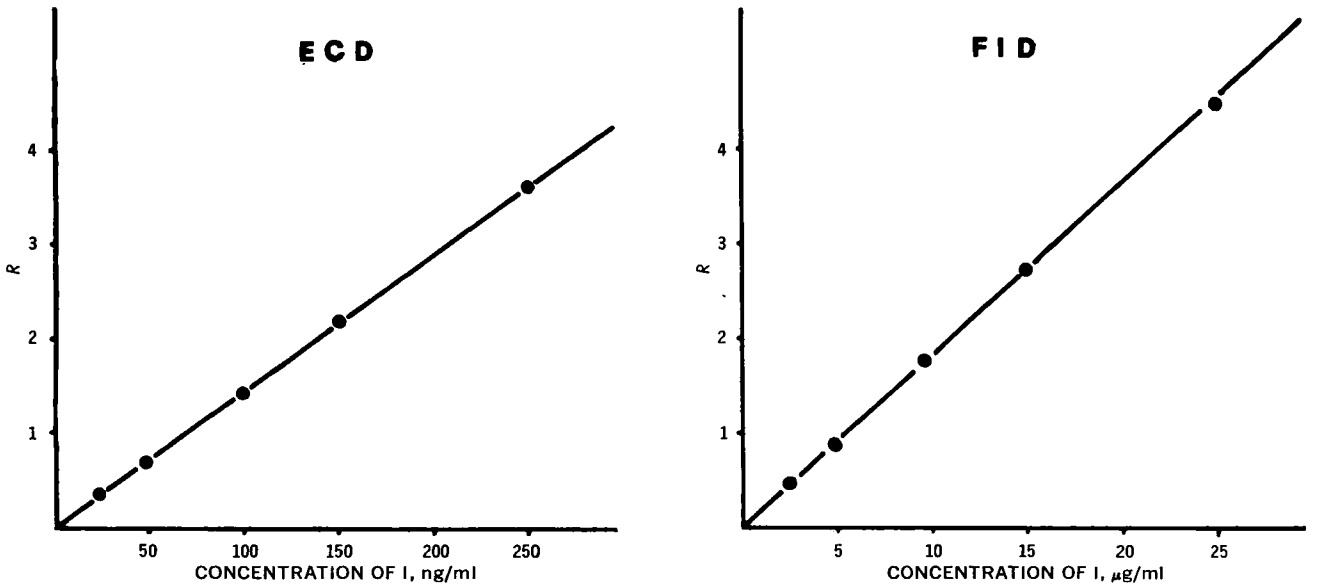


Figure 3—*Standard calibration curves of I to the internal standard II.* R = *the peak area ratio of I to II.*

tions of 25 to 250 ng/ml of specimen with the electron-capture detector and from 2.5 to 25 μg/ml of specimen with the flame-ionization detector.

Calibration curves from urine and liver are not reported because they were identical to those of serum, due to the same recovery after extraction (Table I). All these results are also valid when II is determined by utilizing I as an internal standard.

The chemical identity of the peaks obtained in the GLC analysis of I and II after reaction with trifluoroacetic anhydride was established by means of GLC–mass spectroscopy. The mass spectra (Fig. 4) show molecular ions at *m/e* 356, which means that only one trifluoroacetyl group is present in the molecules.

The results of the distribution in serum and liver of I are reported in Fig. 5. The uptake of the drug by the liver seems to occur very rapidly, since liver levels of the unchanged I are always higher than in serum. Indeed, at 5 min the drug concentration in liver is more than double that in serum. However, the half-life calculated within 30 and 120 min by the regression line of the logarithms of drug concentration *versus* time is not statistically different for serum and liver.

In the urine, I excretion proceeds very rapidly within the 1st hr, but the excretion of the unchanged drug is complete at 24 hr after

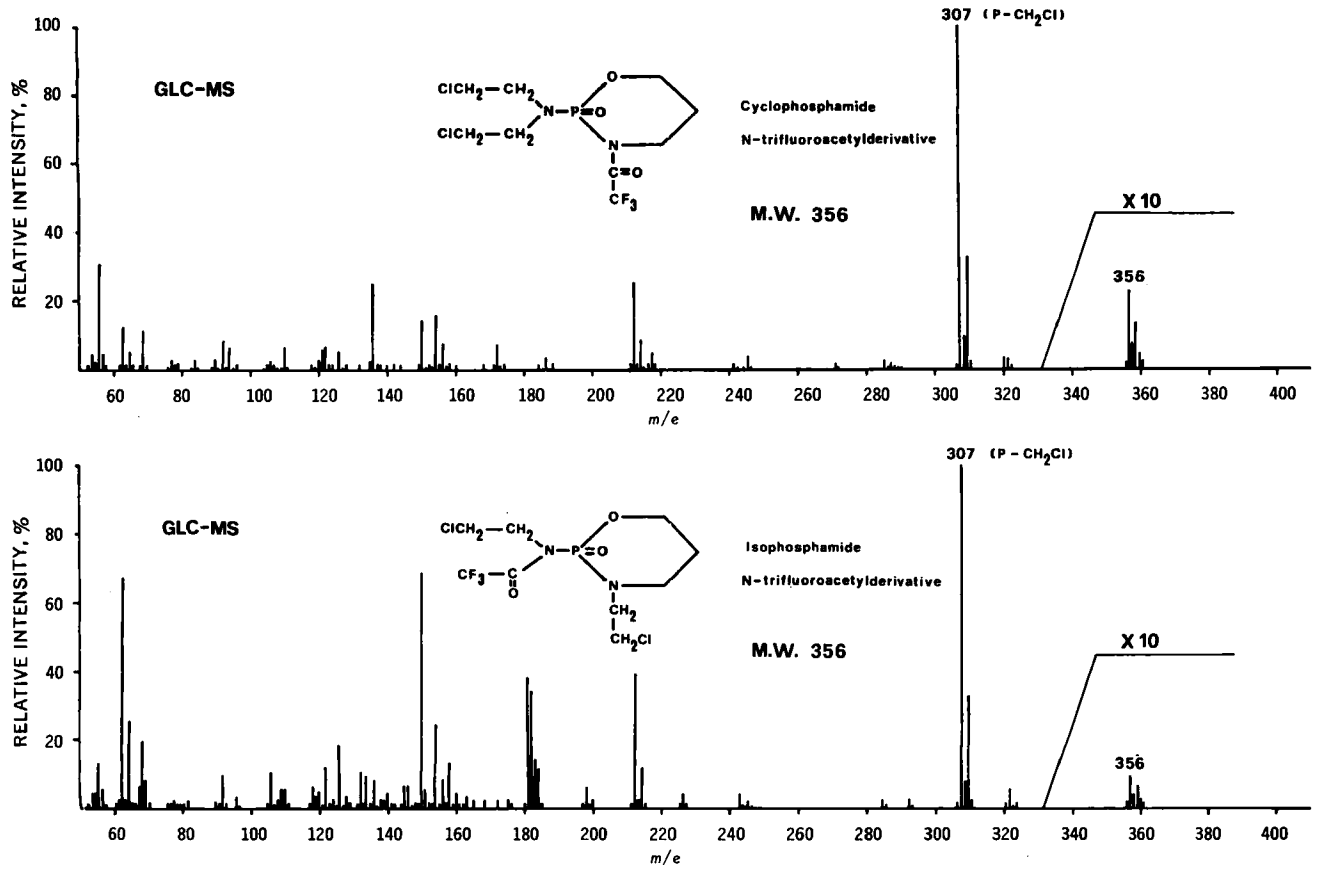


Figure 4—*Mass spectra of the trifluoroacetyl derivatives of I and II obtained by GLC–mass spectroscopy analysis.*

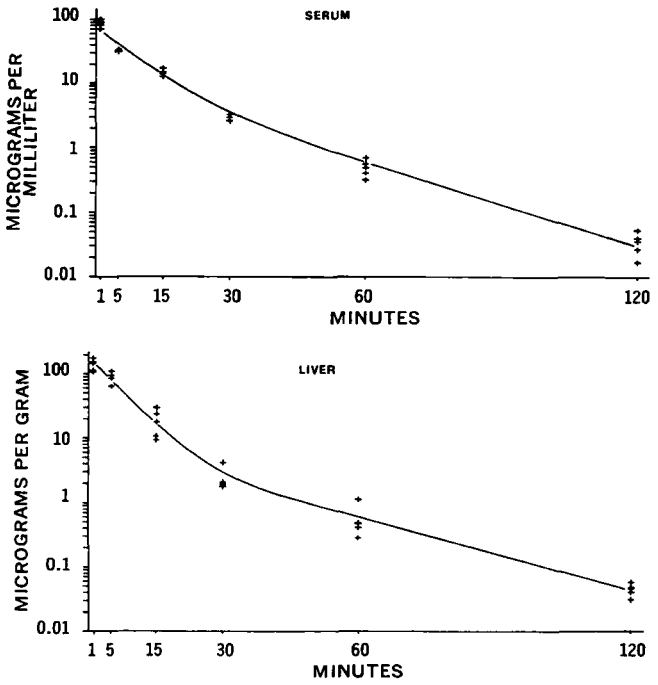


Figure 5—*Levels of I in serum and liver at different times after intravenous injection. Each point is the average of five determinations. Measurements at 1, 5, and 15 min were performed by using a flame-ionization detector, while measurements at 30, 60, and 120 min were performed by using an electron-capture detector. The pharmacokinetic parameters for serum were: half-life β, $t_{1/2}$ (min), 95% confidence limits 13 min 55 sec (12 min 45 sec–15 min 25 sec); volume of distribution,* Vd *(liter/kg), 95% confidence limits 0.076 (0.053–0.111); area under the curve, $\int_0^\infty$ Cp dt (μg/ml × min), 663.07; and total body clearance (liter/min/kg), 0.128. The pharmacokinetic parameters for liver were: half-life β, $t_{1/2}$ (min), 95% confidence limits 15 min 55 sec (14 min 10 sec–18 min 20 sec); and area under the curve, $\int_0^\infty$ Cp dt (μg/ml × min), 1255.31.*

the administration (Table II); a very low percentage (5%) of the unchanged I is excreted in the urine. Experiments are in progress to apply this technique to pharmacokinetic studies of I in tumor-bearing animals.

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[x] To whom inquiries should be directed.

Cholinergic Activity of 2-Azabicyclo[2.2.2]octane Analogs of Acetylcholine

RONALD F. BORNE[x], C. RANDALL CLARK, and I. W. WATERS*

Abstract □ The *cis*- and *trans*-isomers of 5- and 6-acetoxy-2-methyl-2-azabicyclo[2.2.2]octane and the corresponding methiodide salts were synthesized and evaluated for cholinergic activity. The stereochemistry of the intermediate tertiary aminoalcohols is discussed. In the guinea pig ileum assay, all compounds were inactive except two, one of which was an effective competitive antagonist of acetylcholine while the other demonstrated agonist properties.

Keyphrases □ Acetylcholine, 2-azabicyclo[2.2.2]octane analogs—synthesized and screened for cholinergic activity □ 2-Azabicyclo[2.2.2]octane acetylcholine analogs—synthesized and screened for cholinergic activity □ Cholinergic activity—2-azabicyclo-[2.2.2]octane analogs of acetylcholine

In continuing studies on the conformational requirements for drug–receptor interactions, some conformationally restricted analogs of acetylcholine were prepared. The 2-azabicyclo[2.2.2]octane ring system was used to restrict the conformation of the choline portion of the molecule.

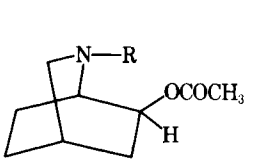
Data compiled on the conformation of acetylcholine both in the crystal form and in water solution point to a synclinal *(sc)* preferred conformation of the N—C—C—O linkage (1–5). Based on these results, Chothia (6) proposed that the nicotinic and muscarinic receptors interact with two different sides of acetylcholine when the transmitter is in a *sc*-conformation. Thus, this approach to the cholinergic receptor is to use the preferred conformation of acetylcholine to explain its action at the nicotinic and muscarinic receptors.

Beers and Reich (7) took a different approach which led to the opposite conclusion. They examined a series of agonist and antagonist molecules, looking for common dimensional features and seeking conformations of acetylcholine that suited these dimensions; they concluded that acetylcholine is present in the antiperiplanar *(ap)* conformation at both receptors. These two receptor hypotheses are cited to point out the still many unanswered questions regarding the active conformation of acetylcholine and the nature of the cholinergic receptor.

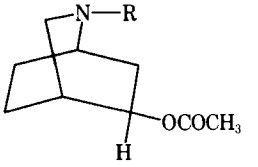
Isomers I-methiodide, II-methiodide, III-methiodide, and IV-methiodide were prepared as conformationally restricted analogs of acetylcholine. Isomers I-methiodide and II-methiodide are related to the *sc*-conformation of acetylcholine, while III-methiodide and IV-methiodide are related to the *ap*-conformation of acetylcholine. Isomers I-methiodide and III-methiodide are ethanolamine analogs, while II-methiodide and IV-methiodide are propanolamine analogs. The maintenance of equivalent stereochemical positions of the pharmacophoric groups in both ethanol and propanolamine derivatives gives this ring system a distinct advantage over other rigid systems used in conformationally restricted analog studies. Isomers I-methiodide and III-methiodide were previously prepared and tested for cholinergic activity (8) and were found to be inactive. However, in rigid analog studies, it is important to compare the relative biological activities within a given series of compounds; thus, these isomers were examined along with II-methiodide and IV-methiodide to complete the study of all isomers of this system.

The work of Cho *et al.* (9) showed some interesting relationships between tertiary amine acetates and their corresponding methiodides. Within a series of compounds in which the tertiary amine derivatives were more effective cholinergics than the methiodide analogs, the basic nitrogen was contained in a ring; in pairs of compounds in which the reverse was true, the nitrogen was attached to an aliphatic chain. These differences have been rationalized in terms of the conformations of the two types of compounds in solution and upon the assumption that a *trans*-conformation of acetylcholine is necessary for muscarinic activity.

The hypothesis of Cho *et al.* (9) was that aliphatic tertiary amines tend to form cyclic conformations *(i.e., sc)* in solution and are, therefore, inactive. Since rigid tertiary amine acetates held in an extended conformation cannot form a cyclic conformation, they are usually more active than their methiodides. Pharmacological evaluation of I, II, III, and IV was conducted to examine these ideas within the 2-azabicyclo[2.2.2]octane ring system. Isomers I and II are fixed in a rigid *cis*-relationship, while III and IV are fixed in a *trans*-relationship to the nitrogen atom. A comparison of the pharmacological activities of these isomers and their methiodides could provide some useful information regarding the nature of drug–receptor interactions at the muscarinic receptors.



I: R = CH_3



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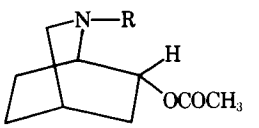
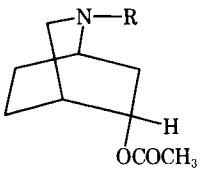
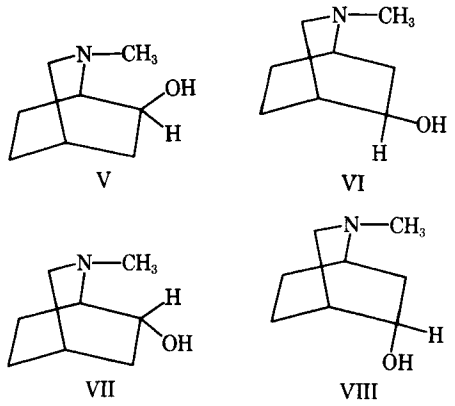


III: R = CH_3



IV: R = CH_3



DISCUSSION

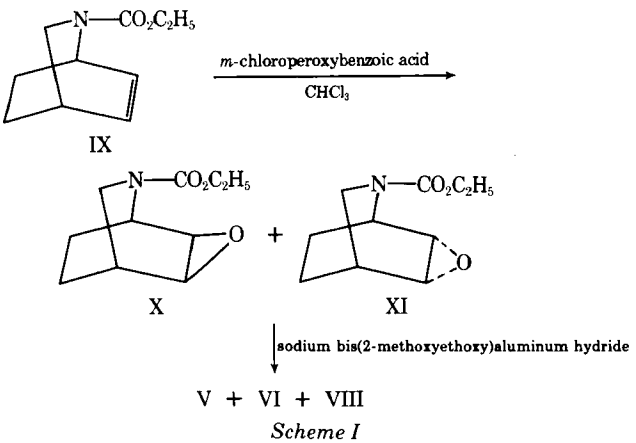
Chemistry—The necessary starting materials in this investigation were the four isomeric aminoalcohols V, VI, VII, and VIII. The stereospecific synthesis of VII, previously reported (10), was a critical step in the determination of the relative stereochemistry and the position of the hydroxy group in the four isomers.

The epoxidation of the olefin, IX (Scheme I), gave a 1:1 mixture of epoxides X and XI, which is consistent with previous work (11). Reduction of the epoxide mixture with sodium bis(2-methoxyethoxy)aluminum hydride or lithium aluminum hydride gave isolatable quantities of VI and VIII plus a small amount of V, as shown by GLC analysis of the product mixture. Since VII had been previously prepared and identified (10) as the 6-*trans*-hydroxy isomer, the *trans*-hydroxy isomer isolated in this reaction must be at the 5-position of the bicyclic ring because only hydroxy groups at the 5- or 6-position can result from normal hydride reduction of epoxides X and XI.

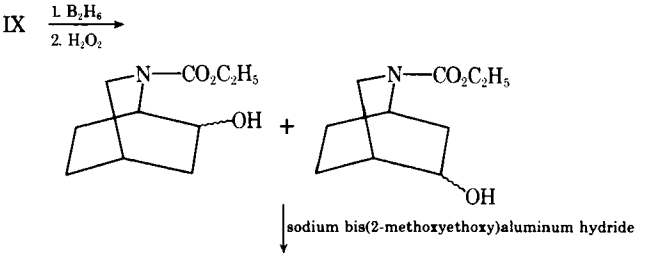
A previous study (10) reported the preparation of the 6-*cis*-hydroxy isomer, V. Therefore, the isolation of a second *cis*-hydroxy isomer from epoxide reduction must be at the 5-position (VI). Compound V was obtained as the major product of the reaction sequence outlined in Scheme II. Isomers VI and VIII can be obtained also *via* the addition of mercuric acetate to IX (12) followed by lithium aluminum hydride reduction of the intermediates.

The proof of the relative stereochemistry of the hydroxy group in these four isomers was accomplished *via* IR spectral analysis of a series of dilute solutions of each isomer. In isomers V and VI the hydroxy groups are *cis* to the ring nitrogen and are capable of intramolecular hydrogen bonding. The hydroxy groups in VII and VIII are *trans* to the nitrogen atom of the ring and intramolecular hydrogen bonding is not possible in these two isomers. The IR spectra of V, VI, VII, and VIII were taken as 1% and 0.01 and 0.002 *M* solutions in carbon tetrachloride. A 0.005 *M* solution is regarded as sufficiently dilute to exclude all intermolecular association (13). The results of the dilution studies and other physical data are shown in Table I.

A 0.002 *M* solution of VII and VIII, each in carbon tetrachloride, showed unassociated hydroxy absorption at 3600–3650 cm^{-1}. The *cis*-alcohols (V and VI) showed some unexpected differences in the



Scheme I



Scheme II

hydroxy region of the IR spectrum. A 0.002 *M* solution of V showed broad hydroxy absorption below 3500 cm^{-1}, indicating intramolecular hydrogen bonding and, therefore, a *cis*-relationship of hydroxy and nitrogen. A solution of VI at 0.002 *M* in carbon tetrachloride showed absorption for both types of hydroxy groups: a peak at 3600–3650 cm^{-1} for free hydroxy and a peak at 3500 cm^{-1} for associated hydroxy.

The unexpected results obtained for VI can be rationalized by comparing the structures of V and VI. Although the bicyclic ring is rigid, a small amount of twisting about the bonds of the ring is possible and this twisting affects V and VI in different ways. The twisting in V produces little change in the intramolecular distance from nitrogen to oxygen as determined from Dreiding models. A distance change of only 0.1 Å was observed for two conformations of V. However, larger distance changes are possible for VI. The interatomic distances can vary from 3.1 to 3.9 Å, a difference of 0.8 Å. It has been shown that the average distance from nitrogen to oxygen in a nitrogen to hydroxy group hydrogen bond is 2.8 ± 0.1 Å (14). The conformation of VI that permits a nitrogen to oxygen distance of 3.1 Å approximates the distance required for intramolecular hydrogen bonding and, thus, the hydroxy absorption at 3500 cm^{-1}. However, the conformation in which the nitrogen to oxygen distance is 3.9 Å does not permit intramolecular hydrogen bonding; thus a peak is observed at 3640 cm^{-1}, indicating free hydroxy absorption.

All cholinergic agents (I–IV and the corresponding methiodides) were prepared by the same method, using a previously stereochemically defined aminoalcohol (V, VI, VII, or VIII). The synthesis of III and III-methiodide as outlined in Scheme III is illustrative of the approach followed. The acetates I–IV were prepared using acetic anhydride and isolated by column chromatography on silica gel. The corresponding methiodides of I–IV were obtained from the acetates using methyl iodide.

Nelson and Wilson (8) noted that the NMR spectra of I-methiodide indicated that the methyl groups attached to nitrogen were not equivalent. They observed two singlets corresponding to the two methyl groups for I-methiodide and a singlet for the two methyl groups in III-methiodide. Since all four of the isomeric acetate methiodides were obtained in the present study, this unexpected observation was investigated. The signal for the two methyl groups attached to nitrogen appeared as a singlet only in III-methiodide. The methyl signals for I-methiodide, II-methiodide, and IV-methiodide appeared as two singlets, one for each of the methyl groups attached to nitrogen. The chemical shift difference ($\Delta\delta$) between the two signals varied with each isomer as shown in Table II.

Nelson and Wilson (8) postulated that the nonequivalent environment was produced by a long-range effect of the carbonyl oxygen of the acetate group. In some preliminary studies on the methiodides of the free alcohols, this nonequivalence of methyl groups also was observed; thus, the carbonyl oxygen is not the only factor involved. The oxygen attached directly to the bicyclic ring appears to contribute greatly to this effect. However, it is difficult

Table I—Physical Properties of V–VIII

Isomer	Boiling Point (20 mm)	Picrate Melting Point	OH_ν cm^{-1} (0.002 *M*)
V	106–110°	259–260°	3450
VI	96–99°	241–243°	3640 and 3500
VII	124–127°	230–231°	3640
VIII	131–133°	264–266°	3640

to determine if this effect is due to shielding or deshielding.

Pharmacology—Compounds I–IV and I-methiodide–IV-methiodide were tested for cholinergic activity as the racemic mixtures on the guinea pig ileum. All tertiary amine acetates tested were inactive at a concentration of 1×10^{-3} *M*. The inactivity of these derivatives is not consistent with the postulation of Cho *et al.* (9). The methiodide salts of the 6-acetoxy derivatives (I and III) were also inactive at 1×10^{-3} *M*. These results are consistent with the previous report of Nelson and Wilson (8) who reported these analogs to be inactive in the rabbit ileum preparation.

Surprising results were obtained with the 5-acetoxy quaternary salts. The methiodide salt of the 5-*cis*-isomer (II) was inactive as a cholinergic agonist but competitively antagonized the effects of acetylcholine. When using the method of Gaddum (15), a pA_2 value of 4.4 was determined for II-methiodide. On the other hand, the methiodide salt of the 5-*trans*-isomer (IV) was found to possess cholinergic agonist activity at a 1×10^{-4} *M* concentration equivalent to the response produced by a 2.1×10^{-6} *M* solution of acetylcholine. Thus, IV-methiodide has an equipotent molar concentration of 47.6 compared to acetylcholine, with an intrinsic activity of 1.0. The action of IV-methiodide was completely blocked in the presence of 1.6 μg/ml of atropine. However, a 1-mg/ml solution of hexamethonium blocked approximately 80% of the contractile effects of IV-methiodide, indicating that IV-methiodide, is acting primarily at parasympathetic ganglia.

It is obvious that the relative configuration of the acetoxy groups in II-methiodide and IV-methiodide has a significant effect on biological activity. Seldom does one observe the large changes in biological activity among conformational isomers represented by II-methiodide and IV-methiodide. In addition to the separation of agonist and antagonist properties in these acetylcholine analogs, it is also somewhat surprising that cholinergic activity is absent in the ethanolamine series (I-methiodide and III-methiodide) but is present to opposite extremes in the propanolamine series. Acetyl-γ-homocholine is much less active than acetylcholine at both parasympathetic ganglia and postganglionic parasympathetic sites of action (16). Examination of Dreiding models of II-methiodide and IV-methiodide indicates that the average interatomic distance from nitrogen to ester oxygen is 3.5 Å in II-methiodide and 4.2 Å in IV-methiodide. The interatomic distance from nitrogen to carbonyl oxygen in IV-methiodide varies from 4.0 to 6.2 Å since the carbonyl group is free to adopt a number of conformations with respect to the ring system.

According to Beers and Reich (7), nicotinic activity is dependent upon a positive charge and a hydrogen acceptor group separated by 5.9 Å while a distance of 4.4 Å between these groups is postulated for muscarinic activity. In IV-methiodide, the ester oxygen to nitrogen distance is similar to that proposed for muscarinic activity and the carbonyl oxygen to nitrogen distance is in agreement with that proposed for nicotinic activity. Only the nitrogen and carbonyl oxygen are capable of assuming these interatomic distances in II-methiodide.

More detailed pharmacological investigations are in progress, particularly with respect to the pharmacological properties exhibited by IV-methiodide. Since the pharmacological results presented here for the methiodide salts of II and IV were conducted on the racemic mixtures, resolution of enantiomers and pharmacological testing of the optical isomers are also being explored.

EXPERIMENTAL[1]

6-*cis*-Hydroxy-2-methyl-2-azabicyclo[2.2.2]octane (V)—To a solution of 2-carbethoxy-2-azabicyclo[2.2.2]octa-5-ene (IX, 28.7 g, 0.15 mole) (17) in 60 ml of tetrahydrofuran was added sodium borohydride (4.2 g, 0.11 mole), followed by the dropwise addition of dimethyl sulfate (13.8 g, 0.11 mole) in 40 ml of tetrahydrofuran. The solution was maintained at 40° for 12 hr and then cooled to 5°. Water (20 ml) was added followed by 32 ml of 3 *N* NaOH and 32 ml of 30% hydrogen peroxide. The aqueous layer was saturated with sodium chloride and the organic layer was separated, washed with saturated sodium chloride (3 × 60 ml), dried over magnesium sulfate, and evaporated to give 25.5 g of a yellow oil.

A solution of 12.0 g of this oil in 75 ml of benzene was added slowly to 80 ml of sodium bis(2-methoxyethoxy)aluminum hydride (0.57 mole), and the resulting mixture was refluxed for 3 hr. Excess hydride was destroyed with ethanol and water, the resulting mixture was filtered, and the filtrate was dried over magnesium sulfate and evaporated. The residue was distilled to give 3.0 g of a clear liquid, bp 106–110° (20 mm); IR (0.002 *M* CCl_4): 3480 cm^{-1} (OH, associated); NMR (CCl_4): δ 1.06–2.7 [broad signals, 12, bicyclic envelope and N—CH_3 (s, 2.4)], 2.9–3.4 (broad signal, 1, H at C_1), and 3.5–4.1 (s over a broad signal, 2, OH and H at C_6). The picrate was prepared in the normal manner, mp 259–261°. The pot residue from distillation was shown by GLC to be a mixture of V and VIII.

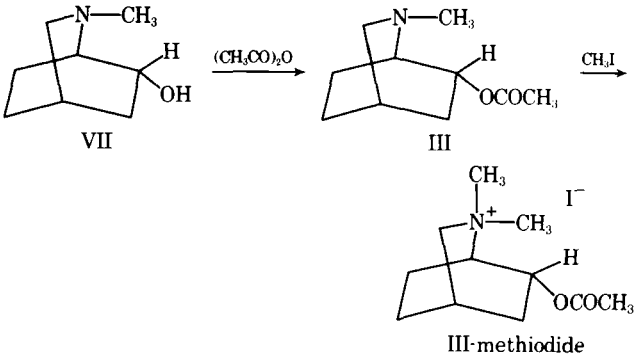
6-*cis*-Acetoxy-2-methyl-2-azabicyclo[2.2.2]octane (I)—A solution of V (1.0 g, 0.007 mole) and 5 ml of acetic anhydride in 20 ml of benzene was stirred at room temperature for 12 hr. Water (50 ml) was added and stirring continued for 1 hr. The solution was made basic with potassium carbonate and extracted with ether. The ether extracts were combined, dried over magnesium sulfate, and evaporated. The resulting oil was chromatographed on 5.0 g of silica gel, with benzene as eluent, to yield the desired ester (0.8 g, 60%) as a clear liquid; IR (CCl_4): 1740 cm^{-1} (C=O); NMR (CCl_4): δ 2.5 (s, 3, N—CH_3) and 2.17 (s, 3, $COCH_3$).

6-*cis*-Acetoxy-2-methyl-2-azabicyclo[2.2.2]octane Methiodide (I-Methiodide)—A solution of I (1.0 g, 0.005 mole) and 5 ml of methyl iodide in 20 ml of dry benzene was stirred at room temperature for 2 hr. The mixture was filtered and the solid was dried and recrystallized from ethanol to give white needles (1.0 g, 62%), mp 165–167°; IR (KBr): 1750 cm^{-1} (C=O); NMR (D_2O): δ 2.17 (s, 3, $COCH_3$), 3.23 (s, 3, N—CH_3), and 3.33 (s, 3, N—CH_3).

Anal.—Calc. for $C_{11}H_{20}INO_2$: C, 40.63; H, 6.20; N, 4.30. Found: C, 40.50; H, 6.25; N, 4.32.

6-*trans*-Acetoxy-2-methyl-2-azabicyclo[2.2.2]octane (III)—A solution of 2.0 g (0.014 mole) of VII, prepared as described previously (10), was treated with 10 ml of acetic anhydride in a manner similar to that described for the synthesis of I. Chromatography of the crude acetate on silica gel gave 1.4 g (55%) of III as a clear liquid; IR (CCl_4): 1740 cm^{-1} (C=O); NMR (CCl_4): δ 2.51 (s, 3, $COCH_3$) and 2.50 (s, 3, N—CH_3).

6-*trans*-Acetoxy-2-methyl-2-azabicyclo[2.2.2]octane Methiodide (III-Methiodide)—A solution of 1.0 g (0.005 mole) of III and 10 ml of methyl iodide in 20 ml of dry benzene was stirred at



VII III III-methiodide

Scheme III

Table II—NMR Data

Isomer	Δδ[a], ppm
I-Methiodide	0.10
II-Methiodide	0.13
III-Methiodide	0.00
IV-Methiodide	0.08

[a] Chemical shift difference between methyl signals.

[1] Melting points were determined on a Thomas-Hoover Uni-Melt apparatus and are corrected. IR spectra were determined with a Perkin-Elmer model 257 or a Beckman IR-33 spectrophotometer. Solution spectra were taken in sodium chloride cells of widths 1.058 mm (reference) and 1.09 mm (sample). Dilution studies were performed using matched sodium chloride cells of widths 10 mm for 0.01 *M* solutions and 25 mm for 0.002 *M* solutions. All NMR spectra were obtained on a Jeolco model C-60-HL spectrometer, and all values are reported in parts per million from tetramethylsilane or sodium 4,4-dimethyl-4-silapentanesulfonate as internal standards. Elemental analyses were performed by Chemalytics, Inc., Tempe, Ariz. GLC was performed on a Varian Aerograph model 600D chromatograph equipped with a flame-ionization detector and a 1.5-m × 0.3-cm (5-ft × 0.125-in.) column containing 3% SE-30 on Varaport 30 (100–120 mesh). Sodium bis(2-methoxyethoxy)aluminum hydride was purchased from Aldrich Chemical Co.

room temperature for 12 hr. The solvent was evaporated and the residue was recrystallized from methanol–ethyl acetate to yield a colorless solid (1.1 g, 68%), mp 165–166° [lit. (8) mp 165–165.5°]; IR (KBr): 1745 cm^{-1} (C=O); NMR (D_2O): δ 2.20 (s, 3, $COCH_3$) and 3.50 (s, 6, N—CH_3).

Anal.—Calc. for $C_{11}H_{20}INO_2$: C, 40.63; H, 6.20; N, 4.30. Found: C, 40.66; H, 6.18; N, 4.21.

5-*cis*-Acetoxy-2-methyl-2-azabicyclo[2.2.2]octane (II)—A solution of 1.5 g (0.01 mole) of VI, prepared as previously described (10), was treated with 5 ml of acetic anhydride in a manner similar to that described for the preparation of I. Chromatography of the crude acetate on silica gel afforded 1.1 g (62%) of the desired ester as a clear liquid; IR (CCl_4): 1745 cm^{-1} (C=O); NMR (CCl_4): δ 2.15 (s, 3, $COCH_3$) and 2.52 (s, 3, N—CH_3).

5-*cis*-Acetoxy-2-methyl-2-azabicyclo[2.2.2]octane Methiodide (II-Methiodide)—A solution of II (1.0 g, 0.005 mole) and 5 ml of methyl iodide in 25 ml of dry benzene was stirred at room temperature for 3 hr. The resulting solid was collected and recrystallized from ethanol to give a white solid (0.5 g, 31%), mp 175–176°; IR (KBr): 1740 cm^{-1} (C=O); NMR (D_2O): δ 2.20 (s, 3, $COCH_3$), 3.30 (s, 3, N—CH_3), and 3.43 (s, 3, N—CH_3).

Anal.—Calc. for $C_{11}H_{20}INO_2$: C, 40.63; H, 6.20; N, 4.30. Found: C, 40.33; H, 6.15; N, 4.52.

5-*trans*-Acetoxy-2-methyl-2-azabicyclo[2.2.2]octane (IV)—A solution of 2.0 g (0.014 mole) of VIII, prepared as previously described (10), was treated with 10 ml of acetic anhydride in a manner similar to that described for the preparation of I. Chromatography of the crude acetate on silica gel gave the desired ester as a clear liquid; IR (CCl_4): 1745 cm^{-1} (C=O); NMR (CCl_4): δ 2.13 (s, 3, $COCH_3$) and 2.41 (s, 3, N—CH_3).

5-*trans*-Acetoxy-2-methyl-2-azabicyclo[2.2.2]octane Methiodide (IV-Methiodide)—A solution of 1.0 g (0.005 mole) of IV and 5 ml of methyl iodide in 25 ml of anhydrous methanol was stirred at room temperature for 6 hr. The solvent was evaporated and the residue was recrystallized from ethanol to give 0.9 g (56%) of IV-methiodide, mp 168–171°; IR (KBr): 1735 cm^{-1} (C=O); NMR (D_2O): δ 2.20 (s, 3, $COCH_3$), 3.24 (s, 3, N—CH_3), and 3.32 (s, 3, N—CH_3).

Anal.—Calc. for $C_{11}H_{20}INO_2$: C, 40.63; H, 6.20; N, 4.30. Found: C, 40.68; H, 6.08; N, 4.13.

Pharmacological Methods—The isolated guinea pig ileum assay (18) was employed to screen the cholinergic effects of I–IV and I-methiodide–IV-methiodide.The terminal ileum from guinea pigs (300–500 g) was cut into 2–3-cm strips and suspended in Tyrode's solution in a 30-ml organ bath. The solution was aerated with 95% oxygen and 5% carbon dioxide at 37°. Contractions of the ileum were recorded on a physiograph[2]. A dose–response curve for acetylcholine was determined with each piece of ileum before assaying the test compounds. Reported drug concentrations represent the final concentration in the organ bath. Compounds producing no contraction at 1×10^{-3} *M* were considered inactive. Evidence that II-methiodide competitively antagonized the effects of acetylcholine was determined by the parallel shift in the dose–response curve toward higher concentrations of acetylcholine in the presence of 1×10^{-4} *M* II-methiodide.

[2] E and M model 4.

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* Department of Pharmacology, School of Pharmacy, University of Mississippi, University, MS 38677

[x] To whom inquiries should be directed.

Stability of Tetrahydrocannabinols I

EDWARD R. GARRETT ˣ and JOSEF TSAU

Abstract □ Δ^9-Tetrahydrocannabinol, as monitored by flame-ionization GLC at various temperatures, degrades by a biphasic semilogarithmic curve with time in acidic aqueous solutions (<1 mg/liter) below pH 4 to GLC-observable products with separate retention times and the degradations are specific hydrogen-ion catalyzed. The products are considered as Δ^8-tetrahydrocannabinol, P_1, P_2, and P_3 and can be observed and isolated by TLC. These products do not appear above pH 4 in the neutral region, and these degradations are primarily first order, are not biphasic, and are pH independent. The half-life of Δ^9-tetrahydrocannabinol is about 15 min at 37° and pH 1, typical stomach conditions. The product P_1 may give rise to cannabinol by the GLC and TLC procedures since the IR, UV, TLC, NMR, and GLC of thin-layer chromatographed P_1 and cannabinol are coincident, but chloroform extracts do not show the higher absorbances expected if the product that forms in solution to give P_1 is cannabinol. The products P_2 and P_3, isolated by TLC, are consistent with the expected properties of Δ^9-hydroxycannabidiol and 9-hydroxycannabinol, respectively, by IR, UV, NMR, and mass spectroscopy. The final amounts of Δ^8-tetrahydrocannabinol, P_1, P_2, and P_3 are in a constant ratio independent of pH below pH 4. Δ^9-Tetrahydrocannabinol, as monitored by flame-ionization GLC, degrades solely by a first-order process to an equilibrium with P_2 and P_3 at acidic pH values and the process is specific hydrogen-ion catalyzed. The equilibrium appears to be independent of pH below pH 4 and is the same when TLC-isolated P_2 or P_3 is used as the starting material. It follows that the acid-catalyzed isolated double-bond migration favors Δ^8-tetrahydrocannabinol over the Δ^9 compound, and it is most probable that the equilibrating P_2 and P_3 are results of water addition to the isolated double bond and ether solvolysis. The product that gives rise to the P_1 retention time that ultimately gives cannabinol is structurally indeterminate at present.

Keyphrases □ Tetrahydrocannabinols—stability, effects of pH, isolation and identification of degradation products, mechanisms, flame-ionization GLC □ Stability—tetrahydrocannabinols, effect of pH, isolation and identification of degradation products, flame-ionization GLC □ GLC—monitoring of tetrahydrocannabinol stability

The pKa', aqueous solubility and anomalous glass-binding properties of Δ^9-tetrahydrocannabinol were published recently (1). In addition, a preliminary study on the aqueous acidic degradation of Δ^9-tetrahydrocannabinol was presented using the developed GLC analytical methods (2).

The quantified kinetics and stability of the tetrahydrocannabinols in aqueous medium as a function of pH need to be known for the development of elegant dosage forms and for insight into the possible chemical transformations and their rates that can occur in biological fluids. This paper presents the quantitative kinetic studies used to determine the rate–pH profiles of Δ^9- and Δ^8-tetrahydrocannabinols. Evidence is also presented for the structures and kinetics of degradation of the major GLC detectable products of tetrahydrocannabinol degradation.

EXPERIMENTAL

Materials—Both Δ^9-tetrahydrocannabinol[1] and Δ^8-tetrahydrocannabinol[2] were used directly or after TLC purification. Various degradation products were obtained by preparative TLC as will be described. Other chemicals were of analytical reagent grade. The concentrated hydrochloric acid was twice preextracted with chloroform. Double-distilled water stored in glass containers was used.

Kinetic Procedures—Appropriate amounts of 2 *N* NaCl were added to prepared solutions of hydrochloric acid, buffer, or sodium hydroxide of known concentrations to adjust the ionic strength to 0.05 except when the inherent ionic strength exceeded 0.05 as with the concentrated hydrochloric acid solutions. An aliquot (0.5 ml) of an ethanolic solution of 1 mg of Δ^9-tetrahydrocannabinol/ml was introduced into 1 liter of solution after it was purged with nitrogen and the erlenmeyer flask was equilibrated to the temperature of the reaction. At various time intervals and after immediate and vigorous shaking, 20 or 25 ml of solution was delivered quickly to a 125-ml separator by a glass dispenser[3]. The reactions in strongly acid solutions were quenched with phosphate buffer solution, which adjusted the pH to 6. The reactions at pH values above 9 were quenched with 2 *N* acetic acid, which adjusted the pH to 5. The aliquots were extracted by vigorous shaking for 2–3 min with 20 ml of chloroform; they were then separated and extracted again with 10 ml of chloroform. The chloroform extracts, combined in a 50-ml conical glass centrifuge tube, were evaporated to dryness below 45° under a stream of nitrogen. Chloroform (0.10 ml) containing 3.0 μg of tetraphenylethylene/ml as an internal standard was added, and an additional 5 ml of chloroform was used to rinse down the inner surfaces of the tube. The chloroform solution again was evaporated to dryness and the residue was reconstituted in 100 μl of chloroform. After vigorous shaking on a vortex shaker, a 0.5- or 1.0-μl aliquot was injected into the chromatograph for analysis by the flame-ionization GLC procedure described previously (2).

The conditions for the gas chromatograph[4] with flame-ionization detection were: column temperature, 240°; detector and injection temperature, 260°; and 3% OV-225 on Gas Chrom Q, 100–120 mesh, for column material. The nitrogen flow rate was 50 ml/min and the hydrogen flow rate was 26 ml/min.

The methods of determining the pH values of the solutions were reported previously (3).

Preparation and Separation of Acidic Degradation Products—Fifty milliliters of an ethanolic solution of 1 mg of Δ^9-tetrahydrocannabinol/ml was added to a gallon glass bottle containing a 20% alcohol–water solution of 0.1 *N* HCl equilibrated at 60°. After 5 days, the mixture was extracted twice with 4 liters of chloroform; the combined extracts were evaporated to about 1 ml, streaked on preparative TLC sheets[5], and developed for 15 cm with cyclohexane–acetone (10:1). The R_f values that corresponded to Δ^8-tetrahydrocannabinol, Product 1 (P_1), Product 2 (P_2), and Product 3 (P_3) were located with a short wavelength UV light. The pertinent strips were eluted with chloroform to remove Δ^8-tetrahydrocannabinol and P_1 and with 20% methanol in chloroform to remove P_2 and P_3, followed by vacuum filtration to obtain the drug samples of the various compounds. The separation between P_2 and P_3 was effected by three repeated TLC developments separated by drying, using cyclohexane–acetone (10:1) with elution from the chromatogram strips with 20% ethanol in chloroform.

[1] S.S.C. lot 61591 furnished by the National Institute of Mental Health, Department of Health, Education, and Welfare, U.S. Public Health Service, Bethesda, MD 20014

[2] S.S.C. lot 61656.

[3] CaLab, California Laboratory Supply, Emoryville, CA 94608

[4] Varian model 2100.

[5] Chrom AR 1000, Mallinckrodt Chemical Co., St. Louis, MO 63160

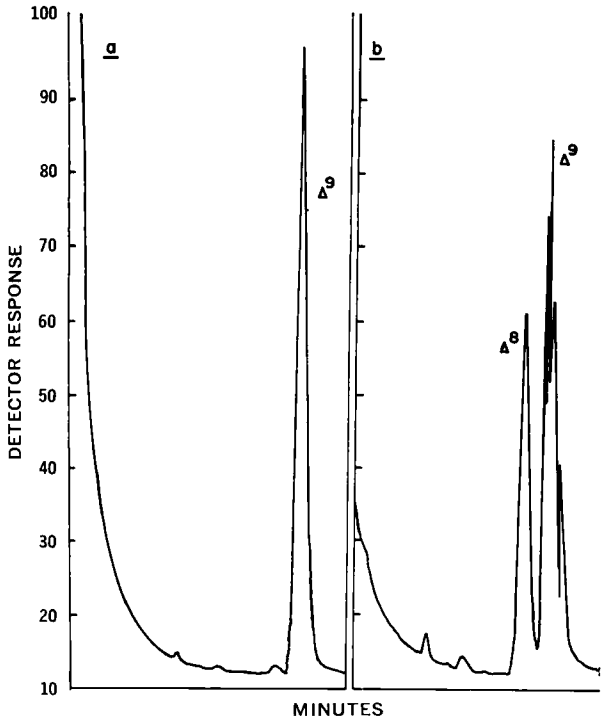


Figure 1—*Gas chromatograms with flame-ionization detection of chloroform-eluted* (a) *lower and* (b) *upper halves of the separated spot with the same* R_f *value as* Δ^9*-tetrahydrocannabinol obtained by TLC of impure* Δ^9*-tetrahydrocannabinol after development with either pure cyclohexane (four repetitive developments to 15 cm separated by drying) or with cyclohexane–acetone (10:1). The impurity of the same retention time as* Δ^8*-tetrahydrocannabinol was observed in the eluted upper half* (b) *of the spot. The segment of* (b) *containing the* Δ^9 *peak was expanded four times in relation to the greater portion of the chromatogram.*

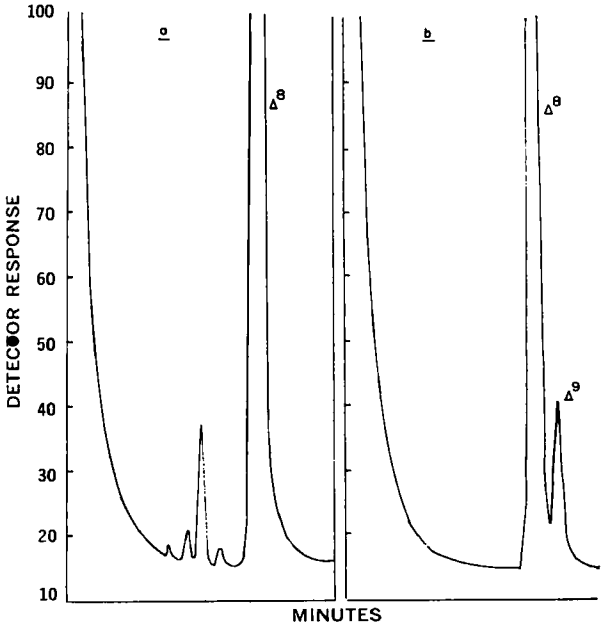


Figure 2—*Gas chromatograms with flame-ionization detection of chloroform-eluted* (a) *upper and* (b) *lower halves of the separated spot with the same* R_f *values as* Δ^8*-tetrahydrocannabinol obtained by TLC of the obtained impure* Δ^8*-tetrahydrocannabinol after development to 15 cm with cyclohexane–acetone (10:1). The impurity of the same retention time as* Δ^9*-tetrahydrocannabinol was observed in the eluted lower half* (b) *of the spot. Further chloroform elution of the lower half of the TLC-developed spot obtained from the chloroform-eluted half spot that gave* (a) *and of the upper half of the TLC spot obtained from the chloroform-eluted half spot that gave* (b) *resulted in chromatographically pure* Δ^8*-tetrahydrocannabinol.*

Separation between large amounts of Δ^8-tetrahydrocannabinol and P_1 was effected by three repeated TLC developments, using cyclohexane–acetone (50:1) with chloroform elution from the chromatogram strips.

Purification of Δ^9- and Δ^8-Tetrahydrocannabinols by TLC—The described TLC method was also used for the purification of Δ^9- and Δ^8-tetrahydrocannabinols. The Δ^9-tetrahydrocannabinol strip obtained from impure material[1] was cut longitudinally in half and the lower half of the strip contained the purified Δ^9-tetrahydrocannabinol extractable by chloroform. The impure Δ^8-tetrahydrocannabinol[2] was also thin layered, and the strip containing it was halved similarly. The strip halves were extracted by chloroform, which was evaporated subsequently at 40° to a small volume (about 100 μl) under a stream of nitrogen. The top half of the strip contained impurities of lower GLC retention times, and the lower half contained an impurity of higher GLC retention time, which most probably was Δ^9-tetrahydrocannabinol. When the condensed chloroform solutions were streaked on a TLC sheet for a second development and the pertinent strips were subsequently halved, purified Δ^8-tetrahydrocannabinol could be extracted from the lower half of the strip obtained from the chloroform extract of the upper half of the strip obtained from the first TLC development and from the upper half of the strip obtained from the chloroform extract of the lower half of the strip obtained from the first TLC development.

Radioactive Monitoring of Tetrahydrocannabinol Degradation—A 0.06 *N* HCl solution (150 ml) was equilibrated at 60.8° in a glass-stoppered 200-ml erlenmeyer flask. Solutions of 2.57×10^6 dpm/ml of ^{14}C-Δ^9-tetrahydrocannabinol (0.40 ml) and 1 mg of purified Δ^9-tetrahydrocannabinol/ml in methanol (0.15 ml) were added. Aliquots (24 ml) were removed at various times and extracted with chloroform, and 1 μl of the final 100 μl of chloroform solution containing the compound and its degradation products from each was assayed by GLC as in the kinetic procedures. The remaining chloroform solution (99 μl out of 100 μl) plus small amounts of chloroform rinses of the container was spotted on a TLC plate[6], which was developed for 15 cm with cyclohexane–acetone (10:1). The radioactivity of the developed plates was quantified by a radiochromatogram scanner[7]. Controls for the system were run with Δ^8- and Δ^9-tetrahydrocannabinols and the degradation products P_1, P_2, and P_3 obtained from the previously described preparative TLC.

Monitoring of UV Spectra of Acid-Degrading Δ^9-Tetrahydrocannabinol—One milliliter of 1 mg of purified Δ^9-tetrahydrocannabinol/ml of ethanol was added to 1 liter of 0.12 *N* HCl maintained at 60.8°. Aliquots (60 ml) were taken after vigorous shaking at 1, 3, 6, 10, 20, 30, 50, 80, and 930 min and twice extracted with 20 ml of chloroform. The combined chloroform extracts were evaporated under nitrogen at 40°, the residue was reconstituted in 2.5 ml of ethanol, and the UV spectrum[8] was monitored. An aliquot (2.0 ml) of this solution was evaporated, 0.1 ml of a chloroform solution of the internal standard tetraphenylethylene was added, and this final solution was analyzed by GLC.

RESULTS AND DISCUSSION

Purification of Δ^9- and Δ^8-Tetrahydrocannabinols by TLC—The Δ^9-tetrahydrocannabinol obtained[1] showed an additional easily distinguishable major peak with the same retention time as Δ^8-tetrahydrocannabinol on flame-ionization GLC. It had 10% of the peak height of the Δ^9 compound. Four other impurities were identifiable by peak separation at 0.5–2% of the peak height of Δ^9-tetrahydrocannabinol (one of the higher peaks had the retention time of cannabinol), and eight others were detectable at the 0.1% level.

TLC (as described under *Experimental*), even with four succes-

[6] Eastman chromagram sheet 6061, Eastman Co., Rochester, N.Y.
[7] Packard model 7201.
[8] Cary model 15, Applied Physics Co., Monrovia, Calif.

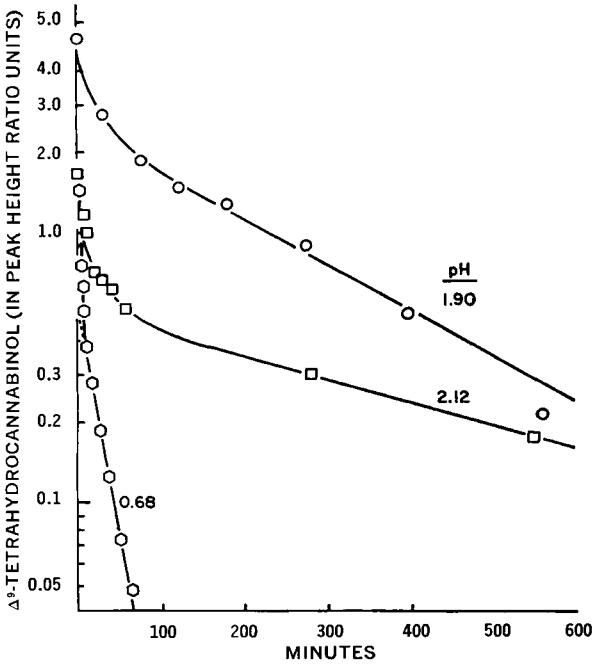


Figure 3—*Semilogarithmic plots of the ratio of the height of the GLC peak for Δ⁹-tetrahydrocannabinol to the peak height of the internal standard tetraphenylethylene against time for the degradation of aqueous solutions (about 1 mg/liter) of purified Δ⁹-tetrahydrocannabinol at 60.8° and at the specified pH values.*

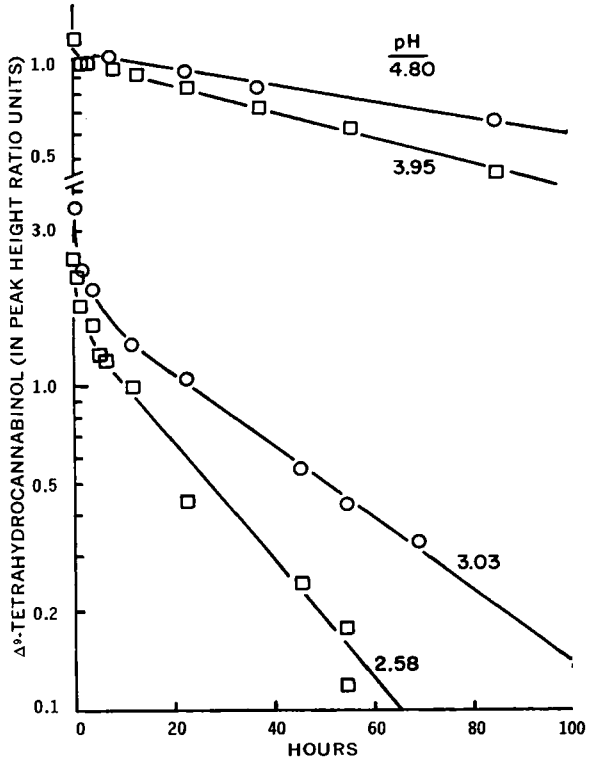


Figure 4—*Semilogarithmic plots of the ratio of the height of the GLC peak for Δ⁹-tetrahydrocannabinol to the peak height of the internal standard tetraphenylethylene against time for the degradation of aqueous solutions (about 1 mg/liter) of purified Δ⁹-tetrahydrocannabinol at 60.8° and at the specified pH values.*

sive developments to 15 cm separated by drying which completely separated the Δ^8- and Δ^9-tetrahydrocannabinol spots, was unable to produce a Δ^9 spot uncontaminated by Δ^8. When this spot was eluted with chloroform and assayed by GLC, a peak with the retention time of Δ^8-tetrahydrocannabinol was still observed. When eluted with chloroform, the lower half of the spot gave a minimal amount of impurity (Fig. 1*a*) whereas the upper half retained it on flame-ionization GLC (Fig. 1*b*).

The Δ^8-tetrahydrocannabinol obtained[2] was even more impure on flame-ionization GLC. Elution of the halves of the TLC spot with the same R_f value as Δ^8-tetrahydrocannabinol showed, by GLC analysis, that the impurities in each half differed. The upper half had the impurities of a smaller retention time (Fig. 2*a*) and the lower half had the impurities of a higher retention time of Δ^9-tetrahydrocannabinol (Fig. 2*b*). A second TLC development of each chloroform-eluted half, with subsequent chloroform elution of the pertinent halves, gave pure Δ^8-tetrahydrocannabinol by GLC.

Kinetics of Δ⁹-Tetrahydrocannabinol Disappearance in Aqueous Solution—The kinetics for nitrogen-purged solutions of purified Δ^9-tetrahydrocannabinol at concentrations below its solubility, *i.e.*, <1 mg/liter, were followed at various pH values from the chloroform extracts of the degrading solution by monitoring the decrease of the ratio of the peak height at the Δ^9 retention time (Fig. 1*a*) obtained with the gas chromatograph using flame-ionization detection (1, 2) to the peak height of the internal standard tetraphenylethylene as a function of time at several constant temperatures. The calibration curves of the peak height ratio against concentration of Δ^9- and Δ^8-tetrahydrocannabinols were linear. Typical semilogarithmic plots of the obtained data at various pH values are given in Figs. 3–5 for studies at 60.8°. These plots are not completely linear, especially in the more highly acidic region below pH 3; they indicate two sequential reactions where the concentration of Δ^9-tetrahydrocannabinol conforms to the time (t) dependency of:

$$C = C_1e^{-k_1t} + C_2e^{-k_2t} \qquad \text{(Eq. 1)}$$

The k_2 values were estimated from the terminal linear slopes of such plots in accordance with:

$$\log C = \log C_2 - k_2t/2.303 \qquad \text{(Eq. 2)}$$

where $\log C_2$ is the extrapolated intercept.

The k_1 values were estimated from the linear slopes of the feathered plots in accordance with:

$$\log (C - C_2e^{-k_2t}) = \log C_1 - k_1t/2.303 \qquad \text{(Eq. 3)}$$

where $\log C_1$ is the intercept of the semilogarithmic plots of the difference between the observed concentration and the antilogarithms of values obtained by the extrapolation of the terminal linear segment of the plot of $\log C$ against time. Above pH 4.0, a sim-

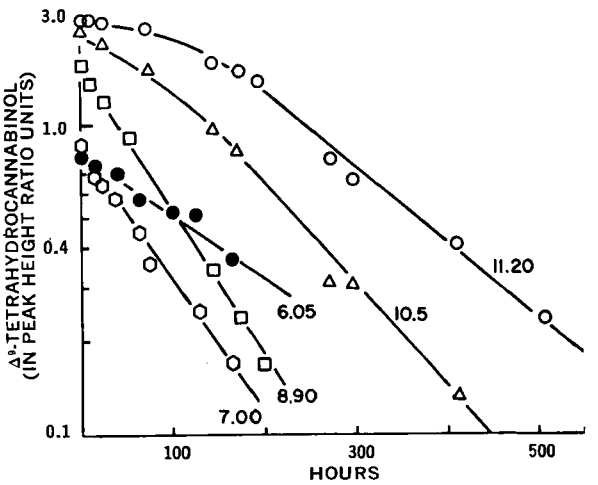


Figure 5—*Semilogarithmic plots of the ratio of the height of the GLC peak for Δ⁹-tetrahydrocannabinol to the peak height of the internal standard tetraphenylethylene against time for the degradation of aqueous solutions (about 1 mg/liter) of purified Δ⁹-tetrahydrocannabinol at 60.8° and at the specified pH values.*

Table I—Rate Constants (in Minutes^{-1}) for the Degradation of Δ^9-Tetrahydrocannabinol in Aqueous Solutions at 0.05 Ionic Strength when Possible in Accordance with $C = C_1e^{-k_1t} + C_2e^{-k_2t}$ below pH 4 and in Accordance with $C = C_0e^{-kt}$ above pH 4

60.8°						70.0°					
pH	10^2k_1	10^4k_2	10^4k	C_1[a]	C_2[a]	pH	10^2k_1	10^4k_2	10^4k	C_1[a]	C_2[a]
0.68	59	398	—	0.7	0.3	1.68	25	243	—	0.7	0.3
1.40	10	85	—	0.7	0.3	2.50	4.3	30.4	—	0.7	0.3
1.90[b]	6.6	36.3	—	0.7	0.3	4.0	—	3.37	—	0.3	0.7
2.12	5.6	20.3	—	—	—	6.95	—	—	2.88	0.0	1.0
2.58	1.1	7.74	—	0.6	0.4	7.97	—	—	3.15	0.0	1.0
3.03	—	4.67	—	0.5	0.5	—	—	—	—	—	—
3.95[c]	—	1.51	—	0.2	0.8	—	—	—	—	—	—
4.80	—	—	1.03	—	—			45.0°			
5.25	—	—	0.66	—	1.0						
6.05	—	—	0.75	—	1.0	0.70	25	99.1	—	0.6	0.4
7.00	—	—	1.70	—	1.0	1.50	3.7	26.7	—	—	—
7.90	—	—	1.90	—	1.0	2.50	3.5	2.73	—	—	—
8.90	—	—	1.82	—	1.0						
9.68	—	—	1.69	—	1.0						
10.5	0.029	—	1.43	—	—						
11.2	0.027	—	1.00	—	—						

[a] Estimated as the fraction of total initial concentration $C_0 = C_1 + C_2$. [b] No significant differences among rate constants evaluated at 0.05 and 0.01 ionic strength. [c] No significant differences among rate constants evaluated at 0.017, 0.051, and 0.10 *M* acetate buffer.

ple first-order decay of Δ^9-tetrahydrocannabinol was observed, which can be characterized by the rate constant k. The rate constants obtained are listed in Table I.

The log k–pH profiles for the apparent first-order rate constants k_1, k_2, and k for the degradation of Δ^9-tetrahydrocannabinol are given in Fig. 6. There are greater errors in the estimates of k_1 from the feathering process.

Except for a few points in the 60.8° plot between pH 4.5 and 6.5 that fall below the reasonably expected pH-independent degradation rate:

$$k = k_0 f_u \qquad \text{(Eq. 4)}$$

The data for k are consistent with the premise that the solvolytic degradation in this pH range is due to the unionized fraction, f_u, of the drug where the pKa′ of Δ^9-tetrahydrocannabinol is 10.6 at 25° (1).

The data for k_1 and k_2 appear to conform to:

$$k_i = k_{H_i^+} a_{H^+} \qquad \text{(Eq. 5)}$$

since:

$$\log k_i = \log k_{H_i^+} - \text{pH} \qquad \text{(Eq. 6)}$$

where:

$$\text{pH} = -\log a_{H^+} \qquad \text{(Eq. 7)}$$

and a_{H^+} is the hydrogen-ion activity.

The values of $k_{H_1^+}$ and $k_{H_2^+}$ were obtained from the antilogarithms of the intercepts of the respective log k *versus* pH plots at the various temperatures; the $k = k_0$ values were estimated from the plateau values between the pH range of 7–9. The microscopic rate constants and their derived Arrhenius parameters are given in Table II. Preliminary studies (1) on acid degradation of Δ^9-tetrahydrocannabinol in 0.01–0.5 $\bar{N}$ HCl gave an apparent $k_{H_2^+}$ value at 55° of 0.12 liter/mole/min, which is consistent with the values listed in Table II.

The few experimental points that fall below the fit to the postulated Eq. 4 at 60.8° between pH 4.5 and 6.5 are difficult to reconcile with existing theory since Δ^9-tetrahydrocannabinol has no pKa′ in this range. This anomaly must either be attributed to some technological artifact or to the buffers exerting an inhibition of degradation rates. However, there were no significant differences among rate constants at pH 3.95 evaluated over a fivefold range of acetate buffer concentrations or a fivefold range of ionic strength at pH 1.90.

A reasonable explanation for the observed biphasic phenomena below pH 4.0 is that the first phase characterized by the apparent first-order rate constant k_1 represents the loss of Δ^9-tetrahydrocannabinol to various products (Scheme I), which includes a *Y* that fortuitously has the same GLC retention time as Δ^9-tetrahydrocannabinol when extracted from the reaction mixture by the specified procedures. This product *Y* subsequently vanishes by a first-order rate at a constant acidity characterized by the rate constant k_2. This would imply that Eqs. 4 and 5 could be combined so that:

$$k_1 = [k_{H_1^+} a_{H^+} + k_0] f_u \qquad \text{(Eq. 8)}$$

where the estimated microscopic rate constants are given in Table II and the dependence of Eq. 8 on k_1 at 60.8° is demonstrated by the dotted line in Fig. 6.

$$\Delta^9\text{-tetrahydrocannabinol} \xrightarrow{k_1} \text{products} + Y \qquad Y \xrightarrow{k_2} Z$$

Scheme I

The fact that only one phase exists above pH 4.0 may be rationalized by postulating that *Y* is only produced by hydrogen-ion attack on the Δ^9-tetrahydrocannabinol whereas the pH-independent degradation above this pH value does not produce *Y*.

Alternatively, the fact that two sequential reactions predominated in the degradation of Δ^9-tetrahydrocannabinol below pH 4 (Figs. 3 and 4) but only the slower phase appeared to persist at the higher pH values to pH 10 can be rationalized by Scheme II.

$$\text{products} \xleftarrow{k_2} \left(\Delta^9\text{-tetrahydrocannabinol} \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} X\right)$$

Scheme II

Then:

$$k_1 = k_1' + k_{-1}' \qquad \text{(Eq. 9)}$$

and (4):

$$k_2 = \frac{k_2}{1 + k_1'/k_{-1}'} = \frac{k_2'}{1 + [X]_{eq}/[\Delta^9]_{eq}} =$$

$$\frac{k_2'}{1 + C_1/C_2} = \frac{k_2'}{1 + 2.333} = 0.3k_2' \qquad \text{(Eq. 10)}$$

from the rough estimate of the C_1 and C_2 values given in Table I.

Scheme II proposes that the GLC observed Δ^9-tetrahydrocannabinol readily equilibrates with a compound *X* where the sum of the backward and forward rate constants, k_1 (Eq. 9), is given by the rate dependency of Eq. 5 and that both microscopic rate constants k_1' and k_{-1}' would have to be hydrogen-ion dependent so that their ratio is not. The C_1 and C_2 values (Table I) obtained from the intercepts of the phases in the plots of Figs. 3 and 4 permit estimates of:

$$k_1'/k_{-1}' = [X]_{eq}/[\Delta^9]_{eq} = C_1/C_2 = 0.7/0.3 = 2.33 \qquad \text{(Eq. 11)}$$

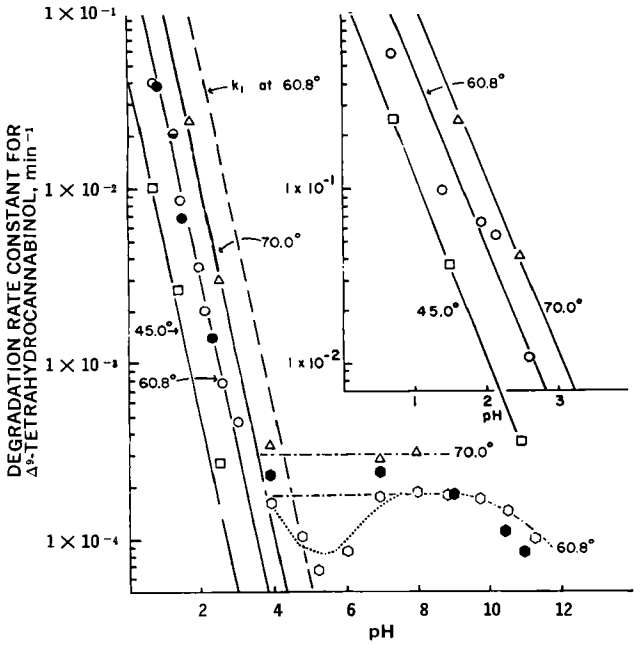


Figure 6—*Log* k–*pH profiles for the degradation of* Δ^9*-tetrahydrocannabinol at several temperatures, where the concentration could be expressed as* $C = C_1e^{-k_1t} + C_2e^{-k_2t}$ *as a function of time,* t*, in minutes. The open symbols were obtained from studies on material purified by TLC, and the solid symbols were obtained from studies on unpurified material. The half-open symbol was obtained from a study on a synthesized mixture of purified* Δ^9*- and* Δ^8*-tetrahydrocannabinols. The larger graph is for the smaller rate constant,* k_2*, and inset is for the larger rate constant,* k_1*, obtained by graphical analysis or appropriate feathering of the plots of the logarithms of the GLC peak height ratios of* Δ^9*-tetrahydrocannabinol to the internal standard tetraphenylethylene against time. For purposes of comparison with the smaller rate constant,* k_2*, the dashed line for* k_1 *at 60.8° is inserted in the larger graph. The dot-dashed lines represent rate constants from reactions at neutral pH values that do not show biphasic semilogarithmic plots.*

so that the concentration of X at the high acidities is more than twice that of Δ^9-tetrahydrocannabinol when equilibrium is established in acidic solutions.

If the apparent rapid equilibration of Δ^9-tetrahydrocannabinol with X characterized by k_1 (Eqs. 5 and 9) is only catalyzed by hydrogen ions but its degradation to products characterized by k_2 (Eq. 10) is not pH dependent above pH 4, then Eqs. 4 and 5 could be combined to:

$$k_2 = [k_{H_2^+}a_{H^+} + k_0]f_u \qquad \text{(Eq. 12)}$$

The rate of loss of Δ^9-tetrahydrocannabinol to products would exceed the postulated rate of its equilibration to X above this pH value and the rapid loss characterized by k_1 would tend to disappear at the higher pH values.

The half-life of Δ^9-tetrahydrocannabinol at pH 1 and 37° can be estimated from the k_1 data of Tables I and II as approximately 15 min and implies that the drug may be significantly degraded in the normal stomach.

GLC and TLC Characterization of Products of Δ^9-Tetrahydrocannabinol in Acid Solution—When Δ^9-tetrahydrocannabinol reacted in aqueous acid solution was analyzed by the stated extraction and GLC procedures, five distinct peaks were observed by flame-ionization detection. Figure 7 shows typical chromatograms of aliquots of solutions of Δ^9-tetrahydrocannabinol at less than 1.0 mg/liter reacted in 0.1 *N* HCl for 4 days at 60.8°. Under the stated experimental conditions, the retention times of the first two peaks, other than the internal standard tetraphenylethylene at 1.3 min, corresponded to Δ^8-tetrahydrocannabinol (2.1 min) and Δ^9-tetrahydrocannabinol (2.34 min). Subsequently, three others were observed and were labeled P_1 (3.5 min), P_2 (4.2 min), and P_3 (5.22 min). The chromatograms of Fig. 7 were obtained from reacting solutions of purified and nonpurified Δ^9-tetrahydrocannabinol in aqueous and in 20% ethanol solutions. There was some difference between the effect of 0 and 20% ethanol on the relative heights of the observed peaks in that the presence of alcohol appeared to favor the products with retention times assigned to Δ^8- and Δ^9-tetrahydrocannabinols. This fact is consistent with the previously stated rationalization (Scheme I) that a product Y may result with the same retention time as Δ^9-tetrahydrocannabinol. Alcohol content did not appear to affect the relative amounts of P_1, P_2, and P_3. The yields of P_2, P_3, and a product with the same retention time as Δ^8-tetrahydrocannabinol increased and the yield of P_1 decreased when impure Δ^9-tetrahydrocannabinol was used as the starting material rather than the purified compound.

Table II—Microscopic Rate Constants (in Liters per Mole per Minute) for the Degradation of Δ^9-Tetrahydrocannabinol in Aqueous Solutions in Accordance with $k_1 = k_{H_1^+}a_{H^+}$ and $k_2 = k_{H_2^+}a_{H^+}$, where $a_{H^+} = 10^{-pH}$ and where k is Independent of pH

Temperature	$k_{H_1^+}$	$k_{H_2^+}$	10^4k
45.0°	1.2	0.045	—
60.8°	5.0	0.265	1.80
70.0°	12.0	1.00	3.05
ΔH_a, kcal/mole	21	23	13
log P, min^{-1}	14.4	14.6	4.9

When the spontaneously formed aqueous emulsion (1) of the original impure Δ^9-tetrahydrocannabinol was reacted in 0.1 *N* HCl at 60° for 5 days and the extracts were analyzed by GLC, the primary product had the same retention time as Δ^8-tetrahydrocannabinol and appeared to have relatively the same peak height ratio to the internal standard as did the Δ^9-tetrahydrocannabinol in the original solution. The peak heights assigned to P_1, P_2, and P_3 were relatively small and formed slowly. The primary Δ^9-tetrahydrocannabinol transformation apparently is to Δ^8, not only in acidified hydrocarbon solutions (5–7) but in the hydrocarbon phase formed

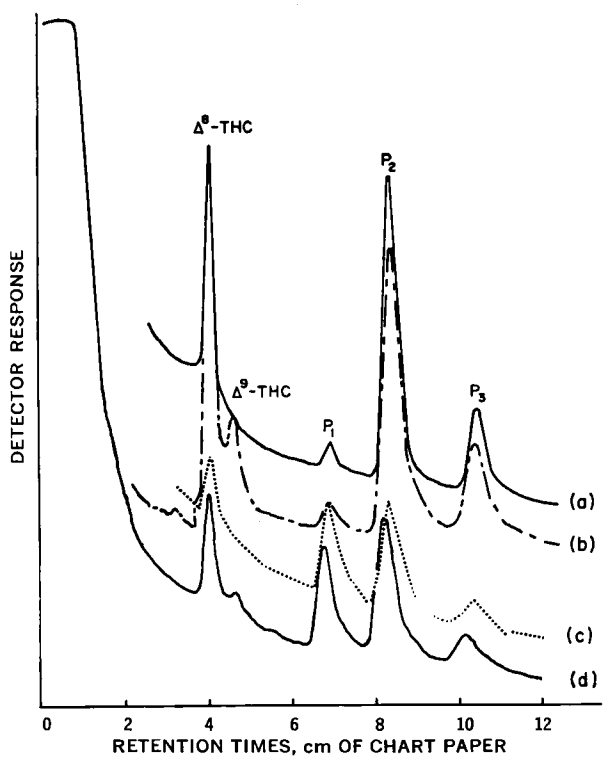


Figure 7—*Gas chromatograms of chloroform-extracted acid-degraded solutions of* Δ^9*-tetrahydrocannabinol (*Δ^9*-THC) in 0.1* N *HCl at 60.8° for 4 days for* (a *and* b) *unpurified compound and* (c *and* d) *TLC-purified compound in 0% ethanol* (a *and* c) *and 20% ethanol* (b *and* d).

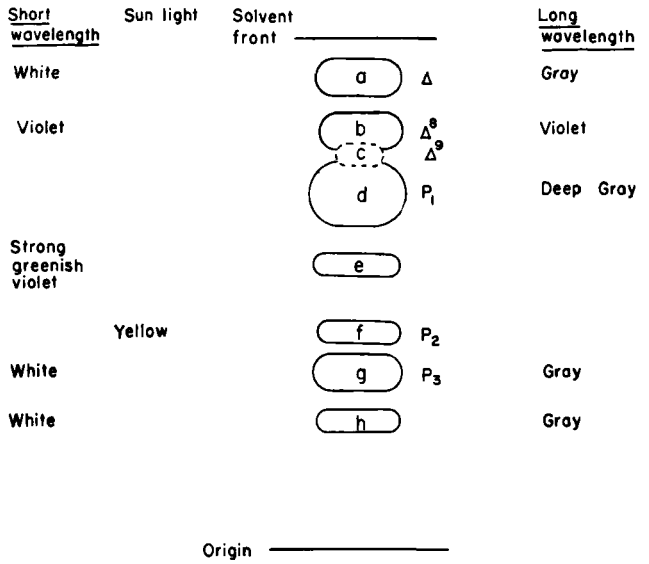
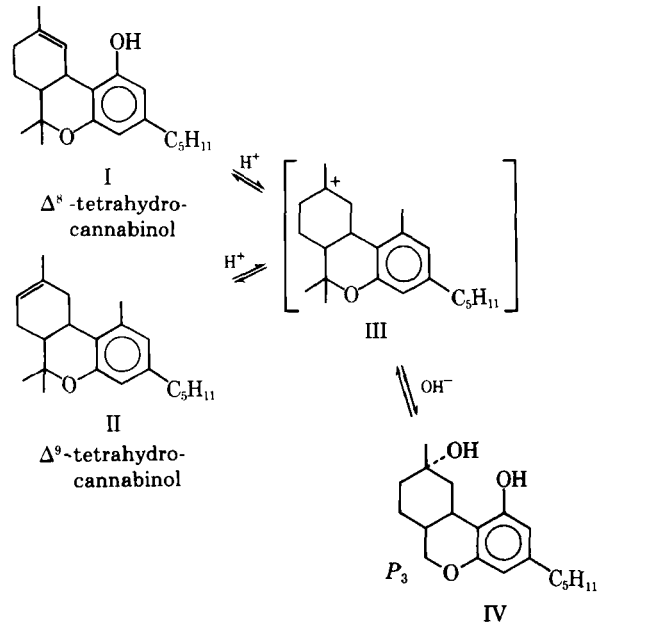


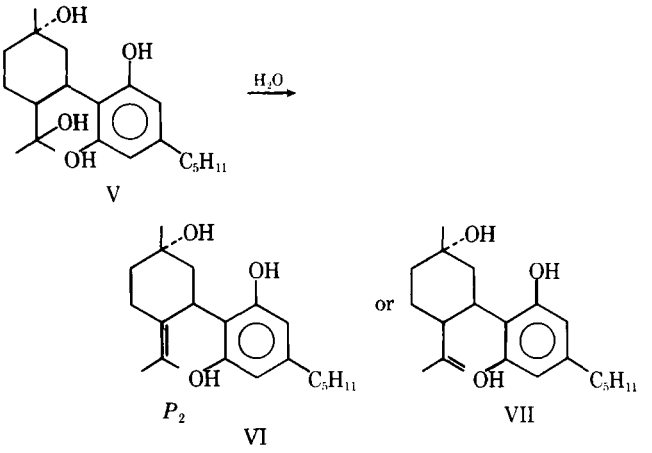
Figure 8—*Thin-layer chromatogram, developed with cyclohexane–acetone (10:1), of chloroform extracts of degraded unpurified Δ⁹-tetrahydrocannabinol (12.5 mg/liter in 20% ethanolic 0.1 N HCl after 5 days). Elution of the pertinent spots and subsequent GLC analyses identified Δ⁸-tetrahydrocannabinol* (b), P_1 (d), P_2 (g), *and* P_3 (h). *The three additional spots observed when the starting material was impure were* (a), *which gave irregular GLC peaks (Fig. 2a), and* (e) *and* (f), *which did not give observable GLC peaks on elution. The dashed spot* (c) *was where Δ⁹-tetrahydrocannabinol would appear if present.*

by its own emulsification (1). The fact that the peak height of Δ^8-tetrahydrocannabinol increased with the increasing alcohol content of true solutions is consistent with the premise that P_1, P_2, and P_3 result from the attack of the components of water on tetrahydrocannabinol in true solution.

TLC [Chrom AR, cyclohexane–acetone (10:1)] effectively separated the chloroform-extracted products of a 20% ethanol solution of Δ^9-tetrahydrocannabinol (12.5 mg/liter) reacted in 0.1 *N* HCl at 61° for 5 days. The spots of R_f values 0.86 *(b)*, 0.80 *(c)*, 0.75 *(d)*, 0.39 *(g)*, and 0.28 *(h)* (Fig. 8), when eluted and gas chromatographed, permitted their identification with the retention times consistent with Δ^8-tetrahydrocannabinol, Δ^9-tetrahydrocannabinol, P_1, P_2, and P_3, respectively. When impure Δ^9-tetrahydrocannabinol was used as the starting material, three additional spots were observed (Fig. 8). One spot *(a)* of R_f 0.97 near the solvent front, when eluted, gave the irregular GLC peaks prior to the Δ^8-tetrahydrocannabinol peak observed when the top half of the spot for Δ^8-tetrahydrocannabinol, obtained by TLC from impure Δ^8-tetrahydrocannabinol, was eluted and gas chromatographed (Fig. 2*a*). A second spot *(e)* of R_f 0.59 showed strong greenish violet fluorescence under short UV wavelength (254 nm); this fluorescence decayed rapidly in air. The third spot *(f)* appeared yellow. Neither spot *(e)* nor spot *(f)* gave an observable GLC peak on elution.



Scheme III



Scheme IV

Characterization of Separated Products—The products were separated and purified by preparative TLC. The separated product, as well as the impurity in the original impure sample of Δ^9-tetrahydrocannabinol that had the same GLC retention time as Δ^8-tetrahydrocannabinol, had the same UV (in methanol) and NMR spectra as pure Δ^8-tetrahydrocannabinol (5, 6).

Tentative Identification of Cannabinol as P_1—The GLC retention times of the TLC isolated P_1 (3.5 min at 245°, 9.5 min at 220°) were coincident with those of cannabinol. Product P_1 and cannabinol had the same R_f values in four different TLC systems: benzene (0.88), chloroform (0.94), cyclohexane (0.04), and cyclohexane–acetone (10:1) (0.79). No spots were observed with either material when isoamyl alcohol was used as the developing solvent. A yellow band was observed at R_f 0.34, and there appeared to be a band at the solvent front in both cases.

The UV spectra[8] of P_1 and cannabinol in methanol had two absorption maxima at 220 and 285 nm, and the ratio of their molar absorptivities was the same ($\epsilon_{220}/\epsilon_{285}$: cannabinol 1.98, P_1 2.18). The IR spectra[8] of P_1 and cannabinol (5) in KBr pellets were coincident. It is well known that both Δ^9- and Δ^8-tetrahydrocannabinols form cannabinol on chloranil dehydrogenation (6).

Tentative Identification of 9-Hydroxycannabidiol as P_2—The UV spectrum[8] of P_2 in methanol had two absorption maxima (275 and 282 nm) very similar to those of Δ^9- and Δ^8-tetrahydrocannabinols, cannabicyclol, cannabigeral, and cannabidiol (5). However, both the IR[9] (Fig. 9*a*) and NMR[10] (Fig. 10*a*) spectra of P_2 exclude its being any of these compounds (5). NMR shows a singlet at δ 6.1 for the two hydrogens on the phenol ring, indicating that these two hydrogens are in the same environment. This can happen only when the ether ring is opened as in cannabinidiol (5). NMR also suggests that the vinylic hydrogen of the double bond at position 9 is absent in P_2 and that the CH_3 peak adjacent to the double bond originally located at δ 1.68 for Δ^9-tetrahydrocannabinol has disappeared. The product P_2 has a strong mass peak at *m/e* 332, which can be assigned to the molecular weight of P_2 which is consistent with the NMR evidence for water addition to the isolated double bond. The mass spectrum[11] pattern of P_2 is similar to that of Δ^8-tetrahydrocannabinol since both compounds have

[9] Beckman Acculab 3.
[10] Varian A60 NMR spectrometer.
[11] AEI MS 30 mass spectrometer combined with a DS10 data system, Scientific Apparatus Corp., Manchester, England.

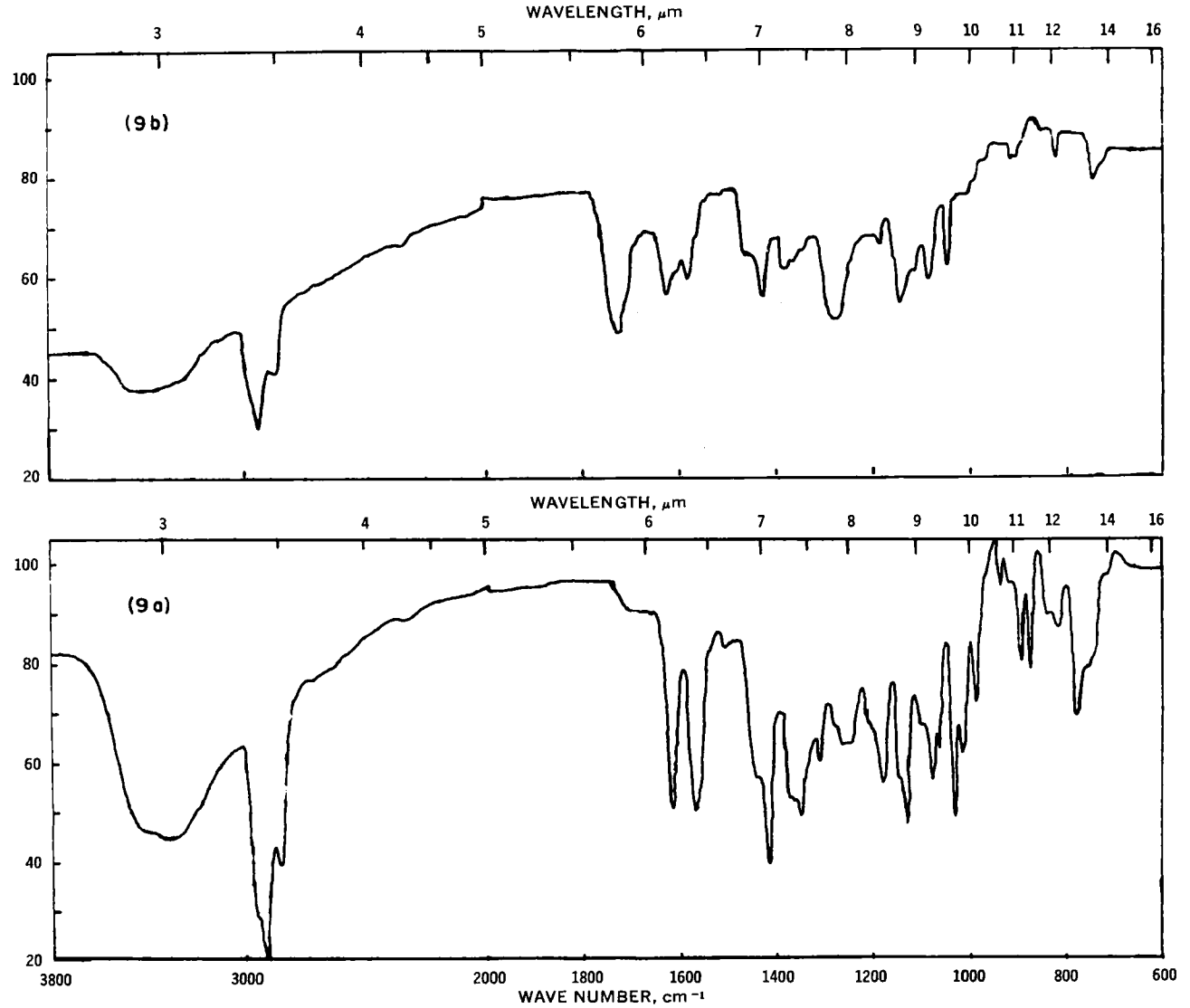


Figure 9—*IR spectra in KBr pellets of* (a) P_2 *and* (b) P_3, *isolated by and extracted from thin-layer chromatograms.*

strong fragment peaks at 314, 299, 271, 258, 243, 231, and 193.

The evidence strongly suggests that P_2 results from: *(a)* the specific hydrogen-ion-catalyzed addition of a water molecule to the nonconjugated double bond of Δ^9- and Δ^8-tetrahydrocannabinols, and *(b)* the opening of the internal ether as shown in Scheme III. The hydrolysis of the ether linkage with subsequent dehydration may result in two possible molecular structures (Scheme IV). Since no $=CH_2$ hydrogens were indicated in the NMR spectrum (about δ 4.7) (5), Structure VI can be assigned to P_2.

Tentative Identification of 9-Hydroxytetracannabinol as P_3—The NMR[10] spectrum of P_3 (Fig. 10*b*) also suggests that the nonconjugated double bond of tetrahydrocannabinol has been saturated since peaks assigned to the vinylic hydrogen and to the CH_3 group adjacent to the double bond (δ 1.68) are missing. The mass spectrum[11] (332 peak) also suggests the addition of a water molecule to tetrahydrocannabinol. The UV[8] and IR[9] (Fig. 9*b*) spectra of P_3 are similar to those of Δ^9-tetrahydrocannabinol and imply that P_3 results from the mechanisms proposed in Scheme III for the production of IV. It is well known that addition to the isolated double bond occurs when cannabidiol is heated in ethanol with dilute hydrochloric acid (5).

Possible Relationship among Yields of P_1, P_2, P_3, and Δ^8-Tetrahydrocannabinol from Δ^9-Tetrahydrocannabinol—The use in GLC of a ratio of the peak height given by a specific compound at its retention time to the peak height of a constant amount of internal standard at its retention time is a standard procedure to establish an analytical calibration curve for the assay of amounts of the compound. A peak height ratio of another compound to the internal standard that is the same as that for the initial compound does not necessarily imply that the amounts of both compounds assayed are the same. In general, a compound of a longer retention time will demonstrate a lower peak height and ratio than a compound with a shorter retention time and of the same quantity since the distribution or spreading of the band of material emerging from the column will be greater. Also, even with the same retention times under a given set of chromatographic conditions, the magnitudes of the detector response for each unit amount of compound may vary. In the specific case of flame-ionization detection, the detector response per unit amount of compound will vary with the burning efficiency of each compound.

If the chemical structures and burning efficiencies of Δ^8- and Δ^9-tetrahydrocannabinols and their GLC-observed products, P_1, P_2, and P_3, are assumed to be similar, and if the same peak heights at the same retention time are assumed to be measures of equivalent amounts of different compounds, it may be possible to correct the peak heights of different retention times to the same retention time to compare yields of GLC-observed products from a given reactant.

Equal amounts of Δ^9- and Δ^8-tetrahydrocannabinols were injected into the gas chromatograph at different column temperatures, and the relative peak heights were obtained as a function of retention time (Fig. 11). The ratios of the relative peak heights of Δ^9- and Δ^8-tetrahydrocannabinols at their normal retention times to their relative peak heights at the retention time of Δ^9- and Δ^8-

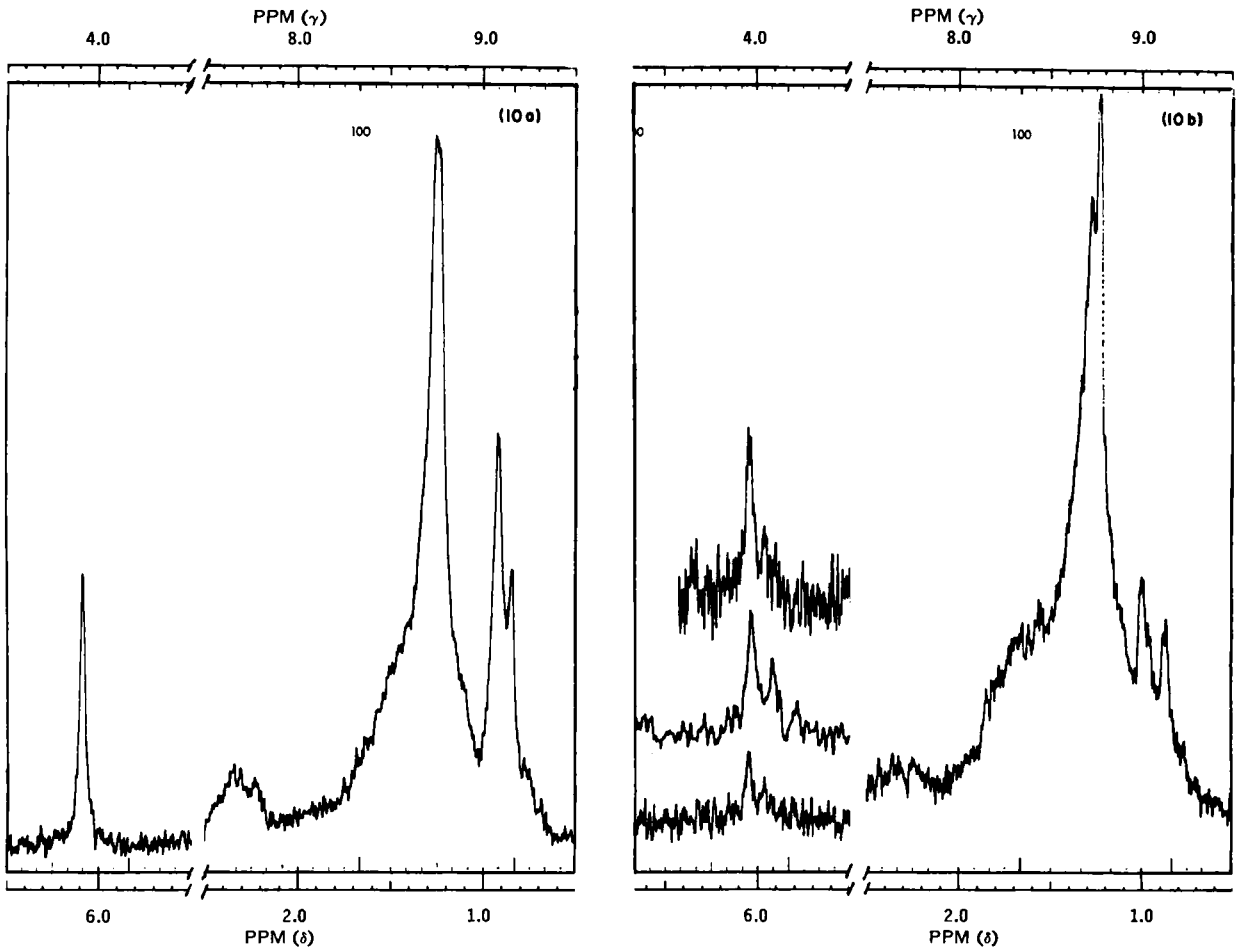


Figure 10—*NMR spectra of* (a) P_2 *and* (b) P_3, *isolated by and extracted from thin-layer chromatograms.*

tetrahydrocannabinols, P_1, P_2, and P_3 were calculated. If the assumptions underlying this approach are correct, the ratios in parentheses are the values necessary for multiplying the peak heights or peak height ratios of Δ^8-tetrahydrocannabinol (0.73), P_1 (1.6), P_2 (2.0), and P_3 (2.6) to relate their amounts to equivalent amounts of Δ^9-tetrahydrocannabinol in the reacting mixture. The ratios in parentheses are the values necessary to multiply the peak heights or peak height ratios of Δ^9-tetrahydrocannabinol (1.4), P_1 (2.2), P_2 (2.8), and P_3 (3.7) to relate their amounts to equivalents of Δ^8-tetrahydrocannabinol reactant. The peak height/amount values obtained from the GLC calibration curves of Δ^9- and Δ^8-tetrahydrocannabinols were 0.155 and 0.182/μg, respectively, which gives a ratio of 0.85 which is in the vicinity of the 0.73 factor obtained by the described procedure.

Production of GLC-Observed Δ^8-Tetrahydrocannabinol, P_1, P_2, and P_3 from Degradation of Purified Δ^9-Tetrahydrocannabinol in Aqueous Solution—The appearance of the peak heights at the GLC retention times of Δ^8-tetrahydrocannabinol, P_1, P_2, and P_3 and the loss of peak height of Δ^9-tetrahydrocannabinol of the chloroform-extracted material (Fig. 7) were observed with time when Δ^9-tetrahydrocannabinol was maintained in aqueous solution at constant temperature. Typical graphs are given in Figs. 12–14 where the actual peak height ratios (to the internal standard tetraphenylethylene) of Δ^9-tetrahydrocannabinol, P_1, P_2, and P_3 were multiplied by the appropriate factors that would correct the diminutions of their peak height ratios at higher retention times on the presumption that equivalent peak height ratios at the same retention time as Δ^9-tetrahydrocannabinol would give reliable estimates of relative amounts of products and precursor.

The ratios of the amounts of products and their relative yields from Δ^9-tetrahydrocannabinol were reasonably the same within the errors of measurement at all pH values below 4 and their rates of appearance were proportional to the hydrogen-ion concentration. This was confirmed by the negative linear slopes of approximately unity of the logarithms of initial rates of peak height appearance of products plotted against pH up to pH 3.0.

In general, the rates of appearance of P_1 and the Δ^8 compound paralleled the initial fast phase (k_1) of loss of the peak for Δ^9-tetrahydrocannabinol and seemed to be greater than the rates of appearance of P_2 and P_3. In several cases, there may have been an anomalous discontinuity in the rate of appearance of the P_1 peak (dashed lines in Figs. 12–14) which could only be explained by the fact that two products have the same retention time where the more rapidly appearing compound is farther degraded or equilibrated to some other at that or some other retention time.

No significant formation of Δ^8-tetrahydrocannabinol, P_1, P_2, and P_3 was observed above pH 4 until pH 8.9, beyond which pH the production of small amounts of Δ^8-tetrahydrocannabinol was again observed.

Patterns of Acidic Degradation of P_2, P_3, and Δ^8-Tetrahydrocannabinol—Pertinent spots obtained from TLC of acid-degraded aqueous solutions of Δ^9-tetrahydrocannabinol were extracted, and the isolated compounds had the GLC retention times of P_2 and P_3. These materials, as well as Δ^8-tetrahydrocannabinol, were reacted in aqueous solutions of pH 0.79 at 60.8°, and the corrected peak height ratios of each compound with time are plotted in Fig. 15. The premise for such plots is that if each compound had the same retention time as Δ^8-tetrahydrocannabinol, the corrected peak height ratios would be proportional to the number of molecules observed. When starting with P_2 and P_3, the sum of the corrected peak height ratios for all those materials at any time was reasonably close to the corrected peak height ratio for P_2 and P_3 at zero time. This was not true when Δ^8-tetrahydrocannabinol was the starting material.

The final ratios of the corrected peak height ratios with time at 60.8° and pH 0.79 (Fig. 15) for the various starting materials are for P_3: $P_2/\Delta^8 = 5.3$, $P_3/\Delta^8 = 2.7$, and $P_2/P_3 = 2.0$; for P_2: $P_2/\Delta^8 = 6.2$, $P_3/\Delta^8 = 2.0$, and $P_2/P_3 = 2.0$; and for Δ^8: $P_2/\Delta^8 = 5.3$, $P_3/\Delta^8 =$

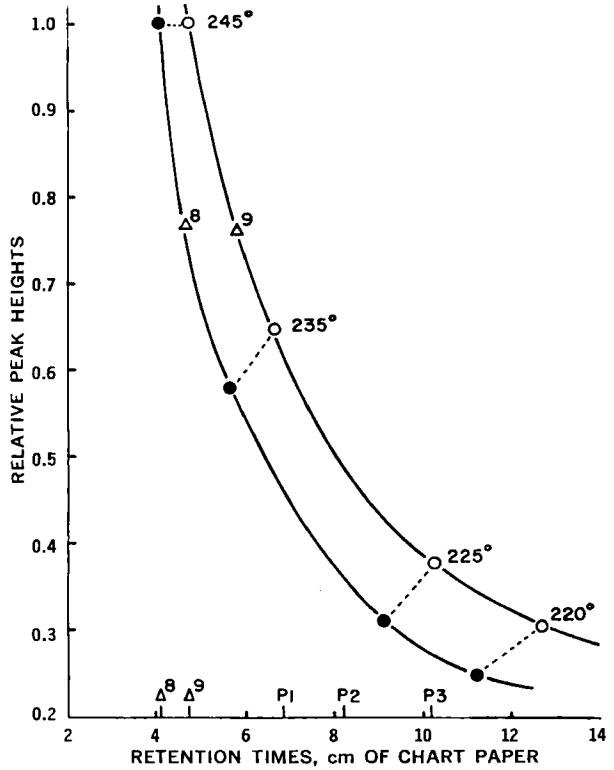


Figure 11—*Plots of peak heights of Δ⁸- and Δ⁹-tetrahydrocannabinols at various column temperatures relative to their respective peak heights at a column temperature of 245° against observed retention times. It can be assumed that the factor necessary to multiply the peak height of a product at its normal retention time with a 245° column temperature to equate it to an equivalent amount of Δ⁹- (or Δ⁸-) tetrahydrocannabinol is determined by the reciprocal of the relative peak height of Δ⁹- (or Δ⁸-) tetrahydrocannabinol at the retention time of the product under the conditions of a 245° column temperature. The labelings for the marks above the abscissa give the retention times for the stated product.*

2.5, and P_2/P_3 = 2.1. At pH 0.56 and 60.8° for P_3, P_2/Δ^8 = 5.0, P_3/Δ^8 = 2.2, and P_2/P_3 = 2.3. At pH 0.26 and 60.8° for Δ^8, P_2/Δ^8 = 3.9, P_3/Δ^8 = 1.6, and P_2/P_3 = 2.4. At pH 1.49 and 60.8° for Δ^8, P_2/Δ^8 = 5.1, P_3/Δ^8 = 2.4, and P_2/P_3 = 2.1. The ratios were remarkably consistent for the studies at these several pH values, well within the errors of measurement, and gave averages of P_2/Δ^8 = 5.1, P_3/Δ^8 = 2.1, and P_2/P_3 = 2.0. Therefore, it is reasonable to conclude that these values represent the final relationships of the equilibria that occur among Δ⁸-tetrahydrocannabinol, P_2, and P_3 in acidic solutions.

The degradation of Δ⁸-tetrahydrocannabinol in acidic solutions appears to be first order, although there may have been an initial lag at some higher pH values such as at pH 2.1 (Fig. 16). The apparent first-order rate constants are plotted semilogarithmically against pH in the inset of Fig. 16 and demonstrate a linear slope of unity in accordance with Eqs. 5 and 6, indicative of specific hydrogen-ion catalysis where $k_{H^+} = 3.6 \times 10^{-3}$ liter/mole/min at 60.8°, which is 0.0007 of the $k_{H_1^+}$ and 0.0135 of the $k_{H_2^+}$ for Δ⁹-tetrahydrocannabinol under the same conditions.

When Δ⁹-tetrahydrocannabinol was reacted in acidified solutions at 60.8°, the observed final P_2/Δ^8 ratios were for several pH values (pH, P_2/Δ^8): 0.68, 5.1 (Fig. 12); 1.40, 4.5 (Fig. 13); and 2.12, 6.0 (Fig. 14). These values are consistent with the results of the Δ⁸-tetrahydrocannabinol studies and indicate that the Δ⁸ product from Δ⁹ degradation forms similar equilibria among P_2 and P_3. The production of P_3 was increasing beyond the time the studies were terminated, which would be consistent with the transformation rates of Δ⁸-tetrahydrocannabinol, so the final P_3/Δ^8 ratio was difficult to estimate. The available data were consistent with a final value of 2.0–3.0 for this ratio. For example, at pH 2.12 at 60.8°, where P_3 was analyzed for longer times than are given in Fig. 14, the corrected peak height ratios for P_3 were 0.102 at 3300 min and 0.144 at 5800 min, which are P_3/Δ^8 final ratios of 1.7 and 2.4, respectively.

Available Evidence and Possible Transformations of Δ⁹-Tetrahydrocannabinol in Aqueous Solutions—The fact that an apparent final equilibrium exists among Δ⁸-tetrahydrocannabinol, P_2, and P_3 in acid solutions, coupled with the evidence (Schemes III and IV) for the structures of P_2 and P_3, indicates that there is an equilibration in the presence of acid among compounds with an intact and hydrolyzed ether linkage and among compounds with a hydrated and isolated double bond.

The fact that Δ⁹-tetrahydrocannabinol degrades in acid solution to an additional product P_1 as well as to Δ⁸ (Figs. 12–14) seems indisputable, but it is difficult on mechanistic grounds to assign P_1 to cannabinol even though chromatographic evidence so indicates.

In one 60.8° kinetic study of Δ⁹-tetrahydrocannabinol in aqueous 0.12 *N* HCl, there was no significant increase in the UV absorbance of the ethanolic reconstitutions of the dried chloroform extracts with time up to 930 min, when significant P_1 was observed by GLC (Figs. 12–14). Since (5) cannabinol has a molar absorptivity (ϵ_{285} = 18,000) more than 10 times that of Δ⁹-tetrahydrocannabinol (ϵ_{280} = 1050), there should have been an enormous increase in absorbance if cannabinol had formed directly from Δ⁹-tetrahydrocannabinol.

A possible rationalization of these contradictions would be that Δ⁹-tetrahydrocannabinol does transform into molecules with solvolyzed ether linkages and/or hydrated double bonds analogous to those prepared with Δ⁸-tetrahydrocannabinol. One of these, P_1, may have the same GLC retention time as cannabinol or is trans-

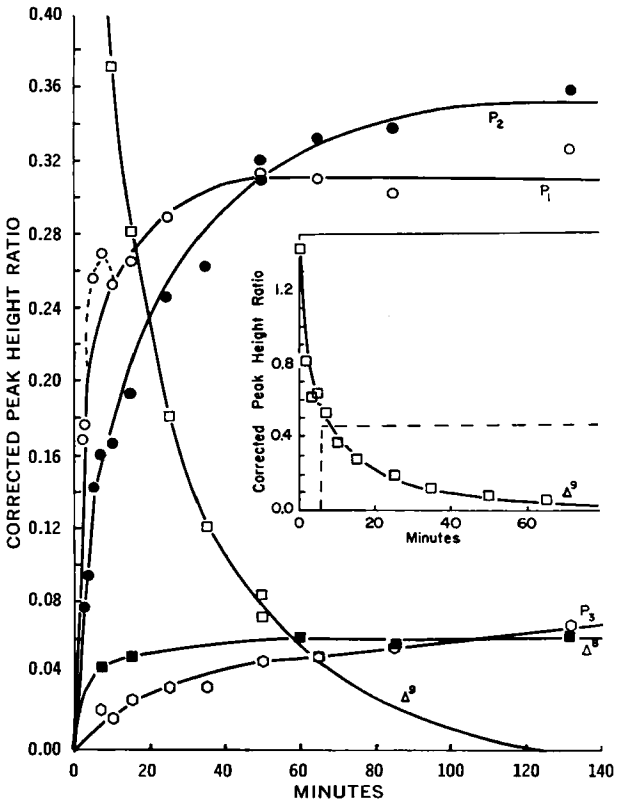


Figure 12—*Plots of the peak height ratio to that of the internal standard tetraphenylethylene against time of Δ⁹-tetrahydrocannabinol reacted at 60.8° in aqueous solutions at pH 0.68 and the corrected peak height ratios of the products of Δ⁸-tetrahydrocannabinol, P_1, P_2, and P_3 observed by GLC. The observed peak height ratios of these latter products were multiplied by 0.73, 1.6, 2.0, and 2.6, respectively, on the presumption that the resultant values would be equivalent to the peak height ratio of Δ⁹-tetrahydrocannabinol. The insets are condensed plots for Δ⁹-tetrahydrocannabinol, and the dashed lines encompass those values plotted in the larger graphs.*

Table III—Percent of Total Radioactivity of Original Δ^9-Tetrahydrocannabinol at Various R_f Values Corresponding to Products of Degradation in 0.06 *N* HCl at 60.8°[a]

Reaction Time, min	$\Delta^8 + \Delta^9$	P_1	$P_2 + P_3$	Subtotal	Origin	Total	Residual
5	64		11	75	6	81	19
15	42	10	22	76	14	86	14
40	26	19	22	67	19	86	14
120	24	18	36	78	13	91	9
1130	11	18	50	79	10	89	11
5760	13	21	44	78	11	88	12

[a] The thin-layer chromatograms of chloroform-extracted samples taken with time were monitored by a radio chromatogram scanner. The areas of the pertinent peaks of the chromatogram were cut and weighed.

formed to cannabinol after injection and travel through the column. Similarly, TLC separation and elution may permit its oxidation to cannabinol. The apparent reproducible maximum on the side of the curve (dashed lines in Figs. 12–14) for the production of material with the retention time characterized as P_1 from Δ^9-tetrahydrocannabinol could indicate the rapid formation of several products of the same retention time where one of them degrades further. The problem of proper characterization of the compounds that give rise to the P_1 retention time needs further elucidation.

The biphasic nature of the semilogarithmic plots of Δ^9-tetrahydrocannabinol degrading in acid solutions against time (Figs. 3 and 4) either may implicate the rapid production of a compound, *Y* (Scheme I), that has the same GLC retention time as Δ^9-tetrahydrocannabinol and that may be slowly transformed further or may implicate the rapid equilibration of Δ^9-tetrahydrocannabinol with a compound, *X* (Scheme II), and the slower further irreversible degradation of one or both compounds. Both explanations are consistent with transformations of Δ^9-tetrahydrocannabinol by hydrogen-ion-catalyzed double-bond migration to Δ^8-tetrahydrocannabinol, hydrogen-ion-catalyzed hydrolysis of the ether linkage (IV → V) with possible subsequent dehydration (Scheme IV), and acid-catalyzed hydration of the isolated Δ^9 double bond (Scheme III).

For both Schemes I and II, *Y* or *X* could be products of Δ^9-tetrahydrocannabinol resulting from a hydrolyzed ether linkage, VIII (Scheme V), or its dehydrated analogs, IX or X. Any one of these could undergo specific hydrogen-ion-catalyzed attack to form an activated complex, III, III′, III″, or III‴, which may further produce the 9-hydroxy analogs, IV, V, VI, or VII, on hydroxylation. Δ^8-Tetrahydrocannabinol does not produce Δ^9-tetrahydrocannabinol or P_1 but produces equilibrating P_2 and P_3, tentatively

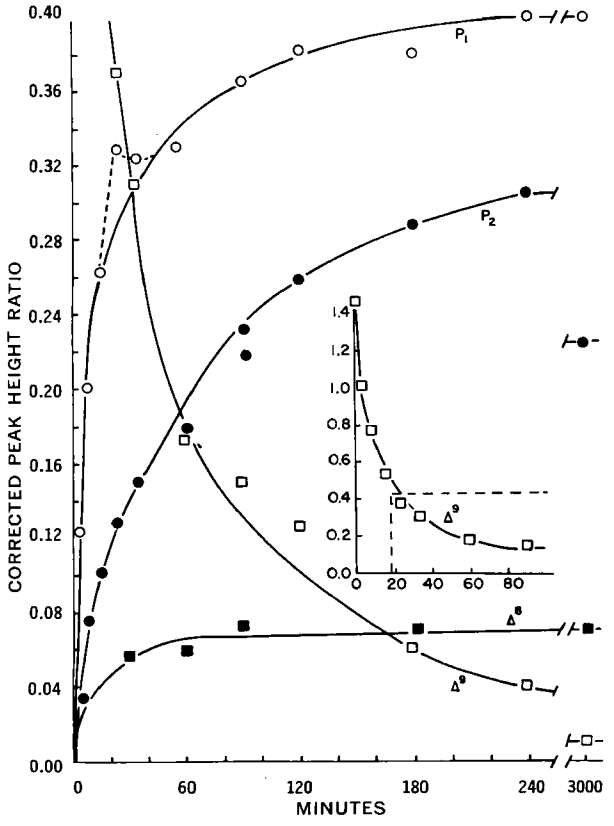


Figure 13—*Plots of the peak height ratio to that of the internal standard tetraphenylethylene against time of Δ^9-tetrahydrocannabinol reacted at 60.8° in aqueous solutions at pH 1.40 and the corrected peak height ratios of the products of Δ^8-tetrahydrocannabinol, P_1, P_2, and P_3 observed by GLC. The observed peak height ratios of these latter products were multiplied by 0.73, 1.6, 2.0, and 2.6, respectively, on the presumption that the resultant values would be equivalent to the peak height ratio of Δ^9-tetrahydrocannabinol. The insets are condensed plots for Δ^9-tetrahydrocannabinol, and the dashed lines encompass those values plotted in the larger graphs.*

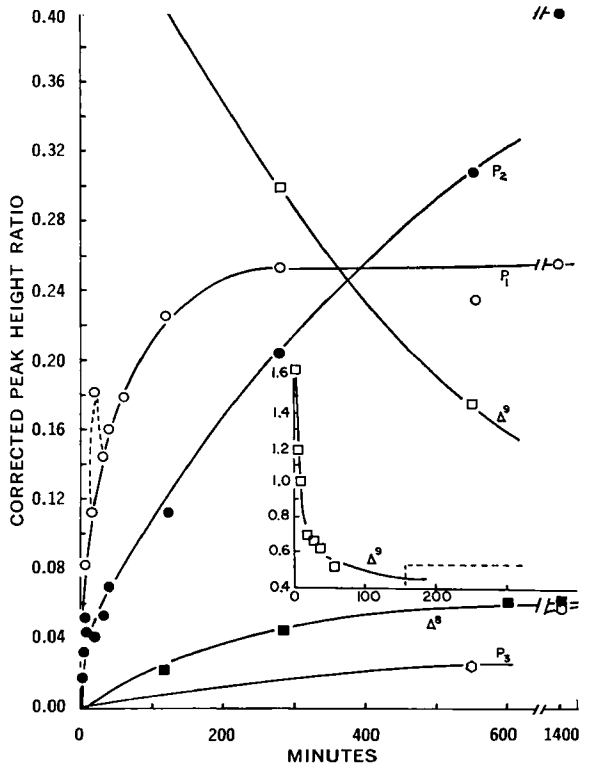


Figure 14—*Plots of the peak height ratio to that of the internal standard tetraphenylethylene against time of Δ^9-tetrahydrocannabinol reacted at 60.8° in aqueous solutions at pH 2.12 and the corrected peak height ratios of the products of Δ^8-tetrahydrocannabinol, P_1, P_2, and P_3 observed by GLC. The observed peak height ratios of these latter products were multiplied by 0.73, 1.6, 2.0, and 2.6, respectively, on the presumption that the resultant values would be equivalent to the peak height ratio of Δ^9-tetrahydrocannabinol. The insets are condensed plots for Δ^9-tetrahydrocannabinol, and the dashed lines encompass those values plotted in the larger graphs.*

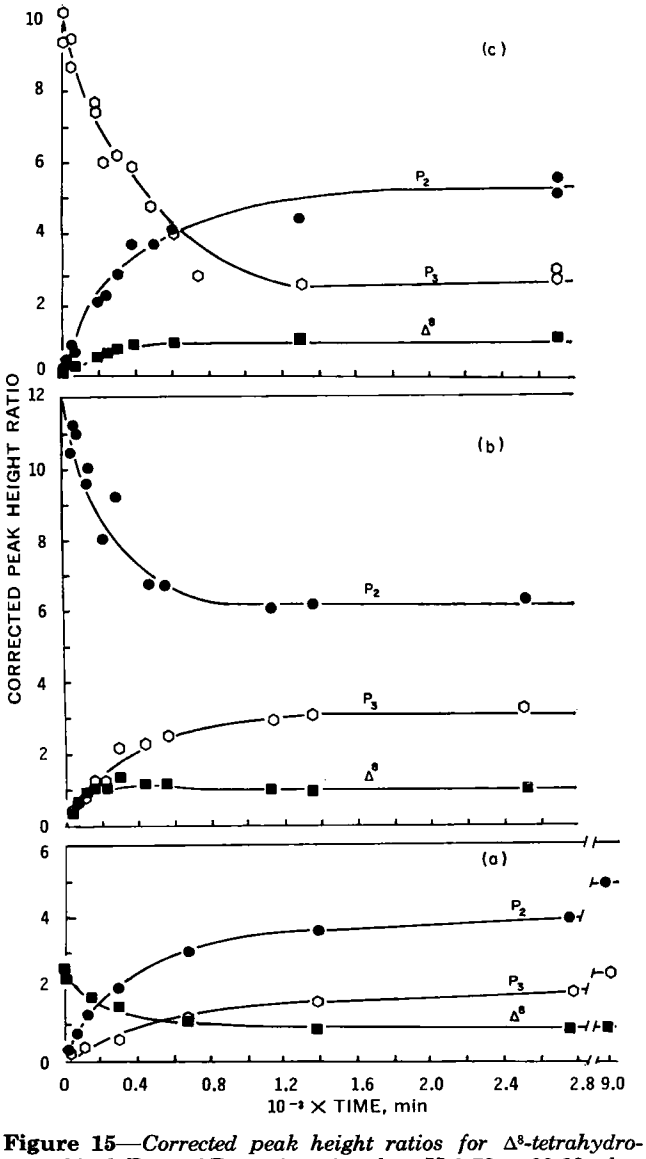


Figure 15—*Corrected peak height ratios for Δ⁸-tetrahydrocannabinol, P_2, and P_3 against time for pH 0.79 at 60.8° when the initial reactant, as material isolated by TLC, was* (a) *Δ⁸-tetrahydrocannabinol,* (b) *P_2, and* (c) *P_3 and their observed initial peak height ratios were 1.0, 1.0, and 1.8, respectively. The corrected peak height ratios were calculated on the premise that a unit peak height ratio of P_2 and P_3 would represent the same number of molecules as one unit of peak height ratio of Δ⁸-tetrahydrocannabinol when all three compounds had the same retention times. This would necessitate multiplying the observed peak height ratios at the observed retention times by 1.0 for Δ⁸-tetrahydrocannabinol, 2.8 for P_2, and 3.7 for P_3 in each graph. Each resultant ordinate value for each graph was actually multiplied by an appropriate factor so that the final value of the corrected peak height ratio for Δ⁸-tetrahydrocannabinol with time would be unity and the other corrected peak height ratios in each graph would be plotted relative to this value for proper comparison among the various graphs when either Δ⁸-tetrahydrocannabinol, P_2, or P_3 was used as the starting material. Thus, if Y is the actual final value of the peak height ratio of Δ⁸-tetrahydrocannabinol, the peak height values of P_2 at a given time in a particular graph were multiplied by 2.8/Y, those of P_3 were multiplied by 3.7/Y, and those of Δ⁸-tetrahydrocannabinol were multiplied by 1.0/Y. The actual values of Y were:* (a) *0.37,* (b) *0.24, and* (c) *0.64.*

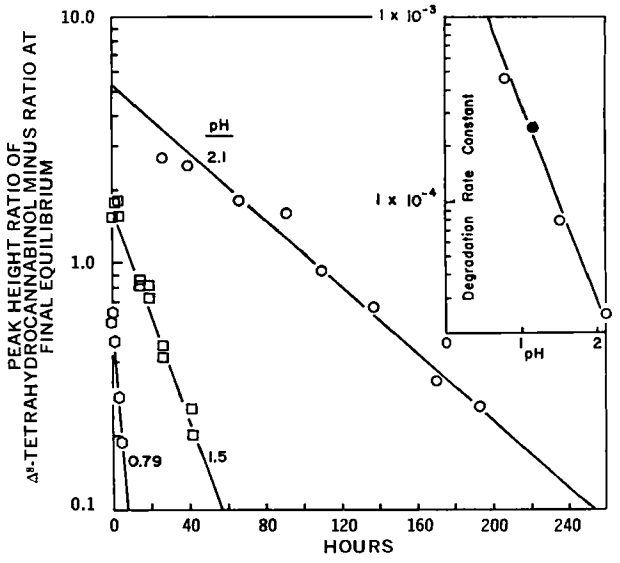


Figure 16—*Semilogarithmic plots with time of the difference between observed peak height ratios of Δ⁸-tetrahydrocannabinol at any time and the equilibrium value for degradations at various pH values. The inset is the log k–pH profile for these degradations plus a synthetic mixture (solid symbol) of Δ⁸- and Δ⁹-tetrahydrocannabinols.*

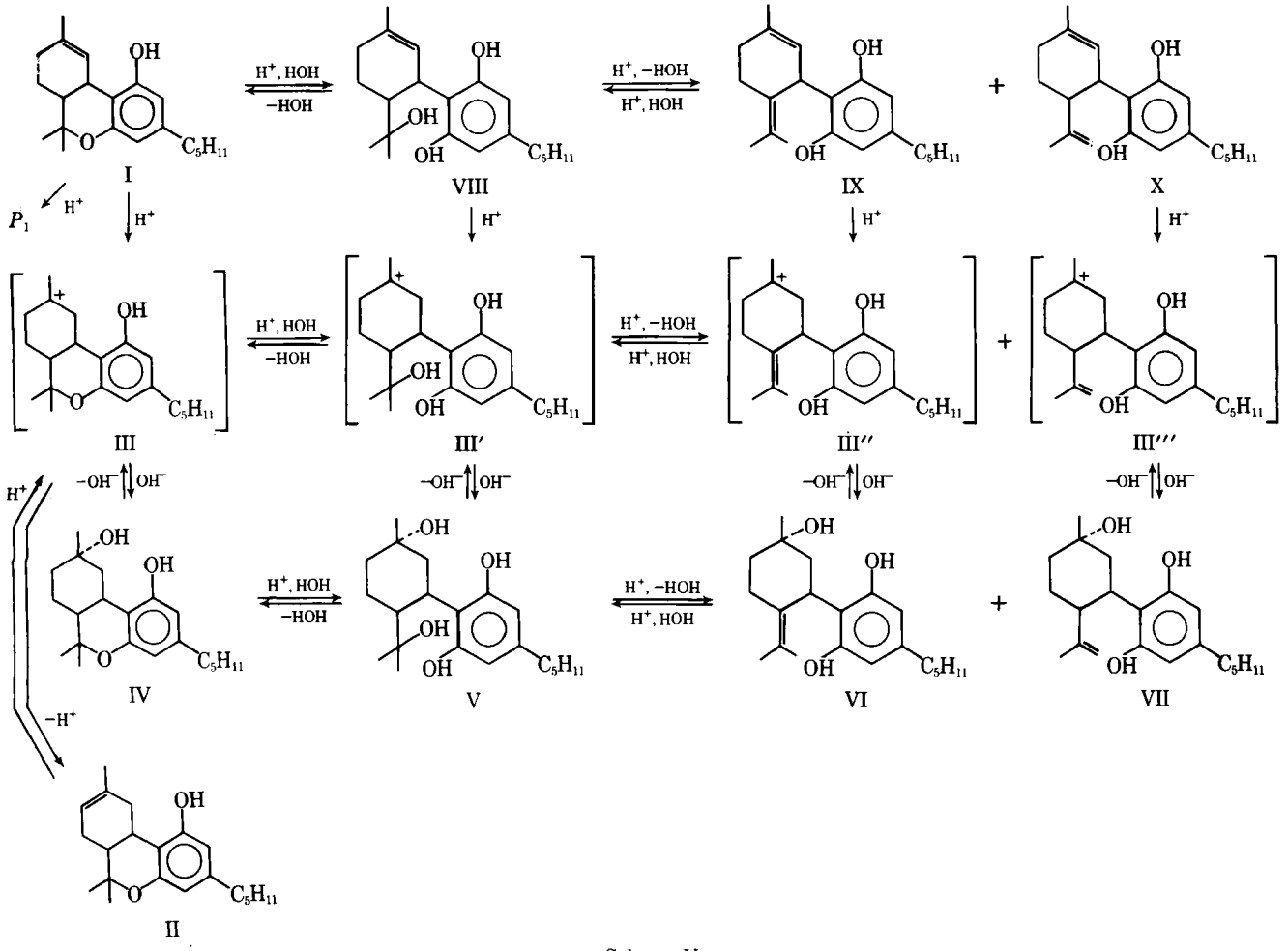
assigned to VI and IV, respectively; Δ⁹-tetrahydrocannabinol produces P_1, P_2, P_3, and Δ⁸-tetrahydrocannabinol. Thus, it is reasonable to postulate that the final product at the P_1 retention time is only a product of Δ⁹-tetrahydrocannabinol and not of Δ⁸-tetrahydrocannabinol and that P_1 and the equilibrating P_2, P_3, and Δ⁸-tetrahydrocannabinol are formed irreversibly from Δ⁹-tetrahydrocannabinol (Scheme V). Therefore, it may be concluded that the hydrogen-ion-catalyzed migration of the double bond favors the Δ⁸-isomer thermodynamically and that P_1 is not in equilibrium with it, P_2, or P_3. This does not exclude a preliminary equilibration among I, VIII, IX, and X where any one of these latter three may initially appear at the retention time of P_1.

The fact that Δ⁹- or Δ⁸-tetrahydrocannabinol in the more neutral pH regions does not produce Δ⁸-tetrahydrocannabinol, P_1, P_2, or P_3 implies that these products are results of hydrogen-ion attack and can be assigned to the k_1 and/or k_2 processes, where pH dependency is characterized by Eqs. 5 and 6. The pH-independent degradation characterization of Δ⁹-tetrahydrocannabinol by k of Eq. 4 must produce a product that was not observed by these GLC procedures.

The transformations of Scheme V readily explain why the major product of Δ⁹-tetrahydrocannabinol in acidified organic solvents or in self-emulsions is Δ⁸-tetrahydrocannabinol since the ether solvolyses and isolated double bond additions require water. In aqueous organic solutions, the activity of water would be lessened and tetrahydrocannabinol would also be favored over the products of hydrolysis or hydration (Fig. 7).

The sums of the radioactivities of the TLC spots assigned to acid-degrading Δ⁹- and Δ⁸-tetrahydrocannabinols, P_1, P_2, and P_3 (Table III) account for approximately 75% of the initial radioactivity of Δ⁹-tetrahydrocannabinol so that it can be concluded that the compounds at these R_f values comprise the most significant products of acid degradation. This does not necessarily mean that the peaks observed from the elution of these spots include the total number of radiolabeled molecules in the spots, since a compound of similar R_f value may be nondetectable by GLC. In fact, the inability of the sums of the observable corrected peak heights to remain invariant with time of degradation indicates that such may be the case. Approximately 12% of the total radioactivity remained at the origin and 12% could not be accounted for by the designated spots and the origin for all times of degradation. However, the fact that 86–90% of the total radioactivity (Table III) was extracted by chloroform from the reacting solution shows that there is a high extraction efficiency of all possible products.

Degradations of Δ⁹-Tetrahydrocannabinol in Alkaline Solutions—A few studies on Δ⁹-tetrahydrocannabinol degradation were conducted at highly alkaline pH values (Fig. 5) under nitrogen. Significant time lags were found at pH 10.5, 11.2, and 12.2 be-



Scheme V

fore the loss of compound became truly first order. In the last case there was little significant change in Δ^9-tetrahydrocannabinol for as long as 200 hr at 60.8°.

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[x] To whom inquiries should be directed.

Identification and Synthesis of Di-(1-phenylisopropyl)methylamine, an Impurity in Illicit Methamphetamine

KEITH BAILEY *x, JACQUES G. BOULANGER ‡, DONALD LEGAULT *, and STÉPHANE L. TAILLEFER ‡

Abstract □ The isolation, purification, and identification of di-(1-phenylisopropyl)methylamine, present in illicit samples of methamphetamine, are described. The identity of the substance was confirmed by synthesis. It is commonly present in amounts ranging between 3 and 15% relative to methamphetamine.

Keyphrases □ Di-(1-phenylisopropyl)methylamine—identification in illicit methamphetamine, NMR, IR, and mass spectroscopy □ Methamphetamine—identification and synthesis of di-(1-phenylisopropyl)methylamine as an impurity in illicit samples

Methamphetamine (I) is a drug popularly used and readily available on the illicit market. It is synthesized in clandestine laboratories on the North American continent and may contain various contaminants. A major impurity in illicit methamphetamine was recently identified as *N*-methyl-*N*-(α-methylphenylethyl)formamide (*N*-formylmethamphetamine, II) (1). A second substance in illicit methamphetamine has now been isolated and identified spectroscopically as di-(1-phenylisopropyl)methylamine (III). The identity was confirmed by synthesis of III.

EXPERIMENTAL[1]

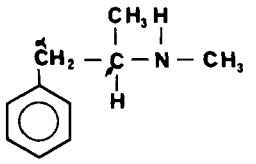
Isolation and Purification of III—The sample of illicit methamphetamine hydrochloride (about 1 g) was dissolved in 0.5 *N* HCl (about 20 ml) and extracted with ether. The ethereal extract contained the *N*-formylmethamphetamine (II) present (1). The aqueous phase was extracted with chloroform and the chloroform was evaporated, leaving a residue of crude III-HCl which, after triturating and washing with ether, had a melting range of 125–184°. (Methamphetamine could be recovered from the aqueous phase.) The material was recrystallized from a mixture of isopropanol and hexane, which raised the melting point to 183–192°, but scarcely changed the proton NMR or IR spectra. The free base was liberated from the salt with potassium carbonate solution, extracted into ether, and recovered as an almost colorless oil.

Synthesis of III—*d,l*-Amphetamine (2.70 g) and phenylacetone (2.68 g) were mixed in ethanol (20 ml) and kept at room temperature for 65 hr. The resulting solution was hydrogenated overnight in a Parr apparatus using 10% palladium-on-carbon (100 mg) as catalyst and an initial hydrogen pressure of 40 psi. The solution was filtered, concentrated, and adsorbed onto a column of silica gel (250 g) packed with chloroform. Elution with chloroform (2 liters) afforded unidentified products; elution with 5% methanol in chloroform yielded di-(1-phenylisopropyl)amine (IV, 360 mg), which was kept with formic acid (90%, 1 ml) and formaldehyde solution (36%, 1.4 ml) at 80° overnight.

The mixture was cooled, made basic with 2 *N* NaOH, and extracted with ether. The extract was dried, concentrated, and adsorbed onto silica gel packed with hexane in a Pasteur pipet. Elution with hexane afforded the tertiary amine III (60 mg), which was identical by NMR, IR, mass spectrometry, and GLC analyses with the free base isolated from illicit methamphetamine. Attempts to convert the base into a crystalline hydrochloride were not successful. A product chromatographically and spectroscopically indistinguishable from synthesized III resulted when the reaction sequence was applied to *d*-amphetamine.

RESULTS AND DISCUSSION

The isolated solid was readily soluble in water, giving a solution of pH about 5 (to test papers) which produced a whitish precipitate (soluble in ammonium hydroxide solution) on adding silver nitrate solution. Therefore, the solid was suspected to be the hydrochloride salt of an amphetamine derivative. The NMR spectrum of the salt, mp 125–184°, is shown in Fig. 1, and the effect of adding deuterium oxide is illustrated in Fig. 2.



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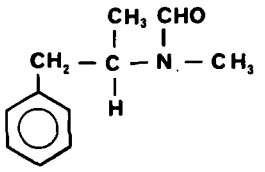

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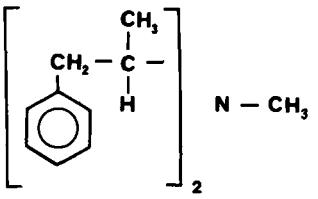
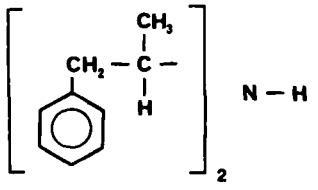


III



IV

[1] GLC was carried out on a Bendix 2500 instrument fitted with 1.8-m (6-ft) glass columns containing 3% OV-1 on Chromosorb W-H-P, 80–100 mesh, and on a Varian Aerograph 1520 fitted with 1.8-m (6-ft) glass columns containing 5% OV-7 on 80–100-mesh Chromosorb W; the carrier gas was nitrogen at 30 ml/min. Optical rotation was measured on a Perkin-Elmer 141 polarimeter (courtesy of Carleton University, Ottawa, Canada). NMR spectra were recorded at 40° on a Varian A-60A spectrometer using solutions in deuterochloroform containing tetramethylsilane as the internal standard. IR spectra were measured on a Unicam SP 1000 spectrophotometer using natural films between sodium chloride plates for the free bases and mineral oil mulls for the salts. A Hitachi Perkin-Elmer RMU-6L mass spectrometer was used for determining mass spectra, with the probe at 160° and an ionizing voltage of 70 ev.

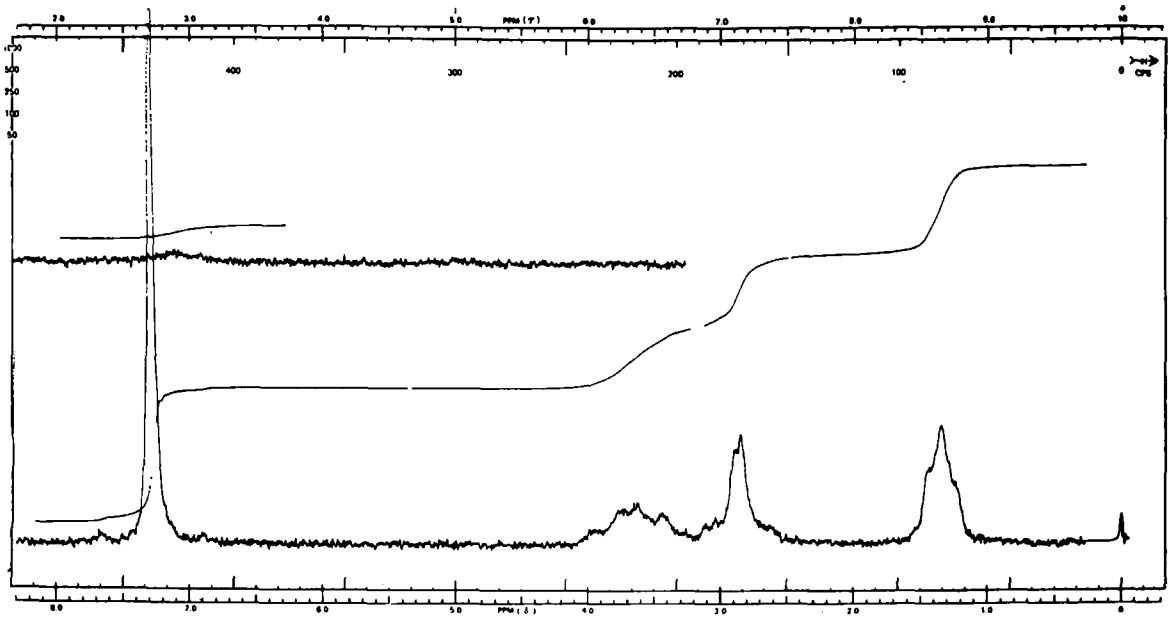


Figure 1—*NMR spectrum of di-(1-phenylisopropyl)methylamine hydrochloride in $CDCl_3$.*

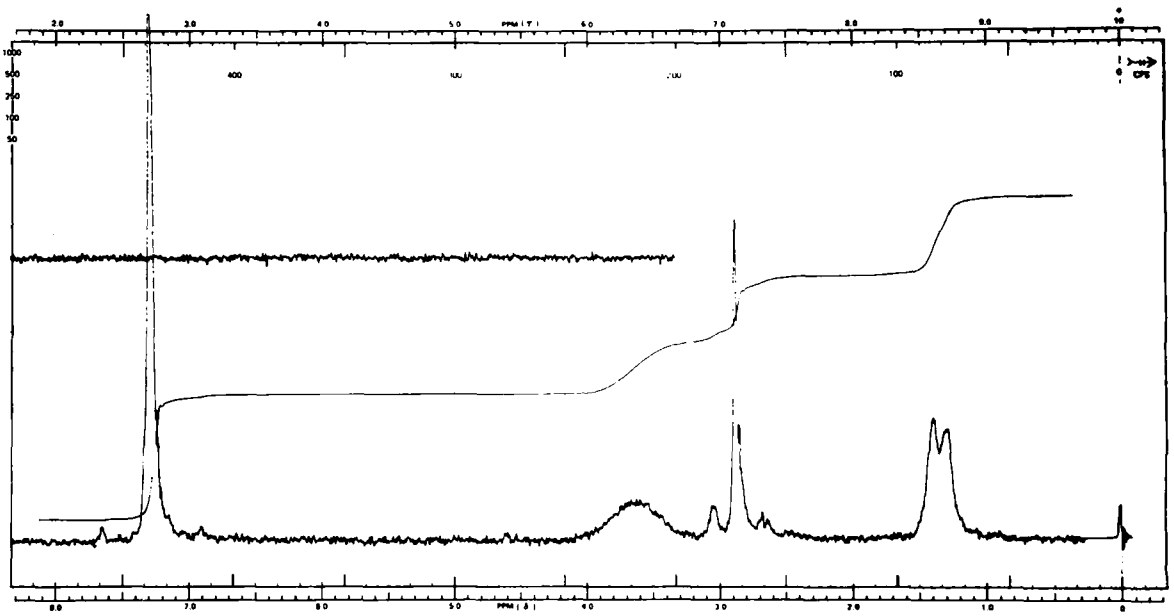


Figure 2—*NMR spectrum of di-(1-phenylisopropyl)methylamine hydrochloride in $CDCl_3$ with D_2O added.*

In Fig. 1, the smallest integration occurs at δ 12.1 (ppm downfield of tetramethylsilane) and is assigned to $^+$N—H; the remaining signals then account for about 26 protons and, with reference to Structure III, are assigned as follows: δ 7.2–7.35 (10), protons of two equivalent monosubstituted aromatic rings; 2.5–4.1 (10), α, β, and N—CH_3 protons (N—CH_3 at 2.86 ppm; the integration between 2.5 and 4.1 ppm indicates that there is one more proton present which could be due to moisture); and 1.35 (six), β-CH_3 protons.

The spectrum of the free base is shown in Fig. 3 and assignments are: δ 7.2, aromatic ring protons; about 2.3–3.3, α, β, and N—CH_3 protons (N—CH_3 at 2.87 ppm); and 0.96, β-CH_3 protons. Protonated III (Fig. 1) shows coupling between the $^+$NH and the β-CH_3 and the $^+$NH and the N—CH_3 protons, which is lost on adding D_2O (Fig. 2) or liberation of the base (Fig. 3), with the β—CH_3 and N—CH_3 then appearing as a doublet and singlet, respectively. The small signal at δ 1.27 in Fig. 3 arises from the long-chain aliphatic protons of portions of an impurity. Apart from this, the NMR spectra of the base III synthesized from either *d*- or *d,l*-amphetamine were identical.

The IR spectrum of the salt, mp 125–184°, was unchanged on recrystallization (Fig. 4). The IR spectra of the base from either illicit drug or synthetic III starting from *d,l*- or *d*-amphetamine were essentially identical (Fig. 5). Of particular note are the bands at 2450 cm^{-1} in Fig. 4, assigned to $^+$N—H stretching, the lack of N—H stretching bands (about 3300–3500 cm^{-1}) in Fig. 5, and the bands at about 700 and 745 cm^{-1} in Figs. 4 and 5, indicative of a monosubstituted benzene ring (2).

Virtually identical mass spectra resulted from the salt or free base of illicit or synthetic III from *d,l*- or *d*-amphetamine (Fig. 6). The molecular ion for III is at *m/e* 267. The very intense peak at *m/e* 176 and other major peaks are compatible with the cracking pattern for amphetamines (3, 4) depicted in Fig. 7 for Structure III.

The GLC retention times on OV-7 were the same for nonrecrystallized and recrystallized salts and the free base of illicit or synthetic III.

Mixtures of illicit and synthetic III were not separated. The method for illicit methamphetamine synthesis (1) would be expected to give an optically inactive product, and no optical rota-

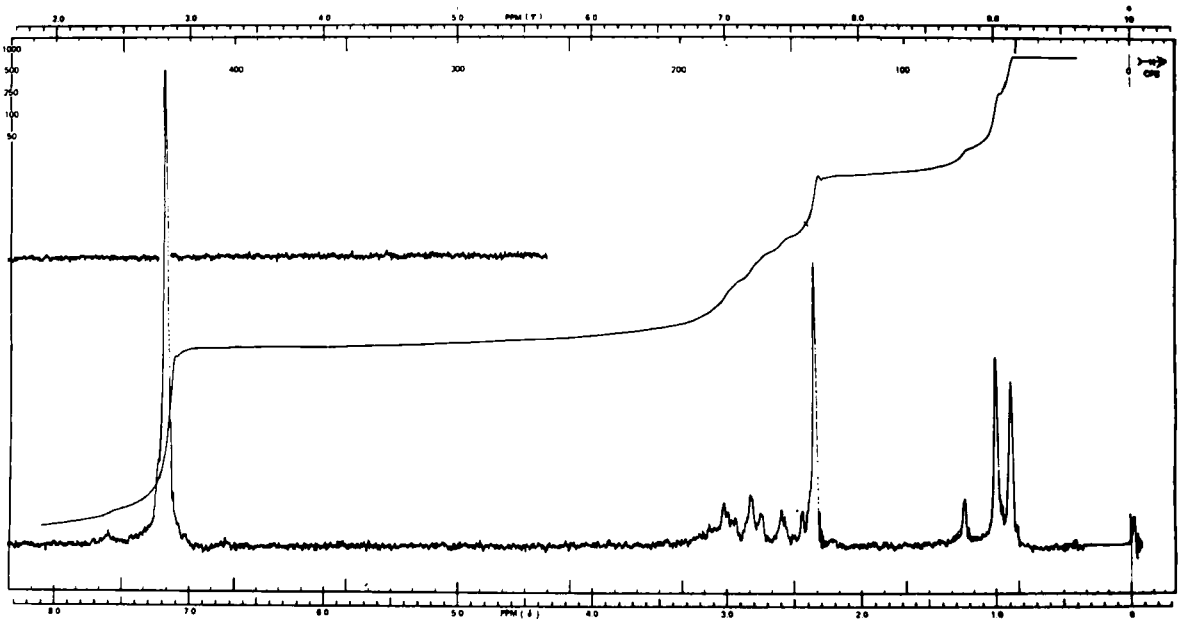


Figure 3—*NMR spectrum of di-(1-phenylisopropyl)methylamine base in $CDCl_3$.*

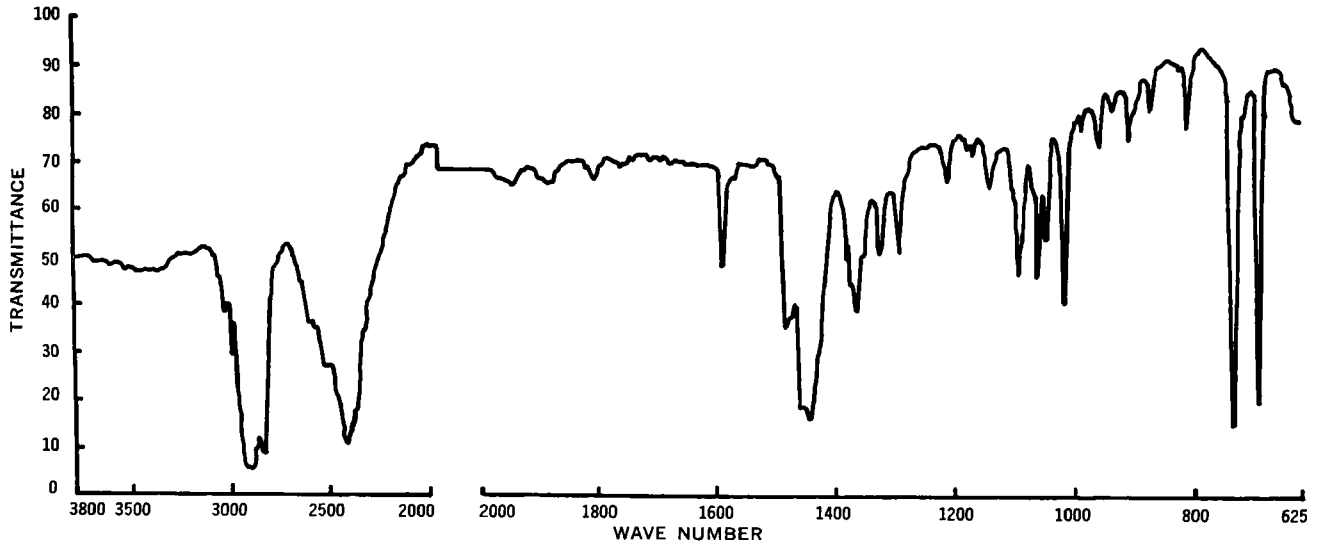


Figure 4—*IR spectrum of di-(1-phenylisopropyl)methylamine hydrochloride in mineral oil.*

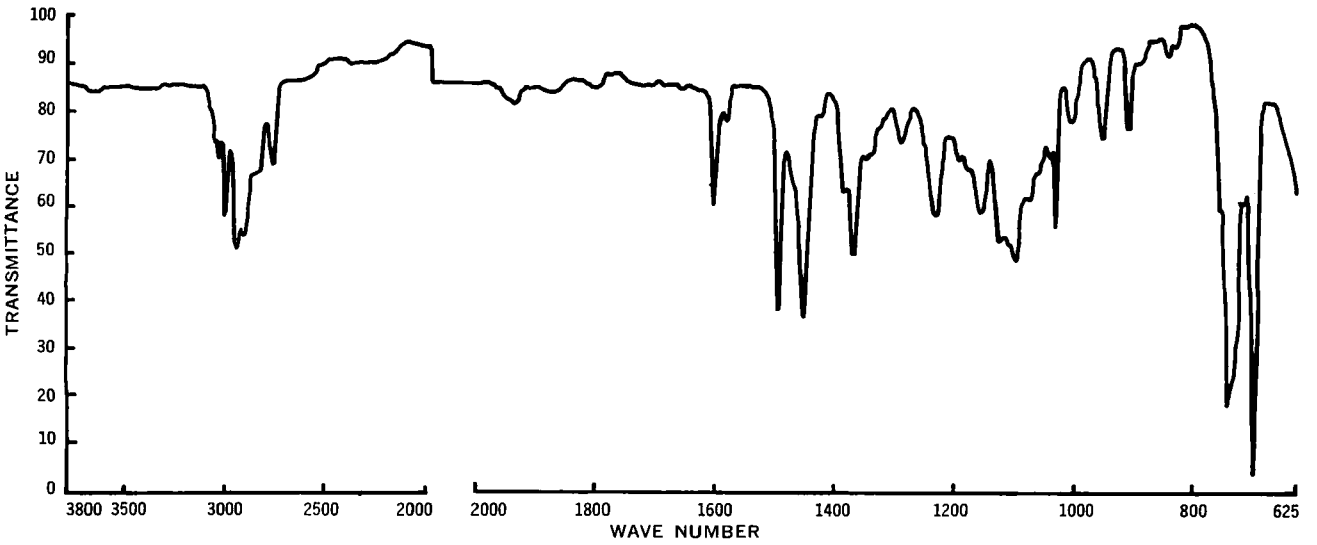


Figure 5—*IR spectrum of di-(1-phenylisopropyl)methylamine base as film.*

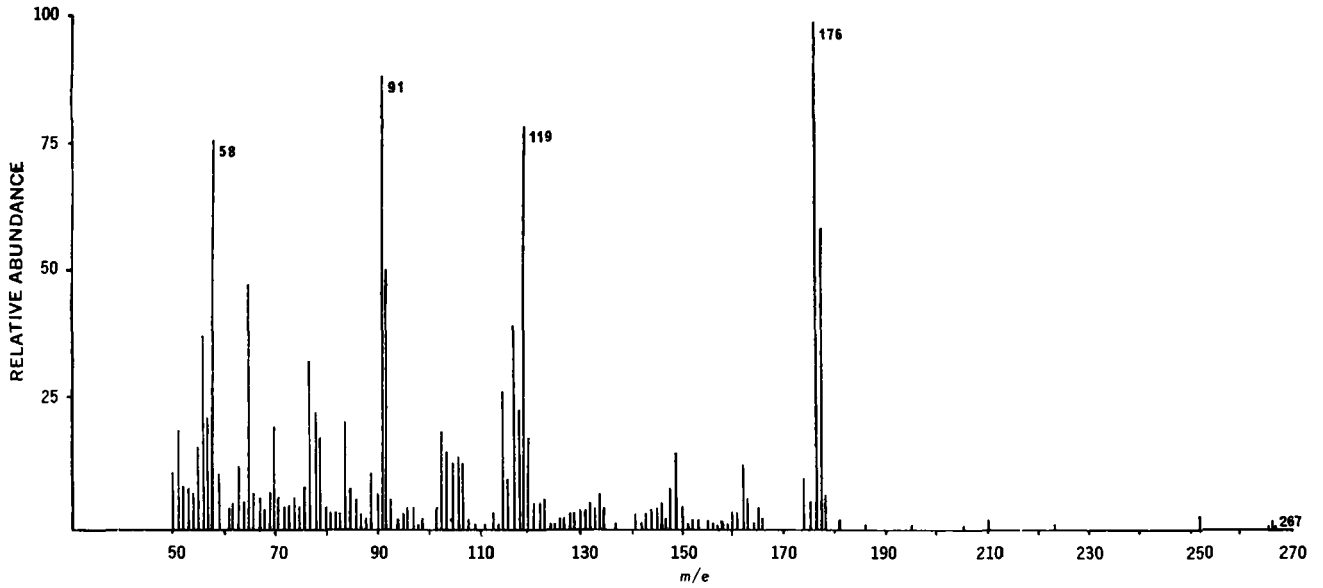


Figure 6—*Normalized mass spectrum of di-(1-phenylisopropyl)methylamine.*

tion was found for methanolic solutions of illicit III at the sodium d line. The synthesis described here, starting from *d,l*-amphetamine, would give a racemic mixture of the *d*- and *l*- and *meso*-forms of III; attempts to convert this mixture into a crystalline hydrochloride salt were not successful. Elemental analyses of the illicit salt, mp 125–184° (found: C, 72.29; H, 8.76) and mp 183–192° (found: C, 72.35; H, 8.53), were indicative of water content (for III, $C_{19}H_{25}N{\cdot}HCl{\cdot}\frac{1}{2}H_2O$ requires C, 72.90; H, 8.70), in corroboration of the NMR result already described.

The impurity in illicit methamphetamine is concluded to be di-(1-phenylisopropyl)methylamine (III). Quantitation by GLC of eight illicit methamphetamine samples received recently indicated that between 3 and 15% of III was present relative to methamphetamine.

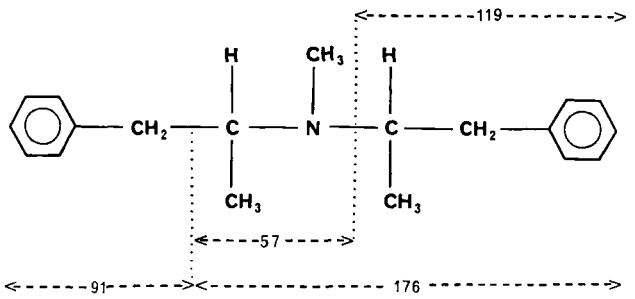


Figure 7—*Amphetamine cracking pattern for di-(1-phenylisopropyl)methylamine.*

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ˣ To whom inquiries should be directed.

GLC Determination of Warfarin in Human Plasma

DAVID G. KAISER ˣ **and ROBERT S. MARTIN**

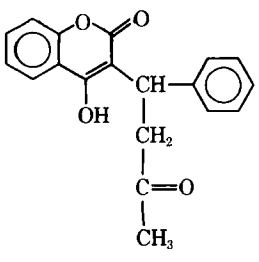
Abstract □ To evaluate the pharmacokinetics and potential drug interactions at relatively low doses (*i.e.*, 7.5 mg), a method for the determination of warfarin in human plasma was required. A sensitive and specific procedure was developed based on: (*a*) ethylene chloride extraction of the acidified specimen, (*b*) TLC of the ethylene chloride extract residue, (*c*) formation of the pentafluorobenzyl derivatives of the materials eluted from the thin-layer chromatogram, and (*d*) quantification of the pentafluorobenzyl derivatives by GLC, utilizing electron-capture detection. The lower level of assay detection sensitivity for measurement of intact warfarin in plasma is 0.02 μg/ml. Statistical analyses indicate a recovery of 98.8%, with a standard deviation of ±10.9%. IR and mass spectrometric analyses, in conjunction with GLC, confirmed that the intact drug was being measured and that no interferences from blank plasma were observed. The procedure was successfully applied to drug absorption studies in humans.

Keyphrases □ Warfarin—GLC determination in human plasma □ Anticoagulants—GLC determination of warfarin in human plasma □ GLC—analysis, warfarin in human plasma

The spectrophotometric method (1) for measurement of warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] (I) in human plasma has been utilized extensively (2–8) to study the absorption, distribution, and metabolism of this potent anticoagulant drug after single-dose oral administration of relatively large amounts (*i.e.*, 25–100 mg). Similarly, the fluorometric method (9) was employed to study the enantiomers of I in humans after single-dose oral administration of 15–100 mg (10). TLC, in conjunction with spectrophotometric or fluorometric techniques, has been used to determine or improve assay specificity for measurement of the intact drug and its metabolites in biological matrixes (6, 10–15).

Recently, the GLC behavior of several coumarin anticoagulants as well as their trimethylsilyl ethers, acetates, trichloroacetates, and trifluoroacetates was reported (16, 17), suggesting that derivatization of I with an electron-capturing group followed by GLC electron-capture detection might be useful for measuring relatively low drug concentrations in plasma specimens (*e.g.*, <1 μg/ml).

To study the pharmacokinetics and potential drug interactions with other therapeutic agents, after single- and multiple-dose oral administrations of relatively low amounts of I (*viz.*, 7.5 mg), a highly sensitive and specific GLC method for measurement of the intact drug in plasma was developed. Subsequent to these investigations, a high-pressure liquid chromatographic method was reported for measurement of I in human plasma (18). After single-dose oral administration of 0.75 mg drug/kg body weight, plasma I concentrations ranged from 1 to 10 μg/ml.



I

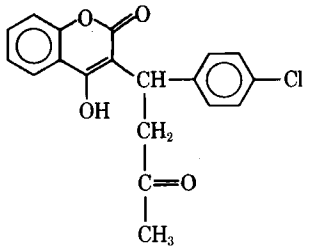
EXPERIMENTAL

Reagents and Materials—Warfarin (I) was used as supplied[1]. The 3-(α-acetonyl-*p*-chlorobenzyl)-4-hydroxycoumarin[2] (II), selected as an internal standard, was purified by chromatography on a column of silica gel 60[3] (4.8 × 35 cm) using a solvent system composed of 10% (v/v) acetone and ethylene chloride. Acetone, ethylene chloride, hexane, and methanol, distilled in glass, were used as supplied[4]. Stock solutions of I in 0.01 *N* aqueous sodium hydroxide (100 μg/ml), II in acetone (100 μg/ml), pentafluorobenzyl bromide[5] in acetone (0.1 ml/100 ml), aqueous sodium hydroxide (0.01 *N*), and aqueous sulfuric acid (3 *N*) were stored in glass containers. All other chemicals were analytical reagent grade. Phenyl methyl silicone fluid (OV-17) on 80–100-mesh Gas Chrom Q (1% w/w) was used as supplied[6].

Instrumentation—A two-speed reciprocating shaker[7] was used for shaking the samples in the horizontal position. A mixer[8] was used to aid in preparing the samples and eluting the zones removed from thin-layer chromatograms. GLC measurements were made with a chromatograph[9] equipped with a nickel-63 electron-capture detector and a −0.1–1.0-mv dual-pen recorder[10]. The detector was operated at 14.5 v in the dc mode. All cylinders of gases (*i.e.*, nitrogen) used for chromatography were fitted with filters containing molecular sieve 4A.

TLC—All analytical chromatography was conducted on thin layers (250 μm) of silica gel F_{254}[11], ascendingly developed in a solvent system composed of 10% (v/v) acetone and ethylene chloride. The separated materials were visualized by irradiation of the plates with a short wavelength (254 nm) UV lamp. Under these conditions, I and II have the same R_f value of 0.40.

GLC—All chromatography was conducted using U-shaped glass columns (0.61 m × 3 mm i.d.) packed with 1% (w/w) OV-17 on 80–100-mesh Gas Chrom Q. All newly prepared columns were preconditioned at 275° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and electron-capture detector were maintained iso-



II

[1] S. B. Penick & Co., Chicago, Ill.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] EM Laboratories, Inc., Elmsford, N.Y.
[4] Burdick & Jackson Labs, Inc., Muskegon, Mich.
[5] Pierce Chemical Co., Rockford, Ill.
[6] Applied Science Labs. Inc., State College, Pa.
[7] Eberbach & Sons, Ann Arbor, Mich.
[8] Vortex model K-500, Scientific Industries, Queens Village, N.Y.
[9] Tracor model MT-220, Tracor, Inc., Austin, Tex.
[10] Westronics, Inc., Fort Worth, Tex.
[11] Brinkmann Instruments, Westbury, N.Y.

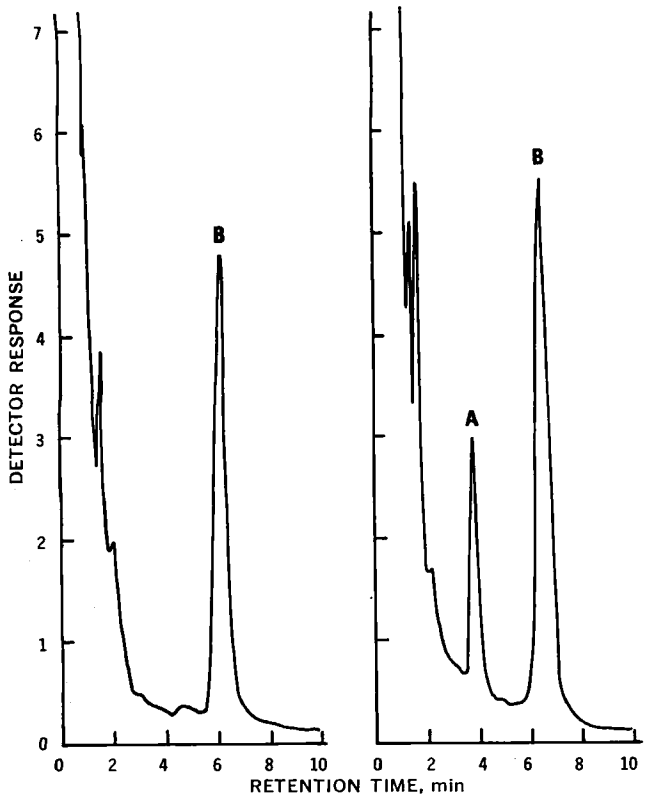


Figure 1—*Gas–liquid chromatograms of human plasma extracts. Left: normal plasma specimen. Right: plasma specimen from subject at 2 hr after single-dose oral administration of 7.5 mg warfarin. Key: A, pentafluorobenzyl derivative of warfarin; and B, II-pentafluorobenzyl derivative.*

thermally at 240, 275, and 285°, respectively. Flow rates of nitrogen gas, used as carrier and purge, were maintained at 75 and 30 ml/min, respectively. Under these conditions, the pentafluorobenzyl derivatives of I and II had retention times of 3.6 and 6.1 min, respectively (Fig. 1).

Synthesis of Standard Materials—Place 100 mg of I in a suitable reaction flask containing a mechanical stirrer and reflux condenser. Add 10 ml of acetone, 0.5 ml of undiluted pentafluorobenzyl bromide, and 1 g of anhydrous potassium carbonate and heat in a water bath at 60° for 5 hr. Evaporate to dryness with a gentle stream of nitrogen gas. Add 2 ml of water and 5 ml of cyclohexane. Shake for 10 min and allow the phases to separate. Transfer the cyclohexane to a 15-ml glass-stoppered centrifuge tube. Repeat the extraction with three additional 5-ml portions of cyclohexane and evaporate the pooled cyclohexane extracts to dryness with nitrogen gas.

Reconstitute the cyclohexane extract residue in 10 ml of 10%

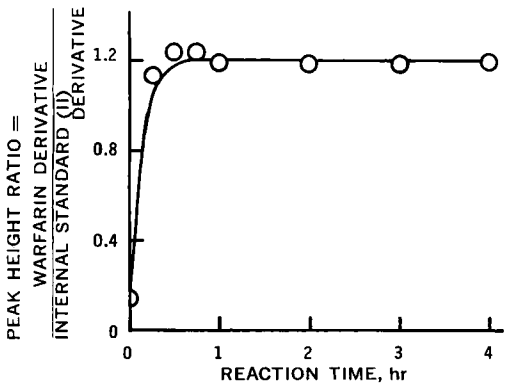


Figure 2—*Effect of reaction time on formation of warfarin pentafluorobenzyl derivative.*

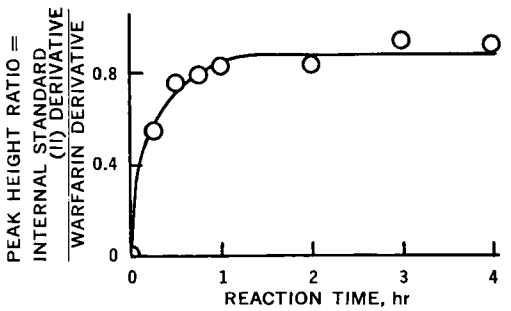


Figure 3—*Effect of reaction time on formation of internal standard pentafluorobenzyl derivative.*

(v/v) acetone in ethylene chloride. Apply the solution to a silica gel column (4.8 × 35 cm) and develop at a flow rate of 3 ml/min with solvent composed of 10% (v/v) acetone in ethylene chloride. Collect 10-ml fractions and monitor each fraction by TLC (*vide supra*). Pool fractions 22–31, remove the solvent under reduced pressure (15 mm), and recrystallize from methanol–ether and water. The pentafluorobenzyl derivative of I is isolated as white crystalline material, mp 107–108°.

Anal.—Calc. for $C_{26}H_{17}F_5O_4$: C, 63.93; H, 3.52; F, 19.45. Found: C, 63.60; H, 3.77; F, 19.68.

Synthesize standard material for identification of the II-pentafluorobenzyl derivative, utilizing the same general reaction conditions as described for I. Recrystallize from methanol–ether and water. The pentafluorobenzyl derivative of II is isolated as white crystals, mp 137–138°.

Anal.—Calc. for $C_{26}H_{16}ClF_5O_4$: C, 59.72; H, 3.09; Cl, 6.78; F, 18.17. Found: C, 60.25; H, 3.37; Cl, 6.58; F, 17.56.

Assay Procedure—*Preparation of Plasma Standards*—Pipet 0.1-ml aliquots of the II stock solution, diluted to 20 μg/ml, into a series of 15-ml glass-stoppered centrifuge tubes. Evaporate to dryness with a gentle stream of nitrogen gas. Pipet 0.5-ml aliquots of I stock solution, diluted to 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 μg/ml, into successive centrifuge tubes containing the internal standard. Add 1 ml of control plasma to each centrifuge tube and mix well. Prepare an appropriate blank. Extract all standards in the same manner as described later for the plasma specimens.

Preparation of Samples—Pipet 0.1-ml aliquots of the II stock solution (20 μg/ml) into a series of centrifuge tubes and evaporate to dryness with a gentle stream of nitrogen gas. Add 0.5 ml of aqueous sodium hydroxide (0.01 *N*) and 1 ml of plasma and mix well. Add 0.5 ml of 3 *N* aqueous sulfuric acid and 10 ml of ethylene chloride and shake in the horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm and aspirate off the aqueous layer. Transfer an 8-ml aliquot of the ethylene chloride layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with a gentle stream of nitrogen gas. Wash down the walls of the centrifuge tube with 1 ml of ethylene chloride and evaporate to dryness with nitrogen.

Reconstitute the ethylene chloride extract residues from the plasma standards and samples in 100 μl of ethylene chloride. To-

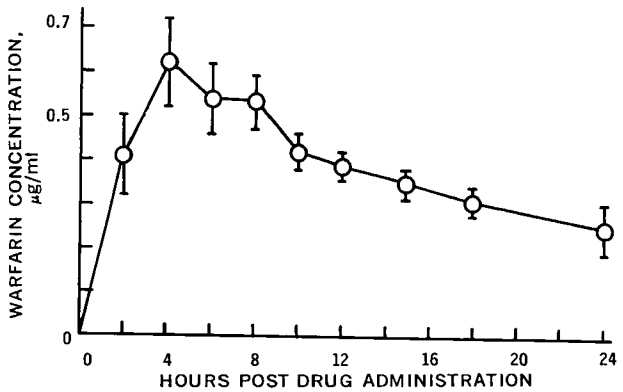


Figure 4—*Mean* (±SEM) *plasma concentrations of warfarin* versus *time in humans* (n = 6) *after single-dose oral administration of 7.5 mg of drug as compressed tablets.*

gether with appropriate standards, spot all of each sample on thin layers of silica gel F_{254} (*vide supra*). Ascendingly develop each plate for a distance of 165 mm, air dry all chromatograms thoroughly at room temperature (24°), and visualize the zones by irradiation with a 254-nm UV lamp. Scrape the zones corresponding to I into separate glass-stoppered centrifuge tubes. Add 2 ml methanol to each tube, shake for 10 min in a horizontal position, and centrifuge for 10 min at 2000 rpm. Transfer 1 ml of the methanol layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with nitrogen gas.

Add 25 mg anhydrous potassium carbonate and a 0.5-ml aliquot of the pentafluorobenzyl bromide stock solution to each centrifuge tube. Stopper tightly and heat in a water bath at 60° for 1 hr. Evaporate to dryness with nitrogen gas. Add 1 ml of water and 2 ml of hexane, shake for 10 min, and allow the phases to separate. Inject a 1-μl aliquot of the hexane layer for analysis into the chromatograph.

Calculations—The peak heights for I- and II-pentafluorobenzyl derivatives are measured. Peak height ratios are obtained by dividing the peak height of the I-pentafluorobenzyl derivative by the peak height of the II-pentafluorobenzyl derivative. Calibration curves from known concentrations of I in plasma are prepared by plotting peak height ratios *versus* I concentration, expressed as micrograms per milliliter. Values for unknown concentrations of I in plasma specimens, obtained in the same manner, are then read directly from the graph or calculated from the slope of the standard curve.

Drug Administration to Humans—*Informed written consent* was obtained from each of six normal human male volunteers prior to participation in the study. All subjects were between the ages of 27 and 51 years and ranged in body weight from 64.1 to 91.8 kg and in height from 1.63 to 1.85 m. All subjects fasted for 16 hr prior to drug administration. Each subject received three tablets (each tablet containing 2.5 mg of I) and 8 fl oz of water. Food was withheld for an additional 2 hr. Blood specimens (10 ml) were withdrawn into heparinized syringes at predetermined time intervals from 0 to 24 hr after drug administration. The plasma was harvested and stored at −18°.

RESULTS AND DISCUSSION

Synthesis and Identification of Pentafluorobenzyl Derivatives—The utility of pentafluorobenzyl bromide as a reagent for the microdetermination of phenols, mercaptans, and organic acids in water (19, 20), as well as a potent nonsteroidal anti-inflammatory agent in human plasma (21), has been reported. A series of samples containing known amounts of I and II were prepared to determine optimal reaction times for the formation of the pentafluorobenzyl derivatives. In studies with I, known amounts of the II-pentafluorobenzyl derivative were added as internal standard. Similarly, in studies with II, known amounts of the I-pentafluorobenzyl derivative were added as internal standard. The results indicated that derivative formation for both compounds was completed within 1 hr (Figs. 2 and 3). In the presence of water and hexane, the derivatives were stable for at least 96 hr.

Synthesis of standard materials showed that the pentafluorobenzyl derivatives of I and II were crystalline at room temperature. GLC, using a solid sample injector and flame-ionization detector, indicated that the pentafluorobenzyl derivatives of I and II submitted for elemental analysis were greater than 99 and 98% pure, respectively. IR and mass spectrometric analyses supported the proposed structures. GLC, in conjunction with mass spectrometry, confirmed that the pentafluorobenzyl derivatives of I and II chromatographed as the intact molecules.

Assay Sensitivity and Specificity—At a sensitivity of 1.6×10^{-11} amp/mv, 0.52 ng of I as its pentafluorobenzyl derivative produced a full-scale response. However, under the assay conditions, the lower limit of detection sensitivity for I in extracts of human plasma is 0.02 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under the assay conditions described, a linear relationship between detector response and concentration is obtained for I over the range of 0–1 μg/ml. Quantification of plasma specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, showed that the material responding to the assay was identical to the known I-pentafluorobenzyl derivative.

Recovery Experiments—Plasma was added to known amounts of I and II contained in centrifuge tubes. The samples were thoroughly mixed and extracted with ethylene chloride. All extract residues were chromatographed on thin layers of silica gel F_{254}, and the zones corresponding to I and II were eluted. The pentafluorobenzyl derivatives were prepared and analyzed *via* GLC. The results indicated that recoveries of I (98.8 ± 10.9%) and II (95.6 ± 3.7%) from plasma were essentially quantitative as compared to an unextracted standard curve of the derivatives.

Plasma Levels of Warfarin in Humans—Results from measurement of plasma I concentrations in six normal human subjects, after single-dose oral administration of 7.5 mg of drug (as three 2.5-mg tablets), demonstrated the utility of the analytical methodology (Fig. 4). A peak mean (±*SEM*) level of I (0.62 ± 0.10 μg/ml) was observed at 4 hr after drug administration. Substantial amounts of intact drug (0.25 ± 0.06 μg/ml) were found in the 24-hr plasma specimens, indicating slow drug disappearance from peripheral circulation. The combined results from these investigations showed that the GLC method could be used for: (*a*) evaluating pharmacokinetics, and (*b*) evaluating potential drug interactions after oral administration of relatively low doses of I (*viz.*, 7.5 mg) to humans.

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[x] To whom inquiries should be directed.

Drug Absorption Analysis from Pharmacological Data III: Influence of Polymers and pH on Transcorneal Biophasic Availability and Mydriatic Response of Tropicamide

VICTOR F. SMOLEN ˣ and RONALD D. SCHOENWALD *

Abstract □ Pharmacological data were employed to determine the time course of transcorneal absorption of the mydriatic drug tropicamide directly to its vicinal site(s) of action (biophase) and peripheral dissipation by other routes following ophthalmic dosing. The method is described and applied to the elucidation of the influence of vehicle composition. Semilogarithmic plots of the time variation of unabsorbed drug were linear; this finding allowed the evaluation of apparent first-order rate constants to characterize the biophasic availability of the drug in each dose studied. The effects of including an anionogenic, cationogenic, and nonionic polymer in the ophthalmic vehicles, buffered at pH 5.0 and 7.4, on the transcorneal absorption and pharmacological response behavior of tropicamide are presented. The concentration of each polymer in the vehicles was adjusted to obtain the same vehicle viscosities in the range of 4.4–16.5 cps. No general patterns of strongly significant effects were found to be attributable to viscosity and the inclusion of the polymers in the vehicles.

Keyphrases □ Ophthalmic drugs—polymer and pH effects on transcorneal biophasic availability and mydriatic response of tropicamide □ Tropicamide—effects of anionogenic, cationogenic, and nonionic polymers and pH on bioavailability and mydriatic response □ Mydriatic drugs—influence of vehicle composition □ Drug absorption—influence of polymers and pH on tropicamide bioavailability □ Bioavailability—tropicamide, polymer and pH effects on transcorneal biophasic availability and mydriatic response

The importance of controlling the pharmacological response characteristics of ophthalmic drugs, particularly mydriatic agents, becomes apparent from considering the wide range of uses of these drugs and the potential hazards that can result from systemic toxicities and unduly prolonged effects (1–3).

Previous studies (4–7) on control of the characteristics of ophthalmic drug response have been concerned with the addition of nonionic polymers, *e.g.*, methylcellulose, to the ophthalmic vehicle. Such effects of polymeric adjuvants have been commonly attributed to an enhanced viscosity of the vehicle causing an increased contact time of the drug with the corneal surface; this influences the amount and rate at which the drug is absorbed to its local sites of action. Adjuvants can also induce effects, independent of viscosity, specific to their chemical nature. This is exemplified by the reported (8) effect of the replacement of magnesium sulfate by sodium sulfate in an atropine preparation; the duration of the mydriatic response dramatically decreased from 26 to 1 hr. It appears that the composition of the ophthalmic vehicle can provide a means by which the solute permeability properties of tissue barriers and, therefore, characteristics of the pharmacological response to ophthalmic drugs may be controlled and ultimately optimized.

A means by which the transference of a drug from its site of administration to its site of action can be studied is a useful tool in approaching this objective. Methods of computing drug absorption have been described (9) and developed (10). However, these methods of drug absorption analysis require the detection of the drug in the blood, urine, or tissues and are not generally applicable for the study of drug absorption from an ophthalmic site of administration. However, the use of pharmacological data for the determination of the cumulative amounts of drug absorbed from a site of administration to the site(s) of action at any time following dosing by any route, as previously described (11–17), can be used. This technique was applied in the present study to determine if vehicle viscosity, pH, and the addition of polymeric materials can influence the pharmacological response and bioavailability characteristics of the mydriatic drug tropicamide following ophthalmic dosing.

Table I—Physical Properties of Polymers

Property	Polymer A[a]	Polymer B[b]	Polymer C[c]
Functionality	Neutral	Anionic	Cationic
Concentrations, expressed as cps (37°)	4.4	5.0	4.4
	8.2	8.1	8.2
	12.0	12.5	11.7
	16.4	16.5	16.7
Viscosity[d] of aqueous solutions at 25°			
0.5%	80	—	—
1.0%	325	1000	1400
2.0%	50,000	—	—

[a] Methylcellulose HG, supplied by Dow Chemical Co., Midland, Mich. [b] Reten A-1, supplied by Hercules, Inc., Wilmington, Del. [c] Reten 205, supplied by Hercules, Inc., Wilmington, Del. [d] Brookfield viscometer.

THEORETICAL

Determination of Transcorneal Biophasic Availability—The details of the manner in which the results of monitoring the time variation of mydriatic response intensity can be employed to determine biophasic drug availability behavior following ophthalmic dosing have been well described and confirmed for tropicamide (11–13). Briefly, however, the time course of the relative cumulative amounts, A_t, of tropicamide that become transcorneally absorbed and traverse the biophase can be computed using Eq. 1:

$$A_t = \frac{Q_B + K_{BO'} \int_0^t Q_B \, dt}{K_{BO'} \int_0^t Q_B \, dt} \qquad \text{(Eq. 1)}$$

where $K_{BO'}$ is an overall elimination constant from the biophase. The value of $K_{BO'}$ was previously reported (11, 13) as 0.051 min^{-1}. The biophasic drug levels, Q_B, are obtained by transforming observed mydriatic response intensities through a dose–effect curve (11–17).

When the clearance of the drug from its site of administration can be described to occur by apparent first-order processes, as is the present case (13), the slope of a linear plot constructed in ac-

Table II—Mydriatic Response Characteristics of Tropicamide following Ophthalmic Dosing[a]

pH of Vehicle	Tropicamide Concentration, % w/v	Onset	t_{max}[b]	Maximum Intensity	Duration	t_m[c]	t_B[d]
7.4	0.010	10.5	17.0	0.603	58.5	13.3	17.7
7.4	0.015	8.8	20.5	0.722	61.0	21.3	15.7
7.4	0.020	9.2	22.5	0.788	73.3	16.0	19.8
7.4	0.030	10.2	27.0	0.936	86.0	24.6	13.3
5.0	0.015	6.1	14.5	0.695	79.9	47.3	21.8

[a] The solutions were approximately isotonic; 0.02 ml of each was instilled into the cul-de-sac of rabbits. Each parameter listed is the average of four determinations on different rabbits. [b] The t_{max} is the time corresponding to the maximum intensity. [c] The t_m is the apparent mydriatic response dissipation half-life and represents least-squares regression values. [d] The t_B is the apparent biophasic drug elimination half-life and represents least-squares regression values.

cordance with Eq. 2 provides a means of evaluating the dissipation rate constant, K_D:

$$\log (1 - A_t) = -K_D t \qquad \text{(Eq. 2)}$$

The dissipation of the drug from its site of administration can be described to occur by the parallel processes of transcorneal absorption and peripheral loss. The latter process can be attributed to volume loss of drug through the lacrimal nasal duct, scleral absorption, and flow of aqueous humor. The dissipation rate constant can be resolved into two contributing constants, K_{AB} and K_{AP}, which describe the dissipation of the drug by transcorneal and peripheral loss routes, respectively; *i.e.*, $K_D = K_{AB} + K_{AP}$. The separate evaluation of each constant is accomplished using Eqs. 2 and 3:

$$\frac{K_{AB}}{K_{AB} + K_{AP}} = \frac{\int_0^\infty Q_B \, dt}{D_e} \qquad \text{(Eq. 3)}$$

where D_e represents an intravenously equivalent ophthalmic dose obtained by extrapolating a plot of log Q_B *versus* time, obtained from the results of ophthalmic dosing, to an ordinate value corresponding to a time of 1.88 min. The basis for this procedure and the rationale for this treatment were fully described previously (13).

EXPERIMENTAL

Materials—Isotonic, phosphate-buffered, tropicamide[1] solutions were prepared in concentrations of 0.010, 0.015, 0.020, and 0.030% (w/v) at pH 7.4; a concentration of 0.015% was prepared at pH 5.0. These solutions were employed for ophthalmic drug administration. Additional ophthalmic solutions were prepared containing tropicamide at pH 7.4 and 5.0 in combination with an anionic, cationic, or nonionic polymer. The polymers selected are listed in Table I along with viscosities of the vehicles. The charged anionic and cationic polymers are linear, water-soluble, polyelectrolytes with molecular weights exceeding 10^6; they retain their ionic functionality over a pH range of 2–12. The concentration of tropicamide in all polymer solutions was 0.015%; the viscosities of the vehicles were determined[2] at 37°. Although these ionogenic polymers are not cleared or recommended for other than experimental use, their presence in the vehicles caused no observable eye irritation or lacrimation.

Four, 3–4-month old, male New Zealand white rabbits were chosen on the basis of: *(a)* their similar pupillary response to varying light intensities, *(b)* the similitude of their mydriatic response to ophthalmic and intravenous doses of tropicamide, and *(c)* the clarity of their pupil definition. This screening procedure served to minimize intersubject variation.

Measurement of Pupillary Response—The experimental arrangement and methodology utilized for the measurement of the rabbit's pupillary diameter have been described previously (11).

The mydriatic response intensity, I, was related to the experimentally determined pupil diameters by Eq. 4:

$$I = \frac{d_t - d_0}{d_0} \qquad \text{(Eq. 4)}$$

where d_t represents the pupil diameter at time t following administration of the drug, and d_0 represents the pupil diameter at time zero.

RESULTS

Mydriatic Response Behavior of Tropicamide—Following ophthalmic dosing, the pupillary diameter was monitored with time and the observed measurements were transformed into mydriatic response intensities, I, using Eq. 4. Values for pharmacological response characteristics, *i.e.*, onset, maximum intensity, time of maximum intensity (t_{max}), and duration of response for each ophthalmic solution employed, are listed in Tables II and III. Onset is defined as the period of time following dosing that is necessary for one-half the maximal mydriatic response to be attained; duration is defined as the period of time between the onset and the time of reappearance of one-half the maximum intensity. Each value listed in the table represents the average of four determinations on different rabbits.

A Student t-test was performed to determine the level of significance for differences appearing in the tables relative to the pH 7.4, 0.015% tropicamide solution without polymer which was used as a standard. When employing a 95% statistical confidence level, only the values of 6.1 and 14.5 min obtained for onset and t_{max}, respectively, with a polymer-free vehicle of pH 5.0 were significantly different from the standard, which provided values of 8.8 and 20.5 min. Although some trends can be found in the results, none of the remaining treatments was significantly different from the standard.

Transformation of experimentally acquired I values into biophasic drug levels, Q_B, was accomplished through the use of the dose–response curve reported earlier (Ref. 11, Fig. 4). Biophasic half-lives, computed from the slopes of the latter, linear segments of plots of log Q_B *versus* time, for each dose and pH are included in Table II. The mydriatic half-life, similarly obtained from log I *versus* time plots, and the drug biophasic elimination half-life, although related, need not necessarily be the same. For any given drug, the precise nature of the relationship between the decay of I and biophasic drug levels depends upon the form of the dose–response relationship (13).

Transcorneal Biophasic Availability—If following ophthalmic administration the dissipation of tropicamide from the surface of the eye can be described by Eq. 3, then a semilogarithmic plot of the fractional amount of drug unabsorbed, $1 - A_t$, *versus* time should be linear. Values of $1 - A_t$ were computed[3] and plotted semilogarithmically (as shown in Figs. 1 and 2 of Ref. 13) for the polymer-free pH 7.4 and 5.0 vehicles. The slope of the regression lines in such plots represents the total proportional rate of loss, K_D, of drug from the site of administration. These values are listed in Table IV along with the linear correlation coefficients of the plots from which they were derived. The transcorneal biophasic availability and peripheral loss constants, obtained with the use of Eq. 3, are also listed in Table IV.

The linearity of the semilogarithmic plots of $1 - A_t$ *versus* time, as evidenced by the values of the linear correlation coefficients, indicates that the transcorneal absorption and peripheral loss of tropicamide occurs by an apparent first-order process, such as passive diffusion, and justifies the computation of the proportional rate constants. Table IV also contains percent biophasic availability, %

[1] Lot No. 2092, supplied by Alcon Laboratories, Fort Worth, Tex.
[2] Brookfield viscometer. A U-L adapter to the viscometer was employed for viscosities of 0–100 cps.
[3] CDC 6500 digital computer.

Table III—Influence of Polymers, Viscosity, and pH of the Vehicle on Mydriatic Response Characteristics of Tropicamide following Ophthalmic Dosing[a]

pH of Vehicle	Solution Administered to Eye: Tropicamide (0.015%) in Presence of Polymer[b]	Onset	t_{max}[c]	Maximum Intensity	Duration
7.4	A, 4.4 cps	9.3	18.0	0.731	71.0
7.4	A, 8.2 cps	8.8	17.5	0.700	60.5
7.4	A, 12.0 cps	10.5	21.0	0.679	67.1
7.4	A, 16.4 cps	9.75	20.0	0.634	69.5
7.4	B, 5.0 cps	12.0	19.0	0.625	63.0
7.4	B, 8.1 cps	8.5	18.5	0.668	74.8
7.4	B, 12.5 cps	10.8	22.0	0.665	58.5
7.4	B, 16.5 cps	9.5	21.5	0.697	66.5
7.4	C, 4.4 cps	9.0	22.5	0.679	67.0
7.4	C, 8.2 cps	10.3	22.5	0.727	69.0
7.4	C, 11.7 cps	11.0	22.0	0.756	80.8
7.4	C, 16.7 cps	10.3	22.0	0.667	67.3
5.0	A, 12.4 cps	8.8	20.5	0.754	76.8
5.0	B, 12.2 cps	8.8	24.5	0.729	65.0
5.0	C, 12.6 cps	12.0	21.5	0.545	61.3

[a] The solutions were approximately isotonic; 0.02 ml of each was instilled into the cul-de-sac of rabbits. Each parameter is the average of four determinations on different rabbits. [b] The polymers are nonionic methylcellulose (A), anionic Reten A-1 (B), and cationic Reten 205 (C). [c] The t_{max} is the time corresponding to the maximum intensity.

BA, values, which represent the percent of the drug dissipated from the surface of the eye which is transcorneally absorbed to reach its sites of action in the biophase. The percent biophasic availability values were computed as equal to $K_{AB}/(K_{AB} + K_{AP}) \times 100$, as discussed previously (13).

The values for percent biophasic availability for tropicamide without the addition of polymeric materials for each dose at pH 7.4 indicate that a range of 45.3–74.9% of the ophthalmic dose was transcorneally absorbed directly into the biophase and the remaining 25.1–54.7% of the dose was dissipated from the surface of the eye by peripheral routes. Also included in Table IV are values of percent biophasic availability for tropicamide at 0.015% as a function of viscosity, polymer functionality, and pH.

DISCUSSION

Influence of pH and Viscosity on Biophasic Availability and Pharmacological Response Behavior—When consideration of "active transport" is neglected, it can be said that solutes traverse tissue barriers by passive diffusion through interstitial spaces or by migration involving tissue binding sites, which can be described as facilitated diffusion (18). Tropicamide is essentially 100% unionized at pH 7.4 (pKa ~ 5). Considering the relative lack of hydrogen bonding sites on the tropicamide molecule and its relative nonpolarity, the contribution of a facilitated diffusion mechanism to the tissue transport of this drug may be expected to be nonexistent or negligible (19, 20). From these considerations and from the linearity of the log $(1 - A_t)$ *versus* time plots, tropicamide is apparently absorbed from the ophthalmic administration site into the biophase by passive diffusion at both pH 7.4 and 5.0. As previously reported (13), a comparison of the pharmacological and biokinetic parameters for the 0.015% solutions of tropicamide without polymer at pH 5.0 and 7.4 showed their differences to be statistically insignificant at the 95% level of confidence. However, the onset, percent biophasic availability, and t_{max} effects exhibited statistical differences between confidence levels of 75 and 92%. The probability level of the difference might have been greater if more than four animals had been studied.

Since the acidic pK of tropicamide is approximately 5.0, a relatively more rapid availability of the drug into the biophase may be expected to occur at pH 7.4, relative to pH 5.0, due to the relatively higher concentration of the more permeable unionized form of the drug (19, 20). However, the time of maximum response for the 0.015% tropicamide vehicle without polymer at pH 5.0 occurred at 13 min as compared to 22 min at pH 7.4. These differences were statistically significant at the 95% confidence level and are antithetical to expectations based on a pH–partition hypothesis (20). A comparison of the absorption rate constants under the same conditions indicated that overall absorption from the surface of the eye was not statistically different.

The increased percent biophasic availability observed at pH 5.0 for 0.015% tropicamide without polymers in the vehicle was equal to 73.8%, relative to 43.3% at pH 7.4. As discussed earlier (13), this difference may be speculatively attributed to the interaction of tropicamide cations with anionic binding sites affixed to the colloids composing the corneal tissue (21).

As shown in Table IV, no systematic effects of viscosity in the

Table IV—Influence of Polymers, Viscosity, and pH of the Vehicle on the Biophasic Availability Characteristics of Tropicamide from Ophthalmic Doses[a]

pH of Vehicle	Solution Administered to Eye	$K_{AB} + K_{AP}$	r^b	K_{AB}	K_{AP}	%*BA*[c]
	Tropicamide Alone					
7.4	0.010	0.0687	0.9766	0.0348	0.0340	50.3
7.4	0.015	0.0528	0.7475	0.0230	0.0300	43.3
7.4	0.020	0.0163	0.9965	0.0454	0.0159	74.9
7.4	0.030	0.0387	0.9905	0.0294	0.0093	47.5
5.0	0.015	0.0667	0.9937	0.0461	0.0206	73.8
	0.015% Tropicamide with Polymer[d]					
7.4	A, 4.4 cps	0.0691	0.9887	0.0422	0.0269	65.6
7.4	A, 8.2 cps	0.0694	0.9772	0.0314	0.0381	49.1
7.4	A, 12.0 cps	0.0426	0.9164	0.0250	0.0176	41.2
7.4	A, 16.4 cps	0.0691	0.9655	0.0245	0.0446	38.6
7.4	B, 5.0 cps	0.0569	0.9355	0.0159	0.0420	31.3
7.4	B, 8.1 cps	0.0390	0.9625	0.0193	0.0197	40.4
7.4	B, 12.5 cps	0.0677	0.9463	0.0218	0.0459	35.8
7.4	B, 16.5 cps	0.0673	0.9502	0.0300	0.0373	49.7
7.4	C, 4.4 cps	0.0403	0.9757	0.0234	0.0179	59.1
7.4	C, 8.2 cps	0.0740	0.9919	0.0483	0.0257	69.5
7.4	C, 11.7 cps	0.0516	0.9946	0.0328	0.0188	70.8
7.4	C, 16.7 cps	0.0640	0.9698	0.0260	0.0381	43.8
5.6	A, 12.4 cps	0.0520	0.9954	0.6410	0.1100	85.1
5.0	B, 12.2 cps	0.0559	0.9000	0.0389	0.0179	75.2
5.0	C, 12.6 cps	0.0400	0.9784	0.0110	0.0290	29.4

[a] The solutions were approximately isotonic; 0.02 ml of each was instilled into the cul-de-sac of rabbits. Each parameter is the average of four determinations on different rabbits. [b] Pearson *r* linear correlation coefficients for plots of log $(1 - A_t)$ *versus* time. [c] The values of percent biophasic availability represent the percent of the dose of drug transcorneally absorbed into the biophase. [d] The concentration of tropicamide in all the polymer vehicles was 0.015%. The polymers are nonionic methylcellulose (A), anionic Reten A-1 (B), and cationic Reten 205 (C).

ranges of 4.4 and 16.5 cps on the absorption characteristics of tropicamide were apparent. Previous studies showed that an increase in viscosity of an aqueous ophthalmic solution results in an increase in contact time between the solution and the cornea, which enables a greater fraction of drug to be absorbed; the pharmacological effects are, therefore, enhanced. However, a minimum concentration of polymer that significantly increases the pharmacological effects is not obvious for any particular drug.

For example, Mueller and Deardorff (5) used rabbits in a study of 0.1 and 1% methylcellulose solutions, which were approximately 14 and 230 cps, respectively, and found that 0.1% homatropine hydrobromide produced a response comparable to that produced by an aqueous solution of the drug alone. Blaug and Canada (4), on the other hand, found that for 0.02% solutions of scopolamine hydrobromide, viscosities above 22.5 cps significantly increased the time required for the pupillary diameter to return to control levels. Mice were used in these studies. In summary, it appears that the effect of methylcellulose as an adjuvant in ophthalmics is tenuous and appears to depend on the drug as well as the test animal used.

Influence of Polymers on Biokinetic and Pharmacological Characteristics of Tropicamide—It was considered that, in addition to the nonspecific effects of vehicle viscosity, the polymers could influence the biophasic availability of the drug by effects whose magnitude would depend on their specific chemical nature. Their effects could include: *(a)* reversible polymer–drug interactions which, by virtue of the large molecular size of the polymers, could retard the diffusive peripheral loss of interacted drug while concomitantly diminishing the rate(s) of biophasic drug availability; and *(b)* the mediation of drug interactions with the tissue surfaces exposed to the drug upon its instillation into the eye. Since such interactions have been shown (21–23) to modulate pharmacological behavior, their promotion or inhibition may be expected to influence biophasic availability.

However, inspection of Tables III and IV indicates that the inclusion of polymers may have some apparently systematic effects on biophasic availability, even though these are not significant at the $p < 0.05$ level of confidence; therefore, the present results preclude any firm conclusions concerning postulated mechanisms for the observed behavior of the systems. Among the most significant effects observed were those produced by the cationic polymer at a vehicle pH of 5.0; the confidence levels for t_{max}, K_{AB}, and percent biophasic availability are between 90 and 95%. If these effects can be considered other than random, they indicate an overall diminution in apparent drug transference rates which is opposite to that expected on the basis of a nonspecific Donnan-type exclusion of the ionized drug which would arise from the presence of relatively immobile charges on the polymer (21). The alternative would be to attribute the observed changes to the specific nature of the polymer. Interestingly, the results do not substantiate the formation of any unabsorbable complexes which could be expected to be formed between the anionic polymer and the cationic drug at pH 5.0.

CONCLUSIONS

Unlike the profound influence of the addition of small quantities of polymeric adjuvants to carbachol, for example (22, 23), the results with tropicamide indicate that the addition of polymers and vehicle viscosity within the range of 4.4–16.5 cps produces no significant influence on the drug transference and pharmacological behavior of tropicamide. However, some trends in the results appear to indicate that several tested polymers (at pH 5 only) could be inducing a slight effect by interacting with tissues at the absorption site and/or with the drug itself.

In view of the uncertainty of the effects of methylcellulose and the other polymers on ophthalmic drug effectiveness and the problems associated with their presence (24), it is best to consider carefully any addition of methylcellulose and other polymeric materials to ophthalmic vehicles except where a promoted effectiveness of the drug can be clearly demonstrated.

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* Present address: Alcon Laboratories, Fort Worth, TX 76101

x To whom inquiries should be directed.

NMR Assay of Diastereoisomers of 7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one Using Deuterated Tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III Shift Reagent

PHILIP REISBERG[x], **IAN A. BRENNER, and JEROME I. BODIN**

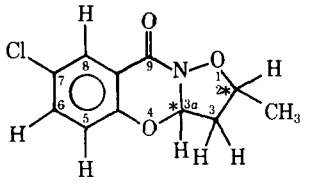
Abstract □ A simple, rapid, and quantitative NMR method was developed for the determination of the ratio of the diastereoisomers present in 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one (I). Deuterated tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III shift reagent causes the doublet assigned to the protons of the 2-methyl group, which normally appear at about 1.5 ppm, to split into a pair of doublets and shifts them downfield to about 3 ppm. Each new doublet represents one racemic diastereoisomeric pair present in I. The ratio of the pairs was determined by NMR integration of the areas under the respective doublets by calculating their proportions relative to the total area under the doublets. When a series of prepared mixtures of the isolated pairs was analyzed and their ratios were determined, the results obtained were within 1% of their actual values.

Keyphrases □ 7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-b)(1,3)benzoxazin-9-one diastereoisomers—NMR analysis, deuterated tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III shift reagent □ Shift reagents—deuterated tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III for NMR analysis of diastereoisomers of 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one □ Tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)-europium III shift reagent—NMR analysis of 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one diastereoisomers □ NMR—analysis, 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo(3,2-b)(1,3)benzoxazin-9-one diastereoisomers

7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-b)(1,3)benzoxazin-9-one[1] (I) is a member of a new class of anti-inflammatory compounds first synthesized by Reisner and Ludwig (1, 2). It is of particular chemical interest since it is a member of a newly reported heterocyclic ring system. The presence of an isoxazole ring contiguous to a 1,3-benzoxazine ring is the unique feature of the system. Because of the presence, at positions 2 and 3*a* (starred), of two asymmetric carbon atoms, four isomers theoretically should occur, resulting in two pairs of diastereoisomers, each consisting of two enantiomers.

DISCUSSION

A quantitative method to determine the proportions of each diastereoisomeric pair present in I was required. Separation by column chromatography was feasible but proved to be long, arduous, and difficult to reproduce quantitatively. However, preparative separation by column chromatography was performed to isolate the two diastereoisomeric pairs, which were designated as the α- and β-forms of I. Expectedly, they had different properties, *e.g.*, melting range, R_f value by TLC, and NMR spectrum.



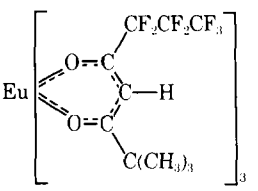
I

It was thought that the difference in their NMR spectra might be used for quantitative analysis. In 1966, van der Vlies *et al.* (3) reported the determination of the ratio of the diastereoisomeric pairs of cyclandelate by NMR spectroscopy. They found a difference in the positions of the peaks in the methyl proton region of the NMR spectrum, which enabled them to determine the ratio by measuring peak heights.

Compound I presented a somewhat different problem since the NMR spectra of its two diastereoisomeric pairs (Fig. 1) do not contain a suitable group for such use. Although there are distinct differences in the splitting patterns of the protons at 2, 3, and 3*a* in the isolated pairs, it is not possible to distinguish the two in combination. In Fig. 2 the spectrum of I appears almost the same as that of the α-form with some slight contamination by the β-form. There is a faint indication of two methyl proton absorption doublets, but these are too close to be of any value for quantitative purposes. A procedure for their separation was required if the NMR method was to be successful.

The use of paramagnetic complexing agents to produce large chemical shifts was reported by Hinckley (4), who observed large shifts in the NMR spectrum of cholesterol in the presence of the pyridine complex of tris(dipivaloylmethanato)europium III (II). Sanders and Williams (5) improved on this method by using the pyridine-free complex of II and increased the observed shifts by a factor of four. Many other lanthanide complexes were investigated, with the result that europium and praseodymium complexes were found to be most effective and to cause the least line broadening. Fortunately, these were complementary in that europium complexes produce a downfield shift, while praseodymium complexes produce an upfield shift. The magnitude of the shift depends on the relative molar proportions of the sample and lanthanide shift reagent used.

Initial work in this laboratory involved the use of the new complex, tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium III (III), first reported by Rondeau and Sievers (6). This compound is more soluble and is a better complexing agent



III

[1] Previously referred to in the literature as W-2395.

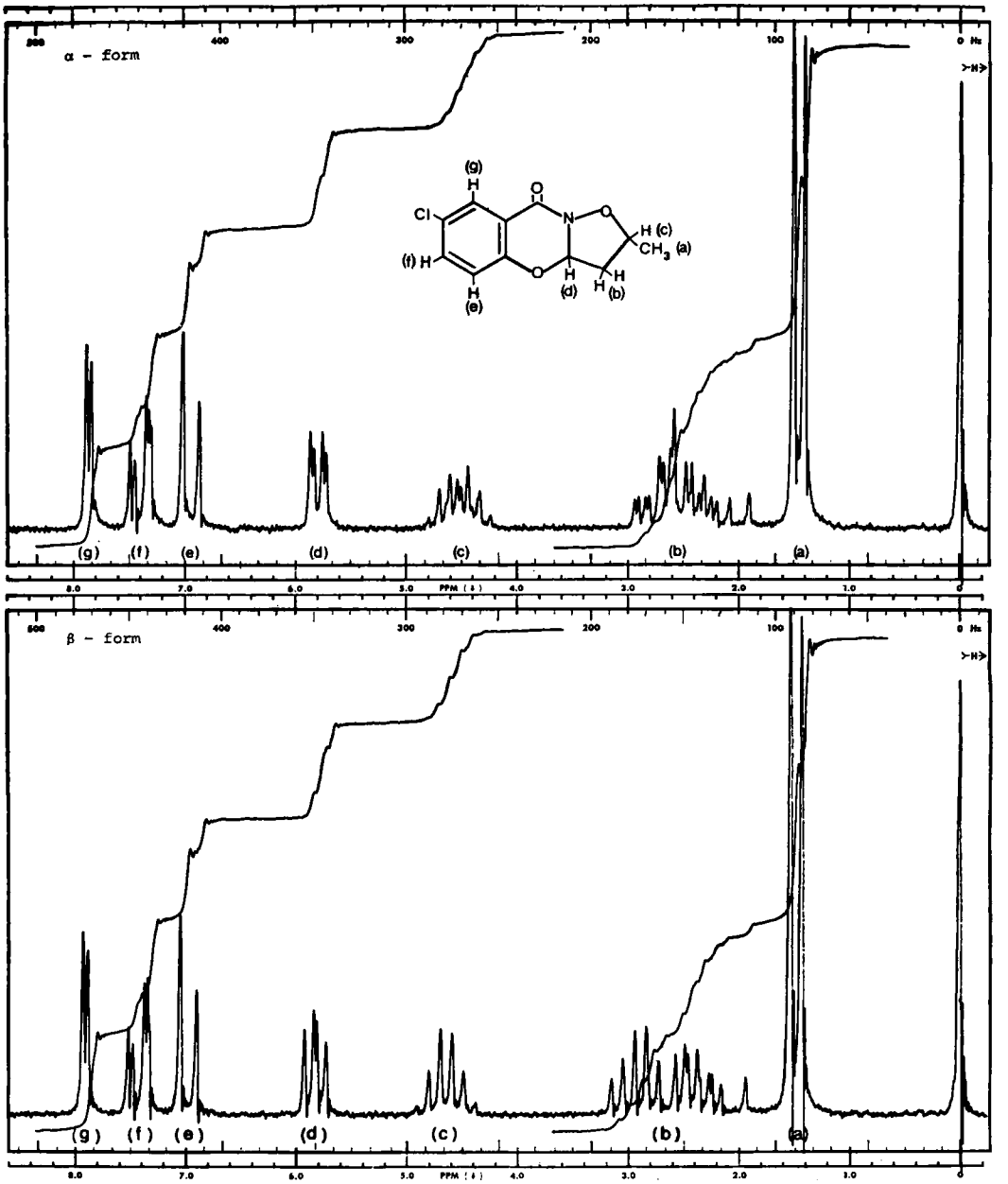


Figure 1—*NMR spectra of α- and β-forms of I in $CDCl_3$.*

than previous lanthanide shift reagents due to increased Lewis acid activity. A slight difficulty due to the absorption of the protons of the nine methyl groups in the 1.0–2.0-ppm region has recently been circumvented by the introduction of the deuterated III compound. The latter reagent, which absorbs only slightly in the 1.0–2.0-ppm region, was considered the reagent of choice.

EXPERIMENTAL

Apparatus—Spectra were obtained at 60 MHz using an analytical spectrophotometer[2]. A sweep time of 250 sec and a sweep width of 500 Hz were used, unless indicated otherwise; the δ-scale was used throughout. Tetramethylsilane (IV) (1% v/v) in deuterated chloroform[3] was used as the internal reference from which to measure chemical shifts.

Reagents and Chemicals—*Compound I*—The purity (sum of the α- and β-forms) was determined by dissolving an accurately weighed sample (about 500 mg) in 50 ml of 0.1 *N* NaOH, refluxing on a hot plate for 5 min, and back-titrating with 0.1 *N* H_2SO_4 using phenolphthalein TS as the indicator. Each milliliter of 0.1 *N* NaOH is equivalent to 23.97 mg of I.

α- and β-Forms—Compound I was separated into its two diastereoisomeric pairs (α and β) by absorption column chromatography on alumina[4] using ethyl acetate–ether (1:1) as the eluent. The presence of the two pairs in the eluate fractions was monitored by TLC. Samples of the eluate were spotted on silica gel plates[5], developed with ether–ethyl acetate (1:1), dried, and viewed under shortwave UV light. The initial fractions, which gave a single spot at R_f 0.48, were collected and evaporated to dryness. The residues were recrystallized from trichloroethylene and the product was assigned the designation α. Subsequent eluates, which gave a single spot on TLC plates at R_f 0.30, were also collected and evaporated to dryness. The product was recrystallized from methanol and given the designation β. Eluates giving more than one spot by TLC were rechromatographed until a fraction was obtained that gave only one spot.

[2] Varian T-60.
[3] Norell Chemical Co., Landing, N.J.
[4] Woelm neutral activity 1.
[5] F254, E. Merck.

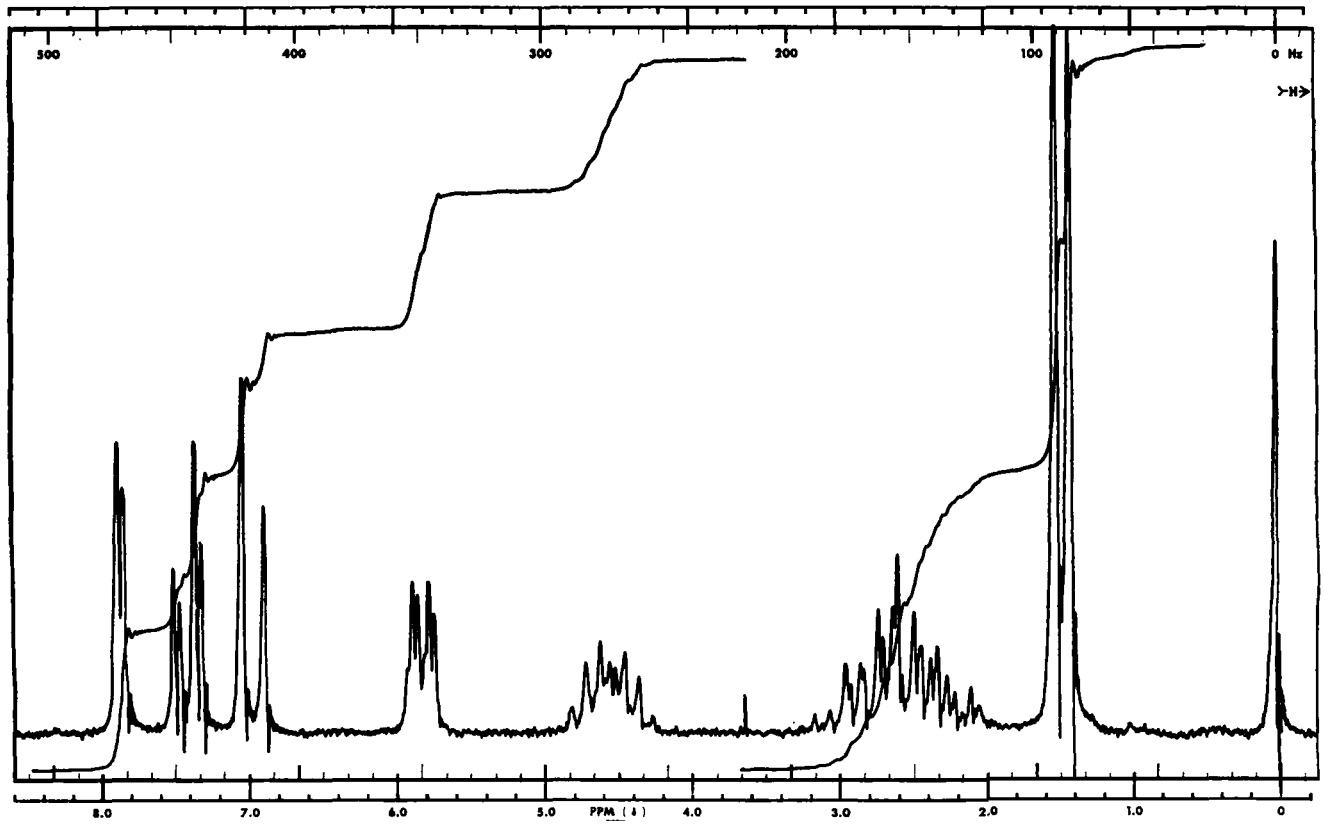


Figure 2—*NMR spectrum of I in $CDCl_3$.*

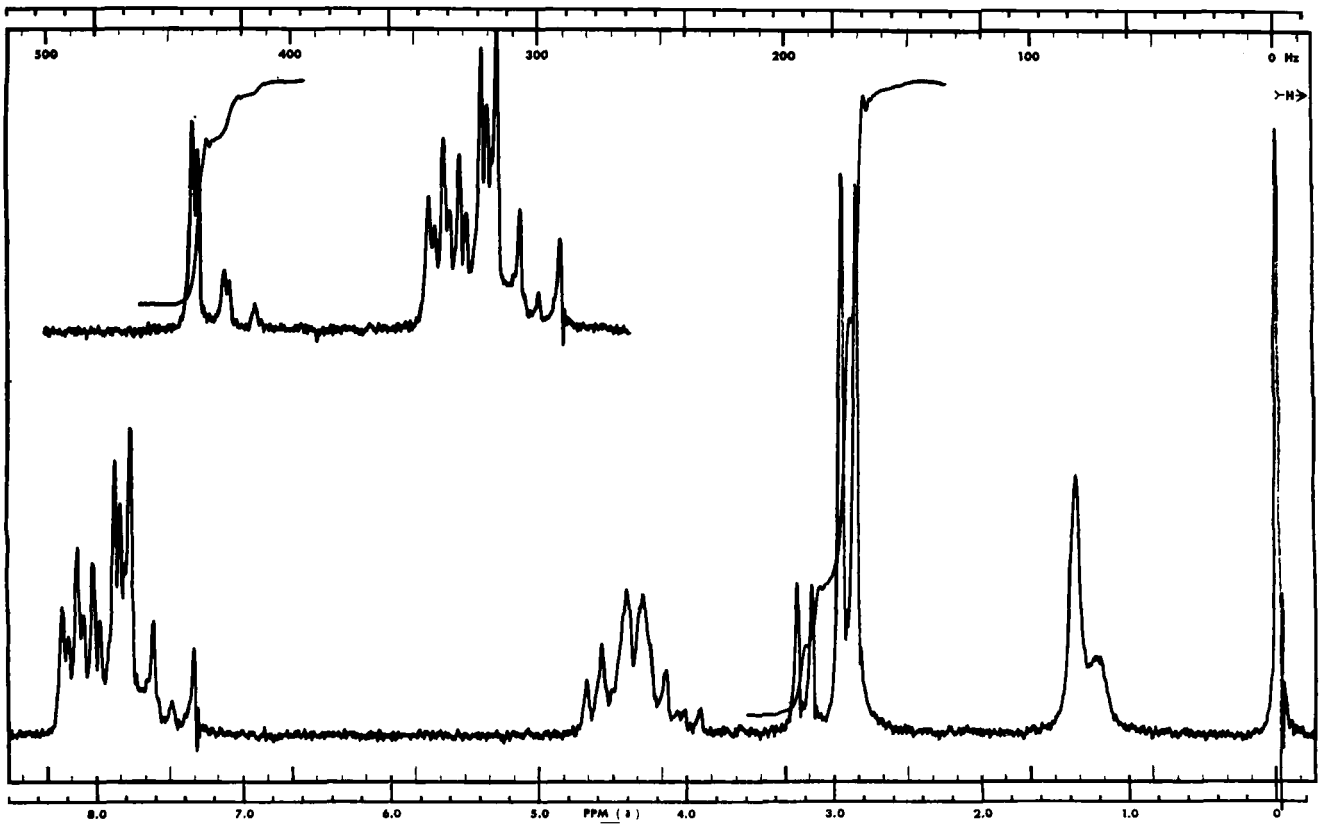
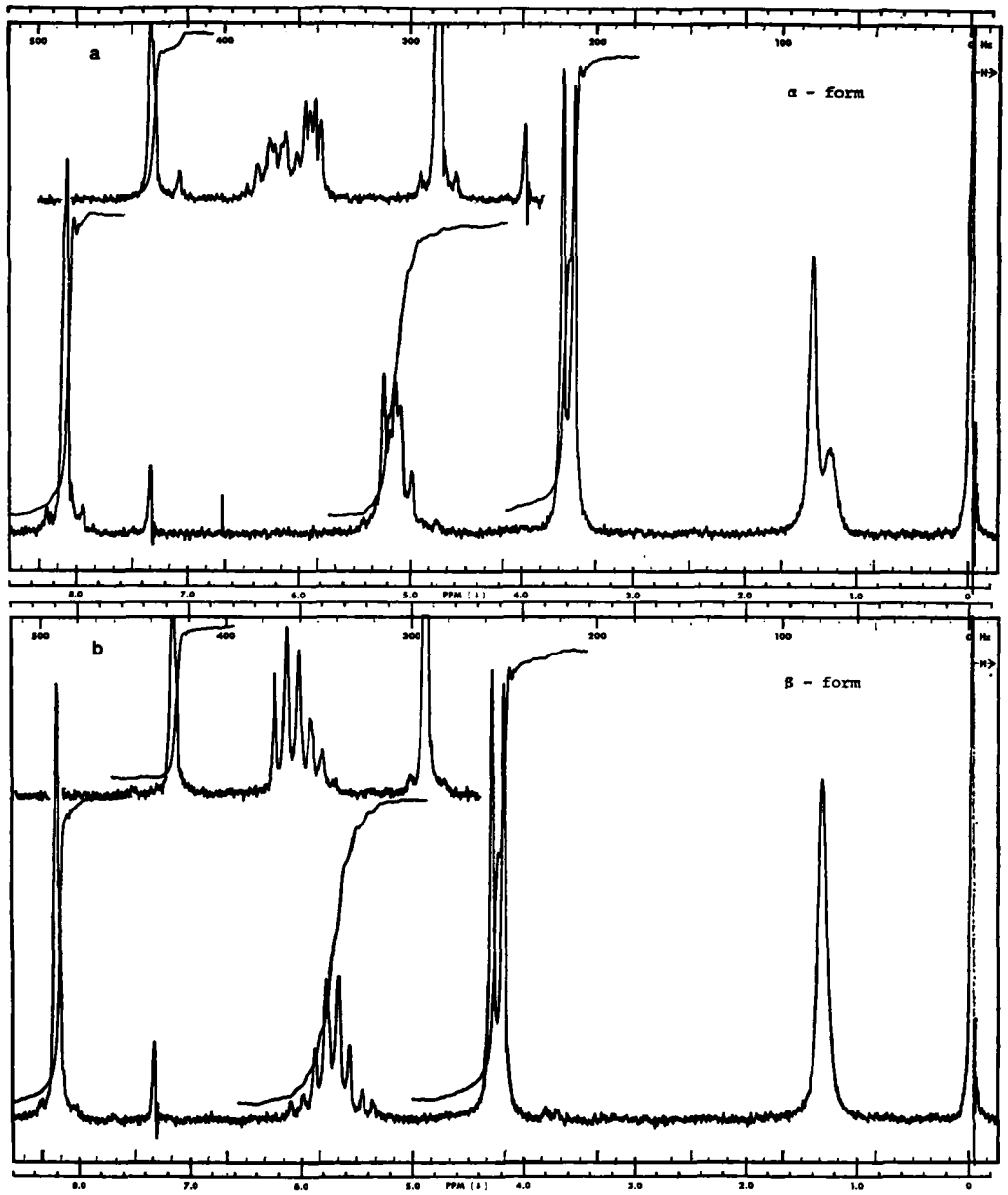


Figure 3—*NMR spectrum of I + deuterated III in $CDCl_3$.*



igure 4—(a) *NMR spectrum of 0.14 mmole α-form + 0.14 mmole deuterated III in $CDCl_3$.* (b) *NMR spectrum of 0.14 mmole form + 0.14 mmole deuterated III in $CDCl_3$.*

Lanthanide Shift Reagent—This was prepared by mixing 2105 g of deuterated III with 3.53 ml of deuterated chloroform, ntaining 1% of IV, and centrifuging the mixture to remove un-ssolved material. This concentration was found to be optimal.

Procedure—*Method A*—Solutions of 60 mg of I and the α- and forms in 0.45 ml of deuterated chloroform containing 1% of IV ere prepared separately in NMR tubes[6], and their NMR spectra ere obtained (Figs. 1 and 2).

Method B—About 33 mg (0.14 mmole) of I and the α- and β-rms were dissolved separately in 0.45 ml of lanthanide shift re-ent, and their NMR spectra were obtained (Figs. 3 and 4).

Method C—Prepared samples of I were made by accurately eighing the quantities shown in Table I, dissolving them in 0.45 l of the lanthanide shift reagent, and obtaining their NMR spec-a. The spectra were expanded to a 250- and 100-Hz sweep width d integrated five times through the region of interest (2.5–3.0 pm). The mean of the integrated values was used for quantitative lculations (Figs. 5 and 6).

Calculation—The percentage composition of each diastereoisomeric pair was determined as follows:

$$\% \alpha = \frac{A_1 \times 100}{A_1 + A_2} \quad \text{(Eq. 1)}$$

$$\% \beta = \frac{A_2 \times 100}{A_1 + A_2} \quad \text{(Eq. 2)}$$

where A_1 = area of upfield doublet (at about 30 ppm) and A_2 = area of downfield doublet (at about 3.3 ppm).

RESULTS

The NMR spectra of I and the α- and β-forms are shown in Figs. 1 and 2. The NMR signal for the protons of the methyl group at 1.5 ppm is split into a doublet by the proton on the adjacent carbon atom. The chemical shift for these protons is essentially the same for all three compounds.

Upon addition of the deuterated III shift reagent to I, a large downfield shift in the NMR spectrum was observed for all peaks due to the protons of ring 3 (Fig. 3). However, unexpectedly, the signal for the protons of the methyl doublet was shifted unequally

[6] No. 507, Wilmad Glass Co., Buena, N.J.

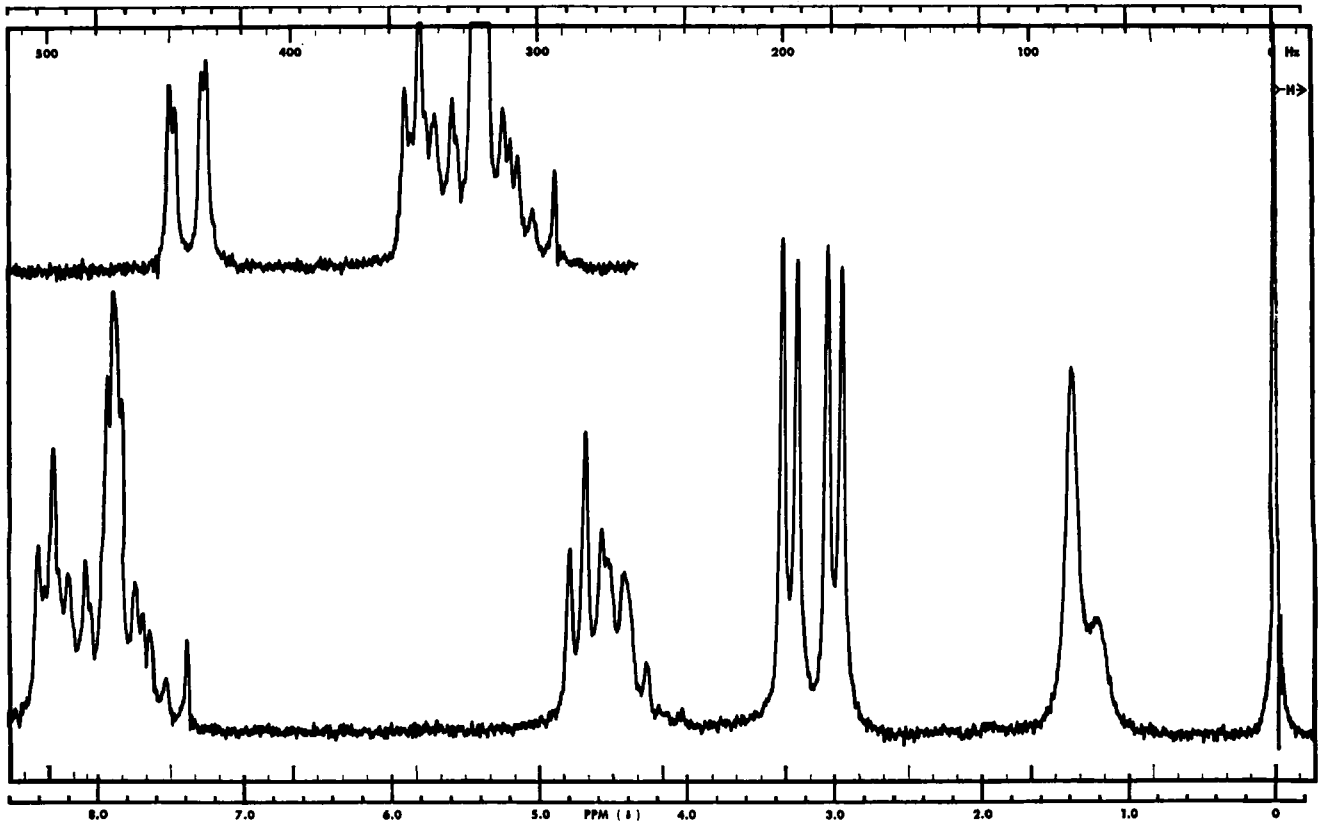


Figure 5—*NMR spectrum of prepared mixture of I (1:1 α-form–β-form) + deuterated III in $CDCl_3$.*

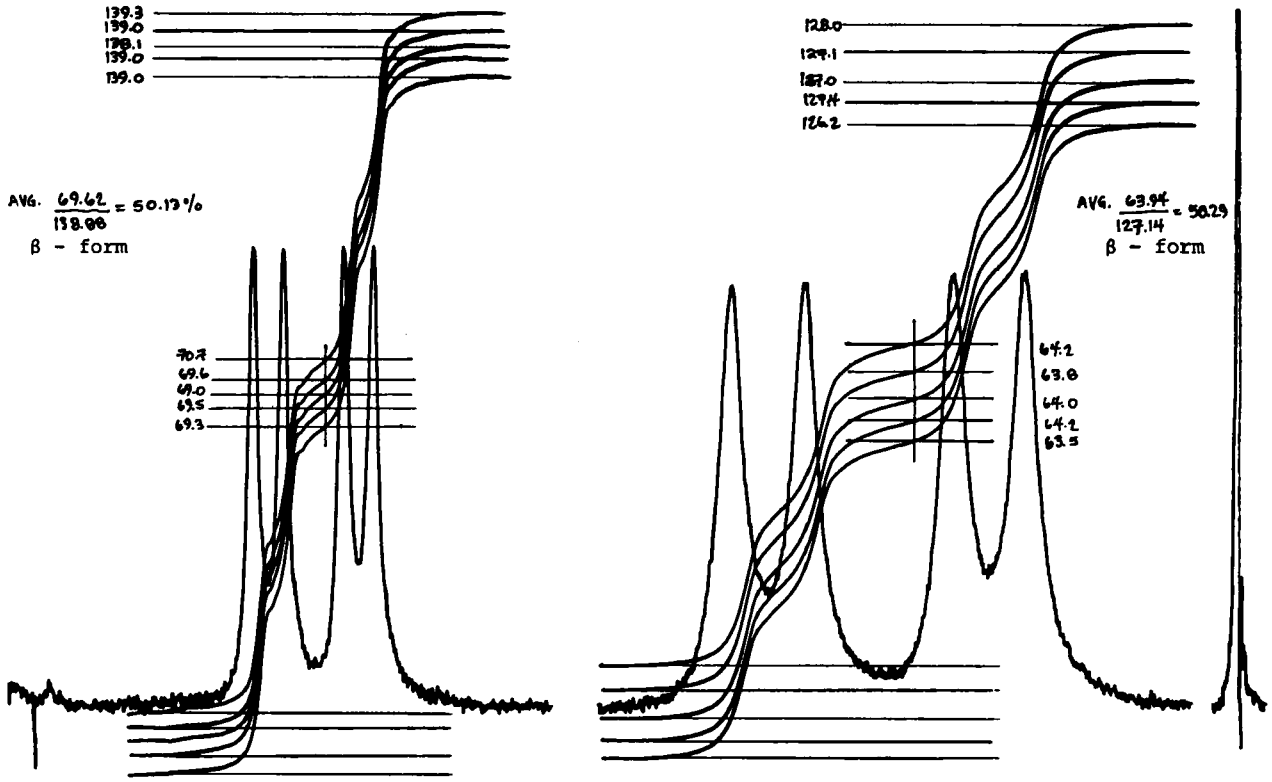


Figure 6—*The 250-Hz (left) and 100-Hz (right) sweep width expansion of methyl absorbances in Fig. 5.*

and was separated into two distinct doublets at about 3 ppm. Each doublet represents the methyl group present in either the α- or β-form. The NMR signals for the other protons in ring 3 were not separated but were still overlapping; one group had been shifted completely into the aromatic region.

This downfield shift probably results from the coordination of the deuterated III with a pair of electrons on oxygen or nitrogen of the isoxazole ring. The difference in the NMR signal of the methyl

Table I—Analysis of Prepared Samples of Compound I by NMR

Sample	α-Form, mg	β-Form, mg	Percent α	Percent α Found	Percent Recovered
1	57.50	3.20	94.73	94.88	100.17
2	54.12	6.31	89.56	89.78	100.25
3	42.66	19.00	69.19	69.80	100.88
4	30.38	31.20	49.33	49.79	100.93
5	28.62	33.52	46.06	46.08	100.04
6	23.80	36.38	39.55	39.86	100.78
		Mean	100.51		
		SD	0.40		

protons is probably due to the difference in the geometry of the two diastereoisomeric pairs and the manner in which the lanthanide shift reagent contacts them. This produces a greater effect in one case than in the other.

Positive identification of each methyl doublet was determined (Fig. 4). Under the same conditions, the NMR signal for the protons of the methyl doublet of the β-form was shifted further downfield than was that for the α-form. This finding confirms what was apparent from the examination of Fig. 3 where the downfield doublet is much smaller than the one upfield.

Table I reports the results of NMR analyses of prepared samples of I. Figures 5 and 6 show the NMR spectra of one of these mixtures, their expansion, and their integration. The recovery studies of these samples resulted in a mean of 100.51% with a standard deviation of ±0.40. There appears to be a constant positive bias which could result from the slight contamination of the β-form with the α-form. This is quite reasonable because α was easily obtained in pure form while β was separated from α only with great difficulty.

Two commercial size batches of I were made by the same process and gave ratios of 83.3% α to 16.7% of β and 82.9% α to 17.2% of β, respectively. The overall purity of these batches was 99.7% as determined by the method given in the *Experimental* section.

The accuracy and simplicity of this NMR method make it very useful and rapid for determining the relative proportions of the diastereoisomeric pairs of I.

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Progesterone Injection Assay by Liquid Chromatography

ROBERT H. KING, LEE T. GRADY [x], and JEANNE T. REAMER

Abstract □ An assay was developed for the progesterone content of injections composed of refined, fixed oils as the primary solvent. Reversed-phase, high-pressure liquid chromatography separates the progesterone from all other known ingredients following initial extraction into aqueous ethanol. Methyltestosterone was found to be a desirable internal standard, and peaks were measured photometrically. Twelve injection samples and six standards can be completed in 1 day. A system suitability test was defined. Evidence is presented for the recovery, specificity, accuracy, and precision of the method.

Keyphrases □ Progesterone injection—analysis by high-pressure liquid chromatography □ Steroids—analysis of progesterone injection by high-pressure liquid chromatography □ High-pressure liquid chromatography—analysis, progesterone injection

The official assay for progesterone in oil is a gravimetric determination of the double 2,4-dinitrophenylhydrazone adduct (1). This assay is unspecific, measuring any ketosteroid content, and serious problems have been observed with the quality of the precipitating reagent and with formation of the precipitate. A new assay with notably improved specificity was required in light of apparent problems with marketed items.

Analytical approaches to drugs in oil-based pharmaceuticals (2) and the pharmaceutical aspects of high-pressure liquid chromatography (HPLC) (3) have been reviewed. Specific, but cumbersome, progesterone determinations have been advanced including an IR partition column assay (4) and an extraction method followed by GLC (5). The purposes of this study were to develop and to define a specific and simple assay that utilizes the advantages offered by HPLC in the analysis of steroids.

EXPERIMENTAL

All reagents were USP, NF, or ACS grade. Spectroquality 2-propanol was purchased. Samples of commercial progesterone injection NF were obtained locally and from manufacturers. Fixed oils were obtained locally.

Chromatographic System—The instrument[1] used a 1-m by

[1] DuPont model 830 high-pressure liquid chromatograph, fitted with circulating warm-air oven and low-pressure mercury UV photometer.

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Sample	α-Form, mg	β-Form, mg	Percent α	Percent α Found	Percent Recovered
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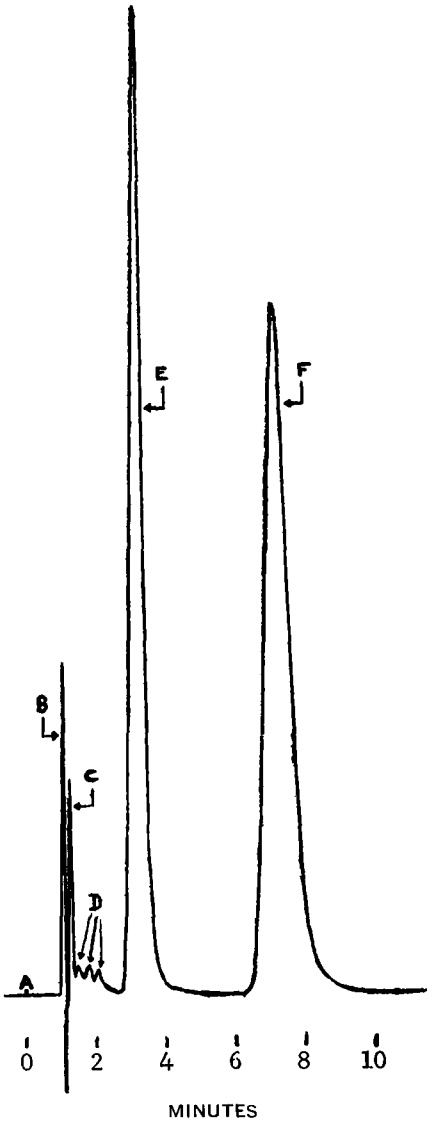

Figure 1—*Product A, 50 mg/ml through procedure. Key: A, inject; B, extracted material from injectable; C, solvent; D, minor impurities; E, methyltestosterone; and F, progesterone.*

2.1-mm i.d., stainless steel column packed with spherical glass microbeads, nominally 30 μm in diameter, with a thin porous coating to which is chemically bonded 1% (w/w) octadecylsilane[2]. The mobile phase was degassed, membrane-filtered[3], 18% 2-propanol–distilled water (v/v) at a flow rate between 0.8 and 1.2 ml/min at about 42–44°. A low-pressure mercury, 254-nm photometer was used as the detector, and an electronic digital integrator[4] was used for area and retention time data.

Internal Standard Solution—Transfer about 165 mg of NF methyltestosterone reference standard, accurately weighed, into a 25-ml volumetric flask, add sufficient 85% ethanol to dissolve it, dilute to volume with 85% ethanol, and mix.

Assay Preparation—Transfer, by calibrated tuberculin syringe, a volume of progesterone injection equivalent to about 25 mg of progesterone into a 15-ml Teflon-lined screw-capped centrifuge tube. Extract with three 5-ml portions of 85% ethanol, shake gently for 10 min, centrifuge[5] for 15 min or until the alcohol layer is clear, and carefully transfer the upper alcoholic layers by suitable Pasteur pipet to a 25-ml Teflon-lined screw-capped test tube, taking care to rinse the transfer device with about 1 ml of 85% ethanol after the first extraction. To the combined alcohol layers, add 2.0 ml, by pipet, of internal standard solution and mix well to obtain about 18 ml of a solution approximately 1.39 μg/μl in progesterone and 0.62 μg/μl in methyltestosterone.

Standard Curve—Transfer four portions of NF progesterone reference standard, about 20, 22.5, 27.5, and 30 mg, accurately weighed, to individual Teflon-lined screw-capped centrifuge tubes and dissolve each in about 15–16 ml of 85% ethanol. Add 2.0 ml of the internal standard solution by pipet and mix. Inject 5 μl from each solution into the chromatographic system. Determine the peak area or heights of the progesterone, P_p, and methyltestosterone, P_{mt}, and calculate the ratio, R_s, by the formula P_p/P_{mt}. Plot the standard curve of the R_s values against the amount, in milligrams, of NF progesterone contained in each portion.

System Suitability—Chromatograph six to 10 injections of a standard solution, containing approximately 1.5 mg/ml progesterone and 0.75 mg/ml methyltestosterone, and measure the peaks as directed for the standard curve. The analytical system is suitable for conducting the assay if: (*a*) the relative standard deviation (*RSD*), calculated by the formula 100 × (standard deviation/mean ratio), for the ratio R_s does not exceed 1.5%; (*b*) the resolution factor between peaks is not less than 3.5; (*c*) the tailing factor (sum of the distances from peak center to the leading edge and to the tailing edge divided by twice the distance from peak center to the leading edge), measured at 5% of peak height of the progesterone

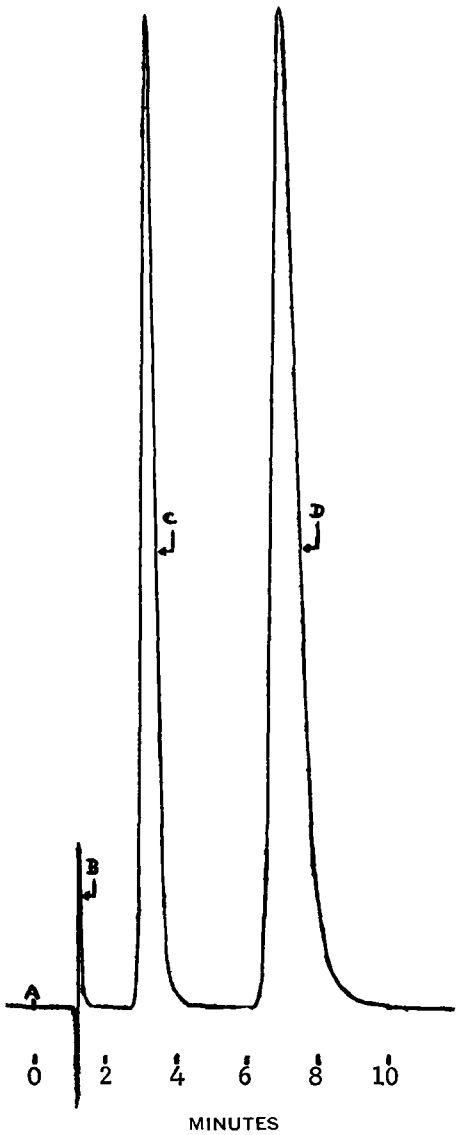

Figure 2—*Standard solution. Key: A, inject; B, solvent; C, methyltestosterone; and D, progesterone.*

[2] ODS Permaphase on Zipax, from the Instrument Products Division, DuPont and Co., Wilmington, Del.
[3] Gelman DM Metricel, 0.45 μm.
[4] Infotronics CRS-204 digital integrator.
[5] International model UV was used at about 1000 RCF.

Table I—Assay Results[a]

Manufacturer, Vial Volume, ml	Labeled Strength, mg/ml	HPLC[b] Peak Areas Mean Recovery, %	*RSD*, %	*n*	HPLC[b] Peak Heights Mean Recovery, %	*RSD*, %	*n*	Gravimetric[c,d,e] Mean Recovery, %	*RSD*, %	*n*
A[f] 1	25	78.4	0.23	4	79.5	0.48	4	91.2	0.95	4
								85.9[h]	0.80	6
A[g] 1	50	84.0	3.10	3	84.9	0.24	4	83.1	0.80	2
		86.4	1.48	3	85.4	0.65	3	—	—	—
B 10	25	90.9	0.60	4	90.4	0.17	4	86.4	0.46	3
		90.2	0.06	3	90.1	0.06	3	—	—	—
B 10	50	100.4	0.81	4	100.8	0.36	6	100.9	1.87	3
		100.7	0.29	4	100.4	0.46	5	—	—	—
C 10	25	101.7	0.33	7	102.1	0.56	7	100.5	0.45	3
		101.5	0.60	5	101.6	0.34	5	—	—	—

[a] As percent of label claim. [b] *n* is the number of repetitive injections of assay preparation. [c] Reference standard in sesame oil, 25.03 mg/ml, yielded a mean recovery of 100.3% with an *RSD* of 0.56% (six determinations), independent of lot of reagent. [d] Matheson, Coleman and Bell reagent, 1972. [e] *n* is the number of times the injection was assayed. [f] Small amount of extraneous material was observed on solvent front tail, but it did not interfere with quantitation. [g] Area ratios (by integrator) affected by extraneous material present on solvent front tail. [h] Eastman reagent, 1968 and 1970.

Table II—Standard Recovery from Oil[a,b,c]

Progesterone, mg	Sample	Peak Areas Recovery, %	Mean, %	*RSD*, %	Peak Heights[d] Recovery, %	Mean, %	*RSD*, %
15	1	98.9, 99.3	99.9	0.97	100.5, 100.9	100.8	0.30
	2	100.6, 100.9			101.2, 100.7		
25	1	98.9, 99.1, 99.6	99.2	0.36	99.8, 99.6	99.7	0.42
	2	98.7, 99.2, 99.0			100.2, 100.1, 100.0		
	3	98.8, 99.1			98.9, 99.0		
	4	99.4, 99.2, 99.2			99.5, 99.7, 99.5		
35	1	99.4, 99.8	100.0	0.56	100.1, 100.4	100.2	0.21
	2	100.2, 100.7			100.4, 100.0		
40	1	98.9, 99.5	99.7	0.60	99.1, 99.6	99.7	0.60
	2	99.9, 100.3			99.5, 100.5		
50	1	100.3, 99.9	99.9	0.37	99.7, 100.1	100.4	0.65
	2	99.4			100.6		
	3	100.0			101.2		
	2[e]	99.1, 99.3	99.4	0.30	99.8, 99.7	100.0	0.47
	3[e]	99.3, 99.8			100.7, 99.8		
60	1	100.2, 100.9	100.6	0.49	100.8, 100.7, 101.9	101.1	0.66
Total[f]			99.9	0.45	—	100.3	0.57

[a] Progesterone weighed directly into centrifuge tube and dissolved in sesame oil. Samples extracted and compared to nonextracted standards (injected in duplicate), using slope of resultant graph to convert ratios to milligrams progesterone; samples prepared over a 2-day period. [b] Except where noted, 2.0 ml of internal standard solution was added. [c] Area ratios of same solutions did not change over ~6-day period, but height ratios of the same solutions decreased ~2.5% over the 6-day period. [d] Minimum peak height (for 15-mg standards) approximately 8.4 cm. [e] 4.0 ml of internal standard solution added to these solutions to approximate 25-mg ratios. [f] Grand totals for recovery based on 2.0-ml internal standard addition only.

does not exceed 2.0; and (*d*) the capacity factor, *k'*, for the methyltestosterone is not less than 1.5.

Procedure—Inject 5.0 μl of the assay preparation into the chromatographic system and obtain a chromatogram and ratio as directed under for the standard curve. Read from the standard curve the quantity, in milligrams, of $C_{21}H_{30}O_2$ in the volume of the injection taken. The column should be rinsed with 100% 2-propanol for 30 min after ~40–60 injections to avoid any possible overloading of the column with extracted oil.

Tocopherols—To identify the extractable UV-absorbing oil constituents, an extract was prepared from equal volumes of oil and 85% ethanol. Thin-layer chromatograms of 500-μl streaks (under nitrogen) on fluorescent silica gel[6] were developed in (S_1) methanol–benzene (1:99) or (S_2) cyclohexane–ether (4:1). The bands were visualized with short wavelength (254 nm) UV light to simulate the detector of the high-pressure liquid chromatograph. The plates were then sprayed with antimony trichloride (6), antimony pentachloride (7), or phosphomolybdic acid (6) to observe color reactions. Individual bands visible under short wavelength UV light were scraped off the plates, extracted into 85% ethanol, filtered, and rechromatographed by HPLC to correlate the relative mobilities of the TLC bands with HPLC retention time as well as the color reaction to the spray reagents.

Only one band was observed to have a retention time similar to either methyltestosterone or progesterone. The relative TLC mobilities and reaction to the spray reagent suggested that this band and one other contained tocopherols. A better separation (8) of the tocopherols was achieved using (S_3) light petroleum–isopropyl ether–acetone–ether–acetic acid (85:12:4:1:1). Using this system, the two bands were separated into four components, all of which reacted similarly to the spray reagents as reported in the literature, and approximated the reported mobilities of α-, β-, γ-, and δ-tocopherols. The UV spectra of the isolated components were recorded and these were identical, having a minimum at 254 nm and maxima at 327, 287, and 236 nm, resembling the spectra of the tocopherols. δ-Tocopherol appears to constitute approximately one-third of the mixture. The solutions of each tocopherol were concentrated and analyzed by HPLC, and δ-tocopherol was confirmed to be the component of the oil that was being extracted and detected by HPLC just after the progesterone peak.

Progesterone Derivative—The 2,4-dinitrophenylhydrazone adducts of the progesterone in each commercial item and of the NF reference standard were examined by TLC using several systems, particularly chloroform–benzene (3:1) on silica gel. In each

[6] Merck silica gel F-254 precoated glass plates (Brinkmann), 250-μm layer.

Table III—Chromatographic Parameters (12, 14)

Column[a]	Peak	Retention Time, min	Capacity[b] Factor, k'	α[c]	Theoretical Plates, n[d]	Tailing Factor[e]	Resolution Factor, R_s[f]
I	Methyltestosterone	3.4	1.9	2.8	434	1.2	4.0
	Progesterone	7.4	5.3		470	1.7	
II	Methyltestosterone	9.8	4.5	3.7	204	3.1	3.6
	Progesterone	31.1	16.4		189	5.5	
III	Methyltestosterone	3.5	1.7	3.1	670	1.1	5.2
	Progesterone	8.2	5.3		661	1.3	
IV	Methyltestosterone	2.7	1.1	4.0	597	1.2	4.4
	Progesterone	6.8	4.3		355	1.6	

[a] I = ODS Permaphase, 44°, 18% 2-propanol–water, 75 atm; II = ODS Permaphase, 24°, 18% 2-propanol–water, 100 atm; III = second ODS Permaphase, 44°, 18% 2-propanol–water, 65 atm; and IV = HCP Permaphase, 34°, 17% 2-propanol–water, 110 atm. All were 1 m with DuPont Zipax as solid support. DuPont models 820 and 830 HPLC and UV 254-nm detector were used. [b] $k' = (T_r - T_0)/T_0$. [c] $\alpha = k'_2/k'_1$. [d] $n = 5.54\ [(T_r/(W_{h/2})]^2$. [e] Tailing factor = width at 5% peak height/2a, where a is the distance from peak center to the leading edge, measured at 5% peak height. [f] $R_s = [2(T_{r2} - T_{r1})]/[1.699(W_{1h/2} + W_{2h/2})]$, with retention and width in same graphical units.

case, only two spots, the known (9) *syn-* and *anti-*isomers, were observed and this finding was confirmed by UV and IR spectroscopy.

RESULTS AND DISCUSSION

Table I lists the results of assays of commercial items from three manufacturers. Substantial manufacturing problems apparently exist for this item (Table I and Fig. 1), both in potency and purity of ingredients. Both methods give accurate and precise assays of oil solutions of NF progesterone reference standard, but the more specific HPLC assay results are not always in agreement with the unspecific gravimetric assay results, particularly when subpotent samples are analyzed.

Quantitative Aspects—Replicate injections of a single solution of progesterone and methyltestosterone reference standards, 25 and 13.2 mg, respectively, in 17 ml of 85% ethanol, and calculation of R_s gave 0.34% *RSD* (n = 7) for electronically measured peak areas and 0.2% *RSD* for peak heights (Fig. 2). Replicate assays (n = 6) of a standard solution of 25 mg/ml progesterone in sesame oil[7] (7) also showed excellent precision, 0.43% *RSD* by area and 0.37% *RSD* by peak heights, and a mean recovery of 100.2% assayed against standard solutions in 85% ethanol. The stability of the system was indicated by the 0.5% *RSD* of a single standard solution over 1 day. It is concluded that the method is precise. Electronic area measurement is preferred for its speed and accuracy.

Recovery of an extracted standard compared to an unextracted standard was complete and unbiased (Table II) for the entire 15–60-mg/ml concentration range. Both the extracted and direct standard graphs showed a highly linear response, with linear regression coefficients ≥ 0.998 for each of nine completely separate standard graphs and no difference in either slope or intercept attributable to the extraction procedure. Chromatographic dynamics cause a slightly displaced y- (ratio) intercept (+5% for one octadecylsilane column and −4% for another, based on 25 mg ratio = 100%)—this would be an individual characteristic of such mixed absorption–partition columns so a standard graph rather than a one-point standard is required for maximum accuracy in the procedure. All progesterone is recovered (85.1, 13.1, 1.6, and 0.2% by HPLC assay of consecutive extractions), consistent with the fact that the slopes and intercepts obtained from extracted and nonextracted standards are identical.

Complete recovery of drug, in conjunction with the substantial specificity inherent in a chromatographic analysis, and the lack of significant known interferences define this as an accurate method.

Assay and standard preparations are stable in the screw-capped containers. Area ratios for 1-week-old solutions, both standard and sample, remained unchanged from the original ratio. Peak height ratios of the same aged solutions, however, showed an absolute 2.5% decrease in R_s at the end of 1 week, but the column at that point had an accumulation of uneluted oil on it. The same solution after 1 month on a freshly rinsed column (100% 2-propanol for 30 min) also gave the same peak height ratio as the original ratio (within 0.3%).

Hydroalcoholic extractants are well established (2, 5) in the recovery of polar drugs, including progesterone, from fixed oils. Hexane usually is mixed with the oil layer to effect a hexane–aqueous ethanol partition system for the oil *versus* the drug. The addition of a hydrocarbon was dispensed with for these reasons. After separation, the 85% ethanol layer is on top, the more convenient place for centrifuge tube methods; the oil readily breaks into fine droplets on shaking, and its viscosity is not so great as to present a mass-transfer problem during extraction. Small volumes were chosen to allow assay of unit doses, to avoid a concentration step in the procedure, and to benefit from centrifuge tube handling in preference to separators. Solubility of progesterone in the 85% ethanol extractant was not limiting, because a level in excess of 75 mg/ml at 24° was found, and the solubility[8] was still 33.5 mg/ml in the even more aqueous 70% ethanol. Additional partitions were necessary if the water content of the extractant was increased much beyond 15%.

Modification of the procedure can allow even faster analyses, such as single partitions directly into the internal standard solution with incorporation of oil solutions of the reference standard (or direct mixing). Conservation of reference standards can be effected by evaporating aliquots of progesterone extract and standard solutions and redissolving the residues in small volumes of the internal standard solution; *i.e.*, collect 3 × 5-ml extracts in a 25-ml volumetric flask and dilute to volume, transfer and evaporate a 2-ml (25-mg/ml) or 1-ml (50-mg/ml) aliquot, redissolve in 1 ml of 1.05 mg/ml methyltestosterone in ethanol, and inject 3 μl. Also, multiple weighings of progesterone can be replaced by a single solution of 2 mg/ml, pipeting 10–15-ml aliquots with dilution to be about 15–16 ml.

Chromatographic Aspects—HPLC is recognized as the method of choice for steroids. Resting on the extensive foundation forged by GC, applications of liquid chromatography to pharmaceutical problems have been precipitous (3, 10). Purity evaluation of progesterone has been suggested (11), using microparticulate silica adsorption chromatography, but the impurities (more polar) were in the tail of the progesterone peak which is less desirable in purity work. The reversed-phase column chosen for the assay reported here was selected precisely because of its known reliability and consistent success in steroid separations. Indeed, the retention and elution of progesterone in such a system already have been reported[9] (10).

The data in Table III were obtained with an octadecylsilane column, 18% 2-propanol–water, and ~1-ml/min flow. Chromatographic parameters are defined elsewhere (3, 12). Although ~35% ethanol–water or ~40% methanol–water on another octadecylsilane column was found satisfactory for chromatographic separation, 2-propanol–water is recommended due to the higher affinity of the oil for this alcohol, which makes column equilibration simpler after a 100% 2-propanol rinse. Another type of hydrocarbon polymer[10] (Column IV, Table III) also gave usable parameters, and a chromatographic precision of 0.6% *RSD* was obtained even at 22°

[7] Welch, Holme sesame oil, lot 5124A.

[8] N. Diding, O. Wallen, and B. Öhrner, personal communications.

[9] Unpublished Drug Standards Laboratory Report 71-735, February 8, 1972, "Progesterone, NF Reference Standard," used gradient elution of 2-propanol in water, 20–40%, and isocratic elution with 20% aqueous 2-propanol on ODS Permaphase, 55°.

[10] HCP Permaphase on Zipax, 1.0 m.

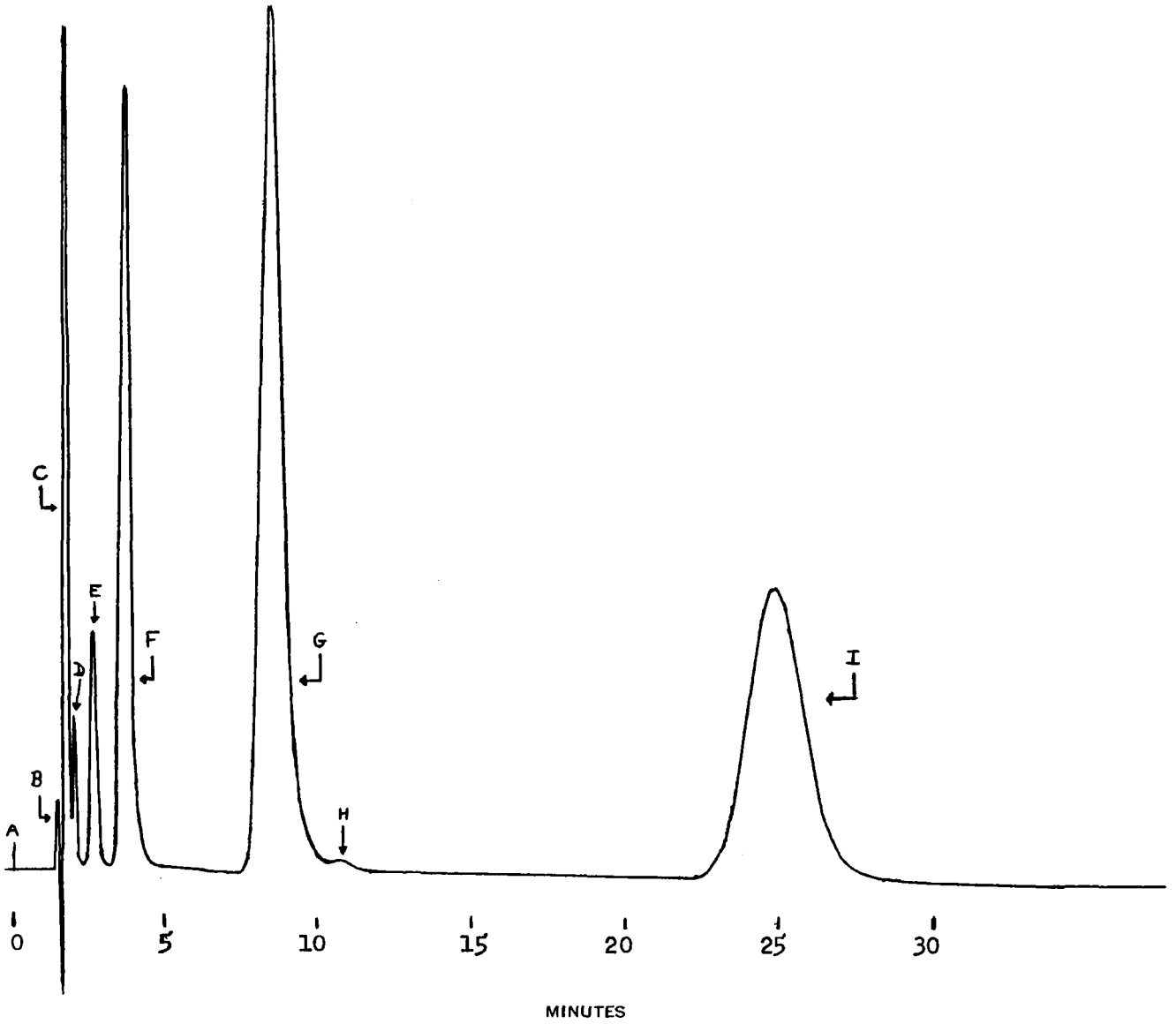

Figure 3—*Some components in oil injections. Extracted sample of "cold-pressed" oil which contained known components of commercial oil injectables. Key: A, inject; B, extracted material from sesame oil; C, solvent, benzyl alcohol, and phenol; D, impurity from benzyl benzoate; E, propylparaben; F, methyltestosterone; G, progesterone; H, δ-tocopherol,* k′ = ~*7.3; and I, benzyl benzoate,* k′ = ~*18.*

with this column (20% 2-propanol–water). Thus, this reversed-phase liquid chromatography approach promises substantial reliability and broad applicability.

Methyltestosterone was chosen as the internal standard because the chromophore and other molecular aspects were similar to progesterone, and it exhibited complete separation from the solvent front, progesterone, and oil impurities. Testosterone, in contrast, eluted too soon after the solvent effects on the baseline to allow reliable quantitation. About 20 steroid reference standards, many with the same chromophore, were examined to identify a suitable internal standard. As a corollary of the inherent specificity of the HPLC system, only a few [testosterone, norethindrone (k' = 1.0), methyltestosterone, and norethindrone acetate (k' = 5.3)] were retained beyond the solvent front and still eluted within a workable time frame. Methyltestosterone was the most desirable of these, being well separated from polar ingredients, progesterone, and oil components.

Solubility of sesame oil in 85% ethanol was found gravimetrically to be on the order of 1 mg/g, which predicts accumulation of about 4 μg of uneluted oil on the column per injection. In addition to the peak height ratio change mentioned, the retention times of progesterone and methyltestosterone were increased by approximately 30 sec during the 150 injections over 1 week's time, but with no apparent change in precision or recovery. Nevertheless, it is desirable to wash the column with 100% 2-propanol for 30 min after 40–60 injections to avoid loading the column with extracted oil.

System Suitability—A system suitability test was developed out of the experiences gathered during method development. In the present instance, more than one stationary phase may be suitable, although reexamination of other known components would be necessary before using a different packing. Furthermore, the analyst should have substantial additional flexibility in choosing temperatures, pressures, and concentrations of 2-propanol in water and still be able to establish a proven suitable system and an acceptable turnaround time.

System suitability testing was envisioned here several years ago as a mechanism to reconcile the requirement of highly defined methodology for official assays to the laboratory realities of wide variations in chromatographic supplies, instrumentation, range of operating conditions, and working times. The applicability of such tests, of course, extends beyond chromatography to other complex assays.

The underlying concept is that the chromatograph (make, plumbing, detectors, electronics, and recorder), packing (chemical type, source, batch, manipulations, and pretreatment), samples

(solvent, concentration, and injection size), operating conditions (temperature, flow rate, composition of mobile phase, and turnaround time), and even the analyst's technique constitute a *single system* and, as such, is amenable to an overall test of *system function*. Such a test reduces to a limited series of numbers and a precise, official language. Once a laboratory achieves a suitable system, straightforward performance of an assay procedure may be anticipated, presuming that classical chemical operations not covered in the test (extractions, concentrations, *etc.*) are performed normally. *In practice*, such testing must be based on adequate experience acquired during method development and evaluation with emphasis on the physical chemistry of chromatography; this probably is inherent in the work habits of experienced chromatographers. One other practical aspect is the presumption that the originating laboratory does not set forth a test based on such unusual materials, methods, or fortuitous chromatography that few other laboratories are likely to establish suitable systems.

Other Components—(See Fig. 3 for a composite.) A distinct advantage of this reversed-phase approach is that polar impurities (or perhaps decomposition products) of progesterone will elute first so that their presence is recognized without prolonging the turnaround time of the analysis. Indeed, one of the 50-mg/ml products, Product A, showed at least three minor polar components (Fig. 1). The product itself was distinctly more yellow or amber colored than the other commercial items or the various fixed oil samples. Expiration dates were not observed on most items. The identity of these components in commercal progesterone is not known with certainty, but work[11] is in progress in other laboratories which may establish their identity.

Benzyl alcohol, benzyl benzoate, phenol, ethanol, chlorobutanol, and propylparaben are the other labeled ingredients of the available pharmaceuticals tested. Chlorobutanol moves with the solvent front and is otherwise undetected. Benzyl alcohol and phenol also elute immediately and are detected by the UV photometer (Fig. 3). Benzyl benzoate elutes after progesterone (α = 3–4) in the octadecylsilane system and has, therefore, the effect of prolonging the turnaround time when present because it partitions into the 85% alcohol extractant. Propylparaben elutes just prior to methyltestosterone but gives baseline separation.

Normal, that is to say "refined," fixed oils[12] do not give any interfering peaks in this procedure, and presumably the compendial items are selected from refined fixed oils. One commercial product gave evidence of unrefined oil components, and a sample of sesame oil[7] of undetermined status was found to yield a small peak just after the progesterone peak which, if unobserved, could bias the (uncorrected) assay of the 25-mg/ml item by +0.3% if measured by electronic area devices with certain settings. Peak height data would not be affected to the same extent, depending on the efficiency and α-values of individual columns. To test the worst possible situation, a sample of health-food (unrefined and, therefore, noncompendial) sesame oil[13], so-called "cold-pressed" oil, was obtained, and a potential error of about +0.95% on progesterone data for the low-strength item was observed (Fig. 3). TLC of the oil allowed collection of the individual bands. Correlations were made between TLC and HPLC mobilities, and color reaction and UV spectra were determined. The interfering component appears to be δ-tocopherol. More polar oil constituents, *i.e.*, those less mobile on silica gel than tocopherols, all eluted in the solvent front in this reversed-phase liquid chromatography system. Van Niekerk's (13) liquid chromatography–adsorption system would be preferred for tocopherol analysis by HPLC. Stowe's (8) thin-layer system was found to be effective for separating the four tocopherols.

[11] E. Smith, personal communication.

[12] Such as Wesson or Kraft food-grade oils from cottonseed and soybeans and Planters peanut oil.

[13] Hain cold-pressed sesame oil, purchased from Mother Nature's Store, McLean, Va.

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[x] To whom inquiries should be directed.

GLC Determination of Clindamycin and Related Compounds

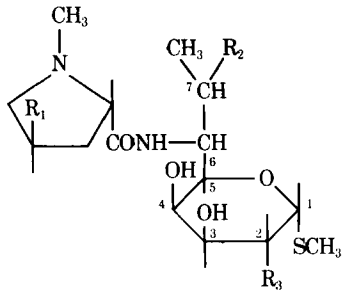
LEO W. BROWN

Abstract □ GLC assays were developed for clindamycin hydrochloride, clindamycin 2-phosphate, clindamycin 2-palmitate hydrochloride, and formulations containing these compounds. Procedures for the determination of possible related trace contaminants were also developed for evaluating the purity of these antibiotics. Different derivatives and GLC behavior of these compounds are discussed.

Keyphrases □ Clindamycin and related compounds—GLC determination of trace contaminants and impurities □ GLC—analysis, clindamycin and related compounds, determination of trace contaminants and impurities

Clindamycin (I), an antibiotic synthesized from lincomycin (II), is produced by bacterial fermentation. It has been assayed and characterized by degradation pathways by GLC (1, 2). Clindamycin 2-phosphate[1] (III) and clindamycin 2-palmitate[1] (IV) are synthesized from clindamycin. The former was developed for use in sterile injection preparations, eliciting a relatively pain-free response at the injection site in comparison with clindamycin hydrochloride. The latter is a microbiologically inactive palmitate ester of clindamycin and is readily cleaved *in vivo* to the active clindamycin. It is devoid of the characteristic bitter taste of clindamycin hydrochloride, making it suitable for oral pediatric formulations (3).

The present study was aimed at developing methods to characterize and quantitate clindamycin hydrochloride, clindamycin 2-phosphate, and clindamycin 2-palmitate hydrochloride in bulk quantities and formulations. This study involved detection of possible trace contaminants such as lincomycin, epilincomycin (V), epiclindamycin (VI), and clindamycin B (VII) in clindamycin hydrochloride. The same trace impurities would be present as the corresponding esters in clindamycin 2-phosphate and clindamycin 2-palmitate hydrochloride, since these two antibiotics are synthesized from bulk clindamycin hydrochloride. Assays of clindamycin hydrochloride and clindamycin 2-palmitate hydrochloride involved derivatization of the compounds followed by GLC. Since derivatized clindamycin 2-phosphate could not be chromatographed satisfactorily, it was enzymatically hydrolyzed to clindamycin prior to derivatization and GLC.



	R_1	R_2	R_3
I:	*n*-propyl	Cl (*S*)	OH
II:	*n*-propyl	OH (*R*)	OH
III:	*n*-propyl	Cl (*S*)	phosphate
IV:	*n*-propyl	Cl (*S*)	palmitate
V:	*n*-propyl	OH (*S*)	OH
VI:	*n*-propyl	Cl (*R*)	OH
VII:	ethyl	Cl (*S*)	OH

EXPERIMENTAL

Clindamycin Hydrochloride—*GLC Conditions*—A gas chromatograph[2] equipped with a flame-ionization detector was used. The U-shaped column was glass, 3 mm i.d. × 61 cm, packed with 3% OV-17 on 60–80-mesh Gas Chrom Q. The following temperatures were maintained: column, 170°; detector, 200°; and flash heater, 170°. The carrier gas (helium) was maintained at a flow of 60 ml/min at the operating temperature. The sample volume injected was 1 μl, with the attenuation adjusted to give at least 50% of full scale on the recorder. The sample was injected directly into the glass column.

Internal Standard Solution—A chloroform solution containing approximately 2 mg of hexacosane/ml was prepared.

Reference Standard and Bulk Drug Preparation—Accurately weighed bulk drug and reference standard samples of approximately 5 mg were placed in 15-ml glass-stoppered centrifuge tubes, 2.0 ml of the internal standard solution was added, and the solutions were then treated as described under *Procedure*.

Reference Standard and Hard-Filled Capsules Preparation—The average capsule fill weight was determined, and a sample equivalent to about 13 mg of clindamycin hydrochloride was accurately weighed into a 15-ml stoppered centrifuge tube. A reference standard clindamycin hydrochloride sample was prepared in the same manner. To the centrifuge tubes were added 6.0 ml of the internal standard solution and 6.0 ml of 1% sodium carbonate solution. The tubes were shaken vigorously and centrifuged. Then the top aqueous layers were discarded and approximately 2 g of anhydrous sodium sulfate was added to dry the chloroform layers. Two-milliliter aliquots of the chloroform solutions were placed into 15-ml glass-stoppered centrifuge tubes and treated as described under *Procedure*.

Procedure—To the reference and sample tubes was added 0.5 ml of trifluoroacetic anhydride. After swirling to mix thoroughly, the tubes were stoppered and placed in a heating block or oil bath to an approximate depth of 5 cm so that the upper part of the centrifuge tube acted as a reflux condenser. The tubes were heated at 45° for 30 min. On removal from the heating block, approximately 1 mg of anhydrous sodium carbonate was added to each tube and the tubes were allowed to stand for approximately 30 min before chromatography.

Clindamycin 2-Phosphate—*GLC Conditions*—The conditions were the same as for clindamycin hydrochloride.

Internal Standard Solution—A chloroform solution containing approximately 0.45 mg of hexacosane/ml was prepared.

pH 9 Borate Buffer with Magnesium—To a 1-liter volumetric

[1] The USAN generic name clindamycin phosphate refers to the 2-phosphate, and the generic name clindamycin palmitate refers to the 2-palmitate.

[2] F & M 402.

Table I—Impurity Results of Seven Replicate Samples of a Research Lot of Clindamycin Hydrochloride

Impurity	Average Percent	Coefficient of Variation
Epilincomycin	2.30	5.80
Lincomycin	0.225	17.2
Clindamycin B	2.20	1.95
Epiclindamycin	0.656	12.8

flask were added 3.1 g of boric acid and 500 ml of water. After mixing, 21 ml of 1 *N* sodium hydroxide and 10 ml of 0.1 *M* magnesium chloride were added. The solution was diluted to volume with water and shaken.

Reference Standard Solution—Approximately 9.5 mg of clindamycin hydrochloride hydrate was accurately weighed and placed in a 35-ml stoppered centrifuge tube; then 20 ml of pH 9 borate buffer was added.

Sample Preparation—A sterile solution of clindamycin 2-phosphate (150 mg clindamycin base equivalents/ml) was diluted with borate buffer to produce a solution containing approximately 0.4 mg of clindamycin base/ml. Twenty-five milliliters of this solution was added to a 50-ml stoppered centrifuge tube, 10 ml of chloroform was added, and the tube was shaken vigorously for 15 min. Then the tube was centrifuged and a 20-ml portion of the aqueous solution was removed with a pipet and placed in a 35-ml centrifuge tube (chloroform layer was used for determination of free clindamycin).

Procedure—To the 35-ml tubes was added 50 units of intestinal alkaline phosphatase[3]. The tubes were allowed to stand until the phosphatase dissolved and then were placed in a 37° water bath for 2.5 hr. After the tubes were removed from the water bath and cooled, 10 ml of the internal standard solution was added to each tube. The tubes were shaken vigorously for 15 min and centrifuged. Then the clear aqueous layer was discarded and the tubes were centrifuged again if an emulsion layer was present.

The remaining aqueous layer was removed by suction, and 3.0 ml of the chloroform layers was transferred to 4-ml tablet vials containing approximately 1 g of anhydrous sodium sulfate. After shaking the vials to dry the chloroform solutions, 1.0-ml aliquots of the solutions were transferred to 15-ml stoppered centrifuge tubes and 0.25 ml of trifluoroacetic anhydride was added to each. They were placed in a 45° heating block for 30 min. On removal from the heating block, approximately 1 mg of anhydrous sodium carbonate granules was added to each tube and the solutions were chromatographed.

Clindamycin 2-Palmitate Hydrochloride—*GLC Conditions*—The same conditions as for clindamycin hydrochloride were used, except the column was packed with 1% SE-54 on 60–80-mesh Diatoport S. The following temperatures were maintained: column, 260°; detector, 285°; and flash heater, 260°.

Internal Standard Solution—A chloroform solution was prepared containing approximately 5 mg of cholesteryl benzoate/ml.

Reference and Bulk Drug Preparation—Approximately 20 mg each of reference and bulk drug clindamycin 2-palmitate hydrochloride was accurately weighed and transferred to 15-ml stoppered centrifuge tubes, and 1 ml of the internal standard solution was added.

Flavored Granules Sample Preparation—A flavored granules sample, containing approximately 80 mg of clindamycin 2-palmitate hydrochloride, was accurately weighed into a 15-ml centrifuge tube. Four milliliters of the internal standard solution was added, and the mixture was shaken vigorously and centrifuged. A 1.0-ml aliquot of the clear solution was transferred to another 15-ml centrifuge tube.

Procedure—One milliliter of pyridine and 0.5 ml of acetic anhydride were added and the tubes (reference and sample) were gently swirled for complete mixing. They were stoppered and placed in a heating block as in the procedure for clindamycin hydrochloride, except that the heating was at 100° for 2.5 hr. The solutions were removed from the block, allowed to cool, and chromatographed.

[3] Worthington Biochemical Corp.

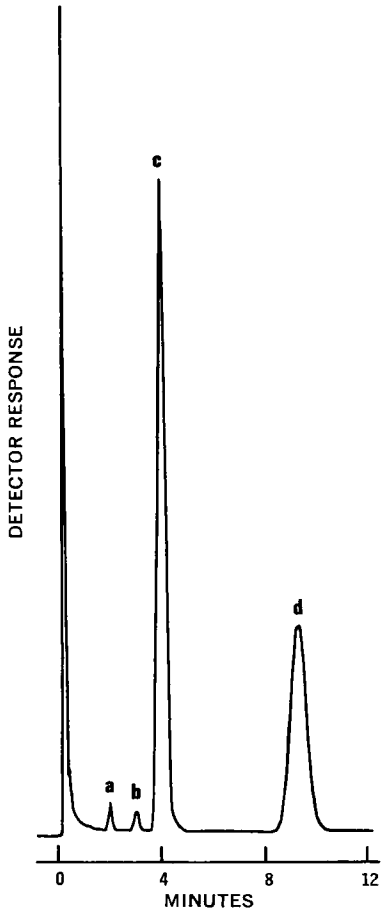


Figure 1—*Chromatogram of: (a) trifluoroacetylated epilincomycin, (b) trifluoroacetylated clindamycin B, (c) trifluoroacetylated clindamycin, and (d) hexacosane as internal standard.*

Calculations—The percent purity was calculated using the equation:

$$\% \text{ purity} = \frac{R_1}{R_2} \times \frac{W_2}{W_1} \times F \times 100 \qquad \text{(Eq. 1)}$$

where R_1 = peak area ratio of sample peak to internal standard peak, R_2 = peak area ratio of reference peak to internal standard peak, W_1 = weight in milligrams of sample, W_2 = weight in milligrams of reference, and F = dilution and conversion factor.

RESULTS AND DISCUSSION

Clindamycin Hydrochloride—Due to the polar groups on the clindamycin molecule, derivatization was necessary for GLC. GLC–mass spectrometry[4] indicated that trifluoroacetylated clindamycin chromatographs intact as the derivatized free base containing three trifluoroacetyl groups.

A chromatogram showing impurities in a research lot of clindamycin hydrochloride is shown in Fig. 1. When present, lincomycin appears between the epilincomycin and clindamycin B peaks and epiclindamycin appears on the tailing edge of the clindamycin peak. Impurity results (Table I) were obtained using peak area percent (without an internal standard), in which the total area of all peaks was assigned to be 100%. As can be seen in Table I, less measurement variation was obtained when the impurity was present in larger amount. GLC weight-response ratios for authentic samples of epilincomycin, epiclindamycin, and clindamycin B relative to clindamycin were determined to be approximately equal.

Linearity and complete extraction of clindamycin were obtained

[4] LKB 9000 mass spectrometer.

Table II—Precision of Replicate Samples of Three Clindamycin Formulations

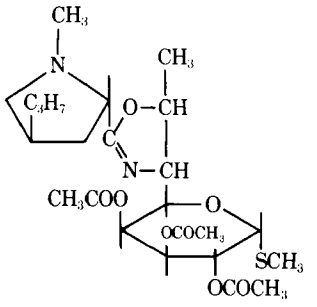
Sample, mg or ml	Clindamycin Base Found, % of Theory
150-mg Clindamycin Hydrochloride Capsules	
134.89	98.5
138.94	101.2
139.83	101.8
135.37	99.5
146.92	102.7
137.03	99.1
Average	100.5 *CV* 1.7%
150-mg/ml Sterile Solution of Clindamycin 2-Phosphate	
1.0	103.0
1.0	100.0
1.0	102.0
1.0	102.0
1.0	102.0
1.0	103.0
Average	102.0 *CV* 0.8%
Flavored Granules of Clindamycin 2-Palmitate	
616.71	100.8
719.90	103.8
755.28	103.0
865.65	102.4
881.77	103.8
790.61	101.4
Average	102.5 *CV* 1.2%

for the hard-filled capsule formulation over the range of ±50% of label. The coefficient of variation of the assay using six replicate samples was less than 2% (Table II). Correlation between this chromatographic procedure and bioassay was excellent. A chromatogram obtained using this procedure is shown in Fig. 1.

In earlier experiments with acetylated clindamycin, using a heavy load column at higher temperature (10% SE-30 column at 250–300°), a relatively small peak appeared on the chromatogram in addition to the large main peak. This small peak had a shorter retention time and tailed badly into the main peak, suggesting on-column degradation. To clarify this finding, the main chromatographic peak was collected in a glass capillary tube and reinjected. Again, the small peak was obtained in addition to the main peak, indicating that the small peak was due to on-column degradation and not to an impurity appearing because of better separation on the heavy load column.

GLC–mass spectroscopy of this degradation peak indicated a molecular weight 36 mass units less than derivatized clindamycin free base, with no evidence of chlorine isotope. The loss of 36 mass units apparently was due to the loss of hydrogen chloride from the molecule. Further characterization of the degradation peak was obtained by IR spectroscopy on a collected sample. IR results showed a loss of amide bands and the appearance of a C=N band when compared with the spectra of acetylated clindamycin. Structure VIII seems to satisfy the data for this on-column degradation peak.

Assay of clindamycin hydrochloride using the acetyl derivative was satisfactory, but separation of epilincomycin from epiclindamycin was not complete (Fig. 2). Higher temperature and longer



VIII

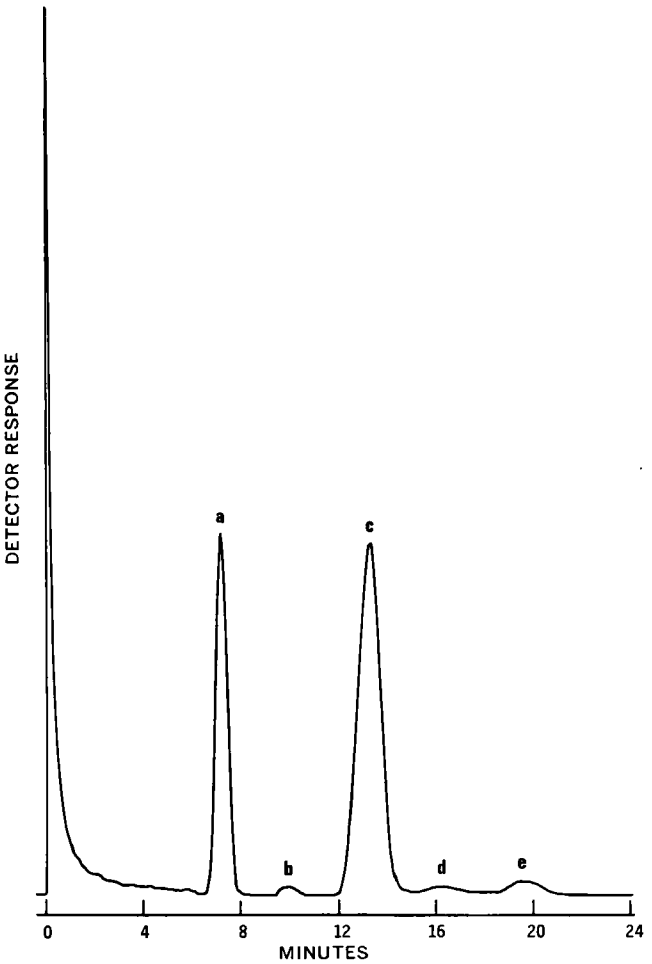


Figure 2—*Chromatogram of: (a) cholestane as internal standard, (b) acetylated clindamycin B, (c) acetylated clindamycin, (d) acetylated epiclindamycin and/or acetylated epilincomycin, and (e) acetylated lincomycin.*

heating time were required with the acetyl derivative procedure. In other experiments using a silylated derivative, clindamycin hydrochloride was not completely separated from lincomycin.

Clindamycin 2-Phosphate—Since this clindamycin ester could not be satisfactorily chromatographed even in a derivatized form, it was hydrolyzed to clindamycin using alkaline phosphatase enzyme, trifluoroacetylated, and then measured by GLC. The coefficient of variation obtained by assaying six replicate samples of sterile solution formulation was approximately 1% (Table II). Clindamycin hydrochloride was used as the reference in the assay instead of clindamycin 2-phosphate mainly because clindamycin 2-phosphate is quite hygroscopic, making it difficult to weigh. However, clindamycin 2-phosphate reference was used to demonstrate complete enzyme hydrolysis.

Impurities present in clindamycin hydrochloride can also be present in their ester form in clindamycin 2-phosphate and detected after hydrolysis (Fig. 1). In addition, it is possible for clindamycin 3-phosphate and clindamycin 4-phosphate to be formed in the synthesis from clindamycin hydrochloride. To determine the specificity of the assay in their presence, authentic samples of these possible impurities were carried through the assay procedure. Clindamycin 3-phosphate was only 37% hydrolyzed and clindamycin 4-phosphate was less than 1% hydrolyzed in the 2.5-hr incubation time of the procedure. Clindamycin hydrochloride, present as an impurity in clindamycin 2-phosphate, is removed in the chloroform wash of the procedure and can be assayed after concentration of the chloroform wash.

Clindamycin 2-Palmitate Hydrochloride—This ester was derivatized with acetic anhydride before GLC. Derivatizing agents such as trifluoroacetic anhydride, *n*-butyl boronic acid, and silylation reagents were more rapid but GLC was erratic. On some col-

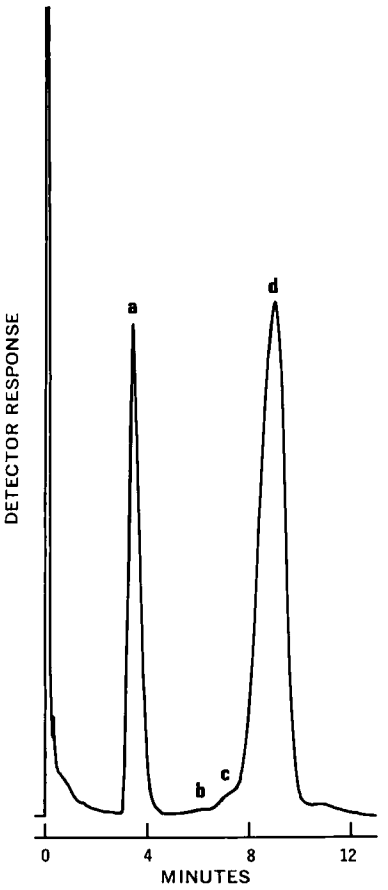


Figure 3—*Chromatogram of: (a) cholesteryl benzoate as internal standard, (b) acetylated clindamycin 4-palmitate, (c) acetylated clindamycin 3-palmitate and/or acetylated clindamycin B 2-palmitate, and (d) acetylated clindamycin 2-palmitate.*

umns a large shoulder appeared on the main chromatographic peak. Prior experience chromatographing acetylated clindamycin at elevated temperature suggested that acetylated clindamycin 2-palmitate may not chromatograph intact but as a cyclic structure, analogous to that proposed for the on-column degradation peak of acetylated clindamycin, and that the shoulder sometimes seen might be due to incomplete degradation to the cyclic compound. GLC–mass spectroscopy obtained on an acetylated clindamycin 2-palmitate peak showed a parent ion at *m/e* 711, indicating the presence of two acetyl groups and loss of hydrogen chloride. Again, no isotopic chlorine peaks were observed. Introduction of a derivatized clindamycin 2-palmitate sample by direct probe into the mass spectrometer produced a molecular ion of 747, showing that loss of HCl in using GLC–mass spectroscopy was due to the column and not the spectrometer. Since no impurity of this nature was detected in clindamycin 2-palmitate hydrochloride drug lots, the GLC peak obtained by on-column degradation of the derivatized ester was acceptable for quantitation. Assay of bulk drug or flavored granules gave a coefficient of variation of approximately 1.2% (Table II).

This compound also has several possible impurities which must be considered in the assay procedure. Clindamycin, clindamycin B 2-palmitate, clindamycin 3-palmitate, and clindamycin 4-palmitate are some impurities found in research lots of clindamycin 2-palmitate. As shown in Fig. 3, clindamycin 4-palmitate is completely separated from clindamycin 2-palmitate while clindamycin 3-palmitate appears as a shoulder on the front side of the clindamycin 2-palmitate peak. Clindamycin B 2-palmitate also appears as a shoulder on the front side; consequently, the assay procedure does not distinguish between these two impurities. The amount of clindamycin B 2-palmitate has been determined by hydrolyzing the sample with strong base, extracting, and then chromatographing, using the trifluoroacetyl derivative procedure as for clindamycin. The shoulder on the back side of the main peak in Fig. 3 is believed to be due to derivatized epiclindamycin 2-palmitate and/or epilincomycin 2-palmitate, analogous to that for acetylated clindamycin in Fig. 2.

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GLC Assay of Sorbitol as Cyclic *n*-Butylboronate

MICHAEL P. RABINOWITZ ˣ, PHILIP REISBERG, and JEROME I. BODIN

Abstract □ A simple, rapid, and quantitative GLC procedure was developed for the determination of sorbitol in solid and aqueous solution forms. The method utilizes the ability of *n*-butylboronic acid to convert sorbitol quantitatively to the cyclic *n*-butylboronate ester. Preparation of the sample is simple, and the reaction goes rapidly to completion. Baseline separation and peak symmetry are obtained for sorbitol, mannitol, and galactitol. A procedure for establishing a sorbitol reference standard is presented.

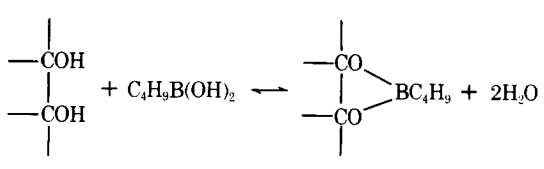
Keyphrases □ Sorbitol—GLC analysis as the cyclic *n*-butylboronate □ *n*-Butylboronic acid—conversion of sorbitol to cyclic *n*-butylboronate ester, GLC analysis □ GLC—analysis, sorbitol as the cyclic *n*-butylboronate

Sorbitol is a naturally occurring, straight-chain hexahydric alcohol commonly incorporated in pharmaceutical products as a humectant and sweetener (1). It is also used as a chemical intermediate in the synthesis of ascorbic acid and is of particular importance as a food additive for dietary purposes (2). Medically, it has been used as an osmotic diuretic and cathartic and for parenteral alimentation (3).

Many reported analytical methods for the quantitative and qualitative determination of sorbitol, sorbitol solutions, and similar alditols are based on refractometry (4, 5), polarimetry of molybdate complexes (6, 7), coulometry (8), colorimetry (9), and titration after periodate oxidation (10). Some of these methods are rapid, others are laborious, and none are specific for sorbitol. Chromatographic methods such as ion exchange (10, 11), paper (12, 13), thin layer (10, 14–16), and adsorption column (1, 17) offer some degree of specificity but are also laborious or time consuming.

DISCUSSION

At the present time, GLC offers the best means of analyzing sorbitol and sorbitol solutions quantitatively with a measure of selectivity and rapidity. Many methods have been proposed based on the conversion of monosaccharides and alditols to silyl and acetate derivatives (18–24), but the numerous disadvantages of these methods have been cited. The silylation procedure necessitates the complete removal of water from the sample before reaction, requires extended reaction times, and does not completely separate sorbitol from mannitol and galactitol. Conversion of the alditols to the hexaacetate derivatives shortens the reaction time considerably, and the derivatives are separated when chromatographed. However, this procedure is still time consuming due to the necessity of either removing water from the sample by rotary evaporation (25) or removing the solvent (pyridine) after reaction to assure that no tailing of peaks will occur (26).



diol + *n*-butylboronic acid dioldibutylboronate

Scheme I

Alkaneboronic acids were recently introduced as aids for the volatilization of various organic compounds (27, 28). Eisenberg (29) reported the successful application of *n*-butylboronic acid for GLC of several sugars and alditols, including sorbitol, mannitol, and galactitol. The proposed reaction is shown in Scheme I (29).

This paper describes the determination of sorbitol in powder form and in aqueous solutions as the cyclic *n*-butylboronate. The sorbitol boronate is formed readily by combining a known weight of sample with a solution of *n*-butylboronic acid in pyridine, containing methyl nonadecanoate as the internal standard. Studies performed in this laboratory indicate that the reaction goes to completion almost immediately. The liquid phase used is a recently introduced cyanoalkyl phenyl silicone which is versatile and stable and exhibits virtually no bleeding of the liquid phase when used at the operating parameters described.

The absence of a suitable sorbitol reference standard of verified composition has hindered the development of a truly quantitative method for the determination of sorbitol. Therefore, a selected lot of crystalline sorbitol was analyzed for total polyhydric alcohol content, using sodium arsenite as the titrant after periodate oxidation, and then for the sorbitol portion of the total by GLC without the internal standard. Such a standard, when properly stored, is stable for several months.

Data demonstrating the linearity of response with changes in concentration, reproducibility, and completeness of recovery are presented.

EXPERIMENTAL

Operational Parameters—A gas chromatograph[1] with hydrogen flame-ionization detectors was used. The column was glass with a U-type configuration, 183 cm long by 4 mm i.d., packed with 3% cyanoalkyl phenyl silicone liquid phase on a silanized diatomaceous earth support, 80–100 mesh. The column was conditioned for 18 hr at 250° with carrier gas flow. Operating parameters were: column temperature, 205°; detector temperature, 260°; injection port temperature, 245°; and carrier gas (nitrogen) flow rate, 50 ml/min. Hydrogen and air flows were adjusted to obtain maximum efficiency as recommended by the manufacturer; the range selector was set at 10 and attenuation was adjusted to obtain at least a 50% recorder scale response for major peaks. All peak areas were measured with an electronic integrator[2].

Reagents and Chemicals—The liquid phase, 50% cyanoalkyl 50% phenyl silicone (SILAR 5CP)[3], the silanized diatomaceous earth support (Gas Chrom Q)[3], and methyl nonadecanoate were used as supplied[3]. Sorbitol standard was crystalline USP grade[4]. *n*-Butylboronic acid, 99+%[5], was kept moist with water until ready for use. Pyridine (ACS reagent grade) was used without further purification.

Reagent–Internal Standard Preparation—An amount of *n*-butylboronic acid (from which the excess water had been removed by light vacuum filtration) was dissolved in sufficient pyridine to yield a final concentration of 10 mg/ml. Methyl nonadecanoate was added to a portion of the *n*-butylboronic acid stock solution so that the concentration of internal standard in the reagent solution was 2 mg/ml.

Sample Preparation—An accurately weighed sample, containing 130–160 mg of sorbitol, was transferred to a 100-ml volumetric flask and was dissolved in and diluted to volume with water. A 1.0-ml aliquot, transferred to a suitable glass vial, was taken to

[1] Model 402, Hewlett-Packard.
[2] Model CSI-208E, Columbia Scientific Industries.
[3] Applied Science Laboratories, State College, Pa.
[4] Pfizer, Inc., New York, N.Y.
[5] Aldrich Chemical Co., Milwaukee, Wis.

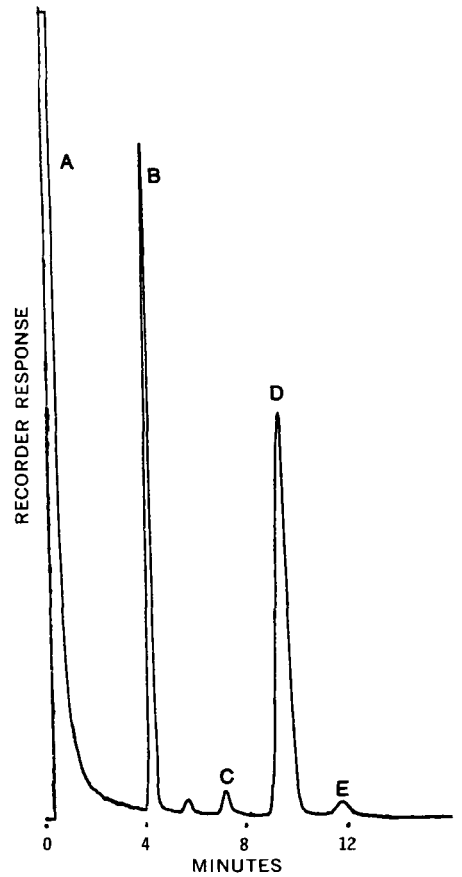


Figure 1—*Sample chromatogram showing relative retention times of sorbitol, mannitol, and galactitol as the* n*-butylboronates and the internal standard methyl nonadecanoate. Key: A, solvent; B, methyl nonadecanoate; C, mannitol; D, sorbitol; and E, galactitol.*

dryness in a vacuum oven at 40–60°. The residue was reconstituted with a 1.0-ml aliquot of the reagent–internal standard preparation. A 1.0-μl sample was injected into the chromatograph and allowed to elute for about 12 min (Fig. 1).

Standard Preparation—Sorbitol reference standard was accurately weighed and diluted with water to a concentration of 1.5–1.7 mg/ml. A 1.0-ml aliquot was transferred to a suitable vial and was taken to dryness in a vacuum oven at 40–60°. The residue was reconstituted with a 1.0-ml aliquot of the reagent–internal standard solution. A 1.0-μl sample was injected into the chromatograph and allowed to elute for about 12 min.

Sorbitol Standard Potency—A batch of sorbitol USP, crystalline grade, was selected as an arbitrary working standard since an official reference standard of known potency is not available. Total polyhydric alcohol content was determined by titration of a 0.40-mg/ml solution of sorbitol standard in water with 0.05 *N* sodium arsenite, as described in the USP XVIII (1, p. 680). The sorbitol content of the standard was determined by an area normalization calculation applied to GLC determinations made using the operating parameters described but without the internal standard. The purity of the sorbitol standard was calculated as the total polyhydric alcohol content times the sorbitol content determined by GLC without the internal standard.

RESULTS

The analytical procedure was designed so that sorbitol solutions of any concentrations may be easily analyzed. No difficulty with the analysis due to the presence of water was encountered when the reagent–internal standard preparation was reacted with a 1-ml aliquot of a diluted sample solution and injected directly. However, the long-term deteriorating effect of routinely injecting water onto a column with a silanized support was considered. Taking the sample aliquot to dryness (or near dryness) in a vacuum oven allows the assembly and manipulation of a large number of samples, which may then be stored until ready for analysis. The almost immediate completeness of the reaction also suggests the possibility of a semiautomated GLC procedure.

Table I—GLC Analysis of Sorbitol Solution USP

Run Number	Sorbitol, % Found (w/w)
1	65.4
2	65.6
3	65.7
4	65.6
5	65.7
6	65.6
Mean	65.6
σ	±0.1

n-Butylboronic acid, when stored under water (1 ml/g of reagent) to inhibit degradation (30), is quite stable. The reagent–internal standard preparation has been used in this laboratory for as long as a month without signs of degradation. Although excess water is removed before dissolving *n*-butylboronic acid in pyridine, the reagent is still slightly damp. Water is also generated in the reaction of the reagent with the alditols. The small amount of water present has not caused any noticeable change in the column characteristics over several months of repeated use.

Eisenberg and others (29–31) noted that extended storage of *n*-butylboronate derivatives in the reaction medium may lead to possible degradation of the derivative.

Standard solutions of sorbitol, ranging from 25 to 125% of the concentration levels to be expected in a typical analysis, were chromatographed; a plot of the ratio of areas *versus* concentration (Fig. 2) shows that the chromatographic response is linear over the entire range.

Replicate determinations of a commercial sample of sorbitol solution, 70%, USP (Table I), were made. The standard deviation calculated indicates excellent precision for the analytical procedure.

A recovery study was performed by analyzing solutions of known concentration prepared from the reference standard which had been analyzed as previously described (Table II).

A standard chromatogram (Fig. 3) and a typical sample chro-

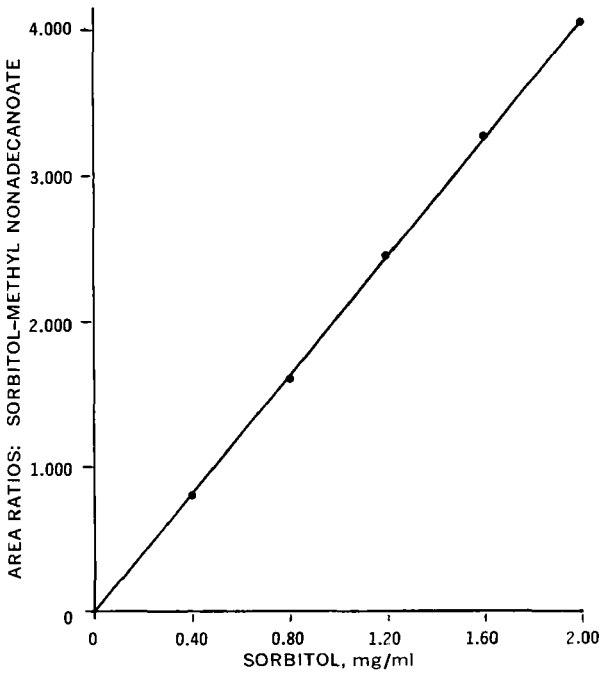


Figure 2—*Linearity of response (ratio of areas) with concentration. Concentration is in milligrams per milliliter with the internal standard as a constant. (See text for other conditions.)*

Table II—Sorbitol Recovery Study

Amount Present, % (w/w)	Experimentally Found, % (w/w)	Recovery, %
70.4	70.8	100.6
70.0	69.7	99.6
68.2	67.8	99.4
70.1	69.6	99.3
70.1	69.7	99.4
69.2	69.5	100.4
Mean	—	99.8

matogram (Fig. 1) show that the sorbitol, mannitol, and galactitol cyclic *n*-butylboronates are well separated and that the peaks show excellent symmetrical configuration. It is believed that the 5- or 6-membered rings formed in the reaction with the *n*-butylboronic acid greatly accentuate the small steric differences between the alditols. A sufficient separation occurs between sorbitol, mannitol, and galactitol, thereby permitting a rapid chromatographic analysis. Relative retention times (to internal standard methyl nonadecanoate, retention time of 4.20 min) are: sorbitol, 2.25; mannitol, 1.71; and galactitol, 2.80. Although linearity response and recovery studies were not done for mannitol or galactitol, quantitative analysis of these two alditols probably could be easily accomplished using the method and parameters described.

The liquid phase chosen is a recently introduced (31) 50% cyanoalkyl 50% phenyl silicone[3] of moderate polarity. It has been found to be stable at relatively high temperatures and exhibits little or no bleeding of the liquid phase at operating temperatures. Despite its use for the analysis of several other compounds and without the use of an oxygen scrubber for the carrier gas, the retention and relative retention times of sorbitol, mannitol, and galactitol, with a particular column, have remained constant over several months.

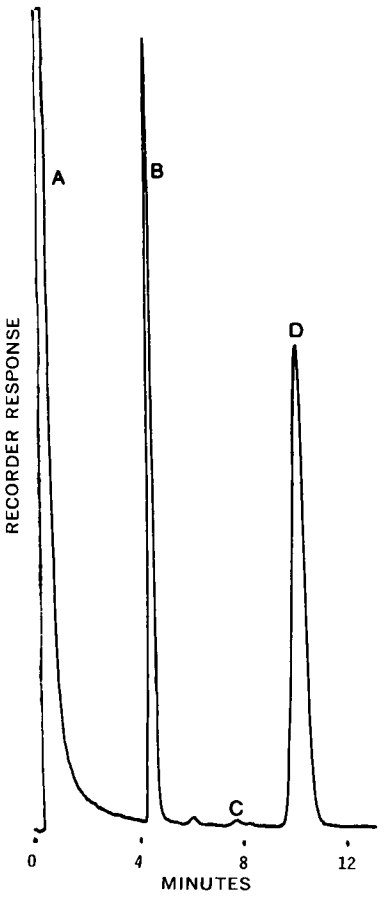


Figure 3—*Standard chromatogram showing relative retention times of sorbitol and mannitol as* n*-butylboronates and the internal standard methyl nonadecanoate. Key: A, solvent; B, methyl nonadecanoate; C, mannitol; and D, sorbitol.*

Table III—Comparison of USP and Proposed GLC Methods

Commercial Sample	Sorbitol Found, % (w/w)	
	GLC	USP
1	68.2	67.7
2	68.4	67.9
3	64.6	64.1
4	64.6	64.3
5	93.9	94.4

Samples from several commercial suppliers were analyzed, and the results were compared to those obtained by the USP (1) procedure (Table III). The USP analyses were performed and certified by the suppliers. The analysis of components of the standard need not be done for each analysis if the standard is kept tightly sealed and placed in a desiccator over silica gel. Total polyhydric alcohol analysis over 6 months has shown a variation of less than 0.05%. Analysis for sorbitol in the reference standard (by GLC without the internal standard) has varied only slightly more over the same period.

SUMMARY

The analytical procedure described overcomes the difficulties previously encountered in reported GLC analyses of sorbitol. Sample preparation and reaction with the derivatizing agent, *n*-butylboronic acid, are simple and rapidly accomplished. Analyses are reproducible and accurate and compare favorably with the cumbersome compendial (1) procedure. A method that enables each laboratory to certify and maintain its own sorbitol reference standard is presented.

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[x] To whom inquiries should be directed.

Effect of Maleic Acid in Compendial UV Absorption Assays for Antihistamine Maleate Salts

WILLIAM M. MENT[x] and HELEN S. NAVIASKY

Abstract □ Standard recoveries averaging 89.9% are reported for the NF XIII brompheniramine maleate tablet assay procedure, which employs UV comparison of unidentical sample and standard molecular species in the same medium. The data presented indicate that these low assay values are attributable to the chromophoric properties of the maleic acid moiety and its ability to protonate the α-pyridyl chromophore of the brompheniramine molecule in a neutral organic solvent.

Keyphrases □ Maleic acid—effect on compendial UV absorption assay for antihistamine maleate salts □ Maleate salts of antihistamines—effect of maleic acid on compendial UV absorption assay □ Antihistamine maleate salts—effect of maleic acid in compendial UV absorption assays □ UV absorption—effect of maleic acid in compendial assays for antihistamine maleate salts

Several current compendial monographs contain assay procedures for organic nitrogenous base maleate salts in which the UV absorbance of a sample solution of an extracted base is compared with that of a standard solution of its maleate salt in the same solvent. Recoveries of about 97–98% were previously reported (1) for dexbrompheniramine, dexchlorpheniramine, and chlorpheniramine maleate reference standards obtained by using one method of the Association of Official Analytical Chemists (2) and three monograph assays of NF XIII (3), all of which employ such methodology. These erroneously low assay values, resulting from UV comparisons of unidentical molecular species, have been shown to be caused by the UV absorption properties of maleic acid in 0.1 *N* HCl and 0.1 *N* H_2SO_4, where measurements are made at about 264 nm (1).

In extending these studies of similar antihistamine maleate assays in official monographs, the NF XIII assay for brompheniramine maleate (I) tablets (3, p. 107) was tested for percent recovery values. This NF procedure involves sample extraction of the free amine from alkaline aqueous solution into chloroform. The UV absorbances of the sample solution and of a standard solution of I, diluted directly to the desired concentration in the same medium, are then determined and compared. This method differs from other antihistamine maleate assays previously tested by the authors in that UV measurements of the extracted sample amine base and directly diluted standard amine salt are made in an organic solvent (chloroform) rather than in aqueous acid solution.

Recoveries ranging from 89.3 to 90.5% were ob-

Table I—Recovery Data for Brompheniramine, Chlorpheniramine, and Pheniramine Maleates Reference Standards Using the NF XIII Brompheniramine Maleate Tablet Assay

Salt	Source	Solvent Used for Standard Dissolution	Recovery, %
Brompheniramine maleate	Commercial	Chloroform	90.2
Brompheniramine maleate	Commercial	Chloroform	89.3
Brompheniramine maleate	Commercial	Chloroform (sodium hydroxide treated)	89.7
Brompheniramine maleate	NF	Chloroform (sodium hydroxide treated)	90.5
			Average 89.9
Chlorpheniramine maleate	USP	Chloroform	88.6
Chlorpheniramine maleate	USP	Chloroform (sodium hydroxide treated)	89.0
			Average 88.8
Pheniramine maleate	Commercial	Chloroform	89.5
Pheniramine maleate	Commercial	Chloroform (sodium hydroxide treated)	89.9
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Chlorpheniramine maleate	USP	Chloroform (sodium hydroxide treated)	89.0
			Average 88.8
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Pheniramine maleate	Commercial	Chloroform (sodium hydroxide treated)	89.9
			Average 89.7

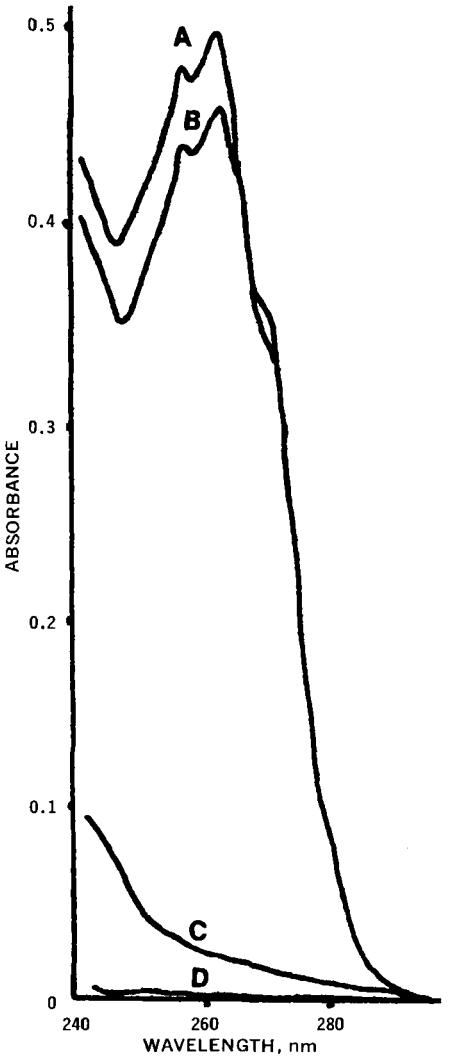

Figure 1—*UV spectra of: A, brompheniramine maleate diluted directly to final concentration of 0.0412 mg/ml in chloroform, alkali treated as per NF assay (3, p. 107); B, brompheniramine at a concentration equivalent to 0.0420 mg/ml of the maleate salt in chloroform, after extraction of brompheniramine maleate by NF assay (3, p. 107); C, maleic acid (0.0112 mg/ml) in chloroform, alkali treated as per NF assay (3, p. 107); and D, chloroform, alkali treated as per NF assay (3, p. 107),* versus *similarly treated chloroform.*

tained for reference standards by the assay for I tablets. These results are of particular significance because of the 95–105% assay limits established by the NF for label-declared amounts of I tablets. In addition, the content uniformity test in this monograph utilizes the same analytical method employed for the composite assay.

The purposes of this paper are to: *(a)* report analytical recovery data for antihistamine maleates obtained by compendial assays which may give erroneous results due to the maleic acid effect (1), and *(b)* show that the significantly and uniquely lower recoveries obtained in the NF assay for I tablets may be explained by bonding (protonation) effects between maleic acid and the α-pyridyl chromophore in the brompheniramine molecule, in addition to the chromophoric properties of the maleic acid moiety itself.

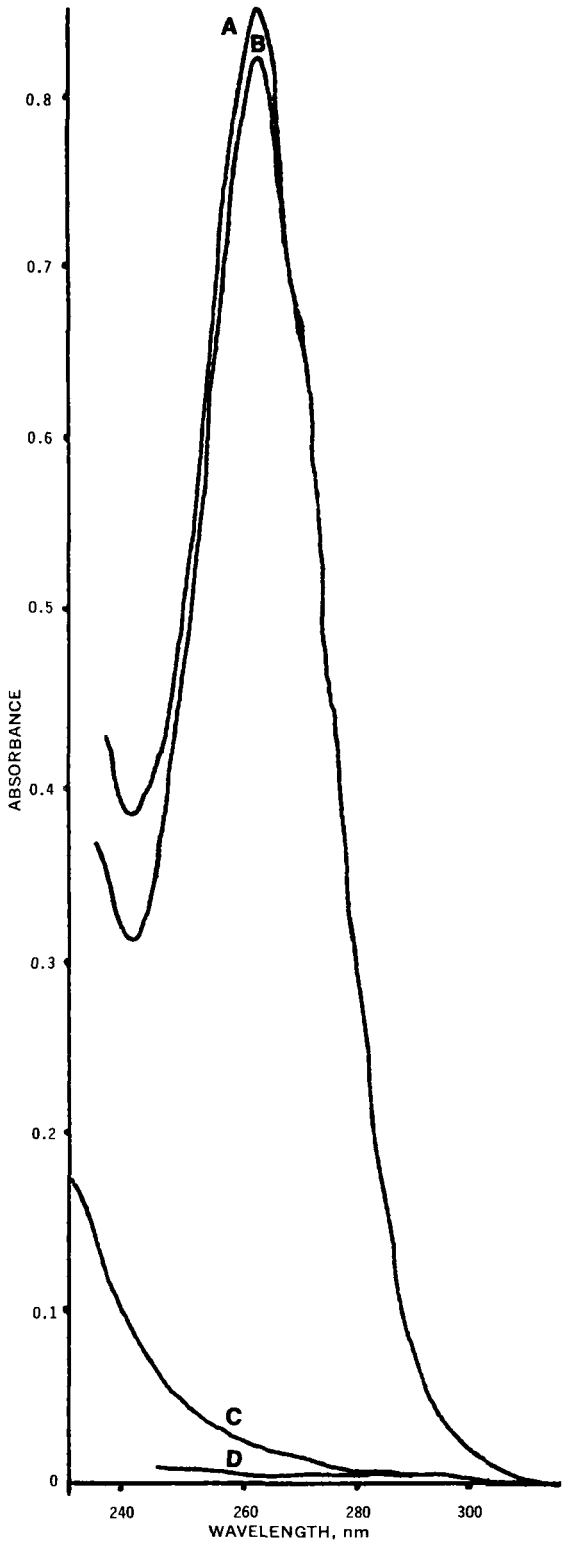

Figure 2—*UV spectra of: A, chlorpheniramine maleate directly diluted to final concentration of 0.0406 mg/ml in hydrochloric acid (1 in 100), solvent treated as per USP assay (4); B, chlorpheniramine at a concentration equivalent to 0.0402 mg/ml of the maleate salt in hydrochloric acid (1 in 100) after extraction of chlorpheniramine maleate by USP assay (4); C, maleic acid (0.01205 mg/ml) in hydrochloric acid (1 in 100), solvent treated as per USP assay (4); and D, hydrochloric acid (1 in 100), solvent treated as per USP assay (4)* versus *similarly treated hydrochloric acid (1 in 100).*

EXPERIMENTAL

Monograph-specified amounts of NF and commercially obtained brompheniramine maleate reference standards were analyzed by the NF XIII assay procedure for I tablets. The commercial standard employed assayed 99.8% by direct dilution and UV comparison with the NF reference standard.

Maleic acid solutions in equimolar concentrations in the appropriate solvents were prepared to determine the contribution of maleic acid to total antihistamine maleate UV absorbance at wavelengths specified in the monograph. The maleic acid employed assayed 99.7% by the NF XIII titrimetric method (3, p. 921).

Spectrograde solvents, when available, were employed in the UV determinations. All spectra were obtained on a recording spectrophotometer[1], using 1-cm matched quartz spectrophotometric cells. All recovery and standard solutions were scanned *versus* their corresponding solvents.

RESULTS AND DISCUSSION

Recoveries averaging 89.9% were obtained in four separate determinations, using the NF assay for I tablets (Table I). UV examination of additional solvent extracts indicated that the free base was completely removed from alkaline aqueous solution by the five 35-ml chloroform extractions specified in the method. Typical UV spectra obtained are presented in Fig. 1.

The NF specifies that UV measurements of sample and standard are to be made "in the same medium." Since this phrase may be subject to interpretation, recovery extracts were compared to standards directly diluted to the desired concentration in both chloroform and chloroform treated with alkali as specified in the monograph. No difference in results was noted with either medium (Table I).

Maleic acid in equimolar concentrations in chloroform and alkali-treated chloroform was found to contribute 3.5–5% to the total UV absorption of I at 263 nm (Fig. 1). (Because of its difficult solubility in chloroform, the maleic acid was initially dissolved in about 1 ml of methanol and diluted to volume with chloroform or alkali-treated chloroform.)

Recoveries of chlorpheniramine maleate and pheniramine maleate reference standards by the NF assay for I tablets were also found to be 88–90% (Table I); maleic acid itself contributed about 4% to the total UV absorbance of the maleate salts of each compound in equimolar amounts in sodium hydroxide-treated chloroform.

Aliquots of the chloroform recovery extracts for all three antihistamines from the I tablet assay were back-extracted with 0.1 *N* H_2SO_4, and the UV absorbances (at 264 nm) of the 0.1 *N* H_2SO_4 extracts were compared to those obtained for the respective antihistamine maleate standards diluted directly to final concentration with chloroform-saturated 0.1 *N* H_2SO_4. Recoveries ranging from 95.8 to 98.6% were obtained. Similarly, 0.1 *N* H_2SO_4 solutions obtained from back-extractions of I–chloroform recovery solutions, when compared with I standards originally directly diluted with alkali-treated chloroform and back-extracted with 0.1 *N* H_2SO_4, gave recoveries of 96.0–96.7%. These results parallel those obtained by the NF XIII assay for dexbrompheniramine maleate tablets (3, p. 213) and for other monograph assays previously tested (1). A possible explanation is that sample (recovery) and standard molecular species may be equally protonated by the highly acidic media. The only effect on UV absorption (and thus the cause of low recoveries) under these conditions is the absence of the maleic acid chromophore from the sample solution. [The authors also obtained standard recoveries averaging 97.6% by the USP XVIII assay for chlorpheniramine maleate elixir (4), in which UV absorptions of sample free amine and standard maleate salt in aqueous acid are also compared. Equimolar amounts of maleic acid were found to contribute 1.8–3.2% of the total absorption at 264 nm in hydrochloric acid (1 in 100). Typical UV spectra are reproduced in Fig. 2.]

These data indicate that elimination of the maleic acid chromophore from the final brompheniramine sample solution as a result of the extraction procedure is not alone sufficient to account for the approximately 10% low recoveries obtained. An investigation

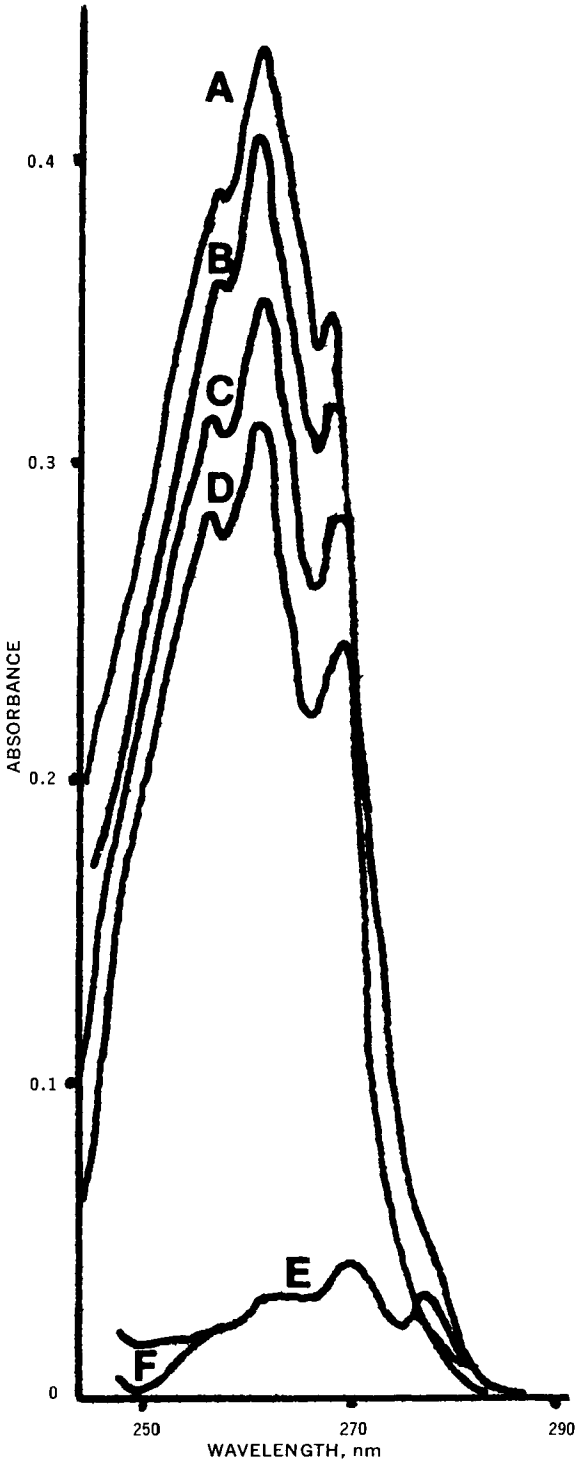

Figure 3—*UV spectra of: A,* p-*bromotoluene (0.0169 mg/ml), α-methylpyridine (0.00933 mg/ml), and maleic acid (0.0116 mg/ml)* versus *maleic acid (0.0116 mg/ml); B, α-methylpyridine (0.0169 mg/ml) and maleic acid (0.0116 mg/ml)* versus *maleic acid (0.0116 mg/ml); C,* p-*bromotoluene (0.0169 mg/ml) and α-methylpyridine (0.00933 mg/ml)* versus *chloroform; D, α-methylpyridine (0.00933 mg/ml)* versus *chloroform; E,* p-*bromotoluene (0.0169 mg/ml) and maleic acid (0.0116 mg/ml)* versus *maleic acid (0.0116 mg/ml); and F,* p-*bromotoluene (0.0169 mg/ml)* versus *chloroform. Spectra of chloroform* versus *chloroform and of maleic acid (0.0169 mg/ml)* versus *maleic acid (0.0169 mg/ml) follow the wavelength axis. All solutions are in chloroform. Concentrations are approximately* 1×10^{-4} *mM.*

[1] Cary model 15, Cary Instruments, Monrovia, Calif.

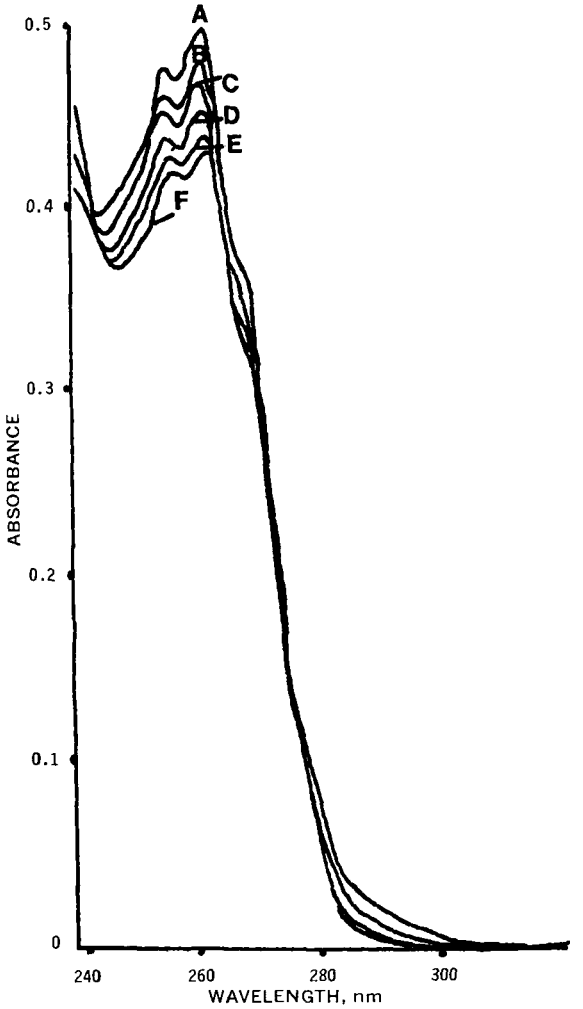

Figure 4—*UV spectra of: A, brompheniramine (0.938 × 10^{-4} mM) and maleic acid (10.05 × 10^{-4} mM) versus maleic acid (10.05 × 10^{-4} mM); B, brompheniramine (0.938 × 10^{-4} mM) and maleic acid (5.023 × 10^{-4} mM) versus maleic acid (5.023 × 10^{-4} mM); C, brompheniramine (0.938 × 10^{-4} mM) and maleic acid (0.942 × 10^{-4} mM) versus maleic acid (0.942 × 10^{-4} mM); D, brompheniramine (0.938 × 10^{-4} mM) and maleic acid (0.628 × 10^{-4} mM) versus maleic acid (0.628 × 10^{-4} mM); E, brompheniramine (0.938 × 10^{-4} mM) and maleic acid (0.314 × 10^{-4} mM) versus maleic acid (0.314 × 10^{-4} mM); and F, brompheniramine (0.938 × 10^{-4} mM) versus chloroform. Spectra of chloroform versus chloroform and of maleic acid (0.942 × 10^{-4} mM) versus maleic acid (0.942 × 10^{-4} mM) follow the wavelength axis. All solutions are in chloroform.*

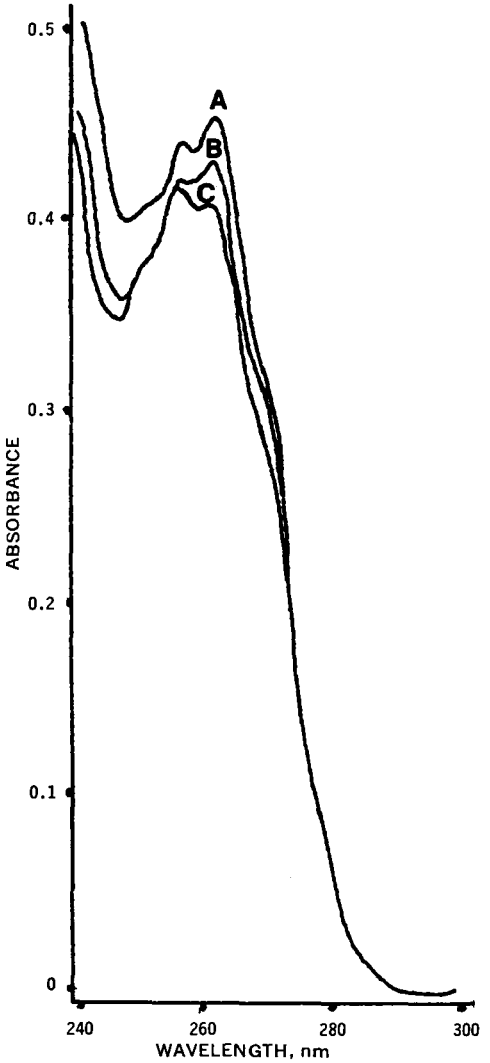

Figure 5—*UV spectra of brompheniramine at a concentration equivalent to 0.0410 mg/ml of the maleate salt in: A, chloroform; B, methylene chloride; and C,* n-C_6H_{14}.

was undertaken to explain the greater reduction in UV absorption observed for the free amine as compared to the maleate salt form when a neutral solvent rather than aqueous acid is employed as the analytical medium.

An unshared pair of electrons on the nitrogen atom in the pyridine ring of the brompheniramine molecule serves as a potential site for proton addition with formation of a pyridinium ion (5). Protonation of pyridine has been reported to enhance the intensity of the $\pi \rightarrow \pi^*$ band (B-Band) (6, 7). Halverson and Hirt (8) obtained a somewhat increased UV absorption in the 250–260-nm region for the ethbromide salt of pyridine as opposed to the parent compound when measured in ethanol, and they found increasing pyridine UV absorption in the same region in solvents of increasing hydrogen-bonding ability. Levine (9) reported that alkaloids containing a basic nitrogen atom in the chromophoric group should be completely protonated in quantitating such drugs by UV measurements, since absorption intensity and wavelength of the maxima vary with degree of ionization. Also, Furman (10) reported increased pyridine and chlorpheniramine UV absorptivities in aqueous acid as opposed to base due to pyridine nitrogen protonation in acid. These data suggest that, should maleic acid be capable of protonating the α-pyridyl chromophore in the I molecule, a hyperchromic effect on UV absorptivity would be expected, accounting for a portion of the observed increased absorption of the salt over the free base form in neutral solvents.

Brompheniramine is a dibasic amine. The nitrogen in the aliphatic side-chain tertiary amine group is more strongly basic than the pyridine nitrogen and would be expected to be preferentially protonated to form the amine salt (11). However, this nitrogen is sufficiently far removed from the active chromophoric site so that any protonation effect here will little influence the UV absorption of the compound. However, brompheniramine maleate is a salt of a dicarboxylic acid with only one of the acidic hydrogens entering into salt formation with the amino group (11). Under certain conditions, such as dissolution in neutral organic solvents, the one proton from the dicarboxylic acid, which is not bonded to the aliphatic amine, might be expected to be available to weakly protonate a chromophoric pyridine nitrogen. Experiments conducted in this laboratory support that argument.

Brompheniramine contains two chromophoric groups, the *p*-bromotoluyl and α-methylpyridyl moieties, both of which absorb in the same UV region. UV spectra of equimolar concentrations of commercially obtained *p*-bromotoluene and α-methylpyridine in chloroform show that the *p*-bromotoluyl moiety contributed less than 10% to the total UV absorption of the combined species at

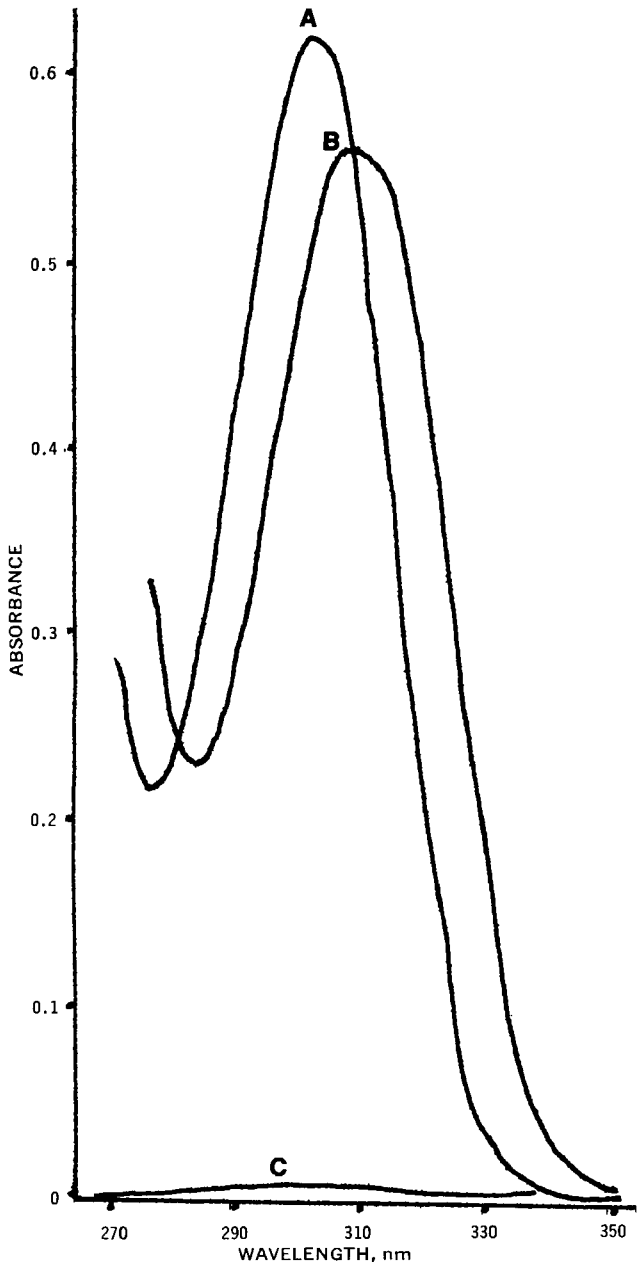

Figure 6—*UV spectra of: A, methapyrilene hydrochloride at a concentration of 0.0398 mg/ml in chloroform; B, methapyrilene at a concentration equivalent to 0.0398 mg/ml of methapyrilene hydrochloride in chloroform; and C, chloroform* versus *chloroform.*

263 nm (Fig. 3). Addition of equimolar quantities of maleic acid to equimolar amounts of both species resulted in a significant increase (about 28%) in the absorption of the α-methylpyridine chromophore at 263 nm and a negligible increase in the UV absorption of *p*-bromotoluene (Fig. 3). The spectra were obtained by scanning *versus* the same maleic acid–chloroform solution to eliminate UV absorption contributed by the maleic acid chromophore. These data indicate that the affected chromophore as well as the site of protonation is the pyridine portion of the brompheniramine molecule. Moreover, they show that the weak carboxylic acid functions of maleic acid are able to exert a measurable effect on the chromophoric properties of the α-methylpyridine system by protonating, at least partly, the pyridine nitrogen.

To provide proof that maleic acid can protonate the pyridyl system in brompheniramine slightly and thus enhance absorptivity at about 263 nm in chloroform, maleic acid in chloroform was added in increasing amounts to the free amine in chloroform and the UV

Table II—Effect of Various Amounts of Maleic Acid on UV Absorption of Brompheniramine

Molar Ratio, Maleic Acid to Brompheniramine (Approximate)	Absorbance of Brompheniramine (Maleate)	Absorbance Increase, %
0.00	0.435	—
0.33	0.442	1.6
0.67	0.455	4.6
1.0	0.469	7.8
5	0.482	10.8
10	0.499	14.7

spectra were recorded. The brompheniramine solution was prepared by dissolving reference standard I in chloroform, extracting with aqueous base to remove the maleic acid, and diluting to volume with chloroform. Increasingly larger aliquots of maleic acid solution were added to a fixed volume of the brompheniramine solution to achieve several molar ratios of maleic acid to brompheniramine, ranging from 0.0 to about 10. All maleic acid–brompheniramine solutions were diluted to the same final volume with chloroform prior to UV scanning. Maleic acid solutions of corresponding concentrations were placed in the reference compartment of the spectrophotometer as previously noted, and the UV spectra of the brompheniramine–maleic acid solutions were recorded by difference measurement (Fig. 4). (Maleic acid was initially dissolved in 1 ml of methanol and diluted to volume with chloroform. Methanol at this concentration was found not to alter the UV spectrum of chloroform.)

Increasing absorption was obtained for brompheniramine with increasing amounts of maleic acid (Table II and Fig. 4). Increased absorption at λ_{max} 263 nm was most pronounced at maleic acid–

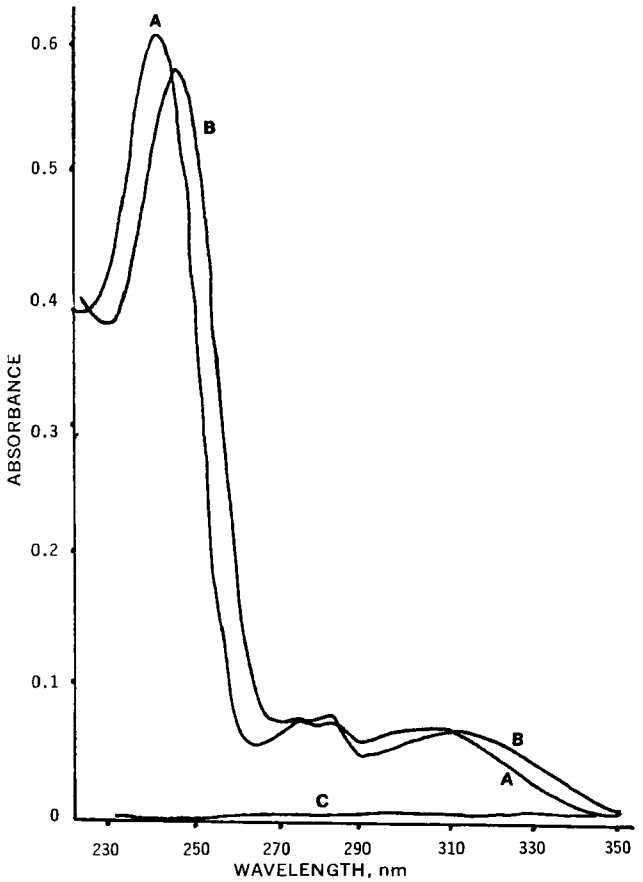

Figure 7—*UV spectra of: A, thonzylamine hydrochloride at a concentration of 0.00835 mg/ml in methylene chloride; B, thonzylamine at a concentration equivalent to 0.00835 mg/ml of thonzylamine hydrochloride in methylene chloride; and C, methylene chloride* versus *methylene chloride.*

brompheniramine ratios of 0.0:1.0. At ratios >1.0, an absorption increase was still exhibited but was lower in magnitude per unit change in ratio. Since the spectra were obtained in such a manner as to cancel out UV absorption due to the maleic acid chromophore, the enhanced absorption observed is explained by interaction of maleic acid with the α-pyridyl chromophore of brompheniramine.

The increase in UV absorption is about four times greater for equimolar amounts of α-methylpyridine and maleic acid in chloroform than for equimolar amounts of brompheniramine and maleic acid in chloroform (Figs. 3 and 4). The difference is apparently due to the presence of the aliphatic amine in brompheniramine, which reduces the degree of protonation of the pyridine nitrogen by preferentially bonding with maleic acid. However, the second ionization of the maleate monosalt is apparently of sufficient magnitude to accomplish weak protonation of the pyridine nitrogen and to produce a measurable effect on the UV absorption of brompheniramine.

To observe the effect of varying degrees of protonation on the UV absorption of brompheniramine, UV spectra were obtained for brompheniramine base in three organic solvents of differing H-donating (H-bonding) abilities (Fig. 5). Identical aliquots from the same I standard stock solution made basic with sodium hydroxide were separately extracted with chloroform, methylene chloride, and *n*-hexane as in the NF assay for I tablets. The extracts were diluted to the same final concentration with the appropriate solvent, and their UV spectra were recorded by scanning *versus* similarly treated solvent blanks. UV tests for completeness of extraction showed total extraction of the brompheniramine base with each solvent using the five 35-ml portions of solvent specified in the method.

Increased UV absorption was exhibited with increasing H-bonding ability of the solvent. Highest brompheniramine absorption at 263 nm was obtained using the best H-donating solvent, chloroform; the absorption was about 5 and 10% lower in the poorer H-bonding solvents, methylene chloride and *n*-hexane, respectively. In addition, brompheniramine and its maleate salt exhibit about twice the UV absorption (with loss of spectral detail) in more highly protonated environments, such as 0.1 *N* HCl, than in chloroform. This is evidenced by the concentrations of the UV solutions used in the NF assay for dexbrompheniramine compared to those for brompheniramine maleate tablets (see also Figs. 1 and 2). These data indicate that the greater the "degree of protonation" of the pyridine chromophore by its surrounding environment, the greater is the increase in UV absorption of the brompheniramine molecule.

Other organic nitrogenous base drugs exhibit different UV spectra for their free amine and salt forms when both are determined in the same neutral organic solvent. Methapyrilene hydrochloride and thonzylamine hydrochloride exhibit increased UV absorptions at somewhat lower wavelengths than their free base forms when measured in chloroform and methylene chloride, respectively (Figs. 6 and 7). In these studies, aqueous base was employed to remove the hydrochloric acid from aliquots of standard stock solutions of the drugs prepared in organic solvents. The free amine-containing solutions were diluted with corresponding organic solvents to a final concentration equivalent to that of their respective standard salt solutions and the UV spectra were recorded. Since these drugs are salts of a nonchromophore-containing acid, the only influence on UV absorption must be due to bonding effects between the proton donating hydrochloric acid and amine chromophores.

CONCLUSIONS

The data indicate that low standard recoveries obtained by the NF XIII assay for brompheniramine maleate tablets are attributable to the chromophoric properties of the maleic acid moiety and to the ability of maleic acid to protonate the α-pyridyl chromophore when in the amine maleate salt form and measured in neutral organic solvents.

Two recommendations appear to offer the best remedies for achieving increased analytical accuracy for present compendial assays of antihistamine maleate preparations that compare the UV absorptions of sample and standard solutions of unidentical molecular species at wavelengths below 280 nm: *(a)* employ an experimentally determined factor in the calculations which relates the absorbance of the standard maleate salt to the absorbance of an equivalent weight of the free amine[2], or *(b)* extract standards in the same manner as samples[3].

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ˣ To whom inquiries should be directed.

[2] Joseph Levine, Director, Division of Drug Chemistry, Office of Pharmaceutical Research and Testing, Bureau of Drugs, Food and Drug Administration, June 1973, personal communication.

[3] The assay procedures for brompheniramine maleate tablets, dexbrompheniramine maleate tablets, dexchlorpheniramine maleate syrup, and dexchlorpheniramine maleate tablets were revised in the Fifth Supplement to the National Formulary, 13th ed., to include extraction of standards in a similar manner as samples.

Generation of Particulate Matter in Large-Volume Parenteral Containers

R. J. WHITLOW, T. E. NEEDHAM ˣ, and L. A. LUZZI *

Abstract □ Polyvinyl chloride and glass large-volume parenteral containers were examined to determine the effects of agitation, storage, and additive handling on the generation of subvisual particulate matter. It was found that particles in flexible plastic containers can be generated by continuous shaking, with the maximum number of particles recorded after 30 hr of shaking. The particles were in the range of 2.33–5.02 μm in diameter, with very few particles recorded above 5 μm. It was also established that the generation and subsequent disappearance of particles during storage after an interval of continuous shaking are not only functions of time but also of temperature.

Keyphrases □ Plastic parenteral containers—particulate contamination, simulated handling conditions □ Large-volume parenteral containers—particulate contamination, simulated handling conditions □ Parenteral containers, large volume—particulate contamination, simulated handling conditions □ Contamination—large-volume plastic and glass parenteral containers

In recent years, intravenous fluids have played an increasingly significant role as a drug delivery system in patient treatment (1). With the greater utilization of this dosage form, it is important to examine the fluids and containers employed. Parenteral solutions should be relatively free of contamination by particulate matter because particulate matter can produce harmful effects such as pulmonary microemboli, thrombi, or granulomas in the patient (1). Twenty-five cases of pulmonary arterial lesions in premature infants were characterized by macrophage reactions around foreign bodies, mainly cotton fibers (2). Platelet agglutination and the formation of thrombi were observed (3) in rabbits following injection of particles 200–500 Å in diameter; an increase in the number and size of particles increased the possibility of the side reactions.

For years, parenteral solutions have been routinely packaged in glass. Glass containers have at times contributed to contamination in intravenous fluids, and there has been substantial evidence (4) that many particles found in glass-contained intravenous fluid have been generated from the rubber closure during or after sterilization or during storage (1). It has also been shown that glass flaking or leaching of particles can occur from the walls of glass containers.

Recently, several types of plastic containers have been introduced in an attempt to prevent particulate contamination of intravenous liquids from the containers. Solutions packaged in flexible plastic polyvinyl chloride containers were reported to contain fewer particles than solutions packaged in glass containers (5). Flexible plastic intravenous solution containers have also been reported to have a lower incidence of contamination under actual use conditions (6).

A preliminary investigation (7) showed that normal saline stored in disposable plastic ampuls had fewer particles than the corresponding material in rubber-plugged glass bottles. The results were recorded as counts per milliliter for particles above 1.3 μm and were in the order of 1000–5000. The all-plastic flexible containers yielded counts of 200–600/ml. More recently, Groves (8) reported results involving particulate contamination of saline packed in polyethylene and polypropylene containers. The polypropylene containers were more heavily contaminated and the contamination increased after sterilization by autoclaving. Solutions in polyethylene ampuls were reported to have a low degree of particulate contamination (4, 7, 9).

A study of intravenous fluids contained in plastic containers was reported; in 18 samples of normal saline solution in 1000-ml plastic containers, the average number of particles over 5 μm was 137 as compared to the number of particles in glass containers that ranged from 243 for one manufacturer to 1751 for another manufacturer.

Since glass containers for intravenous fluids were reported to be a source of particulate matter contamination and since this contamination can be harmful to the patient, the development of an all-plastic container was thought to be the solution to contamination problems. Unfortunately, the plastic containers may have their own source of contamination which might be harmful to the patient (11). Plastic containers are growing in popularity because they can be stored, handled, and disposed of more easily than glass containers (10); therefore, it is important that these containers be studied closely. As yet, the research data on contamination from plastic bags are incomplete.

The objective of this study was to determine the effects of agitation, storage, and additive handling on the generation of subvisual particulate matter in polyvinyl chloride and glass liter containers.

EXPERIMENTAL

Materials and Equipment—The following were employed: polyvinyl chloride plastic containers[1] of 0.9% sodium chloride injection USP, 1000-ml units; parenteral administration sets[2], Code

[1] Viaflex containers, lot NP61X1, Travenol Laboratories, Morton Grove, Ill.

[2] Lot B103L1, Travenol Laboratories, Morton Grove, Ill.

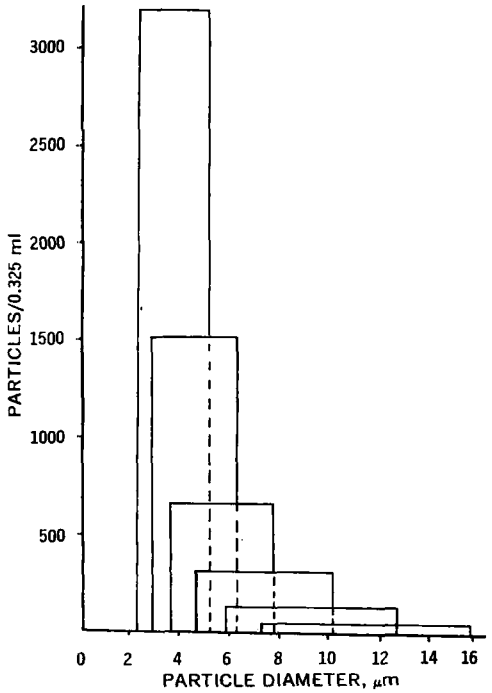

Figure 1—*Number of particles in various size ranges found in plastic bags shaken for 24 hr.*

2C0001; glass containers of 0.9% sodium chloride injection[3] USP, 1000-ml units; disposable venoclysis set[4]; laminar flow unit[4]; and gyrotory shaker[5].

An electronic particle counter in combination with a 100-channel channelyzer[6] was used to examine changes in particulate matter of the intravenous fluid. Four different size aperture tubes, 30, 50, 70, and 100 μm, were available for the experiments. The particle counter has the capability of reproducibly counting particles that are 2–40% of the size of the aperture tube (13). Since a preliminary study indicated that the greater percentage of particles was in the range of 2–6 μm, the 50-μm aperture tube was used.

Prior to counting each sample, the diluent was used to obtain the background counts; it was also used to rinse the aperture tube between samples. A detergent cleaner was used to clean and soak the aperture tube periodically and at the end of each day.

Three counts were taken for each sample, and the average was recorded. Preliminary results showed that, to obtain reproducibility, a 30-sec sampling time, equivalent to 0.325 ml, was necessary for each count.

Statistical Determination of Number of Bags per Experiment—The appropriate number of bags for each experiment was derived statistically from preliminary results. Zero counts were made on 20 control bags, and it was found that no one bag had a zero count greater than 300 particles between 2.33 and 5.02 μm/0.325 ml. The following formula was used to obtain the number of bags needed at a 95% confidence interval (14):

$$1.96(\sigma)/\sqrt{n} = L \qquad \text{(Eq. 1)}$$

where 1.96 is the representative value for a 95% confidence interval, σ is the standard deviation of the control bags, and L is the range limit of the amount of error that can be tolerated in the estimate.

With the considerations of time and expense for the study, as well as the inherent error of the instrumentation when counting 300 particles (12), it was decided to use 40 bags for each test. The values from two out of every 10 bags were plotted using the X–Y recorder integrated into the counting system to preserve and compare typical particle-size distributions.

Sampling Technique—Each plastic liter container was removed from its outer protective wrapper and was assembled with the administration set. The glass bottles were assembled with the disposable venoclysis administration sets. The tubing on the administration sets was shortened for easier sampling. All samplings were done in a laminar flow unit. Samples were collected in 20-ml plastic vials. Prior to each sampling, the administration set was flushed and the accuvette was rinsed twice with a portion of the intravenous fluid to be sampled. This procedure was followed to eliminate the possibility of particulate contamination from the tubing of the administration set and/or from the plastic vials.

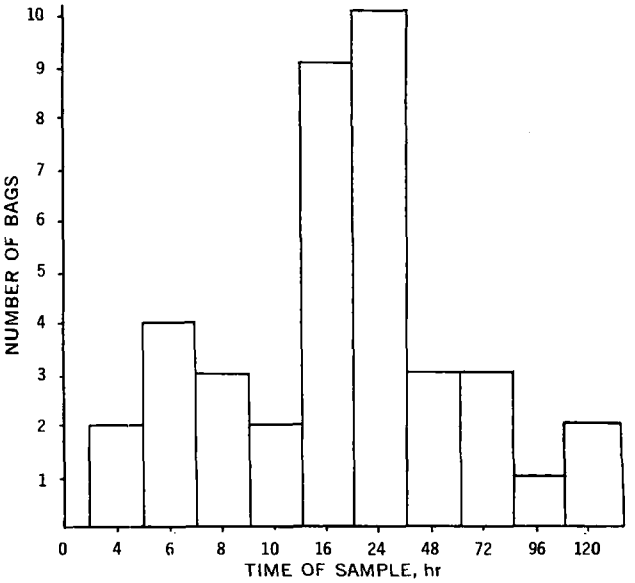

Figure 2—*Number of plastic bags exhibiting maximum particle counts at indicated time during shaking test.*

Shaking Test—A zero count was made on each bag or glass bottle before it was subjected to any test. Forty bags were sequentially placed in a gyrotory shaker and shaken for 120 hr. Samples were taken and counts were made at times 0, 1, 3, 5, 10, 20, and 40 min and 1, 2, 4, 6, 8, 12, 16, 24, 48, 72, 96, and 120 hr.

The same shaking and sampling procedures were followed for the 10 glass bottles used for the test. Since preliminary results had shown that there was a smaller variation among individual glass bottles than among the plastic containers, a smaller number of glass bottles was needed per test.

Drop Test—The plastic liter containers were individually dropped once from a measured height of 1.5 m (5 ft). The containers were dropped so that each container would have a different point of impact. This test was used to simulate accidental dropping during handling. A zero count was made prior to the test, and samples were taken and counted after dropping.

Aging Test—From preliminary results it was seen that the number of particles maximized on the average at 29.64 hr of shaking. Thereafter, 30 hr was used as the $t = 0$ reference time for resting containers. Groups of 40 bags were shaken for 30 hr and then stored at room temperature, 35, 45, and 55°.

Following the shaking period, containers were sampled until the number of particles declined to the range of zero counts. For storage conditions at room temperature, samples were taken at intervals up to 5 weeks. In the case of 35, 45, and 55° storage conditions, counts were taken up to 4 weeks.

At each sampling and just prior to sampling, the container was turned "end for end" one time. This procedure was followed to assure that a representative sample was withdrawn from the container.

Simulated Addition Test—To simulate particles that might be generated during agitation and after addition of an additive, the plastic liter containers were inverted (end to end) 5 times to simulate the mixing procedure.

RESULTS AND DISCUSSION

The intent of this study was to compare the particulate contami-

[3] Lot G173A1, Baxter Laboratories, Morton Grove, Ill.
[4] Abbott Laboratories, North Chicago, Ill.
[5] Model G-25, New Brunswick Scientific Co., New Brunswick, N.J.
[6] Model ZB with P64, Coulter Electronics, Hialeah, Fla.

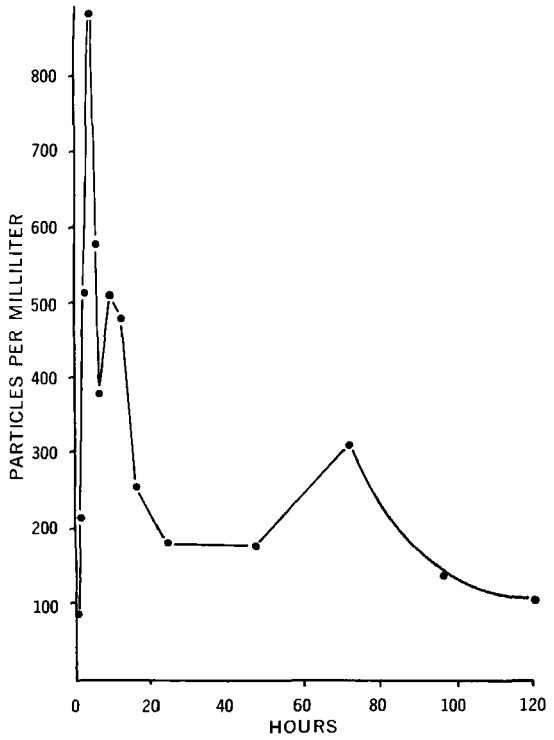

Figure 3—*Mean number of particles between 2.33 and 5.02 μm found in 10 glass containers shaken through 120 hr.*

nation emanating from flexible plastic intravenous fluid containers made from polyvinyl chloride and glass intravenous containers. The study was designed to simulate conditions that might lead to the generation of particulate matter.

Particle-Size Determination—Figure 1 represents a study in which six bags were shaken for 24 hr and sampled for particulate contamination over the size range of 2.33–40.12 μm in diameter. Figure 1 shows that the number of particles decreased as the diameter of the particle size increased in size, and that the greatest number of particles was in the lowest range studied. Throughout the study, the number of particles varied considerably from bag to bag. This is substantiated by previous reports (3) which showed that solutions vary, in degree of particulate contamination, among manufacturers and among different solutions from the same manufacturer. Due to instrumentation, the results were necessarily obtained by collecting data with overlapping sizes. Although samples were examined for particles up to 40.12 μm, no significant number of particles was found beyond 10 μm. It is possible that the particles measured above 10 μm may actually have been the result of agglomeration of several smaller particles. The data shown in Fig. 1 are similar to and agree with an earlier study (15) in which very few particles of greater than 5 μm were present in intravenous fluid bags of "all plastic" construction. Due to the results seen in Fig. 1, the remaining particle counts were performed in the 2.33–5.02-μm range.

Shaking Test—The purpose of the shaking test was to simulate travel or transport conditions for the intravenous fluid containers and was designed to determine if vigorous shaking contributes to particle contamination. Figure 2 shows that the maximum number of particles from plastic bags shaken for 120 hr was observed at 29.64 hr. This average peak time of particle appearance was calculated by using a weighted average of the highest particle count obtained from each bag during continued shaking for 120 hr. For ease of manipulation and calculation, bags were considered to peak at 30 hr of shaking and the results are reported as the mean number of particles per milliliter. Figure 2 represents the average of 40 bags shaken over the 120-hr test period. The peaks represent the average number of particles found at each sampling time, and there is a definite peak between 15 and 40 hr.

Since there is evidence (5–7) eluding to the fact that there are fewer particles in flexible plastic containers compared to glass containers, it was thought appropriate to establish a baseline for the glass containers under identical conditions with the plastic containers. The results from shaking glass bottles (Fig. 3) were obtained during 120 hr of shaking; highest counts for particles between 2.33 and 5.02 μm were produced after 2 hr of shaking. This is in sharp contrast to plastic containers where the number of particles was greatest at 30 hr. Figure 3 also shows that the glass bottles peaked with a particle count of 893/ml after 2 hr of shaking. Again, this finding is in contrast to plastic containers which showed an average maximum of approximately 25,000 particles/ml.

Table I—Numbers of Particles Found in Polyvinyl Chloride Containers and Glass Containers at Times Equal to Maximum Particle Generation

Time	Particles per Milliliter	
	Polyvinyl Chloride Bags	Glass Bottles
0	345	87
2 hr	16,122	893[a]
30 hr	24,308[b]	150

[a] Maximum number of particles in glass containers. [b] Maximum number of particles in plastic containers.

After approximately 2 hr of continued shaking, the glass bottles showed a decrease in particles. The total number of particles continued to decrease until 72 hr where there was an apparent increase. The number of particles continued to decrease thereafter. Although the glass bottles showed an increase in particles, the total number of particles never equaled the number found in the plastic containers. Apparently, as reported earlier (1), the particles found generated from the glass containers were a result of shedding from the rubber closures and/or leaching from the glass itself.

Table I shows a comparison of the number of particles found in both plastic and glass containers. The total number of particles in the 2.33–5.02-μm range in the glass bottles was significantly less than the total number in the plastic bags at all times. The glass bottles showed a 10-fold increase after 2 hr of shaking. The plastic bags showed an approximately 50-fold increase in 2 hr and a 70-fold increase in particle counts in 30 hr, while during the same time the glass containers showed a decrease in particles to less than a twofold increase from time zero.

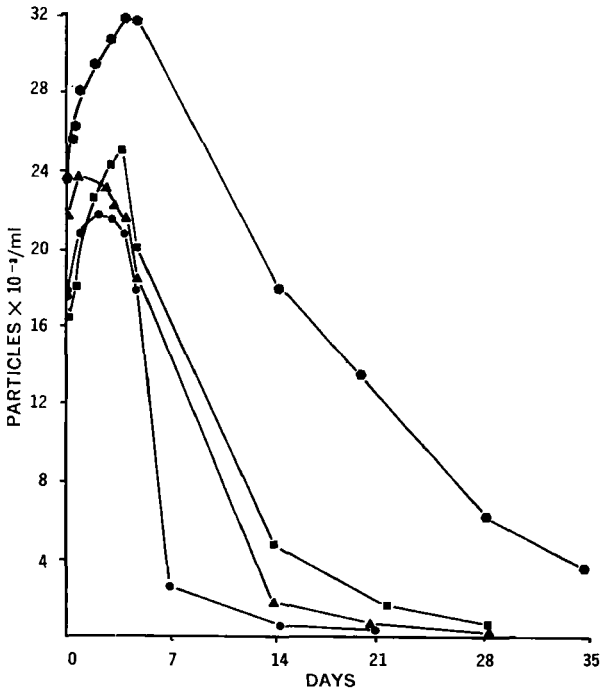

Figure 4—*Number of particles between 2.33 and 5.02 μm found in resting plastic bags after shaking for 30 hr as a function of time and temperature. Key: ■, 35°; ●, 45°; ▲, 55°; and ●, room temperature.*

Drop Test—Initial dropping tests, when carried out as previously described, showed no significant change in the number of particles. Additional tests were made on bags tossed and handled roughly for 1–2 min. These results also failed to establish an appreciable change in number of particles.

Aging Test—For the aging test, it was desirable that the bags contain a maximum number of particles due to shaking to establish what effects storage may have on the appearance and disappearance of particles. Therefore, prior to storage the plastic bags were shaken for 30 hr to generate particles.

To obtain significant results, 160 plastic containers were shaken for 30 hr each. These containers were divided into four batches to be stored at room temperature, 35, 45, and 55°. In each case, base counts were established at the start of the rest or storage periods and are shown as time equal zero in Fig. 4. For all cases and at all storage conditions, each container showed an initial increase in particles. In Fig. 4, particles per milliliter are plotted *versus* time. The resting bags stored at room temperature showed an increase in particles from time zero to 120 hr, and the resting bags stored at 35° showed an increase in particles from time zero to 96 hr. For the bags stored at 45°, an increase in particles from time zero to 48 hr was recorded, and an increase in particles to 24 hr was found for bags stored at 55°. From Fig. 4 it may also be seen that bags stored at the lower temperatures showed a longer period of sustained increase in particles while this was not as apparent in bags stored at 45 and 55°.

It is apparent from Fig. 4 that there was a general increase in the number of particles at all temperatures, with the degree of increase and the time over which the increases was recorded varying directly with temperature. A general decrease in particles, at what appears to be an essentially uniform rate, takes place after 120 hr of resting. At this time there is no clear explanation for these phenomena.

Since the number of particles generated in the bottles after 30 hr of shaking was so small and was almost identical to the number of particles observed before shaking, any changes in the average number of particles on aging would be within the interbottle variation and, therefore, not significant. In addition, it was found that with no shaking and after resting for 30 days in the original container, approximately 90 particles/ml in the 2.33–5.02-μm range were found in the glass containers.

Simulated Addition Test—The simulated addition test was performed to examine the potential particulate generation that might occur after the addition of an additive to the intravenous fluid containers. The turning end to end of both the bags and bottles failed to show any significant increase in particle contamination.

In summary, it may be seen that particles in polyvinyl chloride plastic containers can be generated by continuous shaking. It was established that plastic bags, even of the same lot number, varied considerably in the number of particles generated and that very few particles were found to be larger than 5 μm in diameter, with the greater number of particles in the lower range of 2.33–5.02 μm.

In all cases, glass bottles produced fewer particles than did plastic bags under similar conditions.

The results indicate that the storage conditions of the container after agitation can contribute to the time span involved in additional generation and the subsequent reduction in the number of particles.

During this study, it was established that significant numbers of particles could not be generated by mere handling of the containers such as turning end for end, nor was there a significant generation of particles by dropping the plastic containers from a height of 1.5 m (5 ft).

Therefore, from this study it may be concluded that by continuous shaking, particles can be generated in polyvinyl chloride plastic containers. These results are important because the travel or transport and storage conditions of the plastic containers must be considered to play a significant role in particle contamination of intravenous fluids.

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* Present address: School of Pharmacy, Medical Center, West Virginia University, Morgantown, WV 26506

ˣ To whom inquiries should be directed.

Fluorocarbon Aerosol Propellants II: Loss in Containers and Its Implications for Quantitative Analysis

WIN L. CHIOU [x] and JAUE-HUEY HSIAO

Abstract □ Loss of the three most commonly used fluorocarbon aerosol propellants from various types of containers with different stoppers as a function of time, temperature, and initial concentration was investigated. The loss process was found to continue for at least 3 days. The decreasing order of loss from serum bottles with three types of stoppers was usually: lacquer-coated rubber stopper < regular (plain) rubber stopper < silicone stopper. Significant loss was also with storage in commercially available vacuum tubes. Such mechanisms for loss as adsorption, absorption, complexation, and permeation are discussed.

Keyphrases □ Propellants (fluorocarbon aerosol)—loss from various containers as a function of time, temperature, and initial concentration, relationship to analysis □ Aerosols—loss of three fluorocarbon propellants from various containers, effects of time, temperature, and concentration □ Fluorocarbon propellants, aerosol—loss from various types of containers, effects of time, temperature, and concentration, mechanisms

Fluorocarbon aerosol propellants such as trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane are widely used in aerosol products. Their potential toxicity to humans and animals has been a subject of intensive study in the past few years (1–18). Concern over the potential acute toxicity to susceptible asthmatic patients after inhalation of bronchodilator aerosol products was recently raised (19).

The quantitative determination of concentrations of these propellants in air and biological samples is important in pharmacological and toxicological studies. In a previous study (16) concentrations of the propellants stored in serum bottles sealed with plain rubber or lacquer-coated rubber stopper and aluminum cap could decrease after storage. Since various types of sealed containers such as serum bottles (16, 18, 20, 21) and vacuum containers[1] (22, 23) are generally required to keep propellant samples during and before assay, and since any loss of propellants from the containers would result in an underestimation of the concentrations, it was decided to perform a systematic and quantitative study of the loss of the propellants stored in various types of containers. Effects of storage temperature and propellant concentration on the extent of loss were also explored.

EXPERIMENTAL

Materials—Only the most commonly used fluorocarbon aerosol propellants, trichloromonofluoromethane[2], dichlorodifluoromethane[2], and dichlorotetrafluoroethane[2], were investigated. Their common stock solution in distilled water was prepared according to the method described previously (16).

[1] Vacutainers, 2, 3, 5, and two 10-ml draw, Catalog No. 4850 3272, 4851 3273, 4880 3275, 4707 3200 TGS, and 4716 3200 KA, Becton Dickinson and Co., Rutherford, N.J.

[2] Supplied by E.I. duPont de Nemours and Co., Wilmington, Del.

Table I—Average Percent Loss of Trichloromonofluoromethane Stored in Various Containers at Room Temperature as a Function of Time

Type of Container	Percent of Loss at Various Times				
	2 hr	6 hr	24 hr	48 hr	72 hr
5-ml serum bottle with regular rubber stopper	14.0	24.0	43.0	49.0	59.0
5-ml serum bottle with lacquer-coated stopper	8.0	21.5	26.0	36.4	49.0
5-ml serum bottle with silicone stopper	16.0	40.2	87.5	93.0	99.0
15-ml serum bottle with lacquer-coated stopper	5.2	8.3	15.5	33.0	38.0
50-ml serum bottle with lacquer-coated stopper	1.5	1.7	3.2	6.1	7.8
2-ml vacuum container	10.1	23.0	38.5	51.5	59.6
3-ml vacuum container	8.6	18.6	31.4	43.6	52.0
5-ml vacuum container	1.4	15.0	23.6	37.7	40.0
10-ml vacuum container	3.1	21.5	29.2	35.6	37.3
10-ml vacuum container without silicone coating	5.3	27.3	38.0	42.7	47.0

Three sizes of serum bottles[3], 5, 15, and 50 ml, were used. The diameter of the internal opening of the 5-ml bottle is 13 mm, and that of the 15- and 50-ml bottles is 20 mm. Three types of stoppers were used: *(a)* regular, uncoated rubber stopper[4], *(b)* lacquer-coated, gray rubber stopper[4], and *(c)* silicone stopper[4]. The serum bottle was sealed with a stopper and an aluminum cap[5]. Four different sizes, 2, 3, 5, and 10 ml, of plain vacuum containers[1] with interior silicone coating were studied. A 10-ml vacuum container[1] without interior silicone coating was also studied for comparison.

Propellant Loss Studies at Room Temperature—An appropriate amount of propellant solution in distilled water was introduced into each sealed container through the stopper using a 10-μl syringe[6]. The containers were then usually shaken in a vortex mixer[7] for 5–10 min. The 50-ml bottles were shaken for 30 min to reach an equilibrium for propellant distribution in the bottles. Since the propellants studied exist in the gaseous state at 25° and their partition coefficients between the aqueous phase and the air phase are low (18), essentially all of the propellants added would distribute homogeneously in the container. The concentrations of the propellants in the container were determined by drawing certain volumes of the gas sample, with a 50- or 100-μl gastight syringe[6] equipped with an adaptor[8], which were then injected directly onto the GLC column. The concentrations determined immediately after shaking were assumed to represent the initial concentrations of the propellants in the container and were used to estimate the percent of the propellants lost during storage.

Several samples were obtained and analyzed in duplicate by the GLC method at appropriate times (usually at 2, 6, 24, 48, and 72 hr) after the beginning of the study. They were highly reproducible (within 2% from the mean). Three containers were used for study on each type of container. Intercontainer variation in percent of propellant lost at various times was generally insignificant. Since it was found that the extent of propellant loss might depend on the

[3] Wheaton Scientific Co., Millville, N.J.

[4] Regular uncoated rubber stopper, V-32; lacquer-coated rubber stopper, V-32, 324, Y-40; and silicone stopper, V-32, X-9711; West Co., Phoenixville, Pa.

[5] Catalog No. 224182 and 224183, Wheaton Scientific Co., Millville, N.J.

[6] Hamilton.

[7] Vortex-Genie mixer, Cat. No. 12-812-V1, Fisher Scientific Co., Springfield, Mass.

[8] Chaney.

Table II—Average Percent Loss of Dichlorodifluoromethane Stored in Various Containers at Room Temperature as a Function of Time

Type of Container	Percent of Loss at Various Times				
	2 hr	6 hr	24 hr	48 hr	72 hr
5-ml serum bottle with regular rubber stopper	5.1	12.5	17.2	28.8	38.9
5-ml serum bottle with lacquer-coated stopper	5.3	10.9	14.5	23.8	48.0
5-ml serum bottle with silicone stopper	10.0	22.4	57.0	84.4	93.0
15-ml serum bottle with lacquer-coated stopper	0.5	1.0	2.0	3.1	4.0
50-ml serum bottle with lacquer-coated stopper	0.4	0.5	0.8	1.0	1.2
2-ml vacuum container	6.9	16.8	19.3	23.2	25.7
3-ml vacuum container	5.5	12.0	14.0	20.5	23.6
5-ml vacuum container	0.4	10.5	18.2	28.9	33.4
10-ml vacuum container	0.7	15.0	19.1	23.9	26.5
10-ml vacuum container without silicone coating	1.7	10.1	18.0	27.7	31.4

initial concentration of the propellant in the container, unless otherwise indicated, the initial concentration for each propellant used in all studies was approximately the same: around 0.2 μg/ml for trichloromonofluoromethane, 0.3 μg/ml for dichlorodifluoromethane, and 1.4 μg/ml for dichlorotetrafluoroethane. All of these studies were conducted at room temperature (25 ± 1.0°).

Effect of Concentration on Loss—Since the lacquer-coated rubber stoppers have been routinely used in this laboratory (16, 18), the effect of different concentrations of the propellants on the extent of loss was only studied in 5-ml serum bottles sealed with that type of stopper and regular aluminum cap. Two concentrations of each propellant, differing by a factor of 10, were studied in six bottles. The lower concentrations of the three propellants used were similar to those described in the room temperature studies. Samples were analyzed for propellant concentrations at various times.

Effect of Temperature on Loss—Only the 5-ml serum bottles sealed with lacquer-coated rubber stopper were used. Approximately the same amounts of the three propellants as described for the other variables were injected into six bottles. Three bottles were stored at room temperature and the remaining three bottles were stored at 5° in a refrigerator. To prevent possible contamination of the propellants in the refrigerator (refrigerants generally used are fluorocarbons), the serum bottles were kept in a sealed 240-ml (8-oz) ointment jar. Prior to sampling for propellant analysis, the container was warmed in a 37° water bath for 5 min and then shaken in a vortex mixer for 5 min.

Permeation Study—This study was designed to determine whether permeation through the stopper could contribute to the loss of the propellants from the container. Three empty 5-ml serum bottles, each sealed with a different type of stopper, were stored in a 240-ml (8-oz) ointment jar containing initially about 1.8 μg/ml (w/v) of trichloromonofluoromethane, 3.9 μg/ml of dichlorodifluoromethane, and 15.3 μg/ml of dichlorotetrafluoroethane. After 24 hr of storage at room temperature, air samples inside the sealed serum bottles were obtained and analyzed for propellant contents. This study was performed in duplicate.

Table III—Average Percent Loss of Dichlorotetrafluoroethane Stored in Various Containers at Room Temperature as a Function of Time

Type of Container	Percent of Loss at Various Times				
	2 hr	6 hr	24 hr	48 hr	72 hr
5-ml serum bottle with regular rubber stopper	5.7	13.0	18.1	23.0	31.9
5-ml serum bottle with lacquer-coated stopper	4.5	10.4	13.3	28.9	38.0
5-ml serum bottle with silicone stopper	9.4	23.7	56.0	82.2	91.0
15-ml serum bottle with lacquer-coated stopper	0.6	1.1	2.5	4.2	5.3
50-ml serum bottle with lacquer-coated stopper	0.5	0.6	1.0	1.1	1.2
2-ml vacuum container	5.5	17.5	20.7	28.7	31.8
3-ml vacuum container	5.3	13.8	17.6	22.4	27.3
5-ml vacuum container	1.8	12.0	24.2	29.7	32.2
10-ml vacuum container	1.9	6.0	15.6	19.5	20.7
10-ml vacuum container without silicone coating	2.9	14.1	20.9	22.1	31.5

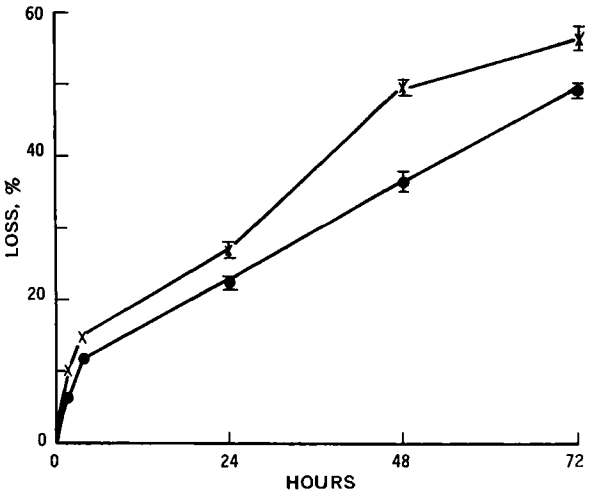

Figure 1—*Effect of concentration on the percent loss of trichloromonofluoromethane from 5-ml serum bottle sealed with lacquer-coated stopper. Key: ×, 57.3 ng/ml; and ●, 5.3 ng/ml.*

GLC Analysis—The instrument and conditions for the GLC analysis were essentially the same as those reported previously (16), except that the detector temperature was raised to 200°. This change in detector temperature increased the detector sensitivity for the three propellants by approximately 50% and reduced the sensitivity for the cyclohexane used to prepare the standard solution of propellants. The propellant standard solution in cyclohexane stored in a 0.94-liter (1-qt) amber glass bottle with screw-cap was stable for at least several months at room temperature.

RESULTS AND DISCUSSION

The results of the average cumulative percentage of loss at various times of the three propellants from 10 different types of containers at room temperature are summarized in Tables I–III.

The loss of all three propellants from most of the containers continued for at least 3 days. The only exception was the 50-ml serum bottle sealed with a lacquer-coated rubber stopper, from which only a negligible loss could be detected for dichlorodifluoromethane and dichlorotetrafluoroethane after 3 days of storage. The loss of trichloromonofluoromethane from this container (6% in 3 days) was lower than from all of the other types of containers. These data indicate that this type of container should be used to store propellant samples for long periods. The rates of loss during the first few hours of study were generally greater than at later times.

With the 5-ml serum bottles, the use of both regular rubber and lacquer-coated rubber stoppers resulted in almost the same extent of loss of dichlorodifluoromethane and dichlorotetrafluoroethane within the first 24 hr. At 48 and 72 hr, the lacquer-coated rubber stopper appeared to permit a more extensive loss of both propellants. The silicone-type stopper permitted a most extensive loss of all three propellants. For trichloromonofluoromethane, the rate of loss followed first-order kinetics with a half-life of 8.8 hr. The kinetics for dichlorodifluoromethane and dichlorotetrafluoroethane, however, seem to be complex and are beyond the scope of this investigation. The vacuum containers coated with silicone appeared to permit less loss than did those not coated with silicone. These findings are in agreement with the general observation that interactions (adsorption, absorption, and complexation) of most chemicals with glass and polymers such as plastics and rubber will be retarded by coating with a silicone layer.

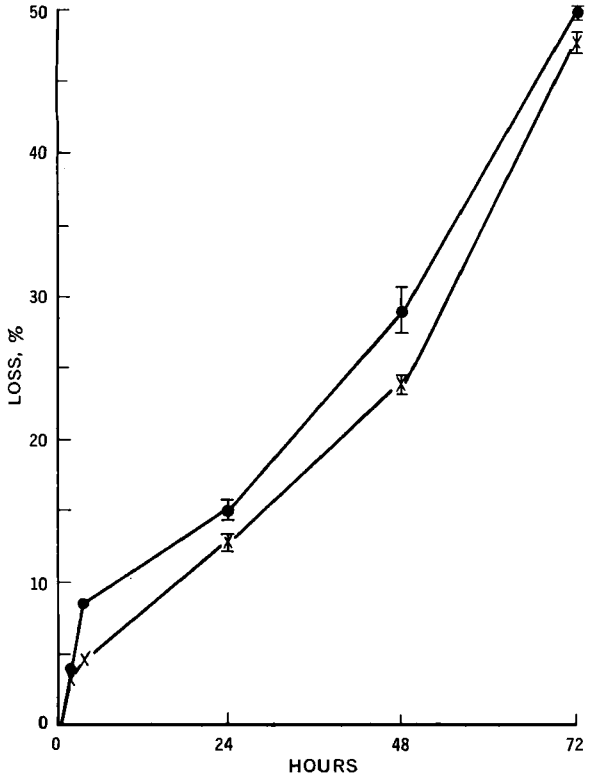

Figure 2—*Effect of concentration on the percent loss of dichlorodifluoromethane from 5-ml serum bottle sealed with lacquer-coated stopper. Key: ×, 319.8 ng/ml; and ●, 32.8 ng/ml.*

Trichloromonofluoromethane appears to be lost more readily than the other two propellants from all 10 types of containers. Approximately 93% was lost in 3 days from 5-ml serum bottles sealed with silicone stoppers. Generally, the larger the size of container, the smaller was the percentage loss of the propellants. This may be partly due to the fact that, on the average, the propellant molecules in the larger size container will take a longer time to travel to contact the surface of the stopper. It may be also attributed to the smaller surface area of stopper exposed to the propellants per unit volume of container in a larger size. For example, one can estimate that the relative surface area per unit of volume for a 50-ml serum bottle is only about 23.6% of that for a 5-ml serum bottle.

Several possible mechanisms can be speculated to explain the extensive and prolonged loss of propellants from the containers. They include: *(a)* adsorption onto the surface of the stopper; *(b)* absorption into the matrix of the stopper; *(c)* complexation between propellants and some ingredients of the stopper; *(d)* permeation to the outside of the container through the matrix space of the stopper, which is generally made of polymeric materials; and *(e)* permeation through the holes made after repeated puncturing by a syringe for sampling.

The loss of propellant from the holes that resulted from repeated puncturing can be ruled out because stoppers initially punctured 10 times with a 22-gauge needle, which is larger than the needle used in the studies, also produced the same extent of loss of the three propellants. The possible loss of propellant due to permeation through the matrix space of stoppers was suggested in the permeation study where considerable amounts of the three propellants could be detected in the sealed empty serum bottles. The bottles sealed with silicone stoppers contained higher concentrations of the propellants than did the ones sealed with other types of stopper. This finding is in agreement with the loss study in which the bottles sealed with silicone stoppers had the highest percent of loss. Since air is essentially not permeable through the rubber stopper of a vacuum container, as demonstrated by its vacuum state over a period of years, and since the molecular weights of the propellants are much greater than those of the constituents of air, it is concluded that permeation was not a cause of the loss from

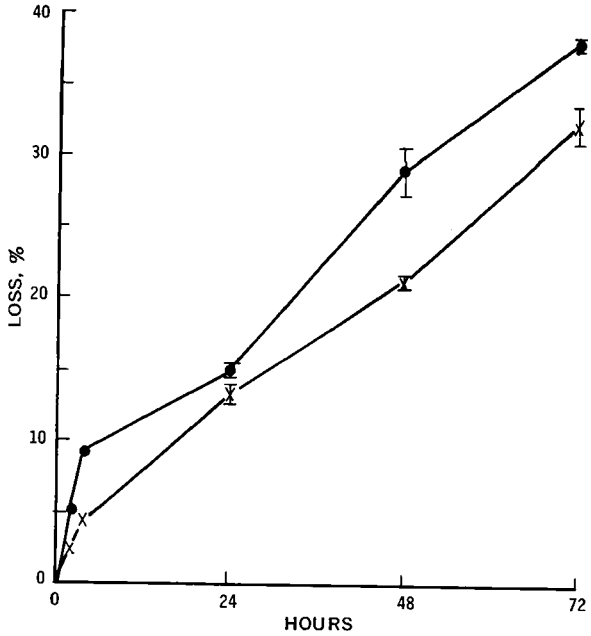

Figure 3—*Effect of concentration on the percent loss of dichlorotetrafluoromethane from 5-ml serum bottle sealed with lacquer-coated stopper. Key: ×, 692.7 ng/ml; and ●, 75.0 ng/ml.*

that type of container in the present study.

The adsorption phenomenon is generally a very fast or instantaneous process (24, 25). The observed loss of the propellants from the containers is, however, a slow process. For example, the extent of loss of trichloromonofluoromethane at 72 hr from 5-ml serum bottles sealed with lacquer-coated stoppers was about 4.5 times higher than that found at 2 hr. The loss of dichlorotetrafluoroethane from a 5-ml vacuum container at 72 hr was about 30 times higher than that at 2 hr. These results indicate that adsorption is not a major cause. Since the absorption and complexation

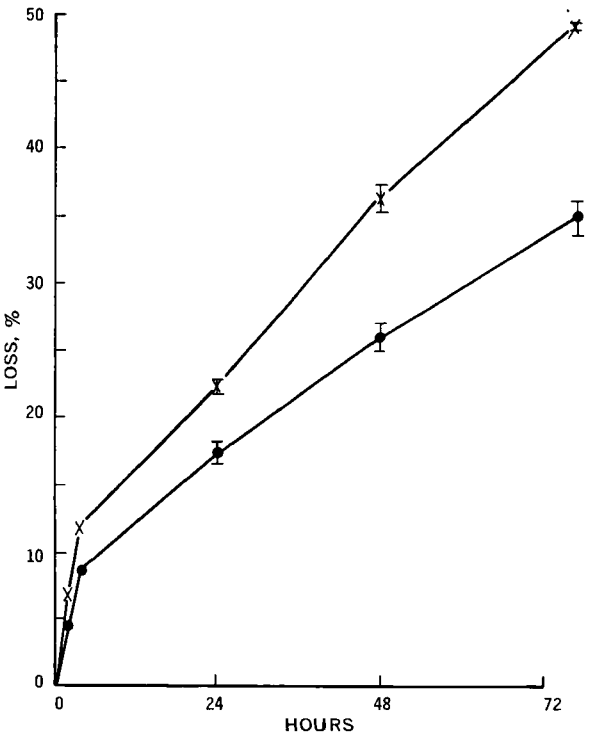

Figure 4—*Effect of temperature on percent loss of trichloromonofluoromethane from 5-ml serum bottle sealed with lacquer-coated stopper. Key: ×, at 25 ± 1°; and ●, at 5 ± 0.5°.*

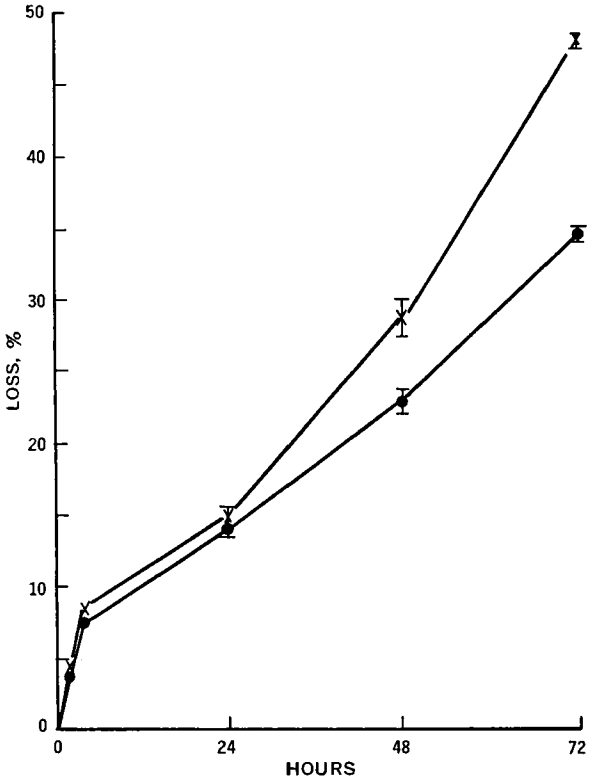

Figure 5—*Effect of temperature on percent loss of dichlorodifluoromethane from 5-ml serum bottle sealed with lacquer-coated stopper. Key: ×, at 25 ± 1°; and ●, at 5 ± 0.5°.*

are usually slow processes, rate limited by the diffusion rate of molecules in the matrix (25), it is believed that they are the major factors causing prolonged loss of the propellants from various containers. The possibility of physicochemical interaction between stoppers and propellants is further substantiated by the profound swelling of stoppers when a large amount of pure liquid trichloromonofluoromethane was stored in various containers. Many common fluorocarbons, including the three studied here, have been

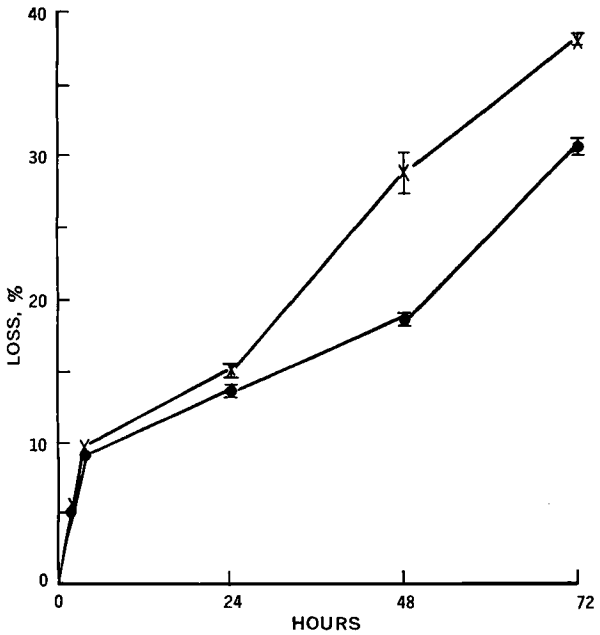

Figure 6—*Effect of temperature on percent loss of dichlorotetrafluoroethane from 5-ml serum bottle sealed with lacquer-coated stopper. Key: ×, at 25 ± 1°; and ●, at 5 ± 0.5°.*

shown to cause swelling of several elastomers such as natural rubber (26). As much as 300 ml of halothane, a fluorocarbon anesthetic gas, could be absorbed into the rubber tubing in the anesthetic breathing circuit during anesthesia and transferred subsequently and unintentionally to a patient administered a different general anesthetic through the same circuit (27).

The effects of concentration of the propellants on the extent of loss from 5-ml serum bottles sealed with lacquer-coated rubber stoppers are shown in Figs. 1–3. For dichlorodifluoromethane and dichlorotetrafluoroethane, the cumulative percents of loss from higher concentrations at 48 hr are significantly lower than those from lower concentrations ($p < 0.025$ using the Student t test). At 72 hr, however, the difference is not as significant ($p > 0.10$ for dichlorodifluoromethane; $p > 0.15$ for dichlorotetrafluoroethane). Such a concentration-dependent effect is consistent with the saturable absorption or interaction phenomenon as observed with drug–plastic interactions (25). On the contrary, the higher concentration of trichloromonofluoromethane gave rise to a higher percent of loss compared to the lower concentration ($p < 0.0005$), probably due to a strong initial chemical interaction between the propellant and the rubber resulting in a state more favorable for further interaction. A similar effect was also found at 5°.

The effects of storage temperature on the extent of loss are shown in Figs. 4–6. At 48 and 72 hr, the loss for three propellants was smaller ($p < 0.025$) when stored at 5°, primarily because of the lower diffusion rate of the molecules at lower temperatures. Again these data are consistent with absorption or complexation theory (25). The cumulative loss at 72 hr was between 30 and 35% for the three propellants. A higher percentage of loss would be anticipated with longer storage. The extent of loss observed in the present lower temperature study is quite surprising since the temperature was well below the boiling point (bp 23.7°) for trichloromonofluoromethane and just slightly above the boiling point (4.1°) for dichlorotetrafluoroethane. It is doubtful that the loss can be completely avoided even with storage at a much lower temperature.

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Effect of Small-Scale Preparation Techniques on Diffusion of Salicylic Acid from Various Ointment Bases

C. W. WHITWORTH [x] and A. F. ASKER

Abstract □ The effect of small-scale preparation techniques on the diffusion of salicylic acid from ointment bases was studied. The method of mechanical incorporation of the drug in cold petrolatum base, using a spatula, appeared to result in higher rates of salicylic acid diffusion than those encountered with ointments prepared by fusion, regardless of drug concentration and the presence or absence of a surfactant. A similar effect was produced in the case of a 10% salicylic acid ointment made with a water-in-oil emulsion base.

Keyphrases □ Ointments—effect of small-scale preparation techniques on salicylic acid diffusion, petrolatum bases with and without surfactant and water-in-oil emulsion base □ Salicylic acid—diffusion from bases, effect of small-scale ointment preparation techniques □ Diffusion—salicylic acid from ointment bases prepared by small-scale techniques

The effect of various liquids on the diffusion of salicylic acid from ointments was reported previously (1). A literature review revealed that several reports have been published on the influence of numerous additives on the diffusion and absorption of drugs from ointments (2–7). However, it appears that little or no attention has been paid to the effect of preparation methods of ointments on the diffusion of the incorporated drug.

The aim of this investigation was to study the effect of small-scale preparation techniques of ointments on the diffusion of salicylic acid from some ointment bases. Mechanical incorporation of the drug using a spatula and the fusion process were investigated.

EXPERIMENTAL

Materials—Salicylic acid[1] USP, white petrolatum[2] USP, and sorbitan monooleate[3] were used as supplied. Other ingredients were official or analytical grades.

Preparation of Ointments—The bases used were white petrolatum, white petrolatum containing 2% sorbitan monooleate, white petrolatum containing 5% sorbitan monooleate, and a water-in-oil base consisting of 64% white petrolatum, 6% sorbitan monooleate, and 30% distilled water.

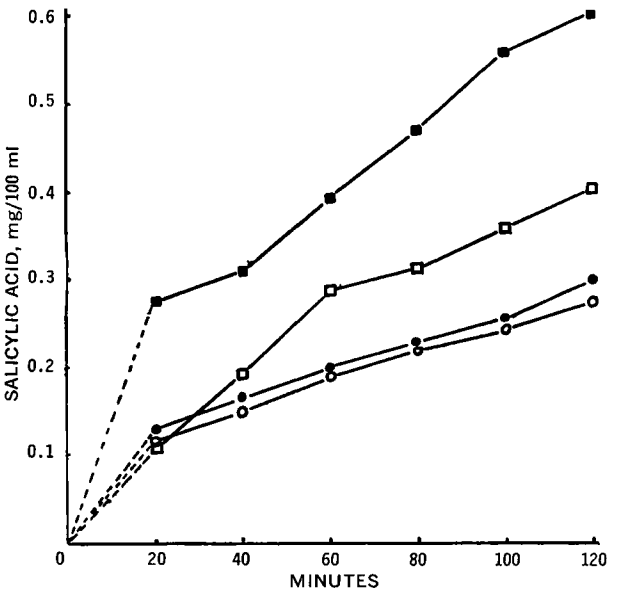

Figure 1—*Diffusion of salicylic acid from white petrolatum. Key: ■, 10% prepared by mechanical incorporation; □, 10% prepared by fusion; ●, 5% prepared by mechanical incorporation; and ○, 5% prepared by fusion.*

Mechanical Incorporation—The salicylic acid as supplied in fine powder was triturated with 150 g of the base on a glass slab using a spatula until a smooth homogeneous ointment was obtained.

Fusion—Each oleaginous base was first melted on a water bath at 70°. Salicylic acid was then gradually added to the molten material and stirred constantly until the ointment cooled to room temperature. In case of the ointments prepared with the water-in-oil base, white petrolatum and sorbitan monooleate were mixed and melted on a water bath at 70°. Salicylic acid was then added and dispersed in this fatty base at 70°. The distilled water, previously heated to 70°, was incorporated while stirring was maintained until the ointment cooled to room temperature.

Diffusion—The techniques used were the same as described previously (1). The amount of salicylic acid diffused into 100 ml of distilled water was determined by measuring the absorbance on a spectrophotometer[4] at 297 nm.

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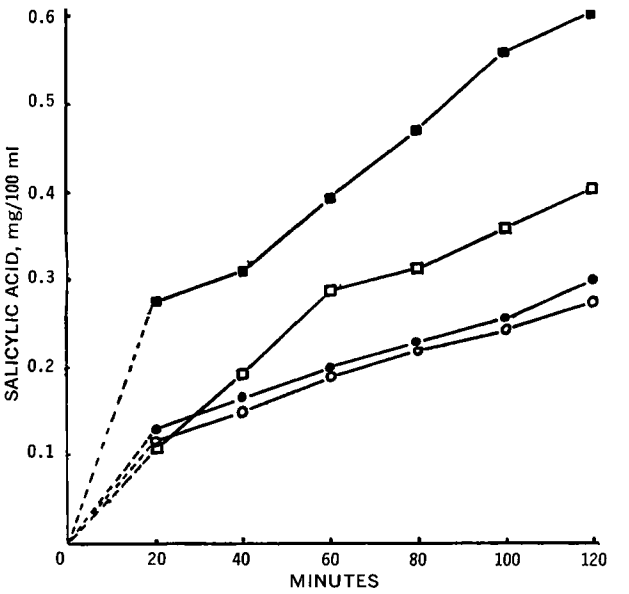

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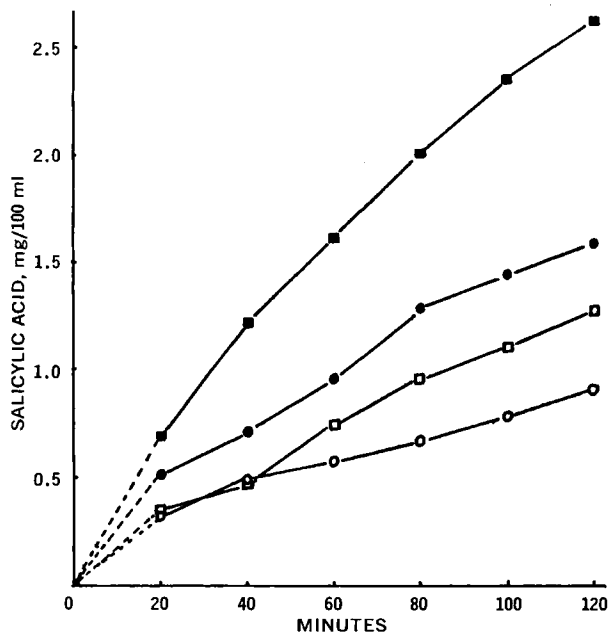

Figure 2—*Diffusion of salicylic acid from white petrolatum containing 5% sorbitan monooleate. Key: see Fig. 1.*

DISCUSSION AND CONCLUSIONS

The release characteristics of salicylic acid from the various ointment bases over 2 hr are illustrated in Figs. 1–3. The increase in concentration of the aqueous diffusion medium at varying time intervals is used to assess the rate of drug release from the ointments.

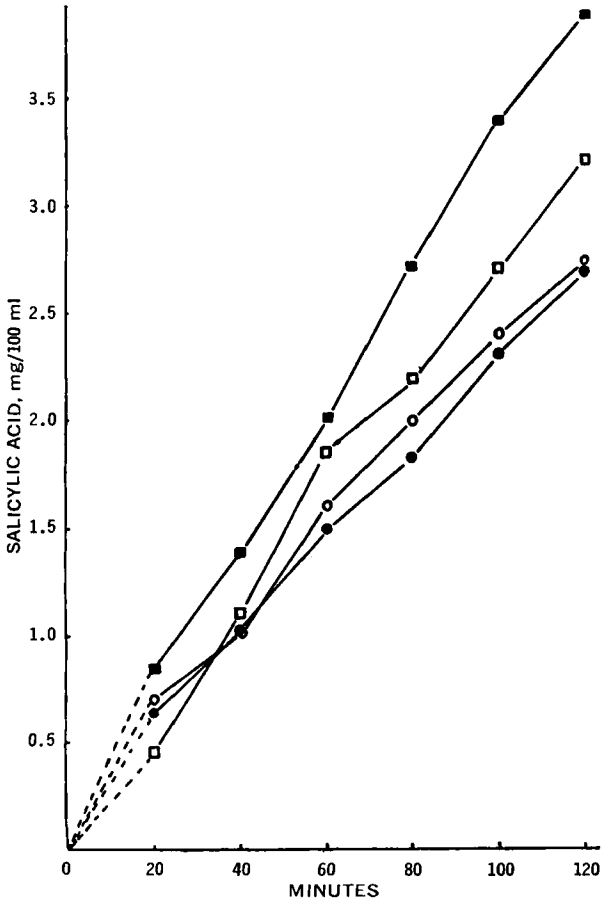

Figure 3—*Diffusion of salicylic acid from a water-in-oil base. Key: see Fig. 1.*

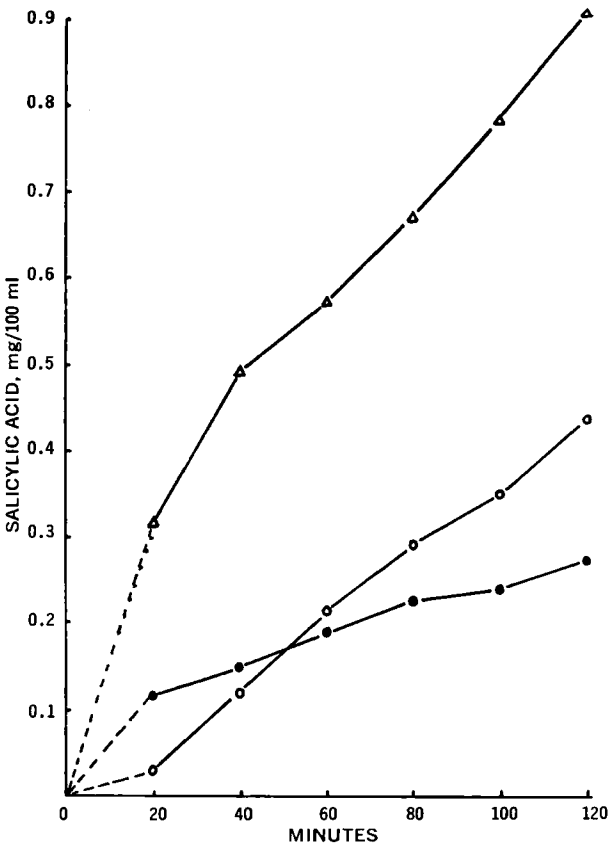

Figure 4—*Effect of sorbitan monooleate on the diffusion of salicylic acid from a 5% ointment in white petrolatum prepared by fusion. Key: ●, no surfactant; ○, 2% surfactant; and △, 5% surfactant.*

It appears from Figs. 1 and 2 that in the case of the oleaginous petrolatum bases the mechanical incorporation techniques using a spatula gave a higher rate of drug release than that obtained with the fusion method, regardless of drug concentration and the presence or absence of a surfactant. This result may be due to the molten base possibly being much less viscous and more flowable than the nonfused base. Consequently, the molten base should be more effective in wetting, adhering to, and coating the incorporated salicylic acid particles, thus resulting in retardation of the diffusion rate of the drug. This was confirmed by a simple qualitative test whereby the comparative rapidity of coloration of the ointments with ferric chloride solution was noted. Two equal portions of the ointments prepared by the two techniques were filled into small plastic vial caps. One drop of ferric chloride solution was then added, and the rate of appearance of a blue color was followed. The development of a blue color appeared to be faster for ointments prepared by mechanical incorporation using a spatula than for ointments prepared by the fusion techniques.

It has been reported (8) that heating petrolatum to its melting point permits rearrangement of its crystal network so that it exhibits its initial thixotropic condition after cooling. Consequently, the difference in release patterns of salicylic acid from the ointments prepared by the two methods could not be attributed to a change in the crystalline structure of petrolatum as a result of fusion.

Figure 3 also shows that mechanical incorporation techniques using a spatula resulted in a higher rate of salicylic acid diffusion from a water-in-oil emulsion base containing 10% salicylic acid. However, with the 5% salicylic acid ointment, no significant difference could be noted. The reason might be that at the lower concentration of salicylic acid, the diffusion-retarding effect produced by the fusion method might have been balanced by the diffusion-enhancing effect demonstrated by the surfactant (Fig. 4). The solubi-

lizing effect of sorbitan monooleate on salicylic acid may account for its higher rate of diffusion from bases containing this surfactant (Figs. 2–4). However, at the higher drug concentration, a relatively greater proportion of salicylic acid particles could not be effectively surrounded by the nonfused base; therefore, the increase in diffusion rate was apparently due mainly to this factor, which would probably outweigh the surfactant effect.

Figures 1–3 also show that greater drug concentration produced higher rates of diffusion in both methods used to make the ointments. This is in agreement with the results obtained by Lockie and Sprowls (9) who found that the rate of diffusion of iodine and sulfathiazole from ointments increased with increasing drug concentration within a certain range. Undoubtedly, the solubility of a drug in an ointment base plays a major role in the diffusion or release of the drug from the base (10). The faster diffusion demonstrated by bases with higher concentrations of salicylic acid apparently reflects this solubility effect. The higher the concentration of salicylic acid, the more will be left undissolved and not effectively coated by the base.

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JOHN L. HOGG and R. L. SCHOWEN [x]

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Keyphrases □ Acetylcholine perchlorates, deuterium labeled—synthesis □ Deuterium-labeled acetylcholine perchlorates—synthesis

The enzyme acetylcholinesterase catalyzes the hydrolysis of the natural substrate acetylcholine in carrying out its central role in the function of the nervous system (1). Studies in this laboratory required the syntheses of four specifically labeled acetylcholine salts: $CD_3CO_2CH_2CH_2N^+(CH_3)_3ClO_4^-$, I; $CH_3CO_2CH_2CH_2N^+(CD_3)_3ClO_4^-$, II; $CH_3CO_2CD_2CH_2N^+(CH_3)_3ClO_4^-$, III; and $CH_3CO_2CH_2CD_2N^+(CH_3)_3ClO_4^-$, IV.

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DISCUSSION

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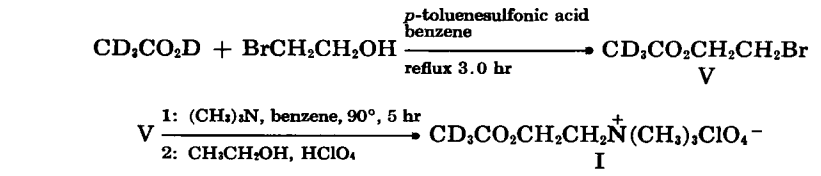
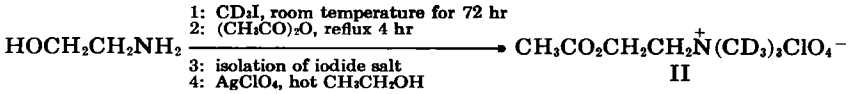
DISCUSSION

The two methods used for the preparation of the ethylene bromohydrins gave very low yields, even though the reactions were re-



Scheme I



Scheme II

peated many times taking every possible precaution. The compounds could not be isolated in a very pure state, and the yields of the subsequent esterification reactions were lower than model reactions would have predicted. The addition of aluminum chloride to the reduction mixture did appear to improve the yield of the reduction (7). The preparation of ethylene chlorohydrins in relatively high yields was reported (8) from the acid chlorides or ethyl esters, and these yields could be repeated almost exactly, which assured that no gross error was being made in experimental technique. This procedure might have offered a solution to the problems mentioned, except that the final quaternization gave yields of only 5–10%. Continuous extraction of the water phase from the workup of the reduction according to Nystrom (9) gave little improvement.

Methods for the preparation of Compounds I–IV are given in Schemes I–IV, respectively.

EXPERIMENTAL[1]

2-Bromoethyl Acetate-d_3 (V)—A reaction flask, fitted with a graduated 2-ml Dean-Starke trap, a microcondenser, and a calcium sulfate drying tube in series, was charged with 2.50 g (3.90×10^{-2} mole) of acetic acid-d_4 (99.5% minimum isotopic purity)[2]. To this was added 4.90 g (3.92×10^{-2} mole) of 2-bromoethanol dissolved in 2.5 ml of reagent grade benzene and 0.15 g (7×10^{-4} mole) of *p*-toluenesulfonic acid monohydrate. An additional 2.5 ml of benzene was used to fill the side arm of the trap to its maximum capacity. The reaction solution was heated at reflux, with magnetic stirring, for 3.0 hr, during which time 0.71 ml of water (3.9×10^{-2} mole) was collected. After cooling, the reaction solution was distilled at atmospheric pressure, with fractions distilling below 150° being discarded. The distillation afforded 4.66 g (70%) of V, bp 159–161° [lit. (10) bp 162–163° for the nondeuterated analog]; IR (CCl_4): 2975 and 1730 cm^{-1}; NMR (CCl_4): δ 2.05 (~0.1H, s)[3], 3.45 (2H, t), and 4.30 (2H, t).

Acetyl-d_3-choline Perchlorate (I)—According to the procedure of Fourneau and Page (3), a pressure bottle, precooled to −15° in an ice–salt bath, was charged with 4.50 g (2.64×10^{-2} mole) of V in 25.0 ml of dry benzene. To this was added 7.71 g (1.31×10^{-1} mole) of anhydrous trimethylamine, and the pressure bottle was sealed. The solution was allowed to warm to room temperature and was then heated, with magnetic stirring, in a silicone oil bath at 90° for 5 hr. The flask was then cooled to −15°, opened cautiously, and allowed to warm to room temperature gradually. Concentration of the reaction solution at reduced pressure gave approximately 5.1 g of slightly wet crystals, which were then dissolved in 38 ml of absolute ethanol and treated with 3.87 g (2.70×10^{-2} mole) of 70% aqueous perchloric acid with stirring and cooling in an ice bath. The resultant white, feathery crystals were stored in the mother liquor overnight in the refrigerator, collected *via* suction filtration, washed with cold ether, and dried *in vacuo* to give 4.67 g (71%) of I, mp 110.0–111.5°. Recrystallization from absolute ethanol gave 4.10 g, mp 113.5–114.5° [lit. (6) mp 116–117° for the nondeuterated analog]; IR (mineral oil): 2941, 2033, and 1733 cm^{-1}; NMR (D_2O): δ 3.60 (9H, s), 4.15 (2H, m), and 4.95 (2H, m).

Anal.[4]—Calc. for $C_7H_{13}D_3ClNO_6$: C, 33.81; "H," 6.49; Cl, 14.26; N, 5.63; atom-% excess D, 18.75. Found: C, 33.97; "H," 6.62; Cl, 13.92; N, 5.78; atom-% excess D, 17.55. These values convert to 93.7% deuteration at the desired site.

***N*-$(CD_3)_3$-Acetylcholine Perchlorate (II)**—According to the procedure of du Vigneaud *et al.* (4), a reaction flask was precooled to dry ice–acetone temperature and charged with 1.40 g (2.92×10^{-2} mole) of ethanolamine. To this was added 5.0 g (3.44×10^{-2} mole) of methyl-d_3 iodide (99.5 atom-% D)[5] and the flask was immediately topped with a condenser and calcium sulfate drying tube. The solution was then allowed to warm to room temperature, at which point it began to reflux spontaneously for about 10 min. After 72 hr of magnetic stirring at room temperature, the viscous orange reaction solution was treated with 7.18 g (7.02×10^{-2} mole) of acetic anhydride (11). The resultant mixture was heated at reflux for 4 hr, cooled to room temperature, and concentrated *in vacuo*. The resultant red oil was treated with 1.50 ml of absolute ethanol to give pale-yellow crystals. Isolation of these crystals followed by two successive recrystallizations from absolute ethanol gave 1.17 g of *N*-$(CD_3)_3$-acetylcholine iodide, which was slowly added to a hot solution of 0.87 g (4.2×10^{-3} mole) of silver perchlorate in 25 ml of absolute ethanol. The yellow silver iodide, which immediately precipitated, was removed by suction filtration of the hot ethanol solution. The clear supernatant solution was allowed to cool to give, after two recrystallizations from hot ethanol, 0.66 g (23%) of II, mp 113.5–114.5° [lit. (6) mp 116–117° for the nondeuterated analog]; NMR (D_2O): δ 2.45 (3H, s), 4.05 (2H, m), and 4.80 (2H, m).

Anal.—Calc. for $C_7H_9D_9ClNO_6$: C, 33.01; "H," 6.33; Cl, 13.92; N, 5.50; atom-% excess D, 56.25. Found: C, 33.41; "H," 6.78; Cl, 13.64; N, 5.50; atom-% excess D, 54.00. These values convert to 96.00% deuteration at the desired site.

1,1-d_2-2-Bromoethanol (VI)—An erlenmeyer flask was charged with 2.50 g (5.98×10^{-2} mole) of lithium aluminum deuteride-d_4 (99 atom-% D)[5] and 7.97 g (5.98×10^{-2} mole) of anhydrous aluminum chloride and stoppered. A three-necked flask was fitted with a pressure-equalizing addition funnel, a condenser and drying tube, and a 20.3-cm section of 2.54-cm diameter (0.3-cm walls) rubber tubing. The erlenmeyer flask containing the deuteride and chloride was fitted into the end of the tubing and clamped so as to bend the tubing shut. The reaction flask was then charged with 50 ml of rigorously dried ether (distilled over lithium aluminum hydride) using a syringe. The reaction flask was cooled in a dry ice–acetone bath, and the lithium aluminum deuteride–aluminum chloride mixture was cautiously added, with magnetic stirring of the ether solution, using the tubing as a solid addition device. The dry ice–acetone bath was removed and the slurry was treated with 10.0 g (5.98×10^{-2} mole) of ethyl bromoacetate over 15 min. The resultant mixture was then stirred at room temperature for 1 hr and treated with 27.5 ml of water followed by 27.5 ml of 6 *N* sulfuric acid. The aqueous phase was separated, saturated with sodi-

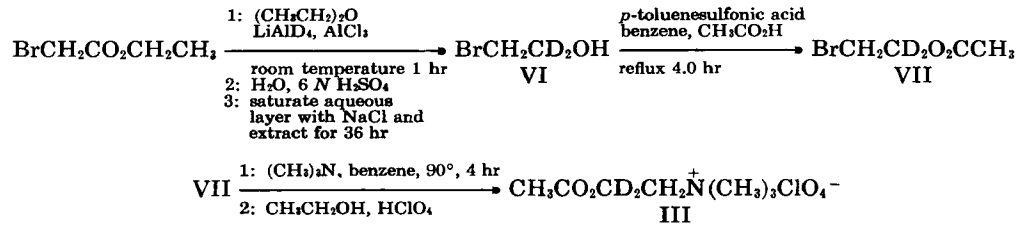
[1] IR spectra were taken on a Perkin-Elmer IR-8, NMR spectra were taken on a Varian T-60, and melting points were determined on a Thomas Hoover apparatus and are uncorrected. Elemental analyses were performed by Mr. Josef Nemeth.

[2] Diaprep.

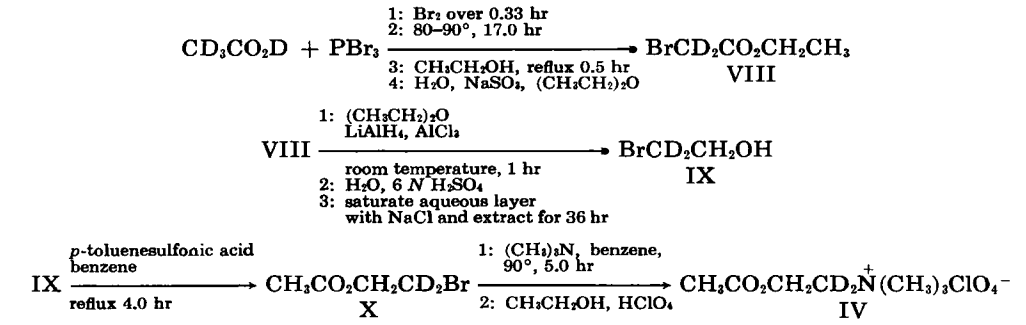
[3] Based on the assumption that the other two signals represented a total of 4H.

[4] The "H" means that the H and D were both considered together, since the gravimetric method used by Mr. Nemeth does not distinguish between H and D. The atom-% excess D values were used to calculate the percent deuteration of the samples.

[5] International Chemical and Nuclear Corp.



Scheme III



Scheme IV

um chloride, and continuously extracted with ether for 36 hr. The combined ether phases were dried over magnesium sulfate, filtered, concentrated, and distilled to give 4.73 g (62%) of VI[6], bp 65–68° (33 mm) [lit. (12) bp 55–59° (22 mm) for the nondeuterated analog].

1,1-d_2-2-Bromoethyl Acetate (VII)—According to the same procedure given for the preparation of V, 4.60 g (3.62×10^{-2} mole) of VI was reacted with 2.19 g (3.65×10^{-2} mole) of acetic acid and 0.1 g (5×10^{-4} mole) of *p*-toluenesulfonic acid monohydrate in benzene for 4.5 hr. Only about 90% of the theoretical amount of water was collected. Distillation as before gave 2.57 g (42%) of VII, bp 160–162° [lit. (10) bp 162–163° for the nondeuterated analog]; IR (CCl_4): 3012, 2160, 1742, and 1706 cm^{-1}; NMR (CCl_4): δ 2.10 (3H, s), 3.50 (1H, s), and 4.35 (1H, s).

Acetylcholine-1,1-d_2 Perchlorate (III)—According to the procedure described for the preparation of I, 1.10 g (6.50×10^{-3} mole) of VII and 6.71 g (1.13×10^{-1} mole) of anhydrous trimethylamine were heated in 20 ml of benzene for 4 hr. Treatment of the resultant concentrated solution in 15 ml of absolute ethanol with 0.75 ml of 70% aqueous perchloric acid allowed isolation of the compound as feathery, white crystals. Successive recrystallizations from ethanol yielded 0.1308 g (8%) of III, mp 111–112° [lit. (6) mp 116–117° for the nondeuterated analog].

Anal.—Calc. for $C_7H_{14}D_2ClNO_6$: C, 33.94; "H," 5.69; Cl, 14.31; N, 5.65; atom-% excess D, 12.50. Found: C, 34.42 and 34.41; "H," 6.47 and 6.51; Cl, 14.33; N, 5.84; atom-% excess D, 12.10. This converts to 99.6% deuteration at the desired site.

Ethyl Bromoacetate-d_2 (VIII)—A reaction flask was charged with 5.00 g (7.80×10^{-2} mole) of acetic acid-d_4 (99.5 atom-% D)[5] and 21.2 g (7.81×10^{-2} mole) of phosphorus tribromide to give a colorless cloudy solution (13). To this was added, over 20 min, 31.20 g (1.90×10^{-1} mole) of bromine. This gave an initial cloudy yellow solution, which began to reflux spontaneously about 5 min into the addition. The resultant blood-red solution was heated at 80–90° for 17 hr. The reaction solution was then treated with 50 ml of absolute ethanol while cooling in an ice bath and heated for 30 min more. The solution was then cooled to room temperature and treated with about 100 ml of water containing enough sodium sulfite to give a colorless solution. This was then extracted with ether, with the ether phases being dried over sodium sulfate. Concentration and distillation gave 6.28 g (48%) of VIII, bp 154–156° [lit. (14) bp 159° for the nondeuterated analog]; NMR ($CDCl_3$): δ 1.35 (3H, t) and 4.30 (2H, q).

2,2-d_2-2-Bromoethanol (IX)—According to the same procedure given for the preparation of VI, 6.10 g (3.60×10^{-2} mole) of VIII, 1.37 g (3.60×10^{-2} mole) of lithium aluminum hydride, and 4.80 g (3.60×10^{-2} mole) of anhydrous aluminum chloride were reacted to give 1.95 g (43%) of IX[7], bp 63.0–64.5° (30 mm) [lit. (12) bp 56–57° (20 mm) for the nondeuterated analog].

2,2-d_2-2-Bromoethyl Acetate (X)—According to the procedure reported for the preparation of VII, 1.70 g (1.34×10^{-2} mole) of IX was allowed to react with 0.81 g (1.35×10^{-2} mole) of acetic acid. Compound X was collected in a yield of 1.04 g (46%), bp 157–159° [lit. (10) bp 161.5° for the nondeuterated analog]; IR (CCl_4): 3497, 2941, 2169, and 1742 cm^{-1}; NMR (CCl_4) δ 1.65 (3H, s), 3.25 (1H, s), and 3.70 (1H, s).

Acetylcholine-2,2-d_2 Perchlorate (IV)—According to the procedure described for the preparation of I, 1.0 g (5.93×10^{-3} mole) of X and 6.71 g (1.13×10^{-1} mole) of anhydrous trimethylamine were heated for 5 hr in 20 ml of benzene. Treatment of the resultant concentrated solution with 70% aqueous perchloric acid as before and recrystallization of the resultant white, feathery crystals gave 0.50 g (34%) of IV, mp 113.5–114.5° [lit. (6) mp 116–117° for the nondeuterated analog].

Anal.—Calc. for $C_7H_{14}D_2ClNO_6$: C, 33.95; "H," 6.50; Cl, 14.31; N, 5.66; atom-% excess D, 12.50. Found: C, 33.80; "H," 6.39; Cl, 14.17; N, 5.62; atom-% excess D, 12.45. This converts to 99.6% deuteration at the desired site.

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[6] This compound was also prepared in 38% yield by lithium aluminum deuteride reduction of bromoacetyl bromide.

[7] This compound was also prepared in 35% yield by lithium aluminum hydride reduction of deuterated bromoacetyl bromide which had been prepared by the action of bromine and red phosphorus on acetic acid-d_4.

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Euparone, a New Benzofuran from *Ruscus aculeatus* L.

MAHMOUD A. ELSOHLY, NORMAN J. DOORENBOS *, MAYNARD W. QUIMBY *, JOSEPH E. KNAPP, DAVID J. SLATKIN, and PAUL L. SCHIFF, Jr. [x]

Abstract □ The structure of euparone, a new benzofuran from *Ruscus aculeatus*, as 2,5-diacetyl-6-hydroxybenzofuran was determined by spectral means and conversion to euparone methyl ether (*O*-methyleuparone).

Keyphrases □ *Ruscus aculeatus* L. constituents—structure determination of euparone □ Euparone (2,5-diacetyl-6-hydroxybenzofuran)—structure determination □ 2,5-Diacetyl-6-hydroxybenzofuran—structure determination □ Benzofurans—from *R. aculeatus*, structure determination

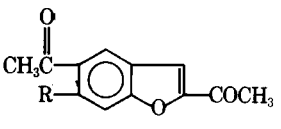
Ruscus aculeatus (Liliaceae), also known as Butcher's Broom or Knee-Holly, is a low evergreen shrub of southern and western Europe and southern United States (1). It has been used medicinally as a diuretic (2) and an anti-inflammatory (3) agent, as well as to treat hemorrhoids (4) and to prevent atherosclerosis (5) and circulatory insufficiency (6). Extracts of various *Ruscus* species have been shown to contain saponins (7) and flavonoids (8). This paper reports the isolation and structure determination of euparone, a new benzofuran.

DISCUSSION

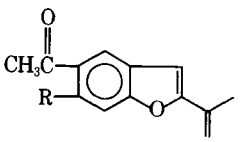
Euparone (I) was isolated as yellow rhomboid crystals from an ethanolic extract of the roots of *R. aculeatus*. Some pertinent data are: $C_{12}H_{10}O_4$; mp 143° ($CHCl_3$–C_2H_5OH); $[\alpha]_D$ 0° (c 1.0, $CHCl_3$); $\lambda_{max}(CH_3OH)$: 228 (sh) (log ϵ 4.19), 233 (4.21), 254 (4.37), 266 (sh) (4.35), 303 (4.11), and 348 (4.07) nm; λ_{max}(0.1 *N* methanolic KOH): 210 (log ϵ 4.32), 244 (4.00), 260 (sh) (3.82), 291 (3.66), 343 (3.78), and 403 (4.01) nm; ν_{max}(KBr): 3150 (bonded OH), 1667 (CO), and 1635 (CO) cm^{-1}; $\delta_{60\ MHz}(CDCl_3)$: 2.57 (s, 3H, $COCH_3$), 2.70 (s, 3H, $COCH_3$), 7.10 (s, 1H, Ar), 7.50 (s, 1H, Ar), 8.23 (s, 1H, Ar), and 12.66 (s, 1H, OH); mass spectroscopy M^+: *m/e* 218 (59%), 203 (100), and 43 (18). The spectral data were indicative of a phenolic 2,5,6-trisubstituted benzofuran containing two acetyl groups, one of which (1635 cm^{-1}) was *ortho* to a phenolic hydroxy (δ 12.66) group (9–12). Euparone gave a positive iodoform reaction characteristic of a methyl ketone. Treatment of euparone with acetic anhydride and pyridine afforded a monoacetate (II), $C_{14}H_{12}O_5$; mp 126° ($CHCl_3$–C_2H_5OH); $\lambda_{max}(CH_3OH)$: 227 (sh) (log ϵ 3.97), 249 (4.20), 287 (3.98), and 320 (sh) (3.68) nm; ν_{max}(KBr): 1765 (ArO-$COCH_3$) and 1665 (CO) cm^{-1}; $\delta_{60MHz}(CDCl_3)$: 2.36 (s, 3H, $COCH_3$), 2.58 (s, 6H, $2COCH_3$), 7.31 (s, 1H, Ar), 7.50 (s, 1H, Ar), and 8.15 (s, 1H, Ar); mass spectroscopy M^+: *m/e* 260 (2%), 218 (88), 203 (100), and 43 (30).

A further indication of the bonded nature of the hydroxy group of euparone was the failure of the compound to form a methyl ether on treatment with diazomethane. However, treatment of euparone with methyl iodide and silver oxide in refluxing chloroform afforded *O*-methyleuparone (euparone methyl ether) (III), $C_{13}H_{12}O_4$; mp 135–136° ($CHCl_3$–CH_3OH); $\lambda_{max}(CH_3OH)$: 232 (log ϵ 4.11), 245 (sh) (4.16), 251 (4.19), 299 (4.07), and 330 (4.11) nm; ν_{max}(KBr): 1685 (CO) and 1665 (CO) cm^{-1}; $\delta_{60\ MHz}(CDCl_3)$: 2.56 (s, 3H, $COCH_3$), 2.63 (s, 3H, $COCH_3$), 3.97 (s, 3H, OCH_3), 7.09 (s, 1H, Ar), 7.46 (s, 1H, Ar), and 8.05 (s, 1H, Ar); mass spectroscopy M^+: *m/e* 232 (48%), 217 (100), 202 (14), and 43 (28). This compound was identical with an authentic sample of euparone methyl ether by direct comparison (UV, IR, NMR, mass spectroscopy, melting point, and mixed melting point).

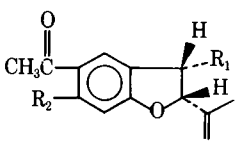
To the knowledge of the authors, this is the first reported isolation of euparone from nature. Euparone methyl ether (*O*-methyleuparone) (III), the first 2-acetylbenzofuran of nature, was first isolated from *Encelia californica* (Compositae) and was so named because of the obvious structural relationship to its probable 2-isopropenyl precursor, euparin (IV), also present in *E. californica* (13). Other 2,5-disubstituted benzofurans of nature include 2,5-diacetylbenzofuran (V) from *Aplopappus* species (14); tremetone (VI), dehydrotremetone (VII), and hydroxytremetone (VIII) (as a group formerly called tremetol) from *Eupatorium* (15, 16) and *Ap-*



I: R = OH
II: R = $OCOCH_3$
III: R = OCH_3
V: R = H



IV: R = OH
VII: R = H



VI: $R_1 = R_2 = H$
VIII: $R_1 = H, R_2 = OH$
IX: $R_1 = OH, R_2 = H$

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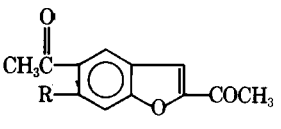
Ruscus aculeatus (Liliaceae), also known as Butcher's Broom or Knee-Holly, is a low evergreen shrub of southern and western Europe and southern United States (1). It has been used medicinally as a diuretic (2) and an anti-inflammatory (3) agent, as well as to treat hemorrhoids (4) and to prevent atherosclerosis (5) and circulatory insufficiency (6). Extracts of various *Ruscus* species have been shown to contain saponins (7) and flavonoids (8). This paper reports the isolation and structure determination of euparone, a new benzofuran.

DISCUSSION

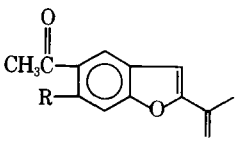
Euparone (I) was isolated as yellow rhomboid crystals from an ethanolic extract of the roots of *R. aculeatus*. Some pertinent data are: $C_{12}H_{10}O_4$; mp 143° ($CHCl_3$–C_2H_5OH); $[\alpha]_D$ 0° (c 1.0, $CHCl_3$); $\lambda_{max}(CH_3OH)$: 228 (sh) (log ϵ 4.19), 233 (4.21), 254 (4.37), 266 (sh) (4.35), 303 (4.11), and 348 (4.07) nm; λ_{max}(0.1 *N* methanolic KOH): 210 (log ϵ 4.32), 244 (4.00), 260 (sh) (3.82), 291 (3.66), 343 (3.78), and 403 (4.01) nm; ν_{max}(KBr): 3150 (bonded OH), 1667 (CO), and 1635 (CO) cm^{-1}; $\delta_{60\ MHz}(CDCl_3)$: 2.57 (s, 3H, $COCH_3$), 2.70 (s, 3H, $COCH_3$), 7.10 (s, 1H, Ar), 7.50 (s, 1H, Ar), 8.23 (s, 1H, Ar), and 12.66 (s, 1H, OH); mass spectroscopy M^+: *m/e* 218 (59%), 203 (100), and 43 (18). The spectral data were indicative of a phenolic 2,5,6-trisubstituted benzofuran containing two acetyl groups, one of which (1635 cm^{-1}) was *ortho* to a phenolic hydroxy (δ 12.66) group (9–12). Euparone gave a positive iodoform reaction characteristic of a methyl ketone. Treatment of euparone with acetic anhydride and pyridine afforded a monoacetate (II), $C_{14}H_{12}O_5$; mp 126° ($CHCl_3$–C_2H_5OH); $\lambda_{max}(CH_3OH)$: 227 (sh) (log ϵ 3.97), 249 (4.20), 287 (3.98), and 320 (sh) (3.68) nm; ν_{max}(KBr): 1765 (ArO-$COCH_3$) and 1665 (CO) cm^{-1}; $\delta_{60MHz}(CDCl_3)$: 2.36 (s, 3H, $COCH_3$), 2.58 (s, 6H, $2COCH_3$), 7.31 (s, 1H, Ar), 7.50 (s, 1H, Ar), and 8.15 (s, 1H, Ar); mass spectroscopy M^+: *m/e* 260 (2%), 218 (88), 203 (100), and 43 (30).

A further indication of the bonded nature of the hydroxy group of euparone was the failure of the compound to form a methyl ether on treatment with diazomethane. However, treatment of euparone with methyl iodide and silver oxide in refluxing chloroform afforded *O*-methyleuparone (euparone methyl ether) (III), $C_{13}H_{12}O_4$; mp 135–136° ($CHCl_3$–CH_3OH); $\lambda_{max}(CH_3OH)$: 232 (log ϵ 4.11), 245 (sh) (4.16), 251 (4.19), 299 (4.07), and 330 (4.11) nm; ν_{max}(KBr): 1685 (CO) and 1665 (CO) cm^{-1}; $\delta_{60\ MHz}(CDCl_3)$: 2.56 (s, 3H, $COCH_3$), 2.63 (s, 3H, $COCH_3$), 3.97 (s, 3H, OCH_3), 7.09 (s, 1H, Ar), 7.46 (s, 1H, Ar), and 8.05 (s, 1H, Ar); mass spectroscopy M^+: *m/e* 232 (48%), 217 (100), 202 (14), and 43 (28). This compound was identical with an authentic sample of euparone methyl ether by direct comparison (UV, IR, NMR, mass spectroscopy, melting point, and mixed melting point).

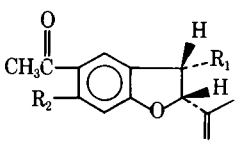
To the knowledge of the authors, this is the first reported isolation of euparone from nature. Euparone methyl ether (*O*-methyleuparone) (III), the first 2-acetylbenzofuran of nature, was first isolated from *Encelia californica* (Compositae) and was so named because of the obvious structural relationship to its probable 2-isopropenyl precursor, euparin (IV), also present in *E. californica* (13). Other 2,5-disubstituted benzofurans of nature include 2,5-diacetylbenzofuran (V) from *Aplopappus* species (14); tremetone (VI), dehydrotremetone (VII), and hydroxytremetone (VIII) (as a group formerly called tremetol) from *Eupatorium* (15, 16) and *Ap-*



I: R = OH
II: R = $OCOCH_3$
III: R = OCH_3
V: R = H



IV: R = OH
VII: R = H



VI: $R_1 = R_2 = H$
VIII: $R_1 = H, R_2 = OH$
IX: $R_1 = OH, R_2 = H$

lopappus species (16–18); and toxol (IX) from *Aplopappus heterophyllus* (18).

EXPERIMENTAL[1]

Isolation of Euparone—The detailed isolation procedure of euparone and other constituents of *R. aculeatus*[2] will be reported later.

Acetylation of Euparone—To euparone (30 mg) in pyridine (2 ml) was added acetic anhydride (2 ml). The mixture was stirred overnight at room temperature, poured into ice water (10 ml), neutralized with sodium bicarbonate, and extracted three times with chloroform (15 ml). The combined chloroform extracts were shaken with diluted H_2SO_4 (10%) (15 ml), washed with water (10 ml), dried (anhydrous sodium sulfate), filtered, and evaporated to leave *O*-acetyleuparone (II), which crystallized from chloroform–ethanol as pale-yellow plates (28 mg), mp 126°; $\lambda_{max}(CH_3OH)$: 227 (sh) (log ϵ 3.97), 249 (4.20), 287 (3.98), and 320 (sh) (3.68) nm; ν_{max}(KBr): 1765, 1685 (sh), 1665, 1610, 1555, 1420, 1350, 1305, 1285, 1240, 1190, 1135, 1070, 1035, 960, 915, 870, 845, and 620 cm^{-1}; $\delta_{60\ MHz}(CDCl_3)$: 2.36 (s, 3H), 2.58 (s, 6H), 7.31 (s, 1H), 7.50 (s, 1H), and 8.15 (s, 1H); mass spectroscopy M^+: *m/e* 260 (2%), 218 (88), 203 (100), and 43 (30).

Methylation of Euparone—To a solution of euparone (25 mg) in chloroform (3 ml) were added silver oxide (50 mg) and methyl iodide (2 ml). The mixture was refluxed for 4 hr, after which additional methyl iodide (2 ml) and silver oxide (50 mg) were added. Then the mixture was refluxed for 4 additional hr, the reaction mixture was filtered, and the filtrate was evaporated to dryness. Treatment of the residue with chloroform–methanol afforded euparone methyl ether (*O*-methyleuparone) (III) as yellowish rosettes (20 mg), mp 135–136°; $\lambda_{max}(CH_3OH)$: 232 (log ϵ 4.11), 245 (sh) (4.16), 251 (4.19), 299 (4.07), and 330 (4.11) nm; ν_{max}(KBr): 3120, 3090, 3020, 3000, 2955, 2930, 1685, 1665, 1620, 1585, 1560, 1475, 1450, 1425, 1410, 1352, 1310, 1293, 1245, 1235, 1192, 1175, 1150, 1075, 1040, 1025, 1003, 960, 920, 905, 855, 812, and 630 cm^{-1}; $\delta_{60\ MHz}(CDCl_3)$: 2.56 (s, 3H), 2.63 (s, 3H), 3.97 (s, 3H), 7.09 (s, 1H), 7.46 (s, 1H), and 8.05 (s, 1H); mass spectroscopy M^+: *m/e* 232 (48%), 217 (100), 202 (14), and 43 (28).

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[1] Melting points were taken on a Thomas-Hoover Uni-melt capillary apparatus and are corrected. The IR spectra were determined on a Perkin-Elmer model 257 or 137 spectrometer in KBr pellets. The UV spectra were obtained on a Perkin-Elmer model 202 recording spectrometer. Optical rotations were measured in a Rudolph polarimeter. Mass spectra were taken with a LKB-9000 mass spectrometer. The NMR spectra were obtained in $CDCl_3$, with tetramethylsilane as the internal standard, on a Hitachi Perkin-Elmer R-24 60-MHz high-resolution spectrometer.

[2] Lot BRC 926, S. B. Penick and Co., New York, N.Y.

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* Present address: Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 36877

ˣ To whom inquiries should be directed.

GLC Determination of Papaverine in Biological Fluids

D. E. GUTTMAN †, H. B. KOSTENBAUDER ˣ, G. R. WILKINSON *, and P. H. DUBÉ

Abstract □ A procedure for the determination of papaverine in blood plasma or urine, based on ion-pair extraction with diethylhexyl phosphate and GLC, permits specific determination of papaverine at levels as low as 0.01 μg/ml. The applicability of this method for bioavailability studies of papaverine after acute administration of oral dosage forms is demonstrated.

Keyphrases □ Papaverine—GLC determination in biological fluids □ GLC—analysis, papaverine in biological fluids

Few methods for the estimation of papaverine in biological fluids such as blood, plasma, and urine have been described. The turbidimetric method utilizing phosphomolybdic acid (1) is not specific for papaverine in the presence of its metabolites. Specificity has been demonstrated for the differential spectrophotometric method combined with a heptane extraction procedure (2) but, unfortunately, the sensitivity of this method (about 0.5 μg) precludes its use for the measurement of papaverine concentrations present in biological fluids after acute oral administration.

A procedure based upon ion-pair extraction and GLC is reported here; it is specific and permits the measurement of papaverine levels down to 0.01 μg/ml in a 5-ml sample. The suitability of the method for evaluating the acute bioavailability of orally administered dosage forms is also described.

Because papaverine is a weak base (pKa ≈ 6.4), its extraction from an aqueous solution by an organic solvent would be expected to be optimal under alkaline conditions. Preliminary studies supported this prediction since an extraction greater than 90% was achieved with a single partition from a pH 12 solution into an equal volume of ether. However, subsequent concentration and GLC of the extract yielded gross interfering peaks with respect to both papaverine and the internal standard. Attempts to reduce this interference by traditional techniques such as buffer washing and/or repeated back-extraction were unsuccessful. However, the inclusion of an ion-pair extraction step with diethylhexyl phosphate and extraction of papaverine as its protonated species from an aqueous phase adjusted to pH 4.0 yielded chromatograms free from interfering peaks.

EXPERIMENTAL

To 5 ml of plasma in a 13-ml centrifuge tube were added 1 ml of internal standard solution (dibucaine hydrochloride, 5 μg of base/ml in distilled water) and 1 ml of 5 *N* NaOH. The solution was extracted with 2 × 5 ml of freshly distilled ether (analytical grade) by gently shaking for 5 min and then centrifuging for the same time. The ether extracts were transferred and combined in a separate centrifuge tube containing 4 ml of 0.2 *M* acetate buffer (pH 4.0).

After extracting, under the same time conditions, the ether was discarded and the aqueous phase was shaken with 2 × 5 ml of ethereal 0.1 *N* diethylhexyl phosphate solution. The ethereal phase was aspirated off, shaken with 3 ml of 1 *N* HCl, and then discarded. The aqueous phase was made alkaline with 1 ml of 5 *N* NaOH and extracted with 3 ml of ether. The final extract was evaporated to dryness in a 10-ml centrifuge tube, using a water bath maintained at 42°, and the residue was reconstituted with 4–12 μl of analytical grade carbon disulfide. Depending upon the expected concentration of papaverine, from 1 to 2 μl of this solution was injected into the gas chromatograph.

The relatively high molecular weight of papaverine necessitated the use of a lightly loaded column and high temperature conditions if the analysis was to be completed in a reasonable time. A gas chromatograph[1] equipped with a flame-ionization detector was employed. The column was Pyrex glass, 1.8 m (6 ft) × 2 mm i.d., packed with 80–100-mesh, dimethyldichlorosilane-treated, calcined diatomaceous earth coated with 1% phenyl (50%) methyl sili-

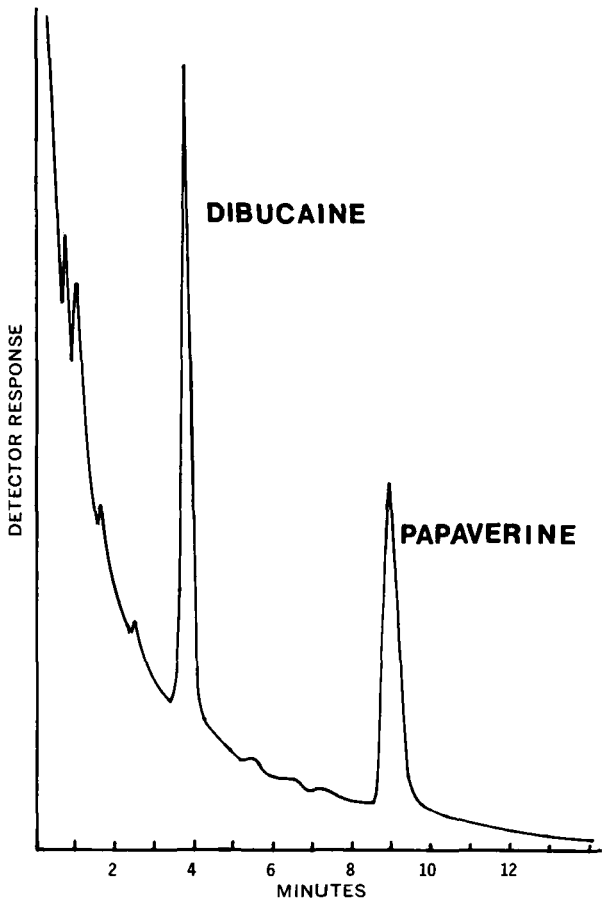

Figure 1—*Chromatogram obtained by spiking blank plasma with dibucaine hydrochloride at 1.0 μg/ml and papaverine hydrochloride at 1.0 μg/ml.*

[1] Varian Aerograph, model 1740, Varian Instrument Division, Palo Alto, Calif.

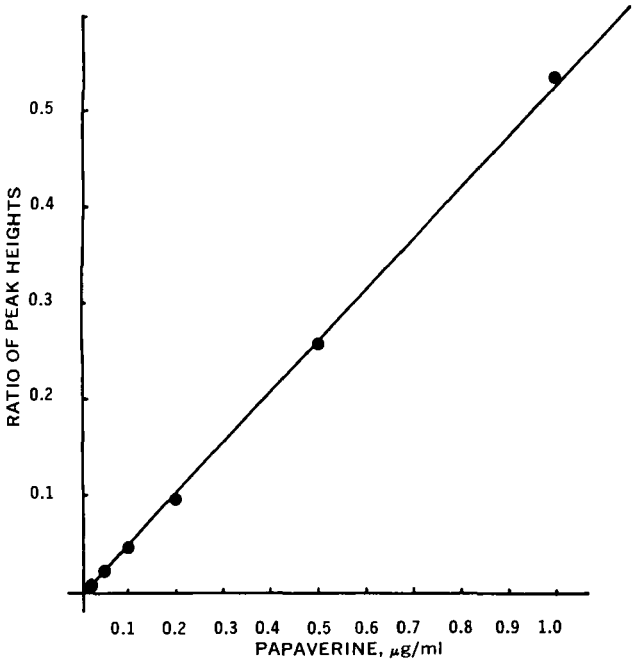

Figure 2—*Typical standard curve obtained from spiked plasma samples.*

cone fluid[2]. The column was conditioned at 300° with a nitrogen carrier gas flow for 8 hr and then overnight under the following operating conditions: oven temperature, 275°; injection port temperature, 285°; detector oven temperature, 300°; carrier gas flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min; and air flow rate, 300 ml/min. The column was silanized[3] *in situ* initially and then periodically.

To reduce further any potential adsorption of papaverine by the column, three injections of a concentrated ethereal solution of papaverine and dibucaine were made prior to any series of analyses. The plasma concentration of papaverine was obtained by calculating the peak height ratio of the drug to that of the internal standard and relating this to a standard curve, which was prepared daily over the concentration range of 0.01–2 μg of papaverine/ml.

RESULTS AND DISCUSSION

Well-resolved, sharp, and symmetrical peaks were obtained for both dibucaine and papaverine with retention times of 3.9 and 9.1 min, respectively (Fig. 1). Extraction of blank plasma samples revealed no interfering peaks in the region of the retention time of either papaverine or dibucaine. A typical standard curve is shown in Fig. 2, and excellent day-to-day reproducibility of the slope of the curve was obtained. Data for five replicate determinations on spiked plasma samples indicate a relative standard deviation of 5.1% at 0.5 μg/ml and 14.4% at 0.05 μg/ml.

The plasma concentrations obtained after the oral administration of 2 × 150-mg papaverine hydrochloride capsules to two male volunteer subjects are shown in Fig. 3 and indicate that the drug is rapidly absorbed since peak levels were seen within 2 hr. Terminal phase half-lives for these two subjects were 1.0 and 1.4 hr. Assay results on urine samples were in agreement with the previous observation (2) that less than 1% of the dose is recoverable in urine as

[2] Gas Chrom Q coated with 1% OV-17, Applied Science Laboratories, State College, Pa.

[3] Silyl 8, Pierce Chemical Co., Rockford, Ill.

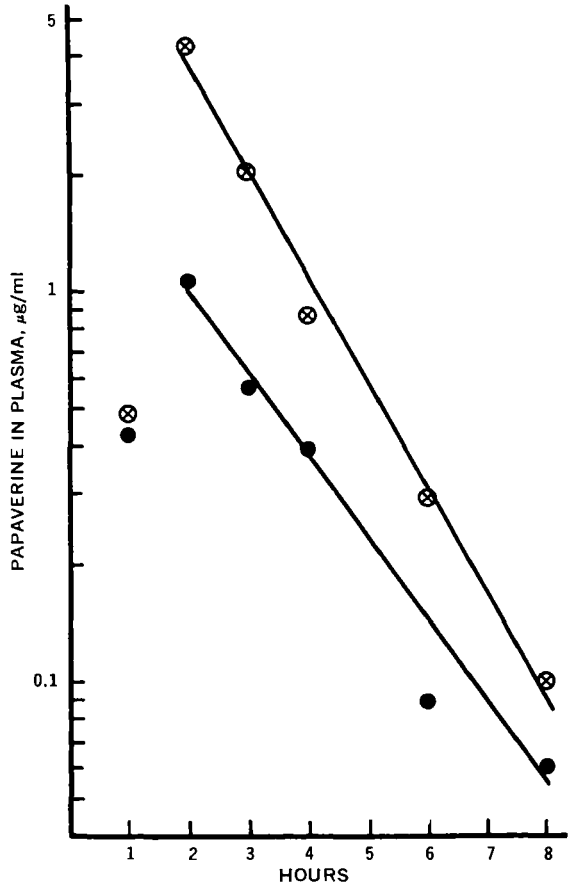

Figure 3—*Plasma papaverine levels, expressed as papaverine hydrochloride, in two human subjects following oral administration of 2 × 150-mg papaverine hydrochloride capsules.*

intact papaverine. The almost negligible urinary recovery of intact papaverine indicates that phenolic metabolites of papaverine do not interfere with the described assay procedure.

This assay procedure was found suitable for determination of papaverine plasma levels up to 12 hr after administration of 300 mg of papaverine hydrochloride in timed-release form. These studies will be reported separately.

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* Present address: Department of Pharmacology, School of Medicine, Vanderbilt University, Nashville, TN 37232

† Deceased.

× To whom inquiries should be directed.

Preparation of 2-Methyl-3-(2-hydroxymethyl)phenyl-4(3*H*)-quinazalone: A Metabolite of Methaqualone

JAMES A. CELLA

Abstract □ The preparation of 2-methyl-3-(2-hydroxymethyl)-phenyl-4(3*H*)-quinazalone (I*b*), a metabolite of methaqualone [2-methyl-3-*o*-tolyl-4(3*H*)-quinazalone] (I*a*), is described. Bromination of I*a* is accomplished with *N*-bromosuccinimide in refluxing carbon tetrachloride. Solvolysis of this bromide (I*f*) in buffered diglyme–water affords I*b* in 86% yield.

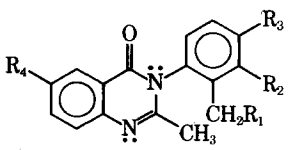
Keyphrases □ 2-Methyl-3-(2-hydroxymethyl)phenyl-4(3*H*)-quinazalone—methaqualone metabolite, synthesis □ Methaqualone—synthesis of metabolite 2-methyl-3-(2-hydroxymethyl)-phenyl-4(3*H*)-quinazalone

Methaqualone [2-methyl-3-*o*-tolyl-4(3*H*)-quinazolinone] (I*a*), a sedative hypnotic and often abused drug[1] (1), is detoxified by nonspecific hydroxylation; its principal metabolites are monohydroxy compounds I*b*–I*e* (2–4). The detection and quantification of these metabolites require standard samples for comparison. While the preparation of I*c*–I*e* is straightforward and has been described elsewhere (4, 5), no report has described the preparation of 2-methyl-3-(2-hydroxymethyl)phenyl-4(3*H*)-quinazalone (I*b*). Accordingly, a facile two-step preparation of I*b* is reported here.

Reaction of I*a* with a slight excess of *N*-bromosuccinimide in refluxing carbon tetrachloride afforded 2-methyl-3-(2-bromomethyl)phenyl-4(3*H*)-quinazalone (I*f*) in good yield. Solvolysis of I*f* in refluxing diglyme–water buffered with sodium carbonate afforded I*b* in 86% yield. The structure of I*b* was confirmed by spectroscopic and elemental analyses. Verification of I*b* as a metabolite of methaqualone has been accomplished by TLC (2, 3) and GLC (6, 7).

EXPERIMENTAL[2]

2-Methyl-3-(2-bromomethyl)phenyl-4(3*H*)-quinazalone (I*f*)—A solution of 250 mg (1 mmole) of methaqualone, 178 mg (1 mmole) of *N*-bromosuccinimide, and a trace of benzoyl peroxide in 25 ml of carbon tetrachloride was refluxed for 6 hr. Progress of the reaction was monitored by GLC. The reaction had to be reinitiated from time to time by addition of small amounts of benzoyl peroxide. Toward the end of the reaction, a slight excess of *N*-bromosuccinimide was added to convert more of the remaining methaqualone to product. The reaction was stopped when about 95% of the methaqualone had reacted. (Longer reaction times gave increased amounts of dibromination product at the expense of the desired product.)



I*a*: $R_1, R_2, R_3, R_4 = H$
I*b*: $R_2, R_3, R_4 = H; R_1 = OH$
I*c*: $R_1, R_3, R_4 = H; R_2 = OH$
I*d*: $R_1, R_2, R_4 = H; R_3 = OH$
I*e*: $R_1, R_2, R_3 = H; R_4 = OH$
I*f*: $R_2, R_3, R_4 = H; R_1 = Br$

The cooled mixture was shaken with 1.5 *N* sodium carbonate and brine and then dried by passage through a cone of anhydrous sodium sulfate. Evaporation of solvent afforded an oil (345 mg), the major (90–95%) component of which was shown by combined GLC–mass spectroscopy to be I*f*.

2-Methyl-3-(2-hydroxymethyl)phenyl-4(3*H*)-quinazalone (I*b*)—Crude I*f* was dissolved in 30 ml of a diglyme–1.5 *N* Na_2CO_3 mixture (5:1), and the resulting solution was refluxed for 6 hr. The cooled solution was diluted with an equal volume of water and extracted with 3 × 30 ml of chloroform. The dried (sodium sulfate) extracts were concentrated, and the residue was chromatographed on 10 g of silica gel to afford 230 mg (86%) of I*b*, mp 110–112° [lit. (8) mp 109–110°]; NMR ($CDCl_3$): δ 7.42 (m, 8, ArH), 4.22 (s, 2, CH_2OH), and 2.01 (s, 3, CH_3) ppm; IR (KBr): 6.02 ($\nu_{C=O}$) and 3.0 (ν_{OH}) μm; mass spectrum: *m/e* 267 (molecular ion) and 160 (loss of $ArCH_2OH$ from molecular ion).

Anal.—Calc. for $C_{16}H_{14}N_2O_2$: C, 72.2; H, 5.3; N, 10.5. Found: C, 72.0; H, 5.3; N, 10.4.

[1] Trade names are Quaalude (Rorer), Sopor (Arnar-Stone), Optimil (Wallace), Parest (Parke-Davis), and Somnafac (Cooper).

[2] Melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. IR spectra were recorded on a Beckman IR 5A spectrophotometer using sodium chloride disks or potassium chloride pellets. NMR spectra were recorded on a Varian A-60 spectrometer. Mass spectra were determined on a Hewlett-Packard 5930-A quadrupole mass spectrometer. GC was performed on an F&M model 402 high efficiency gas chromatograph, using a 1.8-m (6-ft) glass column packed with 3% OV-1 on 80–100-mesh Supelcoport. Microanalysis was performed by Mr. Joseph Alicino, New Hope, PA 18938. Methaqualone was obtained as a gift from Wm. H. Rorer & Co., Inc.

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GLC Analysis of Bis(2-ethylhexyl) Phthalate Plasticizer in Tissue and Plasma

DAVID B. ROLL ˣ, JOHN D. DOUGLAS, and ROBERT V. PETERSEN

Abstract □ A method for the GLC analysis of the plasticizer, bis(2-ethylhexyl) phthalate, in lung, liver, spleen, and plasma in levels as low as 1 mg % is described. The method employs an internal standard, bis(*n*-octyl) phthalate, which greatly simplifies the analysis. The possibility of contamination of biological samples with plasticizers in the laboratory is discussed briefly.

Keyphrases □ Bis(2-ethylhexyl) phthalate—GLC analysis in tissue and plasma □ Phthalate plasticizers, bis(2-ethylhexyl)—GLC analysis in tissues and plasma □ Plasticizers, bis(2-ethylhexyl) phthalate—GLC analysis in tissue and plasma □ GLC—analysis, bis(2-ethylhexyl) phthalate in tissue and plasma

Phthalic acid esters are widely used as plasticizers for polyvinyl chloride plastics. In particular, bis(2-ethylhexyl) phthalate (I) is contained in plastics utilized in the fabrication of medical devices such as blood storage bags, blood administration assemblies, hemodialysis units, and cardiopulmonary bypass units where it may represent 40% or more of the total weight of the finished plastic (1). Marcel and Noel (2) and Jaeger and Rubin (3) were the first to identify the presence of I in human blood stored in plastic bags. Since this discovery, considerable interest has developed regarding the potential toxicogenic effects of I and other phthalate esters, not only as the result of leaching from plastic medical devices but also from environmental contamination resulting from the many industrial and commercial uses of plastics (4).

During studies in this laboratory on the distribution and metabolism of I in experimental animals, it became apparent that its accurate quantitation in various organs by GLC was difficult with available techniques. Extraction of various tissue samples employing the method of Jaeger and Rubin (5) was not satisfactory. Attempted quantitation in which the GLC peak heights of accurately measured volumes of standard solutions of I were compared with those of solutions containing unknown amounts was unsatisfactory. Duplicate runs on the same tissue showed wide discrepancies due at least in part to the inherent difficulty in accurately measuring small samples for injection and to the fact that an average of 22 ± 8% was lost on extraction. These results suggested that a more reliable method of analysis might employ an internal standard. The validity of the use of an internal standard is discussed elsewhere (6), but it should be noted that once a suitable method has been developed, it is no longer necessary to be concerned with the complete recovery of extraction solvents nor with accurate measurement of the quantity injected. The internal standard chosen was bis(*n*-octyl) phthalate (II). The peak height ratio of I to II, when plotted against varying concentrations of I at constant concentration of II, was shown to be linear. Furthermore, it was shown that the peak height ratio of I to II was the same after addition to tissue and extraction in the manner described here as it was prior to tissue extraction. In other words, during the extraction process the relative amounts of both compounds lost were the same. Similar solubility characteristics of the internal standard and the substance analyzed, *i.e.*, II and I, respectively, are important criteria in the choice of an internal standard (7), and these results demonstrated that II is an excellent choice. Another case of an internal standard being employed in the GLC analysis of biological samples for I was reported (8), but it was applied only to blood and is unsuitable for tissue analysis due to interfering peaks. A recent paper described the determination of I in human plasma, but the method is more cumbersome and time consuming in that it involves column chromatographic separation of the plasticizer from the plasma, followed by GLC (9). The method is, however, more sensitive than the one described here.

EXPERIMENTAL

The following method is satisfactory for the analysis of I in many tissues, the extraction portion of the procedure being a modification of the method employed by Jaeger and Rubin (5). To 1–5 g of tissue (depending on whether the sample is likely to contain relatively large or small quantities of I) was added 1 ml of a chloroform solution containing 1.0, 0.25, or 0.1 mg of II[1] (the amount depending on the anticipated level of I). The tissue was then homogenized with 10 ml of methanol[2]–chloroform[2] (1:2), and the homogenizer was washed twice with 10-ml portions of the same solvent. The homogenate and washes were combined, shaken for 1 hr, placed in a separator containing an equal volume of 0.9% saline solution, and shaken. The lower layer (chloroform) was retained, the saline was washed once with 5 ml of methanol–chloroform (1:2), and this wash was combined with the chloroform layer. To the chloroform was added 2–3 g of silica gel[3] followed by shaking and suction filtration[4].

The sample was reduced to dryness under a stream of nitrogen, redissolved in 25 ml of methanol, centrifuged at 1000×*g* for 10 min, transferred to another tube, and reduced to dryness under a stream of nitrogen. Then the residue was dissolved in 1 ml of chloroform and analyzed by injection of 2–6 μl into a gas chromatograph[5] equipped with a flame-ionization detector employing a 1.82-m × 4-mm i.d. coiled glass column containing 3% OV-210 on 80–100-mesh Chromosorb WHP[6] under the following conditions: oven temperature, 225°; injector and detector temperature, 295°; and helium flow rate, 60 ml/min. A sensitivity of 1×10^{-9} was adequate for samples containing 1.0 or 0.25 mg of II; for samples containing only 0.1 mg of II, a sensitivity of 1×10^{-10} was required. The analysis was quite satisfactory for tissue containing a low lipid content such as liver, lung, and spleen. However, in the analysis of

[1] Eastman Organic Chemicals, Rochester, N.Y.
[2] Nanograde, Mallinckrodt Chemical Works, St. Louis, Mo.
[3] Analyzed reagent grade, J. T. Baker Co., Phillipsburg, N.J.
[4] Whatman No. 4 qualitative filter paper, U. & R. Balson Limited.
[5] Packard 7401, Packard Instrument Co., Downers Grove, Ill.
[6] Supelco, Inc., Bellefonte, Pa.

Table I—Comparison of Peak Height Ratio and Peak Height Methods in Spiked Lung Tissue

Actual Content of I, mg %	I:II	Peak Height
30	31	35
60	61	103
80	82	68
100	101	193
150	148	182
160	163	165

tissue containing large amounts of lipid, such as brain and fat, especially at the lower concentration of II, quantitation is difficult if not impossible due to the presence of substances possessing similar retention times to that of I and/or II.

For blood, plasma, or serum, extraction of 1 ml of the sample (after the addition of internal standard) with 30 ml of chloroform, followed by aspiration of the upper layer, evaporation to dryness, redissolution to 1 ml with chloroform, and subsequent injection was much faster than the tissue method with no loss of accuracy.

The I–II peak height ratio of the sample was compared to those obtained by graphing the peak height ratio of I–II standards *versus* concentration of I[1]. The standards employed contained varying amounts of I and equal quantities of II, the amount of the latter being the same as that added to the sample initially. Under these conditions the retention times of I and II were about 4 and 7 min, respectively, varying somewhat with changes in such parameters as column pressure and density of column packing.

RESULTS AND DISCUSSION

Table I illustrates the superiority of the peak height ratio method. Various samples of I were prepared in chloroform which also contained a constant amount of II (1 mg/ml of chloroform). The quantities of I chosen were within the range often found in lung tissue of animals injected intravenously with this compound. One milliliter of each of these samples was added to homogenized lung tissue and extracted, and the values were compared with those obtained by using only the peak height of I compared to the peak height of standards. The precision of the analysis for I in a concentration of 1–10 mg % was approximately ±20%, whereas duplicate samples were usually reproducible to within ±10% in concentrations above this level.

In another experiment, a mongrel dog (11 kg) was infused intravenously over about 30 min with I, prepared by sonication in dog plasma, at a dose of 25 mg/kg. Six hours later the dog was sacrificed with sodium pentobarbital[7], and the lung and liver were removed and minced. These tissues were then divided into seven approximately equal samples, stored in a refrigerator freezer at −11.5°, and analyzed weekly. The results (Table II) demonstrate that the ratio method yields quite reproducible results and indicate that there is little appreciable deterioration of I in the samples when stored under these conditions since the difference between the first and last assays for both tissues is within the experimental error given. The liver samples were determined with II at the 0.25-mg/ml level, whereas the lung samples were analyzed with the internal standard at a concentration of 1.0-mg/ml.

The minimum detectable level of I in lung, liver, and spleen tissues as well as plasma is about 1 mg %. However, the difficulties encountered in the analysis of samples containing less than 5 mg % should be emphasized. At a sensitivity of 1×10^{-10}, with II present at the 0.1-mg/ml level, standards containing I in the range of approximately 1–10 mg % were suitable. At these low levels of I and high sensitivity, it is essential that adequate measures be taken to assure the least possible contamination from glassware, solvents, *etc.*, and that careful blank determinations be performed. It has been found in this laboratory that small amounts of phthalates are present in silicic acid as well as in some solvents and filter paper. Other investigators (10) made similar observations. The described analytical procedure avoids the use of phase-separating filter paper which has been found to contain phthalates (10). Even when all of these precautions were taken, blanks still contained approximately 0.2 mg %. All traces of phthalate, however, can be removed by soaking glassware overnight in concentrated sulfuric acid. When attempting to detect I at these levels, it is also important to be careful in the storage and handling of the sample so that it is protected from contamination. For example, samples should not be stored in screw-top glass containers if the cap is lined with polyvinyl chloride or metal foil. All-glass containers are preferable.

[7] Pentathesia, Wittney and Co., Denver, Colo.

Table II—Amount (mg %) of I Present in Dog Lung and Liver Tissue

Sample	Liver	Lung
1	11.6	143.0
2	10.9	129.3
3	10.2	135.2
4	7.6	133.1
5	9.3	127.4
6	9.7	137.9
7	9.8	129.3
Mean	9.9	133.6
SD	1.3	5.6

There are many reports of the occurrence of small quantities of phthalate esters in numerous samples of biological origin. In light of the facts presented, the possibility exists that, at least in some of these cases, the contamination occurred in the laboratory and not in the environment.

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[x] To whom inquiries should be directed.

Evaluation of Dimethylpolysiloxane Fluids as Vehicles for Various Pharmaceuticals I: Effect on Stability and Dissolution of Aspirin

A. F. ASKER ˣ and C. W. WHITWORTH

Abstract □ The physicochemical effects of dimethylpolysiloxane fluids as vehicles for aspirin on its stability and release characteristics were evaluated. Aspirin was extraordinarily stable in the silicone fluids. No decomposition was noted during 8 weeks of storage at 4 and 26°, and less than 0.5% degradation was detected after 8 weeks of storage at 45°. Moreover, the dissolution patterns of aspirin suspensions in the silicone fluids appeared to be quite satisfactory as compared with that of plain aspirin powder.

Keyphrases □ Dimethylpolysiloxane vehicles—effect on stability and dissolution of aspirin □ Aspirin suspensions—effect of dimethylpolysiloxane vehicles on stability and dissolution □ Silicone fluids, dimethylpolysiloxane—effect on stability and dissolution of aspirin suspensions

In a previous study (1), it was found that aspirin decomposed rapidly when incorporated into various polyethylene glycol bases. The degradation was attributed, at least in part, to a transesterification reaction between aspirin and the polyethylene glycols. Reduction of the number of free hydroxy groups of the polyethylene glycols through the formation of acetylated glycols and methoxyglycols resulted in significant retardation of aspirin decomposition (2). However, while developing a stable and palatable liquid preparation of aspirin, the authors explored the potentiality of using dimethylpolysiloxane fluids as vehicles for aspirin because of their apparent inherent advantages over the commonly used nonaqueous vehicles.

Dimethylpolysiloxane fluids are odorless, colorless, tasteless, and strongly hydrophobic, and they possess very great chemical, thermal, and oxidative stability. They are superior to fixed oils in that they do not develop rancidity with aging. They have low viscosity–temperature coefficients, and their viscosity can be tailored to meet specific applications (3, 4). Dimethylpolysiloxane fluids have been reported to be very low in toxicity (5–9). Medical-grade silicones are widely used in tissue implants (10) and as inhalation sprays for the treatment of pulmonary edema involving frothing of fluid in the upper respiratory tract (11). They are pharmacologically inactive when given orally (12) and do not appear to possess any adverse effects when ingested (4). Their oral use as antiflatulents has been reported (11, 12). Silicones also have been used in tablet and capsule coatings and to fill in hypoplastic body areas for cosmetic purposes (11). In food processing, food-grade dimethylpolysiloxane is used for defoaming (13).

Turnbull and Avis (3) stated that the dimethylpolysiloxane fluids appear to possess most of the characteristics of an ideal nonaqueous solvent. The use of silicone fluids as vehicles for the intramuscular administration of drugs has been reported (3, 14, 15).

Apart from these properties of dimethylpolysiloxane fluids, their hydrophobic nature suggest that they may tend to retard the decomposition and enhance the absorption of drugs such as aspirin. Furthermore, in a recent study, dimethylpolysiloxane significantly protected rat gastric mucosa from the irritant effects of aspirin (12).

The objective of this study was to explore the physicochemical effects of dimethylpolysiloxane fluids, when utilized as vehicles for aspirin, on the stability and release characteristics of this drug.

EXPERIMENTAL

Materials—Dimethylpolysiloxane fluids[1] were used as received from the commercial source. Aspirin[2] and salicylic acid[3] were of USP quality. Chloroform[4] was spectrograde, and other ingredients were USP or analytical grades.

Stability Studies—Quantities of aspirin (0.3 g) were placed into 40-ml amber glass vials, each containing 9.7 g of the silicone fluid. The tightly screw-capped vials were divided into three groups for storage at 4, 26, and 45°, and the samples were assayed at various intervals.

Analytical Method—UV spectrophotometric analysis (2, 16, 17) was employed to measure aspirin and salicylic acid. The contents of each vial were transferred quantitatively into a 100-ml volumetric flask using chloroform containing 1% acetic acid and then diluted to volume with the chloroform–acetic acid mixture. Clear solutions were obtained since the silicone fluids used were miscible (1:10) in chloroform (4). Then 1 ml of this solution was pipeted into a 50-ml volumetric flask and diluted to volume with the chloroform–acetic acid mixture. The absorbance of this solution was read[5] at 280 nm for aspirin and 310 nm for salicylic acid using the appropriate blanks. The silicone fluids did not interfere with the assay. At the two wavelengths indicated, standard curves were prepared for aspirin and salicylic acid. The quantification of aspirin and salicylic acid was based on the standard method of simultaneous spectrophotometric determinations.

Dissolution Studies—A rotating-bottle apparatus, similar to that used by Asker and Becker (18), was used. Screw-capped centrifuge tubes, each of 50-ml capacity, were utilized. Into each tube, 0.1 g of aspirin (passed through a 100-mesh sieve) was dispersed in 3 g of the silicone fluid. Then 25 ml of 0.1 *N* HCl at 37° was introduced. The tubes were allowed to rotate at 45 rpm in a water bath maintained at 37°. Two tubes were removed at each time interval for duplicate determination of the quantity of aspirin released. One tube containing the silicone and hydrochloric acid but no drug served as a blank. Two other tubes containing the drug and the dissolution medium but without silicone were used as the control for comparison.

The contents of each tube were transferred to a small separator. An appropriate volume of the aqueous layer was separated, filtered, and subjected to the assay procedure described by Javaid and Cadwallader (19). The absorbance of the solution was read[5] at 303 nm.

[1] Trade-marketed by Dow Corning Corp. as DC 200 silicone fluid, 350 centistokes; DC 200 silicone fluid, food grade, 350 centistokes; DC 360 medical silicone fluid, 20 centistokes; and DC 360 medical silicone fluid, 50 centistokes.

[2] Merck and Co.

[3] Fisher Scientific Co.

[4] J. T. Baker Chemical Co.

[5] Cary model 118 spectrophotometer.

Table I—Mole Percent Decomposition of Aspirin[a] with Time in the Silicone Fluids

Weeks of Storage	Mole Percent of Salicylic Acid Formed	
	200 Fluid[b], 45°	200 Fluid, Food Grade[b], 45°
0	0	0
1	0	0
2	0	0
3	0	0
4	0	0
5	0.16	0.20
6	0.19	0.20
7	0.40	0.37
8	0.43	0.40

[a] Expressed as moles of salicylic acid formed per 100 moles of aspirin. [b] No salicylic acid formed at 4 and 26°.

RESULTS AND DISCUSSION

The use of nonaqueous vehicles to enhance the stability of aspirin liquid preparations has been reported (2, 16). Due to the outstanding advantages of the silicone fluids as nonaqueous vehicles, it was decided to explore their physicochemical effects on the stability and dissolution rate of aspirin incorporated into them.

Table I shows that aspirin was extraordinarily stable in the silicone 200 fluids. No decomposition was noted during 8 weeks of storage at 4 and 26°, and only less than 0.5% degradation was detected after 8 weeks of storage at 45°. This negligible amount of aspirin decomposition could have been caused by traces of moisture that contaminated the dry aspirin during weighing and preparing the samples. The results obtained support previous findings on the enhanced stability of aspirin in nonpolar vehicles (2, 17).

Figure 1 shows the overall dissolution profiles of aspirin suspended in the silicone 360 medical fluids of the viscosity grades 20 and 50 centistokes. The silicone 360 medical fluid is chemically identical to the 200 fluid but more refined, and the medical literature references to the 200 fluid also apply to the 360 medical fluid (4). The dissolution data indicate that more than 90% of the aspirin was released during the first 6 min when aspirin was suspended in either the 20- or the 50-centistokes viscosity-grade silicone fluids. Plain powder aspirin, on the other hand, demonstrated 100% release in 6 min. The difference can be attributed to the time required for the aqueous dissolution medium to extract the aspirin particles suspended in the highly hydrophobic silicone fluids. Incorporation of a surfactant in the dissolution medium to lower its surface tension to a value corresponding to gastric fluid would be expected to enhance the dissolution rate of aspirin from the silicone fluids. Such expectation is based on previous results (20–22). Further studies on the effect of surface tension of the dissolution medium on the release of aspirin from the silicone fluids would be necessary before drawing a final conclusion. However, the dissolution patterns of aspirin suspensions in the silicone fluids obtained in this study appear to be quite satisfactory as compared with that of plain aspirin. It appears also from Fig. 1 that the viscosity of the silicone fluid influenced the rate of aspirin dissolution. The higher the viscosity of the silicone fluid, the less amount of aspirin is released during the first 20 min. This is, of course, expected since higher viscosity values would retard effective extraction of the drug by the aqueous dissolution medium.

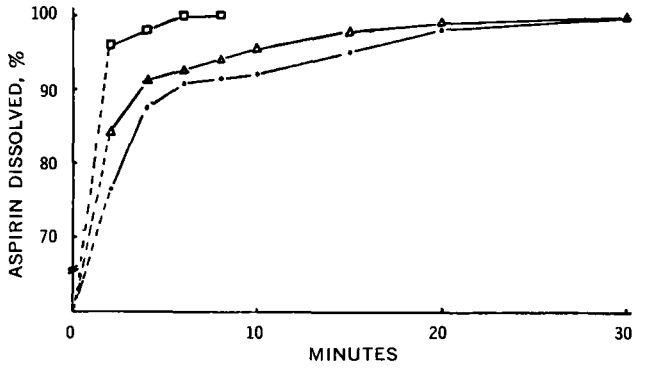

Figure 1—*Dissolution of aspirin in 0.1* N *HCl from aspirin suspensions in silicone fluids. Key:* △, *silicone fluid, 20 centistokes;* ●, *silicone fluid, 50 centistokes; and* □, *aspirin powder.*

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[x] To whom inquiries should be directed.

Metabolites of *Aspergillus cervinus* Massee (Moniliaceae)

H. N. ELSOHLY, D. J. SLATKIN, P. L. SCHIFF, Jr., and J. E. KNAPP [x]

Abstract □ An investigation of the metabolites produced by *Aspergillus cervinus* Massee (Moniliaceae), when grown in submerged culture on a malt extract medium, resulted in the isolation and identification of the quinol terremutin and 3,6-dihydroxy-2,5-toluquinone.

Keyphrases □ *Aspergillus cervinus* Massee (Moniliaceae)—isolation and identification of terremutin and 3,6-dihydroxy-2,5-toluquinone □ Terremutin—isolation and identification from *A. cervinus* □ 3,6-Dihydroxy-2,5-toluquinone—isolation and identification from *A. cervinus*

The genus *Aspergillus* (Moniliaceae) may be divided into 18 groups based on cultural and morphological considerations (1). In accordance with this division, *Aspergillus cervinus* Massee belongs to the *A. cervinus* group, a group consisting of four species and having an ill-defined place within the genus. Similarities between members of this group and members of other groups within the genus makes exact placement difficult. *A. cervinus* bears some similarities to *A. terreus* and was at one time assigned to its group (2). Since *A. cervinus* had not been studied phytochemically and since this knowledge could be of help to its taxonomy, the metabolites of this organism were studied.

DISCUSSION

For this study, *A. cervinus* was grown in a malt extract medium by submerged fermentation. At harvest the mycelium was removed by filtration, and the clear broth was acidified and extracted with ethyl acetate. Concentration of the ethyl acetate extract afforded terremutin (I) as a crystalline mass. Terremutin has previously been isolated only from cultures of a mutant strain of *A. terreus* (3).

Chromatography of the mother liquors from the crystallization of terremutin over silicic acid afforded 3,6-dihydroxy-2,5-toluquinone (II). This compound has been obtained as a degradation product of actinomycin C (4), has been isolated from cultures of *A. fumigatus* (5), and has been prepared synthetically (6).

Minute quantities of several other metabolites have been detected in cultures of *A. cervinus* by TLC, and work is underway to isolate and identify them.

The isolation of terremutin from *A. cervinus* tends to indicate a relationship to *A. terreus*, while the isolation of 3,6-dihydroxy-2,5-toluquinone could indicate a relationship to *A. fumigatus*. Thus, the taxonomical position of *A. cervinus* within the genus *Aspergillus* must remain as it is until further work establishes it firmly.

EXPERIMENTAL[1]

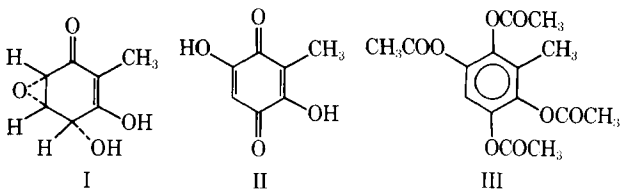
Organism—*A. cervinus*[2] (ATCC 16915) was maintained on slopes of Mycophil[3] agar.

Cultural Conditions—The organism was grown in a medium consisting of malt extract[4] (20 g), glucose[5] (20 g), Phytone[3] (1 g), and distilled water (1 liter). Five liters of this medium was distributed in 125-ml quantities into 40, 250-ml, wide-mouth erlenmeyer flasks; the flasks were stoppered with foam plugs[6] and autoclaved at 15 psi for 20 min. Each flask was inoculated with 1 ml of a spore suspension prepared by adding 10 ml of sterile 1:10,000 sodium lauryl sulfate solution to a heavily sporulated agar slope of *A. cervinus*. The flasks were incubated in the dark on a rotary shaker at 150 rpm for 14 days at 25–30°.

Isolation of Terremutin (I)—The contents of the flasks were pooled and filtered, and the filtrate was acidified to pH 2–3 with concentrated hydrochloric acid and extracted with 3 × 5-liter portions of ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulfate and concentrated *in vacuo* at 40° to about 25 ml. A crystalline mass (1.7 g) was deposited overnight. Recrystallization of this material first from methanol and then from ethyl acetate afforded large prisms of I (1.56 g), mp 146–147°; $[\alpha]_D^{25.5}$ −264.2° (c 1.00, methanol); λ_{max} (CH_3OH): 272 (log ε 4.03) and 315 (sh)(3.77) nm, with a bathochromic shift of the 272-nm peak to 310 nm in 0.01 *N* methanolic potassium hydroxide; ν_{max} (KBr): 3320, 3123, 2953, 1630, 1400, and 1015 cm^{-1}; NMR (at 60 MHz in D_2O): δ 1.68 (s, 3H, CCH_3), 3.65 (d, 1H, J = 4 Hz), 3.85 (d, 1H, J = 4 Hz, epoxide H), and 4.78 (s, 1H, OCH). The mass spectrum gave significant peaks at *m/e* 156 (1.5%) (M^+), 73 (30), 71 (43), and 56 (100).

The isolated material was identical by melting point, mixed melting point, IR, UV, NMR, and mass spectroscopic analyses to an authentic sample of terremutin.

Isolation of 3,6-Dihydroxy-2,5-toluquinone (II)—The ethyl acetate mother liquors from the initial crystallization of terremutin were evaporated to give a residue (6.40 g). This residue was chromatographed over a column of silicic acid[7] (220 g) and eluted with benzene (2-liter fractions). The third and fourth fractions were combined and evaporated to yield an orange residue (190 mg). Crystallization of this material from benzene–methanol afforded orange scales of II (126 mg), mp 160–165° [lit. (4) mp 157–163° and (6) mp 177°]; λ_{max} (CH_3OH): 287 (log ε 3.56) and 420 (2.38) nm [lit. (5) λ_{max} 282 and 420 nm]; ν_{max} (KBr): 3320, 2940, 2860, 1610, 1370, 1310, 1170, 1040, 855, 765, and 700 cm^{-1}; NMR (at 60 MHz in trifluoroacetic acid): δ 2.05 (s, 3H, CCH_3) and 6.29 (s, 1H, quinone H). The mass spectrum gave significant peaks at *m/e*



I II III

[1] Melting points were taken on a Thomas-Hoover Uni-Melt capillary apparatus and are corrected. IR spectra were determined on a Beckman model IR-8 spectrometer in KBr pellets. UV spectra were obtained on a Perkin-Elmer model 202 recording spectrometer. NMR spectra were obtained on a Perkin-Elmer model R-24 spectrometer in D_2O or trifluoroacetic acid solutions. Optical rotations were measured in a Rudolph polarimeter. Mass spectra were obtained with an LKB-9000 mass spectrometer. Fermentations were carried out on a New Brunswick scientific model G-10 gyrotory shaker.

[2] Obtained from the American Type Culture Collection.

[3] Baltimore Biological Laboratory, Baltimore, Md.

[4] Fisher Scientific Co., Pittsburgh, Pa.

[5] Dextrose, laboratory guide, Fisher Scientific Co.

[6] DiSP., Scientific Products, Edison, N.J.

[7] Mallinckrodt Chemical Works, St. Louis, Mo.

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[x] To whom inquiries should be directed.

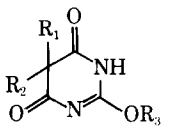
Synthesis of 5,5-Diethyl-2-ethoxytetrahydro-4,6-pyrimidinedione

R. BOUCHE

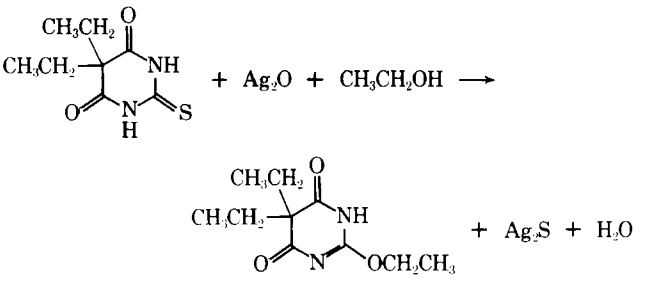
Abstract □ The original synthesis of 5,5-diethyl-2-ethoxytetrahydro-4,6-pyrimidinedione, a new pyrimidine derivative, is described. Evidence of its structure is given.

Keyphrases □ 5,5-Diethyl-2-ethoxytetrahydro-4,6-pyrimidinedione—synthesis, structure determination □ Pyrimidine derivatives—synthesis of 5,5-diethyl-2-ethoxytetrahydro-4,6-pyrimidinedione, structure determination

Alkoxy tetrahydro-4,6-pyrimidinediones (I), related to the family of barbituric acid derivatives, were prepared by a number of workers, starting from isourea alkyl ethers and condensing with substituted malonic esters or nitriles (1–3). They have also been obtained as by-products of the methylation of barbituric acid derivatives (4, 5). These synthesis methods reported require several steps. In the present paper, a new synthesis is reported for a one-step preparation in which silver oxide acts upon 5,5-diethyl-2-thiobarbituric acid in ethanol to give a new pyrimidine derivative, 5,5-diethyl-2-ethoxytetrahydro-4,6-pyrimidinedione



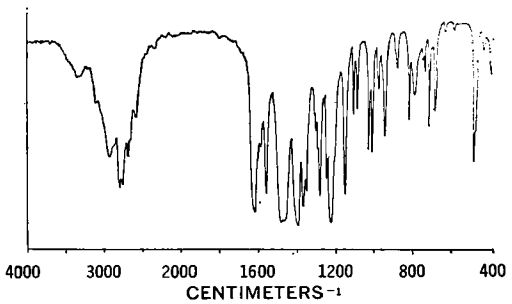
I



II

Scheme I

(II) (Scheme I). This reaction is similar to the general reaction of amides with silver oxide to form a salt (6) which is readily transformed into imidocarboxylic



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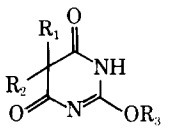
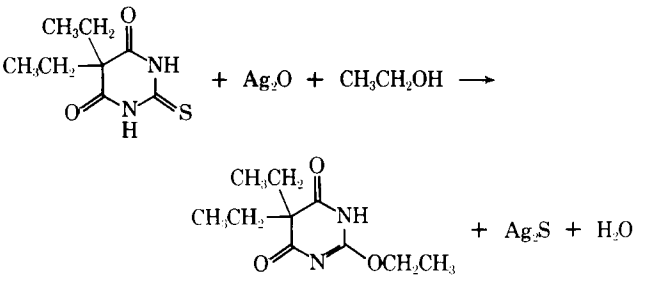


I



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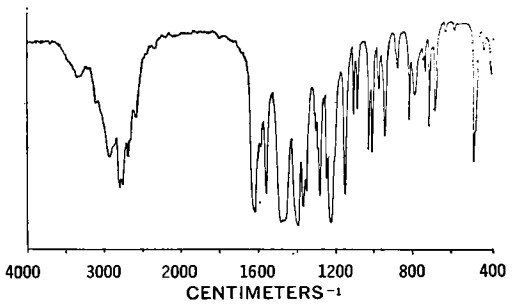

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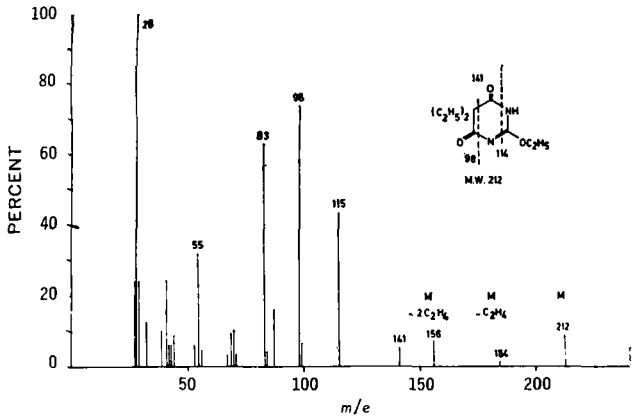

Figure 2—*Mass spectrum of II.*

acid derivatives by the action of a nucleophilic reagent.

A preliminary screening in mice using intraperitoneal administration suggested that the new compound produces skeletal muscle relaxation.

EXPERIMENTAL

To a solution of 1.84 g (0.01 mole) of 5,5-diethyl-2-thiobarbituric acid in 50 ml of absolute ethanol was added 2.6 g (0.03 mole) of silver oxide. The suspension was refluxed for 24 hr. After cooling and adding 2.5 g of talcum to adsorb excess silver oxide and silver sulfide, the mixture was filtered, the filtrate was evaporated to dryness, and the residue (1.9 g) was purified by TLC on silica gel with a solvent mixture of acetone–chloroform (1:9), R_f 0.37, corrected mp 77°.

The IR spectrum of II in chloroform showed two CO absorption bands at 1735 and 1700 cm^{-1} as well as a strong CN absorption at 1590 cm^{-1}. The spectrum taken in a KBr pellet is presented in Fig. 1. The NMR spectrum of II in deuterochloroform showed a badly resolved triplet of the NH proton at 1.02 ppm (τ); the quadruplets and the triplets of the ethoxy groups at 5.44 and 8.59 ppm, respectively; and the two ethyl groups at 7.98 and 9.16 ppm. In the mass spectrum (Fig. 2) were found the molecular ion peak at *m/e* 212 (calc. mol. wt. 212.26) and the usual pattern of fragmentation of the barbituric ring (7) with ion fragments resulting from the loss of a first ethylene molecule at *m/e* 156 and of an ethylisocyanate molecule at *m/e* 141.

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New Compounds: Unusual Chlorination of 2,3-Dimethyl-1,2,4-benzothiadiazine 1,1-Dioxide

GIANLUIGI SOLDATI

Abstract □ Chlorination of 2,3-dimethyl-1,2,4-benzothiadiazine 1,1-dioxide and its 6-chloro derivative with chlorine in dimethylformamide yielded a C-chlorinated end-product.

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The reported chlorination (1, 2) of 3-alkyl-2*H*-1,2,4-benzothiadiazine 1,1-dioxide (I) yields the corresponding 7-chloro derivative (II). Similarly, 3-oxo-3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide (III) yields a 7-chloro derivative (III*a*) or a 5,7-dichloro derivative (III*b*), depending on the reaction conditions.

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Since such an intermediate is not possible in compounds of type IV, the present investigation suggests that the chlorination of these compounds in dimethylformamide undergoes a similar course of reaction as that of toluene or methylpyridine (5) when chlorinated with molecular chlorine.

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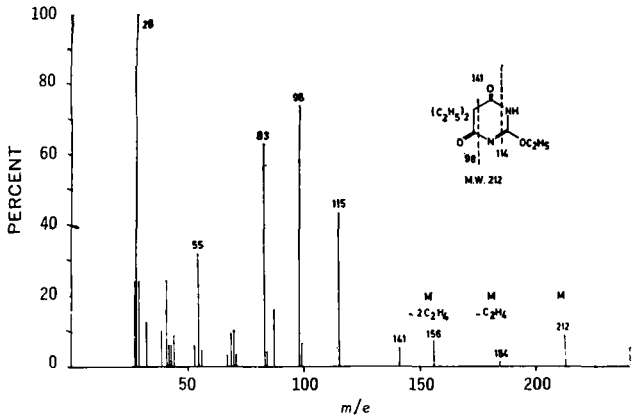

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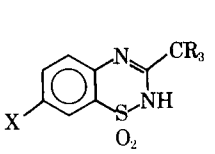

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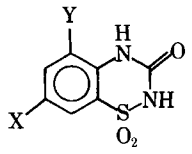
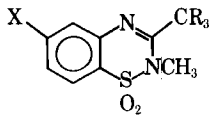
II: R = alkyl, X = Cl



III: X = H, Y = H
IIIa: X = Cl, Y = H
IIIb: X = Cl, Y = Cl



IV: R = H, X = H
IVa: R = Cl, X = H
V: R = H, X = Cl
Va: R = Cl, X = Cl

was removed and the dark-yellow solution was allowed to stand for 0.5 hr. The reaction mixture was then poured into crushed ice and the solid material was collected and washed several times with water. Recrystallization from methanol gave crystals, mp 135–137°. NMR analysis in acetone-d_6 gave a proton count of seven, showing the four protons for the benzene ring at 8 ppm and a singlet (three protons) at 3.75 ppm for the methyl group in the 2-position.

Anal.—Calc. for $C_9H_7Cl_3N_2O_2S$: C, 34.47; H, 2.25; N, 8.93. Found: C, 34.86; H, 2.21; N, 8.95.

6-Chloro-2-methyl-3-trichloromethyl-1,2,4-benzothiadiazine 1,1-Dioxide (Va)—Compound V (16 g) was chlorinated in 50 ml of dimethylformamide at 55–80° with 16 g of chlorine. Recrystallization of the crude substance from benzene–hexane gave Va, mp 145–146°. NMR analysis in acetone-d_6 showed a singlet at 3.75 ppm for the methyl group and three protons for the ring at 7.75–8.20 ppm.

Anal.—Calc. for $C_9H_6Cl_4N_2O_2S$: C, 31.06; H, 1.74; N, 8.05. Found: C, 31.23; H, 1.92; N, 7.81.

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COMMUNICATIONS

Hydroxymethylfuraldimines: Possible Intermediates in Maillard Reaction

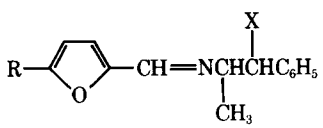
Keyphrases □ Hydroxymethylfuraldimines—possible intermediates in Maillard reaction, relationship to browning of dextroamphetamine sulfate □ Dextroamphetamine sulfate—hydroxymethylfuraldimines as possible intermediates in Maillard reaction □ Maillard reaction—hydroxymethylfuraldimines as possible intermediates, relationship to browning of dextroamphetamine sulfate

To the Editor:

Recently, Blaug and Huang (1) reported that dextroamphetamine sulfate yields a brown precipitate when heated with dextrates in solution. This brown precipitate was postulated as the imine (Ia) resulting from condensation of amphetamine with 5-hydroxymethylfurfural on the basis of elemental analysis, a strong band at 1650 cm^{-1} in the IR spectrum, and TLC.

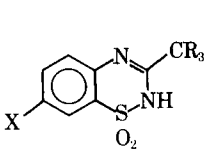
During investigations conducted in this laboratory, it became of interest to prepare Ia and a few other 2-furanecarboxaldimines (Ib–Id) for spectral and reactivity comparisons. These compounds were readily prepared by warming benzene (Ib–Id) or ethanol (Ia) solutions of the amine and the furfural and were unambiguously characterized by their IR, UV (Table I), and NMR spectra[1] (Figs. 1a–1d). Compounds Ib–Id were readily purified by either vacuum distillation (Ic) or crystallization (Ib and Id).

Since extensive decomposition of Ia occurred on attempted distillation, the method of choice for its preparation involved employing a slight excess of



	R	X
Ia:	CH_2OH	H
Ib:	CH_2OH	OH
Ic:	H	H
Id:	H	OH

[1] Determined on a Varian T-60 or Varian XL-100.



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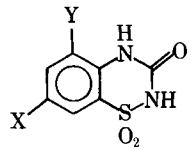

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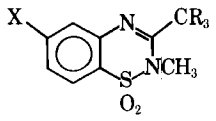

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V: R = H, X = Cl
Va: R = Cl, X = Cl

was removed and the dark-yellow solution was allowed to stand for 0.5 hr. The reaction mixture was then poured into crushed ice and the solid material was collected and washed several times with water. Recrystallization from methanol gave crystals, mp 135–137°. NMR analysis in acetone-d_6 gave a proton count of seven, showing the four protons for the benzene ring at 8 ppm and a singlet (three protons) at 3.75 ppm for the methyl group in the 2-position.

Anal.—Calc. for $C_9H_7Cl_3N_2O_2S$: C, 34.47; H, 2.25; N, 8.93. Found: C, 34.86; H, 2.21; N, 8.95.

6-Chloro-2-methyl-3-trichloromethyl-1,2,4-benzothiadiazine 1,1-Dioxide (Va)—Compound V (16 g) was chlorinated in 50 ml of dimethylformamide at 55–80° with 16 g of chlorine. Recrystallization of the crude substance from benzene–hexane gave Va, mp 145–146°. NMR analysis in acetone-d_6 showed a singlet at 3.75 ppm for the methyl group and three protons for the ring at 7.75–8.20 ppm.

Anal.—Calc. for $C_9H_6Cl_4N_2O_2S$: C, 31.06; H, 1.74; N, 8.05. Found: C, 31.23; H, 1.92; N, 7.81.

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COMMUNICATIONS

Hydroxymethylfuraldimines: Possible Intermediates in Maillard Reaction

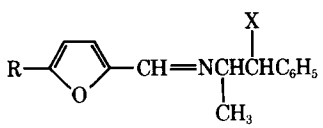
Keyphrases □ Hydroxymethylfuraldimines—possible intermediates in Maillard reaction, relationship to browning of dextroamphetamine sulfate □ Dextroamphetamine sulfate—hydroxymethylfuraldimines as possible intermediates in Maillard reaction □ Maillard reaction—hydroxymethylfuraldimines as possible intermediates, relationship to browning of dextroamphetamine sulfate

To the Editor:

Recently, Blaug and Huang (1) reported that dextroamphetamine sulfate yields a brown precipitate when heated with dextrates in solution. This brown precipitate was postulated as the imine (Ia) resulting from condensation of amphetamine with 5-hydroxymethylfurfural on the basis of elemental analysis, a strong band at 1650 cm^{-1} in the IR spectrum, and TLC.

During investigations conducted in this laboratory, it became of interest to prepare Ia and a few other 2-furanecarboxaldimines (Ib–Id) for spectral and reactivity comparisons. These compounds were readily prepared by warming benzene (Ib–Id) or ethanol (Ia) solutions of the amine and the furfural and were unambiguously characterized by their IR, UV (Table I), and NMR spectra[1] (Figs. 1a–1d). Compounds Ib–Id were readily purified by either vacuum distillation (Ic) or crystallization (Ib and Id).

Since extensive decomposition of Ia occurred on attempted distillation, the method of choice for its preparation involved employing a slight excess of



	R	X
Ia:	CH_2OH	H
Ib:	CH_2OH	OH
Ic:	H	H
Id:	H	OH

[1] Determined on a Varian T-60 or Varian XL-100.

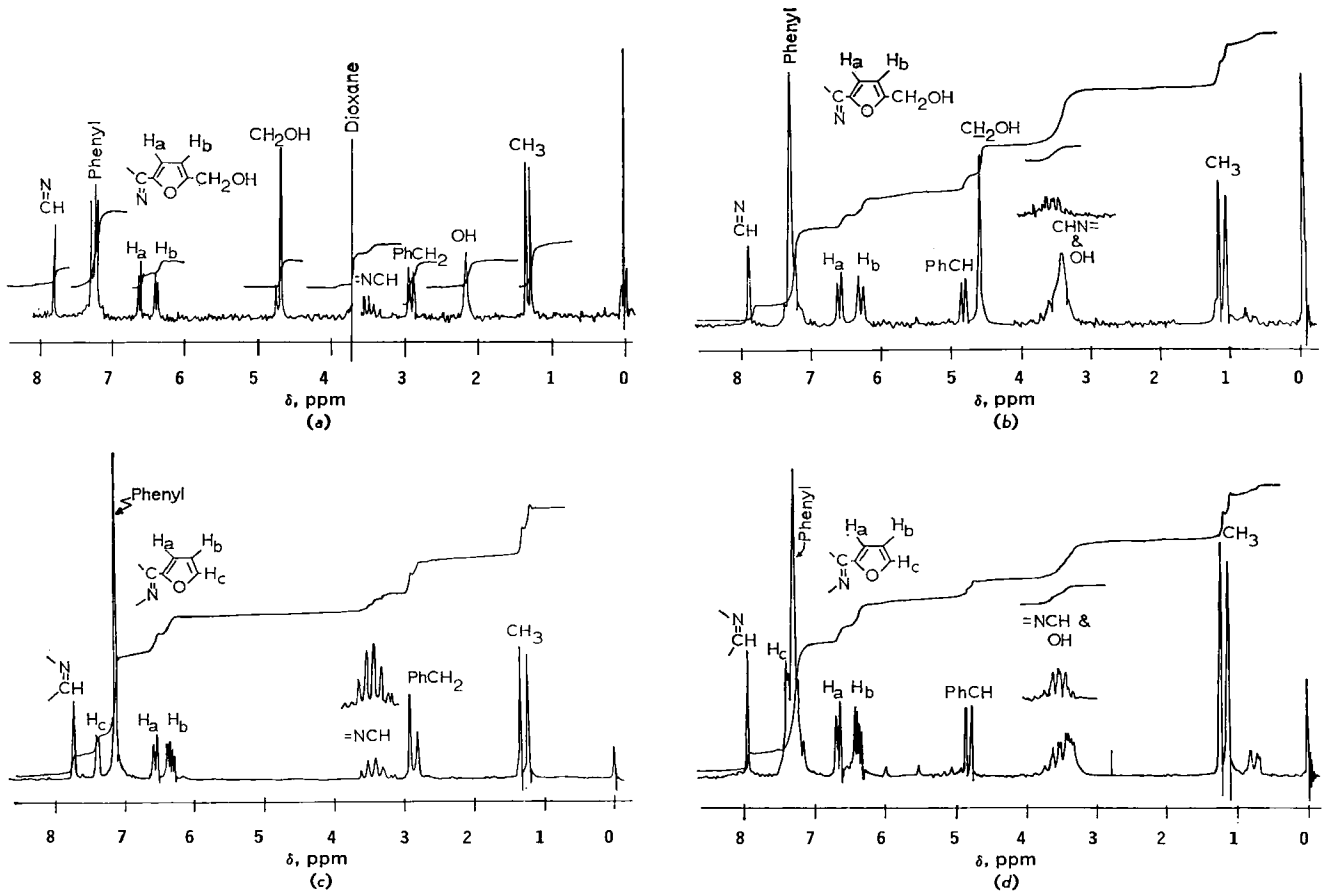


Figure 1—*Proton magnetic resonance spectra of furaldimines. Key:* a, *Ia at 100 MHz;* b, *Ib at 60 MHz (insert: D_2O added);* c, *Ic at 60 MHz; and* d, *Id at 60 MHz (insert: D_2O added).*

dextroamphetamine. Separation of I*a* from excess amphetamine was then obtained by dry column chromatography on silica gel employing dioxane as the eluant. Satisfactory elemental analyses were obtained for I*b*–I*d*; however, the elemental analysis and NMR spectroscopy indicated significant traces of dioxane in I*a*. A GC–mass spectrum of I*a* is shown in Fig. 2.

Several discrepancies were observed in comparing I*a* and the brown precipitate, which was prepared at pH 8 by heating an aqueous solution of 1% dextroamphetamine sulfate and 10% dextrose for 5 days at 60°. The brown precipitate was solid, while I*a* was a pale-yellow oil; the IR and UV spectra (Fig. 3) were different; and TLC confirmed the nonidentity of these substances (Fig. 4).

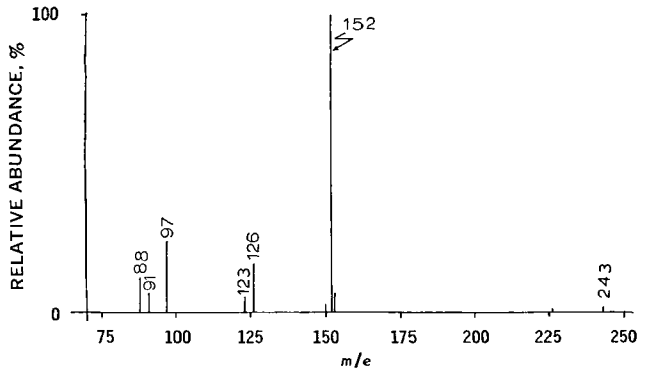


Figure 2—*GC–mass spectrum of* I*a*.

The UV spectrum of I*a* closely approximated that of 5-hydroxymethylfurfural (Table I). Indeed, the UV spectra of all Schiff bases prepared closely resembled those of the corresponding furfural, a result which is expected with Schiff bases unless the C=N is involved in transmitting conjugation between the portions of the molecule derived from the carbonyl and amine.

Blaug and Huang (1) also reported a peak of maximum absorbance at 298 nm, which apparently behaves in a manner similar to the 320-nm band reported by Duvall *et al.* (2). An aqueous solution of I*a* showed maximum absorbance at 282 nm, which may be due either to intact I*a* or to the hydroxymethylfurfural resulting from hydrolysis of I*a*. Consequently, it appears that the 298-nm band is not due to I*a*. One

Table I—Significant Spectrometric Features of Furfural Derivatives

Compound	λ_{max}[a] ($\epsilon \times 10^{-3}$)	υC=N
5-Hydroxymethylfurfural	280 (15.5)	—
I*a*	277 (18.7)	1640[b]
I*b*	277 (18.3)	1640[c]
2-Furaldehyde	270 (14.6)	—
I*c*	268 (18.8)	1645[b]
I*d*	269 (17.7)	1640[c]

[a] UV spectra were recorded in ethanol with about 5.0×10^{-5} *M* solutions. Wavelengths are reported in nanometers. [b] Determined on a neat film. [c] Determined on a mineral oil mull.

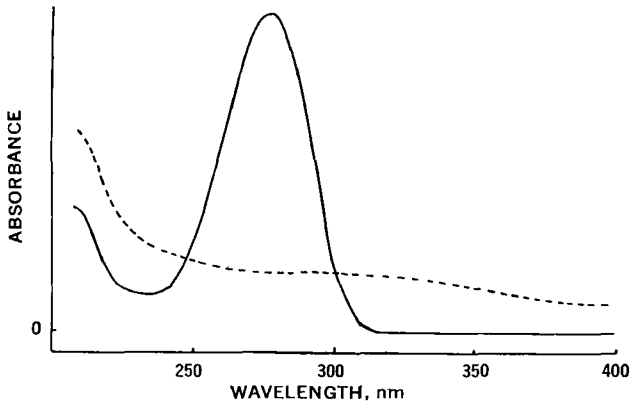


Figure 3—*Absorption spectrum of Ia* (—) *and brown precipitate obtained by heating 1% dextroamphetamine sulfate and 10% dextrose for 5 days at 60°* (- - - -). *Both spectra were determined in ethanol solution.*

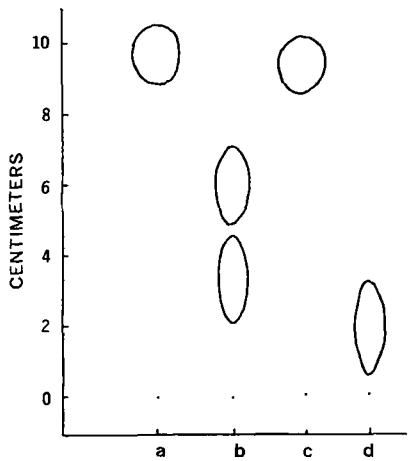


Figure 4—*Thin-layer chromatogram of dextroamphetamine sulfate–dextrose and Schiff-base solutions, developed in the lower phase of a mixture of ethyl acetate–pyridine–water (2:1:2) (see Ref. 1). Key: a, fresh 5-hydroxymethylfurfural; b, ethanol solution of precipitate from 1% dextroamphetamine sulfate and 10% dextrose solution heated at 60° for 5 days; c, Ia; and d, amphetamine base.*

would also expect Ia to absorb at longer wavelength than an amphetamine–sugar Schiff base (3). Consequently, it appears that neither the 298-nm band (1) nor the 320-nm band (2) is due to either Ia or the amphetamine–sugar Schiff base, although either of these may be *intermediates* involved in the browning reaction.

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Robert H. Higgins

Dorsey Laboratories
Division of Sandoz, Inc.
Lincoln, NE 68501

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Effect of Vehicles on Reduction of Brain Norepinephrine by α-Methyltyrosine

Keyphrases □ Methylcellulose—vehicle effect on α-methyl-*p*-tyrosine reduction of brain norepinephrine, compared to polysorbate 80 □ α-Methyl-*p*-tyrosine reduction of brain norepinephrine—vehicle effect, methylcellulose, polysorbate 80 □ Norepinephrine reduction by α-methyl-*p*-tyrosine—vehicle effect, methylcellulose, polysorbate 80

To the Editor:

Investigators seeking to elucidate the functional significance of brain norepinephrine have been aided by the development of the specific enzyme inhibitor, α-methyl-*p*-tyrosine (1). Spector *et al.* (2) demonstrated that α-methyl-*p*-tyrosine selectively inhibits tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of the catecholamines, and thereby depletes these amines from the brain. α-Methyl-*p*-tyrosine has been employed to estimate the turnover rate and the turnover time of the catecholamines (3) in various body tissues including brain.

Bernard and Paolino (4), employing 80 mg/kg α-methyl-*p*-tyrosine suspended in 5% polysorbate 80, were able to lower the brain norepinephrine levels of adult male mice (C57BL/6J) by 52%, 4 hr following intraperitoneal injection. Similar results were obtained in wild rats (5) when using 250 mg/kg α-methyl-*p*-tyrosine. These depleted levels are similar to those obtained by other investigators employing other vehicles or acidification of aqueous solutions to increase the solubility of this enzyme inhibitor (6–9). The purpose of our experiment was to determine if a suspending agent such as 1% methylcellulose 400

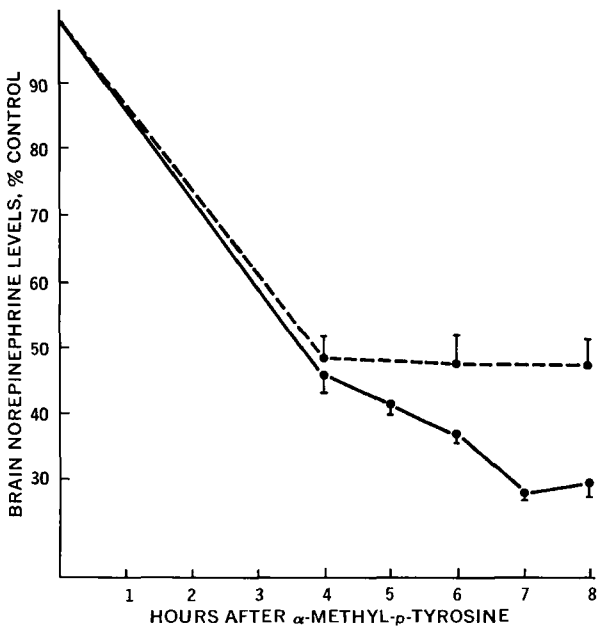
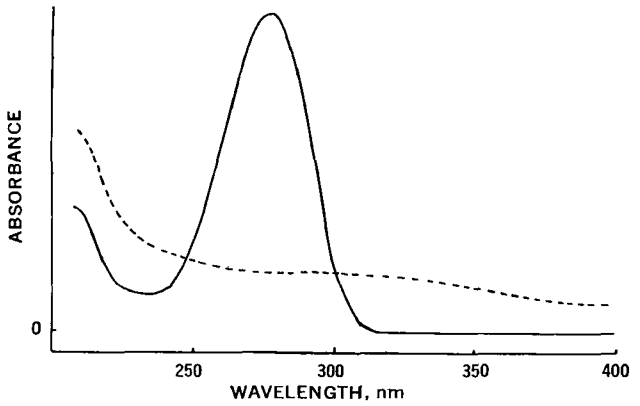


Figure 1—*Adult male mice were injected with α-methyl-p-tyrosine (80 mg/kg ip) in either 5% polysorbate 80* (- - - -) *or 1% methylcellulose 400* (—). *At various time intervals following injection, the animals were decapitated and their brains were removed and assayed for norepinephrine. Each data point represents the mean ± standard error* ($3 \leq n \leq 8$).



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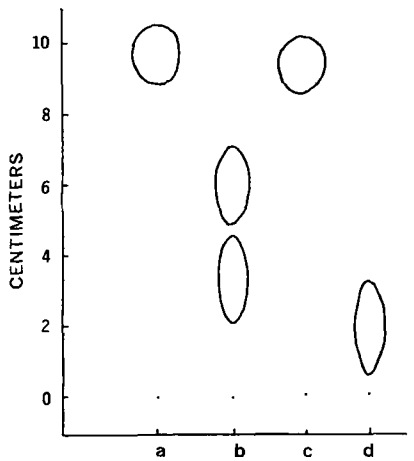

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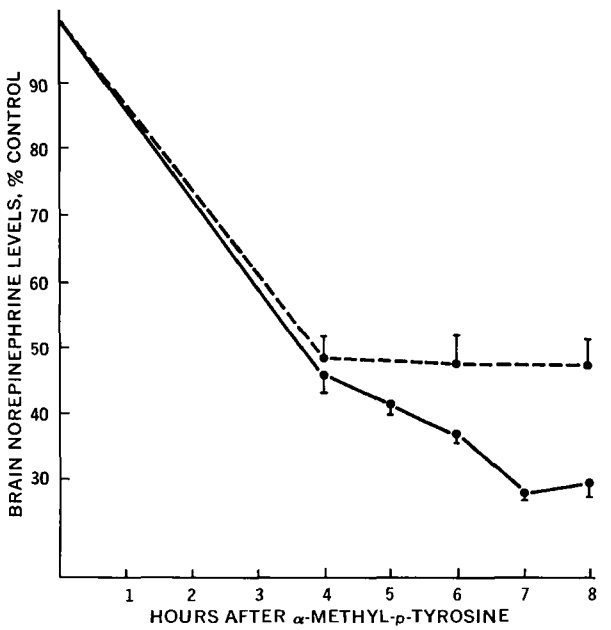

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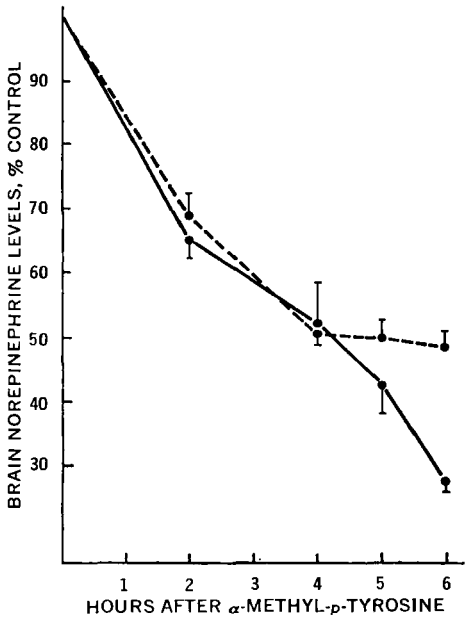

Figure 2—*Adult male rats were injected with α-methyl-p-tyrosine (250 mg/kg ip) in either 5% polysorbate 80 (- - - -) or 1% methylcellulose (—). At various time intervals following injection, the animals were decapitated and their brains were removed and assayed for norepinephrine. Each data point represents the mean ± standard error (3 ≤ n ≤ 8).*

would increase the effectiveness of α-methyl-*p*-tyrosine in reducing brain norepinephrine levels.

The time course for the decline of brain norepinephrine following an injection of α-methyl-*p*-tyrosine was determined in both male albino ICR mice and Wistar rats. Mice were injected with 80 mg/kg ip and rats with 250 mg/kg ip in either 5% polysorbate 80 or 1% methylcellulose 400. Animals were decapitated at various time intervals following injections, their brains were removed, and the norepinephrine content was analyzed by spectrophotofluorometry (4). We observed in rats and mice that 1% methylcellulose 400 significantly reduced brain norepinephrine levels after the 4-hr asymptote seen with polysorbate 80 (Figs. 1 and 2).

One possible interpretation of these data is that the actual amount of α-methyl-*p*-tyrosine being injected into the intraperitoneal cavity was less with polysorbate 80 than with methylcellulose because of the poorer suspending characteristics of polysorbate 80. To check this hypothesis, suspensions of 1% methylcellulose and 5% polysorbate 80 containing either 4.8 mg/ml (80 mg/kg for mice) or 125 mg/ml (250 mg/kg for rats) of α-methyl-*p*-tyrosine were prepared. By using the typical injection procedure, aliquots were withdrawn and injected into previously weighed vials. The liquid was then evaporated, the appropriate vehicle controls were subtracted, and the final α-methyl-*p*-tyrosine weight was compared with the expected injected weight. The results indicated that at the lower concentration (4.8 mg/ml), both 1% methylcellulose and 5% polysorbate 80 delivered approximately the expected amount (2.4 mg). At the higher concentration (125 mg/ml), however, polysorbate 80 delivered significantly less α-methyl-*p*-tyrosine than did methylcellulose ($p < 0.001$; 71% of the expected dose of 125 mg)[1]. This discrepancy in injected α-methyl-*p*-tyrosine at the higher dose does not explain the results obtained in rats (Fig. 2) since polysorbate 80 still delivered 90% of the dose which completely inhibits tyrosine hydroxylase (3), and it fails to explain similar results obtained in mice (Fig. 1). However, the effectiveness of polysorbate 80 apparently decreases when employed as a vehicle for increasing amounts of α-methyl-*p*-tyrosine.

An alternative explanation for the differences observed between polysorbate 80 and methylcellulose in both the high and low doses is that even though the quantity delivered may be similar (as in mice), the amounts of α-methyl-*p*-tyrosine absorbed from the intraperitoneal cavity are less with polysorbate 80. It is well known that many physical properties of various surfactant solutions change at the critical micelle concentration (CMC). In fact, several investigators employing polysorbate 80 (10, 11) or polysorbate 20 (10–14) reported enhanced absorption of drugs across biological membranes when the CMC was not exceeded. Beyond this level, decreases in absorption could be explained as follows: *(a)* a micellar solution consists of two phases; *(b)* the partition ratio of drug between the micellar phase and the aqueous phase is constant, independent of drug concentration; and *(c)* absorption of the drug incorporated in the micelle is negligible. When the CMC was exceeded in the studies, there was a significant increase in the drug–micelle complexation, thereby decreasing the effective concentration of the drug available for absorption. This resulted in a decreased drug absorption rate (15).

Five percent polysorbate 80 exceeds the CMC. Therefore, if α-methyl-*p*-tyrosine formed micelles with polysorbate 80, a further increase in the concentration of the surfactant would probably not enhance absorption. This possible decrease in absorption of α-methyl-*p*-tyrosine would explain the results of Figs. 1 and 2, since the inhibition of tyrosine hydroxylase is known to be dose dependent (1). Although further experimentation will be required to confirm this α-methyl-*p*-tyrosine–micellar complexation theory, such a hypothesis does explain the pharmacological effects reported herein.

This paper presents evidence that 1% methylcellulose 400, when used as a vehicle for delivery of α-methyl-*p*-tyrosine, will result in a greater lowering of mouse and rat brain norepinephrine levels than either polysorbate 80 or some other presently used agents. This is accomplished without the necessity of acidifying the medium (and thus stressing the animal) to increase solubility. Since stress alters brain amine metabolism (16) and, therefore, might be expected to modify normal steady-state brain norepinephrine dynamics, this is an important factor for consideration.

[1] The higher concentration of 125 mg/ml in polysorbate 80 consistently delivered approximately 71% of the expected dose until the number of injections exceeded 75% of the vial volume. Thereafter, the injection concentration increased until it equaled and then exceeded the expected amount.

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B. K. Bernard ˣ
E. R. Finkelstein

Section of Pharmacology and Toxicology
School of Pharmacy
University of Connecticut
Storrs, CT 06268

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ˣ To whom inquiries should be directed.

Influence of Solute Properties on Release of *p*-Aminobenzoic Acid Esters from Silicone Rubber: Theoretical Considerations

Keyphrases □ Silicone matrix—chain-length effect on *in vitro* release of *p*-aminobenzoates, theory, equations □ *p*-Aminobenzoic acid esters—*in vitro* release from silicone matrix, effect of solute properties, chain length, theory, equations □ Chain-length effect—*in vitro* release of *p*-aminobenzoates from silicone matrix, theory, equations

To the Editor:

The use of silicone rubber as a carrier for therapeutic agents is well documented (1–5). The release of drug from such an inert matrix is dependent upon certain solute properties, *i.e.*, solubility and diffusivity, along with other parameters independent of the particular drug in question. Examples of the latter are the geometry of the matrix, the diffusion layer thickness, the amount of drug per unit volume in the matrix, the particle size, and the filler content.

A mathematical model describing drug release from homogeneous and heterogeneous systems was originally presented by Higuchi (6). Within the assumptions of the model, equations were derived that predict a linear dependence of the amount released, Q, upon the square root of time, $t^{1/2}$. Subsequently, the model was extended and equations were derived that consider the solvent boundary diffusion layer as an additional diffusional pathway (7). According to this extended model, plots of Q *versus* $t^{1/2}$ may not be linear during the early stages of the release process. This has been experimentally verified (8) with a series of progestins. Nonlinear behavior was also observed for chlormadinone acetate (9) and for ethynodiol diacetate (10). However, a systematic evaluation of the influence of the properties of a homologous series on the release rate has not been reported.

This communication utilizes these derived mathematical relationships to describe the effect of chain length of esters of *p*-aminobenzoic acid on the *in vitro* release of drug from a silicone matrix. The assumptions and conditions of the model are: *(a)* pseudo-steady state exists; *(b)* diffusion is rate controlling rather than dissolution of drug particles; *(c)* total concentration of drug within the matrix, A, is substantially greater than its solubility, C_s, in the matrix phase, *i.e.*, $A \gg C_s$; *(d)* transport of the drug species occurs through the matrix phase; and *(e)* ideal sink conditions exist in the dissolution media. The amount released per unit area, Q, from a planar surface as a function of time, t, is given by the following expression (7):

$$Q = \frac{-D_s h_a K A \epsilon}{D_a \tau} + \left[\left(\frac{D_s h_a K A \epsilon}{D_a \tau}\right)^2 + \frac{2AD_s C_s \epsilon t}{\tau}\right]^{1/2} \quad \text{(Eq. 1)}$$

where:

A = total concentration of drug in matrix (milligrams per square centimeter)
D_a = diffusion coefficient in aqueous phase (square centimeter per minute)
D_s = diffusion coefficient in matrix phase (square centimeter per minute)
K = partition coefficient (C_s/C_a)
C_a = solubility (milligrams per milliliter) in dissolution media
C_s = solubility in matrix phase (milligrams per milliliter)
h_a = boundary diffusion layer (centimeters)
ϵ = volume fraction
τ = tortuosity

Differentiating Q with respect to time yields the rate equation:

$$\frac{dQ}{dt} = \text{rate} = \frac{\alpha C_s}{2(\beta^2 K^2 + \alpha C_s t)^{1/2}} \quad \text{(Eq. 2a)}$$

where:

$$\alpha = \frac{2AD_s\epsilon}{\tau} \quad \text{(Eq. 2b)}$$

$$\beta = \frac{D_s h_a A \epsilon}{D_a \tau} \quad \text{(Eq. 2c)}$$

For a homologous series of moderate chain length, D_s and D_a can be considered to be relatively constant (11); thus, α and β are constant for a given set of experimental conditions. For this situation, the release rate (Eq. 2*a*) is dependent only upon C_s and C_a (or, alternatively, C_s and K). As the carbon chain length

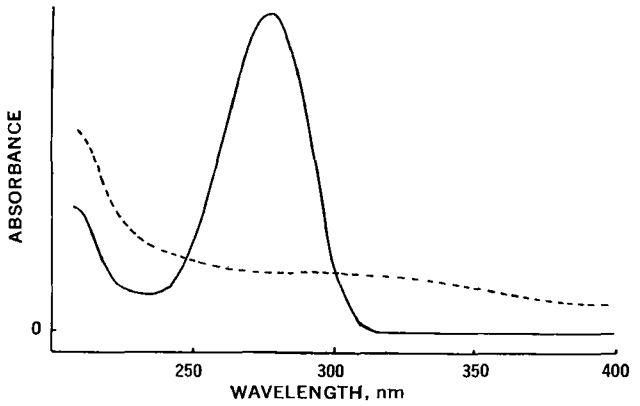

Figure 3—*Absorption spectrum of Ia* (—) *and brown precipitate obtained by heating 1% dextroamphetamine sulfate and 10% dextrose for 5 days at 60°* (- - - -). *Both spectra were determined in ethanol solution.*

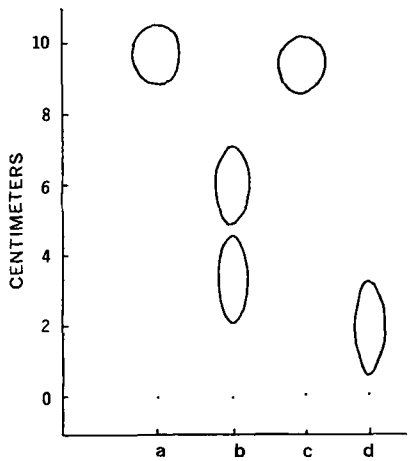

Figure 4—*Thin-layer chromatogram of dextroamphetamine sulfate–dextrose and Schiff-base solutions, developed in the lower phase of a mixture of ethyl acetate–pyridine–water (2:1:2) (see Ref. 1). Key: a, fresh 5-hydroxymethylfurfural; b, ethanol solution of precipitate from 1% dextroamphetamine sulfate and 10% dextrose solution heated at 60° for 5 days; c, Ia; and d, amphetamine base.*

would also expect Ia to absorb at longer wavelength than an amphetamine–sugar Schiff base (3). Consequently, it appears that neither the 298-nm band (1) nor the 320-nm band (2) is due to either Ia or the amphetamine–sugar Schiff base, although either of these may be *intermediates* involved in the browning reaction.

(1) S. M. Blaug and W. T. Huang, *J. Pharm. Sci.*, **62**, 652(1973).

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Robert H. Higgins

Dorsey Laboratories
Division of Sandoz, Inc.
Lincoln, NE 68501

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Effect of Vehicles on Reduction of Brain Norepinephrine by α-Methyltyrosine

Keyphrases □ Methylcellulose—vehicle effect on α-methyl-*p*-tyrosine reduction of brain norepinephrine, compared to polysorbate 80 □ α-Methyl-*p*-tyrosine reduction of brain norepinephrine—vehicle effect, methylcellulose, polysorbate 80 □ Norepinephrine reduction by α-methyl-*p*-tyrosine—vehicle effect, methylcellulose, polysorbate 80

To the Editor:

Investigators seeking to elucidate the functional significance of brain norepinephrine have been aided by the development of the specific enzyme inhibitor, α-methyl-*p*-tyrosine (1). Spector *et al.* (2) demonstrated that α-methyl-*p*-tyrosine selectively inhibits tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of the catecholamines, and thereby depletes these amines from the brain. α-Methyl-*p*-tyrosine has been employed to estimate the turnover rate and the turnover time of the catecholamines (3) in various body tissues including brain.

Bernard and Paolino (4), employing 80 mg/kg α-methyl-*p*-tyrosine suspended in 5% polysorbate 80, were able to lower the brain norepinephrine levels of adult male mice (C57BL/6J) by 52%, 4 hr following intraperitoneal injection. Similar results were obtained in wild rats (5) when using 250 mg/kg α-methyl-*p*-tyrosine. These depleted levels are similar to those obtained by other investigators employing other vehicles or acidification of aqueous solutions to increase the solubility of this enzyme inhibitor (6–9). The purpose of our experiment was to determine if a suspending agent such as 1% methylcellulose 400

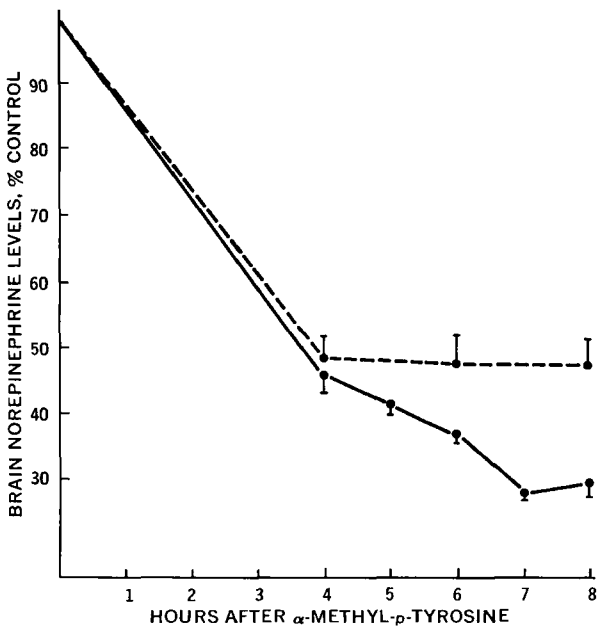

Figure 1—*Adult male mice were injected with α-methyl-p-tyrosine (80 mg/kg ip) in either 5% polysorbate 80* (- - - -) *or 1% methylcellulose 400* (—). *At various time intervals following injection, the animals were decapitated and their brains were removed and assayed for norepinephrine. Each data point represents the mean ± standard error* ($3 \leq n \leq 8$).

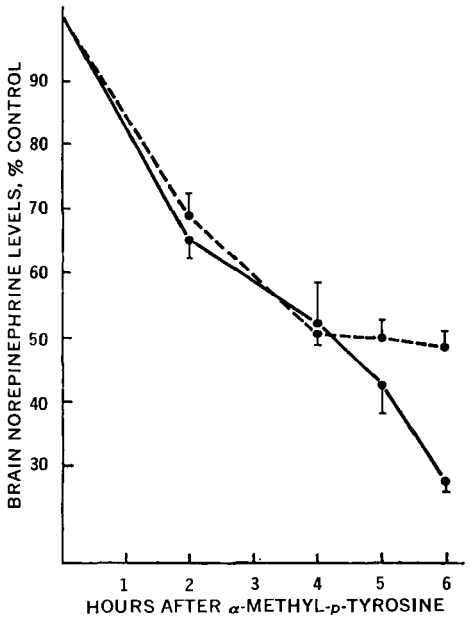

Figure 2—*Adult male rats were injected with α-methyl-p-tyrosine (250 mg/kg ip) in either 5% polysorbate 80 (- - - -) or 1% methylcellulose (—). At various time intervals following injection, the animals were decapitated and their brains were removed and assayed for norepinephrine. Each data point represents the mean ± standard error (3 ≤ n ≤ 8).*

would increase the effectiveness of α-methyl-*p*-tyrosine in reducing brain norepinephrine levels.

The time course for the decline of brain norepinephrine following an injection of α-methyl-*p*-tyrosine was determined in both male albino ICR mice and Wistar rats. Mice were injected with 80 mg/kg ip and rats with 250 mg/kg ip in either 5% polysorbate 80 or 1% methylcellulose 400. Animals were decapitated at various time intervals following injections, their brains were removed, and the norepinephrine content was analyzed by spectrophotofluorometry (4). We observed in rats and mice that 1% methylcellulose 400 significantly reduced brain norepinephrine levels after the 4-hr asymptote seen with polysorbate 80 (Figs. 1 and 2).

One possible interpretation of these data is that the actual amount of α-methyl-*p*-tyrosine being injected into the intraperitoneal cavity was less with polysorbate 80 than with methylcellulose because of the poorer suspending characteristics of polysorbate 80. To check this hypothesis, suspensions of 1% methylcellulose and 5% polysorbate 80 containing either 4.8 mg/ml (80 mg/kg for mice) or 125 mg/ml (250 mg/kg for rats) of α-methyl-*p*-tyrosine were prepared. By using the typical injection procedure, aliquots were withdrawn and injected into previously weighed vials. The liquid was then evaporated, the appropriate vehicle controls were subtracted, and the final α-methyl-*p*-tyrosine weight was compared with the expected injected weight. The results indicated that at the lower concentration (4.8 mg/ml), both 1% methylcellulose and 5% polysorbate 80 delivered approximately the expected amount (2.4 mg). At the higher concentration (125 mg/ml), however, polysorbate 80 delivered significantly less α-methyl-*p*-tyrosine than did methylcellulose ($p < 0.001$; 71% of the expected dose of 125 mg)[1]. This discrepancy in injected α-methyl-*p*-tyrosine at the higher dose does not explain the results obtained in rats (Fig. 2) since polysorbate 80 still delivered 90% of the dose which completely inhibits tyrosine hydroxylase (3), and it fails to explain similar results obtained in mice (Fig. 1). However, the effectiveness of polysorbate 80 apparently decreases when employed as a vehicle for increasing amounts of α-methyl-*p*-tyrosine.

An alternative explanation for the differences observed between polysorbate 80 and methylcellulose in both the high and low doses is that even though the quantity delivered may be similar (as in mice), the amounts of α-methyl-*p*-tyrosine absorbed from the intraperitoneal cavity are less with polysorbate 80. It is well known that many physical properties of various surfactant solutions change at the critical micelle concentration (CMC). In fact, several investigators employing polysorbate 80 (10, 11) or polysorbate 20 (10–14) reported enhanced absorption of drugs across biological membranes when the CMC was not exceeded. Beyond this level, decreases in absorption could be explained as follows: *(a)* a micellar solution consists of two phases; *(b)* the partition ratio of drug between the micellar phase and the aqueous phase is constant, independent of drug concentration; and *(c)* absorption of the drug incorporated in the micelle is negligible. When the CMC was exceeded in the studies, there was a significant increase in the drug–micelle complexation, thereby decreasing the effective concentration of the drug available for absorption. This resulted in a decreased drug absorption rate (15).

Five percent polysorbate 80 exceeds the CMC. Therefore, if α-methyl-*p*-tyrosine formed micelles with polysorbate 80, a further increase in the concentration of the surfactant would probably not enhance absorption. This possible decrease in absorption of α-methyl-*p*-tyrosine would explain the results of Figs. 1 and 2, since the inhibition of tyrosine hydroxylase is known to be dose dependent (1). Although further experimentation will be required to confirm this α-methyl-*p*-tyrosine–micellar complexation theory, such a hypothesis does explain the pharmacological effects reported herein.

This paper presents evidence that 1% methylcellulose 400, when used as a vehicle for delivery of α-methyl-*p*-tyrosine, will result in a greater lowering of mouse and rat brain norepinephrine levels than either polysorbate 80 or some other presently used agents. This is accomplished without the necessity of acidifying the medium (and thus stressing the animal) to increase solubility. Since stress alters brain amine metabolism (16) and, therefore, might be expected to modify normal steady-state brain norepinephrine dynamics, this is an important factor for consideration.

[1] The higher concentration of 125 mg/ml in polysorbate 80 consistently delivered approximately 71% of the expected dose until the number of injections exceeded 75% of the vial volume. Thereafter, the injection concentration increased until it equaled and then exceeded the expected amount.

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B. K. Bernard ˣ
E. R. Finkelstein

Section of Pharmacology and Toxicology
School of Pharmacy
University of Connecticut
Storrs, CT 06268

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Influence of Solute Properties on Release of *p*-Aminobenzoic Acid Esters from Silicone Rubber: Theoretical Considerations

Keyphrases □ Silicone matrix—chain-length effect on *in vitro* release of *p*-aminobenzoates, theory, equations □ *p*-Aminobenzoic acid esters—*in vitro* release from silicone matrix, effect of solute properties, chain length, theory, equations □ Chain-length effect—*in vitro* release of *p*-aminobenzoates from silicone matrix, theory, equations

To the Editor:

The use of silicone rubber as a carrier for therapeutic agents is well documented (1–5). The release of drug from such an inert matrix is dependent upon certain solute properties, *i.e.*, solubility and diffusivity, along with other parameters independent of the particular drug in question. Examples of the latter are the geometry of the matrix, the diffusion layer thickness, the amount of drug per unit volume in the matrix, the particle size, and the filler content.

A mathematical model describing drug release from homogeneous and heterogeneous systems was originally presented by Higuchi (6). Within the assumptions of the model, equations were derived that predict a linear dependence of the amount released, Q, upon the square root of time, $t^{1/2}$. Subsequently, the model was extended and equations were derived that consider the solvent boundary diffusion layer as an additional diffusional pathway (7). According to this extended model, plots of Q *versus* $t^{1/2}$ may not be linear during the early stages of the release process. This has been experimentally verified (8) with a series of progestins. Nonlinear behavior was also observed for chlormadinone acetate (9) and for ethynodiol diacetate (10). However, a systematic evaluation of the influence of the properties of a homologous series on the release rate has not been reported.

This communication utilizes these derived mathematical relationships to describe the effect of chain length of esters of *p*-aminobenzoic acid on the *in vitro* release of drug from a silicone matrix. The assumptions and conditions of the model are: *(a)* pseudo-steady state exists; *(b)* diffusion is rate controlling rather than dissolution of drug particles; *(c)* total concentration of drug within the matrix, A, is substantially greater than its solubility, C_s, in the matrix phase, *i.e.*, $A \gg C_s$; *(d)* transport of the drug species occurs through the matrix phase; and *(e)* ideal sink conditions exist in the dissolution media. The amount released per unit area, Q, from a planar surface as a function of time, t, is given by the following expression (7):

$$Q = \frac{-D_s h_a K A \epsilon}{D_a \tau} + \left[\left(\frac{D_s h_a K A \epsilon}{D_a \tau}\right)^2 + \frac{2AD_s C_s \epsilon t}{\tau}\right]^{1/2} \quad \text{(Eq. 1)}$$

where:

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Table I—Solubility (Milligrams per Milliliter) Data for Esters of *p*-Aminobenzoic Acid (12)

Ester	Water (C_a)	Silicone Oil (C_s)	Partition Coefficient (K)
Methyl	3.82	0.79	0.208
Ethyl	1.68	1.38	0.817
Propyl	8.42×10^{-1}	2.31	2.75
Butyl	3.32×10^{-1}	3.44	1.03×10^{1}
Pentyl	9.32×10^{-2}	3.69	3.95×10^{1}
Hexyl	2.37×10^{-2}	2.48	1.05×10^{2}
Heptyl	5.88×10^{-3}	2.35	4.00×10^{2}
Octyl	9.96×10^{-4}	1.48	1.48×10^{3}

is increased regularly in the homologous series, the ratio of C_s/C_a (which is approximately equivalent to the partition coefficient K) changes in an incremental fashion; *i.e.*, $K_n = K_0\, 10^{\pi n}$, where K_n is the partition coefficient for the *p*-aminobenzoic acid ester of chain length n, K_0 is the partition coefficient of a hypothetical reference member with no methylene groups, and π is the logarithmic change of the partition coefficient per methylene unit (11). Since π is dependent only upon the matrix and solvent properties, it is constant for all homologous series. For silicone oil–water, $\pi = 0.54$ (12). If the log of C_a also changes in a regular fashion with chain length, then $C_s = f[C(s_0), n]$, where $C(s_0)$ is the solubility of the hypothetical homolog with no methylene groups and f is the appropriate simple function (11). For the purposes of this communication, it suffices to deal with C_s and K directly as expressed by Eq. 2*a*.

Table I shows reported (12) solubility data for the *p*-aminobenzoic acid esters. Theoretical release rate data were generated for each homolog in the series

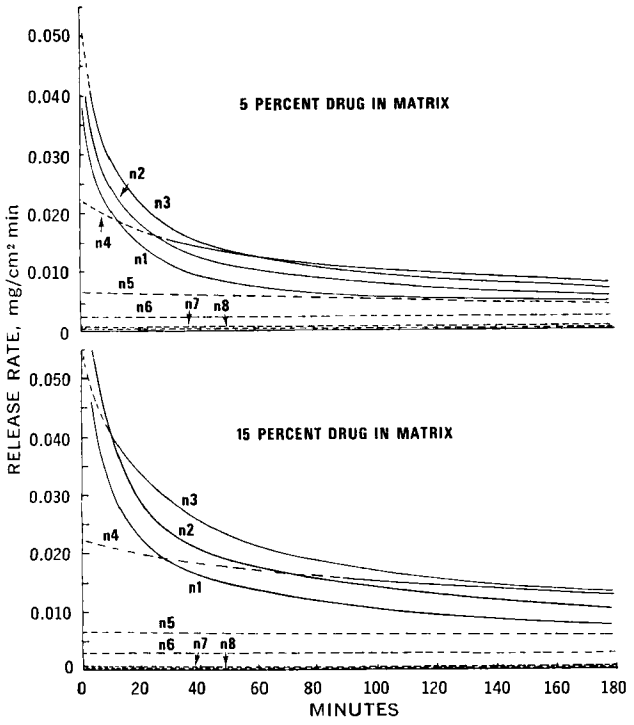

Figure 1—In vitro *theoretical release rates of esters of* p-*aminobenzoic acid from silicone rubber disks at two different concentrations of drug within the matrix* (n = *number of carbons in alkyl chain*).

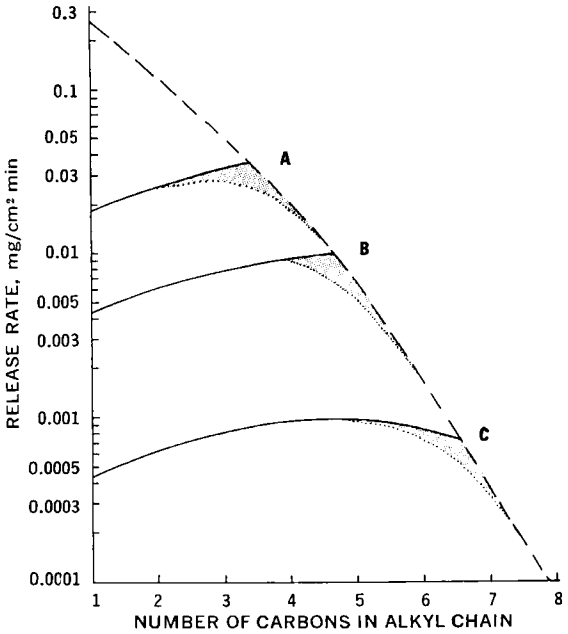

Figure 2—In vitro *theoretical release rates of esters of* p-*aminobenzoic acid as a function of chain length at three different times: A, 10 min; B, 3 hr; and C, 12.5 days. Key: ---, diffusion layer control rate (Eq. 7);* ——, *matrix control rate (Eq. 6); and* · · ·, *expected rate (Eq.* 2a).

with the assumption that C_s can be approximated by the solubility of the ester in silicone oil, and by setting:

$$\frac{D_a}{h_a} = 0.68 \times 10^{-1}\ \frac{\text{cm}}{\text{min}} \qquad \text{(Eq. 3)}^1$$

$$D_s = 1.6 \times 10^{-4}\ \frac{\text{cm}^2}{\text{min}} \qquad \text{(Eq. 4)}^2$$

$$A = 55 \text{ or } 165 \text{ mg/ml} \qquad \text{(Eq. 5)}^3$$

with ϵ and τ assumed to be unity[4].

Figure 1 shows the theoretical release rate profile as a function of time for the esters (methyl through octyl) at two different concentrations of drug within the matrix. The shape of the curves and the magnitude of the release rates are clearly dependent upon chain length. From methyl to butyl, rates decrease significantly with time due to drug transport through an increasing thickness of the depleted drug layer (7). At long times, butyl is released faster because it has a higher matrix solubility than the shorter chain congeners. In other words, at long times, Eq. 2*a* reduces to:

$$\text{rate} = \left(\frac{AD_sC_s\epsilon}{2t\tau}\right)^{1/2} \qquad \text{(Eq. 6)}$$

This is the matrix-controlled case as proposed by Higuchi (6). At higher chain lengths (pentyl through octyl), release rates are essentially constant, approximating a zero-order process. This is described as dif-

[1] A reasonable average value for D_a in dilute aqueous media for the homologous series calculated from the Sutherland-Einstein (13) equation is 4.8×10^{-4} cm²/min, while in a well-stirred system h_a may be around 70×10^{-4} cm (7).

[2] Value taken from Ref. 11.

[3] Equivalent to 5 or 15% (w/w) with the density of the matrix equal to 1.1.

[4] The values of ϵ and τ depend upon the heterogeneity of the matrix, *e.g.*, the filler content. For simplicity, they are assumed to be unity. Data have been presented that show that this is a reasonable assumption for certain matrixes (8, 14).

fusion layer control since diffusion away from the matrix is rate controlling. This behavior results when K is large, *i.e.*, $\beta^2K^2 \gg \alpha C_s t$, and Eq. 2*a* simplifies to:

$$\text{rate} = \frac{D_a C_a}{h_a} \qquad \text{(Eq. 7)}$$

The dashed lines in Fig. 1 indicate that period for which the diffusion layer contribution to the overall release is substantial (*i.e.*, until the time when $\alpha C_s t = \beta^2K^2$), while the continuous lines represent that period when the release is primarily under matrix control. The crossover of the curves results because the transition of diffusion layer to matrix control occurs at different times. Comparison of the 5 and 15% loading doses indicates that release rates are independent of drug concentration at longer chain lengths. However, the shorter chain lengths do exhibit a dependence on concentration which follows Eq. 6 at long times. Also, at the higher concentration, diffusion layer control is operative for a longer time (Fig. 1).

Figure 2 shows the applicability of Eq. 2*a* from a different viewpoint. Here, rate is plotted as a function of the number of carbons *(n)* in the alkyl chain. The dashed line is the rate calculated from Eq. 7 and is directly proportional to C_a. The continuous line is the rate calculated from Eq. 6 and is proportional to $(C_s)^{1/2}$ at a given time. The expected rate given by Eq. 2*a* is represented by the dotted line, and the shaded area is the transition region.

Based upon the model discussed here, factors such as particle size or polymorphic forms of the drug which may influence C_s and/or C_a would be expected to alter the release profile. A forthcoming publication[5] will experimentally test the applicability of these equations.

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T. J. Roseman ˣ
S. H. Yalkowsky

The Upjohn Company
Kalamazoo, MI 49001

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ˣ To whom inquiries should be directed.

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Bioavailability of Digoxin in Presence of Antacids

Keyphrases □ Digoxin—bioavailability in presence of antacids □ Antacids—effect on bioavailability of digoxin □ Bioavailability—digoxin, effect of concurrent antacid administration

To the Editor:

Considerable attention has recently been focused on the problem of bioavailability of digoxin (1–5), and several factors have been reported as responsible for the observed therapeutic effect (6–8). This communication reports the effect of some antacids on the dissolution of digoxin tablets[1] in an attempt to predict the bioavailability of the drug.

Recently, a number of reports (4, 5, 8) confirmed the existence of a close correlation between *in vitro* dissolution and the plasma digoxin level. Shaw *et al.* (4), using seven brands of digoxin tablets, found a good correlation between the percentage of dissolution at 30 min and the plasma digoxin level. Fraser *et al.* (5) reported that both the amount of digoxin dissolved in 1 hr and the reciprocal of the time for 50% dissolution ($1/t_{50\%}$) agreed well with the bioavailability data as computed from the mean area under the serum concentration–time curve. Therefore, dissolution experiments were carried out in the present work to reflect bioavailability.

The dissolution apparatus and procedure adopted were as reported previously (4). The liquid antacid preparation was incorporated in the dissolution medium (water), and an aliquot of 5 ml per digoxin tablet was used. Dissolution tests were performed at 37 ±

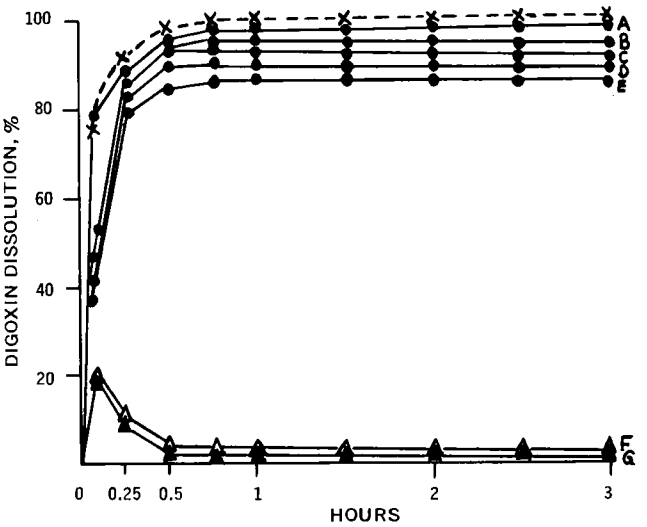

Figure 1—*Effect of some liquid antacid preparations on the dissolution rate of digoxin tablets at 37 ± 0.2° (average of four replicates). Key: – – –, dissolution in the absence of antacids; and ——, dissolution in the presence of antacids. For key to antacid preparations, see Table I.*

[1] Lanoxin tablets (0.25 mg), Batch 2042 X, Burroughs Wellcome & Co., Kent, England.

fusion layer control since diffusion away from the matrix is rate controlling. This behavior results when K is large, *i.e.*, $\beta^2K^2 \gg \alpha C_s t$, and Eq. 2*a* simplifies to:

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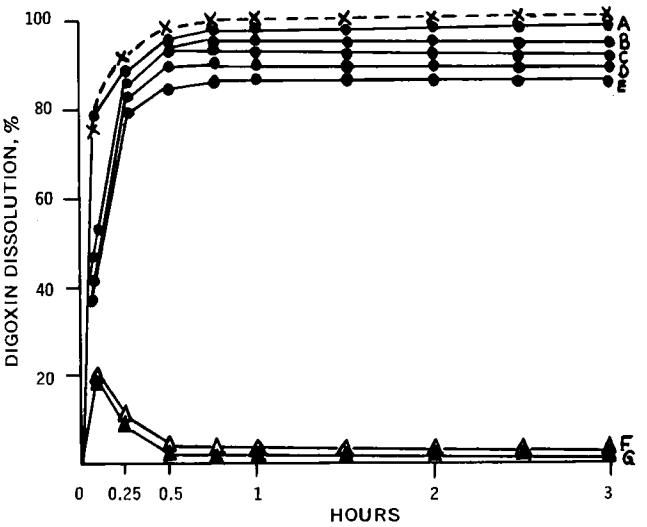

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[1] Lanoxin tablets (0.25 mg), Batch 2042 X, Burroughs Wellcome & Co., Kent, England.

Table I—Effect of Some Antacid Preparations on Dissolution of Digoxin Tablets at 37 ± 0.2°

Antacid Product[a]	Composition (per 10 ml)			Dissolution Data		
				Percent Digoxin Dissolved after		
	Aluminium Hydroxide	Magnesium Hydroxide	Magnesium Trisilicate	0.5 hr	1 hr	$1/t_{50\%}$
A	9.8 ml (as gel)	0.170 g	—	95.1	97.8	0.250
B	0.43 g (as dried gel)	0.160 g	—	91.8	93.1	0.250
C	10 ml (as gel)	—	—	89.8	92.1	0.167
D	9.5 ml (as gel)	0.200 g	—	85.7	91.8	0.167
E	0.44 g (as dried gel)	0.195 g	—	86.3	89.6	0.143
F	—	—	0.50 g	9.8	1.4	<0.001
G	0.62 g (as dried gel)	—	1.24 g	7.9	0.0	<0.001

[a] A = Dijex suspension (Batch No. 7 V), Boots, Nottingham, England; B = Simeco suspension (Batch No. 3720076), Wyeth Lab., Inc., Philadelphia, Pa. (this preparation also contains activated dimethicone); C = Aludrox gel (Batch No. C. IG42), J. Wyeth & Brothers Ltd., Hants, England; D = Polycrol Forte gel (Batch No. 71/2), Nicholas Lab. Ltd., Slough, England; E = Maalox suspension (Batch No. 98508), W. H. Rorer, Inc., Fort Washington, Pa.; F = magnesium trisilicate mixture BPC (this preparation also contains sodium bicarbonate and light magnesium carbonate, the latter having no adsorptive effect on digoxin); and G = Gelusil suspension (Batch No. 705038), W. R. Warners & Co., Ltd., Hampshire, England.

0.2°, and samples were withdrawn periodically over 3 hr. Fresh aliquots of the dissolution medium were added each time to maintain constant volume. The digoxin content in the centrifuged sample was determined by a modification of the European Pharmacopoeia method (9). The surpernate, obtained after centrifugation of the sample, was extracted with chloroform (4 × 10 ml); after evaporation, the residue was dissolved in alcohol and the alkaline sodium picrate solution was added. The color intensity was measured at 490 nm using a spectrophotometer[2] and was compared with a blank. Digoxin powder[3] BP was used as the standard.

Table I shows the compositions of the antacids used, the percentages dissolution of digoxin after 0.5 and 1 hr, and the values of $1/t_{50\%}$. Figure 1 shows the effect of the antacid preparations on dissolution profile over 3 hr. Dissolution of the tablets in the "plain" dissolution medium is shown for comparison. In the presence of antacid preparations containing no magnesium trisilicate, only a relatively slight reduction in dissolution occurred. The presence of magnesium trisilicate in both Products F and G appeared to be responsible for the observed drastic suppression of dissolution. After 1 hr, less than 5% of the labeled digoxin was found in solution (Fig. 1). Values of $1/t_{50\%}$ were, therefore, infinitesimal since the 50% dissolution was not attained even after 12 hr. In the plain dissolution medium, the tablets gave 99.2% dissolution after 1 hr and the time for 50% dissolution was less than 5 min.

The disappearance of digoxin from solution in the presence of magnesium trisilicate-containing antacids may be attributable to the adsorption of the drug on magnesium trisilicate. Preliminary results showed that digoxin was adsorbed on magnesium trisilicate, the value of monolayer adsorption being 0.93 mg/g. Other ingredients of the antacid preparations tested gave negligible adsorption. Magnesium trisilicate has been reported to adsorb a number of drugs including steroids (10–13).

Attempts were made to elute the digoxin adsorbed by both Products F and G. Various elution media were used, with pH values between 1.2 and 8.0. (Hydrochloric acid was used to adjust the pH.) At 37 ± 0.2°, elution of the adsorbed digoxin was insignificant since a maximum of 15% was eluted after 6 hr. In the light of the confirmed correlation between digoxin dissolution and bioavailability, the concurrent administration of oral digoxin and antacids containing magnesium trisilicate may impair the bioavailability of the drug. Investigations are underway in this laboratory to study, in more detail, the *in vitro* adsorption and *in vivo* implications.

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Saleh A. H. Khalil

Department of
Pharmacy and Pharmacology
University of Benin
Benin City, Nigeria

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[3] Burroughs Wellcome & Co., Kent, England.

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Preparation and Curarimimetic Activity of (+)-Isotubocurarine

Keyphrases □ (+)-Isotubocurarine—preparation and curarimimetic activity □ Curarimimetic activity—(+)-isotubocurarine □ Neuromuscular blocking activity—stereochemical requirements, preparation and curarimimetic activity of (+)-isotubocurarine

To the Editor:

We have been interested in the stereochemical requirements for nondepolarizing neuromuscular junction blockade of the voluntary nervous system for some time. King (1), who initially determined the structure of (+)-tubocurarine as I*a* and later isolated and tested (−)-tubocurarine (2), reported a 20–60 times lower activity on the rat diaphragm–phrenic nerve preparation for the latter enantiomer. The rather marked difference in activity and the scarcity of research directed toward this facet of neuromuscular junction blocking agents have been noted (3). Recently, we submitted several reports (4–6) bearing on the problem.

Our present interest was prompted by a report (7) indicating that the structure of (+)-tubocurarine was I*b* instead of I*a*. These investigators found that not only was (+)-tubocurarine represented by I*b* but that (+)-chondrocurarine must have the formula I*a*. The finding that I*b* was a monotertiary, monoquaternary species has been disquieting in view of the generally accepted belief that a bisquaternary form was necessary for potent blocking activity. Although no ready explanation for the potency of I*b* has been forthcoming, Waser (8), noting the easy protonation of the tertiary nitrogen, appears to imply that the resultant dicationic species is somewhat comparable to I*a*.

Since our interest has been directed toward relating the influence of an asymmetric carbon of specific configuration adjacent to the quaternary moiety to neuromuscular junction blocking potency, we felt it would be of interest to prepare what we term "(+)-isotubocurarine" (I*c*). Compound I*c* would have the reverse order of quaternization to that in I*b* and would provide an isomeric tubocurarine in which the only structural change would be that the quaternary moiety would be adjacent to a center with the *S*- rather than the *R*-configuration as in I*b*. Obtaining the relative potencies of I*b* and I*c* would help to answer the question as to whether Waser's implied explanation for the activity of I*b* has substance. If it has merit, there should be only a minor potency difference, dependent on pKa differences, between the two rather similar tertiary nitrogens of I*b* and I*c* derived from (+)-tubocurine [now known to be (+)-chondrocurine (7)]. But if a significant potency difference were found, it could indicate that there is importance to the configuration of the carbon atom adjacent to the quaternary head.

(+)-Isotubocurarine was obtained from (+)-tubocurine, which had been prepared by the demethylation procedure of Shamma *et al.* (9) involving the dequaternization of I*b* with sodium thiophenoxide. The ditertiary base had a melting point of 235–237° and an $[\alpha]_D^{20}$ of +220° (c 1.0, 0.1 *N* HCl). Shamma *et al.* (9) reported a melting point of 222.5–223.5°, and an $[\alpha]_D$ of +221° (c 1.15, 0.1 *N* HCl) for (+)-tubocurine, although (+)-chondrocurine is reported (10) to have a melting point of 232–234° and an $[\alpha]_D^{24}$ of +200° (c 0.5, 0.1 *N* HCl), the two compounds being identical. A direct comparison by mixed melting point, TLC, and spectral (IR and UV) methods showed complete agreement[1]. The discrepancy in melting point is presently not explicable since the sample supplied to us, in our apparatus, gave a melting point of 227–231°. Since analytical data (C, H, N) on our (+)-tubocurine were also in accord with the assigned formula, we believed our compound to be suitable for further experiments.

(+)-Tubocurine, in a large volume of acetone, was treated with 0.5 *M* equivalent of a dilute acetone solution of methyl iodide in increments over approximately 0.5 hr. The reaction mixture, evaporated to dryness, was dissolved in a minimum amount of methanol and crystallization of a large portion of the excess (+)-tubocurine was induced. The mother liquor was evaporated under reduced pressure to provide a yellowish-white powder.

TLC examination of this powder on silica gel, using a mixture of 10% aqueous ammonia–methanol–ethyl acetate–isopropyl alcohol (2:2:1:1), showed four spots with R_f values of 0.83, 0.43, 0.26, and 0.05. Of these, the spots with R_f values of 0.83, 0.43, and 0.05 were shown to correspond to authentic (+)-tubocurine,(+)-tubocurarine chloride neutralized with sodium bicarbonate, and the expected bisquaternary base, respectively. On this basis, the spot with R_f value 0.26 was concluded to represent the isomeric tubocurarine (I*c*). Judging from the intensity of the spot at R_f 0.26 compared to that at R_f 0.43, the former was in the major amount.

A methanolic solution of the mixture subjected to TLC analysis was then passed through an ion-exchange resin[2] (chloride cycle). No iodide ion was detected in the effluent, which was evaporated to give a residue. This residue was dissolved in methanol and distributed on a small portion of alumina which was then air dried. The dried residue was placed on top of a column of grade V neutral alumina and eluted with ethyl acetate to provide the residual amounts of (+)-tubocurine in the early fractions. Continued elution with a mixture of ethyl acetate–methanol, followed

[1] The comparisons were made possible through the cooperation of Dr. M. Shamma, Pennsylvania State University.
[2] Amberlite IRA 410.

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Since our interest has been directed toward relating the influence of an asymmetric carbon of specific configuration adjacent to the quaternary moiety to neuromuscular junction blocking potency, we felt it would be of interest to prepare what we term "(+)-isotubocurarine" (I*c*). Compound I*c* would have the reverse order of quaternization to that in I*b* and would provide an isomeric tubocurarine in which the only structural change would be that the quaternary moiety would be adjacent to a center with the *S*- rather than the *R*-configuration as in I*b*. Obtaining the relative potencies of I*b* and I*c* would help to answer the question as to whether Waser's implied explanation for the activity of I*b* has substance. If it has merit, there should be only a minor potency difference, dependent on pKa differences, between the two rather similar tertiary nitrogens of I*b* and I*c* derived from (+)-tubocurine [now known to be (+)-chondrocurine (7)]. But if a significant potency difference were found, it could indicate that there is importance to the configuration of the carbon atom adjacent to the quaternary head.

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(+)-Tubocurine, in a large volume of acetone, was treated with 0.5 *M* equivalent of a dilute acetone solution of methyl iodide in increments over approximately 0.5 hr. The reaction mixture, evaporated to dryness, was dissolved in a minimum amount of methanol and crystallization of a large portion of the excess (+)-tubocurine was induced. The mother liquor was evaporated under reduced pressure to provide a yellowish-white powder.

TLC examination of this powder on silica gel, using a mixture of 10% aqueous ammonia–methanol–ethyl acetate–isopropyl alcohol (2:2:1:1), showed four spots with R_f values of 0.83, 0.43, 0.26, and 0.05. Of these, the spots with R_f values of 0.83, 0.43, and 0.05 were shown to correspond to authentic (+)-tubocurine,(+)-tubocurarine chloride neutralized with sodium bicarbonate, and the expected bisquaternary base, respectively. On this basis, the spot with R_f value 0.26 was concluded to represent the isomeric tubocurarine (I*c*). Judging from the intensity of the spot at R_f 0.26 compared to that at R_f 0.43, the former was in the major amount.

A methanolic solution of the mixture subjected to TLC analysis was then passed through an ion-exchange resin[2] (chloride cycle). No iodide ion was detected in the effluent, which was evaporated to give a residue. This residue was dissolved in methanol and distributed on a small portion of alumina which was then air dried. The dried residue was placed on top of a column of grade V neutral alumina and eluted with ethyl acetate to provide the residual amounts of (+)-tubocurine in the early fractions. Continued elution with a mixture of ethyl acetate–methanol, followed

[1] The comparisons were made possible through the cooperation of Dr. M. Shamma, Pennsylvania State University.
[2] Amberlite IRA 410.

by enrichment of this solvent with aqueous ammonia, separated the remaining components on the column into two yellow-colored bands. Extrusion of the column contents enabled mechanical separation of the two bands. The slower moving band provided a residue that was identified as I*a* by TLC. The faster moving band, however, proved to be a mixture of I*b* and I*c* when examined by TLC.

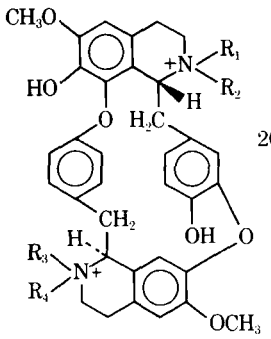
The latter band was extracted with hot methanol and treated with a slight excess of 1 *N* HCl, and the resultant solution was evaporated to dryness. The residue was crystallized from absolute ethanol or absolute ethanol–methanol to give a white crystalline product, mp 260° (frothing and dec.); $[\alpha]D^{20}$ +218.9° (c 1.0, CH_3OH). The NMR (D_2O, sodium 2,2-dimethyl-2-silapentane-5-sulfonate as external standard) showed: δ 2.62 (s, 3H, NCH_3), 2.83–3.78 [18H, 12H aliphatic and 6H $N(CH_3)_2$], 3.84 (s, 6H, OCH_3), and aromatic protons 6.16–7.33 (10H). The IR spectrum of I*c* was remarkably similar to that of I*b*, with minor differences only at 3245, 1080, 1025, and 990 cm^{-1}. The UV absorption spectrum showed λ_{max} (log ϵ): 225 (4.58) and 282 (3.95) nm. The analytical data corresponded to $C_{37}H_{42}N_2O_6 \cdot 3H_2O$.

That I*c* possessed an unmodified structural skeleton except for the specific monoquaternization was evident from its ready conversion to the known bisquaternary, chondrocurarine chloride (I*a*). This was done by treating I*c* (as free base) with excess methyl iodide and passing the resultant product through an ion-exchange resin[2] (chloride cycle) to give, on work-up, a product identical with I*a*, as shown by direct comparison with an authentic sample[3].

The neuromuscular junction blocking potency of I*c* in comparison to I*b* was determined using the cat tongue–hypoglossal nerve preparation previously described (4)[4]. The potency of I*c* appears to be approximately double that of I*b*, with ED_{50} values of 0.029 mg/kg (low 0.024, high 0.035) and 0.067 mg/kg (low 0.055, high 0.102), respectively. Previously, we reported the following values for I*b*: 0.09 and 0.15 mg/kg (Refs. 4 and 5, respectively) using the same test method. That the blocking mechanisms are of the same type is indicated by parallelism of the dose–response curves of I*b* and I*c*.

The pH-stat method (10), employed in our earlier work (4), was used to determine whether the difference in blocking potency could be due to preferential acetylcholinesterase inhibition by I*b* which, presumably, would reduce its apparent blocking ability. No significant differences in inhibitory potency were observed between I*b* and I*c*.

From our previous experiences in determining preferential binding of similar monoquaternary neuromuscular junction blocking agents by blood components (4), it seems unlikely that this factor could be involved. Therefore, a monoquaternary moiety in a blocking agent adjacent to an asymmetric carbon atom of the *S*-configuration seems to endow the molecule with greater potency than if it is adjacent to one of the *R*-configuration. This selectivity is reminiscent of that found for enantiomeric monoquaternaries (4) where the agents with an *S*-configuration were invariably more potent by an approximately 2:1 ratio. The fact that I*b* and I*c* are 1-substituted tetrahydroisoquinoline derivatives, formally similar to those in the cited study (4), is of interest. The difference in blocking potency between I*b* and I*c* suggests also that protonation of the tertiary amine moiety to give a dicationic form similar to I*a* is probably not a satisfactory explanation of the observed activities. This is further reinforced by a report (11) concerning the lack of activity of (+)-chondrocurine (also confirmed by our work) which, undoubtedly, would exist largely in the dicationic form at physiological pH.



I*a*: $R_1 = R_2 = R_3 = R_4 = CH_3$
I*b*: $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$
I*c*: $R_1 = R_3 = R_4 = CH_3$; $R_2 = H$

In conclusion, it appears that the adjacency of the quaternary moiety of I*c* to a center of *S*-configuration rather than to an *R*-configuration as in I*b* leads to an approximate doubling of blocking potency. The reasons for this difference in potency are, no doubt, receptor related since they are not due to preferential acetylcholinesterase inhibition nor, probably, to preferential binding to blood components.

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(9) M. Shamma, N. C. Deno, and J. I. Remar, *Tetrahedron Lett.*, **1966**, 1375.
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(11) J. D. Dutcher, *Ann. N.Y. Acad. Sci.*, **54**, 326(1951).

Taito O. Soine [x]
Janette Naghaway

Department of Medicinal Chemistry
College of Pharmacy
University of Minnesota
Minneapolis, MN 55455

[3] Provided by Mr. A. O. Geiszler, Scientific Division, Abbott Laboratories, North Chicago, IL 60064, as sample A-41785, Ref. 3710-207.

[4] We are indebted to Dr. T. Brody, Chairman, Department of Pharmacology, Michigan State University, East Lansing, MI 48823, for providing the laboratory manual describing the test procedure used in their teaching laboratories.

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Nonaqueous Titration of Procainamide Hydrochloride

Keyphrases □ Procainamide hydrochloride—nonaqueous titration, interference of acetic anhydride □ Titration, nonaqueous—procainamide hydrochloride, interference of acetic anhydride

To the Editor:

The official assay for procainamide hydrochloride (1) involves visual nonaqueous titration, with perchloric acid as the titrant and glacial acetic acid as the titration solvent. Mercuric acetate is added to permit the titration of the tertiary amine hydrochloride portion of the molecule. Therefore, both the aromatic amine and the tertiary amine groups are titrated, and two inflections in the titration curve are obtained (Fig. 1). The first inflection is due to the aromatic amine group while the second is attributable to the tertiary amine hydrochloride group. When crystal violet indicator is used in conjunction with a potentiometric titration, the visual end-point corresponds to the second break in the curve.

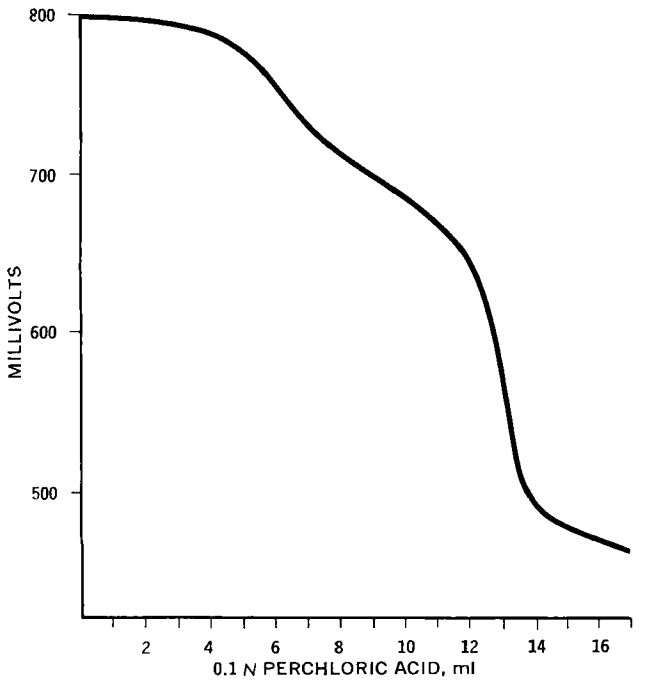

Figure 1—*Typical titration curve for procainamide hydrochloride, using glacial acetic acid as the titration solvent and perchloric acid as the titrant. Titration was performed in the presence of mercuric acetate.*

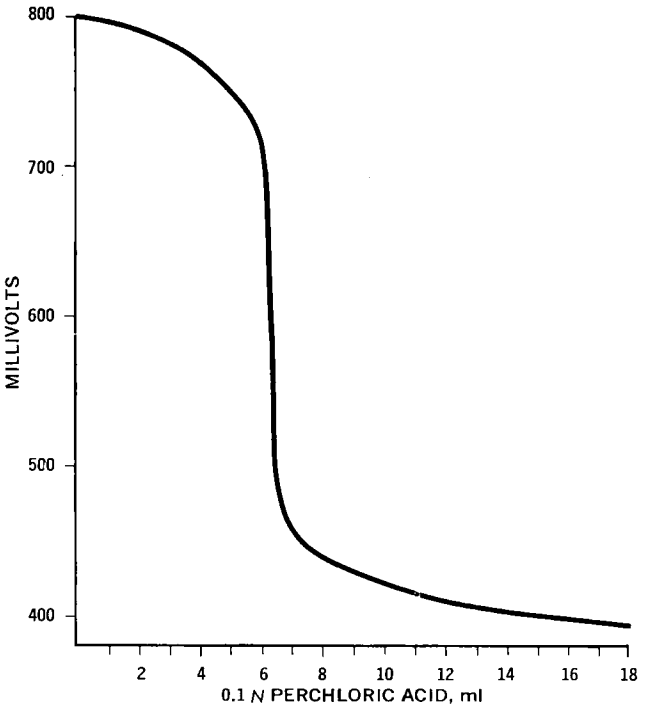

Figure 2—*Typical titration curve for procainamide hydrochloride using acetic anhydride–acetic acid (1:19) as the titration solvent mixture and perchloric acid as the titrant. Titration was performed in the presence of mercuric acetate.*

Acetic anhydride, a potential contaminant of glacial acetic acid, interferes with the first end-point in the titration by acetylating the aromatic amine group and rendering it nontitratable with perchloric acid. If 50 ppm acetic anhydride is present in the titration solvent, a decrease in the percent recovery of over 3% can theoretically be expected when the USP (1) assay directions are followed. However, in the presence of an excess of acetic anhydride (at least a sufficient amount to acetylate completely the aromatic amine group), an excellent titration curve is obtained; it demonstrates a single inflection due to the amine hydrochloride portion of the molecule (Fig. 2).

When the assay directions specified (1) for procainamide hydrochloride were followed, only one inflection was obtained in the titration curve, which resembled Fig. 2, when the titration solvent contained in excess of 0.5% acetic anhydride. In the presence of 0–0.5% acetic anhydride, the volume of perchloric acid required to reach the first potentiometric end-point decreased with an increase in the acetic anhydride content of the titration solvent. The best visual end-points were obtained when the solvent contained 2–25% acetic anhydride. For example, the percent recovery for a series of 10 analyses was 100.6 ± 0.30 when the titration solvent contained 5% acetic anhydride.

To preclude an error in the assay of procainamide hydrochloride due to the presence of acetic anhydride as a contaminant in glacial acetic acid, it is suggested that the titration solvent contain sufficient acetic anhydride to assure complete acetylation of the aromatic amine group. This precaution is taken (perhaps fortuitously) in the official assay for chloro-

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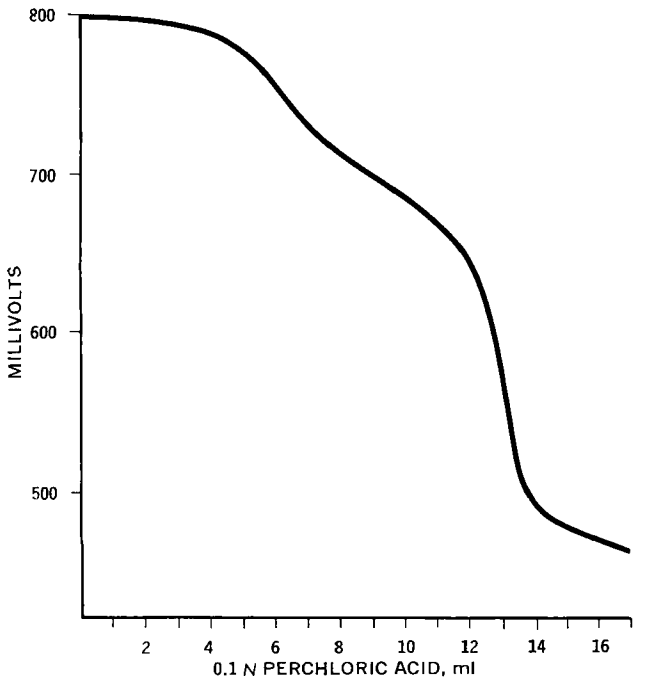

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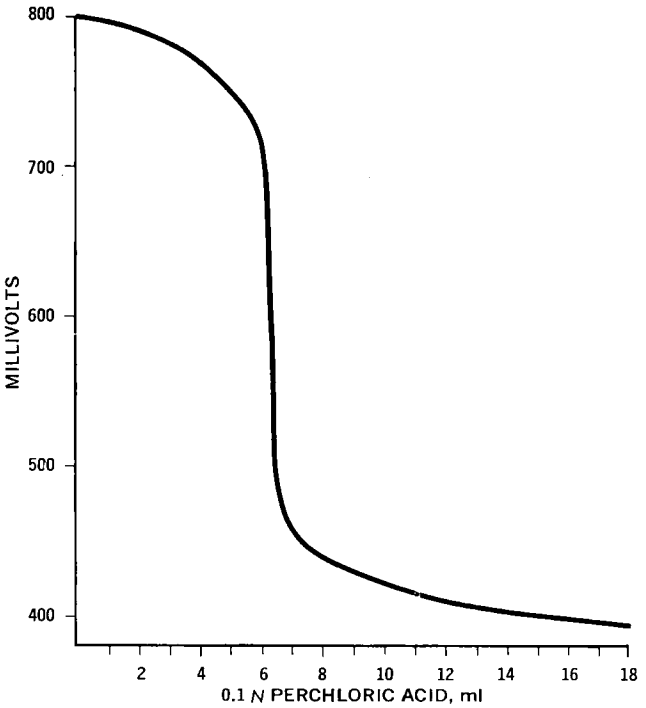

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Martin I. Blake [x]
David W. Bode
Harold J. Rhodes

Department of Pharmacy
University of Illinois
Chicago, IL 60612

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[x] To whom inquiries should be directed.

Albumin Microspheres as Vehicles for Achieving Specificity in Drug Delivery

Keyphrases □ Albumin microspheres—vehicles for specific drug delivery systems □ Microspheres, albumin—vehicles for specific drug delivery systems □ Drug delivery systems—albumin microspheres, vehicle for mercaptopurine delivery

To the Editor:

There is a growing need in modern day drug therapy to develop methods of drug delivery that are highly cell or tissue specific. Cancer chemotherapy, for example, demands that drugs with pronounced systemic toxicities be delivered to target tissues in precise dosages and with minimal interaction with normal tissues. Past approaches to achieving specificity have centered around structural modification of the drug, alterations in route of administration or dose regimen, and efforts to physically position a drug delivery device, such as a polymeric implant, in the closest possible proximity to the target tissue.

The phagocytic cells of the reticuloendothelial system provide a physiological means of achieving cell and tissue specificity in drug delivery that has enormous potential. Radiologists utilize the phagocytic activity of these cells to achieve specificity in the delivery of radiolabeled albumin and sulfur colloids (1). Gregoriadis (2) entrapped anticancer agents within phospholipid vesicles, reported the phagocytic uptake of the vesicles by the liver in rats, and mentioned the possibility of directing drug-containing vesicles to other cell types by manipulation of the vesicle surface. Trouet *et al.* (3) reported reduced toxicity and increased effectiveness in the treatment of leukemia when DNA complexes of the anticancer agent daunomycin are pinocytized from solution. In an attempt to find a phagocytizable carrier capable of accommodating a wide variety of drugs, the anticancer agent mercaptopurine was entrapped within human albumin microspheres.

Table I—Incorporation of Mercaptopurine-8-^{14}C Hydrate in Human Albumin Microspheres

Total Drug Available, μg/mg Albumin	Drug Associated with Microspheres[a], μg/ml Albumin	Entrapment, %
4.00[b]	3.46	86.5
4.00	3.59	87.3
8.00[c]	1.57	—
10.5	1.32	—

[a] Average of two determinations. [b] Drug present in emulsion prior to denaturation (1 mg/ml in internal phase). [c] Drug added to previously prepared albumin microspheres (1 mg/ml).

Albumin microspheres provide a potentially useful means of delivering drugs to endocytic cells because they are physically and chemically stable, rapidly removed from the vascular system by phagocytosis, amenable to preparation in large batches, nonantigenic, metabolizable, and capable of accommodating a wide variety of drug molecules in a relatively nonspecific fashion. Human serum albumin microspheres containing the anticancer agent mercaptopurine were prepared by a modification of the method of Scheffel *et al.* (4). One milliliter of a 27% dimethylformamide–water solution containing 1 mg of mercaptopurine-8-^{14}C hydrate[1] and 250 mg of human serum albumin[2] was emulsified with 100 ml of cottonseed oil USP by repeated passage through a hand-operated homogenizer[3]. The emulsion was added with constant stirring to an additional 100 ml of oil that had been heated to 175°, held at that temperature for 10 min, and cooled. No degradation of mercaptopurine could be detected in dimethylformamide–water solutions kept at 175° in sealed ampuls for similar periods.

Two hundred milliliters of ether was then added and the suspension was filtered through a 0.5-μm filter[4]. The spheres were washed repeatedly with ether to remove residual oil and dried. The quantity of drug entrapped was determined by digesting the spheres in 0.5 *N* NaOH and counting on a liquid scintillation counter[5], using appropriate internal standards. Over 85% of the mercaptopurine present in the original emulsion was associated with the microspheres (Table I).

Since albumin would be expected to adsorb drug from solution, an experiment was performed to determine how much mercaptopurine could be adsorbed onto previously prepared drug-free microspheres. The microspheres were suspended in a 27% dimethylformamide–normal saline solution containing 1 mg mercaptopurine-8-^{14}C hydrate/ml (the same concentration present in the internal phase of the emulsion used to prepare drug-containing spheres), stirred well, centrifuged, and washed five times with a large excess of distilled water. About 40% as much drug was associated with the spheres as when drug was incorporated in the original emulsion (Table I).

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Table II—*In Vitro* Release of Mercaptopurine-8-^{14}C from Human Albumin Microspheres

Suspension Medium	Drug Released from Spheres, %	
	2 hr	24 hr
Buffer alone[a]	15.7	16.0
Buffer and dimethylformamide	17.6	21.0
Buffer, dimethylformamide, and unlabeled mercaptopurine (1 mg/ml)	39.8	42.8

[a] Isotonic, buffered saline, pH 7.4, containing 0.1% polysorbate 80.

The ability of drug-free spheres to adsorb mercaptopurine indicated that a substantial percentage of the drug entrapped from the emulsion might be present on the surface of the microspheres. A wash-off study was conducted to determine the loss of drug from microspheres prepared from a drug emulsion. The spheres (5 mg/ml) were suspended in isotonic, buffered saline solutions (pH 7.4) with and without unlabeled mercaptopurine. After centrifugation, the supernatant liquids were assayed for displaced radiolabeled drug (Table II). Only 16% of the drug was lost from the spheres into buffered saline in 24 hr, but about 40% could be exchanged for unlabeled mercaptopurine. The latter value is in accord with the percentage of drug that appeared to be surface associated in the adsorption study.

The quantity of drug entrapped in this system was limited by the low aqueous solubility of the drug. Preliminary experiments with the anticancer agent daunomycin hydrochloride indicated that a larger quantity of more water-soluble drugs can be entrapped without recourse to mixed solvent systems. Daunomycin hydrochloride was entrapped to the extent of 8 μg/mg albumin, about twice the mercaptopurine entrapment, and preliminary results indicate that this quantity can be increased considerably. Figure 1 shows a scanning electron micrograph of human albumin microspheres containing daunomycin hydrochloride. They range in size from about 0.2 to about 1.2 μm and are representative of the type of physical system discussed for mercaptopurine. They have not been sonicated, a process that produces a smaller, more uniform particle size.

Albumin microspheres containing chemotherapeutic agents may well be potentially useful in the treatment of cancer and fungal or bacterial infestations of the reticuloendothelial system (histoplasmosis and typhoid fever) and in the delivery of immunosuppressive agents. A particulate system containing an antimicrobial drug might, for example, effectively breach the barrier to drug transport provided by the macrophage in typhoid fever and give better access to the bacillus. Several reports indicated that the rate of clearance from the blood of human cancer patients of

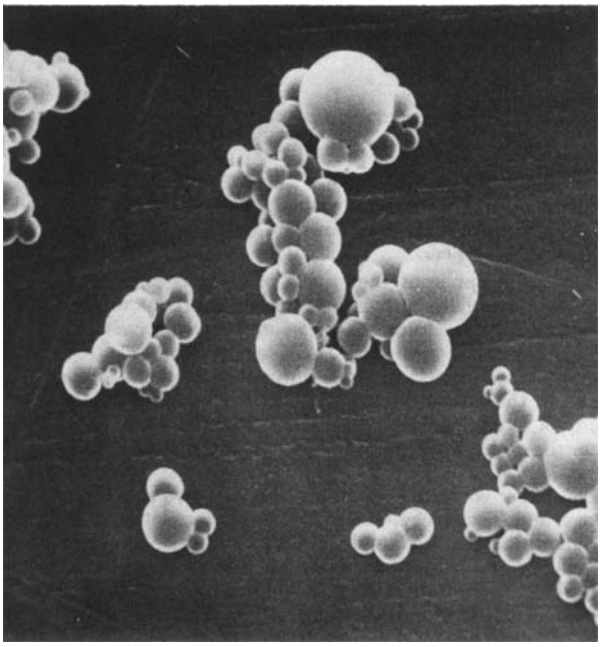

Figure 1—*Scanning electron micrograph (10,000×) of human serum albumin microspheres containing daunomycin hydrochloride (0.8 mg/100 mg albumin). Particles were dried from an ether suspension producing the observed aggregation.*

intravenously administered colloids, such as aggregated human albumin and lipid test emulsions, is proportional to the extent of neoplasia (5, 6).

It is possible that preferential uptake into such tissues as liver or bone marrow might be achieved and required total doses as well as systemic side effects of anticancer agents might be reduced. A study is in progress to determine the fate of intravenously administered microspheres containing radiolabeled mercaptopurine in mice and the kinetics of the release of mercaptopurine from the spheres *in vivo*.

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Paul A. Kramer

Department of Industrial and Physical Pharmacy
School of Pharmacy and Pharmacal Sciences
Purdue University
West Lafayette, IN 47907

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REVIEWS

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The paper-covered book is divided into five sections: Pharmacology and Toxicology, Reproduction, Interrelation between Experimental Toxicology and Clinical Studies, Mutagenicity, and Toxicology and the Future of Drug Therapy. A welcome new section, Free Communications, includes 11 papers on original research in toxicology. All sections are in English except one short paper on a method for determining biological values.

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Pharmacologists, toxicologists, and even clinical pharmacists would find this review very informative and useful.

Reviewed by Charles L. Winek
School of Pharmacy
Duquesne University
Pittsburgh, PA 15219

1974 Supplement to Evaluations of Drug Interactions. Prepared by the Drug Interactions Project of the American Pharmaceutical Association with the assistance of the American Dental Association, the American Medical Association, the American Society of Hospital Pharmacists, the Food and Drug Administration, and the National Library of Medicine. Published by the American Pharmaceutical Association, 1974. iv + 66 pages, 14 × 23 cm. Price $2.00.

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DRUGS ANONYMOUS?

We make no claim of originality with respect to the title of this editorial. In fact, it was the title of a booklet published in May 1967 by the Pharmaceutical Manufacturers Association. This booklet was a real eye-catcher, with a group of dressed but faceless mannequins prominently displayed on both its front and back covers. The theme of the booklet was the importance of knowing the identity of a drug's manufacturer or source, and the booklet advocated the use of brand names or trademarks as the ideal formula for accomplishing this aim.

In addition to its clever title, *Drugs Anonymous?*, and the imaginative cover design, the text of the booklet made a rather convincing case for the sponsor's argument that you darn well better know who produces the drug product you prescribe or dispense. Sure, the well-informed and sophisticated reader could find the usual flaws in the conclusions drawn from their presentation, but overall it must have been persuasive to the average reader.

Well, we couldn't help but be reminded of this booklet—and its title—during the past few years as a number of states have sought to enact statutes requiring that the name of the *actual manufacturer* (*i.e.*, not just the *distributor*) of the dosage form be included on the drug product label. After all, the industry in its booklet has told us how important it is to know "exactly what drug preparation" is used, to "identify a specific medicine," to "identify the manufacturer," and to "specify the source of the product desired."

Having read all this in their booklet, and other publications, believing souls might naturally expect that the PMA would be leading the charge up the steps of the state capitol to encourage the legislature to pass such legislation. But strangely enough this did not happen, either in California—where such legislation was originally proposed about four years ago—or in other states which subsequently considered similar bills.

As a matter of fact, the lobbying, the maneuvering, and the chicanery which occurred in California, all in an effort to defeat, abort, or emasculate this legislation, were truly astounding! The state pharmaceutical association was so mystified by it that they published an editorial in the *California Pharmacist* entitled "What Kind of Games Are Being Played?"

And who was behind this entire effort to obstruct, defeat, and then repeal the legislation to require manufacturer identification? None other than the drug industry, and in particular the PMA!

In light of the enormous commitment of their legal staff, their lobbying resources, their field network, and their communications system that PMA made in this effort, it was obvious that they must have felt compelling reasons to oppose and fight the requirement of manufacturer disclosure.

And sure enough, there were! Passage and implementation of the California law enabled the California Pharmaceutical Association to compile and publish lists of who made what and for whom. The revelations were comparable to a pharmaceutical version of the "Watergate Tapes." It turns out that the tetracycline capsules from 13 different companies actually are all made by a single firm, that 18 different versions of chloral hydrate capsules are likewise made by a single manufacturer, and so on down the line.

"Drugs anonymous," indeed! Or, at least they were anonymous until the California people flushed them out for the first time. At the time this column is being written, we have heard rumors, now that the cat is out of the bag, that the drug industry plans to drop its opposition to a *federal* requirement of manufacturer identification. This approach would equally satisfy the need for information, and it would make more sense for all concerned to have a uniform national requirement.

However, whatever may be the eventual outcome, pharmacy, medicine, and the public owe a debt of gratitude to CPhA and the California legislature for providing the means and for taking the action to sweep away the cloak of secrecy concealing the true origin and source of a major portion of the drug supply upon which practitioners have relied and have trustingly placed their faith. —EGF

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REVIEW ARTICLE

Synthesis and Biological Activity of Semisynthetic Digitalis Analogs

RICHARD THOMAS[x], JOHN BOUTAGY, and ALEX GELBART

Keyphrases □ Digitalis—synthesis and biological activity of semisynthetic analogs, review □ Cardiotonic steroids—synthesis and biological activity of semisynthetic digitalis analogs, review □ Structure–activity relationships—digitalis analogs, model for digitalis receptor, review □ Guanylhydrazones—synthesis and biological activity, review □ Receptors, digitalis—model proposed, structure–activity relationships of digitalis-like compounds, review □ Steroids, cardiotonic and cardioactive guanylhydrazones—synthesis and biological activity, structure–activity relationships, digitalis receptor proposed, review

CONTENTS

Properties of Naturally Occurring Cardiotonic Steroids 1651
Development of Synthetic Methods 1652
Structural Modifications of Cardiotonic Genins 1653
Modification of 17β-Lactone Ring 1653
Replacement of Lactone Ring with Open-Chain Structures 1656
Modification of Steroid System 1657
Attachment of Butenolide Ring 1657
Introduction and Modification of Other Functional Groups 1658
Modification of Pharmacokinetic Properties 1660
Steroidal Guanylhydrazones and Other Cardioactive Compounds 1661
Biological Activity 1666
Interrelation of Biological Effects 1668
Na^+,K^+-ATPase as Digitalis Receptor 1671
Dissociation of Inotropic Effects from Inhibition of Na^+,K^+-ATPase 1673
Structure–Activity Relationships 1675
Model for Digitalis Receptor 1675
Relationship between Cardiotonic Activity and Inhibition of Na^+, K^+-ATPase 1679
Conclusions 1680
References 1680

The cardiac glycosides are one of the most important and widely used groups of drugs in clinical medicine. These vegetable drugs act on the failing heart to produce a marked inotropic effect, and for this purpose they are unrivaled by any synthetic or semisynthetic substitute. The cardiac glycosides are also one of the most toxic groups of substances in general clinical use.

In a recent prospective study, Beller *et al.* (1) examined 931 consecutive patients admitted to the medical service at the Boston City Hospital. They found that 15% of the patients admitted were taking digitalis on admission; of these, 23% showed definite manifestations of digitalis toxicity and a further 6% showed possible digitalis toxicity. The mortality rates for the various groups of patients were: *(a)* patients receiving digitalis on admission and showing definite signs of digitalis toxicity, 41%; *(b)* patients receiving digitalis on admission and showing no signs of digitalis toxicity, 17%; and *(c)* patients not receiving digitalis, 9%.

Many factors other than the use of digitalis would have contributed to these mortality rates, but the results of this survey, one of the few prospective studies available, generally confirm previously reported rates of digitalis toxicity. Beller *et al.* (1) cited seven other studies in which the mortality rates of patients who

developed digitalis toxicity ranged from 7 to 50% (average of 22%). Recent accounts of the clinical pharmacology and toxicology of cardiac glycosides are given in Refs. 2–11.

The incidence of digitalis intoxication may be increasing (12). This apparent trend was discussed by Beller *et al.* (1) who cited several possible contributory factors, including an increase in the proportion of the population at risk. Older people generally show a higher rate of digitalis toxicity to "normal" doses of digitalis, possibly because of decreased kidney function (13). However, Beller and associates believe that the increase in the *reported* incidence of digitalis toxicity is due largely to improved methods of surveillance and to improved diagnosis of toxicity based on a more adequate knowledge of the electrocardiography of arrhythmias.

The need for an effective but safer substitute for digitalis is thus obvious. But there is another reason for the current interest in digitalis and digitalis-like compounds. The cardiac glycosides are unique specific inhibitors of the pumping mechanism which transports sodium and potassium across cell membranes against the electrochemical gradient. The pumping entity has been identified as a spatially oriented, membrane-bound adenosine triphosphatase which requires the presence of magnesium ions and is activated by changes in the concentrations of sodium and potassium ions. It is specifically inhibited by ouabain and other biologically active cardiac glycosides and related cardiotonic steroids. This enzyme, henceforth referred to as Na^+,K^+-ATPase, has been the subject of approximately 1500 published scientific papers in the 10-year period prior to 1972 [figure cited by Schwartz *et al.* (14) in their recent review].

The current interest in Na^+,K^+-ATPase arises because of its key role in controlling cell physiology and because it is the putative receptor mediating the effects of cardiac glycosides on myocardial contractility. A characteristic feature of almost all living cells is that the concentration of potassium is high in the intracellular fluid and low in the extracellular fluid and that the reverse applies in the case of sodium. The cell usually achieves this situation by actively exchanging three intracellular ions of sodium for two extracellular ions of potassium. The cell thereby undergoes a net loss of ions. It is believed that the development of this capacity was an essential step in the evolution of life. For millions of years, the primoidal cell must have struggled with the problem of accumulation of water as a result of the osmotic effects of nondiffusible organic molecules. The cell may have responded by developing a mechanism (Na^+,K^+-ATPase) whereby a net loss of ions was achieved (15). In doing so, it would have established an electropotential difference across the cell membrane and laid the basis for such excitatory events as the propagation of impulses in nerve and muscle. Other membrane phenomena that have been linked directly or indirectly with Na^+,K^+-ATPase activity include amino acid transport, sugar transport, antilipolytic effects, transport of *p*-aminohippuric acid, and transport of biogenic amines. (For references, see Ref. 14.)

Considerable evidence supports the theory, first proposed by Repke and Portius (16), that the effects of cardiac glycosides on heart muscle contractility result from the inhibition of myocardial Na^+,K^+-ATPase. There is also much evidence to the contrary, including the fact that the cardiac glycosides seem to exert a wide range of primary effects, ranging from the release of intracellular bound calcium to direct effects on the polymerization of actin and myosin (17). The investigator is thus faced with the extremely difficult task of correlating one of several events at the biochemical level with events at the physiological level. One important body of evidence that links Na^+,K^+-ATPase with the active transport of Na^+ and K^+ and with the effects of cardiac glycosides on heart muscle contractility is the analysis of structure–activity relationships. A consistent parallelism has been claimed to exist between cardiotonic activity and the ability of cardiac glycosides to inhibit both Na^+,K^+-ATPase and the active transport of Na^+ and K^+. However, these structure–activity relationships are less impressive when viewed against the limited range of structural modifications that have been made of the basic cardiac glycoside molecule.

All that has been said so far emphasizes the need for a more extensive search for digitalis-like compounds. The objectives of the medicinal chemist in this area may be defined as follows:

1. To develop cardiotonic steroids with an improved therapeutic index. (This is based on the assumption that toxic and therapeutic effects can be separated.)
2. To contribute to the study of the mode of action of cardiac glycosides by developing systematic and graded series of compounds for use in structure–activity relationship studies. (The present authors believe that they have achieved this objective with respect to defining the essential structural features of the C-17β-lactone ring and its biologically active isosteres, as discussed under *Structure–Activity Relationships.*)
3. To search for new types of therapeutic agents based on the selective inhibition of Na^+,K^+-ATPase in tissues other than the heart.

The factors limiting such studies have been attributed to the difficulties in the chemistry of the cardiac glycosides. According to Thorp and Cobbin (18): ". . . the problem is an enormous one since the chemistry of the cardiac glycosides is so difficult. It is no exaggeration to say that the work of fifty chemists might be insufficient to occupy the full time of one pharmacologist." On the basis of personal experience, it is suggested that the ratio should be about 10 to one to synthesize and screen a series of analogs.

The purpose of the present review is to give an account of the major semisynthetic modifications that have been made on the cardiotonic steroids. Only brief mention is made of the many studies of structure–activity relationships among the naturally occurring cardiotonic steroids since this field has been covered by numerous reviews which will be cited. The mode of action of cardiotonic steroids will be discussed in some detail, even though there are many

up-to-date and far more comprehensive reviews of this subject. The reason for including this section is to highlight those aspects that are relevant to the interpretation of structure–activity relationships and the development of receptor concepts.

The review concludes with an account of the authors' own work in replacing the 17β-lactone with a series of open-chain substituents. On the basis of this work, a model is proposed for that part of the "digitalis receptor" that accommodates the lactone and its biologically active isosteres.

PROPERTIES OF NATURALLY OCCURRING CARDIOTONIC STEROIDS

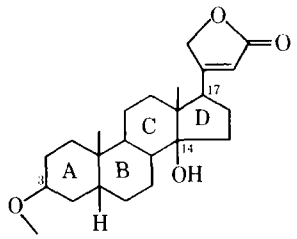
Any discussion of structure–activity relationships among cardiac glycosides and related compounds must take note of the problems associated with the determination of cardiotonic and cardiotoxic activity. Considerable variations in both actual and relative potency are observed when compounds are compared using different test preparations and different species (19).

The common practice of using toxicity measurements for the initial assessment of potency of cardioactive compounds can be misleading (19, 20). Wolff *et al.* (21) showed that newly synthesized compounds that displayed cardioactive properties when tested by the cat toxicity method showed no cardiotonic properties when tested on the isolated rabbit atrium preparation. This finding does not imply a separation of the two facets of digitalis-like activity but rather that the cardiotoxic properties measured were not of the digitalis-like type.

The significance of reported cardiotonic activity is thus difficult to assess, at least in quantitative terms. The determination of digitalis-like activity of new compounds needs to be based on a spectrum of test procedures, and a selection of well-tested reference compounds must be included in the test program. Details of various biological procedures used in estimating cardiotonic activity were extensively reviewed (18). Factors that modify the action of cardiotonic steroids on heart function also were reviewed recently (17).

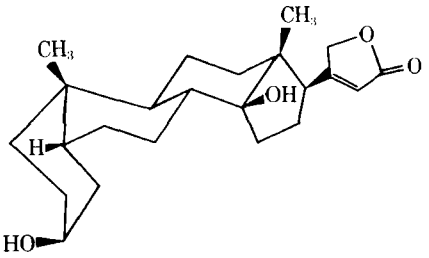
Within the constraints discussed, the following generalizations may be made. Qualitatively, the physiological activities of the naturally occurring digitalis-like compounds are similar, implying a fundamental similarity in their mechanism of action. Quantitatively, individual members of this class of compounds exhibit differences in potency and duration of action. However, the therapeutic ratios of most, if not all, of these compounds are similar, supporting the widely held beliefs that the toxic effects of digitalis-like compounds are an extension of the positive inotropic effects and, hence, that it would not be possible to improve therapeutic ratios by molecular modification. These assumptions have yet to be adequately tested, but it must be emphasized that the term therapeutic ratio has no absolute meaning and will vary according to what portions of the log dose–response curves are compared.

Digitoxin (a cardenolide) (I), digitoxigenin (II), and bufalin (a bufadienolide) (III) are representative examples of naturally occurring cardiotonic steroids. The biological activity of these molecules is thought to arise as a result of a specific interaction between a receptor and the aglycone (or genin) portion of the molecule. If the glycoside is cleaved, the genin retains activity whereas the isolated sugars are devoid of cardiotonic effects (22). However, the presence of the sugars is thought to retard detoxification effectively and to confer on the molecule certain physical properties important for uptake and distribution (23). These observations are important in the design of new cardiotonic steroids since a molecule with a free hydroxy group at C-3 may undergo rapid metabolism and show no useful activity (23). In other words, modification of the side chain at C-3 is an appropriate means of varying the pharmacokinetic properties of cardiotonic steroids.

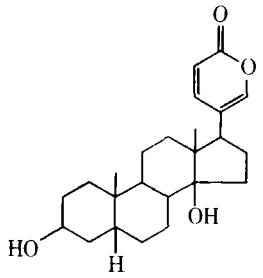


(digitoxose)$_3$

I: digitoxin (cardenolide)



II: digitoxigenin



III: bufalin (bufadienolide)

The stereochemistry of the steroid portion of cardiac glycosides is unusual in that the C/D and usually the A/B ring junctions have the *cis*-configuration (II). The general chemistry of the cardiac glycosides and the genins has been well documented (24, 25), and the chemistry of the sugar residues of natural and semisynthetic glycosides has been reviewed (26, 27).

For many years, studies on structure–activity relationships of cardiac glycosides have been limited to the determination of relative potency ratios of naturally occurring glycosides or to relatively minor semisynthetic modifications of functional groups com-

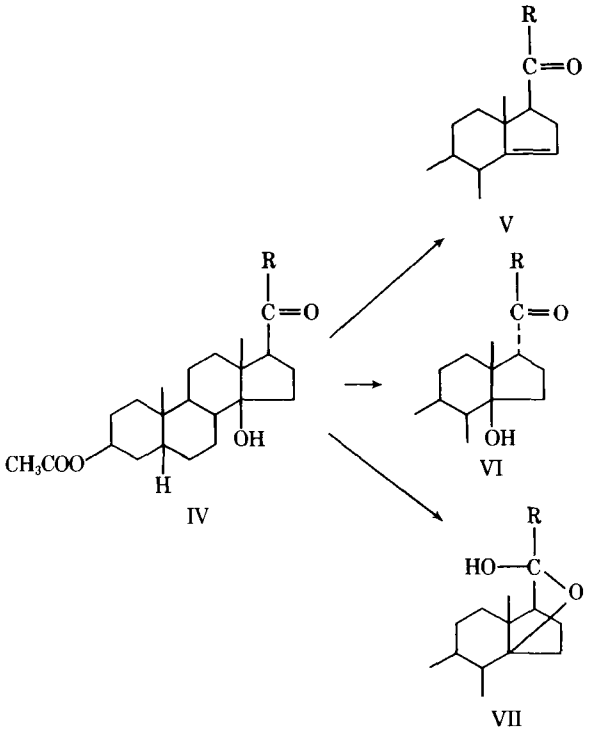
mon to this class. The early work in this field [reviewed by Tamm (22) and Chen (28)] established three structural features thought to be associated with cardiotonic activity (29):

1. *The unsaturated lactone at C-17.* This is present either as a butenolide ring in the cardenolides (*e.g.*, I and II) or as a 2-pyrone ring in the bufadienolides (*e.g.*, III). Saturation of the lactone ring results in considerable loss in activity (19, 30).

2. *The stereochemistry of the steroid molecule.* A C/D *cis*-configuration in conjunction with β-orientation of the lactone ring at C-17 is the basic stereochemical requirement for cardiotonic activity. Alteration of the stereochemistry at either of these centers results in abolition of activity. The 17α-cardenolides, for example, are inactive (22), as is the recently synthesized 14α-digitoxigenin (31). The 14α,17α-cardenolides also have been shown to be devoid of activity (32). The *cis*-configuration of the A/B ring junction, when present, confers maximum activity. Epimerization to the 5α-configuration, as found in uzarigenin (5α-digitoxigenin), results in a reduction in potency. Uzarigenin is approximately one-half as potent as digitoxigenin (22).

3. *The hydroxy groups at C-3 and C-14.* Potency is greatest when the β-hydroxy group at C-3 is combined as a glycoside and is generally reduced when this group is oxidized or esterified (19). Epimerization to the 3α-configuration leads to almost complete loss in activity (23). Recent studies on the importance of the oxygen function at C-3 showed that 3-desoxydigitoxigenin, synthesized independently by Zürcher *et al.* (31) and by Saito *et al.* (33), is almost equipotent with digitoxigenin when tested for its effects on isolated cardiac muscle (34) or for its inhibitory action on Na^+,K^+-ATPase (31). This indicates that the 3β-hydroxy is not an indispensable requirement for cardiotonic activity.

Likewise, the 14β-hydroxy group is not an absolute requirement for activity, but its presence enhances potency. This group may be replaced by a 14β,15β-oxido group as occurs in resibufogenin or in 14β,15β-epoxy-β-anhydrodigitoxigenin with the retention of activity (35–37). The fact that a 14β-oxygen function is not an essential requirement for cardiotonic activity was confirmed by the synthesis of 14-desoxy-14β-uzarigenin (38), which was shown to possess a definite cardiotonic activity, somewhat less than that of uzarigenin (39).



Scheme I

DEVELOPMENT OF SYNTHETIC METHODS

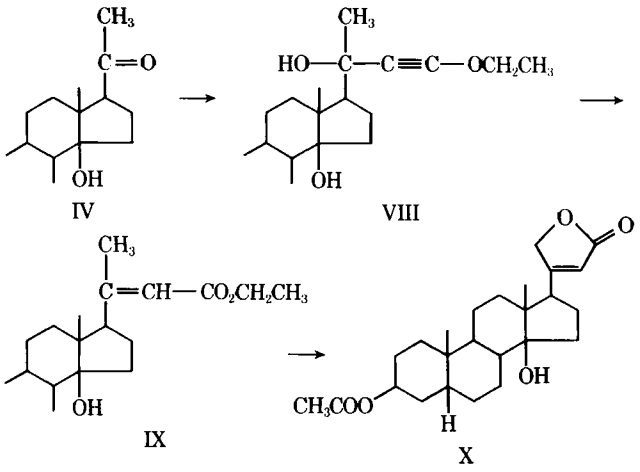
The difficulties presented by the chemistry of the cardioactive genins are demonstrated by the considerable amount of work carried out over many years aimed at the total synthesis of naturally occurring cardenolides. In the 1940's, Ruzika, Plattner, and colleagues and Elderfield and coworkers achieved efficient methods for the synthesis of the 17β-butenolide ring and the introduction of the 14β-hydroxy group into model substances. But the goal of introducing both of these functional groups into the one steroid molecule while still maintaining the C/D ring junction in the *cis*-configuration was not achieved.

These studies were resumed in 1956, and in 1962 the successful synthesis of digitoxigenin (II) was reported (40). In a review, Sondheimer (41) drew attention to three potential difficulties involved in the construction of the 17β-lactone from 14β-hydroxy-20-oxosteroids (Scheme I). First, 14β-hydroxy steroids (IV) are readily dehydrated with acids to give the corresponding Δ^{14}-compounds (V), and no practical method for protection of this group is known*. Reactions in which strong Lewis acids are present, *e.g.*, the Reformatsky reaction, are, therefore, excluded. Second, the 17β-side chain of 14β-hydroxy steroids exists in a thermodynamically unstable configuration, and conversion to the more stable 17α-isomer (VI) occurs if possible. Third, interaction between the 14β-hydroxy group and the 17β-side chain can take place and, in the case of 20-oxosteroids, the relatively unreactive hemiketal (VII) is produced.

Sondheimer's successful synthesis was eventually achieved by reacting the ketone (IV, R = CH_3) with lithium ethoxyacetylide in tetrahydrofuran followed by mild treatment of the ethoxyacetylenic carbinol (VIII) with dilute acid. Oxidation of the resulting α,β-unsaturated ester (IX) with selenium dioxide resulted in about 30% yield of digitoxigenin acetate (X) (Scheme II). Since the work of Sondheimer, other successful syntheses of naturally occurring cardenolides have been reported, and some useful synthetic methods have emerged (42–45).

Similarly, much work has also been directed at the total syntheses of the second group of cardioactive

**Note added in press:* It was recently shown that the 14β-hydroxy group of digitoxigenin can be acetylated using isopropenyl acetate and *p*-toluenesulfonic acid (method developed by R. Rawson, Syntex Research, Palo Alto, Calif., personal communication).



Scheme II

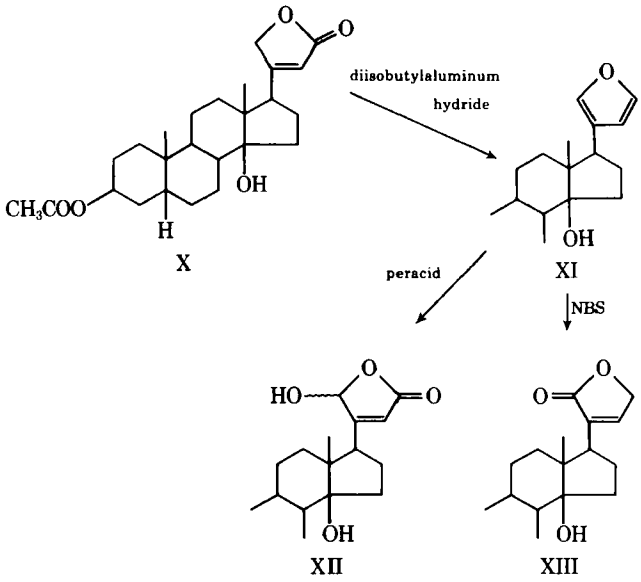
steroids, the bufadienolides. Only recently has this goal been achieved (46) by the synthesis of bufalin (III) and resibufogenin from common steroids. Following this report, other workers have also reported the successful syntheses of naturally occurring bufadienolides and other steroidal 5′-substituted 2-pyrones (47–50).

STRUCTURAL MODIFICATIONS OF CARDIOTONIC GENINS

The value of steroids as hormones and as hormonal-type drugs is probably due to the fact that the deployment of a number of simple functional groups around the rigid steroid moiety enables a wide variety of unique coding systems to be achieved. It is not surprising then that the bulk of the efforts devoted to the chemical modification of cardioactive genins has been designed to delineate the effects of the various substituents and functional groups on physiological potency. Recent publications containing somewhat limited reviews on new cardioactive steroids have been published (51–58).

The major emphasis in this section centers on the chemistry of new compounds synthesized as potential digitalis-like substances. Brief reference to biological effects is given where the information is available and seems of significance. In many cases, biological data were not given or, in the case of compounds found in the patents section of *Chemical Abstracts*, were not readily accessible. The transformation of cardioactive genins into structural isomers of steroid hormones has also received some attention (59, 60); but since these compounds are not cardiac stimulant substances, they will not be discussed further.

Modification of 17β-Lactone Ring—This functional group is common to all cardiac glycosides, and several studies have established its importance in the biological activity of this compound class. Until recently, only a few modifications of the lactone ring had been made, including saturation of the double bond and epimerization to the 17α-configuration. Methods of replacing the lactone ring with other isosteric hetero rings have been investigated (61). A route has been devised to convert the butenolide ring of the cardenolide genins to a furan ring bonded at

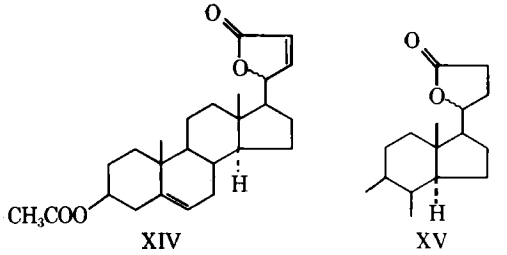


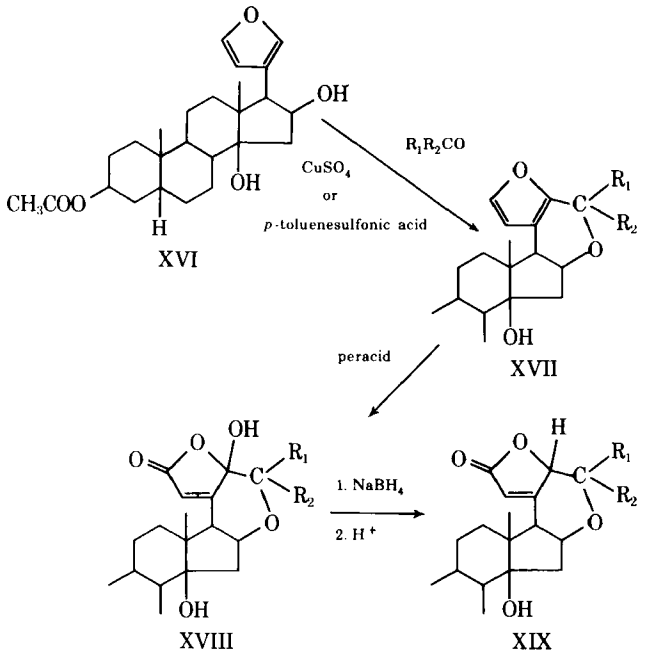
Scheme III

the 3-position to C-17 of the steroid (62) (XI). For example, diisobutylaluminum hydride reduction of digitoxigenin 3-acetate (X) gave the corresponding 17β-(3-furyl) derivative (XI). Such a compound can be oxidized in different ways to give either the 21-hydroxy derivative (XII) of the starting cardenolide or an isomeric cardenolide (XIII) (Scheme III) (63). The latter compound is of particular interest because it differs from the natural cardenolide only in the position of attachment of the butenolide ring to the steroid nucleus.

Several 17β-(3-furyl) steroids derived from naturally occurring cardenolides have been prepared for biological evaluation (64). Minesita *et al.* (65) reported that 17β-(3-furyl) steroids such as XI exhibit cardiotonic activity comparable to the cardenolides. This suggests that the unsaturated lactone is not essential for physiological activity and can be substituted by an isosteric hetero ring. Similarly, many isomeric cardenolides have been prepared by oxidation of 17-furyl steroids (66). Deghenghi (53) reported that the isomeric cardenolides such as XIII increased the force of myocardial contraction in dogs with pentobarbital-induced heart failure, with a potency comparable to digitoxigenin (II). It was also claimed that this compound elicited an inotropic activity at a dose considerably smaller than that producing toxic effects. This property is interesting because the main problem involving the clinical use of cardiac glycosides is related to their narrow therapeutic ratio.

Pettit *et al.* (67) described another isomeric cardenolide (XIV) in which the lactone ring was substi-





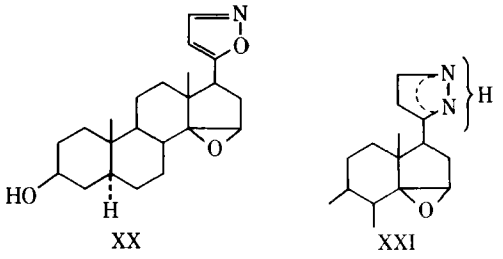
Scheme IV

tuted as the γ-carbon instead of the β- or α-carbon found, respectively, in the natural cardenolides or in the previously described isomeric cardenolide (XIII). Compound XIV and its butenolide analog (XV) (68) also differ from the natural series in that they lack an hydroxy group at C-14 and the C/D ring junction has the *trans*-configuration. Both these compounds were biologically inactive.

Further investigation of the 17β-furyl steroids (69) showed that 17β-(3-furyl) derivatives of 16β-hydroxycardenolides (XVI) underwent a novel condensation with simple carbonyl compounds in the presence of copper sulfate or *p*-toluenesulfonic acid to form the bicyclic derivative (XVII). Oxidation of XVII with peracid, as previously described, gave the lactol (XVIII) which, on reduction with sodium borohydride, gave the bicyclic derivative (XIX) which incorporates the original unsaturated lactone ring (Scheme IV). The biological activity of these compounds was not reported.

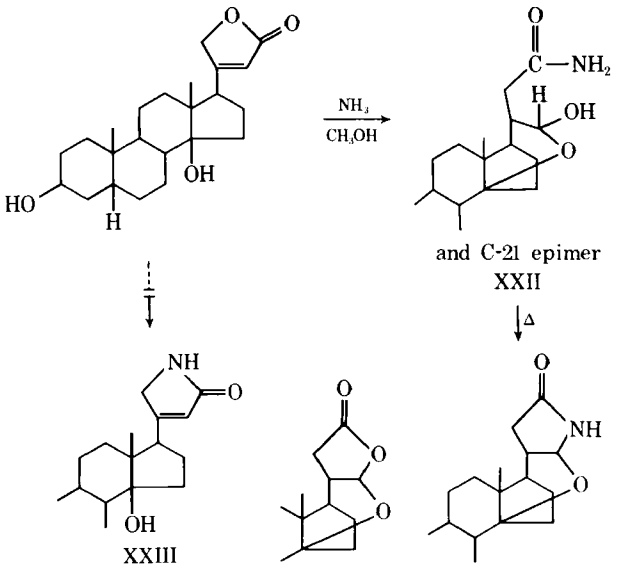
Nambara *et al.* (70) recently described the preparation of 17β-(5-isoxazolyl) and 17β-(3-pyrazolyl) derivatives of 14β,15β-oxidosteroids (XX and XXI, respectively) as potential cardiotonic compounds. In each case the lactone ring of the original cardenolide was replaced with an isosteric hetero ring. Both these compounds possess the 14β,17β-configuration essential for physiological activity in the natural cardenolides. For biological comparison, the 17α,14β-isomers were also prepared[1]. These workers felt that biological assay of the 14,17-*cis*-steroids (XX and XXI) may indicate the significance of the oxygen atom attached to C-21 of the cardioactive cardenolides.

A lactam analog (XXIV) of isodigitoxigenin has been described (71, 72). It was obtained by ammonolysis of digitoxigenin in methanol solution to give the lactol amide (XXII) which, on heating at 200° or on treatment with acid, cyclized to give the lactam (XXIV) (Scheme V). In no instance was the γ-crotonolactam (XXIII) obtained, probably due to the tendency of cardenolides to cyclize to the "iso" form in basic medium. Isodigitoxigenin (XXV) was considered to be an intermediate in the reaction.



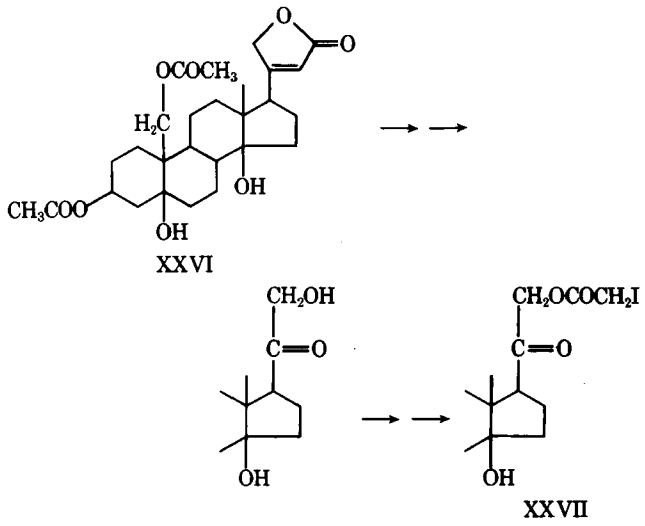
The influence of the lactam (XXIV) on the response elicited by physiologically active cardenolides on three different biological systems was examined. Although the lactam was devoid of cardiotonic activity, it inhibited the early inotropic response of acetyldigitoxigenin on isolated guinea pig atria. However, the effect of acetyldigitoxigenin on cat toxicity or on Na^+,K^+-dependent ATPase was not affected by the lactam. It was thought that the lactam may inhibit the mechanism responsible for transport of cardiotonic steroids across the cell membrane and, hence, increases the time required for the cardenolide to reach optimal concentrations at the inotropic receptor. Examination of acetylisodigitoxigenin (XXV) revealed that this compound also inhibited the positive inotropic response of guinea pig atria to cardenolides. Compounds of this nature (73) may prove useful in elucidating the mode of action of cardenolides.

Further studies relating to the role of the lactone ring in the biological activity of cardiac glycosides followed the discovery (74) that the 20-oxo-21-iodoacetate (XXVII) derived from the degradation of strophanthidol diacetate (XXVI) (Scheme VI) exhibited cardioactivity when tested on cats. The activity of



Scheme V

[1] The compounds prepared by Nambara *et al.* (70), described here and elsewhere in this review, were tested on the isolated frog heart preparation and found to be inactive (personal communication).



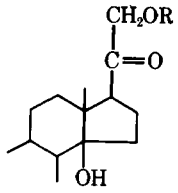
XXVI XXVII

Scheme VI

this compound was originally interpreted as a cardiotonic effect, resulting from the alkylating properties of the iodoacetate group. This finding led to the synthesis of a number of related compounds (75) in which the butenolide ring of strophanthidol and digitoxigenin was replaced with functional groups considered capable of reacting with sulfhydryl groups (XXVIII and XXIX). The basis of design of these compounds was the hypotheses (76) that alkylation of essential sulfhydryl groups on the receptor may be involved in drug action and that the unsaturated lactone or the iodoacetate group may perform this function.

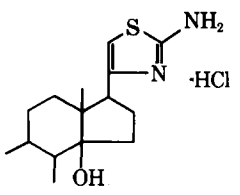
An analogous series of C-17α-compounds was prepared together with another series of steroids containing the same alkylating groups attached to different steroid moieties. Tests on isolated rabbit atria showed that all compounds prepared were devoid of cardiotonic activity. It was concluded that the observed cardioactivity, when tested on cats, was a cardiotoxic action unrelated to any cardiotonic effects. Such findings emphasize the care required when interpreting biological data on new compounds.

Other modified cardenolides capable of acting as alkylating agents have also been described (77). These compounds possess a maleimide function at

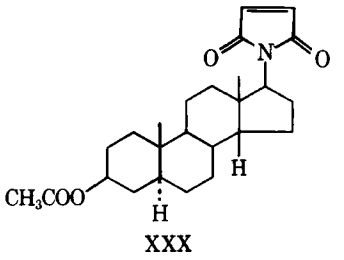


R_1 = $COCH_2Cl$, $COCH=CH_2$, COC_2H_5, or $CO(CH_2)_2CO_2H$

XXVIII



XXIX

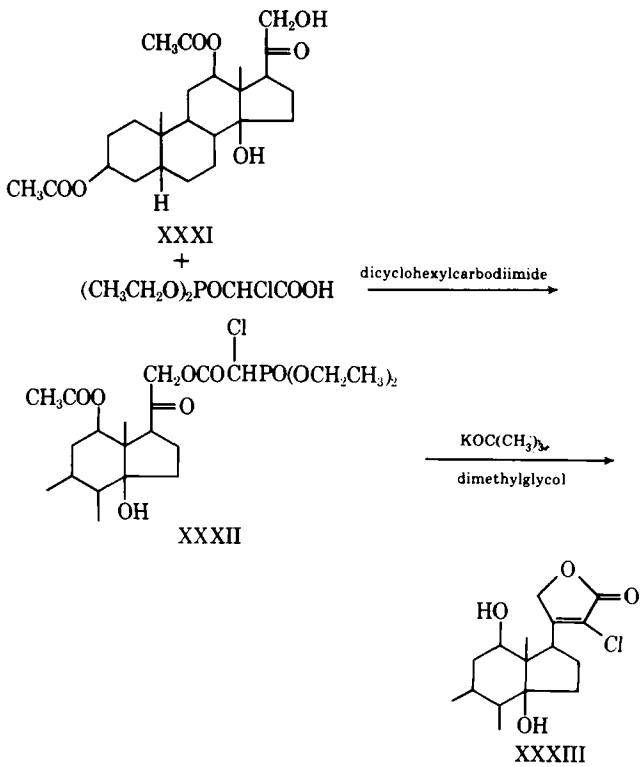


XXX

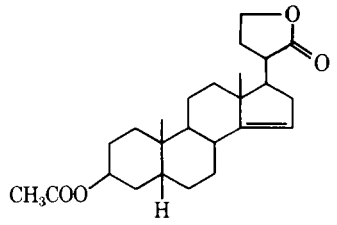
C-17, which is another functional group considered capable of reacting with sulfhydryl groups. Compound XXX fulfills the stereochemical requirements at C-14 and C-17 for cardiotonic activity. Biological evaluation has yet to be reported, but following the results obtained (75) it seems unlikely that alkylation of sulfhydryl groups is responsible for cardiotonic activity.

The introduction of halogen, alkyl, and alkoxy substituents into the 22-position of the lactone ring has been reported (78). The compounds were prepared by condensing the 21-hydroxy-5β-pregn-20-one derivatives (XXXI) of a series of cardiotonic steroids with appropriate phosphonate reagents followed by intramolecular PO-activated cyclization. A typical sequence is shown in Scheme VII. The authors briefly referred to the biological activity of these compounds, stating that contraction-promoting activity was increased by introduction of a fluoro group and decreased by introduction of a methoxy group. These observations are compatible with the model for the digitalis receptor proposed under *Structure–Activity Relationships.*

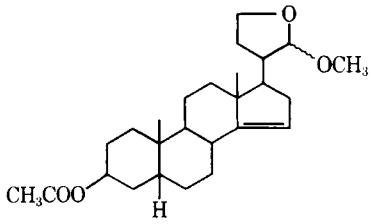
Isomeric cardanolides (XXXIV and XXXV) have



Scheme VII



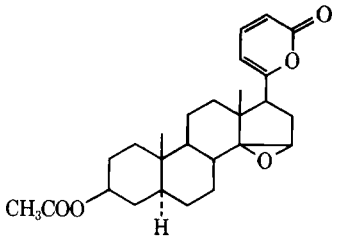
XXXIV



XXXV

been prepared and found to be weak inhibitors of Na^+,K^+-ATPase and Mg^{+2}-ATPase (79).

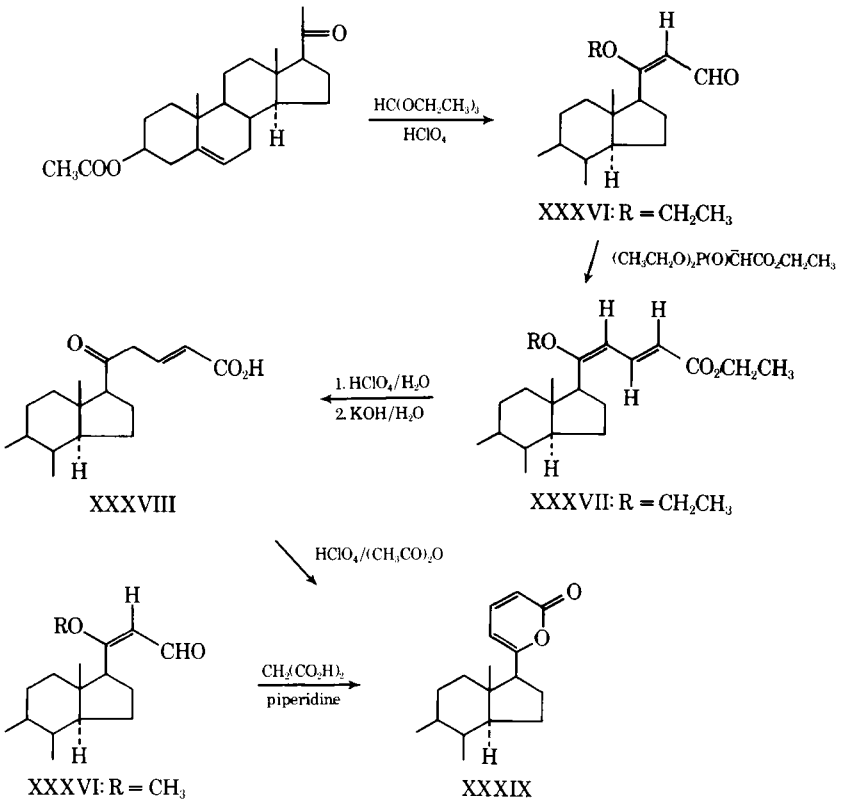
The 5′-substituted 2-pyrone ring of the naturally occurring bufadienolide genins has also received some attention, but few modifications of this group have been made. Their biological characteristics, including anticancer activity, have promoted the development of synthetic approaches to isomeric bufadienolides in which the 2-pyrone ring is linked at C-17 to one of three alternative positions (3′, 4′, or 6′). Details of the synthesis of 6′-isobufadienolides (XXXIX) were described (80) but no biological data on the compounds prepared were given. Condensation of the enol ether-aldehyde (XXXVI) with the ethoxycarbonylmethyldiethylphosphonate carbanion gave the *cis,trans*-ester (XXXVII) which, after hydrolysis to the unsaturated keto-acid (XXXVIII), readily cyclized to give the 6′-isobufadienolide (XXXIX) (Scheme VIII). Better yields of XXXIX were obtained directly from the enol ether-aldehyde (XXXVI) by condensation with malonic acid in the presence of a secondary amine (Scheme VIII). The 6′-isobufadienolides synthesized were of the C/D *trans*-series, and it is unlikely that they possess cardiotonic activity.



XL

The malonic acid condensation with steroidal enol ether-aldehydes such as XXXVI has been investigated (81–84) in attempts to prepare 6′-isobufadienolides with differing configurations at C-14 and C-17. Successful synthesis of 14β,15β-oxido-6′-isobufadienolide (XL) (82) gave a compound that was stereochemically related to the natural genins, but no biological data were included in the report.

Replacement of Lactone Ring with Open-Chain Structures—As part of a general study on the mode of action of cardiotonic steroids, Boutagy and Thomas recently replaced the lactone ring of digitoxigenin with a series of analogous open-chain structures (85–87). The purpose of this particular study was to define structure–activity relationships



Scheme VIII

and to generate a series of new compounds with potential value as therapeutic agents and as tools for exploring and correlating the biochemical and physiological manifestations of digitalis-like activity.

The general plan of the series of compounds synthesized is shown in Scheme IX. Digitoxigenin (II) was first converted to a 17β-formyl steroid (XLIV), which was the key intermediate for the series of compounds. This intermediate was then condensed with a series of phosphonate reagents or with various hydrazine derivatives, such as guanylhydrazine, to give the required compounds. The following types of changes were attempted:

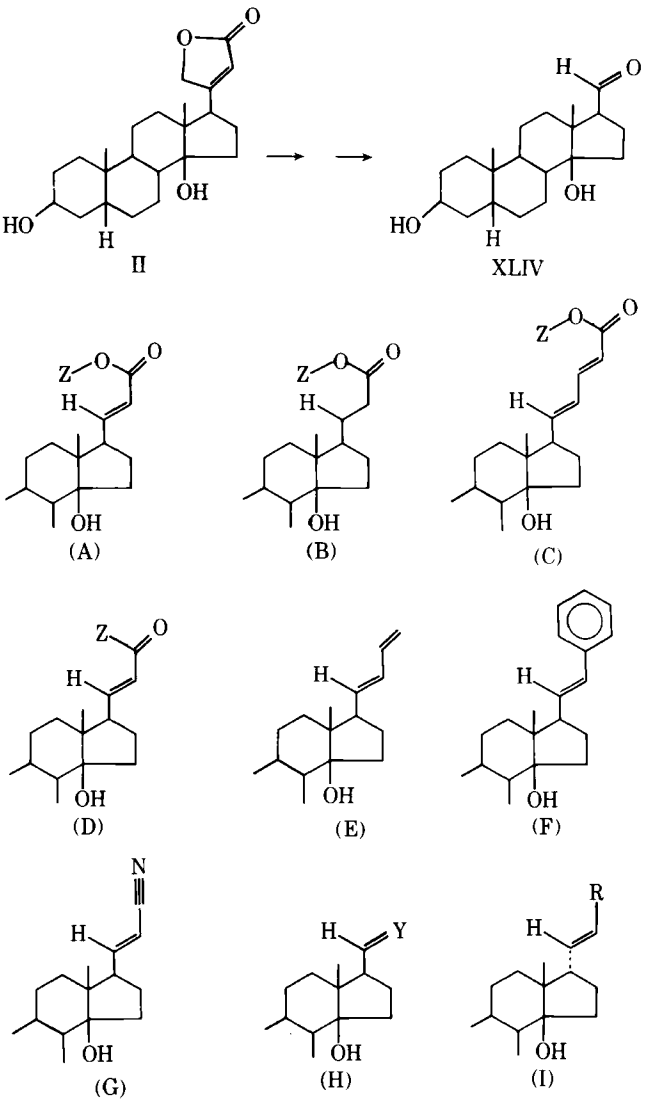
1. Replacement of the lactone by a *trans*-α,β-unsaturated ester (or acid) moiety (A), the bulk of which was varied by systematically increasing the size of group Z and by introducing substituents on the α-carbon atom.
2. Abolition of the conjugation of the carbonyl group (B).
3. Extension of the conjugation of the carbonyl group and expansion of the distance between the carbonyl oxygen and ring D (C). The bulk of the alkoxy moiety was changed by varying the size of group Z.
4. The role of the ether oxygen of (A) was studied by replacing the alkoxy group with an alkyl or aryl group or groups containing nitrogen (D).
5. Both the ether oxygen and carbonyl group were replaced by other electron dense groups such as C=C< (E), aryl (F), and C≡N (G).
6. The C-20 carbon atom was attached directly to a hetero atom (H), for example by preparing the hydrazone and related derivatives.
7. The importance of the 17β-configuration was studied by preparing a similar series of compounds possessing the 17α-configuration (I).

Most compounds described were prepared by condensing the C-17 formyl intermediate (XLIV) with a variety of phosphonate reagents. These reagents, if they are of the appropriate type, will condense with carbonyl compounds under relatively mild conditions to form olefins in good yield. Under most conditions, the predominant product is the *trans*-olefin, a necessary property if the products are to be isosteric with the butenolide ring. The mechanism and scope of olefin synthesis using phosphonate carbanions were reviewed recently (88).

The reaction sequence used for the preparation of the intermediate etianaldehyde (XLIV) and for a typical α,β-unsaturated ester is shown in Scheme X (85).

Other reactions used for the preparation of C-17β-*trans*-(E)-unsaturated esters and derivatives are shown in Schemes XI and XII (86). Other compounds prepared in this study are shown in Schemes XIII–XVII (87).

The compounds shown in Schemes IX–XVII were tested for their ability to induce a positive inotropic effect in guinea pig hearts[2] and for their ability to inhibit guinea pig atrial Na^+,K^+-ATPase (89). A spectrum of activities was observed, and the results were



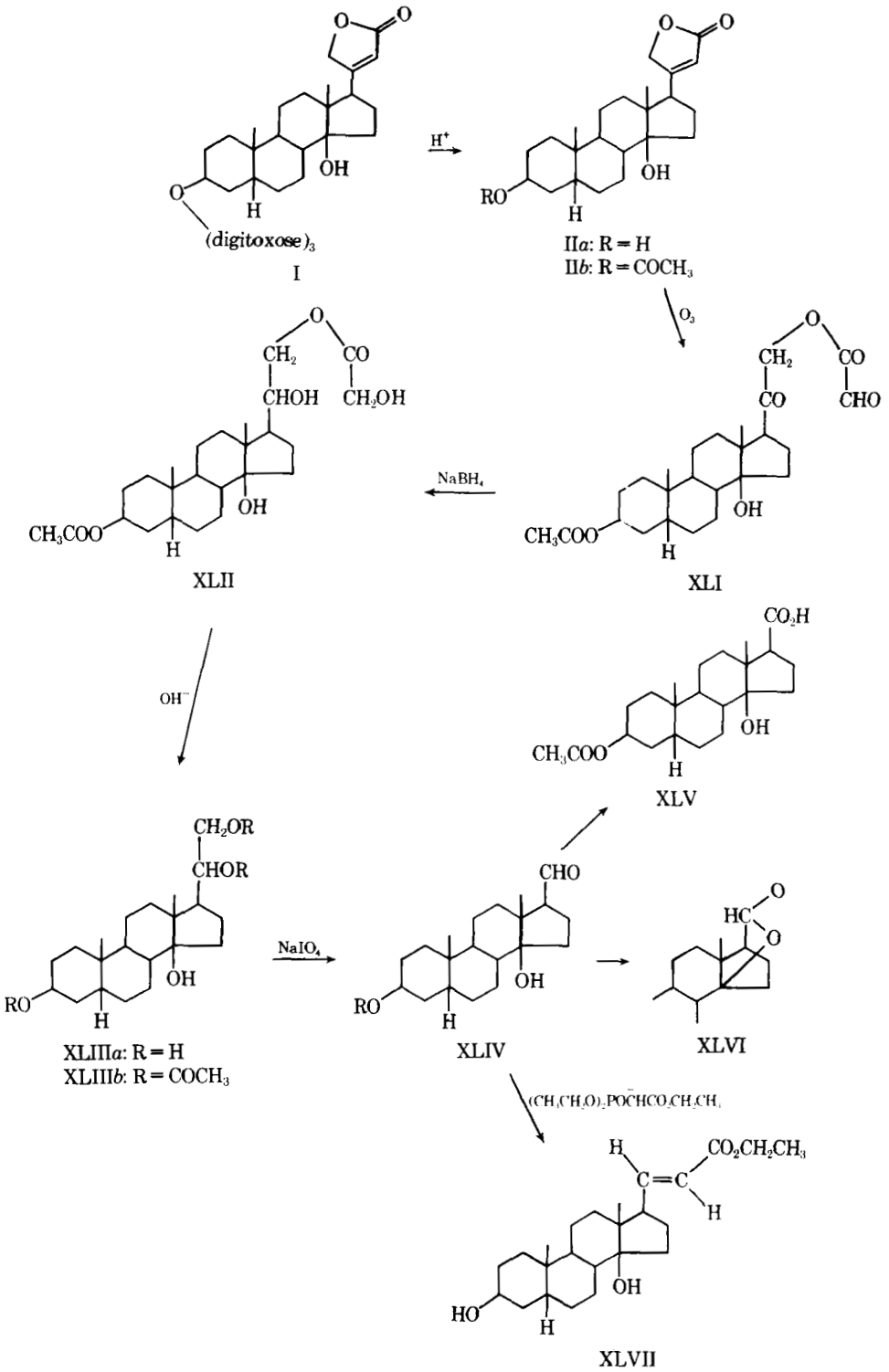
Scheme IX—Classification of digitoxigenin analogs prepared by Boutagy and Thomas (85–87)

used to infer a model of that part of the digitalis receptor that accommodates the C-17 side chain of cardiotonic steroids. These results are discussed further under *Structure–Activity Relationships.*

Modification of Steroid System—Analogs containing an unsaturated lactone attached to the C-17 position of a modified steroid system may be prepared either by attaching the lactone to an appropriate steroid or by modifying one of the many naturally occurring cardenolides or bufadienolides. Numerous such compounds have been synthesized, and many are described in the general reviews already cited. This review will deal only with recent developments in this field.

Attachment of Butenolide Ring—Two novel and relatively simple methods have emerged that were effective in the synthesis of the butenolide ring at C-17 of various steroid nuclei. The first method involves the use of a Wittig or phosphonate modification of the Wittig reaction with 20-oxo-21-hydroxy steroids (LXXX) followed by an intramolecular cyclization (90–93). A variation of this method involves a direct intramolecular Wittig-type condensation using 20-

[2] To be reported.



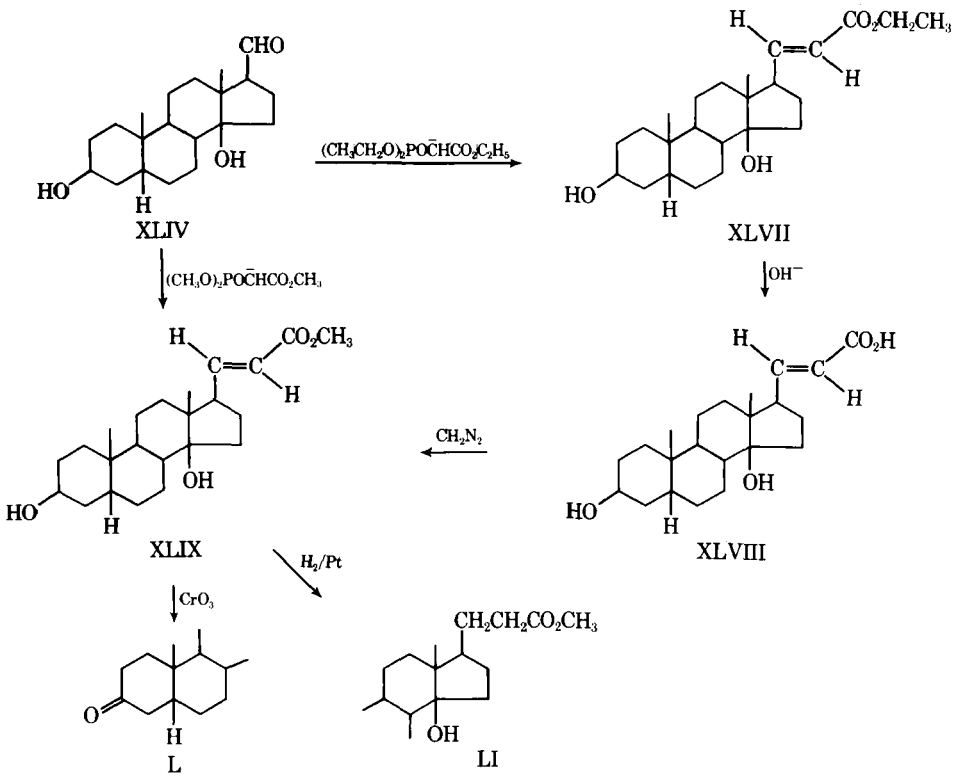
Scheme X—Synthetic sequence to the etianaldehyde (XLIV) and α,β-unsaturated ester (XLVII) (85)

oxo-21-acyl steroids such as LXXXI and LXXXV (94, 95) (Scheme XVIII).

The second method utilizes peracid oxidation of 17-furyl steroids (LXXXVI) (which are readily accessible from 17-oxo steroids) to form 21-hydroxycardenolides (LXXXVII) (53). Reduction of the latter with sodium borohydride, as previously described, gives the cardenolide (LXXXVIII) (Scheme XIX).

Introduction and Modification of Other Functional Groups—The introduction of various substituents into the steroid nucleus and modification at molecular centers associated with cardiac activity have produced numerous new compounds, some of which were discussed under *Properties of Naturally Occurring Cardiotonic Steroids*. Some recent work will now be reviewed.

The synthesis of a cardiotonic glycoside (XC) with an aromatic ring A has been described (96). The compound was prepared from the 3,14β-dihydroxy-19-norcarda-$\Delta^{1,3,5(10),20(22)}$-tetraenolide (LXXXIX) (97)



Scheme XI—Synthesis of C-17-trans-(E)-α,β-unsaturated esters and derivatives (86)

by glycosidation with acetobromoglucose and silver oxide (Scheme XX). No biological activity was reported.

Compound XC is of potential interest because of a previous report (98) that the $\Delta^{1,4}$-3-oxo derivative of digitoxigenin (XCI) may have an improved therapeutic ratio.

Danieli *et al.* (99) described the synthesis of a 17α-hydroxycardenolide (XCII) by selenium dioxide oxidation of digitoxigenin 3-acetate (Scheme XXI). The 17α-hydroxy group was shown not to influence the cardiotonic activity of digitoxigenin, although inversion of the two C-17 substituents abolished activity (33).

The biological consequences of inverting the C-16 hydroxy group of gitoxin to produce 16-epigitoxin has been studied (100). Compared with gitoxin (XCIII) and ouabain, the epi compound showed a greater difference between doses, producing minimum and maximum inotropic effects as well as having a greater influence upon myocardial contractility and a lesser effect on the pacemaker activity of the Purkinje conductive system. The authors made the important conclusion that the therapeutic and toxic properties of digitalis-like compounds may be partially dissociated by this type of chemical modification. 16-Epigitoxin was prepared by treatment of gitoxin with strong alkali and water (Scheme XXII) (100).

The pharmacological consequences of introducing oxygen functions at C-15 were recently reviewed (57). 15-Oxodigitoxigenin (XCV) was shown to be biologically active whereas 15α-hydroxydigitoxigenin (XCVI) was inactive (35, 36). One study (101) showed that 14α-artebufogenin (XCVII) was inactive whereas the 14β-isomer (XCVIII) was active. The corresponding butenolide analogs (XCIX and C) were both inactive. Chen and Henderson (102) made similar studies.

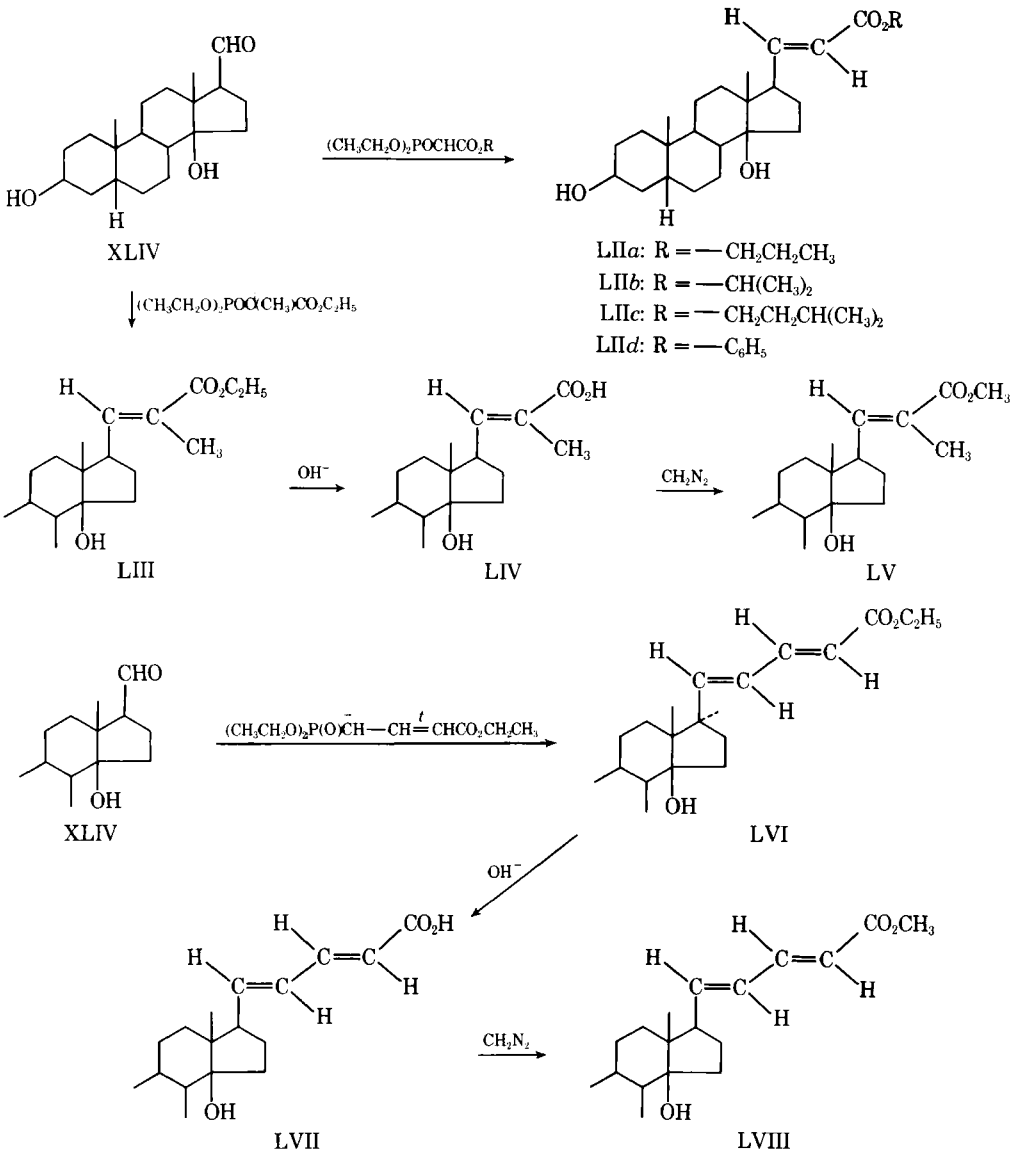
Recently, Okada and Saito (103) described the synthesis of a series of 15α- and 15β-hydroxycardenolides. The series included compounds with the usual digitalis-type stereochemistry, together with C-17 epimers and compounds with the *trans*-C/D junction. No biological activity was reported.

Wolff and Ho (104, 105) described the synthesis of 19-halo and 5,10-cyclo derivatives of strophanthidin. Several other derivatives of strophanthidin were synthesized and tested (106); the 3-haloacetate, 3-diazoacetate, and 19-oxime displayed considerable biological activity when tested on isolated cardiac muscle, Na^+,K^+-dependent ATPase, and tumor cells in tissue culture (cytotoxic activity).

Studies by Hokin *et al.* (107) showed that the 3-haloacetates of strophanthidin (CI) were biological alkylating agents and acted as active site-directed irreversible inhibitors of Na^+,K^+-ATPase.

Similar alkylating agents were prepared (108) from digitoxigenin, digoxigenin, 15α-hydroxydigoxigenin, and Δ^{14}-anhydrodigitoxigenin. All compounds were effective irreversible inhibitors of Na^+,K^+-ATPase. The fact that 15α-bromoacetoxydigitoxigenin 3-acetate was active whereas the parent 15α-hydroxy compound was inactive is interesting in view of the belief that α-substituents may interfere with drug–receptor interactions.

Significant cardiotonic activity was found in the 3,3-ethylenedioxy and the 3-tetrahydropyranyl ether derivatives of digitoxigenin and digoxigenin (12β-hydroxydigitoxigenin) (109). Another series of ketal



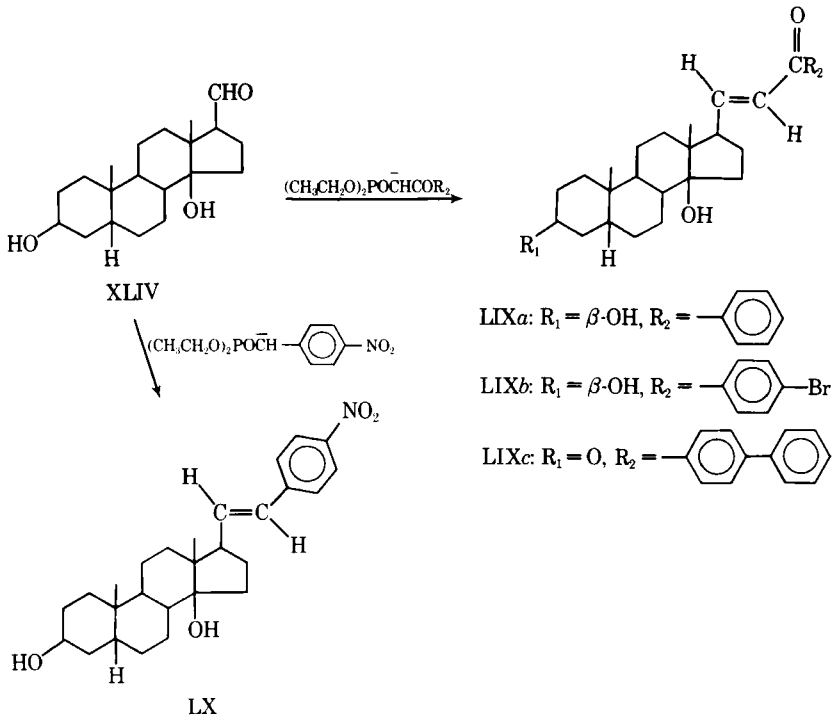
Scheme XII—Synthesis of C-17β-α,β-unsaturated esters and derivatives (86)

derivatives of 3-oxocardenolides was reported in the patent literature (110).

Other chemical modifications of cardiac genins which appeared recently include the 19-cyanomethylene derivative of strophanthidin and related 19-oxo genins (111); the thio analog of 14,15-epoxy genins (112); the 16-methyl- or 16-cyano-substituted cardenolides (32); the 14,15-*cis*-diol derivatives of digitoxigenin (113); the alkoxy and cyclocarbonate derivatives of gitoxin (114); the suberoxylalanine ester of digitoxigenin (115); the cardenolide nicotinates (116), nitrates (117), and glycerates (118); the 3-pyranyl ether derivatives of cardenolides (119), and some 4-chloro derivatives of cardenolides (120). The substitution of the 3-hydroxy group of digitoxigenin with a series of simple nitrogen-containing functions has been described (57), as has the preparation of glycosides of amino-sugars (121–123).

Modification of Pharmacokinetic Properties—Most compounds discussed in the preceding sections were synthesized with the objective of defining structure–activity relationships with respect to the pharmacodynamic properties of cardiotonic steroids. Recently, there has been much interest in the study of the pharmacokinetics[3] of digitalis (124–126) as a means of improving the therapeutic properties of these agents and reducing the incidence of toxicity. Part of the problem of digitalis toxicity arises because of the unsatisfactory nature of the physical properties of the commonly used cardiotonic steroids. Those glycosides, such as digoxin, that are sufficiently nonpolar to be effectively absorbed show a high degree of accumulation (127). The pharmacokinetic properties of the ideal cardiotonic steroid were recently defined (58) as being *(a)* complete absorption, *(b)* rapid onset of action, and *(c)* reduced accumulation as a result of an increased rate of elimination.

[3] The term pharmacokinetics refers to all factors (absorption, metabolism, excretion, *etc.*) that control the rate and the extent to which the drug reaches its site of action. The term pharmacodynamics refers to all processes involved in the interaction of the drug with the responding system and the subsequent manifestation of response.



Scheme XIII—Synthesis of C-17β-α,β-unsaturated keto and aryl derivatives (87)

Based on previous experience, these properties should be found in compounds that have been chemically modified so as to increase their hydrophobic character but which, after or during absorption, are converted to more polar derivatives which are rapidly excreted. Recently, there have been several attempts to synthesize such compounds.

One of the first attempts to modify deliberately the pharmacokinetic properties of cardiac glycosides was the work of Megges and Repke (128), who studied the penta-acetyl derivative of gitoxin (16β-hydroxydigitoxin). Because of its low lipid solubility, gitoxin is very poorly absorbed and is virtually inactive when given by the oral route. Conversion to the penta-acetyl derivative gave a compound whose rate and extent of absorption exceeded those of the highly lipid-soluble digitoxin. During and after absorption, the compound was rapidly deacetylated to yield the parent compound, gitoxin, which was then eliminated at a much faster rate than digitoxin. A similar rationale was applied in the design of two recently introduced derivatives of digoxin: β-acetyldigoxin (4‴-acetyldigoxin) (CII*b*) and β-methyldigoxin (4‴-methyldigoxin) (CII*c*).

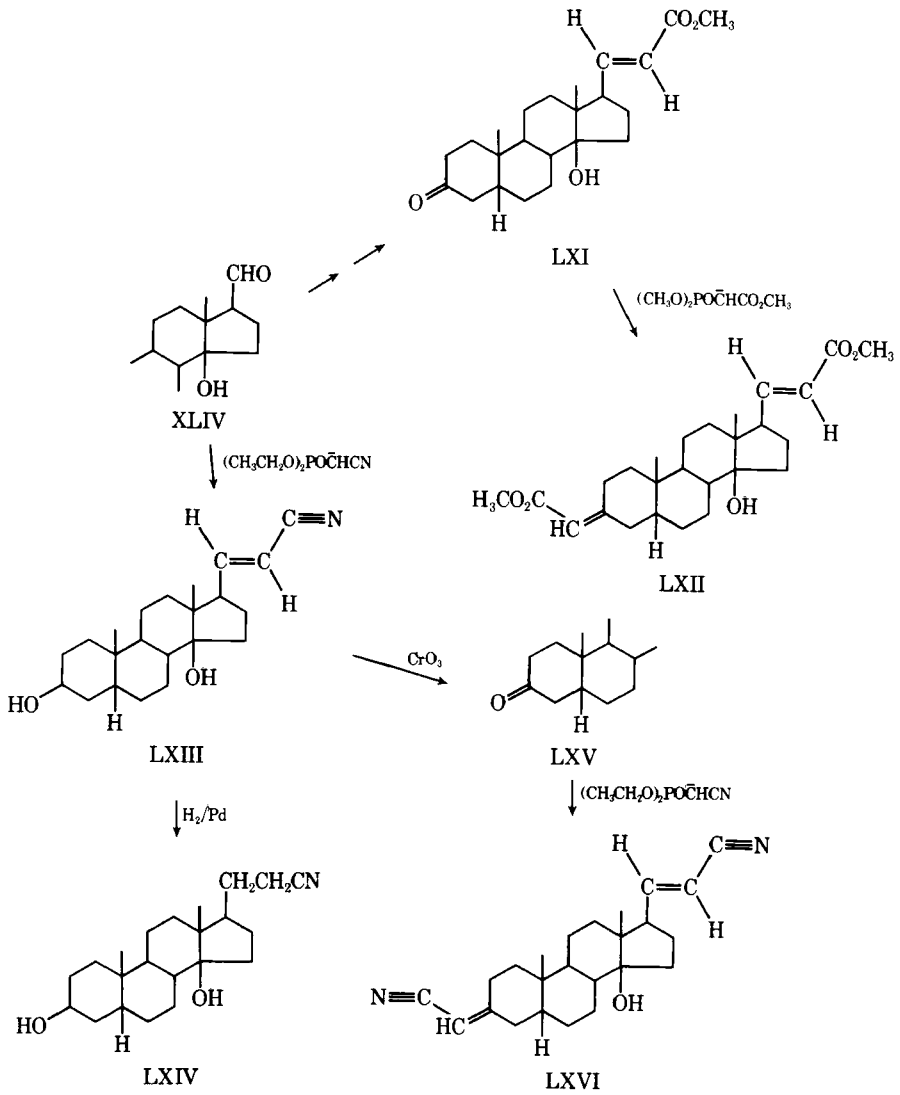
The development and biological evaluation of β-methyldigoxin, together with a series of related alkyl and acyl derivatives of the sugar portions of cardiac glycosides, were recently reviewed (58). It has been clearly demonstrated that the methylation of a single hydroxy group in digoxin leads to a pronounced increase in the rate at which the drug is absorbed. Several small clinical trials (129) indicated that β-methyldigoxin is effective and well tolerated. However, the observation that the drug is only slowly demethylated (130, 131) could mean that more extensive studies may reveal that the drug is more toxic than the parent digoxin because of an increased tendency to accumulate.

The pharmacokinetics of tritiated β-acetyldigoxin have been studied in humans (132); the drug was better absorbed than digoxin. Rapid deacetylation apparently occurred during or after absorption, since no trace of β-acetyldigoxin was found in plasma or urine following oral administration. By contrast, Rietbrock *et al.* (131) found no significant difference in the rat between the plasma levels of radioactivity following the intraduodenal administration of radioactive digoxin and β-acetyldigoxin. They did find that β-methyldigoxin was more rapidly and more extensively absorbed, but the plasma half-life of this drug was twice as long as that of digoxin (10 hr compared with 4.5 hr). The biliary clearance of β-methyldigoxin was only about one-third that of digoxin when observed over 12 hr.

The naturally occurring glycoside lanatoside C possesses an acetyl group which may be thought to endow the molecule with some of the desirable pharmacokinetic features described earlier. However, studies (133, 134) showed that lanatoside C is apparently converted to digoxin in the GI tract and that only the resulting digoxin is absorbed in appreciable quantities. These conclusions were subsequently confirmed (135).

STEROIDAL GUANYLHYDRAZONES AND OTHER CARDIOACTIVE COMPOUNDS

Cardiotonic activity has been claimed for compounds possessing none of the unique structural features associated with the biologically active cardiac glycosides. Of particular interest are the 3,20-bisgu-



Scheme XIV—Synthesis of monosubstituted and disubstituted unsaturated ester and nitrile derivatives (87)

anylhydrazone derivatives of prednisone (CIII) and prednisolone (CIV). These compounds were first reported in 1964 (136, 137) as a new class with digitalis-like activity. Both of these componds were subjected to various tests for digitalis-like activity and were shown to have high activity with respect to positive inotropic effects and inhibition of Na^+,K^+-ATPase. A systematic search of the patent literature since that time revealed a large number of steroidal guanylhydrazones and related molecules which have been synthesized as potential cardioactive compounds (138–146).

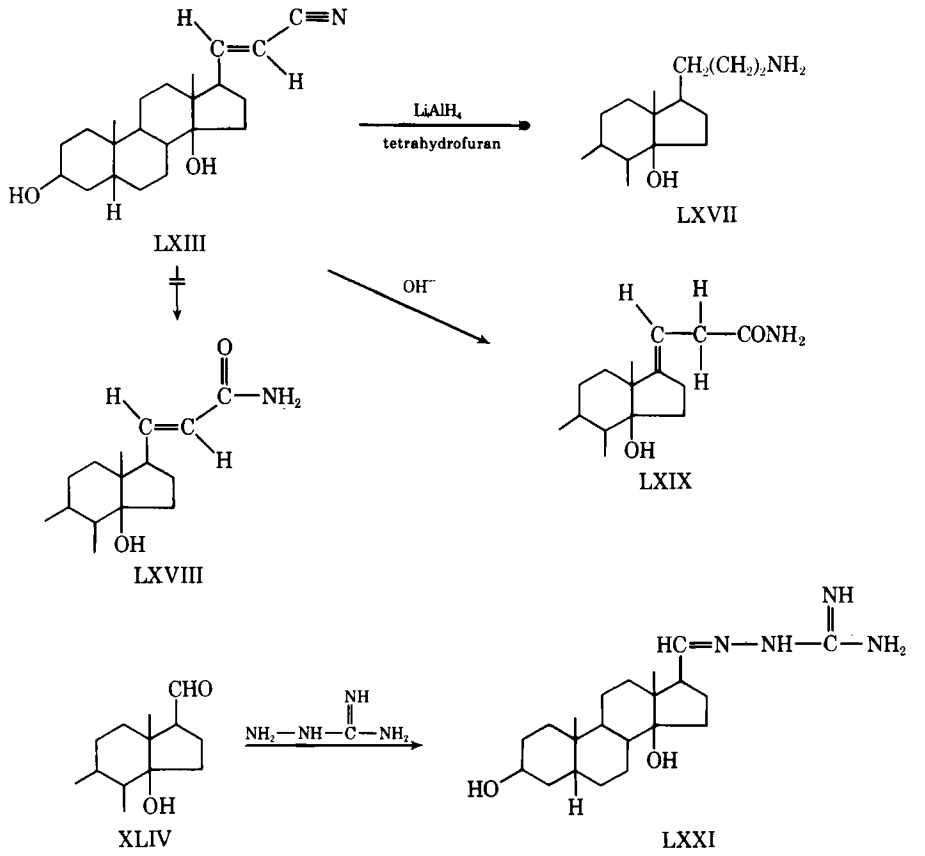
A review of the chemistry and biological activity of the guanylhydrazones was recently published (147). This review drew attention to several important features concerning the structure–activity relationships of these compounds. First, a wide range of steroidal 3,20-bisguanylhydrazones has been shown to exert a positive inotropic action on cardiac muscle; compared with the cardiac glycosides, their activity is not so significantly affected by changes in the stereochemistry of the steroid nucleus or of the substituents on the steroid. The bisguanylhydrazone derivatives of 5β- or 5α-pregnanedione do not differ significantly in activity.

Second, changing the distance between the two guanylhydrazone groups by changing the positions of their attachment to the steroid nucleus does not, within certain limits, alter the activity of the molecule to any great extent. Furthermore, some bisguanylhydrazones of nonsteroid molecules also show cardiotonic activity. For example, some diphenylbisguanylhydrazones and bisguanylhydrazone derivatives of di- and tricyclic molecules proved to be active.

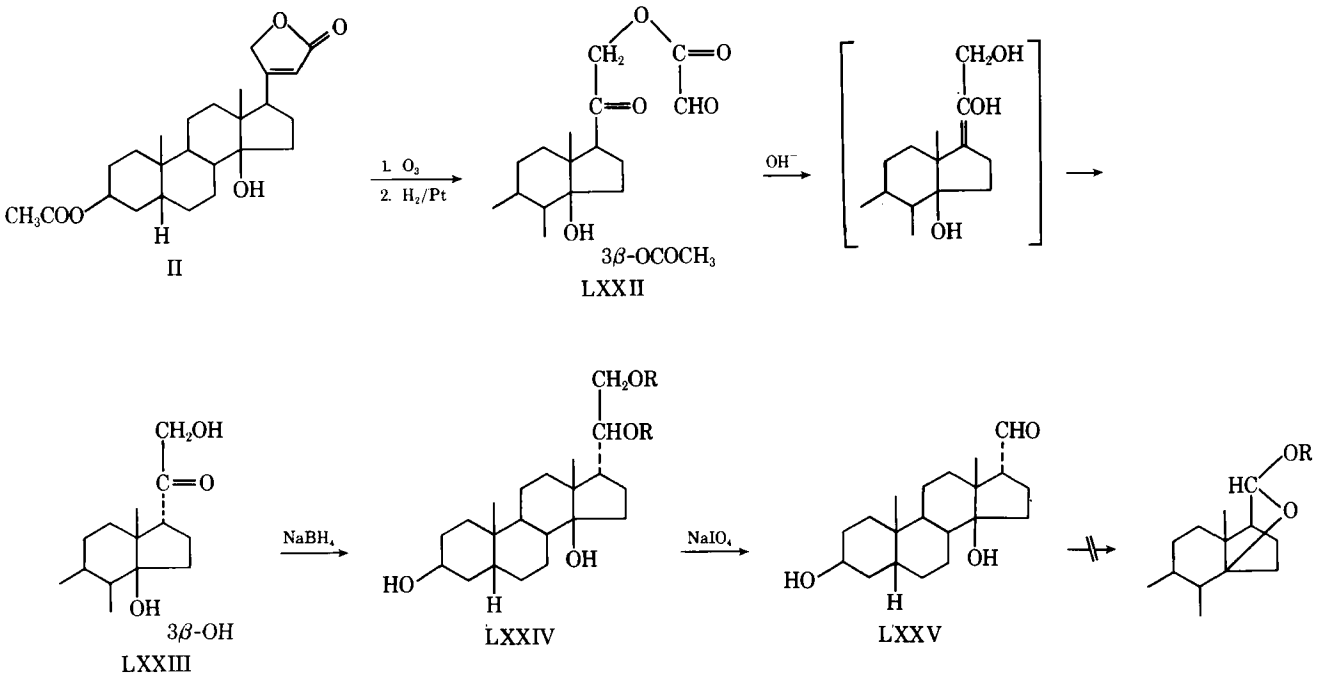
Third, certain steroidal mono- and trisguanylhydrazones are active. Of particular interest is the guanylhydrazone of 3-oxodigitoxigenin (CV) which is cardioactive whereas the guanylhydrazone derivative (CVI) of the corresponding 14-desoxy-14α-cardenolide is devoid of activity.

Fourth, hydrogenation of the guanylhydrazone group to give the diaminoguanidine derivative produced no significant loss in activity. However, the replacement of the guanylhydrazone moiety with other related groups, such as the hydrazine or the *S*-methylisothiosemicarbazone groups, gave inactive compounds.

The absorption, distribution, and other phar-



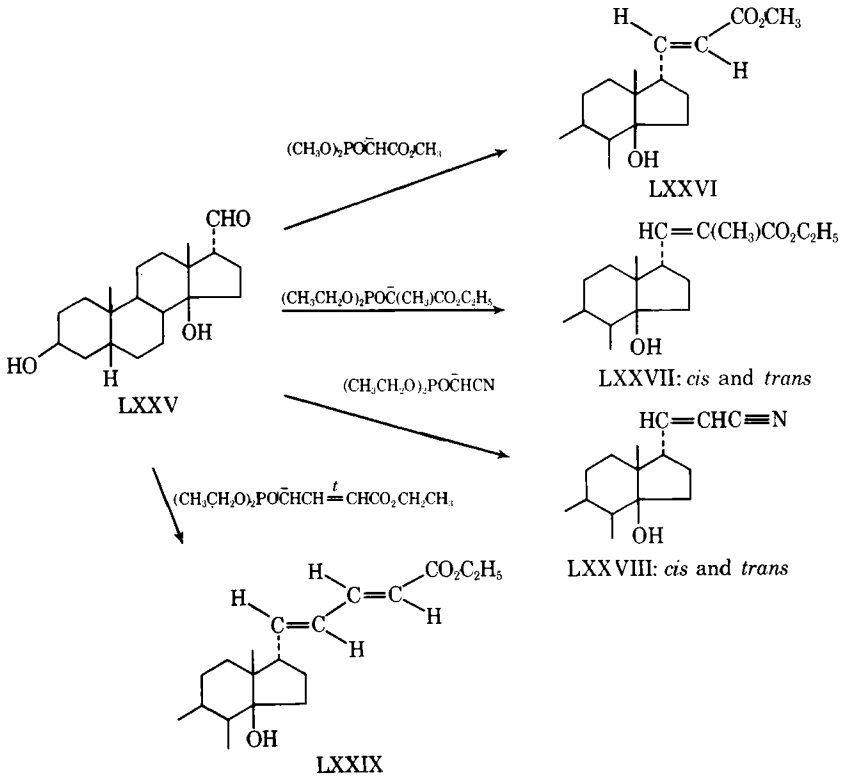
Scheme XV—Synthesis of C-17 amine, amide, and guanylhydrazone derivatives (87)



Scheme XVI—Synthetic sequence to the 17α-etianaldehyde (LXXV) (87)

macokinetic properties of the steroidal guanylhydrazones have been investigated *in vivo* (148) using ^{14}C-labeled molecules.

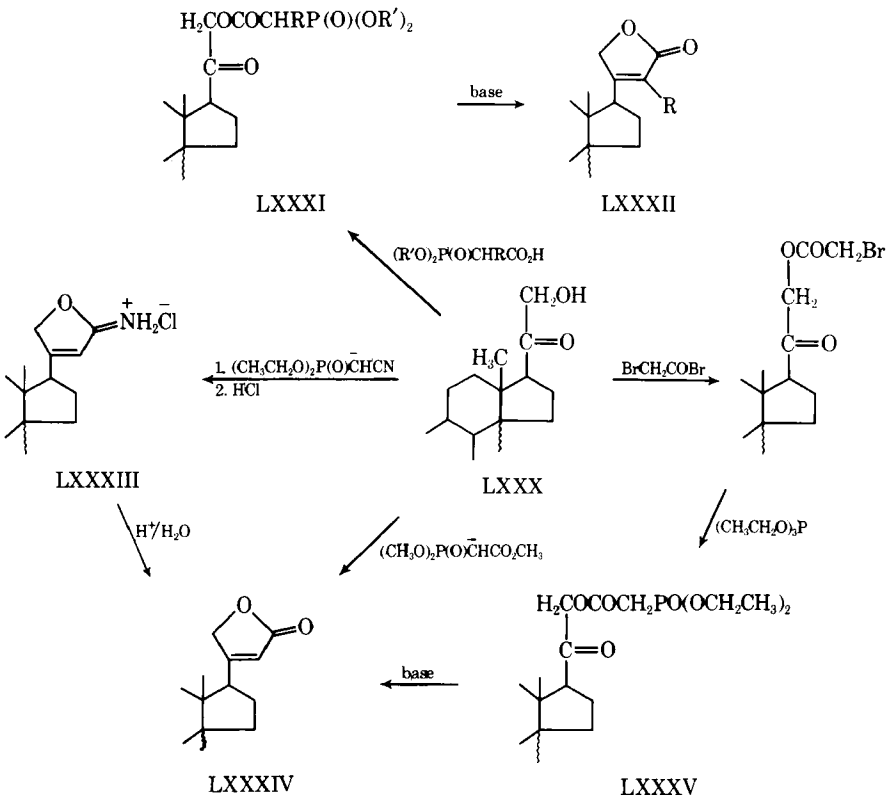
The pharmacology of bisguanylhydrazones in laboratory animals and in humans has been reviewed (149). The 3,20-bisguanylhydrazones of all steroids tested in animals were found to exert a powerful positive inotropic effect. The compounds also showed a marked ability to inhibit Na^+,K^+-ATPase, and the ranking order of the compounds in terms of the two



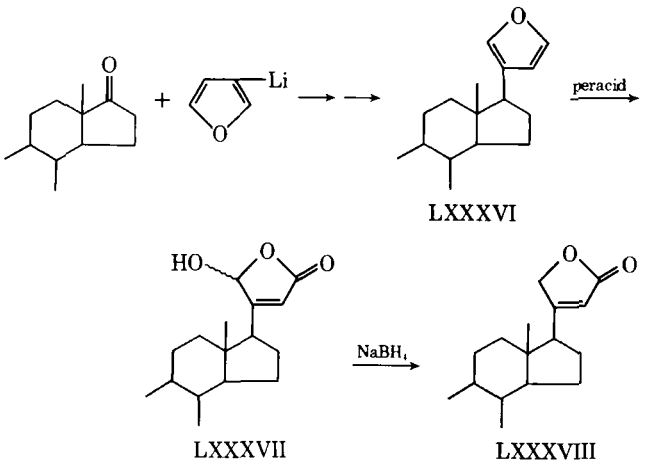
Scheme XVII—Synthesis of C-17α-analogs of digitoxigenin (87)

biological effects was the same. However, for individual compounds the ratio of the molar concentration required to produce a positive inotropic effect to that required to produce 50% inhibition of Na^+,K^+-ATPase varied from approximately 1:1 to 1:40. This may imply a separation of the two biological effects and will be discussed later.

The reader who refers to this review (149) should



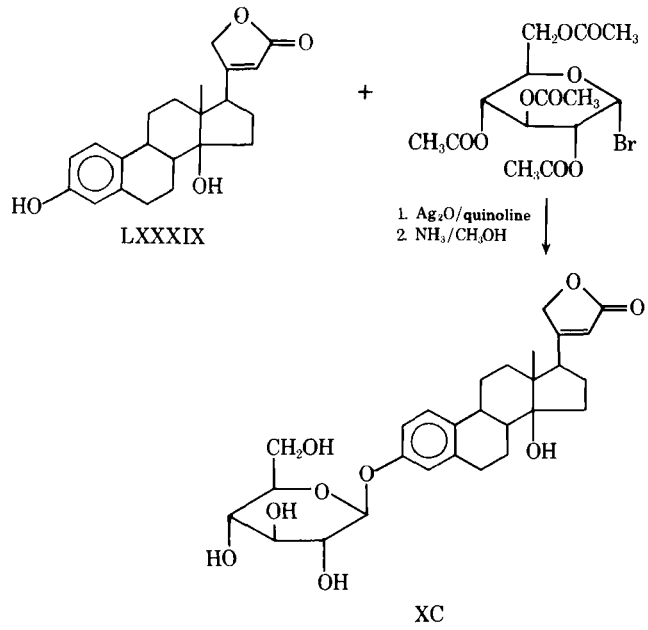
Scheme XVIII—Synthetic sequences to the C-17β-butenolide ring



LXXXVI LXXXVII LXXXVIII

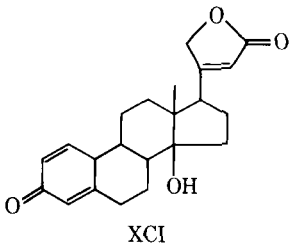
Scheme XIX

note that inotropic effects are related to concentrations expressed as grams per milliliter whereas inhibition of Na^+,K^+-ATPase is related to moles per liter (Ref. 149, Fig. 13). Furthermore, drug concentrations that produce positive inotropic effects refer to those at the foot of the log dose–response curves (threshold values). It seems also that maximum inotropic effects occur at doses approximately 100 times the concentrations given in the tables and that the maximum increase in the force of contraction was approximately 20–30% that of the predrug value. The therapeutic ratios of the 3,20-bisguanylhydrazones were approximately twice as favorable as those of the naturally occurring cardiac glycosides. The half-time for duration of contractility effects varied from less than 15 min in the case of progesterone-3,20-bisguanylhydrazone to 400 min with triamcinolone-3,20-bisguanylhydrazone. These differences were not related to the half-lives of elimination which were approximately 10–12 hr in all cases (based on elimination of total radioactivity following intravenous administration of radioactive drug).



LXXXIX XC

Scheme XX



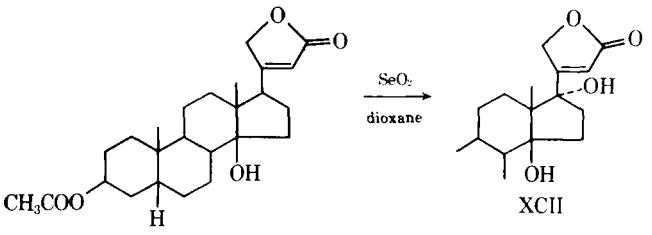
XCI

All effects described refer to studies in the guinea pig. On the basis of these studies, triamcinolone-3,20-bisguanylhydrazone was selected as the most promising drug for studies in humans. Ten patients with indications for digitalis were given 10 mg iv of the triamcinolone derivative. Cardiac output was increased by an average of 32%, and the rate of circulation was increased by an average of 16%. The heart rate was slowed but no bradycardia was observed. Unfortunately, the duration of action was too brief and the author concluded that the drug could not be regarded as a suitable substitute for digitalis.

Greeff and Schlieper (150) compared the biological effects of prednisolone-3,20-bisguanylhydrazone in humans, rats, rabbits, and guinea pigs. The authors found that the log dose–response curves for force of contraction in the isolated auricle of humans, rabbits, and rats were almost identical (half-maximum effects at approximately 6×10^{-5} *M*) whereas comparable effects in the guinea pig occurred at approximately 4×10^{-7} *M*. By contrast, the log dose–response curves for inhibition of erythrocyte Na^+,K^+-ATPase from humans, rabbits, and rats were distributed over a 100-fold range in concentration. Since Repke (23) showed that erythrocyte Na^+,K^+-ATPase is a suitable model for the myocardial enzyme, these results may signify that inhibition of Na^+,K^+-ATPase is not related to positive inotropic effects. (Compare Figs. 1–3.)

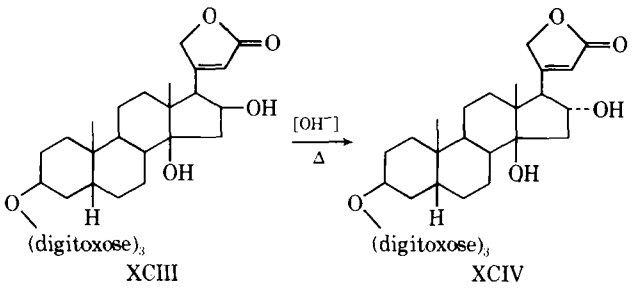
The *Erythrophleum* alkaloid cassaine (CVII) has been recognized for many years as having a powerful digitalis-like action on heart muscle (151). There appears to be little structural resemblance between cassaine and the cardiac steroids. While the unsaturated lactone ring is an important functional group for biological activity in the cardiac glycosides, the biological properties of cassaine depend on the presence of the unsaturated aminoethyl ester group. Hydrolysis of this group to give the corresponding acid destroys activity (152).

Clarke and associates (153–155) described the synthesis of simplified analogs of cassaine as part of a study to determine the role of the skeletal structure and the various substituents of cassaine in the pro-



XCII

Scheme XXI



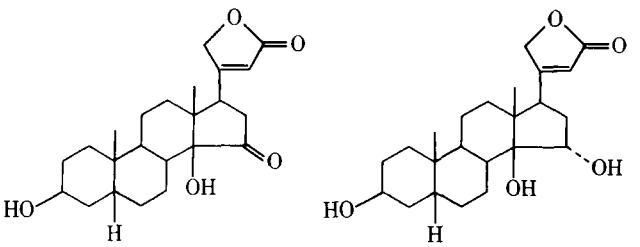
XCIII XCIV

Scheme XXII

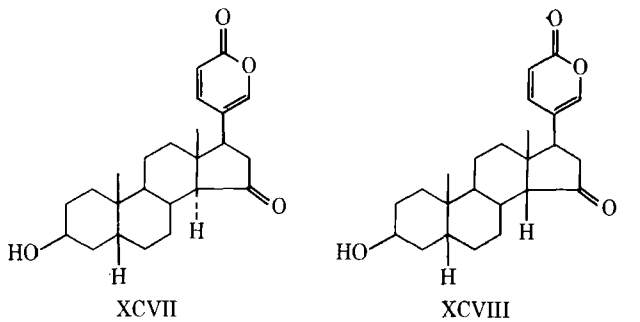
motion of cardiotonic activity. Some of the analogs prepared (CVIII, CIX, and CX) were shown to be active but less potent than cassaine when tested for cardiotonic activity on isolated cardiac muscle or in the intact dog.

There are several other groups of cardiotonic alkaloids. The biological properties of these compounds, along with those of the *Erythrophleum* group, have been reviewed (156) and are considered to be of no therapeutic interest because of their association with many other pharmacological effects.

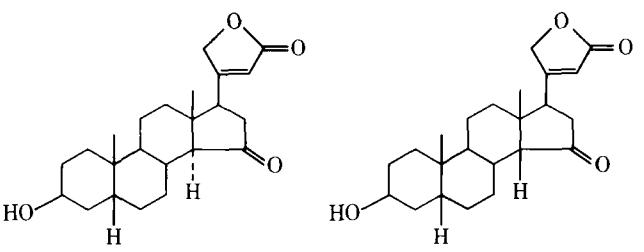
In view of the importance of the dimethylaminoethyl group in the activity of cassaine, it is interesting to note that esterification of bile acids with dimethylaminoethanol (157) imparts a slight digitalis-like activity in spite of the configurational differences between the bile acids and the cardiac genins. Recently, Brown *et al.* (158, 159) patented a new method to introduce the dimethylaminoethoxycarbonylmethylene function at different positions on the



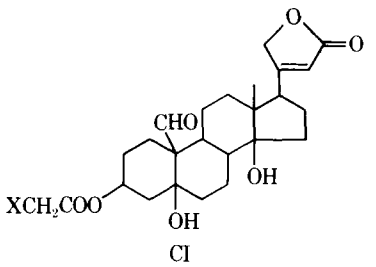
XCV XCVI



XCVII XCVIII



XCIX C



CI

steroid nuclei. On this basis, many such derivatives were prepared and assessed for cardiotonic activity. Compounds of the CXI type and other dimethylaminoethoxycarbonylmethylene steroids were claimed to be cardioactive, but no biological details were given in the abstract.

Digitalis-like activity has been claimed (160) for some adrenocorticoid steroids. However, there is no general acceptance of this idea because it appears that the effects of these compounds are slight and of doubtful significance.

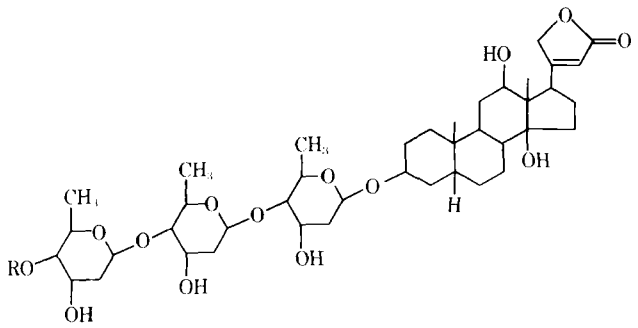
The studies described indicate that digitalis-like activity is not limited to compounds possessing the specific requirements deduced by the classical studies described previously. A logical extension of this conclusion is that cardiotonic activity may be found in nonrigid molecules of the appropriate type. Recently, Inhoffen *et al.* (161) described the synthesis of a "pseudosteroid" (CXII) which, on testing, was found to have a negative inotropic effect.

A variety of aromatic and aliphatic bisguanylhydrazones (*e.g.*, CXIII, CXIV, and CXV) were reported (149) to have a weak inotropic effect on guinea pig heart.

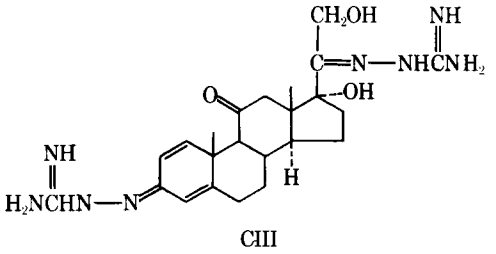
To conclude this section, mention should be made of the wide variety of nonsteroidal compounds known to inhibit Na^+,K^+-ATPase. Discussion of these compounds is beyond the scope of this review and the reader is referred to a recent comprehensive review (162) for an account of this subject.

BIOLOGICAL ACTIVITY

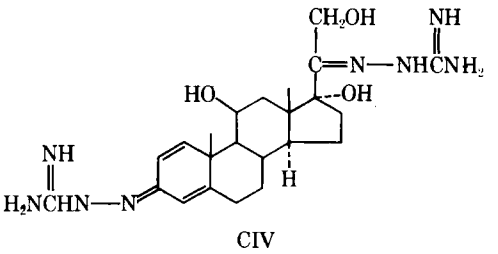
The interpretation of drug action in terms of biochemical effects arising from interactions with specific receptors has much philosophical appeal and, in some cases, is of practical value in the design of new drugs. Therefore, it is appropriate to outline current concepts of the mode of action of cardiotonic ste-



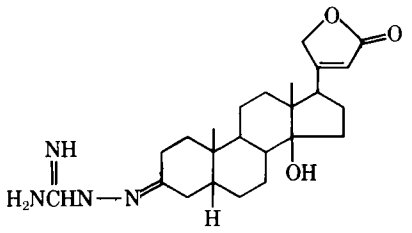
CII*a*: R = H (digoxin)
CII*b*: R = $COCH_3$ (β-acetyldigoxin)
CII*c*: R = CH_3 (β-methyldigoxin)



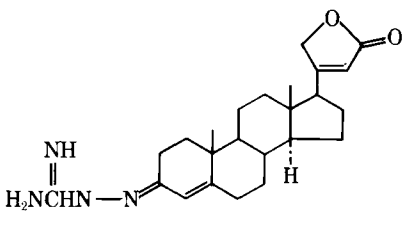
CIII



CIV



CV



CVI

roids, particularly as these relate to the interpretation of structure–activity relationships and to the biological evaluation of new cardioactive substances.

The basic problem is one that always applies in the study of the biochemical pharmacology of drug action, namely the difficulty of correlating events at different levels of biological organization. Cardiotonic steroids are known to cause effects at three biological levels: at the enzyme or subcellular level, at the cellular level, and at the organ or tissue level.

The biochemical effects of cardiotonic steroids have been the subject of considerable study. In 1961, at the First International Pharmacological Meeting, the effects of cardiotonic steroids on a wide range of biochemical systems were described. In many cases, reasonable circumstantial evidence was presented to link these biochemical effects with effects on heart muscle contractility (163). Recent accounts of the subcellular effects of cardiotonic steroids were presented (14, 17), and these reviews suggested that the effects of cardiotonic steroids on myocardial contractility arise as a result of one of the following mechanisms: *(a)* inhibition of Na^+,K^+-ATPase, *(b)* increase in intracellular free Ca^{+2} unrelated to effects on Na^+,K^+-ATPase, and *(c)* increase in intracellular free Ca^{+2} as a consequence of inhibition of Na^+,K^+-ATPase.

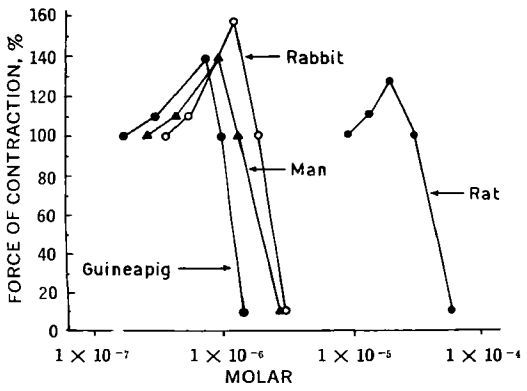


Figure 1—*Influence of K-strophanthin on the force of contraction of the myocardium of different species. (After Greef and Schlieper, Ref. 150.)*

Other reported subcellular effects of cardiotonic steroids, including direct effects on contractile proteins and modification of myocardial energy metabolism, seem to have no *in vivo* significance or are secondary to effects on contractility (17).

At the cellular level, cardiotonic steroids directly or indirectly affect the transport and distribution of a

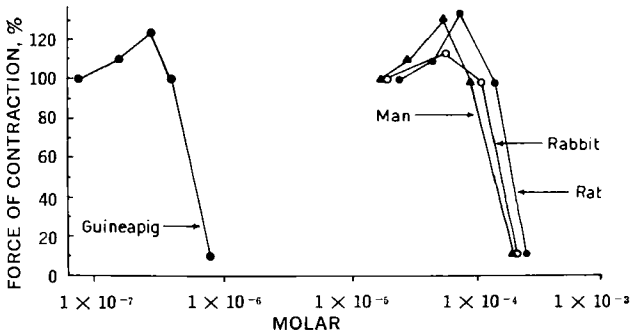


Figure 2—*Influence of prednisolone-3,20-bisguanylhydrazone on the force of contraction of the myocardium of different species. (After Greef and Schlieper, Ref. 150.)*

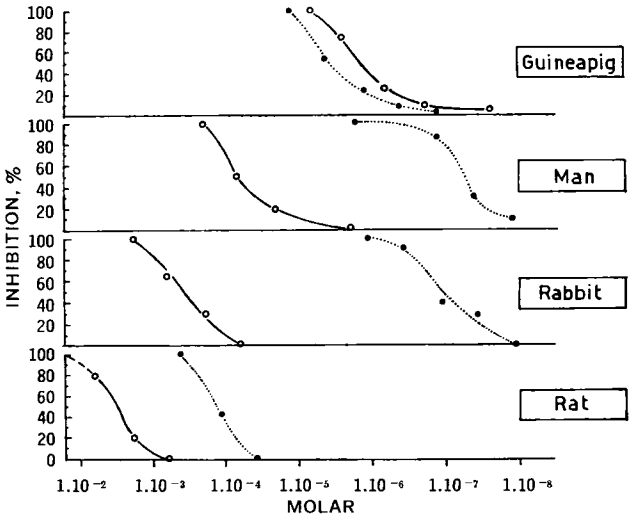
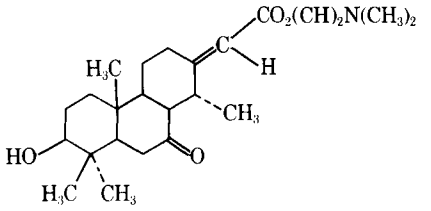
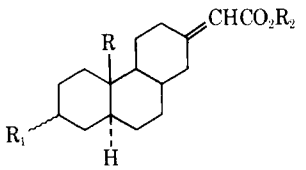


Figure 3—*Influence of K-strophanthin (. . .) and prednisolone-3,20-bisguanylhydrazone (—) on the active transport of Na^+ and K^+ in cooled erythrocytes of different species. (After Greef and Schlieper, Ref. 150.)*

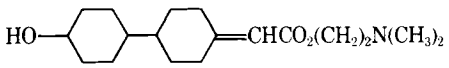


CVII

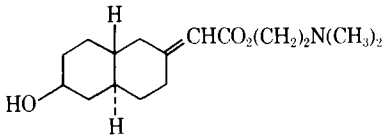


CVIII: R = H or CH_3

R_1	R_2
H	$(CH_2)_2N(CH_3)_2$
$NNH(C{=}NH)NH_2$	$(CH_2)_2N(CH_3)_2$
O	$(CH_2)_nN(CH_3)_2$, $n = 2$-4
α-OH or β-OH	$(CH_2)_nN(CH_3)_2$, $n = 2$-4
β-OH	$(CH_2)_2N(R'_3)_2$, $R' = H, CH_3, C_2H_5$



CIX

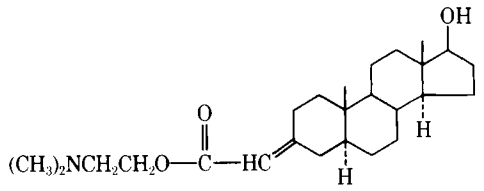


CX

wide range of substances including monovalent and divalent cations and possibly carbohydrates, amino acids, biogenic amines, and lipids. The reader is referred to a recent review for an account of these aspects (14).

At the organ level, the most striking effect is on the heart. The complex nature of the pharmacological effects of cardiotonic steroids on the failing and nonfailing heart has been documented (17, 18, 164). Recent studies emphasized that the rate of onset and intensity of the effects of cardiotonic steroids on myocardial contractility may be significantly affected by various factors such as temperature, frequency of stimulation, and concentrations of Na^+,K^+ and Ca^{+2}. (See Refs. 17 and 164 for a critical discussion of these effects.) Furthermore, it is now apparent that the older view which held that the positive inotropic effects of cardiotonic steroids occurred *in situ* only in failing hearts is no longer tenable (17). Failure to recognize this fact may have been due to the development of compensatory effects in the normal heart. All of these observations have obvious significance in the determination of structure–activity relationships. In addition to effects on the heart, cardiotonic steroids also have a pharmacodynamic action in the central nervous system and in the kidney.

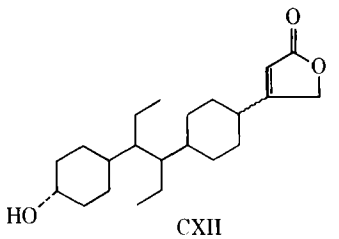
Interrelation of Biological Effects—The possible links between the effects of cardiotonic steroids at the various levels of biological organization will now be considered. At the outset, it must be emphasized that there is much controversy in the literature. This arises partly because of variations in experimental parameters and partly because of the inherent difficulties that arise when the intact functioning system is disturbed by the experimental probe. To quote Lee and Klaus "... to date, the enormous information about the effects of cardiac glycosides has not provided a clear mechanism of positive inotropism on a reasonably firm experimental basis" (17). What follows is the present authors' selection of what appears to be the most adequately established conclusions. Almost every point, however, is in dispute, and the reader is referred to the previously cited reviews and to others to be cited for a critical appraisal.



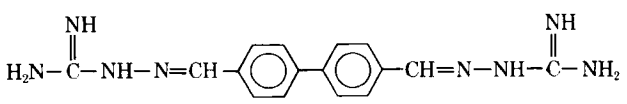
CXI

As previously mentioned, the purpose of this review is to direct the medicinal chemist to relevant sections of the voluminous literature on the mode of action of cardiotonic steroids. It is advisable to start with an appreciation of the morphology and physiology of the heart and of the events involved in excitation–contraction phenomena. This should then be followed by an appreciation of what is meant by positive inotropism and of the methods used to measure this phenomenon. The possible mode of action of cardiotonic steroids should then be considered against this background. The present authors found a number of reviews useful with respect to these concepts (165, 166, 164, 167–169, 14, 17, 170; the reviews are probably best read in the order given).

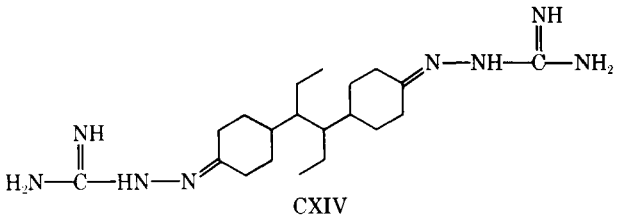
With the hope that it would be a further guide to the reader, a "systems analysis" for the coupling of myocardial excitation and contraction was prepared (Scheme XXIII). The scheme was prepared by incorporating the conclusions of many authors, and it includes much that is hypothetical and controversial.



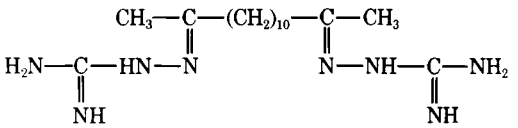
CXII



CXIII



CXIV



CXV

The scheme can thus be regarded as an eclective model and should be considered more as a guide to the literature than a guide to the heart.

Some aspects of the proposed model can be regarded as well established. These include:

1. Excitation leads to an influx of Na^+ and an efflux of K^+, and this in turn leads to depolarization of the plasma membrane of the sarcolemma. (The sarcolemma is a double-membrane structure that surrounds and invaginates the myoplasm. It consists of an inner or plasma membrane and an outer or basement "membrane" or external lamina composed of a dense mat of fine filaments.)

2. As a consequence of 1, there is an increase in the concentration of free or "activator" Ca^{+2} in the vicinity of the myofibrils (the contractile elements within the cell).

3. When the level of the free Ca^{+2} exceeds a critical value, it begins to antagonize the inhibitory action of troponin. (In the resting state, the existence of an actin–troponin–tropomyosin–myosin complex constitutes a barrier against the formation of actomyosin.)

4. Antagonism of troponin by Ca^{+2} permits the formation of the energetically stable actomyosin complex, with resulting contraction of the myocardium, using energy supplied by ATP.

5. Relaxation (uncoupling of actin and myosin) requires an input of energy (supplied by ATP) and sequestration of activator Ca^{+2} by some internal store, which is probably located in the sarcoplasmic reticulum. [The sarcoplasmic reticulum is a longitudinal system of fine interconnecting tubules that permeate the myoplasm and come in close contact with the myofibrils, T-tubules, and sarcolemma. The T-tubules are invaginations (mainly lateral) of the sarcolemma.]

6. Repolarization results from activation of Na^+,K^+-ATPase. The latter is located in the plasma membrane of the sarcolemma and is activated when the internal concentration of Na^+ (Na_i^+) and the external concentrations of K^+ (K_o^+) rise above a critical level. The system thus constitutes a feedback control.

7. The biological effects of cardiac glycosides result from interaction with some component on the external face of the plasma membrane. Since the plasma membrane invaginates (as T-tubules), it is thus possible for cardiac glycosides to interact with structures deep within the myocardial cell.

8. Cardiac glycosides combine with the external face of the Na^+,K^+-ATPase macromolecule (or complex of macromolecules) and inhibit enzyme activity by an allosteric mechanism.

9. The toxic effects of cardiac glycosides are a direct consequence of extensive inhibition of Na^+,K^+-ATPase.

The following aspects of the analysis shown in Scheme XXIII are disputed.

1. There is no general agreement on the nature of the link, if any, between Na^+ influx and Ca^{+2} uptake.

2. The origin of activator Ca^{+2} is probably the most controversial aspect of the link between excitation and contraction. Some investigators believe that activator Ca^{+2} is derived primarily from the "internal stores" located probably in the sarcoplasmic reticulum, whereas others believe that the prime or sole source is either from some superficial store located in or close to the plasma membrane or from the extracellular fluid. The interpretation of data is complicated by the fact that there seems to be no simple relationship between the concentrations of free intracellular Ca^{+2} and the development of muscle tension.

3. The interconnection between Ca^{+2} influx and the various stores of Ca^{+2} is poorly understood.

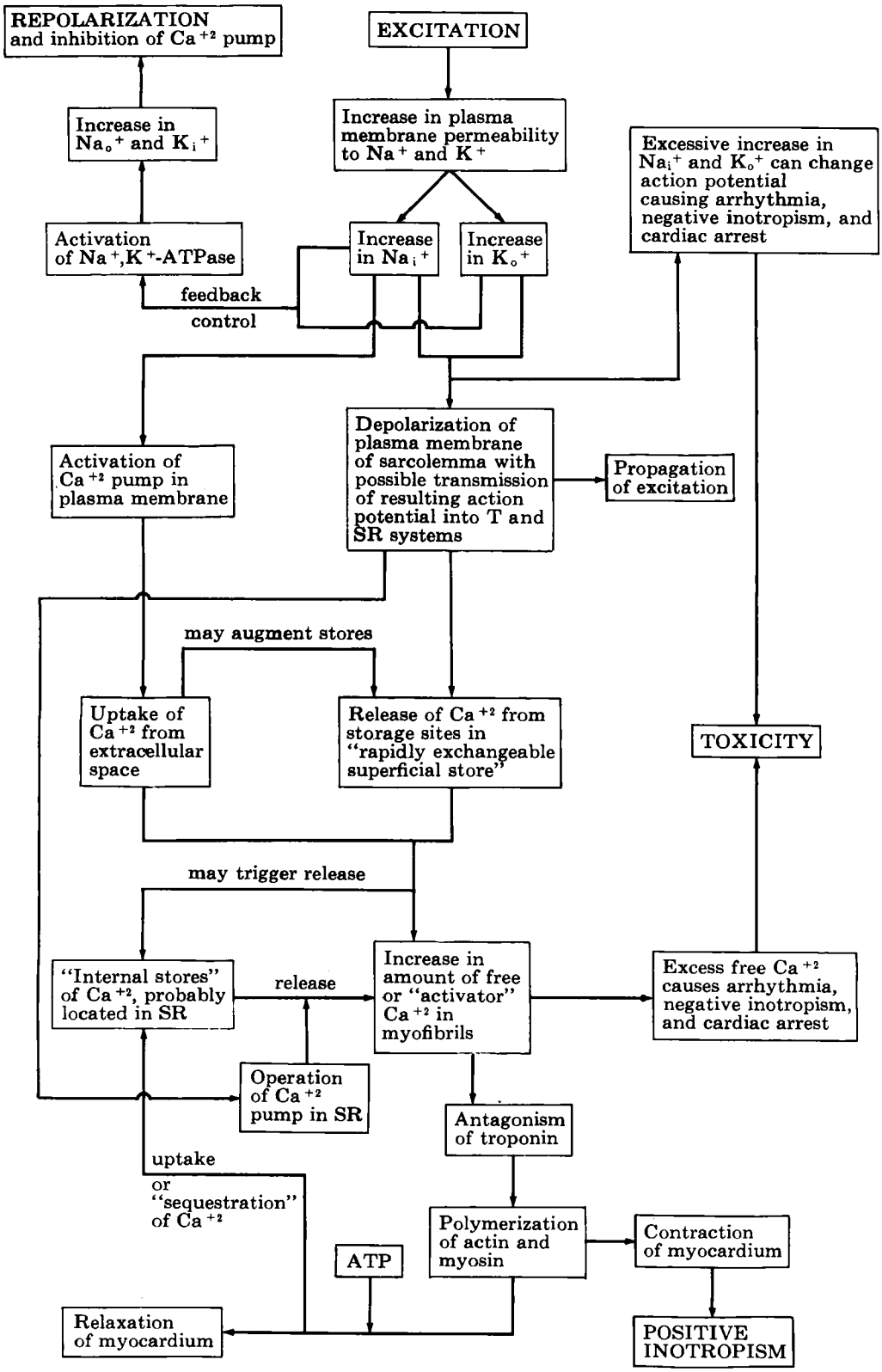
4. The mechanism of the inotropic action of cardiac glycosides is the subject of much dispute. While there is general agreement that the toxic effects of cardiac glycosides result from inhibition of Na^+,K^+-ATPase and are accompanied by a net influx of Na^+ and efflux of K^+, no such agreement exists with respect to the effects of therapeutic doses of digitalis.

5. Except for the activation of Na^+,K^+-ATPase, the various feedback, augmenting, and triggering mechanisms shown in Scheme XXIII are largely conjecture.

References supporting these statements may be found in the previously cited reviews (14, 17, 164–170).

While the biochemical basis of the inotropic effects of cardiotonic steroids is still unresolved, the bulk of recent work indicates that therapeutic doses of digitalis-like compounds affect cation fluxes in a manner consistent with the conclusion that inhibition of Na^+,K^+-ATPase is the primary event that leads, through an increase in activator Ca^{+2}, to the positive inotropic effect. Two important qualifications must now be applied to this statement.

First, the methods used to isolate Na^+,K^+-ATPase discard most of the enzyme activity during the process of partial purification. Therefore, it is possible to hypothesize that there exists a small subfraction of "Na^+,K^+-ATPase" which has acquired some specialized role and which may be the entity mediating the therapeutic effects of digitalis. This "fraction," which may be discarded during purification, may even have lost its ability to transport Na^+ and K^+. It may, for example, be located in the T-tubules where these



Scheme XXIII—Eclectic model for coupling of excitation and contraction in myocardium (14, 17, 164–170)

come in close contact with the sarcoplasmic reticulum and hence may play some role in triggering the release of the internal stores of Ca^{+2}.

The second qualification is really a corollary of the first. It is possible that the apparent correlation between the therapeutic effects of cardiac glycosides and inhibition of Na^+,K^+-ATPase may simply reflect the possibility that the enzyme is acting as a "model" receptor. This problem is common in biochemical pharmacology. For example, it may be found that a group of substances stabilizes lysosomal membranes and that this effect reflects structure–activity rela-

tionships as these apply to some therapeutic effect. This does not necessarily mean that the therapeutic effect results from stabilization of lysosomal membranes. It is equally possible that the therapeutic effect results from the stabilization of some other membrane structure and that the lysosomal membranes are simply a suitable model for this effect. Likewise, the effects of cardiotonic steroids on Na^+,K^+-ATPase may simply be reflecting some other (therapeutically related) drug–receptor interaction.

As mentioned earlier, Na^+,K^+-ATPase was probably elaborated very early in the evolution of life and it would not be surprising if the heart had adapted a portion of this enzyme for some specialized role. If so, it might explain the unique effects of cardiac glycosides on the heart. Digitalis-sensitive Na^+,K^+-ATPase is found in most, if not all, of the body's cells. In addition, the basic essentials of excitation–contraction coupling depicted in Scheme XXIII apply equally to skeletal muscle. What then is the basis of the unique effects of digitalis on the heart? It is inadequate, in fact it is tautological, to simply say that the unique effects of digitalis on the heart reflect the fact that the heart is different from other organs. What needs to be known is the nature of the difference that forms the basis of the selective action of digitalis.

Studies of the microstructure of cardiac cells have led to the suggestion [discussed by Langer (170)] that the source of activator Ca^{+2} is different from that in skeletal muscle. This could well be the basis of the selective action. Besch and Schwartz (171) hypothesized that the inotropic effects of digitalis-like compounds may not be consequential to changes in Na^+ and K^+ flux but may arise because the interaction of cardiotonic steroids with the Na^+,K^+-ATPase macromolecule may induce conformational changes in neighboring regions of the plasma membrane, with consequential release of a hypothetical store of Ca^{+2}.

Much significance has been given to the studies of Baker *et al.* (172) who used the perfused squid axon (from which the axoplasm had been previously extruded) to obtain direct evidence that Ca^{+2} influx was increased when the concentration of Na^+ was decreased on the outside of the membrane or increased on the inside. These results, while supporting the proposition that inhibition of Na^+,K^+-ATPase promotes the uptake of Ca^{+2}, do not, for obvious reasons, explain the unique effects of digitalis on the heart.

Any comprehensive theory of digitalis action must also incorporate an explanation of the fact that insulin potentiates the action of cardiotonic steroids (173) whereas thyroxine raises the threshold for digitalis toxicity (174). All of these comments focus attention on the important role played by the cell membrane in regulating intracellular events. Langer (170) has emphasized this point and his quotation from Whaley and associates (175) is worth repeating: "... there is a growing awareness that many cellular functions are directly influenced or 'controlled' by macromolecules 'outside' the cell, either as components of the plasma membrane, or as cell surface-associated materials, or as components of intercellular matrices." The manner by which these mechanisms operate is largely unknown.

Knowledge of the interior of the cell is equally limited. In 1972, Schwartz *et al.* (14) wrote: "We have practically no concept of the inside of a living cell." In 1945, Needham (176) wrote: "How, for instance, does an egg know which of its ends is which; which end is to be the front of the future animal and which end the back? If a transparent egg of a sea-urchin, for example, is centrifuged ... the contents of the egg are largely stratified, different sorts of fat, granules, *etc.*, coming together in layers. Yet the further development is not in the least affected. The egg will shortly afterwards bud forth its new small cells in exactly the same place as it would if nothing had been done to it. It 'knows' which end is which, and your throwing things about inside it has not in the least confused it. It looks as if it had a 'crystal lattice' inside it, like one of those rigid cat's cradles of coloured balls which you can see in the South Kensington museums." Modern research has confirmed that that which we call the "soluble fraction" of the cytoplasm is, in fact, a highly structured system, but we are still at the very threshold of comprehending its intricate nature.

The study of the biological effects of cardiac glycosides should thus be seen to have a much broader significance than the mere elucidation of a therapeutic effect. Increasingly, biologists are using cardiac glycosides as tools to study membrane phenomena in a wide variety of tissues. Further discussion of this fascinating subject is beyond the scope of the present review, but the medicinal chemist has played an invaluable role in providing the biologist with chemical probes with which to study the molecular basis of life. Our present knowledge of protein synthesis, for example, would not have been gained without such drugs as puromycin and other antibiotics, antimetabolites, and biological alkylating agents.

Na^+,K^+-ATPase as Digitalis Receptor—Irrespective of the role of Na^+,K^+-ATPase in the mediation of effects on myocardial contractility, two conclusions are beyond dispute: the enzyme contains a receptor for digitalis-like compounds, and the enzyme plays a vital role in most, if not all, of the body's cells. It is appropriate then that medicinal chemists should develop new series of compounds with selective effects on Na^+,K^+-ATPase. Such studies may yield new drugs with effects other than on the heart and may further assist the biologist in elucidating the role of this enzyme in controlling cell ecology. As an aid to the medicinal chemist, some of the important properties of Na^+,K^+-ATPase are listed here. But this is not a critical appraisal, and the reader is referred to the various specialist reviews [particularly that of Schwartz *et al.* (14)] for a comprehensive review of the literature.

The Na^+,K^+-ATPase referred to in this review has already been defined as a membrane-bound, Mg^{+2}-dependent ATPase which is activated by Na^+ and K^+ and specifically inhibited by ouabain and other cardiotonically active cardiac glycosides. At least

three other membrane-bound ATPases have been identified in heart muscle cells (177), but only Na^+,K^+-ATPase (sometimes referred to as transport-ATPase) shows all of the listed properties. Cytochemical studies (177, 178) indicate that Na^+,K^+-ATPase is located in the plasma membrane of the sarcolemma. Biochemical techniques used to isolate and measure Na^+,K^+-ATPase activity have been described in detail (179).

The enzyme appears to be an intrinsic part of the cell membrane, and no enzyme activity passes into solution when cells are extensively homogenized. Treatment of the enzyme suspension to remove lipids produces a soluble protein preparation with reduced ability to bind cardiac glycosides and no Na^+,K^+-ATPase activity. The addition of phosphatidylserine to the solubilized protein produces a soluble complex which binds ouabain and splits ATP in a manner comparable to the particulate preparation (180). The similarity between the properties of the reconstituted soluble enzyme complex and those of membrane fragment preparations led Chipperfield and Whittam (180) to conclude that phosphatidylserine may be closely involved in the *in situ* operation of Na^+,K^+-ATPase. These results are in accord with other work (*e.g.*, Ref. 181), indicating that the enzyme is lipoprotein in nature.

Estimates of the molecular weight of Na^+,K^+-ATPase range from 250,000 to 1,000,000 daltons. These values were obtained using the method of radiation inactivation (182, 183) and by various techniques of gel filtration and centrifugation (184, 185). Divergence in estimates reflects the limitations of the various techniques and the fact that the enzyme probably consists of subunits. What is more important is that these estimates show that the enzyme is of sufficient size to penetrate the full width of the plasma membrane and thus has the necessary dimensions to act as the cation carrier in the active transport of Na^+ and K^+. There is much evidence to suggest that hydrolysis of ATP (which provides the energy for active cation transport) takes place on the internal face of the enzyme (186) and that the sodium and potassium binding sites are located, respectively, on the inner and outer surfaces of the enzyme (187). Various models have been suggested to depict the mechanical transfer of Na^+ and K^+, but these generally lack experimental verification (188). There is, however, abundant evidence that the enzyme can and does undergo controlled conformational changes and that these changes form the basis of active transport (14).

There have been extensive studies of the kinetics of ATP hydrolysis by Na^+,K^+-ATPase, including studies of the effects of cardiac glycosides. The interpretation of the data from these studies is by no means straightforward, and the relevance of some work is questionable since there is good reason to believe that the properties of the cation pump are significantly influenced by many features of the *in situ* environment. The following sequence may be applicable to the intact system.

Rising concentrations of Na^+ on the inside of the cell membrane result in the binding of this ligand to specific cation-binding sites on the inner face of the enzyme. The energy changes consequent to the uptake of Na^+ ions could be manifested in several ways including changes in charge distribution, changes in enzyme conformation, and changes in the structure of bound water. Whatever the mechanism, the uptake of Na^+ leads to the binding of one molecule of ATP to an active site on the inner face of the enzyme. Stage one is thus the formation of an enzyme–ATP complex. This is followed very rapidly by Mg^{+2}-catalyzed hydrolysis of ATP to produce a phosphorylated enzyme (stage two). What happens next is unknown, but it is assumed that the high energy released by the hydrolysis of ATP results in a major conformation change or series of changes, the net result of which is the transfer of Na^+ across the membrane and subsequent release into the "extracellular fluid" (in the heart this would be into the space between the plasma and basement membranes of the sarcolemma). The release of Na^+ terminates stage three.

The energy or conformational changes consequent to stage three result in the generation of specific K^+ binding sites on the outer face of the enzyme. These sites may be the same or different from those that release Na^+. The uptake of K^+ by the "new" cation-binding sites (stage four) results in further changes, which culminate in dephosphorylation of the enzyme (stage five). The energy changes consequent to dephosphorylation induce another series of major conformational changes in the enzyme with the result that K^+ is transferred across the membrane and released into the cytoplasm (stage six).

This sequence incorporates suggestions by Albers *et al.* (189) and Schönfeld *et al.* (190). The former workers proposed that the conformational changes occurring in the cycle are of the *cis/trans*-variety but there seems to be no experimental justification for this suggestion. The controversial role of Mg^{+2} ions seems to have been settled by Schönfeld *et al.* (190) who showed that an enzyme–ATP complex will form in the presence of Na^+ in Mg^{+2}-free medium containing cyclohexyldiaminetetraacetate to chelate traces of contaminating Mg^{+2} ions. Addition of Mg^{+2} was found to be necessary for phosphorylation of the enzyme (stage two in the sequence). Furthermore, studies in some tissues, such as erythrocytes, indicate that the hydrolysis of one molecule of ATP is coupled with an inward movement of two ions of K^+ and an outward movement of three ions of Na^+ (191). Studies of the stoichiometry of ion transport in the myocardium have yielded conflicting results (14).

Na^+,K^+-ATPase is inhibited by cardiac glycosides in concentrations comparable to those associated with the therapeutic and toxic effects of these substances. Inhibition appears to result from interaction of the drug with the external face of the enzyme (192). This is of particular interest in view of recent direct evidence that the inotropic effects of cardiac glycosides are mediated by drug acting on the outer surface of the membrane. This evidence was obtained using albumin-bound glycoside and glycoside specific antibodies (170). Since a substantial body of evidence

(*e.g.*, Ref. 186) indicates that hydrolysis of ATP takes place on the inner surface of the membrane, it would appear that inhibition of hydrolysis by cardiac glycosides is the result of an allosteric mechanism. Using a variety of techniques, Schwartz *et al.* (14) obtained good evidence that the interaction of ouabain with membrane fragments containing Na^+,K^+-ATPase leads to conformational changes of gross magnitude.

Matsui and Schwartz (193) also demonstrated that only physiologically active cardiotonic steroids can compete with ^3H-digoxin for receptor sites on the enzyme. The conditions that determine maximum binding of cardiac glycosides to the enzyme have been examined by many groups and have been shown to parallel both *in vivo* and *in vitro* inhibition of enzyme activity (189, 194). This evidence, together with a large volume of similar studies (see previously cited reviews for references), has firmly established the existence of a "digitalis receptor" as part of the macromolecular complex constituting the Na^+,K^+-pump.

Mathematical models (rate equations) have been formulated to describe the complex interaction of Na^+,K^+-ATPase with its many ligands (Na^+,K^+, ATP, Mg^{+2}, and ouabain) (190, 195, 196). It is clear from these studies that binding affinities are interdependent and vary with varying ligand concentrations. The system is further complicated by the fact that Na^+ inhibits K^+ binding at the K^+ binding site and vice versa. In the *in vivo* situation, the existence of an intact membrane modifies this situation. In spite of the complexities of interpretation, several conclusions seem applicable to the *in vivo* state:

1. It has been corroborated that conformational changes accompany the binding of ligands.

2. It is clear that the digitalis receptor is generated during the cyclical series of conformational changes constituting the normal operation of the pump. The receptor is probably not part of any of the actual cation-carrying moieties but is so constituted that its occupation by a cardiotonic steroid stabilizes one of the intermediate phosphorylated states of the enzyme.

3. There is some uncertainty as to the actual stage in the pump cycle at which the digitalis receptor appears, but the bulk of evidence favors its appearance at the stage where ATP is bound but before phosphorylation has occurred. The receptor may persist through several intermediate stages prior to the release of Na^+. If so, its occupation at any of the various stages could lead to different forms of the drug–enzyme complex. It is also possible that two quite distinct digitalis-binding sites may be generated during the pump cycle. Lindenmayer and Schwartz (196) believed that there is essentially only one species of ouabain–receptor complex. Schönfeld and associates (190) believed that there are least four distinct states of the binding conformation for ouabain, and Taniguchi and Iida (197) used Scatchard plot analysis to detect two distinct ouabain-binding sites in Na^+,K^+-ATPase.

Albers *et al.* (189) showed that the rate at which cardiotonic steroids are bound to the enzyme is inversely proportional to the number of sugars and hydroxy groups, and this was interpreted as inferring that the receptor may be hydrophobic in character. The same workers also showed (189) that the enzyme–ouabain complex can be phosphorylated by orthophosphate whereas the native enzyme requires the presence of a more effective leaving group (the adenine dinucleotide) for what is presumably a nucleophilic substitution reaction with phosphate.

The following scheme has thus emerged. Cardiotonic steroids inhibit Na^+,K^+-ATPase by combining with a hydrophobic patch on the extracellular face of the enzyme. This combination induces conformational changes which are relayed across the full width of the plasma membrane. These changes profoundly affect the reactivity of the cardinal site on the intracellular surface so that the energy barrier for phosphorylation is lowered and the stability of the resulting phosphorylated enzyme is increased to such a level that the pump is "frozen" at this stage in its cycle. The original hydrophobic patch is itself not a consistent component of the enzyme surface but is generated as a result of the binding of Na^+ and ATP to the inner surface of the membrane.

Since this review is intended primarily for chemists, it is appropriate to point out that conformational interactions of the type depicted here are fully in accord with modern concepts of molecular biology. As far back as 1945, Needham (176) wrote of the "ceaseless interchange [by which] the pattern of the body is fully maintained." A fascinating example is that of the amoeba which moves by continuously tearing down and building up its plasma membrane, the "old" membrane material "disappearing" into the cytoplasm.

Dissociation of Inotropic Effects from Inhibition of Na^+,K^+-ATPase—An apparent contradiction in the literature concerns the widely different reports of the stability of the drug–enzyme complex. According to Lindenmayer and Schwartz (196) and Albers *et al.* (189), the complex is virtually irreversible. In fact, the series of binding studies carried out by Schwartz and associates is based on the assumption that no dissociation of drug occurs under nonequilibrium conditions. In one such study (198), ouabain was administered to dogs in sufficient dosage to produce maximum inotropic effect. The hearts were then excised and Na^+,K^+-ATPase was extracted and subjected to partial purification under nonequilibrium conditions. The enzyme was found to possess only 40% of the activity of control enzyme, presumably because of bound ouabain. The authors stated that qualitatively similar results were obtained when the enzyme was subjected to more extensive purification procedures involving treatment with deoxycholate and prolonged extraction with concentrated sodium iodide solutions. By contrast, Yoda (199) reported that when ouabain–Na^+,K^+-ATPase complexes, which had been preincubated for 10 min, were subjected to 10-fold dilution, the apparent first-order dissociation rate constant was 0.42 hr^{-1}. This value was associated with an initial enzyme inhibition of 72%. Somewhat similar results were obtained for a selection of other monoglycosides.

Yoda's results were obtained under conditions in

Table I—Composite Table of Results Selected from Okita *et al.* (207)

Cardiotonic Steroid	Approximate Mean Increase in Force of Contraction, % of Control	Period Required to Washout Positive Inotropic Effect, min	Mean Percent Inhibition of Na^+,K^+-ATPase Isolated *Ex Vivo* after Washout Period
Strophanthidin-3-bromoacetate	30	30–45	29
Strophanthidin	30	40–60	38
Ouabain	30	60–90	39

which binding was promoted in the presence of Mg^{+2} and inorganic phosphate (P_i) as the only ligands. This is in accord with studies (200) indicating that significant ouabain binding occurs in the presence of either Mg^{+2} + ATP + Na^+ or Mg^{+2} + P_i or Mn^{+2} and that similar conformational changes may be associated with all three sets of binding conditions. Studies similar to those of Yoda were described in the previously cited comprehensive report (190) and are in substantial agreement with Yoda's results. The latter authors claimed that the release of ouabain from its complex with Na^+,K^+-ATPase is independent of whether phosphorylation was produced with ATP + Mg^{+2} + Na^+ or with P_i + Na^+. Schönfeld *et al.* (190) acknowledged that these conclusions are not supported by some other workers (201, 202) but cited different experimental techniques as the basis for lack of agreement. In another study (203), it was suggested that ouabain-induced enzyme inhibition was directly proportional to binding in the presence of K^+ and that the interaction of digitalis with the enzyme under optimal conditions may proceed through an intermediate state in which the drug was loosely bound and then to a more stable tightly bound complex. However, the same report suggested that the stable complex may also form as a result of binding in the presence of only Mg^{+2} and P_i.

In spite of this controversy, the authors of this present review accept the general conclusion reached by Allen *et al.* (203) that inhibition of Na^+,K^+-ATPase under *in vivo* conditions is associated with the formation of an extremely stable, relatively irreversible, drug–enzyme complex. This conclusion is important in view of the relative ease with which the inotropic effects of cardiac glycosides, particularly the more water-soluble types such as ouabain, can be washed out of organ bath preparations of myocardium (including whole heart).

In 1966–1967, one of the present authors worked for 10 months in the laboratory of Dr. George T. Okita. During that period, studies (204, 205) were initiated which are of considerable relevance to the issues raised. The experiments were based on the observations of Hokin *et al.* (206) that strophanthidin 3-haloacetates acted as active site-directed irreversible inhibitors of brain Na^+,K^+-ATPase. Hokin *et al.* found that these alkylating agents rapidly combined with the receptor to form a reversible complex, which was then more slowly converted to an irreversible complex, the conversion being complete within 3 hr. In the study of Okita and associates, guinea pig and rabbit atria were driven for 3 hr in a medium containing sufficient strophanthidol 3-bromoacetate-19-^3H to cause a 30–40% increase in the force of contraction. The atrial preparations were then washed, and it was found that the drug effect on contractility was rapidly removed (half-life for loss of drug effect from rabbit atria was 7 min compared with a half-life for drug loss of 240 min). To confirm that all contractility receptors had been cleared of drug, the ouabain dose–response curve was determined for the washed preparation and found to be identical to that of a properly constituted control.

The following explanation was offered to account for the dissociation of drug effect and drug half-life. Since the drug must have been associated with the contractility receptors for 3 hr (a 30–40% increase in force of contraction was recorded for all of this period) and since this drug effect was rapidly and completely reversed by washout, the "contractility" receptor, unlike Na^+,K^+-ATPase, was incapable of being alkylated and was different from Na^+,K^+-ATPase. This conclusion was subject to the following limitations:

1. The concentrations that Hokin *et al.* (206) found necessary for irreversible alkylation were at least one magnitude greater than those used in Okita's work.
2. The mode of action of alkylating cardiotonic steroids may be different from that of nonalkylating steroids.
3. The atrium may not be representative of the whole myocardium.

These limitations were overcome in a recent study (207). In addition to using an alkylating agent, the effects of strophanthidin and ouabain were also examined. The experiments were carried out using the Langendorff heart preparation, and Na^+,K^+-ATPase activity was assayed both before and after the washout period. The main features of Okita's work are shown in Table I, which is based on Ref. 207.

The results shown in Table I clearly infer a separation of positive inotropic effects from inhibition of Na^+,K^+-ATPase. However, recent studies similar to those described by Okita *et al.* have failed to demonstrate the separation of effects observed by the latter (A. Schwartz, private communication). Since both groups of workers apparently commenced washout procedures before the phase of drug equilibration was complete and also used isolation techniques (K^+-free media) that were not optimum for minimizing dissociation of the enzyme-drug complex, more work in this area is needed.

In 1973, Goldman *et al.* (208) published the results of an *in vivo* study which they claimed supported Repke's hypothesis. These authors studied the effects of digoxin in normokalemic and hyperkalemic dogs and found that both the positive inotropic effects and the degree of inhibition of Na^+,K^+-ATPase were significantly reduced in the hyperkalemic dog as compared with the normokalemic animal (K^+ is known to inhibit the ability of cardiotonic steroids to

inhibit Na^+,K^+-ATPase; see Refs. 14 and 17 for a discussion of this effect). The main features of the Goldman *et al.* study are shown in Table II.

Table II—Composite Table of Results Selected from Goldman *et al.* (208)

Parameter	Approximate Percent by which Parameter Was Reduced in Hyperkalemic Dogs as Compared with Normokalemic Dogs
Positive inotropic effect (*dp/dt*)	82
Drug concentration in left ventricle, ng/mg	23
Drug bound to microsomal fraction, ng/mg	30
Percent inhibition of Na^+,K^+-ATPase	37

STRUCTURE–ACTIVITY RELATIONSHIPS

Structure–activity relationships are studied for various reasons: to provide empirical guidelines for the synthesis of new compounds; to seek evidence for the existence of a receptor; and to establish degrees of correlation between biochemical, physiological, therapeutic, and toxic effects associated with the drug. For all of these purposes, structure–activity relationship analyses can prove both useful and misleading and should always be interpreted with caution. Most correlations between biological effects are based on circumstantial evidence; the danger is that once a theory, which seems reasonable, has become widely accepted, all new evidence is conceptualized in terms of the theory and alternative explanations are not sought. Even such a well-established concept as the role of the cholinergic system in nerve transmission can be turned "on its head," and one group of workers (209, 210) rejected the theory that acetylcholine is involved in synaptic transmission.

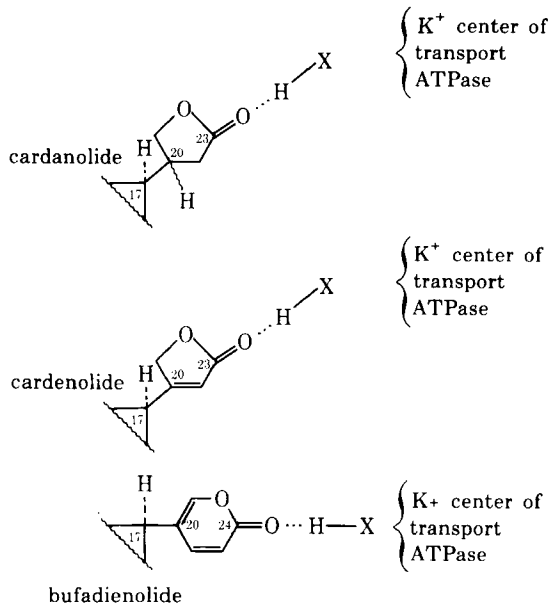
The first step in any structure–activity relationship analysis is to decide, if possible, whether the drug is acting by a specific (receptor-mediated) mechanism or by a nonspecific mechanism (such as membrane swelling). Drugs that act by a nonspecific mechanism may still be selective in action if their physical properties cause them to concentrate in particular parts of the body. The barbiturates, for example, are thought by some workers to act nonspecifically by selective effects (probably membrane swelling) which result in depression of the arousal center in the brain (211). Slight modifications to the structure of barbiturate molecules can subtly alter the physical properties of these drugs so that their selective effect is manifested elsewhere in the brain and the body convulses.

The activity of drugs that act by a receptor-mediated mechanism usually show great sensitivity to minor changes in structure. This sensitivity to structural changes applies in the case of the phenothiazine antipsychotics; yet there is great controversy as to whether these drugs act by a specific or nonspecific mechanism (212). In fact, the phenothiazine antipsychotics are a good group of drugs with which to compare the cardiac glycosides. There is much dispute as to the molecular and biochemical basis of antipsychotic activity. Many properly established, but conflicting, correlations have been made, including one that is often used in the screening of antipsychotics, namely the good correlation that exists between blocking of the conditioned-avoidance response in the rat and antipsychotic activity in humans. Here at least it is obvious that one is not dealing with a cause-and-effect relationship but with an associative relationship that reflects some feature common to both species but separately connected with the two phenomena. This is not an unusual situation and, in philosophical terms, it reflects one of the most important scientific concepts of the 20th century, namely the quantum theory which states, in general terms, that nature has achieved the vast complexity of the universe by utilizing a relatively small number of basic building blocks. It is the *organization* of the building blocks that constitutes the essential features of different systems, but it is often only the building blocks that the probes detect.

Model for Digitalis Receptor—It is generally accepted, and it is probably true, that the physiological effects of the cardiac glycosides are mediated by an interaction with a specific receptor. This conclusion is based on the high potency, high selectivity, and apparently specific structural requirements of digitalis. The idea is supported by the existence of at least one type of identifiable digitalis receptor, Na^+,K^+-ATPase. One reliable indicator of a receptor-mediated mechanism, the existence of a structually related competitive antagonist, has not been found for digitalis. Nevertheless, it will be assumed that the biological effects of cardiotonic steroids result from the interaction of these drugs with one or more closely related receptors.

According to the classical studies of the structure–activity relationships of digitalis, the entire molecule seems to take part in the drug–receptor interaction. Thus, the 17β-lactone ring, the unique stereochemistry of the digitalis steroid moiety, and the nature of the substituent at C-3 were all shown to be important for activity. It was also known that the inactive 17β-H isomers of cardiac glycosides were not effective as inhibitors of the active 17α-H isomers, so the determining factors in structure requirements were probably concerned with what is now called affinity (binding) rather than intrinsic activity (factors that affect the nature of the drug–receptor complex).

In 1963, Repke (213) showed that similar relationships applied to the binding of active cardiotonic steroids to Na^+,K^+-ATPase. Concentrations of 17β-H isomers up to 10,000 times those of the active 17α-H isomers were without effect on the latters' log dose–response curves for enzyme inhibition. Repke concluded that the lactone ring played a key role in the drug–receptor interaction and that unless the ring was correctly orientated with respect to the rest of the molecule, no binding at all took place. Repke fur-



Scheme XXIV—Representation of hypothetical one-point attachment of digitalis molecule to receptor enzyme by hydrogen bridge binding (16)

ther hypothesized that the lactone ring was bound to the receptor by a single-point attachment comprising a hydrogen bond, the strength of which was proportional to the fractional negative charge on the carbonyl group. Thus, the increasing capacity of the cardanolide, cardenolide, and bufadienolide systems to form hydrogen bonds (Scheme XXIV), was shown to parallel ability to inhibit Na^+,K^+-ATPase (213).

Repke recognized that a single-point attachment involving a hydrogen bond would have negligible binding force. He proposed (16) that the β-face of the steroid system interacted with a complementary patch on the receptor surface and that this interaction, through an accumulation of van der Waals forces, provided most of the binding energy. The role of the α,β-unsaturated lactone was thus envisaged as one that directed the molecule toward the receptor by the formation of a hydrogen bond acting over a relatively long distance; but once the molecule was correctly in position, the shorter range van der Waals bonds became the chief binding force.

The role that Repke assigned to the steroid ring is almost certainly correct since lactone rings *per se* are inactive. Forces that bind the steroid, however, may be other than van der Waals forces and could involve conformational changes in the receptor surface of the type discussed by Belleau (214), in which binding results as a consequence of a shift by a macromolecule to a more thermodynamically stable state with the drug molecule becoming locked within a fold of the receptor.

The role that Repke described for the lactone also seems reasonable, but consideration needs to be given to whether a single hydrogen bond is adequate to initiate the formation of the drug–receptor complex. After all, the possibilities for hydrogen bond formation in the body are immense, and hydrogen bonding with water must first be disrupted before the lactone can react with the receptor. Moreover, the difference in the ability of cardenolides and cardanolides to form hydrogen bonds is not an adequate explanation, in terms of Repke's theory, to explain the observed differences in biological potency. It is therefore hypothesized (89, 215) that the α,β-unsaturated lactone binds by a reinforced hydrogen bond and that such a possibility is not available for the cardanolides. Some evidence for this proposal will now be examined.

To study further the role of the lactone in controlling the biological activity of cardiotonic steroids, we have replaced the lactone ring of digitoxigenin (II) with a series of open-chain structures of varying electronic and steric similarity to the lactone (XLVII–LXXIX). These compounds were tested for their ability to inhibit guinea pig myocardial Na^+,K^+-ATPase (89) and for their ability to affect guinea pig myocardial contractility (215). A selection of the results is shown in Table III.

The results in Table III show that a range of potency was observed, varying from compounds with activities comparable to those of the parent molecule, digitoxigenin, to compounds that were inactive or that produced a negative inotropic effect. We have shown that it is possible to replace the butenolide ring with certain open-chain structures, namely, $-CH=CHCOOCH_3$ (XLIX), $-CH=CH-CN$ (LXIII), and $-CH=N-NH-C(NH)NH_2$ (LXXI), and still retain considerable potency (Table III). All of these compounds increased the force of myocardial contraction by approximately 120% at maximum therapeutic doses (Table IV). Compounds of the 17α-series (*e.g.*, LXXVI) were inactive or virtually inactive, confirming Repke's hypothesis. Reduction of the Δ^{20}-double bond (*e.g.*, LI) abolished activity.

Table III—Comparison of Effects on Guinea Pig Atrial Contractility and Guinea Pig Myocardial Na^+,K^+-ATPase (89, 215)

Compound	Positive Inotropic Effect (Potency Relative to Digitoxigenin)[a]	Inhibition of Na^+,K^+-ATPase: Potency Relative to Digitoxigenin[a]	Inhibition of Na^+,K^+-ATPase: Inhibition at 10^{-4} M[a], %
Digitoxigenin (II*a*)	1.0	1.0	100
Methyl ester (XLIX)	0.49	1.3	100
Nitrile (LXIII)	0.66	1.1	100
Guanylhydrazone (LXXI)	0.20	0.14	76
Branched ester (LV)	0.04	0.09	47[b]
Ethyl ester (XLVII)	0.03	0.09	44[b]
Isopropyl ester (LII*b*)	0.005	0.06	55
Phenyl ester (LIX*a*)	0.005	0.12	50[b]
Diene ester (LVIII)	0.004	0.03	33
17α-Methyl ester (LXXVI)	0.003	Inactive	Inactive
Reduced methyl ester (LI)	Inactive	0.03	24[b]
Acid (XLVIII)	Inactive	Inactive	Inactive
Semicarbazone	Inactive	Inactive	Inactive
Phenyl ketone (LIX*a*)	Negative inotropic effect	0.06	30[b]

[a] Calculated from the log dose–response curve. [b] Percent inhibition at 10^{-5} M. Inhibition at 10^{-4} M was not read from the dose–response curve because of suspected insolubility at or above this concentration.

Increasing the bulk of the alkoxy group (*e.g.*, XLVII and LII*b*) greatly reduced activity. Increasing the distance between the electronegative carbonyl group and the steroid system by means of an extended (diene) chain (*e.g.*, LVIII) was also associated with marked loss in potency. The presence of the carbonyl group was shown not to be an essential requirement for activity since the α,β-unsaturated nitrile (LXIII) and the guanylhydrazone (LXXI) were both potent substances.

Any interpretation of these results must include an evaluation of the relative importance of both the steric and electronic consequences of the changes described as well as the possibility that reduction in activity may be associated with unfavorable interactions with the rest of the molecule or with the receptor surface. These factors were considered in a detailed analysis of the results published elsewhere (215). If, for the moment, consideration of the guanylhydrazone (LXXI) is excluded, then the results indicate that all that is required for activity is the following *coplanar* arrangement of atoms in the side chain —CH=CH—C(—)=A (where A = a hetero atom).

Our analysis also indicated that this side chain probably interacted with the receptor by means of a two-point attachment, involving perhaps a hydrogen bond at one of the points as shown in Fig. 4.

The model shown in Fig. 4 is compatible with our observation that reduction of the C-20 double bond led to total or almost total loss in activity since the reduced compound lacked both the fractional positive charge at C-20 and the coplanar arrangement of atoms. The model would also account for the great loss in activity observed when a methyl group was substituted at C-21 (LV), since it may be presumed that the methyl group would physically prevent the close association needed to establish an effective two-point attachment. This explanation is also compatible with the results of Eberlein *et al.* (78) who found that the introduction of fluorine into the C-21 position of the lactone ring gave an active compound whereas introduction of a methoxy group at C-21 (XXXIII) greatly reduced activity. Fluorine has approximately the same size as hydrogen.

If it may be further supposed that the whole of the C-17 side chain fits into a "cleft" in the enzyme surface, then it is possible to explain the influence of R′ (Fig. 4) on biological activity. When R′ does not exceed a critical size, no unfavorable steric interactions occur and activity reaches a maximum. This condition operates in the case of the nitrile (LXIII), where R′ is absent, and in the case of the methyl ester (XLIX), where R′ is methoxy. It also applies to digitoxigenin (II*a*), where R′ is equivalent to methoxy. When R′ is ethoxy (XLVII), steric interactions occur with the edge of the cleft and activity is greatly reduced. Further increases in the size of R′ lead to no further appreciable fall in activity, indicating that steric interactions reach an unfavorable maximum once the size of R′ exceeds methoxy. The concept of drug molecules fitting into and interacting with clefts in biological macromolecules is well established. Actinomycin D, for example, is believed to fit into the lesser groove of DNA.

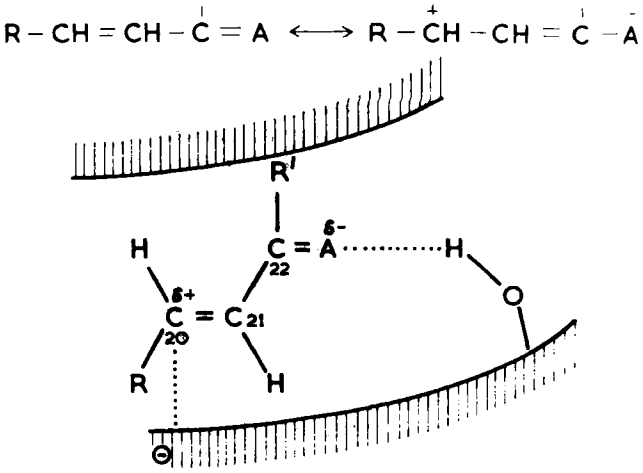


Figure 4—*Proposed interaction of the C-17 side chain of active cardenolide analogs with the "digitalis" receptor. The side chain is shown as lying within a cleft on the enzyme surface. Binding is depicted as involving two points on the side chain, the electron-rich hetero atom (A) and the electron-deficient C-20. The origin of the charge distribution in the side chain is shown by the resonance structures drawn above the receptor model. The scheme is compatible with structure–activity relationship analyses based on both inhibition of Na^+,K^+-ATPase (89) and positive inotropic effects (215).*

Finally, the model incorporates the observation that the ester group, as present in the lactone ring of digitoxigenin (II*a*) and in the methyl ester (XLIX), is not essential for activity since the nitrile (LXIII) has comparable activity.

The side chain of the acid (XLVIII) has some features common to those of the active compounds:

methyl ester (XLIX): —CH=CH—C(OCH$_3$)=O (active)

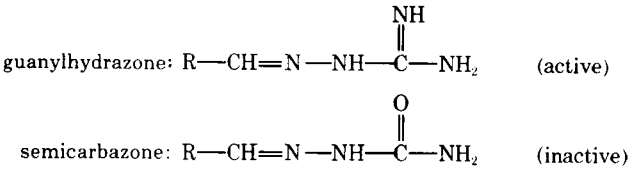
nitrile (LXIII): —CH=CH—C≡N (active)

acid (XLVIII): —CH=CH—C(=O)—O$^-$ (inactive)

The acid meets the stereochemical requirements of the model but, under physiological conditions, it lacks a fractional positive charge at C-20 and has a full negative charge distributed over the terminal oxygens. The model thus predicts the inactivity of the acid since, although the acid can very effectively hydrogen bond, the full negative charge would be repelled by the negative charge proposed for the receptor. The fact that compounds with electron-rich groups, such as the α,β-unsaturated nitrile (LXIII) or ester (XLIX), can interact with the receptor is not incompatible with this reasoning since, in the case of the ester and nitrile, the fractional negative charge is neutralized by the formation of a hydrogen bond and as such contributes to binding.

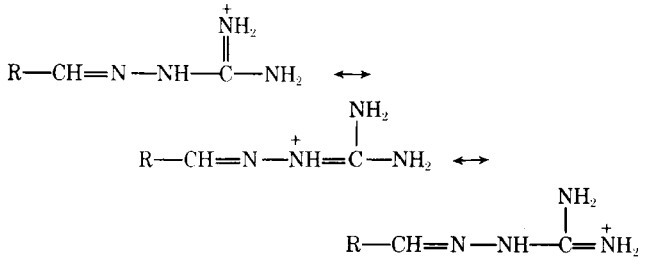
Further indirect evidence for the existence of a substantial negative charge on the receptor surface was obtained from a consideration of the activity of the guanylhydrazone (LXXI), which was approxi-

mately one-fifth as potent as digitoxigenin. Under physiological conditions, the guanylhydrazone group would exist as a cation with the positive charge dispersed over most of the side chain. The electron-rich group thought to be necessary in the case of the other active compounds is thus absent in the guanylhydrazone cation. Since we found that the log dose–response curve of the guanylhydrazone was parallel to those of the other active compounds, including digitoxigenin, we suggested that the guanylhydrazone cation interacted with the proposed receptor by the formation of a single-point attachment involving an ion-pair interaction. The fact that the nonionized semicarbazone analog was inactive supports the suggestion that electronic interactions between the side chain and the receptor are essential to the activity of cardiotonic steroids. The close similarity between the two side chains is shown below:



The guanylhydrazone group is a strong base because it can accept a proton to produce a cation with the positive charge distributed over all three nitrogens of the terminal guanidine portion of the side chain (Scheme XXV).

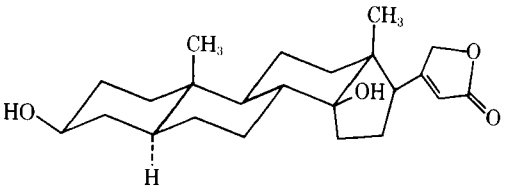
This line of reasoning can be extended to provide an explanation for the cardiotonic activity of the bisguanylhydrazones of prednisolone and related steroids (see *Steroidal Guanylhydrazones and Other Cardioactive Compounds*). The steroid systems of these compounds do not possess the properties predicted by Repke for the formation of effective van der Waals forces, so the main source of binding energy proposed by Repke for digitalis-like steroids is greatly diminished or absent. If we now propose that there exists a second anionic site on the receptor surface in the vicinity of the area that binds the C-3 region of the steroid, then the binding of the prednisolone-type derivatives can be accounted for in terms of two ion-pair interactions. As discussed previously, the stereochemistry of the steroid system is less critical for the 3,20-bisguanylhydrazones than for digitalis-like compounds. Also, since the positive charge of the guanylhydrazone group is distributed over three atoms, it is possible to allow a considerable latitude in the distance between the two guanylhydrazone groups and still align a positive charge with the fixed



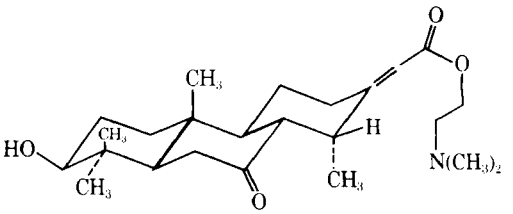
Scheme XXV

anionic sites on the receptor. Our proposal that there is a strong nucleophilic group (perhaps a full anion) in the region of the receptor which corresponds to the C-3 portion of the steroid is supported by the fact that this region can be alkylated by 3-haloacetyl derivatives of cardiotonic steroids (107).

It remains now to account for the activity of the other main group of cardiotonic substances, the *Erythropleum* alkaloids. The structural formula of the principal member of this group, cassaine (CVII), is shown here together with that of the cardiotonic steroid uzarigenin (CXVI).



CXVI: uzarigenin



CVII: cassaine

The structure–activity relationships of cassaine and related semisynthetic analogs have been studied (153–155) and are compatible with the proposed model for the digitalis receptor (Fig. 4). Cassaine and its active analogs possess the required >C=CH—C(—)=A grouping (in this case an ester). Activity is lost when the ester is hydrolyzed to the free acid or if the double bond is reduced. Activity is greatly reduced when a methyl group is attached to the double-bonded carbon atom (analogous to C-21 in the cardiotonic steroids). It will be recalled that we accounted for a similar fall in the activity of the digitoxigenin analog (LV) by postulating that the methyl branch at the double bond interfered with the formation of the two-point attachment to the receptor.

There is one significant way in which the structure–activity relationships of the cassaine analogs differ from those of digitoxigenin, namely that the length of the alkoxy group (R′, Fig. 4) is critical for the digitoxigenin series but not for the cassaine series. However, if the width of the cassaine alkoxy groups is increased by replacing the terminal *N*-methyl groups with ethyl groups, almost all activity is lost. Thus, steric features of the receptor seem to be limiting the scope of molecular modification for both series, but the limitation is expressed in a different way. It is possible to still retain the idea of the side chain fitting into a cleft as depicted in Fig. 4 by assuming that the alkoxy group of cassaine lies along the axis of the cleft rather than across it. This assumption is not unreasonable in view of the difference in the manner of attachment of the cassaine side chain to the ring system.

In attempting to correlate structure with activity, we have made the simplest assumption, namely that all of the three groups of cardiotonic substances (digitalis-like steroids, bisguanylhydrazones, and cassaine-like alkaloids) interact with the same receptor. While we have no definite proof for this assumption, the proposal is consistent with structure–activity relationship analyses for the three groups. Our proposals for the nature of the receptor were based on correlations with cardiotonic activity but, in the case of our own work at least, apply equally well to inhibition of Na^+,K^+-ATPase (Table III).

We have shown that cardiotonic activity is associated with certain steric and electronic features of the C-17 side chain (or its equivalent in the cassaine alkaloids). These features suggest that the side chain may fit within a cleft or depression in the receptor surface and interact with an anionic site. This interaction may involve a single ion-pair association or a two-point attachment consisting of a weak electrostatic interaction reinforced by a hydrogen bond. It is possible that the electronic interaction promotes a redistribution of charge density and that this may play some role in the allosteric mechanism suggested for digitalis-like activity (see *Biological Activity*). It would not be possible for the binding forces depicted in our model (Fig. 4) to account for the pseudo irreversible binding associated with at least some aspects of digitalis-like activity. The remaining portions of the molecule must thus play some highly significant role in binding. It is possible that the receptor itself undergoes "accommodating perturbations" (214) when it interacts with cardiotonic substances, and this may provide part of the binding energy.

Relationship between Cardiotonic Activity and Inhibition of Na^+,K^+-ATPase—In 1961, at the First International Pharmacological Conference, Repke (23) proposed that inhibition of Na^+,K^+-ATPase may be the biochemical basis for the effects of digitalis on myocardial contractility. This theory was further developed at the Second International Conference (213) when Repke showed that there were many parallels between the two biological effects of digitalis. The ouabain log dose–response curve for inhibition of guinea pig myocardial Na^+,K^+-ATPase was shown to encompass the range of concentrations producing inotropic and toxic effects. It was also shown that both biological responses were affected in a similar way by hypothermia and by changes in the concentrations of K^+, H^+, Ca^{+2}, Na^+, and Li^+.

While these effects are significant, they are too general to establish firmly a cause-and-effect relationship. Repke, therefore, compared the concentration of drug required to inhibit guinea pig Na^+,K^+-ATPase with the dose (micromoles per kilogram) required to produce lethal effects in the intact cat (see Ref. 216 for a recent presentation of these data). This comparison was carried out for 21 digitalis-like compounds. The results showed in a general way that compounds highly toxic in the cat were powerful inhibitors of myocardial Na^+,K^+-ATPase in the guinea pig. Since the therapeutic ratios of most cardiac glycosides are similar, it may also be inferred that a similar parallelism exists between inhibition of the enzyme and positive inotropic effects.

Table IV—Comparison of Na^+,K^+-ATPase Inhibition with Stimulant Activity on Guinea Pig Atrial Muscle (All Values Were Read from the Log Dose–Response Curve)

Compound	Concentration, $\times 10^{-7}$ *M*	Inhibition of Na^+,K^+-ATPase, % ± Mean Deviation	Increase in Force of Contraction of Atria, ± *SE*
Digitoxigenin (II*a*)	3.2	10 ± 5	25 ± 5
	9.0	20 ± 3	75 ± 12
	21	30 ± 2	130 ± 21
	33	40 ± 4	Toxicity
	65	50 ± 2	Toxicity
Methyl ester (XLIX)	3.2	10 ± 5	10 ± 3
	10	20 ± 3	40 ± 8
	20	30 ± 4	80 ± 10
	31	40 ± 3	115 ± 15
	65	50 ± 2	Toxicity
Nitrile (LXIII)	4.0	10 ± 2	15 ± 3
	10	20 ± 2	55 ± 10
	21	30 ± 2	105 ± 12
	33	40 ± 2	Toxicity
	63	50 ± 2	Toxicity
Guanylhydrazone (LXXI)	50	10 ± 1	75 ± 7
	100	20 ± 2	120 ± 12
	200	30 ± 2	Toxicity
	310	40 ± 2	Toxicity

This conclusion is supported by our own studies (Table III) in which we compared the positive inotropic effects of a series of analogs of digitoxigenin with ability to inhibit Na^+,K^+-ATPase. The ranking order and relative potencies are in good overall agreement for the two biological effects but a strict quantitative relationship was not observed (Table IV).

Since the results in Table IV were obtained using two different biological preparations, the differences in relative potency may reflect differences in transport and distribution phenomena for the two systems. Alternatively, our results could be taken at "face value" and the conclusion drawn that the same percent of enzyme inhibition is associated with different degrees of positive inotropism and hence that the two effects were not causally related.

Repke *et al.* (217) studied species differences with respect to response to ouabain. They compared inhibition of Na^+,K^+-ATPase with positive inotropic and lethal effects in eight species. With one exception, the frog, each species showed the same rank order of sensitivity to ouabain for the three parameters. Quantitatively, the sensitivity scales were similar for each parameter measured for a particular species, but strict ratios were not preserved. Nevertheless, the study represents one of the most significant pieces of evidence in support of a cause-and-effect relationship between inhibition of the enzyme and effects on contractility.

Repke (23) also showed that the cardiotoxic effects of cardiac glycosides in humans and rats parallel inhibition of Na^+,K^+-ATPase from erythrocytes of the respective species. He also found that the sensitivity of human erythrocyte Na^+,K^+-ATPase reflects the changes in cardiotoxic effects that accompany modification of the structures of various cardiotonic ste-

roids. In other words, erythrocyte Na^+,K^+-ATPase reflects the properties of the digitalis receptor in the heart. The question that we have not been able to answer satisfactorily is whether myocardial Na^+,K^+-ATPase is also just a suitable model for the "contractility" receptor or whether it is the actual receptor.

CONCLUSIONS

In spite of considerable effort, it has not been possible to explain satisfactorily the mode of action of cardiac glycosides or to find a suitable, less toxic substitute. There is some evidence that structural modification may improve the therapeutic index, but clinical evidence has yet to be obtained. There have been several attempts to modify toxicity by changing the pharmacokinetic properties of digitalis, but adequate clinical data to substantiate these claims have yet to be published.

The question of whether inhibition of Na^+,K^+-ATPase is the biochemical basis for the effects of digitalis on myocardial contractility has yet to be resolved. There is no doubt that there is a remarkable series of parallelisms between the effects of cardiotonic steroids on the two biological resposes. Dose ranges, structure–activity relationship analyses, and species differences all give general support for a cause-and-effect relationship. This is further substantiated by a large body of biochemical data, which shows that a digitalis receptor is present in the enzyme complex and that the conditions for binding to this receptor and for inhibiting the enzyme parallel those that determine the magnitude of effects on contractility.

On the other hand, it is known that a number of substances will inhibit the enzyme without affecting myocardial contractility. It has also been claimed that it is possible to wash out the inotropic effect and still leave the drug bound to the enzyme receptor. Finally, there is evidence that a strict quantitative relationship between ability to inhibit the enzyme and to produce a positive inotropic effect may not apply.

It seems then that the data can be interpreted in four different ways:

1. Na^+,K^+-ATPase is the receptor for contractility but an inotropic response only occurs when the enzyme is inhibited in a particular way (for example, by digoxin and not by oligomycin). The digitalis-like mode of inhibition may induce a particular type of conformational change which triggers some mechanism (such as the release of Ca^{+2}) which then mediates the inotropic response.

2. The contractility receptor is contained by a subfraction of the cell's Na^+,K^+-ATPase content. This specialized form of the enzyme has an explicit role in controlling myocardial contractility and forms readily reversible complexes with cardiotonic steroids. The enzyme comprises only a minute proportion of the cell's total Na^+,K^+-ATPase content.

3. The contractility receptor is not Na^+,K^+-ATPase but has evolved from it and closely resembles it in many ways. Na^+,K^+-ATPase is thus a suitable model for this receptor.

4. Cardiotonic steroids act by combining with a receptor present within the myoplasm. The drugs enter the cell by a process of active transport which requires Na^+,K^+-ATPase. Combination with the transport receptor is the rate-determining step. Under *in vivo* conditions, therapeutic doses of digitalis-like substances do not inhibit the enzyme, although this is shown under *in vitro* conditions. There is some evidence (218) that the action of cardiac glycosides depends upon active transport into the myocardium.

The present authors believe that the second proposal is the most likely, but it is recognized that the stage at which the study of cardiotonic steroids has reached is not, to paraphrase Churchill, at the beginning of the end but simply at the end of the beginning.

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[x] To whom inquiries should be directed.

Protein Binding and Erythrocyte Partitioning of Disopyramide and Its Monodealkylated Metabolite

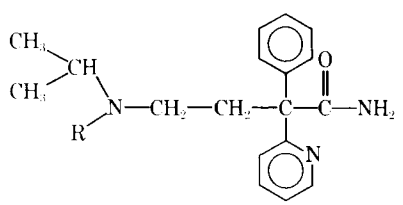
PETER H. HINDERLING, JANINE BRES, and EDWARD R. GARRETT ˣ

Abstract □ The plasma protein binding of disopyramide, determined by equilibrium dialysis and ultrafiltration, is saturable; the fraction bound to human plasma protein varied from 0.05 to 0.65 with decreasing plasma concentration. The modified Scatchard plots are curved and indicate either the existence of more than one class of independent binding sites or a set of binding sites, where the binding of one molecule of the drug decreases the affinity of another to a previously equivalent binding site. The presence of high concentrations of the monodealkylated metabolite of disopyramide lowered the binding of the parent drug significantly. The fraction of the metabolite bound was less than disopyramide and varied between 0.05 and 0.10. A high concentration of disopyramide decreased the metabolite binding significantly. The red blood cell partitionings of unbound disopyramide (D = 1.2) and the metabolite (D = 1.4) in plasma, studied by centrifugal and ultrafiltration methods, were concentration independent and unaltered by the presence of high concentrations of each other. Equilibration between plasma and red blood cells was quickly obtained for disopyramide but not for the metabolite. Reequilibration of red blood cells containing the drugs gave the same partition coefficients.

Keyphrases □ Disopyramide and monodealkylated metabolite—protein binding and erythrocyte partitioning, equilibrium dialysis and ultrafiltration, Scatchard plots □ Protein binding—disopyramide and monodealkylated metabolite, equilibrium dialysis and ultrafiltration, Scatchard plots □ Erythrocyte partitioning—disopyramide and monodealkylated metabolite

Disopyramide[1] [4-diisopropylamino-2-phenyl-(2-pyridyl)butyramide] (I) is a new and effective antiarrhythmic drug (1–3). It is excreted unchanged and as the monodealkylated metabolite [4-isopropylamino-2-phenyl-(2-pyridyl)butyramide] (II) in humans[2].

The kinetics of drugs in, and their effects on, the body can only be described fully when drug properties in the subcompartments of the central compartment are understood. Evidence exists that the transfer kinetics of drugs are primarily dependent on the concentration of unbound or free, and possibly unionized, drug in plasma water (4). Too frequently, steady-state conditions are assumed within the central compartment without experimental evidence, even though it has been shown that the erythrocytes may act as a "deep compartment" for several drugs, with low rates of transfer with the plasma (5) (Scheme I).



I: R = $(CH_3)_2$—CH
II: R = H

For proper evaluation of studies on the pharmacokinetics and pharmacodynamics of disopyramide[2], it was necessary to delineate the protein binding and red blood cell partitioning of disopyramide and its metabolite separately and in the presence of each other.

This paper compares the methods of equilibrium dialysis and ultrafiltration in the study of protein binding and the methods of centrifugation and ultrafiltration in erythrocyte partitioning. The methods of analysis were spectrofluorometry and liquid scintillation spectrophotometry of labeled drug. It will be shown that disopyramide has unusual binding properties to plasma proteins which, if not considered properly, would lead to the anomalous aberrations of nonlinear pharmacokinetics. These properties suggest a saturable binding to one set of binding sites with high affinity and a less saturable set of binding sites with lower affinity where the fraction bound is a function of the concentration of free drug and/or metabolite in the plasma water.

EXPERIMENTAL[3]

Reagents—Disopyramide phosphate[1] in sterile aqueous solution, ^{14}C-disopyramide phosphate[1], and ^{14}C-monodealkylated metabolite[1] were used.

The radiolabeled disopyramide was synthesized by the method described by Karim *et al.* (6) and had a specific activity of 19.5 μCi/mg. The ^{14}C-labeled monodealkylated metabolite was obtained by isolation from the urine of volunteers who had received the labeled parent drug. Pure metabolite, with a specific activity of 0.16 μCi/mg, was obtained by the method of Karim *et al.* (6).

[1] Norpace is the phosphate salt of disopyramide, G. D. Searle & Co., Skokie, Ill.

[2] P. H. Hinderling and E. R. Garrett, manuscript in preparation.

[3] A Foci model MK-1 spectrofluorometer (Farrand Optical Co., New York, N.Y.) was used in the analysis of disopyramide. A Beckman model LS 100 scintillation counter (Beckman Instruments, Fullerton, Calif.), efficiency determined by an external standard method, and a Packard Tri-Carb 3003 scintillation counter (Packard Instruments Co., Downers Grove, Ill.), efficiency determined by the channel ratio method, were used for the measurement of labeled disopyramide and the monodealkylated metabolite. The pKa′ was determined with a recording titrator, type TTT 1c/SBR^{2}c (Radiometer, Copenhagen, Denmark) equipped with a combination electrode (Corning, New York, N.Y.). Centriflo membrane ultrafilters with conical supports and tubes (Amicon Corp., Lexington, Mass.) were used for ultrafiltration; Visking membranes (Visking Corp.) were used for equilibrium dialysis. A model MB microhematocrit centrifuge (International Equipment Co., Needham Heights, Mass.), tubes (Yankee, Clay Adams, Division of Becton, Dickinson and Co., Parsippany, N.J.), and a microcapillary reader (International Equipment Co., Needham Heights, Mass.) were used to determine the hematocrit.

The liquid scintillation reagent used was Aquasol, New England Nuclear, Boston, Mass. *tert*-Butyl hydroperoxide was obtained from Matheson, Coleman and Bell, Norwood, Ohio.

Heparinized blood and human plasma[4] with known protein fractions were obtained from healthy volunteers who had no prior drug intake. All organic solvents used were of analytical reagent grade.

pKa' Determination of Disopyramide—The differential potentiometric method of Parke and Davis (8) was applied to the titration of 10.0 ml of a sterile aqueous disopyramide solution (3.0 mg/ml = 6.86×10^{-3} *M*) in 0.0045 *N* HCl with 0.0050 *N* NaOH. A solution of 10.0 ml of 0.0045 *N* HCl was titrated with 0.0050 *N* NaOH as the blank.

Analytical Procedures—The spectrofluorometric assay described by Ranney *et al.* (9) was used. This method depends on the fluorescence exhibited by disopyramide in strong acid solution at 410 nm, activated at 275 nm. The original procedure was modified in the measurement of plasma drug concentration to precipitate the plasma proteins by trichloroacetic acid. Plasma (3.0 ml) and water (0.5 ml) were mixed in a centrifuge tube, which was shaken and centrifuged for 2 min. Neutralization was effected by the addition of 1.5 ml of 2.5 *N* NaOH. An aliquot (2.0 ml) of the supernatant solution was carried through the methylene chloride (10.0 ml) extraction procedure. Separate calibration curves were prepared for drug in phosphate buffer solutions and/or plasma in a concentration range between 5×10^{-6} and 2.74×10^{-5} *M* disopyramide. This precipitation of plasma proteins decreased the background significantly.

The liquid scintillation spectrophotometry for ^{14}C-disopyramide and its ^{14}C-metabolite used duplicate aliquots of 100–500 μl of plasma or plasma water pipeted into liquid scintillation vials containing 10 ml of a scintillation reagent. The samples were measured for 10 or 20 min, and the measurements were repeated four times. Corrections were made for background and efficiency. The efficiency was determined either by using an external standard or by the channel ratio method (10), depending on the instrument used.

Plasma water aliquots with slight degrees of hemolysis were subjected to a bleaching procedure prior to measurement to minimize color quenching. Ten to 25 μl of 1 *N* NaOH was added to the plasma water aliquots placed in liquid scintillation vials. The vials were then kept at 37° for 2 hr and for an additional 8 hr after the addition of 10 μl of *tert*-butyl hydroperoxide. Subsequently, 10 ml of a scintillation reagent was added to each vial and the counts were measured. Corrections were made for background and efficiency.

Protein Binding of Disopyramide and Its Monodealkylated Metabolite—Equilibrium dialysis and ultrafiltration were used to study the protein binding of disopyramide in human plasma. A three-chamber dialysis apparatus (11) was used for equilibrium dialysis. The chambers were separated by two membranes. Plasma was placed in the middle chamber, disopyramide in sterile pH 7.4 isotonic phosphate buffer (5.50×10^{-5}–1.10×10^{-3} *M*) was placed in one outer chamber, and sterile buffer was placed in the other. The apparatus had been sterilized previously by steam in an autoclave, except for the membranes and O-rings which were sterilized under UV light. Dialysis was effected for 48 hr at 4° and for 16 hr at 37 and 25°, respectively.

The achievement of equilibrium was ascertained by the equivalence of disopyramide concentrations in both outer chambers. Disopyramide was assayed by the spectrofluorometric method in both outer chambers and in the middle chamber. A pH of 7.15–7.45 was maintained in all three chambers and was determined at the beginning and end of the experiment. The total drug concentration in plasma (middle chamber) at equilibrium ranged between 2.89×10^{-4} and 1.87×10^{-6} *M*. Preliminary experiments showed no significant membrane binding of disopyramide.

In the ultrafiltration studies, spiked plasma aliquots were half-filtered through high flux cone membranes. Half-filtration was preferred, because complete filtration of plasma with precipitation in the cone from a substrate containing plasma might tend to trap and inhibit the free passage of other soluble plasma components (12–14). Total drug concentrations (bound and unbound) in spiked plasma prior to filtration and the free drug concentration in the generated filtrate were determined either by liquid scintillation spectrophotometry or by spectrofluorometry. The protein binding of disopyramide was studied in the concentration range of 4.25×10^{-4}–5.66×10^{-6} *M*. It can be assumed that the plasma filtrates are equivalent to plasma water; the absence of protein was ascertained in the plasma filtrates (15).

The experiments were conducted at 25°, and the pH was kept within 7.30–7.45 unless otherwise specified. The reproducibility of the results was ascertained by the simultaneous ultrafiltration of each concentration of the drug through two to four different cone membranes.

Preliminary experiments indicated a membrane binding of disopyramide. Two different procedures were used to quantify and overcome this problem.

Procedure A—Plasma water was generated by filtering plasma through high flux cone membranes. Aliquots of plasma water were spiked with drug in the concentration range of 4.25×10^{-4}–3.68×10^{-7} *M* and were half-filtered to quantify the membrane binding as a function of the drug concentration. An apparent concentration-dependent membrane binding ranging from 25 to 45% was found. The membrane binding was significantly decreased to 5.4% (±4.8%) when this procedure was repeated with the same cone membranes filled with identically spiked fresh plasma water aliquots. These findings indicated that the membrane binding was saturable. All membranes used in the definitive protein binding experiments were presaturated routinely with drug by half-filtrations of plasma water spiked with an appropriate drug concentration. Half-filtrations of plasma and plasma water were reached after centrifugations of 18–20 and 3–4 min, respectively, at 1000 rpm.

Procedure B—The magnitude of the membrane binding of disopyramide was estimated from a calibration curve obtained when disopyramide-spiked pH 7.4 phosphate buffer solutions (5.00×10^{-6}–2.76×10^{-3} *M*) were filtered through high flux membranes at 2000 rpm for 5 min. These definitive protein binding experiments were performed without presaturation of the membranes. The influence of pH on binding properties of human plasma was the specific goal in this part of the study. Human plasma was adjusted to pH 7.4, 8.0, and 6.7 prior to filtration.

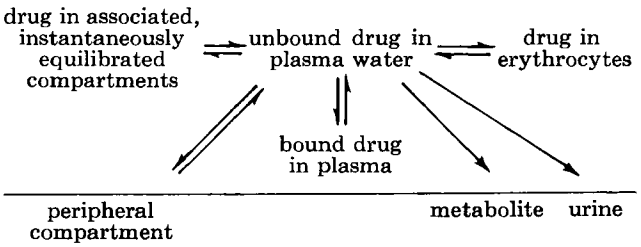
Protein Binding of Disopyramide in Presence of Its Monodealkylated Metabolite in Human Plasma—The ultrafiltration procedure was applied. The protein binding of the ^{14}C-labeled parent drug in the presence of its unlabeled metabolite was investigated at seven different molar concentration ratios of the parent drug to its metabolite, ranging from 9.63 to 0.0045. The concentrations of the labeled parent drug were determined by liquid scintillation spectrophotometry in plasma and plasma water. The extent of the membrane binding of the parent drug in the presence of the metabolite was determined separately at each concentration ratio. The membranes used in the definitive protein binding experiments were saturated by half-filtration of plasma water spiked with an appropriate concentration of both the parent drug and the metabolite.

Protein Binding of the Monodealkylated Metabolite in Human Plasma—The ultrafiltration procedure was used. A significant and apparently concentration-independent membrane binding was found for the metabolite in the magnitude of 20.4% (±3.7%) when spiked plasma water aliquots (2.71×10^{-4}–1.18×10^{-5} *M*) were half-filtered through the membranes. Repeated filtrations of identically spiked plasma water aliquots through the same cone membranes reduced the membrane binding of the metabolite to 1.9% (±1.9%). The membranes used in the definitive experiments were presaturated by one half-filtration of plasma water spiked with appropriate metabolite concentrations.

Protein Binding of the Monodealkylated Metabolite in Presence of Disopyramide in Human Plasma—The ultrafiltration procedure was applied. The protein binding of labeled metabolite in the presence of the unlabeled parent drug was investigated at one concentration ratio: 0.080 of the metabolite to the parent drug. The concentrations of the labeled metabolite in plasma and plasma water were determined by liquid scintillation spectrophotometry. The membranes used in the definitive protein binding experiments were presaturated with appropriate concentrations of the metabolite and the parent drug.

Red Cell–Plasma Water Partition Coefficient of Disopyramide and Its Monodealkylated Metabolite—The determination of the red blood cell partitioning of disopyramide was done in whole blood and red blood cell suspensions in both plasma water and pH 7.4 phosphate buffer. The red blood cell suspensions were prepared from fresh red blood cells separated from plasma by cen-

[4] Blood Bank, Shands Teaching Hospital and Clinics, Gainesville, Fla.



Scheme I—Drug in central compartment

trifugation and washed twice with sterile isotonic sodium chloride solution. After another centrifugation, they were suspended in either buffer or plasma water; the latter was obtained by filtering the separated plasma through the high flux membranes.

Whole blood and red blood cell suspensions were spiked with disopyramide to yield total drug concentrations in the range of 5.50×10^{-5}–8.23×10^{-7} *M*. The hematocrit was routinely determined on the solutions using a microcentrifuge with capillary tubes. The drug concentrations were measured in plasma, plasma water, or buffer by spectrofluorometry or liquid scintillation spectrophotometry after the red blood cells were separated by centrifugation or ultrafiltration through high flux cone membranes. The experiments were carried out at 25°, and the pH was kept between 7.30 and 7.50 and between 7.00 and 7.85 in the red blood cell suspensions and whole blood, respectively.

The red blood cell partitioning of disopyramide as a function of various concentrations of the drug was studied by spiking whole blood and red blood cell buffer suspensions with drug to yield total drug concentrations between 5.50×10^{-5} and 8.23×10^{-7} *M*. Equilibration was allowed for 1 hr. The samples were shaken frequently and subsequently centrifuged for 1 hr for half-filtration. The drug concentrations were measured in plasma, plasma water, and buffer by spectrofluorometry.

The red blood cell partitioning of disopyramide was studied as a function of time by spiking the red blood cell–plasma water suspensions with labeled drug to obtain three different total drug concentrations: 1.58×10^{-4}, 8.23×10^{-5}, and 8.23×10^{-6} *M*. After drug addition, the samples were shaken immediately for 2 min and later at regular time intervals. Aliquots were taken and centrifuged, and the plasma water separated at 5, 6, 9, 18, 33, and 63 min after spiking. The drug concentrations in plasma water were measured by liquid scintillation spectrophotometry. The hematocrit determinations were made on the residual suspension after each aliquot was taken. It was assumed that the obtained hematocrit values reflected those in the removed aliquots. This assumption was confirmed by the consistency among the determined hematocrit values, indicating that the samples were thoroughly mixed at any given time during the experiments. The hematocrit value was determined and the obtained values were corrected by a factor of 0.98 to account for trapped plasma (16).

The repartitioning of disopyramide in the red blood cells into plasma water as a function of time was studied by adding aliquots of the concentrated red blood cells with known hematocrit, obtained from the disopyramide–red blood cell partitioning experiments, to fresh plasma water. The new red blood cell–plasma water suspensions were shaken for 2 min and later at regular time intervals. Samples were taken and centrifuged, and the plasma water separated at 5 and 9 min after spiking. Hematocrit determinations were made on the residual suspensions. The drug concentrations in plasma water were determined by liquid scintillation spectrophotometry.

The red blood cell partitioning of disopyramide in the presence of the monodealkylated metabolite was studied by spiking red blood cell–plasma water suspensions with labeled disopyramide and unlabeled metabolite to yield molar concentration ratios of 0.35 and 0.17 of the parent drug to the metabolite. Aliquots were taken and centrifuged, and the plasma water separated at 5, 9, 33, and 63 min after spiking with the drug. The concentration of the labeled parent drug in plasma water was measured by liquid scintillation spectrophotometry.

The partitioning of the monodealkylated metabolite between red blood cells and plasma water was studied by spiking red blood cell–plasma water suspensions with the labeled metabolite. The plasma water aliquots were separated by centrifugation, and the

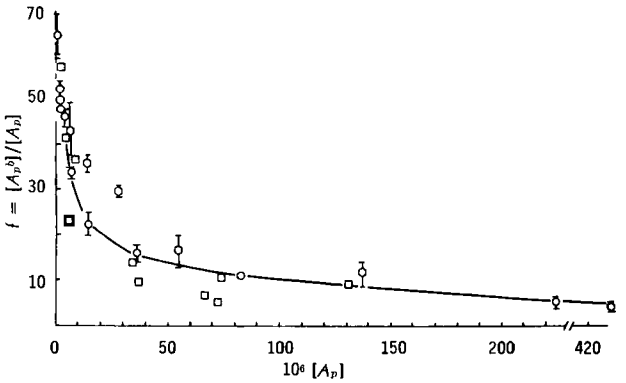


Figure 1—*Plots of the fraction,* f, *of the total drug in plasma that is bound to protein,* $[A_p^b]/[A_p]$, *against the total concentration of drug in the plasma,* $[A_p]$, *for disopyramide as obtained by equilibrium dialysis* (□) *and ultrafiltration through pretreated* (○) *and untreated* (○) *membranes. The standard deviations of the ultrafiltration data are represented by vertical lines through the points. The curve drawn through the points was calculated from an equation given by Hart (20):*

$$f = [A_p^b]/[A_p] = ([A_p^b]/[A_p^u])(1 - f) = \left[\frac{n_1P_1K_1}{K_1[A_p^u] + 1} + \frac{n_2P_2K_2}{K_2[A_p^u] + 1}\right] \times (1 - f)$$

The binding parameters n_1P_1, K_1, n_2P_2, *and* K_2 *were obtained by graphical analysis, where* $[A_p^u]$ *is the concentration of unbound drug in the plasma.*

metabolite concentrations were measured by liquid scintillation spectrophotometry. The procedures and conditions regarding pH, temperature, and hematocrit measurements were the same as for the experiments with the parent drug.

The red blood cell–plasma water partitioning of the monodealkylated metabolite as a function of different concentrations was studied by spiking red blood cell–plasma water suspensions with the metabolite to yield the following concentrations: 1.20×10^{-5}, 8.00×10^{-6}, and 4.00×10^{-6} *M*. The red blood cell–plasma water partitioning of the metabolite as a function of time was studied in the suspensions containing these three concentrations. Aliquots were taken, centrifuged, and separated at 4, 5, 6, 7, 8, 9, 15, 36, and 66 min after spiking.

The repartitioning of the metabolite in the red blood cells into plasma water was studied by adding aliquots of the concentrated red blood cells with known hematocrit, obtained from the metabolite red blood cell partitioning experiments, to fresh plasma water. The new red blood cell–plasma water suspensions were shaken for 2 min and later at regular time intervals. A sample was taken and centrifuged, and the plasma water separated at 66 min after spiking. The red blood cell–plasma water partitioning of the metabolite in the presence of disopyramide was studied in red blood cell–plasma water suspensions spiked with the labeled metabolite and unlabeled parent drug in a molar concentration ratio of 0.86 of the metabolite to the parent drug. Aliquots were taken and centrifuged, and the plasma water separated at 8, 36, and 66 min after spiking.

RESULTS AND DISCUSSION

Protein Binding of Disopyramide—The interaction between a ligand and protein can be formulated as (17):

$$n[A_p^u] + [P] \overset{K}{\rightleftharpoons} [A_nP]$$

Scheme II

where $[A_p^u]$ is the concentration of free or unbound drug in plasma, K is the association constant for the binding of a molecule of drug to one of the equivalent n binding sites of the protein molecule, and $[P]$ is the concentration of protein. Then:

$$[A_p^u] + [A_p^b] = [A_p] \qquad \text{(Eq. 1)}$$

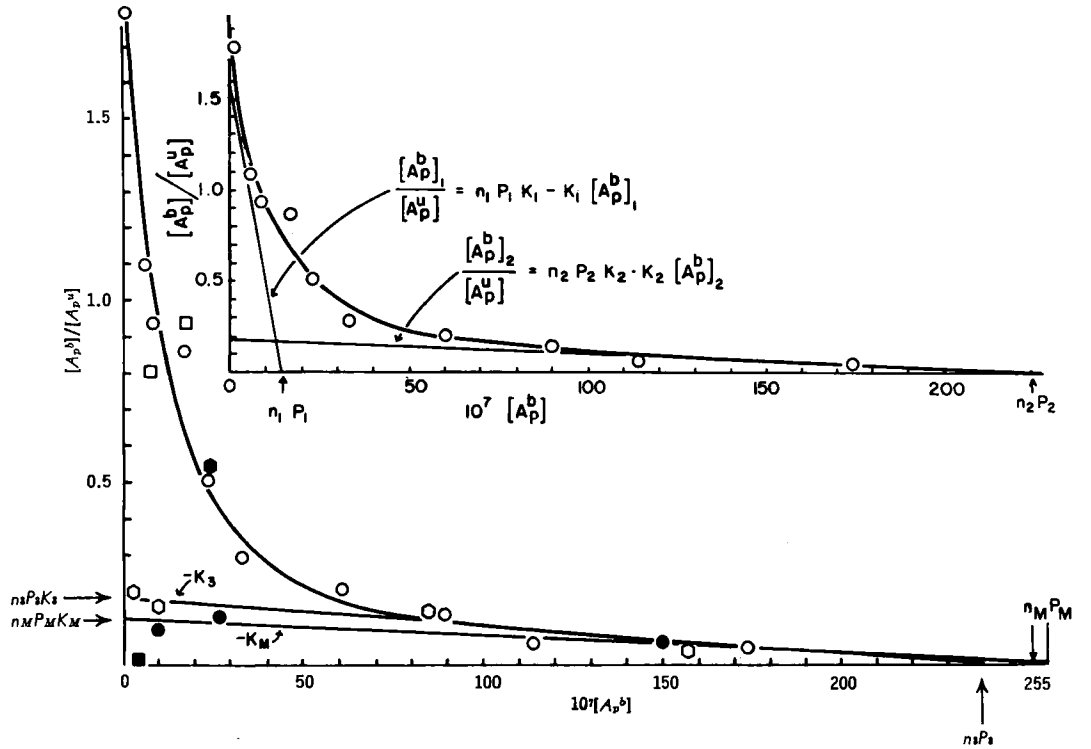


Figure 2—*Modified Scatchard plots for disopyramide and the monodealkylated metabolite in the absence and presence of each other. Key:* ○, *disopyramide alone* ($4.25 \times 10^{-4} - 3.68 \times 10^{-7}$ M); *disopyramide in the presence of different concentrations of metabolite such as* (○) 5.08×10^{-4} M, (●) 2.54×10^{-6} M, *and* (□) 5.08×10^{-7} M; ●, *metabolite alone* ($2.71 \times 10^{-4} - 1.18 \times 10^{-5}$ M); *and* ■, *metabolite* (2.36×10^{-5} M) *in the presence of* 2.97×10^{-4} M *disopyramide. The inset is a modified Scatchard plot of disopyramide with an example of the graphical analysis according to Rosenthal (18) on the premise of the existence of two independent classes of binding sites. Two straight lines,* $[A_p{}^b]_1/[A_p{}^u] = n_1K_1P_1 - K_1[A_p{}^b]$ *and* $[A_p{}^b]_2/[A_p{}^u] = n_2K_2P_2 - K_2[A_p{}^b]_2$, *with the respective slopes of* $-K_1$ *and* $-K_2$, *the respective intercepts on the abscissa of* n_1P_1 *and* n_2P_2, *and the respective intercepts on the ordinate of* $n_1P_1K_1$ *and* $n_2K_2P_2$, *were obtained. The determined binding parameters for disopyramide alone were* $K_1 = 1.00 \times 10^6$/M, $n_1P_1 = 1.60 \times 10^{-6}$ M, $K_2 = 8.44 \times 10^3$/M, *and* $n_2P_2 = 2.25 \times 10^{-5}$ M. *The determined binding parameters for the monodealkylated metabolite alone were* $K_m = 5.39 \times 10^3$/M, *and* $n_mP_m = 2.55 \times 10^{-5}$ M. *For disopyramide in the presence of a high concentration of metabolite* (3.80×10^{-4} M), *they were* $K_3 = 7.98 \times 10^3$/M *and* $n_3P_3 = 2.38 \times 10^{-5}$ M.

where $[A_p{}^b]$ is the concentration of drug bound to protein, and $[A_p]$ is the total drug concentration (bound and unbound) in plasma.

The Langmuir isotherm describes the interaction between small molecules and protein for any given concentration ratio (14) and any number of classes of binding sites:

$$r = \sum_1^i \frac{n_i K_i [A_p{}^u]}{1 + K_i [A_p{}^u]} \qquad \text{(Eq. 2)}$$

where r is the ratio $[A_p{}^b]/[P]$ of the molar concentration of drug bound to protein and the total molar concentration of protein, n_i is number of binding sites in the ith class, and K_i is the association constant for the binding of drug in the ith class. If r, the moles of drug bound per mole of protein, is only proportional to drug concentration, this implies that $K_i[A_p{}^u] \ll 1$ in Eq. 2. Thus, the numbers of binding sites of low affinity are in great excess, so the fraction of the drug bound to protein does not significantly vary with drug concentration and the process appears nonsaturable.

Equation 2 for an ith class of binding sites may be arranged to:

$$r/[A_p{}^u] = n_iK_i - K_ir \qquad \text{(Eq. 3)}$$

and the Scatchard plot (14) of $r/[A_p{}^u]$ against r permits the estimation of n_i and K_i from the slope and intercept. If the nature and/or concentration of the protein is not known, Eq. 3 can be modified (18) to:

$$[A_p{}^b]_i/[A_p{}^u] = n_iK_iP_i - K_i[A_p{}^b]_i \qquad \text{(Eq. 4)}$$

where a plot of $[A_p{}^b]/[A_p{}^u]$ against $[A_p{}^b]$ permits the estimation of K_i and n_iP_i from the slope and intercept. Linear Scatchard or modified Scatchard plots indicate one class of independent binding sites. Curvature in these plots, however, may indicate that there is more than one class of binding sites with different association constants and/or that a class of binding sites exists where the binding of one molecule of the drug affects the affinity of another to a previously equivalent binding site. The fraction of drug bound to a class of independent binding sites can be expressed as:

$$f = \frac{[A_p{}^b]}{[A_p]} = 1/(1 + [A_p{}^u]/n_i[P_i] + 1/n_iK_i[P_i]) \qquad \text{(Eq. 5)}$$

and if the process is nonsaturable, so that the fraction bound is invariant with the concentration of unbound drug, $[A_p{}^u]/n[P_i]$ must be negligible relative to the other two factors in the denominator.

Plots of the fraction of drug bound to plasma protein, f, against the concentration of total drug in the plasma, $[A_p]$, are given in Fig. 1 for the data obtained by equilibrium dialysis and ultrafiltration experiments.

There is good agreement between the data obtained by equilibrium dialysis and ultrafiltration through presaturated cone membranes. When ultrafiltration studies were conducted with untreated membranes, consistently higher values were obtained for f, even when corrections were made for the binding of drug from filtered phosphate buffer solutions. The use of phosphate buffer solutions of drug rather than plasma water solutions leads to an underestimation of the drug fraction bound to the membrane, which results in an overestimation of the fraction bound to plasma protein. It can be argued that phosphate may compete with the parent drug for "binding sites" on the membrane as has been demonstrated to occur between phosphate and small molecules for albumin (19).

The fraction bound to plasma, f, is clearly concentration dependent and increased from 0.05 to 0.65 as $[A_p{}^u]$ decreased from 2.13×10^{-4} to 1.31×10^{-7} M (Fig. 1), indicating saturation of the available binding sites.

The modified Scatchard plot (Fig. 2) for disopyramide is not linear and implies at least two classes of independent binding sites with different affinities or at least one class of binding sites, where

Table I—Influence of Plasma pH on Protein Binding of Disopyramide[a]

Disopyramide Concentration	Fraction of Disopyramide Bound		
	pH 7.5	pH 8.0	pH 6.7
2.70×10^{-4}	0.12, 0.07	0.10	0.08
1.30×10^{-4}	0.10, 0.10	0.15	0.07
5.50×10^{-5}	0.20, 0.13	0.18	0.05
2.74×10^{-5}	0.31, 0.28	0.30	0.15
1.37×10^{-5}	0.35, 0.37	0.36	0.15
5.50×10^{-6}	0.49	0.42	0.29
2.70×10^{-6}	—	0.50	0.12

[a] Ultrafiltrations were performed through untreated membranes.

the binding of one molecule of the drug decreases the affinity of another to a previous equivalent binding site. Graphical and algebraic methods are available (18, 20) to analyze the binding data in accordance with the former hypothesis. A graphical analysis of the data obtained by ultrafiltration with presaturated membranes is shown in the inset of Fig. 2, with the assumption of two independent sets of binding sites. Two straight lines were obtained for $i = 1$ and $i = 2$ of Eq. 4 with negative slopes, $-K_1$ and $-K_2$, indicating that both postulated classes of binding sites are saturable, one more readily than the other. The values obtained for the respective binding parameters were $K_1 = 1.00 \times 10^6/M$, $n_1P_1 = 1.60 \times 10^{-6}$ M, $K_2 = 8.44 \times 10^3/M$, and $n_2P_2 = 2.25 \times 10^{-5}$ M.

Plasma Protein Binding of Disopyramide and Its Monodealkylated Metabolite in Presence of Each Other—The metabolite data could be fitted by a straight line in the modified Scatchard plot of Fig. 2 and indicated that the metabolite may be bound to one class of binding sites without mutual interaction within the concentration range studied. The binding parameters were $K_M = 5.39 \times 10^3/M$ and $n_MP_M = 2.55 \times 10^{-5}$ M. When disopyramide was added at the relatively high concentration of 2.94×10^{-4} M for a molar ratio of metabolite to parent drug of 0.080, the ability of plasma protein to bind the metabolite was significantly decreased.

The protein binding characteristics of disopyramide in the absence and presence of the metabolite (II) are shown in the modified Scatchard plots of Fig. 2. The molar concentration ratios of parent drug and metabolite were varied between 0.0034 and 7.20. At high concentrations of the metabolite (5.08×10^{-4} M) and low molar ratios of parent drug to metabolite (0.0034, 0.014, 0.14, and 0.84), the binding capacity of the plasma protein for disopyramide was significantly decreased and the data points of the parent drug were fitted by a single straight line where $K_3 = 7.98 \times 10^3/M$ and $n_3P_3 = 2.38 \times 10^{-5}$ M. This indicated that, on the premise of two classes of independent binding sites, the availability of a more readily saturable class of binding sites was decreased. There appeared to be no significant interaction between parent drug and metabolite at lower concentrations of the metabolite (2.54×10^{-6} -5.08×10^{-7} M) where the molar ratios of parent drug to metabolite were higher (7.20, 3.37, and 2.70). These latter circumstances corresponded to those found in pharmacokinetic studies of disopyramide in humans[2].

These facts imply that the parent drug may be bound to two different classes of independent sites, or to at least one class of binding sites where the binding of one molecule of the drug decreases the affinity of another to a previous equivalent binding site. The binding of the metabolite, studied only over a limited concentration range since the available radiolabeled specific activity was low, was consistent with binding to a single class of independent sites. The binding parameters (K_m and n_mP_m) were similar to those obtained for the parent drug (K_2 and n_2P_2) for the less saturable class of binding sites or for all concentrations of disopyramide in the presence of high metabolite concentration (K_3 and n_3P_3).

The interaction between disopyramide and its metabolite that affects the protein binding of each other can be explained by a competition for common binding sites or by conformational changes of the protein molecule induced by the binding of one compound to its specific binding site that modifies or inhibits the binding of the other to its own.

Table II—Effect of Temperature on Plasma Protein Binding of Disopyramide from Equilibrium Dialysis

Disopyramide Concentration	Fraction of Disopyramide Bound	
	37°	25°
2.89×10^{-4}	—	0.07
2.68×10^{-4}	0.00	—
1.42×10^{-4}	0.04	—
1.31×10^{-4}	—	0.09
9.24×10^{-6}	0.36	0.35
2.90×10^{-6}	0.50	0.43

Table III—Effect of Concentration, Time, and Presence of the Monodealkylated Metabolite on Partition Coefficient, D, of Disopyramide into Red Blood Cells as Analyzed after Centrifugation of Red Blood Cell Suspensions in Plasma Water

Time after Spiking, min	10^5 $[A_B]$ Disopyramide	10^4 $[A_B]$ Metabolite	D[a]
5	15.8		1.30
33			1.24
63			1.19
Average			1.24 ± 0.050
5	15.8	4.50	1.21
33			1.19
63			1.30
Average			1.23 ± 0.053
5	8.23		1.25
9			1.23
Average			1.24 ± 0.014
5	8.23	4.74	1.24
9			1.31
Average			1.27 ± 0.050
6	0.823		1.13
18			1.27
33			1.28
Average			1.23 ± 0.080

[a] $D = [A_{RBC}]/[A_{p^u}]$ is the ratio of disopyramide concentration in the erythrocytes to the concentration of free or unbound drug in the plasma.

Influence of pH on Plasma Protein Binding of Disopyramide—The plasma protein binding of disopyramide was the same for plasma pH values of 7.4 and 8.0 but decreased significantly at pH 6.7 for the entire studied concentration range of 2.74×10^{-4}–2.70×10^{-6} M (Table I).

The fact of decreased binding capacity at pH 6.7 may be most readily explained by a net increase in positive charge on the protein to inhibit the binding of the protonated disopyramide. Of course, lowering of the pH to 6.7 could cause conformational changes in the interacting protein fractions (21) that lessen binding affinity.

The fact that there was no systematic decrease of binding with pH indicates that it is difficult to explain the observed data by assuming that only unionized drug (pKa′ = 10.45) is bound, since the ratios of unionized to ionized species would be 0.00017 at pH 6.7, 0.001 at pH 7.45, and 0.0036 at pH 8.0.

Influence of Temperature on Plasma Protein Binding—No significant difference in f, the fraction of disopyramide bound to plasma proteins, at pH 7.45 was found between 25 and 37° using equilibrium dialysis (Table II). Small temperature dependence of binding is characteristic for an interaction between oppositely charged species (22).

Red Blood Cell Partitioning of Disopyramide—The red blood cell–plasma partition coefficient of a drug, on the presumption that only unbound drug in the plasma can diffuse into the red blood cells, can be defined (17) as:

$$D = [A_{RBC}]/[A_{p^u}] = \{[A_B]/[A_{p^u}](1 - H) - [A_{p^b}]/[A_{p^u}] - 1\}(1 - H)/H \quad \text{(Eq. 6)}$$

Table IV—Average Values of the Partition Coefficient, D ($\pm\sigma$), for the Partitioning of Unbound Disopyramide in Plasma into Red Blood Cells[a]

1	2	3	4	5
1.24 ± 0.050	1.17 ± 0.15	1.12 ± 0.14	1.10 ± 0.12	1.04[b] ± 0.06

[a] 1 = Red blood cell–plasma water suspensions by liquid scintillation spectrophotometry, after separation by centrifugation; 2 = whole blood by spectrofluorometry, after separation by centrifugation; 3 = red blood cell–phosphate buffer suspensions by spectrofluorometry, after separation by centrifugation; 4 = whole blood by spectrofluorometry, after separation by ultrafiltration; and 5 = repartitioning from red blood cells into plasma water by liquid scintillation spectrophotometry after separation by centrifugation. [b] Resultant $[A_B] = 3.20 \times 10^{-5}$ M.

$$D = [A_{RBC}]/[A_p{}^u] = \{[A_B]/[A_p]\}(1 - f)(1 - H) - f/(1 - f) - 1\}(1 - H)/H \quad \text{(Eq. 7)}$$

where $[A_{RBC}]$ is the total drug concentration in the red blood cells; $[A_p{}^u]$ and $[A_p]$ are the drug concentrations in plasma, free and total, respectively; $[A_B]$ is the total concentration of a drug in whole blood or red blood cell suspension; H is the hematocrit; $(1 - H)$ is the plasmacrit; and f is the fraction of drug bound to plasma protein.

In a red blood cell–buffer or red blood cell–plasma water suspension, D can be calculated from Eq. 6 where $[A_p{}^b] = 0$. The consistency of the partition coefficients of disopyramide (Table III) with time after spiking of red blood cell suspensions in plasma water shows that the equilibrium of drug between plasma water and red blood cells is reached in less time than is needed for the performance of the initial mixing and subsequent centrifugation of the samples. The presence of high concentrations of the metabolite to give molar ratios of 0.35 and 0.17 of disopyramide to the metabolite had no significant effect on disopyramide partitioning. The values for $D = [A_{RBC}]/[A_p{}^u]$ were constant and concentration independent. However, if a partition coefficient were obtained for the partitioning of disopyramide between total plasma concentration and red blood cell concentration so that $D^* = [A_{RBC}]/[A_p]$, the resultant values would be concentration dependent and would decrease as f increases. For example, D^* at $f = 0.10$ is 1.11 but at $f = 0.33$ and 0.65, it would be 0.82 and 0.45, respectively.

When the values of D were obtained from disopyramide addition to whole blood and the fraction bound, f, was taken into account (Eq. 6), excellent agreement was shown (Table IV) with the values obtained from plasma–red blood cell and phosphate buffer–red blood cell suspensions. It was shown also that when disopyramide was repartitioned from red blood cells into plasma water, the obtained D values were the same.

The partition coefficients, D, for disopyramide were consistently larger than 1.0. This demonstrates an additional binding to, or greater solubility in, the red blood cells than can be accounted for on the assumption that the volume of the erythrocytes contains only an aqueous phase wherein the disopyramide has the same chemical activity as in plasma water.

Table V—Effect of Concentration, Time, and Presence of Disopyramide on the Partition Coefficient, D, of the Monodealkylated Metabolite into Red Blood Cells as Analyzed after Centrifugation of Red Blood Cell Suspension in Plasma Water

Time after Spiking, min	$[A_B]$ Metabolite	$[A_B]$ Disopyramide	D^a Metabolite
8	1.20×10^{-4}		0.81
36	1.20×10^{-4}		1.34
66	1.20×10^{-4}		1.42
8	1.20×10^{-4}	2.56×10^{-4}	0.82
36	1.20×10^{-4}	2.56×10^{-4}	1.37
66	1.20×10^{-4}	2.56×10^{-4}	1.43
5	8.00×10^{-5}		0.96
9	8.00×10^{-5}		1.30
66	8.00×10^{-5}		1.42
4.5	4.00×10^{-5}		0.13
7	4.00×10^{-5}		1.19
14	4.00×10^{-5}		1.12

[a] $D = [A_{RBC}]/[A_p{}^u]$ is the ratio of the monodealkylated metabolite concentration in the erythrocytes to the concentration of free or unbound drug in plasma. The average value of D at 66 min was 1.42 ± 0.005. On repartitioning of red blood cells containing monodealkylated disopyramide, the obtained D was 1.23 after 66 min, when the resultant $[A_B] = 3.30 \times 10^{-5}$ M.

Red Blood Cell Partitioning of Metabolite—Equilibration of the monodealkylated metabolite between red blood cells and plasma was reached after a significant delay (Table V) and appeared to be independent of metabolite concentration. The presence of disopyramide for a molar ratio of 0.47 of metabolite to parent drug did not affect the partitioning of the metabolite. An equivalent result was obtained when the metabolite was partitioned from the red blood cells into the plasma. The average D value of 1.4 obtained for the metabolite was indicative of an additional binding and/or sequestration over simple distribution into the water phase of the red blood cells.

The difference between equilibrium between plasma and red blood cells for disopyramide and metabolite may be explained by the fact that the metabolite has reduced lipophilicity due to its lessened number of alkyls, which may significantly slow down its diffusion through the lipid-like membrane of the red cells (23).

Pharmacokinetic Implications—Since the protein binding of disopyramide is concentration dependent, the pharmacokinetics of disopyramide, when assayed as total concentration in plasma, could not be linear if only the free fraction of drug in plasma diffuses, is excreted, or is metabolized by first-order processes. However, such kinetics are more amenable to linearization if the pharmacokinetic model is based on concentration of unbound drug in the plasma and if the overall elimination processes are assumed to be first order. This hypothesis was challenged and confirmed by the fact that red blood cell partitioning coefficients were invariant only when based on the premise that the unbound fraction of drug in the plasma partitions. In essence, this corresponds to an isolated two-compartment system *in vitro*, which would be difficult to evaluate *in vivo*.

The determination of a time-dependent partitioning of a drug into red blood cells is vital for establishing a valid pharmacokinetic model. It is apparent that the blood compartment cannot be considered as kinetically homogeneous with respect to the metabolite. The red blood cells can actually represent a kinetically definable separate compartment with slow drug influx, a fact that must be considered in the forthcoming pharmacokinetic analysis.

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[x] To whom inquiries should be directed.

Urinary Excretion of Amitriptyline *N*-Oxide in Humans

GIUSEPPE SANTAGOSTINO [x], ROBERTO MAFFEI FACINO, and DEMETRIO PIRILLO

Abstract □ The excretion rate of amitriptyline *N*-oxide was measured in healthy human subjects after the administration of a single oral dose of amitriptyline. The total amount of this metabolite excreted accounted for about 1% of the dose. It appears in the urine soon after administration of the parent compound but has practically disappeared by the 9th hr after treatment. The role of this metabolite in amitriptyline degradation in humans is discussed.

Keyphrases □ Amitriptyline—excretion of *N*-oxide urinary metabolite in humans □ Metabolism—urinary excretion of amitriptyline *N*-oxide in humans □ Biotransformation—urinary excretion of amitriptyline *N*-oxide in humans

Excretion of the major metabolites of amitriptyline and related drugs has been extensively studied in humans (1, 2). There exists, however, an interesting minor metabolic route, that of *N*-oxidation. The mechanism of formation, metabolism, and excretion of these *N*-oxides was investigated (3–6) both *in vitro* and *in vivo* in rats and guinea pigs. However, in humans, studies of the excretion of these compounds have, thus far, been limited to imipramine *N*-oxide (7, 8).

Because of previous work in this laboratory on amitriptyline metabolism, interest also existed in its metabolism to the *N*-oxide (9). According to Bickel and coworkers (3–6), some reasons why this minor pathway is of general interest are:

1. The natural occurrence of *N*-oxides in plant and animal tissues has posed interesting problems as to the biochemistry and function of these compounds in biological systems.

2. *N*-Oxides are metabolites of many tertiary amine drugs and have been postulated as intermediates in *N*-dealkylation.

3. Certain *N*-oxides possibly formed in mammalian tissues act as antimetabolites or carcinogens and are postulated as possible inducers of spontaneous cancer (10).

This paper reports studies on the excretion rate of amitriptyline *N*-oxide in humans. On the basis of these data and the results of previous work (9) on amitriptyline metabolism in humans, the probable role of this metabolite in the degradative pathway of the drug is discussed.

EXPERIMENTAL[1]

Synthesis of Amitriptyline *N*-Oxide—The synthesis of amitriptyline *N*-oxide was performed according to the method of van Steen (11), with the following modifications. Five grams of amitriptyline base, 10,11-dihydro-*N*,*N*-dimethyl-5*H*-dibenzo[*a*,*d*]cycloheptene-$\Delta^{5\gamma}$-propylamine, was dissolved in 20 ml of anhydrous methanol. Then 5 ml of 30% H_2O_2 was added to the solution, dropwise with stirring, and the solution was kept at 0° for a few days. After the further addition of 3 or 4 ml of 30% H_2O_2 and subsequent storage at 0° for 5 days, the excess hydrogen peroxide was cautiously decomposed with an aqueous suspension of 10% Pt black. This mixture, kept in ice, was freed from Pt black by filtration, and the filtrate was concentrated under reduced pressure at 30°. The solid residue was twice recrystallized from a mixture of methyl chloride–anhydrous ether (1:1) and colorless crystals of pure amitriptyline *N*-oxide, mp 114–115°, were obtained in an 88% yield. Amitriptyline *N*-oxide in the original method of van Steen melts at 99–106°. The IR spectrum of amitriptyline *N*-oxide was identical to that of amitriptyline except for the absence of the signal of the NH-band between 2400 and 2550 cm^{-1}. The UV spectrum of the compound, dissolved in methanol, showed the absorption maximum at 238 nm characteristic of amitriptyline.

Characterization of Amitriptyline *N*-Oxide—*TLC*—Glass plates (10 × 20 cm) were spread with a mixture of silica gel G[2]–fluorescent indicator[3]–distilled water (1:0.04:2); the thickness was 0.3 mm. Standards of amitriptyline *N*-oxide, amitriptyline, and the other amitriptyline metabolites known to occur in humans (1) (10-hydroxyamitriptyline, nortriptyline, desmethylnortriptyline, and 10-hydroxynortriptyline) were chromatographed by the ascending technique in the following solvent systems: A, isopropanol–ethyl acetate–concentrated ammonium hydroxide (45:5:1); B, chloroform–methanol (4:1); and C, benzene–dioxane–water–concentrated ammonium hydroxide (62.5:35:1.8:0.7).

The detection of amitriptyline and its metabolites was performed with a UV light (254 nm); the R_f values are listed in Table I. In all solvent systems tested, amitriptyline *N*-oxide possessed

[1] All chemicals and reagents (analytical grade) were obtained from Merck, Darmstadt, Germany. Amitriptyline, nortriptyline, desmethylnortriptyline, 10-hydroxyamitriptyline, and 10-hydroxynortriptyline were supplied by Merck, Sharp & Dohme, Pavia, Italy.
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[x] To whom inquiries should be directed.

Urinary Excretion of Amitriptyline *N*-Oxide in Humans

GIUSEPPE SANTAGOSTINO [x], ROBERTO MAFFEI FACINO, and DEMETRIO PIRILLO

Abstract □ The excretion rate of amitriptyline *N*-oxide was measured in healthy human subjects after the administration of a single oral dose of amitriptyline. The total amount of this metabolite excreted accounted for about 1% of the dose. It appears in the urine soon after administration of the parent compound but has practically disappeared by the 9th hr after treatment. The role of this metabolite in amitriptyline degradation in humans is discussed.

Keyphrases □ Amitriptyline—excretion of *N*-oxide urinary metabolite in humans □ Metabolism—urinary excretion of amitriptyline *N*-oxide in humans □ Biotransformation—urinary excretion of amitriptyline *N*-oxide in humans

Excretion of the major metabolites of amitriptyline and related drugs has been extensively studied in humans (1, 2). There exists, however, an interesting minor metabolic route, that of *N*-oxidation. The mechanism of formation, metabolism, and excretion of these *N*-oxides was investigated (3–6) both *in vitro* and *in vivo* in rats and guinea pigs. However, in humans, studies of the excretion of these compounds have, thus far, been limited to imipramine *N*-oxide (7, 8).

Because of previous work in this laboratory on amitriptyline metabolism, interest also existed in its metabolism to the *N*-oxide (9). According to Bickel and coworkers (3–6), some reasons why this minor pathway is of general interest are:

1. The natural occurrence of *N*-oxides in plant and animal tissues has posed interesting problems as to the biochemistry and function of these compounds in biological systems.

2. *N*-Oxides are metabolites of many tertiary amine drugs and have been postulated as intermediates in *N*-dealkylation.

3. Certain *N*-oxides possibly formed in mammalian tissues act as antimetabolites or carcinogens and are postulated as possible inducers of spontaneous cancer (10).

This paper reports studies on the excretion rate of amitriptyline *N*-oxide in humans. On the basis of these data and the results of previous work (9) on amitriptyline metabolism in humans, the probable role of this metabolite in the degradative pathway of the drug is discussed.

EXPERIMENTAL[1]

Synthesis of Amitriptyline *N*-Oxide—The synthesis of amitriptyline *N*-oxide was performed according to the method of van Steen (11), with the following modifications. Five grams of amitriptyline base, 10,11-dihydro-*N*,*N*-dimethyl-5*H*-dibenzo[*a*,*d*]cycloheptene-$\Delta^{5\gamma}$-propylamine, was dissolved in 20 ml of anhydrous methanol. Then 5 ml of 30% H_2O_2 was added to the solution, dropwise with stirring, and the solution was kept at 0° for a few days. After the further addition of 3 or 4 ml of 30% H_2O_2 and subsequent storage at 0° for 5 days, the excess hydrogen peroxide was cautiously decomposed with an aqueous suspension of 10% Pt black. This mixture, kept in ice, was freed from Pt black by filtration, and the filtrate was concentrated under reduced pressure at 30°. The solid residue was twice recrystallized from a mixture of methyl chloride–anhydrous ether (1:1) and colorless crystals of pure amitriptyline *N*-oxide, mp 114–115°, were obtained in an 88% yield. Amitriptyline *N*-oxide in the original method of van Steen melts at 99–106°. The IR spectrum of amitriptyline *N*-oxide was identical to that of amitriptyline except for the absence of the signal of the NH-band between 2400 and 2550 cm^{-1}. The UV spectrum of the compound, dissolved in methanol, showed the absorption maximum at 238 nm characteristic of amitriptyline.

Characterization of Amitriptyline *N*-Oxide—*TLC*—Glass plates (10 × 20 cm) were spread with a mixture of silica gel G[2]–fluorescent indicator[3]–distilled water (1:0.04:2); the thickness was 0.3 mm. Standards of amitriptyline *N*-oxide, amitriptyline, and the other amitriptyline metabolites known to occur in humans (1) (10-hydroxyamitriptyline, nortriptyline, desmethylnortriptyline, and 10-hydroxynortriptyline) were chromatographed by the ascending technique in the following solvent systems: A, isopropanol–ethyl acetate–concentrated ammonium hydroxide (45:5:1); B, chloroform–methanol (4:1); and C, benzene–dioxane–water–concentrated ammonium hydroxide (62.5:35:1.8:0.7).

The detection of amitriptyline and its metabolites was performed with a UV light (254 nm); the R_f values are listed in Table I. In all solvent systems tested, amitriptyline *N*-oxide possessed

[1] All chemicals and reagents (analytical grade) were obtained from Merck, Darmstadt, Germany. Amitriptyline, nortriptyline, desmethylnortriptyline, 10-hydroxyamitriptyline, and 10-hydroxynortriptyline were supplied by Merck, Sharp & Dohme, Pavia, Italy.
[2] Merck, Darmstadt, Germany.
[3] Woelm, Eschwege, Germany.

Table I—R_f Values of Amitriptyline and Its Metabolites in Solvent Systems A, B, and C[a]

	A	B	C
Amitriptyline	0.80	0.75	0.77
10-Hydroxyamitriptyline	0.75	0.48	0.52
Nortriptyline	0.49	0.32	0.25
Desmethylnortriptyline	0.52	0.30	0.24
10-Hydroxynortriptyline	0.29	0.16	0.14
Amitriptyline *N*-oxide	0.08	0.27	0.02

[a] A, isopropanol–ethyl acetate–concentrated ammonium hydroxide (45:5:1); B, chloroform–methanol (4:1); and C, benzene–dioxane–water–concentrated ammonium hydroxide (62.5:35:1.8:0.7).

different R_f values from those of amitriptyline and the other amitriptyline metabolites.

Reduction with Titanium Trichloride to Amitriptyline—Amitriptyline *N*-oxide (1 mg/ml water) was mixed with 1 ml of 2 *N* HCl and 0.6 ml of 13% (w/v) $TiCl_3$ and incubated for 30 min in the dark at room temperature. At the end of the reaction, the mixture was adjusted to pH 10 with 5 *N* NaOH and extracted with five volumes of 1,2-dichloroethane. The extract was concentrated to dryness *in vacuo*, taken up in 500 μl of methanol, and submitted to chromatographic analysis in the various solvent systems. Only the spot of amitriptyline was present on the chromatogram, indicating that the *N*-oxide had been quantitatively reduced to amitriptyline.

Solvent Extraction—For the extraction of amitriptyline *N*-oxide from the aqueous phase, the following organic solvents were tested: 1,2-dichloroethane, chloroform, ether, and *n*-heptane. The compound was extracted from 0.1 *M* buffered solutions; the buffers used were: acetate, pH 5; phosphate, pH 7; and glycine, pH 9 and 11.

Previous to extraction, the organic phases were presaturated by shaking with the same volume of the corresponding aqueous phase for 10 min. Amitriptyline *N*-oxide was dissolved in 10 ml of aqueous buffer solution (10 μg/ml) and extracted by shaking with 10 ml of the organic solvent for 30 min at 20°. Drug concentrations were determined by UV spectrophotometry at 238 nm (the absorption maximum of amitriptyline *N*-oxide) in the aqueous phase before extraction and in both phases after extraction. The concentrations were evaluated with a linear standard curve. Five experiments were run for each buffer–solvent combination.

Figure 1 shows that the passage of amitriptyline *N*-oxide from the aqueous solutions into the organic solvents varied over a wide range (6–97%) depending on the solvent. The extractability of amitriptyline *N*-oxide into the organic solvents did not seem to be influenced by the pH of the aqueous phase.

Of the four solvents tested, chloroform extracted the most amitriptyline *N*-oxide, but it also extracted other compounds (*i.e.*, urinary pigments) which interfered in the chromatographic separation of the metabolite. Thus, the urines were extracted with 1,2-dichloroethane, which extracts a high proportion (82%) of this metabolite and less of the impurities.

Isolation of Amitriptyline *N*-Oxide from Urines—Immediately after collection, 50 ml of each urine sample was acidified with acetic acid and buffered with 0.1 *M* acetate buffer to pH 5. Then the samples were twice extracted with five volumes of 1,2-dichloroethane. The organic layers were collected, dried over anhydrous sodium sulfate, filtered, and concentrated to dryness *in vacuo* at 30°. The residue was taken up in 1 ml of methanol. The extract was subjected to two-dimensional TLC in Solvent Systems A and B.

For two-dimensional separation, Solvent System A was run first and the plate was air dried before developing in the second dimension with the other solvent system. The spot corresponding to amitriptyline *N*-oxide, by the criterion of R_f values, was eluted with water and its identity was confirmed by reduction by titanium trichloride and UV and IR spectra.

Quantitative Determination and Percent Recoveries from Urines—The quantitative determination of amitriptyline *N*-oxide isolated from urines was performed as follows. After the chromatographic run, the silica gel spot of amitriptyline *N*-oxide was scraped off and extracted by shaking for 15 min with 2.5 ml of 0.1 *M* acetate buffer (pH 5)–methanol (3:1). After centrifugation for 5 min (4000 rpm), the absorbance at 238 nm of the extract was determined *versus* a silica gel blank. The corresponding concentration was determined from a calibration curve of known amounts of the synthesized standard that had been applied to silica gel plates. This curve was linear in the range from 0.5 to 10 μg/ml; 5 μg of amitriptyline *N*-oxide had an absorbance of about 0.08. This quantitation method proved highly reproducible. When amitriptyline *N*-oxide was added to normal urine in a concentration of 1 μg/ml and incubated for 6, 12, or 24 hr at 37°, recovery was 94 ± 1.6% (mean ± *SE* of five experiments).

Table II—Urinary Excretion of Amitriptyline *N*-Oxide by Human Subjects

Subject	Sex	Weight, kg	Age, years	12 hr[a]	24 hr[a]
1	M	74	23	0.92	1.04
2	M	70	30	0.99	1.08
3	M	79	24	0.88	1.00
4	M	71	22	0.94	1.05
Mean ± *SE*		73.5	24.7	0.93 ± 0.02	1.04 ± 0.02
5	F	60	22	0.98	1.14
6	F	56	26	1.06	1.24
7	F	54	24	1.05	1.18
8	F	65	27	0.96	1.05
Mean ± *SE*		58.7	24.7	1.01 ± 0.02	1.15 ± 0.04[b]

[a] Data in the last two columns represent the total amount of the metabolite excreted (expressed as percent of the dose of amitriptyline, 50 mg, oral) by the 12th or 24th hr after administration. [b] $p < 0.05$ when compared with the male value; Student's *t* test.

Drug Administration and Urine Collection—Eight volunteers, four females and four males (Table II), with normal gastroenteric, circulatory, hepatic, and renal functions (as determined by routine clinical tests) participated in the study. The subjects had not taken any drug for at least 4 weeks before the study. Amitriptyline hydrochloride[4] was administered orally as a single 50-mg

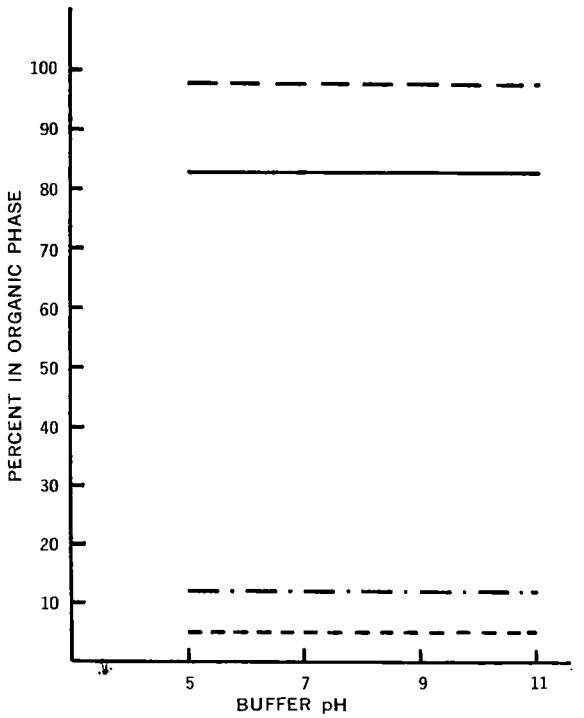

Figure 1—*Extractability of amitriptyline* N*-oxide by various solvents. Amitriptyline* N*-oxide (10 μg/ml) in various aqueous buffers (see* Experimental*) was extracted with an equal volume of 1,2-dichloroethane* (——), *chloroform* (— — —), *ether* (—·—·—), *or* n*-heptane* (— — — —).

[4] Tryptizol, Merck, Pavia, Italy.

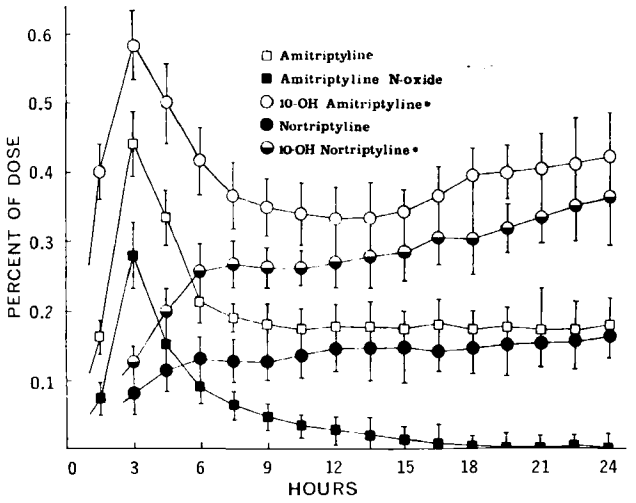

Figure 2—*Urinary excretion of amitriptyline and its metabolites. The data cover the first 24 hr after administration of amitriptyline (50 mg po). Each bar represents the percent of the dose (mean ± SE from four male subjects) present as each metabolite at the given time points. Key: *, conjugated + unconjugated fraction.*

tablet. The drug was always given in the morning to the fasted subjects.

The urine from each volunteer was collected at time intervals of 1.5 hr on the day of drug administration and then at intervals of 24 hr up to 180 hr. Each urine sample was divided into two portions, one for the determination of amitriptyline *N*-oxide and the other for the determination of unmodified amitriptyline and the related principal metabolites known to occur in humans (1) (10-hydroxyamitriptyline, nortriptyline, desmethylnortriptyline, and 10-hydroxynortriptyline). These determinations were done by methods reported previously (12).

RESULTS AND DISCUSSION

The urinary levels of amitriptyline *N*-oxide in male and female volunteers 12 and 24 hr after administration are reported in Table II. The total amount of amitriptyline *N*-oxide excreted by the subjects represents 1.04 ± 0.02% (in males) and 1.15 ± 0.04% (in females) of the administered dose of amitriptyline. Of this amount, 90% is excreted during the first 12 hr. The difference between males and females, although statistically significant, is small enough to be attributable to differences in distribution of the drug within subjects of different body weights. Therefore, there is no clear indication of a sex difference in synthesis of this metabolite. These findings are in agreement with results of studies with drugs chemically related to amitriptyline. Christiansen *et al.* (8) and Goldenberg *et al.* (13) observed that, in humans, about 1% of administered imipramine and promazine was excreted as the respective *N*-oxide. The extremely low excretion of amitriptyline *N*-oxide, when compared to that of the other amitriptyline metabolites (Table III), demonstrates that the *N*-oxidation of amitriptyline is a minor metabolic pathway of the drug in humans.

Figure 2 shows the excretion rate patterns, for four male subjects, of amitriptyline, amitriptyline *N*-oxide, and other amitryptyline metabolites during the first 24 hr after drug administration. The hydroxylated and *N*-demethylated metabolites of amitryptyline represent the products of the major metabolic pathways of the drug in humans (1).

The most polar metabolite is 10-hydroxyamitriptyline, which appears early in urine and is almost entirely glucuronide conjugated. Similarly, amitriptyline *N*-oxide appears early and this behavior correlates with its polar character due to the N—O bond (10). Unlike the other compounds, however, this metabolite soon disappears completely from urine, although considerable levels of the parent drug are still present in the body and all of the other amitriptyline metabolites continue to be excreted.

Table III—Excretion[a] of Amitriptyline and Its Metabolites

Amitriptyline	5.0 ± 0.4
10-Hydroxyamitriptyline	27.6 ± 1.6
Nortriptyline + desmethylnortriptyline	6.2 ± 0.5
10-Hydroxynortriptyline	41.4 ± 4.1
Amitriptyline *N*-oxide	1.04 ± 0.02

[a] Data are expressed as percentages (mean ± *SE* for four male subjects) of the administered dose (50 mg amitriptyline po) excreted over 180 hr after administration.

The disappearance of amitriptyline *N*-oxide from urine may be interpreted in various ways:

1. Formation of this metabolite ceases when the concentration of the parent drug in the body falls below a critical level. If so, the *N*-oxidation could represent an "emergency" route of amitriptyline metabolism in humans.
2. Amitriptyline *N*-oxide is continuously formed in the presence of the parent drug in an amount proportional to its concentration in the body, but most of it undergoes further metabolic transformations so that only a small fraction of the amount formed appears in the urine.

In either case, one might expect to see an "escape" by this metabolite into the urine early after administration; this could correspond to the first passage of the parent compound through the liver when both the concentration in this organ and the rate of metabolism would be maximal. Once the concentration of the parent compound declines due to metabolism and distribution throughout the body, the rate of formation of amitriptyline *N*-oxide would also decline and less of it might escape further metabolism.

Amitriptyline *N*-oxide is known to undergo further metabolism in the rat (5). It has been reported that this compound, which is formed by microsomal enzymes, undergoes reduction to amitriptyline or *N*-dealkylation to nortriptyline, either within the cell or in the circulating blood. If these processes take place also in humans, amitriptyline *N*-oxide should be considered as an intermediate product rather than as a terminal metabolite in amitriptyline metabolism.

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[x] To whom inquiries should be directed.

Bioavailability of 14 Nitrofurantoin Products

M. C. MEYER ˣ, G. W. A. SLYWKA, R. E. DANN, and P. L. WHYATT *

Abstract □ Single lots of 14 commercially available 50- and 100-mg nitrofurantoin products were evaluated *in vitro* and *in vivo*. All products tested met USP XVIII specifications for drug content, disintegration time, and dissolution rate. Statistically significant differences were observed in bioavailability, as determined from crossover urinary excretion studies in 14 human volunteers. The cumulative amount of drug excreted and the duration of therapeutic urine levels were compared. No useful correlation was observed between the extent of urinary excretion and either the disintegration or dissolution characteristics of the dosage forms tested.

Keyphrases □ Nitrofurantoin—bioavailability of 14 commercially available 50- and 100-mg products, attempted *in vivo–in vitro* correlations □ Bioavailability—14 commercially available 50- and 100-mg products, correlations between urinary excretion and disintegration/dissolution characteristics attempted

Numerous recent reports (1–4) provided evidence to support the contention that not all commercially available products containing a given drug will necessarily exhibit equivalent bioavailability. While compendial assay procedures may indicate that each product has essentially identical content, the rate and extent of absorption of the drug from the dosage form following oral administration may differ significantly from product to product.

The urinary tract, antibacterial drug nitrofurantoin is one therapeutic agent that has exhibited variable bioavailability between chemically equivalent products (5–8). In recognition of a potential for bioavailability problems, nitrofurantoin was considered in the recent APhA Bioavailability Pilot Project (9).

The present report deals with a crossover comparison of the bioavailability of 14 nitrofurantoin products. Urinary excretion studies were employed since this drug is excreted in the urine approximately 40% as unchanged drug following oral administration in humans (5–8), and the urinary tract is the site of therapeutic activity. The drug has a half-life in humans of less than 1 hr (5), and clearance is not dependent on urinary pH (8).

EXPERIMENTAL

Analysis of Urine Samples—A method nearly identical to that of Conklin and Hollifield (10) was employed in the determination of nitrofurantoin excreted in the urine. The method involved acidification of 0.5 ml of urine with 2.0 ml of 0.2 *N* HCl and extraction with 5.0 ml of nitromethane. Three milliliters of the nitromethane layer was combined with 0.5 ml of 0.04 *M* hyamine hydroxide in absolute methanol. The absorbance of the resulting solution was determined at 400 nm against a blank sample prepared by carrying 0.5 ml of distilled water through the extraction procedure. Blank urine samples, collected prior to drug administration, were also analyzed; each drug absorbance reading was corrected for the blank urine value.

Product Composite Assay—Twenty tablets or the contents of 20 capsules of each product were finely powdered and six aliquots, each equivalent to approximately 50 mg of nitrofurantoin, were accurately weighed. Approximately 10 ml of dimethylformamide (I) was added to each portion. Following thorough mixing, the samples were filtered through a glass-fiber filter, with adequate rinsing to ensure quantitative transfer. The resulting filtrate was diluted to 50 ml with I, and 1 ml of this solution was diluted to 25 ml with a 10% aqueous solution of I. One-milliliter samples of these solutions were then carried through the assay extraction procedure employed for the urine samples. Blank values were derived from the extraction of 1.0 ml of a 10% aqueous solution of I.

Content Uniformity—The contents of 10 individual capsules or 10 tablets of each 50- or 100-mg product were crushed into fine powders, and each individual powder was assayed as described in the composite assay, except that the 100-mg nitrofurantoin samples were diluted to 50 ml, prior to extraction, with a 10% solution of I.

USP Disintegration Test—Six dosage units of all 14 products were subjected to the USP XVIII disintegration test. The tests were carried out in distilled water at 37° with disks, using a USP tablet disintegration tester[1].

Dissolution Test—Six dosage units of all 14 products were subjected to the USP XVIII dissolution test. The determinations were made in a six-chamber dissolution apparatus[2] at 37°. Each dosage unit was placed into a wire-mesh dissolution basket and submersed in 900 ml of pH 7.2 phosphate buffer. The basket was rotated at 100 rpm; 5-ml samples were withdrawn at 5, 10, 15, 20, 30, 40, 50, 60, 75, and 90 min after the basket was introduced into the buffer solution. The withdrawn samples were assayed for nitrofurantoin content by diluting 1.0 ml of each sample to 10.0 ml with buffer and determining the absorbance at 380 nm, using a pH 7.2 phosphate buffer as the blank.

Clinical Study Protocol—The average age of the subjects[3] was 24 years (range 21–32), the average weight was 78.7 kg (range 61.2–106.6), and the average height was 175.6 cm (range 167.6–190.5). Each volunteer was screened regarding general health and any known drug allergies. In addition, all subjects underwent a hematologic and SMA 12/60 blood analysis to ensure inclusion of only those subjects in good health. Each subject gave written informed consent to participate in the study.

All subjects were instructed to refrain from taking any medication during the study. Each subject was given a single dose of a nitrofurantoin product once a week for 14 weeks. The dose was taken in the morning, following an overnight fast. No food or liquid, other than water, was permitted until 4 hr following ingestion of the dose. A blank urine sample was voided prior to taking the dose, and urine samples were collected at 1, 1.5, 2, 3, 4, 6, and 12 hr postadministration.

The total volume of the sample was recorded, along with the time of collection, and at least a 10-ml portion of the sample was placed into a small glass vial with a plastic snap-cap. The samples were frozen until the day of analysis. At the time of assay, the sample was thawed and analyzed for nitrofurantoin concentration in the previously described manner.

The sequence in which each subject received each drug each week is summarized in Table I. The sequence follows essentially the pattern suggested by Williams (11) and is designed to minimize the influence of any cumulative or residual effects of preceding doses. Due to technical difficulties, during the 1st week of the study the precise design (11) was not employed. Each subject repeated his 1st week drug following his 14th week drug, and the results of the 1st week were discarded. Furthermore, Subject 14 was dosed partially out of sequence. However, residual or cumulative effects were not anticipated due to the short half-life of the drug and the 1-week interval between doses.

[1] SGA Scientific Inc., Bloomfield, N.J.
[2] Hansen Research Corp., Northridge, Calif.
[3] Fourteen paid male volunteers were recruited from the student body of the University of Tennessee College of Pharmacy.

Table I—Experimental Design for Nitrofurantoin Bioavailability Evaluation[a]

Subject	Week													
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th
1	2	14	3	13	4	12	5	11	6	10	7	9	8	1
2	3	1	4	14	5	13	6	12	7	11	8	10	9	2
3	4	2	5	1	6	14	7	13	8	12	9	11	10	3
4	5	3	6	2	7	1	8	14	9	13	10	12	11	5
5	6	4	7	3	8	2	9	1	10	14	11	13	12	5
6	7	5	8	4	9	3	10	2	11	1	12	14	13	6
7	8	6	9	5	10	4	11	3	12	2	13	1	14	7
8	9	7	10	6	11	5	12	4	13	3	14	2	1	8
9	10	8	11	7	12	6	13	5	14	4	1	3	2	9
10	11	9	12	8	13	7	14	6	1	5	2	4	3	10
11	12	10	13	9	14	8	1	7	2	6	3	5	4	11
12	13	11	14	10	1	9	2	8	3	7	4	6	5	12
13	14	12	1	11	2	10	3	9	4	8	5	7	6	13
14	1	13	12	3	11	2	4	10	5	9	6	8	7	14

[a] Each number within the matrix corresponds to a specific product lot: No. 1 was 50-mg tablets from Ketchum Laboratories (Lot 204145); No. 2 was 50-mg tablets from Lederle Laboratories (Lot 286-108); No. 3 was 100-mg tablets from Ketchum Laboratories (Lot 2011460); No. 4 was 100-mg tablets from McKesson Laboratories (Lot 2B039); No. 5 was 100-mg tablets from Purepac Pharmaceutical Co. (Lot 1061460); No. 6 was 100-mg tablets from Eaton Laboratories (Lot 698627); No. 7 was 100-mg macrocrystal capsules from Eaton Laboratories (Lot 697203); No. 8 was 50-mg tablets from Purepac Pharmaceutical Co. (Lot 090009); No. 9 was 50-mg tablets from Eaton Laboratories (Lot 693775); No. 10 was 50-mg macrocrystal capsules from Eaton Laboratories (Lot 698613); No. 11 was 50-mg tablets from McKesson Laboratories (Lot 2J784); No. 12 was 100-mg tablets from Lederle Laboratories (Lot 286-104); No. 13 was 100-mg Zambon tablets from Wolins Pharmacal (Lot 24015); and No. 14 was 50-mg Zambon tablets from Wolins Pharmacal (Lot 24732).

Selection of Nitrofurantoin Products—Table I also summarizes the nitrofurantoin products evaluated. Six manufacturers or distributors are represented, with a single lot of at least two product strengths tested for each. In each instance the products tested were purchased directly from a local pharmacy or drug wholesaler to ensure that the products were representative of those currently available for dispensing.

RESULTS AND DISCUSSION

Product Composite Assay—The results of the composite analyses of the 14 product lots are summarized in Table II, expressed in terms of the percent of labeled content actually found by analysis. Each of the 14 products was within the prescribed USP XVIII limits of 95.0–105.0% of the labeled content.

Content Uniformity Analysis—The USP XVIII requires that the assay of 10 individual tablets of each lot should indicate that not less than nine of the tablets are within 85–115% of the average of the tolerances specified in the potency definition in the monograph and that the content of none of the tablets falls outside the limits of 75–125% of that average. Table III summarizes the results of the content uniformity determination of 10 tablets or capsules of each of the 14 nitrofurantoin product lots. In no instance did any of the products fail to meet the USP XVIII specifications.

Disintegration Testing—The USP XVIII requires the testing of six tablets of each product lot for disintegration properties and specifies that none of the six tablets should fail to disintegrate in less than 30 min. If one or two tablets fail the test, an additional 12 tablets are to be tested and not less than 16 of the tablets must disintegrate within 30 min. As may be seen from Table IV, all 14 products tested disintegrated well within the 30-min time limit, with the mean times ranging from 1.3 to 16.4 min.

Dissolution Rate Testing—The USP XVIII nitrofurantoin monograph specifies that the time required for 60% of the labeled amount to dissolve is not less than 60 min. This is in contrast to USP XVIII monographs for other drugs, which require that a certain percent of the dose must dissolve within a specified period. Inconsistencies in the USP XVIII dissolution rate specifications for nitrofurantoin were recently discussed (12). For nitrofurantoin, if one or two dosage units fail to meet the requirements, the test is repeated with six additional dosage units, and not less than 10 of the 12 units tested must pass.

Table V summarizes the dissolution data obtained for each of the 14 products. With the exception of Products 5 and 8, all products were significantly less than 50% dissolved at the 60-min sampling time and, therefore, met the USP specifications. Because two of the six tablets tested for Product 8 were more than 60% dissolved after 60 min, and five tablets of Product 5 were close to 60% dissolved after this time, an additional six tablets of each of these products were tested. Of the 12 total tablets tested for Products 5 and 8, three and four tablets, respectively, dissolved more rapidly than permitted by the USP XVIII specifications.

Strictly speaking, these two products failed to meet the USP requirements for dissolution, but the clinical significance of this specification is debatable. The apparent intent of the USP dissolution test is to provide for a relatively slowly dissolving dosage form,

Table II—Twenty Tablet USP Composite Assay for Nitrofurantoin Products

Product[a]	Dose, mg	Mean Percent of Labeled Content[b]	*SD*
9	50	105.43	1.64
14	50	105.15	1.78
10	50	104.67	2.30
12	100	102.92	1.41
11	50	102.89	1.15
6	100	102.83	1.15
8	50	102.60	3.88
4	100	102.41	1.79
5	100	101.91	2.71
1	50	101.50	1.91
13	100	101.08	2.74
7	100	100.06	2.09
3	100	99.40	1.66
2	50	96.12	3.50

[a] See Table I for product code numbers. [b] Mean of six determinations.

Table III—USP XVIII Content Uniformity Analysis of Nitrofurantoin Products

Product[a]	Dose, mg	Mean Percent of Labeled Content[b]	*SD*
9	50	107.07	2.09
7	100	106.71	4.63
14	50	106.41	4.01
10	50	106.01	6.40
5	100	104.70	3.74
8	50	103.99	4.21
12	100	103.55	2.99
4	100	103.54	6.30
6	100	103.26	3.38
11	50	102.56	3.71
13	100	101.06	3.26
3	100	100.96	6.76
1	50	100.84	3.84
2	50	98.12	3.42

[a] See Table I for product code numbers. [b] Mean of six determinations.

Table IV—USP XVIII Disintegration Test for Nitrofurantoin Products

Product[a]	Dose, mg	Mean Disintegration Time[b], min	*SD*
8	50	1.30	0.26
5	100	8.26	3.43
9	50	8.96	1.40
10	50	8.98	0.62
2	50	9.97	2.57
14	50	10.20	1.56
6	100	11.26	3.70
7	100	11.27	0.65
12	100	12.10	1.93
4	100	12.30	1.58
11	50	13.38	3.23
13	100	13.64	1.09
3	100	15.94	0.59
1	50	16.42	1.36

[a] See Table I for product code number. [b] Mean of six determinations.

Table VI—One-Way Analysis of Variance of Percent Dissolution of Nitrofurantoin Products after 90 min[a]

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	*F* Ratio
Between products	13	38.84	2.99	66.98
Within products	70	3.12	0.04	
Total	83	41.97		

Rank Order of Products in Terms of Mean Percent Dissolved after 90 min[b] (Least to Most Dissolved Product)[c]

(4) 14 (13) 11 (3) 2 9 1 (12) (6) 10 (7) (5) 8

[a] Using Cochran's test for homogeneity of variance. All data were subjected to a ln (X) transformation because of a significant nonadditivity in the untransformed data. [b] Products ranked on the basis of the Newman–Keuls *a posteriori* test. Products underlined by the same line are not significantly different ($p > 0.01$). [c] See Table I for product code numbers; 100-mg products are indicated by parentheses.

which presumably will be less prone to elicit GI distress in susceptible individuals. In the present study, 196 doses of nitrofurantoin were administered and only one incident of nausea and vomiting was reported.

Table VI summarizes the statistical analysis of the dissolution data, comparing the mean percent dissolved from each of the 14 products after 90 min. Because of a significant nonadditivity in the dissolution data, the analysis was conducted on natural log transformed data which reduced but did not eliminate the nonadditivity. The analysis indicates that the dissolution data may be approximately divided into three dissolution groups, with Products 8, 5, 7, 10, and 6 being relatively rapidly dissolved, Products 4, 14, 13, 11, and 3 being relatively slowly dissolved, and the remainder having an intermediate dissolution rate.

Urinary Excretion Studies—Figure 1 summarizes the average data from the urinary excretion studies of all 14 products in the 14 subjects. The mean cumulative percent of nitrofurantoin excreted for Products 14 and 11 was less than half that observed following administration of Product 9. A two-way analysis of variance of the urinary excretion data was conducted for each urine sampling time. At the early sampling times, 1 through 3 hr postadministration, the data exhibited considerable variability between subjects for each product, as indicated by a relatively large standard deviation and relative standard deviation. As the sampling continued through 12 hr, the intersubject variability continually declined. From 3 hr on, Products 14, 11, 10, and 7 began to emerge as being less bioavailable than the other products being evaluated. Table VII summarizes the two-way analysis of variance for products at the 12-hr sampling time.

The week's effect (in conjunction with the product and subject effects) was examined using a general linear regression model and, as expected, was not significant for any analysis ($p = 0.05$). Therefore, the week's sums of squares, with its accompanying degrees of freedom, was added into the error terms. Products 14 and 11 were significantly ($p < 0.01$) less bioavailable than the other products. While Products 10 and 7 were the next least bioavailable, the differences were not statistically significant compared to the other products tested.

There was no apparent relationship between the dose (50 or 100 mg) and the percent of drug excreted. The two products exhibiting the poorest bioavailability were 50-mg tablets. However, studies of the 100-mg tablets obtained from the same sources as the 50-mg tablets indicated satisfactory bioavailability. In the absence of knowledge of the components of the formulations and the manufacturing process, it is not possible to explain the observed differences in bioavailability.

In addition to examining the cumulative percent of nitrofurantoin excreted following administration of each product, the urine levels of nitrofurantoin were compared. Previous reports indicated that a urine concentration of about 30 μg/ml should be effective against 90% of the strains of *Escherichia coli*, although levels of 75 μg/ml may be required against some resistant strains (13). Urine nitrofurantoin concentrations at each sampling time, for each product, in each subject, were plotted *versus* the time each sample was collected. The graphical points were connected by straight lines, and the duration of time for the maintenance of the 30- and 75-μg/ml levels was measured from each graph. The procedure, while somewhat imprecise due to unequal sampling intervals, still afforded the opportunity to identify further two products that

Table V—USP XVIII Dissolution Test of Nitrofurantoin Products

Product[a]	Dose, mg	Mean Percent Dissolved[b] 15 min	30 min	60 min	90 min
8	50	28.66 (1.65)	41.98 (3.45)	57.58 (3.59)	69.71 (4.31)
5R	100	21.97 (8.53)	39.53 (8.63)	57.43 (9.14)	66.90 (8.05)
5	100	19.99 (6.23)	37.72 (4.96)	55.11 (6.25)	65.12 (3.98)
8R	50	25.36 (4.69	38.03 (6.90)	52.20 (9.11)	62.03 (9.62)
7	100	13.03 (2.12)	24.81 (4.29)	39.66 (7.05)	50.26 (8.93)
10	50	12.61 (1.93)	22.77 (2.46)	38.31 (4.12)	47.46 (4.79)
6	100	9.66 (1.42)	19.71 (2.88)	34.56 (2.74)	45.18 (3.68)
1	50	1.80 (0.51)	7.39 (3.54)	28.19 (10.83)	38.34 (14.75)
12	100	3.66 (1.44)	16.08 (3.96)	29.49 (4.90)	36.94 (4.75)
9	50	3.86 (1.23)	9.21 (2.48)	17.86 (5.49)	24.82 (6.64)
2	50	1.98 (2.29)	8.21 (4.81)	18.37 (6.83)	24.23 (9.13)
3	100	1.01 (0.34)	3.54 (1.30)	11.13 (2.77)	16.66 (3.05)
11	50	3.42 (0.82)	5.78 (1.58)	9.99 (1.55)	12.75 (1.92)
13	100	3.34 (0.39)	5.17 (0.93)	9.70 (2.25)	12.47 (2.87)
4	100	2.42 (1.13)	4.67 (2.06)	7.52 (1.96)	9.67 (2.42)
14	50	3.21 (0.93)	4.60 (0.56)	7.60 (0.68)	9.54 (1.05)

[a] See Table I for product code numbers; 5R and 8R represent repeat determinations. [b] Mean of six determinations. Data are expressed in terms of percent of labeled content, with standard deviations in parentheses.

Table VII—Two-Way Analysis of Variance of Cumulative Percent of Nitrofurantoin Excreted 12 hr Postadministration Using Tukey's Test for Nonadditivity[a]

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	*F* Ratio
Subjects	5.84	13	0.45	5.22
Products	18.45	13	1.42	16.50
Nonadditivity	0.22	1	0.22	2.50
Balance	14.46	168	0.09	
Total	38.96	195		

Rank Order of Products 12 hr Postadministration[b] (Least to Most Bioavailable Product)[c]

14 11 10 (7) (3) (12) 2 (4) (6) (13) 1 8 (5) 9

[a] All data were subjected to a ln $(X + 0.01)$ transformation because of a significant nonadditivity in the untransformed data. [b] Products ranked on the basis of the Newman–Keuls *a posteriori* test. Products underlined by the same line are not significantly different ($p > 0.01$). [c] See Table I for product code numbers; 100-mg products are indicated by parentheses.

Table VIII—Duration (Hours) for Maintenance of Urine Nitrofurantoin Levels of 30 or 75 μg/ml

Product[a]	Dose, mg	Duration, hr[b] 30 μg/ml	75 μg/ml
6	100	5.50 (1.63)	2.32 (1.58)
4	100	5.27 (2.07)	1.82 (1.89)
3	100	4.93 (2.06)	2.48 (1.66)
12	100	4.87 (2.14)	2.20 (1.66)
5	100	4.79 (2.38)	2.60 (2.13)
13	100	4.77 (2.29)	2.23 (2.05)
7	100	4.69 (1.68)	1.91 (1.55)
9	50	3.88 (1.98)	1.30 (1.47)
1	50	3.30 (2.27)	1.34 (1.33)
8	50	3.11 (1.37)	1.56 (1.30)
10	50	2.59 (2.18)	0.50 (0.99)
2	50	2.38 (1.92)	0.99 (1.41)
14	50	0.51 (0.98)	0.11 (0.41)
11	50	0.49 (0.81)	0.10 (0.26)

[a] See Table I for product code numbers. [b] Mean value for 14 subjects, with standard deviation in parentheses.

provided relatively low urine drug levels. Table VIII summarizes the mean duration that each drug product maintained the specified urine levels, and Tables IX and X give the results of the two-way analysis of variance of the duration data. Products 14 and 11 provided a significantly ($p < 0.01$) shorter duration of the 30-μg/ml drug level, with no statistically significant difference between the other products tested. Products 14 and 11 also provided a shorter duration of the 75-μg/ml drug level ($p < 0.01$).

Differences observed between the various other products are summarized in Tables IX and X. While Product 7 was ranked as being relatively poor in terms of cumulative percent excreted, it provided for a duration of urine drug levels comparable to the other products tested. Even though this product was less completely available for absorption, the rate and extent of absorption were apparently sufficient to provide adequate urine drug levels. The 100-mg products provided the highest levels, as would be expected if the assumed direct relationship between dose absorbed and quantity of drug excreted was valid.

It appears that the relatively poor bioavailability of Products 14 and 11, as determined from total cumulative percent of drug excreted and the relatively short duration of urine levels recognized as clinically effective, would strongly suggest that these two products may not compare favorably with the other products tested in terms of therapeutic equivalency.

***In Vitro–In Vivo* Correlations**—Attempts were made to correlate the mean *in vitro* disintegration time of samples of each of the 14 nitrofurantoin products with the mean cumulative percent of nitrofurantoin excreted in the urine after 12 hr. While Fig. 2 indicates the expected trend of a decreasing cumulative percent excreted with increasing disintegration time, the correlation ($r = -0.23$) is not sufficient to provide for a reliable prediction of nitrofurantoin bioavailability from the disintegration test. The correlation of mean disintegration time with cumulative percent excreted after 1 and 2 hr was slightly improved, $r = -0.32$ and $r = -0.36$, respectively, but was insufficient for accurate prediction of *in vivo* availability.

Correlations between bioavailability and dissolution rate of solid dosage forms have been reported to be more meaningful than correlations with disintegration time for certain drugs (14, 15). Figure

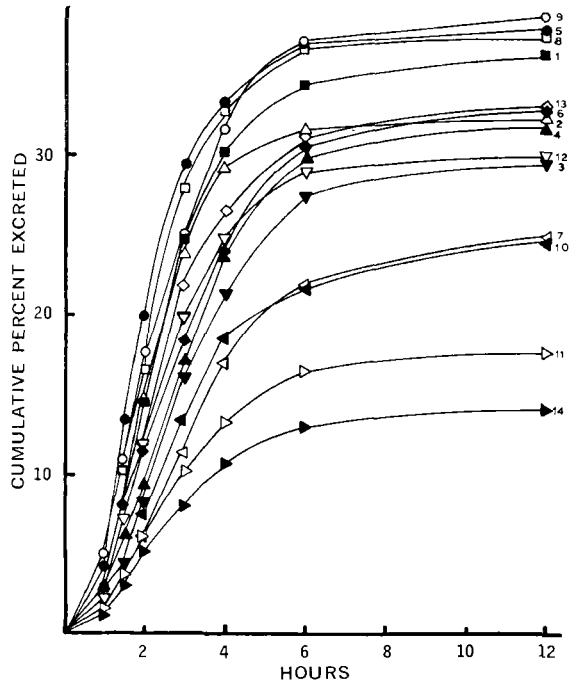

Figure 1—*Mean cumulative percent of nitrofurantoin excreted following the oral administration of 14 nitrofurantoin products. Product code numbers (Table I) appear beside the 12-hr sample. Each data point is the mean cumulative percent excreted for all 14 subjects.*

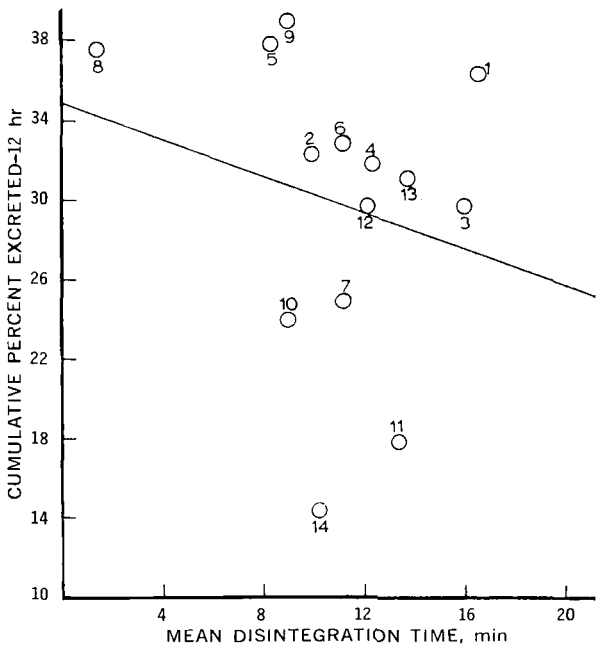

Figure 2—*Correlation of mean disintegration time with cumulative percent of nitrofurantoin excreted after 12 hr. Cumulative percent excreted values are mean values from 14 subjects; disintegration times are means of six determinations. Product code numbers (Table I) appear beside each data point.*

Table IX—Two-Way Analysis of Variance for Duration of 30-μg/ml Urine Levels[a]

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	*F* Ratio
Subjects	17.82	13	1.37	0.55
Products	447.68	13	34.44	13.80
Nonadditivity	0.93	1	0.93	0.37
Balance	419.17	168	2.50	
Total	885.59	195		

Rank Order of Products[b] (Least to Greatest Duration of 30-μg/ml Urine Level by Product)[c]

14 11 10 2 1 8 (12) 9 (5) (13) (7) (3) (4) (6)

[a] All data were subjected to a ln $(X + 0.01)$ transformation because of a significant nonadditivity, using Tukey's test, in the untransformed data. [b] Products ranked on the basis of the Newman–Keuls *a posteriori* test. Products underlined by the same line are not significantly different ($p > 0.01$). [c] See Table I for product code numbers; 100-mg products are indicated by parentheses.

Table X—Two-Way Analysis of Variance for Duration of 75-μg/ml Urine Levels[a]

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	*F* Ratio
Subjects	32.97	13	2.54	0.50
Products	406.19	13	31.25	6.11
Nonadditivity	22.54	1	22.54	4.41
Balance	858.64	168	5.11	
Total	1320.34	195		

Rank Order of Products[b] (Least to Greatest Duration of 75-μg/ml Urine Levels by Product)[c]

14 11 10 2 9 1 (4) 8 (5) (7) (13) (12) (6) (3)

[a] All data were subjected to a ln $(X + 0.01)$ transformation because of a significant nonadditivity, using Tukey's test, in the untransformed data. [b] Products ranked on the basis of the Newman–Keuls *a posteriori* test. Products underlined by the same line are not significantly different ($p > 0.01$). [c] See Table I for product code numbers; 100-mg products are indicated by parentheses.

3, however, shows the relatively poor correlation ($r = 0.45$) for mean percent of nitrofurantoin dissolved after 90 min *versus* cumulative percent excreted after 12 hr. As was the case with the attempted correlation with disintegration time, the *in vitro* test was not a reliable predictor of *in vivo* availability. Even poorer results were obtain for attempted correlations between cumulative percent excreted after 12 hr and mean percent dissolved after 15 min ($r = 0.29$), 30 min ($r = 0.36$), and 60 min ($r = 0.43$). Thus, while the attempted correlations indicated that the quantity of nitrofurantoin absorbed increased with an increasing dissolution rate, the prediction of *in vivo* bioavailability was not sufficiently precise to permit application of the *in vitro* testing procedure to evaluate the products.

SUMMARY AND CONCLUSIONS

The results of the evaluation of single lots of 14 different nitrofurantoin products indicated that all products tested exhibited chemical and physical properties that were within acceptable limits, as specified by USP XVIII. Urinary excretion determinations revealed that two lots were significantly less bioavailable than the others tested. Two products available in the form of macrocrystals were less bioavailable than the majority of the products tested, but urine drug levels appeared adequate, and differences between these products and the 10 most readily absorbed products were not statistically significant. No adequate correlation was observed between the extent of *in vivo* bioavailability and either the *in vitro* disintegration time or dissolution rate.

While the present investigation does not consider the issue of whether other lots of a nitrofurantoin product from a given company will necessarily be identical to the lots tested, the results do indicate that certain companies have marketed products that satisfy present USP specifications but exhibit unsatisfactory bioavailability.

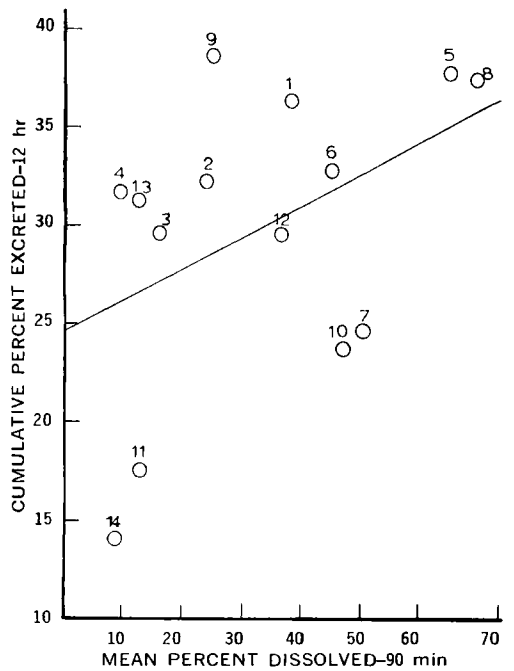

Figure 3—*Correlation of mean percent dissolved after 90 min with cumulative percent excreted after 12 hr. Cumulative percent excreted values are mean values from 14 subjects; dissolution values are means of six determinations. Product code numbers (Table I) appear beside each data point.*

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* Department of Molecular Biology, College of Pharmacy, University of Tennessee Medical Units, Memphis, TN 38163

ˣ To whom inquiries should be directed.

Critical Evaluation of Use of Effective Protein Fractions in Developing Pharmacokinetic Models for Drug Distribution

DANIEL SHEN and MILO GIBALDI ˣ

Abstract □ A critical evaluation of the concept of effective protein fractions, which was previously utilized in physiologically based pharmacokinetic models to describe the binding of barbiturates to plasma and various tissues, is presented. The calculation of effective protein fractions requires as a minimum the extrapolation of *in vitro* binding parameters from one concentration of protein to another. Based on available literature data, it is shown that thiopental binding parameters vary markedly with the concentration of bovine serum albumin and that such concentration dependency cannot be predicted readily. Similar anomalous protein binding behavior has been reported for other drugs. Consequently, the use of effective protein fractions in developing pharmacokinetic models for drug distribution may not generally be feasible. The apparent successful use of such fractions in the case of thiopental appears to be fortuitous. Errors incurred in the extrapolation of the binding parameters may be compensated for by errors introduced by the experimental method in which the original binding data were obtained as well as interspecies differences in protein binding.

Keyphrases □ Protein fractions, effective—evaluation of use in developing drug distribution pharmacokinetic models □ Pharmacokinetic models—effective protein fraction, barbiturate binding, evaluation of use, equations □ Drug distribution—pharmacokinetic models, evaluation of effective protein fraction □ Binding parameters—evaluation of use of effective protein fraction in pharmacokinetic drug distribution models

In recent years the utility of physiologically based pharmacokinetic models has been demonstrated in describing quantitatively the distribution and elimination of barbiturates (1, 2), methotrexate (3–5), and cytarabine (cytosine arabinoside) (6) in various species. These models are uniquely different from the conventional compartmental models because all parameters involved have a specific physiological and/or physicochemical basis.

The development of such models carried the exciting implication that, in principle, based on *in vitro* tissue binding and partition studies together with well-documented physiological parameters such as organ weight and blood flow rate, it may be possible to make *a priori* predictions of drug distribution in the body. Excretion parameters such as biliary and renal clearances can be obtained from appropriate animal data. With the cytarabine model, an initial attempt was made to incorporate *in vitro* enzyme kinetic data in quantitating metabolism. It is evident that the development of physiologically and anatomically realistic models offers the possibility of a systematic, rational approach in extrapolating animal data to humans by appropriate scale-up of parameters.

DISCUSSION

In general, the four-compartment model proposed for barbiturates (1) and, in particular, for thiopental (2) represents the basic framework of all physiological models. The body is divided into several well-defined anatomical regions: blood, viscera, lean, and adipose compartments. Each body region further consists of a blood pool in equilibrium with the respective tissue. Within each compartment, except for the adipose tissue, the drug is present in both freely diffusible and "protein"[1]-bound form. Mathematical resolution of the model, therefore, requires binding parameters relating the free and bound concentrations in the various compartments.

In principle, the binding relationships should be readily obtainable by *in vitro* binding studies with plasma and various tissues. Abundant information on drug–plasma protein interaction already exists in the literature, whereas tissue binding data are meager or nonexistent. Furthermore, tissue proteins are poorly defined. Tissue samples are often too insoluble, and only their homogenates or centrifugal fractions can be studied. But plasma binding studies can easily be performed with whole plasma, plasma protein fractions, or crystalline serum albumin. The latter is often preferred because it is commercially available in pure form, is well characterized, and constitutes about 50–65% of total plasma protein, *i.e.*, 3.5–5.5% by weight of plasma.

Binding data obtained with an isolated protein such as albumin can be analyzed according to the physical law of mass action. Multiple classes of noninteracting binding sites are often assumed, and

[1] The term "protein," as used in this article, refers to those components in various tissues and blood to which the drug is bound. These components include proteins and other macromolecules.

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The development of such models carried the exciting implication that, in principle, based on *in vitro* tissue binding and partition studies together with well-documented physiological parameters such as organ weight and blood flow rate, it may be possible to make *a priori* predictions of drug distribution in the body. Excretion parameters such as biliary and renal clearances can be obtained from appropriate animal data. With the cytarabine model, an initial attempt was made to incorporate *in vitro* enzyme kinetic data in quantitating metabolism. It is evident that the development of physiologically and anatomically realistic models offers the possibility of a systematic, rational approach in extrapolating animal data to humans by appropriate scale-up of parameters.

DISCUSSION

In general, the four-compartment model proposed for barbiturates (1) and, in particular, for thiopental (2) represents the basic framework of all physiological models. The body is divided into several well-defined anatomical regions: blood, viscera, lean, and adipose compartments. Each body region further consists of a blood pool in equilibrium with the respective tissue. Within each compartment, except for the adipose tissue, the drug is present in both freely diffusible and "protein"[1]-bound form. Mathematical resolution of the model, therefore, requires binding parameters relating the free and bound concentrations in the various compartments.

In principle, the binding relationships should be readily obtainable by *in vitro* binding studies with plasma and various tissues. Abundant information on drug–plasma protein interaction already exists in the literature, whereas tissue binding data are meager or nonexistent. Furthermore, tissue proteins are poorly defined. Tissue samples are often too insoluble, and only their homogenates or centrifugal fractions can be studied. But plasma binding studies can easily be performed with whole plasma, plasma protein fractions, or crystalline serum albumin. The latter is often preferred because it is commercially available in pure form, is well characterized, and constitutes about 50–65% of total plasma protein, *i.e.*, 3.5–5.5% by weight of plasma.

Binding data obtained with an isolated protein such as albumin can be analyzed according to the physical law of mass action. Multiple classes of noninteracting binding sites are often assumed, and

[1] The term "protein," as used in this article, refers to those components in various tissues and blood to which the drug is bound. These components include proteins and other macromolecules.

Table I Total Protein Content of Various Fresh Soft Tissues in Humans[a]

Tissue	Total Protein, %
Plasma	6.95
Red blood cell	36.8
Brain (whole)	10.5
Heart	16.0
Liver	17.0
Kidney	18.0
Muscle	18.5

[a] From Ref. 27.

parameters such as the association constant and binding capacity associated with each class of site can be calculated by graphical techniques (7, 8) and computer analysis (9, 10).

In view of the lack of information on tissue binding of the barbiturates and thiopental, Dedrick and Bischoff (1) introduced the concept of "effective protein fractions" to deal with the mathematical implications of tissue binding. Through rigorous examination of the concept of an effective protein fraction, it became clear that care must be exercised when *in vivo–in vitro* correlation of protein binding is attempted. The general practice in binding experiments is to vary the total drug concentration over the range of pharmacological interest while keeping the albumin concentration constant. The extent of binding, *i.e.*, the bound and/or unbound concentration, is then measured. The binding parameters obtained from such a study are often assumed to be applicable at all other albumin levels.

It will be shown that this assumption may at times be incorrect. The significance of this problem will be illustrated with readily available thiopental binding data. It will also be demonstrated that such unpredictable deviations affect the calculation of the various effective protein fractions.

Effective Protein Fraction—A detailed study of the binding of thiopental to 1% bovine serum albumin, using the method of ultrafiltration, was reported (11). The binding data can be described by a two-term, Langmuir-type equation:

$$x = \frac{B_1K_1C}{1 + K_1C} + \frac{B_2K_2C}{1 + K_2C} \qquad \text{(Eq. 1)}$$

The same definitions as used by Dedrick and Bischoff (1) are adopted here, where x = bound concentration, C = free concentration, B = maximum binding capacity, and K = equilibrium association constant. The subscripts denote the two different classes of noninteracting binding sites. The following values were assigned (2): B_1 = 18,400 μmoles/liter, B_2 = 305,000 μmoles/liter, K_1 = 0.060 liter/μmole, and K_2 = 0.000625 liter/μmole.

In the absence of additional information, Bischoff and Dedrick explored the approximation that the binding characteristics for all proteins in the body are the same as those of bovine serum albumin. They then had to consider the question of how much protein is present in the different tissues. The normal total protein content of various soft tissues ranges from 10 to 20% by weight (Table I). Obviously, various kinds of proteins have different binding affinities toward thiopental and not all proteins would be accessible to the drug. Furthermore, tissue components other than proteins can be responsible for binding.

Based on the total protein content of various tissues listed in Table I, thiopental concentrations in blood and tissue compartments were simulated using Bischoff and Dedrick's (2) model. The same dose and input function were used. The predicted blood and muscle concentrations are compared to the experimental data of Brodie *et al.* (12) in Fig. 1 and a substantial overestimate is apparent. Less obvious, but equally significant, is that neither the time required to attain distribution equilibrium nor the predicted tissue-to-blood ratios agree with the experimental data. High metabolic clearance values have to be assigned to bring the blood concentration profile back into the proper perspective. However, even under these conditions the rank order of the tissue-to-blood distribution ratio cannot be corrected. It is apparent that only part of the tissue proteins are *effective* in binding the drug.

Goldbaum and Smith (11) reported the fraction of thiopental bound to various rabbit organ homogenates at an initial concentration of 0.5 m*M*. Dedrick and Bischoff (1) showed that for each data point a mass balance equation can be written:

$$C_t = fC + (1 - f)x \qquad \text{(Eq. 2)}$$

where C_t, the total drug concentration, was assumed to be that present initially, *i.e*, 0.5 m*M*; and C and x, free and bound drug concentrations, respectively, are related by Eq. 1. The term f is a dimensionless parameter defined as the fraction of water in tissue (2). To be more precise, the term $(1 - f)$ is an estimate of the effective fraction of protein and f is simply the remaining fraction of a volume of tissue homogenate. Free drug is assumed to be distributed homogeneously in this nonprotein fraction. Therefore, Eq. 2 was rewritten as:

$$C_t = (1 - f_p)C + f_px \qquad \text{(Eq. 3)}$$

where f_p is the effective protein fraction. The bound concentration, x from Eq. 1 has units of millimoles of drug bound per kilogram of protein. To convert this into the proper units of concentration (*i.e.*, millimoles of drug bound per liter of tissue homogenate), a density of unity has been assumed. By rearranging Eq. 3, an algebraic expression for f_p can be obtained:

$$f_p = \frac{C_t - C}{x - C} \qquad \text{(Eq. 4)}$$

Free drug concentration can be calculated from the fraction bound data of Goldbaum and Smith (11), since:

$$C = (1 - \text{fraction bound})C_t \qquad \text{(Eq. 5)}$$

By combining Eqs. 1, 4, and 5, f_p values for each rabbit tissue studied were calculated (Table II). When these values were compared to those provided in Ref. 1 (Table C2), it immediately became apparent that all of the present values except for the undiluted plasma were one-fifth those calculated by Dedrick and Bischoff (1). Subsequently, referring back to Goldbaum and Smith's original paper (11), it was noted that the rabbit blood and tissue samples were homogenized and diluted to five volumes with phosphate buffer before ultrafiltration. This explains the fivefold difference between the sets of values.

Attention was then drawn to the fact that fraction bound values

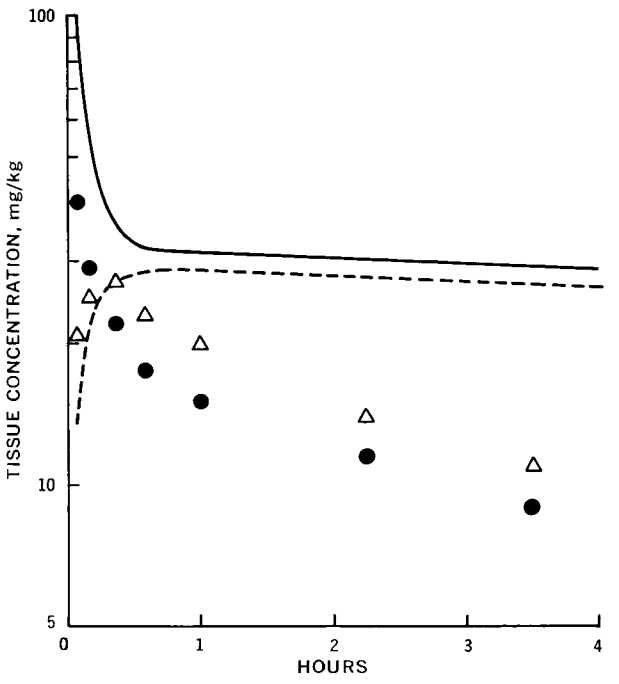

Figure 1—*Predicted concentrations of thiopental in blood* (——) *and muscle* (– – –) *using total protein concentration in various tissues with the four-compartment physiological model of Bischoff and Dedrick (2). Blood* (●) *and muscle* (△) *concentrations of thiopental in a dog obtained from Brodie* et al. *(12) are included for comparison.*

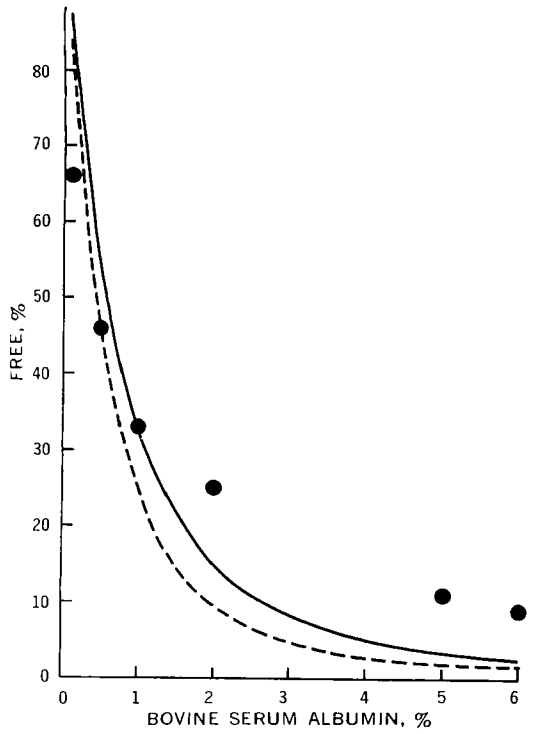

Figure 2—*Effect of bovine serum albumin concentration on the percent of unbound thiopental. Key:* ●, *data obtained from Goldbaum and Smith (11);* ——, *predicted values at a total drug concentration of 1 m*M*;* ***and*** – – –, *predicted values at a total drug concentration of 0.5 m*M.

for both diluted (five times) and undiluted plasma were available in the paper by Goldbaum and Smith (11). One would expect that values of effective protein fractions calculated for both diluted and undiluted plasma should be identical. However, after calculating an f_p value and applying a correction factor of 5, the diluted plasma data yielded a fraction value of 0.04255. This new effective protein fraction for plasma is about three times larger than that calculated from the undiluted plasma data—*viz.*, 0.0148. The discrepancy prompted a close examination of the thiopental binding data.

Dependence of Binding Parameters on Protein Concentration—The key assumption in the calculation of effective protein fractions for thiopental lies in the dilution factor. If the fraction represents a real mass of binding material, the fivefold correction is appropriate. However, the adjustment for dilution also involves the implicit assumption that the binding parameters remain unchanged throughout the extrapolated range of albumin concentration. In the study by Goldbaum and Smith (11), the effect of varying bovine serum albumin concentration on the fraction bound of a 1 m*M* thiopental solution is also reported. Those data are reproduced here in Fig. 2 as percent of free drug *versus* percent bovine serum albumin. The validity of applying binding parameters from one protein level to another in the case of thiopental can be ascertained by simulating the fraction of free drug as a function of protein concentration utilizing the binding parameters obtained at the 1% albumin level and comparing the calculated fraction to the actual values obtained.

Given the total drug concentration and a set of binding parameters, the concentration of free drug can be calculated as follows. The effective protein fraction, f_p in Eq. 3 is replaced with P_t, which now represents the concentration of albumin. To be consistent, P_t has to be expressed in kilograms of albumin per liter of solution. By substituting Eq. 1 into Eq. 3 and expanding the resulting expression, a third degree polynomial equation in free concentration, C, is obtained:

$$(1 - P_t)\delta C^3 + [(1 - P_t)\gamma + \beta P_t - \delta C_t]C^2 + [(1 - P_t) + \alpha P_t - \gamma C_t]C - C_t = 0 \quad \text{(Eq. 6)}$$

where $\alpha = B_1K_1 + B_2K_2$, $\beta = B_1K_1K_2 + B_2K_2K_1$, $\gamma = K_1 + K_2$, and $\delta = K_1K_2$.

Table II—Calculated and Reported Values of Effective Protein Fractions

Tissue	Fraction Bound[a]	Calculated f_p[b]	Reported f_p[c]
Muscle	0.45	0.00364	0.0175
Liver	0.66	0.00719	0.0362
Red blood cell	0.40	0.00306	0.0151
Heart	0.62	0.00631	0.0301
Kidney	0.53	0.00473	0.0237
Brain	0.50	0.00429	0.0212
Plasma, undiluted	0.85	0.01480	0.0145
Plasma, diluted	0.71	0.00851	—[d]

[a] Rabbit tissue homogenate data obtained by Goldbaum and Smith (11). [b] Calculated from Eqs. 1, 4, and 5. [c] Values tabulated in Table C2 of Ref. 1. [d] An effective protein fraction based on diluted (five times) plasma was not calculated.

A similar equation was derived by Hart (13), who established that equations of this type have only one positive real root which corresponds to the actual concentration of the unbound drug. Numerical solutions of Eq. 6 at various protein concentrations, P_t, while the total concentration, C_t, is held constant can readily be found by conventional methods of successive approximation on a digital computer. Solutions were generated for total drug concentrations of 1 and 0.5 m*M*. The predicted free concentration, expressed in terms of percent free over the appropriate range of albumin concentration, is shown with the experimental data in Fig. 2. Except for coincidence with the 1% data point, the predicted curve at an initial concentration of 1 m*M* provides a poor fit of the data.

At albumin concentrations of 5 and 6%, where the percentage of free drug is very small, the differences between predicted and observed values are more than threefold. Similar experimental data at 0.5 m*M*, the initial concentration at which the rabbit tissue binding study was carried out, are not available. However, based on the comparison at a total concentration of 1 m*M*, one can certainly conclude that the binding parameters obtained with the 1% bovine serum albumin solution cannot be applied, with any degree of confidence, at any other protein concentration.

The dependency of the binding parameters on protein concentration can best be depicted by transposing Goldbaum and Smith's (11) data onto a Scatchard plot (Fig. 3). A calculated curve based on the 1% binding parameters is included in Fig. 3. The experimental points lie nearly on a straight line with a positive slope, indicating a continuous decrease in either binding capacity B or association constant K, or both, as the concentration of albumin is increased at a fixed total drug concentration. It is apparent that no one average set of binding constants, *i.e.*, no one Scatchard curve, could fit all points.

Brunkhorst and Hess (14) observed very similar binding behavior with cortisol toward both human and bovine serum albumin. These investigators did not offer any definite explanations for the anomalous binding characteristics. They did mention the possibility that various quantities of competitive "inhibitors" may be present. Recently, Crooks and Brown (15) provided an example where the dependence of binding parameters on protein concentration is merely an artifact due to the particular buffer used. Different Scatchard plots were obtained for tolbutamide with 1 and 2% human serum albumin in tromethamine buffer. No such difference was observed when phosphate buffer was used. The investigators surmised that components of the tromethamine buffer may be competing with the drug for the same binding sites, leading to the observed dependency. In an early work of Klotz and Urquhart (16), a drastic decrease in the amount of methyl orange bound per mole of bovine serum albumin at the same free ligand concentration was observed when the protein concentration was increased fivefold, from 0.2 to 1.0%. Thus, the type of protein concentration dependency exhibited by thiopental may be more general than is now realized.

One less desirable feature of the ultrafiltration technique is that the volume of the drug–protein solution is continually reduced as part of the aqueous portion is filtered. The protein concentration increases as the filtration process progresses. Therefore, a binding equilibrium may not be attained throughout the filtration. In usual

practice, only a small portion of the solution is filtered, *i.e.*, less than 20%, so that the overall effect on protein concentration may be assumed to be negligible. Perhaps due to limitations of the assay, Goldbaum and Smith (11) chose to filter approximately one-half of the initial volume. This procedure effectively doubles the albumin concentration, and the total drug concentration remaining is different from that present initially. These changes, however, were not taken into consideration by Goldbaum and Smith. The bound concentration was simply taken to be the difference between the initial and the ultrafiltrate drug concentrations.

If one assumes, as a first approximation, that the concentration of thiopental in the ultrafiltrate represents the equilibrium free concentration in the drug–protein solution at the end of the filtration process, one can recalculate a more likely total drug concentration. The correction does not change the Scatchard plot in Fig. 3, since both bound drug and bovine serum albumin were concentrated to the same extent; *i.e.*, the variable r is not affected. The percent free data shown in Fig. 2, however, will be different. The total drug concentration after ultrafiltration will be higher than the initial concentration and will vary according to the amount of albumin present. Nevertheless, simulations based on these assumptions reveal a lack of agreement, similar to Fig. 2, between the percent free drug values as calculated from the binding parameters and those values calculated from the observed results.

Curvature in a Scatchard plot of drug–protein binding data has often been interpreted as evidence for heterogeneity of binding sites. However, Koshland (17) pointed out that the nonlinearity may also be a result of the conformational change of the macromolecule induced by an initial association with small molecules. In other words, interaction between binding sites occurs. The phenomenon is often referred to as cooperativity. There is ample evidence (18, 19) suggesting that albumin is a rather flexible molecule and can undergo extensive reversible conformational alterations. If the binding of each molecule of drug makes it more difficult for the next molecule to bind, *i.e.*, a negative cooperative effect, a downward concave Scatchard curve similar to the 1% predicted curve shown in Fig. 3 would be expected.

In fact, an opposite upward concave curve as a result of positive cooperativity has been observed with the binding of nicotinamide adenine dinucleotide to yeast glyceraldehyde 3-phosphate dehydrogenase (20). More recently, similar behavior was observed with the binding of acetylcholine to an acetylcholine specific receptor protein (21). One may perhaps infer from this discussion that a positive type of cooperative effect may explain the unusual Scatchard plot with thiopental. However, the reported positive cooperative effects have all been observed at one protein or receptor concentration and there is no reason why, in the case of thiopental, positive cooperative effects are only observed when the protein level is varied. An alternative rationalization could be that the serum bovine albumin undergoes "molecular aggregation." As the concentration of albumin increases, less of the binding surface per molecule becomes available, resulting in a decrease of affinity for the drug.

Regardless of the reasons for the deviant binding characteristic, the dependency of thiopental binding parameters on protein concentration offers a plausible explanation for the threefold difference in the value of an effective protein fraction when calculated from either diluted or undiluted rabbit plasma. Similar differences probably occur with the rest of the tissue data so that all of the effective protein fractions provided by Bischoff and Dedrick, except for undiluted plasma, may have been overestimated. The dilution factor is probably around 1.7 rather than 5.

In spite of the inappropriateness of the fivefold correction, the particular set of effective protein fractions listed by Bischoff and Dedrick provided excellent fit of the mathematical model to experimental data. When the effective protein fractions are decreased to one-third of the reported values, substantial changes in the concentration–time profiles occur. The values provided by these investigators appear to be unique. Bischoff and Dedrick followed Goldbaum and Smith in adopting the initial thiopental concentration added to the various rabbit tissue homogenates as the total drug concentration, C_t. When corrections were attempted to account for the excessive ultrafiltration, the effective protein fractions increased to about twice those of the previous values (Table II). When these new fractions were further multiplied by a dilution factor of 1.7, the values come very close to the numbers used by Bischoff and Dedrick. It appears that, by a fortuitous circumstance, the error incurred by the fivefold correction may partly be offset by an underestimation of the total drug concentration in the rabbit tissue homogenates.

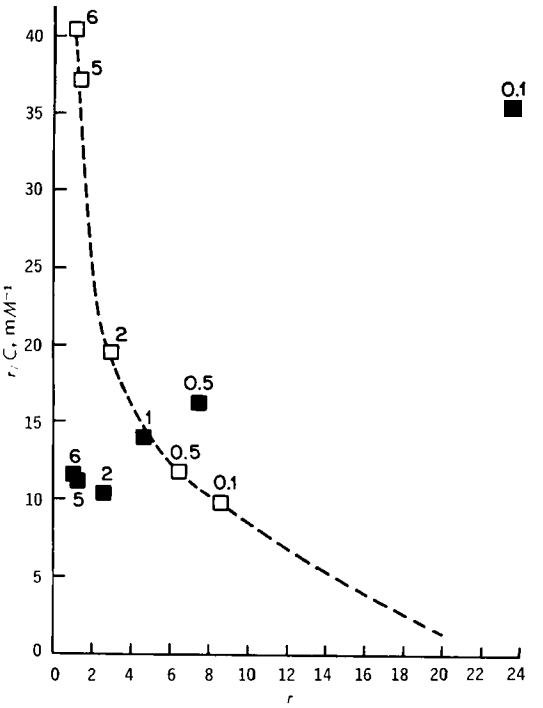

Figure 3—*Scatchard plot of fraction bound values of thiopental obtained by ultrafiltration at various bovine serum albumin concentrations. The initial concentration of thiopental was 1 m*M*. Key: – – –, calculated curve based on binding parameters obtained with 1% bovine serum albumin solution;* ■*, experimental points; and* □*, theoretical points. Numerals accompanying each point indicate the protein concentrations. The term* r *is defined as the ratio of millimoles of drug bound to millimoles of albumin, and* C *is the free concentration of drug. The molecular weight of albumin is assumed to be 68,000.*

Interspecies Differences in Protein Binding—It is well recognized that significant interspecies differences often exist with plasma protein binding. Examples include various sulfonamides (22), salicylates (23), and desipramine (24). Differences in both the quantity and binding characteristics of different plasma proteins contribute to the interspecies variability. An interesting example of the significance of interspecies differences in protein binding can be found with warfarin (25). The association constant of warfarin to human plasma albumin is six times that of canine plasma albumin. This difference has been used to explain why phenylbutazone potentiates the anticoagulant effect of warfarin in humans through protein displacement, while in dogs the enzyme-inducing effect is predominant.

In view of reported interspecies differences, it is instructive to consider the sources of the thiopental binding data used by Bischoff and Dedrick. The isotherm was based on *bovine* serum albumin. *Rabbit* organs were used for determination of tissue binding. The predicted results of the model were eventually compared to *human* and *dog* data. A total of four animal species was involved. Kane and Smith (26) studied the binding of thiopental to human plasma by equilibrium dialysis. Their study suggested that at the same free concentrations bovine serum albumin may bind five to eight times more thiopental than human plasma. Hence, interspecies variation in protein binding may be offered as another possible explanation as to why the particular set of effective protein fractions used for thiopental (1) happened to provide good prediction of thiopental distribution in dogs and humans.

CONCLUSION

As is apparent from the foregoing discussion, *in vivo–in vitro* correlation of tissue protein binding is fraught with limitations and

difficulties. Since one cannot perform binding studies with homogenized tissues without dilution, care must be exercised when extrapolation of binding relationships from one protein level to another is required. Likewise, human tissue is difficult to obtain and uncertainty exists when animal tissue data are substituted. The problem of interspecies variability in binding characteristics of protein may, in some ways, be analogous to that encountered with biotransformation processes.

Therefore, the use of an effective protein fraction to account for tissue binding does not appear to be feasible if unpredictable binding behavior similar to that displayed by thiopental is found. At present, the most practical approach to handling protein binding with respect to modeling appears to be the use of equilibrium tissue-to-plasma distribution ratios as was done with the methotrexate models (3, 4). The distribution ratios can be measured or estimated from *in vivo* animal data. It must be considered, however, that the distribution ratio will vary as a function of concentration if nonlinearity in binding occurs.

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[x] To whom inquiries should be directed.

ADDENDUM

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This will be a brief commentary on the issues discussed by Shen and Gibaldi. To begin, we concur with their overall results, many of which were known to us when we published the first work, on barbiturate pharmacokinetics, that utilized the notion of "effective protein fraction." Their simulation shown in Fig. 1, where total tissue protein values are used, certainly emphasizes the fact that smaller values—effective protein fractions—are needed to predict the drug distribution properly. These types of results were what naturally led us to formulate the whole concept. Furthermore, we would now probably favor the use of "effective protein amounts" and define total concentration similarly to Krüger-Thiemer:

$$C_T = wC + \bar{p}x \qquad \text{(Eq. A1)}$$

where w is fraction water (usually approximately known) and p is the effective protein concentration. This obviates assuming unity densities, and there is no need for the two coefficients to be fractions that add to unity. The net results are similar, but fewer approximations of principles are required.

The results shown for thiopental in Fig. 2 of this Shen and Gibaldi article were also known to us; in fact, similar discrepancies are also true for the other barbiturates, even the weakly bound barbital. This was our reason for including plasma in the effective protein table rather than using the actual protein fractions, that one would hope would have worked for this tissue. In retrospect, these details perhaps should also have been published; Shen and Gibaldi, by describing this technique in greater detail, have performed a useful service for those actually wishing to use it.

To be more specific, the effective protein concept assumes that: *(a)* binding can be represented by an isotherm, $x(C)$, on a thermodynamically intensive basis (*i.e.*, per unit protein mass), multiplied by a protein concentration; and *(b)* intensive isotherms for nonspecific binding would be the same (at least approximately) for all body tissues (the mixing of data for different species was done due to the lack of more complete data and should naturally be avoided if at all possible). The first should be true if there are no interactions, say at higher protein concentrations. This, unfortunately, does not seem to be true in the physiological range for plasma and indicates a gap in our knowledge of protein binding. We were interested in the additional evidence and references provided by Shen and Gibaldi. The second could not be checked by us, since complete isotherm data were only available for plasma proteins (in fact, bovine serum albumin) and not for any tissue homogenates. It would seem that tissue differences for nonspecific binding would not be as severe as for metabolism where, presumably, very specif-

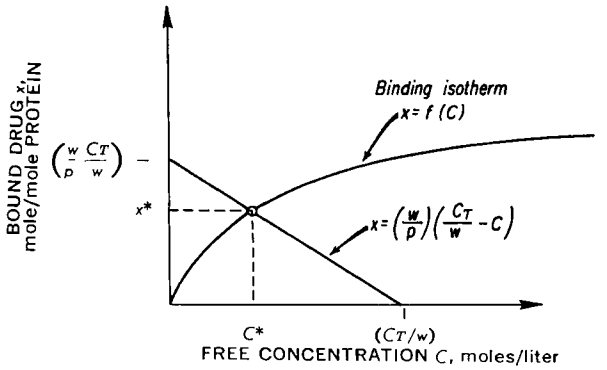

Figure A1—*Illustration of a graphical procedure for the estimation of free concentration of drug for a given total drug and protein concentration.*

ic sites are involved. However, these still remain open conjectures.

A brief description of a useful graphical technique to perform the calculations might also be of interest. The equation for total concentration can be rearranged to give:

$$x = \frac{w}{p}\left(\frac{C_T}{w} - C\right)$$
$$= f(C), \text{ binding isotherm} \qquad \text{(Eq. A2)}$$

This expression is a straight line; if it is plotted on the same graph as $x = x(C)$, the intersection provides the desired value of free concentration corresponding to a (given) total concentration of drug present (see Fig. A1). Shen and Gibaldi used an iterative computer routine to determine the solution of the nonlinear algebraic equation, but the simple graphical technique often provides answers of adequate accuracy, or they could be used as starting values for an iterative computer solution.

To conclude, the questions raised by Shen and Gibaldi concerning the effective protein concentration concept are valid and were the basis of our choice of the term "effective." We did not feel that the data we used (1954 vintage) were sufficiently precise, nor was there a wide enough range of information, to be worth more detailed analysis. We had hoped that more extensive and detailed investigations would be done to test the conjectures. It does seem, however, that good simulations and predictions of tissue drug levels are possible, both from our own work and that of others during the intervening several years. As discussed by Shen and Gibaldi, the alternate method of utilizing a tissue–plasma distribution ratio, $R(c)$ varying with concentration for nonlinear binding and estimated from *in vivo* data, is probably the best approach in light of our present knowledge of binding.

However, this approach has limitations: *(a)* a fairly large amount of *in vivo* biopsy data is required, since predictions from *in vitro* binding isotherm data are presumably not used; and *(b)* interesting effects, such as binding saturation and/or competitive binding of two drugs, cannot be predicted. Therefore, it is felt that the effective protein concentration concept has valuable and unique capabilities for pharmacokinetic simulations, and it is hoped that further development of it, along with fundamental binding studies with physiological protein concentrations, will lead to a more firm quantitative basis for handling drug binding.

Kenneth B. Bischoff

School of Chemical Engineering
Cornell University
Ithaca, N.Y.

Robert L. Dedrick

Biomedical Engineering and
Instrumentation Branch
National Institutes of Health
Bethesda, Md.

Mechanistic Studies on Effect of Cholate Concentration on Cholesterol Uptake by Isolated Rabbit Intestine

ANWAR B. BIKHAZI *x and JEAN JACQUES HAJJAR ‡

Abstract □ A novel apparatus was designed to measure the uptake of cholesterol by mounted and isolated segments of rabbit intestine. The system maintained constant hydrodynamic conditions at the exposed mucosal surfaces. Cholesterol uptake was studied in a series of Ringer's solutions containing different cholate concentrations. The data indicated a substantial effect of cholate on cholesterol transport. A simple semi-infinite sink model in conjunction with theoretical equations on a first-order transport process was proposed. First-order uptake rate constants for the early experimental data points were calculated for the various cholesterol–cholate systems. At 4.64 m*M* cholate, there was approximately a 190 times increase in the relative initial uptake rate compared to the 0 m*M* cholate level. Above the CMC of cholate, there was a decrease in the cholesterol uptake. The diffusion coefficients of cholesterol in the various cholate media varied between 3.6 and 13.0 × 10^{-6} cm^2/sec, which were in the range of 4.0 × 10^{-6} cm^2/sec calculated by the Stokes–Einstein approximation. The calculated permeability coefficients of cholesterol, below and above the CMC of cholate, were on the order of 0.8–9.0 × 10^{-2} cm/sec.

Keyphrases □ Cholate—effect on cholesterol uptake, isolated rabbit intestine, new apparatus described and tested, CMC and permeability coefficients □ Cholesterol—effect of cholate concentration on uptake by isolated rabbit intestine, new apparatus described and tested, CMC and permeability coefficients □ Transport rates—effect of cholate concentration on cholesterol uptake by isolated rabbit intestine, new apparatus described and tested □ Permeability—effect of cholate concentration on cholesterol uptake by isolated rabbit intestine, new apparatus described and tested

The mechanism of cholesterol transport across the intestine has been under considerable debate for the past few years. Several investigators have emphasized the role that bile salts play in the enhancement of cholesterol absorption (1, 2). Their observations led to the prevalent view that micellar solubilization is essential for cholesterol absorption.

Sylvèn and Borgström (3) proposed a possible model for cholesterol transport through the intestinal barrier. This model was later expanded (4) at a slightly higher mechanistic level. These models, however, need stronger *in vivo* experimental support, particularly with respect to the mechanism of cholesterol transport at submicellar as well as micellar concentration levels of bile salts. Hofmann (5) estimated the critical micelle concentration (CMC) of bile salts to be in the range of 2–3 m*M*. Rough calculations, considering the bile salt concentration in gallbladder bile, the volume of bile secreted per meal, and the dilution of bile salts in the intestinal volume and con-

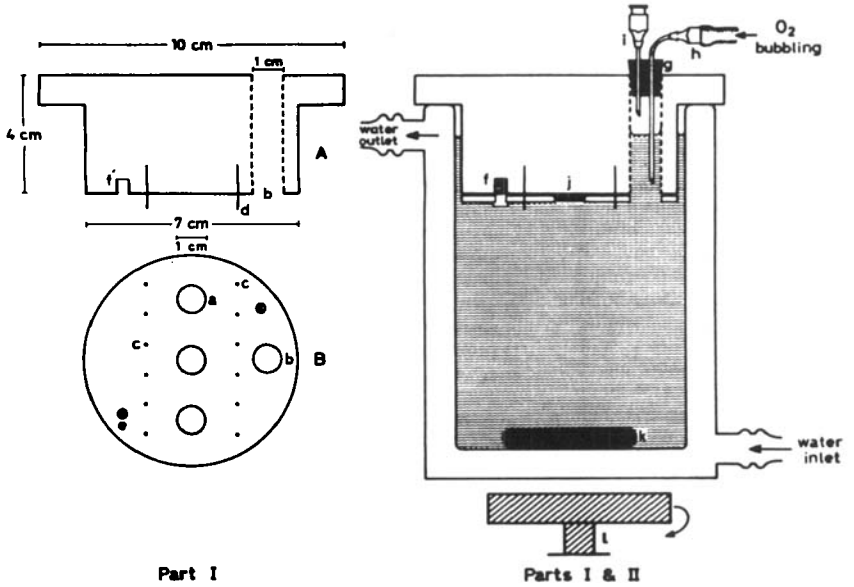

Figure 1—*Uptake apparatus. Part I consists of components A and B to be attached together with screws f through openings e and f'. Openings a appear only in component B to expose 1-cm diameter of intestine as shown in j. Needles d penetrate through openings c to hold tight the intestinal pieces. Hypodermic needles h and i are the inlet and outlet of oxygen gas bubbles attached through cork g on top of sampling opening b; k is a Teflon-coated magnetic bar, and l is a magnetized shaft of the synchronous motor.*

tents, resulted in numbers indicating that intestinal fluids contain bile salt micelles (the concentration of bile salts in the intestinal content is around 2–3 m*M*).

The present work is an attempt to establish quantitatively a means of measuring cholesterol uptake by the intestine.

EXPERIMENTAL

Treatment of Animals and Description of Apparatus—Mongrel rabbits were utilized and sacrificed by intravenous injection of sodium pentobarbital (6). The abdomen was opened rapidly and segments of the ileum were resected out, slit open along the mesenteric border, and washed free of intestinal contents with cold Ringer's solution, pH 6.90 (140 m*M* NaCl, 1.2 m*M* $CaCl_2$, and 1.2 m*M* $MgCl_2$). Three segments were mounted on component A of Part I of the apparatus (Fig. 1). Then component B of Part I immediately was gently placed over component A and firmly attached to it using two screws. This step resulted in exposing three 1-cm diameter portions of mucosal surfaces. Part I of the apparatus then was submerged in a constant-temperature (37 ± 0.1°) water-jacketed beaker which contained a preequilibrated Ringer's solution with a known amount of cholesterol-4-^{14}C (10^{-9} g/ml)[1] and/or cholic-^{3}H acid (7×10^{-9} g/ml)[2] and the desired concentration of sodium cholate[3] (biochemical purity). Into this solution, 95% O_2–5% CO_2 was gently bubbled through opening b of Part I of the apparatus with the help of a hypodermic needle attached through a Teflon tube to an oxygen tank. The bubbling was very cautiously controlled so as not to disturb the stirring of the system and thereby alter the diffusional mechanisms at the surfaces of the membranes. The Ringer's solution in the apparatus was constantly stirred before and during the experiments by utilizing a Teflon-coated magnetic bar rotating at 120 rpm with a synchronous constant-speed motor[4] placed beneath the beaker. This synchronous stirring process assured constant hydrodynamic conditions at the exposed surfaces of the intestine.

Sampling and Assay of Samples—To measure the rate of disappearance of cholesterol from the system, 1-ml samples were taken through opening b of Part I at different time intervals. These samples were assayed for cholesterol-4-^{14}C and/or cholic-^{3}H acid in a three-channel liquid scintillation spectrometer[5] using Bray's solution (7). Control experiments were done to measure the adsorption of cholesterol on glass and on Leucite in the absence of the intestinal segments. The amount of solute adsorbed in the presence of Ringer's solution was found to be negligible for a 1-hr period.

Determination of Cholate CMC in Ringer's Solution—The CMC of sodium cholate was determined in Ringer's solution employing the volume-per-drop method. Sodium cholate concentrations ranging from 0 to 23.2 m*M* were freshly prepared in Ringer's solution. A Teflon-tipped buret was vertically assembled. The level of each cholate solution in the buret was adjusted to be the same at the beginning of every count. The number of droplets delivered from the buret per 5-ml volume was counted for each surfactant solution.

Measurement of Diffusion Layer Thickness—The method of Howard (8) was used in measuring the diffusion layer thickness along the intestinal mucosa at a 120-rpm stirring rate in the aqueous bulk phase. A 0.005% *p*-nitrotoluene stock solution in Ringer's solution was prepared. The procedure described under *Treatment of Animals and Description of Apparatus* was employed, except that the *p*-nitrotoluene stock solution was put in the water-jacketed beaker replacing the cholesterol–cholate solution. Two-milliliter samples were taken at different time intervals for 90 min. The samples were then diluted with 10 ml of water and read against the proper blank in a spectrophotometer[6] at λ_{max} 285 nm. The concentrations of the samples were calculated from standard Beer's law curves for *p*-nitrotoluene.

Calculations and Treatment of Data—Figure 2 describes a simple semi-infinite sink model which assumes constant stirring in the aqueous phase resulting in a diffusion layer region. This region is effectively stagnant, and a quasi-steady-state constant hydrodynamic condition is assumed to exist in it. At the intestine–water interface, a concentration-independent partition coefficient, K, exists at equilibrium. In this model it can be assumed that the diffusion coefficient of the solute in the aqueous diffusion layer is D_w. The following equation describes the situation:

$$\frac{dC_w}{dt} = -\frac{D_w A}{hV_w}(C_w - C_l) \qquad \text{(Eq. 1)}$$

where C_w is the bulk solute concentration at any time t, C_l is the solute concentration at the intestinal mucosa at any time t, D_w is the aqueous solute diffusion coefficient, A is the total surface area of exposed mucosal surface, h is the diffusion layer thickness, and V_w is the aqueous bulk volume.

It is assumed that steady state is achieved early in the experiments and that the data obtained are employed in the calculation and treatment of the results. It is also assumed that during the experiments the intestine is still viable. This is supported by comparable experiments on the transport properties of rabbit intestines showing viability for periods longer than 1 hr (9). If this is so, then

[1] Radiochemical Center, Amersham, Buckinghamshire, England.
[2] New England Nuclear, Boston, Mass.
[3] Nutritional Biochemicals Corp., Cleveland, Ohio.
[4] Motor Division, Hurst Manufacturing Corp., Princeton, Ind.
[5] Packard Tri-Carb model 3320, Packard Instrument Co., Downers Grove, Ill.
[6] Perkin-Elmer model 202, Perkin-Elmer Corp., Norwalk, Conn.

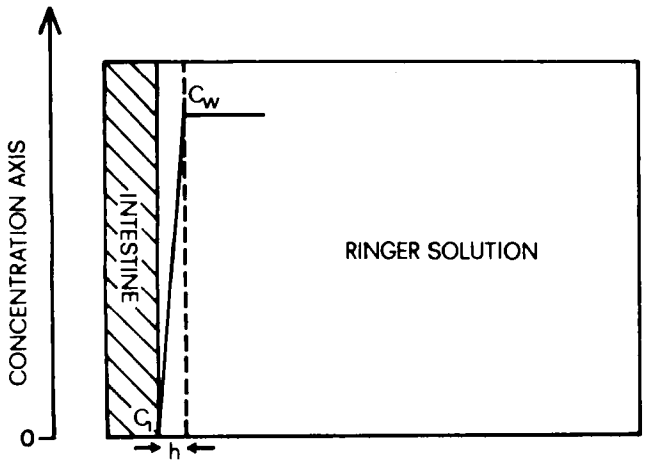

Figure 2—*Simple semi-infinite sink model of solute transport across an aqueous diffusion layer of thickness* h *into the mucosa.* C_w *is the aqueous bulk concentration;* C_I *is the interfacial (Ringer's solution–mucosal) aqueous concentration. The solid line in the Ringer's solution and aqueous diffusion layer represents condition at time equals zero.*

Eq. 1 can be written as:

$$\frac{dC_w}{dt} = -\frac{D_w A}{hV_w}C_w \qquad \text{(Eq. 2)}$$

After separating the variables and integrating, one can obtain:

$$\ln C_w = -\frac{D_w A}{hV_w}t + \text{constant} \qquad \text{(Eq. 3)}$$

A plot of C_w *versus* t on semilogarithmic graph paper yields a straight line whose slope $(-D_wA/hV_w)$ is the uptake rate constant. Since the calculated value is the initial slope, the constant isolated is designated as the initial uptake rate constant. Furthermore, when the partition coefficient K of the solute between the intestinal mucosa and the aqueous bulk phase is relatively large, the data are expected to deviate from linearity and Eq. 3 only applies for the first few initial points.

The diffusion layer thickness is calculated from the results obtained when *p*-nitrotoluene is used as the solute in Ringer's solution. The aqueous diffusion coefficient of *p*-nitrotoluene is reported to be about 1.1×10^{-6} cm²/sec (8). Figure 3 is a semilogarithmic plot of *p*-nitrotoluene concentration remaining in the diffusion medium *versus* time in minutes. The best straight line joining the data points is drawn. When equating the slope to $-(D_wA/hV_w)$, substituting for $D_w = 1.1 \times 10^{-6}$ cm²/sec, $V_w = 175$ ml, and $A = 1.61$ cm², and changing the slope from minute^{-1} to second^{-1}, the value of h was found to be 0.00046 cm.

For the uptake of cholesterol, the slopes of the lines from the semilogarithmically plotted data are equated to $-(D_wA/hV_w)$. The values of A, h, and V_w are also known; D_w for cholesterol is calculated in the different sodium cholate systems. The permeability coefficient, $P = (D_w/h)$, is also calculated.

Figure 4 reports the results on the determination of the CMC of sodium cholate in water and in Ringer's solution. The semilogarithmic plot of the number of droplets delivered per 5-ml volume at constant rate *versus* cholate concentration in m*M* resulted in

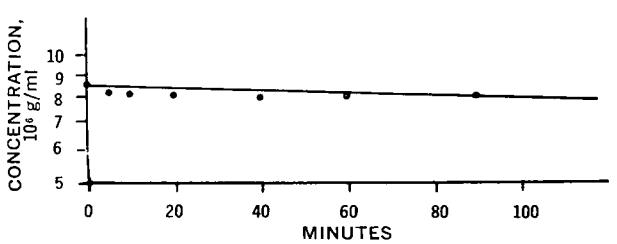

Figure 3—*Semilogarithmic plot of* p*-nitrotoluene concentration remaining in the diffusion medium* versus *time in minutes.*

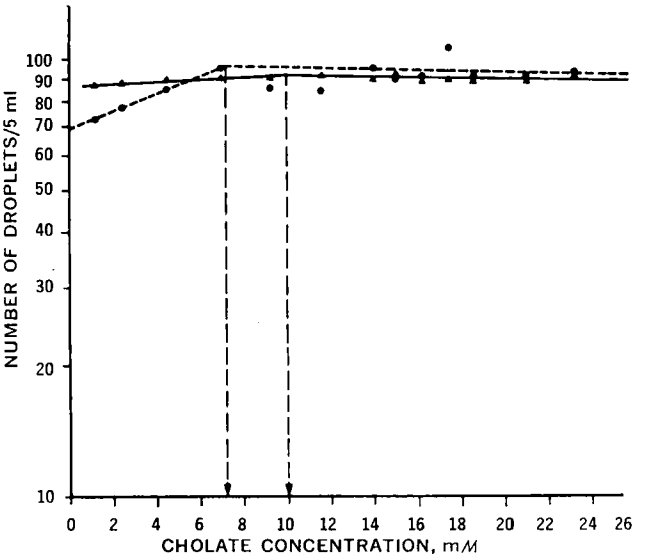

Figure 4—*Semilogarithmic plot of number of droplets/5 ml* versus *cholate concentration in* m**M**. *Key:* ●, *data points when cholate is dissolved in water (the CMC determined is indicated with an arrow on the concentration axis and is 7.2* m**M**); *and* ▲, *data points when cholate is dissolved in Ringer's solution (the CMC determined is indicated with an arrow on the concentration axis and is 10* m**M**).

points that could be linearly joined by two separate lines. One line joins the low cholate concentration data, and the other line joins the higher concentration data. The two lines intercept each other at the CMC as shown in the figure. The CMC of the cholate–water system is around 7.2 m*M*, while that of the cholate–Ringer's solution system is around 10 m*M*. These results agree with data reported previously (10).

RESULTS

Uptake of Cholesterol-4-^{14}C Utilizing a Water-Jacketed Beaker of V_w = 370 ml—Experiments were run utilizing the same amount of cholesterol in Ringer's solution but altering the cholate concentration (pH of all cholate solutions studied ranged between 6.9 and 7.3). Figure 5 shows a representative plot of cholesterol (in counts/10 min) remaining in solution *versus* time in minutes. Each point in the graph represents at least three experimental runs. At 1.16, 2.32, and 4.64 m*M* cholate concentrations,

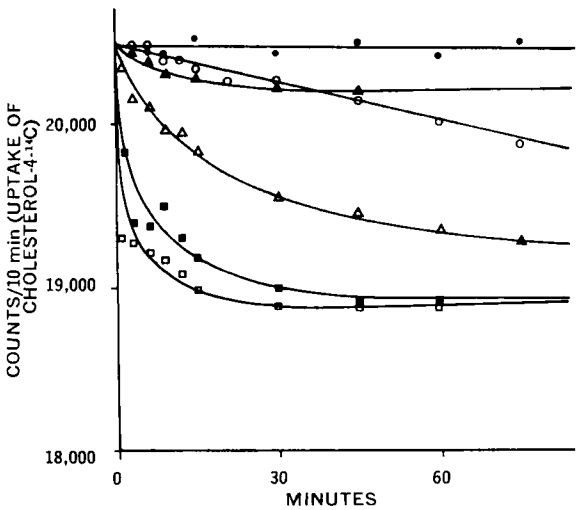

Figure 5—*Plot of cholesterol-4-*^{14}*C in counts/10 min left over in solution* versus *time in minutes when* V_w *= 370 ml. Key:* ○, *0* m**M** *sodium cholate;* △, *1.16* m**M** *sodium cholate;* ■, *2.32* m**M** *sodium cholate;* □, *4.64* m**M** *sodium cholate;* ▲, *11.16* m**M** *sodium cholate; and* ●, *17.40* m**M** *sodium cholate.*

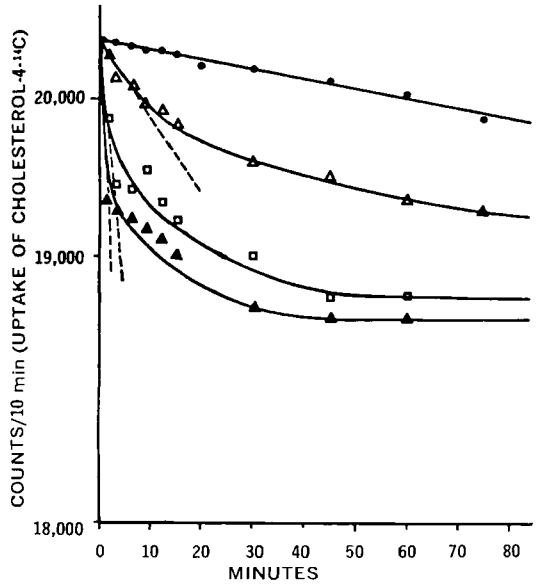

Figure 6—*Semilogarithmic plot of cholesterol-4-^{14}C in counts/10 min left over in solution* versus *time in minutes when* V_w = *370 ml for some representative experiments. Key:* ●, *0 mM sodium cholate;* △, *1.16 mM sodium cholate;* □ *2.32 mM sodium cholate; and* ▲, *4.64 mM sodium cholate. The dotted lines are those joining the early experimental data points. The slopes of these lines were employed in the treatment of data.*

there was an increase in the rate of cholesterol uptake as compared to the rate at 0 m*M* cholate concentration. This increase continued even at 6.38, 8.12, and 11.16 m*M* cholate concentration (Table I). The latter rates represent a decreasing trend when compared to those of 1.16, 2.32, and 4.64 m*M* levels of cholate. At 17.4 m*M* cholate, there seemed to be little or no uptake of cholesterol for 75 min.

Figure 6 represents a semilogarithmic plot of the concentration of cholesterol *versus* time for the 0, 1.16, 2.32, and 4.64 m*M* levels of cholate. The dotted lines are extensions of the straight lines passing through the early data points. The slopes of these lines and similar lines for the 6.38, 8.12, 11.16, and 17.40 m*M* levels were calculated and are reported in Table I. As indicated in the table there

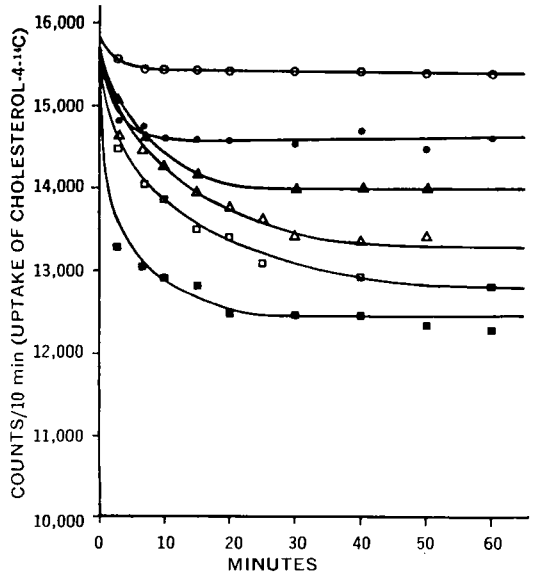

Figure 7—*Plot of cholesterol-4-^{14}C in counts/10 min left over in solution* versus *time in minutes when* V_w = *175 ml. Key:* △, *0 mM sodium cholate;* □, *1.16 mM sodium cholate;* ■, *2.32 mM sodium cholate;* ●, *8.12 mM sodium cholate;* ▲, *17.40 mM sodium cholate; and* ○, *23.20 mM sodium cholate.*

Table I—Estimated Initial Rates and Relative Initial Rates of Cholesterol Uptake into Rabbit Intestine as a Function of Cholate Concentration

Cholate in Ringer's Solution, m*M*	pH	Initial Rate Constant[a], hr^{-1}	Relative Initial Rate Constant[b]
0.00	6.90	0.024	1.00
1.16	6.92	0.166	6.90
2.32	7.00	0.976	40.70
4.64	7.00	4.560	190.00
8.12	7.05	0.244	10.17
11.16	7.15	0.059	2.50
17.40	7.30	0.000	0.00

[a] Calculated from the slope of the line from the semilogarithmic plot passing through the first few experimental data points. [b] Calculated by dividing the initial rate of the system containing sodium cholate by the initial rate of the system containing 0 m*M* cholate.

was an increase in the relative initial rate constant as a function of cholate concentration, with a maximum being reached roughly around 4.64 m*M* of cholate. Below and above this cholate concentration, the relative initial rate constants showed a decline as compared to the maximum value.

Uptake of Cholesterol-4-^{14}C Utilizing a Water-Jacketed Beaker of V_w = 175 ml—Figures 7 and 8 are representative plots of the uptake of cholesterol in Ringer's solution with different cholate concentrations. Both plots are similar to Figs. 5 and 6. The differences are indicated in the initial rate constants and not in their relative values. This is expected due to the differences in the diffusion layer thicknesses.

Uptake of Cholic-^{3}H Acid Utilizing a Water-Jacketed Beaker of V_w = 175 ml—There was no significant cholic acid uptake by the rabbit intestine. This was observed in all cholate concentrations.

DISCUSSION

A novelty of such experiments resides in finding the effect of

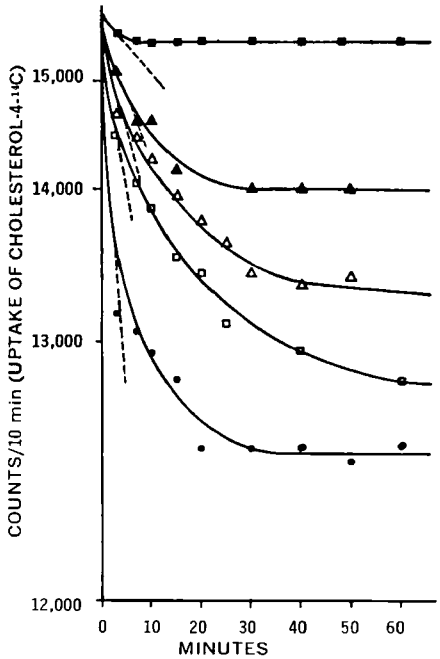

Figure 8—*Semilogarithmic plot of cholesterol-4-^{14}C in counts/10 min left over in solution* versus *time in minutes when* V_w = *370 ml for some representative experiments. Key;* △, *0 mM sodium cholate;* □, *1.16 mM sodium cholate;* ●, *2.32 mM sodium cholate;* ▲, *17.40 mM sodium cholate; and* ■, *23.20 mM sodium cholate. The dotted lines are those joining the early experimental data points. The slopes of these lines were employed in the treatment of data.*

Table II—Estimated Diffusion and Permeability Coefficients of Cholesterol Uptake into Rabbit Intestine as a Function of Cholate Concentration

Cholate in Ringer's Solution, m*M*	Initial Rate Constant, $\cdot$ hr^{-1}	Diffusion Coefficient, cm^2/sec	Permeability Coefficient, cm/sec
0.00	0.928	1.30×10^{-5}	3.0×10^{-2}
1.16	1.499	2.10×10^{-5}	4.5×10^{-2}
2.32	2.929	4.10×10^{-5}	9.0×10^{-2}
8.12	1.392	1.90×10^{-5}	4.1×10^{-2}
17.40	0.644	9.00×10^{-6}	1.9×10^{-2}
23.20	0.260	3.60×10^{-6}	0.8×10^{-2}

some bile salt constituents on the transport of cholesterol by the intestinal epithelium. The question that could be raised here is whether cholate (one bile salt constituent) enhances cholesterol transport. Figure 5 and Table I clearly indicate that there is a substantial increase in the initial uptake rate of cholesterol across the intestinal barrier between the 1 and 10 m*M* cholate concentration range.

The CMC of sodium cholate in water was estimated to be in the range of 7.2 m*M* when determined by the volume-per-drop method, and the CMC of cholate in Ringer's solution was found to be around 10 m*M* (Fig. 4). Hofmann and Borgström (11) reported the CMC to be in the range of 20 m*M* of cholate when the latter acted as a solubilizing agent for 20-methylcholanthrene. With this in mind and considering the conditions of the intestinal content, it can be suggested that the CMC of cholate in the intestine is somewhere in the range of 10–20 m*M*. The data in Table I indicate that below the CMC the transport rate is fast and that close to the CMC the rate is declining; it is close to nil at around 18 m*M* when micelles are in existence.

Figures 7 and 8 further support the data. Because of the faster rate of transport of cholesterol in the V_w = 175-ml beaker (compare the third column of Table I with the second column of Table II), it was easier to measure in it the diffusion layer thickness along the intestinal surface at a 120-rpm stirring rate. Table II presents the calculated diffusion coefficients of cholesterol in the different cholate solutions in Ringer's solution. Howard (8) reported the value of the diffusion coefficient, D_w, of cholesterol at 30° in water to be around 2.0×10^{-6} cm^2/sec. From the Stokes–Einstein approximation, the calculated value of D_w of cholesterol was around 4.0×10^{-6} cm^2/sec. In this study (Table II), the calculated diffusion coefficient of cholesterol in 0 m*M* cholate and at 37° was around 13.0×10^{-6} cm^2/sec, which is in agreement with the reported data. As the cholate concentration increased, the value of the diffusion coefficient also increased. At higher cholate concentrations, there was a decrease in the D_w values; this decrease was maintained at and above the CMC of sodium cholate.

The calculated values of the permeability coefficients (Table II) agreed fairly well with the sequence of the results. There was roughly a 10 times decrease in the permeability coefficient value between the 2.32 and 23.2 m*M* cholate concentration levels. The differences obtained in the permeability coefficient values support the fact that this is a highly sensitive system. The values indicate that slight changes in the cholate concentrations showed significant changes in the permeability coefficients of cholesterol uptake.

The following explanation is given for the role that cholate plays on the uptake of cholesterol by the intestinal mucosa. The cholate–cholesterol interaction varies with the concentration of cholate in the system. Below the CMC, this interaction could be, for example, nil, a 1:1 interaction, a 2:1 interaction, a 1:2 interaction, *etc.* Therefore, the concentration of free cholesterol in the medium is not altered drastically. This suggests that cholate may alter the permeability of the intestinal barrier (12) and, consequently, the effect of cholate at submicellar concentration levels. At or above the CMC, the formation of micelles yields stronger cholesterol–cholate interactions. The concentration of cholesterol in the micelles would be higher than in the aqueous surroundings, resulting in a higher partitioning tendency between the hydrophobic part of the micelle and its environment. Experiments with the cholic acid–cholate system ruled out the possibility that micelles are taken up by the mucosa for 1 hr. These experimental findings suggest that micelle formation retards cholesterol uptake by the mucosal surface of the intestine.

CONCLUSION

The data reported show that the uptake of cholesterol by the isolated rabbit intestine is enhanced in the presence of sodium cholate. The design of the apparatus and the geometry of the system made it possible to control the temperature, solute concentration, cholate concentration, and hydrodynamics. With these factors controlled, it was possible to determine quantitatively the initial rate constants, diffusion coefficients, and permeability coefficients of cholesterol during its passage across the mucosal surface of the rabbit intestine.

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ˣ To whom inquiries should be directed.

Decalin Analogs of Phenethylamines as Inhibitors of Amine Uptake by Rabbit Platelets I: Uptake and Distribution of Histamine

JOUKO TUOMISTO *§, EDWARD E. SMISSMAN ‡†, THOMAS L. PAZDERNIK ‡ˣ, and EDWARD J. WALASZEK *

Abstract □ The importance of conformation with regard to the inhibition of histamine uptake by platelets was studied utilizing different conformationally rigid and semirigid inhibitors. Rabbit blood platelets were incubated in plasma for 80 min with 10^{-6} *M* ^{14}C-histamine, and the total accumulation of radioactivity with or without inhibitors was determined. Four different series were studied. The parent substances were β-phenethylamine, *N*-isopropyl-β-phenethylamine, β-hydroxy-β-phenethylamine, and *N*-isopropyl-β-hydroxy-β-phenethylamine. These structures were made conformationally rigid by incorporating the functional groups into the 2- and 3-positions of the *trans*-decalin molecule. The semirigid systems were studied using cyclohexane and *erythro*- and *threo*-butane analogs of the parent compounds. In three of the four decalin series, the most active conformation was found to have an axial phenyl and equatorial amino group. In one series, both phenyl and amino groups were equatorial in the most active isomer. Thus in all cases the active conformation possessed a *gauche*-relationship. The semirigid systems did not give consistent results, which could be due partly to their flexibility which makes it impossible to deduce the principal conformation in the binding mode. Most substances studied were racemic mixtures; the *d*- and *l*-forms of amphetamine were tested separately, and the *d*-form was slightly more active at low concentrations. The preferred conformation for uptake inhibition in most cases appears to have the phenyl and amino groups at a dihedral angle of approximately 60°, but the differences are not outstanding, which may at least in part be due to the addition of a bulky lipophilic group to the phenethylamine structures.

Keyphrases □ Phenethylamines, decalin analogs—conformationally rigid and semirigid inhibitors of amine uptake by rabbit platelets, uptake and distribution of histamine □ Histamine uptake inhibitors—conformationally rigid and semirigid inhibitors, decalin analogs of phenethylamines, uptake and distribution of histamine by rabbit platelets □ Decalin analogs of phenethylamines—uptake and distribution of histamine by rabbit platelets, conformation effects □ Structure-activity relationships—decalin analogs of phenethylamines–histamine inhibition, uptake and distribution of histamine by rabbit platelets

As a step to the characterization of the chemical structure of receptors, one recent approach has been to determine the stereochemical requirements for the effective agonist or competitive antagonist. This approach can help to ascertain the distances between the active groups of the receptor molecule, to predict some details of the receptor surface, to help explain differences in the effects of closely related substances, and to predict the efficacy of a certain drug on that particular receptor.

A great amount of work has been reported on both cholinergic and adrenergic receptors (1). The clear difference between *d*- and *l*-enantiomers of norepinephrine as adrenergic agonists is well known, and some attempts have been made to determine the possible conformational requirements. In addition to the binding to adrenergic receptors, there appear to be stereochemical requirements for norepinephrine reuptake as well. In rat heart, *l*-norepinephrine is more effectively taken up than *d*-norepinephrine (2), and the *d*-enantiomer of amphetamine is a more potent norepinephrine uptake inhibitor than the *l*-form (3). Likewise, one of four possible methylphenidate stereoisomers is by far the most active in inhibiting norepinephrine uptake by aortic strips (4). Some studies using conformationally rigid molecules were performed recently on norepinephrine uptake by various sympathetically innervated tissues[1].

In the present study an attempt was made to determine the possible stereoselectivity of another kind of uptake, that of biogenic amines by blood platelets. Histamine, which is probably taken up by the same mechanism as 5-hydroxytryptamine and catecholamines (5), was chosen as the indicator; the inhibition of its uptake by various compounds was measured. Unlike the monoamines, histamine is not metabolized or only slowly metabolized by rabbit platelets[2]. Thus, it is easy to measure changes in its intracellular distribution and to calculate whether the inhibition of uptake is taking place on the outer membrane of the cell or at the granular level (6).

Monoamines are not as suitable for the purpose because mitochondrial monoamine oxidase effectively metabolizes the portion accumulating in the cytoplasma if the granular uptake is blocked (7). However, histamine is metabolized by plasma histamine and if uptake inhibitors also have an effect on the metabolism, care must be taken in interpreting the results. In some cases, results of histamine uptake were compared with those of 5-hydroxytryptamine uptake to exclude secondary effects of metabolism.

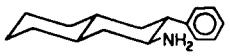
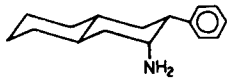
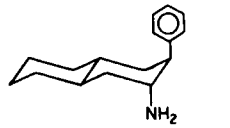
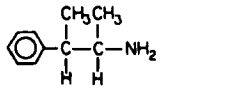
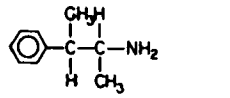
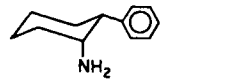
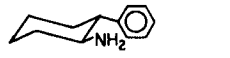
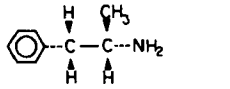
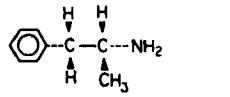
EXPERIMENTAL

Male albino rabbits, 1.8–3.0 kg, were bled, and platelet-rich plasma was prepared as previously described (8). Radioactive histamine was added to ice-cold plasma to give the final concentration of 10^{-6} *M*. Two-milliliter portions of plasma were immediately placed in polypropylene incubation tubes, which contained the substances to be tested in 0.2 ml saline or, in the case of poorly water-soluble substances, in 3–33% dimethyl sulfoxide in saline. The control samples were always incubated with the corresponding solvent. Dimethyl sulfoxide did not interfere to any detectable extent with the uptake (0–10% difference between the dimethyl sulfoxide control and the saline control).

After 80 min of incubation at 37° in oxygen with 5% CO_2, the platelets were sedimented by centrifugation for 30 min at 2000×*g* or for 5 min at 20,000×*g* and then were washed once with 2 ml of saline to remove the extracellular radioactivity. Excess saline

[1] M. Hava, E. E. Smissman, and S. El-Antably, in preparation.
[2] Unpublished results.

Table I—Inhibition of ^{14}C-Histamine Uptake in Percent of Control by β-Phenethylamine-like Compounds Made Conformationally Rigid by Incorporating Them into a *trans*-Decalin Structure[a]

Inhibitor	Structure	10^{-5} *M*	10^{-4} *M*	10^{-3} *M*
1. 2(e)-Amino-3(e)-phenyl-*trans*-decalin		24.2 ± 2.2 (6)	49.7 ± 2.5 (8)	94.8 (3)
2. 2(a)-Amino-3(e)-phenyl-*trans*-decalin		20.4 ± 1.1 (5)	38.0 ± 2.4 (8)	92.4 (3)
3. 2(e)-Amino-3(a)-phenyl-*trans*-decalin		8.6 ± 1.3 (5)	29.1 ± 2.4 (8)	77.9 (3)
4. 2(a)-Amino-3(a)-phenyl-*trans*-decalin		17.5 ± 0.7 (6)	38.7 ± 1.8 (8)	94.6 (3)
5. *erythro*-2-Amino-3-phenylbutane		19.9 ± 2.0 (5)	48.6 ± 2.1 (6)	77.3 (1)
6. *threo*-2-Amino-3-phenylbutane		13.2 ± 3.3 (5)	35.7 ± 3.1 (6)	69.3 (1)
7. *cis*-1-Amino-2-phenylcyclohexane		8.3 ± 0.7 (4)	33.0 ± 1.8 (5)	69.2 (2)
8. *trans*-1-Amino-2-phenylcyclohexane		11.8 ± 1.1 (4)	35.6 ± 1.5 (5)	71.0 (2)
9. *d*-(*S*)-Amphetamine		25.4 ± 2.6 (3)	51.8 ± 1.5 (6)	79.1 ± 1.5 (4)
10. *l*-(*R*)-Amphetamine		21.3 ± 1.3 (6)	52.8 ± 1.6 (6)	80.8 ± 1.7 (4)

[a] Platelets were incubated for 80 min at 37° under an atmosphere of oxygen with 5% CO_2. Values are means ± standard error. Number of experiments is given in parentheses.

above the platelet pellet was removed by wiping with filter paper. Washed platelets were solubilized[3], 5 ml of scintillation fluid [0.4% diphenyloxazole and 0.01% 1,4-bis(5-phenyloxazol-2-yl)benzene in toluene] was added, and the radioactivity was counted[4]. Results are given as percent inhibition compared with a control sample incubated with the solvent under the same conditions. All experiments were performed in duplicate, and the difference between duplicates was usually 0–2%.

For intracellular studies, 5-ml samples were incubated as described. Four milliliters of 0.32 *M* sucrose was pipetted onto the final washed platelet pellets and the platelets were homogenized ultrasonically[5]. Unbroken cells and other larger particles were sedimented at 2500×*g* for 20 min, and a coarse granular fraction was sedimented at 18,500×*g* for 30 min (average *g* values) (9). The two sediments and the final supernate were extracted with butanol according to the histamine assay of Shore *et al.* (10). To 0.5 ml of the final acid phase, 5 ml of scintillation fluid (11) [0.4% diphenyloxazole and 0.01% 1,4-bis(5-phenyloxazol-2-yl)benzene in equal parts of toluene and ethylene glycol monoethyl ether] was added, and the radioactivity was counted as described. In all cases at least 10,000 counts and in most at least 20,000 counts were made.

Partition Studies—The methanesulfonate salt was dissolved in 50 ml of Sørensen's phosphate buffer (pH 7.30), previously washed with the appropriate organic solvent (heptane or octanol). In the cases where the free amine was used, excess methanesulfonic acid was added to solubilize the amine and the pH was adjusted to 7.30 using 0.1 *N* NaOH.

These solutions were placed in a 60-ml separator containing a pipetted sample of an organic solvent (heptane or octanol) previously shaken with the buffer. The solutions were shaken mechanically for 22 hr. Concentrations of amines before and after shaking were determined spectrophotometrically[6]. The apparent partition coefficients were then determined by:

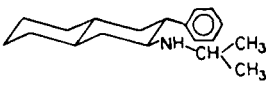
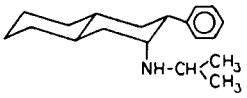
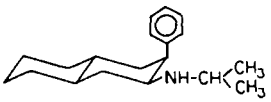
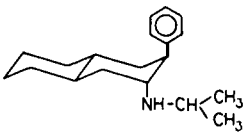
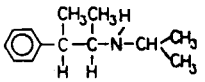
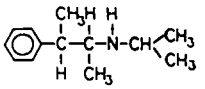
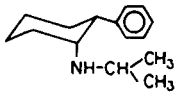
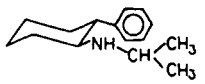
$$K_{app} = \frac{C_{init} - C_{final}}{C_{final}} \times \frac{V_w}{V_{org}} \qquad \text{(Eq. 1)}$$

where K_{app} = apparent partition coefficient, C = concentration in water layer, V_w = volume of water layer, and V_{org} = volume of organic layer.

Chemicals—The *d*- and *l*-forms of amphetamine sulfate[7], β-phenethylamine hydrochloride[8], β-hydroxy-β-phenethylamine (2-

[3] Soluene 100.
[4] Packard Tri-Carb scintillation counter.
[5] Sonifier cell disruptor, model W140D, microtip, setting 3, 1 min.
[6] Cary 14 spectrophotometer.
[7] Sigma Chemical Co.
[8] Calbiochem.

Table II—Inhibition of ^{14}C-Histamine Uptake by *N*-Isopropyl-β-phenethylamine-like Compounds[a]

Inhibitor	Structure	10^{-5} *M*	10^{-4} *M*	10^{-3} *M*
12. 2(e)-Isopropylamino-3(e)-phenyl-*trans*-decalin		18.0 ± 1.7 (5)	41.7 ± 2.0 (7)	86.2 (3)
13. 2(a)-Isopropylamino-3(e)-phenyl-*trans*-decalin		15.7 ± 2.1 (5)	32.0 ± 2.3 (7)	62.2 (3)
14. 2(e)-Isopropylamino-3(a)-phenyl-*trans*-decalin		16.5 ± 1.9 (5)	60.1 ± 1.8 (7)	95.6 (3)
15. 2(a)-Isopropylamino-3(a)-phenyl-*trans*-decalin		14.8 ± 1.7 (5)	36.9 ± 2.1 (7)	92.3 (3)
16. *erythro*-2-Isopropylamino-3-phenylbutane		12.3 ± 1.5 (6)	31.4 ± 2.3 (7)	54.4 ± 2.8 (4)
17. *threo*-2-Isopropylamino-3-phenylbutane		13.3 ± 1.5 (6)	33.2 ± 2.4 (6)	56.1 ± 1.9 (4)
18. *cis*-1-Isopropylamino-2-phenylcyclohexane		19.2 ± 1.6 (6)	40.8 ± 1.5 (6)	69.4 ± 1.4 (3)
19. *trans*-1-Isopropylamino-2-phenylcyclohexane		15.4 ± 1.5 (6)	35.7 ± 1.5 (6)	69.0 ± 2.0 (3)

[a] For details see Table I.

amino-1-phenethanol)[9], *l*-norepinephrine[9], histamine (ring-2-^{14}C) hydrochloride[10], and scintillation chemicals[11] were obtained from commercial sources. Other chemicals were synthesized as follows: Compounds 1–8 as methanesulfonates, 12–18 as hydrochlorides, 31–34 by the method of Smissman and Pazdernik (12, 13), 20–23 by the method of Smissman and Gastrock (14), and 24–30 as hydrochlorides by the method of Smissman and El-Antably (15).

RESULTS AND DISCUSSION

Amphetamine-like compounds were effective amine-uptake inhibiting agents. In this model, little evidence was found for any marked difference between the *d*- and *l*-forms of amphetamine, although in low concentrations the *d*-(*S*)-enantiomer was slightly more effective ($p < 0.05$) (Table I). In the semirigid system, β-methylamphetamine (2-amino-3-phenylbutane), the *erythro*-form, was more active (10^{-5} *M*, $p < 0.01$; 10^{-4} *M*, $p < 0.001$), but the difference was not great (Compounds 5 and 6). Because of bulky methyl groups, the transoid conformation of the phenyl ring and nitrogen would be favored for the *erythro*-form, while for the *threo*-form the preferred conformation is *gauche*. In the other semirigid system, with the amphetamine structure incorporated in cyclohexane, there was very little difference ($p > 0.05$) in activity between cisoid and transoid forms (Compounds 7 and 8). In an almost completely rigid system, where the structure is incorporated in *trans*-decalin, the most active isomer has phenyl and amino groups in the diequatorial positions (Compound 1), with the weakest compound (Compound 3) having the phenyl group axial and the amino function equatorial.

N-Isopropyl derivatives of the same substances gave slightly different results (Table II). There was little difference in semirigid systems, although the *threo*-structure seemed to be slightly more potent. In the decalin derivatives the cisoid compound with axial phenyl and equatorial amino was clearly the most effective in higher concentrations, although at 10^{-5} *M* concentration the diequatorial was the most effective.

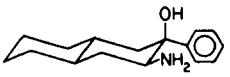
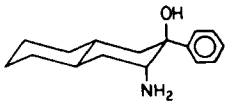
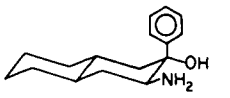
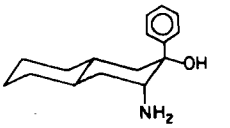
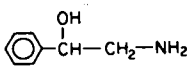
Adding a hydroxy group in the β-position appeared to decrease the effect of these β-phenethylamine derivatives, both in the parent compounds and in the decalin system. Of the β-hydroxy-β-phenethylamine derivatives (Table III), by far the most active compound was the cisoid derivative with the axial phenyl and equatorial amino group. This compound was comparable in potency to the parent compound. The *N*-isopropyl derivatives of these gave similar results (Table IV). Very little difference was found between *erythro*- and *threo*-forms of the parent compound (Compounds 29 and 30).

To determine whether various decalin structures without a complete phenethylamine structure would be active, various substances were studied (Table V). 2-Amino-*trans*-decalins as such were as potent histamine uptake inhibitors as some of the β-phenethylamine derivatives. On the other hand, 2-*trans*-decalol was without any effect and 3(a)-phenyl-2(a)-*trans*-decalol caused inhibition only at the highest concentration used.

To ascertain the localization of action of these compounds, the effects of some of them were studied on ^{14}C-histamine distribution in the platelet (Figs. 1 and 2). Reserpine was used as a substance acting mainly at the granular level. It caused a complete change in the intracellular distribution of histamine at concentrations that inhibit the total uptake only slightly or not at all. Both amphetamines and 2(e)-amino-3(a)-phenyl-3-*trans*-decalol at low concentrations caused an inhibition of uptake and only in higher concentrations did they begin to influence the distribution of histamine.

[9] Mann Research Laboratories.
[10] Amersham/Searle Corp.
[11] Packard Instrument Co.

Table III—Inhibition of ^{14}C-Histamine Uptake by β-Hydroxy-β-phenethylamine-like Compounds[a]

Inhibitor	Structure	10^{-4} *M*	10^{-3} *M*
20. 2(e)-Amino-3(e)-phenyl-3-*trans*-decalol		17.5 ± 1.3 (7)	36.4 ± 3.3 (9)
21. 2(a)-Amino-3(e)-phenyl-3-*trans*-decalol		13.4 ± 3.0 (6)	34.5 ± 3.0 (7)
22. 2(e)-Amino-3(a)-phenyl-3-*trans*-decalol		35.9 ± 3.2 (10)	80.0 ± 2.9 (11)
23. 2(a)-Amino-3(a)-phenyl-3-*trans*-decalol		7.6 ± 3.1 (7)	31.9 ± 3.0 (9)
24. β-Hydroxy-β-phenethylamine		35.4 ± 2.3 (8)	72.5 ± 0.9 (9)

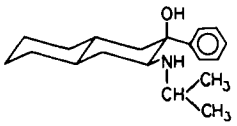
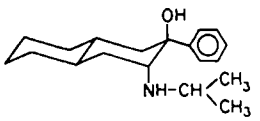
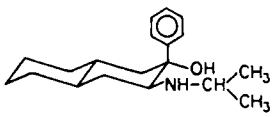
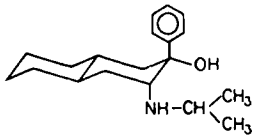
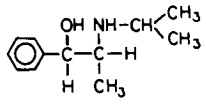
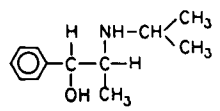
[a] For details see Table I.

It is concluded that they do not inhibit the uptake secondarily due to the inhibition of storage, because reserpine causes much less inhibition of uptake although the distribution change indicating storage failure is quite evident. The phenethylamines studied appear to act primarily on the outer cell membrane. In high concentrations they affect the granules as well, but this effect does not contribute greatly to the inhibition of the total amine uptake.

To check the possibility of platelet damage, electron microscopy was performed. After 80 min of incubation with 10^{-4} *M* 2(e)-amino-3(a)-phenyl-3-*trans*-decalol (Compound 22), no electron microscopic evidence of damage was seen.

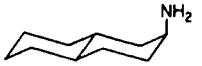
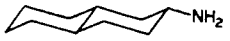
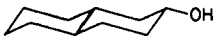
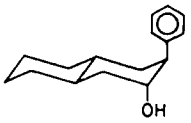
The lipid–buffer partition values of some compounds are presented in Table VI. All decalin derivatives were much more lipid soluble at physiological pH than were the parent phenethylamines. The differences among the stereoisomers are marked. Thus, the solubility and the conformation must be considered for the effect

Table IV—Inhibition of ^{14}C-Histamine Uptake by *N*-Isopropyl-β-hydroxy-β-phenethylamine-like Compounds[a]

Inhibitor	Structure	10^{-4} *M*	10^{-3} *M*
25. 2(e)-Isopropylamino-3(e)-phenyl-3-*trans*-decalol		24.1 ± 1.2 (7)	56.5 ± 0.9 (7)
26. 2(a)-Isopropylamino-3(e)-phenyl-3-*trans*-decalol		22.4 ± 1.1 (7)	56.3 ± 1.3 (7)
27. 2(e)-Isopropylamino-3(a)-phenyl-3-*trans*-decalol		41.2 ± 1.3 (7)	75.2 ± 1.7 (7)
28. 2(a)-Isopropylamino-3(a)-phenyl-3-*trans*-decalol		17.8 ± 1.4 (6)	51.8 ± 1.7 (7)
29. *erythro*-2-(*N*-Isopropylamino)-1-phenylpropanol		27.0 ± 1.1 (8)	52.9 ± 1.5 (11)
30. *threo*-2-(*N*-Isopropylamino)-1-phenylpropanol		24.8 ± 1.7 (8)	56.6 ± 0.8 (11)

[a] For details see Table I.

Table V—Inhibition of ^{14}C-Histamine Uptake by Some Substituted *trans*-Decalins[a]

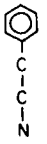
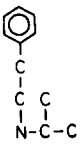
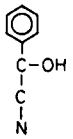
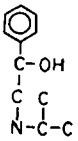
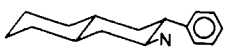
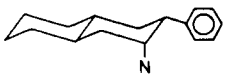
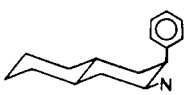
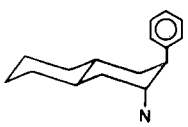
Inhibitor	Structure	10^{-5} *M*	10^{-4} *M*	10^{-3} *M*
31. 3(a)-Amino-*trans*-decalin		14.8 ± 1.4 (6)	30.1 ± 3.2 (6)	60.4 ± 1.7 (3)
32. 3(e)-Amino-*trans*-decalin		15.1 ± 2.6 (5)	42.0 ± 3.1 (6)	76.0 ± 1.7 (5)
33. 3(e)-*trans*-Decalol		—	1.8 ± 1.1 (5)	−3.0 ± 2.6 (5)
34. 3(a)-Phenyl-2(a)-*trans*-decalol		—	2.8 ± 1.1 (5)	16.3 ± 4.9 (7)

[a] For details see Table I.

Table VI—Comparison of Inhibition of ^{14}C-Histamine Uptake with Partition Coefficients

Inhibitor	Partition Coefficient		Inhibition, % at 10^{-4} *M*
	Octanol–Buffer	Heptane–Buffer	
Amphetamine			
9, 10	0.39 ± 0.08	0.051 ± 0.003	51.8 ± 1.5
Butanes			
5. *erythro*	0.59 ± 0.09	0.072 ± 0.005	48.6 ± 2.1
6. *threo*	0.47 ± 0.04	0.040 ± 0.004	35.7 ± 3.1
Hexanes			
7. *cis*	1.51 ± 0.10	0.072 ± 0.022	35.6 ± 1.5
8. *trans*	3.07 ± 0.74	0.25 ± 0.05	33.0 ± 1.8
Decalins			
1. NH_2 = e, C_6H_5 = e	>100	6.75 ± 0.77	49.7 ± 2.5
2. NH_2 = a, C_6H_5 = e	50.9 ± 5.4	10.0 ± 0.55	38.0 ± 2.4
3. NH_2 = e, C_6H_5 = a	5.1 ± 2.0	1.51 ± 0.1	29.1 ± 2.4
4. NH_2 = a, C_6H_5 = a	69.0 ± 10.5	3.49 ± 0.37	38.7 ± 1.8
20. NH_2 = e, C_6H_5 = e	58.6 ± 9.3	—	17.5 ± 1.3
21. NH_2 = a, C_6H_5 = e	>300	—	13.4 ± 3.0
22. NH_2 = e, C_6H_5 = a	∞	—	35.9 ± 3.2
23. NH_2 = a, C_6H_5 = a	47.1 ± 2.1	—	7.6 ± 3.1

Table VII—Summary of Uptake Inhibition by Compounds with Decalin Structure at a Concentration of 10^{-4} *M* (The Structure of the Phenethylamine Moiety is Shown at the Top and the Conformation at the Left Margin; the Most Active Isomer Is Underlined)

				
	<u>49.7</u>	41.8	17.5	24.1
	38.0	32.0	13.4	22.4
	29.1	<u>60.1</u>	<u>35.9</u>	<u>41.2</u>
	38.7	36.9	7.6	17.8
Parent substance (closest choice)	51.8 52.8	31.4 33.2	35.4	24.8 27.0

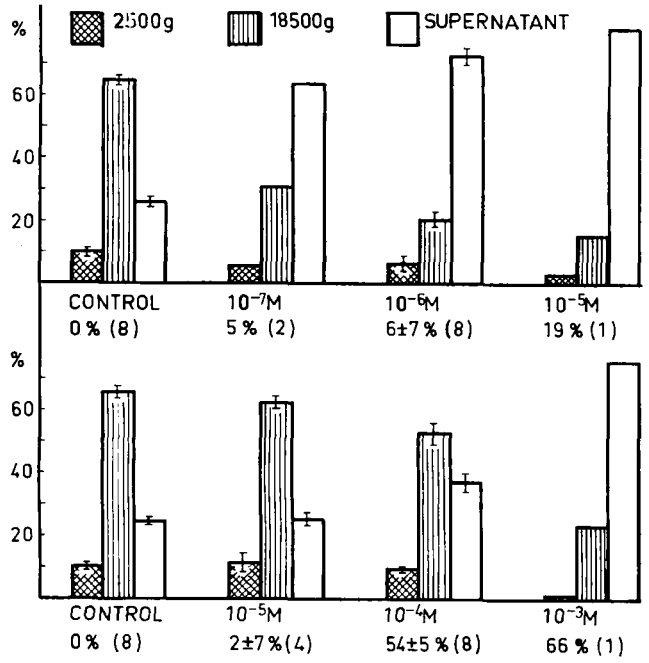


Figure 1—*Intracellular distribution of ^{14}C-histamine in platelets after 80 min of incubation with or without different concentrations of reserpine (upper line) or 2(e)-amino-3(a)-phenyl-3-*trans*-decalol (Compound 22, lower line). Inhibition of uptake in percent is shown below the respective columns. Distribution is given in percent and the sum of three columns in one group is always 100. Values are means ± standard error. Number of experiments is given in the parentheses.*

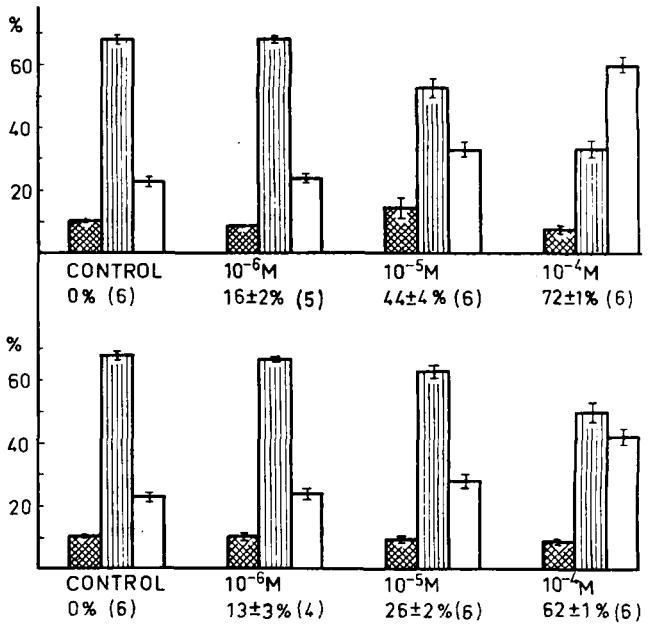


Figure 2—*Intracellular distribution of ^{14}C-histamine in platelets incubated with or without* d-*enantiomer of amphetamine (upper line) or* l-*enantiomer (lower line). Other conditions are as in Fig. 1.*

on the uptake inhibition. Compound 3, which had an exceptionally low inhibitory activity, was less lipid and more water soluble than its isomers.

There are some inherent weaknesses in the present approach. First, analogs of 5-hydroxytryptamine would be the most logical substances to study because the affinity of the parent substance for the platelet carrier is evidently much higher than that of phenethylamines. However, these compounds are difficult to obtain synthetically. Second, as shown by the experiments with 2-amino-*trans*-decalins, even substances without the phenyl ring are moderately effective. Therefore, for each compound there may be two modes of binding to the receptor: one utilizing the phenyl ring and the other the decalin moiety. In those cases where the parent substance is not very active, the "background inhibition" caused by the aminodecalin portion of the analog is so high that the conformational differences are not evident or may be distorted.

Third, the decalin derivatives are very lipid soluble compared with phenethylamines or other biogenic amines. This is probably not crucial in agonist–antagonist studies on the cholinergic or adrenergic receptors, but in uptake inhibition it definitely must be considered, since one is dealing with a transport process through a cell membrane of mainly lipid character. If the inhibitor is very lipid soluble, it may accumulate in the lipid membrane and saturate the carrier to such a degree that it is not available to transport the biogenic amines, which would render the inhibition noncompetitive. This would also cause differences among isomers because the more lipid-soluble isomers tend to be the more active.

Because the affinity of histamine to the carrier seems to be relatively low and the uptake quite slow and because histamine metabolism in plasma might influence the results in the relatively long incubations needed, a full survey of the kinetics of the uptake was impractical. However, some preliminary experiments suggest that the inhibition by decalin derivatives may be noncompetitive. A more detailed study is being performed with ^{14}C-5-hydroxytryptamine as a substrate, incubating it for 1 min with some inhibitors under the conditions described here. The results will be presented separately (16).

Decalin-type inhibitors seemed to cause mainly noncompetitive inhibition. Norepinephrine was clearly competitive and amphetamine mainly competitive, but it appeared to change the V_{max} slightly, possibly indicating some noncompetitive component. This finding could be interpreted as meaning that more lipid-soluble substances tend to cause the noncompetitive type of inhibition. This would also be in agreement with the suggestions (17) that primary binding takes place through the positively charged nitrogen and that the weaker secondary binding might be different in saturated, lipid-soluble hydrocarbons from that of catechol or heterocyclic rings. In the latter case, van der Waals-type forces might be operating; in the first case, hydrophobic binding would be the most probable.

Generally speaking, the results seem to suggest that the decalin derivatives with phenyl and amino groups in *gauche*-conformation are more active (Table VII). In three out of four cases the most active stereoisomer had an axial phenyl and equatorial amino group. In the fourth case the most active was the transoid diequatorial isomer. In all of the aforementioned compounds, the *gauche*-conformation (~60°) between the amino and phenyl groups existed.

Of the β-methylamphetamines, the *erythro*-form was more effective than the *threo*-form. The *N*-isopropyl-β-methylamphetamines gave no significant difference between *threo*- and *erythro*-forms. In the cyclohexane derivatives, no significant difference was observed between the *cis*- and *trans*-forms.

Other studies suggest that substances having phenyl and amino groups held rigidly in a *gauche*-conformation are effectively transported. Debrisoquin is effectively taken up by the platelets and, although it is probably not stored by the platelet granules, a concentration gradient of about 20 is found between the platelet and the incubation medium (18). It also inhibits 5-hydroxytryptamine uptake competitively (19). In preliminary studies[2], a number of tetrahydroisoquinoline derivatives inhibited histamine uptake; harmaline, which has an indole ring instead of a phenyl ring, was even more effective. This may be related to the higher activity of 5-hydroxytryptamine. However, these experiments did not give clear enough results to exclude the activity of *anti*-conformations. If *anti*-compounds are also transported, the carrier must have a flexible binding site to accommodate both *gauche*- and *anti*-conformations, which may have as much as 1.5 Å difference in the distance of the nitrogen from the aromatic ring.

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§ Present address: Department of Pharmacology, University of Helsinki, Helsinki, Finland.

† Deceased.

ˣ To whom inquiries should be directed. Present address: Department of Pharmacology, University of Kansas Medical Center, Kansas City, KS 66103

Decalin Analogs of Phenethylamines as Inhibitors of Amine Uptake by Rabbit Platelets II: Uptake of 5-Hydroxytryptamine

JOUKO TUOMISTO *§, EDWARD J. WALASZEK *, EDWARD E. SMISSMAN ‡†, and THOMAS L. PAZDERNIK ‡ˣ

Abstract □ The steric aspects of the uptake of 5-hydroxytryptamine by rabbit platelets were studied utilizing the conformationally rigid decalin analogs of ephedrine and amphetamine. These analogs or their parent compounds were added to platelet-rich plasma, and their influence on the uptake of ^{14}C-5-hydroxytryptamine was determined along with the kinetics of the process. Among the β-phenethanolamine-type compounds, the isomer having an axial phenyl and equatorial amino function was the most effective inhibitor and the isomer in which the phenyl and amino functions were both axial was the weakest inhibitor of uptake. In the β-phenethylamine series, the same cisoid isomer [(a) phenyl, (e) NH_2] was the weakest inhibitor and the remaining three isomers were equipotent. The most effective cisoid decalin isomer of phenethanolamine displayed a mixed type of inhibition. The inhibition by amphetamine or β-phenethanolamine was also of a mixed type but was closer to competitive than to noncompetitive inhibition. Norepinephrine was a competitive inhibitor. The differences were suggested to be due to different binding of the aromatic ring. It was also suggested that lipid solubility causes the decalin derivatives to accumulate in the cell membrane and bind part of the carrier so that it is inaccessible to the substrate. Different lipid solubility would then explain the inconsistency of the most active conformation in different series. In essence, these results are in agreement with the inhibition of histamine uptake by rabbit platelets, which was earlier studied using a number of rigid and semirigid phenethylamine derivatives.

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Experiments concerning stereochemical aspects of the inhibition of histamine uptake by platelets have been reported from this laboratory (1). Several amines which were conformationally rigid substituted *trans*-decalins were studied. In general, a cisoid conformation in which the phenyl group is axial and the amine group is equatorial appeared to be more favorable for uptake inhibition. In three out of four cases, the most effective isomer had an axial phenyl and an equatorial amino group. Among β-phenethylamine-like decalin isomers, the axial phenyl and equatorial amino isomer was the least active; the most active had a diaxial structure.

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To determine whether these findings were due to

secondary factors, such as effects on histamine metabolism during the long incubation, it was decided to utilize another substrate, 5-hydroxytryptamine. 5-Hydroxytryptamine is taken up rapidly, thus making it possible to use a very short incubation period which would minimize the effects of metabolism. A study of the kinetics of uptake is more facile with 5-hydroxytryptamine as compared to histamine because the active uptake is rapid, the saturation concentration is much sharper, and the role of diffusion is less than in the long incubations with histamine. Therefore, the information obtained as to competitiveness or noncompetitiveness of uptake inhibition is more reliable.

EXPERIMENTAL

Platelet-rich plasma of male rabbits, 2.2–3.0 kg, was prepared as described earlier (2). It contained 522,000–1,100,000 platelets/mm^3. The substances to be tested were pipetted into polypropylene centrifuge tubes in 0.2 ml of saline or 3% dimethyl sulfoxide in saline. Then, ^{14}C-5-hydroxytryptamine was added to a pool of ice-cold plasma to give the final concentration of 10^{-7} *M*. Two-milliliter plasma samples were rapidly added to the polypropylene tubes and incubated at 37° for 5 min. The incubation was terminated by placing the tubes in an ice bath, after which they were centrifuged for 30 min at 2000×*g*. The pellet was washed with 2 ml of saline and centrifuged again. The supernate was discarded, the tube was dried of excess saline with filter paper, and the pellet was solubilized[1]. The total radioactivity was counted[2] with a scintillation fluid containing 0.4% diphenyloxazole and 0.01% 1,4-bis(5-phenyloxazol-2-yl)benzene in toluene.

To study the reaction kinetics, a 1-min incubation period was used to represent the initial velocity of ^{14}C-5-hydroxytryptamine (10^{-7} *M*) and a necessary amount of cold carrier was used. The incubation in these experiments was terminated by adding 5 ml of ice-cold saline and centrifuging the tubes immediately for 5 min at 20,000×*g*. The pellet was washed with 2 ml of ice-cold saline, solubilized, and counted.

The noncommercial decalin derivatives and 2-amino-1-phenethanol were synthesized[3] as described earlier (3, 4). Dextroamphetamine sulfate[4], 5-hydroxytryptamine creatinine sulfate[4], β-phenethylamine hydrochloride[5], 5-hydroxytryptamine-3-^{14}C creatinine sulfate[6], and the scintillation chemicals[7] were obtained from commercial sources.

For calculating the reaction kinetics, Lineweaver–Burk (5) and Eadie–Hofstee (6, 7) plots were used. The standard error of the mean was calculated where necessary.

RESULTS AND DISCUSSION

Rate of Uptake—5-Hydroxytryptamine uptake under the conditions described was extremely rapid (Fig. 1). Depending on the platelet count in plasma, around 40% of the added total amount of ^{14}C-5-hydroxytryptamine was taken up during the 1st min, after which the rate decreased to give an uptake of 80% in the first 10 min. When the inhibitor was present, the rate was affected to the greatest extent at the start, with the uptake continuing for a longer time before reaching a plateau; thus the inhibition calculated after a 10-min incubation was very slight.

Inhibition of Uptake—All β-hydroxy-β-phenethylamine-like compounds inhibited 5-hydroxytryptamine uptake, and the most effective was the decalol derivative with an axial phenyl and equatorial amino group (Compound 3, Table I). The transoid isomer (Compound 4) was the weakest. All isomers except Compound 4 were more effective than β-hydroxy-β-phenethylamine itself.

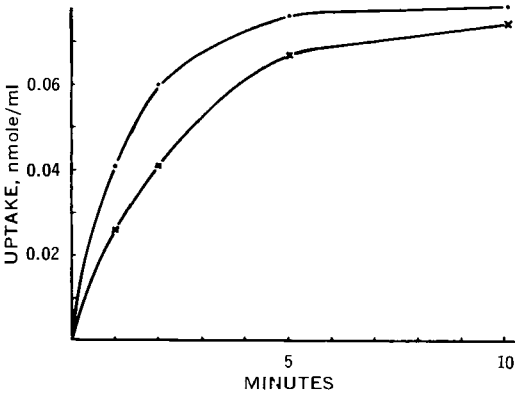


Figure 1—*Time course of 5-hydroxytryptamine uptake by rabbit platelets incubated in plasma. Added ^{14}C-5-hydroxytryptamine concentration was 10^{-7} M, or 0.1 nmole/ml. The result is given as nanomoles taken up by platelets in the same volume. Upper curve is without an inhibitor. Lower curve is with 10^{-4} M 2(e)-amino-3(a)-phenyl-3-*trans*-decalol. (Mean of two duplicate virtually identical results.)*

Among β-phenethylamine-like compounds, the isomer with the same conformation as Compound 3 was the least active and about equal to the parent compound. All other compounds, two *gauche* and one *anti*, were equipotent.

Reaction Kinetics—When 1-min incubations were used to indicate the initial velocity and accumulation was plotted against 5-hydroxytryptamine concentration, a two-phase curve was obtained. At low concentrations, up to about 10^{-6} *M*, there appeared to be a saturable process; in high concentrations (from about 10^{-6} *M* to at least 10^{-4} *M*), a linear process existed (Fig. 2). The latter was interpreted to be due to diffusion of 5-hydroxytryptamine into the cells. The linear part of the curve was extrapolated to the ordi-

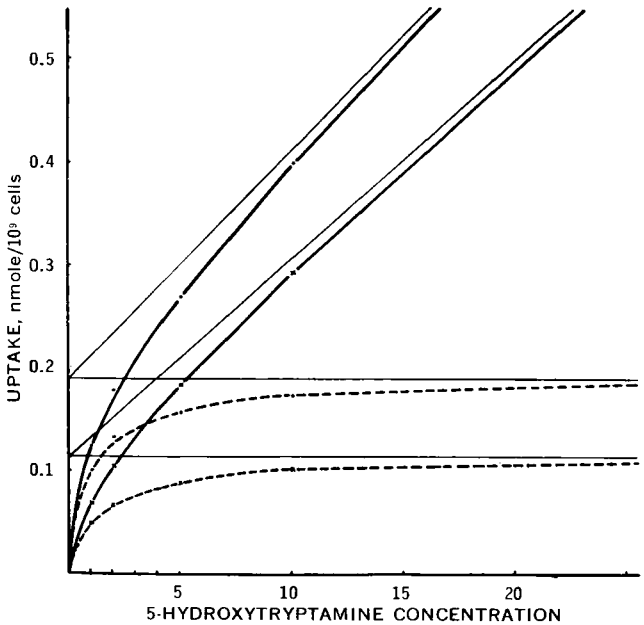
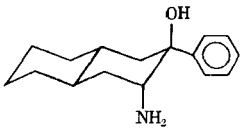
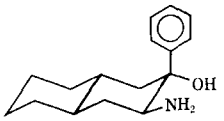
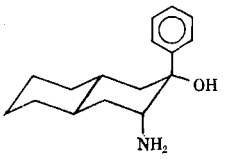
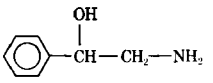
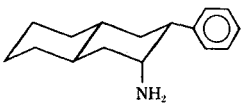
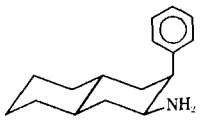
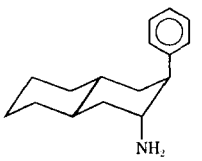
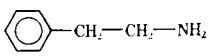


Figure 2—*Uptake of 5-hydroxytryptamine plotted against 5-hydroxytryptamine concentration. The thick solid lines represent the total uptake, the upper line without inhibitor and the lower line with 10^{-4} M 2(e)-amino-3(a)-phenyl-3-*trans*-decalol. The curves continued linear up to 10^{-4} M 5-hydroxytryptamine concentration, the highest concentration tested. The thin angles confine the linear part of uptake, which then is subtracted from the total, and the broken lines show the saturable part supposedly representing the active uptake without or with the inhibitor, respectively. The uptake is given per 10^9 platelets. (Mean of four duplicate experiments.)*

[1] Soluene 100.
[2] Packard Tri-Carb scintillation counter.
[3] Department of Medicinal Chemistry, University of Kansas.
[4] Sigma Chemical Co.
[5] Calbiochem.
[6] Amersham/Searle.
[7] Packard Instrument Co.

Table I—Inhibition of ^{14}C-5-Hydroxytryptamine Uptake in Percent by Some Conformationally Rigid Amino-*trans*-Decalins[a]

Inhibitor	Structure	Inhibition, %
1. 2(e)-Amino-3(e)-phenyl-3-*trans*-decalol		26.0 ± 2.3
2. 2(a)-Amino-3(e)-phenyl-3-*trans*-decalol		23.0 ± 2.9
3. 2(e)-Amino-3(a)-phenyl-3-*trans*-decalol		39.8 ± 3.1
4. 2(a)-Amino-3(a)-phenyl-3-*trans*-decalol		11.1 ± 3.4
5. β-Hydroxy-β-phenethylamine		16.0 ± 4.0
6. 2(e)-Amino-3(e)-phenyl-*trans*-decalin		58.5 ± 4.4
7. 2(a)-Amino-3(e)-phenyl-*trans*-decalin		58.4 ± 6.8
8. 2(e)-Amino-3(a)-phenyl-*trans*-decalin		25.3 ± 6.7
9. 2(a)-Amino-3(a)-phenyl-*trans*-decalin		60.8 ± 4.4
10. β-Phenethylamine		23.8 ± 8.7

[a] Rabbit blood platelets were incubated for 5 min at 37°. Values are means ± standard error of five experiments. Inhibitor concentration was 10^{-4} *M*.

nate, and this line was used for calculating the diffusion at different concentrations. The calculated diffusion was then subtracted from the total accumulation; as a result, a typical saturation curve was obtained (Fig. 2).

These new curves were used for Lineweaver–Burk and Eadie–Hofstee plots (Fig. 3) to determine the reaction kinetics. Without an inhibitor, both plots gave a V_{max} of 0.18 nmole/10^9 platelets/min and K_m of 0.85×10^{-7} *M* when the results were corrected to the platelet count of 10^9 platelets/ml plasma. With 10^{-4} *M* 2(e)-amino-3(a)-phenyl-3-*trans*-decalol (Compound 3), the V_{max} was 0.11 nmole/10^9 platelets/min and K_m was 1.4×10^{-7} *M*. So there was a change both in V_{max} and in K_m, indicating a mixed type of inhibition. On the other hand, norepinephrine under similar conditions showed competitive kinetics with no change in V_{max} (Fig. 4). Amphetamine (Fig. 5) and β-hydroxy-β-phenethylamine (Fig. 6) both showed a mixed type of kinetics but with less change in V_{max} than in K_m. Thus, they appear to be closer to competitive inhibitors than the decalin derivative.

The V_{max} and K_m values of the first series of experiments were lower than the others (compare Figs. 3 and 4–6, respectively). This finding is probably due to the use of a thicker tube in the first experiments, which caused a slower rise in temperature when the tubes were placed into the water bath; thus the actual incubation time was shorter than in the later experiments. (The reason for change of tubes was the purchase of a new centrifuge with slightly different tubes.)

The two series of decalin derivatives gave almost identical results for 5-hydroxytryptamine uptake compared with histamine uptake by platelets. Therefore, it can be assumed that the effect is on the uptake process itself and not on amine metabolism.

The noncompetitive component of uptake inhibition may mean that these compounds bind differently to the amine carrier compared with the substrate. However, they might also bind similarly, if it is assumed that their high lipid solubility causes them to accumulate on the cell membrane. If the freely moving receptor of the carrier were occupied by the excess decalin derivative inside the membrane so that it could not attract the polar substrate molecules outside the membrane in the incubation medium, the inhibition would evidently be partly noncompetitive regardless of the actual mode of binding.

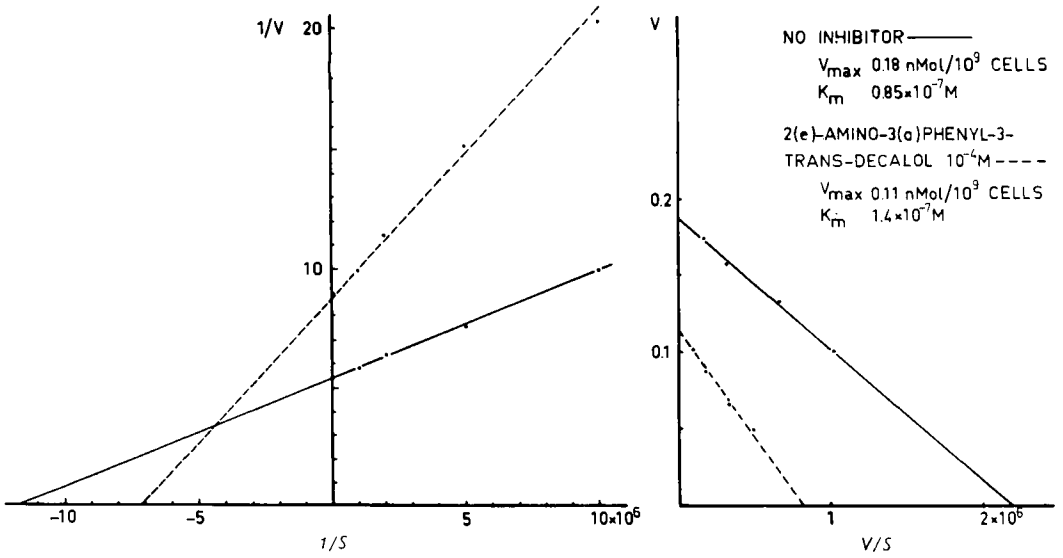


Figure 3—*Lineweaver–Burk plot (left) and Eadie–Hofstee plot (right) drawn on the basis of the saturable process of the same material as in Fig. 2.*

On the other hand, experiments with various amines inhibiting histamine uptake suggest that binding may be different. Heterocyclic amines might bind by ionic forces and van der Waals forces, but aliphatic amines may bind by ionic forces and hydrophobic forces (8). The saturated decalin structure would evidently then belong to the last category. The decalin derivatives of amines might thus have two possible ways of binding, one using the phenethylamine moiety and the other using the amino-*trans*-decalin moiety.

Since the suggested ionic binding through the amino nitrogen appears to be by far the most important mode of binding, it is possible that the amine function alone would be enough to inhibit uptake if the concentration is high enough. Thus, it may not be necessary that the secondary binding site, *i.e.*, the suggested hydrophobic site, be present in the carrier molecule. If this is the case, the results with highly lipid-soluble uptake inhibitors, such as the decalin derivatives, do not give a reliable indication of conformational preferences. Therefore, the differences in lipid solubility and other physicochemical properties could be the most important aspects of the activity. This would explain the inconsistency of the most effective conformation in different series of decalin derivatives as histamine uptake inhibitors (1) and with 5-hydroxytryptamine uptake inhibitors as shown in this study. The possibility of one-point inhibition is favored by the earlier finding that plain amino-*trans*-decalin is only a slightly weaker inhibitor than the 2-amino-3-phenyl-*trans*-decalins (1). Therefore, the presence of a phenyl group increases the potency but it is not necessary for activity and its influence is small enough that in some cases it may be negated by opposing factors.

It is necessary to incubate for a very short period to obtain a reliable initial velocity, and it is absolutely necessary to subtract the linear part of the uptake. If these steps are omitted, even results with decalin derivatives may show an apparently competitive type of inhibition, at least in Lineweaver–Burk plots. If the concentration is too high and/or incubation prolonged, one is dealing with a complex situation where inhibition on the outer membrane, metabolism of excess amine by mitochondrial monoamine oxidase, and inhibition at the granular level all have their own role. In these circumstances, one might have several zero-order and first-order processes taking place at the same time. Actually an incubation time of less than 1 min would be preferable, but using this technique it would not be possible to maintain the incubation time constant in different tubes.

In most early studies, amphetamine has been considered a purely competitive inhibitor. It would be important to know if this is due to a longer incubation time and/or to the higher concentrations used. If one accepts the model of binding suggested earlier (8), the differences among the amphetamines and the catecholamines or heterocyclic amines could be explained since the benzyl ring of amphetamine would bind in part through hydrophobic forces, in contrast to pure van der Waals-type binding of catechol, indole, or imidazole rings.

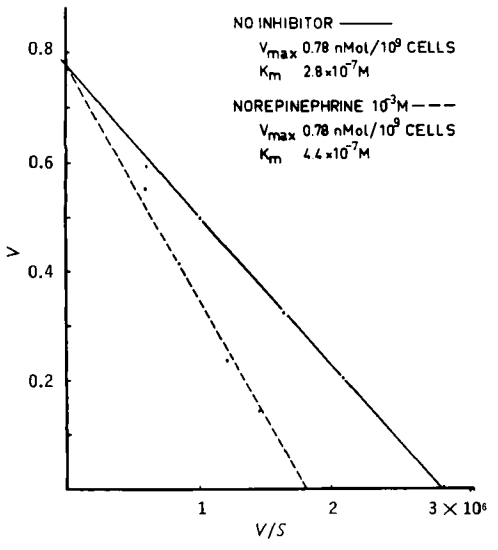


Figure 4—*Kinetics of 5-hydroxytryptamine uptake with norepinephrine as an inhibitor; Eadie–Hofstee plot according to the same principles as in Fig. 3. (Mean of four duplicate experiments.)*

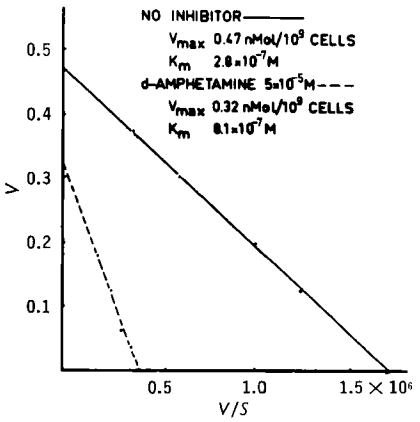


Figure 5—*Kinetics of 5-hydroxytryptamine uptake with dextroamphetamine as an inhibitor. (Mean of four duplicate experiments.)*

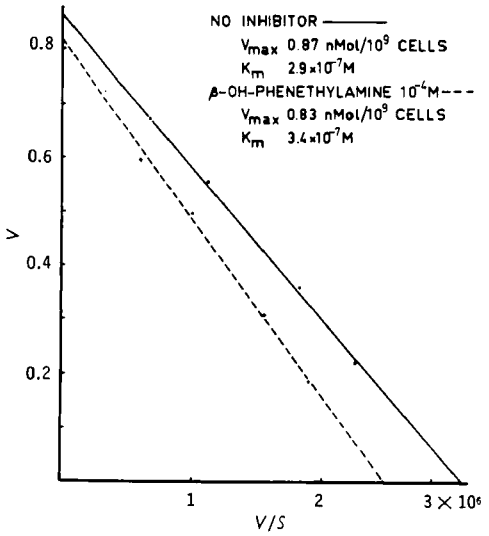


Figure 6—*Kinetics of 5-hydroxytryptamine uptake with β-hydroxy-β-phenethylamine as an inhibitor. (Mean of four duplicate experiments.)*

In conclusion, it is felt at this phase of the investigation that partly noncompetitive uptake inhibitors with large nonpolar groups may attach to the transport carrier in a relatively nonspecific manner and, in some cases, only through the amino group, which seems to be the only absolute requirement for the inhibition. The relatively high activity in the cases studied might be explained by the accumulation of the inhibitor in high concentrations at the cell membrane. To prove conclusively that one conformation of phenethylamine-type inhibitors is actually the most effective, relatively small molecular weight compounds that possess a lipid solubility not too different from the parent compound should be investigated.

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[§] Present address: Department of Pharmacology, University of Helsinki, Helsinki, Finland.

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Distribution of ^{14}C-Lomustine (^{14}C-CCNU)-Derived Radioactivity following Intravenous Administration of Three Potential Clinical Formulations to Rabbits

CHARLES L. LITTERST *x, EDWARD G. MIMNAUGH *, ALAN C. COWLES [‡], THEODORE E. GRAM *, and ANTHONY M. GUARINO *

Abstract □ ^{14}C-Lomustine (CCNU) was administered intravenously to rabbits in three potential clinically useful vehicles of propylene glycol–ethanol (4:1), fat emulsion, and vegetable oil emulsion; the organ distribution of the radioactivity was followed for 24 hr. Bile and fat were the only tissues showing a consistent vehicle-dependent change in the distribution of radioactivity, and in these two instances the fat emulsion and the vegetable oil emulsion both produced the same distribution pattern. Liver, kidney, and lung tissue consistently contained the highest amounts of radioactivity. By 12 hr after dosing, essentially all radioactivity had been eliminated by the animals. Binding of radioactive drug to plasma proteins was found to be twice as great *in vitro* (93%) as *in vivo* (51%).

Keyphrases □ Lomustine, radiolabeled—distribution of radioactivity, three vehicles tested, intravenous administration, rabbits □ 1-(2-Chloroethyl)-3-(cyclohexyl-1-^{14}C)-1-nitrosourea (lomustine)—distribution of radioactivity, three vehicles compared, intravenous administration, rabbits □ Antineoplastic agents—radiolabeled lomustine, effect of three vehicles on distribution of radioactivity, intravenous administration, rabbits

Lomustine, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU, I), is a potent antineoplastic drug utilized in the treatment of Hodgkins disease and other solid tumors (1). Although it shares the nitrosourea structure with several other drugs, I differs from other nitrosoureas in having no satisfactory parenteral dose formulation. In experimental animals and humans, I has been administered in propylene glycol–

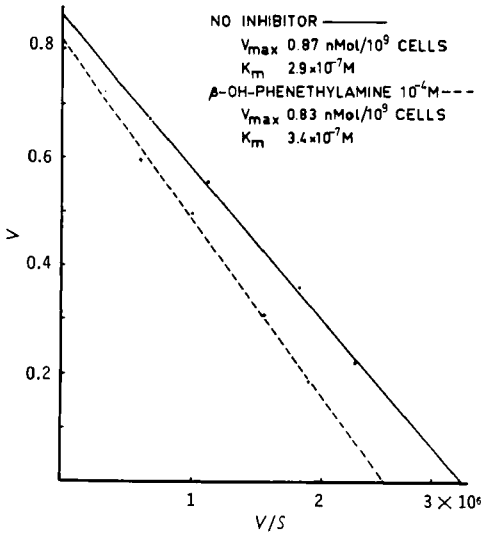

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Table I—Tissue–Blood Ratio of Radioactivity in Tissues Containing More Drug-Derived Radioactivity than Was Present in Blood

Tissue	Vehicle[a]	Hours 0.2	1	4	12	24
Liver	C	1.86	2.78	2.04	1.00	0.86
	B	1.77	2.51	4.31	0.91	1.07
	A	2.00	2.63	3.67	0.91	0.76
Lungs	C	1.42	1.37	1.20	0.67	0.64
	B	1.36	1.17	1.25	0.64	0.53
	A	1.41	1.39	1.36	0.73	0.44
Kidneys	C	3.37	3.51	2.56	2.44	1.43
	B	3.18	3.80	3.44	1.41	1.20
	A	4.01	3.44	2.64	1.50	0.88
Adrenal	C	1.65	0.98	0.67	0.44	0.50
	B	1.54	0.84	0.81	0.68	0.47
	A	1.58	0.73	0.69	0.64	0.44
Mucosa	C	1.27	0.92	1.42	0.63	0.64
	B	1.24	1.12	1.53	0.82	0.60
	A	1.27	1.09	1.83	0.64	0.36
Bile	C	1.50	13.56	12.82	5.44	1.36
	B	1.55	10.28	8.50	18.23	0.73
	A	3.35	30.33	23.53	20.45	6.08
Serosa	C	0.86	0.91	1.42	0.52	0.36
	B	0.89	0.86	1.00	0.54	0.53
	A	0.86	0.91	1.11	0.54	0.36
Fat	C	0.64	1.22	1.33	1.30	0.36
	B	0.63	1.15	1.03	0.82	0.34
	A	0.51	0.58	0.67	0.68	0.36
Urine	C	13.18	26.11	176.67	69.82	37.28
	B	3.51	793.88	183.56	159.86	28.00
	A	2.65	238.16	229.95	102.26	61.71
Absolute Amounts of Recovered Drug Equivalents in Blood, μg/ml						
	Vehicle A	1.56 ± 0.03	0.79 ± 0.06	0.36 ± 0.03	0.22 ± 0.04	0.25 ± 0.04
	Vehicle B	1.80 ± 0.33	0.81 ± 0.07	0.36 ± 0.05	0.22 ± 0.04	0.15 ± 0.04
	Vehicle C	1.68 ± 0.05	0.87 ± 0.14	0.45 ± 0.06	0.27 ± 0.07	0.14 ± 0.04

[a] A = propylene glycol–ethanol (4:1); B = fat emulsion (Intralipid)–ethanol (9:1); and C = vegetable oil emulsion (Emulphor)–ethanol–0.85% NaCl (1:1:18).

ethanol (4:1); in humans it is given either orally or intravenously utilizing a fat emulsion[1]. The effectiveness of the oral formulation of I is limited by the drug-related toxicity seen at therapeutic doses, and the utility of the emulsion is hampered by such a short stability that the drug must be prepared for administration at the bedside using a cumbersome mixing procedure.

Recently, Davignon *et al.* (2) experimented with a polyethoxylated vegetable oil emulsifier[2] as a possible vehicle for parenteral administration of nitrosoureas in humans. Preliminary studies in humans utilizing this vehicle are already commencing[3], based in part on results reported here on the similarity of drug distribution between the vegetable oil emulsifying agent[2] and traditional clinical vehicles. The present investigation was conducted to provide information concerning the distribution of radioactivity derived from ^{14}C-I after intravenous administration of the drug to rabbits in each of three vehicles.

EXPERIMENTAL

Drug—^{14}C-I was labeled in the 1-position of the cyclohexyl ring[4] (specific activity 12.626 mCi/mmole). The radiochemical purity of the drug was in excess of 99%, and the drug was chemically pure. The drug was made up weekly in absolute ethanol at a concentration of 0.37 mg/ml (54.05 μCi/mg) and stored at −30° until use. In solution, I has been shown (2) to be stable for 7 days at 0°. On the day of the experiment, 0.5 ml of the ethanol solution was mixed with 9.8 mg of nonradioactive I and diluted to 10 ml with one of three vehicles: Vehicle A, ethanol–propylene glycol (1:4 v/v); Vehicle B, ethanol–fat emulsion[5] (1:9 v/v); and Vehicle C, ethanol–polyethoxylated vegetable oil[6]–0.85% NaCl (1:1:18 v/v).

Animal Handling—Compound I was administered to male New Zealand rabbits, 1.4–1.9 kg, through a marginal ear vein at a dose of 1 mg/kg; this dose approximates the clinical dosage of this drug. Each milligram of drug contained 1 μCi of radioactivity, and 1 ml of the drug solution was administered/kg of body weight. At 10 min or 1, 4, 12, or 24 hr after injection, the rabbits were killed and the organ distribution of radioactivity was determined. Five rabbits received I dissolved in each vehicle at each time point.

Approximately 2 min prior to sacrifice, blood was drawn from the heart, using ethylenediaminetetraacetic acid-coated evacuated blood collection tubes[7]. The animal then was killed by the injection of a 30-ml bolus of air into the marginal ear vein contralateral to that used for drug injection. The following tissues were removed: testes, kidneys, adrenals, perirenal fat, spleen, stomach, small intestine, liver, suprasternal muscle, lungs, heart, brain, and lumbar spinal cord. An incision was made along the lesser curvature of the stomach and the organ was everted and washed free of food. The contents of the small intestine were manually expressed, a 20–25-cm segment of jejunum was incised, and the mucosa was removed using a glass microscope slide. In addition to the mucosa,

[1] Intralipid, Vitrum Corp., Stockholm, Sweden.
[2] Emulphor, GAF Corp., New York, N.Y.
[3] M. D. Walker, Baltimore Cancer Research Center, National Cancer Institute, Baltimore, Md., personal communication.
[4] Synthesized by Monsanto Chemical Co. under Contract NIH-NCI-72-3715 and provided by Dr. R. Engle, National Cancer Institute.
[5] Fat emulsion containing, in water, soybean oil (10% w/v), glycerol (2.25% w/v), and egg yolk phospholipid (1.2% w/v); Vitrum Corp., Stockholm, Sweden.
[6] Polyethoxylated vegetable oil; Emulphor, EL 620, GAF Corp, New York, N.Y.
[7] Vacutainer, Becton-Dickinson Co., Rutherford, N.J.

Table II—Tissue–Blood Ratio[a] of Radioactivity in Tissues Containing Less Drug-Derived Radioactivity than Was Present in Blood

Tissue	Vehicle[b]	Hours 0.2	1	4	12	24
Heart	C	0.68	0.72	0.58	0.37	0.29
	B	0.62	0.62	0.78	0.45	0.53
	A	0.65	0.66	0.28	0.32	0.28
Spleen	C	0.65	0.70	0.73	0.59	0.50
	B	0.52	0.60	0.75	0.41	0.33
	A	0.53	0.59	0.61	0.45	0.28
Testes	C	0.67	0.77	0.73	0.56	0.50
	B	0.56	0.72	0.78	0.54	0.60
	A	0.68	0.73	0.69	0.59	0.44
Brain	C	0.40	0.46	0.51	0.37	0.21
	B	0.38	0.52	0.67	0.36	0.33
	A	0.42	0.52	0.61	0.45	0.16
Spinal cord	C	0.51	0.44	0.47	0.41	0.29
	B	0.48	0.33	0.58	0.41	0.20
	A	0.51	0.38	0.53	0.45	0.20
Muscle	C	0.54	0.52	0.58	0.41	0.21
	B	0.46	0.58	0.69	0.32	0.53
	A	0.46	0.57	0.56	0.45	0.28
Stomach	C	0.58	0.63	0.73	0.41	0.43
	B	0.46	0.56	0.78	0.36	0.53
	A	0.57	0.68	0.92	0.50	0.28
Small intestine	C	0.81	0.99	0.93	0.63	0.57
	B	0.59	1.00	1.28	0.73	0.40
	A	0.89	0.99	1.06	0.68	0.36

[a] Absolute amounts of drug products per milliliter of blood are the same as listed in Table I. [b] See Footnote *a* in Table I for vehicle identification.

the underlying submucosal–serosal portion of the intestine also was assayed. Each organ was weighed and a random sample was analyzed for radioactivity.

Urine and bile were collected from their respective bladders at the time of sacrifice. For time points longer than 4 hr, passed urine was collected and measured and the catch pans were rinsed. Rinses and urine were pooled prior to counting and calculations were based on the original volume of urine. Although determined separately, bladder urine data were combined with passed urine data for presentation in Table I.

Analysis of Radioactivity—Tissue samples (90–150 mg) were placed in glass scintillation vials containing 2 ml of a tissue solubilizer[8]. The tissue was minced with scissors and the capped vials were placed in a shaking water bath at 38° until the tissues dissolved. Vials were cooled to room temperature; then 18 ml of a toluene-based scintillation solution containing 4.2 mg/ml of 2,5-diphenyloxazole and 52.5 mg/ml of 1,4-bis[2-(5-phenyloxazolyl)]benzene was added and the radioactivity was determined[9]. Background disintegrations per minute were subtracted from each sample prior to any mathematical manipulation. The data were punched on cards and calculations were done by computer[10]. Differences between the two experimental vehicles and the clinically used propylene glycol vehicle were determined by a two-tailed *t* test with a level of significance of $p \leq 0.05$.

Plasma Binding—*In Vivo*—^{14}C-I in each vehicle was administered intravenously to four rabbits; 30–40 ml of blood was drawn by cardiac puncture 30 min later. The plasma was separated by centrifugation and a 3-ml aliquot of plasma was placed in an ultrafiltration membrane cone[11] and centrifuged at 1000×*g* for 30 min as described previously (3). The whole plasma and the resulting ultrafiltrate were analyzed for radioactivity as described for the other rabbit tissues.

In Vitro—Blood was drawn from an untreated rabbit and the plasma was separated. Then 8.3 μg of ^{14}C-I was added per milliliter of plasma, and the protein binding was analyzed as described. A range (4.0–8.3 μg/ml) of concentrations was studied *in vitro* with equivalent results; because of the associated better counting statistics, the higher values are reported here. The *in vitro* binding was determined on four replicate samples for each vehicle.

[8] NCS, Amersham Searle Corp., Arlington Heights, Ill.
[9] Radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation spectrometer.
[10] FORTRAN IV program run on an IBM 370 computer.
[11] Centriflo, Amicon Corp., Lexington, Mass.

Table III—Binding of Radioactivity to Plasma Protein of Rabbits following Intravenous Administration of ^{14}C-I in Three Vehicles[a]

Vehicle[b]	Percent Bound *In Vitro*	*In Vivo*
A	92.3 ± 1.2	52.9 ± 7.4
B	93.9 ± 1.1	51.7 ± 2.1
C	93.5 ± 1.4	49.7 ± 2.0

[a] Data are means ± standard deviation of radioactivity bound to proteins for four replicates where the drug was added *in vitro* or administered *in vivo* as described under *Experimental*. [b] See Footnote *a* in Table I for vehicle identification.

RESULTS

Vehicle Influence on Disposition of Radioactivity following Injection of ^{14}C-I—In general, there were no marked differences in the disposition of radioactivity from ^{14}C-I as a function of the vehicle employed (Tables I and II). Although statistically significant differences were noted at isolated time points in various organs, there were, with two notable exceptions, no consistent differences in tissue levels of drug-derived radioactivity among Vehicles A, B, and C.

The exceptions to this generalization were substernal fat and bile. The biliary excretion of radioactivity was significantly greater with Vehicle A than for either emulsion. In addition, the two emulsions partitioned radioactivity more readily into fat than did Vehicle A. The explanation for these differences in distribution is obscure, but an alteration in disposition of metabolites is unlikely because tissues such as brain and spinal cord, which are essentially lipid depots, showed no change in distribution.

Organ Distribution of Radioactivity following ^{14}C-I Administration—When considered in terms of tissue–blood ratios, it is clear that only the organs of excretion (liver, kidneys, and lungs) concentrated radioactivity to any appreciable extent after intravenous administration of ^{14}C-I (Table I). In addition, at selected times after dosing, the adrenal glands and the mucosal surface of the small intestine also showed amounts of drug-derived radioactivity that were appreciably greater than the radioactivity found in the blood. The reason for the great difference in amounts of drug products localized in mucosal as opposed to serosal surfaces of the small intestine is unknown but may reflect relative blood flow because radioactivity in the serosal portion reflects radioactivity seen in muscle (Table II). The radioactivity in heart, spleen, adrenals, testes, brain, spinal cord, fat, muscle, and gut did not differ appreciably from blood at most time points.

The organs accounting for the majority of the radioactivity were those having large concentrations of radioactivity (*e.g.*, kidneys), those of large mass but small concentration of radioactivity (*e.g.*, small intestine), and those with a large concentration of radioactivity and a large mass (*e.g.*, liver). The largest amounts of radioactivity were found mainly in organs associated with the excretion of I and its biotransformation products.

Binding of ^{14}C-I to Plasma Proteins—In addition to determining the tissue distribution of radioactivity from I, the plasma binding of radioactivity derived from parent and biotransformation products was studied (Table III). Although all three vehicles gave identical results both *in vivo* and *in vitro*, the *in vivo* binding results differed considerably from the *in vitro* binding. When ^{14}C-I was added to plasma *in vitro*, only 7% of the radioactivity was recovered in the ultrafiltrate, indicating that the label was 93% bound to plasma proteins. When the drug was administered to rabbits and the binding was determined in the resulting plasma, 49% of the radioactivity was recovered in the ultrafiltrate, indicating that 51% of the label was bound in the plasma.

DISCUSSION

Relative to Vehicle A, neither Vehicle B nor Vehicle C appreciа-

bly altered the distribution pattern of the nitrosourea-derived radioactivity. In the two instances where differences were noted between Vehicle A and the emulsifiers, both emulsifiers produced similar results. Because Vehicle A is not preferred clinically, the fact that Vehicles B and C produced equivalent distribution patterns for the radioactivity should be encouraging to those interested in the practical usefulness of Vehicle C as a parenteral formulation for nitrosoureas and other lipid-soluble drugs.

Other investigators (4, 5) showed that urinary excretion is a major route by which biotransformation products of I and other nitrosoureas are cleared from the body in mice, monkeys, and humans, and this is confirmed by the finding of large tissue–blood ratios of radioactivity in the kidneys and urine of rabbits, especially during the first 4 hr after drug administration. Moreover, relatively high concentrations of the isotope in bile (Table I) indicate that this clearance route is of significance in the rabbit. Similar observations have been made for other species (6).

Knowledge of the tissue concentrations of antitumor drugs might be used to help explain specific organ toxicity or unique tissue uptake by certain organs and to suggest hitherto unconsidered therapeutic implications. Thus, the high concentrations of drug-derived radioactivity in the lungs support the use of I in the treatment of lung neoplasms (7, 8). The high levels of radioactivity recovered in the liver and kidneys correlate well with organ toxicity data for nitrosoureas. Thus, Oliverio (9) reported hepatic and renal damage in dogs after a single oral dose of I, and Thompson and Larson (10) showed liver damage in rats following a single oral dose of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, II). In addition, Lu and Larson (11) showed a decrease in hepatic microsomal metabolism of pentobarbital in male rats following intraperitoneal administration of II.

The chemical instability of the nitrosourea antitumor drugs as a class has been abundantly documented (5, 12, 13), and numerous studies have established that these agents are orally effective in the treatment of certain kinds of human neoplastic disease (14, 15). For example, a recent investigation (5) considered the disposition of I and its methyl analog in humans following their oral administration and found that with neither of these agents could unchanged parent drug be detected in the plasma at any time after drug administration. These studies imply that unchanged I *per se* is not the active carcinostatic chemical species (5) but that this activity may reside in one or more of its metabolites and/or degradation products. Unfortunately, I is known to give rise to numerous metabolic or degradation products, and the active carcinostatic species has not yet been identified (5). For these reasons, the data from this study are reported in terms of the tissue–blood ratio of total radioactivity, and an attempt was not made to differentiate this radioactivity into unchanged I and a number of chemically distinct fractions.

The results reported here for protein binding of I-derived radioactivity agree well with other results from nitrosoureas. Loo *et al.* (13) reported that II was bound to the extent of 80% to proteins from human plasma *in vitro*, while Oliverio *et al.* (6) estimated an initial binding of I to dog plasma protein of 45% which increased to 60% 6 hr after treatment. The discrepancy found between *in vitro* and *in vivo* binding of I to plasma components is of interest (Table III). More drug or drug products appear to be bound *in vitro* than *in vivo*, suggesting that a dynamic process is responsible for the *in vivo* decrease in binding. The rapid metabolism and clearance of I might be suggested as the basis for the binding decrease *in vivo*.

The clinical usefulness of I could be greatly enhanced with a convenient vehicle for intravenous administration. Propylene glycol-based vehicles are common for I administration but possess obvious toxicity problems. The fat emulsion has little of the propylene glycol toxicity, but the emulsion it forms with I has an extremely short stability and must be prepared and administered immediately. Vehicle C is a potentially useful vehicle for human intravenous administration of I due to its convenience, its low acute toxicity (16), and a pattern of drug distribution consistent with that produced by currently used parenteral vehicles.

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ˣ To whom inquiries should be directed.

Sweet Taste Receptor Studies Using Model Interaction Energy Calculations

HANS-DIETER HÖLTJE*x and LEMONT B. KIER‡

Abstract □ Model interaction energy calculations using the monopoles-bond polarizabilities method are used to explain the different sweetness levels in a series of 2-amino-4-nitrobenzenes. The receptor model is isomorphic with the actual receptor feature.

Keyphrases □ Sweetness—model interaction energy calculations used to study sweet taste receptor □ Receptors, sweet taste—studied using model interaction energy calculations □ Monopoles-bond polarizabilities method—model interaction energy calculations of sweetness levels of 2-amino-4-nitrobenzenes □ 2-Amino-4-nitrobenzenes—determination of sweetness levels, model interaction energy calculations

A recent report (1) discussed the structural features of sweet-tasting molecules responsible for imparting the gustatory response. By beginning with the A–H/B theory (2) and recognizing the operation of stereospecificity in some sweet-tasting molecules (3), the location and nature of a third pharmacophore feature were sought. As a consequence of some conformational predictions using molecular orbital calculations, the pharmacophore was predicted to appear as shown in Scheme I (1).

The nature of this third structural feature, *X*, found in all major sweet-tasting molecules, was predicted to be a predominantly dispersion binding moiety. Support for this view came from the comparison of sweet taste potency, in a nitroaniline series, with a summed bond polarizability parameter approximating the ability of a particular moiety to engage in a dispersion bond. This comparison was made to describe in a more fundamental way the process involved, relative to a substituent parameter statistical comparison previously made with the same compounds (4). A very good correlation was found (r = 0.950 and r = 0.968) between the group polarizability and sweetness potency from two separate taste tests (5, 6).

The conclusion (1) that the third binding site of the sweet taste pharmacophore is engaging in a dispersion bond at a receptor site suggests that actual energies of interaction may be calculable using models of the receptor site.

BACKGROUND

In a recent study (7), a new approach to receptor mapping was initiated in which energies of interaction of parts of drug molecules were calculated, with model compounds simulating possible receptor features. This *receptor mapping using model interaction calculations* involves the assessment of the energy *versus* distance characteristics of various drug moieties in an interacting state, with possible receptor features. If the change of activity in a drug series can be ascribed primarily to the change in interaction energy (or affinity) at a particular drug moiety, then it may be possible to obtain a good correlation with activity if a receptor model simulating the actual physical situation is picked.

By using such an approach, the interaction energies and their changes due to molecular modification were calculated for the trimethylammonium and ammonium groups interacting with a series of small molecules, surrogate for amino acid side chains (7). The study led to the prediction that a reasonable choice of a receptor feature complimentary to the trimethylammonium group in acetylcholine is not a carboxylate or other polar moiety but is more likely an aromatic ring as found in phenylalanine, histidine, or tryptophan.

With the sweet-tasting nitroanilines, there is an opportunity to employ this same technique, receptor mapping using model interaction calculations, in a closely related series. In this study, the authors sought to identify receptor models giving interaction energies with the *X* pharmacophore feature in the nitroanilines correlating with the range of taste potency.

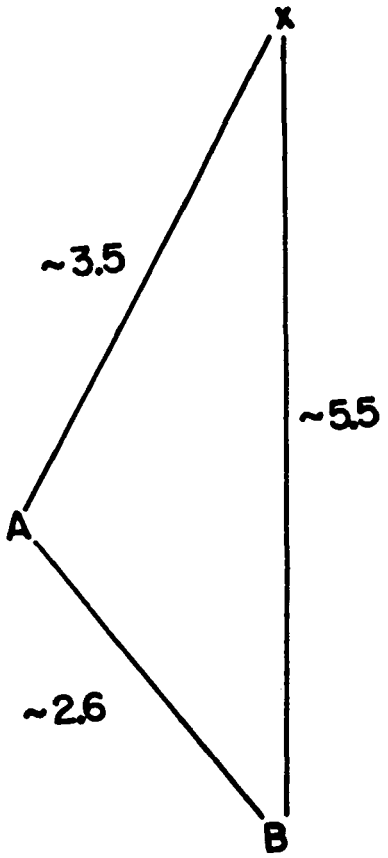
This nitroaniline series was examined previously using the extrathermodynamic approach to seek correlation with the sweetness level (4); a multicomponent equation was optimized when contributions representing an electronic influence and a hydrophobic bonding constant were included. It was found that the sweetness level depended upon the relative level of these contributions plus steric effects. In the present approach, the authors sought to visualize, quantitate, and correlate the enthalpy of interaction to determine if an alternative hypothesis to hydrophobic bonding can be supported.

The study model assumes that the A–H and B features of the sweet nitroanilines are anchored at the receptor with sufficient energy that the initial atom of the *X* moiety, the attached phenyl carbon atom, must be at a constant distance and relationship to the receptor feature complimentary to *X*. It is further assumed that preferred conformation of the *X* moiety is retained throughout the interaction. The sweet nitroanilines studied are shown in Table I. The general procedure is to select receptor models, derived from amino acid side chains, and to calculate the interaction energies between the *X* moiety and the receptor model for various modes of approach.

Whether some form of hydration of the receptor site or the model compound occurs should be considered. The nature and extent of solvation around molecular groups or atoms are not well understood. Arguments can be made for extensive or negligible intimate water interaction with various groups. In this case, it is felt that the point may be essentially moot for the following reasons. If only one water molecule intervened between the two interacting surfaces, then the van der Waals radii of the group and the water itself would prohibit an approach closer than about 8 Å, resulting in a negligible interaction energy between the groups *per se*. The energy of interaction involving the water and each interacting group, at 8 Å separation, would really be no more than the interaction energy of each isolated group and a complexed water molecule. Since a biological effect is measured, and since it is assumed that it is due largely to an interaction, it follows that the situation must involve the approach of two molecular groups, closer than 8 Å, in the absence of an intervening water molecule.

The favorable entropy change of water molecules surrounding the nonpolar portions of the sweet agent and the receptor may favor the association of these groups. The complexity of assessing this relative effect through this series is prohibitive. In essence, the model assumes a relatively constant influence from this sector while attempting to quantitate and correlate the enthalpic effect due to the interaction with the biological activity.

The finding of a good correlation between relative interaction energies at a common *X*–receptor model distance and sweetness level implies that the model is a good one, simulating what may be the actual receptor environment. Caution must be used in claiming that the receptor feature interacting with *X* has been identified. This may not be the actual case. However, the reported findings



Scheme I—Pattern of atoms imparting a sweet taste (glucophore)

identify a good candidate for that receptor feature, and this model may be useful in predicting and explaining the activity levels of new compounds.

This study considered only the amino acids as likely candidates for several reasons. The idea that a protein molecule is a good candidate for a receptor has wide currency. In the case of taste, some preliminary studies have, in fact, led to the isolation of a protein from the tongue which responds to sweet molecules in a parallel manner to their taste level (8). A firmer and, at the same time, a more general reason is that the amino acids in a protein are capable of significant perturbations by interacting with small ligands. Thus, the close approach of a ligand to a side chain can lead to a conformational perturbation in the side chain and in that residue backbone. This can easily lead to the massive disruption or alteration of the function of the entire protein, since its infrastructure is highly dependent upon influences across space in a folded molecule.

The steroid as a receptor model is nowhere near as good a choice. It is definitely rigid and unperturbable, except at positions joining it to other molecules. Furthermore, the steroid does not repeat itself in any kind of a mosaic, so the alteration of one steroid molecule will not result in an amplification or propagation of the effect through a macromolecule.

The phospholipid as a receptor candidate is a poorer choice for reasons similar to those described for the steroids. In addition, the phosphate group, being highly charged, presents a rather insensitive, nonselective feature to an approaching ligand. The high contribution of coulombic forces in the interaction with any ligand would not lead to a significant degree of discrimination between one ligand and another. Thus, the gradation in binding energies and the consequent gradation in biological effects observed would not be expected.

EXPERIMENTAL

For the calculation of the energies of interaction between the sweet compounds and the receptor models, the monopoles-bond polarizabilities method (9) was employed. The interaction energy is written in terms of the charge distributions of the two interacting molecules, approximated as point charges centered on the nucleus, and the polarizabilities of the bonds. These charges have been calculated by the CNDO–MO method (10). To this approach is added the repulsive energy component as the charge distributions begin to overlap. The interaction energy is the sum of the electrostatic (E_e), polarization (E_p), dispersion (E_d), and repulsion (E_r) energies.

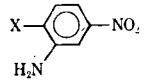
The electrostatic energy, E_e, is the work required or the energy gained when two charged points approach each other. The charge associated with each atom is assumed to be centered on the nucleus. The total electrostatic energy for two interacting molecules is then the sum of all pairs of atoms in the two molecules. Charges of like sign are repulsive while opposite signed atoms are attractive. This energy is related to the first power of the distance between two atoms, and it has a significant value even at long distances.

The polarization energy, E_p, is a measure of the distortion produced in the electron cloud of a bond by the presence of a charged atom in its vicinity. The ease with which a cloud of electrons can be perturbed is the polarizability. The magnitude depends on the relative freedom of the peripheral electrons from the influence of the atom nucleus. The distortion or polarization of the electrons in a bond by a nearby charged atom leads to an attraction that is related to the third power of the distance between the atom and the bond. This distance is a vector from the atom pointing to the midpoint of the bond. The energy of interaction of this type is a summation between all atom-bond pairs in the two molecules.

The dispersion energy, E_d, is the attractive force derived from the charge fluctuation in an orbital inducing a charge fluctuation in a nearby orbital. The two dipoles thus formed are complimentary and are attractive. The influence is related to the sixth power of the distance and is, therefore, highly dependent upon proximity and molecular similarity. The phenomenon is prominent in neutral nonpolar molecules. The ability of the peripheral electrons in the molecule to oscillate or be perturbed by induction is dependent upon their polarizability in the molecule. This molecular polarizability is approximated by the ionization potential of the molecule, *i.e.*, the energy required to remove an electron. The energy is derived from a summation over all atoms in both molecules.

All of these interactions would yield, upon calculation, an increasing energy of binding as atoms or molecules approached. Even coalescence of nuclei would calculate to be a favored process. But in reality, a repulsion occurs as the atoms approach. The repulsive energy quickly rises as atoms penetrate each other's electron domains or van der Waals radii. It is necessary then to correct the attractive energies with an expression simulating the repulsive energy, E_r. This equation is empirically derived, using the van der

Table I—Sweetness–Structure Relationships among 2-Amino-4-nitrobenzenes and Total Calculated Interaction Energies



X	Log Sweetness Relative to Sucrose	Calculated Total Binding Energy, at 4.25 Å Distance, kcal/mole (Scheme II)
OC_2H_7	3.61	5.40
$OCH_2CH{=}CH_2$	3.30	5.25
I	3.10	4.93
OCH_2CH_3	2.98	4.91
Br	2.90	3.79
$OCH(CH_3)_2$	2.78	4.91
Cl	2.60	3.59
OCH_3	2.34	4.22
CH_3	2.34	3.96
F	1.50	3.24
H	1.50	3.11

Table II—Contributions to Total Interaction Energy in Table I (kilocalories per mole)

R	E_e	E_p	E_d	E_r	E (total)
OC_3H_7	−0.129	−0.041	−6.545	+1.318	−5.397
$OCH_2CH{=}CH_2$	−0.122	−0.040	−6.136	+1.051	−5.254
I	+0.234	−0.032	−6.558	+1.425	−4.931
OCH_2CH_3	−0.115	−0.046	−5.921	+1.168	−4.914
Br	+0.225	−0.026	−5.056	+1.063	−3.794
$OCH(CH_3)_2$	−0.115	−0.046	−5.921	+1.168	−4.914
Cl	−0.200	−0.025	−4.609	+0.841	−3.593
OCH_3	−0.099	−0.051	−4.969	+0.902	−4.214
CH_3	−0.220	−0.045	−4.645	+0.948	−3.962
F	−0.191	−0.100	−3.607	+0.657	−3.241
H	−0.190	−0.036	−3.514	+0.632	−3.108

Waals radii in its formulation. These repulsions are summed over all interacting atoms in both molecules.

These calculations were used to predict interaction energies between DNA base pairs in the pioneering work of Rein and his collaborators (11–13). Hoyland and Kier (14) used it in the first application to the successful prediction of a molecular conformation, prostaglandin E_1.

The interaction energy between the two molecules can be written approximately as a sum of first- and second-order perturbation terms. The monopole-bond polarizabilities method of Claverie and Rein (9), as elaborated by Huron and Claverie (15), was utilized for the actual numerical calculations. Within this approximation, the interaction energy can be written in terms of the charge distributions of the two molecules approximated by point charges centered at the nuclei and the polarizabilities of the bonds, together with an algorithm for computing the repulsive energy component as the charge distribution begins to overlap. The actual working equations will be discussed.

For convenience, the two molecules are designated by subscripts 1 and 2. Let N_i and B_i be the number of atoms and bonds, respectively, in molecule i. Then the long-range interaction energy can be written as a sum of electrostatic *(E_e)*, polarization *(E_p)*, and dispersion *(E_d)* components. The explicit formulas are as follows:

$$E_e = \sum_{i=1}^{N_1}\sum_{j=1}^{N_2}\frac{q_i q_j}{R_{ij}} \qquad \text{(Eq. 1)}$$

$$E_p = -\frac{1}{2}\sum_{k=1}^{B_1}\boldsymbol{E}_k\bar{\mathbf{A}}_k\boldsymbol{E}_k - \frac{1}{2}\sum_{l=1}^{B_2}\boldsymbol{E}_l\bar{\mathbf{A}}_l\boldsymbol{E}_l \qquad \text{(Eq. 2)}$$

$$E_d = -\frac{1}{4}\times\frac{I_1 I_2}{I_1 + I_2}\sum_{k=1}^{B_1}\sum_{l=1}^{B_2}R_{kl}^{-6}\mathrm{Tr}(\bar{\bar{\mathbf{T}}}_{kl}\bar{\mathbf{A}}_k\bar{\bar{\mathbf{T}}}_{kl}\bar{\mathbf{A}}_l) \qquad \text{(Eq. 3)}$$

In Eq. 1, q_i is the charge at nucleus i and R_{ij} is the distance between the nuclei i and j. The quantity $\boldsymbol{E}_k$ in Eq. 2 is the electric field at the center of bond k (in molecule 1) due to the monopole charges q_j of molecule 2:

$$\boldsymbol{E}_k = \sum_{j=1}^{N_2} q_j \mathbf{R}_{jk} R_{jk}^{-3} \qquad \text{(Eq. 4)}$$

where R_{jk} is the distance from nucleus j to the midpoint of bond k, and $\mathbf{R}_{jk}$ is the vector of magnitude R_{jk} pointing from center j to the bond midpoint k. The quantity $\bar{\mathbf{A}}_k$ is the polarizability tensor for bond k.

The dispersion equation, Eq. 3, contains the average excitation energies, I_1 and I_2, approximated by the ionization potentials and a factor X which corrects for the fact that the usual London equation ($X = 1$) gives a result that is too small. The quantity R_{kl} is the distance between the midpoints of bonds k and l, and the tensor $\bar{\bar{\mathbf{T}}}_{kl}$ is defined as:

$$\bar{\bar{\mathbf{T}}}_{kl} = 3\frac{\mathbf{R}_{kl}\times\mathbf{R}_{kl}}{R_{kl}^2} - 1 \qquad \text{(Eq. 5)}$$

where 1 is the unit matrix. The symbol Tr in Eq. 3 indicates the trace of the quantity in parenthesis.

The final contribution that must be considered is the repulsion resulting from overlap of the charge distribution of the two molecules, designated as E_r. The Kitaygorodski (16) repulsion derived empirically from crystal energies of hydrocarbons, but slightly modified by Huron and Claverie (15), was used. This relationship is:

$$E_r = 30{,}000\sum_{i=1}^{N_1}\sum_{j=1}^{N_2}\exp[-5.5R_{ij}(V_iV_j)^{-1/2}] \qquad \text{(Eq. 6)}$$

where V_i is the van der Waals radius of atom i.

Standard bond lengths and angles are assumed. The ionization potentials are derived from the CNDO/2 calculations. The polarizabilities are taken from Denbigh (17) or leFevre (18). The van der Waals radii are as follows: H = 1.2, C = 1.6, C (aromatic) = 1.8, N = 1.5, O = 1.4, F = 1.4, Cl = 1.8, Br = 2.0, and I = 2.2.

Several preliminary calculations were made using the derivatives listed in Table I and amino acid side-chain models. A correlation was sought between the interaction energies at a constant X–receptor distance through the series and the sweetness level. Only the model compound surrogate for the tryptophan side chain gave a significant correlation. Accordingly, this receptor model was examined further by varying the mode of approach of X to the 3-methylindole surface. The mode of interaction and the X–receptor distance was optimized to give the best correlation to total interaction energy and sweetness.

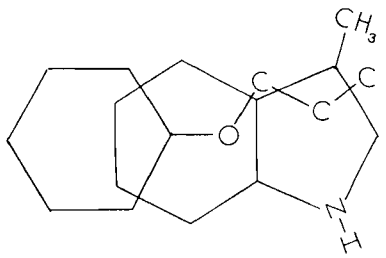
A second criterion was used to optimize the X–receptor interaction model. If it is assumed that the sweetness level in this series is due entirely to the affinity of X for the receptor, then the potency ratio of the extremes in the series can be related to the difference in binding energies of the extreme cases with the equation:

$$\Delta E = -RT\ \ln\ \rho \qquad \text{(Eq. 7)}$$

The term ρ is the potency ratio, 127 in the present case. This expression has been used (19, 20) to relate affinities to potencies. From the relative potencies in Table I, the difference in binding energies necessary to account for the extreme potency difference was calculated to be 3.0 kcal/mole. These interaction calculations should agree as closely as possible with this range of binding energies for the two extreme cases of sweetness potency to ensure that the model is reasonable.

RESULTS

The calculations show that 3-methylindole, simulating the side chain of tryptophan, is the best receptor model for the X moiety of sweet nitroanilines among several amino acid side-chain models. The optimum relationship between X and this model is shown in Scheme II. Table I lists the total calculated interaction energies for each compound at the optimum X–receptor distance of 4.25 Å;



Scheme II—Optimum mode of interaction of the X moiety of the sweet nitroanilines and 3-methylindole, simulating tryptophan as a receptor site. The X in this case is propoxy.

Table II lists the contributions of the electrostatic, polarization, dispersion, and repulsion energies to the total energy (Table I). The mentioned distance separates the phenyl ring carbon attached to X and the plane of the indole ring.

Relating the energies to the sweetness level (Table I) gives a correlation coefficient of $r = 0.887$. The interaction energy difference predicted between the two extreme cases is 2.3 kcal/mole, compared to the 3.0 kcal/mole predicted from the expression previously described.

DISCUSSION

The calculations reveal a fairly good correlation relating a biological activity with a single, relatively fundamental, molecular property. The property is a dynamic one, namely the total interaction energy with a model compound simulating the receptor feature. Accordingly, it can be concluded that the choice of receptor is fairly good under the circumstances.

The interaction energy difference calculated for the two extreme sweetness cases, 2.3 kcal/mole, is of the same magnitude as the energy difference predicted from the thermodynamic expression, 3.0 kcal/mole. The latter value presumes that all biological activity variation is due to differences in the energies of binding.

An interesting observation can be made relating to the value of the correlation and the extremes of binding energy predicted in the series. The correlation coefficient, $r = 0.887$, indicates that 79% of the variation has been accounted for in the relationship. By comparing the calculated interaction energy spread of 2.3 kcal/mole with the energy spread predicted from the thermodynamic expression of 3.0 kcal/mole, it can be said that the results imply that 77% of the biological activity ratio of the extreme cases is a result of interaction energies considered in the model.

The similarity of these values, derived from independent aspects of the study, indicates that the model is correctly simulating the part of the drug–receptor binding that can be ascribed to the calculated interaction energies. From Table II it is clear that the overwhelming contribution to those energies is, as predicted, due to dispersion forces. Electrostatic forces are relatively small.

The potential value of these results and of the general method in other cases lies in the possibility that a model can now be constructed that is isomorphic with the actual drug–receptor system. Such a model may be useful in explaining activity and designing new drugs.

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Keyphrases □ Warfarin—GLC determination in plasma □ GLC—analysis, warfarin in plasma

Analysis of warfarin from biological fluids by spectrophotometric (1), fluorometric (2, 3), and TLC (4) methods have been described. The O'Reilly *et al.* (1) method, used by several investigators (3, 5–8), was modified by Welling *et al.* (9) to make it more sensitive. This modified method has been successfully employed to study the *in vivo* and *in vitro* availability of commercial warfarin tablets (10).

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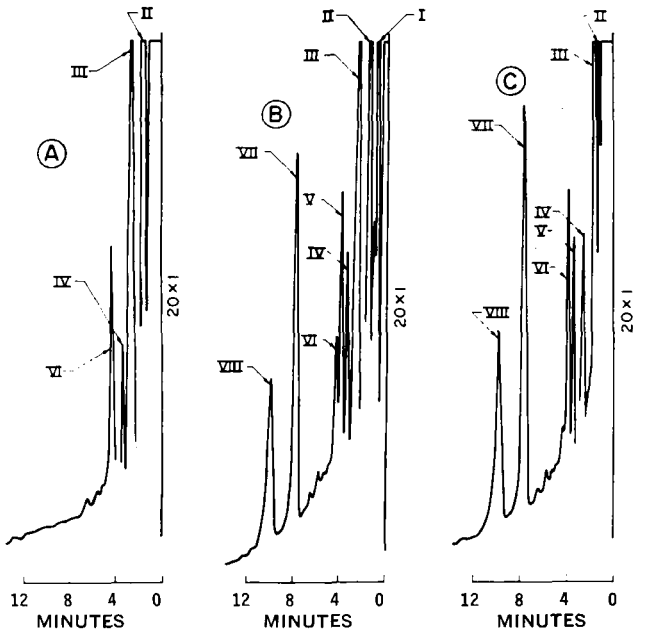


Figure 1—*Gas chromatogram of human plasma. Key: A, control plasma; B, plasma containing 1.25 μg/ml of warfarin and 5.0 μg/ml of phenylbutazone; and C, plasma from a human volunteer who has been given 20 mg of sodium warfarin. Peaks V and VII are peaks of methylated phenylbutazone, and peak VIII is methylated warfarin. Peaks I–IV and VI are due to endogenous materials in plasma.*

These methods are either tedious (4) or require large amounts of plasma samples (9). To study the bioavailability of commercial warfarin formulations on the Canadian market, a specific and sensitive GLC method for measuring plasma levels of warfarin following single doses has been developed.

EXPERIMENTAL

Reagents—Ethylene dichloride[1] and ether[2] were glass-distilled prior to use. Stock solutions containing 100 μg/ml of warfarin[3] (I) were prepared weekly in distilled water and stored in the dark in a refrigerator. Appropriate dilutions (0.25–8.00 μg/ml) were made daily before use. Aqueous solutions of the internal standard phenylbutazone[4] (II), containing 100 μg/ml, were prepared as reported previously (11) and diluted daily to 10 μg/ml with phosphate buffer[5] (pH 7.2). Spectral grade carbon disulfide[5], 1 *N* NaOH, and 3 and 5 *N* HCl were employed. An ethereal solution of diazomethane was synthesized from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[6]. Warfarin metabolites[7], namely 3-[α-(2-hydroxypropyl)-benzyl]-4-hydroxycoumarin (two diastereoisomers), 6-hydroxywarfarin, and 7-hydroxywarfarin, were used.

Plasma Level Study—Sodium warfarin[8], 20 mg, was administered separately to three healthy male volunteers weighing 71, 82, and 89 kg. Blood samples, 10 ml, were withdrawn from the cubital vein, using heparinized evacuated tubes[9], at 10 appropriate time intervals after dosing. To compare the UV procedure (9) with the GLC method, 40 ml of blood was obtained from Volunteer 1 (71 kg) at 1, 5, 8, and 24 hr after drug administration. The blood samples were centrifuged and the plasma was transferred to another tube before storing at −10°.

[1] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[2] Mallinckrodt Chemical Works Ltd., Montreal, Quebec, Canada.
[3] Warfarin Sodium, Warner-Lambert, Scarborough, Ontario, Canada.
[4] Butazolidin, Geigy Pharmaceuticals, Montreal, Quebec, Canada.
[5] Fisher Scientific Co. Ltd., Montreal, Quebec, Canada.
[6] Diazald, Aldrich Chemical Co., Milwaukee, WI 53233
[7] Donated by Dr. K. K. Chan, School of Pharmacy and Medicine, University of Southern California, Los Angeles, Calif.
[8] Coumadin Tablets, Endo Drugs Ltd., Montreal, Quebec, Canada.
[9] Vacutainers, Becton Dickinson Co., Mississauga, Ontario, Canada.

Table I—Recovery of Warfarin and Phenylbutazone from Plasma

Micrograms Added to 1 ml Plasma	*n*	Mean Micrograms Recovered	Mean Percent Recovery	*SD* of Percent Recovery
Recovery of Phenylbutazone				
1.25	3	0.70	55.40	1.25
2.50	3	1.40	55.73	1.61
Mean = 55.57 ± 1.30%				
Recovery of Warfarin				
1.25	3	1.18	94.67	0.92
2.50	3	2.36	94.27	0.92
Mean = 94.47 ± 0.85%				

Table II—Estimation of Warfarin Added to Plasma by GLC

Added Warfarin, μg	*n*	Mean Peak Height Ratio Warfarin/Phenylbutazone	*SD*	*CV*
0.25	8	0.09	0.005	5.52
0.50	7	0.16	0.008	5.00
1.00	8	0.34	0.010	3.07
2.00	7	0.70	0.019	2.73
4.00	8	1.37	0.016	1.17
8.00	7	2.75	0.039	1.40
Mean *CV* = 3.15				
$y = mx$ where $m = 0.344 \pm 0.002$				
$r^2 = 1.000$				

Extraction of Warfarin—To a 2-ml plasma sample ("spiked" or from dosed volunteers) in a glass-stoppered centrifuge tube[10] (42 ml) are added 1 ml of II (5.0 μg/ml), 2 ml of 3 *N* HCl, and 20 ml of ethylene dichloride. The samples are stoppered and extracted by shaking at an angle of 30° for 10 min at 180 cpm on a flat-bed shaker[11]. After centrifugation at 3000 rpm for 10 min, the aqueous layer is removed by aspiration and 18 ml of the ethylene dichloride layer is transferred into another glass-stoppered centrifuge tube (42 ml) containing 10 ml of 1 *N* NaOH.

The extraction is repeated (shake 15 min, centrifuge 10 min) and 9 ml of the sodium hydroxide is transferred to another glass-stoppered tube (42 ml) containing 3 ml of 5 *N* HCl. The tube is swirled gently and the extraction is carried out with another 20 ml of ethylene dichloride for 20 min. The aqueous layer is removed by aspiration and the extraction is repeated for 5 min with 5 ml of phosphate buffer (pH 7.2).

After discarding the phosphate layer by aspiration, 18 ml of ethylene dichloride is transferred to a glass-stoppered centrifuge tube[10] (50 ml). The ethylene dichloride extract is evaporated[12] under a stream of dry nitrogen at 85° to a volume of approximately 4 ml. A crystal of anhydrous calcium chloride[5] is added and the tube is swirled gently. The extract is transferred quantitatively into a custom-made evaporating tube (12) containing a small anti-bumping granule[13] and evaporated as before to dryness.

The tube is allowed to cool to room temperature and the residue is dissolved in 100 μl of ethereal diazomethane by mixing[14] for 30 sec. Then the tube is left to stand for 10 min and the reaction mixture is then evaporated under a stream of dry nitrogen at room temperature. The evaporated extract is redissolved in 25 μl of car-

[10] Wilkens-Anderson Co., Chicago, Ill.
[11] Eberbach Corp., Ann Arbor, Mich.
[12] Thermolyne Dri-Bath, Fisher Scientific Co. Ltd., Montreal, Quebec, Canada.
[13] British Drug House, Toronto, Ontario, Canada.
[14] Vortex Genie Mixer, Fisher Scientific Co. Ltd., Montreal, Quebec, Canada.

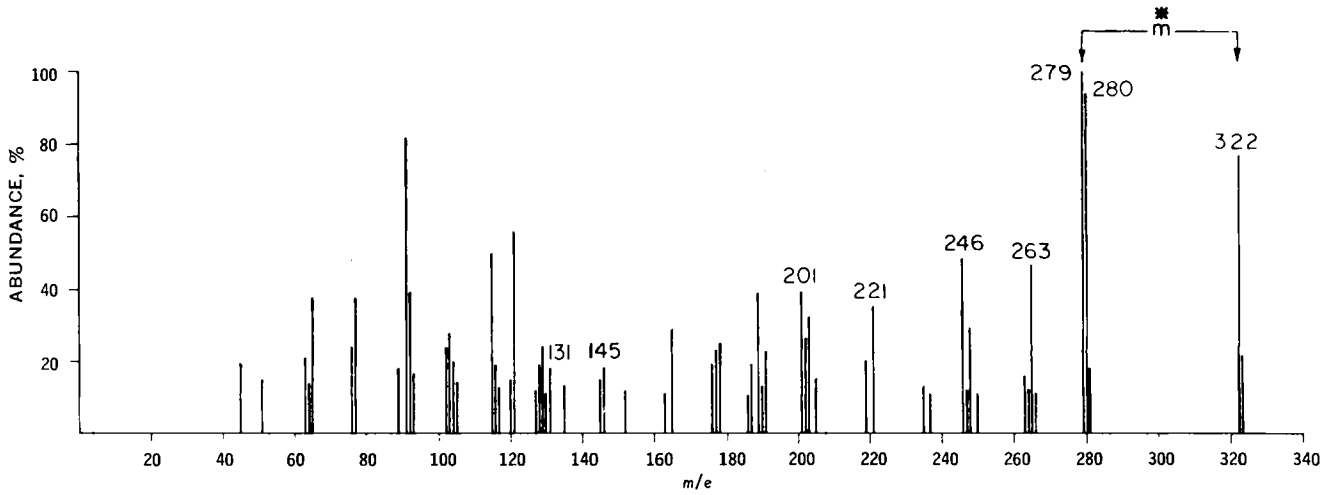


Figure 2—*GLC–mass spectrum of methylated warfarin.*

bon disulfide by mixing[14] for 30 sec and injected (2–3 μl) into the gas chromatograph[15].

GLC—A gas chromatograph[15] equipped with a flame-ionization detector was employed. The column was a spiral glass tubing[15], 1.8 m (6 ft) × 0.25 cm (0.10 in.) i.d., silanized with a 5% solution of trimethylchlorosilane[16] and hexamethyldisilazane[16] (1:1) in ether. It was packed with 5.0% phenyl methyl dimethyl silicone[17] coated on acid-washed, dimethylchlorosilane-treated, 80–100-mesh, high performance flux-calcined diatomite support[18].

The column was conditioned by injecting 25 μl of a mixture of *N',O*-bis(trimethylsilyl)acetamide, trimethylsilyl diethylamine, and hexamethyldisilazane[19] and maintaining the column at 300° for 18 hr with low nitrogen flow. The injection port, detector, and oven temperatures were 280, 270, and 260°, respectively. Nitrogen, as a carrier gas, was maintained at a flow rate of 63 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response.

Calculations—Peak height ratios were calculated by dividing the height of the peak due to warfarin (9.8 min) by that of phenylbutazone (7.7 min). Calibration curves were constructed from the results of spiked control plasma samples by plotting the peak height ratios against the concentration of warfarin (micrograms per milliliter of plasma).

RESULTS AND DISCUSSION

Methylation of warfarin with diazomethane gave one peak on GLC analysis with a retention time of 9.8 min (peak VIII, Fig. 1B). Methylation of phenylbutazone with diazomethane gave two peaks on GLC with retention times of 3.9 and 7.7 min (peaks V and VII, Fig. 1B). The heights of the respective peaks were in the ratio of 0.7:1.0. This ratio was independent of oven temperature and sample size. The second peak (VII), which was well separated from the peaks of endogenous material in plasma, was used for quantification.

To establish the structure of the compound giving rise to the peak (VIII, Fig. 1B) from diazomethane treatment of warfarin, eluates from the GLC column were fed directly into the mass spectrometer[20] and the mass spectrum of VIII was recorded. The mass spectrum (Fig. 2) showed a molecular ion at *m/e* 322 and abundant ions at *m/e* 280, 279, 265, 263, 246, 235, 221, 219, 203, 202, 201, 145, and 131. Structures I–XIV have been postulated for these ions. These fragmentations are in agreement with some of those proposed by Trager *et al.* (13) and suggest that the methylated derivative of warfarin has the structure 3-(α-acetonylbenzyl)-4-methoxycoumarin. The combined GLC–mass spectral evidence was obtained for the major peak (VII, retention time of 7.7 min) of the internal standard in the present study and was consistent (molecular ion at *m/e* 322 and other diagnostic ions at *m/e* 77, 183, 266, and 279) with the structure 1,2-diphenyl-3-methoxy-4-*n*-butyl-5-oxopyrazoline (14).

Attempts to prepare trimethylsilyl ethers with hexamethyldisi-

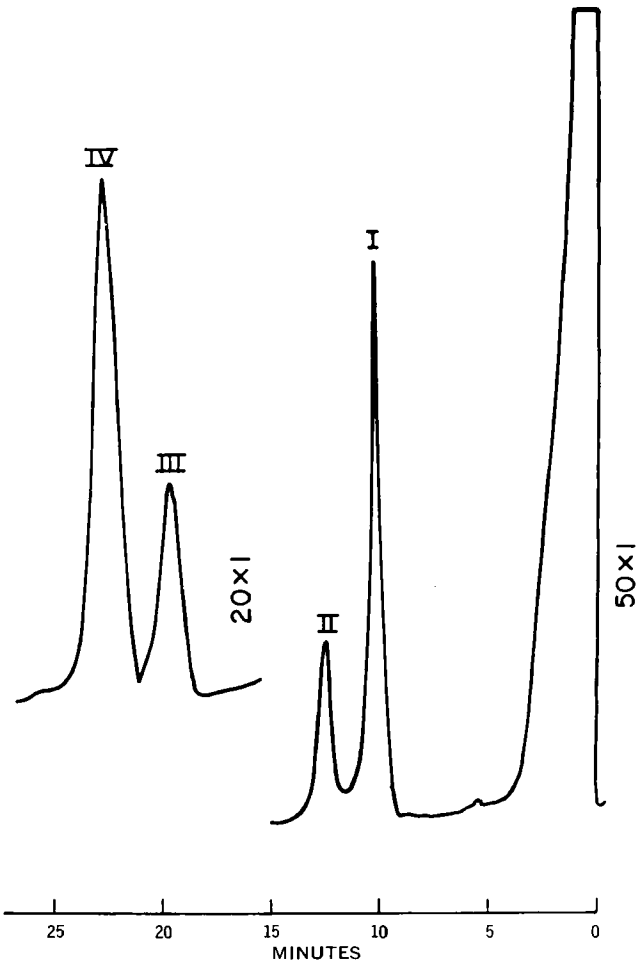
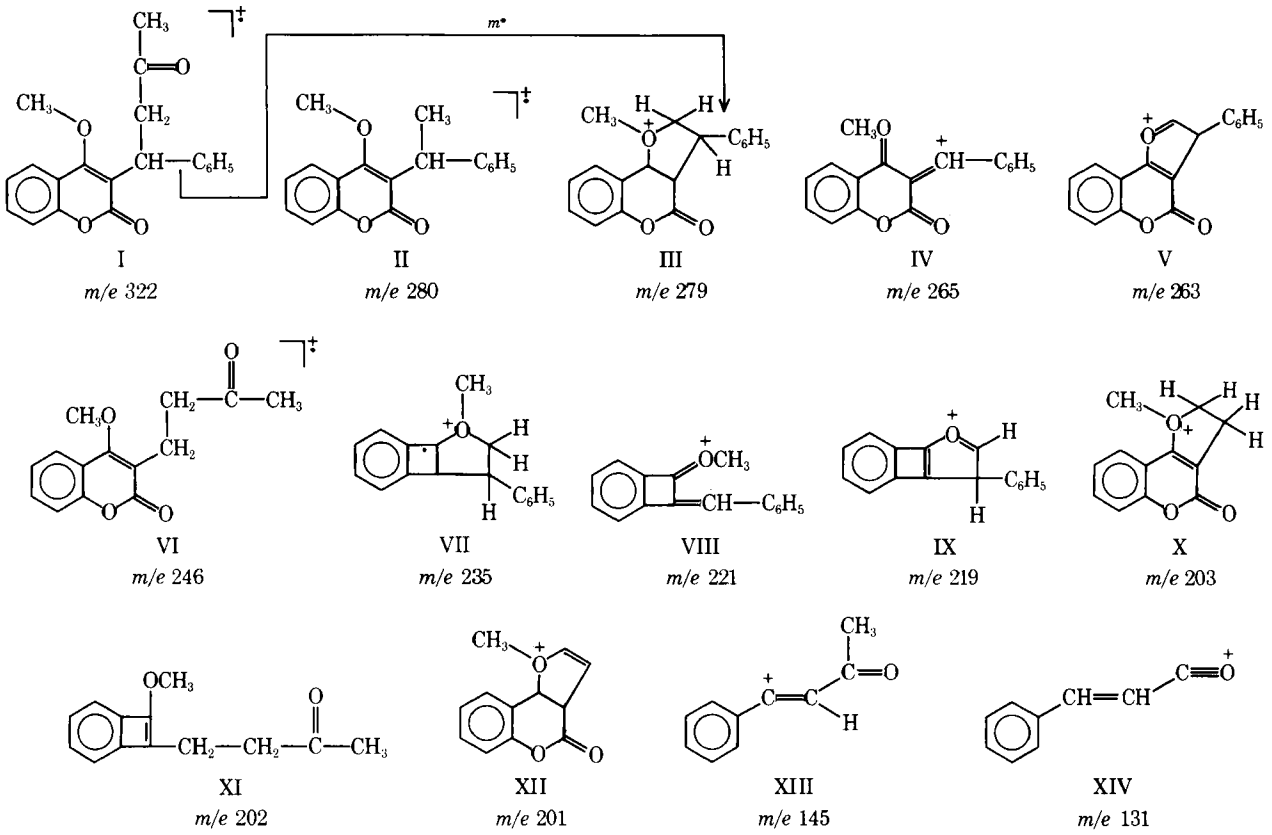


Figure 3—*Gas chromatogram of methylated warfarin metabolites. Key: peaks I and II, warfarin alcohols (two diastereoisomers); peak III, methylated 6-hydroxywarfarin; and peak IV, methylated 7-hydroxywarfarin.*

[15] Model F/11, Perkin-Elmer, Montreal, Quebec, Canada.
[16] Pierce Chemical Co., Rockford, Ill.
[17] OV-7, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[18] Chromasorb W, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[19] Silyl-8, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[20] Perkin-Elmer model 900 gas chromatograph attached to a Hitachi Perkin-Elmer model RMSU mass spectrometer through a jet separator.



lazane in pyridine according to the method of Deckert (15) led to the breakdown of warfarin with no reproducibility. Silylation of warfarin with other silylating agents such as bis(trimethylsilyl)trifluoroacetamide[16], a mixture of trimethylsilylimidazole, bis(trimethylsilyl)acetamide, and trimethylchlorosilane[21], and a mixture of hexamethyldisilazane and trimethylchlorosilane[22] in solvents such as pyridine[16], dimethyl sulfoxide[16], acetonitrile[16], and dimethylformamide[16] led either to extensive breakdown of the drug or to formation of the trimethylsilyl derivative to which the flame-ionization detector was not sensitive enough for plasma level determinations following single doses of warfarin (20 mg).

Attempts to prepare the methoxime derivative of warfarin with methoxyamine hydrochloride[16] followed by silylation were successful. However, these derivatives did not significantly increase the sensitivity of the flame-ionization detector to warfarin. Flash-heater methylation of warfarin using trimethylanilinium hydroxide (0.2 *M*)[23] in methanol and subsequent GLC analysis gave symmetrical and well-defined peaks. However, on-column degradation of the derivative with time was observed. Since the degradation was not reproducible, this procedure of derivatization was not employed.

Ethereal diazomethane was effective in the methylation of warfarin and other coumarin derivatives such as phenprocoumon[24] and acenocoumarol[25]—the later two being investigated as possible internal standards for the assay of warfarin. These derivatives gave sharp well-defined GLC peaks. Phenprocoumon could not be employed as an internal standard because a peak from endogenous material in the plasma interfered with the peak of methylated phenprocoumon on GLC. Acenocoumarol was discarded as a possible internal standard for the assay of warfarin because its methylated derivative had a much longer retention time than that of warfarin, thereby increasing the time of analysis. However, since methylated phenylbutazone gave a GLC peak with the desired retention time and had similar partition characteristics to warfarin, it was chosen as an internal standard for the assay of warfarin.

Several other liquid phases such as saturated hydrocarbon lubricant[26], methyl silicone gum[27], and phenyl methyl silicone fluid[28] were tested, but the peak of methylated warfarin was either not sharp or lacked the response as compared to that obtained on phenyl methyl dimethyl silicone[17]. Carbon disulfide employed as the injection solvent rendered a very small solvent peak which greatly improved the analysis. Plasma extracts, when left unreacted, were much less stable than those reacted immediately after extraction. The plasma extracts that had been reacted with diazomethane were stable up to 8 hr at room temperature.

Figure 1A shows a typical chromatogram obtained by processing

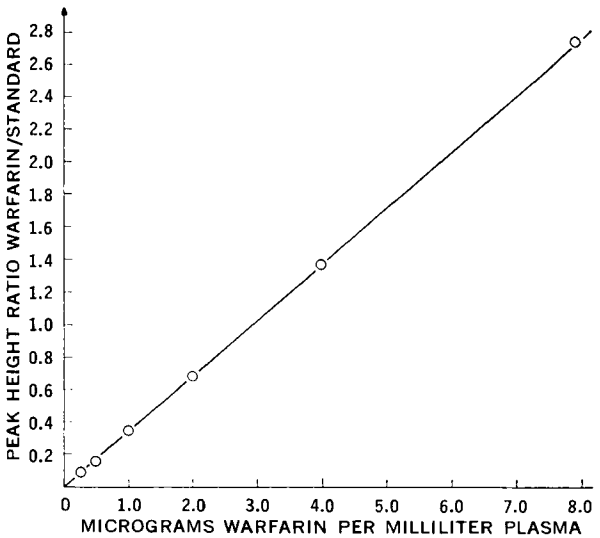


Figure 4—*Calibration curve of warfarin extracted from human plasma. Each point is a mean of seven determinations.*

[21] Tri-Sil TBT, Pierce Chemical Co., Rockford, Ill.
[22] Tri-Sil-Concentrate, Pierce Chemical Co., Rockford, Ill.
[23] Methelute, Pierce Chemical Co., Rockford, Ill.
[24] Marcumar, Hoffmann–La Roche Inc., Montreal, Quebec, Canada.
[25] Sintrom, Geigy Pharmaceuticals, Montreal, Quebec, Canada.

[26] Apiezon-L, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[27] SE-30, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.
[28] OV-17, Chromatographic Specialties Ltd., Brockville, Ontario, Canada.

Table III—Comparison of Modified O'Reilly UV Assay (9) and GLC Assay of Warfarin from Plasma of a Dosed Volunteer following a Single 20-mg Dose

Hours after Ingestion	UV Method		GLC Method	
	Concentration, μg	Mean	Concentration, μg	Mean
1	2.52	2.46	2.72	2.73
	2.40		2.74	
	2.13	2.12	2.11	2.12
5	2.10		2.13	
8	1.94	1.92	1.96	1.99
	1.89		2.02	
24	1.41	1.37	1.16	1.20
	1.32		1.23	

fresh blank plasma as described in the *Experimental* section. The extraneous peaks (I–IV and VI) were observed in chromatograms of all human plasma samples. A chromatogram obtained when the method was applied to spiked plasma containing 1.25 μg/ml of warfarin and 5.0 μg/ml of phenylbutazone is shown in Fig. 1B, where it is clear that the extraneous peaks (I–IV and VI) do not interfere with the peaks from the internal standard (VII, retention time of 7.7 min) or warfarin (VIII, retention time of 9.8 min).

Figure 1C shows a chromatogram from a 2-ml plasma sample (25 hr after ingestion) from a volunteer who received 20 mg of sodium warfarin[8]. The time required for analysis is 12 min. The metabolites of warfarin, namely 3-[α-(2-hydroxypropyl)benzyl]-4-hydroxycoumarin (two diastereoisomers), 6-hydroxywarfarin, and 7-hydroxywarfarin (13), do not interfere with the assay since, on methylation with diazomethane (Fig. 3), they gave retention times of 10.5, 12.5, 19.6, and 22.7 min, respectively. No metabolites were detected in plasma samples of dosed volunteers (n = 10) following single doses of 20 mg of sodium warfarin[8], an observation that confirms the findings of Welling *et al.* (9).

The response of the flame-ionization detector to methylated warfarin was linear with concentration in the range of 0.25–8.00 μg/ml. The peak height ratio of the drug and the internal standard was used as the index of detector performance and overall efficiency of the analytical procedure. The overall recoveries of warfarin and phenylbutazone from plasma were of the order of 94.47 ± 0.85 and 55.57 ± 1.30%, respectively (Table I).

The accuracy and precision of the GLC assay are demonstrated in Table II. Results are based on at least seven determinations of each warfarin concentration, ranging from 0.25 to 8.00 μg/ml, which were treated as described under *Experimental*. The overall coefficient of variation was 3.15%. Figure 4 shows the calibration curve obtained by plotting the peak height ratio of methylated warfarin–phenylbutazone *versus* the concentration of warfarin. The plot is a straight line ($y = mx$) over the concentration range of 0.25–8.00 μg warfarin/ml plasma. A mean slope value of 0.344 ± 0.002 ($r^2 = 1$) was obtained.

The GLC procedure was compared with the modified O'Reilly assay (9). Routine analysis by both methods of duplicate plasma samples from a human volunteer (Volunteer 1, 71 kg) who was administered 20 mg of sodium warfarin[8] gave good agreement with an overall difference of 6.75% (Table III).

Application of the method to plasma level determinations is demonstrated in Fig. 5. A 20-mg dose of sodium warfarin[8] (2 × 10-mg tablets) was given to two healthy male volunteers (82 and 89 kg), and plasma was withdrawn at intervals over 96 hr and analyzed for the drug by the described GLC method.

The described GLC procedure is sensitive for the determination of warfarin. It offers the advantage that the plasma samples required are not large. It is specific since it clearly distinguishes the drug from its metabolites and therefore can be employed for single- as well as multiple-dose pharmacokinetic studies.

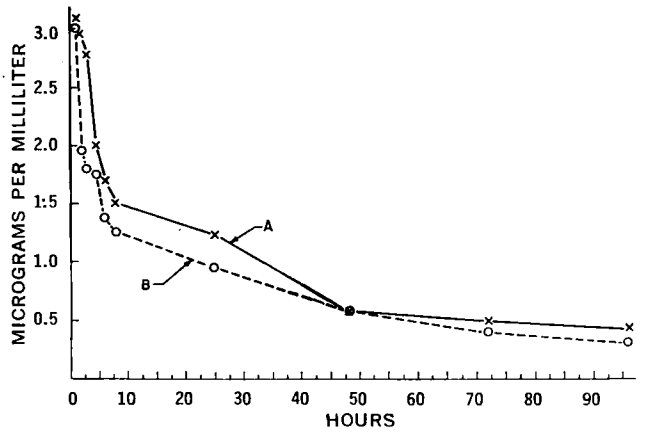


Figure 5—*Warfarin concentrations in two human volunteers following a single oral dose of 20 mg of sodium warfarin. Key: A, Volunteer 2 (82 kg); and B, Volunteer 3 (89 kg).*

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[x] To whom inquiries should be directed.

Aromatic Hydroxylation and Alkyl Oxidation in Metabolism of Mitotane (*o,p'*-DDD) in Humans

V. D. REIF*§, J. E. SINSHEIMER*x, J. C. WARD*¶, and D. E. SCHTEINGART‡

Abstract □ Ether extracts of urine from patients treated with mitotane (*o,p'*-DDD) were methylated with diazomethane. Five derivatives of metabolites were detected and identified by comparison of GLC retention times, mass spectra, and IR spectra with synthetic reference compounds. The derivatives are: methyl *o,p'*-dichlorodiphenylacetate, methyl 1-(2-chloro-4-methoxyphenyl)-1-(4-chlorophenyl)acetate, methyl 1-(2-chloro-3-methoxyphenyl)-1-(4-chlorophenyl)acetate, methyl 1-(2-chloro-3,4-dimethoxy)-1-(4-chlorophenyl)acetate, and *o,p'*-dichlorodiphenylacetylglycine methyl ester. The synthesis and mass, IR, and NMR spectra of reference compounds are given. Metabolite levels over a period of time in urine samples of two patients were quantitated by GLC.

Keyphrases □ Mitotane—aromatic hydroxylation and alkyl oxidation during metabolism, humans, detection and identification of metabolites □ *o,p'*-DDD (mitotane)—aromatic hydroxylation and alkyl oxidation during metabolism, humans, detection and identification of metabolites □ Metabolism—mitotane in humans, aromatic hydroxylation and alkyl oxidation, detection and identification of metabolites

Mitotane[1], 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD) (I), has been shown to produce atrophy of the adrenal cortex, inhibition of ACTH stimulation of steroidogenesis, and alteration of extraadrenal cortisol metabolism (1). Its utility in the treatment of Cushing's syndrome secondary to either adrenal cortical carcinoma (2) or hyperfunction (3) has led to Food and Drug Administration (FDA) approval for human use, although knowledge is limited as to its mode of action, side effects, and biotransformation.

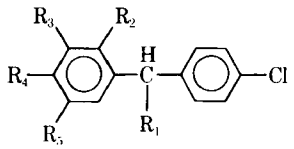
Mitotane shows a species difference in its effects on the adrenal cortex. It causes adrenal atrophy in dogs and humans, but it is inactive in rats, mice, rabbits, and monkeys (4). The compound can inhibit adrenal glucose 6-phosphate dehydrogenase activity in the dog and ACTH stimulation of steroidogenesis *in vivo* but not *in vitro* (1, 5). These findings might suggest that a metabolite of I rather than the compound itself is responsible for its activity.

In addition, 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethane (*p,p'*-DDD)[2] (II) is relatively inactive, while 1-(*m*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*m,p'*-DDD)[3] (III) shows adrenolytic activity in the dog (6). A difference in lipid solubility (7), a free *para*-position, or a biotransformation to give a *para*-hydroxylated species might be responsible for the difference in activity. Biotransformation of an analogous compound, 2-chlorophenoxyacetic acid, in *Aspergillus niger* gives hydroxylation at primarily the 4- and 5-positions (8).

Previously, *o,p'*-dichlorodiphenylacetic acid (*o,p'*-DDA) (IV*a*) was identified in these laboratories as a metabolite of I in humans (9), but ring hydroxylation of I has not been reported. This study was undertaken to detect and identify other metabolites of I. Urine from patients with adrenal cortical carcinoma being treated with I was used as the source of these metabolites.

RESULTS

Ether extracts of urine were divided into: (*a*) a neutral fraction, (*b*) a sodium hydroxide-soluble fraction, and (*c*) a sodium bicarbonate-soluble fraction (Scheme I). The fractions were methylated with diazomethane and examined by GLC–mass spectroscopy. A metabolite of I was believed to be present when unique GLC peaks were present in urine fractions from treated patients but not in the corresponding fractions from urine of control subjects. The chlorinated character of the compounds was initially indicated by their characteristic mass spectra in the molecular ion region of such compounds. No chlorinated compounds were found in the sodium hydroxide and neutral fractions. However, the presence of the



I: $R_1 = CHCl_2$; $R_2 = Cl$; R_3, R_4, $R_5 = H$
II: $R_1 = CHCl_2$; $R_4 = Cl$; R_2, R_3, $R_5 = H$
III: $R_1 = CHCl_2$; $R_3 = Cl$; R_2, R_4, $R_5 = H$

IV: $R_1 = CO_2CH_3$; $R_2 = Cl$; R_3, R_4, $R_5 = H$
IV*a*: $R_1 = CO_2H$; $R_2 = Cl$; R_3, R_4, $R_5 = H$

VII: $R_1 = CO_2CH_3$; $R_2 = Cl$; R_3, $R_4 = H$; $R_5 = OCH_3$
VII*a*: $R_1 = CO_2H$; $R_2 = Cl$; R_3, $R_4 = H$; $R_5 = OH$

VIII: $R_1 = CO_2CH_3$; $R_2 = Cl$; R_3, $R_5 = H$; $R_4 = OCH_3$
VIII*a*: $R_1 = CO_2H$; $R_2 = Cl$; R_3, $R_5 = H$; $R_4 = OH$

X: $R_1 = CO_2CH_3$; $R_2 = OCH_3$; R_3, $R_5 = H$; $R_4 = Cl$
X*a*: $R_1 = CO_2H$; $R_2 = OH$; R_3, $R_5 = H$; $R_4 = Cl$

XI: $R_1 = CO_2CH_3$; $R_2 = Cl$; $R_3 = OCH_3$; R_4, $R_5 = H$
XI*a*: $R_1 = CO_2H$; $R_2 = Cl$; $R_3 = OH$; R_4, $R_5 = H$

XII: $R_1 = CO_2CH_3$; $R_2 = Cl$; $R_3 = H$; R_4, $R_5 = OCH_3$
XII*a*: $R_1 = CO_2H$; $R_2 = Cl$; $R_3 = H$; $R_4 = OCH_3$; $R_5 = OH$
XII*b*: $R_1 = CO_2H$; $R_2 = Cl$; $R_3 = H$; $R_4 = OH$; $R_5 = OCH_3$

XIII: $R_1 = CO_2CH_3$; $R_2 = Cl$; R_3, $R_4 = OCH_3$; $R_5 = H$
XIII*a*: $R_1 = CO_2H$; $R_2 = Cl$; $R_3 = OCH_3$; $R_4 = OH$; $R_5 = H$
XIII*b*: $R_1 = CO_2H$; $R_2 = Cl$; $R_3 = OH$; $R_4 = OCH_3$; $R_5 = H$

XIV: $R_1 = CONHCH_2CO_2CH_3$; $R_2 = Cl$; R_3, R_4, $R_5 = H$
XIV*a*: $R_1 = CONHCH_2CO_2H$; $R_2 = Cl$; R_3, R_4, $R_5 = H$

[1] Preferred USAN chemical name is 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.
[2] Preferred Merck chemical name is 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane.
[3] Preferred Merck chemical name is 1,1-dichloro-2-(*m*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

Table I—GLC Retention Times (Minutes) of Methylated Metabolites[a]

Column	o,p'-Dichlorodiphenylacetic Acid Methyl Ester (IV)	Bis(p-chlorophenyl)acetic Acid Methyl Ester[b]	Derivatives of o,p'-Dichlorodiphenylacetic Acid (IVa)					
			5-Methoxy (VII)	4-Methoxy (VIII)	3-Methoxy (XI)	4,5-Methoxy (XII)	3,4-Methoxy (XIII)	Glycine Conjugate (XIV)
3% OV-1[c]	6.5	7.4	11.3	11.3	11.3	14.5	14.5	18.7
3% OV-17[d]	24.3	—[e]	32.6	34.1	35.5	39.7	40.7	—[f]
3% OV-25[d]	25.6	—[e]	33.9	36.2	38.5	42.6	44.1	—[f]

[a] Retention times are for reference standards that cochromatograph with urine extract peaks. [b] Internal standard. [c] Column temperature at 190° for 4 min followed by a 4°/min program. See text, Footnote 6, for other conditions. [d] Column temperature at 170° with a 2°/min program. See text, Footnote 6, for other conditions. [e] Not determined in this system. [f] Glycine conjugate is not detected under these conditions.

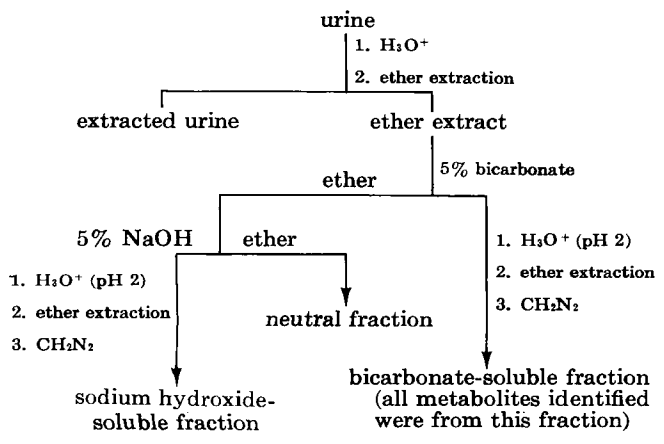
methyl ester of o,p'-dichlorodiphenylacetic acid (IV) was detected as arising from the sodium bicarbonate fraction. The methyl derivatives of hydroxylated, dihydroxylated, and glycine-conjugated derivatives of IVa were also detected. The structures of these derivatives were determined by comparison of GLC retention time, mass spectra, and IR spectra with synthetic reference compounds.

Condensation of the appropriate chloromethoxymandelic acids (V and VI) with chlorobenzene was attempted to synthesize the 5-methoxy-substituted (VII) and the 4-methoxy-substituted (VIII) methyl esters of o,p'-dichlorodiphenylacetic acid. This procedure was analogous to the reported (10) synthesis of the methyl ester of o,p'-dichlorodiphenylacetic acid. Preparation of the mandelic acids was similar to that reported for mandelic acid (11).

The preparation of the 5-methoxy compound (VII) was successful, but the condensation of 2-chloro-4-methoxymandelic acid with chlorobenzene failed to yield any material that could be identified as VIII, the 4-methoxy-substituted compound. However, VIII was obtained by methylation of a condensation product of 4-chloromandelic acid with 3-chlorophenol. As anticipated, substitution also occurred *para* to the chloro group of 3-chlorophenol. This product was present as the lactone of 2-hydroxy-substituted bis(p-chlorophenyl)acetic acid (Xa) in the reaction fraction, which is insoluble in sodium bicarbonate solution. Compound Xa, characterized as the methylated Compound X, is of interest since it is a potential metabolite of the insecticide DDT[4] [1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane, p,p'-DDT]. Hydroxylation at the 2-position of 4-chlorophenoxyacetic acid has been reported (8).

Figure 1 shows a GLC separation on an OV-25 column of a typical methylated urine extract. The relatively small peak B (less than 10% of C) observed in several bicarbonate-soluble urine fractions cochromatographed with the synthetic 5-methoxy compound (VII). However, insufficient amounts were present to confirm these results by mass spectroscopy.

Peak C corresponded in GLC retention time as well as in its mass spectrum to the synthetic 4-methoxy compound (VIII). A urine extract in which the ratio of D to C was less than 5% was used to isolate C by preparative GLC. The IR spectra from this material corresponded to that of synthetic VIII but not VII or XI.



Scheme I—Partition of urine ether extract

Acetylation of the urine extract followed by methylation (12) gave a GLC peak whose mass spectrum indicated the methyl esterified, *O*-acetoxy derivative of the 4-hydroxy compound (VIIIa). However, no Compound VIII was detected, thus indicating that Metabolite C is actually present in urine as VIIIa and not its methoxy derivative.

The mass spectrum of peak D was similar to that of peak C except for the addition of a fragment at mass 289, which indicated loss of one chlorine from the parent compound. The GLC retention time and mass spectrum of the synthetic 3-methoxy compound[5] (XI) (13) were identical to those of peak D.

The methylated derivative of the dihydroxy metabolite present in the urine extract was originally thought to be XII, the 4,5-dimethoxy isomer, based upon GLC on an OV-1 column. However, cochromatography of synthetic XII with the urine extract on OV-17 and OV-25 columns indicated a retention time corresponding to peak E of Fig. 1 and not F, the dimethoxy metabolite peak. Compound XIII, the 3,4-disubstituted isomer, synthesized by Feil *et al.* (13), cochromatographed with peak F. Peak E may be the 4,5-isomer present in amounts of less than 15% of F. However, because of the low amounts present and its close proximity to F, it was not possible to confirm E to be the dimethoxy Compound XII by mass spectroscopy.

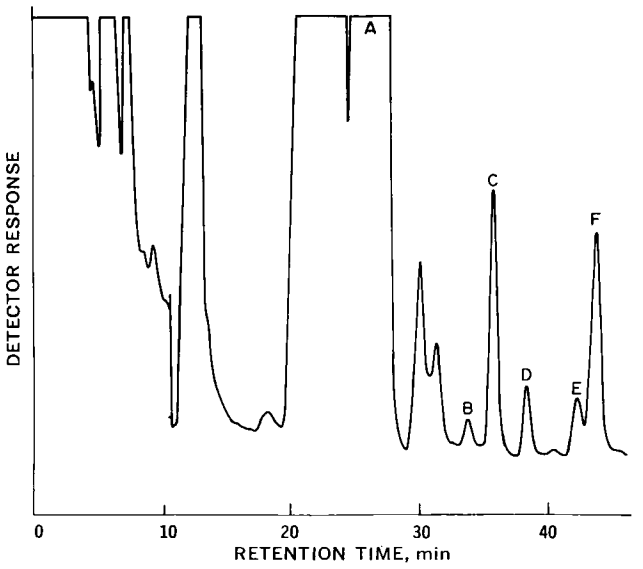


Figure 1—*GLC trace of a typical urine ether extract with retention times in minutes, OV-25 column at 170° with 2°/min program. The indicated peaks cochromatographed with the following compounds: A, methyl* o,p'-*dichlorodiphenylacetate (IV); B, methyl 1-(2-chloro-5-methoxyphenyl)-1-(4-chlorophenyl)acetate (VII); C, methyl 1-(2-chloro-4-methoxyphenyl)-1-(4-chlorophenyl)acetate (VIII); D, methyl 1-(2-chloro-3-methoxyphenyl)-1-(4-chlorophenyl)acetate (XI); E, methyl 1-(2-chloro-4,5-dimethoxyphenyl)-1-(4-chlorophenyl)-acetate (XII); and F, methyl 1-(2-chloro-3,4-dimethoxyphenyl)-1-(4-chlorophenyl)acetate (XIII).*

[4] Preferred Merck chemical name is 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane.

[5] Supplied by V. Feil, U. S. Department of Agriculture.

Table II—Quantitation by GLC of Metabolites of Mitotane in Urine from Patient 1 (M.B.)[a]

Days of Administration	*o,p'*-Dichlorodiphenylacetic Acid (IV*a*)	*o,p'*-Dichlorodiphenylacetic Acid Derivatives			
		Monohydroxy (VIII*a* + XI*a*)	Hydroxy Methoxy (XIII*a* + XIII*b*)	Glycine Conjugate (XIV*a*)	Total
134	0.045 (18.2)	0.015 (5.9)	0.010 (3.5)	0.011 (3.8)	0.081 (31.4)
134[b]	0.073 (29.9)	0.051 (19.5)	0.020 (7.1)	0 (0)	0.144 (56.5)
253	0.034 (10.0)	0.024 (6.6)	0.015 (3.8)	0.014 (3.5)	0.087 (23.9)
290	0.021 (7.7)	0.008 (2.9)	0.011 (3.7)	0.012 (3.8)	0.052 (18.1)
399	0.060 (11.8)	0.017 (3.2)	0.019 (3.3)	0.013 (2.9)	0.109 (21.2)
401	0.046 (11.9)	0.017 (4.2)	0.038 (8.6)	0.028 (6.3)	0.129 (31.0)

[a] Values are milligrams of metabolite per milligram of creatinine excreted. Values in parentheses represent percent excretion of the daily dose of 2 g. [b] Values found after acid hydrolysis of urine.

It is difficult to differentiate between the mass spectra of the dimethoxy isomers XII and XIII. Likewise, the IR spectra of synthetic XII, synthetic XIII, and the metabolite isolated by preparative GLC are similar. A discernable difference is that both the metabolite and XIII have IR bands at 1270 and 1290 cm^{-1} while XII has bands at 1310, 1270, and 1250 cm^{-1}.

Acetylation of urine extract followed by methylation gave a GLC peak whose mass spectrum indicated the methyl esterified, *O*-acetoxy derivative of XIII*a* and/or XIII*b*. The literature (13–15) suggests that XIII would most likely be originally present in urine as one or both of the monomethylated catechols XIII*a* and XIII*b*.

The IR and mass spectrum and the GLC retention time of synthetic methylated glycine conjugate XIV are identical to those of the compound in urine extracts, confirming the presence of the glycine conjugate of *o,p'*-dichlorodiphenylacetic acid as a urinary metabolite of mitotane.

Table I gives GLC retention times on three different columns for peaks in the urine extract that cochromatographed with the appropriate synthetic standards and, except in the cases of VII and XII, were confirmed with the appropriate mass spectra. In these programmed temperature determinations, an increase in height of a particular peak when a synthetic compound was added to and coinjected with the urine extract was a more reliable indicator of equal retention times than separate determinations. The first set of conditions was used for metabolite quantitation and includes the retention time for the methyl ester of the internal standard bis(*p*-chlorophenyl)acetic acid. Recovery of the internal standard through the extraction, methylation, and chromatographic processes was 22.4–55.6%.

Tables II and III list amounts of the four metabolites quantitated by GLC from human urine extracts. Both patients were female and suffering from adrenal carcinoma. Patient 1 (M.B.) was given 6 g of mitotane/day for 85 days, at which time the dose was lowered to 2 g/day. Patient 2 (N.P.) started with a dose of 6 g/day and after 145 days the regimen was changed to 2 g/day. The monohydroxy values given represent a sum of the 4-hydroxy (VIII) and 3-hydroxy (XI) compounds present. When using GLC conditions that separate VIII and XI, the percent of monohydroxy present as XI in several urine extracts ranged from less than 5 to 20%.

DISCUSSION

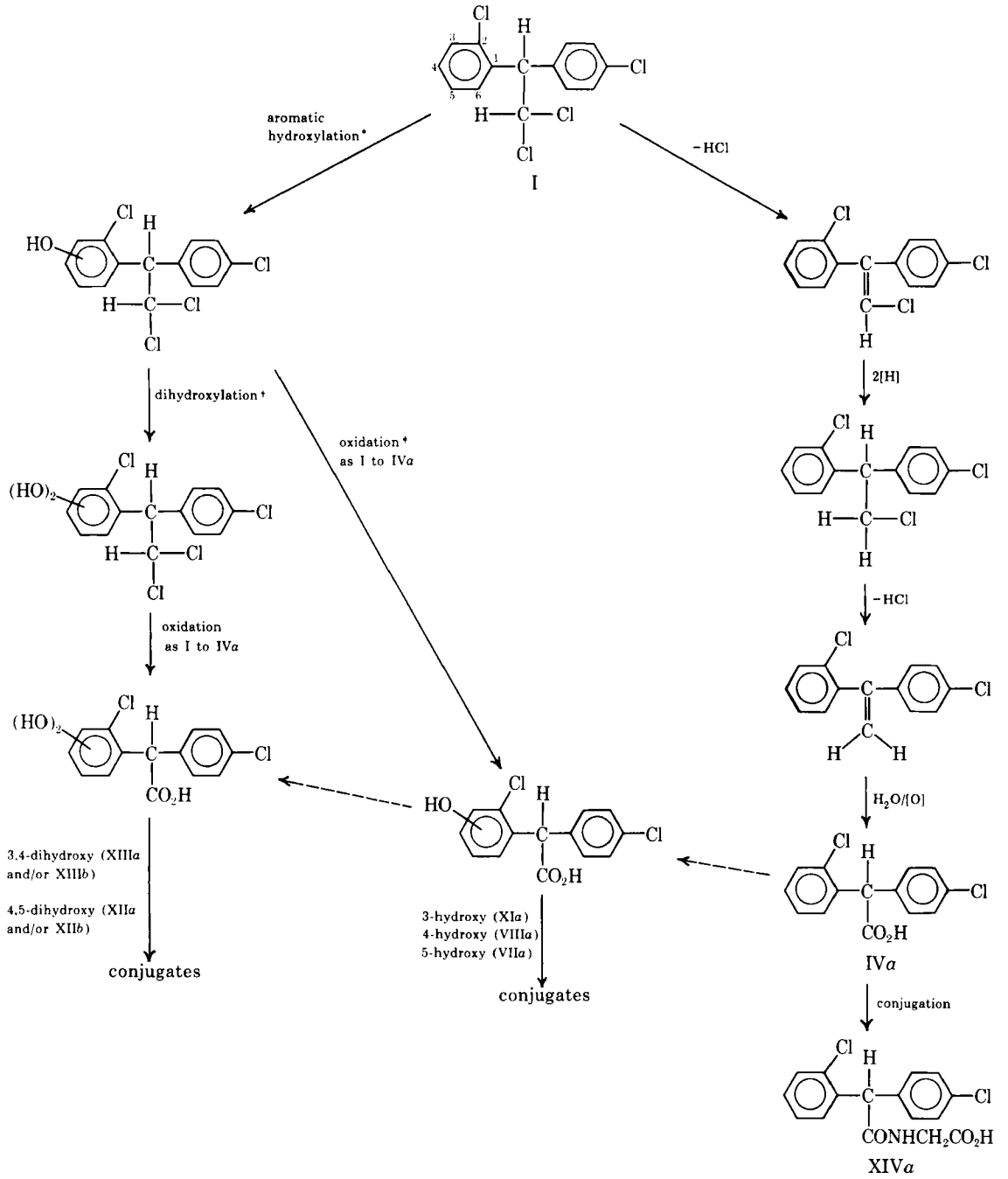
Recently, Feil *et al.* (13) identified a number of metabolites of the related compound 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane (*o,p'*-DDT) in rat feces which could also be metabolites of mitotane. They reported the hydroxylated carboxylic acid derivatives VII*a*, VIII*a*, XI*a*, and XIII*a*. Also reported were hydroxylated metabolites with an intact dichloro side chain and serine and glycine conjugates of the carboxylic acid IV*a*.

Based upon their work and that previously reported for analogous *o,p'*- and *p,p'*-dichlorodiphenyl-chlorinated ethane insecticides, it is reasonable to postulate the metabolic pathways for mi-

Table III—Quantitation by GLC of Metabolites of Mitotane in Urine from Patient 2 (N.P.)[a]

Days of Administration	*o,p'*-Dichlorodiphenylacetic Acid (IV*a*)	*o,p'*-Dichlorodiphenylacetic Acid Derivatives			
		Monohydroxy (VIII*a* + XI*a*)	Hydroxy Methoxy (XIII*a* + XIII*b*)	Glycine Conjugate (XIV*a*)	Total
15	0.006 (1.0)	0.002 (0.4)	0 (0)	0 (0)	0.008 (1.4)
21	0.077 (3.1)	0.036 (1.4)	0.037 (1.3)	0.027 (0.9)	0.177 (6.7)
34	0.013 (1.2)	0.006 (0.5)	0.005 (0.4)	—	0.024 (2.1)
100	0.012 (1.2)	0.005 (0.5)	0.006 (0.5)	0.005 (0.4)	0.028 (2.6)
100[b]	0.051 (5.0)	0.020 (1.9)	0.039 (3.4)	0 (0)	0.110 (10.3)
113	0.034 (2.2)	0.015 (0.9)	0.014 (0.8)	0.010 (0.6)	0.073 (4.5)
154	0.022 (3.8)	0.010 (1.6)	0.009 (1.3)	0.009 (1.3)	0.050 (8.0)
168	0.017 (5.8)	0.003 (0.9)	0.003 (1.0)	0.011 (3.2)	0.034 (10.9)
168[b]	0.075 (24.6)	0.023 (7.0)	0.052 (14.8)	0 (0)	0.150 (46.4)

[a] Values are milligrams of metabolite per milligram creatinine excreted. Values in parentheses represent percent excretion of the dose given on that day. Daily dose was 6 g until Day 145 when it was reduced to 2 g because of severe GI side effects to the patient. [b] Values found after acid hydrolysis of urine.



*Scheme II—Possible metabolic pathways for mitotane. Compounds IV*a, *VII*a, *VIII*a, *XI*a, *XII*a, *XII*b, *XIII*a, *XIII*b, *and XIV*a *are indicated in the present study.* * *Hydroxylation has been reported at the 3- or 5-position and* † *dihydroxylation has been reported at 3- and 4-positions for the metabolism of 1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2,2-trichloroethane in the rat (13).* ‡ *Dehalogenation and oxidation as reported for the chlorinated phenylethane insecticides (16).*

totane to be as outlined in Scheme II. This scheme is supported in the present study with indications for the presence of the carboxylic acid derivatives IV*a*, VII*a*, VIII*a*, XI*a*, XII*a*, XII*b*, XIII*a*, and XIII*b* as urinary metabolites of mitotane in humans. Unchanged mitotane was not detected in the urine. No metabolites with alkyl side chains intact nor intermediates toward oxidation to the carboxylic acid metabolites were isolated. Also there is no information as to relative extent of hydroxylation before or after side-chain oxidation.

Hydroxylation is primarily at the 4-position (VIII*a*) but is also at the 3-position (XI*a*). Dihydroxylation results in the 3,4-disubstituted isomers XIII*a* and/or XIII*b*. The presence of small amounts of 5-hydroxy (VII*a*) and 4,5-dihydroxy (XII*a* and/or XII*b*) metabolites is indicated by GLC studies only.

Acid hydrolysis of urine (Tables II and III) prior to extraction led to a higher percentage value of excretion for *o,p'*-dichlorodiphenylacetic acid (IV*a*) and its hydroxylated derivatives. Conjugates, other than the glycine derivative of IV*a* (XIV*a*) actually isolated, are indicated by the hydrolysis data. That is, the glycine conjugate does not account for the total increase of IV*a* nor the increase in the hydroxylated metabolites VIII*a* and XIII*a* or XIII*b* after hydrolysis.

Such conjugation is consistent with literature reports for analogous compounds. Thus, in addition to the serine and glycine conjugates of the carboxylic acid IV*a* reported by Feil *et al.* (13), serine and aspartic acid conjugates of bis(*p*-chlorophenyl)acetic acid have been reported (17) in the rat. Also the mouse and hamster are reported to conjugate this compound with alanine and glycine (18).

The quantitation data obtained did not exhibit any outstanding trends such as the appearance or disappearance of a given metabo-

lite. The variation in metabolite yield could be related to the method of analysis used and other factors such as fluctuating health, physical activity, and additional therapy received by the patient. Metabolite yields were expressed as a function of the creatinine excreted to correct for possible differences in renal clearance. These data show variations similar to that calculated as percent yield of daily dose administered.

In Patient 1, total metabolites detected before hydrolysis ranged from 18 to 31% of the dose administered on the day of urine collection. In Patient 2, they ranged from 1 to 11%. Hydrolysis data indicated a 56.5% total excretion for Patient 1; for Patient 2, excretions of 10.3 and 46.4% were indicated. These values are higher than Moy's (19) report that 10% of an oral daily dose of mitotane was excreted in the urine. He attributed 60% of the dose to unabsorbed material, with the remainder explained by tissue storage and biliary excretion.

EXPERIMENTAL[6]

2-Chloro-5-methoxymandelic Acid (V)—A saturated sodium bisulfite solution (20 ml) was added over 10 min to a stirred mixture of 3.0 g (0.059 mole) of sodium cyanide, 10 ml of water, and 10.0 g (0.059 mole) of 2-chloro-5-methoxybenzaldehyde (20). During this addition, 18.0 g of ice was added. The mandelonitrile layer was separated and placed in concentrated hydrochloric acid at room temperature for 18 hr, with subsequent boiling for 5 hr. The hydrolysis solution was extracted with ether, the ether was removed *in vacuo*, and the residue was recrystallized three times from benzene to give white crystals, mp 102–103.5°, 7.32 g (57.6%); IR (KBr): 3400 (OH), 3000, 1700 (C=O), 1575, 1460, 1425, 1250, 1175, 1140, 1100, 1030, 925, 855, 815, 725, 645, and 605 cm^{-1}; NMR (acetone-d_6): δ 7.40 (1H, d, $J_{4,3}$ = 9.0 Hz, ArH), 7.22 (1H, d, $J_{4,6}$ = 3.0 Hz, ArH), 6.95 (1H, d of d, $J_{3,4}$ = 9.0 Hz, $J_{6,4}$ = 3.0 Hz, ArH), 5.63 (1H, s, CH), and 3.82 (3H, s, OCH_3).

Anal.—Calc. for $C_9H_9ClO_4$: C, 49.90; H, 4.19. Found: C, 49.78; H, 4.02.

2-Chloro-4-methoxymandelic Acid (VI)—The same procedure as for V was used. Amounts of reactants were 0.75 g (0.015 mole) of sodium cyanide, 2.49 ml of water, 2.50 g (0.015 mole) of 2-chloro-4-methoxybenzaldehyde (21), 4.25 ml of saturated sodium bisulfite solution, and 4.5 g of ice. The crude VI obtained was recrystallized three times in benzene to give white crystals, mp 108.5–110.0°, 1.26 g (39.3%); IR (KBr): 3460 (OH), 3070, 1730 (C=O), 1590, 1470, 1300, 1245, 1190, 1070, 1045, 930, 890, 820, 730, 710, 610, and 490 cm^{-1}; NMR (acetone-d_6): δ 7.53 (1H, d, $J_{5,6}$ = 8.0 Hz, ArH), 7.03 (1H, d, $J_{5,3}$ = 2.5 Hz, ArH), 6.98 (1H, d of d, $J_{6,5}$ = 8.0 Hz, $J_{3,5}$ = 2.5 Hz, ArH), 5.58 (1H, s, CH), and 3.85 (1H, s, OCH_3).

Anal.—Calc. for $C_9H_9ClO_4$: C, 49.90; H, 4.19. Found: C, 49.70; H, 4.29.

Methyl 1-(2-Chloro-5-methoxyphenyl)-1-(4-chlorophenyl)acetate (VII)—A flask, with magnetic stirring bar, containing a mixture of 2.32 g (0.0107 mole) of V and 1.44 g (0.0129 mole) of chlorobenzene was placed in an ice bath and 5 ml of concentrated sulfuric acid was added over 15 min. After 1 hr the ice bath was removed and the reaction was continued for 2.5 hr at room temperature. Ice was added to the flask, the mixture was extracted with ether, and the ether layer was extracted with 3 × 30 ml of 5% $NaHCO_3$. The sodium bicarbonate was acidified and extracted with ether, which was dried over sodium sulfate and treated with ethereal diazomethane. The ether was removed *in vacuo* and the residue was distilled, bp 165–170° (0.500 mm), 1.33 g (39.1%); IR (NaCl plates): 3010, 2950, 2840, 1725 (C=O), 1575, 1550, 1450, 1300, 1200, 1165, 1095, 1065, 1030, 1025, 815, 755, 635, and 505 cm^{-1}; NMR ($CDCl_3$): δ 7.31 (4H, s, ArH), 7.31 (1H, d, $J_{4,3}$ = 8.5 Hz, ArH), 6.90 (1H, d, $J_{4,6}$ = 3.0 Hz, ArH), 6.76 (1H, d of d, $J_{3,4}$ = 8.5 Hz, $J_{6,4}$ = 3.0 Hz, ArH), 5.47 (1H, s, CH), 3.72 (1H, s, OCH_3), and 3.70 (1H, s, OCH_3); mass spectroscopy (70 ev): *m/e* (relative intensity) 326 (26) M + 2, 325 (7) M + 1, 324 (38) M^+, 291 (7), 289 (21) M^+ − Cl, 267 (65), 266 (8), 265 (100) M − $C_2H_3O_2$, 232 (4), 231 (5), 230 (12), 229 (12), 217 (5), 215 (15), 195 (33), 186 (12), 163 (4), and 152 (34).

Anal.—Calc. for $C_{16}H_{14}Cl_2O_3$: C, 59.10; H, 4.34. Found: C, 59.15; H, 4.34.

Methyl 1-(2-Chloro-4-methoxyphenyl)-1-(4-chlorophenyl)-acetate (VIII)—In a mortar, 4.95 g (0.039 mole) of 3-chlorophenol and 6.00 g (0.032 mole) of 4-chloromandelic acid were mixed and then transferred to a flask placed in ice. Over 15 min, 15 ml of concentrated sulfuric acid was added while the mixture was stirred with a glass rod. The reaction was continued in ice for 1 hr and at room temperature for 3 hr with magnetic stirring. Workup was as in the preparation of VII. Treatment of the sodium bicarbonate-soluble fraction with diazomethane overnight gave a residue of 833 mg after solvent evaporation. With the aid of a Craig microdistillation apparatus, a fraction, bp 170–180° (0.500 mm), was obtained and recrystallized three times with methanol to give VIII, mp 82.5–84.0°; IR (KBr): 3100, 2950, 1710 (C=O), 1575, 1550, 1460, 1300, 1235, 1210, 1165, 1095, 1045, 1020, 1000, 860, 845, 765, 685, 565, and 495 cm^{-1}; NMR ($CDCl_3$): δ 7.32 (4H, s, ArH), 7.24 (1H, d, $J_{5,6}$ = 7.0 Hz, ArH), 7.01 (1H, d, $J_{5,3}$ = 2.5 Hz, ArH), 6.82 (1H, d of d, $J_{3,5}$ = 2.5 Hz, $J_{6,5}$ = 7.0 Hz, ArH), 5.43 (1H, s, CH), 3.80 (3H, s, OCH_3), and 3.77 (3H, s, OCH_3); mass spectroscopy (70 ev): *m/e* (relative intensity) 326 (13) M + 2, 325 (6) M + 1, 324 (19) M^+, 267 (67), 266 (17), 265 (100) M − $C_2H_3O_2$, 231 (9), 230 (8), 229 (13), 199 (5), 187 (15), 186 (11), 165 (5), 163 (4), and 152 (4).

Anal.—Calc. for $C_{16}H_{14}Cl_2O_3$: C, 59.10; H, 4.34. Found: C, 58.91; H, 4.40.

The crude methylated sodium bicarbonate-soluble fraction contained 65.6 mg of VIII (0.63% yield) as quantitated by GLC (OV-17 column at 250°). The fraction also contained 148.6 mg of methyl 4-chlorobenzoate, identified by its mass spectrum and GLC retention time comparisons and quantitated on OV-17 at 140° with 2°/min program.

The crude unmethylated sodium bicarbonate-soluble fraction was chromatographed on commercial 2-mm × 20-cm × 20-cm silica gel F-254 preparative TLC plates[7] with an acetic acid–chloroform (4:96 v/v) solvent system and development twice to 15 cm. Three bands and material at the origin were detected *via* fluorescent quenching at 254 nm. After separation on four plates with 100 mg of material on each, the bands were extracted with acetone and the solvent was removed. Residue from band one (R_f 0.64) sublimed to give 48.3 mg of 4-chlorobenzoic acid, identified by comparing its NMR and IR spectra with commercial material. The residue from band three (R_f 0.15) was isolated as an oil. The NMR ($CDCl_3$) indicated VIII*a*: δ 7.40 (4H, s, ArH), 7.25 (1H, d, J = 8.5 Hz, ArH), 7.03 (1H, d, J = 8.5 Hz, ArH), 6.87 (1H, d of d, J = 2.5 Hz, J = 8.5 Hz, ArH), and 5.47 (1H, s, CH). Methylation of the residue with diazomethane and crystallization with methanol gave 24.7 mg of VIII.

1-(2-Hydroxy-4-chlorophenyl)-1-(4-chlorophenyl)acetic Acid Lactone (IX)—The ether fraction remaining after the sodium bicarbonate extraction in the preparation of VIII was dried over sodium sulfate. The ether was removed *in vacuo* and the residue was recrystallized three times with methanol, mp 119–121°, 1.91 g (21.4%); IR (KBr): 3100, 1795 (C=O), 1600, 1460, 1230, 1195, 1180, 1135, 1090, 1055, 1015, 920, 855, 810, 800, and 500 cm^{-1}; NMR ($CDCl_3$): δ 7.28 (7H, m, with major peaks at 7.34, 7.23, and 7.22, ArH) and 4.87 (1H, s, CH); mass spectroscopy (70 ev): *m/e* (relative intensity) 280 (12) M + 2, 279 (4) M + 1, 278 (18) M^+, 251 (12), 250 (4), 249 (17) M − CHO, 217 (33), 216 (15), 215 (100) M − CO − Cl, 201 (2), 199 (16), 186 (5), 163 (4), 152 (28), and 125 (8).

Anal.—Calc. for $C_{14}H_8Cl_2O_2$: C, 60.24; H, 2.89. Found: C, 60.12; H, 2.91.

Methyl 1-(2-Methoxy-4-chlorophenyl)-1-(4-chlorophenyl)acetate (X)—Compound IX (1.91 g) was refluxed in 5% NaOH for 5 min. The sodium hydroxide was acidified with 10% HCl and

[6] A Loenco 160 GLC instrument equipped with flame-ionization detector and a 3% OV-17 on Gas Chrom Q (100–120) stainless steel column [1.8 m × 0.3 cm (6 ft × 0.125 in.)], with 30 ml/min helium carrier gas flow, was used where retention time determinations and quantitation involved use of this column. A 3% OV-25 on Chromasorb G (100–120) stainless steel column [1.8 m × 0.3 cm (6 ft × 0.125 in.)], with 30 ml/min helium carrier gas flow, was also used for retention time determinations. Preparative work was on a Varian Aerograph 90-P equipped with thermocouple detector and a 3% SE-30 on Gas Chrom Q (100–120) stainless steel column [1.5 m × 0.6 cm (5 ft × 0.25 in.)], with a helium carrier flow of 60 ml/min. Quantitation of metabolite levels was on a Hewlett-Packard 5750 equipped with a flame detector and a 3% OV-1 on Gas Chrom Q (100–120) stainless steel column [1.8 m × 0.3 cm (6 ft × 0.125 in.)], with a helium carrier flow rate of 65 ml/min. A DuPont 21-490 single-focusing instrument with the Loenco 160 GLC instrument was used for GLC–mass spectroscopy determinations.

[7] Brinkmann.

extracted with ether. The ether layer was dried over sodium sulfate, the solvent was removed, and the residue was isolated as an oil. NMR ($CDCl_3$) indicated Xa: δ 7.32 (4H, s, ArH), 6.87 (3H, m, ArH), and 5.18 (1H, s, CH). Diazomethane treatment of the residue and recrystallization four times with methanol gave crystals, mp 75–77°, 1.68 g (74.7% based on IX); IR (KBr): 3100, 2950, 1700 (C=O), 1560, 1475, 1375, 1305, 1250, 1215, 1165, 1115, 1090, 1030, 1020, 1000, 890, 845, 770, and 500 cm^{-1}; NMR ($CDCl_3$): δ 7.33 (4H, s, ArH), 7.02 (1H, s, ArH), 6.98 (1H, d, J = 2.0 Hz, ArH), 6.93 (1H, d, J = 2.0 Hz, ArH), 5.27 (1H, s, CH), 3.83 (3H, s, OCH_3), and 3.75 (3H, s, OCH_3); mass spectroscopy (70 ev): *m/e* (relative intensity) 326 (19) M + 2, 325 (6) M + 1, 324 (29) M^+, 295 (8), 293 (12) M − CH_3O, 267 (68), 266 (19), 265 (100) M − $C_2H_3O_2$, 251 (6), 249 (7), 231 (3), 230 (3), 229 (5), 217 (6), 215 (17), 199 (8), 197 (4), 195 (4), 186 (7), 165 (21), 152 (19), and 125 (16).

Anal.—Calc. for $C_{16}H_{14}Cl_2O_3$: C, 59.10; H, 4.34. Found: C, 59.10; H, 4.27.

Methyl 1-(2-Chloro-4,5-dimethoxyphenyl)-1-(4-chlorophenyl)acetate (XII)—Following the procedure used for VII, 2.00 g (0.0107 mole) of 4-chloromandelic acid and 2.22 g (0.0129 mole) of 4-chloroveratrole, prepared by dimethyl sulfate methylation of 4-chlorocatechol (22), were used. The sodium bicarbonate-soluble residue obtained was sublimed under vacuum and recrystallized twice with isopropanol to give white crystals, mp 81–83°, 1.32 g (38.3%); IR (KBr): 2950, 1725 (C=O), 1475, 1375, 1310, 1270, 1250, 1210, 1160, 1090, 1030, 1010, 965, 925, 860, 840, 780, and 755 cm^{-1}; NMR ($CDCl_3$): δ 7.30 (4H, s, ArH), 6.94 (2H, s, ArH), 5.47 (1H, s, CH), 3.86 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), and 3.78 (3H, s, OCH_3); mass spectroscopy (70 ev): *m/e* (relative intensity) 356 (17) M + 2, 355 (4) M +1, 354 (26) M^+, 324 (3), 322 (4), 297 (65), 295 (100) M − $C_2H_3O_2$, 261 (4), 259 (9) M − $C_2H_3O_2$ − HCl, 247 (4), 245 (13) M − $ClC_2H_3O_2$ − CH_3, 255 (13), 202 (7), 199 (9), 182 (9), 181 (9), 173 (9), 152 (11), 139 (17), and 125 (13).

Anal.—Calc. for $C_{17}H_{16}Cl_2O_4$: C, 57.48; H, 4.54. Found: C, 57.34; H, 4.59.

***o,p'*-Dichlorodiphenylacetyl Glycine Methyl Ester (XIV)**—Starting material IVa was synthesized according to literature methods (10). A solution of 0.35 g (0.00125 mole) of IVa, 0.16 g (0.00125 mole) of glycine methyl ester hydrochloride, and 0.054 g (0.00125 mole) of triethylamine in 25 ml of benzene–methanol (4:1) was added to 0.32 g (0.0013 mole) of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (23). The solution was stirred at room temperature for 5 hr, washed with 10 ml of water, and then washed with 2 × 10 ml of 5% HCl. After removal *in vacuo* of the reaction solvent, the residue was recrystallized in isopropanol to give white crystals, mp 135–137°, 0.296 g (67.8%); IR (KBr): 3275, 3075, 2930, 1725 (C=O), 1625 (C=O), 1525, 1220, 1050, 810, and 760 cm^{-1}; NMR ($CDCl_3$): δ 7.36 (8H, s, ArH), 5.46 (1H, s, CH), 4.14 (2H, d, N—CH_2), and 3.78 (3H, s, OCH_3); mass spectroscopy (70 ev): *m/e* (relative intensity) 318 (26) M + 2 − Cl, 317 (17), 316 (71) M^+ − Cl, 237 (36), 236 (24), 235 (55) M^+ − $C_4H_6O_3N$, 203 (12), 201 (43), 199 (29), 165 (71), 116 (69), and 88 (100).

Anal.—Calc. for $C_{17}H_{15}Cl_2NO_3$: C, 57.97; H, 4.29; N, 3.98. Found: C, 57.69; H, 4.40; N, 4.00.

Urine Extraction—Urine collected over 24 hr was pooled, and approximately 800-ml aliquots of this urine were adjusted to pH 2 with 10% HCl and continuously extracted with ether for 48 hr. The ether layer was reduced to 150 ml and extracted with 4 × 100 ml of 5% $NaHCO_3$. The sodium bicarbonate layer was acidified with 10% HCl and extracted with 4 × 200 ml ether. The ether phase was dried over sodium sulfate and treated with diazomethane followed by solvent evaporation. The resulting residue was taken up in 1 ml chloroform and used for GLC–mass spectroscopy.

The ether layer remaining after sodium bicarbonate extraction was reextracted with 4 × 100 ml of 5% NaOH. The sodium hydroxide extract was treated as the sodium bicarbonate extract above. The ether layer remaining after sodium hydroxide extraction was dried, methylated, and reduced to a residue which was taken up in 1 ml of chloroform. This procedure is outlined in Scheme I.

Quantitation of Metabolites—A known amount (25–50 mg/100 ml of urine) of bis(*p*-chlorophenyl)acetic acid (*p,p'*-DDA)[8] was added as an internal standard to 100–800-ml aliquots of urine. The urine was extracted and the methylated sodium bicarbonate fraction was prepared as already described. Standard solutions (5–10 mg/ml chloroform) of synthetic IV, VIII, XII, XIV, and the methyl ester of the internal standard were used for calibration. The 4-methoxy (VIII) and 3-methoxy (XI) compounds cochromatographed and gave an equal response of peak height *versus* amount injected. Synthetic VIII was used to quantitate the mixture of the two. The 4,5-dimethoxy synthetic standard (XII) was used to quantitate the 3,4-dimethoxy metabolite (XIII) because only XII was available as a crystalline compound of known purity. It is assumed that detector response *versus* amount injected is the same for both compounds. Amounts of standard solution were injected such that peak heights of the standard and the compound in urine extract were similar. Within the range of amounts injected, the graph of peak height *versus* amount injected was linear. GLC conditions, using an OV-1 column, are given in Table I.

The amounts of metabolites found in urine extracts were corrected by multiplying the ratio of the internal standard added to unextracted urine to the amount detected by GLC in urine extract. Values of milligrams metabolite extracted per milligram creatinine present were calculated. Creatinine determinations were by the method of Taussky (24). Creatinine and milligram metabolite values used are averages of three determinations. The percent daily dose excreted was calculated by dividing the milligrams metabolite excreted in a 24-hr urine sample by the dose in milligrams given on that day.

Hydrolysis—The internal standard bis(*p*-chlorophenyl)acetic acid was added to patient urine, which was then hydrolyzed by refluxing with an equal volume of concentrated hydrochloric acid for 4 hr. The hydrolyzed urine was extracted and a methylated sodium bicarbonate fraction was obtained and quantitated by the methods already described.

Methylation—Ethereal diazomethane was prepared by literature methods (25). Excess diazomethane was added to the sample dissolved in methanol and ether (1:1) and allowed to stand for 24 hr, analogous to literature procedures (12).

[8] Aldrich.

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Phosphorus–Nitrogen Compounds XVIII: Hydrazides and Thiosemicarbazides

L. A. CATES[x] and T. L. LEMKE

Abstract □ Phenylphosphono(thio)dihydrazides and phosphorylated thiosemicarbazides were synthesized for anticancer testing, and some of these agents displayed chelation properties. Reactions involving methylhydrazine resulted in substitution of N^1-protons. A study of diphenylphosphoro- and phosphoramidomethylhydrazides also indicated that the N^1-phosphorylated isomers are predominately formed.

Keyphrases □ Phosphorus–nitrogen compounds—synthesis of hydrazides and thiosemicarbazides as potential anticancer agents □ Nitrogen–phosphorus compounds—synthesis of hydrazides and thiosemicarbazides as potential anticancer agents □ Thiosemicarbazides, phosphorylated—synthesis as potential anticancer agents □ Hydrazides—synthesis of phenylphosphono(thio)dihydrazides as potential anticancer agents □ Anticancer agents, potential—synthesis of phenylphosphono(thio)dihydrazides and phosphorylated thiosemicarbazides

The hydrazino moiety frequently appears in agents possessing antitumor activity. The presence of this grouping in some of these compounds, such as procarbazine, indole-3-propionic acid hydrazide, α-hydrazino-ω-cyclohexylbutyric acid, 5-hydrazide 1-glutamic acid, and 2-(4-nitroso-7-oxo-1,3,5-cycloheptatrien-1-yl)isonicotinic acid hydrazide (1), is obvious, while in other agents its occurrence is less conspicuous. Various thiadiazole, pyrazole, triazeno, and azapurine oncolytics also contain the N—N bond. In a few cases, such as procarbazine and heterocyclic 2-carboxaldehyde thiosemicarbazones, the hydrazino grouping is believed to be involved in the cytotoxic process *via* redox reactivity (2) or chelation (3), respectively. Even hydrazine sulfate inhibits Walker carcinosarcoma by 28–94%, possibly through gluconeogenesis interference (4).

The confirmed activity of 4,4′,4″-phosphinylidyne trisemicarbazide[1] (5) in Walker carcinosarcoma encouraged the preparation of additional hydrazine compounds (I–XII) as potential antitumor agents. Six of the products can be classified as thiosemicarbazides, including a derivative (XII) of the anticancer agent 2-amino-1,3,4-thiadiazole, which contains a comparable structure in cyclic form. The remaining compounds are hydrazides (I–V and XI), including one containing a urethan portion (V) and a water-soluble dihydrazide (XI). This latter product may find interest in Walker carcinosarcoma in view of the probable greater sensitivity of this tumor toward hydrophilic drugs (6), while the methylhydrazides, I and II, were considered of interest in view of the established activity of procarbazine.

DISCUSSION

During this investigation, particular attention was directed toward the reaction involving the monophosphorylation of methylhydrazine. This reactant possesses two nitrogens with replaceable hydrogens, and both N^1- or N^2-substitution products are possible (Scheme I). Theoretically, the more nucleophilic N^1 should preferentially undergo substitution. Debo (7), however, reported that N^2-phosphorylation occurred when phosphorochloridates were reacted with methylhydrazine in sodium carbonate solution. Support for this assignment is found in work with the reactions of methylhydrazine with esters and anhydrides of carboxylic acids (8). This study showed that, while anhydrides form mainly 1-acyl-1-methylhydrazides, esters yield chiefly 1-acyl-2-methylhydrazides, with the percentage of these latter isomers increasing with the size of the acyl group of the esters.

Debo's synthesis using diphenylphosphorochloridate was repeated during this study and the reaction products were examined by NMR spectroscopy. Of the three methylhydrazides reported by

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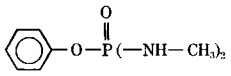
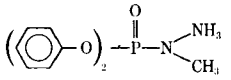
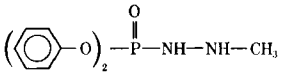
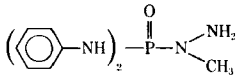
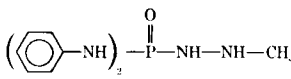
Table I—NMR Spectra of Isomeric Hydrazides and Phenyl *N,N'*-Dimethylphosphorodiamidate[a]

Compound	Structure	δ, ppm	Assignment	Signal[b]	Protons	*J*, Hz
XIII		2.50 7.22	N-CH_3 Ar-H	d s	6 5	12 —
XIV		2.97 7.35	N-CH_3 Ar-H	d s	3 10	8 —
XV		2.53 7.35	N-CH_3 Ar-H	s s	0.3 10	— —
XVI		2.87 6.28 7.04	N-CH_3 Ar-N-H Ar-H	d d s	3 2 10	12 14 —
XVII		2.34 6.28 7.04	N-CH_3 Ar-N-H Ar-H	s d s	0.3 2 10	— 14 —

[a] Taken on Varian T-60 or EM 360 spectrometer using deuterated chloroform as the solvent and tetramethylsilane as the internal reference. The N-H signals, other than anilino-H, are generally broad and poorly defined. [b] Singlet and doublet are abbreviated s and d, respectively.

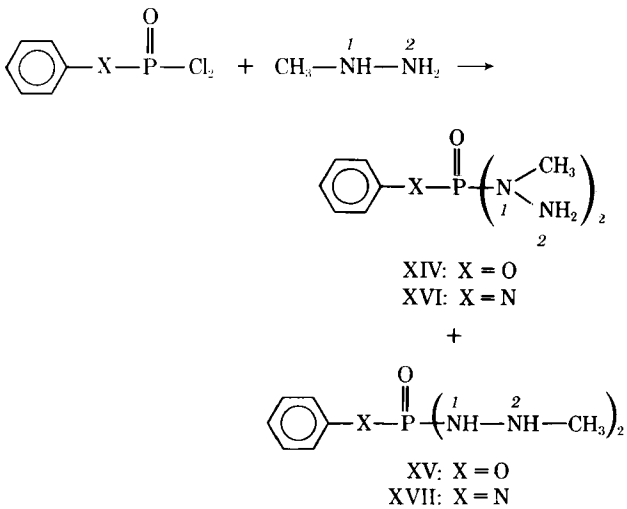
Debo, the bulkier diphenyl ester derivative was chosen as more likely to result in the N^2-substituted product. Interpretation of the NMR findings is based on the investigation of Nielsen and Sisler (9), who reported the spectra of phosphorus hydrazides but confined their study to unsymmetrical dimethyl-, trimethyl-, and dimethylethylhydrazines, each of which permits only one product.

Hydrazides resulting from monophosphorylation of dimethylhydrazine possess the N^1-H system which gives doublets (δ3.7, 3.8, 4.0, and 4.5). The N^2-methyl protons, however, were not split by phosphorus and showed singlets at δ2.4, 2.5, 2.5, 2.6, and 3.2. However, when a methyl or methylene is attached to N^1, as in the case of one 1,1,2-trimethyl- and two 1,1-dimethyl-2-ethylhydrazides, these protons gave a doublet (δ2.5) and quadruplets (δ3.3 and 3.3), respectively. Phenyl *N,N'*-dimethylphosphorodiamidate (XIII) was prepared for NMR study and showed similar splitting (Table I). Therefore, N^1-H, N^1-CH_2-, and N^1-CH_3 protons are split by phosphorus while N^2-methyl protons are not.

When the unpurified, water-insoluble oil product from the Debo synthesis was examined, it showed a doublet, assigned to XIV, and a nonreplaceable singlet (Table I and Fig. 1). This latter signal was assigned to the methyl protons of the N^2-substituted product, XV, with the amount of this material estimated by integration of the signals to be 10%. The same results were obtained from the crude products synthesized by the reaction of diphenylphosphonate–carbon tetrachloride–methylhydrazine and of diphenylphosphorodiamidic chloride–methylhydrazine (XVI and XVII, Table I). TLC of the mixture of XIV and XV gave two spots attributable to each isomer. Column chromatographic separation was only partially successful and provided for the isolation of only XIV. In addition, spectra of the pure products isolated from the reaction of phenylphosphonic dichloride (I), phenylphosphonothioic dichloride (II), and methylhydrazine showed only doublets for the methyl protons. Unsymmetrical hydrazide XI provides a compound containing both P—N—CH_3 and P—N—N—CH_3 systems, and its NMR spectrum shows a doublet (two methyls) and a singlet (four methyls) (Table II).

These data demonstrate that the percent composition of a mixture of isomers can readily be estimated by NMR spectroscopy. With regard to selective phosphorylation of a monosubstituted hydrazine, a similar situation was encountered when *N,N*-dimethylphosphoramidic dichloride and phenylphosphorodichloridate were reacted with 2-hydroxyethylhydrazine. The 3-*N*-amino-1,3,2-oxazaphospholidines were shown, *via* IR and NMR spectroscopy and derivatization, to result from substitution on the secondary nitrogen (10).

The new hydrazides (I–V and XI) and thiosemicarbazides (VI–X and XII) were synthesized *via* conventional substitution and condensation reactions, respectively. The dihydrazide V was the product from an attempt at the synthesis of the monohydrazide, a



XIV: X = O
XVI: X = N

+

XV: X = O
XVII: X = N

Scheme I

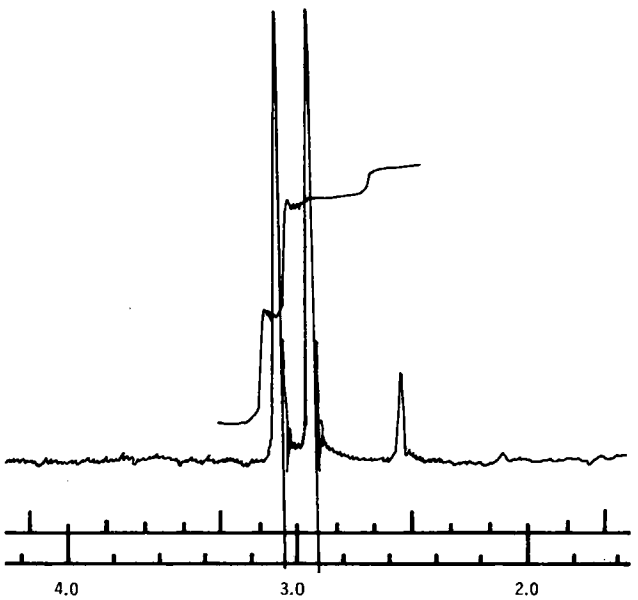
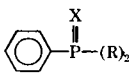
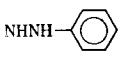
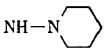
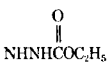
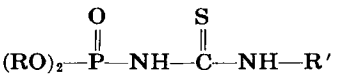
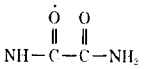
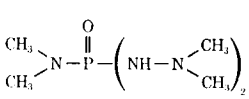
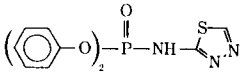


Figure 1—*Portion of an NMR spectrum produced by a mixture of isomers XIV and XV. The solvent was $CDCl_3$ and D_2O.*

Table II—Hydrazides and Thiosemicarbazides

Compound	X	R	Melting Point[a]	Formula	Analysis, %[b]		NMR Spectra[c]				
					Calc.	Found	δ, ppm	Assignment	Signal	Protons	J, Hz
											
I	O		139–143° dec.	$C_8H_{15}N_4OP$	C 44.86 H 7.06 N 26.16	45.10 7.07 26.06	2.91 7.50 to 8.14	$N\text{-}CH_3$ Ar-H	d m	6 5	9 —
II	S		96–99°	$C_8H_{15}N_4PS$	C 41.73 H 6.57 N 24.33	41.92 6.50 24.37	2.82 7.31 to 8.32	$N\text{-}CH_3$ Ar-H	d m	6 5	12 —
III	O		188–190° dec.	$C_{18}H_{19}N_4OP$	C 63.90 H 5.66 N 16.56	63.60 5.66 16.50	6.94 to 7.72	Ar-H	m	—	—
IV	O		179–180°	$C_{16}H_{27}N_4OP$	C 59.61 H 8.44 N 17.38	59.78 8.55 17.22	1.59 2.62 7.17 to 7.62 7.75 to 8.26	C-H C-H Ar-H Ar-H	m m m m	12 8 3 2	— — — —
V	O		197–198° dec.	$C_{12}H_{19}N_4O_5P$	C 43.64 H 5.80 N 16.96	43.70 5.88 16.91	1.22 4.07 7.26 to 8.13	C-H C-H Ar-H	t q m	6 4 5	7 6 —
											
	R	R′									
VI	CH_3CH_2		153°	$C_9H_{20}N_3C_4PS$	C 36.36 H 6.78 N 14.13	36.47 6.81 14.05	1.32 2.88 3.74 3.89 to 4.99	C-H C-H[d] C-H[d] C-H	t m m m	6 4 4 4	7 — — —
VII			167–168° dec.	$C_{17}H_{20}N_3O_4PS$	N 11.13	10.71	2.60 3.71 7.30	C-H[d] C-H[d] Ar-H	m m s	4 4 10	— — —
VIII	CH_3CH_2		129°	$C_{10}H_{22}N_3O_3PS$	C 40.67 H 7.51 N 14.23	40.79 7.55 14.16	0.99 to 1.58 1.71 2.86 3.93 to 4.51	C-H C-H[e] C-H[e] C-H	m m m m	6 6 4 4	— — — —
IX			161–162° dec.	$C_{18}H_{22}N_3O_3PS$	C 55.23 H 5.67 N 10.73	55.34 5.72 10.65	1.55 2.72 7.31	C-H C-H Ar-H	m m s	6 4 10	— — —
X			153–154° dec.	$C_{15}H_{15}N_4O_5PS$	C 45.69 H 3.83 N 14.21	45.47 3.98 14.04	6.63 to 7.82	Ar-H	m	—	—
				Miscellaneous							
XI			96–98°	$C_6H_{20}N_3OP$	C 34.44 H 9.63 N 33.47	34.32 9.72 33.59	2.58 2.75	$N\text{-}CH_3$ $N\text{-}CH_3$	s d	12 6	— 9
XII			158° dec.	$C_{14}H_{12}N_3O_3PS$	C 50.45 H 3.63 N 12.61	50.80 3.68 12.62	7.37 to 7.73 8.23	Ar-H C-H	m d	10 1	— 2

[a] Performed on a Fisher-Johns apparatus and are uncorrected. [b] Performed by Atlantic Microlab, Inc., Atlanta, Ga. [c] Taken on Varian T-60 or EM 360 spectrometer using deuterated chloroform (II, V, VIII, X, and XII) or deuterated dimethyl sulfoxide (I, II, IV, VI, VII, IX, and XI) as the solvent and tetramethylsilane as the internal reference. Generally, N-H signals were indistinct and are not reported. Singlet, doublet, triplet, quartet, and multiplet splittings are abbreviated s, d, t, q, and m, respectively. [d] Morpholino-H. [e] Piperidino-H.

proposed candidate for intramolecular dehydrohalogenation to the imide derivatives.

In view of the antitumor activity associated with chelation properties (3), the ability of six derivatives to complex Co^{+2}, Cu^{+2}, Fe^{+3}, and Ni^{+2} ions was demonstrated. Those agents displaying ligand properties were IV–VI (Co, Cu, Ni), VII and IX (Co, Ni), and VIII (Co, Fe, Ni). The thioic analog of III (X = S) was reported (11) to have some activity against E0771 tumor in mice.

Samples of Compounds I–XII have been submitted to the National Cancer Institute for antitumor testing.

EXPERIMENTAL

Phenylphosphono(thio)dihydrazides (I–V)—Solutions of 1 mole of phenylphosphonic dichloride or phenylphosphonothioic dichloride in ether were added to ethereal solutions of 4 moles of methylhydrazine (I and II), a mixture of 2 moles of phenylhydrazine (III) or *N*-aminopiperidine (IV) and 2 moles of triethylamine, or 2 moles of ethyl carbazate (V) placed in a three-necked, round-bottom flask equipped with dropping funnel and condenser fitted with a drying tube. The dichlorides were added at a rate sufficient to maintain reflux conditions. The reaction mixtures were filtered, the precipitates were washed with water, and any insoluble material was added to the filtrates which were then spin evaporated. The residues were crystallized from petroleum ether (bp 30–60°) for I, benzene for II, or ethanol for III–V.

Phosphorylated Thiosemicarbazides (VI–X)—Equal molar quantities of either diethyl- or diphenylphosphoroisothiocyanatidate were added to solutions of *N*-aminomorpholine (VI and VII), *N*-aminopiperidine (ether) (VIII and IX), or oxamic dihydrazide (acetonitrile) (X). After remaining at ambient temperature for 24 hr, the precipitates that formed were collected on a filter and crystallized from ethanol.

***N,N*-Dimethyl-N^2,N^2-dimethylphosphoramidodihydrazide (XI)**—A mixture of 1 mole of *N,N*-dimethylphosphoramidic dichloride (12) and 4 moles of 1,1-dimethylhydrazine in ether was refluxed for 4 hr and then filtered. The filtrate was spin evaporated to yield an oil, which precipitated as crystals from its ethereal solution. An IR spectrum[2] of XI showed absorptions consistent with this structure.

Diphenyl *N*-2-(1,3,4-Thiadiazolyl)phosphoramidate (XII)—One mole of diphenylphosphorochloridate was added to a dioxane solution of 1 mole each of 2-amino-1,3,4-thiadiazole and triethylamine. The filtrate from the reaction mixture was treated with water to yield a precipitate, which was collected on a filter and crystallized from ethanol.

Isomeric Diphenylphosphoro- and Phosphoramidomethylhydrazides (XIV–XVII)—The reaction between diphenylphosphorochloridate and methylhydrazine in 20% sodium carbonate solution was conducted according to the procedure of Debo (7). The resultant semisolid was dissolved in chloroform, and the solution was dried over calcium sulfate and filtered. The filtrate was spin evaporated; samples of the residue were then used for NMR spectroscopic examination, TLC, column chromatography, and purification. An NMR spectrum of the crude product was obtained, and the pertinent absorptions are shown in Fig. 1 and Table I.

Spectra of both starting materials, chloroform solvent, deuterated chloroform, and deuterium oxide were run separately and showed no peaks in this region. TLC [methanol–chloroform (1:10)] of the crude product and reactants revealed two spots not attributable to the latter. A sample placed on a silica gel column was eluted with chloroform to yield XIV and then with methanol. The latter eluate gave a material whose NMR spectrum failed to show aromatic proton absorption. Crystallization of the crude product from ether gave XIV, mp 53–54°. The unpurified product obtained from the reaction between diphenylphosphonate, carbon tetrachloride, and dimethylamine (13) gave results identical to those reported here.

Methylhydrazine (2 moles) in acetonitrile was added dropwise with stirring and cooling at 0–5° to an acetonitrile solution of *N,N*-diphenylphosphorochloridate (14). After remaining at ambient temperature for 48 hr, the reaction mixture was filtered. Spin evaporation of the filtrate produced a brown semisolid. NMR spectroscopic examination (Table I) and TLC [methanol–chloroform (1:10)] of this material indicated that it consisted of XVI and XVII. Crystallization of the crude product from ethanol–water yielded white crystals of XVI.

Phenyl *N,N*-Dimethylphosphorodiamidate (XIII)—This compound was synthesized according to the procedure of Audrieth and Toy (15).

Chelation Studies—To 2 ml of 1% ethanol solutions of V–IX were added 0.2 ml of 1% ethanol solutions of cobaltous, cupric, ferric, or nickelous chloride followed by 0.05 ml of 10% alcoholic potassium hydroxide. Solutions of ethyl carbazate, *N*-aminopiperidine, and *N*-aminomorpholine were treated in the same manner. The formation of colors in the former tests, prior to and after the addition of base, as compared to those involving starting materials was interpreted as the ability to chelate the metal ions.

[2] Beckman IR-8 spectrometer.

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[x] To whom inquiries should be directed.

Monoamine Oxidase Inhibitory and Anticonvulsant Properties of 1,2,4-Trisubstituted 5-Imidazolones

MAHIMA VERMA*, ARVIND K. CHATURVEDI*, ANSHUMALI CHAUDHARI*, and SURENDRA S. PARMAR*‡×

Abstract □ Several 1,2,4-trisubstituted 5-imidazolones were synthesized and characterized by their sharp melting points and elemental analyses. All substituted imidazolones inhibited activity of monoamine oxidase in rat brain homogenate. Preincubation of the brain homogenates with these substituted imidazolones, prior to the addition of the substrate (kynuramine), in no way altered the degree of enzyme inhibition. On further evaluation, one of these compounds indicated the competitive nature of inhibition of monoamine oxidase. All substituted imidazolones possessed anticonvulsant activity ranging from 20 to 70% at a dose of 100 mg/kg; however, this activity was unrelated to their monoamine oxidase inhibitory effectiveness.

Keyphrases □ 5-Imidazolones, 1,2,4-trisubstituted—synthesis, anticonvulsant activity and relationship to inhibition of monoamine oxidase □ Structure–activity relationships—5-imidazolones, anticonvulsant activity, rats, inhibition of brain monoamine oxidase □ Anticonvulsant activity—1,2,4-trisubstituted 5-imidazolones □ Monoamine oxidase inhibition—1,2,4-trisubstituted 5-imidazolones

Hydrazine derivatives (1), cinnamides (2), and styrylquinoliniums (3) have been shown to inhibit monoamine oxidase [EC 1.4.3.4 monoamine: O_2 oxidoreductase (deaminating)] *in vitro*. The high degree of inhibition possessed by styrylquinoliniums was proposed to be related to the presence of the styryl group and, more specifically, to the ethylenic moiety (3, 4). Inhibitors of the enzyme monoamine oxidase have also been reported to possess antidepressant (5) and anticonvulsant (6, 7) properties. Certain derivatives of imidazole have also been reported to possess anticonvulsant activity (8, 9).

The ability of imidazolecarboxyhydrazides to manifest monoamine oxidase inhibitory activity during their evaluation as possible psychopharmacological agents (10–12) led to the synthesis of 1,2,4-trisubstituted 5-imidazolones, having a substituted benzylidene moiety at position 4 of the imidazolone nucleus, as monoamine oxidase inhibitors. In the present study, attempts were made to correlate the *in vitro* monoamine oxidase inhibitory effectiveness of these imidazolones with their behavioral effects and anticonvulsant activity as a function of their chemical structure.

CHEMISTRY

The various 1,2,4-trisubstituted 5-imidazolones were synthesized by the route outlined in Scheme I. Aceturic acid (I*a*), synthesized by the acetylation of glycine, was treated with acetic anhydride in the presence of anhydrous sodium acetate and then with suitable aromatic aldehydes to obtain the corresponding substituted oxazolones (I*e*). The reaction proceeds by an initial cyclization of the acylaminoacetic acid (I*b*), which tautomerizes into an intermediate (I*c*) and is followed by the Perkin-type condensation of the aldehyde with the active methylene unit of I*d*. The oxazolones (I*e*) were heated in equimolar ratio with the appropriate ethyl *p*-aminobenzoate in an oil bath at 140°; this procedure resulted in the formation of the substituted benzylidene-5-imidazolones (Compounds I–IX), which were converted into their corresponding hydrazides (Compounds X–XVIII) by refluxing with hydrazine hydrate (99–100%) in absolute ethanol.

EXPERIMENTAL[1]

Aceturic Acid (I*a*)—Compound I*a* was synthesized by the acetylation of glycine according to a method reported earlier (13).

2-Methyl-4-substituted Benzylidene-5-oxazolones (I*e*)—A mixture of the appropriate aromatic aldehyde (0.38 mole), powdered aceturic acid (0.24 mole), freshly fused and powdered sodium acetate (0.27 mole), and acetic anhydride (0.65 mole) was heated on an electric hot plate with occasional shaking until all solids went into solution. The resulting solution was warmed on the steam bath for 1 hr, cooled, and allowed to stand overnight in a refrigerator. The solid mass which separated was collected, washed with 60 ml of cold water, dried, and used without further purification for subsequent reaction (13).

1-Carboethoxy-2-methyl-4-substituted Benzylidene-5-imidazolones (I–IX)—The appropriate oxazolone was heated with an equimolar quantity of the suitable ethyl *p*-aminobenzoate in an oil bath at 140° for 10–20 min. The jelly-like mass which separated (14) was recrystallized from suitable solvents. The various substituted imidazolones were characterized by their sharp melting points and elemental analyses (Table I).

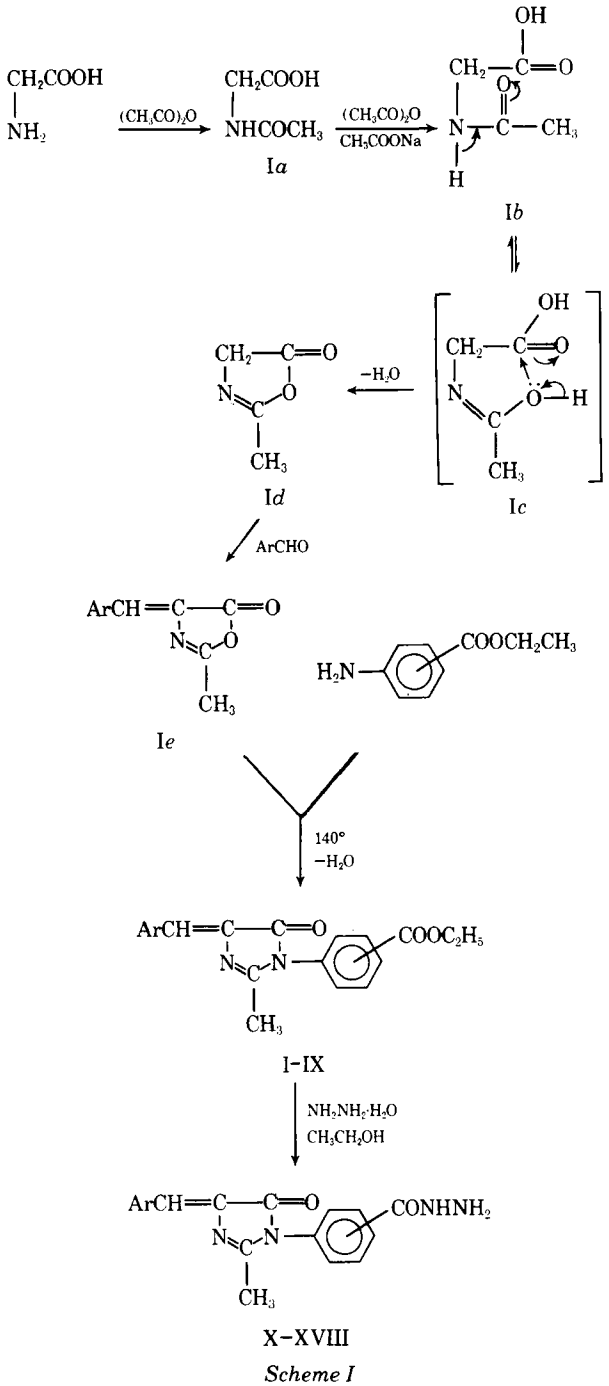
1-Carbohydrazino-2-methyl-4-substituted Benzylidene-5-imidazolones (X–XVIII)—A mixture of 0.1 mole of the appropriate 1-carboethoxy-2-methyl-4-substituted benzylidene-5-imidazolone (I–IX) and 0.2 mole of hydrazine hydrate (99–100%) in absolute ethanol was refluxed on a steam bath for 8–10 hr. Excess ethanol was removed by distillation under reduced pressure, and the hydrazides which separated were collected by filtration, dried, and recrystallized from ethanol. The various 1-carbohydrazino-2-methyl-4-substituted benzylidene-5-imidazolones (Table I) were characterized by their sharp melting points and elemental analyses.

Determination of Monoamine Oxidase Activity—Monoamine oxidase activity of rat brain homogenate was determined by a spectrophotofluorometric method, using kynuramine as the substrate (15). Male albino rats, 100–150 g, were allowed food and water *ad libitum* and were sacrificed by decapitation. The brains were removed immediately and homogenized[2] in the ratio of 1:9 (w/v) in ice-cold 0.25 *M* sucrose. The monoamine oxidase activity of rat brain homogenate was determined by incubation at 37° in air for 30 min. The reaction mixture in a total volume of 3 ml consisted of 0.5 ml of phosphate buffer (0.2 *M*, pH 7.5), 0.1 m*M* kynuramine, and 0.5 ml of brain homogenate (equivalent to 10 mg wet weight of the tissue).

The various 1-carboethoxy-2-methyl-4-substituted benzylidene-5-imidazolones and 1-carbohydrazino-2-methyl-4-substituted benzylidene-5-imidazolones were dissolved in propylene glycol (100%) and added to the brain homogenate to produce a final concentration of 0.25 m*M*. An equivalent amount of propylene glycol was added to the control tubes and the mixture was incubated for 10 min before the addition of kynuramine. The mixture, after addition of kynuramine, was further incubated for 30 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (w/v), and the precipitated proteins were removed by centrifugation.

[1] All compounds were analyzed for carbon, hydrogen, and nitrogen. Melting points were taken in open capillary tubes with partial immersion thermometer and are corrected.

[2] Potter–Elvehjem homogenizer.



Scheme I

Suitable 1-ml aliquots of the supernatant solution were taken in 2 ml of 1 *N* NaOH solution and were assayed for 4-hydroxyquinoline, formed during oxidative deamination of kynuramine by rat brain homogenate. The 4-hydroxyquinoline formed was measured fluorometrically[3], using activating light of 315 nm and measuring fluorescence at the maximum of 380 nm. An increase in absorbance provided a direct measurement of 4-hydroxyquinoline formation, which was taken as an index of the monoamine oxidase activity. The percentage inhibition was calculated from the decrease observed in absorbance, and this provided an index of the inhibitory property of these substituted imidazolones.

In preincubation studies, rat brain homogenate, in the incubation mixture, was incubated with or without substituted imidazolones at 37° for 10, 20, and 30 min prior to the addition of kynuramine. The zero-time experiments represent those in which substituted imidazolones and kynuramine were added simultaneously to the reaction mixture containing appropriate brain homogenate preparation. The I_{50} values (concentrations producing 50% inhibition) were determined graphically from the values obtained for the inhibition of rat brain monoamine oxidase by the use of different concentrations of these imidazolones. In the present study, the nature of the enzyme inhibition caused by XVII was evaluated by the graphic method of Lineweaver and Burk (16) as modified by Dixon (17).

[3] Aminco-Bowman.

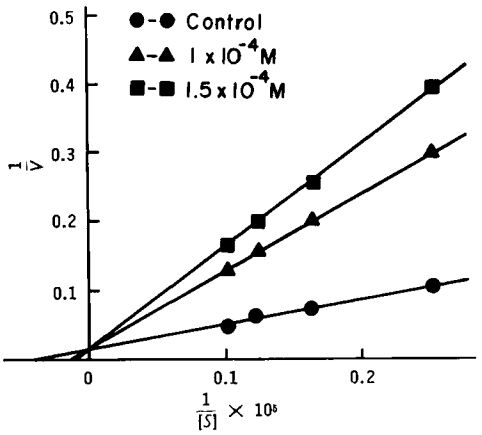


Figure 1—*Competitive inhibition of rat brain monoamine oxidase by 1-*m*-carbohydrazino-2-methyl-4-*p*-chlorobenzylidene-5-imidazolone (Compound XVII). Assay procedure and the contents of the reaction mixture are described in the text.* [S] *denotes molar concentration of kynuramine, and 1/V represents reciprocal of the change in percent absorbance/10 mg fresh tissue wet weight/30 min. Key:* ●, *control;* ▲, *0.1 m*M *imidazolone; and* ■, *0.15 m*M *imidazolone. The* K_m *value for kynuramine was found to be 0.22 m*M.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined against pentylenetetrazol-induced seizures in mice, 25–30 g, of either sex. The mice were divided into groups of 10, keeping the group weights as near the same as possible. All compounds were suspended in 5% aqueous gum acacia, devoid of anticonvulsant activity, to give a concentration of 0.25% (w/v). An arbitrary dose of 100 mg/kg ip of imidazolones was administered to 10 mice. The mice were then injected with pentylenetetrazol (90 mg/kg sc) 4 hr (2) after the administration of the test compounds. This dose of pentylenetetrazol has been shown to produce convulsions in almost all untreated mice and to exhibit 100% mortality during 24 hr. No mortality was observed during 24 hr in animals treated with 100 mg/kg of the imidazolones alone.

The mice were observed for 60 min for the occurrence of seizures. An episode of clonic spasm persisting for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks and tremulousness were not counted. Mice devoid of threshold convulsions during 60 min were considered protected. The number of mice protected in each group was recorded, and the anticonvulsant activity of these imidazolones was represented as percent protection. The animals were then observed for 24 hr, and the mortality of mice in each group was recorded.

Determination of Behavioral Effects—The effects of these imidazolones were investigated in albino mice, 25–30 g, by administration of a dose of 100 mg/kg ip.

RESULTS AND DISCUSSION

All imidazolones except III inhibited rat brain monoamine oxidase when used at a final concentration of 0.25 m*M* (Table II), where most of the 1-carbohydrazino-2-methyl-4-substituted benzylidene-5-imidazolones exhibited greater inhibition as compared to their corresponding precursors, 1-carboethoxy-2-methyl-4-substituted benzylidene-5-imidazolones. Such an increase in the inhibitory effectiveness of hydrazide derivatives was also reflected by their I_{50} values. In both of these series of imidazolones, with the exception of XI, the imidazolones possessing the *m*-$COOC_2H_5$ or *m*-$CONHNH_2$ substituent at position R′ of their structure produced higher inhibition as compared to their corresponding *ortho*-

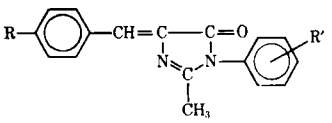


Table I—Physical Constants of 1-Carboethoxy/Carbohydrazino-2-methyl-4-substituted Benzylidene-5-imidazolones

Compound	R	R′	Recrystallization Solvent	Melting Point[a]	Yield, %	Molecular Formula	Analysis, %	
							Calc.	Found
I	H	*o*-$COOC_2H_5$	Benzene–petroleum ether (60–80°)	185°	42	$C_{20}H_{18}N_2O_3$	C 71.85 H 5.38 N 8.38	72.11 5.77 8.54
II	H	*m*-$COOC_2H_5$	Benzene–petroleum ether (60–80°)	113–115°	43	$C_{20}H_{18}N_2O_3$	C 71.85 H 5.38 N 8.38	71.71 5.45 8.58
III	H	*p*-$COOC_2H_5$	Ethanol	197–199°	47	$C_{20}H_{18}N_2O_3$	C 71.85 H 5.38 N 8.38	71.53 5.21 8.34
IV	OCH_3	*o*-$COOC_2H_5$	Acetone–ethanol	215–216°	40	$C_{21}H_{20}N_2O_4$	C 69.23 H 5.49 N 7.69	69.37 5.67 7.54
V	OCH_3	*m*-$COOC_2H_5$	Acetone–ethanol	211–214°	54	$C_{21}H_{20}N_2O_4$	C 69.23 H 5.49 N 7.69	69.43 5.31 7.79
VI	OCH_3	*p*-$COOC_2H_5$	Acetone–ethanol	220–223°	53	$C_{21}H_{20}N_2O_4$	C 69.23 H 5.49 N 7.69	69.48 5.53 7.34
VII	Cl	*o*-$COOC_2H_5$	Ethanol	203–206°	43	$C_{20}H_{17}ClN_2O_3$	C 65.12 H 4.61 N 7.59	64.97 4.35 7.68
VIII	Cl	*m*-$COOC_2H_5$	Ethanol	210–211°	57	$C_{20}H_{17}ClN_2O_3$	C 65.12 H 4.61 N 7.59	65.00 5.01 7.44
IX	Cl	*p*-$COOC_2H_5$	Ethanol	199–202°	61	$C_{20}H_{17}ClN_2O_3$	C 65.12 H 4.61 N 7.59	65.34 4.52 7.50
X	H	*o*-$CONHNH_2$	Ethanol	154°	40	$C_{18}H_{16}N_4O_2$	C 67.50 H 5.00 N 17.50	67.32 4.98 17.47
XI	H	*m*-$CONHNH_2$	Ethanol	199–202°	46	$C_{18}H_{16}N_4O_2$	C 67.50 H 5.00 N 17.50	67.42 5.37 17.71
XII	H	*p*-$CONHNH_2$	Ethanol	215–218°	56	$C_{18}H_{16}N_4O_2$	C 67.50 H 5.00 N 17.50	66.92 4.75 17.32
XIII	OCH_3	*o*-$CONHNH_2$	Ethanol	89°	38	$C_{19}H_{18}N_4O_3$	C 65.14 H 5.14 N 16.00	65.45 5.43 15.88
XIV	OCH_3	*m*-$CONHNH_2$	Ethanol	68°	50	$C_{19}H_{18}N_4O_3$	C 65.14 H 5.14 N 16.00	65.08 5.35 15.78
XV	OCH_3	*p*-$CONHNH_2$	Ethanol	78–80°	57	$C_{19}H_{18}N_4O_3$	C 65.14 H 5.14 N 16.00	65.54 5.03 15.78
XVI	Cl	*o*-$CONHNH_2$	Ethanol	114°	40	$C_{18}H_{15}ClN_4O_2$	C 60.93 H 4.23 N 15.79	60.72 4.52 15.72
XVII	Cl	*m*-$CONHNH_2$	Ethanol	125–127°	53	$C_{18}H_{15}ClN_4O_2$	C 60.93 H 4.23 N 15.79	61.21 4.37 15.85
XVIII	Cl	*p*-$CONHNH_2$	Ethanol	108–111°	50	$C_{18}H_{15}ClN_4O_2$	C 60.93 H 4.23 N 15.79	61.32 4.33 15.67

[a] Melting points were taken in open capillary tubes with a partial immersion thermometer and are corrected.

and *para*-substituted imidazolones. In general, substitution of a OCH_3 (IV–VI and XIII–XV) or Cl (VII–IX and XVI–XVIII) substituent at position R increased the monoamine oxidase inhibitory property of these imidazolones; with VII, however, the degree of enzyme inhibition decreased by the introduction of Cl substituent at the R position.

The monoamine oxidase inhibitory property of 1-carboethoxy-2-methyl-4-substituted benzylidene-5-imidazolones could presumably account for the presence of the benzylidene group, *i.e.*, the styryl group or, more specifically, the ethylenic moiety, at position 4 of the imidazolone nucleus (3). Increased activity of 1-carbohydrazino-2-methyl-4-substituted benzylidene-5-imidazolones may presumably be due to the additive monoamine oxidase inhibitory effectiveness of both the styryl and the hydrazide groups. Preincubation of these imidazolones for varying times prior to the addition of the substrate in *in vitro* studies in no way altered the degree of monoamine oxidase inhibition. These studies thus indicated a rapidly reversible and competitive nature of the inactivation by imidazolones (Table III). This finding was further supported by kinetic studies with XVII, which revealed the competitive nature of inhibition of rat brain monoamine oxidase (Fig. 1). The intercept at the $1/S$ axis was taken as $-1/K_m$; the value of 0.22 mM was obtained as the Michaelis constant (K_m) in these experiments, using kynuramine as the substrate.

All 1,2,4-trisubstituted 5-imidazolones possessed anticonvulsant activity, and the protection of pentylenetetrazol-induced convulsions ranged from 10 to 70%, with maximum anticonvulsant activity observed with VI (Table II). These results, unlike the monoamine oxidase inhibitory effects, indicated a decrease in anticonvulsant activity of 1-carboethoxy-2-methyl-4-substituted benzyli-

Table II—Monoamine Oxidase Inhibitory, Anticonvulsant Activity, and Behavioral Effects of 1-Carboethoxy/Carbohydrazino-2-methyl-4-substituted Benzylidene-5-imidazolones

Compound	Monoamine Oxidase Inhibition[a]		Anticonvulsant Activity[b], % Protection	Pentylenetetrazol Mortality[b], % Protection after 24 hr	Behavioral Effects[c]
	% Inhibition	I_{50} Values, mM			
I	41.2 ± 0.4	0.32	50	20	A,B,C
II	54.4 ± 0.6	0.22	40	40	C
III	Nil	—	40	70	A,B
IV	60.7 ± 0.1	0.20	20	70	A,C
V	69.2 ± 0.2	0.19	40	50	A,C
VI	65.0 ± 0.2	0.17	70	50	A
VII	20.6 ± 0.5	0.62	20	80	A,C
VIII	64.2 ± 0.6	0.17	40	70	A,B,C
IX	52.5 ± 0.4	0.23	60	40	B,C
X	46.8 ± 0.3	0.29	40	80	C
XI	28.0 ± 0.5	0.47	30	60	Nil
XII	35.5 ± 0.4	0.38	20	80	C
XIII	66.2 ± 0.1	0.16	40	80	A,C,D
XIV	72.5 ± 1.0	0.14	50	80	A,C,D
XV	60.7 ± 0.8	0.20	40	70	A,C,D
XVI	73.0 ± 0.4	0.13	10	80	Nil
XVII	91.7 ± 0.5	0.09	20	70	A,C,E
XVIII	61.7 ± 0.3	0.21	30	70	Nil

[a] Vessel contents and assay procedures are described in the text. Each experiment was done in triplicate, and the values are the mean values of three separate experiments with ±standard error of the mean. Kynuramine and imidazolones were used at a final concentration of 0.1 and 0.25 mM, respectively. The enzyme activity observed in the absence of imidazolones ranged from 20.1 to 24.7% change in transmission/10 mg fresh tissue weight/30 min. [b] Screening procedures for the determination of anticonvulsant activity are described in the text. Mortality in pentylenetetrazol-treated animals was observed during 24 hr. [c] Behavioral effects were observed at a dose of 100 mg/kg where A = general excitation, B = increased motor activity, C = circling movement, D = ataxia, and E = slight depression of spontaneous motor activity.

Table III—Preincubation Studies with 1-Carboethoxy/Carbohydrazino-2-methyl-4-substituted Benzylidene-5-imidazolones to Study Monoamine Oxidase Inhibition

Compound	Preincubation Time, min			
	0	10	20	30
	Monoamine Oxidase Inhibition[a], %			
I	40.3 ± 0.4	40.9 ± 0.4	39.5 ± 0.2	41.8 ± 0.4
II	54.0 ± 0.3	55.1 ± 0.2	54.9 ± 0.1	54.9 ± 0.3
III	Nil	Nil	Nil	Nil
IV	59.8 ± 0.7	59.9 ± 0.6	60.5 ± 0.2	61.0 ± 0.1
V	69.2 ± 0.2	69.4 ± 0.3	70.0 ± 0.7	69.8 ± 0.3
VI	65.4 ± 0.3	65.6 ± 0.4	64.9 ± 0.8	64.7 ± 0.6
VII	20.7 ± 0.4	20.5 ± 0.4	20.9 ± 0.4	21.0 ± 0.1
VIII	63.9 ± 0.2	64.1 ± 0.3	64.3 ± 0.5	64.7 ± 0.5
IX	52.3 ± 0.1	52.6 ± 0.6	53.0 ± 0.3	53.2 ± 0.4
X	46.7 ± 0.4	46.5 ± 0.3	46.2 ± 0.3	46.7 ± 0.2
XI	28.0 ± 0.7	28.8 ± 0.2	27.9 ± 0.4	28.4 ± 0.3
XII	35.1 ± 0.7	36.2 ± 0.4	35.4 ± 0.2	36.2 ± 0.1
XIII	65.9 ± 0.5	66.1 ± 0.3	65.8 ± 0.5	66.7 ± 0.9
XIV	72.7 ± 0.6	71.9 ± 0.4	72.3 ± 0.2	72.9 ± 1.0
XV	59.9 ± 0.4	60.3 ± 0.8	60.8 ± 0.7	60.5 ± 0.9
XVI	72.9 ± 0.1	73.4 ± 0.4	72.7 ± 0.3	72.5 ± 0.4
XVII	90.8 ± 0.8	91.2 ± 0.3	91.8 ± 0.6	92.0 ± 0.2
XVIII	61.2 ± 0.3	61.7 ± 0.2	62.0 ± 0.4	62.3 ± 0.8

[a] Contents of the reaction mixture and the assay procedures are as indicated in Table II. The enzyme preparations were incubated with the imidazolones for varying times before the addition of kynuramine. Zero-time experiments indicate that both kynuramine and the imidazolones were added to the reaction mixture containing enzyme preparation simultaneously.

dene-5-imidazolones by their conversion into their corresponding 1-carbohydrazino-2-methyl-4-substituted benzylidene-5-imidazolones, with the exception of IV and V where a slight increase in anticonvulsant activity was observed (XIII and XIV). Data on anticonvulsant activity of these compounds and their 24-hr pentylenetetrazol-induced mortality did not indicate an association between increased protection from convulsions and decreased mortality in experimental animals.

With many of these imidazolones, the behavioral effects indicated were general excitation, increased motor activity, and the circling movement. But XI, XVI, and XVIII were devoid of any appreciable behavioral effects at the dose tested. Ataxia was observed only with XIII–XV, while slight depression of the spontaneous motor activity was observed with XVII.

The results from this study demonstrate, with only a few exceptions, some correlation between the biological activities and the chemical structures of these 1,2,4-trisubstituted 5-imidazolones. The hydrazides are better inhibitors than the esters for monoamine oxidase, whereas the reverse is true for anticonvulsant activity. In addition, there is a clear indication that the monoamine oxidase inhibitory activity of the *meta*-compounds is greater than that of the corresponding *ortho*- and *para*-compounds and that the enzyme inhibitory activity increases by substitution of OCH_3 or Cl. However, these studies failed to provide any definite correlation between monoamine oxidase inhibitory property of these imidazolones and their anticonvulsant activity. It is hoped that detailed pharmacological and toxicological studies and investigations of the effects of these compounds on other enzyme systems may reflect a biochemical basis for the anticonvulsant activity of these 1,2,4-trisubstituted imidazolones.

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JOHN STERLING, STEPHEN COX, and W. G. HANEY ˣ

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Spectrophotofluorometric procedures have a high daily capacity for sample analysis, are more amenable to application by less sophisticated personnel than are GLC techniques, and generally are more precise. Therefore, the development of such an assay for procainamide as a possible substitute for the colorimetric assay is of interest.

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A general procedure for the microanalysis of drugs containing the primary aromatic amino substituent was presented previously (9). This procedure is based on selective reaction of fluorescamine with the primary aromatic amino substituent and subsequent de-

termination of the derivative by spectrophotofluorometry. This report presents results of the application of this procedure to the analysis of procainamide in plasma. To allow an investigation of the specificity of the analytical procedure, a modified GLC assay was developed, and results of the two procedures were compared. Finally, results and utility of the well-accepted colorimetric procedure were compared and contrasted with those of the GLC and spectrophotofluorometric procedures.

EXPERIMENTAL

Apparatus—A spectrophotofluorometer[1] was equipped as described previously (9). Operating conditions were also the same, except that the band passes were maintained at 2.0 and 1.0 mm for excitation and emission wavelengths, respectively.

A gas chromatograph[2] with isothermal control and flame-ionization detector was used. Columns (1.82 m, spiral-shaped, borosilicate glass) were packed with 100–120-mesh, acid-washed, flux-calcined diatomite coated with 0.5% polyamide[3] and 6% (3,3,3-trifluoropropyl)methylsilicone. Prior to use, the column was conditioned at 250° for 24 hr. Injector port (fitted with glass injector sleeve), column, and detector temperatures were 270, 235, and 280°, respectively. Gas flow rates were: hydrogen, 40 ml/min; air, 225 ml/min; and nitrogen as carrier gas, 40 ml/min. Peak areas were determined using an automatic electronic digital integrator[4] with printout.

A spectrophotometer[5] was used for colorimetric determinations.

Reagents and Solutions—Dibucaine hydrochloride was obtained as a USP reference standard. Standard solutions of dibucaine (20.0 mg/100 ml) and procainamide were prepared in distilled water, and the pH was adjusted to 5.0 with 0.1 *N* HCl and/or 0.1 *N* NaOH. These solutions were diluted quantitatively prior to use. For recovery studies, a solution of dibucaine (2.125 μg/ml) was prepared by extracting a solution of dibucaine in pH 9.5 borate buffer (10.0 ml) with chloroform (80 ml) and adding chloroform to a volume of 100.00 ml.

Other reagents and solutions were obtained or prepared as previously indicated (13).

Preparation of Calibration Curves—Calibration curves for the GLC assay were prepared by mixing solutions (1.0–20.0 μg/ml in 1.0 ml) of procainamide with internal standard solution (1.0 ml of 8.5 μg/ml), pH 9.5 borate buffer (0.5 ml), and methylene dichloride (8 ml). This mixture was shaken for 2 min and centrifuged for 3 min at 2000 rpm. The methylene dichloride extract was filtered through phase-separating filter paper, and the filtrate was evaporated to dryness in a water bath under a stream of dry nitrogen. The residue was reconstituted in carbon disulfide (50 μl), and a volume (3–5 μl) of this solution was injected into the chromatograph. The ratio of the area of the peak representing procainamide to that representing dibucaine was plotted against the concentration of procainamide in the solution. Calibration curves were prepared on 10 consecutive days.

Calibration curves for spectrophotofluorometric studies were prepared by mixing solutions (1.0–20.0 μg/ml in 1.0 ml) of procainamide with pH 5.5 phosphate buffer (0.5 ml). Fluorescamine solution (0.5 ml of a solution of 25 mg/100 ml in acetone) was added with shaking. After 7 min, fluorescence of the solution was determined at 400 and 498 nm for excitation and emission wavelengths, respectively, against a reagent blank. Calibration curves were prepared on 10 consecutive days.

Calibration curves for colorimetric determinations were prepared by adding various quantities of procainamide solution to pooled plasma and treating the spiked samples as previously noted (3).

Extraction—Procainamide solution was added to plasma (20.0 ml) to give concentrations of 2.0–20.0 μg/ml. An aliquot (2.0 ml) of this plasma was mixed with pH 9.5 borate buffer (1.0 ml), extracted with methylene dichloride (16.0 ml), and centrifuged at 2000 rpm for 3 min. The extract was passed through phase-separating filter paper. An aliquot (6.0 ml) was removed, evaporated to dryness, and reconstituted with pH 5.5 phosphate buffer. Fluorescence of the solution was determined against a reagent blank.

An aliquot (6.0 ml) of the remaining methylene dichloride extract was combined with the solution of dibucaine in chloroform (3.0 ml), evaporated to dryness, reconstituted with carbon disulfide (50 μl), and injected into the chromatograph. Recoveries were calculated by correcting for the volume of extract taken and by reference to previously derived calibration curves.

Accuracy and Precision—Procainamide solution was added to plasma (90.0 ml) to give concentrations of 2.0–20.0 μg/ml, together with sufficient dibucaine solution to give a final concentration of 8.5 μg/ml and pH 5.5 phosphate buffer (6.0 ml). Aliquots (1.0 ml) of these solutions were added to vials, and the vials were capped and maintained at −6°. On 10 consecutive days, the contents of nine vials were thawed, and three each were analyzed for procaina-

[1] American Instruments Co.
[2] Varian model 575.
[3] Versamid 900, Analabs, North Haven, Conn.
[4] Varian model 475.
[5] Cary model 118.

Table I—Efficiency of Extraction of Procainamide from Plasma as Determined by GLC and Spectrophotofluorometry

Method	Procainamide Added, μg	Procainamide Recovered, μg	Recovery, %	*SD*, %[a]
GLC	2.00	1.97	98.50	5.43
Spectrophotofluorometry	2.00	2.02	101.00	2.73
GLC	4.00	4.13	103.75	4.51
Spectrophotofluorometry	4.00	3.96	98.75	2.30
GLC	8.00	7.85	98.12	1.84
Spectrophotofluorometry	8.00	7.93	99.12	1.94
GLC	12.00	12.31	102.58	2.47
Spectrophotofluorometry	12.00	12.14	101.16	2.11
GLC	16.00	15.53	97.06	4.14
Spectrophotofluorometry	16.00	15.74	98.30	1.76
GLC	20.00	21.06	105.33	5.91
Spectrophotofluorometry	20.00	20.40	102.00	2.14

[a] $n = 10$.

Table II—Accuracy and Precision of Three Methods of Procainamide Determination

Method	Procainamide Quantity, μg	Procainamide Found, μg	Recovery, %	*SD*, %[a]
GLC	2.00	1.94	97.00	7.57
Spectrophotofluorometry	2.00	2.02	101.00	3.41
Colorimetry	2.00	2.09	104.50	9.84
GLC	4.00	4.23	105.75	6.90
Spectrophotofluorometry	4.00	4.13	103.25	4.11
Colorimetry	4.00	3.81	95.25	8.72
GLC	8.00	8.31	103.87	5.74
Spectrophotofluorometry	8.00	8.07	100.87	3.02
Colorimetry	8.00	8.39	104.87	6.59
GLC	12.00	12.60	105.00	6.94
Spectrophotofluorometry	12.00	11.71	97.59	3.17
Colorimetry	12.00	11.55	96.25	6.87
GLC	16.00	16.03	100.19	8.42
Spectrophotofluorometry	16.00	15.87	99.19	2.54
Colorimetry	16.00	15.34	95.88	7.22

[a] $n = 30$.

Table III—Procainamide Concentration in Plasma as Determined by Three Methods

Sample	Other Drugs	Apparent Procainamide Concentration[a]		
		GLC	Spectrophotofluorometry	Colorimetry
1	Nitrofurantoin, methaqualone	5.40	5.12	5.59
2	Digoxin, glutethimide	7.21	6.84	7.36
3	Chlordiazepoxide, codeine	2.74	2.92	4.16
4	Tolbutamide, diazepam	6.14	6.23	5.71
5	Aspirin, phenacetin, caffeine, propoxyphene	2.10	2.34	2.61
6	Dicumarol, erythromycin	6.41	6.29	6.53
7	Aspirin, codeine, chlordiazepoxide	5.37	5.54	8.97
8	Methaqualone, chlorothiazide	10.74	9.96	10.92

[a] Expressed in micrograms per milliliter.

mide content by spectrophotofluorometry, colorimetry, and GLC.

Patient Plasma Procainamide—Blood from patients taking procainamide was mixed with pH 5.5 phosphate buffer (1.0 ml) and centrifuged, and the plasma was stored at −6°. At the time of analysis, samples were thawed and split into aliquots (1.0 ml). Dibucaine solution (1.0 ml of a 8.5 μg/ml solution) was added to the aliquot to be analyzed by GLC, and analysis of each aliquot by spectrophotofluorometry, colorimetry, and GLC proceeded as described.

RESULTS AND DISCUSSION

The selectivity of the spectrophotofluorometric procedure was examined by comparing results of the method with those obtained from a GLC procedure. Nonlinear calibration curves and incomplete extraction necessitated modifications in the previously proposed GLC procedure (8). Since it appeared that nonlinearity was due to adsorption of procainamide onto the solid support, a more polar stationary phase and a higher liquid load were used. In addition, a small amount of a polyamide was used to coat the solid support prior to coating with (3,3,3-trifluoropropyl)methylsilicone. The polyamide in this capacity acts to deactivate potential adsorptive sites on the solid support (10). The packing material was stable over 3 months.

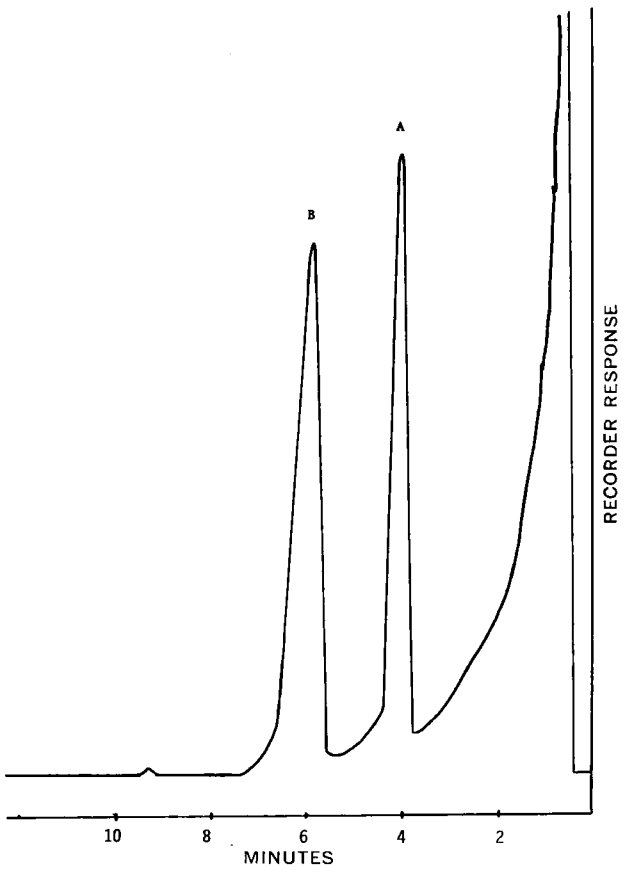

Figure 1—*Chromatographic trace of an extract of plasma containing procainamide (6.5 μg/ml, A) and internal standard (8.5 μg/ml, B). (See text for chromatographic conditions.)*

Calibration curves for the GLC assay were prepared on 10 consecutive days, and the standard deviation of the curve slope was 1.6%. The average correlation coefficient was 1.022, and the average y-intercept was +0.007.

Dibucaine was used as an extracted internal standard in the GLC assay. Because of the use of dibucaine in drug therapy, it may not be the internal standard of choice in routine GLC determinations of procainamide in plasma. However, it is well suited for this comparative study, since it does not interfere with results of the spectrophotofluorometric or colorimetric assay. Therefore, it may be added to plasma before the sample is split into three aliquots for analysis, ensuring that differences in results obtained *via* the three procedures are due to differences in analytical procedures rather than to differences in sampling.

Calibration curves for the spectrophotofluorometric assay were prepared on 10 consecutive days. The standard deviation of the slope of the curve was 0.72%, the average correlation coefficient was 1.009, and the average y-intercept was −0.005.

Results of the investigation of extraction efficiency as determined by GLC and by spectrophotofluorometry are presented in Table I. The therapeutic concentration of procainamide in plasma has been estimated to be 4–10 μg/ml (3). Extraction efficiency in this range is satisfactory as determined by either method. However, results of the GLC assay are much less precise at 2.0, 16.0, and 20.0 μg/ml than results at other concentrations and results of the spectrophotofluorometric assay. This finding appears to reflect a diminution in analytical precision as the difference between the area of procainamide and the internal standard increases rather than erratic recovery.

Results of the study designed to examine day-to-day accuracy and precision of each of the three analytical procedures are presented in Table II. While the precision of the results obtained both by the GLC and spectrophotofluorometric procedures is less than that obtained in the extraction studies, this difference is larger with the GLC method. The precision of the colorimetric method is much less than was previously reported (3). While other factors may contribute to this discrepancy, one major consideration is that precision, in the latter study, was determined by analyzing many samples in 1 day. Results gathered on numerous days apparently would yield more meaningful information concerning precision, since this approach more closely approximates conditions under which the assay will be performed. The precision of results obtained by GLC analysis is comparable to that found previously (8).

Since the study was performed on plasma for 10 consecutive days, it is noteworthy that plasma concentration of procainamide

on Day 1 was not significantly different from that on Day 10, as determined by each of the three procedures. Procainamide in plasma stored under the conditions in this study appears to be stable for at least 6 weeks. However, in initial studies, a large quantity of plasma was spiked with procainamide, frozen, and thawed daily before samples were taken. When using this procedure, 21% of procainamide degraded by the 7th day. This result necessitated freezing procainamide in separate aliquots for analysis.

The specificity of the colorimetric and spectrophotofluorometric assays was investigated by determining apparant procainamide concentration in plasma of patients taking various drugs. Results were compared with results of the GLC assay (Table III).

Interferences in the colorimetric assay would be limited to those substances that possess a primary aromatic amine substituent or that have significant absorption at 550 nm and are extracted from the plasma. While fluorescamine will react with any primary amine under the proper conditions, its reactivity is limited in this procedure by adjusting the pH of the reaction medium to 5.5. Under these conditions, primary aliphatic amines do not react with fluorescamine (9). Thus, the selectivity of the two spectral procedures should be comparable.

Results of the three procedures differ significantly in only two cases, both of which were characterized by apparently higher levels of procainamide as determined by colorimetry. Since both samples were obtained from patients taking chlordiazepoxide, the potential interference of chlordiazepoxide in the colorimetric determination was investigated by using colorimetry to determine apparent procainamide levels in the plasma of patients taking only chlordiazepoxide. For three such patients, apparent procainamide levels were 2.12, 3.47, and 1.87 μg/ml. The explanation of this interference is not apparent, since known metabolites of the drug should not participate in the colorimetric reaction.

The utility of the procedures was also investigated by using each of the three methods to determine the procainamide concentration in the plasma of 17 patients taking only procainamide. Concentrations ranged from 1.23 to 15.31 μg/ml, and none of the values obtained by the three procedures differed by more than 17%.

The proposed spectrophotofluorometric procedure has several important advantages over the colorimetric procedure. Since extraction is quantitative, it is not necessary to extract spiked plasma samples to prepare calibration curves. Solutions (pH 5.5 phosphate buffer, pH 9.5 borate buffer, and fluorescamine) used in the assay are stable for at least 6 weeks, as opposed to the instability of the sodium nitrite and ammonium sulfamate solutions used for the colorimetric procedure. The proposed procedure appears to be somewhat more specific than the colorimetric procedure, as evidenced by results obtained from plasma of patients taking chlordiazepoxide. Precision of results obtained by the spectrophotofluorometric procedure is greater than that obtained by colorimetry. Finally, time is much less critical in this procedure, since the fluorescence has been shown to be stable for up to 24 hr (9), in contrast to the instability of the chromophore in the colorimetric procedure (7).

With respect to the GLC assay, a major advantage is the increased capacity for sample analysis by spectrophotofluorometry. Fifteen samples can easily be processed in 2 hr by a single technician. It is a simple, easily mastered procedure. Additionally, precision is greater than precision of the GLC assay. The spectrophotofluorometric procedure should not, however, be applied to analysis of procainamide in plasma of patients also taking other drugs (such as sulfonamides) that contain the primary aromatic amino substituent.

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[x] To whom inquiries should be directed.

Release of a Water-Soluble Drug from a Wax Matrix Timed-Release Tablet

FRANK W. GOODHART ˣ, ROBERT H. McCOY, and FRED C. NINGER

Abstract □ The purpose of this work was to characterize the release of drug from a wax matrix tablet under relatively mild agitation conditions. It was found that phenylpropanolamine hydrochloride was released from a typical wax matrix by a diffusion mechanism. After an initial rapid release of drug from the tablet, the amount dissolved was proportional to the square root of time. The advance of the solvent front into the tablet matrix was also proportional to the square root of time. Compression force was not a major factor affecting drug release. Data on drug release from a single tablet face compared to release from a totally exposed tablet indicated that drug release is proportional to the total surface area.

Keyphrases □ Timed-release tablets, wax matrix—water-soluble drug release (phenylpropanolamine hydrochloride), mild agitation conditions, mechanisms □ Phenylpropanolamine hydrochloride—release rate from wax matrix timed-release tablet, mechanisms □ Drug release—phenylpropanolamine hydrochloride release rate from wax matrix timed-release tablet □ Wax matrix (timed-release tablet)—release rate of phenylpropanolamine hydrochloride, mechanisms

Numerous compositions have been suggested for sustaining drug release over a long period (1, 2). However, the fabrication of such compositions has been largely empirical and little work has been carried out to explain the mechanism involved. Usually some *in vitro* procedure is used to characterize drug release from the dosage form. It is often assumed that a 30–50% *in vitro* drug release in 1 hr is good and that a continuous drug release over 5–7 hr is satisfactory, but only recently has any correlation between *in vitro* drug release and *in vivo* performance been reported (3).

Ideally, a sustained-release preparation should provide part of the drug at the absorption site quickly to achieve sufficient absorption to produce a rapid therapeutic response. The balance of the drug should be provided at a sufficient rate to maintain pharmacological activity. In the most simple case, *i.e.*, a one-compartment open model with essentially 100% drug absorption and total urinary excretion *via* first-order kinetics, the drug should be provided to the blood at a rate equal to the rate loss from that compartment (4). Examples of the effect of zero- and first-order drug dissolution on resulting blood levels (one-compartment model) were well described (4) using analog computational methods, and some shortcomings of certain drug release patterns were pointed out.

A timed-release tablet granulation may typically contain 20–30% of a mixture of wax and fatty acid. The balance of the formulation is drug and various diluents. The usual practice for formulating a sustained-action tablet is to layer an immediate-release dose with the slow releasing wax matrix or to place the wax matrix as a core in a press-coated tablet. These methods should then provide an immediate drug blood level that is maintained by the balance of the drug being released over some prolonged period.

The *in vitro* drug release characteristics of timed-release tablets may be determined by various methods which can generally be classified as high agitation or low agitation. With high agitation, the total amount of drug released is a function of the amount released by diffusion plus the amount eroded away. This type of release is obtained from the rotating-bottle method (5) or the basket-rack assembly (6). *In vitro* release by these methods causes tablets to be gradually worn away until the entire mass is broken into small particles, at which time the drug is considered to be 100% released. More gentle methods, such as circulating fluid over a tablet contained in a well, do not cause tablet erosion; drug is released entirely by a diffusional process, typically over 8 or 10 hr for a water-soluble material. Above a certain stirring speed, the drug release rate should be independent of revolutions per minute if it is diffusion controlled.

An inherent problem in timed-release medication is to obtain maximum drug availability. Analysis of some data where sustained-action and immediate-release forms of drug were compared indicates that part of the dose is unavailable; the drug release might be too slow and the full dose is not being utilized. One objective of the present study was to obtain *in vitro* dissolution characteristics that might logically lead to the use of a satisfactory input function for *in vivo* evaluation and correlation. Another objective was to develop a method for logical characterization of the release properties of various tablet formulations. Because of the need for more knowledge of the formulation of timed-release dosage forms, the following work was carried out using phenylpropanolamine hydrochloride in a wax matrix tablet as a typical system.

EXPERIMENTAL

Preparation of Phenylpropanolamine Hydrochloride Tablets—A timed-release granulation was prepared according to the formula shown in Table I. Single-layer tablets weighing 1.080 g, representing 150 mg of phenylpropanolamine hydrochloride, were compressed[1] using 15.9-mm (0.63-in.), flat-faced, beveled edge punches and dies. Tablets were made using compression forces of 226.8, 453.6, 544.3, and 907.2 kg. Measurements of tablet breaking strength and thickness resulted in the values shown in Table II.

***In Vitro* Release Procedure**—Dissolution of tablets subjected to various compression forces was studied. Special attention was directed toward tablets compressed at 544.3 kg, since they met the requirements of a proposed formulation with respect to hardness. A tablet and capsule dissolution apparatus (7) was employed using a platform with a center well. Four hundred milliliters of 0.1 *N* HCl was placed in the jacketed beaker maintained at 37.5 ± 0.5°.

[1] Carver press.

Table I—Formula for Preparation of Phenylpropanolamine Hydrochloride Timed-Release Tablets

Ingredients	Per 1000 Tablets, g
Phenylpropanolamine hydrochloride NF	150.0
Wax–fat mixture	202.5
Diluent	721.5
Calcium stearate NF	4.5
Colloidal silicon dioxide NF	1.5
	1080.0

When the solution reached equilibrium temperature, a tablet was introduced into the center well of the platform. Dissolution was initiated using a constant stirring rate of 75 rpm. One-milliliter samples were removed *via* pipet in the outlet port. One milliliter of fresh 0.1 *N* HCl was added to maintain constant volume. Samples were collected throughout the dissolution study at various time intervals and assayed. Phenylpropanolamine hydrochloride was analyzed using the periodate oxidation procedure and read spectrophotometrically as benzaldehyde in heptane (8).

A dissolution study of a tablet-in-the-die (compression force of 544.3 kg) was made for comparison of drug release from a fixed surface area. The penetration rate of the dissolution media into the tablets compressed using 544.3 kg of force was determined. Seven tablets were placed on a dissolution platform containing seven wells and placed into the dissolution apparatus. Tablets were removed at intervals coinciding with the dissolution rate studies. Excess water on the surface of the tablet was absorbed with absorbent paper. The tablet thickness was carefully measured so as not to compress the hydrated layer. The hydrated layer was removed from both sides of the tablet with a scalpel, and the thickness of the dry part of the tablet was measured.

RESULTS AND DISCUSSION

The cumulative amount of drug released for tablets made using 226.8 kg of compression force is given in Table III, together with the tablet position during dissolution. The dissolution data for tablets made at various compression forces are shown in Fig. 1. A comparison of the data of the various compression forces tested showed little difference; however, the tablets did not vary greatly in thickness. All tablets showed a relatively fast initial drug release (33–40%) during the 1st hr followed by a slower release. About 80% drug was released in 6–7 hr.

The mechanism of drug release from a wax matrix was discussed in detail previously (9). In the treatment of those data, it was assumed that drug release was either first order or a diffusion-controlled process. One indication of the mechanism can be obtained using a plot of the logarithm of the percent remaining in the matrix against time. A first-order release would be linear, while the diffusion-controlled process would generate an S-shaped curve (9).

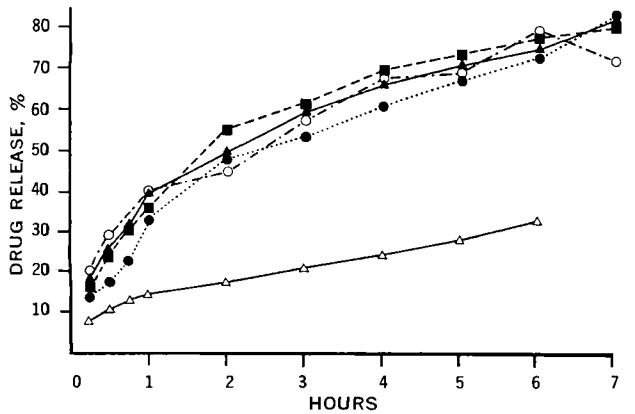

Figure 1—*Effect of compression force on release profiles of phenylpropanolamine from wax matrix tablets. Compression force was:* ●, *226.8 kg;* ○, *453.6 kg;* ▲, *544.3 kg;* ■, *907.2 kg; and* △, *544.3 kg (tablet-in-die).*

Table II—Compression Conditions and Physical Properties of Timed-Release Phenylpropanolamine Hydrochloride Tablets

Compression Force, kg	Breaking Strength[a], kg/in.^{2}	Thickness, mm
226.8	9.6	4.88
453.6	16.5	4.62
544.3	19.0	4.39
907.2	25.5	4.27

[a] Measured on modified Strong-Cobb tablet hardness tester.

Extended studies up to 10 hr were performed on the tablets compressed at 544.3 kg, and the data obtained were used to explain the mechanism of drug release.

The logarithm of the percent of drug remaining in the matrix was plotted against time (Fig. 2) and produced an S-shaped curve, indicating a lack of conformance to first-order kinetics. Therefore, an attempt was made to determine whether the drug release could be described by a diffusion equation proposed by Higuchi (10):

$$Q = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t} \qquad \text{(Eq. 1)}$$

where Q is the amount of drug released per unit area of the tablet exposed to the solvent, D is the diffusion coefficient of the drug in the permeating fluid, ϵ is the porosity of the matrix, τ is the tortuosity of the matrix, A is the concentration of solid drug in the matrix, C_s is the solubility of drug in the dissolution medium, and t is time. In this work, $2A$ exceeded ϵC_s by a factor of at least three, thereby justifying the use of this particular diffusion equation. This includes porosity due to air and water-soluble ingredients including the drug. If:

$$K = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s} \qquad \text{(Eq. 2)}$$

then:

$$Q = Kt^{1/2} \qquad \text{(Eq. 3)}$$

For a diffusion-controlled mechanism as postulated here, a plot of the amount of drug release *versus* the square root of time should be linear as predicted by Eq. 3. The data were graphed in this manner (Fig. 3) for tablets made at various compression forces, and a linear plot of percent drug release was obtained for times greater than 1 hr. It is evident that the differences in tablet thickness and hardness resulting from different compression conditions employed did not cause more than 10% variation in the release of phenylpropanolamine hydrochloride at any particular time interval. Therefore, under the mild agitation conditions of testing, drug

Table III—Release of Phenylpropanolamine Hydrochloride from Wax Matrix Tablets[a]

Hours	Tablet Position	Cumulative Amount Released		
		Percent	Q', mg	Q, mg/cm^2
0.25	Stationary	13.5	20.2	3.29
0.50	Stationary	17.6	26.4	4.29
0.75	Stationary	22.8	34.2	5.56
1.0	Sliding[b]	33.0	49.5	8.05
2.0	Sliding[b]	48.4	72.6	11.81
3.0	Slight elevation	53.8	80.7	13.12
4.0	Slight elevation	61.3	92.0	14.96
5.0	Rotation	68.0	102.0	16.59
6.0	Floating below propeller	74.0	111.0	18.05
7.0	Broken by propeller	84.0	126.0	20.49

[a] Compression force of 226.8 kg; data from two trials. [b] Density of tablet begins to approach density of the dissolution medium.

Table IV—Comparison of Dissolution Rate per Area with Compression Force for Free Tablets and Tablet-in-a-Die

Hours	Compression Force, kg	Release Rate, mg/hr cm²	Percent Release	Tablet Position
0–1	226.8	8.05	33.0	Sliding
0–1	453.6	9.76	40.0	Stationary
0–1	544.3	9.64	39.5	Stationary
0–1	907.2	10.41	36.0	Stationary
0–1	544.3 (tablet-in-die)	10.75	14.4	Stationary
2–4	226.8	2.28	48.4–61.3	Slight elevating
2–4	453.6	2.27	45.3–68.2	Sliding
2–4	544.3	2.19	49.3–66.7	Sliding
2–4	907.2	2.74	54.9–69.8	Sliding
2–4	544.3 (tablet-in-die)	2.78	17.8–24.5	Stationary

release does not appear to be affected by compression force over the ranges studied.

Evaluation of the slope, K in Fig. 3, with the aid of Eq. 3 gave a value of 6.35 mg/cm² $hr^{1/2}$, equivalent to 26.6% $hr^{1/2}$ for the composite results of all runs. The factors comprising K are given by Eq. 2; these are diffusion coefficient, porosity, tortuosity, concentration of drug in the matrix, and solubility of drug in the dissolution medium. Drug solubility is a constant in any particular dissolution fluid. Therefore, drug release from a wax matrix can be altered by altering the factors comprising K. Generally, K is modified by a change in formulation such as increased or decreased wax content or a change in diluent. For instance, compressional force might be expected to change the porosity, ϵ, and hence K; however, in this work, K was not significantly affected by change in compressional force. Further characterization of K for various systems would allow some general conclusions to be made regarding drug release as a function of formulation variables.

Another equation may be used to calculate the value of K. By multiplying both sides of Eq. 3 by the surface area, S, of the tablet from which drug is released:

$$QS = KSt^{1/2} \qquad \text{(Eq. 4)}$$

or:

$$Q' = KSt^{1/2} \qquad \text{(Eq. 5)}$$

where Q' is the amount of drug released.

Differentiation of Eq. 5 with respect to time gives (9):

$$\frac{dQ'}{dt} = \frac{KS}{2t^{1/2}} \qquad \text{(Eq. 6)}$$

Substitution of Q'/KS for $t^{1/2}$ results in:

$$\frac{dQ'}{dt} = \frac{K^2S^2}{2Q'} \qquad \text{(Eq. 7)}$$

Equation 7 states that, for a diffusion-controlled process, the rate of drug release is linearly related to $1/Q'$, the reciprocal of the amount of drug release. Release rates that expressed milligrams of drug release as a function of time were calculated at the various time intervals. A plot of these release rates, dQ'/dt *versus* $1/Q'$, gives a slope of $K^2S^2/2$. The constant, K, was calculated from the slope and found to be 8.83. This value does not compare exactly with the K of 6.35 calculated according to Eq. 4. However, the lack of smoothness of the graph of amount released *versus* time probably accounts for the discrepancy.

Tablets undergoing dissolution were generally stationary in the well for the first few hours. Some movement then occurred because of the loss of drug and diluent. At the end of 6–7 hr, tablets began to rotate in the well as the tablet density began to approach the density of the dissolution medium. Tablets throughout the experiments maintained their original shape; no erosion of any kind occurred.

Drug Release from a Fixed Surface Area—The dissolution of a tablet-in-a-die, compressed at 544.3 kg, was required for a strict evaluation of dissolution rates per unit area. The area available for release from the total single tablet surface and from a single face of the tablet was 6.35 and 1.98 cm², respectively. Dissolution rates of phenylpropanolamine hydrochloride from a tablet-in-a-die was determined by the same method as described for free tablets. The die containing the tablet was lowered into the dissolution medium in an upright position, creating an air space between

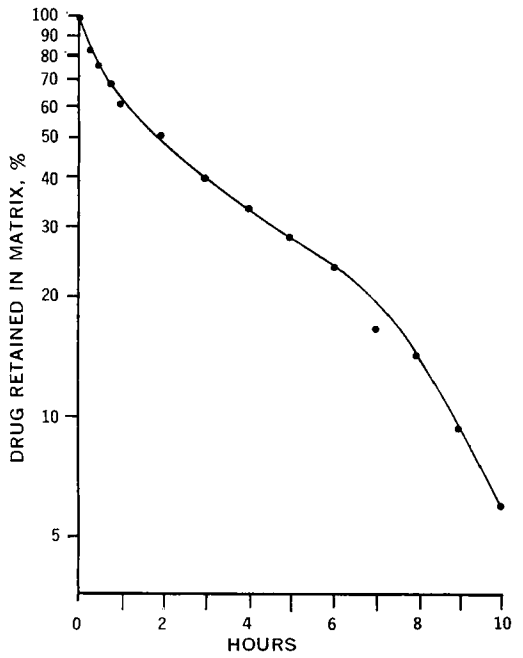

Figure 2—*Release of phenylpropanolamine hydrochloride from a wax matrix tablet. Compression force was 544.3 kg.*

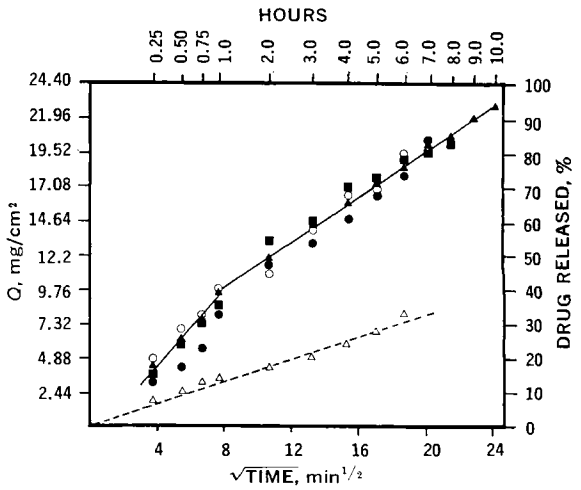

Figure 3—*Amount of drug release* versus *the square root of time using phenylpropanolamine hydrochloride 150-mg wax matrix tablet. Compression force was:* ●, *226.8 kg;* ○, *453.6 kg;* ▲, *544.3 kg;* ■, *907.2 kg; and* △, *544.3 kg (tablet-in-die).*

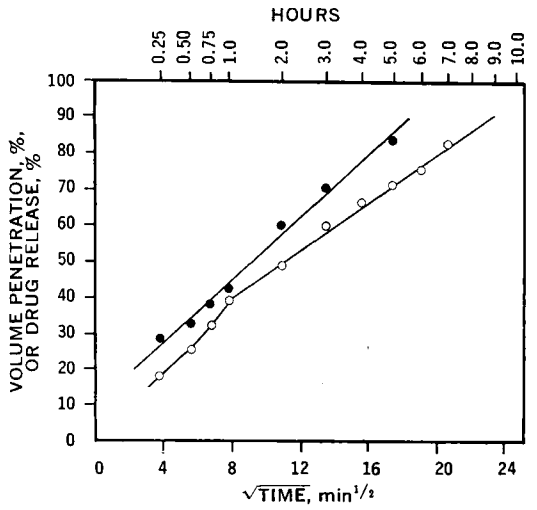

Figure 4—*Comparison of fluid penetration and percent drug release from a phenylpropanolamine hydrochloride 150-mg wax matrix tablet. Compression force was 544.3 kg. Key: ●, percent volume penetration of dissolution fluid; and ○, percent drug release.*

the lower tablet surface and the medium. This procedure assured that the upper surface was the only releasing surface. Adjustment of stirring height was made to provide the same tablet to propeller distance as used for the free tablet.

The pattern of drug release for the tablet-in-a-die was linear with the square root of time throughout the entire period (Fig. 3). A comparison of dissolution rates (Q *versus* t) was made for free tablets and the tablet-in-a-die compressed at 544.3 kg (Table IV). Comparisons in rates were made for the 1-hr time period and the average 2–4-hr time period. These rates for all conditions, various compressional forces, and various surface areas compare favorably, indicating that diffusion in free tablets is occurring through both top and bottom surfaces and the sides. Therefore, the dissolution apparatus does not restrict diffusion from any part of the tablet surface.

Penetration of Dissolution Media into Tablets—Wax matrix tablets are slowly permeated by the dissolution media as a function of time. The percent of volume penetration and percent drug release as a function of $t^{1/2}$ is shown in Fig. 4. It can be seen that some correlation exists between drug release and fluid penetration and that volume penetrated is proportional to the $t^{1/2}$. In the earlier time period, drug release lags about 1 hr behind fluid penetration. In later time periods, the lag time increases. An extrapolation of penetration and drug release curves results in a time period of about 7 hr for complete penetration and about 11.5 hr for complete drug release.

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GLC Determination of γ-Hydroxyphenylbutazone in Plasma

K. K. MIDHA [x], I. J. McGILVERAY, and C. CHARETTE

Abstract □ A recently described GLC method for the determination of phenylbutazone and its major metabolite, oxyphenbutazone [1-phenyl-2-(*p*-hydroxyphenyl)-3,5-dioxo-4-*n*-butylpyrazolidine], in plasma was extended to the estimation of the second major metabolite, γ-hydroxyphenylbutazone [1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)pyrazolidine]. The method is sensitive to 1.0 μg/ml.

Keyphrases □ γ-Hydroxyphenylbutazone—GLC determination in plasma □ Phenylbutazone metabolites—GLC determination of γ-hydroxyphenylbutazone in plasma □ GLC—analysis, γ-hydroxyphenylbutazone in plasma

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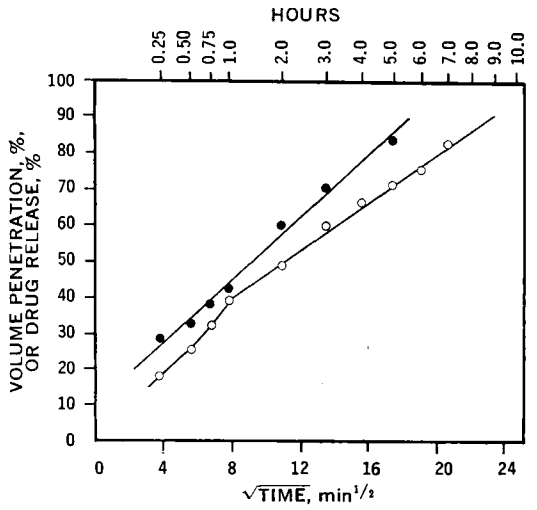

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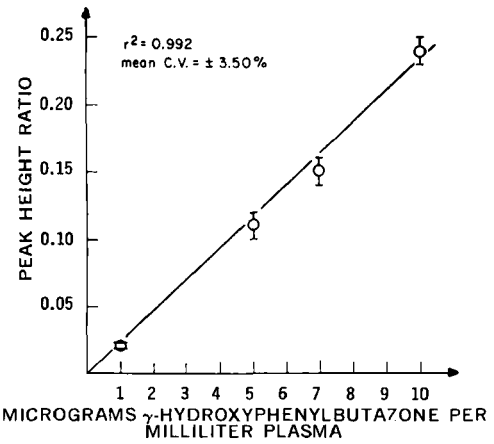

Figure 1—*Calibration curve of γ-hydroxyphenylbutazone extracted from human plasma. Each point is a mean of at least four determinations.*

butazone[1], and γ-hydroxyphenylbutazone[2] were obtained. All chromatographic materials[3] were purchased commercially. Stock solutions of γ-hydroxyphenylbutazone containing 50 μg/ml were prepared and diluted to the required concentrations with methanol. Stock solutions of oxyphenbutazone and the internal standard, 5-(4-hydroxyphenyl)-5-phenylhydantoin, were prepared as described previously (2).

Plasma Level Study—Phenylbutazone (2 × 100-mg tablets[4]) was administered to a healthy male volunteer (31 years, 75 kg). Three weeks after the single dose, this subject was given a 100-mg dose of phenylbutazone[4] every 12 hr for 7 days (9:00 a.m. and 9:00 p.m., 15 doses total). Samples of blood (10 ml) were withdrawn from the cubital vein using heparinized vacuum tubes[5] at 15 appropriate time intervals after dosing. The blood samples were centrifuged and plasma was removed and stored at −10°. Plasma phenylbutazone and oxyphenbutazone were determined as previously reported (2, 3).

GENERAL PROCEDURE

Extraction—The extraction procedure and GLC analysis, following flash-heater methylation with trimethylanilinium hydroxide, were similar to those described for oxyphenbutazone (2). The plasma sample (1 ml), to which 1 ml of internal standard [5-(4-hydroxyphenyl)-5-phenylhydantoin in 0.01 *N* NaOH (1.11 μg/ml)] was added, was first extracted with ether to remove interfering substances and then with *n*-heptane under acidic conditions to separate the phenylbutazone. The aqueous acidic residue from which the phenylbutazone had been selectively removed was shaken again with ether to extract oxyphenbutazone and γ-hydroxyphenylbutazone, which were analyzed by flash-heater methylation on a gas chromatograph.

GLC—GLC was effected on a gas chromatograph[6] equipped with a flame-ionization detector. A stainless steel column [1.8 m × 0.3 cm (6 ft × 0.125 in.) o.d.], packed with 5% phenyl methyl dimethyl silicone[7] on acid-washed, dimethylchlorosilane-treated, high performance flux-calcined diatomite support[8], 80–100 mesh, was used. The column was conditioned by injecting 25–50 μl of column conditioner[9] [mixture of *N,O*-bis(trimethylsilyl)acetamide, trimethylsilyldiethylamine, and hexamethyldisilazane] and maintaining the column at 300° for 18 hr with low nitrogen flow.

The injection port and detector temperatures were 320° and the column oven temperature was 270°. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min. Hydrogen and compressed air flow rates were adjusted to give maximum response. The retention times of γ-hydroxyphenylbutazone (peak III) and the internal standard (peak IV) were 10.6 and 12.0 min, respectively.

Calculation—Peak height ratios were calculated by dividing the height of peak III (10.6 min), due to methylated γ-hydroxyphenylbutazone, by the height of peak IV (12.0 min), due to the methylated internal standard. By using spiked plasma, calibration curves were constructed by plotting the peak height ratios against their respective concentrations of γ-hydroxyphenylbutazone (micrograms per milliliter) (Fig. 1).

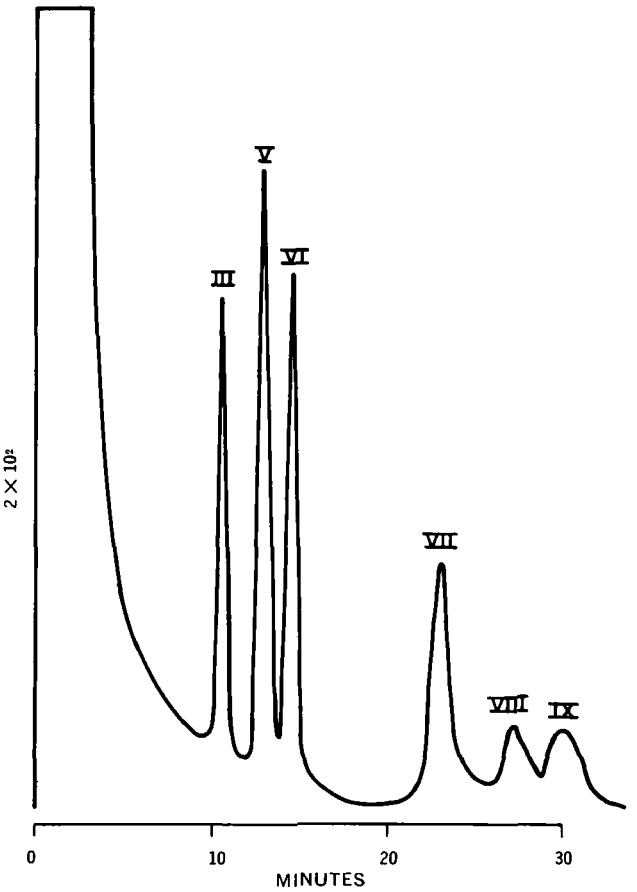

Figure 2—*Gas chromatogram of flash-heater methylated γ-hydroxyphenylbutazone (peaks III, V, VII, and VIII) and oxyphenbutazone (peaks VI and IX)—no extraction.*

RESULTS AND DISCUSSION

Flash-heater methylation of γ-hydroxyphenylbutazone with trimethylanilinium hydroxide gave four peaks with retention times of 10.6, 12.9, 23.1, and 27.1 min (Fig. 2, peaks III, V, VII, and VIII, respectively). To establish the structures of the compounds giving rise to these peaks, the eluates from the GLC column were fed directly into the mass spectrometer[10] and a mass spectrum of each peak was recorded. The mass spectrum of the compound giving rise to peak III showed a molecular ion at *m/e* 352 and other diagnostic ions, which indicated the structure of the methylated derivative to be 1,2-diphenyl-3,5-dioxo-4-methyl-4-(3-methoxy)butylpyrazolidine.

The mass spectra of the other peaks (V, VII, and VIII) showed them to be methylated breakdown products of γ-hydroxyphenylbutazone. The respective peak height ratios of the three breakdown products giving rise to peaks V, VII, and VIII varied with the concentration of flash-heater methylated γ-hydroxyphenylbutazone. Under the described GLC conditions, the height of peak III was observed to be proportional to the concentration of γ-hydrox-

[2] Dr. A. Sallmann, Ciba-Geigy, Switzerland.
[3] Chromatographic Specialties, Brockville, Ontario, Canada.
[4] Butazolidin, Geigy Pharmaceuticals, Montréal, Canada.
[5] Vacutainers, Becton Dickinson & Co., Mississauga, Ontario, Canada.
[6] Model F/11, Perkin-Elmer, Canada.
[7] OV-7, Chromatographic Specialties, Brockville, Ontario, Canada.
[8] Chromosorb W, Chromatographic Specialties, Brockville, Ontario, Canada.
[9] Silyl-8, Pierce Chemical Co., Rockford, Ill.
[10] Hitachi Perkin-Elmer model RMS4 mass spectrometer with Perkin-Elmer gas chromatograph model 990.

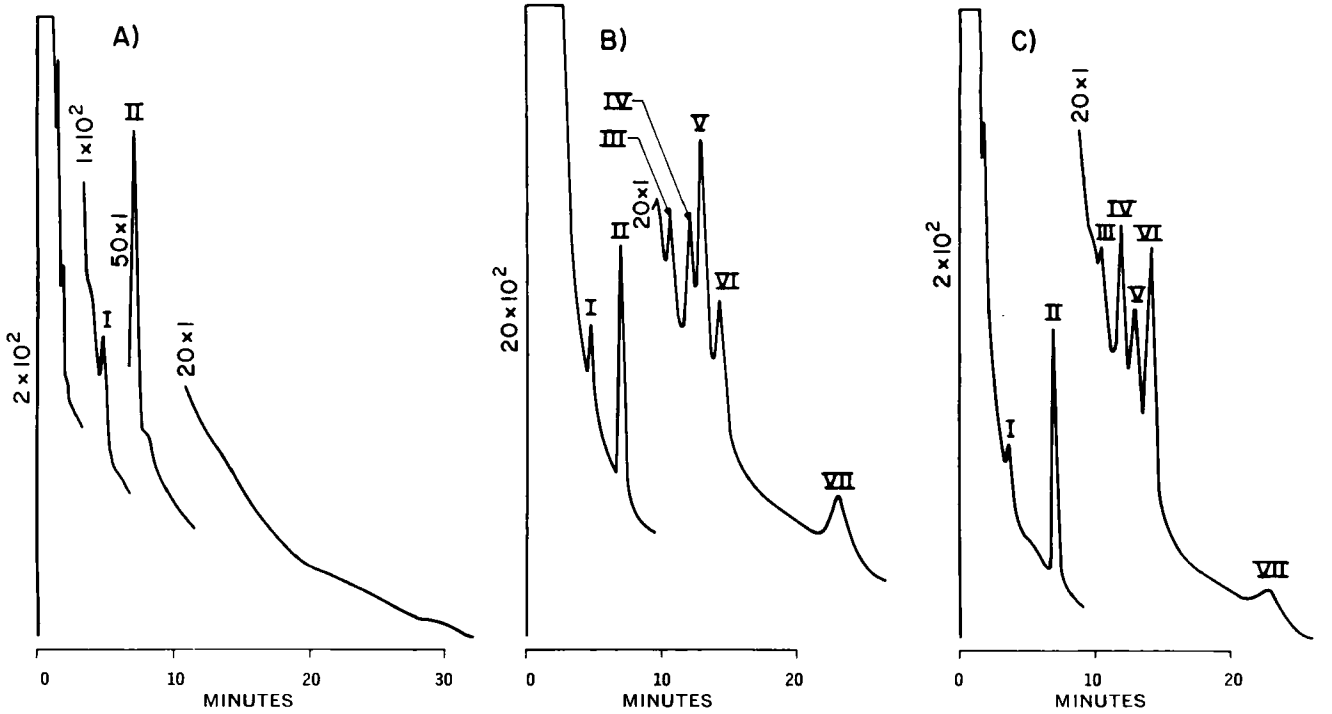

Figure 3—*Gas chromatograms of human plasma. Key: A, control plasma, endogenous material (I and II); B, plasma containing 11.2 μg/ml γ-hydroxyphenylbutazone (III, V, and VII), 7.76 μg/ml oxyphenbutazone (VI), and 1.11 μg/ml internal standard (IV); and C, plasma from a human volunteer who was given 15 × 100-mg tablets of phenylbutazone, 6.00 μg/ml of γ-hydroxyphenylbutazone, and 12.5 μg/ml of oxyphenbutazone.*

yphenylbutazone added to plasma. Therefore, peak III was used for quantitation.

Peaks VI and IX (Fig. 2) with retention times of 14.4 and 30.0 min, respectively, are due to flash-heater methylation of oxyphenbutazone, the other major metabolite of phenylbutazone.

The concentration of the compound giving rise to peak III varied with the injection port temperature. This temperature was optimized to give the maximum quantity of the methylated product (peak III).

Figure 3A shows a typical chromatogram obtained by processing fresh blank plasma but omitting the internal standard. The extraneous peaks I and II were observed in chromatograms of plasma from all human subjects (4), including pooled plasma. These extraneous peaks do not interfere with peaks due to methylated γ-hydroxyphenylbutazone (peak III) and internal standard (peak IV), as shown in the chromatogram for spiked plasma (1 ml) (Fig. 3B) containing γ-hydroxyphenylbutazone (11.2 μg) and oxyphenbutazone (7.76 μg). Phenylbutazone and oxyphenbutazone do not interfere in the assay, because phenylbutazone is removed by partitioning into *n*-heptane and flash-heater methylation of oxyphenbutazone gives peaks VI and IX separated from the methylated γ-hydroxyphenylbutazone. Figure 3C shows the chromatogram of a sample (175 hr after first dose) from a subject who had been given 100 mg phenylbutazone every 12 hr for 15 doses. The concentration of γ-hydroxyphenylbutazone was estimated to be 6.0 μg/ml.

The response of the flame-ionization detector to γ-hydroxyphenylbutazone was linear in the 1–10-μg/ml range (Fig. 1). Calibration curves were tested in the presence of various amounts of oxyphenbutazone, and no change in the slope of γ-hydroxyphenylbutazone was observed. The efficiency of extraction of γ-hydroxyphenylbutazone in this procedure was low but consistent. The overall recovery from plasma at 4 and 10 μg/ml was 20.8 ± 0.9% in the presence of oxyphenbutazone (1–20 μg). Several attempts were made to improve the recovery by employing different solvents, such as methylene chloride and ethylene chloride. However, there were large variations in the recovery, attributed either to the breakdown of γ-hydroxyphenylbutazone or to carryover of trace amounts of acid which would inhibit flash-heater methylation.

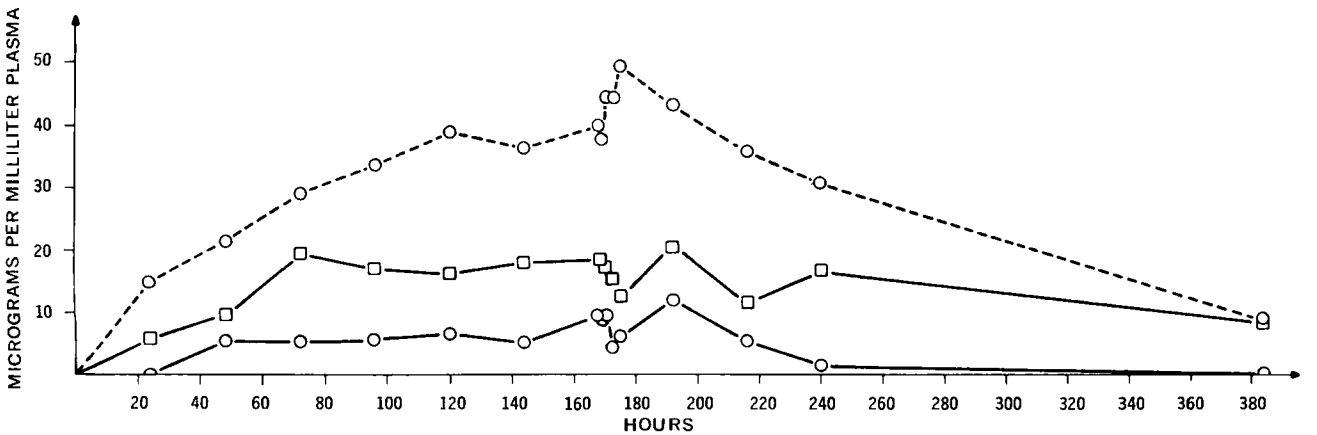

Figure 4—*Phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone concentrations in a human volunteer following multiple oral doses of 15 × 100-mg tablets of phenylbutazone. Key: O---O, phenylbutazone; □—□, oxyphenbutazone; and O—O, γ-hydroxyphenylbutazone.*

Application of the method to plasma level determination of γ-hydroxyphenylbutazone by GLC is shown in Fig. 4. This profile was assembled following multiple doses of phenylbutazone to this subject (15 × 100 mg). Following single doses of phenylbutazone (up to 400 mg) in six subjects, γ-hydroxyphenylbutazone could not be detected.

The described GLC method is sensitive and specific for γ-hydroxyphenylbutazone. It offers the advantage that both metabolites (γ-hydroxyphenylbutazone and oxyphenbutazone) can be determined simultaneously following phenylbutazone administration.

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Abstract □ Plasma salicylates, salicylic acid and acetylsalicylic acid (aspirin), were estimated as their respective trimethylsilyl derivatives by GLC. The method involved the use of fluoride and cooling to minimize the hydrolysis of aspirin in whole blood. Following solvent extraction, internal standards were added to each plasma extract. The extracts were evaporated to dryness and the residues were trimethylsilylated with hexamethyldisilazane in acetone. Small portions of the reaction mixture were chromatographed and the salicylates were quantitated. The components eluting from the chromatograph were identified by IR spectrophotometry and mass spectrometry.

Keyphrases □ Salicylic acid and aspirin—simultaneous GLC determination in plasma □ Aspirin and salicylic acid—simultaneous GLC determination in plasma □ GLC—analysis, simultaneous, salicylic acid and aspirin in plasma

Numerous methods have been described for the determination of salicylic acid and aspirin in pharmaceuticals and in biological media. Of these published techniques, surprisingly few have been developed for the simultaneous and direct determination of aspirin and salicylic acid in a single sample.

Among the techniques employed in the past is a large group of colorimetric estimations involving complexation with ferric ion (1–9). Other colorimetric assays involve diazotization with *p*-nitroaniline and nitrous acid (10), use of Folin and Ciocalteu phenol reagent (11), complexation with cupric ion in nitrous acid (13–15), or estimation as the nitro derivative (16).

Collectively, colorimetric assays are quite nonspecific. Plasma blank values are often high; blanks equivalent to 240 μg of salicylic acid/ml have been reported (11). Common plasma constituents such as salicyluric acid (1, 12), gentisic acid, uric acid, tyrosine, and tryptophan (12) interfere with colorimetric assays.

Furthermore, this entire group of assays determines salicylic acid only; to estimate aspirin, hydrolysis to salicylic acid and a second determination are required. The difference between the two salicylic acid concentrations is assumed to be due to aspirin.

A second analytical method is the group of UV assays. By these techniques, either salicylic acid alone or salicylic acid plus aspirin may be determined. Salicylic acid has been estimated, either in combination with aspirin (17) or individually following a physical separation step (18–21).

Both salicylates have been determined simultaneously by use of the pH-dependent shift in their individual absorption spectra (22–24). This hypsochromic shift technique results in some overlap of the absorption spectra of salicylic acid and aspirin, and corrections may be required for these spectral interferences (23, 24). The individual determination of salicylic acid is subject to interference from aspirin, gentisic acid, and 2,6-dihydroxybenzoic acid (19).

Salicylates have also been estimated fluorometrically. Aspirin may be estimated directly (25) or, more commonly, as salicylic acid. This has been accomplished by direct hydrolysis (26) or after a separation step followed by hydrolysis (27–30). Fluorometric assays generally require a hydrolytic procedure and a dual determination to estimate aspirin. Another problem is the high and variable background fluores-

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cence, which may be minimized by use of blank plasma from each individual subject (26).

The most specific quantitative methods employed to date are chromatographic estimations. TLC (31, 32) and liquid chromatography (33) have been used, but most quantitative chromatographic procedures for salicylates involve GLC. Estimation of aspirin and salicylic acid as the free acids by this technique (34–36) is difficult due to their low vapor pressures and the presence of polar functional groups which cause absorption and tailing. Masking the functional groups by derivatization makes these molecules much less polar, more volatile, and, consequently, more amenable to GLC analysis. Among the derivatives that have been prepared quantitatively are the methyl ester or methyl ether derivatives (37–39) and the trimethylsilyl derivatives (40–46).

The most promising of the mentioned methods appears to be GLC because:

1. It permits the estimation of both compounds simultaneously (35, 37–39).
2. GLC assays are more rapid than are the colorimetric, UV, and fluorometric assays because of the elimination of the hydrolytic step and dual determination. As little as 3 min is required to estimate both salicylates (38).
3. It is the most specific of the methods mentioned. Even similar compounds such as benzoic acid (41), 3-hydroxybenzoic acid (41), 4-hydroxybenzoic acid (41), 2,5-dihydroxybenzoic acid (38), 2,3-dihydroxybenzoic acid (38), 2,3,4-trihydroxybenzoic acid (38), and phenacetin (34) exhibit different retention times from the salicylates and, consequently, do not affect their estimation.
4. GLC methods are quite sensitive and compare favorably with fluorometric procedures. For example, 0.4 μg of aspirin/ml may be estimated in the presence of a vast excess of salicylic acid (43).

The generation and use of methylating agents may be hazardous. For this reason and to provide an alternative method of analysis, it was decided to develop a procedure for the simultaneous GLC estimation of salicylic acid and aspirin as their trimethylsilyl derivatives.

EXPERIMENTAL

Materials—The following were used: salicylic acid[1], *n*-butyl benzoate[1], acetone[1], aspirin[2] (recrystallized from benzene prior to use), ethyl anisate[3], chloroform[4], and hexamethyldisilazane and other silylating reagents[5].

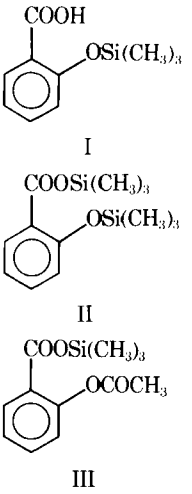
Apparatus—All routine salicylate analyses were conducted on a gas chromatograph[6] equipped with dual flame-ionization detectors. The signal from the detectors was fed into a strip-chart recorder[7], with a 1.0-mv full-scale response. Dual coiled glass columns, 1.8 m (6 ft) in length and 4 mm (i.d.), containing 3% OV-25 coated onto 60–80-mesh Chromosorb G (regular), were used.

The operating temperatures were: injectors, 240°; oven, 160° (isothermal); and detectors, 240°. The gas flow rates were: helium, 55 ml/min; hydrogen, 40 ml/min; and air, approximately 600 ml/min.

For the IR study, a gas chromatograph[8] equipped with thermal conductivity detectors was used to separate and collect the salicylate derivatives. These fractions were incorporated into micro- (1.5 mm diameter) potassium bromide pellets and scanned on a grating IR spectrophotometer[9].

A gas chromatograph[10] coupled to a mass spectrometer[11] was used in the mass spectrometric study.

In both the IR and mass spectrometric studies, the columns and operating conditions were identical to those mentioned previously.



RESULTS AND DISCUSSION

Silylation Studies—The structure of salicylic acid suggests that it should be possible to obtain up to three individual trimeth-

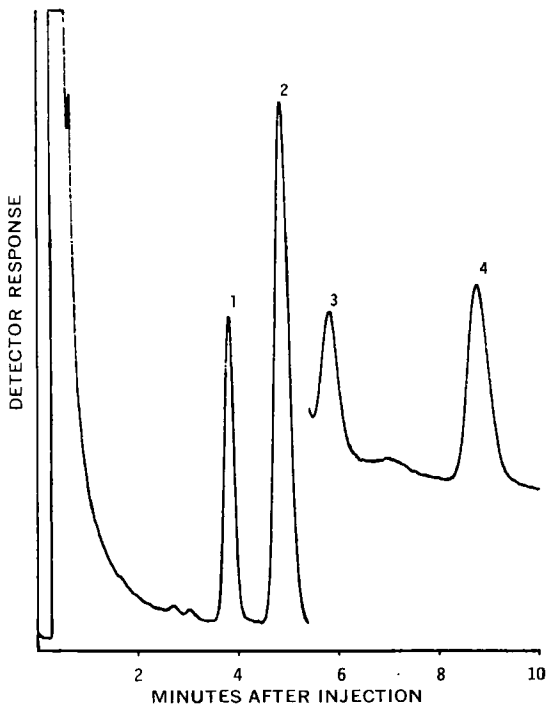


Figure 1—*Typical chromatogram. Key: 1,* n*-butyl benzoate; 2, trimethylsilyl 2-trimethylsiloxybenzoate (II); 3, ethyl anisate; and 4, trimethylsilyl 2-acetoxybenzoate (III).*

[1] Fisher Scientific Co.
[2] British Drug Houses.
[3] Eastman Organic Chemicals.
[4] Matheson, Coleman and Bell.
[5] Pierce Chemical Co.
[6] Perkin-Elmer model F-11.
[7] Hewlett-Packard model 7127A.
[8] Hewlett Packard model 5700A.
[9] Perkin-Elmer model 627.
[10] Varian model 1200.
[11] AEI-MS2.

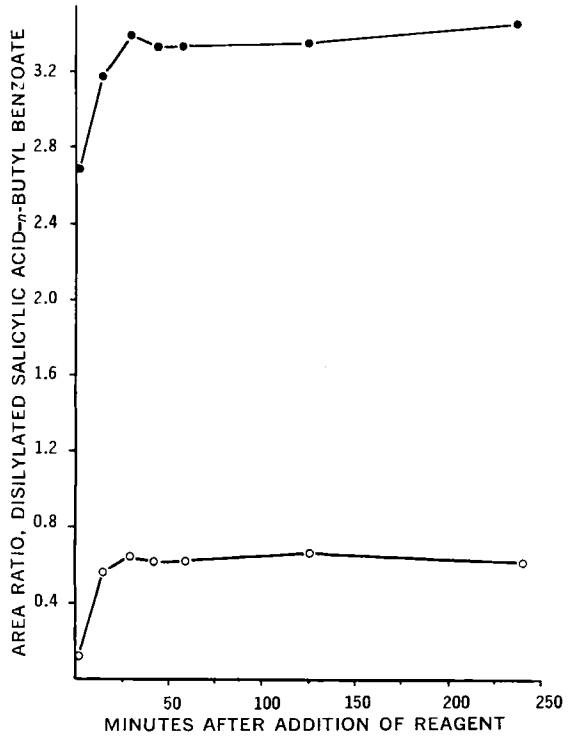


Figure 2—*Time required for disilylation of salicylic acid. Key:* ●, *150 μg of salicylic acid and 25 μg of* n-*butyl benzoate; and* O, *25 μg of salicylic acid and 25 μg of* n-*butyl benzoate.*

ylsilylated (or silylated) derivatives. Multiple GLC peaks have been reported from attempts to silylate salicylic acid (39, 42). Furthermore, two of the three possible structures, namely 2-trimethylsiloxybenzoic acid (I) and trimethylsilyl 2-trimethylsiloxybenzoate (II), were isolated and characterized (44–46). In contrast, aspirin can only produce one silylated derivative, trimethylsilyl 2-acetoxybenzoate (III). The molecular weights and chromatographic retention times of these silylated derivatives are as follows: I (mol. wt. = 210; retention time = 2.1 min), II (mol. wt. = 282; retention time = 4.9 min), and III (mol. wt. = 252; retention time = 8.6 min).

A typical chromatogram is depicted in Fig. 1. Peak 2 is II, and peak 1 is its internal standard, *n*-butyl benzoate. The break in the trace between peaks 2 and 3 is due to a change of attenuation required to increase the size of the subsequent peaks. Peak 3 is ethyl anisate, the internal standard for peak 4 (III).

Time for Reaction—To check for completeness of silylation, the disilylation of salicylic acid and the monosilylation of aspirin were monitored. Two concentrations of salicylic acid (25 and 150 μg) and of aspirin (5 and 25 μg) were studied for 250 min after the addition of silylating reagent (Figs. 2 and 3). It is apparent from Figs. 2 and 3 that the silylation reactions run to completion in approximately 50 min since there are no appreciable increases in peak area ratios after that time.

Furthermore, both II and III synthesized in this fashion are stable in the reaction mixture for over 4 hr after the addition of reagents. In subsequent studies, 60 min was allowed to elapse between the addition of reagent and injection of a portion of the mixture into the chromatograph.

Silylating Reagent—Several common silylating reagents and reagent mixtures were investigated to determine the most useful: *(a)* 100 μl of hexamethyldisilazane, *(b)* 100 μl of *N,O*-bis(trimethylsilyl)acetamide, *(c)* 100 μl of trimethylchlorosilane, *(d)* 50 μl of hexamethyldisilazane and 50 μl of trimethylchlorosilane, and *(e)* 80 μl of hexamethyldisilazane and 20 μl of trimethylchlorosilane.

Fixed quantities of salicylic acid (150 μg) and of aspirin (25 μg) were silylated with each reagent at room temperature. Neither *N,O*-bis(trimethylsilyl)acetamide nor trimethylchlorosilane produced as large a peak area ratio for salicylic acid in 60 min as did hexamethyldisilazane. Mixtures of hexamethyldisilazane and trimethylchlorosilane showed no advantage over hexamethyldisilazane alone, and the extent of silylation appeared to be dependent upon the amount of hexamethyldisilazane present. Consequently, hexamethyldisilazane was selected as the silylating reagent.

Solvent for Silylation—Choice of solvents for silylation reactions and subsequent GLC is limited to those that are inert to silylation and elute rapidly from the column. The latter stipulation excludes most tertiary amines, even those of low molecular weight such as trimethylamine and triethylamine.

Using fixed quantities of salicylic acid (150 μg) and of aspirin (25 μg) and 100 μl of hexamethyldisilazane, various solvents were investigated. Dioxane, chloroform, *n*-hexane, carbon disulfide, and ether all produced less than a quantitative synthesis of II, as evidenced by the presence of a I peak after 60 min. Dimethylformamide, pyridine, and acetone catalyzed quantitative synthesis of both II and III, but the first two solvents tailed badly on chromatographing; consequently, acetone was selected as the solvent.

Presence of Moisture—The effects of moisture on the silylation of 150 μg of salicylic acid and 25 μg of aspirin in 100 μl of acetone were investigated. Salicylic acid tolerated about 1.5% moisture, while aspirin was more sensitive and decreased peak area ratios were observed with 1.0% moisture. Water in the reaction mixture was slow to elute off the column.

Preliminary experiments suggested that 25 μl of hexamethyldisilazane was sufficient to silylate a mixture of 150 μg of salicylic acid and 25 μg of aspirin in 100 μl of acetone. On this basis, 100 μl of hexamethyldisilazane should silylate 600 μg of salicylic acid plus 100 μg of aspirin.

IR Studies—The silylated salicylates were separated and collected for these studies by preparative GLC. The GLC effluent was condensed in small glass capillaries, and the capillary contents were incorporated into a potassium bromide microdisk (1.5 mm diameter). When used with a beam condenser, reliable spectra were obtained from these disks.

These spectra were consistent with those reported earlier for I

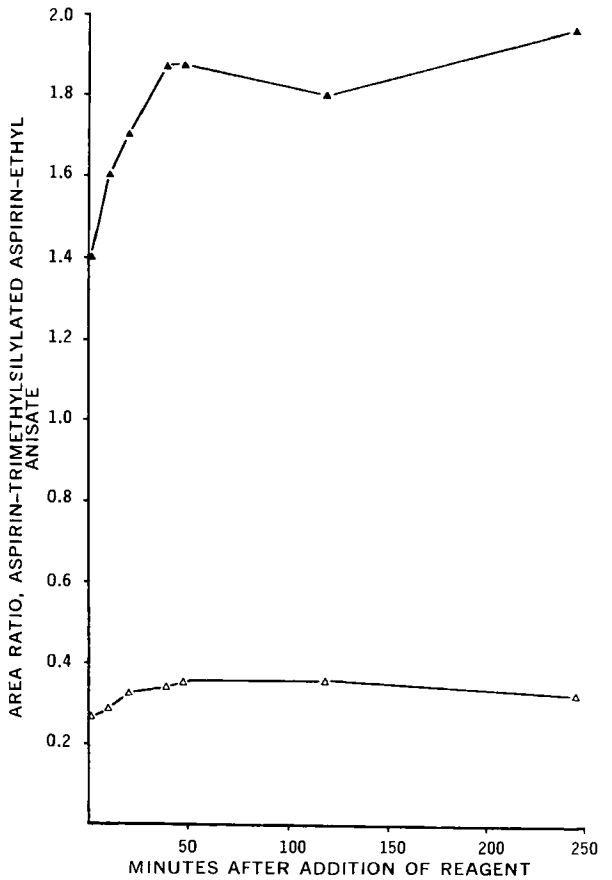


Figure 3—*Time required for monosilylation of aspirin. Key:* ▲, *25 μg of aspirin and 0.5 μg of ethyl anisate; and* △, *5 μg of aspirin and 0.5 μg of ethyl anisate.*

Table I—Effects of Fluoride and Cooling on the Hydrolysis of Aspirin in Plasma

Conditions		Salicylic Acid, μg Present	Aspirin Hydrolyzed, μg	Percent of Added Aspirin Hydrolyzed
Potassium Fluoride	Temperature			
−	37°	6.07	7.92	31.7
−	1°	3.35	4.37	17.5
+	37°	3.62	4.72	18.9
+	1°	2.46	3.21	12.8

and II (45, 46). Some difficulty was encountered with the II spectra. This compound appeared to decompose to I during collection and disk manufacture, as evidenced by the gradual diminution of the peaks at 1710, 1077, and 920 cm^{-1} and by the appearance of a peak at 3240 cm^{-1}. In fact, within 30 min of collection of supposedly pure II, the IR spectrum was indistinguishable from that of I.

GLC–Mass Spectrometry—Mass spectral data, from combined GLC–mass spectrometry, were obtained at both 20 and 70 ev. The mass spectra of I were consistent with the structure proposed here. A 20-ev spectrum showed characteristic ions at *m/e* 210 (5.4%, M^+), 195 (17.3%, M − 15$^+$), 151 (5.5%, M − 15 − CO_2^+), and 75 [12.5%, $(CH_3)_2Si{=}O^+H$], and 73 [8.7%, $(CH_3)_3Si^+$]. In addition, the 70-ev spectrum of this compound had an ion at *m/e* 45 (30.8%), shown by accurate mass measurement to be $COOH^+$.

Both the 20- and 70-ev scans of II showed the following characteristic ions: *m/e* 267 (77.4%, M − 15$^+$), 209 [5.5%, M − $Si(CH_3)_3^+$], 193 [4.9%, M − $OSi(CH_3)_3^+$], and 73 [100.0%, $(CH_3)_3Si^+$]. These ions are consistent with a previously published spectrum of the disilylated derivative of salicylic acid (44) and support the proposed structure.

The mass spectrum of III at 20 ev showed ions of *m/e* 193 (23.0%, M − $OCOCH_3^+$) and 73 [100.0%, $(CH_3)_3Si^+$]. In addition, the corresponding 70-ev spectrum had the following ions: *m/e* 210 [15.2%, $(M - CH_2{=}C{=}O)^+$], 195 (44.8%, M − 42 − 15$^+$), and 135 [20.0%, M − $COOSi(CH_3)_3^+$]. The loss of $CH_2{=}C{=}O$ (42 amu) is characteristic of acetates.

Plasma Extraction Studies—*Acidifying Agent*—Unacidified 1-ml plasma samples containing known quantities of salicylic acid and aspirin yielded approximately 4.0% of the salicylic acid and 6.0% of the aspirin present when extracted into an organic solvent. These extremely low recoveries clearly indicated the need to lower the pH of the plasma to facilitate partitioning into the organic phase. Potassium bisulfate solutions have been employed successfully (43) as acidifying agents, and several concentrations were investigated here. The optimal concentration of potassium bisulfate in terms of recovery of added salicylates was 10% (w/v). In subsequent experiments, 1.0-ml plasma samples were acidified with 1.0 ml of 10% potassium bisulfate solution. The pH of this acidified plasma was approximately 1.0.

Extraction Solvent—Several solvents were screened in an attempt to obtain quantitative recovery of added salicylates. A single extraction procedure was selected to minimize handling and the time required for analysis. The solvents investigated were ether, methylene chloride, ethylene dichloride, and chloroform. The highest and most consistent recoveries of salicylate added to blank plasma were obtained with chloroform and ethylene dichloride.

Chloroform was chosen over ethylene dichloride on the basis of its higher volatility and ease of removal by flash evaporation. Although ether was relatively easy to remove, it was difficult to work with due to its volatility. It also extracted appreciable quantities of moisture from plasma. Chloroform offered the best compromise between volatility and extraction of moisture.

Subsequently, all 1.0-ml plasma samples were acidified with 1.0 ml of 10% potassium bisulfate solution in 15-ml glass-stoppered centrifuge tubes. Five milliliters of chloroform was added to each tube, and these were then agitated on a mechanical shaker for 15 min. The resulting emulsions were centrifuged, and 4.0 ml of the chloroform solution was uniformly recovered. These extracts were transferred into tubes containing 25 μg of *n*-butyl benzoate and 0.5 μg of ethyl anisate as internal standards. The contents of each tube were carefully flash evaporated to dryness and then silylated for 60 min at room temperature with 100 μl of hexamethyldisilazane in 100 μl of acetone.

Standard Calibration Curves—Calibration curves were constructed for both salicylic acid (*versus n*-butyl benzoate) and aspirin (*versus* ethyl anisate) by adding known and varying amounts of each salicylate to 1.0-ml portions of plasma. The salicylates were then extracted as outlined, and fixed quantities of internal standards were added. After evaporation and silylation, the chromatographic peak area ratios of II–*n*-butyl benzoate and III–ethyl anisate were plotted against the known weight ratios.

Aspirin Hydrolysis—It had previously been demonstrated that the presence of fluoride in whole blood samples and the cooling of the samples after drawing the blood resulted in considerable reduction of the hydrolysis of aspirin in the samples (43). The effects of fluoride and cooling were investigated here as follows.

Whole blood samples were drawn from normal human volunteers into glass tubes containing 50 units of heparin, free from preservatives. Common preservatives such as benzyl alcohol may be extracted, silylated, and subsequently chromatographed. The blood was immediately centrifuged, and the plasma was pipetted off and pooled at room temperature.

Four 10-ml aliquots of the pooled plasma were withdrawn. Two of the aliquots of the pooled plasma were warmed to 37° and the other two were cooled to 1° on ice. Then 50 μl of a potassium fluoride solution (25 mg of potassium fluoride) was added to one warmed and one cooled aliquot. Subsequently 250 μg of aspirin was added in ether to each 10-ml aliquot. The ether layer was gently evaporated and the samples were mixed and frozen immediately on dry ice. The total time that elapsed between the addition of the aspirin and freezing of the samples was 10 min.

For the analysis, six 1.0-ml aliquots were assayed for aspirin and salicylic acid. The results (Table I) show that the presence of fluoride and the cooling of the samples each reduced the extent of hydrolysis, but that the combination of fluoride plus cooling was best. When these results are combined with those given below, it is evident that approximately 4.3% of the aspirin hydrolysis observed occurred during the freezing, storage, and thawing process.

Storage of Samples—Previous experimenters observed that plasma samples containing aspirin could be stored on dry ice for as long as 1 month without loss of salicylic acid or aspirin (43). In this experiment, 35 ml of pooled heparinized plasma containing fluoride was used. The plasma was cooled to 1°, and 25 μg of aspirin/ml of plasma was added. The plasma was well mixed and divided into four equal portions; one was analyzed immediately and the others were rapidly frozen and stored on dry ice. Individual por-

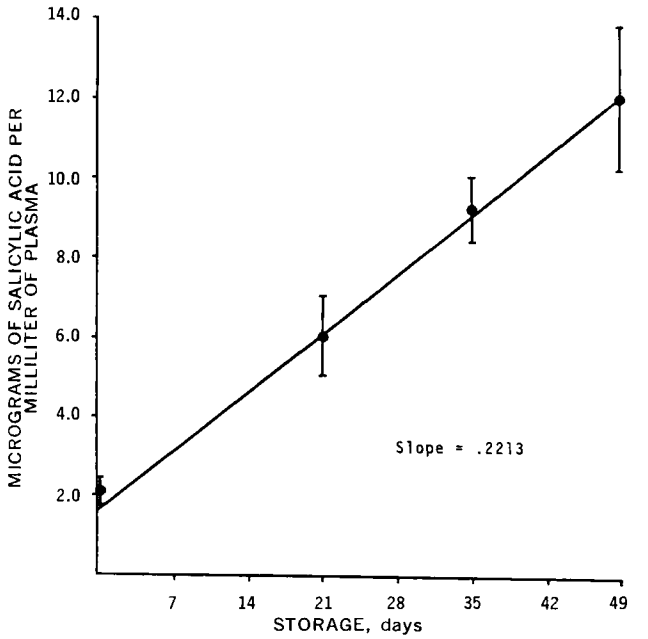


Figure 4—*Effects of storage on decomposition of aspirin. Each point on the curve represents the mean of seven determinations with standard deviation.*

tions were thawed and analyzed for salicylic acid and aspirin after 21, 35, and 49 days of storage.

The results of this experiment (Fig. 4) showed that approximately 8.5% of the aspirin was hydrolyzed during the extraction and handling procedure prior to estimation. The half-life of aspirin in frozen plasma, assuming aspirin decomposition to salicylic acid, was calculated to be 23.5 days and the corresponding rate constant was 0.02949 day^{-1}.

The conclusion to be drawn from this experiment is that plasma samples containing aspirin should be assayed as soon as possible after drawing.

CONCLUSIONS

The method presented enables the simultaneous estimation of salicylic acid and aspirin in a single sample. One-milliliter plasma samples are conveniently assayed, although the procedure is adaptable to smaller volumes. The limits of detection are approximately 1.0 μg of salicylic acid/ml plasma and 0.5 μg of aspirin/ml plasma. A correction should be made to allow for aspirin hydrolyzed during storage and extraction.

This method is currently being applied to the determination of the comparative bioavailabilities of various dosage forms of aspirin in arthritic patients.

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[x] To whom inquiries should be directed.

Inhalation of 2,3,5-Triiodobenzoic Acid by Normal and Sulfur Dioxide-Exposed Rats

H. K. MORGAN *, S. M. SHAW ˣ, J. E. CHRISTIAN, and W. W. CARLTON

Abstract □ Exposure to ^{125}I-labeled 2,3,5-triiodobenzoic acid resulted in deposition of the material in the lungs of rats. The initial uptake was 20.85% followed by removal at an exponential rate. The residency half-life in the lungs was 17.7 min. Exposure of animals to sulfur dioxide for 4 consecutive days, 4 hr/day, prior to inhalation of the labeled compound did not provide statistically significant differences in the parameters studied.

Keyphrases □ 2,3,5-Triiodobenzoic acid, radiolabeled—inhalation and half-life in lungs, normal and sulfur dioxide-exposed rats □ Sulfur dioxide—effect on residency time of 2,3,5-triiodobenzoic acid in lungs of rats □ Inhalation—radiolabeled 2,3,5-triiodobenzoic acid, normal and sulfur dioxide-exposed rats, residency time in lungs

The substance 2,3,5-triiodobenzoic acid has been shown to affect the growth and development of certain plants. Greer and Anderson (1) reported that treatment of soybean plants with this chemical at the beginning of flowering resulted in an increased seed yield.

While the uptake and fate of 2,3,5-triiodobenzoic acid following oral administration have been studied in various species (2–5), no information is available relevant to lung uptake and retention following inhalation of the compound. In the present investigation, the degree of uptake and loss of inhaled ^{125}I-labeled 2,3,5-triiodobenzoic acid in the lungs of rats was studied. In addition, the consequence of exposure to sulfur dioxide was determined. Sulfur dioxide-induced alterations in the uptake and rate of removal of inhaled particles have been studied with varying results (6–8). Sulfur dioxide-induced alterations in the physiological response of the lung could affect the uptake, loss, and subsequent hazard of 2,3,5-triiodobenzoic acid from inhalation.

EXPERIMENTAL

For this investigation, 2,3,5-triiodobenzoic acid labeled in the 2-position with ^{125}I was employed in the production of the dry labeled aerosol. The labeled material was synthesized in a manner similar to that described previously (4, 9). Approximately 100 mCi of ^{125}I in the form of sodium iodide[1] was used in the synthesis. Purification of the final material was achieved by TLC (3, 4), employing a fluorescent silica gel[2] and a developing solution consisting of petroleum ether (bp 30–60°)–propionic acid (10:1). The labeled material was removed from the gel with hot ethanol and was found to be 97.2% pure. Approximately 0.25 g of labeled 2,3,5-triiodobenzoic acid containing about 10 mCi of ^{125}I was dissolved in 10.0 ml of ethanol, and the resulting solution was used in the aerosol generator.

The radioactive aerosol was produced with a Lucite jet and generator in a manner similar to that of Johnson and Ziemer (10). Measurement of aerosol diameter was performed with a seven-stage cascade impactor[3]. Calibration and operation of the impactor were performed according to methods described previously (10, 11).

The sampling flow rate through the impactor was 105–120 ml/min, which approximates the breathing rate of rats (12). The aerosol was sampled for 3 min during each exposure period. Each cover slip from each stage in the cascade impactor was removed and analyzed by crystal scintillation spectrometry for the amount of impinged radioactive aerosol. The percent less than stated size for each impactor stage was calculated from the results obtained for the cover slips, and the activity median aerodynamic diameter and corresponding geometric standard deviation of the aerosol were determined from these data.

For the entire investigation, the activity median aerodynamic diameter values ranged between 0.31 and 0.51 μm, with the standard deviations ranging from 2.45 to 3.09 μm. The average value was 0.41 μm and the average of the standard deviations was 2.73 μm. The standard deviation of the average activity median aerodynamic diameter value was 0.074 μm at the 68% confidence level. The diameter of the aerosol for each aerosol run was within 2 *SD* of the average. Therefore, variation in biological data obtained in the various portions of the investigation probably was not caused by different particle sizes.

A Lucite chamber (38 × 38 × 15 cm) was used for exposing the animals to sulfur dioxide. The environmental chamber was divided into five segments, thus allowing five animals to be kept separated during exposure. Compressed air mixed with 10% sulfur dioxide[4] was passed through the closed chamber at a flow rate of about 4.5 liters/min. The exhaust air–gas mixture was analyzed 3 hr after initiation of sulfur dioxide exposure by passing the exhaust through a trapping solution. The trapping solution was analyzed colorimetrically by the West-Gaecke method in a similar manner as described by Stern (13).

The major portion of the investigation was conducted in two phases. Phase I dealt with the determination of the percent uptake and residency half-life of inhaled 2,3,5-triiodobenzoic acid in the lungs of 200–300-g male laboratory rats[5] exposed only to an aerosol of the labeled material. For each run, a group of five animals was anesthetized with a freshly prepared solution of sodium pentobarbital and chloral hydrate (10). The anesthetized animals were placed in a special small animal aerosol exposure chamber[6]. Operational procedures for the exposure chamber were conducted in a similar manner as described previously (10, 14).

After a 6-min aerosol inhalation period, an animal was decapitated at 15, 30, 45, 60, and 75 min postexposure. The amount of inhaled ^{125}I, expressed as corrected counts per minute, in the skinned head, excreta, left and right lungs, and residual carcass was determined by crystal scintillation spectrometry. Although the chamber design prevented contamination of the body of the animal, the skin was removed from the head because it became contaminated with the radioactive aerosol during the exposure period. The total inhaled radioactivity for each animal was determined by the summation of counts per minute for all samples composing that animal.

The percent of total inhaled radioactivity remaining in the left and right lungs at the various postexposure time periods was calculated. Percents at the various time periods were plotted on semilog paper, and a line of best fit was statistically determined along with the equation describing the line. From the equation of the

[1] New England Nuclear Corp., Boston, Mass.
[2] Adsorbosil-1P, Applied Science Laboratories, Inc., State College, Pa.
[3] Supplied by the Lovelace Foundation, Albuquerque, N.M., who also supplied the effective cut-off diameters for the various stages.
[4] Matheson Co., Inc., Joliet, Ill.
[5] Sprague–Dawley strain supplied by Laboratory Supply, Indianapolis, Ind.
[6] Designed by the Lovelace Foundation.

Table I—Percent Lung Retention[a] of Labeled 2,3,5-Triiodobenzoic Acid following Inhalation

Post-exposure Time, min[b]	Study A			Study B			Study C		
	Left Lung	Right Lung	Total Lung	Left Lung	Right Lung	Total Lung	Left Lung	Right Lung	Total Lung
Labeled Compound Only									
15	4.23	7.05	11.28	6.07	9.33	15.40	7.50	4.06	6.56
30	1.79	3.29	5.08	1.68	3.26	4.94	1.78	3.21	4.99
45	0.67	1.27	1.94	1.63	2.56	4.19	1.34	2.71	4.05
60	0.53	0.80	1.33	0.43	1.00	1.41	0.69	1.18	1.81
75	0.20	0.31	0.51	0.19	0.89	1.08	0.25	0.38	0.63
Sulfur Dioxide and Labeled Compound[c]									
15	5.14	7.38	12.52	6.97	7.84	14.81	2.48	4.45	6.93
30	2.60	4.37	6.97	2.20	3.40	5.60	1.51	2.54	4.05
45	1.36	1.75	3.11	0.76	1.30	2.06	1.07	1.83	2.90
60	0.87	1.79	2.66	0.70	1.21	1.91	0.31	0.55	0.86
75	0.52	0.80	1.32	0.47	0.61	1.08	0.30	0.49	0.79

[a] Percent in each lung was based on the total amount of inhaled labeled 2,3,5-triiodobenzoic acid found in the entire animal, including excreta and excluding skin on the head. [b] One animal at each time period for each study. [c] The average concentration of four sulfur dioxide exposures for Studies A, B, and C was 429, 370, and 325 ppm, respectively.

line, the initial percent uptake at time zero was determined and the residency half-life was calculated from the slope of the line. Slope of the line was also used to express the rate of removal of radioactive compound from the lungs. Three values for each time period were obtained by repeating Phase I three times.

Phase II was the determination of the uptake and residency half-life of inhaled labeled aerosol in the lungs of rats after the animals were exposed to sulfur dioxide. The animals were subjected to the sulfur dioxide gas in the chamber described previously. The concentration of sulfur dioxide in the exhaust air was approximately 400 ppm for each sulfur dioxide exposure. Each group of five animals was exposed to the gas for 4 hr daily for 4 consecutive days.

One hour following the fourth exposure to sulfur dioxide, each group of five animals was subjected to the aerosol of labeled 2,3,5-triiodobenzoic acid. Following the same procedures as in Phase I, the initial percent uptake and residency half-life of inhaled labeled aerosol in the lungs were determined for animals in Phase II. Three values for each time period were obtained by conducting Phase II three times.

Gross autoradiography techniques were employed to determine the approximate location and loss of inhaled labeled 2,3,5-triiodobenzoic acid within the lungs at the various postexposure time periods. Two separate groups of five animals were treated identically as the animals in Phases I and II. The lungs from each animal were exposed to X-ray film[7] while frozen. All photographic processing procedures were kept constant to allow correlation in intensity of the darkened X-ray with the amount of inhaled labeled aerosol.

A pathology study was performed to determine if either 2,3,5-triiodobenzoic acid or sulfur dioxide produced any pathoanatomic alterations of the lung tissue. One group of five animals was exposed to the labeled aerosol while another group received sulfur dioxide and the labeled aerosol as described previously. Lungs were removed and prepared for sectioning. The tissue slices were stained with eosin–hematoxylin and a glycoprotein stain. The staining solutions and techniques employed were described by Luna (15). Lungs from control animals were treated similarly for comparative purposes.

RESULTS AND DISCUSSION

Table I lists the percent lung retention of 2,3,5-triiodobenzoic acid at the various postexposure time periods for animals exposed only to the labeled compound and animals exposed to sulfur dioxide previous to 2,3,5-triiodobenzoic acid administration. Studies A, B, and C represent the three individual runs performed for each phase. It can be seen from the data that inhaled compound reached both the left and right lungs. Also, the amount of labeled compound in the lungs decreased as time elapsed following exposure. The data indicate that the amount of inhaled compound was always greater in the right lung than in the left lung. This occurrence may be explained by the greater mass of the right lung.

The data in Table I were expressed as individual exponential equations for the left, right, and total lungs for Studies A, B, and C. The general equation $Y = Ae^{-BX}$ represents each line, where Y is the percent retention, A is the ordinate intercept, B is the slope of the line, and X is time in minutes. The intercept of the equation is equal to the initial percent uptake at postexposure time zero.

For rats exposed only to labeled 2,3,5-triiodobenzoic acid, the percent uptake for the total lung for Studies A, B, and C was 22.75, 24.72, and 15.09%, respectively. The average of these values was 20.85 ± 5.09% at the 68% confidence level. The residency half-life of labeled 2,3,5-triiodobenzoic acid in the lung was calculated from individual exponential equations. The slope of each line from each equation was found to be equal; thus, the residency half-life for each line was equal. The average of the nine residency half-life values was 17.7 min.

From the nine equations for animals exposed to sulfur dioxide previous to labeled 2,3,5-triiodobenzoic acid, the average residency half-life value was 15.8 min. The total percent uptake at time zero, as obtained from the intercept of the equation, was 20.30, 21.47, and 13.07% for Studies A, B, and C, respectively, with an average value of 18.28 ± 4.55% at the 68% confidence level. The uptake of 2,3,5-triiodobenzoic acid for animals exposed only to labeled compound was statistically ($p = 0.10$) equal to the value obtained for animals receiving sulfur dioxide previous to inhalation of the labeled material. Sulfur dioxide produced no observable effect upon the percent uptake of inhaled compound at time zero in the lungs of rats.

Figure 1 represents a compilation of the data for animals exposed to labeled 2,3,5-triiodobenzoic acid only as well as those receiving sulfur dioxide before exposure to the labeled compound. Each line in Fig. 1 was obtained by statistically determining a line of best fit for the corresponding 15 data points from Studies A, B, and C (Table I). Because of the magnitude of values, the 90 data points were omitted from the figure.

Statistical comparisons between the total, right, and left lungs within and between each phase were conducted by a reported method (16). These comparisons showed that the slopes of the lines within each phase and between each phase were equal. Thus, the slopes of the lines representing the rate of removal of 2,3,5-triiodobenzoic acid from the lungs were equal not only among the total, right, and left lungs within each phase but also between each phase. Because the slopes were equal, the residency half-life value of 17.7 min for animals exposed to labeled 2,3,5-triiodobenzoic acid only was equal to the 15.8-min half-life value for animals receiving sulfur dioxide previous to inhalation of the labeled compound.

The fact that inhaled 2,3,5-triiodobenzoic acid was removed from the lungs in all animals exposed to the labeled compound was substantiated by the gross autoradiography study. The intensity of the darkened area on the X-ray film was the greatest at the 15-min postexposure time period, and there was a continuous decrease in

[7] Kodak No-Screen, Eastman Kodak Co., Rochester, N.Y.

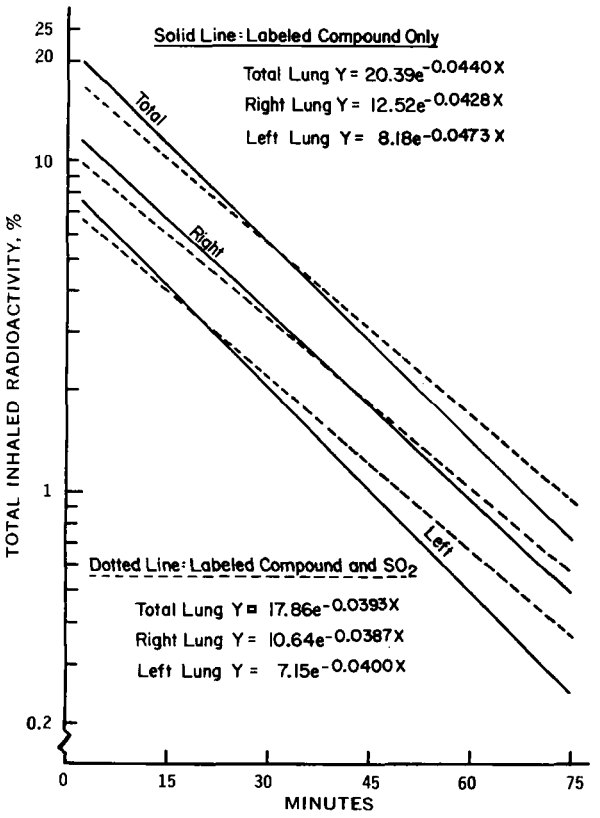

Figure 1—*Percent of the total labeled 2,3,5-triiodobenzoic acid remaining in the lung at various postexposure time periods for pooled values of all three studies in each phase. Each line represents 15 data points.*

intensity for each subsequent time period. Since the intensity for the 60- and 75-min postexposure time periods was negligible, satisfactory photographs for publication were not feasible.

Histopathological examination of lung tissues from animals exposed to either labeled 2,3,5-triiodobenzoic acid or sulfur dioxide was performed. No pathoanatomic alterations were observed when the lung tissues were compared to the controls. It was concluded that neither the labeled compound nor sulfur dioxide at these experimental conditions produced pathological changes in the lungs of rats exposed to either substance.

Since other studies (17, 18) showed that sulfur dioxide at the concentrations used in this investigation will immobilize ciliary movement, it would seem that ciliary movement is not responsible for the removal of the dry labeled aerosol from the lungs in the present study. However, the rate of removal of labeled 2,3,5-triiodobenzoic acid was rapid and may be due to the biological solubility of the substance. Although 2,3,5-triiodobenzoic acid may be a possible environmental pollutant, damage to the lung stemming from inhalation of the compound is not likely since the substance is removed rapidly from the lungs.

The results of the investigation show that inhalation of 2,3,5-triiodobenzoic acid is a very important route by which the compound may be assimilated during application to crops. Furthermore, sulfur dioxide inhalation did not influence the uptake and subsequent removal of the compound from the lung.

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* Present address: Nuclear Medicine Division, University of Texas Medical Branch, Galveston, TX 77550

ˣ To whom inquiries should be directed.

Liquid Chromatography in Pharmaceutical Analysis II: Determination of a Reserpine–Chlorothiazide Mixture

I. L. HONIGBERG ˣ, J. T. STEWART, A. P. SMITH, R. D. PLUNKETT, and D. W. HESTER

Abstract □ Operating conditions are described for the qualitative and quantitative analysis of a reserpine–chlorothiazide mixture in two steps by high-pressure liquid chromatography. Each compound was chromatographed on an octadecyl column using a 55:45 mixture (pH 5.6) of absolute methanol–aqueous 0.5% ammonium chloride at a flow rate of 2.0 ml/min (2000 psig). Each determination can be achieved in approximately 60 min with an accuracy of 3–5%.

Keyphrases □ Reserpine–chlorothiazide—high-pressure liquid chromatography analysis □ Chlorothiazide–reserpine—high-pressure liquid chromatography analysis □ High-pressure liquid chromatography—analysis, reserpine–chlorothiazide mixture □ Liquid chromatography, high pressure—analysis, reserpine–chlorothiazide mixture

The use of commercially available high-pressure liquid chromatography (HPLC) systems in the analysis of multicomponent pharmaceutical dosage forms has been the subject of investigations in this laboratory. The successful separation and quantification of drugs in cough–cold mixtures by HPLC have been reported recently (1). In a continuation of the applicability of liquid chromatography in pharmaceutical analysis, an in-depth study of the separability of antihypertensive agents (2) was undertaken. A direct result of this work led to the investigation of the single-dose analysis of the antihypertensive combination, reserpine–chlorothiazide, by HPLC. Some analytical problems associated with this mixture include instability of reserpine in solution and a 2000-fold difference in concentration of the ingredients.

Urbanyi and O'Connell (3) reviewed the problems involved in the analysis of reserpine in combination with hydralazine and chlorothiazide or hydrochlorothiazide, and they used a fluorometric procedure based on oxidation of reserpine with vanadium pentoxide. Other fluorescence (4, 5) and colorimetric (6, 7) methods have been reported, but these techniques are subject to interferences from components present in the samples. Chu (8) used an ion-exchange separation followed by spectrophotometry to analyze chlorothiazide in the presence of other drugs such as methyldopa. Other reported methods for chlorothiazide mixtures use bromometric or bromochlorometric titrimetry (9), nonaqueous titrimetry (10, 11), and spectrophotometry (12).

The determination of reserpine–chlorothiazide mixtures by HPLC overcomes or circumvents many shortcomings in the reported methods. This paper reports a two-step liquid chromatographic analysis of reserpine–chlorothiazide, and a quantitative study is described for a typical mixture. The preparation of samples is simple and rapid, with the analysis time requiring less than 20 min for each component. The overall analysis time for both components in the simulated dosage form including the extraction procedure is approximately 60 min.

EXPERIMENTAL[1]

Reagents and Chemicals—Powdered samples of reserpine[2] and chlorothiazide[3] were used in the analytical procedure and in preparation of standard curves. All other chemicals and solvents were the highest grade of the commercially available materials.

Mobile Phase—The mobile phase, consisting of 55:45 absolute methanol–0.5% aqueous ammonium chloride, was prepared fresh daily. The pH of the solution was 5.6.

Internal Standard Solutions—Stock internal standard solutions consisting of 1 mg/ml of phenanthrene in chloroform and 4.996 mg/ml of phenanthrene in absolute methanol were prepared. Phenanthrene powder, mp 98–99°, was purified before use by Method 1 of Phillips (13).

Standard Solutions for Calibration Curves—A stock solution of reserpine (3.125 mg/ml) was prepared in chloroform. Volumes of 1.0, 2.0, and 4.0 ml of the stock solution were each placed in 100-ml volumetric flasks. One milliliter of the internal standard stock solution in chloroform was pipetted into each flask, and the solution was diluted to volume with chloroform. These solutions were made and used within 24 hr. If the solutions were stored overnight, they were kept at 4°.

Into individual 250-ml volumetric flasks were added accurately weighed quantities of 125, 250, and 375 mg of chlorothiazide powder. Five milliliters of internal standard stock solution in absolute methanol and 2.0 ml of internal standard solution in chloroform were pipetted into each flask and the solution was diluted to volume with absolute methanol.

The three concentrations of each drug were subjected to a linear regression analysis, and the slope and intercept were calculated (Table I).

Procedure for Simulated Dosage Form—The simulated dosage form was assayed in two steps. Into a 12-ml centrifuge tube containing 250 mg of chlorothiazide powder was added 2.0 ml of chloroform, which contained 0.125 mg of reserpine and 0.02 mg of internal standard. The mixture was centrifuged for 5 min at 10,000 rpm and the supernatant liquid was analyzed for reserpine.

In the second step, the mixture was resuspended and transferred to a 250-ml volumetric flask with 10 10-ml washings of absolute methanol. To this was added 5.0 ml of internal standard stock solution in methanol, and the solution was diluted to volume with absolute methanol.

[1] A Waters Associates liquid chromatograph (model ALC 202), equipped with an M-6000 pump, a UV monitor, an Infotronics integrator (model CRS-204) with digital printout, and Waters packed columns, 1.22 m long × 2.3 mm i.d., was used.

[2] Ciba Pharmaceutical Co., Summit, N.J.

[3] Merck Sharp and Dohme, Rahway, N.J.

Table I—Calibration Data for Standard Drug Solutions

Compound	Final Concentration, mg	D/IS Ratio[a]	Slope	Intercept	$r \pm s$
Reserpine	0.0625[b]	0.2290 ± 0.018[c]	3.2281	0.0303	0.9999 ±0.004
	0.1250	0.4384 ± 0.044			
	0.2500	0.8358 ± 0.036			
Chlorothiazide	125.0[d]	0.1925 ± 0.0069	0.00152	0.0042	0.9999 ±0.002
	250.0	0.3874 ± 0.0144			
	375.0	0.5727 ± 0.0447			

[a] Data represent five to eight replicate injections of standard solutions; D/IS is the ratio of the integrated area of the drug at some concentration divided by the integrated area of phenanthrene at concentrations of 0.01 and 0.1 mg/ml for reserpine and chlorothiazide, respectively. [b] Total mg/2-ml sample. [c] Confidence limits at $p = 0.05$. [d] Total mg/250-ml sample.

Conditions for Chromatographic Separation and Quantification—The degassed mobile phase was pumped through a column containing a monomolecular layer of octadecyltrichlorosilane (C_{18}), chemically bonded to a high efficiency pellicular packing consisting of solid glass cores with a porous silica surface[4], at a flow rate of 2.0 ml/min (2000 psig) at room temperature until a stable baseline was obtained. For the standard solutions and the analysis of chlorothiazide in the congeneric dosage form, replicate 20-μl injections of each solution were made using a 25-μl syringe[5]. For the analyses of reserpine in the dosage form, replicate 50-μl injections were made using a 100-μl syringe[5,6]. The chart recorder provided a record of the elution of the drugs from the column as peaks on a chromatogram. In all cases, the solute was measured by digital integration of the peak area[1].

RESULTS AND DISCUSSION

The analysis of the two-component dosage form, reserpine–chlorothiazide, necessitated the examination of three separate analysis problems: *(a)* the development of a set of operating parameters of HPLC that would separate the two components without peak overlap, *(b)* the detection and quantification of each component at the level found in the dosage form, and *(c)* the development of a series of analytical steps in which the detector response remains linear over the concentration range of each compound in the dosage form.

The operating parameters studied showed that the compounds were separated best on an octadecyl column using a 55:45 mixture (pH 5.6) of absolute methanol–aqueous 0.5% ammonium chloride (2). A flow rate of 2.0 ml/min, which resulted in a pressure of 2000 psig at room temperature, was the most satisfactory since separations could be obtained in less than 20 min. UV detection was employed since both reserpine and chlorothiazide are strong absorbers and the detector provided maximum sensitivity for the low concentration levels of reserpine in the dosage form.

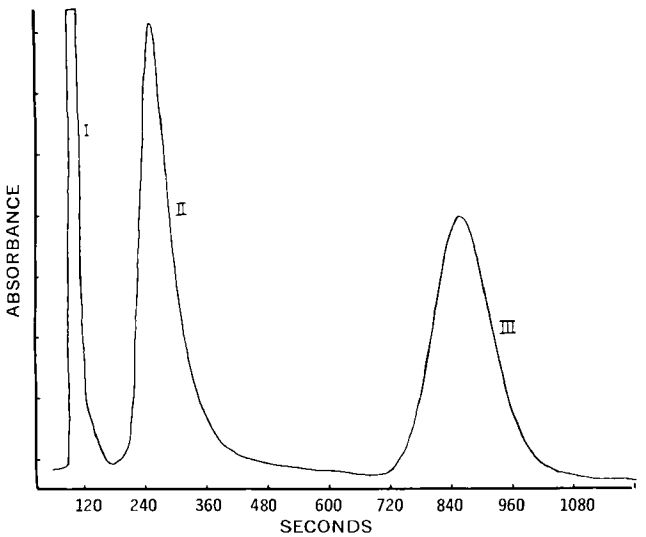

Figure 1—*Liquid chromatogram of reserpine analysis in first step of simulated dosage form on octadecyl column with 55:45 absolute methanol–aqueous 0.5% ammonium chloride. Key: I, chloroform; II, reserpine; and III, phenanthrene.*

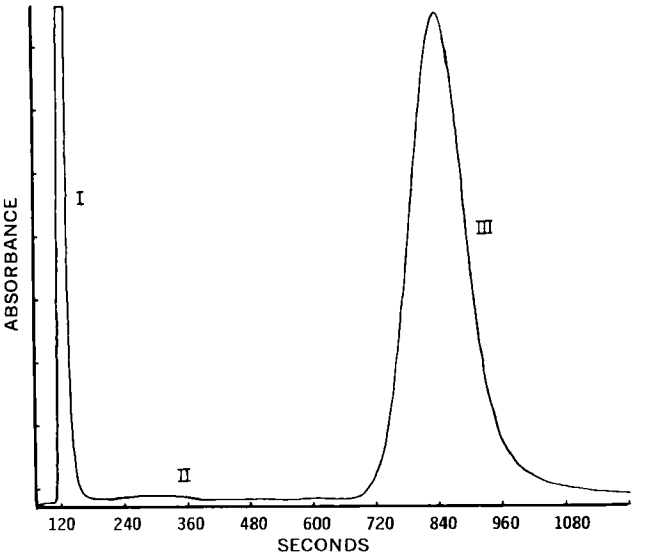

Figure 2—*Liquid chromatogram of chlorothiazide analysis in second step of simulated dosage form on octadecyl column with 55:45 absolute methanol–aqueous 0.5% ammonium chloride. Key: I, chlorothiazide; II, reserpine; and III, phenanthrene.*

Various concentrations of standard solutions of reserpine and chlorothiazide were chromatographed using the octadecyl column (Figs. 1 and 2). Phenanthrene was added to each solution as the internal standard. The area under the curve for each peak on the chromatograms was integrated digitally. The ratio of each peak area to the area of the internal standard was calculated for each chromatogram. A linear regression line of these data at three concentrations of each drug gave the slope, intercept, and correlation coefficient for each calibration curve (Table I).

A simulated dosage form containing quantities of each drug similar to those found in commercial products was analyzed sequentially in two steps due to the 2000-fold difference in concentration of the two ingredients. In the initial step, 2 ml of chloroform containing the internal standard was added to solubilize the reserpine. The mixture was centrifuged for 5 min and the supernatant liquid was analyzed for reserpine (Fig. 1). The shoulder shown on the chloroform peak is thought to be due to minute quantities of chlorothiazide soluble in the chloroform.

In the second step of the simulated dosage form assay, the reserpine–chlorothiazide mixture was quantitatively transferred with absolute methanol. Additional internal standard solution in methanol was added and the final solution was analyzed for chlorothiazide (Fig. 2).

Attempts to use a single solvent for the simultaneous assay of

[4] Corasil/C_{18}, 37–50 μm, Waters Associates, Milford, Mass.
[5] Model B-110, Precision Sampling Corp., Baton Rouge, La.
[6] To improve precision of analysis of reserpine in the dosage form, it was necessay to increase the total quantity of drug in each analysis; therefore, 50-μl injections of reserpine were employed.

Table II—Analysis of Reserpine–Chlorothiazide in Known Mixture

Mixture	Added, mg	Amount Found[a], mg	Accuracy, %
Reserpine	0.125	0.120 ± 0.013[b]	3.8
Chlorothiazide	250.000	260.600 ± 4.886	4.2

[a] Based on five replicate determinations of known mixture. [b] Confidence limits at $p = 0.05$.

both drugs in the simulated dosage form were unsuccessful. Solvents such as acetonitrile and methanol solubilized both reserpine and chlorothiazide, but the ratio of chlorothiazide to reserpine was so large that the two components could not be resolved completely. This difficulty was resolved by the sequential use of the two solvents, chloroform and methanol, in the analytical scheme. Reserpine was very soluble in chloroform whereas chlorothiazide was essentially insoluble. Peak overlap of the two drugs was avoided, thus enabling determination of microgram quantities of reserpine in the presence of milligram concentrations of chlorothiazide. In addition, the volume of chloroform could be reduced, thus enhancing the detectability of reserpine in a more concentrated solution. Methanol was then added to solubilize the undissolved chlorothiazide and the analysis was completed.

The ratios of drug peak areas/internal standard peak areas (D/IS) were calculated for each drug. The constants (slope and intercept) for the linear regression equation shown in Table I were used to solve for drug concentration [D/IS = slope (concentration) + intercept]. The calculations were performed on a programmable calculator[7].

The data in Table II demonstrate the quantitative results obtained for the simulated dosage form. The utility of HPLC in the analysis of the reserpine–chlorothiazide mixture is clearly demonstrated, with an accuracy of 3–5% (14).

[7] Olivetti-Underwood programma 101.

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Ion-Pair Extraction and Precipitation Methods for Ethambutol Determination

E. H. GIRGIS [x], Z. GAD, and M. MARAM

Abstract □ A sensitive method for determining ethambutol (20–100 μg) in aqueous solutions and tablets is described, using bromthymol blue as a complex-forming agent. Extraction of the complex in chloroform as well as in methylene chloride or ethylene dichloride is accomplished readily at an optimum pH of 7. A stoichiometric relationship of 1:2 between ethambutol and the acid dye is proved. Cycloserine, isoniazid, and sodium aminosalicylate do not interfere with the assay. The reineckate precipitation method for ethambutol determination is compared and its direineckate derivative is identified.

Keyphrases □ Ethambutol hydrochloride—analysis, ion-pair extraction and precipitation methods □ Ion-pair extraction—bromthymol blue–ethambutol complex, precipitation and analysis □ Acid-dye technique—ion-pair extraction of ethambutol–bromthymol blue complex, precipitation and analysis □ Bromthymol blue—ion-pair extraction of ethambutol and analysis

Oral administration of ethambutol hydrochloride, (+)-2,2′-(ethylenediimino)-di-1-butanol dihydrochloride, currently marketed as the dextro form, is used in the treatment of tuberculosis (1, 2). Colorimetric methods for determination of ethambutol as the copper complex (3, 4) and as the reineckate derivative (5) were reported previously. The chelation of ethambutol with copper required either a nonaqueous medium or a critical amount of excess alkali to produce a stable color with low absorptivity, and small amounts could not be determined with sufficient accuracy. In the present study the acid-dye technique formerly adopted for the microdetermination of various pharmaceutical amines (6–12) was applied to assay ethambutol and compared with the reineckate assay method after being optimized.

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Table I—Effect of Different Solvents on Extraction of Ethambutol–Dye Complex at pH 7

Solvent	Absorbance (1 cm)[a]
Chloroform	0.63
Methylene chloride	0.63
Ethylene dichloride	0.63
Benzene	0.33
Carbon tetrachloride	0.11
Hexane	0.00

[a] Mean value of three experiments obtained from 5-ml aliquots of dilute ethambutol hydrochloride in 20 ml of solvent.

EXPERIMENTAL

Materials—The following were used: ethambutol, crystalline commercial product[1]; bromthymol blue[2]; chloroform; methylene chloride; ethylene dichloride; benzene; carbon tetrachloride; and hexane. All were BP or analytical reagent grade.

Reagents—The following were used: buffers, pH 2–10 (BP); solutions of bromthymol blue (6.4×10^{-4} *M*) and of dilute ethambutol hydrochloride (0.72×10^{-4} *M*) in the required buffers; solutions of ethambutol hydrochloride (6.4×10^{-4} *M*) in the buffers (pH 4.8 and 7); and a saturated solution of ammonium reineckate and a diluted 1% solution of the reagent, freshly prepared.

Preparation of Test Sample Solutions—Twenty ethambutol tablets were weighed and finely powdered. In the acid-dye assay, an aliquot of the powder, accurately weighed, was dissolved, filtered, and diluted with the pH 7 buffer to make approximately 20 μg of ethambutol/ml. In the reineckate assay, an aliquot of the powder, accurately weighed, was dissolved, filtered, and diluted with 0.1 *N* HCl to give approximately 1–2 mg of ethambutol/ml.

Acid-Dye Assay Procedure—The prepared test sample solution of ethambutol (3 ml, ~60 μg) was added to 4 ml of bromthymol blue in the pH 7 buffer contained in a 125-ml separator and diluted to 14 ml with the pH 7 buffer solution. The aqueous solution was shaken gently for 2 min with 25 ml of chloroform, and the separated chloroform layer was centrifuged. A blank experiment was carried out using 3 ml of the pH 7 buffer in place of the test sample solution. The absorbance of the yellow-colored extract was measured at 410 nm against chloroform from the blank experiment. The mean value of three experiments was taken, and the exact amount of ethambutol hydrochloride was plotted from a calibration curve.

Determination of Stoichiometric Relationship—Aliquots (3 ml) of variable proportions of ethambutol and bromthymol blue (6.4×10^{-4} *M*) in the buffered solutions (pH 4.8 or 7) were delivered into six dry separators and diluted with 4-ml aliquots of the pH 4.8 or 7 buffer. Each solution was shaken thoroughly for 2 min with 25 ml of chloroform. The separated organic phases were centrifuged and measured at 410 nm against chloroform from the blank experiment in the absence of ethambutol.

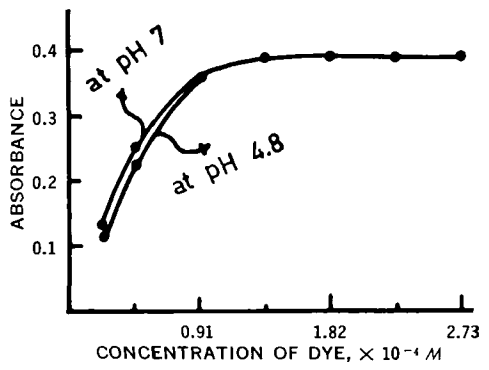

Figure 1—*Effect of dye concentration on color formation at pH 4.8 and 7 at fixed ethambutol hydrochloride concentration of 1.54 × 10⁻⁵ M.*

[1] Assayed by nonaqueous titration against standard perchloric acid in acetic acid to give 99.53% result.
[2] Merck.

Table II—Effect of Buffer Concentration on Extraction of Ethambutol–Dye Complex

Buffer Concentration, *M*	Absorbance (1 cm) of	
	Blank/Chloroform	Complex[a]/Blank
0.05	0.025	0.39
0.10	0.040	0.39
0.15	0.060	0.39
0.20	0.110	0.39

[a] Results obtained from 3-ml aliquots of dilute ethambutol solution at pH 7 in 20 ml of solvent.

Reineckate Assay Procedure—The prepared test sample solution of ethambutol (25 ml, ~50 mg) was precipitated by adding, with continuous stirring, 25 ml of saturated ammonium reineckate solution. The mixture was set aside for 30 min and then filtered through a previously dried and tared sintered-glass crucible (porosity 4). The precipitated reineckate was completely transferred onto the filter and washed successively with two 5-ml portions of the diluted reagent of ammonium reineckate and 5 ml of ice-cold water.

In the gravimetric determination, each gram of the dried precipitate at 105° is equivalent to 0.3292 g of ethambutol hydrochloride. For the colorimetric determination, the washed precipitate was dissolved in an appropriate volume of methanol and the absorbance was read at 525 nm. The exact amount of ethambutol hydrochloride was determined from a calibration curve.

RESULTS AND DISCUSSION

Ion-Pair Extraction of Ethambutol—Maximum extraction of the dye complex in chloroform was achieved at pH 4.8–7, and not more than gentle shaking was needed to extract the complex readily in chloroform at a pH value approximating 7. Table I demonstrates that methylene chloride and ethylene dichloride are equally effective in extracting the complex. A spectrophotometric scanning of the yellow color in the visible region showed a maximum absorbance at 410 nm, and the color was stable for 2 hr at 20 and 40°. An increase in buffer concentration from 0.05 to 0.2 *M* at pH 7 (Table II) did not influence extraction of the complex, although high blank values were produced with the higher buffer concentrations.

Effect of Dye Concentrations on Absorbance of Complex—Different dye concentrations were made from bromthymol blue (6.4×10^{-4} *M*) solutions in pH 4.8 or 7 buffer; 3-ml aliquots of the dilute ethambutol solution at the same pH were added to each and they were diluted to 14 ml with the buffer used. Extraction was

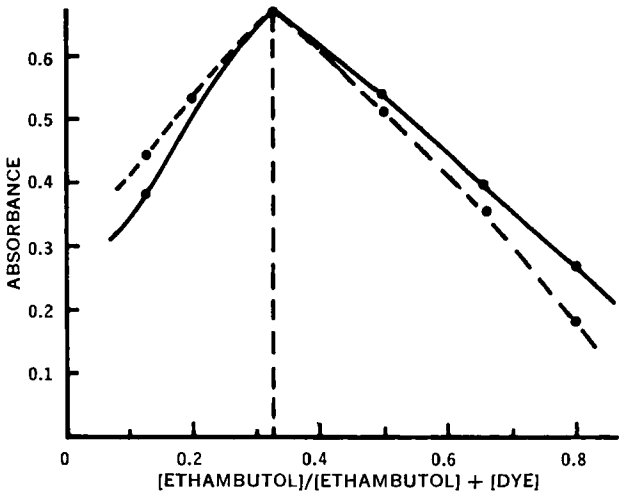

Figure 2—*Continuous variation curve obtained from solutions of ethambutol hydrochloride and bromthymol blue (6.4×10^{-4} M) at pH 4.8 (---) and 7 (——).*

Table III—Acid-Dye and Reineckate Assay Results of Ethambutol Hydrochloride Tablets (250 mg)

Acid-Dye Assay		Reineckate Assay			
		Gravimetric		Colorimetric	
Found per Tablet, mg	Found, %	Found per Tablet, mg	Found, %	Found per Tablet, mg	Found, %
252.15	100.86	252.00	100.80	253.22	101.28
253.82	101.53	252.00	100.80	253.22	101.28
253.82	101.53	259.00	103.60	253.22	101.28
253.82	101.53	251.95	100.78	250.00	100.00
250.00	100.00	250.85	100.34	250.00	100.00
250.00	100.00	—	—	—	—
Mean	100.90		101.26		100.73
% *SD*	±0.75		±1.30		±0.64

Table IV—Weight Ratios between Assayed Ethambutol Hydrochloride and Reineckate Derivatives

Weight of Ethambutol Hydrochloride, mg	Weight of Reineckate Derivative, mg, Produced by Adding Excess Reagent and Leaving for		Ratio between Ethambutol Hydrochloride and Reineckate Derivative
	30 min	60 min	
21.0	63.77	—	1:3.037
51.6	—	157.7	1:3.056
50.0	151.50	—	1:3.030
100.4	—	304.6	1:3.033

Table V—Reineckate Assay of Ethambutol Hydrochloride in Bulk Material

Sample, mg	Amount of Ethambutol Hydrochloride			
	Gravimetric		Colorimetric	
	Found, mg	Recovery, %	Found, mg	Recovery, %
40	40.10	100.25	40	100
50	49.90	99.80	50	100
60.3	60.60	100.49	60	99.5
100	100.08	100.08	100	100
101	100.70	99.70	101.2	100.19
	Average	100.064	Average	99.938
	% *SD*	±0.336	% *SD*	±0.24

then effected by thorough shaking with 20 ml of chloroform for 2 min, and absorbances of the yellow color were measured against their blanks. Figure 1 shows that a 1.82×10^{-4} *M* dye concentration is sufficient to bring maximum absorbance at a constant ethambutol hydrochloride concentration of 1.54×10^{-5} *M* in the aqueous phase.

Stoichiometric Balance—Figure 2 represents the stoichiometric relationship of 1:2 between ethambutol and the acid dye attributed to the presence of two basic centers in ethambutol.

Adherence to Beer's Law—The yellow complex of ethambutol hydrochloride (20–100 μg) in chloroform, assayed by the acid-dye method, showed adherence to Beer's law.

Acid-Dye Assay of Ethambutol Tablets—Comparison of the acid-dye results with those of the reineckate method for ethambutol tablets (Table III) indicates good precision, agreement between results, and no interference from tablet excipients (6). Microamounts of ethambutol could be determined by the acid-dye technique with a fairly rapid and simple procedure.

Analysis of Ethambutol in Presence of Cycloserine, Isoniazid, or Sodium Aminosalicylate—A preliminary experiment carried out by the acid-dye method on a 5-ml aliquot solution of cycloserine, isoniazid, or sodium aminosalicylate (600 μg) showed no formation of a colored complex in chloroform. Consequently, on assaying ethambutol (60 μg) in the presence of either former solution, no interferences occurred and ethambutol was easily and quantitatively extracted.

Reineckate Precipitation of Ethambutol—An acid solution of ethambutol was required for an optimum precipitation of ethambutol at room temperature by the reineckate reagent. With the unacidified solution, the precipitated reineckate was not easily filtered; with solutions made slightly ammoniacal, no precipitation occurred. The separated crystals of ethambutol reineckate from an acid solution at room temperature showed the same melting point (177–180° dec.) as crystals separated at 70°. Washed and dried crystals of ethambutol reineckate showed no loss in weight when heated for 3 hr at 105°.

Table IV illustrates that 1 g of ethambutol hydrochloride is equivalent to nearly 3.037 g of ethambutol reineckate, indicating a direineckate formation. Unlike most dibasic compounds (13), ethambutol yielded the direineckate derivative in an acid solution at 70°.

A comparison between the gravimetric and colorimetric reineckate assay results of ethambutol bulk material (Table V) indicated good precision and recovery within the range of 40–100 mg of the material.

UV Absorbance of Ethambutol Direineckate—The absorbance of an ethambutol reineckate methanolic solution (1.6 mg/100 ml) in the UV region showed two maxima at 235 and 310 nm, attributed to the reineckate moiety (13) and independent of ethambutol itself which had no absorption in this region. It was found that a methanolic solution of ethambutol reineckate containing 0.6–1.6 mg in 100 ml of methanol obeyed Beer's law at 310 nm.

CONCLUSION

A sensitive acid-dye technique is applied for assaying ethambutol (20–100 μg) in aqueous solutions and in tablets through the formation of a complex with bromthymol blue, which is extracted readily in chloroform at pH 7. The method is more sensitive and rapid than the reineckate method. Moreover, it is adequate for selective extraction of ethambutol in the presence of cycloserine, isoniazid, or sodium aminosalicylate. The reineckate precipitation of ethambutol is optimized and the direineckate derivative (mp 177–180°) is heat stable at 105° and shows, in methanolic solution, the two typical maxima in the UV region ascribed to the reineckate moiety.

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[x] To whom inquiries should be directed.

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SOBHI A. SOLIMAN [x], HASSAN ABDINE, and NASHAAT A. ZAKHARI

Abstract □ An indirect nonaqueous titrimetric method was devised for the determination of the hydrochlorides of nitrogenous bases. The chloride-ion interference was prevented without the use of a mercuric acetate reagent. The method depends on the treatment of a solution of the hydrochloride of the organic base with an excess of standard aceteous perchloric acid solution and the hydrogen chloride displaced is removed by boiling. Residual excess perchloric acid is determined by titration against the basic titrant sodium acetate in glacial acetic acid, using either potentiometric or visual end-point detection with a crystal violet indicator. The proposed method was applied successfully to the determination of several hydrochlorides of nitrogenous bases. The mean percent recoveries obtained indicate that the proposed method is equivalent in accuracy and precision to the nonaqueous titrimetric method most commonly used by official compendia for this determination. Potentiometric titrations showed that the point of the maximum inflection in the titration curves coincided with the appearance of the violet color of the indicator.

Keyphrases □ Nitrogenous bases, hydrochlorides—indirect nonaqueous titration, method compared to compendial methods □ Nonaqueous titration, indirect—hydrochlorides of nitrogenous bases, compared to compendial methods □ Titrations, indirect nonaqueous—hydrochlorides of nitrogenous bases, compared to compendial methods

Many titrimetric methods have been developed for the determination of nitrogenous bases in aqueous solutions, and some have been accepted by various compendia. However, titrations of the nitrogenous bases in nonaqueous media allow much more accurate results and are quite simple. The nitrogenous bases may be extracted from an aqueous solution by a nonpolar solvent and determined directly without further isolation.

In the titration of the hydrochlorides of the nitrogenous bases in nonaqueous media, perchloric acid displaces hydrochloric acid from its salt. In an acetic acid medium, hydrogen chloride is only a little less acidic than perchloric acid, which makes the displacement reaction not quantitative and renders the method unsuitable for quantitative determination. Therefore, in the titration of the hydrochlorides of nitrogenous bases, there must be some specific technique to avoid the difficulty arising from the presence of the chloride ion.

Some procedures have been suggested to overcome this difficulty. An extraction titration method (1) was developed to determine a number of alkaloid hydrochlorides. Some hydrochlorides of nitrogenous bases were determined (2) by the titration of their acidic components against standard sodium methoxide solution in anhydrous pyridine.

A limited number of the hydrochlorides of organic bases and some alkali metals and ammonium halides were titrated directly in boiling glacial acetic acid against aceteous perchloric acid solution, using different indicators (3). The titrated solution had to be boiled to remove the hydrogen halides displaced during titration. However, no attempt was made to obtain extremely accurate quantitative results; rather, information was sought as to how some anions may influence analytical results.

An ingenious method was developed (4) for determining the hydrochlorides of nitrogenous bases in which the halide interference was prevented by using excess mercuric acetate reagent. The investigators used standard perchloric acid in dioxane as the titrant, with either potentiometric or visual end-point detection. This method has proved to be efficient for eliminating halide-ion interference and, therefore, has been adopted by many official compendia for the determination of the hydrochlorides of nitrogenous bases. The method has also been applied to the determination of some alkaloid halides (5, 6), using 0.01 *N* perchloric acid solution as the titrant and crystal violet as the indicator, and the hydrochlorides of several antihistamines (7) by potentiometric titration against standard tosylic acid solution in chloroform. The hydrochlorides of some phenothiazine derivatives were also determined (8) either in acetone using a mercuric acetate reagent or in a mixture of hexane and acetone after extracting the base from an aqueous potassium hydroxide solution.

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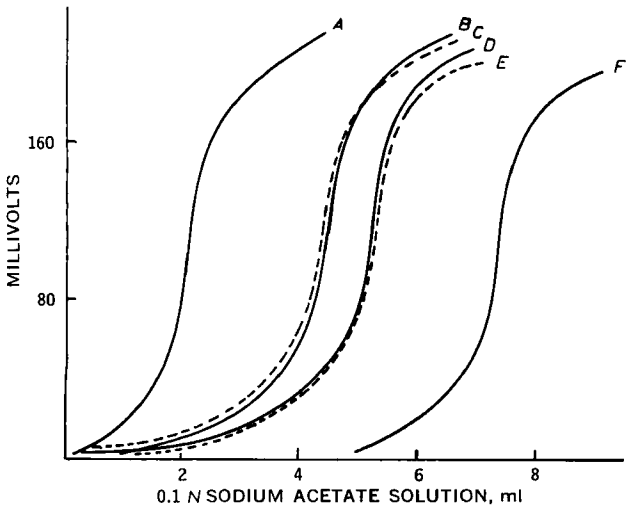

Figure 1—*Potentiometric titration of the hydrochlorides of the nitrogenous bases in glacial acetic acid by the proposed method. Key: A, quinine hydrochloride; B, ephedrine hydrochloride; C, phenylephrine hydrochloride; D, diphenhydramine hydrochloride; E, papaverine hydrochloride; and F, chlorpromazine hydrochloride.*

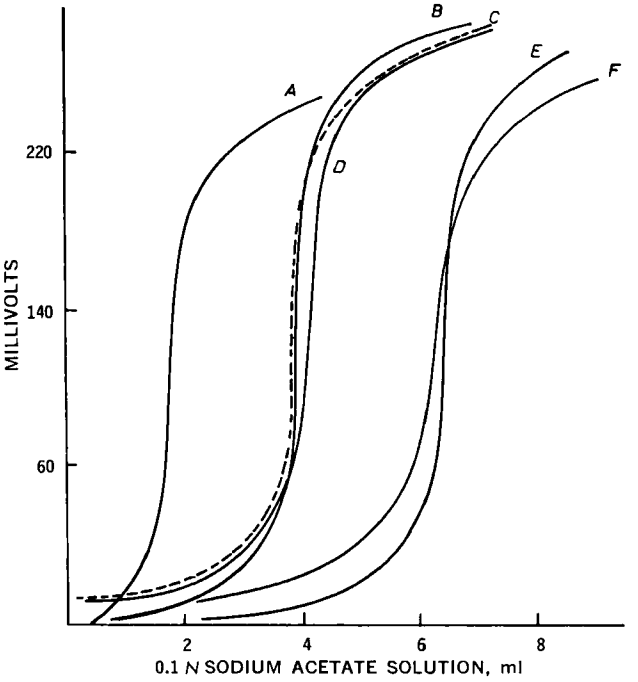

Figure 2—*Potentiometric titration of the hydrochlorides of the nitrogenous bases in dioxane–glacial acetic acid mixture by the proposed method. Key: A, quinine hydrochloride; B, ephedrine hydrochloride; C, diphenhydramine hydrochloride; D, phenylephrine hydrochloride; E, papaverine hydrochloride; and F, chlorpromazine hydrochloride.*

ous titrimetric method was devised for the determination of the hydrochlorides of nitrogenous bases in which the basic titrant sodium acetate was used. The difficulty arising from chloride ions was overcome without the use of a mercuric acetate reagent.

EXPERIMENTAL

Apparatus and Materials—The following were used: a suitable titrimeter[1] equipped with a combination electrode[2], a magnetic stirrer, a microburet (capacity 10 ml) graduated to 0.02 ml, glacial acetic acid[3], acetic anhydride[3], dioxane[4], 70% perchloric acid[5], mercuric acetate[5], ascorbic acid[6], sodium acetate[7], potassium acid phthalate[8], chlorpromazine hydrochloride[8], papaverine hydrochloride[9], ephedrine hydrochloride[9], quinine hydrochloride[10], diphenhydramine hydrochloride[11], and phenylephrine hydrochloride (BP grade).

Solutions—The following reagents were prepared in glacial acetic acid: 0.1 *N* perchloric acid solution, 0.1 *N* sodium acetate solution, 5% (w/v) mercuric acetate solution, and 0.2% (w/v) crystal violet indicator solution.

The perchloric acid solution was standardized by potentiometric titration against potassium acid phthalate, previously dried at 110° for 2 hr, in glacial acetic acid.

For comparison, the hydrochlorides of the nitrogenous bases investigated were determined by the method of Pifer and Wollish (4), where mercuric acetate was used to liberate the base. All investigated compounds behaved as monoacidic bases, taking up 1 equivalent of acid/mole, except quinine hydrochloride where 2 equivalents were taken up.

Determination of Hydrochlorides of Nitrogenous Bases by Proposed Indirect Method—The accurately weighed sample (50.0–400.0 mg) was placed in a 150-ml erlenmeyer flask with 40 ml of glacial acetic acid. The sample was allowed to dissolve with the aid of gentle heat, if necessary. A known excess of 0.1 *N* aceteous perchloric acid solution (10–20 ml) was added, and the flask was placed on a hot plate at 100–120° for 30 min. The solution was cooled, 2 drops of crystal violet indicator solution were added, and the titration was performed against 0.1 *N* aceteous sodium acetate solution to the first violet color change.

Potentiometric titrations were carried out in a 250-ml beaker. The solution, prepared as described and magnetically stirred, was titrated against standard aceteous sodium acetate solution. The end-point was determined from the inflection in the titration curve. The appearance of the violet color was found to coincide with the maximum change in millivolts reading. Potentiometric titrations were also performed in a glacial acetic acid–dioxane solvent system by adding an equal volume of dioxane to the contents of the beaker just before titration.

Determination of Chlorpromazine Hydrochloride—An accurately weighed sample of chlorpromazine hydrochloride (55.0–300.0 mg) was dissolved in 30 ml of glacial acetic acid. Standard aceteous perchloric acid solution was added and the solution was heated as mentioned previously. Ten milliliters of glacial acetic acid and enough ascorbic acid (1.0–3.0 g) to decolorize the developed red color (9) were added. The solution was titrated against standard aceteous sodium acetate solution using crystal violet indicator as mentioned.

RESULTS AND DISCUSSION

The most commonly used method for the determination of the hydrochlorides of nitrogenous bases is that of Pifer and Wollish (4). This method has been adopted by official compendia such as USP XVIII (10), BP 1968 (11), and NF XIII (12) for the determination of many hydrochlorides of organic bases. According to this method, the use of mercuric acetate, which binds the halide ion present, thus liberating the nitrogenous base, is necessary to make the acid–base reaction between perchloric acid and the hydrochloride of the organic base quantitative. The resulting mercuric halide and the excess mercuric acetate, both being nonionized, do not interfere with the determination.

However, in the proposed method the liberation of the base (B) from its hydrochloride salt is achieved by heating the salt under investigation with an excess of 0.1 *N* aceteous perchloric acid solution, whereby the base perchlorate and hydrogen chloride are

[1] Pye model 79.
[2] Pye catalog No. 461 E07, series No. 546022.
[3] El-Nasr Pharmaceutical Chemicals Co., A.R.E.
[4] VEB Laborchemie Apolda, Germany.
[5] Merck.
[6] Roche.
[7] British Drug Houses Ltd., England.
[8] Rhone-Poulenc, France.
[9] Chemishe Fabriken, Ludwigshafen am Rhein.
[10] Carnegies of Welwyn Ltd., England.
[11] Kongo Chemicals Co., Japan.

Table I—Nonaqueous Titration of Hydrochlorides of Nitrogenous Bases by Proposed Indirect Method and the Direct Method[a]

Proposed Indirect Method			Direct Method		
Weight, mg	Weight Found, mg	Recovery, %	Weight, mg	Weight Found, mg	Recovery, %
		Papaverine Hydrochloride			
404.0	401.4	99.36	403.2	399.1	98.98
299.7	301.1	100.47	198.5	196.9	99.19
200.4	201.1	100.35	142.5	142.0	99.65
154.9	155.6	100.45	89.9	89.4	99.44
95.7	95.7	100.00			
59.5	60.0	100.84			
Mean percent recovery (p' = 0.05) 100.24 ± 0.53			Mean percent recovery (p' = 0.05) 99.31 ± 0.47		
		Quinine Hydrochloride			
302.6	300.6	99.34	406.1	412.8	101.65
154.7	154.4	99.81	302.2	306.9	101.55
151.8	152.0	100.13	165.3	167.5	101.33
103.0	105.1	102.04	100.0	101.6	101.60
102.7	105.1	102.34	54.4	55.2	101.47
50.0	49.9	99.80			
Mean percent recovery (p' = 0.05) 100.58 ± 1.34			Mean percent recovery (p' = 0.05) 101.52 ± 0.15		
		Ephedrine Hydrochloride			
200.6	198.9	99.15	201.2	198.4	98.61
152.3	150.7	98.95	164.0	162.8	99.27
121.0	121.2	100.16	118.3	117.3	99.15
101.8	102.6	100.78	100.8	99.4	98.61
52.7	52.3	99.24	68.5	67.7	98.83
			51.0	50.8	99.62
Mean percent recovery (p' = 0.05) 99.66 ± 0.97			Mean percent recovery (p' = 0.05) 99.01 ± 0.42		
		Diphenhydramine Hydrochloride			
272.7	273.7	100.37	309.3	307.2	99.32
193.1	191.9	99.38	212.7	213.1	100.19
134.0	132.1	98.58	175.1	175.7	100.34
105.0	103.7	98.76	153.5	154.3	100.52
56.0	55.3	98.75	104.5	105.1	100.57
			76.5	77.1	100.78
Mean percent recovery (p' = 0.05) 99.17 ± 0.91			Mean percent recovery (p' = 0.05) 100.29 ± 0.54		
		Phenylephrine Hydrochloride			
300.0	299.7	99.90	272.3	271.2	99.60
198.8	197.2	99.19	211.3	210.2	99.67
158.7	157.2	99.05	162.5	161.5	99.38
121.3	121.6	100.25	125.6	125.6	100.00
100.0	99.3	99.30	80.2	79.9	99.62
			64.5	65.0	100.77
Mean percent recovery (p' = 0.05) 99.54 ± 0.64			Mean percent recovery (p' = 0.05) 99.84 ± 0.52		

[a] Pifer and Wollish method, Ref. 4.

formed (Scheme I).

$$BH^+Cl^- + HClO_4 \rightleftharpoons BH^+ClO_4^- + HCl$$

Scheme I

The thermodynamic activity of hydrogen chloride at any given concentration in glacial acetic acid is much higher than that in water (3) and, therefore, volatilization from such a nonaqueous solvent takes place more readily. Under the conditions of the proposed method, the liberated hydrogen chloride is readily removed by volatilization as it is formed, thus making the reaction between the base and the protonated acetic acid quantitative. The protonated acetic acid formed by the reaction of perchloric acid with this solvent (Scheme II) is a stronger acid than the conjugate acid of the titrated base, consequently acting effectively as a proton donor when titrated with standard sodium acetate solution.

$$CH_3COOH + HClO_4 \rightleftharpoons CH_3COOH_2^+ClO_4^-$$

Scheme II

An indicator that fulfills the requirement that its pK_1 value lies midway between the pKa values of the conjugate acids of the titrated bases and the protonated acetic acid will be suitable to effect titration of these bases visually (3).

In the direct titration of the hydrochlorides of nitrogenous bases, apart from the type of solvent and standard solution employed, the main factors influencing the shape of the potentiometric titration curve and hence the sharpness of the color change of the indicator are the strength and concentration of the base being titrated. Since the indirect proposed procedure involves the titration of excess standard perchloric acid against standard sodium acetate solution irrespective of the base titrated, the method has the advantage that the strength and concentration of the base play a minor role in determining the sharpness of the indicator color change as well as the magnitude of potential break in the titration curve.

The potentiometric titration curves of the compounds studied in glacial acetic acid and in dioxane–glacial acetic acid mixture are shown in Figs. 1 and 2, respectively. In glacial acetic acid solvent the magnitudes of the breaks at the end-point range from 200 to 250 mv/ml of titrant and the shapes of the curves are identical. In dioxane–glacial acetic acid mixture, however, the magnitudes are

Table II—Determination of Chlorpromazine Hydrochloride According to Proposed Indirect Method, Direct Method of USP XVIII, and BP 1968 Method

Number	Weight, mg	Weight Found, mg	Recovery, %	USP XVIII[a]	BP 1968[a]
1	299.5	297.9	99.46		
2	196.9	198.9	101.01		
3	139.6	138.8	99.43		
4	102.8	104.0	101.17		
5	57.2	56.7	99.12		
Mean percent recovery ($p' = 0.05$)		100.04 ± 1.20		100.70 ± 0.81	100.57 ± 1.05

[a] Mean percent recoveries were calculated from five determinations; data were reported by the authors in Ref. 9.

higher, ranging from 670 to 810 mv/ml, and the shapes are also almost identical. Such a narrow range of potential breaks and identical shapes of titration curves in each solvent system may support the fact that in the proposed method the shape of the curve, the magnitude of its break, and, hence, the sharpness of the indicator color change at the end-point are independent of the strength and the concentration of the base under investigation.

In addition, the proposed method has the advantage that it does not necessitate the use of mercuric acetate to liberate the base from its hydrochloride salt as in the Pifer and Wollish method (4). The repeated heating in the course of titration (3) has also been avoided.

The proposed method and the Pifer and Wollish (4) method were applied to the determination of the hydrochlorides of papaverine, quinine, ephedrine, diphenhydramine, phenylephrine, and chlorpromazine, which represent some general chemical structures having different pharmacological actions (Tables I and II). The mean percent recoveries ($p' = 0.05$) obtained by applying the proposed method to the determination of the hydrochlorides of papaverine, quinine, ephedrine, diphenhydramine, and phenylephrine were 100.24 ± 0.53, 100.58 ± 1.34, 99.66 ± 0.97, 99.17 ± 0.91, and 99.54 ± 0.64, respectively, compared with 99.31 ± 0.47, 101.52 ± 0.15, 99.01 ± 0.42, 100.29 ± 0.54, and 99.84 ± 0.52, respectively, when the Pifer and Wollish method was applied.

The determination of chlorpromazine hydrochloride was carried out according to the proposed method. However, ascorbic acid was used (9), as mentioned under *Experimental*, to prevent the formation of the red-colored semiquinoid free radical which interferes with the visual end-point detection. The data obtained are listed in Table II. A mean percent recovery of 100.04 ± 1.2 was obtained compared with 100.70 ± 0.81 and 100.57 ± 1.05 when the Pifer and Wollish method, adopted by the USP XVIII (10), and the BP 1968 method, respectively, were employed. Statistical analysis of the data listed in Tables I and II indicated that the proposed method is almost equivalent in accuracy and precision to the Pifer and Wollish method.

It is advisable that the number of equivalents of standard perchloric acid be twice that of the hydrochloride of the nitrogenous base to be determined. When the weights of the hydrochlorides of papaverine, quinine, and phenylephrine are larger than 400 mg, caution should be exercised not to exceed the 30-min heating time with perchloric acid; otherwise the titrated solution may develop a yellow color which may interfere with the visual detection of the end-point. However, in all cases the heating time should not be less than 15 min.

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[x] To whom inquiries should be directed.

Colorimetric Determination of Betamethasone Benzoate in Topical Gel Preparation by Lewbart–Mattox Method

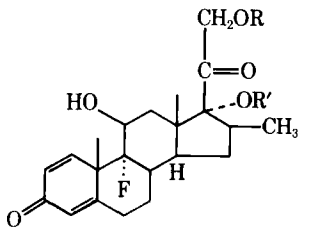
LESTER CHAFETZ ˣ, DIMITRI C. TSILIFONIS, and CHRISTINA MORAN

Abstract □ Betamethasone benzoate, a 17-substituted ester, is determined by preliminary oxidation of the 17α-ketol function (−$COCH_2OH$) to a glyoxal (−COCHO), which is then condensed with phenylhydrazine in acid to provide a yellow chromophore. The method is selective for betamethasone benzoate in the presence of its acyl migration product, the 21-benzoate, which is the only degradation product detected in formulations. Observations on the chemistry and scope of the method are presented.

Keyphrases □ Betamethasone benzoate—topical gel preparation, colorimetric determination by Lewbart–Mattox method □ Lewbart–Mattox method—colorimetric determination of betamethasone benzoate in topical gel preparation □ Colorimetry—determination, betamethasone benzoate in topical gel preparation by Lewbart–Mattox method

Betamethasone benzoate[1] (II) is the 17-substituted ester of betamethasone (I). It is a potent new synthetic corticosteroid and has been formulated in topical gel preparations. Long-term stability studies on these preparations, monitored by TLC, have shown only small increases in the concentration of betamethasone 21-benzoate (III), which, along with betamethasone alcohol, may be detectable in samples of the drug substance as a synthesis process impurity. Recent kinetic studies, using temperatures as high as 85°, confirmed that acyl migration to the 21-benzoate is the only important degradation route for the steroid.

A convenient colorimetric assay for gel preparations of betamethasone benzoate, selective in the presence of its rearrangement product, was developed. The method is based on chemistry described by Lewbart and Mattox (1), requiring preliminary oxidation of the 17-ketol function (−$COCH_2OH$) to a glyoxal (−COCHO), followed by reaction with acidic phenylhydrazine to form a Porter–Silber chromophore. The use of this method in the analysis of pharmaceuticals apparently has not been reported previously. A description of the procedure and observations on its scope are presented.



I: R = R′ = H
II: R = H, R′ = COC_6H_5
III: R = COC_6H_5, R′ = H

[1] USAN name for 9-fluoro-11β-17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione 17-benzoate.

EXPERIMENTAL

Materials—Betamethasone benzoate[2], betamethasone[2], betamethasone 21-benzoate[2], betamethasone benzoate reference standard[3], and corticosterone[4] were used. Other steroids tested were of USP or NF grade. The assays were performed on clear gel preparations[5], which declare 0.025% betamethasone benzoate, carboxyvinyl polymer, disodium edetate, propylene glycol, diisopropanolamine, purified water, and alcohol.

Assay—*Sulfuric Acid Reagent*—Cautiously add 80 ml of concentrated sulfuric acid to 20 ml of water.

Cupric Acetate Solution—Dissolve about 100 mg of cupric acetate monohydrate in methanol and dilute to 100 ml. The solution is usable for at least 1 week, despite possible color changes and precipitation.

Methanol–Acid—Add 2 volumes of the sulfuric acid reagent to 1 volume of methanol.

Phenylhydrazine Reagent—Prepare fresh as needed by dissolving 20 mg of colorless phenylhydrazine hydrochloride in 20 ml of the sulfuric acid reagent.

Standard Preparation—Dissolve an accurately weighed amount of betamethasone benzoate reference standard in methanol and dilute quantitatively and stepwise to obtain a solution containing about 50 μg/ml.

Assay Preparation—Transfer an accurately weighed portion of the gel, equivalent to about 0.5 mg of betamethasone benzoate, to a 125-ml separator containing 20 ml of water. Add 1 ml of saturated sodium acetate solution, mix thoroughly, and extract with three 25-ml portions of chloroform, shaking about 2 min with each portion.

Combine the extracts in a second separator, wash with one 10-ml portion of water, and filter the extracts through paper into a conical flask with the aid of several small portions of chloroform. Evaporate the chloroform nearly to dryness on the steam bath with the aid of a current of air, and remove the last traces of solvent without heating. Dissolve the residue in exactly 10.0 ml of methanol.

Procedure—Transfer two 1-ml portions of the assay preparation and two 1-ml portions of the standard preparation to separate 15-ml glass-stoppered tubes. Add 0.1 ml of the cupric acetate solution to each tube, mix, and let stand open to air for 20 min with occasional mixing. Place the tubes in an ice bath, and add 2.0 ml of the phenylhydrazine reagent to one tube from the assay preparation and one from the standard preparation, allowing the reagent to flow down the sidewall of the tube without mixing. Similarly, add 2.0 ml of the sulfuric acid reagent to the remaining tubes, which serve as blanks.

Stopper the tubes, mix the contents carefully (heat is evolved), and heat the tubes in a constant-temperature bath at 60° for 15 min. Cool the tubes, add 2.0 ml of methanol–acid to each, and mix. Then determine the absorbances of the solutions from assay and standard preparations, against their respective blanks, in 1-cm cells at the wavelength of maximum absorbance at about 425 nm.

[2] Obtained from the Warner-Vister Steroid Research Institute, Casatenovo (Como), Italy.

[3] Prepared by the Analytical and Physical Chemistry Department, Warner-Lambert Research Institute.

[4] Pregnen-11β,21-diol-3,20-dione, Mann Research Laboratories.

[5] Benisone Gel, Warner-Chilcott Laboratories, Morris Plains, NJ 07950; Flurobate Gel, Texas Pharmacal Co., San Antonio, TX 78215

Table I—Data from Reactions in Thermostatted Cells for Various Corticosteroids

Steroid	Temperature	Time to Constant A	a, liters/g cm	ϵ, liters/mole cm
Betamethasone benzoate	25°	20 hr	29.5	14,600
	60°	15 min	25.3	12,600
Corticosterone	25°	15 min	15.9	5,500
Hydrocortisone	25°	70 min	14.4	6,200
Triamcinolone acetonide	25°	60 min	9.45	4,100

Calculate the percentage of betamethasone benzoate, by weight, from the formula $(C/W)(A_U/A_S)$, where C is the concentration, in micrograms per milliliter, of betamethasone benzoate reference standard in the standard preparation; W is the weight, in milligrams, of the portion of gel taken; and A_U and A_S are the absorbances of the solutions from the assay and standard preparations, respectively.

Chromatographic Method—Evaporate 4.0 ml of the assay preparation to dryness in a conical tube with the aid of a current of nitrogen, and take up the residue in 0.2 ml of a mixture of chloroform–methanol (7:3). Prepare comparison solutions, in the same solvent mixture, containing 0.2 mg of betamethasone/ml, 0.2 mg of betamethasone 21-benzoate/ml, and 1.0 mg of betamethasone benzoate reference standard/ml. Activate a 20 × 20-cm TLC plate, coated with a 0.25-mm layer of silica gel GF, by heating at 105° for 30 min. Mark equidistant points 2 cm above one edge of the plate, and apply the test solution and standards, all in 5-μl increments, according to the following scheme:

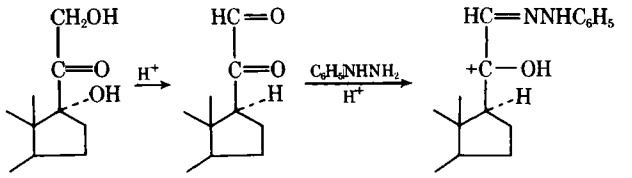
	solutions spotted, μl				
points	1	2	3	4	5
betamethasone	5	10	—	—	25
betamethasone 21-benzoate	5	10	—	—	25
betamethasone benzoate	90	80	—	100	50
reference standard	—	—	100	—	—
test solution					

Develop the plate in a chamber previously equilibrated with a solvent mixture of benzene–acetone–methanol (75:25:4) until it has ascended about 15 cm, remove the plate, and examine under shortwave UV light. The R_f values are about 0.4 for betamethasone, 0.6 for betamethasone benzoate, and 0.7 for betamethasone 21-benzoate. Estimate the concentration of betamethasone and betamethasone 21-benzoate in the test solution (lane 3) by comparison with the corresponding standards in lane 1 (1%), lane 2 (2%), and lane 5 (5%), viewing the plate under shortwave UV light.

Rate of Color Development—The time required to attain constant absorbance in the chromogenic reaction was determined for betamethasone benzoate and some other corticosteroids, selected for comparison, in 1-cm cells in the thermostatted[6] cell compartment of a double-beam spectrophotometer[7], set at 425 nm and equipped with a strip-chart recorder[8]. Test solutions, phenylhydrazine reagent, and sulfuric acid reagent were chilled in an ice bath before mixing in the spectrophotometer cell, and the absorbances were recorded without further dilution with methanol–acid.

RESULTS AND DISCUSSION

Chemistry of Colorimetric Method—The Porter–Silber (2) reaction, production of a yellow chromophore by reaction of a corticosteroid with phenylhydrazine in sulfuric acid, is restricted to corticosteroids with a 17-dihydroxyacetone side chain. The reaction has been shown (3, 4) to proceed in two stages: *(a)* the acid-catalyzed rearrangement of the dihydroxyacetone moiety to a 17-deoxy-20-one-21-ol, and *(b)* condensation of this glyoxal with phenylhydrazine to form a 21-phenylhydrazone (Scheme I).



Scheme I

Although many esters of corticosteroids, *e.g.*, hydrocortisone 21-acetate, can be determined by the Porter–Silber reaction, neither betamethasone benzoate nor its 21-benzoate isomer gives any color in the reaction, indicating that the ester is stable to hydrolysis under the strongly acid reaction conditions.

Lewbart and Mattox (1) extended the Porter–Silber reaction to the detection and microdetermination of pure 17-deoxy-α-ketolic steroids. Their modification consists of producing the requisite glyoxal reactant by oxidation with cupric acetate, followed by reaction with phenylhydrazine (Scheme II).

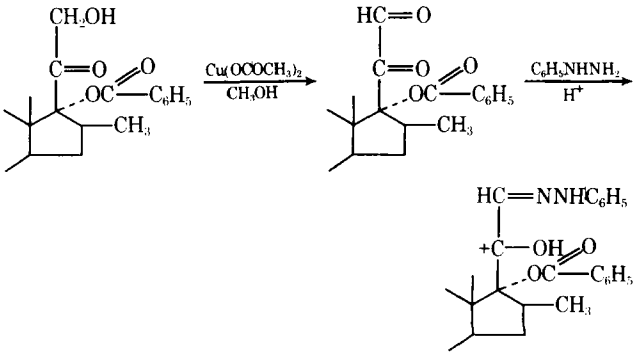
Betamethasone 21-benzoate, since it cannot be oxidized to a glyoxal, does not interfere. Betamethasone alcohol is chromogenic in the reaction; however, the molar absorptivity of the color produced with it is only about 40% that of betamethasone benzoate. Betamethasone undergoes the normal Porter–Silber reaction; however, it gives only about one-tenth the absorptivity without preliminary cupric acetate oxidation that it provides in the assay method, all other reaction parameters being identical.

Stability of Betamethasone Benzoate—Gardi (5) reviewed the chemistry of 17-monoesters of corticosteroids. He noted that these compounds undergo facile rearrangement to 21-esters. Studies on a number of different formulations of betamethasone benzoate revealed acyl migration to the 21-benzoate as the only degradation route detectable. Since the 21-benzoate does not interfere in the colorimetric assay, the method is stability indicating.

Two additional routes of decomposition were considered when these studies were initiated: ester hydrolysis and α-ketol group oxidation. Neither has been observed. Betamethasone, which might be formed by ester hydrolysis, has been estimated at less than 1–2% of initial betamethasone benzoate concentration throughout stability studies of its formulations. Increases in betamethasone concentration have been seen in high temperature kinetic studies on gels only when more than half of the 17-benzoate rearranged. As noted, the benzoate ester is stable to the strong acid medium of the Porter–Silber reaction. No evidence of oxidation has been discerned during 5 years of stability studies on diverse formulations.

It is remarkable, in this connection, that conditions prescribed for blue tetrazolium colorimetry, which is an oxidation reaction, for betamethasone and its esters official in NF (6) require heating at 45 or 50° while the reaction is carried out at room temperature in the general method for corticosteroid drugs. One may speculate that the 16β-methyl group shields the ketol group from attack.

Scope of Reaction—Although betamethasone benzoate is converted smoothly to the glyoxal with cupric acetate, the chromogenic reaction occurs much more sluggishly with it than with any other corticosteroid tested. In experiments where the reaction was conducted in the thermostatted cell of a recording spectrophotom-



Scheme II

[6] A Haake circulating constant-temperature bath was used.
[7] Coleman model 124.
[8] Bausch & Lomb VOM 5.

Table II—Differential Absorbance Readings of Placebo Gel Extracts

Trial	1	2	3	4	5	6	Reagent Blank
Sample weight, g	1.9488	2.6514	2.0021	1.9674	1.7296	2.4704	–
A (425 nm)	0.026	0.024	0.018	0.022	0.020	0.019	0.023

eter, constant absorbance was obtained only after 20 hr at 25° or after 15 min at 60°; however, the absorptivity was lower for the heated sample (Table I).

The absorptivities obtained for betamethasone benzoate in these experiments were calculated using the nominal volume of 3.1 ml, and they are lower than is generally obtained by the procedure in the assay (see *Experimental*). Corticosterone, lacking a 17-hydroxy function, does not undergo the normal Porter–Silber reaction. Triamcinolone acetonide gives only about one-tenth the color obtained with hydrocortisone in the Porter–Silber reaction (7); however, it appears to react as readily as hydrocortisone under the Lewbart and Mattox conditions. The method appears to be applicable to any corticosteroid with a 20-one-21-ol side chain, but time and temperature parameters should be determined for each compound.

Recovery of Betamethasone Benzoate from Gels—Recovery experiments were performed by weighing 2-g portions of gel placebo formulations into separators containing 20 ml of water, adding exactly 1.0 ml of a methanol solution of betamethasone benzoate reference standard, containing 0.50 mg/ml, and following the assay procedure. Values of 102.1, 100.0, 101.2, 97.3, 101.2, and 102.9% of the amount added were obtained, averaging 100.8%. The relative standard deviation calculated for the six trials was 1.98%.

Absence of Excipient Interference—The proposed method affords a differential measurement of both sample and standard, using a reagent without phenylhydrazine as the blank. This technique compensates for any chromophores that might be induced by reagent acidity, but it does not correct for chromogenic reactions of phenylhydrazine with excipients. As a check on this point, six trials were made on placebo gel (Table II). It is evident that there is no relationship between the differential absorbance readings and the amount of gel taken nor any difference between these and the reagent blank. It was concluded, therefore, that there is no excipient interference in the method.

Linearity and Reproducibility—Plots of absorbance against concentration have been rectilinear when determined by any one chemist at one time. Absorptivities calculated for standards run by one chemist over several months ranged from 27.4 to 33.9, with a mean of 30.9 liters/g cm, in 16 determinations. Another chemist, in six trials at various times, obtained absorptivities ranging from 32.2 to 34.4, with a mean of 33.0. Six chemists, determining absorptivities on the same standard, obtained values ranging from 28.7 to 33.5, with a mean value of 31.5.

SUMMARY AND CONCLUSIONS

A novel and selective method for the colorimetric determination of betamethasone benzoate in a topical gel formulation is described. The procedure is derived from the Lewbart–Mattox (1) modification of the Porter–Silber reaction (2), which consists of preliminary oxidation of the 17α-ketol function with methanolic cupric acetate and condensation of the resulting glyoxal with phenylhydrazine. The method is selective for betamethasone benzoate in the presence of its 21-benzoate rearrangement product, the only decomposition product detected in stability studies of formulations. The glyoxal formed from betamethasone benzoate reacts much more slowly with Porter–Silber reagent than those from corticosterone, hydrocortisone, and triamcinolone acetonide. The method affords satisfactory recovery and precision values.

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[x] To whom inquiries should be directed.

Effect of Formulation and Processing Techniques on Release of Salicylic Acid from Ointments

A. F. ASKER * and C. W. WHITWORTH ˣ

Abstract □ The effects of variation of drug particle size, fusion temperature of the base, and ointment milling on the release of salicylic acid were investigated. The larger the drug particle size, the higher was the amount of salicylic acid released from petrolatum base. The fusion temperature of the base also appeared to influence the rate of drug release, suggesting an optimum temperature to be considered. Ointments prepared by fusion at 70 or 90° demonstrated lower rates of drug release than those produced by mechanical incorporation. Moreover, salicylic acid was found to sublime during the ointment preparation by fusion at 90°. Homogenization of the ointment in a three-roller mill enhanced the rate of drug release from emulsion bases but not from petrolatum base.

Keyphrases □ Salicylic acid release from ointments—effects of drug particle size, temperature of fusion of the base, and milling process □ Ointments, salicylic acid—effects of drug particle size, temperature of fusion of the base, and milling process on drug release □ Drug release, salicylic acid from ointments—effects of drug particle size, temperature of fusion of the base, and milling process

From the standpoint of therapeutics, one important attribute of an ointment is its ability to release its active ingredient. Studies on the effect of various additives on drug release from different ointment bases have been reported (1–7). However, it appears from literature reports that the manufacture of ointments is generally carried out on an empirical basis following traditional techniques, and that no attempt has been made to study the effect of the variables encountered in formulating and processing ointments on the release of the incorporated drug.

A previous report (8) indicated that there was a significant difference in the release of salicylic acid from ointments prepared by fusion and mechanical incorporation using a spatula. The aim of this study was to investigate the effect of variation of drug particle size, fusion temperature of the base, and ointment milling on the release of salicylic acid.

EXPERIMENTAL

Materials—Salicylic acid[1], cetyl alcohol[1], white petrolatum USP[2], sorbitan monostearate[3], and polyethylene sorbitan monooleate[4] were obtained from commercial sources. Chloroform[5] was spectrograde. Other materials were official or analytical grades.

Drug Release Studies—Diffusion techniques have been extensively employed to measure drug release from heterogeneous preparations such as ointments (9) and emulsions (10). The techniques used in this study were the same as those described previously (1). The amount of salicylic acid diffused into 40 ml of distilled water at room temperature was determined by measuring the absorbance on a spectrophotometer[6] at 297 nm.

Assay of Salicylic Acid Ointments Made with Petrolatum Base—About 100 mg of the ointment was dissolved in chloroform, and the mixture was transferred into a 100-ml volumetric flask and diluted to volume with chloroform. Salicylic acid was determined by measuring the absorbance of this solution at 308 nm against an appropriate blank.

Effect of Particle Size of Salicylic Acid on Its Diffusion from White Petrolatum—Salicylic acid powder was classified using a set of standard sieves. Particles of 68-, 90-, and 127-μm average size were separated and used.

Three batches, each of 1 kg of 2% salicylic acid ointment, were made from the three portions of salicylic acid powder having different particle size. White petrolatum was first fused at 50° in the bowl of a laboratory model planetary, power-driven, kitchen aid

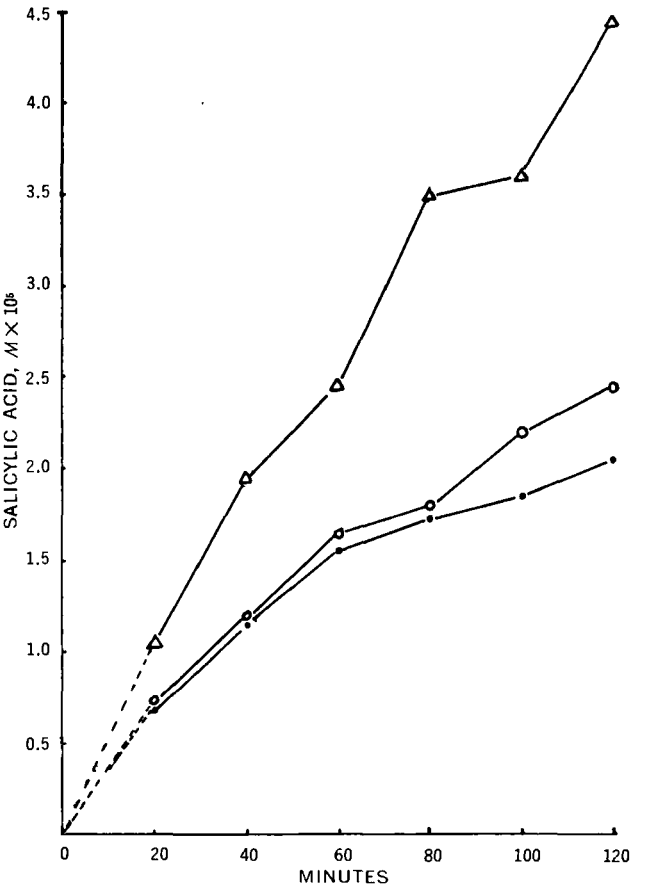

Figure 1—*Effect of particle size of salicylic acid on its diffusion from a 2% ointment in white petrolatum prepared by fusion at 50°. Key:* ●, *68 μm;* ○, *90 μm; and* △, *127 μm.*

[1] Fisher Scientific Co.
[2] Oils Inc., Paterson, N.J.
[3] Span 60, Atlas Chemical Industries, Wilmington, Del.
[4] Tween 80, Atlas Chemical Industries, Wilmington, Del.
[5] J. T. Baker Chemical Co.
[6] Cary model 118 recording spectrophotometer.

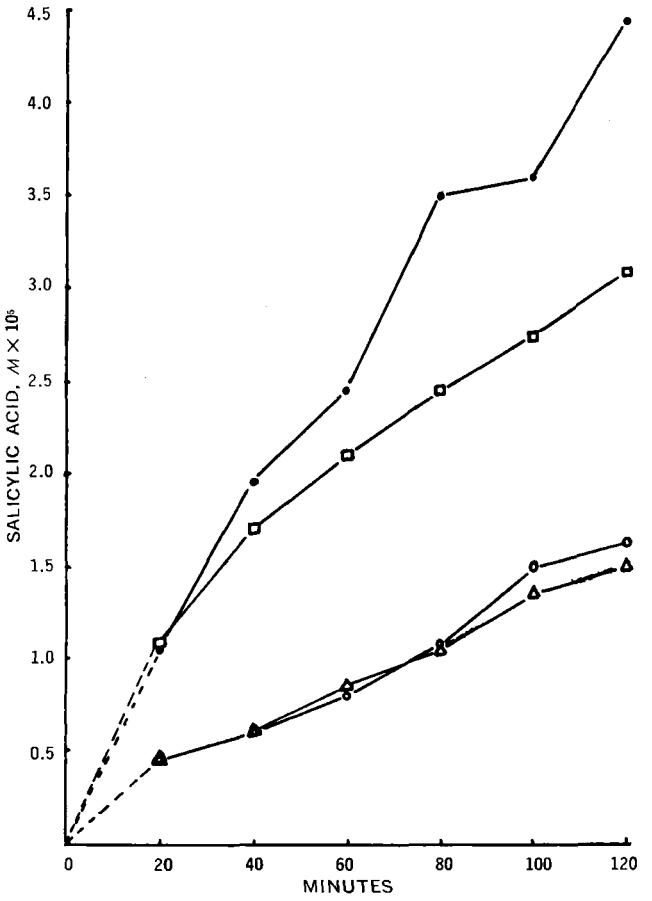

Figure 2—*Effect of fusion temperature of white petrolatum on the diffusion of salicylic acid. Key:* □, *control prepared by mechanical incorporation at room temperature;* ●, *50°;* ○, *70°; and* △, *90°.*

mixer[7]. Salicylic acid was then added while stirring was maintained at a fixed speed using the wire-type beater.

Heavier mixing blades were not utilized to avoid any change of particle size of the drug as a result of the grinding action produced by heavier blades. Stirring was continued until the ointment cooled to room temperature. Diffusion of salicylic acid from the three batches of ointment was determined the next day.

Effect of Variation of Temperature of Fusion of White Petrolatum on Diffusion of Salicylic Acid—Three batches of 2% salicylic acid ointment were prepared by fusing petrolatum at three different temperatures (50, 70, and 90°) in a manner similar to that already described. Diffusion of the salicylic acid from each batch was determined the next day.

Salicylic acid has been reported to sublime at 76° (11). Gore *et al.* (12) found that the loss of salicylic acid by sublimation could occur even at moderately elevated temperatures of 40, 50, and 70°. Therefore, it was decided to investigate the possible loss of salicylic acid as a result of sublimation. This was done by preparing the ointment at the three temperatures in a bowl covered with aluminum foil. Any deposition of salicylic acid on the foil was taken as an indication of sublimation. Moreover, the ointment was assayed to determine the extent of loss of salicylic acid due to sublimation.

Effect of Milling—The effect of homogenization of 2% salicylic acid ointment prepared with various bases was studied. The bases used were: *(a)* white petrolatum; *(b)* a water-in-oil-base consisting of 64% white petrolatum, 6% sorbitan monostearate, and 30% distilled water; and *(c)* an oil-in-water base consisting of 25% cetyl alcohol, 25% petrolatum, 12% glycerin, 5% polyoxyethylene sorbitan monooleate, and 33% distilled water.

All ointments were prepared by mechanical incorporation of the drug in the base at room temperature. Half the quantity of each batch of ointment was milled by passing once through a three-roller mill[8] maintained at constant operating conditions. The second half of the ointment was not milled and served as a control for comparison. Diffusion of salicylic acid from each ointment was then determined.

DISCUSSION

An examination of many ointment formulas reveals that the fusion process is practically always used in the preparation of an ointment in large-scale manufacturing. Therefore, more emphasis was placed in this study on the effect of formulation on the diffusion of salicylic acid from ointments prepared by fusion.

The release characteristics of salicylic acid from the various ointments over 2 hr are illustrated in Figs. 1–3. The increase in drug concentration of the aqueous diffusion medium at varying time intervals was used to assess the rate of drug release from the ointments.

It appears from Fig. 1 that the larger the particle size of salicylic acid, the higher was the amount of salicylic acid diffused from the petrolatum base. There was no appreciable difference in drug diffusion between particles of 68- and 90-μm average size, possibly due to the relatively narrower difference in particle size. The results may be explained by the gel-like structure of the base and its thixotropic properties (13), which would cause the smaller particles of salicylic acid to be entrapped more effectively within the crys-

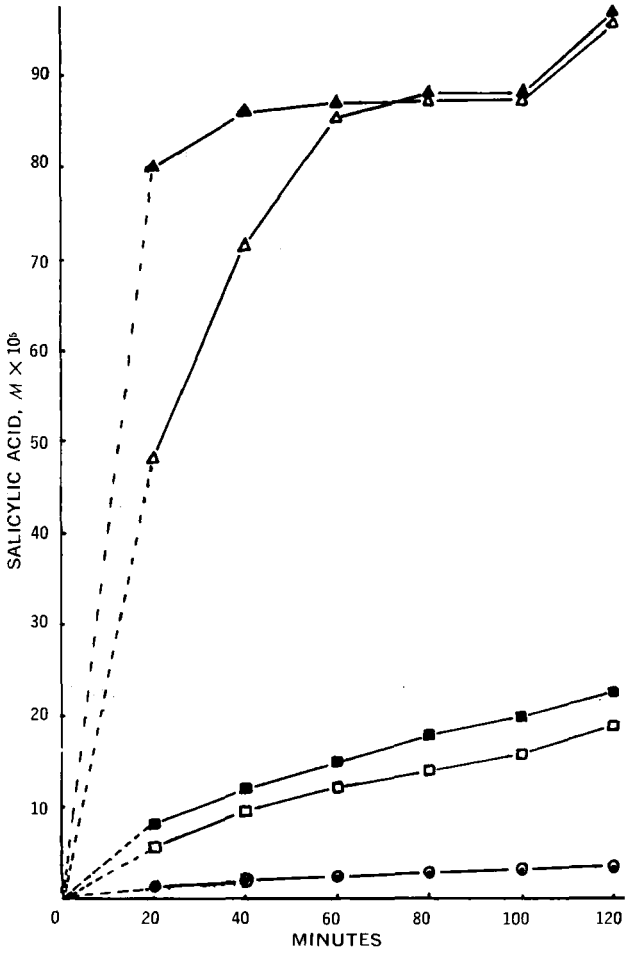

Figure 3—*Effect of milling on the diffusion of salicylic acid from various ointments prepared by mechanical incorporation. Open points represent the nonmilled ointments while the filled points represent the milled ointments. Key:* ▲, △, *oil-in-water base;* ■, □, *water-in-oil base; and* ●, ○, *white petrolatum base.*

[7] Hobart Manufacturing Co., Troy, Ohio.

[8] Erweka, West Germany.

talline hydrophobic structural network of the petroleum jelly. These findings are in agreement with those of Buckwalter and Dickison (14), who found that small particles of procaine penicillin G suspended in oily vehicles gelled with aluminum stearate were superior to large particles in delaying absorption. This finding was attributed to the reduction in rate of solution of the suspended solid from the thixotropic vehicle.

The effect of variation of fusion temperature of petrolatum on the diffusion of salicylic acid is illustrated in Fig. 2. It appears that white petrolatum fused at 50° before incorporation of the drug gave a higher rate of drug diffusion than that demonstrated when petrolatum was fused at 70 or 90°. This may be explained on the basis that, on heating petrolatum at temperatures higher than 50°, it became a more flowable and less viscous liquid that would more effectively wet, strongly adhere to, and coat the suspended drug particles. Such difference in diffusion rate could not be attributed to any changes in the crystalline structure of petrolatum produced as a result of the fusion process. This is based on the fact that heating petrolatum to its melting point permits a rearrangement of the crystal network, so that the product, after cooling, exhibits its initial thixotropic condition (13).

Ointments prepared by fusion at 50°, however, demonstrated higher rates of diffusion than those of the ointments prepared by mechanical incorporation at room temperature. The reason might be that the less viscous base maintained at 50° could have enhanced aggregation of salicylic acid particles. This would be expected to increase the diffusion rate due to an increase of particle size (Fig. 1). This effect, however, was not exhibited by the ointments prepared at the higher temperatures of 70 and 90°, probably due to better dispersion of the drug particles caused by the greatly reduced viscosity of the base. The slower release rates demonstrated by ointments made by fusion at 70°, as compared with those prepared by mechanical incorporation at room temperature, confirm the results previously reported for the effect of small-scale preparation techniques of ointments on the release of salicylic acid (8). It appears, therefore, that the optimum temperature of fusion should be considered in the preparation of ointments by fusion.

Salicylic acid was found to sublime and collect on the bowl cover as tiny crystalline needles in the case of ointments prepared at 90°. No sublimation was noted for ointments prepared at 50 or 70°. The loss of salicylic acid due to sublimation at 90° was 1.5%.

Figure 3 shows the effect of homogenization of salicylic acid ointment on its diffusion from the various bases. It is evident that milling enhanced the rate of drug diffusion from the emulsion bases, but no apparent difference in diffusion rate was noted for petrolatum base. This could be due to an increase in the degree of dispersion and/or solubility of salicylic acid in the emulsion phases as a result of homogenization.

It can be also seen that the emulsion-type ointments were superior to white petrolatum in salicylic acid release. The oil-in-water type base gave a better release than the water-in-oil type. These results are in agreement with those already published (3).

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Fluorocarbon Aerosol Propellants III: Effect of Water Vapor on Sensitivity of Electron-Capture Detector during GC Analysis

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Abstract □ The quantitative depressive effects of the presence of various amounts of water in samples injected onto a GC column on the detector response to three fluorocarbon aerosol propellants were investigated.

Keyphrases □ Propellants (fluorocarbon aerosol)—effect of water vapor on sensitivity of electron-capture detector, GC analysis □ Aerosols—fluorocarbon propellants, effect of water vapor on sensitivity of electron-capture detector, GC analysis □ Fluorocarbon aerosol propellants—effect of water vapor on sensitivity of electron-capture detector, GC analysis □ GC—effect of water vapor on sensitivity of electron-capture detector during analysis of fluorocarbon aerosol propellants

The presence of water vapor has been known to affect adversely the performance of ionization detectors in GC analysis. Lovelock (1) stated that the contamination of water in the carrier gas was objectionable. Although its presence could not be immediately detected, it could lead to a serious reduction in the detector sensitivity. It was shown (2) that the sensitivity of a macro-argon detector would be reduced 10-fold by a change in water vapor concentration from 30 to 1000 ppm (v/v). It was also shown (3) that

talline hydrophobic structural network of the petroleum jelly. These findings are in agreement with those of Buckwalter and Dickison (14), who found that small particles of procaine penicillin G suspended in oily vehicles gelled with aluminum stearate were superior to large particles in delaying absorption. This finding was attributed to the reduction in rate of solution of the suspended solid from the thixotropic vehicle.

The effect of variation of fusion temperature of petrolatum on the diffusion of salicylic acid is illustrated in Fig. 2. It appears that white petrolatum fused at 50° before incorporation of the drug gave a higher rate of drug diffusion than that demonstrated when petrolatum was fused at 70 or 90°. This may be explained on the basis that, on heating petrolatum at temperatures higher than 50°, it became a more flowable and less viscous liquid that would more effectively wet, strongly adhere to, and coat the suspended drug particles. Such difference in diffusion rate could not be attributed to any changes in the crystalline structure of petrolatum produced as a result of the fusion process. This is based on the fact that heating petrolatum to its melting point permits a rearrangement of the crystal network, so that the product, after cooling, exhibits its initial thixotropic condition (13).

Ointments prepared by fusion at 50°, however, demonstrated higher rates of diffusion than those of the ointments prepared by mechanical incorporation at room temperature. The reason might be that the less viscous base maintained at 50° could have enhanced aggregation of salicylic acid particles. This would be expected to increase the diffusion rate due to an increase of particle size (Fig. 1). This effect, however, was not exhibited by the ointments prepared at the higher temperatures of 70 and 90°, probably due to better dispersion of the drug particles caused by the greatly reduced viscosity of the base. The slower release rates demonstrated by ointments made by fusion at 70°, as compared with those prepared by mechanical incorporation at room temperature, confirm the results previously reported for the effect of small-scale preparation techniques of ointments on the release of salicylic acid (8). It appears, therefore, that the optimum temperature of fusion should be considered in the preparation of ointments by fusion.

Salicylic acid was found to sublime and collect on the bowl cover as tiny crystalline needles in the case of ointments prepared at 90°. No sublimation was noted for ointments prepared at 50 or 70°. The loss of salicylic acid due to sublimation at 90° was 1.5%.

Figure 3 shows the effect of homogenization of salicylic acid ointment on its diffusion from the various bases. It is evident that milling enhanced the rate of drug diffusion from the emulsion bases, but no apparent difference in diffusion rate was noted for petrolatum base. This could be due to an increase in the degree of dispersion and/or solubility of salicylic acid in the emulsion phases as a result of homogenization.

It can be also seen that the emulsion-type ointments were superior to white petrolatum in salicylic acid release. The oil-in-water type base gave a better release than the water-in-oil type. These results are in agreement with those already published (3).

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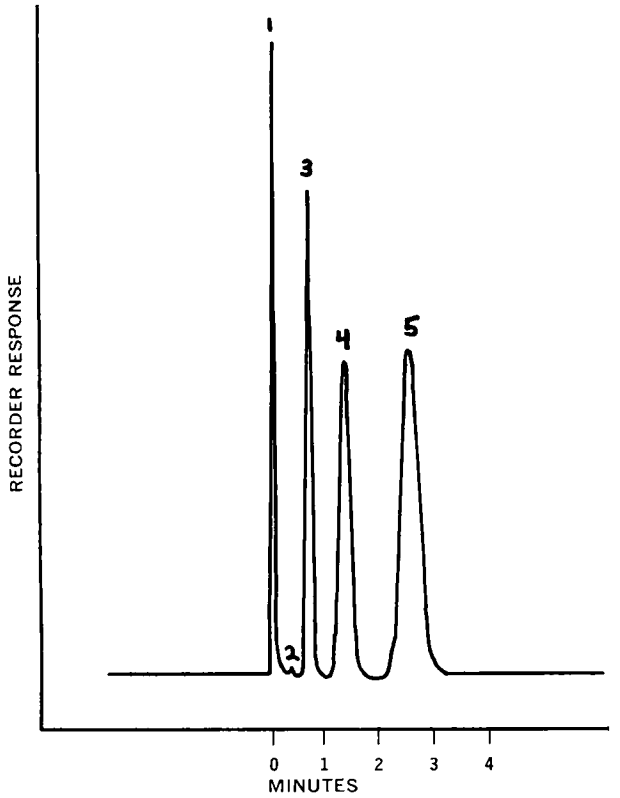

Figure 1—*Typical gas chromatogram of a mixture of three propellants and a negligible amount of water vapor. Key: 1, air; 2, water vapor; 3, dichlorodifluoromethane; 4, dichlorotetrafluoroethane; and 5, trichloromonofluoromethane.*

a slight increase in water vapor concentration from 8 to 10 ppm resulted in a 20% reduction in the response of the same type of detector to ether.

Aerosol products have been widely used for household, cosmetic, pharmaceutical, and other purposes. The possible toxicity of the most commonly used aerosol propellants, fluorinated hydrocarbons, has been a subject of intensive research and controversy (4–8). Quantitation of these fluorocarbon propellants in biological samples could be most sensitively performed by the GC method using an electron-capture detector (8–13). Although the technique of direct injection of blood samples onto GC columns has been used (9, 10, 12), it can result in serious contamination of the column and therefore require frequent changes of the column. Whether the presence of a large amount of water in blood or other aqueous samples could affect the sensitivity of electron-capture detectors toward these fluorocarbon propellants has not been reported. A quantitative study was undertaken to determine the extent of such an effect. The results of the present investigation can also be applied to other types of compounds using this kind of detector.

EXPERIMENTAL

Materials—Only the three most commonly used fluorocarbon aerosol propellants were used: trichloromonofluoromethane[1] (bp 23.7°), dichlorodifluoromethane[1] (bp −29.8°), and dichlorotetrafluoroethane[1] (bp 4.1°). Common stock solutions of the three propellants in distilled water and cyclohexane were prepared according to the method described previously (12).

Procedure—Ten microliters of the propellant stock solution in distilled water was injected into a 15-ml serum bottle[2] sealed with a flange-type, lacquer-coated rubber stopper[3] and aluminum cap. The bottle was shaken in a vortex mixer[4] for 30 min. Fifty microliters of the equilibrated air sample was then taken from the bottle, using a 100-μl gas-tight syringe[5], and injected onto the column of the gas chromatograph. Duplicate samples and injections were made and constant readings were obtained.

To measure the reduction in response of the electron-capture detector toward propellants in the presence of different amounts of water, 50 μl of the air sample from the equilibrated bottle was transferred to a 100-μl gas-tight syringe followed by drawing a certain amount of distilled water (usually less than 1 μl) into the same syringe, and the mixture was injected onto the column immediately. This process was repeated several times when the normal detector response was recovered. Different peak heights corresponding to different amounts of water vapor and three propellants were obtained on each injection. The peak height of the 1-μl water vapor response at the same GC condition was obtained by direct injection of exactly 1 μl of distilled water, using a 1-μl syringe[5], onto the GC column.

The duration of the water vapor effect on the reduction of detector response toward the three propellants was determined by direct injection of 0.5 μl of distilled water onto the column of the gas chromatograph immediately followed by the injection of 50 μl of air sample taken from the bottle containing equilibrated propellants. Injection of air samples obtained at various times was car-

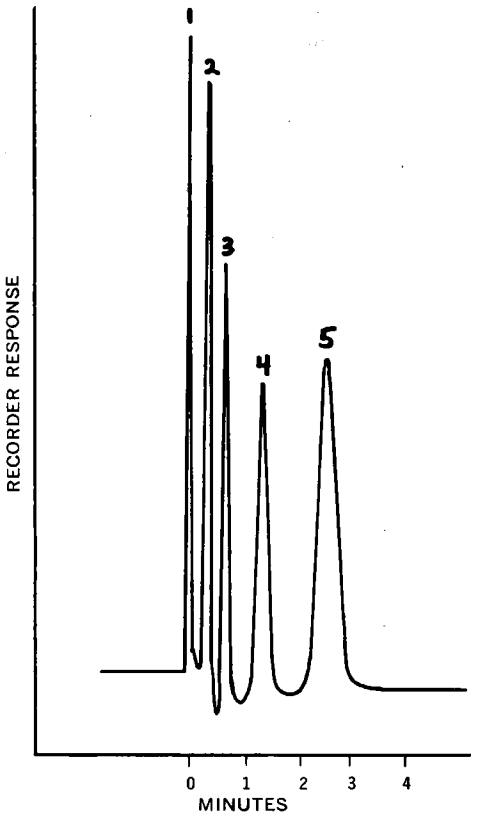

Figure 2—*Typical gas chromatogram of a mixture of three propellants and a significant amount of water vapor. Key: 1, air; 2, water vapor; 3, dichlorodifluoromethane; 4, dichlorotetrafluoroethane; and 5, trichloromonofluoromethane.*

[1] Supplied by DuPont de Nemours and Co., Wilmington, Del.

[2] Wheaton Scientific, Millville, N.J.

[3] West Co., Phoenixville, Pa.

[4] Vortex-Genie mixer, Cat. No. 12-812-V1, Fisher Scientific Co., Springfield, Mass.

[5] Hamilton.

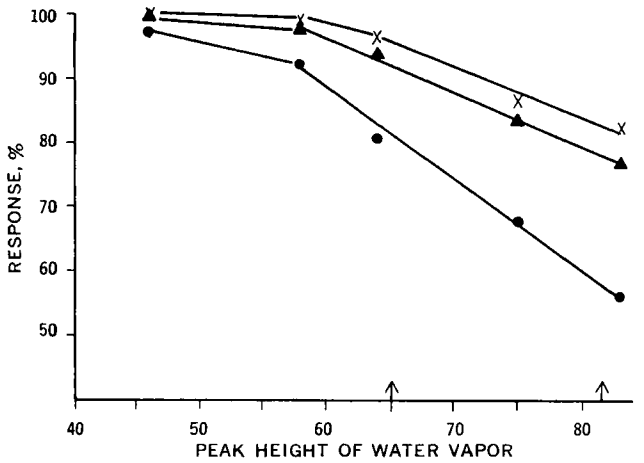

Figure 3—*Percent of maximum response for three propellants in the presence of different amounts of water vapor. Key: ×, trichloromonofluoromethane; ●, dichlorodifluoromethane; and ▲, dichlorotetrafluoroethane. Two arrows on the abscissa correspond to 0.5 and 1.0 μl of water injected.*

ried out until constant peak heights were obtained (about 90 min). In a separate study, no significant loss of the three propellants from the bottle in 2 hr was found.

Instrumentation—The GC unit[6] was equipped with a tritium-foil (150 μCi) electron-capture detector and a 1.8-m (6-ft) U-shaped glass column (4 mm i.d.) packed with Porapak Q[7]. The conditions of the GC analyses were: attenuation, 64×10^{-10}; column temperature, 170°; injection port temperature, 175°; detector temperature, 200°; and carrier gas (nitrogen) flow rate, 85 ml/min.

RESULTS AND DISCUSSION

A typical chromatogram for the three propellants in the presence of a negligible amount of water vapor (10 μl of aqueous propellant solution added to a 5-ml sealed empty serum bottle) obtained at the GC conditions described previously is shown in Fig. 1. The retention times for water, dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane were 0.38, 0.72, 1.40, and 2.58 min, respectively. The retention times of the three propellants were not changed by the presence of a large amount of water. However, the baseline of the chromatogram was shifted toward the negative direction and then returned back gradually to the original position (Fig. 2). The lowering of the baseline is much more pronounced if the chromatograph is run at a higher sensitivity or lower attenuation. Sometimes a sharp overshooting in the baseline between the peaks of water and of dichlorodifluoromethane was observed, and deflection appeared at the point where the signal of dichlorodifluoromethane started. In such a case, the deflection point was used to measure the peak height of the propellant, although the peak height measurement is generally used to quantitate the concentration of the propellant. If the injection of the sample is not completed rapidly, the broad water peak could also overlap the signal of dichlorodifluoromethane, resulting in poor resolution and inaccurate estimate of the propellant concentration.

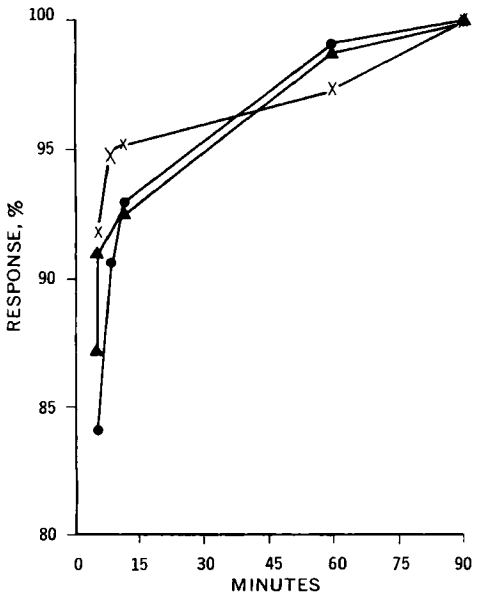

Figure 4—*Recovery of detector response toward three propellants as a function of time after an injection of 0.5 μl of distilled water. Key: ×, trichloromonofluoromethane; ●, dichlorodifluoromethane; and ▲, dichlorotetrafluoroethane.*

The presence of a large quantity of water injected into the column can also decrease the detector sensitivity toward the propellants. Figure 3 shows the relationship between the percentages of the maximum response for the three propellants and the amounts of water present in samples, expressed in terms of its peak height on the chromatogram. One unit of peak height corresponds to 0.254 cm (0.1 in.) of peak height on the 25.4-cm (10-in.) chart paper under the GC conditions described previously. The peak heights of water from normal atmospheric air samples were negligible, and injections of 0.5 and 1.0 μl would give rise to 65 and 81.5 units of peak height, respectively, indicating the existence of nonlinear response. The relative responses in the presence of 1 μl of water were 58.2, 78.5, and 83.4% for dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane, respectively, compared to their responses in the presence of a negligible amount of water. These data indicate that the magnitude of reduction in detector sensitivity is inversely proportional to the length of retention time. The data in Fig. 3 also show that the reduction in detector sensitivity would not be pronounced in the presence of only a small amount of water.

Figure 4 shows the recovery of the detector response toward the three propellants as a function of time after an injection of 0.5 μl of distilled water. The recovery rates were found to be higher in the first 15 min, and total recovery occurred in 90 min. Such a time- and water content-dependent detector response as observed in this study should be of importance to researchers using direct injection onto GC columns of biological or aqueous samples. The mechanisms for these findings are not known. It is thought that adsorption of water molecules on the surface of tritium foil may cause a temporary inhibition of β-emission and, hence, the sensitivity of the electron-capture detector, because the response of the detector is directly proportional to the amount of radioactivity (β-emission).

In a recent study (13) from this laboratory on the partition coefficients of the three propellants between distilled water or normal saline and air, the propellant concentrations in both aqueous and air phases at the equilibrium state were measured by injecting the samples directly onto the column. The partition coefficients (water–air or normal saline–air) obtained were 0.284, 0.06, and 0.026 for trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane, respectively. Since 1 μl of aqueous sample was injected, an underestimate of the propellant concentration is expected in light of the results from the present study. After correction for the reduced detector sensitivity, these partition coefficients should be 0.340, 0.103, and 0.031. To confirm these results, propellants in the aqueous phase were extracted into cyclohexane, and the concentrations in cyclohexane were analyzed by direct injection onto the column. The partition coefficients obtained by this extraction method were 0.346, 0.104, and 0.032 for the three propellants; these values are in excellent agreement with the data after correction.

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Influence of Drug Concentration on *In Vitro* Release of Salicylic Acid from Ointment Bases

F. BOTTARI, G. DI COLO, E. NANNIPIERI, M. F. SAETTONE [x], and M. F. SERAFINI

Abstract □ The effect of drug concentration on *in vitro* release of salicylic acid from a series of ointment bases was investigated. The bases were commercially available vehicles containing lanolin and/or lanolin derivatives and formed stable water-in-oil or oil-in-water emulsions. Release tests were performed both with the anhydrous and with the emulsion forms of the bases, at varying salicylic acid concentrations (0.5–5.0% w/v), and involved the use of silicone rubber membranes. The release of salicylic acid from the bases was in agreement with a reported diffusional model. A linear relationship between release rate, $q/\sqrt{t}$, and drug concentration in ointments existed when the drug was completely dissolved in the vehicles. The method reported is of potential utility for the determination of drug solubility in ointments and for the evaluation of the optimal drug concentration in topical vehicles. The relationships among type of vehicle, drug concentration, drug solubility, and release rate are discussed.

Keyphrases □ Vehicles (lanolin and lanolin derivatives)—*in vitro* release of salicylic acid, influence of drug concentration □ Ointment bases—influence of drug concentration on *in vitro* release of salicylic acid □ Drug concentration—influence on *in vitro* release of salicylic acid from ointment bases □ Lanolin and derivatives as ointment vehicles—influence of drug concentration on *in vitro* release of salicylic acid

It is generally recognized that a topical vehicle or base may affect drug penetration by modifying the permeability of the skin barrier phase and by releasing the drug to the skin in adequate amounts at a sufficient rate (1–3). A series of physicochemical factors, both pertaining to the drug and to the vehicle, appear to be involved in the latter process (4–6). Although several investigations have been directed toward determining these factors and elucidating their role in release, many unexplored points still exist whose study might prove profitable. The present paper is concerned with an *in vitro* study of the influence of drug concentration in different topical vehicles on release.

THEORETICAL

In the case of a membrane separating the donor and receptor phases, the release process may obey two different kinetic laws, depending on the resistance offered by the membrane to drug penetration. The relevant mathematical relationships have been developed and mainly investigated by T. Higuchi, W. I. Higuchi, and their coworkers (7–11). When the membrane offers little resistance to drug penetration (as may occur with injured skin or with some artificial membranes), large concentration gradients develop in the donor phase, and diffusional migration of the drug within the vehicle constitutes the slowest step in the release process. The following equations, derived from Fick's law, have been found to describe adequately the rate of release of drugs from ointment bases under these conditions (7, 10). The first equation refers to uniform solutions of drugs in ointments:

$$Q = q/A = 2C\sqrt{Dt/\pi} \qquad \text{(Eq. 1)}$$

where Q is the amount of drug (q) released to the sink at time t per unit area (A) of contact, D is the diffusion coefficient of drug in the vehicle, and C is the initial concentration of drug in the vehicle, expressed in units per milliliter. The second equation refers to suspension-type ointments:

$$Q = q/A = \sqrt{Dt(2C - Cs)Cs} \qquad \text{(Eq. 2)}$$

where C is the total drug concentration, and Cs is the solubility of drug in ointment; both values are expressed in units per milliliter. Equations 1 and 2 predict that plots of the amounts of drug released with $\sqrt{t}$ will give straight lines passing through the origin. The origin as intercept may not be observed in some cases because of the lag time phenomenon (12).

The preceding model is based on a series of simplifying assumptions: *(a)* only a single drug species is important in the base; *(b)* the diffusion coefficient is constant with respect to both time and position in the base; *(c)* the drug alone is allowed to diffuse out of the base; *(d)* the drug is rapidly removed upon reaching the base–membrane interface, and the receiving phase is a "perfect sink"; *(e)* the percent drug released is not too large (<30%) in the case of solutions; and *(f)* C is substantially greater than Cs in the case of suspensions.

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$$Q = q/A = \sqrt{Dt(2C - Cs)Cs} \qquad \text{(Eq. 2)}$$

where C is the total drug concentration, and Cs is the solubility of drug in ointment; both values are expressed in units per milliliter. Equations 1 and 2 predict that plots of the amounts of drug released with $\sqrt{t}$ will give straight lines passing through the origin. The origin as intercept may not be observed in some cases because of the lag time phenomenon (12).

The preceding model is based on a series of simplifying assumptions: *(a)* only a single drug species is important in the base; *(b)* the diffusion coefficient is constant with respect to both time and position in the base; *(c)* the drug alone is allowed to diffuse out of the base; *(d)* the drug is rapidly removed upon reaching the base–membrane interface, and the receiving phase is a "perfect sink"; *(e)* the percent drug released is not too large (<30%) in the case of solutions; and *(f)* C is substantially greater than Cs in the case of suspensions.

The assumption that D must be constant with respect to both time and position is a serious limitation, because in many situa-

tions involving emulsions the diffusion coefficient is not constant but varies with concentration. Koizumi and Higuchi (12) were able to show that even if D is concentration dependent the release pattern is the same as where D is constant. The equation for the amount of drug released becomes:

$$Q = 2a\sqrt{t} \qquad \text{(Eq. 3)}$$

where a is a constant, dependent on the initial drug concentration and on a function describing the change of D.

Rearranging Eq. 1:

$$q/\sqrt{t} = 2AC\sqrt{D/\pi} \qquad \text{(Eq. 4)}$$

shows the relationship existing between the release rate, $q/\sqrt{t}$, and C, the overall concentration of drug dissolved in ointment. According to Eq. 4, plots of release rates observed at different drug concentrations against the corresponding concentrations should be linear, with zero intercept and slope = $2A\sqrt{D/\pi}$. Such a linear relationship should hold until C exceeds the effective solubility, Cs. In the presence of suspended drug, release conditions described by Eq. 2 should prevail; accordingly, a different relationship between overall drug concentration and release rate might be observed. One main purpose of the present study was to test the scope and usefulness of Eq. 4 in a series of practical cases.

EXPERIMENTAL

Materials—Finely powdered, reagent grade salicylic acid[1] was used. Hydrophilic ointment and hydrophilic petrolatum, used as reference bases, were prepared according to USP XVIII. Dimethyl polysiloxane[2] (silicone rubber) sheeting, in a labeled thickness of 5 mil, was used as a membrane material. More than 40 commercial ointment bases, containing lanolin and/or lanolin derivatives, were submitted to a preliminary screening aimed at selecting the bases forming stable emulsions on admixture with an equal weight of water. An emulsion was considered stable if it showed no phase separation after storage at 40° for 30 days. The following bases were selected.

A. A mixture of sterols and sterol esters derived from wool grease and refined lanolin[3,4].

B. A balanced blend of cosmetic grade, anhydrous lanolin USP reacted with ethylene oxide plus *p*-oxybenzoates and antioxidants[3,5].

C. A mixture of pure, natural lanolin sterols in free alcohol form, free of fatty acids and esters and of preservatives[3,6].

D. A blend of sterols, fatty alcohols, and partial esters of fatty alcohols, waxes, and mineral oils[3,7].

E. A mixture of 30- and 75-mole ethoxylated lanolin (about 30% of total) plus hydrocarbon oils and cetyl and stearyl alcohols[3,8].

F. A mixture of cholesterol, alcohols, and esters obtained from lanolin (60% of total) in an oily vehicle analogous to human sebum[3,9].

Apparatus—The release of salicylic acid through silicone rubber membranes was investigated using a specially designed stainless steel cell (Fig. 1). The capacity of the cell was 5.0 ml, and the diameter of the available area for diffusion was 50 mm. For use, the filled cell was placed in a jacketed beaker (internal height 11.0 cm, internal diameter 10.0 cm) connected with a constant-temperature bath and circulator. The solution external to the cell was stirred using a 300-rpm synchronous motor[10]. The system was designed to produce reasonably fast release rates and to avoid absorption of salicylic acid by the cell material.

Preparation of Ointments—Emulsions were prepared by slowly adding distilled water, heated at 70°, to an equal weight of base, heated at the same temperature, while stirring. After cooling to room temperature, the emulsions were roll milled[11] twice. They are

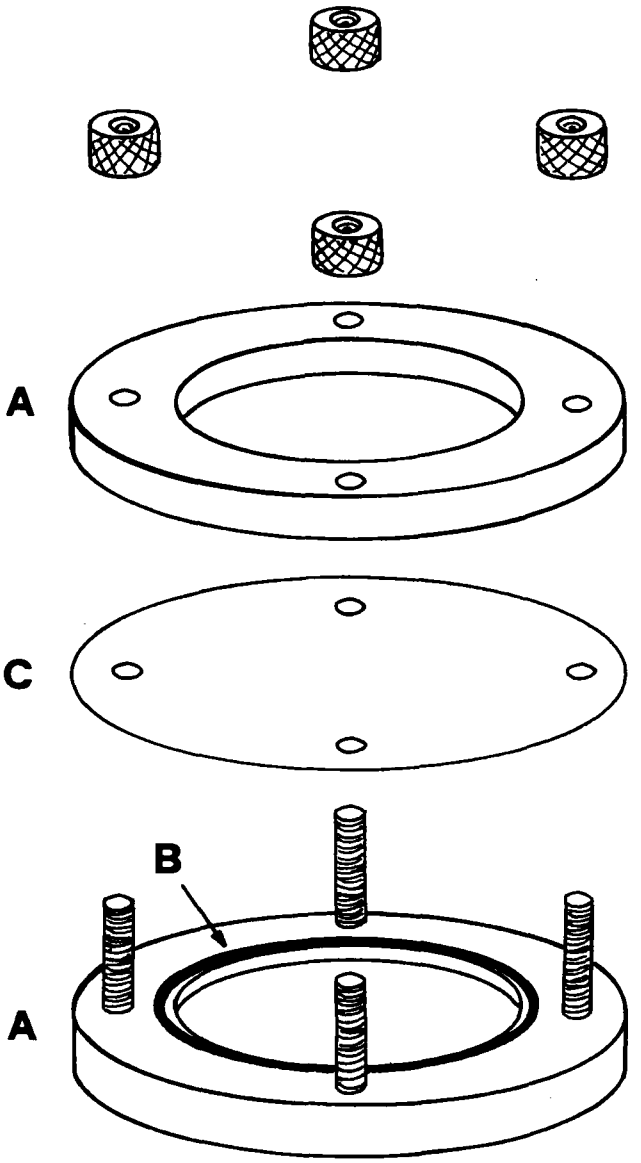

Figure 1—*Diffusion cell used for release experiments. Key: A, stainless steel cell body and plate; B, O-ring gasket; and C, silicone rubber membrane.*

indicated as X/H_2O, where X is the symbol of the anhydrous base. Salicylic acid (final concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 w/v) was incorporated in anhydrous and emulsion bases by levigation, using a mortar and pestle. The resulting ointments were roll milled twice and stored at room temperature. Prior to use, each ointment was kept 2 days at 30°.

Solubility of Salicylic Acid in Anhydrous Bases—The method described by Mayer and Kedvessy (13) was followed with minor modifications. Finely powdered salicylic acid was stirred into each base at 80° in a series of concentrations ranging from 0.5 to 7.0% (w/v), with 0.2-g increments up to 3.0% and 0.5-g increments up to the maximum concentration of 7.0%. After cooling to room temperature, the ointments were roll milled twice and stored in a thermostatic oven at 30 ± 0.1°. After 30 days, they were examined using a 40× microscope[12] for the presence of suspended particles. The solubility value, Cs, was taken as the highest concentration at which no suspended crystals were visible.

Release from Ointments—The cell was filled with an ointment; the excess was removed with a spatula to produce an even surface; and the membrane, which had been presoaked in water for at least 12 hr, was carefully placed and pressed on the ointment. A

[1] Carlo Erba, Milano, Italy.
[2] Silastic, Medical Products Division, Dow Corning Corp., Midland, Mich.
[3] List of main components as given by the technical literature.
[4] Falba, Pfaltz & Bauer, Inc., Flushing, N.Y.
[5] Lanobase S. E., Lanaetex Products, Inc., Elizabeth, N.J.
[6] Nimcolan 11, Malmstrom Chemical Corp., Linden, N.J.
[7] Protegin X, Th. Goldschmidt A. G., Essen, West Germany.
[8] Sebase, Westbrook Lanolin Co., Bradford, England.
[9] Sebolan, Croda Ltd., London, England.
[10] Crouzet S. A., Paris, France.
[11] Erweka GmbH, Frankfurt am Main, West Germany.
[12] Zeiss Zoom III Stereomicroscope.

new membrane was used for each release run. The upper part of the cell was then assembled, thus securing the membrane in place. Prewarmed 0.01 *N* NaOH (200 ml) was introduced into the jacketed beaker, into which the cell was immediately placed, and stirring was initiated. At intervals, 100-ml portions of the solution were removed and replaced at once with an equal amount of prewarmed 0.01 *N* NaOH. This procedure allowed a direct spectrophotometric[13] determination (297 nm) of salicylic acid in the samples, thus simplifying the calculations. Sodium hydroxide was added to avoid retrodiffusion of the drug into the membrane, although the periodic removal of solution was sufficient to ensure sink conditions. Blank runs, performed on all bases, demonstrated the absence of diffusable substances, which might interfere with the measurements.

Statistical Evaluation of Data—The release results obtained with all bases and with the reference bases, containing 1.0 and 3.0% (w/v) salicylic acid, were compared to detect statistical differences between individual bases. Each experiment was repeated at least four times, and the average values obtained for each experimental point were used to draw the individual q *versus* $\sqrt{t}$ plots (*e.g.*, Figs. 2 and 3). For each experiment, statistical differences were assessed by employing the Student t test and comparing experimental points corresponding to identical values on the time axis. A significant difference between two bases was assumed to exist if all experimental points, except the first pair (1 hr), were significantly different at the 5% level.

RESULTS AND DISCUSSION

Release Rate Studies—The experiments of salicylic acid release from the bases investigated were carried out over 6 hr at concentrations ranging from 0.5 to 5.0% (w/v). In all cases, linear plots were obtained when the amount of salicylic acid released to the aqueous phase was plotted *versus* the square root of time. The release patterns obtained from the anhydrous and from the emulsion bases containing 1.0% salicylic acid are illustrated in Figs. 2 and 3, respectively. The data obtained with the reference bases are also included for comparison. In no case did the plots in Figs. 2 and 3 pass through the origin. Several examples reported in the literature also exhibit a lag time for the release of a drug across an artificial membrane (12, 14–17), and this phenomenon has been associated with the presence of the membrane separating the bulk phase and sink.

As indicated by Koizumi and Higuchi (12), two distinct lag times may be operative in similar cases; one is the classical lag time of Barrer (18), which is a measure of the time required for the absorption of drug by the membrane, and another results from a diffusion coefficient of the drug in the membrane smaller than that in the bulk phase. Steady-state diffusion experiments (not reported here) of salicylic acid in aqueous solution through a 5-mil silicone rubber membrane have shown Barrer's lag time to be of the order of a few seconds. The lag time observed in the present case, about 20 min, should, therefore, result from slow membrane diffusion[14]. The slower diffusivity of the permeant in the membrane is probably due to a greater rigidity of the polymeric units forming the silicone film, compared with the materials of the bulk, and to adsorption of the diffusant on the siliceous filler in the membrane. Indeed, the diffusion process is known to be slower when diffusion is accompanied by adsorption onto internal sites (19). The presence of a small but definite lag time probably indicates nonperfect sink conditions at the vehicle–membrane interface. However, even if this system does not comply exactly with the theoretical model, it should approximate the theoretical requirements, at least within the scope of the present investigation.

Release was faster from the emulsions than from the corresponding anhydrous bases, in agreement with other workers (1, 4, 20–23). Among emulsion bases, there was no clear trend to faster release from oil-in-water compared with water-in-oil types. This appears to confirm the reports of Barrett *et al.* (24) and of Sarkany *et al.* (25), who found no marked difference in skin penetration of methylnicotinate and of betamethasone valerate, respectively, from oil-in-water or water-in-oil creams. The anhydrous base, E, giving rise to a water-in-oil emulsion, showed the fastest release. It is possible that the absence of high hydrophilic–lipophilic balance emulsifiers, favoring formation of oil-in-water emulsions and endowed with a great interactive nature for salicylic acid (26–28), might have favored a faster release from this base. Release from

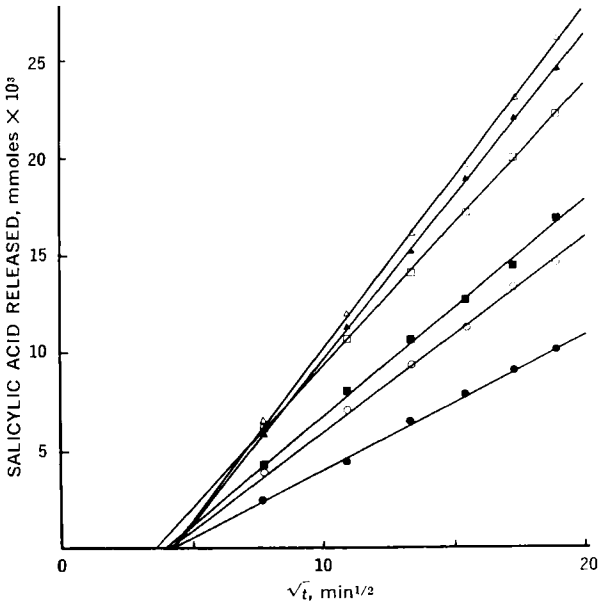

Figure 2—*Release of salicylic acid from anhydrous 1.0% (w/v) ointments at 30°. Key: △, D (and F, not shown for clarity of graph); ▲, hydrophilic petrolatum; □, A; ■, C; ○, E; and ●, B.*

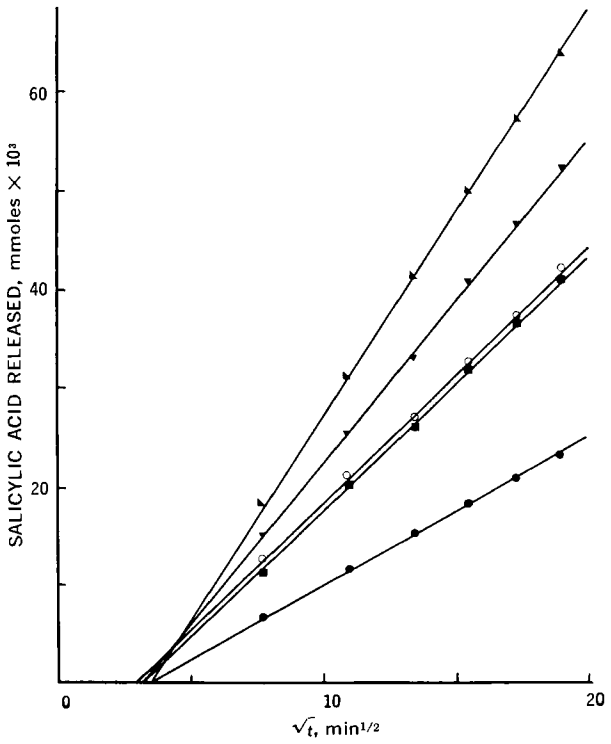

Figure 3—*Release of salicylic acid from 1.0% (w/v) emulsion-type ointments at 30°. Key: ▲, F–H_2O; ▼, hydrophilic ointment, A–H_2O, and D–H_2O (last two not shown for clarity of graph); ○, E–H_2O; ■, C–H_2O; and ●, B–H_2O.*

[13] Zeiss PMQ II spectrophotometer.

[14] The very small differences observed for the individual lag times probably originated from the different diffusion coefficients of the drug in the various bases. Slight modifications of the membrane by some ingredient of the bases might also contribute to the observed differences. Neither factor, however, should affect the validity of the experimental model. A study on membrane effects (drug transport through and partition with the membrane) in this model is now in progress.

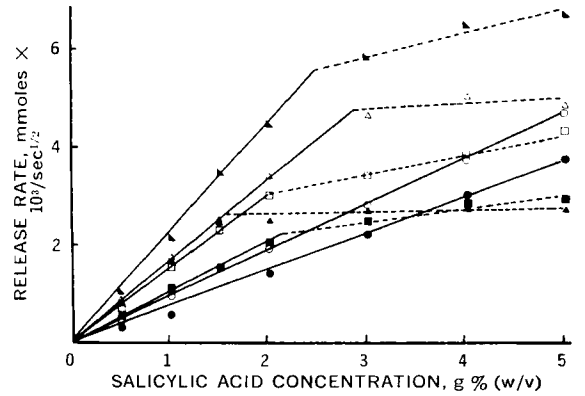

Figure 4—*Plot showing the effect on release rate of increasing concentration of salicylic acid in anhydrous ointments. Key: ▼, F; △, D; ○, E; □, A; ●, B; ■, C; and ▲, hydrophilic petrolatum.*

hydrophilic ointment was faster than from hydrophilic petrolatum, in agreement with *in vitro* (4) and *in vivo* (29) results.

Drug Concentration-Release Rate Relationship—To test the validity of Eq. 4, the release rates, $q/\sqrt{t}$, of the ointments containing salicylic acid in the 0.5–5.0% (w/v) concentration range were plotted *versus* the corresponding concentrations (Figs. 4 and 5, anhydrous bases and emulsions, respectively). Linear plots with zero intercept, as predicted by Eq. 4, were obtained only from two bases (B and E). The solubility of salicylic acid in the anhydrous form of these bases exceeded 7.0% (w/v), and, in all probability, the drug was soluble in the corresponding emulsions up to the maximum concentration tested in the release experiments (5.0%). The $q/\sqrt{t}$ *versus* C graphs of all other anhydrous bases, in which a lower solubility of salicylic acid had been verified, showed a linear portion with zero intercept, followed by a sudden change in slope in correspondence to a point on the abscissa coinciding with the solubility limit of salicylic acid, determined visually (*cf.*, Table I). This finding also appears to agree with Eq. 4, which predicts a linear dependency of $q/\sqrt{t}$ on C when C is below the solubility of the drug in the ointment. The emulsions gave similar graphs, showing slope changes at lower drug concentration with respect to the corresponding anhydrous bases. It might be assumed that these changes also correspond to the maximum solubility, Cs, of the drug in the emulsions. These lower "saturation" values agree with the fact that solubility of salicylic acid in the emulsions ought to be lower than in the corresponding anhydrous bases because of the low solubility of the drug in water. The "solubility" values of salicylic acid in all ointments, in grams percent (w/v), obtained graphically from the plots are reported in Table I. The agreement between these values and those independently obtained for the anhydrous bases is quite satisfactory. The apparent diffusion coefficients, D', of salicylic acid in all ointments, calculated with Eq. 4 from the first portions of the $q/\sqrt{t}$ *versus* C plots, are also reported in Table I.

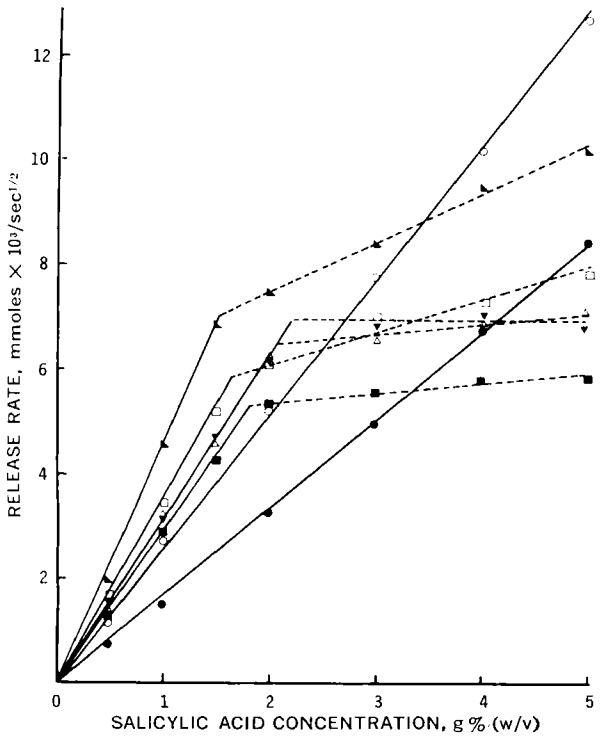

Figure 5—*Plot showing the effect on release rate of increasing concentration of salicylic acid in emulsion-type ointments. Key: ○, E–H₂O; ▲, F–H₂O; □, A–H₂O; △, D–H₂O; ▼, hydrophilic ointment; ■, C–H₂O; and ●, B–H₂O.*

Table I—Solubility and Apparent Diffusion Coefficients of Salicylic Acid in Ointments

Base (Emulsion Type)[a]	Solubility, g % (w/v)		Apparent Diffusion Coefficient, D', $cm^2\ sec^{-1} \times 10^6$
	Microscopic Data	Release Data	
A	2.00	2.00	0.89
A–H_2O (oil-in-water)	—	1.60	4.95
B	7.00	5.00	0.23
B–H_2O (oil-in-water)	—	5.00	1.09
C	2.00	2.15	0.42
C–H_2O (water-in-oil)	—	1.80	3.45
D	3.00	2.90	1.05
D–H_2O (water-in-oil)	—	2.05	4.00
E	7.00	5.00	0.33
E–H_2O (oil-in-water)	—	5.00	2.69
F	2.50	2.45	1.99
F–H_2O (water-in-oil)	—	1.50	8.53
Hydrophilic petrolatum	1.50	1.55	1.20
Hydrophilic ointment (oil-in-water)	—	2.20	4.10

[a] Determined by the electrical conductivity method.

Whether the slope changes observed with the emulsions also correspond to the saturation of the vehicles by the drug might be open to some doubt. Indeed, an essential condition for the validity of Eq. 4 is the constancy of the diffusion coefficient, and this factor has been found to be concentration dependent in the case of some emulsions (12). However, the linearity of the plots within a definite concentration range and their zero intercept might indicate that D is sufficiently concentration independent in the present case to not interfere too seriously with the application of Eq. 4 or, at least, that there is a linear dependency of $2a$ (Eq. 3) on C. This point, however, might deserve further investigation. If it is assumed that the change in slope in the $q/\sqrt{t}$ *versus* C graphs is related to the saturation of the vehicles by the drug rather than to a sudden change of D with increasing concentration (particularly in the case of emulsions), application of Eq. 4 might yield useful information on the solubility of drugs in ointments in cases where visual methods for determining solubility are not applicable (emulsions or otherwise opaque vehicles) and might give direct insight on the effect on the release rate of drug concentration and solubility in vehicles.

Adherence to Eq. 2 of saturated ointments was not satisfactory in most cases. One essential condition for the validity of Eq. 2 is that the amount of drug present per unit volume, C, be substantially greater than the solubility, Cs; this condition was probably approximated only at the highest concentrations tested. An inadequate dissolution rate of the suspended particles (9, 15) might also be responsible for the unsatisfactory adherence to Eq. 2; this factor seems evidenced by the independence of release rate on drug concentration shown by many saturated ointments. An approximate zero-order dependence of release on drug concentration for saturated systems was observed by others (15).

Figures 4 and 5 clearly show that release proceeds most efficiently only within the solubility range; after saturation, a further increase of drug concentration may have little effect on the release rate. Therefore, the presence of excess undissolved drug in a vehi-

Table II—Groups of Bases Showing Release Rates Not Statistically Different at the 5% Probability Level

Salicylic Acid Concentration	
1.0% (w/v)	3.0% (w/v)
Hydrophilic ointment; D–H_2O	Hydrophilic ointment; D–H_2O; A–H_2O
C–H_2O; E–H_2O	B–H_2O; D
Hydrophilic petrolatum; D; B–H_2O	Hydrophilic petrolatum; E

cle may be wasteful in cases where the rate of release, rather than the total amount of released drug, is therapeutically important. The solubility factor assumes a paramount importance when release rates of different bases are compared. One base might show, with respect to a reference base in which the drug is more soluble, faster release rates at a low drug concentration and slower rates at a higher drug concentration. The release rate at 1% salicylic acid was faster for D–H_2O than for E–H_2O, while the opposite occurred at the 3% concentration. Thus, comparison of release properties made at a single drug concentration, with no concern for solubility, may offer only a partial view of the drug-releasing potentiality of different vehicles.

Statistical Evaluation of Data—Table II summarizes the results of the statistical tests. Only groups of bases giving release results that are not statistically different at the 5% level are indicated. One objective of the tests was to assess whether relative identities in release rates within groups would be maintained at different drug concentrations (1.0 and 3.0%). Only one pair of ointments [hydrophilic ointment–(D–H_2O)] released salicylic acid at relatively identical rates at both concentrations. Most bases in Table II showing release rates not statistically different at one concentration had dissimilar rates at the other, and vice versa. This effect, presumably due to saturation of the vehicle by the drug, was discussed in a previous section. At the 3% concentration, salicylic acid was not completely dissolved in most bases (*cf.*, Table I).

CONCLUSIONS

The present results, although needing further experimental support, are indicative of a practical method for obtaining information on the solubility of drugs in topical vehicles and on interactions between drugs and vehicles that might influence drug absorption. The results indicate that the solubility of a drug in the vehicle plays an important role in release and should be carefully considered when selecting or developing a base. Such knowledge may be essential for the selection of the most therapeutically effective base and for the determination of the most effective drug concentration in a given base.

During the present investigation, several commercial "lanolin" bases were examined, and their different release characteristics were pointed out. The different release rates observed at low drug concentration might possibly be rationalized in terms of different diffusivity of the drug in the various bases. An investigation on the correlations between the composition (and/or structure) of the bases and drug diffusivity within them was beyond the scope of the present work, but the problem might deserve further study.

Some bases showed particularly good release characteristics when compared with the standard vehicles, hydrophilic ointment and hydrophilic petrolatum. It is possible that the same bases might show a similar action *in vivo*, in analogy with other fatty substances (30) and water-in-oil emulsifiers (31) acting as "sorption promotors" (32). Further *in vivo* experiments have been planned to verify this hypothesis.

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[x] To whom inquiries should be directed.

Hydrolytic Degradation of Methaqualone

JEROME J. ZALIPSKY ˣ, DAHYABHAI M. PATEL, and N. H. REAVEY-CANTWELL

Abstract □ The degradation products from acid and base hydrolysis of methaqualone, 2-methyl-3-*o*-tolyl-4(3*H*)-quinazolinone, were isolated and identified by combined methods using liquid–liquid extraction, TLC, GLC, and IR, UV, and visible spectroscopy. Examination of the degradation products resulting from the hydrolysis of methaqualone revealed that, in boiling acidic or basic media, anthranilic acid, *o*-toluidine, and acetic acid were the major degradation components. The formation of the other degradation products, *N*-acetylanthranilic acid and 2-aminobenzo-*o*-toluidide, was acid–base strength dependent. The degradation of methaqualone in basic or acidic media followed different reaction pathways, but the primary decomposition was due to hydrolytic cleavage. The relationship between the degradation product, 2-aminobenzo-*o*-toluidide, and 2-nitrobenzo-*o*-toluidide, a metabolite of methaqualone in humans, is discussed.

Keyphrases □ Methaqualone—hydrolytic degradation, acid and base hydrolysis, isolation and identification of degradation products □ Hydrolysis—methaqualone, isolation and identification of degradation products, acid and basic media □ Degradation, methaqualone—acid and basic media, isolation and identification of products

In recent years there has been considerable interest in the pharmacology and chemistry of quinazolinone derivatives (1), and their hypnotic and sedative effects are of clinical interest (2–5). Methaqualone, 2-methyl-3-*o*-tolyl-4(3*H*)-quinazolinone, is marketed both as a hypnotic and a sedative under various trade names. A monograph for it has been included in the British Pharmacopoeia (6). Although there have been several reports on the metabolic (7–9) and analytical (10–12) chemistry and bioavailability (13, 14) of methaqualone, little information is available concerning the degradative chemistry of this entity.

The purposes of this study were: *(a)* to investigate the degradative profile of methaqualone following acid and base hydrolysis, *(b)* to isolate and characterize the degradation products, and *(c)* to determine any differences in the formation products. This study was also conducted to find a possible correlation between *in vitro* degradation product(s) and *in vivo* metabolites (15).

EXPERIMENTAL

Apparatus—A dual-column gas chromatograph[1] was used with a 1.8-m × 0.3-cm (6-ft × 0.125-in.) o.d. stainless steel column (Column 1) packed with 10% UC W-98 Diatoport S, 80–100 mesh; with a 1.8-m × 0.3-cm (6-ft × 0.125-in.) o.d. stainless steel column (Column 2) packed with 5% neopentyl glycol succinate on Gas Chrom Z, 100–120 mesh; or with a 1.8-m × 0.3-cm (6-ft × 0.125-in.) o.d. stainless steel column (Column 3) packed with 10% Apiezon L on Diatoport S[2], 60–80 mesh. Flame-ionization detectors were used with nitrogen as a carrier gas (30 ml/min). The areas under the peaks were measured by electronic integrator.

IR spectral studies were carried out on a spectrophotometer[3] using KBr pellets. UV and visible spectra were determined on a recording spectrophotometer[4]. Conditions for TLC are described in Table I.

Procedure—A mixture of 1 g of methaqualone[5] and either 30 ml of 1.0 *N* NaOH, 30 ml of 0.1 *N* NaOH, or 30 ml of 1.2 *N* HCl was boiled separately in three round-bottom flasks for 7 hr. For each mixture, the volatile components were distilled through a horizontally positioned condenser into 20 ml of 0.1 *N* HCl (Solution A); the cooled reaction mixture was basified using 10% NaOH and extracted five times with 10-ml portions of ether, and the ether extracts were combined (Solution B); and the aqueous layer was acidified with 5 ml of 6 *N* HCl (Solution C).

Solutions A from the 1 *N* and 0.1 *N* NaOH reaction mixtures, after evaporation to dryness, each gave a crystalline solid. The solids were examined by UV (aqueous solution), TLC, and GLC using Column 2 (oven temperature 150°, injection temperature 180°, and detector temperature 200°). A reference sample of *o*-toluidine hydrochloride[6] was similarly analyzed.

Solutions B, after evaporation to dryness (under vacuum), gave solids which were analyzed by GLC using Column 1 (oven temperature 205°, injection temperature 230°, and detector temperature 250°) and by TLC. In addition, each solid was diazotized (16) and coupled with *N*-(1-naphthyl)ethylenediamine dihydrochloride[7], and the visible spectrum of the resulting purple solution was obtained. The reference samples of methaqualone and 2-aminobenzo-*o*-toluidide[5] were similarly analyzed.

Solutions C were extracted with 10 ml of chloroform, and each extract was analyzed by GLC using Column 3 (oven temperature 75°, injection temperature 150°, and detector temperature 200°). A reference sample solution of acetic acid in chloroform was similarly chromatographed. The aqueous layer of each Solution C was evaporated to dryness under a stream of air, and the resulting solid residue was dried at 60° for 3 hr. The residue was triturated with five 10-ml portions of ether; the ether extract, on treatment with 10 ml of a saturated hydrochloric acid–ether solution, gave a white precipitate which was subsequently filtered, washed, and dried at 60° for 2 hr. The ether filtrate was evaporated to dryness under a vacuum and the resulting solid was dried at 60° for 2 hr. The residue from the aqueous layer of each Solution C, the precipitate, and the solid recovered from the filtrate were examined by UV (methanol solution), TLC, and IR. The reference samples of anthranilic acid[6] and *N*-acetylanthranilic acid[6] were similarly analyzed.

RESULTS AND DISCUSSION

The isolation, identification, and confirmation of the degradation products of methaqualone following hydrolysis in basic and acidic media were accomplished by the use of the liquid–liquid extraction procedure followed by instrumental analyses. The degradation products of base and acid hydrolysis are presented in Scheme I.

Degradation Products from Boiling 1 *N* NaOH Solution—The condensed component trapped in 0.1 *N* HCl solution (Solution A) yielded 75 mg of solid I. The UV spectrum of I was compa-

[1] Hewlett-Packard model 7620A.
[2] Applied Science Laboratory, State College, Pa.
[3] Perkin-Elmer model 221.
[4] Cary model 14.
[5] William H. Rorer, Fort Washington, Pa.
[6] Eastman Kodak Co., Rochester, N.Y.
[7] Matheson, Coleman and Bell, East Rutherford, N.J.

Table I—TLC of Degradation Products

Degradation Product (Solvent System)[a]	Solution A (R_f)			Solution B (R_f)			Solution C (R_f)		
	1 *N* NaOH	0.1 *N* NaOH	1.2 *N* HCl	1 *N* NaOH	0.1 *N* NaOH	1.2 *N* HCl	1 *N* NaOH	0.1 *N* NaOH	1.2 *N* HCl
Anthranilic acid (A)	—	—	—	—	—	—	0.28	0.28	0.28
o-Toluidine (B)	0.15	0.15	—	—	—	—	—	—	0.15
2-Aminobenzo-*o*-toluidide (C)	—	—	—	0.88	0.88	—	—	—	—
N-Acetylanthranilic acid (D)	—	—	—	—	—	—	—	0.24	—
Unreacted methaqualone (C)	—	—	—	0.93	0.93	0.93	—	—	—

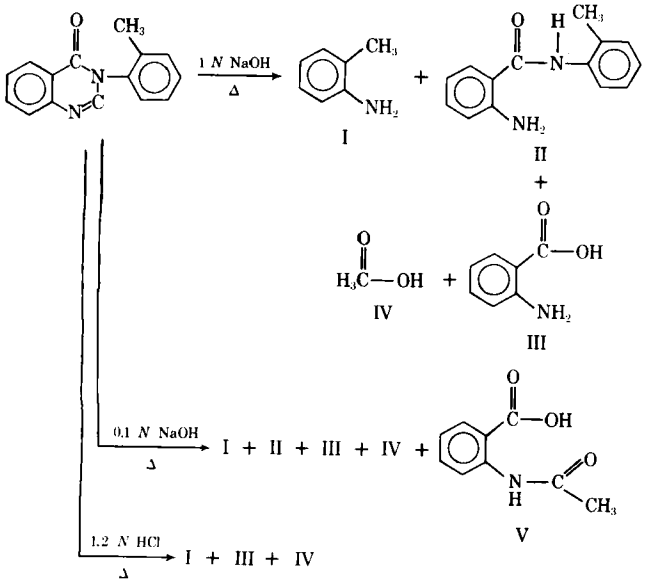
[a] A = ethanol–water (48:52), MN polyamide plate; B = chloroform–cyclohexane–acetic acid (50:40:10), silica gel plate; C = ether plus 2 drops of ammonium hydroxide, MN polyamide plate; and D = ethanol–water–acetic acid (48:52:10), MN polyamide plate. Chromatogram of silica gel G plate (Brinkmann Instruments, Inc., Westbury, N.Y.) was visualized in an iodine chamber. Chromatograms of Polyamide-11 (UV 254) plates (Brinkmann Instruments, Inc., Westbury, N.Y.) were visualized under short UV light.

rable with the reference spectrum of *o*-toluidine hydrochloride, showing three absorption maxima at 229, 266, and 279 nm. In addition, I was identified as *o*-toluidine by GLC (retention time of 3.25 min), TLC (Table I), and IR analysis (2860, 2620, 1600, 1500, 1145, and 752 cm^{-1}).

The ether extract (Solution B), after evaporation to dryness, yielded 772 mg of a solid containing a mixture of two components—80% of unreacted methaqualone and 20% of II; the latter was identified as 2-aminobenzo-*o*-toluidide by GLC with a retention time of 25.72 min (methaqualone = 22.82 min), TLC (Table I), and visible spectroscopy with a maximum absorption at 549 nm.

The aqueous acidified solution (Solution C) contained two components—anthranilic acid hydrochloride (III) and acetic acid (IV). Compound III was obtained after Solution C was extracted with chloroform and evaporated to dryness. The UV spectrum of III had two absorption maxima at 247 and 334 nm. Additional confirmation was obtained from TLC (Table I) and IR analysis (2975, 1690, 1460, 1390, 1215, 1101, 792, 758, and 751 cm^{-1}). Compound IV was extracted into chloroform and identified by GLC using Column 3 (retention time of 2.60 min).

Degradation Products from Boiling 0.1 *N* NaOH Solution—The separation and the identification of the degradation products resulting from the hydrolysis of methaqualone in 0.1 *N* NaOH solution were followed by the stated procedures. When analyzing Solutions A, B, and C, it was found that the yield of the degradation products decreased and a new product *N*-acetylanthranilic acid (V) had been formed. Solution A contained only a few milligrams of *o*-toluidine (I), and Solution B yielded only 6% of 2-aminobenzo-*o*-toluidide (II) and 94% of unreacted methaqualone as determined by GLC. Solution C contained a mixture of three components, which were identified as anthranilic acid hydrochloride (III), acetic acid (IV), and *N*-acetylanthranilic acid (V). Only a few milligrams of *N*-acetylanthranilic acid was found in the filtrate (ether extract) from which the anthranilic acid had been removed. The UV spectrum of V showed two absorption maxima at 252 and 302 nm. Additional confirmation was obtained from TLC (Table I) and IR analysis (3200, 2600, 1692, 1605, 1585, 1510, 1228, 790, and 768 cm^{-1}).



Scheme I

Degradation Products from Boiling 1.2 *N* HCl Solution—The hydrolysis of methaqualone in an acidic medium, in contrast to basic media, showed more extensive bond cleavage. The degradation products were identified as *o*-toluidine (I), anthranilic acid (III), and acetic acid (IV) by the stated methods. No other components, *e.g.*, *N*-acetylanthranilic acid or 2-aminobenzo-*o*-toluidide, which had been found in the basic media were present.

SUMMARY

A profile of the degradation products of methaqualone undergoing hydrolysis in boiling strong acid (1.2 *N* HCl) and strong bases (1.0 and 0.1 *N* NaOH) has been established. Under these stringent conditions, the low yields of the degradation products show that the molecule is relatively stable. None of the degradation products was comparable with the metabolites of methaqualone studied by Preuss *et al.* (17) and Nowak *et al.* (18). However, it was of interest to note that 2-aminobenzo-*o*-toluidide is the amino analog of a metabolite, 2-nitrobenzo-*o*-toluidide, isolated and identified by Murata and Yamamoto (15).

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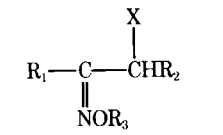
Abstract □ 2-Alkoxyiminoalkylmercaptans were treated with appropriate reagents by established procedures to prepare the corresponding "Bunte salts," 2-(2-alkoxyiminoalkylthio)-2-imidazolines, 2-(2-alkoxyimino)alkyl dithiocarbonates, and ethyl 2-alkoxyiminoalkyl trithiocarbonates. Selected compounds were screened for radioprotective activity, and none was found to have significant activity.

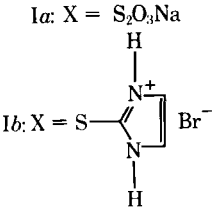
Keyphrases □ 2-Alkoxyiminoalkylmercaptan derivatives—synthesized as potential radioprotective agents □ Radioprotective activity—2-alkoxyiminoalkylmercaptan derivatives synthesized and screened □ Antiradiation agents, potential—synthesis and screening of 2-alkoxyiminoalkylmercaptan derivatives

The grouping of atoms N—C—C—S is apparently a good pharmacophore for antiradiation activity, since cysteamine (2-mercaptoethylamine) derivatives are among the most effective prophylactics against radiation damage. Since 2-alkoxyiminoalkyl bromides can be prepared by reacting *O*-alkyl ethers of aldoximes and ketoximes with *N*-bromosuccinimide (1), it was felt that they could be readily converted to 2-alkoxyiminomercaptan derivatives (I) incorporating the radioprotective pharmacophore. The bromides were reacted with sodium thiosulfate, ethylenethiourea, sodium *O*-ethyl dithiocarbonate, and sodium ethyl trithiocarbonate to yield "Bunte salts" (I*a*), 2-(2-alkoxyiminoalkylthio)-2-imidazolines (I*b*), ethyl *S*-(2-alkoxyimino) alkyl dithiocarbonates (I*c*), and ethyl 2-alkoxyiminoalkyl trithiocarbonates (I*d*), respectively, by established procedures (2–5). The yields and physical data of the compounds prepared are shown in Table I.



I

I*a*: X = S_2O_3Na



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I*c*: X = $SCOSC_2H_5$

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Compounds III, XII, XVI, XX, XXI, XXV, and XXVIII were evaluated for radioprotective activity[1]. The test method was described (6) previously.

EXPERIMENTAL

Bunte Salts—The preparation of sodium *S*-(3-ethoxyimino-2-butyl)thiosulfate illustrates the general procedure.

A solution of 24.8 g (0.1 mole) of sodium thiosulfate pentahydrate in 50 ml of water was mixed with a solution of 19.4 g (0.1 mole) of 3-bromobutanone oxime *O*-ethyl ether in 30 ml of 95% ethanol. The mixture was stirred while heating with steam at reflux for 25 min. The resulting homogeneous solution was then evaporated to dryness under reduced pressure. The residue was extracted with 40 ml of ether, and the solution was cooled in the refrigerator overnight. The white amorphous crystals that formed were collected and dried in air to yield 4.4 g of the title compound. The mother liquor was taken to dryness on the flash evaporator, and the residue was taken up in 95% ethanol and treated with ether. The 7.2 g of product that precipitated melted at 162–164° dec.; total yield, 11.6 g (56.3%). Minor changes in the workup were all that distinguished the preparative methods for the various Bunte salts.

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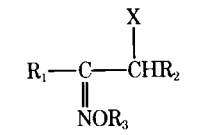

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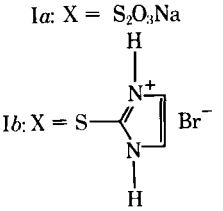

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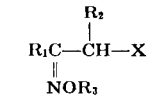
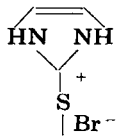
Table I—2-Alkoxyiminomercaptan Derivatives



Compound	R_1	R_2	R_3	X	Boiling Point or Melting Point (with Decomposition)	Yield, %	Composition	Refractive Index
I	CH_3	H	C_2H_5		180–185°	94	$C_5H_{10}NNaO_4S_2$	—
II	CH_3	CH_3	C_2H_5		162–164°	56	$C_6H_{12}NNaO_4S_2$	—
III	H	C_2H_5	C_2H_5		128–130°	70.5	$C_6H_{12}NNaO_4S_2$	—
IV	—$(CH_2)_3$—		C_2H_5		220–222°	71	$C_7H_{12}NNaO_4S_2$	—
V	—$(CH_2)_4$—		C_2H_5		154–157°	44	$C_8H_{14}NNaO_4S_2$	—
VI	CH_3	C_2H_5	C_2H_5	S_2O_3Na	139–140°	54	$C_7H_{16}NNaO_5S_2$[a]	—
VII	C_6H_5	H	C_2H_5		135–136°	25	$C_{10}H_{12}NNaO_4S_2$	—
VIII	CH_3	H	CH_3		164–165°	97	$C_4H_8NNaO_4S_2$	—
IX	C_6H_5	H	CH_3		156–158°	92	$C_9H_{12}NNaO_5S_2$[a,b]	—
X	—$(CH_2)_3$—		CH_3		185–190°	76	$C_6H_{10}NNaO_4S_2$	—
XI	CH_3	CH_3	CH_3		151–153°	53	$C_5H_{12}NNaO_5S_2$[a]	—
XII	CH_3	H	C_2H_5		117–119°[c]	89	$C_8H_{16}BrN_3OS$	—
XIII	C_6H_5	H	C_2H_5		133–135°[c]	57	$C_{13}H_{18}BrN_3OS$	—
XIV	CH_3	H	CH_3		168–170°	90	$C_7H_{14}BrN_3OS$	—
XV	C_6H_5	H	CH_3		175–176°	86	$C_{12}H_{16}BrN_3OS$	—
XVI	C_6H_5	H	C_2H_5	$SCOSC_2H_5$	132–134°/0.1 torr	35	$C_{13}H_{17}NO_2S_2$	n_D^{27} 1.5852
XVII	C_6H_5	H	C_2H_5	$SCS_2C_2H_5$	174–176°/0.1 torr	41	$C_{13}H_{17}NOS_3$[d]	$n_D^{21.5}$ 1.6354
XVIII	C_6H_5	H	CH_3	$SCOSC_2H_5$	128–130°/0.025 torr	56	$C_{12}H_{15}NO_2S_2$[e]	n_D^{22} 1.5998
XIX	C_6H_5	H	CH_3	$SCS_2C_2H_5$	145–147°/0.025 torr	42	$C_{12}H_{15}NOS_3$[f]	$n_D^{21.5}$ 1.6477
XX	CH_3	CH_3	C_2H_5	$SCOSC_2H_5$	78–80°/0.02 torr	68	$C_9H_{17}NO_2S_2$	$n_D^{29.5}$ 1.5232
XXI	CH_3	CH_3	C_2H_5	$SCS_2C_2H_5$	90–92°/0.02 torr	31	$C_9H_{17}NOS_3$	$n_D^{21.5}$ 1.5815
XXII	CH_3	CH_3	CH_3	$SCOSC_2H_5$	75–77°/0.025 torr	54	$C_8H_{15}NO_2S_2$	n_D^{22} 1.5350
XXIII	CH_3	CH_3	CH_3	$SCS_2C_2H_5$	85–87°/0.025 torr	51	$C_8H_{15}NOS_3$[g]	n_D^{22} 1.5934
XXIV	CH_3	H	C_2H_5	$SCOSC_2H_5$	154–156°/26 torr	60	$C_8H_{15}NO_2S_2$[h]	n_D^{27} 1.5294
XXV	CH_3	H	C_2H_5	$SCS_2C_2H_5$	96–98°/0.03 torr	52	$C_8H_{15}NOS_3$	$n_D^{21.5}$ 1.6239
XXVI	CH_3	H	CH_3	$SCOSC_2H_5$	72–74°/0.02 torr	58	$C_7H_{13}NO_2S_2$	n_D^{22} 1.5420
XXVII	CH_3	H	CH_3	$SCS_2C_2H_5$	93–95°/0.02 torr	56	$C_7H_{13}NOS_3$[i]	n_D^{22} 1.6044
XXVIII	—$(CH_2)_3$—		C_2H_5	$SCOSC_2H_5$	100–102°/0.03 torr	59	$C_{10}H_{17}NO_2S_2$	$n_D^{29.5}$ 1.5460

[a] Analyzed as the monohydrate. [b] Calc. for: C, 35.87; N, 4.65. Found: C, 35.22; N, 4.26. [c] Melt without decomposition. [d] Calc. for: C, 52.17. Found: C, 53.09. [e] Calc. for: C, 53.53; S, 23.79. Found: C, 52.88; S, 23.33. [f] Calc. for: S, 33.68. Found: 32.11. [g] Calc. for: N, 3.91. Found: 5.71. [h] Calc. for: S, 28.96. Found: 29.98. [i] Calc. for: S, 43.05. Found: 42.76.

Table II—Analytical Data for 2-Alkoxyiminomercaptan Derivatives

	Analysis							
	Calculated				Found			
Compound	C	H	N	S	C	H	N	S
I	25.53	4.28	5.95	27.26	25.38	4.41	5.87	27.36
II	28.91	4.85	5.62	25.72	28.67	4.74	5.67	25.61
III	28.91	4.85	5.62	25.72	28.97	4.91	5.76	25.48
IV	32.18	4.63	5.36	24.54	32.11	4.83	5.53	24.42
V	34.90	5.13	5.09	23.29	34.84	5.28	5.22	23.19
VI	29.89	5.73	4.98	22.79	30.04	5.81	5.11	22.73
VII	40.40	4.07	4.71	21.57	40.25	4.10	4.65	21.63
VIII	21.72	3.65	6.33	28.99	21.64	3.80	6.25	28.84
IX	35.87	4.01	4.65	21.28	35.22	3.80	4.26	21.05
X	29.15	4.08	5.66	25.93	29.21	4.18	5.78	25.90
XI	23.71	4.78	5.53	25.32	23.49	4.59	5.59	25.44
XII	34.05	5.71	14.89	11.36	34.18	5.64	14.79	11.31
XIII	45.35	5.27	12.21	9.31	45.41	5.39	12.27	9.30
XIV	31.35	5.26	15.67	11.96	31.46	5.51	15.53	11.90
XV	43.64	4.88	12.72	9.71	43.85	5.01	12.70	9.75
XVI	55.12	6.00	4.94	22.61	55.09	6.00	5.08	22.68
XVII	52.17	5.68	4.68	32.10	53.09	5.79	4.59	32.05
XVIII	53.53	5.57	5.20	23.79	52.88	5.66	5.33	23.33
XIX	50.52	5.26	4.87	33.68	50.61	5.34	4.67	32.11
XX	45.91	7.23	5.94	27.23	46.11	7.34	6.09	27.24
XXI	43.20	6.77	5.60	38.40	43.52	6.32	5.56	38.33
XXII	43.42	6.78	6.33	28.96	43.90	6.89	5.98	29.00
XXIII	40.51	6.33	3.91	40.51	40.56	6.49	5.71	40.50
XXIV	43.43	6.79	6.33	28.96	43.49	6.52	6.32	29.98
XXV	40.51	6.32	5.91	40.51	40.54	6.47	5.98	40.35
XXVI	40.58	6.28	6.76	30.91	41.79	6.11	6.61	30.54
XXVII	37.66	5.82	6.28	43.05	37.56	5.71	6.26	42.73
XXVIII	48.58	6.96	5.67	25.91	48.75	6.83	5.93	26.06

was then crystallized from 15 ml of isopropyl alcohol to yield 5 g (89%) of white plates, mp 117–119°.

Ethyl 2-Methoxyiminoalkyl Trithiocarbonates—Into a 100-ml round-bottom flask was placed 4 g (0.025 mole) of sodium ethyl trithiocarbonate in 20 ml of acetone, and 5.7 g (0.025 mole) of the *O*-methyl ether of ω-bromoacetophenone oxime in 10 ml of acetone was added with stirring. A white solid precipitate formed immediately and the solution became warm. The addition of 80 ml of water caused an oil to separate while the original precipitate went into solution. The oil was extracted with 15-ml portions of ether, and the extracts were combined and dried over anhydrous sodium sulfate. The ether was removed by distillation, and the remaining oil was distilled under reduced pressure.

Ethyl *S*-(2-Methoxyimino) Alkyl Dithiocarbonates—Into a round-bottom flask was placed 3.65 g (0.025 mole) of sodium *O*-methyl dithiocarbonate in 20 ml of acetone. To the stirred solution was added 5.7 g (0.025 mole) of the *O*- ethyl ether of ω-bromoacetophenone oxime in 10 ml of acetone. A white solid precipitate formed immediately and the solution became warm. When 80 ml of water was added, an oil separated while the original precipitate went into solution. The oil was then extracted with three 15-ml portions of ether. The extracts were combined and dried over anhydrous sodium sulfate. The ether was removed by distillation, and the remaining oil was distilled under reduced pressure.

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Suppression of Benzoic Acid Adsorption on Sulfamethazine by Polyvinylpyrrolidone: Effect of Contact Time

R. N. NASIPURI and SALEH A. H. KHALIL ˣ

Abstract □ The suppressive effect of polyvinylpyrrolidone on benzoic acid adsorption by sulfamethazine was found to be time dependent. First-order plots showed that benzoic acid apparently diffused from the bulk to the sulfonamide surface through the polymeric "protective" film; therefore, the effect was dependent on the polymer–sulfonamide ratio. An increase in the polymer concentration slowed down the rate of benzoic acid migration. This effect cannot be attributable solely to an increase in bulk viscosity since nonpolymeric viscosity-imparting agents, *e.g.*, glycerol and syrup, did not appreciably inhibit adsorption.

Keyphrases □ Benzoic acid—suppression of adsorption on sulfamethazine by polyvinylpyrrolidone, effect of contact time □ Sulfamethazine—adsorption of benzoic acid, suppression by polyvinylpyrrolidone, effect of contact time □ Polyvinylpyrrolidone—suppression of benzoic acid adsorption on sulfamethazine, effect of contact time □ Adsorption—benzoic acid on sulfamethazine, suppression by polyvinylpyrrolidone, effect of contact time

Protective colloids have been used to inhibit some surface phenomena such as crystal growth and adsorption of solutes onto solid surfaces. Polyvinylpyrrolidone appears to be exceptionally effective for these purposes. For example, the polymer was effective in suppressing the crystal growth of sulfathiazole (1) and inhibiting the interconversion of two forms of sulfaline (sulfamethoxypyrazine) (2). The suppressive effect of polyvinylpyrrolidone on cyanocobalamin adsorption by talc was reported (3). Recently, the authors found that, in the presence of as little as 5 mg % of this polymer, benzoic acid adsorption on sulfamethazine was completely inhibited (4, 5).

Table I—Lag Period (in Hours)[a] at Various Sulfamethazine–Polyvinylpyrrolidone Concentrations

Polyvinylpyrrolidone Concentration, g %	Sulfamethazine Concentration, g %				
	0.1	0.4	1	4	10
0.02	2	1	1	1	1
0.04	4	2	2	2	1
0.10	8	6	4	4	2
0.20	10	10	6	4	4
0.50	20	12	8	6	4
1.00	24	16	12	8	8

[a] Average of four runs (±0.2 hr).

The effect of time on the suppressive effect of protective polymers has received little attention. The present report concerns the effect of contact time on the suppressive role of polyvinylpyrrolidone on benzoic acid adsorption by sulfamethazine.

EXPERIMENTAL

Materials—Sulfamethazine[1], BP grade, was used; the powder had a mean volume–surface diameter of 32.6 μm. Benzoic acid[2] was of BP quality and the polyvinylpyrrolidone[3] sample had an av-

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Table II—Effect of Various Sulfamethazine and Polyvinylpyrrolidone Concentrations on Rate Constant K of Benzoic Acid Adsorption

Sulfamethazine Concentration, g %[a]	Polymer–Sulfonamide Ratio[a]	$K \times 10^2$, hr^{-1}	Polyvinylpyrrolidone Concentration, g %[b]	Polymer–Sulfonamide Ratio[b]	$K \times 10^2$, hr^{-1}
0.1	1.40×10^{-3}	2.20	0.02	0.14×10^{-4}	9.79
0.4	0.34×10^{-3}	5.46	0.04	0.28×10^{-4}	8.66
1.0	0.14×10^{-3}	6.45	0.10	0.70×10^{-4}	5.60
4.0	0.34×10^{-4}	8.36	0.20	0.14×10^{-3}	3.80
10.0	0.14×10^{-4}	9.79	0.50	0.35×10^{-3}	3.65
—	—	—	1.00	0.70×10^{-3}	3.20
—	—	—	2.00	1.40×10^{-3}	0.75

[a] At a constant polyvinylpyrrolidone concentration of 0.02 g %; the average molecular weight of the polymer was taken as 40,000. [b] At a constant sulfamethazine concentration of 10 g %.

erage molecular weight of 40,000. Glycerol[2] and tragacanth[2] were of BP grade and the syrup was prepared according to the BP.

Methods—The adsorption experiments and the determination of benzoic acid were carried out as previously reported (4). Equilibrium dialysis experiments[4] were performed at 24 ± 0.2° following the procedure described previously (4).

The effect of contact time on the adsorption of benzoic acid in the sulfamethazine–polyvinylpyrrolidone system was studied by equilibrating the system at 24 ± 0.2° in an oscillating water bath. Samples were withdrawn at various intervals and filtered through a sintered-glass funnel[5], and the benzoic acid that remained unadsorbed was determined.

RESULTS AND DISCUSSION

First-order plots (Figs. 1 and 2) show a gradual decrease in the percentage of unadsorbed benzoic acid in systems containing varying concentrations of sulfamethazine and polyvinylpyrrolidone. This decrease suggests that benzoic acid was gradually adsorbed by the sulfonamide (4, 5) or possibly complexed with the polymer. In all systems studied, the percentage of "free" benzoic acid was unchanged in the 1st hr. This "lag period" varied from 1 to 24 hr, depending on the composition of the system (Table I).

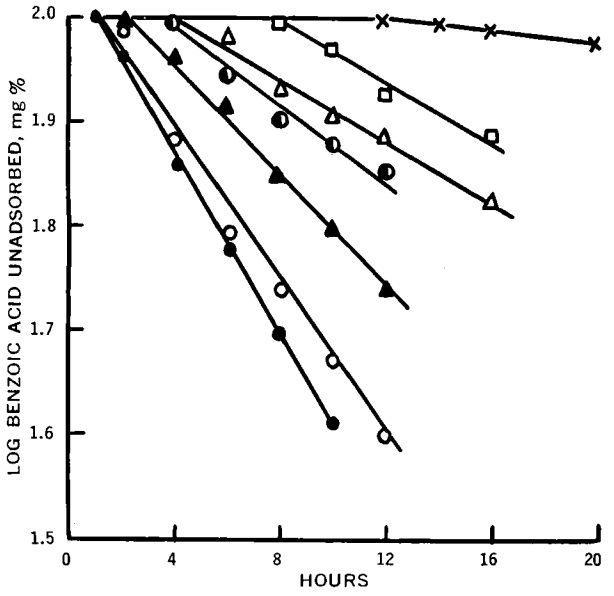

Figure 1—*Effect of time on the suppressive effect of varying concentrations of polyvinylpyrrolidone on benzoic acid adsorption by sulfamethazine. The concentrations of polyvinylpyrrolidone (g %) were: ●, 0.02; ○, 0.04; ▲, 0.10; ◐, 0.20; △, 0.5; □, 1.0; and ×, 2.0. The concentration of sulfamethazine in the system was 10 g %.*

[4] Visking tubing, The Scientific Instrument Centre Ltd., London, England.
[5] Jena 39 G.3.

At a constant sulfamethazine concentration (10% w/w), an increase in the polyvinypyrrolidone concentration resulted in an increase in the protective effect, as evidenced from the changes in the slopes of the linear plots of Fig. 1 and the values of the rate constant, K (Table II). The lag period was also extended from 1 to 24 hr for systems containing 0.02 and 2 g % polyvinylpyrrolidone, respectively. Increasing the sulfonamide content at a constant polymer concentration (0.02 g %) produced a relatively minor reduction of the lag period from 2 to 1 hr, but the slopes of the linear plots (Fig. 2) and the computed K values (Table II) suggest a decrease in the protective effect. The latter seemed to be affected by the molecular ratio of polyvinylpyrrolidone to sulfamethazine. Generally, a decrease in this ratio resulted in an increase in the values of the rate constant; as the ratio increased, the values of K decreased (Table II).

Since the majority of protective colloids are viscosity-imparting agents, their protective effect on the adsorption process may be attributed to: *(a)* the formation of a protective film at the adsorbent surface, *(b)* decreased diffusion of the adsorbate due to an increase in the bulk viscosity and environmental viscosity around the particle, and/or *(c)* possible interaction between the polymer and the adsorbate.

Polyvinylpyrrolidone has been reported to exert its protective effect by being preferentially adsorbed at the solid surface (1, 3).

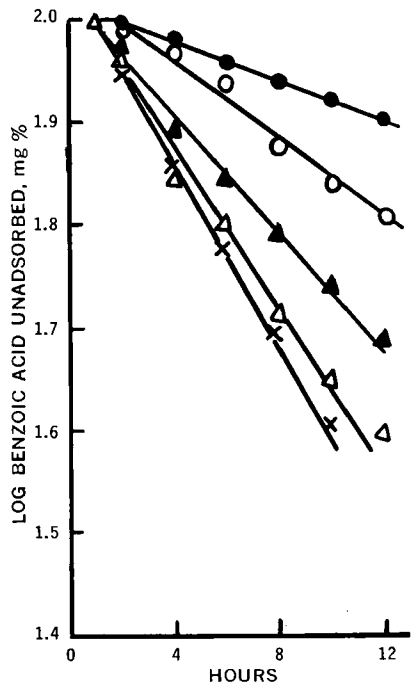

Figure 2—*Effect of time on the suppressive effect of polyvinylpyrrolidone on benzoic acid adsorption by sulfamethazine. The concentrations of sulfamethazine (g %) were: ●, 0.1; ○, 0.4; ▲, 1.0; △, 4.0; and ×, 10. The concentration of polyvinylpyrrolidone in the system was 0.02 g %.*

Table III—Comparative Suppressive Effects of Polyvinylpyrrolidone, Glycerol, Syrup BP, and Compound Tragacanth Powder BP on Benzoic Acid Adsorption by Sulfamethazine[a] (Contact Time of 1 hr)

	Polyvinylpyrrolidone		Glycerol		Syrup		Compound Tragacanth Powder	
Concentration, g %	0.02	2.0	10	50	10	50	2	4
Relative viscosity, η_{rel}[b]	1.03	1.27	1.49	4.65	1.42	3.19	4.01	12.21
Benzoic acid unadsorbed, mg %	99.9	100	7.6	11.9	6.9	8.9	27.2	45.9

[a] Initial benzoic acid concentration of 0.1 g %; initial sulfamethazine concentration of 4 g %. [b] Using an Ostwald viscometer at 24 ± 0.2°.

However, its effect on increasing the viscosity cannot be ruled out. To test this point, the effect of two nonpolymeric viscosity-imparting agents (glycerol and syrup BP) on benzoic acid adsorption by sulfamethazine was examined. The data obtained (Table III) were compared with the effect produced by two polymeric materials (compound tragacanth powder BP and polyvinylpyrrolidone). Although the relative viscosities of both glycerol and syrup (each at a concentration of 10 and 50% w/w) are higher than that of the highest polyvinylpyrrolidone concentration used, the protective effect of the former agents was negligible. The percentages of benzoic acid unadsorbed were about 12 and 9 (mg %) in systems containing 50 % (w/w) of glycerol and syrup, respectively. In the presence of polyvinylpyrrolidone, not more than 0.1 mg % benzoic acid was adsorbed in systems containing 0.02 g % of the polymer. Compound tragacanth powder had an intermediate protective effect (Table III). It can then be concluded that the suppressive role of polyvinylpyrrolidone cannot be attributed to an increase in bulk viscosity.

Dialysis experiments designed to test the possible interaction between benzoic acid and polyvinylpyrrolidone revealed that, in accordance with the data of Higuchi and Kuramoto (6), a negligible interaction occurred at a polymer concentration below 0.1 g %. When the polymer concentration was increased, a gradual increase in the interaction between benzoic acid (0.1 g %) and polyvinylpyrrolidone occurred, but this amounted to about 5% at a polymer concentration of 1%. This cannot solely account for the observed suppression in the adsorption.

In conclusion, the suppressive effect of protective polymers, *e.g.*, polyvinylpyrrolidone, on adsorption should be assessed taking into consideration the effect of time. In some instances, an increase in contact time may result in a reduction of the protective action of the polymer due to the transfer of the adsorbate from the bulk to the adsorbent surface.

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[x] To whom inquiries should be directed. Present address: Department of Pharmacy and Pharmacology, Faculty of Medicine and Pharmacy, University of Benin, Benin City, Nigeria.

Stability of Aspirin in Liquid and Semisolid Bases IV: Polyethylene Glycol 400 Diacetate and Triethylene Glycol Diacetate

C. W. WHITWORTH[x] and A. F. ASKER

Abstract □ The stability of aspirin in polyethylene glycol 400 diacetate and triethylene glycol diacetate was studied at 4, 26, and 45°. Degradation of aspirin in these vehicles was temperature dependent and appeared to follow a first-order rate. The results supported a previous conclusion of enhanced stability of aspirin in the substituted derivatives of polyethylene glycols.

Keyphrases □ Aspirin—stability in liquid and semisolid bases, polyethylene glycol 400 diacetate and triethylene glycol diacetate □ Polyethylene glycol 400 diacetate—stability of aspirin at three temperatures □ Triethylene glycol diacetate—stability of aspirin at three temperatures □ Stability—aspirin in polyethylene glycol 400 diacetate and triethylene glycol diacetate

A previous paper (1) on the decomposition of aspirin in polyethylene glycols provided data showing that degradation was due, at least in part, to a transesterification reaction between aspirin and polyethylene glycols. Blocking free hydroxy groups on the polyethylene glycols retarded the decomposition of aspirin (2). The purpose of this work was to study the degradation of aspirin in commercial substituted derivatives of polyethylene glycol, polyethylene glycol 400 diacetate and triethylene glycol diacetate.

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[x] To whom inquiries should be directed. Present address: Department of Pharmacy and Pharmacology, Faculty of Medicine and Pharmacy, University of Benin, Benin City, Nigeria.

Stability of Aspirin in Liquid and Semisolid Bases IV: Polyethylene Glycol 400 Diacetate and Triethylene Glycol Diacetate

C. W. WHITWORTH[x] and A. F. ASKER

Abstract □ The stability of aspirin in polyethylene glycol 400 diacetate and triethylene glycol diacetate was studied at 4, 26, and 45°. Degradation of aspirin in these vehicles was temperature dependent and appeared to follow a first-order rate. The results supported a previous conclusion of enhanced stability of aspirin in the substituted derivatives of polyethylene glycols.

Keyphrases □ Aspirin—stability in liquid and semisolid bases, polyethylene glycol 400 diacetate and triethylene glycol diacetate □ Polyethylene glycol 400 diacetate—stability of aspirin at three temperatures □ Triethylene glycol diacetate—stability of aspirin at three temperatures □ Stability—aspirin in polyethylene glycol 400 diacetate and triethylene glycol diacetate

A previous paper (1) on the decomposition of aspirin in polyethylene glycols provided data showing that degradation was due, at least in part, to a transesterification reaction between aspirin and polyethylene glycols. Blocking free hydroxy groups on the polyethylene glycols retarded the decomposition of aspirin (2). The purpose of this work was to study the degradation of aspirin in commercial substituted derivatives of polyethylene glycol, polyethylene glycol 400 diacetate and triethylene glycol diacetate.

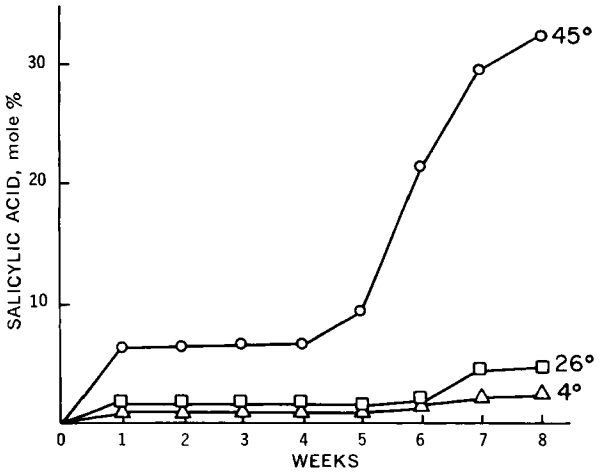

Figure 1—*Rate of appearance of salicylic acid* versus *time for a 5% solution of aspirin in polyethylene glycol 400 diacetate.*

EXPERIMENTAL

Materials—Aspirin USP[1] and salicylic acid[1] were used as received. Polyethylene glycol 400 diacetate[2] and triethylene glycol diacetate[2] were supplied by the manufacturer. Chloroform[3] was spectrograde.

Sample Preparation—The samples were prepared by dissolving the aspirin in either polyethylene glycol diacetate or triethylene glycol diacetate at room temperature to give solutions of 5 and 10% (w/v) of aspirin. Five milliliters of each solution was placed in airtight amber glass vials with screw caps and the solutions were stored at 4, 26, and 45°.

Analytical Method—UV spectrophotometric analysis, as reported by Tinker and McBay (3), was employed to measure aspirin and salicylic acid. Samples were taken at specific intervals and dissolved in 100 ml of chloroform containing 1% acetic acid. A further appropriate dilution was made, and the absorbance of the dilution was read[4] at 280 nm for aspirin and 310 nm for salicylic acid. At these wavelengths, standard curves were prepared for aspirin and salicylic acid. The quantification of aspirin and salicylic acid was based on the standard method of simultaneous spectrophotometric determinations.

RESULTS AND DISCUSSION

Aspirin undergoes degradation in polyethylene glycol at least in part *via* a transesterification reaction (1). The use of substituted derivatives of polyethylene glycols such as the acetylated glycol and the methoxyglycol was found to enhance the stability of aspirin (2).

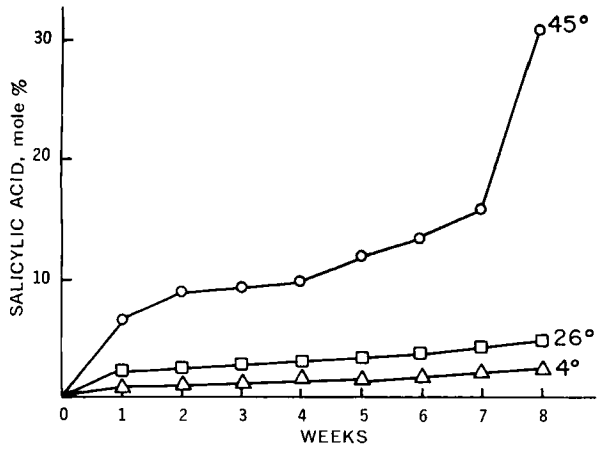

Figure 2—*Rate of appearance of salicylic acid* versus *time for a 10% solution of aspirin in polyethylene glycol 400 diacetate.*

[1] Merck & Co., Rahway, N.J.
[2] Union Carbide Corp., New York, N.Y.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Cary model 118 spectrophotometer.

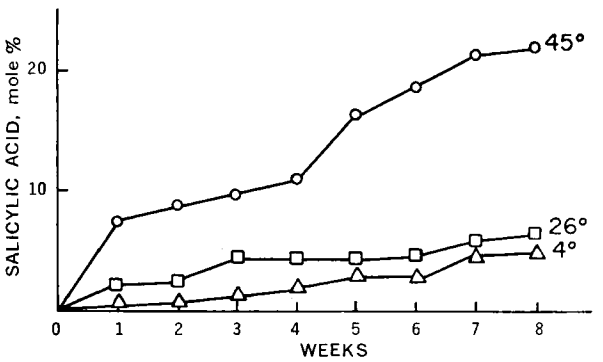

Figure 3—*Rate of appearance of salicylic acid* versus *time for a 5% solution of aspirin in triethylene glycol diacetate.*

Figures 1–4 show the overall profiles of aspirin degradation in the two vehicles studied at three temperatures as indicated by the rate of appearance of salicylic acid. It is evident from the figures that the decomposition of aspirin was temperature dependent.

Table I shows the amount of aspirin remaining in the two vehicles after storage for 8 weeks at three temperatures. A previous publication (4) reported that the decomposition of aspirin in polyethylene glycol 400 was such that the amounts of aspirin remaining after storage at 4 and 24° for 8 weeks were 98.1 and 92.4%, respectively. However, at 45°, the amount of aspirin remaining after 39 days was 69.4%. In the present study, the average amounts of aspirin remaining after 42 days of storage at 45° were 82.67% in polyethylene glycol 400 diacetate and 78.21% in triethylene glycol diacetate. The results indicate that the substituted derivatives of polyethylene glycols have a stabilizing effect on aspirin, thus supporting data published previously (2, 4, 5).

Figures 5 and 6 show apparent first-order degradation of aspirin at 45° in polyethylene glycol 400 diacetate and triethylene glycol diacetate, respectively. Almost identical degradation patterns and essentially the same slope were obtained regardless of the initial concentration of aspirin used, thus verifying first-order dependence of the reaction with respect to aspirin. The reaction velocity constants for the degradation of aspirin in polyethylene glycol 400 diacetate and triethylene glycol diacetate were 2.95/day and 85.27 $\times 10^{-2}$/day, respectively. The velocity constant for the degradation of aspirin in polyethylene glycol 400 diacetate was based on the data taken from the 5th to the 8th week of storage since the rate of degradation during the initial period of storage was relatively slow.

The method of least squares was used to obtain the curves of Figs. 5 and 6. Data for the decomposition of aspirin at 4 and 26° were not treated graphically in a similar fashion since the samples stored at these temperatures were satisfactory (degradation was

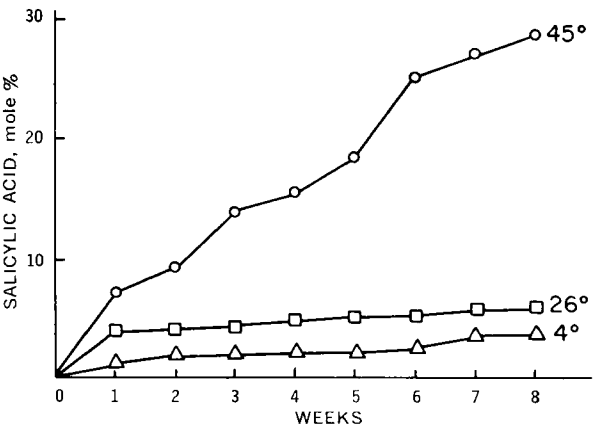

Figure 4—*Rate of appearance of salicylic acid* versus *time for a 10% solution of aspirin in triethylene glycol diacetate.*

Table I—Percent of Aspirin Remaining after 8 Weeks of Storage at Three Temperatures

Temperature	Polyethylene Glycol 400 Diacetate		Triethylene Glycol Diacetate	
	5%	10%	5%	10%
4°	98.65	97.80	95.38	96.50
26°	95.49	95.44	93.81	94.69
45°	67.77	69.00	77.19	72.06

less than 5% in the case of polyethylene glycol 400 diacetate and less that 7% in the case of triethylene glycol diacetate after storage for 8 weeks).

Tsakalotos and Horsch (6, 7) found that acetic acid increased the rate of decomposition of aspirin. Kelly (8) also indicated that hydrolysis of aspirin is catalyzed by the acetate ion, a considerably more powerful nucleophile than water. Therefore, it may be speculated that the higher rate of aspirin decomposition in polyethylene glycol 400 diacetate during the last 5–8 weeks might be due to the hydrolysis of this vehicle. This hydrolysis would result in the formation of the more polar polyethylene glycol 400 and acetic acid.

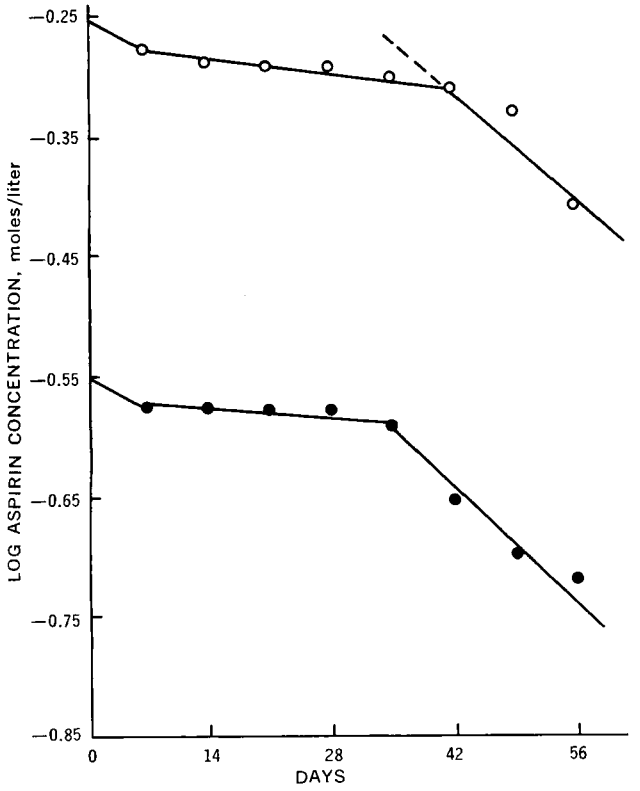

Figure 5—*First-order plot for the degradation of aspirin at 45° in polyethylene glycol 400 diacetate. Key: ○, 10% solution; and ●, 5% solution.*

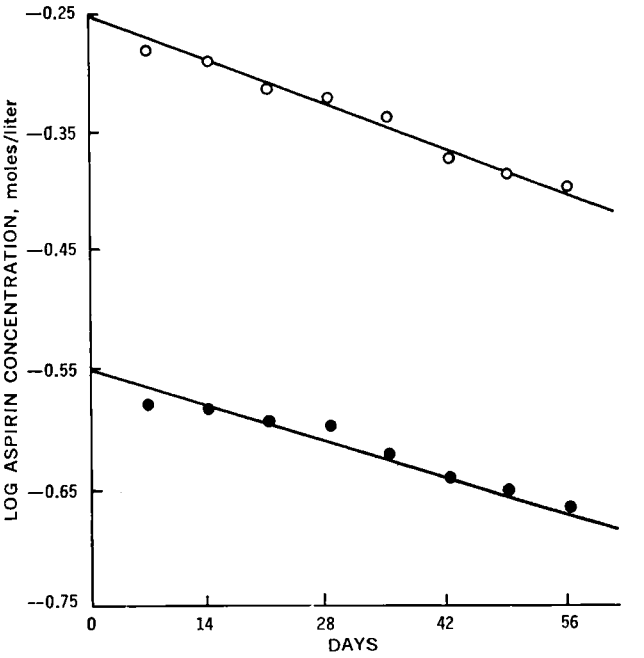

Figure 6—*First-order plot for the degradation of aspirin at 45° in triethylene glycol diacetate. Key: ○, 10% solution; and ●, 5% solution.*

These hydrolytic products are expected to enhance the decomposition of aspirin, especially in the presence of traces of moisture. This moisture could have contaminated the dry aspirin during preparation of the samples and then gradually diffused throughout the relatively viscous vehicle during storage at 45°.

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[x]To whom inquiries should be directed.

Quantitative Fluorometric TLC Procedure for Determination of Hydroflumethiazide in Biological Fluids

Y. GARCEAU, I. DAVIS, and J. HASEGAWA ˣ

Abstract □ A rapid, sensitive, and specific fluorometric TLC procedure for the estimation of hydroflumethiazide in human biological fluids is described. The drug was removed from plasma in a single extraction step and applied to a TLC plate of silica gel. After development, the plate was scanned in a spectrodensitometer equipped to measure fluorescence below 400 nm. Quantitation was achieved by comparing the areas under the peaks obtained from the unknowns to those obtained from standards applied to the same plate. The limit of detection was 10 ng of drug/ml of plasma. A mean recovery value of 99.7% was obtained when the drug was extracted from spiked human plasma samples, while 97.5% of drug was recovered from spiked human urine samples. To test the validity of the method, a beagle dog was given an intravenous dose of hydroflumethiazide, and postadministration blood and urine samples were collected and analyzed.

Keyphrases □ Hydroflumethiazide—fluorometric TLC analysis in plasma and urine □ TLC, spectrofluorodensitometry—analysis, hydroflumethiazide in plasma and urine □ Spectrofluorodensitometry—analysis, hydroflumethiazide

Although the benzothiadiazine diuretics have been in use for more than 10 years, the scientific literature contains little information on specific assay methods for the determination of these drugs in biological fluids. Procedures have been described for the determination of chlorothiazide (1), hydrochlorothiazide (2), and bendroflumethiazide (3), but they relied on nonspecific colorimetric reactions, involving diazotization and coupling to form an azo dye, and they lacked sensitivity. Qualitative procedures using UV spectrophotometry, paper chromatography, and TLC have also been reported (4, 5).

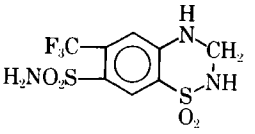
Hydroflumethiazide (I) is a potent diuretic, about 10 times as effective as chlorothiazide on a weight basis. A literature survey failed to reveal any published report on a specific assay method for hydroflumethiazide in biological fluids. This paper describes a quantitative fluorometric TLC procedure for the estimation of this drug in human plasma and urine.

EXPERIMENTAL

Materials and Chemicals—TLC plates (20 × 20 cm) of silica gel[1] were used. No activation was required. The plates were divided into 20 channels of 1-cm width with a scoring device[2]. Samples were applied to the plates with 10-, 25-, or 50-μl syringes[3].

Stock solutions of hydroflumethiazide[4] were prepared by dissolving accurately weighed amounts of drug in methanol. Working standards were made by appropriate dilutions of the stock standards. All solvents and chemicals were analytical reagent grade.

Procedure for Assay of Plasma and Urine Samples—One-milliliter samples of human plasma were spiked with aliquots of 50



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μl of standard solutions of hydroflumethiazide. The amounts of drug added were chosen to cover the concentration range of 0.05–1.00 μg/ml of plasma. To each plasma sample, 0.5 g of sodium chloride was added and the mixture was extracted for 15 min on a mechanical shaker[5] with 15 ml of a solution of ether–ethylene dichloride (1:1).

After centrifugation, the organic layer was transferred and dried for 5 min with 1.5 g of sodium sulfate. A 10-ml aliquot from the organic layer was transferred to a clean conical tube and evaporated to dryness[6] under nitrogen at 40°. The inside of the tube was washed with 1 ml of the extracting solvent mixture, and the solution was again evaporated to dryness.

The dry residue was dissolved in 100 μl of chloroform–acetone (7:3 v/v). Aliquots from this solution, 25–50 μl, were applied to prescored TLC plates, generally in duplicate. Standards of hydroflumethiazide of three different concentrations were also spotted so that a calibration line was included on each plate.

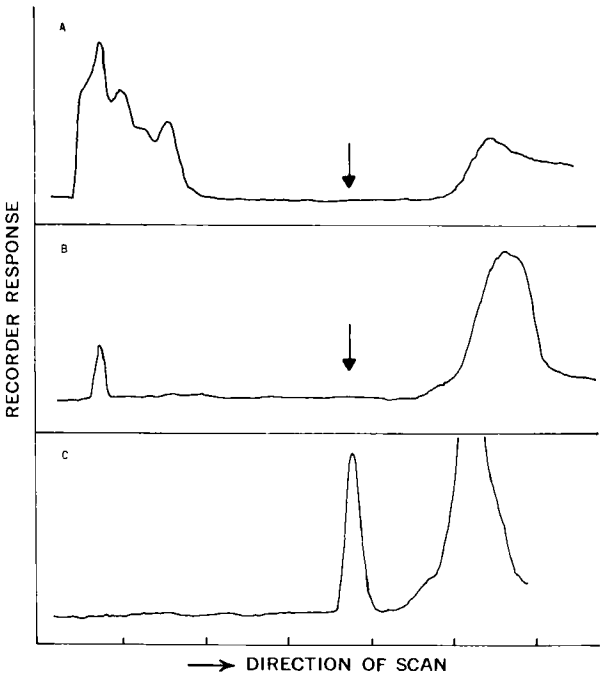

Figure 1—*Typical chromatograms. Key: A, blank human urine; B, extract of blank human plasma (R_f value of hydroflumethiazide is shown by arrow); and C, extract of a human plasma sample spiked with hydroflumethiazide at a concentration of 0.2 μg/ml. Additional peaks represent urine or plasma components.*

[1] Sil G-25-22, Brinkmann Instruments Inc.
[2] SDA 320, Schoeffel Instrument Corp.
[3] Hamilton Co.
[4] Linson Ltd., Dublin, Ireland.
[5] Eberbach, Ann Arbor, Mich.
[6] N-Evap, Organomation Assoc.

Table I—Summary of Recovery Experiments

Amounts Spiked, μg/ml	Amounts Recovered, μg/ml		
	Run 1	Run 2	Run 3
	Plasma		
0.05	0.048	0.048	0.048
0.10	0.093	0.096	0.096
0.20	0.204	0.197	0.204
0.40	0.401	0.428	0.390
0.60	0.612	0.636	0.606
0.80	0.762	0.756	0.780
1.00	1.044	1.056	1.068
Mean recovery: 99.7% *SD*: 4.4%			
	Urine		
5.0	4.7	5.0	5.0
10.0	9.5	9.5	9.5
20.0	20.0	20.5	20.0
30.0	28.0	29.0	29.5
40.0	38.5	39.0	39.5
Mean recovery: 97.5% *SD*: 2.7%			

Urine samples were analyzed directly without any extraction step. Spiked human urine samples were prepared in the concentration range of 5–40 μg/ml. Unspiked samples served as blanks. Duplicate aliquots of 10 μl were spotted directly on a TLC plate. Standards of at least three different concentrations were also spotted.

TLC—The plates were developed in a saturated tank containing 100 ml of ethyl acetate–methanol–0.1 *N* ammonium hydroxide (96:2:2). After developing 15 cm, the plates were air dried. They were then scanned in a spectrodensitometer[7] equipped with a density computer[8]. The light source was a xenon mercury 200-w lamp[9].

The instrument was operated in the fluorescence mode. The excitation wavelength was 265 nm. Emission was filtered with a UV-transmitting, visible-absorbing filter[10] having a transmission band

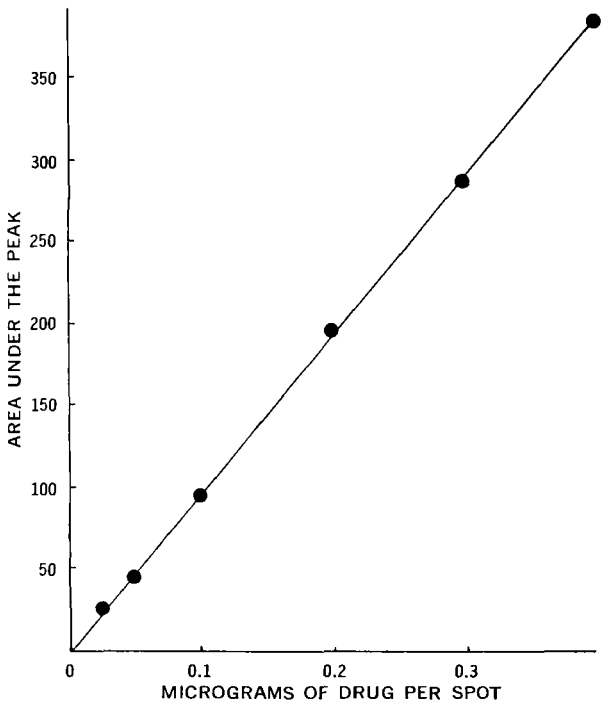

Figure 2—*Typical calibration line for hydroflumethiazide.*

[7] Model SD 3000, Schoeffel Instrument Corp.
[8] Model SDC 300, Schoeffel Instrument Corp.
[9] Hanovia Lamp Division, Camrand Precision Ind.
[10] Corning, C.S. 7.51.

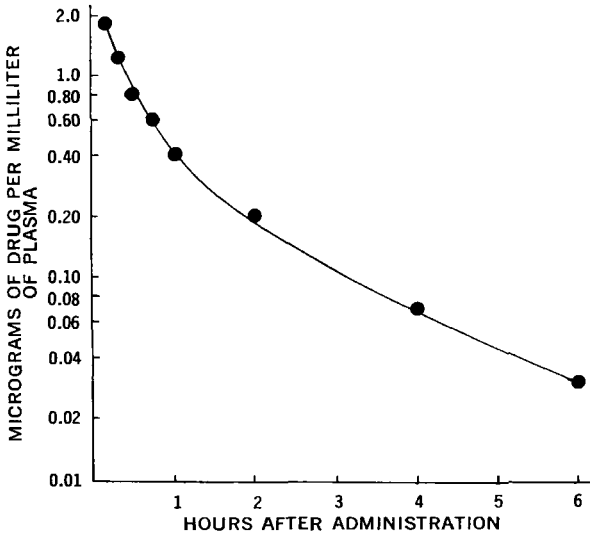

Figure 3—*Plasma levels of hydroflumethiazide following intravenous administration of a 15-mg dose to a beagle dog.*

between 290 and 420 nm, with maximum transmission at 365 nm. Gain was set at 700 on the arbitrary scale of the instrument, and the density computer was set at O.D. 0.2. Scanning and chart speeds were 10.2 cm (4 in.)/min.

***In Vivo* Experiment**—To assess the applicability of the method, a beagle dog was given 15 mg of hydroflumethiazide intravenously (3 ml of a 5-mg/ml solution in 0.02 *N* NaOH). Heparinized blood samples were collected prior to and at 10, 20, 30, and 45 min and at 1, 2, 4, and 6 hr after injection. The blood samples were centrifuged immediately, and the plasma was frozen until analyzed. Urine was collected for 24 hr after administration.

RESULTS AND DISCUSSION

Hydroflumethiazide fluoresces strongly, and its emission peak is below 400 nm on a thin layer of silica gel. Since the spectrodensitometer used was not originally equipped for fluorescence analysis below 400 nm, it was adapted to that purpose by using a UV filter transmitting between 290 and 420 nm. This filter accomplished the dual purpose of filtering off the excitation energy and passing the fluorescence emission. It also filtered off visible fluorescence, thus increasing specificity.

Under the experimental conditions described, hydroflumethiazide had an R_f value of 0.52 and was separated from a known degradation product, 2,4-disulfamyl-5-trifluoromethylaniline, R_f 0.64. Hydroflumethiazide is not known to be metabolized in humans. It was well separated from plasma or urine components (Fig. 1) and from the common diuretics chlorothiazide, R_f 0.25, and hydrochlorothiazide, R_f 0.44. The latter compounds do not fluoresce but can be detected on a TLC plate of silica gel from their UV absorption at 290 nm. The lowest detectable amount of these substances is around 50 ng.

A typical calibration line is shown in Fig. 2. The correlation coefficient was 0.999.

Recovery experiments are summarized in Table I. Recoveries from spiked human plasma samples ranged from 93 to 107%, and the mean was 99.7 ± 4.4% *SD*, n = 21. Recoveries from spiked human urine samples ranged from 94 to 103%, and the mean was 97.5 ± 2.7% *SD*, n = 15.

Plasma samples spiked with hydroflumethiazide were allowed to stand at room temperature for up to 24 hr before extraction to check for degradation and possible recovery loss. Recovery was constant for the period of time tested.

Under the conditions described, the instrument can reliably integrate an "area under the peak" of 10 units, which sets the limit of detection to 5 ng of hydroflumethiazide/spot. This sensitivity corresponds to 10 ng of drug/ml of plasma or 0.5 μg/ml of urine. Urine samples were applied directly to the TLC plates and much greater sensitivity could be obtained by including the extraction step used for plasma. However, this was considered unnecessary. The method is adequate to quantitate hydroflumethiazide in plas-

ma or urine following administration of a single 50-mg oral dose to humans[11].

An *in vivo* study was conducted to confirm the applicability of the method. A plot of plasma levels of hydroflumethiazide following a single intravenous injection to a beagle dog is shown in Fig. 3. The curve shows a typical biphasic pattern, with a half-life of 10 min for the α-phase and of 90 min for the β-phase. The amount of drug excreted in a 24-hr urine collection was 8.6 mg or 57% of the dose. The reason for incomplete excretion is not known at present.

The analytical method was developed using human plasma and urine while the pilot experiment was conducted in dogs. However, dog plasma and urine were free of interfering impurities, as were the human fluids.

The procedure described in this paper is accurate and reproducible and has a high degree of sensitivity. It is quick and easy to carry out. One analyst can assay 20–25 plasma samples in a normal working day.

[11] Unpublished data.

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[x] To whom inquiries should be directed.

Diphenylhydantoin Microdetermination in Serum and Plasma by UV Spectrophotometry

JACK E. WALLACE [x] and HORACE E. HAMILTON

Abstract □ A modified UV spectrophotometric procedure for determining diphenylhydantoin in 0.1–0.2 ml of serum is described. Improvement of previously described techniques was accomplished by modifying the extraction conditions and refluxing assembly. Continuous extraction of the oxidized diphenylhydantoin provided the maximum yield of benzophenone, resulting in optimal sensitivity as well as reproducibility of analytical values. Reliable clinical assays are achieved on 0.1 ml of serum containing 0.5 μg of diphenylhydantoin. Recovery of the drug from biological fluids is approximately 94%.

Keyphrases □ Diphenylhydantion—microdetermination in serum and plasma, UV spectrophotometry □ Microdetermination—diphenylhydantoin in serum and plasma, UV spectrophotometry □ UV spectrophotometry—microdetermination of diphenylhydantoin in plasma and serum

The requirement for plasma levels of diphenylhydantoin in the clinical management of epileptic patients has been well documented (1–4). Earlier colorimetric and spectrophotometric methods for determining this common anticonvulsant were plagued by interference from other drugs, necessitating extensive separation techniques (5–7). Wallace *et al.* (8) developed the first sensitive and specific spectrophotometric determination for diphenylhydantoin not requiring preliminary separation from other drugs, a technique based upon the oxidation of the parent drug to benzophenone. Numerous modifications of that method have been published (4, 9–14), with a primary objective of determining the drug in as little as 0.4–2.0 ml of specimen (4, 12–14).

GLC techniques are available both for the determination of unchanged diphenylhydantoin (15–18) and derivatized diphenylhydantoin (19–25). GLC techniques are often complicated and usually demand a time interval exceeding that required for a spectrophotometric scan. Additionally, the lability of the hydantoins makes them highly susceptible to degradation at the injection temperature conditions of the chromatograph.

The present report describes two modifications of the spectrophotometric analysis: one utilizing conventional condensers and requiring 1 ml of plasma and the other utilizing a modified reflux condenser[1] and requiring only 0.1–0.2 ml plasma. The methods, in addition to providing a greater sensitivity than that achieved by available methods, eliminate the time-consuming evaporation of chloroform required in the Lee (4) and Saitoh (13) methods. Additionally, a far greater product stability than exists in the "bomb oxidation techniques" is obtained.

EXPERIMENTAL

Apparatus—Reflux condensers were mounted on a support[2]. The semimicrodetermination utilizes a conventional condenser[3]; the microdetermination utilizes a previously described "external cold finger" reflux condenser[1]. Heating mantles[4], 270 w, 500-ml ca-

[1] Unpublished data.
[2] Flexaframe.
[3] Allihn.
[4] Glas-Col.

ma or urine following administration of a single 50-mg oral dose to humans[11].

An *in vivo* study was conducted to confirm the applicability of the method. A plot of plasma levels of hydroflumethiazide following a single intravenous injection to a beagle dog is shown in Fig. 3. The curve shows a typical biphasic pattern, with a half-life of 10 min for the α-phase and of 90 min for the β-phase. The amount of drug excreted in a 24-hr urine collection was 8.6 mg or 57% of the dose. The reason for incomplete excretion is not known at present.

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Keyphrases □ Diphenylhydantion—microdetermination in serum and plasma, UV spectrophotometry □ Microdetermination—diphenylhydantoin in serum and plasma, UV spectrophotometry □ UV spectrophotometry—microdetermination of diphenylhydantoin in plasma and serum

The requirement for plasma levels of diphenylhydantoin in the clinical management of epileptic patients has been well documented (1–4). Earlier colorimetric and spectrophotometric methods for determining this common anticonvulsant were plagued by interference from other drugs, necessitating extensive separation techniques (5–7). Wallace *et al.* (8) developed the first sensitive and specific spectrophotometric determination for diphenylhydantoin not requiring preliminary separation from other drugs, a technique based upon the oxidation of the parent drug to benzophenone. Numerous modifications of that method have been published (4, 9–14), with a primary objective of determining the drug in as little as 0.4–2.0 ml of specimen (4, 12–14).

GLC techniques are available both for the determination of unchanged diphenylhydantoin (15–18) and derivatized diphenylhydantoin (19–25). GLC techniques are often complicated and usually demand a time interval exceeding that required for a spectrophotometric scan. Additionally, the lability of the hydantoins makes them highly susceptible to degradation at the injection temperature conditions of the chromatograph.

The present report describes two modifications of the spectrophotometric analysis: one utilizing conventional condensers and requiring 1 ml of plasma and the other utilizing a modified reflux condenser[1] and requiring only 0.1–0.2 ml plasma. The methods, in addition to providing a greater sensitivity than that achieved by available methods, eliminate the time-consuming evaporation of chloroform required in the Lee (4) and Saitoh (13) methods. Additionally, a far greater product stability than exists in the "bomb oxidation techniques" is obtained.

EXPERIMENTAL

Apparatus—Reflux condensers were mounted on a support[2]. The semimicrodetermination utilizes a conventional condenser[3]; the microdetermination utilizes a previously described "external cold finger" reflux condenser[1]. Heating mantles[4], 270 w, 500-ml ca-

[1] Unpublished data.
[2] Flexaframe.
[3] Allihn.
[4] Glas-Col.

Table I—Diphenylhydantoin Determination by Semimicroprocedure

Concentration[a], μg/ml	Absorbance[b,c]	Absorbance/ Concentration	Recovery[c], %
2.5	0.057 ± 0.009	0.0764	103.5 ± 15.8
5	0.105 ± 0.003	0.0700	94.9 ± 3.1
10	0.211 ± 0.003	0.0702	95.2 ± 1.0
20	0.421 ± 0.005	0.0701	95.0 ± 1.1
30	0.620 ± 0.018	0.0689	93.3 ± 2.7
40	0.810 ± 0.020	0.0675	91.4 ± 2.3
60	1.267 ± 0.036	0.0703	95.3 ± 2.7
80	1.655 ± 0.016	0.0691	93.6 ± 1.3
Mean		0.0703 ± 0.0044 (6.3%)	95.3 ± 6.0 (94.1 ± 2.0 over the range 5–80 μg/ml)

[a] Determinations performed on 1.0 ml plasma. [b] Absorbance at 247 nm, adjusted for mean plasma blank of 0.012. [c] Mean of triplicate determinations ± standard deviation.

pacity, were positioned upon magnetic stirrers beneath the condenser. Six reflux units were attached to a single variable transformer[5] which applied voltage to each heating mantle through the use of a multiple-electric outlet[6]. A similar outlet box applied line voltage to each of the six magnetic stirrers. A convenient reflux system may be achieved by mounting two sets of six reflux units each from a single support matrix, one set to the front and the other to the rear. The use of BB's or shot pellets as a heat transfer medium allows the heating mantles to be used with varying sizes of flasks.

Spectrophotometric measurements were performed on a ratio-recording spectrophotometer[7] equipped with a three-cell multiposition mount with microcell holder. Quartz microcells, 10 mm square × 25 mm, 0.4-ml capacity, were utilized for the microdeterminations; quartz cells, 10 mm square × 45 mm, 3.5-ml capacity, were utilized for the semimicrodeterminations.

Procedure—*Microdetermination*—Five milliliters of ethylene dichloride, 0.1–0.2 ml of plasma, serum, or whole blood, and 0.1 ml of 1 *N* HCl are pipetted into a 15-ml glass-stoppered tube. The tube is stoppered, shaken vigorously for 1 min, and, following removal of the stopper, centrifuged for 2 min at 2000 rpm. Then 4.5 ml of the ethylene dichloride layer is transferred to a second tube and similarly extracted into 2.5 ml of 5 *N* NaOH. Two milliliters of the sodium hydroxide extract, approximately 0.2 g of potassium permanganate, 1.0 ml of spectrograde *n*-heptane, and a 1.3-cm (0.5-in.) Teflon-coated magnetic stirring bar are placed in a 50-ml round-bottom flask fitted with a 24/40 ground-glass joint.

Semimicrodetermination—With 25 ml of ethylene dichloride, 1 ml of plasma or serum, and 0.5 ml of 1 *N* HCl, extraction and centrifugation are performed as already described in a 50-ml round-bottom centrifuge tube. The aqueous layer may be aspirated and the solvent decanted into a stoppered 50-ml graduated cylinder, or a known quantity, *i.e.*, 24 ml, may be transferred *via* pipet to a second 50-ml centrifuge tube. Ten milliliters of 5 *N* NaOH is added and the tubes are stoppered, shaken vigorously for 1–2 min, and subsequently centrifuged for 2 min at 2000 rpm. Nine milliliters of the aqueous layer is transferred to a 50-ml round-bottom flask, to which are added a magnetic stirring bar, 3 ml of *n*-heptane, and approximately 0.2 g of potassium permanganate.

The boiling flask is attached to the reflux condenser and secured into a heating mantle, which is supported by the top surface of a magnetic stirrer. After the mixture has refluxed with vigorous stirring for 25 min, optimally achieved with 40 v ac applied to the heating mantles, the heating mantles are removed and the flasks are allowed to cool for 5–10 min. Then 10–25 ml of water is poured into the flasks through the open end of the reflux columns.

The flasks are removed, sufficient water is added to float the heptane layer just into the neck of the flask, and the heptane layer is transferred to the quartz cells. The heptane layer is scanned spectrophotometrically over the 310–230-nm range, or the absorbance is measured at 247 nm. Since all standards and unknowns are treated identically, calculations are accomplished by application of a simple ratio:

$$\frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times \text{concentration of standard} = \text{concentration of specimen} \quad \text{(Eq. 1)}$$

If different volumes of specimen and standard are used, or if different volumes of solvent are recovered, appropriate corrections must be made in the final calculation.

Comparison of Oxidation Techniques—For evaluation of the nonreflux oxidation (13), 4 ml of 5 *N* NaOH containing diphenylhydantoin and 0.2 g of potassium permanganate were alternately placed into a 15-ml tube having a ground-glass stopper or into a 5-ml long-stem glass ampul which was then hermetically sealed. The stoppered tube or sealed ampul was immersed in a boiling water bath for 5–25 min, after which it was removed and cooled to room temperature. Then the contents were extracted with 2 ml heptane and scanned spectrophotometrically.

RESULTS AND DISCUSSION

Both the semimicro- and microprocedures provide quantitative

Table II—Diphenylhydantoin Determination by Microprocedure

Concentration[a], μg/ml	Absorbance[b]	Absorbance/ Concentration	Recovery, %
5	0.050 ± 0.008	0.0694	94.4
10	0.099 ± 0.002	0.0688	93.5
20	0.196 ± 0.002	0.0680	92.6
30	0.302 ± 0.012	0.0699	95.1
40	0.392 ± 0.003	0.0680	92.6
60	0.598 ± 0.007	0.0692	94.1
80	0.793 ± 0.017	0.0688	93.7
Mean		0.0689 ± 0.0027	93.7 ± 3.6

[a] Triplicate plasma determinations performed on 0.2-ml specimens. [b] Absorbance at 247 nm ± standard deviation, adjusted for mean plasma blank of 0.022.

Table III—Reproducibility Achieved with Different Volumes of Heptane in Reflux

Volume of Heptane, ml	250-ml Flask[a,b], Standard Condenser	50-ml Flask[a,b], Standard Condenser	50-ml Flask[a,c], External Cold Finger
0.50	—[d]	—[d]	11.6
0.75	—[d]	—[d]	3.8
1.00	5.6	6.4	2.0
3.00	0.8	0.5	—[d]

[a] Percent relative standard deviation, mean of triplicate determinations. [b] Nine milliliters of 5 *N* NaOH, 10 μg diphenylhydantoin/ml heptane. [c] Two milliliters of 5 *N* NaOH, 10 μg diphenylhydantoin/ml heptane. [d] Not determined.

[5] Staco.
[6] CRC Multi-lectric.
[7] Beckman Acta CIII.

Table IV—Comparison of Reflux and Nonreflux Oxidation Techniques

Oxidation Technique[a]	Absorbance/ Concentration[b], μg/ml
Sealed ampul, 5 min	0.0051 ± 0.0008
Sealed ampul, 25 min	0.0377 ± 0.0004
Glass-stoppered tube, 5 min	0.0536 ± 0.0022
Glass-stoppered tube, 25 min	0.0393 ± 0.0053
Reflux[c], 25 min	0.0770 ± 0.0021

[a] Twenty micrograms diphenylhydantoin either refluxed in the presence of alkaline permanganate and heptane or incubated in alkaline permanganate and subsequently extracted with heptane. [b] Mean of triplicate determinations. [c] Semimicroreflux; *i.e.*, 3 ml heptane, Allihn condenser.

recovery of diphenylhydantoin at subtherapeutic through toxic levels from small volumes of plasma (Tables I and II, respectively). Wallace *et al.* (8) previously demonstrated that the alkaline permanganate oxidation, utilized in conjunction with an acidic extraction, is a highly specific determination for diphenylhydantoin, free of interference from other drugs, drug metabolites, or naturally occurring constituents. Previous spectrophotometric determinations for diphenylhydantoin utilized large boiling flasks and solvent volumes for reflux, resulting in an extensive dilution of the benzophenone product. Use of 50-ml boiling flasks with the condenser[3] requires only 3 ml of heptane, which results in a significant increase in sensitivity. However, a further decrease in the solvent volume to 2 ml, when using the standard condenser, more than doubles the coefficient of variation that is achieved with 3 ml of solvent, apparently due to an insufficient amount of liquid solvent available for the continuous extraction of benzophenone.

Utilization of an "external cold finger" reflux condenser induces a more rapid condensation of solvent vapors, which permits the utilization of smaller volumes of solvent and results in a enhanced sensitivity (Table III). The cold finger condenser was developed for working with extremely small amounts of refluxing solvent[1]. It essentially involves the projection of the cooling surface of the condenser to a point only 2.54 cm (1 in.) above the boiling liquid.

It was demonstrated previously (14) that, within wide limits, the amount of potassium permanganate (0.1–0.4 g) and the molarity of the sodium hydroxide (5–11 *M*) are not critical for the quantitative oxidation of diphenylhydantoin. For the present micro- and semimicromodification, the amount of potassium permanganate should exceed 0.05 g and the concentration of the alkali should exceed 5.0 *M*.

Certain investigators (13) employed a nonreflux alkaline permanganate oxidation of diphenylhydantoin, in which the aqueous layer is extracted with solvent subsequent to, rather than concurrent with, the oxidative reactions. Comparative studies in this laboratory indicate that the observed absorbance–concentration ratio is significantly less in the nonreflux techniques, and a marked decrease in absorbance is observed if the time of incubation is prolonged only slightly past optimum at temperatures exceeding 90° (Table IV). It was suggested (13) that these observations were a result of the volatile nature of the product, benzophenone, and that the losses occurred through the spaces between the ground-glass joint and the glass stopper of the tubes.

A decreased yield of benzophenone was achieved if the magnetic stirrers were temporarily turned off during reflux. When considering also the high boiling point of benzophenone, it is apparent that the decreased sensitivity obtained in the nonreflux technique is a consequence of overoxidation of benzophenone (product degradation), rather than its volatility.

A comparison of the absorbance–concentration values obtained with the nonreflux technique, utilizing both glass-stoppered tubes and hermetically sealed ampuls (to prohibit any benzophenone volatility), with those achieved by the reflux technique further indicates that the decreased sensitivity of the nonreflux procedure is not a consequence of the volatility of benzophenone (Table IV). The sensitivity achieved with the ampul and tube samples at 5 min of incubation was 72 and 70%, respectively, of that achieved by the reflux procedure; both the ampul and tube samples exhibited a similar decrease in sensitivity with increasing duration of incubation.

Although the nonreflux technique does not provide the sensitivity obtained through reflux, it does offer the advantages of not requiring the more expensive reflux apparatus and of achieving its optimum sensitivity in less time than that required for the reflux technique. Thus, for laboratories having a limited budget and not possessing reflux apparatus, the nonreflux technique may be the most appropriate method. But for those laboratories possessing the necessary apparatus, the enhanced sensitivity and reproducibility achieved by the reflux method more than offset the slight additional time of analysis. The utilization of the cold finger condenser makes for a micromethod that is unequaled in sensitivity by the sealed oxidation techniques.

Table V—Diphenylhydantoin Levels in Patients' Plasma Level (Micrograms per Milliliter)

Patient	Micromethod[a]	Macromethod[b]
1	24.5, 25.3	25.1
2	19.3, 21.5	20.0
3	3.5, 5.6	—[c]
4	28.2, 29.1	28.9
5	0.6, 1.1	3.5
6	8.9, 10.0	8.7
7	30.3, 30.5	29.0
8	13.2, 13.5	13.2
9	0, 0.5	0
10	18.4, 19.1	19.4
11	8.5, 10.3	11.2
12	30.4, 32.5	28.9
13	22.5, 24.0	—[c]
14	0, 0	4.2

Mean difference between duplicate determinations—±1.0 μg/ml

Mean difference between macro- and microdetermination—±1.3 μg/ml

[a] Microdeterminations performed in duplicate in this laboratory. [b] Macromethod (14) performed in a public hospital laboratory in single determinations. [c] Sample size insufficient for macrodetermination.

To demonstrate the clinical application and effectiveness of the proposed techniques, 14 patient specimens were analyzed in duplicate by the microprocedure (Table V). The mean difference between duplicate determinations was 1.0 μg/ml, the mean concentration being 15.4 μg/ml. The diphenylhydantoin levels had been obtained on 12 of the specimens (single determination only) at a public hospital laboratory utilizing an earlier modification of the Wallace *et al.* (14) method; the mean difference between levels in this study and the hospital levels was 1.3 μg/ml.

The proposed micromethod provides the most sensitive, reliable, and reproducible values of any available spectrophotometric method capable of determining diphenylhydantoin in 0.1 ml of serum. The limit of accurate quantitation for 0.1–2.0-ml specimens is 0.5 μg/specimen.

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* To whom inquiries should be directed.

Antitumor Activity and Cardiac Stimulatory Effects of Constituents of *Anthopleura elegantissima*

R. J. QUINN *, MIDORI KASHIWAGI ‡, TED R. NORTON ‡ x, S. SHIBATA ‡, M. KUCHII ‡, and R. E. MOORE *

Abstract □ A crude aqueous ethanolic extract of the sea anemone, *Anthopleura elegantissima* (Brandt), displayed activity against two experimental mouse tumors (P-388 lymphocytic leukemia and Ehrlich ascites tumor). In addition, it displayed a positive inotropic effect on isolated rat atria. Preliminary separations established that the three biological activities are due to three different constituents of this marine organism.

Keyphrases □ *Anthopleura elegantissima* (Brandt)—separation of constituents, screened for antitumor activity and cardiac stimulatory effects □ Sea anemone—separation of constituents, screened for antitumor activity and cardiac stimulatory effects □ Antitumor activity—constituents of *A. elegantissima* screened □ Cardiac stimulatory activity—constituents of *A. elegantissima* screened

The antitumor activity of a crude extract of the sea anemone, *Anthopleura xanthogrammica* (Brandt) (1), and the cardiac stimulatory effects of extracts of both *A. xanthogrammica* and *A. elegantissima* (2) have been reported. An aqueous ethanolic extract of *A. elegantissima*[1] has been found active against P-388 lymphocytic leukemia (P-388) and Ehrlich ascites tumor in mice and produces a positive inotropic effect on isolated rabbit auricles (Table I). These three effects are due to three different constituents obtained in a preliminary fractionation of the crude extract.

Gel permeation chromatography using Resin 1[2] separates the P-388 active material, which is not retarded, from the Ehrlich ascites and heart stimulant substances, which are retarded. The P-388 active material is further purified using Resin 2[3] and the Ehrlich ascites and heart stimulant substances are separated from each other using cellulose chromatography.

Taurine was also isolated from the *A. elegantissima* extract. Taurine was first isolated from a marine organism from the red algae *Ptilota pectinata, Porphyra umbilicalis,* and *Gelidium cartilagineum* (3). *N,N*-Dimethyltaurine was isolated previously from the red alga *Furcellaria fastigiata* (4), while both the *N*-methyl- and *N,N*-dimethyltaurines were shown to be constituents of the sponge *Calyx nereis* (5); the trimethyl derivative, taurobetaine, was found in the gorgonian *Briareum asbestinum* (6).

RESULTS AND DISCUSSION

A typical chromatogram, monitored at 280 nm, of the crude extract on Resin 1 is shown in Fig. 1. Fraction A (V_e/V_0 0.86–1.50) was active against P-388, Fraction C (V_e/V_0 1.73–1.97) displayed a strong positive inotropic effect on isolated rat atria (2) and some inhibition of Ehrlich ascites, and Fraction D (V_e/V_0 1.97–2.25) inhibited Ehrlich ascites tumor.

Further gel permeation chromatography of Fraction A was achieved on Resin 2 (Fig. 2). The P-388 active constituent separated from the large amount of 280-nm absorbing material at V_e/V_0 0.83–1.31 but was distributed over a large region (Fraction H, V_e/V_0 1.31–2.35). In comparison to the crude extract, Fraction H showed a marked improvement in activity against P-388.

Fraction C was separated further by cellulose chromatography (Fig. 3). The Ehrlich ascites active material was eluted almost at

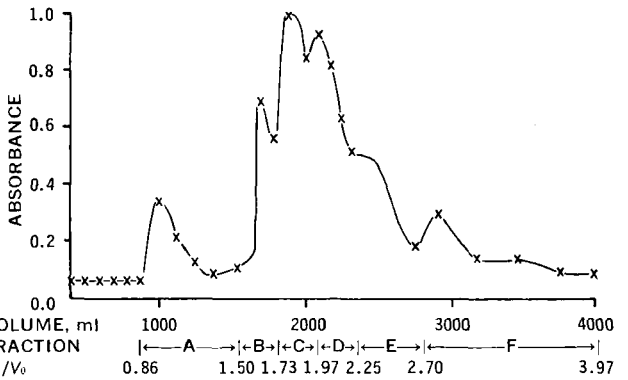

Figure 1—*Chromatography of crude extract on Resin 1.*

[1] The authors thank Dr. Cadet Hand, Director, Bodega Marine Laboratory, Bodega Bay, Calif., for supplying a collection of the animals.
[2] Sephadex G-25.
[3] Sephadex G-75.

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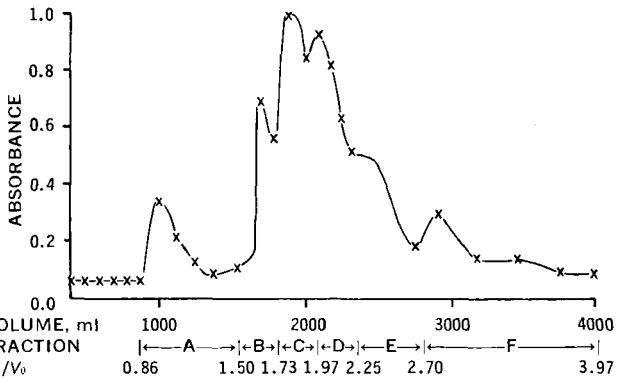

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[2] Sephadex G-25.
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Table I—Biological Results

Fraction	P-388 Leukemia[a,b] Dose, mg/kg	P-388 Leukemia[a,b] T/C, %	Ehrlich Ascites[b] Dose, mg/kg	Ehrlich Ascites[b] Survivors at 30 Days % Alive	Ehrlich Ascites[b] Survivors at 30 Days % Nonascitic	Heart Stimulant[c]
Crude extract	300	Toxic	2.5	80	60	Strong positive inotropic effect at 100 μg/ml
	200	125[a]	1.25	40	40	
		129[a]	0.8	40	20	
		107[b]	0.4	0	0	
	160	155[b]				
	133	133[a]				
		116[a]				
	106	114[b]				
	100	118[b]				
	88	125[a]				
		129[a]				
	70	102[b]				
A	320	Toxic (0.5 hr)	50	50	50	Weak effect at 91 μg/ml
12% of crude	160	140[b]	25	50	25	
extract	80	128[b]	3.0	0	0	
	50	125[b]	0.76	0	0	
	40	137[b]				
	25	137[b]				
	12.5	126[b]				
C	B,C,D,E,F combined		8	25	25	Threshold 2.58 μg/ml, maximum 25.8 μg/ml
33% of crude	310	106[b]	2.56	60	40	
extract			2.2	40	20	
			2	0	0	
			0.64	80	40	
D	B,C,D,E,F combined		3.3	50	25	Weak at 129 μg/ml
16% of crude	310	106[b]	0.83	25	25	
extract						
H	22.5	157[b]				
16.6% of A	6.75	146[b]				
2% of crude	2.0	131.5[b]				
extract						
L			2.2	80	80	Inactive
			0.43	40	40	
M			3.7	40	40	Inactive
			0.74	40	40	
O–R			O-9.5	0	0	Positive inotropic effect
			P-12.3	0	0	
			R-25.4	0	0	

[a] Data from the National Cancer Institute screening program through the courtesy of Dr. Jonathan L. Hartwell. Schedule of 1 dose/day × 9 days. [b] Standard screening procedure used in this laboratory; for general procedure, see Ref. 7. Schedule of 1 dose/day × 10 days. [c] Determined using the isolated atria of live rat hearts as described in Ref. 2.

the solvent front (Fractions L and M), while the heart stimulant was eluted later (Fractions O–R). The bioassay results are given in Table I. Fraction L gave the best observable inhibition of Ehrlich ascites in that 80% of the treated mice were cured, *i.e.*, no abdominal distension at 30 days. Fraction M showed less Ehrlich ascites activity. TLC of Fractions L and M on cellulose HF[4] with 76% aqueous ethanol gave two ninhydrin-positive spots at R_f 0.64 and 0.56. Examination of the Ehrlich ascites active Fraction D revealed the presence of two faint spots with the same R_f values.

The P-388 active material is excluded from Resin 1 and, therefore, should have a molecular weight greater than 5000. But the Ehrlich ascites active and heart-stimulating constituents may be small molecules since Fraction C was retarded by Resin 1 and contains taurine, which is isolated from Fraction S from the cellulose chromatography.

The three biological activities displayed by a crude extract of *A. elegantissima*, as described here, have been shown to be due to three different constituents. Further purification of these drugs is continuing.

EXPERIMENTAL

A. elegantissima specimens were collected from Bodega Bay, Calif.[1], preserved in 95% ethanol, and stored at −10° prior to extraction.

Wet, drained anemones (8.7 kg) were homogenized batchwise in a blender with 36 liters of 28.5% ethanol, combined with the 4.6 liters of storage liquor, and allowed to stand at room temperature for 2 days. Most of the supernatant liquid was decanted, and the remaining extract was separated from the solids by centrifugation (2000 rpm, 5 min).

The combined liquors were concentrated under reduced pressure at ≦50° to 7 liters. The concentrate was clarified by centrifugation (20,000×*g*, 10 min), decanted through a plug of glass wool to remove a small amount of orange oil floating on the surface, and lyophilized to give 500 g of a yellow hygroscopic solid.

The combined marc from both centrifugation steps was resuspended in 7 liters of 28.5% ethanol and allowed to stand, with occa-

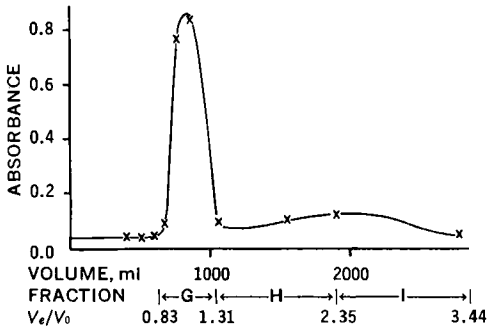

Figure 2—*Chromatography of Fraction A on Resin 2.*

[4] Merck.

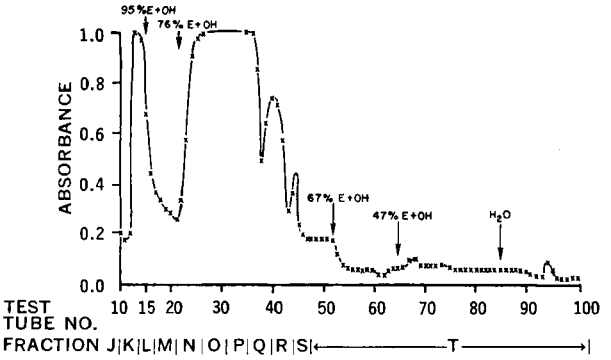

Figure 3—*Chromatography of Fraction C on cellulose.*

sional shaking, for 2 weeks. After decantation, centrifugation (2000 rpm, 10 min), and filtration through a plug of glass wool, the solution was lyophilized to give 79 g of a yellow hygroscopic solid.

The crude extract (20 g) was dissolved in 100 ml of water with the aid of sonication for 2 min and shaken vigorously with 100 ml of chloroform. Centrifugation (20,000×*g*, 15 min) of this mixture gave a brown aqueous layer, a yellow chloroform layer, and some insoluble solid at the interface. The aqueous layer was removed, recentrifuged, and then lyophilized to give the water-soluble portion of the crude extract, 16.5 g of a yellow solid.

Chromatography on Resin 1—The water-soluble portion of the crude extract (3.00 g) in 20 ml of water was chromatographed on a 49 × 8-cm column of Resin 1 (fine, 500 g). The void volume was determined to be 1025 ml with 20 ml of 0.15% blue dextran. Elution was with water saturated with chloroform at a rate of 6.5 ml/min (7.7 ml hr^{-1} cm^{-2}). The column effluent was monitored using a recording UV monitor at 280-nm wavelength. The collected fractions were flash evaporated (≤50°) to reduce volume and lyophilized (Fig. 1). The weights of the active fractions were: A, 441 mg; C, 1220 mg; and D, 589 mg.

Chromatography on Resin 2—Fraction A (2.00 g in 20 ml of water) was chromatographed on a 49 × 8-cm column of Resin 2 (180 g) with a 810-ml void volume. Elution was with water saturated with chloroform at a rate of 5.25 ml/min (6.3 ml hr^{-1} cm^{-2}). The column effluent was monitored at 280 nm. Fraction H fluoresced yellow green with longwave UV light and Fraction I fluoresced blue. The fractions collected were flash evaporated (≤50°) to reduce volume and lyophilized (Fig. 2). The P-388 activity was found in Fraction H, which weighed 333 mg.

Chromatography on Cellulose—A slurry of 1.00 g of Fraction C and about 0.5 g of cellulose in 10 ml of water was lyophilized. This mixture, slurried in 95% ethanol, was mounted on top of a 38 × 1.5-cm column of 20 g of cellulose MN 300[5] packed in 95% ethanol. Elution was initiated with 300 ml of 95% ethanol, followed successively with 500 ml of 76% ethanol, 200 ml of 67% ethanol, 322 ml of 47% ethanol, and 200 ml of water (Fig. 3).

Fraction S, on standing, deposited long colorless needles, which were collected by filtration, washed with 95% ethanol, and dried to give 16.3 mg of solids. The NMR and IR spectra were superimposable with an authentic sample of taurine.

The collected fractions were evaporated to remove the ethanol and lyophilized. The weights of the active fractions were: L, 27.1 mg; and M, 45.9 mg.

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[x] To whom inquiries should be directed.

[5] Machery, Nagel and Co.

Syntheses and Antimicrobial Properties of Isothiocyanate Derivatives

MISAO KOJIMA

Abstract □ Nine reaction products of isothiocyanates with cysteine, *N*-(*N*-substituted-thiocarbamoyl)cysteines, and eight reaction products of isothiocyanates with 2,3-dimercapto-1-propanol, 2,3-bis(substituted-thiocarbamoylthio)-1-propanols, were synthesized. The antimicrobial activity of these compounds was investigated.

Keyphrases □ Isothiocyanate derivatives—synthesized and screened for antimicrobial activity □ Cysteine, reaction products with isothiocyanates—synthesized and screened for antimicrobial activity □ 2,3-Dimercapto-1-propanol, reaction products with isothiocyanates—synthesized and screened for antimicrobial activity □ Antimicrobial activity—isothiocyanate derivatives synthesized and screened

Some naturally occurring isothiocyanates with antimicrobial activity are known (1–4), and their biological activity is based on the isothiocyanate group (—N=C=S) which reacts with the functional group of the peptide (5). However, little has been reported on the antimicrobial activity of the derivatives of isothiocyanates (6).

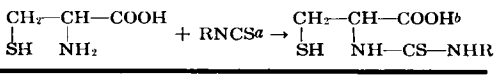
This paper is concerned with the antimicrobial properties of chemically related compounds in which the isothiocyanate group is bound to aliphatic and aromatic radicals.

DISCUSSION

The experimental compounds were synthesized in this laboratory. Tables I and II show the characteristics of these new compounds.

All of the following strains of microorganisms, except *Lactobacillus hiochi*, were grown on glucose–bouillon liquid medium (pH

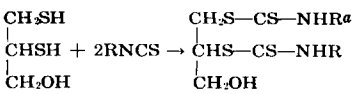
Table I—Characteristics of *N*-(*N*-Substituted-thiocarbamoyl)cysteines



Compound	R	Melting[c] Point	Formula	Analysis, % Calc.	Analysis, % Found	Solubility in Water, mg/ml
1	CH_3CH_2	185° dec.	$C_6H_{12}N_2O_2S_2$	C 34.62 H 5.81 N 13.46 S 30.75	34.67 5.99 13.56 30.97	1.0
2	$CH_3(CH_2)_2$	180° dec.	$C_7H_{14}N_2O_2S_2$	C 37.84 H 6.35 N 12.61 S 28.80	38.18 6.32 12.83 28.05	0.5
3	$(CH_3)_2CH$	205° dec.	$C_7H_{14}N_2O_2S_2$	C 37.84 H 6.35 N 12.61 S 28.80	37.52 6.23 12.66 28.71	0.5
4	$CH_3(CH_2)_3$	200° dec.	$C_8H_{16}N_2O_2S_2$	C 40.67 H 6.83 N 11.86 S 27.10	40.43 6.80 11.81 26.62	0.25
5	$(CH_3)_2CHCH_2$	190° dec.	$C_8H_{16}N_2O_2S_2$	C 40.67 H 6.83 N 11.86 S 27.10	40.60 6.83 11.86 27.09	1.0
6	C_6H_5	160° dec.	$C_{10}H_{12}N_2O_2S_2$	C 46.88 H 4.72 N 10.93 S 25.00	46.79 4.42 10.72 24.97	0.5
7	$C_6H_5CH_2$	190° dec.	$C_{11}H_{14}N_2O_2S_2$	C 48.89 H 5.22 N 10.37 S 23.02	48.61 5.27 10.34 23.55	0.25
8	$C_6H_5(CH_2)_2$	191° dec.	$C_{12}H_{16}N_2O_2S_2$	C 50.70 H 5.67 N 9.86 S 22.76	50.17 5.67 9.95 22.41	0.5
9	p-ClC_6H_4	166–167.5°	$C_{10}H_{11}ClN_2O_2S_2$	C 41.32 H 3.78 N 9.63 S 22.06	41.10 3.80 9.74 22.16	0.1

[a] Obtained commercially or synthesized from corresponding primary amines by the method of Mumm and Richter (8). [b] *N*-(*N*-Allylthiocarbamoyl)cysteine (7) is the sole known compound of this type. These compounds gave a red color with the nitroprusside test, demonstrating the free sulfhydryl group. In the IR spectrum of these compounds, a weak band near 2600 cm^{-1} indicated the presence of a sulfhydryl group. [c] Melting points are uncorrected.

Table II—Characteristics of 2,3-Bis[alkyl (alkenyl, aryl, and aralkyl)-thiocarbamoylthio]-1-propanols



Compound	R	Melting[b] Point	Formula	Analysis, %		Solubility in Water, mg/ml
				Calc.	Found	
10	CH_3	125° dec.	$C_7H_{14}N_2OS_4$	C 31.11 H 5.19 N 10.39 S 47.40	31.09 5.30 9.97 47.47	1.0
11	$CH_3(CH_2)_2$	113–114°	$C_{11}H_{22}N_2OS_4$	C 40.45 H 6.79 N 8.58 S 39.27	40.47 6.70 8.32 38.94	0.1
12	$(CH_3)_2CHCH_2$	102–103°	$C_{13}H_{26}N_2OS_4$	C 44.07 H 7.34 N 7.90 S 36.72	43.88 7.34 7.36 36.44	0.1
13	$(CH_3)_2CH(CH_2)_2$	131–132°	$C_{15}H_{30}N_2OS_4$	C 47.08 H 7.91 N 7.33 S 33.51	46.92 7.95 7.42 33.06	0.1
14	CH_2CHCH_2	119.5–120°	$C_{11}H_{18}N_2OS_4$	C 40.99 H 5.63 N 8.69 S 39.72	41.03 5.31 8.61 39.50	0.03
15	$C_6H_5CH_2$	140.5–141°	$C_{19}H_{22}N_2OS_4$	C 54.01 H 5.20 N 6.63 S 30.35	53.83 5.20 6.71 30.66	0.1
16	$C_6H_5(CH_2)_2$	139–139.5°	$C_{21}H_{26}N_2OS_4$	C 55.99 H 5.77 N 6.22 S 28.46	55.91 5.63 6.24 28.86	0.1
17	p-ClC_6H_4	137–138°	$C_{17}H_{16}Cl_2N_2OS_4$	C 44.06 H 3.45 N 6.04 S 27.67	44.10 3.39 6.31 27.61	0.03

[a] These compounds were negative to the nitroprusside test. In the IR spectrum, no peak occurred near 2600 cm^{-1}, indicating the absence of a sulfhydryl group. [b] Melting points are uncorrected.

Table III—Culture Conditions for Tested Microorganisms

Strain	Temperature	Days
Lactobacillus hiochi No. 2[a]	30°	10
Streptococcus aureus FDA 209P	37°	2
Pseudomonas aeruginosa IFO 3080	37°	2
Saccharomyces cerevisiae IFO 0599	25°	3
Aspergillus niger ATCC 9642	25°	3

[a] Isolated in the Research Laboratory, Takeda Pharmaceutical Industries Ltd. of Japan.

6.0). *L. hiochi* No. 2 was grown on a liquid medium (pH 4.5) which contained 1% glucose, 1% yeast extract, 1% polypeptone, and 13% ethanol.

Table III shows tested microorganisms and culture conditions. The antimicrobial activity of a solution or suspension of these compounds is shown as the minimum inhibitory concentration (MIC) (micrograms per milliliter) in Table IV.

Compounds 7, 8, 9, 14, and 17 exhibited the highest antimicrobial activity, especially against yeast and mold, while Compounds 7 and 8 showed the highest potency for all tested microorganisms. However, these results were unsatisfactory (Table IV), because of the low solubilities of the compounds in water. Therefore, nonionic

Table IV—Effects of Derivatives of Isothiocyanates on Microorganism Growth

Compound	MIC				
	Lactobacillus hiochi	*Streptococcus aureus*	*Pseudomonas aeruginosa*	*Saccharomyces cerevisiae*	*Aspergillus niger*
1	1000	1000	500	500	500
2	1000	1000	1000	>1000	1000
3	1000	>1000	>1000	>1000	1000
4	1000	1000	1000	1000	1000
5	1000	>1000	>1000	>1000	>1000
6	1000	1000	1000	500	1000
7	250	250	250	30	7.8
8	>1000	250	1000	30	15.6
9	>1000	>1000	>1000	>1000	1000
10	1000	>1000	>1000	>1000	130
11	1000	>1000	>1000	>1000	>1000
12	1000	>1000	>1000	>1000	>1000
13	1000	>1000	>1000	>1000	>1000
14	1000	>1000	250	130	60
15	>1000	>1000	>1000	500	500
16	1000	1000	1000	1000	1000
17	1000	500	1000	62.5	31.3

Table V—Effects of Compounds 7 and 8, in which Sorbitan Fatty Acid Esters were Added, on Microorganism Growth

Sorbitan Ester	MIC, μg/ml			
	Streptococcus aureus		*Aspergillus niger*	
	Compound 7	Compound 8	Compound 7	Compound 8
None (control)	250	250	7.8	15.6
Monolaurate	<7.8	<7.8	7.8	15.6
Monopalmitate	125	125	7.8	15.6
Monostearate	250	125	7.8	15.6
Tristearate	62.5	125	15.6	15.6
Monooleate	125	250	7.8	15.6
Trioleate	62.5	125	7.8	31.3

surfactants were employed as solubilizing agents.

Table V shows the MIC of surfactants plus Compounds 7 and 8. The surfactants used had no antimicrobial activity. Only for *Streptococcus aureus* were the compounds with added surfactants more effective than those compounds alone. The sorbitan monolaurate suspensions of the compounds had good MIC values, below 7.8 μg/ml. Since the MIC's of Compounds 7 and 8 alone were 250 μg/ml, the compounds with the sorbitan fatty acid esters may have enhanced activities.

EXPERIMENTAL

***N*-(*N*-Substituted-thiocarbamoyl)cysteines**—These compounds were obtained by the method of Todrick and Walker (7).

2,3-Bis(substituted-thiocarbamoylthio)-1-propanols—2,3-Dimercapto-1-propanol was dissolved in about 30 parts of water. Two equimolar quantities of the corresponding isothiocyanate were added and gentle shaking was maintained for 3 hr, whereupon a yellow granular solid started to separate. The mixture was stored overnight and filtered, and the solid was recrystallized from ethanol.

In the preparation of 2,3-bis[aryl(aralkyl)-thiocarbamoylthio]-1-propanol, the same procedure was employed except that dimethyl sulfoxide was used instead of water.

Antimicrobial Activity—The antimicrobial activity was determined by determining the MIC (micrograms per milliliter) macroscopically or microscopically using the normal dilution method.

The concentration of each surfactant was 1 mg/ml of liquid medium (pH 6.0).

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Transport Mechanisms of β-Lactam Antibiotics across Everted Rat Gut

STANLEY C. PENZOTTI, Jr.[x], and JOHN W. POOLE

Abstract □ Penicillins and cephalosporins, both monobasic and amphoteric, were tested for active transfer by measurement of the ratio of drug between serosal and mucosal solutions in a standard everted rat gut preparation. In no instance did any of these β-lactam antibiotics demonstrate active transport. Parallel experiments with glucose, a compound known to be actively transported in this preparation, always resulted in a positive ratio. A linear relationship between rate of transfer and concentration for two compounds, sodium ampicillin and sodium nafcillin, provided further evidence for passive transport. Although sodium nafcillin showed an apparent saturable process between 20 and 40 mg/ml, surface tension measurements showed that the CMC for sodium nafcillin lies within this range.

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Since penicillins are known to be actively secreted into the renal tubules (1–3) and actively transported from the cerebrospinal fluid to the blood (4), the possibility that there may also be a specialized process in the intestinal absorption of β-lactam antibiotics must be considered. Earlier *in vitro* studies with an everted gut preparation indicated that the transport of phenoxymethyl penicillin and phenoxybenzyl penicillin (phenbenicillin) is a saturable process (5, 6), although additional studies did not produce evidence of active transport (6).

In the present work, the everted rat intestinal sac was employed to investigate the transport mechanism of several penicillins and cephalosporins, in-

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In the present work, the everted rat intestinal sac was employed to investigate the transport mechanism of several penicillins and cephalosporins, in-

Table I—Final Serosal-to-Mucosal Concentration Ratio across the Everted Rat Intestinal Sac, pH 7.4 Krebs–Henseleit Buffer, 37°

β-Lactam, 2 mg/ml	Serosal-to-Mucosal Concentration Ratio[a]					
	Nonsaturated			Saturated		
	20 min	40 min	60 min	20 min	40 min	60 min
Potassium penicillin G	0.93	0.87	0.74	0.92	0.81	0.69
Potassium phenoxymethyl penicillin	0.93	0.86	0.84	0.87	0.85	0.70
Sodium ampicillin	0.89	0.83	0.70	0.87	0.82	0.80
Sodium cyclacillin	0.91	0.89	0.91	0.92	0.88	0.86
Sodium nafcillin	0.89	0.88	0.69	0.85	0.77	0.63
Sodium dicloxacillin	0.84	0.74	0.60	0.94	0.81	0.79
Pivampicillin	0.95	0.84	0.84	0.90	0.71	0.71
Cephalexin monohydrate	0.93	0.75	0.72	1.05	0.89	0.83
Cephaloglycin dihydrate	0.61	0.57	0.50	0.72	0.64	0.76
Cephaloridine	0.92	0.88	0.93	1.00	1.04	0.73
Sodium cephalothin	0.87	0.87	0.64	0.84	0.75	0.64
Glucose[b], 1.8 mg/ml	1.33 ±0.19	1.73 ±0.39	2.43 ±0.76	1.44 ±0.20	1.84 ±0.36	2.54 ±0.71

[a] Average of duplicate sacs. [b] Average of 22 sacs ± *SD*.

cluding sodium ampicillin, sodium cyclacillin, cephalexin, and cephaloglycin. These four compounds are amphoteric β-lactams, exhibiting many physical–chemical properties common to amino acids (7) which are known to be actively taken up from the intestine (8).

EXPERIMENTAL

A large section of small intestine, starting approximately 15 cm below the pyloric sphincter, was removed from an ether-anesthetized rat, rinsed with iced saline, and everted. Three 10-cm sections were used from each of two animals. Each section was tied at one end, filled with 2 ml of pH 7.4 Krebs–Henseleit bicarbonate buffer (9) containing the drug at 2 mg/ml and 10 m*M* glucose, and then tied at the other end to form a sac. The sacs were suspended in a large test tube containing 100 ml of the same drug and glucose-containing buffer solution; *i.e.*, equal concentrations were established on both sides of the membrane. There were two sacs per tube, and each tube was continually gassed with 5% carbon dioxide in oxygen and maintained at 37°.

At specified intervals, the sacs were removed and the outsides were rinsed with saline and blotted dry. The inside (serosal) contents were removed and assayed for drug and glucose content. At the same time, a sample from the outside solution (mucosal side) was withdrawn for assay. A similar set of experiments was conducted with sacs that had been incubated previously in an identical aerated drug solution for 1 hr and thus saturated with drug on both the mucosal and serosal sides. These tissues were designated as treated segments.

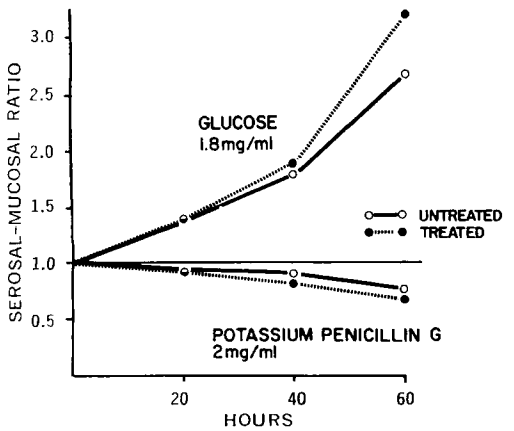

Figure 1—*Serosal-to-mucosal concentration ratios for glucose and potassium penicillin G in everted rat intestine after three periods of incubation (20, 40, and 60 min). Both drug-saturated (treated) and unsaturated (untreated) readings are shown. Each point represents the average of two preparations.*

The final serosal-to-mucosal concentration ratios were determined, and a ratio of greater than 1 was used as an indication of active transport. All determinations were done in duplicate. The 20- and 60-min incubations were carried out on one sac from the distal portion and one sac from the proximal portion of the intestine, and the 40-min incubation was done on the two middle sections. At no time was there any detectable difference in the behavior of segments from different portions of the intestine.

Two compounds, sodium ampicillin and sodium nafcillin, were selected for additional study to determine if the transport rate is proportional to the initial concentration. Glucose, a model compound for active transport (10), was included with each antibiotic tested to demonstrate the metabolic viability of the preparation. In these experiments the everted sacs, 15 cm in length, were filled with 3 ml of drug-free Krebs bicarbonate buffer and incubated in 100 ml of buffer containing the drug at the desired concentration. The transfer of the drug was followed with time, and the mucosal and serosal drug contents were measured as before at the end of 20 or 30 min.

In all cases the β-lactam antibiotics were assayed using the iodometric titration procedure (11). Glucose was determined enzymatically[1], and surface tension measurements were made with a surface tensiometer[2] at room temperature in the same buffer.

The penicillins studied[3] were potassium penicillin G, potassium phenoxymethyl penicillin (potassium penicillin V), sodium ampicillin, sodium cyclacillin, sodium dicloxacillin, sodium nafcillin, and pivampicillin. The cephalosporins[4] were sodium cephalothin, cephaloridine, cephaloglycin dihydrate, and cephalexin monohydrate. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

Figure 1 shows the results for glucose and for potassium penicillin G, a typical monobasic penicillin, when the ratios of serosal-to-mucosal concentrations were examined at 20, 40, and 60 min. With both the treated and untreated preparations for glucose, there was a continuous accumulation of drug on the serosal side. With potassium penicillin G, however, no accumulation against a concentration gradient occurred at any of the three periods studied. In this case, and in fact for each drug studied, there was a slight decrease in the ratio with time, and the value was almost always less than 1. This result was due to a decrease in concentration on the inside or serosal side of the membrane and not to any increase on the mucosal side. The decrease was originally thought to be due to drug

[1] Using the Glucostat reagent, Glucostat TM, Worthington Biochemical Corp., Freehold, N.J.
[2] Fisher Surface Tensiomat, Fisher Scientific Co., Pittsburgh, Pa.
[3] Wyeth Laboratories, Radnor, Pa.
[4] Eli Lilly and Co., Indianapolis, Ind.

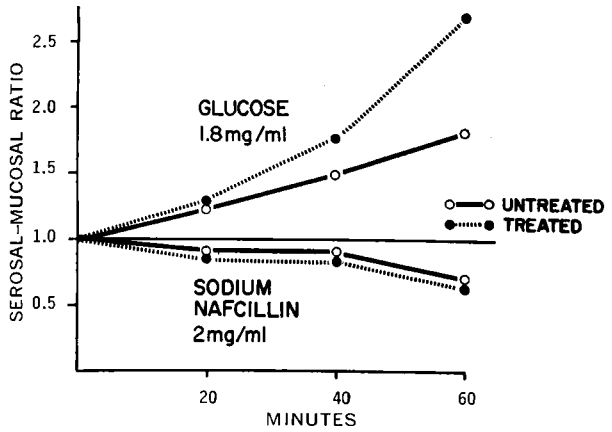

Figure 2—*Serosal-to-mucosal concentration ratios for glucose and sodium nafcillin in everted rat intestine after three periods of incubation (20, 40, and 60 min). Both drug-saturated (treated) and unsaturated (untreated) readings are shown. Each point represents the average of two preparations.*

binding to the tissue surface which, because of the smaller volume on the serosal side, might have a significant effect on the final concentration. However, as can be seen from the dashed lines (Fig. 1), there was also a similar decrease in the treated (saturated) preparations. Moreover, glucose behaved the same way regardless of treatment.

Figure 2 illustrates the results for a penicillinase-resistant compound, sodium nafcillin. The behavior of this drug was similar to that of potassium penicillin G; *i.e.*, a decrease in the ratio with time occurred with both the treated and untreated segments. Again, glucose accumulated against a concentration gradient.

Table I summarizes the data on all of the compounds run. In all trials, glucose accumulated on the serosal side at 2–2.5 times its initial concentration. Control studies in the absence of any antibiotic gave similar results. Although the serosal-to-mucosal concentration ratios appear greater with the saturated segment, the unsaturated values compared to the saturated values at each time period were found not to be significantly different by the Student *t* test. The reason for the apparently higher ratios lies in the fact that the initial mucosal glucose concentrations were lower in the saturated segments due, most likely, to the metabolism of glucose by the unsaturated segments run previously. Although there was a corresponding decrease in the amount accumulated on the serosal side in the saturated guts, the decrease was proportionally less than the decrease in the mucosal concentration, so a slightly higher serosal-to-mucosal concentration ratio resulted.

The β-lactam antibiotics (except in three instances) exhibited ratios of less than 1, whether treated (saturated) or not. The three exceptions were the 20-min saturated value for cephalexin monohydrate and the 20- and 40-min saturated ratios for cephaloridine. Even in these cases, the values at the end of 1 hr were less than 1. The reason for the decrease is not known. Previous workers with

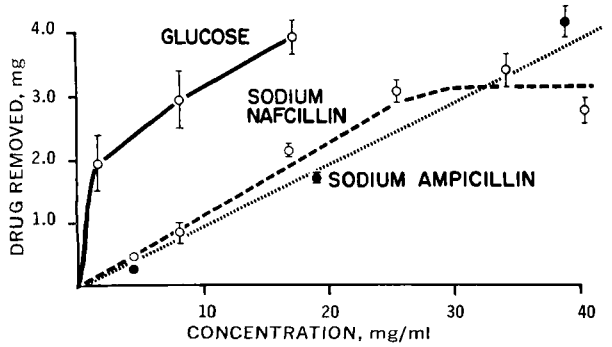

Figure 3—*Total amount of drug (milligrams) transferred across the everted rat intestine in 20 min (glucose and sodium ampicillin) or 30 min (sodium nafcillin) at various concentrations. Each point represents the average of at least two experiments, with the bars representing the range.*

Table II—Clearance Rates for Transport across Everted Rat Intestinal Sacs at Various Concentrations

Compound	Initial Concentration, mg/ml	Clearance Rate, ml/hr
Sodium ampicillin	1.0	0.26[a]
	4.2	0.27
	19.0	0.28
	38.7	0.32
Sodium nafcillin	1.0	0.21[a]
	4.2	0.23
	8.0	0.21
	16.8	0.26
	25.2	0.24
	34.3	0.20
	40.5	0.14
Glucose	1.4	4.04
	8.0	1.10
	17.0	0.69

[a] Studies run in the absence of glucose.

everted gut preparations have shown that in the presence of glucose there is a net transfer of water of between 0.1 and 0.3 ml/cm/hr from the mucosal to the serosal side (12). Therefore, if the transport of the antibiotic lags behind, a dilution could occur on the serosal side and could account for the lower values found.

Further evidence for passive transport was provided by studies of the effect of increasing concentration on transfer rate. Figure 3 shows the amount transferred in 20 min for sodium ampicillin and glucose and in 30 min for sodium nafcillin. Glucose gave the expected result for an active transport process since the amount removed did not increase proportionately with the increase in concentration. Data for sodium ampicillin were typical for a compound that is passively transported; *i.e.*, there was a linear response to the increasing concentration. Results for sodium nafcillin were indicative of something more than the simple passive transport suggested by previous data for this compound. Although there was an apparent linear increase in the amount transferred up to approximately 25–30 mg/ml, a leveling of the rate was observed above this concentration.

These data can be expressed in another fashion, *i.e.*, as clearance rates (Table II). The clearance rate was calculated by dividing the amount transferred in a unit time (here normalized to 1 hr) by the initial concentration. A constant clearance rate indicates passive transport. As can be seen, the values for glucose fell off dramatically, decreasing from approximately 4 ml/hr at 1.4 mg/ml to 0.7 ml/hr at the highest concentration (17 mg/ml), thus indicating active transport. The rates for sodium ampicillin remained constant over a 10-fold change in concentration. For sodium nafcillin the values were essentially constant up to 25–30 mg/ml but started decreasing above this concentration. Control studies for both antibiotics run in the absence of glucose demonstrated that glucose did not affect their transport.

If it is assumed from the sodium nafcillin data that no active process is occurring at the lower concentrations, then some change

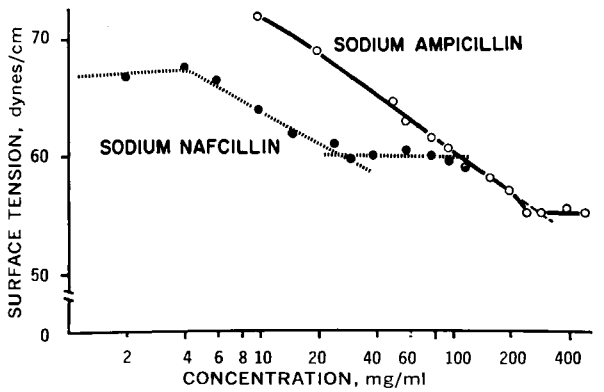

Figure 4—*Surface tension (dynes per centimeter) of sodium ampicillin and sodium nafcillin at various concentrations.*

in the form of the drug must be taking place at the higher concentrations. Evidence in the literature indicates that a variety of surface-active compounds may undergo association (13), and when such association takes place the monomer concentration can no longer be equated to stoichiometric concentrations. Certain β-lactam antibiotics have shown surface-active properties (14).

Figure 4 shows a plot of log concentration *versus* surface tension for the sodium salts of nafcillin and ampicillin. The apparent critical micelle concentration (CMC) for sodium nafcillin (the break in the plot) is in the range of 20–30 mg/ml, the same point where saturation of the transport was observed. Sodium ampicillin, on the other hand, has a CMC above the concentrations used in the transport studies.

In summary, these studies show that the β-lactam antibiotics (both penicillins and cephalosporins) are not transported across the everted rat gut by any specialized transport mechanism. This was true for both penicillinase-sensitive and penicillinase-resistant compounds in this series, as well as for compounds (amphoteric) resembling amino acids. In addition, the results obtained with the model compound, glucose, demonstrate the utility of the everted rat gut for this type of investigation.

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New Carcinostatic Agent with Possible Selective Activity on Tumor Cells

I. NABIH [x] and A. ZAYED

Abstract □ The nitrogen mustard analog of the antitumor agent 1,2-cyclohexeno-4-(β-diethylaminoethylamino)thiaxanthone was synthesized. Its biological activity in experimental tumors *in vivo* and on human leukemia *in vitro* is described.

Keyphrases □ Nitrogen mustard analog of 1,2-cyclohexeno-4-(β-diethylaminoethylamino)thiaxanthone—synthesis and screening for carcinostatic and antitumor activities □ 1,2-Cyclohexeno-4-(β-diethylaminoethylamino)thiaxanthone, nitrogen mustard analog—synthesized and screened for carcinostatic and antitumor activities □ Antitumor activity—synthesis and screening of nitrogen mustard analog of 1,2-cyclohexeno-4-(β-diethylaminoethylamino)-thiaxanthone

Previous articles (1, 2) reported the antitumor activity of a new group of compounds related to the cyclohexenothiaxanthones. These are mainly represented by the three isomers 1,2-cyclohexeno-4-(β-diethylaminoethylamino)thiaxanthone (I), 2-(β-diethylaminoethylamino)-3,4-cyclohexenothiaxanthone (II), and 1-(β-diethylaminoethylamino)-3,4-cyclohexenothiaxanthone (III).

DISCUSSION

Structurally, the cyclohexenothiaxanthone system incorporates the thiaxanthone skeleton along with that of tetrahydronaphthalene. The rational for this combination is that the thiaxanthone tends to polarization due to resonance (3), thus indicating separation of charges and high dipole moments. This physical property would permit a selective attack on the tumor cells, avoiding the normal ones, at the appropriate concentrations, since the cellular membranes of the former carry considerably higher negative charges than their normal homologs (4, 5). Studies indicate that the thiaxanthone moiety in these compounds could be used as a carrier or a target-seeking device for the selective attack on tumor cells through electrostatic attraction.

The other structural part of the molecule, the tetralin, tends to attach molecular oxygen in an oxygenation process (6). Such oxygen carriers could affect the glycolysis in the cell by oxidation of the essential SH-groups of some enzymes necessary for cell growth and replication. In addition, the tetralins could undergo biological transformation to naphthoquinone-type structures, which are known to depress cell division.

In experimental tumors of the Ehrlich ascites carcinoma type, mammary carcinoma, Maloney virus lymphoma, and mouse ascites leukemia, the three isomers showed pronounced antitumor effects (1, 2). *In vitro* experiments on cells of these tumor types, as well as on leucocytes from several forms of human leukemia, showed that the compounds possessed varying but definite damaging effects on malignant cells.

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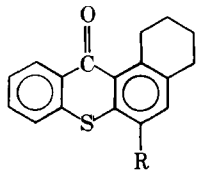

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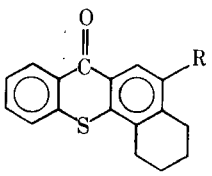

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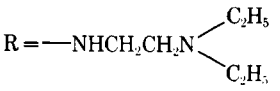

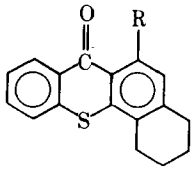

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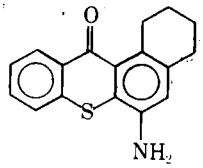

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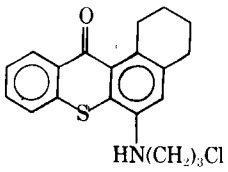

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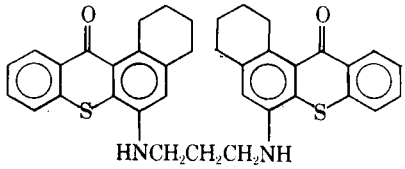
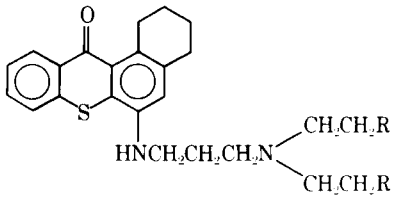


VI



VII: R = OH
VIII: R = Cl

cerning the so-called antimalarial mustards as possible chemotherapeutic agents in the management of neoplastic diseases (7). In chloroquine and quinacrine, which possess side chains that terminate in tertiary diethylamino groups, both terminal hydrogen atoms on each group were substituted with chlorine to give the nitrogen mustard analogs. In experimental and clinical trials, both analogs showed pronounced antitumor activity (8).

This finding initiated the synthesis of the nitrogen mustard analog of the compound, I. It was synthesized with three carbon atoms between the two nitrogens of the side chain. Compounds with increased number of carbon atoms between the nitrogens of the side chain of the antimalarials nitrogen mustard analogs showed increased antitumor activity and decreased host toxicity (9).

Reaction of 1,2-cyclohexeno-4-aminothiaxanthone (IV) (1) with 1-bromo-3-chloropropane in the presence of sodium acetate gave a mixture of 1,2-cyclohexeno-4-(β-chloropropylamino)thiaxanthone (V) and 4,4′-bis(1,2-cyclohexenothiaxanthone)-1,3-diaminopropane (VI). When this reaction was carried out in boiling *n*-butanol, V was the predominant product. Condensation of V with diethanolamine in the presence of sodium acetate gave 4-bis(2′-hydroxyethyl)aminopropylamino-1,2-cyclohexenothiaxanthone (VII). This compound, upon treatment with phosphorus oxychloride or thionyl chloride, gave the desired product, 4-bis(2′-chloroethyl)aminopropylamino-1,2-cyclohexenothiaxanthone (VIII).

EXPERIMENTAL

Biological—The product VIII is slightly soluble in water but is freely soluble in 70% ethanol. It was used as a colloidal suspension (through dissolution in ethanol and then dilution with water up to 8%).

In *in vivo* experiments, tumors of the Ehrlich ascites carcinoma, mammary carcinoma, and Maloney virus lymphoma cell lines were used for tests in mice, along with the appropriate controls. Treatment began on the 3rd day postinoculation of 10^6 cells of Ehrlich ascites carcinoma and 10^4 cells of mammary carcinoma and Maloney virus lymphoma types in the proper mice per host strains. (For Ehrlich ascites carcinoma type, CBA mice strains were used; for mammary carcinoma and Maloney virus lymphoma types, AxSn mice strains were used.) Doses were 60 mg/kg body weight daily for 5 consecutive days. In the treated mice, tumors did not develop throughout the follow-up 6-week postinoculation observation. In controls, mice that had been similarly inoculated but not treated developed tumors.

In the *in vitro* experiments, human leucocytes from acute myeloblast leukemia were used as substrates for the test compound. The leucocytes were collected and suspended in a balanced salt solution. Normal leucocytes collected from normal blood were used in the same manner as controls to test for the selectivity of the compound.

The compound to be tested was added as a colloidal suspension in 8% ethanol to correspond to two different concentrations of 0.2 and 0.5 mg/ml of the total medium. The normal leucocytes used as a control were also suspended in a balanced salt solution diluted with 8% ethanol and similarly treated at both concentration levels.

Examinations were made of dead and living leucocytes 24 hr after the addition of the material at both concentrations to the normal leucocytes and to the malignant ones. For differentiation between living and dead cells, trypan blue staining solution was used in aliquots taken from the treated leucocytes. This test showed that all malignant leucocytes were dead at both concentrations. Only a few normal cells were dead (10–15%) at both concentrations.

These results indicate that a certain selectivity exists for the uptake of the compound by the malignant leucocytes, which is not seen with the normal ones.

Chemical[1]—*2-Cyclohexeno-4-(β-chloropropylamino)thiaxanthone (V) and 4,4-Bis(1,2-cyclohexenothiaxanthone)-1,3-diaminopropane (VI)*—A mixture of IV (1) (3 g, 0.01 mole), 1-bromo-3-chloropropane (1.8 g, 0.01 mole), and sodium acetate (1.5 g, 0.01 mole) was heated at 150° for 3 hr. The gummy product was washed with sodium carbonate solution and water and extracted with ethyl acetate. The ethyl acetate was distilled and the residue was recrystallized twice from ethanol to give 1.4 g (37%) of V, mp 130–132°.

Anal.—Calc. for $C_{20}H_{20}ClNOS$: C, 67.12; H, 5.63; Cl, 9.90; N, 3.91. Found: C, 66.97; H, 5.56; Cl, 10.02; N, 3.83.

The ethyl acetate-insoluble residue fraction was recrystallized from benzene to give 1.2 g (19%) of VI, mp 220–222°.

Anal.—Calc. for $C_{37}H_{34}N_2O_2S_2$: C, 73.72; H, 5.69; N, 4.65. Found: C, 73.68; H, 5.65; N, 4.59.

The reaction was repeated under the following conditions. The same mixture of reactants was heated under reflux in *n*-butanol (30 ml) for 10 hr. The butanol was removed by steam distillation and the residue was recrystallized from ethanol to give 2.9 g (75%) of V.

4-Bis(2′-hydroxyethyl)aminopropylamino-1,2-cyclohexenothiaxanthone (VII)—A mixture of V (3.3 g, 0.0092 mole), diethanolamine (1 ml), and sodium acetate (2 g) was heated at 150° for 5 hr. The reaction product was washed with sodium carbonate solution and water and recrystallized from ethanol to give 3 g (84%) of VII, mp 151–153°.

Anal.—Calc. for $C_{24}H_{30}N_2O_3S$: C, 67.57; H, 7.07; N, 6.58. Found: C, 67.48; H, 6.98; N, 6.56.

[1] Melting points were taken in open capillary tubes by the use of a sulfuric acid bath and Gallenkamp melting-point apparatus and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, National Research Center, Cairo, U.A.R. IR spectra were determined on a UR 10 Zeiss-Jena IR, using KBr pellets.

4-Bis(2'-chloroethyl)aminopropylamino-1,2-cyclohexenothiaxanthone (VIII)—Compound VII (2 g, 0.0047 mole) was refluxed with phosphorus oxychloride (6 ml) for 3 hr, and excess oxychloride was removed under reduced pressure. The product was washed with sodium carbonate solution and water and recrystallized from benzene–petroleum ether to give 1.8 g (84%) of VIII, mp 160–162°.

Anal.—Calc. for $C_{24}H_{28}Cl_2N_2OS$: C, 62.26; H, 6.10; Cl, 15.32; N, 6.05. Found: C, 62.28; H, 6.17; Cl, 14.98; N, 5.97.

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[x] To whom inquiries should be directed. Present address: Karolinska Institutet, Medicinska Nobelinstitutet, Laboratorium för Enzymforskning, Solnavägen 1, S-104 01 Stockholm 60, Sweden.

COMMUNICATIONS

Evaluation of Mathematical Models for Diffusion from Semisolids

Keyphrases □ Diffusion, semisolids—evaluation of mathematical models □ Semisolids—evaluation of mathematical models for diffusion of drugs from semisolid systems

To the Editor:

Recently, Ayres and Laskar (1) reported an evaluation of models used to study the release of drugs from semisolid systems. Their analysis encompassed literature data (2–7) obtained on diverse experimental systems containing suspended or dissolved drugs in the form of gels, ointments, and emulsions. We find ourselves critical of both the approach and methods used in this analysis.

To establish the framework for our objections, it is necessary to present again the fundamental equations governing release of drugs from such systems. For semisolid systems initially containing uniformly dissolved drug in a homogeneous base, the amount of drug released in one dimension is given by Eq. 1 (8):

$$Q = hC_0\left[1 - \frac{8}{\pi^2}\sum_{m=0}^{\infty}\frac{1}{(2m+1)^2}\exp\left(-\frac{D(2m+1)^2\pi^2t}{4h^2}\right)\right] \quad \text{(Eq. 1)}$$

where:

Q = amount of drug released per unit area of application
h = thickness of vehicle layer
C_0 = initial concentration of drug in the vehicle
D = diffusion constant of drug in the vehicle
t = time after application
m = integer, with values from 0 to ∞

Equation 1, which is derived from Fick's second law of diffusion, is a valid expression for release from one side of a layer of vehicle containing drug in solution as long as the following assumptions are met (8):

1. There is a single drug in true solution and initially uniformly distributed throughout the vehicle.
2. The composition of the vehicle remains fixed during the diffusion process, *i.e.*, components other than the drug do not leave or enter the vehicle phase.
3. The diffusion constant of the drug is independent of time and position in the vehicle.
4. The drug reaching the receptor side of the vehicle layer is cleared rapidly.

For most practical applications of Eq. 1, a simplified form (Eq. 2) may be used up to about 30% drug release (8):

$$Q = 2C_0\left(\frac{Dt}{\pi}\right)^{1/2} \quad \text{(Eq. 2)}$$

Higuchi (9) derived an equation (Eq. 3) for studying the rate of drug release from ointment bases containing drugs in suspension. This equation relates the amount of drug released to time and other variables of the system:

$$Q = (2C_0 - C_s)\sqrt{Dt\left/1 + \frac{2(C_0 - C_s)}{C_s}\right.} \quad \text{(Eq. 3)}$$

where C_s = solubility of drug as units per centimeter^3 in the external phase of the ointment, and D = diffusion constant of drug in the external phase of the ointment.

The operative boundary conditions for the use of Eq. 3 are as follows: *(a)* the suspended drug is in a

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To establish the framework for our objections, it is necessary to present again the fundamental equations governing release of drugs from such systems. For semisolid systems initially containing uniformly dissolved drug in a homogeneous base, the amount of drug released in one dimension is given by Eq. 1 (8):

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The operative boundary conditions for the use of Eq. 3 are as follows: *(a)* the suspended drug is in a

fine state such that the particles are much smaller in diameter than the thickness of the applied layer; *(b)* the amount of drug, C_0, per unit volume is substantially greater than C_s, the solubility of the drug per unit volume of the vehicle; and *(c)* the surface to which the drug ointment is applied is immiscible with respect to the ointment and constitutes the perfect sink for the released drug.

For the common suspension case of $C_s \ll C_0$, Eq. 3 simplifies to Eq. 4:

$$Q = \sqrt{2C_0DC_st} \qquad \text{(Eq. 4)}$$

Ayres and Laskar (1) obtained Eq. 5 by ignoring the $m > 0$ summation terms in Eq. 1:

$$M_t = M_\infty - \frac{8M_\infty}{\pi^2}\exp\left(-\frac{D\pi^2 t}{A}\right) \qquad \text{(Eq. 5)}$$

where M_t = amount diffused up to time t, M_∞ = amount diffused to time infinity, and A = area over which diffusion occurs. No boundary conditions were specified.

To apply Eq. 1 or any simplified form, linear (Eq. 2) or exponential (Eq. 5), the experimental system must meet the basic assumptions of the physical model. These equations may be applied to a single drug in true solution and initially uniformly distributed throughout the vehicle. If the drug is suspended as fine particles in a semisolid system and the system is described as in the derivation of Eq. 3, then this equation or its simplified form (Eq. 4) may be used in obtaining physical parameters.

The experimental system should either meet the restrictions of the solution model (Eq. 1) or the suspension model (Eq. 3). It was stated (1) that Eq. 3 may take the form of Eq. 2 used in the analysis of diffusion of medicaments from semisolid dosage forms. This is totally incorrect and fundamentally wrong, because Eqs. 2 and 3 deal with distinctly different physical models.

The fluocinolone acetonide release data (2) from 20% propylene glycol–80% water, gelled with carboxypolymethylene[1] containing 0.025% drug, were analyzed in an attempt to show that the nonlinear curve-fitting procedures using Eq. 5 provided better fit compared to Eq. 2. The gel system in question contained drug in the form of a suspension at initial time and at some later time due to depletion of the solid drug; the gel did not contain drug in the form of a suspension. At the end of the experiment, almost 80% of the initial amount was released from the gel. This experiment was not designed to test either the solution model or the suspension model. Poulsen *et al.* (2) reported these data to demonstrate the effect of vehicle composition on the release of fluocinolone acetonide. Therefore, a basic flaw in the cited report (1) is the use of an incorrect physical model in the analysis of these data.

Similarly, these authors (1) incorrectly analyzed the published *in vitro* release data of salicylic acid (3%) in hydrophilic cream and white petrolatum USP (5). Since the salicylic acid was present as a suspended material, equations describing the solution case cannot be applied.

One assumption made in the derivation of Eq. 1 and its simplified form (Eq. 2) was that the diffusion constant must be constant with respect to time and position. In many situations involving emulsions, the diffusion constant is not constant but varies with concentration. Koizumi and Higuchi (3) analyzed this practical and important case by studying the release of an amine–amine hydrochloride solute mixture in water-in-oil emulsion into an aqueous sink. For an emulsion case, a general functional expression for the effective diffusion constant was obtained:

$$D_e = f(k_i, v_i, v_c, D_i, D_c) \qquad \text{(Eq. 6)}$$

The volumes of the internal phase, v_i, and the continuous phase, v_c, are assumed constant for a given system. Even when the diffusion constants of the internal phase, D_i, and the continuous phase, D_c, are constant, the effective diffusion constant, D_e, is a function of the partition coefficient of the internal phase, k_i, which may be concentration dependent. It was theoretically shown that even if the diffusion constant is concentration dependent, the release pattern is exactly the same as the constant D case.

The experimental system (3) used to study the release of pyridine from water-in-oil emulsion separated the unstirred emulsion from the well-stirred sink. The assumptions that the sink is perfect and that the drug concentration at the emulsion–sink interface is always maintained at zero were revised subsequently (4). A physical model considering the cases when the partition coefficient between emulsion and membrane is unity and not unity was presented. The effective diffusion constant, D_m, through the unstirred layer in and around the membrane was calculated by conducting experiments in which both the emulsion and sink were stirred. The effective diffusion constant in the emulsion was calculated by either the Bruggeman or Wagner–Wiener equation. The comparisons between experiment and theory were in excellent agreement when the effects of the membrane and stirring were considered in the physical model.

Ayres and Laskar (1) took one set of pyridine release data from a water-in-oil emulsion system (3) and analyzed these data using Eqs. 2 and 5. Since more rigorous physical models were in the literature, it is not clear what these authors achieved by using curve-fitting procedures. Specifically, the authors not only ignored the rigorous physical models appropriate for studying the release of drugs from emulsion systems but also overlooked the basic assumptions made in deriving Eq. 1.

In conclusion, our understanding of drug release from semisolid systems has evolved to a point where the physical model approach has been quite useful in helping to explain and to predict the roles of various factors involved in the transport phenomena. The meaningful application of this technique, however, requires that the models and the mathematics em-

[1] Carbopol.

ployed are appropriate from the physical standpoint. Statistical analyses and curve-fitting procedures used without regard for the physical basis in the equations are of less than doubtful value in this area of biopharmaceutics.

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Z. T. Chowhan [x]
B. J. Poulsen
Syntex Research
Institute of Pharmaceutical Sciences
Palo Alto, CA 94304

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[x] To whom inquiries should be directed.

Development of a Stable Sublingual Nitroglycerin Tablet I: Interaction of Nitroglycerin with Selected Macromolecules

Keyphrases □ Nitroglycerin—stable sublingual tablet, formulated with macromolecules □ Tablets, nitroglycerin sublingual—development of stable tablet, nitroglycerin interacted with selected macromolecules □ Formulation—development of stable sublingual nitroglycerin tablet, interaction of nitroglycerin with macromolecules

To the Editor:

There has recently been intense research activity in the pharmaceutical aspects of the angina drug nitroglycerin (1–3). The high volatility of nitroglycerin leads to a loss of drug to the environment during patient storage and use (4). Consequently, the Food and Drug Administration (FDA) has now instituted regulations governing the dispensing and the types of containers that may be used for sublingual nitroglycerin tablets, and the two most popular brands available in this country were recently reformulated for improved stability (2, 5).

We have found that although these products demonstrate somewhat better stability and content uniformity characteristics compared to previous formulations under certain conditions, their stability under more severe, but not unanticipated storage conditions, *e.g.*, when the tablets are exposed to the atmosphere in a semiclosed or open container, is inadequate. There is, therefore, a need to develop a nitroglycerin sublingual tablet whose stability can be assured over a sufficiently long period without any stringent storage conditions imposed on the patient and inconvenient dispensing restrictions imposed on the pharmacist.

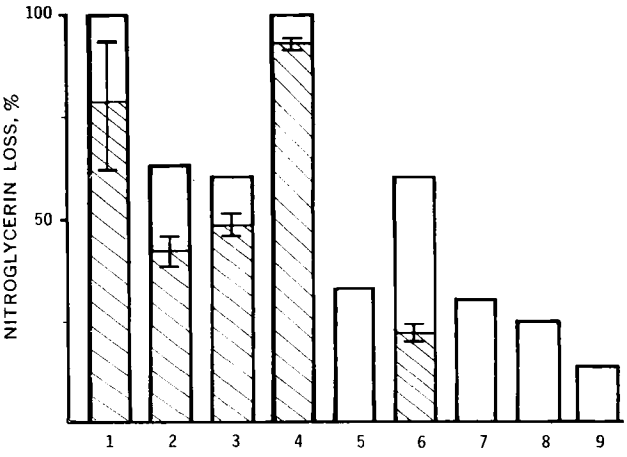

Figure 1—*Percentage nitroglycerin loss in different powder mixes after storage under vacuum (shaded) and under ambient (open) conditions. Key: 1, control; 2, Brand A; 3, Brand B; 4, 20% gelatin; 5, 20% hydroxypropyl cellulose; 6, 1% polyvinylpyrrolidone; 7, 3% polyvinylpyrrolidone; 8, 5% polyvinylpyrrolidone; and 9, 10% polyvinylpyrrolidone. Relative standard deviations for the shaded columns are indicated by bars, but those for the open columns are too small to be shown.*

The present communication reports our initial studies on the lowering of the thermodynamic activity of nitroglycerin when dispersed in powders and sublingual tablets with different macromolecules. Experimental sublingual tablets containing polyvinylpyrrolidone showed vastly improved stability over existing commercial brands.

Figure 1 shows the percentage nitroglycerin loss in different powder mixes under two sets of "accelerated" experimental conditions: *(a)* completely exposed to the atmosphere for 9 days at ambient temperatures (open columns), and *(b)* under vacuum in a desiccator over an activated charcoal bed at 1–3 torr for 4 hr at ambient temperatures (shaded columns). Commercial tablets (Brands A[1] and B[2]) were purchased locally and were ground up to approximately comparable particle size. The control powder contained 0.6 mg of nitroglycerin[3] in 30 mg of lactose, and the test powders contained the designated percent (w/w) of macromolecules as an additive.

Triplicate samples of the powder mixes were assayed prior to and after the respective treatment by the kinetic assay method recently developed in this laboratory (1). Data from the vacuum study represent composites, with a minimum of two runs for each powder mix carried out on different days. It is evident from Fig. 1 that the two accelerated stability test conditions showed similar rank-order correlation

[1] Eli Lilly & Co., 0.6-mg tablets, Lot 6VP80A.
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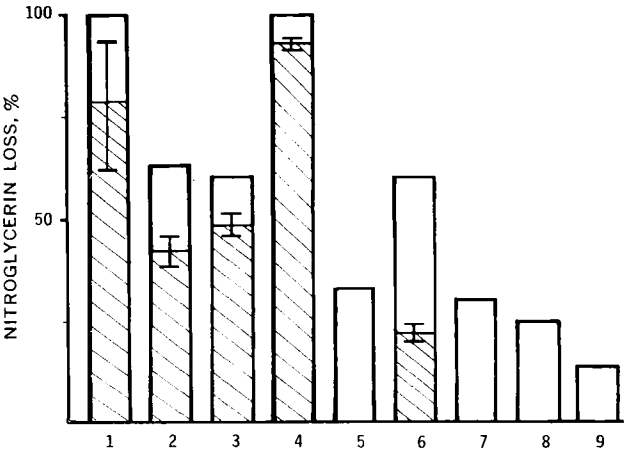

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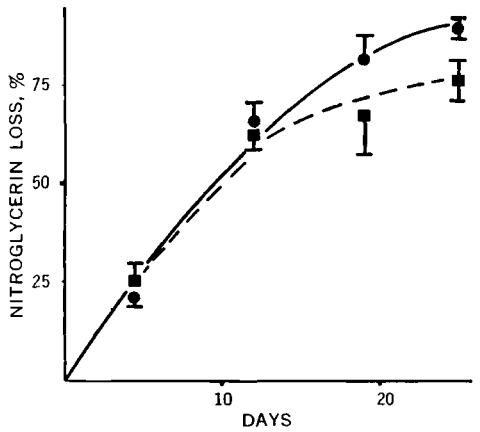

Figure 2—*Percentage loss of nitroglycerin from commercial tablets. Key:* ■, *Brand A; and* ●, *Brand B. Bars indicate relative standard deviations.*

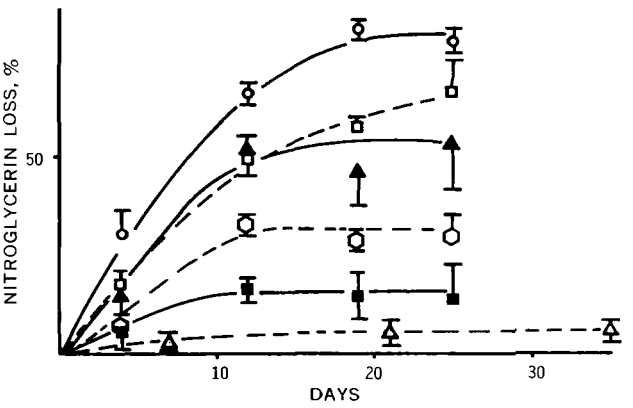

Figure 3—*Percentage loss of nitroglycerin from experimental sublingual tablets containing various percentages of polyvinylpyrrolidone. Key:* ○, *0%;* □, *1%;* ▲, *3%;* ◌, *5%;* ■, *10%; and* △, *15%. Bars indicate relative standard deviations.*

for the relative stability of nitroglycerin in the powder mixes studied. Powders from the commercial tablets were more stable than the control and that containing 20% gelatin but were less stable than those containing hydroxypropyl cellulose and polyvinylpyrrolidone[4].

Although the vacuum method revealed a better stability for the powder mix containing 1% polyvinylpyrrolidone as compared to the commercial lots, it was less sensitive in distinguishing the relative stability of powder mixes containing 20% hydroxypropyl cellulose and 3, 5, and 10% polyvinylpyrrolidone, all of which lost no nitroglycerin after the prescribed period. The atmospheric exposure method, however, showed that preparations containing 3% or more polyvinylpyrrolidone were more stable than that containing 20% hydroxypropyl cellulose and that the stability increased with increasing polyvinylpyrrolidone content. These simple preliminary accelerated stability tests led to the selection of polyvinylpyrrolidone as a stabilizer in preparing experimental sublingual tablets for further stability screening.

Experimental sublingual tablets with different polyvinylpyrrolidone concentrations (0–15% w/w) were prepared by molding, using an aqueous alcohol solvent mix for granulation. The tablet size ranged from about 75 to 90 mg, with approximately 0.6 mg of nitroglycerin/tablet. The stability of these tablets and commercial lots was monitored for about 1 month at various times after they were placed in open petri dishes at ambient temperatures. The petri dishes had to be placed at least 61 cm (2 ft) from each other or the more stable tablets gained in nitroglycerin content at the expense of their less stable neighbors. At least five tablets were assayed each time.

Figures 2 and 3 show the percentage nitroglycerin loss from commercial and experimental tablets, respectively. Consistent with the results of the accelerated powder studies, the experimental molded tablets containing 3% or more of polyvinylpyrrolidone were much more stable than the commercial lots tested. When the polyvinylpyrrolidone content was high, the loss of nitroglycerin reached a plateau after about 15 days. In fact, tablets containing 10 and 15% polyvinylpyrrolidone were still within the USP specifications after nearly 1 month of complete exposure to the atmosphere.

The mechanism of interaction between polyvinylpyrrolidone and nitroglycerin is probably of electronic origin since organic nitrates are known to be electron acceptors (6) and polyvinylpyrrolidone is an electron donor (7). This solid phase interaction lowers the fugacity of nitroglycerin in the dosage form, but the complex formed between nitroglycerin and polyvinylpyrrolidone is most likely to be extremely unstable in an aqueous environment because of preferential solvation of polyvinylpyrrolidone molecules by water. The solid phase stabilization, therefore, is not expected to affect the bioavailability of nitroglycerin significantly. When experimental tablets containing 10% polyvinylpyrrolidone were dissolved in water and extracted with octanol, the amount of nitroglycerin recovered from the organic phase was the same as that recovered from control systems containing Brands A and B.

Detailed *in vitro* dissolution and *in vivo* studies in humans are now underway to ascertain the bioavailability of these stabilized nitroglycerin sublingual tablets.

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Ho-Leung Fung ˣ
S. K. Yap
C. T. Rhodes

Department of Pharmaceutics
State University of New York at Buffalo
Buffalo, NY 14214
Received July 19, 1974.

[4] Plasdone C-30, GAF Corp.

Accepted for publication August 15, 1974.
Supported by the Pharmaceutical Division, Fisons Corp., Bedford, Mass.
[x] To whom inquiries should be directed.

Absolute Drug Bioavailability: Approximation without Comparison to Parenteral Dose for Compounds Exhibiting Perturbable Renal Clearance

Keyphrases □ Bioavailability—approximation of absolute drug bioavailability without comparison to parenteral dose for compounds exhibiting a perturbable renal clearance □ Drug bioavailability—approximation without comparison to parenteral dose, compounds exhibiting perturbable renal clearance

To the Editor:

It is generally accepted that the absolute bioavailability of a drug dosage form can only be determined by comparison with a parenteral dose. However, ethical and legal considerations prevent injection of many compounds, greatly inhibiting the acquisition of bioavailability data for new drugs.

The principal purpose of this communication is to demonstrate that it is possible to approximate the absolute bioavailability of a large class of drugs (1), those whose renal clearance is perturbable, without the administration of a parenteral dose. As an example, consider an agent that exhibits an area under the plasma concentration–time curve of AUC under condition X (*e.g.*, coadministration of a urinary acidifying agent) and of AUC' under condition Y (*e.g.*, coadministration of urinary alkalinizing agent). Similarly, let Cl_B, Cl_R, and Cl_{NR} equal the total body clearance, mean renal clearance, and nonrenal clearance, respectively, during condition X, and let the prime notation indicate their values under condition Y. Finally, let D equal the dose administered and F equal the fraction of the dose that is absorbed. The following analysis assumes that: (*a*) the system is linear, and (*b*) Cl_{NR}, F, and intercompartment transfer constants (if any) are independent of the perturbation of renal clearance.

Since:

$$Cl_B = Cl_R + Cl_{NR} \qquad \text{(Eq. 1)}$$

and:

$$Cl_B' = Cl_R' + Cl_{NR} \qquad \text{(Eq. 2)}$$

by letting:

$$Cl_R - Cl_R' = \Delta Cl_R \qquad \text{(Eq. 3)}$$

and:

$$Cl_B - Cl_B' = \Delta Cl_B \qquad \text{(Eq. 4)}$$

it follows that:

$$\Delta Cl_R = \Delta Cl_B = Cl_B - Cl_B' \qquad \text{(Eq. 5)}$$

From the model independent equation:

$$\text{total body clearance} = \frac{DF}{\text{area under the plasma concentration-time curve}} \qquad \text{(Eq. 6)}$$

and Eq. 5, it is apparent that:

$$\Delta Cl_R = \frac{DF}{AUC} - \frac{DF}{AUC'} \qquad \text{(Eq. 7)}$$

or:

$$F = \frac{\Delta Cl_R}{D}\left[\frac{(AUC)\ (AUC')}{AUC' - AUC}\right] \qquad \text{(Eq. 8)}$$

Since all terms on the right-hand side of Eq. 8 can be determined experimentally without reference to a parenteral dose, it follows that absolute bioavailability may be approximated by this method given the validity of the listed assumptions.

(1) A. M. Asatoor, D. R. London, M. D. Milne, and M. L. Simenoff, *Brit. J. Pharmacol.*, **20**, 285(1963).

David Lalka [x]
Hal Feldman

Research Laboratories
Astra Pharmaceutical Products Inc.
Worcester, MA 01606

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[x] To whom inquiries should be directed.

Gastric Irritation and Bleeding after Drug Administration

Keyphrases □ Gastric irritation and bleeding—effects of aspirin, phenylbutazone, methyl salicylate, salicylic acid, and triamcinolone, powder and solution forms, effects of polyethylene glycol bases, rats □ Bleeding, gastric—effects of various drugs under varying experimental conditions, rats □ Drug administration—extent of gastric irritation and bleeding caused by aspirin, phenylbutazone, methyl salicylate, salicylic acid, and triamcinolone, powder and solution forms, effects of polyethylene glycol bases, rats

To the Editor:

Many drugs are known to be irritating to the stomach and GI tract, and some have been shown to produce gastric ulceration and bleeding (1–3). This communication describes results obtained in a study to determine if various drugs would induce bleeding or ulceration in the stomach of rats under various experimental conditions.

Seventy rats, 130–150 g, were divided into groups (four to six rats in each group). The rats were fasted in screen-bottom cages for 24 hr prior to drug administration. During the fast, water was allowed *ad libitum*. The animals then received the following drugs

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David Lalka [x]
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David Lalka [x]
Hal Feldman

Research Laboratories
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Seventy rats, 130–150 g, were divided into groups (four to six rats in each group). The rats were fasted in screen-bottom cages for 24 hr prior to drug administration. During the fast, water was allowed *ad libitum*. The animals then received the following drugs

by means of an oral stomach tube: 0.5 ml of a solution of aspirin (100 mg/ml) in polyethylene glycol 400, 0.5 ml of a solution of salicylic acid (100 mg/ml) in polyethylene glycol 400, 0.5 ml of 95% alcohol, 0.5 ml of methyl salicylate, 0.5 ml of acetylated and methoxypolyethylene glycol 350, 0.5 ml of a solution of phenylbutazone (20 mg/ml) in polyethylene glycol 400, and 0.5 ml of a solution of caffeine (20 mg/ml) in water. One group of rats received the solution of aspirin in polyethylene glycol rectally.

Drugs were also administered directly into the stomach in powder form; a device for such administration of powder was made in this laboratory. It consisted of a 20-gauge, flexible, hollow stainless steel cannula (about 10 cm) with a piece of tubing fitted to the end. Tubing dimensions were 3.2 mm o.d., 2.4 mm i.d., and 15 mm length. Prior to the drug administration, paraffin oil was applied to the surface of tubing. The tubing was packed with the drugs in powder form, and the powder was forced out into the stomach by depressing the plunger connected to the flexible cannula. Drugs administered in this manner were triamcinolone, phenylbutazone, and aspirin. To study the effect of food in the stomach on the gastric bleeding, one group of rats was allowed food at least 1 hr before the administration of aspirin powder and aspirin solution.

One hour after administration of the various drugs, each group of rats was sacrificed with chloroform. The stomach was removed, opened along the line of lesser curvature, and observed for the presence of bleeding or ulceration.

In the rats that received the aspirin solution orally, an average of four or five lesions and some slight bleeding were observed. There was evidence of ulceration and bleeding in the rats that received 25 mg of aspirin in powder form. The rats that received the solution of salicylic acid had a large amount of bleeding, and gastric lesions were produced.

The alcohol produced a large extent of irritation and erythema of the stomach mucosa but no bleeding or lesions. This result is in contrast to the finding of Morris *et al.* (4), who reported that the administration of 0.3 ml of 30% ethanol solution orally produced gastric lesions in the stomach of the rat. The number and severity of the lesions were similar to those produced by the administration of aspirin solution.

Rats that received acetylated and methoxypolyethylene glycol base showed no bleeding or lesion production, with the exception of one rat in the group. There was extensive dilatation of the blood vessels of the stomach of one rat following administration of acetylated polyethylene glycol base. The substituted polyethylene glycol 400 was used to see whether chemical alteration of the base has any effect on gastric irritation. The administration of methyl salicylate produced some slight redness and irritation of the stomach mucosa, but no bleeding or ulceration was observed.

The administration of phenylbutazone in a solution produced four or five lesions in the stomach of the rat. This result is similar to that obtained with the aspirin solution in this study. However, Alphin and Droppleman (5) reported that phenylbutazone produced less ulceration and bleeding in the cat than were produced by aspirin. Phenylbutazone is known as a possible ulcerogenic drug, and its ulcerogenic effect was suggested to be due to reduced mucosal resistance to the erosive action of acid and pepsin.

When phenylbutazone (25 mg) was administered in the powder form, no gastric bleeding and lesions were found in the rats. This was due to poor dissolution of the drug in the stomach; phenylbutazone powder was found in the stomach of the rat after 1 hr.

After the administration of a caffeine solution, there was no evidence of bleeding or the presence of lesions. Little or no irritation was produced by the caffeine on the stomach mucosa of the rat.

None of the rats given triamcinolone powder (20 mg) showed any evidence of bleeding or ulceration of the stomach. Triamcinolone is also known to be ulcerogenic in susceptible humans.

In the final group of rats, food was allowed at least 1 hr before the administration of aspirin solution or aspirin powder. No bleeding or ulceration was seen in any of these rats.

This study indicates that common drugs can cause the production of lesions in the stomach of the rat. One of the most widely used compounds by humans, aspirin, produced irritation, bleeding, and lesion formation upon administration of aspirin powder or solution by mouth in the dose studied. Since aspirin is freely soluble in polyethylene glycol base, this observation suggests that aspirin, when administered in a solution, can cause as much gastric bleeding and ulceration as the administration of a solid form of aspirin. Polyethylene glycol base did not seem to prevent gastric lesions produced by aspirin.

Little or no bleeding or lesion production was seen in the rats that received the aspirin solution rectally. The presence of food in the stomach appears to reduce bleeding and ulcer production usually caused by aspirin.

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J. G. Strom
H. W. Jun [x]

School of Pharmacy
University of Georgia
Athens, GA 30602

Received May 13, 1974.
Accepted for publication August 13, 1974.
[x] To whom inquiries should be directed.

REVIEWS

The *Vinca* Alkaloids: Botany, Chemistry, and Pharmacology. Edited by WILLIAM I. TAYLOR and NORMAN R. FARNSWORTH. Dekker, New York, NY 10016, 1973. 357 pp. 16 × 24 cm. Price $32.75.

The dedication to Jozef Mokry is most appropriate. I knew him for but a short while during which time it was my privilege to become his friend. His untimely death in 1966 ended the brilliant career of a dedicated scientist. He was indeed a gentleman and a gentle man.

The editors have done an excellent job in selecting an outstanding group of contributors to review all pertinent aspects of this genus. The taxonomy is lucidly presented and should settle once and for all the *Vinca–Catharanthus* controversy. The volume includes material presented at the 1964 symposium on the subject, sponsored by the American Society of Pharmacognosy, and follows the developments for the next 6–7 years, the latest cited reference being 1971.

This volume was apparently a long time in the making, and as a result the accomplishments of the early '70's are not included. While this may detract somewhat from its value, it is thorough for the years covered. This reviewer has never been enamored with the photo-offset process of printing, especially at the price of $32.75. However, I still consider this volume to be a worthy addition to the library of the serious alkaloid researcher.

Reviewed by Gordon H. Svoboda
Eli Lilly and Co.
Indianapolis, IN 46206

Methods of Analysis of Anti-Epileptic Drugs. Edited by J. W. A. MEIJER, H. MEINARDI, C. GARDNER-THORPE, and E. VAN DER KLEIJN. American Elsevier Co., Inc., 52 Vanderbilt Ave., New York, NY 10017, 1973. xvi + 258 pp. 17 × 25 cm. Price $26.50.

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The book contains a list of the chemical and proprietary names of the common antiepileptic drugs which would be useful to both the clinician and analyst alike.

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Reviewed by Dennis J. Weber
Drug Metabolism Research
Physical and Analytical Chemistry
The Upjohn Company
Kalamazoo, MI 49001

Temporal Aspects of Therapeutics. Edited by J. URQUHART and F. E. YATES. Plenum Press, Div. of Plenum Publishing Corp., 227 West 17th St., New York, NY 10011, 1973. 213 pp. 16.8 × 25.7 cm. Price $14.50.

This book, the second volume of a series, represents the proceedings of a conference held October 18-20, 1972 and sponsored by the innovative Alza Corporation of Palo Alto, Calif. It speaks to biochemists, pharmacologists, and endocrinologists about the need for more polyparameter quantitative experiments and their interpretation by multivariate analysis. While simple versions of such experiments are becoming common in the area of pharmacokinetics, they are rarely conducted and published by the above specialists. Such experiments allow for the construction of dynamic mathematical models from which extrapolations can be made relevant to normal/abnormal life processes, the interaction of drugs and life processes, and the interaction of multidrug and multilife processes. Insights achieved from such extrapolations demand further polyparameter experiments, *etc.* This volume focuses on only one such dynamic parameter, time, and proceeds from basics up to the design of effective drug delivery systems. Listing of chapter headings (authors) indicates the logical progression of the material presented: The Biological Time Scale (H. J. Morowitz), Temporal and Hierarchical Organization in Biosystems (F. E. Yates and A. S. Iberall), Time and Timelessness in Biological Clocks (A. T. Winfree), Sequential Assembly of Virus Particles (D. Kaiser), Temporal Aspects of Macromolecular Synthesis in Eukaryotic Cells (E. B. Thompson), Hormonal Control of the Menstrual Cycle and Ovulation in the Rhesus Monkey (E. Knobil), Pharmacokinetic Aspects of Controlled Drug Delivery Systems (G. Levy), Cell Proliferation Characteristics and Cancer Chemotherapy (S. Perry), Chronopharmacology in the Treatment of Hypertension with Diuretics (W. J. Meyer, III, C. S. Delea, and F. C. Bartter), Testosterone Polydimethylsiloxane Implants and Contraception in Male Rabbits (L. L. Ewing, C. Desjardins, and L. G. Stratton), and Progress Towards an Implantable Glucose Sensor and an Artificial Beta Cell (J. S. Soeldner, K. W. Chang, S. Aisenberg, and J. M. Hiebert). Each presentation is accompanied by abundant references and is followed by a round-table discussion by the participants. These discussions are often more illuminating than the corresponding presentations. This volume is an excellent scientific book and is highly recommended. Its language is both academic and technical. It will not appeal to those individuals operating pragmatically in the traditional random mode. A practicing physician at the conference made the following remark at the end of the first paper and was not heard from again, "For God's sake, please try to make things as simple as possible."

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Drug Metabolism Research
Physical and Analytical Chemistry
The Upjohn Company
Kalamazoo, MI 49001

Temporal Aspects of Therapeutics. Edited by J. URQUHART and F. E. YATES. Plenum Press, Div. of Plenum Publishing Corp., 227 West 17th St., New York, NY 10011, 1973. 213 pp. 16.8 × 25.7 cm. Price $14.50.

This book, the second volume of a series, represents the proceedings of a conference held October 18-20, 1972 and sponsored by the innovative Alza Corporation of Palo Alto, Calif. It speaks to biochemists, pharmacologists, and endocrinologists about the need for more polyparameter quantitative experiments and their interpretation by multivariate analysis. While simple versions of such experiments are becoming common in the area of pharmacokinetics, they are rarely conducted and published by the above specialists. Such experiments allow for the construction of dynamic mathematical models from which extrapolations can be made relevant to normal/abnormal life processes, the interaction of drugs and life processes, and the interaction of multidrug and multilife processes. Insights achieved from such extrapolations demand further polyparameter experiments, *etc.* This volume focuses on only one such dynamic parameter, time, and proceeds from basics up to the design of effective drug delivery systems. Listing of chapter headings (authors) indicates the logical progression of the material presented: The Biological Time Scale (H. J. Morowitz), Temporal and Hierarchical Organization in Biosystems (F. E. Yates and A. S. Iberall), Time and Timelessness in Biological Clocks (A. T. Winfree), Sequential Assembly of Virus Particles (D. Kaiser), Temporal Aspects of Macromolecular Synthesis in Eukaryotic Cells (E. B. Thompson), Hormonal Control of the Menstrual Cycle and Ovulation in the Rhesus Monkey (E. Knobil), Pharmacokinetic Aspects of Controlled Drug Delivery Systems (G. Levy), Cell Proliferation Characteristics and Cancer Chemotherapy (S. Perry), Chronopharmacology in the Treatment of Hypertension with Diuretics (W. J. Meyer, III, C. S. Delea, and F. C. Bartter), Testosterone Polydimethylsiloxane Implants and Contraception in Male Rabbits (L. L. Ewing, C. Desjardins, and L. G. Stratton), and Progress Towards an Implantable Glucose Sensor and an Artificial Beta Cell (J. S. Soeldner, K. W. Chang, S. Aisenberg, and J. M. Hiebert). Each presentation is accompanied by abundant references and is followed by a round-table discussion by the participants. These discussions are often more illuminating than the corresponding presentations. This volume is an excellent scientific book and is highly recommended. Its language is both academic and technical. It will not appeal to those individuals operating pragmatically in the traditional random mode. A practicing physician at the conference made the following remark at the end of the first paper and was not heard from again, "For God's sake, please try to make things as simple as possible."

Reviewed by Marvin H. Malone
Department of Physiology-Pharmacology
School of Pharmacy
University of the Pacific
Stockton, CA 95204

REVIEWS

The *Vinca* Alkaloids: Botany, Chemistry, and Pharmacology. Edited by WILLIAM I. TAYLOR and NORMAN R. FARNSWORTH. Dekker, New York, NY 10016, 1973. 357 pp. 16 × 24 cm. Price $32.75.

The dedication to Jozef Mokry is most appropriate. I knew him for but a short while during which time it was my privilege to become his friend. His untimely death in 1966 ended the brilliant career of a dedicated scientist. He was indeed a gentleman and a gentle man.

The editors have done an excellent job in selecting an outstanding group of contributors to review all pertinent aspects of this genus. The taxonomy is lucidly presented and should settle once and for all the *Vinca–Catharanthus* controversy. The volume includes material presented at the 1964 symposium on the subject, sponsored by the American Society of Pharmacognosy, and follows the developments for the next 6–7 years, the latest cited reference being 1971.

This volume was apparently a long time in the making, and as a result the accomplishments of the early '70's are not included. While this may detract somewhat from its value, it is thorough for the years covered. This reviewer has never been enamored with the photo-offset process of printing, especially at the price of $32.75. However, I still consider this volume to be a worthy addition to the library of the serious alkaloid researcher.

Reviewed by Gordon H. Svoboda
Eli Lilly and Co.
Indianapolis, IN 46206

Methods of Analysis of Anti-Epileptic Drugs. Edited by J. W. A. MEIJER, H. MEINARDI, C. GARDNER-THORPE, and E. VAN DER KLEIJN. American Elsevier Co., Inc., 52 Vanderbilt Ave., New York, NY 10017, 1973. xvi + 258 pp. 17 × 25 cm. Price $26.50.

This book reports on the proceedings of the Workshop on the Determination of Anti-Epileptic Drugs in Body Fluids held at Noordwijkerhaut, The Netherlands, April 1972. The book is divided into four parts and each part contains from four to eight papers. There are reference and discussion sections at the end of each paper. There are a total of 23 papers given in the volume. At the end of each of the four main sections there is a more complete discussion on the papers given.

Part I gives the rationale for the need to determine blood levels of antiepileptic drugs to determine dosage schedules properly. The section also gives some pharmacokinetic concepts as they relate to epilepsy. The other three sections give detailed reports on spectrophotometric, fluorometric, thin-layer, and gas chromatographic analyses of antiepileptic drugs. The problems of derivatization, interference by metabolites, and the effect of multiple-drug therapy on analytical procedures are frequent topics of discussion in the book. A very wide and complete list of antiepileptic drugs is discussed and detailed. Analytical procedures for all drugs commonly used, whether alone or in multiple-drug therapy, are given. Gas chromatography is by far the preferred analytical technique.

The book contains a list of the chemical and proprietary names of the common antiepileptic drugs which would be useful to both the clinician and analyst alike.

There is a section summarizing the various analytical procedures as to drugs analyzed, extraction procedures, gas chromatographic conditions, retention times, and average blood levels found in patients. There is also a cumulative bibliography section and a subject index.

Anyone who has an interest in the drug therapy of epileptics or the anaylsis of blood levels of antiepileptic drugs will find this book to be very useful. The book is an excellent summary of the current state of the analysis of antiepileptic drugs and details the problems that still exist in the field.

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Reviewed by Marvin H. Malone
Department of Physiology-Pharmacology
School of Pharmacy
University of the Pacific
Stockton, CA 95204

Journal of Pharmaceutical Sciences

DECEMBER 1974

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QUANTITATIVE FORMULA DISCLOSURE

Last month in this column we wrote about "Drugs Anonymous," and we expressed our thoughts on the desirability of a requirement that the identity of the actual manufacturer of the drug product be included on the label of the article.

After that column had gone to the printer, but before its final publication, the Pharmaceutical Manufacturers Association announced a new policy adopted by its Board. Since PMA had formerly been the leading opponent to such a requirement, this change of policy—which now favors such a national legislative requirement—constitutes a very dramatic reversal on the part of this influential organization within the drug industry. Consequently, we are pleased to note this positive step forward and compliment the organization for its willingness to swallow pride and adopt this position which has been long advocated by APhA and a number of other pharmacy groups.

Consideration of this subject brings us to the next logical question. Namely, now that we know who makes a product, how do we know what is in it?

This latter problem has not been associated with prescription legend drug products, but it has been a significant shortcoming relative to the labeling of nonprescription or over-the-counter (OTC) drug products. The law requires only that active ingredients be named or listed in the drug label, and there is no obligation to provide quantitative information. Consequently, a very large proportion of OTC drug products have made no quantitative disclosure of their content.

Our February 1971 editorial was devoted to a discussion of this situation under the title "'Patent' Medicines vis-à-vis An Enlightened Society." That column specifically described the frequent difficulty in obtaining drug composition information for inclusion in the Association's publication the *Handbook of Non-Prescription Drugs*. It also criticized the intransigence of many firms within the proprietary drug industry: "... *furthermore, this practice (of keeping secret formulations) is tacitly supported, if not avidly defended, by much of the industry manufacturing these products.*"

APhA has persisted in its efforts to overcome this situation. It has done so by urging the proprietary drug industry voluntarily to adopt policies and practices which would accomplish the intended purpose and also by making such recommendations to legislative and regulatory agencies whenever the opportunity arose. For example, such a suggestion was included in the filing APhA made on the proposed regulations FDA issued concerning OTC antacid products. However, when the antacid regulations were issued in final form this summer, the Commissioner concluded that, despite its desirability, FDA did not have authority to issue such a requirement under present law. Hence, he could only request "... *that manufacturers voluntarily place such information on their label ...*."

But again there is welcome news. Late this past summer, APhA was informed that the Board of Directors of the Proprietary Association had made a policy decision to recommend to all PA member firms that they list the quantity of all active ingredients on the labels of all OTC medicines they market.

Whether as a result of their trade association recommendation or not—we have no way of knowing—we have already noted a decided swing toward inclusion of this information on OTC drug product labels.

Indeed, one major company's recent annual report repeatedly emphasized, in pictorial form and by way of prominently displayed quotations, their dedication to providing just this type of information. The entire front cover of this imaginatively prepared report is devoted to a one-sentence quote from the company's founder which was written forty-one years earlier, and starts out, "*Medicines should be good formulas, fairly priced and truthfully advertised ...*."

There is an implication that during the intervening years individual proprietary drug manufacturers may have deviated from these goals to varying degrees. Adoption of, and implementation of, the voluntary decision to reveal the quantitative formulas of their products indicate that this industry is recognizing its consumer and professional responsibilities. This first step is an encouraging sign; it is hoped that more will follow.

Edward G. Feldmann

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REVIEW ARTICLE

Pharmaceutical and Cosmetic Aerosols

JOHN J. SCIARRA

Keyphrases □ Aerosols, pharmaceutical and cosmetic—product formulation, components, manufacturing and packaging, testing, toxicity, and abuse, review □ Pharmaceutical aerosols—product formulation components, manufacturing and packaging, testing, toxicity, and abuse, review □ Cosmetic aerosols—product formulation components, manufacturing and packaging, testing, toxicity, and abuse, review □ Dosage forms, aerosols—literature review of pharmaceutical and cosmetic aerosols □ Toxicity—pharmaceutical and cosmetic aerosols, review

CONTENTS

Product Formulation 1816
Systems 1816
Propellants 1817
Formulation 1818
Solutions 1818
Dispersions and Powders 1820
Emulsions 1822
Semisolids 1824
Aerosol Components 1825
Valves 1825
Applicators and Fitments 1826
Containers 1826
Manufacturing and Packaging of Aerosols 1827
Testing of Aerosol Products 1827
General Testing 1827
Analytical Procedures 1828
Evaluation of Foams 1828
Particle-Size Analysis 1828
Other Methods 1831
Toxicity Considerations 1831
Deliberate Product Abuse 1831
Propellant Toxicity 1831
Product Toxicity 1833
Conclusion 1833

Aerosol science and technology have been extended to numerous different products over the past 10–15 years, as evidenced by the fact that during 1973 over 2.9 billion aerosol units were produced in the United States and Canada (1). This amount, together with the over 1.5 billion units produced in the rest of the world, makes an impressive record based upon units produced (2). However, not so impressive when compared to these figures is the total production of pharmaceutical aerosols during the past years. In 1973 this production amounted to approximately 45 million units (1). Included were such products as antiasthmatic sprays, burn treatments, room vaporizers, antiseptics, oral anesthetics, contraceptives, fungicides, and various dermatological products. These product types are representative of the nature of pharmaceuticals packaged in aerosol form.

If one were to include in this category many of the products classified as "cosmetics," then the product figures as well as product categories present quite a different picture. Products such as antiperspirants and deodorants, feminine hygiene sprays, and various skin products accounted for over 500 million units during this same year (1). The development and formulation, as well as the manufacture and distribution of many of these cosmetic products, have come within the sphere of interest of the "pharmaceutical scientist" and the pharmaceutical industry due to diversification within the pharmaceutical industry as well as acquisition of pharmaceutical concerns by cosmetic companies and vice versa.

The first textbook devoted exclusively to the subject of aerosol science and technology appeared in 1958 and was authored by Herzka and Pickthall (3).

This was followed by the second edition in 1961 (4). In 1961, Shepherd (5) edited a comparable aerosol textbook, and other aerosol textbooks have appeared over the years (6–11). While all of these publications cover the aerosol field, the text by Johnsen *et al.* (10) is intended primarily as a reference to many practical aspects of aerosol technology such as crimping of valves, quality control systems, and container and valve specifications. The Sanders book (9) is intended as a teaching tool, while the Sciarra and Stoller text (11) covers the teaching aspects and serves as a reference for the development of various types of aerosol products. Many chapters on the subject of pharmaceutical and/or cosmetic aerosols have appeared in these as well as other books (12–21).

Aerosols as a dosage form have been accepted by both the USP (22) and the NF (23). In addition to containing definitions and test procedures in regard to aerosol dosage forms, the NF contains monographs for several commonly used propellants including trichloromonofluoromethane (24), dichlorodifluoromethane (25), and dichlorotetrafluoroethane (26).

Various medicinal agents have been formulated as aerosol dosage forms. These aerosols have been administered orally for either inhalation therapy or local activity in the lungs and externally to treat various skin conditions.

Several investigators have commented that pharmaceutical aerosols have not been developed and used to the same extent as other aerosols. According to a recent report, inhalation bronchial dilators are probably the largest single category of aerosol pharmaceuticals sold in pharmacies and hospitals, followed by antiseptic sprays and proprietary asthma control preparations. The fatalities that occurred in England and other European countries with these preparations and the ensuing unfavorable publicity probably accounted for a decrease in the sale of inhalation aerosol products (27). Stolley (28) indicated that these mortalities were due to the fact that most of these preparations contained a fairly high dosage of isoproterenol. The most frequently prescribed nebulizers in the United Kingdom contained a concentration of isoproterenol five times greater than that generally used in the United States. These preparations delivered a dose of active ingredient ranging from 0.08 to 0.40 mg/spray. Others (29, 30) have indicated different reasons for the difference in the mortality rate between these countries.

The development and growth of pharmaceutical aerosols have been, and always will be, relatively slow compared to the growth of other aerosols such as shave creams, insecticides, antiperspirants, deodorants, and room deodorants (31–34). According to Parisse (33): "Toxicological information concerning the inhalation of drugs is extremely sparse. Although a New Drug Application will most probably be required for any compound selected, it should be less burdensome to work with those drugs which have good toxicological histories in other dosage forms." He also indicated that the effective concentration for the drug when given by inhalation may be different from the concentration used in other dosage forms.

Other properties such as solubility of the drug in secretions, hygroscopicity of the drug, particle-size distribution, and selection of the propellant should also be considered. Pick (34) indicated that, with the exception of inhalation products for bronchospasms, the real potential for pharmaceutical aerosols has not been exploited. In addition, other problems such as the need for pharmaceutical grade components and FDA regulations resulted in a slower growth rate for pharmaceutical aerosols.

Research leading to the development and/or improvement of pharmaceutical aerosols has greatly increased during the past 10 years. This research has centered around several areas such as product formulation, aerosol components, propellant and product toxicity, and manufacturing and packaging technology including quality control and testing procedures.

PRODUCT FORMULATION

Pharmaceutical aerosols have been formulated as solutions, suspensions, emulsions, or semisolids (35–38).

Solution aerosols consist of solutions of active ingredients dissolved directly in the liquefied gas propellant or in a mixture of a cosolvent and propellant. These products are generally dispensed as a spray forming fairly small (about 0.5–1.0 μm) droplets to fairly large ones (about 50–100 μm), depending upon the valve characteristics and the nature and amount of the propellant.

Suspensions consist of dispersions or suspensions of the active ingredient in a mixture of the propellant, solvents, and suitable dispersing agents. These products are also dispersed as a fine spray with varying degrees of wetness, depending upon the solvents and propellants used. Emulsions can be dispensed either as a foam or spray, depending upon the specific formulation. Generally, water-in-oil-type emulsions (propellant is in the oil phase) are dispensed as fine droplets while oil-in-water types are dispensed as a foam. The stability of the foam can vary greatly, from very stiff to quick breaking. Semisolid formulations are generally dispersed in their original state, with the exception of some newer postfoaming gels.

Systems—Various systems have been used to dispense pharmaceutical aerosols. The use of conventional aerosol systems for various aerosol products is widespread and well known. These systems are capable of dispensing selected products as sprays or foams and depend upon the presence of from 5 to 95% of liquefied gas propellant. In certain instances, compressed gases have been utilized to accomplish the same end effect. While the liquefied gas propellant is chiefly of the fluorinated hydrocarbon type, hydrocarbons have been used to advantage in some systems.

While most aerosol products in present day usage utilize one of these systems, many products cannot utilize these systems for numerous reasons. The viscosity of the product, the incompatibility of the product concentrate and propellant, and the desired dispensing characteristic of the finished product represent a few of the limitations of these systems. Several additional systems are available and are character-

Table I[a]—Ostwald Solubility Coefficient at 21.1°

Solvent	Solubility Coefficient: Nitrous Oxide	Solubility Coefficient: Carbon Dioxide
Water	0.6	0.85
Acetone	5.92	6.95
Ethanol	2.96	2.84
Methanol	3.20	3.51
Chloroform	5.54	3.6
Acetic acid	4.77	5.1
Methyl acetate	—	6.4
Ether	7.65	6.32
n-Pentane	4.13	1.94
Carbon tetrachloride	2.5	4.28
Ethylene bromide	2.7	2.1
Glycerin	1.2	0.03
Ethylene chloride	3.2	3.7

[a] From Ref. 52.

ized by providing for a physical separation of the propellant and product.

Many designs have been submitted for these systems, but only two basic ones have been accepted: the barrier packs and the siphon systems. Barrier packs utilize a plastic bag or piston to separate the product from the propellant, while siphon systems utilize a propellant cartridge and an outer nonpressurized container holding the product. While both systems separate the propellant from the product, they differ in several respects. In barrier packs, only the product is delivered and there is never any contact between the product and propellant (except for any propellant or product that may permeate through the plastic bag or piston). Since the propellant is located within the product container in siphon systems, the outer container no longer needs to be made from the materials used in the manufacture of conventional aerosol containers. With the absence of pressure, this container can be made of plastic and other similar materials in addition to metal and glass and there is a greater freedom in design. These systems and their potential application to pharmaceutical systems have been reviewed extensively in the literature (39–46).

Sciarra (47) discussed the potential application of codispensing systems for pharmaceuticals. Any reaction resulting in precipitation, liquefication, oxidation–reduction, hydrolysis, or the evolution of a gas is a candidate for codispensing. This system was developed during the late 1960's (48) and has been used to dispense two incompatible ingredients, such as hydrogen peroxide and a reducing agent, resulting in the liberation of sufficient heat. When combined with a shave cream formulation, a hot foam is produced. Figure 1 illustrates the temperature rise noted using different concentrations of reducing agent (49).

At the present time, shave foams are heated by the reaction of an oxidizing agent with a reducing agent. The reducing agent, such as a sulfite, thiosulfate, urea, or thiourea, is placed with the external phase consisting of soap solution and propellant while the oxidizing agent, such as hydrogen peroxide, is kept within a plastic bag attached to the valve.

Hair dyes have also been developed in this system where the dye and propellant are in the external phase and the ammonium hydroxide is kept within

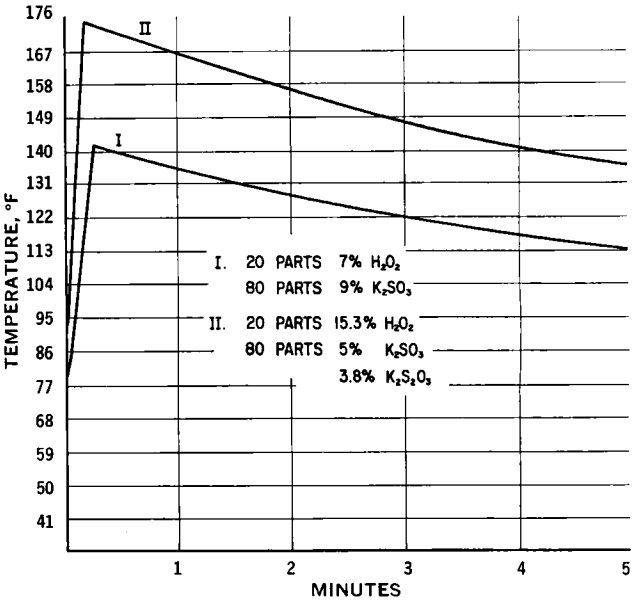

Figure 1—*Heat production from hydrogen peroxide (H_2O_2) reacted with potassium sulfite (K_2SO_3) and potassium thiosulfate ($K_2S_2O_3$). (From Ref. 49.)*

the bag. An antiperspirant preparation containing a high concentration of aluminum salt also utilizes this system. One phase contains the aluminum salt while the other phase contains the solvents and propellant.

Propellants—One important component of the aerosol dosage form is the propellant. Even though investigations were carried out using compressed gases as possible propellants for aerosols, liquefied gases and, in particular, fluorinated hydrocarbons still remain the propellant of choice for most applications. Hostier (50) described the various classifications of propellants along with methods for determining gas concentration, solubility, temperature effect, and the influence of pressure. Holzner (51) suggested the use of carbon dioxide and nitrous oxide in combination with fluorinated hydrocarbons. Since these gases are somewhat soluble in different aerosol systems, they tend to give a fine dispersion of the product and also to compensate partially for the drop in pressure brought about by a change in volume as the product is dispensed.

Hsu and Campbell (52) developed several equations which can be used to calculate the solubility of compressed gases in a given formulation. Based upon the Ostwald solubility coefficient (λ) for a particular solvent:

$$\lambda = \frac{\text{volume of gas dissolved}}{\text{volume of liquid}} \qquad \text{(Eq. 1)}$$

the volume of compressed gas dissolved in a given volume of liquid can be determined. Table I gives the Ostwald solubility coefficient for some commonly used solvents. The coefficient can easily be determined using a pressure container partially filled with a known volume of the liquid. A sufficient amount of gas is added to saturate the liquid, and the Ostwald coefficient is calculated from the following equation:

$$\lambda = \frac{1}{X}\left(\frac{WRT}{V_c MP} + X - 1\right) \qquad \text{(Eq. 2)}$$

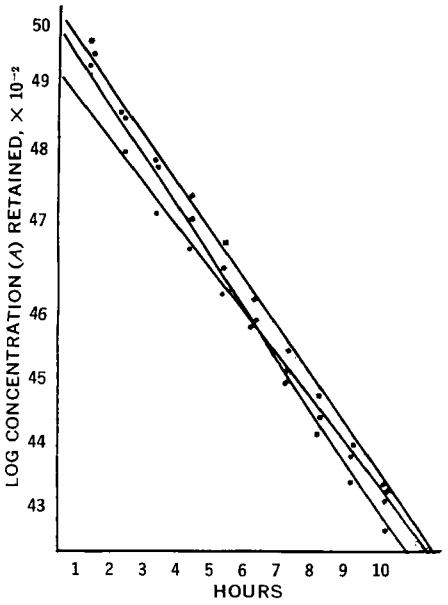

Figure 2—*Release of gentian violet into different solvents (Emerez 1533 film containing hexadecyl alcohol, 10% w/w). Key: ●, desorbing solvent, 0.45% sodium chloride solution; □, desorbing solvent, 0.45% sodium sulfate anhydrous solution; and *, desorbing solvent, potassium phosphate buffer solution, pH 7. (From Ref. 53.)*

where λ = Ostwald solubility coefficient, X = volume of liquid/volume of container, W = weight of propellant gas (grams), R = gas constant (82.06 ml atm mol^{-1} deg^{-1}), T = temperature (°K), V_c = volume of container (milliliters), M = molecular weight of the propellant, and P = total pressure (atmospheres).

By rearranging Eq. 2 and solving for W, the weight of compressed gas required to saturate the liquid at a given pressure can be calculated:

$$W = \frac{V_c MP}{RT}(X\lambda + 1 - X) \qquad \text{(Eq. 3)}$$

These equations are applicable to the formulation of emulsion systems where a foam is desired using a soluble compressed gas. The pressure drop from compressed gas systems as the contents are utilized can be calculated from a modification of Eq. 3 to yield:

$$P_2 = P_1 \Big/ \left(1 + \frac{\Delta X}{X\lambda - \Delta X\lambda + 1 - X}\right) \qquad \text{(Eq. 4)}$$

where P_1 = pressure in container before dispensing (atmospheres), and P_2 = pressure in container after dispensing (atmospheres).

Formulation—*Solutions*—Various studies have been conducted on materials used in the aerosol dosage form. Sciarra and Gidwani (53) determined the influence of polymeric films such as ethylcellulose, polyamide resins, and acrylic resins together with plasticizers, including hexadecyl alcohol and tributyl citrate, upon the rate of release of gentian violet. The films were prepared from an aerosol and cast upon a mercury surface. They determined that this release followed first-order kinetics (Fig. 2). The polyamide film was successful in producing a timed-release film, while several of the other films showed a delay in the release of gentian violet. These properties were then related to film hardness, modulus of elasticity and flexibility, and alkali resistance. The effect of electrolytes upon this release is shown in Fig. 3.

Proposed kinetics for the release of cetylpyridinium chloride and benzalkonium chloride were discussed (54). These drugs were dissolved in films cast from solutions of several film-forming agents. The dried films were then exposed to a desorbing solution of demineralized water and demineralized water–sodium chloride; the rate of the release is shown in Figs. 4–6.

All data presented in this study (54), with the exception of data on the mixed polymer, showed that the drug release followed first-order kinetics. For *in vitro* drug release to be exponential, the plot of log A *versus* time should yield a straight line, and the y-intercept at zero time should correspond to the initial concentration. But the typical apparent first-order profiles show that the values of the y-intercepts are, in fact, less than the predicted values. It seemed apparent from the drug release profiles that one portion of the drug is released immediately and the other portion exponentially. The initial release is faster than the remainder, which follows first-order kinetics, because of the presence of surface drug which may be ignored. There is, therefore, a lag time before the rate follows a first-order process. Hence the linear portion of the curve is the rate-determining step for the drug release. Since the rate release during the first 60 min (lag time) was indeterminate, the rate-limiting step permitted the determination of relative rate constants and half-lives for the drugs studied. Two processes possibly may be involved: transportation of the drug by hydration followed by release of drug from the film surface.

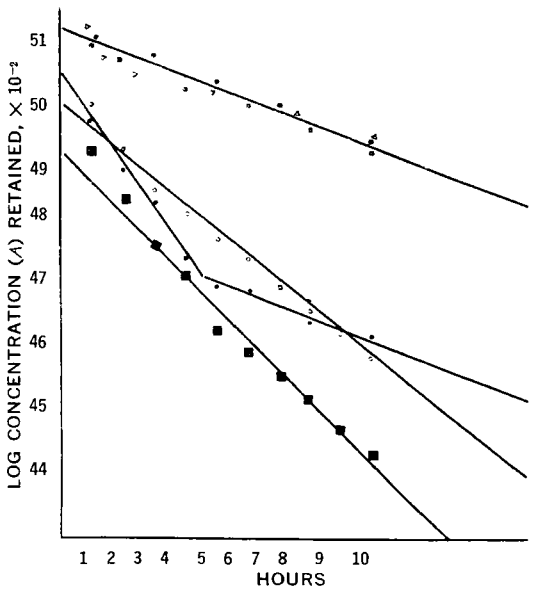

Figure 3—*Release of gentian violet from various films (solvent, 0.45% sodium chloride solution). Key: ●, Emerez 1540, hexadecyl alcohol, 10% w/w; ■, Emerez 1540, hexadecyl alcohol, 30% w/w; □, Emerez 1540, Citroflex-4, 10% w/w; *, Emerez 1540, Amerchol-L 101, 10% w/w; ○, Emerez 1536, unplasticized; and △, Carboset 525, unplasticized. (From Ref. 53.)*

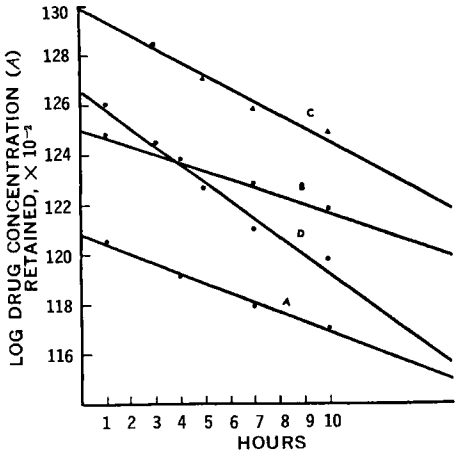

Figure 4—*Apparent first-order profile for ethylcellulose film containing hexadecyl alcohol, 30 PHR (parts of plasticizer per 100 parts of polymer). Key: A, cetylpyridinium chloride/demineralized water; B, cetylpyridinium chloride/0.225% sodium chloride; C, benzalkonium chloride/0.225% sodium chloride; and D, benzalkonium chloride/demineralized water. (From Ref. 54.)*

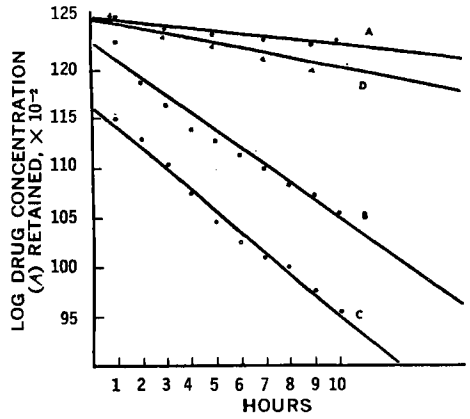

Figure 5—*Apparent first-order profile for cetylpyridinium chloride. Key: A, hexadecyl alcohol, 10 PHR/0.45% sodium chloride; B, hexadecyl alcohol, 10 PHR/demineralized water; C, tributyl citrate, 10 PHR/demineralized water; and D, tributyl citrate, 10 PHR/0.45% sodium chloride. (From Ref. 54.)*

Such drug release may be expressed mathematically by the following equations:

$$\text{total amount released} = \text{amount released immediately} + \text{amount released exponentially} \quad \text{(Eq. 5)}$$

$$C_t = A_0 C_i + A C_e \quad \text{(Eq. 6)}$$

where C_t = total amount of drug released at any time t, A_0 = original amount of drug in the film, C_i = fraction of original amount of drug released immediately, A = amount of drug remaining in the film, and C_e = fraction of remaining drug released exponentially.

Further studies (55) were concerned with the effect of plasticizer concentration on the water vapor transmission of film-forming agents.

The selection of a polymeric film for application as a protective film to the skin is determined by considering various physicochemical and biological properties. These include hardness, modulus of elasticity, alkali resistance, water vapor transmission, and stability to degradation from exposure to UV radiation. These properties were evaluated to determine the use of certain film plasticizers for application to the body *via* an aerosol spray.

A film-forming polymer suitable for application to injured skin should be permeable to water vapor so as to decrease the possibility of anaerobic bacterium growth in the wound vicinity. However, some conditions may require the use of an occlusive dressing. Components added to film-forming agents as a part of the formulation may affect the rate of water vapor transmission. The effects of several plasticizers, such as diacetin, ethyl phthalate (diethyl phthalate), triethyl citrate, and acetyltriethyl citrate, on the water vapor transmission of cellulose acetate films were reported (56).

The rate of water vapor transmission *(WVT)* of a film between two specified parallel surfaces is dependent upon the film, the plasticizer and its concentration, and film thickness. The water vapor transmission can be calculated from the following:

$$WVT = (g)(24/t)(a) \quad \text{(Eq. 7)}$$

where g = weight of loss or gain (grams), t = time (hours) during which loss or gain in g was observed, a = exposed area of the specimen (square inches), and WVT = rate of water vapor transmission (expressed in g/in^2/24 hr).

Flux *(F)* can be calculated from:

$$F = \frac{W \times T}{A \times 0.1} \quad \text{(Eq. 8)}$$

where W = weight of water vapor permeating (milligrams), T = film thickness, and A = effective area of exposed film.

The permeability coefficient *(P_c)* is defined as the milligrams of water vapor that permeates through a 0.1-mm thick film, per unit area in square centime-

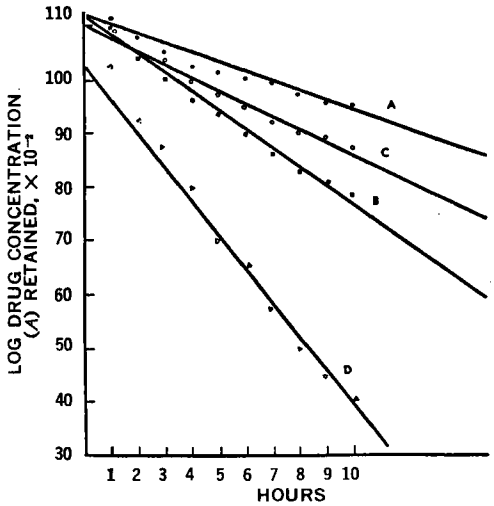

Figure 6—*Apparent first-order profile for benzalkonium chloride. Key: A, hexadecyl alcohol, 10 PHR/0.45% sodium chloride; B, hexadecyl alcohol, 10 PHR/demineralized water; C, tributyl citrate, 10 PHR/0.45% sodium chloride; and D, tributyl citrate, 10 PHR/demineralized water. (From Ref. 54.)*

Table II[a]—Water Vapor Transmission of Films Prepared from Ethylcellulose at 37°

Plasticizer[b]	Hours	Flux (*F*) I, mg	II, mg	Mean, mg
Unplasticized	24	151.3	151.5	151.4
	48	275.9	277.6	276.8
	72	410.9	412.0	411.4
	96	537.0	547.8	542.4
Tributyl citrate, 10 PHR	24	84.0	84.9	84.4
	48	162.7	162.4	162.5
	72	220.0	221.4	220.7
	96	281.0	279.5	280.3
Tributyl citrate, 20 PHR	24	120.2	121.1	120.6
	48	269.6	270.6	270.1
	72	450.2	451.8	450.5
	96	645.8	647.4	646.6
Tributyl citrate, 10 PHR; hexadecyl alcohol, 10 PHR	24	254.5	253.7	254.1
	48	572.1	573.9	573.0
	72	869.5	871.6	870.6
	96	1177.0	1179.6	1178.3
Tributyl citrate, 15 PHR; hexadecyl alcohol, 15 PHR	24	110.0	93.4	101.7
	49	219.6	176.4	198.0
	72	343.5	270.7	307.1
	96	473.5	338.0	405.7

[a] From Ref. 55. [b] PHR indicates parts of plasticizer per 100 parts of polymer.

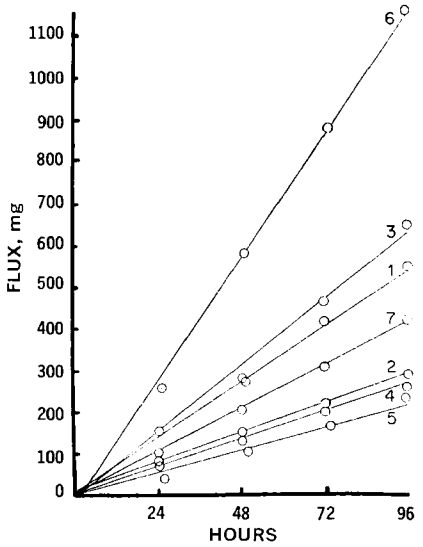

Figure 7—*Water vapor transmission of ethylcellulose films. Key: 1, unplasticized; 2, tributyl citrate (10 PHR); 3, tributyl citrate (20 PHR); 4, hexadecyl alcohol (10 PHR); 5, hexadecyl alcohol (20 PHR); 6, tributyl citrate (10 PHR)/hexadecyl alcohol (10 PHR); and 7, tributyl citrate (15 PHR)/hexadecyl alcohol (15 PHR). (From Ref. 55.)*

ters, per unit pressure drop every 24 hr, following a steady state of diffusion under the experimental conditions of temperature and pressure. The water vapor transmission of films prepared from two film-forming agents is given in Tables II and III and Figs. 7 and 8.

The hardness and tackiness of various film-forming agents were also studied using a pendulum hardness tester (57). The films of uniform thickness required for this type of determination were prepared with the aid of a centrifugal apparatus. Eckardt (57) examined various acid esters of ethylcellulose, polyvinylpyrrolidone–vinyl acetate, and other substances. In addition to moisture, the results suggested that sebum, plasticizer, and fragrance can greatly affect the hardness of these film formers used in aerosols.

Table III[a]—Water Vapor Transmission of Films Prepared from Polyamide Resin at 37°

Plasticizer[b]	Hours	Flux (*F*) I, mg	II, mg	Mean, mg
Unplasticized	24	4.91	4.74	4.83
	48	10.95	11.00	10.96
	72	14.81	14.86	14.79
	96	20.06	19.90	19.90
Tributyl citrate, 10 PHR	24	8.94	9.90	9.02
	48	17.36	17.49	17.37
	72	29.21	28.60	28.90
	96	41.90	42.17	41.98
Tributyl citrate, 20 PHR	24	8.03	8.23	8.13
	48	16.88	17.09	16.86
	72	27.64	27.85	27.45
	96	35.84	36.25	36.04
Hexadecyl alcohol, 20 PHR	24	7.39	6.46	6.88
	48	15.66	14.87	15.23
	72	22.09	22.02	22.05
	96	32.37	32.11	32.19
Tributyl citrate, 10 PHR; hexadecyl alcohol, 10 PHR	24	12.17	12.28	12.22
	48	24.19	24.38	24.28
	72	37.97	38.18	38.03
	96	50.06	50.22	50.14

[a] From Ref. 55. [b] PHR indicates parts of plasticizer per 100 parts of polymer.

The spreading of hair spray resins when applied to hair was discussed from the point of view of the wettability of hair fibers (58). The wettability depends on the magnitude of the surface tension of the solution compared with the critical surface tension of the hair fibers. Measurements of both of these quantities showed that, for the commonly employed hair spray solvents, complete wettability (zero contact angle) invariably occurs, giving rise to spontaneous spreading on the fiber surface. The rate of spreading was shown to depend mainly on the viscosity of the solution, the rate of evaporation of the solvent, and the rate of increase in the solution viscosity with concentration due to evaporation. The Washburn equation describing the rate of capillary penetration of liquids into porous systems was also shown to be insufficient when dealing with volatile fluids. A new equation, a modification of the Washburn equation, allowing for the effects of solvent evaporation and increasing solution viscosity with evaporation, was described and shown to be in good agreement with experimental data for various resin solutions spreading in a bundle of human hairs.

Dispersions and Powders—Very few references have appeared in the pharmaceutical literature regarding the development of inhalation aerosols. For the most part, epinephrine bitartrate or isoproterenol sulfate or hydrochloride has been used as a dispersion in the propellant. Epinephrine hydrochloride has also been used in solution form for this purpose. Not until 1960 were several reports published concerning the development and evaluation of these products (59, 60). These reports were concerned with the uniformity of dispensing each dose from the aerosol package, particle-size distribution, and analytical control methods, and they represent an early attempt

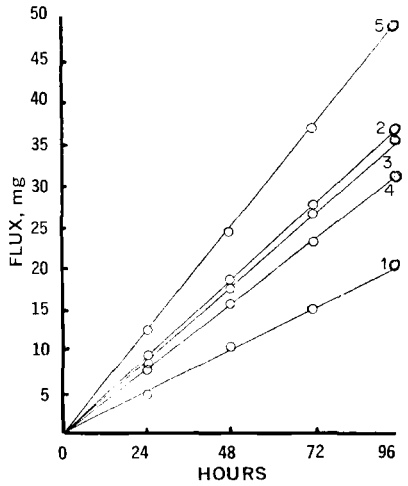

Figure 8—*Water vapor transmission of polyamide resin films. Key: 1, unplasticized; 2, tributyl citrate (10 PHR); 3, tributyl citrate (20 PHR); 4, hexadecyl alcohol (20 PHR); and 5, tributyl citrate (10 PHR)/hexadecyl alcohol (10 PHR). (From Ref. 55.)*

to establish some standards for this dosage form. Several of these procedures and suggested standards were reflected in NF XIII specifications and standards.

Various epinephrine salts were investigated as alternatives to the bitartrate and hydrochloride (61). The maleate, malate, and fumarate were synthesized and formulated as both suspension and solution-type aerosols. The solubility and stability of each salt were determined. The bitartrate and malate were the least soluble in the fluorocarbon propellant, while the maleate and fumarate showed a higher degree of solubility. The decomposition that took place followed first-order kinetics, and epinephrine maleate and bitartrate seemed to be more stable than the other salts of epinephrine studied.

Additional studies (62) showed the development and pharmacological action of epinephrine bitartrate in the rabbit eye. A formulation consisting of 0.5% epinephrine bitartrate in 0.5% hydroxypropyl methylcellulose was compared to a 0.5% epinephrine bitartrate preparation in a fluorocarbon propellant. Figure 9 illustrates the effect of each preparation upon intraocular pressure and pupil size. Based upon chemical analysis, it was noted that only 13% of the calculated dose of epinephrine was delivered to the eye by the aerosol adaptor used, even though similar pharmacological effects were obtained with the aerosol. This was taken as an indication that more epinephrine than was actually needed was being used. Modified Draize eye irritation studies did not reveal any signs of irritation following aerosol administration of the vehicle or the drug. The investigators concluded that this lack of irritation, along with the desired pharmacological effect, indicates the possible usefulness of administering ophthalmic drugs by means of an aerosol dosage form.

Most aerosols intended for inhalation therapy contain a fairly low concentration of solid particles. In many instances, the maximum concentration rarely exceeds 1%. Other solids have been dispensed in aerosol form and include the so-called powder aerosols. The concentration of powder in these cases is about 10–15%, although it is possible to produce a system with from 85 to 90% powder. Several reviews on this subject have appeared over the past few years. Most authors agree that powder aerosols have the potential of presenting serious formulation problems in the areas of valve clogging, particle-size growth and agglomeration, caking and sedimentation, and ease of redispersion (63–65).

According to Herzka (64), the three basic reasons for the most serious problem, valve clogging, are large and/or needle-shaped particles, the presence of only partially soluble resinous or crystalline materials in the product, and the agglomerative sedimentation of the products. The design of the valve was also discussed. Isopropyl myristate, in a concentration of 0.5–2.0%, was suggested as a lubricating agent to prevent valve clogging as well as agglomeration of particles. According to Elvin (65), needle- and plate-shaped particles tend to present a greater clogging potential than spherical ones. These particles should generally be in the 35–40-μm range, while spherical particles can be as large as 75 μm. To aid in resuspending the powders, a bulking agent such as a silica gel can also be added. In this investigation, various silica derivatives were studied to determine the relative effect of each.

Many studies with powder systems utilize aluminum chlorhydroxide formulations as antiperspirants. Crotty *et al.* (66) investigated the physical behavior of this solid in aerosol formulations. Their study in-

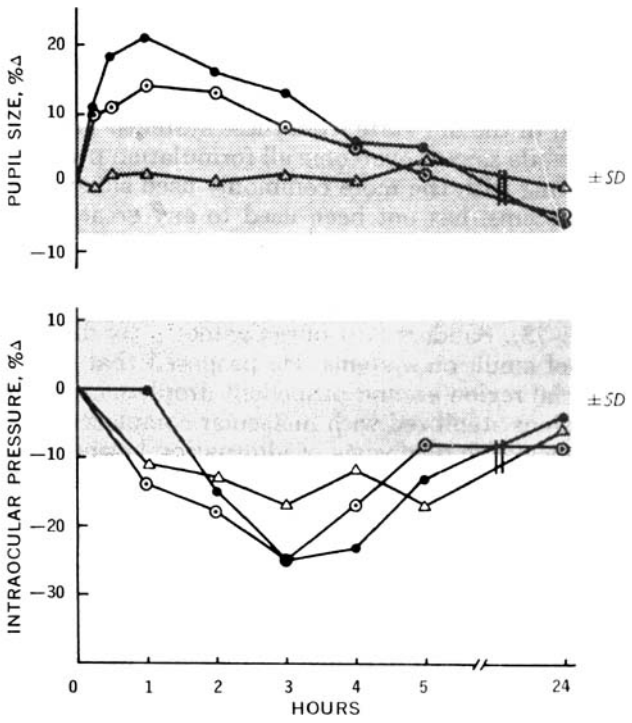

Figure 9—*Intraocular pressure and pupil size after drug administration [epinephrine bitartrate (0.5%) in: ●, hydroxypropyl methylcellulose; ⊙, fluorocarbon; and △, saline]. Each point represents the percent change of the mean values of from six to 12 eyes for each time period. The standard deviation about the control mean is based on 54 observations. (From Ref. 62.)*

Table IV[a]—Stability of Aqueous Aerosol Systems[b] by Polyoxyethylene Fatty Ethers

Composition: Polyoxyethylene Fatty Ether	Water Solubility	Weight Percent in Aqueous Phase	Hydrophilic–Lipophilic Balance Value	Stability, min: Emulsion[c]	Stability, min: Foam[d]
Polyoxyethylene (4) lauryl ether	Dispersible	1.8	9.7	<1	<5
Polyoxyethylene (23) lauryl ether	Soluble	6.0	16.9	>1	<5
Polyoxyethylene (3) cetyl ether	Insoluble	1.6	5.3	>30	>120
Polyoxyethylene (10) cetyl ether	Dispersible	3.3	12.9	<1	<15
Polyoxyethylene (20) cetyl ether	Soluble	5.4	15.7	<1	<5
Polyoxyethylene (2) stearyl ether	Insoluble	1.8	4.9	>30	>120
Polyoxyethylene (10) stearyl ether	Dispersible	3.4	12.4	<1	<15
Polyoxyethylene (20) stearyl ether	Dispersible	5.5	15.3	<5	<5

[a] From Ref. 79. [b] Aerosol formulation: 90% aqueous phase–10% Freon 12/Freon 114 (40:60) propellant. Freon is du Pont's registered trademark for its fluorocarbons. [c] Time to initial phase separation after shaking. [d] Time to first indication of foam collapse.

cluded the use of fuming silica as a dispersing agent for aluminum chlorhydroxide. The effects of moisture, polarity, and particle size of the substituents upon viscosity and homogeneity were also studied.

The use of hexadecyl alcohol and other emollients to aid in the suspension and redispersion of the aluminum chlorhydroxide was suggested (67), as was the use of colloidal silica and isopropyl myristate along with passing the slurry through a colloid mill (68). The conclusion in the latter study was that the use of this mill would prevent agglomeration of the aluminum chlorhydroxide particles and avoid valve clogging. The use of alcohol-soluble aluminum salts as deodorants and antiperspirants was studied, and it was concluded that such use was not feasible or involved a great deal of uncertainty as to formulation and stability (69, 70).

While most aerosol powder systems contained a fairly low percentage of solids (up to 15%) and a large amount of propellant, a system containing a minor portion of liquefied gas propellant and a major portion of powder was developed (71). The powder is delivered in the dry state from these systems. This system, while said to overcome all formulation problems occurring with the more commonly used slurry powder systems, has not been used to any great extent (72).

Emulsions—The formulation of emulsions for dispensing as aerosol foams has been studied extensively (73–78). Sanders (79) investigated many different aerosol emulsion systems. He proposed that the interfacial region around propellant droplets in aerosol emulsions stabilized with molecular complexes is polymolecular and consists of alternating layers of oriented water molecules and bimolecular layers of the molecular complex. He further noted that the molecular complex has a liquid crystal structure which is insoluble in any of the phases, resulting in its concentration at the interface. According to Sanders, this phenomenon has a stabilizing effect upon the foam. He further stated that these liquid crystals in aqueous systems are often responsible for the pearlescence occurring in creams.

The relationship between the solubility of a series of polyoxyethylene fatty ethers and their effectiveness as stabilizers for aerosol emulsions and foams is shown in Table IV. The polyoxyethylene ethers producing the most stable foam were polyoxyethylene (2) stearyl and polyoxyethylene (2) cetyl ethers. These were the only ethers found to be insoluble in water. Sanders concluded that the best stabilizers for aerosol foams were those with a low solubility in the aqueous phase and low solubility or insolubility in the propellant phase. However, the stabilizers must be dispersible to some extent in the propellant phase. For systems containing an aqueous alcohol phase, Sanders also noted the importance of solubility of the surfactant in the aqueous alcohol phase. Since increasing the concentration of ethanol in these systems increases the solubility of the surfactant, the more stable foams are produced at low alcohol concentrations. However, this technique is used to produce the quick-breaking foam systems.

Emulsions containing mineral oil or glycol as the nonaqueous phase were also investigated. Table V illustrates the relationship between foam stability and solubility of the surfactant in polyethylene glycol 400 while Table VI shows this same relationship with mineral oil foam systems. Mineral oil foam systems differed from the glycol systems in that the propellant was soluble in mineral oil. For the most part, however, it seems that the most stable foams were produced with surfactants that were least soluble in either polyethylene glycol 400 or mineral oil.

Table V[a]—Foam Stability and Surfactant Solubility in Polyethylene Glycol 400 Foams[b]

Surfactant	Solubility of Surfactant Glycol	Foam Stability
Ethoxylated stearyl alcohol	Insoluble	Stable foam
Polyoxyethylene (4) lauryl ether	Soluble	No foam
Polyoxyethylene (23) lauryl ether	Insoluble	Stable foam
Polyoxyethylene (2) cetyl ether	Insoluble	Stable foam
Polyoxyethylene (10) cetyl ether	Soluble	No foam
Polyoxyethylene (20) cetyl ether	Insoluble	Stable foam
Polyoxyethylene (2) stearyl ether	Insoluble	Stable foam
Polyoxyethylene (10) stearyl ether	Insoluble	Stable foam
Poloxyethylene (2) oleyl ether	Soluble	No foam
Polyoxyethylene (10) oleyl ether	Soluble	No foam

[a] From Ref. 79. [b] Aerosol formulation: 86% polyethylene glycol 400–4% surfactant–10% Freon 12/Freon 114 (40:60) propellant.

Table VI[a]—Surfactant Solubility and Foam Stability in Mineral Oil Systems[b]

Surfactant	Surfactant Solubility in Mineral Oil	Foam Stability
Ethoxylated stearyl alcohol	Insoluble	Stable
Polyoxyethylene (4) lauryl ether	Soluble	No foam
Polyoxyethylene (2) cetyl ether	Soluble	No foam
Polyoxyethylene (10) cetyl ether	Insoluble	No foam
Polyoxyethylene (2) stearyl ether	Insoluble	Stable
Polyoxyethylene (10) stearyl ether	Insoluble	No foam
Polyoxyethylene (2) oleyl ether	Soluble	No foam

[a] From Ref. 79. [b] Aerosol formulation: 79% mineral oil–6% surfactant–15% Freon 12 propellant.

An emulsifier system of octanoic acid and 1-aminooctane was used in a study of liquid crystalline phases in aerosol formulations (80). Both propellant and acid showed complete solubility in the system containing the least amount of water. A two-phase area was formed, showing the immiscibility of the propellant, dichlorotetrafluoroethane, with the water–amine solution. As the water content was increased, similar solubility relationships were seen, except that the two-phase area of immiscibility between the propellant and the water–amine layer increased. This two-phase portion representing the area of immiscibility of propellant with the water–amine layer was referred to as the liquid crystalline phase (Fig. 10). In going from a low water content to a higher water content, the liquid crystalline phase increases (Fig. 11).

Further studies along these same lines (81) revealed the fact that emulsion compositions containing both a liquid crystalline phase and a liquid phase gave rise to foams with high stability compared to foams produced from compositions where only a liquid phase was present. According to Friberg and coworkers (82, 83), foam stability is related to the presence of liquid crystalline phases in which the emulsifiers, water, and oil phases are all associated into an ordered structure, and not as indicated by Sanders (79). Sanders indicated that the foam stability was related to the formation of a molecular complex. Sanders evaluated the foams on the basis of phase separation and ultimate collapse of the foam, while Jederström *et al.* (81) evaluated the foam on the basis of foam height. Differences in the type of surfactant used as well as the composition of aqueous phase–surfactant–propellant phase could have influenced the results.

Based upon the results of this latest study (81), it is suggested that emulsifiers and stabilizers for aerosol foams be evaluated by noting the composition of the emulsion prior to the addition of the propellant. A liquid crystalline phase should be present, and this phase should show some miscibility with the propellant.

The pearlescence occurring with certain water-soluble polyoxyethylene fatty ethers and fatty alcohols

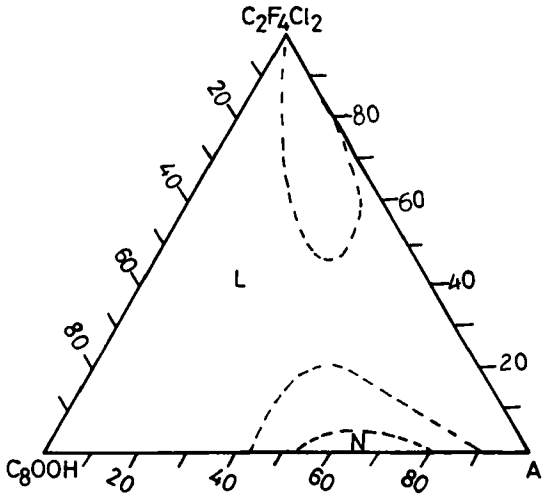

Figure 10—*Phase regions in the system of octanoic acid–propellant–water/amine [water (19%), 1-aminooctane (81%)] (C_8OOH–$C_2Cl_2F_4$–A). Key: L, isotropic solution; and N, liquid crystalline phase, "neat phase." (From Ref. 80.)*

was also studied (84) and took place only when the surfactant would concentrate in the aqueous phase. The incompatibility of certain pearlescent structures with fluorinated hydrocarbon propellants was observed. The triethanolamine myristate–propellant system was also investigated (85). The properties of the foam produced from two emulsions with different degrees of stability were compared to those of the emulsion. A glass pressure cell was developed so that the emulsions could be viewed microscopically. Microscopic and visual observations of the two systems showed that the surfactant system producing emulsified propellant droplets with the smaller diameters also produced foams with an initially smaller bubble size and a slower increase in bubble size after discharge. Greater stability was noted in systems that showed a smaller emulsified droplet.

Various methods for the production of these emul-

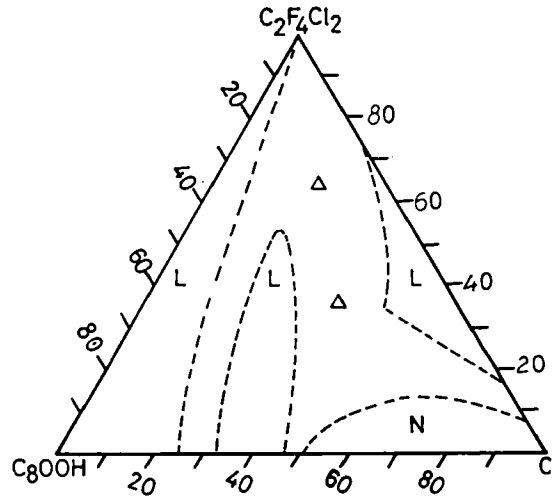

Figure 11—*Phase regions in the system of octanoic acid–propellant–water/amine [water (33%), 1-aminooctane (67%)] (C_8OOH–$C_2Cl_2F_4$–C). Key: L, isotropic solution; N, liquid crystalline phase, "neat phase"; and Δ, three-phase area indication. (From Ref. 80.)*

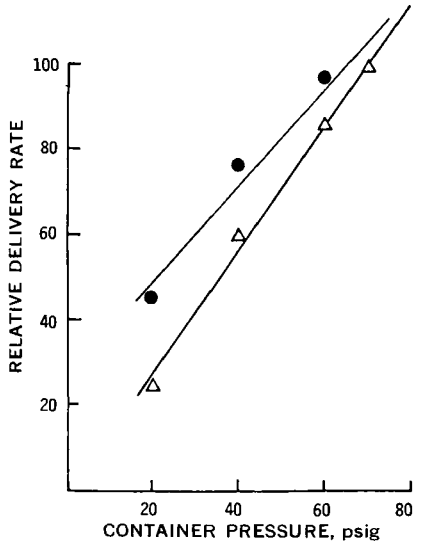

Figure 12—*Plot of the relative delivery rate* versus *container pressure for selected sodium carboxymethylcellulose solutions* (●, *2%; and* △, *3%*). (*From Ref. 93.*)

sions were also studied (86). Photomicrographs and phase separation times were used to judge the quality of the emulsion. Aerosols were prepared for the best and the poorest emulsions produced by adding the propellant to the concentrate. Concentrates with the smallest droplet size and the longest separation times produced the most stable aerosols. These stable aerosols also produced the most stable foams. Sanders (86) accounted for this stability on the basis of the formation of a triethanolamine myristate–myristic acid complex during the initial addition of the aqueous phase.

Depending upon the nature of the propellant, surfactant, and aqueous or nonaqueous phase, foams of differing viscosity and consistency could be produced. In addition, such foams described as sparkling, shiny, expanding, collapsing, periscoping, and crackling could be produced (87). These foams are described as being suitable for use for a variety of cosmetic and pharmaceutical products. The aerosol

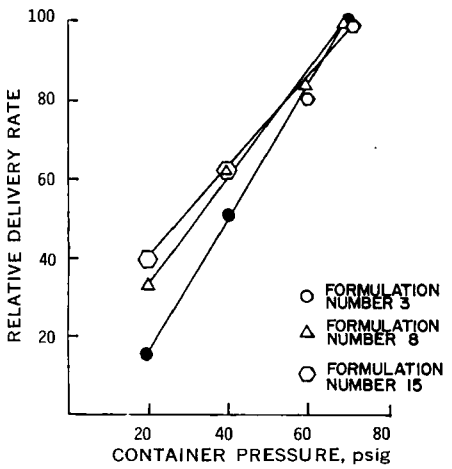

Figure 13—*Plot of the relative delivery rate* versus *container pressure for selected formulations. Formulation Number 3 is representative of a typical cream, Number 8 is a lotion, and Number 15 is a suspension.* (*From Ref. 93.*)

quick-breaking foam is based upon a system consisting of alcohol, water, surfactant, and propellant (88). As the propellant vaporizes, the foam is formed since the surfactant comes out of solution due to the loss of propellant. Under the action of skin heat, the surfactant partially dissolves, causing the foam to collapse. Rubbing of the foam destroys the crystal lattice and results in the same effect. A more detailed study of aqueous alcohol aerosol foams reports on the solubility of the propellant in water–ethanol solutions and the effect of various surfactants upon this solubility. Other solvents such as acetone, diethylene glycol, ethylene glycol, glycerin, propylene glycol, and isopropyl myristate were also studied (89).

Lemlich (90) reviewed the physical aspects of foam. Bubble size, shape, fit, and movement, along with methods for measuring the liquid content and bubble size, were indicated.

The effect of nonionic emulsifiers, buffer, pH, and hydrophilic–lipophilic balance values in the stability of emulsions used to produce aerosol foams was studied (91). A series of emulsions containing a high water content was prepared and studied for stability. These workers noted that the Sorenson buffer system was superior to an acetate buffer system as related to emulsion stability. Evidence indicated the existence of an unfavorable pH-dependent acetate-ion effect on emulsion stability.

Semisolids—Semisolids are dispensed from pressurized systems. Generally, ointments, creams, gels, and similar materials make up this dosage form. Recently, these products have been successfully dispensed from "barrier pack" pressurized systems. The flow properties of the semisolid as it passes from the container and through the valve openings is especially critical. Fisher and Sheth (92, 93) studied the effect of product rheology and pressure upon the delivery rate of a model cream, lotion, suspension, and polymer solution from one such barrier pack[1]. Flow curves were developed for each model system studied. The delivery rate was also determined in terms of grams discharged per second. As one would predict, the delivery rate increased with pressure, although the rate of increase was not linear with the increase in pressure. The concept of relative delivery rate was proposed by these workers and included comparing the delivery rate at a specific pressure to the delivery rate at the highest test pressure. In this study, 70 psig was used as the highest pressure and the equation used was:

$$R = \frac{\text{delivery rate at a specific pressure}}{\text{delivery rate at 70 psig}} \times 100 \quad \text{(Eq. 9)}$$

where R = relative delivery rate.

Plots of these values for each model are shown in Figs. 12 and 13. The rheological properties of the polymer solution were determined at different pressures (Fig. 14).

A novel approach to the dispensing of gels from

[1] Consisting of an accordian-like pleated plastic bag placed inside a standard three-piece aerosol can (Continental Can Co., New York, N.Y.).

Table VII[a]—Mean Dose Delivered (Milligrams) from Meter Valves at Various Levels of Container Emptying

Level of Emptying	Type of Meter Valve[b]				
	E501	V501	V501-DA	R501-EC	S501-EC
			Formulation I		
Initial	68.0	69.0	73.0	68.3	87.6
10%	68.1	69.2	75.2	68.7	88.7
50%	68.5	69.7	76.2	69.6	91.3
80%	68.9	69.8	77.4	70.5	92.9
Total mean	68.3	69.4	76.1	69.3	90.1
Coefficient of variation, %	2.08	4.90	5.73	2.22	3.80
			Formulation II		
Initial	65.4	65.8	75.4	69.7	89.4
10%	66.0	65.6	76.2	70.6	90.8
50%	68.0	67.4	77.3	71.6	92.9
80%	68.5	67.2	78.3	72.3	94.6
Total mean	66.9	66.5	76.7	71.0	91.9
Coefficient of variation, %	2.48	5.26	1.51	2.43	2.87
			Formulation III		
Initial	38.4	41.0	43.4	40.1	43.3
10%	37.4	38.7	43.0	39.3	47.8
50%	37.3	37.9	41.8	37.8	46.9
80%	36.8	36.9	40.0	36.8	45.5
Total mean	37.5	38.9	42.0	38.5	47.1
Coefficient of variation, %	5.01	3.78	3.33	3.94	5.41
			Formulation IV		
Initial	64.2	71.1	79.6	72.7	96.7
10%	64.1	70.7	79.2	73.0	97.7
50%	64.0	70.2	79.0	72.8	97.1
80%	63.9	70.1	79.4	72.8	97.6
Total mean	64.1	70.5	79.3	72.8	97.0
Coefficient of variation, %	1.5	1.73	1.28	1.61	1.42

[a] From Ref. 96. [b] E501 = 50 μl, Emson Research, Inc.; V501 = 50 μl, VCA (upright); V501-DA = 50 μl, VCA (inverted); R501-EC = 50 μl, Riker Labs. (inverted); and S501-EC = 50 μl, experimental valve (inverted).

pressurized systems was developed (94) and consisted of a gel packaged in an enclosed bag. In addition to the gel, a liquid with a low boiling point, such as dichlorotetrafluoroethane[2] or pentane, is incorporated into the gel. A propellant such as dichlorodifluoromethane[3] or a mixture of isobutane–propane is added on the outside of the bag and supplies the dispensing pressure. When emitted, the gel is in a ribbon-like structure but foams as the low boiling solvent escapes due to the warmth of the hand or by rubbing. This principle has been used for foaming shave gels.

AEROSOL COMPONENTS

Valves—The delivery of an exact dosage from an aerosol package is largely controlled by the valve design. However, the nature of the formulation and the nature of the propellant must be considered. The number of doses per container and the amount per dose are important standards for inhalation and oral aerosols (95). A comparison between commercially available meter valves using identical formulations and evaluation methods was undertaken (96, 97) to generate data that might result in standards for meter valve performance and dose variation. In addition to the actual dose of medication dispensed, dose-to-dose variation at different levels of container emptying must be considered since the difference between the first dose dispensed and the last dose can be significant.

Fractionation of the propellant blend does occur as the contents are used and could lead to increased variation (Table VII). Formulations I and II contained dichlorodifluoromethane–dichlorotetrafluoroethane (50:50 and 75:25, respectively), while Formulation III contained 50% dichlorodifluoromethane–dichlorotetrafluoroethane (50:50) together with 50% of absolute alcohol. Formulation IV contained only octafluorocyclobutane[4] so no fractionation could occur. From Table VII, it can be readily seen that Formulation IV gave the smallest amount of variation as the container was emptied. This study was completed using formulations that did not contain any active ingredients. Since the formulation will affect not only the dose dispensed but also the dose-to-dose variation, final studies of the valve must include an evaluation of the finished preparation.

While not used in metered valves, the presence of a vapor tap in the valve can result in fractionation occurring not only with propellant blends but also with propellant–concentrate ratios. Flanner and Matera (98) determined the effect of vapor tap valves in the ratio of propellant to concentrate in several cosmetic aerosol formulations. They noted that fractionation of the propellant blend would occur after 75% of the product was consumed. Valves used in this study were of the continuous spray type. Changes were also noted in the ratio of the propellant to the concentrate. As the size of the vapor tap orifice increased, the ratio of propellant to product decreased.

[2] Propellant 114.
[3] Propellant 12.
[4] Propellant C-318.

Table VIII[a]—Fill Tolerances for Propellant 1-Chloro-1,1-difluoroethane–Trichloromonofluoromethane (50:50) Systems

Number of Units Analyzed	Nominal Fill, g	Mean Fill, g	*SD*, g	Experimental Range, g	Tolerance Limits[b], g		Tolerance Limit Spread, g
					Lower	Upper	
50	10	9.517	0.193	1.052	8.914	10.120	1.206
50	20	20.695	0.432	2.026	19.345	22.045	2.700
50	30	30.346	0.407	2.595	29.074	31.618	2.544
50	40	40.671	0.471	2.853	39.199	42.43	2.944
50	60	60.525	0.585	2.989	58.696	62.354	3.658
50	80	80.752	0.358	2.180	79.633	81.871	2.238
50	100	101.152	0.532	3.052	99.489	102.815	3.326
50	115	114.171	0.572	3.006	112.383	115.959	3.576

[a] From Ref. 123. [b] The tolerance limits will be expected to contain 99% of the population with 95% confidence.

Further studies (99) led to the conclusion that fractionation was least where the ratio of valve body orifice to vapor tap was greatest. When alcohol was present in the formulation, its concentration in the total product increased the most with the smallest valve body orifice and the least with the largest valve body orifice.

Applicators and Fitments—A review (100) of various fitments applicable for use with pharmaceutical aerosols indicated a variety of different designs. These are used to dispense oral and topical aerosols. Applicators suitable for use with aerosols designed to be applied to the vagina, ear, nose, and eye were also described.

One problem associated with the use of oral aerosols by asthmatics is their failure to coordinate the release of medication with inspiration. This problem has become rather serious with children and the elderly. A device has been developed that automatically dispenses the medication as the patient inhales, and the operation of this system was described (100, 101). One study (102) reported that this device showed a statistically greater improvement in patients as compared to the standard inhaler. Since the valve operates only when the patient achieves a flow rate of about 40–50 liters/min, one is assured that the medication is released at the proper moment.

Although not used with pressurized systems, a device has been developed for use with dry powder aerosols (103). This inhaler utilizes a powder contained in a gelatin capsule. The capsule is punctured and the powder is then carried into the inspired air during inspiration. Since this unit is used with dry powders, the dosage is not limited except by the size of the capsule used. This inhaler also achieves coordination of inspiratory effort and inhalant administration.

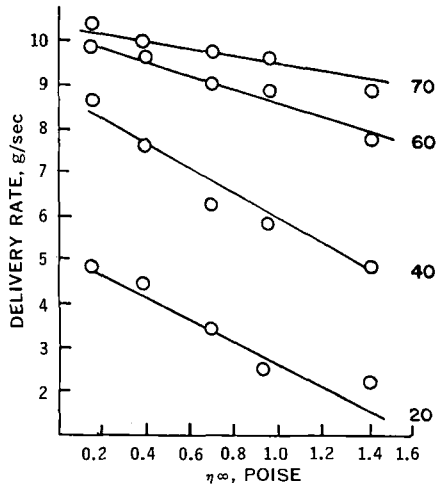

Figure 14—*Plot of* $\eta\infty$ versus *delivery rate for sodium carboxymethylcellulose solution at pressures of 20–70 psig. (From Ref. 93.)*

Containers—A review of the literature concerning developments in aerosol container technology for pharmaceutical aerosols over the past 10–15 years reveals little change. Several reviews appeared (104–108), and additional articles were concerned with various aspects of container corrosion (109–112). Giggard (113) discussed some developments in aerosol can linings. To decrease the danger of attack upon the product by the tin plate or steel of the container, an internal lining is generally applied to the flat tin plate. Following fabrication of the container, an additional coating of organic lining is added over the side seam.

While solder of varying composition has been used to seal the body of a fabricated can, a recent development made available a container electrically welded and not soldered (114). This development may find application for pharmaceutical aerosols where the presence of solder can lead to incompatibility between the container and the product. In addition, the presence of pieces of loose solder in the container can add to the severity of this problem and lead to valve clogging.

Aerosol containers made from aluminum have been used to package many different pharmaceutical aerosols. Since these containers are drawn from a single slug of aluminum, there are no side seams and the container is less prone to leakage. Additionally, aluminum is less subject to attack than tin plate aerosol containers. However, aluminum aerosol containers packaged with ethanol are subject to corrosion under certain conditions. It is known that aluminum will react with anhydrous ethanol to form aluminum alcoholate (115) (Scheme I):

$$2Al + 6C_2H_5OH \longrightarrow 2(C_2H_5O)_3Al + 3H_2$$

Scheme I

Further studies on the relationship between this reaction and the composition of the aluminum used to fabricate the container were carried out by Yamamoto *et al.* (116). Since these workers noted perfora-

Table IX[a]—Fill Tolerances for Dichlorodifluoromethane System

Number of Units Analyzed	Propellant Line, psig	Nominal Fill, g	Mean Fill, g	*SD*, g	Experimental Range, g	Tolerance Limits[b], g		Tolerance Limit Spread, g
						Lower	Upper	
49	500	5	5.556	0.596	2.430	3.691	7.421	3.730
48	500	20	20.078	0.744	3.361	17.743	22.413	4.670
43	500	40	39.931	0.596	3.546	38.038	41.824	3.786
46	500	47	46.690	0.583	2.663	44.847	48.533	3.686
48	500	60	59.020	0.603	3.592	57.127	60.913	3.786
50	500	80	80.289	0.480	2.209	78.789	81.789	3.000
46	900	20	19.313	0.527	2.415	17.647	20.790	3.143
43	900	60	58.469	0.455	2.203	57.024	59.914	2.890
50	900	80	80.312	0.432	0.255	78.962	81.662	2.700

[a] From Ref. 123. [b] The tolerance limits will be expected to contain 99% of the population with 95% confidence.

tions taking place in containers packaged with trichloromonofluoromethane[5] and ethanol and not in the aluminum containers packaged with dichlorodifluoromethane, they concluded that the corrosion taking place was apparently due to the presence of ethanol in combination with trichloromonofluoromethane. They also noted that corrosion was a function of not only the alloy used to prepare the aluminum slug but also of the method used to fabricate the container.

The development of aerosol containers with a pressure relief device was discussed (117, 118). These devices would release the excess pressure if a rapid build-up in pressure in an aerosol container occurred due to a fire or some other cause of heat. One such device, accepted by the Department of Transportation for use with aerosols, consists of a number of scores placed at precisely located points around the top of the aerosol container. Under high internal pressure, the top of the container will buckle upward, opening the scores and allowing the gas to escape slowly.

MANUFACTURING AND PACKAGING OF AEROSOLS

The packaging of pharmaceutical and cosmetic aerosols generally does not involve any special techniques. However, since these products are used topically or orally, special precautions must be taken to ensure that they are manufactured and packaged according to specification and that there is no contamination of the product with either foreign materials or microbial organisms. In regard to the latter, several reports were published as to the need for and the setup of aseptic filling lines for aerosols.

Joyner (119, 120) suggested the addition of ethylene oxide to the container to sterilize the product. He indicated that this method has not been fully accepted by various governmental agencies. The filling of pharmaceutical aerosols under aseptic conditions has been reported (121, 122). Methods useful for sterilizing each component, the equipment, and the final package were indicated. While many will agree upon the importance of aseptic filling, most facilities used to package pharmaceutical aerosols are kept separate from other filling lines and, in many instances, the line is completely glass enclosed.

In a study designed to determine the fill tolerances and the effect of variables upon the packaging of pharmaceutical and cosmetic aerosols, it was noted that the under-the-cap filler produced satisfactory results (123). Various propellant systems as well as filling pressures were studied. Tables VIII and IX indicate the results obtained using two different propellant systems. Based upon these results, the investigators concluded that only a variation in propellant line pressure from 500 to 900 psig was of practical significance. It was also noted that the fill tolerances of the dichlorodifluoromethane system was greater than the fill tolerance of the 1-chloro-1,1-difluoroethane[6]–trichloromonofluoromethane (50:50) system.

The application of various quality control systems to aerosols was discussed (124–131). The importance of in-process quality controls was stressed since part of the actual manufacture takes place during the packaging operation as the product concentrate and propellant are mixed at this time. Specifications for containers, valves, sampling, acceptable quality levels (AQL), control charts, and other aspects were covered specifically for aerosol products.

TESTING OF AEROSOL PRODUCTS

In addition to general testing procedures used to determine the acceptability of the nonaerosol portion of the pharmaceutical and cosmetic aerosol (concentrate), special test procedures have been developed for the aerosol package as well as the evaluation of the final product. These areas of specialization include particle-size distribution of those products dispensed as sprays, rheological examination of foams and semisolids, and special analytical techniques to obtain a quantitative measure of the stability of the final package.

General Testing—Various test procedures have been developed by the Chemical Specialties Manufacturers Association (132). Several of these methods have been modified for use specifically with pharmaceutical aerosols and appear in NF XIII (133). Delivery rate, leak testing, pressure testing, sampling apparatus, and moisture determination represent those test procedures applied to most aerosols. Additional tests may be indicated for specific aerosols. Table X

[5] Propellant 11.

[6] Propellant 142b.

Table X[a]—Testing of Pharmaceutical Aerosols

Product	Test Procedure			
	Delivery Rate	Leak Testing	Pressure Testing	Other
Dexamethasone aerosol	Yes	Yes	Yes	—
Isoproterenol sulfate aerosol	No	Yes	No	Unit spray content particle size
Povidone-iodine aerosol	No	Yes	Yes	—
Thimerosal aerosol	No	Yes	Yes	—
Triacetin aerosol	No	Yes	Yes	—
Triamcinolone acetonide aerosol	No	Yes	Yes	—

[a] From Ref. 134.

indicates the tests required for some pharmaceutical aerosols included in the NF. Additional test procedures were reviewed (60, 135, 136).

Analytical Procedures—Since the aerosol package is pressurized, the procedures generally used to obtain a sample for analysis cannot be used without modification. A sampling chamber, which can also be used as a titration vessel, was utilized to assay aerosols containing benzocaine, epinephrine bitartrate, ergotamine tartrate, isoproterenol hydrochloride, prednisolone, cyclomethycaine hydrochloride, and pramoxine (137). The results indicated that this chamber could be used to obtain a representative sample of the product; in those cases where a titration was involved, the vessel could also serve for the titration.

A pressure attachment for a rotational rheometer was described (138), and this device made possible the evaluation of pharmaceutical and cosmetic foams. Various soap concentrates were pressurized and the flow characteristics of the foam produced from each emulsion were evaluated using a pressure rheometer. These foams showed pseudoplastic properties which could not always be related to the flow properties of the resultant foams. Sanders (85) described a glass pressure cell which could be used to observe foams under a microscope. He noted that the pressure cell was most satisfactory when used with a stable emulsion system.

Gas chromatography has been used extensively to assay pharmaceutical and cosmetic aerosols. Cohen (139) investigated the accurate sampling and analysis of volatile components of aerosols used in inhalation therapy. By utilizing a pressure syringe, a representative sample of the aerosol was obtained which could then be injected into the gas chromatograph. Air, dichlorodifluoromethane, water, dichlorotetrafluoroethane, ethanol, and ethylene glycol monomethyl ether were separated by this method. When these results were compared to those from other analytical procedures, differences of less than 1% were noted and in most cases these differences were less than 0.5%.

Other investigators applied gas chromatography to the analysis of aerosols containing volatile oils (140) and medicinal amines (141). In both cases, it was noted that these constituents could be easily separated by gas chromatography and gave results comparable to results obtained using other analytical techniques. A method that could be used to separate 18 volatile components common to aerosol formulations was developed (142). By utilizing gas chromatography with thermal conductivity detection, mixtures of alcohols, hydrocarbons, chlorofluorocarbons, and aliphatic halides were separated. The use of vinyl chloride as an internal standard was advantageous because it gave an improvement in the quantitative results.

Methylene chloride in hair sprays was determined using an aerosol transfer button, polyethylene tubing, and a hypodermic needle as a sampling device (143). The sample was introduced into a small serum bottle and transferred to the gas chromatograph with a high pressure liquid sampling syringe. Other studies (144, 145) utilized gas chromatography in conjunction with IR analysis for the detection and analysis of propellants.

Evaluation of Foams—Several methods were developed to evaluate foams, and the various methods used were reviewed (146). Foam density and the effect of propellant substitution and addition of nonpolar constituents upon the foams were determined (147). It was concluded that increasing the concentration of propellant will result in increased foam consistency. Foam consistency increased with an increase in soap concentration. Further studies on foams included foam stability and effect of shear (148). When using a rotational viscometer, it was noted that pressurized foams exhibited a marked decrease in foam consistency with time and that the rate of decrease was independent of formulation. It was concluded that pressurized foams are plastic in nature.

The final study in this series was concerned with changes in propellant concentration as the contents of an emulsion aerosol were discharged (Table XI) (149). This was related to changes in foam consistency. Based upon the results, it can be noted that fractionation of propellant blends will take place along with a change in the ratio of propellant to soap concentrate.

A photographic technique for the analysis of bubble size of foams was studied (150), and foam stiffness was measured (151) using a curd tension meter. The relationship between foam stiffness and density with product discharge was noted. Propellant composition was found to affect both stiffness and foam density.

Particle-Size Analysis—Depending upon the size range of the various particles found in an aerosol spray, several methods can be used for the measurement of particle size. The techniques may be complicated by the properties of the particles as well as the difficulty encountered in sampling the spray to obtain a representative sample. The term particle size generally refers to solid particles, while droplet size refers to liquid particles. In many instances, however, the terms have been used interchangeably.

For medicinal aerosols, the size of both liquid and

Table XI[a]—Computer Data for Emptying of a Pressurized Foam Container Employing a 57:43 (w/w) Mixture of Dichlorodifluoromethane and Dichlorotetrafluoroethane (10% w/w) as Propellant

Dichlorodifluoromethane		Dichlorotetrafluoroethane		Total Emulsion, g	Total Pressure, psia	Amount Removed, g
Parts, g	% (w/w)	Parts, g	% (w/w)			
		Initial Concentrations				
5.700	5.700	4.300	4.300	100.000	0.000	0.0
		Concentrations after Vapor Space Filling				
5.420	5.439	4.229	4.244	99.649	64.550	0.0
		Removing 1 g/Actuation				
5.420	5.439	4.229	4.244	99.649	64.550	0.0
4.701	5.258	3.757	4.202	89.417	64.237	10.0
4.005	5.058	3.288	4.153	79.188	63.885	20.0
3.335	4.837	2.823	4.093	68.961	63.484	30.0
2.694	4.587	2.361	4.020	58.737	63.022	40.0
2.087	4.302	1.904	3.925	48.517	62.482	50.0
1.519	3.968	1.454	3.796	38.302	61.841	60.0
1.000	3.562	1.013	3.607	28.093	61.068	70.0
0.542	3.035	0.589	3.334	18.913	60.228	80.0
0.200	2.594	0.185	2.406	7.721	58.402	90.0
		Removing 5 g/Actuation				
5.420	5.439	4.229	4.244	99.649	64.550	0.0
4.696	5.252	3.757	4.202	89.416	64.358	10.0
3.997	5.047	3.287	4.152	79.187	64.012	20.0
3.322	4.818	2.821	4.091	68.959	63.616	30.0
2.678	4.559	2.359	4.016	58.735	63.159	40.0
2.067	4.261	1.901	3.918	48.514	62.622	50.0
1.497	3.909	1.449	3.784	38.299	61.980	60.0
0.976	3.476	1.006	3.584	28.090	61.196	70.0
0.519	2.903	0.579	3.236	17.891	60.217	80.0
0.149	1.939	0.183	2.380	7.705	58.983	90.0
		Removing 10 g/Actuation				
5.420	5.439	4.229	4.244	99.649	64.550	0.0
4.690	5.245	3.757	4.202	89.415	64.520	10.0
3.985	5.032	3.287	4.151	79.185	64.172	20.0
3.306	4.794	2.819	4.089	68.957	63.786	30.0
2.656	4.523	2.356	4.011	58.732	63.337	40.0
2.041	4.207	1.897	3.910	48.511	62.808	50.0
1.466	3.830	1.443	3.769	38.295	62.179	60.0
0.943	3.358	0.998	3.554	28.086	61.380	70.0
0.485	2.714	0.565	3.163	17.887	60.365	80.0
0.120	1.568	0.159	2.070	7.704	59.003	90.0

[a] From Ref. 149.

solid particles may be involved from the time the particles leave the nozzle of the aerosol package to the time the particles are deposited onto the desired surface of the respiratory tract. In many instances, the active ingredient is a solid such as epinephrine bitartrate, sulfate, or hydrochloride; isoproterenol sulfate or hydrochloride; and ergotamine tartrate; this solid is either dissolved in a solvent such as water and alcohol or suspended directly in the propellant. In any event, the ultimate result is that the solvents and propellant vaporize, leaving behind the solid particles of active ingredient. These solid particles, which must be finely subdivided initially, are generally in the range of from 1 to 10 μm and, in most cases, are from 3 to 4 μm in diameter.

Many techniques and instruments have been devised to measure particle size in aerosol systems. Several methods used for aerosols include microscopy, sedimentation, impaction and inertial techniques, and optical methods. Direct methods include the use of microscopes and sieves. Indirect methods are based on the measurement of some property of the particle that is related to its size such as sedimentation velocity, density, viscosity of medium, light-scattering ability, and susceptibility to impaction. Many indirect methods are used to a greater extent than the direct methods since they are more convenient and are less time consuming.

Several review articles covered many of these methods. Licht (152) presented a theoretical treatment of movement of aerosol particles, indicating that the movement was governed primarily by the influence of gravity, drag, inertia, diffusion, and electrostatic charges. It is the combined effect of these forces that determines the path of the particle which, in turn, can be related to the ultimate deposition of the particle. Furthermore, Licht stated that particles less than 50 μm in diameter will be suspended in a free space and that particles greater than 10 μm but less than 50 μm in diameter will have the greatest tendency to deposit upon a surface. This will then take advantage of the inertial effects. Since particles greater than 10 μm will probably not enter the respiratory system, particles should be greater than 10 μm in diameter where inhalation toxicity is a consideration.

Deposition of particles on flat surfaces was also discussed (153). Factors affecting particle-size distribution of aerosol sprays were covered (154). The influence of the valve and propellant upon particle size

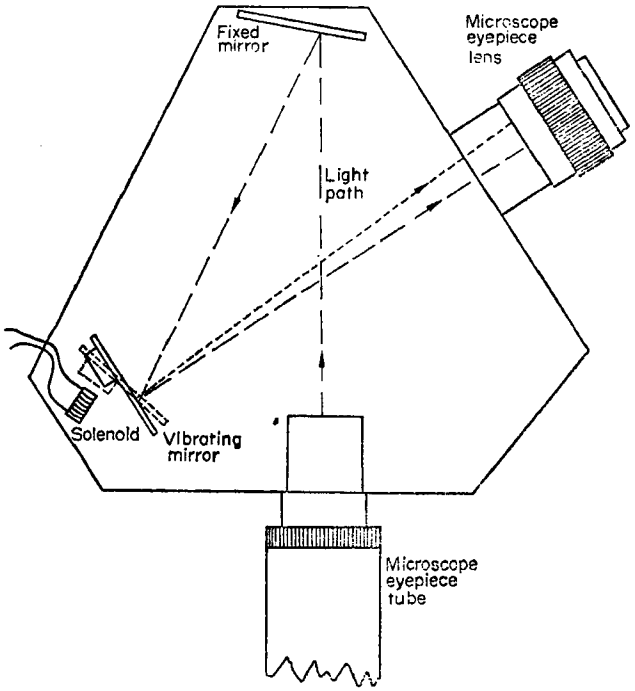

Figure 15—*Cut-away drawing of vibrator unit showing optical system. (From Ref. 159.)*

was determined using high speed electronic photography. Gidwani (155) covered the theoretical aspects of particle-size distribution of powder aerosols. He compared the collection of solid particles on a nest of screens to the collection of particles on the glass slides of a cascade impactor and indicated that the impaction technique was an extension of the sieving technique.

Of these methods, an impaction technique utilizing the cascade impactor has been used to the greatest extent for aerosol products. Polli *et al.* (156) used this technique for determining the influence of drug particle size, drug concentration, surfactant concentration, valve, vapor pressure, and propellant temperature upon the particle-size distribution of inhalation-type aerosols. Based upon the results, it was concluded that the particle size of the spray may be decreased by reducing drug particle size, drug concentration, and orifice of the valve. An increase in vapor pressure and propellant temperature also reduced the particle size. The inclusion of a surfactant in the formulation aided in reducing the particle size.

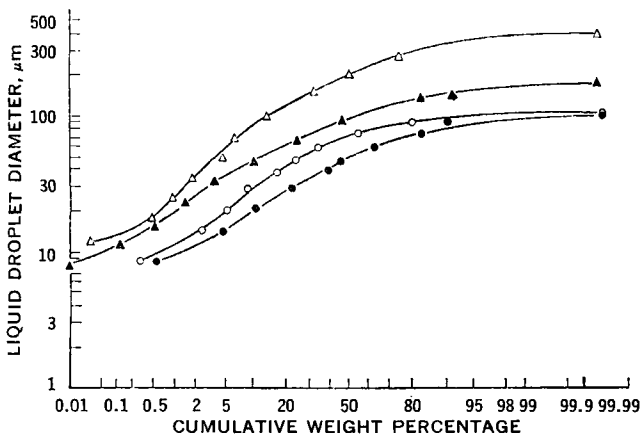

Figure 16—*Particle-size distributions of hair sprays packed at different pressures. Key: △, 145 kNm^{-2}; ▲, 179 kNm^{-2}; ○, 276 kNm^{-2}; and ●, 393 kNm^{-2}. (From Ref. 159.)*

Studies by Sciarra *et al.* (157) utilized the cascade impactor as a screening method for different formulations to select the one producing the least number of particles in the respirable range (less than 10 μm in diameter). The effects of valve orifice and propellant concentration upon the particle-size distribution have been shown. Sciarra and Adelman (158) showed the usefulness of adding a fluorescent tracer to the aerosol prior to the determination of the particle-size distribution of the product. Gussman *et al.* (159) modified the cascade impactor so that samples containing a high volume of airborne particulate matter could be analyzed. With this high volume sampler, these investigators were able to classify aerosol particles between 1 and 20 μm.

Recently, a modification of the microscopic method for measuring the size of particles was utilized to measure the particle-size distribution of a hair spray (160). This method was based upon an extension of the conventional microscopic method using the double-image principle. Particles in the range of from 1 to 250 μm can be classified with an accuracy of ±2%. The system used is shown in Fig. 15, and Fig. 16 illustrates the results obtained using different propellant combinations. The method enables one to count particles on a microscopic slide at the rate of about 2000 particles/hr.

A fairly new development for measuring particle-size distribution of aerosols involves the use of a pulsed ruby laser (wavelength of 0.6943 μm) and a specially designed holographic camera. The laser is fired through the sample to produce the hologram. Particle images then can be reconstructed and viewed on a TV monitor at magnifications up to 1500×. Particles as small as 1.4 μm can be viewed (161).

Particles will deposit in the respiratory tract by inertial impaction. This acts upon particles ranging in diameter from a few microns to over 100 μm. The rate of elimination of particles is a function of their size and mass, as well as the diameter of the air passageways. Particles larger than 5 μm in diameter are prevented from entering the lungs. Particles between 0.5 and 5.0 μm tend to fall against the alveolar walls and mix with the alveolar fluid (162). It is generally agreed that particles larger than 20 μm fail to go beyond the terminal bronchioles, those 6 μm in diameter are removed before they reach the lower alveolar ducts, and almost all particles 2 μm and larger are removed in the lower alveolar ducts (163). There is only a 50% chance for the deposition of particles 1 μm in size. Particles smaller than 1 μm are expired. Idson (162) stated that the particle size for treatment of pulmonary disease should be in the region of 3 μm. For systemic action, particles smaller than 2 μm would be desirable. Dautrebande (164) stated that the depth of penetration increases with decreasing particle size and that the percentage of pulmonary retention increases with increasing particle size. The

relationship between particle size and deposition in the lungs was indicated by many investigators (165–168).

Other Methods—To compare the relative proportion of the doses administered from various aerosol units, Kirk (169, 170) designed a system that could measure the ability of the particles released from an aerosol dosage form to penetrate an artificial system of wet-lined tubes. This system was intended to simulate the upper part of the respiratory tract. Using this system, both solution- and suspension-type aerosols containing isoproterenol were compared as to mean penetrative efficiency using different applicators. In addition, the results were compared with *in vivo* tests conducted on 28 patients. Both clinical and *in vitro* results were comparable, indicating the suitability of the method for the evaluation of the formulations and applicators used with oral aerosols. Kirk also noted that suspension-type formulations were more efficient than those based upon solution in a cosolvent.

A model lung chamber was designed (171) for the evaluation of oral inhalation aerosols. It was shown by evaluating the deposition of materials of known particle size and several commercially available oral aerosols that this lung chamber could be used to determine the efficacy of various aerosol formulations. A vacuum of 30.4 cm (12 in.) of mercury was used based on air flow through the model lung. The deposition of particles in the different compartments was measured as a function of vacuum. Less deviation was noted at this vacuum reading than at 7.6 or 50.8 cm of mercury.

Various workers studied the chilling effect of aerosols upon the skin. Broderick and Flanner (172) and Dunne (173) developed an *in vitro* method for determining the change in temperature upon a surface as it is sprayed with propellant. These methods utilized an aluminum foil-covered surface containing a temperature-recording sensor. Based upon these results, they determined the relative chilling effect of several commonly used propellants alone and in combination with each other as well as with suitable solvents.

A quantitative evaluation of the chilling effect was made (174). The developed method is based on the calculation of the instantaneous cooling rate of a specified thermistor probe exposed to the spray in the test chamber. Cooling curves for the propellants were determined and plotted as probe temperature *versus* time (seconds). A plot of log $(T_t - T_m)$ *versus* time produces a straight line (T_t is the temperature of the probe at time T, and T_m is the minimum probe temperature). Based upon this relationship, the following equation is applicable:

$$\frac{-dT_t}{dt} = K(T_t - T_m) \qquad \text{(Eq. 10)}$$

When integrated between initial probe temperature, T_0, and probe temperature, T_t, at time t, one obtains:

$$\log \frac{T_0 - T_m}{T_t - T_m} = \frac{kt}{2.303} \qquad \text{(Eq. 11)}$$

The constant, k, is the first-order cooling rate constant and is obtained from the plot. From these data, the chill index, I_c, may be computed for each propellant as well as propellant blends and mixtures of propellants with solvents according to the equation:

$$I_c = k(T_n - T_m) = 0.693(T_n - T_m)/t_{0.5^c} \qquad \text{(Eq. 12)}$$

where T_n is the normal body temperature.

Kabasakalian (175) noted the dispensing efficiency of some commercially available nonmetered topical aerosols to be low as compared to ointments, creams, and lotions. This is in contrast to the many reports indicating the efficient application of topical aerosols. In this study, pickup efficiency was used as a criterion for the evaluation of the spray aerosol. A method for determining pickup efficiency has been described in the literature (176) and is commonly used with nonpharmaceutical residual spray-type products. Based upon results obtained using this test method, Kabasakalian noted that time, temperature, and distance from the target area were all important parameters related to pickup efficiency. He also noted a great variation in pickup efficiency at room temperature for some typical pharmaceutical aerosols.

TOXICITY CONSIDERATIONS

The fluorinated hydrocarbons are generally considered to be inert and nontoxic when used as directed. Several complete reviews of this subject were given (177–179). It is on the basis of their nontoxic nature that the fluorinated hydrocarbons have been widely used as refrigerants as well as for aerosol propellants.

DELIBERATE PRODUCT ABUSE

Since 1967 when persons started deliberately to inhale the concentrated vapors of the propellant, attention has been directed toward the toxicity of the propellant. To date, over 100 deaths have been attributed to the deliberate inhalation of aerosols. Death has been attributed to freezing of the larynx, lack of oxygen, and abnormal heart rhythms induced by the simultaneous inhalation of the vapors in high concentration and the secretion of adrenalin in response to excitement, fear, or exertion. Bass (180) collected records of 110 sudden sniffing deaths; he reported that the excessive inhalation of volatile hydrocarbons from aerosols and various chemicals used in glues, paint thinner, gasoline, *etc.*, may be the cause of these sudden deaths (181).

Not only have products containing pure propellant been subjected to abuse (182), but so have products such as hair sprays, deodorants, and antiperspirants (183). There are many other references to this deliberate abuse of propellants either alone or in combination with other ingredients, and the "aerosol industry" has mounted an extensive educational program aimed at the high school population.

Propellant Toxicity—This topic is most contro-

Table XII—Some Physical Properties of Propellants

Propellant	Chemical Structure	Molecular Weight	Boiling Point °F	Boiling Point °C	Vapor Pressure, psia 70°F	Solubility in Water, wt %
Trichloromonofluoromethane	CCl_3F	137.4	74.8	23.8	13.4	0.11[a]
Dichlorodifluoromethane	CCl_2F_2	120.9	−21.6	−29.8	84.9	0.028[a]
Dichlorotetrafluoroethane	$CClF_2—CClF_2$	170.9	38.8	3.8	1.8	0.013[a]
1-Chloro-1,1-difluoroethane	CH_3CClF_2	100.5	15.1	−9.4	43.8	0.14[b]
1,1-Difluoroethane	CH_3CHF_2	66.05	−11.2	−24.0	76.4	0.32[b]

[a] At 1 atm pressure and 22° (77° F). [b] At 1 atm pressure and 21° (70° F).

versial at the present time. Ever since Taylor and Harris (184) reported the cardiotoxicity of aerosol propellants, a flood of reports have indicated either that the propellants were toxic or safe. Taylor and Harris noted that the fluoroalkane gases used to propel aerosols were toxic to the heart of mice, sensitizing them to asphyxia-induced sinus bradycardia, atrioventricular block, and T-wave depression. The propellants were postulated to possess a spectrum of cardiotoxic effects capable of causing bradyarrhythmias, tachyarrhythmias, or myocardial depression. Taylor and Harris concluded that the fluorinated hydrocarbons can no longer be considered inert. Further studies (185–187) reached the same conclusion.

In reply to the Taylor and Harris report, Zapp (188, 189) reported that the lack of oxygen, not cardiac toxicity of the fluorocarbon propellants, was the probable cause of death of the mice. The fluorocarbon propellants have very low order toxicity on the scale of comparative toxicities. The vapors of the fluorocarbon propellants produce narcotic effects if inhaled at a high enough concentration. Deliberate misuse by collecting and inhaling concentrated vapors of propellants in the absence of oxygen probably causes death (181, 189). Azar *et al.* (190) concluded from their study that: "from the practical point of view, it is important to note that the induction of cardiac sensitization or asphyxia requires the deliberate inhalation of high concentrations of propellants—much higher concentrations than those generated when aerosol products, including asthma inhalers, are used as directed." They also noted that there was no evidence of any form of chronic cardiac toxicity. Similar conclusions were reported by others (191–193).

Typical of the many studies following the Taylor and Harris report is one by Flowers and Horan (194).

Table XIII[a]—Blood Concentrations of Trichloromonofluoromethane

Minutes	Blood Concentration, μg/ml: Patient 1 (Three Puffs), Arterial	Patient 2 (Six Puffs), Arterial	Patient 2 (Six Puffs), Venous
0.5	—	0.63	—
1	1.7	0.39	—
2	0.5	0.39	0.20
3	0.35	—	—
4	—	—	0.22

[a] From Ref. 196.

These workers were able to produce cardiac arrhythmias in dogs exposed to aerosols containing fluorinated hydrocarbons as propellants. They indicated that the studies were carried out in an atmosphere containing normal arterial oxygen tension, carbon dioxide tension, and other elements necessary to sustain life. They also showed that trichloromonofluoromethane was readily absorbed into the blood after inhalation. The less volatile propellants are thought to be absorbed to a greater extent than the more volatile compounds such as dichlorodifluoromethane.

Typical of the studies reporting on the absence of sensitization of the myocardium to arrhythmias induced by asphyxia is a report by Egle *et al.* (195). These workers were not able to duplicate the results reported by Taylor and Harris (184). According to Egle *et al.*, their results do not support the contention that inhalation of haloalkane propellants under the conditions employed by Taylor and Harris makes cardiac activity in mice more sensitive to the effects of asphyxia. Further aspects of their study led to the conclusion that the propellants would not sensitize the heart to catecholamines in the generally used concentrations, but that higher concentrations of propellant could result in this effect.

Other studies have been directed toward determination of the concentration of fluorocarbon in the bloodstream following inhalation. A literature search reveals little information as to the presence of a known amount of fluorocarbon in the body. However, the need for this type of information has not been definitely determined.

The detection of fluorocarbons in body fluids is not an easy task. The physical properties of the propellants are such that their detection would be difficult. Their relatively low boiling point, high vapor pressure, and low aqueous solubility tend to minimize their presence in body fluids in sufficient concentration so that they can be detected (Table XII).

As can be seen from Table XII, of the propellants listed, only trichloromonofluoromethane is likely to be found in any reasonable amount in the bloodstream. 1-Chloro-1,1-difluoroethane and 1,1-difluoroethane[7] have higher water solubilities but also lower boiling points and would tend to escape from the bloodstream.

A compound does not have to be in the bloodstream to exert a toxic reaction. Volatile materials such as these gases can act in the lungs and locally

[7] Propellant 152a.

Table XIV[a]—Peak Blood Levels of Fluorocarbons in Dogs after Aerosol Administration[b]

Fluorocarbon	Peak Level, μg/ml		Peak Arterial Level as Percent of Administered Dose[c]	
	10 Actuations	5 Actuations	10 Actuations	5 Actuations
Trichloromonofluoromethane				
Arterial	22.3 ± 1.0	13.2 ± 1.4	15.9	8.89
Venous	6.22 ± 2.6	2.45 ± 0.29		
Dichlorodifluoromethane				
Arterial	6.17 ± 0.38	3.16 ± 0.06	4.41	4.51
Venous	1.54 ± 0.84	0.56 ± 0.04		
Trichlorotrifluoroethane				
Arterial	11.56 ± 1.78	6.43 ± 0.61	8.26	9.19
Venous	2.96 ± 1.40	0.79 ± 0.06		
Dichlorotetrafluoroethane				
Arterial	3.80 ± 0.52	2.32 ± 0.12	2.71	3.31
Venous	0.87 ± 0.41	0.26 ± 0[d]		

[a] From Ref. 197. [b] Five or 10 actuations of an aerosol mixture containing 25% (w/w) of trichloromonofluoromethane, dichlorodifluoromethane, trichlorotrifluoroethane, and dichlorotetrafluoroethane were administered to three or four dogs, respectively. One actuation delivered 16.8 mg of each fluorocarbon. The results are expressed as the mean ± *SE* of the peak fluorocarbon blood level determined for three or four dogs. [c] Calculated for a 15-kg dog with 8% of body weight as blood volume. [d] Mean ± *SE* from two dogs.

upon the respiratory system. However, for systemic toxicity to occur, the material must be present in the bloodstream and from there be carried to the site of action. The recent reports in which it was noted that propellants were found in the bloodstream of patients and dogs gave rise to additional investigations.

Since the amount of propellant that may be present in the bloodstream is very small, analytical techniques capable of detecting fairly low levels of fluorocarbons were investigated. Gas chromatography was useful for this purpose. Dollery *et al.* (196) used gas chromatography with a nickel-63 electron-capture detector; Shargel and Koss (197) also used an electron-capture detector, but the source of the electrons was from tritium. Under these conditions, both groups were able to detect fluorocarbons in the bloodstream in the quantities noted in Tables XIII and XIV. An examination of these two tables indicates that generally trichloromonofluoromethane is detected in a greater concentration than the other propellants.

Product Toxicity—For many years, aerosols containing epinephrine or isoproterenol have been the center of controversy. Restrictions were placed on the labeling of aerosol preparations containing isoproterenol in 1968 (198–202) when several reports of death through alleged overuse of isoproterenol aerosols came to light. The Federal Register of June 18, 1968 indicated that all inhalation preparations containing isoproterenol must bear the following statement:

> *Occasional patients have been reported to develop severe paradoxical airway resistance with repeated, excessive use of isoproterenol inhalation preparations. The cause of this refractory state is unknown. It is advisable that in such instances the use of this preparation be discontinued immediately and alternative therapy instituted, since in the reported cases the patients did not respond to other forms of therapy until the drug was withdrawn.*
>
> *Deaths have been reported following excessive use of isoproterenol inhalation preparations, and the exact cause is unknown. Cardiac arrest was noted in several instances.*

Since 1968, reports continued to appear in the medical literature as to adverse reaction (30, 203–206). Recently, in addition to isoproterenol preparations, epinephrine aerosols were said to cause similar reactions when overused. As a result, the Federal Register of April 15, 1972, published that similar warning statements would now be required for epinephrine inhalation preparations.

The reports showed that severe paradoxical bronchoconstriction episodes, but no deaths, occurred as a result of excessive and repeated use of epinephrine inhalation preparations. Thus, only the first warning statement as indicated for isoproterenol inhalation preparations must be included and not the statement concerned with deaths following excessive use.

Since the time when the first aerosol product was used commercially in the late 1940's, reports have appeared as to the possible hazard of inhaling the particles. Today, many reports have been issued as to the potential hazard of using these products. Many of these reports have been issued by various consumer groups, and these findings were recently summarized (207). Cambridge (208) reviewed the toxicity noted with hair sprays in the early 1950's as well as some recent studies. Different household aerosol products were studied by Marier *et al.* (209). With an exposure to fluorocarbons of about 10 times greater than normal, these workers were not able to detect any measurable fluorocarbon blood levels nor any indication of toxicity.

Other aerosols have become suspect in various toxic manifestations. Feminine hygiene sprays, medicated vaporizer sprays, and adhesive sprays have been subjected to scrutiny by various consumer and governmental agencies. Their status, as well as the status of other aerosols, is currently under attack due to suspected toxicity. A massive research program is underway at present, and hopefully the results of these reports will shed light on this highly controversial subject.

CONCLUSION

The aerosol dosage form has been accepted by both

the medical and pharmaceutical professions as another means for the administration of drugs orally, topically, or into one of the body cavities. As noted by the number of original research articles appearing in various publications, there is a great deal of interest in furthering the use of this dosage form. However, there is need for additional research and development before it can fully realize its potential.

The administration of many drugs for inhalation therapy by means of a pressured system represents a rather large area in need of development. With the exception of drugs intended to relieve the symptoms of asthma, this dosage form has not been applied to many other drugs. Encouraging, however, is the fact that the literature does give reports on the use of insulin (210) and other drugs (211–215) by inhalation therapy. These drugs represent a potential for further application. The use of antibiotic aerosols, with or without a plastic dressing, following surgery has also been indicated (216–218). Additional applications include contraceptives (219), topical anesthetics (220–221), and antimycotics (222–223). These represent only a few of many other applications which have been studied but may not have been fully developed.

New techniques for the formulation need to be developed. Codispensing systems and barrier packs have not been used to any great extent. Microencapsulation techniques, while widely used in the pharmaceutical industry, recently have been applied to aerosol systems (224, 225). As these and other systems are developed, many advantages of the aerosol dosage form can be fully realized.

At the present time, the toxicity potential of aerosols and the hazards, if any, that they may cause are important problems. The Food and Drug Administration and other governmental and private agencies are concerned with these problems. Further studies are indicated to determine the toxicity of these products. Particle-size distribution and inhalation and the effect that they have upon the human body must be determined. Current studies are directed toward this end.

Publications by the Food and Drug Administration (Federal Register of March 7, 1973) indicated the "proposal regarding warning statements" to be included on the label of all food, drug, and cosmetic aerosol products containing "halocarbons" and, to a lesser extent, hydrocarbons.

The proposed regulations mandate the inclusion on the label of these products the following statements:

Warning—Do not inhale directly; deliberate inhalation of contents can cause death.

or:

Warning—Use only as directed; intentional misuse by deliberately concentrating and inhaling the contents can be harmful or fatal.

In addition, pharmaceutical aerosols must contain the following:

Warning—Avoid inhaling. Keep away from eyes or other mucous membranes.

The statement "avoid inhaling" is not necessary for preparations specifically designed for use by inhalation, and the phrase "or other mucous membranes" is not necessary for preparations specifically designed for use in mucous membranes. These exemptions have been included for oral inhalants such as the antiasthmatics.

These statements are to be included along with the previously required statements as to contents under pressure, keep out of reach of children, do not incinerate or puncture container, *etc.*

Pharmaceutical aerosols represent only a small segment of all dosage forms available. However, the application of all areas of research is required to develop and manufacture the finished product. While most manufacturing and packaging of these aerosols are left to outside contract facilities, the need for the marketer to develop suitable quality control procedures and packaging specifications is apparent. These procedures have been available and are widely used, although there is need for greater sophistication of the methods and specifications. Metered valves along with delivery of dosage, uniformity of dosage, valve leakage, *etc.*, are all factors that must be satisfactorily considered before the product can be introduced into the marketplace.

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Present address: Brooklyn College of Pharmacy, Long Island University, Brooklyn, NY 11216

RESEARCH ARTICLES

Spectrofluorodensitometric Determination of Flurazepam and Its Major Metabolites in Blood

J. ARTHUR F. de SILVA ˣ, IHOR BEKERSKY, and CARL V. PUGLISI

Abstract □ The spectrofluorodensitometric assay for the determination of flurazepam and its major metabolites in blood involves selective extraction of flurazepam and its major metabolites into ether and hydrolysis in acid to their respective benzophenones, which are cyclized in dimethylformamide–potassium carbonate to their respective 9-acridanone derivatives and separated by TLC. The fluorescence of the 9-acridanones is measured directly on the chromatoplate, using a scanning TLC analyzer in the reflectance mode. The limits of detection of the assay are 0.5–1.0 ng of each compound/ml of blood using a 4-ml specimen/analysis.

Keyphrases □ Flurazepam and major metabolites—fluorometric TLC analysis in blood □ TLC, spectrofluorodensitometry—analysis, flurazepam and metabolites in blood □ Spectrofluorodensitometry—analysis, flurazepam and metabolites

The quantitation of organic compounds separated by TLC has usually been done after elution of the component into a suitable solvent, followed by the use of an appropriate physicochemical assay procedure (1). These methods impart a high degree of flexibility and specificity for quantitation. However, the precision of the assay requires minimal physical loss of the silica gel during its transfer from the chromatoplate and maximal elution recovery of the compound.

Direct quantitation of compounds on a chromatoplate using scanning chromatogram analyzers has the distinct advantages of greater precision, sensitivity, and time saving (2–5). The advantages and disadvantages of quantitative measurements made in the transmission *versus* the reflectance modes of operation for spectrophotodensitometry and spectrofluorodensitometry have been extensively discussed (6–13). The use of these direct scanning techniques for the quantitation of drugs has also been well documented (11–16).

A spectrofluorometric assay for the determination of flurazepam[1] (I) and its major metabolites in blood and urine, the hydroxyethyl (II) and *N*-desalkyl (III) analogs, was reported (17), which uses the fluorescence of their respective 9-acridanone derivatives (18, 19) for quantitation after TLC separation. The chemical structures and reactions of flurazepam (I) and its major metabolites are shown in Scheme I.

The use of a direct scanning chromatogram analyzer enabled the simultaneous quantitation of the 9-acridanones with greater precision and sensitivity because the component to be quantitated is concentrated over a small surface area. The three compounds are completely resolved from each other and from interfering biological contaminants and migrate as distinct fluorescent spots (Fig. 1). They are quantitated by scanning the chromatoplate at a fixed excitation wavelength (383 nm) and measuring the fluorescence emitted (at 457 nm) at a 45° angle of reflectance (20).

[1] Flurazepam, 7-chloro-1-[2-(diethylamino)ethyl]-5-(*o*-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one dihydrochloride, is the active drug in the pharmaceutical formulation Dalmane, Hoffmann-La Roche Inc., Nutley, N.J.

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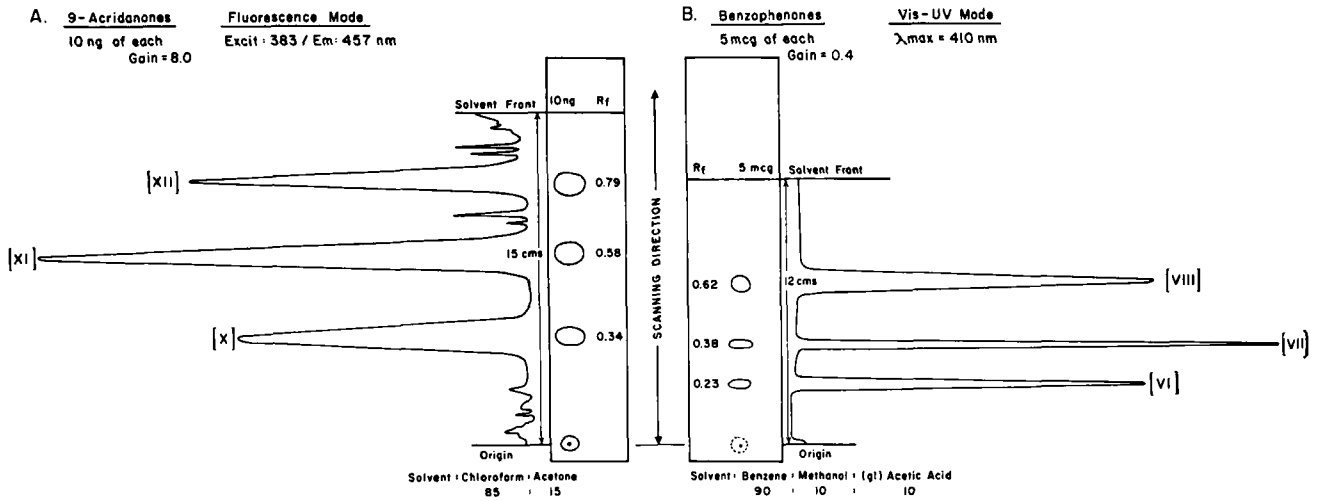


Figure 1—*Thin-layer chromatograms and densitometric scans of: (A) the 9-acridanones determined in the fluorescence mode, and (B) the benzophenones determined in the visual–UV mode.*

The limits of detection are 0.5–1.0 ng each of I, II, and III per milliliter of blood using a 4-ml specimen/analysis.

Blood levels of flurazepam and its major metabolites were determined in two subjects following a single 30-mg dose of flurazepam administered orally.

EXPERIMENTAL

Instrumentation—A TLC spectrophotodensitometer[2] equipped with a 150-w, dc xenon arc energy source was used for direct scanning in the 45° reflectance mode (20). The instrument is equipped with motor-driven dual monochromators for excitation and emission measurements and for automatic wavelength scanning of the excitation and emission spectra of a component directly on the chromatoplate.

The following instrumental parameters were used for the measurement of fluorescence emission of the 9-acridanones.

Fluorescence Mode—The excitation monochromator was set at 383 nm; the exciter leg (beam condenser) has a 0.500-in. reducer (spacer) and a No. 7-54 optical filter. The emission or analyzer monochromator was set at 457 nm, and a No. 3-73 optical filter was used with the 300–800-nm transmission lens in the analyzer leg without any reducers. A sensitive photomultiplier[3] was used in conjunction with 10-nm slits at the analyzer (Table I).

The chromatoplate was scanned spatially at a rate of 4 in./min in the single-beam recording mode, with the recorder chart speed also set at 4 in./min. A baseline controller connected between the electrometer output and the recorder was essential for adjusting the baseline above electrical zero for accurate quantitation. This adapter was retrofitted to this instrument to correct a drift problem.

Calibration—Authentic standards of known concentrations of the three acridanones were spotted as standard mixtures on the plate and developed on the same plate as the biological specimens to minimize the plate variables (Fig. 1A). These standards were used to calibrate the response of the instrument and to adjust it according to the expected concentration range of the unknowns.

Absorption (Visual-UV) Mode—This mode is proposed for the analysis of the benzophenone (VII) produced by hydrolysis of the major metabolite (II), present in urine as a glucuronide conjugate. Although the benzophenones are intensely yellow and can be quantitated at 400 nm, greater sensitivity can be achieved by using the major UV band at 258 nm where the absorbance is about 4–5 times that at 400 nm (17). In this mode, the amount of the excitation energy reflected from the surface of the spot (*i.e.*, not absorbed by the compound) is measured at a 45° angle.

For this purpose, the emission monochromator was removed and the photomultiplier housing, containing a UV-sensitive photomultiplier tube[4], was installed in its place. The excitation monochromator was set at 258 nm, and this energy was incident upon the plate through the beam condenser lens (200–375 nm) in the exciter leg containing a 0.625-in. reducer (spacer) and no optical filters. The emission monochromator was removed and the analyzer leg was used with the 200–350-nm lens, in conjunction with a 0.125-in. reducer and a No. 7-54 optical filter. A 10-nm entrance slit was used with the 1P-28 photomultiplier (Table I).

Chromatoplates—Precoated 20 × 20-cm silica gel G chromatoplates of 60-μm particle size and 250-μm bed thickness were used[5]. The samples were applied to the origin of the chromatogram using calibrated 50-μl disposable pipets.

Analysis of Blood Specimens by Fluorodensitometry of Acridanones—The blood specimens are processed exactly as previously described (17), along with a set of standards of the authentic compounds I, II, and III added to blood as a mixture covering the concentration range of 10, 50, or 100 ng of each compound. These standards are used for the determination of each compound in the unknowns. Mixtures (10, 50, or 100 ng) of authentic standards of the 9-acridanones X, XI, and XII are applied to the chromatoplate as the reference standards for calibrating the instrument and as markers for locating the 9-acridanones in the biological extracts.

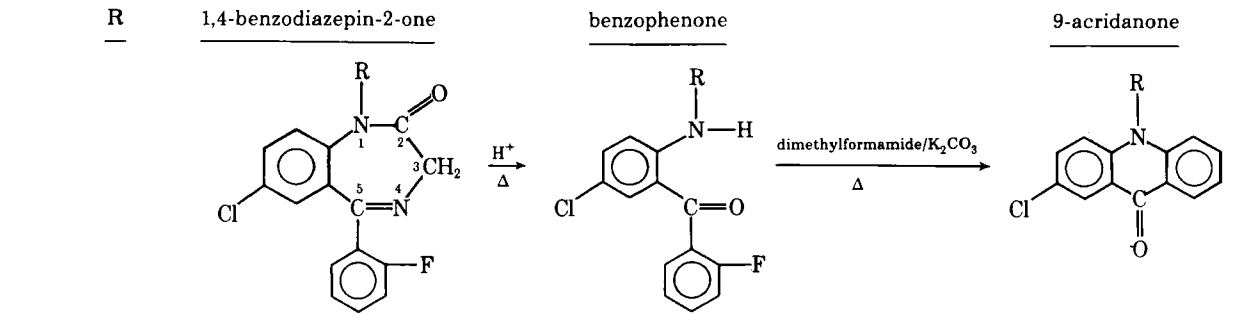
The residues containing the 9-acridanones from the blood extracts are dissolved in 100 μl of chloroform, transferred onto 20 × 20-cm precoated silica gel G thin-layer chromatoplates, and developed in chloroform–acetone (85:15) as described (17) to separate the three 9-acridanones X, XI, and XII (Scheme I). The plate is air dried and examined under short- and longwave UV light, and the respective 9-acridanones (X, R_f 0.34; XI, R_f 0.58; and XII, R_f 0.79) (Fig. 1A) are located visually as intensely fluorescent blue-green spots by reference to the R_f values of the 100-ng mixture of the authentic acridanone standards run alongside the sample extracts.

Since the intrinsic fluorescence of each 9-acridanone is approximately equal, the amplifier gain is set to give 95–98% full-scale deflection on the recorder pen for the 100-ng standard of any one of the acridanones and then scanned to check the fluorescence emission of the other two for full-scale pen response. The chromatoplate is scanned in the fluorescence mode described and the fluorescence emission of each compound is recorded as symmetrical Gaussian-shaped peaks (Fig. 1A). The peak area [peak height (centimeters) × width (centimeters) at half-height], which is determined using either the slope baseline technique or electronic digital integrations, is proportional to concentration (nanograms) of the acridanone.

[2] Model 137190 visual-UV chromatogram analyzer, Farrand Optical Co., Mount Vernon, N.Y.

[3] The RCA 1P-21 photomultiplier used is a specially selected tube (purchased from Farrand) for maximum sensitivity.

[4] RCA 1P-28 or equivalent photomultiplier tube.



R	1,4-benzodiazepin-2-one	benzophenone	9-acridanone
$-(CH_2)_2N(C_2H_5)_2$	I	VI	X
$-CH_2CH_2OH$	II	VII	XI
$-H$	III	VIII	XII
$-H$	IV, 3>CHOH	VIII	XII
$-CH_2COOH$	V	IX	XIII

Scheme I—Chemical reactions of N-1-alkyl-substituted o-fluoro-1,4-benzodiazepin-2-ones (see Ref. 17)

Table I—Instrumental Parameters for the Operation of the Visual–UV TLC Analyzer[a]

Function		Excitation System			Detection System				
		Excitation–Beam–Condenser			Analyzer–Draw		Detector		
Mode	Excitation Wavelength, nm	Lens, nm	Reducer	Optical Filter	Reducer	Optical Filter	Exit Slit, nm	Emission Wavelength, nm	Photomultiplier
Fluorescence	383	375–800	0.500 in.	No. 7-54	None	No. 3-73	10	457[b]	IP-21
Visual–UV	258 (UV)	200–375	0.625 in.	None	0.125 in.	No. 7-54	10	258[c]	IP-28
	410 (visual)	375–800	0.250 in.	None	None	None	10	410[c]	IP-28

[a] Farrand analyzer. [b] With emission monochromator in position. [c] With emission monochromator removed and 1P-28 photomultiplier in its place.

A calibration curve of peak area (square centimeters) *versus* concentration (nanograms) of each acridanone derivative obtained from the respective added authentic standards of I, II, and III (Fig. 2A) is constructed, from which the concentration of these compounds in the unknowns can be determined by interpolation.

The overall recovery of the added authentic standards of I, II, and III is determined by comparison of the fluorescence emission (as peak area) of their respective recovered 9-acridanones against that of the respective authentic standards scanned on the chromatoplate. Percent recovery should be determined routinely as a check on the reproducibility of the assay.

Analysis of Benzophenones in Urine by Spectrophotodensitometry—The major urinary metabolite (II), which accounts for 40–55% of a given dose, is present as a glucuronide conjugate along with minor amounts of I, III, IV, and V (Scheme I). To 1.0 ml of urine, add 5 ml of 6 *N* HCl, mix well, and extract with 2 × 10 ml of ether to remove interfering substances. The aqueous phase is hydrolyzed, and the benzophenones formed are extracted from the hydrolysate as previously described (17) and analyzed by TLC but with the following modifications.

Dissolve the ether residues containing the basic benzophenones (extracts C and D) (Scheme II in Ref. 17) in 100 μl of acetone, transfer a 25-μl aliquot of the extract onto a 20 × 20-cm silica gel G chromatoplate[5] as before, and develop as described (17). The concentration of each component in the unknowns is determined by comparison to 5.0 or 10.0 μg of their respective authentic standards recovered from urine as previously described (17).

The sensitivity of the instrument for the benzophenones in the visual–UV mode is 0.5–1.0 μg measured at 258 nm. The major urinary metabolite (II) is usually present in sufficient concentration to be readily quantitated as its benzophenone (VII) (R_f 0.38), whereas the amounts of VI (R_f 0.23) and VIII (R_f 0.62) (Fig. 1B) present may be at the sensitivity limit of the instrument and not accurately measurable. In this event, the benzophenones can be eluted separately into methanol, cyclized to the 9-acridanone derivatives, and quantitated fluorometrically as described (17).

Table II—Reproducibility (Mean and Standard Deviation) of the Measurement of Varying Concentrations of the 9-Acridanones on the Same Chromatoplate

Concentration Applied to Plate, ng	Instrument Gain Setting	Peak Area, cm^2 [a]		
		X	XI	XII
1	10	0.71 ± 0.06	0.87 ± 0.07	2.34 ± 0.16
10	3	1.86 ± 0.13	2.75 ± 0.09	4.00 ± 0.18
25	1.5	2.52 ± 0.24	3.75 ± 0.05	5.00 ± 0.24
50	1.5	5.18 ± 0.33	6.64 ± 0.16	9.05 ± 0.25
100	0.75	4.68 ± 0.07	6.63 ± 0.08	7.50 ± 0.14

[a] Mean of three individual measurements.

RESULTS AND DISCUSSION

Method Conditions—The spectrophotodensitometer[2] used can be operated only in the reflectance mode (20) and is claimed to have certain advantages over measurement in the transmission mode, which is possible with other commercial instruments[6]. The most significant advantages of spectrofluorodensitometry in the reflectance mode are the minimization of energy losses due to internal absorption or self-quenching due to the thickness of the silica gel bed and transmission losses through a glass plate.

Commercially available TLC plates of 250-μm bed thickness and 60-μm particle-size silica gel G[5] gave reproducible data with respect to reproducibility of peak area measurement of varying concentrations of the 9-acridanones on the same chromatoplate (Table II) and between chromatoplates (Table III). The reproducibility data of these fluorometric measurements using the reflectance mode are in acceptable agreement with data reported using

[5] Chromatoplates were manufactured by E. Merck (F_{254}), Darmstadt, Germany, and marketed by Brinkmann, Westbury, N.Y.

[6] *E.g.*, Zeiss (2, 3) and Schoeffel (4, 6, 12, 13).

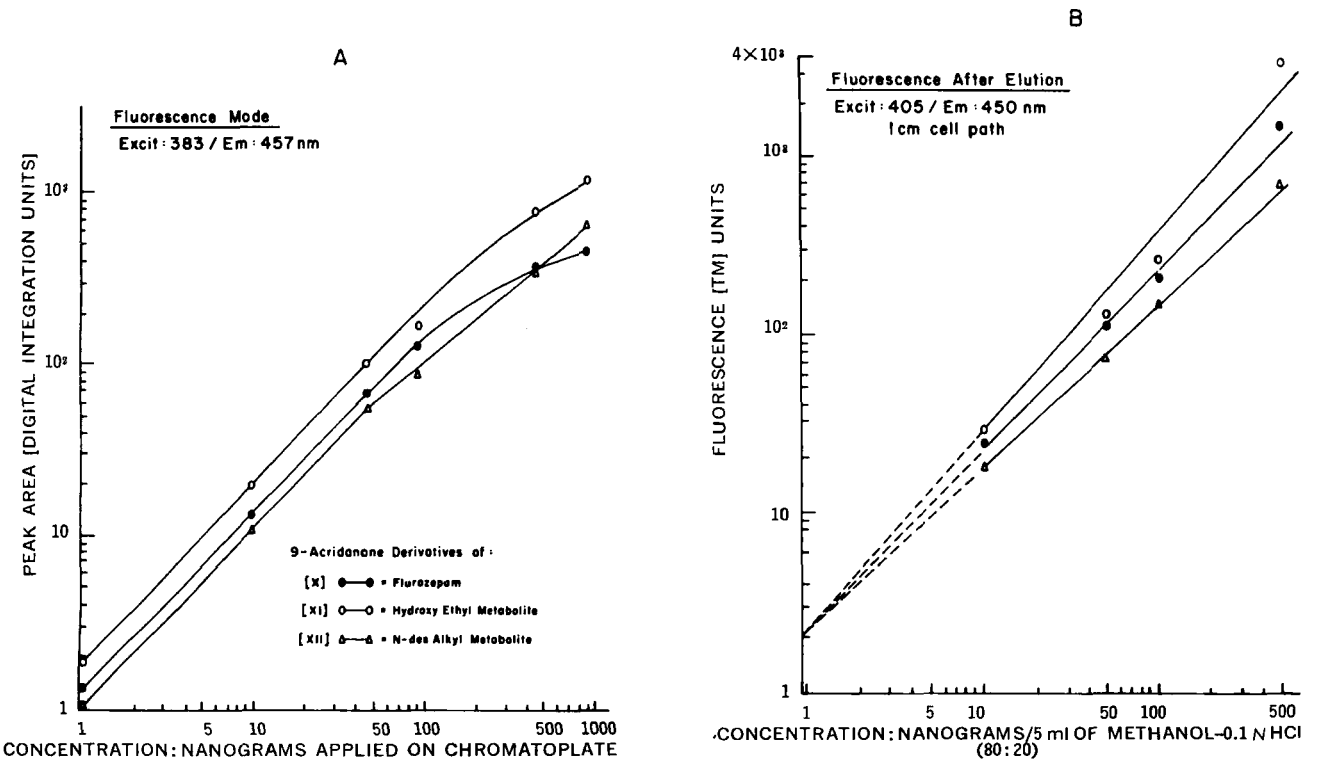


Figure 2—*Calibration curves of the 9-acridanones determined by: (A) spectrofluorodensitometry, and (B) solution fluorometry after elution.*

other equipment in the same mode and similar chromatoplates (2–6, 12, 13, 21, 22). It is essential to run at least one set of standards on each chromatoplate for purposes of calibration and quantitation of unknowns run on the same plate.

Blood levels of flurazepam following 30-mg therapeutic doses are barely detectable (23) by solution fluorescence (after elution); however, they can be quantitated with much greater precision by direct scanning fluorometry. The signal-to-noise ratio at the gain setting used to measure these levels is sufficient that further amplification of the signal can be employed without significantly increasing the background. Consequently, flurazepam levels as low as 0.1–0.2 ng/ml of blood (using a 4-ml specimen/assay) may be detected by direct scanning. This is feasible mainly due to the high intrinsic fluorescence of the 9-acridanones and the absence of spurious fluorescent impurities from the biological extracts that may interfere with the measurement.

The acceptable agreement seen in the blood levels of each component measured by both methods (Fig. 3) is especially encouraging with respect to the saving in analysis time, realized by eliminating the elution step, and the higher sensitivity (almost a fourfold increase) and precision attainable using the scanner. Although solution fluorescence measurements have a much wider linear dynamic range of concentration than direct scanning (Fig. 2), the latter enjoys certain distinct advantages with respect to minimization of solvent interaction and quenching effects, concentration of the compound to be measured in a small well-defined band, and a superior signal-to-noise ratio which can be further amplified to increase sensitivity. These factors offset the disadvantages of a limited linear dynamic range and the need to run reference standards for calibration of each chromatoplate to compensate for any variability of fluorescence emission between plates (4, 12, 13, 21).

Table III—Reproducibility (Mean and Standard Deviation) of the Measurement of Varying Concentrations of the 9-Acridanones Measured on Different Chromatoplates

Concentration Applied to Plate, ng	Peak Area, cm²[a]		
	X	XI	XII
1	0.24 ± 0.01	0.29 ± 0.02	0.30 ± 0.04
10	2.40 ± 0.05	3.35 ± 0.13	3.20 ± 0.17
25	5.58 ± 0.26	8.43 ± 0.26	7.63 ± 0.06
50	11.40 ± 0.34	16.03 ± 0.75	14.87 ± 0.15
100	21.70 ± 1.68	30.70 ± 0.86	27.75 ± 1.85

[a] Mean of individual peak area measurements [corrected to the same instrument gain factor (3.0) made on three separate plates].

The analysis of urinary metabolites by scanning in either the fluorescence or visual-UV mode is hindered by significant amounts of endogenous UV-absorbing or fluorescent components which interfere with their quantitation. Although washing the acidified urine with ether prior to hydrolysis removes significant amounts of UV-absorbing endogenous phenolic and indolic acids, sufficient amounts of UV-absorbing and fluorescent components are still extracted into ether after hydrolysis from the alkalinized urine. The use of an aliquot of the total extract for TLC separation reduces the amount of interfering substances but at the expense of sensitivity.

The benzophenones are intensely yellow in color and can also be quantitated in solution at 415 nm after elution into methanol (17). The low energy output of the xenon continuum at 415 nm reduces the sensitivity limits of detection, thereby nullifying the potential utility of direct scanning. It was evident that extensive sample clean-up was required prior to TLC analysis to enable precise and sensitive quantitation at 258 nm by direct scanning. Due to these limitations and also to the fact that the benzophenone of metabolite II was the major measurable component present, it was more convenient to elute this component and quantitate it in solution as previously described (17).

Application of Method to Clinical Specimens—Two subjects (C.R. and N.S.), free of previous benzodiazepine therapy, were selected and administered a single 30-mg dose of flurazepam[1] orally. Blood specimens were drawn prior to medication (control) and at 1, 3, 6, 12, and 24 hr postmedication. The fluorescence of the 9-acridanone derivatives separated by TLC analysis was first determined using the direct scanning TLC analyzer and then redetermined in solution after the compounds were eluted from the silica gel into methanol–0.1 *N* HCl (80:20) as described (17).

The blood level data obtained by the two procedures for flurazepam and its major blood metabolites (II and III) in these two subjects are shown in Fig. 3. Measurable levels of all three components were seen in both subjects. The amount of flurazepam in

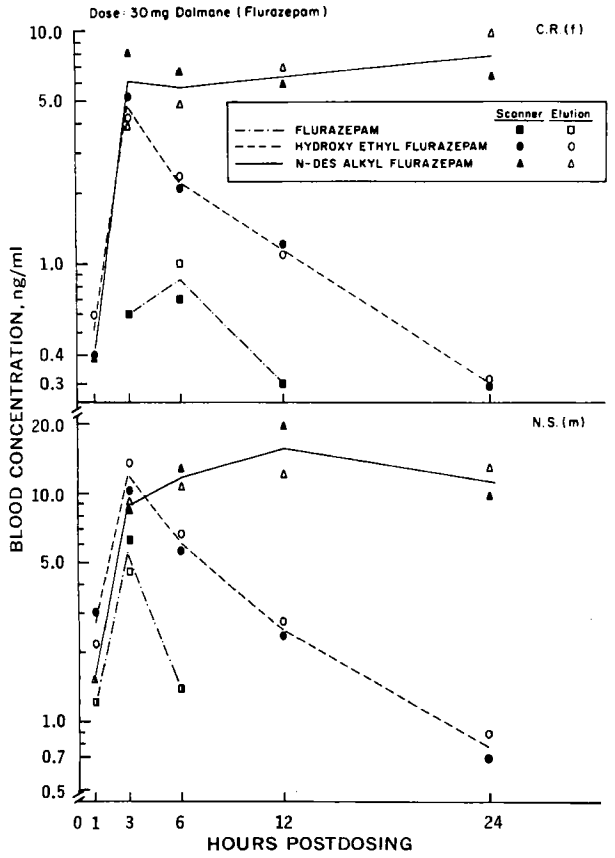


Figure 3—*Blood levels of flurazepam and its major blood metabolites in humans determined by fluorometry after elution* versus *scanning fluorodensitometry.*

each subject was very low, being at about the limit of detectability by elution, although measurable with adequate sensitivity by scanning densitometry. In Subject C.R., trace flurazepam levels of 1 ng or less/ml of blood were seen between 3 and 12 hr; in Subject N.S., a maximum flurazepam level (mean = 5 ng/ml) was seen at 3 hr and declined to nonmeasurable amounts by 12 hr.

The amounts of metabolites II and III, however, were readily measurable by both methods 1 hr after dosing. In both patients the hydroxyethyl metabolite (II) showed maximum levels at 3 hr, declined thereafter, and were measurable up to 24 hr. The concentration of *N*-desalkyl flurazepam (III), the major metabolite, increased with time, reaching a mean concentration of 10–12 ng/ml at 24 hr.

In another study (23) in four subjects following chronic administration of a single 30-mg oral dose daily for 14 consecutive days, the *N*-desalkyl metabolite (III) was the major metabolite in blood, reached steady-state levels after 7 days, and was eliminated with an "apparent" half-life ranging from 47 to 100 hr.

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[x] To whom inquiries should be directed.

Tissue Metabolites of Trifluoperazine, Fluphenazine, Prochlorperazine, and Perphenazine in the Rat: Identification and Synthesis

URSULA BREYER ×, AXEL PROX, RUDOLF BERTELE, and HANS JÖRG GAERTNER

Abstract □ Rats were treated chronically with increasing doses of trifluoperazine, prochlorperazine, fluphenazine, or perphenazine *per os*, and drug metabolites were extracted from their livers, kidneys, and lungs. Fluphenazine metabolites were also obtained from tissues of a dog treated with repeated oral doses. Isolation and separation of the compounds were achieved by TLC. Besides the parent drugs and the derivatives formed by removal of the terminal *N*-alkyl group, tissues contained metabolites resulting from degradation of the piperazine ring to form an ethylenediamine structure. This biotransformation occurred with and without concomitant removal of the terminal *N*-alkyl group, so that monosubstituted and symmetrically disubstituted ethylenediamine derivatives were produced from each drug. The structures of the metabolites were elucidated by mass spectrometry and confirmed by chemical synthesis.

Keyphrases □ Phenothiazines (trifluoperazine, fluphenazine, prochlorperazine, perphenazine)—isolation of drugs and metabolites in rat tissue, identification by mass spectrometry, synthesis □ Trifluoperazine and metabolites—isolated from rat tissue, identified by mass spectrometry, synthesis □ Fluphenazine and metabolites—isolated from rat and dog tissues, identified by mass spectrometry, synthesis □ Prochlorperazine and metabolites—isolated from rat tissue, identified by mass spectrometry, synthesis □ Perphenazine and metabolites—isolated from rat tissue, identified by mass spectrometry, synthesis □ Metabolism of phenothiazines—isolation and identification of drug and metabolites from rat tissue □ Piperazine-substituted phenothiazines—isolation and identification of rat tissue metabolites, ethylenediamine derivatives □ Mass spectroscopy—identification, metabolites of trifluoperazine, fluphenazine, prochlorperazine, and perphenazine

Until recently, biotransformation reactions on the piperazine ring contained in drugs were not known. In 1955, Bangham (1) noted: "This is not surprising, since the piperazine ring does not occur in nature and it is unlikely that the body would break down or use such a ring structure."

However, upon chronic administration of the neuroleptic drug perazine to rats, a metabolite was found to accumulate; this metabolite was identified as *N*-[γ-(phenothiazinyl-10)-propyl]ethylenediamine (2). This showed that the piperazine ring can be partially degraded to produce an ethylenediamine structure. The same biotransformation reaction occurred with the antihistamines chlorcyclizine and meclizine (3) and with a thrombocyte aggregation inhibitor (4).

The present paper describes the identification of analogous products resulting from biodegradation of ring-substituted phenothiazine drugs that contain a piperazine ring in the side chain. In contrast to observations on perazine, the ring cleavage occurred to a considerable extent with retention of the methyl or the hydroxyethyl group on the terminal nitrogen atom.

Some results were reported previously (5).

EXPERIMENTAL

Drugs—Trifluoperazine hydrochloride[1], fluphenazine hydrochloride[2], prochlorperazine edisylate[3], and perphenazine (free base)[4] were used.

Animal Treatment—Male Wistar rats, 280–370 g, were daily given aqueous solutions of the drugs as salts by gavage. The doses were slowly increased during 2–10 weeks. Expressed as free base, they were: trifluoperazine, 5–40 mg/kg; fluphenazine, 2–40 mg/kg; prochlorperazine, 5–50 mg/kg; and perphenazine, 2–50 mg/kg. A female beagle dog was given 15–40 mg/kg fluphenazine daily in capsules. Animals were sacrificed 24 hr after the last drug dose.

Isolation of Metabolites from Tissues—Animal tissue (liver, kidney, or lung, 5 g) was extracted as described previously (2, 6). When large quantities were to be obtained for mass spectrometric investigation, mostly liver tissue was used. The purification of the metabolites by TLC on silica gel[5] was performed according to Breyer (6), except that after washing the plates with chloroform–isopropanol (10:1), they were developed in Solvent A (Table I).

For the separation of the ethylenediamine derivatives, Solvent B was used. Prior to mass spectrometry, the metabolites were chromatographed in Solvent B on TLC plates prewashed with hexane and methanol–ammonia (20:1). In this case, elution from the gel was carried out by partitioning between 2 *N* ammonia and benzene.

γ-(2-Trifluoromethylphenothiazinyl-10)-propyl Chloride and γ-(2-Chlorophenothiazinyl-10)-propyl Chloride—These compounds were prepared according to Sherlock and Sperber (7) and freed from colored impurities by chromatography on a column of silica gel[6], with petroleum ether (bp 50–75°)–benzene (10:1).

***N*-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]piperazine (I*a*) and the 2-Chloro Analog (I*b*)**—Compounds I*a* and I*b* were obtained as described previously (8).

***N*-[γ-(2-Chlorophenothiazinyl-10)-propyl]ethylenediamine (III*b*) as Dimalonate**—Compound III*b* was prepared as follows: γ-(2-Chlorophenothiazinyl-10)-propyl chloride (2.5 g) in 10 ml of dry dioxane was added within 1 hr with stirring to 4 ml of ethylenediamine kept at 90°. After 3 hr, the mixture was concentrated *in vacuo* and the residue was distributed between 1 *N* NaOH and chloroform. The reaction product was extracted from the chloroform into 1 *N* acetic acid; after washing with chloroform and alkalinization, it was back-extracted into fresh chloroform.

Following evaporation of the solvent, the product was dissolved in 10 ml of dioxane and added dropwise with stirring to a solution of malonic acid (2.08 g) in 50 ml of absolute ether. The crystalline precipitate was washed with ether and recrystallized twice from absolute ethanol, resulting in nearly colorless needles, mp 158.5–159.5°.

Anal.—Calc. for $C_{17}H_{20}ClN_3S \cdot 2C_3H_4O_4$ (542.0): C, 50.97; H, 5.21; Cl, 6.54; N, 7.75; S, 5.92. Found: C, 50.62; H, 5.23; Cl, 6.53; N, 7.51; S, 5.84.

***N*-[γ-(2-Chlorophenothiazinyl-10)-propyl]-*N'*-methylethylenediamine (II*b*) as Dimalonate**—Compound II*b* was prepared as follows. γ-(2-Chlorophenothiazinyl-10)-propyl chloride (7 g), dissolved in 55 ml of dimethyl sulfoxide, was added within 2 hr with stirring to 19 ml of *N*-methylethylenediamine heated to 90–95°. After 30 min the starting material was no longer detectable

[1] Jatroneural, Röhm & Haas, Darmstadt, Germany.
[2] Lyogen, Byk Gulden Lomberg, Konstanz, Germany.
[3] Témentil, Rhône-Poulenc, Paris, France.
[4] Decentan, Merck, Darmstadt, Germany.
[5] Kieselgel GF_{254}, Merck, Darmstadt, Germany.
[6] Kieselgel 60, Merck, Darmstadt, Germany.

[TLC in petroleum ether–benzene (9:1)]. Following concentration *in vacuo* and distribution between 1 *N* NaOH and chloroform, the product was extracted into 2 *N* acetic acid.

The alkalinized solution was extracted with chloroform which, upon evaporation, left 6.45 g of a colorless oil. As TLC in Solvent A showed, this oil consisted of two isomers, the nondesired unsymmetrically disubstituted ethylenediamine (ninhydrin positive) being in excess. The separation was achieved by column chromatography on 100 g of silica gel with absolute acetone, which eluted the desired II*b* only (2.1 g). The dimalonate was obtained as described for III*b*; the salt was crystallized four times from absolute ethanol, yielding 1.6 g of fine colorless needles, mp 156–157.5°.

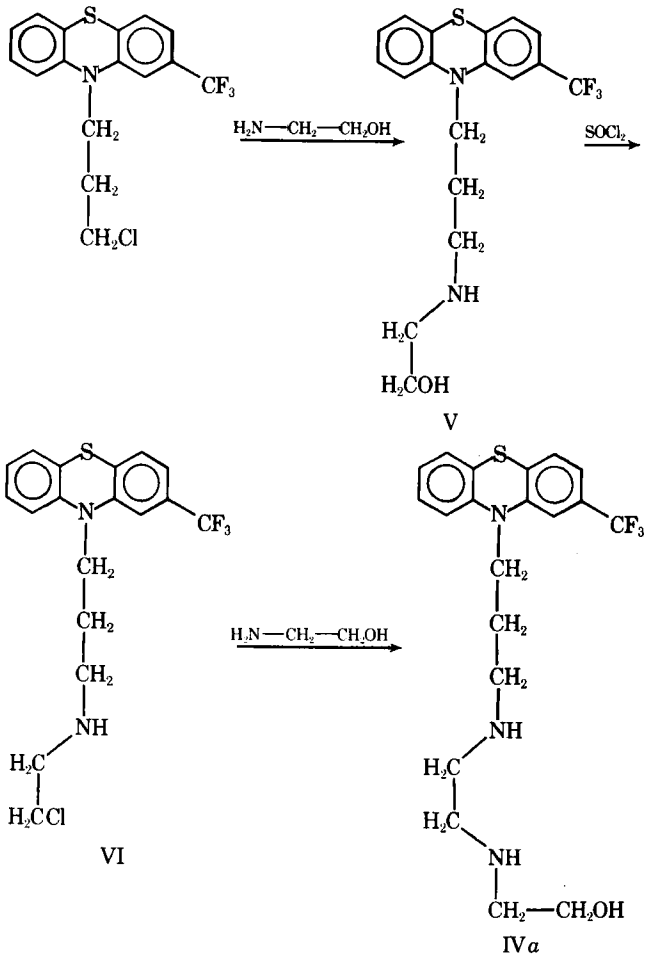
Anal.—Calc. for $C_{18}H_{22}ClN_3S \cdot 2C_3H_4O_4$ (556.0): C, 51.84; H, 5.44; N, 7.56. Found: C, 51.78; H, 5.32; N, 7.41.

***N*-[γ-(2-Chlorophenothiazinyl-10)-propyl]-*N'*-(β-hydroxyethyl)ethylenediamine (IV*b*)**—The reaction between γ-(2-chlorophenothiazinyl-10)-propyl chloride (1 g) and *N*-(β-hydroxyethyl)ethylenediamine (8 g) was carried out in analogy to the preparation of II*b*. Since the two isomers obtained could not be separated by column chromatography with acetone, a small quantity was purified by TLC in Solvents A and B, yielding a colorless oil. In both solvents, the nondesired *N,N*-disubstituted isomer had a higher R_f value than the desired one. It was present in a lower quantity.

***N*-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]ethylenediamine (III*a*) as Dimalonate**—The preparation of III*a* was according to the procedure used for the synthesis of III*b*. The dimalonate was crystallized three times from isopropanol, yielding colorless crystals, mp 141–142°.

Anal.—Calc. for $C_{18}H_{20}F_3N_3S \cdot 2C_3H_4O_4$ (575.6): C, 50.08; H, 4.90; N, 7.30; S, 5.57. Found: C, 50.19; H, 5.20; N, 7.17; S, 5.46.

***N*-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]-*N'*-methylethylenediamine (II*a*)**—A small quantity of Compound II*a* was prepared by reacting γ-(2-trifluoromethylphenothiazinyl-10)-propyl chloride with an excess of *N*-methylethylenediamine. The nondesired isomer was removed by TLC in Solvents A and B.



*Scheme I—Synthesis of IV*a. *For chemical names of the compounds, see text.*

Table I—R_f Values of Piperazine-Substituted Phenothiazine Drugs and of Their Tissue Metabolites on Silica Gel

Compound	R_f Value in Solvent[a] A	B
Trifluoperazine	0.86	0.81
N-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]piperazine (CF_3-PPP) (I*a*)	0.44	0.50
N-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]-*N'*-methylethylenediamine (CF_3-PPMED) (II*a*)	0.20	0.49
N-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]ethylenediamine (CF_3-PPED) (III*a*)	0.20	0.70
Fluphenazine	0.76	0.90
N-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]-*N'*-(β-hydroxyethyl)ethylenediamine (CF_3-PPHED) (IV*a*)	0.20	0.54
Prochlorperazine	0.86	0.81
N-[γ-(2-Chlorophenothiazinyl-10)-propyl]piperazine (Cl-PPP) (I*b*)	0.42	0.43
N-[γ-(2-Chlorophenothiazinyl-10)-propyl]-*N'*-methylethylenediamine (Cl-PPMED) (II*b*)	0.15	0.42
N-[γ-(2-Chlorophenothiazinyl-10)-propyl]ethylenediamine (Cl-PPED) (III*b*)	0.15	0.63
Perphenazine	0.76	0.90
N-[γ-(2-Chlorophenothiazinyl-10)-propyl]-*N'*-(β-hydroxyethyl)ethylenediamine (Cl-PPHED) (IV*b*)	0.15	0.47

[a] A = isopropanol–chloroform–25% ammonia–water (32:16:2:1); B = acetone–isopropanol–1 *N* ammonia (27:21:12).

***N*¹-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]-*N*³-methylimidazolidine**—A solution of II*a* (0.2 mg) in 0.5 ml of methanol was mixed with 0.02 ml of 4% aqueous formaldehyde. After 30 min the solution was concentrated and the residue was distributed between 2 *N* ammonia and benzene. TLC of the organic layer showed a single compound with R_f 0.80 in Solvent A and 0.83 in Solvent B.

***N*-[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]-*N'*-(β-hydroxyethyl)ethylenediamine (IV*a*) (Scheme I)**—*N*-[γ-*(2-Trifluoromethylphenothiazinyl-10)-propyl*]-*N*-*(β-hydroxyethyl)amine (V) as Hydrochloride*—Compound V was prepared as follows. γ-(2-Trifluoromethylphenothiazinyl-10)-propyl chloride (0.93 g) in 10 ml of dimethyl sulfoxide was added within 45 min to 3 ml of ethanolamine kept at 90°. After 30 min, the mixture was concentrated *in vacuo* and the residue was taken up in chloroform. The solution was washed several times with 1 *N* NaOH and water and subsequently shaken with 6 ml of 1 *N* HCl. The V-HCl remained in the chloroform layer, which was washed with 1% sodium chloride solution and evaporated, leaving a nearly colorless oil (0.85 g).

N-*[γ-(2-Trifluoromethylphenothiazinyl-10)-propyl]*-*N*-*(β-chloroethyl)amine (VI) as Hydrochloride*—To V-HCl (0.85 g) in 8 ml of dry chloroform was added 0.49 g thionyl chloride. The solution was left overnight at room temperature and then warmed for 3.5 hr to 40° and for 1 hr to 50°. The solvent was removed *in vacuo* and the residue was twice brought to dryness following addition of benzene.

*IV*a *as Dimalonate*—Crude VI-HCl (0.74 g) in 10 ml of dimethyl sulfoxide was added within 15 min to 3 ml of ethanolamine kept at 80° (3 hr) and at 90° (2.5 hr). Following evaporation, the residue was dissolved in ethylene dichloride and washed with 1 *N* NaOH. The product was extracted into 2 *N* acetic acid and back-extracted into ethylene dichloride after alkalinization, and the solvent was removed, leaving 0.55 g of a light-yellow oil.

According to TLC in Solvent A, small quantities only of impuri-

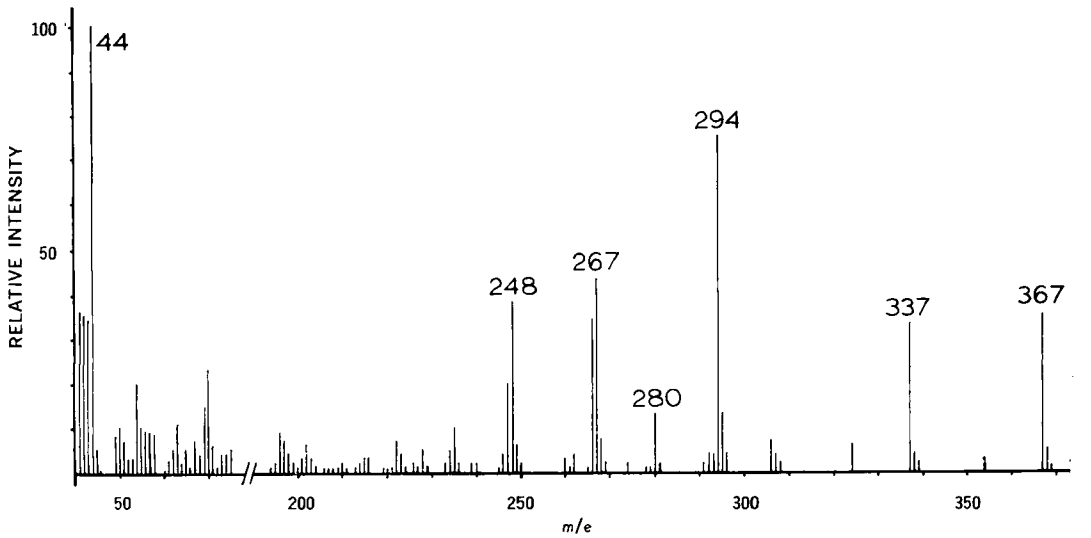


Figure 1—*Mass spectrum (70 ev) of IIIa (M^+, 367) isolated from the livers of rats treated with trifluoperazine.*

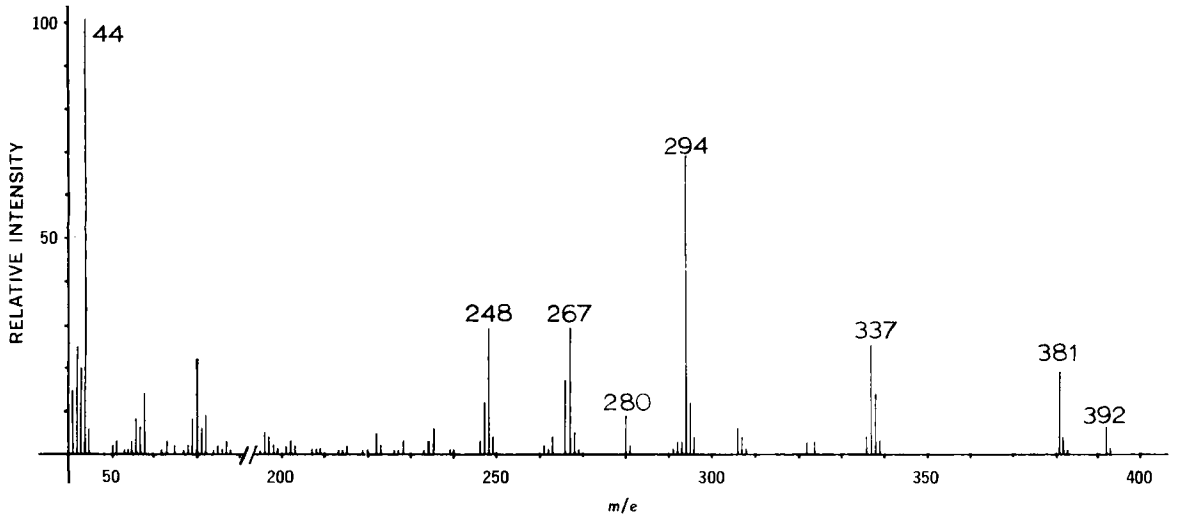


Figure 2—*Mass spectrum (70 ev) of IIa (M^+, 381) isolated from the livers of rats treated with trifluoperazine.*

ties were admixed with IV*a*, but a crystalline malonate could not be obtained. Therefore, part of the product was subjected to preparative TLC in Solvent A, followed by isolation of the compound by partitioning between 2 *N* ammonia and chloroform. From the residue of the organic layer, a colorless dimalonate was prepared and recrystallized from isopropanol, mp 144.5–146°.

Anal.—Calc. for $C_{20}H_{24}F_3N_3OS \cdot 2C_3H_4O_4$ (619.6): C, 50.40; H, 5.21; N, 6.78. Found: C, 50.97; H, 5.24; N, 6.97.

For mass spectrometric investigation, synthetic compounds obtained as salts were converted to the free bases by addition of ammonia to their solutions in water. They were extracted into benzene and the extracts were evaporated.

Mass Spectrometry—High- and low-resolution mass spectra were recorded[7]. All samples were directly introduced into the ion source, the temperature of which varied between 120 and 150°. A trap current of 500 and 100 μamp was used for recording the high- and low-resolution mass spectra, respectively. High-resolution mass spectrometry was performed on-line with a 16-sec scan and a resolving power better than 10,000. All mass measurements were within 10 ppm.

RESULTS

In tissues of animals that had received trifluoperazine, fluphenazine, prochlorperazine, or perphenazine, the parent drugs were consistently present, as were the metabolites produced by removal of the terminal *N*-alkyl group, I*a* and I*b*. The identification of these compounds was achieved by TLC comparisons in Solvents A and B (Table I).

A further band on chromatograms of tissue extracts in Solvent A could be resolved into two main components in Solvent B. The one with the higher R_f value was similar to *N*-[γ-(phenothiazinyl-10)-propyl]ethylenediamine, a perazine metabolite (2), in its chromatographic properties. In addition, it was ninhydrin positive, and the product formed from trifluoperazine proved to be identical to that resulting from fluphenazine; the same applied to the metabolites of prochlorperazine and perphenazine with similar TLC behavior. Therefore, these compounds were assumed to be the monosubstituted ethylenediamines III*a* and III*b*, respectively.

The metabolites exhibiting lower R_f values in Solvent B differed from one another; therefore, the terminal *N*-alkyl group was assumed to be still present. On the other hand, their low mobility in Solvent A pointed to the presence of two secondary (or primary) amino groups, so that symmetrically disubstituted ethylenediamines (II*a*, II*b*, IV*a*, and IV*b*) were taken into consideration. The mass spectra of the isolated metabolites proved that the proposed structures were correct. Further confirmation came from chromatographic and mass spectrometric comparison with synthetic compounds, mostly prepared by reacting a 2-substituted phenothiazinylpropyl chloride with ethylenediamine, *N*-methylethylenediamine, or *N*-(β-hydroxyethyl)ethylenediamine.

The mass spectra of the metabolites deriving from trifluoperazine and fluphenazine are shown in Figs. 1–3. The presence of sev-

[7] MS 902 S-DS 30 instrument, A.E.I., Manchester, England.

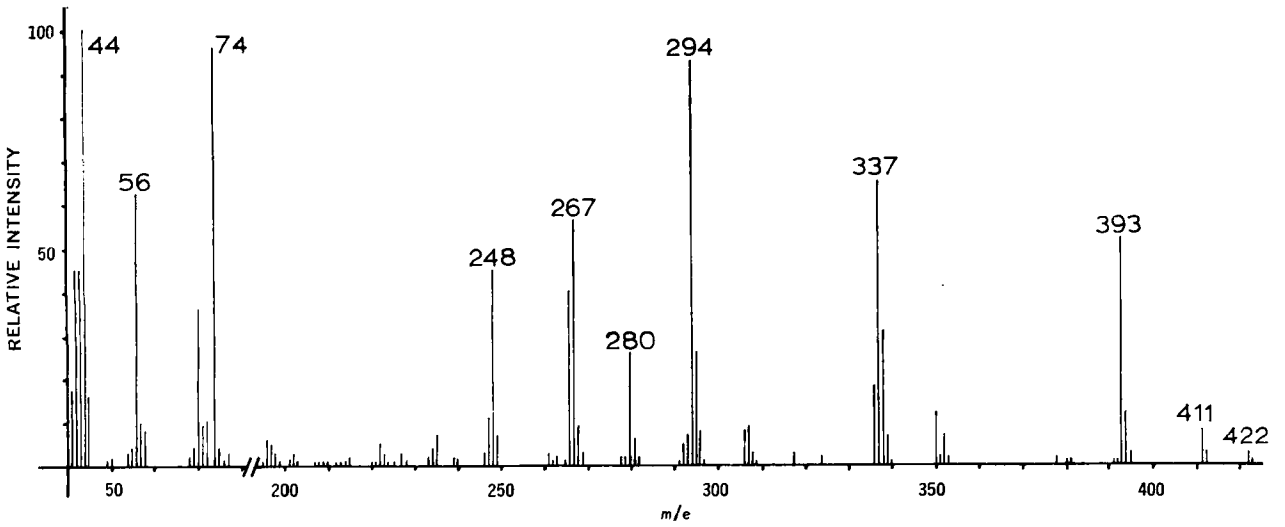


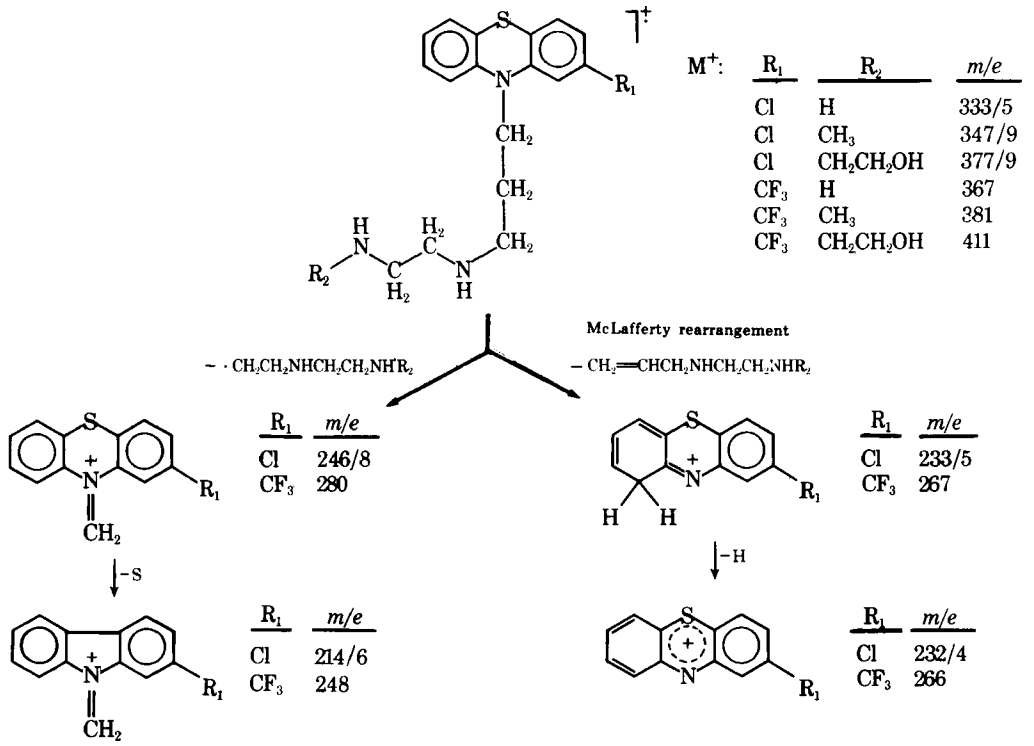
Figure 3—*Mass spectrum (70 ev) of IV*a (*M*$^+$, *411*) *isolated from the liver of a dog treated with fluphenazine.*

eral functional groups in the side chain at the phenothiazine nitrogen atom complicates the breakdown of the metabolites in the mass spectrometer. In particular, the substituent R_2 at the ethylenediamine group affects the mode of their fragmentation. Thus, the unambiguous elucidation of the metabolite structures using their mass spectra only is somewhat complicated. However, all metabolites can be immediately recognized as phenothiazine derivatives by the occurrence of abundant ions of the 2-substituted phenothiazine moiety in their mass spectra (9, 10).

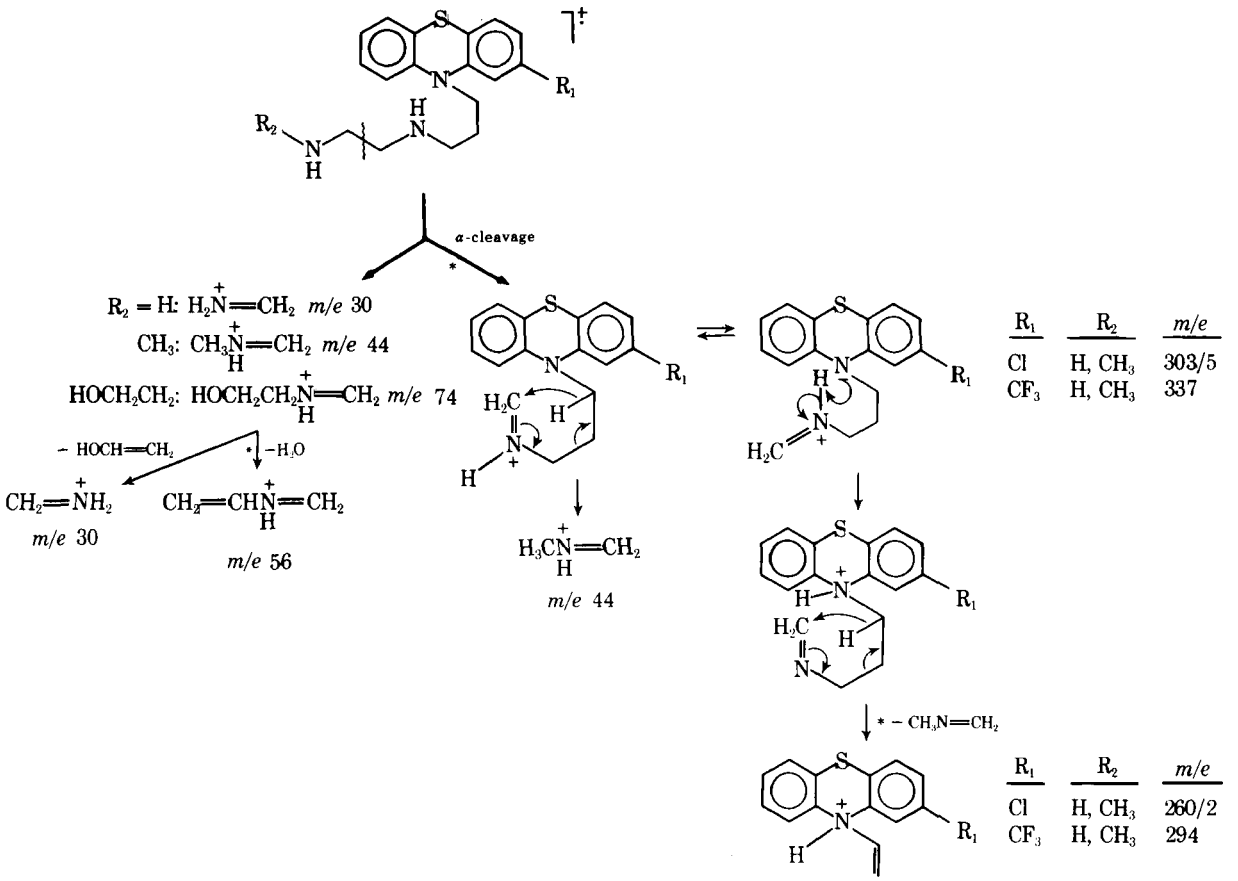
As depicted in Scheme II, the elimination of the *N*-side chain by a McLafferty rearrangement leads to the formation of the 2-substituted phenothiazine radical ion (R_1 = Cl, *m/e* 233/5; R_1 = CF_3, *m/e* 267), which further loses a hydrogen atom. Besides this ion doublet, the less abundant fragment ion formed by a simple α-cleavage is observed (R_1 = Cl, *m/e* 246/8; R_1 = CF_3, *m/e* 280). It is characterized by the elimination of the sulfur atom from the phenothiazine ring system (R_1 = Cl, *m/e* 214/6; R_1 = CF_3, *m/e* 248). The size of the side chain in the phenothiazine metabolites can thus be calculated using these ions and the corresponding molecular ions. The intensity of the molecular ions varies according to the ethylenediamine substituent R_2. In all cases where R_2 represents a hydrogen atom or a methyl group, quite abundant molecular ions are observed (Figs. 1 and 2).

As can be expected, the ethylenediamine moiety induces a strong α-cleavage of the ethylene C—C bond, with the possibility of charge retention at either of the nitrogen atoms. Some ions in the low mass range derive from this primary cleavage, *i.e.*, *m/e* 74 (C_3H_8NO) and in part *m/e* 44 (C_2H_6N) and *m/e* 30 (CH_4N). As illustrated in Scheme III, the ion *m/e* 74 not only eliminates a water molecule but also acetaldehyde as a neutral fragment, giving rise to the occurrence of the ions *m/e* 56 (C_3H_6N) and *m/e* 30 (CH_4N), respectively.

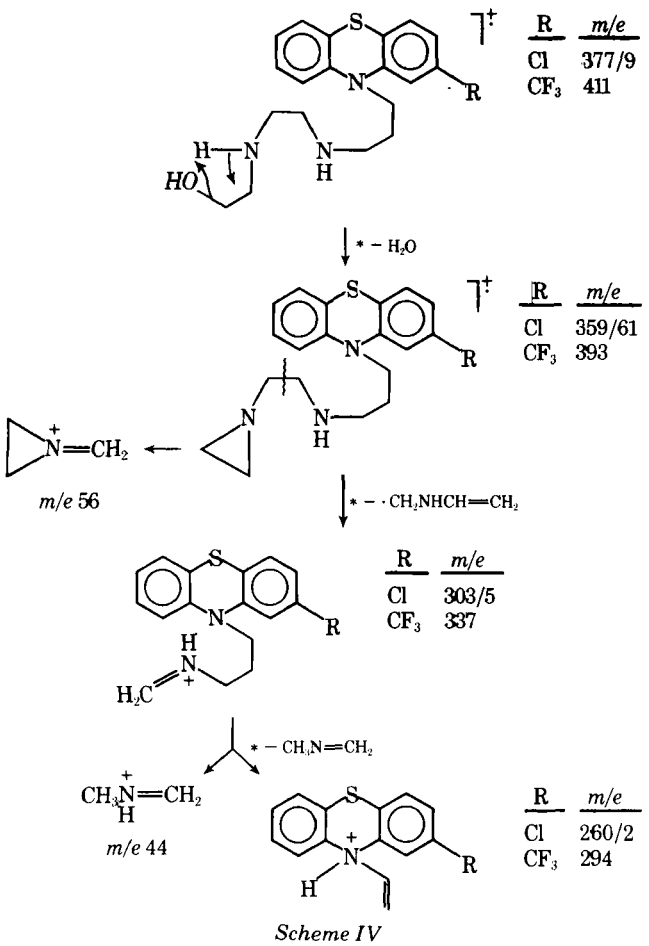
Thus, the ion *m/e* 30 may also derive from other processes than a primary α-cleavage. Therefore, an abundant ion *m/e* 30 in the mass spectra of the metabolites does not necessarily imply the presence of a primary amino group. The same is true for the very



Scheme II



Scheme III



Scheme IV

intense fragment ion *m/e* 44, which is found in the mass spectra of all metabolites regardless of the nature of the substituent R_2 at the ethylenediamine group.

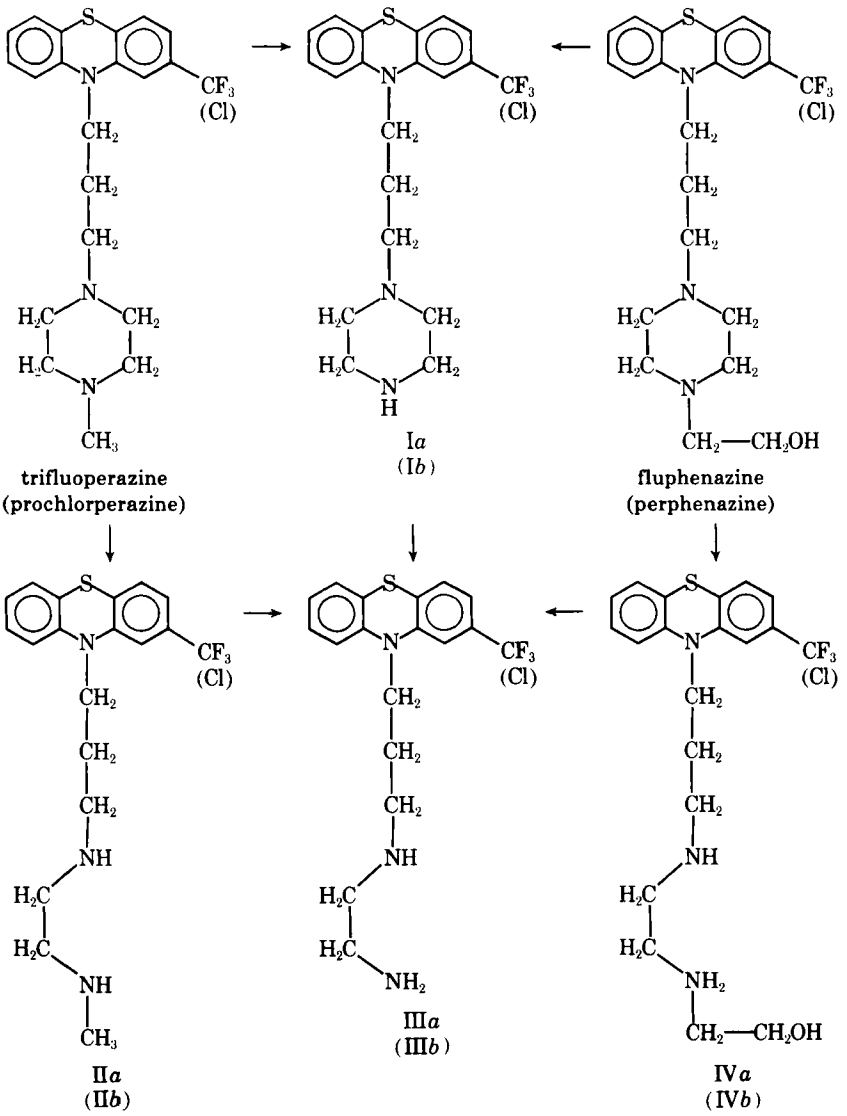
The counterions of the initial α-cleavage are only observed when the substituent R_2 is a hydrogen atom or a methyl group. As visualized in Scheme III, these ions decompose in two ways. Hydrogen rearrangement caused by the immonium ion generates the very abundant fragment ion *m/e* 44 (C_2H_6N). Thus, a methyl substituent (R_2) at the ethylenediamine group cannot be deduced from the observation of this fragment ion.

Charge exchange between the two nitrogen atoms by a proton transfer triggers the elimination of a $CH_3—N=CH_2$ molecule (43 amu). This gives rise to the formation of one of the most abundant ions in the mass spectra of the phenothiazine metabolites (R_1 = Cl, *m/e* 260/2; $R_1 = CF_3$, *m/e* 294). As indicated in Scheme III by an asterisk (*), a strong metastable ion can be observed for this process. Following the outlined degradation process, the structure of the *N*-side chain can be deduced without any ambiguity.

In contrast, the loss of a water molecule is the primary process in the mass spectra of the metabolites having a hydroxyethyl group at the ethylenediamine moiety. This elimination is the reason for the low intensity of the corresponding molecular ions (Fig. 3). Several possibilities can be envisaged to explain this elimination process, but only one has been depicted in Scheme IV. α-Cleavage of the resulting ions (R_1 = Cl, *m/e* 359/61; $R_1 = CF_3$, *m/e* 393) leads to the formation of the fragment ion *m/e* 56 (C_3H_6N) and the elimination of a C_3H_6N radical as well. The further breakdown of the ions (R_1 = Cl, *m/e* 303/5; $R_1 = CF_3$, *m/e* 337) generated by the loss of a C_3H_6N radical has already been outlined (Scheme III).

In conclusion, the structures of the phenothiazine metabolites can be elucidated by their mass spectra, taking into account the degradation processes and the resulting different fragment ions. The mass spectra of the corresponding synthetic compounds were identical with those obtained using the isolated metabolites (Figs. 1-3).

However, the mass spectrometric analysis was complicated by the presence of impurities showing higher masses than the molecular ions, namely, M + 11 and M + 12. The impurities were present in the synthetic products as well as in the metabolites isolated



Scheme V—Biodegradation of piperazine-substituted phenothiazine drugs to tissue metabolites. For names of the compounds, see Table I.

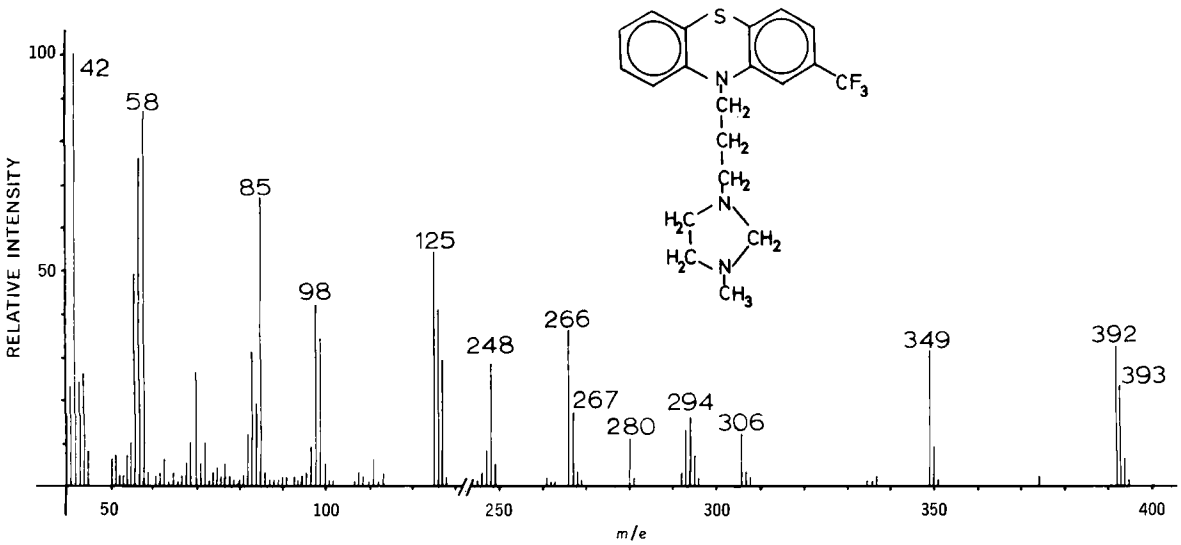


Figure 4—*Mass spectrum (70 ev) of* N¹-[γ-(*2-trifluoromethylphenothiazinyl-10*)-*propyl*]-N³-*methylimidazolidine* (M^+, *393*).

from tissues, although they occurred in greater quantities in the metabolites. In the case of II*a*, the contaminant could be shown to be N^1-[γ-(2-trifluoromethylphenothiazinyl-10)-propyl]-N^3-methylimidazolidine. Therefore, it was prepared from II*a* and formaldehyde and found to form very readily. Its mass spectrum is shown in Fig. 4.

The impurities increased with time upon storage of the samples at room temperature. Therefore, it can be concluded that the contaminants had formed *in vitro* in some stage of the preparation of the samples for mass spectrometry and were not due to additional metabolites in the tissues.

Based on the results of these investigations, the reaction sequences shown in Scheme V are suggested to be operative *in vivo*, leading to tissue metabolites of the four drugs. According to Scheme V, the monosubstituted ethylenediamines III*a* and III*b* originate by two different routes. Ring degradation starting from the terminally dealkylated metabolite has been demonstrated using desmethyl-perazine as a precursor of *N*-[γ-(phenothiazinyl-10)-propyl]ethylenediamine (6). On the other hand, terminal *N*-dealkylation of a *N,N'*-disubstituted ethylenediamine could be shown to occur by administering II*b* to rats. After 12 hr, their livers contained small quantities of III*b* in addition to II*b*.

Piperazine ring degradation to ethylenediamines is not confined to rats, since the tissues of the dog treated with fluphenazine contained the same metabolites as rat tissues. The identity was proven by TLC and mass spectrometric comparison of III*a* and IV*a* isolated from dog and rat liver.

DISCUSSION

The piperazine ring cleavage demonstrated here can be assumed to proceed *via* two consecutive *N*-dealkylation reactions, but intermediates confirming this concept have, until now, not been detected. Due to rapid aromatic hydroxylation of the drugs, the ring degradation pathway is probably a minor one in the total biotransformation in the rat. *In vitro*, it could not be demonstrated (8).

In vivo experiments on fluphenazine revealed the sulfoxide and the 7-hydroxy derivative as major excretion products in acutely and chronically treated dogs (11, 12), but elimination studies following repeated doses did not give an indication of an accumulation of these metabolites[8]. Rats given a single dose of one of the four drugs investigated here excreted in urine nearly exclusively the sulfoxide and the terminally dealkylated sulfoxide (8).

In long-term treatment with high doses, however, the ethylenediamine derivatives represent an important fraction of total tissue metabolites due to their tendency to accumulate[8] (6). Under the conditions used in the present study, their concentrations are comparable to or higher than those of the parent drugs and the dealkylation products I*a* and I*b*. Details on their kinetics in chronically treated animals will be published subsequently[8].

Pharmacological tests have not yet been carried out on the ethylenediamine metabolites. Even if they prove to be inactive by themselves, a possible significance lies in their ability to serve as precursors for biodegradation to phenothiazinylpropylamines that are metabolites common to dimethylamino-substituted and piperazine-substituted phenothiazine drugs (9).

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[x] To whom inquiries should be directed.

Nicotine Base Permeation through Silicone Elastomers: Comparison of Dimethylpolysiloxane and Trifluoropropylmethylpolysiloxane Systems

T. S. GAGINELLA, P. G. WELLING[x], and P. BASS

Abstract □ The rate of release of nicotine base from several silicone polymer systems was studied relative to diffusivity and solubility characteristics. Tubes composed of dimethylpolysiloxane released nicotine base rapidly: >75% in 4 hr. The same tubing with two external fluorosilicone laminations reduced release to about 40% in 4 hr. Tubes made of the silicone derivative trifluoropropylmethylpolysiloxane caused a substantial reduction in transmission of nicotine base. Drug permeability through dimethylpolysiloxane was >20 times more rapid than through the trifluoropropylmethylpolysiloxane system. Solubility of nicotine base in the dimethylpolysiloxane and trifluoropropylmethyl derivative membranes was estimated to be 201.4 and 112.9 mg/ml, respectively. The decreased permeability through the trifluoropropylmethylpolysiloxane polymer appears to be due to reduced diffusivity and solubility. The lower diffusivity cannot be explained on the basis of an increased effective diffusional path length since tortuosity values of about 1.0 were calculated for both polymers. Drug adsorption to filler and greater molecule–polymer interaction within the free volume matrix of the trifluoropropylmethylpolysiloxane polymer appear to be the predominant factors retarding the efflux of the nicotine. The release process was found to obey first-order kinetics by utilizing sigma-minus plots. It appears that the dimethylpolysiloxane membrane is much less suitable than the trifluoropropylmethyl derivative if one wishes to use nicotine experimentally as a timed-release preparation. Of major import is that, for other small molecules having physical–chemical characteristics similar to nicotine base, trifluoropropylmethylpolysiloxane polymers may be the most suitable drug vehicles if timed release is desirable.

Keyphrases □ Silicone elastomers—nicotine base permeation, comparison of dimethylpolysiloxane and trifluoropropylmethylpolysiloxane systems, diffusivity and solubility characteristics, application to timed-release preparations □ Nicotine base—permeation, comparison of dimethylpolysiloxane and trifluoropropylmethylpolysiloxane systems, timed-release formulation implications, diffusivity and solubility characteristics □ Dimethylpolysiloxane—nicotine base permeation, compared to trifluoropropylmethylpolysiloxane, diffusivity and solubility characteristics, timed-release formulations considered □ Trifluoropropylmethylpolysiloxane—nicotine base permeation, compared to dimethylpolysiloxane, diffusivity and solubility characteristics, timed-release formulations considered □ Timed-release formulations—comparison of dimethylpolysiloxane and trifluoropropylmethylpolysiloxane as vehicles, nicotine base permeation

In 1962, Ballard and Nelson (1) discussed the processes controlling the release of solid drugs from pellets used as subcutaneous implants. More recent research in this area has revolved around newer drug delivery systems, such as those prepared from silicone polymers, since these can provide timed release of many types of drugs (2–7). An added advantage of the use of silicone materials is that they produce minimal adverse tissue reactions when surgically implanted (8).

Higuchi (9) presented theoretical considerations for the timed release of solid drugs in matrixes. Steroid drugs can be incorporated into silicone matrixes and are then slowly released (3). Contrary to this, a small molecule like nicotine base diffuses through these membranes very rapidly (10). Most studies involving silicone membrane materials utilized the dimethylpolysiloxane elastomer.

Several other derivatives of polysiloxane are available and these exhibit differential permeability characteristics relative to various permeant molecules. For example, trifluoropropylmethylpolysiloxane membranes have been found to be less permeable than dimethylpolysiloxane to some steroids (11), thus reducing their release rate. Membranes composed of this type of material might be expected to reduce the release rate of a drug like nicotine similarly.

The studies reported here evaluate the potential for polysiloxane polymer membranes, prepared as tubes, to release nicotine base at a predictable rate over an extended period. Such tubes are easy to implant in animals and could be used to study chronic effects of nicotine and other drugs on biochemical and physiological functions.

EXPERIMENTAL

General Considerations—Transmission of a drug through a given synthetic membrane is dependent upon several factors. One important factor is the concentration gradient across the membrane, since diffusion through these membranes appears to follow Fick's law for various types of drugs (12–15). Permeability *(P)* through a *planar* silicone membrane can be described in terms of diffusion and solubility by Eq. 1 for the case where pure drug is initially in contact with the membrane:

$$P = D \times S \qquad \text{(Eq. 1)}$$

Here D is the apparent diffusivity (diffusion coefficient) in units of square centimeters per second, and S is the solubility of the permeant substance in the membrane phase. Units of milligrams per centimeter per second are usually given to the permeability. Diffusivity is a property of the permeant molecular size and temperature (16), in addition to the type and structure of the polymer membrane used (17, 18). Diffusivity and solubility are parameters that can be obtained experimentally and then used to estimate permeability values.

The diffusivity of a permeant molecule through a *hollow cylinder* of a silicone polymer can be calculated from Eq. 2 (19, 20):

$$D = \frac{(b^2 - a^2) + [(a^2 + b^2)] \ln a/b}{4L(\ln a/b)} \qquad \text{(Eq. 2)}$$

The designations a and b are the internal and external radii (centimeters) of the cylinder, respectively; L is the lag time (seconds) before steady-state diffusion is reached. A value for L can be obtained graphically by extrapolation of the tangent to the curve of the amount of permeant diffused *versus* time to the time *(x)* intercept (20).

If one assumes that at any given point along the inside cylinder wall a single permeant molecule "sees" the surface it enters as being planar, then a simplified expression (21) may be applied to calculate the diffusivity:

$$D = \frac{h^2}{6L} \qquad \text{(Eq. 3)}$$

where D is again the diffusivity (square centimeters per second), h is the membrane thickness (centimeters), and L is the lag time (seconds) before steady-state diffusion is reached. It will be shown later that diffusivities calculated from either Eq. 2 or 3 agree with one another very well.

Solubility of the permeant in the membrane also plays a significant part in the overall permeation process (13, 22). For polar or unsaturated molecules, an accurate value for this parameter is not easily determined, since commercial silicone rubbers generally contain porous fillers that may adsorb or sequester permeant molecules (23). An apparent solubility may be estimated by equilibration of the given membrane material (without filler) with a solution of the permeant.

Samples of the solution can be assayed over time and a solubility value can be obtained at equilibrium and used with diffusivity to calculate permeation through the matrix. In filled membranes, corrections for the volume fraction of filler and the adsorptive capacity also can be applied if the filler is known and is available in pure form.

Materials—The following three types of heat-vulcanized tubings were studied[1]: *(a)* dimethylpolysiloxane, *(b)* dimethylpolysiloxane with two external fluorosilicone laminations, and *(c)* trifluoropropylmethylpolysiloxane. Nicotine base[2] (grade 1, 99% pure) was constituted with *N*-methyl-^{14}C-nicotine base[3] (26 mCi/mmole) prior to incorporation into the silicone tubes. The aqueous desorbing medium (pH 7.4) was composed of 0.022 *M* $NaH_2PO_4 \cdot H_2O$ and 0.104 *M* Na_2HPO_4.

Methods—Dimethylpolysiloxane tubings, with and without two external fluorosilicone laminates, were prepared in lengths of 3 cm containing 25 μl (25.24 mg = 0.155 mmole) of the liquid ^{14}C-nicotine base (specific activity of 1.61 mCi/mmole). The ends were sealed with steel plugs immediately prior to suspension in the desorbing solution. Tubes of trifluoropropylmethylpolysiloxane were prepared[4] by heat vulcanization in special molds; one end of the tube was sealed in this process. These tubes were filled with 50 μl (50.48 mg = 0.310 mmole) of ^{14}C-nicotine base, and the open end was sealed with special room temperature vulcanizing silicone cement[5].

After preparation, each tube was suspended with silk thread into a 125-ml flask containing 100 ml of the pH 7.4 desorbing buffer. Flasks were maintained at 37° and shaken (about 70 oscillations/min) in an incubator[6]. Duplicate 100-μl samples were taken from the desorbing solution at specific time intervals and counted in a liquid scintillation spectrometer[7] using a commercial scintillation fluid[8] suitable for aqueous samples. All counts per minute were corrected to disintegrations per minute by internal standardization with ^{14}C-toluene (70–75% efficiency). The percent of nicotine base released at any given time was calculated from the total nicotine activity initially within the tube and the values obtained at sampling times.

Ionized compounds are known not to permeate silicone membranes (12, 13). Nicotine base has pK values of 3.10 and 8.01 at 25° (24). Therefore, nicotine entering the desorbing solution at pH 7.4 would be primarily in the form of the monovalent pyrrolidinium cation and thus unable to pass back through the membrane effectively *via* retrograde diffusion. In addition, factors such as the large volume (100 ml) of desorbing medium relative to the amount (25–50 mg) and the solubility (>1 g/ml) of nicotine base in the buffer indicate that sink conditions could be assumed to exist.

For determination of the solubility of nicotine in the polymers studied, standard pieces of dimethylpolysiloxane and trifluoropropylmethylpolysiloxane were cut from specially prepared *fillerless* sheets of uniform thickness. Each piece was placed in a glass scintillation vial and covered completely with pure nicotine base. Duplicate vials for each polymer were prepared in this manner.

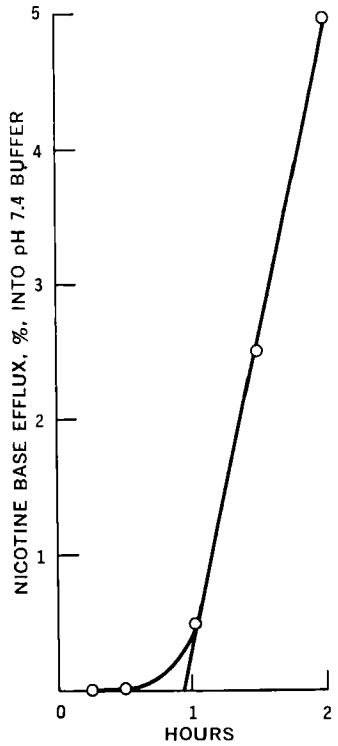

Figure 1—*Plot of steady-state portion of diffusion curve for nicotine base release from trifluoropropylmethylpolysiloxane tubing into pH 7.4 buffer at 37°. The intercept on the time axis is the lag time, estimated to be 55 min.*

The vials were shaken in an incubator[6] at 37°; they were removed periodically, blotted dry, and weighed until constant weight was obtained (about 8 days). The specific gravity of each polymer was determined by water displacement, and the solubility of nicotine was calculated (expressed as per milliliter of polymer volume and as per gram of polymer mass).

RESULTS

Diffusivity and Permeability—Lag times were estimated by extrapolation of linear portions of percent release *versus* time curves, according to the method of Barrer (21). Nicotine base permeated the plain (unlaminated) dimethylpolysiloxane membrane very rapidly, with an estimated lag time of only 2 min. An extrapolated value of about 26 min was obtained for the fluorosilicone-laminated membrane. The trifluoropropylmethyl derivative membrane delayed initial release most effectively and had an estimated lag time of 55 min (Fig. 1 and Table I).

Apparent diffusivity values were calculated for the three types of tubings using Eqs. 2 and 3. Diffusivity decreased in the fluorosilicone-laminated membrane compared to unlaminated dimethylpolysiloxane and decreased still further in the trifluoropropylmethyl polymer. Agreement between values calculated with Eqs. 2 and 3 was good (Table I), thus establishing the validity for application of either equation to determine nicotine base diffusivity under the present experimental conditions.

Perhaps more precise diffusivity values might have been calculated if fillerless tubings had been available for these studies; Most (23) claimed that filled materials may yield less "true" values when the lag time method is utilized. However, the tubings contained approximately equal amounts of filler so that comparisons between the tubings under these conditions are valid.

For calculation of diffusivities using Eq. 2, the internal *(a)* and external *(b)* radii of the tubings were used to determine the D_1 values shown in Table I. These radii were (in centimeters): a = 0.075 and b = 0.125 for dimethylpolysiloxane, a = 0.060 and b = 0.180 for fluorosilicone-laminated dimethylpolysiloxane, and a = 0.119 and b = 0.193 for the trifluoropropylmethylpolysiloxane tubing.

Solubility of nicotine base in the dimethylpolysiloxane and the trifluoropropylmethylpolysiloxane membranes (cured without filler) was determined to be 201.40 (194.60) and 112.90 mg/ml (88.90 mg/g) at 37°, respectively. The specific gravity of each type of

[1] All silicone membrane materials used were gifts from Dow Corning Corp., Midland, Mich.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] International Chemical and Nuclear Corp., Cleveland, OH 44128
[4] At the Dow Corning Corp.
[5] Due to the small surface area of the sealed ends of the trifluoropropylmethylpolysiloxane tubes relative to total surface area, diffusion of drug through the ends was considered negligible.
[6] Dubnoff.
[7] Packard 2002, Packard Instruments, Downers Grove, Ill.
[8] Insta-Gel, Packard Instruments, Downers Grove, Ill.

Table I—Comparison of Apparent Nicotine Base Diffusivity at 37° in Three Types of Silicone Elastomeric Tubings

Tubing Type	Wall Thickness, cm	Lag Time, sec	D_1, cm²/sec[a]	D_2, cm²/sec[b]	Difference[c], %	Permeability, mg/cm/sec[d]
Dimethylpolysiloxane	0.050	120	3.47×10^{-6}	3.42×10^{-6}	1.44	6.95×10^{-4}
Dimethylpolysiloxane + fluorosilicone laminates	0.120	1560	1.54×10^{-6}	1.57×10^{-6}	1.95	3.14×10^{-4}
Trifluoropropylmethyl-polysiloxane	0.074	3300	2.69×10^{-7}	2.78×10^{-7}	3.34	3.09×10^{-5}

[a] D_1 is the apparent diffusivity calculated from Eq. 2 (19). [b] D_2 is the apparent diffusivity calculated from Eq. 3 (20). [c] $(D_1 - D_2)/D_1 \times 100$. [d] Calculated using the mean value, $(D_1 + D_2)/2$.

cured polymer was determined to be 0.966 and 1.270 g/ml for the dimethylpolysiloxane and trifluoropropylmethyl polymers, respectively. The assumption was made that the solubility of nicotine in the dimethylpolysiloxane polymer could be used for calculation of the permeability parameter in the case of the dimethylpolysiloxane fluorosilicone-laminated system.

Permeability values for each system were estimated with Eq. 1 from the experimentally determined solubilities and the *mean* diffusivity ($D_1 + D_2/2$) values (Table I). The mean values used in the calculation were: dimethylpolysiloxane = 3.45×10^{-6} cm²/sec, fluorosilicone laminated = 1.56×10^{-6} cm²/sec, and trifluoropropylmethylpolysiloxane = 2.74×10^{-7} cm²/sec.

There was a considerable reduction in permeability through the trifluoropropylmethyl derivative membrane compared to dimethylpolysiloxane: 3.09×10^{-5} *versus* 6.95×10^{-4} mg/cm/sec. The fluorosilicone-laminated tubing gave an intermediate permeability value of 3.14×10^{-4} mg/cm/sec (Table I).

First-Order Release Characteristics—In addition to studying the physical parameters of diffusivity and permeability, the release kinetics of nicotine base from these systems were determined. Initially, plotting the cumulative percent of nicotine base released *versus* time for the dimethylpolysiloxane and the laminated systems illustrated that the laminated tubing released only about 65% of the nicotine after 16 hr compared to about 92% from the plain dimethylpolysiloxane system over the same period (Fig. 2).

Further analysis was attempted by graphing data for the trifluoropropylmethylpolysiloxane system as a sigma-minus plot (26, 27). In this type of plot, the log of the amount of nicotine base remaining to be released at any given time $(A^\infty - A)$ is plotted as a linear regression on time in days (Fig. 3). An excellent linear relationship was obtained ($r = -0.999$) for release into pH 7.4 buffer. From this plot, an apparent rate constant (K) was calculated from the relation $K = 2.303 \times$ slope. The K value derived in this manner was used to calculate the amount of nicotine released at specific times, assuming first-order kinetic processes and using the following standard expression:

$$A = A^\infty[1 - e^{-Kt}] \qquad \text{(Eq. 4)}$$

Here A is the cumulative amount of nicotine base released at any time t, and A^∞ is the total amount released from the system. A plot of values calculated according to this first-order relationship gave essentially the same curve as that generated experimentally (Fig. 4). A first-order rate constant of 0.357 day^{-1} was obtained for the release from the trifluoropropylmethylpolysiloxane tubing. Therefore, nicotine base release from this system appears to obey first-order rate kinetics.

In a separate experiment the efflux of nicotine base from the trifluoropropylmethyl polymer system into *n*-octanol was studied and analyzed in the same manner. In this case, there was again excellent agreement between the experimental values and those calculated assuming first-order release kinetics (Fig. 5).

DISCUSSION

Nicotine base is an oily liquid at room temperature and was present in this form in the tubings used. This compound has a substantial ability to dissolve directly in the silicone polymer membranes discussed. Some steroid derivatives such as medroxyprogesterone acetate are released slowly when the solid form is incorporated into dimethylpolysiloxane membrane systems (25). In the case of solid drugs, the permeation process is thought to be dissolution rate controlled rather than diffusion controlled (22). Unlike steroids, the permeation rate for nicotine base was rapid; this apparently is due to its good solubility and high diffusivity within the dimethylpolysiloxane polymer. Thus, release of this small molecule in its liquid state appears to be diffusion controlled.

External fluorosilicone lamination of the dimethylpolysiloxane

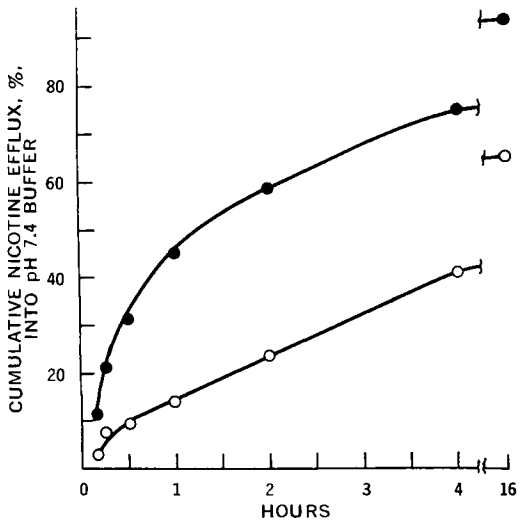

Figure 2—*Plot of the cumulative amount of nicotine base released from unlaminated dimethylpolysiloxane* (●) *and dimethylpolysiloxane + fluorosilicone-laminated polysiloxane* (○) *tubings into pH 7.4 buffer at 37°.*

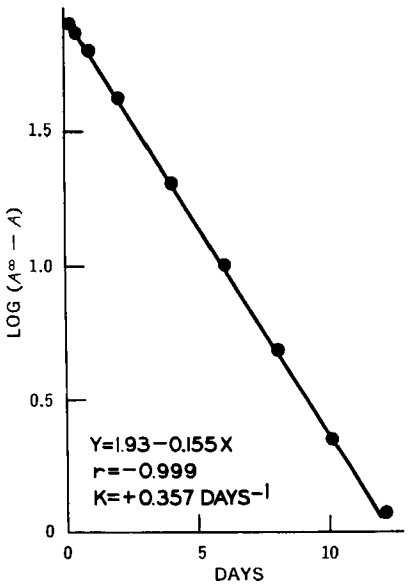

Figure 3—*Sigma-minus plot of the percent of nicotine base remaining to be released from trifluoropropylmethylpolysiloxane tubing into pH 7.4 buffer at 37°* ($A^\infty = 97\%$).

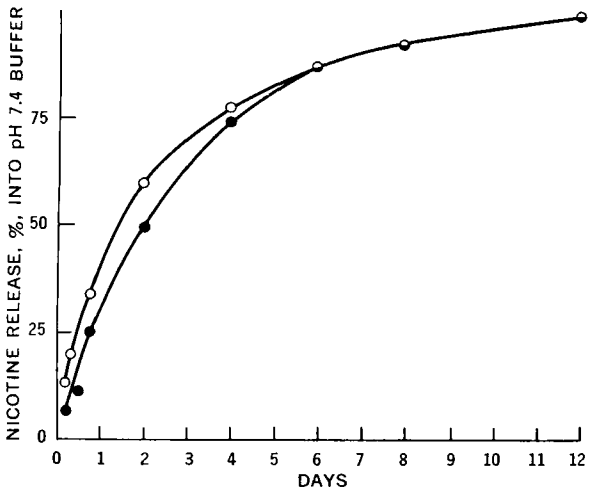

Figure 4—*Comparison of experimentally determined (actual) release* (○) *of nicotine base from trifluoropropylmethylpolysiloxane into pH 7.4 buffer with theoretical release* (●) *calculated with Eq. 4 (see text).*

membrane reduced permeability. It is not clear from these experiments whether the fluorosilicone layers retarded permeation by decreasing the diffusivity of nicotine or by acting as a final barrier in which the nicotine base was less soluble compared to the bulk membrane. The laminations comprise only a minor component of the overall membrane thickness, as reflected by the relatively small reduction in permeability.

A considerable reduction in permeability was obtained with the trifluoropropylmethyl derivative of polysiloxane. The permeability value for transport through the trifluoropropylmethylpolysiloxane membrane was less than 5% of that through the dimethylpolysiloxane system. A similar reduction in release of nicotine from the trifluoropropylmethylpolysiloxane compared to dimethylpolysiloxane tubes was recently shown *in vivo* after subcutaneous dimethylpolysiloxane implantation (28).

Various interactions within the membrane determine the overall release rate of a given drug. For nicotine, the reduction in permeation through the trifluoropropylmethylpolysiloxane membrane appears to be derived from reductions in both diffusivity and solubility. Reduced diffusivity may arise from greater molecular interaction of the nicotine with the trifluoropropyl or the methyl side-chain moieties of the polysiloxane matrix.

Filler effects may also be responsible for reduced diffusivity (23). The filler in these tubings was present in approximately equal amounts (±5%) in each type of membrane studied, but further details as to the exact filler type, particle size, exact quantity incorporated, and specific catalyst used in preparation were not

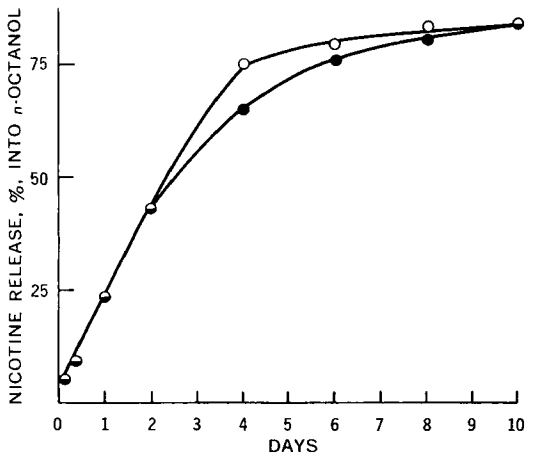

Figure 5—*Comparison of experimentally observed release* (○) *of nicotine base from trifluoropropylmethylpolysiloxane into n-octanol at 37° with theoretical release* (●) *calculated with Eq. 4 (see text).*

available. Flynn and Roseman (29) have given an expression for estimation of lag times in heterogeneous dimethylpolysiloxane barriers when filler effects may be operative. Their expression (Eq. 5) includes a tortuosity term, τ, which reflects an apparent "increased average diffusional path" for a permeant:

$$L = \frac{\tau^2 h^2}{6D} \qquad \text{(Eq. 5)}$$

Rearrangement gives the expression for calculating tortuosity:

$$\tau = \left(\frac{L6D}{h^2}\right)^{1/2} \qquad \text{(Eq. 6)}$$

By using this equation, tortuosity values of 0.970 and 1.01 were calculated for the dimethylpolysiloxane and trifluoropropylmethylpolysiloxane membranes, respectively. A value of $\tau = 1.00$ indicates that the diffusional path length is not effectively reduced within the matrix; thus the reduced diffusivity of nicotine could be due to adsorption by the filler. However, since the tubings contained about the same quantity of filler, the reduced diffusivity in the trifluoropropylmethyl polymer system may be due more to molecule–polymer interactions in the free volume phase of the matrix.

Sigma-minus plots to show first-order release kinetics are useful for obtaining rate constants. These K values may then be used to estimate the rate of permeation through silicone membrane systems more directly, without determining diffusivities, solubilities, and permeabilities. When comparing release into pH 7.4 buffer and *n*-octanol, it was noted that there was a slightly reduced rate of efflux into the *n*-octanol and a lesser maximum value was attained. These rate differences were minor, but the diminished values for the *n*-octanol case may be related to a change in sink conditions, *i.e.*, from the aqueous to the lipoidal external phase. Some retrograde partitioning (and diffusion) of *uncharged* nicotine could also have been occurring between this lipoid-desorbing medium and the nonpolar siloxane membrane.

Analysis of permeation based on diffusivity and first-order release indicates that the dimethylpolysiloxane membrane material is not suitable for prolonging the release of nicotine base or perhaps other small molecules having similar physical–chemical characteristics. The trifluoropropylmethylpolysiloxane membrane, however, does delay release of this compound effectively. By altering membrane thickness and length of this type of tubing, liquid nicotine may be administered over prolonged periods from subdermal implants.

Other salt forms of nicotine may also be of use if they can be shown to be released from silicone membrane systems. Copermeants may similarly be useful to modify transport through such membranes, since these can exert various influences by complexation externally or within the polymer phase (30, 31).

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Charge-Transfer Complexes in Alkaloid Assay

A. M. TAHA [x], A. K. S. AHMAD *, C. S. GOMAA, and H. M. EL-FATATRY *

Abstract □ The intense charge-transfer UV bands in the spectra of molecular complexes of iodine with amines were utilized in a sensitive spectrophotometric assay of alkaloids. Solutions of the alkaloids in chloroform or carbon tetrachloride when mixed with iodine in the same solvent exhibited blue-shifted iodine bands in the 270–305-nm range. Owing to the high molar absorptivities of the complexes, significant increases in sensitivity, accuracy, and precision were observed upon quantitation. The method is directly applicable to the purified alkaloid-containing chloroform fractions obtained in pharmacopeial assays or isolated from plant extracts and drug formulations in the general concentration range of 10^{-5}–10^{-6} M with a relative standard deviation of 0.015. The method is particularly recommended for assaying the tropane alkaloids and other weak UV-absorbing alkaloids such as ephedrine, codeine, and sparteine.

Keyphrases □ Alkaloids—spectrophotometric assay, charge-transfer complexes □ Charge-transfer complexes—alkaloid spectrophotometric assay □ Complexes, charge transfer—alkaloid spectrophotometric assay □ Spectrophotometry—alkaloid assay, charge-transfer complexes

The decolorization of bromine and iodine by many alkaloids is well known (1–4), and colorimetric methods based upon the residual color on adding excess reagent have been developed (4). In some cases, decolorization is due to iodination of the ring, *e.g.*, epinephrine and isoproterenol (4). However, the true nature of this more general reaction as a charge-transfer complex formation between the nitrogen of the alkaloid as the n-donor and the halogen molecule as the σ-acceptor was not recognized by early investigators.

The formation of complexes between electron donors and acceptors is an important phenomenon. Many molecular complexes are colored and give rise to new absorption bands in the electronic spectra. Although a new absorption band in the UV spectra of iodine and benzene solutions was recognized early as characteristic of the molecular complex between benzene and iodine (5), only after Mulliken (6) propounded the charge-transfer theory could the various features of the spectra and other properties of such molecular complexes be understood fully.

Amines are excellent n-donors, and charge-transfer complexes of these compounds with halogens and pseudohalogens have been reported (7–11). However, emphasis was placed on the study of the complexes by various physical methods and the determination of thermodynamic constants rather than on quantitative implications. Furthermore, recent comprehensive monographs on charge-transfer complexes (10, 11) are devoid of explicit reference to alkaloid–halogen complexes.

This work is a preliminary report on charge-transfer complexes of iodine with alkaloids and their utilization in a sensitive assay of many alkaloids, particularly those with very low original absorptivities. The determination of equilibrium constants for some selected alkaloids is also described.

EXPERIMENTAL[1]

Alkaloids—Analytical reagent or pharmaceutical grade alkaloids and alkaloidal salts, which passed compendial limits and possessed the correct physical constants, were used.

[1] A constant-temperature water bath was used in the determination of equilibrium constants. Spectra were made on SP 8000 Pye-Unicam spectrophotometer.

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Table I—Peak Position and Intensity for Selected Alkaloids

Alkaloid	Solvent[b]	Position and Intensity of Peak[a]						
		Complex with Iodine		Complex with Solvent		Reported (16)		
		λ, nm	($A^{1\%}_{1cm}$)	λ, nm	($A^{1\%}_{1cm}$)	λ, nm	($A^{1\%}_{1cm}$)	Solvent
Atropine	A	270	(285)	265	(35)	264	(6)	D
		410	(95)	—	—	—	—	—
	B	280	(390)			End absorption		
Scopolamine	B	270	(33)	270	(18)	257	(14)	D
N-Butylscopolammonium bromide	A	280	(1050)	265	(20)	276	(11)	D
Sparteine	C	228	(250)	230	(6)	214	(3.7)	E
		295	(150)					
Ephedrine	B	294	(60)	260	(16)	256	(11.5)	E
Codeine	B	285	(500)	292[c]	(200)	284	(52.5)	E
Quinine	A	280	(180)	285	(140)	278	(133)	E
		340	(200)	340	(165)	332	(163)	
Cinchonidine	B	280	(630)	280	(360)	—	—	
		300	(580)	300	(326)	315	(320)	E
Strychnine	A	258	(429)	258	(400)	255	(315)	D
Brucine	A	270	(440)	270	(350)	267	(302)	E
Emetine	A	293	(220)	290	(210)	285	(208)	D
Papaverine	A	285	(240)	285	(230)	284	(193)	F
		320	(155)	320	(129)	310	(253)	
Reserpine	A	—	—			267	(239)	E
		295	(480)			294	(150)	
		355	(300)			—	—	

[a] Value of A (1%, 1 cm) is the average of five determinations. [b] A = chloroform, B = carbon tetrachloride, C = cyclohexane, D = 0.1 *N* H_2SO_4, E = ethanol, and F = 0.1 *N* HCl. [c] Plateau from 292 to cutoff point (see Fig. 2).

If not available commercially, some alkaloidal free bases were prepared from the pure salts by liberation with appropriate alkali (4) followed by recrystallization, with a purity check by melting-point determination and TLC. Alternatively, an amount of the salt corresponding to the required weight of the free base was treated with alkali and quantitatively extracted with chloroform. The washed and dried (anhydrous sodium sulfate) extract was diluted to the required concentration.

Reagents—Resublimed analytical reagent grade iodine was sublimed again under nitrogen and kept over phosphorus pentoxide. The following spectral grade solvents were utilized: cyclohexane, chloroform (ethanol free), and carbon tetrachloride.

Qualitative Screening for Charge-Transfer Complex Formation—One milliliter of the alkaloid solution in chloroform (1 mg/ml) was mixed at room temperature with an equal volume of iodine solution in chloroform (0.1 mg/ml), and the effect was observed. The mixture was diluted to 50 ml with the same solvent and then warmed to 45–50°, and the effects of dilution and heat upon reversion of color back to violet were noted.

Molecular Ratio of Reactants in Complex—The Job method of continuous variation (12) was employed. With atropine as a model, master solutions of the base [0.145 mg (0.0005 mole)/ml] and iodine [0.127 mg (0.0005 mole)/ml] were prepared in chloroform. A series of 10-ml quantities of mixtures of the master solutions of atropine and iodine was made up comprising complementary proportions of the two solutions (0:10, 1:9, ... 9:1, 10:0) in 10-ml volumetric flasks. The mixtures were allowed to stand in a thermostated water bath at 20 ± 0.5° for 30 min. After this period, the absorbances of the solutions were measured at 270 nm.

A similar experiment was performed for sparteine in cyclohexane using a master solution containing 0.117 mg base/ml and measurement at 295 nm.

Determination of Molar Absorptivity and Association Constant—*Rose–Drago (13) Plot*—A series of alkaloid solutions in chloroform was prepared (1.5, 3.0, 4.5, and 6.0 × 10^{-4} *M*). These solutions and an iodine solution in chloroform (4 × 10^{-5} *M*) were placed in a thermostated water bath at 20 ± 0.5° for 30 min. Five milliliters of each alkaloid solution was mixed with an equal volume of the iodine solution. The mixture was quickly placed in a 1-cm quartz cell and the absorbance was read at λ_{AD}, the wavelength of maximum absorption of the complex, previously determined for the alkaloid (*e.g.*, 270 nm for atropine).

The same experiment was repeated in carbon tetrachloride with measurement at the proper λ_{AD} (280 nm for atropine). In each case, the values computed from the Rose–Drago (13) equation (Eq. 1) for each set of solutions were used to plot a curve of $(K_{AD}{}^c)^{-1}$ *versus* random values of $\epsilon_{AD}{}^\lambda$ (20,000-30,000 for atropine, 40,000–50,000 for brucine, *etc.*):

$$(K_{AD}{}^c)^{-1} = \frac{A}{\epsilon_{AD}{}^\lambda} - a - d + \frac{ad}{A}\epsilon_{AD}{}^\lambda \qquad \text{(Eq. 1)}$$

where:

$(K_{AD}{}^c)^{-1}$ = reciprocal of association constant of charge-transfer complex at 20°
A = absorbance value at concentration (d)
$\epsilon_{AD}{}^\lambda$ = molar absorptivity of charge-transfer complex
a = concentration of acceptor (0.00004 *M*)
d = concentration of donor, moles liter^{-1}

Benesi–Hildebrand (14) Plot—The values of A, a, and d obtained in the previous experiment were utilized to plot $1/d$ values *versus* a/A using the Benesi–Hildebrand (14) equation:

$$(a/A) = [(1/K_{AD}{}^c \cdot \epsilon_{AD}{}^\lambda)\cdot(1/d)] + (1/\epsilon_{AD}{}^\lambda) \qquad \text{(Eq. 2)}$$

The intercept with the ordinate gives $(\epsilon_{AD}{}^\lambda)^{-1}$ and the gradient is equal to $[(\epsilon_{AD}{}^\lambda)(K_{AD}{}^c)]^{-1}$.

Nagakura's (15) Formula—When there was overlapping absorption of the alkaloid with the complex, Nagakura's (15) formula was used to evaluate $K_{AD}{}^c$:

$$K_{AD}{}^c = [d(A_0 - A') + d'(A - A_0)]/[dd'(A' - A)] \qquad \text{(Eq. 3)}$$

where A_0, A, and A' are the absorbances measured at λ_{AD} of solutions whose concentrations of the acceptor are equal and whose donor concentrations are zero, d, and d', respectively.

Quantitative Analysis—The A values (1%, 1 cm) of the alkaloid in the complex are first evaluated from the absorbance values of solutions containing definite amounts of the alkaloid in the selected solvent mixed with iodine solution in the same solvent. The obtained A values (1%, 1 cm) are utilized for computation of the concentration of unknown solutions whose absorbance is determined. Alternatively, standard curves can be constructed by plotting observed absorbance values *versus* the volume taken of equimolar concentrations of the various alkaloids (5 × 10^{-4} *M*) mixed with the iodine solution (4 × 10^{-4} *M*). In all cases, Beer's law held for the system.

Assay of Alkaloids—For assay of alkaloids in crude drugs, galenicals, isolates, principles, and pharmaceutical preparations, the appropriate methods of preparing the sample, liberation of the base, solvent extraction, and purification are first performed fol-

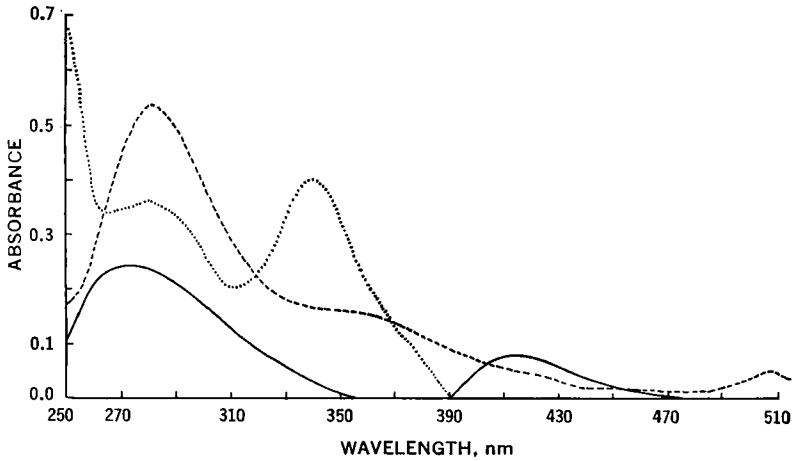

Figure 1—*Absorption spectra of charge-transfer complexes of some alkaloids with iodine in chloroform (iodine concentration = 4 × 10^{-4} M). Key: ———, atropine, 2 × 10^{-4} M; – – – – –, N-butylscopolammonium bromide, 1.2 × 10^{-5} M; and · · · · · ·, quinine, 3.2 × 10^{-4} M.*

lowing standard procedures (4, 16, 17) or compendial directions. The final determination of the alkaloidal content is then made by mixing the properly diluted chloroform solution of the alkaloid (10^{-5}–10^{-4} *M*, corresponding to 2–100 μg/ml depending on the molecular weight) with the iodine solution in chloroform (4 × 10^{-4} *M*) and measuring at the wavelength of maximum absorption of the charge-transfer complex previously determined using the pure alkaloid. The concentration of the alkaloid in the preparation is then calculated from the *A* values (1%, 1 cm) by comparison with a standard or from a calibration curve constructed in the usual manner.

RESULTS AND DISCUSSION

Qualitative Screening for Charge-Transfer Complex Formation—The immediate change of the violet color of iodine in chloroform to lemon yellow or yellowish purple upon reaction with alkaloids was taken as suggestive of charge-transfer complex formation which justified scanning in the UV range for the new band. The complex formation is distinguished from other slow oxidation or substitution reactions of the halogen with alkaloids by being practically instantaneous in analogy to ionic reactions (10).

Further confirmation of the charge-transfer nature of the reaction was obtained on extracting the alkaloid from the complex by shaking with aqueous mineral acid, whereby the violet color of iodine in chloroform was restored. The following compounds were tested and gave positive response: atropine, hyoscyamine, *N*-butylscopolammonium bromide, hyoscyamine hydrobromide, homatropine hydrobromide, ephedrine, codeine, morphine, papaverine, papaverine hydrochloride, quinine, cinchonidine, strychnine, brucine, emetine, and pilocarpine. Negative visual response was obtained with scopolamine, colchicine, and 8-hydroxyquinoline (oxine).

With compounds showing positive response, the degree of stability of the charge-transfer complex varied, as suggested from the extent of reversion of the violet color of iodine upon dilution and warming to 50°. Emetine, strychnine, and, to a lesser extent, quinine and codeine gave the most stable complexes which resisted dissociation on dilution and heating.

Failure of colchicine to give a response confirms that a sufficiently basic nitrogen is necessary to sustain the charge-transfer complex, since the nitrogen of this alkaloid is in amide linkage. Engagement of the lone pair of the nitrogen in hydrogen bonding as in oxine leads to the same effect. In the latter case, however, the negative response may also be due to the steric hindrance.

The apparent negative response of scopolamine was found to be only visual, since instrumental scanning showed a positive but weak response. The weak reactivity of this compound may be explained on the basis of steric hindrance by the epoxide bridge in scopolamine to the approach of the bulky iodine molecule for complex formation with the nitrogen.

The positive response of the hydrohalide salts of some alkaloids is at first sight paradoxical owing to the engagement of the nitrogen lone pair responsible for the charge-transfer complex formation with the proton of the acid. However, the positive response may be readily explained on the basis of interaction of iodine with the halide anion in the salt, leading to a trihalide complex.

Peak Position and Intensity with Various Alkaloids—The results for a number of selected alkaloids are shown in Table I, and some are illustrated in Figs. 1 and 2. The results are to be compared with reported data (16) for the same compound in ethanol or 0.1 *N* acid, as shown in the last column of Table I.

It is clear from Table I that the *A* values (1%, 1 cm) for the iodine-complexed alkaloids are much higher than those for the uncomplexed compounds in ethanol or acid. This result is due to the strong charge-transfer band of the complex which is weak or may be absent in the spectrum of the donor and exists in the visible spectrum of iodine (10). The outcome is an overall increase in the *A* values (1%, 1 cm) from a high of about 100 times for *N*-butylscopolammonium bromide to a low of 1.1 times the uncomplexed value, as in the case of emetine.

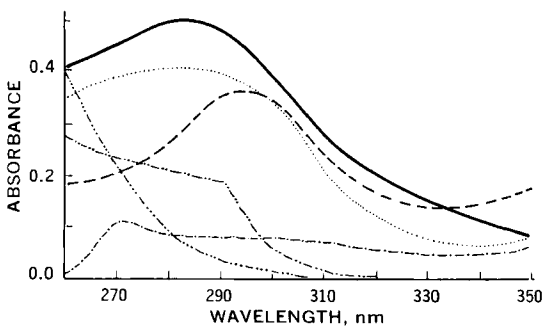

Figure 2—*Absorption spectra of charge-transfer complexes of some alkaloids with iodine in carbon tetrachloride. Key: ———, codeine, 1 × 10^{-4} M; – – – – –, ephedrine, 1.6 × 10^{-3} M; · · · · · ·, atropine, 2.4 × 10^{-4} M; – · – · –, scopolamine, 1.2 × 10^{-3} M; – · · – · · –, codeine, same concentration without iodine; and – · · · – · · · –, atropine, same concentration without iodine.*

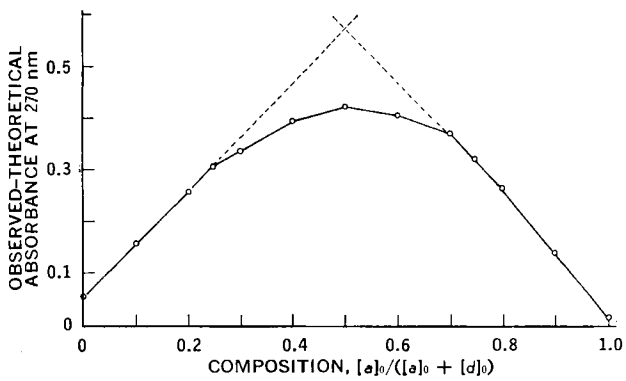

Figure 3—*Continuous variation curve for atropine–iodine complex in chloroform.*

Table II—Electrical Properties of Some Solvents (24)

Solvent	Specific Conductance, $ohm^{-1}\ cm^{-1}$	Dielectric Constant	Dipole Moment, Debye Units, 10^{-18} esu
Chloroform	1×10^{-10}	4.8	1.15
Carbon tetrachloride	4×10^{-18}	2.24	0.0
Cyclohexane	7×10^{-18}	2.02	0.0
Hexane	1×10^{-16}	1.88	0.85
Benzene	4.4×10^{-17}	2.28	0.0
Ether	3×10^{-16}	4.33	1.15
Ethanol	1.35×10^{-9}	24.55	1.66

More interesting is the increased A values (1%, 1 cm) of the alkaloids in the polyhalogenated solvents (without iodine) over the values in ethanol or 0.1 N acid. This finding was taken as suggestive of charge-transfer complex formation between the alkaloidal bases as donors and the polyhalogenated solvents as acceptors. This is in line with reported data (18, 19) concerning the contact charge-transfer pairs of amines with halomethanes, which are characterized by low association constants (20).

On the basis of these findings, it may be suggested that the high solubilities of the relatively polar alkaloidal molecules in these essentially "fat" solvents may be due to charge-transfer interactions. Electron donor–acceptor interaction is recognized as one important intermolecular force contributing to the power of dissolution of many solvents (21). In support of this assumption, the high solubilities of alkaloids in these low conductance, low dielectric constant solvents are to be compared with the lower solubilities of the same compounds in other organic solvents such as hexane, cyclohexane, benzene, and ether (16, 22, 23) which possess comparable electrical properties (Tables II and III). More striking is the high solubilities of alkaloids and some alkaloidal salts in chloroform over their solubility in the essentially more polar ethanol.

Contact pairs with solvent may also explain the fading of the charge-transfer complex color with some alkaloids on dilution with chloroform, because the solvent in massive amounts will compete with iodine as an acceptor in spite of the low stability of the solvent complex.

Alkaloid–Iodine Ratio in Complex—When using atropine as a model, the application of the Job method of continuous variation (12) indicated a 1:1 donor–acceptor ratio (Fig. 3). This finding was anticipated on the basis of the presence of one donating nitrogen in atropine. In the case of sparteine, with two identical nitrogen atoms, a 2:1 donor–acceptor ratio was found.

Molar Absorptivities and Association Constants—These were evaluated at 20° utilizing the well-established Rose–Drago (Eq. 1) (10, 11, 13) and Benesi–Hildebrand (Eq. 2) (10, 14) plots. The graphs for atropine are illustrated in Figs. 4 and 5, respectively.

From these curves the value of $\epsilon_{AD}{}^{\lambda}$ for the atropine–iodine complex at 20° was found to be 24,400 ± 120 in chloroform. Plots

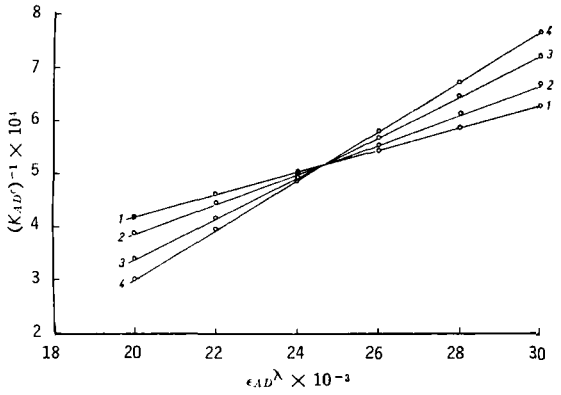

Figure 4—*Graphical solution of Rose–Drago equation (Eq. 1) for a series of atropine–iodine solutions in chloroform (iodine concentration = 4×10^{-5} M). Key (atropine concentration): Set 1, 1.5; Set 2, 3.0; Set 3, 4.5; and Set 4, 6.0×10^{-4} M.*

Table III—Solubilities of Selected Alkaloids in Some Solvents (16)

Alkaloid	Solubility in Chloroform	Ethanol	Ether
Atropine	1:1	1:3	1:60
Quinine	1:1.6	1:17	1:70
Strychnine	1:6	1:250	1:5500
Codeine	1:2	1:2	1:50
Reserpine	1:6	1:2000	Insoluble
Papaverine hydrochloride	1:10	1:120	Insoluble
Hyoscyamine hydrobromide	1:1.7	1:2.5	Very slightly soluble

involving solutions in carbon tetrachloride suggested a higher value of 26,700 ± 160 at 20°.

The association constant, K_{AD^c}, extrapolated from the same plots was also higher in carbon tetrachloride, being 2300 ± 50 liters $mole^{-1}$, and lower in chloroform, 1920 ± 30 liters $mole^{-1}$, at 20° for atropine. These data are in line with the values of association constants of other charge-transfer complexes previously reported (10, 11) in these two solvents which are generally higher in carbon tetrachloride. Similar plots gave a value of $\epsilon_{AD}{}^{\lambda}$ of 45,000 ± 200 and K_{AD^c} of 4430 ± 140 liters $mole^{-1}$ at 20° for *N*-butylscopolammonium bromide in chloroform.

When there is significant contribution at λ_{AD} by the absorption of the alkaloid, as is the case with the strong UV absorbers quinine and brucine, values of K_{AD^c} were evaluated from Nagakura's (15) formula (Eq. 3). Thus, the values for quinine are $\epsilon_{AD}{}^{\lambda}$ of 38,200 ± 200 and K_{AD^c} of 2460 ± 50 liters $mole^{-1}$ at 20° in chloroform, while for brucine $\epsilon_{AD}{}^{\lambda}$ was found to be 48,000 ± 220 and K_{AD^c} 4100 ± 100 liters $mole^{-1}$ at 20° in chloroform.

These relatively high values of association constants compare with the reported values for analogous complexes, *e.g.*, triethylamine–iodine with a K_{AD^c} of 5100 ± 130 at 25° (13) and tri-*n*-butylamine–iodine with a K_{AD^c} of 1600 at 20° (10, 11). The high values are common to complexes between σ-acceptors, such as iodine, and *n*-donors where the intermolecular overlap may be considerable.

Quantitation and Linearity of Beer's Plot—In the determination of the association constant and the molar absorptivity by the Benesi–Hildebrand plot, the equation is valid only when $[d] \gg [a]$. This may lead to anomalies and apparent deviations from Beer's law by the complex species (10). The effect of large donor concentration, expressed in the Benesi–Hildebrand equation, will have its counterpart in solutions where $[d] \ll [a]$. Consequently, the optimum conditions to minimize these deviations will be when $[d] \approx [a]$. It is under such conditions that the anomalies disappear. For this reason, in quantitative work the concentration of iodine was kept in the same order of magnitude as that of the alkaloid. A typical case is the 5×10^{-4} *M* iodine solution and 3×10^{-4} *M* atro-

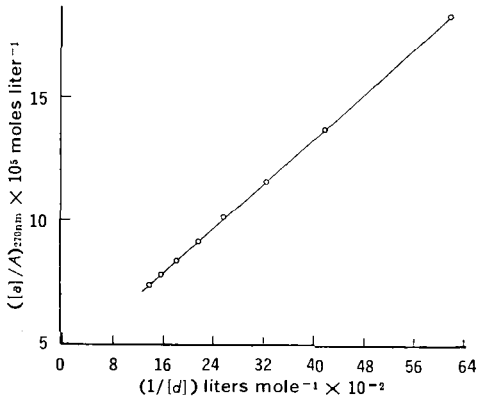

Figure 5—*Benesi–Hildebrand plot for atropine–iodine complex in chloroform at 270 nm. The donor concentration [d] was 1.6, 2.4, 3.2, 4.0, 4.8, 6.0, or 7.2 $\times 10^{-4}$ M. The acceptor concentration [a] was 2×10^{-5} M. The A_{270nm} was the absorbance of the complex at λ_{AD} (270 nm).*

pine solution in chloroform. Under such conditions, perfectly linear Beer's plots were obtained and were quite suitable for quantitation.

Accuracy and Precision—Standard solutions of the alkaloids prepared from the free base, or by quantitative recovery from the salts, when subjected to this procedure afforded results that were accurate to within 1.3% of the true values and were reproducible to ±1.5% at 95% confidence limits.

Assay of Alkaloids—The application of the charge-transfer method to various alkaloidal preparations following preliminary separation by standard and compendial methods afforded data of the same accuracy and precision stipulated. This justifies the adoption of the method to substitute ordinary spectrophotometric assays in routine alkaloidal analysis, since the extent of extra experimental work is negligible while sensitivity is greatly improved. The method is particularly attractive when the alkaloid concerned is an originally weak UV absorber. Thus, significantly improved recoveries were obtained in the assay of preparations containing atropine and other tropane alkaloids, *N*-butylscopolammonium bromide, ephedrine, and codeine.

Limitations—The same limitations of the direct spectrophotometric measurement of alkaloids (4) also apply in this method. Good recoveries are dependent upon the skill in the isolation of the free alkaloid from the preparation. However, an added advantage over the direct method, which balances some of these limitations, is the enhanced sensitivity, accuracy, and precision owing to the high molar absorptivities of the charge-transfer complexes.

SUMMARY

The intense charge-transfer UV bands in the spectra of molecular complexes of iodine with amines were utilized in a sensitive spectrophotometric assay of alkaloids. Solutions of the alkaloids in chloroform or carbon tetrachloride, when mixed with iodine in the same solvent, exhibited blue-shifted iodine bands in the 270–305-nm range.

Using the Job method of continuous variation, the molar ratio of iodine–alkaloid in the complex was found to be 1:1 with monobasic compounds and 1:2 with dibasic compounds.

Molar absorptivities of the charge-transfer complexes, $\epsilon_{AD}{}^{\lambda}$, for selected alkaloids ranged from 24,400 to 48,000, as determined by the Rose–Drago and Benesi–Hildebrand plots. Association constants of the complexes, $K_{AD}{}^{c}$, extrapolated from the same plots or computed from Nagakura's formula, varied from a low of 1920 ± 30 liters mole^{-1} for atropine in chloroform at 20° to a high of 4100 ± 100 liters mole^{-1} for brucine in chloroform at 20° and were solvent dependent, being higher in carbon tetrachloride (2300 ± 50 liters mole^{-1} for atropine at 20°).

The data obtained suggest the formation of similar but weak charge-transfer contact pairs between the alkaloids and the polyhalogen solvents. These weak complexes may account for the presence of moderate absorption maxima in the spectra of alkaloids in chloroform and carbon tetrachloride, which are weak or absent in ethanol, and for the high solubilities of the basic alkaloids in these low conductance solvents as compared to ethanol.

The original *A* values (1%, 1 cm) of the uncomplexed alkaloids showed an increase in the charge-transfer band ranging from a high of 50 times the original value (tropane alkaloids) to a low of 1.2 times (quinine and emetine), with red shift of the alkaloid band in most cases. This increase in absorbance was reflected in proportionate increases in sensitivity, accuracy, and precision upon quantitation.

The method is directly applicable to the purified alkaloid-containing chloroform fractions obtained in pharmacopeial assays or isolated from plant extracts and drug formulations in the general concentration range of 10^{-4}–10^{-5} *M* with a relative standard deviation of 0.015. The method is particularly recommended for assaying the tropane alkaloids and other weak UV-absorbing alkaloids such as ephedrine, codeine, and sparteine.

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* Present address: Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr-Al-Aini, Cairo, Egypt.

[x] To whom inquiries should be directed.

Solubility of Nonelectrolytes in Polar Solvents II: Solubility of Aliphatic Alcohols in Water

G. L. AMIDON[x], S. H. YALKOWSKY*, and S. LEUNG[‡]

Abstract □ The surface areas for 73 aliphatic hydrocarbons and alcohols, for which solubility and boiling point data are available, were calculated. The solubility of the alcohols and hydrocarbons can be quantitatively related to a combination of the hydrocarbon and hydroxyl surface areas of the molecules. The boiling points of the alcohols also correlated well with these two parameters. Isomeric alcohols, having a lower hydrocarbon surface area and/or a lower hydroxyl group surface area, are more soluble in water. The correlations show that branching, cyclization, and positional isomerism can be accounted for without introducing additional terms.

Keyphrases □ Solubility—73 aliphatic alcohols in water, correlated with surface areas and boiling points □ Alcohols, aliphatic—solubility in water, correlated with surface areas and boiling points □ Aliphatic hydrocarbons and alcohols—solubility of 73 determined, correlated with surface areas and boiling points □ Surface areas—73 aliphatic hydrocarbons and alcohols, correlated with solubility in water and boiling points

The ability to predict the effects of even simple structural modifications on aqueous solubility could be of great value in the design of improved drugs and drug delivery systems. At present, theoretical descriptions of solubility are mainly restricted to either nonpolar solutes in nonpolar solvents (1) or to salts and other highly polar compounds in water and other polar solvents (2) and are not generally applicable to most compounds of pharmaceutical interest.

In this paper, the solubility of semipolar and nonpolar compounds in water is discussed in terms of the molecular surface area of the solute and a corresponding "interfacial tension" term. This approach, initially suggested by Langmuir (3) and discussed further (4–6) for aqueous systems, was recently shown (7) to be successful in correlating the solubilities of hydrocarbons in water.

In the present paper, this approach is applied to the aqueous solubilities of primary, secondary, and tertiary linear and branched alcohols. While the primary purpose is to analyze the effects of relatively simple structural variations on the water solubility of alcohols, the approach appears to be generally applicable to semipolar and nonpolar compounds of arbitrary structure.

THEORETICAL

Ideal Solutions—In an ideal solution, one in which the activity equals the mole fraction over the entire composition range, there is no change in enthalpy or volume on mixing. The only term contributing to the free energy of mixing is the entropy of mixing. For two liquids, this quantity is always negative and results in infinite miscibility. From a molecular viewpoint, the molecules must be alike insofar as they are under the same forces in the mixture as in the pure liquids.

If one component of the mixture is a solid at the temperature of interest, the ideal solubility may be a good approximation to the actual solubility, provided that the molecules are "sufficiently" alike, *e.g.*, naphthalene in benzene. In this case, the solubility process may be considered as the sum of the two steps in Scheme I:

solid solute ⟶ supercooled liquid solute

supercooled liquid solute ⟶ solution

Scheme I

If the second step is considered as an ideal process, $\Delta G = RT \ln X$ for this step. The free energy change for the first step is obtained by integrating $(\partial \Delta G/\partial T)_p$ from T_m (the melting point) to the temperature of interest, T. When assuming that the enthalpy and entropy changes are constant over this temperature range, the free energy change for this step is $\Delta G = \Delta H_f[(T_m - T)/T_m]$.

The total free energy change is $\Delta G = \Delta H_f[(T_m - T)/T_m] + RT \ln X$ and at equilibrium ($\Delta G = 0$) the ideal solubility is given by Eq. 1:

$$\log X = \frac{-\Delta H_f}{2.3RT}\left(\frac{T_m - T}{T_m}\right) \qquad \text{(Eq. 1)}$$

where X is the mole fraction of the solute, ΔH_f is the heat of fusion, T_m is the melting point, and T is the temperature of interest (below the melting point).

Regular Solution Approach—The next step in solution theory is to admit an excess enthalpy while maintaining the random or ideal entropy of mixing (*i.e.*, zero excess entropy of mixing). This is the regular solution approach (1, 8). In this model, the enthalpy (energy) of transfer of a mole of liquid 2 from pure liquid to solution is set equal to $v_2 \phi_1^2(\delta_1 - \delta_2)^2$, where v_2 is the molar volume of liquid 2, ϕ_1 is the volume fraction of liquid 1, and δ is the solubility parameter that equals the square root of the energy of vaporization divided by the molar volume.

Therefore, the free energy of transfer of a mole of liquid solute 2 from pure liquid 2 to solution is:

$$\Delta \bar{G}_2 = RT \ln a_2 = v_2\phi_1^2(\delta_2 - \delta_1)^2 + RT \ln X_2 \qquad \text{(Eq. 2)}$$

or:

$$RT \ln (a_2/X_2) = v_2\phi_1^2(\delta_2 - \delta_1)^2 \qquad \text{(Eq. 3)}$$

For a dilute solution ($\phi_1 = 1$), this becomes:

$$RT \ln (a_2/X_2) = v_2(\delta_2 - \delta_1)^2 \qquad \text{(Eq. 4)}$$

or:

$$RT \ln X_2 = -v_2(\delta_2 - \delta_1)^2 \qquad \text{(Eq. 5)}$$

for the solubility of two immiscible liquids. If solute 2 is a solid at the temperature of interest, the correction discussed for the ideal solution case must be applied. For a pure solid solute in equilibrium with saturated solution, the solubility is given by:

$$\ln X_2 = -\frac{\Delta H_f(T_m - T)}{RT_mT} - v_2\phi_1^2(\delta_2 - \delta_1)^2 \qquad \text{(Eq. 6)}$$

(using the pure supercooled liquid as the standard state).

This approach is applicable to nonpolar compounds but must be further modified for polar substances. Four modifications have been proposed: *(a)* divide the solubility parameter into polar (τ) and nonpolar (λ) parts, *(b)* incorporate a factor to account for deviation from the geometric mean rule, *(c)* determine a correction factor for the entropy of mixing to account for differences in molecular size (Flory–Huggins correction term), and *(d)* include a correction term for the additional entropy effects associated with hydrogen bonding substances (1).

Since all of these factors must be considered for aqueous sys-

tems, this approach is of limited predictive value due to the lack of adequate estimates of the various terms.

Molecular Surface Area Approach—An alternative approach to aqueous solutions (9–11) suggests that the number of water molecules that can be packed around the solute molecule plays an important role in the theoretical calculation of the thermodynamic properties of the solution. Hence, the molecular surface area of the solute is an important parameter in the theory. Hermann (7) applied this approach to the solubilities of hydrocarbons in water with good results. For an arbitrary semipolar molecule, the total molecular surface area can be divided into polar and nonpolar parts. The contribution of each part to the solubility can then be calculated by multiplying the respective surface areas by the appropriate free energy per unit area terms.

This approach has the advantage that, once the free energy per unit area terms for various functional groups are known, an estimate of the solubility of a new structure can be made simply by calculating the molecular (group) surface area(s). An additional advantage over other group contribution methods is that the effects of branching and stereochemistry (*e.g.*, diastereoisomer solubility differences) are accounted for without resorting to different factors for each structural variation.

Historically, Langmuir (3) was the first to approach solubility from a consideration of the molecular surface area of a molecule. He split the energy of vaporization into three parts, polar–polar, nonpolar–nonpolar, and nonpolar–polar interactions, and calculated the energy of vaporization as a sum of the fractional surface areas times the corresponding "interfacial energies." A solution of two different species was treated in a similar manner by considering the various interfacial energies. The mutual solubilities of two immiscible liquids could then be calculated.

Scatchard (8) noted that Langmuir's equation could be put into the same form as that of the regular solution theory by replacing the energy density with energy per unit surface area and the volume fraction by surface fraction. However, he criticized two assumptions in Langmuir's approach; one is that the surface of contact between two surfaces is proportional to the product of the surfaces, and the second is the assumption that the surface area of any molecule is proportional to the two-thirds power of its volume. The second criticism is resolved using the surface area approach developed by Hermann (7) and discussed here. The first criticism, while valid in general, is not necessary for dilute solutions of semipolar compounds in water since each solute is only surrounded by water molecules; *i.e.*, solute–solute interactions are negligible.

A related approach (4–6, 10, 11), based on a cavity model for the solvent, considers the energy required to create a cavity in the solvent as an important determinant of the solubility of a gas molecule in the solvent and the surface area of the molecule enters into the calculation along with the surface tension of the solvent. This approach has been used successfully for water (10) as well as other solvents (6, 12). Similar approaches to the complexation in liquid systems have also been used successfully (13, 14).

Comparison of Area and Volume Theories—Returning to the analogy between the regular solution approach and Langmuir's approach suggested by Scatchard (8), the equation for the solubility of one liquid in another according to regular solution theory is:

$$RT \ln X_2 = -v_2\phi_1^2(\delta_2 - \delta_1)^2 \qquad \text{(Eq. 7)}$$

Assuming low solubility ($\phi_1 = 1$), the analogy gives:

$$RT \ln X_2 = -SA_2(\gamma_2^{1/2} - \gamma_1^{1/2})^2 \qquad \text{(Eq. 8)}$$

where SA_2 is the surface area of molecule 2, and γ_1 and γ_2 are the surface tensions of liquids 1 and 2, respectively. The term in parenthesis $(\gamma_2^{1/2} - \gamma_1^{1/2})^2$ resembles Antonow's rule for the interfacial tension, γ_{12} (15). This then suggests that for two very different liquids, where an experimental interfacial tension can be measured, the solubility prediction may be improved by using:

$$RT \ln X_2 = (-SA_2)(\gamma_{12}) \qquad \text{(Eq. 9)}$$

This equation can also be developed by considering the work of cohesion of the two pure liquids (W_{11} and W_{22}) and the work of adhesion (W_{12}). The reversible work required to remove a solute molecule from bulk liquid to vapor is one-half the work of cohesion times the surface area, $(\frac{1}{2} W_{11})(SA)$. The work required to create a cavity in the solvent is $(\frac{1}{2}W_{22})(SA)$. The free energy change in placing the solute molecule into the solvent cavity is $(-\gamma_{12})(SA)$. Thus, the reversible work for the process of transferring a solute molecule from bulk liquid to solution is: $(\frac{1}{2}W_{11} + \frac{1}{2}W_{22} - W_{12})(SA)$.

At this point all energy and entropy terms have been accounted for in the transfer process (unitary terms). However, the solute is still fixed in position in solution. Thus, the cratic (mixing) term of the molecule (or molecule plus cosphere) must be added to obtain the total free energy change (2):

$$\Delta\overline{G}_2 = RT \ln a_2 = (\frac{1}{2}W_{11} + \frac{1}{2}W_{22} - W_{12})(SA_2) + RT \ln X_2 \qquad \text{(Eq. 10)}$$

For a pure liquid in equilibrium with solution, the solubility is then given by:

$$RT \ln X_2 = -(\frac{1}{2}W_{11} + \frac{1}{2}W_{22} - W_{12})(SA_2) \qquad \text{(Eq. 11)}$$

If the work of adhesion and cohesion terms are replaced with the bulk interfacial tensions, the solubility expression becomes:

$$RT \ln X_2 = (-\gamma_{12})(SA_2) \qquad \text{(Eq. 12)}$$

which is identical to Eq. 9. Implicit in this approach are the requirements that the solution be dilute, so the solute–solute interaction terms in solution can be neglected, and that the molecular cospheres do not overlap.

Microscopic Surface Tension—A second point to consider is the use of bulk surface tensions for molecular cavities. It is well known that for surfaces of molecular dimensions, a curvature correction must be applied (15). In general, this has the effect of lowering the surface tension at a curved surface from that of a bulk planar surface. In this approach, the curvature correction for the pure liquid phase surface tensions, γ_1 and γ_2, need not be considered since they cancel out of the final equation. However, the interfacial tension term, γ_{12}, that remains must be considered.

If, as discussed later, a molecule is divided into groups, the only meaningful interfacial tension terms would be those between the hydrophobic portions of the molecule and water. In the case of aliphatic hydrocarbon portions, the experimental bulk hydrocarbon–water interfacial tension is about 50 ergs/cm^2 [and relatively independent of the actual hydrocarbon (16)] and should provide an upper limit for the slope of ln (sol) *versus* hydrocarbon surface area plot. From the significant structure theory applied to the solution of hydrocarbons in water (11), the interfacial tension is calculated to be about 15 ergs/cm^2. Hence, the curvature correction is significant.

However, the actual energetics of cavity formation are considerably more complex than the preceding discussion, based on free energies, would suggest (particularly for the short-chain compounds). An analysis similar to this for the energy and entropy changes would suggest that, since the bulk hydrocarbon–water interfacial tension decreases with increasing temperature (17), the entropy of cavity formation as well as the energy of surface formation would be positive.

On the other hand, transfer of small hydrocarbons from nonpolar liquids to water is accompanied by large negative entropies and small heat effects (18). These effects are usually attributed to water structuring; however, it is not clear whether this effect is intrinsic to the creation of a small cavity in water or to the introduction of the nonpolar solute into the cavity (19). For the longer chain length alcohols (lengths greater than four carbons), the entropy effect levels off, with more than two-thirds of the free energy of solution coming from the enthalpy of solution (20). This is in line with the notion that interfacial effects should increase with increasing chain lengths (21) and the approach taken in this work.

Molecular Surface Area—The definition of molecular surface area adopted in this paper is that of Hermann (7). A molecule is considered as a collection of spheres with each radius located at the nuclear center. To each radius on the solute (alcohol) molecule, a radius for the solvent (water) is added to give a surface shown schematically in Fig. 1 for ethanol and *tert*-butanol. This approach has the desirable property of eliminating from the total surface area of a molecule those areas not exposed (or accessible) to the solvent.

Table I—Experimental Solubilities, Boiling Points, Surface Area Results, and Predicted Solubilities

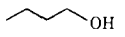
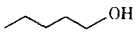
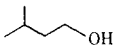
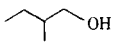
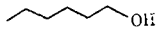
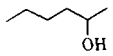
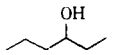
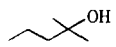
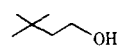
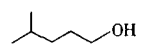
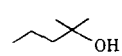
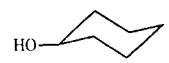
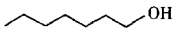
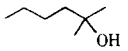
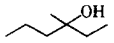
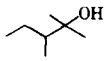
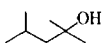
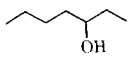
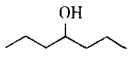
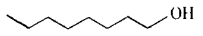
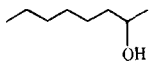
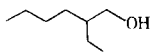
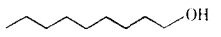
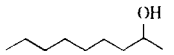
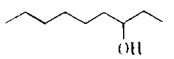
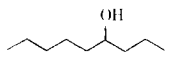
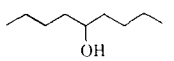
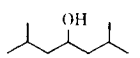
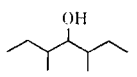
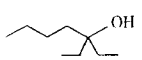
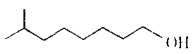
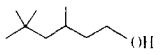
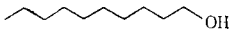
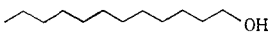
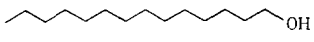
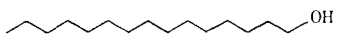
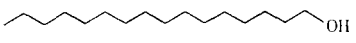
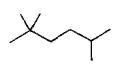
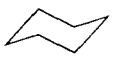
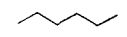
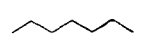
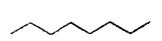
Number	Compound	Structure	Solubility[a], molal	*TSA*[b], Å^2	*OHSA*, Å	*Tb*[c]	Predicted Solubility[d], molal
1	*n*-Butanol		1.006 (1.1)	272.1	59.2	117.7°	0.821
2	2-Methyl-1-propanol		1.023	263.8	52.4	107.9°	1.299
3	2-Butanol		1.068	264.1	42.8	99.5°	1.496
4	*n*-Pentanol		2.6 (2.5) × 10^{-1}	303.9	59.2	137.8°	2.09 × 10^{-1}
5	3-Methyl-1-butanol		3.11 × 10^{-1}	291.4	59.2	131.2°	3.57 × 10^{-1}
6	2-Methyl-1-butanol		3.47 × 10^{-1}	289.4	52.4	128.7°	4.33 × 10^{-1}
7	2-Pentanol		5.3 × 10^{-1}	295.9	42.8	119°	3.81 × 10^{-1}
8	3-Pentanol		6.15 × 10^{-1}	293.5	36.0	115.3°	4.70 × 10^{-1}
9	3-Methyl-2-butanol		6.67 × 10^{-1}	284.3	40.2	111.5°	6.54 × 10^{-1}
10	2-Methyl-2-butanol		1.403	282.5	38.6	102.0°	7.25 × 10^{-1}
11	2,2-Dimethyl-1-propanol[e,f]		4.11 × 10^{-1} [5.24 × 10^{-1}]	283.5	47.3	113.1°	6.05 × 10^{-1}
12	*n*-Hexanol		6.14 (6.1, 6.6) × 10^{-2}	335.7	59.2	157°	5.32 × 10^{-2}
13	2-Hexanol		1.36 (1.69) × 10^{-1}	327.7	42.8	139.9°	9.69 × 10^{-2}
14	3-Hexanol		1.6 × 10^{-1}	325.3	36.0	135.4°	1.20 × 10^{-1}
15	3-Methyl-3-pentanol		4.36 × 10^{-1}	305.8	32.7	122.4°	2.91 × 10^{-1}
16	2-Methyl-2-pentanol		3.27 × 10^{-1}	314.3	38.6	121.4°	1.84 × 10^{-1}
17	2-Methyl-3-pentanol		2 × 10^{-1}	314.3	37.9	126.5°	1.86 × 10^{-1}
18	3-Methyl-2-pentanol		1.94 × 10^{-1}	311.3	45.1	134.2°	1.89 × 10^{-1}
19	2,3-Dimethyl-2-butanol		4.27 × 10^{-1}	301.2	34.77	118.6°	3.44 × 10^{-1}
20	3,3-Dimethyl-1-butanol		7.5 × 10^{-2}	307.5	59.2	143°	1.79 × 10^{-1}
21	3,3-Dimethyl-2-butanol		2.44 × 10^{-1}	296.7	36.6	120°	4.05 × 10^{-1}
22	4-Methyl-1-pentanol		1.02 × 10^{-1}	323.0	59.2	151.8°	9.20 × 10^{-2}
23	4-Methyl-2-pentanol		1.63 × 10^{-1}	314.9	41.5	131.7°	1.71 × 10^{-1}
24	2-Ethyl-1-butanol		6.16 × 10^{-2}	308.6	51.1	146.5°	1.93 × 10^{-1}
25	Cyclohexanol		3.83 × 10^{-1}	290.5	49.6	161°	4.3 × 10^{-1}

Table I—*Continued*

Number	Compound	Structure	Solubility[a], molal	*TSA*[b], Å²	*OHSA*, A	*Tb*[c]	Predicted Solubility[d], molal
26	*n*-Heptanol		1.55 (1.45, 1.7) × 10^{-2}	367.5	59.2	176.3°	1.36 × 10^{-2}
27	2-Methyl-2-hexanol		8.43 × 10^{-2}	346.1	38.6	142.5°	4.69 × 10^{-2}
28	3-Methyl-3-hexanol		1.04 × 10^{-1}	337.7	32.7	142.4°	7.41 × 10^{-2}
29	3-Ethyl-3-pentanol		1.47 × 10^{-1}	324.4	32.7	142.5°	1.31 × 10^{-1}
30	2,3-Dimethyl-2-pentanol		1.35 × 10^{-1}	323.8	38.6	139.7°	1.22 × 10^{-1}
31	2,3-Dimethyl-3-pentanol		1.44 × 10^{-1}	321.8	32.7	139°	1.46 × 10^{-1}
32	2,4-Dimethyl-2-pentanol		1.17 × 10^{-1}	328.6	28.1	133°	1.17 × 10^{-1}
33	2,4-Dimethyl-3-pentanol		6.07 × 10^{-2}	331.7	33.9	138.8°	9.39 × 10^{-2}
34	2,2-Dimethyl-3-pentanol		7.11 × 10^{-2}	326.1	29.8	136°	1.27 × 10^{-1}
35	3-Heptanol		4.1 × 10^{-2}	57.1	36.0	156.8°	3.05 × 10^{-2}
36	4-Heptanol		4.09 × 10^{-2}	357.1	36.0	155.0°	3.05 × 10^{-2}
37	1-Octanol		4.51 (3.68, 4.1) × 10^{-3}	399.4	59.2	195.2°	3.45 × 10^{-3}
38	2,2,3-Trimethyl-3-pentanol		5.33 × 10^{-2}	335.2	26.7	152.5°	9.05 × 10^{-2}
39	2-Octanol		8.6 × 10^{-3}	391.0	42.8	179.8°	6.39 × 10^{-3}
40	2-Ethyl-1-hexanol		6.76 × 10^{-3}	371.3	51.1	184.6°	1.31 × 10^{-2}
41	1-Nonanol		1.0 (0.934) × 10^{-3}	431.2	59.2	213.1°	8.79 × 10^{-4}
42	2-Nonanol		1.8 × 10^{-3}	423.2	42.8	198.5°	1.60 × 10^{-3}
43	3-Nonanol		2.2 × 10^{-3}	420.8	36.0	194.7°	1.97 × 10^{-3}
44	4-Nonanol		2.6 × 10^{-3}	420.8	36.0	193°	1.97 × 10^{-3}
45	5-Nonanol		3.2 × 10^{-3}	420.8	36.0	195.1°	1.97 × 10^{-3}
46	2,6-Dimethyl-4-heptanol		3.1 × 10^{-3}	394.0	33.4	178°	6.50 × 10^{-3}
47	3,5-Dimethyl-4-heptanol		5.0 × 10^{-3}	379.3	29.8	187°	1.29 × 10^{-2}
48	2,2-Diethyl-1-pentanol		3.8 × 10^{-3}	372.5	44.6	192°	1.38 × 10^{-2}

(*continued*)

Table I—*Continued*

Number	Compound	Structure	Solubility[a], molal	*TSA*[b], Å^2	*OHSA*, A	*Tb*[c]	Predicted Solubility[d], molal
49	7-Methyl-1-octanol		3.2×10^{-3}	418.7	59.2	206°	1.50×10^{-3}
50	3,5,5-Trimethyl-1-hexanol		3.12×10^{-3}	376.6	59.2	193.0°	9.19×10^{-3}
51	1-Decanol		$2.0\ (2.69) \times 10^{-4}$	463	59.2	230.2°	2.24×10^{-4}
52	1-Dodecanol		2.3×10^{-5}	527	592	—	1.43×10^{-5}
53	1-Tetradecanol[e]		1.46×10^{-6} [2.84×10^{-6}]	591	59.2	263.5°	9.38×10^{-7}
54	1-Pentadecanol[e]		4.5×10^{-7} [1.02×10^{-6}]	623	59.2	—	2.36×10^{-7}
55	1-Hexadecanol[e]		1.7×10^{-7} [4.55×10^{-7}]	655	59.2	—	5.87×10^{-8}
56	*n*-Butane		2.34×10^{-3}	255.2	—	—	1.43×10^{-3}
57	Isobutane		2.83×10^{-3}	249.1	—	—	1.86×10^{-3}
58	*n*-Pentane		5.37×10^{-4}	287.0	—	—	3.65×10^{-4}
59	2-Methylbutane		6.61×10^{-4}	274.6	—	—	6.21×10^{-4}
60	3-Methylpentane		1.48×10^{-4}	300.1	—	—	2.08×10^{-4}
61	Neopentane		7.48×10^{-4}	270.1	—	—	7.52×10^{-4}
62	2,2-Dimethylbutane		2.14×10^{-4}	290.8	—	—	3.10×10^{-4}
63	2,4-Dimethylpentane		4.07×10^{-5}	324.7	—	—	7.20×10^{-5}
64	2,2,4-Trimethylpentane		7.48×10^{-5}	338.9	—	—	3.91×10^{-5}
65	2,2,5-Trimethylhexane		8.95×10^{-6}	373.0	—	—	9.03×10^{-6}
66	Cyclohexane		6.61×10^{-4}	279.1	—	—	5.11×10^{-4}
67	(e)-Methylcyclohexane		1.41×10^{-4}	304.9	—	—	1.69×10^{-4}
68	1-*cis*-2-Dimethylcyclohexane		5.38×10^{-5}	315.5	—	—	1.07×10^{-4}
69	Cycloheptane		3.05×10^{-4}	301.9	—	—	1.92×10^{-4}
70	Cyclooctane		7.05×10^{-5}	322.58	—	—	7.89×10^{-4}
71	*n*-Hexane		1.11×10^{-5}	319	—	—	1.23×10^{-4}
72	*n*-Heptane		2.93×10^{-5}	351	—	—	2.33×10^{-5}
73	*n*-Octane		5.79×10^{-6}	383	—	—	5.87×10^{-6}

[a] Solubilities were taken from the following references and references cited therein: C. Hansch, J. E. Quinlar, and G. L. Lawrence, *J. Org. Chem.*, **33**, 347 (1968); G. H. Bell, *Chem. Phys. Lipids*, **10**, 1(1973); J. R. Hommelen, *J. Colloid. Sci.*, **14**, 385(1959); R. Vochten and G. Petre, *J. Colloid. Interface Sci.*, **42**, 320(1973); and C. McAuliffe, *J. Phys. Chem.*, **70**, 1267(1966). The first listed values were used in the regression analysis, values in parentheses are other reported values. [b] Hydrocarbon surface areas were taken from Ref. 7. [c] Reference 23. [d] From Eq. 16 in text. [e] Solids at room temperature; the solubility in brackets [ ] is the solubility of the pure supercooled liquid at 25°. [f] The ΔH_f for this compound was calculated from the cryoscopic constant (29).

Another advantage of this method is that individual atom contributions to the total area are calculated. This allows the total area of a molecule to be divided into group contributions (*e.g.*, hydrocarbon and hydroxyl group portions for alcohols), and the group contribution to the solubility can be evaluated. Standard bond angles and distances were used for all molecules considered in this study (22). The van der Waals radii were taken to be C = 1.6 Å, H = 1.2 Å, O = 1.4 Å, and H_2O = 1.5 Å (solvent radius). The

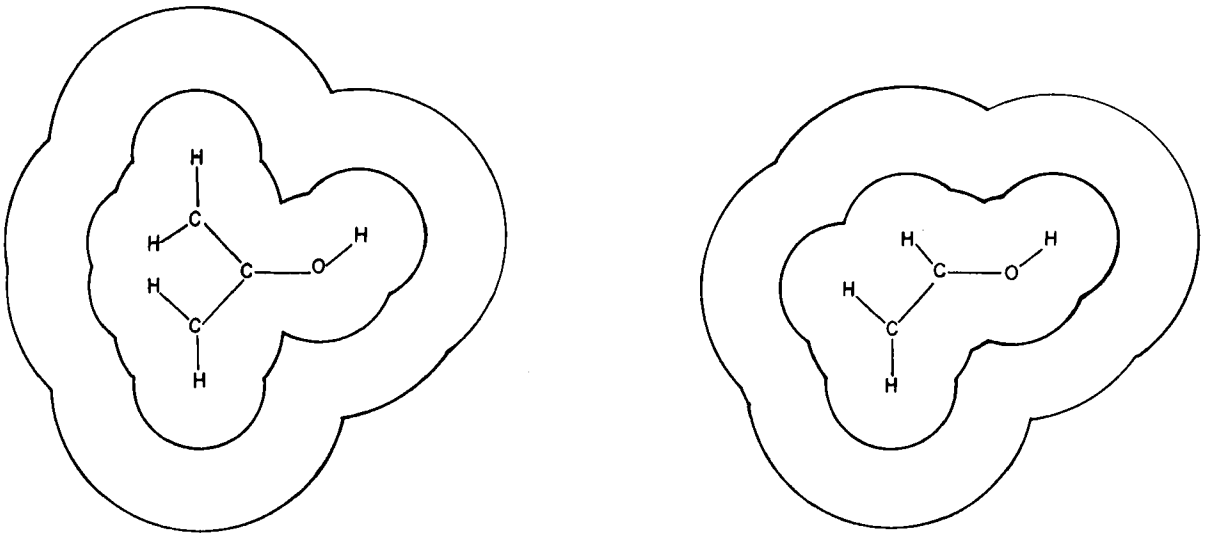


Figure 1—*Planar sections of* tert-*butanol* (a) *and ethanol* (b). *The inner surface represents surface area with the van der Waals radius of the solvent equal to zero. The outer surface includes a 1.5 Å solvent radius.*

main chain of all compounds was placed in an all-*trans*-conformation.

RESULTS

Solubility and Boiling Point Data—The solubilities of the alcohols considered are given in Table I. The overall decrease in solubility with increasing hydrophobicity is obvious. There are, however, more subtle changes in solubility with structure which can best be illustrated by considering a set of isomers such as the pentanols (Compounds 4–11).

From Table I, it is apparent that the primary pentanols are less soluble than the secondary pentanols and that the tertiary pentanols have the greatest solubilities in this group. It is also evident that, for the primary pentanols (Compounds 4–6 and 11), branching of the normal chain increases solubility; it can also be seen that 3-pentanol is more soluble than 2-pentanol, which is more soluble than 1-pentanol. These same trends are present in each set of isomers in Table I, *i.e.*, butanols, hexanols, heptanols, octanols, and nonanols.

In addition, the boiling points of the alcohols are listed in Table I. These values are also dependent upon the described structural features. In general, boiling points increase with chain length and decrease with branching and substitution on the α-carbon.

Surface Area—The surface area of each alcohol was calculated as discussed[1]. The total surface areas *(TSA)* as well as the hydroxyl group areas *(OHSA)*, the sum of the oxygen and hydroxyl hydrogen exposed areas, are presented in Table I. From these values it is apparent that substitution on either the α- or β-carbon reduces the *OHSA* from its value in a normal alcohol (*OHSA* = 59.2 Å^2). Qualitatively, the surface area, solubility, and boiling point seem to be dependent on the same structural features.

DISCUSSION

Before discussing the relationship between solubility and surface area, it should be noted that Compounds 11 and 51–53 are solids at room temperature. Since the solubility estimate refers to that of the supercooled liquid, the observed solubility for these compounds must be corrected by multiplying by the factor $\exp[+\Delta H_f(T_m - T)/RT_mT]$ (see Eq. 2).

The heat of fusion data were taken from Wilhoit and Zwolinski (23). The corrected solubilities are given in parentheses in Table I.

To evaluate the proposed relationship between solubility and surface area, regression analysis was performed on the 51 alcohols, Compounds 1–55 in Table I omitting the solid alcohols. This analysis gives:

$$\ln(\text{sol}) = -0.043TSA + 11.78 \qquad \begin{matrix} r & s \\ 0.974 & 0.499 \end{matrix} \qquad \text{(Eq. 13)}$$

where sol is the molar solubility, *TSA* is the total surface area in Å^2, r is the correlation coefficient, and s is the standard error.

If the hydroxyl group were to make a constant contribution to the solubility, a correlation with hydrocarbon surface area, $HYSA = TSA - OHSA$ should give a similar correlation. However, the analysis gives:

$$\ln(\text{sol}) = -0.0396HYSA + 8.94 \qquad \begin{matrix} r & s \\ 0.94 & 0.706 \end{matrix} \qquad \text{(Eq. 14)}$$

which is considerably poorer than the correlation with *TSA*. This can be observed in Fig. 2 where there is considerably more scatter in the *HYSA* plot than in the *TSA* plot.

A third analysis, which includes both the *HYSA* and *OHSA*, gives the following equation:

$$\ln(\text{sol}) = -0.043HYSA - 0.060OHSA + 12.41 \qquad \begin{matrix} r & s \\ 0.978 & 0.462 \end{matrix} \qquad \text{(Eq. 15)}$$

which in spite of having an additional parameter is not significantly better than Eq. 13. This suggests that the hydroxyl group is making a variable contribution to the solubility.

To investigate the hydroxyl group contribution to the solubility more thoroughly, regression analysis was performed using the solubility data for both the alcohols and hydrocarbons in Table I. The analysis gives the following equation:

$$\ln(\text{sol}) = -0.0430HYSA + 8.003IOH - 0.0586OHSA + 4.420 \qquad \begin{matrix} r & s \\ 0.992 & 0.452 \end{matrix} \qquad \text{(Eq. 16)}$$

where the *IOH* term equals 1 if the compound is an alcohol and zero if the hydroxyl group is not present[2]. The predicted solubilities in Table I were calculated using this equation, and Fig. 3 shows a plot of ln (sol) observed *versus* ln (sol) predicted. This equation gives an excellent estimate of the solubilities of the alcohols and hydrocarbons in Table I without the need to include additional correction factors for branching, cyclization, positional isomers, *etc.*

[1] The computer program utilized in this study was a modified version of a program written by Dr. Robert Hermann. The original program (QCPE 225) was obtained through the Quantum Chemistry Program Exchange, Chemistry Department, Indiana University, Bloomington, Ind.

[2] It would equal zero or the number of (independent) hydroxyl groups in a polyhydroxy alcohol.

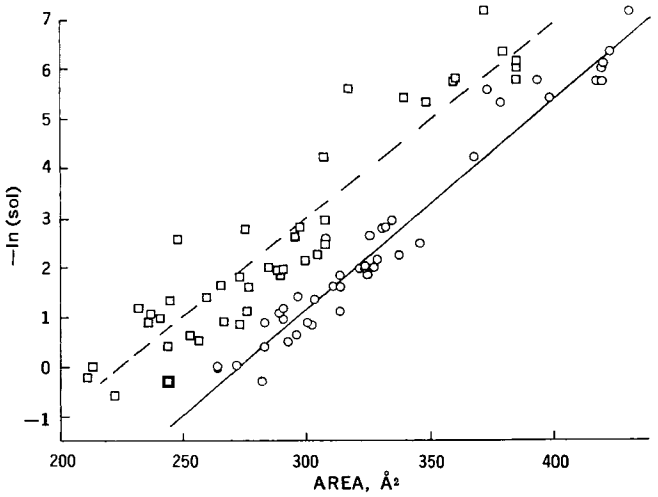


Figure 2—*Plot of* $-ln$ *(sol)* versus *total surface area* (○) *and hydrocarbon surface area* (□) *for the alcohols.*

In view of the success of Eq. 16, a discussion of each term in the equation is appropriate. The *HYSA* term represents the incremental effect on solubility (per Å²) of increasing the hydrocarbon surface area in the molecule. The coefficient of this term is negative, as expected; when converted into a corresponding interfacial tension (see Eq. 9), it gives a value of 17.7 ergs/cm². While this coefficient is only an adjusted parameter from Eq. 16, if it is considered as a microscopic interfacial tension, it is well within the range expected for such a term as discussed.

The constant, 4.420, in Eq. 16 is related to the concentration units used for the solubility data. Equation 9 predicts a zero intercept on a mole fraction basis; converting to a molal concentration scale gives an approximate theoretical intercept of 4.0 (on a natural log scale), in good agreement with the observed intercept.

The *IOH* and *OHSA* terms collectively represent the effect of the hydroxyl group on the solubility of the hydrocarbon (there is also an effect on the *HYSA* term as discussed later). If for comparative purposes the normal alcohols are considered as reference alcohols with a constant *OHSA* equal to 59.2 Å² (Table I), the effect of structural variation in the alcohol on its solubility can be discussed on a comparative basis. For this purpose, it is convenient to rearrange the *IOH* and *OHSA* terms to give:

$$8.003IOH - 0.0586OHSA = 0.0767NOHSA + 0.0586(NOHSA - OHSA) \quad \text{(Eq. 17)}$$

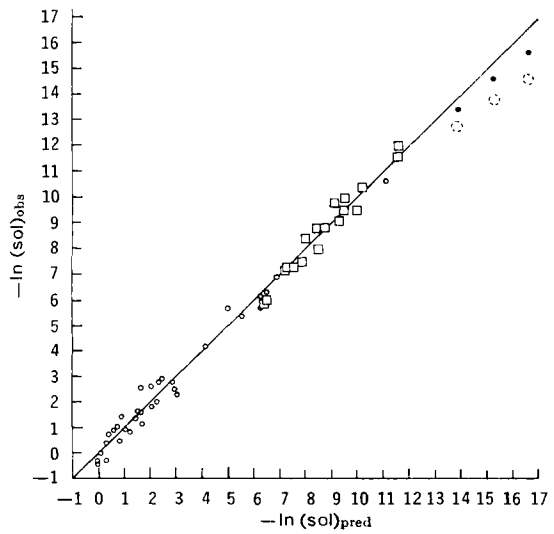


Figure 3—*Plot of* $-ln$ *(sol) observed* versus $-ln$ *(sol) predicted, from Eq. 16, for alcohols* (○) *and hydrocarbons* (□). *Solid circles* (●, *experimental solubility*) *and dashed circles* (◌, *solubility of pure supercooled liquids*) *are the points for the 14-, 15-, and 16 carbon alcohols* (*see text*).

Table II—Free Energy Contribution of Hydroxyl Group to Solution Process

OH Position	ΔG, kcal/molal		
	Loss of *HYSA*[a]	Presence of OH[a]	Total
1°	−1.12	−2.69	−3.81
2°	−0.78	−3.49	−4.27
3°	−0.70	−3.59	−4.29

[a] This division will vary somewhat depending on the structure of the hydrocarbon portion of the alcohol and the position of the OH.

where *NOHSA* represents the exposed surface area of the hydroxyl group in the normal alcohol (59.2 Å²). By referring to the right-hand side of Eq. 17, it can be seen that, as expected, in the normal position the hydroxyl group increases the solubility since *NOHSA* = *OHSA* = 59.2 and the coefficient of the *NOHSA* term is positive. This corresponds to a free energy contribution to solubility of −2.69 kcal/mole for the addition of the hydroxyl group to the hydrocarbon, with the *HYSA* term remaining constant.

However, when the hydroxyl group is added to a hydrocarbon, the *HYSA* of the molecule is reduced by about 43 Å² for the normal alcohols (compare normal hydrocarbons and alcohols in Table I). This gives an additional free energy increment of −1.12 kcal/mole for a total free energy increment of −3.81 kcal/mole to the solubility for the addition of a hydroxyl group to a normal hydrocarbon in the 1-position (see also Table II).

For the isomeric alcohols, the *OHSA* term is, in general, less than 59.2 (Table I). Referring to Eq. 17, this indicates that the higher solubility of the isomeric alcohols over the normal alcohols is due to a reduced *OHSA*, since the *OHSA* is less than the *NOHSA* and the coefficient of this term, +0.0586, is positive, indicating a positive contribution to the solubility. This reduction in the *OHSA* for the isomers is a result of the hydroxyl group being less exposed than in the normal position. Since this reduced accessibility of the hydroxyl group, in other than normal alcohol, would not be expected to favor its interaction with water, the effect of branching, *etc.*, would seem to reside in its effect on the properties of the pure liquid alcohol phase (or possible electronic effects).

Alcohols are known to be associated through hydrogen bonding in the liquid state, with the association decreasing in the order primary, secondary, and tertiary due to steric limitations (24, 25). The effect of this reduced association would be to increase the vapor pressure and reduce the boiling point. In addition to the steric effects, these structural changes also affect the proton-donating and proton-accepting abilities of the hydroxyl group through electronic changes.

Experimental data suggest that branching reduces the proton-donating ability of the hydroxyl group and increases the proton-accepting ability (24). While the net effect of the electronic changes on the interaction of the alcohol with itself and with water is not certain, the steric effect on the self-association of the alcohol appears to be a dominant factor in the formation of alcohol-water mixtures (25).

The overall correlation of boiling point with total surface area

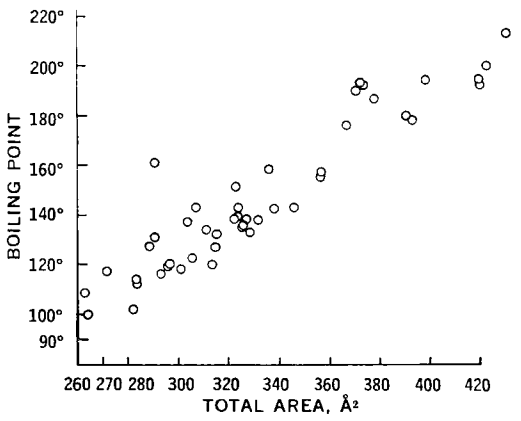


Figure 4—*Plot of boiling point* versus *total surface area for the alcohols.*

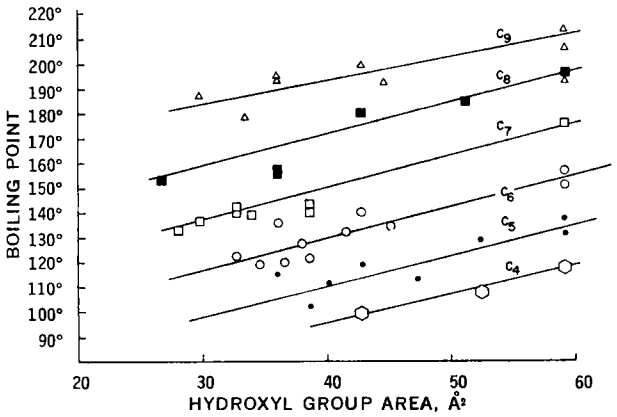


Figure 5—*Plot of boiling point* versus *hydroxyl group surface area for the various chain length alcohols.*

(TSA) is apparent in Fig. 4. To analyze further the effect of the *OHSA* term on the properties of the pure liquid alcohols, a regression analysis was performed on the boiling point data (Table I) which gives:

$$\text{boiling point} = 0.632HYSA + 1.182OHSA - 87.78 \qquad r = 0.982 \quad s = 6.37 \qquad \text{(Eq. 18)}$$

The positive coefficient of the *OHSA* term indicates that reducing the *OHSA* (relative to that of a normal alcohol) reduces the boiling point. This effect is shown in Fig. 5, where for a given number of carbon atoms the decrease in boiling point with decreasing *OHSA* is very evident. Hence, the increased solubility of, for example, 2-pentanol (Compound 7) over 1-pentanol (Compound 4) is due to both a reduction in the hydrocarbon area and a reduction in the hydroxyl surface area.

The free energy contributions of the hydroxyl group to the solubility of a given chain length hydrocarbon are summarized in Table II. The contribution from the reduction of hydrocarbon surface area was calculated by comparing the appropriate alcohols and hydrocarbons in Table I. The contribution of the hydroxyl group is the sum of the *IOH* and *OHSA* terms in Eq. 16. While the division will vary somewhat depending on the structure of the hydrocarbon portion of the alcohol, it is evident from Table II that secondary and tertiary hydroxyl groups cause a larger relative increase in solubility than primary hydroxyl groups. This effect is due presumably to the reduced association in the pure liquid alcohol as discussed earlier.

Returning to the overall correlation of the solubilities of the alcohols and hydrocarbons (Fig. 3), it can be observed that the solubilities of the C_{14}, C_{15}, and C_{16} alcohols (Compounds 53–55, Table I) lie below the predicted line, indicating a higher aqueous solubility than predicted. For *n*-hexadecanol, the predicted solubility (Table I and Eq. 16) is 6.3×10^{-8} *M* while the solubility of the pure supercooled liquid is 4.6×10^{-7} *M*. Hence, the normal C_{16} alcohol is about 10-fold more soluble than expected on the basis of the extrapolation from the shorter chain alcohols (C_4–C_{12}). Aside from possible experimental error, two of the possible reasons are self-association and intramolecular coiling of the hydrocarbon chain.

Recent experimental data (26) indicate that the free energy of transfer of *n*-alkyl carboxylic acids from heptane to aqueous buffer is linear for all acids up to behenic acid ($C_{21}H_{43}COOH$), suggesting that preferential coiling does not occur in either phase. On the other hand, dimerization of fatty acid anions is known to occur in aqueous solutions (21, 27). Using the estimated dimerization constant for an uncharged dimer (assuming only dimers exist) to calculate the solubility of the 1-hexadecanol monomer gives a solubility estimate of 1.67×10^{-7} *M*. This accounts for about half of the observed difference between the experimental and predicted values.

Since the formation of higher aggregates is more likely to occur for the alcohols than the fatty acid anions, this estimated solubility of the monomer is likely to be an overestimate. While more accurate estimates cannot be made at present, it appears that self-association can account for most, if not all, of the observed deviation in the solubility of the longer chain (C_{14}, C_{15}, and C_{16}) alcohols. However, the possibility of some preferential coiling cannot be completely eliminated.

Table III—Methylene and Methyl Group Surface Areas in 1-Nonanol

CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-OH
9 8 7 6 5 4 3 2 1

Group	Exposed Surface Area
OH	59.15
CH_2 (1)	45.43
CH_2 (2)	39.80
CH_2 (3)	31.82
CH_2 (4)	31.82
CH_2 (5)	31.82
CH_2 (6)	31.82
CH_2 (7)	31.82
CH_2 (8)	42.75
CH_3 (9)	84.92
Total	431.15

It has frequently been noted that a methyl group and a methylene unit should make different contributions to the free energy of solution (28). The method of surface area calculation employed in this study allows an easy assessment of these differences. Table III presents the group contributions to the total surface area of *n*-nonanol. From this table it is clear that the exposed surface area of a terminal methyl group (84.9 Å²) is about three times that of the methylene unit (31.8 Å²). From the slope of Eq. 16, these values correspond to contributions to the free energy of solution of 810 cal/mole/methylene group and 2.16 kcal/mole/terminal methyl group. Clustered methyl groups such as in *tert*-butanol or neopentanol have somewhat lower surface areas than a terminal group. It may also be noted in Table III that not all of the methylene units are identical.

While this study was limited to those alcohols showing limited miscibility with water, the results can be extrapolated to the miscible alcohols. Table IV presents the experimental and theoretically calculated activity coefficients for the miscible as well as some immiscible alcohols studied. The agreement is good, with a general tendency to overestimate the activity coefficient. However, particularly for the lower molecular weight alcohols, the surface area approach must be viewed as only providing good estimates of the free energy of solution since, as discussed previously, a direct application of the interfacial approach fails to give good estimates of the enthalpy and entropy of solution.

SUMMARY

The aqueous solubilities of 73 aliphatic alcohols and hydrocarbons can be correlated with a simple linear equation involving the functional group surface areas, and the coefficients of each term

Table IV—Experimental and Predicted Activity Coefficients of Alcohols at Infinite Dilution

Alcohol	Activity Coefficients, *M*	
	Experimental[a]	Predicted
Methanol	3.42×10^{-2}	1.75×10^{-2}
Ethanol	8.54×10^{-2}	7.89×10^{-2}
Propanol	0.250	0.310
Isopropanol	0.157	0.252
n-Butanol	0.923	1.22
2-Butanol	0.500	0.668
tert-Butanol	0.237	0.550
n-Pentanol	3.12	4.78
2-Pentanol	2.05	2.62
n-Octanol	1.98×10^{2}	2.90×10^{2}
2-Octanol	1.35×10^{2}	1.56×10^{2}
n-Decanol	3.78×10^{3}	4.46×10^{3}

[a] R. A. Pierotti, C. H. Deal, and E. L. Derr, *Ind. Eng. Chem.*, **51**, 95 (1959), and supplementary tables. Mole fraction activity coefficients were converted to molar activity coefficients using partial molar volumes calculated from Ref. 30.

can be related to other physical–chemical data. The analysis indicates that the hydroxyl group contribution to the solubility is variable and depends significantly on the properties of the pure liquid alcohols. Aliphatic alcohols of varying structure are conveniently handled without the need to introduce additional parameters for branching, cyclization, *etc.* The method appears to be general enough to be extended to other functional groups as well as more complex organic molecules with limited solubility in water.

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* Pharmacy Research, The Upjohn Co., Kalamazoo, MI 49001

‡ Undergraduate Research Assistant, School of Pharmacy, University of Wisconsin, Madison, WI 53706

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Vanadyl Complexes of Antiamebic Drugs: Electronic Absorption Spectra

R. ABU-EITTAH ˣ, M. M. SEIF EL-NASR, and M. M. AMER

Abstract □ The complexing properties of the antiamebic drugs iodochlorhydroxyquin and diiodohydroxyquin with vanadium as VO^{+2} were investigated using spectrophotometric methods. The composition of the complexes and their stability constants were determined by more than one procedure. When ethanol was the solvent and the pH of the solution was 2.0–6.0, the 1:2 complexes were predominant.

Keyphrases □ Iodochlorhydroxyquin—complexing properties, electronic absorption spectra of vanadyl complexes □ Diiodohydroxyquin—complexing properties, electronic absorption spectra of vanadyl complexes □ Vanadyl complexes—iodochlorhydroxyquin and diiodohydroxyquin, complexing properties investigated using electronic absorption spectra □ Complexes—vanadium with iodochlorhydroxyquin and diiodohydroxyquin, complexing properties investigated using electronic absorption spectra

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Quinoline derivatives are strong chelating agents. At pH 2.5, the 1:2 iron (III)–chiniofon was obtained with log K_f 7.5 (5). The same ligand forms different complexes with Co^{+2}, Ni^{+2}, Mo^{+4} (6, 7), and V^{+5}

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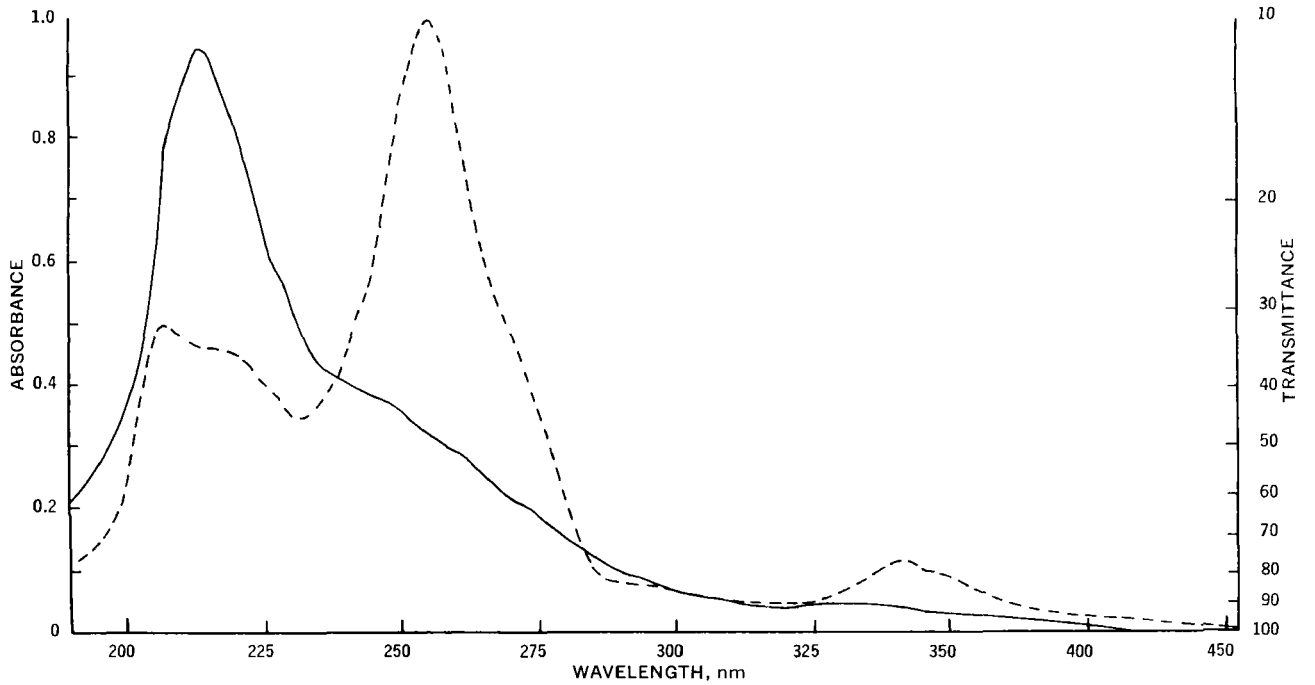


Figure 1—*Electronic absorption spectra of vanadyl sulfate* (——) *and iodochlorhydroxyquin* (– – –) *in ethanol.*

With Mo^{+4}, a yellow complex was obtained and extracted quantitatively with cyclohexane. Complexation of some substituted 8-hydroxyquinolines with transition metal ions has been investigated using different methods (8–15).

Vanadium as VO^{+2} was determined spectrophotometrically using ferron (chiniofon) (16). Vanadium ions are thought to have toxic effects, and 8-hydroxyquinolines have tuberculostatic, antibacterial, and fungitoxic effects. In this work the complexing properties of VO^{+2} with some 8-hydroxyquinolines were investigated using 96% ethanol as a solvent. Vanadyl quinolates may have tuberculostatic and fungitoxic activities as have copper quinolates (17, 18).

EXPERIMENTAL

Materials—Iodochlorhydroxyquin[1] (5-chloro-7-iodo-8-quinolinol) and diiodohydroxyquin[1] (5,7-diiodo-8-quinolinol) were purified by repetitive crystallization from purified ethanol. Vanadyl sulfate[2] solution was prepared in 96% ethanol[2] and standardized potentiometrically (19).

Apparatus and Method—Electronic absorption spectra were scanned[3] using 1.0-cm fused silica cells. To determine the composition and stability constant of the complexes, solutions of the metal ion and ligands (in 96% ethanol) were mixed just before scanning the spectra.

In the slope ratio method, two sets of experiments were carried out. In the first, the concentration of VO^{+2} was kept constant at 1.0×10^{-3} *M* and in excess of the iodochlorhydroxyquin concentration, which was varied from 4.0×10^{-5} to 1.0×10^{-4} *M*. In the second set the iodochlorhydroxyquin concentration was kept constant at 1.0×10^{-3} *M* and in excess of that of vanadyl sulfate, which was varied from 4.0×10^{-5} to 1.0×10^{-4} *M*. The spectra of the two sets of solutions were scanned. In the molar ratio method, the [vanadyl]–[reagent] ratios varied from 1:1 to 1:10.

The acid dissociation constant of iodochlorhydroxyquin was determined potentiometrically by titrating 2×10^{-3} *M* solution (in ethanol) with 2×10^{-2} *M* alcoholic potassium hydroxide (20).

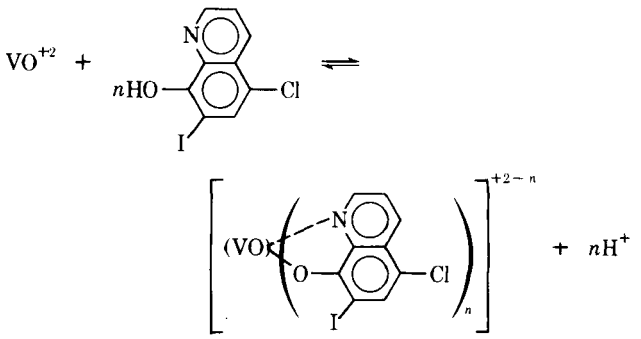
The electronic absorption spectra of the precipitated complexes were scanned in 96% ethanol and found to be similar to those obtained on mixing ligands–vanadyl sulfate solutions. IR spectra[4] of the dried precipitated complexes were investigated.

The solution pH was adjusted using Britton (21) and Robinson modified universal buffer and was measured on a precision pH meter[5], using the millivolt scale and calculating the corresponding pH values.

RESULTS AND DISCUSSION

Iodochlorhydroxyquin–Vanadium (IV) Complexes—*Absorption Spectra in Ethanol*—The spectrum of vanadyl sulfate ($VOSO_4$) (Fig. 1) shows a shoulder at ≃250 nm and an intense band at 215 nm. Iodochlorhydroxyquin shows no absorbance in the visible region (Fig. 1) but has some bands in the UV region (λ_{max} at 340, 255, and less than 220 nm). Dilute solutions of iodochlorhydroxyquin are pale yellow, and those of vanadyl sulfate are blue. When both are mixed, a yellow to red color is obtained and a new band appears in the spectra of the mixture (λ_{max} 410 nm) in addition to a long wavelength shoulder in the region of 500 nm (Fig. 2).

The reaction between iodochlorhydroxyquin and vanadyl ion possibly proceeds as shown in Scheme I. To investigate the nature



Scheme I

[1] RP grade reagents, obtained from Rhone-Poulenc.
[2] Prolabo grade reagent.
[3] Unicam SP8000 spectrophotometer.
[4] As KBr pellets with a Spektromom 2000.
[5] Radelkis type OP-205.

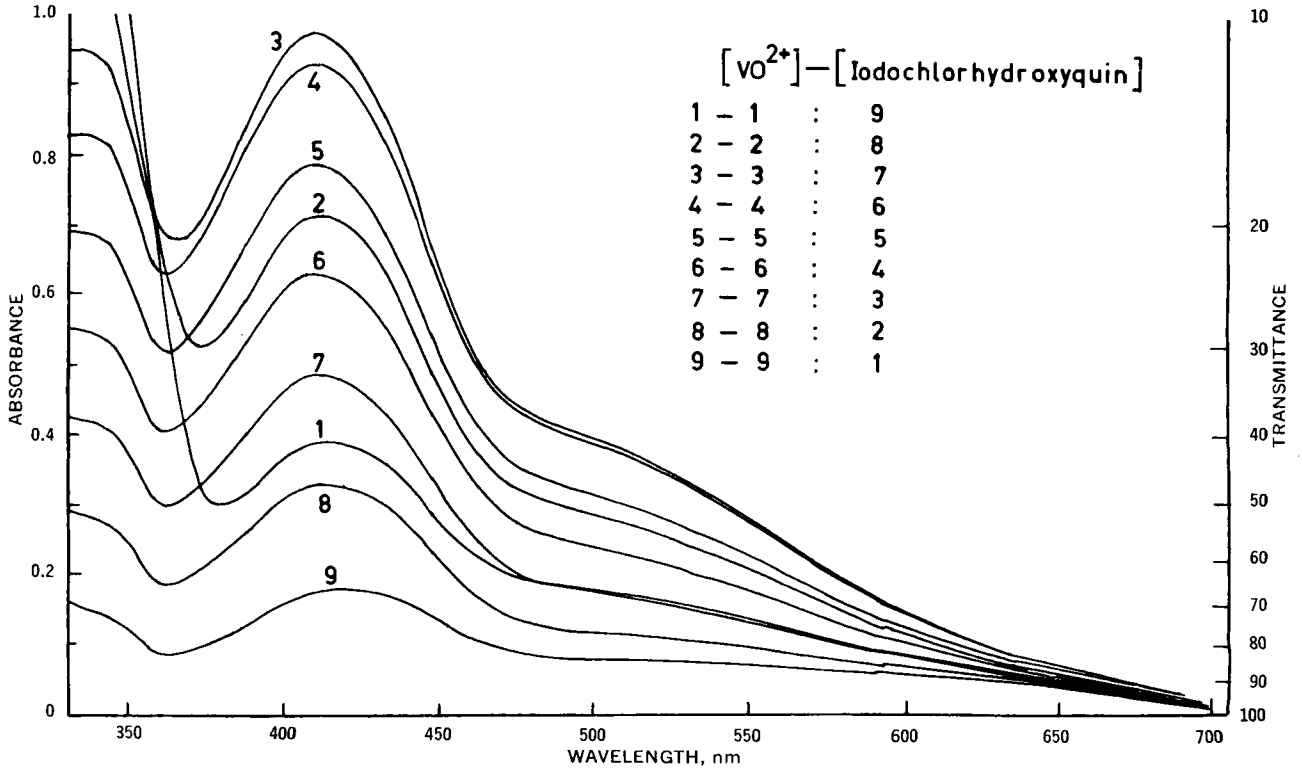


Figure 2—*Electronic absorption spectra of vanadyl sulfate–iodochlorhydroxyquin solutions of constant total concentration.*

of the chelate, Job's (22) method was followed. The spectra of vanadyl sulfate–iodochlorhydroxyquin solutions of total constant concentration were scanned (Fig. 2). The intensity of the 410-nm band was dependent on both vanadyl and ligand concentrations. The absorbance at 410 nm was plotted *versus* the mole fraction of the ligand (Fig. 3), and the results indicate that the predominant complex is the 1:2 vanadyl sulfate–iodochlorhydroxyquin chelate.

Job's (22) method also was carried out at different pH values (Fig. 4); a small shift of the complex band maximum and an increase in its intensity appeared. The results (Fig. 4) indicate that the 1:2 complex is predominant within the pH range of 2.0–6.0.

To confirm these results, the Harvey and Manning (23) slope ratio method was used to determine the composition of the iodochlorhydroxyquin–vanadyl complex (Table I).

Plots of absorbance *versus* the concentration of the variable gave straight lines (Fig. 5*a*) with slope ratios of 1:2.1, indicating that the predominant complex at the concentrations used is 1:2. The mole ratio (24) method was also used to confirm the composi-

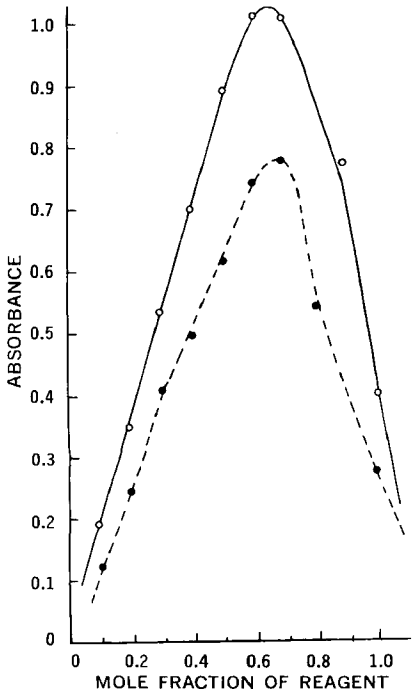


Figure 3—*Application of the continuous variation method to vanadyl sulfate complexes. Key: ○, reagent is diiodohydroxyquin; and ●, reagent is iodochlorhydroxyquin.*

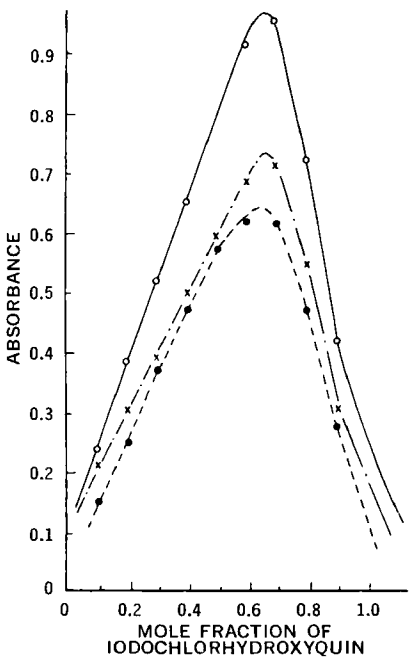


Figure 4—*Application of the continuous variation method to vanadyl sulfate–iodochlorhydroxyquin solutions at different pH values. Key: ○, pH 6.0, λ_{max} 395 nm; ×, pH 4.1, λ_{max} 398 nm; and ●, pH 2.6, λ_{max} 400 nm.*

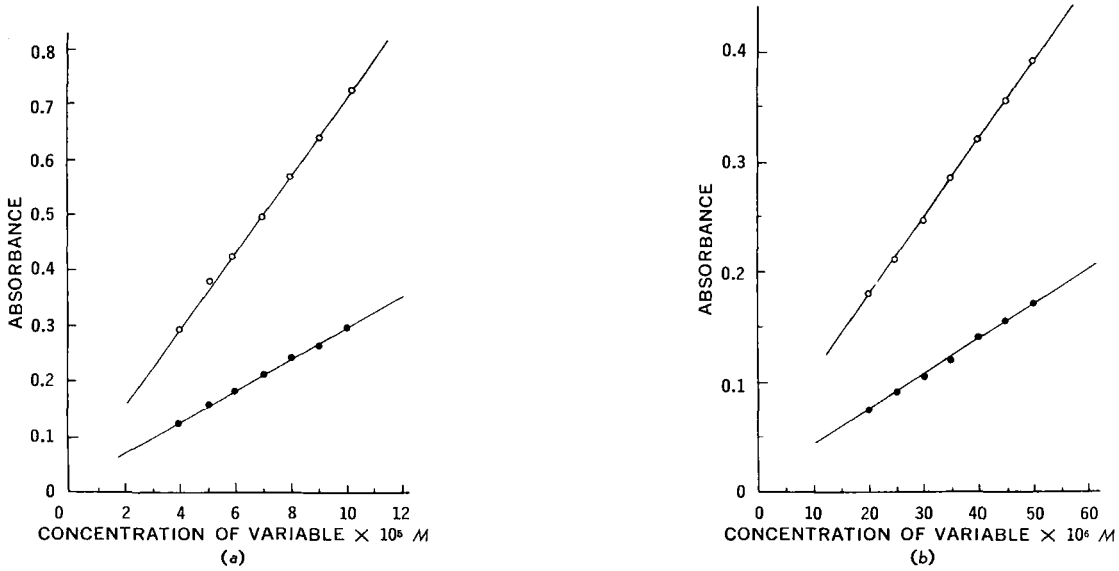


Figure 5—*Application of the slope ratio method to:* (a) *vanadyl sulfate–iodochlorhydroxyquin system and* (b) *vanadyl sulfate–diiodohydroxyquin system. Key* (a): ○, *[iodochlorhydroxyquin] is constant; and* ●, *[VO^{+2}] is constant. Key* (b): ○, *[diiodohydroxyquin] is constant; and* ●, *[VO^{+2}] is constant.*

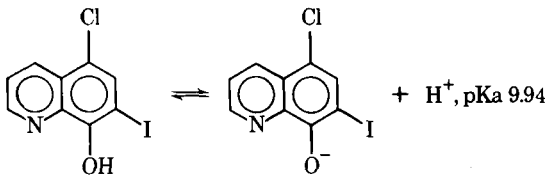
tion of the complex. As Fig. 6 indicates, the predominant complex is the 1:2.

Stability Constant of Vanadyl–Iodochlorhydroxyquin Complexes—The acid dissociation constant of the reagent was determined potentiometrically (Table II) and calculated from the equation:

$$\text{pH} = \text{pKa} + \log\frac{[\text{L}^-]}{[\text{HL}]} \qquad \text{(Eq. 1)}$$

The average value of pKa was 9.94.

The reaction between vanadyl ion and iodochlorhydroxyquin was investigated in the pH range of 1.5–2.0. In this range, concentration of the quinolate ion *(a)* is negligible (Scheme II), meaning that the complexing species is probably the drug iodochlorhydroxyquin itself and not its anion. Similar results were obtained in the kinetic study of complexing Ni^{+2} with 8-hydroxyquinoline and 8-hydroxyquinoline-5-sulfonate (25). Attempts to calculate the free ligand concentration $[L^-]$ and hence the ligand number $\bar{n}$, using spectrophotometric methods, were unsuccessful. As a result, approximate methods were used to calculate the stability constant of the 1:2 vanadyl–iodochlorhydroxyquin complex (23).



(a)

Scheme II

Consider the equilibrium in Scheme III:

$$[\text{VO(R)}_n]^{+2} \rightleftharpoons \text{VO}^{+2} + nR$$

Scheme III

If the degree of dissociation is α and the concentration of the complex is C, then the instability constant is given by:

$$K' = (\alpha C)(n\alpha C)^n/C(1-\alpha) \qquad \text{(Eq. 2)}$$

The value of n is 2, and C will be equal to the initial concentration of vanadyl sulfate. The value of α is given by:

$$\alpha = A_m - A_s/A_m \qquad \text{(Eq. 3)}$$

where A_m is the absorbance when all the vanadyl present is complexed and A_s is the absorbance at the stoichiometric molar ratio of R to vanadyl ion in the complex (R = ligand).

The absorption spectra of solutions containing 1:1 to 1:30 vanadyl–ligand ratios were scanned. In Fig. 7*a*, absorbance is plotted against the number of moles of iodochlorhydroxyquin per mole of vanadyl sulfate for different vanadyl concentrations. From Fig. 7*a*, α was computed; and using Eq. 2, K' (instability constant) and K (stability constant) were computed. The average value of the stability constant for the complex was 1.1×10^9, giving $\log \beta_2 = 9.04$. Other attempts to obtain an estimate of the overall stability constant β_2 were tried.

From Fig. 7*a*, the molar absorptivity of the complex ϵ_2 was computed. At the maximum absorbance the concentration of the complex was assumed to be that of the total metal (vanadyl sulfate). From the values of ϵ_2 and absorbances, the concentrations of the complex were computed; by knowing the total metal and ligand concentration, β_2 was calculated (Table III).

IR Spectra—IR spectra of the precipitated complexes were compared with those of the free ligands (Fig. 8). The stretching —OH band of diiodohydroxyquin appeared as a relatively broad band in the region of 3000 cm^{-1}, indicating strong hydrogen bonding. On complexation, the band was further broadened and red shifted. In the region of 1600 cm^{-1}, the band's characteristics of the C=N group were shifted on complexation. The same observation was made when iodochlorhydroxyquin was the ligand.

Diiodohydroxyquin–Vanadyl Complexes—To confirm these

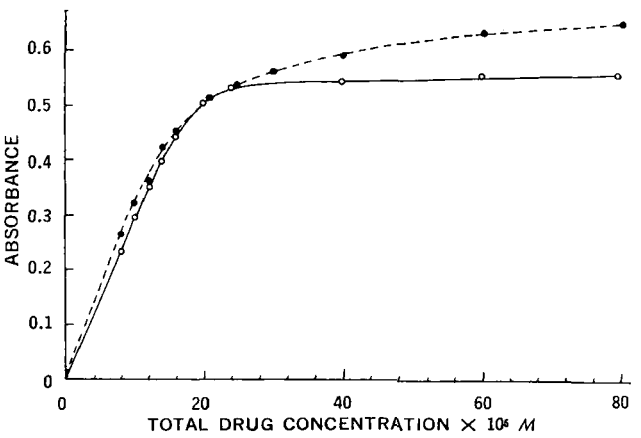


Figure 6—*Absorbance as a function of drug concentration at a constant vanadyl-ion concentration. Key:* ○, *using iodochlorhydroxyquin; and* ●, *using diiodohydroxyquin.*

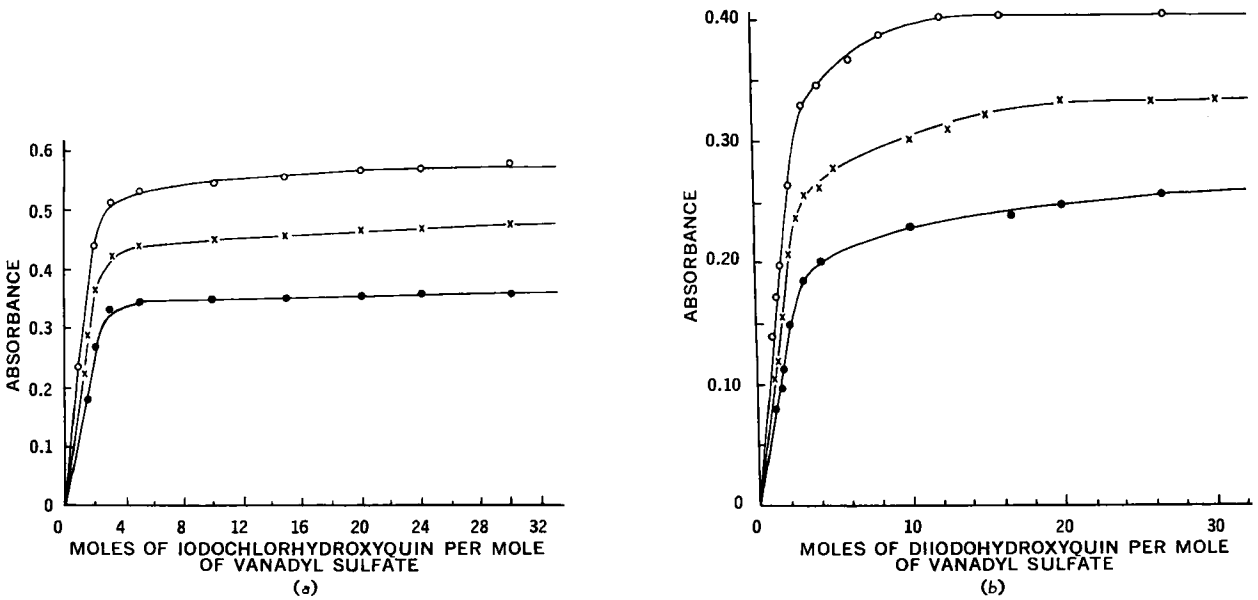


Figure 7—(a) *Variation of absorbance of vanadyl sulfate–iodochlorhydroxyquin with iodochlorhydroxyquin concentration. Key:* ○, $[VO^{+2}] = 7.5 \times 10^{-5}$ M; ×, $[VO^{+2}] = 6.25 \times 10^{-5}$ M; *and* ●, $[VO^{+2}] = 5.0 \times 10^{-5}$ M. (b) *Variation of absorbance of vanadyl sulfate–diiodohydroxyquin with diiodohydroxyquin concentration. Key:* ○, $[VO^{+2}] = 5 \times 10^{-5}$ M; ×, $[VO^{+2}] = 4 \times 10^{-5}$ M; *and* ●, $[VO^{+2}] = 3 \times 10^{-5}$ M.

results, the complexing properties of diiodohydroxyquin with vanadyl ion were investigated. The procedure used previously was adopted. The electronic structure of the two ligands is similar, yet differences in the electronegativities of iodine and chlorine may have some effects on the chelating properties of the drug.

The acid dissociation constant of diiodohydroxyquin was determined in ethanol, and an average value of 10.15 was obtained for pKa. This means that at pH 1.5-2.0 the concentration of the quinolate ion is negligible and that the ligand is the drug itself. Job's (22) method (Fig. 3) indicates that the 1:2 vanadyl–diiodohydroxyquin chelate is obtained. The complex obtained has an absorption band of its own with λ_{max} 414 nm. The slope ratio (Fig. 5*b*) and mole ratio (Fig. 6) methods indicate also that the 1:2 complex predominates.

The instability constant (*K'*) of the 1:2 vanadyl–diiodohydroxyquin complex was computed as before. Data needed for this calculation were taken from a plot (Fig. 7*b*) of absorbance *versus* the number of moles of diiodohydroxyquin per mole of vanadyl sulfate. The computed values of *K'* were used to calculate β_2, and an average value of 1.8×10^9 was obtained ($\log \beta_2 = 9.25$) (Table III).

CONCLUSION

These results indicate that iodochlorhydroxyquin and diiodohy-

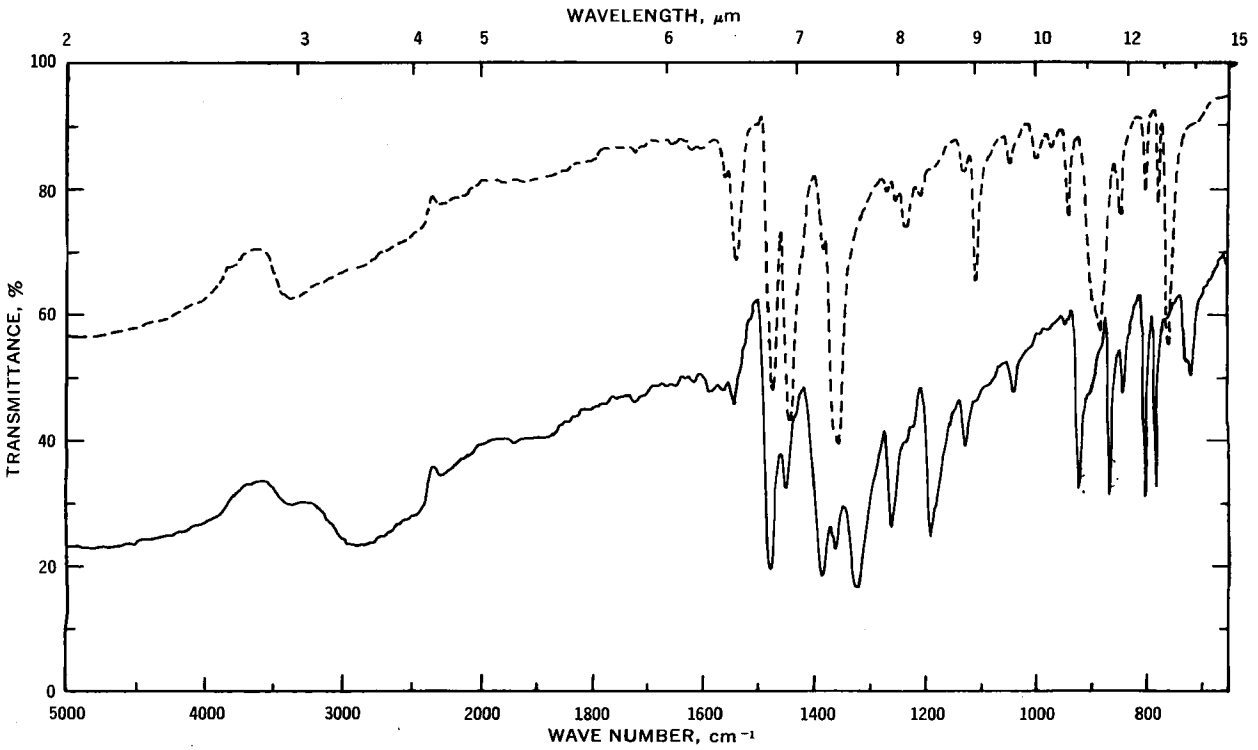


Figure 8—*IR spectra of diiodohydroxyquin* (——) *and diiodohydroxyquin–vanadyl sulfate* (– – –) *complex.*

Table I—Variation of Absorbance at 410 nm with Concentration of Iodochlorhydroxyquin and Vanadyl Sulfate

$[VO^{+2}] = 1.0 \times 10^{-3}$ M		Iodochlorhydroxyquin $= 1.0 \times 10^{-3}$ M	
[Iodochlorhydroxyquin] $\times 10^5$	Absorbance (410 nm)	$[VO^{+2}] \times 10^5$	Absorbance (410 nm)
4.0	0.125	4.0	0.295
5.0	0.155	5.0	0.385
6.0	0.185	6.0	0.425
7.0	0.215	7.0	0.495
8.0	0.245	8.0	0.570
9.0	0.265	9.0	0.640
10.0	0.295	10.0	0.735

Table II—Potentiometric Titration of Iodochlorhydroxyquin

Alcoholic Potassium Hydroxide, ml	pH	[HL], mg	$[L^-]$, mg	$\log \frac{[HL]}{[L^-]}$	pKa
0.5	9.046	27.335	3.255	0.9242	9.9702
1.0	9.380	24.080	6.510	0.5680	9.9480
1.5	9.580	20.830	9.760	0.3293	9.9093
2.0	9.780	17.570	13.020	0.1302	9.9102
2.5	9.930	14.315	16.275	−0.0557	9.9743
3.1	10.210	10.400	20.190	−0.2881	9.9219
3.5	10.310	7.805	22.785	−0.4651	9.9449
4.0	10.560	4.550	26.040	−0.7577	9.8023[a]

[a] Neglected.

Table III—Values Obtained for the Log β_2 at Different Metal and Ligand Concentrations

Total $[VO^{+2}] \times 10^5$ M	Total Iodochlorhydroxyquin Concentration $\times 10^5$ M	Log β_2	Total $[VO^{+2}] \times 10^5$ M	Total Diiodohydroxyquin Concentration $\times 10^5$ M	Log β_2
7.0	35	8.104	1.0	10	8.669
8.0	40	7.954	2.0	20	8.608
9.0	45	7.913	3.0	30	8.624

droxyquin form strong complexes with vanadyl ion, even in distinct acid media. The predominant complexes were the 1:2 complexes. The complex of diiodohydroxyquin is slightly more stable and has a band maximum at a longer wavelength than iodochlorhydroxyquin. These differences are attributed to the differences in electronegativities of chlorine and iodine. Since the complexes were obtained in an acid medium (pH 1.5–2.0), the chelating agent is probably the drug itself and not its anion.

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[x] To whom inquiries should be directed. Present address: Chemistry Department, Faculty of Science, Kuwait University, Kuwait.

Constituents of *Cannabis sativa* L. VII: Use of Silyl Derivatives in Routine Analysis

CARLTON E. TURNER ˣ, KATHY W. HADLEY, JANIS HENRY, and M. LEONARD MOLE

Abstract □ Naturally occurring cannabinoids previously impossible to separate and analyze were quantitated on a routine basis using silylation. Relative retention times of many silylated cannabinoids are reported for the first time.

Keyphrases □ Cannabinoids—silylation and GLC separation □ *Cannabis sativa* L.—silylation and GLC separation of cannabinoids □ GLC—analysis, *C. sativa* constituents

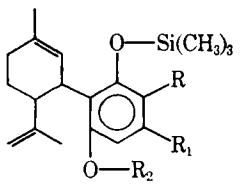
Claussen *et al.* (1) used trimethylsilyl derivatives to separate some cannabinoids from their corresponding carboxylic acid analogs. However, at the time of this report, isolation of cannabinoids with subsequent identification by synthesis and combined GLC–mass spectrometry was in an embryonic state. Thus, Claussen *et al.* (1) were unable to identify many peaks in the chromatogram.

Later, Heaysman *et al.* (2) examined the constituents of *Cannabis* using silyl derivatives, and they reported retention times for the trimethylsilyl ethers of cannabidiol, (−)-Δ^9-*trans*-tetrahydrocannabinol, and cannabinol. Caddy and Fish (3) compared the GLC separation obtained for underivatized cannabidiol, (−)-Δ^9-*trans*-tetrahydrocannabinol, and cannabinol with their trimethylsilyl and trifluoroacetyl derivatives. Other investigators (4, 5) described a trimethylsilyl method for the analysis of cannabidiol, (−)-Δ^9-*trans*-tetrahydrocannabinol, and (−)-Δ^9-*trans*-tetrahydrocannabinolic acid.

Paris and Paris (6) reported the identification of cannabidiolic acid as its trimethylsilyl ester–ether derivative in a sample of hashish, and the use of silyl derivatives to obtain a clear and discrete separation of synthetic cannabidiol and cannabichromene was reported (7); separation of the bis(trimethylsilyl) ether of synthetic cannabidiol from the mono(trimethylsilyl) ether was also obtained. The use of trimethylsilyl derivatives to separate the equatorial and axial isomers of synthetic hexahydrocannabinol was also reported (8).

Therefore, the potential application of trimethylsilyl derivatives in routine separation of naturally occurring cannabinoids not conveniently separated by other means (7) and the need for a routine and practical method of catholic use for assaying acid derivatives of naturally occurring cannabinoids (I–XVIII) with pentyl and propyl side chains prompted this investigation.

The investigation of methyl homologs (9), which also exist in fresh *Cannabis* plant material as their corresponding acid derivatives, and other trace cannabinoids not routinely found in *Cannabis sativa* L. plant material will not be discussed.



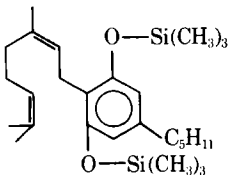
number	cannabinoid	R	R_1	R_2
I	cannabidivarin	H	C_3H_7	$-Si(CH_3)_3$
II	cannabidivarin	H	C_3H_7	H
III	cannabidiol	H	C_5H_{11}	$—Si(CH_3)_3$
VII	cannabidiol	H	C_5H_{11}	H
VIII	cannabidivarinic acid	$CO_2Si(CH_3)_3$	C_3H_7	$—Si(CH_3)_3$
XIV	cannabidiolic acid	$CO_2Si(CH_3)_3$	C_5H_{11}	$—Si(CH_3)_3$

EXPERIMENTAL

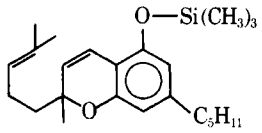
Samples used were grown in Mississippi from seed obtained through the National Institute on Drug Abuse (NIDA), the United Nations, and the U.S. Department of Justice Drug Enforcement Administration (DEA) and from researchers throughout the world. Plant material was grown in 1972[1]. All plant samples were from aboveground plant parts.

Routine GLC Analyses—Three 1-g samples were extracted simultaneously with 40 ml of spectrograde chloroform. Resulting solutions were allowed to stand at room temperature for 1 hr. During the hour, each sample was shaken for approximately 15 sec at 20-min intervals. The plant material was then removed by filtration and the mother liquor was concentrated *in vacuo* at ambient temperature to a greenish paste void of solvent.

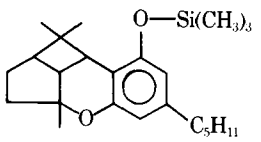
At this point, 1.5 ml of an ethanolic solution containing 10 mg/ml of androst-4-ene-3,17-dione was added as the internal standard. Continuous vibration from an ultrasonic vibrator was then applied until all resin was in solution. Usually this 15 mg of solution of the internal standard was adequate. Routinely, 0.2 μl of the resulting solution was injected with 0.2 μl of ethanol as a flush solvent. In these laboratories, this method provides excellent results.



XIII: bis(trimethylsilyl)ether of cannabigerol

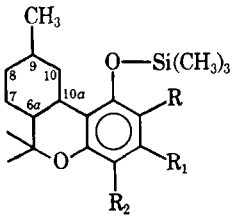


VI: trimethylsilyl ether of cannabichromene



X: trimethylsilyl ether of cannabicyclol

[1] *Cannabis* herbarium specimens are stored in the Herbarium, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677



number	cannabinoid	other	R	R_1	R_2
IV	(−)-Δ^9-*trans*-tetrahydrocannabivarin	$\Delta^{9,10}$	H	C_3H_7	H
XVI	(−)-Δ^9-*trans*-tetrahydrocannabivarinic acid	$\Delta^{9,10}$	$CO_2Si(CH_3)_3$	C_3H_7	H
IX	(−)-Δ^8-*trans*-tetrahydrocannabinol	$\Delta^{8,9}$	H	C_5H_{11}	H
XI	(−)-Δ^9-*trans*-tetrahydrocannabinol	$\Delta^{9,10}$	H	C_5H_{11}	H
XVII	(−)-Δ^9-*trans*-tetrahydrocannabinolic acid A	$\Delta^{9,10}$	$CO_2Si(CH_3)_3$	C_5H_{11}	H
XVIII	(−)-Δ^9-*trans*-tetrahydrocannabinolic acid B	$\Delta^{9,10}$	H	C_5H_{11}	$CO_2Si(CH_3)_3$
V	hexahydrocannabinol (C_9 methyl axial)	—	H	C_5H_{11}	H
XII	hexahydrocannabinol (C_9 methyl equatorial)	—	H	C_5H_{11}	H
XV	cannabinol	$\Delta^{6a,7}$ $\Delta^{8,9}$ $\Delta^{10,10a}$	H	C_5H_{11}	H

Analyses were performed using gas chromatographs[2] equipped with hydrogen flame-ionization detectors and operated isothermally at 210°. Inlet and detector temperatures were 240 and 260°, respectively. Glass columns, 0.64 cm (0.25 in.) o.d. and 2 mm i.d. × 2.43 m (8 ft), were packed with 2% OV-17 (high purity polar methyl silicone; approximately 30,000 mol. wt.) on 100–120-mesh Chromosorb WHP. Nitrogen was used as the carrier gas at a flow rate of 10–30 ml/min, depending upon separation and instrument requirements. Usually the head pressure was between 26 and 40 psi.

Peak area measurements were made with a computer[3]. The peak area, measured in millivolts, was compared with the peak area of the internal standard. Relative response factors obtained from synthetic and natural cannabinoids were prerequisites for reproducibility and accuracy.

Silylation of Plant Extract—Plant material was processed as described for the routine GLC analyses. After the greenish paste void of solvent was obtained, 1.5 ml of anhydrous pyridine containing 10 mg/ml of androst-4-ene-3,17-dione was added. Continuous vibration from an ultrasonic vibrator was then carried out until all resin was in solution. At this point, 0.5 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane[4] was added.

The reaction mixture was heated, using a heating mantle, for approximately 10 min at 80°. Then 0.2 μl of the reaction mixture was routinely injected. Decarboxylation of cannabinoid acids was accomplished by heating the plant material extracts at 110° for 1.5 hr prior to silylation. A positive nitrogen head pressure can be used to prevent oxidation of certain cannabinoids. However, the nitrogen head pressure is not mandatory for good results when decarboxylating extracts, but it is recommended if plant material is heated prior to extraction.

GLC–mass spectrometry and TLC were used as supportive tools for identification and assignment of previously unknown peaks (10). Fragmentation patterns of silyl ester–ether derivatives of cannabinoids and electrovoltage–mass fragment intensity graphs (9) will be presented elsewhere.

RESULTS AND DISCUSSION

Betts and Holloway (11) reported the formation of trimethylsilyl ethers of cannabinoids by an overnight reaction of hexamethyldisilazane and a drop of trimethylchlorosilane. They observed that cannabidiol reacted more slowly than the other cannabinoids; thus, the long reaction time reported by this research group allowed sufficient time for cannabidiol to react and for trace amounts of ammonium chloride formed as a by-product to precipitate.

Claussen *et al.* (1) also preferred overnight reactions, whereas Makita and Wells (12) and Heaysman *et al.* (2) used a shorter reaction time. These investigators employed hexamethyldisilazane and trimethylchlorosilane with a solvent such as anhydrous pyridine or anhydrous isopropylamine. Claussen *et al.* (1) used the trimethylsilyl derivative of cannabidiol as an internal standard, whereas Heaysman *et al.* (2) used anthracene, which has a retention time between silylated cannabidiol and Δ^9-tetrahydrocannabinol. Betts and Holloway (11) used the hydrocarbon *n*-eicosane as an internal standard.

Davis *et al.* (5) first reported the use of androst-4-ene-3,17-

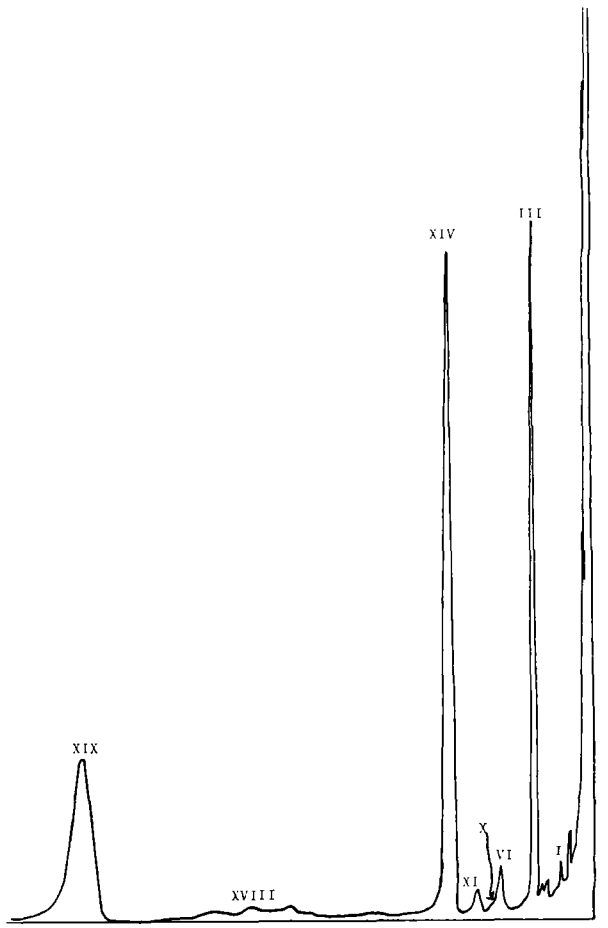


Figure 1—*Gas chromatogram of silylated male Turkish (TU-A) C. sativa L. showing cannabidivarin disilylated (I), cannabidiol disilylated (III), cannabichromene (VI), cannabicyclol (X), (−)-Δ^9-trans-tetrahydrocannabinol (XI), cannabidiolic acid trisilylated ester–ether (XIV), (−)-Δ^9-trans-tetrahydrocannabinolic acid B disilylated ester–ether (XVIII), and androst-4-ene-3,17-dione (XIX), the internal standard.*

[2] Beckman GC-45, GC-72-5, and GC-65.
[3] Digital, PDP-8.
[4] BSTFA with 1% TMCS, Pierce Chemical Co. or Regis Chemical Co.

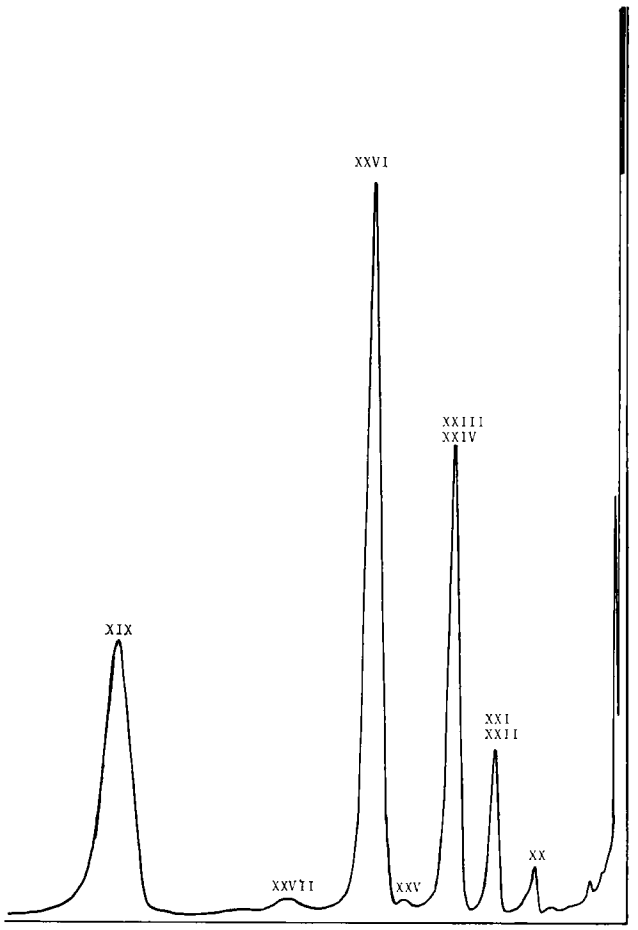


Figure 2—*An OV-17 chromatogram of underivatized female Afghanistan* Cannabis *[AF-B(1)/C-71] showing cannabidivarin (XX), (−)-Δ⁹-trans-tetrahydrocannabivarin (XXI), cannabicyclol (XXII), cannabichromene (XXIII), cannabidiol (XXIV), (−)-Δ⁸-trans-tetrahydrocannabinol (XXV), (−)-Δ⁹-trans-tetrahydrocannabinol (XXVI), cannabinol (XXVII), and androst-4-ene-3,17-dione (XIX).*

dione as an internal standard. Subsequently, this standard has gained in popularity since it can be used in routine quantitation of cannabinoids as well as quantitation provided by the trimethylsilyl method. Other investigators (4, 5) also used androst-4-ene-3,17-dione. Silylation was accomplished by these groups with bis-*N,O*-(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane in anhydrous pyridine.

When using this method to quantitate cannabidiol and cannabichromene, it was found that cannabichromene was present in all variants examined and that, as reported (7), cannabidiol was absent in some variants. For example, Fig. 1 of Turkish (TU-A) *Cannabis* shows a peak (VI) for cannabichromene. Although Fig. 1 is a chromatogram of silylated fresh plant material, when decarboxylation was carried out and assignments of the peaks were made using instrumentation as described in the *Experimental* section, cannabichromene accounted for 4.8% of the peak classically labeled cannabidiol when a routine analysis was performed using an OV-17 column.

Figure 2, a classical chromatogram of Afghanistan *Cannabis* AF-B(1)/C-71, shows the separation obtained using OV-17 columns with underivatized cannabinoids. Cannabidiol and cannabichromene are under one peak and are routinely reported as cannabidiol. In this plant sample [AF-B(1)/C-71], when using decarboxylated and silylated plant material, it was ascertained that the peak labeled cannabichromene and cannabidiol in Fig. 2 contains 81.2% cannabichromene and 18.8% cannabidiol. Figure 3 of decarboxylated and silylated AF-B(1)/C-71 also shows the presence of hexahydrocannabinol (C_9 methyl equatorial) (XII) and a slight amount of the C_9 axial isomer; the latter is not labeled but it is the shoulder preceding cannabichromene (VI).

The presence of hexahydrocannabinol in a plant sample stored at 50° for 2 years was postulated by Turner *et al.* (8). Since this particular sample was heated at 110° for longer than 1.5 hr and since no hexahydrocannabinol is observed in Fig. 2, which is a routine analysis of AF-B(1)/C-71, these data support the previous postulation (8) that hexahydrocannabinol can be formed in plant material by heating. Hexahydrocannabinol (C_9 methyl equatorial) probably would not be detected by an untrained individual, since its relative retention time is near that of silylated cannabigerol (XIII). However, by observing the routine chromatogram of AF-B(1)/C-71 (Fig. 2) and then Fig. 4, it is evident that cannabigerol is not present in this plant sample.

Figure 5, a chromatogram of an Indian variant (IN-F) which was decarboxylated and silylated, shows a large cannabicyclol peak (X). This was common in certain samples containing significant amounts of cannabichromene. Rather uniquely, however, some samples containing large amounts of cannabichromene do not, when silylated, show significant amounts of cannabicyclol. Cannabicyclol, on the other hand, does always appear under the peak routinely labeled (−)-Δ⁹-*trans*-tetrahydrocannabivarin in underivatized analyses of plant materials containing cannabichromene (Fig. 2). Moreover, it is always present when synthetic cannabichromene is subjected to GLC analyses. This points to an unex-

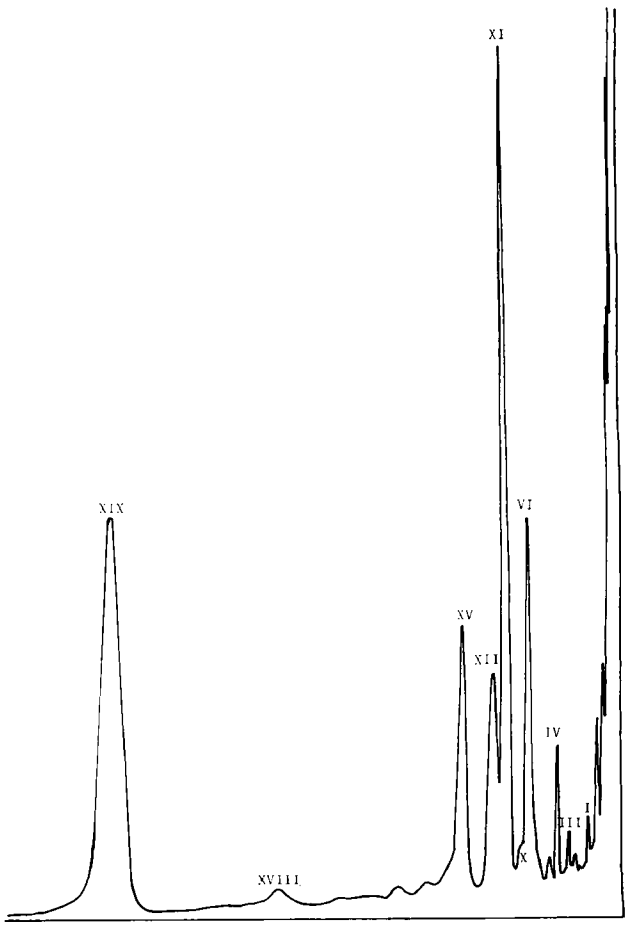


Figure 3—*Chromatogram of decarboxylated and silylated female Afghanistan* Cannabis *[AF-B(1)/C-71] showing cannabidivarin disilylated (I), cannabidiol disilylated (III), (−)-Δ⁹-trans-tetrahydrocannabivarin (IV), cannabichromene (VI), cannabicyclol (X), (−)-Δ⁹-trans-tetrahydrocannabinol (XI), hexahydrocannabinol (C_9 methyl equatorial) (XII), cannabinol (XV), (−)-Δ⁹-trans-tetrahydrocannabinolic acid B disilylated ester–ether (XVIII), and the internal standard (XIX). (−)-Δ⁹-trans-Tetrahydrocannabinolic acid B (XVIII), not as readily decarboxylated as is acid A, is observed here; it is under acid A (XVII) in Fig. 4.*

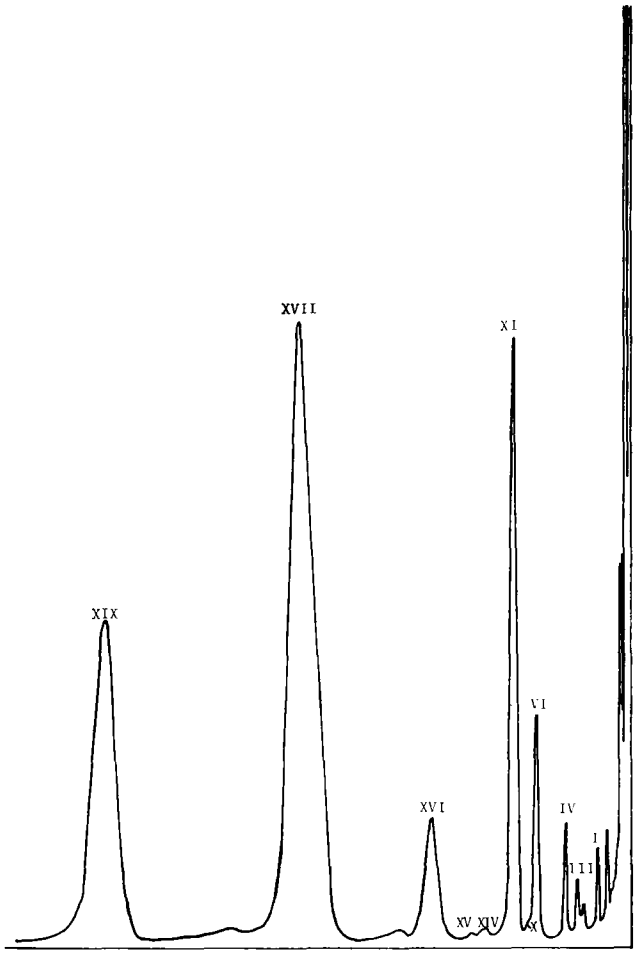


Figure 4—*Chromatogram of silylated female Afghanistan* Cannabis [*AF-B(1)/C-71*] *showing cannabidivarin disilylated* (*I*), *cannabidiol disilylated* (*III*), (−)-Δ^9-trans-*tetrahydrocannabivarin* (*IV*), *cannabichromene* (*VI*), *cannabicyclol* (*X*), (−)-Δ^9-trans-*tetrahydrocannabinol* (*XI*), *cannabidiolic acid* (*XIV*), *cannabinol* (*XV*), (−)-Δ^9-trans-*tetrahydrocannabinolic acid A* (*XVII*), *and androst-4-ene-3,17-dione* (*XIX*).

plained phenomenon: certain plant samples containing a high cannabichromene content show more cannabicyclol than others.

For example, the plant material in Fig. 3, AF-B(1)/C-71, contains only a relatively small amount of cannabicyclol, even after being heated to such extremes that hexahydrocannabinol was formed. The plant material in Fig. 5, IN-F, contains more cannabicyclol. When a normalization analysis was run on AF-B(1)/C-71 and IN-F, the percent of cannabichromene in regard to all cannabinoids present was 12.87 and 18.48%, respectively. Subsequently, cannabicyclol for AF-B(1)/C-71 and IN-F was 4.28 and 2.92%, respectively. This phenomenon was observed in many other variants.

Cyclization of cannabichromene in the injection port of the gas chromatograph is known (7). Silylation prevents any observable cyclization since a TLC-purified sample of cannabichromene can be silylated and analyzed without any cannabicyclol being formed. Thus, it seems that other undefined physical parameters must be specific in *Cannabis* of different variants. Since *C. sativa* L. from different geographical locations (13, 14) exhibits pronounced chemical and morphological differences, it is possible for many other factors to influence this observed phenomenon.

Cannabicyclol and (−)-Δ^9-*trans*-tetrahydrocannabivarin are not separated in routine underivatized analyses (Fig. 2) but can be separated using the silyl method (Figs. 4 and 5). And, as previously reported (4–6), acid derivatives of cannabinoids can be semiquantitated using a silyl procedure. In these laboratories, using the procedure described, it is possible to analyze routinely for (−)-Δ^9-*trans*-tetrahydrocannabivarinic acid and (−)-Δ^9-*trans*-tetrahydrocannabinolic acids A and B. Acid B has only been isolated and

Table I—Relative Retention Times of Trimethylsilyl Derivatives of Cannabinoids[a]

Derivative	Relative Retention Time
Cannabidivarin disilylated	0.07
Cannabidivarin monosilylated	0.11
Cannabidiol disilylated	0.11
(−)-Δ^9-*trans*-Tetrahydrocannabivarin	0.12
Hexahydrocannabinol (C_9 methyl axial)	0.16
Cannabichromene	0.17
Cannabidiol monosilylated	0.18
Cannabidivarinic acid	0.18
(−)-Δ^8-*trans*-Tetrahydrocannabinol	0.20
Cannabicyclol	0.21
(−)-Δ^9-*trans*-Tetrahydrocannabinol	0.22
Hexahydrocannabinol (C_9 methyl equatorial)	0.22
Cannabigerol disilylated	0.26
Cannabidiolic acid	0.28
Cannabinol	0.31
(−)-Δ^9-*trans*-Tetrahydrocannabivarinic acid	0.38
(−)-Δ^9-*trans*-Tetrahydrocannabinolic acid A	0.64
(−)-Δ^9-*trans*-Tetrahydrocannabinolic acid B	0.68
Androst-4-ene-3,17-dione	1.00

[a] Relative retention times were obtained from synthetic cannabinoids supplied by the National Institute on Drug Abuse. Cannabinoids not available were isolated from *Cannabis sativa* L. Peak assignments were based on data from synthetic and natural cannabinoids. A GLC–mass spectrometry system was used to confirm assignment of peaks; see Refs. 9, 10, 13, and 14 for methods and additional data.

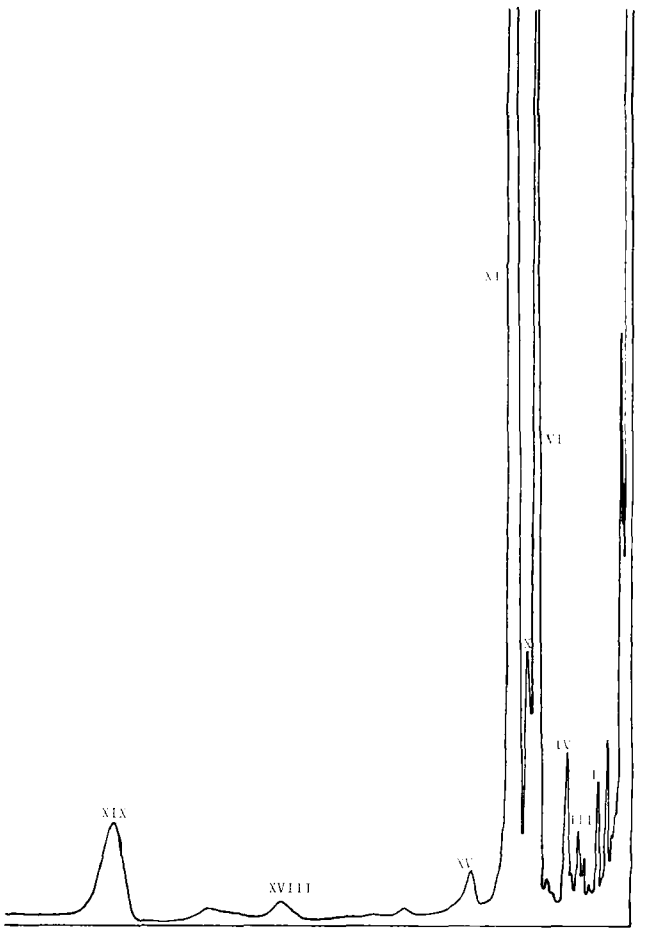


Figure 5—*Gas chromatogram of an Indian variant of* Cannabis (*IN-F*). *This decarboxylated and silylated sample shows cannabidivarin disilylated* (*I*), *cannabidiol disilylated* (*III*), (−)-Δ^9-trans-*tetrahydrocannabivarin* (*IV*), *cannabichromene* (*VI*), *cannabicyclol* (*X*), (−)-Δ^9-trans-*tetrahydrocannabinol* (*XI*), *cannabinol* (*XV*), (−)-Δ^9-trans-*tetrahydrocannabinolic acid B* (*XVIII*), *and androst-4-ene-3,17-dione* (*XIX*).

Table II—Relative Retention Times of Underivatized Cannabinoids and Other Components Found in *Cannabis*

Component	Relative Retention Time
Olivetol	0.04
Cannabidivarin	0.18
Tetrahydrocannabivarin	0.26
Cannabicyclol	0.26
Cannabichromene	0.34
Cannabivarin	0.34
Cannabidiol	0.34
Hexahydrocannabinol	0.37
Cannabigerol monomethyl ether	0.38
$\Delta^{9,11}$-Tetrahydrocannabinol (exocyclic)	0.41
(−)-Δ^{8}-*trans*-Tetrahydrocannabinol	0.44
Cannabielsoin	0.48
(−)-Δ^{9}-*trans*-Tetrahydrocannabinol	0.49
Cannabigerol	0.57
Cannabinol	0.63
C_{29}-Hydrocarbon	0.67
Androst-4-ene-3,17-dione (Δ^{4}-dione)	1.00

identified once from hashish (15). Recent work in these laboratories, however, has resulted in acid B being isolated from fresh *Cannabis* (16). These findings will be the subject of a forthcoming report. Additionally, it is possible to analyze for cannabidivarinic acid and cannabidiolic acid. Cannabichromene acid is not consistently observed in *Cannabis*, even when a large content of cannabichromene is confirmed by TLC, mass spectroscopy, and the silyl GLC method.

In these laboratories, quantitation of cannabinoids using the trimethylsilyl method was slightly less accurate than the underivatized procedure, since response factors of silyl cannabinoids are difficult to obtain and may vary greatly. Thus, these authors prefer to use "semiquantitative" when referring to data obtained by the silyl method. Underivatized cannabinoids in these laboratories, using good analytical techniques and proper response factors (see Table III), can be quantitated with better than 98% accuracy. Accuracy using the silyl procedure is from 93 to 95%[5].

Although the silyl method used may be semiquantitative, it affords better results than methods employing no internal standard or an external standard. Thus, for laboratories wishing to separate cannabinoids not routinely separated by conventional GLC, the silyl method is sufficient unless accuracy greater than 95% is required.

Table I shows the relative retention times of all cannabinoids routinely analyzed by these laboratories as their trimethylsilyl ethers or ester–ethers; Table II gives the relative retention times of cannabinoids as their free phenols. All relative retention times are based on the same internal standard and chromatographic conditions (7). By using these retention times, it is possible, in most cases, to identify most cannabinoids. For best results, an all-glass system is preferred. Stainless steel columns consistently give results 20–30% below the glass system in these laboratories. Presilylated stainless steel columns reduce this apparent adsorption to approximately 15%.

De Zeeuw *et al.* (17) showed that hydrocarbons are present in *Cannabis* and can possibly distort analytical data. Extraction of *Cannabis* with chloroform to date has not indicated the presence of significant amounts of hydrocarbons when the extract is silylated. A major source of error, however, in preparing silyl derivatives is exposure of the reaction mixture to rubber septums, which produces many peaks that appear to be cannabinoids. This disadvantage can be negated by proper laboratory procedures.

[5] The accuracy was obtained after correlating data from over 6500 samples analyzed in these laboratories during the calendar year of 1973. These samples included synthetic, herbal, hash, cookies, and other samples prepared by outside sources. Data obtained from silylated cannabinoids were consistently 3–5% lower than from unsilylated cannabinoids. Additionally, the 3–5% range was observed whether peaks were calculated by peak height times width at half-height or by computer. Response factors were used in all analyses.

Table III—Relative Response Factors of Silylated and Unsilylated Synthetic Cannabinoids[a]

Cannabinoid	Unsilylated	Silylated
Cannabidiol	1.07	0.68
(−)-Δ^{9}-*trans*-Tetrahydrocannabinol	1.05	0.80
Cannabinol	0.97	0.72
Androst-4-ene-3,17-dione (internal standard)	1.00	1.00

[a] Volume injected was 0.25 μl. Response factors vary with each instrument in these laboratories.

SUMMARY

The silyl procedure described can be used as a routine method for quantitating cannabinoids not quantitated by other means. Accuracy is between 93 and 95%. Cannabinoids, whether free phenols or the carboxylic acid derivatives, can be quantitated by this method.

Data generated by this method indicate that cannabichromene, previously thought to be a minor component in *Cannabis*, is more abundant than cannabidiol in many variants.

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[x] To whom inquiries should be directed.

Comparative Binding of Disopyramide Phosphate and Quinidine Sulfate to Human Plasma Proteins

YIE W. CHIEN *x, HOWARD J. LAMBERT *, and AZIZ KARIM ‡

Abstract □ Disopyramide phosphate and quinidine sulfate are pharmacologically related, but chemically unrelated, antiarrhythmic agents. Two groups of independent binding sites were observed for each drug after protein binding studies in human plasma and human serum albumin solutions. The number of binding sites per protein molecule and their corresponding binding affinities were computed. The magnitude of N_1K_1, the fraction of binding, and the relative lipophilicity are quantitatively correlated. Competitive binding studies demonstrated that these two drugs do not compete for the same binding sites on protein molecules.

Keyphrases □ Disopyramide phosphate—binding to human plasma proteins compared to quinidine sulfate □ Quinidine sulfate—binding to human plasma proteins compared to disopyramide phosphate □ Binding—comparison of disopyramide phosphate and quinidine sulfate to human plasma proteins □ Protein binding—comparison of binding sites of disopyramide phosphate and quinidine sulfate, human plasma proteins

Disopyramide phosphate (I) has been reported to be two to three times as potent as quinidine sulfate in reversing cardiac arrhythmias (1). Table I lists the relative oral and intravenous mean effective dosages in canine atrial arrhythmias induced by aconitine (Scherf) or *via* crush–stimulation[1].

In a recently completed study comparing the activities of disopyramide phosphate and quinidine sulfate (II) against human ventricular and supraventricular arrhythmias, patients given 150 mg of disopyramide phosphate showed statistically equivalent therapeutic responses to patients receiving 325 mg of quinidine sulfate[1]. Both drugs were given every 6 hr, a time interval that approximates a single half-life for both drugs[1].

The interactions of drugs with plasma proteins in blood have been recognized for many years as an important parameter in drug availability, drug efficacy, and drug transport (2). It has been established that the interactions between drug and plasma proteins affect the concentration of unbound drug in the general circulation and limit the availability of drug to target tissues (3). This paper reports observations on the comparative binding of disopyramide phosphate and quinidine sulfate to human plasma proteins and discusses the potential biopharmaceutic implications of the binding profiles observed.

EXPERIMENTAL

Materials—Human plasma was collected by centrifuging[2] whole blood at 2500 rpm and 4° for 20 min. Human serum albumin[3] (fraction V, fatty acid poor) was prepared at a concentration of 7.028×10^{-4} *M* in isotonic 0.1 *M* phosphate buffer (pH 7.4). Disopyramide phosphate[4] and quinidine sulfate[5] were used as obtained.

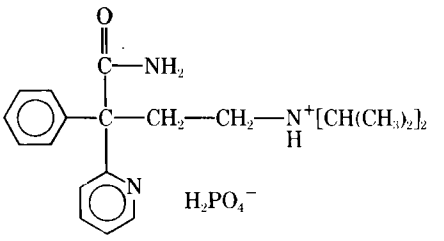
Table I—Effect of Disopyramide Phosphate and Quinidine Sulfate on Reversal of Canine Atrial Arrhythmias

Route	Drug	Mean Effective Dose, mg/kg: Aconitine	Mean Effective Dose, mg/kg: Crush–Stimulation
Intravenous	Disopyramide phosphate	2.7	5.8
Intravenous	Quinidine sulfate	8.7	11.8
Oral	Disopyramide phosphate	15	20
Oral	Quinidine sulfate	35	50

Binding Studies—Drug solutions, $1.6–8.0 \times 10^{-4}$ *M*, were prepared in isotonic 0.1 *M* phosphate buffer (pH 7.4) immediately prior to binding measurements. After mixing well, 1–5 ml of drug solution was added to 5 ml of either human plasma or human serum albumin solutions, and buffer was added to a final volume of 10 ml. The resultant mixture was equilibrated, poured into membrane ultrafilters[6], and then centrifuged[2] at 1250 rpm and 4° for 30 min.

The filtrates were assayed spectrophotometrically, and the absorbance at 260 (disopyramide phosphate) and 334 (quinidine sulfate) nm was recorded for calculating the free drug concentration. Drug solution in the absence of proteins was also investigated at the same time to correct for drug loss by membrane adsorption. The average quantities of drug adsorbed were 8 and 20% for disopyramide phosphate and quinidine sulfate, respectively.

Competitive Binding Studies—The procedure for studying the competitive binding of disopyramide phosphate and quinidine sulfate to human plasma proteins was essentially the same as described earlier, except that the concentration of one drug was maintained at 8×10^{-5} *M* while the concentration of the other was



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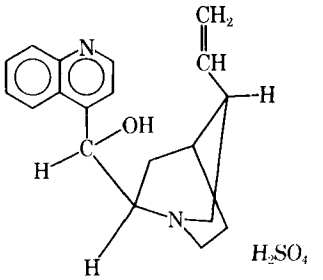

II

[1] Unpublished data.
[2] Sorvall RC-3 automatic refrigerated centrifuge.
[3] Nutritional Biochemical Corp., Cleveland, Ohio.
[4] Supplied by the Chemical Research Department of this laboratory.
[5] J. T. Baker Chemical Co., Glen Ellyn, Ill.
[6] Centriflo, model CF 50A, Amicon Corp., Lexington, Mass.

Table II—Binding of Disopyramide Phosphate and Quinidine Sulfate to Human Plasma

[Drug] $\times 10^5$ M	β Values[a], % ± SD		
	Disopyramide Phosphate	Quinidine Sulfate	Ratio[b]
1.6	30.65 ± 4.4	74.7 ± 1.4	2.44
3.2	29.8 ± 7.5	70.8 ± 3.2	2.38
4.8	26.4 ± 16.5	63.5 ± 2.0	2.41
6.4	22.3 ± 3.75	58.2 ± 3.2	2.61
			2.46 (±0.11)

[a] The fraction of drug bound, β, is defined by Eq. 1 (n = 4). [b] Ratio of the β values for quinidine sulfate over those for disopyramide phosphate at corresponding drug concentration (n = 4).

Table III—Binding of Disopyramide Phosphate and Quinidine Sulfate to Human Serum Albumin (Fraction V)

[Drug] $\times 10^5$ M	β Values[a], % ± SD		
	Disopyramide Phosphate	Quinidine Sulfate	Ratio[b]
1.6	33.01 ± 2.6	71.1 ± 5.3	2.154
3.2	30.96 ± 5.7	59.5 ± 5.5	1.922
4.8	21.1 ± 13.6	56.3 ± 1.4	2.668
6.4	22.7 ± 7.2	55.45 ± 1.33	2.443
			2.3 (±0.36)

[a] The fraction of drug bound, β, is defined by Eq. 1 (n = 5). [b] Ratio of the β values for quinidine sulfate over those for disopyramide phosphate at corresponding drug concentration (n = 4).

varied from 1.6 to 8.0 × 10^{-5} M. Since the spectrophotometric peaks for disopyramide phosphate (λ_{max} = 260 nm) and quinidine sulfate (λ_{max} = 334 nm) are well defined and clearly separated from one another, independent computation of free drug concentration of one drug in the presence of another was facilitated.

Partition Studies—A drug concentration of 8 × 10^{-5} M was freshly prepared in 1-octanol-saturated phosphate buffer (0.1 M, pH 7.4, isotonic). Ten milliliters was shaken and equilibrated with 10 ml of phosphate buffer-saturated 1-octanol until a clear phase separation was established (at least 5 hr). The drug concentrations in the buffer phase before and after equilibration were measured spectrophotometrically and utilized to estimate the magnitude of the partition coefficient.

RESULTS AND DISCUSSION

The fraction of drug bound to a given concentration of plasma proteins, β, was estimated from the difference in drug concentration recovered from the filtrate in the absence and in the presence of plasma proteins by using the following equations:

$$\beta\ (\%) = \frac{[D]_B}{[D]_T} \times 100 \qquad \text{(Eq. 1}a\text{)}$$

$$\beta\ (\%) = \frac{[D]_T - [D]_F}{[D]_T} \times 100 \qquad \text{(Eq. 1}b\text{)}$$

where $[D]_T$ and $[D]_F$ are the drug concentrations recovered in the absence and in the presence of proteins, respectively, and $[D]_B$ is the drug concentration bound to protein molecules.

As seen in Table II, both disopyramide phosphate and quinidine sulfate were bound to different degrees by human plasma proteins (final concentration of 36.3 × 10^{-5} M). When the concentration of drug was increased from 1.6 × 10^{-5} to 6.4 × 10^{-5} M, the β values for disopyramide phosphate and quinidine sulfate decreased. Table II shows that the β values for quinidine sulfate were 2.5-fold greater than those for disopyramide phosphate at all drug concentrations investigated. Since drug–protein interaction decreased the concentration of free drug and only the free drug species is membrane permeable, then the availability for permeation of quinidine sulfate at equimolar plasma concentration should be approximately 2.5-fold less than that of disopyramide phosphate.

As stated previously, disopyramide phosphate and quinidine sulfate are pharmacologically related, but chemically unrelated, antiarrhythmic agents. A series of binding studies with several concentrations of one drug in the presence of a fixed concentration (8 × 10^{-5} M) of the other was performed to evaluate the potential for competitive binding interactions (4). Plots of the reciprocal of the binding ratio *versus* the reciprocal of the free drug concentration (5) were constructed to determine if disopyramide phosphate and quinidine sulfate were competing for the same binding site (Figs. 1 and 2). The same magnitude of slope and the different extrapolated $1/\gamma$ intercepts in the absence and the presence of disopyramide phosphate (vice versa) clearly demonstrated that these two drugs do not compete for the same binding site (6). Therefore, the probability for displacement of one drug by the other upon simultaneous administration is markedly diminished.

The correlation between the binding data in Table III (human serum albumin) and those in Table II (human plasma) indicates that albumin was the primary molecule responsible for the observed binding of both disopyramide phosphate and quinidine sulfate.

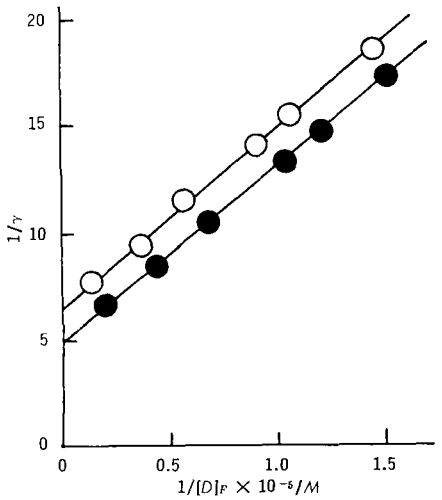

Figure 1—*Relationship between the reciprocal of the binding ratio ($1/\gamma$) and the reciprocal of the free quinidine sulfate concentration ($1/[D]_F$) at low γ values ($\gamma \leq 0.15$) in the absence (○) and in the presence (●) of disopyramide phosphate (8 × 10^{-5} M). Equivalent slopes were obtained.*

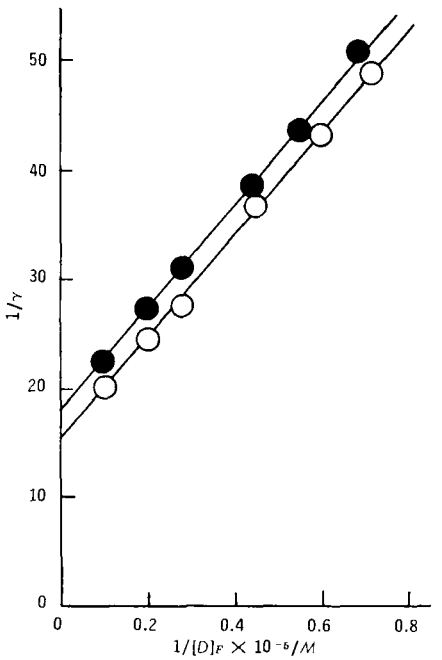

Figure 2—*Relationship between the reciprocal of the binding ratio ($1/\gamma$) and the reciprocal of the free disopyramide phosphate concentration ($1/[D]_F$) at low γ values ($\gamma \leq 0.05$) in the absence (○) and in the presence (●) of quinidine sulfate (8 × 10^{-5} M). Equivalent slopes were obtained.*

Table IV—Human Serum Albumin Binding Parameters for Disopyramide Phosphate and Quinidine Sulfate

[Drug] × 10^5 M[a]	Disopyramide Phosphate		Quinidine Sulfate	
	γ[b]	$\gamma/[D]_F$[c] ($\times 10^{-2}/M$)	γ[b]	$\gamma/[D]_F$[c] ($\times 10^{-2}/M$)
40	0.120	3.354	0.336	11.94
24	0.0945	4.568	0.247	16.11
16	—	—	0.199	22.06
8	0.0581	9.746	0.119	31.15
6.4	0.0497	10.684	0.100	34.4
4.8	0.0468	14.817	0.078	37.9
3.2	0.0330	16.164	—	—
2.4	—	—	0.041	43.4
1.6	—	—	0.031	60.8

[a] The drug concentration added initially. [b] The binding ratio, γ, is given by $[D]_B/[HSA]$, where $[D]_B$ and [HSA] are the concentrations of drug bound and of human serum albumin added, respectively. [c] $[D]_F$ is defined as the concentration of free drug in the drug–protein mixture.

fate. The data also support the use of aqueous solutions of human serum albumin (HSA) for the mechanistic analysis of drug–protein interaction between disopyramide phosphate and quinidine sulfate.

To perform a Scatchard analysis of the drug–protein interaction, several studies were carried out for both drugs over a wide concentration range (more than 20-fold) with a physiological concentration (3.514×10^{-4} M) of human serum albumin. A representative set of data is illustrated in Table IV. As expected, with a fixed number of albumin binding sites, when the concentration of drug was decreased, the magnitude of the binding ratio, $\gamma = ([D]_B/[HSA])$, decreased and the value of $\gamma/[D]_F$ increased. A computer program (6) based on Eq. 2 (7) was applied to calculate the number of binding sites in (N_1 and N_2) binding groups and their corresponding binding affinities (K_1 and K_2):

$$\gamma = \frac{N_1K_1[D]_F}{1 + K_1[D]_F} + \frac{N_2K_2[D]_F}{1 + K_2[D]_F} \qquad \text{(Eq. 2)}$$

The results are shown in Table V. It is apparent that disopyramide phosphate was bound mainly to the binding sites in the N_1 binding group while quinidine sulfate showed predominant binding to the N_2 group. Furthermore, quinidine sulfate had much higher binding affinities (K_1 = 12,844.5 and K_2 = 3422.5) than disopyramide phosphate (K_1 = 4618.1 and K_2 = 457.2). These observations are in agreement with the observation reported earlier (Tables II and III) that quinidine sulfate was bound to protein molecules 2.4-fold more than disopyramide phosphate.

In the dosage range (3–10 mg/kg) used for antiarrhythmic therapy, the resultant blood levels (10^{-5}–10^{-6} M) of disopyramide phosphate and quinidine sulfate[1] will interact primarily with the N_1 binding group. The results in Table V indicate that the affinity between the binding sites in the N_1 group and quinidine sulfate is 2.78-fold stronger than disopyramide phosphate (from 12,844.5 to 4618.1), even though serum albumins have approximately the same number of binding sites in the N_1 group available to both disopyramide phosphate (3.51) and quinidine sulfate (3.42). Therefore, the magnitude of the N_1K_1 value for quinidine sulfate (43,928.2) is about 2.7-fold greater than that for disopyramide phosphate (16,209.5). The 2.46-fold higher β value for quinidine sulfate than for disopyramide phosphate (Tables II and III) is a quantitative reflection of the 2.7-fold difference in the magnitude of the N_1K_1 values.

Table V—Comparison on Binding Characteristics to Human Serum Albumin between Disopyramide Phosphate and Quinidine Sulfate

Binding Parameters	Disopyramide Phosphate	Quinidine Sulfate
Groups of binding sites		
N_1	3.51	3.42
N_2	0.81	9.88
Binding affinities		
K_1	4618.1	12,844.5
K_2	457.2	3,422.5
N_1K_1[a]	16,209.5	43,928.2

[a] Ratio of N_1K_1 value for quinidine sulfate over that for disopyramide phosphate was 2.7.

Table VI—Physicochemical Parameters for Disopyramide Phosphate and Quinidine Sulfate

Drug	Partition Coefficient[a]	Lipophilicity[b]
Disopyramide phosphate	0.66 (±0.03)	−0.181
Quinidine sulfate	129.9 (±4.5)	+2.114

[a] Measured in 1-octanol–phosphate buffer (pH 7.4) (n = 3). [b] Lipophilicity = log (partition coefficient).

The results of partitioning studies in the system, 1-octanol–phosphate buffer at pH 7.4 (Table VI), demonstrate that quinidine sulfate had a much higher lipophilicity (2.114) than disopyramide phosphate (−0.181). The relative lipophilicity (8), Hansch parameter (π), was estimated as follows:

$$\pi = \log (\text{p.c.})_X - \log (\text{p.c.})_H = 2.114 - (-0.181) = 2.295 \qquad \text{(Eq. 3)}$$

The 2.3–2.5-fold higher drug–protein interaction observed for quinidine sulfate when compared to disopyramide phosphate (Tables II and III) may possibly be correlated with the higher π value for the former (9).

The ratio of percent drug retained by the membrane ultrafilters (quinidine sulfate–disopyramide phosphate) was also 2.5 (20%:8%). Further work with a series of disopyramide derivatives is underway to evaluate the correlation between hydrophobic, nonspecific protein binding and adsorption to polymer membranes.

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[x] To whom inquiries should be directed.

Water-Soluble Derivatives of 3-Oxy-substituted 1,4-Benzodiazepines

ABRAHAM NUDELMAN ˣ, R. J. McCAULLY, and S. C. BELL

Abstract □ Several acid addition salts of amino ester derivatives of 3-oxy-substituted 1,4-benzodiazepines were prepared and tested for water solubility, stability, and pH. The monomethanesulfonic acid salts were the most suitable for parenteral administration.

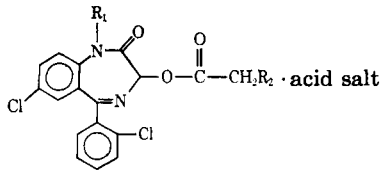
Keyphrases □ 1,4-Benzodiazepines, 3-oxy-substituted—water-soluble derivatives synthesized and tested for water solubility, stability, and pH □ Oxazepam—synthesis of aminoacetyl derivatives, tested for water solubility, stability, and pH □ Lorazepam—synthesis of aminoacetyl derivatives, tested for water solubility, stability, and pH □ Acid salts—synthesis of water-soluble derivatives of 3-oxy-substituted 1,4-benzodiazepines, tested for water solubility, stability, and pH

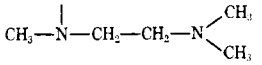
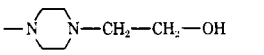
Recent efforts (1) in the area of 3-hydroxy-1,4-benzodiazepines have been directed to the preparation of water-soluble derivatives suitable for parenteral administration. Aqueous 1,4-benzodiazepine solutions would be expected to have the distinct advantage of diminishing the incidence of pain and the subsequent venous inflammation often encountered with injectable formulations of water-insoluble 1,4-benzodiazepines.

RESULTS AND DISCUSSION

The approach involved the preparation of derivatives of two known 3-hydroxy-1,4-benzodiazepines, oxazepam and lorazepam (I), whose pharmacological properties have been established and shown to be useful. The hydroxy groups used in the formation of the derivatives lent themselves to the formation of amino ester salts, which *in vivo* presumably hydrolyze to the starting 3-hydroxy-1,4-benzodiazepines. A number of derivatives that showed a range of water solubilities are listed in Table I.

Table I—Water-Soluble Derivatives of



Compound	R_1	R_2	Acid Salt
III	H		HCl
IV	CH_3		HCl
V (*a–d*)	H		2HCl[a], HCl[b], CH_3SO_3H[c], maleate[d]
VI (*a–c*)	CH_3		2HCl[a], HCl[b], CH_3SO_3H[c]
VII	H		2HCl
VIII	H		2HCl

The morpholino derivatives III and IV had somewhat lower solubilities and tended to hydrolyze rather readily. The diamine derivatives V–VIII as hydrochlorides showed considerably higher solubilities but had the disadvantage of forming very acidic aqueous solutions. This undesirable acidity was reduced to a large extent by the preparation of the monomethanesulfonic acid salts (Table II).

Table II—pH of Aqueous Solution of V at 5 mg/ml Concentration

Salt	pH
2HCl	2.3
HCl	3.7
CH_3SO_3H	4.9
Maleate	4.3

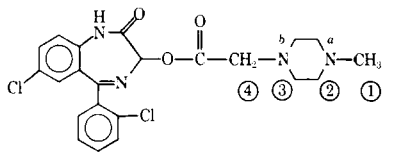
The methanesulfonic acid salts were also much more soluble than the hydrochlorides. Solutions of 200–300 mg/ml of either Vc or VIc could be prepared without difficulty.

The higher solubilities and stabilities of the monohydrochlorides and monomethanesulfonates of V and VI compared to the hydrochlorides of the morpholino derivatives III and IV may be attributed to the position of protonation (Table III). In the piperazine derivatives V and VI, protonation took place on the *a* nitrogen, as clearly indicated by the NMR spectra; in the morpholino derivatives, only the *b* nitrogen was available for protonation.

The synthetic sequences employed to prepare the compounds are outlined in Schemes I–IV. An efficient procedure for the methylation of I to II (Scheme I) was developed, in which equimolar amounts of base and methylating agent were used. When excess base and methyl iodide were used, two additional by-products (IX and X) were formed. The base-catalyzed tautomerization of I to XI in the presence of excess base also proceeded rapidly (2–4).

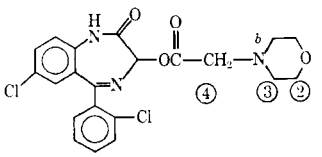
The intermediate Compounds XII and XIII were prepared by two alternative methods. Compound XII was obtained by an *N*-oxide rearrangement (2–5) of XIV (6, 7) when treated with chlo-

Table III—NMR Chemical Shifts (δ) of III and V



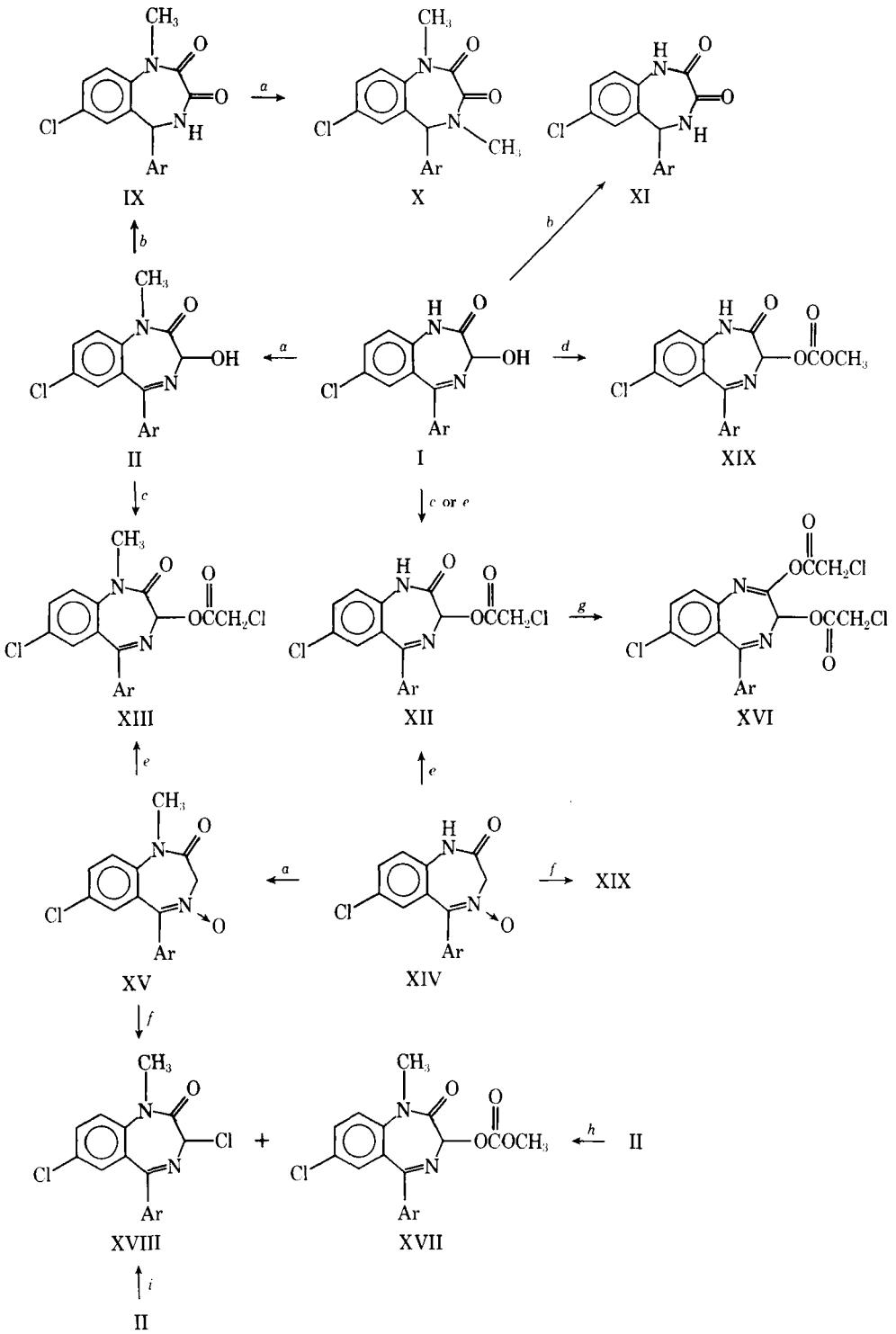
V

	①	②	③	④
Free base	2.27	2.55	2.70	3.57
Monohydrochloride	2.81	3.35	3.05	3.67
Dihydrochloride	2.80	3.40	3.40	4.20



III

Monohydrochloride	3.5	4.0	4.67



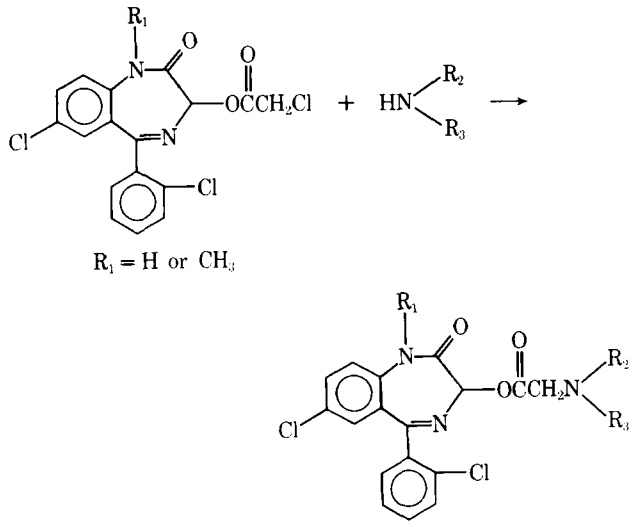
Scheme I—Key: Ar, o-chlorophenyl; a, *potassium hydroxide and methyl iodide;* b, *potassium hydroxide;* c, *chloroacetyl chloride and 4-dimethylaminopyridine;* d, *methyl chloroformate and 4-dimethylaminopyridine;* e, *chloroacetic anhydride;* f, *methyl chloroformate;* g, *chloroacetyl chloride;* h, *methyl chloroformate and lutidine; and* i, *thionyl chloride.*

roacetic anhydride. Methylation of XIV (8) followed by an analogous rearrangement gave XIII. Alternatively, XII and XIII were obtained from I and II by chloroacetylation with chloroacetyl chloride in the presence of 4-dimethylaminopyridine (9) or directly with chloroacetic anhydride. When an excess of chloroacetyl chloride was used, an additional product, XVI (derived from diacylation), was obtained.

When the analogous *N*-oxide rearrangement of XV with methyl chloroformate was carried out, a mixture of the carbonate XVII and the chloro Compound XVIII (1) was obtained. The carbonate, uncontaminated with XVIII, could be prepared from the 3-hydroxy Compound II upon treatment with methyl chloroformate. The 3-chloro derivative was alternatively prepared from II and thionyl chloride (1).

The final step in the preparation of the amine derivatives III–VIII involved the treatment of XII and XIII with the secondary amines (Scheme II).

The alternative direct acylation of I with the activated amino acid XXII (10) was also feasible (Scheme III). The required amino acid XXI could be prepared *via* the ester XXIV or directly from chloroacetic acid and the amine XX. When a large excess of chloroacetic acid was used, Compound XXIII was the only product.



Scheme II

However, equimolar quantities of chloroacid and amine afforded a mixture of XXI and XXIII.

The formation of XXIII could be avoided by the initial reaction of XX with ethyl chloroacetate to form the ester XXIV (11), which readily hydrolyzed in hot water.

The methanesulfonates Vc and VIc and the maleate V*d* were obtained by treatment of V and VI with equimolar amounts of the appropriate acids. The dihydrochlorides were best prepared by treating the free base with an excess of ethereal hydrogen chloride. The simplest procedure for the preparation of the monohydrochlorides involved the admixture of equimolar amounts of the dihydrochlorides and their respective free bases (Scheme IV).

The pharmacological activity of III–VIII is similar to that of I and II (12).

EXPERIMENTAL

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one (II)—A 3-liter erlenmeyer flask equipped with a magnetic stirrer was charged with 160 g (0.5 mole) of I, 1500 ml of tetrahydrofuran, and a solution of 33.0 g (0.5 mole) of potassium hydroxide (85%) in 300 ml of water. Iodomethane (75.0 g, 5.3 moles) was added to the reaction and stirring was continued for 1.5 hr. Since the reaction mixture was still alkaline, 15 ml of dimethyl sulfate was added. The mixture was stirred for an additional hour, filtered, and concentrated *in vacuo*. The residue was filtered and washed successively with 500 ml of water and 300 ml of ethanol. The product, mp 205–207° [lit. (1) mp 205–207°], after drying in a vacuum oven overnight, weighed 104 g.

Concentration of the filtrate afforded an additional 42.0 g of crude product, mp 180–195°, which was recrystallized by adding a warm tetrahydrofuran solution of the crude material to 600 ml of boiling ethanol and concentrating the resulting solution until crystals began to separate (300 ml). The recrystallized product, mp 209–211°, weighed 25.3 g. Further concentration of the filtrate afforded 6.7 g of crystalline material, mp 190–194°. The total yield was 81%.

7-Chloro-5-(*o*-chlorophenyl)-1-methyl-4,5-dihydro-2*H*-1,4-benzodiazepine-2,3(1*H*)-dione (IX) and 7-Chloro-5-(*o*-chlorophenyl)-4,5-dihydro-1,4-dimethyl-1*H*-1,4-benzodiazepine-2,3-dione (X)—When the previous reaction was carried out in the presence of excess base and excess methylating agent, two additional products (IX and X) were isolated in low yield. Compound IX had mp 239–241°; NMR [$(D_3C)_2SO$]: ppm (δ) 3.28 (s, 3), 5.82 (s, 1), and 7.2–7.7 (m, 7).

Anal.—Calc. for $C_{16}H_{12}Cl_2N_2O_2$: C, 57.32; H, 3.60; N, 8.36. Found: C, 57.52; H, 3.66; N, 8.41.

Compound X had mp 255–258°; NMR [$(D_3C)_2SO$]: ppm (δ) 3.10 (s, 3), 3.20 (s, 3), 6.00 (s, 1), and 6.9–8 (m, 7).

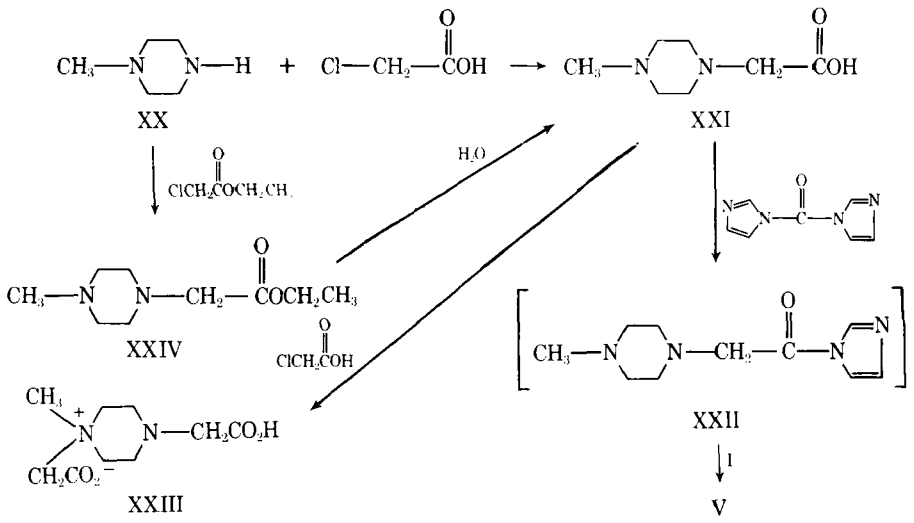
Anal.—Calc. for $C_{17}H_{14}Cl_2N_2O_2$: C, 58.47; H, 4.04; Cl, 20.31; N, 8.02. Found: C, 58.62; H, 4.11; Cl, 20.31; N, 8.07.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one Chloroacetate (XIII)—*Method I*—This compound was prepared by the same procedure as described for XII but from 7-chloro-5-(*o*-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one 4-oxide (XV) (6.7 g, 0.02 mole) and chloroacetic anhydride (12 g, 0.07 mole), giving 6.8 g (82.6% yield), mp 172–174°; NMR ($CDCl_3$): ppm (δ) 3.51 (s, 3), 4.41 (s, 2), 6.15 (s, 1), 7.19 (d, 1), and 7.4–8 (m, 6).

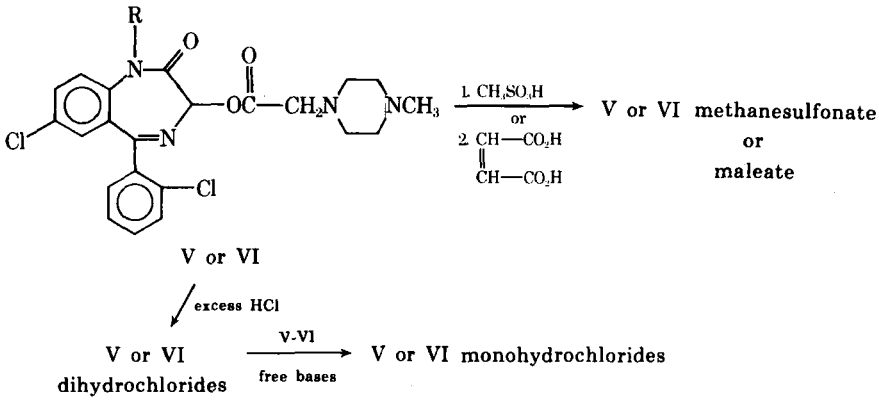
Anal.—Calc. for $C_{18}H_{13}Cl_3N_2O_3$: C, 52.52; H, 3.18; Cl, 25.84; N, 6.80. Found: C, 52.27; H, 3.37; Cl, 25.72; N, 6.61.

Method II—To a solution of II (10 g, 0.03 mole) and 4-dimethylaminopyridine (3.66 g, 0.03 mole) in 200 ml of tetrahydrofuran was added chloroacetyl chloride (3.39 g, 0.03 mole). A very heavy precipitate formed instantly. After 10 min of stirring, no trace of starting material could be detected by TLC (silica plates using ether as eluent). The precipitate was filtered off and the filtrate was flash evaporated, giving an oil which crystallized upon addition of ether, yielding 12.1 g (99%) of product. The melting point and the IR and NMR spectra were identical to those described above.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one Chloroacetate (XII)—*Method I*—A mixture of 7-chloro-5-(*o*-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide (XIV) (16 g, 0.05 mole) and chloroacetic anhydride (25 g, 0.145 mole) was heated, with stirring, at 100–110° for 2.5 hr. Ether was then added and the mixture was stirred for 10 min. The solid obtained (14.7 g, 75% yield) was filtered, washed with ether, and recrystallized from methylene chloride–ether, giving XII, mp 232–234°; NMR [$(D_3C)_2SO$]: ppm (δ) 4.72 (s, 2), 6.10 (s, 1), 7.17 (d, 1), and 7.4–8 (m, 6).



Scheme III



Scheme IV

Anal.—Calc. for $C_{17}H_{11}Cl_3N_2O_3$: C, 51.35; H, 2.79; N, 7.05. Found: C, 51.26; H, 2.83; N, 6.94.

Method II—Compound XII was prepared from I by a procedure similar to that described for the preparation of XIII. The product had properties identical to those of XII obtained from the *N*-oxide XIV.

7-Chloro-5-(*o*-chlorophenyl)-3*H*-1,4-benzodiazepine-2,3-diol Bis(chloroacetate) (XVI)—When XIV was treated with an excess of chloroacetyl chloride, the dichloroacetylated compound XVI was obtained in low yield. Recrystallization from acetone gave a product with a melting point of 212–213.5°; NMR [$(D_3C)_2SO$]: ppm (δ) 4.69 (s, 2), 5.05 (s, 2), 6.31 (s, 1), 7.3 (broad s, 1), and 7.4–7.9 (m, 6).

Anal.—Calc. for $C_{19}H_{12}Cl_4N_2O_4$: C, 48.13; H, 2.55; Cl, 29.91; N, 5.91. Found: C, 48.23; H, 2.62; Cl, 30.25; N, 5.89.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one Carbonate Methyl Ester (XIX)—*Method I*—A mixture of XIV (5 g, 0.0155 mole) and 100 ml of methyl chloroformate was refluxed for 3 hr. A small amount of undissolved material was filtered off and the filtrate was flash evaporated. The residue was dissolved in 10 ml of methylene chloride and allowed to crystallize. The obtained solid, 2 g (30% yield), was filtered, washed with a small amount of cold methylene chloride, and dried, giving XIX, mp 223–225°; NMR [$(D_3C)_2SO$]: ppm (δ) 3.82 (s, 3), 5.82 (s, 1), 7.05 (d, 1), and 7.3–7.9 (m, 6).

Anal.—Calc. for $C_{17}H_{12}Cl_2N_2O_4$: C, 53.84; H, 3.19; N, 7.39. Found: C, 53.40; H, 3.22; N, 7.19.

Method II—To a solution of 3.21 g (0.01 mole) of I and 1.5 g (0.0123 mole) of dimethylaminopyridine in 100 ml of anhydrous tetrahydrofuran was added 2 ml of methyl chloroformate. A heavy precipitate formed immediately. The mixture was stirred at room temperature for 18 hr. The solid was filtered and the filtrate was flash evaporated to a light-yellow foam. The foam was dissolved in 15 ml of methylene chloride and allowed to crystallize. The obtained solid, 2.8 g (72% yield), had the same melting point and IR and NMR spectra as those obtained for the product prepared by Method I.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one Carbonate Methyl Ester (XVII)—To a solution of II (16.7 g, 0.05 mole) in 300 ml of tetrahydrofuran were added 10 ml of methyl chloroformate and 10 ml of 2,6-lutidine. The reaction mixture was stirred overnight. TLC on silica gel, with ether as eluent, indicated the presence of unreacted starting material. The precipitate was filtered and a fresh excess of methyl chloroformate and 2,6-lutidine was added.

This process was repeated five times until only traces of 3-hydroxy compound could be detected. The solution was then flash evaporated and the residue was taken up in ethyl acetate and washed several times with water. The solution was concentrated to 50 ml, whereupon it crystallized, yielding 12.5 g (64%) of product, mp 193–194.5; NMR ($CDCl_3$): ppm (δ) 3.50 (s, 3), 3.89 (s, 3), 5.90 (s, 1), 7.08 (d, 1), and 7.3–7.9 (m, 6).

Anal.—Calc. for $C_{18}H_{14}Cl_2N_2O_4$: C, 54.98; H, 3.59; Cl, 18.03; N, 7.13. Found: C, 55.08; H, 3.63; Cl, 18.12; N, 7.15.

3,7-Dichloro-5-(*o*-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (XVIII)—A mixture of II (22 g, 0.066 mole) and 50 ml of thionyl chloride was refluxed for 0.5 hr. The excess thionyl chloride was flash evaporated and the residue was recrystallized from acetonitrile, giving 21 g (90% yield) of XVIII, mp 217–219° [lit. (1) mp 217–219°]; NMR ($CDCl_3$): ppm (δ) 3.58 (s, 3), 5.80 (s, 1), 7.18 (d, 1), and 7.2–8 (m, 6).

XVIII (1) and XVII from XV—A mixture of XV (2.5 g, 7.5 mmoles) and methyl chloroformate (50 ml) was refluxed for 2 hr, whereupon no trace of starting *N*-oxide XV could be detected by TLC [ether–pentane (8.5:1.5) on silica gel plates]. The TLC showed the presence of two compounds in approximately equal ratio. The faster moving compound had an R_f value identical to that of XVIII (1). The solvent was flash evaporated and the residue was chromatographed on 100 g of silica gel. The 3-chloro Compound XVIII was eluted initially, followed by the 3-methyl carbonate XVII, 0.4 g (13% yield), mp 193–194.5°; NMR ($CDCl_3$): ppm (δ) 3.56 (s, 3), 3.96 (s, 3), 6.01 (s, 1), 7.24 (d, 1), and 7.4–8 (m, 6).

Anal.—Calc. for $C_{18}H_{14}Cl_2N_2O_4$: C, 54.98; H, 3.59; N, 7.13. Found: C, 54.90; H, 3.76; N, 7.27.

7-Chloro-5-(*o*-chlorophenyl)-4,5-dihydro-2*H*-1,4-benzodiazepine-2,3(1*H*)-dione (XI)—The title compound was obtained in low yield upon treatment of I with an excess of base, analogous to the conversion of II to IX, mp 330–331°; NMR [$(D_3C)_2SO$]: ppm (δ) 5.92 (s, 1) and 7.1–7.9 (m, 7).

Anal.—Calc. for $C_{15}H_{10}Cl_2N_2O_2$: C, 56.10; H, 3.14; N, 8.72. Found: C, 55.91; H, 3.29; N, 9.03.

4-Methyl-1-piperazineacetic Acid (XXI)—*Method I*—A solution of *N*-methylpiperazine (XX) (15 g, 0.15 mole) and chloroacetic acid (4.7 g, 0.05 mole) in 50 ml of water was stirred for 18 hr. The solution was then placed on a column containing 300 g of resin[1] in its basic form. The column was washed with distilled water until the pH of the eluent was neutral; then the column was eluted with 10% hydrochloric acid. A total of 900 ml of the acid eluent was collected and rechromatographed through a column containing 300 g of resin[2] in its acidic form.

The column was washed with distilled water until no more chloride ion was eluted (as indicated by the absence of a precipitate with silver nitrate) and was then eluted with 10% ammonium hydroxide until 600 ml of basic eluent was collected. The eluted solution, when flash evaporated, gave 3.7 g (46% yield) of crystalline product (recrystallized from methanol–ether), mp 159.5–161°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.25 (s, 3), 2.5 (broad m, 4), 2.7 (broad m, 4), and 3.15 (s, 2).

Anal.—Calc. for $C_7H_{14}N_2O_2 \cdot \frac{1}{4}H_2O$: C, 51.67; H, 8.97; N, 17.22. Found: C, 51.69; H, 8.98; N, 16.81.

Method II—A solution of ethyl 4-methyl-1-piperazineacetate (XXIV) (3.2 g, 0.0172 mole) in 75 ml of water was refluxed for 2 hr. The solvent was flash evaporated and the residue was crystallized from methanol–ether, giving 2.6 g (95%) of the free acid, with melting point and NMR spectra identical to those reported in Method I.

1,4-Dicarboxymethyl-1-methylpiperazinium Hydroxide Inner Salt and Its Sodium Salt (XXIII)—To a solution of chloroacetic acid (19 g, 0.02 mole) in 10 ml of water was added a solution of sodium hydroxide (8 g, 0.02 mole) in 10 ml of water. The solution was stirred for 0.5 hr, and XX (10 g, 0.01 mole) was added. The solution was stirred for 18 hr at room temperature and was

[1] Dowex 1-X 8.
[2] Dowex 50 W-X2.

then chromatographed on 900 g of resin[2] (acidic form). The column was washed with distilled water until no more chloride ion was obtained, as indicated by silver nitrate treatment, and was then eluted with 3 *N* ammonium hydroxide until 1800 ml of basic eluent was collected. Flash evaporation gave a foam which, when mixed with absolute ethanol and added to methylene chloride, gave 9.2 g (42% yield) of product as a white crystalline solid. Recrystallization from water–methanol afforded pure product, mp 306–307°; NMR (D_2O): ppm (δ) 3.43 (s, 3), 3.7 (broad m, 4) [superimposed at 3.8 (s, 2)], 4 (broad m, 4), and 4.12 (s, 2).

Anal.—Calc. for $C_9H_{16}N_2O_4$: C, 49.99; H, 7.46; N, 12.96. Found: C, 50.13; H, 7.64; N, 12.73.

Treatment of this 1,4-dicarboxymethyl-1-methylpiperazinium hydroxide inner salt with an equivalent amount of sodium hydroxide, followed by recrystallization from water–methanol, gave the corresponding sodium salt in 90% yield, mp 159–162°; NMR (D_2O): ppm (δ) 3.1 (broad m, 4), 3.25 (s, 2), 3.35 (s, 2), 3.8 (broad m, 4), and 4.0 (s, 2).

Anal.—Calc. for $C_9H_{15}N_2NaO_4{\cdot}2H_2O$: C, 39.42; H, 6.98; N, 10.21. Found: C, 39.28; H, 7.06; N, 10.13.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one 4-Methyl-1-piperazineacetate Dihydrochloride (V*a*)—*Method I (from XII)*—A solution of XII (3 g, 7.5 mmoles) and XX (3 ml) in 50 ml of anhydrous tetrahydrofuran was stirred for 16 hr at room temperature. The solvent was flash evaporated and the residue, dissolved in methylene chloride, was washed repeatedly with saturated aqueous sodium chloride. The organic phase was dried and concentrated to a total volume of 5 ml, which was then added to a vigorously stirred solution of gaseous hydrogen chloride in ether. The obtained solid was recrystallized from methanol–ether, giving 1.5 g of V*a* (35% yield), mp 202–204° dec.; NMR [$(D_3C)_2SO$]: ppm (δ) 2.80 (s, 3), 3.40 (broad s, 8), 4.23 (s, 2), 5.98 (s, 1), 6.96 (d, 1), and 7.2–7.8 (m, 6).

Anal.—Calc. for $C_{22}H_{22}Cl_2N_4O_2{\cdot}2HCl{\cdot}2H_2O$: C, 46.33; H, 4.95; H_2O, 6.32; N, 9.82. Found: C, 46.11; H, 4.34; H_2O, 6.40; N, 9.85.

Method II (from XXI)—To a solution obtained by warming a mixture of 4-methyl-1-piperazineacetic acid (0.79 g, 5 mmoles) and anhydrous dimethylformamide (10 ml) was added 1,1-carbonyldiimidazole (0.81 g, 0.5 mmole). A solution of I (1.6 g, 0.5 mmole) in 50 ml of anhydrous tetrahydrofuran was added. The reaction mixture was stirred at room temperature for 80 hr and then flash evaporated, giving an oil. The residue was mixed with ethyl acetate and washed three times with 100 ml of water.

The organic phase was dried and evaporated, giving an oil. The oil was dissolved in methylene chloride (3 ml), and the solution was added to a solution of gaseous hydrogen chloride in ether. A precipitate was obtained which, when recrystallized from methanol–ether, gave 1.2 g (42% yield) of the desired hydrochloride, mp 218–220°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.80 (s, 3), 3.40 (broad s, 8), 4.23 (s, 2), 5.98 (s, 1), 6.96 (d, 1), and 7.2–7.8 (m, 6).

Anal.—Calc. for $C_{22}H_{22}Cl_2N_4O_3{\cdot}2HCl{\cdot}H_2O$: C, 47.84; H, 4.75; N, 10.15. Found: C, 47.51; H, 4.57; N, 9.85.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one 4-Methyl-1-piperazineacetate Dihydrochloride (VI*a*)—This compound was prepared as described in the synthesis of V*a* but from XIII (5 g, 0.012 mole) and *N*-methylpiperazine. The product (2.5 g, 36% yield) decomposed above 210°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.82 (s, 3), 3.43 (broad s, 11), 4.27 (s, 2), 6.00 (s, 1), 7.07 (d, 1), and 7.4–8 (m, 6).

Anal.—Calc. for $C_{23}H_{24}Cl_2N_4O_3{\cdot}2HCl{\cdot}1\frac{1}{2}H_2O$: C, 48.02; H, 5.08; N, 9.74. Found: C, 47.88; H, 4.89; N, 9.78.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one 4-Morpholineacetate Ester Hydrochloride (III)—This compound was prepared by a procedure similar to the one described in the synthesis of V but from XII (3 g, 7.5 mmoles) and morpholine. The product obtained (1.3 g, 40% yield) had a melting point of 255–257° dec.; NMR [$(D_3C)_2SO$]: ppm (δ) 3.5 (broad s, 4), 4.0 (broad s, 4), 4.67 (s, 2), 6.17 (s, 1), 7.11 (d, 1), and 7.5–8 (m, 6).

Anal.—Calc. for $C_{21}H_{19}Cl_2N_3O_4{\cdot}HCl{\cdot}H_2O$: C, 50.17; H, 4.41; Cl, 21.15; N, 8.36. Found: C, 50.43; H, 4.06; Cl, 21.39; N, 8.55.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one 4-Morpholineacetate Hydrochloride (IV)—This compound was prepared by a procedure similar to the one described in the synthesis of V but from XIII (3.5 g, 8.5 mmoles) and morpholine. The product (2.7 g, 61% yield) had a melting point of 237–238°; NMR [$(D_3C)_2SO$]: ppm (δ) 3.52 (broad s, 7), 4.05 (broad s, 4), 4.75 (s, 2), 6.26 (s, 1), 7.27 (d, 1), and 7.7–8 (m, 6).

Anal.—Calc. for $C_{22}H_{21}Cl_2N_3O_4{\cdot}HCl{\cdot}H_2O$: C, 51.13; H, 4.68; N, 8.13. Found: C, 51.41; H, 4.60; N, 8.11.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one 4-Methyl-1-piperazineacetate Hydrochloride (V*b*)—An aqueous solution of V*a* (29 g, 0.05 mole) was neutralized with an excess of 1 *N* sodium hydroxide and extracted with methylene chloride. The organic phase was dried and evaporated, giving V as a fine white powder, 18.5 g (80% yield); NMR ($CDCl_3$): ppm (δ) 2.27 (s, 3), 2.55 (broad s, 4), 2.70 (broad s, 4), 3.57 (s, 2), 6.08 (s, 1), 7.08 (d, 1), and 7.2–7.9 (m, 6).

A solution of equimolar quantities of V*a* and the above-obtained free base (2.3 mmoles of each) in methanol was stirred at room temperature for 30 min. The solvent was flash evaporated and the residual foam was recrystallized from methanol–ether, giving 2 g (82% yield) of white solid, mp 233–234°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.81 (s, 3), 3.05 (broad m, 4), 3.35 (broad m, 4), 3.67 (s, 2), 5.95 (s, 1), 7.00 (d, 1), and 7.3–7.8 (m, 6).

Anal.—Calc. for $C_{22}H_{22}Cl_2N_4O_3{\cdot}HCl{\cdot}H_2O$: C, 51.22; H, 4.89; Cl, 20.62; N, 10.86. Found: C, 51.44; H, 4.56; Cl, 20.91; N, 10.74.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one 4-Methyl-1-piperazineacetate Hydrochloride (VI*b*)—An aqueous solution of VI*a* (11.6 g, 0.02 mole) was neutralized with an excess of 1 *N* sodium hydroxide and extracted with methylene chloride. The organic phase was dried and evaporated, giving VI as a fine powder, 9.1 g (94.6% yield); NMR ($CDCl_3$): ppm (δ) 2.27 (s, 3), 2.57 (broad m, 4), 2.65 (broad m, 4), 3.46 (s, 3), 3.55 (s, 2), 6.10 (s, 1), 7.10 (d, 1), and 7.2–7.9 (m, 6).

A solution of equimolar quantities of VI*a* and the above-obtained free base (20 mmoles of each) in methanol was stirred at room temperature for 30 min. The solvent was flash evaporated and the residual foam was recrystallized from methanol–ether, giving 15 g (71% yield) of solid, mp 270–271°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.87 (s, 3), 3.1 (broad m, 4), 3.4 (broad m, 4), 3.49 (s, 3), 3.70 (s, 2), 6.07 (s, 1), 7.12 (d, 1), and 7.5–7.9 (m, 6).

Anal.—Calc. for $C_{23}H_{24}Cl_3N_4O_3{\cdot}HCl{\cdot}H_2O$: C, 51.13; H, 5.14; Cl, 20.08; N, 10.57. Found: C, 52.34; H, 4.75; Cl, 20.64; N, 10.56.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one 4-Methyl-1-piperazineacetate Methanesulfonate (V*c*)—To a solution of 7-chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one 4-methyl-1-piperazineacetate (V) (2.3 g, 5 mmoles), prepared as described, was added methanesulfonic acid (0.48 g, 5 mmoles). The solution was stirred at room temperature for 30 min. The solvent was then flash evaporated and the residue was recrystallized from methanol–ether, giving 2.3 g (80% yield) of white crystalline product, which did not melt sharply but slowly decomposed above 160°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.42 (s, 3), 2.82 (s, 2), 3.0 (broad m, 4), 3.4 (broad m, 4), 3.65 (s, 2), 5.95 (s, 1), 7.05 (d, 1), and 7.2–7.9 (m, 6).

Anal.—Calc. for $C_{22}H_{22}Cl_2N_4O_3{\cdot}CH_4SO_3{\cdot}H_2O$: C, 48.00; H, 4.90; Cl, 12.32; N, 9.74. Found: C, 48.08; H, 4.71; Cl, 12.62; N, 9.41.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one 4-Methyl-1-piperazineacetate Methanesulfonate (VI*c*)—To a solution of 7-chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-1-methyl-2*H*-1,4-benzodiazepin-2-one 4-methyl-1-piperazineacetate (VI) (197.5 g, 0.4 mole), prepared as described, was added methanesulfonic acid (38.8 g, 0.4 mole). The solution was stirred at room temperature for 30 min. The solvent was then flash evaporated and the residue was recrystallized from methanol–ether, giving 196 g (85.5% yield) of white crystalline product, mp 238–241°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.48 (s, 3), 2.87 (s, 3), 3 (broad m, 4), 3.3 (broad m, 4), 3.48 (s, 3), 3.68 (s, 2), 6.02 (s, 1), 7.10 (m, 1), and 7.5–7.95 (m, 6).

Anal.—Calc. for $C_{23}H_{24}Cl_2N_4O_3{\cdot}CH_4SO_3$: C, 50.44; H, 4.94; Cl, 12.41; N, 9.80; S, 5.61. Found: C, 50.32; H, 4.94; Cl, 12.41; N, 9.77; S, 5.67.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one 4-Methyl-1-piperazineacetate Maleate (V*d*)—To a methanolic solution of V (2.3 g, 5 mmoles), prepared as described, was added maleic acid (0.58 g, 5 mmoles). The solution was stirred at room temperature for 30 min. The solvent was then flash evaporated and the residue was recrystallized from methanol–ether, giving 1.5 g (51% yield) of white crystalline product, which did not melt sharply but slowly decomposed above

125°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.92 (s, 3), 3.0 (broad s, 4), 3.4 (broad s, 4), 3.63 (s, 2), 6.02 (s, 1), 6.30 (s, 2), 7.1 (d, 1), and 7.3–7.9 (m, 6).

Anal.—Calc. for $C_{22}H_{22}Cl_2N_4O_3 \cdot C_4H_4O_4 \cdot \frac{1}{2}H_2O$: C, 53.25; H, 4.64; Cl, 12.09; N, 9.55. Found: C, 52.96; H, 4.53; Cl, 11.96; N, 9.46.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one *N*-(2-Dimethylaminoethyl)-*N*-methylglycinate Dihydrochloride (VII)—The title compound was prepared by the same procedure as that described in the preparation of V*a* but from XII and *N,N,N'*-trimethylethylenediamine. The product was obtained from ethanol–ether in 20% yield, mp 221–223° dec.; NMR [$(D_3C)_2SO$]: ppm (δ) 2.95 (s, 6), 3.00 (s, 3), 3.65 (broad s, 4), 4.52 (broad s, 2), 6.10 (s, 1), 7.02 (d, 1), and 7.4–7.8 (m, 6).

Anal.—Calc. for $C_{22}H_{24}Cl_2N_4O_3 \cdot 2HCl \cdot 2H_2O$: C, 46.17; H, 5.28; N, 9.79. Found: C, 45.96; H, 5.36; N, 9.85.

7-Chloro-5-(*o*-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one 4-(Hydroxyethyl)-1-piperazineacetate Dihydrochloride (VIII)—The title compound was prepared as described for VII but from XII and *N*-(2-hydroxyethyl)piperazine. The product, obtained in 46% yield from ethanol–ether, became dark above 195° and decomposed above 202°; NMR [$(D_3C)_2SO$]: ppm (δ) 3.1–4.2 (broad m, 16), 4.55 (broad s, 2), 6.03 (s, 1), 7.01 (d, 1), and 7.3–7.8 (m, 6).

Anal.—Calc. for $C_{23}H_{26}Cl_4N_4O_4 \cdot 2HCl \cdot 2\frac{1}{2}H_2O$: C, 45.33; H, 5.13; H_2O, 7.39; N, 9.29. Found: C, 45.34; H, 4.70; H_2O, 7.39; N, 9.33.

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GLC Determination of Propranolol, Other β-Blocking Drugs, and Metabolites in Biological Fluids and Tissues

THOMAS WALLE

Abstract □ A highly sensitive and specific electron-capture GLC method was developed for β-blocking drugs, including propranolol, oxprenolol, alprenolol, pronethalol, dichloroisoproterenol, practolol, and sotalol, and several of their metabolites. The drugs are separated and detected as their trifluoroacetyl derivatives. Minimum detectable amounts ranged from 0.1 to 1.1 pg. The chemical structures of the derivatives were confirmed by GLC–mass spectrometry. Propranolol was extracted from plasma by either a single- or a back-extraction procedure, with oxprenolol as the internal standard. The minimum detectable concentration was 0.1 ng/ml plasma. The standard curve was linear from 0.5 to 500 ng/ml. Applications of this method demonstrated quantitatively detectable plasma propranolol levels 24 hr after a small (0.05 mg/kg) intravenous dose. Accurate plasma determinations could be made in patients receiving numerous other drugs. The method was shown to be applicable to propranolol and two metabolites, 1-(α-naphthoxy)-2,3-propylene glycol and *N*-desisopropylpropranolol, in tissue. Determinations of picogram amounts of oxprenolol and its four major urinary metabolites demonstrate the general applicability of the method to all β-blocking drugs and most of their metabolites.

Keyphrases □ Propranolol and two metabolites—GLC determination in biological fluids and tissues □ β-Blocking drugs—electron-capture GLC determination of propranolol, oxprenoiol, alprenolol, pronethalol, dichloroisoproterenol, practolol, sotalol, and several metabolites in biological fluids and tissues □ GLC—determination of propranolol, other β-blocking drugs, and metabolites in biological fluids and tissues

Large variations in response to β-blocking drug therapy have been demonstrated in numerous clinical studies, making the task of evaluating the clinical efficacy of this chemical and pharmacological class of compounds extremely difficult. These variations have been attributed in part to individual differences in the metabolic disposition of these drugs.

A better understanding of variations in the metabolic disposition of β-blocking drugs during treatment requires reliable measurements of these drugs in plasma, other biological fluids, and tissues. Most β-blocking drugs are extensively metabolized (1, 2),

125°; NMR [$(D_3C)_2SO$]: ppm (δ) 2.92 (s, 3), 3.0 (broad s, 4), 3.4 (broad s, 4), 3.63 (s, 2), 6.02 (s, 1), 6.30 (s, 2), 7.1 (d, 1), and 7.3–7.9 (m, 6).

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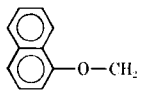
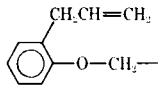
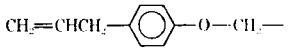
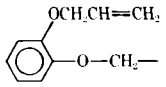
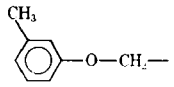
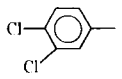
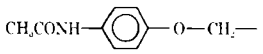
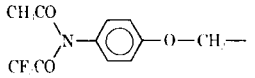
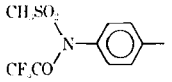
A better understanding of variations in the metabolic disposition of β-blocking drugs during treatment requires reliable measurements of these drugs in plasma, other biological fluids, and tissues. Most β-blocking drugs are extensively metabolized (1, 2),

Table I—Electron-Capture Response and Relative Retention Times for Trifluoroacetyl Derivatives of β-Blocking Drugs

$R-CH(OCOCF_3)CH_2N(COCF_3)CH(CH_3)_2$		MDQ		
		Moles per Second $\times 10^{-16}$	Picograms[a]	Relative Retention[b]
	Propranolol	2.0	0.5	8.90
	Alprenolol	2.8	0.7	2.20
	H 64/52	3.2	0.8	3.00
	Oxprenolol	4.3	1.1	3.30
	Toliprolol	3.8	0.9	1.48
	Pronethalol	2.6	0.6	3.87
	Dichloroisoproterenol	0.5	0.1	1.59
	Practolol	2.9	0.7	26.3
	Practolol	2.8	0.7	7.2
	Sotalol	2.4	0.7	7.0

[a] MDQ (minimum detectable quantity) expressed in picograms is valid at t_R = 3 min for a column with 3800 theoretical plates. [b] Using 1-bromonaphthalene (t_R = 1.00 min) as the reference compound.

and some of the resulting metabolites have important pharmacological properties (3, 4). Therefore, a method for drug determination should include such metabolites if possible.

Several methods have been reported for quantitative determinations of β-blocking drugs. Propranolol and sotalol have been determined fluorometrically (5, 6), practolol has been determined colorimetrically (7), and alprenolol has been determined by GLC (8). A recently published method for propranolol also utilizes GLC (9). The specificity of the fluorometric method for propranolol has been demonstrated to be inadequate[1]. The fluorometric method for sotalol and the colorimetric method for practolol are limited with respect to both specificity and sensitivity. None of the methods has been demonstrated to be applicable to the determination of metabolites.

This investigation describes the improvement and extension of the electron-capture GLC method originally reported for alprenolol (8) to include propranolol and eight other β-blocking drugs.

Applications demonstrate the method to be useful for both plasma, tissue, and urine determinations and to include drug metabolites.

EXPERIMENTAL

Reagents—Trifluoroacetic anhydride[2] in 1-ml ampuls was used as the derivatization reagent. To protect the anhydride from hydrolysis by moist air after opening the ampuls, it was stored in airtight 3-ml vials[3]. Trimethylamine (1 *M*) in benzene was prepared from gaseous trimethylamine[4]. Nanograde benzene[5] was used without prior distillation. Sulfuric acid (0.2 *N*), sodium hydroxide (5 *N*), and pH 6.0 phosphate buffer (0.5 *M*) were prepared with glass-distilled water and stored in glass bottles.

Propranolol[6], alprenolol[7], 1-(*p*-allylphenoxy)-3-isopropylamino-2-propanol[8], oxprenolol[9], 1-(*m*-toloxy)-3-isopropylamino-2-propanol[10], pronethalol[6], dichloroisoproterenol[6], and sotalol[9] were obtained as their hydrochlorides and practolol[6] was obtained as the free base from commercial sources. Their purity was checked by GLC as the trifluoroacetyl derivatives and by TLC. The chemical structures of their trifluoroacetyl derivatives are shown in Table I.

[1] K. Walle and T. Walle, to be published.

[2] Pierce Chemical Co., Rockford, Ill.
[3] Reacti-Vials, Pierce Chemical Co.
[4] Eastman-Kodak Co., Rochester, N.Y.
[5] Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Imperial Chemical Industries, Ltd., Wilmslow, Great Britain.
[7] AB Hässle, Mölndal, Sweden
[8] H 64/52, AB Hässle.
[9] William S. Merrell Co., Cincinnati, Ohio.
[10] Toliprolol, ICI 45763, Imperial Chemical Industries, Ltd.

Glassware—All glassware was cleaned in chromic acid. Glass-stoppered 10-ml centrifuge tubes were then silanized with dimethyldichlorosilane[2] and rinsed with methanol and toluene. Glassware treated this way could be used repeatedly for several months. Between such treatments, all glassware was cleaned in a 2% cleaning solution[11].

Preparation of Derivatives—Propranolol as its hydrochloride or as its free base after extraction was taken up in 50–200 μl of benzene in glass-stoppered 10-ml centrifuge tubes. Twenty-five microliters of trimethylamine (1 *M*) in benzene and 50 μl of trifluoroacetic anhydride were added. The tubes, after being tightly stoppered, were heated for 5 min in a 50° water bath. After cooling, the reaction mixtures were shaken vigorously for 30 sec with 1 ml of pH 6.0 phosphate buffer (0.5 *M*) and centrifuged. Part of the benzene phase (1–5 μl) was taken for electron-capture GLC analysis.

The completeness of the reaction under various conditions was studied with 1-bromonaphthalene[12] as the internal standard. Extended reaction times from 5 to 60 min with varying amounts of reagents [10–75 μl of trimethylamine (1 *M*) in benzene and 10–75 μl of trifluoroacetic anhydride] gave identical recoveries, indicating quantitative derivatization conditions for propranolol. The same conditions also gave quantitative reactions with alprenolol, 1-(*p*-allylphenoxy)-3-isopropylamino-2-propanol, oxprenolol, 1-(*m*-tolyoxy)-3-isopropylamino-2-propanol, pronethalol, and dichloroisoproterenol.

Quantitative derivatization of practolol and sotalol required 75 μl of trimethylamine (1 *M*) in benzene and 75 μl of trifluoroacetic anhydride. The final wash was made with 2 ml of pH 6.0 phosphate buffer (0.5 *M*).

GLC—A gas chromatograph[13] equipped with a ^{63}Ni electron-capture detector was used. A 150-cm glass column with an internal diameter of 2 mm was packed with 2% OV-17 on resilanized (10) Chromosorb W,AW-DMCS (80–100 mesh). The nitrogen flow rate was 30 ml/min. (The column was conditioned at 250° for 72 hr.) The injector temperature was 230°, the column temperature was 170°, and the detector temperature was 270°.

The ^{63}Ni detector gave a linear response for quantities ranging from 15 to 800 pg of trifluoroacetylated propranolol.

GLC–Mass Spectrometry—The combination instrument[14] was operated at an accelerating voltage of 3.5 kv, an ionization voltage of 20 ev, and a trap current of 65 μamp. The column, 90 cm long and 2 mm i.d., was packed with the same liquid phase and support as already described. The instrument was equipped with an accelerator voltage alternator for multiple-ion detection (11).

Propranolol Determinations in Plasma—*Single Extraction*—To samples of 1.00 ml of plasma were added 50–100 μl of an aqueous solution of the internal standard (oxprenolol hydrochloride) and 0.1 ml of 5 *N* NaOH (pH 13) in a glass-stoppered 10-ml centrifuge tube. The sample was shaken with 3 ml of benzene for 5 min and centrifuged at 3000 rpm for 5 min.

The benzene phase was transferred to another tube and evaporated to a small volume (50 μl) with a gentle stream of nitrogen at 70°. Derivatization and buffer wash were done according to procedures described under *Preparation of Derivatives*. For electron-capture analysis, 1–5-μl volumes of the final benzene phase were injected.

Back Extraction—The initial benzene phase from the single-extraction procedure (3 ml) was transferred to a tube containing 1 ml of 0.2 *N* H_2SO_4. The tube was shaken for 1 min and centrifuged. The acidic aqueous phase was transferred to another tube, made alkaline (pH 13) with 5 *N* NaOH, and shaken with 3 ml of benzene. The benzene phase was then treated according to the single-extraction procedure.

Standard Curve—One-milliliter control plasma samples were spiked with propranolol hydrochloride and extracted following either the single-extraction procedure (5–15 ng/ml) or the back-extraction procedure (0.5–4 ng/ml), using 25 ng/ml oxprenolol hydrochloride as internal standard. Peak area ratios of propranolol–oxprenolol were measured and plotted as a function of propranolol concentration.

[11] Micro, International Products Corp., Trenton, N.J.
[12] Pfalz and Bauer, Inc., Flushing, N.Y.
[13] Varian model 1440, Varian-Aerograph, Walnut Creek, Calif.
[14] LKB 9000 S, LKB Instruments, Inc., Rockville, Md.

Table II—Plasma Propranolol Levels after Chronic Oral Administration in Humans

Daily Oral Dose, mg	Propranolol[a], ng/ml
120	40 ± 4
120	77 ± 3
160	26 ± 2
160	72 ± 2
240	38 ± 3
320	67 ± 6

[a] Mean of four determinations.

Plasma Determinations—A dose of 3.3 mg of propranolol hydrochloride was given intravenously to a 72-year-old patient. Five-milliliter heparinized blood samples were drawn 0.5, 1, 1.5, 2, 3, 6, 12, 18, and 24 hr after the dose. After separating the plasma by centrifugation (15 min at 6000×*g*), 1-ml samples were taken for analysis following the back-extraction procedure.

Blood samples from six patients on chronic oral propranolol therapy, 120–320 mg daily in four divided doses, were drawn 2 hr after the morning dose (Table II). These samples were handled identically. The patients were treated with numerous other drugs concomitantly including methyldopa, digoxin, furosemide, aldactone, guanethidine, allopurinol, propoxyphene, chlordiazepoxide, and dicyclomine.

Propranolol and Metabolites in Heart Tissue—Two hundred milligrams of heart tissue (wet weight) from a propranolol-treated blood-perfused dog heart–lung preparation (12) was homogenized[15] in 4 ml of ice-cold 0.4 *N* $HClO_4$. After centrifugation, the supernate was taken for analysis following the single-extraction procedure, with 6 ml of benzene as the extractant.

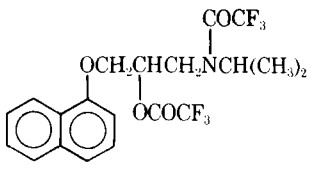
Electron-Capture Response—Trifluoroacetyl derivatives of propranolol and eight other β-blocking drugs, prepared according to procedures described under *Preparation of Derivatives*, were analyzed in triplicate, either alone with 1-bromonaphthalene as the reference compound or in mixtures. The electron affinity is expressed as the minimum detectable quantity in moles per second × 10^{-16} (10). The minimum detectable quantity, defined as the quantity that gives a signal three times the background noise level, was determined for 1-bromonaphthalene (2.6×10^{-16} mole/sec).

The response of the trifluoroacetylated drugs was determined in relation to this compound. Detector cell temperatures ranging from 200 to 270° did not significantly affect the sensitivity. The effect of the detector cell temperature on the electron-capture detector response was reviewed by Wentworth and Chen (13). The higher detector temperature, 270°, reduced contamination of the detector.

Oxprenolol and Oxprenolol Metabolites in Rat Urine—One-milliliter urine samples from rats treated with 10 mg of oxprenolol hydrochloride intraperitoneally were hydrolyzed with β-glucuronidase and sulfatase[16] (2). Ten microliters of these hydrolysates was taken for analysis following the single-extraction procedure but extraction was with ethyl acetate at pH 10.

RESULTS AND DISCUSSION

Preparation of Derivatives—Trifluoroacetylation of propranolol with trifluoroacetic anhydride, as described in the *Experimental* section, yields a derivative containing two trifluoroacetyl groups in the side chain:



This structure has been confirmed by GLC–mass spectrometry (14). The derivative has excellent GLC properties and gives a very high response on an electron-capture detector. The minimum de-

[15] Polytron, Brinkmann Instruments, Inc., Westbury, N.Y.
[16] Glusulase, Endo Laboratories, Garden City, N.Y.

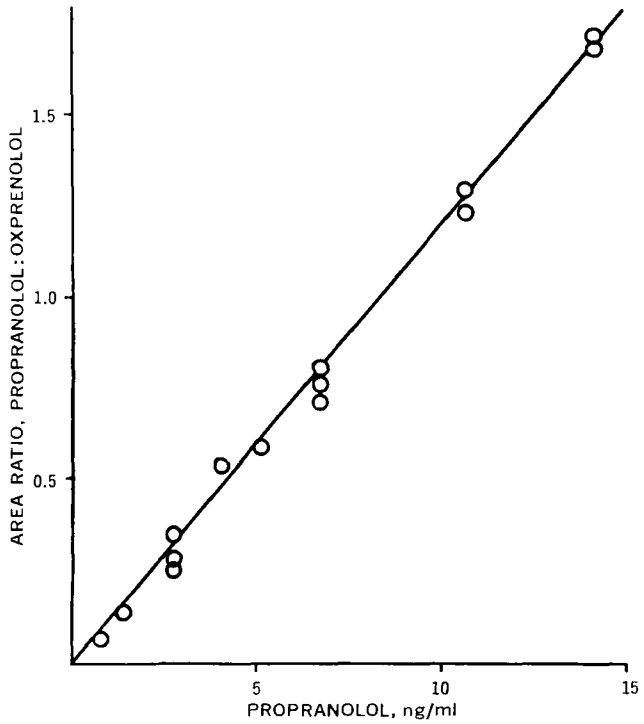


Figure 1—*Standard curve for the determination of propranolol in plasma. Single-extraction procedure for concentrations of 5–15 ng/ml, and back-extraction procedure for concentrations of 0.5–4 ng/ml, with oxprenolol as the internal standard. Each point represents the mean of two to five independent determinations. The curve is obtained by the method of least squares from a total number of 38 determinations.*

tectable quantity (10) is 2.0×10^{-16} mole/sec, corresponding to 0.5 pg under the GLC conditions used.

Heptafluorobutyric anhydride[2] as a reagent yielded the corresponding derivative with two heptafluorobutyryl groups in the side chain. This derivative was only slightly more sensitive, 0.9×10^{-16} mole/sec. This finding agrees with sensitivities reported for other similar trifluoroacetyl and heptafluorobutyryl derivatives of bifunctional molecules (10, 15).

Heptafluorobutyric anhydride was selected as the reagent for propranolol by Di Salle *et al.* (9). Trifluoroacetylation in this study, however, provided much higher selectivity and thereby

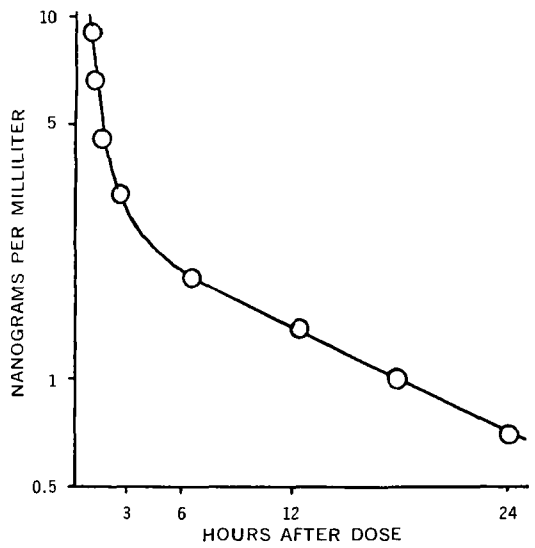


Figure 2—*Plasma propranolol levels after a 3.3-mg iv dose, 0.05 mg/kg, in a 72-year-old man. Each point represents the mean of three determinations.*

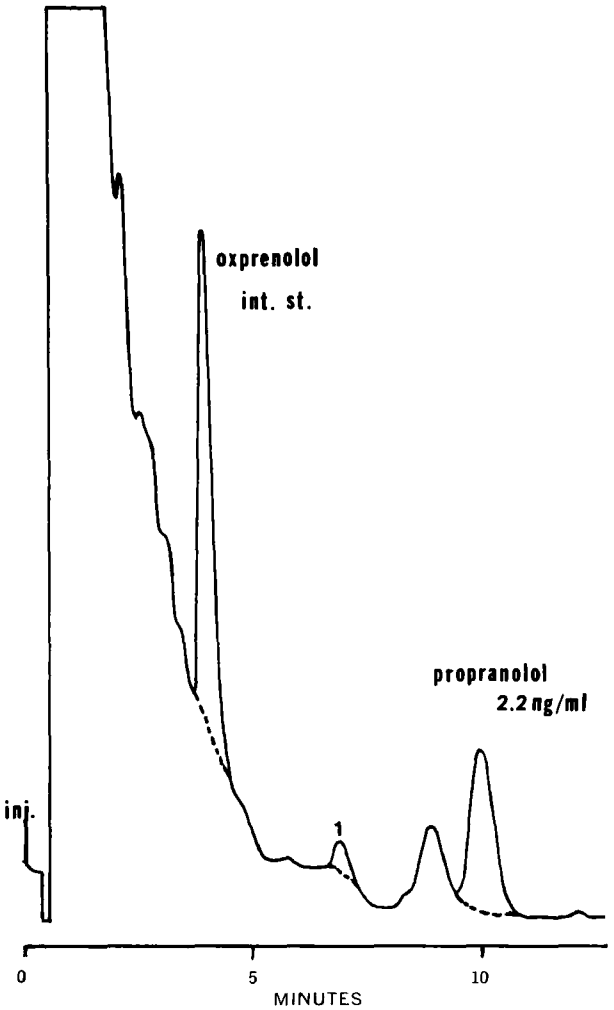


Figure 3—*Gas chromatogram of a plasma extract (back extraction) obtained 6 hr after the dose from the patient in Fig. 2. 1 = N-desisopropylpropranolol. Dashed lines represent the background noise from control plasma.*

higher sensitivity than heptafluorobutyrylation for determinations in complex biological extracts. Coextracted endogenous monofunctional amino, alcoholic, or phenolic compounds with heptafluorobutyric anhydride yield derivatives that have a high response on an electron-capture detector (10, 16) while the response of the corresponding derivatives with trifluoroacetic anhydride is only 1/100 to 1/1000, making the trifluoroacetyl derivatives less likely to interfere (15, 16).

The trifluoroacetylation reaction is quantitative in less than 5 min using trimethylamine as the catalyst. The effect of trimethylamine on the acylation of primary and secondary amines, alcohols, and phenols was described earlier (10, 16). Excess reagent is removed by shaking the reaction mixture with a pH 6.0 phosphate buffer (16). Under these conditions, the derivative is stable for at least 24 hr.

Interference in the derivatization reaction by free acid in the trifluoroacetic anhydride reagent, as earlier reported for heptafluorobutyric anhydride (10), was observed. By careful handling of the trifluoroacetic anhydride reagent according to the *Experimental* section, this problem can be avoided.

Plasma Propranolol Determinations—Propranolol is quantitatively (>95%) extracted from plasma at high pH with benzene. Oxprenolol, used as internal standard, was added to the plasma before extraction. This procedure (single extraction) including the GLC separation takes 45 min, permitting analysis of 12–15 samples/day on a single-column instrument. For very low concentrations (<5 ng/ml), a back-extraction clean-up procedure may be necessary. The back-extraction clean-up procedure removes neutral compounds from the initial benzene extract that may interfere

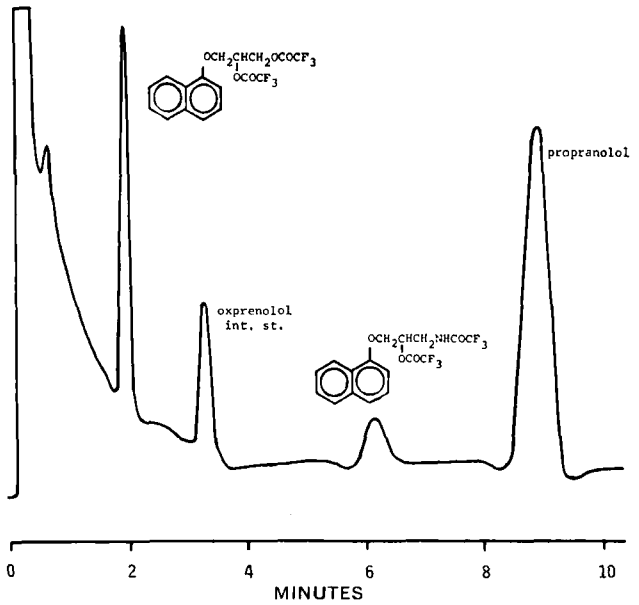


Figure 4—*Gas chromatogram of a heart tissue extract (single extraction) obtained from a propranolol-treated blood-perfused dog heart–lung preparation. Two metabolites, 1-(α-naphthoxy)-2,3-propylene glycol and* N*-desisopropylpropranolol, are shown.*

in the analysis and is particularly valuable for the analysis of plasma samples from patients taking numerous drugs. The back-extraction procedure including GLC takes about 70 min.

Figure 1 shows the standard curve obtained for propranolol determinations in plasma with oxprenolol as the internal standard, following both the single-extraction procedure (5–15 ng/ml) and the back-extraction procedure (0.5–4 ng/ml). The results obtained from the two procedures fit the same standard curve. The curve is linear within a 30-fold range and goes through the origin. The minimum detectable concentration is about 0.1 ng/ml of plasma.

The precision of the method after repeated analysis is about ±15% at the 3-ng/ml level (back extraction, $n = 8$) and about ±8% at the 25-ng/ml level (single extraction, $n = 8$). The standard curve can be extended to 500 ng of propranolol/ml of plasma by limiting the amount of trifluoroacetyl derivative injected to 800 pg (see *Experimental*).

By using this method, the plasma levels of propranolol were followed for 24 hr in a 72-year-old patient receiving 3.3 mg of propranolol (0.05 mg/kg) intravenously (Fig. 2). Propranolol was still detectable (0.7 ng/ml) 24 hr after the administration. The calculated plasma half-life of 12 hr greatly exceeds that reported for normal volunteers (5). Whether this unusually long half-life is related to the physical condition of the patient or to the use of the more sensitive method, permitting a more accurate determination of the second phase of the biphasic elimination curve, is not known.

A GLC tracing of a 6-hr plasma sample (back extraction) from this patient is shown in Fig. 3. In addition to propranolol and the internal standard, a major metabolite of propranolol in blood, *N*-desisopropylpropranolol (2), is recognized. Criteria of identity of propranolol include retention time and characteristic molecular ion and base peak by multiple-ion detection GLC–mass spectrometry. Other peaks in the chromatogram are normal constituents of human plasma under the conditions used. The absence of interfering compounds was tested by repetitive scanning analysis.

Table II demonstrates plasma concentrations in six patients treated chronically with 120–320 mg of propranolol hydrochloride orally per day[17]. The patients were treated with numerous other drugs as well. Concentrations ranging from 26 to 77 ng/ml were obtained with the back-extraction method, with a precision ranging from 2.8 to 10%. No interferences from other drugs were observed.

Propranolol and Metabolites in Tissues—The method is applicable to tissue as well as to plasma samples. Figure 4 shows a gas chromatogram of a heart tissue extract. The tissue was obtained from a propranolol-treated (10 mg of propranolol hydrochloride) blood-perfused dog heart–lung preparation (12). In addition to propranolol, two pharmacologically active propranolol metabolites, 1-(α-naphthoxy)-2,3-propylene glycol (4) and *N*-desisopropylpropranolol[18], were detected. Criteria of identity for these two metabolites include retention times and the presence of characteristic molecular ions and base peaks by multiple-ion detection GLC–mass spectrometry. The presence of the two metabolites reflects extrahepatic metabolism (12).

Two trifluoroacetyl groups are incorporated in these metabolites, giving them an electron-capture detector response the same as or higher than propranolol. This should be true for any metabolite of propranolol containing either an alcohol and an amino or two alcohol groups in the side chain (2, 17). Noticeable too is the fairly clean GLC tracing of heart tissue, indicating very little interference from endogenous material. Propranolol and the two metabolites have also been measured in a rat liver microsomal preparation using the same method (18).

Preliminary investigations demonstrated that two other metabolites of propranolol, the active 4-hydroxypropranolol (3) and the ring hydroxylated 1-(α-naphthoxy)-2,3-propylene glycol, can also be determined using a modification of the same method.

Applications to Other β-Blocking Drugs and Their Metabolites—Trifluoroacetylation of several other β-blocking drugs, using the same derivatization conditions as for propranolol, yielded derivatives with high electron-capture detector response. Retention times and minimum detectable quantity of these derivatives are given in Table I. The peak symmetry was excellent even

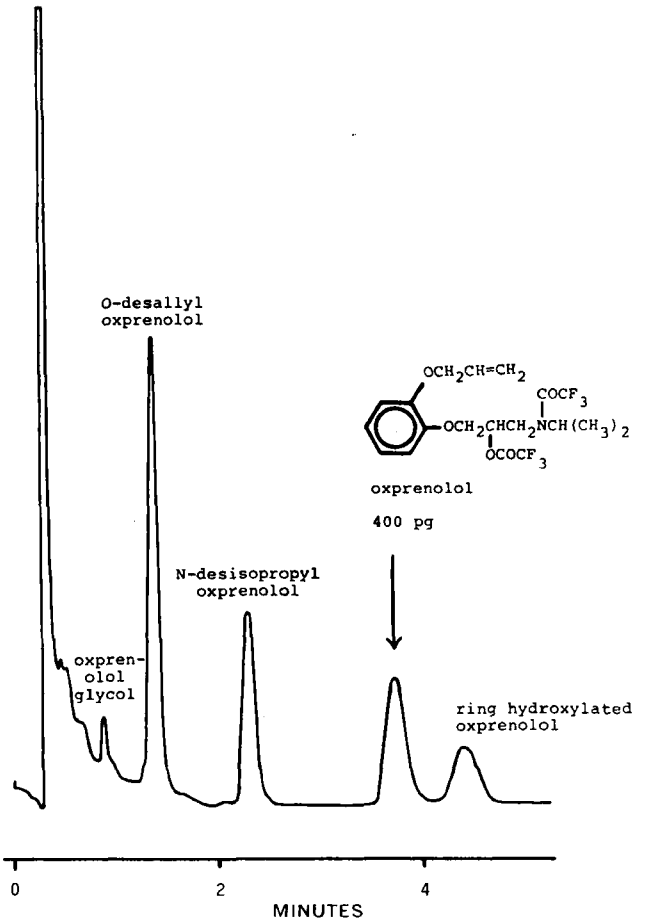


Figure 5—*Gas chromatogram of an extract (single extraction, pH 9) of hydrolyzed urine (10 μl) obtained from a rat administered 10 mg of oxprenolol hydrochloride. For identification of metabolites, see Ref. 1.*

[17] T. Walle, E. Conradi, K. Walle, and T. Gaffney, to be published.

[18] Pharmacological activity. J. Pruett, unpublished data from this laboratory.

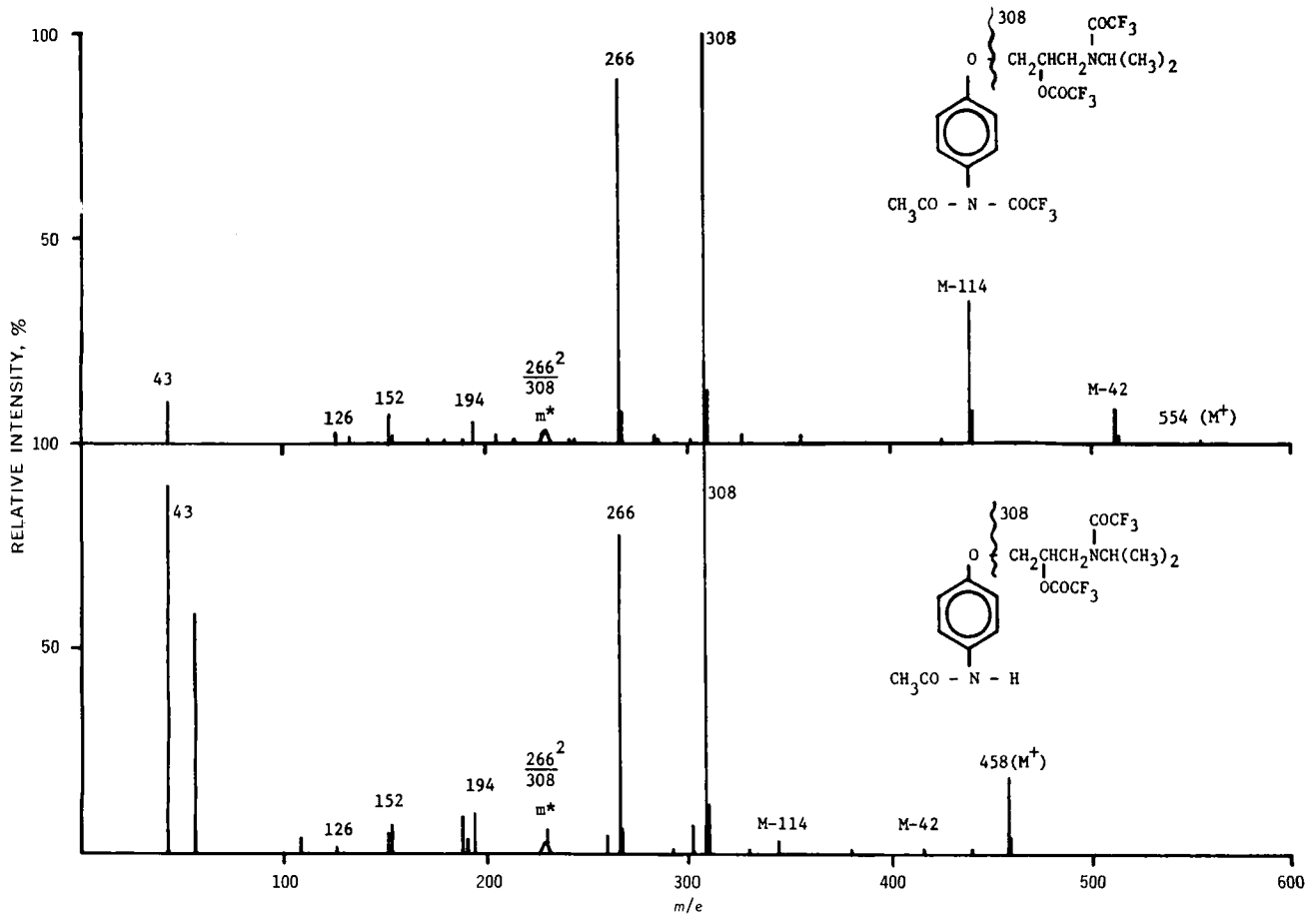


Figure 6—*Normalized mass spectra of the di- (bottom) and tri- (top) trifluoroacetyl derivatives of practolol.*

in low picogram quantities. Dichloroisoproterenol, with the contribution of its two chlorine atoms, could be detected down to 0.1 pg. Minimum detectable amounts for the other β-blocking drugs had a narrow range of 0.5–1.1 pg, demonstrating the trifluoroacetylated β-blocking side chain to be the single most important contributor to the high electron-capture response. The chemical structures of most of these derivatives were confirmed earlier by GLC–mass spectrometry (14).

Isothermal separation of all nine β-blocking drugs in Table I could be accomplished in about 10 min, with the shortest retention time (1.48 min) for 1-(*m*-toloxy)-3-isopropylamino-2-propanol. Separation factors of more than 1.10 were obtained for all of these drugs except dichloroisoproterenol–1-(*m*-toloxy)-3-isopropylamino-2-propanol (1.07) and practolol–sotalol (1.03).

The extraction conditions described for propranolol from plasma and tissues can also be applied to alprenolol, 1-(*p*-allylphenoxy)-3-isopropylamino-2-propanol, oxprenolol, 1-(*m*-toloxy)-3-isopropylamino-2-propanol, pronethalol, and dichloroisoproterenol. It can also be applied to metabolites of these drugs, if optimum pH for their extraction is considered. Thus, picogram quantities of oxprenolol and its four major urinary metabolites in rats (ring hydroxylated oxprenolol, *N*-desisopropyloxprenolol, *O*-desallyloxprenolol, and a glycolic oxprenolol metabolite) have been simultaneously determined after extraction with ethyl acetate at pH 10 (Fig. 5). The chemical structures of these metabolites were described in other studies (1)[19].

Fast (<5 min) and quantitative derivatization of practolol and sotalol required larger quantities of both trimethylamine [75 μl of trimethylamine (1 *M*) in benzene] and trifluoroacetic anhydride (75 μl). Under these conditions, practolol formed a tri-trifluoroacetyl derivative with the third trifluoroacetyl group incorporated in the acetamide moiety (Table I). The mass spectrum of this derivative is shown in Fig. 6 (top half). The molecular ion at *m/e* 554 is very weak. Distinct fragment ions are seen at *m/e* 512, M − 42 (CH_2CO), and at *m/e* 440, M − 114 (CF_3COOH). The base peak at *m/e* 308 and most of the remaining fragment ions are characteristic of all aryloxy β-blocking drugs (14).

This derivative is stable for at least 8 hr under the conditions used. Prolonged storage (>24 hr) of the derivative in contact with the pH 6.0 buffer quantitatively yields the corresponding di-trifluoroacetyl derivative (Table I). Figure 6 (bottom half) shows the mass spectrum of this derivative. The molecular ion at *m/e* 458 is prominent. Characteristic ions at *m/e* 416, M − 42 (CH_2CO), and at *m/e* 344, M − 114 (CF_3COOH), are obtained (*cf.*, spectrum on top) in addition to ions characteristic of the β-blocking side chain (14). Note the large difference in retention times between the di- and tri-trifluoroacetyl derivatives (Table I) and also the similarity in electron-capture response.

Like practolol, sotalol forms a tri-trifluoroacetyl derivative with the third trifluoroacetyl group incorporated in the sulfonamide moiety (Table I). Its mass spectrum is shown in Fig. 7. The molecular ion at *m/e* 560 is weak. Distinct fragment ions are obtained at *m/e* 481, M − 79 (CH_3SO_2), and at *m/e* 446, M − 114 (CF_3COOH). The base peak at *m/e* 168 is a result of α-cleavage of the major side chain. The tri-trifluoroacetyl derivative of sotalol is stable in contact with pH 6.0 buffer for at least 8 hr. Extended storage (>24 hr) results in partial hydrolysis, loss of the trifluoroacetyl group from the sulfonamide moiety, and loss of trifluoroacetic acid from the β-blocking side chain.

Since practolol and sotalol are considerably less lipophilic than the other β-blocking drugs, neither compound can be quantitatively extracted with benzene. Both can, however, be extracted with ethyl acetate.

SUMMARY

A highly sensitive and specific electron-capture GLC method for propranolol was described. The drug is extracted from plasma fol-

[19] D. Garteiz, Division of Clinical Pharmacology, University of Cincinnati College of Medicine, Cincinnati, OH 45219, personal communication.

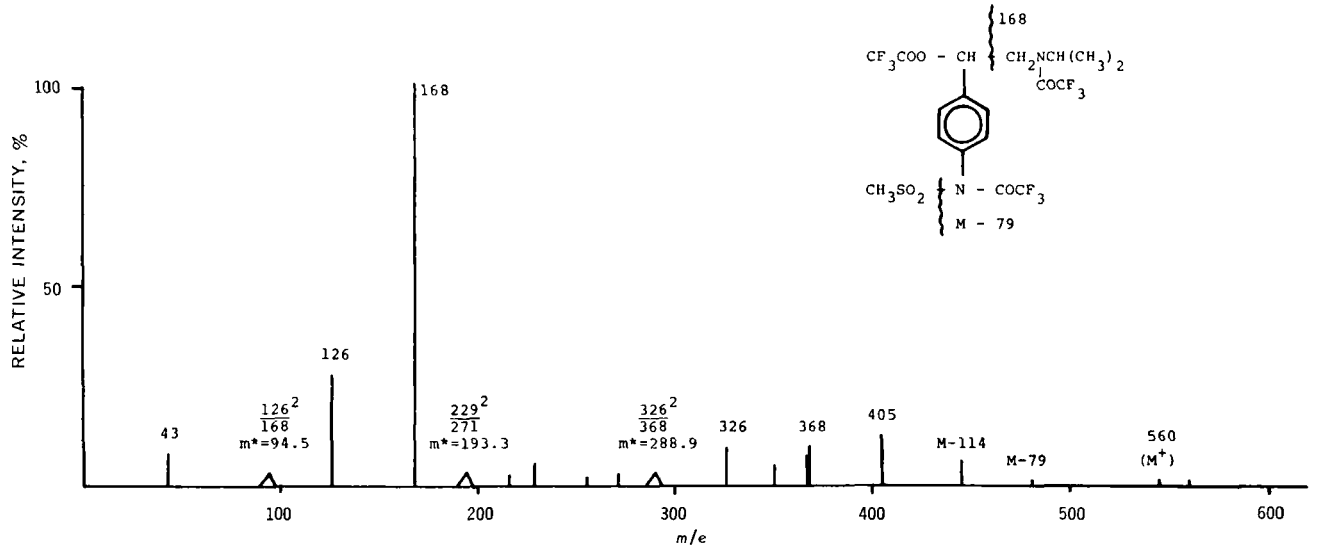


Figure 7—*Normalized mass spectrum of the tri-trifluoroacetyl derivative of sotalol.*

lowing either a single- or a back-extraction procedure, with oxprenolol as the internal standard. Extracted propranolol and internal standard are separated and detected as their di-trifluoroacetyl derivatives. The minimum detectable amount of propranolol is 0.5 pg. The minimum detectable concentration of propranolol in plasma is 0.1 ng/ml. The standard curve is linear from 0.5 to 500 ng/ml.

Applications of the method demonstrated quantitatively detectable plasma propranolol levels 24 hr after a small (0.05 mg/kg) intravenous dose. Accurate plasma determinations could be made in patients receiving numerous other drugs. The method is applicable to propranolol and two metabolites, 1-(α-naphthoxy)-2,3-propylene glycol and *N*-desisopropylpropranolol, in heart tissue.

The electron-capture response and retention times were also reported for eight other β-blocking drugs. Alprenolol, 1-(*p*-allylphenoxy)-3-isopropylamino-2-propanol, oxprenolol, 1-(*m*-toloxy)-3-isopropylamino-2-propanol, pronethalol, and dichloroisoproterenol form di-trifluoroacetyl derivatives. Practolol and sotalol form tri-trifluoroacetyl derivatives with incorporation of the third trifluoroacetyl group in the acetamide and sulfonamide moieties, respectively. The chemical structures of these derivatives were confirmed by GLC–mass spectrometry. Minimum detectable amounts of these derivatives by electron-capture detection ranged from 0.1 to 1.1 pg. The method for propranolol was shown to be applicable to the separation of picogram quantities of oxprenolol and four of its major urinary metabolites.

These results strongly indicate that the method described is generally applicable to quantitative determinations of all β-blocking drugs and most of their metabolites in body fluids and tissues. Trifluoroacetylation of biological extracts has already proved most valuable in the identification of numerous propranolol and oxprenolol metabolites by GLC–mass spectrometry (1, 2, 4, 18).

The high sensitivity of the method will permit more accurate half-life determinations, particularly after low doses, and will permit analysis of very small sample sizes, *e.g.*, serial blood samples from infants or biopsy samples. It should be possible to do complete pharmacokinetic studies of this entire chemical and pharmacological class of compounds, including their metabolites. Application of the method could lead to a better understanding of the complex metabolic disposition and pharmacology of this class of compounds and may be a useful aid in clinical drug therapy.

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Present address: Department of Pharmacology, Medical University of South Carolina, Charleston, SC 29401

Evaluation of Telemetry in Determining Toxicity of Aerosol Preparations

M. A. DORATO, C. O. WARD [x], and J. J. SCIARRA

Abstract □ The effect of selected aerosol products and fluorinated hydrocarbon propellants on the heart rate and respiration of rats was determined in whole body exposure chambers by biotelemetry. Electrocardiogram and respiratory transmitters were used to broadcast selected FM signals to a nearby receiver and physiological recorder for visual display. Each rat was subjected to a single 360-min exposure of the aerosol product or propellant, kept under observation for toxicological responses, and then examined for histopathological signs of toxicity. The fluoroalkane propellants used depressed the heart rate in high concentrations. First degree atrioventricular block and T wave inversion were also observed in some animals. The propellants tested also had a depressant effect on respiratory rate and minute volume. The selected aerosol product produced similar effects but at concentrations much higher than would be experienced during normal consumer use. Histopathological examination revealed greater tissue damage with the aerosol product than with the propellants alone. The cardiac and respiratory abnormalities occurred only after exposure to high concentrations of the fluoroalkane propellants. Also, the pathology report gave no indication of the existence of "thesaurosis" or pulmonary granulomatosis.

Keyphrases □ Telemetry—evaluation as technique for determining toxicity of aerosol preparations □ Toxicity, aerosols—evaluation of telemetry as technique for determination □ Aerosols—evaluation of telemetry for determining toxicity □ Propellants, aerosols—evaluation of telemetry for determining toxicity □ Fluorinated hydrocarbons—use of telemetry in evaluating toxicity of aerosol preparations

In the last decade there has been a significant increase in the marketing and use of aerosol products for medicinal, cosmetic, and household purposes. This has led to the occasional questioning, by both consumer and professional organizations, of the safety of the various aerosol preparations commercially available. Recently, charges of cardiac and pulmonary toxicity have been leveled at certain commercial aerosol preparations. The ability of any particular product to produce injury, and the risk or probability that the injury will occur, must be carefully considered in the toxicological evaluation of commercial aerosols.

Bass (1), for example, reported several cases of sudden "sniffing death" from aerosol abuse, while Taylor and Harris (2) demonstrated that asphyxia could lead to cardiac arrhythmias in animals following the inhalation of some aerosol propellants. Similar toxic effects were also reported for some cosmetic aerosols. Flowers and Horan (3) reported arrhythmias in dogs following inhalation of fluoroalkane propellants despite careful maintenance of normal arterial oxygen tension and pH. Kilen and Harris (4) reported a direct depressant effect of dichlorodifluoromethane on the contractility *in vitro* of the isolated rat papillary muscle.

In addition, Reinhardt *et al.* (5) reported that the cardiac sensitization was a possible mechanism for the aerosol sniffing deaths. In subsequent experiments, Taylor *et al.* (6) proposed that either sensitization of the myocardium to catecholamines or a direct depressant action on the myocardium might explain the cardiotoxicity of the fluorocarbon propellants.

Table I—Particle-Size Distribution of Antiperspirant Preparation

Diameter of Particle, μm	Mass of Collected Material, g	Cumulative Mass, g	Cumulative Weight, %
1	0.0416	0.0024	1.895
2	0.0254	0.0088	6.932
4	0.0327	0.0269	21.309
8	0.0182	0.0597	47.134
16	0.0064	0.0851	67.156
32	0.0024	0.1267	100.00

It is well known that the deposition of aerosols in the respiratory tract is a function of several variables (7–10): particle size and shape, density of particles, hygroscopicity, anatomical pattern of the respiratory tract of the test animal, and pathological changes in respiratory patterns (11). All of these variables must be considered when evaluating a possible test system or toxicological data concerning a particular aerosol product.

Bergman *et al.* (12) first indicated the possibility of a connection between the use of aerosols and granulomatous lesions in the lungs; their findings were later supported by others (13–15). Ward (16) indicated that certain aerosol deodorants might be the cause of pulmonary lesions in susceptible users.

Other workers (17–20), however, failed to produce any of these pulmonary lesions, thesaurosis, or granulomatosis in experimental animals. Many of the variables between experimental animals and humans could explain these discrepancies. There are obvious differences in the anatomy and physiology of the respiratory systems of small animals and humans (15). In general, the dimensions of the upper respiratory structures decrease and the tortuosity increases as the size of the animal decreases. The ratio of surface area to volume of the upper respiratory tract also increases. Therefore, the inherent efficiency of the upper respiratory tract in trapping small particles increases as the animal becomes smaller.

Hatch and Gross (21) stated that the pulmonary air spaces are not remarkably smaller in small animals *versus* humans. The lesser lung volume in small animals is accounted for by fewer, rather than smaller, alveoli. However, because of the higher respiratory efficiency, the fraction of inhaled particles deposited in the pulmonary air spaces is lower in small animals for particles greater than 1 μm in diameter (22).

Table II—Mean Heart Rate and Mean Percent Change in Heart Rate of Rats following 360 min of Aerosol Exposure

Treatment[a]	Mean Heart Rate[b]	p Value[c]	Mean Percent Change in Heart Rate[d]	p Value
Dichlorodifluoromethane[e]	389.10 ± 23.50	0.02	−9.71 ± 5.28	0.001
Dichlorodifluoromethane–trichloromonofluoromethane[f]	399.91 ± 25.92	0.001	−7.99 ± 6.08	0.02
Antiperspirant[g]	384.00 ± 31.32	0.02	−7.54 ± 7.61	0.05
Control I	416.46 ± 6.75	—	−0.30 ± 1.62	—
Control II	444.28 ± 6.44	—	−0.96 ± 1.45	—

[a] Each group was composed of five animals. [b] Beats per minute ± standard deviation. [c] For significance, $p < 0.05$. [d] Percent change ± standard deviation. [e] Compared with Control I. [f] Compared with Control II. [g] Compared with Control I.

In humans, particles between 0.5 and 5.0 μm in diameter are the optimum size for deposition in the lung (7). As additional evidence, it would be desirable to reproduce the reported human lesions in laboratory animals; failure to do so, however, does not constitute proof that the material is harmless to humans (14).

Because of the apparent drawbacks in methodology used by these and other investigators to record cardiovascular and respiratory function during aerosol exposure, the present investigation was undertaken to evaluate the potential of a new technique, telemetry, as a means for recording vital signs during the exposure of laboratory animals to propellants and/or specific aerosol products. It was hypothesized that telemetry could have possible advantages over the more common exposure techniques available and that experimentation was necessary to prove or disprove these possible advantages.

EXPERIMENTAL

The aerosol preparations used were prepared by the cold filling process (23). The antiperspirant preparation used was prepared according to the following formula (24) (percent w/w): aluminum chlorhydroxide[1], 35.0; hexachlorophene[2], 2.0; isopropyl myristate, 56.0; fumed silica[3], 6.0; and butylated hydroxytoluene, 1.0.

The aerosol consisted of 10% by weight of concentrate and 90% by weight of dichlorodifluoromethane[4]–trichloromonofluoromethane[5] (50:50). Before pressurizing, a magnetic stirring bar was placed in the container to facilitate mixing. The particle-size distribution of this preparation was determined using an impaction technique (25, 26)[6]. The spray rate was determined by a weight-by-difference method.

The exposure apparatus consisted of a 30.48 × 30.48 × 30.48 cm (1 cu ft) chamber. The air flow through the chamber was adjusted to approximately 2.5 liters/min. An automatic aerosol delivery device (27) mounted on a magnetic stirrer served both to activate and to mix the aerosol preparations.

Male Sprague–Dawley rats (200–300 g) were trained to wear a 2.54-cm wide elastic harness (28), containing either an electrocardiogram (ECG) or respiratory transmitter. In addition to the preconditioning, a period of adjustment was required to allow the animals to adapt to the transmitter, harness, and chamber. Sixty minutes was found to be appropriate for this purpose.

The animals were exposed individually to the various test agents. The automatic aerosol delivery apparatus was adjusted to deliver a 7.5-sec spray every 10 min for 6 hr (18, 29). The animals were exposed to three preparations: an antiperspirant preparation, dichlorodifluoromethane, and a blend of dichlorodifluoromethane–trichloromonofluoromethane (50:50). During the exposure, either ECG or respiratory recordings were made. The animals were observed for weight changes and other toxicological manifestations for 7 days; they were then sacrificed and selected tissues were excised for histopathological examination.

An additional group of male Sprague–Dawley rats was exposed individually three times a day to a 15-sec spray of the aerosol antiperspirant for 30 days. The animals were exposed singly and allowed to remain in a sealed chamber for 15 min following each spray (17, 30). These animals were observed for weight changes.

Analysis of the fluoroalkane propellant concentration in the chamber was determined by GC[7], using a thermal conductivity cell. The injection port temperature was set at 140°, the detector temperature was 158°, the filament current was 200 mamp, and the attenuator was set at 2×. Helium was used as the carrier gas at a flow of 40 ml/min. Two glass columns were used. The first was 2.4 m × 0.3 cm (8 ft × 0.125 in.), packed with 10% polyethylene glycol 20M on 80–100-mesh Aerograph A-3-55. The second was 1.8 m (6 ft) of 80–100-mesh Poropak Q. The columns were operated at a temperature of 120°. A strip-chart recorder was used at a chart speed of 1.7 cm (0.66 in.)/min.

RESULTS AND DISCUSSION

GC analysis yielded a chamber concentration of 304.54 mg/liter for the propellant blend of dichlorodifluoromethane–trichloromonofluoromethane (50:50), 382.51 mg/liter for dichlorodifluoromethane, and 219.69 mg/liter for the propellant contained in the antiperspirant preparation.

The particle-size distribution of the antiperspirant preparation is shown in Table I. Based upon the cumulative weight percent, the mass median diameter of the particles is approximately 8 μm. Approximately 21% of the particles are 4 μm in diameter or smaller and, therefore, are in the respirable range (7).

The effects of impingement of particulate matter on the fur of experimental animals should be considered in every inhalation experiment; it probably explains the excessive lowering of concentration that occurs when the volume of animal used is in excess of 5% of the chamber volume (31). Calandra and Kay (17), for example, reported that experimental animals weighing 200–300 g occupy a volume between 1 and 2 liters. Thus, to keep the ratio of chamber to animal volume as large as possible, the rats were exposed individually in this study.

A temperature range of 21–23° and a relative humidity of approximately 50% have been suggested as suitable exposure conditions for most animals; rats and mice thrive at somewhat higher temperatures (32). Thus, the conditions of this study adhered as closely to these guidelines as possible.

The effects of the fluoroalkane propellants on heart rate are shown in Table II. The dichlorodifluoromethane and the antiperspirant groups were compared with Control I, and the rats exposed to the dichlorodifluoromethane–trichloromonofluoromethane blend were compared with Control II. All treatment groups exhibited a significant percent decrease in heart rate. When compared with the antiperspirant group, dichlorodifluoromethane and dichlorodifluoromethane–trichloromonofluoromethane did not significantly decrease the heart rate on a percent basis (p values of <0.6 and <0.9, respectively).

[1] Chlorhydrol, Impalable, Reheis Chemical Co., Berkeley Heights, N.J.
[2] Since the time this study was initiated, the Food and Drug Administration has placed restrictions on the use of this compound.
[3] Cab-O-Sil M-5, Cabot Corp., Boston, Mass.
[4] Propellant 12.
[5] Propellant 11.
[6] Cascade Impactor, model CI-S-6, Scientific Advances, Inc., Columbus, Ohio.
[7] Aerograph model 350 B chromatograph.

Table III—Mean Percent Change in Respiratory Rate and Minute Volume of Rats following 360 min of Aerosol Exposure

Treatment[a]	Mean Percent Change in Respiratory Rate[b]	p Value[c]	Mean Percent Change in Minute Volume[b]	p Value[c]
Dichlorodifluoromethane	−3.57 ± 3.68	0.5	−3.52 ± 3.69	0.4
Dichlorodifluoromethane–trichloromonofluoromethane	−7.42 ± 4.08	0.05	−7.29 ± 4.13	0.02
Antiperspirant	−4.11 ± 2.89	0.3	−4.09 ± 2.90	0.2
Control	−1.99 ± 3.52	—	−1.58 ± 3.67	—

[a] Each group was composed of five animals. [b] Mean percent change ± standard deviation. [c] For significance, $p < 0.05$.

Table IV—Effects of Aerosol Propellants and a Selected Aerosol Product on 7-Day Weights of Rats following a Single 360-min Aerosol Exposure

Treatment[a]	Mean Weight[b]	p Value	Mean Percent Daily Weight Gain[b]	p Value[c]	Mean Percent Change[b]	p Value[c]
Dichlorodifluoromethane	263.07 ± 22.50	0.7	3.5 ± 1.9	0.7	13.4 ± 9.7	0.7
Dichlorodifluoromethane–trichloromonofluoromethane	258.74 ± 22.75	0.4	3.5 ± 1.7	0.6	12.9 ± 9.9	0.8
Antiperspirant	259.80 ± 8.51	0.3	−1.5 ± 2.5	0.01	−4.1 ± 3.1	0.001
Control	269.07 ± 19.96	—	3.0 ± 1.6	—	11.2 ± 8.2	—

[a] Each group was composed of five animals. [b] Mean ± standard deviation. [c] For significance, $p < 0.05$.

The fluoroalkane propellants produced significant changes in heart rate in concentrations of approximately 200, 300, and 400 mg/liter, with the 50:50 combination of dichlorodifluoromethane–trichloromonofluoromethane producing the most significant decrease in heart rate ($p < 0.001$).

The control heart rates were shown to vary slightly, as might be expected (28) when measuring heart rate for prolonged time intervals. Mean decreases in heart rate of 0.30 ± 1.62 and 0.96 ± 1.45% were shown for Controls I and II, respectively, after 360 min in the exposure chamber. The rats exposed to dichlorodifluoromethane showed a mean decrease in heart rate of 9.71 ± 5.28%, while those exposed to the propellant blend and the antiperspirant preparation showed mean decreases in heart rate of 7.99 ± 6.08 and 7.54 ± 7.61%, respectively.

The animals exposed to the antiperspirant preparation showed the greatest overall depression in heart rate, 18.02%. The group exposed to dichlorodifluoromethane exhibited a net decrease in heart rate of 11.04%, while those exposed to the propellant blend exhibited a net decrease of 16.21%. The toxicity of fluoroalkane propellants has been reported to vary inversely with the strength of the carbon–fluorine bond; the strength of the bond also varies with the number of fluorine atoms in the molecule (33).

In accordance with these findings, trichloromonofluoromethane was reported to be more toxic than dichlorodifluoromethane (34). The present study reflects this concept in that addition of trichloromonofluoromethane to dichlorodifluoromethane amplified the myocardial depression. However, neither pure propellant nor the propellant blend produced a significant decrease in heart rate ($p < 0.4$); the percent decrease in heart rate was also not significantly different ($p < 0.6$).

High concentrations of fluoroalkane propellants have been reported to produce anesthesia, narcosis, cardiac arrhythmias, cardiac sensitization, and death (5, 34–36). Bradycardia, cardiac arrhythmias typical of first degree A-V block, inversion of the T wave, loss of the P wave, lengthening of the QRS interval, and death were reported (37) as results of exposure to dichlorodifluoromethane–trichloromonofluoromethane (50:50) in concentrations of 200 and 400 mg/liter. Arrhythmias, bradycardia, and T wave depression were also reported by other investigators (2, 3, 6).

The present study agrees with these reports of bradycardia produced by the propellants, either alone or in combination. The bradycardia observed was consistent with the direct depressant effect of dichlorodifluoromethane on myocardial contractility reported by Kilen and Harris (4). The present study showed transient arrhythmias, which mainly occurred between 300 and 360 min of exposure. The observed increase in the P-R interval was likely a reflection of first degree A-V block (38). As the bradycardia continued, the QRS interval was increased over normal values. The P wave, which is recorded as soon as the impulse leaves the S-A node (39), disappeared, indicating diminution of atrial activation, such as S-A block or atrial fibrillation (40). An inverted T wave, which was only seen sporadically in response to the propellant blend, indicates abnormal conduction through the A-V node (39).

All rats in the treated groups exhibited ataxia and hypomotility after approximately 200 min of aerosol exposure. Only animals exposed to the propellant blend showed tremors, salivation, and a kyphotic position. Recovery always occurred within 5 min after removal from the exposure chamber, and no deaths were recorded during either the 360-min exposure or the 7-day observation that followed.

The effects of inhalation of either propellants or the antiperspirant formula on respiration are shown in Table III. Of the various treatments used, only the propellant blend produced significant changes in the rate and volume of respiration. As with heart rate, the control respiratory rate was shown to have a slight variation throughout the exposure period; the net decrease in respiratory rate was 1.99 ± 3.52%.

The propellant blend alone produced a significant decrease in respiratory minute volume ($p < 0.02$). The present study demonstrated a concentration-dependent decline in respiratory rate with the propellant blend, since 300 mg/liter but not 200 mg/liter produced a significant depression; no significant effects were produced by 400 mg/liter of dichlorodifluoromethane.

The effects of inhalation of the various aerosols on weight gain are shown in Tables IV and V. It is well known that weight loss can serve as an index of toxicity (41, 42). Only the antiperspirant preparation produced any significant weight loss ($p < 0.001$) or de-

Table V—Mean Starting and Ending Weights of Rats following 30 Days of Three Times a Day Aerosol Exposure

Treatment[a]	Mean Starting Weight[b]	p Value[c]	Mean Ending Weight[b]	p Value[c]
Antiperspirant	243.9 ± 10.5	0.5	299.6 ± 40.2	0.001
Control	249.0 ± 15.2	—	440.1 ± 55.1	

[a] Each group was composed of eight animals. [b] In grams ± standard deviation. [c] For significance, $p < 0.05$.

Table VI—Histopathological Examination of Selected Rat Tissue following Aerosol Exposure[a]

Pathological Diagnosis	Dichlorodifluoromethane[b]	Dichlorodifluoromethane–Trichloromonofluoromethane[b]	Antiperspirant[b]	Control[b]
Passive pulmonary congestion	0/5	0/5	0/5	2/5
Nonspecific hepatitis	0/5	0/5	0/5	1/5
Toxic hepatitis (possible)	0/5	0/5	4/5	0/5
Acute reactive splenitis	0/5	0/5	3/5	0/5
Interstitial nephritis	0/5	0/5	1/5	0/5
Acute liver congestion	0/5	0/5	1/5	0/5
Interstitial edema of the heart	0/5	0/5	1/5	0/5
Acute pulmonary congestion	0/5	0/5	2/5	0/5
Nonspecific pneumonitis	4/5	3/5	2/5	1/5
Bronchopneumonia	1/5	0/5	0/5	1/5

[a] Examinations performed by G. Forman, Director of Pathology, Central Health Laboratories, Wantagh, N.Y. [b] Number responding/number tested.

crease in weight gain ($p < 0.001$). Because the propellants themselves did not produce depression of weight gain, they cannot be implicated in the changes produced by the antiperspirant preparation. It follows, then, that particulate matter may produce a toxic sequela. The animals exposed to dichlorodifluoromethane and dichlorodifluoromethane–trichloromonofluoromethane exhibited a greater increase in weight gain than did the control group. This finding is consistent with the reported (42) weight gain increases in animals treated with fluoroalkane propellants.

The results of the histopathological examination of selected tissues are summarized in Table VI. In the opinion of many investigators (43, 44), there is no animal of choice for inhalation experiments. Small rodents are known, for instance, to be subject to respiratory infections, yet they are often used in inhalation experiments (2, 17). In this study, using rats, it was shown that the antiperspirant preparation produced the greatest histopathological changes. Some responses were toxic hepatitis, acute reactive splenitis, interstitial nephritis, acute congestion of liver, interstitial edema of the heart, acute pulmonary congestion, and nonspecific pneumonitis; no pulmonary granulomatosis was observed.

The animals exposed to the fluoroalkane propellants showed only a nonspecific pneumonitis; bronchopneumonia was reported in one rat exposed to dichlorodifluoromethane. The control group showed similar lung pathology; thus no significant pathology was found in rats exposed to the fluoroalkane propellants. This would appear to suggest that the propellants themselves do not produce pulmonary and systemic pathology in rats exposed to commercial aerosols, as has been suggested for other preparations (15).

CONCLUSIONS

The use of telemetry facilitates the measurement of heart and respiratory rates in the unrestrained animal. Furthermore, there are no trailing wires and the animals do not have to be removed from the test atmosphere for recordings to be made, as is often the case when vital functions are recorded in other ways. The latter advantage is very important since the test animals invariably recover from any visible effects of the aerosol product almost immediately when removed from the exposure chamber. Although a period of adjustment is necessary before the animals will easily accept the transmitter pack, once adaptation to the presence of the pack does occur, the only limitation to the length of continuous telemetric recording is the life of the batteries powering the transmitter.

These studies should in no way be construed to imply that aerosol products used by the consumer will produce similar toxic effects. The doses and techniques used were selected to produce toxicity so the value of telemetric monitoring could be evaluated. Thus, the doses and exposure periods are considerably higher than would likely be experienced by a consumer of any commercial aerosol.

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[x] To whom inquiries should be directed.

Mass Spectral Analysis of Medicinal Pyrazolidinediones

R. A. LOCOCK [x], R. E. MOSKALYK, L. G. CHATTEN, and L. M. LUNDY *

Abstract □ For the facilitation of mass spectral analysis of medicinal pyrazolidinediones, fragmentation pathways of phenylbutazone, oxyphenbutazone, and sulfinpyrazone were established by means of deuterium labeling, metastable peaks, and accurate mass determinations. The major pathways are the McLafferty rearrangement of the molecular ion and formation of azobenzene and substituted azobenzene ions. The mass spectra of 1,2-diphenyl-3,5-pyrazolidinedione and the 4-methyl and 4,4-dimethyl derivatives are also discussed.

Keyphrases □ Pyrazolidinediones, medicinal—mass spectra, fragmentation pathways, mechanisms □ Phenylbutazone—mass spectrum, fragmentation pathways, mechanisms □ Oxyphenbutazone—mass spectrum, fragmentation pathways, mechanisms □ Sulfinpyrazone—mass spectrum, fragmentation pathways, mechanisms □ Mass spectroscopy—medicinal pyrazolidinediones, fragmentation pathways

Although medicinal pyrazolidinediones such as phenylbutazone have received wide attention in the past 20 years, only a few reports of their mass spectra have appeared recently (1–3). This technique was used for the characterization of degradation products of phenylbutazone (1, 2), but no details of the fragmentation pathways of phenylbutazone or the degradation products were described.

The fragmentation patterns of phenylbutazone, oxyphenbutazone, and their *O*-methyl and C-methyl derivatives were described (3), but that investigation did not include verification by evidence of metastable ions, accurate mass determinations, or use of labeled derivatives.

This paper presents the mass fragmentation patterns of 1,2-diphenyl-3,5-pyrazolidinediones of medicinal importance: phenylbutazone, oxyphenbutazone, and sulfinpyrazone, as well as some simpler model derivatives. The establishment of characteristic mass spectral patterns and fragmentation schemes for these compounds may facilitate further characterization of pharmaceutical degradation products, metabolic studies, and forensic analysis of this important class of medicinals.

EXPERIMENTAL[1]

All mass spectra were recorded on a mass spectrometer[2] at an ionizing potential of 70 ev. The samples were introduced *via* the direct probe and were vaporized at temperatures between 150 and 200°. Accurate mass measurements were made by the peak matching technique.

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[2] AEI MS-9.

[3] Gift of Geigy Pharmaceuticals.

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[x] To whom inquiries should be directed.

Mass Spectral Analysis of Medicinal Pyrazolidinediones

R. A. LOCOCK [x], R. E. MOSKALYK, L. G. CHATTEN, and L. M. LUNDY *

Abstract □ For the facilitation of mass spectral analysis of medicinal pyrazolidinediones, fragmentation pathways of phenylbutazone, oxyphenbutazone, and sulfinpyrazone were established by means of deuterium labeling, metastable peaks, and accurate mass determinations. The major pathways are the McLafferty rearrangement of the molecular ion and formation of azobenzene and substituted azobenzene ions. The mass spectra of 1,2-diphenyl-3,5-pyrazolidinedione and the 4-methyl and 4,4-dimethyl derivatives are also discussed.

Keyphrases □ Pyrazolidinediones, medicinal—mass spectra, fragmentation pathways, mechanisms □ Phenylbutazone—mass spectrum, fragmentation pathways, mechanisms □ Oxyphenbutazone—mass spectrum, fragmentation pathways, mechanisms □ Sulfinpyrazone—mass spectrum, fragmentation pathways, mechanisms □ Mass spectroscopy—medicinal pyrazolidinediones, fragmentation pathways

Although medicinal pyrazolidinediones such as phenylbutazone have received wide attention in the past 20 years, only a few reports of their mass spectra have appeared recently (1–3). This technique was used for the characterization of degradation products of phenylbutazone (1, 2), but no details of the fragmentation pathways of phenylbutazone or the degradation products were described.

The fragmentation patterns of phenylbutazone, oxyphenbutazone, and their *O*-methyl and C-methyl derivatives were described (3), but that investigation did not include verification by evidence of metastable ions, accurate mass determinations, or use of labeled derivatives.

This paper presents the mass fragmentation patterns of 1,2-diphenyl-3,5-pyrazolidinediones of medicinal importance: phenylbutazone, oxyphenbutazone, and sulfinpyrazone, as well as some simpler model derivatives. The establishment of characteristic mass spectral patterns and fragmentation schemes for these compounds may facilitate further characterization of pharmaceutical degradation products, metabolic studies, and forensic analysis of this important class of medicinals.

EXPERIMENTAL[1]

All mass spectra were recorded on a mass spectrometer[2] at an ionizing potential of 70 ev. The samples were introduced *via* the direct probe and were vaporized at temperatures between 150 and 200°. Accurate mass measurements were made by the peak matching technique.

Phenylbutazone[3] (I), Oxyphenbutazone[3] (II), and Sulfinpyrazone[3] (III)—Compounds I–III had literature melting points and gave NMR and IR spectra consistent with their structures. Official assays gave values in excess of 98% for each compound. They were used without further purification.

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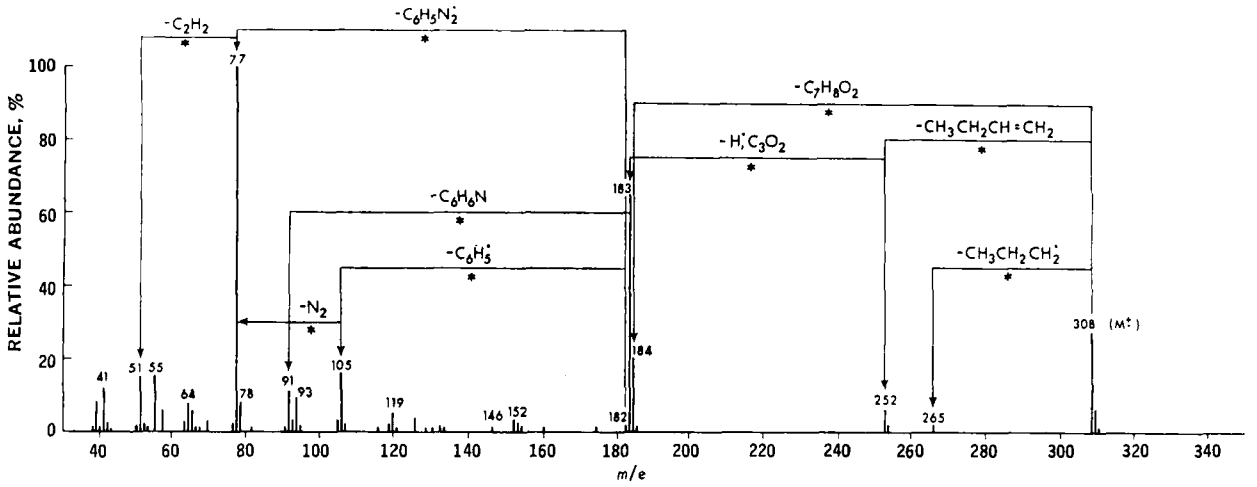


Figure 1—*Mass spectrum of phenylbutazone.*

1,2-Diphenyl-3,5-pyrazolidinedione (IV)—Compound IV was prepared following the method of Pesin *et al.* (4), mp 180–181° (ethanol) [lit. mp 173.5° (4) and 178° (5)]; IR (KBr): 1753 and 1722 (νC=O) cm^{-1}; NMR ($CDCl_3$): 2.68 (s, 10 p., phenyl) and 6.62 (s, 2 p., CH_2) τ.

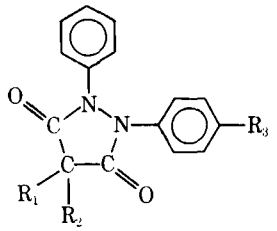
4-Methyl-1,2-diphenyl-3,5-pyrazolidinedione (V)—Compound V was prepared by an adaptation of the method reported for phenylbutazone (6). Sodium metal (0.9 g) was dissolved in 30 ml of anhydrous ethanol, and 7.5 g of methyl diethylmalonate and 6.4 g of 1,2-diphenylhydrazine were added. The mixture was refluxed for 12 hr, the progress of the reaction being followed by TLC (hexane–ethyl acetate, 2:1). The alcohol was distilled off and the residue was heated at 130° overnight.

After cooling, 30 ml of water was added and the resulting mixture was filtered. The filtrate was made acidic with 10% HCl, and the resulting precipitate was collected and recrystallized from 10% ethanol, mp 115° [lit. (7) mp 114–116°]; IR (KBr): 1753 and 1725 (νC=O) cm^{-1}; NMR ($CDCl_3$): 2.65 (s, 10 p., phenyl), 6.61 (q, 1 p., C-4 H), and 8.43 (d, 3 p., C-4 methyl) τ.

4,4-Dimethyl-1,2-diphenyl-3,5-pyrazolidinedione (VI)—Compound VI was prepared by a modification of the method described for the methylation of phenylbutazone (6). To a solution of 1.5 g of V in 40 ml of 1% alcoholic potassium hydroxide solution was added 3.1 g of methyl iodide. The resulting solution was refluxed with stirring for 3 days, after which time the solution turned a dark brown and a precipitate had formed. The precipitate was collected and recrystallized from 60% ethanol to give a white crystalline product, mp 134–135°; IR (KBr): 1755 and 1725 (νC=O) cm^{-1}; NMR ($CDCl_3$): 2.69 (s, 10 p., phenyl) and 8.52 (s, 6 p., C-4 methyl groups) τ.

Anal.—Calc. for $C_{17}H_{16}N_2O_2$: C, 72.84; H, 5.75; N, 9.99. Found: C, 72.58; H, 6.02; N, 9.84.

The deuterated Compounds I*a* and IV*a* were prepared by refluxing a solution of the parent compound in tetrahydrofuran containing deuterium oxide for 4 hr, followed by removal of the solvents *in vacuo*. The exchange was monitored by the disappearance of the C-4 proton absorption in the NMR.

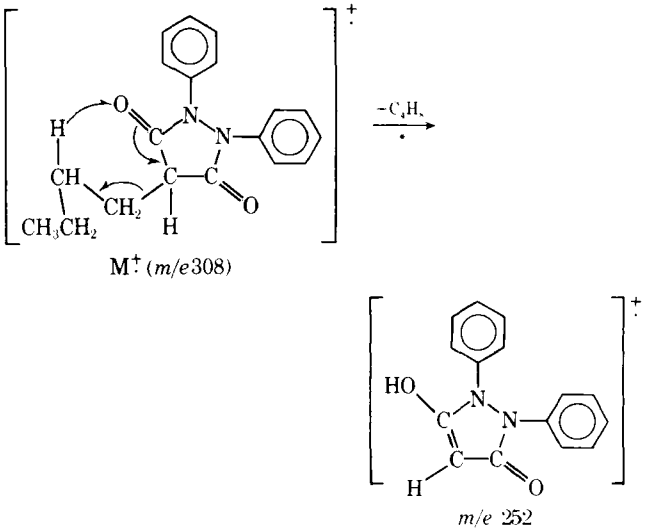


compound	R_1	R_2	R_3
phenylbutazone (I)	H	$CH_3CH_2CH_2CH_2$	H
phenylbutazone deuterated (I*a*)	D	$CH_3CH_2CH_2CH_2$	H
oxyphenbutazone (II)	H	$CH_3CH_2CH_2CH_2$	OH
sulfinpyrazone (III)	H	$C_6H_5SOCH_2CH_2$	H
1,2-diphenyl-3,5-pyrazolidinedione (IV)	H	H	H
1,2-diphenyl-3,5-pyrazolidinedione deuterated (IV*a*)	D	D	H
4-methyl-1,2-diphenyl-3,5-pyrazolidinedione (V)	H	CH_3	H
4,4-dimethyl-1,2-diphenyl-3,5-pyrazolidinedione (VI)	CH_3	CH_3	H

RESULTS AND DISCUSSION

The mass spectra of the pyrazolidinediones, I–VI, are reproduced in Figs. 1–6. As observed by Unterhalt (3), phenylbutazone, oxyphenbutazone, and sulfinpyrazone undergo the McLafferty rearrangement to give a radical ion at *m/e* 252 (*m/e* 268 in oxyphenbutazone). Metastable ions are present in all three spectra to indicate that this is a direct fragmentation from the molecular ion with the loss of the elements of butene or $C_6H_5SOCHCH_2$ for sulfinpyrazone. This fragmentation is presented in Scheme I for phenylbutazone.

A minor fragmentation pathway for the molecular ions of phenylbutazone and oxyphenbutazone, also substantiated by the presence of metastable ions, is the loss of a propyl radical from the butyl side chain of the molecular ion to give ions at *m/e* 265 (1.2%, phenylbutazone) or *m/e* 281 (3.2%, oxyphenbutazone). In the spectrum of sulfinpyrazone, strong peaks at *m/e* 279 (20.0%) and



Scheme I—McLafferty rearrangement of phenylbutazone

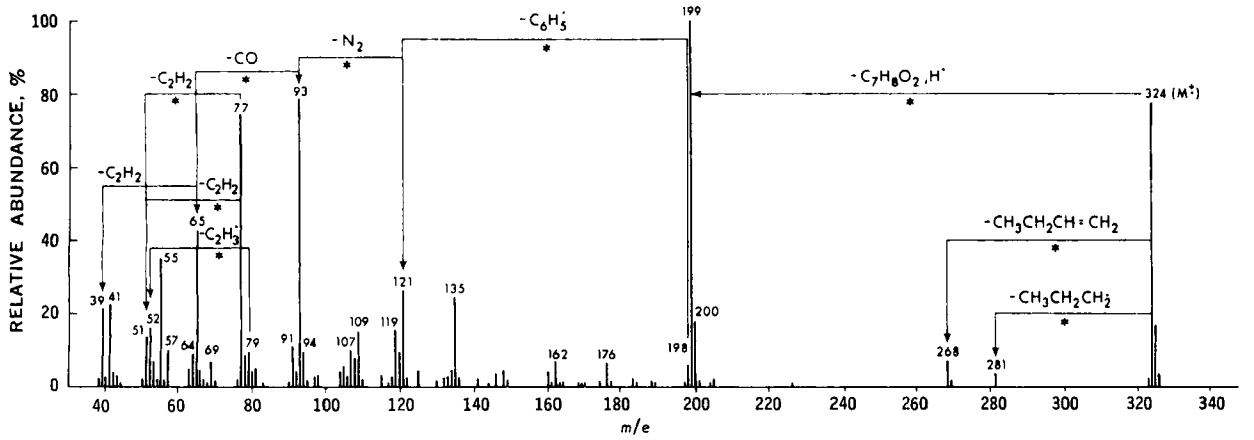


Figure 2—*Mass spectrum of oxyphenbutazone.*

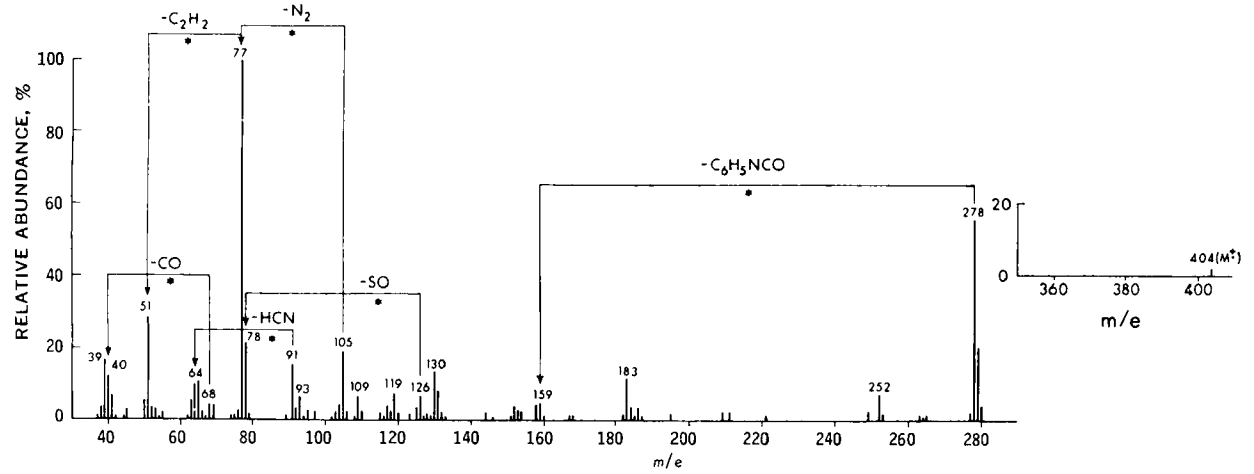


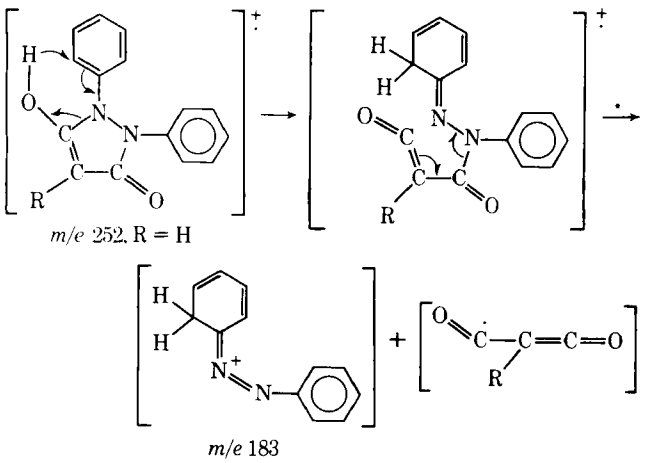
Figure 3—*Mass spectrum of sulfinpyrazone.*

at *m/e* 278 (55.4%) represent the loss of C_6H_5SO and C_6H_5SOH from the side chain of the sulfinpyrazone molecular ion. No metastable ion is present in the spectra for this fragmentation. An ion occurring at *m/e* 125 was identified as the phenylsulfoxide ion, $(C_6H_5SO)^+$, by accurate mass measurement (125.0041 measured, 125.0061 calculated).

The most characteristic fragments in the mass spectra of all pyrazolidinediones studied were a series of peaks at *m/e* 182, 183, and 184 (198, 199, and 200 in oxyphenbutazone). The relative abundances of these peaks are given in Table I.

The peak at *m/e* 182 can be attributed to the formation of the azobenzene radical ion, $(C_6H_5N_2C_6H_5)^+$. Metastable evidence for the origin of this ion could not be found in any spectra except that for Compound VI, in which the *m/e* 182 ion was formed from the molecular ion, M^+ (*m/e* 280) → *m/e* 182, (m* observed, 118.4; m* calculated, 118.3).

The ion at *m/e* 183 can originate from the molecular ion or the McLafferty rearrangement radical ion (*m/e* 252) by hydrogen transfer as depicted in Scheme II. Metastable ions at 133.0 (calculated 132.9) were present in the spectra of I and IV to indicate that the fragment ion at *m/e* 183 arose from the ion at *m/e* 252. In the spectrum of oxyphenbutazone, the equivalent peak, *m/e* 199, apparently is derived from the molecular ion, M^+ (*m/e* 324) → *m/e* 199 (m* observed, 122.3; m* calculated, 122.2). This is also the case in the spectrum of Compound V: M^+ (*m/e* 266) → *m/e* 183 (m* observed, 126.0; m* calculated, 125.9).



Scheme II—Mechanism for formation of ion at m/e *183*

Table I—Relative Abundances (Percent) of Fragment Ions in the Range *m/e* 182–185

Compound	*m/e* 182[a]	*m/e* 183[a]	*m/e* 184[a]	*m/e* 185
I	2.3	65.1	19.8	2.1
I*a*	2.9	32.4	42.6	14.0
II	6.8 (*m/e* 198)	100.0 (*m/e* 199)	17.7 (*m/e* 200)	—
III	1.9	11.2	3.2	—
IV	5.8	100.0	35.8	4.0
IV*a*[b]	1.1	7.2	70.4	13.7
V	4.1	50.0 $C_{12}H_{11}N_2$	6.8 $C_{12}H_{12}N_2$	—
VI	14.4 $C_{12}H_{10}N_2$	3.7 $C_{12}H_{11}N_2$	—	—

[a] Where formula followed percent relative abundance, the identity was established by accurate mass measurement. [b] This spectrum also contained an ion at *m/e* 186 (13.0%).

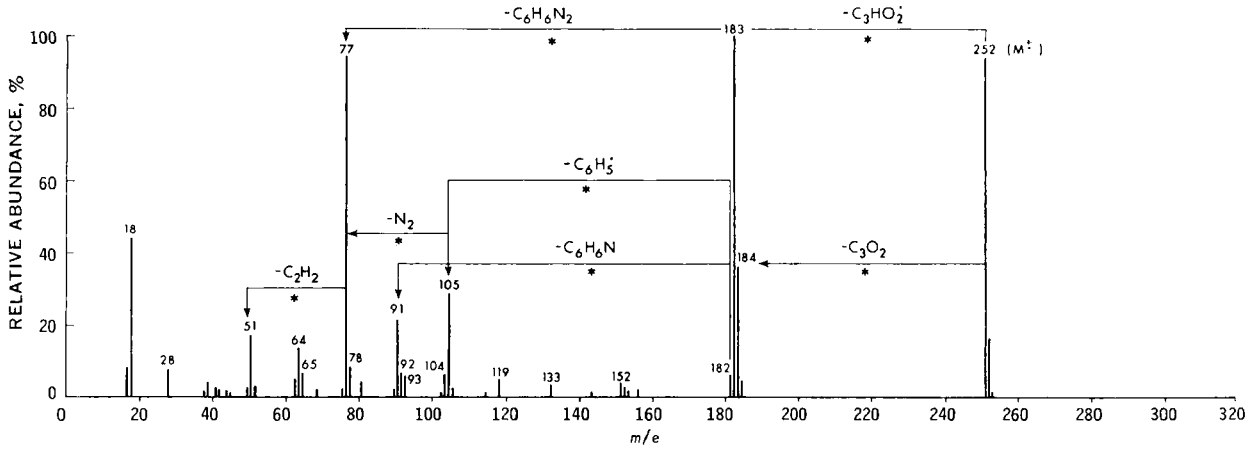


Figure 4—*Mass spectrum of 1,2-diphenyl-3,5-pyrazolidinedione.*

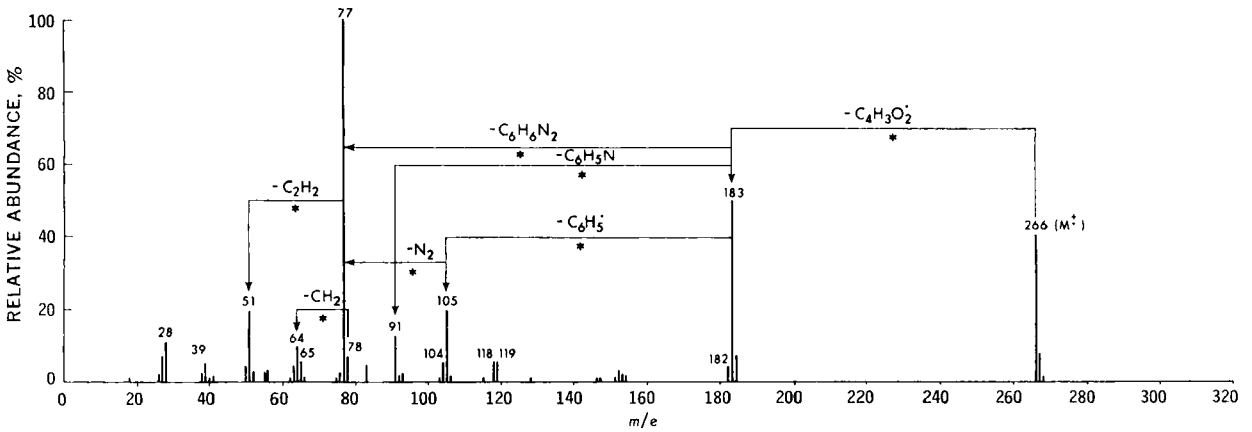
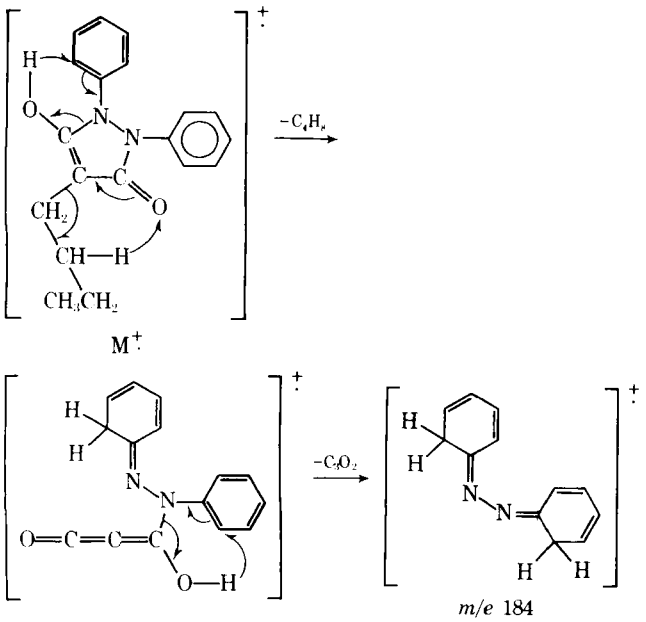


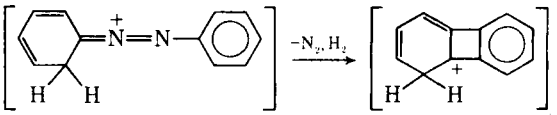
Figure 5—*Mass spectrum of 4-methyl-1,2-diphenyl-3,5-pyrazolidinedione.*

Compound VI was examined with the expectation that there would not be a peak at *m/e* 183, since neither enolization nor a McLafferty rearrangement is possible with this compound. Although it is only present to a small extent (3.7%), an accurate mass determination did establish that this ion had the composition $C_{12}H_{11}N_2$ and was not a simple isotope peak. It would appear that intermolecular hydrogen transfer may be involved in the formation of this ion in the spectrum of VI.

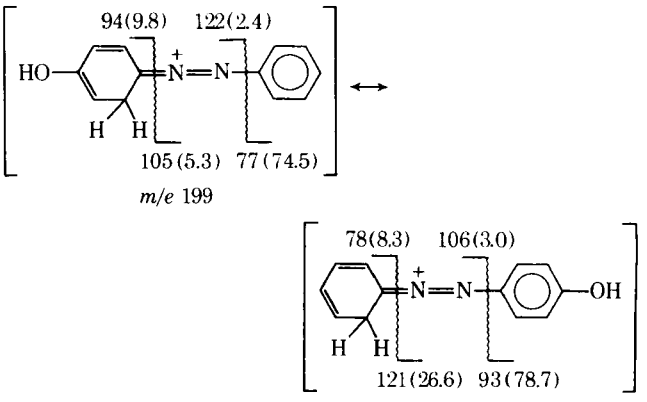
The ion at *m/e* 184 may arise from the transfer of two hydrogens to *ortho* positions of the aromatic rings (Scheme III). Metastable ions were present for the formation of this ion from the mo-



Scheme III—Formation of ion at m/e *184, $C_{12}H_{12}N_2$, from the molecular ion*



Scheme IV—Formation of ion at m/e *153 from the ion at* m/e *183*



Scheme V—Ions arising from the m/e *199 ion*

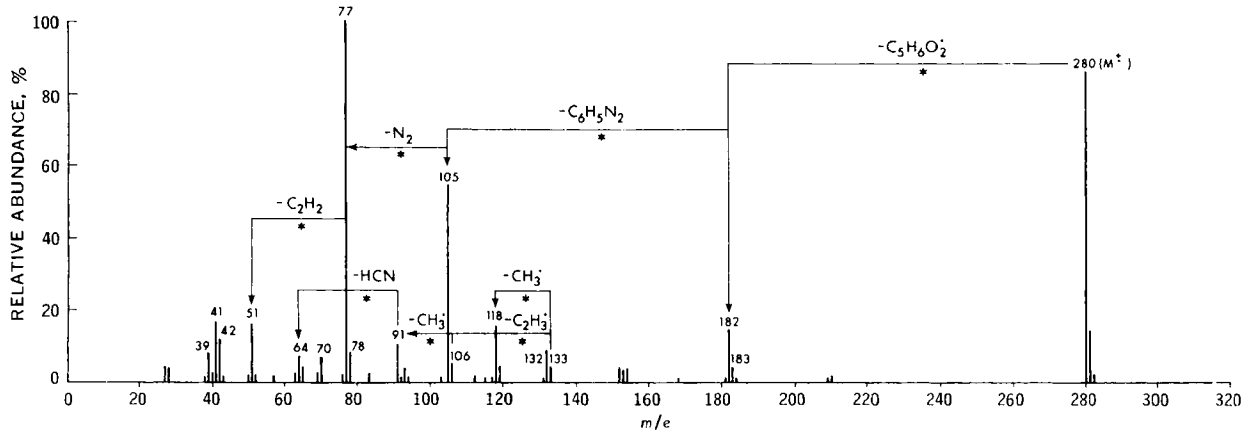


Figure 6— *Mass spectrum of 4,4-dimethyl-1,2-diphenyl-3,5-pyrazolidinedione.*

lecular ion in the spectra of I [M^+, *m/e* 308 → *m/e* 184 (m* observed, 110.0; m* calculated, 109.9)] and IV [M^+, *m/e* 252 → *m/e* 184 (m* observed, 134.3; m* calculated, 134.4)].

The peak at *m/e* 184 in the spectrum of V cannot be explained by Scheme III and, although of low relative abundance (6.8%), it did have the composition $C_{12}H_{12}N_2$. It may also arise from an intermolecular hydrogen transfer mechanism. There is no peak at *m/e* 184 in the spectrum of VI, as would be predicted, since there are no hydrogen atoms for transfer in the 4-position of the pyrazolidinedione nucleus.

Deuteration of phenylbutazone and 1,2-diphenyl-3,5-pyrazolidinedione yielded Ia and IV*a*, respectively. Peaks in the mass spectra of these deuterated derivatives are consistent with the mechanism given in Scheme III. Comparison of the peaks in the *m/e* 182–185 region in the spectra of I and Ia demonstrates that the peak at *m/e* 182 is almost unchanged in percent relative abundance upon deuteration. Deuteration decreases the intensity of the peak at *m/e* 183 and increases the intensity of the *m/e* 184 peak by almost exactly one-half, which may be explained by the observation that in the deuterated compound (Ia) there is now equal opportunity for the formation of $C_{12}H_{11}N_2$, *m/e* 183, and $C_{12}H_{10}DN_2$, *m/e* 184, from the McLafferty ion, *m/e* 252, or the enol form of the molecular ion. A significant peak at *m/e* 185 (14.0%) likewise corresponds to $C_{12}H_{11}DN_2$.

A similar comparison can be made for analogous peaks in the spectra of IV and IV*a*. A peak at *m/e* 186, 13.0% relative abundance, corresponding to $C_{12}H_{10}D_2N_2$, occurs in the mass spectrum of IV*a*.

Skeletal rearrangement ions of *m/e* 152, 153, and 154 were observed in all compounds studied. In oxyphenbutazone, these occur at *m/e* 168, 169, and 170. These ions may be assumed to be due to the loss of nitrogen and hydrogen from the azobenzene radical ion. Such rearrangement ions were described (8) in a study of mass spectra of azobenzenes. The peak at *m/e* 153 in all spectra probably arises from an equivalent rearrangement of the *m/e* 183 ion (Scheme IV).

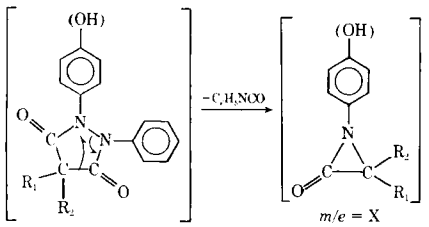
Other ions in the spectra of the pyrazolidinediones that may arise from the azobenzene radical ion are the result of the loss of a phenyl radical, $C_6H_5^{\cdot}$, to give $C_6H_5N_2^+$, *m/e* 105, which subsequently may lose nitrogen to give $C_6H_5^+$, *m/e* 77. The azobenzene radical ion may also lose $C_6H_5N_2^{\cdot}$ as a radical to give $C_6H_5^+$, *m/e* 77. These fragmentations were documented for azobenzene (8).

A series of analogous ions may be generated from the *m/e* 199 ion in the mass spectrum of II (Scheme V).

A fragmentation from the *m/e* 183 ion, which yields ions at *m/e* 92 and 91, may be due to the fission of the two nitrogen atoms (Scheme VI).

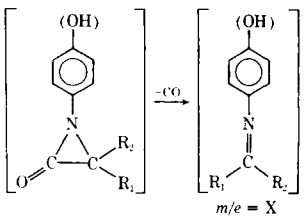
The ion of mass 91 (ionized phenyl nitrene) has been observed in the mass spectra of 3-phenyl-5-pyrazolone (9), substituted pyrazolone azomethine dyes (10), and 4-acyl-substituted 1-phenyl-3-methyl-5-pyrazolones (11). The $C_6H_6N^+$ ion, *m/e* 92, has also been observed in the mass spectra of substituted pyrazolones (11). In

Table II—Fragmentations Involving Loss of C_6H_5NCO as a Neutral Molecule



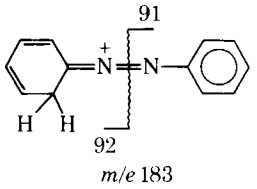
R_1	R_2	X	Compound (Relative Abundance, %)
H	H	133	IV (2.6)
D	D	135	IV*a* (2.2)
CH_2	H	146	I (1.0)
		[162]	[II (6.8)]
CH_2	D	147	Ia (0.7)
CH_2CH	H	159	III (4.2)
CH_2CH_2	H	160	I (1.2), III (1.3)
		[176]	[II (3.7)]
CH_2CH_2	D	161	Ia (0.6)

Table III—Ions Resulting from Loss of CO after Loss of Neutral Phenyl Isocyanate



R_1	R_2	X (Relative Abundance, %)	Compound
H	H	105 (28.3) m* obs. 82.9, calc. 82.9	IV
D	D	107 (3.0) m* obs. 85.0, calc. 84.8	IV*a*
H	CH_2	118 (2.5) m* obs. 95.4, calc. 95.4	III
H	CH_2	118 (5.4)	V
D	CH_2	119 (5.4)	Ia
H	CH_2CH	131 (8.1)	III
H	CH_2CH_2	148 (4.1)	II (*p*-OH)
H	CH_2CH_2	132 (1.0)	I
D	CH_2CH_2	133 (0.9)	Ia
CH_3	CH_3	133 (4.1)	VI[a]

[a] *m/e* 133 in the spectrum of VI may lose $H^{\cdot}$ to yield *m/e* 132 (8.7%), $C_9H_{10}N$ (132.0808 measured, 132.0813 calculated).



m/e 183

Scheme VI—Fragmentation of the m/e *183 ion*

the spectrum of oxyphenbutazone, analogous peaks were seen at *m/e* 108 (7.6%), $(C_6H_6NO)^+$, and at *m/e* 107 (11.2%), $(C_6H_5NO)^+$.

A characteristic fragmentation of all pyrazolidinediones studied was the loss of the elements of phenyl isocyanate either to form the radical ion $(C_6H_5NCO)^+$, *m/e* 119 [$(HOC_6H_5NCO)^+$, *m/e* 135 in the case of II] or as a neutral molecule. These pathways have been observed in the mass spectrum of aminopyrine (12). Fragmentations involving the loss of phenyl isocyanate as a neutral molecule are listed in Table II.

The ions (X, Table II) resulting from the loss of the elements of phenyl isocyanate as a neutral molecule may then lose CO to give ions $C_6H_5NCR_1R_2$ (Table III).

The remaining peaks in the low mass range, below *m/e* 100, are common to substituted aromatic systems, and the fragmentations observed are illustrated in Figs. 1–6.

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Kinetics of Dehydration of Epitetracycline in Solution

BETTY-ANN HOENER, THEODORE D. SOKOLOSKI [x], LESTER A. MITSCHER, and LOUIS MALSPEIS

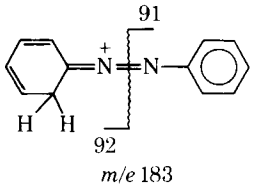
Abstract □ The dehydration kinetics of epitetracycline in solution to form epianhydrotetracycline were studied using UV and visible spectrophotometry. The reaction was found to be first order with respect to epitetracycline and hydronium-ion concentrations. The activation energy for the reaction was 28.3 kcal/mole at pH 2.0 in 0.1 *M* potassium chloride solutions. Dehydration of epitetracycline at pH 2.0 and 70° was found to be slower than that for tetracycline under similar solution conditions, although the activation energy for both reactants is essentially the same. This result is explicable on the basis of conformational differences in the molecules. This paper represents a portion of studies of the rates of various degradation reactions of tetracycline that lead to the toxic material epianhydrotetracycline.

Keyphrases □ Epitetracycline—dehydration kinetics in solution, activation energy, UV and visible spectrophotometry □ Dehydration—epitetracycline to epianhydrotetracycline kinetics in solution, activation energy, UV and visible spectrophotometry □ Epianhydrotetracycline—formation from epitetracycline, dehydration kinetics in solution, activation energy, UV and visible spectrophotometry □ Kinetics, dehydration—epitetracycline in solution

Studies show that commercially available tetracycline products contain significant amounts of degradation products of the antibiotic (1–3). This might be expected because tetracyclines can degrade through at least four different pathways: epimerization, dehydration, hydrolysis, and oxidation. Since the first two reactions are the most commonly encountered, they have been of specific interest for study. In solution at acid pH, two pathways connect tetracycline and 4-epianhydrotetracycline, as shown in Scheme I.

Epimerization about carbon-4 in tetracycline leads to inactive, nontoxic epitetracycline (4). The kinetics of this reaction were studied by other workers (4–7). Epimerization, which is a reversible first-order process, occurs most rapidly between pH 3 and 5. Dehydration and aromatization of the C-ring of tetracycline follow pseudo-first-order kinetics, leading to anhydrotetracycline, which is inactive *in vivo* and nontoxic. This reaction occurs in solution at very low pH (8) and in the solid state under thermal conditions (9).

There are two important steps in the overall degradation of tetracycline, whose kinetic characteristics have not as yet been studied in solution or in the solid state. These are the epimerization of anhydrotetracycline and the dehydration of epitetracycline. Both reactions lead to the inactive, but toxic, epi-



m/e 183

Scheme VI—Fragmentation of the m/e *183 ion*

the spectrum of oxyphenbutazone, analogous peaks were seen at *m/e* 108 (7.6%), $(C_6H_6NO)^+$, and at *m/e* 107 (11.2%), $(C_6H_5NO)^+$.

A characteristic fragmentation of all pyrazolidinediones studied was the loss of the elements of phenyl isocyanate either to form the radical ion $(C_6H_5NCO)^+$, *m/e* 119 [$(HOC_6H_5NCO)^+$, *m/e* 135 in the case of II] or as a neutral molecule. These pathways have been observed in the mass spectrum of aminopyrine (12). Fragmentations involving the loss of phenyl isocyanate as a neutral molecule are listed in Table II.

The ions (X, Table II) resulting from the loss of the elements of phenyl isocyanate as a neutral molecule may then lose CO to give ions $C_6H_5NCR_1R_2$ (Table III).

The remaining peaks in the low mass range, below *m/e* 100, are common to substituted aromatic systems, and the fragmentations observed are illustrated in Figs. 1–6.

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Abstract □ The dehydration kinetics of epitetracycline in solution to form epianhydrotetracycline were studied using UV and visible spectrophotometry. The reaction was found to be first order with respect to epitetracycline and hydronium-ion concentrations. The activation energy for the reaction was 28.3 kcal/mole at pH 2.0 in 0.1 *M* potassium chloride solutions. Dehydration of epitetracycline at pH 2.0 and 70° was found to be slower than that for tetracycline under similar solution conditions, although the activation energy for both reactants is essentially the same. This result is explicable on the basis of conformational differences in the molecules. This paper represents a portion of studies of the rates of various degradation reactions of tetracycline that lead to the toxic material epianhydrotetracycline.

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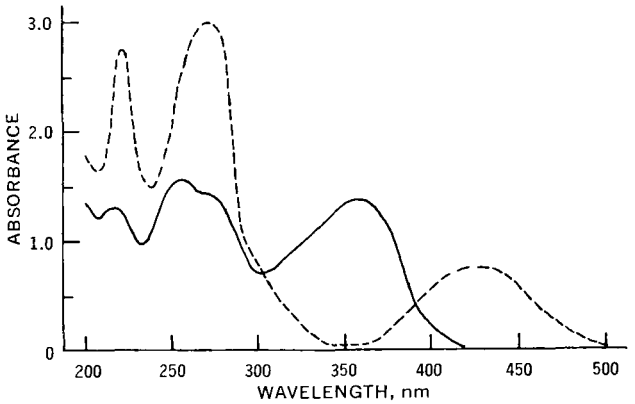


Figure 1—*UV and visible spectra of epitetracycline (——) and epianhydrotetracycline (– – –) at a concentration of 1 × 10^{-4} M in 0.1 M KCl, pH 2.0, using a 1-cm cell.*

anhydrotetracycline. To supply some of the missing kinetic parameters, studies on the effects of pH and temperature on the dehydration of epitetracycline solutions were conducted.

Epianhydrotetracycline formation may be conveniently and accurately studied by the appearance of an absorption peak at 427 nm or through the loss of epitetracycline, as reflected by the decrease in the absorption peak at 356 nm (Fig. 1). Analogous to the dehydration of tetracycline, epianhydrotetracycline formation is the only reaction occurring at pH 2 or less (8). In addition to the effects of pH and temperature, the influence of citric acid on the rate of dehydration of epitetracycline is reported, since citric acid has been shown to enhance markedly the degradation of tetracycline powder (1).

EXPERIMENTAL

Authentic samples of 4-epitetracycline[1], 4-epianhydrotetracycline[2], and tetracycline[3] were used as obtained. All other chemicals were reagent grade. Solutions were prepared in demineralized, double-distilled water, again distilled over ethylenediaminetetraacetic acid. All reaction solutions were 0.1 *M* in potassium chloride, and hydrochloric acid was added to adjust the pH[4]. In one experiment, citric acid at a concentration of 0.001 *M* was added to the 0.1 *M* KCl solution before adjusting the pH.

Solutions of 4-epitetracycline were sealed in ampuls for the studies carried out at 70.7° and pH 2.00. All other reactions were carried out in a sealed reaction vessel (Fig. 2). The 98.5° reaction was carried out using a boiling water bath, and those at 89.8 and 70.7° were carried out using an oil bath[5]. For those reactions carried out in the reaction vessel (Fig. 2), samples of about 4 ml were withdrawn *via* the syringe and placed in test tubes immersed in ice to stop the reaction. The time of the sample was taken as the time of placement in the test tube.

The absorbances of the samples were determined[6] after warming to room temperature. When ampuls were used, they were removed from the constant-temperature bath at appropriate time intervals and chilled in an ice bath. The ampuls were then opened, 5 ml was withdrawn and diluted to 10 ml with cold 0.1 *M* KCl–HCl solution, and the absorbance was measured at room temperature.

The absorbances of all epitetracycline solutions at concentrations ranging from 8.3×10^{-5} to 1.82×10^{-4} *M* were read at ambient temperatures at 356 and 427 nm, using a 1-cm cell. These absorbances measure the content of epitetracycline (ϵ_{356} = 13,800, ϵ_{427} = 0) and epianhydrotetracycline (ϵ_{427} = 5190, ϵ_{356} = 736). The absorption spectra for these compounds are given in Fig. 1. For the tetracycline solutions, absorbances at 356 nm measure the content of tetracycline and those at 433 nm measure the content of anhydrotetracycline (8).

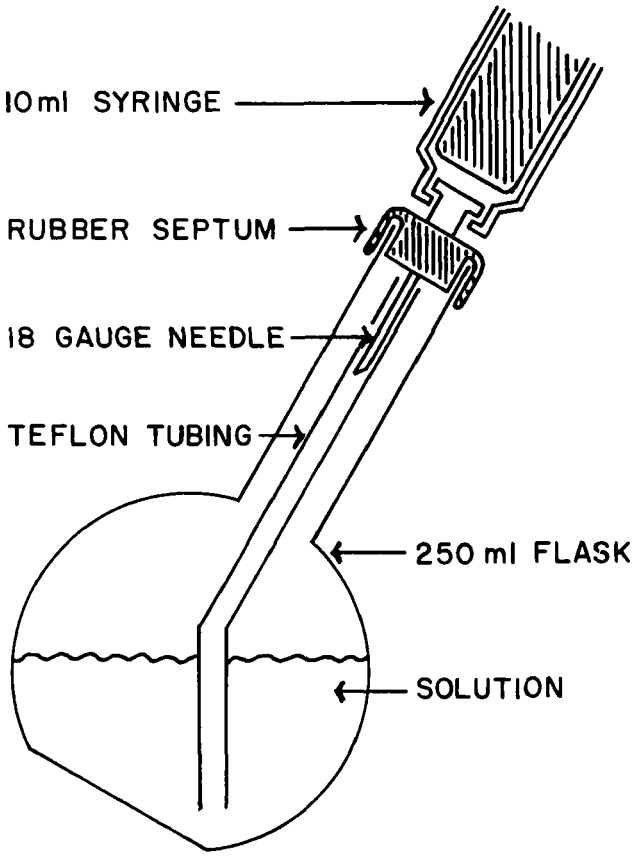
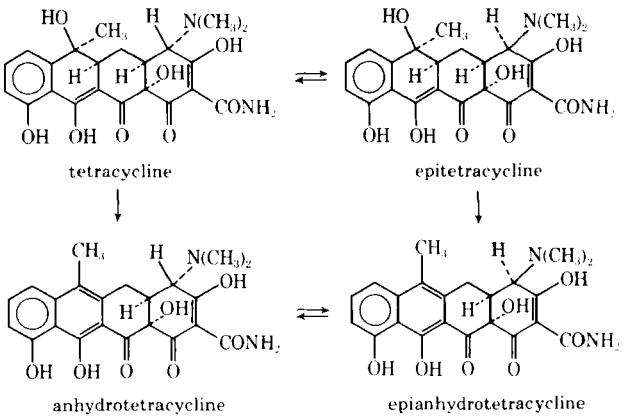


Figure 2—*Schematic representation of the reaction vessel used to study the dehydration of epitetracycline and tetracycline. The flask is immersed in a constant-temperature bath.*

RESULTS

Semilogarithmic plots of the difference between absorbance at a given time and absorbance at infinite time (determined after about 10 half-lives) at 356 nm and vice versa at 427 nm of the epitetracycline solutions gave an excellent linear relation. Figure 3 shows the results obtained at pH 2.00 and 70.7° for both wavelengths.



Scheme I

[1] Ammonium salt, Batch 430, British Pharmacopoeia Commission.
[2] Hydrochloride salt, Batch 429, British Pharmacopoeia Commission.
[3] Hydrochloride salt, Lot 48379-171, Lederle Laboratories.
[4] The pH was determined using a Sargent-Welch pH meter, model DR, standardized using pH 4.00 buffer, Lot 69M 388C 6471, Ohio State Reagent Laboratory.
[5] Sargent Thermonitor model ST.
[6] Cary model 16 spectrophotometer.

Table I—Pseudo-First-Order Rate Constants for Dehydration of Epitetracycline or Tetracycline at Various Temperatures at pH 2.0

Reactant[a]	Temperature	Rate Constant, $min^{-1} \times 10^2 \pm SD$[b] at 356 nm	at 427[c] or 433 nm[d]
Epitetracycline	98.5 ± 0.2°	5.59[e] ± 0.09	5.59[e] ± 0.06
Epitetracycline	89.8 ± 0.2°	3.08 ± 0.04	3.21 ± 0.06
Epitetracycline	80.0 ± 0.2°	1.18 ± 0.02	1.27 ± 0.03
Epitetracycline	70.7 ± 0.2°	0.395[e] ± 0.079	0.426[e] ± 0.084
Epitetracycline + 0.001 *M* citric acid	70.7 ± 0.2°	0.424 ± 0.023	0.460 ± 0.073
Tetracycline	70.7 ± 0.2°	0.553[e] ± 0.039	0.599 ± 0.069

[a] In 0.1 *M* KCl–HCl, pH 2.00. [b] Determined by linear regression analysis from the data of a semilogarithmic plot. [c] For epitetracycline. [d] For tetracycline. [e] Average of two determinations. The average precision obtained between all pairs of determinations was ±6.5%.

Also included in Fig. 3 are the results of an experiment under the same conditions utilizing tetracycline.

The pseudo-first-order rate constants at various temperatures at pH 2.0 are given in Table I. Also included in Table I is the study made in the presence of citric acid. An Arrhenius plot of the epitetracycline dehydration data gave an activation energy, E_a, of 28.3 ± 2.4 kcal/mole. The pseudo-first-order rate constants for the reaction at 70.7° at three different pH values are presented in Table II. A log (rate constant) *versus* pH plot gave a slope of −0.91 ± 0.1.

DISCUSSION

The dehydration of epitetracycline in solution at low pH to form epianhydrotetracycline has been shown to be first order with respect to epitetracycline. A pH–rate profile also has shown the reaction to be first order with respect to hydronium ion at below pH 2.0. The error limits found for the log rate constant–pH relation (Table II) may be due to the limited number of pH values that could be used and the change in total ionic strength over the pH range used.

Unlike the situation with tetracycline powder (1), citric acid at this low concentration had no catalytic effect on the dehydration of epitetracycline solutions. A most interesting result was that the rate of dehydration of epitetracycline was significantly less than that of tetracycline under similar conditions (Fig. 3). The rate constant obtained in this study for tetracycline is in agreement with that of Pryves (8). These epimers differ in the configuration of the dimethylamino group on carbon-4 in the A-ring of tetracycline. It seems likely that the explanation for the considerable difference in the rate for the two tetracycline epimers can be found in either the difference in electrostatic screening provided by the protonated dimethylamino function which is oriented differently in space relative to the 6β-hydroxy group in the two isomers or through the operation of the "Barton effect," *i.e.*, conformational transmission (10–12).

The transition state for dehydration is favorable in either isomer because the elements of water are located antiparallel and *trans* to each other and the 6β-hydroxy group is tertiary, benzylic, and axial. Furthermore, the canonical form of the β-diketo system at C-11, 11*a*, and 12, in which C-11 is olefinic, gives the C-ring a naphthaleneoid geometry at the outset (I).

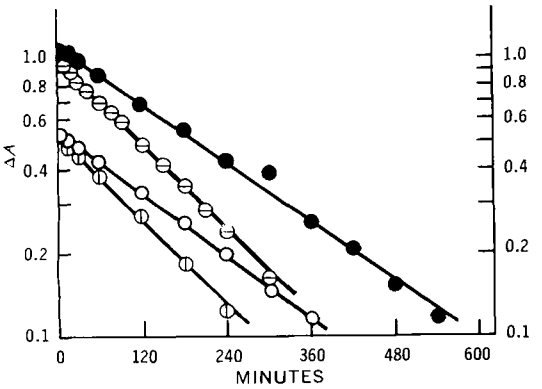
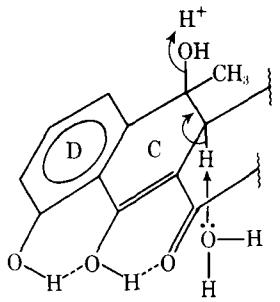


Figure 3—*Semilogarithmic plot of absorbance change* versus *time for epitetracycline* (● *at 356 nm and* ○ *at 427 nm*) *and tetracycline* (⊖ *at 356 nm and* ⊙ *at 433 nm*) *at 70° and pH 2.0.*

As in the conformation adopted by chlortetracycline hydrochloride in the crystal and supported by solution NMR and circular dichroism studies, the dimethylamino function in tetracycline (II) is axial and it is located on the side of the molecule opposite the 6β-hydroxy group and screened from it by the sigma framework (13, 14).

In 4-epitetracycline (III), the equitorial dimethylamino group is much closer to the 6β-hydroxy group (14) and would be expected to inhibit the approach of a hydronium ion more successfully than would tetracycline itself. Depending on the extent to which this effect would be operative, it would decrease the rate of dehydration of 4-epitetracycline relative to tetracycline and thus be in accord with the experimental findings.

The Barton, or conformational transmission, effect has been thoroughly substantiated over the years with numerous examples (10–12). It has been found in numerous steroids that, in addition



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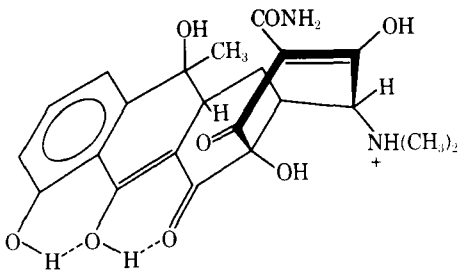

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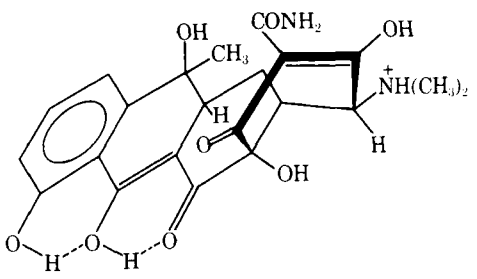

III

Table II—Pseudo-First-Order Rate Constants for Dehydration of Epitetracycline at Several pH Values at 70.7°

pH[a]	Rate Constant, $min^{-1} \times 10^2 \pm SD$[b] at 356 nm	Rate Constant, $min^{-1} \times 10^2 \pm SD$[b] at 427 nm
2.00	0.395 ± 0.079[c]	0.426 ± 0.084[c]
1.53	1.03 ± 0.01	1.04 ± 0.01
1.04	3.19 ± 0.03	3.15 ± 0.01

[a] In 0.1 *M* KCl. [b] Determined by linear regression analysis from the data of a semilogarithmic plot. [c] Average of two determinations. The average precision obtained for these data sets was ±1.5%.

to expected electronic effects, conformational distortion in remote portions of a molecule, caused by epimeric constituents and the like, can be transmitted through the sigma framework of as many as four rings and significantly affect the rate and even steric outcome of reactions occurring at relatively great distances from the site of isomerization (15–25).

Detailed X-ray, NMR, and circular dichroism studies (13, 14) have shown that the A-ring of tetracyclines is somewhat distorted in acidic solutions because of the necessity of relieving a transannular 1,3-diaxial interaction between the 12*a*-hydroxy and protonated dimethylamino group at C-4. This apparently provides the driving force for epimerization at C-4, since epimerization relieves the interaction and releases the strain distortion of ring A. Transmission of the different strain in these two isomers from the A-ring through three to four carbon atoms to the 5*α*-hydrogen and 6*β*-hydroxy groups, whose geometry relative to one another plays a dominant role in the dehydration reaction, could conceivably result in rate differences of the magnitude observed in this study (Table I).

The involvement of steric effects in rationalizing the differential rates of dehydration of tetracycline and epitetracycline is further supported by noting that the activation energy for epitetracycline dehydration (28 kcal/mole) is essentially the same as that for the dehydration of tetracycline (27 kcal/mole) (8).

These studies reporting the solution kinetics of the dehydration of epitetracycline represent one factor in the overall degradation processes available to tetracycline in dilute aqueous solutions and in the solid state. Studies are underway to determine the rate of epimerization of anhydrotetracycline in solution to complete the solution phase picture.

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[x] To whom inquiries should be directed.

Lignans from *Larrea divaricata*

OLE GISVOLD [x] **and EMIL THAKER**

Abstract □ A phytochemical reinvestigation of the phenolics of *Larrea divaricata* led to the isolation and characterization of dihydroguaiaretic acid, norisoguaiacin, and 3′-demethoxyisoguaiacin. Partially demethylated dihydroguaiaretic acid was detected *via* the use of partially demethylated dihydroguaiaretic acid synthesized from dihydroguaiaretic acid. Techniques are given for the preparation of norisoguaiacin, dihydroguaiaretic acid, and nordihydroguaiaretic acid. Sodium molybdate complexes were used to effect the isolation and preparation of norisoguaiacin. Some biological properties of the phenolic compounds studied are given.

Keyphrases □ *Larrea divaricata*—isolation and characterization of dihydroguaiaretic acid, norisoguaiacin, and 3′-demethoxyisoguaiacin, biological screening □ Lignans from *L. divaricata*—isolation and characterization of phenolics, biological screening □ Anticancer agents, potential—isolation and characterization of dihydroguaiaretic acid, norisoguaiacin, and 3′-demethoxyisoguaiacin from *L. divaricata*, screening

Previous phytochemical investigations of *Larrea divaricata* resulted in the isolation of nordihydroguaiaretic acid (1) and two incompletely characterized flavones (2). Subsequent to its isolation and characterization, nordihydroguaiaretic acid was used as an antioxidant. A review of its properties has been reported (3) and its activities at the molecular level are outstanding (4, 5).

Several years ago a reinvestigation of the phenolics of *L. divaricata* was initiated to resolve the complex mixture indicated by preliminary TLC. These studies were stimulated by the report (6) that marked regression of a malignant melanoma occurred in an elderly man who had consumed a tea prepared from *L. divaricata* over several months.

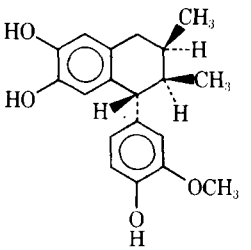
Because nordihydroguaiaretic acid is a lignan, one would suspect that biogenetically related compounds might be present in the phenolics of *L. divaricata*. Four such compounds were isolated: norisoguaiacin (I), a lignan containing one catechol moiety and probably formed from the cyclization of guaiaretic acid followed by partial demethylation or by the cyclization of partially demethylated guaiaretic acid, which is more lipid soluble than nordihydroguaiaretic acid; dihydroguaiaretic acid (II); partially demethylated dihydroguaiaretic acid[1] (III), conceivably a biogenetic intermediate in the biosynthesis of nordihydroguaiaretic acid; and a minor product, 3′-demethoxyisoguaiacin (IV).

EXPERIMENTAL

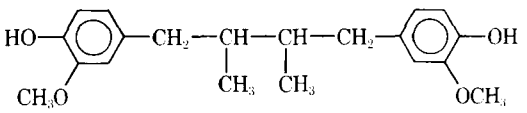
TLC analyses using silica gel were used to follow the isolations and to detect the purity of the isolates. The mobile phase used was benzene–isopropyl alcohol–acetic acid–water (25:5:2:10).

Isolation of Nordihydroguaiaretic Acid—A primary extract of 50 g of the leaves and very small twigs of *L. divaricata*[2] was prepared by shaking overnight with 500 ml of toluene containing 10% ether, 10% isopropyl ether, 10% acetone, 7.5% methyl ethyl ketone, 10% methanol, 10% ethanol, or 7.5% isopropyl alcohol. This primary extract was concentrated under vacuum using a rotovapor pump, a bath temperature of 50°, and a temperature inside the distilling flask of 25°. A final volume of 250 ml was desirable in most cases. During the concentration, considerable amounts of resinous material separated containing most of the degradation products, flavonoid pigments, and some less soluble phenolics. Upon standing or following centrifugation, a clear solution separated and was seeded with nordihydroguaiaretic acid crystallized from toluene. In some cases, nordihydroguaiaretic acid began to crystallize in a few hours; in other instances, this occurred after standing overnight. Even upon prolonged standing, not all of the nordihydroguaiaretic acid crystallized. It may be almost colorless or various shades of yellow, depending on the amount of flavonoids present. The mother liquor also contains the more lipid-soluble phenolics, pigments, waxes, and possibly other lipids.

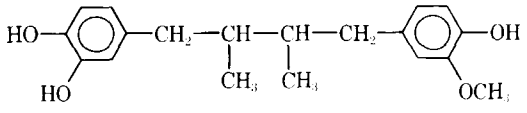
The insoluble substances that separated when the primary extract was concentrated contained considerable amounts of nordihydroguaiaretic acid together with norisoguaiacin, partially demethylated dihydroguaiaretic acid, dihydroguaiaretic acid, yel-



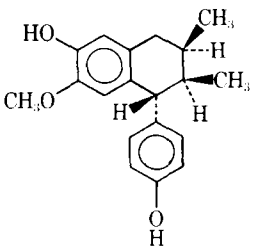
I: norisoguaiacin



II: dihydroguaiaretic acid



III: partially demethylated dihydroguaiaretic acid



IV: 3′-demethoxyisoguaiacin

[1] From a nomenclature standpoint, this new phenolic should be called nordihydroguaiaretic acid and the nordihydroguaiaretic acid of the literature would better be named bisnordihydroguaiaretic acid.

[2] American West Botanical Co., Tucson, Ariz. Voucher specimens have been deposited in the Botany Department, University of Minnesota, Minneapolis, Minn.

low pigments, and, no doubt, minor amounts of other lignans. These substances were dissolved in 30 ml of ether and diluted with 500 ml of toluene. This solution was processed in the same way as was the original primary extract with similar results. This process was repeated as often as necessary to obtain some crystalline nordihydroguaiaretic acid from the toluene concentrate. The amount obtained depended on the source of the original *L. divaricata*.

The NMR spectrum for nordihydroguaiaretic acid tetramethyl ether in deuterated chloroform was as follows: a sharp doublet centering at δ 0.87 (J = 6.5 Hz) and integrating for six protons, a complex multiplet ranging from δ 1.5 to 2.0 and integrating for two methine protons, a multiplet of eight lines ranging from δ 2.04 to 3.04 and integrating for four methylene protons, a sharp singlet at δ 3.85 accounting for 12 methoxy protons, and a multiplet between δ 6.5 and 7.0 and integrating for six aromatic protons. These data support the known structure for nordihydroguaiaretic acid tetramethyl ether.

Preparation of Norisoguaiacin—Toluene solutions of the phenolics of *L. divaricata* from which much of the nordihydroguaiaretic acid had separated were concentrated *in vacuo* to remove all of the solvent. The resinous mass obtained was dissolved in ethylene dichloride and diluted with three parts of ligroin[3] to obtain a saturated solution containing about 1% of phenolics. After standing overnight, 400 ml of this solution, now free of nordihydroguaiaretic acid, was shaken with 2 × 100 ml of 5% sodium molybdate containing 0.4% sodium hydroxide and 3% sodium pyrosulfite[8]. These extractions removed, as red molybdate complexes, all phenolics containing a catechol moiety together with very small amounts of other phenolics that accompanied these aqueous molybdate extractions, probably as a mechanical dispersion. The latter were removed readily in a liquid–liquid extraction apparatus using *n*-hexane[4]. Norisoguaiacin was recovered from its molybdate complex by continuous extraction with ether. This ether extract was decolorized by charcoal; TLC, using silica gel, showed the presence of only one spot. Norisoguaiacin was crystallized directly from this preparation.

Norisoguaiacin is soluble in ether, and the addition of *n*-hexane followed by the slow removal of ether yields a crystalline product, mp 148–149°. It gave a single spot on a silica gel TLC plate and a single peak by GLC, using the trimethylsilyl ethers and a OV-1 column. The IR spectrum showed aromatic bands at 1500 and 1600 cm^{-1} and phenolic hydroxyls at 3500–3600 cm^{-1}. The NMR spectrum in deuterated acetone or deuterated methanol showed a doublet of a CH_3 group centering at δ 0.87 (J = 6.5 Hz), a complex multiplet ranging from δ 1.50 to 3.2, a sharp doublet at δ 3.62 (J = 6.5 Hz), a sharp singlet centering at δ 3.75, a multiplet ranging from δ 6.2 to 6.85, and a sharp singlet at δ 7.18 that can be exchanged with D_2O. The mass spectrum gave a molecular ion peak at 314.

Anal.—Calc. for $C_{19}H_{22}O_4$: C, 72.61; H, 7.00. Found: C, 72.90; H, 6.86. Methoxyl—Calc.: 9.87. Found: 9.87.

Preparation of Norisoguaiacin Trimethyl Ether (Dimethylisoguaiacin)—Norisoguaiacin was methylated in the usual way using potassium carbonate and dimethyl sulfate. The crude methylated product was recrystallized several times from methanol, mp 100–101°, $[\alpha]D^{23}$ −46° (c 5.2% in $CHCl_3$). The NMR spectrum revealed a doublet centering at δ 0.90 (J = 6.5 Hz) and integrating for six protons, a multiplet ranging from δ 1.50 to 3.24 and integrating for four protons, three singlets appearing between δ 3.50 and 4.00 and integrating for 13 protons (*i.e.*, 12 methoxy protons and one dibenzylic proton), and aromatic protons appearing as multiplets between δ 6.24 and 6.95 and integrating for five protons.

Anal.—Calc. for $C_{22}H_{28}O_4$: C, 74.15; H, 7.86. Found: C, 74.25; H, 8.06. Methoxyl—Calc.: 34.83. Found: 34.27.

The physical constants of trimethylnorisoguaiacin agree with those of dimethylisoguaiacin in the literature (9). The NMR data reported are new and support the stereochemistry previously reported for dimethylisoguaiacin.

Norisoguaiacin formed a triphenylurethane derivative when prepared from phenylisocyanate in the usual way, mp 137–139°.

Anal.—Calc. for $C_{40}H_{37}N_3O_7$: C, 71.52; H, 5.55; N, 6.26. Found: C, 71.39; H, 5.78; N, 6.05.

Partial Demethylation of Dihydroguaiaretic Acid—Dihydroguaiaretic acid was partially demethylated with boiling hydrobromic acid (48%). Recovery of the half-demethylated dihydroguaiaretic acid in 40% yield was effected in a manner similar to that used for the preparation of norisoguaiacin, mp 87–88°, $[\alpha]D^{25}$ 0°.

Partially demethylated dihydroguaiaretic acid readily formed a triphenylurethane derivative when prepared in the usual way, mp 198–200°.

Anal.—Calc. for $C_{40}H_{39}N_3O_7$: C, 71.02; H, 5.80. Found: C, 70.62; H, 6.07.

Partially Demethylated Dihydroguaiaretic Acid—The mother liquors obtained from the isolation of crystalline norisoguaiacin yielded some amorphous resinous material. This material, although it gave only one spot on a TLC silica gel plate, refused to crystallize. However, it gave two peaks of about equal intensity by GLC using the trimethylsilyl ethers and an OV-1 column. The retention time of one peak was identical to that obtained for the trimethylsilyl ether of norisoguaiacin and the other was identical to that obtained for the trimethylsilyl ether of the partially demethylated dihydroguaiaretic acid.

Preparation of 3′-Demethoxyisoguaiacin—The ethylene dichloride–ligroin (1:3) solution that had been treated with the aqueous sodium molybdate contained much dihydroguaiaretic acid and what appeared to be a fair amount of a phenolic that was somewhat slower moving on a TLC silica gel plate than was dihydroguaiaretic acid. Preparative thick-layer chromatography, using silica gel and the same mobile phase utilized for the TLC studies, led to the isolation of several hundred milligrams of an ether-soluble phenolic which was crystallized from hot 5% aqueous acetic acid. Fine needles were obtained, mp 96–98°.

Anal.—Calc. for $C_{19}H_{22}O_3$: C, 76.51; H, 7.38. Found: C, 76.40; H, 7.50.

The NMR spectrum for 3′-demethoxyisoguaiacin supported the presence and stereochemistry of a tetrahydronaphthalene moiety that is the same as that found in norisoguaiacin. It also showed the presence of only one methoxy group and two phenolic hydroxy groups.

The mass spectrum showed a molecular ion peak at 298. The fragmentation pattern supported the presence of a methyl group at each of the 2- and 3-positions of the hydroaromatic ring of this lignan because a significant peak was obtained at M−56. This also was the case with norisoguaiacin.

Isolation of Dihydroguaiaretic Acid—The toluene–*n*-hexane (1:2) solutions saturated with the phenolics from which some norisoguaiacin had separated was diluted with *n*-hexane until a ratio of 1 part of toluene to 5 parts of *n*-hexane was obtained. The total volume first used was 1000 ml. It was shaken with 30-ml portions of 5% aqueous sodium carbonate until very small amounts of extractives were obtained. The organic solution then was washed once with 25 ml of 5% potassium biphosphate. Removal of the solvents under vacuum left a resinous mass which, upon standing several days, developed large amounts of crystalline material. This material appeared to be about 95% of one substance by GLC and TLC analyses. This crystalline mass was stirred with a small volume of toluene and *n*-hexane (1:1) and filtered. The crystalline mass was washed on the filter with small amounts of this solvent mixture. This product was recrystallized from ether and *n*-hexane, mp 88° [lit. (10) mp 88°]; the mixed melting point with an authentic specimen of *meso*-dihydroguaiaretic acid showed no depression.

Anal.—Calc. for $C_{20}H_{26}O_4$: C, 72.7; H, 7.9. Found: C, 73.13; H, 8.02.

Dihydroguaiaretic acid formed a diphenylurethane derivative when prepared from phenylisocyanate in the usual way, mp 165°.

Anal.—Calc. for $C_{34}H_{36}N_2O_6$: C, 71.81; H, 6.38; N, 4.93. Found: C, 71.71; H, 6.54; N, 5.14.

Preparation of *meso*-Diethyldihydroguaiaretic Acid—Dihydroguaiaretic acid was ethylated in the usual way using potassium carbonate and diethyl sulfate. Recrystallizations from methanol gave white needles, mp 98–99° [lit. (11) mp 98–99°]. The NMR spectrum revealed a sharp doublet centering at δ 0.87 (J = 6.5 Hz) and integrating for six protons of the two methyl groups in the two ethoxy groups, a complex multiplet ranging from δ 1.5 to 2.0 of the methine protons and overlapped by this triplet, a multiplet of eight lines ranging from δ 2.04 to 3.04 and integrating for four methylene protons, a sharp singlet at δ 3.85 accounting for six

[3] Apcothinner, Apco Oil Corp., Oklahoma City, Okla.
[4] Skellysolve-B.

methoxy protons, a quartet centering at δ 4.08 and partially overlapped by the methoxy resonance at δ 3.85 accounting for four methylene protons of the ethoxy groups, and a multiplet of aromatic protons ranging from δ 6.50 to 7.00 accounting for six protons. These data support the known structure for this compound.

GLC—A gas chromatograph[5] equipped with a hydrogen flame-ionization detector and a circular stainless steel column, 1.8 m (6 ft) long and 0.3 cm (0.12 in.) o.d., was used. The column packing was Chromosorb 40 (60–80 mesh) coated with 3% OV-1; the injection port and flame-ionization detector temperatures were both 310° and the oven temperature was 215°. The carrier gas (nitrogen), hydrogen, and air flow rates were 30, 35, and 500 ml/min, respectively.

The free phenolics of *L. divaricata* could not be used because they decomposed. Their trimethylsilyl ethers were suitable, and silylation of the total phenolics gave at least nine peaks. The peak due to nordihydroguaiaretic acid was the largest, followed by an almost equivalent peak for dihydroguaiaretic acid. The other peaks were progressively much smaller and were due to norisoguaiacin, partially demethylated dihydroguaiaretic acid, probably other biogenetically related lignans, and the flavonoids. Demethylation of the total phenolics followed by silylation and GLC analysis revealed one major peak, nordihydroguaiaretic acid, and a second smaller peak, bisnorisoguaiacin. Similar results were obtained from the completely methylated phenolic resin examined by GLC. These results indicate that, other than the flavonoids, the phenolics of *L. divaricata* have only two carbon skeletons, *i.e.*, the one present in nordihydroguaiaretic acid and the one found in norisoguaiacin. It also indicated that most lignans present contain catechol or partially methylated catechol moieties.

The retention times of the silyl ethers of nordihydroguaiaretic acid, dihydroguaiaretic acid, guaiaretic acid, norisoguaiacin, and partially demethylated dihydroguaiaretic acid are 11.5, 8.6, 9.35, 6.5, and 10.8 min, respectively. Those of tetramethylnordihydroguaiaretic acid and dimethylisoguaiacin are 6.9 and 5.0 min, respectively.

Biological Activity—The minimum inhibitory concentration of norisoguaiacin for *Streptococcus* sp., *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* was 100 μg/ml in tryptone agar[6]. It is as active against the beef heart mitochondrial NADH oxidase system and succinoxidase system as nordihydroguaiaretic acid (12).

The minimum inhibitory concentration of dihydroguaiaretic acid for *Strep.* sp., *Staph. aureus*, and *B. subtilis* was 10 μg/ml in tryptone soy agar.

Partially demethylated dihydroguaiaretic acid is as active against the beef heart mitochondrial NADH system as is nordihydroguaiaretic acid or norisoguaiacin.

Antioxidant Activity—Using the active oxygen method stability test at 110°, with lard having a stability of 6 hr, 0.01% norisoguaiacin gave a stability of 55.5 hr whereas the stability of nordihydroguaiaretic acid at the same concentration was 73 hr[7].

DISCUSSION

The total phenolics together with small amounts of lipid substances produced by *L. divaricata* were 16% from older plants and 21% from young and vigorously growing plants collected in the vicinity of Tucson, Ariz. This was determined by extracting the dried small twigs and leaves with toluene containing 10% ether.

Norisoguaiacin and partially demethylated dihydroguaiaretic acid are as active as nordihydroguaiaretic acid against the beef heart mitochondrial NADH oxidase system and succinoxidase system as is nordihydroguaiaretic acid. Thus, the biscatechol structure found in nordihydroguaiaretic acid is not an essential requirement for the described activities. Nordihydroguaiaretic acid has been called the "Penicillin of hydroquinones and the most potent cancer antimetabolite in vitro" (4). It also is effective *in vivo* against Ehrlich ascites tumor in mice when combined with ascorbic acid. In view of the reported anticancer activity for nordihydroguaiaretic acid, it is desirable to determine if dihydroguaiaretic acid, norisoguaiacin, 3′-demethoxyisoguaiacin, or partially demethylated dihydroguaiaretic acid possesses similar properties.

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[x] To whom inquiries should be directed.

[5] Perkin-Elmer 900.

[6] S. R. Rolfing, 3M Co., St. Paul, Minn., personal communication.

[7] W. E. Lundberg, Hormel Institute, Austin, Minn., personal communication.

Labilization of C—N Linkages by Dibenzocyclohepten-5-yl Ring System

H. V. MAULDING ˣ, A. F. MICHAELIS, and J. P. NAZARENO

Abstract □ The hydrolysis of several 5-aminodibenzo[*a,d*]cycloheptenes is discussed. The relative reactivity of the various compounds was closely related to the amino substituents at position 5 of the tricyclic nucleus. Both secondary and tertiary amines were prepared. They showed instability in aqueous solution below the pKa with invariant velocity constants at pH 0.15–7.0. This is indicative of spontaneous decomposition of the molecule *via* a carbonium ion or through the kinetically equivalent solvent attack on C_5 of the dibenzocycloheptene ring system. The fully aromatic ring system leads to a two magnitude enhancement of the velocity constants as compared to those of the corresponding 10,11-dihydrodibenzo[*a,d*]cycloheptenes. The cycloheptene nucleus is unstable when the adjacent nitrogen is cationic. When positively charged, the compounds undergo decomposition by nucleophilic attack of hydroxylic oxygen either on a carbonium-ion-like substrate or the protonated parent compound. The rate law may be written as $k_{obs} = k_0\ [H^+]/([H^+] + K_a)$. These results indicate cleavage of the C—N bond to be S_N1 in character. Degradation was primarily to the bis-ether by way of the 5-hydroxy intermediate, with evidence of traces of the ketone. Kinetic analyses were by the acid–dye procedure, utilizing methyl orange as the anionic component. This method is specific for the reactions involved and yields first-order plots showing linearity through several half-lives.

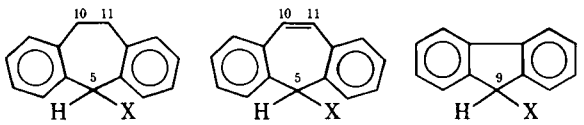
Keyphrases □ 5-Aminodibenzo[*a,d*]cycloheptenes—hydrolysis rates, compared to cycloheptanes, effect of 5-amino substituents □ Dibenzo[*a,d*]cycloheptenes—hydrolysis rates compared to cycloheptanes, effect of 5-amino substituents □ Hydrolysis—dibenzo[*a,d*]cycloheptenes, effect of 5-amino substituents

Dibenzo[*a,d*]cycloheptenes and their 10,11-dihydro analogs have served as the nucleus for numerous medicinal agents and possess an isosteric relationship with the phenothiazines (1). Dibenzo[*a,d*]cycloheptanes (I) exhibit antihistaminic activity (2, 3) while the analogous dibenzocycloheptenes (II) are active as anticonvulsants (4–6).

It was previously reported (7) that 5-aminodibenzo[*a,d*]cycloheptanes (I) hydrolyze in aqueous solutions at pH values below the pKa, *i.e.*, where an appreciable fraction of the amine is protonated. The intriguing feature of a highly labile carbon–nitrogen bond at C_5 (Structures I and II) was further investigated in this work. The effect of unsaturation in the seven-membered ring was examined quantitatively.

Several substituted 9-aminofluorenes (III) were studied with no indication of instability under the conditions utilized (8).

Molecules of Structure II show approximately a 100-fold increase in reactivity in aqueous acid over the 10,11-dihydro analogs (I). The products of the transformation are primarily the bis-ether and some alcoholic intermediate, with small amounts of ketone probably formed by air oxidation of the alcohol.



X = mono- or disubstituted aliphatic or cyclic amine

I II III

EXPERIMENTAL

Preparation of Compounds—The compounds prepared are listed in Tables I and II. The general procedures are illustrated with specific examples.

*5-Chloro-5*H*-dibenzo[*a,d*]cycloheptene*—A solution of 5-hydroxy-5*H*-dibenzo[*a,d*]cycloheptene[1] (5 g, 0.024 mole) was prepared in toluene (44 ml, dried over phosphorus pentoxide), with stirring, in a 100-ml round-bottom flask. The red material accumulating in the flask was removed by decantation of the solution into a second flask. Then thionyl chloride (2.08 ml) was added dropwise to the solution and the solution was cooled in an ice bath. The contents were allowed to come to room temperature for 30 min. The solvent was removed *in vacuo*, with the chloride being utilized without further purification.

*5-(1-Morpholino)-5*H*-dibenzo[*a,d*]cycloheptene (Compound 7, Table I)*—Solid 5-chloro-5*H*-dibenzo[*a,d*]cycloheptene (3.4 g) was added with stirring to a solution of morpholine (30 ml). After 15 min, the solution was heated on a steam bath for 30 min, followed by removal of solvent *in vacuo*. The semisolid residue was treated with 50 ml of 0.1 *N* NaOH. The basic solution was taken up in a separator and extracted with 2 × 50 ml of ether.

The ether extract was shaken out with 2 × 50 ml of distilled water and then extracted with 3 × 30-ml portions of 0.1 *N* HCl. The acid solution was neutralized with concentrated ammonia, with the resultant solid being extracted into ether and dried over anhydrous sodium sulfate. The ether was evaporated and the material was crystallized from methanol, yielding 1.9 g, 45%, mp 116–118° [lit. (9) mp 116–118°].

Hydrochlorides—These salts were prepared by dropwise addition of ethereal hydrochloric acid to an ethereal solution of amine. The white precipitate was collected and then recrystallized from methanol–ether (Table I).

9-Pyrrolidinofluorene (Compound 3, Table II)—A mixture of 2-chlorofluorene[1] (10 g, 0.05 mole) and pyrrolidine (3.5 g, 0.05 mole) was refluxed in 50 ml of benzene overnight. The solvent was removed under reduced pressure. The resultant mass was treated with 50 ml of 0.1 *N* NaOH followed by three 50-ml portions of distilled water. The solid was dissolved in methanol with heating and crystallized once from hot methanol, yielding 5.2 g, mp 86–90°.

9-Pyrrolidinofluorene Methiodide (Compound 4, Table II)—Two hundred milligrams of 9-pyrrolidinofluorene, 10 ml of methyl iodide, and 25 ml of acetonitrile were heated overnight at 50° (oil bath). The solvents were removed and the resultant material was crystallized from methanol–ether, yielding 112 mg, mp 191–192° dec.

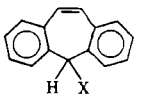
Solubility Analysis—*Method A*—Fifty milligrams of 5-pyrrolidinodibenzocycloheptene (Compound 5, Table I) was placed in a stoppered glass vial with the addition of 10 ml of buffer solution. Samples were turned at 20 rpm in a constant-temperature water bath at 23.6 ± 0.1°. Five-milliliter samples were withdrawn by filter pipet and monitored in the UV at 290 nm.

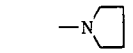
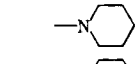
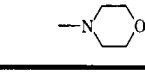
Method B—Ten milligrams of the propyl compound (Compound 3, Table I) was dissolved in 1 ml of water. Nine milliliters of buffer was added followed after 1 hr by filtration. The filtrate was monitored at 290 nm, and the pH was measured[2].

TLC of 5-Aminodibenzocycloheptenes and 9-Aminofluo-

[1] Aldrich Chemicals, Milwaukee, Wis.
[2] Metrohm pH meter.

Table I—Physical Constants of 5-Aminodibenzocycloheptenes



Compound	X	Melting Point	Yield, %	Molecular Weight	Molar Absorptivity, $\epsilon \times 10^{3}$[a]
1	$-N(C_2H_5)_2 \cdot HCl$	180–184°	32[b]	299.9	25.2
2	$-NH(CH_2CH{=}CH_2) \cdot HCl$	192–194°	20[b]	283.8	20.5
3	$-NH(CH_2CH_2CH_3) \cdot HCl$	115–117°	22[b]	285.8	26.7
4	$-NCH_3(CH_2CH_2OH)$	105–106°	18[c]	263.3	24.7
5		130–131°	54[d]	261.4	26.8
6		115–117°	39[d]	273.4	26.9
7		116–118°	45[d]	275.4	—[e]

[a] Absorptivity measured by employing a 4-ml sample (0.25–0.05 mg/ml), buffering at pH 3.44, and adding 25 ml of methyl orange solution, followed by extraction into 100 ml of chloroform. Plots of absorbance *versus* concentration then were prepared from these data. The ϵ values were calculated from moles of amine and absorbance of colored complex indicative of methyl orange. [b] Recrystallized from methanol–ether, Ref. 9. [c] Recrystallized from aqueous methanol. [d] Recrystallized from methanol, Ref. 9. [e] No satisfactory formation of colored complex.

renes (Tables I and II)—The compounds in Table I were chromatographed, using 10 μl of a 1–2% solution. Several solvent systems on silica gel GF[3] plates were used with a development distance of 15 cm. These are listed as follows (system, table, compound, and R_f): chloroform–methanol (98:2), I, 1, 0.15; chloroform–methanol (98:2), I, 5, 0.4; chloroform–methanol (98:2), I, 4, 0.2; chloroform–methanol (98:2), I, 6, 0.9; chloroform, I, 6, 0.45; chloroform–methanol (90:10), I, 1, 0.65; chloroform–methanol (90:10), I, 2, 0.95; chloroform–methanol (90:10), I, 4, 0.75; chloroform–methanol (90:10), I, 5, 0.85; chloroform, II, 1, 0.5; chloroform, II, 2, 0.4; and chloroform, II, 3, 0.2. The amines could be visualized by short-wave UV light (255 nm) as well as by means of Dragendorff reagents or bromcresol purple (15 mg/100 ml of ethanol).

Solution Preparation and Solvents—The pH 3.44 citrate buffer and methyl orange solution were prepared as previously stated (7).

Other solvents and chemicals were of reagent quality and were used without further treatment. Buffers were made to ionic strength of 0.2 except where specified.

Kinetic Procedures—A solution containing 25 mg of each compound was placed in 100 ml of 0.1 *N* HCl in volumetric flasks previously equilibrated at the specified temperatures, giving about 10^{-3} *M* solutions. Four-milliliter aliquots of the acidic solution were periodically withdrawn and neutralized with 0.1 *N* NaOH (4 ml) followed by 4 ml of pH 3.44 citrate buffer and 25 ml of methyl orange solution.

The aqueous layer was extracted three times with 30-ml portions of chloroform in a separator, and the chloroform solutions were collected and diluted to 100 ml in a volumetric flask. The absorption spectra of the yellow-colored complexes were recorded on a spectrophotometer over the 350–500-nm range.

Other kinetic procedures were carried out by the same analytical method after adjusting the pH of the aliquot drawn from the reaction flask to 3.44 prior to addition of methyl orange indicator.

The reaction products gave no color attributable to ion-pair formation with methyl orange when extracted using 10^{-3} *M* concentrations. Additionally, the intensity of the colorimetric analysis was not altered when the by-products were mixed at 10^{-3} *M* with solutions of the intact molecules.

Preparation and Properties of Reaction Products—*5-Hydroxydibenzo[a,d]cycloheptene (IV)*—One hundred milligrams of 5-morpholinodibenzo[*a,d*]cycloheptene (Compound 6, Table I) was heated for 24 hr at 23.6° in 100 ml of 0.1 *N* HCl. The white solid was filtered, dried, and streaked (in 0.5 ml of chloroform) on preparative silica gel GF plates[3] with development in chloroform. The spot corresponding to alcohol (R_f 0.3) was scraped off and eluted with chloroform, followed by filtration and evaporation, yielding 10 mg of a white powder, mp 121–123°, corresponding to the authentic alcohol[1], mp 122.5–124°. Mixed melting points of the two compounds resulted in no depression.

The UV spectrum was the same as that of the authentic alcohol. TLC of both the authentic alcohol and the product isolated in: (*a*) chloroform–diethylamine (99:1), (*b*) alcohol USP, and (*c*) ether–petroleum ether–methanol (20:80:1) gave identical R_f values of 0.3, 0.5, and 0.85, respectively.

Anal.—Calc. for $C_{30}H_{22}O$: C, 86.5; H, 5.8. Found: C, 86.4; H, 5.7.

Bis(5H-dibenzo[a,d]cyclohepten-5-yl) Ether (V)—One hundred milligrams of the morpholinodibenzocycloheptene (Compound 6, Table I) was heated at 80° in 100 ml of 0.1 *N* HCl for 24 hr. The white precipitate was filtered and dried, yielding 41 mg (39%), mp 202–208°. Washing with cold petroleum ether removed contaminating ketone and alcohol as ascertained by TLC in chloroform–diethylamine (99:1), with the resultant product melting at 208–211° [lit. (10) mp 210–212°].

TLC in chloroform showed traces of alcohol and ketone. Mass spectroscopy showed a molecular ion at 406, giving two fragments at $M^+ = 211$ and 195.

Anal.—Calc. for $C_{30}H_{22}O$: C, 90.4; H, 5.6. Found: C, 90.1; H, 5.5.

5H-Dibenzo[a,d]cyclohepten-5-one (VI)—Degraded solutions contained a spot on TLC attributable to the ketone. This compound was not identified *per se* but may be present as a result of air oxidation of the alcohol. Solutions containing the decomposition products of the amine exhibited fluorescent spots of identical R_f to the authentic ketone[1] when subjected to TLC in the solvent systems previously listed as applicable to the alcohol (IV).

RESULTS AND DISCUSSION

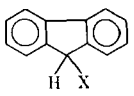
Several 5-aminodibenzo[*a,d*]cycloheptanes were recently reported to undergo cleavage at the C—N bond in aqueous acid by a route that may be visualized as proceeding *via* a carbonium ion or a kinetically equivalent mechanism (7). From the standpoint of resonance-stabilized structures, these 5-aminodibenzocycloheptanes (I) allow spreading of the positive charge over seven carbon atoms, presumably leading to the observed high reactivity. The 5-aminodibenzocycloheptenes (II) should be expected to be even more unstable in aqueous media due to the possibility of charge distribution over nine carbon atoms—the entire ring system—of the tricyclic skeleton, the center of which is composed of a potential tropylium ion.

Tropylium ions are among the most stable carbonium ions yet prepared. This longevity is partially due to distribution of six π-electrons evenly over the π-orbitals comprising the heptagonal ring, thus fulfilling the criteria for aromaticity (11, 12). More stable cations are generated when a number of resonance structures can be drawn indicative of distribution of the positive charge over several atoms rather than its confinement to a single atom.

Preparation of Compounds—Amines involved in this investigation were prepared from literature methods (6). The sequence

[3] Analtech, Newark, Del.

Table II—Physical Constants of 9-Aminofluorenes



Compound	X	Melting Point	Yield, %	Molecular Weight	Molar Absorptivity, $\epsilon \times 10^{3a}$	Analysis, % Calc.	Found
1		86–90°	44[b]	235.3	30.0	C 86.8 H 7.3 N 6.0	86.4 7.7 5.9
2		92–96°	21[b]	247.3	30.2	C 87.4 H 6.9 N 5.7	87.1 7.0 5.5
3		145–149°	37[b]	251.3	—[c]	C 81.2 H 6.8 N 5.6	81.7 6.8 5.6
4		191–192°	47[d]	377.3	27.3	C 57.3 H 5.4 N 3.7 I 33.6	57.4 5.5 3.5 33.0

[a] Absorptivity was measured by employing a 4-ml sample (0.25–0.05 mg/ml), buffering at pH 3.44, and adding 25 ml of methyl orange solution, followed by extraction into 100 ml of chloroform. Plots of absorbance *versus* concentration were then prepared from these data. The absorbance of the colored complex (indicative of methyl orange) was plotted *versus* the concentration of the amine (moles per liter) to determine ϵ. [b] Recrystallized from methanol. [c] No complex formation. [d] Recrystallized from methanol–ether.

consisted of conversion of the 5-hydroxy analog[1] into the chloride on treatment with equimolar thionyl chloride in toluene followed by amination with the appropriate primary or secondary amine. The 5-aminodibenzocycloheptenes (II) were then crystallized or converted to the hydrochlorides for further utilization. The compounds studied along with pertinent physical data are listed in Table I.

Table II gives some 9-aminofluorenes prepared for solvolytic investigation.

Evaluation of Rate Constants—Decomposition of the various 5-aminodibenzocycloheptenes (II) in aqueous solution (pH 7 and below) was monitored by colorimetry using the acid–dye method with methyl orange as the anionic component (7, 13). Complexes were of the amines (Tables I and II) and methyl orange (1:1). The molecularity was determined by extraction of a fixed quantity of amine in pH 3.44 buffer with increasing amounts of methyl orange indicator (7). Small discrepancies from the equimolar ratio were the result of volatile impurities—likely water—in the methyl orange indicator. Figure 1 represents a typical plot for ascertaining the molecularity of the complex.

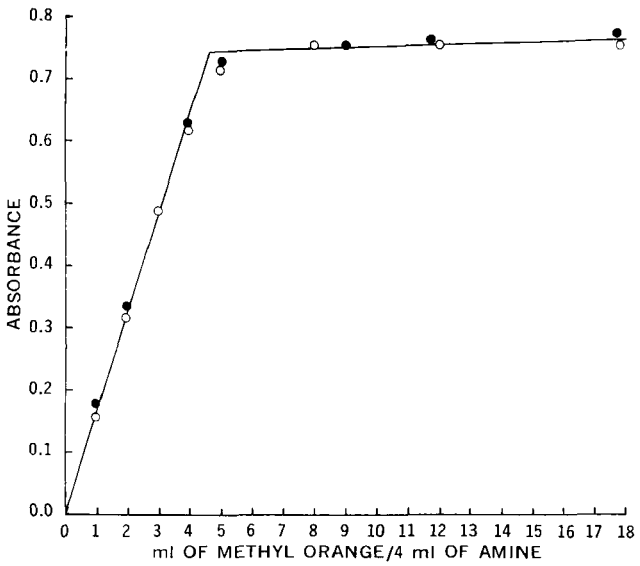
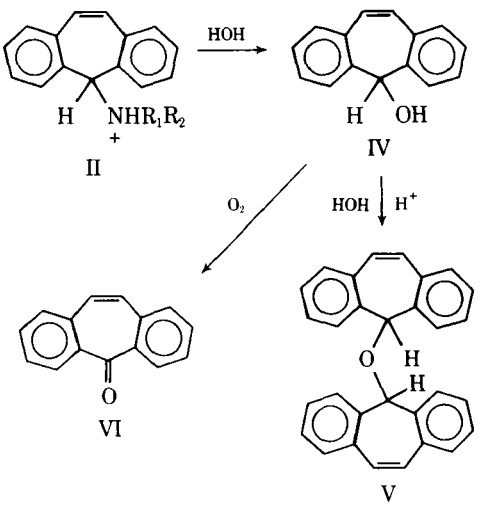


Figure 1—*Determination of the molecularity of the 5-pyrrolidinodibenzocycloheptene complex (Compound 5, Table I) by means of adding increasing volumes of 7.6 × 10⁻⁴ M methyl orange indicator solution to 4-ml portions of 7.6 × 10⁻⁴ M amine in pH 3.44 buffer. Point of intersection of the lines is indicative of the ml of methyl orange/4 ml of equimolar amine. The value is a little greater than 4 ml of methyl orange, which may be attributed to volatile impurities (possibly water) found in the reagent methyl orange. Black and white circles represent two different experiments.*

The principal products of the solvolysis in water (Scheme I) were the alcohol (IV), the bis-ether (V), and the ketone (VI). The allyl and propyl derivatives (Compounds 2 and 3, Table I) gave a fourth substance, which was separated by TLC but not identified. The transformations shown in Scheme I are analogous to those previously reported for the 10,11-dihydro analogs (7). At lower temperatures the alcohol predominates, while at 70° and above the ether is the primary product.

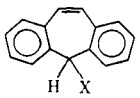
Scheme I illustrates the general reaction sequence, although the formation of the ether (V) has not been proven to go entirely through the alcohol. Some ether could conceivably result from reaction of the amine (II) with the alcohol (7).

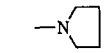
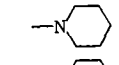
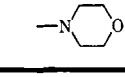
The ketone (VI) was present in trace amounts, possibly due to air oxidation of the alcohol (IV), while the latter serves as an intermediate. None of the materials formed on hydrolysis (IV, V, and VI) interferes with the analytical procedure when mixed with reactant amines in equimolar concentrations.



Scheme I

Table III—Substituent Effect of Substituted Amino Group on Rate Constants for Hydrolysis of 5-Aminodibenzo[*a*,*d*]cycloheptenes, about 8 × 10^{-4} *M*[a], in 0.1 *N* HCl



Compound	X	k_{obs}, hr^{-1}[b] 80°	70°	60°	50°
1	$-N(C_2H_5)_2 \cdot HCl$	(45.0)[c]	(10.3)[c]	2.5[d]	0.53
2	$-NH(CH_2CH{=}CH_2) \cdot HCl$	3.1	0.84	0.24	0.043
3	$-NH(CH_2CH_2CH_3) \cdot HCl$	0.89	0.21	0.065	0.011
4	$-NCH_3(CH_2CH_2OH)$[e]	(120.0)[c]	(32.0)[c]	7.6	2.1
5		0.27	0.054	0.013	0.0023
6		1.7	0.46	0.12	0.020
7		—[f]	—	—	—

[a] Initial concentration of 25 mg/100 ml with 4-ml samples withdrawn and analyzed as specified in the *Experimental* section. [b] Rate constants were determined in triplicate. Constants listed are the average of three runs with no deviations greater than ±15% of the arithmetic mean. [c] Values were determined by extrapolation of log k_{obs} = constant − $Ea/2.303RT$. [d] Other values for the diethylamino compound were: 40°, 0.11; and 23.6°, 0.059. [e] Other value: 0.58, 40°. [f] Reaction extremely rapid with $t_{1/3}$ of about 30 min at 23.6° as estimated from TLC plates.

First-order disappearance of starting material was followed by:

$$\log(A - A_\infty) = \log(A_0 - A_\infty) - k_{obs}t/2.303 \quad \text{(Eq. 1)}$$

where A_0, A, and A_∞ are absorbances of the ion-pair in chloroform at times zero, t, and infinity, respectively, and k_{obs} is the apparent or observed first-order rate constant in $hours^{-1}$.

Figure 2 illustrates typical plots of log absorbance *versus* time for the decomposition of the 5-aminodibenzocycloheptenes (Table I). Residual absorbances, A_∞, of less than 2.5% of the initial A_0 were observed. These were primarily blank extractions of slight amounts of indicator into the chloroform. Graphs of the first-order process were linear through up to six half-lives. Readings were taken at 340 ± 2 nm, with blank determinations being subtracted from the absorbances found.

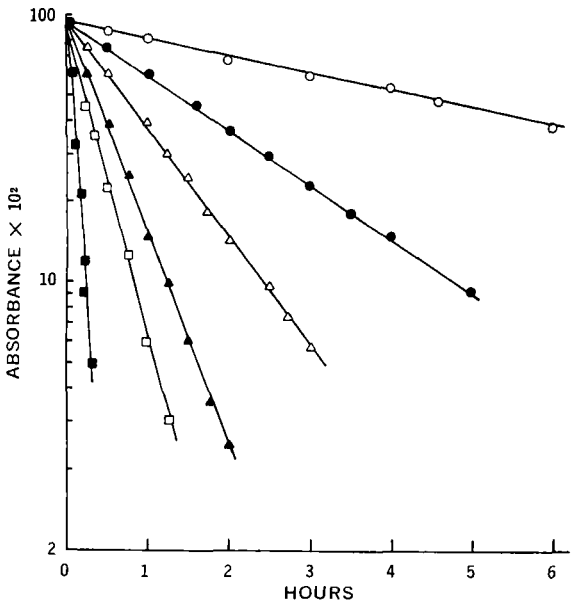


Figure 2—*First-order plots for hydrolysis of several 5-aminodibenzocycloheptenes in 0.1 N HCl at 80° unless otherwise indicated. Twenty-five milligrams of substrate was dissolved in 100 ml of 0.1 N HCl followed by taking a 4-ml aliquot, adjusting the pH to 3.44, and adding 25 ml of methyl orange solution. The complex was then extracted into 100 ml of chloroform and monitored as a function of time. Compounds studied were: ■, —$NCH_3CH_2CH_2OH$, 50°; □, —$NHCH_2CH{=}CH_2$; ▲, piperidino; △, —$NHCH_2CH_2CH_3$; ●, —$N(CH_2CH_3)_2$, 50°; and ○, pyrrolidino. Values for velocity constants are listed in Table III.*

Concentrations at 2.5 mg/100 ml solution were monitored, employing longer path length cells and/or less chloroform volume for extraction. Values for the rate constants were the same within experimental error as those obtained with 25 mg of amine/100 ml of solution. Centrifugation was circumvented by allowing the chloroform solutions to clarify for 5–24 hr prior to analysis.

The morpholino derivative (Compound 7, Table I) decomposed quickly under ambient conditions but failed to form an extractable complex with methyl orange. It was more readily extractable from pH 3.44 buffer into chloroform than the piperidino congener (Compound 6, Table I). The morpholino was completely removed on one extraction, while the piperidino showed measurable absorbance in the aqueous layer after three extractions (15 ml of chloroform–15 ml of pH 3.44 buffer).

When equimolar amounts of amine and methyl orange were prepared (7 × 10^{-4} *M*) followed by shake-out with equal volumes of chloroform, the morpholino passed into the organic layer while the dye remained in the aqueous layer; however, the piperidino formed a readily extractable complex. This extractability of noncomplexed amine into the organic layer and subsequent failure of acid–dye interaction has been described elsewhere (14).

The oxygen atom *per se* is not the cause of this phenomenon because the —$NCH_3(CH_2CH_2OH)$ analog responds in the normal manner. The pKa values of all compounds in Table I should be similar enough to eliminate this as the factor causing the spurious result.

The 9-aminofluorenes (Table II) exhibited no tendency to degrade on prolonged treatment at 80°, with the morpholino again failing to complex with the dye.

pKa Estimation—Equilibrium solubility analysis of 5-propylaminodibenzo[*a*,*d*]cycloheptene (Compound 3, Table I) and 5-pyrrolidinodibenzo[*a*,*d*]cycloheptene (Compound 5, Table I) was carried out for approximation of the pKa's (15). An excess of the pyrrolidino analog was allowed to equilibrate with various buffers

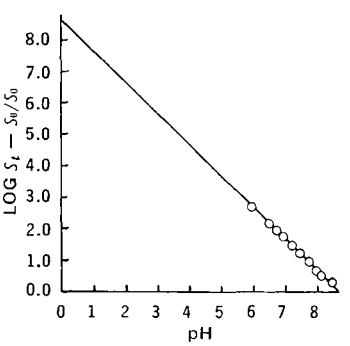
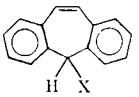


Figure 3—*Plot of log* $S_t - S_0/S_0$ *versus pH from Eq. 5. This figure represents data obtained from solubility analysis of the 5-pyrrolidinodibenzo*[a,d]*cycloheptene (Compound 5, Table I) at 23.6°. Point of intersection with the* y*-axis is the pKa value, slope* = −1. S_t *is solubility of protonated and neutral species, with* S_0 *being solubility of neutral or uncharged base.*

Table IV—Observed First-Order Rate Constants (k_{obs}, hr^{-1}) for Decomposition of 5-Aminodibenzo[*a*,*d*]cycloheptenes in Aqueous Solution[a,b]



Compound	X	Temperature	pH[c,d] 0.1	1.1	2	3.4	5	6	7	Water
1	$-N(C_2H_5)_2 \cdot HCl$	60°	0.52	0.53	—	—	0.49	—	0.48	0.44
2	$-NH(CH_2CH{=}CH_2) \cdot HCl$	70°	—	0.84	—	0.89	0.78	—	0.80	0.82
3	$-NH(CH_2CH_2CH_3) \cdot HCl$	80°	0.89	0.88	0.82	0.91	—	—	0.71	0.77
4	$-NCH_3(CH_2CH_2OH)$	50°	—	2.1	—	—	—	2.3	—	2.0
5		80°	0.28	0.29	—	0.31	—	—	0.27	0.22
6		80°	1.82	1.89	1.71	1.74	1.68	1.76	1.79	1.61
7		—	—[e]	—	—	—	—	—	—	—

[a] Followed by acid–dye method, initial concentration of 25 mg/100 ml except at pH 7 where 10 mg/100 ml was used with extraction of complex into 50 ml of chloroform. See *Experimental* section. [b] Most kinetic runs were carried out in duplicate, with results reported as averages of total number of runs. [c] The pH values of buffers were measured on a Metrohm pH meter. The pH values of hydrochloric acid solutions were determined from activity coefficients employing Ref. 19. The slight variation with temperature of the solutions was not considered. [d] The pH 1.1 and 0.1 buffers were made from 0.1 and 1.0 *N* HCl, respectively. The pH 2 buffer was made from 0.05 *M* $NaH_2PO_4 \cdot H_2O$ with H_3PO_4 added to give the proper pH. The pH 5 buffer was made to ionic strength 0.1 from sodium hydroxide on addition of acetic acid. The pH 3.44 buffer was made as stated in Ref. 7. The pH 6 and 7 buffers were prepared from 0.05 *M* $Na_2HPO_4 \cdot 7H_2O$ by addition of hydrochloric acid. [e] Could not be assayed by acid–dye method. [f] Compounds 4, 5, and 6 were dissolved by addition of equimolar hydrochloric acid.

in a constant-temperature bath, 23.6°, overnight followed by UV analysis of the filtrate. The morpholino and propylamino were dissolved in 0.1 *N* HCl and water prior to the addition of buffer. Instability prevented their long equilibration.

Amines and their salts may be treated in a manner similar to that recently discussed for acids and their anions (16). The ionization constant, K_a, for a protonated amine may be expressed as:

$$K_a = \frac{[B][H^+]}{[BH^+]} \qquad \text{(Eq. 2)}$$

or, if certain assumptions are made, can be stated in terms of solubilities (15):

$$K_a = \frac{[H^+]S_0}{[S_{BH^+}]} \qquad \text{(Eq. 3)}$$

when:

$$S_t = S_0 + S_{BH^+} \qquad \text{(Eq. 4)}$$

where S_t, S_0, and S_{BH^+} represent total solubility, solubility of the neutral molecule, and solubility of the protonated amine, respectively, in moles per liter.

By combining Eqs. 3 and 4 and writing the result in terms of negative logarithms, the relationship:

$$\log\frac{S_t - S_0}{S_0} = \text{pKa} - \text{pH} \qquad \text{(Eq. 5)}$$

is derived for the solubility of the undissociated amine in combination with its protonated form, where S_0 is constant regardless of pH.

Plotting $\log S_t - S_0/S_0$ on the ordinate against pH on the abscissa, one obtains a straight line with slope −1. The *y*-intercept is the pKa. Figure 3 illustrates an example utilizing the 5-pyrrolidino analog.

The other compounds (Table I) could not be studied by this equilibration procedure due to their tendency to degrade under ambient conditions. However, assuming rapid equilibration, the propylamine hydrochloride (Compound 3, Table I) was dissolved in a minimal volume of water with addition of buffer, followed by filtration and analysis of the filtrate. The morpholine (Compound 7, Table I) was treated analogously except for dissolution in 0.1 *N* HCl preceding addition of buffer with pKa = 7.5 ± 0.3 at 21°. The pKa values for the propylamino and pyrrolidino derivatives determined at 23.6° were 8.9 ± 0.3 and 8.8 ± 0.3, respectively.

The usual methods for pKa evaluation were not applicable because of: (*a*) the extreme insolubility of the free base precluding titration, (*b*) the lack of spectral shifts in the UV region on conversion of protonated to nonprotonated species, and (*c*) the inability to make determinations kinetically due to the poor solubility of the unionized base.

Application of Eq. 5 as shown in Fig. 3 gives a pKa value of 8.9 ± 0.3. The nature of the equation allows the point of intersection with the abscissa to give the pKa value. Examination of a graph of milligrams per milliliter in solution, S_t, against pH reveals a pKa approximately the same as that obtained from Eq. 5. The pKa is the point where $S_t = 2 \times S_0$; S_0 was estimated from the asymptote at higher pH values where practically all amine is present as the neutral species. Figure 4 is an example of the data obtained from the tedious solubility experiment with the propylamine derivative.

Reaction Routes—Tables III and IV present experimental kinetic data. The expression describing the observed velocity constant for hydrolysis of the 5-aminodibenzocycloheptenes may be given as:

$$k_{obs} = k_0 f_{BH^+} = k_0 \frac{[H^+]}{[H^+] + K_a} \qquad \text{(Eq. 6)}$$

when the fraction of neutral amine is appreciable or $k_{obs} = k_0$ when the fraction of cationic amine is 1, $f_{BH^+} = 1$. Equations of this sort are indicative of water reaction with substrate (17, 18).

The compounds investigated showed an invariant rate constant at a given temperature in water. At pH 7, the value for the velocity constant was the same, within experimental error, as that in 1 *N* HCl (Table IV). Due to the low solubility of the partially protonated compounds, only a small percentage of uncharged substrate was present, leading to the apparently identical rate constants obtained at higher hydrogen-ion concentrations.

The velocity constants were a function of temperature, solvent (water), nature of substrate, and protonation of the amino group. When hydrochloride salts were dissolved in distilled water, the rate constants were the same as those in aqueous acidic or buffer solutions as long as the nitrogen was cationic (Table IV). No ionic strength or buffer effects were noted.

Decomposition of the protonated amine (Compound II, Scheme I) appears as a spontaneous process at a specified temperature in aqueous solution. No color was observed as indicative of free carbonium ions in nonaqueous media, but it is unlikely that complete ionization would occur in water. A concerted mechanism might be expected where the solvent interacts to some extent with the substrate at all phases of the hydrolysis.

Arrhenius Parameters—Figure 5 exemplifies plots from the

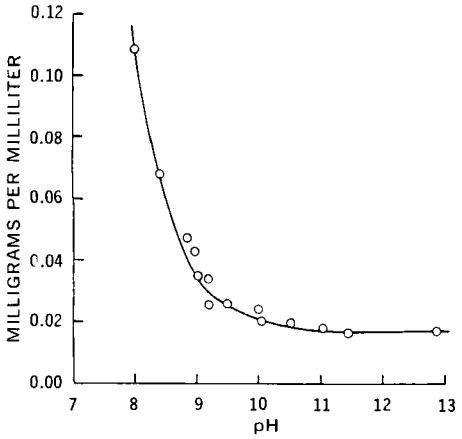


Figure 4—*Plot of solubility of 5-propylaminodibenzocycloheptene (Compound 3, Table I)* versus *pH, 23.6°. The pKa value is equivalent to point where total solubility equals twice the observed solubility at pH 12–13;* i.e., $S_t = 2S_0 = pKa$.

Arrhenius equation:

$$\ln k_{obs} = Ea/RT + \text{constant} \qquad \text{(Eq. 7)}$$

for several compounds in Table III. The apparent energies of activation are invariant for the pH ranges studied (0.1–7). The *Ea* values for the compounds under scrutiny (Fig. 5) lie between 29.8 and 32.6 kcal/mole, so it appears that the activated complexes are quite similar in all cases within the realm of experimental error.

Relative Reactivities—Removal of two hydrogen atoms and subsequent aromatization of the dibenzocycloheptane nucleus bring about almost a 100-fold enhancement of the rate constants, $k_{\text{cycloheptene}}/k_{\text{cycloheptane}}$, for the molecules with the same amino substituents at position 5 of the ring. The k_{ene}/k_{ane} values are 79, 87, and 204 for the diethylamino, pyrrolidino, and piperidino analogs, respectively, within the rate constant data for the 10,11-dihydro congeners taken from previously published results (7). That

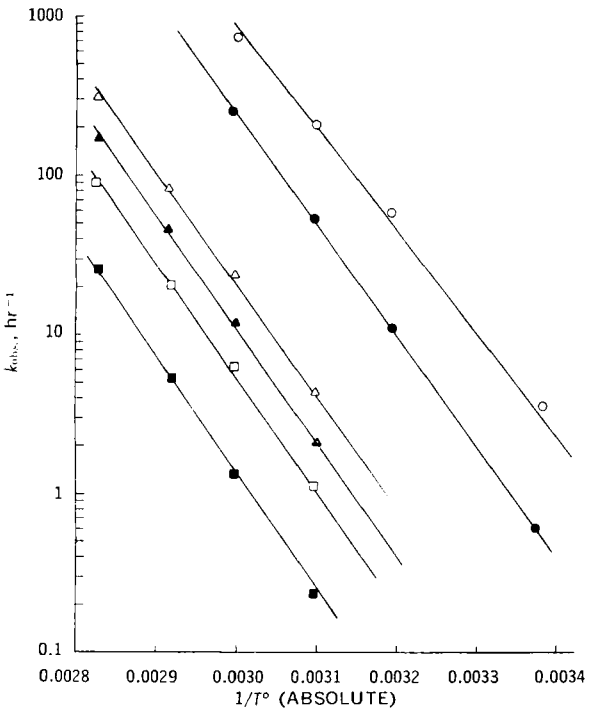


Figure 5—*Arrhenius plots for various compounds in 0.1* N *HCl. Key:* ■, $-NC_4H_8$; ▲, $-NC_5H_{10}$; □, $-NH(C_3H_8)$; △, $-NHCH_2CH{=}CH_2$; ●, $-N(CH_2CH_3)_2$; *and* ○, $-NCH_3(CH_2CH_2OH)$. *Compounds included are listed in Table III.*

the fully aromatic structures (II) react so rapidly is not as disconcerting as the fact that the hydrogenated analogs (I) react at all, because the opportunity for formation of the tropylium ion is not afforded in these molecules.

Another unexpected finding is that the k_{obs}–pH relationships (Table IV) give no evidence of an increased reaction rate as the pH scale is ascended, contrary to recently published papers dealing with tropylium ions (20–23). These data reinforce the previously stated idea that the rate-determining step is ionization or some equivalent of the 5-amino functionality, with concomitant hydroxylation by the nucleophilic solvent, water, leading to the ether and alcohol as primary products (7). In light of recent work (21, 22), it is surprising that as the pH increases the affinity of the intermediate for OH^- ions does not increase the rate constant. Other nucleophilic agents such as methanol interacted even more quickly with the protonated amines, and this will be the subject of a later report.

The initial reaction of Scheme I with water attack on the 5-amino position may be analogous to the reaction of water with alkyl iodide or other halide to give the alcohol. This series represents a unique class of transformations, probably greatly facilitated by the potential tropylium ion flanked by two phenyl rings, thus allowing dispersion of the charge over the entire framework of the ring system. The protonated amines serve as unlikely leaving groups, producing electron-deficient intermediates.

CONCLUSIONS

A series of 5-aminodibenzocycloheptenes was prepared and investigated kinetically. The velocity constants are two magnitudes larger than those for the corresponding 5-aminodibenzocycloheptanes. This can be explained on the basis of complete dispersion of the potential positive charge generated on or during cleavage of the C—N bond over the entire tricyclic skeleton.

The rate law was found to be $k_{obs} = k_0\,[H^+]/[H^+] + K_a$ and can be simplified to $k_{obs} = k_0$ at pH values below 7 where the amines are practically 100% protonated. This type of kinetic result indicates a S_N1 mechanism, although rigid proof of the proposed reaction type is difficult.

These compounds further illustrate cases where a carbon–nitrogen linkage is easily cleaved in aqueous solution, and the reaction class is rather reminiscent of hydrolysis of certain alkyl halides in aqueous solution.

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Keyphrases □ Colestipol hydrochloride—*in vitro* binding to various drugs, effects of ionic strength, pH, and competing ion, compared to cholestyramine □ Binding—colestipol hydrochloride and various drugs *in vitro*, effects of ionic strength, pH, and competing ion, compared to cholestyramine □ Drug binding—colestipol hydrochloride and various drugs *in vitro*, effects of ionic strength, pH, and competing ion, compared to cholestyramine □ Cholesterol-reducing agents—*in vitro* binding of various drugs to colestipol hydrochloride

Colestipol hydrochloride is a copolymer that indirectly lowers serum cholesterol in experimental animals and humans and is believed to do so through binding bile acids and their conjugates in the GI tract (1). Cholestyramine (a bile salt-sequestering agent) interferes with the absorption of thyroxine in humans (2) and other drugs in rats and dogs (3). It was conceivable that concurrently ingested drugs would be similarly bound to colestipol hydrochloride and thus be poorly absorbed.

To identify potential drug–polymer interaction problems prior to *in vivo* binding studies, the comparative *in vitro* binding to colestipol hydrochloride and cholestyramine was determined for different drugs. Initial drug–polymer ratios typical of clinical usage were employed under experimental conditions (pH and ionic strength) approximating those of the distal portion of the ileum, one of the later absorbing sites in the GI tract.

EXPERIMENTAL

Materials—Sodium taurocholate-7-^{3}H had a specific activity of 46.3 mCi/mmole[1], and sodium taurocholate-24-^{14}C had a specific activity of 6.6 μCi/mmole[2]. Unlabeled sodium taurocholate[3] was used as carrier. These compounds were homogeneous by TLC. Tetracycline-7-^{3}H hydrochloride (1200 mCi/mmole[4]), niacin-6-^{14}C (26.2 mCi/mmole[4]), D-biotin-carbonyl-^{14}C (57.5 mCi/mmole[4]), chlorpropamide-^{35}S (1.8 mCi/mmole[4]), potassium benzyl penicillin-^{14}C (20–40 mCi/mmole[4]), phenobarbital-2-^{14}C (2.97 mCi/mmole), and cholesterol-26-^{14}C (46 mCi/mmole[5]) were of 95–99% radiochemical purity as determined by paper or thin-layer chromatography.

The binding of these compounds to colestipol hydrochloride[6] and to cholestyramine[7] was studied.

[1] Supplied by Nuclear Research Chemicals Co., Orlando, Fla.
[2] Prepared by Dr. R. C. Thomas, The Upjohn Co., Kalamazoo, Mich.
[3] Grade A, Calbiochem Co., Los Angeles, Calif.
[4] Amersham/Searle Co., Chicago, Ill.
[5] New England Nuclear, Boston, Mass.
[6] Colestid, supplied by The Upjohn Co., Kalamazoo, Mich.
[7] Cuemid, Merck, Sharp and Dohme, West Point, Pa.

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The following buffers (all concentrations are final concentrations) were used: sodium phosphate containing 0.15 *M* phosphate with μ = 0.4 and pH 7.5; sodium phosphate containing 0.23 *M* phosphate with μ = 0.4 and pH 6.5; sodium acetate containing 0.15 *M* sodium acetate adjusted to pH 6.0 with acetic acid; imidazole hydrochloride containing 0.15 *N* HCl adjusted to the following pH's with imidazole, pH 5.95, 6.58, 6.98, 7.47, and 7.96; tromethamine–sodium chloride containing 0.05 *N* HCl and 0.1 *N* NaCl adjusted to pH 7.5 with tromethamine (Buffer I, μ = 0.15). The buffer employed in a particular experiment is indicated in the tables.

Apparatus—The UV spectra of free unlabeled drugs were measured with a spectrophotometer[8]. The radioactivity of free labeled drugs was measured with scintillation counters[9].

Methods—Fifteen milliliters of the appropriate buffer, 100 mg of colestipol hydrochloride (or cholestyramine), and an amount of bile acid conjugate (or drug whose binding was to be tested) were added to a 50-ml glass-stoppered centrifuge tube (round bottom).

The amount of sequestrant and drug added corresponded to 1/150 of the daily human dose (unlabeled drug was added to labeled drug to make up the required amounts). The samples were vigorously mixed at 275 oscillations/min during equilibration. Periodically, the shaker[10] was stopped and the tubes were shaken manually so that grains of resin adhering to the sides of the tubes were washed down. In most experiments, the samples were equilibrated for 1 hr at 23.7°. Other times are indicated in the text.

After equilibration, the samples were centrifuged at about 1000×*g* for 5 min. With radioactive samples, 0.5 ml of supernate was mixed with 15 ml of scintillator[11] [toluene–dioxane–methanol (350:350:210 by volume) containing 73 g of 2,5-diphenyloxazole and 0.08 g of 1,4-bis-2-(5-phenyloxazolyl)benzene/liter] and counted. For the zero time control, 0.5 ml of solution that had not been exposed to polymer was counted. For the unlabeled drugs, *e.g.*, vitamin A, the amount of drug in the supernate was measured by the absorbance of the solution at the wavelength of maximum absorptivity. The percent drug bound (B) at various equilibration times was calculated according to the formula:

$$B = 100(1 - C/C_0) \qquad \text{(Eq. 1)}$$

where C is the number of radioactive counts or absorbance (concentration) of free drug in the supernate after equilibration, and C_0 is the radioactive count or absorbance (concentration) in the absence of polymer.

RESULTS AND DISCUSSION

The binding of various drugs was studied in different buffers, at different pH values, and, in some cases, at different concentrations of drug. As model compounds, aspirin and taurocholate were studied more intensively.

Binding of Taurocholate at Different Equilibration Times—The binding of taurocholate to colestipol hydrochloride was sufficiently rapid that the binding process was about 94% complete in 60 min (Buffer I, μ = 0.15 and pH 7.5). Equilibration was established in about 3 hr (Fig. 1), and the time required was independent of buffer ionic strength in the range studied (0.01–0.25). With cholestyramine, taurocholate required 1–2 hr to establish equilibrium (Fig. 2). To approximate the degree of equilibration that might be encountered *in vivo*, the equilibration time in most experiments was limited to 1 hr.

Ionic Strength—The binding of taurocholate was found to decrease with increasing ionic strength (2) (Figs. 1 and 2) and was probably due to competition of the other ions for the same sites or to shielding. Such relationships with ionic strength suggest that at least some binding occurs between oppositely charged particles (4). Since taurocholate is negatively charged at pH 7.5, colestipol hydrochloride and cholestyramine must be positively charged according to this hypothesis. In the case of cholestyramine, this expectation is confirmed since it is a copolymer of styrene cross-linked

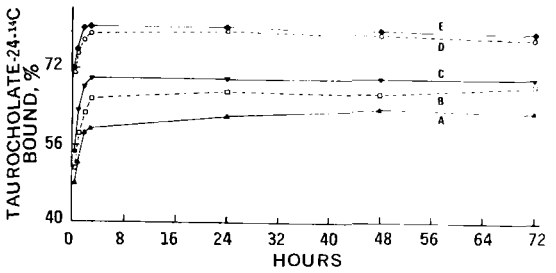

Figure 1—*Effect of ionic strength at pH 7.5 on the binding of taurocholate to colestipol hydrochloride. Key: A, μ = 0.25, tromethamine, 0.05 N HCl, 0.2 N NaCl; B, μ = 0.15, phosphate; C, μ = 0.15, tromethamine, 0.05 N HCl, 0.1 N NaCl; D, μ = 0.05, tromethamine, 0.05 N HCl; and E, water.*

with 2% divinylbenzene[12] and is a strongly basic anion-exchange resin with quaternary ammonium functional groups (5). The ion-exchange group in colestipol hydrochloride can be considered to be weakly basic secondary and tertiary amines.

An ionic strength of 0.15 approximates the lower limit of ionic strength of GI secretions. Gastric juice, gastric mucus, pancreatic juice, hepatic duct bile, and jejunal secretions are nearly isotonic (6) with blood plasma. Almost all of the osmotic pressure is contributed by monovalent ions—*viz.*, Na^+, H^+, Cl^-, and HCO_3^-. Thus, the lower limit of the ionic strength of GI secretions is approximately equal to that of physiological saline. The contribution of polyvalent ions such as proteins to ionic strength could be substantial (about 0.1) but would depend upon the kind of protein, pH, and ion concentration. Ingested substances would further alter the ionic strength to many other values. An ionic strength of 0.15 was employed as a standard condition.

Binding of Taurocholate and Aspirin at Different pH's—To study the effect of pH on the binding of a drug and bile acid, the binding of aspirin and taurocholate was studied in imidazole hydrochloride buffers (ionic strength of 0.15). The pH values were varied from 5.95 to 7.96. The effect of pH on taurocholate was much greater than on aspirin. As the pH was increased over this range, aspirin binding (60-min values) to colestipol hydrochloride decreased from 28.3 to 20.9%. For taurocholate, the binding (60-min values) in this system decreased from 20.4 to 7% as the pH was increased over the same range.

As shown in Fig. 3, the data suggested that a weak basic ionizing group in colestipol hydrochloride, with a pKa in the region of pH 7.2–7.3, was involved in the binding of taurocholate and aspirin. At pH 7.5, the binding contribution by this group was relatively minimal. Since some residual binding was still present around pH 8, some groups could be strongly basic amines or quaternary nitrogens.

In fasting humans, pH's between 2.5 and 7.5 in the duodenum, between 6.5 and 7.5 in the jejunum, and between 3.5 and 8 in the ileum have been recorded (7). For fed humans, the pH values ranged from 5.3 to 8 in these regions but tended to increase from 6

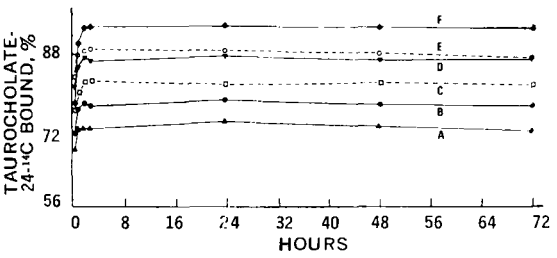

Figure 2—*Effect of ionic strength at pH 7.5 on the binding of taurocholate to cholestyramine. Key: A, μ = 0.25, tromethamine, 0.05 N HCl, 0.2 N NaCl; B, μ = 0.15, tromethamine, 0.05 N HCl, 0.1 N NaCl; C, μ = 0.10, tromethamine, 0.05 N HCl, 0.05 N NaCl; D, μ = 0.05, tromethamine, 0.05 N HCl; E, μ = 0.15, phosphate; and F, water.*

[8] Hitachi model 124, supplied by Coleman Instruments Co., Maywood, Ill.

[9] Models 3375 and 314EX2A, Packard Instrument Co., Downers Grove, Ill.

[10] Utility shaker, Clay Adams, New York, N.Y.

[11] Diotol, supplied by Burdick and Jackson, Muskegon, Mich.

[12] Dowex 1-X2.

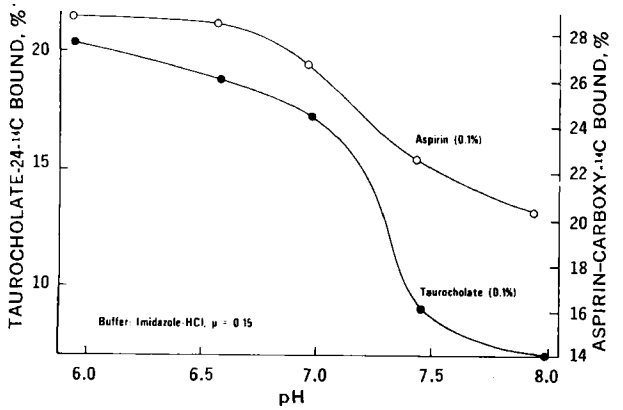

Figure 3—*Binding to colestipol hydrochloride as a function of pH.*

to 7 or 8 as the intestinal contents proceeded from the duodenum to the lower ileum (6). Binding of *ionized* acidic compounds is expected to be greater at the lower pH's and would be dependent on the relative positions of the compound and colestipol hydrochloride in the GI tract.

Weak acids, which must be unionized to be efficiently absorbed, should tend to be bound less to colestipol hydrochloride when the pH in the tract is below the pKa of the weak acid. It is expected that drugs bound at the lower pH's (when the drug–polymer combination is at positions in the tract near the stomach) would tend to be released as lower portions of the small intestine and higher pH's are reached. If a drug is more strongly bound at the higher pH's, then the determination at the higher pH's would be an estimate of the maximum binding.

A pH of 7.5 was chosen to estimate the amount of drug binding that might be expected at the distal portion of the ileum, where bile acid conjugate absorption takes place. Since absorption of the drug may have taken place at prior points in the GI tract, this binding value represents the "last chance" binding for drug not previously absorbed and is an estimate of the upper limit of the *dose* of the drug bound.

Aspirin Binding as a Function of Aspirin Concentration—The percent binding of taurocholate to colestipol hydrochloride is highly concentration dependent. It was of interest to determine if the binding of aspirin (a model compound) to colestipol hydrochloride would also be concentration dependent in the same range, 1.85–46.3 m*M*. In this concentration range, the binding of aspirin

Table I—Effect of Sodium Taurocholate, Monoolein, and Oleic Acid on Drug Binding to Colestipol Hydrochloride

Drug	Input Concentration, mg/ml	Percent Drug Bound after 1 hr Equilibration: Buffer Alone[a]	Percent Drug Bound after 1 hr Equilibration: Buffer + Sodium Taurocholate, Monoolein, and Oleic Acid
Chlorpropamide-^{35}S	0.16	6.4	−5.9
Niacin-6-^{14}C	0.13	5.5	2.3
Biotin-^{14}C	0.05	0.5	−1.7
Aspirin (UV 266 nm)	1.67	10.8[b]	—[c]
Salicylic acid (UV 229 nm)	1.67	28.8	—[c]
Ascorbic acid (UV 264 nm)	0.553	4.5	—[c]
Benzyl penicillin-^{14}C	0.27	20.0	10.6
Tetracycline-7-^{3}H hydrochloride	0.27	48.3	30.7

[a] Buffer: tromethamine, 0.05 *N* HCl, and 0.1 *N* NaCl with a final pH of 7.5. [b] This value was corrected for the 10.5% (relative to the initial molar concentration of aspirin) salicylic acid formed. [c] Experiment not run.

Table II—Effect of Sodium Taurocholate, Monoolein, and Oleic Acid on Drug Binding to Cholestyramine

Drug	Concentration, mg/ml	Percent Drug Bound after 1 hr of Equilibration: Buffer Alone[a]	Percent Drug Bound after 1 hr of Equilibration: Buffer + Sodium Taurocholate, Monoolein, and Oleic Acid
Chlorpropamide-^{35}S	0.16	53.5	31.8
Niacin-6-^{14}C	0.13	9.4	−0.8
Biotin-^{14}C	0.05	11.8	−1.1
Aspirin (UV 266 nm)	1.67	17.0[b]	—[c]
Salicylic acid (UV 229 nm)	1.67	60.2	—[c]
Ascorbic acid (UV 264 nm)	0.553	6.4	—[c]
Benzyl penicillin-^{14}C	0.27	43.8	17.6
Tetracycline-7-^{3}H hydrochloride	0.27	55.8	42.8

[a] Buffer: tromethamine, 0.05 *N* HCl, and 0.1 *N* NaCl with a final pH of 7.5. [b] This value was corrected for the 3.5% (relative to the initial molar concentration of aspirin) salicylic acid formed. [c] Experiment not run.

(together with any small amount of salicylic acid formed) to colestipol hydrochloride (average 8.9%, range 8.1–9.4% bound, n = 11) was not greatly concentration dependent, if at all.

This finding may indicate that aspirin (unlike taurocholate or glycocholate) does not undergo transformation to a new molecular species (such as micelles or dimers) when the concentration is increased and/or that the new molecular species have the same selectivity coefficient.

Since the binding of drugs to the copolymers is expected to be concentration dependent, the binding was studied where the relative amounts of drug to copolymer approximate the ratio of the daily therapeutic dose of drug and copolymer (except for compounds with assay end-points of low absorptivity and where higher concentrations were required).

Choice of Suitable Buffer for Drug Comparisons—Ion-exchange resins bind some ions more than others and, on the basis of mass action, another ion can displace a given ion from a binding site. Therefore, drug binding could vary with the ionic environment. The initial objective was to find a buffer that would satisfactorily control pH and not greatly inhibit binding.

Chlorpropamide-^{35}S, niacin-6-^{14}C, biotin-^{14}C, ascorbic acid, aspirin, phenobarbital-^{14}C, and taurocholate-24-^{14}C were bound to colestipol hydrochloride to an extent less than or equal to 6.2% in sodium phosphate buffer at pH 6.5 or 7.5 (ionic strength equal to 0.4). Binding of the drugs tended to be less at the lower pH. The binding of taurocholate greatly increased (from an average of 0.9 to 21.9%) when the ionic strength of the phosphate buffer was reduced from 0.4 to 0.004 (pH 6.5).

At the same ionic strength and pH (μ = 0.15 and pH 7.5), sodium phosphate buffer reduced taurocholate binding to colestipol hydrochloride more than Buffer I (Fig. 1). When cholestyramine was used as a binding agent, the reverse was true. On an equimolar basis, however, the presence of phosphate greatly reduced taurocholate binding to both colestipol hydrochloride and cholestyramine below that found when other anions (chloride or acetate) were substituted. Since the binding of most compounds in phosphate buffers appeared to be relatively low, another buffering system was sought as a standard system for drug comparisons.

The binding of drugs to colestipol hydrochloride and cholestyramine was studied in Buffer I at pH 7.5 and μ = 0.15 (Tables I and II). The binding of the drugs to colestipol hydrochloride varied from zero for lincomycin hydrochloride to as high as 59% for warfarin. Except for warfarin and tetracycline hydrochloride, less than 30% of the initial inputs of the drugs was bound to colestipol hydrochloride. In this system, the drugs studied were more greatly bound by cholestyramine than by colestipol hydrochloride (Tables I and II).

The relatively strong binding of tetracycline to the polymers is

Table III—Effect of Monoolein, Oleic Acid, and Taurocholate on the Binding of Phenobarbital to Colestipol Hydrochloride

Compounds Present[a]					Taurocholate Binding		Phenobarbital Binding	
Colestipol	Phenobarbital	Taurocholate	Monoolein	Oleic Acid	Free, dpm	% Bound	Free, dpm	% Bound
+	+	−	−	−	—	—	6315	14.3
+	+	+	−	−	20,436	55.8	6250	15.2
+	+	+	+	−	18,235	60.6	6305	14.5
+	+	+	+	+	29,724	35.7	6353	13.8
+	+	−	−	+	—	—	6277	13.8
+	−	+	−	−	20,378	55.9	—	—
+	−	+	+	−	18,098	60.9	—	—
+	−	+	+	+	29,497	36.2	—	—
+	−	+	−	+	29,922	35.3	—	—
+	+	+	−	+	29,505	36.2	6357	13.7
−	+	+	−	−	46,258	—	7370	—

[a] Plus = compounds added; minus = compounds not added. Final concentrations of the compounds added were: 0.5% sodium taurocholate-^{3}H, 0.00067% phenobarbital-^{14}C, 0.025% monoolein, and 0.140% oleic acid. The solutions were buffered by tromethamine, 0.067 *N* HCl, and 0.067 *M* NaCl at pH 7.5. There was 100 mg of colestipol hydrochloride in each 15 ml of solution.

Table IV—Effect of Monoolein, Oleic Acid, and Taurocholate on the Binding of Phenobarbital to Cholestyramine

Compounds Present[a]					Taurocholate Binding		Phenobarbital Binding	
Cholestyramine	Phenobarbital	Taurocholate	Monoolein	Oleic Acid	Free, dpm	% Bound	Free, dpm	% Bound
+	+	−	−	−	—	—	4257	42.2
+	+	+	−	−	12,715	72.5	5203	29.4
+	+	+	+	−	11,835	74.4	5134	30.3
+	+	+	+	+	12,671	72.6	5650	23.3
+	+	−	−	+	—	—	4570	38.0
+	−	+	−	−	12,270	73.4	—	—
+	−	+	+	−	12,350	73.3	—	—
+	−	+	+	+	11,759	74.6	—	—
+	−	+	−	+	11,164	75.9	—	—
+	+	+	−	+	12,771	72.3	5581	24.2
−	+	+	−	−	46,258	—	7370	—

[a] Plus = compounds added; minus = compounds not added. Final concentrations of the compounds were: 0.5% sodium taurocholate-^{3}H, 0.00067% phenobarbital-^{14}C, 0.025% monoolein, and 0.140% oleic acid. The solutions were buffered by 0.067 μ tromethamine, 0.067 *N* HCl, and 0.067 *M* NaCl at pH 7.5. There was 100 mg of cholestyramine for each 15 ml of solution.

interesting since tetracycline would only be partially negatively charged at pH 7.5 with pKa's at 7.82 and 9.57 (8). Whether the binding involves the nitrogen hydrogens of the polymers and the two 1,3-diketones of tetracycline to form rings through ionic, hydrogen bonding, and van der Waals' forces is not known. Another possibility is that the ionized tetracycline molecules form relatively insoluble salts with the polymers and the binding reaction continues as additional tetracycline associates until a final equilibrium is established.

The final pH's after equilibration of the drugs and the polymers was within 0.1–0.2 of a pH unit from pH 7.5. Thus, the Buffer I system was considered satisfactory and was employed in further studies.

Table V—Effect of Tromethamine, Monoolein, and Oleic Acid on Taurocholate-^{3}H Binding to Colestipol Hydrochloride[a]

T	Sodium Chloride	Monoolein	Oleic Acid	Final pH	Supernatant Radioactivity, dpm	Percent Bound
−	2+	+	+	8.95	19,614	17.7
+	+	+	+	7.90	15,219	36.2
+	+	−	−	8.00	17,655	26.0
+	+	−	+	7.90	22,624	5.1
+	+	8+	−	7.90	16,379	31.3
+	+	−	−	8.10	23,844[b]	—

[a] The 15 ml of solution consisted of 0.05 *M* $NaHCO_3$ plus the following compounds. T: plus is 0.05 *N* HCl + tromethamine to pH 7.5; sodium chloride: plus is 0.05 *N*, 2+ is 0.1 *N*; monoolein: plus is 3.8 mg (0.025% final concentration), 8+ is 26.4 mg (0.176% final concentration); oleic acid: 21 mg (0.140% final concentration); sodium taurocholate: 40 g (4.96 m*M* or 0.267% final concentration). Minus is no addition of the compound was made. [b] No copolymer present.

Reproducibility of Binding Experiments—To obtain an estimate of the precision of two different levels of drug binding, advantage was taken of the fact that taurocholate binding to colestipol hydrochloride is dependent on taurocholate concentration. For 15% binding, the standard deviation was 1.0% (absolute); for 55% binding, the standard deviation was 2.7%.

Effect of Taurocholate, Monoolein, and Oleic Acid on Drug Binding: Binding of Aqueous Soluble Compounds—Since taurocholate, monoolein, and oleic acid together form micelles (9), it was of interest to determine if the binding of some drugs would be increased by the presence of these substances. As shown in Tables I and II, chlorpropamide-^{35}S, niacin-6-^{14}C, biotin-^{14}C, benzyl penicillin-^{14}C, and tetracycline-7-^{3}H hydrochloride were each bound less by cholestyramine or colestipol hydrochloride when sodium

Table VI—Effect of Tromethamine, Monoolein, and Oleic Acid on Taurocholate-^{3}H Binding to Cholestyramine[a]

T	Sodium Chloride	Monoolein	Oleic Acid	Final pH	Supernatant Radioactivity, dpm	Percent Bound
−	2+	+	+	8.88	4199	82.7
+	+	+	+	8.08	4237	82.2
+	+	−	−	8.09	5955	75.0
+	+	−	+	8.95	4099	82.8
+	+	8+	−	8.10	6026	74.7
+	+	−	−	8.10	23,844[b]	—

[a] The 15 ml of solution consisted of 0.05 *M* $NaHCO_3$ plus the following compounds. T: plus is 0.05 *N* HCl + tromethamine to pH 7.5; sodium chloride: plus is 0.05 *N*, 2+ is 0.1 *N*; monoolein: plus is 3.8 mg, 8+ is 26.4 mg; oleic acid: 21 mg (0.140% final concentration); sodium taurocholate: 40 mg (4.96 m*M* or 0.267% final concentration). Minus is no addition of the compound was made. [b] No resin present.

Table VII—Colestipol Hydrochloride Precipitation or Binding of Fat-Soluble Vitamins Dissolved in Micelles

Vitamin	Initial Concentration, μg/ml	Equilibration Time, hr	Percent Taurocholate-^{3}H[a] Bound	Percent Vitamin Bound or Precipitate[b]
A_1[c]	8.8	1.0	60.7	96
		2.0	63.8	96
D_2[c]	9.1	1.0	60.5	104
		2.0	64.2	99
K_1[d]	1300	1.0	62.7	58
		2.0	66.0	85

[a] ^{3}H radioactivity. [b] Absorbance of the free vitamin was measured. [c] Solubilizing agent: 1% sodium taurocholate-^{3}H in Buffer I (μ = 0.15 and pH 7.5). [d] Solubilizing agent: 1% sodium taurocholate-^{3}H and 2.0 mg/ml monoolein in Buffer I (μ = 0.15 and pH 7.5).

taurocholate, monoolein, and oleic acid were present in combination. Taurocholate, monoolein, and oleic acid, alone or in combination, were tested as factors in altering phenobarbital binding to cholestyramine or to colestipol hydrochloride. Both taurocholate ($p < 0.05$) and oleic acid ($p < 0.01$) significantly reduced (Table IV) binding of phenobarbital to cholestyramine. Only oleic acid was significant ($p < 0.01$) in reducing the binding of phenobarbital to colestipol hydrochloride (Table III).

The decreased binding could be the result of decreased availability of binding sites (for anions) on the polymers, *i.e.*, a reduction in the number of binding sites and/or polarity of the medium. These results plus those shown in Tables I and II indicated that the *in vivo* binding of these water-soluble drugs may be expected to decrease when ingested together with fatty foods.

The separate and combined effects of monoolein and oleic acid on the binding of taurocholate were investigated. Taurocholate binding to colestipol hydrochloride increased with the composition of the media as follows (Tables III and V): buffer + oleic acid < buffer + monoolein + oleic acid < buffer < buffer + monoolein. Oleic acid decreased ($p < 0.01$), while monoolein increased ($p < 0.05$), the binding of taurocholate to colestipol hydrochloride. The data are compatible with: *(a)* greater binding of taurocholate as micelles to colestipol hydrochloride, with taurocholate micelles having less affinity than taurocholate–monoolein micelles; and *(b)* oleic acid competing for the binding sites of colestipol hydrochloride. The results suggest that the *in vivo* binding of taurocholate may be increased by monoglycerides derived from food and decreased by fatty acids.

The binding of taurocholate to cholestyramine was less affected by the presence of monoolein or oleic acid (Table IV) with taurocholate at a concentration of 0.5%. The range of binding values was only 3.6% (median 73.4%). At the lower concentration of 0.267%, the range of values was 8.1% (median 82.2%) (Table VI). The greatest binding was observed when oleic acid was present ($p < 0.01$) with or without monoolein.

Such observations are compatible with oleic acid increasing slightly the binding of taurocholate as micelles of taurocholate–oleic acid or taurocholate–oleic acid–monoolein. Monoolein alone did not affect ($p > 0.05$) the binding of taurocholate to cholestyramine. Thus, under these conditions, micelles do not play as great a relative role in the binding of taurocholate to cholestyramine and/or their affinity for cholestyramine is not greatly different from single taurocholate molecules.

Table VIII—Cholestyramine Precipitation or Binding of Fat-Soluble Vitamins Dissolved in Micelles

Vitamin	Initial Concentration, μg/ml	Equilibration Time, hr	Percent Taurocholate-^{3}H[a] Bound	Percent Vitamin Bound or Precipitated[b]
A_1[c]	8.8	1.0	73.4	96
		2.0	74.2	94
D_2[c]	9.1	1.0	74.0	106
		2.0	75.4	104
K_1[d]	1300	1.0	75.1	81
		2.0	76.0	82

[a] ^{3}H radioactivity. [b] Absorbance of the free vitamin was measured. [c] Solubilizing agent: 1% sodium taurocholate-^{3}H in Buffer I (μ = 0.15 and pH 7.5). [d] Solubilizing agent: 1% sodium taurocholate-^{3}H and 2.0 mg/ml monoolein in Buffer I (μ = 0.15 and pH 7.5).

Table IX—Effect of Different Concentrations of Taurocholate on Colestipol Hydrochloride Precipitation or Binding of Vitamin A_1

Supernatant Taurocholate Concentration[a]		Colestipol Hydrochloride, mg	Vitamin A_1 in Supernate: Absorbance minus Blank	Percent Bound
Input, %	After Equilibration, %			
1.0	0.35–0.38	100.0	0.019	98.7
1.0	1.0	0.0	1.46 (9.4 μg/ml)	
2.5	1.75	100.0	1.29	16.2
2.5	2.5	0.0	1.54 (9.9 μg/ml)	

[a] Buffer I (μ = 0.15 and pH 7.5).

Binding of Compounds with Low Aqueous Solubility—The binding of a substance is difficult to study in water when that substance is not very water soluble, *e.g.*, fat-soluble vitamins, cholesterol, fats, and nonpolar drugs.

Bile salts have been implicated in the GI absorption of lipid-soluble vitamin D (10, 11), fats (12, 13), and certain drugs of low aqueous solubility (14). Bile salts have been postulated to increase the aqueous solubility of a compound by forming micelles which entrap or envelop the otherwise "insoluble" compound. Conceivably, such micelles could be bound by an ion-exchange resin.

Vitamins A_1, D_2, and K_1—The possible binding of the lipid-soluble vitamins to colestipol hydrochloride and cholestyramine in the presence of bile salts was studied. Sodium taurocholate (1%, about 18 m*M*) was added to solubilize vitamins A_1 and D_2. The further addition of monoolein to taurocholate was required to improve the solubility of vitamin K_1. After equilibration with colestipol hydrochloride and cholestyramine, the vitamins were almost entirely absent from the supernates in both copolymer experiments (Tables VII and VIII). Such results could occur if the vitamins were being tightly bound by the copolymers and/or if the water solubility of the vitamin–taurocholate micelles were being lowered by the loss of taurocholate in the supernates to the copolymers.

To check whether the amount of taurocholate was a factor, the removal of vitamin A_1 from the supernate was measured in the presence of 1 and 2.5% (initial concentration) sodium taurocholate plus 100 mg of colestipol hydrochloride (Table IX). If the final taurocholate concentration were not a factor limiting the solubility of the vitamin–taurocholate micelles, nearly quantitative binding would be expected for both concentrations of taurocholate. With the higher initial concentration of 2.5% taurocholate, the removal

Table X—Extent of Vitamin A_1 Precipitation when the Concentration of Sodium Taurocholate is Lowered

Final Sodium Taurocholate Concentration, %	Vitamin A_1 Absorbance of the Solution		Percent Vitamin A_1 Precipitated Relative to Initial Absorbance of 2.5% Solution (1.464)
	After Dilution[a]	After Shaking (1 hr)	
1.75	1.46 (9.4 μg/ml)	1.45	0.7
1.00	1.46	1.45	1.0
0.40	1.35	1.17	20.1

[a] All solutions had equal inputs of vitamin A_1; Buffer I (μ = 0.15 and pH 7.5).

Table XI—Effect of Different Concentrations of Taurocholate on Precipitation or Binding of Cholesterol-^{14}C to Colestipol Hydrochloride

Input Supernatant Sodium Taurocholate Concentration[a], %	Colestipol Hydrochloride, mg	^{14}C dpm minus Background[b]	Percent Binding or Precipitation
0.4	0.0	580	8.2
1.00	0.0	637	−0.8
1.75	0.0	615 } Mean = 632	2.7 } Mean = 0%
2.50	0.0	643	−1.7 (by definition)
1.00	100.0	20	96.8
2.50	100.0	454	28.2

[a] Buffer I (μ = 0.15 and pH 7.5). [b] Input cholesterol concentration: 4 ng/ml.

of vitamin A_1 from the supernate was reduced to only 16.2%; therefore, taurocholate concentration was a factor in the removal of vitamin A_1 from the supernate.

Since the taurocholate concentration in the supernate decreased from 2.5% to about 1.75% after equilibration, an adequate amount of taurocholate was present for retaining the vitamin in a micellar solution. Thus, the 16.2% removal of vitamin A_1 from the supernate with the higher taurocholate concentration likely represents nonionic adsorption to the taurocholate–colestipol hydrochloride surface or binding of taurocholate–vitamin A micelles to colestipol hydrochloride.

About 30% taurocholate was bound when the initial concentration was 2.5%. If the initial ratio of vitamin A_1 to taurocholate were maintained after equilibration with the copolymer, then the binding of vitamin A_1 would also have been 30%. Since the binding was less than this value, the act of binding taurocholate to the copolymer decreased the micellar association of vitamin A_1 with the taurocholate in the copolymer.

Since the presence of taurocholate helped to solubilize vitamin A, the extent that vitamin A_1 precipitated external to the copolymer due to the removal of taurocholate was estimated by the following experiment. Vitamin A_1 was dissolved in 7.5% sodium taurocholate and diluted into 0.4, 1.0, and 1.75% sodium taurocholate (final concentrations) with Buffer I (μ = 0.15 and pH 7.5). The 0.4 and 1.75% values corresponded to the equilibrated supernatant sodium taurocholate concentrations that would be obtained when 1.0 and 2.5% sodium taurocholate solutions are equilibrated with 100 mg of colestipol hydrochloride.

The initial input of vitamin A_1 was chosen to approximate the concentrations employed in the "binding" experiment. If the binding phenomenon previously observed had been due to precipitation of vitamin external to the copolymer, then almost quantitative vitamin A_1 precipitation should occur for the 0.4% sodium taurocholate concentration, and 16% vitamin A_1 precipitation should occur for the 1.75% sodium taurocholate concentration.

The results (Table X) indicated that less vitamin A_1 precipitated in the absence of colestipol hydrochloride than would be expected if the binding observed in the presence of the copolymer were due only to precipitation external to the copolymer. The vitamin A_1 remaining in the supernate in the absence of colestipol hydrochloride is an indication of the solubility of vitamin A_1 in the presence of the different concentrations of sodium taurocholate. The final concentrations of vitamin A_1 left in the supernate after equilibration with colestipol hydrochloride (Table XI) do not exceed the solubility limit of vitamin A_1 at any of the final concentrations of taurocholate in the supernate. Thus, the values for the "percent bound" represent the amount bound to the copolymer only and no precipitation of the vitamin likely occurred external to the copolymer under the experimental conditions.

In the GI tract, the presence of ingested lipids should help solubilize vitamin A and hence reduce any precipitation of the fat-soluble vitamin external to colestipol hydrochloride. The binding of the vitamin to the copolymer depends on the concentration of bile salts present and the competition for copolymer–bile salt sites by other lipids.

Cholestyramine bound vitamin D_3-^{3}H to a considerable extent *in vivo* and *in vitro* (10). *In vitro*, 100% of vitamin D_3-^{3}H was bound by cholestyramine (100 mg) in the presence of taurocholate (20 m*M*). However, further additions of linoleic acid and monoolein decreased the amount of the vitamin bound. At levels of cholestyramine sufficient to cause steatorrhea, absorption of vitamin D_3-^{3}H in rats was decreased from a control value of 65 ± 4% to 28 ± 14.7% ($p < 0.001$). In dicumarol-treated dogs, low doses of cholestyramine (201 mg/kg/day) did not affect absorption of vitamin K_1, but doses of 1 g/kg and greater decreased and delayed absorption of the vitamin (15).

When 2% cholestyramine (equivalent to 12–15 g/day as percentage of food intake in humans) was added to a diet marginal in vitamin K activity, prothrombin times of chicks slightly lengthened after 2 weeks but not after 4 weeks (16). Vitamin K stores were lower in chicks fed cholestyramine. Body weight gains of weanling rats were reduced when 2% cholestyramine was added to their high-fat diet containing only growth-limiting amounts of vitamin A. When larger dietary amounts of vitamin A were given, no effect on weight gains was observed but liver storage of the vitamin was reduced. Thus, vitamin deficiency was avoided when the vitamin intake was more than minimal (16).

Although the *in vitro* and *in vivo* binding of lipid-soluble compounds by bile acid sequestrants can occur, clinical signs of deficiency are uncommon at therapeutic hypocholesterolemic doses (17, 18). No abnormalities in prothrombin times or other evidence of fat-soluble vitamin deficiencies occurred in healthy male subjects on normal diets and 12–15 g of colestipol hydrochloride/day[13]. After a year of colestipol hydrochloride administration to dogs at a maximum dose equivalent to approximately 10 times the human therapeutic dose of 15 g/day, no laboratory or clinical signs of fat-soluble vitamin deficiencies occurred (19). The utilization of fat-soluble vitamins was not evidently impaired in weanling rats fed laboratory food[14] containing up to 2000 mg of colestipol hydrochloride/kg daily for 18 months[15].

Cholesterol-^{14}C—The binding or precipitation of cholesterol-^{14}C was studied in the presence of taurocholate (1 and 2.5% initial

Table XII—Recovery of Water-Soluble Drug Bound to Colestipol Hydrochloride after Four Equilibrations with Fresh Buffer

Drug	Input Concentration, mg/ml	Assay	Percent Bound[a] in 1 hr	Percent Recovery after Four Washes[a]
Tetracycline hydrochloride	1.42	UV (361 nm)	30	98
Sulfadiazine	0.0203	UV (255 nm)	23	100
Benzyl penicillin	0.50	^{14}C	23	102
Lincomycin hydrochloride	0.568	^{14}C	0	100
Warfarin	0.070	UV (305 nm)	59	85

[a] Buffer and wash: tromethamine + 0.05 *N* HCl + 0.1 *N* NaCl (pH 7.5).

[13] Dr. K. Gundersen cited in Ref. 19.
[14] Purina.
[15] Dr. H. D. Webster cited in Ref. 19.

concentration) and colestipol hydrochloride (Table XI). Results were similar to those found for the lipid-soluble vitamins. Quantitative precipitation or binding of cholesterol-^{14}C was observed in the presence of an initial taurocholate concentration of 1%, but only 28% was precipitated or bound on or in colestipol hydrochloride in the presence of an initial concentration of 2.5% sodium taurocholate. Only 8% of the cholesterol-^{14}C precipitated in 0.4% sodium taurocholate in the absence of colestipol hydrochloride (the supernatant concentration of sodium taurocholate was 0.4% after 15 ml of initially 1% sodium taurocholate was equilibrated with 100 mg of colestipol hydrochloride).

No cholesterol-^{14}C precipitated in the absence of colestipol hydrochloride when the concentration of sodium taurocholate was reduced to 1.75% (the supernatant concentration of sodium taurocholate after 15 ml of initially 2.5% sodium taurocholate was equilibrated with 100 mg of colestipol hydrochloride). Thus, cholesterol-^{14}C likely did not precipitate and the binding values found represent cholesterol-^{14}C bound to colestipol hydrochloride. The binding is markedly dependent on sodium taurocholate concentration. The binding of cholesterol to colestipol hydrochloride in the presence of bile salt provides another mechanism by which cholesterol may be removed from enterohepatic circulation.

Recovery of Aqueous-Soluble Drugs Bound to Colestipol Hydrochloride—In the GI tract, drugs bound to colestipol hydrochloride would be repeatedly bathed by fluids. Some unbound drug would be continuously removed by GI absorption. This process was simulated by determining the recovery of colestipol-bound drug after repeated equilibration with fresh buffer. Drugs were equilibrated with colestipol hydrochloride in Buffer I for 1 hr, and the amount of unbound drug was determined. The clear portion of the solution was passed through 45-μm filters[16] and the filtrate was saved.

Fifteen milliliters of Buffer I and the filter just used were added to the centrifuge tube containing the colestipol hydrochloride and any drug that was bound. The contents of the centrifuge tube were reequilibrated by shaking for 15 min and then the material was again filtered with a fresh filter. In this manner the colestipol hydrochloride and bound drug were washed four times. The results of such experiments are shown in Table XII. Some of these drugs were among the strongest bound of the aqueous-soluble drugs tested. All were recovered quantitatively in the filtrate except for warfarin, and even warfarin was 85% recovered. Thus, the binding of these drugs to colestipol is completely or largely reversible and colestipol hydrochloride may, to some extent, serve as a depot source of drug.

In the GI tract, where the concentration of free drug is reduced by absorption and the GI contents compete for binding sites, the final percentage of the *dose* of ingested drug remaining on the copolymer at the ileum and beyond is expected to be less than the percentage of drug bound to the copolymer in the *in vitro* experiments (single equilibration). Indeed, Phillips *et al.* (20) found that the area under the serum concentration–time curve of aspirin-^{14}C, sulfadiazine-^{35}S, or phenobarbital-2-^{14}C in rats was not affected by concurrent ingestion of therapeutic doses of colestipol hydrochloride. Three times the therapeutic dose of colestipol hydrochloride was required before the bioavailability of aspirin-^{14}C was decreased by only 15% and those of sulfadiazine-^{35}S and phenobarbital-2-^{14}C were affected little or not at all.

[16] HA Millipore.

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[x] To whom inquiries should be directed.

Determination of Drugs in Dosage Forms by Difference Spectrophotometry

THOMAS D. DOYLE ˣ and FRANK R. FAZZARI

Abstract □ The technique of difference spectrophotometry was applied to the rapid assay of numerous pharmaceuticals, without prior separation from other materials. Contributions to absorbance by interferences were automatically canceled; characteristic isosbestic points provided proof of this. Factors affecting selection of optimum pH were investigated, and reactions such as hydrolysis or formation of Schiff bases were utilized to produce spectral shifts. These and other aspects of the technique are discussed in detail.

Keyphrases □ Drugs—analysis in dosage forms, difference spectrophotometry, pH selection, hydrolysis, and Schiff-base formation considered, examples □ Spectrophotometry, difference—analysis of drugs in dosage forms, pH selection, hydrolysis, and Schiff-base formation considered, examples □ Difference spectrophotometry—analysis of drugs in dosage forms, pH selection, hydrolysis, and Schiff-base formation considered, examples □ Analysis—drugs in dosage forms, difference spectrophotometry

Electronic absorption spectrophotometry is the technique most extensively used in official methods as the measurement step for the quantitative assay of drugs in dosage forms. Although this use is in part a result of the rapidity and simplicity of the method, these advantages are somewhat negated by the requirement (1) that the actual determinative step be preceded by an isolation procedure, since tablet excipients, a syrup medium, or other substances may contribute to the total absorbance. It has been shown, for example, that simply dissolving trifluoperazine tablets and reading the absorbance of the solution gave errors of up to 5% due to background absorbance of excipients (2). Errors of such magnitude may not always be detected by simple inspection of the spectrum.

As an alternative to preliminary separation, the authors explored the use of difference spectrophotometry[1]; this technique provides for automatic cancellation of interferences while retaining the inherent convenience of direct reading. The method is applicable to all substances that can be made to undergo reproducible spectral changes.

The method is not new; notable examples of its application to drug analysis include assays for estradiol (3), morphine (4), caffeine (5), and corticosteroids (6, 7). Some elegant procedures for drug mixtures that undergo multiple spectral changes have been described (8, 9). However, the technique has not yet received widespread application nor understanding and is frequently misapplied.

This paper reports the difference spectrophotometric assay of numerous acidic, basic, and amphoteric drug substances. It is shown how detailed knowledge of ionization behavior is essential for utilization of pH-induced spectral shifts or how, alternatively, shifts may be effected without change in pH. It is demonstrated how isosbestic points, characteristic of most difference spectra, provide information concerning cancellation of interferences, purity of ionization or reaction processes, and location of optimum wavelengths for analysis of mixtures.

EXPERIMENTAL

All spectra were obtained on a recording spectrophotometer[2], using 1-cm silica cells.

Stock solutions were prepared by direct dissolution of the dosage form to give a concentration of, typically, from two to 10 times the final desired concentration. Water was the preferred solvent, but alcohol or dimethyl sulfoxide was used for preliminary solution, when required, without introducing any complication.

Duplicate aliquots of the stock solution were treated, each in a different manner, by addition of, *e.g.*, acid to one aliquot and base, buffer, other reagent, or nothing to the other. Both aliquots were then diluted to an identical final concentration, giving two sample solutions, A and B. Duplicate aliquots of a standard stock solution were similarly treated. When the buffer or other reagent had a detectable absorbance at wavelengths of interest, blank Solutions A and B were also prepared.

The zero point of the recording spectrophotometer was raised manually to permit recording of minima. The baseline was obtained by reading blank against blank. Sample Solution A was placed in the sample compartment, sample Solution B was placed in the reference compartment (or vice versa), and the difference spectrum was recorded. The standard difference spectrum was likewise scanned, superimposed on the sample spectrum to facilitate observance of isosbestic points.

Calculations of sample concentration were then made as in conventional spectrophotometry, using the recorded absorbance of the maximum, minimum, or amplitude.

RESULTS AND DISCUSSION

General Considerations—In double-beam spectrophotometry, a reference solution is scanned simultaneously with the sample solution to compensate for any opacity of cell, solvent, or added reagents. Ideally, the reference and sample solutions should be identical in all respects except for the presence of the analyte. In practice, when analyzing pharmaceutical samples, there is always uncertainty concerning interfering materials which may have accompanied the sample and which remain uncompensated for by the reference solution.

[1] The technique has been variously known as the $\Delta\epsilon$ method, differential spectrophotometry, and (incorrectly) derivative spectrophotometry. Difference spectrophotometry has also been used and is favored here since it best connotes the simple subtractive nature of the instrumental process.

[2] Cary 15 or 118, Cary Instruments, Monrovia, CA 91016

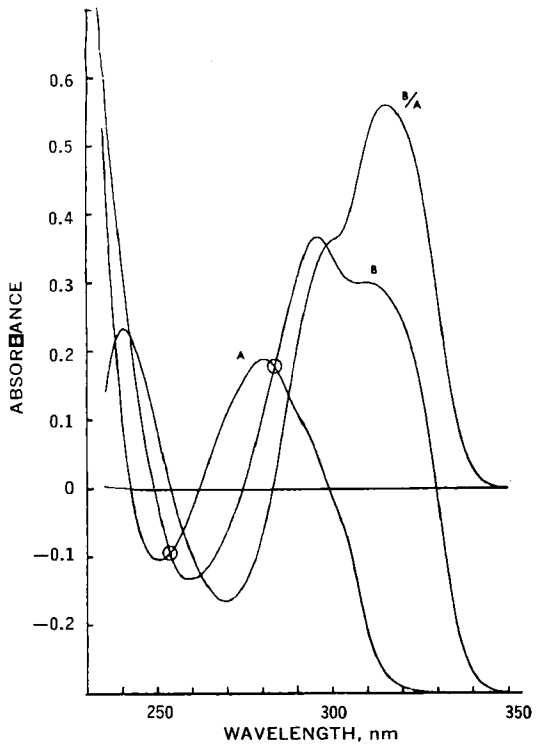


Figure 1—*Conventional and difference spectra of benzthiazide (about 15 μg/ml). Key: A, acidic solution; B, basic solution; and B/A, difference spectrum of basic* versus *acidic solutions. Note the isosbestic points (circled) at 287 and 255 nm, corresponding to intersection of the difference spectrum with the baseline.*

Difference spectrophotometry provides an approximation of the ideal reference solution by employing an aliquot of the sample solution itself as reference, adjusted by change in pH or other parameter but containing both the substance being analyzed and all extraneous substances at exactly the same concentrations as the sample. If the pH or other variation causes an alteration in the spectrum of the sample, the instrument records this as a characteristic difference spectrum. If other materials present are unaffected by the change in conditions, their contribution to the total absorbance of each solution will be identical and their effect will be exactly canceled.

Figure 1 shows the conventional spectra of the acidic drug benzthiazide in aqueous acid and in aqueous base, together with the difference spectrum recorded as base *versus* acid. Here and elsewhere, the zero point of the difference spectrum has been manually raised, so that minima as well as maxima may be observed. The choice of sample and reference solutions is essentially arbitrary, making the distinction between maximum and minimum meaningless. The difference in absorbance between an adjacent maximum and minimum may be termed the amplitude.

Since both the basic and acidic solutions obey Beer's law at all wavelengths, it is evident that the difference of these two spectra does likewise. This difference is precisely what the instrument records automatically; the amplitude may therefore be used for quantitative determinations in exactly the same manner that simple absorbance is used in conventional spectra.

Figure 2 shows the difference spectra of standard benzthiazide at various concentrations. The 313-nm maximum, the 271-nm minimum, and the amplitude between these peaks all gave linear absorbance–concentration plots, as expected. It is usually more convenient and always more sensitive to employ the amplitude rather than either of the individual peaks. However, when a buffer or other reagent added to one of the two solutions has a detectable, uncompensated absorbance over the wavelength interval involved, as blank readings will demonstrate, it is simpler to take readings of a single peak relative to the baseline rather than to make the necessary corrections for calculation of the amplitude.

Spectral changes that are apparently minor may, nevertheless, produce useful difference spectra. Figure 3 shows the conventional spectra of hydrochlorothiazide in acid and base. The maxima are shifted by only 6 nm, and the absorbances at these maxima differ by only 12%. However, the difference spectrum, superimposed on the figure and determined at the same concentration, gives an amplitude that is fully 53% as sensitive as the acid spectrum. Most of the amplitude is due to the peak at 339 nm, which occurs in a region where the absorbances of both acid and base spectra are rapidly increasing. In spite of this, operation of the spectrophotometer at normal scan speeds gave reproducible results, identical with those obtained at slow scan.

Isosbestic Points—The principal advantage of difference spectrophotometry lies in its potential for the cancellation of interferences; therefore, it is necessary to have some indication that this has been accomplished in a given case. The isosbestic points usually present in difference spectra provide a useful test for such cancellation.

In Fig. 1, for benzthiazide, the conventional acidic and basic spectra intersect at 287 and 255 nm, indicating that the two forms have identical absorbance at these isosbestic wavelengths. In the difference spectrum, the net absorbance at these wavelengths will be zero; this will be true for any concentration of the drug (Fig. 2). Therefore, sample and standard curves may be checked for maintenance of isosbestic points even if they are not at the same, or even similar, concentrations. This provides a powerful test for spectral (but not physical) purity.

If a sample difference spectrum does intersect the baseline at all points predicted by the standard, then this confirms that interferences, if present, have canceled at these points and strongly suggests that this is true throughout the spectrum. It does not constitute absolute proof. An extraneous substance could exhibit a zero difference absorbance at the indicated wavelengths but a positive absorbance at the wavelength of measurement. This would be improbable, however. Failure to maintain one or more points conclusively demonstrates that sample and standard solutions differ in some respect (in addition to any concentration difference, which does not affect the points). Some other substance that also undergoes spectral shifts must be present, necessitating a preliminary isolation or alternative assay procedure.

When it is desired to use this criterion as a test for spectral purity, it is convenient to record baseline, standard, and sample curves superimposed. Maintenance of the points may then be seen at a glance, as shown in Fig. 4.

Isosbestic points also find many uses in the development and selection of optimum analytical conditions, as discussed in succeeding sections.

Choice of pH—In most examples studied, the spectral changes are induced by simple reversible ionization of groups directly con-

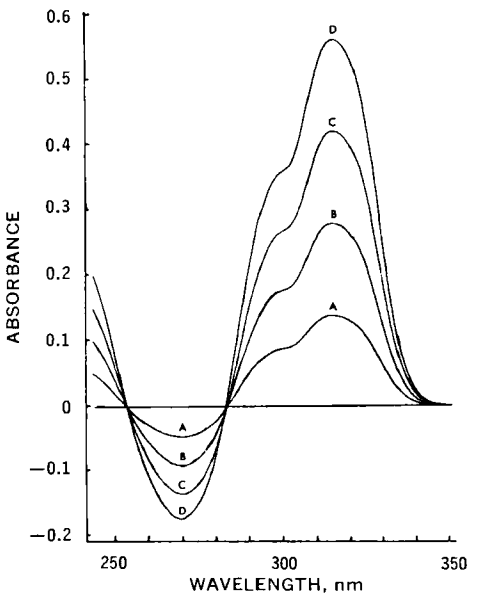


Figure 2—*Difference spectra used to verify Beer's law relation for benzthiazide (basic* versus *acidic solution for concentrations of: A, 5; B, 10; C, 15; and D, 20 μg/ml).*

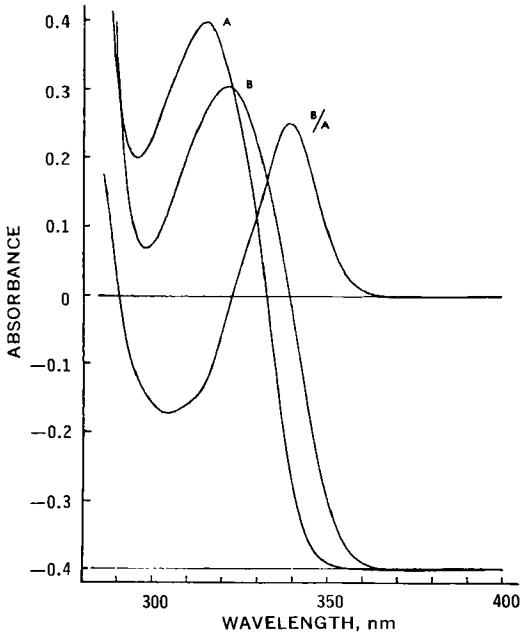


Figure 3—*Conventional and difference spectra of hydrochlorothiazide (about 20 μg/ml). Key: A, acidic solution; B, basic solution; and B/A, difference spectrum of basic* versus *acidic solutions.*

jugated to a chromophore. It is important that the acid and base solutions be selected so that both are at least two pH units removed from the pKa, on opposite sides of this value. For monofunctional compounds, this will ensure that each solution represents a single species of at least 99% spectral purity. At pH values closer to the pKa, small pH changes may result in appreciable changes in the difference spectrum.

Williams and Zak (10) described a difference spectra method for the analysis of barbiturates which employs solutions at pH 13.5 and 10.5. This method depends on the ionization of the monoanion to the dianion as the pH is raised. However, at pH 13.5, the dianion is present to an extent of less than 90% for most barbiturates; small differences in pH between standard and sample solutions would thus lead to serious errors.

For routine assay, the authors prefer to utilize the spectral change between the free barbiturate (read in dilute hydrochloric acid) and the monoanion (read at pH 10.5). The species so obtained are at least 99% pure. The use of these conditions does have

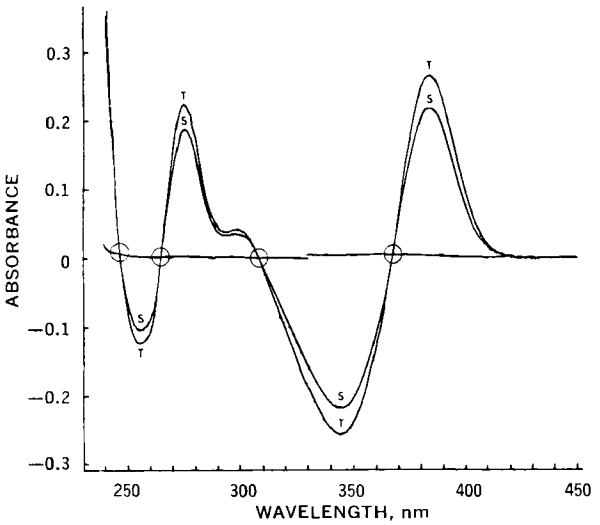


Figure 4—*Difference spectrum of triamterene, basic* versus *acidic solutions (about 2.5 μg/ml in aqueous alcohol). Key: S, standard solution; and T, direct dissolution of ground tablets. Note the presence of four isosbestic points.*

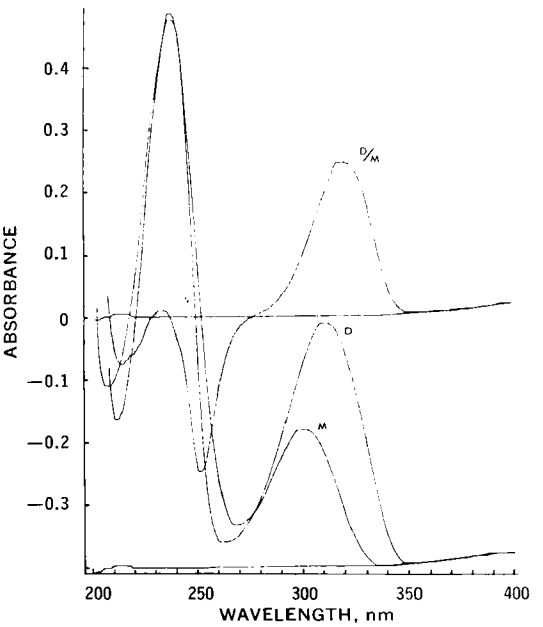


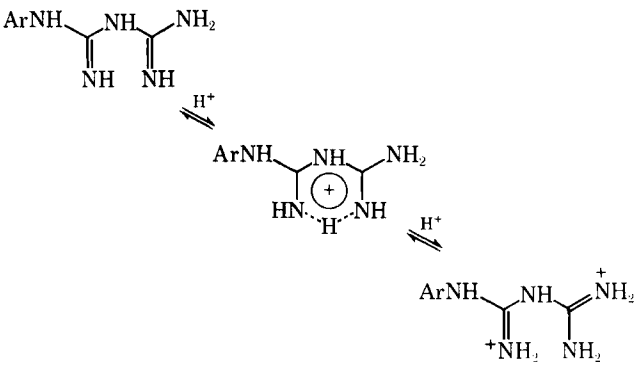
Figure 5—*Conventional and difference spectra of methapyrilene (about 25 μg/ml). Key: M, monocationic species at pH ~7; and D, dication at pH ~1. The pH's were chosen to utilize the ionization on the 2-aminopyridine ring of methapyrilene.*

a drawback in that the acid spectrum is essentially featureless so that no isosbestic points are observed and the difference spectrum is primarily that of the monoanion. However, the desired cancellation of interferences is still expected, making the procedure superior to simple reading against a blank. On the other hand, additives such as parabens, which may be present, undergo spectral changes due to ionization of the phenolic hydroxyl (pKa ~10), interfering with the assay by either of the discussed variations.

For polyfunctional compounds, a detailed knowledge of the ionization constants may be necessary to select optimum conditions. Goodman *et al.* (11) determined methapyrilene by a difference spectra method utilizing measurements at pH 10.6 and 7.45. This pH range includes the ionization of the aliphatic amine function of methapyrilene (pKa_1 = 8.9); ionization of this group produces a usable but relatively small change in the spectrum of the remote 2-aminopyridine portion of the molecule.

In contrast, the authors determined methapyrilene in hydrochloric acid and neutral solutions (Fig. 5), taking advantage of the ionization of the pyridine ring itself (pKa_2 of methapyrilene = 3.8) and thus obtaining a sensitivity six times greater than that reported by Goodman *et al.* (11).

Phenformin hydrochloride in neutral solution displays an intense peak at 233 nm, due apparently to a delocalized pseudo six-membered ring of the protonated biguanidine group (Scheme I). This group is so basic ($pKa_1 \sim 13$) that solutions with an effective pH > 15 would be required to obtain the pure free phenformin species. By contrast, a second proton is readily accepted in acid so-



Scheme I

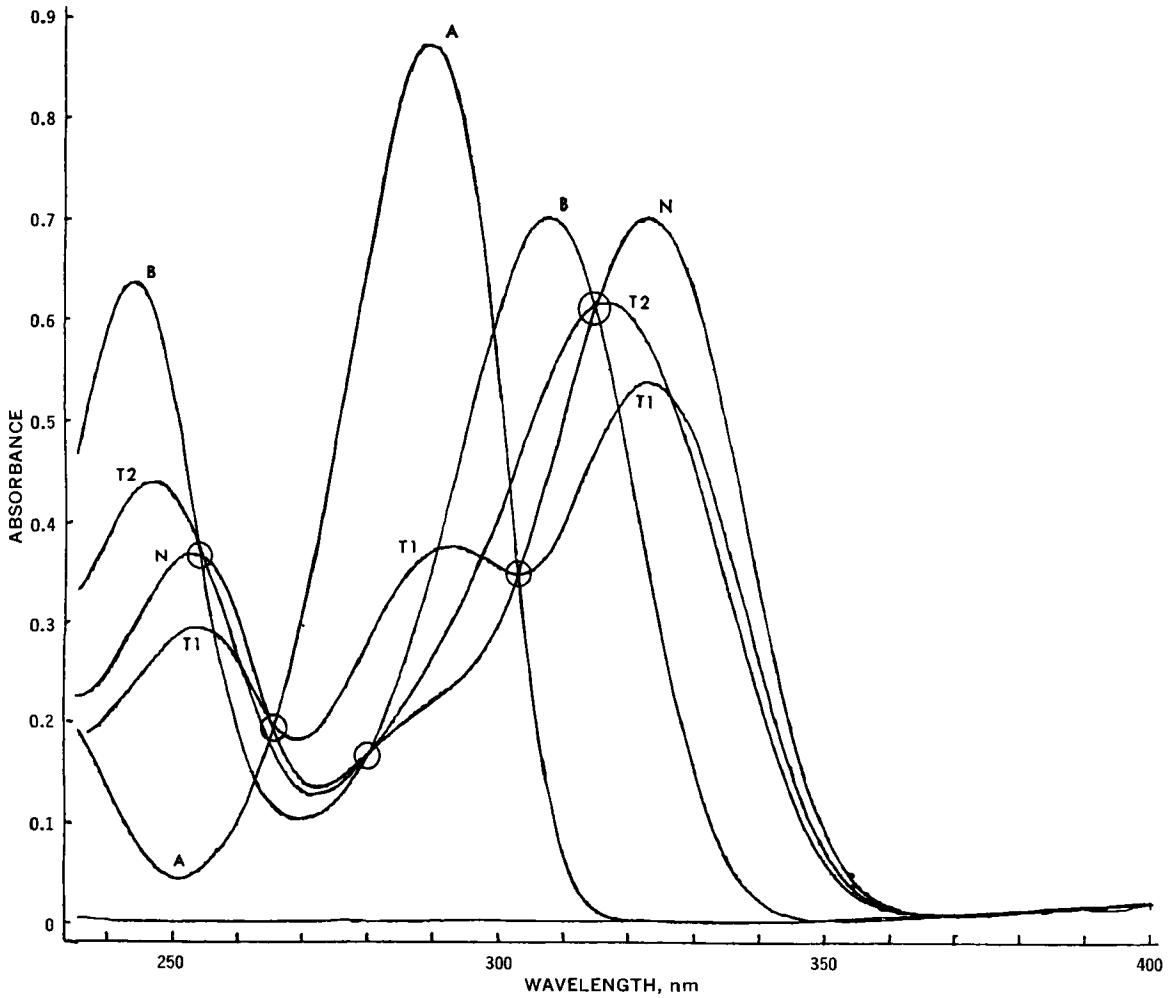


Figure 6—*Conventional spectra showing transformations due to multiple ionization of the amphoteric drug pyridoxine. All solutions are at identical concentrations of about 20 μg/ml. Key: A, pH 1; T1, pH 5; N, pH 6.6; T2, pH 9; and B, pH 13. The isosbestic points (circled) at 303 and 266 nm correspond to the transformation of the cation to neutral species; those at 315, 280, and 255 nm are associated with the further ionization to the anion.*

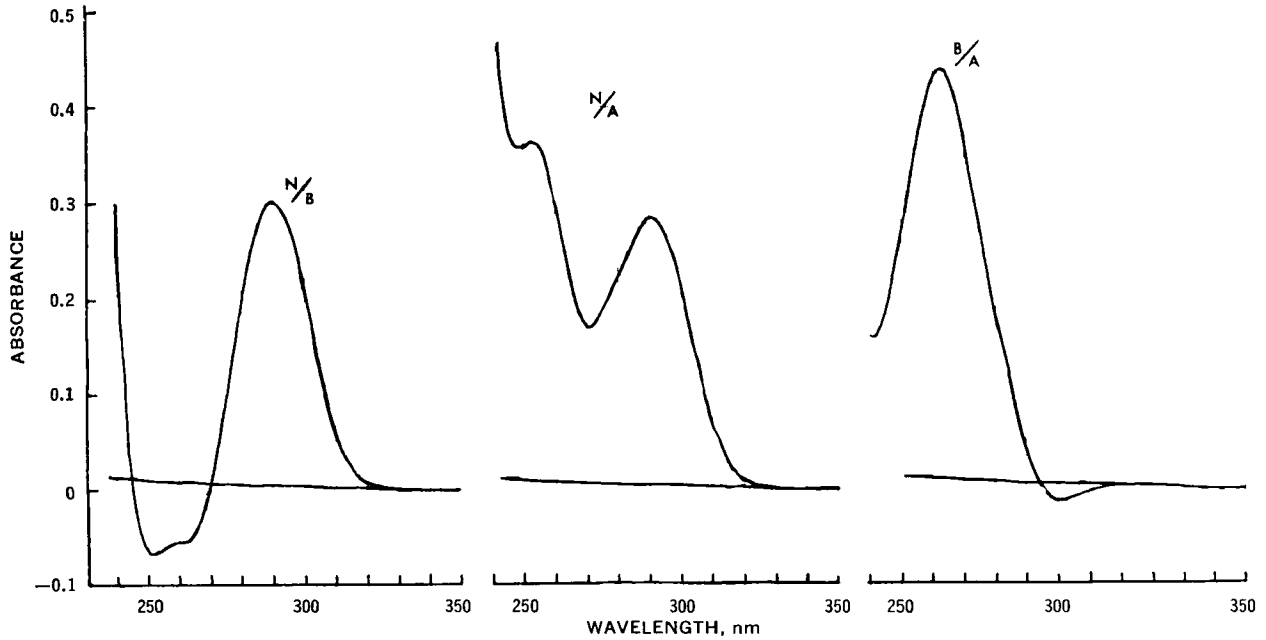


Figure 7—*Three difference spectra for sulfathiazole in aqueous alcohol. Key: A, acid; B, base; and N, neutral. All solutions are at identical concentrations of about 10 μg/ml.*

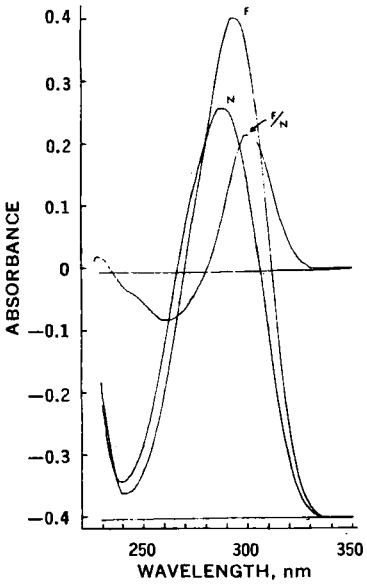


Figure 8—*Conventional and difference spectra of procaine, utilizing Schiff-base formation. All solutions are at identical pH (neutral) and concentration (about 10 μg/ml). Key: N, no reagent; and F, formaldehyde added.*

lution ($pKa_2 \sim 3$) with loss of absorbance; a neutral solution read against a solution with pH < 1 provides an effective assay method.

The amphoteric drug pyridoxine is highly soluble in water at all pH values and cannot be extracted by organic solvents. Figure 6 shows the complex spectral changes this compound undergoes as a function of pH. Simple ionization of the basic nitrogen and of the acidic aromatic hydroxyl fully accounts for these changes. This is demonstrated by the maintenance of isosbestic points at 303 and 266 nm in going from pH 1 to 6.6, together with the loss of these points and the appearance of a second set of points at 315, 280, and 255 nm as the pH is further increased from 6.6 to 13.

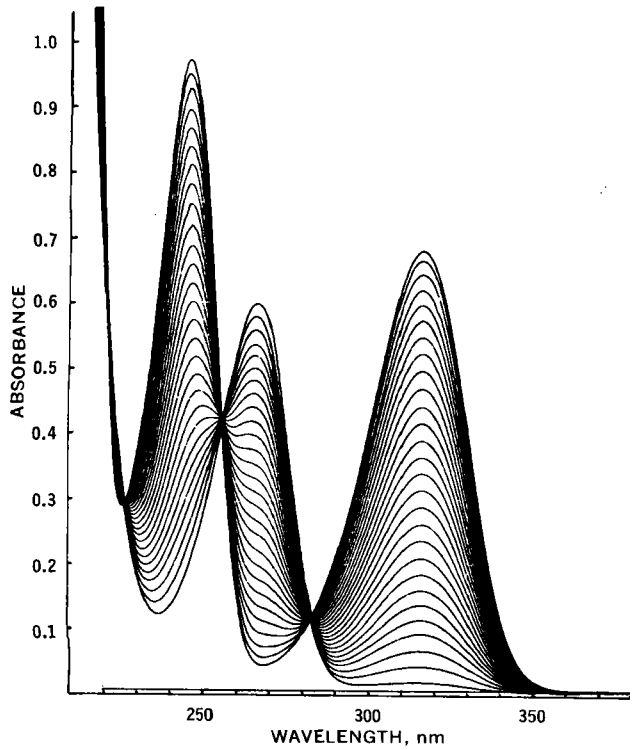


Figure 9—*Hydrolysis of pyridostigmine bromide in dilute alkali. Twenty-eight conventional scans were taken over 2 hr from the inception to the completion of the reaction, during which time the starting material (peak at 266 nm) was gradually replaced by the product (peaks at 246 and 316 nm). Note the isosbestic points which demonstrate lack of complicating side reactions. Rate of hydrolysis is controlled by the concentration of alkali.*

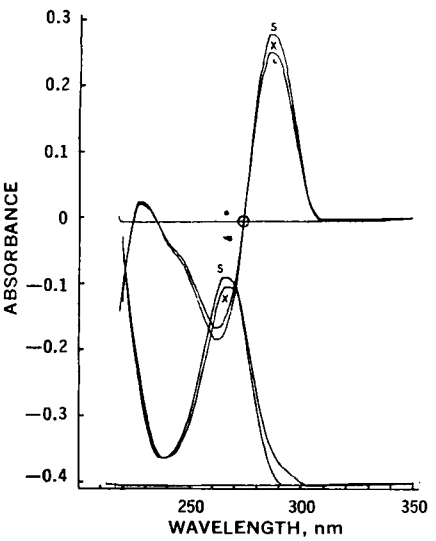


Figure 10—*Analysis of a partially decomposed sample of pyridostigmine bromide. Key: X, sample with 10% decomposition; and S, standard solution. The lower curves show the conventional spectra; the upper curves show the difference spectra using the hydrolytic method (see text).*

Recording the curves and determining the pH of each of the five solutions in Fig. 6 provided sufficient data to estimate the ionization constants of pyridoxine; pKa_1 was found to be 4.85 for the basic nitrogen, and pKa_2 was calculated as 9.07 for the acidic hydroxyl. Thus, the difference spectrum taken in the usual hydrochloric acid and sodium hydroxide solutions is known to result from pure cationic and anionic species and may, therefore, be used with confidence despite the complexity of the intermediate transformations.

Sulfathiazole is representative of a large class of amphoteric sulfonamides which may be analyzed by this method. The ionization constants are generally available in the literature; therefore, the pH's required to obtain pure species may be ascertained beforehand. Most of these compounds are relatively insoluble in water; preparation of the stock solution in 50% alcohol avoids this problem.

Figure 7 shows the difference curves obtained by the three possible pairings of cationic, neutral, and anionic species of sulfathia-

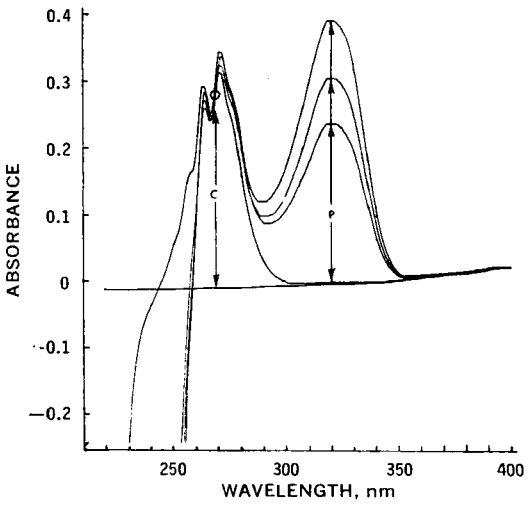


Figure 11—*Difference spectra analysis of the mixture of chlorpheniramine (C) and pyrilamine (P). Four solutions with constant concentrations of chlorpheniramine and varying concentrations of pyrilamine are shown scanned as acid* versus *neutral. The constant absorbance at 268 nm provides a measure of chlorpheniramine; pyrilamine is measured at 320 nm.*

zole. All gave useful spectra of comparable sensitivity; the neutral *versus* acid curve lacks an isosbestic point and is unsuited for the detection of interferences.

Reaction Methods—While most instances of difference spectra involve pH effects, spectral changes may also result from factors other than ionization. These permit sample and reference solutions to be prepared at an identical pH, with improved likelihood for cancellation of interferences. For example, the chromophore in the A ring of corticosteroids was reduced with sodium borohydride, and the intact drug was scanned against the reduction product (6, 7).

The authors found that the reaction of formaldehyde with primary aromatic amines, which affords Schiff bases, proceeds rapidly and reproducibly at room temperature and alters the spectra of such pharmaceutical substances as many of the local anesthetics and sulfa drugs. Figure 8 shows a typical result for procaine. Tetracaine, often combined with procaine, is a secondary amine; it does not react and thus cancels out of the difference spectrum so that no separation is required to assay for procaine in this mixture.

The most suitable reactions are rapid, clean, and complete and employ mild reagents that are transparent over the spectral region of interest. If both the drug and its reaction product have distinct spectra of comparable intensity, then isosbestic points should be produced. A reaction illustrating all of these desirable features is the alkaline hydrolysis of pyridostigmine bromide. Figure 9 shows consecutive spectra obtained during hydrolysis in dilute sodium hydroxide. The isosbestic points at 285, 258, and 228 nm demonstrate that the reaction is clean; pseudo-first-order kinetics computed from the consecutive scans were used to establish the completeness of reaction.

The hydrolysis product in alkaline solution can be read against the intact neutral drug, but a superior procedure is to adjust the reaction solution back to neutral after complete hydrolysis. The spectrum shifts again but remains distinct from that of the original compound. One solution is prepared by mixing drug, sodium hydroxide, and then excess acid after reaction. The other, unreacted, solution is prepared by mixing the same amounts of sodium hydroxide, excess acid, and then drug. Although the only difference is the order of addition of the reagents, a pronounced, reproducible difference spectrum results.

The assay of a sample of pyridostigmine bromide containing 10% hydrolyzed material by this procedure is shown in Fig. 10. Also shown is the conventional spectrum of the same mixture. The theoretical result is that the decomposed portion will not be detected by the difference method, as demonstrated by the maintenance of isosbestic points in Fig. 10, so that the true assay for intact drug alone is obtained.

On the other hand, the analyst would remain unaware of the presence of the decomposition product. It could be detected by comparison of the conventional spectrum with a standard, as shown. This conventional spectrum alone would, however, give a false assay value. The methods thus complement each other.

Mixtures of Drugs—Assay of mixtures of drugs by difference spectrophotometry, where more than one drug undergoes spectral shifts, constitutes a special challenge which can sometimes be met. The analysis of analgesics (8, 9) established the guidelines for such problems. Figure 11 illustrates an example from this laboratory for the antihistamine combination of chlorpheniramine and pyrilamine. Both have second ionization constants (pKa_2) of about 4.

The difference spectrum of pyrilamine exhibits an isosbestic point at 268 nm; this is the wavelength of choice for the analysis of the other component, chlorpheniramine, since the contribution of pyrilamine to the net absorbance at this wavelength is always zero. Chlorpheniramine has a difference curve with maximum at 272 nm. Readings are taken not at this maximum but at 268 nm on a slope and are best done with the instrument set at fixed wavelength at 268 nm.

In general, such methods for mixtures require meticulous technique and depend on the fortuitous juxtaposition of an isosbestic point of one compound with a maximum of another. By contrast, difference spectra methods for single-component dosage forms are usually simple and rugged.

CONCLUSIONS

Although only slightly more elaborate than direct dissolve-and-read methods, difference spectrophotometry has the advantages of potential cancellation of interferences and of an automatic test for such cancellation. Although it is not meant to supplant traditional extraction or chromatographic procedures, it can be argued that it achieves spectrophotometric rather than physical isolation of the analyzed substance. It is recommended especially for rapid routine assay of drugs with solubility characteristics that make preliminary separation difficult or impractical.

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[x] To whom inquiries should be directed.

Influence of Sunscreening Agents on Color Stability of Tablets Coated with Certified Dyes II: FD&C Blue No. 1

B. R. HAJRATWALA

Abstract □ The influence of protective coatings of six sunscreening agents (glyceryl *p*-aminobenzoate, benzocaine, 2-ethoxyethyl *p*-methoxycinnamate, *m*-homomenthyl salicylate, *n*-octyl salicylate, and amyl salicylate) upon the photostability of FD&C Blue No. 1 used to color coat tablets was studied. Alcohol film-, modified sugar-, and film-coating methods were used to apply sunscreening agents. Tablets were exposed to 1000 foot-candles of light. Visual observations as well as UV spectrophotometric determinations were made. A kinetic study, using the Kubelka–Munk equation, was made and approximate shelflives of various colored tablets were calculated. No sunscreening agent exhibited significant protective action for FD&C Blue No. 1.

Keyphrases □ Sunscreening agents—effect on color stability (shelflife) of tablets coated with FD&C Blue No. 1 □ Tablets—three methods for coating with sunscreening agents, effect of agents on color stability (shelflife) of FD&C Blue No. 1 □ Dyes, color stability of coated tablets—effect of sunscreening agents on FD&C Blue No. 1

The influence of six sunscreening agents (of salicylate, benzoate, and cinnamate types) on the color stability of tablets coated with FD&C Red No. 3 was reported previously (1). Three different methods were developed for the application of these sunscreening agents. The degree of protection was dependent not only upon the type of sunscreening agent but also upon the method by which it was applied.

In recent years, considerable attention has been directed toward the improvement and understanding of color stability. Such studies have been stimulated by the decertification of many colors. The approach commonly used to improve the light stability of a medicinal agent or to prevent color fading is to place it in a dark container. Such a solution is not always desirable, nor is it always effective.

UV absorbers (sunscreening agents) have been reported (1–5) to have a degree of effectiveness in preventing or slowing color fading under varying conditions of light exposure. Basically five types of UV absorbers have been found useful: benzoates, benzophenones, benzotriazoles, cinnamates, and salicylates. Benzoates in pharmaceuticals (1), benzophenones in textiles (6) and in pharmaceuticals (4, 5), benzotriazoles in plastics (7), and cinnamates (1) and salicylates in pharmaceuticals (1) and in plastics (7, 8) have been used for preventing color fading with some success. The toxicity of many of these UV absorbers is unknown and the reactions, if any, occurring among the dye, the UV absorber, and other "inert" or active pharmaceutical ingredients are unknown. Such reaction products may be toxic.

The purposes of this study were to evaluate the influence of selected sunscreening agents on tablets coated with FD&C Blue No. 1 and to predict the color shelflife of tablets.

EXPERIMENTAL

Materials—The six sunscreening agents used were glyceryl *p*-aminobenzoate[1], benzocaine, 2-ethoxyethyl *p*-methoxycinnamate[2], *m*-homomenthyl salicylate, *n*-octyl salicylate, and amyl salicylate[3]. The dye used was FD&C Blue No. 1. All compounds were used as received without further purification.

Procedure—The preparation of tablets, application of sunscreening agents, equipment, exposure to light, and determination of sunscreening agents per tablet were reported previously (1).

Coating Procedure—Ten subcoats and 25 smoothing coats were applied to core tablets, in lots of 6 kg, using medium strength gelatin syrup and white subcoating dusting powder. After tablets were completely dried, the color coating was applied. Plain coating syrup (170 g of sucrose and 100 ml of deionized water) and color syrup (0.300 g of dye in 100 ml of plain coating syrup) in the following ratios were applied: seven coats of 25 ml each in 40:1 parts, followed by 10 coats of 27 ml each in 30:1 parts, followed by six coats of 30 ml each in 20:1 parts, and finally 15 coats of 30 ml each in 10:1 parts of plain syrup and color syrup, respectively.

Application of Sunscreening Agents—Three coating techniques, alcohol film, modified sugar, and film, were used to apply sunscreening agents to color-coated tablets. Observations regarding the "wet" feeling of tablets coated with glyceryl *p*-aminobenzoate, turbid solutions for modified sugar-coating method, and mottled appearances in the case of tablets coated by the film-coating method with salicylates were similar to those reported (1). In earlier experiments it was noted that tablets coated with glyceryl *p*-aminobenzoate or benzocaine changed hue when exposed to light. To see whether such a hue change could be prevented, a protective coat with the following composition was applied:

cellulose acetate phthalate	5%
polyethylene glycol 4000	2%
acetone	43%
95% ethanol	*q.s.*

Five coats were applied to two sets of tablets, one containing glyceryl *p*-aminobenzoate and the other containing benzocaine.

Measurement of Color Change—Adequate samples of each set of color-coated tablets were exposed to light. Samples were withdrawn from the light cabinet, maintained at 1000 foot-candles intensity of light and 26 ± 2°, at designated time intervals. The reflectance of the tablets was measured using the transmittance scale. The spectrophotometer[4] and its reflectance attachment unit (Fig. 1) were used to measure the reflectance of individual tablets.

[1] Escalol-106, Van Dyke & Co. Inc.
[2] Giv-Tan-F, Sindar Corp.
[3] Amyl Salicylate Extra, Fritsche Brother Inc.
[4] Beckman DU.

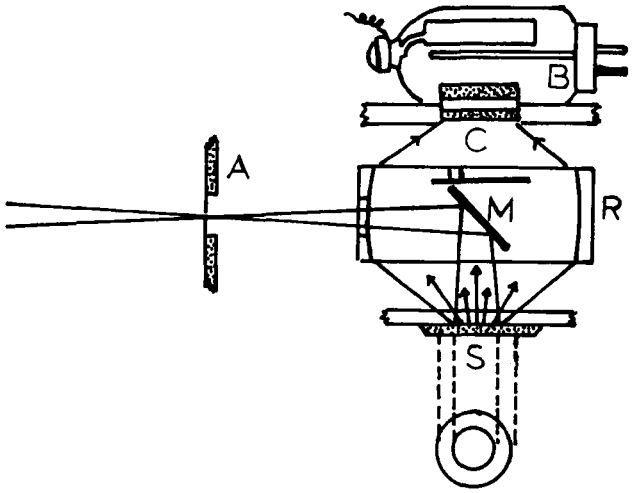

Figure 1—*Schematic diagram of the reflectance attachment for the spectrophotometer. Key: A, exit slit; B, phototube; C, diffusing plate; M, mirror; R, ellipsoidal metallic mirror ring; and S, sample.*

Two magnesium carbonate blocks [5.1 × 3.6 × 1.8 cm (2 × 1.4 × 0.7 in.)] were fitted to the compartments of the reflectance attachment. The block in the front compartment, with a concave cavity (Fig. 2), was used to hold the sample tablet while the rear compartment block was used as a reference standard. The depth of the cavity was one-half the thickness of the tablet, with a tolerance of less than 0.04 cm (0.015 in.). The cavity was centered in the magnesium carbonate block so that the tablet was always centered in front of the aperture windows of the instrument. By properly marking this position, it could be easily reproduced.

Measurements were made between 540 and 700 nm for each sample at appropriate wavelength intervals and at the maximum for the dye (640 nm) (Fig. 3). Evaluation of the color of the tablets by visual observation was carried out as previously reported (1).

RESULTS AND DISCUSSION

The color fading of FD&C Blue No. 1 followed an apparent first-order rate up to 20,000 foot-candles days. The fading followed the Kubelka–Munk equation:

$$\theta_t = \theta_t' e^{-kt I} \qquad \text{(Eq. 1)}$$

where $\theta_t = (1 - R)^2/2R$, where R is the reflectance at the absorbance maximum at time t; I is the intensity in foot-candles, k is the apparent first-order rate constant; and θ_t' is θ_t at time $t = 0$. Figure 4 shows typical plots of θ_t *versus* (time and intensity).

A hypsochromic shift of maximum from 640 to 630 nm occurred after approximately 3 days of exposure to 1000 foot-candles of light (Fig. 3). By this time, the tablets had lost most of their color (visible to naked eye). Therefore, when plotting θ_t *versus* (time × intensity), the values of time were taken only up to 4 days. The data up to 40 days show biexponential behavior and follow Eq. 2:

$$\theta_t = \theta_{t_1} e^{-k_1 t I} + \theta_{t_2} e^{-k_2 t I} \qquad \text{(Eq. 2)}$$

where $\theta_{t_1} + \theta_{t_2} = \theta_t'$.

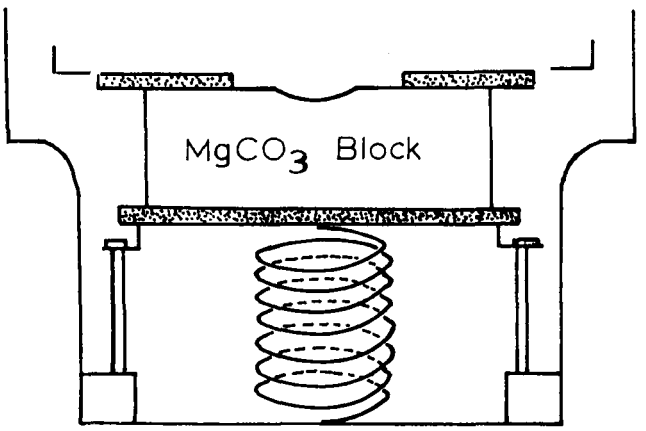

Figure 2—*Tablet arrangement with magnesium carbonate block for reflectance attachment.*

Table I—Summary of Visual Observations of Tablets Coated with FD&C Blue No. 1 Exposed to an Intensity of 1000 Foot-Candles of Light over 40 Days[a]

Sunscreening Agent	Coating Film		
	Alcohol Film	Modified Sugar	Film
Control	Noticeable fading becoming objectionable within 5 days and becoming almost colorless within 10–20 days		
Glyceryl *p*-aminobenzoate	1A, S	1	1A, S
Glyceryl *p*-aminobenzoate (p.c.)	Not studied	Not studied	1A, S
Benzocaine	2	1	1A
Benzocaine (p.c.)	Not studied	Not studied	1A
2-Ethoxyethyl *p*-methoxycinnamate	2, S	2	2, S
m-Homomenthyl salicylate (n.p.)	2, S	Not studied	Not studied
m-Homomenthyl salicylate	2, S	2	2A
n-Octyl salicylate	2	2	2A
Amyl salicylate	2	2	2A

[a] 1 = fading less than control, 1A = fading less than control with change of hue, 2 = fading similar to control, 2A = fading similar to control but with mottled appearance due to cracked film, S = brilliant shine, p.c. = contains protective coat, and n.p. = not polished (all others polished).

The units for both rate constants are foot-candles^{-1} days^{-1}. The second rate constant could represent: *(a)* changes occurring at or within the reflectance surface without any dye (9), *(b)* the possibility of reactions between the dye and its decomposition product, atmosphere, or inert ingredients of tablet (10), or *(c)* the porous nature of tablet coating. This is in general agreement with other workers (10–12). Equation 1 satisfactorily covers the color change within 4 days. After about 4 days, although a reflectance reading is obtained, the tablets appear white or "colorless" and fall past the objectionable fading period. Since the present objectives of the study were therefore met, the rate constant k_2 was not evaluated.

Visual observations were made on all tablets (Table I). The time at which objectionable fading or appearance was seen, in the opinion of the author, was also noted. The corresponding value of θ_t is obtained from plots of log θ_t *versus* (time × intensity). Thus, all values of Eq. 1 are known at $I = 1000$ foot-candles. The time at which objectionable fading occurs can then be calculated at a lower intensity. The value of $I = 50$ foot-candles as the light intensity of a well-illuminated room (1) was used for calculating approximate shelflife in days for minimum acceptable value of θ_t. Table II shows the apparent first-order rate constants and the shelflife values.

An unexplained phenomenon was observed in the reflectance curve of tablets coated with FD&C Blue No. 1 by the film-coating method (Fig. 5). When the photocell was changed at 625-nm wavelength, a hypsochromic effect was observed. This hypsochromic effect decreased upon exposure to light with time and became zero after about 20 days, but by this time the tablets had almost no visible color left. This phenomenon was observed in all FD&C Blue No. 1 tablets coated by the film-coating method, with or without sunscreening agents, but not with any other groups of tablets.

Analysis of tablets for the amount of sunscreening agent per tablet (based on average of three assays) and of thickness of coatings (based on average of 25 readings) was carried out as previously reported, and results were in excellent agreement with the values reported previously (1).

Table II—Rate Constants for Fading of Color-Coated Tablets with FD&C Blue No. 1 and Approximate Sheflife to Objectionable Fading at 50 Foot-Candles[a]

Sunscreening Agent	Alcohol Film Coating		Modified Sugar Coating		Film Coating	
	$k \times 10^4$, (foot-candles day)$^{-1}$	Shelflife, days $\times 10^{-2}$	$k \times 10^4$, (foot-candles day)$^{-1}$	Shelflife, days $\times 10^{-2}$	$k \times 10^4$, (foot-candles day)$^{-1}$	Shelflife, days $\times 10^{-2}$
Control	2.7	0.3	3.2	0.4	1.7	0.6
Glyceryl *p*-aminobenzoate	2.6	6.3	1.8	0.5	1.4	0
Glyceryl *p*-aminobenzoate (p.c.)	*a*	*a*	*a*	*a*	1.6	0
Benzocaine	3.4	0.3	2.1	0.4	1.2	0.5
Benzocaine (p.c.)	*a*	*a*	*a*	*a*	1.7	0.4
2-Ethoxyethyl *p*-methoxycinnamate	3.3	0.3	3.6	0.4	1.7	0.6
m-Homomenthyl salicylate (n.p.)	5.0	0.2	*a*	*a*	*a*	*a*
m-Homomenthyl salicylate	3.5	0.3	2.8	0.4	3.0	0
n-Octyl salicylate	3.5	0.3	2.8	0.4	3.2	0
Amyl salicylate	3.2	0.3	2.8	0.4	3.2	0

[a] p.c. = protective coat, n.p. = not polished, and *a* = not studied.

Control—The FD&C Blue No. 1 undergoes fading at nearly three times the rate of FD&C Red No. 3 (1). The fading of FD&C Blue No. 1 was shown (10) to be more stable (5–13 times, depending upon the type and intensity of light) than FD&C Red No. 3. One possible explanation for such a difference in results is the difference in overall composition of tablets, *i.e.*, the background material. Lachman *et al.* (10) included the dye and UV absorber within the granulation, while in this study they were applied to tablets as coatings. In the previous study (10) the rate constants were obtained from plots of log absorbance *versus* time whereas in this study the Kubelka–Munk equation was used. The order of fading of the two dyes reported in the present study is in agreement with a study by Vaidya (5).

Glyceryl *p*-Aminobenzoate—When tablets were coated by either alcohol film coating or film coating, a distinct change in hue (blue to green) was noticed. In both cases the tablets had a brilliant shine (prior to polishing). The protective coat does not appear to have any appreciable effect. Although the intensity of color change was slightly smaller than the one without protective coat, it was not significant. The rate constant decreased nearly by half in the case of the film-coating method but this was offset by an almost immediate change of hue. No work was carried out to determine the reaction involved or to isolate a complex if any.

Benzocaine—When the tablets were coated by the film-coating method, a distinct change of hue (blue to green) was observed after about 5 days of light exposure. The shade of hue was very different from the hue change produced by glyceryl *p*-aminobenzoate. A protective coat was applied as described before, without any apparent advantage.

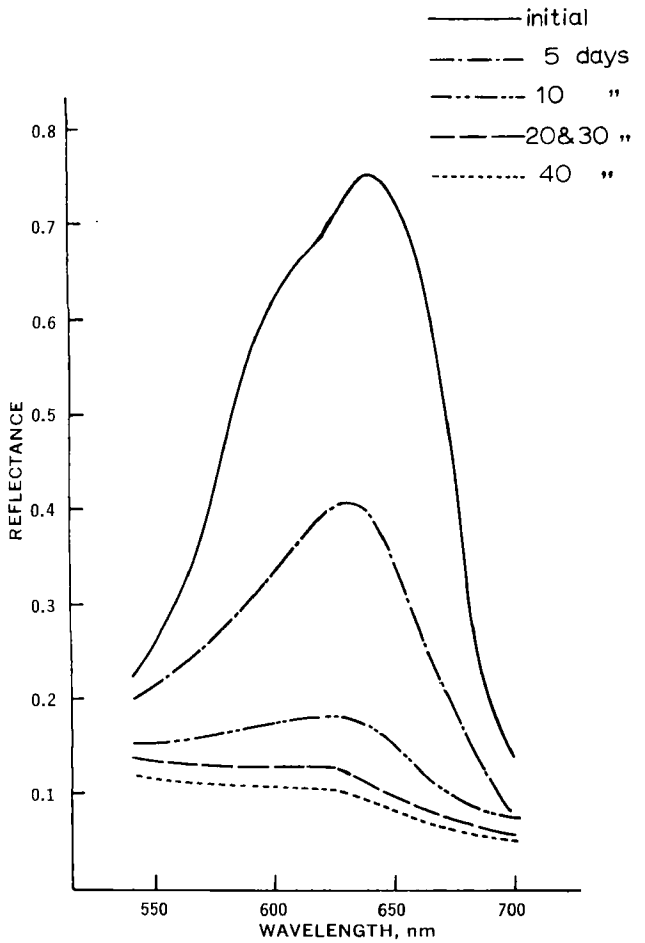

Figure 3—*Visible absorption spectra of control tablets color coated with FD&C Blue No. 1 by alcohol film coating, after intervals of storage under 1000 foot-candles of illumination.*

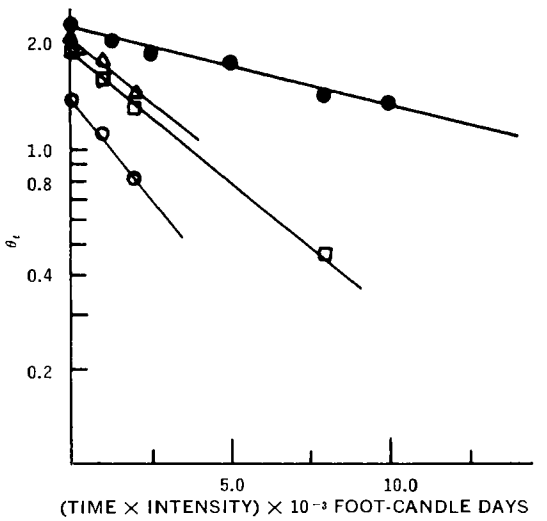

Figure 4—*Plots of* θ_t versus *the product of time and intensity for color-coated tablets. Key:* ●, *FD&C Red No.3, benzocaine, film coating;* △, *FD&C Blue No. 1, benzocaine, modified sugar coating;* □, *FD&C Blue No. 1, 2-ethoxyethyl* p*-methoxycinnamate, film coating; and* ○, *FD&C Blue No. 1, amyl salicylate, film coating.*

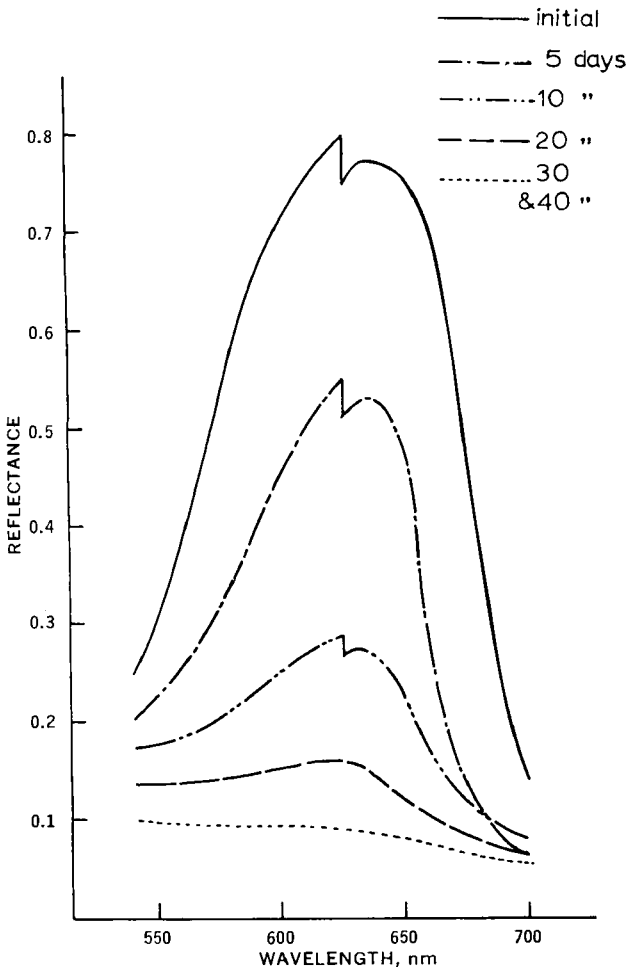

Figure 5—*Visible absorption spectra of tablets color coated with FD&C Blue No. 1 with 2-ethoxyethyl* p-*methoxycinnamate by film-coating technique, after intervals of storage under 1000 foot-candles of illumination.*

2-Ethoxyethyl *p*-Methoxycinnamate—A brilliant shine was observed when tablets were coated by the alcohol film-coating method or by the film-coating method. This did not alter the shelflife or the rate constant significantly. This compound does not appear effective as a protectant for FD&C Blue No. 1.

Salicylates—Tablets coated by the film-coating method for all salicylates appeared mottled after 24 hr of drying, due to cracking of film. When compared to control tablets, all salicylates were equally ineffective as light protectants.

None of the six sunscreening agents employed in the present study exhibited protective action for FD&C Blue No. 1 when applied in concentrations ranging from around 10 to 400 μg/tablet by the alcohol film-, modified sugar-, or film-coating method.

SUMMARY

The photostability effect of six sunscreening agents (glyceryl *p*-aminobenzoate, benzocaine, 2-ethoxyethyl *p*-methoxycinnamate, *m*-homomenthyl salicylate, *n*-octyl salicylate, and amyl salicylate) on FD&C Blue No. 1 was evaluated by exposing tablets to intense measured light.

The sunscreening agents were applied by alcohol film-, modified sugar-, and film-coating methods. The film-coating method employed was not satisfactory for glyceryl *p*-aminobenzoate (with or without protective coat), benzocaine, and all salicylates used in this study. The alcohol film-coating method was also not satisfactory for glyceryl *p*-aminobenzoate.

The rate constant for FD&C Blue No. 1 was about three times greater than for FD&C Red No. 3. Red No. 3 thus shows relatively greater fastness to light when applied in the form of a coating. None of the sunscreening agents applied by any of the three methods exhibited significant protective action for FD&C Blue No. 1.

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Present address: Department of Pharmacy, University of Otago, Dunedin, New Zealand.

System for Automated Determination of Dissolution Rate

J. B. JOHNSON[x], P. G. KENNEDY, and S. H. RUBIN

Abstract □ An automated dissolution rate apparatus meeting requirements of the USP–NF dissolution test and applicable to various other agitation systems in common usage is described. The equipment allows simultaneous determination of the dissolution rate of six unit doses, enabling evaluation of a statistically significant number of samples. The system, composed of readily available standardized components, has a disposable filter unit and can measure the concentration of dissolution solutions in the 0.020–3.000 absorbance range. Dose-to-dose uniformity and stability of dissolution characteristics are shown for several products. Data relating the USP–NF agitation system to the beaker–stirrer (Levy) method are presented. Dissolution rates of sulfisoxazole formulations are correlated with *in vivo* availability, and the adverse effects of inadequate packaging and severe environmental storage conditions on dissolution rate are demonstrated.

Keyphrases □ Dissolution rate determination—automated analysis system described, applications discussed □ Automated analysis—dissolution rate testing, equipment described, applications discussed

It is generally accepted that the rate at which a drug dissolves from the dosage form can be the limiting factor governing its bioavailability in many instances. The correlation between *in vitro* dissolution rates and *in vivo* efficacy remains to be established for most drugs.

The current issues of the official compendia describe an *in vitro* dissolution test and apparatus (1, 2) which, while probably not applicable to all dosage formulations, do constitute the basis for a standardized procedure. Based on data obtained over 2 years in this laboratory, the test does provide discrimination among solid dosages of differing bioavailability when agitation speed and dissolving solvent are properly defined.

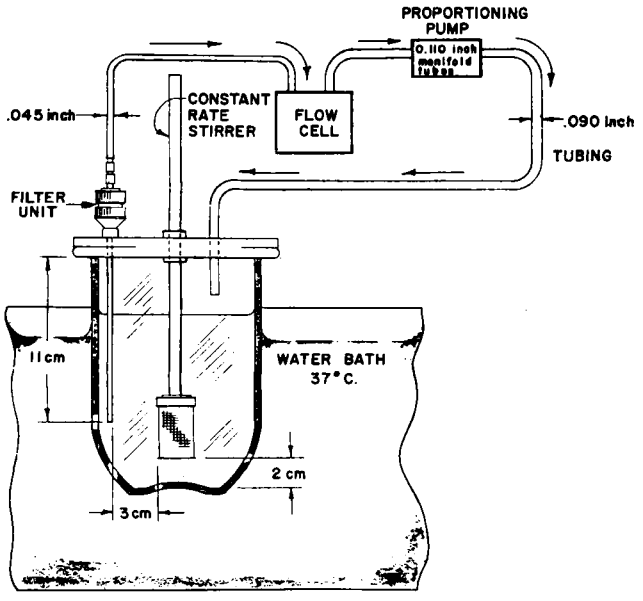

Figure 1—*Flow diagram of apparatus and filter unit assembly with USP–NF Method I dissolution vessel.*

A comparison of the USP–NF system with that described by Levy and Hayes (3), a method widely quoted in the literature, shows that meaningful *in vitro–in vivo* correlations can be established using either test procedure. Both afford a gentle agitation of the dosage unit and ensure minimum abrasion of the sample; however, the USP apparatus appears more widely adaptable since it can better handle the less dense forms such as floating tablets and capsules.

To obtain dissolution profiles on statistically significant samplings of each batch, an automated system was developed with the following significant features:

1. Direct printout (as percent dissolved) of six simultaneous dissolution rate determinations using a completely automated measuring system.
2. Monitoring system containing a minimum volume of dissolving solvent to allow low flow rates, thus eliminating extraneous agitation of the samples.
3. Instrumentation utilizing standard, commercially available, low-cost components.

The instrumentation is adaptable to the USP–NF and Levy agitation systems as well as many others (4, 5). It is adaptable to all single drugs that exhibit a sufficient visible or UV spectrum and to combinations of drugs where the individual spectra lend themselves to simultaneous measurements at different wavelengths. Dissolution rate determinations of combination drugs and single-component dosages whose assay requires measurement at more than one wavelength are made possible by the use of a spectrophotometer equipped with an automatic wavelength programmer.

A major obstacle to be overcome in any continuous flow system is that of assuring the measurement of a clear solution, free of extraneous particles which would cause turbidity in the solution and cloud the surfaces of the flow cells. This was accomplished by the incorporation of filter units into the system which utilize disposable Teflon or polyvinyl inserts and allow the selection of a filter to provide adequate filtration of the solution while minimizing difficulties that could be caused by a poor flow rate through the measuring system.

The entire system as described maintains conditions of agitation exactly as specified in the official test method (USP–NF Method I). All sample agitation is provided by precise, mechanically controlled stirring of the sample basket.

Elimination of any extraneous turbulence within the dissolution vessel is assured by maintaining a low

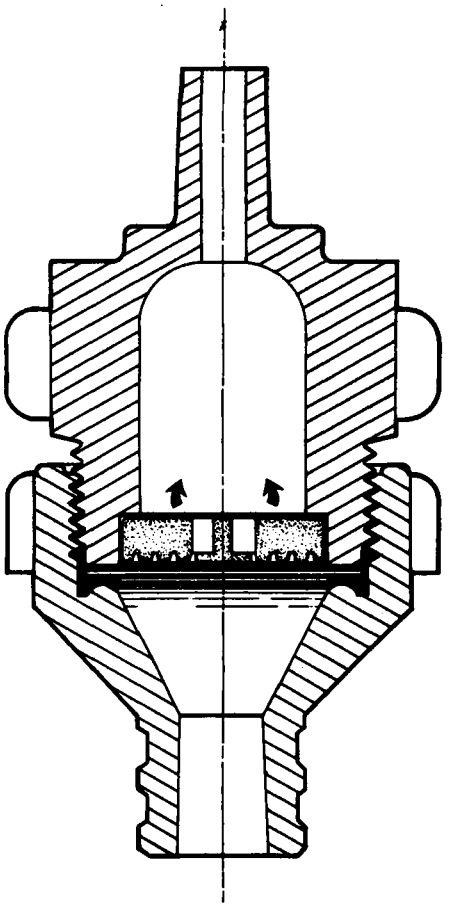

Figure 2—*Cross-sectional drawing of filter unit assembly.*

volume of fluid in the measuring system so as to allow a pumping rate through the flow cell of approximately 2 ml/min. This also minimizes the chances of error caused by eddy currents in the solution being measured in the flow cell itself.

EXPERIMENTAL

Apparatus—Instrumentation for the simultaneous monitoring of six dissolution rate determinations was assembled from commercially available components consisting of a spectrophotometer[1] with automatic wavelength programmer and turret assembly[2], a proportioning pump[3], and a multispeed dissolution tester[4]. A constant temperature of 37° is maintained by a water bath[5]. A digital display and printer[6] and a linear recorder[7] were incorporated to provide data directly as "percent dissolved." The dissolution solvent is filtered through Teflon or polyvinyl filters[8]. The proportioning pump is incorporated into the system on the return side of each of the six flow cells[9] utilized in the turret assembly (Fig. 1).

The filter unit employed in the system (Fig. 2) has the advantages that the Teflon or polyvinyl filter inserts can be discarded after each test, eliminating cleaning, and that various porosities can be selected for each application to eliminate clogging and maximize solvent flow. An 11-cm glass tube having an inside diameter of 2.5 mm is attached to the sampling orifice of the filter unit. Alternative agitation systems, such as described in NF XIII, Method II, and the beaker–stirrer assembly of Levy and Hayes were studied in place of the multidissolution tester (USP–NF) by simply inserting the filter units in the respective dissolution vessels specified for those systems in place of the USP dissolution flask.

Agitation speeds between 25 and 250 rpm are provided by a variable speed multidissolution tester, which incorporates a tachometer to facilitate precise speed adjustment. A standard model spectrophotometer was modified to allow direct digital printout in percent drug dissolved. The use of standard, commercially available components both for the agitation and measuring systems minimizes the cost of the unit; however, modifications of both the printer and spectrophotometer are necessary.

Circuitry changes in the spectrophotometer were made so that a single "print" impulse is transmitted to the printer, which corresponds to the maximum absorbance obtained while a particular cell is in the light path. Modifications were also made in the printer circuitry to allow for recycling after each series of six printouts. Light path dwell times for the individual cells were adjusted to give 2.5- and 5-min cycles, allowing a measurement for each of the six dissolution tests to be recorded at any multiple of 2.5 or 5 min.

Method—Normally, 900 ml of simulated gastric, intestinal fluid or other dissolving solvent is heated to 37° and placed in the glass vessels specified in the the USP–NF test. Filter units are placed in position through the vessel covers parallel to and approximately 3 cm from the side of the sample basket. The solutions are drawn from the flask through the filters and the flow cells by a proportioning pump and are returned to the vessels at a flow rate of approximately 2 ml/min. The total volume in the measuring system is 4.8 ml.

At the appropriate wavelength setting, each cell is adjusted to zero absorbance using variable beam attenuators, modified by enlarging the bore to allow more reliable positioning. To set the concentration mode, the spectrophotometer is set in the single-beam position with cell 1 in the light path. The absorbance representing

Table I—Tubing Selection

Configuration	Delivery Tubing Diameter, in.	Pump Tubing Diameter, in.	Seconds to Cell
1	0.045	0.073	30–35
2	0.073	0.073	>60
3	0.073	0.090	60
4	0.045	0.110	20

Table II—Manual and Automated Dissolution Rates Using 50-mg Chlorprothixene Tablets

Minutes	Percent Dissolved	
	Manual	Automated
5	2	1
10	19	17
15	84	85
20	97	97

Table III—Diffusion Rate within the Dissolution Vessel (100 rpm)

Station	Time to Indicate 100% Diffusion, sec	Percent of Theory at Maximum
1	110	102
2	140	100
3	130	99
4	110	100
5	90	102
6	120	99
Average	117	100
ts: 95%	±43	±2.8

[1] Beckman model DB GT spectrophotometer.
[2] Spectrophotometer model DB GT P/N 1405 and turret assembly P/N 565810, Beckman Instruments, Mountainside, N.J.
[3] Autoanalyzer proportioning pump PP2 105-A200-1, Technicon Instrument Corp., Tarrytown, N.Y.
[4] Model T1044-20, Hanson Research Corp., North Ridge, Calif.
[5] Lab-Line Instruments, Melrose Park, Ill.
[6] Digital display P/N 573230 and printer model 3115, Beckman Instruments, Mountainside, N.J.
[7] Model 100500, Beckman Instruments, Mountainside, N.J.
[8] Swinnex-13 filter units (polypropylene) SX00-01300, Millipore Corp., Bedford, Mass.
[9] Using 0.1- and 1-cm flowthrough cells QS170 and QS175, Hellma Cells Inc., Jamaica, N.Y.

Table IV—Cell-to-Cell Variation

Cell	Percent of Assay Value					
	1	2	3	4	5	6
Cycle 1	101	101	101	100	100	101
Cycle 2	101	102	100	100	101	101
Cycle 3	101	102	100	100	101	101
Average: 101 *ts:* 95% ± 0.9						

100% theoretical dissolution is set on the digital display using the dynode voltage control, and the digital display is switched to the "conc" mode. A concentration count of 100 or 1000 is then fixed, which corresponds to 100%. The spectrophotometer is reset to double-beam operation. Six tablets are placed in individual 40-mesh stainless steel baskets and attached to the spindles of the agitator. The baskets are immersed in sequence at the appropriate time intervals into the dissolving medium and properly positioned in the flask.

To obtain optimum flow conditions the minimum volume within the system, various tubing configurations were studied (Table I). Configuration 4 was chosen, because it provides a minimum lag time in delivering the dissolving solution to the flow cell.

RESULTS

Dissolution rates obtained by the automated system and those obtained by manual measurement agree. Dissolution of sugar-coated tablets containing 50 mg of chlorprothixene was studied both manually and in the automated apparatus (Table II).

The time for the entire system to reach equilibrium and the reproducibility of the actual measurement were studied by introducing 10 ml of a standard solution containing 250 mg of levodopa to each of the six stations. The time for the system to indicate complete diffusion and the accuracy of six determinations were evaluated (Table III).

Considering the accuracy and reproducibility of the instrumentation, real differences in the dissolution rates of unit doses within a batch can be measured if these exceed ±2.8% of the average assay. The small variations shown in Column 3 of Table III are indicative of limitations in the mixing action within the USP–NF dissolution vessel. Actual variation caused by the instrumentation is negligible, as demonstrated by the values obtained when a single solution was passed through each of the six flow cell channels (Table IV). Here no significant variations (less than 1%) due to cell positioning or electrical imbalance were found within three repetitive cycles.

The intertablet variations obtained with a typical batch of 500-mg levodopa tablets and with a 2-mg benzodiazepine tablet are shown in Table V.

Variations in this range present no problems, especially with levodopa, since the drug is given in large doses, each dose containing several tablets. However, excessive tablet-to-tablet variations could present problems with other drugs given as a single low dose where a rapid therapeutic response is required, as exemplified by benzodiazepine.

A comparison of dissolution rates of levodopa tablets, using the USP–NF agitation system and the Levy and Hayes system, shows rank-order correlation between the two methods based on the average of six tablet determinations. The range of the individual tests was much greater with the Levy agitation system, undoubtedly because of the less defined parameters it provides for positioning of the dose and specific design of the vessel (6). Data obtained at a moderate agitation speed of 60 rpm by both methods are shown in Table VI.

Table V—USP–NF *In Vitro* Dissolution Rate in Simulated Gastric Fluid

Minutes	500-mg Levodopa Tablet			2-mg Benzodiazepine Tablet		
	Average, %	Range, %	*ts:* 95%	Average, %	Range, %	*ts:* 95%
2.5	48	40–57	15	22	15–36	17
5	85	78–89	10	69	63–74	11
10	97	90–101	10	83	78–87	7
15	99	94–101	7	91	85–97	11
20	100	97–102	4	100	94–105	11
30	100	98–102	4	—	—	—

Table VI—USP–NF and Levy Agitation (60 rpm) Using 500-mg Levodopa Tablets

Minutes	USP–NF		Levy	
	Average, %	Range[a], %	Average, %	Range[a], %
2.5	17	9–26	16	9–26
5	38	21–64	30	20–42
10	78	57–84	53	31–74
15	87	79–91	67	39–90
20	90	86–94	73	46–96
30	94	91–96	81	70–100

[a] Six determinations.

APPLICATIONS

As typical examples illustrating the use of the methodology described, the following are considered: *(a)* a packaging problem relating to sulfisoxazole tablets, and *(b)* a more sophisticated metabolic problem that attempts to relate the complex metabolic behavior of levodopa, which is extensively and rapidly biotransformed in the body, to the conditions of *in vitro* dissolution.

Correlation of *in vitro* dissolution rates and blood levels in the dog was examined using the USP–NF method of agitation (Method I) with sulfisoxazole tablets that had been packaged inadequately in polyvinyl chloride unit dose blisters and exposed to elevated conditions of temperature and relative humidity (37°, 85% relative humidity) for 1 month. These tablets showed considerable moisture pickup and greatly reduced *in vitro* rates of dissolution (Sample B). The reduction in dissolution rate was apparent when either 0.84 *N* HCl or simulated intestinal fluid was used as the dis-

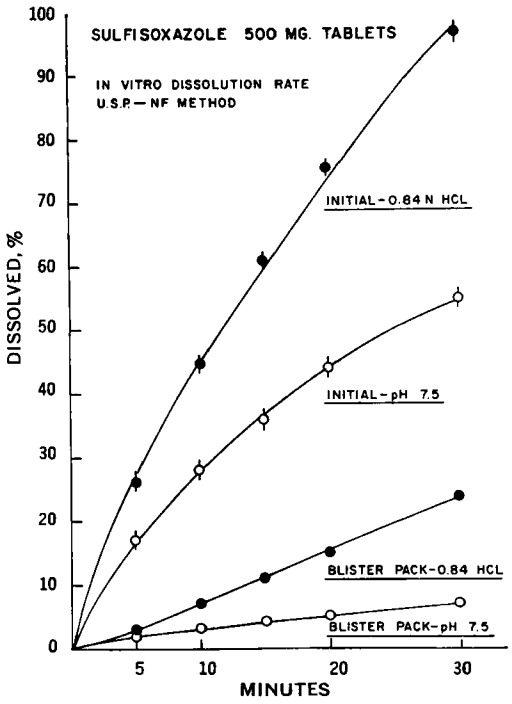

Figure 3—In vitro *dissolution rate of 500-mg sulfisoxazole tablets using USP–NF Method I. Key: ● and ○, Sample A, control tablets; and ● and ○, Sample B, tablets exposed to 37° and 85% relative humidity in inadequate packaging.*

Table VII—Plasma Levodopa Micrograms per Milliliter at 30–300 min

	Minutes						
	30	60	90	120	160	240	300
Formulation A	0.58	0.52	0.69	0.69	0.48	0.27	0.14
Mean ± *SE*	±0.12	±0.08	±0.13	±0.20	±0.08	±0.04	±0.02
Formulation B	0.44	0.56	0.61	0.52	0.47	0.34	0.15
Mean ± *SE*	+0.14	±0.12	±0.15	±0.10	±0.08	±0.07	±0.01

solution solvent, both of which provided sink conditions for the test (Fig. 3).

Samples of these tablets along with a control sample (Sample A) were administered to two beagle dogs in a crossover study[10], and blood levels were determined over 24 hr (Fig. 4). The measurements suggest some rank-order correlation, as shown by a plot of the dissolution rate $T_{25\%}$ *versus* the reciprocal of the area under the blood level curve (Fig. 5). However, the radical reduction in *in vitro* dissolution (Method I) was not reflected in any drastic change in bioavailability.

The importance of providing gentle agitation of the dosage form has been demonstrated (7); however, caution must be exercised in reducing the degree of agitation to a level where observed differences do reflect significant differences in bioavailability. To illustrate, dissolution rates of two different solid dosage formulations of levodopa were determined, using agitation speeds of 50 or 100 rpm with either 0.01 or 0.1 *N* HCl, respectively, as the dissolving solvents. Dissolution rates determined using a 50-rpm agitation speed in 0.01 *N* HCl showed differences between the formulations (Fig. 6), depending upon use of $T_{50\%}$ as contrasted with use of $T_{80\%}$ as the critical points. At a speed of 100 rpm in 0.1 *N* HCl, no such differences were observed.

Withey (8) observed wide variations when dissolving a single sodium chloride crystal at an agitation speed of 50 rpm. The large coefficient of variation reported reflects the nondisintegrating nature of the sample and the small percentage of sodium chloride dissolved at the low agitation speed of 50 rpm and illustrates the importance of selecting the proper speed for each dosage form based on correlations with *in vivo* data.

Biologically, crossover blood level studies in humans failed to show any significant differences between these two formulations, corroborating the results of the second test (Table VII).

Further evidence of the validity of the second set of test parameters was provided by correlations with human blood levels obtained on experimental formulations (C and D), which showed marked *in vitro* differences using the higher agitation speed and 0.1 *N* HCl as the dissolving solvent (Fig. 7). Correlation of $T_{50\%}$ *in vitro* dissolution data and the time required to reach peak blood levels is shown in Fig. 8[11]. This example serves to emphasize the importance of having critical, unique biological data available before choosing a suitable dissolution test for control purposes.

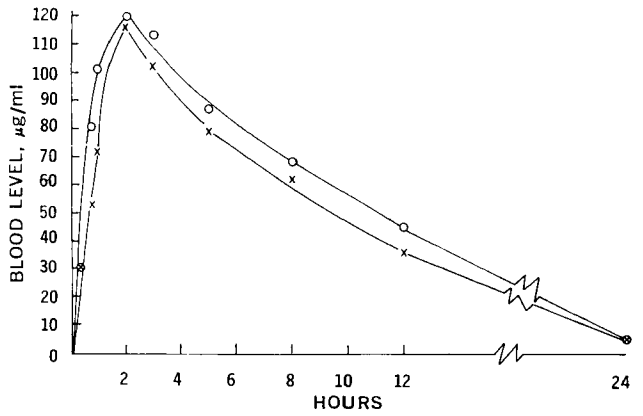

Figure 4—*Comparative drug blood levels in dogs using 500-mg sulfisoxazole tablets. Key: ○, Sample A; and ×, Sample B.*

DISCUSSION

An automated system that will determine the dissolution rate of six unit doses simultaneously was designed and assembled from commercially available, moderately priced components. The instrumentation has been used routinely over 2 years with good utility. High precision and reproducibility within the measuring system are achieved by maintaining low volume and flow rates, thus allowing measurement of real differences in dissolution characteristics between dosage forms, if these exceed ±3%. The Levy method was shown to give results in the same rank order as the USP–NF Method I, although with poorer reproducibility due to the lack of precise definition within the dissolution vessel.

The filter units allow selection of the proper filter porosity that provides adequate flow rates within the system commensurate with solution clarity. The need for extensive cleaning between sample runs is eliminated by the use of disposable inserts. The low volume measuring system minimizes the amount of solution required to be drawn through the filter units, thus eliminating the need for elaborating anticlogging systems (9), and makes exact absorbance measurement possible by preventing the formation of eddy currents within the flow cells.

Dissolution rates of dosage forms can be markedly affected by exposure to improper storage conditions. The official test currently used for sulfisoxazole appears to present a poor measure of the true bioavailability of the drug, since dosages that fail the test specification provide adequate therapeutic blood levels in dogs. The differences between the control and package sample are in the same order as those reported (10) in a study comparing oral solutions of sulfisoxazole to acceptable tablet formulations.

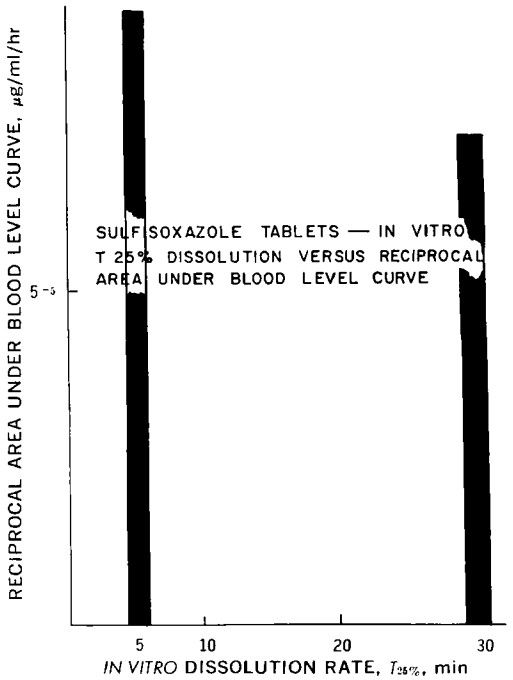

Figure 5—In vitro $T_{25\%}$ *dissolution* versus *reciprocal of the area under blood level curve for 500-mg sulfisoxazole tablets. Key: left, Sample A; and right, Sample B.*

[10] S. Kaplan, Hoffmann-La Roche Inc., Nutley, N.J., personal communication.

[11] C. Joseph, Hoffmann-La Roche Inc., Nutley, N.J., personal communication.

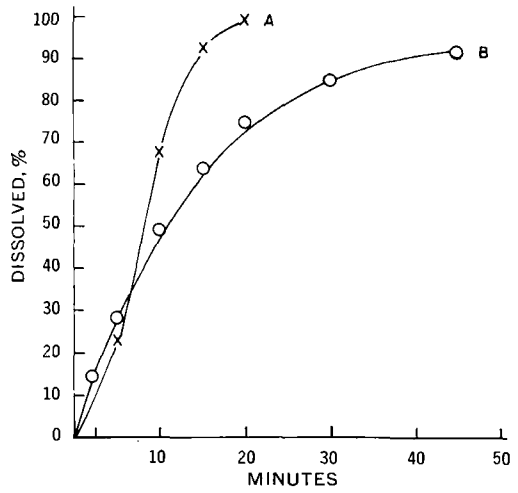

Figure 6—In vitro *dissolution rate of 500-mg levodopa Formulations A and B using USP–NF Method I, 50 rpm, and 0.01* N *HCl.*

The adequacy of a final package configuration should always be evaluated with regard to maintaining a drug's bioavailability. In the case of sulfisoxazole tablets, it was shown that polyvinyl chloride blister packages will not provide adequate protection to ensure compliance with current USP specification on exposure to 37° and 85% relative humidity conditions of storage. However, in this instance the biological significance of the specification is questionable.

It is also apparent that overly discriminatory *in vitro* tests can be designed that indicate differences in dissolution rates between formulations that have no biological significance, as illustrated by the first test described for levodopa dosages. This is not to say that a significant *in vitro* test is not possible, but only that its selection should be based soundly on *in vivo* correlation and not established arbitrarily. The second set of test parameters described for levodopa provide such a correlation and represent a meaningful evaluation.

SUMMARY

An automated dissolution rate apparatus that meets the requirements of the USP–NF dissolution test and is applicable to various other agitation systems in common usage has been assembled. The equipment allows simultaneous determination of the dissolution rate of six unit doses, enabling evaluation of a statistically significant number of samples. Dose-to-dose uniformity and stability of dissolution characteristics are shown for several products.

Data relating the USP–NF agitation to the beaker–stirrer (Levy) method are presented. The authors prefer the current USP–NF method because of its better defined parameters and concur with the findings of other investigators (11) that it is the simplest and most versatile of the available methods. Dissolution rates of levodopa formulations and, to some extent, sulfisoxazole formulations were found to correlate with *in vivo* availability when the *in vitro* test was properly designed.

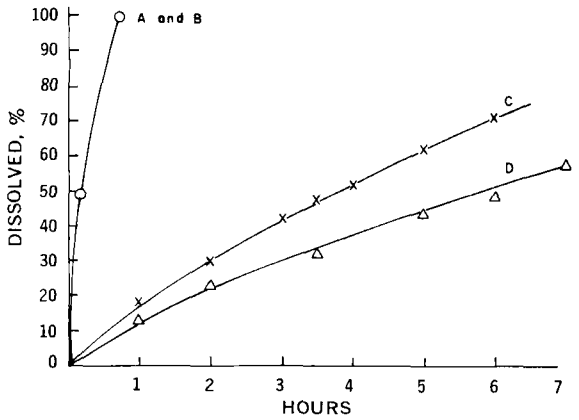

Figure 7—In vitro *dissolution rate of 500-mg levodopa formulations using USP–NF Method I, 100 rpm, and 0.1* N *HCl. Key:* ○, *Samples A and B;* ×, *Sample C; and* △, *Sample D.*

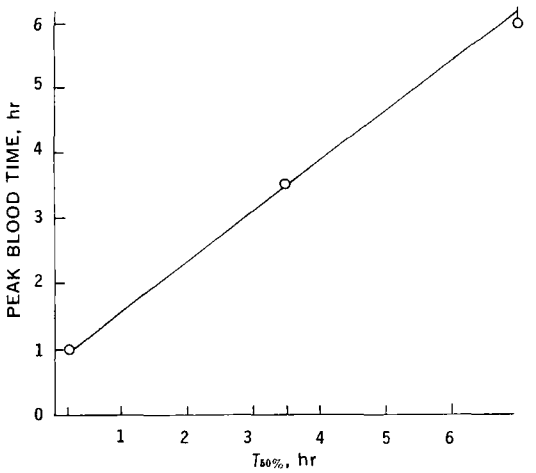

Figure 8—*Levodopa tablets,* in vitro $T_{50\%}$ *dissolution value* versus *time in hours to reach peak blood level.*

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[x]To whom inquiries should be directed.

Preliminary Study of Potential Antiarrhythmic Effects of *Crataegus monogyna*

EMMANUEL B. THOMPSON[x], GEORGE H. AYNILIAN, PAUL GORA, and NORMAN R. FARNSWORTH

Abstract □ Crude extracts of the bark and leaves of *Crataegus monogyna* were tested in rabbits for potential antiarrhythmic activity. Four fractions (A contained the petroleum ether solubles, B contained the 95% ethanol solubles, C contained the chloroform solubles, and D contained the water solubles), which were infused 5 min after arrhythmia had been induced by intravenous injection of 10 μg/kg of aconitine, did not cause the arrhythmia to revert to normal cardiac rhythm. However, when the maximal tolerated amounts of either Fraction B or D of the bark or leaves were first infused, higher doses of aconitine than those used in control animals were required to generate arrhythmias in the treated rabbits. The present study showed that the extracts from bark and leaves of *C. monogyna* possess a prophylactic antiarrhythmic activity.

Keyphrases □ *Crataegus monogyna* L. (Rosaceae)—antiarrhythmic effects of crude extracts of bark and leaves □ Medicinal plants—*C. monogyna*, crude extracts of leaves and bark screened for antiarrhythmic effects □ Antiarrhythmic activity—*C. monogyna* leaf and bark extracts screened

Several species of the genus *Crataegus*, commonly called Hawthorn, have been studied for their potential medicinal value. Claims of pharmacological activity range from curative agents in hypertension to the treatment of certain cardiac disorders. As early as 1896, Jennings (1) advocated the use of tincture of *Crataegus oxyacantha* as an adjuvant to, and even a substitute for, *Digitalis* in the treatment of cardiac disorders. A different species, *Crataegus pentagyna*, was reported by previous investigators to possess an effect on the isolated mammalian heart (2–4). A later report (5) referred to the antiarrhythmic effectiveness of *C. pentagyna* against aconitine-induced arrhythmias in rabbits.

In 1949, Neugebauer (6) reported that *Crataegus monogyna* contained a substance that was intensely active on the heart. Some years later, Andreeva (7) showed that extracts of *C. monogyna* elicited sedative activity and a positive inotropic effect on the frog heart, the major part of the activity being found in infusions of the flowers and bark. Nikolov and Ivanov (8), in 1969, reported that a flavonoid mixture from *C. monogyna* improved cardiac activity in experimental animals.

Although other *Crataegus* species have been shown to prevent cardiac arrhythmias (2–4), this effect has not been demonstrated for *C. monogyna*. Thus, it was decided to carry out a preliminary pharmacological investigation on extracts of the leaves and bark of *C. monogyna* to determine whether they possess antiarrhythmic activity.

EXPERIMENTAL[1]

Preparation of Extracts—One kilogram of coarsely powdered leaves of *C. monogyna* was continuously extracted with petroleum ether (bp 30–60°) in a soxhlet extractor until the final extract was colorless. Evaporation of the petroleum ether *in vacuo* yielded 2 g of an oily residue.

The defatted powder was air dried and macerated for 24 hr with 95% ethanol and percolated slowly until the eluate was colorless. Evaporation of the ethanol *in vacuo* yielded a 35-g residue, of which 12 g was set aside for testing. The remaining 23 g was taken up in 100 ml of water and extracted four times with 50-ml portions of chloroform. After the separation of the two layers, the chloroform extract was dried over anhydrous sodium sulfate and taken to dryness *in vacuo*. The yield was 12.3 g. Lyophilization of the aqueous fraction yielded 9.6 g of residue. Fraction A contained the petroleum ether solubles, Fraction B contained the 95% ethanol solubles, Fraction C contained the chloroform solubles, and Fraction D contained the water solubles.

Similarly, 1 kg of coarsely powdered bark of *C. monogyna* yielded 0.5 g of Fraction A and 40 g of Fraction B, of which 25 g was partitioned between chloroform and water. Fraction C weighed 14 g while Fraction D weighed 11 g.

Solubilization of Plant Extracts—Solutions of the various fractions of *C. monogyna* used for infusion in the experimental animals were prepared by dissolving the extract in an appropriate solvent to obtain a concentration of 50 mg/ml, from which dilutions were made as needed. One gram each of Fractions D and B of the bark and leaves, respectively, was dissolved in 20 ml of a solvent mixture. The mixture contained a sufficient amount of dimethyl sulfoxide to dissolve the solid material, and the final volume was made up to 20 ml with distilled water to obtain the desired concentration (50 mg/ml).

Preparation of Animals—Adult female albino rabbits, weighing 2.0–3.0 kg, were used. Each animal was anesthetized with 25 mg/kg iv sodium pentobarbital[2]. A femoral artery and vein were each cannulated for monitoring arterial blood pressure and for drug administration, respectively. Conventional limb lead II was used to monitor the ECG, from which heart rate was derived. Antiarrhythmic activity of *C. monogyna* was tested against aconitine[3]-induced arrhythmias, using a modification of the method introduced by Scherf (9). Initially, the arrhythmogenic dose of aconitine was established by intravenous sequential injections of 10 μg/kg at intervals of 15 min until arrhythmia developed. When the induced arrhythmia was well sustained, about 30 ml or more of the test solution of the crude extract of *C. monogyna* was infused intravenously at a rate of 0.75 ml/min by means of an infusion pump[4]. Infusion was discontinued when normal sinus rhythm was restored or when death resulted.

In another group of rabbits, 30 ml of the appropriate crude frac-

[1] The leaves and bark of *C. monogyna* L. (Rosaceae) were collected from plants cultivated at the Morton Arboretum, Lisle, Ill., in July 1973. The plant material was air dried and milled to a coarse powder. The identity of this plant material was confirmed by Mr. Floyd Swink of the Morton Arboretum, and a voucher specimen (SP-2510) from the collection has been deposited in the herbarium of the Department of Pharmacognosy and Pharmacology, University of Illinois at the Medical Center.

[2] Sodium Nembutal, Abbott.

[3] Pfaltz Bauer Inc., Flushing, N.Y.

[4] Harvard compact infusion pump model 975.

Table I—Aconitine-Induced Arrhythmia after *C. monogyna* Infusion

Extract Infused (Concentration)	Number of Animals	Amount of Extract Infused	Total Amount of Aconitine Injected	Number of Animals Exhibiting Arrhythmias/Number Tested	Protection, %
Control	5		10 ± 2.0[b]	5/5	0
Fraction D of bark (10 mg/ml)	8	375 ± 50[a]	53 ± 16[b]	3/8	63
Fraction B of bark (10–20 mg/ml)	6	340 ± 88[a]	60 ± 12[b]	2/6	67
Fraction D of leaves (10–20 mg/ml)	6	300 ± 1.0[a]	35 ± 8[b]	3/6	50
Fraction B of leaves (10–20 mg/ml)	2	600 ± 1.0[a]	35 ± 10[b]	2/2	0

[a] Mean ± standard error (milligrams). [b] Mean ± standard error, micrograms per kilogram aconitine.

tion (10–20 mg/ml) was infused into the experimental animals at a rate of 0.75 ml/min, while the controls received either equivalent amounts of saline or the appropriate solvent in which the fraction was dissolved (a mixture of dimethyl sulfoxide and water). Thirty minutes after the infusion was completed, each animal was challenged with the test dose of aconitine (10 μg/kg) at regular intervals of 15 min for no longer than 90–95 min or until arrhythmia developed. The index of protection was taken as a lack of the development of abnormal cardiac rhythm during aconitine injection. The total amount of aconitine administered was then recorded.

RESULTS AND DISCUSSION

The arrhythmogenic dose of aconitine determined in five rabbits was 10.0 ± 2.0 μg/kg. These arrhythmias occurred within 9–12 min after the initial injection of aconitine. The cardiac disturbances included isolated ventricular extrasystoles at the start, which developed into runs of ventricular tachycardia and, in a few instances, ventricular fibrillation. Only in one animal did a reversion of the arrhythmia to normal sinus rhythm occur with the infusion of Fraction D of the bark of *C. monogyna* (50 mg/ml). Fraction D of the leaves and Fractions B of the leaves and bark failed to abort the aconitine-induced disturbance. None of the Fractions A, B, and C of *C. monogyna* bark and leaves tested reversed aconitine-induced arrhythmia.

In the prophylactic test for antiarrhythmic activity, certain fractions of *C. monogyna* demonstrated protection, while others did not (Table I). Death occurred almost instantaneously upon start of the infusion of either Fraction A or C from the bark or leaves. These fractions did not afford any degree of protection at any dose. Considerable protection was observed with Fraction D of the bark since minimal arrhythmias developed with the injection of aconitine. Moderate protection was afforded by Fraction D of the leaves and Fraction B of the bark; the arrhythmia seen was of short duration and occurred only after very high doses of aconitine had been injected. Since earlier experiments had established that the mean arrhythmogenic dose of aconitine in five rabbits was 10.0 ± 2.0 μg/kg iv, the large amounts of aconitine which the rabbits tolerated after the infusion of these fractions suggested a protective antiarrhythmic effect of the principles in both the leaves and bark (Table I).

Only two of 22 rabbits infused with the various fractions (especially Fraction D) were completely protected since there was no occurrence of arrhythmia after infusion of *C. monogyna*. However, the other animals exhibited a moderate degree of protection since large amounts of aconitine (greater than control amounts) did induce mild arrhythmia in a few of these treated rabbits.

The weak protective activity observed in *C. monogyna* may be attributed partly to the fact that all studies in this laboratory were conducted with crude extracts of the various fractions. Thus, the presence of extraneous substances may have masked the true activity of the principles present in the plant parts tested. However, the test conducted (5) with isolates of plant material, which included saponins, flavonoids, and anthocyanins, from *C. pentagyna*, demonstrated antiarrhythmic activity. Reports of past investigations of other species of *Crataegus* demonstrated pharmacological activity in the flowers, leaves, and fruit (7). The present study showed that the bark also possesses active principles.

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[x]To whom inquiries should be directed.

Cocaine Content of *Erythroxylum* Species

G. H. AYNILIAN *, J. A. DUKE ‡, W. A. GENTNER §, and N. R. FARNSWORTH *x

Abstract □ Concentrations of cocaine ranging from 0.00008 to 0.00882% were found in herbarium specimens of seven of eight species of the genus *Erythroxylum* that were examined by GLC. A sample of *E. coca* which was at least 44 years old contained 0.03% cocaine.

Keyphrases □ *Erythroxylum* species—cocaine content of herbarium specimens, GLC □ Cocaine—content of herbarium specimens, GLC □ GLC—analysis, cocaine content of herbarium specimens of *Erythroxylum* species

According to Engler (1) there are about 200 known species of the genus *Erythroxylum*. Although *E. coca* is well known as the major source of cocaine, knowledge as to the distribution of this alkaloid in other *Erythroxylum* species is limited to its occurrence in *E. lucidum*, *E. coca* var. *spruceanum (E. truxillense)*, and *E. novogranatense (E. coca* var. *novogranatense)* (2, 3).

The total alkaloid content of *E. coca* leaves varies from 0.5 to 1.5%, but Javan leaves have been reported to contain as much as 1.0–2.5% total bases (4). Bolivian *E. coca* leaves reportedly contain 70–80% of their total alkaloids as cocaine, whereas Peruvian leaves contain only 50% of their total alkaloids as cocaine (4). It has been reported that the alkaloid content of coca leaves rapidly diminishes during storage and is practically lost after about 7 months (4).

This report presents results from the analyses of single leaves of nine *Erythroxylum* species, including a specimen of *E. coca* stored for at least 44 years. With the exception of *E. coca*, all samples were obtained from herbarium specimens. No data were found indicating that the herbarium specimens had been treated with fumigants, such as mercuric chloride or formalin, which might have affected the analytical results. Quantitation of data was by means of GLC.

EXPERIMENTAL

Alkaloid Extraction—Pulverized leaf samples of *Erythroxylum* species weighing from 19 to 200 mg were processed for alkaloids by maceration with 10% ammoniacal chloroform for 24 hr in separate 5-ml erlenmeyer flasks. The solutions thus obtained were filtered through cotton-plugged Pasteur pipets, and the filtrates were dried over anhydrous sodium sulfate and then with a stream of nitrogen. For GLC analyses, suitable solutions of the crude total alkaloids were prepared in acetone so that each 1.0 ml represented the extractive from 0.2 g of air-dried leaf material.

GLC—A gas chromatograph[1] equipped with a flame-ionization detector was employed for the analyses. A 1.8-m, 0.6-cm (6-ft, 0.25-in.) coiled glass column was packed with 5% OV-101 on Gas Chrom Q, 100–120 mesh[2]. The column bath temperature was maintained at 215°, the injector port was heated to 250°, and the detector temperature was set at 235°. Helium was employed as the carrier gas at a flow rate of 50 ml/min. The internal standard was tetraphenylethylene, and samples of 1–2 μl were injected.

For the quantitative analyses of the cocaine in the extracts, a modification of the method employed by Chu and Mika (5) for the analysis of tropane alkaloids was used. Accordingly, separate acetone solutions containing known quantities of cocaine were prepared and the internal standard solution was added. The solutions were chromatographed and the integrated peak areas of cocaine and tetraphenylethylene were obtained. A standard curve for cocaine was established by plotting the peak areas of cocaine *versus* the weight (Fig. 1). The reproducibility of the standard curve was determined by the analysis of freshly prepared solutions of cocaine on alternate days. The quantity of cocaine in the leaf sample extracts was determined by computing the cocaine peak area from the chromatogram and obtaining the corresponding weight of cocaine from the standard curve. The resulting value was then converted to cocaine concentration as a percentage of air-dried powered leaf sample (Table I).

RESULTS AND DISCUSSION

The relative retention time (rR_t) for reference cocaine was 0.46, with the retention time for internal standard being 7.32 min. Under the experimental conditions described, the minimal quantity of cocaine detectable was 0.077 μg.

Table I presents the results of the quantitative analyses of the *Erythroxylum* species examined. The data represent the average of two separate analyses. Additional analyses could not be performed due to the restricted sample sizes.

Trace quantities of cocaine, ranging from 0.00008 to 0.00882%, were found in the leaves of *E. campestre*, *E. deciduum*, *E. novogranatense*, *E. panamense*, *E. pelleterianum*, and *E. pulchrum*. The plant samples had been collected during 1930–1968. Cocaine could not be detected in *E. citrifolium* (1966) or *E. rufum* (1929 and 1950).

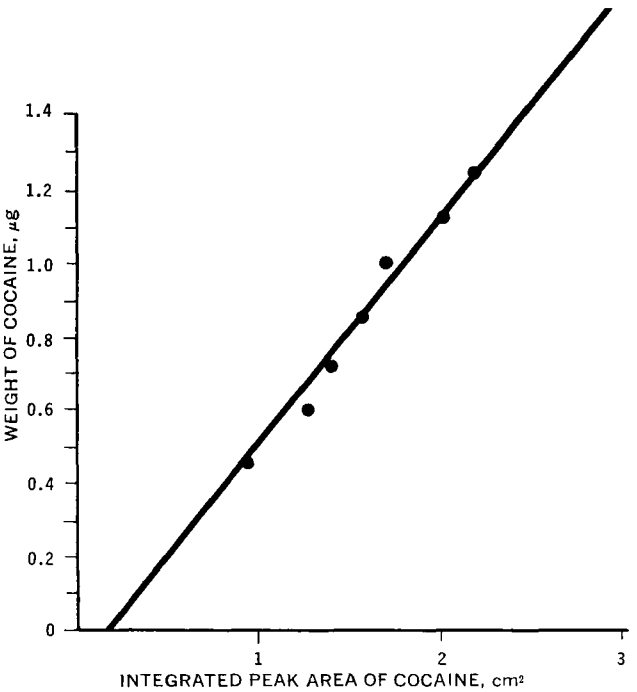

Figure 1—*Standard curve for cocaine.*

[1] Perkin-Elmer model 881.
[2] Applied Science Laboratories, State College, Pa.

Table I—Concentration of Cocaine in Nine Species of *Erythroxylum* as Determined by GLC

Species	Collector	Specimen Number	Locality	Herbarium[a]	Collection Date	Cocaine[b], %
E. campestre A. St. Hil.	P. Dusen	16421	Brazil	MO	?	0.00014
E. citrifolium A. St. Hil.	W. H. Lewis *et al.*	254	Panama	MO	1966	n.d.
E. deciduum A. St. Hil.	L. O. Williams	7424	Brazil	MO	1945	n.d.
E. deciduum A. St. Hil.	L. B. Smith & Klein	7452	Brazil	NY	1956	0.00080
E. novogranatense (Morris) Hieron	R. E. Schultes	—	Cuba	NY	1955	0.00882
E. novogranatense (Morris) Hieron	Br. Daniel	15584	Colombia	OA	1961	0.00216
E. panamense Turcz.	T. B. Croat	5519	Canal Zone	MO	1968	0.00121
E. panamense Turcz.	T. B. Croat	4879	Canal Zone	MO	1968	0.00141
E. pelleterianum A. St. Hil.	Y. Mexia	4364	Brazil	MO	1930	0.00123
E. pulchrum A. St. Hil.	Riedel	390	Brazil	MO	?	0.00008
E. rufum Cav.	E. L. Ekman	11609	Santo Domingo	AA	1929	n.d.
E. rufum Cav.	R. A. Howard	12247	Dominican Republic	AA	1950	n.d.
E. coca Lam.[c]	J. A. Duke	—	—	—	1974	0.53
E. coca Lam.	—	—	—	—	<1930	0.03

[a] MO = Missouri Botanical Gardens, NY = New York Botanical Gardens, OA = Oaks Ames Economic Herbarium, Harvard University; and AA = Arnold Arboretum Herbarium, Harvard University. [b] Single leaves were analyzed, with data being expressed as the mean percent of two determinations; n.d. = cocaine not detected. [c] Leaf material from a 2-year-old living plant was collected and analyzed within 48 hr.

A sample of *E. coca* leaf analyzed within 2 days from the time of removal from the living plant contained 0.53% of cocaine, whereas a dried and stored sample at least 44 years old still contained 0.03% cocaine.

Studies are currently in progress to determine the decomposition rate of cocaine in stored *E. coca* leaves, as well as the natural variability of this alkaloid in various populations of *E. coca*.

REFERENCES

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ROBERT J. ALAIMO [x] and MARVIN M. GOLDENBERG

Abstract □ The synthesis and anti-inflammatory evaluation of a number of 2-(substituted amino)quinolizinium bromides, including some previously unreported analogs, are described. The more active compounds in inhibiting carrageenin-induced edema formation are those containing a *p*-alkoxyanilino group in the 2-position of the quinolizinium ring.

Keyphrases □ Quinolizinium bromides, 2-(substituted amino)—synthesis and anti-inflammatory evaluation □ Aminoquinolizinium bromides (2-substituted)—synthesis and anti-inflammatory evaluation □ Anti-inflammatory activity—evaluation of 38 2-(substituted amino)quinolizinium bromides

Previously, the synthesis and antiparasitic screening of a series of 2-(substituted amino)quinolizinium bromides were reported (1). Many of these compounds (III–XL) exhibited a significant degree of anthelmintic activity. The primary anti-inflammatory evaluation of this series of compounds, including some previously unpublished analogs, is reported herein. Compounds III–XL, when examined in the carrageenin-induced rat paw edema assay, inhibited edema formation as much as 76%.

The previously reported (1) synthesis involves the reaction of an appropriately substituted amine with either of the two intermediates, 2-bromoquinolizinium bromide (I) or 2-bromo-6-methylquinolizinium

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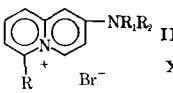
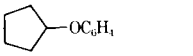
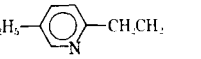
The previously reported (1) synthesis involves the reaction of an appropriately substituted amine with either of the two intermediates, 2-bromoquinolizinium bromide (I) or 2-bromo-6-methylquinolizinium

Table I—Anti-Inflammatory Evaluation of Certain Quinolizinium Bromides



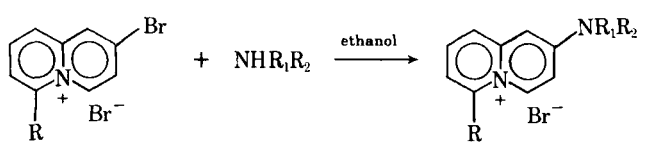
III–XXXIV: R = H
XXXV–XL: R = CH_3

Compound	R_1	R_2	Inhibition of Edema Formation[a], %
III	H	C_6H_5	59
IV	H	4-BrC_6H_4	26
V	H	4-$C_6H_5N{=}N{-}C_6H_4$	39
VI	H	4-$(CH_3)_2NC_6H_4$	62
VII	H	4-$(C_2H_5)_2NC_6H_4$	58
VIII	H	4-$C_2H_5OC_6H_4$	44
IX	H	4-$CH_3CH_2CH_2OC_6H_4$	68
X	H	4-$(CH_3)_2CHOC_6H_4$	41
XI	H	4-$(CH_3)_2CHCH_2OC_6H_4$	76
XII	H	4-$CH_3(CH_2)_3OC_6H_4$	26
XIII	H	4-$CH_3(CH_2)_7OC_6H_4$	44
XIV	H	4-$HC{\equiv}CCH_2OC_6H_4$	17
XV	H	4-$CH_2{=}CHCH_2OC_6H_4$	67
XVI	H	4-$C_6H_5OC_6H_4$	31
XVII	H		67
XVIII	H	3-$CH_3CHOHC_6H_4$	0
XIX	H	2-$CH_3OC_6H_4$	9
XX	H	2-$C_2H_5OC_6H_4$	21
XXI	H	3,4-$(CH_3)_2C_6H_3$	50
XXII	H	3,4-$(CH_3O)_2C_6H_3$	8
XXIII	H	2,4-$(CH_3O)_2C_6H_3$	29
XXIV	H	2-CH_3—4-$CH_3OC_6H_3$	46
XXV	H	2,4,6-$(CH_3O)_3C_6H_2$	19
XXVI	H	2,4-$(CH_3O)_2$—5-ClC_6H_2	0
XXVII	H	2,5-$(CH_3O)_2$—4-ClC_6H_2	2
XXVIII	H	2,5-$(C_2H_5O)_2C_6H_3$	34
XXIX	H	2-CH_2O—5-$CH_3C_6H_3$	51
XXX	H	$HOCH_2CHOHCH_2$	41
XXXI	$HOCH_2CH_2$	$HOCH_2CH_2$	0
XXXII	$CH_3CH_2OCH_2CH_2$	$CH_2CH_2OCH_2CH_2$	3
XXXIII	NH_2		22
XXXIV	H	3-$CF_3C_6H_4$	53
XXXV	H	4-$CH_3OC_6H_4$	12
XXXVI	H	4-$C_2H_5OC_6H_4$	44
XXXVII	H	4-$CH_3SC_6H_4$	9
XXXVIII	H	2,4-$(CH_3O)_2C_6H_3$	22
XXXIX	NH_2	$HOCH_2CH_2$	34
XL	$CH_2{=}CHCH_2$	$CH_2{=}CHCH_2$	34
XLI	Phenylbutazone[b]		59

[a] Compared to control (nondrug treated) hindpaw 4 hr after carrageenin administration. Compounds administered at 300 mg/kg. [b] Phenylbutazone administered at 100 mg/kg.

bromide (II) (Scheme I).

The compounds were tested for anti-inflammatory activity according to the carrageenin method reported by Winter *et al.* (2). Each compound was suspended in distilled water by sonification and administered perorally, 300 mg/kg in three male Wistar rats, 1 hr prior to subplantar injection of 0.05 ml of a 1% solution of carrageenin[1] into the left hindfoot. The percentage reduction of edema formation (as compared to a nondrug-treated control) in the rat hindpaw was recorded 4 hr after carrageenin administration. For comparison, the reference anti-inflammatory drug phenylbutazone (XLI) was tested (Table I).



I: R = H
II: R = CH_3

III–XXXIV: R = H
XXXV–XL: R = CH_3

Scheme I

The more active compounds exhibiting a high degree of reduction of the carrageenin-induced edema formation had a substituted anilino group in the 2-position of the quinolizinium ring. The presence of the methyl group in the 6-position (XXXVI and XXXVIII) did not appear to alter the degree of anti-inflammatory activity when compared with the corresponding demethyl compounds (VIII and XXIII).

Substitution on the anilino ring with alkoxy or dialkylamino groups in the 4-position resulted in the highest degree of anti-inflammatory activity (VI, VII, IX, XI, XV, and XVII), and the active quinolizinium compounds did not exhibit overt signs of toxicity, central depression, or lethality in doses that exerted marked anti-inflammatory activity.

[1] Viscarin.

Table II—Analytical and Physical Data for New Compounds

Compound	Melting Point	Yield, %	Recrystallization Solvent	Formula	Analysis, % Calc.	Found
V	239–240°	90	C_2H_5OH	$C_{21}H_{17}BrN_4$	C 62.23	62.07
					H 4.23	4.29
					Br 19.72	19.67
XXX	166–168°	60	C_2H_5OH	$C_{12}H_{15}BrN_2O_2$	C 48.17	48.42
					H 5.05	5.01
					Br 26.71	26.64
XXXIX	209–210°	92	C_2H_5OH	$C_{12}H_{16}BrN_3O$	C 48.33	48.48
					H 5.41	5.28
					Br 26.80	26.81

EXPERIMENTAL[2]

2-(*m*-Trifluoromethylanilino)quinolizinium bromide (XXXIV) was prepared as follows. To a solution of 2-bromoquinolizinium bromide (I) (1) (45 g, 0.15 mole) in ethanol (600 ml) was added *m*-trifluoromethylaniline (50 g, 0.30 mole). The stirred mixture was boiled under reflux for 5 hr, then treated with charcoal, and filtered. The product was precipitated from the filtrate by the addition of anhydrous ether.

The crude product weighed 42 g (76%). Recrystallization from isopropanol–ether provided analytically pure material, mp 229–230°.

Anal.—Calc. for $C_{10}H_{12}BrF_3N_2$: C, 52.03; H, 3.28; N, 7.59. Found: C, 51.96; H, 3.25; N, 7.61.

All other 2-(substituted amino)quinolizinium bromides were prepared similarly. Compounds V, XXX, XXXIV, and XXXIX have not been reported previously; the analytical and physical data for V, XXX, and XXXIX are shown in Table II. Analytical and physical data for all other compounds (Table I) were reported previously (1).

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Halothane Uptake by Coacervate Systems

W. F. STANASZEK *[x], R. S. LEVINSON *, and B. ECANOW [‡]

Abstract □ Two aggregated coacervae systems (acacia–gelatin and gelatin–benzalkonium chloride) were analyzed for gas sorption of halothane with a gas chromatograph, using a modified tonometer as an absorption chamber. Similar studies were performed on each system after the coacervate had been broken or "dissolved." Differences in absorption or solubilizing effect between coacervates and dissolved coacervates were noted. A significant halothane gas uptake was observed in the highly structured coacervate system.

Keyphrases □ Halothane—uptake by acacia–gelatin and gelatin–benzalkonium chloride aggregated coacervate systems, uptake compared to broken coacervate systems □ Coacervate systems—halothane uptake by acacia–gelatin and gelatin–benzalkonium chloride systems and similar broken systems □ Acacia–gelatin coacervate system—halothane uptake, compared to similar broken system □ Gelatin–benzalkonium chloride system—halothane uptake, compared to similar broken system

The importance of solvent abilities in structured systems as well as a renewed interest in liquid crystalline states suggested that it would be of value to investigate the solvent abilities of a coacervate in contrast to the solvent abilities of an analogous "broken" coacervate. Coacervates may be considered to be coagulated systems, while "dissolved" or broken coacervates can be considered noncoagulated systems. In this study, a unique ability of coacervate systems to attract and solubilize nonpolar gases in a polar medium is discussed.

The term coagulation, as used here, refers to a type of aggregate characterized by film–film bonded particulates. The term coacervation denotes the formation of a liquid precipitate by the mutual coagulation of hydrophilic colloids (1). The definition of coacervation has been more elaborately discussed (2) as a phenomenon of the separation of colloidal solutions into two or more immiscible liquid phases. The electrostatic and thermodynamic properties of a colloidal solution can be varied in such a way that the system separates into two amorphous liquid layers. One layer contains most of the colloid (the coacervate), while the second layer is colloid poor (the equilibrium liquid). The coacervate appears in the form of

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The importance of solvent abilities in structured systems as well as a renewed interest in liquid crystalline states suggested that it would be of value to investigate the solvent abilities of a coacervate in contrast to the solvent abilities of an analogous "broken" coacervate. Coacervates may be considered to be coagulated systems, while "dissolved" or broken coacervates can be considered noncoagulated systems. In this study, a unique ability of coacervate systems to attract and solubilize nonpolar gases in a polar medium is discussed.

The term coagulation, as used here, refers to a type of aggregate characterized by film–film bonded particulates. The term coacervation denotes the formation of a liquid precipitate by the mutual coagulation of hydrophilic colloids (1). The definition of coacervation has been more elaborately discussed (2) as a phenomenon of the separation of colloidal solutions into two or more immiscible liquid phases. The electrostatic and thermodynamic properties of a colloidal solution can be varied in such a way that the system separates into two amorphous liquid layers. One layer contains most of the colloid (the coacervate), while the second layer is colloid poor (the equilibrium liquid). The coacervate appears in the form of

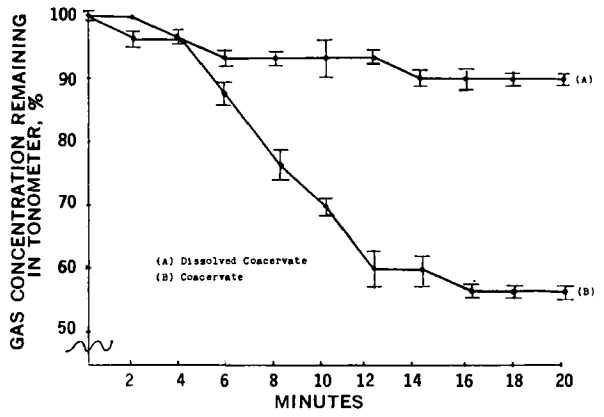

Figure 1—*Halothane gas sorption by gelatin–benzalkonium preparations.*

droplets which coalesce rapidly to form one homogeneous, colloid-rich layer (2).

EXPERIMENTAL

Preparation—Two aggregated coacervate systems (acacia–gelatin and gelatin–benzalkonium chloride) were analyzed for their ability to attract halothane vapor. Similar studies were performed on each system after the coacervate had been broken or dissolved.

A solution of 6.0% (w/v) acacia and 5% (w/v) gelatin comprised the acacia–gelatin coacervates[1]. Three milliliters of 0.2 *N* NaOH, pH 9.95, was added to a portion of the coacervate which had the effect of breaking or dissolving the structure.

The gelatin–benzalkonium chloride coacervate was a solution of 2% gelatin and 0.6% benzalkonium chloride at a pH > 9. To break the coacervate, 1.7 ml of concentrated hydrochloric acid was added to a portion of the coacervate. The major difference between a coacervate and a "dissolved coacervate" is that the coacervate, if mixed with the equilibrium liquid, will maintain its immiscibility and volume, whereas when the dissolved coacervate is mixed with the equilibrium liquid, a clear single-phase system is obtained.

Halothane was originally in the liquid state. In preparation for the desired vapor volume, the following formula was used:

$$\frac{(A)(B)(C)}{(D)(E)} = \text{volume of liquid anesthetic (milliliters)} \quad \text{(Eq. 1)}$$

where A = percent of gas concentration, B = volume (milliliters) of tonometer, C = molecular weight of anesthetic agent, D = specific gravity of anesthetic agent, and E = gas constant (22,400 ml).

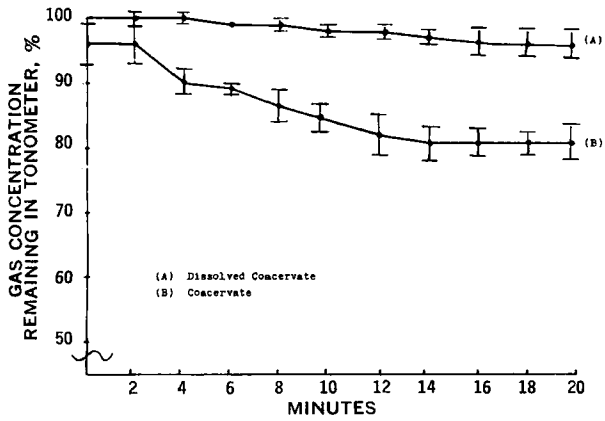

Figure 2—*Halothane gas sorption by acacia–gelatin preparations.*

[1] Acacia–gelatin and gelatin–benzalkonium chloride preparations were obtained through the courtesy of Dr. Harun Takkuri, College of Pharmacy, University of Illinois.

Table I—Statistical Statement

Acacia–Gelatin System: Dissolved Coacervate *versus* Coacervate Halothane Sorption		Gelatin–Benzalkonium Chloride System: Dissolved Coacervate *versus* Coacervate Halothane Sorption	
Minutes	$t_{[4]}$	Minutes	$t_{[4]}$
0	1.98[a]	0	1.03[a]
2	2.34[a]	2	5.51[a]
4	9.91[b]	4	2.45[a]
6	29.16[b]	6	5.07[a]
8	8.91[b]	8	13.10[b]
10	13.60[b]	10	34.03[b]
12	9.19[b]	12	21.11[b]
14	10.56[b]	14	28.85[b]
16	10.61[b]	16	9.71[b]
18	11.36[b]	18	68.93[b]
20	8.98[b]	20	49.05[b]

[a] Not significant. [b] $p < 0.001$.

Equipment[2]—Gas uptake was followed by GLC analysis. A modified tonometer (3), constructed of glass and fitted with rubber septums, was used as an absorption chamber. The tonometer was designed in both 50- and 150-ml sizes for enclosing the gas samples and in a 300-ml size for use as the preparation and storage facility.

Ten-milliliter samples of each coacervate and dissolved coacervate were placed individually in a 50-ml modified tonometer. A 10-ml aliquot of halothane vapor at ambient temperature and pressure was added for gas uptake observations. Since the gelatin–benzalkonium chloride coacervate was a gel at room temperature, it was necessary to heat it in a water bath to 40° before transfer to the tonometer. Once transferred, it was allowed to reach room temperature before the gas absorption experiments.

Gas syringes were used to transfer the gas volume from the preparation tonometer to that containing the coacervate or broken coacervate. These syringes were fitted with 22-gauge, 6.4-cm (2.5-in.) needles. Gastight syringes[3] (100 μl), fitted with Teflon hub needles [24 gauge, 3.8 cm (1.5 in.)], were used for removal of halothane vapor samples from the second tonometer and for injection of samples into the gas chromatograph.

A gas chromatograph with dual hydrogen flame-ionization detectors was utilized to follow gas uptake. The hydrogen flow rate was maintained at 10 ml/min, air at 350 ml/min, and nitrogen at 60 ml/min. The stainless steel columns used for analysis of gas uptake were 1.8 m × 0.64 cm (6 ft × 0.25 in.) and were packed with 25% silicone gum rubber (SE-30) on Anakron (70–80 mesh).

Operating temperatures used in the gas chromatograph were: injection port, 165°; column, 125°; and internal detector, 130°. Gas analysis samples were removed at approximately 1-min intervals for 20 min.

RESULTS AND DISCUSSION

The tonometer gas concentration (microliters per milliliter) in the vapor phase above the liquid samples *versus* the time (minutes) was followed graphically. It was determined during experimentation that 20 min was sufficient for equilibrium to occur. For presentation purposes, graphic data have been arranged to indicate the percentage of gas concentration remaining in the tonometer *versus* time (minutes). Figure 1 represents the gelatin–benzalkonium chloride coacervate system, and Fig. 2 represents the acacia–gelatin coacervate system.

Results (Table I) indicate that a significant uptake occurred with the intact coacervate system, considerably greater than the degree of solubilization observed in the dissolved or broken system. This significant uptake of halothane is probably indicative of

[2] Sources of the materials were as follows: WCLID 1670 model gas chromatograph, Warner-Chilcott Laboratories, Richmond, Calif.; columns, HCL Scientific Inc., Rockford, Ill.; and halothane (Fluothane), Ayerst Laboratories, New York, N.Y.

[3] Hamilton.

the presence of a highly structured, nonpolar system, similar to surfactant micelles in a polar medium.

Further ramifications are currently under study in these laboratories.

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ˣ To whom inquiries should be directed.

Electron-Capture GLC Determination of Blood Levels of 7-Chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one in Humans and Its Urinary Excretion as Lorazepam Determined by Differential Pulse Polarography

J. ARTHUR F. de SILVA ˣ, IHOR BEKERSKY, and MARVIN A. BROOKS

Abstract □ This report presents data to demonstrate the clinical utility of a previously published electron-capture GLC assay in the measurement of blood levels of 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one in humans following a single oral 4-mg dose. It also describes a differential pulse polarographic assay for the quantitation of its major urinary metabolite, lorazepam, in humans. This assay has greater sensitivity and specificity than the previously published Bratton–Marshall spectrophotometric assay.

Keyphrases □ 7-Chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one—electron-capture GLC determination of human blood levels □ Lorazepam—urinary metabolite of 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one, determined by differential pulse polarography □ GLC, electron capture—determination, 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one in human blood samples □ Differential pulse polarography—determination, lorazepam as urinary metabolite of 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one in humans

The 1,4-benzodiazepine class of compounds are clinically important as tranquilizers, hypnotics, and muscle relaxants (1). 7-Chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one (I) was synthesized by Sternbach *et al.* (2) and is currently undergoing clinical evaluation as an antianxiety agent. A sensitive and specific electron-capture GLC assay for the determination of I and its major metabolite, lorazepam (II), was published previously (3)[1].

This report presents data to demonstrate the clinical utility of the electron-capture GLC assay in the measurement of blood levels of I in humans following a single oral 4-mg dose. It also describes a differential pulse polarographic assay for the quantitation of lorazepam. The polarographic analysis of oxazepam and lorazepam in pharmaceutical formulations was described previously (4, 5).

EXPERIMENTAL

Clinical Protocol—A pilot blood level study was conducted in three subjects[2] following the administration of a single 4-mg oral dose (as 2 × 2-mg tablets), and whole blood specimens were collected as indicated in Table I. Urine specimens were collected as indicated in Table II. The oxalated blood specimens and urine specimens were stored frozen until analyzed.

Analysis of Specimens—Blood and urine levels of I were determined by the published electron-capture GLC assay (3), which has sensitivity limits of 0.5–1.0 ng/ml for I and of 4–5 ng/ml for II in blood or urine using a 15-mCi nickel-63 electron-capture detector. The major urinary metabolite in humans (lorazepam) is present mainly as a glucuronide conjugate (6). It is extracted from urine following deconjugation with glucuronidase–sulfatase and is analyzed by differential pulse polarography (Fig. 1), with a sensitivity limit of 50 ng/ml for II in urine using a 5-ml specimen/analysis.

Differential Pulse Polarographic Analysis of II—Lorazepam (II) is present in the urine mainly as a glucuronide and/or sulfate conjugate; small amounts of I are also excreted. The urine specimen is first extracted at pH 9.0 with ether[3], which quantitatively removes I and any unconjugated lorazepam, and is processed for electron-capture GLC analysis (3). The specimen is then titrated to pH 5.3, incubated with glusulase enzyme[4] (2% by volume) to deconjugate II, and then extracted into ether after adjusting the pH to 9.0 as previously described (3).

Internal standards of 200, 400, or 1000 ng of II are added to the control urine specimens prior to incubation and are carried through the entire procedure. After incubation, the samples are cooled to room temperature and the pH is adjusted to 9.0 using a pH meter by titrating the sample dropwise with 6 *N* NaOH. This

[1] Consult Fig. 1 and Table I in this reference for the chemical structures and nomenclature of Compounds I, II, and V.

[2] Conducted under the supervision of Dr. James D. Moore at the Deer Lodge Research Unit, Deer Lodge, Mont.

[3] Diethyl, absolute, analytical reagent grade, Mallinckrodt.

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[3] Diethyl, absolute, analytical reagent grade, Mallinckrodt.

[4] Endo Laboratories, Garden City, Long Island, N.Y.

Table I—Blood Levels[a] of *I* in Humans following a Single Oral Dose in a Tablet Formulation

Time	Subject 1 (68.1 kg), Dose of 0.059 mg/kg	Subject 2 (68.1 kg), Dose of 0.059 mg/kg	Subject 3 (73.6 kg), Dose of 0.054 mg/kg
	ng of I/ml of Blood		
15 min	2.0	1.8	0.7
30 min	4.0	6.0	3.4
1 hr	4.4	7.7	5.6
2 hr	4.8	15.5	5.9
4 hr	7.4	12.8	5.7

[a] Determined by electron-capture GLC analysis. Sensitivity limit = 0.5–1.0 ng of I/ml of blood.

solution is transferred quantitatively into a 50-ml centrifuge tube and extracted twice with 15 ml of ether by shaking for 10 min and then centrifuging for 5 min.

The ether extracts are combined in a 15-ml conical centrifuge tube by successive transfer and evaporation to dryness. The residues are solubilized with 100 μl of methanol, dissolved in 2 ml of 0.1 *N* HCl, and deaerated for 1 min with nitrogen bubbled through the sample with a sintered-glass (fritted) tube. Then the solution is transferred into a semimicropolarographic cell and analyzed by differential pulse polarography.

Conditions for Polarographic Analysis—A polarographic analyzer[5] equipped with a drop timer[6] was used in conjuction with a three-electrode semimicropolarographic cell comprised of a dropping mercury electrode, a saturated calomel electrode, and a platinum wire as the auxiliary electrode, as previously described (7). The drop time was 1.0 sec, and the drop rate was 2.74 mg/sec where $(m^{2/3})(t^{1/6}) = (1.958\ mg^{2/3})(sec^{-1/2})$.

The current range was set at 0.5 or 1 μamp for a peak response of full-scale deflection, the scan range was 1.5 v, and the scan rate

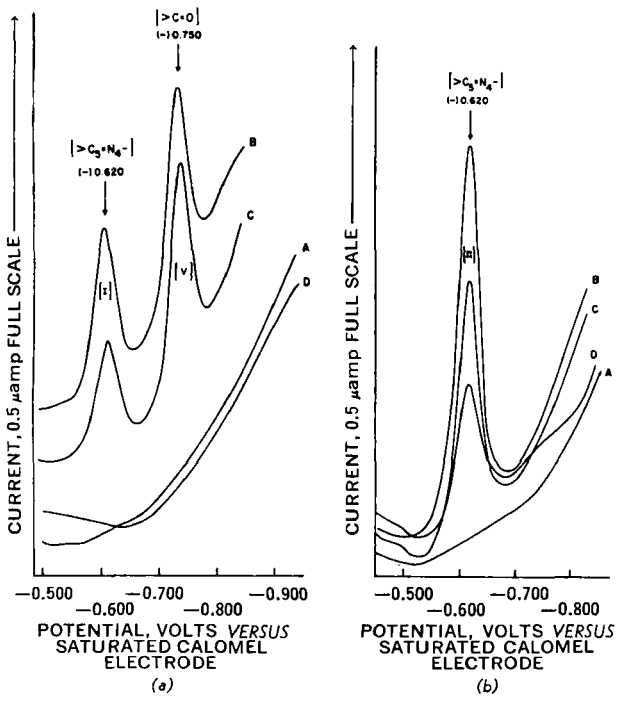
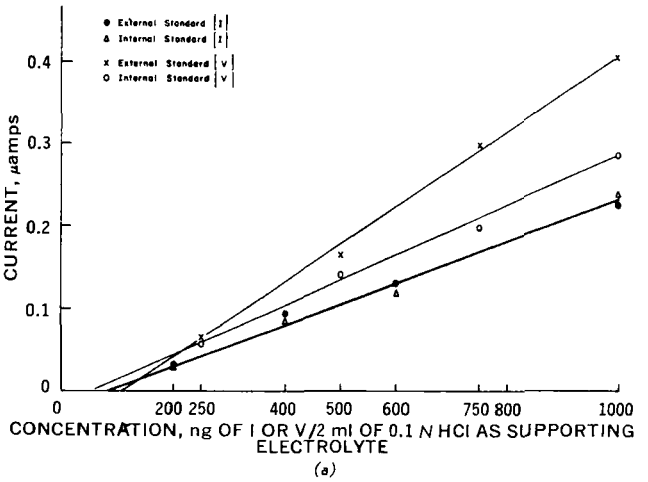


Figure 1—*Differential pulse polarograms:* (a) *Compound I and its benzophenone* (*V*), *and* (b) *II obtained in 0.1* N *HCl as the supporting electrolyte. Key: A, control urine blank; B, authentic reference standards; C, authentic standards recovered from urine; and D, patient urine, 0–6 hr postdosing fraction.*



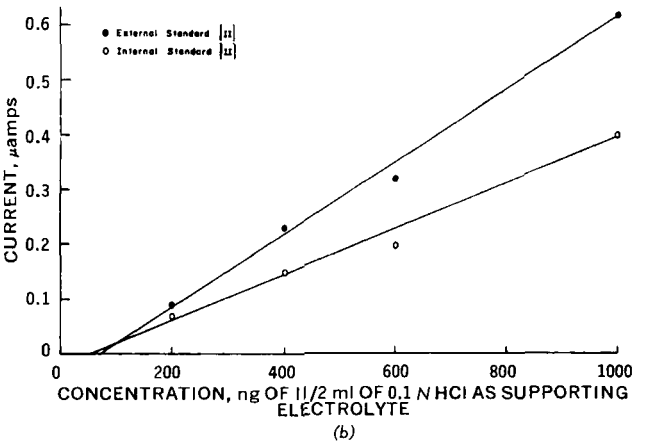


Figure 2—*Calibration curves for the differential pulse polarographic analysis:* (a) *Compound I and its benzophenone* (*V*), *and* (b) *II.*

was 2 mv/sec. The samples were scanned between −0.500 and −0.900 v *versus* the saturated calomel electrode and the polarograms were obtained on an X–Y recorder[7]. The analytical peak due to the reduction of the azomethine (>C_5=N_4—) group of II occurs at −0.620 v *versus* the saturated calomel electrode (Fig. 1*b*).

Calculations—The current (microamperes) resulting from II and the overall recovery of II are determined as described previously (8, 9). The concentration of II in the unknowns is determined by interpolation from the internal standard curve (Fig. 2*b*).

RESULTS AND DISCUSSION

Differential pulse polarography has been successfully applied to the determination of several classes of drugs in biological fluids (10) in their intact form such as benzodiazepines (7), nitroimidazoles (11), and pyrimidine-containing compounds (9); others such as glibornuride (a tolylsulfonylurea) (8), phenobarbital, and diphenylhydantoin (12) have been determined as their nitro derivatives. The 1,4-benzodiazepin-2-ones can be quantitated in the submicrogram range due to the ease of reduction of the azomethine group in dilute acids and the relatively high sensitivity (microamperes per microgram) achieved.

Consequently, I and II can be selectively extracted and quantitated with a minimum of cleanup because the coextracted impurities do not interfere in the potential region of interest. The use of a semimicropolarographic cell (7), with a minimum sample volume of 2 ml compared to that of 5 ml with the original cell (9), and the

[5] PAR model 174, Princeton Applied Research Corp., Princeton, N.J.
[6] PAR model 172A, Princeton Applied Research Corp., Princeton, N.J.
[7] Omnigraph model 2200-3-3, Houston Instruments, Bellaire, Tex.

Table II—Urinary Levels of I and II-Glucuronide in Humans

Excretion Period, hr	Subject 1		Subject 2		Subject 3	
	Total Excreted as I[a], μg	Total Excreted as II[b], μg	Total Excreted as I[a], μg	Total Excreted as II[b], μg	Total Excreted as I[a], μg	Total Excreted as II[b], μg
0–6	1.94	16.5	1.01	14.8	1.49	30.8
6–12	1.86	27.2	2.46	35.0	0.79	44.1
12–24	2.95	75.5	3.54	81.6	0.99	41.8

[a] Determined by electron-capture GLC as free or directly extractable drug. [b] Determined by differential pulse polarography after deconjugation with glucuronidase–sulfatase.

use of a longer drop time of 1 sec and a slower scan speed of 2 mv/sec have all increased the reduction current generated and, therefore, the overall sensitivity of the assay.

Both I and II can be quantitated in the 150–250-ng range by differential pulse polarography at a reduction potential (E_p) equal to −0.620 v *versus* the saturated calomel electrode, with an overall recovery of 95 ± 5.0% for I and 65 ± 5.0% for II, using ether as the extracting solvent.

The overall recovery of II can be increased significantly (>85%) by using either ethyl acetate or methylene chloride (dichloromethane); however, it is achieved at the expense of greatly increased impurities being coextracted with the compounds of interest and precludes direct polarographic analysis without extensive cleanup of the sample.

Differential pulse polarographic analysis of I and its benzophenone (V) as a mixture is possible because of the separation of the reduction peaks of the azomethine group of I at −0.620 v *versus* the saturated calomel electrode and of the carbonyl group of V at −0.750 v *versus* the saturated calomel electrode (Fig. 1*a*). They can be quantitated at concentrations greater than 150 ng of each/2 ml of 0.1 *N* HCl as the supporting electrolyte (Fig. 2*a*).

Blood specimens obtained over 4 hr following the oral administration of a single 4-mg dose of I were analyzed by electron-capture GLC (3) (Table I). The drug was measurable throughout the 15-min to 4-hr postdosing interval, demonstrating the clinical utility of the assay. No measurable level of unconjugated lorazepam (II) was seen in the blood at any time interval. 2-Amino-5,2′-dichlorobenzophenone (V) and 4-(2-chlorophenyl)-6-chloroquinazolin-2(1*H*)-one (IV) (3), which were reported to be minor metabolites of II in humans (13), were not detected by electron-capture GLC in this study.

The urinary excretion of I and II in humans was determined by electron-capture GLC and differential pulse polarographic analysis, respectively (Table II).

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[x] To whom inquiries should be directed.

Dose–Response Predictability of Urinary Bladder Hyperplasia by *N*-2-Fluorenylacetamide Feeding in Mice: Its Modification by Sex

THOMAS J. HALEY ˣ, G. SCHIEFERSTEIN, W. E. JAQUES, JOHN FARMER, C. FRITH, and R. W. SPRAWLS

Abstract □ A serial sacrifice experiment involving urinary bladder hyperplasia, produced by feeding 8–86 ppm of *N*-2-fluorenylacetamide for 90 days to BALB/c mice, showed a dose–response relationship for males but not females. The information from this study was used in a statistical design to predict the percent of hyperplasia in relation to time and dose. A formula for establishing the confidence limits of the curve was derived. Sex-dependent spontaneous pericardial fibrosis and mineralization were also observed, with the male response being twice that of the female.

Keyphrases □ *N*-2-Fluorenylacetamide—production of urinary bladder hyperplasia in mice, dose–response predictability, modification by sex □ Urinary bladder hyperplasia—*N*-2-fluorenylacetamide feeding in mice, dose–response predictability, modification by sex □ Hyperplasia, urinary bladder—effects of feeding *N*-2-fluorenylacetamide to BALB/c mice, dose–response predictability, modification by sex

Haley *et al.* (1) reported a dose–response-related urinary bladder hyperplasia in both sexes of C57BL/6 and BALB/c strain mice fed *N*-2-fluorenylacetamide at dietary concentrations ranging from 0.001 to 0.05%. Clayson *et al.* (2) previously reported a similar observation after feeding the chemical at a concentration of 0.03% for 2 weeks. However, only two of 12 C57 × IF male mice responded, and the hyperplastic index was 68 for the treated and 58 for the controls by 11 weeks. The development of urinary bladder hyperplasia has been reinvestigated utilizing larger numbers of animals and five different concentrations of *N*-2-fluorenylacetamide.

EXPERIMENTAL

BALB/c strain mice, 1680, equally divided between the sexes were used. The animals were maintained in quarters thermostatically regulated to 24° (72 ± 5°F) with free access to food and water. The mice weighed from 20 to 30 g. The sexes were randomized as to cages and racks. The groups were composed of 480, 480, 240, 160, and 80 animals, equally divided by sex and fed the dietary concentrations of *N*-2-fluorenylacetamide in meal[1] shown in Table I. *N*-2-Fluorenylacetamide dietary content was determined by the methods of Bowman and King (3). There were 240 controls.

Sacrifice periods were 1, 3, 6, 9, and 13 weeks from the start of the experiment, and 336 animals were sacrificed at each time interval. All animals were necropsied and the following tissues were examined microscopically after staining with hematoxylin–eosin: lungs, heart, aorta, thymus, skeletal muscle, kidneys, adrenal, liver, spleen, gallbladder, pancreas, cerebellum, spinal cord, stomach, colon, ileum, duodenum, lymph node, salivary glands, lacrimal gland, eye, harderian gland, thyroid, trachea, esophagus, skin, tongue, sternum, testes, epididymus, preputial gland, seminal vesicle, coagulating gland, ovary, uterus, mammary gland, urinary bladder, prostate, pituitary, and any unidentified mass. The hyperplastic index at 13 weeks was calculated by the method of Clayson *et al.* (2) (Fig. 1). Hyperplastic bladder epithelium is scored as follows: 5 points for six to eight cell layers, 3 points for three to five cell layers, and 1 point for doubtful hyperplasia.

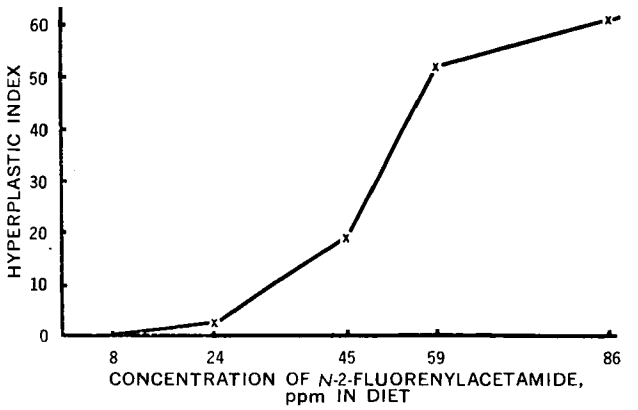

Figure 1—*Hyperplastic index for BALB/c male mice ingesting* N*-2-fluorenylacetamide in the diet for 13 weeks. Females gave indexes of 7.5 and 21.2 after ingesting 59 and 86 ppm, respectively, for the same time interval. Both sexes had a hyperplastic bladder epithelium of three to five cell layers, equivalent to a value of 3 points. The formula of Clayson* et al. *(2),* N/n, *where* N *is the total number points per group of animals and* n *is the number of mice per group, was used to calculate the plotted values.*

The urinary bladder hyperplasia response is of a quantal nature and was recorded for different dose levels and different sacrifice times. Therefore, it seemed appropriate to attempt to quantify this dose, response, and time relationship in a plane whose equation is:

$$Y = a + bX + cZ \qquad \text{(Eq. 1)}$$

when Y is the probit of the response, X is $\log_{10}$ (dose), and Z is $\log_{10}$ (time). The mathematics for such a model was discussed by Finney (4).

If one considers a fixed response (say 50% hyperplasia), then the resulting linear equation may be solved for dose for a given time or

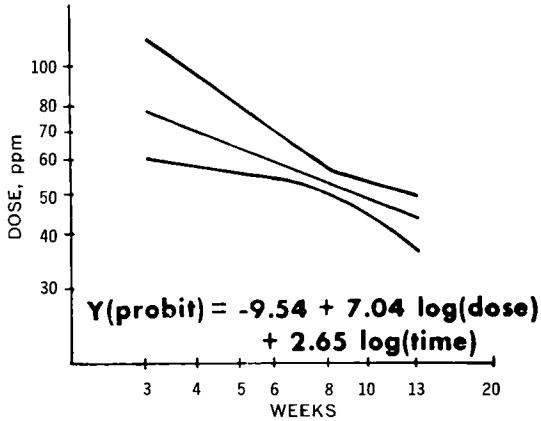

Figure 2—*A plot of* $5.00 = -9.54 + 7.04 \log (dose) + 2.65 \log (time)$ *with appropriate confidence limits on dose.*

[1] Purina.

Table I—Incidence of Urinary Bladder Hyperplasia in BALB/c Strain Mice Fed *N*-2-Fluorenylacetamide

Sacrifice Interval, weeks	Sex	Dose of *N*-2-Fluorenylacetamide, ppm					
		0	8	24	45	59	86
1	♂	0/24[a]	0/48	0/48	0/24	0/16	0/8
	♀	0/24	0/48	0/48	0/24	0/16	0/8
3	♂	0/23	0/47	0/48	1/24	2/16	6/8
	♀	0/24	0/48	0/48	0/24	0/16	0/8
6	♂	0/24	0/47	0/48	2/24	11/16	8/8
	♀	0/24	1/47[b]	0/47	0/24	0/16	0/8
9	♂	0/23	0/47	2/48	5/22	11/15	8/8
	♀	0/24	0/47	0/46	0/24	0/16	2/8
13	♂	0/24	0/46	3/44	7/23	13/15	8/8
	♀	0/24	0/47	0/47	0/24	2/16	3/8

[a] Number responding out of total number in group. [b] Animal had cystitis.

time for a given dose and confidence limits placed on either. This may be accomplished by considering the region of $(Y - Y_0) \geq t^2$ var (Y), where Y_0 is a fixed response and:

$$\text{var}\,(Y) = 1/Snw + X^2/Snwx^2 + 2XZ/SnwXZ + Z^2/SnwZ^2 \quad \text{(Eq. 2)}$$

$$X = X - \bar{X} \quad \text{(Eq. 3)}$$

$$Z = Z - \bar{Z} \quad \text{(Eq. 4)}$$

By replacing $Y = Y + bX + cZ$, expanding this, and taking the equality, one has a quadratic in X or Z. When fixing Z and solving the quadratic in X, the solution is:

$$X = \bar{X} + \frac{-B \pm \text{root}\,(B^2 - 4AC)}{2A} \quad \text{(Eq. 5)}$$

where:

$A = b^2 - t^2/SnwX^2$
$B = 2(bw - t^2Z/SnwXZ)$
$w = \bar{Y} + cZ - Y_0$
$C = (w^2 - t^2/Snw - t^2Z^2/SnwZ^2)$

and the terms are from the usual maximum likelihood probit solution.

If one then increments the time variable (Z), upper and lower confidence limits are produced for the dose variable (X). This may be repeated for any fixed response or contour (Y_0).

RESULTS

Table I shows the time sequence for the development of urinary bladder hyperplasia in BALB/c mice fed dietary concentrations of *N*-2-fluorenylacetamide ranging from 8 to 86 ppm. The first response was observed at 3 weeks and was seen only in the males. Moreover, the response became progressively greater with each sacrifice interval as this sex responded with increased frequency at lower dosage levels. Only at the two higher dosage levels and at the 13th-week interval was hyperplasia observed in the females. The one female that responded at 8 ppm at the 6th-week interval was suffering from acute cystitis, a condition that produces hyperplasia. The difference in response between the sexes might involve metabolic conversion of *N*-2-fluorenylacetamide to its *N*-hydroxy derivative, the proximate carcinogen (4), with the females showing a lower conversion rate.

There is apparently a critical level of *N*-2-fluorenylacetamide ingestion involving dosage rate, time, and total dose for the development of urinary bladder hyperplasia. Figure 2 indicates the interplay of these factors in the calculation of hyperplasia responses. The model from the *Experimental* section was fit to male hyperplasia data for 3–9 weeks and doses ranging from 24 to 86 ppm. The estimated model was:

$$Y\,(\text{probit}) = -9.54 + 7.04\,\log\,(\text{dose}) + 2.65\,\log\,(\text{time}) \quad \text{(Eq. 6)}$$

The model was an adequate representation of the data as expressed with the χ^2 goodness of fit with 13 *df*, $X_{13}^2 = 20.27$. The 95% confidence intervals on dose for the 50% response contour are shown in Fig. 2.

Other than urinary bladder hyperplasia, the only consistent histopathological findings were spontaneous pericardial fibrosis and mineralization, apparently unrelated to chemical ingestion because they occurred with equal frequency in the controls. The overall incidence was males 218/824 (26.4%) and females 115/829 (13.8%). All other tissues examined did not differ from the control tissues.

DISCUSSION

Reports of *N*-2-fluorenylacetamide inducing urinary bladder hyperplasia in mice have been confirmed (1, 2). In addition, it has been shown that the level below 300 ppm is equally effective in inducing this pathological change in the urinary bladder epithelium. The sex difference in response was similar to the observation of liver tumors in male but not in female mice (5).

Calcareous pericarditis was previously reported for several inbred strains of mice: DBA, C, CBA, C3H, A, and BALB/c (6–9). The calcified lesions varied from 77 to 94% for the first four strains, but figures were not given for the BALB/c strain. In contrast to the observation of a greater incidence of the lesion in male BALB/c mice, the other strains showed no sex difference.

The successful fitting of a model to the data for male hyperplasia gives hope that such a model may also be fit to tumor data and that the time factor can be given appropriate consideration in extrapolating to safe doses.

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[x] To whom inquiries should be directed.

Anticonvulsant Activity and Selective Inhibition of Nicotinamide Adenine Dinucleotide-Dependent Oxidations by 1,3,5-Trisubstituted Pyrazolines

S. P. SINGH *, A. CHAUDHARI *, JAYANTI P. BARTHWAL *, and SURENDRA S. PARMAR *‡x

Abstract □ Several 3-(3-acetoamino)phenyl-1,5-substituted phenyl-Δ^2-pyrazolines were synthesized and evaluated for their anticonvulsant activity. All substituted pyrazolines exhibited anticonvulsant activity, which was reflected by 30–80% protection observed against pentylenetetrazol-induced seizures. Most of these substituted pyrazolines inhibited selectively the *in vitro* oxidation of substrates requiring nicotinamide adenine dinucleotide (NAD dependent) by rat brain homogenates. In the present study, the anticonvulsant activity possessed by these substituted pyrazolines was found to bear no relationship with their ability to inhibit selectively the NAD-dependent oxidations.

Keyphrases □ Pyrazolines, 1,3,5-trisubstituted—synthesis, anticonvulsant activity, relationship to NAD-dependent oxidations □ Structure–activity relationships—pyrazolines–anticonvulsant activity, rats □ Anticonvulsant activity—1,3,5-trisubstituted pyrazolines □ Nicotinamide adenine dinucleotide-dependent oxidations—inhibition by 1,3,5-trisubstituted pyrazolines

Diverse pharmacological properties exhibited by pyrazole derivatives include tranquilizing, psychoanaleptic, muscle relaxant (1), hypnotic (2), and anticonvulsant (3, 4) activities. Furthermore, selective inhibition of nicotinamide adenine dinucleotide- (NAD) dependent oxidation of pyruvic acid and substrates of the tricarboxylic acid cycle by 2-methyl-3-*o*-tolyl-4-quinazolone (5, 6) possessing hypnotic (7) and anticonvulsant (8) properties led to the synthesis of some 1,3,5-trisubstituted pyrazolines.

In the present study, substituted pyrazolines were synthesized as possible anticonvulsants to investigate correlation between anticonvulsant activity possessed by these pyrazolines with their ability to inhibit the respiratory activity of rat brain homogenates as a basis of their biochemical mechanism of action.

EXPERIMENTAL[1]

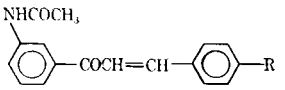
3-Acetoaminoacetophenone—This compound was prepared by heating 3-aminoacetophenone with acetic anhydride according to the method reported earlier (9).

Substituted Benzal-3-acetoaminoacetophenones—3-Acetoaminoacetophenone (0.022 mole) was gradually added, with continuous stirring, to a solution of sodium hydroxide (0.027 mole) in 12 ml of water and 20 ml of ethanol. To this mixture was added the appropriate aldehyde (0.022 mole) with stirring, and the temperature was maintained at 25°. The mixture was stirred vigorously until it resulted into a thick mass and the stirring was no longer effective, and it was kept overnight in a refrigerator.

The solid product which separated was filtered, washed with water until the washings were neutral to litmus and then with 20 ml of ice-cold ethanol, dried, and recrystallized from ethanol.

[1] *All compounds were analyzed for their carbon, hydrogen, and nitrogen content. Melting points were taken in open capillary tubes with partial immersion thermometer and are corrected.*

Table I—Physical Constants of Substituted Benzal-3-acetoaminoacetophenones



R	Melting Point[a]	Yield, %	Formula	Analysis, %	Calc.	Found
H	166°	80	$C_{17}H_{15}NO_2$	C	76.98	76.59
				H	5.66	5.33
				N	5.28	5.21
Cl	180°	77	$C_{17}H_{14}ClNO_2$	C	68.11	68.25
				H	4.67	4.68
				N	4.67	4.63
					4.67	
OCH_3	154°	85	$C_{18}H_{17}NO_3$	C	73.22	73.37
				H	5.76	5.82
				N	4.74	4.80

[a] All melting points were taken in open capillary tubes and are corrected.

These substituted benzal-3-acetoaminoacetophenones were characterized by their sharp melting points and elemental analyses (Table I).

3-(3-Acetoamino)phenyl-1,5-substituted Phenyl-Δ^2-pyrazolines—To a solution of a suitable phenylhydrazine (0.003 mole) in 10 ml of acetic acid was added the appropriate substituted benzal-3-acetoaminoacetophenone (0.003 mole), and the mixture was refluxed on a free flame for 3 hr. The resultant solution was cooled and diluted with water. The solid which separated was filtered, washed with water, dried, and recrystallized from ethanol. All pyrazolines (Table II) were characterized by their sharp melting points and elemental analyses.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined against pentylenetetrazol-induced convulsions in mice of either sex weighing between 25 and 30 g. The mice were divided into groups of 10, keeping the group weights as near the same as possible. All compounds were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compounds were administered in a dose of 100 mg/kg ip to a group of 10 animals.

Four hours after the administration of the test compounds, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol was shown to produce convulsions in almost all untreated mice, and the mice were also found to exhibit 100% mortality during 24 hr. No mortality was observed during 24 hr in animals treated with 100 mg/kg of the substituted pyrazolines alone.

The mice were observed 60 min for the occurrence of seizures. An episode of clonic spasm persisting for at least 5 sec was considered a threshold convulsion. Transient intermittent jerks and tremulousness were not counted. Animals devoid of threshold convulsions during the 60-min period were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of the substituted pyrazolines was represented as percent protection. The animals were then observed for 24 hr and their mortality was recorded.

Assay of Respiratory Activity of Rat Brain Homogenate[2]—

[2] Commercial chemicals were used in the present study. Sodium pyruvate, monosodium α-ketoglutarate, sodium β-hydroxybutyrate, NADH, sodium succinate, adenosine monophosphate (sodium salt), and cytochrome c were obtained from Sigma Chemical Co., St. Louis, Mo. Other common chemicals were obtained from the British Drug House, Bombay, India.

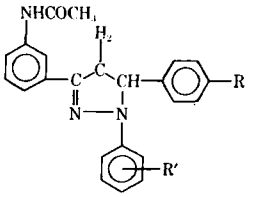


Table II—Physical Constants of 3-(3-Acetoamino)phenyl-1,5-substituted Phenyl-Δ^2-pyrazolines

Compound	R	R′	Melting Point[a]	Yield, %	Formula	Analysis, %	Calc.	Found
I	H	H	125°	72	$C_{23}H_{21}N_3O$	C	77.46	77.52
						H	5.91	5.88
						N	11.83	11.79
II	H	2-NO_2	100°	60	$C_{23}H_{20}N_4O_3$	C	69.00	69.04
						H	5.00	5.22
						N	14.00	14.30
III	H	4-NO_2	235°	76	$C_{23}H_{20}N_4O_3$	C	69.00	69.12
						H	5.00	5.08
						N	14.00	14.23
IV	H	2,4-$(NO_2)_2$	144°	68	$C_{23}H_{19}N_5O_5$	C	62.02	62.18
						H	4.27	4.31
						N	15.73	15.66
V	Cl	H	205°	80	$C_{23}H_{20}ClN_3O$	C	70.86	70.91
						H	5.13	5.22
						N	10.78	10.64
VI	Cl	2-NO_2	160°	63	$C_{23}H_{19}ClN_4O_3$	C	63.52	63.52
						H	4.37	4.34
						N	12.88	12.85
VII	Cl	4-NO_2	142°	78	$C_{23}H_{19}ClN_4O_3$	C	63.52	63.58
						H	4.37	4.41
						N	12.88	12.85
VIII	Cl	2,4-$(NO_2)_2$	165°	65	$C_{23}H_{18}ClN_5O_5$	C	57.56	57.53
						H	3.75	3.79
						N	14.60	14.68
IX	OCH_3	H	190°	80	$C_{24}H_{23}N_3O_2$	C	74.80	74.55
						H	5.97	5.89
						N	10.91	10.76
X	OCH_3	2-NO_2	156°	64	$C_{24}H_{22}N_4O_4$	C	66.98	66.92
						H	5.11	5.15
						N	13.02	13.27
XI	OCH_3	4-NO_2	250°	75	$C_{24}H_{22}N_4O_4$	C	66.98	66.89
						H	5.11	5.18
						N	13.02	13.00
XII	OCH_3	2,4-$(NO_2)_2$	140°	66	$C_{24}H_{21}N_5O_6$	C	60.63	60.68
						H	4.43	4.47
						N	14.73	14.55

[a] Melting points were taken in open capillary tubes and are corrected.

Male albino rats (100–150 g), kept on *ad libitum* diet, were used in all experiments: Rat brains isolated from decapitated animals were immediately homogenized in ice-cold 0.25 *M* sucrose in a homogenizer[3] in a ratio of 1:9 (w/v). All incubations were carried out at 37°, and the oxygen uptake was measured by the conventional Warburg manometric technique, using air as the gas phase (5).

Fresh brain homogenate, equivalent to 125 mg wet weight, was added to the chilled Warburg vessels containing 6.7 mmoles of magnesium sulfate, 20 mmoles of sodium hydrogen phosphate buffer solution (pH 7.4), 1 mmole of adenosine monophosphate (sodium salt), 33 mmoles of potassium chloride, and 500 μg of cytochrome c in a final volume of 3 ml unless otherwise stated. The central well contained 0.2 ml of 20% KOH solution. Pyruvate, α-ketoglutarate, β-hydroxybutyrate, and succinate were used at a final concentration of 10 m*M*, while the concentration of NADH was 0.5 m*M*. It was presumed that the endogenous NAD, present in the homogenate, was sufficient for these oxidative processes. All compounds under assay were dissolved in propylene glycol (100%), and an equal volume of the solvent was added to the control vessels.

RESULTS AND DISCUSSION

As evident from Table III, all pyrazolines possessed anticonvulsant activity and the degree of protection afforded by these compounds against pentylenetetrazol-induced convulsions ranged from 30 to 80%. Maximum protection was observed with compounds possessing 4-anisyl and 2,4-dinitrophenyl substituents at positions 1 and 5, respectively, of the pyrazoline nucleus (Compound XII). In general, pyrazolines possessing a 2,4-dinitrophenyl substituent at position 5 of the pyrazoline moiety (Compounds IV, VIII, and XII) possessed greater anticonvulsant activity. Introduction of a 4-chloro or 4-methoxy substituent on the phenyl nucleus attached to position 1 of the pyrazoline moiety (Compounds V–VIII and IX–XII) produced greater protection from pentylenetetrazol-induced seizures as compared to their respective unsubstituted 1-phenyl pyrazolines (Compounds I–IV).

Data on anticonvulsant activity of these pyrazolines and 24-hr pentylenetetrazol-induced mortality, in general, indicated a trend of some association between increased protection from convulsions and decreased pentylenetetrazol mortality in experimental animals. None of these pyrazolines exhibited any appreciable sedative or central nervous system-depressant effect or 24-hr mortality in a dose of 100 mg/kg as used in the present investigation.

All pyrazolines, except Compounds III, V, and IX, selectively inhibited NAD-dependent oxidation of pyruvate, α-ketoglutarate, β-hydroxybutyrate, and NADH by rat brain homogenates. On the other hand, NAD-independent oxidation of succinate remained unaltered. At present, it is difficult to explain the inability of Compounds III, V, and IX to affect NAD-dependent oxidations, and this property was not consistent with the nature of the substituents on the phenyl nuclei attached to positions 1 and 5 of the pyrazoline moiety. The ability of these pyrazolines to inhibit the oxidation of NADH provides evidence regarding the possible inactivation of the process of the electron transfer in the electron transport

[3] Potter–Elvehjem.

Table III—Anticonvulsant Activity and Inhibition of Respiratory Activity of Rat Brain Homogenate by 3-(3-Acetoamino)-phenyl-1,5-substituted Phenyl-Δ^2-pyrazolines

Compound	Anticonvulsant Activity[a], % Protection	Mortality[b] after 24 hr, %	Inhibition, %[c]			
			Pyruvate	α-Ketoglutarate	β-Hydroxybutyrate	NADH
I	30	50	37.0 ± 0.7	31.6 ± 0.6	46.5 ± 0.8	47.6 ± 0.6
II	40	30	59.0 ± 1.0	76.8 ± 0.8	61.6 ± 1.1	74.7 ± 0.8
III	30	60	Nil	Nil	Nil	Nil
IV	40	30	72.7 ± 0.5	75.3 ± 0.1	68.8 ± 1.1	69.9 ± 1.5
V	60	20	Nil	Nil	Nil	Nil
VI	60	Nil	70.7 ± 1.2	77.3 ± 0.9	84.2 ± 1.7	80.0 ± 1.8
VII	40	60	59.4 ± 0.9	61.1 ± 1.6	68.5 ± 0.3	56.2 ± 0.9
VIII	70	Nil	72.3 ± 1.3	75.3 ± 1.5	76.9 ± 0.9	62.3 ± 1.8
IX	40	40	Nil	Nil	Nil	Nil
X	50	20	39.3 ± 1.5	47.7 ± 1.2	66.8 ± 1.0	46.2 ± 1.2
XI	60	30	29.6 ± 0.9	14.5 ± 0.9	47.5 ± 1.1	34.8 ± 0.8
XII	80	10	50.2 ± 0.7	66.8 ± 1.3	79.8 ± 0.6	53.5 ± 0.7

[a] Screening procedures for the determination of anticonvulsant activity were described in the text. Substituted pyrazolines were administered at 100 mg/kg 4 hr before the administration of pentylenetetrazol. [b] Represents mortality during 24 hr in each group of animals administered pentylenetetrazol. [c] Each experiment was done in duplicate. All values represent mean values of percent inhibition with ± standard error of the mean from three separate experiments. Inhibition was determined by the decrease in the oxygen uptake/125 mg wet tissue weight/hr. The final concentrations of the various substrates, NADH, and substituted pyrazolines were 10, 0.5, and 1 m*M*, respectively. Vessel contents and assay procedures are described in the text.

chain by acting presumably at the site of transfer of electrons from NADH to flavine adenine dinucleotide. In the present study the selective inhibition of NAD-dependent oxidations by these pyrazolines was unrelated to their structure, so a definite structure–activity relationship was not exhibited (Table III).

These observations failed to provide any correlation between *in vitro* selective inhibition of NAD-dependent oxidations by these pyrazolines and their anticonvulsant activity. A detailed pharmacological and biochemical study using other purified enzyme systems may reflect the cellular basis for the anticonvulsant activity of these substituted pyrazolines.

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[x] To whom inquiries should be directed (at the University of North Dakota).

Chemistry and Biological Activity of *N*-Substituted Hydroxylamines

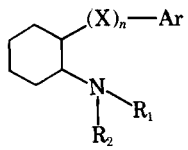
J. W. LEWIS ˣ, P. L. MYERS, J. A. ORMEROD, and C. F. C. SMITH

Abstract □ The synthesis and pharmacological properties of a novel series of *N*-substituted hydroxylamines are described. No useful CNS or cardiovascular activity was observed.

Keyphrases □ Hydroxylamines, *N*-substituted—synthesis and biological activity □ CNS activity—synthesis and screening of *N*-substituted hydroxylamines □ Cardiovascular activity—synthesis and screening of *N*-substituted hydroxylamines

Pharmacological activity was found in a series of cyclohexylamines (I) having either an aryl group or an aryl group linked through an oxygen or a carbon atom in the 2-position. 2-Phenyl-1-*N*-pyrrolidinocyclohexane (I*a*) is a member of a series of cholinesterase inhibitors (1) for which locomotor activity depression in mice has also been claimed (2). The aryloxycyclohexylamine series exemplified by I*b* displays antihistaminic, spasmolytic, and anesthetic properties (3, 4). The primary activity of the arylhydroxymethyl series (I*c*) is diuresis (5), whereas acetylated derivatives of I*c* show central nervous system (CNS) depressant activity (6).

The 2-substituted cyclohexylamines have now been extended by the synthesis of a series of arylhydroxylamine derivatives (I*d*) isosteric with I*c*. Since the Ar—N(OH)—C—C—N grouping was previously unknown, some open chain analogs of I*d* were also prepared for pharmacological screening.

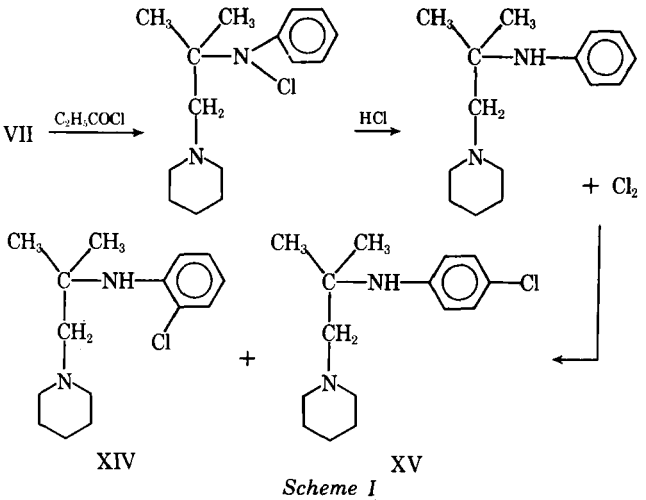


I*a*: Ar = C_6H_5; $(X)_n$ = O; $R_1 + R_2$ = —$(CH_2)_4$—
I*b*: Ar = C_6H_5; $(X)_n$ = O; $R_1 = R_2 = C_2H_5$
I*c*: Ar = 4-$OCH_3C_6H_4$; X_n = CH(OH); $R_1 + R_2$ = —$(CH_2)_5$—
I*d*: $(X)_n$ = N(OH)

CHEMISTRY

The *N*-substituted hydroxylamines (Table I) were prepared by condensation of nitrosobenzene or 2,6-dichloronitrosobenzene with the appropriate enamine, followed by reduction of this crude reaction product with sodium borohydride as previously described (7).

Attempts to convert some hydroxylamines to their *N*-acyloxy derivatives with the appropriate acid chloride or anhydride generally resulted in the isolation of a complex mixture of products. In the case of hydroxylamine (VII), reaction with propionyl chloride in refluxing chloroform gave the diamines XIV and XV. Formation of XIV and XV must occur by an analogous mechanism to that encountered in the Orton rearrangement (8) as shown in Scheme I. The trace of hydrochloric acid necessary to catalyze the reaction is presumably derived from partial hydrolysis of the acid chloride.



Scheme I

With methyl isocyanate, VIII gave the expected product (XVI), but the instability of this compound and related analogs prepared by the same method rendered them unsuitable derivatives for pharmacological screening.

The 2-arylhydroxylaminocyclohexylamines are believed to have the stereochemistry shown in XVII. This follows from consideration of the NMR spectrum of the crystalline diamine (XVIII) derived by hydrogenation of IV. This spectrum shows a narrow signal ($W_{1/2}$ = 10 Hz) for H_2, which eliminates the possibility of any 1,2-axial–axial interactions with adjacent protons and infers an axial configuration for the phenylhydroxylamino grouping. If it is assumed that the pyrrolidine substituent will have attained the more stable equatorial configuration in the formation of IV and hence, XVIII, the only possible arrangement of the two substituents is *cis*. An analogous synthesis of 1-*N*-pyrrolidino-2-phenylcyclohexane *via* the enamine derived from 2-phenylcyclohexanone has been shown[1] to give the *cis*-product by comparision with authentic samples of both the *cis*- and *trans*-isomers prepared by unequivocal routes.

DISCUSSION

All compounds were examined for CNS activity (9–15) and some for antihypertensive (16) and anticholinesterase (17) activity (Table II). Weak CNS depressant action was shown by II and VII, which potentiated barbiturate sleeping times at ED_{50}'s of 18 and 23 mg/kg, respectively, on oral administration in mice. Several hydroxylamines, at doses of 100 mg/kg, caused appreciable falls in blood pressure on intraperitoneal administration to rats in which hypertension had been induced by subcutaneous injections of desoxycorticosterone acetate (16). However, concurrent falls in heart rate were always observed and were sufficiently high in most cases to induce death in the animal subject.

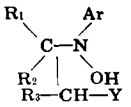
These results suggest that these substances exert their hypotensive action by a nonspecific smooth muscle and nervous depressant effect. Their anticholinesterase activity compared unfavorably with that of physostigmine, which produced 50% inhibition at 3.6×10^{-7} *M* under the same screening conditions (17).

EXPERIMENTAL

Melting points were determined with a hot-stage apparatus[2] and

[1] M. J. Readhead, Reckitt and Colman Pharmaceutical Division, 1973, personal communication.
[2] Kofler.

Table I—*N*-Arylhydroxylamines

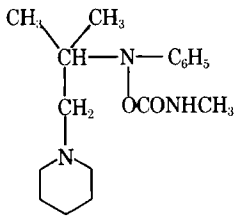


Compound[a]	R_1	R_2	R_3	Ar	Y	Method	Yield, %	Recrystallization Solvent	Melting Points (dec.)	Analysis, %	
										Calc.	Found
II	H	$-(CH_2)_4-$		C_6H_5	Morpholine	A	45	Ethanol–ether	218–221°	C 61.42	61.29
										H 8.05	7.68
										N 8.95	8.90
III	H	$-(CH_2)_4-$		C_6H_5	Piperidine	B	76	Ethanol	201–203°	C 65.68	65.45
										H 8.75	9.09
										N 9.01	8.63
IV	H	$-(CH_2)_4-$		C_6H_5	Pyrrolidine	B	17	Ethanol	219–220°	C 64.74	64.52
										H 8.48	8.61
										N 9.43	9.23
V	H	$-(CH_2)_4-$		C_6H_5	1-Methyl-piperazine	A	25	Methanol–ether	~230° (var.)	C 54.94[b]	55.22
										H 8.15	8.15
										N 11.32	11.57
VI	H	$-(CH_2)_4-$		$2,6\text{-}Cl_2C_6H_3$	Pyrrolidine	A	23	Ethanol–ether	~196° (var.)	C 52.55	52.46
										H 6.34	6.23
										N 7.66	7.83
VII	CH_3	CH_3	H	C_6H_5	Piperidine	B	92	Ethanol–ether	134.5–135.5°	C 72.54	72.77
										H 9.74	9.84
										N 11.28	11.28
VIII	CH_3	CH_3	H	C_6H_5	Pyrrolidine	B	86	Ether–light petroleum	112.5–113°	C 71.75	71.64
										H 9.46	9.65
										N 11.96	12.24
IX	CH_3	CH_3	H	$2,6\text{-}Cl_2C_6H_3$	Piperidine	A	17	Ethanol–ether	~265° (var.)	C 50.92	51.11
										H 6.56	6.52
										N 7.92	8.19
X	CH_3	CH_3	H	$2,6\text{-}Cl_2C_6H_3$	Pyrrolidine	A	37	Ethanol–ether	152–152°	C 49.50	49.32
										H 6.23	6.25
										N 8.25	8.25
XI	CH_3	H	H	C_6H_5	Piperidine	B	12	Ether–light petroleum	81–82°	C 71.75	71.71
										H 9.46	9.55
										N 11.96	12.05
XII	$-(CH_2)_5-$		H	C_6H_5	Morpholine	A	21	Ethanol–ether	185.5–187°	C 62.46	62.63
										H 8.32	8.30
										N 8.57	8.69
XIII	$-(CH_2)_5-$		H	C_6H_5	Piperidine	A	58	Ethanol–ether	174–175°	C 66.52	66.51
										H 8.99	8.92
										N 8.62	8.50

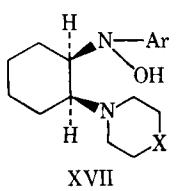
[a] All compounds are in the form of monohydrochloride salts except VII, VIII, and XI (free bases) and V (dihydrochloride salt). [b] Analyzed as hemihydrate.

are uncorrected. The structures of all compounds were assigned on the basis of compatible IR and NMR spectra.

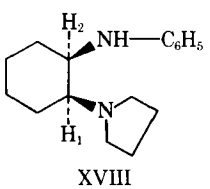
Hydroxylamines (Table I)—*Procedure A:* N-*(2-Morpholinocyclohexyl)phenylhydroxylamine (II)*—1-Morpholin-1-ylcyclohexene (16.7 g, 0.1 mole) in dry benzene (50 ml) was added over 20 min to a stirred suspension of nitrosobenzene (10.7 g, 0.1 mole) in dry benzene at 0–5°. The mixture was stirred at this temperature for a further 10 min and then allowed to warm up to room temperature. A color change from green to brown was observed, and the nitrosobenzene gradually dissolved as reaction took place. Stirring was continued for 10 min after all the nitrosobenzene had dissolved, and the solution was poured into an ice-cold mixture of sodium borohydride (3.8 g, 0.1 mole) in ethanol (50 ml). After standing for 15 hr, water (200 ml) followed by 2 *N* HCl was added. The solution was made alkaline with concentrated ammonium hydroxide and extracted with ether (2 × 100 ml). The extracts were combined, washed with water, dried (sodium sulfate), and evaporated; the residual oil was redissolved in ether and treated with ethereal dry hydrochloric acid until no further precipitation occurred. The precipitate was dried *in vacuo* and crystallized from ethanol–ether to give 9.0 g (45%) of II, mp 218–221°; IR ν_{max} (KBr): 3250 (N—OH) and 1595 (ArC=C) cm^{-1}.



XVI



XVII



XVIII

Procedure B: N-*[2-(2-Methyl-1-pyrrolidinopropyl)]phenylhydroxylamine (VIII)*—1-Pyrrolidin-1-ylisobutene (12.5 g, 100 mmoles) in dry toluene (50 ml) was added to a stirred suspension of nitrosobenzene (10.7 g, 0.1 mole) in dry toluene (50 ml) at −60°. The mixture was allowed to warm slowly in the cooling bath until all nitrosobenzene had reacted, and it was then poured into a suspension of sodium borohydride (3.8 g, 0.1 mole) in ethanol (50 ml) at −20°. Isolation (as in Procedure A) gave a white solid, which was recrystallized from ether–light petroleum, bp 40–60°, to give 20.4 g (86%) of VIII, mp 112.5–113°; IR ν_{max} (KBr): 3240 (N—OH) and 1596 (ArC=C) cm^{-1}.

2′ - Chloro - *N* - [2 - (2 - methyl - 1 - piperidinopropyl)]aniline (XIV) and Its 4′-Chloro Isomer (XV)—The hydroxylamine (VII) (5.7 g) and propionyl chloride (3.2 g) in chloroform (40 ml) were heated under reflux for 2.5 hr. The chloroform solution was washed with sodium bicarbonate and water and dried (sodium sulfate). Evaporation gave an oil which was chromatographed on alumina (Grade I). Elution with light petroleum (1:19) gave the diamine (1.01 g); NMR ($CDCl_3$): δ 1.29 (s, 6, $2CH_3$), 6.3–7.3 [m, 4, ArH, including a dd (1, J = 7 Hz, Ar 3H)]. The hydrochloride salt was recrystallized from ethanol–ether to give 0.7 g of XIV, mp

Table II—Comparative Pharmacological Data

Compound	CNS Tests, ED_{50}, mg/kg po Administration							Mean Fall in Blood Pressure[a], Desoxycorticosterone Acetate Intraperitoneal Administration	Anticholinesterase[b], % Inhibition
	Phenylquinone-Induced Writhing (Ref. 9)	Ataxia (Ref. 10)	Reserpinized Hypothermia (Ref. 11)	Strychnine Antagonism (Ref. 12)	Pentylenetetrazol Antagonism (Ref. 13)	Maximal Electroshock (Ref. 14)	Barbiturate-Induced Sleeping (Ref. 15)		
II	>300	>300	>100	>300	>300	>300	18	31% (100 mg/kg) cyanosis	9 (10^{-4} *M*)
III	>300	>300	>100	>300	>300	>300	~300	T[c] (100 mg/kg) 20% (10 mg/kg)	
IV	>100	>300	>100	>100	>100	>100	100		22 (10^{-4} *M*)
V	>100	>100	>100	>100	>100	>100	—	32% (100 mg/kg) cyanosis	
VI	>200	>200	>100	>200	>200	115	—		Inactive (10^{-4} *M*)
VII	>200	>200	>200	>200	>200	>200	23	Inactive (100 mg/kg)	13 (10^{-4} *M*)
VIII	>150	>150	>150	>150	>150	>150	—	T (100 mg/kg) 17% (10 mg/kg)	14 (10^{-4} *M*)
IX	>300	>300	>100	>300	>300	>300	—		
X	>100	>100	>100	>100	>100	>100	—	T (100 mg/kg)	15 (10^{-4} *M*)
XI	148	>200	>200	>200	>200	>200	>100		
XII	~300	>300	>300	>300	>300	>300	~100		9 (10^{-4} *M*)
XIII	86	>300	>300	>300	>300	>300	>100		

[a] Modified procedure to that described in Ref. 16. [b] Reference 17; concentration of acetylcholine is 0.01 *M*. [c] T = toxic.

166–166.5°. Elution with ether–light petroleum (2:3) gave the diamine (1.68 g); NMR ($CDCl_3$): δ 1.20 (s, 6, $2CH_3$) and 7.0 (A_2B_2q, 4, ArH). The hydrochloride salt (XV) was recrystallized from ethanol–ether (0.96 g), mp 167–168.5°.

***O*-Methylcarbamate of *N*-[2-(2-Methyl-1-pyrrolidinopropyl)]phenylhydroxylamine (XVI)**—The hydroxylamine (VIII) (1.5 g) and methyl isocyanate (0.7 g) in dry benzene (20 ml) were stirred at room temperature for 30 min. The residue, after removal of solvent, was crystallized from ether–light petroleum, bp 40–60°, to give XVI (1.38 g), mp varied dec. (Found: C, 65.85; H, 8.68; N, 14.71. $C_{16}H_{25}N_3O_2$ requires C, 65.95; H, 8.65; N, 14.42); IR ν_{max} (KBr): 3375 (NH), 1728 (C=O), and 1600 (ArC=C) cm^{-1}.

1-Anilino-2-pyrrolidinocyclohexane (XVIII)—The free base derived from the hydroxylamine (IV) (4.36 g, 0.016 mole) was hydrogenated in ethanol solution, using 10% palladium-on-charcoal (0.87 g) as the catalyst (hydrogen uptake 1.05 moles). After filtering and washing the catalyst with ethanol, the combined filtrates were evaporated under reduced pressure. The residue was crystallized from ethanol–water to yield 2.9 g (67%) of XVIII, mp 88–89° (Found: C, 78.71; H, 9.82; N, 1169. $C_{16}H_{24}N_2$ requires C, 78.63; H, 9.90; N, 11.46); IR ν_{max} (KBr): 3350 (NH) and 1602 (ArC=C) cm^{-1}.

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[x] To whom inquiries should be directed.

GLC Determination of Chlorpheniramine in Human Plasma

S. HANNA [x] and A. TANG

Abstract □ A specific and quantitative GLC method for the determination of chlorpheniramine in human plasma is described. The procedure involves the alkalinization of plasma samples followed by extraction with chloroform at pH 11. The sensitivity of the method is such that 0.1 μg of material can be detected in 1 ml of plasma. The method was used in a crossover study for comparative bioavailability of chlorpheniramine maleate from syrup formulations.

Keyphrases □ Chlorpheniramine—GLC determination in human plasma ■ GLC—analysis, chlorpheniramine in human plasma

Chlorpheniramine maleate is a widely used antihistaminic drug (1–3). Although it has been in use for many years, reports of its decomposition in animals (4), urinary excretion (5, 6), urinary metabolites (7), and blood levels in humans (8) have been appearing only recently.

Many sophisticated analytical methods have been published for the quantitation of the drug either *in vitro* from its pharmaceutical preparations or *in vivo* for its therapeutic and bioavailability evaluation. Among these are paper chromatography, TLC (8–10), spectrophotometry (5, 11), and fluorometry (8) procedures; however, these methods either lack the required specificity and sensitivity or are too elaborate and lengthy. Other GLC methods reported (12–14) were either not "adapted" for biological specimens or are time consuming.

This paper describes a sensitive and specific GLC method for chlorpheniramine determination in human plasma under conditions where only chlorpheniramine maleate was orally administered. The method was successfully applied in a crossover study

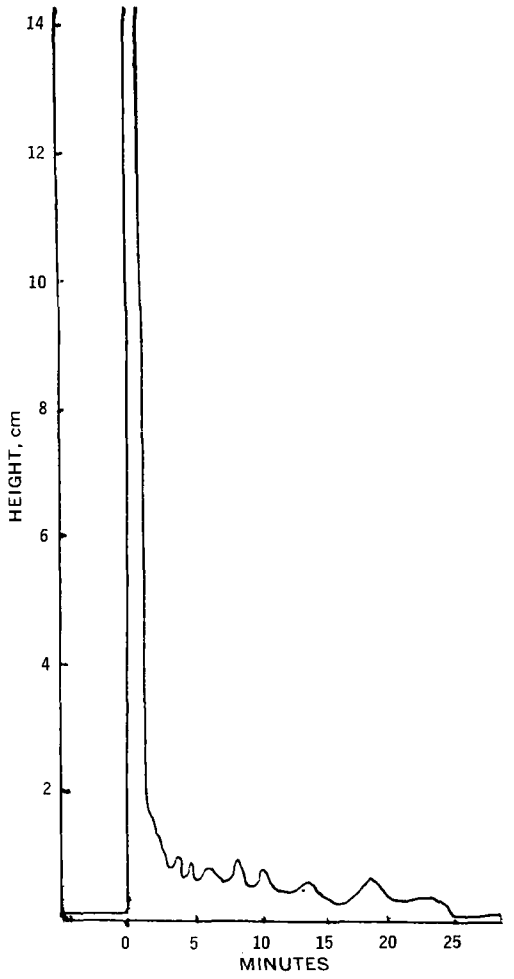

Figure 1—*Gas chromatogram showing plasma extract from Subject A before chlorpheniramine administration. Curve represents 2-μl injection of 50 μl of chloroform extract.*

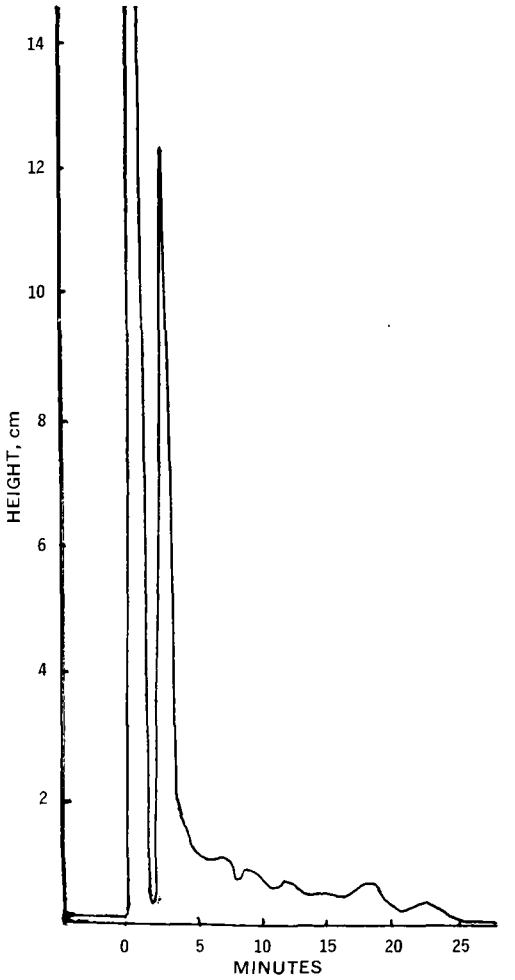

Figure 2—*Gas chromatogram showing plasma extract from Subject A plus 0.6 μg of chlorpheniramine maleate USP reference standard before chlorpheniramine administration. Curve represents 2-μl injection of 50 μl of chloroform extract.*

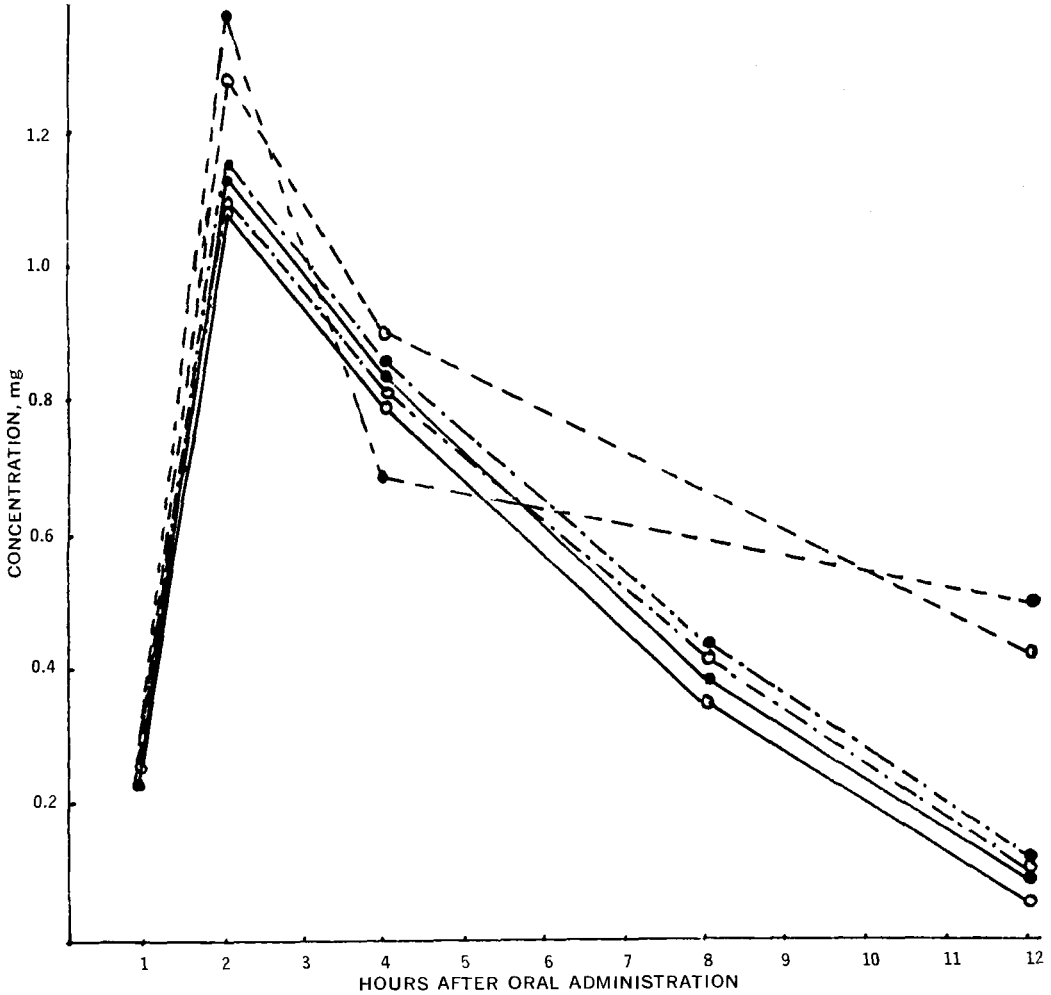

Figure 3—*Chlorpheniramine plasma levels after 4 mg oral administration. Key:* ○, *new formula, 4 mg chlorpheniramine maleate;* ●, *commercial product, 4 mg chlorpheniramine maleate;* ——, *Subject A;* – – –, *Subject B; and* ·–·–·, *Subject C.*

for comparative bioavailability of chlorpheniramine maleate from syrup formulations.

EXPERIMENTAL

Reagents and Chemicals—The reagents and chemicals used were: sodium hydroxide, chloroform, and sodium oxalate, all analyzed reagents[1]; SE-30[2], 60–80-mesh Chromosorb W DMCS[3]; and chlorpheniramine maleate USP reference standard.

Instrumentation—A gas chromatograph[4] equipped with dual hydrogen flame-ionization detectors was used for GLC analysis, using a 1.8 m (6 ft) long and 0.3 cm (0.125 in.) o.d. stainless steel column packed with 5% SE-30 on 60–80-mesh Chromosorb W DMCS. The following conditions were used: column temperature, 250°; injection port temperature, 270°; detector block temperature, 250°; hydrogen flow rate, 25 ml/min; air flow rate, 250 ml/min; and nitrogen carrier gas flow rate, 50 ml/min. Chromatograms were recorded on a 1-mv recorder[5].

Determination of Chlorpheniramine in Human Plasma—Three male Caucasian subjects, 27–38 years of age, in normal state of health as determined by prior physical and laboratory medical examination, were used. Each was administered an oral dosage of 8 ml of syrup (equivalent to 4 mg of chlorpheniramine maleate). At least 96 hr elapsed between the administration of the new formula and the commercial samples[6]. Two milliliters of blood was taken from each individual at 1, 2, 4, 8, and 12 hr after administration.

Prior to the administration of each syrup, an equal volume of blood was taken from each individual and extracted in the same way as the test samples. A portion of the chloroform extract of the normal plasma was used as a blank when injected alone into the gas chromatograph (Fig. 1), while the second portion was used for quantitative calculation. Two to three drops of 4% sodium oxalate aqueous solution were added to each blood sample and the plasma was immediately separated by centrifuging. The plasma was made alkaline (pH 11) by adding drops of 10% sodium hydroxide aqueous solution. The drug was extracted from the alkaline plasma with 2 × 15 ml of chloroform.

The chloroform extracts were pooled and evaporated to dryness under vacuum at 60° and the residue was dissolved in 50 μl of chloroform. Two microliters of this solution was injected into the gas chromatograph previously set up as mentioned. The injection technique of Rader and Aranda (15) was used to eliminate the need for an internal standard. Since the peaks obtained were sharp and narrow and their retention time was less than 5 min, calculations were based on peak heights.

The chlorpheniramine plasma level of the test blood samples was calculated by the volume of blood in the whole body to give a total plasma level of the body, using the equation of Wasserman *et al.* (16), for each determination.

The validity of this method was verified by adding various known amounts of chlorpheniramine maleate USP reference standard to the blank plasma. This plasma was extracted according to the described method, and 2 μl was injected into the gas chromatograph. A linear relationship was observed when peak heights were plotted *versus* amounts injected. The range of linearity was found to extend from 0.1 to 0.7 μg. Several samples of plasma taken at 1, 2, 4, 8, and 12 hr were pooled, extracted, and chromatographed.

The material obtained in the chlorpheniramine peak was

[1] J. T. Baker Chemicals Co.
[2] Varian Aerograph.
[3] Johns-Manville.
[4] Varian Aerograph model 204-IC.
[5] Honeywell Electronik-16.
[6] Chlor-Trimeton Syrup, Schering Corp., Bloomfield, NJ 17003

trapped and analyzed with a mass spectrophotometer[7]. The mass spectrum of the collected chlorpheniramine peak was identical with that obtained from a USP reference standard. The absence of the two mass peaks at *m/e* 44 and 30 in the mass spectrum supported the absence of the two known chlorpheniramine metabolite structures, *N*-monodesmethyl and *N*-didesmethyl, from the collected peak of the gas chromatograms (7).

A summary of the recovery results of chlorpheniramine obtained with human plasma is presented in Table I. A typical chromatogram is shown in Fig. 2.

RESULTS AND DISCUSSION

The quantity of chlorpheniramine found in the plasma of the three subjects after oral administration is shown in Table II. The pattern of chlorpheniramine plasma level is shown in Fig. 3.

There was a sharp rise in chlorpheniramine plasma levels from zero to 2 hr after the administration of 4 mg of the drug of both comparative samples and then a smooth, gradual decline from 2 to 12 hr (Figs. 4 and 5). No plasma samples were taken after this 12-hr sample period.

The total percentage of recovery of chlorpheniramine in plasma of each individual after the oral dosage compared favorably for both the newly developed formula and the commercial product samples.

The standard deviations of the plasma recovery of chlorpheniramine in three subjects compared favorably with the two samples (Table II). The *t* value for the *t* test of significance between the two samples was calculated with the pooled variance and found to be 0.1221. The degree of freedom in the pooled standard deviation was 28; in the *t* table (17), for $p = 0.05$, $df = 28$, *t* was given as 2.048. The calculated *t* was much less than this value. Therefore, it is concluded that there is no significant difference between the newly developed formula and the commercial sample taken as a standard in this investigation.

Table I—Recovery of Chlorpheniramine after *In Vitro* Addition to Human Plasma

Added, μg/ml	Mean Recovery[a], μg/ml	± *SD*
1	1.10	±0.25
3	2.99	±0.248
5	4.99	±0.303
7	7.12	±0.286

[a] Average of five determinations.

CONCLUSIONS

A specific and sensitive GLC method for the determination of chlorpheniramine in human plasma was presented. When used in a two-way crossover bioavailability study on plasma samples of the subjects under test, the method showed that the drug was easily absorbed into the blood from its oral syrup formulations at a dose level of 4 mg. The difference between the bioavailability results of

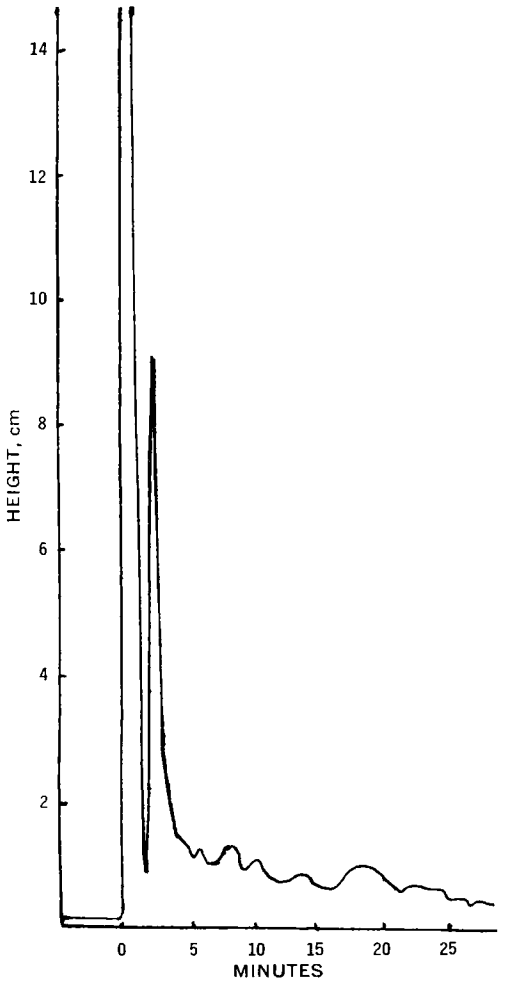

Figure 4—*Gas chromatogram showing plasma sample for Subject A 2 hr after oral administration of the new formula. Curve represents 2-μl injection of 50 μl of chloroform extract.*

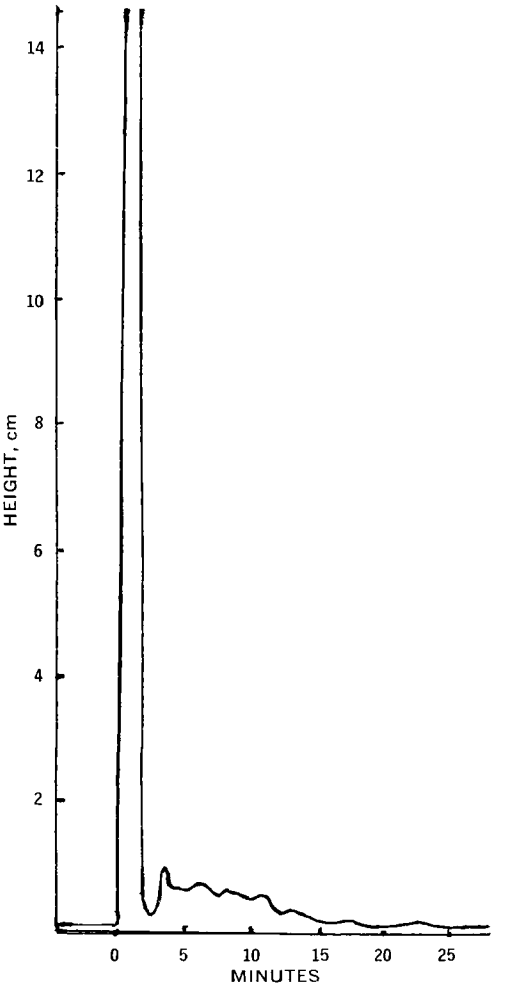

Figure 5—*Gas chromatogram showing plasma sample for Subject A 12 hr after oral administration of the commercial product. Curve represents 2-μl injection of 50 μl of chloroform extract.*

[7] Associated Electrical Industry mass spectrometer 902.

Table II—Chlorpheniramine Plasma Levels after 4 mg Oral Administration

Subject[a]	Sample[b]	Concentration, μg/2 μl[c] 1 hr	2 hr	4 hr	8 hr	12 hr	Amount, mg, in Whole Body Plasma in 12 hr[d]	Total Recovery, %	Standard Deviation of Three Subjects	t^e Value
A	a	0.1087	0.4414	0.3268	0.1400	0.0280	2.567	64.4	a ± 0.150	0.1221
38, 79	b	0.1129	0.4500	0.3354	0.1511	0.0425	2.688	67.2	b ± 0.153	0.1221
B	a	0.0780	0.3690	0.2565	—[f]	0.1185	2.836	70.9	a ± 0.150	0.1221
27, 110	b	0.0720	0.3948	0.1995	—[f]	0.1443	2.796	69.9	b ± 0.153	0.1221
C	a	0.1087	0.4421	0.3112	0.1575	0.0488	2.636	65.9	a ± 0.150	0.1221
33, 78	b	0.1125	0.4579	0.3431	0.1684	0.0521	2.792	69.8	b ± 0.153	0.1221

[a] The first number is for age (years), and the second number is for body weight (kilograms). [b] a = new formula, and b = commercial product. [c] Obtained by calculation from the reading values of that of USP reference standard. [d] Obtained by calculating the volume of blood in the whole body to give a total plasma level of body. [e] t Test of significance where $t = (\bar{x}_1 - \bar{x}_2)/S\sqrt{(n_1n_2/n_1 + n_2)}$; t value calculated with the pooled variance of the two syrup samples. [f] No blood sample was taken.

the newly developed formula and the commercial product taken as a standard in this investigation was insignificant.

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[x] To whom inquiries should be directed. Present address: Endo Laboratories, Garden City, NY 11530

Time-Dependent Effect of Oral Procainamide in Patients with Exertional Arrhythmias

Keyphrases □ Procainamide, oral—time-dependent effect, patients with exertional arrhythmias □ Arrhythmias, exertional—time-dependent effect of oral procainamide □ Antiarrhythmic agents—time-dependent effect of oral procainamide in patients with exertional arrhythmias

To the Editor:

The direct translation of procainamide pharmacokinetic data such as absorption and elimination half-lives into practical dosage regimen recommendations involves the assumption that procainamide is reversibly acting, *i.e.*, its antiarrhythmic effect is directly related to its presence in plasma (1). Studies (2–4) have established effective plasma concentration ranges of 4–8 and 4–10 μg/ml in hospitalized patients with acute myocardial infarction or coronary artery disease.

In a more recent study, Gey *et al.* (5) studied the relationship between procainamide plasma level and prevalence of *exertional* arrhythmias in ambulatory patients and established that the same plasma level range, 4–8 μg/ml, was effective also in suppressing most arrhythmias. The drug was administered in single oral doses and its antiarrhythmic effect was assessed before and 1 hr after drug administration, during maximal treadmill exercise testing, and during the immediate recovery period.

Nine patients performed a third exercise test at 2.5 hr, after receiving a 15-mg/kg dose, to assess the time dependency of the antiarrhythmic effect. In four patients there was a tendency for arrhythmias to reappear as plasma levels decreased between 1 and 2.5 hr, but this effect was not statistically significant (5). The phenomenon of time dependency was pursued further, and the additional information obtained to date is presented here.

Six patients who exhibited only partial suppression of arrhythmias at the 1-hr exercise test, following a 15-mg/kg dose, were tested again using a 22.5-mg/kg dose with exercise tests before and at 1.0 and 2.5 hr after drug administration. The methods of ECG recording and analysis as well as of procainamide plasma determination were the same as those previously described (5). The individual patient data are shown in Table I.

When mean plasma concentration rose from 0 to 8.86 μg/ml at 1 hr, the mean prevalence of arrhythmia dropped from 164 to 15 ($p < 0.05$). The corresponding decrease in the severity index was from 12.8 to 5.8 ($p < 0.01$). All patients exhibited more than 78% reduction in prevalence of arrhythmia, but only one (C. F.) showed total suppression.

Of greater significance, when mean plasma levels dropped to 6.93 μg/ml at 2.5 hr, the corresponding arrhythmia count increased to a mean value of 48, a statistically significant increase ($p < 0.016$). In other words, between 1 and 2.5 hr, a 20% decrease in plasma level was accompanied by more than a 200% increase in arrhythmia count. Similarly, the severity index increased by 32%. An examination of individual patient data shows that there was only one exception to this pattern (H. E., severity data).

These results lend support to the hypothesis that, in the patients studied, procainamide is reversibly acting against *exertional* arrhythmias during the single-dose *oral* administration. (The possible role played by metabolites cannot be assessed in view of the total lack of quantitative kinetic information.)

These findings have several therapeutic consequences:

1. When only temporary protection from exertional arrhythmias is required, *e.g.*, joggers, a single oral dose (15 or 22.5 mg/kg) might be sufficient.
2. If protection is required on a 24-hr basis (patients exhibiting similar arrhythmias at rest), the short biological half-life of procainamide, 2.5–4.5 hr (2, 3), would require short dosing intervals (3–4 hr).
3. The approach of a standard maximal treadmill exercise test (6) in conjunction with plasma level

Table I—Plasma Concentrations, Prevalence of Arrhythmia[a], and Severity following a 22.5-mg/kg Oral Dose of Procainamide

	Control		1 hr			2.5 hr		
Patient	Prevalence of Arrhythmia	Severity Index	Prevalence of Arrhythmia	Severity Index	Plasma Concentration, μg/ml	Prevalence of Arrhythmia	Severity Index	Plasma Concentration, μg/ml
A. G.	137	15	29	11	8.89	55	11	6.24
C. F.	55	13	0	0	8.67	5	2	7.37
H. E.	113	12	16	10	8.85	41	8	6.05
O. N.	79	9	6	5	7.91	26	9	7.21
R. L.	467	15	24	4	11.73	91	9	8.25
H. J.	133	13	16	5	7.13	70	7	6.48
Mean ± *SD*	164 ± 152	12.8 ± 2.2	15 ± 11	5.8 ± 4.1	8.86 ± 1.56	48 ± 31	7.7 ± 3.1	6.93 ± 0.83

[a] Number of premature ventricular beats during exercise and the immediate recovery period.

measurements provides an objective and rapid method of optimizing dosage regimens of procainamide for individual patients.

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Rene H. Levy [x]

Department of Pharmaceutical Sciences
School of Pharmacy
University of Washington
Seattle, WA 98195

George O. Gey
Robert A. Bruce

Division of Cardiology
School of Medicine
University of Washington
Seattle, WA 98195

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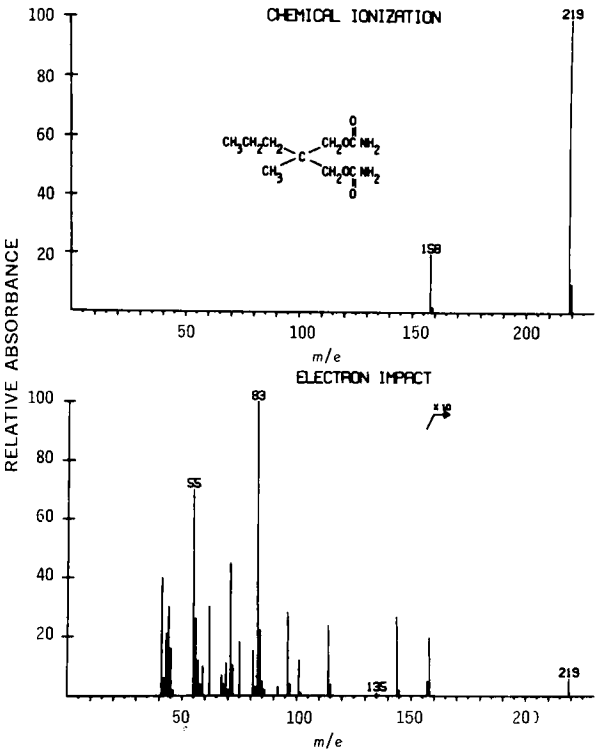

Figure 1—*Comparison of the normalized electron-impact and chemical ionization mass spectra of Compound Ia. All other dicarbamates listed in Table I gave similar spectra.*

Combined Application of High-Resolution Chemical Ionization and Electron-Impact Mass Spectrometry to Medicinal Dicarbamates

Keyphrases □ Mass spectrometry, electron impact and high-resolution chemical ionization—application to medicinal dicarbamates □ Dicarbamates, medicinal—combined application of high-resolution chemical ionization and electron-impact mass spectrometry

To the Editor:

A recent report on the application of field desorption mass spectrometry to a series of medicinal dicarbamates to determine the molecular weight (1) prompts us to communicate earlier results on the application of chemical ionization mass spectrometry to a series of structurally similar dicarbamates (2).

Several electron-impact mass spectral studies of monocarbamates have appeared (3–7). It has been pointed out that, in addition to having relatively predictable spectra, the volatility of carbamates, the small amounts of sample required for analysis, and the wealth of structural information contained in the spectra make mass spectrometry an excellent tool for structural elucidation (3). In addition, it was noted that relatively obvious molecular ions were obtained and that no recombination peaks were detected (3).

When a series of 10 dicarbamates having the general structure, I (Scheme I), were examined by high-resolution electron-impact mass spectrometry, spectra rich in readily interpretable fragment ions were obtained but molecular ions were conspicuously absent. Instead, peaks of less than 1% relative abundance were observed at *m/e* (M + 1) in all instances. Due to the difficulty in obtaining high-resolution data on the very weak M + 1 ions along with the desirability of directly determining the molecular formula[1], an investigation of the chemical ionization mass spectrometry was undertaken.

In an effort to minimize fragmentation, the combination of a low inlet temperature of 100°[2] and a mild chemical ionization reagent, isobutane, was used. In all chemical ionization (C.I.) spectra, the MH^+ ion was the most intense ion, with the only significant fragment ion arising from the direct loss of carbamic acid from the MH^+ ion[3]. Fast-scan high-resolution data were readily obtained.

A comparison of the spectra obtained in the chemical ionization and electron-impact modes for Ia [R_1 = CH_3, R_2 = $CH_2CH_2CH_3$ (meprobamate)] is given in Fig. 1 and is typical of all dicarbamates investigated.

[1] With only low-resolution data, the recombination M + 1 ion could be misinterpreted as the molecular ion.
[2] Dicarbamates are susceptible to thermal decomposition at higher inlet temperatures.
[3] The direct loss was established from metastable ions.

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Rene H. Levy [x]

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School of Pharmacy
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Seattle, WA 98195

George O. Gey
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Division of Cardiology
School of Medicine
University of Washington
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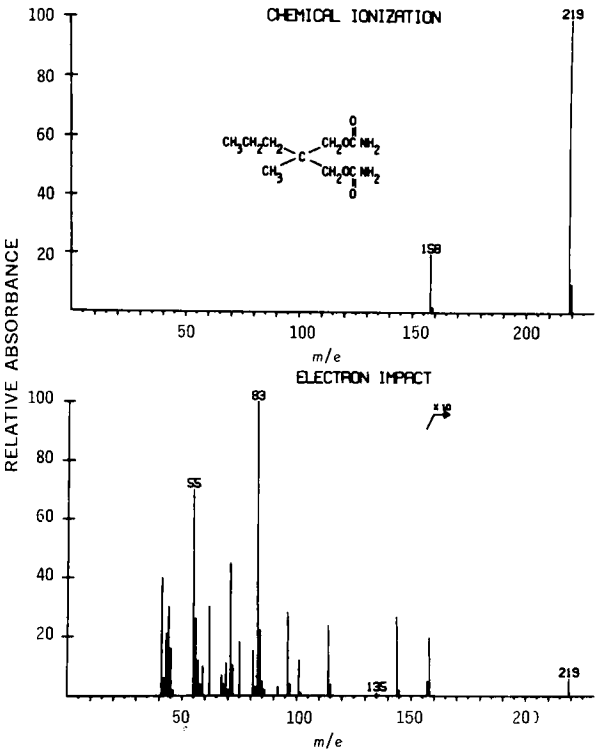

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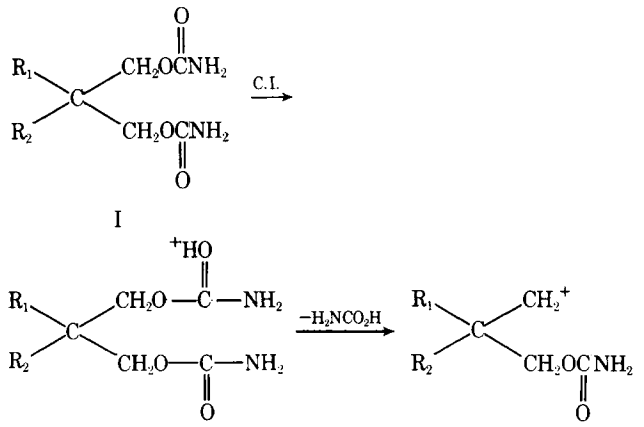
[3] The direct loss was established from metastable ions.

Table I—High-Resolution Chemical Ionization[a,b] Mass Spectral Data for MH^+ Ions

R_1	R_2	Elemental Composition	Theoretical	Found	Relative Intensity: Chemical Ionization	Relative Intensity: Electron Impact[c]
CH_3	$CH_3CH_2CH_2$	$C_9H_{19}N_2O_4$	219.1344	219.1361	100%	0%
CH_3	$ClCH_2CH_2CH_2$	$C_9H_{18}ClN_2O_4$	253.0954	253.0925	100%	0%
$CH_3CH_2CH_2$	$ClCH_2$	$C_9H_{18}ClN_2O_4$	253.0954	253.0970	100%	0%
CH_3CH_2	$ClCH_2$	$C_8H_{16}ClN_2O_4$	239.0815	239.0798	100%	0%
$CH_2=CHCH_2$	H	$C_8H_{15}N_2O_4$	203.0998	203.1031	100%	0%
$CH_3CH_2OCH_2$	$CH_2=CHCH_2$	$C_{11}H_{21}N_2O_5$	261.1445	261.1440	100%	0%
$CH_3OCH_2CH_2$	CH_3	$C_9H_{19}N_2O_5$	235.1293	235.1276	100%	0%
CH_3OCH_2	CH_3OCH_2	$C_9H_{19}N_2O_6$	251.1238	251.1229	100%	0%
$CH_3CH_2OCH_2$	CH_3	$C_9H_{19}N_2O_5$	235.1293	235.1301	100%	0%
$CH_3OCH(CH_3)$	CH_3	$C_9H_{19}N_2O_5$	235.1293	235.1287	100%	0%

[a] 500 ev. [b] Perfluorokerosene-H was used as an internal standard for exact mass measurements. [c] Molecular ion, M^+.



I

Scheme I

High-resolution data were obtained in both modes, so the combined use of the two modes provides a very potent method for structural elucidation of dicarbamates. The electron-impact spectra are rich in readily interpretable fragment ions from which much detailed structural information can be derived, while the high-resolution chemical ionization mode allows an unequivocal determination of the molecular formula. In Table I, the exact measured mass of all MH^+ ions is listed and compared with the theoretical mass.

Although the application of field desportion mass spectrometry to dicarbamates allows the determination of an unequivocal molecular weight (1), chemical ionization mass spectrometry offers a distinct advantage in the precise determination of molecular formula and ease of application[4].

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[4] High-resolution data were not obtained by Rouse and Brent (1). At present, considerable effort and time are required to obtain field desorption mass spectra.

George L. Nelson [x]

Department of Chemistry
Saint Joseph's College
Philadelphia, PA 19131

Charles F. Kuhlman
T. L. Chang

Wyeth Institute for Medicinal Research
Radnor, PA 19087

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[x] To whom inquiries should be directed.

Powder Mixing by Frictional Pressure: Specific Example of Use of Ordered Mixing

Keyphrases □ Powder mixing—frictional pressure, example of use of ordered mixing □ Mixing, powder—frictional pressure, example of use of ordered mixing □ Dissolution rate—effect of ordered mixing

To the Editor:

A recent study (1) reported the effect of different methods of preparing triturations of either digoxin or hydrocortisone with lactose on the dissolution rates of these drugs. It was demonstrated that frictionally deposited drug, *i.e.*, where the drugs were spread over the surface of the diluent by frictional pressure in a mortar and pestle, gave the highest dissolution rate. The other trituration methods investigated were simple blending by bottle tumbling, simple blending with ground drug, and solvent deposition.

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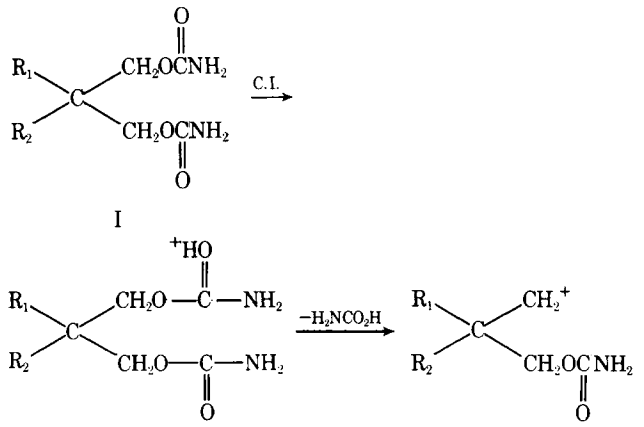

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High-resolution data were obtained in both modes, so the combined use of the two modes provides a very potent method for structural elucidation of dicarbamates. The electron-impact spectra are rich in readily interpretable fragment ions from which much detailed structural information can be derived, while the high-resolution chemical ionization mode allows an unequivocal determination of the molecular formula. In Table I, the exact measured mass of all MH^+ ions is listed and compared with the theoretical mass.

Although the application of field desportion mass spectrometry to dicarbamates allows the determination of an unequivocal molecular weight (1), chemical ionization mass spectrometry offers a distinct advantage in the precise determination of molecular formula and ease of application[4].

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(3) C. P. Lewis, *Anal. Chem.*, **36**, 1582(1964).

(4) *Ibid.*, **36**, 176(1964).

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[4] High-resolution data were not obtained by Rouse and Brent (1). At present, considerable effort and time are required to obtain field desorption mass spectra.

George L. Nelson [x]

Department of Chemistry
Saint Joseph's College
Philadelphia, PA 19131

Charles F. Kuhlman
T. L. Chang

Wyeth Institute for Medicinal Research
Radnor, PA 19087

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Accepted for publication September 11, 1974.
Adapted in part from the M. S. thesis of C. F. Kuhlman.
[x] To whom inquiries should be directed.

Powder Mixing by Frictional Pressure: Specific Example of Use of Ordered Mixing

Keyphrases □ Powder mixing—frictional pressure, example of use of ordered mixing □ Mixing, powder—frictional pressure, example of use of ordered mixing □ Dissolution rate—effect of ordered mixing

To the Editor:

A recent study (1) reported the effect of different methods of preparing triturations of either digoxin or hydrocortisone with lactose on the dissolution rates of these drugs. It was demonstrated that frictionally deposited drug, *i.e.*, where the drugs were spread over the surface of the diluent by frictional pressure in a mortar and pestle, gave the highest dissolution rate. The other trituration methods investigated were simple blending by bottle tumbling, simple blending with ground drug, and solvent deposition.

In view of the fact that the trituration prepared using spreading by frictional pressure gave the highest dissolution rate, it was concluded that this method of powder mixing was worthy of further study. Since frictional forces are used at various stages in the preparation of dosage forms in milling, blending, slugging, granulating, and tableting, an explanation of batch variations in the dissolution rates of tablets and capsules may be forthcoming from such a study.

Where a film of drug is spread over the surface of the diluent material, some degree of order is being introduced to the powder mixture. This is different from the random mixing of noncohesive powders, from which powder mixing theory has been developed. To distinguish between these two forms of powder mixing, the terms random mixing (for the mixing or shuffling of noncohesive particles) and ordered mixing (for the mixing, spreading, or coating by cohesive particles) have been used (2).

Random mixing has been more widely studied due to the ease of handling systems of noncohesive particles, whereas ordered mixing probably occurs widely in actual powder mixing practice, where cohesive particles usually have to be employed. This is particularly the case in pharmaceutical systems requiring a high degree of homogeneity and high dissolution rates, both of which can only be satisfied by the use of fine cohesive particles.

Just as the completely randomized system in random mixing is only approached in practice (3–5), in ordered mixing the completely ordered system would be difficult to attain. It would require homosized diluent particles with a layer of equal thickness (or an equal number of homosized particles) of drug. Ordered mixing requires the use of cohesive or spreadable material and, for most materials, is dependent upon particle size. For large noncohesive particles, mixing is predominantly random. As the particle size is reduced, the cohesiveness, as measured by angle of repose, flow through an orifice, shear cell, *etc.*, increases until the mixing is predominantly by the ordered mechanism. The transition occurs in the region of 100 μm, depending on the hardness and surface properties of the material.

The standard deviation of the theoretical, completely randomized binary mixture, σ_R, decreases with increasing sample size, M, according to:

$$\sigma_R = \left(\frac{xyw}{M}\right)^{0.5} \qquad \text{(Eq. 1)}$$

where x and y are the proportions of the two homosized ingredients of particle weight w. In contrast, the completely ordered mixture would have zero standard deviation until the sample weight, M, was reduced to such an extent as to contain less than one single particle of diluent and associated drug adhering or spread onto it.

Of the methods used for the formation of the digoxin–lactose and hydrocortisone–lactose triturations (1), the solvent deposition method may also be classified as an ordered process, since a thin film of drug should be deposited at the surface. The success or failure of this method to produce improved dissolution rates depends largely on the drying conditions and solution migration. Thus, a good dispersion may be obtained, such as aspirin deposited on a lactose–starch mixture, resulting in increased dissolution rates (6).

It is apparent that both decreased particle size and greater dispersion of the drug with the diluent are required for increased dissolution rates. Ordered mixing may give better results than random mixing, but only at the cost of the increased energy input required to bring about this degree of order. The use of frictional pressure to induce spreading of the drug over the diluent is one example of ordered mixing, where with extra energy input, more efficient dissolution rates have been obtained.

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John A. Hersey

School of Pharmaceutics
Victorian College of Pharmacy
381 Royal Parade, Parkville
Victoria, 3052, Australia

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Particle-Size Requirements Related to Content Uniformity of Solid Dosage Forms

Keyphrases □ Particle size—requirements related to content uniformity of solid dosage forms, particle-size limit compared to particle-size distribution □ Content uniformity, solid dosage forms—particle-size limit and particle-size distribution discussed

To the Editor:

In a recent publication (1), the particle-size requirements to achieve satisfactory content uniformity in a compound tablet were discussed. An example was given for a three-component system containing 50, 5, and 0.5% of the respective drugs with the requirement that these percentages be present in the final 100-mg tablet within ±10% of the nominal values at the 99.7% probability level. The resultant σ values related to the tolerance range of ±10% were

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wrongly stated, and the subsequently evaluated particle-size requirements based on randomized mixtures are consequently in error.

Using the same notation (1), the system containing the three components P, Q, and R present in a 100-mg tablet at the 50, 5, and 0.5% levels is considered here. The tolerance of ±10% for a nominal value of 50% drug gives an acceptance range of 50 ± 5.0%. At the 99.7% probability level then:

$$3\sigma_{|A(P)|} = 5.0 \qquad \text{(Eq. 1}a\text{)}$$

Similarly, for Q and R the acceptance ranges equal 5 ± 0.5% and 0.5 ± 0.05%, respectively. Hence:

$$3\sigma_{|A(Q)|} = 0.5 \qquad \text{(Eq. 1}b\text{)}$$

$$3\sigma_{|A(R)|} = 0.05 \qquad \text{(Eq. 1}c\text{)}$$

The effective mean particle weight (w) in each case can be evaluated from the equation (1):

$$\sigma_A = \{g(100 - g)w/W\}^{1/2} \qquad \text{(Eq. 2)}$$

where W is the sample weight (100 mg), and g is the percentage weight of the active ingredient. The results give:

$$w_P = 0.1111 \text{ mg} \qquad \text{(Eq. 3}a\text{)}$$

$$w_Q = 0.005847 \text{ mg} \qquad \text{(Eq. 3}b\text{)}$$

$$w_R = 0.0005584 \text{ mg} \qquad \text{(Eq. 3}c\text{)}$$

When assuming spherical particles of density 1.2 g/cm^3, the effective mean particle diameters become:

$$d_{(P)} = 561\ \mu\text{m} \qquad \text{(Eq. 4}a\text{)}$$

$$d_{(Q)} = 210\ \mu\text{m} \qquad \text{(Eq. 4}b\text{)}$$

$$d_{(R)} = 96\ \mu\text{m} \qquad \text{(Eq. 4}c\text{)}$$

The results can be confirmed using Eq. 9 of Ref. 2, which is an expression for the coefficient of variation of drug content in a two-component mixture:

$$C_v = 100\left(\frac{\pi\rho}{6G}\right)^{1/2}\left(\sum fd^3\right)^{1/2} \qquad \text{(Eq. 5)}$$

where ρ = drug density (grams per cubic centimeter), G = weight of drug per sample (grams), d = diameter of spherical drug particle (centimeters), and f = weight fraction of drug existing with mean particle diameter d.

Equation 5 is based on a simplification of Stange's (3) equations and holds for two-component mixtures containing up to 1% by weight of drug. For calculation purposes, the three components P, Q, and R can be dealt with separately, thus reducing the problem to consideration of a mixture containing one active ingredient with excipient. Hence, in the case of R (0.5%), Eq. 5 may be used to calculate $d_{(R)}$. In this case, where mean particle diameters are being considered, Σfd^3 becomes d^3 and:

$$C_v = 100\left(\frac{\pi\rho d^3}{6G}\right)^{1/2} \qquad \text{(Eq. 6)}$$

For a tolerance of ±10%, the coefficient of variation, C_v, will be 3.333%. With 100-mg tablets, the weight of drug per sample, G, in case R will be 0.0005 g. When the density is taken as 1.2 g/cm^3, the calculated value for $d_{(R)}$ from Eq. 6 is 96 μm, the same result as Eq. 4c, using the correct σ value.

Table I—Data for Determining Equivalent Particle-Size Distribution

Diameter, μm	Percent by Weight Greater than Stated Diameter	Mean Fraction Particle Diameter, d_i, μm	Weight Fraction, f_i
250	0		
		225	0.03
200	3		
		175	0.04
150	7		
		135	0.02
120	9		
		105	0.11
90	20		
		75	0.3
60	50		
		50	0.2
40	70		
		30	0.2
20	90		
		10	0.1
0	100		

In the cases of components P and Q, Eq. 5 does not give the correct solution since the drug concentrations are too high. With the assumption that the drug and excipient have similar particle-size distributions, that is the effective mean particle weights are equal, Stange's equation for a two-component mixture (see Eq. 13 of Ref. 2) reduces to:

$$C_v = 100\left(\frac{Y\pi\rho d^3}{6G}\right)^{1/2} \qquad \text{(Eq. 7)}$$

where Y = weight fraction of excipient in mixture, and d = effective mean particle diameter of drug.

Equation 7 holds for all drug concentrations and may be used to calculate $d_{(P)}$ and $d_{(Q)}$. For 100-mg tablets containing only component Q (5%) and excipient, Y = 0.95 and the weight of drug, G, per tablet will be 0.005 g. Assuming a density of 1.2 g/cm^3, the value of $d_{(Q)}$ obtained from Eq. 7 equals 210 μm. Similarly, in the case of component P, Y = 0.5 and G will be 0.05 g, giving a value of $d_{(P)}$ from Eq. 7 equal to 561 μm. The results confirm the corrected values given in Eqs. 4a and 4b and also demonstrate the equivalence of the two methods for calculating the effective mean particle diameters necessary to produce a product of specified content uniformity.

According to these results, this would necessitate an overall reduction in particle size to 96 μm and not 5 μm as stated in Ref. 1.

Practically speaking, components of a mixture are very rarely, if ever, homogeneous with respect to particle size, so it would be more instructive to consider the problem in terms of the particle-size distribution desirable for the component instead of a single particle-size limit.

It is possible to interpret the calculated value of $d_{(R)}$ in terms of an equivalent particle-size distribution as shown in Table I. From Table I:

$$\sum f_i d_i^3 = 889{,}702.5\ \mu\text{m}^3 \qquad \text{(Eq. 8)}$$

In a distribution the effective mean particle weight can be written:

$$w = (f_1 w_1 + f_2 w_2 + \ldots f_n w_n) \quad \text{(Eq. 9)}$$

where f_1 is the weight fraction of particles of weight, w_1, *etc.* For spherical particles:

$$w = \frac{\pi\rho}{6} \sum_1^n f_i d_i^3 \quad \text{(Eq. 10)}$$

In the case where particles are the same size, Eq. 10 becomes:

$$w = \frac{\pi\rho d^3}{6} \quad \text{(Eq. 11)}$$

For Eqs. 10 and 11 to be equivalent:

$$d^3 = \sum_1^n f_i d_i^3 \quad \text{(Eq. 12)}$$

Substituting the result from Eq. 8 into Eq. 12 gives $d = 96.2$ μm. Hence, the particle-size distribution given in the example, which was determined by trial and error, is equivalent to the calculated value of $d_{(R)}$ of 96 μm. This distribution is typical of the kind of result encountered in practice. Use of such a particle-size distribution for all active ingredients would allow a safety margin for ingredients *P* and *Q* but, in the case of *R*, would necessitate achieving a truly random mixture to fulfill the desired tolerance range of ±10%.

In practice, a random mix is not always achieved and it may be desirable to introduce an additional safety margin for the lowest concentration drug, *R*. Hersey *et al.* (1) did this in effect by setting the calculated effective mean particle-size limit for *R* as the maximum particle-size limit for the mixture. Alternatively, the coefficient of variation used in Eq. 7 could be set at a lower value than that corresponding to the specified tolerance range of ±10%. For example, instead of 3.333%, a C_v value of 2.5% could be used which would give $d_{(R)}$ from Eq. 7 equal to 79 μm. An equivalent particle-size distribution corresponding to this value of $d_{(R)}$ would contain a considerable fraction above the proposed maximum limit of 96 μm (1) while still incorporating a safety margin to allow for the occurrence of nonrandomized mixing.

In conclusion, converting the particle-size limit into an equivalent particle-size distribution increases the utility of the calculations and provides a more convenient guideline in the practical situation. Additionally, a particle-size distribution of a drug obtained on recrystallization or precipitation or after milling can be tested for its suitability with regard to content uniformity by evaluating $\Sigma f_i d_i^3$ and comparing this value with the value of d^3 derived from Eq. 2 or 7.

(1) J. A. Hersey, P. Cook, M. Smyth, E. A. Bishop, and E. A. Clark, *J. Pharm. Sci.*, **63**, 408(1974).
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M. C. R. Johnson
Upjohn Limited
Crawley, Sussex
United Kingdom

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I wish to thank Dr. J. Hersey for his helpful comments.

Definitive GLC Method of Identifying Cocaine

Keyphrases □ Cocaine—definitive GLC identification □ Trimethylanilinium hydroxide—on-column methylation of cocaine, GLC identification □ GLC—identification, cocaine

To the Editor:

The identification of underivatized cocaine by GLC can be misinterpreted and erroneously reported as pentazocine, levorphanol, or methaqualone when using programmed or isothermal temperatures on 7% OV-17[1]. TLC can also pose problems and lead to the report of a false positive for methadone instead of cocaine (1). Many laboratories are combining mass spectrometry with GLC to provide a more definitive instrumental method for identifying drugs such as cocaine (2); however, many laboratories cannot afford a mass spectrometer and, therefore, more definitive GLC methods of analysis are desirable.

In view of these problems encountered when employing GLC or TLC as a means of identifying cocaine, we wish to report a novel, definitive GLC method of identifying cocaine *via* an on-column GLC reaction under methylation reaction conditions that is applicable to confirming the presence of cocaine in various legitimate and illegitimate dosage forms. In our laboratory we have routinely used trimethylanilinium hydroxide in methanol as a methylating reagent for GLC analysis of anticonvulsant drugs in body fluids (3, 4). We anticipated that this methylating reagent would have an interesting on-column

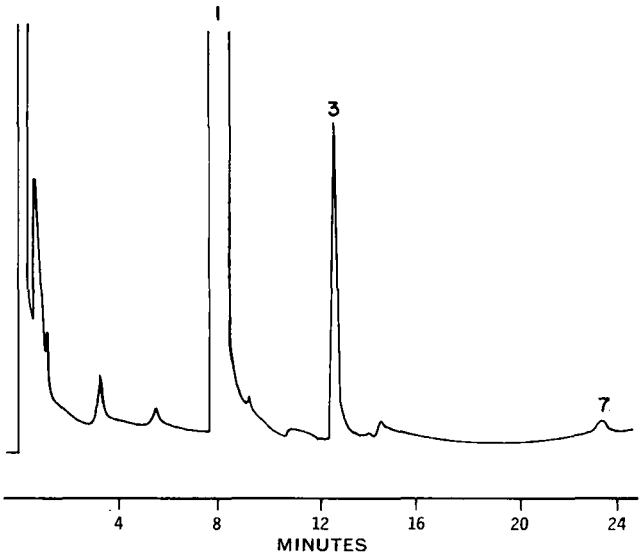

Figure 1—*Characteristic chromatogram representing an on-column reaction of cocaine (1.5 μg) and trimethylanilinium hydroxide (no time lapse after adding the methylating reagent to cocaine). See Table I for identification of the peaks.*

[1] In our laboratory, these drugs have retention times similar to cocaine under programmed and isothermal conditions and are extracted concurrently with cocaine at basic pH.

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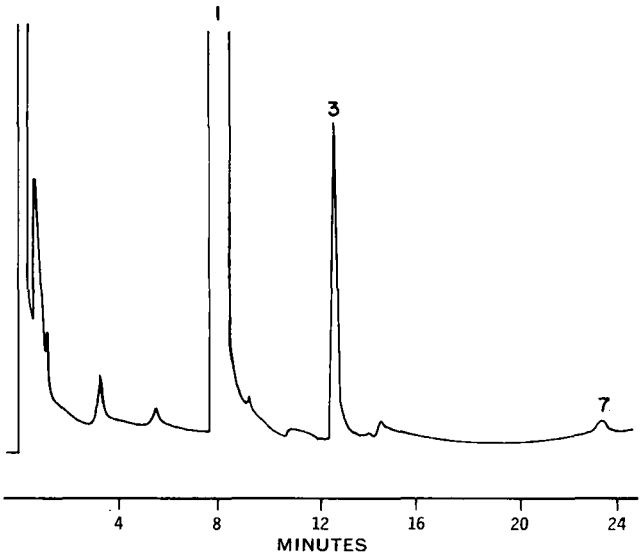

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Table I—Relative GLC Retention Times of Cocaine and Some Commonly Abused Drugs after On-Column Reaction[a]

Drug	R_t, min
Cocaine[b,c]	Peak 1 (8.0), *N,N*-dimethylaniline Peak 2 (10.4), possible Hoffman elimination product Peak 3 (12.5), ecgonidine methyl ester Peak 4 (13.9), under investigation Peak 5 (14.2), ecgonine methyl ester (under investigation) Peak 6 (17.2), under investigation Peak 7 (23.3), cocaine
Amphetamine	8.3
Benzocaine	15.7, 16.8, 17.6, 18.2
Codeine	27.5
Heroin	24.1, 27.2
Levorphanol	21.4
Lidocaine	16.6, 18.5
Methaqualone	23.2
Morphine	27.5
Pentazocine	21.9
Phencyclidine	18.2
Phenobarbital	12.8, 14.3, 14.5, 18.4
Procaine	17.5, 20.8, 21.7, 23.1
Quinine	34.0, 37.0
Secobarbital	15.4

[a] *Under programmed temperature* GLC conditions. [b] The number of chromatographic peaks seen after on-column reaction depends on the concentration of cocaine and the time lapse between addition of the methylating reagent and its on-column injection (see Figs. 1–3). [c] Similar results can be obtained on 3% OV-17 by reducing the nitrogen flow rate from 60 to 30 ml/min and changing the programmed column conditions from 50–250° (10°/min) to 50–250° (8°/min).

reaction with an unusual bicyclic diester tertiary amine structure such as cocaine, through possible ester cleavage and methylation, as well as serve as a definitive confirmatory method by converting it into one or more identifiable derivatives.

All GLC injections were made on a dual-channel instrument[2] equipped with four hydrogen flame detectors. The 1.83-m (6-ft) U-shaped glass columns (2 mm i.d.) were packed with 7% OV-17 on 80–100-mesh Chromosorb W[3]. Operating temperatures were: injector port, 275°; column (isothermal), 250°; column, 50–250° (programmed at 10°/min); and detector, 275°. Flow rates (milliliters per minute) were: nitrogen, 60; air, 300; and hydrogen, 40. Instrumental attenuation was 8×10^{-10}. Under these conditions, reference standard underivatized cocaine had retention times of 23.3 and 2.9 min under programmed and isothermal column temperatures, respectively.

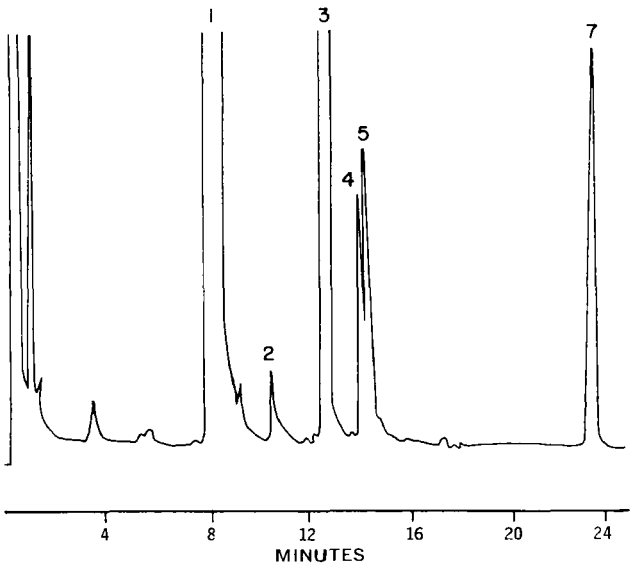

Figure 2—*Chromatogram representing an on-column reaction of cocaine (21 μg) and trimethylanilinium hydroxide (no time lapse after adding the methylating reagent to cocaine). See Table I for identification of the peaks.*

The reaction of cocaine with trimethylanilinium hydroxide solution was initially studied by adding 50 μl of a 2 *M* methanolic trimethylanilinium hydroxide solution[4] (3) to 0.15 mg of cocaine and immediately injecting 0.5 μl (1.5 μg) into the chromatograph under programmed temperature conditions. The resulting chromatogram is illustrated in Fig. 1. Peak 1 is *N,N*-dimethylaniline, a product derived from trimethylanilinium hydroxide during the methylation reaction.

Utilizing a preparative gas chromatograph, we trapped peak 3 and obtained its mass spectrum. Peak 3 was identified as ecgonidine methyl ester (molecular ion, *m/e* 181; base peak, *m/e* 152). Its mass spectrum is identical to the corresponding methylated and trapped reference standard ecgonidine[5]. Ecgonidine methyl ester (peak 3) appeared to be the most characteristic product of on-column reaction between cocaine and trimethylanilinium hydroxide and was observed at all concentrations regardless of the length of time elapsing between addition of the methylating reagent and its on-column injection (peak 3 in Figs. 1–3).

Figure 2 is a representative chromatogram depicting the cocaine–trimethylanilinium hydroxide reaction using a larger amount of cocaine (21 μg), in which case the cocaine–trimethylanilinium hydroxide solution was injected immediately after adding the methylating reagent to cocaine. Mass spectral analysis of peak 2 indicates it to be a potential double Hoffman elimination product, although its structural determination is inconclusive at this time.

Figure 3 represents the chromatogram of the same cocaine–trimethylanilinium hydroxide solution after standing at room temperature for 24 hr. An increase in the concentration of peak 2 (possible Hoffman elimination product) occurred when cocaine was allowed to stand in the alkaline trimethylanilinium hydroxide solution for 1–24 hr (compare Figs. 2 and 3). Peaks 4 and 6 are presently under investigation, and peak 7 is cocaine.

Based on preliminary mass spectral analysis, peak 5 appears to be the ecgonine methyl ester derivative (molecular ion, *m/e* 199; base peak, *m/e* 152). Therefore, to minimize the number of products formed, in-

[2] Varian 2100.

[3] OV-17 and Chromosorb W were obtained from Applied Science Laboratories, Inc., State College, Pa. The 7% OV-17 was prepared in our laboratory using conventional methods (2.1 g OV-17/30 g Chromosorb W).

[4] Trimethylanilinium hydroxide (0.1 *M* in methanol) (trimethylphenylammonium hydroxide) was obtained from Eastman Kodak Co., Eastman Organic Chemicals Division. Trimethylanilinium hydroxide (2 *M* in methanol) was prepared in our laboratory by concentrating 100 ml of 0.1 *M* reagent to 5 ml.

[5] K & K Laboratories, Inc., Plainview, N.Y.

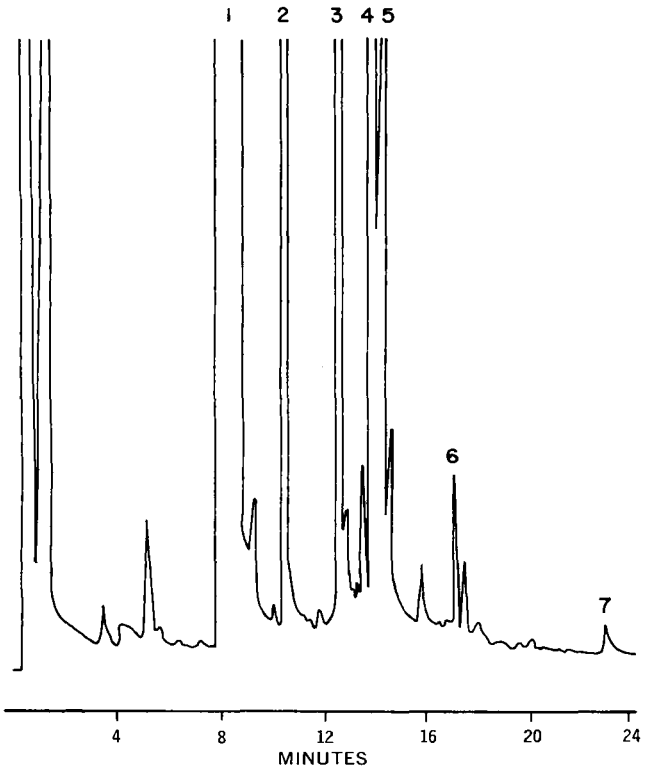

Figure 3—*Chromatogram representing an on-column reaction of cocaine (21 μg) and trimethylanilinium hydroxide (24-hr time lapse after adding the methylating reagent to cocaine). See Table I for identification of the peaks.*

jection of the solution should be performed immediately after adding the methylating reagent to the suspected cocaine residue.

We also wish to report the relative GLC retention times of other commonly abused drugs frequently extracted at alkaline pH ranges (pH 8–10) which could possibly interfere with the confirmation of cocaine by this method. These values are reported in Table I and represent the relative retention times of the product(s) produced using the same on-column reaction conditions performed with cocaine. Of the drugs examined, phenobarbital is the only one that could possibly interfere with the characteristic ecgonidine methyl ester peak (peak 3) under programmed temperature conditions. However, there is no interference between cocaine and phenobarbital when underivatized cocaine is analyzed under isothermal temperature conditions.

To test this method further, we analyzed a simulated street sample containing 6% cocaine hydrochloride, 19% quinine, and 75% dextrose. This analysis was carried out by performing a conventional alkaline extraction (pH 10) of 2.7 mg of the sample with chloroform–isopropanol (3:1), evaporation of the solvent, addition of 50 μl of trimethylanilinium hydroxide to the residue, and immediate injection of 1 μl. The resultant chromatogram resembled Fig. 1, with no interference from quinine.

In summary, we believe this method will be of value as a definitive confirmatory screening test for cocaine after first tentatively identifying underivatized cocaine using isothermal or programmed GLC temperature conditions.

(1) D. Bayse, N. Radin, D. S. Lewis, and G. Guerrent, "Proficiency Testing, Toxicology-Drug Abuse Survey III," U.S. Department of Health, Education, and Welfare, Public Health Service, Aug. 1973, p. 13.

(2) B. S. Finkle, D. M. Taylor, and E. J. Bonelli, *J. Chromatogr. Sci.*, **10**, 312(1972).

(3) R. H. Hammer, B. J. Wilder, R. R. Streiff, and A. Mayersdorff, *J. Pharm. Sci.*, **60**, 327(1971).

(4) R. J. Perchalski, K. N. Scott, B. J. Wilder, and R. H. Hammer, *ibid.*, **62**, 1735(1973).

Richard H. Hammer ˣ
James L. Templeton
H. L. Panzik

Department of Pharmaceutical Chemistry
College of Pharmacy
University of Florida
Gainesville, FL 32610

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ˣ To whom inquiries should be directed.

REVIEWS

Chromatography of Antibiotics. By G. H. WAGMAN and M. J. WEINSTEIN. Journal of Chromatography Library Volume 1, American Elsevier, 52 Vanderbilt Ave., New York, NY 10017, 1973. 238 pp. 20 × 27 cm. Price $26.00.

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The rest of the book is devoted to a literature search on the TLC, paper chromatography, electrophoresis, countercurrent distribution, and GLC of most known antibiotics. The procedures are presented in a concise, standardized, outline form. Only essential details are presented. However, separation and R_f data reported in the original papers are detailed. Literature references are included, which is helpful for obtaining more details on the procedures cited. In my opinion, this part of the book would have been strengthened if the authors had included some of their own experiences in chromatography. Perhaps they should have indicated which methods they have used and what merits these methods possessed.

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Most workers in chromatography will find this a handy, concise compendium of the methods used to chromatograph antibiotics. Scientists searching for a method for separating antibiotics will probably consult this volume before any other.

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National Center for Antibiotics Analysis
Washington, DC 20204

Pharmacology of Gastrointestinal Motility and Secretion, Volumes I and II. PAMELA HOLTON, Section Editor. Pergamon Press Ltd., Headington Hill Hall, Oxford, England, 1974. lv + 665 pp. 14 × 22 cm. Price $54.00

The two volumes represent the continued effort of the International Encyclopedia of Pharmacology and Therapeutics to provide a critical and comprehensive survey of present knowledge in various specific areas.

Volume I contains 10 chapters and each chapter represents a complete presentation of a single subject. The subject matter is related to that of other chapters but is presented so that each chapter can stand by itself in an encyclopedic manner.

Salivary secretion and factors influencing it are presented in Chapter 1. Histamine and its effects on gastric acid secretion in the rat and nonrodents are discussed extensively in Chapters 2 and 3, respectively. Chapters 4 and 5, respectively, concern effects of substances on gastric acid secretion in animals and humans. Experimental methods for the bioassays are included. Chapter 6 presents extensively the pharmacology of isolated amphibian gastric mucosa, secretory stimulation, and inhibition by various agents. The chemical structure and actions of gastrin and other naturally occurring proteins are discussed in Chapter 7. Chapter 8 deals with the effects of the catecholamines on gastric secretion and its constituents in both animals and humans. Chapter 9 discusses drugs used in the treatment of peptic ulcer and briefly the pathophysiology of peptic ulcer, while Chapter 10 concerns the pathogenesis of gastric ulcers largely from the aspect of mucosal blood flow. Experimental ulcer models are described.

Volume II contains Chapters 11 through 18. Chapter 11 discusses the chemistry, physiology, and clinical testing application of secretin, whereas Chapter 12 similarly deals with cholecystokinin. Chapter 13 deals with the use of isolated GI muscle, the actual bioassay procedures, solutions utilized, and methods of determining drug action. Various drugs are included and their effects are discussed. Chapter 14 presents drug treatment for the many disorders of motility of the human GI tract, along with a brief discussion of the disorders themselves. Chapter 15 is an extensive presentation of the pharmacology of GI motility, including myogenic and neurogenic methods. The role of adrenyl cyclase in the activity of the GI tract is presented in Chapter 16 along with a valuable addendum. Chapter 17 deals with the action of carbonic anhydrase inhibitors at different levels of the GI tract. Chapter 18, the concluding chapter, deals with some endocrine tumors capable of producing GI symptoms; tests, diagnosis, and treatment are presented.

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Chemically Induced Magnetic Polarization. Edited by A. R. LEPLEY and G. L. CLOSS. Wiley, 605 Third Ave., New York, NY 10016, 1973. 416 pp. 16 × 24 cm. Price $19.95.

Actualités de Dermopharmacologie. By H. THIERS and J. COTTE. Centre Europeen de Dermopharmacologie, B. P. n° 10 Lyon-Mouche, 69342 Lyon Cedex 2, France, 1973. 295 pp. 16 × 24 cm. *(French)*

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ERRATA

In the article titled "Disintegration of Tablets" (1), the following correction should be made:

On page 1710, Ref. 203 should read:

(203) J. K. C. Yen, *Can. Pharm. J.*, **97**(11), 25(1964).

(1) W. Lowenthal, *J. Pharm. Sci.*, **61**, 1695(1972).

In the article titled "Tetracycline Epimerization Kinetics Utilizing NMR Spectrometry" (1), the following corrections should be made:

On page 259, the figure appearing above the legend for Fig. 3 should appear above the legend for Fig. 4, and the figure appearing above the legend for Fig. 4 should appear above the legend for Fig. 3.

(1) K. D. Schlecht and C. W. Frank, *J. Pharm. Sci.*, **62**, 258(1973).

In the article titled "Stability of Solids and Solid Dosage Forms" (1), the following corrections should be made:

On page 1, column 2, Eq. 5 should read:

$$x = 1 - [1 - (k_3 t/r_0)]^3$$

On page 8, column 1, Eq. 37 should read:

$$C = S[1 - e^{-\alpha l}]/(\alpha l)$$

(1) J. T. Carstensen, *J. Pharm. Sci.*, **63**, 1(1974).

In the article titled "Magnesium Lauryl Sulfate in Tableting: Effect on Ejection Force and Compressibility" (1), the following correction should be made:

On page 290, in Fig. 2, the first two graduation markings on the ordinate axis, starting from the origin, should read 10 and 20 instead of 5 and 10, respectively.

(1) A. M. Salpekar and L. L. Augsburger, *J. Pharm. Sci.*, **63**, 289(1974).

In the article titled "Identification and Quantitative Determination of Phenothiazine Drugs in Urine Samples of Psychiatric Patients" (1), the following correction should be made:

On page 393, column 1, Eq. 2 should read:

$$C_u = \frac{5}{v}\frac{1}{k}\frac{A_x'}{A_s'} w_s$$

(1) A. P. De Leenheer, *J. Pharm. Sci.*, **63**, 389(1974).

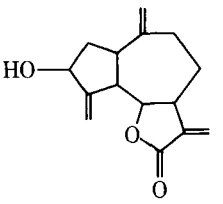
In the article titled "Condensing Effect of Cholesterol: A Physical Model" (1), the following correction should be made:

On page 860, in Figs. 2–5, the vertical axes should be labeled A_m rather than π.

(1) J. L. Zatz, *J. Pharm. Sci.*, **63**, 858(1974).

In the article titled "Tumor-Inhibitory Agent from *Zaluzania robinsonii* (Compositae)" (1), the following correction should be made:

On page 1321, column 2, the structure for I should be:



(1) S. D. Jolad, R. M. Wiedhopf, and J. R. Cole, *J. Pharm. Sci.*, **63**, 1321(1974).